Enzyme-Mediated and Mechanistic Investigations of Tetrahydroisoquinoline Synthesis through the Pictet-Spengler Reaction

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Enzyme-Mediated and Mechanistic Investigations of Tetrahydroisoquinoline Synthesis through the Pictet-Spengler Reaction

by

Jordan Elise Fauser

A Thesis
Submitted in
Partial Fulfillment of the
Requirements for the Degree of
Master of Science

Department of Chemistry
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Chicago, Illinois
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This work is dedicated to my family for their constant support and encouragement of my scientific endeavors.
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Chapter 1: Introduction

Alkaloids in Medicinal Chemistry

An alkaloid is a class of secondary plant metabolites characterized by a heterocycle, typically containing a basic nitrogen, derived from an amino acid. The first uses of plant derived alkaloids for medicinal purposes can be dated back to ancient Mesopotamia. Alkaloids contain biological activity and have historically been used as medicinal treatments, recreational drugs, and many alkaloids are poisonous. These secondary metabolites were most likely produced by plants as a deterrent for herbivorous mammals and as such have a greater tendency to be bitter and poisonous. However, due to their high probability to possess biological activity, these small molecules have been of great interest to medicinal chemists in developing libraries to identify new potential medicines. Currently alkaloids are used as stimulants (caffeine), vasodilators (norcoclaurine), antimalarial (quinine), hypoglycemic agents (berberine), pain management (morphine), and many others. The reason for the multitude of potential applications of alkaloids is the diversity of naturally produced alkaloids. Common ring structures found in alkaloids are shown in Figure 1.

![Common alkaloid ring structures](image)

The alkaloids of interest for this research are tetrahydroisoquinolines (THI), their derivatives, and other isoquinoline derivatives. One particular isoquinoline derived alkaloid is norcoclaurine. (Figure 2)
Norcoclaurine has shown pharmacological benefits as a β-2-adrenergic receptor agonist in rodent models. It is also a precursor for a host of other natural products with potential pharmacological benefits, such as berberine. Derivatives of norcoclaurine and subsequent alkaloids from natural pathways could potentially provide new functionality to these compounds or enhance known functionality. For example, the addition of halogens to berberine resulted in a decreased IC$_{50}$ of berberine and an increase in glucose lowering capabilities in cell lines.\(^8\)

Full synthesis of these alkaloids and their derivatives pose several challenges.\(^9\) However, some of these synthetic challenges may be alleviated by using an enzyme-mediated synthesis followed by precursor directed biosynthesis in *Berberis vulgaris* cells. Previous attempts by the Maresh lab to create berberine derivatives through cell feeding experiments with dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) encountered difficulties with substrate degradation. Synthesis of the more stable norcoclaurine derivatives prior to feeding into the *Berberis vulgaris* cells may solve the issue of substrate degradation.\(^{10}\) Norcoclaurine derivatives can be synthesized using an enzyme-mediated synthesis of dopamine or L-DOPA derivatives followed by a Pictet-Spengler condensation with 4-HPAA.

The investigations reported in the following chapters address the potential use of tyrosinase in halogenated catechol and halogenated THI synthesis. The oxidation of natural substrates such as tyramine and tyrosine and halogenated substrates by tyrosinase was performed. The unfavorable equilibrium of the oxidation reaction was overcome through the coupling of the enzyme reaction and a Pictet-Spengler reaction with either propionaldehyde or 4-hydroxyphenylacetaldehyde. The result of the coupling of the reactions was the formation of various halogenated and un-halogenated THIs.
The Pictet-Spengler mechanism for the reaction of 3-hydroxy phenethylamine and an aldehyde in the presence of a buffer is shown in Scheme 1. The synthesis of the THI, norcoclaurine involves the Pictet-Spengler condensation. This mechanism is the primary reaction used in the synthesis of tetrahydroisoquinolines from phenethylamines. This reaction is enzyme catalyzed in plants but can also be carried out as a chemical reaction. Pesnot et al. proposed a mechanism for phosphate catalysis of the Pictet-Spengler condensation in 3-hydroxyphenethylamines. This mechanism was further investigated through computational modeling to propose the likely role of inorganic phosphate in the catalysis of this reaction by Parra and Maresh. Kinetic data on the phosphate catalysis of the Pictet-Spengler condensation could potentially provide information on the rate-determining step and role of phosphate in catalysis. This mechanism involves both acid and base catalysis from the buffer. A kinetic evaluation varying the pH and concentration of the buffer could provide insight as to whether the reaction primarily acid or base-catalyzed. Determination of the rate-limiting step can be done using isotopic labeling of the starting material, in particular, the use of deuterium. Previous studies of this reaction mechanism indicate possible base catalysis for the rate-limiting
Base catalysis could potentially indicate that steps 5 or 6 in Scheme 1 may be the rate-determining step. The kinetic isotope effect can effectively distinguish which of these two steps is the rate-limiting step. Steps 5 and 6 are an electrophilic aromatic substitution. In the first step of an electrophilic aromatic substitutions the hybridization change of the carbon bonded to the heavy isotope results in a secondary isotope effect. Whereas, in the second step (step 6) deprotonation occurs to re-establish aromaticity. The breaking of a carbon-hydrogen bond results in a primary kinetic isotope effect.

This reaction of 3-hydroxyphenethylamines with an aldehyde primarily results in cyclization at the para position, however the observation of the regioisomer in which cyclization occurs at the ortho position has been reported. Furthermore, the formation of the regioisomer is pH dependent; where a more acidic pH exhibits a reduction in the percent of the ortho isomer. The ratio of this isomer and the target THI also appears to vary with the concentration of phosphate present, implying that phosphate may play a role in conformational selectivity of this reaction. The combined effect of phosphate and pH control of regioisomer formation could assist in developing an efficient method to only produce the desired isomer.

Figure 3: THI and isoTHI framework

For the research presented in the following chapters, the Pictet-Spengler reaction between 3-hydroxyphenethylamine and propionaldehyde was evaluated through kinetic and computational methods. The potential catalytic role of the monobasic and dibasic phosphate species in the reaction was evaluated as well as the potential role of each of these species in the regioselectivity of the reaction. As a comparison to verify the phosphate specific catalyst was conducted by repeating the kinetic experiments with an organic buffer of similar pKa, Maleic Acid buffer. To supplement the observed dependence of regioisomer formation on phosphate buffer concentration and pH, a computational analysis of the intrinsic reaction coordinate for the final step of regioisomer formation in the presence of various catalysts was conducted.

Chapter 2: Tyrosinase Mediated Synthesis of Norcoclaurine

Introduction

Tyrosinase is a prolific catechol oxidase, with phenolase and catecholase activity, found in a wide variety of organisms. The active site of tyrosinase is a binuclear copper center with
two oxo bridges and six coordinating histidines.\textsuperscript{15} Tyrosinase’s active site is highly conserved, however the tyrosinase found in white button mushrooms most closely resembles the tyrosinase found in mammals compared to tyrosinase from other organisms.\textsuperscript{15-17} This enzyme is involved in the oxidation of tyrosine to L-DOPA and the subsequent oxidation to dopaquinone, through the use of molecular oxygen as a substrate, in the melanin production pathway. Tyrosinase can also act on dopaquinone to form dopachrome, both of these products are unstable and can lead to polymerization and thus the formation of melanin.\textsuperscript{17, 24-25} Ascorbic acid can act as a reducing agent to reverse this process of over-oxidation by tyrosinase and yield the desired catechol. Other methods to control further oxidation by tyrosinase is by controlling the levels of hydrogen peroxide. H$_2$O$_2$ is produced by tyrosinase during the oxidation of DOPA to dopaquinone, thus high concentrations can be inhibitory, however H$_2$O$_2$ is also a cofactor in subsequent oxidations and activates tyrosinase at lower concentrations.\textsuperscript{26} Due to its involvement in melanin production and association with reactive oxygen species, tyrosinase has historically been investigated in the context of inhibition for melanoma control.\textsuperscript{15-17, 24-25}

However, in recent years tyrosinase has become an enzyme of interest in the field of biotechnology. This enzyme has been used as a phenolic scavenger for waste water treatment, as a phenol and diphenol biological sensor, and as a synthetic tool for L-DOPA synthesis.\textsuperscript{18-23} Tyrosinase has low substrate specificity,\textsuperscript{15-25} making it a prime target for use with non-natural substrates. Tyrosinase exhibits equivalent activity with tyrosine and tyramine. As dopamine is a precursor for norcoclarine, we propose that this enzyme may be able to accept halogenated tyramine or tyrosine as alternative substrates to form halogenated dopamine or L-DOPA respectively. Use of this enzymatic process to form these products alleviates some of the synthetic challenges in full synthesis of halogenated dopamine or L-DOPA.

![Scheme 2: Phenolase and catecholase activity of tyrosinase on natural substrates tyrosine and L-DOPA with the concurrent reduction of dopaquinone to L-DOPA by ascorbic acid.](image)

There are some potential drawbacks to using tyrosinase as a synthetic tool however. Tyrosinase is involved in the first three steps of the melanin production pathway. As the formation of the catechol is the first step in this series of reactions, yield of the catechol is typically low. Were the goal strictly to produce the halogenated catechol, tyrosinase would not be an effective synthetic tool. The reason for the low yield is an unfavorable equilibrium in the
catechol formation. In the cell this equilibrium would be overcome by continuous oxidation resulting in the removal of the catechol, acting as a driving force for continued oxidation of tyrosine. Based on Le Chatelier’s Principle, the removal of a product drives a reaction in equilibrium forward to produce more of the product and use more of the reactant to reestablish equilibrium. Using this concept, the unfavorable equilibrium may be overcome through the use of coupling reactions. The addition of an aldehyde to the reaction solution would result in the Pictet-Spengler reaction between the catechol and the aldehyde. This reaction would result in the formation of THI. In mild aqueous conditions, the Pictet-Spengler reaction will only occur upon the oxidation of the aromatic ring. As a result the aldehyde will only react with the catechol and have no effect on the phenol. This reaction coupling accomplishes two goals, first to overcome the enzyme equilibrium and secondly to produce the desired THI.

In this chapter the use of tyrosinase from various sources as crude extract or cross-linked enzyme aggregate to oxidize tyramine and tyrosine followed by the Pictet-Spengler reaction with 4-HPAA or propionaldehyde. The extraction procedure for tyrosinase from white-button mushrooms, avocados, and sweet potatoes was optimized as well as the cross-linking procedure to produce an insoluble protein aggregate. Using these methods to acquire tyrosinase, the enzyme-mediated oxidation and Pictet-Spengler reaction were attempted to synthesize various THIs and drive the coupled reaction to completion. This was attempted using natural substrates as a verification that this synthetic technique was feasible.

General Methods

HPLC-UV Analysis Methods

Several different HPLC methods were used to analyze the various reaction used throughout these projects. The stationary phase of the column was not changed throughout the experiments. However, the mobile phase solvent gradients were varied to best suit the reactions analyzed. Reactions were monitored using high pressure liquid chromatography on a Waters Acquity Ultra Performance Liquid Chromatography instrument with a photodiode array detector (HPLC-UV) with an Acquity HPLC BEH C18 column (1.7 µm, 2.1 x 50 mm) at 25 °C.

Linear Gradient 0 to 20 Percent

The solvent gradient used in this method is as follows: 0.4 mL/min; 0% acetonitrile (MeCN) in 0.1% aqueous trifluoroacetic acid (TFA) for 1.0 minute, 0-20% MeCN in 0.1% TFA over 4.25 minutes, 20-70% MeCN in 0.1% TFA over 1.0 minute, hold at 70% MeCN in 0.1% TFA for 1.0 minute. This method involves a brief one minute delay at the start of the method, the 0 to 20% gradient with the delay was used for the kinetics reactions. A method without this initial delay was used for much of the tyrosinase reaction monitoring with unhalogenated substrates.

Linear Gradient 0 to 30 Percent
The solvent gradient used in this method is as follows: 0.4 mL/min; 0% acetonitrile (MeCN) in 0.1% aqueous trifluoroacetic acid (TFA) initially, 0-30% MeCN in 0.1 % TFA over 11 minutes, 30-0% MeCN in 0.1% TFA over 0.5 minute, hold at 0% MeCN in 0.1% TFA for 1.0 minute. This method was primarily used to analyze the tyrosinase reactions with the halogenated substrates.

Isocratic Method 2.5 Percent

The solvent gradient used in this method is as follows: 0.4 mL/min; 0-2.5% acetonitrile (MeCN) in 0.1% aqueous trifluoroacetic acid (TFA) for 1 minute, 2.5% MeCN in 0.1 % TFA over 9 minutes, 2.5-13% MeCN in 0.1% TFA over 2 minutes, 13-0% MeCN in 0.1% TFA over 2 minutes, hold at 0% MeCN in 0.1% TFA for 1.0 minute. This method was used for monitoring the decarboxylation of halogenated tyrosine to improve the separation between the tyrosine and tyramine.

Enzyme Extraction

Enzyme extraction from Agricus bisporus

Tyrosinase was extracted from white button mushrooms using the Duckworth and Coleman method of tyrosinase extraction with optimized methods. White button mushrooms were finely chopped as a method of mechanical cell lysis. The chopped mushrooms were then frozen in liquid nitrogen to more effectively lyse the cells and increase the yield of enzyme extracted. To further increase yield the frozen mushroom was ground in a mortar. Three milliliters of a 50 mM NaH$_2$PO$_4$ at a pH of 6.5 extraction solution were added per gram of mushrooms and allowed to equilibrate at 4 °C for one hour. Following equilibration the extract solution underwent gravity filtration. This solution was centrifuged at 20,000 xg and 4 °C for 15 minutes. The supernatant was collected and stored at -20 °C if not immediately used.

Enzyme extraction from Persea americana

Using the methods presented by Vieira et al. with slight modifications, tyrosinase was extracted from avocados. Avocados were peeled and the seed was removed, the flesh was finely chopped as a method of mechanical cell lysis. The chopped avocados were then frozen in liquid nitrogen to more effectively lyse the cells and increase the yield of enzyme extracted. To further increase yield the frozen avocado was blended in the presence of the extraction solution. Three milliliters of a 50 mM NaH$_2$PO$_4$ at a pH of 6.5 extraction solution were added per gram of avocado and allowed to equilibrate at 4 °C for one hour. Following equilibration the extract solution underwent rapid filtration through a cheese cloth. This solution was centrifuged at 20,000 xg and 4 °C for 15 minutes. The supernatant was collected and stored at -20 °C if not immediately used.

Enzyme extraction from Ipomoea batatas
Sweet potatoes were peeled and finely chopped as a method of mechanical cell lysis. The chopped sweet potatoes were then frozen in liquid nitrogen to more effectively lyse the cells and increase the yield of enzyme extracted. To further increase yield the frozen sweet potato was blended in 100 milliliters of 0.1 M NaH$_2$PO$_4$ at a pH of 7.0 per 25 grams of potato. This puree was allowed to equilibrate at 4 °C for one hour. Following equilibration the potato extract was rapidly filtered through a cheese cloth. To remove some of the starches and phenolic compounds present in the potato extract, 0.1 g of gelatin was dissolved in 10 mL of deionized water and the gelatin solution was added to the extract. The solution was gently mixed and filtered to remove the coagulant. This solution was centrifuged at 20,000 xg and 4 °C for 15 minutes. The supernatant was collected and stored at -20 °C if not immediately used.

**Protein Cross-linking**

To improve pH and thermal stability of the enzyme, to allow for continuous re-use of the catalyst, the protein was crosslinked to form an insoluble aggregate. This cross-linked enzyme aggregate (CLEA) also reduces the noise peaks in HPLC chromatograms caused by absorbance from protein and contaminant in the crude mushroom extract. The aggregate is also easily filtered out of the reaction solution for easy isolation of target compounds. The protein aggregate is formed using the previously described extraction procedures to obtain crude enzyme extract and the methods proposed by Xu et al. for protein corsslinking.$^{18}$ To precipitate the protein, 40% wt/vol ammonium sulfate was added slowly to the crude extract, while stirring at 4 °C. This solution was then stirred for 10 minutes at 4 °C. Glutaraldehyde was used to cross-link the precipitated protein. Two milliliters of glutaraldehyde per 50 mL of solution were added dropwise to the solution while stirring at 4 °C. The solution remained stirring at 4 °C for 12 hours to allow for protein aggregation. Following cross-linking, the solution was centrifuged at 20,000 x g for 15 minutes at 4 °C. The aggregate and supernatant were filtered under vacuum and washed with 50 mM NaH$_2$PO$_4$ buffer, pH 6.5. The aggregate was then dried and ground to a fine powder. The aggregate was stored at 4 °C for up to a month if not used immediately.

**Enzyme-mediated Synthesis**

*Enzymatic synthesis of Dopamine and L-DOPA using crude enzyme extract*

The crude enzyme extract acquired using the previously mentioned methods could be used to oxidize tyrosine or tyramine. For the conversion of 1 mmol of substrate, 100 mL of the crude extract was added to an Erlenmeyer flask containing 50 mL of 50 mM monobasic sodium phosphate buffer pH 6.5. An equivalent of ascorbic acid was added to the reaction solution reduce any produced quinone to the desired catechol. The reaction progress was monitored via HPLC and chromatograms were extracted at 280 nm as well as 225 nm. Using the chromatograms extracted at 225 nm reduces the noise peaks from the contaminant protein in the crude enzyme extract, it also increases sensitivity for lower concentrations of compounds.

*Enzymatic synthesis of Dopamine and L-DOPA using Cross-linked Enzyme Aggregate (CLEA)
For the conversion of 1 mmol of substrate, 50 mg of the CLEA was added to an Erlenmeyer flask containing 100 mL of 50 mM monobasic sodium phosphate buffer pH 6.5. An equivalence of ascorbic acid was added to the reaction solution reduce any produced quinone to the desired catechol. The reaction progress was monitored via HPLC and chromatograms were extracted at 280 nm as well as 225 nm. This enzymatic reaction reaches an unfavorable equilibrium at approximately 20% conversion of the phenolic amine to the desired catechol. In an attempt to alleviate this equilibrium and drive the reaction forward to completion 4-HPAA was added to the enzyme reaction solution. The addition of 4-HPPA resulted in the Pictet-Spengler condensation of the catechol and the aldehyde.

Synthesis of 4-hydroxyphenylacetaldehyde

Sodium Hypochlorite oxidation of Tyrosine

1 mmol of L-Tyrosine was dissolved in 250 mL of 10 mM sodium phosphate buffer at pH 7.0. To the vigorously stirring tyrosine solution, an equivalence of NaOCl in a 0.1 M solution was added dropwise over 10 minutes using a syringe pump. The reaction was carried out at 37 °C with continued stirring, reaction progress was monitored via HPLC and quenched after approximately 2 hours. The solution was then extracted with dichloromethane (DCM) and washed with brine. The solution was then dried over solid MgSO₄ and filtered. The solvent was removed under reduced pressure in a cold water bath.

Parikh-Doering Synthesis of 4-HPAA

The starting material, 4-(2-hydroxyethyl)phenol, was dissolved in approximately 5 ml of dimethyl sulfoxide (DMSO) per 10 mmol reagent. Then 2 equivalents of trimethylamine were added. The reaction flask was purged with N₂ and cooled to 0 °C. In a separate dry flask, 2 equivalents of SO₃-pyridine complex were dissolved in approximately 7 mL of DMSO per 20 mmol of SO₃. Dissolved SO₃-pyridine complex was added to main reaction flask dropwise via syringe over 10 min, under continued N₂ purge. After the addition of the SO₃-pyridine complex, the reaction was carried out at room temperature and reaction progress was monitored via HPLC. The compounds were separable on the BEH C18 column using a gradient of 0-20% acetonitrile in 0.1% (trifluoroacetic acid) TFA over 5 min. Upon the reaction slowing, an extra 0.5 equivalents of SO₃-Pyridine in DMSO was added. Once reaction reached 95-97% complete or side products appeared, the reaction was cooled to 0 °C and 3 equivalents of H₂O were added to quench the reaction. To improve extraction efficiency, 5-6 volume equivalents of brine were added to the quenched solution. The aqueous layer was extracted with 1:1 DCM:THF (tetrahydrofuran) solution (3 X 25 mL for 10 mmol scale). The solvent was evaporated under reduced pressure with ice-water bath cooling.

Investigation of Tyrosinase influence on the Pictet-Spengler reaction

The Pictet-Spengler reaction between dopamine and an aldehyde results in the formation of two regio-isomers, THI and isoTHI. Throughout the investigation of the enzyme-mediated synthesis followed by Pictet-Spengler condensation, low yields of the isoTHI were
observed. To determine if the tyrosinase was involved in controlling the isomer formation, several experiments were conducted.

The first series of experiments conducted involved varying the concentration of phosphate in a series of enzymatic reactions. Using the previously described method to set up the enzymatic reaction, eight reaction solutions were prepared using 1 M, 0.5 M, 0.1 M, 0.075 M, 0.05 M, 0.025 M, 0.01 M and 0.001 M phosphate buffer at pH 6.5. Tyrosinase CLEA was added to each of these reaction solutions as well as 1 mmol of Tyramine, and 1.5 mmol of Ascorbic Acid. Upon the production of dopamine 4-HPAA was added to the reaction solution. However, due to the variations in the phosphate buffer concentration, several of the reaction solutions exhibited lower enzymatic activity, and reliable data could not be obtained.

A second set of reactions were prepared to attempt to elucidate the role, if any, of tyrosinase in the regioselectivity of the reaction. The first reaction solution was prepared with 75 mL of 50 mM Phosphate buffer at pH 6.50, 10 mmol of dopamine, 10 mmol of ascorbic acid, and excess propionaldehyde. Propionaldehyde was used for this investigation because it is readily available and the Pictet-Spengler reaction of dopamine and propionaldehyde exhibits the same isomeric formation behavior as the reaction of dopamine and 4-HPAA. The second reaction solution was prepared with 35mL of the tyrosinase extraction solution in 50 mM phosphate buffer pH 6.50, 40 mL of 50 mM phosphate buffer at pH 6.50, 10 mmol of tyramine, and 10 mmol of ascorbic acid. The reaction was monitored via HPLC until dopamine was produced. At this point excess propionaldehyde was added to the reaction solution. The presence of the propionaldehyde drove the enzymatic reaction forward to completion over the course of 10 hours. The reactions were monitored via HPLC-UV and chromatograms were extracted at wavelengths of 280 nm and 225 nm. Both wavelengths were used in analysis because the proteins in the extract solution absorb heavily at 280 nm and make peak identification difficult. However, dopamine absorbs better at 280 nm and increased peak intensity is observed for dopamine, thus both wavelengths were useful for analysis.

Results and Discussion
Synthesis of 4-hydroxyphenylacetaldehyde (4-HPAA)

Two synthetic techniques were used to generate 4-HPAA. Each of these techniques has their own complications, mostly due to the instability of 4-HPAA and the frequency with which the reaction results in side products. The first was the synthesis of 4-HPAA from tyrosine and sodium hypochlorite as described in a paper published by the Maresh et al. This was a very simple reaction, however can only be used to synthesize approximately 50 mg of 4-HPAA at a time and the extraction procedure requires large quantities of solvent, making this an inefficient method for the production of a large amount of starting material. The second was the Parikh-Doering synthesis of 4-HPAA from tyrosol. This synthetic method was more challenging and poses some issues with purification of 4-HPAA from side products and starting material. This technique, however allowed for a larger scale synthesis and thus was the optimal
method for the purpose of the enzyme-mediated synthesis of norcoclaurine. With 4-HPPA synthesized, the tyrosinase reaction was optimized.

Optimization of Tyrosinase Reaction Methods

Several experiments were conducted to optimize the enzyme-mediated synthesis of tetrahyrdoisoquinolines from tyramine and tyrosine. Initially the activity of the CLEAs were evaluated compared to the crude enzyme extract. In these experiments the longevity of the CLEA activity was compared to the longevity of the crude extract. Little difference was observed in these trials. The primary benefit of the CLEA is the ease of removing the enzyme upon reaction completion and the decreased noise on the HPLC chromatograms. Another benefit of the facile removal of the enzyme CLEA from the reaction solution is the reusability of the CLEAs for repeat experiments. The CLEAs maintained activity at 27 °C for approximately a week and could be used for repeat experiments for the duration of that time period.

The pH of the extraction buffer and reaction solution were also optimized for most rapid rate of tyrosine or tyramine conversion to L-DOPA or dopamine respectively. A comparative analysis of six samples was conducted. Tyrosinase was extracted from white button mushrooms and the pH of the sodium phosphate was varied; pH 6.0, 6.5, and 7.0 were used. The aggregation procedure was carried out for each of these extracted solutions. The pH 7.0 extract yielded no precipitate and thus was not used for further experimentation. The CLEAs obtained with an extraction buffer at pH 6.0 and 6.5 were then added to three reaction solutions with varied pH (pH 6.0, 6.5, 7). The CLEAs were added 10 mg of aggregate per 10 mL of buffer with 2 mmol of tyrosine and 2 molar equivalents of ascorbic acid. These reactions were monitored over the course of 4 hours, the conversion was compared as a ratio of starting material peak area and product peak area. Standard curves of tyramine and dopamine were created to quantify the reaction yield. From this experiment, the enzyme extraction in a buffer of pH 6 and a reaction pH of 6.0 appeared to be the most effective, however, the enzyme extraction in a buffer of pH 6.5 yielded more aggregate. The conversion of tyrosine to L-DOPA was comparable at approximately 20% conversion for both of these combinations.

Enzyme-mediated synthesis of Unhalogenated Tetrahydroisoquinolines

The enzyme-mediated oxidation of tyrosine or tyramine results in an unfavorable equilibrium in which there is minimal conversion of the substrate to the product. In the natural biochemical pathway the catechol formed through the first oxidation would again be oxidized to form a quinone. This quinone would then be used in subsequent steps in the melanin synthesis pathway thus driving the tyrosine oxidation forward. However, in our synthetic system, there is no driving force to overcome this unfavorable equilibrium. This issue was addressed by coupling the enzymatic reaction with the Pictet-Spengler reaction to form THI.

Following the optimization of the procedure the enzyme was extracted and CLEAs were produced. Tyramine or tyrosine were dissolved in monobasic phosphate buffer of pH 6.5 and either the crude enzyme extract or the CLEA. The reaction was monitored until equilibrium was
reached approximately 4 hours into the reaction. Upon reaching equilibrium, 4-HPAA was added to the reaction solution. Due to the instability of 4-HPAA at higher temperatures, the 4-HPAA was added slowly over time and the reaction was monitored via HPLC. Through this reaction, norcoclaurine and a carboxylated form of norcoclaurine were formed. (Figure 2).

Figure 3: HPLC chromatogram of the reaction progress of a one-pot enzyme-mediated synthesis of norcoclaurine. Reaction carried out in 50 mM NaH₂PO₄ buffer pH 6.5 with tyrosinase crude extract from white-button mushrooms. Chromatogram extracted at 225 nm to minimize the peaks caused by the crude enzyme extract.

Unfortunately, due to the challenges with synthesis of 4-HPAA and the instability of 4-HPAA at higher temperatures, pushing the reaction to completion is very challenging. Propionaldehyde was used to examine the reaction because it is more readily available than 4-HPAA and does not degrade as readily as 4-HPAA. This test revealed that upon addition of an aldehyde in excess to the enzymatic reaction solution the oxidation reaction was driven to completion and THI and isoTHI were formed (Figure 3). The continued addition of the unhalogenated amine substrate to the reaction solution with the continued addition of excess aldehyde results in continuous formation of THI. The identification of THI in the enzymatic reaction was verified using a control reaction of dopamine and propionaldehyde in phosphate buffer at the same reaction conditions. (Figure 4)
Figure 4: Coupled enzymatic reaction and Pictet-Spengler reaction for the conversion of Tyramine $\rightarrow$ Dopamine and Dopamine + Aldehyde $\rightarrow$ THI. Analyzed via HPLC-UV at 225 nm using a 0-20% solvent gradient. Retention time for Dopamine: 1.5 min, Tyramine: 1.9 min, THI: 2.3 min, and isoTHI: 2.9 min. A: Enzymatic reaction after 2.5 h with the addition of propionaldehyde. B: Enzymatic reaction after 17 h with the addition of propionaldehyde. Peaks starred are a result of the crude enzyme extract.
Figure 5: Pictet-Spengler reaction between dopamine and propionaldehyde in 50 mM monobasic phosphate buffer pH 6.5 after 2.5 hrs. Analyzed via HPLC-UV at 225 nm using a 0-20% solvent gradient. Retention time for Dopamine: 1.5 min, THI: 2.3 min, and isoTHI: 2.9 min.

Impact of Tyrosinase on Tetrahydrossoquinoline isomer Formation

Using the method previously described, the ratio of THI to isoTHI formed in the presence and absence of tyrosinase was evaluated. The peak area ratios were evaluated once each of the reactions were complete. Based on this data, the tyrosinase does not appear to play a significant role in the regioselectivity of the Pictet-Spengler reaction occurring in the reaction solution. The low percentage of isoTHI observed in these enzymatic reactions is likely due to the concentration of phosphate as well as the more acidic pH, which is verified by the comparative reaction with no enzyme present.

Table 1: Relative Peak Area Percentages for THI and isoTHI formation in Absence and Presence of Tyrosinase

<table>
<thead>
<tr>
<th>Species</th>
<th>Peak Area Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosinase Present</td>
<td></td>
</tr>
<tr>
<td>Tyramine</td>
<td>4.2 %</td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.0 %</td>
</tr>
<tr>
<td>THI</td>
<td>90.4 %</td>
</tr>
<tr>
<td>isoTHI</td>
<td>5.4 %</td>
</tr>
<tr>
<td>No Tyrosinase</td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.0 %</td>
</tr>
<tr>
<td>THI</td>
<td>97.1 %</td>
</tr>
<tr>
<td>isoTHI</td>
<td>2.9 %</td>
</tr>
</tbody>
</table>
The ratio of peak areas were also evaluated in enzymatic reactions with varied phosphate concentration at a constant pH. In many of these reactions the enzyme catalyzed oxidation occurred very slowly and the reaction could not be driven to completion before the enzyme activity was completely lost. As a result this data was highly inconclusive. There was no observable trend in THI versus isoTHI formation in the reactions that did proceed to completion.

Summary

These initial results verified the use of the enzyme-mediated synthesis of norcoclaurine and a carboxylated norcoclaurine in phosphate buffer. The enzyme aggregate was suspended in phosphate buffer and the natural tyrosine substrate was added. This substrate was used to ensure that the aggregate maintained the same activity as the crude enzyme extract. Upon the formation of L-DOPA, 4-HPAA was added to the reaction mixture to determine if the Pictet-Spengler reaction could occur in the presence of the enzyme. This resulted in the formation of a tetrahydroisoquinoline (THI) and a potential isomer of this THI. Following this verification of the proposed enzyme-mediated synthesis, a repeat experiment was conducted with tyramine. This resulted in the formation of dopamine and upon the addition of 4-HPAA, norcoclaurine. These two experiments were used as controls to verify that the enzyme-mediated synthesis can occur with natural substrates. These experiments were also used to optimize the phosphate concentration and buffer pH and compare the activity of the crude enzyme extract and the cross-linked enzyme aggregate. Upon verification that the coupled enzyme oxidation and Pictet-Spengler reaction results in THI formation the synthetic technique was applied to halogenated substrates.

Chapter 3: Synthesis of Halogenated Substrates and Halogenated THI

Introduction

Using the techniques described in Chapter 2, we propose that selectively halogenated THI can be synthesized. The halogenated THI then has the potential to be used in precursor directed biosynthesis experiments. Selective halogenation of tyrosine and tyramine to use as substrates for oxidation by tyrosinase could yield either halogenated L-DOPA or halogenated dopamine. If these compounds are accepted as alternative substrates, we expect an unfavorable equilibrium. As in the prior chapter, this limitation will be overcome by the addition of the aldehyde to couple oxidation to the Pictet-Spengler reaction, thus producing THI. Once an equivalent of the aldehyde is added to drive the unfavorable oxidation reaction to completion, the enzyme can be filtered out and the THI can be isolated from the aqueous reaction solution. Halogenated norcoclaurine derivatives will eventually be supplied to *Berberis vulgaris* cell lines to explore their capacity to produce berberine derivatives.
Scheme 3: Proposed synthesis of Halogenated berberine from halogenated tyramine. (X: Cl, Br) Oxidation of Tyramine specifically halogenated in the 3 position by tyrosinase to form halogenated dopamine. The Pictet–Spengler reaction of halogenated dopamine and 4-hydroxy phenylacetaldehyde in the presence of 50mM monobasic sodium phosphate buffer pH 6.5 resulting in the formation of halogenated norcoclaurine. Followed by biosynthesis of halogenated berberine from halogenated norcoclaurine through the use of Berberis vulgaris cell lines.

Methods

Synthesis of Halogenated Tyrosine

Using the methods previously applied in the Maresh lab, tyrosine was selectively mono-halogenated.$^{10,28,29}$

**Synthesis of 2-amino-3 (3-chloro-4-hydroxyphenyl)propanoic acid (3-Chloro-tyrosine)**

Tyrosine (5 mmol) was dissolved in approximately 6 mL of glacial acetic acid in a round bottom flask. To this reaction solution 5.5 mmol of SO$_2$Cl$_2$ was added dropwise. The reaction proceeded for 5 hours before the precipitate was filtered under vacuum. The precipitate was washed with glacial acetic acid (4 x 25 mL) and dried under vacuum. The compound was characterized using proton NMR.

**Synthesis of 2-amino-3 (3-chloro-4-hydroxyphenyl)propanoic acid (3-Bromo-tyrosine)**

A solution containing (30 mmol) of tyrosine in 25 mL of glacial acetic acid and 15 mL of a 33% solution of HBr in acetic acid (60 mmol), was prepared. A solution of excess bromine (33 mmol) in 25 mL of glacial acetic acid was then added to the previously prepared solution over a period of 3 hours, and the resulting solution was allowed to stir for 24 hours. Upon completion of the reaction the solid precipitate was collected by vacuum filtration, washed with glacial acetic acid (3 x 25 mL) and diethyl ether (3 x 25 mL) yielding an orange powder. The compound was verified using proton NMR.
Synthesis of Halogenated Tyramine

Using the methods described by Bovonsombat et al. for para-selective halogenation of tyramine, several halogenated tyramine compounds were synthesized.\(^\text{27}\)

**Synthesis of 3-Chlorotyramine**

A solution of 12.5 mmol of tyramine in 300 mL of acetonitrile with 2 equivalents (25 mmol) of p-toluenesulfonic acid was created. The solution was stirred for 5 minutes before an equivalent (12.5 mmol) of n-chlorosuccinimide was added to the reaction mixture. The reaction was carried out at room temperature for 2 hours before the white precipitate was vacuum filtered and washed with acetonitrile (2 x 20 mL). The reaction was monitored via HPLC-UV and formation of monohalogenated tyramine initially detected by HPLC and further confirmed by proton NMR.

**Synthesis of 3-Bromotyramine**

A solution of 12.5 mmol of tyramine in 300 mL of acetonitrile with 2 equivalents (25 mmol) of p-toluene sulfonic acid was created. The solution was stirred for 5 minutes before an equivalent (12.5 mmol) of n-bromosuccinimide was added to the reaction mixture. The reaction was carried out at room temperature for 2 hours before the white precipitate was vacuum filtered and washed with acetonitrile (2 x 20 mL). The reaction was monitored via HPLC-UV and formation of monohalogenated tyramine initially detected by HPLC and further confirmed by proton NMR.

**Synthesis of 3-Iodotyramine**

A solution of 12.5 mmol of tyramine in 300 mL of acetonitrile with 2 equivalents (25 mmol) of p-toluene sulfonic acid. The solution was stirred for 5 minutes before an equivalent (12.5 mmol) of n-iodosuccinimide was added to the reaction mixture. The reaction was carried out at room temperature for 2 hours before the white precipitate was vacuum filtered and washed with acetonitrile (2x20 mL). The reaction was monitored via HPLC-UV revealing the formation of mono-iodinated and potentially di-iodinated tyramine.

**Purification of 3-Chlorotyramine**

The 3-chlorotyramine was adsorbed onto silica gel and loaded onto a column. The p-toluene sulfonic acid was separated from the chlorotyramine using dichloromethane and methanol with 4% triethylamine at a linear gradient of 0-30%. The first peak was p-toluene sulfonic acid and the second peak was chlorotyramine the fractions were collected and the solvent was evaporated under reduced pressure.

**Decarboxylation of Tyrosine**

Tyrosine (1 mmol) was dissolved in 50 mL of cyclohexanol with catalytic amounts of 2-cyclohexen-1-one. The reaction was refluxed at 160 °C and monitored via HPLC every 15 minutes over the course of an hour.
Decarboxylation of Chlorotyrosine

Chlorotyrosine synthesized using the previous methods (0.5 mmol) was dissolved in 25 mL of cyclohexanol with 2% v/v 2-cyclohexen-1-one. This reaction was refluxed at 150 °C for three and a half hours, until the reaction had gone to completion. The reaction was monitored via HPLC.

Selective Recrystallization of Cl-Tyramine

Several recrystallization strategies were attempted. The halogenated tyramine was recrystallized in tetrahydrofuran (THF) with no success. The solid formed through the recrystallization was washed in propanol and PET ether in an attempt to selectively remove the tosylate from the solid, this was also unsuccessful in removing the tosylate group. Attempts to protonate the tosylate group prior to recrystallization were also made with no success. Similarly, concentrated NaOH was added during recrystallization. This did not aid in selectively recrystallizing Cl-Tyramine.

The halogenated tyramine and tosylate salt was dissolved in chloroform and extracted with water and HCl. Tosic Acid and halogenated tyramine were present in both the organic and the aqueous layer.

Results and Discussion

Halogenation of tyrosine and tyramine

Relatively simple synthetic techniques for the halogenation of tyrosine and tyramine have already been developed.\textsuperscript{10,27-29} However, purification of halogenated tyramine from these synthetic techniques was less successful due to difficulties with selective recrystallization as well as chromatographically separating. The solubility of tosic acid and halogenated tyramine were too similar to allow for selective recrystallization of the halogenated tyramine. As a result chromatographic methods were attempted, and chlorotyramine was successfully separated from the tosic acid. However, the brominated tyramine exhibited polarity too similar to tosic acid to effectively separate using the same chromatographic methods used for chlorotyramine. Several different techniques were attempted to produce the mono-brominated tyramine with potentially easier purification. Forming a benzoate salt instead of tosylate salt would potentially improve the ease of purification due to the different solubility of benzoate and tosylate. Upon the addition of n-bromosuccidimide (NBS), the reaction turned black and no distinguishable peak was present on the HPLC chromatogram. Another method to potentially monohalogenate tyramine without the use of tosic acid was to slowly add the NBS to a cold reaction, this was done in hopes that the more favorable halogenation in the 5 position would occur and minimal dehalogenation would take place. This too was unsuccessful and tyramine was brominated in both the 5 and 2 positions. Chlorinated tyramine was synthesized and purified successfully. Brominated tyramine was never successfully purified after being synthesized. Iodination of tyramine was also attempted using the same procedure as chlorination and bromination, however the reaction yielded mono-iodinated tyramine and a side product, potentially di-
iodinated tyramine, which were not easily separated. The synthetic strategy used to form the halogenated tyramine results in a salt of tosic acid and the halogenated tyramine. As a result of these complications with separating halogenated tyramines from tosic acid, a different approach to synthesis of halogenated tyramine was needed. For this reason the decarboxylation of halogenated tyrosine to form halogenated tyramine was attempted.

**Enzymatic Synthesis of Halogenated Dopamine and L-DOPA**

To evaluate if tyrosinase can accept halogenated substrates, the halogenated tyrosine was added to the enzyme extract solution or a solution of CLEAs in sodium phosphate buffer. Both chlorotyrosine and bromotyrosine were used in the reaction mixture and both successfully produced halogenated L-DOPA.

Since the most effective procedure for the synthesis of halogenated tyramine produced a salt mixture of halogenated tyramine and p-toluenesulfonic acid, this salt was added to the enzyme solution to evaluate whether or not purification of the halogenated tyramine was necessary. The enzymatic reaction proceeded significantly slower than under the equivalent conditions with either un-halogenated tyramine or the purified chlorinated tyramine. The p-toluenesulfonic acid apparently inhibits tyrosinase reaction. The inhibition mechanism was not specifically determined, but likely competes with substrate binding. The HPLC-UV chromatograms were extracted at 280 nm. Although typically the sensitivity of the instrument is greater at 225 nm, making this an ideal wavelength for measuring species of low concentration, the halogenated dopamine does not absorb significantly at 225 nm and thus the peak intensity was significantly reduced. The enzyme-mediated oxidation of Br-Tyramine was less successful than the oxidation of Cl-Tyramine. The Br-Tyramine reaction proceeded significantly slower than that of the Cl-Tyramine resulting in approximately 3% conversion of Br-Tyramine to Br-Dopamine over the course of 45 h. In comparison, the reaction with Cl-Tyramine resulted in approximately 10% conversion to Cl-Dopamine over the course of 45 h.
Figure 6: HPLC chromatogram of the formation of 5-Cl-Dopamine from 3-Cl-Tyramine by tyrosinase CLEA from white-button mushrooms in a 50 mM Phosphate buffer solution after 45 h. Chromatogram extracted at 280 nm.

Figure 7: HPLC chromatogram of the formation of 5-Br-Dopamine from 3-Br-Tyramine by tyrosinase CLEA from white-button mushrooms in a 50 mM Phosphate buffer solution after 45 h. Chromatogram extracted at 280 nm.

The chlorinated tyramine, purified using column chromatography, was added to the pH 6.5 buffer containing either the crude enzyme extract or the CLEA in the presence of l-
ascorbate and successfully yielded chlorinated dopamine. All of these methods did successfully yield the desired catechol indicating that the presence of a halogen does not prevent substrate binding in tyrosinase. This result indicates that the use of tyrosinase to synthesize halogenated dopamine is plausible. Despite the unfavorable equilibrium, this method can be used as the first step in the eventual synthesis of halogenated tetrahydroisoquinolines.

**Enzyme-mediated Synthesis of halogenated Tetrahydroisoquinolines**

Adding an aldehyde to the reaction mixture containing the halogenated dopamine or L-DOPA would result in the Pictet-Spengler Condensation and formation of halogenated THI as well as isoTHI. Using Le Chatelier’s principle, the reaction of the aldehyde with the product drives the enzyme-mediated oxidation forward by removing the halogenated catechol product. This reaction is also driven forward by continuously increasing the phenolic substrate concentration. Through the combination of these effects, halogenated THI and isoTHI can be produced on a large enough scale to eventually be used for precursor directed biosynthesis. (Appendix B)

The Pictet-Spengler reaction of the halogenated catechol and an aldehyde progresses at a slower rate than the reaction with the unhalogenated substrate. There are a variety of reasons that this could be the case. Most likely the reaction was slowed due to the presence of the halogen at the reaction site. This potentially creates some steric hindrance in the cyclization reaction. The formation of brominated THI was not observed in the reaction solution as it was currently designed. As bromine is larger than chlorine it likely creates an enhanced effect on the slowed rate of reaction due to steric interactions. Furthermore MM2 calculations showed some energetic favorability for the isoTHI over the THI with a halogen present. This could be a further reason for the slowed reaction rate. As shown in the kinetic evaluation of the Pictet-Spengler mechanism, the rate of formation of the isoTHI is slower than that of the THI. These hypotheses require further investigation. Computational comparisons of the reaction coordinates with and without the halogen may provide insight into the favored isomer. Similarly extraction and characterization of the THI product could provide more insight.

**Conclusions and Future Work**

The techniques described in this chapter provide a foundation for future experiments in generating derivatives of tyramine, dopamine, tyrosine, L-DOPA, norcoclaurine, berberine, and a series of other THIs depending on the aldehyde added to the solution. From these derivatives, a library of potential biologically active compounds can be created. This relatively facile one-pot synthesis of THIs is a new potential method for the synthesis of more complex natural products and their derivatives.

This work is still in the early stages of development and could be greatly expanded upon. The current data provides proof that tyrosinase extracted from white-button mushrooms, avocados, or sweet potatoes can be used to synthesize halogenated catechols from halogenated hydroxyphenethylamines and the addition of an aldehyde to the reaction solution.
yields halogenated THIs. However, the products of these reactions have not yet been used in feeding experiments to yield halogenated berberine, nor have any of these compounds been evaluated for biological activity. Similarly, the production of halogenated norcoclaurine specifically has not been highly successful due to complications in synthesizing 4-hydroxyphenylacetaldehyde. Were this aldehyde produced on a larger scale, the production of norcoclaurine would not pose significant challenges. The addition of 4-HPAA would need to be incremental to prevent substrate degradation, though the enzymatic reaction can be driven forward through this incremental addition if the initial phenolic substrate was added at the same time.

The Pictet-Spengler reaction of the halogenated catechol and an aldehyde progresses at a slower rate than the reaction with the unhalogenated substrate. There are a variety of reasons that this could be the case. Most likely the reaction is slowed due to the presence of the halogen at the reaction site. The halogen potentially creates steric hindrance preventing the Pictet-Spengler cyclization. The formation of brominated THI was not observed in the reaction solution as it was currently designed. As bromine is larger than chlorine it likely creates an enhanced effect on the slowed rate of reaction due to steric interactions. The current understanding of catalytic mechanism of phosphate in the Pictet-Spengler reaction places the phosphate in the same region of space as the halogen, as such the catalysis of the Pictet-Spengler may be disrupted with the halogenated substrates. Furthermore MM2 calculations showed some energetic favorability for the isoTHI over the THI with a halogen present. This could be a further reason for the slowed reaction rate. As shown in the kinetic evaluation in Chepater 4 and 5 of the Pictet-Spengler mechanism, the rate of formation of the isoTHI is slower than that of the THI. These hypotheses require further investigation. Computational comparisons of the reaction coordinates with and without the halogen may provide insight into the favored isomer. Similarly isolation and characterization of the THI product could provide more insight.

In the subsequent chapters the role of phosphate in the Pictet-Spengler mechanism will be evaluated. By improving understanding of the catalytic mechanism of phosphate in this reaction, as well as the regioselective effect of the catalyst, optimizations of synthetic methods like those described in Chapter 2 and 3 can be performed.

Chapter 4: Kinetic analysis of the Pictet-Spengler in Phosphate

Introduction

Tetrahydroisoquinoines are primarily synthesized through the Pictet-Spengler reaction, including many alkaloids with potential medicinal uses.\textsuperscript{11,30} The Pictet-Spengler mechanism is a multistep reaction scheme. The first step involves the condensation reaction between an aromatic ethylamine and an aldehyde which results in the formation of an iminium cation. This then results in the formation of a heterocyclic ring through a two-step electrophilic aromatic substitution. With indoles this reaction occurs under mild conditions, however
phenethylamines typically require harsher reaction conditions such as strong acids or high temperatures.\(^{31}\) 3-hydroxy-phenethylamine can react under mild, aqueous conditions due to the electron donating group in the meta position to the ethylamine. Pesnot et al. suggested that other electron donating groups in this position could also facilitate cyclization.\(^{11}\)

![Scheme 4: Pictet-Spengler mechanism with proposed mechanism for regio-isomer formation through shared iminium intermediate](image)

Pesnot et al. also suggested that phosphate may play a specific role in catalysis. Two potential reaction pathways were proposed, one involving nucleophilic attack of the phosphate on the iminium ion to form a reactive aminophosphate species that is proposed to be more electrophilic than the iminium itself.\(^{11}\) In an alternative proposal, phosphate acts as a base, activating the ring for the addition at the para position. After cyclization, phosphate also acts as a general base, abstracting the ring proton to reestablish aromaticity. These various proposals for the specific role of phosphate catalysis have been evaluated computationally. Computational models found a lowest energy pathway. This pathway involves the coordination of $\text{HPO}_4^{2-}$ with the meta position hydroxyl group and the coordination of $\text{H}_2\text{PO}_4^-$ with the iminium at the site of cyclization.\(^{30}\)

Beyond catalysis, phosphate has been observed to influence regioselectivity in the Pictet-Spengler reaction. Previous studies revealed there is a dependence of the ratio of THI to isoTHI on the concentration of phosphate present in the reaction.
Given that buffer catalysis plays an important role in the Pictet-Spengler mechanism, studying the way that each species influences the rate may provide evidence to support or refute the computational mechanistic proposal and/or the proposals of Pesnot.

The contributors to the observed rate constant are shown below. By observing the effect changing each contributor has on overall rate, the role of the buffer in catalysis can be better explained.

\[ k_{\text{obs}} = k_1 [H^+] + k_2 [OH^-] + k_a [BH^+] + k_b [B] + k_3 \]  \hspace{1cm} \text{Eq. 1} \]

The rate constants correspond to each potential type of catalysis; \( k_1 \) and \( k_2 \) are the rate constants associated with the proton and hydroxide concentration respectively, \( k_a \) and \( k_b \) are the general acid and base rate constants respectively, and \( k_3 \) is the rate constant for the uncatalyzed reaction. The general acid or base catalysis can be determined by comparing the rates of reactions conducted in a buffer with varied pH but with species of constant pKa and constant phosphate concentration. Varying buffer concentration with constant pKa and constant pH can provide information on the specific catalysis by the buffer species. If the reaction is acid/ base specifically catalyzed there will be no dependence of the rate on the concentration of buffer. Whereas dependence of the rate on the concentration of buffer is indicative of general acid/ base catalysis. The Pictet-Spengler mechanism as shown is both acid and base-catalyzed. Due to the observed dependence of the rate constant on the concentration of the buffer, general acid/ base catalysis is likely the primary contributor to the rate-limiting step.

By measuring the \( k_{\text{obs}} \) as a pseudo-first order rate constant at constant pH and ionic strength while varying the buffer concentration, the dependence of the reaction on the buffer species can be determined. An expression for the rate constant at constant pH and varied buffer concentration is given by equation 5.

\[ k_0 = k_1 [H^+] + k_2 [OH^-] + k_3 \]  \hspace{1cm} \text{Eq. 2} \]

The relationship between the acidic/basic buffer species and the \([H^+]\) and total buffer concentration \( B_t \) can be used to further elucidate the catalytic role of the buffer in the mechanism. The expression for the fraction of acid or base in the buffer can be derived from the equilibrium expression and given in terms of the acid dissociation constant and the concentration of \( H^+ \).

\[ F_b = \frac{[B]}{[B_t]} = \frac{K_a}{K_a + [H^+]} \]  \hspace{1cm} \text{Eq. 3a} \\
\[ F_a = \frac{[BH^+]}{[B_t]} = \frac{[H^+]}{K_a + [H^+]} \]  \hspace{1cm} \text{Eq. 3b}
The relationships shown in equations 6a and 6b depicting the expression for the fraction of basic or acidic buffer species in solution can be substituted into equation 4 to describe the observed rate constant exclusively in terms of the buffer concentration.

\[ k_{\text{obs}} = k_o + (k_b F_b + k_a F_a) B_t \quad \text{Eq. 4} \]

This provides a linear expression for the observed rate constant in terms of the total buffer concentration. Due to \( F_b + F_a = 1 \) the expression can be written with the dependence on only the total buffer concentration and the concentration of base.

\[ k_{\text{obs}} = k_o + (k_a + (k_b - k_a) F_b) B_t \quad \text{Eq. 5} \]

Equation 8 gives a linear relationship between the observed rate constant and the total concentration of base, the \( y \)-intercept is \( k_0 \) at constant pH. Rearranging this equation gives a linear expression that can be used to determine the \( k_a \) and \( k_b \) at constant pH.

\[ \frac{k_{\text{obs}} - k_o}{B_t} = k_a + (k_b - k_a) F_b \quad \text{Eq. 6} \]

Methods

Preparation of Phosphate Buffers and Standard Solutions

Twelve phosphate buffers were created for the kinetic analysis of the role of phosphate in the Pictet-Spengler reaction. Initially a 0.200 M stock solution of monobasic sodium phosphate was created. From this stock solution the twelve reaction buffers were created: three different concentrations with varied pH. All reaction buffers were created to have a constant ionic strength of 0.122 using NaCl. The pH of each buffer was adjusted to consistent final values using concentrated base. As a result there were four 20 mM phosphate buffers with pH 6.31, 6.59, 7.00, and 7.29; four 40 mM phosphate buffers with pH 6.29, 6.60, 7.00, and 7.30; and four 80 mM phosphate buffers with pH 6.29, 6.59, 6.99, and 7.29.

Standard solutions of 3-(2-aminoethyl)phenol (m-tyramine, called such for the hydroxyl group in the meta position) and propionaldehyde (propanal) were prepared. A 99.98 mM solution of m-tyramine was created by gravimetrically weighing 0.1736 g of m-tyramine and dissolving it in 10 mL of Milli-Q water. Prior to preparing the standard solution, propanal was purified to remove propionic acid contaminant as a product of the reaction of propanal and molecular oxygen. Sodium bicarbonate was added to propanal and the solution was shaken vigorously using a shaker for 30 minutes. Calcium chloride was then added to dry the propanal, followed by distillation under N\(_2\). The solution of propanal was then prepared by gravimetrically weighing 5.808 g of purified propanal into a 50 mL volumetric flask.

Determination of m-Tyramine area extinction coefficient

To obtain meaningful data from the reaction kinetics experiment, the concentration of each species in the reaction must be determined from the HPLC-UV chromatograms. At a
specific wavelength the area under the peak can be converted to a concentration using a known extinction coefficient ($\alpha$).

$$C_x = \frac{\alpha_x \times Area\lambda}{b}$$  \hspace{1cm} Eq. 7

Using a standard curve with known concentrations of m-tyramine the extinction coefficient for m-tyramine was determined. Twelve serial dilutions of a 0.5 mM m-tyramine solution were created and analyzed using HPLC-UV. Using this analytic technique the standard curve for m-tyramine was determined. The extinction coefficient for 280 nm was determined to be $1.077 \pm 0.002 \times 10^{-6}$ mM·µV⁻¹·s⁻¹.

![Figure 8: The standard calibration curve for m-tyramine to determine the extinction coefficient for m-tyramine at 280 nm. The equation of the line is given by $[m\text{-Tyramine}]=1.075 \pm 0.004 \times 10^{-6}$ mM·µV (Peak Area) with $R^2=1.0$](image)

**Determination of THI and isoTHI area Extinction coefficients**

A standard curve for m-tyramine was created using standard procedures. However, neither the THI nor the side product assumed to be an isomer of the tetrahydroisoquinoline have been successfully isolated from this reaction. As such a different method was used to determine the extinction coefficients for both the THI and iso-THI. This method utilized the varying ratio of iso-THI to THI with varied phosphate concentration. Three controls of 0.5 mM m-tyramine in milli-Q water were created to account for any fluctuations in concentration due to pipetting error. These controls did not include propionaldehyde and were used to determine the starting concentration of m-tyramine. Five reaction solutions were created with 0.5 mM m-tyramine.
100 mM propionaldehyde, and five different concentrations of phosphate buffer at a pH of 7.0. The higher pH was chosen because the reaction appears to be base-catalyzed and a higher pH results in a faster reaction and, as the rate of this reaction is not of great importance, a faster reaction was desirable. The phosphate concentrations used were 400 mM, 100 mM, 50 mM, 25 mM, and 5 mM. These five reaction solutions were allowed to go to completion before analyzing via HPLC. For each concentration of phosphate buffer 9 HPLC injections were conducted. Lower concentrations of phosphate resulted in increased production of iso-THI. Using these ratios and the known initial concentration of m-tyramine, as well as the known extinction coefficient for m-tyramine a non-linear curve fit can be used in IGOR to simultaneously find the extinction coefficient for both THI and iso-THI. The equation used assumes the sum of the concentration of the starting material and each product at any given point in the reaction is equal to the initial concentration of the starting material. Using these methods, the extinction coefficients for all species in the reaction were determined.

\[
[m\text{Tyramine}]_{\text{start}} = \alpha_{m\text{Tyr}}Area_{m\text{tyr}} + \alpha_{THI}Area_{m\text{tyr}} + \alpha_{iso}Area_{m\text{tyr}} \quad \text{Eq. 8}
\]

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<th>Species</th>
<th>Extinction Coefficients (280 nm)</th>
<th>Extinction Coefficients (250 nm)</th>
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<td>m-Tyramine</td>
<td>1.077 ± 0.002 x10^{-6} mM·µV^{-1}·s</td>
<td>4.769 ± 0.001 x10^{-7} mM·µV^{-1}·s</td>
</tr>
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<td>THI</td>
<td>9.30 ± 0.02 x10^{-7} mM·µV^{-1}·s</td>
<td>2.27 ± 0.01 x10^{-7} mM·µV^{-1}·s</td>
</tr>
<tr>
<td>isoTHI</td>
<td>9.4 ± 0.2 x10^{-7} mM·µV^{-1}·s</td>
<td>3.7 ± 0.3 x10^{-7} mM·µV^{-1}·s</td>
</tr>
</tbody>
</table>

**Kinetic Analysis via HPLC**

All pipettes were calibrated before each experiment to ensure accuracy and precision in the species concentrations. These calibrations were conducted using Milli-Q water at room temperature and accounting for the density of water at room temperature. The kinetic measurements were obtained via HPLC-UV. The autosampler of the HPLC was set to 23 °C and an injection volume of 5 µL was used for each sample. In a 2 mL glass HPLC sample vial, 1029.5 µL of a given phosphate buffer and 5.5 µL of m-tyramine was then added to the reaction. Then 65 µL of propionaldehyde was added and the vial was capped, shaken, and placed in the autosampler for injection. The reaction start time was recorded using the clock on the instrument computer. All time points were recorded by the instrument software using this clock. As such this allowed for an accurate start time and accounts for the approximately 60 s delay between the reactions start time and the injection. These reactions were carried out under pseudo-first order kinetics due to the excess aldehyde. Through the addition of extreme excess aldehyde the rate became dependent on the concentration of the m-tyramine rather than both the tyramine and the propionaldehyde. The rate of the second order reaction is given below. Due to the reaction being initially treated as a pseudo-first order reaction with respect
to the m-Tyramine concentration, the rate constants include the concentration of aldehyde, which can be factored out.

\[
Rate = k_{obs}[m-Tyramine][Propionaldehyde]
\]  \hspace{1cm} Eq. 9

The rate constants were obtained using COPASI. In COPASI the kinetic data was processed using parameter estimation method with Hooke & Jeeves analysis over 100 iterations and a tolerance of 1x10^{-9}. Hooke and Jeeves analysis was used to obtain the best possible convergence of the calculated curve to the experimental data with the lowest tolerance. Due to the excess propionaldehyde the reaction was treated as irreversible first order forming two products. Using COPASI to process the data yielded two rate constants for each of the competing reactions.

\[
mTyramine \xrightarrow{k_1} THI
\]
\[
mTyramine \xrightarrow{k_2} isoTHI
\]  \hspace{1cm} Eq. 10

**Preparation of [2,4,5,6-^2H]- m-Tyramine**

The deuterated m-tyramine was synthesized using methods described by Vining et al.\(^{35}\) 3-(2-aminoethyl)phenol hydrochloride (65 mg) was added to a sealed 48 mL high-pressure reaction tube (Ace Glass 8648 Pressure Tube with PFTE-glass plunger valve) with 6.0 mL of 99.99% D\(_2\)O (Isotech Labs). The tube was sealed and the liquid was frozen in liquid nitrogen. The tube was attached to a freeze dryer (Labconco FreeZone 1) and evacuated for 30 minutes at 0.050 Torr. The tube was sealed and placed in a 210 °C sand bath for 24 hours. The excess solvent was removed in vacuo on a Büchi RotoVapor in a 40 °C water bath. This procedure was repeated 3 times using 1.0 mL of 99.99% D\(_2\)O per 10 mg of 3-(2-aminoethyl)phenol. The resulting white crystalline solid had \(^1\)H-NMR spectra identical to natural 3-(2-aminoethyl)phenol, but with no ortho or para aromatic protons.

![Figure 9: Deuterated m-tyramine with aromatic protons replaced with \(^2\)H](image)

**Results and Discussion**

The Pictet-Spenger reaction between 3-hydroxyphenethylamines and aldehydes can occur in an aqueous environment due to the electron donating hydroxyl group in the meta position. This mechanism in mild, aqueous environment is mediated by both acidic and basic species at various steps in the reaction mechanism, making the use of a buffer as a catalyst ideal. Previous studies indicated that the reaction is primarily base-catalyzed, furthermore, these studies indicated that phosphate played a role in increasing the rate of the reaction.
Evaluating the catalytic role of phosphate in this mechanism can provide vital information on controlling the rate of reaction.

**Phosphate Specific Catalysis**

The potential role of phosphate specific catalysis was evaluated by varying the concentration of the phosphate in the reaction solutions while keeping the pH and the ionic strength constant. As is evident in Figure 9 below, there is a relationship between the observed rate constant with respect to the phosphate concentration, as well as a relationship between pH and the observed rate constant. Increasing phosphate concentration results in an increased rate of reaction. Similarly, increasing the pH of the reaction buffer increases the overall rate of the reaction. The effect of change in pH will be investigated in further detail in subsequent sections. However, from this initial plot, an interesting observation can be made. With the increase in pH, there appears to be an increase in slope of the line. This could be indicative of an important catalytic role for HPO$_4^{2-}$ in the reaction mechanism. This observation agrees with previous studies, both experimental and computational. The $y$-intercept of each of these lines gives the $k_0$ for each constant pH, as shown in equation 4.

![Figure 10](image-url)

*Figure 10: The observed rate constant plotted with respect to phosphate concentration. Each series corresponds to a specific pH: pH 7.3 (◊) the equation of the line $k_{obs} = 1.465 \pm 0.005 \times 10^{-7}$ mM$^{-2}$s$^{-1}$ [Phosphate] + 7.1 \pm 0.2 \times 10^{-7}$ with $R^2 = 0.99997$, pH 7.0 (Δ) the equation of the line $k_{obs} = 9.4 \pm 0.1 \times 10^{-8}$ mM$^{-2}$s$^{-1}$ [Phosphate] + 1.17 \pm 0.06 \times 10^{-6}$ with $R^2 = 0.9995$, pH 6.6 (○) equation of the line $k_{obs} = 5.2 \pm 0.3 \times 10^{-8}$ mM$^{-2}$s$^{-1}$ [Phosphate] + 9 \pm 1 \times 10^{-7}$ with $R^2 = 0.99463$, pH 6.3 (o) equation of the line $k_{obs} = 3.7 \pm 0.4 \times 10^{-8}$ mM$^{-2}$s$^{-1}$ [Phosphate] + 4 \pm 1 \times 10^{-7}$ with $R^2 = 0.97965$.*

The overall concentration of phosphate appears to play a role in the catalysis of the Pictet-Spengler reaction. There also appears to be a relationship between the concentration of
phosphate and the formation of isoTHI versus THI. An interesting observation could be made from the plot of the ratio of THI to isoTHI and total phosphate concentration. As the concentration increased there appeared to be an increase in THI formed versus the isoTHI. However, the ratios relative to each phosphate concentration did not converge to a single point. Rather, within each concentration of phosphate the ratio of THI to isoTHI increased with respect to decreasing pH. This indicated that there may be a potential regioselective role that $H_2PO_4^-$ plays in the Pictet-Spengler mechanism. The relationship between the acidic phosphate species, monobasic phosphate ($H_2PO_4^-$) or the basic phosphate species, dibasic phosphate ($HPO_4^{2-}$) and the ratio of the isomers being formed in the reaction was investigated.

![Figure 11: A plot of the ratio of THI to isoTHI versus the concentration of phosphate. A positive correlation exists with increasing phosphate concentration and increased THI. pH 6.3 (black), pH 6.6 (red), pH 7.0 (green), pH 7.3 (blue)](image)

Evaluating the ratio of THI to isoTHI formation versus the acidic phosphate ($H_2PO_4^-$), a trend can be observed. As the concentration of the acidic phosphate species is increased, the ratio of THI to isoTHI increases. However, when the same comparison is made with the basic phosphate species ($HPO_4^{2-}$) a trend cannot be found. This would indicate that diprotic phosphate may play a specific role in controlling the regioselectivity of the reaction. When the same evaluation was conducted using the ratio of the observed rate constants for the formation of THI or isoTHI, the same trend appeared. This indicates that the regiocontrol is a kinetic effect. This also may indicate that there is a shift in the rate-determining step at a certain concentration of the acidic phosphate species.
Figure 12: Plot of the ratio of THI concentration to isoTHI concentration versus the concentration of the acidic phosphate species.

Figure 13: Plot of the ratio of the observed rate constant for m-Tyramine → THI to m-Tyramine → isoTHI (k_{THI}/k_{iso}) versus the concentration of the acidic phosphate species.
The observed relationship between the regioselectivity and the concentration of $\text{H}_2\text{PO}_4^-$ is complimented by the computational data obtained by Parra et al.\textsuperscript{30} In this computational study, both $\text{H}_2\text{PO}_4^-$ and $\text{HPO}_4^{2-}$ were necessary for the catalysis of the Pictet-Spengler reaction. The lowest energy pathway involved the basic phosphate species coordinated with the hydroxyl group in the meta position on the aromatic ring, and the acidic phosphate species coordinated with the iminium.

![Proposed phosphate coordination in the Pictet-Spengler mechanism in the lowest energy pathway based on computational models. Dotted lines represent non-covalent interactions.](image)

Based upon the experimental data, there appears to be specific phosphate catalysis. Not only does phosphate appear to play a role in catalysis, but also appears to play an important role dictating the isomer being formed in the reaction.

**General Acid/Base Catalysis**

To determine if the reaction exhibits general acid or base catalysis and find the $k_a$ and $k_b$ for the Pictet-Spengler reaction several mathematical analyses were conducted. Plotting the observed rate constant versus the concentration of the conjugate base shows a positive trend of rate constant increase with increased concentration of the conjugate base ($\text{HPO}_4^{2-}$). This would indicate that general base catalysis is involved in the reaction mechanism.
Figure 15: Plot of observed rate constant with respect to the concentration of conjugate base. The equation of the line is 
\[ k_{\text{obs}} = 1.8 \pm 0.1 \times 10^{-7} \text{mM}^{-2} \text{s}^{-1} [\text{HPO}_4^{2-}] + 4 \pm 2 \times 10^{-7} \text{ with } R^2 = 0.95713. \]

The linear dependence of the observed rate constant on the concentration of base indicates that the rate-limiting catalytic species is \( \text{HPO}_4^{2-} \) and there does not appear to be dependence on the acidic phosphate species. As such, the equation of the line in the above figure could be represented as follows. Where \( k' \) is the y intercept of the above plot and \( k_b \) is the slope

\[
k_{\text{obs}} = k' + k_b[B] \quad \text{Eq. 11a}
\]

\[
k' = k_1[H^+] + k_2[OH^-] + k_a[BH^+] + k_3 \quad \text{Eq. 11b}
\]

A similar analysis can be conducted for the acidic phosphate species to determine if general acid catalysis occurs. There appears to be no trend in \( \text{H}_2\text{PO}_4^- \) concentration and the observed rate constant. However, an observation can be made that as the pH increases, there is an increase in the observed rate constant. This can be seen with the 3 bands with a negative slope on the plot of the observed rate constant and \( \text{H}_2\text{PO}_4^- \) concentration. Each of these bands correlates to a total phosphate concentration and each point within the band correlates to a specific pH of that phosphate concentration. The higher \( k_{\text{obs}} \) values are observed with the higher pH (lower acidic phosphate species concentration and higher basic phosphate species concentration).
Figure 16: Plot of observed rate constant versus the concentration of the acidic phosphate species. 20mM phosphate buffer (□) pH 7.3, 7.0, 6.6, 6.3. 40mM phosphate buffer (X) pH 7.3, 7.0, 6.6, 6.3. 80mM phosphate buffer (○) pH 7.3, 7.0, 6.6, 6.3.

The relationship observed between the $k_{obs}$ and the concentration of $H_2PO_4^-$ indicates some dependence of the $k_a$ on the basic species. Using equation 4 and assuming that $k_2$ has no dependence on the buffer concentration, an expression can be derived exhibiting the dependence of $k_a$ on $[B]$ by representing $[BH^+]$ and the difference between the total buffer concentration and the conjugate base concentration. By substituting equation 12a into equation 4, equation 12b can be obtained. This is an expression in which the dependence of the general acid rate constant on the concentration of base is adequately represented.

\[
[BH^+] = [B_t] - [B] \quad \text{Eq. 12a}
\]
\[
k_0 = k_2 + k_a([B_t] - [B]) + k_b[B] \quad \text{Eq. 12b}
\]

From these initial analyses, a general base-catalyzed mechanism can infered with minimal influence on rate based upon the concentration of acidic phosphate. The acidic species may still play a role in the mechanism, but based upon the present information, does not appear to be involved in the rate controlling step. A more comprehensive view of the kinetic influences of phosphate on the mechanism can continue to be evaluated through the determination of the general acid and base rate constants. The equation of the line given in the figure below is equation 9. From this plot the $k_a$ and $k_b$ can be determined. The y-intercept gives the $k_a$ and the slope can be used to determine the $k_b$. Using these expressions, $k_b$ was found to be $1.9 \pm 0.2 \times 10^{-7}$ mM$^{-1}$s$^{-1}$ and $k_a$ was found to be $2.7 \pm 0.9 \times 10^{-8}$ mM$^{-1}$s$^{-1}$. There is an order of
magnitude difference between the $k_a$ and $k_b$ indicating that the catalysis from the basic phosphate species contributes more to the observed rate constant.

\[
Slope = k_b - k_a \quad \text{Eq. 13a}
\]

\[
k_b = Slope + k_a \quad \text{Eq. 13b}
\]

If specific acid or base catalysis was the primary catalytic mechanism, the rate of the reaction would not be dependent on the concentration of the buffer. As this is not the case, the reaction appears to be primarily general acid or base-catalyzed.

**Kinetic Isotope Effect**

To further provide information on the rate-limiting step a series of parallel reactions with deuterated m-tyramine were carried out. Through isotopic labeling a kinetic isotope effect (KIE) can be observed. The rate of reaction observed for the deuterated m-tyramine reactions were significantly slower than that of the natural m-tyramine. The observation of a primary KIE indicates that the rate-limiting or a preceding step involves the breaking of the hydrogen bond.
The rate constants for the conversion of m-tyramine to THI and m-tyramine to isoTHI were plotted with respect to the concentration of the basic phosphate species. These plots were compared for the natural m-tyramine and the deuterated m-tyramine to evaluate the KIE. From these plots, the KIE for the conversion of m-tyramine to THI was determined to be 3.6 based on the concentration of HPO$_4^{2-}$. This is indicative of a primary kinetic isotope effect, meaning the rate-limiting step involves the breaking of a C-H bond. By visual inspection, the effect of the isotopic label is apparent through the reduction in the slope.

The calculated $k_H/k_D$ for the conversion of m-tyramine to isoTHI is 0.9, which is indicative of a secondary KIE. However, due to the scatter in the data, this may not be the actual value. There are several factors that could result in the decreased KIE for the formation of the isomer. One possibility is the formation of the isomer is not dependent on the concentration of the base. This possibility was discounted by performing with same analysis with the total phosphate concentration, acidic phosphate concentration. Both of these plots showed no correlation between the observed rate constant ($k_2$) and the phosphate species concentration. To verify that there is a dependence of the $k_2$ on the concentration of the conjugate base a plot of $k_2$ versus the fraction of base was created. This plot showed that there was a positive correlation between the increased fraction of base and the increased $k_2$ value. The correlation appears to fall off at the higher fractions of base and appears to have some dependence on total phosphate concentration at the larger fraction of base. This is most evident at the highest

![Figure 16](image-url)

**Figure 16:** A: The observed rate constant for m-tyramine $\rightarrow$ THI ($k_1$) versus the concentration of HPO$_4^{2-}$ for the deuterated m-tyramine (○) and natural m-tyramine (△). The equation of the line for the natural m-tyramine is $y = 1.9 \pm 0.1 \times 10^{-7}$ mM$^{-2}$s$^{-1} X + 3 \pm 3 \times 10^{-7}$ with $R^2 = 0.96584$. The equation of the line for the deuterated m-tyramine is $5.1 \pm 0.2 \times 10^{-8}$ mM$^{-2}$s$^{-1} X + 3 \pm 5 \times 10^{-8}$ with $R^2 = 0.98547$. B: The plot of $k_{obs}$-$k_0$/$[B]_t$ versus the fraction of base. The equation of the line for natural m-Tyramine is $k_{obs}$-$k_0$/$[B]_t$ = $2.1 \pm 0.2 \times 10^{-7}$ mM$^{-2}$s$^{-1}$ [B]/[B]$_t$ - $2.5 \pm 0.8 \times 10^{-8}$ mM$^{-1}$ s$^{-1}$ with $R^2 = 0.94492$. The equation of the line for the deuterated m-Tyramine is $k_{obs}$-$k_0$/$[B]_t$ = $5.5 \pm 0.5 \times 10^{-8}$ mM$^{-2}$s$^{-1}$ [B]/[B]$_t$ - $5 \pm 3 \times 10^{-8}$ mM$^{-1}$ s$^{-1}$ with $R^2 = 0.91522$. The same evaluation was conducted for the rate of isoTHI formation. There was significant scatter in this plot. While a decreased rate was observed for the deuterated reaction the KIE was not as strong as observed for the overall reaction nor the rate for THI formation.
fraction of base, which corresponds to the highest pH. Another possibility is that the formation of the isomer occurs through a different mechanism with a different rate-limiting step than THI.

All of the HPLC chromatograms were extracted at two wavelengths, 225nm and 280nm. There were several reasons for evaluating the data at two different absorption wavelengths. The chromatograms obtained at 280nm have a reduction of noise compared to the 225nm chromatograms. This is due to the increased sensitivity at 225nm. Increased sensitivity could potentially yield better results for the lower concentrations of each species. The resultant rate constants from both wavelengths were consistent from trial to trial. The rate constants from both wavelengths were used to generate all of the data presented in these chapters to best account for the increased sensitivity at 225 nm and the decreased noise in 280 nm.

Computational Analysis of isoTHI formation

To develop more comprehensive understanding of the formation of the THI regioisomer (isoTHI) computational investigations are currently being conducted. The formation of the target regioisomer (THI) was previously investigated by Parra et al. From these computational studies the diprotic phosphate species coordinating with the iminium and facilitating the cyclization reaction provided the lowest energy structure. Using the same investigative techniques, an analysis of the potential reaction coordinate pathway of isoTHI formation is being conducted. The role of basic and acidic phosphate species on stabilizing the transition state structure is also being evaluated. The transition state geometries have been determined, and energetic evaluations of the transition states provides information of the role of each of these species in the isoTHI formation.
Scheme 5: The reactants and products in the reaction schemes that were evaluated computationally.

The scheme above represents the initial proposal for the isoTHI formation based on previous computational studies. However, upon initial calculations this scheme was discovered to be insufficient in describing the reaction mechanism. The iminium, pre-reaction, species in the presence of a base immediately forms a cyclized species. The optimized structure of the iminium species in the presence of a base cannot be determined due to the favorability of forming a non-planar, non-aromatic heterocyclic species. As such, step one in the scheme below is not being evaluated in this computational study. The energetic favorability of the cyclization in the presence of base indicates that this is not likely the step in the reaction scheme with the greatest energy barrier. Although this is a qualitative observation, it does agree with the kinetic data. The primary kinetic isotope effect indicated that the rearomatization step was the rate-limiting step rather than the cyclization step. These initial geometry optimizations and calculations led to the further investigation of the second step in the reaction scheme below. This is the rearomatization step, predicted to be an intermolecular reaction in the presence of base. However, initial calculations show that in the absence of a second basic species, the rearomatization can occur as an intramolecular reaction. The basic species likely acts as a catalyst, facilitating the hydrogen transfer.
The energy barrier for this intramolecular reaction is very large, however upon the addition of a catalyst, this energy barrier is decreased. To initially test the catalytic mechanism in this reaction, calculations were performed using water as the catalyst to facilitate the transition from species 2 to species 3 in scheme 6. The addition of water as a catalyst resulted in a significant decrease in the energy barrier. Similar to the calculations using water as a catalyst, the introduction of monobasic phosphate resulted in the reduction of the energy barrier. Monobasic phosphate facilitates the transfer of the hydrogen in a manner similar to that shown in Scheme 6. These results indicate that not only is monobasic phosphate a catalyst, effectively lowering the energy barrier, but that phosphate specifically has a pronounced catalytic effect. The energy barrier with no catalyst present was 39.11 kcal/mol, while the addition of water lowered this to 16.16 kcal/mol, the addition of monobasic phosphate resulted in the substantial lowering of the energy barrier to 3.21 kcal/mol.

**Figure 19:** Pre-reaction intermediate, transition state structure, and product for the uncatalyzed reaction with their relative energies for HF/3-21G in gas phase calculations.
These initial calculations were conducted using HF/3-21G in the gas phase. The reaction coordinate for the hydrogen transfer in the absence of a basic species, in the presence of water, in the presence of monobasic phosphate, and in the presence of dibasic phosphate are in progress at the current level of theory and in the gas phase. Following these calculations, a second series of calculations accounting for solvent effects and conducted at a higher level of theory and more complete basis set will be conducted. These computational studies can complement the conclusions drawn from the experimental kinetics study and provide further insight into the catalytic mechanism of each phosphate species in the Pictet-Spengler reaction. In particular, these studies provide novel information about the isoTHI species, for which little data currently exists.

Summary
Through the evaluation of the rates of the Pictet-Spengler reaction in varied phosphate buffer concentrations and pH, several conclusions could be drawn. The first of these is that the Pictet-Spengler reaction with 3-(2-aminoethyl)phenol and propionaldehyde is base-catalyzed at the rate-limiting step, as such the $k_b$ is the major contributor to the observed rate constant. Furthermore, this catalysis is general base catalysis meaning the rate is dependent on the concentration of conjugate base in the buffer rather than strictly hydroxide concentration. To verify general versus specific base catalysis, analysis of varied phosphate concentration at constant pH, a dependence of rate on overall phosphate concentration was observed confirming the assertion. Base catalysis was verified by the positive linear dependence of the observed rate constant on the concentration of conjugate base. The same dependence was not observed with the concentration of acidic phosphate species.

Through this kinetic evaluation, a potential thermodynamic effect of phosphate buffer on the Pictet-Spengler reaction was observed. The ratio of THI to isoTHI was observed to have a positive correlation with the concentration of phosphate buffer indicating a regiospecific control exhibited by the phosphate species. Further inspection led to the observation of a dependence of the ratio on the pH. A plot of the ratio of THI to isoTHI versus the concentration of the acidic and basic phosphate species revealed that there was a positive linear correlation between the regio-control and the concentration of the acidic phosphate species. This relationship implies a potential thermodynamic effect of the acidic phosphate species on THI.
versus isoTHI. Computational studies to evaluate the energetics involved in this potential relationship are currently being conducted.

Finally, a primary kinetic isotope effect was observed in with the deuterated m-tyramine. The primary KIE indicates that the rate-limiting step involved the breaking of a C-H bond. In the Pictet-Spengler mechanism. For this mechanism, the KIE predicts the rate-limiting step is Step 6 in scheme 1. The rearomatization step involves the abstraction of a proton. This is the final step in the reaction mechanism, as such the equation used to describe the observed rate constant is consistent with the data, because all steps preceding the rate-limiting step influence the overall rate of the reaction.

Chapter 5: Kinetic Analysis of the Pictet-Spengler in Maleate

Introduction

Analysis of the Pictet-Spengler mechanism in the presence of phosphate has brought to light interesting potential roles for specific phosphate species in the reaction. To further investigate whether this is a phosphate specific catalytic contribution or a result of catalysis by a buffer with similar pKa a parallel series of reactions were conducted to compare with phosphate buffer. Maleic acid buffer was selected as the buffer to conduct this comparison for several reasons. The pKa of maleic acid is similar enough to phosphate to make a reasonable comparison. Secondly, previous kinetic investigations of the Pictet-Spengler mechanism with dopamine and propionaldehyde found that reactions conducted in maleic acid occurred quickly enough to prevent substrate degradation and the formation of side-products. These studies evaluated the rate of reaction between dopamine and propionaldehyde in Imidizole, BES, and Maleic acid buffers due to their similarities in pKa to phosphate and the work conducted by Pesnot et al. indicating that all of products were formed in all of these buffers. Maleic acid exhibits the fastest rate of reaction of the three buffers investigated, as such this was the buffer selected for a comparative kinetic analysis.

Methods

Preparation of Maleic Acid Buffers

Maleic acid buffers were prepared using the same method as described in chapter 4 for the preparation of the phosphate buffers. Once again twelve buffer solutions were prepared with varied concentrations, pH, and constant ionic strength of 0.212. The constant ionic strength was calculated using the Debye-Huckle relationship similarly to the calculation used for phosphate buffers. The result was the preparation of 20 mM, 40 mM, and 80 mM maleic acid buffers with varied pH 5.3, 5.6, 6.0, and 6.3.

Using the same methods for collecting kinetics data as with the phosphate buffer reactions reported in Chapter 4, each of the reactions with varied pH and concentration of buffer were conducted in triplicate. The kinetic isotope effect was evaluated for the reactions in maleic acid buffer using deuterated m-tyramine.
Results and Discussion

General Acid/Base Catalysis

Similarly to the kinetic analysis with phosphate buffer, a dependence of rate on the concentration of maleic acid buffer was observed. This indicates that similarly to the phosphate buffer, there is general acid or base catalysis. However, the rate constant dependence on concentration is more significant at higher pH. This could be indicative of specific base catalysis in the maleic acid buffers. However, the data for the maleic acid buffer trials exhibits more scatter than the phosphate buffer trials. These reactions took between 4 and 6 days to complete. As a result, there may have been evaporation of the highly volatile propionaldehyde. This evaporation would affect the treatment of the reaction as a pseudo-first order reaction. However, the evaporation should be consistent from one sample to the next because all reactions are conducted at the same temperature under the same conditions. The rate constants obtained for the maleic acid trials are useful relative to one another, though are likely not the absolute rate constants. These difficulties with the maleic data were kept in mind while analyzing data and the conclusions drawn will need to be substantiated with further experiments.

Figure 21: The observed rate constant plotted with respect to maleate concentration. Each series corresponds to a specific pH: pH 6.3 (○) equation of the line \( k_{obs} = 4.6 \pm 0.4 \times 10^{-9} \text{ mM}^{-2}\text{s}^{-1} \text{ [total Buffer]} + 9 \pm 2 \times 10^{-9} \text{ with } R^2 = 0.98625 \), pH 6.0 (Δ) equation of the line \( k_{obs} = 2.0 \pm 0.8 \times 10^{-9} \text{ mM}^{-2}\text{s}^{-1} \text{ [total Buffer]} + 5 \pm 4 \times 10^{-8} \text{ with } R^2 = 0.7268 \), pH 5.6 (□) equation of the line \( k_{obs} = 8 \pm 1 \times 10^{-10} \text{ mM}^{-2}\text{s}^{-1} \text{ [total Buffer]} + 4.6 \pm 0.6 \times 10^{-10} \text{ with } R^2 = 0.94968 \), pH 5.3 (○) equation of the line \( k_{obs} = 3 \pm 2 \times 10^{-10} \text{ mM}^{-2}\text{s}^{-1} \text{ [total Buffer]} + 4 \pm 1 \times 10^{-8} \text{ with } R^2 = 0.37198 \)

A plot of the observed rate constant versus the pH shows a positive correlation between the increased rate constant and the increased pH. This plot merely indicates a positive correlation between increasing base concentration, either general or specific, and increasing rate constant.
This data could be indicative of substantial contributions of both general and specific base catalysis on the overall rate. The inconsecutive trend could also be a result of the loss of propionaldehyde due to the remarkably slow reaction rate in maleic acid buffer. Most likely, the rates for the two lowest buffer concentrations as well as the lower pH buffers were slow enough that external factors introduced too much error for this data to be reliable. This issue could be handled in several ways. First, the reaction could be carried out at higher temperatures to increase the rate of reaction. For this to provide meaningful data for the study at hand, the reaction would have to be carried out at multiple temperatures and an Arrhenius plot would be needed to accurately determine the rate constant for the reactions at room temperature. Another approach would be to increase the concentration of the maleate for each buffer solution. The higher buffer concentration shows the expected trends with rate constants and pH. Possibly by increasing the buffer concentration the effect of increased buffer will counter the effect of low pH and provide meaningful results for the pH range evaluated.

![Figure 22: The plot of the observed rate constant versus the pH with respect to each buffer concentration. 20mM maleic acid buffer (o) y=6x10^-4 mM^-1 s^-1 X – 3x10^-7 with R^2=0.7821. 40mM maleic acid buffer (o) y=9x10^-4 mM^-1 s^-1 X – 5x10^-7 with R^2=0.8908. 80mM maleic acid buffer (X) y=3x10^-4 mM^-1 s^-1 X – 1x10^-6 with R^2=0.9548.](image)

**Acid/ Base Rate Constants**

Using the same methodology as with the phosphate buffer kinetics in Chapter 4, the \( k_b \) and \( k_a \) were calculated. A plot of Equation 9 for the maleate buffers can once again provide the general acid and base rate constants. These values were calculated under the assumption that an errors caused by evaporation of propionaldehyde are consistent in all solutions and thus the comparative analysis of the rates is useful. The relative \( k_b \) and \( k_a \) values were determined to be 7 ± 1x10^-6 mM^-1 s^-1 and 2.3 ± 0.5 x10^-6 mM^-1 s^-1 respectively.
Figure 23: The plot of $k_{\text{obs}} - k_0/B_t$ versus the fraction of base. The line is representative of equation 9 and can be used to determine the general acid and base rate constants. All values plotted on this graph are representative of triplicate trials to obtain the observed rate constant for each reaction in a buffer of varied pH or total phosphate concentration. The equation of the line is $y = 9 \pm 1 \times 10^{-6} \text{ mM}^{-2} \text{s}^{-1} X - 2.3 \pm 0.5 \times 10^{-6}$ with $R^2 = 0.86854$. The $y$-intercept is indicative of $k_a$ and the slope of the line can be used to find $k_b$.

Kinetic Isotope Effect

Using the same methodology as with the phosphate buffers, parallel reaction of the maleic acid buffers with deuterated m-tyramine were performed. A primary kinetic isotope effect was once again expected for the Pictet-Spengler reaction in maleic acid buffer. This evaluation can provide further confirmation that step 6 of Scheme 1, the rearomatization step, is the rate-limiting step in the proposed mechanism. Similarly to the evaluation of the KIE for the phosphate buffer reactions, the overall observed rate constant and observed rate constants for each of the competing reactions were plotted against the concentration of basic maleate species. The overall KIE based on the concentration of the base was determined to be 9.3. This is a very large KIE which was not expected. While this kinetic isotope effect agrees with the anticipated primary kinetic isotope effect, this value is not likely the true KIE value. Due to the slow reaction times, only amplified by the addition of the heavy isotope, the data displayed increased scatter. When the most erroneous data was excluded, the overall KIE became 7.5, while this value is still significantly higher than expected, this confirmed that the exceedingly large KIE is a result of the increased data scattering for the maleic acid trials. However, it is unlikely that with an improved data set the KIE would no longer be a primary KIE. This can be evaluated through repeating the experiment with a set of maleic acid buffers of significantly higher concentrations.
Figure 24: The plot of the observed rate constant for the overall reaction versus the concentration of conjugate base for the isotopically labeled reaction (○) and the natural m-tyramine reaction (Δ). The equation of the line for the natural m-tyramine reaction is \( k_{obs} - \frac{k_0}{[B]_t} = 8.0 \pm 0.9 \times 10^{-6} \text{ mM}^{-2} \text{s}^{-1} [B]/[B]_t - 2.2 \pm 0.5 \times 10^{-7} \) with \( R^2 = 0.86921 \). The equation of the line for the deuterated m-tyramine is \( k_{obs} - \frac{k_0}{[B]_t} = 5 \pm 2 \times 10^{-7} \text{ mM}^{-2} \text{s}^{-1} [B]/[B]_t - 3 \pm 9 \times 10^{-8} \) with \( R^2 = 0.47664 \).

Figure 25: The observed rate constant for m-tyramine →THI \((k_1)\) versus the concentration of conjugate base for the deuterated m-tyramine (○) and natural m-tyramine (Δ). The equation of the line for the natural m-tyramine is \( y = 3.1 \pm 0.7 \times 10^{-9} \text{ mM}^{-2} \text{s}^{-1} X + 2.4 \pm 0.7 \times 10^{-8} \) with \( R^2 = 0.063861 \). The equation of the line for the deuterated m-tyramine is \( y = 7.2 \pm 0.9 \times 10^{-10} \text{ mM}^{-2} \text{s}^{-1} X + 6 \pm 2 \times 10^{-9} \) with \( R^2 = 0.84491 \).

Finally, the same evaluation was conducted for the reaction of m-Tyramine to isoTHI in the presence of maleic acid buffer. This plot also revealed a primary KIE of 3.4. This is still a large
primary KIE but approaches more expected values. However, in the phosphate trials the kinetic isotope effect for the formation of isoTHI from m-tyramine was diminished compared to the overall KIE and the KIE for formation of THI. This consistently diminished KIE for the formation of isoTHI is likely due to increased error due to the low concentrations of isoTHI.

Figure 26: The plot of the observed rate constant for the overall reaction versus the concentration of conjugate base for the isotopically labeled reaction (○) and the natural m-tyramine reaction (△). The equation of the line for the natural m-tyramine reaction is $k_{iso} - k_0 / [B]_t = 5.0 \pm 0.6 \times 10^{-7} \text{mM}^{-1} \text{s}^{-1} [B]/[B]_t - 1.4 \pm 0.3 \times 10^{-7}$ with $R^2 = 0.85163$. The equation of the line for the deuterated m-tyramine is $k_{iso} - k_0 / [B]_t = 8 \pm 3 \times 10^{-8} \text{mM}^{-1} \text{s}^{-1} [B]/[B]_t - 2 \pm 8 \times 10^{-8} R^2 = 0.30791$.

Figure 27: The observed rate constant for m-tyramine $\rightarrow$ isoTHI ($k_2$) versus the concentration of conjugate base for the deuterated m-tyramine (red) and natural m-tyramine (black). The equation of the line for the natural m-tyramine is $y=1.7 \pm 0.4 \times 10^{-10} \text{mM}^{-1} \text{s}^{-1} X + 2.3 \pm 0.4 \times 10^{-8}$ with $R^2=0.64505$. The equation of the line for the deuterated m-tyramine is $y=1.0 \pm 0.2 \times 10^{-10} \text{mM}^{-1} \text{s}^{-1} X + 1.3 \pm 0.6 \times 10^{-8}$ with $R^2=0.65865$.
Comparison of Maleate and Phosphate

The formation of iso-THI was diminished in the presence of diprotic phosphate. The same analysis was conducted for the maleate buffers. The positive correlation between the buffer concentration and the ratio of THI to isoTHI is weak but significant. Similarly, the plot of the ratio of THI to isoTHI versus the acidic maleate species has a weak but significant positive correlation. These correlations agree with previous research indicating a dependence of the formation of the isoTHI on pH. This result hints that the presence of an acidic species in increased concentrations either results in the formation of the THI being more favorable or the isoTHI being less favorable or possible some combination of these effects. The effect of the acidic species is enhanced when this species is diprotic phosphate as can be seen in the figure below, however there is clearly a dependence on pH.

Figure 28: Plot of THI/isoTHI ratio versus total maleic acid buffer concentration. pH 5.3 (red) pH 5.6 (black) pH 6.0 (green) pH 6.3 (blue)
Figure 29: Plot of the ratio of THI concentration to isoTHI concentration versus the concentration of the acidic species. Phosphate acidic species (black) Maleic Acid species (red)

The rate constants for maleate were an order of magnitude smaller than the rate constants for phosphate. Indicating that buffers of a similar pKa do not possess the same catalytic properties as phosphate. From this data, the catalytic role of phosphate in the Pictet-Sengler mechanism is evident. The Pictet-Spengler mechanism for the reaction of 3-hydroxyphenethylamine and propionaldehyde appears to be base-catalyzed, but also appears to exhibit phosphate specific catalysis. The phosphate specific catalysis can be qualitatively evaluated with a comparison of the observed rate constants of maleic acid buffers and phosphate buffers of the same concentration and pH. The plot of the observed rate constants versus the total buffer concentration at pH 6.3 for maleic acid and phosphate are shown below. From this plot the order of magnitude difference between the phosphate rate constants and the maleic acid rate constants is evident.
Figure 30: Plot of the observed rate constants for buffers of three different concentrations (20 mM, 40 mM, 80 mM) at pH 6.3 for phosphate and maleate buffers. The observed rate constants for phosphate (X) at pH 6.3. Equation of the line $y = 1.514 \pm 0.00 \times 10^7$ mM$^{-2}$s$^{-1} \times +1.01 \pm 0.03 \times 10^6$ with $R^2=0.99996$. The observed rate constants for phosphate (X) at pH 6.3. Equation of the line $y = 4.7 \pm 0.4 \times 10^9$ mM$^{-2}$s$^{-1} \times +9 \pm 2 \times 10^{-11}$ with $R^2=0.98625$.

The KIE evaluations for both maleic acid and phosphate buffers revealed a primary kinetic isotope effect. This is indicative that despite the buffer used the Pictet-Spengler reaction between 3-hydroxyphenethylamine and an aldehyde is base-catalyzed, and the rate-determining step is step 6 in Scheme 1.

Summary

From these kinetic evaluations several conclusions can be drawn about the Pictet-Spengler mechanism in with a substituted phenethylamine, information can also be gained about the mechanism of catalysis for this reaction. The comparative analysis of the reaction in the 24 different buffer solutions revealed that reactions in higher pH buffers resulted in faster reaction rates. Similarly, increased buffer concentration also revealed higher reaction rates. Despite this common trend in both maleic acid and phosphate buffers, the rates in the phosphate buffers were significantly faster than those in the maleic buffers. To verify this was not purely a result of increased pH in the phosphate buffers, a comparison of phosphate and maleic buffers at the same concentrations and pH was conducted and. This comparison revealed a magnitude of difference in the observed rate constants between maleic acid and phosphate buffers. From these observations, it can be assumed that this reaction is primarily general base-catalyzed, which is indicated by the dependence on buffer concentration concurrently with pH dependence. This general base catalysis was further substantiated through the observation of a positive linear trend between increasing conjugate base concentration and increasing observed rate constant as well as the positive correlation
between the fraction of base and observed rate constant. Furthermore a phosphate specific catalysis was observed in this mechanism. The phosphate specificity was indicated by the increased observed rate constants with the increase of phosphate concentration, as well as the apparent increase in rate constant in phosphate versus maleic buffers. The observed base catalysis indicates that the rate-limiting step is facilitated by the presence of a basic species. From these analyses the rate-limiting step could be limited down to either the 5th or 6th step in the reaction mechanism. Step 5 involves the deprotonation of the hydroxyl group and resultant cyclization while step 6 involves the deprotonation of the ring to reestablish aromaticity.

Heavy isotope labeling of the aromatic protons on the 3-hydroxyphenethylamine can assist in determining which of the base-catalyzed steps in the reaction mechanism is the rate-limiting step. The observation of a primary KIE indicates the breaking of an isotopically labeled bond is the rate-limiting step. In the Pictet-Spengler mechanism, this is step 6 in which aromaticity is reestablished through the abstraction of a proton. A secondary KIE would indicate that the atom adjacent to the heavy isotope undergoes a change in hybridization. In step 5 the ring closure results in a change in change in the hybridization of a carbon adjacent to a deuterium. In both maleic acid and phosphate buffers a primary KIE was observed, this would indicate that step 6, the rearomatization, is the rate-limiting step.

Despite the challenges with the maleic acid data, the correlations are still observable and can be used for effective comparative analysis.

Chapter 6: Conclusions and Future Work

Tyrosinase Mediated Synthesis of Halogenated Dopamine, L-DOPA, THIs

The purpose of the tyrosinase mediated reaction scheme was to discover a method to produce halogenated norcoclaurine to introduce into Berberis vulgaris cells to biosynthetically produce halogenated berberine. Using norcoclaurine and norcoclaurine derivatives offers some advantages as the precursor scaffold for precursor directed biosynthesis of berberine and berberine derivatives. As has been discussed throughout, 4-HPAA is not stable for long periods of time at higher temperatures. Similarly dopamine oxidizes to a quinone over time at higher temperatures. The lower stability of these two compounds makes them less appealing as precursor scaffolds. However, norcoclaurine exhibits a higher degree of stability and is farther in the biosynthetic pathway than dopamine and 4-HPAA. This minimizes potential issues in the feeding experiments with the halogenated substrates. The berberine biosynthetic pathway is a tyrosine derived pathway, however halogenated tyrosine would not be a suitable due to the high specificity of the enzymes at the beginning step in the biosynthetic pathway. For these reasons a facile synthesis method for halogenated norcoclaurine is required. The tyrosinase mediated, one-pot synthesis, of halogenated norcoclaurine from halogenated tyramine and 4-HPAA could potentially provide this facile method.
A one-pot enzyme-mediated synthesis of THI and their halogenated derivatives was proposed using tyrosinase extracted from a variety of sources in a monobasic sodium phosphate buffer. Both halogenated and unhalogenated phenolic substrates were readily oxidized by the tyrosinase to form their subsequent catechols. Upon the addition of an aldehyde the secondary Pictet-Spengler reaction occurs to form the subsequent tetrahydroisoquinolines. This secondary reaction not only results in the production of the desired product for the feeding experiments, but also acts as a driving force to bring the enzymatic reaction to completion despite the unfavorable equilibrium. The use of this two reaction system was successfully used to drive a reaction with the unhalogenated substrate to completion. However this has not yet been successful with the halogenated substrates. Chlorinated THIs have been formed using this method, however the rate of cyclization as well as the rate of oxidation by tyrosinase appears to be significantly reduced for the halogenated substrates. Originally this rate retardation was believed to be due to the presence of p-toluenesulfonic acid. Tyrosinase inhibition by p-toluenesulfonic acid likely occurred, however a decreased reaction rate was still observed in the halogenated tyrosine and the purified halogenated tyramine. As such, another potential reason for a decreased reaction rate is required. The cyclization reaction may be slower due to steric hindrance caused by the halogen, which would be amplified as the size of the halogen increases. This may indicate why the brominated THI was not formed. The degradation of 4-HPAA at room temperature likely occurs before the cyclization reaction can occur. Using the information on phosphate catalysis of the Pictet-Spengler reaction investigated in the kinetics study, this reaction could be optimized to improve the formation of halogenated THIs. This method could also be used to form THIs with aldehydes that do not degrade or polymerize readily at room temperature.

There are many advantages to this particular synthetic method that justify further investigations to improve the yield of halogenated norcoclaurine or other THIs. This is an aqueous method, providing a method that is both fiscally and environmentally appealing. The use of the cross-linked enzyme aggregate provides a reusable catalyst that can easily be removed from the reaction solution. This results in product in aqueous phosphate buffer, which could then be used for future feeding experiments.

Pictet-Spengler Mechanism

The kinetic and computational analysis of the role of phosphate in the reaction of 3-hydroxyphenethylamine and an aldehyde revealed general base catalysis by the dibasic phosphate species. The effect of the concentration of buffer on the observed rate constant as well as the effect of pH on the observed rate constant contributed to the conclusion that the rate of the reaction was dependent on the concentration of basic phosphate species present in the buffer solution. The cyclization and rearomatization steps in Scheme 1 (Steps 5 and 6) are base mediated or base-catalyzed respectively. The catalysis by basic phosphate species observed in the reaction of 3-hydroxyphenethylamine and propionaldehyde agrees with the computational studies conducted by Dr. Ruben Parra\textsuperscript{30}, in which the presence of the basic
phosphate species effectively lowered the energy barrier for the transition from the intermediate species to the final product, Species 2 to 3 in Scheme 6. This phosphate specific catalysis was further confirmed through the use of a series of maleic acid buffers of the same concentration of phosphate buffers. From this comparative analysis, the rate of reaction in phosphate buffer was significantly greater than that of the maleic acid buffers. To verify that this was indeed due to the concentration of basic phosphate species and not overall pH, a qualitative analysis of the observed rate constants of maleic and phosphate buffers at the same pH was conducted. A significant difference between the observed rate constants of the reaction in maleic and phosphate buffers was observed. To quantify this observation, the $k_b$ values of the reaction in phosphate buffer versus maleic acid buffers were compared and revealed an order of magnitude in difference.

A kinetic isotope experiment was also conducted in the reactions catalyzed by both phosphate and maleic acid. The observed primary kinetic isotope effect in both buffers indicates that the rate-limiting step is Step 6 in Scheme 1. This also indicated that the rate-limiting step was consistent in both buffers and the reaction mechanism was not influenced by the catalyst used. Which was the desired result.

Despite the difficulties experienced with the maleic acid buffers with the slow rate of reaction and resultant evaporation of aldehyde and exhaustion of reaction solution, the use of trials with maleic acid still proved useful as a comparison for the phosphate catalyzed trials. However, the observed rate constants from the maleic acid trials have the potential to be improved. By reducing the scatter and improving the quality of the maleic acid data, the conclusions drawn could be better confirmed. There are several methods that could be used to potentially alleviate the challenges experienced. Firstly, the use of maleic acid buffers of significantly higher concentrations could increase the rate of reaction substantially enough for the reaction to complete in reasonable time. Increasing the reaction rate would avoid the challenges experienced. Another method to increase the reaction rate could be increasing the temperature at which the reaction was carried out, an Arrhenius plot could then be created to determine the rate constant that would be suitable for comparison to the phosphate buffers. However, this would require conducting these experiments at multiple temperatures and may not be the most practical method.

Computational Analysis

An interesting trend was observed with increasing acidic phosphate species and an increase in regiocontrol of the reaction. The same trend was not observed for the maleic acid trials. This would indicate that the acidic phosphate species may have some influence over the regioselectivity of the reaction. This interesting trend led to an investigation of the role of each phosphate species in the mechanism of the regioisomer formation. Through the use of computational methods the role of the each phosphate species, was evaluated. However, these calculations were carried out at a low level of theory and a minimal basis set.
The depth of the calculations should be improved before any substantiated claims can be made about the role of phosphate in the formation of the regioisomer. The apparent hydrogen transfer from the ring to the adjacent phosphate and while a second hydrogen is transferred from the phosphate species to the oxygen on the ring may indicate why the isomer formation may be mediated by the presence of the acidic phosphate species. However, these calculations have only been conducted in the gas phase, thus solvent effects have not been taken into account, and conducted at a low level of theory. These computational evaluations should be repeated using a more complete basis set that allows for increased flexibility in the movement of electrons, such as 6-31+G (d,p). As well as using a higher level of theory, such as a Density functional theory method. Through these computational evaluations an improved understanding of the regioselectivity of the reaction between 3-hydroxyphenethylamine and an aldehyde can be obtained. Understanding regiocontrol in potential pharmaceutical candidates could be vital information for improving synthetic methods.
References

7. Tsukiyama, M; Ueki, T; Yasuda, Y; Kikuchi, H; Akaishi, T; Okumura, H; Abe, K (2009). Beta2-adrenoceptor-mediated tracheal relaxation induced by higenamine from Nandina domestica Thunberg. Planta medica 75 Santa medica


36. Jo, H; Choi, M; Kumar, A; Jung, Y; Kim, S; Yun, J; Kang, J; Kim, Y; Han, S; Jung, J; Cho, J; Lee, K; Kwak, J; Lee, H (2016). Development of novel 1,2,3,4-Tetrahydroquinoline scaffolds as potent NF-κB inhibitors and cytotoxic agents. *ACS Medicinal Chemistry Letters*.


Appendix A: NMR spectra

**Halogenated Tyramines**

**Figure 31:** $^1$H NMR (300 MHz, CDCl$_3$) spectra of brominated tyramine from reaction with n-Bromosuccinimide, tyramine, and p-toluenesulfonic acid.

**Figure 32:** $^1$H NMR (300 MHz, CDCl$_3$) spectra of chlorinated tyramine from reaction with n-Chlorosuccinimide, tyramine, and p-toluenesulfonic acid.
Deuteration of m-Tyramine

Figure 33: $^1$H NMR spectra of deuterated m-tyramine in MeOD.
Appendix B: HPLC Chromatograms

Synthesis of Halogenated Tyramine

Figure 34: HPLC-UV Chromatogram extracted at 225 nm for a linear solvent gradient of 0-30% acetonitrile for tyramine and tosic acid. Retention Times: Tyramine: 1.9 min, Tosic acid: 2.3 min
Figure 35: HPLC-UV Chromatogram extracted at 225 nm for a linear solvent gradient of 0-30% acetonitrile for tyramine and tosic acid. Retention Times: CI-Tyramine: 4.1 min, Tosic acid: 2.3 min

Figure 36: HPLC-UV Chromatogram extracted at 225 nm for a linear solvent gradient of 0-30% acetonitrile for tyramine and tosic acid. Retention Times: Br-Tyramine: 4.9 min, Tosic acid: 2.3 min
Figure 37: HPLC-UV Chromatogram extracted at 225 nm for a linear solvent gradient of 0-30% acetonitrile for tyramine and tosic acid. Retention Times: l-Tyramine: 6.4 min, Toscic acid: 2.3 min
Coupled Enzymatic and Pictet-Spengler Reaction for Halogenated Tyrosine

Figure 38: Enzymatic oxidation of chlorinated tyrosine followed by the Pictet-Spengler reaction with propionaldehyde. At the start of the reaction. HPLC-UV analysis at 225 nm with 0-20% linear solvent gradient.
Figure 39: Enzymatic oxidation of chlorinated tyrosine followed by the Pictet-Spengler reaction with propionaldehyde. After 3 hours. HPLC-UV analysis at 225 nm with 0-20% linear solvent gradient.
Figure 40: Enzymatic oxidation of chlorinated tyrosine followed by the Pictet-Spengler reaction with propionaldehyde. After 4 hours of enzymatic reaction and one hour after the addition of aldehyde. HPLC-UV analysis at 225 nm with 0-20% linear solvent gradient.
Appendix C: Rate Constants and Kinetics Data

Psuedo-First Order Rate constants in Phosphate

Table 3: Copasi generated observed Rate constants for Phosphate Buffer reactions Trial 1

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The pseudo-first order rate constants for the conversion of m-tyramine to THI and m-tyramine to isoTHI for Trial 1 using HPLC-UV data extracted at 280 nm and 225 nm.

Table 4: Copasi generated observed Rate constants for Phosphate Buffer reactions Trial 2

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<td>0.4</td>
<td>4.41</td>
<td>0.4</td>
<td>9.44</td>
<td>0.5</td>
<td>4.52</td>
<td>0.7</td>
</tr>
<tr>
<td>80mM</td>
<td>7.29</td>
<td>13.5</td>
<td>1.0</td>
<td>7.14</td>
<td>1.0</td>
<td>13.85</td>
<td>0.3</td>
<td>7.90</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The pseudo-first order rate constants for the conversion of m-tyramine to THI and m-tyramine to isoTHI for Trial 2 using HPLC-UV data extracted at 280 nm and 225 nm.
### Table 5: Copasi generated observed Rate constants for Phosphate Buffer reactions Trial 3

<table>
<thead>
<tr>
<th>Concentration</th>
<th>pH</th>
<th>$k_{THI}$ ($x10^{-4}$ s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{isoTHI}$ ($x10^{-5}$) s$^{-1}$</th>
<th>% Stdv</th>
<th>$k_{THI}$ ($x10^{-4}$ s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{isoTHI}$ ($x10^{-5}$) s$^{-1}$</th>
<th>% Stdv</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM</td>
<td>6.31</td>
<td>1.205</td>
<td>0.3</td>
<td>0.905</td>
<td>0.3</td>
<td>1.145</td>
<td>0.5</td>
<td>0.925</td>
<td>0.6</td>
</tr>
<tr>
<td>20mM</td>
<td>6.59</td>
<td>2.176</td>
<td>0.4</td>
<td>1.680</td>
<td>0.4</td>
<td>2.10</td>
<td>0.6</td>
<td>1.73</td>
<td>0.7</td>
</tr>
<tr>
<td>20mM</td>
<td>7.00</td>
<td>3.481</td>
<td>0.2</td>
<td>3.205</td>
<td>0.3</td>
<td>3.23</td>
<td>0.5</td>
<td>3.18</td>
<td>0.6</td>
</tr>
<tr>
<td>20mM</td>
<td>7.29</td>
<td>4.098</td>
<td>0.2</td>
<td>4.274</td>
<td>0.2</td>
<td>3.91</td>
<td>0.4</td>
<td>4.38</td>
<td>0.5</td>
</tr>
<tr>
<td>40mM</td>
<td>6.29</td>
<td>2.174</td>
<td>0.2</td>
<td>1.127</td>
<td>0.2</td>
<td>2.07</td>
<td>0.5</td>
<td>1.15</td>
<td>0.6</td>
</tr>
<tr>
<td>40mM</td>
<td>6.60</td>
<td>3.456</td>
<td>0.2</td>
<td>1.987</td>
<td>0.2</td>
<td>3.28</td>
<td>0.5</td>
<td>2.02</td>
<td>0.5</td>
</tr>
<tr>
<td>40mM</td>
<td>7.00</td>
<td>5.63</td>
<td>0.3</td>
<td>3.81</td>
<td>0.4</td>
<td>5.37</td>
<td>0.5</td>
<td>3.93</td>
<td>0.6</td>
</tr>
<tr>
<td>40mM</td>
<td>7.30</td>
<td>7.30</td>
<td>0.2</td>
<td>5.94</td>
<td>0.3</td>
<td>6.9</td>
<td>0.4</td>
<td>6.14</td>
<td>0.6</td>
</tr>
<tr>
<td>80mM</td>
<td>6.29</td>
<td>3.559</td>
<td>0.2</td>
<td>1.382</td>
<td>0.2</td>
<td>3.38</td>
<td>0.5</td>
<td>1.42</td>
<td>0.6</td>
</tr>
<tr>
<td>80mM</td>
<td>6.59</td>
<td>5.62</td>
<td>0.3</td>
<td>2.228</td>
<td>0.4</td>
<td>5.36</td>
<td>0.5</td>
<td>2.33</td>
<td>0.7</td>
</tr>
<tr>
<td>80mM</td>
<td>6.99</td>
<td>9.95</td>
<td>0.3</td>
<td>4.52</td>
<td>0.4</td>
<td>9.48</td>
<td>0.5</td>
<td>4.68</td>
<td>0.6</td>
</tr>
<tr>
<td>80mM</td>
<td>7.29</td>
<td>14.15</td>
<td>0.2</td>
<td>7.36</td>
<td>0.3</td>
<td>13.47</td>
<td>0.4</td>
<td>7.67</td>
<td>0.7</td>
</tr>
</tbody>
</table>

The pseudo-first order rate constants for the conversion of m-tyramine to THI and m-tyramine to isoTHI for Trial 3 using HPLC-UV data extracted at 280 nm and 225 nm.

### Table 6: Copasi generated observed Rate constants for Phosphate Buffer reactions with Deuterated m-tyramine Trial 1

<table>
<thead>
<tr>
<th>Concentration</th>
<th>pH</th>
<th>$k_{THI}$ ($x10^{-4}$ s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{isoTHI}$ ($x10^{-5}$) s$^{-1}$</th>
<th>% Stdv</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM</td>
<td>6.31</td>
<td>0.344</td>
<td>0.7</td>
<td>0.616</td>
<td>0.7</td>
</tr>
<tr>
<td>20mM</td>
<td>6.59</td>
<td>0.592</td>
<td>0.6</td>
<td>1.112</td>
<td>0.7</td>
</tr>
<tr>
<td>20mM</td>
<td>7.00</td>
<td>0.871</td>
<td>0.6</td>
<td>1.87</td>
<td>0.7</td>
</tr>
<tr>
<td>20mM</td>
<td>7.29</td>
<td>1.020</td>
<td>0.6</td>
<td>2.38</td>
<td>0.6</td>
</tr>
<tr>
<td>40mM</td>
<td>6.29</td>
<td>0.622</td>
<td>0.6</td>
<td>0.823</td>
<td>0.6</td>
</tr>
<tr>
<td>40mM</td>
<td>6.60</td>
<td>1.000</td>
<td>0.5</td>
<td>1.488</td>
<td>0.5</td>
</tr>
<tr>
<td>40mM</td>
<td>7.00</td>
<td>1.511</td>
<td>0.5</td>
<td>2.65</td>
<td>0.5</td>
</tr>
<tr>
<td>40mM</td>
<td>7.30</td>
<td>1.92</td>
<td>0.5</td>
<td>3.78</td>
<td>0.5</td>
</tr>
<tr>
<td>80mM</td>
<td>6.29</td>
<td>1.093</td>
<td>0.5</td>
<td>1.022</td>
<td>0.5</td>
</tr>
<tr>
<td>80mM</td>
<td>6.59</td>
<td>1.719</td>
<td>0.5</td>
<td>1.805</td>
<td>0.5</td>
</tr>
<tr>
<td>80mM</td>
<td>6.99</td>
<td>2.70</td>
<td>0.5</td>
<td>3.34</td>
<td>0.6</td>
</tr>
<tr>
<td>80mM</td>
<td>7.29</td>
<td>3.79</td>
<td>0.6</td>
<td>5.45</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The pseudo-first order rate constants for the conversion of deuterated m-tyramine to THI and m-tyramine to isoTHI for Trial 1 using HPLC-UV data extracted at 280 nm and 225 nm.
Table 7: Copasi generated observed Rate constants for Phosphate Buffer reactions with Deuterated m-tyramine Trial 2

<table>
<thead>
<tr>
<th>Concentration</th>
<th>pH</th>
<th>$k_{THI} \times 10^5$ (s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{isoTHI} \times 10^5$ (s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{THI} \times 10^4$ (s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{isoTHI} \times 10^4$ (s$^{-1}$)</th>
<th>% Stdv</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM</td>
<td>6.31</td>
<td>0.329</td>
<td>0.4</td>
<td>0.578</td>
<td>0.5</td>
<td>0.289</td>
<td>0.4</td>
<td>0.577</td>
<td>0.4</td>
</tr>
<tr>
<td>20mM</td>
<td>6.59</td>
<td>0.532</td>
<td>0.5</td>
<td>0.977</td>
<td>0.5</td>
<td>0.484</td>
<td>0.4</td>
<td>0.967</td>
<td>0.4</td>
</tr>
<tr>
<td>20mM</td>
<td>7.00</td>
<td>0.796</td>
<td>0.5</td>
<td>1.674</td>
<td>0.5</td>
<td>0.729</td>
<td>0.3</td>
<td>1.664</td>
<td>0.4</td>
</tr>
<tr>
<td>20mM</td>
<td>7.29</td>
<td>0.946</td>
<td>0.5</td>
<td>2.15</td>
<td>0.5</td>
<td>0.866</td>
<td>0.3</td>
<td>2.144</td>
<td>0.3</td>
</tr>
<tr>
<td>40mM</td>
<td>6.29</td>
<td>0.611</td>
<td>0.5</td>
<td>0.801</td>
<td>0.5</td>
<td>0.557</td>
<td>0.3</td>
<td>0.792</td>
<td>0.4</td>
</tr>
<tr>
<td>40mM</td>
<td>6.60</td>
<td>0.937</td>
<td>0.5</td>
<td>1.38</td>
<td>0.5</td>
<td>0.868</td>
<td>0.2</td>
<td>1.389</td>
<td>0.2</td>
</tr>
<tr>
<td>40mM</td>
<td>7.30</td>
<td>1.92</td>
<td>0.5</td>
<td>3.78</td>
<td>0.5</td>
<td>1.792</td>
<td>0.2</td>
<td>3.803</td>
<td>0.2</td>
</tr>
<tr>
<td>80mM</td>
<td>6.29</td>
<td>1.09</td>
<td>0.5</td>
<td>1.022</td>
<td>0.5</td>
<td>0.999</td>
<td>0.3</td>
<td>1.008</td>
<td>0.3</td>
</tr>
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<td>1.72</td>
<td>0.5</td>
<td>1.805</td>
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<td>1.588</td>
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<td>1.798</td>
<td>0.3</td>
</tr>
<tr>
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<td>0.5</td>
<td>3.34</td>
<td>0.6</td>
<td>2.525</td>
<td>0.3</td>
<td>3.350</td>
<td>0.3</td>
</tr>
<tr>
<td>80mM</td>
<td>7.29</td>
<td>3.79</td>
<td>0.6</td>
<td>5.45</td>
<td>0.6</td>
<td>3.60</td>
<td>0.3</td>
<td>5.55</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The pseudo-first order rate constants for the conversion of deuterated m-tyramine to THI and m-tyramine to isoTHI for Trial 2 using HPLC-UV data extracted at 280 nm and 225 nm.

Table 8: Copasi generated observed Rate constants for Maleic Acid Buffer reactions with m-tyramine Trial 1

Psuedo-First Order Rate Constants in Maleic Acid

<table>
<thead>
<tr>
<th>Concentration</th>
<th>pH</th>
<th>$k_{THI} \times 10^5$ (s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{isoTHI} \times 10^5$ (s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{THI} \times 10^4$ (s$^{-1}$)</th>
<th>% Stdv</th>
<th>$k_{isoTHI} \times 10^4$ (s$^{-1}$)</th>
<th>% Stdv</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM</td>
<td>6.31</td>
<td>3.99</td>
<td>1.2</td>
<td>3.33</td>
<td>1.3</td>
<td>4.01</td>
<td>1.4</td>
<td>3.45</td>
<td>1.5</td>
</tr>
<tr>
<td>20mM</td>
<td>6.59</td>
<td>7.30</td>
<td>0.8</td>
<td>6.16</td>
<td>0.8</td>
<td>7.13</td>
<td>0.8</td>
<td>6.39</td>
<td>0.9</td>
</tr>
<tr>
<td>20mM</td>
<td>7.00</td>
<td>12.5</td>
<td>0.8</td>
<td>10.9</td>
<td>0.8</td>
<td>12.2</td>
<td>3.8</td>
<td>6.95</td>
<td>4.4</td>
</tr>
<tr>
<td>20mM</td>
<td>7.29</td>
<td>11.8</td>
<td>1.2</td>
<td>10.8</td>
<td>1.3</td>
<td>11.4</td>
<td>1.1</td>
<td>11.2</td>
<td>1.2</td>
</tr>
<tr>
<td>40mM</td>
<td>6.29</td>
<td>6.28</td>
<td>1.3</td>
<td>4.81</td>
<td>1.4</td>
<td>6.25</td>
<td>1.4</td>
<td>4.97</td>
<td>1.5</td>
</tr>
<tr>
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<td>11.5</td>
<td>0.9</td>
<td>8.71</td>
<td>1.0</td>
<td>11.1</td>
<td>0.9</td>
<td>9.02</td>
<td>1.0</td>
</tr>
<tr>
<td>40mM</td>
<td>7.00</td>
<td>14.3</td>
<td>1.5</td>
<td>11.3</td>
<td>1.7</td>
<td>13.3</td>
<td>1.5</td>
<td>11.4</td>
<td>1.6</td>
</tr>
<tr>
<td>40mM</td>
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<td>21.6</td>
<td>1.0</td>
<td>17.8</td>
<td>1.0</td>
<td>19.8</td>
<td>0.9</td>
<td>17.7</td>
<td>1.0</td>
</tr>
<tr>
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<td>6.29</td>
<td>7.18</td>
<td>1.3</td>
<td>4.86</td>
<td>1.4</td>
<td>6.93</td>
<td>1.4</td>
<td>5.01</td>
<td>1.5</td>
</tr>
<tr>
<td>80mM</td>
<td>6.59</td>
<td>12.6</td>
<td>0.8</td>
<td>8.24</td>
<td>0.8</td>
<td>11.9</td>
<td>0.8</td>
<td>8.38</td>
<td>0.9</td>
</tr>
<tr>
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<td>26.1</td>
<td>0.8</td>
<td>18.6</td>
<td>1.0</td>
</tr>
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<td>44.4</td>
<td>0.5</td>
<td>32.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The pseudo-first order rate constants for the conversion of m-tyramine to THI and m-tyramine to isoTHI for Trial 1 using HPLC-UV data extracted at 280 nm and 225 nm.
Appendix D: Computational Data

Reaction Coordinate with Energy Relative to Transition State Structure

**Figure 41:** Relative energy (kcal/mol) with respect to reaction coordinate using HF/3-21G for conversion of the intermediate to product through an intramolecular hydrogen transfer in the absence of a catalyst. Energy barrier 39.11 kcal/mol.

**Figure 42:** Relative energy (kcal/mol) with respect to reaction coordinate using HF/3-21G for conversion of the intermediate to product through an intermolecular hydrogen transfer between intermediate and catalyst with water as the catalyst. Energy barrier 16.16 kcal/mol.
Figure 43: Relative energy (kcal/mol) with respect to reaction coordinate using HF/3-21G for conversion of the intermediate to product through an intermolecular hydrogen transfer between intermediate and catalyst with monobasic phosphate as the catalyst. Energy barrier 3.21 kcal/mol.