Synthesis and Evaluation of Unnatural HPAA, Norcoclaurine, and Tyramine Analogues

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SYNTHESIS AND EVALUATION OF UNNATURAL HPAA, NORCOCLAURINE, AND TYRAMINE ANALOGUES

A Thesis

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Requirements for the Degree of

Master of Science

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By:

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This work is dedicated to my grandfather. You have always taught me that it is important to learn as much as I can. That journey has just begun.
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Chapter 1 Introduction

1.1 Natural Products as Sources of Medicinal Compounds

Natural products have always played an important role in medicine dating back to ancient times when plant extracts were first used as therapeutics. Natural products have a great advantage over compounds derived from small molecule libraries as useful leads for medicinal compounds as they have structures that have already been validated and optimized by nature to interact with the cellular machinery that often serves as target for drugs. In the realm of pharmaceuticals natural products play many roles including being used as medicinal agents themselves, as scaffolds for novel compounds, and as starting materials for semisynthetic compounds. Despite a major shift away from natural product discovery towards using combinatorial synthesis and library development approximately 50% of all small molecules approved for use as medicinal agents between the years of 2000 and 2010 were natural products, derived from natural products, influenced by natural products, or served as natural product mimics. Even more impressive is that between 1981 and 2010 approximately 75% of all small molecules approved for use as antibiotics and 79% of all anticancer drugs were in some way influenced by or mimicked a natural product.

Because of the importance of natural products and natural product-like compounds in the field of medicine, chemists are often tasked with the development of methods that can yield the desired compounds. This is often made difficult by the nature of these compounds, which typically contain complicated heterocyclic ring systems and sometimes contain several stereo-centers. Currently there are several approaches commonly used to obtain these compounds. The oldest, and possibly the most obvious approach, is isolation of the natural products from their natural sources. This typically involves cultivating a large amount of the source species, and then using one or many of a variety of separation methods, that are designed to isolate the compound based on its solubility, stability, and volatility. These techniques typically include various forms of extraction and chromatography, and usually have to be used in conjunction with one another or repeated using different conditions. Although this has been used to successfully isolate drug compounds such as penicillin G and morphine, it is often very difficult to achieve due to the complex mixture of compounds present in a living organism. In addition to this, the compound of interest may often be found at very low overall abundance in the tissues, resulting in low yields. This is also compounded with the fact that the isolation process usually involves the destruction of the source organism, which can result in major ecological impacts. An example of this was seen with the anticancer drug Paclitaxel (Taxol™). This compound was originally isolated from the bark of the pacific yew tree, requiring the destruction of the tree, at a yield of only 0.02%, necessitating the destruction of 2500 trees to yield 1 kg of the drug. In addition to this, isolation of the natural product only gives access to the natural product itself, which may not be as medicinally useful as analogues of these compounds. A report by Newman and Cragg, demonstrates the necessity for having a convenient process for developing unnatural analogues to natural compounds, by demonstrating that 28% of all small molecule drugs approved between 1981 and 2010 were derivatives of natural compound products.

The other classic approach to obtaining medicinally useful compounds is through the use of total synthesis. This approach is useful in that it allows a chemist to create analogues of the compound which
may have altered or increased activity. In addition, the total synthesis of the natural compound with altered or removed functional groups has also been useful in elucidating the mechanism of action of some complex natural products.\textsuperscript{5,6} However, due to the same complex nature of natural products, that give them their unique functionality, total synthesis of these compounds is no trivial matter. They often require several steps using a multitude of toxic reagents, resulting in low yields and large amounts of waste. The results of these drawbacks are typically negative environmental impacts as well prohibitively high costs of production at the industrial scale. Once again, Taxol is an excellent example of the problems associated with total synthetic approaches to obtaining natural products with the reported synthesis requiring more than 30 steps and giving an overall yield of 0.05%.\textsuperscript{7,8}

As a result of the inherent difficulties that occur when trying to isolate and synthesize complex natural products, a variety of techniques have been developed in order to either minimize or avoid the complications that arise from natural product isolation and total synthesis. These processes typically manipulate the natural biosynthetic machinery found in the organism that produces the compounds of interest. One of the most common approaches is the use of semi-synthesis, which is the process of extracting a natural product, and then performing chemical reactions to obtain different compounds. This type of approach is useful because it allows for the organism's biosynthetic machinery to build the complex structure, which can then be modified as desired. This approach has ultimately led to the ability to generate libraries of compounds based off of natural products, allowing for a bridge between library development and natural product research, where a gap traditionally exists.\textsuperscript{1,9} Several interesting approaches to this method of drug development have been incorporated including the complexity to diversity approach developed by the Hergenrother group, which transforms complex natural products into libraries through the use of ring distortion reactions to yield compounds with diverse scaffolds.\textsuperscript{10} Although semi-synthesis has been successful for a variety of drugs, it is limited in its ability to yield novel compounds, due to the fact that the researcher is often limited to performing chemistry on only functional groups that are easily accessible.\textsuperscript{11} This type of chemistry is also hampered by the large degree of functionality typically associated with natural products and the researcher is also limited to reactions that can be performed with a high degree of chemo-selectivity.

One of the solutions to the problems associated with semi-synthesis is the use of precursor directed biosynthesis (PDB). PDB is the process of functionalizing precursor compounds of natural products and incorporating these functionalized compounds into the biosynthetic pathway in the hopes that the compounds will be utilized to generate predictable novel unnatural analogues of the natural product of interest.\textsuperscript{1,12} This means that nature might be able to create new libraries of natural products, if a library of precursor compounds could be developed. This is beneficial because the manipulation of these precursor compounds is often easier and cheaper than manipulation of the final product due to the precursors being structurally less complex. This approach has been commonly applied in bacterial systems to generate novel analogues of several natural products including hormaomycin, rapamycin, and pacidamycin.\textsuperscript{13-15} One of the obvious and major problems with this technique arises from the natural preference for the native precursor compounds over unnatural analogues, which may result in reduced yields of the desired unnatural analogues.\textsuperscript{1,12} In addition to this, the presence of the native substrate usually means that there will be a mixture of the natural product and its analogue, which can result in even more complicated purification procedures.\textsuperscript{1} One of the major solutions to these problems is the
use of mutasynthesis, in conjunction with PDB. This technique utilizes molecular engineering to knock out the genes responsible for the natural production of the precursor compounds, so there is no longer competition between the native substrate and the analogue. It can also be used to alter the natural cellular machinery to incorporate the desired unnatural functional group, preventing the need to synthesize the precursor compounds.\textsuperscript{1, 11, 16} These two approaches can also be utilized in conjunction with semi-synthesis, by incorporating an uncommon functional group that has very selective reactivity. For example, the addition of an alkyne or azide to the precursor compound could allow for the use of click-chemistry type alterations of the product. The added benefit of incorporating these types of unnatural functional groups is that the subsequent reactions can be performed bioorthogonally, even in a crude cell lysate, which can allow for alterations to be made prior to purification.\textsuperscript{16} A recent example of this approach involved the generation of analogues of pacidamycin, by incorporating a tryptophan halogenase into the proteome of a strain of \textit{Streptomyces coerulescens} in order introduce 7-chlorotryptophan into the biosynthetic pathway of pacidamycin.\textsuperscript{16} The researchers were then able to perform Suzuki-Miyaura coupling reactions within the crude extract, and found that they obtained good chemo-selectivity, avoiding reaction with the other functional groups on the chlorinated pacidamycin analogue as well as with other compounds in the crude extract.\textsuperscript{16}

2.2 Precursor Directed Biosynthesis of Benzylisoquinoline Alkaloids

Several of the aforementioned techniques along with many others have been used to study many classes of natural products. Of particular interest is a class of natural products, termed alkaloids. Alkaloids are nitrogen-containing natural products derived from amino acids. Most of these compounds are composed of a nitrogen containing heterocycle, and typically include other heteroatoms such as oxygen, forming sophisticated ring structures.\textsuperscript{17} They are most commonly found in plants, although there have been several reports of animal and fungal alkaloids, including many of the toxic compounds found in amphibian skin.\textsuperscript{18} Typically these compounds are classified by the structure of the ring systems that they comprise, including some common ring systems such as the ones shown in Figure 1.1.\textsuperscript{19}

![Figure 1.1. Ring systems commonly found in alkaloids.\textsuperscript{19}](image)

These compounds display a variety of biological properties that are particularly potent to animal species.\textsuperscript{17} As a result of these effects on animals, they have been frequently studied as possible sources of pharmaceuticals, and to date have been shown to display several interesting medicinal properties.
These properties include, but are not limited to: anti-inflammatory (berberine), analgesic (morphine, codeine), stimulant (caffeine), anticancer (vinblastine), muscle relaxant (papaverine), and antibiotic (sanguinarine) effects.\textsuperscript{20}

Due to the medicinal benefits of alkaloids, it would be useful to have access to a library of compounds derived from these compounds in order to screen these for their possible uses as medicinal agents. As such, a convenient method of developing analogues of these complex structures is necessary. Because of this, plant alkaloids present an interesting system for the testing and evaluation of precursor directed biosynthesis. Of particular interest in our lab are benzylisoquinoline alkaloids and those found in the Amaryllidaceae plant family.

Benzylisoquinoline alkaloids (BIAs) present an excellent system for the evaluation of PDB in plant systems due to their potent biological activity, and their use in a variety of drugs such as morphine and codeine. Some of the biological activities that have been observed include analgesic, anti-inflammatory, and antibacterial properties.\textsuperscript{20} In addition to this, the biosynthetic pathways of many of these compounds have been mapped out. This is necessary for planning an efficient PDB experiment since the structures of the precursors must be known, in order to create useful precursor compounds. In the case of BIAs the biosynthesis starts with tyrosine (1), which can end up as either of two compounds, 4-hydroxyphenylpyruvic acid or L-DOPA (Figure 1.2). These two precursors are then converted to either 4-hydroxyphenylacetaldehyde (3) (HPAA) or dopamine (2) respectively.\textsuperscript{21} This is followed by the first committed step in all benzylisoquinoline alkaloid biosyntheses, the Pictet-Spengler condensation of these two compounds to form the tetrahydroisoquinoline alkaloid, (S)-norcoclaurine (4), by the enzyme norcoclaurine synthase (NCS) (Figure 1.2). From here a series of methylations and oxidations occur to form the tetrahydroisoquinoline alkaloid (THIA) (S)-reticuline (5). After this point the pathways can vary depending on the organism, resulting in a variety of diverse and structurally interesting alkaloids such as morphine (Figure 1.3).\textsuperscript{21} In the plant species Berberis vulgaris, this pathway goes to produce the BIA, berberine (6), which is the alkaloid of interest in the present study. The pathway of berberine biosynthesis is continued from (S)-reticuline by a ring closure catalyzed by the berberine bridge enzyme (BBE), forming the alkaloid, (S)-scoulerine, yielding the four ring protoberberine structure.\textsuperscript{21} (S)-Scoulerine is then selectively methylated at one of the phenols by the enzyme, (S)-scoulerine 9-O-methyltransferase (STOM), to yield (S)-tetrahydrocolumbamine.\textsuperscript{21} This can then undergoes a ring closing reaction catalyzed by the enzyme (S)-canadine synthase (CAS), yielding (S)-canadine, which is subsequently oxidized by (S)-tetrahydroberberine oxidase (STOX), yielding berberine (6).\textsuperscript{21}

In addition to the medicinal benefits of berberine, the PDB of berberine analogues also presents an interesting test case for future applications in other plant species as we have access to cell culture that yields large amounts of the alkaloid. Also because the biosynthetic pathway of berberine has been completely mapped out, all of the intermediates and products that would result from the PDB of berberine can be easily predicted. This allows for an easy analysis of crude extracts of plant culture to determine the success of these experiments. Once the PDB of berberine alkaloids has been evaluated, we hope to apply our precursor analogues to other cell species allowing for access of to analogues of several medicinally useful compounds including analgesic morphine, the antitussive noscapine, the muscle relaxant tubocurarine, the antioxidant magnoflorine, and the vasodilator papaverine, as well as several of the other compounds some of which are shown in Figure 1.3.\textsuperscript{21}
Figure 1.2. The biosynthetic pathway of berberine as described by Desgagne-Penix and Facchini. The biosynthesis begins with tyrosine which differentiates into dopamine and HPAA. The red denotes the structural contributions by dopamine and the blue denotes the structural contributions by HPAA. The listed enzyme abbreviations are given by Desgagne-Penix and Facchini, and are repeated here, tyrosine/dopa decarboxylase (TDYC), norcoclaurine synthase (NCS), (R,S)-norcoclaurine 6-O-methyltransferase (6OMT), (S)-coclaurine-N-methyltransferase (CNMT), (S)-N-methylcoclaurine 3′-hydroxylase (NMCH), (S)-3′-hydroxy N-methylcoclaurine 4′-O-methyltransferase (4′OMT), berberine bridge enzyme (BBE), (S)-scoulerine 9′-O-methyltransferase (SOMT), canadine synthase (CAS), (S)-tetrahydroberberine oxidase (STOX).

Figure 1.3 Examples of the various alkaloids available from precursor directed biosynthesis of dopamine, HPAA, and norcoclaurine. The red denotes the structural contributions from dopamine where as the blue denotes the structural contributions from HPAA.
When trying to perform precursor directed biosynthesis, it is necessary to select a precursor scaffold that is: likely to make it into the biosynthetic pathway of the desired compound, unlikely to interfere with the primary metabolism of the host species, and will be relatively easy to synthesize in a cost effective manner. In the case of BIAs, there are several possible precursor compounds that are available for study including tyrosine, dopamine, HPAA, and norcoclaurine. It is also necessary to pick an alteration that will be useful for the purpose of study. In our lab, we decided to introduce halogens in our precursor compounds, based on several observations: their relatively small size makes them more likely to make it through the biosynthetic pathway, they possess unique mass spectral fragmentation patterns make them easy to detect using mass spectrometry (MS) analysis. Additionally, their ability to participate in cross coupling reactions makes them excellent functional groups for post biosynthetic chemo-selective modifications, allowing for potential access to libraries of unique compounds containing the berberine background structure.

Due to the several precursor compounds found in the biosynthetic pathway, there were a variety of precursor options available to us as routes for study. When planning for the PDB of berberine, we wanted to limit the possibilities based on the requirements outlined above, starting with considering only the compounds that were easily synthesized. This left us with the options of analogues of tyrosine, dopamine, HPAA, and norcoclaurine. Although it was considered, tyrosine analogues were eventually eliminated from consideration based on the importance of amino acids to the primary metabolism of plant species. The results of Runguphan et al, confirm that halogenated amino acids are unlikely to be suitable for PDB experiments, based on the increased selectivity of enzymes that deal with the primary metabolites, resulting in a bottleneck, and low yields of the final product. Based on this analysis, our lab chose to focus on dopamine, HPAA (Figure 1.4), and norcoclaurine (Figure 1.5) as the possible precursor scaffolds for the development of berberine analogues. In the present study only the development of HPAA and norcoclaurine analogues is considered.

Figure 1.4. Proposed precursor directed biosynthesis of berberine from HPAA. The HPAA analogue, and the structural contribution of the introduced analogue are shown in blue. X=F, Cl, Br, or I.
Proposed precursor directed biosynthesis of berberine analogues from norcoclaurine. The precursor analogue is and its structural contribution to the berberine product is shown in blue. X=F, Cl, Br, or I.

The synthesis of the native precursor compound HPAA has been published several times. However, the synthesis of the halogenated analogues in the literature at this time. Based on these observations, there were many possible routes to the compounds that were considered and attempted. The synthesis of norcoclaurine has also been described in the literature, via a phosphate catalyzed Pictet-Spengler reaction between dopamine and HPAA. This leads to a possible strategy for the production of halogenated norcoclaurine analogues as well.

1.3 Precursor Directed Biosynthesis of Galanthamine

Another major class of alkaloids for the evaluation of precursor directed biosynthesis is that of the Amaryllidaceae plant family, in particular, the alkaloid, galanthamine. This compound is of particular interest as a candidate for PDB due to its acetylcholinesterase activity, and its approved use as a therapeutic against Alzheimer's disease and other neurodegenerative conditions. In addition to this, the currently reported syntheses of this compound require several steps that offer low degrees of stereoselectivity, making isolation of the compound from its natural source the most efficient route for commercial production. The biosynthesis of this compound is not as well resolved as that of berberine, and the incorporation of halogenated precursors may also be an interesting way to map out the biosynthesis due to their characteristic fragmentation pattern when analyzed by mass spectrometry, which may allow for the determination of some of the unknown intermediates in the biosynthetic pathway. It is hypothesized that the use of halogenated precursor compounds, may have some advantages over isotopically enriched and radioactively labeled precursors, due to the fact that some of the enzymes may work slower with the new analogues, possibly allowing for the buildup of intermediate compounds that are short lived in the natural biosynthetic pathway.

The known biosynthetic pathway of galanthamine begins with the amino acids phenylalanine and tyrosine. Phenylalanine is then turned into cinnamic acid via an elimination reaction catalyzed by the enzyme phenylalanine ammonia lyase (PAL), which is then oxidized to p-
courmaric acid by the P450 dependent enzyme cinnamate 4-hydroxylase (C4H) (Figure 1.6). After this step, less is known about the specific enzymes that perform the individual steps, but it is known that \( p \)-courmaric acid undergoes a second oxidation to form caffeic acid, which is then converted to 3,4-dihydroxybenzaldehyde (DHBA, 8) (Figure 1.6). In parallel, tyrosine is decarboxylated to yield tyramine (9) by the enzyme tyrosine decarboxylase. These two compounds then undergo a reductive amination catalyzed by an unknown enzyme to form the alkaloid norbelladine (10), which is a branching point for the formation of several different alkaloids, some of which can be seen in Figure 1.8. Figure 1.7 details the currently reported biosynthesis of galanthamine which starts by the methylation of norbelladine to give \( O \)-methylnorbelladine. This is thought to undergo a para-ortho oxidative coupling of the two phenyl rings, which is spontaneously followed by a ring formation between the meta phenol at the 3\(^{\prime}\) position of norbelladine, and the 2 position of norbelladine to give the alkaloid \( N \)-demethylnarwedine. This is followed by a stereo specific reduction giving norgalanthamine, which is followed by an \( N \)-methylation to yield galanthamine (Figure 1.7).

![Figure 1.6. The biosynthesis of norbelladine.](image-url)

The biosynthesis of norbelladine begins with the amino acids phenylalanine and tyrosine to eventually form 3,4-dihydroxybenzaldehyde (DHBA) and tyramine respectively. These two compounds are then condensed via a reductive amination of DHBA and tyramine to form norbelladine. The blue denotes the structural contribution from phenylalanine and the red show the structural contribution from tyrosine. The known enzymes in the biosynthetic pathway are phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and tyrosine decarboxylase. Also shown is the numbering scheme used for norbelladine.

![Figure 1.7. The Biosynthesis of galanthamine from O-methylnorbelladine.](image-url)

The blue represents the structural contribution from phenylalanine while the red represents the structural contribution from tyrosine.
Based on what is known about the biosynthetic pathway of galanthamine, the possible precursor scaffolds available for use in PDB type experiments were tyrosine, phenylalanine, DHBA, tyramine, and norbelladine. The amino acids were not considered as possible precursors for the same reason as they were excluded from consideration for berberine analogues, as they are likely to interfere with primary metabolic pathways of the plant and may result in significant bottle necking, preventing formation of the desired analogues. Because the most direct synthetic route to norbelladine is via a reductive amination between DHBA and tyramine, analogues of norbelladine were not considered until after a convenient synthesis of either halogenated DHBA or tyramine analogues was first worked out. Additionally, the catecholic benzaldehyde DHBA is both oxidatively unstable in the presence of molecular oxygen and prone to polymerization. As such it was decided that tyramine analogues were the most suitable for PDB (Figure 1.9). One interesting difference between the PDB of galanthamine versus berberine is that there are two possible analogues that can result from the halogenated tyramine precursors, the structures of which can be seen in Figure 1.9. This means that the analysis of these products is going to be more complex. As with HPAA, there are several possible routes to the desired 3-halotyramines, the details of which can be found in Chapter 4 of the present work.
3,4-dihydroxybenzaldehyde (DHBA) + tyramine → Norbelladine analogue + galanthamine analogue

Figure 1.9. Planned precursor directed biosynthesis of galanthamine analogues from halogenated tyramine precursors. For every tyramine analogue, there are two possible galanthamine analogues that can result. The red represents the structural contributions from the tyramine analogue precursor. X=F, Cl, Br, or I.

1.4 Summary

Natural products have served as important sources of medicinal compounds for centuries due to the large variety of biological activities they possess. Because these compounds typically contain a large degree of functionality and many contain several stereocenters, natural products are notoriously difficult to synthesize often requiring several steps. This means that chemists are often tasked with the development of creative ways to obtain these natural compounds as well as their analogues. One of these approaches is a technique known as precursor directed biosynthesis. This technique involves the synthesis of compounds analogous to the natural precursor compounds found in the biosynthetic pathway of the natural product of interest. Once the precursor analogues are obtained, they are then feed to a cell line known to produce the natural product in the hope of exploiting its biosynthetic machinery to make unnatural analogues of the natural product. In the present work the synthesis of halogenated precursors of both benzylisoquinoline alkaloids (BIAs) and galanthamine is reported. Chapter 2 of this work discusses the synthesis of halogenated HPAA and norcoclaurine analogues for use in PDB experiments of BIAs. Chapter 3 discusses the development of an enzymatic assay for norcoclaurine synthase. Whereas Chapter 4 of this work includes the synthesis of halogenated tyramine analogues to be used in the PDB of unnatural galanthamine analogues. The development of a one-pot synthesis/feeding experiment of both HPAA and norcoclaurine analogues can be found in Chapter 5 of this work.
Chapter 2 HPAA and Norcoclaurine Analogues

2.1 Introduction

When performing PDB type experiments with the intention of developing berberine analogues, there were several possible precursors that could be considered for study. These include tyrosine, HPAA, dopamine, and norcoclaurine analogues. At first glance, halogenated tyrosine analogues seem to be the preferable choice since mono halogenations can be achieved in high yield in just one reaction. However, the results of Runguphan et al. suggest that halogenated amino acids are not suitable for PDB experiments. These results showed that chlorinated tryptophan was unsuitable for use as a substrate for PDB, due to a bottleneck that occurred at the tryptophan decarboxylase, resulting in low alkaloid product turnover. In addition to this, tyrosine is vital to the primary metabolism of most plants, so any competitive inhibition of these vital pathways may also result in cellular toxicity further making them unsuitable for these experiments. Finally, because tyrosine is the precursor to both HPAA and dopamine, it would seem likely that even if there was no bottleneck effect from the halogenated analogues, it is likely that the final berberine products maybe a complex mixture of alkaloids that are halogenated at multiple sites, making the data analysis and product characterization significantly more complicated.

As a result it was decided that HPAA and norcoclaurine analogues would be more suitable for these types of experiments. For the target HPAA analogues several synthetic strategies were considered and attempted, which can be broken down into three main approaches: oxidation of 4-(2-hydroxyethyl)phenol, Wittig olefination of 4-hydroxybenzaldehyde, and an oxidative decarboxylation of tyrosine. Attempts at the reduction and oxidation approaches are detailed in section 2.2 of this chapter, the attempted Wittig olefination can be found in section 2.3, whereas the successful synthesis of the target halogenated HPAA analogues is detailed in section 2.4. In addition to this, a one-pot biomimetic synthesis of halogenated norcoclaurine analogues from the respective halogenated tyrosine analogues is explained in section 2.5 of this chapter.

2.2 Attempts at Oxidation Chemistry

The first synthetic approach explored in the synthesis of the target HPAA analogues was the oxidation of 4-(2-hydroxyethyl)phenol (20), to the desired aldehyde oxidation state. In order to pursue this pathway, the alcohol had to be obtained. Although 20 is commercially available it is also expensive, so we hoped to be able to obtain large amounts of the alcohol cheaply through a synthetic approach from 4-hydroxyphenylacetic acid (12). Two different attempts at this reduction were performed (Scheme 2.1 and 2.2). The first approach we employed was the halogenation of 12, followed by an acid catalyzed esterification of the acid, and then reduction of the ester with LiAlH4 to generate the alcohol. The first halogenation that was attempted was an iodination of 12, using I2 in the presence of NH4OH to yield 3-iodo-4-hydroxyphenylacetic acid 15. Although this reaction produced the desired compound, it was difficult to purify the desired compound from the residual iodine and ammonia salts, often resulting in low yields of <50 %.
However, it was hoped that the acid could be reduced in high yields to give large quantities of 20, making this low-yield halogenation worthwhile. Previous attempts in our lab at reducing the methyl ester directly to the aldehyde with DIBAL-H, were low yielding, and so these were not pursued. We instead hoped that complete reduction to 20 with LiAlH₄ would be more suitable. Although this reaction was successful at producing the desired tyrosol, the reaction was difficult to purify as the aluminum salt side products would often form gelatinous material during work up. After filtration of the gel, crude yields of less than 20 % were obtained. The low yields of this reaction were thought to be due to the desired product being trapped in this gel resulting in product loss during filtration. In addition, GC/MS and ¹H NMR analysis indicated the presence of starting material in the crude product, necessitating additional purification steps and further reducing the yield. As a result of these difficulties, several workups were attempted, including the Fieser workup, which involves the addition of a specific quantity of water equal to the mass of LiAlH₄, followed by an equal volume of 15 % NaOH, which is then followed by a volume of water equal to 3 times the mass of LiAlH₄. This too failed to yield a significant amount of the desired product. Simple quench and extraction with ethyl acetate was also attempted, but this approach also gave poor yields.


Although it is likely that the yields of the purification of these reactions could be increased using phenol protection, this was not attempted as this would add two more steps to the overall scheme, and we were only interested in pursuing this approach if it proved to be cheaper than simply purchasing the resultant alcohol. Instead a second method for production of 20 was looked into. The method reported by Zhou et al. was attempted, as it allows for the protection of the phenol in situ. This procedure utilizes two equivalents of trimethyl borate to increase the reactivity of borane reductions in the presence of phenols without having to protect the phenol. The trimethyl borate presumably forms a complex between the electron rich alcohol, and the electron deficient boron compound, effectively masking the phenol proton from the reducing agent (Scheme 2.2). In addition to this, the reaction generates a reactive acyloxyborohydride compound, which then reacts with dimethyl sulfate to produce an acyloxyborane intermediate that further reacts to form a trialkoxyboroxine intermediate that yields the desired alcohol after undergoing acidic hydrolysis (Scheme 2.2). Although this reaction went to completion when analyzed by GC/MS and ¹H-NMR, the yield after purification was only

X = H, Cl, Br, or I
17.3 % making this approach more expensive than the purchase of the resultant alcohol and thus unsuitable for our applications. As a result of these difficulties, reductive approaches to generating 20 analogues were abandoned.

Scheme 2.2. Reaction mechanism for the reduction of 4-hydroxyphenylacetic acid (12) with trimethyl borate, sodium borohydride, and dimethyl sulfate.\(^{40-42}\) Trimethyl borate reacts with 11 to form the protected diboroester compound (41), which is then reduced by sodium borohydride giving the reactive acyloxyborohydride compound (42). This then reacts with dimethylsulfate to give a acyloxyborane species (43) that decomposes to the respective alcohol under acid hydrolysis.

Due to the difficulties of cheaply synthesizing 20, the compound was instead purchased from a commercial supplier. We hoped to generate the target analogues using the Parikh-Doering oxidation, as it was previously reported to successfully generated HPAA for use in enzymatic studies.\(^{23,43}\) This strategy was hopeful as it would allow for the synthesis of the target compounds in just two steps; a halogenation of 20 followed by the oxidation of these halogenated alcohols to yield the desired HPAA analogues (Scheme 2.3)

Scheme 2.3. Synthesis of HPAA and 3-halogenated HPAA analogues via the Parikh-Doering Oxidation. The values in parentheses indicate impure yields. Conditions: i. NaCl, oxone, acetone/water (0 °C to RT).\(^{44}\) ii. NaBr, oxone, acetone/water (0 °C to RT).\(^{44}\) iii I\(_2\), KI, 40 % dimethyl amine (RT).\(^{45}\) iv. Selectfluor® (RT). v. SO\(_3\)·pyridine complex, triethylamine, DMSO (RT).\(^{25}\) The yields in parentheses represent impure yields.

Initial attempts at this reaction using 20 as the reactant yielded HPAA, albeit at a low yield of 24 % after purification by column chromatography. Although the reaction yield was low, it was hoped that further optimization would increase the yield. Because of this, chlorinated (21), brominated (22), and iodinated (23) analogues of 20 were synthesized. Chlorination and bromination were achieved through the reaction of 20 with the respective sodium halide salt and Oxone\(^\circ\) in acetone. This reaction gave good yields of 72 % for 3-chloro-4-(2-hydroxyethyl)phenol (21) and 79 % for 2-bromo-4-(2-hydroxyethyl)phenol (22) after recrystallization from CH\(_2\)Cl\(_2\). \(^1\)H NMR analysis of the products suggests
that these reactions were successful. Three aromatic protons were observed for each compound, with one of the peaks appearing as a doublet, the second as a doublet of doublets, and the last peak as a singlet that shows a small degree of splitting (Figure 2.1 a and b protons, F, E, and D respectively). The doublet of doublet peak and the singlet that is slightly split most likely result from protons D and E, respectively, as labeled in Figure 2.1. This splitting is evidence that these protons undergo long distance coupling with one another. GC/MS analysis of these compounds was also useful in determining the success of the halogenations, due to the characteristic fragmentation that occurs with chlorinated and brominated compounds. GC/MS analysis of compound 21 displays an m/z of 172 Da, and the characteristic an (M+2)^+ peak found in chlorinated compounds of 174 Da resulting from chlorine having two abundant isotopes, both of which are in agreement with the expected masses of 172.03 and 174.03 Da (Figure 2.2). Likewise, GC/MS analysis of 22 also suggests that the reaction was successful, yielding the expected M^+ of 216 Da and the characteristic (M+2)^+ of 218 Da found in brominated compounds resulting from bromine having two abundant isotopes as well, both of which are in agreement with the expected masses of 215.98 and 217.98 Da (Figure 2.2.b). The same reaction was attempted using sodium iodide, but GC/MS and ^1^H NMR analysis showed that this reaction produced a large amount of the di-iodinated compound. A different approach was used where 20 was allowed to react elemental iodine in the presence of potassium iodide and dimethyl amine, which gave the desired 2-iodo-4-(2-hydroxyethyl)phenol (23) with a yield of 72 %. As with compounds 21 and 22, ^1^H NMR analysis of the product displayed the expected aromatic peaks, as described above (Figure 2.1.c). GC/MS results also confirmed the success of the reaction showing that the product had an M^+ of 264 Da, which was consistent with the expected mass of 264.06 Da (Figure 2.2.c).
Figure 2.1 $^1$H NMR spectra for 2-halogenated-4-(2-hydroxyethyl)phenol compounds. a. $^1$H NMR spectrum for 2-chloro-4-(2-hydroxyethyl)phenol. b. $^1$H NMR spectrum for 2-bromo-4-(2-hydroxyethyl)phenol. c. $^1$H NMR spectrum for 2-iodo-4-(2-hydroxyethyl)phenol. In each case, the labeled protons represent the respectively labeled peaks. In Figure 2.3a and 2.3b, the solvent is acetone-$D_6$, which corresponds to the peaks shown at 2.07 ppm, while the peak at 2.95 ppm most likely corresponds to water. In Figure 2.4.c the solvent is CDCl$_3$, corresponding to the peak at 7.29 ppm, while the peak at 1.56 ppm most likely corresponds to water. The peak trailing off of the water peak at 1.66 ppm may correspond to proton H$_A$, as labeled on the spectra, but is not integrated due to the overlap with the water peak.
Figure 2.2. GC/MS spectra for 2-halogenated-4-(2-hydroxyethyl)phenol compounds. a. GC/MS spectrum for 2-chloro-4-(2-hydroxyethyl)phenol. MS analysis of this compound yielded a $M^+$ of 172 Da and a $(M+2)^+$ of 174 Da consistent with the expected masses of 172.03 and 174.03 Da. b. GC/MS Spectrum for 2-bromo-4-(2-hydroxyethyl)phenol. MS analysis of this compound revealed a $M^+$ of 216 Da and an $(M+2)^+$ of 218 Da, agreeing with the expected values of 215.98 and 217.98 Da. c GC/MS spectra for 2-iodo-4-(2-hydroxyethyl)phenol. GC/MS analysis of this compound yielded a $M^+$ of 264, which agrees with the expected value of 264.06 Da. In Figures 2.5.a and b the insert serves to emphasize the $(M+2)^+$ peaks resulting from the chlorine and bromine ions present in the molecule.

In addition to this, the synthesis of 2-fluoro-4-(2-hydroxyethyl)phenol (24) was also attempted, by reacting 20 with 1-Chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate) (Selectfluor®). GC/MS analysis of the products however, showed two different peaks with a mass consistent with monofluorinated (2-hydroxyethyl)phenol products, suggesting that fluorination was occurring in two different locations on the benzene rings. GC/MS analysis also revealed a peak consistent with a di-fluorinated (2-hydroxyethyl)phenol product, suggesting that this reagent was over fluorinating the compound as well. Finally, $^{19}$F NMR analysis of the products displayed several peaks, confirming that this reaction was not selective for the desired product. HPLC analysis also resulted in a chromatogram showing several peaks that were very close in retention time, suggesting that the side products would not be easily resolved by chromatography. As a result it was decided that this method would not be useful for synthesizing the desired fluorinated compound. As such no further attempts at fluorination were performed.
Although the halogenation reactions on tyrosol proceeded with little difficulty, the subsequent oxidation was still low yielding giving several side products which can be seen in Figure 2.3. This figure shows an HPLC chromatogram of a typical Parikh-Doering oxidation of 20, using two equivalents of sulfur trioxide pyridine, two equivalents of triethyl amine, and DMSO, depicting the undesired side products. The presence of these side products with such close retention times, meant that a difficult and low yielding column was necessary for the purification of these products was necessary.

![HPLC Chromatogram](image)

**Figure 2.3. Reverse phase HPLC chromatogram of a typical Parikh Doering Oxidation of Tyrosol.** This chromatogram was generated by plotting the intensity of the maximum absorbance at each time. As expected, pyridine, DMSO, and HPAA are present along with several other unknown side products.

This oxidation was repeated on compounds 21, 22, and 23 as well using. Column chromatography of the products resulted in a large amount of product loss with yield of 35 %, 6.2 %, and 26 % for 3-chloro-4-hydroxyphenylacetaldehyde (25), 3-bromo-4-hydroxyphenylacetaldehyde (26), and 3-iodo-4-hydroxyphenylacetaldehyde (27) respectively. In addition to this, the products still contained impurities, as seen in $^1$H NMR spectra (Figure 2.4), which show several undesired peaks in each of the spectra.
Figure 2.4. $^1$H NMR spectra for 3-halogenated-4-hydroxyphenylacetaldehyde analogues synthesized using the Parikh-Doering Oxidation. a. $^1$H NMR spectrum for 4-hydroxyphenylacetaldehyde synthesized using the Parikh-Doering Oxidation of tyrosol after column purification. b. $^1$H NMR spectrum for 3-chloro-4-hydroxyphenylacetaldehyde synthesized using the Parikh-Doering Oxidation of 3-chloro-tyrosol after column purification. c. $^1$H NMR spectrum for 3-bromo-4-hydroxyphenylacetaldehyde synthesized using the Parikh-Doering Oxidation of 3-bromo-tyrosol after column purification. d. $^1$H NMR spectrum for 3-iodo-4-hydroxyphenylacetaldehyde synthesized using the Parikh-Doering Oxidation of 3-iodotyrosol after column purification. In each spectrum the peaks labeled with a letter correspond to the respectively labeled peaks in the accompanying image, whereas the unidentified impurities are circled in black. In each case the solvent is CDCl$_3$, corresponding to the peak at 7.29 ppm.

To improve yields, several methods to optimize this reaction were attempted. The first attempt was the addition of pyridine to the reaction mixture which has been suggested to eliminate side products. Pyridine sulfuric acid, a common impurity found in pyridine sulfur trioxide, can react to form side products, and the addition of excess pyridine can quench this reactive species through the formation of the less reactive di-pyridine sulfuric acid. We hoped that this modification would increase yields of HPAA and its analogues, however the yields or purity were not significantly altered. This suggested that the side product formation was not the result of the pyridine sulfuric acid side product. As a result of this, the reaction was then performed at lower dilution of reagents in CH$_2$Cl$_2$ and a reduced temperature of 0 °C. This too, however, did not result in higher quality product. Because of the low yield associated with this synthesis, this approach to the desired compounds was no longer pursued.
2.3 Attempts at Wittig Olefination

Because of the many difficulties surrounding oxidation of 20, the second strategy of Wittig transformation of 4-hydroxybenzaldehyde (28) was pursued. This approach utilized the commercially available Wittig reagent, methoxymethyltriphenylphosphonium chloride, to transform the benzaldehyde into a methoxyvinyl phenol, which can then tautomerize in the presence of strong acid. This approach was previously used by Seganish and DeShong to transform a benzaldehyde into the corresponding phenyl acetaldehyde, and so we hoped that it would be suitable for the synthesis of the desired HPAA analogues. The first step for this route required the synthesis of halogenated 4-methoxybenzaldehyde precursors. These were synthesized from 28 in with good yields (Scheme 2.4). 3-chloro-4-hydroxybenzaldehyde (29) was obtained from the reaction of SO2Cl2 with 28 in acetic acid with a satisfactory yield of 61%. Although the yield was not less than desired, the product of this reaction precipitated from solution after forming. This prevented di-halogenation and also made for easy purification by filtration followed by recrystallization from water, making this reaction easily scalable. 3-chloro-4-methoxybenzaldehyde (31) was then synthesized through a SN2 type reaction between 28 and methyl iodide with a good yield of 79% after extraction and filtration through a silica plug. Likewise, 3-bromo-4-methoxybenzaldehyde (32) was synthesized from 28 in two steps. In this case the protocol reported by Bovosombat et al. for the synthesis of mono-halogenated phenols was followed. This protocol called for the use of p-toluenesulfonic acid (TsOH) to prevent over halogenation of the benzene ring. Over halogenation was prevented through the formation of an intermolecular hydrogen bond between the sulfonic acid and the phenol, which prevents di-halogenation by blocking the other available site (Figure 2.5). This reaction worked well producing 3-bromo-4-hydroxybenzaldehyde (30) with an 86% yield after extraction and recrystallization from water. As with the chlorinated analogue, the brominated compound was also easily methylated through an SN2 reaction with iodomethane which resulted in an 81% yield of 32 after column chromatography.

Scheme 2.4. Synthesis of halogenated benzaldehydes. The yields of each reaction are reported below each of the respective compounds. Conditions: i. SO2Cl2, acetic acid (RT). ii. N-bromosuccinimide, acetonitrile (RT). iii. methyl iodide, CH3Cl2, 2 M NaOH, tetrabutylammoniumhydrogensulfate (RT).
Figure 2.5. Schematic indicating the selectivity for mono halogenation when using NBS and TsOH. The selectivity of this reaction is likely due to the formation of an intermolecular hydrogen bond between TsOH and the phenol, which prevents halogenation of one of the ortho positions by physically blocking that site.\(^\text{37}\)

In order to demonstrate the viability of the Wittig reaction, the commercially available 4-methoxybenzaldehyde was used as a surrogate for the halogenated precursors (Scheme 2.5). The typical procedure for this reaction was to react methoxymethyltriphenylphosphonium chloride with two equivalents of base to form the reactive phosphonium ylide species, and then introduce the aldehyde to the reaction mixture and reflux to produce the methoxyvinyl anisole compound (36) (Scheme 2.5). The reaction however, did not proceed as smoothly as planned, as analysis by GC/MS showed no peaks with the expected m/z of 164 Da. An unknown peak with an m/z of 216 Da was instead observed (Figure 2.6). Attempts to rationalize this peak were made, but were ultimately unsuccessful since the expected products m/z would only be 164 and the side product, triphenylphosphine oxide, has an expected m/z of 278 Da, and would not be expected to fragment into a species with the observed mass. In a further attempt to rationalize this peak, a GC/MS sample of the Wittig reagent was performed to ensure that this was not a contaminant present in the reagent. This sample showed only one peak with an m/z of 262 Da, which is consistent with triphenylphosphine, but not methoxymethyltriphenylphosphonium chloride, which is expected to have an m/z of 308 Da. \(^1\)H NMR sample confirmed that the reagent was good, resulting in the expected fifteen aromatic protons, three methoxy protons, and two methylene protons.

Scheme 2.5. Suggested synthesis of HPAA via Wittig olefination of 4-hydroxybenzaldehyde and related compounds. Conditions i. methoxymethyltriphenylphosphonium chloride, NaH, THF (RT).\(^{48}\) ii. HCl (reflux).\(^{48}\) iii. BBr\(_3\), CH\(_2\)Cl\(_2\) (-78 °C to RT).\(^{49}\)

With all of this evidence in hand it became clear that the problem was with the reaction itself and so the reaction was attempted using several other conditions, which are summed up in Table 2.1. In total, the reaction was performed using 5 different bases, three different solvents, several different temperature conditions and with three different aldehydes. In each case GC/MS analysis consistently showed the same side product of 216 Da and no evidence of the desired compound. Additionally, \(^1\)H NMR analysis suggested that the resulting product was a mixture of several compounds that were not characterized. Because the 216 peak was consistent amongst all of the different conditions even when using different aldehydes, it seems likely that this peak is comes from the Wittig reagent, and not the
aldehyde, suggesting that the Wittig reagent was decomposing rather than reacting with the aldehyde to produce the desired compound. To test this theory, methoxymethyltriphenylphosphonium chloride was reacted in the presence of base without adding any aldehyde, and a GC/MS sample was taken (Figure 2.6). Once again the product with m/z of 216 was observed confirming that this product is a degradation product of the Wittig reagent suggesting that this approach would not be useful in synthesizing the desired HPAA analogues.

Table 2.1. Conditions used in the attempt to optimize the Wittig reaction.

<table>
<thead>
<tr>
<th>Aldehydes</th>
<th>Solvents</th>
<th>Bases</th>
<th>Temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>THF</td>
<td>NaH</td>
<td>0 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LiOH</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Diethyl ether</td>
<td>NaOH</td>
<td>Room temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sodium methoxide</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Methanol</td>
<td>Potassium t-butoxide</td>
<td>Reflux temperature of each solvent</td>
</tr>
</tbody>
</table>

Figure 2.6. GC/MS chromatogram and spectra demonstrating the formation of a methoxymethyltriphenylphosphonium chloride degradation product observed during the attempted Wittig transformation of 4-methoxybenzaldehyde. The unknown side product appears with an m/z of 216, which is not consistent with the molecular weight of any expected intermediate, product, or side product of the Wittig transformation, and it was ultimately discovered to be a product of the degradation of our Wittig reagent.
2.5 Successful Synthesis of HPAA analogues

Next, we attempted to generate HPAA by oxidative decarboxylation of the amino acid tyrosine in the presence of NaOCl under buffered conditions at pH 7. This approach was of particular interest because it was also used to make HPAA for use in enzymatic studies of norcoclaurine synthase. In addition to this, it also benefited from the use of much more environmentally benign reagents rather than using harsh and toxic compounds such as pyridine. With this in mind a new synthetic strategy that involved the halogenation of tyrosine followed by this oxidative decarboxylation was envisioned and pursued (Scheme 2.6).

![Scheme 2.6. Synthetic strategy for the synthesis of HPAA analogues from the oxidative decarboxylation of halogenated tyrosines. Conditions: i. SO$_2$Cl$_2$ acetic acid (RT). ii. HBr, Br$_2$, acetic acid (RT). iii. I$_2$, NH$_4$OH. iv. Selectfluor® methanol, (RT). v. 1 equivalent NaOCl, phosphate buffer pH 7.](image)

GC/MS and $^1$H NMR analysis of the initial attempts at this approach show that the reaction was unsuccessful, resulting in a large quantity of a side product with an m/z of 133 Da, which was determined to be an over-oxidized side product, 4-hydroxybenzylcyanide. This was confirmed by isolation of the product by an ethyl acetate extraction, and $^1$H NMR analysis of the product, which can be seen in Figure 2.7.
Figure 2.7. $^1$H NMR Spectrum for the observed nitrile side product, 4-hydroxybenzylcyanide. In each case the lettered peaks correspond to the lettered protons in the chemical structure.

This observation was problematic as the major product of this reaction was the nitrile rather than the desired aldehyde. The initial protocol suggested a cold addition of 1 equivalent of a household bleach solution to a solution of 10 mM tyrosine in 20 mM phosphate buffer at pH 7. After several attempts at this procedure, it was proposed that the household bleach solution might be a problem due to the different additives found in these solutions such as sodium carbonate, sodium hydroxide, and sodium polyacrylate. A solution of 10-15% reagent grade sodium hypochlorite was used instead. Initially, the presence of the nitrile compound was still observed. The nitrile disappeared, however, when less NaOCl was added, suggesting this reaction is sensitive to the amount of oxidant added. Therefore, the sodium hypochlorite solution was titrated to determine the exact concentration of the 10-15% solution. This was done through the use of an iodimetric titration method. By this method $\Gamma^-$ is oxidized to $I_2$ by OCl$^-$. This $I_2$ then reacts with excess $\Gamma^-$ in solution to form $I_3^-$ (Eq. 2.1). This solution can then be titrated against a standardized solution of sodium thiosulfate, which works to reduce the triiodide anion back to $\Gamma^-$ (Eq. 2.2).

$$\text{OCl}^- + 2H^+ + 3\Gamma^- \rightleftharpoons \text{Cl}^- + I_3^- + H_2O \quad (2.1)$$

$$I_3^- + 2S_2O_3^{2-} \rightleftharpoons 3\Gamma^- + S_4O_6^{2-} \quad (2.2)$$

Titration revealed the concentration of the commercial solution to be 9.16% NaOCl by weight (1.48 M), meaning that 673 µL of this solution contained 1 mmol of NaOCl. In practice, it was found that 700 µL of this solution was sufficient to transform 1 mmol of tyrosine into HPAA without producing the nitrile side product. Although this revelation greatly decreased the amount of the nitrile side product,
following the reaction by HPLC revealed that there were still traces of other unidentified side products produced when the reaction was run at 10 mM tyrosine. These side products however, were not observed when the reaction was performed at 1 mM tyrosine. This suggested that the concentration of tyrosine also affected the purity of the final product, and as such a tyrosine concentration study was performed to determine the maximum concentration that would eliminate side products. Figure 2.8 shows the results at six different concentrations of tyrosine (1, 2, 3, 4, 5, and 8 mM). In each case a 0.1 mM solution of NaOCl was added dropwise at a rate of 1 mL/min via syringe pump in a 37 °C warm room to a tyrosine solution in pH 7 phosphate buffer solution. The reaction was then allowed to stir at 37 °C for 1.5 to 2 hours. The undiluted reaction mixture was then analyzed by reverse phase HPLC. From this experiment, it was determined that 4 mM was the highest concentration that would deliver minimal side products, since it was observed that concentrations of 5 and 8 mM yielded several different peaks other than the desired compound (Figure 2.8). Based on these results, all future results were performed at a maximum concentration of 4 mM tyrosine.

![HPLC chromatograms for the optimization of the oxidative decarboxylation of tyrosine. a. 1 mM tyrosine. b. 2 mM tyrosine. c. 3 mM tyrosine d. 4 mM tyrosine. e. 5 mM tyrosine. f. 8 mM tyrosine. In all cases the](image_url)
starting material, tyrosine, and product, HPAA are labeled, and unknown side products are pointed out for the 5 and 8 mM reaction. In each case the chromatograms were generated by plotting the intensity of the maximum absorbance at each time.

Finally, we examined the dependence of the rate of this reaction on buffer catalysis. As before, one equivalent of NaOCl was added dropwise to tyrosine, but with a series of concentrations of pH 7 phosphate buffer (0-30 mM). Time points for each of these reactions were collected approximately every ten minutes, by quenching a sample of the reaction with DMSO, and then analyzing the reaction via HPLC. The integrated areas of the absorbance peaks at 275 nm of tyrosine and HPAA were integrated and fit to an exponential function in order to determine the observed rate constants of tyrosine disappearance and HPAA appearance. Figure 2.9.a displays what a sample of data from this study looked like, showing the disappearance of tyrosine and the appearance of the HPAA products. In addition to this, the appearance and disappearance of an undetermined intermediate is also seen. Figure 2.9.b and c display the disappearance of tyrosine and the appearance of HPAA at all of the tested concentrations of phosphate.

![Figure 2.9. Sample data for the kinetic study of the oxidative decarboxylation of tyrosine.](image)

**Figure 2.9. Sample data for the kinetic study of the oxidative decarboxylation of tyrosine.** a. Sample data for the reaction at 2 mM tyrosine. The diamonds represent tyrosine, triangles the intermediate, and circles HPAA. b. Plots of tyrosine disappearance. c. Plot of HPAA appearance.

The resulting rate constants were then plotted against the respective concentrations of phosphate buffer to determine the phosphate dependence. Figure 2.10.a shows the observed rate of tyrosine disappearance as a function of phosphate concentration. From this plot, there appears to be a linear dependence of the rate constant of the concentration of the phosphate buffer, however three points ([HPO₄²⁻ = 0,1, and 30 mM]) do not follow the trend line. The low concentrations of phosphate may not fall along this line due to the fact that at these concentrations the reaction may not be buffered, and
the pH may change during the course of the reaction as NaOCl is added and CO₂ is produced. The point at 30 mM may suggest that the increase in the rate constant might level off after 20 mM phosphate since the rates at 20 and 30 mM are approximately equal within their experimental error. Although this data needs to be reproduced for greater confidence, it does suggest that there is some phosphate dependence for the first step of this reaction. Figure 2.10.b however, shows the observed rate constant of HPAA appearance as a function of phosphate buffer. The points for this plot appear to be randomly scattered suggesting that there is no dependence on phosphate concentration for the rate constant of HPAA appearance (the second step of the reaction), thus the overall rate constant of this reaction is independent of the buffer concentration used. These results also suggest that the first step of this reaction occurs faster than the subsequent steps, since the rate of product appearance does not agree with the rates of tyrosine disappearance. If this first step, were the slowest step then we would expect this rate to be equal to the rate of product appearance. Since these two rates are unequal, however, it appears that a step following the decomposition of tyrosine is slower and affects the rate of HPAA appearance. Currently these experiments are being repeated to confirm both of these observations, by using an alternative method that removes the quench procedure by injecting a sample of the reaction directly into the HPLC at regular intervals. This should confirm that the observed trends realistically represent the kinetics of the reaction, and that they are not influenced by the quenching procedure.

Figure 2.10. Results for the kinetic study of the oxidative decarboxylation of tyrosine. a. Observed rate constant dependence of tyrosine decay as a function of phosphate buffer. The points in red correspond to 0, 1, and 30 mM phosphate. Linear regression results of the points between 2 and 25 mM give a relationship of \( k_{obs} = (0.076 \pm 0.002)s^{-1}M^{-1}[\text{phosphate}] + (5.3 \pm 0.3)s^{-1} \). b. Observed rate constant dependence of HPAA appearance as a function of phosphate concentration. In both cases the rate constants were determined by fitting the respective curves in Figure 2.9 to an exponential function. The error bars are the error as calculated by the fitting program.

With the oxidative decarboxylation procedure optimized to yield a single product, a work up and purification procedure needed to be optimized as well. It was hoped that simple extraction would be sufficient to yield pure product since it was expected that the zwitterionic tyrosine would not be soluble in the extraction solvent. Because of this, an ethyl acetate extraction was performed, which yielded the
compound without any tyrosine impurity. However, NMR analysis of the isolated product showed that the compound decomposed at room temperature during the rotary evaporation process. Figure 2.11 shows $^1$H NMR spectra of 3-chloroHPAA, before and after rotary evaporation to remove the observed ethyl acetate. These spectra show the appearance of several new peaks, which were not present in the initial spectrum, suggesting that the compound was decomposing at room temperature. The observation of this decomposition is in agreement with other reports that the aldehyde is not stable in solution, with reported half-lives of < 1hr.\textsuperscript{53} The instability of the product suggested that the product needs to be concentrated quickly and at lower temperature than the heated bath of the rotary evaporator. This was achieved by simply changing the extraction solvent from ethyl acetate to dichloromethane, which has a significantly lower boiling point than ethyl acetate, allowing the solvent to be removed under vacuum while cooling with an ice bath, allowing for greater stabilization of the product.

![Figure 2.11. $^1$H NMR evidence of the instability of halogenated HPAA analogues. a. $^1$H NMR spectrum for 3-chloroHPAA, with ethyl acetate. b. $^1$H NMR spectrum for 3-chloroHPAA after one more hour under vacuum at room temperature. The circles show the new peaks that appeared after being under reduced pressure to remove ethyl acetate impurity. In addition to these new impurities, some of the ethyl acetate still remains. In both cases the peak at 7.29 ppm corresponds to the CDCl$_3$ solvent peak, while the peak at 1.56 ppm is most likely due to water.](image)

With both the reaction and isolation optimized for HPAA, halogenated analogues of tyrosine were prepared for the purpose of synthesizing halogenated HPAA analogues. Chlorination of tyrosine with SO$_2$Cl$_2$ was straightforward as the product precipitated from the solution after halogenation preventing di-halogenation from occurring. This reaction gave a yield of 66 % of compound 41 as the hydrochloride salt after filtration and recrystallization from HCl. Similarly the bromination by Br$_2$ and HBr was also straightforward as the product also precipitated from the solution after halogenation preventing the di-halogenation from occurring, yielding 70 % of compound 42 as the hydrochloride salt after filtration and recrystallization from HCl. Iodination of tyrosine by I$_2$ in NH$_4$OH, was more difficult, yielding only 30 % of 43. This low yield was most likely a result of the fact that the reaction requires the use of a difficult precipitation procedure from water by acidification and removal of the water under reduced pressure until only 10 % remained. It may have been likely that some of the product remained soluble in the remaining aqueous solution, resulting in the lower yield. However, retaining some water was necessary to remove the iodine and ammonium salts from the solution. As with the halogenated
tyrosol compounds, $^1$H NMR was also used to confirm the success of these reactions, since it was expected that the halogenated tyrosines would have similar aromatic splitting as those of the halogenated tyrosol. As expected the $^1$H NMR analysis of these compounds all displayed three aromatic protons, one of which was a singlet (proton E in Figure 2.12), and two of which were doublets (protons F and G in Figure 2.12) suggesting that the desired analogues were successfully synthesized.

Figure 2.12. $^1$H NMR spectra for 3-halogenated tyrosine analogues. a. $^1$H NMR Spectrum for 3-chlorotyrosine. b. $^1$H NMR spectrum for 3-bromotyrosine. c. $^1$H NMR spectrum for 3-iodotyrosine. In each spectrum the peaks labeled with letters correspond to the respectively labeled peaks on the compound. The solvent in each case is D$_2$O, resulting in the large solvent peak at 4.69 ppm as well as the loss of the amine, carboxyl, and phenol protons in each compound.

Once the halogenated tyrosines were synthesized, the corresponding HPAA analogues could be synthesized. The optimized NaOCl procedure, with DCM as the extraction solvent, gave yields of 83, 82, and 62 % for 3-chloroHPAA (25), 3-bromoHPAA (26), 3-iodoHPAA (27) respectively, that were of high purity. $^1$H NMR analysis of each of the compounds suggests that the reactions were successful due to the appearance of the characteristic aldehyde peak at 9.74 ppm for 26, and 27, and at 9.75 ppm for 25 (Peak A in Figure 2.13). In addition to this, there is a loss of the $\alpha$ proton peak in each of the tyrosine starting materials (Peak B in Figure 2.13), as well as a change in the splitting for the methylene peak in the tyrosines, which changed from a multiplet to a doublet, suggesting that it is now only being split by the aldehyde proton (Peak B Figure 2.13). Analysis by GC/MS also confirmed that the expected products were successfully prepared displaying M$^+$ of 136 Da for HPAA, which was consistent with the expected
mass of 136.15 (Figure 2.14.a). The chlorinated compound displayed an M\(^+\) of 170 Da, and an (M+2)\(^+\) peak characteristic of chlorinated compounds 172 Da, both of which agreed with the expected masses of 170.01 and 172.01 Da (Figure 2.14.b). The brominated compound also displayed its characteristic mass peaks giving an M\(^+\) of 214 Da and an (M+2)\(^+\) of 216 Da, which were in agreement with the expected masses of 213.96 and 215.96 Da (Figure 2.14.c). Finally, the iodinated analogue displayed an M\(^+\) of 262 Da, which also agreed with the expected mass of 262.04 Da (Figure 2.14.d).

Figure 2.13. \(^1\)H NMR spectra for 3-halogenated-4-hydroxyphenylacetaldehyde analogues generated via the oxidative decarboxylation of halogenated tyrosine analogues. a. \(^1\)H NMR spectrum for 4-hydroxyphenylacetaldehyde. b. \(^1\)H NMR spectrum for 3-chloro-4-hydroxyphenylacetaldehyde. c. \(^1\)H NMR spectrum for 3-bromo-4-hydroxyphenylacetaldehyde. d.\(^3\)H NMR spectrum for 3-iodo-4-hydroxyphenylacetaldehyde. In each case, the NMR solvent is CDCl\(_3\), which is responsible for the solvent peaks at 7.29 ppm, while the peak at 1.56 ppm is water, and peaks at 5.3 ppm are most likely residual CH\(_2\)Cl\(_2\), and the peak at 2.17 ppm is consistent with acetone contamination.
Figure 2.14. GC/MS spectra for 3-halogenated-4-hydroxyphenylacetaldehyde analogues. a. GC/MS spectrum for HPAA. The M⁺ for this compound was 136 Da, which was in agreement with the calculated value of 136.15 Da. b. GC/MS spectra for 3-chloro-4-hydroxyphenylacetaldehyde. GC/MS analysis of this compound revealed that the resulting M⁺ was 170 with an (M+2)⁺ of 172 Da, both of which are in agreement with the expected values of 170.01 and 172.02 Da. c. GC/MS spectrum for 3-bromo-4-hydroxyphenylacetaldehyde. This analysis revealed that The M⁺ for this compound was 214 Da, and the (M+2)⁺ was 216 Da, both of which are consistent with the expected values of 213.96 and 215.96 Da. d. GC/MS spectrum for 3-iodo-4-hydroxyphenylacetaldehyde. This analysis resulted in a M⁺ of 262 Da which agrees with the expected value of 262.04 Da.
2.6 Synthesis of Norcoclaurine Analogues

Because of the relative instability of HPAA and its analogues, there was concern that they would not be suitable substrates for PDB experiments. These types of experiments require the analogues to be shaken in liquid media for times greater than one week. Ideally, the substrate should be stable for over a week. So in addition to the HPAA analogues, an efficient synthesis of 3-halogenated norcoclaurine analogues (44-46) was also developed. The results of an undergraduate teaching lab at DePaul University served as the starting point for this synthesis. The lab exercise involved the use of norcoclaurine synthase to stereoselectively prepare norcoclaurine analogs from halogenated dopamine compounds and HPAA. After analyzing these results, it was discovered that the negative controls which contained dopamine, HPAA, and the same phosphate buffer, but no enzyme still produced norcoclaurine after incubation in phosphate buffer. These results were also in agreement with the reports by Pesnot et al., which also suggested that phosphate was able to catalyze this transformation yielding racemic tetrahydroisoquinoline products. Based on these results, a biomimetic one-pot synthesis of racemic norcoclaurine analogues was developed, that utilized the NaOCl transformation of tyrosine to HPAA in phosphate buffer (Scheme 2.7). These reactions were performed by reacting tyrosine and the halogenated analogues in the same manner as when producing the HPAA analogues. After the tyrosine was consumed, excess NaOCl was quenched through the addition of 0.5 equivalents of ascorbic acid, in order to promote a reducing environment. This was followed by the addition of an excess of dopamine and the solution was left to stir at 37 °C until the aldehyde was completely consumed, leading to the formation of the respective norcoclaurine analogue.

Scheme 2.7. Synthetic route to norcoclaurine analogues, via the newly developed one-pot synthesis from tyrosine. Also included is the numbering scheme used to name the analogues. Conditions: i. 1 equivalent of NaOCl, phosphate buffer pH 7. ii. 1.2 equivalents of dopamine, phosphate buffer pH 7.

The synthesis itself was straightforward; however it was necessary to develop a method of purification. It was hoped that the purification method of norcoclaurine reported by Bonamore et al. utilizing solid phase activated charcoal extraction to isolate the compound would be applicable to the
halogenated analogues as well.\textsuperscript{50} Although this technique worked to remove the norcoclaurine analogue products from the solution, desorbing the analogues from the carbon proved to be difficult. The reported method of desorption with 0.005 N ethanolic sodium hydroxide to extract the compound from the carbon was unsuccessful in our hands. As a result, several other conditions were considered, including methanolic sodium hydroxide, sodium hydroxide in acetonitrile, ethanolic HCl, methanolic HCl, and HCl in acetonitrile, all of which failed to release the compound as well. Due to the complications of removing the compounds from the activated carbon this approach to purification was abandoned. Simple ethyl acetate extraction resulted in the isolation of the desired compounds. Using this one pot synthesis allowed for quantitative conversion of the aldehyde to the respective norcoclaurine analogue as observed by HPLC measurements, with isolated yields of 28, 45, 48, and 55 \%, for norcoclaurine (4), 3-chloronorcoclaurine (44), 3-bromonorcoclaurine (45), and 3-iodonorcoclaurine (46) respectively. Characterization of the products by \textsuperscript{1}H NMR spectroscopy suggests that these reactions were successful. Due to the loss of the aldehyde peaks at around 9.74 ppm, and the appearance of two new singlet aromatic peaks, as expected from the structure of the product (labeled as G and H in Figure 2.15.a and H and I in Figure 2.15.b-c). The loss of the aldehyde peak, and the appearance of the two singlets is consistent with a reaction between dopamine and the aldehyde since dopamine contains two singlet aromatic protons. In addition to this, there is an appearance of several new alkyl protons in the range of 2.5 - 3.3 ppm, consistent with the expected protons in the unconjugated ring of the norcoclaurine products (protons C-F in Figure 2.15.a and protons D-G in Figure 2.15.b-d). Although these peaks are not well resolved in the spectra, they integrate to values consistent with the expected products, and they appear to be similar to previously reported spectra for norcoclaurine like compounds as reported by Ruff at al. suggesting that the desired compounds were successfully synthesized.\textsuperscript{54}
Figure 2.15 $^1$H NMR spectra for 3-halogenated norcoclaurine analogues. a. $^1$H NMR Spectrum for norcoclaurine. b. $^1$H NMR spectrum for 3-chloronorococlaurine. c. $^1$H NMR spectrum for 3-bromonorococlaurine. d. $^1$H NMR spectrum for 3-iodonorococlaurine. The solvent in Figure 2.15. a, b, and d is methanol-D$_4$, and is responsible for the solvent peaks at 4.8 and 3.6 ppm. The solvent for Figure 2.15.c is DMSO-D$_6$ and is responsible for the solvent peak at 2.56 ppm, whereas the peak at 3.33 ppm is most likely due to water.

The success of the newly developed one-pot synthesis of norcoclaurine and the related analogues was further verified by an enzymatic synthesis of the norcoclaurine and the related analogues using the Pictet-Spengler catalyzing enzyme norcoclaurine synthase. This enzyme is known to catalyze the condensation between HPAA and dopamine to selectively yield (S)-norcoclaurine, and it has also been shown to incorporate a wide variety of phenylacetaldehydes into the corresponding norcoclaurine analogues.$^{23, 49, 54}$ We hoped to verify the production of our norcoclaurine analogues by comparing the HPLC retention times between the chemically synthesized norcoclaurine product and the enzymatically synthesized products. We hoped that this analysis would reveal that the chemically synthesized compounds had the same retention time as the enzymatically synthesized compounds. The results of this analysis can be seen in Figure 2.16, which shows eight different chromatograms. Figure 2.16.a and b show the results for chemically synthesized and enzymatically synthesized norcoclaurine respectively. The identical retention times for both compounds is 2.47 minutes for HPLC condition C (HPLC conditions can be found in Appendix A), suggesting that they are the same compound. Figure 2.16 c and d shows similar data for 3-chloronorococlaurine, showing that the retention time for the chemically synthesized compound is the same as the retention time for the enzymatic product, with both compounds eluting at
1.98 minutes using HPLC condition B. Similarly the retention times for chemically synthesized 3-bromonorcoclaurine and enzyme synthesized 3-bromonorcoclaurine were the same (Figure 2.16 d and e), with the compound eluting at 2.05 minutes (HPLC condition B). This same analysis was also applied to 3-iodonorcoclaurine resulting in equal retention times under HPLC condition B (Figure 2.16 g and h) of 2.21 minutes for both compounds. Based on these results it seems that the reported one-pot synthesis of norcoclaurine and its analogues was successful.

Figure 2.16. HPLC chromatograms for evaluation of the 1-potsynthesis of norcoclaurine and related halogenated analogues from tyrosine and related halogenated tyrosine analogues. a. HPLC chromatogram for norcoclaurine synthesized from tyrosine. The retention time was found to be 2.47 minutes (HPLC condition C). b. HPLC chromatogram for norcoclaurine synthesized using NCS. The retention time was found to be the same as that of the product in Figure 2.16.a. c. HPLC chromatogram for 3-chloronorcoclaurine synthesized from 3-chlorotyrosine. The retention time was found to be 1.98 minutes (HPLC condition B). d. HPLC chromatogram for 3-chloronorcoclaurine synthesized using NCS. The retention time was found to be the same as that of the product in Figure 2.16.c. e. HPLC chromatogram for 3-bromonorcoclaurine synthesized from 3-bromotyrosine. The retention time was found to be 2.05 minutes (HPLC condition B). f. HPLC chromatogram for 3-bromonorcoclaurine synthesized using NCS. The retention time was found to be the same as that of the product in Figure 2.16.e. g. HPLC chromatogram for 3-iodonorcoclaurine synthesized from 3-iodotyrosine. The retention time was found to be 2.21 minutes (HPLC condition B). h. HPLC chromatogram for 3-iodonorcoclaurine synthesized using NCS. The retention time was found to be the same as that of the product in Figure 2.16.g. All of these chromatograms were generated by plotting the intensity of the maximum absorbance at each time.

2.6 Summary

Three different synthetic pathways were attempted and evaluated for their ability to yield the desired 3-halogenated-4-hydroxyphenylacetaldehyde analogues. These reactions included reduction of
the respective 3-halogenated-4-hydroxyphenylacetic acid compounds to the respective alcohols followed by the Parikh-Doering oxidation of those alcohols. A Wittig olefination of 3-halogenated-4-hydroxybenzaldehyde to yield trapped enol intermediates that can yield the desired 3-halogenatedHPAA analogues following acid catalyzed tautomerization was also attempted. A green biomimetic NaOCl mediated oxidative decarboxylation of 3-halogenatedtyrosine compounds was also successfully used to generate the target molecules. Although the synthesis required several optimization steps, it was successfully used to give the desired HPAA analogues cleanly with good yields. This approach yielded the desired HPAA analogues in only two steps with cumulative yields of 55, 57 and 19 % for 3-chloroHPAA, 3-bromoHPAA, and 3-iodoHPAA, respectively. In addition to this, a one-pot biomimetic synthesis of racemic 3-halogenatednorcoclaurine analogues from 3-halogenatedtyrosine compounds was also developed. This used a phosphate mediated Pictet-Spengler condensation between dopamine and 3-halogenatedHPAA synthesized from 3-halogenatedtyrosine. This yielded the desired norcoclaurine analogues in two steps as well, with cumulative yields of 30, 34, and 17 % for 3-chloronorcoclaurine, 3-bromonorcoclaurine, and 3-iodonorcoclaurine respectively.
Chapter 3 Norcoclaurine Synthase

3.1 Introduction

The first committed step to benzylisoquinoline biosynthesis is the Pictet-Spengler condensation of HPAA and dopamine to form the tetrahydroisoquinoline alkaloid norcoclaurine. This reaction is catalyzed by the Pictet-Spenglerase enzyme norcoclaurine synthase (NCS).\textsuperscript{21, 23, 54} As a result of the interesting reaction performed by this enzyme and its importance in the biosynthetic pathway of these natural products, a considerable amount of work has been performed on this enzyme since its discovery in 1981 by Rueffer et al.\textsuperscript{55} The enzyme was originally named (S)-norlaudanosoline synthase because its activity was originally characterized through the observation of the condensation between 3,4-dihydroxyphenylacetaldehyde and dopamine to form the alkaloid, (S)-norlaudanosoline.\textsuperscript{55} It was eventually renamed after it was discovered that the natural condensation performed in the biosynthetic pathway produces norcoclaurine from dopamine and HPAA, rather than norlaudanosoline.\textsuperscript{56} Recently, the mechanism of this enzyme has been proposed by Luk et al.\textsuperscript{23} and supported by crystal structure determination.\textsuperscript{53} Figure 3.1 displays a summary of the mechanism based on these two works. It is believed that the important catalytic residues are; lysine 122 (K122), tyrosine 108 (Y108), and glutamate 110 (E110), and that the reaction proceeds by the activation of the aldehyde substrate by the K122 residue of the enzyme. The activation of the aldehyde allows for the nucleophilic attack by the amine likely forming a Schiff base intermediate. Deprotonation of the hydroxyl group meta to the phenethylamine side chain of dopamine allows for electron density in the ring to undergo nucleophilic attack at the imine carbon, closing the ring. The final step of this mechanism includes the rearomatization of the dopamine ring system, which is facilitated by a proton abstraction by the E110 residue of the enzyme.\textsuperscript{23, 57} The importance of these amino acid residues has been demonstrated through site directed mutations of the enzyme. These experiments have shown that the K122 residue is the most vital for the catalytic activity, with a K122A mutant showing complete loss of catalytic activity.\textsuperscript{53} Additionally, Y108F and E110A mutants have also been tested for their catalytic activity. Both mutants exhibit decreased catalytic activity compared to the wild type enzyme, suggesting that they contribute, but are not necessary for, the catalytic activity of this enzyme.\textsuperscript{53} The crystal structure also elucidates how the enzyme controls the stereochemistry of the product. This can be seen in Figure 3.2, which shows the two substrates aligned in the active site of the enzyme side by side, thus controlling the side of attack by dopamine on the aldehyde substrate. The interactions between the catalytic residues, K122 and E110, can also be seen in Figure 3.2, showing a rationale for the proposed mechanism.
Figure 3.1. Proposed mechanism of the condensation of HPAA and dopamine by NCS based on the results from references 23 and 48. The first step most likely involves the activation of the aldehyde by the K122 residue, followed by a nucleophilic attack by dopamine at the aldehyde. This is then followed by the formation of a Schiff base intermediate after the loss of water. After this point the phenol meta to the phenethylamine functional group is deprotonated allowing for nucleophilic attack by the aromatic ring to occur at the imine, forming the ring. After this occurs, E110 of the enzyme can act as a base to remove the proton and reestablish aromaticity, forming (S)-norcoclaurine.

Figure 3.2. Crystal structural explanation of the observed stereoselectivity and mechanism of NCS. The left image is a close up of the active site of norcoclaurine synthase detailing the interactions between the catalytic residues, K-122, E110, and Y108. It also details the arrangement of dopamine and the aldehyde giving a rationale for the enzyme’s control over stereochemistry. The right image is a close up of the surface of NCS detailing the active site pocket of NCS, and the arrangement of the aldehyde and dopamine substrates. In each case the aldehyde is the non-reactive substrate mimic 4-hydroxybenzaldehyde. Color scheme of stick images, blue=N, red=O, green=C. Hydrogen is not shown for clarity. The crystal structures were determined in reference 48, and both images were generated using PyMOL (PDB ID: 2VQ5).
Recently, an alternative mechanism for the catalytic activity of this enzyme has been proposed by Pesnot et al. based on their work with NCS isolated from *Coptis japonica* (Figure 3.3).\textsuperscript{49} This study utilized a fluorescamine based assay to study the ability of the enzyme to catalyze the Pictet-Spengler reaction between several different aldehyde and amine substrates. Their work revealed that the enzyme can incorporate aldehydes with much larger ring systems. Based on this observation, it seemed unlikely that the stacked confirmation observed in the crystal structure of NCS is actually occurring since it would result in steric clashes. Based on this hypothesis, the researchers performed a series of computational substrate docking studies on the enzyme to discover if there is a lower energy docking arrangement that the substrates can undergo. The results of this study revealed that dopamine most likely docks first and interacts with its meta-hydroxyl, with the amine group pointing towards the opening of the catalytic cavity. This is followed by the docking of HPAA, with the aldehyde pointing towards the amine and the aldehyde’s aromatic ring pointing outwards toward the solvent, thus preventing steric clashes between larger side chains and the enzyme. This is then followed by the deprotonation of the amine by aspartatic acid 141 (D141), making the amine more nucleophilic and poised for attack on the carbonyl. An imine intermediate would then be formed after the loss of water, which is aided by a water channel located near the active site of the enzyme. Evidence of this water channel can be seen in Figure 3.4 a and b, which shows what appears to be a channel through the center of the protein adjacent to the active site that is filled with water molecules. K122 could then deprotonate the meta hydroxyl in the dopamine substrate, catalyzing the ring closure, followed by re-aromatization by E110.
This mechanism differs from the mechanism proposed by Ilari et al. based on a different arrangement of substrates. This mechanism involves the docking of dopamine first with dopamine arranged so that the catechol points towards the inside of the catalytic cavity while the amine points away from this cavity. This is followed by the docking of 4-HPAA in such a manner that the aldehyde points towards the inside of the active site while the aromatic region is in the opening partially exposed to solvent (top left). The catalysis first involves the activation of dopamine through the deprotonation of the amine by D141, followed by nucleophilic attack of the carbonyl by this amine to form the aminol intermediate (top middle). This intermediate then loses water, presumably aided by the existence of a water channel (see Figure 3.4), yielding the imine intermediate (top right). From here the key catalytic residue, K122 can deprotonate the meta hydroxyl of dopamine while D141 stabilizes this formation allowing for the cyclization of the compound (bottom right). The rearomatization of this intermediate is then catalyzed by E110 giving the final (S)-norccoclaurine product (bottom left).
3.2 NCS Assay Development and Results

Because norococlaurine synthase represents the first committed step in benzylisoquinoline synthesis, it was vitally important that our HPAA and dopamine analogues could be utilized by the enzyme to make the respective halogenated norococlaurine analogues. Previous studies of this enzyme found that the enzyme is rather promiscuous and is willing to incorporate several different acetaldehydes into different tetrahydroisoquinoline like products. These include various substituted phenyl acetaldehydes, including 3,4-dimethoxyphenylacetaldehyde. These results were encouraging and an enzymatic assay was developed in order to test the viability of our analogues. The previously reported kinetic assay for NCS utilized phosphate buffer, which was troubling because of the high rate of background reaction associated with this buffer. Because of this, we initially decided to base our assay off of the work by Ruff et al. We were encouraged by their HPLC based approach, using methanol to quench the reaction at different time points, in TRIS buffer.
Based on the previously reported $K_m$ values for this enzyme of $(0.350 \pm 0.048)$ mM for dopamine at 37 °C and pH 7.5, and $(0.288 \pm 0.038)$ mM for HPAA at 37 °C and pH 7.5, in conjunction with the fact that 2 mM was saturating conditions for both substrates, it was decided to perform our assay using sequential 1:1 dilutions of substrate ranging from 0.015 to 2 mM.\textsuperscript{23} We obtained the same recombinant sequence of NCS purified from *Thalictrum flavum* as the one reported and used by Luk et al., cloned into a pET-28a (+) vector as a generous gift from Sarah O''Connor. This vector incorporates an N-terminal His-tag into the protein, to facilitate purification of the protein using nickel resin affinity chromatography as well as a thrombin cleavage site to allow for the removal of the His-tag. The original procedure that was developed followed the report by Ruff et al, using 100 mM TRIS buffer at a pH of 7.0. To do this several solutions of HPAA were made using eight sequential dilutions starting at 2.11 mM and decreasing to 0.016 mM. Then a master mix of an enzyme, buffer, and dopamine solution was made that contained enough of each solution component to perform nine reactions, so that the final buffer concentration would be 100 mM, the final dopamine concentration would be 2 mM, and the final amount of enzyme would be 8.1 µg per assay. To start the reaction, the appropriate amount of this master mix was added to one of the tubes containing a HPAA solution. The kinetics were monitored by HPLC by quenching samples of the reaction every 2 minutes and 40 seconds, removing a 60 µL sample of the reaction and adding 15 µL of methanol containing 0.2 mM naphthalene acetic acid (NAA), with the hopes of denaturing the protein. It was found however, that this quenching procedure was inadequate so the conditions were changed to an 80 µL sample of reaction being quenched by the addition of 120 µL of methanol, which we found to fully quench the reaction.

Once the quenching procedure was worked out, several kinetic runs were performed, using the above conditions. However, with all of these attempts we were never able to reach saturating conditions with the enzyme, and it was observed that a significant side product was produced (Figure 3.5). This side product, however, was not observed with imidazole as the reaction buffer, suggesting that the side reaction was dependent on the use of TRIS. A further look into the use of TRIS buffer for enzymatic assays using aldehyde substrates showed that these issues have been seen before, and result from a condensation between the primary amine in the TRIS and the aldehyde forming an imine, which is then trapped by one of the free hydroxyl groups forming an oxazolidine product as seen in Figure 3.6.\textsuperscript{60} Based on this report, it seems likely that the observed side product is this oxazolidine compound.
Figure 3.5. HPLC chromatogram of an enzyme reaction in 100 mM TRIS at pH 7.0. The intensity of the maximum absorbance at each time is plotted, yielding the observed chromatogram. As expected, dopamine, HPAA, norcoclaurine, and NAA are present along with the appearance of an undesired side product, which is attributed to being an oxazolidine containing compound.

Figure 3.6. Reaction scheme detailing the formation of the observed oxazolidine side product. The first step involves the condensation of the primary amine in TRIS and the aldehyde forming an imine intermediate, followed by the attack of one of the hydroxyl groups at the imine carbon forming an oxazolidine ring.

Due to the appearance of this side product, and the likelihood that it was consuming the aldehyde substrate, a new buffer had to be selected that displayed the useful properties of both low background reaction and no side reactions. In addition to this, we also wanted to see if the background reaction could be quenched by dilution into methanol. As a result a simple assay that would allow for the efficient testing of different buffering systems was needed. This assay was designed around the necessity to use HPLC as the means of detection, and was largely modeled after the design of the previous enzyme kinetic assay reported by Ruff et al. In order to test the relative background reactions of each species, as well as the quenchability of the background reaction, different tubes containing 2 mM solutions of both dopamine and HPAA, were allowed to react in a solution containing between 20-50 mM buffer. This reaction was then injected into the HPLC instrument and the absorbance peak of the norcoclaurine product was monitored at 225 nm, integrated, and plotted against time. Because no calibration curve data exists between the integrated area of norcoclaurine and the concentration of norcoclaurine, these data were not converted into concentration units and instead the slopes of these lines were then taken as the background rate of the Pictet-Spengler condensation. Table 3.1 shows a
synopsis of all the tested buffers including the pH tested, the concentration of buffer, the rate of the reaction, and the relative rate of reaction as compared to phosphate buffer.

In addition to monitoring the background reaction rates, the ability to quench the reaction with methanol was also tested by setting up an identical reaction mixture, followed by quenching an 80 μL sample with 120 μL of methanol. This reaction mixture was then injected into the HPLC instrument and plotted in the same manner as the background reaction rates by monitoring the production of norcoclaurine at 225 nm and plotting the integrated peak area against time. As with the background reaction rates, the slope of this line was taken to be the quenched background rate, however in most cases the rates were nothing more than random scattering, suggesting that the reaction was completely quenched, meaning that this rate is most likely insignificant after quench. Figure 3.7 shows a typical set of data for a 25 mM bicine buffer at pH 7.5, showing both the buffer catalyzed background reaction and the rate of the reaction after methanol quench. Based on this, there is an obvious difference between the background reaction before and after dilution into methanol, suggesting that background reaction is effectively stopped by quenching.

### Table 3.1: Observed background reaction rates for various buffering systems.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Rate (µV*s/s)</th>
<th>Rate After Quench (µV*s /s)</th>
<th>Percent of Phosphate (%) (quenched)</th>
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</thead>
<tbody>
<tr>
<td>25 mM Phosphate</td>
<td>6.5</td>
<td>1106</td>
<td>--</td>
<td>100</td>
</tr>
<tr>
<td>25 mM Bicine</td>
<td>7.5</td>
<td>171</td>
<td>1.6</td>
<td>15 (0.15)</td>
</tr>
<tr>
<td>50 mM TRIS</td>
<td>7.5</td>
<td>78.6</td>
<td>-3.2</td>
<td>7.11 (-0.29)</td>
</tr>
<tr>
<td>25 mM HEPES</td>
<td>7</td>
<td>18</td>
<td>0.9</td>
<td>1.64 (0.08)</td>
</tr>
<tr>
<td>25 mM Imidazole</td>
<td>6.5</td>
<td>7.9</td>
<td>-1.2</td>
<td>0.72 (-0.10)</td>
</tr>
<tr>
<td>20 mM Glycine Amide</td>
<td>7.5</td>
<td>8.2</td>
<td>0.6</td>
<td>0.74 (0.06)</td>
</tr>
<tr>
<td>20 mM Maleic Acid</td>
<td>6.7</td>
<td>52</td>
<td>2.0</td>
<td>4.70 (0.18)</td>
</tr>
</tbody>
</table>
Figure 3.7. Sample background reaction plots for optimizing buffer conditions for NCS enzyme assays. Each reaction was performed using 2 mM of both dopamine and HPAA. The data depicted here is for a 25 mM bicine buffer at pH 7.5. In both cases the progress was monitored using HPLC using UV absorbance detection extracted at 225 nm. The integrated area of the norcoclaurine peak at this wavelength is plotted against time. In each case the slope of the line is taken to be the rate of transformation. The slope of the line for the unquenched bicine reaction is 171 µV*s/s, while the slope of the line for the quenched reaction is 1.6 µV*s/s.

Based on the results shown in Table 3.1 it was decided that the best buffering system is an imidazole buffer at pH 6.5, which showed a very small amount of background reaction, and was fully quenchable using our quench method. The procedure was repeated using a 25 mM imidazole buffering system (pH 6.5) by running a series of reactions each containing 4 mM of the dopamine substrate and varying concentrations of HPAA from 2.1 to 0.13 mM. The reactions were followed by mixing the appropriate amounts of substrates, buffer, and enzyme into a vial and making repeated injections of this solution into the HPLC. The reaction progress was monitored by following the absorbance of the norcoclaurine peak at 225 nm using HPLC. The integrated area of the norcoclaurine peak at 225 nm was then plotted as a function of time to determine the rates of reaction in units of µV*s/s, in lieu of a rate in terms of concentration units as no conversion currently exists. Because concentration and absorbance are linearly related to one another, the rates acquired in these units should be easily converted to more traditional rates in terms of concentration per time once a calibration curve that relates the two has been established. Analysis of the results were performed by plotting these rates as a function of HPAA concentration and using non-linear regression software to fit the data to the Michaelis-Menten equation (eq. 3.1).

\[
v = \frac{V_{\text{max}} [S]}{K_M + [S]} \tag{3.1}
\]

in Eq. 3.1 \(v\) is the rate of the reaction, \(V_{\text{max}}\) is the maximum rate of the reaction after saturation of the enzyme, \([S]\) is the concentration of substrate, and \(K_M\) is the Michaelis constant, and is equal to the concentration of substrate at half saturation. Fitting these results to eq. 3.1 resulted in the curve seen in
Figure 3.8 yielding a $K_M$ value of $(1.1 \pm 0.4) \text{ mM}$ for the aldehyde substrate. This however, is significantly higher than the previously reported value of $(0.288 \pm 0.38) \text{ mM}$.23

**Figure 3.8.** Experimental kinetics data for the NCS enzymatic assay in 25 mM imidazole buffer at pH 6.5 using a continuous injection method. The graph on the left shows the plots used to determine the rates of enzyme catalysis. In each case the production of norcoclaurine was monitored using HPLC by integrating its peak at 225 nm, and plotting the change as a function of time. The slopes were found to be 3280, 2650, 1860, 750, and 310 µV*s/s for 2.1, 1.05, 0.525, 0.263, and 0.131 mM HPAA respectively. These rates were used to generate the Michaelis-Menten Curve on the right for NCS for the substrate HPAA in 25 mM imidazole buffer at pH 6.5. The data were fit to eq. 3.1 resulting in a $K_M$ value of $(1.1 \pm 0.4) \text{ mM}$.

Although the difference in the observed $K_M$ and the reported value could be attributed to the differences in temperature and pH between our study and the reported study, we still wanted to see if we could observe a curve that reached full saturation. So this test was then repeated using methanol quenches to monitor the rate of the reaction, and a higher maximum concentration of HPAA. During this evaluation, the maximum concentration of HPAA was increased to 3.2 mM and eight samples were prepared with concentrations from 0.03 - 3.2 mM HPAA. The progress of this reaction was then monitored by making quenches every 30 seconds by diluting an 80 µL sample of the reaction mixture into 120 µL of methanol, and following the area of the norcoclaurine peak at 225 nm. The data analysis was performed using the procedure stated above, resulting in the plot shown in Figure 3.9. The results of this plot yielded a curve that does not appear to reach the saturation point of the enzyme and a $K_M$ of $(2.6 \pm 0.2) \text{ mM}$, which is an order of magnitude larger than the literature value for this substrate and enzyme.23
Experimental kinetics data for the NCS enzymatic assay in 25 mM imidazole buffer at pH 6.5 using the methanol quench procedure. The graph on the left shows the plots used to determine the rates of enzyme catalysis. The production of norcoclaurine was monitored using HPLC by following and integrating its peak at 225 nm, and change was plotted as a function of time. The slopes were found to be 5330, 3580, 2240, 1370, 680, 340, 180, and 60 µV*s/s for 3.2, 1.6, 0.80, 0.40, 0.20, 0.10, 0.05, and 0.025 mM HPAA, respectively. These rates were used to generate the Michaelis-Menten curve on the right for NCS for the substrate HPAA in 25 mM imidazole buffer at pH 6.5. The data were fit to eq. 3.1 resulting in a $K_m$ value of (2.6 ± 0.2) mM.

Discouraged by this result, we decided to test to see if another buffering system would yield different results. Based on the results shown in Table 3.1, HEPES was chosen based on its low rate of background reaction and quenchability. As with the imidazole studies, these reactions were performed using differing concentrations of HPAA in the presence of a constant concentration of dopamine in a 25 mM HEPES buffer (pH = 7.0). The data analysis was also performed as described above, resulting in the two curves shown in Figure 3.10. In each case, the maximum concentration of HPAA tested was 2.7 mM, and sequential 1:1 dilutions were made ranging from 2.7 - 0.021 mM HPAA, while dopamine was held constant at 2.25 mM. Fitting of the results of Figure 3.10.a and Figure 3.10.b resulted in $K_m$ values of (0.350 ± 0.095) mM and (0.498 ± 0.082) mM respectively. This yielded an average $K_m$ value of (0.42 ± 0.06) mM, which is far closer to the previously reported values of (0.288 ± 0.38) mM at for this enzyme.23
Figure 3.10. Michaelis-Menten curves for HPAA in 25 mM HEPES buffer pH 7.0. Both experiments utilized a range of concentrations from 0.021 to 2.7 mM HPAA, while the reaction was monitored by HPLC with UV detection at 225 nm by integrating the area of the resultant norcoclaurine peaks, and plotting the change in this area against time to obtain a rate. The results were fit to Eq. 3.1 the curve in Figure 3.10.a results in a $K_M$ of $(0.350 \pm 0.095)$ mM whereas the curve in Figure 3.10.b results in a $K_M$ of $(0.498 \pm 0.082)$ mM, yielding an average $K_M$ of $0.42 \pm 0.06$ mM.

Encouraged by these results, the experiments were repeated with the hopes of determining a $K_m$ for dopamine. These experiments were designed to be analogous to the previous assays, but altering the concentration of dopamine while maintaining a constant HPAA concentration. Using the same 25 mM HEPES buffer at pH 7.0, these reactions were repeated using concentrations of dopamine ranging from 0.18 to 2.9 mM, in the presence of 2.25 mM HPAA. Once again the kinetics were monitored by quenching the reactions every 2 minutes and 40 seconds, and monitoring the change in the integrated area of the norcoclaurine absorbance peak at 225 nm, as a function of reaction time. These rates were then plotted against the respective concentration of dopamine, resulting in the curve shown in Figure 3.11.a. This curve, however, did not appear to reach saturation, resulting in a $K_M$ of $(3.9 \pm 0.2)$ mM. Based on the observation that the kinetics never seemed to have reached saturation, a second test was performed using a wider range of dopamine concentrations ranging from 0.033 to 4.2 mM, yielding the curve shown in Figure 3.11.b. Once again the results were discouraging, resulting in another curve that failed to reach saturation and a very large $K_M$ of $(4 \pm 1)$ mM and an average $K_M$ of $(4.0 \pm 0.4)$ mM. Once again, this is an order of magnitude higher than the reported $K_M$ of $(0.350 \pm 0.048)$ mM.$^{23}$
Figure 3.11. Michaelis-Menten curves for dopamine in 25 mM HEPES buffer pH 7.0. In each experiment, the reactions were monitored by HPLC after methanol quench. The results of both curves were then fit to Eq. 3.1 with the data in Figure 3.11.a yielding a $K_M$ of $(3.9 \pm 0.2)$ mM and the data in Figure 3.11.b yielding a $K_M$ of $(4 \pm 1)$ mM.

Once again these results can be explained by the differences in both temperature and pH between the reported assay and the observed values. However, because saturation was never achieved, we decided to test another buffer, 25 mM maleic acid (pH 6.7). As with all of the other assays, these reactions were monitored using the methanol quench procedure along with HPLC separation followed by UV detection of the norcoclaurine peak at 225 nm. The rates were determined by plotting the change in the area of the norcoclaurine peak against time in seconds yielding rates with units of $\mu$V*s/s. With this buffering system, experiments to determine a $K_M$ for both HPAA and dopamine were performed. The HPAA varied experiment tested concentrations of HPAA from 0.032 to 4.0 mM, with methanol quenches being performed every 2 minutes and 40 seconds. Figure 3.12.a displays the curve resulting from this experiment, which after plotting the observed rates against concentration of HPAA and fitting to Eq. 3.1 yielded a $K_M$ of $(1.6 \pm 0.3)$ mM. The dopamine varied experiment was performed and analyzed in an identical manner, with the exception that the concentrations tested ranged from 0.026 to 3.34 mM. Figure 3.12b shows the Michaelis-Menten plot for this reaction, yielding a $K_M$ of $(0.76 \pm 0.08)$ mM.
Figure 3.12. Michaelis-Menten plots for enzyme reactions in Maleic acid buffer. a. Michaelis-Menten plot for HPAA in 25 mM maleic acid buffer pH 6.7. Fitting of this curve to Eq. 3.1 resulted in a $K_M$ of $(1.6 \pm 0.3)$ mM. Figure 3.12.b. Michaelis-Menten plot for dopamine in 25 mM maleic acid buffer pH 6.7. Fitting of this plot resulted in a $K_M$ of $(0.76 \pm 0.08)$ mM. The progress of reaction was monitored by HPLC by monitoring the integrated area of the norcoclaurine peak at 225 nm. The resulting areas were plotted as a function of time yielding the rates in units of $\mu$V*s/s. These rates were then plotted against the concentration of substrate and the curves were fit to Eq. 3.1.

The results of this study were encouraging because this was the first buffering system that appeared to result in saturation of the enzyme for dopamine, and was the first buffering system to yield a reasonable $K_M$ for the dopamine substrate. However, when these experiments were repeated, this trend was not observed, and instead the enzyme reactions were once again found to not reach saturation, and resulted in unreasonably large $K_M$ values. Figure 3.13 displays the result of one of these assays, resulting in a $K_M$ of $(2.6 \pm 0.6)$ mM. After a further analysis of the data, it appeared that each rate was double the rate of the one for the concentration below it, resulting in linear trends rather than the expected hyperbolic trend typically associated with enzyme catalysis. Table 3.2 and Figure 3.14 show a sample of data that followed this trend, yielding a linear Michaelis-Menten plot and a $K_M$ of $(33 \pm 4)$ mM. Based on this result, it appeared that the reaction was dependent only on the concentration of substrate, and it was thought that we were only observing background reaction, and not enzyme catalysis.
Figure 3.13. Michaelis-Menten curve showing results showcasing that the enzyme reactions fail to reach saturation. The \( K_M \) value for this sample was found to be \((2.6 \pm 0.6)\) mM after fitting to Eq. 3.1.

Table 3.2: Concentrations and resulting rates for a dopamine varied experiment.

<table>
<thead>
<tr>
<th>[Dopamine] (mM)</th>
<th>Rate ((\mu V*)s /s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7</td>
<td>2143</td>
</tr>
<tr>
<td>1.9</td>
<td>1152</td>
</tr>
<tr>
<td>0.93</td>
<td>579</td>
</tr>
<tr>
<td>0.46</td>
<td>303</td>
</tr>
<tr>
<td>0.23</td>
<td>148</td>
</tr>
<tr>
<td>0.12</td>
<td>73.9</td>
</tr>
<tr>
<td>0.058</td>
<td>44.3</td>
</tr>
<tr>
<td>0.029</td>
<td>27.2</td>
</tr>
</tbody>
</table>
Figure 3.14. Michaelis-Menten curve for the data in Table 3.2. The data in this plot appear to increase almost linearly rather than hyperbolically, with each rate appearing to be approximately twice the rate of the rate for the concentration below it. The $K_M$ resulting from this data was found to be $(33 \pm 4)$ mM after fitting to Eq. 3.1.

Based on this observation, a new expression of NCS was performed and several enzyme assays were performed, all of them unfortunately yielding similar results where saturation was never reached, yielding greatly inflated $K_M$ values. When looking into the data, more closely it was noticed that the rates were not extrapolating to 0 AU absorbance at 0 seconds time, and that this effect was more pronounced the longer the samples were waiting before injection into the HPLC (Figure 3.15). It was also noticed that even when capped the level of solution in the vials was noticeably lowered after the samples had completed running. Based on this evidence it seemed likely that the methanol was evaporating while the samples were awaiting injection. Based on this knowledge, we decided to incorporate a new quench method into the procedure that prevented the use of volatile organic solvents in the hope that it would result in cleaner data. This method involved the use of 1 M HCl to quench the solution by lowering the pH to <2 by adding 20 µL of 1M HCl into 80 µL of reaction sample. This pH was chosen based on the observation that the background reaction at this pH was negligible even when performed in phosphate buffer, and the enzyme was fully quenched at this pH. Using this procedure another assay was performed. In this case the rates extrapolated back to zero (Figure 3.16), but the reaction still failed to reach saturation and yielded inflated $K_M$ values.
Figure 3.15. Sample kinetic data for an enzyme assay using the previously stated methanol quench procedure. In each case, the rates fail to extrapolate back to zero suggesting that evaporation of the solution is occurring while the samples await injection into the HPLC.

Figure 3.16. Sample kinetic data for an enzyme assay using the newly developed HCl quench method. In each reaction samples are quenched by the addition of 20 µL of 1 M HCl into 80 µL of reaction sample. This data reveals that the rates extrapolate to a value much closer to 0 suggesting that less evaporation is occurring.

Even when the highest concentration of HPAA was increased to an order of magnitude higher than the previously reported saturation point of the enzyme by Luk et al. (10.73 mM), saturation was not achieved, and the resulting $K_M$ was $(6 \pm 1)$ mM (Figure 3.17). These results were disappointing because they suggest that there is something wrong with the enzyme itself. When looking into the
differences between the construct tested in our lab and the one used by Luk et al. It was discovered that our enzyme contained an N-terminal His tag, while the one used by Luk et al. contained a C-terminal tag. A second look at the crystal structure then revealed that the protein crystallizes as a dimer, which was in agreement with the results by Luk et al. that the activity of this protein on the dopamine substrate displays cooperative binding, with a Hill coefficient of 1.98.\textsuperscript{23, 61} The cooperative nature of this enzyme suggests that it behaves as a dimer in solution as well. This was an important discovery since the crystal structure reveals that when dimerized, the N-terminus of the protein is close to the site where the two peptides form a dimer, and it is possible that the extension of the N-terminus by adding a His tag can prevent the protein from dimerizing and decrease its activity. This seems to be supported by our results as we have never observed sigmoidal kinetics consistent with the expected cooperative binding, and a Hill coefficient of \textasciitilde2. Additionally, previously reported observations by Samanani et al. also note that altering the N-terminus of NCS may alter its activity. In this report, recombinant NCS\textDelta10 was cloned into a PET29b vector between the BamHI and XhoI restriction sites. Incorporation into these sites resulted in a C-terminal His tag, and the addition of an approximately 4 kDa peptide chain on the N-terminus. The kinetic studies performed using this protein also resulted in a reduced activity, with $K_M$ values approximately twice those of the natural enzyme.\textsuperscript{61} This suggests that the decreased activity could result from the extended N-terminus, which may prevent the proteins from forming the dimer, and thus preventing its cooperative activity from occurring. Currently we are awaiting the delivery of the exact construct used by Luk et al. in order to determine if the issues we are having with the inconsistency of this enzyme's activity is related to the N-terminal His tag, after which these experiments will be repeated with the hopes of yielding an enzyme that delivers $K_M$ values consistent with those described in the literature. Once that has been achieved, we then hope to characterize the kinetics of our analogues by determining their $K_M$ as well.
Figure 3.17. Kinetics data for an HPAA varied experiment where the highest concentration of HPAA is 10.7 mM. From these data it appears that the enzyme is not saturated even at concentrations an order of magnitude higher than the values reported by Luk et al.\textsuperscript{21}

### 3.3 Evaluation of Unnatural HPAA analogues as Substrates for NCS

Based on the realization that the major issue with these assays can be attributed to issues in the structure of the enzyme, it was decided to abandon any attempts to characterize the enzyme in terms of its $K_M$ and instead an approach similar to the one used by Ruff et al. and Presnot et al. was taken. These studies characterized the ability of the enzyme to turn over the substrate using a single point kinetic approach, where they ran an enzyme reaction using either the native substrate or analogues for a set amount of time and then quenched the reaction and determined the percent turnover of that substrate. Although this information was interesting, the value of a single data point reveals very little about the enzyme itself, and instead we decided to run side by side reactions to gather rate data for the analogues relative to the natural substrate. In order to ensure that the data was as accurate as possible, all of the reactions were performed side by side using solutions containing 1 mM HPAA (or analogue). In order to ensure that a uniform amount of enzyme, dopamine, and buffer were present in each reaction, a large master mix solution containing all three was made. The reactions were then started by the addition of enough of this solution so that the final concentration of dopamine would be 1 mM, final concentration of HEPES would be 100 mM (pH 7.2), and the final concentration of enzyme would be 11 µg per reaction. Each reaction was then quenched at 1 minute time points through the addition of 20 µL of 1 M HCl into 80 µL of reaction. These quenches were then analyzed by HPLC by monitoring the area of the product at 225 nm, and plotting these values as a function of time to yield a rate in units of µV*s/s. These rates were then reported as a percent of the HPAA rate. These experiments were performed in triplicate, with a new HPAA control being run every time, so a direct comparison could be made between runs. The compounds tested were: HPAA (3), 3-chloroHPAA (25), 3-bromoHPAA (26), and 3-iodoHPAA (27). Table 3.3 details the result of this study, in which relative rates of (45 ± 4)%, (63 ± 6)%, and (67 ± 9)% for
analogues 25, 26, and 27, respectively. In addition to this, negative controls were performed, using added buffer rather than enzyme to confirm that the products formed were resulting from enzyme catalysis and not background buffer catalysis. These tests resulted in no detectable norcoclaurine products, suggesting that the products seen were formed through enzyme catalysis. Currently, experiments to further verify the enzyme’s activity and stereoselectivity are being performed using chiral HPLC to separate the two enantiomers. Initial results of these experiments suggest that only the S-enantiomer is present when norcoclaurine is synthesized using enzyme catalysis, further confirming the enzyme’s activity.

**Table 3.3. Rates of tested HPAA analogues relative to the natural substrate HPAA (3).** The reported error is the standard deviation through three trials.

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>Structure</th>
<th>Rate relative to HPAA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td><img src="image" alt="Structure 3" /></td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td><img src="image" alt="Structure 25" /></td>
<td>45 ± 4</td>
</tr>
<tr>
<td>26</td>
<td><img src="image" alt="Structure 26" /></td>
<td>63 ± 6</td>
</tr>
<tr>
<td>27</td>
<td><img src="image" alt="Structure 27" /></td>
<td>67 ± 9</td>
</tr>
</tbody>
</table>

The results of these experiments were interesting because they suggest that the larger and less polar 3-bromo and 3-iodoHPAA analogues are more easily taken up by the enzyme that the smaller more polar 3-chloroHPAA analogue, as seen by approximately a 20 % decrease in the relative rate for this compound over 26 and 27. One possible explanation for this observation is that the less polar analogues have better interactions with the amino acid side chains that make up the HPAA binding site (F99, F80, Y108, L76, and M183). This could result in a much more favorable fit in the active site for these less polar analogues, which may result in favorable activity over that of the more polar chlorinated compound. Even though they display a decreased rate, the results of this study are encouraging, since they suggest that the analogues can successfully be transformed by the enzyme into the respective norcoclaurine analogues, and thus make it through the first committed step of benzylisoquinoline biosynthesis. This suggests that these analogues maybe useful as precursor analogues in PDB type experiments of berberine and related alkaloids.

### 3.4 Summary

Norcoclaurine synthase catalyzes the first committed step to benzylisoquinoline biosynthesis, by catalyzing the stereoselective Pictet-Spengler reaction between dopamine and HPAA. Because of the enzyme’s importance in the biosynthetic pathway of berberine it is vitally important that the
synthesized 3-halogenated HPAA analogues can be transformed into norcoclaurine analogues by the enzyme if they are to be useful in PDB experiments of berberine. Several attempts to develop a useful kinetic assay for this enzyme were performed using HPLC to monitor the reaction. These assays tested different buffers, pH, concentration, and substrate concentrations with the hope of observing Michaelis-Menten saturation, and characterizing the kinetics by determining $K_M$ values for the different substrates. Ultimately these attempts proved unsuccessful, and it appears that the reason for this maybe due to the N-terminal His tag attached to the construct tested in this assay. Instead the rates of the different analogues were measured under identical circumstances. The rates were then reported relative to the rate of the native substrate (HPAA), yielding relative rates of $(45 \pm 4)$, $(63 \pm 6)$, and $(67 \pm 9)$ % of the rate for HPAA. Monitoring of the background reaction yielded no detectable norcoclaurine product suggesting that the observed catalysis was performed by the enzyme. These results suggest that the analogues are capable of entering the biosynthetic pathway of berberine. Currently studies are being performed to demonstrate that the enzyme is yielding enantiomerically pure norcoclaurine through the use of chiral HPLC separation. In addition to this, work is being done to confirm that the N-terminal His tag is responsible for the decreased activity that our construct displays, including the use of thrombin to cleave the His tag. We also plan to obtain the construct used by Luk et al. and perform related experiments using this enzyme as well.
Chapter 4 Tyramine Analogues

4.1 Introduction

In addition to the study of the berberine biosynthetic pathway, it was also desired to study the biosynthesis of galanthamine through the use of precursor directed biosynthesis. Of the several precursor compounds available for study, we decided that tyramine analogues were the most suitable. This meant that an efficient method for producing halogenated tyramine analogues was necessary. In this case, two methods were examined. The first step of both of these pathways was the halogenation of 4-hydroxybenzaldehyde, as explained in section 4.2. The first of these pathways involved converting the synthesized 3-halogenated-hydroxybenzaldehyde compounds (29,30) into the corresponding 3-halogenated-4-hydroxynitrostyrene (47,48), followed by a zinc mediated reduction to the corresponding 3-halogenatedtyramine analogues (49,50) (Scheme 4.2). This method was desirable because it would allow for an efficient synthesis of the desired compounds using only three reactions: halogenations nitro-aldol condensation followed by reduction. The second strategy was modified from the report by Weinstock et al. and also started with 3-halogenated-4-hydroxybenzaldehyde (29,30), with the first step being phenol protection to yield the corresponding 3-halogenated-4-methoxybenzaldehyde compounds (31,32). These compounds could then be reduced to the corresponding 3-halogenated benzyl alcohol (51,52) allowing for the conversion to the corresponding 3-halogenated benzylchloride (53,54), allowing for the an S_n2 type reaction with the cyanide anion to form the respective benzylcyanide (55,56). Finally, the benzylcyanide compounds can be reduced to the 3-halogenated-4-methoxytyramine analogues (57,58), and deprotected to give the desired tyramine analogues (Scheme 4.3). Although this was significantly more steps than the other strategy, each of the reactions to be utilized were reported to have high yields, rapid reaction times, and minimal work up necessary, which made this strategy attractive.

4.2 Chlorination and Bromination of 4-hydroxybenzaldehyde

As previously stated, 28 was the starting material for both synthetic strategies, and the halogenations were performed as described in Chapter 2 using SO_2Cl_2 and NBS for chlorination and bromination, respectively. These reactions went smoothly, producing 3-chloro-4-hydroxybenzaldehyde (29), and 3-bromo-4-hydroxybenzaldehyde (30) with yields of 61 and 86 % respectively (Scheme 4.1). This was due to each reaction having a unique mechanism to promote monohalogenation. In the case of chlorination, the halogenated product precipitates from solution preventing it from further reaction, whereas the reaction with NBS utilizes toluene sulfonic acid as a directing reagent, physically blocking the second halogenation site from being able to react (see chapter 2, Figure 2.5) for a more in depth discussion).

The confirmation of these products was achieved through $^1$H NMR spectroscopy and GC/MS (Figure 4.1 and Figure 4.2). In particular, changes in the aromatic peaks were used to determine that the halogenation was successful. In each case, it was expected that the two aromatic doublets in the starting material would shift to form three aromatic peaks each representing one proton. In addition it was expected that these peaks would include one singlet and two doublets. As expected the $^1$H NMR spectra show these peaks, although the expected singlet (proton B in Figure 4.1) also shows some long distance coupling to the proton on the other side of the aldehyde (proton C in Figure 4.1). This is evidenced by peak B having a small degree of doublet character, while peak C appears as a doublet of doublets. GC/MS data for these compounds also suggests that the desired compounds were produced by these reactions. The mass spectrum for compound 29 yielded an m/z of 156 Da, and the characteristic (M+2)$^+$ peak of 158 Da, that is typically associated with the presence of a chlorine. Similarly, GC/MS analysis for compound 30 was also in agreement with the expectations for this compound, yielding an m/z of 200 Da, with the characteristic (M+2)$^+$ peak at 202 Da, which are in agreement with the expected masses of 199.95 and 201.95 Da for the major isotopes of this compound (Figure 4.2).

Figure 4.1. $^1$H NMR Spectra for 3-halogenated-4-hydroxy benzaldehyde Compounds. a $^1$H NMR Spectrum for 3-chloro-4-hydroxybenzaldehyde. b. $^1$H NMR spectrum for 3-bromo-4-hydroxybenzaldehyde. In both cases the labeled peaks correspond to the labeled protons in the picture.
GC/MS Spectra for 3-halogenated-4-hydroxybenzaldehyde compounds. a. GC/MS spectrum for 3-chloro-4-hydroxybenzaldehyde. Analysis of this compound resulted in a M⁺ of 156 with a percent abundance of 70 % and a (M+2)⁺ peak of 158 with a percent abundance of 21 %, both consistent with the expected values of 156.00 and 157.99 Da. The appearance of a (M+2)⁺ peak that is approximately 30 % of the M⁺ is also consistent with the addition of a chlorine atom, due to chlorine having two major isotopes.

b. GC/MS spectrum for 3-bromo-4-hydroxybenzaldehyde. Analysis of this compound resulted in a M⁺ of 200 with a percent abundance of 55 % and a (M+2)⁺ peak of 202 with a percent abundance of 60 %, both of which are consistent with the expected values of 199.95 and 201.95 Da. The appearance of an (M+2)⁺ peak that is approximately equal in abundance to the M⁺ is also consistent with the incorporation of a bromine atom due to bromine having two major isotopes that are approximately equal in percent abundance.

4.3 Nitro-aldol Attempt at generating tyramine analogues

Once the halogenations were complete, the generation of tyramine analogues using a nitro-aldol approach was evaluated (Scheme 4.2). These reactions were relatively straightforward yielding the 3-halogenated-4-hydroxy nitrostyrenes by reflux of the benzaldehyde in nitromethane in the presence of ammonium acetate, giving excellent yields of 94 % for both 3-chloro-4-hydroxy nitrostyrene (47) and 3-bromo-4-hydroxy nitrostyrene (48) after extraction into diethyl ether and rotary evaporation. As can be seen from the ¹H NMR of these compounds, there is a new set of doublets that are coupled to one another, and appear to be in a conjugated system. For compound 47, these peaks appear as broad doublets at 7.97 – 7.88 and 7.52 ppm, while for compound 48, they appear at 7.92 and 7.53 ppm, for protons labeled as A and B (Figure 4.3).

Figure 4.3. $^1$H NMR spectra for 3-halogenated-4-methoxybenzaldehyde compounds. a. $^1$H NMR spectrum for 3-chloro-4-hydroxyphenylnitrostyrene. b. $^1$H NMR spectrum for 3-bromo-4-hydroxy nitrostyrene. The lettered peaks correspond to each of the respective lettered protons. The solvent used in these spectra is CDCl$_3$ and is responsible for the peak at about 7.29 ppm. The peak at about 1.56 ppm is most likely water.

After successfully synthesizing the two nitrostyrene compounds in high yield, a method of reducing the nitrostyrene to the phenethylamine had to be established. For this procedure a zinc mediated reduction in the presence of HCl was attempted, which utilizes the reaction between zinc and HCl to generate hydrogen gas, which can then serve as the reducing agent for the nitrostyrene compound to yield the respective phenethylamine. Based on previous work performed in our lab, it was known that this reaction is strongly dependent on temperature, and the reaction must be kept at temperatures < 4 °C. Based on these results, the general procedure for this reaction called for a 1:1 solution of concentrated HCl in methanol to be cooled in an ice/salt water bath. In addition to the ice bath, the zinc and nitrostyrene had to be added in alternating portions over 2 hours in order to prevent the reaction temperature from rising too quickly. This reaction was attempted twice and gave inconsistent results. HPLC analysis of the first time it was attempted appeared to yield one major product, but when this was repeated several side products were observed as seen in Figure 4.4. In addition to this, all attempts to purify the desired compound failed, yielding total product loss. This resulted in an inability to characterize the products of this reaction to ensure that the observed product was in fact 3-chlorotyramine. Due to the inconsistencies observed from this reaction, it was decided it would be better to pursue another route to the desired compounds rather than attempt to optimize this reaction.
Figure 4.4. HPLC analysis of the products for the zinc reduction of 3-chlorotyramine. The initial attempt (top) produced one major product, while the second attempt (bottom) produced several side products. In both chromatograms the intensity of the maximum absorbance at each time is plotted.
4.4 Successful Generation of tyramine analogues

After this initial attempt to convert the nitrostyrene products into the respective tyramine analogues, the method of synthesizing phenethylamines reported by Weinstock et al. was attempted. This scheme of reactions also started with 28, which was then halogenated to either 29 or 30. These could then be reduced to the corresponding benzyl alcohols, transformed into benzyl chlorides, extended by one carbon to make the benzyl cyanides, and finally reduced to the corresponding phenethylamines (Scheme 4.3). The first step required the protection of the phenol, which was achieved through an $S_{N}2$ type reaction between the phenol and iodomethane in the presence of NaOH. These reactions were relatively straight forward and gave good yields of 79 and 81 % for compounds 31 and 32, respectively, after filtration through a silica plug. The evidence that this was achieved can be seen in the $^1H$ NMR spectra for these compounds, which display a new singlet peak at 4.02 ppm for compound 31 and 4.00 ppm for compound 32. This new peak integrates to three protons and is within the range of what is expected for a methoxyl proton, suggesting that this new functional group was successfully added (proton E in Figure 4.5 a and b). In addition to this, GC/MS analysis of the products yielded an m/z of 170 Da and a (M+2)$^+$ of 172 Da for compound 20, both of which agreed with the calculated values of 170.01 and 172.01 Da (Figure 4.6.a). GC/MS analysis of compound 32 also yielded a M$^+$ of 214 Da and a (M+2)$^+$ of 216 Da, which agree with the expected values of 213.96 ad 215.96 Da (Figure 4.6.b).

Figure 4.5 $^1$H NMR spectrum for 3-halogenated-4-methoxybenzaldehyde compounds. a. $^1$H NMR spectrum for 3-chloro-4-methoxybenzaldehyde. b. $^1$H NMR spectrum for 3-bromo-4-methoxybenzaldehyde. In each spectrum, the labeled protons correspond with their respective peaks. In both cases, the solvent is CDCl$_3$ resulting in the solvent peak at 7.29 ppm. the solvent peak at 1.56 ppm is most likely due to water.

Figure 4.6 GC/MS spectra for 3-halogenated-4-methoxybenzaldehyde compounds. a. GC/MS spectrum for 3-chloro-4-methoxybenzaldehyde. The M$^+$ for this compound was found to be 170 Da with an (M+2)$^+$ of 172 Da, both of which are consistent with the expected values of 170.01 and 172.01 Da, for this compound. The mass fragments at 169 Da and 171 Da are consistent with the loss of the aldehyde proton for the compound resulting in fragments that are 1 mass unit less, but still showing the characteristic chlorine isotopic abundance. b. GC/MS spectrum for 3-bromo-4-methoxybenzaldehyde. The M$^+$ for this compound is 214 Da with an (M+2)$^+$ of 216 Da, which are both consistent with the expected masses of 213.96 and 215.96 Da. The mass peaks of 213 Da and 215 Da suggest the loss of the aldehyde proton as well, resulting in fragments that are 1 unit less, but still showing the characteristic bromine isotopic abundance. In both spectra the inset is added to emphasize the molecular ions and the characteristic isotopic ratios resulting from the chlorine and bromine atoms.

The next reaction in the series was a reduction of the benzaldehyde to a benzyl alcohol using NaBH$_4$. Once again this reaction worked very well requiring only 0.7 equivalents of the borohydride and 1 hour of stirring at room temperature. The only issue with this reaction was that there were some residual impurities left over after extraction, which can be seen in the $^1$H NMR spectra for these compounds (Figure 4.7 a and b). Because of this, the calculated yields were artificially high values of 120% and 93% for compounds 51 and 52, respectively. Although these impurities were worrisome, it was
discovered that they did not impact the results of the next reaction so they were not dealt with as it was hoped they could be removed more easily after the future steps. The $^1$H NMR spectra for these compounds show that the reductions were successful, as they show a disappearance of the aldehyde peak around 9 ppm, and in both cases a new triplet is observed. The presence of this triplet is consistent with a primary alcohol proton that is being split by the two methylene protons found adjacent to it. In addition to this, GC/MS analysis of compound 51 resulted in an m/z of 172 Da and an $(M+2)^+$ of 174 Da, which is consistent with the molecular weights of 172.03 and 174.03 Da for this compound (Figure 4.8.a). Similarly for compound 52 the GC/MS yielded an m/z of 216 Da with an $(M+2)^+$ of 218 Da, which are also consistent with the expected molecular weights of 215.98 and 217.98 Da (Figure 4.8.b). Additionally, IR spectral results show the presence of a broad peak from 3323 to 3498 cm$^{-1}$ for compound 51 and a broad peak from 3078 to 3489 cm$^{-1}$ for compound 52, both of which are consistent with the expected OH stretch from the new alcohol.

![Figure 4.7 $^1$H NMR spectra for 3-halogenated-4-methoxybenzylalcohol compounds.](image1)

![Figure 4.8 GC/MS spectra for 3-halogenated-4-methoxybenzylalcohol compounds.](image2)
both of which are consistent with the expected molecular weights of 215.98 and 217.98 Da. In both spectra the insert is added to emphasize the characteristic \((M+2)^+\) peaks resulting from the chlorine and bromine atoms.

As with the previous two reactions, the chlorination using thionyl chloride (SOCl₂) also worked very well, giving quantitative conversion of the alcohol to the benzyl chloride within one hour, and isolated yields of 83 and 99 % for compounds 53 and 54, respectively. Evidence for the success of this reaction can be found in the \(^1\text{H}\) NMR spectra for these compounds (Figure 4.9 a and b), both of which show a loss of the triplet peak, suggesting that the alcohol is no longer present. In addition to this, the methylene doublets for compounds 53 and 54, that were doublets due to their splitting by the OH proton are now singlets, suggesting that the alcohol has been replaced by a different functional group that does not contain any protons. Furthermore, GC/MS analysis of the products confirmed the addition of a chorine atom to the compound, resulting in a unique fragmentation pattern. This pattern included \(M^+, (M+2)^+,\) and \((M+4)^+\) peaks for both compounds. For compound 53, the \(M^+\) was 190 Da, the \((M+2)^+\) was 192 Da, and the \((M+4)^+\) was 194 Da (Figure 4.10). These values were consistent with the expected values of 190.00, 191.99, and 193.99 Da, respectively. Similarly compound 54 displayed a \(M^+\) of 234 Da, an \((M+2)^+\) of 236 Da, and an \((M+4)^+\) of 238 Da, all of which are in agreement with the expected values of 233.94, 235.94, and 237.94 Da, respectively (Figure 4.10).

![Figure 4.9](image_url) \(^1\text{H}\) NMR spectra for 3-halogenated-4-methoxybenzylchloride compounds. a \(^1\text{H}\) NMR spectrum for 3-chloro-4-methoxybenzylchloride. b. \(^1\text{H}\) NMR spectrum for 3-bromo-4-methoxybenzylchloride. In each spectrum the labeled protons correspond with their respectively labeled peaks.
Figure 4.10 GC/MS spectra for 3-halogenated-4-methoxybenzylchloride compounds. a. GC/MS spectrum for 3-chloro-4-methoxybenzylchloride. The M⁺ for this compound was found to be 190 Da, with an (M+2)⁺ of 192 Da, and an (M+4)⁺ of 194 Da, all of which agree with the expected masses of 190.00, 191.99, and 193.99 Da. Figure 4.10b. GC/MS spectrum for 3-bromo-4-methoxybenzylchloride. The M⁺ for this compound was found to be 234 Da, with an (M+2)⁺ of 236 Da, and an (M+4)⁺ of 238 Da, all of which are consistent with the expected molecular weights of 233.94, 235.94, and 237.94 Da. In both spectra the appearance of the (M+4)⁺ peak is in agreement with the addition of a chlorine atom to the compound resulting from chlorine's two abundant isotopes. The inserts in both spectra emphasize the complex M⁺, (M+2)⁺, and (M+4)⁺ fragmentation patterns for this compound.

The next reaction involved the use of an S_N2 reaction between the cyanide anion and the halogenated benzyl chloride in order to form the requisite carbon-carbon bond, while simultaneously adding the necessary nitrogen. These reactions too, were fairly straightforward, as they were complete within an hour of adding NaCN and heating to 40 °C, giving excellent yields of 90 % for both compounds 55 and 56. Although the ¹H NMR spectra for these compounds are not significantly different than those of the benzylchloride compounds, it was still informative to see that the methylene peak had a large shift up field (proton A in Figure 4.11 a and b). For compound 53 this peak was found at 4.73 ppm, and was shifted to 3.95 ppm upon conversion to 55, while this peak shifted from 4.73 ppm to 3.93 ppm upon transformation from 54 to 56. In addition to this, mass spectral data for these products were also in agreement with what is expected for the desired compounds. Analysis of compound 55 resulted in an M⁺ of 181 Da, with an (M+2)⁺ of 183 Da, which were in agreement with the expected values of 181.03 and 183.03 Da (Figure 4.12.a). Likewise analysis of compound 56 yielded an M⁺ of 225 Da and an (M+2)⁺ of 227 Da. These values are in agreement with the expected values of 224.98 and 226.98 Da (Figure 4.12.b). In both cases, the appearance of odd molecular weights suggested that a single nitrogen atom was added to the molecules, further suggesting that the desired transformation was successful. Finally, IR spectroscopy for these compounds also suggests that a nitrile is present. The IR analysis of compound 55 displays a peak from 2204 to 2299 cm⁻¹, while compound 56 has a similar peak from 2208 to 2289 cm⁻¹, both of which are consistent with the expected CN triple bond stretch found in nitrile containing compounds, suggesting that the reaction was successful.
Figure 4.11 $^1$H NMR spectra for 3-halogenated-4-methoxybenzylcyanide compounds. a $^1$H NMR spectrum for 3-chloro-4-methoxybenzylcyanide. b $^1$H NMR spectrum for 3-bromo-4-methoxybenzylcyanide. In each spectrum the labeled protons correspond with their respectively labeled peaks.

Figure 4.12 GC/MS spectra for 3-halogenated-4-methoxybenzylcyanide compounds. a GC/MS spectrum for 3-chloro-4-methoxybenzylcyanide. GC/MS analysis of this compound yielded an $M^+$ of 181 Da and an (M+2)$^+$ of 183 Da, both of which are consistent with the expected values of 181.03 and 183.03 Da. In addition to this, the odd molecular ions are also consistent with the incorporation of a single nitrogen atom into the molecule suggesting the desired transformation was successful. b GC/MS spectrum for 3-bromo-4-methoxybenzylcyanide. The resulting $M^+$ and (M+2)$^+$ values were 225 Da and 227 Da respectively, and were in agreement with the expected values of 224.98 and 226.98 Da. Once again the odd molecular ions are in agreement with the incorporation of a single nitrogen atom into the molecule. In both spectra the insert shows a zoomed in picture of the molecular ions.

Once the halogenated benzylcyanides were obtained, they were reduced to the desired phenethylamines by a simple reflux in a solution of BH$_3$-THF complex, which gave the amines as free bases. These were then crystallized as their respective hydrobromide salts giving yields of 51 and 88 % for compounds 57 and 58, respectively. The $^1$H NMR spectra where very useful in determining the success of these reactions, since in both spectra there was a new broad peak that integrated to three protons, which is consistent with the production of a primary amine (proton A in Figure 4.13 a and b). For compound 57 this peak appeared at 7.75 ppm and for compound 58 this peak was found at 7.71 ppm. The IR spectra for these compounds also show the loss of the nitrile peaks at about 2200 cm$^{-1}$, showing that the nitrile starting material was successfully transformed into a different product. The IR
spectrum for compound 57 displayed three new peaks from 3196-3271, 3290-3445, and 3445-3533 cm\(^{-1}\), which are consistent with what would be expected for the N-H stretches in a primary amine with three N-H bonds. The IR spectrum for compound 58, however, was not as well resolved and instead exhibited a single broad peak from 3202-3564 cm\(^{-1}\), which is within the expected range for the N-H stretch. The lack of resolution in this spectrum may indicate that the solvent or the compound may have had some residual water contamination, resulting in the broad peak. Based on these observations, it was determined that the two compounds were successfully synthesized.

![Figure 4.13 ¹H NMR spectra for 3-halogenated-4-methoxyphenethylamine compounds. a. ¹H NMR Spectra for 3-chloro-4-methoxyphenethylamine. b. ¹H NMR spectra for 3-bromo-4-methoxyphenethylamine. In both cases the solvent is DMSO-\(d_6\) resulting in the solvent peak at 2.56 ppm. In Figure 4.13.a. the broad peak at 4.1 ppm is most likely the result of \(\text{H}_3\text{O}^+\) present in the sample, while in Figure 4.13.b. the peak at 3.3 ppm is most likely due to water.](image)

The final step to producing the desired 3-bromo and 3-chloro tyramine analogues was deprotection of the phenol. This reaction was also easily performed for compound 57 through an acid catalyzed \(S_N\)2 type reaction, requiring only reflux of the compound in 48% HBr, followed by the removal of the solvent with a rotary evaporator, giving a yield of 88 % for compound 49. Analysis of the product by \(¹\text{H-NMR}\) spectroscopy confirms that the synthesis successfully yielded the desired 3-chlorotyramine. This can be seen by both the loss of the methoxy protons (proton G in Figure 4.13.a) and the appearance of a new singlet at 10.07 ppm (Proton G in Figure 4.14.a), which is consistent with what would be expected for the phenol proton. Deprotection of 58, however, resulted in a significant side product that was roughly 33 % of the total product obtained by \(¹\text{H NMR}\) spectroscopy. This is evident through the analysis of the product by \(¹\text{H NMR}\), which shows peaks that are consistent with the product, but also some new aromatic peaks as well (Figure 4.14.b). In addition to this, the amine signal (proton A in Figure 4.14) displays an integration value of 4.46, which is about 50 % greater than the expected value of 3. The methylene peaks (protons B and C in Figure 4.14.b) also display greater than expected values of 3.36 and 2.95, both of which are about 50 % greater than the expected value of 2. This suggests that the impurity is a second phenethylamine compound and there is a 2:1 ratio of the desired 3-bromotyramine to the undesired phenethylamine side product. Attempts to purify this compound by column chromatography did not yield a product of any greater purity, but instead resulted in a black oil. This suggests that the acid catalyzed deprotection of this compound is not suitable. Currently, we are exploring other
deprotection options that utilize less harsh conditions to mediate this reaction, including the use of Lewis acids such as BBr$_3$ to catalyze this reaction at low temperatures.

![Figure 4.14](image)

**Figure 4.14 $^1$H NMR spectra for 3-halogenated-4-hydroxyphenethylamine compounds.** a. $^1$H NMR spectrum for 3-chloro-4-hydroxyphenethylamine (3-chlorotyramine). b. $^1$H NMR spectrum for the mixture of products containing 3-bromo-4-hydroxyphenethylamine (3-bromotyramine). The integrated peaks are the peaks that are consistent with what is expected for the desired compound. There also appears to be a major side product which is evident by the appearance of new aromatic peaks. In addition to this, the amine (proton A) and methylene (protons B and C) protons integrate to values approximately 50% greater than their expected values suggesting that the side product is also a phenethylamine, and that there is a 2:1 ratio between the desired and undesired compounds.

### 4.5 Summary

During the synthesis of 3-halogenated tyramines (49 and 50), two different synthetic routes were explored, both of which started with the respective 3-halo-4-hydroxybenzaldehyde (29 and 30). The first procedure started with a nitro-aldol reaction to yield the nitrostyrene products 47 and 48 in good yield, however, the subsequent reduction to yield the 3-halogenated tyramine 49 and 50 compounds were unsuccessful. The alternative pathway tested started with compounds 29 and 30 as well. The phenol versions of these compounds were then protected by the addition of methyl groups using an S$_{\text{N}}$2 reaction between the benzaldehyde starting materials and iodomethane, yielding the 3-halo-4-methoxybenzaldehyde compounds (31 and 32). This protection was necessary for the success of the subsequent reduction, chlorination, cyanation, and nitrile reductions in order to yield the 3-halo-4-methoxyphenethylamine compounds (57 and 58). Deprotection of compound 57 was achieved through simple reflux in concentrated HBr. This yielded the target 3-chlorotyramine in 7 steps, with a cumulative yield of 4%. Deprotection of 58 was not achieved in the same manner, and as such the synthesis of the target molecule 3-bromotyramine was not achieved. However, synthesis of the 4-methoxy-3-bromophenethylamine precursor was achieved in 6 steps with an excellent cumulative yield of 51%. This compound is one step away from yielding the desired compound, which maybe available through the use of Lewis acid mediated S$_{\text{N}}$2 type reaction to yield the desired compound.
Chapter 5 Feeding Experiment Design and Proposed Work

5.1 One-pot Synthesis/Feeding experiment of HPAA and Norcoclaurine Analogues

As previously stated, the synthesis of the various halogenated HPAA and norcoclaurine analogues was performed with the intention of utilizing them as precursors for halogenated berberine analogues. In order for this to be achieved, a method of feeding the compounds had to be developed. The general procedure that was utilized before involved making a 1 mM solution of the analogue in a liquid solution of Gamborg’s media, then filter sterilizing the solution, and suspending *Berberis vulgaris* (cell line PC-464) callus cell culture, purchased from the Leibiniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ), (Figure 5.1) in the resulting solution for one week with shaking. This method was previously shown to work in experiments involving fluorinated dopamine analogues and it was hoped that the process could be adapted to become an extension of the NaOCl synthesis of HPAA analogues, and the one pot synthesis of norcoclaurine analogues. Since both of these reactions are performed utilizing benign reagents in a solution of phosphate buffer, it was hoped that the syntheses of halogenated HPAA and norcoclaurine analogues could be performed at 2 mM concentrations of HPAA using the procedures described in Chapter 2, then be quenched, and the solution diluted directly into an equal volume of doubly-concentrated Gamborg’s media, filter sterilized, and the cells suspended and shaken.

![Figure 5.1. PC 464 cell line used for PDB of unnatural berberine analogues.](image)

Left plate of *B. vulgaris* callus cell culture. Liquid media cultures of *B. Vulgaris* cells used in feeding experiments.
This was performed for each of the halogenated analogues of both HPAA and norcoclaurine by reacting a 2 mM solution of the respective tyrosine analogue with one equivalent of bleach until all of the halogenated tyrosine was consumed when monitored by HPLC. To this solution was added half an equivalent of ascorbic acid to promote a reducing environment in the solution, to quench any unreacted NaOCl, and to promote better stability of the products in solution. Ascorbic acid was chosen as the preservative of choice based on the results of two different reports that showed that ascorbic acid reduces the effects of salinity stress. This type of stress was a concern because the syntheses were performed in a solution of sodium phosphate buffer that is of relatively high concentration (10 mM), which may have adverse effects on the health of the cells. In addition to this, ascorbic acid is necessary for plant health, as it increases the plant’s ability to handle several other kinds of stresses including oxidative stress and damage from wounds and pathogens. Based on these observations, it seemed that the inclusion of this compound in cell media would serve three functions; quenching any unreacted NaOCl, stabilization of the catechol products from oxidation, and promotion of cell health. For the feeding of HPAA analogues, the resulting solution of ~2 mM analogue and 1 mM ascorbic acid was then filter sterilized into an equal volume of 2x Gamborg’s media to yield a solution containing 1 mM analogue, 0.5 mM ascorbic acid, and 5 mM phosphate in a 1 x solution of Gamborg’s media in which the PC-464 cells could be suspended and shaken.

This procedure was also adapted for the feeding of norcoclaurine analogues. This was performed using the same procedure for the preparation of HPAA and followed by an ascorbic acid quench yielding a solution containing 2 mM HPAA analogue and 1 mM ascorbic acid in 10 mM phosphate buffer. To the resulting solution, 1.2 equivalents of dopamine were added, and the reaction was allowed to proceed until all of the aldehyde was fully consumed. This resulted in a solution that was ~ 2 mM norcoclaurine analogue, 1 mM ascorbic acid, and 10 mM phosphate (pH 7.0). This solution was then filter sterilized into an equal volume of 2x media yielding a final solution of 1 mM norcoclaurine analogue, 0.5 mM ascorbic acid, 5 mM phosphate, in 1x solution of Gamborg’s media. A plate of PC-464 cells were then suspended in the resulting analogue-media solutions for a week at room temperature while shaking, after which the solids were separated from the liquids, freeze dried, and then stored at -80 °C, until they could be analyzed.

Currently, this procedure has been applied to the chlorinated and brominated HPAA and norcoclaurine analogues. The resulting cells and media were separated from each other by gravity filtration, and have been freeze dried, are currently stored at - 80 °C awaiting analysis. The proposed plan for analysis involves grinding the frozen cells and media in a solution of methanol in order to extract the organic soluble contents from the plant material and buffer salts. The liquid portion would then be filtered through a 0.22 µm filter and analyzed by high resolution LC/MS. The success of the incorporation of the analogues into the biosynthetic pathway would be determined through high resolution mass analysis of the extracted compounds. It is planned to look for the existence of all the possible intermediates in the biosynthesis of berberine as well as the final berberine product itself (Figure 5.2). If we find that the analogues are successfully incorporated into berberine biosynthetic pathway, then we hope to scale up the procedure and isolate the compound for future analysis. If it turns out that there is a buildup of an intermediate species, suggesting that the next enzyme in the pathway does not accept the analogue, we hope to develop mutant species of B. vulgaris that produce a mutant variety of the enzyme that might be able to incorporate our analogues. This analysis is currently
on hold until a collaboration between our group and a University or group that has access to a high resolution LC/MS, through which the samples could be run, is established.

![Diagram of predicted analogues](image)

**Figure 5.2.** Predicted analogues that may result from PDB experiments of 3-halogenated HPAA and 3-halogenated norcoclaurine analogues. When analyzing the LC/MS results of PDB, the predicted molecular weights of each the possible intermediate analogues would be looked for.

### 5.2 Proposed Feeding Experiments of Tyramine Analogue

In addition to the feeding of berberine precursors, we also hope to feed tyramine analogues in order to produce unnatural galanthamine analogues. This would involve a very similar feeding procedure to that adopted for dopamine analogues previously used in our lab. A sample of cells would be suspended and shaken in a 1 mM solution of the respective halogenated tyramine analogue in Gamborg's media, for one week. Because galanthamine is volatile enough for GC analysis, GC/MS would be utilized as the initial verification of the success of the experiment. This would be followed up by a high resolution LC/MS analysis of the solution for confirmation, looking for all of the possible intermediate and product analogues (Figure 5.3). Unlike the data analysis of berberine analogues, the analysis of these data would be made more difficult because for each tyramine analogue there are two possible resulting galanthamine analogues (Figure 5.3). If successful the hope is to scale up and isolate the galanthamine analogue for future experiments, and if the product does not successfully make it through the pathway, a mutant cell line would be developed. Currently these experiments are awaiting the successful development of callus cell culture for a *Narcissus* species, since all attempts to date have proven to be unsuccessful.
Figure 5.3: Predicted analogues that would arise from precursor directed biosynthesis of tyramine analogues. When analyzing the LC/MS results of PDB, the predicted molecular weights of each the possible intermediate analogues would be looked for.

5.3 Summary

A one-pot synthesis/feeding experiment was developed and performed for the PDB of unnatural berberine analogues for the synthesis and feeding of HPAA and norcoclaurine analogues. To date this procedure has been performed on all of the chlorinated and brominated HPAA and norcoclaurine analogues, with the resultant cells currently awaiting LC/MS analysis. To date no feeding of unnatural tyramine analogues has been performed as we are currently waiting the development of callus cell culture for a Narcissus species. Once this cell culture is attained, we hope to develop a feeding experiment based off the description given, with the hopes of generating unnatural galanthamine analogues.
Appendix A Experimental Procedures

**General Procedures.** All reagents were purchased from commercial suppliers at the highest available purity and used without further purification. Reactions were monitored using high pressure liquid chromatography on a Waters Acquity Ultra Performance liquid chromatography instrument equipped with an Acquity UPLC BEH C18 (1.7 µm) 2.1x100 mm column or an Acquity UPLC BEH C18 (1.7 µm) 2.1x50 mm column. The solvent conditions used for HPLC analysis are as follows: Condition A: 0-70 % acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) in MilliQ water over 10 minutes using the 2.1x100 mm column. Condition B: 0-70 % ACN in 0.1 % TFA over 4.75, holding at 70 % ACN for 0.25 minutes using the 2.1x50 mm column. Condition C: 0-17.5% ACN in 0.1 % TFA over 2.5 minutes followed by 17.5% to 70 % ACN in 0.1 % TFA over 1.5 minutes, holding at 70 % for 1 minutes using the 2.1x50 mm column. Gas Chromatography mass spectrometry analysis of products was performed using a Hewlett Packard HP6890 Gas Chromatography System with an Agilent Technologies 5975 inert mass selective detector. 1H and 19F NMR spectroscopy were performed using a Bruker Avance 300 MHz NMR spectrometer at 300 MHz, and 282 MHz respectively. All 1H NMR peaks are described relative to a tetramethylsilane internal standard. Infrared spectroscopy analysis of reaction products were performed using a ABB FT-IR FTLA 2000 spectrometer. All reactions involving air or water sensitive reagents were performed using flame dried glassware under an inert atmosphere of N2.

![Chemical Structures](image)

**3-iodo- 4-Hydroxyphenylacetic acid (14).** To a stirring solution of 4-hydroxyphenylacetic acid (11) (1.527 g, 10 mmol) in 70 mL of methanol and 40 mL of concentrated NH4OH was added I2 (5.712g, 22.5 mmol) dissolved in ethanol drop wise over 3 hours at 0°C. The reaction was allowed to stir for 1.5 more hours after which the solvent was removed under reduced pressure. The resulting solid was re-suspended in acetone, filtered and the solvent removed under reduced pressure yielding 15 (30%) as a yellow solid. 1H NMR (300 MHz, Acetone-d6) δ 9.06 (s, 1H), 7.70 (d, J = 2.0 Hz, 1H), 7.18 (dd, J = 8.3, 2.1 Hz, 1H), 6.92 (d, J = 8.3 Hz, 1H), 3.54 (s, 2H).LRMS (EI⁺) M/z 278 (M⁺, 55.01) 233 (100) 152 (0.78) 106 (14.7) 78 (9). HPLC retention time 2.52 mins condition B. Absorbance spectra λmax 229 nm minor λmax 284 nm.
**Methyl-2-(4-hydroxyphenyl) acetate (16).** To a stirring solution of 4-hydroxyphenyl acetic acid (12) (1.569g, 10.3 mmol) in methanol (60mL) was added concentrated H$_2$SO$_4$ (80 µL, cat.), and brought to reflux for 2 hours. The resulting solution was allowed to cool to room temp diluted with water and extracted into diethyl ether (3x20mL), and the combined organic layer washed with NaHCO$_3$ (2x20mL), brine (1x20mL), dried over MgSO$_4$ and the solvent removed under reduced pressure to yield 1.71g of (16) (92%) as a clear oil, which was stored in a freezer at -20°C. $^1$H NMR (300 MHz, Acetone d-6) δ 8.3 (s, 1H), 7.15 (d, 2H), 6.75 (d, 2H), 3.65 (s, 3H), 3.53 (s, 2H). LRMS (EI$^+$) M/z 166 (M$^+$, 26.6), 107 (100), 77 (14.6). HPLC retention time 6.86 mins condition A. Absorbance spectra $\lambda_{max}$ 222 nm minor $\lambda_{max}$ 281 nm.

**Ethyl 2-(4-hydroxy-3-chlorophenyl)acetate (13).** To a stirring solution of 3-chloro-4-hydroxyphenyl acetic acid (13) (0.5891 g, 3.14 mmol) in ethanol (50mL) was added concentrated H$_2$SO$_4$ (30 µL, cat.), and brought to reflux for 3 hours. The resulting solution was allowed to cool to room temp diluted with water and extracted into diethyl ether (3x20mL), and the organic layer washed with NaHCO$_3$ (2x20mL), washed with a brine solution (1x20mL) and then dried with MgSO$_4$. The solvent was then removed under reduced pressure to yield 0.636 of (59) (95%) as a clear oil, which was stored in a freezer at -20°C. $^1$H NMR (300 MHz, Acetone-d$_6$) δ 8.70 (s, 1H), 7.30 (d, $J$ = 2.1 Hz, 1H), 7.10 (dd, $J$ = 8.3, 2.1 Hz, 1H), 6.96 (d, $J$ = 8.3 Hz, 1H), 4.11 (q, $J$ = 7.1 Hz, 2H), 3.56 (s, 2H), 1.22 (t, $J$ = 7.1 Hz, 3H). LRMS (EI$^+$) M/z 214 (M$^+$, 23.7), 216 (M+2, 7.8), 143 (32.9), 141 (100), 77 (17.3).

**2-iodo-4-(2-hydroxyethyl)phenol (23).** A solution containing 3-iodo-4-hydroxyphenylacetic acid (15) (0.1334g, 0.5 mmol) and trimethyl borane (114 µL, 1.0 mmol) in 1 mL THF was slowly added over 5 minutes to an ice cold stirring solution of NaBH$_4$ (0.0377g, 1 mmol) in 2 mL THF. Upon completion of this addition, dimethyl sulfate (93 µL, 1 mmol) was slowly added over 5 minutes and the resulting solution was allowed to stir overnight at room temperature, after which the reaction was quenched by the addition of 3 mL of water. The resulting solution was then extracted into ethyl acetate (2X5 mL), the combined organic layers dried over MgSO$_4$, and the solvent removed under reduced pressure, yielding 0.022 g of (23) (17%) as an off white solid. $^1$H NMR (300 MHz, CDCl$_3$) δ 8.86 (s, 1H), 7.61 (d, $J$ = 2.1 Hz, 1H), 7.10 (dd, $J$ = 8.2, 2.1 Hz, 1H), 6.87 (d, $J$ = 8.2 Hz, 1H), 3.73 – 3.65 (m, 3H), 2.70 (t, $J$ = 6.6 Hz, 2H). LRMS (EI$^+$) M/z 306 (M$^+$, 60.6), 307 (M$^{+1}$, 7.3), 248 (7.8), 247 (100), 105 (10.8), 91 (7.3), 90 (19.7), 77 (14.6). HPLC retention time 2.48 mins condition B. Absorbance spectra $\lambda_{max}$ 217 nm minor $\lambda_{max}$ 284 nm.
4-(Methoxymethoxy)benzaldehyde (60).69 0.6106 g of 4-hydroxybenzaldehyde (44) (5 mmol) was dissolved in 30 mL of dry DMF (dried over 3 Å molecular Sieves) and in an oven dried 3 neck round bottom flask under a N₂ atmosphere and cooled to 0 °C. To this solution 0.2106 g of NaH (60% dispersion in mineral oil) (5.1 mmol) was slowly added over 30 minutes. The solution was allowed to warm to room temperature and 0.39 mL of chloromethoxymethyl ether (5.1 mmol) was slowly added via syringe and stirred for 3 hours. The reaction was then quenched with water and stirred for 10 minutes. The solvent was removed under reduced pressure yielding a tan oil, which was purified using dry vacuum column chromatography (Hexanes/EtOAc -0-25%) yielding 0.5545 g of 64 as a clear oil (66.8%). ¹H NMR (300 MHz, Acetone d-6) δ 9.91 (s, 1H), 7.85-7.95 (d, 2H), 7.17-7.28 (d, 2H), 5.29-5.40 (s, 2H), 3.40-3.54 (s, 3H) LRMS (EI⁺) m/z 161 (M⁺, 100), 135 (47), 121 (9), 105 (22), 77 (52), 65 (35), 51 (35).

(E)-1-methoxy-4-(2-methoxyvinyl)benzene(36).47, 48, 70 NaH (60% dispersion in mineral oil, 0.0818g, 2 mmol) was added to a dry flask and washed with hexanes (2 x 10 mL) under N₂ to remove the mineral oil. The resulting solid was suspended in 20 mL of sieve dried diethyl ether and methoxymethyltriphenylphosphonium chloride (0.7845 g, 2.3 mmol) was added slowly and brought to reflux for 30 minutes. The resulting solution was cooled to room temperature and 4-methoxybenzaldehyde (33) was slowly added. The solution was brought back to reflux for 15 hr, then the reaction was then diluted with ether and quenched by washing with H₂O (2 x 20mL). The organic layer was then washed once with 1 M HCl (1 x 20 mL) and once with brine (1 x 20 mL), then dried with MgSO₄. The solvent was removed under reduced pressure yielding a yellow oil, which was not characterized.

2-chloro-4-(2-hydroxyethyl)phenol (21).44 A suspension containing 4-(2-hydroxyethyl)phenol (20) (2.990, 21.6 mmol) and NaCl (1.266 g, 21.6 mmol) in acetone (55 mL) was cooled to 0 °C while a solution of oxone (19.818 g, 32.2 mmol) in water (100 mL) was added drop wise over three hours. The reaction was then allowed to stir overnight at room temperature, after which the reaction was extracted with ethyl acetate (3 x 60 mL) and the combined organic layers washed with brine (1 x 30 mL). The resulting solution was then dried with MgSO₄, filtered and the solvent removed under reduced pressure yielding an amber colored oil. The resulting oil was then dissolved in a minimal amount of hot dichloromethane and cooled yielding 3.735 g of 21 (72 %) as white crystals. ¹H NMR (300 MHz, Acetone-
2-bromo-4-(2-hydroxyethyl)phenol (22). A suspension containing 20 (3.463, 25 mmol) and NaBr (2.589 g, 25.2 mmol) in acetone (55 mL) was cooled to 0 °C while a solution of oxone (23.153 g, 32.2 mmol) in water (100 mL) was added drop wise over three hours. The reaction was then allowed to stir for three additional hours at room temperature, after which the reaction was extracted with ethyl acetate (3 x 60 mL) and the combined organic layers washed with brine (1 x 30 mL). The resulting solution was then dried with MgSO₄, filtered and the solvent removed under reduced pressure yielding an amber colored oil. The resulting oil was then dissolved in a minimal amount of hot dichloromethane and cooled yielding 4.292 g of 20 (79 %) as a white solid. ¹H NMR (300 MHz, Acetone D-6) δ 8.64 (s, 1H), 7.39 (d, J = 2.0 Hz, 1H), 7.07 (dt, J = 8.1, 4.1 Hz, 1H), 6.95 – 6.89 (m, 1H), 3.72 (dd, J = 6.1, 4.4 Hz, 2H), 2.79 – 2.66 (m, 2H). LRMS (EI⁺) m/z 216 (M⁺, 23.8), 217 (M⁺+1, 2.4), 218 (M⁺+2, 24.3 173 (16), 188 (9.0), 187 (97.9), 186 (9.1), 185 (100), 107 (10) 106 (4.4) 78 (9.3), ( 77 (21.7). HPLC retention time 2.31 mins. Absorbance spectra λmax 218 nm minor λmax 281 nm.

2-iodo-4-(2-hydroxyethyl)phenol (23). A solution of I₂ (5.074 g, 20 mmol) and KI (4.374 g, 26 mmol) in 100 mL of a 1:1 water and ethanol was added to a solution containing 4-(2-hydroxyethyl)phenol (20) (2.733 g, 20 mmol) dissolved in 20 mL of a 40% solution of dimethyl ammine. The resulting solution was allowed to stir at room temp for 4 hours after which it was acidified with a 2 M solution a HCl and extracted into ethyl acetate (3x100 mL). The combined organic layers were then washed with a 10% (m/V) solution of sodium thiosulfate (2x50 mL) and brine (1x40mL). The resulting organic layer was then dried with MgSO₄, filtered, and the solvent removed under reduced pressure yielding a brown oil. The resulting oil was dissolved in a minimal amount of hot water and cooled yielding 3.7602 g of 20 (72 %) as white needle like crystals. ¹H NMR (300 MHz, CDCl₃) δ 7.55 (d, J = 2.0 Hz, 2H), 7.12 (dd, J = 8.2, 2.0 Hz, 2H), 6.93 (d, J = 8.3 Hz, 2H), 5.40 (s, 2H), 3.84 (t, J = 6.5 Hz, 5H), 2.84 – 2.63 (m, 5H), 1.60 (d, J = 33.9 Hz, 4H). LRMS (EI⁺) m/z 264 (M⁺ 35.2), 265 (M⁺+1 3.0), 234 (9.8), 233 (100), 128 (1.4), 107 (5.0), 106 (13.9) 78 (7.8), 77 (6.5). HPLC retention time 2.48 mins condition B. Absorbance spectra λmax 217 nm minor λmax 284 nm.
2-fluoro-4-(2-hydroxyethyl)phenol (24). 1-Chloromethyl-4-fluoro-1,4-diazeniabicyclo[2.2.2]octane bis(tetrafluoroborate (Selectfluor®) (0.4666g, 13 mmol) and 20 (0.1620g, 12 mmol) were dissolved in 10 mL of a 1:1 methanol water solution, and allowed to stir for 36 hours, after which, 0.2148 g of Selectfluor® was added and the reaction allowed to stir 12 more hours. The resulting solution was then diluted into 25 mL of water, and extracted into ethyl acetate (3x25mL), the combined organic layers were then washed with 25 mL of a brine solution, dried with MgSO₄, filtered, and the solvent removed under reduced pressure, yielding 0.1771 g of an amber oil. ¹⁹F NMR analysis revealed several peaks suggesting that several fluorinated products were obtained. This was consistent with GCMS analysis, which suggested two different mono and difluorinated compounds were present.

4-hydroxyphenylacetaldehyde (3). In a flame dried flask purged with N₂, sulfur trioxide pyridine complex (3.1140 g, 20 mmol) was dissolved in 15 mL of sieve dried dimethyl sulfoxide. This solution was then canulated into a second flame dried flask purged with N₂ containing 20(0.9513 g, 6.8 mmol) and triethyl amine (3 mL, 21 mmol), dissolved in 15 mL of sieve dried dimethyl sulfoxide, slowly over a period of 15 mins. The resulting solution was allowed to stir at room temperature for 1.5 hours under a N₂ atmosphere, after which it was poured into 100 mL of ice cold water and acidified with 10 mL of 2 M HCl. The resulting solution was extracted into ethyl acetate (3x100 mL), and the combined organic fractions washed with brine (2x100 mL), dried with MgSO₄, filtered and the solvent removed under reduced pressure yielding a crude amber colored oil. The oil was purified by flash chromatography (20% ethyl acetate in hexanes) yielding 0.2272g of 3 (24%) as a clear oil. ¹H NMR (300 MHz, CDCl₃) δ 9.74 (t, J = 2.4 Hz, 1H), 7.15 – 7.06 (m, 2H), 6.90 – 6.82 (m, 2H), 4.80 (s, 1H), 3.63 (dd, J = 8.4, 2.9 Hz, 2H). LRMS (EI⁺)m/z 136 (M⁺1), 137 (M⁺, 1.7), 108 (9.5), 107 (100), 89 (.63), 78 (3.7), 77 (21.6). HPLC retention time 1.63 mins condition B. Absorbance spectra λ max 222 nm minor λ max 276 nm.

3-chloro-4-hydroxyphenylacetaldehyde (25). In a flame dried flask purged with N₂, sulfur trioxide pyridine complex (0.3110 g, 1.9 mmol) was dissolved in 5 mL of sieve dried dimethyl sulfoxide. This solution was then canulated into a second flame dried flask purged with N₂ containing 20 (0.1289 g, 0.75
mmol) and triethyl amine (0.33 mL, 3.7 mmol), dissolved in 5 mL of sieve dried dimethyl sulfoxide, slowly over a period of 15 mins. The resulting solution was allowed to stir at room temperature for 4 hours under a N₂ atmosphere, after which it was poured into 100 mL of ice cold water. The resulting solution was extracted into ethyl acetate (3x50 mL), and the combined organic fractions washed with brine (1x40 mL), dried with MgSO₄, filtered and the solvent removed under reduced pressure yielding a crude amber colored oil. Flash chromatography was performed (0-40% ethyl acetate in hexanes) yielding 0.1274 g of 25 (35.5%). ¹H NMR (300 MHz, CDCl₃) δ 9.74 (t, J = 2.2 Hz, 1H), 7.20 (d, J = 6.4 Hz, 1H), 7.12 – 7.01 (m, 2H), 5.62 (s, 1H), 3.66 (t, J = 11.0 Hz, 2H). LRMS (EI⁺) m/z 170 (M⁺, 5.89), 172 (M+2, 18.8), 143 (32.2), 141 (100), 105 (5.4), 77 (25.6). HPLC retention time 2.17 mins condition B. Absorbance spectra λ_max 222 nm minor λ_max 280 nm.

3-chloro-4-hydroxyphenylacetaldehyde (26). In a flame dried flask purged with N₂, sulfur trioxide pyridine complex (0.897 g, 5.6 mmol) was dissolved in 10 mL of sieve dried dimethyl sulfoxide. This solution was then canulated into a second flame dried flask purged with N₂ containing 20 (0.5858 g, 2.7 mmol) and triethyl amine (0.75 mL, 5.4 mmol), dissolved in 10 mL of sieve dried dimethyl sulfoxide, slowly over a period of 15 mins. The resulting solution was allowed to stir at room temperature for 1 hour under a N₂ atmosphere, after which it was poured into 150 mL of ice cold water. The resulting solution was extracted into ethyl acetate (3x75 mL), and the combined organic fractions washed with brine (1x50 mL), dried with MgSO₄, filtered and the solvent removed under reduced pressure yielding a crude orange oil. Flash chromatography was performed (25% ethyl acetate in hexanes) yielding 0.0361 g of 20 (6.2%). ¹H NMR (300 MHz, CDCl₃) δ 9.72 (d, J = 16.0 Hz, 1H), 7.35 (s, 1H), 7.09 (d, J = 8.5 Hz, 1H), 7.03 (d, J = 8.3 Hz, 1H), 5.61 (s, 1H), 3.61 (d, J = 22.4 Hz, 2H). LRMS (EI⁺) m/z 214 (M⁺, 17.4), 215 (M⁺1, 2.5), 216 (M+2, 18.0), 188 (8.7), 187 (100), 186 (9.7), 185 (97), 107 (6.1), 106 (4.9), 78 (15.6), 77 (26). HPLC retention time 2.33 mins condition B. Absorbance spectra λ_max 223 nm minor λ_max 281 nm.

3-iodo-4-hydroxyphenylacetaldehyde (27). In a flame dried flask purged with N₂, sulfur trioxide pyridine complex (0.869 g, 5.5 mmol) was dissolved in 10 mL of sieve dried dimethyl sulfoxide. This solution was then canulated into a second flame dried flask purged with N₂ containing 23 (0.7060 g, 2.7 mmol) and triethyl amine (0.75 mL, 5.4 mmol), dissolved in 10 mL of sieve dried dimethyl sulfoxide, slowly over a period of 15 mins. The resulting solution was allowed to stir at room temperature for 1 hour under a N₂ atmosphere, after which it was poured into 100 mL of ice cold water. The resulting solution was
extracted into ethyl acetate (3x80 mL), and the combined organic fractions washed with brine (1x100 mL), dried with MgSO₄, filtered and the solvent removed under reduced pressure yielding a crude orange oil. Flash chromatography was performed (30% ethyl acetate in hexanes) yielding 0.1804 g of 27 (26%) as a slightly colored oil. ¹H NMR (300 MHz, CDCl₃) δ 9.74 (t, J = 2.2 Hz, 1H), 7.54 (d, J = 2.0 Hz, 1H), 7.12 (dd, J = 8.3, 2.1 Hz, 1H), 7.01 (d, J = 8.3 Hz, 1H), 5.29 (s, 1H), 3.63 (d, J = 2.2 Hz, 2H). LRMS (EI⁺) 262 (M⁺, 26.6), 263 (M⁺+1, 2.2), 234 (8.4), 233 (100), 136 (0.19), 127 (2.41), 107 (3.5), 106 (16.5), 78 (10), 77 (9). HPLC retention time 2.63 mins condition B. Absorbance spectra λ_max 284 nm.

**3-chloro-tyrosine (41).** Tyrosine (7.567 g, 41.8 mmol) was suspended in 75 mL of glacial acetic acid and SO₂Cl₂ (3.44 mL, 44 mmol) was slowly added to the suspension over 10 minutes. The resulting solution was allowed to stir for 5.5 hours, after which the solid precipitate was collected by vacuum filtration and washed with glacial acetic acid (3x10 mL) and diethyl ether (3x25mL). The resulting product was then recrystallized from boiling concentrated hydrochloric acid yielding 6.910 g of 41 (66%) as the white crystals. ¹H NMR (300 MHz, DMSO-d₆) δ 10.17 (s, 1H), 8.25 (s, 3H), 7.24 (d, J = 2.0 Hz, 1H), 7.02 (d, J = 8.3, 2.1 Hz, 1H), 6.92 (d, J = 8.3 Hz, 1H), 4.14 (s, 1H), 3.00 (t, J = 5.7 Hz, 2H). HPLC retention time 1.56 mins condition B. Absorbance spectra λ_max 222 nm minor λ_max 280 nm.

**3-bromotyrosine (42).** A solution containing 1 (5.541 g, 30.6 mmol) suspend in 25 mL of glacial acetic acid and 15 mL of a 33% solution of HBr in acetic acid (61 mmol), was prepared. A solution of Bromine (1.7 mL, 33.2 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 (3x25 mL) and diethyl ether (3x25mL) yielding an orange powder. The powder was recrystallized from boiling concentrated hydrochloric acid yielding 6.023 g of 42 (70%) as white crystals. ¹H NMR (300 MHz, D₂O) δ 7.40 (d, J = 2.1 Hz, 1H), 7.06 (dd, J = 8.3, 2.1 Hz, 1H), 6.89 (d, J = 8.3 Hz, 1H), 4.12 (d, J = 5.4 Hz, 1H), 3.15 (dd, J = 14.7, 5.6 Hz, 1H), 3.02 (dd, J = 14.8, 7.6 Hz, 1H). HPLC retention time 1.64 mins condition B. Absorbance spectra λ_max 280 nm.
3-iodotyrosine(43). A solution of 1 (4.968 g, 27 mmol) dissolved in concentrated NH₄OH was cooled in an ice water bath, and iodine (7.0325 g, 27.8 mmol) dissolved in 150 mL of ethanol was slowly added dropwise via syringe pump. The resulting solution was allowed to stir overnight, after which the solvent was removed under reduced pressure, yielding an off white solid, which was suspended in 150 mL of ice cold water. The solid was collected by vacuum filtration and re-suspended in ice cold acetone and stirred for 1.5 hours. The resulting solid was collected by vacuum filtration yielding 2.528 g of 43 (30 %) as a white powder. ¹H NMR (300 MHz, D₂O) δ 7.60 (d, J = 2.1 Hz, 1H), 7.12 – 7.03 (m, 1H), 6.83 (d, J = 8.3 Hz, 1H), 3.84 – 3.78 (m, 1H), 3.10 – 3.01 (m, 1H), 2.91 (dd, J = 14.7, 7.8 Hz, 1H). HPLC retention time 1.78 mins condition B. Absorbance spectra λₘₐₓ 283 nm.

Standardization of sodium thiosulfate. A solution of sodium thiosulfate containing 0.025 g NaCO₃ as a preservative was standardized against a 0.03740 M solution of KIO₃ by dissolving approximately 2 g of potassium iodide in 5 mL of 6 M H₂SO₄ and 10 mL of the KIO₃ solution. The resulting solution was titrated with the sodium thiosulfate solution until it was a pale yellow solution, after which a mL of a starch indicator solution was added. The resulting blue solution was then titrated until the solution was clear. The above procedure was repeated 4 times yielding a concentration of (5.14 ±0.05) x 10⁻² M.

Titration of a 10-15% NaOCl. 2.4476 g of a 10-15% solution of sodium hypochlorite was diluted into a 250 mL volumetric flask with Milli Q water. 50 mL of the resulting solution was then added to a flask, and diluted with 50 mL of Milli Q water. To this solution, 10 mL of glacial acetic acid and approximately 2 g of KI were added and mixed thoroughly, yielding a brown solution, which was titrated with a (5.14 ±0.05) x 10⁻² M solution of Na₂S₂O₃ until pale yellow, after which 1 mL of a starch indicator was added. The resulting blue solution was then titrated until the solution became clear. This was repeated 3 times yielding a concentration of 9.16% by mass NaOCl.

4-hydroxyphenylacetaldehyde(3). A solution of 1 (0.189 g, 1 mmol) in 250 mL of 10 mM phosphate pH 7, was warmed in a 37 °C warm room. To this, a solution containing 730 μL of 9.16 % NaOCl (1 molar equivalent) dissolved in 7 mL of Milli Q water was added drop wise over 7 mins via syringe pump with vigorous stirring. This solution was allowed to stir at 37 °C for 2 hours. The resulting solution was extracted into ethyl acetate (3x100 mL), dried over MgSO₄, filtered, and the solvent removed under
reduced pressure yielding 0.0938 g of 3 (66 %) as a brown oil. $^1$H NMR (300 MHz, CDCl$_3$) δ 9.74 (t, $J = 2.4$ Hz, 1H), 7.11 (d, $J = 8.5$ Hz, 2H), 6.89 – 6.82 (m, 2H), 4.75 (s, 1H), 3.64 (d, $J = 2.3$ Hz, 2H). LRMS (EI')m/z 136 (M$^+$, 17.6), 137 (M$^{+1}$, 1.7), 108 (9.5), 107 (100), 89 (.63), 78 (3.7), 77 (21.6). HPLC retention time 1.63 mins condition B. Absorbance spectra $\lambda_{\text{max}}$ 222 nm minor $\lambda_{\text{max}}$ 276 nm.

3-chloro-4-hydroxyphenylacetaldehyde(25). A solution of 41 (0.253g, 1 mmol) in 250 mL of 10 mM phosphate pH 7, was warmed in a 37 °C warm room. To this, a solution containing 70 uL of 9.16 % NaOCl (1 molar equivalent) dissolved in 7 mL of Milli Q water was added drop wise over 7 mins via syringe pump with vigorous stirring. This solution was allowed to stir at 37 °C for 1.5 hours. The resulting solution was extracted into CH$_2$Cl$_2$ (3x100 mL), dried over MgSO$_4$, filtered, and the solvent removed under reduced pressure yielding 0.1426 g of 25 (83 %) as a brown oil. $^1$H NMR (300 MHz, CDCl$_3$) δ 9.75 (t, $J = 2.1$ Hz, 1H), 7.21 (s, 1H), 7.04 (s, 2H), 5.55 (s, 1H), 3.64 (d, $J = 2.1$ Hz, 2H). LRMS (EI')m/z 170 (M$^+$, 5.89), 172 (M$^{+2}$, 18.8), 143 (32.2), 141 (100), 105 (5.4), 77 (25.6). HPLC retention time 2.17 mins condition B. Absorbance spectra $\lambda_{\text{max}}$ 222 nm minor $\lambda_{\text{max}}$ 280 nm.

3-bromo-4-hydroxyphenylacetaldehyde(26). A solution of 42 (0.298g, 1 mmol) in 250 mL of 10 mM phosphate pH 7, was warmed in a 37 °C warm room. To this, a solution containing 700 uL of 9.16 % NaOCl (1 molar equivalent) dissolved in 7 mL of Milli Q water was added drop wise over 7 mins via syringe pump with vigorous stirring. This solution was allowed to stir at 37 °C for 1.5 hours. The resulting solution was extracted into CH$_2$Cl$_2$ (3x100 mL), dried over MgSO$_4$, filtered, and the solvent removed under reduced pressure yielding 0.177 g of 26 (82 %) as a brown oil. $^1$H NMR (300 MHz, CDCl$_3$) δ 9.74 (t, $J = 2.1$ Hz, 1H), 7.35 (t, $J = 2.7$ Hz, 1H), 7.09 (dd, $J = 8.3$, 1.8 Hz, 1H), 7.03 (d, $J = 8.3$ Hz, 1H), 5.58 (s, 1H), 3.64 (d, $J = 2.0$ Hz, 2H). LRMS (EI')m/z 214 (M$^+$, 17.4), 215 (M$^{+1}$, 2.5), 216 (M$^{+2}$, 18.0), 188 (8.7), 187 (100), 186 (9.7), 185 (97), 107 (6.1), 106 (4.9), 78 (15.6), 77 (26). HPLC retention time 2.33 mins condition B. Absorbance spectra $\lambda_{\text{max}}$ 223 nm minor $\lambda_{\text{max}}$ 281 nm.
3-ido-4-hydroxyphenylacetaldehyde (27).

A solution of 43 (0.302 g, 1 mmol) in 250 mL of 10 mM phosphate pH 7, was warmed in a 37 °C warm room. To this, a solution containing 700 uL of 9.16 % NaOCl (1 molar equivalent) dissolved in 7 mL of Milli Q water was added drop wise over 7 mins via syringe pump with vigorous stirring. This solution was allowed to stir at 37 °C for 1.5 hours. The resulting solution was extracted into CH₂Cl₂ (3x20 mL), dried over MgSO₄, filtered, and the solvent removed under reduced pressure yielding 0.160 g of 27 (62 %) as a brown oil. ¹H NMR (300 MHz, CDCl₃) δ 9.74 (t, J = 2.2 Hz, 1H), 7.54 (d, J = 2.0 Hz, 1H), 7.11 (dd, J = 8.3, 2.1 Hz, 1H), 7.00 (d, J = 8.3 Hz, 1H), 5.36 (s, 1H), 3.63 (t, J = 3.2 Hz, 2H). LRMS (EI⁺) 262 (M⁺, 26.6), 263 (M⁺1, 2.2), 234 (8.4), 233 (100), 136 (0.19), 127 (2.41), 107 (3.5), 106 (16.5), 78 (10), 77 (9). HPLC retention time 2.63 mins condition B. Absorbance spectra λmax 284 nm.

Norcoclaurine(4). ²⁵, ²⁶, ⁵⁰

A solution of 3 was generated by dissolving 1 (0.189 g, 1 mmol) in 250 mL of 20 mM phosphate pH 7, was warmed in a 37 °C warm room. To this, a solution containing 700 uL of 9.16 % NaOCl (1 molar equivalent) dissolved in 20 mL of Milli Q water was added drop wise over 20 mins via syringe pump with vigorous stirring. This solution was allowed to stir at 37 °C for 2 hours, and quenched by the addition of ascorbic acid (0.185 g, 1 mmol). Dopamine hydrochloride (2) (0.2913g, 1.5 mmol) was added to the resulting solution, which was stirred until all of the aldehyde was consumed as monitored by HPLC (condition B). The reaction was then extracted into ethyl acetate (3x250 mL), dried with MgSO₄, and the solvent removed under reduced pressure yielding 0.0804 g of 4 (28%) as a yellow solid. ¹H NMR (300 MHz, Methanol-d₄) δ 7.10 (d, J = 8.5 Hz, 2H), 6.77 (d, J = 8.5 Hz, 2H), 6.66 (s, 1H), 6.53 (s, 1H), 3.18 (s, 2H), 2.84 (s, 2H), 2.69 (s, 2H), 2.02 (d, J = 2.9 Hz, 1H). HPLC retention time 1.74 mins condition B. Absorbance spectra λmax 225 nm minor λmax 283 nm.
3-chloronorcoclaurine(44). A solution of 25 was generated by dissolving 41 (0.3098, 1.5 mmol) in 500 mL of 20 mM phosphate solution pH 7 was warmed in a 37 °C warm room. To this, a solution containing 1050 µL of 9.16 % NaOCl (1 molar equivalent) dissolved in 40 mL of Milli Q water was added drop wise over 40 mins via syringe pump with vigorous stirring. This solution was allowed to stir at 37 °C for 2 hours, and quenched by the addition of ascorbic acid (0.161 g, 0.9 mmol). 2 (0.295g, 1.5 mmol) was added to the resulting solution, which was stirred until all of the aldehyde was consumed as monitored by HPLC (condition B). The reaction was then extracted into ethyl acetate (3x250 mL), dried with MgSO₄, and the solvent removed under reduced pressure yielding 0.1955 g of 44 (45%) as a yellow solid. 1H NMR (300 MHz, CDCl₃) δ 7.25 (d, J = 2.1 Hz, 1H), 7.07 – 7.02 (m, 1H), 6.90 (d, J = 8.2 Hz, 1H), 6.64 (s, 1H), 6.56 (s, 1H), 3.29 – 3.16 (m, 3H), 2.98 (d, J = 6.7 Hz, 1H), 2.87 – 2.74 (m, 3H). HPLC retention time 1.98 mins condition B. Absorbance spectra λ_max 284 nm.

3-bromonorcoclaurine(45). A solution of 26 was generated by dissolving 42 (0.5235, 2 mmol) in 500 mL of 20 mM phosphate solution pH 7 was warmed in a 37 °C warm room. To this, a solution containing 1400 µL of 9.16 % NaOCl (1 molar equivalent) dissolved in 20 mL of Milli Q water was added drop wise over 20 mins via syringe pump with vigorous stirring. This solution was allowed to stir at 37 °C for 2 hours, and quenched by the addition of ascorbic acid (0.2100 g, 1.2 mmol). 2 (0.381 g, 2 mmol) was added to the resulting solution, which was stirred until all of the aldehyde was consumed as monitored by HPLC (condition B). The reaction was then extracted into ethyl acetate (3x250 mL), dried with MgSO₄, and the solvent removed under reduced pressure yielding 0.3391 g of 45 (48%) as a tan solid. 1H NMR (300 MHz, CDCl₃) δ 10.20 – 9.87 (m, 1H), 8.73 (s, 1H), 8.68 – 8.50 (m, 1H), 7.40 (d, J = 2.0 Hz, 1H), 7.11 – 7.01 (m, 1H), 6.87 (d, J = 8.2 Hz, 1H), 6.60 (s, 1H), 6.43 (s, 1H), 3.98 – 3.92 (m, 1H), 3.09 – 3.03 (m, 1H), 2.93 (s, 1H), 2.80 – 2.73 (m, 1H), 2.67 (d, J = 13.9 Hz, 1H). HPLC retention time 2.05 mins condition B. Absorbance spectra λ_max 284 nm.

3-iodonorcoclaurine(46). A solution of 27 was generated by dissolving 46 (0.1650, 0.53 mmol) in 250 mL of 20 mM phosphate solution pH 7 was warmed in a 37 °C warm room. To this, a solution containing 375 µL of 9.16 % NaOCl (1 molar equivalent) dissolved in 5 mL of Milli Q water was added drop wise over 5 mins via syringe pump with vigorous stirring. This solution was allowed to stir at 37 °C for 2 hours, and
quenched by the addition of ascorbic acid (0.0949 g, 0.54 mmol). 2 (0.101 g, 0.54 mmol) was added to the resulting solution, which was stirred until all of the aldehyde was consumed as monitored by HPLC (condition B). The reaction was then extracted into ethyl acetate (3x250 mL), dried with MgSO$_4$ and the solvent removed under reduced pressure yielding 0.1179 g of 46 (55%) as a yellow solid. $^1$H NMR (300 MHz, Methanol-D$_4$) $\delta$ 7.64 (d, $J = 2.0$ Hz, 1H), 7.13 – 7.07 (m, 1H), 6.82 (d, $J = 8.2$ Hz, 1H), 6.64 (s, 1H), 6.55 (s, 1H), 3.24 – 3.13 (m, 2H), 2.93 (d, $J = 7.3$ Hz, 1H), 2.83 – 2.68 (m, 3H), 2.03 (s, 1H). HPLC retention time 2.21 mins condition B. Absorbance spectra $\lambda_{\text{max}}$ 285 nm.

\[ \begin{align*}
\text{28} & \rightarrow \\
\text{29}
\end{align*} \]

$\text{3-chloro-4-hydroxybenzaldehyde}(29)$.$^{32}$ 4-hydroxybenzaldehyde (28) (3.130 g, 25.6 mmol) was dissolved in 25 mL of glacial acetic acid and SO$_2$Cl$_2$ (3.82 g, 27.5 mmol) was added all in one portion. The resulting solution was allowed to stir at room temperature for 4 hours, and the resulting precipitate was collected by vacuum filtration. The collected solid was then allowed to dry under vacuum overnight, then recrystallized from boiling water yielding 2.419 g of 29 (61%) as white needle like crystals. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 9.87 (s, 1H), 7.92 (d, $J = 1.9$ Hz, 1H), 7.76 (dd, $J = 8.4$, 1.9 Hz, 1H), 7.18 (d, $J = 8.4$ Hz, 1H), 6.17 (s, 1H). LRMS (EI$^+$)m/z 156 (M$^+$, 70.0), 157 (M$^+$+1, 38.5), 158 (M$^+$+2, 20.94), 155 (100), 129 (6.4), 127 (18.7), 101 (5.9), 99 (19.1), 91 (5.9), 77 (0.42). HPLC retention time 2.538 mins condition B. Absorbance spectra $\lambda_{\text{max}}$ 228 nm minor $\lambda_{\text{max}}$ 275 nm.

\[ \begin{align*}
\text{28} & \rightarrow \\
\text{30}
\end{align*} \]

$\text{3-bromo-4-methoxybenzaldehyde}(30)$.$^{37}$ A solution containing 28 (3.147 g, 25.8 mmol) and p-toluenesulfonic acid (2.309 g, 12.1 mmol) in 120 mL of acetonitrile was allowed to stir for 5 minutes after which, N-bromosuccinimide (4.946 g, 27.8 mmol) was added and the resulting solution was then allowed to stir over night at room temperature. The product was then extracted into diethyl ether (3x100 mL), washed with 10 % sodium thiosulfate (1x100 mL), washed with brine (1x100 mL), dried over MgSO$_4$, filtered, and the solvent removed under reduced pressure. The resulting solid was recrystallized from water yielding 4.428 g of 30 (86 %) as off white needle like crystals. $^1$H NMR (300 MHz, Acetone-d$_6$) $\delta$ 9.86 (s, 1H), 8.09 (t, $J = 2.8$ Hz, 1H), 7.84 – 7.76 (m, 1H), 7.20 (d, $J = 8.4$ Hz, 1H). LRMS (EI$^+$)m/z 200 (M$^+$,55), 201 (M$^+$+1, 90), 202 (M$^+$+2,60), 203 (M$^+$+3, 6.8), 199 (100), 173 (16), 171 (14), 145 (11), 143 (11), 119 (4.6), 92 (19), 79 (2.1). HPLC retention time 2.69 mins condition B. Absorbance spectra $\lambda_{\text{max}}$ 229 nm minor $\lambda_{\text{max}}$ 286 nm.
3-chloro-4-methoxynitrostyrene(47). A solution containing (29) (1.254 g, 8.0 mmol) and ammonium acetate (0.413 g, 5.4 mmol) in 50 mL nitromethane was allowed to reflux for 6 hours. The resulting red solution was then poured into 200 mL of water, extracted into diethyl ether (3x150 mL), washed with brine (1 x 150 mL), dried over MgSO$_4$, filtered and the solvent removed under reduced pressure, yielding 1.508 g of 47 (94%) as a red solid. $^1$H NMR (300 MHz, CDCl$_3$) δ 7.97 – 7.88 (s, 1H), 7.58 (d, $J = 2.1$ Hz, 1H), 7.52 (d, $J = 13.6$ Hz, 1H), 7.43 (dd, $J = 8.6$, 2.1 Hz, 1H), 7.12 (d, $J = 8.5$ Hz, 1H), 5.99 (s, 1H). HPLC retention time 3.30 mins condition B. Absorbance spectra $\lambda_{max}$ 246 nm minor $\lambda_{max}$ 354 nm.

3-bromo-4-methoxynitrostyrene(48). A solution containing (30) (1.656 g, 8.2 mmol) and ammonium acetate (0.950 g, 12.3 mmol) in 50 mL nitromethane was allowed to reflux overnight. The resulting red solution was then poured into 150 mL of water, extracted into diethyl ether (3x150 mL), washed with brine (1 x 100 mL), dried over MgSO$_4$, filtered and the solvent removed under reduced pressure, yielding 1.885 g of 48 (94%) as a red solid. $^1$H NMR (300 MHz, CDCl$_3$) δ 7.92 (d, $J = 13.6$ Hz, 1H), 7.72 (d, $J = 2.1$ Hz, 1H), 7.53 (t, $J = 9.5$ Hz, 1H), 7.47 (dd, $J = 8.5$, 2.1 Hz, 1H), 7.11 (d, $J = 8.5$ Hz, 1H), 5.94 (s, 1H). HPLC retention time 3.37 mins condition B. Absorbance spectra $\lambda_{max}$ 250 nm minor $\lambda_{max}$ 355 nm.

3-chloro-4-hydroxyphenethylamine(52). 47 (0.205 g, 1 mmol) and Zn dust (0.503 g, 7.7 mmol) were slowly added in alternating portions over the course of 1.5 hours to a solution of 1 mL concentrated HCl in 2 mL of methanol at 0 °C. The resulting solution was then incubated at 4 °C and allowed to stir overnight, yielding a thick gray paste that showed several peaks by when monitored by HPLC suggesting that the reaction was not successful.
3-chloro-4-methoxybenzaldehyde (31). A solution of tetrabutylammonium hydrogen sulfate (1.147 g, 3.4 mmol) in 50 mL of 2 M NaOH was added to a solution of 29 (0.935 g, 6.0 mmol) in 30 mL of CH₂Cl₂ while stirring. After the solution became clear, methyl iodide (4.3 mL, 69 mmol) was added and the resulting solution was allowed to stir for 24 hours. The solution was then neutralized through the addition of 25 mL of 6 M HCl, and the organic phase was separated from the aqueous. The aqueous phase was then extracted with DCM (3x50 mL) and the combined organic fractions washed with brine (1x40 mL), dried with MgSO₄, filtered, and the solvent removed under reduced pressure. The resulting crude oil was then purified through flash column chromatography (20% ethyl acetate in hexanes) to yield 0.572 g of 29 (79%) as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 9.90 (d, J = 9.5 Hz, 1H), 7.94 (d, J = 2.0 Hz, 1H), 7.80 (dd, J = 8.5, 2.0 Hz, 1H), 7.07 (d, J = 8.5 Hz, 1H), 4.02 (s, 3H). LRMS (EI⁺) m/z 170 (M⁺, 64.1), 171 (M⁺+1, 37.6), 172 (M⁺+2, 21.4), 173 (M⁺+3, 1.8) 169 (100), 154 (1.0), 141 (8.1), 126 (9.3), 111 (6.3), 99 (13.0), 77 (9.6). HPLC retention time 3.50 mins condition B. Absorbance spectra λ_max 226 nm minor λ_max 274 nm.

3-bromo-4-methoxybenzaldehyde (32). A solution of tetrabutylammonium hydrogen sulfate (3.252 g, 9.6 mmol) in 140 mL of 2 M NaOH was added to a solution of 30 (3.514 g, 17.5 mmol) in 120 mL of CH₂Cl₂ while stirring. After the solution became clear, methyl iodide (11.5 mL, 122 mmol) was added and the resulting solution was allowed to stir for 24 hours. The solution was then neutralized through the addition of 50 mL of 6 M HCl, and the organic phase was separated from the aqueous. The aqueous phase was then extracted with DCM (2X150 mL) and the combined organic dried with MgSO₄, filtered, and the solvent removed under reduced pressure. The resulting crude oil was then purified through dry vacuum column chromatography (50% ethyl acetate in hexanes) to yield 3.678 g of 30 (98 %) as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 9.87 (s, 1H), 8.11 (d, J = 2.0 Hz, 1H), 7.85 (d, J = 8.5, 2.0 Hz, 1H), 7.04 (d, J = 8.5 Hz, 1H), 4.00 (d, J = 8.6 Hz, 3H). LRMS (EI⁺) m/z 214 (M⁺, 74.5), 216 (M⁺+2, 73.6), 217 (M⁺+3, 6.3), 215 (100) 213 (96.2), 187 (6.9), 185 (7.3), 172 (8.7), 170 (8.4), 157 (6.8), 155 (6.0), 145 (10.7), 143 (11.4), 119 (7.9), 104 (3.4), 77 (8.7). HPLC retention time 3.60 mins condition B. Absorbance spectra λ_max 229 nm minor λ_max 275 nm.
**4-methoxybenzylalcohol (61).** Sodium borohydride (0.131 g, 3.5 mmol) was added in one portion to a solution containing 33 (0.7106 g, 5.2 mmol) and the resulting solution was allowed to stir overnight. The reaction was then quenched by the addition of 4 mL of acetone and the solvent was removed under reduced pressure. The resulting oily residue was then dissolved in 50 mL of CH$_2$Cl$_2$ and the organic solution washed with water (2x15 mL). The organic solution was then dried with MgSO$_4$, filtered through a celite plug, and the solvent was removed under reduced pressure yielding 0.458 g of 61 (64%) as a yellow oil that crystallized at -20°C. $^1$H NMR (300 MHz, CDCl$_3$) δ 7.31 (t, $J$ = 7.2 Hz, 2H), 6.95 – 6.86 (m, 2H), 4.65 (d, $J$ = 5.9 Hz, 2H), 3.83 (s, 3H), 1.55 (t, $J$ = 5.9 Hz, 1H). LRMS (EI$^+$) m/z 138 (M$^+$, 100), 139 (M$^+$+1, 8.7), 137 (69) 123 (6.2), 121 (52), 109(73), 107(27), 105 (14.3), 94 (35), 91 (9.5), 79 (18.4), 77 (46). HPLC retention time 2.18 mins condition B. Absorbance spectra $\lambda_{\max}$ 225 nm minor $\lambda_{\max}$ 274 nm.

![Chemical structure of 3-chloro-4-methoxybenzylalcohol](image)

**3-chloro-4-methoxybenzylalcohol (51).** Sodium borohydride (0.101 g, 2.8 mmol) was added in one portion to a solution containing 31 (0.551 g, 3.2 mmol) and the resulting solution was allowed to stir overnight. The reaction was then quenched by the addition of 8 mL of acetone and the solvent was removed under reduced pressure. The resulting oily residue was then dissolved in 75 mL of CH$_2$Cl$_2$ and the organic solution washed with brine (2x50 mL). The combined brine layers were then washed with 75 mL of CH$_2$Cl$_2$ and the combined organic phase was then dried with MgSO$_4$, filtered through a celite plug, and the solvent was removed under reduced pressure yielding 0.689 g of 51 (120%) as a clear oil that crystallized at -20°C. $^1$H NMR (300 MHz, CDCl$_3$) δ 7.42 (d, $J$ = 2.1 Hz, 1H), 7.24 (dd, $J$ = 8.4, 2.1 Hz, 1H), 6.93 (d, $J$ = 8.4 Hz, 1H), 4.63 (d, $J$ = 5.3 Hz, 2H), 3.92 (s, 3H), 1.66 (t, $J$ = 5.7 Hz, 1H). LRMS (EI$^+$) m/z 172 (M$^+$, 100), 173 (M$^+$+1, 18), 174 (M$^+$+2, 33) 171(35), 169 (19), 157(13), 155(33), 143(49), 141 (23), 139 (6.2), 132 (54), 128 (13), 113 (9.1), 108 (36), 107 (23), 105 (8.3), 94 (13.9), 77 (46.4). HPLC retention time 2.70 mins condition B. Absorbance spectra $\lambda_{\max}$ 228 nm minor $\lambda_{\max}$ 274 nm.

![Chemical structure of 3-bromo-4-methoxybenzylalcohol](image)

**3-bromo-4-methoxybenzylalcohol (52).** Sodium borohydride (0.458 g, 12 mmol) was added in one portion to a solution containing 32 (3.6783 g, 17 mmol) and the resulting solution was allowed to stir overnight. The reaction was then quenched by the addition of 30 mL of acetone and the solvent was removed under reduced pressure. The resulting oily residue was then dissolved in 70 mL of water and then acidified with 2 M HCl. The water was then extracted with CH$_2$Cl$_2$ (3x70 mL) and the combined organic solution washed with water (2x30 mL), brine (2x30 mL), dried with MgSO$_4$, filtered, and the solvent was removed under reduced pressure yielding 3.452 g of 52 (93%) as a white crystalline solid. $^1$H NMR (300 MHz, DMSO-d$_6$) δ 7.50 (d, $J$ = 1.9 Hz, 1H), 7.27 (dd, $J$ = 8.4, 1.9 Hz, 1H), 7.06 (d, $J$ = 8.4 Hz, 1H),
5.19 (t, J = 5.7 Hz, 1H), 4.42 (d, J = 5.6 Hz, 2H), 3.83 (s, 3H). LRMS (Ei⁺) m/z 217 (M⁺, 33) 218 (M⁺¹, 93), 219 (M⁺², 7.4), 216 (100), 215 (38) 213 (12), 201 (26), 199 (25), 189 (20), 187 (32), 172 (9.4), 157 (8.8), 137 (50), 109 (37), 108 (74), 105 (15), 94 (29), 89 (15), 78 (22), 77 (52). HPLC retention time 2.88 mins condition B. Absorbance spectra λₘₐₓ 281 nm.

4-methoxybenzylchloride(62). Thionyl chloride (430 µL, 6 mmol) was added to a solution of 61 (0.398 g, 2.9 mmol) in 5 mL of CH₂Cl₂ in three equal portions over a period of 15 minutes. After allowing the solution to stir for 1 hour, the solvent was removed under reduced pressure yielding 0.464 g of 62 (104%) as a clear oil. ¹H NMR (300 MHz, CDCl₃) δ 7.37 – 7.30 (m, 2H), 6.94 – 6.87 (m, 2H), 4.59 (s, 2H), 3.83 (s, 3H). LRMS (El⁺) m/z 156 (M⁺, 17), 157 (M⁺¹, 1.7), 158 (M⁺², 5.4), 122 (9.2), 121 (100), 106 (2.3), 91 (6.6), 77 (14).

3-chloro-4-methoxybenzylchloride(53). Thionyl chloride (570 µL, 8 mmol) was added to a solution of 51 (0.690 g, 4 mmol) in 5 mL of CH₂Cl₂ in three equal portions over a period of 15 minutes. After allowing the solution to stir for 1 hour, the solvent was removed under reduced pressure yielding 0.6362 g of 53 (83%) as a white paste. ¹H NMR (300 MHz, CDCl₃) δ 7.42 (d, J = 2.1 Hz, 1H), 7.24 (dd, J = 8.4, 2.1 Hz, 1H), 6.93 (d, J = 8.4 Hz, 1H), 4.63 (d, J = 5.3 Hz, 2H), 3.92 (s, 3H), 1.66 (t, J = 5.7 Hz, 1H). LRMS (Ei⁺) m/z 190 (M⁺¹, 19), 191 (M⁺, 1.9), 192 (M⁺², 12), 193 (M⁺³, 1.2), 194 (M⁺⁴, 1.9), 157 (32), 156 (9.5), 155 (100), 112 (5.4), 105 (8.6) 89 (4.7), 77 (22).

3-bromo-4-methoxybenzylchloride(54). Thionyl chloride (3 mL, 42 mmol) was added to a solution of 52 (3.431, 15.8 mmol) in 35 mL of dropwise over a period of 15 minutes. After allowing the solution to stir for 1 hour, the solvent was removed under reduced pressure yielding 3.50 g of 54 (100%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ 7.67 (d, J = 2.1 Hz, 1H), 7.44 (dd, J = 8.4, 2.1 Hz, 1H), 7.12 (d, J = 8.5 Hz, 1H), 4.73 (s, 2H), 3.86 (s, 3H). LRMS (Ei⁺) m/z 234 (M⁺¹, 21.3), 236 (M⁺², 28), 237 (M⁺³, 2.6), 238 (M⁺⁴, 6.4), 221 (1.4), 202 (8.6), 201(97), 200 (11), 199 (100), 111 (2.2), 105 (17), 89 (11), 77 (34).
4-methoxybenzylcyanide(63). Sodium cyanide (0.162 g, 3.3 mmol) was ground with a mortar and pestle and added to a solution containing 62 (0.464 g, 3.0 mmol) in 10 mL of DMSO. The resulting solution was then heated in a 40 °C water bath for 1 hour, then allowed to stir over night. The solution was then poured into 25 mL of water, extracted into diethyl ether (3x50 mL), washed with brine (1x50 mL), dried with MgSO_4, and then the solvent removed under reduced pressure yielding 0.466 g of 63 (107 %) as a clear oil. 1H NMR (300 MHz, CDCl_3) δ 7.27 (d, J = 8.6 Hz, 2H), 6.95 (d, J = 8.6 Hz, 2H), 3.94 (s, 2H), 3.75 (s, 3H). HPLC retention time 3.19 mins condition B. Absorbance spectra λ_{max} 225 nm minor λ_{max} 275 nm. LRMS (EI)^+ m/z 147 (M^+,100), 148 (M^+1, 10), 146 (45), 133 (3.3), 132 (38), 117 (8.6), 116 (21), 105 (2.9), 91 (11), 77 (37).

3-chloro-4-methoxybenzylcyanide(55). Sodium cyanide (0.188 g, 3.8 mmol) was ground with a mortar and pestle and added to a solution containing 53 (0.6362 g, 3.3 mmol) in 10 mL of DMSO. The resulting solution was then heated in a 40 °C water bath for 1 hour, then allowed to stir over night. The solution was then poured into 25 mL of water, extracted into diethyl ether (3x50 mL), washed with brine (1x50 mL), dried with MgSO_4, and then the solvent removed under reduced pressure yielding 0.542 g of 55 (90 %) as a white solid. 1H NMR (300 MHz, DMSO-d_6) δ 7.58 (d, J = 2.1 Hz, 1H), 7.35 (dd, J = 8.5, 2.1 Hz, 1H), 7.14 (d, J = 8.5 Hz, 1H), 3.97 (s, 2H), 3.85 (s, 3H). LRMS (EI)^+ m/z 181 (M^+,52), 182 (M^+1, 6.0), 183 (M+2, 17), 184 (M^+3, 1.6), 166 (9.2), 147 (10), 146 (100), 138 (8.4), 116 (4.9), 108 (7.6), 107 (33), 91 (4.8), 75 (8.5). HPLC retention time 3.61 mins condition B. Absorbance spectra λ_{max} 227 nm minor λ_{max} 279 nm.

3-bromo-4-methoxybenzylcyanide(56). Sodium cyanide (0.865 g, 17.6 mmol) was ground with a mortar and pestle and added to a solution containing 54 (3.491 g, 15.8 mmol) in 45 mL of DMSO. The resulting solution was then heated in a 40 °C water bath for 1 hour. The solution was then poured into 150 mL of water, extracted into diethyl ether (3x150 mL), washed with brine (1x150 mL), dried with
MgSO$_4$, and then the solvent removed under reduced pressure yielding 3.191 g of 56 (90 %) as a yellow. $^1$H NMR (300 MHz, DMSO-d$_6$) δ 7.58 (d, $J = 2.1$ Hz, 1H), 7.35 (dd, $J = 8.5$, 2.2 Hz, 1H), 7.14 (d, $J = 8.5$ Hz, 1H), 3.96 (d, $J = 10.4$ Hz, 2H), 3.85 (s, 3H). LRMS (El$^+$)m/z 225 (M$^+$,66), 226 (M$^{+1}$, 9.0), 227 (M+2, 64), 228 (M$^{+3}$, 6.5), 212 (9.0), 210 (8.7), 182 (4.1), 147 (9.8), 146 (100), 130 (4.8), 116 (10), 103 (47), 89 (11), 76 (14). HPLC retention time 3.74 mins condition B. Absorbance spectra $\lambda_{\text{max}}$ 227 nm minor $\lambda_{\text{max}}$ 280 nm.

4-methoxyphenethyamine(64). $^6$ 5 mL of a 1 M borane THF solution was added to a flame dried reflux apparatus, that had previously been purged with N$_2$. To this solution, a solution of 63 (0.361 g, 2.5 mmol) in 5 mL of sieve dried THF was slowly added over 5 minutes, and the resulting solution was brought to reflux for 1.5 hours. The solution was then cooled to room temperature and quenched by the slow addition of 6 mL of methanol, then the solvent was removed under reduced pressure. The crude oil was then dissolved in 25 mL of 2 M HCl and washed with diethyl ether (2x25 mL), then the aqueous phased was basified to pH > 11 by the addition of 5 M NaOH, and extracted with CH$_2$Cl$_2$ (3x50 mL). The combined organic phase was then dried over MgSO$_4$, filtered, and the solvent removed to yield the amine base as a clear oil. To this oil was added 10 mL of 48 % HBr in 5 mL of methanol, and the solvent removed under reduced pressure to yield 0.201 g of 64 (35 %) as an off white solid. $^1$H NMR (300 MHz, DMSO-d$_6$) δ 7.14 (d, $J = 8.3$ Hz, 2H), 6.87 (d, $J = 8.5$ Hz, 2H), 3.81 (s, 3H), 2.95 (t, $J = 6.8$ Hz, 2H), 2.71 (t, $J = 6.8$ Hz, 2H). HPLC retention time 1.80 mins condition B. Absorbance spectra $\lambda_{\text{max}}$ 223 nm minor $\lambda_{\text{max}}$ 275 nm.

3-chloro-4-methoxyphenethyamine(57). $^6$ 4.7 mL of a 1 M borane THF solution was added to a flame dried reflux apparatus, that had previously been purged with N$_2$. To this solution, a solution of 55 (0.422 g, 2.3 mmol) in 5 mL of sieve dried THF was slowly added over 5 minutes, and the resulting solution was brought to reflux for 1.5 hours. The solution was then cooled to room temperature and quenched by the slow addition of 8 mL of methanol, then the solvent was removed under reduced pressure. The crude oil was then dissolved in 25 mL of 2 M HCl and washed with diethyl ether (2x25 mL), then the aqueous phased was basified to pH 11 by the addition of 5 M NaOH, and extracted with CH$_2$Cl$_2$ (3x75 mL). The combined organic phase was then washed with Brine (1x30 mL), dried over MgSO$_4$, filtered and the solvent removed to yield the amine base as a clear oil. To this oil was added 5 mL of 48 % HBr in 10 mL of methanol, and the solvent removed under reduced pressure to yield 0.312 g of 57 (51 %) as a gray solid. $^1$H NMR (300 MHz, DMSO-d$_6$) δ 7.75 (s, 3H), 7.36 (d, $J = 2.0$ Hz, 1H), 7.23 – 7.17 (m, 1H), 7.11 (d, $J =$
8.4 Hz, 1H), 3.84 (s, 3H), 3.09 – 2.96 (m, 2H), 2.80 (t, J = 7.6 Hz, 2H). HPLC retention time 2.17 mins condition B. Absorbance spectra λ<sub>max</sub> 279 nm.

3-bromo-4-methoxyphenethylamine(58). 18 mL of a 1 M borane THF solution was added to a flame dried reflux apparatus, that had previously been purged with N₂. To this solution, a solution of 56 (1.989 g, 8.8 mmol) in 18 mL of sieve dried THF was slowly added over 5 minutes, and the resulting solution was brought to reflux for 1.5 hours. The solution was then cooled to room temperature and quenched by the slow addition of 20 mL of methanol, then the solvent was removed under reduced pressure. The crude oil was then dissolved in 50 mL of 2 M HCl and washed with diethyl ether (2x50 mL), then the aqueous phased was basified to pH 11 by the addition of 5 M NaOH, and extracted with CH₂Cl₂ (3x100 mL). The combined organic phase was then dried over MgSO₄, filtered and the solvent removed to yield the amine base as a yellow oil. To this oil was added 10 mL of 48 % HBr in 10 mL of methanol, and the solvent removed under reduced pressure to yield 2.352 g of 58 (88 %) as a gray solid. ¹H NMR (300 MHz, DMSO-d₆) δ 7.71 (s, 3H), 7.50 (d, J = 2.1 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.08 (d, J = 8.5 Hz, 1H), 3.83 (s, 3H), 3.08 – 2.98 (m, 2H), 2.79 (t, J = 7.7 Hz, 2H). HPLC retention time 2.25 mins condition B. Absorbance spectra λ<sub>max</sub> 222 nm minor λ<sub>max</sub> 280 nm.

4-hydroxyphenethylamine(tyramine)(65). A solution of 64 (0.200 g, 0.86 mmol) in 6 mL of 48% HBr was brought to reflux for 3.5 hours, after which it was allowed to cool to room temperature, diluted into 5 mL of methanol, and the solvent was removed under reduced pressure yielding 0.127 g of 65 (91%) as a tan solid. ¹H NMR (300 MHz, DMSO-d₆) δ 9.31 (s, 1H), 7.71 (s, 3H), 7.04 (d, J = 8.4 Hz, 2H), 6.72 (d, J = 8.4 Hz, 2H), 2.96 (s, 2H), 2.78 – 2.67 (m, 2H). HPLC retention time 1.30 mins condition B. Absorbance spectra λ<sub>max</sub> 221 nm minor λ<sub>max</sub> 275 nm.

3-chloro-4-hydroxyphenethylamine(3-chloro-tyramine)(49). A solution of 57 (0.318 g, 1.3 mmol) in 9 mL of 48% HBr was brought to reflux for 3.5 hours, after which it was allowed to cool to room
temperature, diluted into 5 mL of methanol, and the solvent was removed under reduced pressure yielding 0.285 g of 49 (88 %) as a tan solid. \(^1\)H NMR (300 MHz, DMSO-d\(_6\)) \(\delta\) 10.07 (s, 1H), 7.71 (s, 3H), 7.25 (d, \(J = 1.8\) Hz, 1H), 7.06 – 6.98 (m, 1H), 6.92 (d, \(J = 8.3\) Hz, 1H), 2.99 (s, 2H), 2.74 (t, \(J = 7.6\) Hz, 2H). HPLC retention time 1.55 mins condition B. Absorbance spectra \(\lambda_{\text{max}}\) 220 nm minor \(\lambda_{\text{max}}\) 280 nm.

3-Bromo-4-hydroxyphenethylamine (3-bromo-tyramine) (50). A solution of 58 (1.686 g, 5.4 mmol) in 35 mL of 48% HBr was brought to reflux for 3.5 hours, after which it was allowed to cool to room temperature, diluted into 10 mL of methanol, and the solvent was removed under reduced pressure yielding 0.285 g of 50 (88 %) as an impure brown solid. An attempt to purify the crude solid through column chromatography (0-10 % Methanol and 3 % triethylamine in DCM) yielded a black impure oil.

Feeding experiments for HPAA and norcoclaurine analogues. A 0.2 mM solution of halogenated tyrosine (either chloro, bromo, or iodo) in 10 mM phosphate buffer was warmed to 37 °C and converted into the corresponding HPAA or norcoclaurine as described above. The resulting analogues in phosphate solution was then diluted into an equal volume of 2x Gamborg’s media and filter sterilized through a 0.22 µm filter. Berberis cells were then shaken in this solution at room temperature. After a period of 1 week, the solids were separated from the liquid via gravity filtration, then lyophilized and stored at -80 °C until they were ready for analysis.

General Procedure for the Evaluation of background reaction rates for Pictet-Spengler reactions. 0.5 mM of HPAA and dopamine were mixed in a solution of each respective buffer. The rate of background reaction was determined using HPLC (condition C) by monitoring the area of the resulting norcoclaurine peak at 225 nm. The resulting areas were then plotted as a function of time to yield straight lines. The slopes of these lines were taken to be the rate of background reactions.

General Procedure for Norcoclaurine synthase kinetic assays. Sequential 1:1 dilutions of HPAA (or dopamine), were made so that 8 different concentrations of the substrate were prepared on a log\(_2\) scale. These were then reacted in the presence of a constant concentration of dopamine (or HPAA), buffer (50 mM TRIS, 25 mM imidazole, 25 or 100 mM HEPES, 25 mM maleic acid), and enzyme (8 µg/mL). The reaction was quenched at different time points by adding either 80 µL of the reaction into 120 µL of methanol or 80 µL of reaction into 20 µL of 1 M HCl. The rate of the reaction was determined using HPLC (condition C) by monitoring the area of the resulting norcoclaurine peak at 225 nm. The resulting areas were then plotted as a function of time to yield straight lines, the slopes of which gave the rates in terms of µV*s /s. These rates were then plotted against the concentration of the varied substrate (either HPAA or dopamine), and the resulting curves were fit to the Michaelis-Menten equation (Eq. 3.1), using OriginPro8 (Origin Lab).
General Procedure for the Evaluation of buffers concentrations on activity of Norcocluarine synthase.

Solutions containing 1 mM HPAA and Dopamine were reacted in the presence of a constant concentration of enzyme (8 µg/mL) and differing concentrations of buffer (20-100 mM). The rate of the reaction was determined using HPLC (condition C) by monitoring the area of the resulting norcocluarine peak at 225 nm. The resulting areas were then plotted as a function of time to yield straight lines, the slopes of which gave the rates in terms of µV*s/s. These rates were then plotted against the concentration of the varied buffer to determine if the buffer affected the rate of catalysis.
References

51. Clorox® (Clorox® Regular Bleach.
58. Anonymous (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.


