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Synthesis and Exploration of Dopamine and 4- HPAA Analogs for Norcoclaurine Synthase

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Synthesis and Exploration of Dopamine and 4-HPAA Analogs for Norcoclaurine Synthase

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This work is dedicated to my family, who have helped and encouraged my interest in science only when it was a curious question in my youth. Hopefully I can answer some of your questions now as you answered mine.

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Chapter 1 - Introduction

Early cultures of humans treated contagious diseases and viral infections with natural products extracted from plants. Such treatments are still continued even today as in the case of Artemisinin, which is used to treat malaria, and has been used since 200 BCE in China[1]. More than half of other drugs that are used to treat illnesses since the advent of chemistry are derivatives that come from existing natural products. Further drug development screens large numbers of potential molecules for activity towards a particular disease or disorder, or a general screening for any activity. A single potential target molecule that has shown desired activity is then taken through a labor intensive process of modification and exploration of the structure-activity relationships (SARs). Such procedures often take many years to complete and often do not yield the desired results because of cytotoxicity, side-effects, off-target hits, or low activity.

There are a variety of approaches that help modulate these processes, such as using chemistry that allows for easy modification of a molecule through either click chemistry, microfluidics at industrial scale, or bioengineering. The drug mentioned above, Artemisinin, was obtained from plants until a genetically modified yeast was developed to create a precursor which is used to produce semi-synthetic analogs[2]. Such advances allow for more efficient drug design and drug processing. However, currently humanity is facing a crisis of antibacterial resistance. To combat this rising threat, drug design should not solely rely on natural mimics and natural products. As such, rational drug designs as well as protein design have to be considered as new alternatives. Novel alternatives to natural products have been presented, such as rationally designed drugs that bind to fit receptors, but do not resemble natural products, or are modified proteins or antibodies. Likewise a similar approach for drug creation is precursor directed biosynthesis[3-5], which allows for a creation of precursors that are modified chemically and then taken up through a particular protein pathway to achieve final product. A benefit of this method is high yield of protein modified structures which modern chemistry might not achieve in the near future.

Although the precursor directed biosynthesis approach is not without drawbacks, such as the generation of natural and non-natural product mixtures[6], the total synthesis of complex molecules usually takes a significantly longer time with lower yields. However, utilizing precursor directed biosynthesis to generate a non-natural products as a first step and eventually developing engineered

organisms that produce the desired non-natural product, total synthesis can be avoided for the synthesis of complex natural products. Further, optimization of organisms, media, and conditions for growth and production can allow for higher production and possibly eliminate the need for total synthesis of molecules all together. The significant drawback for biosynthesis using engineered organisms is that it requires large investment of time and resources for its development, specifically in the creation of novel plasmids, growth conditions, and enzymatic conditions; therefore, a target drug should be one with a lasting impact. Artemisinin is a good example since it is currently the fastest and most potent drug that is available for treating malaria. However, recently Artemisinin-resistant strains of the malaria plasmodium have shown up. Likewise due to the threat of antibiotic resistant bacteria, novel drugs are always in demand. Based on a 2011 review, fewer drugs are being produced[7]. Antibiotic resistance is a growing concern[8]. There will always be a need for new drugs and flexible, low cost methods to produce them.

Due to the number of natural products that are used by the pharmaceutical industry, synthetic compounds are usually mimics of already existing compounds or are improved modifications of the already preexisting compounds. The approach for altering already preexisting compounds is not without risk. As mentioned above, once a microbe is resistant to a certain type of drug such as Artemisinin or a β-lactam type antibiotic, it is significantly harder to find treatment since the resistance is usually developed against the mechanism of action of the antibiotic, thus any similar antibiotics which use similar mechanism such as β-lactams are no longer potent or much less potent against the organism. Thus there exists an acute need for novel drugs which can be synthesized quickly and efficiently for testing and exploring their potency. One such approach as mentioned before would be to utilize precursor directed biosynthesis. It is particularly noteworthy that many of the drug candidates and drug leads are isolated from natural sources. Modifications to existing pathways in the form of altered precursors, particularly for precursors which are used in a large amount of naturally found drug molecules, would be useful in order to obtain a large selection of altered drug candidates without utilizing total synthesis.

Plants have been used as medicine for a very long time; particularly there exist classes of chemical structures in plants that are prevalent in many drugs. Structures that contain a basic nitrogen atom in their core are called alkaloids (Figure 1.1). Many of these structures are part of the currently used drugs such as the indolizine and pyrrolidine rings. Tropane rings and benzylisoquinoline, are also present in a variety of drugs. Previous work has successfully employed precursor directed

biosynthesis[9],[10] to obtain non-natural indole alkaloids. Likewise novel work was accomplished[11] in order to use semi-synthesis, procedure where a particular molecule is removed from cell and chemically altered, on modified alkaloids produced in culture using cross coupling reactions.

Benzylisoquinoline compounds, that are present in a variety of useful products like berberine, morphine, sanguinarine, and others, are of great interest because of large amount of different plant cell lines that utilize a precursor of benzylisoquinoline compounds: tetrahydroisoquinonlines (THI). These precursor molecules are found in many of the drugs mentioned before. Thus it would of benefit to modify tetrahydroisoquinoline compounds in order to access a large library of modified drugs and drug leads via different cells. In plant metabolism these THI compounds are a product of dopamine and 4-hydroxyphenacetaldehyde, both of which are derived from the amino acid L-tyrosine.

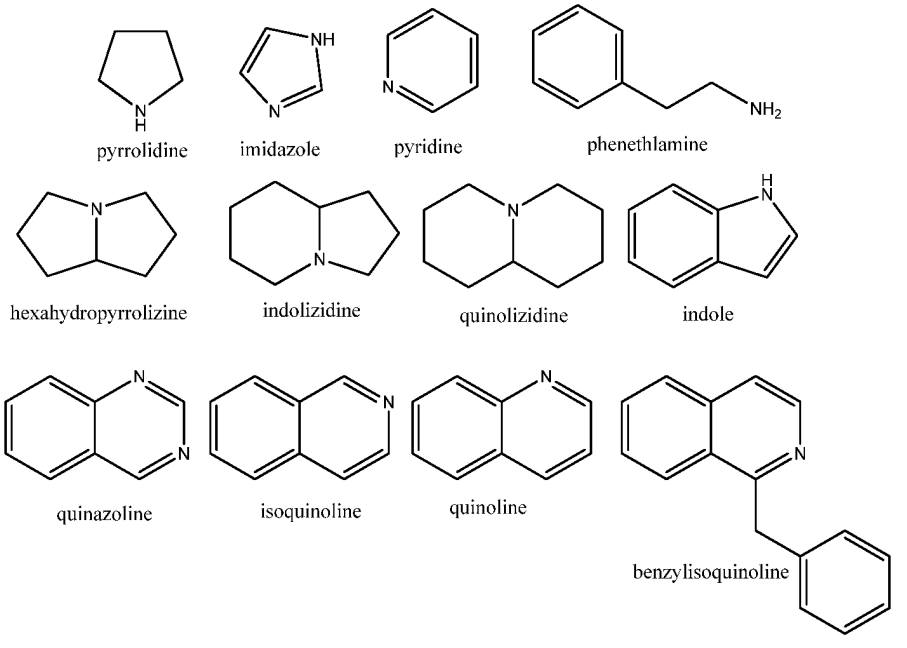
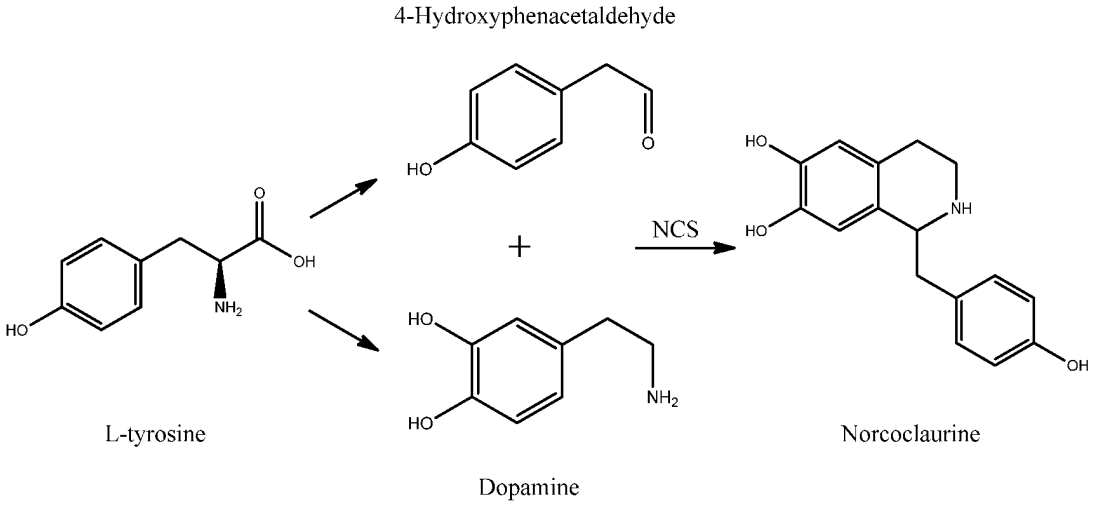


Figure 1.1. **Alkaloidal Cores.** Common types of alkaloid cores found in nature, which are present in potential and currently used drug structures.



Scheme 1.1. **Biosynthesis of Norcoclaurine.** The synthesis of norcoclaurine produced in plant cells by converting tyrosine into 4-hydroxyphenacetaldehyde and dopamine.

Following Scheme 1.1, readily available material like tyrosine can be manipulated as a synthetic precursor for modification of the THI product and even further the benzylisoquinolines. Precursor directed biosynthesis (PDB) utilizes organic chemistry to modify a much smaller component with much simpler chemistry. These chemically altered precursors are then taken-up by plant or bacterial cells which use their cellular machinery, such as enzymes[12], to modify small molecule precursors to complex modified structures. The non-natural starting material chemically altered must not disrupt cell's enzymes' natural activity, such as inhibiting it. Complex structures can be made by optimizing conditions of the solution or media in which cells are present.

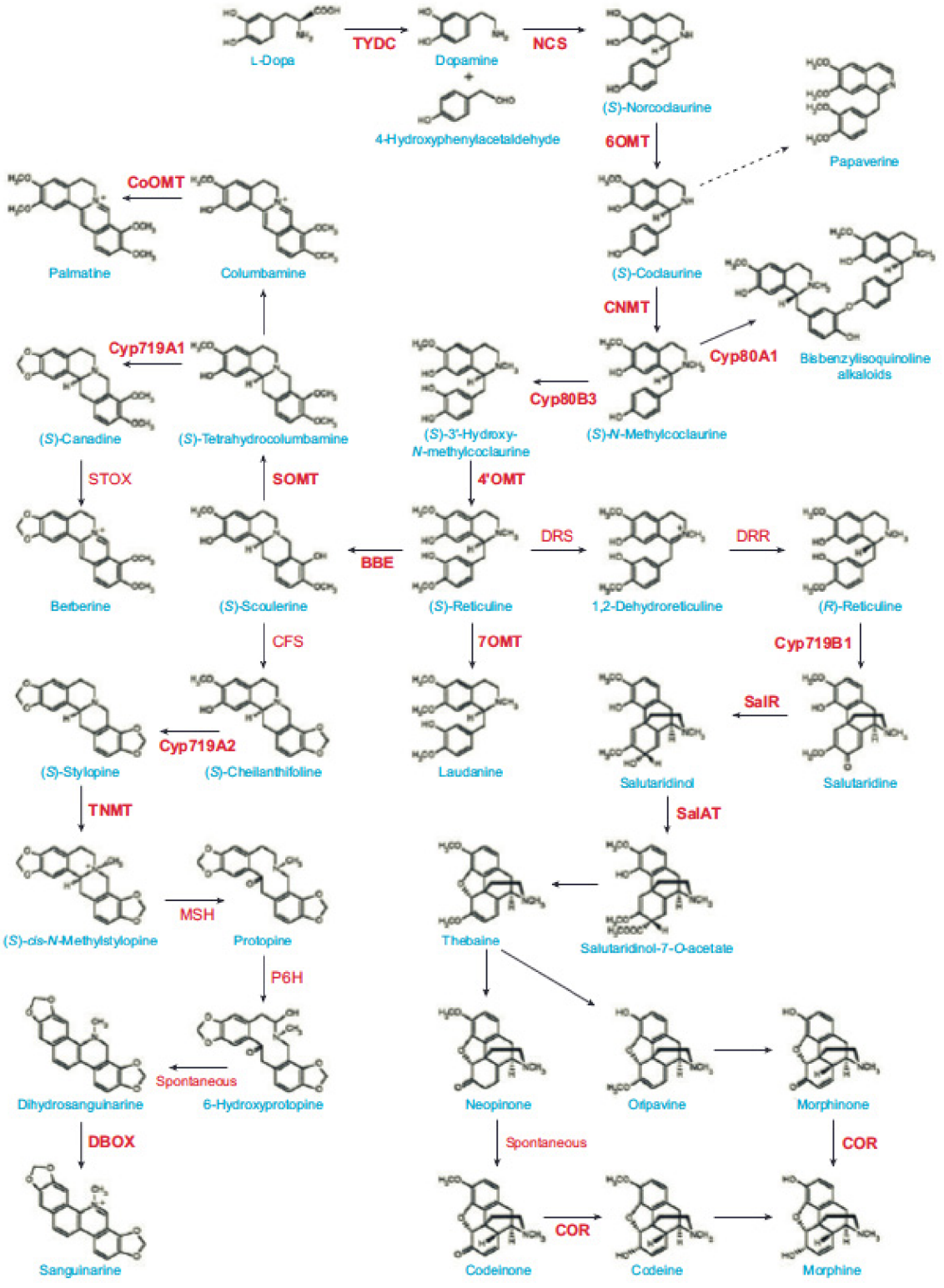
Unfortunately tyrosine is an essential building block in cells for protein structures and other important biochemical processes. Because of this, it is not a viable substrate for alteration and is an unlikely target for precursor directed biosynthesis. However, other relevant useful precursors can be used, which are not involved in biochemical processes that are important to cell proliferation. For this reason precursors such as dopamine and 4-hydroxyphenacetaldehyde (4-HPAA) are useful because they are not part of essential cell processes. Chemically altering dopamine or 4-HPAA will produce analogs that could be used by enzymatic system to produce a diverse library of compounds. The formation of tetrahydroisoquinoline core is present in a large variety of other potential drug leads. In order to test precursor directed biosynthesis a useful target molecule with an easily accessible cell line is necessary. For this purpose pentacyclic molecule berberine was chosen to be used as an example for studies in

precursor directed biosynthesis using altered dopamine and 4-HPAA substrates, which if successful could then be applied to a large variety of potential drugs and drug leads.

Chapter 2 - Halogenated Dopamine Analogs

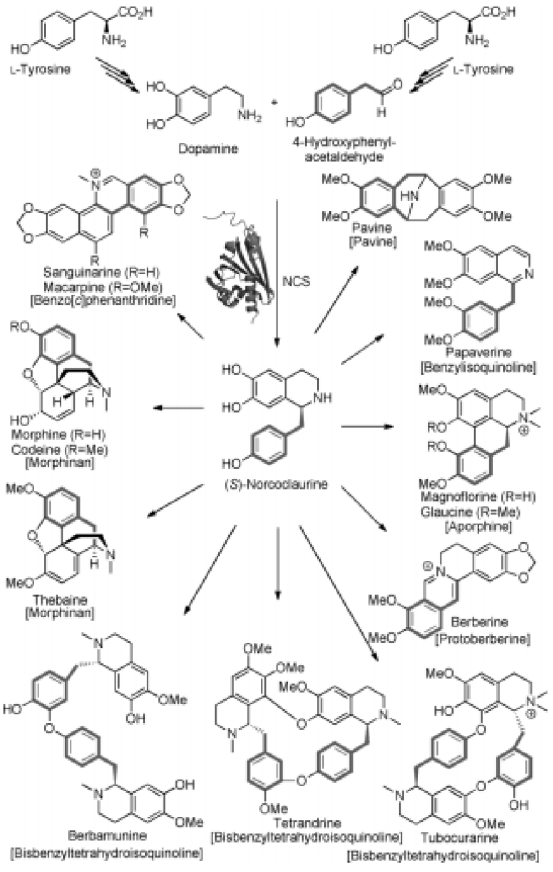
Introduction

There have been several studies on berberine as a potential drug. Although the use of berberine currently is used to stimulate AMP-activated protein kinase (AMPK) in order to decrease insulin resistance and lower blood cholesterol. In conjunction with ongoing AMPK studies, recent experiments have drawn attention to berberine as a lead compound for potential anticancer agents[13, 14] and a compound for several other uses[15], such as antimicrobial properties[16, 17]. Natural products that contain halogens[18] are potentially useful as a lead structure core that could further be modified by organic methods, because of this halogenated berberine analogs could be of interest. However, synthesis of a complex structure like berberine can lead to low yields due to purification and synthetic difficulty. Hence an alternative approach is to create precursors modified with halogens, such as fluorine, chlorine, bromine, and iodine, and push them through berberine producing cells that can uptake and incorporate non-natural substrate into the structure. Here the particular substrate that was chosen for modification is dopamine. By a complex mechanism (Scheme 2.1) dopamine along with 4-hydroxyphenacetaldehyde (4-HPAA) can be up taken by the cells to produce novel berberine analogs.



Scheme 2.1. **Dopamine and 4-HPAA Enzymatic Pathway**. The enzymatic pathway above Ziegler and acchini[19] shows the wide variety of compounds produced from dopamine and 4-HPAA as precursors.

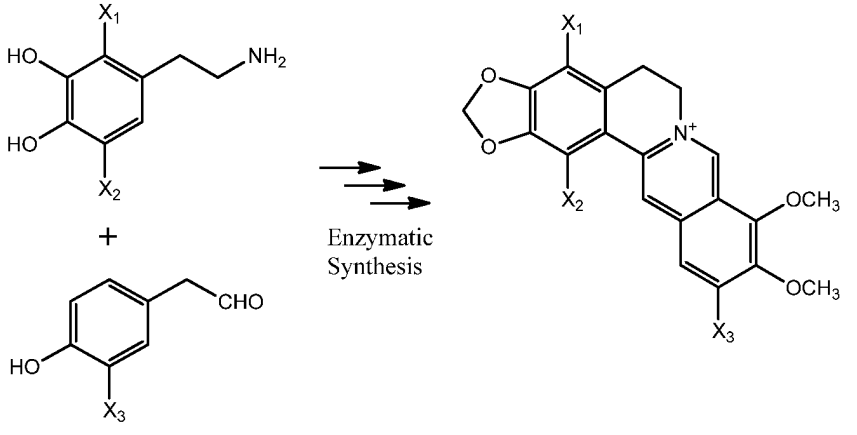
Using this scheme one can see the extent to which a combination of molecules could be created using precursor directed biosynthesis. The most difficult enzyme for this approach is likely norcoclaurine synthetase (NCS), which is the gateway enzyme[20, 21] and thereby responsible for making the scaffold for further enzymatic pathways by catalyzing Pictet-Spengler condensation between dopamine and 4-HPAA (Scheme 2.1) inside cells.



Scheme 2.2. **Enzymatic Scheme for Final Products of Plant Metabolism of Norcoclaurine.** Synthetic and enzymatic scheme presented by Waldmann et. al[22]. Core structure of each final product comes from the synthesis of (*S*)-Norcoclaurine, which in turn comes from bolded 4-HPAA and dopamine when they are joined by NCS.

Recently reported arylations of berberine at the dopamine 2-position as well as 4-HPAA 3-position have been found to improve antibacterial activity[23]. With this in mind, halogenation of dopamine at the 2- and 5- positions provides the possibility to further modify by Stille, Heck, or any other cross coupling reaction following enzymatic pathway to generate halogenated dopamine analogs. The modified positions in the final product can be seen in Scheme 2.3. The addition of halogens at certain positions have been observed to increase activity either by preventing drug from being

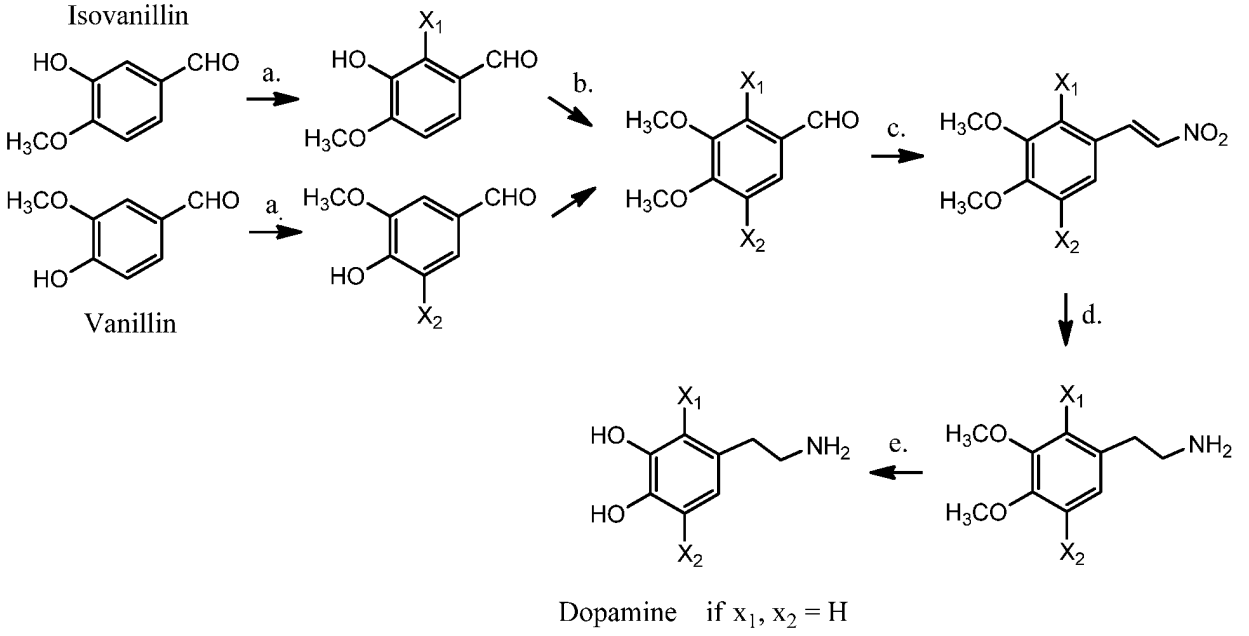
metabolized by the body or by the substituent fitting into the receptor pocket[24]. Similarly, as mentioned above, halogens are useful for completing other reactions such as Stille and Heck coupling reactions along with others such as directed lithium exchange since lithium halogen exchange has different rate of reaction depending on what halogen is present. These possibilities allow for selective manipulation of a complex molecule possibly allowing for quick screening of a large number of analogs with different substituent groups. Likewise halogenation of dopamine and 4-HPAA along with "feeding" to different cell cultures can produce a range of different halogenated drug molecules shown in scheme 1.1. This further enhances the scope of halogenated precursor molecules. If an efficient method is created for halogenation of dopamine and 4-HPAA many of the already known useful chemicals can be modified with halogens through a fully enzymatic pathway allowing for production of some very complex molecules. Equally, if the benefit is great enough for the production of halogenated drugs like berberine, gateway enzymes like NCS can be biologically modified to be more accepting of foreign substrates, allowing for easier production. The great advantage of using berberine stems from the availability of berberine cells which are readily available from public cell culture collections. If the precursor molecules are taken up and produce berberine analogs, this approach could potentially be applied to a variety of other cell systems to produce a large amount of analogs of complex drug molecules.



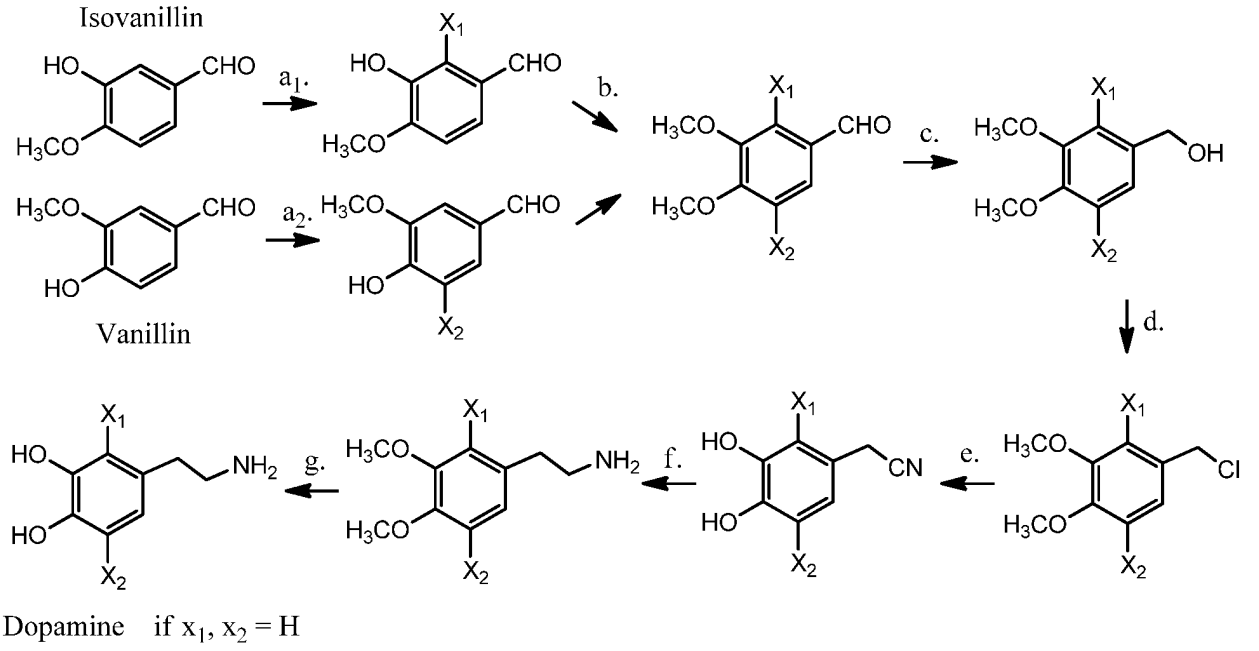
Scheme 2.3. **Enzymatic Synthesis of Berberine of Halogenated Berberine.** The substituted positions X_1, X_2 on dopamine and X_3 on 4-HPAA can be synthetically substituted with fluoro, chloro, bromo, or iodo halogens. Further "fed" to berberine producing cells and if up taken into the pathway the modified positions on berberine are kept.

Experimental/Discussion

Halogenation of dopamine analogs involved different synthetic pathways for fluoro and iodo analogs versus chloro and bromo analogs. Fluorinated analogs of dopamine were previously synthesized in our group[3], and preliminary data showed that when fed to *Berberis vulgaris* cell cultures were able to generate fluorinated berberine. (unpublished data). Therefore, we decided to prepare dopamine analogs containing Cl-, Br-, and I- to test the limits of this pathway. Scheme 2.4 and Scheme 2.5 detail the two alternative approaches to these compounds.



Scheme 2.4. **Synthetic Scheme for Cl- and Br- Analogs.** In the above scheme at step b reactions become identical for both 2- and 5- substituted analogs; therefore, both are shown on the same molecule. The reactions steps are described as following: **a.** NBS or NCS**; b.** CH_3I, DCM, TBAHS, NaOH-H_2O**; c.** NO_2CH_3, NH_4COOH, CH_3COOH, reflux**; d.** Zn/HCl in MeOH**; e.** BBr_3 in DCM, dry DCM.



Scheme 2.5. **Synthetic Scheme for 2- and 5- iodo Analogs.** In the above scheme at step b reactions become identical for both 2- and 5- substituted analogs; therefore, both are shown on the same molecule. The reactions steps are described as following: **a_1.** I_2, HIO_2, KI; **a_2** ICl, NC_5H_6, dark; **b.** CH_3I, DCM, TBAHS, NaOH-H_2O; **c.** EtOH, $NaBH_4$; **d.** DCM, HCl; **e.** NaCN, DMSO; **f.** BH_3•THF in dry THF, reflux; **g.** BBr_3 in DCM, dry DCM.

The reactions described in (Scheme 2.5) for iodine analogs follow a different synthetic pathway than those for chloro or bromo analogs. In the pathway for chloro and bromo analogs, after the Henry reaction in (Scheme 2.4), which uses nitromethane, acetic acid, ammonium acetate, and reflux to convert the aldehyde to a nitrostyrene, iodine gave many side-products and lower yield than the other analogs. Furthermore, reduction using zinc/HCl fully dehalogenates iodine from the molecule. Careful testing has shown that over the course of two hours at -10 °C both the reduction and dehalogenation proceeds cleanly to the final product of dimethoxy dopamine[25] (Figure 2.1). Finally at -20 °C the reaction became trapped in the intermediates and did not proceed to the product.

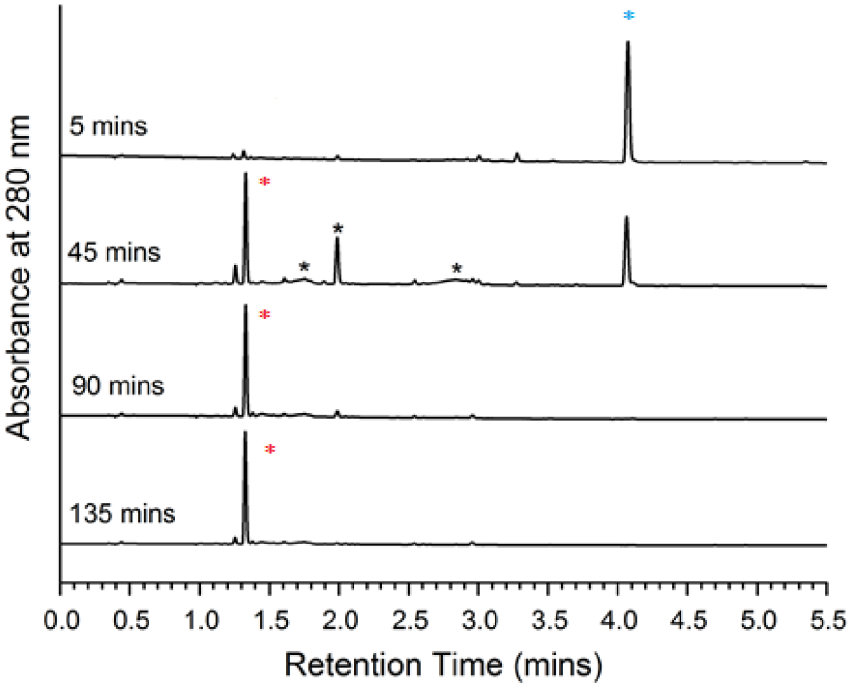


Figure 2.1. **Zinc/HCl Reduction at -10 °C.** HPLC traces for 0, 45, 90 and 135 minutes showing the conversion of 5-iodo-nitrostyrene to dehalogenated methoxyphenethylamine. The reaction proceeds from starting material (blue) to intermediates (black) and final dehalogenated product (red).

A possible explanation for this dehalogenation is the relative size of iodine versus the space where iodine can fill. The structures for qualitative comparison were modeled using ChemBio 3D, and their energies were minimized using HF calculation. As shown in Figure 2.2, the distortion due to iodine forces unfavorable interactions to occur at the methoxy groups. Though it is uncertain, there could be zinc interactions with the lone pairs of the oxygen atoms causing further strain for the iodine atom as the methoxy group would want to be planar. As such, under reductive conditions, iodine was likely disassociated from the molecule and replaced by hydrogen during reduction.

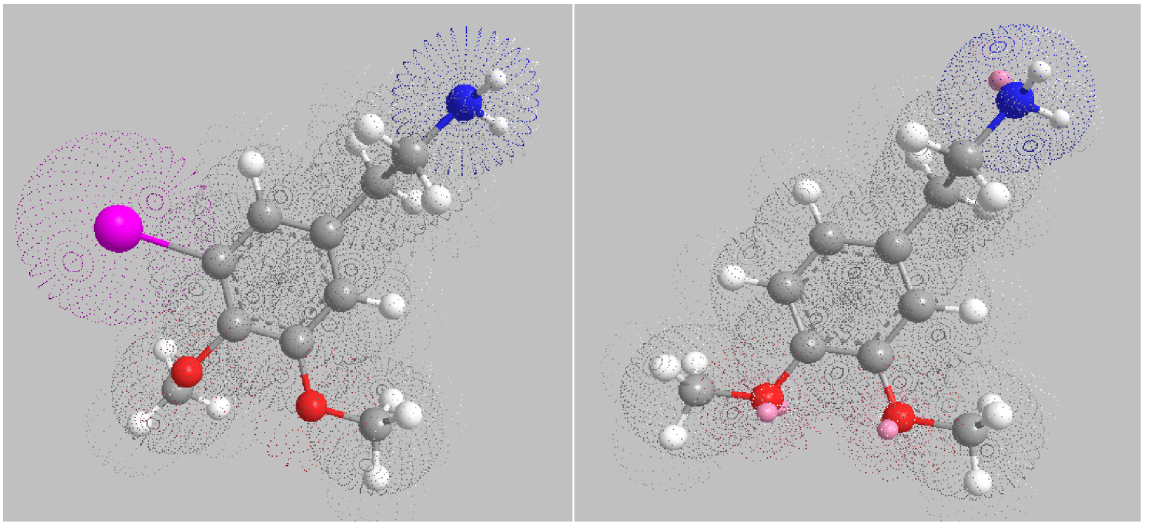
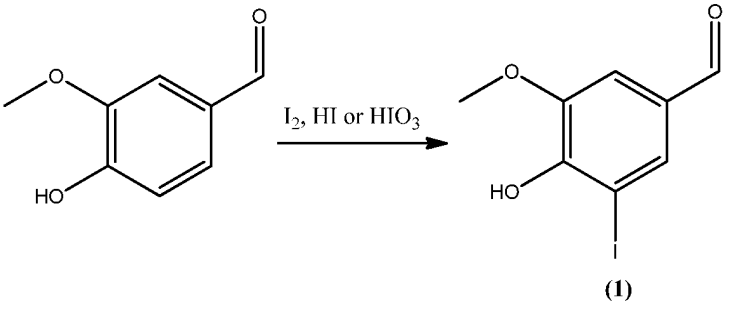


Figure 2.2. **Comparison of Size of 2-Iodo 3,4-dimethoxyphenethylamine vs. 3,4-dimethoxyphenethylamine**. ChemBio 3D generated image of space filling atomic model using relative electron density of the atoms for size comparison. Each structure represents the minimum energy conformation from HF/3-21G quantum mechanical calculation using GAMESS. 5-iodo dimethoxy dopamine is on the left comparative to the dehalogenated dimethoxy dopamine.

Other attempts at reduction of 5-iodo nitrostyrene were made using tin (Sn) reduction conditions. Although the desired product was observed via HPLC analysis the final mixture contained inseparable products as they eluded separation using liquid extraction, recrystallization, and column chromatography methods. The nitrostyrene method was abandoned due to the need for expensive reagents and poor reported yields, hence a different approach was conceived. Avoiding the problem of dehalogenation of the dopamine analog by Zn/HCl, Sn reduction conditions, and costly reagents, a separate synthesis based on 2-fluoro and 5-fluoro synthetic pathway, previously accomplished in our group[3], was used to produce 2- and 5- position substituted iodine analogs.

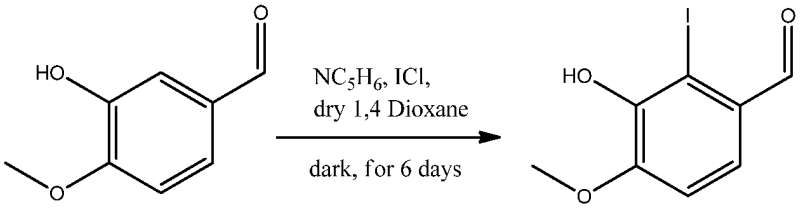
Halogenated Dopamine Analogs:



(1)

Scheme 2.6

Several attempts were made at iodination of the 5- position. Attempts using NaI along with vanillin, water, and oxone proved to be ineffective in generating the desired iodinated compound. Further attempts were abandoned in favor of mixing I_2 with vanillin and iodic acid in EtOH as solvent. This yielded a brown solution with off white precipitate, which was filtered and washed with sodium thiosulfate. Finally, the pure 5-iodovanillin **(1)** was obtained from EtOH recrystallization. An even simpler procedure was to simply grind of vanillin, I_2, and KI with a mortar and pestle to produce crude product that was easily recrystallized from EtOH (97%).

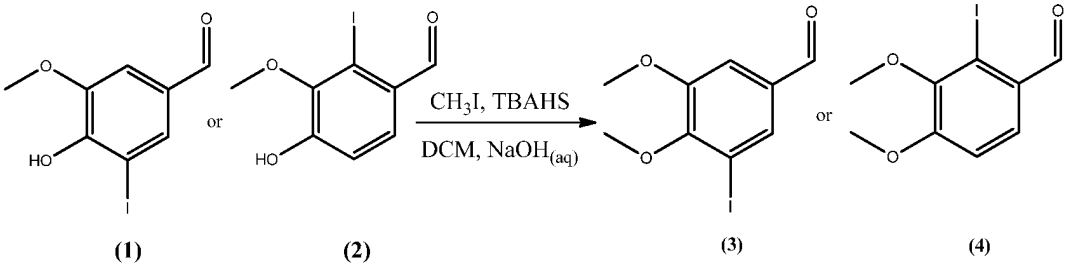


(2)

Scheme 2.7

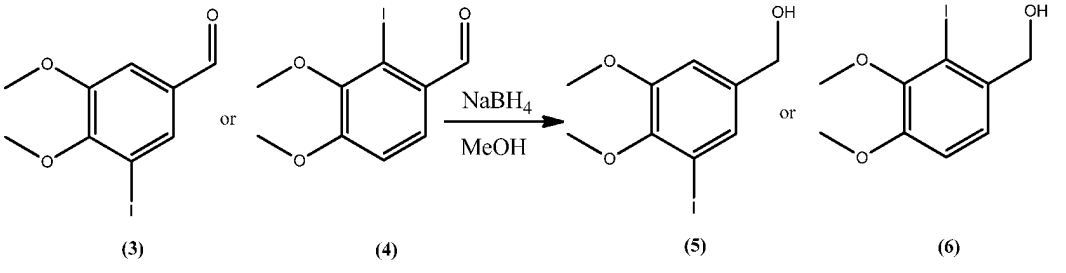
These same techniques were attempted on isovanillin, but with no success. This could be attributed to the congested nature of the 2- position on isovanillin, which is between the aldehyde and hydroxyl groups on the benzene ring. Several other procedures to produce 2-iodo vanillin were tried, including: 1) MeOH and concentrated ammonia solution under 0 °C with slow addition of I_2, 2) isovanillin dissolved in water with I_2 and $NaHCO_3$ under N_2 atmosphere and dark conditions. After testing of these procedures vanillin was used instead of isovanillin to obtain 2-iodo vanillin, which after methylation in step b, would yield identical product if isovanillin was used. Similar conditions were used as those that have worked for vanillin iodination except with the addition of phosphorus pentoxide, yet there was no reaction. Again, this is possibly because of the position and size of the substituent. Using pyridine with dissolved isovanillin was partially successful. When cooled to 0 °C and under dry conditions a mixture of iodine monochloride (I-Cl) in dry 1,4 dioxane was added slowly. The solution was then wrapped in aluminum foil and allowed to react for 6 days. After several extractions and recrystallizations a final product of 2-iodo isovanillin was obtained in a 24% yield. A final attempt at obtaining 2-iodoisovanillin **(2)** was made using I_2 stirred into 1-methylpiperazine. This generated an iodine-methylpiperazine complex, which acted as a source of I^+ for the iodination of isovanillin[26]. After the generating the complex it was dried under reduced pressure and finally added over a two hour period to a mixture of isovanillin and K_2CO_3 in water. After two hours the solution was placed into an ice bath and acidified very slowly with 25% H_2PO_4. The reaction was finally allowed to stand for 24 hours at 4 °C. Unfortunately

no precipitate was observed as the procedure claimed. It is worth noting that many of these procedures, which were tried, have been claimed to produce excellent yield of >90%. Although 2-iodo isovanillin was obtained it was deemed to be not worth the materials and time to try and optimize the iodine monochloride procedure and the 2-iodo compound was simply purchased from Sigma-Aldrich.



Scheme 2.8

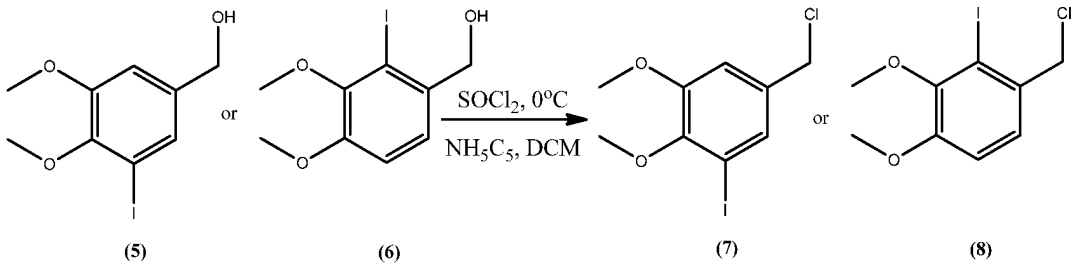
Both 2- and 5- iodo analogs were methylated using the same procedure. A mixture of either 2- or 5- iodo analog in DCM, NaOH and water was allowed to come to equilibrium, at which point tetrabutylammonium hydrogen sulfate (TBAHS) was added followed by methyl iodide (MeI). The reaction was allowed to stir for a minimum of two and half hours after which layers are separated. The aqueous layer was extracted with DCM and combined organic layers were evaporated under reduced atmosphere generally yielding a solid yellow product. TBAHS, which is a phase transfer catalyst and therefore present in both organic and water layers was removed by column chromatography using hexanes: ethyl acetate (4:1). The products, 2-Iodo-3,4-dimethoxybenzaldehyde (97%) **(4)** and 5-Iodo-3,4-dimethoxybenzaldehyde (90%) **(3)**, were obtained as white fluffy solids.



Scheme 2.9

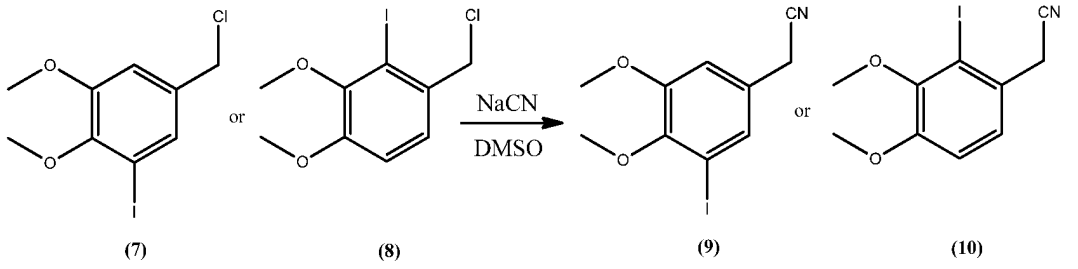
Reduction of the aldehyde product was accomplished by stirring sodium borohydride ($NaBH_4$) in MeOH for a minimum of one hour. The products, 2-Iodo-3,4-dimethoxyphenylmethanol (95%) **(6)** and 5-Iodo-3,4-dimethoxyphenylmethanol (95%) **(5)**, were obtained after the reaction was quenched with acetone and evaporated until half of the volume was left, diluted with water, and extracted using DCM.

The products appear as clear or slightly foggy oils and were used without purification for the following chlorination reaction.



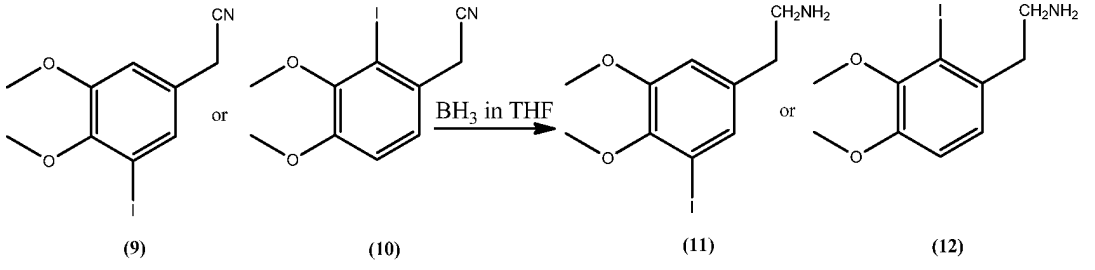
Scheme 2.10

Different procedures were tested to convert the alcohol to the chloride. Stirring in pyridine at 0 °C with a slow addition of thionyl chloride ($SOCl_2$) gave satisfactory yields; however because of the nature of the reagents such as $SOCl_2$ and pyridine a different reaction was preferred. Thionyl chloride reaction with triethylamine (NEt_3) yielded similar results. After a thorough search through the literature another method for chlorination was found and tested. The method utilized the alcohol analog dissolved in benzene in a separatory funnel. Two molar equivalents of concentrated HCl were added to the funnel and reaction was shaken for 10 minutes to yield 5-iodo-3,4-dimethoxybenzylchloride (91%) **(7)**. However, due to the toxicity benzene, other solvents were tried which included: toluene, diethyl ether (Et_2O), DCM, and THF. The best results for chlorination of the 5-iodo-benzyl alcohol were obtained by simply stirring the alcohol in DCM with HCl under vigorous stirring in a round bottom flask. This approach, however, did not produce any reaction for chlorination of the 2-iodo analog. This is perhaps due to the requirement for protonation of the benzyl alcohol to leave as H_2O in forming a stable benzylic carbocation which is further stabilized by methoxy resonance donation. Because 2-iodo is on the neighboring carbon its inductive effects provide interference, which can possibly lead to a much higher energy barrier for water to dissociate. Hence, the previously mentioned method of thionyl chloride with pyridine was used to obtain 2-iodo-3,4-dimethoxybenzylchloride (90%) **(8)**. Further purification was not needed and the products were used for the next reaction after being concentrated under reduced pressure.



Scheme 2.11

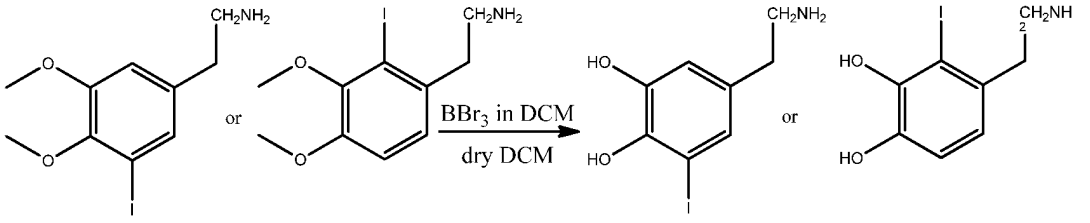
Both 2- and 5- position iodo-benzylchloride were substituted in DMSO with sodium cyanide (NaCN) for a minimum of one hour at which point the mixture was checked for completion by HPLC, extracted with Et_2O and concentrated under reduced pressure. The white solid products, 2-iodo-3,4-dimethoxybenzylchloride (99%) **(10)** and 5-iodo-3,4-dimethoxybenzylchloride (87%) **(9)**, were obtained and used for reduction.



Scheme 2.12

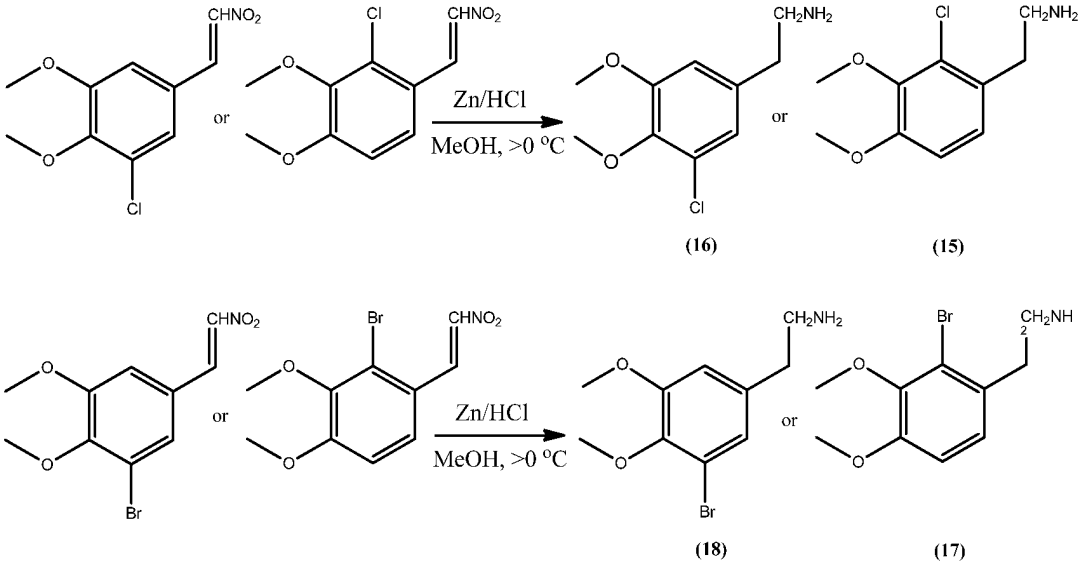
The reduction of nitrile to amine was tested utilizing other methods besides borane reduction (e.g. H_2 with Pd/C under dry conditions). These methods did not yield any favorable reaction. Likewise other reaction conditions such as samarium triiodide (SmI_3), zirconium tetrachloride ($ZrCl_4$), or lithium aluminum hydride ($LiAlH_4$) were considered, but ultimately avoided due to the potential risk of iodine reduction. Therefore, the reduction followed a standard procedure for borane reduction using BH_3•THF in dry THF under inert atmosphere (N_2) with reflux. The extraction of the reduced amine proved to be a challenging. Although the reaction went to completion, the quench of excess BH_3 unless done under 0 °C yielded side products which were inseparable by chromatography and acid/base extractions. There are possibly conditions that could have purified product of the reaction from the contaminants that were present. However, such methods were not pursued and a method for facile quench and extraction procedure was instead optimized. The quench was performed in an ice bath with slow addition of H_2O until foaming in the round bottom flask stopped. The mixture was then exposed to air and acidified with 37% HCl, stirred for an hour, and extracted with DCM if any side products were observed. After

extraction, the mixture was then diluted with H_2O and basified using 30% NaOH solution. The product of the reaction is extracted with 97:3 DCM/MeOH mixture and concentrated under reduced pressure to yield products 2-iodo-3,4-dimethoxyphenethylamine (95%) **(12)** and 5-iodo-3,4-dimethoxyphenethylamine (96%) **(11)**. It should be noted that similar procedures use reflux with HCl instead of stirring for one hour, basifies, extracts with Et_2O, and utilizes flash column chromatography. These steps were tested, but ultimately found unnecessary after optimized extraction conditions.



Scheme 2.13

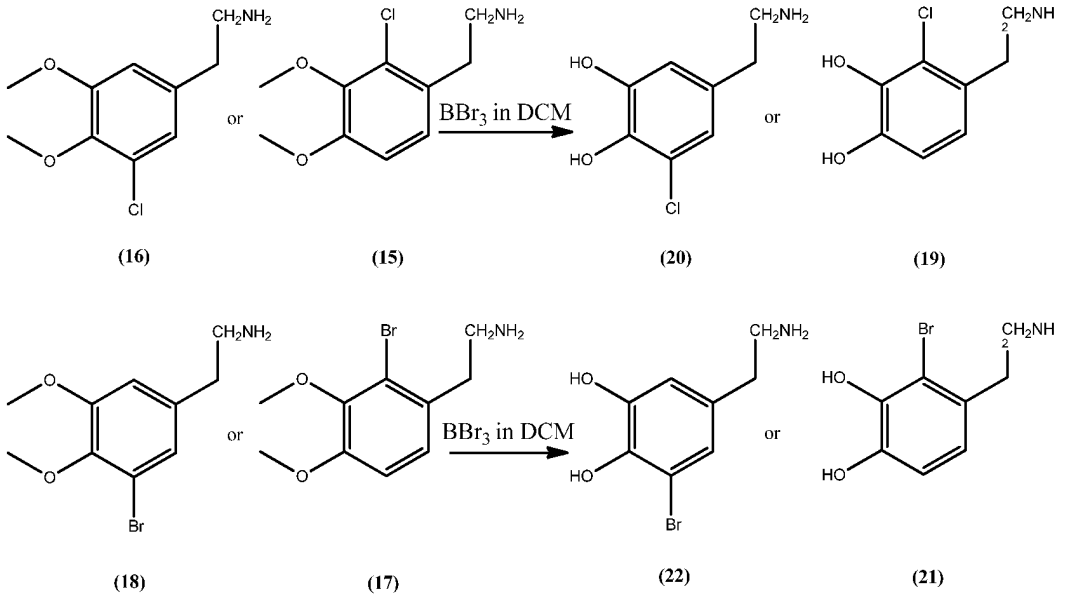
Deprotection to yield the catechol was tested using several conditions, which included: reflux with concentrated HCl, concentrated HBr, and BBr_3. However, only the BBr_3 method fully deprotected without any side products, possibly due to high temperatures necessary for acid deprotection reactions. HPLC traces indicated that these products formed oligomeric products that most likely resulted from the oxidation to highly electrophilic ortho-quinones during the reaction or during evaporation to remove the high boiling acids. HPLC evidence also suggested that dehalogenation occurred with both 2- and 5- iodo compounds under acidic reflux conditions. Acid reflux deprotection was abandoned in lieu of BBr_3 deprotection. The reaction was performed in dry DCM, inert atmosphere (N_2), and ice bath with slow addition of BBr_3. After the addition the reaction was allowed to stir at room temperature for 1.5 hours. The reaction was then quenched with dry methanol in an ice bath and allowed to stir for another 10 minutes. Solvents and boron byproducts (trimethoxy boron, trihydroxy boron) were evaporated under reduced pressure, and the solid product was then washed with Et_2O to obtain white powder for both 2-iodo-dopamine (99%) **(14)** and 5-iodo-dopamine (98%) **(13)**.



Scheme 2.14

Synthesis of 2-, 5- chloro and 2-, 5- bromo according to Scheme 2.4 was performed in our lab[27], by a previous student, to step (d), which involved zinc reduction with HCl. Extensive testing was performed to optimize the reduction of the nitrostyrene starting material to obtain halogenated protected phenethylamines. Initially, dehalogenation was a problem for the chloro and bromo compounds as was for the 2- and 5- iodo compounds. However, the chloro- and bromo- compounds were less prone to dehalogenation and could be reduced under subzero temperature. This is possibly due to the size difference between the halogens which makes the iodo in these conditions more labile than chloro or bromo. Iodo compounds fully dehalogenated at -10 °C conditions (Figure 2.1) and under -20 °C reaction ceased all progress and was stuck in intermediates (with iodo halogen present). This was not observed with chloro and bromo compounds at temperatures below zero. The reaction proceeded with portion-wise addition of starting halogenated nitrostyrene and zinc powder into a stirring solution of methanol and HCl. A bath of ethylene glycol (antifreeze was used as an inexpensive substitute for pure ethylene glycol) with ice, provided adequate cooling (-5 °C) for the reaction to proceed without dehalogenation. Reaction was then quenched with methanolic sodium hydroxide (NaOH pellets dissolved in MeOH) to avoid any water possible which generated side products when present. Basification produced a thick slush from which reduced phenethylamine compounds were extracted after the pH of solution was adjusted to 11. The choice of solvent for extraction remained questionable for a period of time. Several solvents were tested for extraction, such as: DCM, Et_2O, and chloroform ($CHCl_3$). Amongst these solvents it was found that $CHCl_3$ elutes the product in reasonable amount and

avoids contaminants. Diethyl ether also provided similar results; however, after removal of solvent under reduced pressure unknown side products were observed. After evaporation of $CHCl_3$ the products were obtained as pale yellow oils of: 2-chloro-3,4-dimethoxyphenethylamine (99%) **(15)**, 5-chloro-3,4-dimethoxyphenethylamine (93%) **(16)**, 2-bromo-3,4-dimethoxyphenethylamine (73%) **(17)**, and 5-bromo-3,4-dimethoxyphenethylamine (87%) **(18)**.



Scheme 2.15

Last step towards chloro and bromo halogenated dopamine was identical to the iodo pathway which used careful addition of BBr_3, under inert atmosphere (N_2), and dry DCM to deprotect the hydroxyl groups. After quench and extraction procedure described earlier, the final products were obtained as pale yellow oils: 2-chloro-dopamine (88%) **(19)**, 5-chloro-dopamine (98%) **(20)**, 2-bromo-dopamine (94%) **(21)**, and 5-bromo-dopamine (90%) **(22)**.

Notes/Observations:

Optimization of separation of phase transfer catalyst (TBAHS) present during methylation reaction was attempted. Earlier references [28] reported liquid extraction using acid/base conditions. It was found that these conditions were not compatible with dimethoxybenzaldehydes. Optimization of the extraction solvents was attempted by using DCM/MeOH (95:5, 90:10), Et_2O/Hexanes (1:9, 1:10) and EtOAc/Hexanes (20:80, 40:60, 45:55, 50:50, 70:30, 80:20). Progress of separation was monitored by TLC and checked using UV. Staining using *p*-anisaldehyde is also suitable. After thorough testing, extraction

using either 1:4/1:5 EtOAc/Hex yields the highest extraction. Product obtained from extraction should be a white fluffy powder without any discoloration. If discoloration is observed (particularly yellow color) it is possibly due to phase transfer catalyst still being present in the product. Further extraction is then needed.

Purification of $NaBH_4$, chlorination, and nitrile substitution not required. Products from $NaBH_4$ and chlorination yield pale yellow oils. The products need to be thoroughly checked by other methods than visual inspection. Nitrile products, though usually was pure, can be recrystallized from either EtOH or Et_2O.

The protected phenethylamines **(11, 12, 15-18)** can be purified by acid precipitation by dissolving the product in cold Et_2O and adding cold concentrated HCl, HBr, or H_2SO_4 slowly until white precipitate forms. If too much acid is used, some product dissolves in the aqueous layer formed by acid. The white salt can then filtered and dried under vacuum. Product purified this way is suitable for deprotection because of inability to dissolve back into organic solvents that are necessary for the deprotection. For further reactions, salt products must be basified and extracted from aqueous solution.

While the dopamine analogs **(13, 14, 19-22)** were almost exclusively pure some side products were observed to occur in either the zinc reduction (no acid precipitation was used) or the deprotection steps. This result was possibly due to the reaction mixtures inadequately cooled or quenched. Deprotection, water, or excess HCl added for the zinc reduction were also observed to produce contaminants. These side products could be avoided in the final step by using acetonitrile (ACN) or isopropanol with slow addition Et_2O to precipitate dopamine exclusively over the side products. This step, however, produces drastically lower yields since a large amount of dopamine will stay in solution.

Discussion:

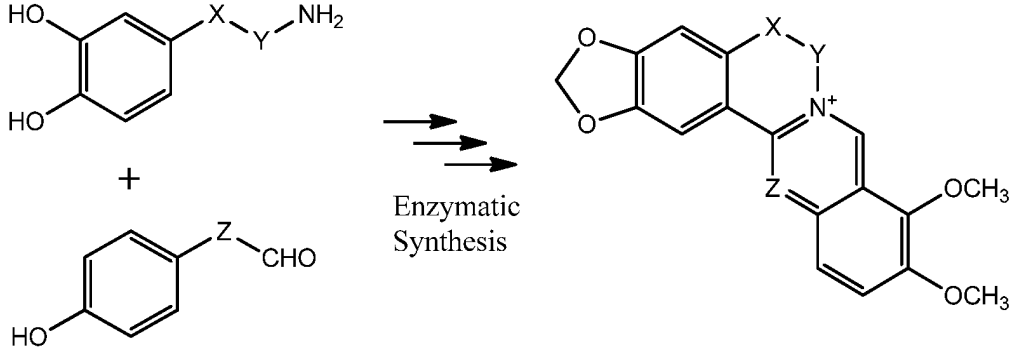
Utilizing the above methods, chloro, bromo, and iodo halogenated dopamine analogs were synthesized for further testing enzymatic activity of NCS on non-natural substrates. Chemically modified dopamine can also be tested if it can produce halogenated berberine analogs. While it is unknown if the halogenated dopamine analogs are taken up into the enzymatic pathways, as mentioned previously fluoro dopamine analogs were observed to be taken up by cells through the enzymatic pathway to produce a fluorinated berberine analog. Cells were incubated in liquid media with the fluorine analog of

dopamine over a period of two weeks. Utilizing similar conditions other halogenated dopamine analogs can be incubated and tested for halogenated berberine analogs. It was confirmed in preliminary studies that norcoclaurine synthase was able to generate THI analogs from halogenated dopamine analogs. This provides a good precedent that other halogenated dopamine analogs can be further taken up by enzymatic pathway. Despite previous reports that norcoclaurine synthase has limited substrate specificity[29], it was observed that NCS is able to take up iodo dopamine analogs and produce halogenated tetrahydroisoquinoline (see Chapter 5).

Chapter 3 - Dopamine Ethyl Linker Analogs

Introduction

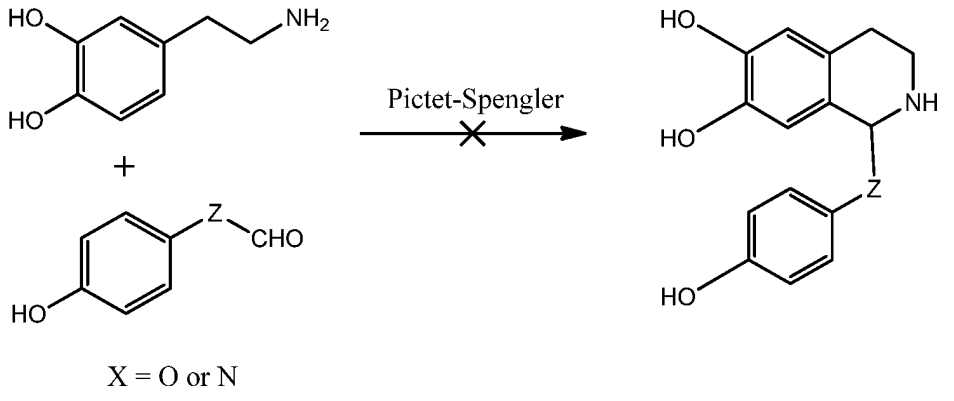
The process of making norcoclaurine and norcoclaurine analogs involves a Pictet-Spengler condensation between the aldehyde 4-HPAA and dopamine. Modifications that are introduced into either 4-HPAA or dopamine can be then incorporated into larger structures via the Pictet-Spengler reaction. The easier target for analog variation is dopamine due to its stability when compared to the 4-HPAA, which cannot handle heat and is not stable for long periods of time even at -80 °C. While halogens are useful for crosslinking and biological activity, there are two more positions that could be varied in dopamine that could produce interesting structural analogs of norcoclaurine and berberine by the norcoclaurine synthase enzyme or Pictet-Spengler reaction. Likewise it is standard substitution in medicinal chemistry to incorporate nitrogen atoms into carbon positions. This can increase or decrease the pharmacokinetic and/or pharmacodynamic properties of the drug lead. Complicated structures like berberine, substitution, which is introduced in dopamine or 4-HPAA is a simple way to obtain berberine ring analogs. The changes that are presented in such a manner allow for complex structure manipulation without total synthesis.



Scheme 3.1. **Substitutions Proposed for Dopamine and 4-HPAA for Enzymatic Synthesis**. The substituted positions X and Y on dopamine and Z on 4-HPAA can be synthetically substituted with a variety of atoms such as O or N. Further "feeding" to berberine producing cells and up take into the pathway, modified positions on berberine are kept providing modified ring structure. The most likely substituent in 4-HPAA would be nitrogen since in the berberine structure the ring containing Z substitution needs to be aromatic.

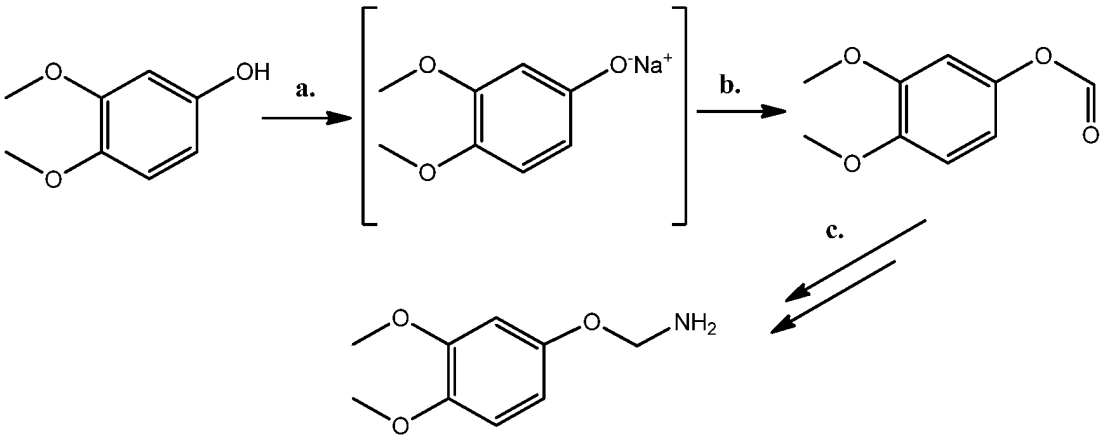
Modifying 4-HPAA presents another challenge as a lack of suitable carbons which could be manipulated. The benzylic carbon presents many challenges such as expensive reagents and possibly

difficult chemistry along with the fact that the aldehyde is not stable. It is possible that substitution of nitrogen or an oxygen atom will lead to producing a formamide which is possibly unreactive or an ester, which will generate hydroquinone after a nucleophilic attack. Hence substitutions at the Z position would not lead to a successful Pictet-Spengler reaction (Scheme 3.2). It is possible to substitute something onto the benzylic position, but as mentioned before such chemistry could be difficult considering that the starting material for 4-HPAA synthesis currently is tyrosine. To place a substituent on the benzylic carbon of 4-HPAA, it would need to undergo several steps involving protecting groups for the aldehyde that could give access to the benzylic position for proton abstraction. It might be simpler to start from a different starting material that is also inexpensive and readily available.

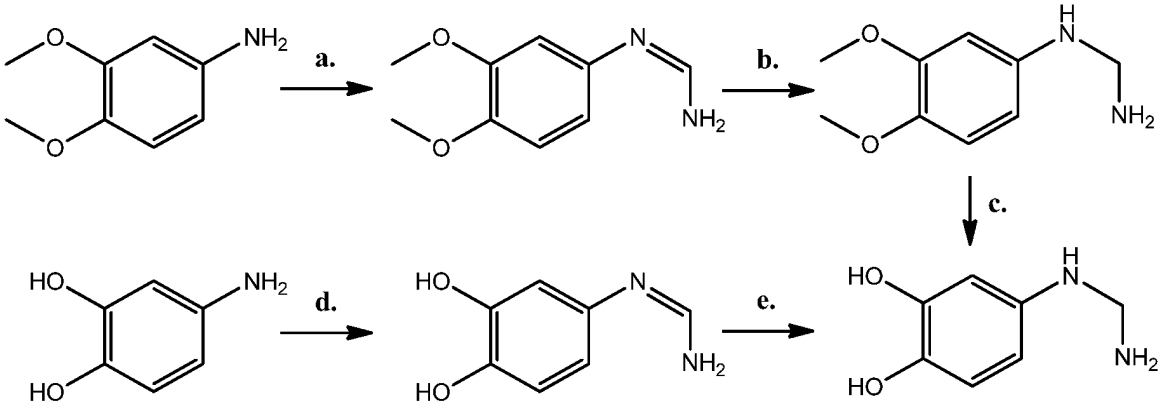


Scheme 3.2. **Proposed Substitution for 4-HPAA Pictet-Spengler Condensation**. The substituted aldehyde should not react in a Pictet-Spengler reaction to yield substituted norcoclaurine analog and will instead degrade to either formylated dopamine or hydroquinone or will not react with the formamide.

Dopamine analogs in the positions indicated in Scheme 3.1 are the best for ring modifications. These modifications can be inserted by similar chemistry that has been performed in the synthesis of the halogenated dopamine analogs. Modification at the Y position present more challenge for structural modification since starting materials have to be different (Scheme 3.1). For Y position substituent as an oxygen atom, the likely position for synthesis start this is something like dimethoxyphenol, which could be reacted to generate dimethoxy sodium phenolate, and reacted with a formyl group. This will generate dimethoxyphenyl formate that could can be reduced to an alcohol or reacted with an amine to generate and imine. It is also possible that the attack by the amine will end up discarding dimethoxy phenol as a leaving group. This presents severe challenges like 4-HPAA for producing structural analogs.



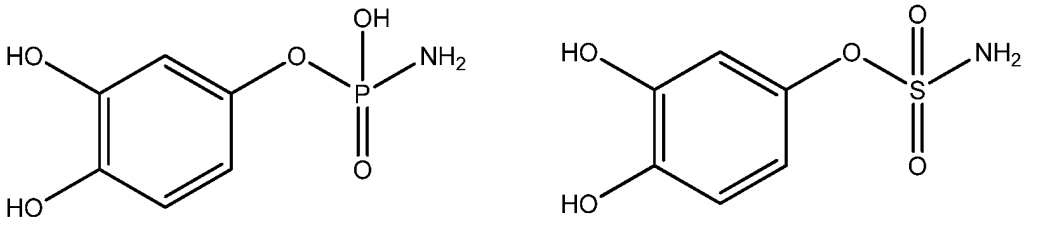
Scheme 3.3. **Proposed Scheme for Oxygen Substitution**. The scheme above shows dopamine position X substitution using oxygen producing a novel dopamine analog. The chemistry to develop the new link between O-C-N has not been observed yet and therefore would provide substantial challenge.



Scheme 3.4. **Proposed Scheme for Nitrogen Substitution**. The scheme above shows dopamine position X substitution using nitrogen producing a novel dopamine analog.

The two analogs of the X position possibly would not be stable in water because of polar charge at the carbon. Instead this position can be substituted as an oxygen atom by reacting dimethoxy sodium phenolate with chlorinated phosphate (Scheme 3.1). Another reaction with thionyl chloride or other chlorinating reagent will generate another phosphochloride to be reacted with ammonia. This will produce dimethoxyphenylphosphamidate, which can further be converted to dihydroxyphenylphosphamidate **(23)** by BBr_3 or similar deprotection methods. The structure produced is not very similar to dopamine, containing two substitutions into its ethyl linker in the form of an oxygen and phosphorus atoms and occupying a much larger area. Structure such as this be interesting to study

for its applications if it could undergo cyclization via Pictet-Spengler to form a phosphate incorporated norcoclaurine analog. These phosphamide analogs of norcoclaurine could be of interest assuming it can be taken-up and incorporated through the enzymatic pathway for evaluating its effect on the berberine activity since it is an incorporation of a radically different group. A similar approach could be applied to obtain incorporated sulfonamides generating dihydroxyphenylsulfamate **(24)** and studied for biological activity. There is precedent[30, 31] for stable sulfonamide-containing drugs that contain either antibacterial or anti-inflammatory properties. It is also known that phosphamide compounds can be used for cancer treatment[32]. It would of interest to compare sulfonamide and phosphamide norcoclaurine structures and use them further in a precursor directed biosynthesis towards novel berberine and other analogs.



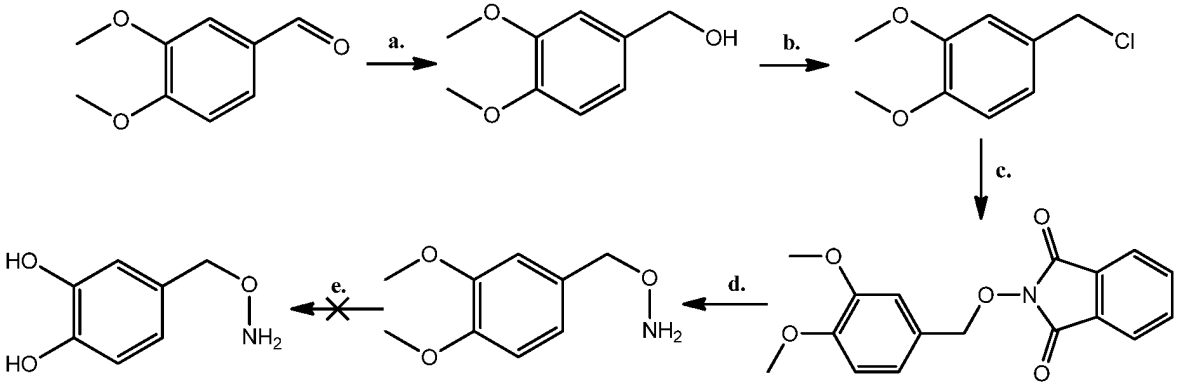
(23) (24)

Scheme 3.5. **Proposed Structures for Phosphamide and Sulfonamide**. Proposed structures for dopamine analogs which could be substituted for the ethyl linker that normally is present in dopamine.

For Y position modifications, synthesis could begin with dimethoxy analine which could be reacted with a formamide to generate dimethoxyphenylformimidamide to be reduced by simple $NaBH_4$ and deprotected using BBr_3 to produce dihydroxyphenylmethanediamine **(25)**. It as well might be likely that 4-aminocatechol can be used without protection groups present thus yielding protection free final product of substituted Y position nitrogen atom (scheme 3.1). This avoids many problems associated with using oxygen in this position due to the reactivity of nitrogen specifically with aldehydes to produce imines which are stable and readily reduced in a variety of methods.

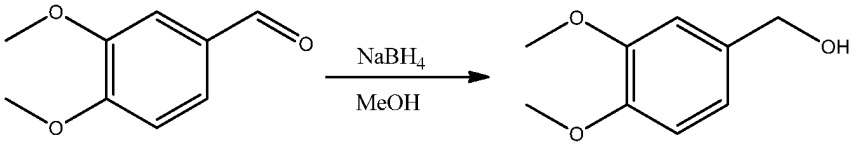
Results and Discussion

O-hydroxylamine Analog Synthesis:



Scheme 3.6. Synthetic Scheme of Hydroxylamine Dopamine. This synthetic scheme represents the first attempted at generating hydroxylamine tail in a dopamine analog. **a.** EtOH, $NaBH_4$; **b.** $SOCl_2$, NEt_3; **c.** o-hydroxypthalimide, THF, heat; **d.** NH_2NH_2, MeOH; **e.** BBr_3-DCM in dry DCM.

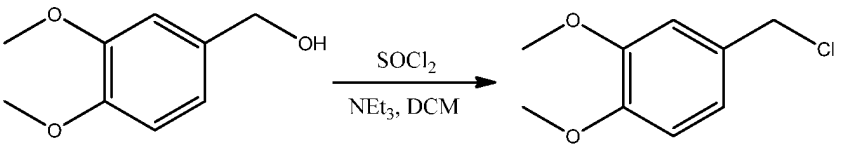
While the X position requires other starting materials than are currently worked on in the experiments, the Y position presented in (Scheme 3.1) can be readily synthesized from 3,4-dimethoxybenzaldehyde **(26)** or 3,4-dihydroxybenzaldehyde **(27)**, which are readily available in large quantities at low expense. The chemistry which can generate O and N substituted products is also less complicated than the chemistry which is involved in producing Y substituted position. These advantages were considered and the synthesis of position Y analogs in the dopamine ethyl linker was pursued. The first analog pursued involved an oxygen atom substitution to generate a hydroxylamine tail instead of the dopamine ethylamine linker according to Scheme 3.6.



Scheme 3.7

Reduction of 3,4-dimethoxybenzaldehyde **(26)** used sodium borohydride in methanol. The reaction was stirred for a minimum of one hour and then quenched with acetone, evaporated until half of the volume was left, diluted with water, and extracted with DCM to obtain the product, 3,4-

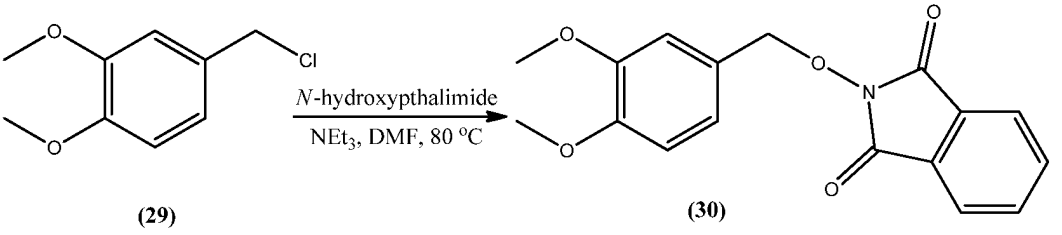
dimethoxyphenylmethanol (97%) **(28)** as clear or slightly cloudy oil and used without purification for chlorination.



(28) **(29)**

Scheme 3.8

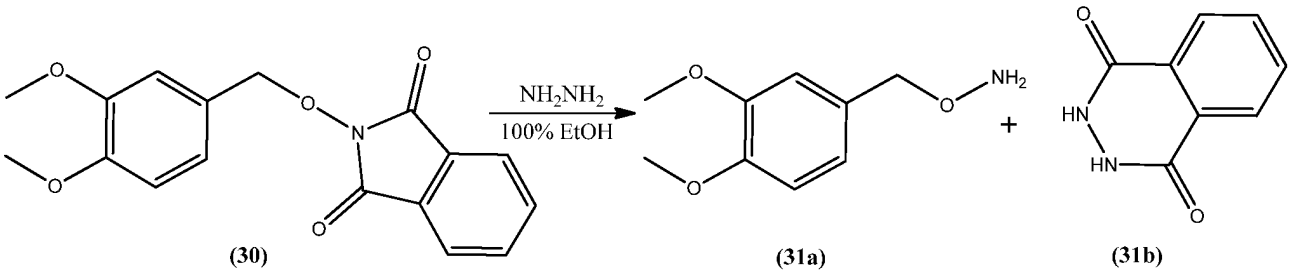
The 1,2-dimethoxyphenylmethanol **(28)** was converted to benzylchloride by thionyl chloride and triethylamine (NEt_3). Starting material was mixed with DCM and NEt_3 and cooled to 0 °C in an ice bath. Once temperature equilibration was complete, thionyl chloride was added slowly to prevent vigorous reaction. After full addition, reaction was allowed to warm up to room temperature and was monitored by HPLC until completion. The reaction was then evaporated under reduced pressure to yield 3,4-dimethoxybenzylchloride (92%) **(29)** as pale yellow oil.



(29) **(30)**

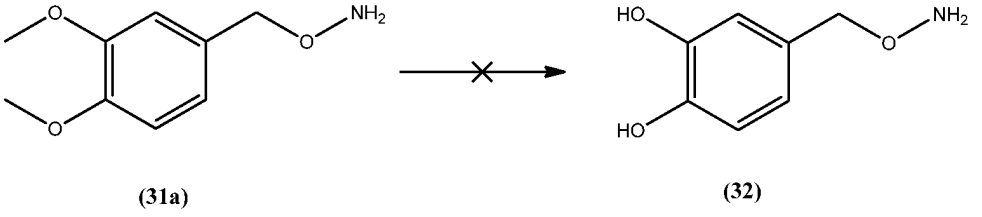
Scheme 3.9

To produce the hydroxylamine tail, a previously described method[33] was used. *N*-hydroxypthalimide was added to a solution of NEt_3 and **29** in dimethylformamide (DMF), heated to 80 °C, and allowed to stir overnight. Reaction was then quenched with H_2O. The formed precipitate was filtered and washed with more H_2O to produce the product, 2-((3,4-dimethoxybenzyl)oxy)isoindoline-1,3-dione (89%) **(30)**.



Scheme 3.10

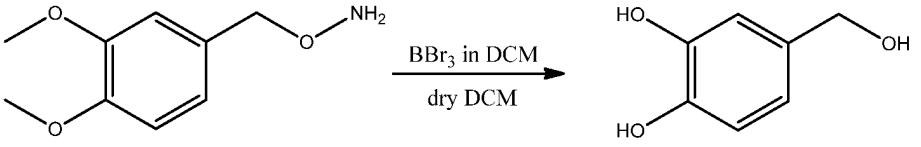
Cleavage of the hydroxypthalimide adduct was accomplished by a previously described method, hydrazinolysis[34], in which hydrazine was added to the suspended starting material **30** in pure ethanol (100% EtOH). After a minute all solid proceeded to dissolve into solution, after which the reaction solution turned transparent and brown in color. Additional five minutes of stirring after solution was clear, the solvents evaporated under reduced pressure. Et_2O was added to the reaction flask and the solid/oil mixture was filtered, solid was washed with Et_2O, and the combined organic layers were evaporated under reduced pressure to produce product 3,4-dimethoxybenzylhydroxylamine (98%) **(31a)**. The solid was characterized as 2,3-dihydrophthalazine-1,4-dione **(31b)**, a byproduct of hydrazinolysis.



Scheme 3.11

Two different methods were tested for the deprotection of **31a** to produce the final product, 3,4-dihydroxybenzylhydroxylamine **(32)**: HBr reflux and BBr_3. HBr reflux proved to be ineffective and generated significant amount of side products that were not characterized. Instead the deprotection was carried out using BBr_3 under inert atmosphere (N_2), and in dry DCM, which yielded a single product peak by HPLC. However, upon addition of dry MeOH as a quenching reagent the product of the reaction formed severe side products. Several attempts were made to optimize the quench procedure including testing H_2O, non-dry MeOH, HCl, and trifluoroacetic acid (TFA). Adding a small amount of HCl to the reaction and allowing it to stir for 10 minutes under inert atmosphere produced no side reactions. The solvent was then evaporated under reduced pressure to generate a white powder. Upon exposure to air

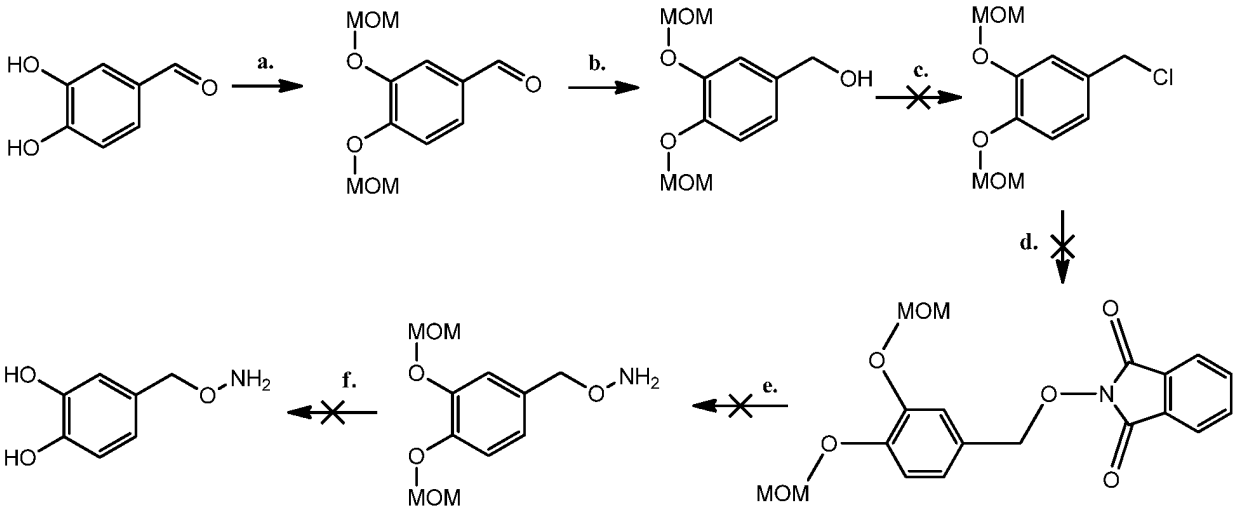
for more than 30 minutes, white compound would proceed to generate side products and deteriorate to form a black solid/oil mixture. The initial conclusion was that the hydroxylamine is not stable and in presence of air forms quinones which are immediately attacked by hydroxylamine tail and finally polymerized in a series of side reactions. However, upon closer inspection by HPLC the actual product of the deprotection reaction was proposed and tested to be 1,2-dihydroxyphenylmethanol **(33)** which was produced when BBr_3 coordinated with the oxygen in the hydroxylamine tail and cleaved the N-O bond (Scheme 3.12). This was further confirmed by reacting 3,4-dihydroxybenzaldehyde **27** with $NaBH_4$ under previously described conditions to generate the reduced alcohol **33**. Sample of sodium borohydride reduction was compared to the deprotection reaction using HPLC, which showed identical retention time and wavelength pattern.



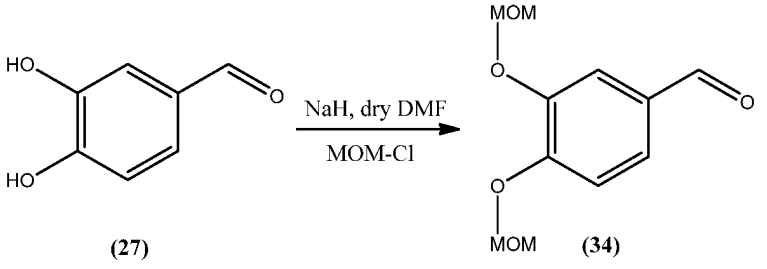
(31a) **(33)**

Scheme 3.12. **Product of Deprotection of 3,4-dimethoxybenzylhydroxylamine.** The reaction utilizing BBr_3 leads to a product formation which was not anticipated yielding a benzalcohol while cleaving the amine group from the structure.

A separate procedure was tested with BCl_3 deprotection of methoxy groups; however, it did not yield a reaction and was thus discarded. $AlCl_3$ was considered as another option for deprotection of the methoxy protecting groups and was rejected as too harsh for the N-O bond to survive. An alternate method was proposed using a different protecting group for the synthesis (scheme 3.12).

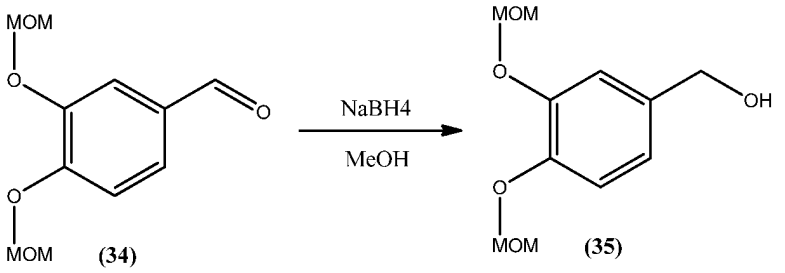


Scheme 3.13. **Synthetic Scheme of Hydroxylamine Dopamine Using MOM**. This synthetic scheme uses protecting group chemistry in the form of chloromethyl methyl ether in order to preserve the O-N bond at the end of the synthesis. **a.** NaH, dry DMF, MOM-Cl; **b.** EtOH, $NaBH_4$; **c.** $SOCl_2$, NEt_3 dry DCM.



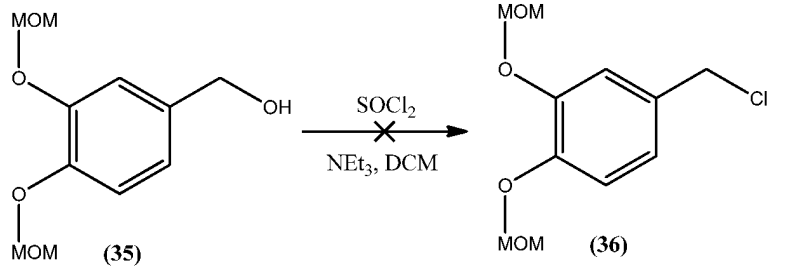
Scheme 3.14

Protection of catechol as chloromethyl methyl ether was carried-out by stirring **27** in dry DMF followed by portion-wise addition of sodium hydride (60% NaH in mineral oil) over a period of 15 minutes. Once the solution changed color from brown to yellow the flask was placed under inert atmosphere and in an ice bath. After equilibration chloromethyl methyl ether (MOM-Cl) was added slowly and allowed to stir for 1.5 hours. The reaction was monitored by HPLC and once fully converted to protected benzaldehyde a solution of 0.1 M NaOH was added as a quench condition. The reaction was then allowed to stir for another 10 minutes. The solution was diluted with H_2O and extracted with DCM. Partially protected starting material was present when analyzed by HPLC. The solution was then evaporated under reduced pressure to yield 3,4-bis(methoxymethoxy)benzaldehyde (86%) **(34)**.



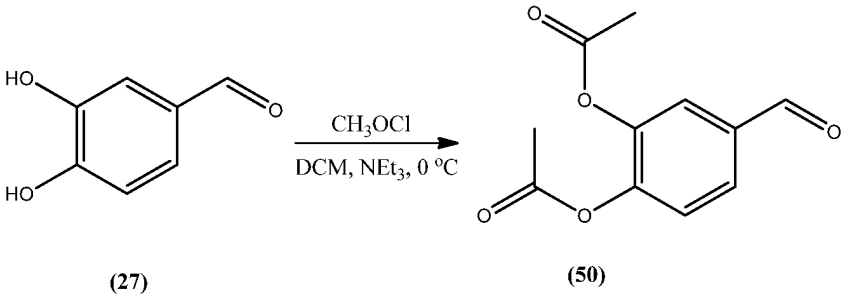
Scheme 3.15

The protected benzaldehyde was then reduced to 3,4-bis(methoxymethoxy)phenylmethanol **(35)** by $NaBH_4$ reduction in excellent isolated yield of 94% (Scheme 3.15). However, the reduction did not proceed to completion and instead was only 96% complete regardless of excess $NaBH_4$ added to push the reaction to completion. It also worth noting that the partially protected reduced contaminants were not soluble in DCM extraction conditions previously described in Chapter 2.



Scheme 3.16

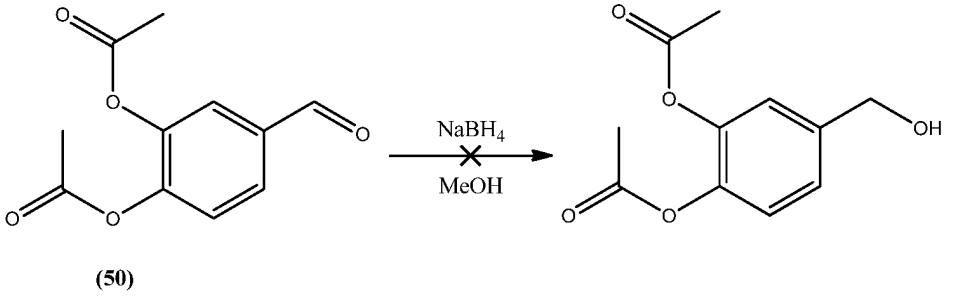
Chlorination of the starting material **35** was carried out using NEt_3 and $SOCl_2$ in dry DCM under inert atmosphere (Scheme 3.16). However, the reaction did not produce a single product, 3,4-bis(methoxymethoxy)benzylchloride **(36)**, and instead produced multitude of side products and reacted very vigorously even when cooled and $SOCl_2$ added exceedingly slowly. Excess base was added to prevent formed protons from thionyl chloride to deprotect the methoxymethoxy protecting group. Still, this did not alleviate the problem of vigorous reaction and side product formation. Ultimately the reaction scheme was abandoned in search of alternate chlorination conditions, possibly using N-chlorosuccinimide[35] and alternative protection conditions.



(27) (50)

Scheme 3.17

Different protection was attempted utilizing acetyl chloride as a protecting groups that was installed onto the starting material **27** (Scheme 3.17). The reaction was started by dissolving starting material in DCM, adding NEt_3, and cooling mixture to 0 °C. After the mixture reached thermal equilibrium a solution of acetyl chloride in DCM was added via addition funnel slowly over 30 minutes and the reaction was allowed to stir at room temperature overnight. Progress was checked by HPLC and the organic solvent was removed under reduced pressure. The resulting product, 4-formyl-1,2-phenylene diacetate (92%) **(50)**, was brown oil which was checked by NMR and used without purification for further reactions.



(50)

Scheme 3.18

Reduction of the aldehyde was attempted by following a similar procedure as mentioned before (Scheme 3.18). However, methanol was replaced with THF because methanol was found to deprotect acetyl groups under the conditions of the reaction. After addition of $NaBH_4$ reaction was stirred for 2 hours. Unfortunately the reaction showed signs of complete deprotection of the acetyl groups as well as a mixture of other products by HPLC. This protection scheme was abandoned.

Notes/Observations:

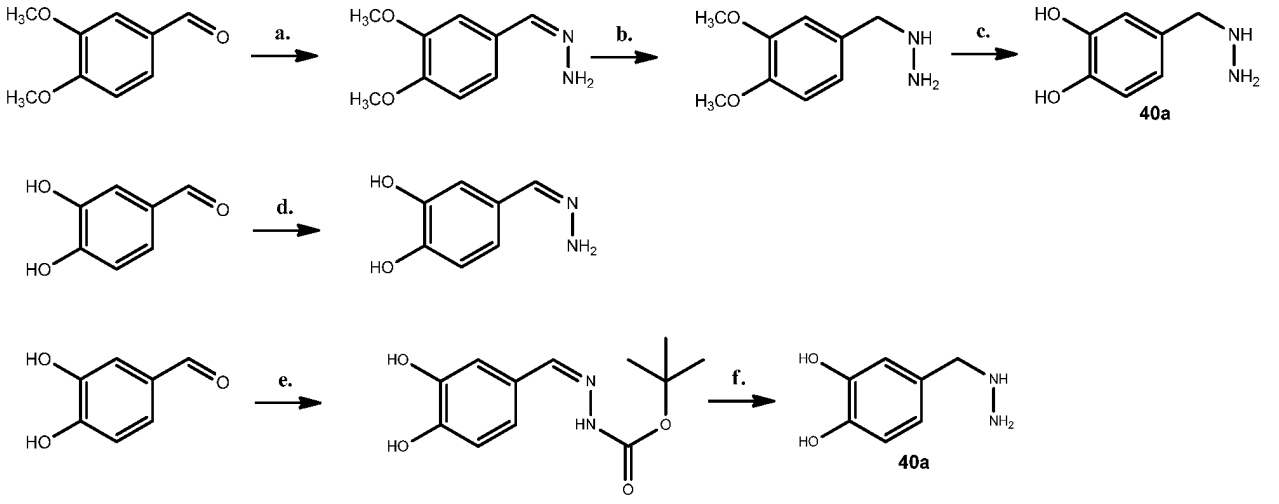
Purification of chlorinated product **29** can be accomplished by evaporating solvent under reduced pressure and then re-dissolving it in Et_2O and petroleum ether (pet. ether) in a -78 °C bath. The product precipitates from solution and can be harvested by filtration.

While the byproduct of the hydrazinolysis **31b** has no use in these sets of experiments it is interesting to note that once analyzed by NMR based on the peaks the structure exists in a half tautomer form where part of it remains in the lactam form while the other half is in the lactim form.

The product of deprotection reaction **33** rapidly decomposes in the presence of air forming ortho-quinones which undergo Michael addition between each other to form polymers. While it would be useful to attempt to synthesize the unprotected alcohol and chlorinate it using thionyl chloride, the actual product **33**, while being extremely unstable, is also insoluble in most solvents that are typically used for thionyl chloride reactions such as THF and DCM, it is likewise insoluble in ethyl acetate (EtOAc), ACN, and EtOH. It is soluble in H_2O and partially soluble in a large quantity of MeOH; however, these solvents are not compatible with thionyl chloride. Likewise it is unknown how thionyl chloride will react with the presence of a catechol.

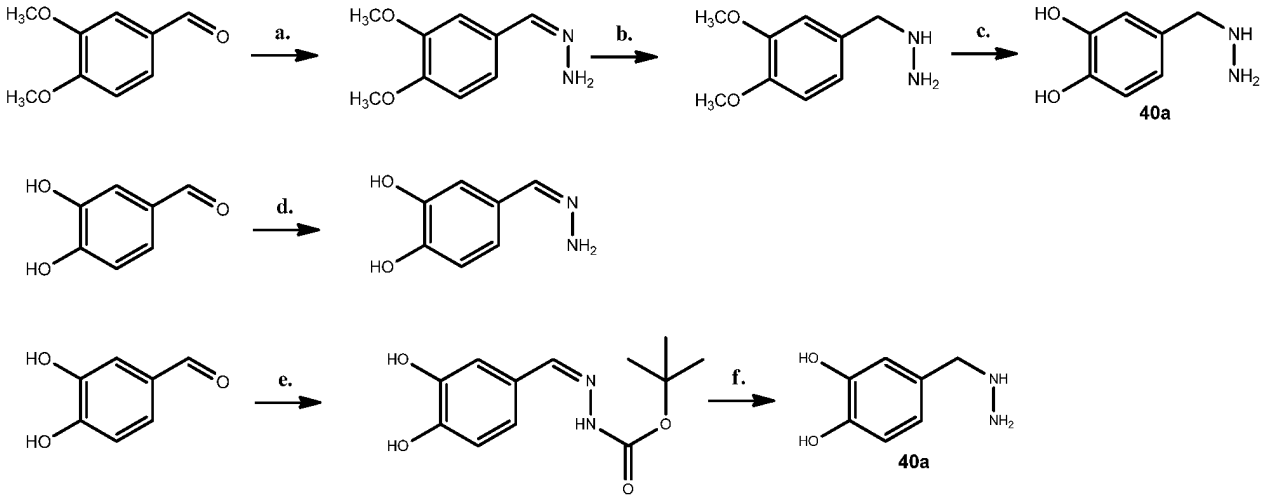
Hydrazine Dopamine Analog Synthesis:

The hydroxylamine substitution presented a challenge due to the problem of the labile N-O bond susceptible to cleavage by reagents that cleave methoxy protecting groups. Further challenges lie in proper protection of the catechol, chlorination for installing hydroxypthalimide adduct, and deprotection. While this is still under investigation, a different substitution at the X position using a nitrogen atom was attempted.

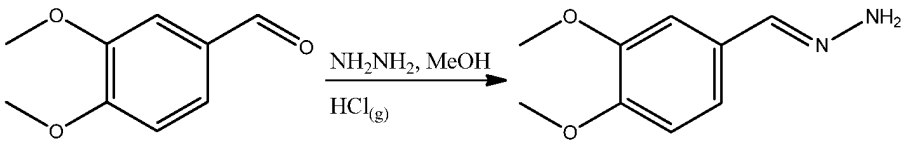


Scheme 3.19. **Synthetic Schemes for Nitrogen Substituted Dopamine**. Several different attempts were used in order to obtain the hydrazide product. The final product was accomplished by using a protected hydrazine derivative which helped avoid unnecessary side reactions which occurred in steps a and d.

The synthesis envisioned for this particular compound utilizes the ability of amines to react with aldehydes to form stable imine compounds that can be reduced to amines to generate the final product with an N-N bond. As shown in



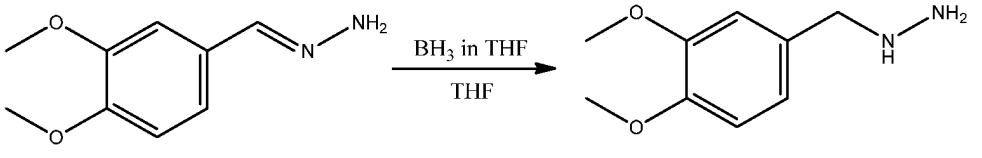
Scheme 3.19, several different attempts were investigated to create the product, **40a**.



(27) (37)

Scheme 3.20

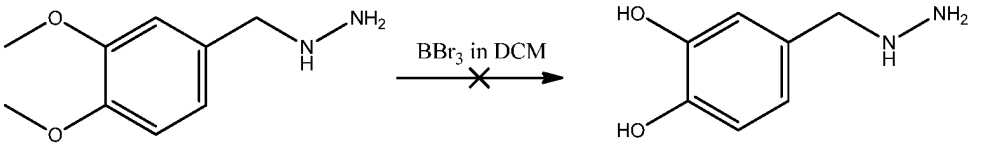
The first attempt to produce **37** followed a very similar route as hydroxylamine analog and utilized **26** as a starting point. To a solution of **26** in MeOH was added hydrazine. The reaction proceeded to generate a multitude of peaks. In attempt to solve this problem dry HCl gas was bubbled into the solution from the reaction of $CaCl_2$ and 37% HCl. Though this lessened the side products that occurred and precipitating a solid product it did not fully solve the problem. The free amine, which does not attack the aldehyde instead attacks another molecule of aldehyde starting material and links the two together through a conjugated system composing of two imines. A portion of the reaction which precipitated contained a salt of the formed imine product, 3,4-dimethoxybenzylidenehydrazine (67%) **(37)**.



(37) (38)

Scheme 3.21

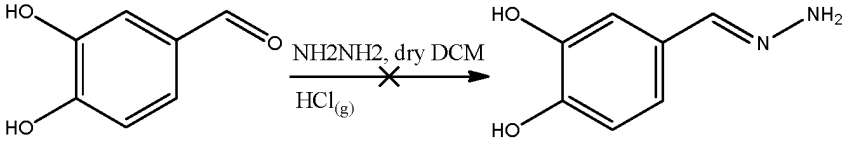
Sodium borohydride ($NaBH_4$) and sodium cyanoborohydride ($NaBH_3CN$) were first employed to reduce **37,** the product of hydrazine condensation (Scheme 3.21). No reaction was observed with either reagent. Instead, borane reduction with BH_3 in THF was carried out to generate 3,4-dimethoxybenzylhydrazine **(38)** isolated yield of 82%.



(38) (40a)

Scheme 3.22

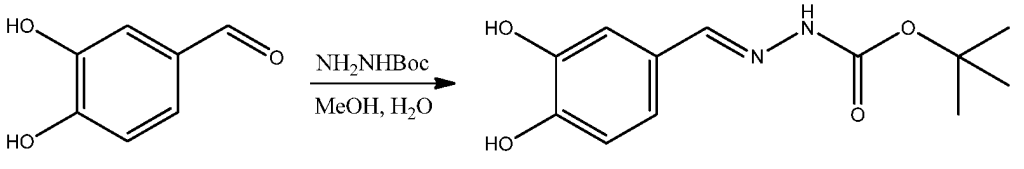
Deprotection was again attempted with HBr reflux (Scheme 3.22). Reaction produced several products while turning black. Instead BBr_3 reaction was attempted to solve this problem. After checking the reaction mixture by HPLC, it was revealed to contain multitude of peaks present with product peak area of around 26% of the mixture, and extraction of the product was not attempted. Instead a different reaction scheme was performed.



(27)

Scheme 3.23

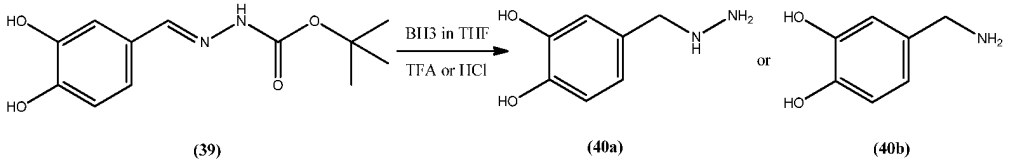
A separate route was tested by using compound **27** as starting material since the catechol should be stable to the proposed reagents, since none of them can activate the oxygen atoms. Compound **27** was dissolved in dry DCM to which hydrazine was added (Scheme 3.23). As soon as hydrazine was added, HCl gas was bubbled through the solution producing a solid yellow colored precipitate. Solution again was tested by HPLC to reveal multiple peaks. The same crosslinking reaction with hydrazine reacting on both ends is most likely to be responsible for formation of side products. Product of this reaction was not isolated.



(27) (39)

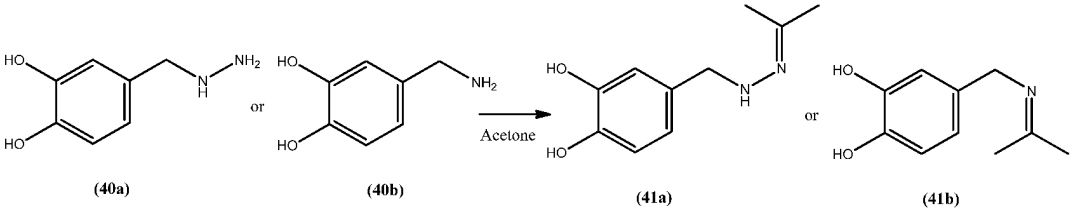
Scheme 3.24

In order to minimize the side reactions that occur when hydrazine is used, a Boc-protected version was purchased. To a solution of starting material **27** dissolved in MeOH a solid *tert*-butyl carbazate was added and the reaction was allowed to stir for 2 hours. The reaction was checked by HPLC and once complete, solvent was removed under reduced pressure to yield the product *tert*-butyl 2-(3,4-dihydroxybenzylidene)hydrazinecarboxylate (91%) **(39)** and used without further purification for the next reaction.



Scheme 3.25

Compound **39** was dissolved into dry THF and BH_3 in THF was added in room temperature (Scheme 3.25). Some minor side products were observed, which could possibly be avoided by using an ice bath. Once the reaction was complete by HPLC, concentrated HCl was added to the reaction and allowed to stir for a 5 minutes. White precipitate was observed to form, which was filtered and dried under vacuum. The product produced was observed to have a small contaminant of unknown structure, possibly from an earlier reaction. However, the product, 1,2-dihydroxyhydrazynylmethylbenzene (92%) **(40a)** was obtained. While NMR data and HPLC data suggested that the analog was made, it remained somewhat ambiguous if the N-N bond was still present or if it was cleaved in the reaction generating 1,2-dihydroxybenzylmethylamine **(40b)**.



Scheme 3.26

To test if the N-N bond was still present compound **40** was condensed with d_6-acetone. This ensures that the terminal amine will react to produce an imine compound. From this compound a simple NMR analysis revealed whether the N-N bond was still intact because the methylene would have had significantly different NMR shift. If the N-N bond was present then the methylene peak should not have a significant shift in the spectra and the hydrogen bond present at the X position substituted

nitrogen should be visible. Yet if the bond was cleaved during previous steps the formation of the imine compound would shift the methylene peak significantly more upfield. Hence compound **40** was stirred in a solution of d^6-acetone to avoid the need for evaporation under reduced pressure. After 10 minutes of stirring the reaction was checked by HPLC to reveal a single product peak with a different retention time and wavelength profile than the starting material. The product of the reaction was analyzed by NMR and concluded to be 4-((2-(propan-2-ylidene)hydrazinyl)methyl)benzene-1,2-diol **(41a)**.

Notes/Observations:

The amount of side reaction produced from compounds **26** and **27** depended on how quickly HCl was bubbled into the solution; however, no matter how quickly HCl gas was added there was still some side reactions which were present.

It is possible that $NaBH_4$ and $NaBH_3CN$ are not strong enough on their own to reduce the imine bond formed and need to be stirred together with some acid. Utilizing BH_3 can one can reduce the bond without significant trouble. Similar compounds containing imine are often reduced with either of the borohydrides; however, it appears that a hydrazinyl imine is not as susceptible to such reduction without the presence of acid. As noted before possible improvements for the reaction can consist of slower addition of BH_3 as well as cooling of the solution. These modifications should prevent the formation of any side products.

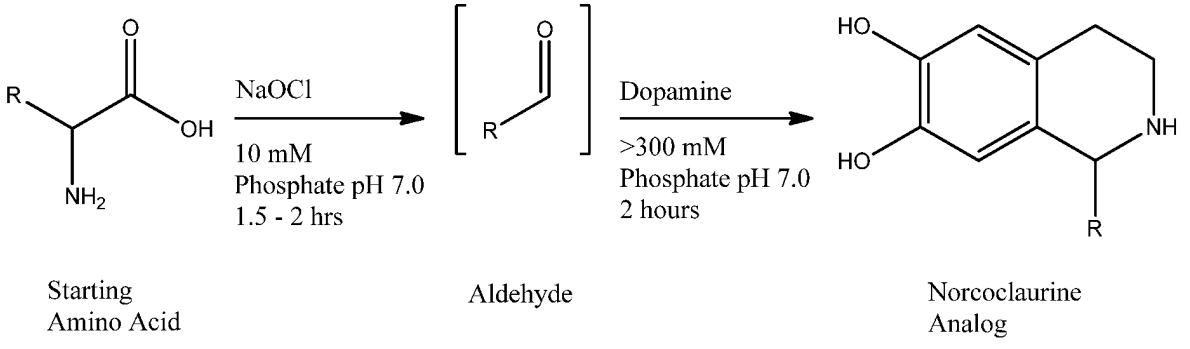
Purification of **41a** can be accomplished by washing it with ACN/Et_2O mixture followed by pure EtOH. Further purification can be attempted by utilizing acid/base conditions and acid precipitation.

After quench with acid and testing by NMR it appears that a about 10% of the borane reduction generated the cleaved product which was evidenced by test reaction using acetone which generated a peak in the region for methylene present next to an imine forming compound **(41b)**.

Chapter 4 - Norcoclaurine Analog Synthesis from 4-HPAA analogs

Introduction

Formation of norcoclaurine (tetrahydroisoquinoline compound or THI) analogs can be accomplished with alternative 4-HPAA analogs. While there are significant limits on alteration of the carbon linker of 4-HPAA, halogenation of 4-HPAA *ortho* to the hydroxyl group is trivial. If the halogenated analogs of 4-HPAA are taken-up by cells, they could enzymatically produce X_3 variants of berberine shown in Scheme 2.3. Again the halogenation of the final product can allow for cross coupling reactions with other groups for structure-activity relationships, space filling inside protein cavities, and stability against metabolism. Likewise, halogenation of 4-HPAA provides functionality to the final product of enzymatic synthesis on the opposite side of the dopamine halogenation.



Scheme 4.1. **Synthetic Scheme for Norcoclaurine Analogs**. The scheme above represents a synthesis utilizing amino acids with bleach to obtain an intermediate aldehyde and react it with dopamine to a corresponding norcoclaurine analog.

One particular route for halogenation of 4-HPAA precursor was published in earlier literature[36] and the method for synthesis of 4-HPAA was likewise published[36]. These methods were improved and expanded upon in our laboratory engendering a method for one pot synthesis of halogenated norcoclaurine analogs as well as a method to produce other 4-HPAA-like analogs. Primarily 4-HPAA is made by using a sodium hypochlorite (bleach) oxidation reaction with L-tyrosine; which provides a relatively quick method (2 hours) for a protection group free reaction (Scheme 4.1). Attempts were made to expand upon this work to include other amino acids for formation of norcoclaurine analogs which provide a variety of potential alternate drugs.

As such, a variety of different amino acids were utilized in the experimental procedure in Scheme 4.1. This generates 4-HPAA analogs which then further react with dopamine for conversion to

norcoclaurine analogs. The aldehyde and target norcoclaurine analogs are shown in Table 4.1. The majority of the aldehydes were not isolated due to the instability of the aldehyde formed through the bleach oxidation. Other aldehydes generated through this reaction method reacted spontaneously with each other to polymerize into a mixture of substances not useful for our purposes.

In the non-enzymatic synthesis of norcoclaurine, a racemic mixture of *S* and *R* enantiomers are generated. During this non-enzymatic synthesis an isomer of norcoclaurine (iso-THI, Figure 4.1) is also produced where the final ring closing occurs on a carbon *ortho* to the hydroxyl group rather than *para*.

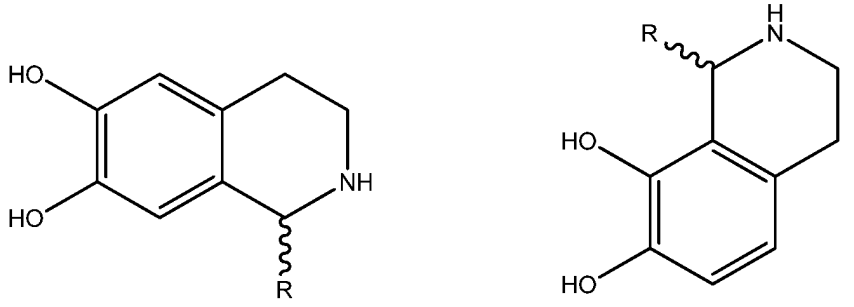
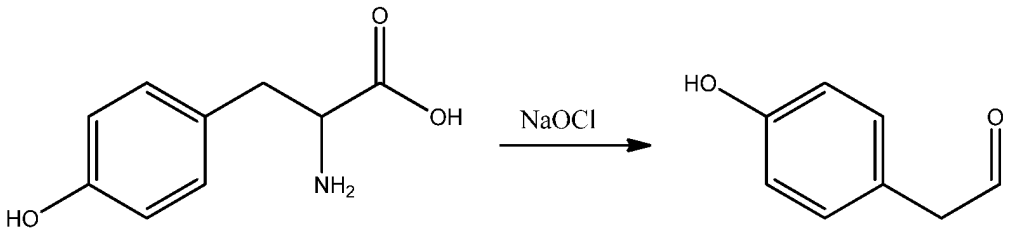


Figure 4.1. **Tetrahydroisoquinoline and Iso-tetrahydroquinoline**. Figure above shows the two different isomers which can be formed by Pictet-Spengler condensation.

Results and Discussion

HPAA Analog Synthesis:

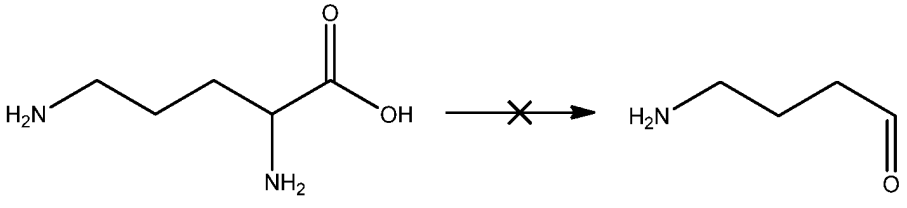
THI was synthesized to varying degrees with various aldehyde analogs. It is interesting to note that the amount of THI appears to be influenced by size, for example large bulky aldehydes primarily form both *S* and *R* enantiomers and do not produce the iso-THI analog. Likewise, the very small aldehydes do not produce much iso-THI.



(42)

Scheme 4.2

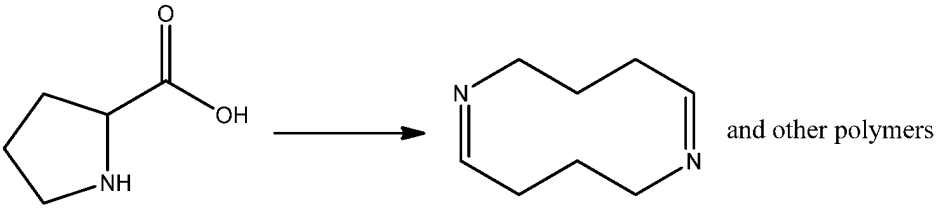
Following the procedure described (see Appendix A, "Bleach oxidation" and "Pictet-Spengler for Tetrahydroisoquinoline synthesis"), L-tyrosine was dissolved in phosphate buffer and warmed to 37 °C, and bleach solution was added over a period of 10 minutes. This reaction was used as a control for comparison for further amino acid bleach oxidation reactions. The mixture was quenched with ascorbic acid after 1.5 hours of reaction and extracted with DCM four times. The solvent was then evaporated under reduced pressure at 0 °C to avoid 4-HPAA polymerization through Aldol condensation and other side reactions. The final product, 4-HPAA **(42)** was pale yellow oil with a distinct rose-like smell. A similar reaction was prepared, however 4-HPAA was not extracted but rather dopamine was added along with high concentration of phosphate buffer. The high concentration of buffer helped catalyze the reaction in order to generate primarily norcoclaurine as opposed to the isomer. This catalysis by phosphate at higher concentrations is responsible for minimization of formation of the isomer, which was tested and published in an earlier article[36].



(43)

Scheme 4.3

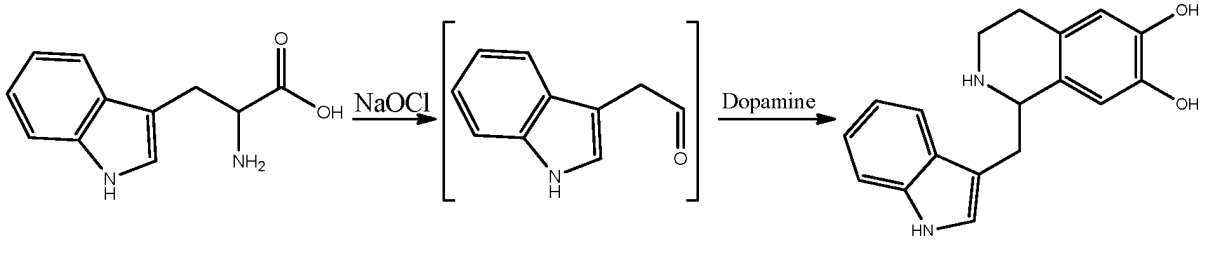
Following the same procedure, L-lysine was dissolved in phosphate buffer and warmed to 37 °C, and bleach solution was added over a period of 10 minutes. This reaction was not expected to succeed due to the amine group present with lysine and when checked by HPLC the reaction contained an assortment of peaks. Reaction was abandoned due to its tendency to generate polymeric and cyclic products, instead of the theoretical target compound **(43)**.



(44)

Scheme 4.4

Following the same procedure, L-Proline was dissolved in phosphate buffer and warmed to 37 °C, and bleach solution was added over a period of 10 minutes. Reaction was monitored by HPLC and quenched with ascorbic acid after 2 hours then extracted with DCM four times. The solvent was then evaporated under reduced pressure at 0 °C. While the product was a single peak on HPLC, NMR did not indicate formation of the expected product. GC-MS was taken to try and discern what the compound was as it did not contain peaks which were expected. Using GCMS the following products were postulated to be present in the reaction mixture: (1*Z*,5*Z*)-2,3,4,7,8,9-hexahydro-1,6-diazecine **(44).** Further experimentation with proline yielded mixtures of compounds which were assumed to be polymers formed from the ring opening and polymerization with present aldehydes.

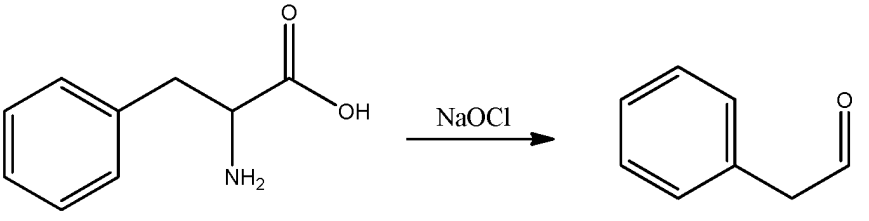


(45)

Scheme 4.5

Following the same procedure, L-Tryptophan was dissolved in phosphate buffer and warmed to 37 °C, and bleach solution was added over a period of 10 minutes. Reaction was monitored by HPLC and quenched with ascorbic acid after 2 hours then extracted with DCM four times. The solvent was then evaporated under reduced pressure at 0 °C. While this product was expected to be easily isolated from phosphate buffer due to the large aromatic ring structure, during extraction and evaporation of solvent the product, 2-(1H-indol-3-yl)acetaldehyde **(45)**, generated side products as a thick brown colored oil. Instead the product was not isolated and a separate reaction was prepared, after 1.5 hours of reaction

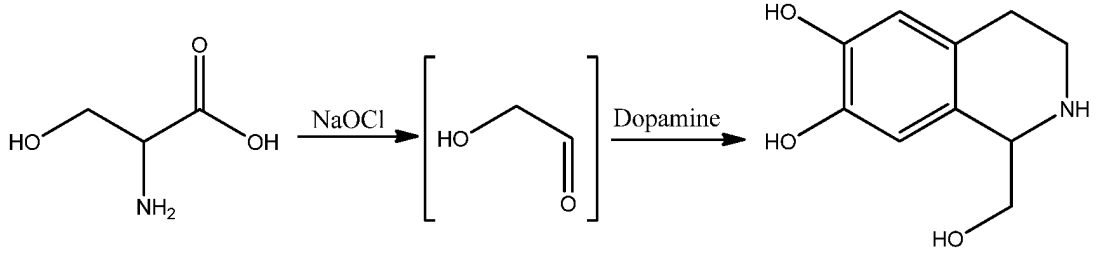
(full conversion) the reaction was quenched with ascorbic acid. Then, dopamine along with high concentration of phosphate buffer (pH 7.0) was added and allowed to react until the aldehyde was consumed. The product of the Pictet-Spengler reaction was attempted to be isolated by using EtOAc as an extraction solvent. However, this did not optimally extract pure product. Instead the reaction generated a purple colored solid which was filtered before extraction, after evaporation under reduced pressure compound **45** was isolated as a pale yellow solid (38%). The yellow solid may be further purified by dissolving in minimal MeOH, adding ~10 volumes of $CHCl_3$, and precipitating with Et_2O to yield a fluffy crystalline solid. Alternatively, the product may also be isolated by using THF and adding brine to the phosphate solution. NMR was taken in two different solvents due to peak overlap with solvent.



(46)

Scheme 4.6

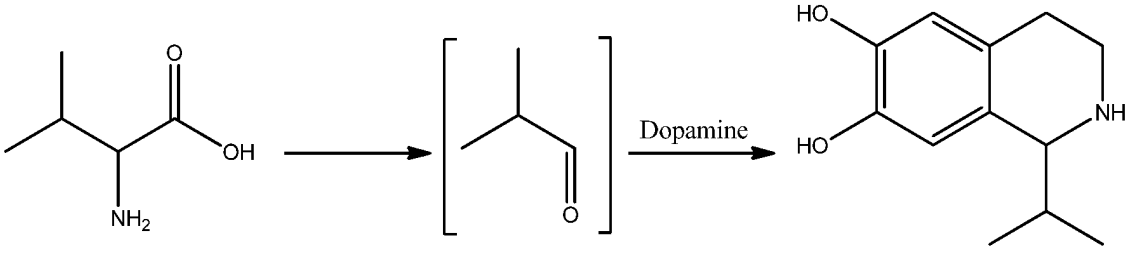
Following the procedure described, L-phenylalanine was dissolved in phosphate buffer and warmed to 37 °C, and bleach solution was added over a period of 10 minutes. The mixture was quenched with ascorbic acid after 2 hours of reaction and extracted with DCM four times, and solvent was evaporated under reduced pressure to yield the product 2-phenylacetaldehyde **(46)** as pale yellow oil (60%).



(47)

Scheme 4.7

Following the procedure described, L-serine was dissolved in phosphate buffer and warmed to 37 °C, and bleach solution was added over a period of 10 minutes. The mixture was quenched with ascorbic acid after 2 hours of reaction the product was not attempted to be extracted due to the aldehyde that was generated being very small (2-hydroxyacetaldehyde) and undetectable by HPLC conditions available. Instead reaction was quenched with ascorbic acid and dopamine along with high concentration of phosphate buffer (pH 7.0) was added and allowed to react for another 2 hours. The product, which was visible by HPLC, was attempted to be extracted using normal extraction procedure. However, the product of the reaction, 1-(hydroxymethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol **(47)**, was more soluble in phosphate buffer than it was in DCM. Other solvents were attempted as extraction conditions including EtOAc, ACN/brine, THF/brine, and $CHCl_3$. None of these solvents worked for extraction; therefore a different tactic was attempted using shell freezing – a technique where the inside contents of a flask are frozen by spinning the solution on a rotary evaporator without vacuum and cooled to freezing using MeOH/dry ice bath. Once the liquid was frozen it was placed under lyophilizer in order to sublime all the water present in the mixture. This process took 4 days to finish. Once this was completed the solid debris was attempted to be dissolved with EtOAc, THF, and DCM. Again the product was not detected in the solution. It is possible that the product was lost during the process of freeze-drying or lyophilizing. As such the product was only monitored by HPLC.

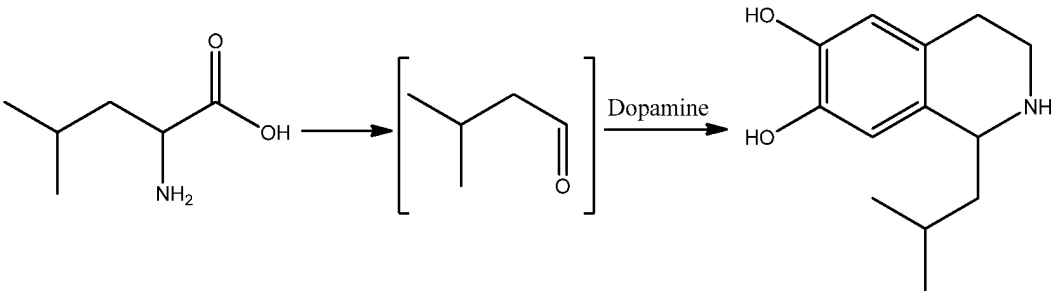


(48)

Scheme 4.8

Following the procedure described, L-valine was dissolved in phosphate buffer and warmed to 37 °C, and bleach solution was added over a period of 10 minutes. The mixture was quenched with ascorbic acid after 2 hours of reaction the product was not attempted to be extracted due to the aldehyde that was generated being very small and undetectable by HPLC methods. Much like with serine, the corresponding aldehyde, isobutyraldehyde was not isolated. Instead following the procedure described earlier corresponding THI, 1-isopropyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol **(48)** was

attempted to be isolated. Again the THI analog was too polar to be isolated from the phosphate buffer even after shell-freezing and lyophilization.



(49)

Scheme 4.9

Following the procedure described, L-leucine was attempted to be converted to the corresponding aldehyde, 3-methylbutanal. The same problem occurred as with previous analogs where the aldehyde could not be detected and the corresponding THI, 1-isobutyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol **(49)**, could not be isolated.

Notes/Observations:

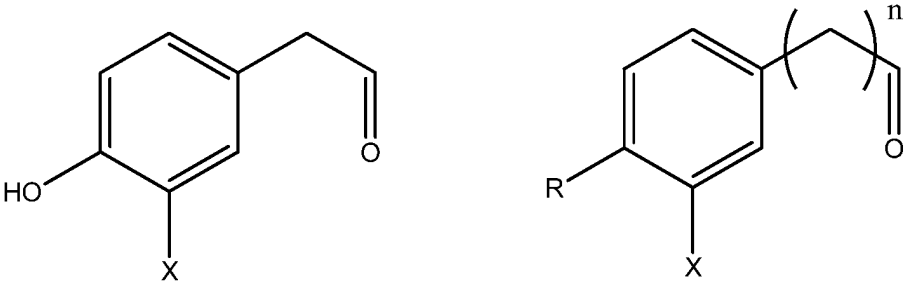
Based on the tested amino acids and the attempted isolation, it is clear that small or polar residues are extremely hard to isolate from phosphate buffer into organic solvents for analysis. In order to separate these compounds a complex column chromatography or acid/base extraction procedure is required. Alternatively, the synthesis could be attempted in a different solvent, which will allow for catalysis of the reaction as well as separation of the aldehyde/norcoclaurine products and side products.

The synthesis of 4-HPAA analogs was dependent on their ability to be extracted into solution for further analysis. Currently there is research being conducted using propionaldehyde as a substitute for 4-HPAA to address the challenges posed by isolation and working with 4-HPAA. However, considerable difficulties still exist in the extraction of 4-HPAA from a reaction which utilizes beach oxidation of amino acids. A possible way to overcome this difficulty is to attempt a partial distillation of the available solution; however, the quantity of aldehyde produced during the reaction remained very small, which again will make isolation difficult. Likewise, there also exists a possibility of Aldol condensation once the aldehyde solution is heated, particularly with ascorbic acid present in solution which can donate a catalytic proton.

It is interesting to note that a cyclic amino acid L-Proline generated a cyclic compound as well as other polymers. If the product could be captured in the partially hydroxylated state, there could be synthetic applications for obtaining an easy bicyclic system from an amino acid in two steps. This presents a possible reduction in cost of synthesis since both bleach and L-Proline are inexpensive starting materials and facile isolation.

Discussion:

A different approach could be utilized with larger substrates containing a large hydrophobic group allowing for facile isolation from water. This was observed for three analogs of tryptophan, tyrosine, and phenylalanine. Proline underwent bleach oxidation followed by ring opening which prevented reduction of amine to aldehyde and instead generated a cyclic compound as a polymer of two proline molecules. Histidine was ignored as a potential candidate due to two present amine groups; one of which can potentially react to attack the aldehyde possibly much like proline or will react to yield enamines. With these limited options, the only other possibility for generating novel norcoclaurine analogs is to buy aldehydes which can be used for synthesis or novel aldehydes could be created. The most practical approach for full exploration of Pictet-Spengler with 4-HPAA analogs would involve a combination of these two factors. Following **figure 4.0** a variety of positions could be changed in the 4-HPAA structure besides utilizing structurally different aldehyde species.



HPAA HPAA variant

Scheme 4.10. Proposed Changes for 4-HPAA. The normal substrate 4-HPAA is presented on the right but it could be changed by altering the structure at the R and X positions as well as adjusting the length of the aldehyde linker.

The previously discussed alteration of the Z position of 4-HPAA shown in Scheme 3.1 and Scheme 3.2 may not react to react in a Pictet-Spengler, either non-enzymatically or enzymatically, because the alterations are too great. However, based on Scheme 4.1 the modifications to 4-HPAA can be facilitated by adding different R substituents alongside halogenation and varying the aldehyde linker length. Particularly interesting would be analogs containing hydrophobic substituents at the R position of Scheme 4.1. There is interest in these hydrophobic compounds due to experiments which tested natural 4-HPAA vs. halogenated 4-HPAA and phenacetaldehyde (Table 4.1).

Table 4.1. Rate of Reaction for 4-HPAA Analogs using norcoclaurine synthase (NCS).[a]

Substrate	Relative rate of Reaction	Error in the Relative Rate of Reaction[c]
4-HPAA[b]	100%[b]	N/A[b]
3-Cl,4-HPAA	24%	4%
3-Br,4--HPAA	40%	8%
3-I,4-HPAA	43%	4%
Phenacetaldehyde[d]	190%	20%

a. The analogs were tested against the natural substrate, 4-HPAA, and were found to have increasing rate of reaction with phenacetaldehyde containing the greatest rate almost doubling that of 4-HPAA substrate.

b. HPAA was assumed to be 100% reactivity without standard deviation.

c. Error was obtained as the standard deviation from three enzyme assays.

d. Phenacetaldehyde was commercially acquired checked against synthesized phenacetaldehyde and then tested against 4-HPAA.

Based on the data that is presented in Table 4.1 it is interesting to note that the rate of phenacetaldehyde is much greater than the natural substrate, likewise it can be noted that as the size of the halogen increases the reaction rate also increases. Exploration of different combinations of R and X substituents would not only provide interesting analogs of norcoclaurine but would also provide insight into the active site of the enzyme, norcoclaurine synthase, responsible for the catalyzing this reaction in plants. More testing is required in isolation of other norcoclaurine analogs produced from other amino acids as well as further investigation of the reactivity when compared to the 4-HPAA natural substrate.

Chapter 5 - Chemically and Enzymatically Mediated Pictet-Spengler using Dopamine Analogs

Introduction

The enzyme norcoclaurine synthase (NCS) catalyzes formation of norcoclaurine and is found in 20% of all flowering plants, and additionally the compound itself undergoes various enzymatic pathways to produce about 2500 other benzoisoquinoline alkaloids (BIA) many of which are active drug agents; such as morphine, papaverine, pavine, sanguinarine, berberine, and others (Scheme 2.1 and Scheme 2.2). Utilizing precursor directed biosynthesis (PDB) one can alter dopamine, 4-HPAA, or norcoclaurine to produce a host of drug analogs by introducing structurally modified analogs of enzymatic pathway precursors and allowing enzymes to generate a structural analog. This kind of approach would limit organic synthesis time, save cost of expensive reagents and solvents, which are sometimes necessary to create complex structures, and avoid utilizing protecting group chemistry. While the precursor directed biosynthesis approach itself is possible, currently organic synthesis of analogs is more practical due to the sheer amount of material one can produce in organic chemistry, making biosynthesis not as practical due to the small amount of natural product that can currently be obtained.

A more practical approach to the biosynthesis would be to create an organism such as bacteria and insert vectors containing genes for all enzymes needed for a particular drug pathway. This is evident as mentioned before in the current synthesis of Artemisinin. Using this approach would be more efficient in conversion from one product to the next and minimize material loss where the plant, as a whole, might use reactants for purposes other than producing the target compound. It is possible to isolate all necessary proteins found in the plant enzymatic pathway, and by placing them in bacterial vectors for purification, utilize them as catalysts in a reaction mixture. This would need to be accomplished by first cloning all protein genes into separate bacterial organisms, optimize their protein expression, and then optimize protein isolation conditions. Finally, the reaction conditions would need to be optimized to achieve the highest enzymatic activity while minimizing inhibition of enzyme and side reactions. Often buffers which can be used for enzyme reactions have side reactions that occur with your target compound or product. While optimization is a long process, after the methods are optimized enzymatic reactions allow for generally full conversion of starting materials to products, assuming there is not an equilibrium which can be established by the enzyme between the amount of starting material and product.

NCS is a vital enzyme to understand since it the gateway enzyme[20, 21, 29] for biological synthesis of BIAs (Scheme 2.1) and a proposed limiting step in synthesis of complex structures available beyond it in the various pathways. The proposed and observed pathways which are available after NCS are complex and depend on which cell/plant material is used to enzymatic feeding of dopamine and 4-HPAA analogs. However, as shown in the Scheme 2.1 all the compounds are generated by the starting material of dopamine and 4-HPAA which form norcoclaurine.

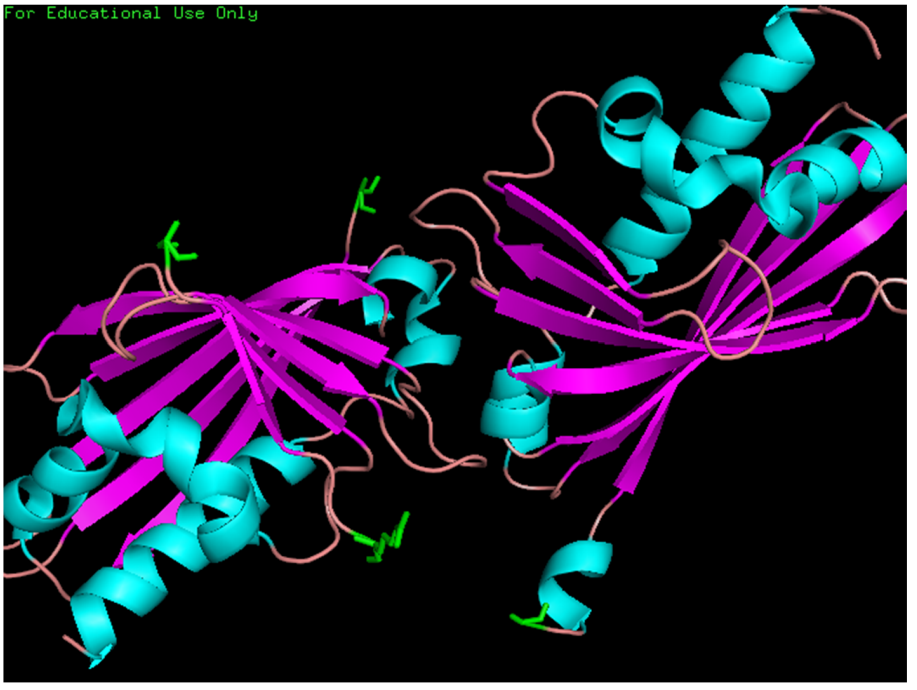


Figure 5.1. **Norcoclaurine Structure.** Above figure shows the disjointed amino acids in green, β-sheets in purple, α-helices in teal, and loops in pink. The structure was obtained from PDB 2VQ5.

To date, the enzyme has not been studied in proper amount of detail. Likewise, there are no reliable crystal structures of norcoclaurine synthase available for protein modeling work (**Error! Reference source not found.**). While the figure shows a general structure of the enzyme, it is missing fifteen residues in the beginning and has a large structural gap between amino acids arginine (**R26**) and glycine (**G27**). This suggests that the structure crystallized and solved by x-ray diffraction is incomplete or misassigned. Other work performed on NCS[20, 37] was incorrect due to issues with experimental procedures. These issues included utilizing a phosphate buffer, which catalyzes background reaction of dopamine and 4-HPAA to a significant degree (Table 5.1), and reactivity of the buffer with starting materials (Figure 5.2).

Results

NCS Reactions and Buffer Optimization:

Table 5.1. Dopamine Conversion without Enzyme.[a]

Time[b] (minutes)	% Dopamine with no NCS present[c]
0	100
15	69
30	57
45	53
150[d]	36

a. Table above represents conversion of dopamine over a span of 2.5 hours monitored by HPLC without the enzyme present in 50 mM phosphate buffer.

b. Time from adding dopamine into the 4-HPAA reaction which followed earlier described protocol for 4-HPAA synthesis starting from L-tyrosine.

c. The percent dopamine was calculated as the measured HPLC peak area (280 nm) of time point divided by starting concentration of dopamine at the time of addition into the reaction flask.

d. The end point of the reaction was obtained when the 4-HPAA was no longer present in the reaction solution as viewed by HPLC at 280 nm.

Table 5.2. Dopamine Conversion with Enzyme.[a]

Time[b] (minutes)	% Dopamine with NCS present[c]
0	100
15	60
30	53
60	47
150[d]	29

a. Table below represents conversion of dopamine over a span of 2.5 hours monitored by HPLC with the enzyme present in 50 mM phosphate buffer.

b. Time from adding dopamine into the 4-HPAA reaction which followed earlier described protocol for 4-HPAA synthesis starting from L-tyrosine.

c. The percent dopamine was calculated as the measured HPLC peak area (280 nm) of time point divided by starting concentration of dopamine at the time of addition into the reaction flask.

d. The end point of the reaction was obtained when the 4-HPAA was no longer present in the reaction solution as viewed by HPLC at 280 nm

Table 5.1 and Table 5.2 it is clear that the background reaction in phosphate is very severe, which is of interest because other buffers tested did not display such high background catalysis of Pictet-Spengler reaction. This particular aspect of the reaction is currently under investigation. However, because of such problems, the method for enzyme assays first had to be optimized with respect to buffer of choice. There were several variables which were necessary to consider: background Pictet-Spengler reaction catalysis by buffer, side reaction with any of the starting materials present, pH buffering capacity for maximum enzymatic activity, and finally enzymatic inhibition by the buffer. Considering pH dependence, activity of NCS was reported to be optimal at pH 7.0, which limited buffer choices to those that could buffer in this specific range. Consulting Good's buffers[38], which are buffering agents useful for biological and biochemical needs that have been tested and described by Norman Good, a few were selected as potential buffers before testing their effects[38]. The buffers selected were chosen for their buffering range capacity as ±1 pH from 7. Testing by Tris buffer revealed, as shown in Figure 5.2 competing side reaction where the Tris base attacks and forms an imine intermediate.

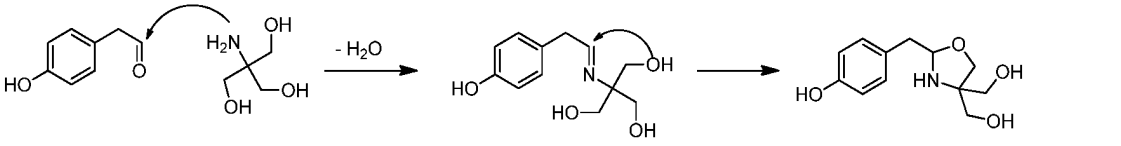


Figure 5.2. **Reaction of Tris with 4-HPAA.** Competing reaction of Tris buffer[36] observed when present with the starting substrate 4-HPAA, leading to consumption of 4-HPAA.

Further buffers selected did not contain primary amine groups. The reaction buffers considered and tested included: ACES, ADA, BES, Bicine, Bis-Tris, HEPES, imidazole, maleic acid, MES, and MOPSO. Further testing was done to ensure that the buffers did not contain background reaction or inhibition of enzyme by performing a series of assays. The procedure for background reaction was established using HPLC to monitor formation of norcoclaurine and compare it with phosphate buffer, which was assumed to be 100%. Testing for inhibition of enzymatic activity was performed as an enzyme assay by preparing solutions of dopamine (1 mM) with 4-HPAA (1 mM) and mixing them with different concentrations of buffer present (20, 40, 60, 80 mM) and monitoring enzymatic activity. If inhibition occurred the buffer was discarded from use for further experiments. Data collected is shown in Table 5.3.

Table 5.3. Buffers Tested for Pictet-Spengler and NCS Reactions.[a]

Buffer	**Rate of Pictet-Spengler reaction (relative to phosphate)** [b]	**Inhibition of NCS**[c]
Phosphate	100%	None
ACES	1.2%	0.32 ± 0.05
ADA	4.3%	None
BES	0.8%	None
Bicine	15.4%	None
Bis-Tris	3.6%	None
HEPES	0.9%	0.27 ± 0.03
Imidazole	0.7%	0.58 ± 0.08
Maleic acid	13.2%	None
MOPSO	1.0%	0.28 ± 0.08
Tris[d]	7.1%	None

a. Buffers listed in the table above were utilized in test reactions for minimizing both side reaction with 4-HPAA and inhibition of NCS

b. Progress of reaction between dopamine and 4-HPAA was monitored by HPLC for product formation.

c. Variety of buffer concentrations were tested for inhibition as a set of known buffer concentrations were varied and the progress of the reaction was monitored by HPLC.

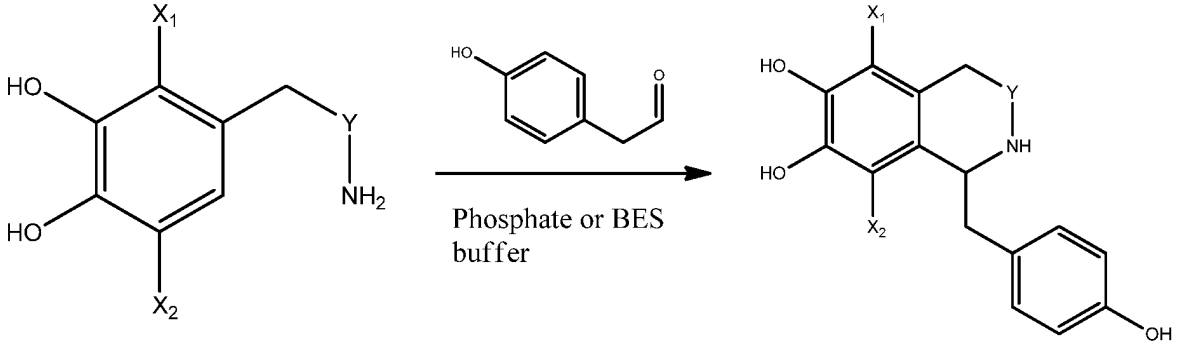
d. Observed side reaction with the starting material of enzyme reactions.

Based on the results available in Table 5.3 several buffers had low rate of the background Pictet-Spengler reaction when compared with phosphate. While most of the buffers contained no inhibition of NCS, it was unfortunate that the ones which contained inhibition of NCS were also ones that exhibited low background reaction. It is interesting to note that, out of the four buffers that inhibit NCS, three contain a ring structure: HEPES, imidazole, and MOPSO, and three contain a sulfonic acid moiety: ACES, HEPES, and MOPSO. While other buffers do not contain a cyclic structure they also are lacking a sulfonic acid group. The buffer which had low background reaction catalysis and no inhibition was BES, which does not contain a cyclic structure but does contain a sulfonic acid moiety. The reactions considered contained identical amount of starting materials as well as enzyme when present.

As such, a plasmid carrying an expressible construct of NCS with an N-terminal hexa-histidine tag was obtained from Dr. Sarah O'Connor (John Innes Centre) and transformed into JM109 cells. The

protein was provided with a His-tag which allowed for possible purification utilizing Ni-NTA column chromatography, which selectively binds His-tags above other protein available. A standard procedure for obtaining NCS enzyme was to grow an overnight culture. The overnight was then added to a large scale culture containing kanamycin (30 µg/mL) flask and grown until OD_{600} of 0.6 was reached (the grown cells were in their log growth phase). At this time, the inducer isopropyl-β-D-galactopyranoside (IPTG), was added to induce overexpression of protein. Cell growth was then continued until OD_{600} reached 3-4, at which point cells were harvested by centrifugation and lysed using sonication. The mixture was then pelleted and the supernatant was decanted and precipitant discarded. The liquid was then pipetted at least 100 times to insure sheering of DNA, which can potentially clog the column. After mixture was pipetted, it was then loaded onto a prepped Ni-NTA column, solution was slowly pushed through the syringe and washed with buffers containing imidazole at low and high concentrations. The low concentration wash removed non-specific binding protein from the column so as to obtain pure NCS enzyme, and the high concentration imidazole was used to elute NCS from the column. While it was found that imidazole inhibits enzymatic processes, it also is the only way to elute from Ni-NTA column without denaturing the enzyme in the process. Likewise, if the inhibitor is present in all samples it is not affecting one experiment differently than the other samples utilizing the same protein fractions. A more detailed description of protein purification is available in Biochemical Methods.

After the optimization of buffers, which could be used for reactions and purification of the enzyme by nickel column chromatography, substrate specificity with regards to dopamine was tested. The reactions were prepared by using a small amount of halogenated dopamine analogs alone with the hydrazine analog of dopamine (Scheme 5.1). Unfortunately, the hydroxylamine analog was not available for testing.



Scheme 5.1. **Synthetic and Enzymatic Synthesis of Norcoclaurine from Dopamine Analogs**. Dopamine analogs were used in 100 mM phosphate and 100 mM BES buffers in order to test their reactivity towards 4-HPAA with and without enzyme present.

Table 5.4: Preliminary Dopamine Analog Reaction Using NCS.[a]

	THI product	
Dopamine Variant[b]	Phosphate[c]	Enzymatic[d]
2-Cl	yes	yes
5-Cl	yes	yes
2-Br	yes	yes
5-Br	yes	Unknown[e]
2-I	yes	yes
5-I	yes	yes
Hydrazinyl	Unknown[f]	Unknown[f]

a. Table above has a collection of preliminary data testing NCS for activity with different dopamine analogs. Data collected is shown in – NMR and HPLC data section.

b. Halogenated dopamine analog used for the experiment.

c. Reaction carried out in 100 mM phosphate buffer at pH 7.0 observed at 280 nm.

d. Enzymatic reaction was carried out in presence of 100 mM BES buffer at pH 7.0 observed at 280 nm.

e. Large amount of unwanted contaminants make it hard to pinpoint peak for 5-Br THI analog.

f. The concentration of the solution was too little to detect THI product by HPLC.

The table above was compiled as preliminary data for the different dopamine analogs tested. While there were some analogs that could not be fully elucidated from the HPLC traces, majority of the

dopamine analogs were taken up by NCS as unnatural substrates and formed corresponding THI products.

Discussion:

While it is uncertain whether the hydroxylamine analog of dopamine can react well with the aldehyde to cyclize, the other dopamine analogs have formed the expected norcoclaurine product as determined by HPLC. The amount of starting material which was available did not present a chance to purify norcoclaurine analogs for proper NMR analysis; however, the reaction mixture was observed by HPLC and retention time of the analogs as well as the wavelength signature were closely related. Similarly, precise kinetic data was also not checked due to time constraints and lack of starting material for the reactions.

Though it was expected that the compounds undergo cyclization with phosphate buffer present, which presents as an excellent catalyst or reaction buffer for the Pictet-Spengler, it is interesting to note that all the compounds underwent cyclization to the norcoclaurine analog with the norcoclaurine synthase present. The reaction mixtures were prepared in parallel where half of the centrifuge tubes contained phosphate buffer without any added norcoclaurine synthase and half contained BES buffer with norcoclaurine synthase. Upon addition of 4-HPAA, the reactions were started and samples were processed on HPLC after 15 minutes since by as enough product forms from either reaction in that time frame. The enzyme appears to be much more tolerable to substrates which exhibit dopamine like characteristics. The enzyme, crystallized with dopamine and 4-HPAA in the active site, shows the catechol hydroxyl groups of dopamine extending outside of the active site while 4-HPAA is buried inside (Figure 5.3).

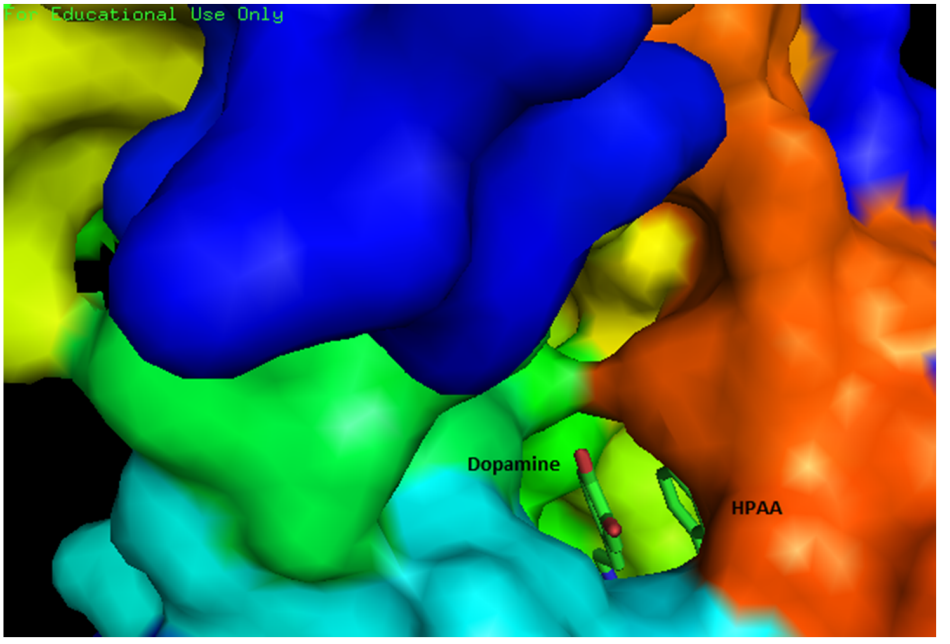


Figure 5.3. **Norcoclaurine Synthase Active Site**. The active site of the NCS is shown above with both 4-HPAA and dopamine present, the dopamine molecule is more outside while the 4-HPAA is buried inside the active site which suggests that dopamine is bound second after 4-HPAA is already in the active site.

It is of interest to determine if larger substrates are able to bind inside the active site. The position of dopamine based on the PDB structure shows that it is facing inside the active site with the amine side chain, which provides information as to how larger substrates my bind to the active site. Substitutions into the ethyl linker of the amine that are relatively small, such as the nitrogen or oxygen, should not cause a significant enough distortion where the substrate is no longer is able to enter the pocket for the cyclization reaction. The larger sulfonamide and phosphamide substrates discussed in Chapter 3 could possibly prove to be a challenge for the enzyme since the size of the group which has to enter is much greater. Likewise, it is uncertain whether the enzyme can catalyze a reaction of a sulfonamide or phosphamide. Alterations to the structure might also be necessary, further increasing the size of the group, making it more bulky and cumbersome for the active site.

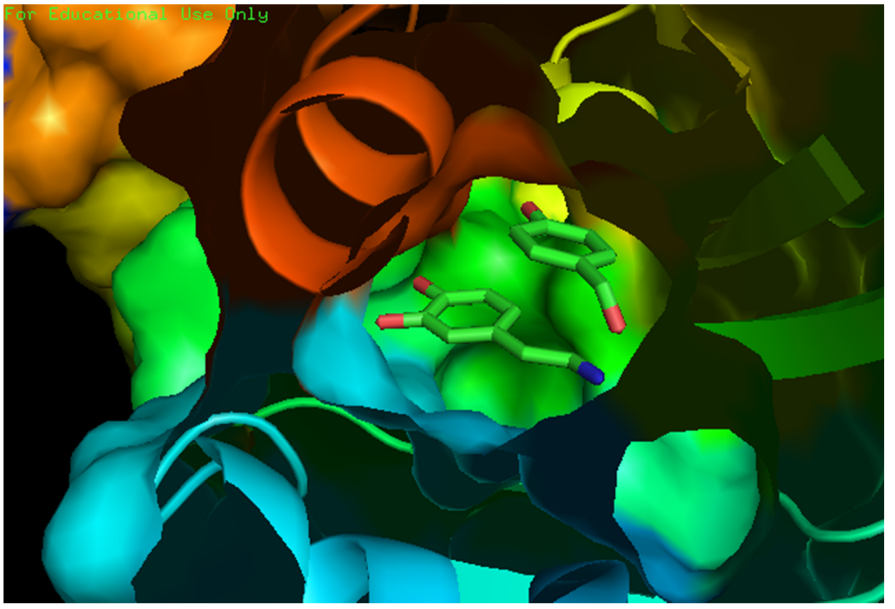


Figure 5.4. **Norcoclaurine Synthase Active Site Uncovered.** The active site of the NCS is shown above with both 4-HPAA and dopamine present uncovered from the residues around it showing that the amine and aldehyde are oriented to each other.

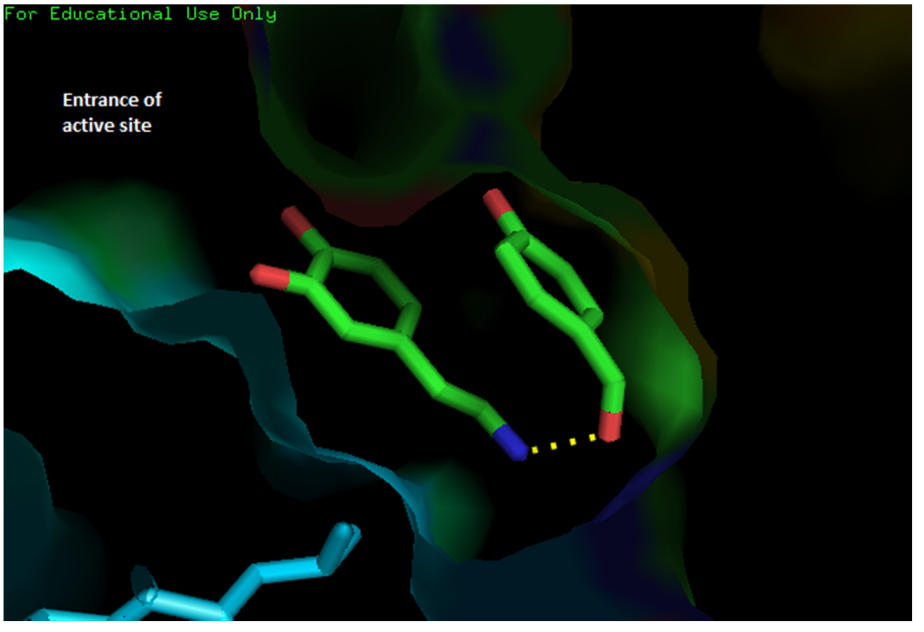


Figure 5.5. **Norcoclaurine Synthase Active Site Space**. The active site of the NCS is shown above with both 4-HPAA and dopamine present, as can be observed the closest amino acid to the dopamine molecule is leucine which is a non-polar residue that could be modified. Likewise there is polar connection which can be seen between the dopamine and 4-HPAA.

Upon closer inspection of the active site, it can be seen that the active site consists of cavities which are hydrophobic in nature and contain no amino acids. The structure, as stated before is questionable in its entirety, at this point provides evidence for a pocket which could be exploited in synthesis to better occupy the active site. Likewise, it is possible that the lysine (**L71**) residue which is shown in Figure 5.5 can be changed by point mutation to a valine or alanine preserving the hydrophobic element, or to a small polar residue like serine or threonine which will help with hydrogen bonding for phosphamide and sulfonamide.

Chapter 6 - Suggestions for Future Work

The research presented here indicates that novel dopamine analogs that are viable candidates for feeding experiments for attempting to obtain halogenated drug compounds such as morphine, berberine, sanguinarine, and others. Another structural analog was synthesized containing a hydrazine tail instead of a normal amine tail present in dopamine and synthesis of a hydroxylamine tail was attempted. Further work needs to be done in order to synthesize the hydroxylamine tail because of the instability of the O-N bond to attempted deprotection conditions. This work will involve using a different protecting group than MOM, methoxy, or acetyl since none of these are compatible with chemistry present to insert O-N bond.

As was previously mentioned, there are ways of synthesizing the corresponding phosphamide and sulfonamide moieties instead of the ethyl chain which can present interesting targets for synthesis and biological activity. Currently there is precedent for norcoclaurine as a drug, which presents a lucrative target for exploration for potential pharmacological benefit. Also, the additional benefit of generating norcoclaurine analogs allows for exploration of modified drugs is demonstrated in Scheme 2.3 for berberine analogs. This precursor-directed biosynthesis procedure can potentially lead to a discovery of other classes of related natural products (Scheme 2.2) with optimized or radically different pharmacological effects.

Another particular avenue of interest not yet explored in these compounds is a combination of different analogs from both dopamine and 4-HPAA synthesis allowing for an even greater amount of norcoclaurine analogs. These analogs can be used to selectively modify large scale molecules like berberine. Particularly this is possible when different halogens or functional groups are combined together allowing for precise control over coupling reactions like the Negishi, Heck, or Suzuki couplings. Currently there is no research focused on exploring these reactions in the context of norcoclaurine analogs or berberine analogs, possibly due to the large amount of synthesis involved in building these scaffolds and the need to develop even more chemistry to modify structures normally used for synthesis. As mentioned before there have been some studies into using halogenated precursors and then further modifying target molecule by cross coupling reactions, yet this approach was not explored fully.

There is also a need for kinetic studies to be finished for obtaining the K_m and V_{max} for the enzyme and our newly prepared dopamine analogs. As mentioned, previous studies had severe faults in their experimental methods which likely lead to incorrect values. The experiments completed by a different graduate student from our lab have failed to reach saturated enzyme rate required for Michaelis-Menten kinetics; however, it was speculated that the reason for that is the His-tag present at the N-terminus of the protein structure. The N-terminus is close to the active site and might act as a steric block for the substrate, thus blocking it from reaching saturated levels of activity. A solution for this is either to obtain untagged protein, which would be difficult to purify, or to obtain a C-terminus tagged enzyme. Likewise, one can also perform cleavage of the His-tag after purification of the enzyme and attempt to utilize that for kinetic assays using dopamine and 4-HPAA.

Finally, the terminal goal of this study would be to incorporate necessary enzymes into a single organism which can take either starting precursors like tyramine or tyrosine synthetically halogenated and produce proteins necessary to convert the precursors to dopamine. Then feed the bacterial solution 4-HPAA and produce norcoclaurine analogs. Optimization of yield of whole pathways into heterologous organisms will be a complex project. However, if any products generated from these precursor-directed biosynthesis yields useful drug candidates, this will ultimately be the best approach for yielding target products.

Appendix A - Experimental Methods

General Synthetic Methods

Methylation:

Following an adaptation of the procedure of McKillop et al.[28] an aqueous solution of 1.20 g of NaOH (30 mmol, 3.0 eq.) in 50 mL of deionized water was added to a stirring solution of 2.31 g (10 mmol) of 2-bromo-isovanillin (2-bromo-3-hydroxy-4-methoxy-benzaldehyde) in 50 mL of dichloromethane. Next, phase transfer catalyst was added, as 3.40 g of either tetrabutylammonium hydrogen sulfate (TBAHS, 10.0 mmol, 1.0 eq.) or recycled catalyst (assuming that the recovered catalyst is tetrabutylammonium hydroxide, 2.6 g is 10 mmol, 1.0 eq). Once dissolved, 17 g (120 mmol, 12 eq.) of methyl iodide was then added to the mixture and the reaction was allowed to stir at room temperature. Reaction progress was monitored by HPLC. As monitored by HPLC, reaction progress generally showed complete turnover to product with no side products by 3 hours, however the solution was typically allowed to stir overnight for convenience. The reaction mixture was extracted with 3 x 50 mL portions of CH_2Cl_2. The combined organic extracts were washed with brine and deionized water, dried over $MgSO_4$, filtered, and concentrated by evaporation under reduced pressure to yield a either a white or yellow solid. To remove catalyst, the solid was first ground to a fine powder with a mortar and pestle. This solid was poured on top of a 2 – 3 cm layer of dry silica gel in a 3 – 4 cm (I.D.) sintered glass fritted Buchner funnel. The solid was extracted with 1:5 ethyl acetate: hexanes (EtOAc:Hex) in 75 mL portions by pouring the solvent mixture over the dry solids with vacuum suction to collect the solution in a round bottom flask. Allow the solids to dry between solvent portions for best separation. The first 1250 mL typically contained 85 - 95% of pure product. The combined eluent was evaporated to dryness under reduced pressure to afford a dense, white, flakey solid (2.06 g, 90%).

Sodium Borohydride Reduction:

Starting material of aldehyde (1 eq.) was dissolved in minimal amount of MeOH or EtOH required to dissolve starting material, and stirred in an ice bath until the mixture was cooled to 0 °C. To the reaction mixture $NaBH_4$ (~1.0 eq.) was added slowly and the reaction was allowed to warm to room temperature. Progress of reaction typically was monitored by UPLC. After reaction was finished the

mixture was cooled to 0 °C and quenched with acetone (0.5 mL). The organic layers are concentrated to half volume *in vacuo*, water was then added in equal amount and the aqueous layers were extracted with (3x25mL) DCM. Organic layers were then combined and concentrated *in vacuo* to yield the alcohol product typically as yellow oil.

Chlorination:

Starting benzyl alcohol was dissolved in DCM and NEt_3 or pyridine (1.5 eq.) was added. The reaction mixture was then cooled to 0 °C and thionyl chloride (1.1 eq.) was added slowly via syringe pump. The reaction was then allowed to warm to room temperature and progress was monitored by UPLC. Upon completion the reaction mixture was evaporated under reduced pressure and resulting oil was triturated with Et_2O/Hexanes. Resulting solid was filtered off and dried under vacuum.

Borane Reduction:

Starting material for the borane reduction was dissolved in dry THF and placed under nitrogen atmosphere and cooled to 0 °C. To the reaction mixture BH_3•THF (1.1 eq.) was added dropwise via syringe, and the solution was allowed to warm up to room temperature (for halogenated dopamine analog the mixture was refluxed). Progress was monitored by UPLC, if the reaction was not progressing additional half equivalent (0.5 eq.) was added to the mixture slowly via syringe. Once complete the reaction was cooled to 0 °C and quenched with 1 mL of H_2O added slowly via syringe followed by 5 mL of concentrated HCl. The solution was allowed to stir for 20 minutes and then diluted with 25 mL of H_2O and basified with concentrated NaOH and the mixture was then extracted three times with 97:3 DCM:MeOH and concentrated *in vacuo* to yield the product as a pale oil. Further purification can be accomplished by dissolving basic extract in cold Et_2O with stirring, concentrated HCl was added dropwise to precipitate the product as an HCl salt. Filtration afforded a white solid.

Nitrostyrene Reduction:

Procedure followed for these compounds is published by our laboratory[27]. For every 1.0 mmol of nitrostyrene, 2 mL of methanol, 800 mg of zinc dust (12 mmol), and 2 mL of 37% HCl (24 mmol) were used. Methanol was vigorously stirred in an ice bath maintained <0 °C (ice/NaCl or freezer-chilled

commercial antifreeze). HCl, zinc dust, and nitrostyrene were slowly added over the course of 30 minutes in alternating small portions taking care that the temperature did not rise above 0 °C. For large-scale reactions (>25 mmol), HCl was added continuously by syringe pump. After addition was complete, any solids on the side were washed into the solution with a small amount of methanol. All starting material was consumed within one hour of complete reagent addition as monitored by HPLC and observed by complete disappearance of the initial yellow color. At this point, an intermediate and the phenethylamine product generally dominate the mixture as observed by HPLC. The intermediate is typically converted to product after 4 hours of total stirring at 0 °C. The reaction is typically complete 4–6 hours after the yellow color has disappeared. The reaction may stir for as long as 16 hours in a 4 °C refrigerator without significant formation of side products. If HPLC is not available to monitor the reaction, we suggest adding an additional 200 mg of zinc dust (3 mmol) and 0.6 mL (7 mmol) of concentrated HCl for every 1.0 mmol of nitrostyrene after 5 hours and stir for an additional hour to ensure complete reaction. Once complete, the excess solid zinc was removed by filtration through filter paper. *Note that filtering through celite, silica gel, or alumina at this stage leads to product decomposition.* The solution was made basic by dropwise addition of saturated sodium hydroxide in methanol, while maintaining the temperature below 5 °C, until the pH was greater than 11 by pH paper. Next, 10 mL of $CHCl_3$ was added (per mmol of reactant). Solid anhydrous $MgSO_4$ was added to dry the organic layer. The organic extract was filtered by filter paper. The remaining paste was extracted two more times with $CHCl_3$ and filtered. The combined organic extracts were evaporated *in vacuo* to yield phenethylamine as amber oil. In cases when oil was not obtained at the final step, the material was dissolved in minimal $CHCl_3$ and the remaining inorganic salts were completely precipitated by addition of diethyl ether. After filtration and evaporation, salt-free oil was obtained.

Deprotection:

Starting material was dissolved in minimal dry DCM and cooled to 0 °C by ice bath under nitrogen atmosphere. After cooling, BBr_3 in DCM (2.2 mL, 1 M solution) was added slowly via syringe to prevent vigorous reaction. The reaction was allowed to warm to room temperature and stirred for 1.5 hours. Progress of the reaction was monitored by UPLC. If the reaction was incomplete, extra 0.2-1.0 equivalents of BBr_3 may be added. The reaction mixture was then cooled again in an ice bath, quenched by slow addition of dry MeOH, and allowed to stir for 10 minutes. The resulting solution was concentrated *in vacuo*. The products were extracted by washing first with Et_2O and then dry MeOH to

remove any borane byproducts and the leftover oil was dried under vacuum. The product can be precipitated by dissolving the oil in minimal acetonitrile followed by addition of minimal Et_2O until precipitation occurs.

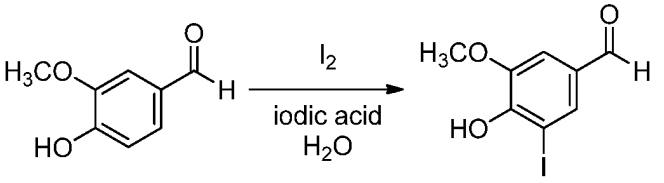
Bleach Oxidation[36]:

To a vigorously stirring solution of amino acid (1 mmol) in 250 mL of sodium phosphate buffer (10 mM, pH 7.0) was added a solution of NaOCl (1 equivalent as a 0.1 M aqueous solution) slowly over 10 minutes via syringe pump in a 37 °C warm room or water bath. The resulting solution was stirred vigorously at this temperature until the reaction was judged to be complete by HPLC (1-2 hours). In general, the reaction may be stopped after 2 hours if monitoring is inconvenient. The solution was then extracted into dichloromethane (4 x 40 mL), washed with brine (2 x 50 mL), dried over $MgSO_4$, and the solvent removed under reduced pressure in an unheated water bath to afford the desired aldehyde.

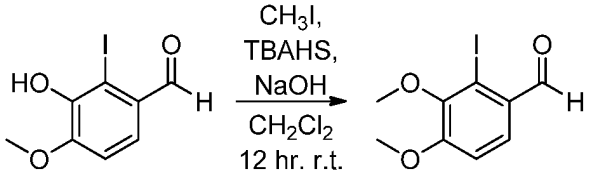
Pictet-Spengler for Tetrahydroisoquinoline synthesis[36]:

Each 1 mmol of amino acid was suspended with 2.0 mL of 1.0 M phosphate buffer at pH 6.9 (this concentrated buffer gives pH 7.0 upon dilution) in 200 mL of purified water at 37 °C. A solution of 1 molar equivalent of sodium hypochlorite (e.g. 700 µL of 9.16 % NaOCl) was dissolved in 10 mL of purified water. The NaOCl was added dropwise over 10 minutes via syringe pump with vigorous stirring by magnetic stir bar. This solution was allowed to stir at 37 °C for 1.5 - 2 hours and was quenched by the addition of ascorbic acid (88 mg, 0.5 mmol). Additional phosphate catalyst, 100 mL of 1.0 M phosphate buffer at pH 6.9, was then added to the solution followed by dopamine hydrochloride (291 mg, 1.5 mmol). The reaction was monitored until all of the aldehyde was consumed as monitored by HPLC. If monitoring is not convenient, all reactions are complete by 2 hours. The reaction was then extracted into ethyl acetate (6 X 50 mL), the combined organic layers were washed brine (2 X 50 mL), dried with $MgSO_4$, and the solvent removed under reduced pressure yielding the product as a solid.

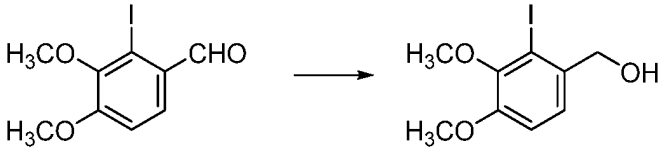
Specific Organic Synthesis Procedures



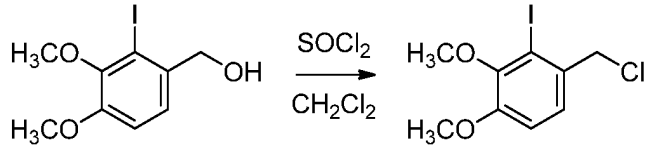
5-Iodo-vanillin (1). Vanillin (38.1 g, 250 mmol) and iodine (25.4 g, 100 mmol) were added to a 1 L round bottom flask containing minimal EtOH. Iodic acid (8.8 g, 50 mmol) was dissolved in minimal amount of water (50 mL), and added to the flask containing the vanillin-iodine mixture. The flask was placed in a water bath at a constant temperature of 35 °C and stirred. The thick consistency of the reaction mixture requires overhead mechanical stirring. While reacting, minimal amounts of EtOH and distilled H_2O were occasionally used to wash solids down the sides of the flask. After 1.5 hours, the reaction was judged to be complete by HPLC. The cream-colored solid was washed in a Buchner funnel with 1.5 L of saturated sodium thiosulfate (NaS_2O_3) and 0.5 L of deionized H_2O. The remaining solid was recrystallized in EtOH yielding 42.2 g (97.7%) of light yellow prism-shaped crystals. **^{1}H NMR (300 MHz, $CDCl_3$)** δ 9.74 (s, 1H), 7.87 (d, *J* = 1.8 Hz, 1H), 7.40 (d, *J* = 1.7 Hz, 1H), 3.89 (s, 3H). **UV-Vis:** 228.7, 297.4 nm.



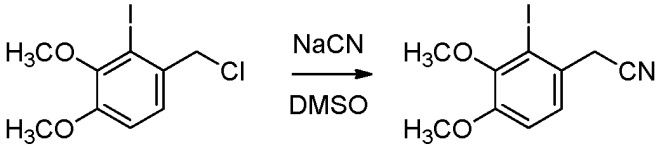
2-Iodo-3,4-dimethoxybenzaldehyde (4). Following methylation procedure from 2.78g (10 mmol) of 2-iodo-isovanillin (2-iodo-3-hydroxy-4-methoxybenzaldehyde) methylation afforded 2.65 g (90%) of a dense, white, flakey solid; **^{1}H-NMR (300 MHz, DMSO-*d*$_6$):** δ 9.90 (s, 1H), 7.64 (d, *J* = 8.6 Hz, 1H), 7.26 (d, *J* = 8.7 Hz, 1H), 3.93 (s, 3H), 3.75 (s, 3H). **UV-Vis**: 227.5, 292.4 nm.



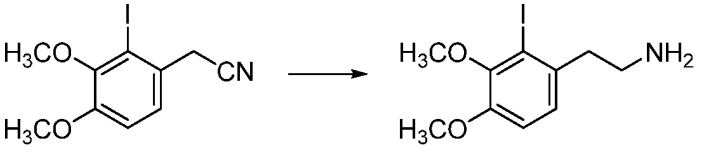
(2-iodo-3,4-dimethoxyphenyl)methanol (6). Following general $NaBH_4$ reduction, 3,4-dimethoxy-5-iodo-benzaldehyde (2.92g, 10 mmol) were reacted to yield the product as yellow oil (2.79 g, 9.5 mmol, 95%). **^{1}H NMR (300 MHz, $CDCl_3$):** δ 7.40 – 7.27 (m, 1H), 7.02 (d, *J* =1.7 Hz, 1H), 4.53 (s, 2H), 3.87 (s, 3H), 3.77 (s, 3H). **UV-Vis:** 216.5, 278.9 nm.



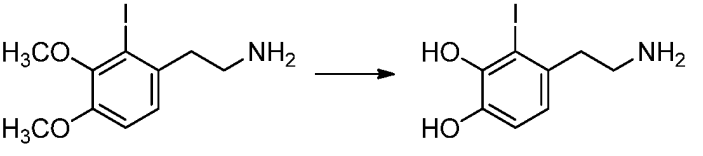
(2-iodo-3,4-dimethoxyphenyl)chloromethane (8). Following general chlorination procedure, (2-iodo-3,4-dimethoxyphenyl)methanol (2.79 g, 9.5 mmol) was reacted to afford a yellow oil (2.92 g, 8.55 mmol, 90%) which crystalized upon standing. UPLC analysis of the crude reaction mixture indicated that ~10% of the starting material did not react during the chlorination procedure. **^{1}H NMR (300 MHz, DMSO-d_6):** δ 7.36 (dd, *J* =8.4, 3.3 Hz, 1H), 7.09 (dd, *J* = 8.5, 3.2 Hz, 1H), 4.80 (d, *J* = 3.2 Hz, 2H), 3.86 – 3.80 (m, 3H), 3.72 – 3.66 (m, 3H). **UV-Vis:** 220.0, 281.4 nm.



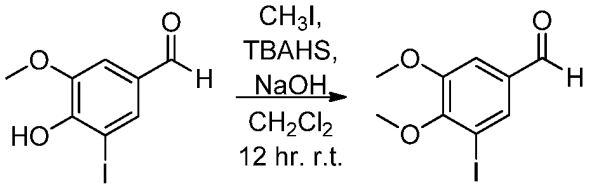
(2-iodo-3,4-dimethoxyphenyl)acetonitrile (10). (2-iodo-3,4-dimethoxyphenyl)chloromethane (2.66 g, 8.55 mmol) was dissolved in 150 mL of DMSO and allowed to stir. Sodium cyanide was added in excess to the mixture and allowed to stir for 2.5 hours. After the reaction was checked by HPLC the mixture was poured into a separating funnel and extracted with diethyl ether three times and washed with brine. The product was concentrated under vacuum to afford a white crystalline solid (2.24 g, 8.52 mmol, 99.6%). **^{1}H NMR (300 MHz, DMSO-d_6):** δ 7.27 (d, *J* = 8.5 Hz, 2H), 7.13 (d, *J* = 8.5 Hz, 2H), 3.96 (d, *J* = 15.8 Hz, 5H), 3.83 (s, 7H). **UV-Vis:** 215.9, 285.7 nm.



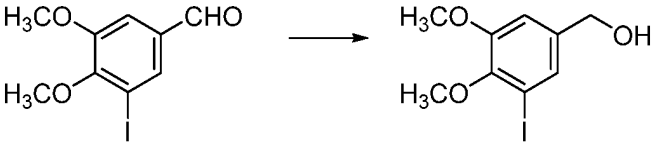
2-iodo-3,4-dimethoxy-phenethylamine (12). Following general borane reduction, (2-iodo-3,4-dimethoxyphenyl)acetonitrile (0.151 g, 0.5 mmol) was reacted to afford a white solid 95% yield. **^{1}H NMR (300 MHz, methanol-d_4):** δ 7.06 (dd, *J* = 10.8, 8.4 Hz, 1H), 6.98 (dd, *J* = 8.4, 3.4 Hz, 1H), 5.51 (s, 3H), 3.88 – 3.83 (m, 3H), 3.80 – 3.76 (m, 3H), 3.27 (t, *J* = 7.2 Hz, 1H), 2.95 – 2.77 (m, 3H). **UV-Vis:** 212.9, 279.5 nm.



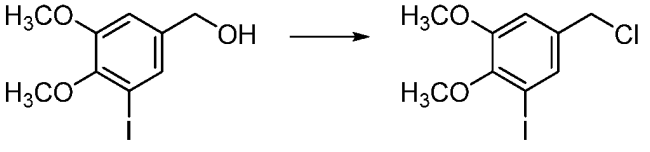
2-iodo-dopamine (14). Following general deprotection procedure, 2-iodo-3,4-dimethoxy-phenethylamine (0.5 mmol) was reacted to yield 2-iodo-dopamine as and oil which solidified under vacuum (99.5%). **^{1}H NMR (300 MHz, methanol-d_4):** δ 6.78 (d, *J* = 8.1 Hz, 1H), 6.71 (d, *J* = 8.1 Hz, 1H), 3.10 (s, 2H), 3.07 (d, *J* = 4.6 Hz, 2H).



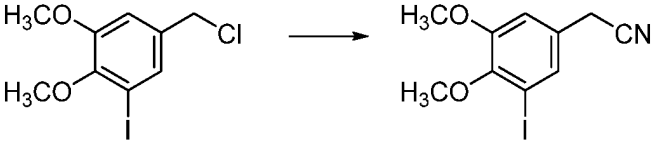
3-Iodo-4,5-dimethoxybenzaldehyde (3). Following methylation procedure **f**rom 2.78g (10 mmol) of 5-iodo-vanillin (3-iodo-4-hydroxy-5-methoxybenzaldehyde) methylation afforded 2.83 g (97%) of a white, fluffy solid; **^{1}H-NMR (300 MHz, DMSO-d_6):** δ). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.40 (d, *J* = 2.4 Hz, 1H), 8.48 (t, *J* = 2.1 Hz, 1H), 8.07 (d, *J* = 1.8 Hz, 1H), 4.49 (d, *J* = 2.3 Hz, 3H), 4.44 (d, *J* = 2.4 Hz, 3H). **UV-Vis:** 228.1, 297.5 nm.



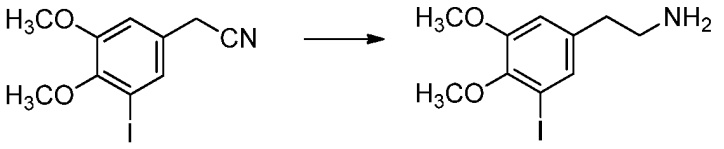
(5-iodo-3,4-dimethoxyphenyl)methanol (5). 3, 4-dimethoxy-5-iodo-benzaladehyde (2.92 g, 10 mmol) was dissolved in minimal EtOH and stirred in an ice bath. After the entire solid had dissolved $NaBH_4$ (3.4 g, ~10 mmol) was added to the mixture. The progress was monitored by HPLC. After, 1.5 hours the complete reaction mixture was concentrated under vacuum to half its volume and poured into a separating funnel containing water. Aqueous layer was extracted three times with DCM. It was then concentrated to give the final product as yellow oil (2.79 g, 9.5 mmol, 95%). **^{1}H NMR (300 MHz, DMSO-d_6):** δ 7.40 – 7.27 (m, 1H), 7.02 (d, *J* = 1.7 Hz, 1H), 4.53 (s, 2H), 3.87 (s, 3H), 3.77 (s, 3H). **UV-Vis:** 214.7, 285.1 nm.



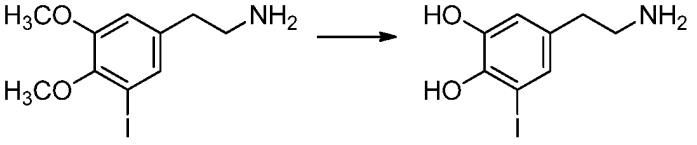
(5-iodo-3,4-dimethoxyphenyl)chloromethane (7). (5-iodo-3,4-dimethoxyphenyl)methanol (2.78 g, 9.4 mmol) obtained from the previous reaction was dissolved in DCM in a separatory funnel. To the reaction mixture HCl was added (2 mL) and the reaction funnel and was shaken for 10 minutes. The organic layer was washed with H_2O and brine. The organic layer was concentrated *in vacuo* to afford yellow colored oil (2.92 g, 8.55 mmol, 91%) which crystalized on standing. **^{1}H NMR (300 MHz, DMSO-d_6):** δ 7.36 (dd, *J* = 8.4, 3.3 Hz, 1H), 7.09 (dd, *J* = 8.5, 3.2 Hz, 1H), 4.80 (d, *J* = 3.2 Hz, 2H), 3.86 – 3.80 (m, 3H), 3.72 – 3.66 (m, 3H). **UV-Vis:** 218.9, 283.8 nm.



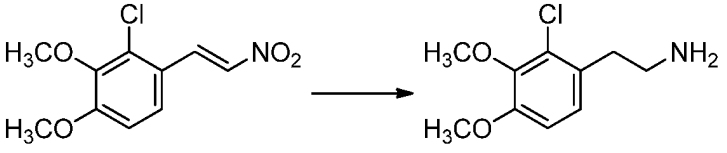
(5-iodo-3,4-dimethoxyphenyl)acetonitrile (9). The product from the previous reaction (2.92 g, 8.55 mmol) was dissolved in 150 mL of DMSO and allowed to stir. Sodium cyanide was added in excess (4.9 g, 10 mmol) to the mixture and allowed to stir for 2.5 hours. The reaction mixture was poured into a separatory funnel and extracted with diethyl ether three times and washed with brine. The product was concentrated *in vacuo* to give a white crystalline solid (2.24 g, 7.4 mmol, 87%). **^{1}H NMR (300 MHz, DMSO-*d*$_6$):** δ 7.31 (d, *J* = 2.1 Hz, 1H), 6.86 (d, *J* = 2.0 Hz, 1H), 3.90 (s, 3H), 3.85 (s, 3H), 3.69 (s, 2H). **UV-Vis:** 216.5, 285.7 nm.



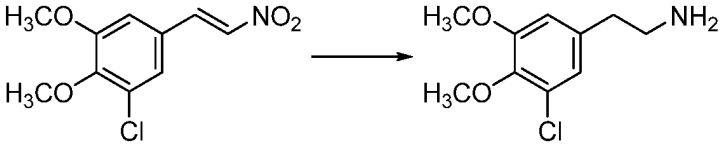
5-iodo-3,4-dimethoxy-phenethylamine (11). Following general borane reduction, 3,4-dimethoxy-5-iodo-benzylnitrile (0.156 g, 0.5 mmol) was reacted yield the product as a pale oil (0.147 g, 0.48 mmol, 96%). **^{1}H NMR (300 MHz, CDCl$_3$):** δ 7.22 (s, 1H), 6.74 (s, 1H), 5.32 (s, 2H), 3.87 (s, 3H), 3.83 (s, 3H). **\UV-Vis:** 214.1, 284.4 nm.



5-iodo-dopamine (13). Following general deprotection procedure, 5-iodo-3,4-dimethoxy-phenethylamine (1 mmol) was reacted to yield 5-iodo-dopamine (0.98 mmol, 98%). Additional precipitation of the dopamine product can be performed from ACN. **^{1}H NMR (300 MHz, methanol-*d*$_4$)**: δ 6.99 (d, *J* = 1.6p Hz, 1H), 6.66 (d, *J* = 1.5p Hz, 1H), 2.89-2.98 (m, 2H), 2.63-2.70 (m, 2H).

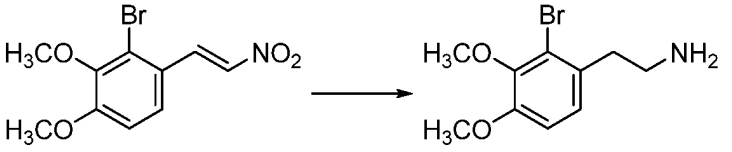


2-chloro-3,4-dimethoxy-phenethylamine (15). Using general nitrostyrene reduction, 517 mg of (*E*)-2-chloro-3,4-dimethoxy-1-(2-nitrovinyl)benzene (2.4 mmol) was reacted to yield 434 mg (74%) as an oil. **^{1}H NMR (300 MHz, methanol-*d*$_4$)** δ 7.01 (d, *J* = 8.4 Hz, 1H), 6.88 (d, *J* = 8.5 Hz 1H), 3.82 (s, 3H), 3.79 (s, 3H), 2.94 (d, *J* = 5.6 Hz, 2H), 2.83 (d, *J* = 5.4 Hz, 2H).

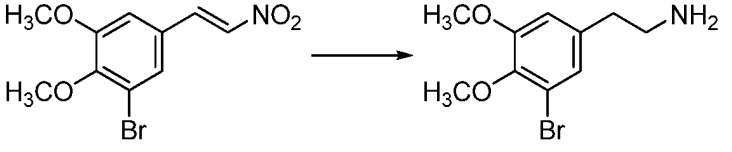


5-chloro-3,4-dimethoxy-phenethylamine (16). Using general nitrostyrene reduction, (*E*)-5-chloro-3,4-dimethoxy-1-(2-nitrovinyl)benzene 487 mg (2.0 mmol) was reacted to yield 376.5 mg (87%) as a yellow

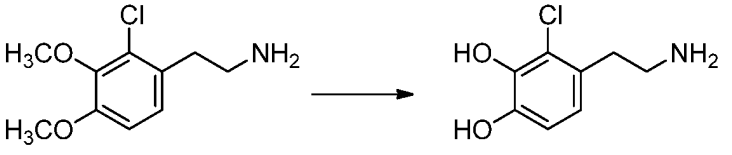
oil. **^{1}H NMR (300 MHz, methanol-d_4)** δ 6.93 (d, *J* = 1.9 Hz, 1H), 6.90 (d, *J* = 1.9 Hz, 1H), 3.90 (s, 3H), 3.81 (s, 3H), 3.14 (t, *J* = 7.5 Hz, 2H), 2.88 (t, *J* = 7.5 Hz, 2H).



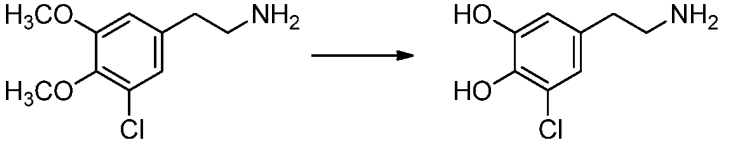
2-bromo-3,4-dimethoxy-phenethylamine (18). Using general nitrostyrene reduction, (*E*)-2-bromo-3,4-dimethoxy-1-(2-nitrovinyl)benzene 288 mg (1.0 mmol) was reacted to yield 201.3 mg (77%) as yellow oil. **^{1}H NMR (300 MHz, methanol-d_4)** δ 6.96 (d, *J* = 8.5 Hz, 1H), 6.80 (d, *J* = 8.5 Hz, 1H), 3.88 (s, 3H), 3.88 (s, 3H), 2.97 (dd, *J* = 10.4, 3.8 Hz, 2H), 2.84 (dd, *J* = 10.5, 3.9 Hz, 2H).



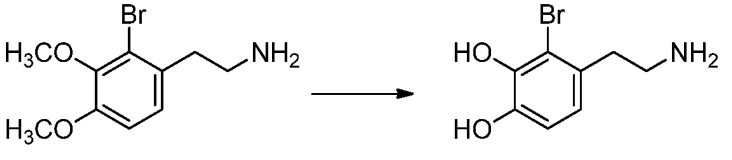
5-bromo-3,4-dimethoxy-phenethylamine (17). Using general nitrostyrene reduction, 288 mg (*E*)-5-bromo-3,4-dimethoxy-1-(2-nitrovinyl)benzene (1.0 mmol) was reacted to yield 216.1 mg (83%) as a yellow colored solid. **^{1}H NMR (300 MHz, methanol-d_4)** δ 7.10 (d, *J* = 1.9 Hz, 1H), 6.96 (d, *J* = 1.9 Hz, 1H), 3.90 (s, 3H), 3.80 (s, 3H), 3.19 (t, *J* = 7.6 Hz, 2H), 2.92 (t, *J* = 7.5 Hz, 2H).



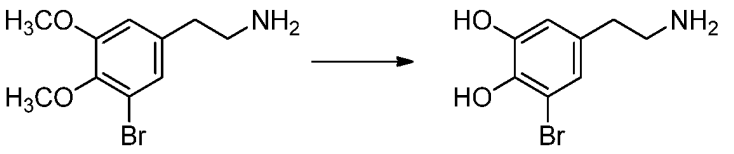
2-chloro-dopamine (19). Following general deprotection, 0.169 g (0.78 mmol) of 2-chloro-3,4-dimethoxy-phenethylamine was reacted as above to yield 0.130 g (88.19%) of product as a yellow oil. **^{1}H NMR (300 MHz, methanol-d_4):** δ 6.81 – 6.71 (m, 2H), 3.16 (d, *J* = 5.1 Hz, 1H), 3.12 – 2.98 (m, 1H).



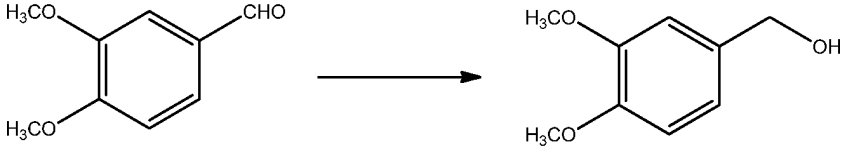
5-chloro-dopamine (20). Following general deprotection, 0.221 g (1 mmol) of 5-chloro-3,4-dimethoxy-phenethylamine was reacted as above to yield 0.191 g (98.9%) as a yellow oil. **^{1}H NMR (300 MHz, methanol-d_4):** δ 6.75 (d, *J* = 2.0 Hz, 1H), 6.68 (d, *J* = 1.9 Hz, 1H), 3.14 (t, *J* = 7.6 Hz, 2H), 2.82 (t, *J* = 7.6 Hz, 2H).



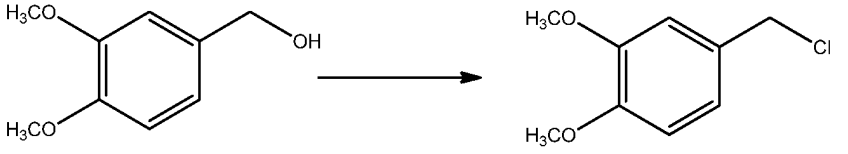
2-bromo-dopamine (22). Following general deprotection, 0.201 g (0.77 mmol) of 2-bromo-3,4-dimethoxy-phenethylamine was reacted as above to yield 0.171 g (94.8%) as a yellow oil. **^{1}H NMR (300 MHz, methanol-d_4)**: δ 6.73 (q, *J* = 8.2 Hz, 2H), 3.20 – 3.09 (m, 1H), 3.08 – 2.97 (m, 1H).



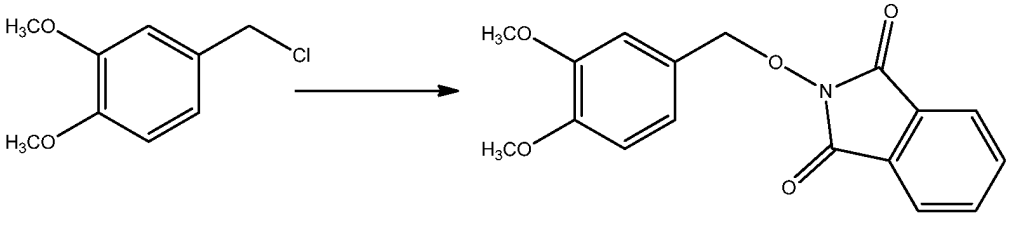
5-bromo-dopamine (21). Following general deprotection, 0.216 g (0.8 mmol) of 5-bromo-3,4-dimethoxy-phenethylamine was reacted as above to yield 0.175 g of the product (90.8%) as yellow oil. **^{1}H NMR (300 MHz, methanol-d_4):** δ 6.90 (d, *J* = 2.0 Hz, 1H), 6.72 (s, 1H), 3.11 (d, *J* = 8.0 Hz, 2H), 2.86 – 2.79 (m, 2H)



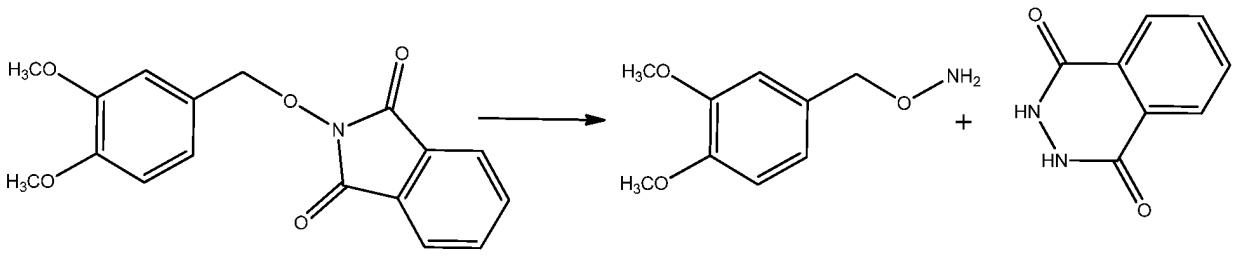
(3,4-dimethoxyphenyl)methanol (28). 1.66 g (10 mmol) of starting material was reacted according to general reduction using $NaBH_4$ to yield the product as a pale oil (9.8 mmol, 98%). NMR was not obtained



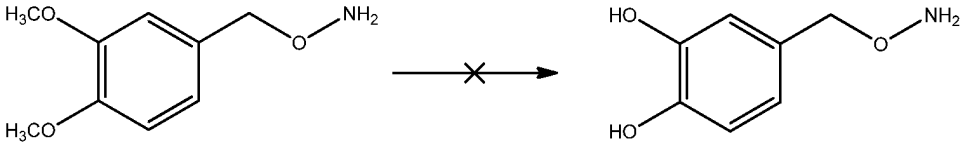
4-(chloromethyl)-1,2-dimethoxybenzene (29). 5 mmol of starting material were chlorinated using general chlorination procedure with NEt_3 to yield the product (4.3 mmol, 87%) as a pale white solid. NMR was not obtained.



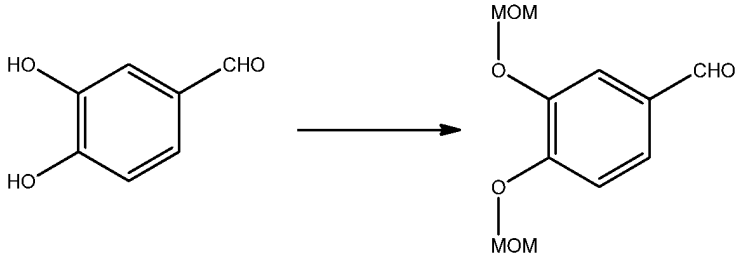
2-((3,4-dimethoxybenzyl)oxy)isoindoline-1,3-dione (30a). Following already established procedure[01] 2 mmol of starting material and NEt_3 (2.4 mmol, 1.2 eq.) were dissolved in 10 mL of DMF and stirred. To the solution *N*-hydroxypthalimide (2.2 mmol, 1.1 eq.) was added and the reaction was heated to 70 °C overnight. Reaction progress was monitored by HPLC. The reaction was observed to be 80% complete at which point the H_2O was added to quench the reaction, which formed precipitate. After filtration precipitate was washed with H_2O to yield the product (1.56 mmol, 78%) as a pale cream colored powder. **^{1}H NMR (300 MHz, CD_3CN)** δ 7.82 (s, 4H), 7.13 (d, *J* = 1.9 Hz, 1H), 7.04 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.92 (d, *J* = 8.2 Hz, 1H), 5.11 (s, 2H), 3.82 (s, 6H).



***O*-(3,4-dimethoxybenzyl)hydroxylamine (31a).** 0.3 g of starting material (1 mmol) were stirred in 100% EtOH and hydrazine (1.5 eq) was added to the solution. Upon addition of hydrazine starting material dissolved into the mixture forming a brown colored clear solution. Following 5-10 minute stirring, solid precipitate was formed, which was filtered off and identified via NMR as the by-product of the reaction. The organic layer was evaporated under reduced pressure and subsequent brown oil was re-dissolved in Et_2O. Any solid which formed was filtered again, and organic layer was again concentrated *in vacuo* to yield the product as a brown clear oil (0.16 g, 0.87 mmol, 87.3%). **^{1}H NMR (300 MHz, CD_3OD)** δ 6.98 (s, 1H), 6.92 (s, 2H), 4.60 (s, 2H), 3.83 (d, J = 3.7 Hz, 6H).

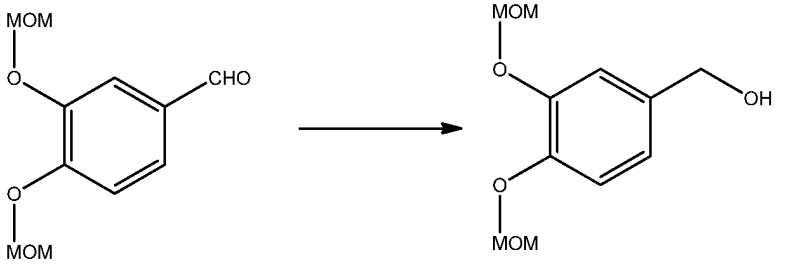


4-((aminooxy)methyl)benzene-1,2-diol (32). 1 mmol of starting material was used following general deprotection method outlined above. The reaction produced a single product peak which was observed by HPLC and isolated by quenching the reaction with HCl gas. White precipitate formed which was filtered off and dissolved in d^3-acetonitrile for NMR. Some side products were observed along with an unknown tri-peak which could not be assigned to any common NMR contaminants. Upon other experimentation detailed in Chapter 3 the product of the reaction was assigned to be 1,2-dihydroxybenzalcohol, which was produced from O-N bond cleavage by BBr_3.

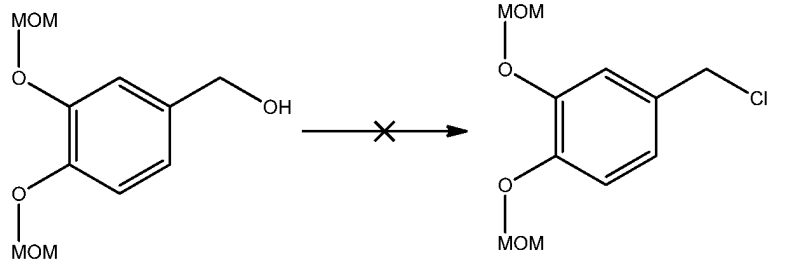


3,4-bis(methoxymethoxy)benzaldehyde (34). 1 mmol of starting material was dissolved in dry DMF, cooled to 0 °C, and 60% NaH in mineral oil suspension (2.2 eq) was added portion-wise. Reaction was observed to change color from clear brown to matte yellow. After addition reaction was de-gassed and

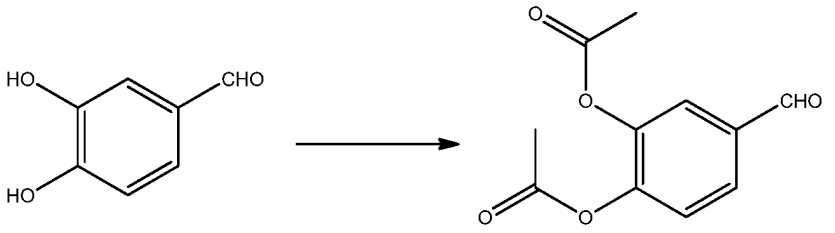
placed under nitrogen atmosphere while maintaining 0 °C. Chloromethyl methyl ether (MOM-Cl) (2.2 eq) was slowly added to the reaction mixture via syringe to prevent vigorous reaction. Solution was then allowed to stir at room temperature for an hour and the reaction progress was monitored by HPLC. Two other peaks (< 15%) were observed along with the product peak, which were attributed to singly protected para- and meta- position alcohols. Reaction was then quenched with 0.1 M solution of NaOH, diluted with 10 mL of brine and extracted with (3x 10 mL) of DCM. Organic layers were combined and evaporated under reduced pressure. The product was pale oil 0.196 g (0.86 mmol, 86%). **^{1}H NMR (300 MHz, DMSO-*d*$_6$)** δ 9.85 (s, 1H), 7.59 (td, *J* = 4.4, 1.9 Hz, 2H), 7.33 – 7.28 (m, 1H), 5.33 (s, 2H), 5.28 (s, 2H), 3.41 (d, *J* = 1.5 Hz, 6H).



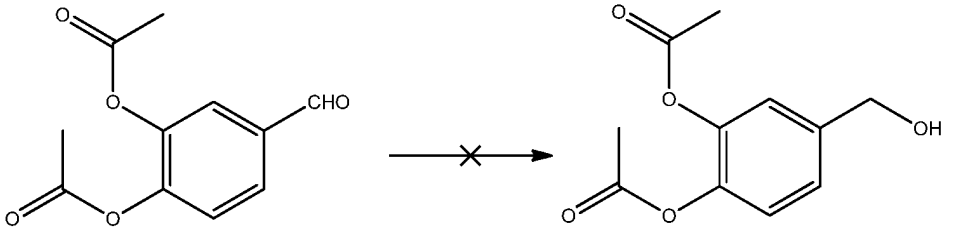
(3,4-bis(methoxymethoxy)phenyl)methanol (35). Following general $NaBH_4$ reduction, 0.86 mmol were reacted to yield an off-color clear oil product (0.83 mmol, 94%). **^{1}H NMR (300 MHz, DMSO-*d*$_6$)** δ 7.06 (dd, *J* = 10.6, 5.0 Hz, 2H), 6.89 (dd, *J* = 8.2, 1.9 Hz, 1H), 5.17 (s, 2H), 5.14 (s, 2H), 4.41 (d, *J* = 4.6 Hz, 2H), 3.41 (s, 3H), 3.40 (s, 3H).



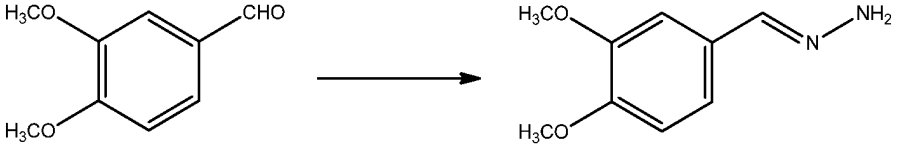
4-(chloromethyl)-1,2-bis(methoxymethoxy)benzene (36). Following general chlorination procedure was attempted; however, reaction was very vigorous even upon slow addition of thionyl chloride with excess amount of base present. Likewise by HPLC analysis reaction produced multiple side products and was not pursued any further.



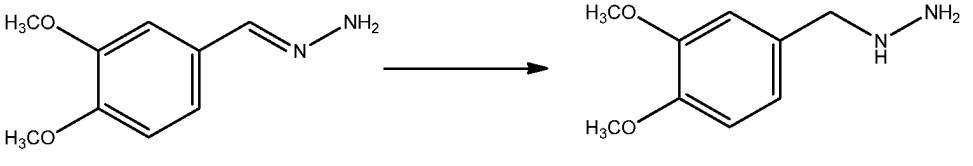
4-formyl-1,2-phenylene diacetate (50). 2 mmol of starting material were dissolved in 10 mL DCM and NEt_3 (4.4 mmol, 2.2 eq.) was added and solution was cooled to 0 °C. To the reaction mixture acetyl chloride (4.2 mmol, 2.1 eq.) dissolved in 20 mL of DCM was added dropwise via addition funnel. Reaction was allowed to stir for 1 hour and progress was monitored by HPLC. Reaction mixture was then diluted with 10 mL of brine and the aqueous layer was extracted with 10 mL of DCM. Combined organic layers were evaporated under reduced pressure to yield the product as clear oil (1.86 mmol, 92%). **^{1}H NMR (300 MHz, DMSO-d_6)** δ 9.99 (s, 1H), 7.90 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.83 (d, *J* = 1.8 Hz, 1H), 7.54 (d, *J* = 8.3 Hz, 1H), 2.32 (d, *J* = 2.0 Hz, 6H).



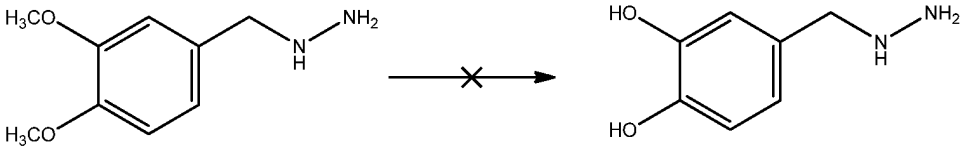
4-(hydroxymethyl)-1,2-phenylene diacetate (51). Reduction of the aldehyde (dissolved in THF instead of MeOH) was attempted according to the standard $NaBH_4$ reduction procedure; however, significant deprotection and side products were observed upon stirring with $NaBH_4$. The reaction was not pursued any further.



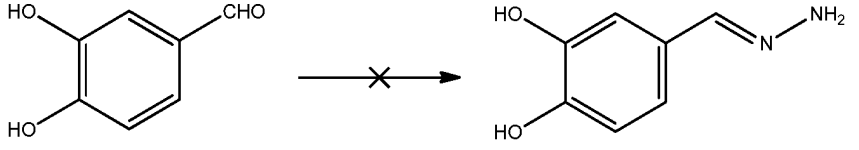
(*E*)-(3,4-dimethoxybenzylidene)hydrazine (37). Starting material (1 mmol) was dissolved in dry THF and cooled to 0 °C. To the reaction mixture (1.1 mmol, 1.1 eq.) of hydrazine was added. After addition dry HCl gas was bubbled through the solution. Formed precipitate was filtered off and dried *in vacuo*. The product was a pale yellow powder (0.65 mmol, 65%). Significant side products were observed by HPLC.



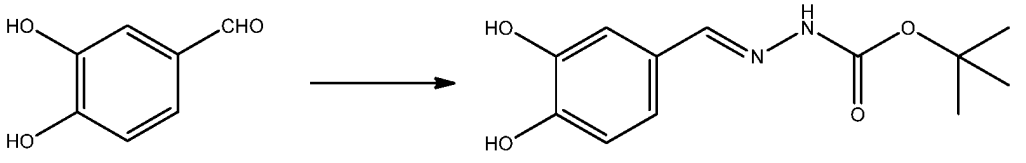
(3,4-dimethoxybenzyl)hydrazine (38). 0.65 mmol of starting material were reacted according to general borane reduction to yield the product as clear oil. Some side products were observed. Further purification was carried out by dissolving the oil in cold Et_2O, and dropwise addition of concentrated HCl until white precipitate formed (0.56 mmol, 86%). **^{1}H NMR (300 MHz, CD_3OD)** δ 7.04 (d, *J* = 14.0 Hz, 2H), 4.13 (s, 1H), 3.88 (s, 2H), 3.88 – 3.84 (m, 2H).



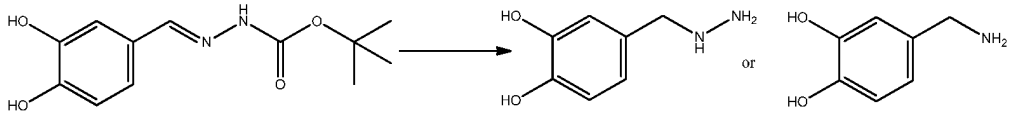
4-(hydrazinylmethyl)benzene-1,2-diol (40a). Deprotection using above described method was attempted; however, significant side products were observed upon reaction with BBr_3 and the reaction was not pursued any further.



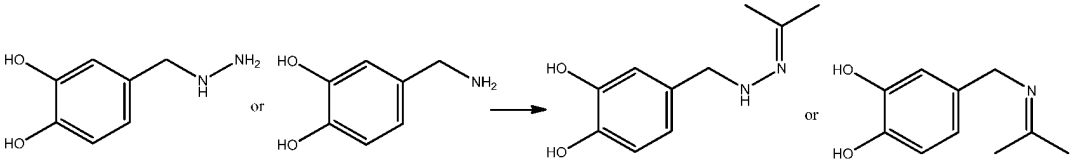
(*E*)-4-(hydrazonomethyl)benzene-1,2-diol. Starting material (1 mmol) was dissolved in dry THF and reaction mixture was cooled to 0 °C. Hydrazine was added (1.1 mmol, 1.1 eq.) to the reaction mixture and HCl gas was bubbled through. Significant side products were observed by HPLC and reaction was not pursued any further.



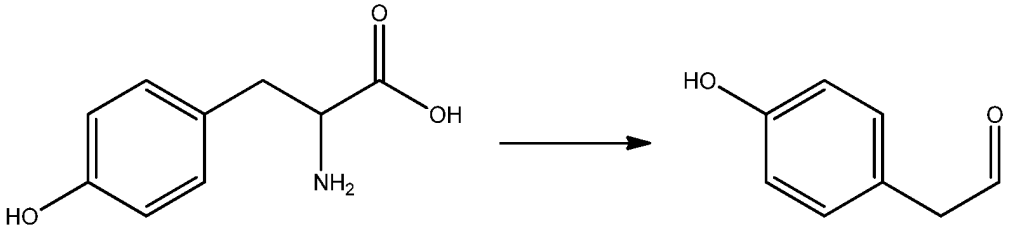
(*E*)-tert-butyl 2-(3,4-dihydroxybenzylidene)hydrazinecarboxylate (39). 2 mmol of starting material were dissolved in minimal MeOH and tert-butyl hydrazinecarboxylate (Boc-hydrazine) (4.2 mmol, 2.1 eq.) was added to the mixture. Reaction was stirred at room temperature and monitored by HPLC. Reaction was deemed to be complete at 2 hours and 0.1 mL of HCl was added. The solution was then concentrated under reduced conditions. Product was clear off-yellow pale oil (1.84 mmol, 92%). **^{1}H NMR (300 MHz, CD_3OD)** δ 7.76 (s, 1H), 7.07 (d, *J* = 1.9 Hz, 1H), 6.86 (dd, *J* = 8.1, 1.9 Hz, 1H), 6.76 (d, *J* = 8.1 Hz, 1H).



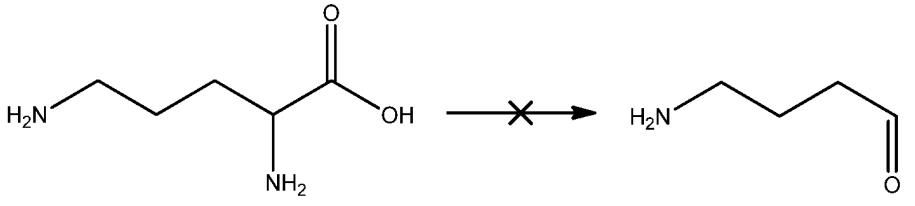
4-(hydrazinylmethyl)benzene-1,2-diol (40a). Following borane reduction procedure 1 mmol of starting material was reacted. After completion the reaction mixture was quenched with concentrated HCl and precipitate was filtered. Product was a white powder (0.91 mmol, 91%). Some side product was observed in the product which was inseparable from the product. **^{1}H NMR (300 MHz, DMSO-d_6)** δ 6.70 (s, 1H), 6.66 (d, *J* = 7.9 Hz, 1H), 6.55 (d, *J* = 7.9 Hz, 1H), 3.57 (s, 2H).



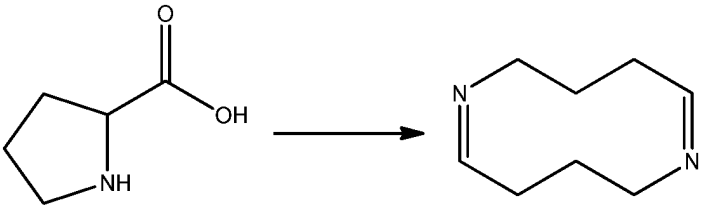
4-((2-(propan-2-ylidene)hydrazinyl)methyl)benzene-1,2-diol (41a). Starting material was dissolved in pure d^6-acetone (0.5 mL) and was allowed to stir for 15 minutes at room temperature. Reaction was then checked by HPLC. Reaction mixture was then used for NMR data. **^{1}H NMR (300 MHz, Acetone-d_6)** δ 8.22 (d, *J* = 18.1 Hz, 1H), 6.37 – 6.03 (m, 3H), 3.54 (d, *J* = 11.2 Hz, 2H).



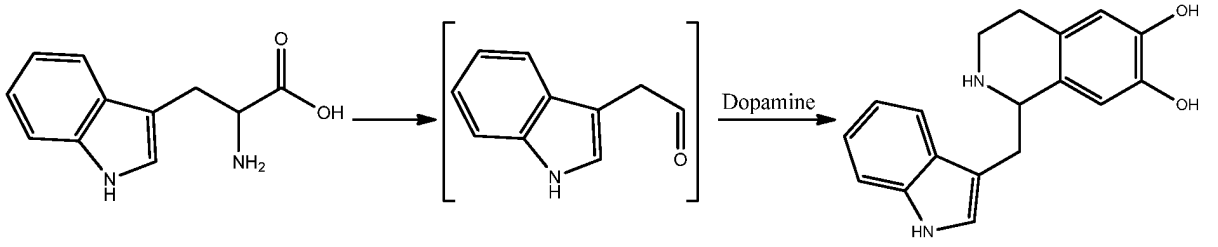
2-(4-hydroxyphenyl)acetaldehyde (42). L-tyrosine (1 mmol) was reacted according to general procedure for bleach oxidation procedure to yield the product as a pale yellow oil (0.66 mmol, 66%), with a distinct rose-like smell. NMR was published in recent publication.



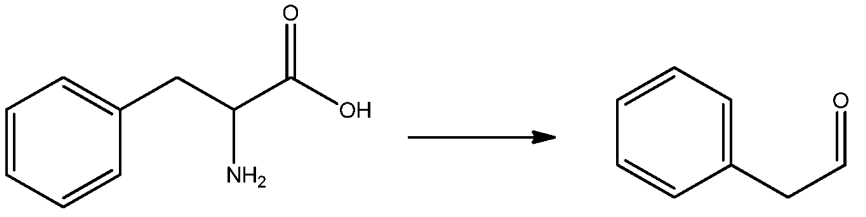
4-aminobutanal (43). L-lysine (1 mmol) was reacted according to general bleach oxidation procedure. Reaction was monitored by HPLC and showed significant side product formation as was expected. It was not pursued any further.



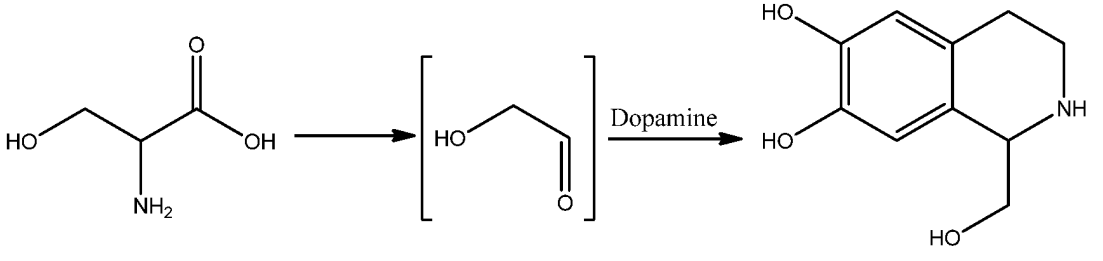
(1*Z*,5*Z*)-2,3,4,7,8,9-hexahydro-1,6-diazecine (44). L-proline (1 mmol) was reacted according to the bleach oxidation procedure. The resulting product was not as was expected and was analyzed by GCMS to reveal the product as dimerized product as well as other polymers.



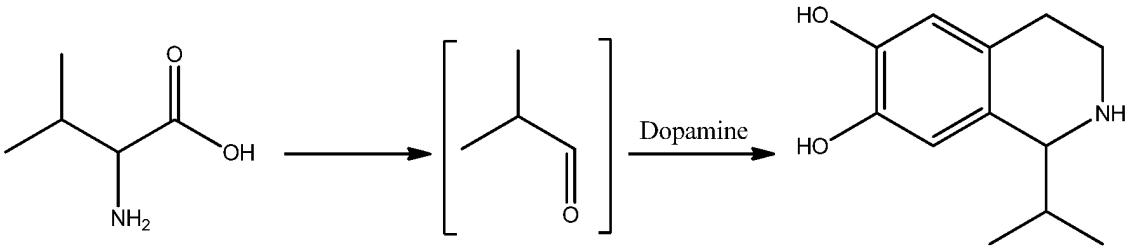
1-((1H-indol-3-yl)methyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (45). L-tryptophan (1 mmol) was reacted according to the tetrahydroisoquinoline synthesis procedure to yield the product (isolated in THF, not EtOAc) as a pale yellow powder. The yellow solid may be further purified by dissolving in minimal methanol, adding 10 volumes of chloroform, and precipitating with diethyl ether to yield a fluffy crystalline solid (0.112g, 38%). NMR was published in recent publication[36].



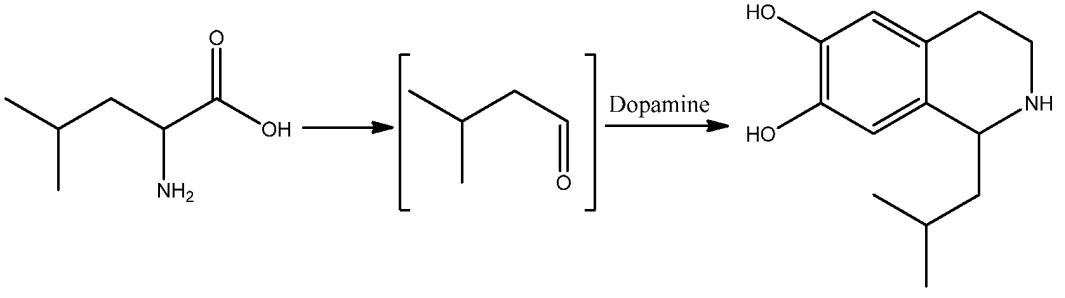
2-phenylacetaldehyde (46). L-phenylalanine (1 mmol) was reacted according to general procedure for bleach oxidation procedure to yield the product as a pale yellow oil (0.71 mmol, 71%). NMR was not obtained. NMR was published in recent publication.



1-(hydroxymethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (47). L-serine was reacted according to the tetrahydroisoquinoline synthesis procedure to yield the product observed by HPLC. However isolation procedures discussed earlier were unsuccessful in extracting the product from aqueous phase.



1-isopropyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol (48). L-valine was reacted according to the tetrahydroisoquinoline synthesis procedure to yield the product observed by HPLC. However isolation procedures discussed earlier were unsuccessful in extracting the product from aqueous phase.



1-isobutyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol (49). L-leucine was reacted according to the tetrahydroisoquinoline synthesis procedure to yield the product observed by HPLC. However isolation procedures discussed earlier were unsuccessful in extracting the product from aqueous phase.

Biochemical Methods

Method for protein purification was previously published in our earlier work[36].

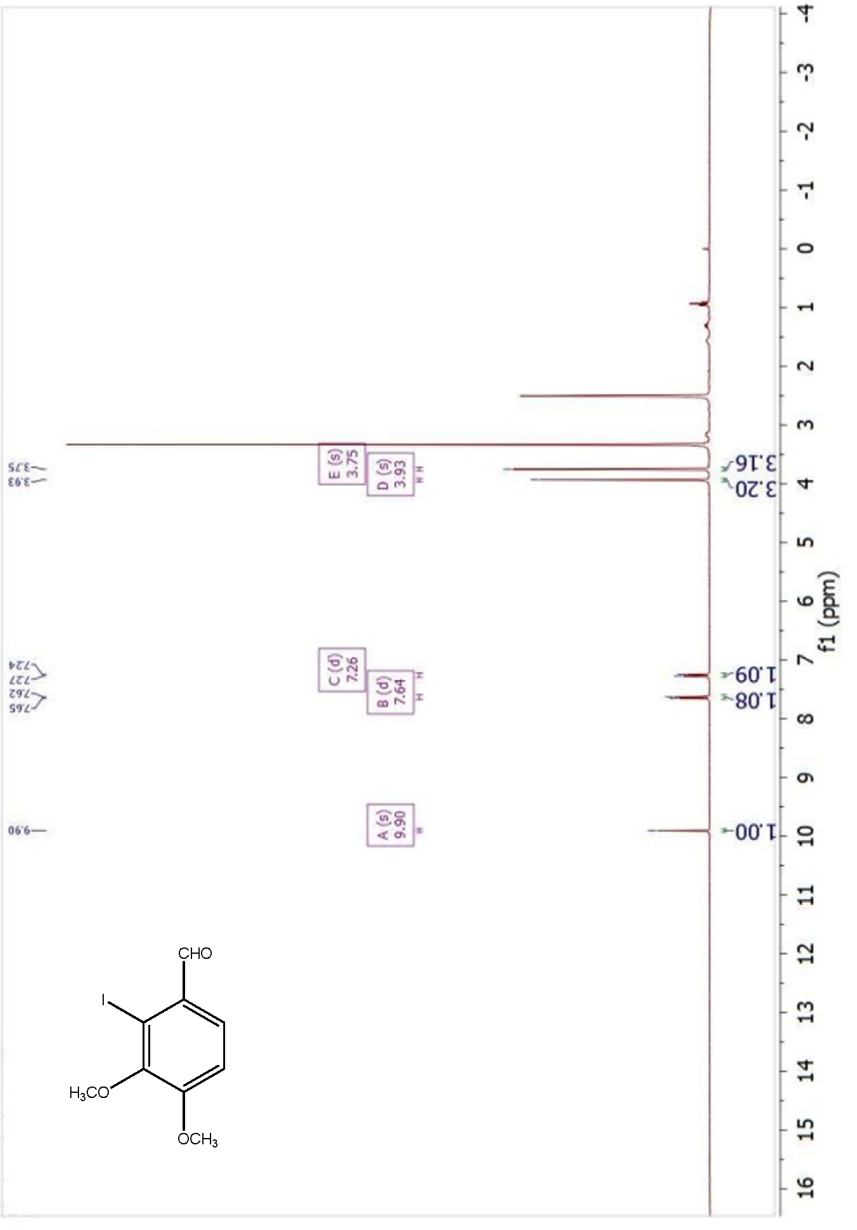
We followed a modification of the procedure described by Luk et al.[29] except using the plasmid construct of Ruff et al.[39] This plasmid contains a codon-optimized sequence of TfNCSΔ19 (5071-5769), *Thalictrum flavum* norcoclaurine synthase with the N-terminal 19 amino acids deleted. This sequence was cloned into the pET-28a(+) vector between the NdeI and EcoRI restriction sites to generate plasmid pNCSa. This plasmid was transformed into BL21(DE3) chemically competent cells (Sigma).

The cells were incubated with shaking at 250 rpm in 50 mL of Luria-Bertani (LB) medium containing BL21(DE3)/pNCSa and 30 µg/mL kanamycin overnight at 37 °C. The overnight cultures were poured into 1.0 L of LB medium containing 30 µg/mL kanamycin and grown at 37 °C with shaking at 250 rpm until an OD_{600} of 0.6 was reached. Cells were induced for overexpression by addition of 238 mg (1 mM) of isopropyl-β-D-galactopyranoside (IPTG), and the cultures were allowed to continue growth at 25 °C until an OD_{600} of 3.0 - 4.0 was reached (~24 h). Cells were harvested by centrifugation at 4000 rcf for 20 min and re-suspended in binding buffer (50 mM phosphate, 20 mM imidazole, 300 mM NaCl at pH 8.0). The cells were lysed by sonication at 250 Watts for 120 sec in cycles of 10 seconds on and 10 seconds of cool down. The cell lysate was clarified by centrifugation at 20,000 rcf for 25 min. The supernatant was drawn through a 10 mL syringe with 22 gauge needle 80 times to shear DNA and filtered through a 0.22 µm membrane filter. A 1 mL GE Healthcare HisTrap FF nickel affinity column was washed with 10 column volumes (CV) of water, 10 CV of binding buffer (50 mM phosphate, 20 mM imidazole, 300 mM NaCl at pH 8.0) to prime the column. The filtered cell lysate was loaded at a rate of 1 mL/min. Binding buffer (10 CV) was passed through the column at 1 mL/min. A wash with wash buffer (20 mM imidazole, 500 mM NaCl at pH 7.8) was used to remove nonspecifically bound proteins until no more flow-through protein eluted, as determined by measurement of A_{280} (~50 CV). Histidine-tagged protein was eluted with 21 CV of elution buffer (300 mM imidazole and 500 mM NaCl at pH 7.0). The enzyme was concentrated by ultrafiltration (Amicon Ultra-4, 10 000 MWCO) to a volume of 500 µL. This solution diluted into equal volume glycerol to yield 3 -7 mg/mL as determined by Bradford assay. The solution was divided into 500 µL aliquots and stored at -20 °C without freezing and with no noticeable loss of activity after several months. The enzyme was not allowed to warm before use.

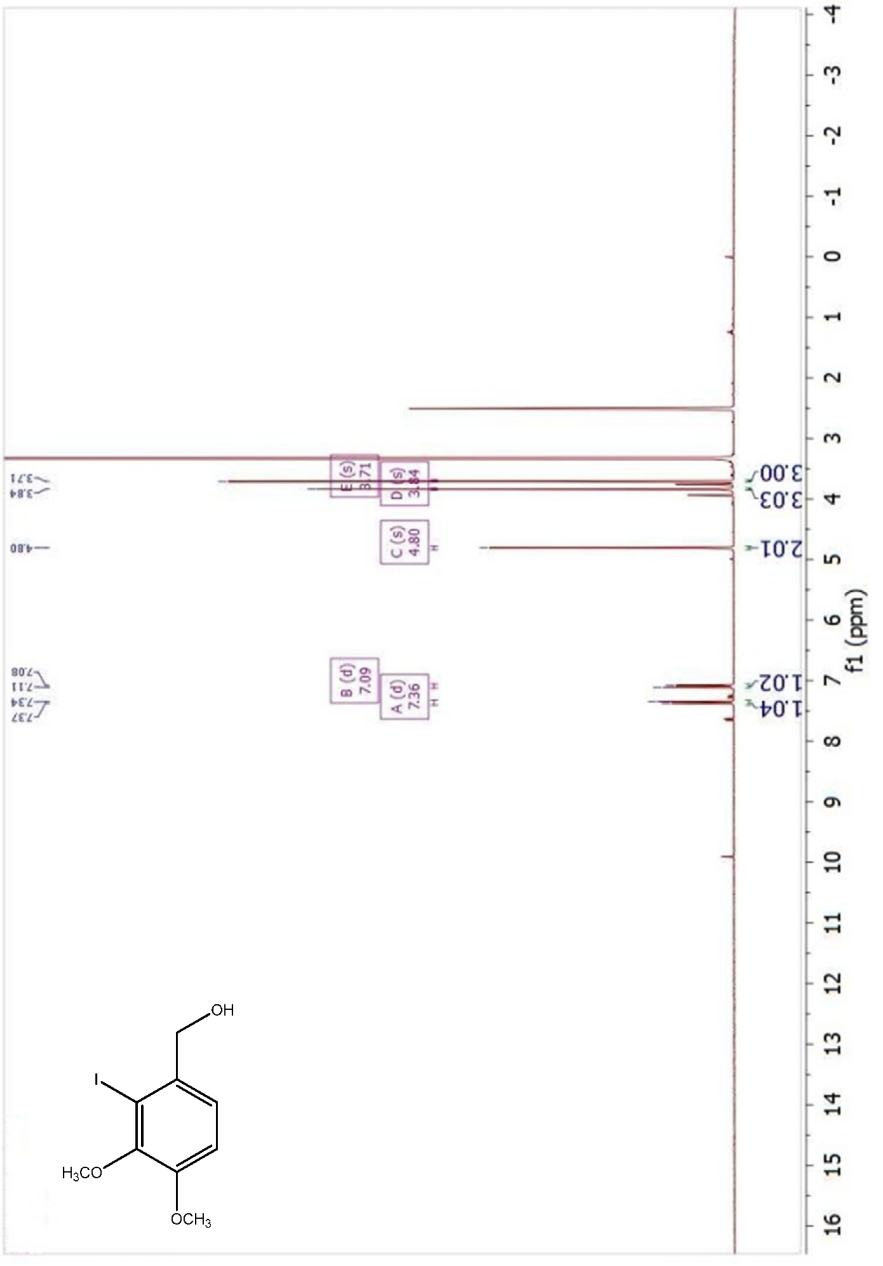
Appendix B – NMR and HPLC data

NMR

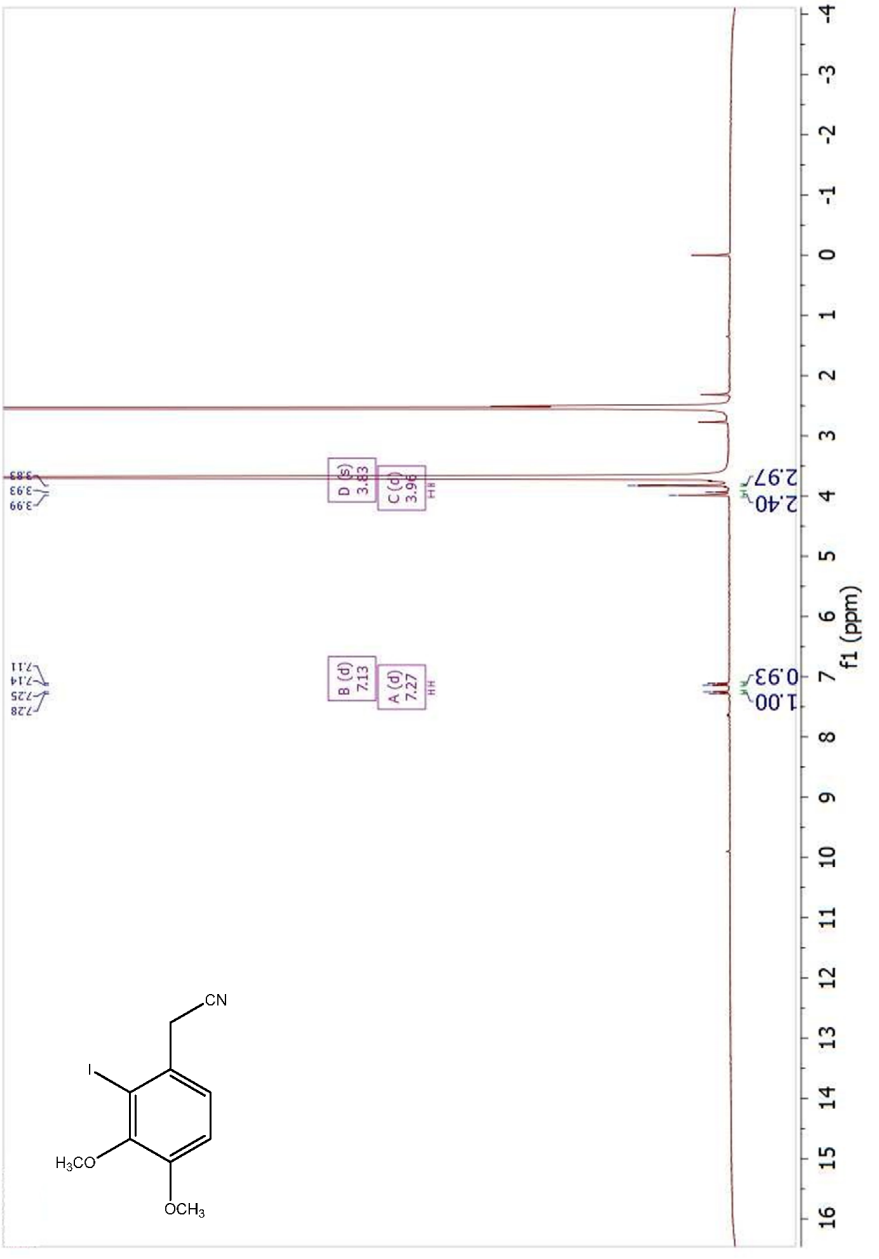
Halogenated Dopamine Analog NMR



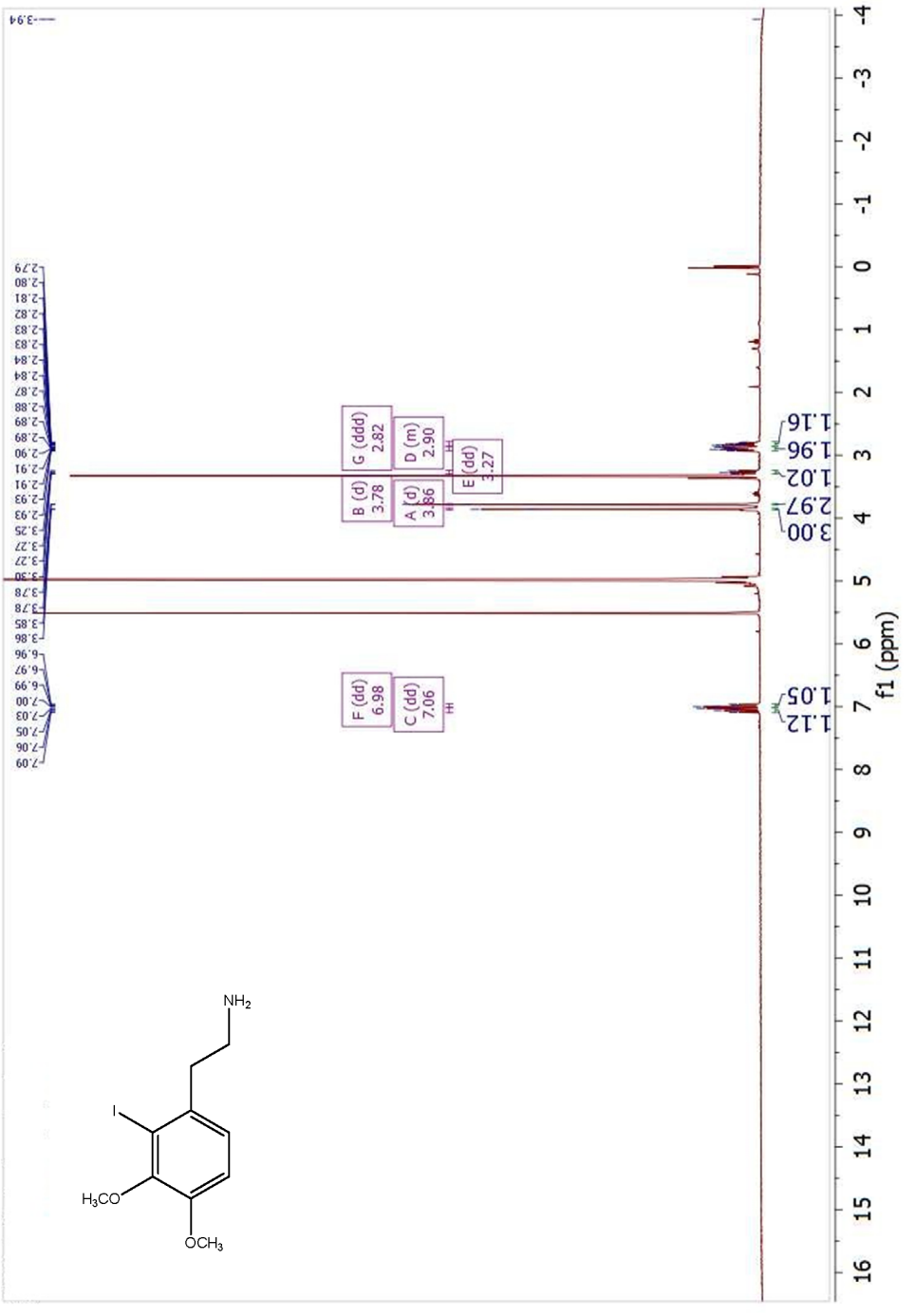
2-Iodo-3,4-dimethoxybenzaldehyde (4). DMSO and H_2O as well as minor other solvent peaks are present.



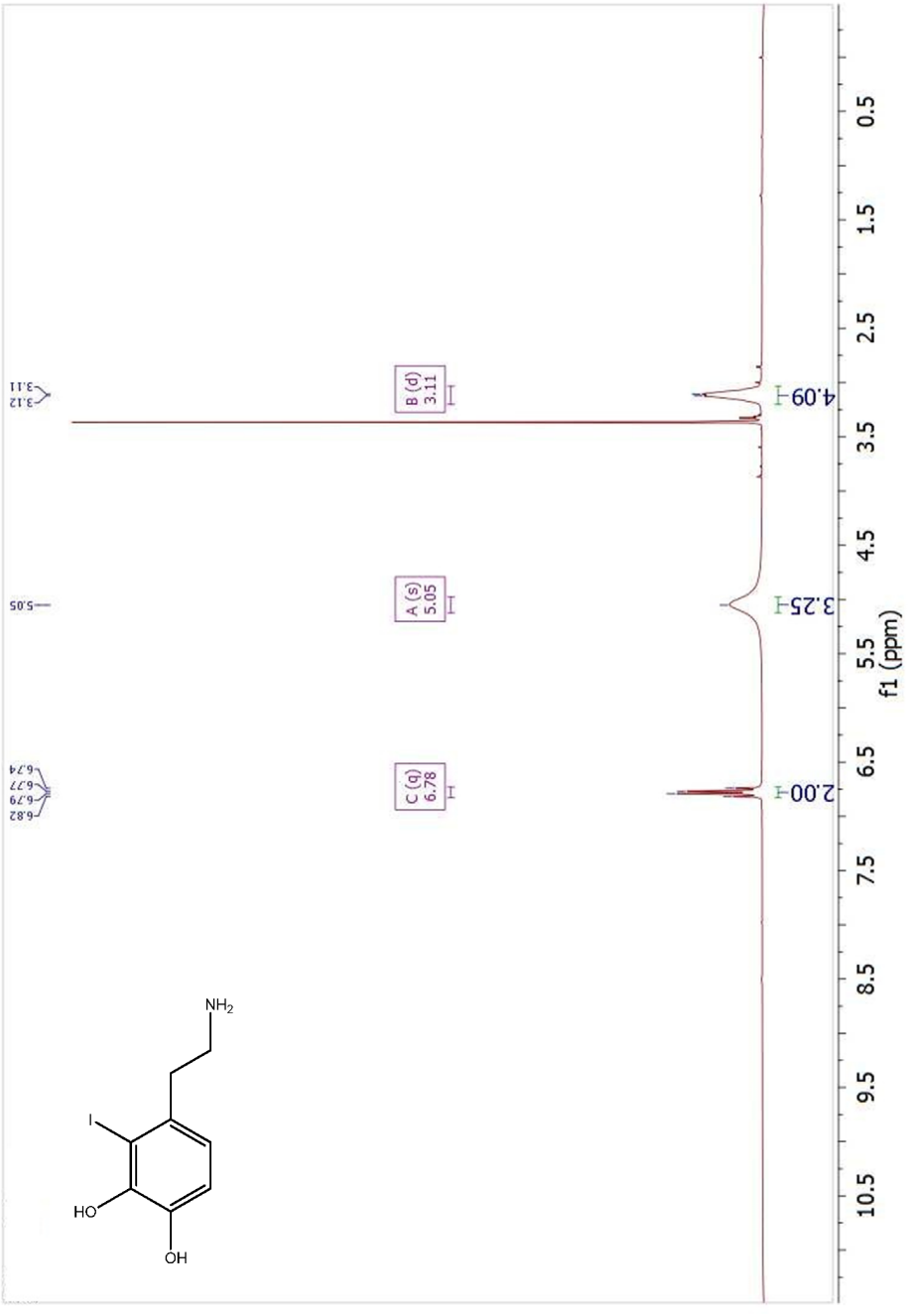
(2-iodo-3,4-dimethoxyphenyl)chloromethane (8). DMSO and H_2O as well as minor other solvent peaks are present.



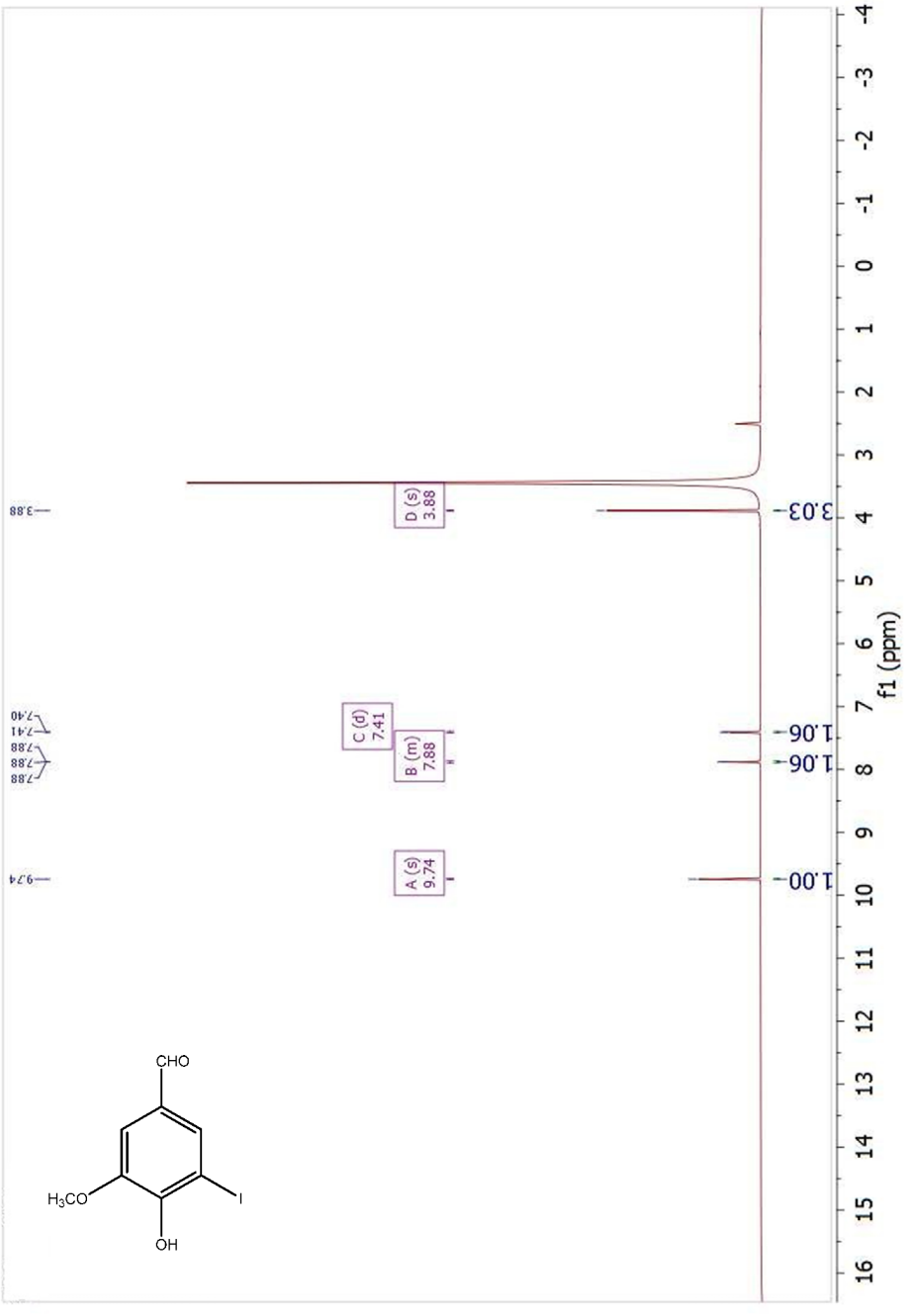
(2-iodo-3,4-dimethoxyphenyl)acetonitrile (10). DMSO and H_2O as well as minor other solvent peaks are present.



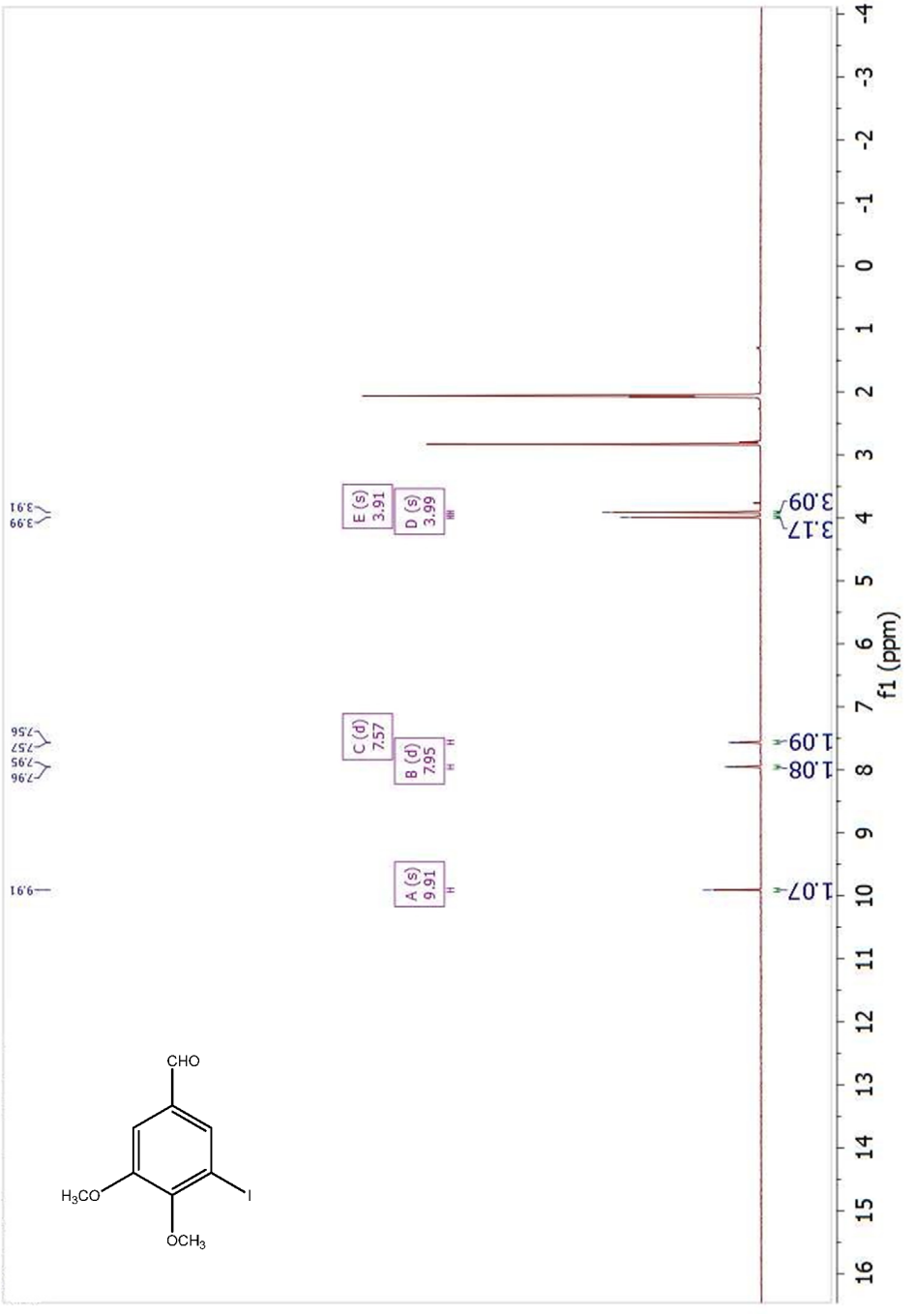
2-iodo-3,4-dimethoxy-phenethylamine (12). MeOD and H_2O, DCM and other solvent peaks are present.



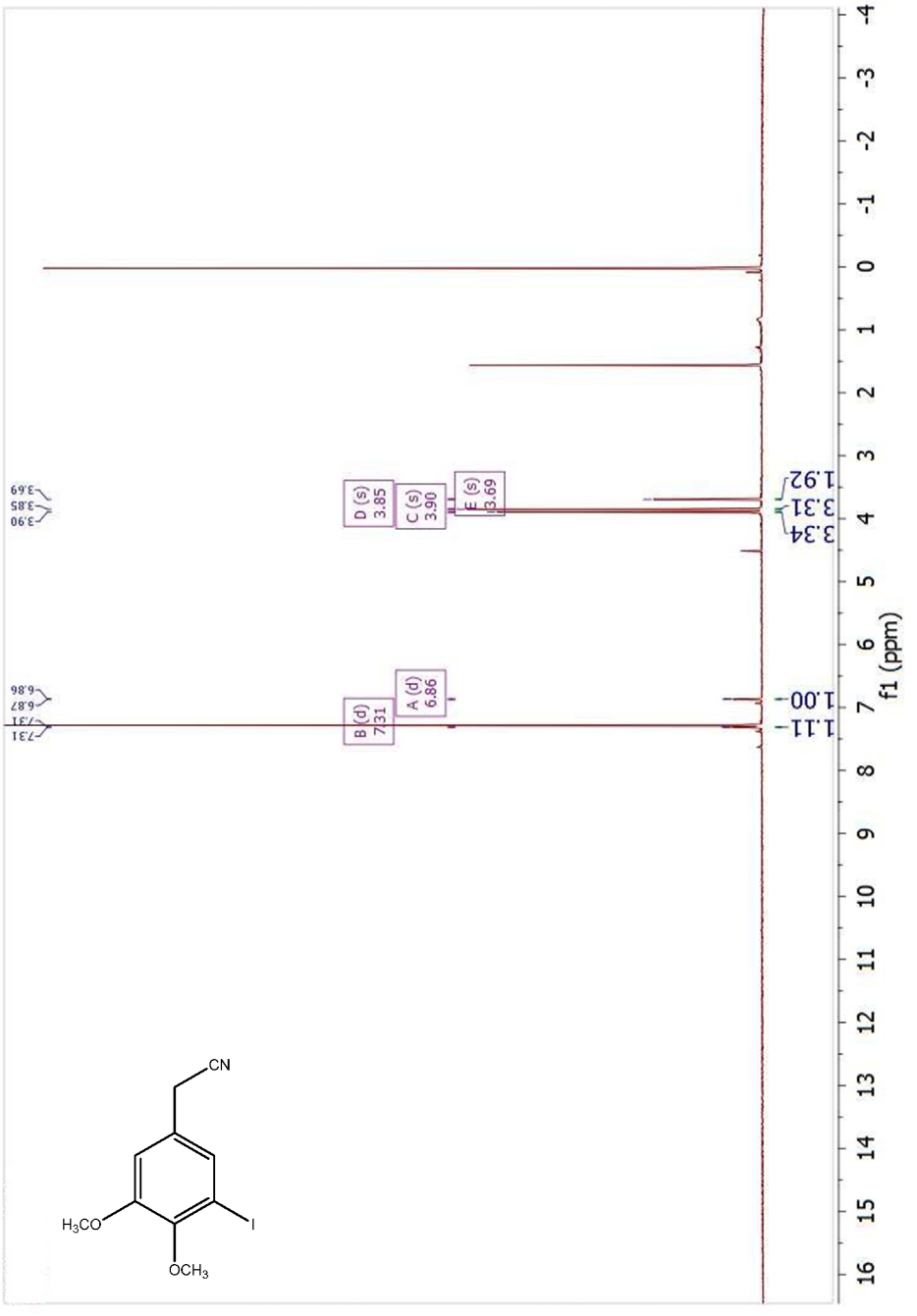
2-iodo-dopamine (14). MeOD is present. H_2O was not present because MeOD was dried in 3 Å sieves.



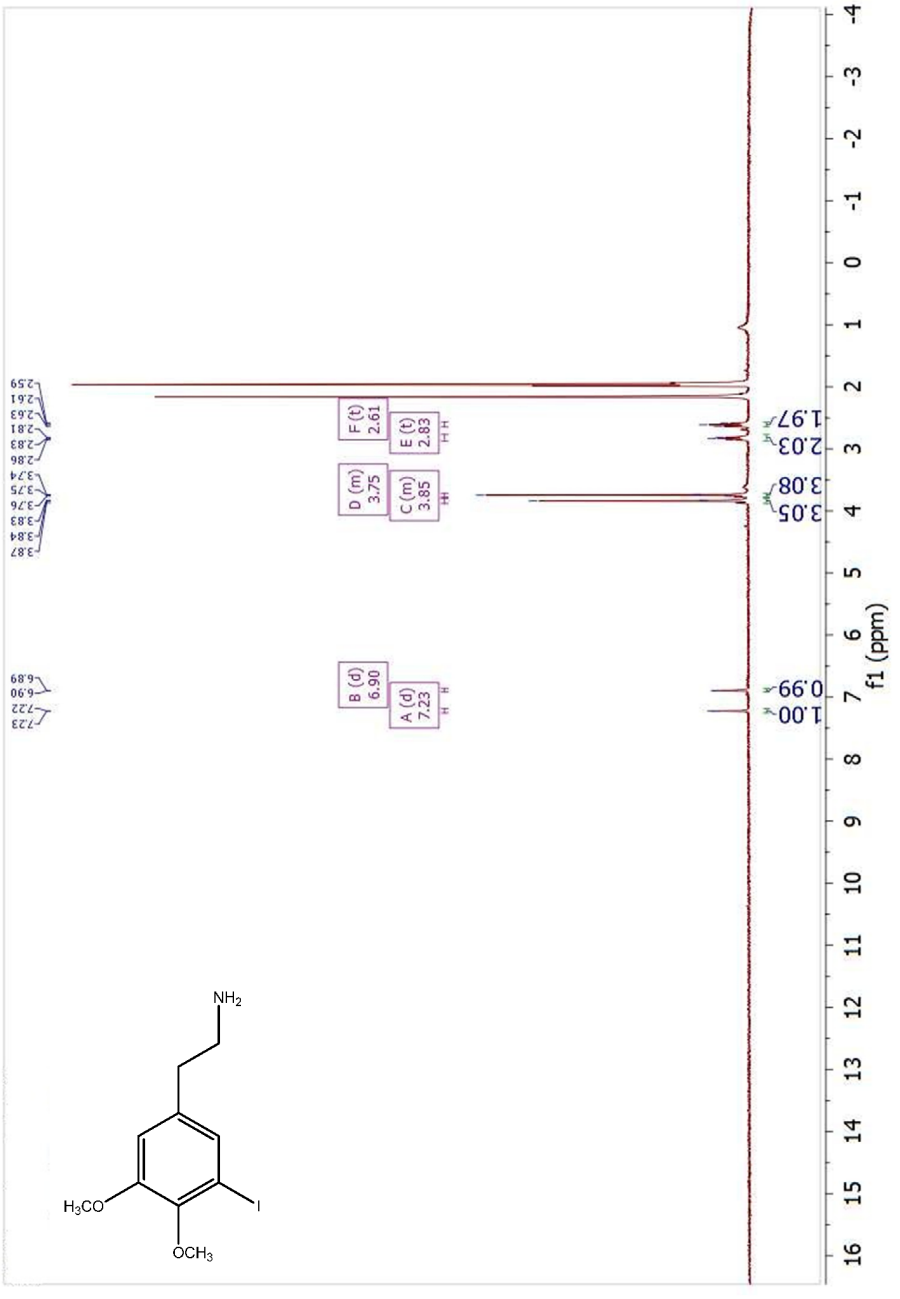
5-Iodo-vanillin (3). DMSO and H_2O solvent peaks are present.



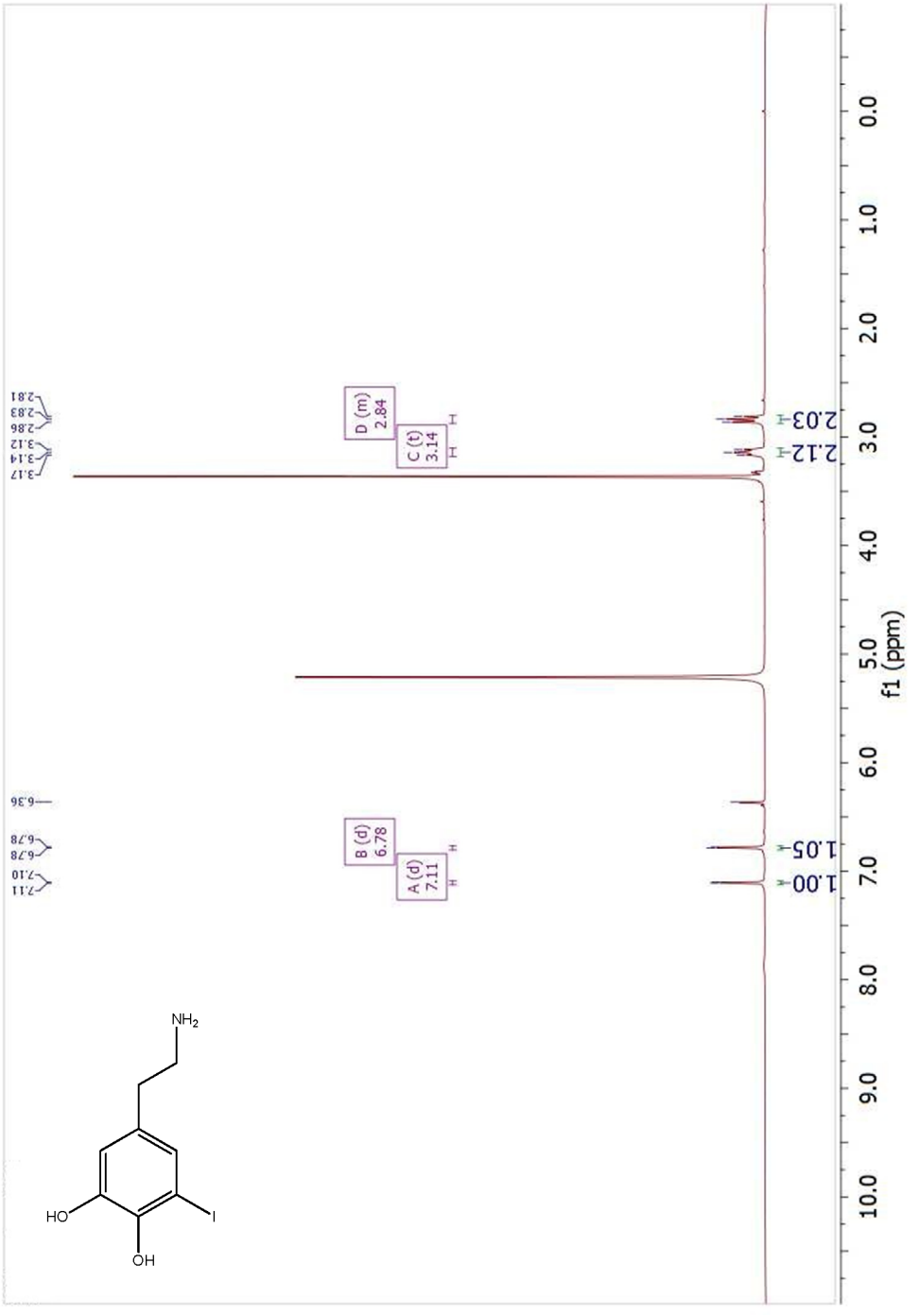
5-Iodo-3,4-dimethoxybenzaldehyde (3). DMSO and H_2O solvent peaks are present.



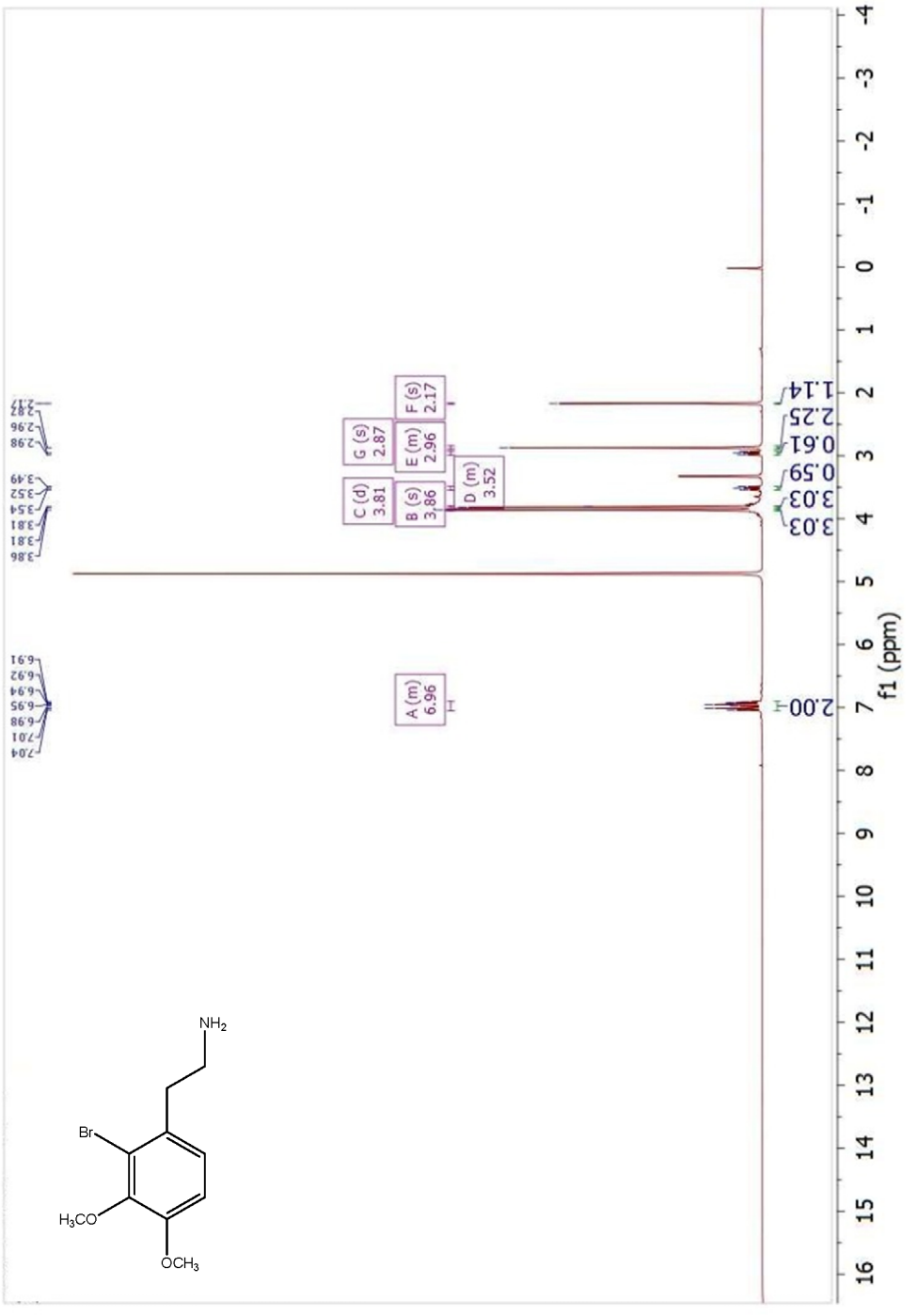
(5-iodo-3,4-dimethoxyphenyl)acetonitrile (9). $CDCl_3$, H_2O, and TMS standard solvent peaks are present.



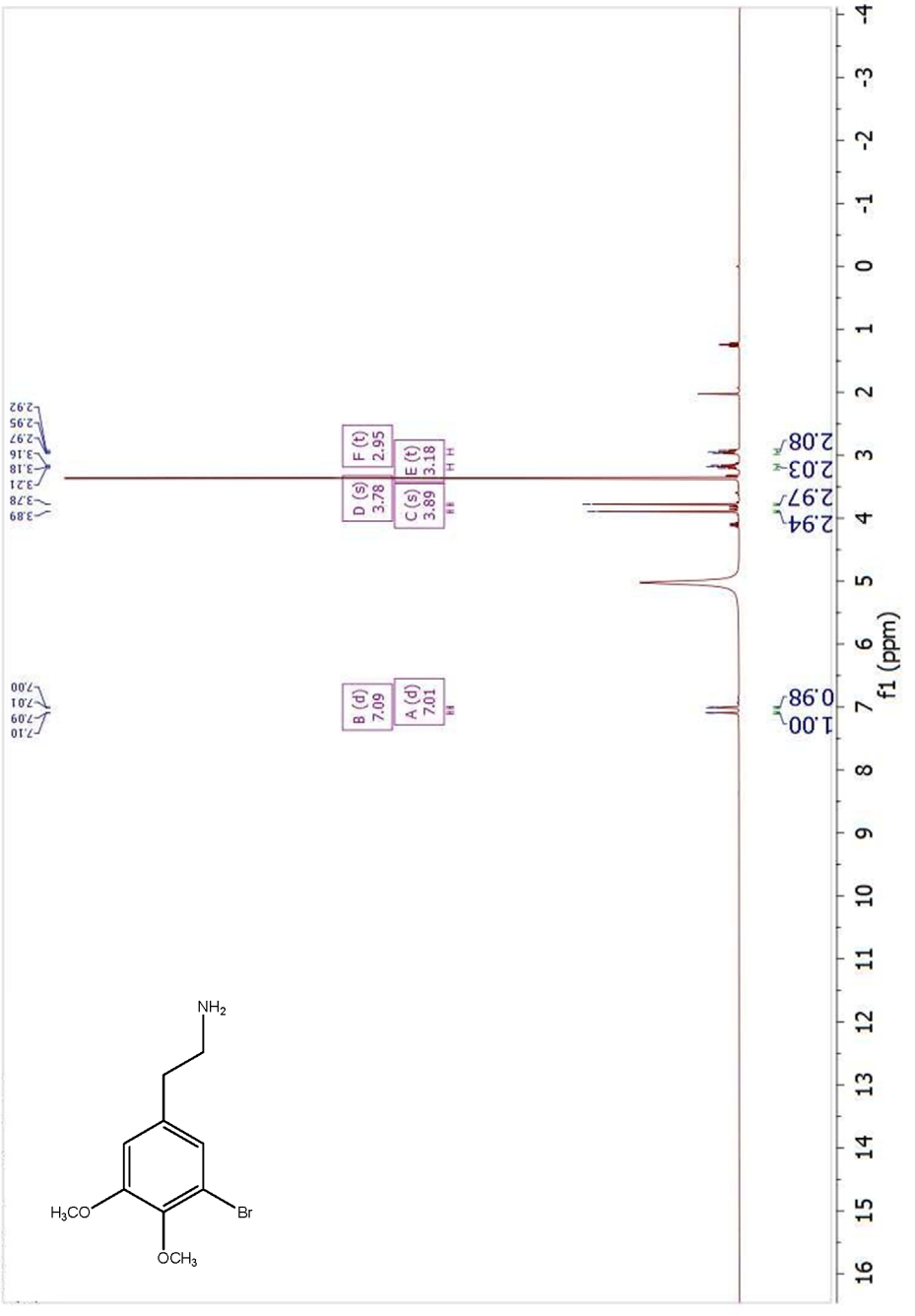
5-iodo-3,4-dimethoxy-phenethylamine (11). Acetonitrile and H_2O solvent peaks are present.



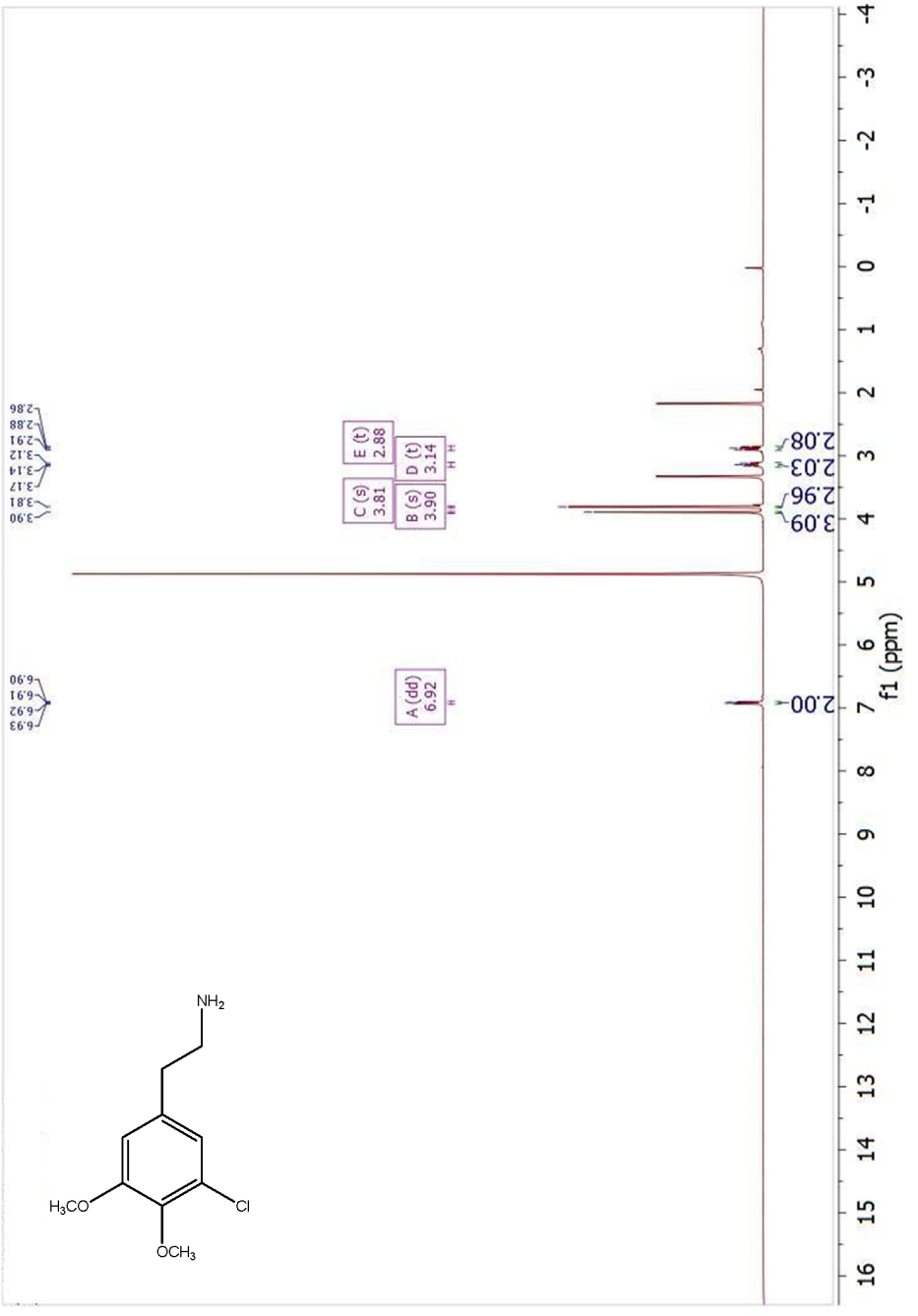
5-iodo-dopamine (13). MeOD and H_2O solvent peaks are present.



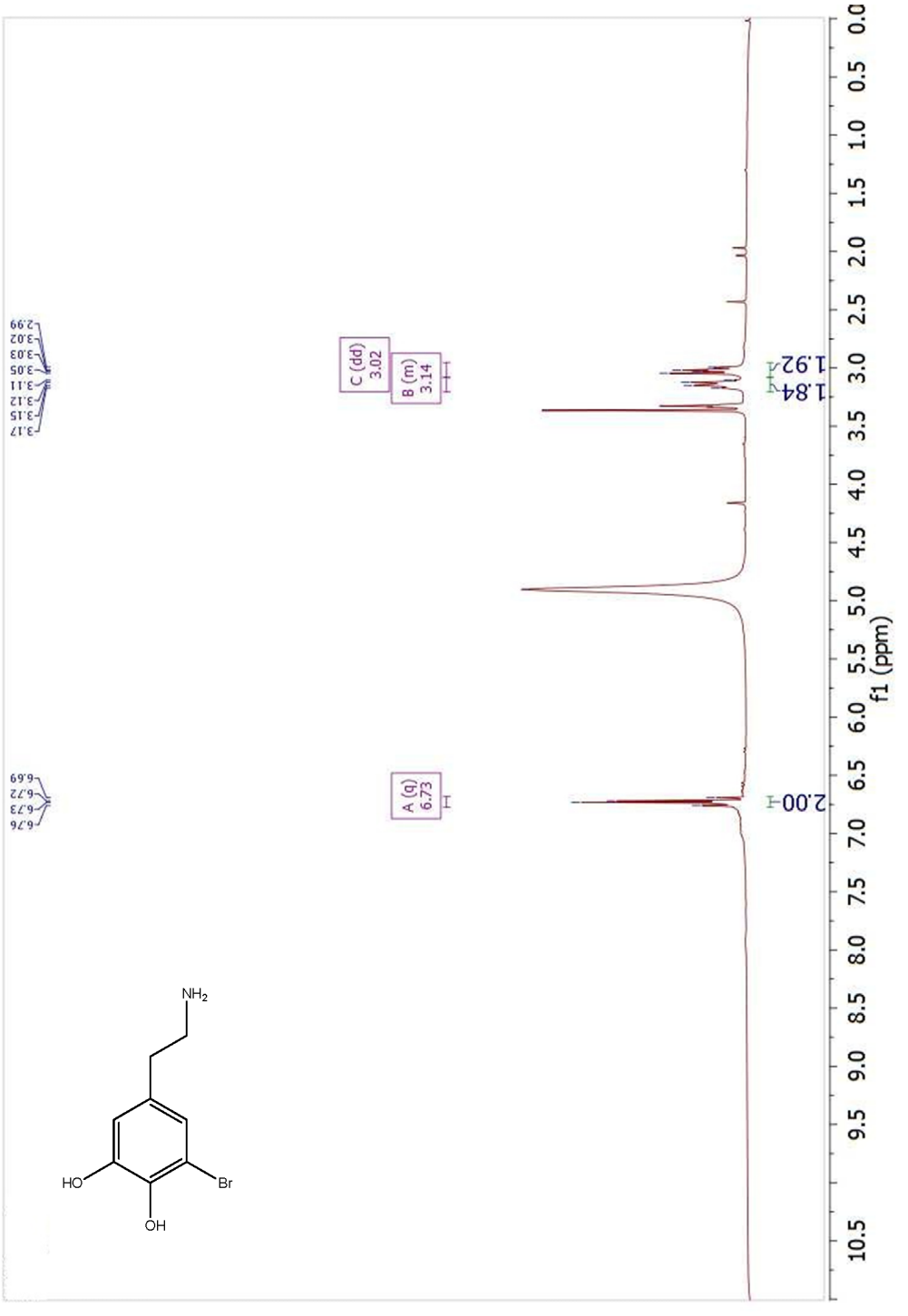
2-bromo-3,4-dimethoxy-phenethylamine (18). MeOD and H_2O as well as TMS solvent peaks are present.



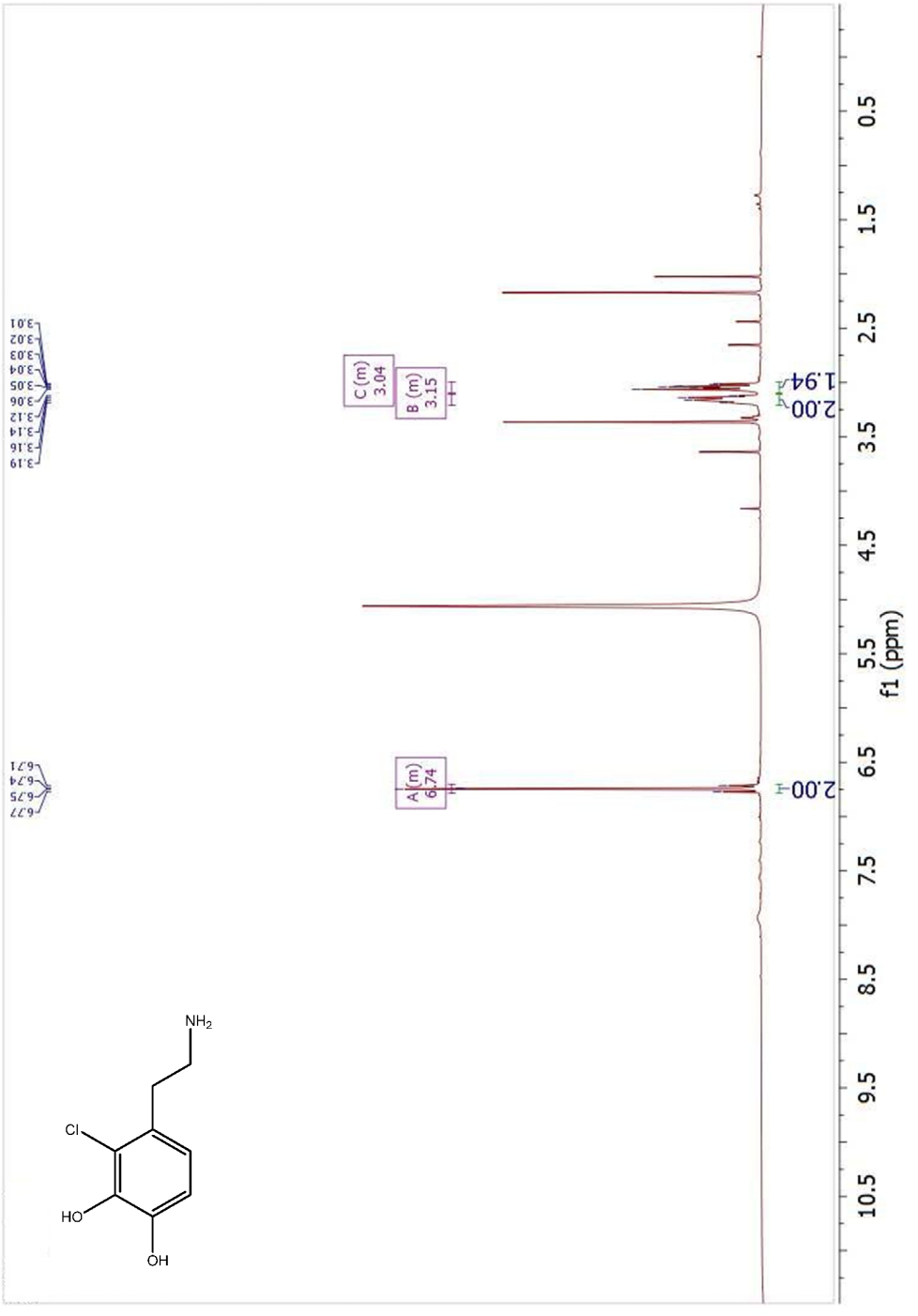
5-bromo-3,4-dimethoxy-phenethylamine (17). MeOD, H_2O, and EtOAc solvent peaks are present.



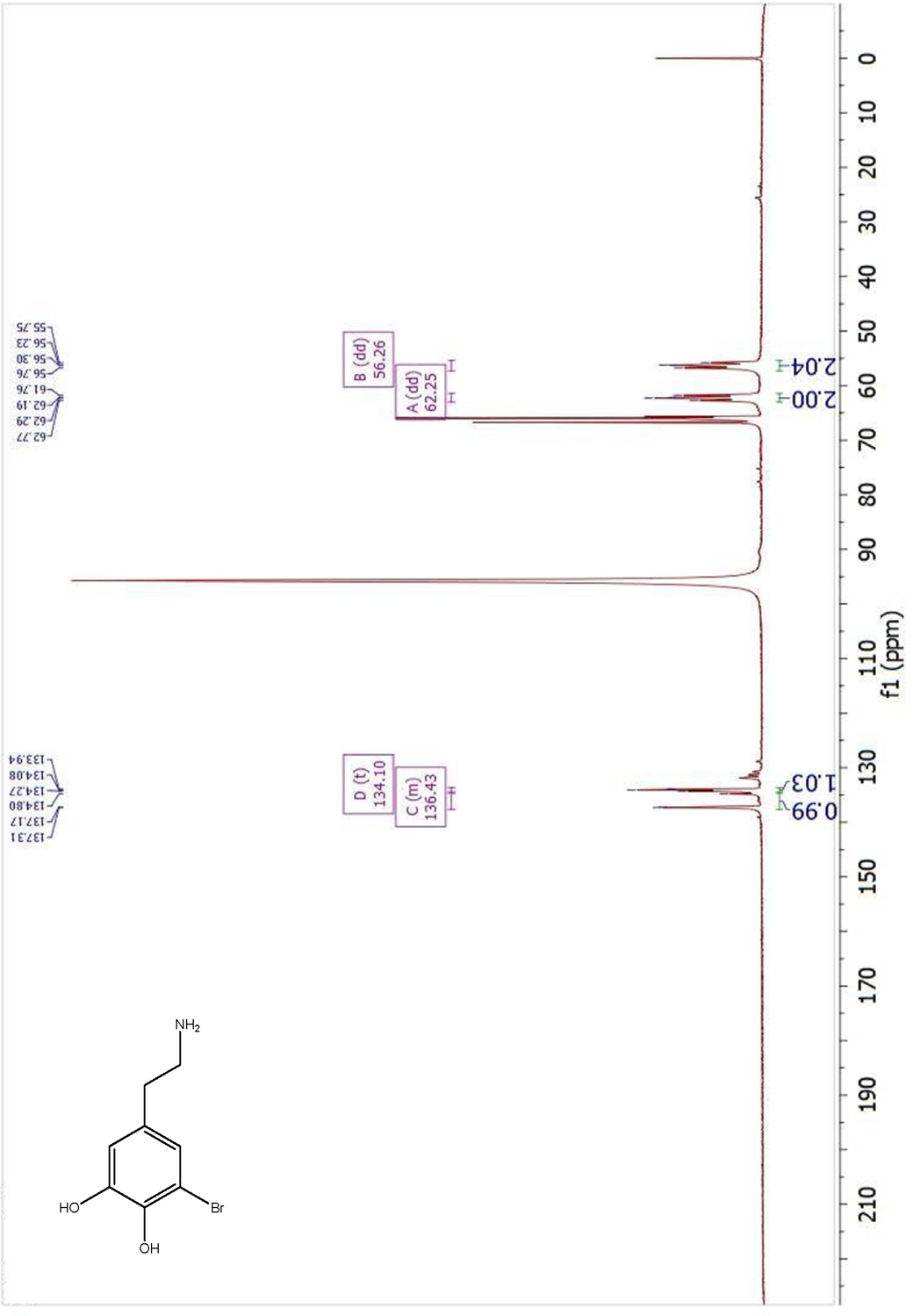
5-chloro-3,4-dimethoxy-phenethylamine (16). MeOD, H_2O, and EtOAc solvent peaks are present.



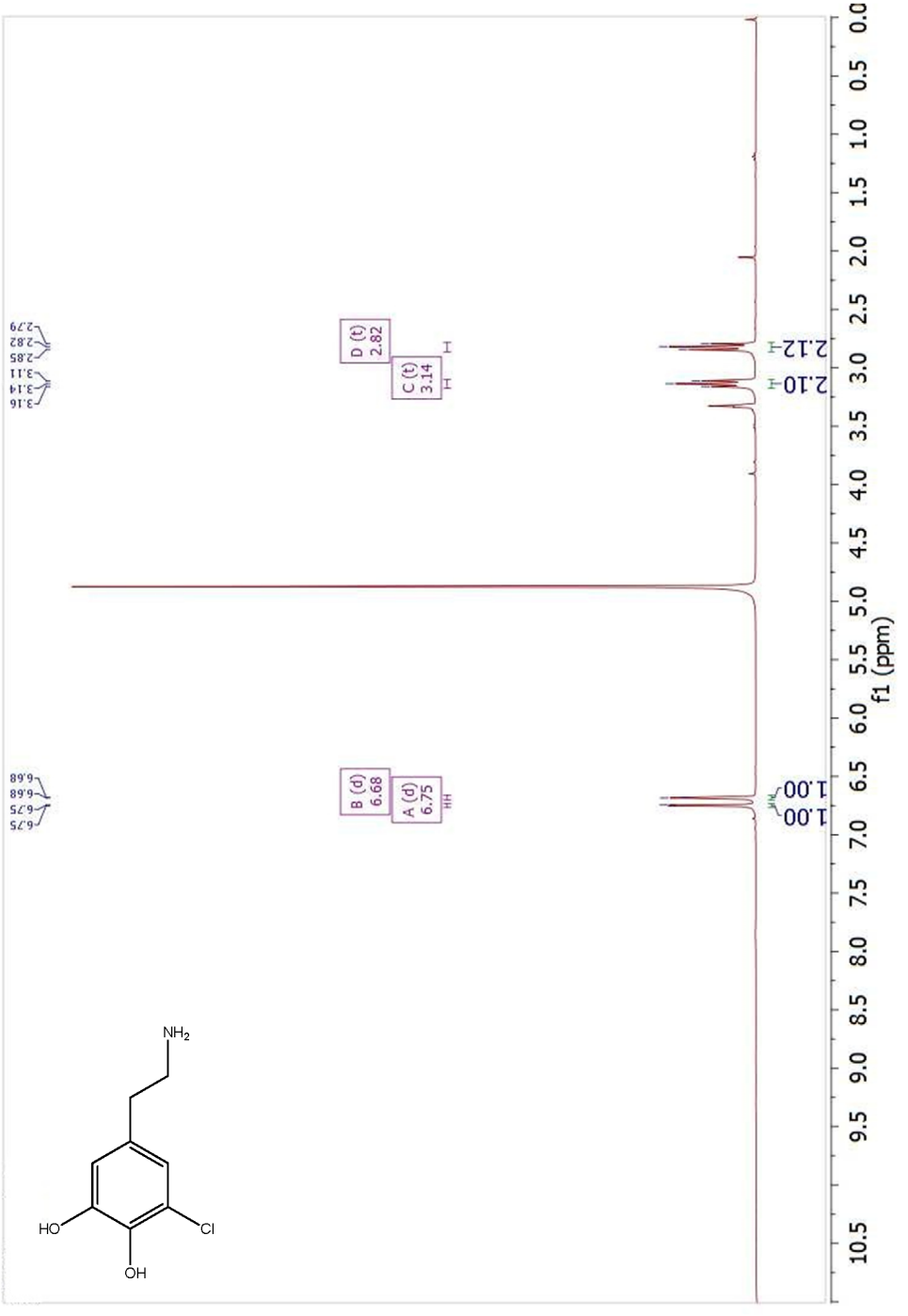
2-bromo-dopamine (22). MeOD and H_2O solvent peaks are present.



2-chloro-dopamine (19). MeOD, H_2O, EtOH, Acetone, and ACN solvent peaks are present.

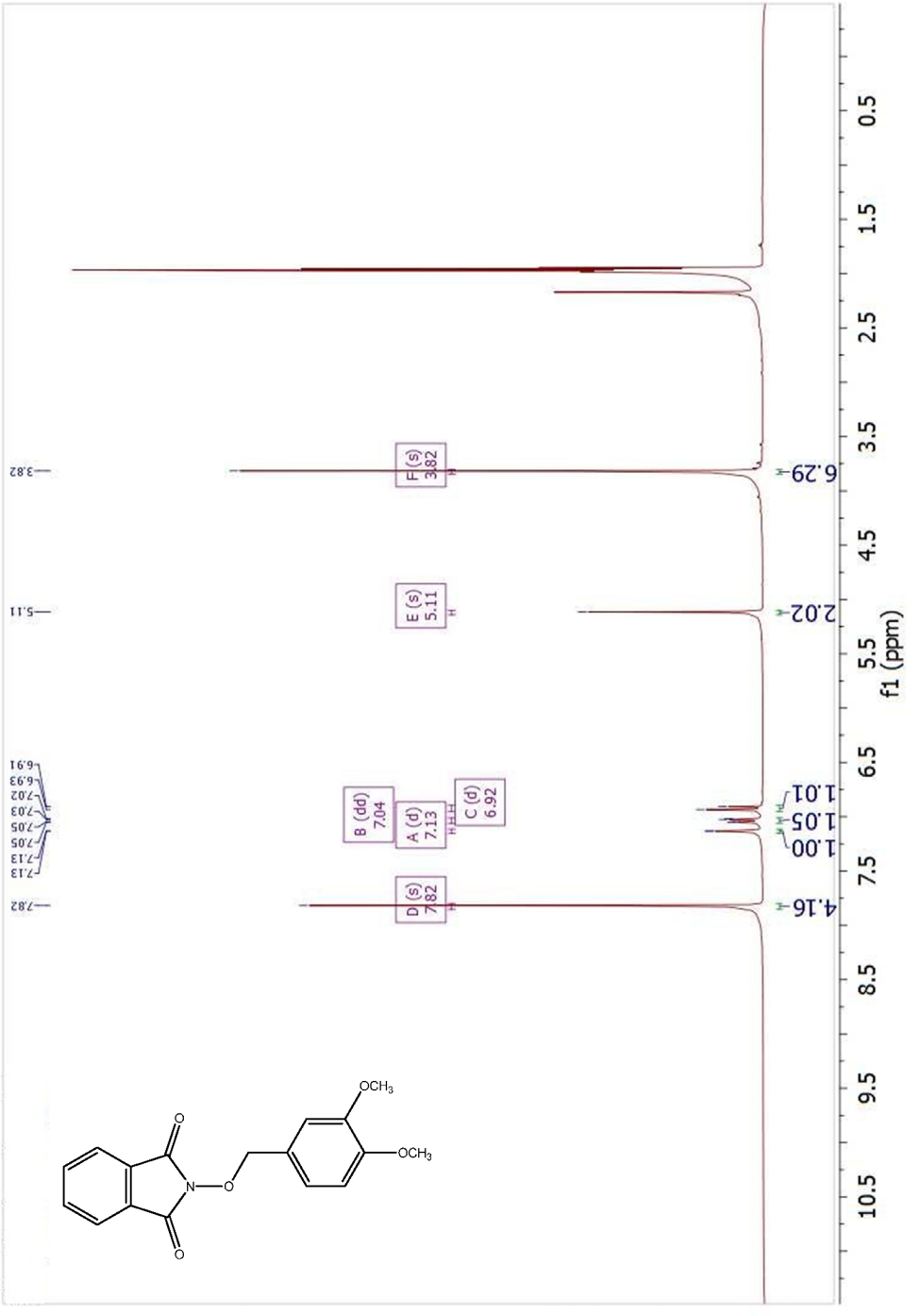


5-bromo-dopamine (21). Spectra data is corrupted and is showing ppm shift of a carbon NMR. Spectra was taken in MeOD, showing MeOD, H_2O, and TMS solvent peaks.

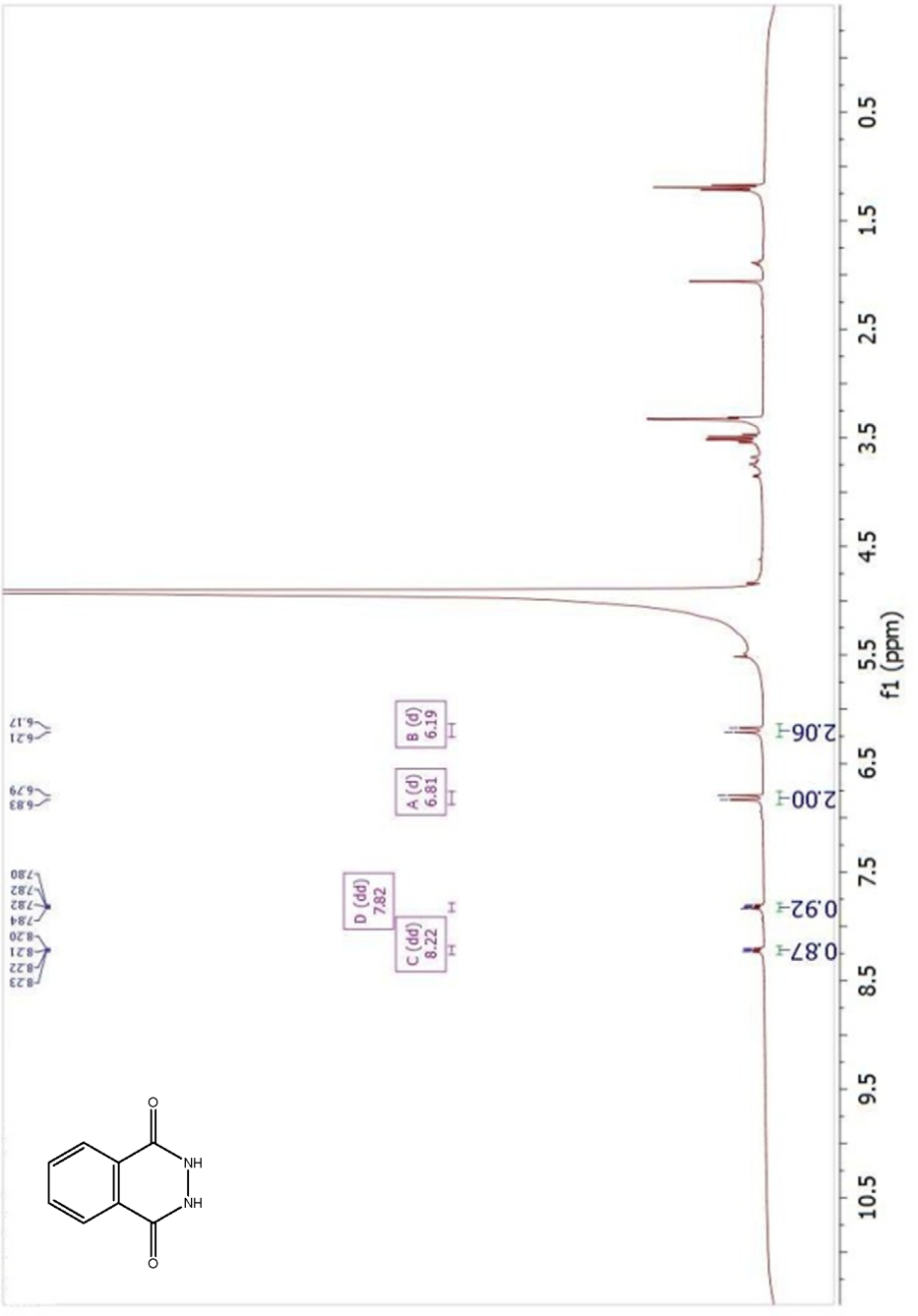


5-chloro-dopamine (20). MeOD and H_2O solvent peaks are present.

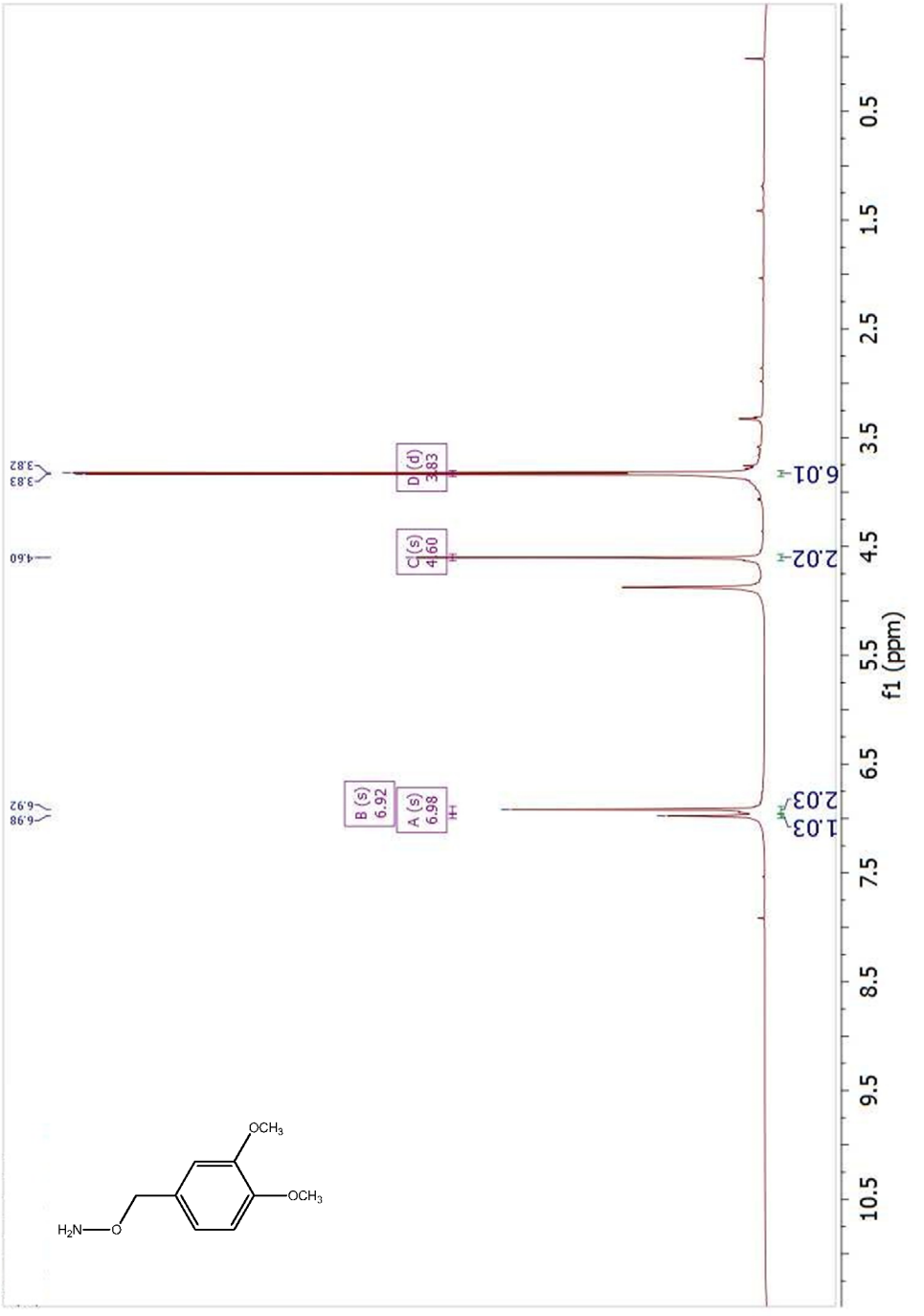
O-hydroxylamine Dopamine Analog NMR



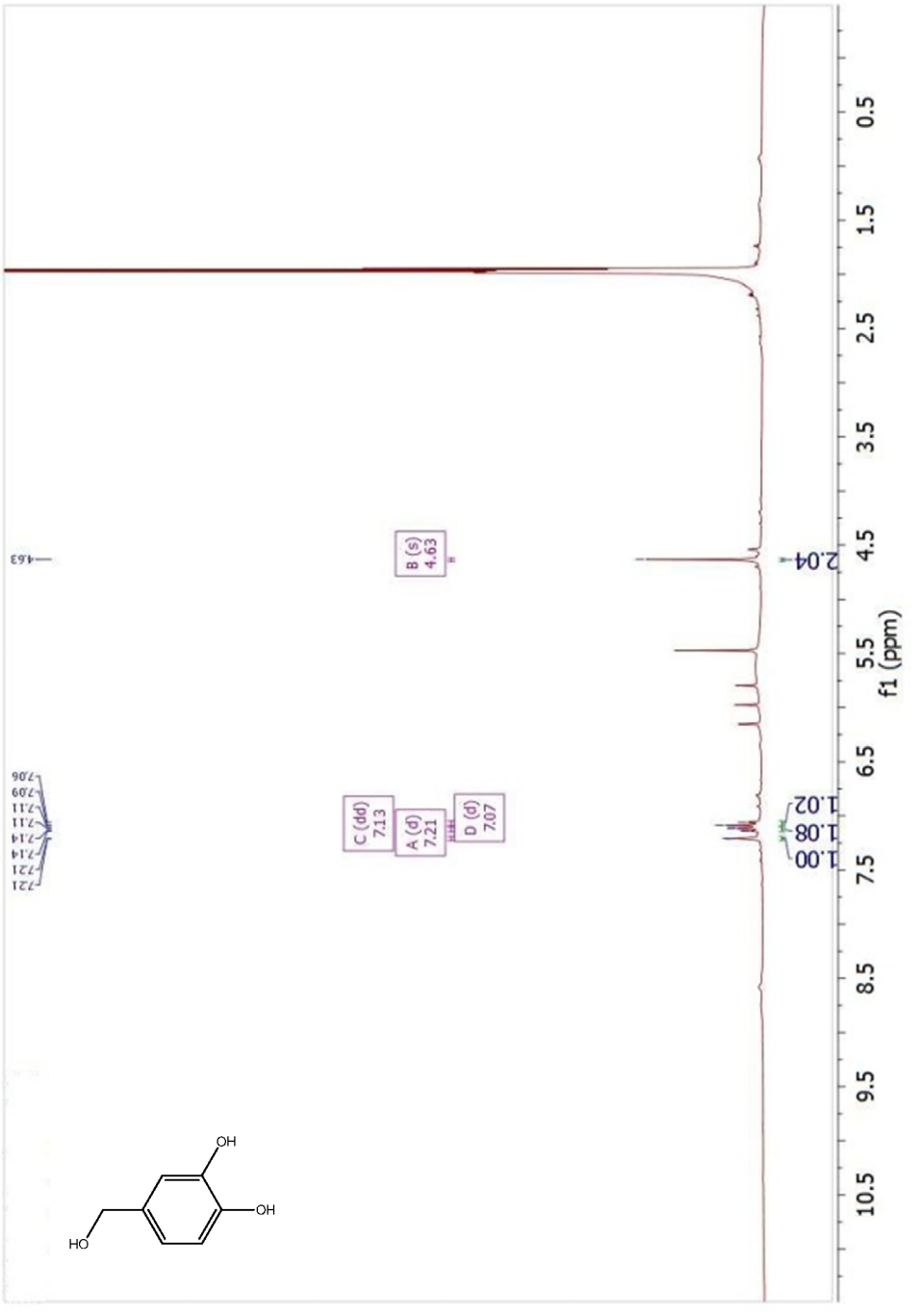
2-((3,4-dimethoxybenzyl)oxy)isoindoline-1,3-dione (30a). Acetone and H_2O solvent peaks are present.



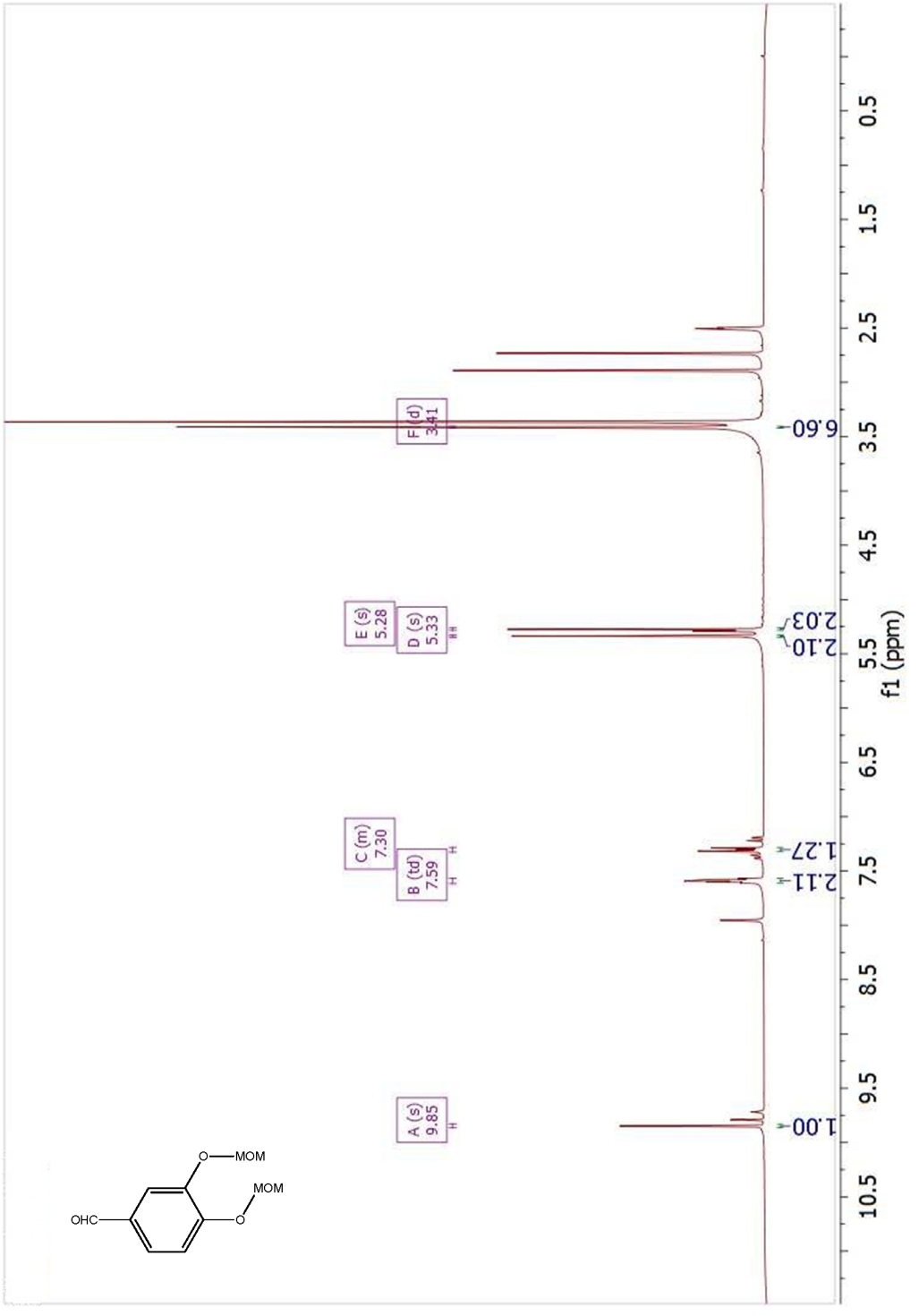
2,3-dihydrophthalazine-1,4-dione (31b). MeOD, H_2O, and other solvent peaks are present. This is the NMR for the byproduct of the hydrazinolysis reaction.



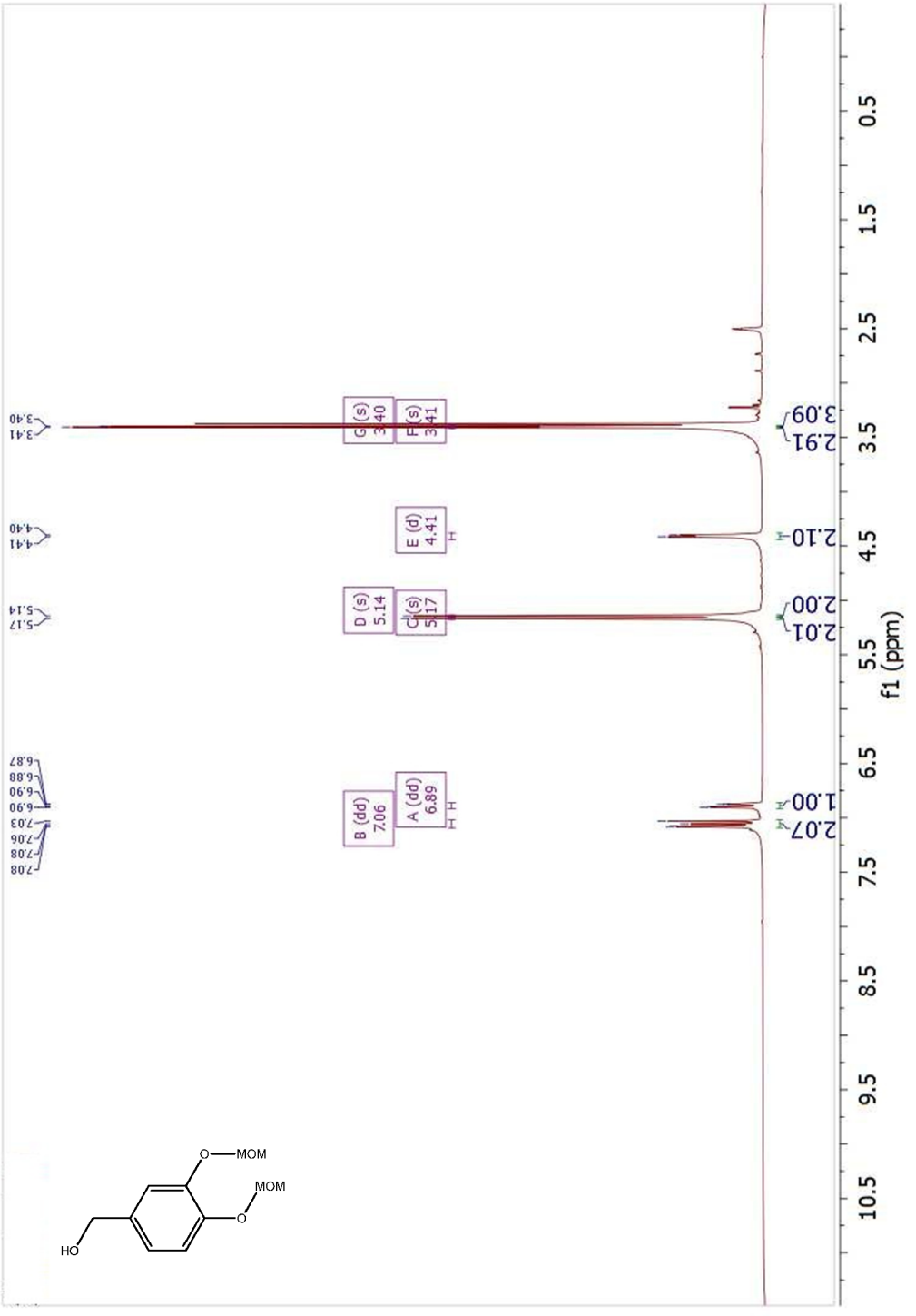
O-(3,4-dimethoxybenzyl)hydroxylamine (31a). MeOD and H_2O solvent peaks are present.



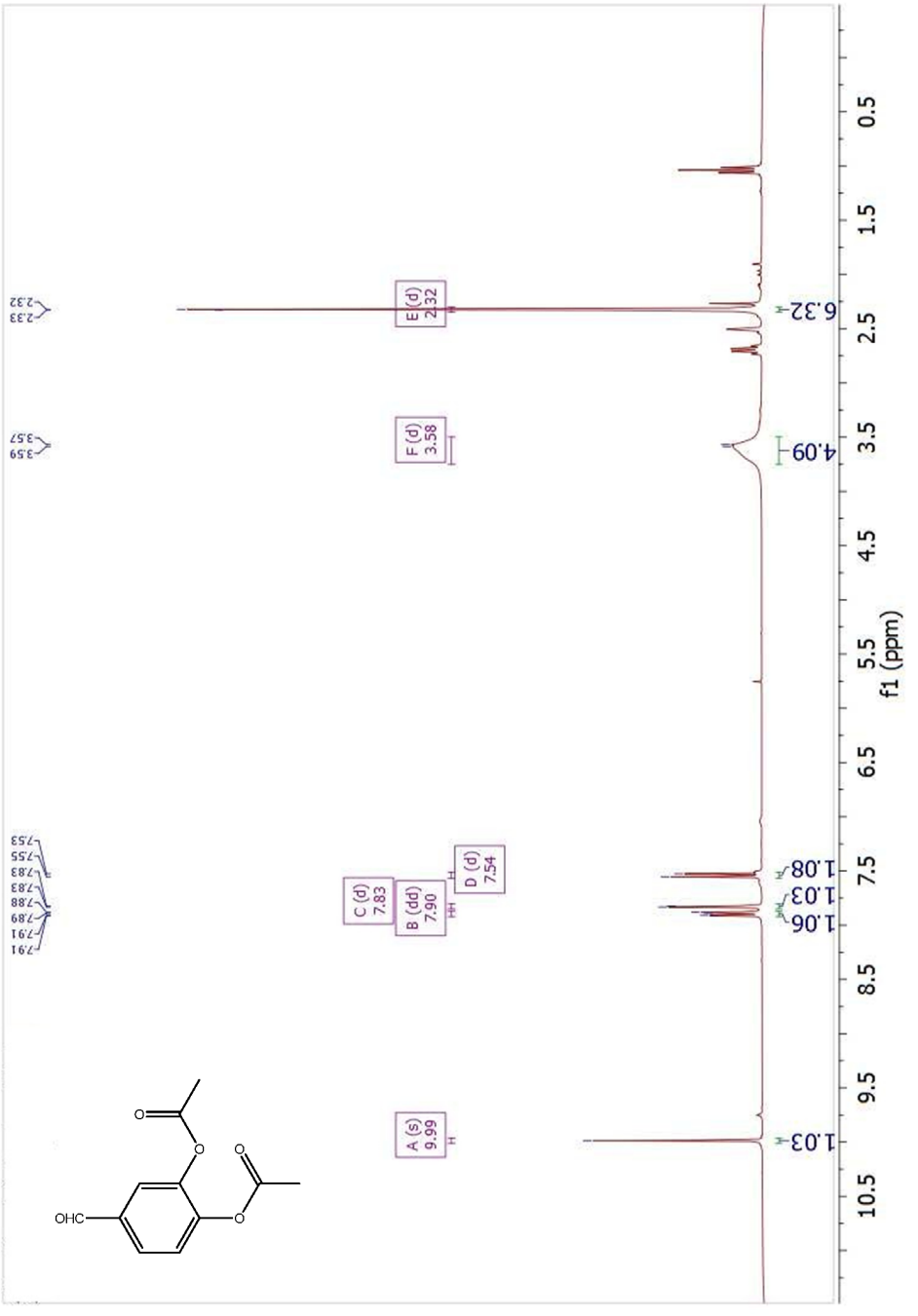
1,2-dihydroxybenzalcohol (33). Acetonitrile, as well as a three peak contamination is present.



3,4-bis(methoxymethoxy)benzaldehyde (34). DMSO, DMF, H_2O solvent peaks are present.

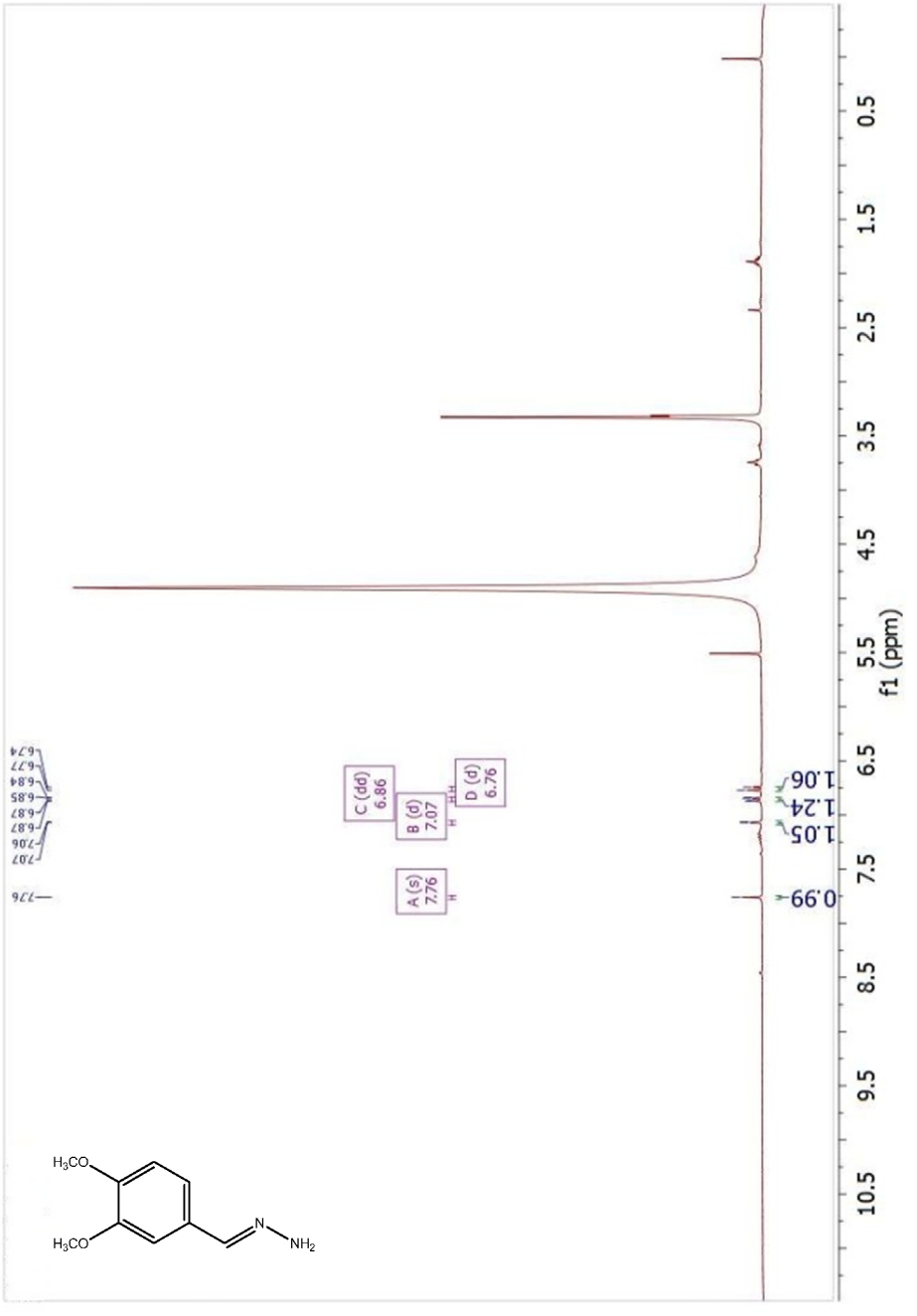


(3,4-bis(methoxymethoxy)phenyl)methanol (35). DMSO and H_2O solvent peaks are present.

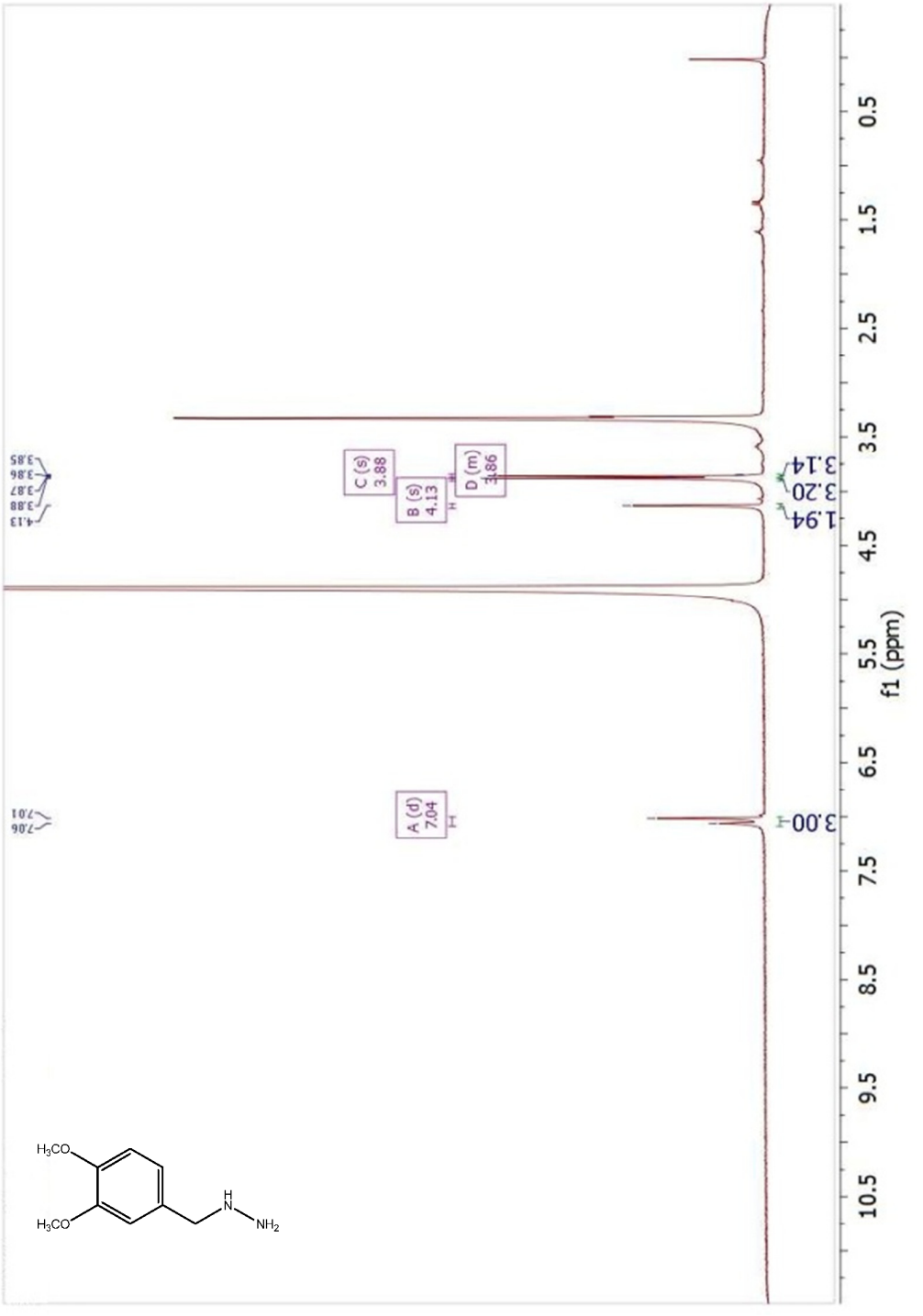


4-formyl-1,2-phenylene diacetate (50). DMSO, integrated peak at 3.5 is H_2O and EtOH are present.

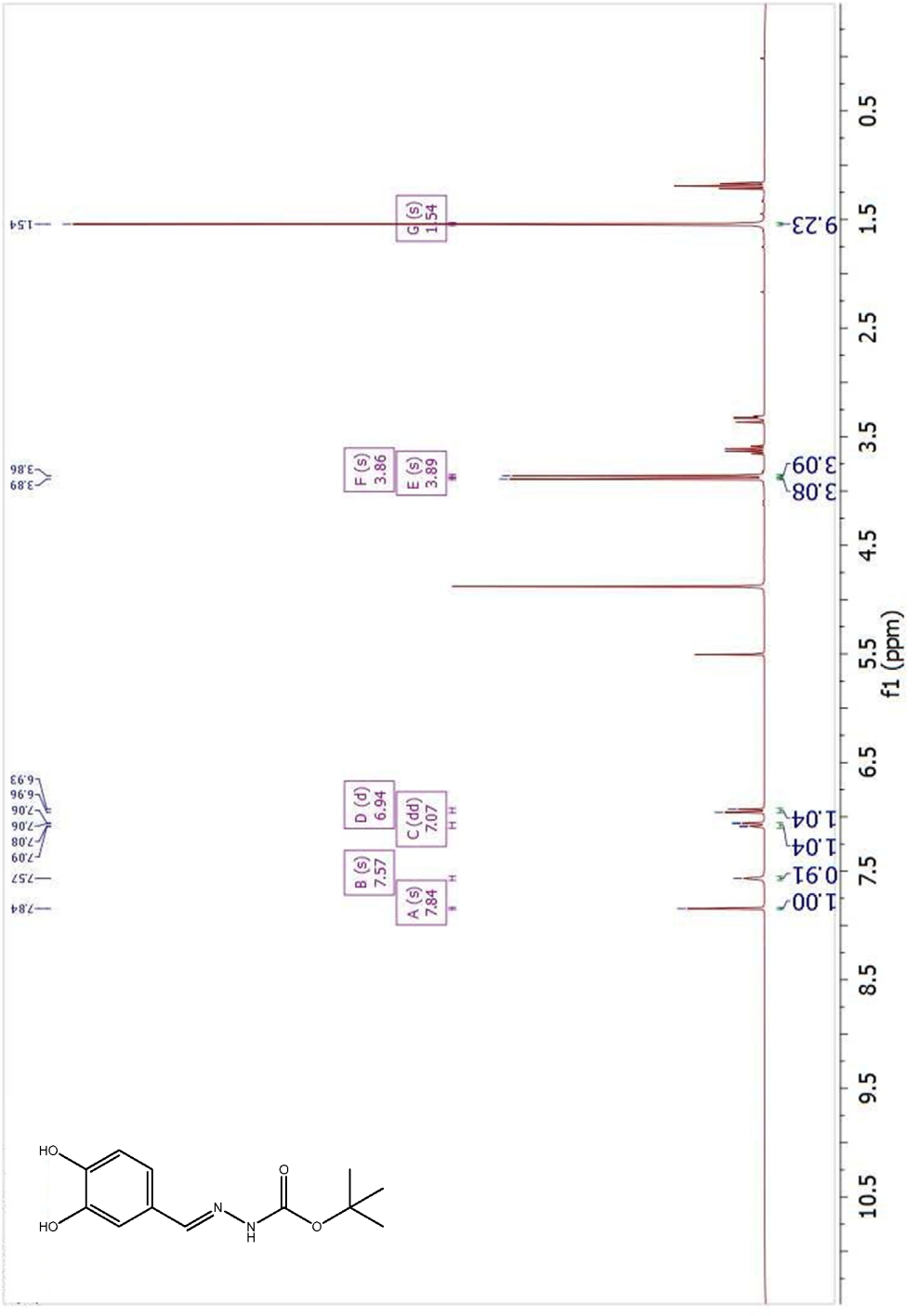
Hydrazine Dopamine Analog NMR



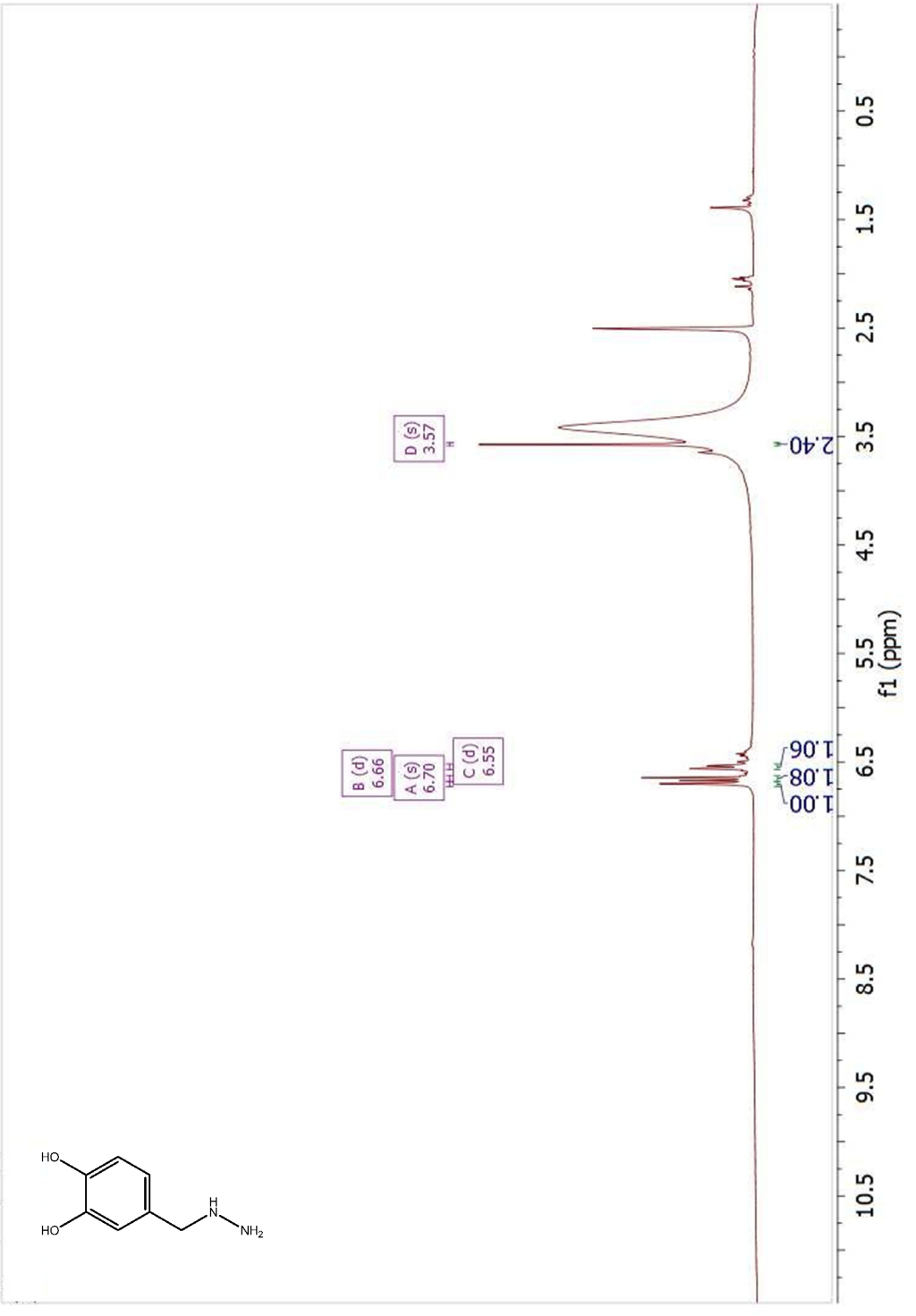
(*E*)-*(3,4-dimethoxybenzylidene)hydrazine (37)* .MeOH and H2O as well as other minor solvent peaks are present.



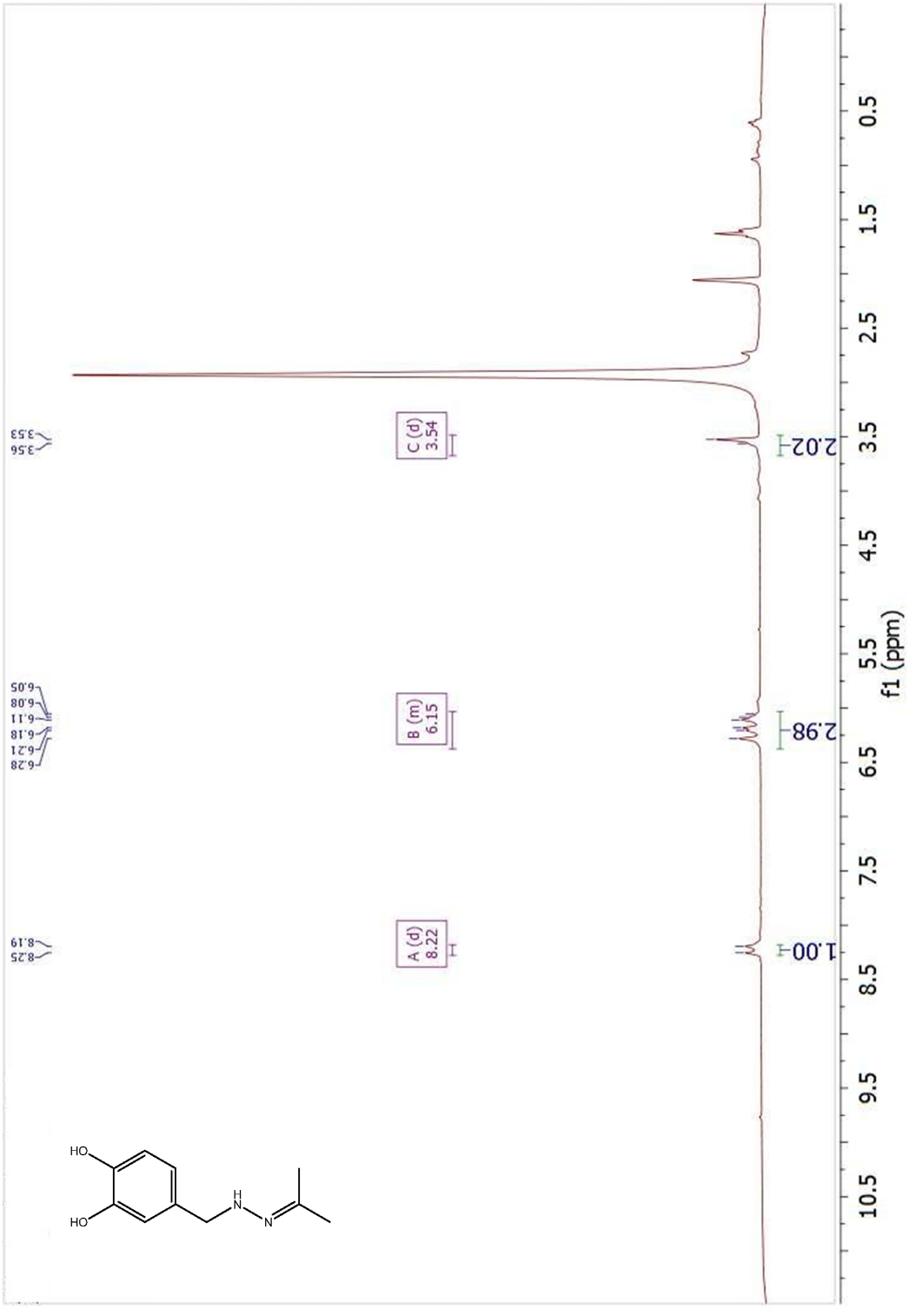
(3,4-dimethoxybenzyl)hydrazine (38). MeOD and H_2O solvent peaks are present.



(E)-tert-butyl 2-(3,4-dihydroxybenzylidene)hydrazinecarboxylate (39). MeOD and H_2O solvent peaks are present as well as THF.



4-(hydrazinylmethyl)benzene-1,2-diol (40a). DMSO and H_2O peaks are present as well as other trace contaminants from reaction.



4-((2-(propan-2-ylidene)hydrazinyl)methyl)benzene-1,2-diol (41a). Acetone, DMSO, and H_2O solvent peaks are present as well as other trace contaminants.

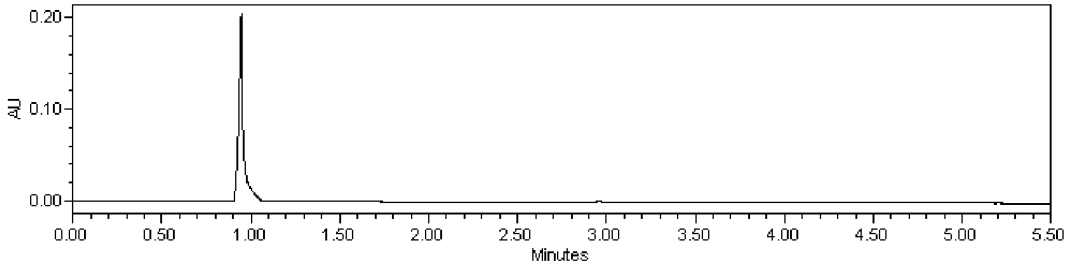
HPLC Data

General notes:

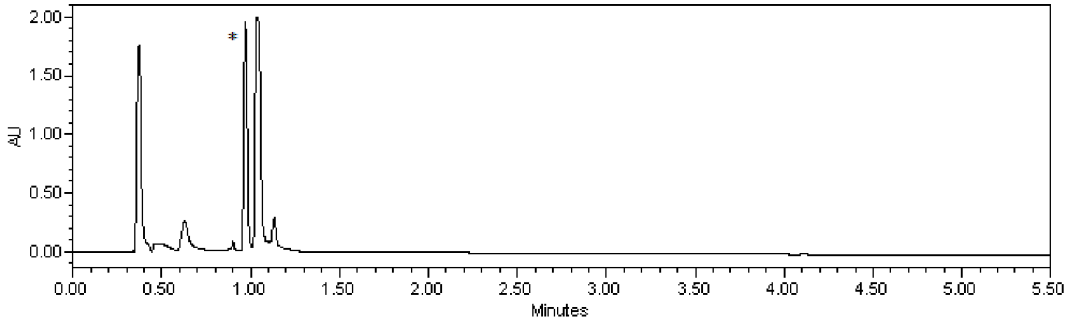
Reactions were monitored using high pressure liquid chromatography on a Waters Acquity Ultra Performance Liquid Chromatography instrument with a photodiode array detector. The solvent and gradient conditions used for HPLC analysis were as follows. Condition A was used for routine analysis. Condition B provided well-resolved peaks for monitoring dopamine disappearance for kinetics. Condition C was used for resolution of chiral products.

- Condition A: Acquity UPLC BEH C18 column (1.7 µm, 2.1x50 mm); 0.4 mL/min; 0-70% acetonitrile in 0.1 % trifluoroacetic acid (TFA) over 4.75 minutes, holding at 70% acetonitrile for 0.25 minutes.
- Condition B: Acquity UPLC BEH C18 column (1.7 µm, 2.1x50 mm); 0.4 mL/min, 0-17.5% acetonitrile in 0.1% TFA over 2.5 minutes followed by 17.5% to 70% acetonitrile in 0.1 % TFA over 1.5 minutes, holding at 70% for 1.0 minute.
- Condition C: Astec Chirobiotic™ T2 column (5 µm, 2.1x150 mm); 0.4 mL/min; isocratic mobile phase composition of 30% acetonitrile, 0.25% triethylamine, and 0.50% acetic acid in methanol.

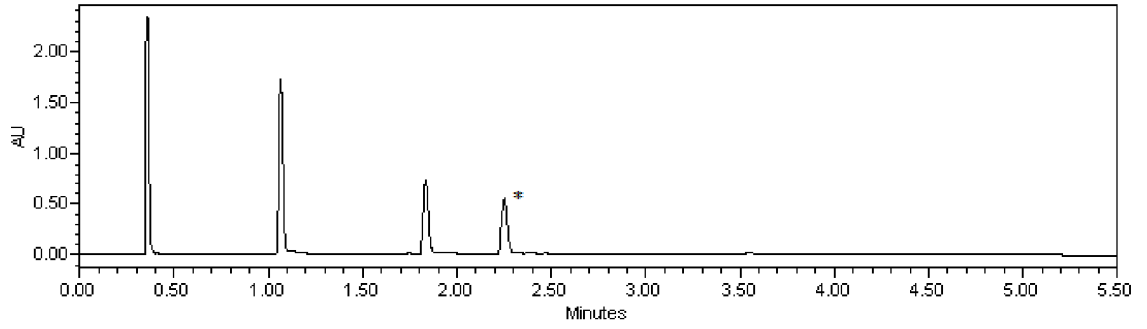
Reactions shown here were monitored using condition B described earlier.



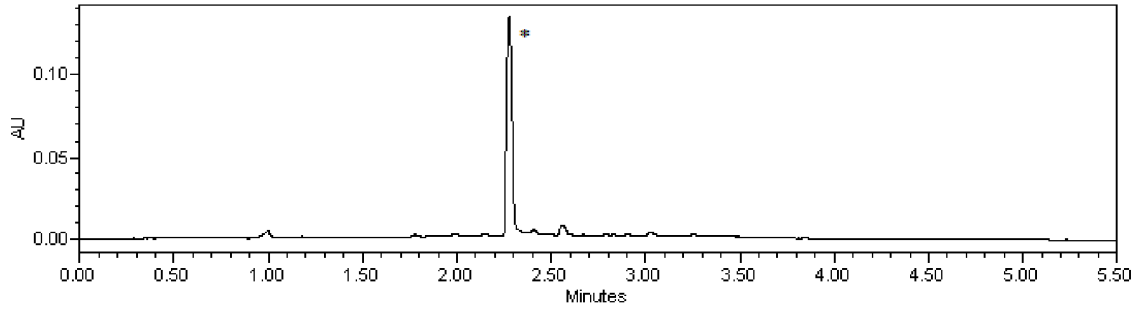
Background Spectra: HPLC background spectrum which doesn't contain a solvent flow peak at 0.4 minutes. All relevant peaks are marked with stars (*).



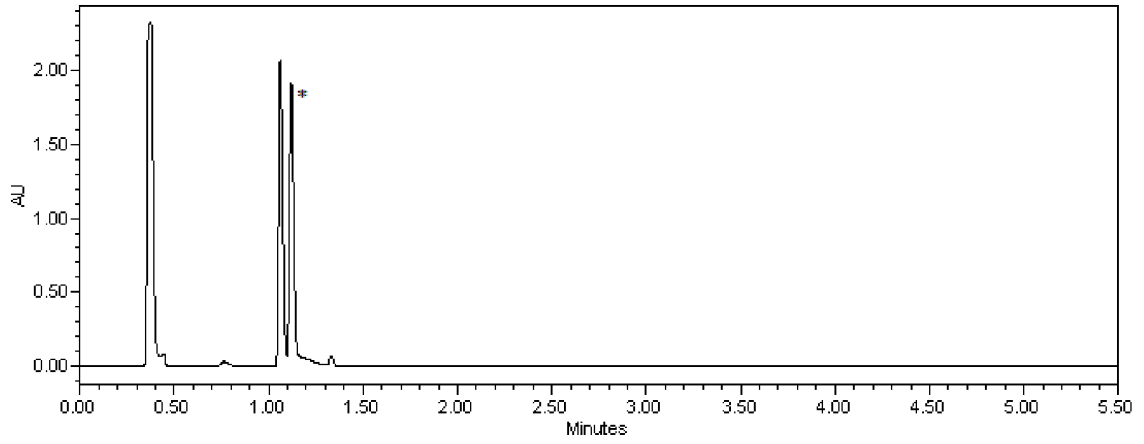
Serine THI: Serine reaction contains some side product reaction along with being more polar than dopamine present at 1.05 minutes.



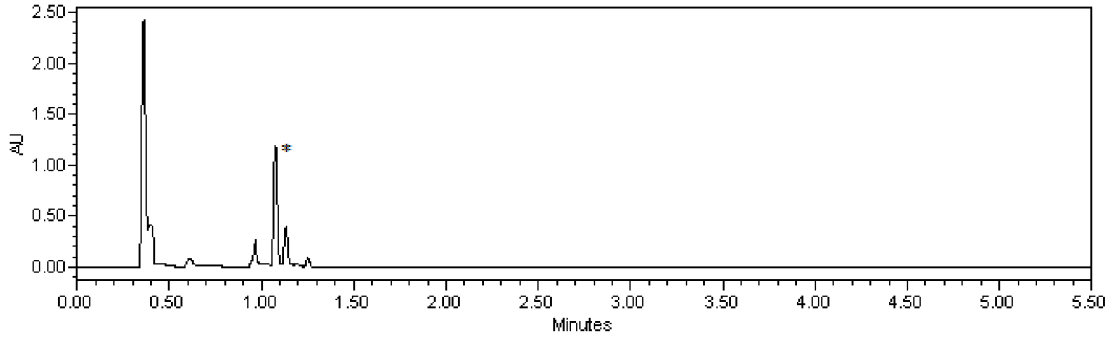
Tryptophan THI: Tryptophan THI reaction contains an unknown side reaction which was further removed by purification.



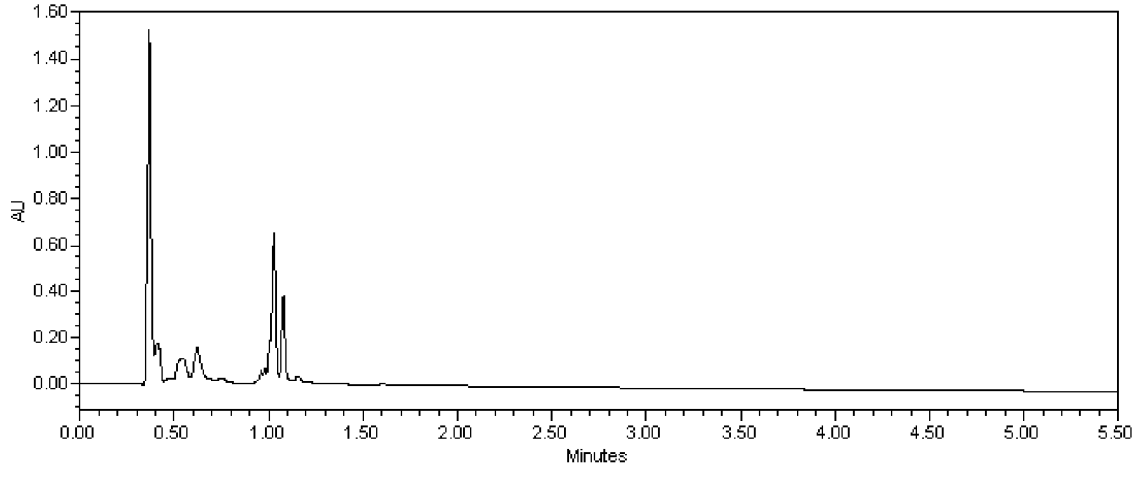
Purified Tryptophan THI: Purification of the tryptophan THI was described earlier.



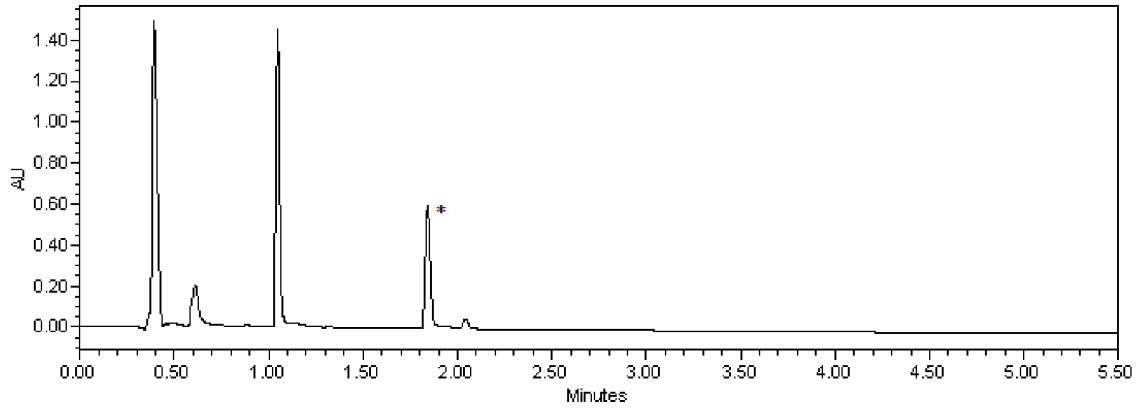
Alanine THI: The product of the reaction overlaps dopamine peak at 1.05 minutes.



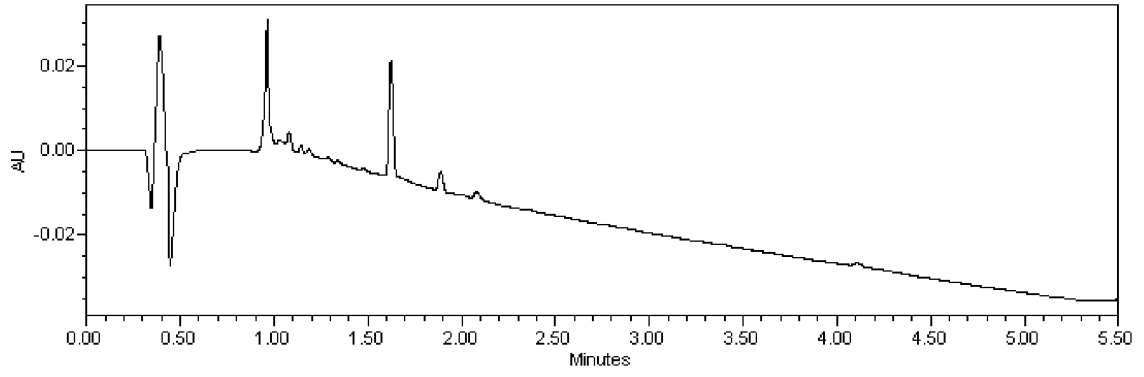
Aspartate Aldehyde Reaction: The reaction producing aspartate aldehyde leads to several side products.



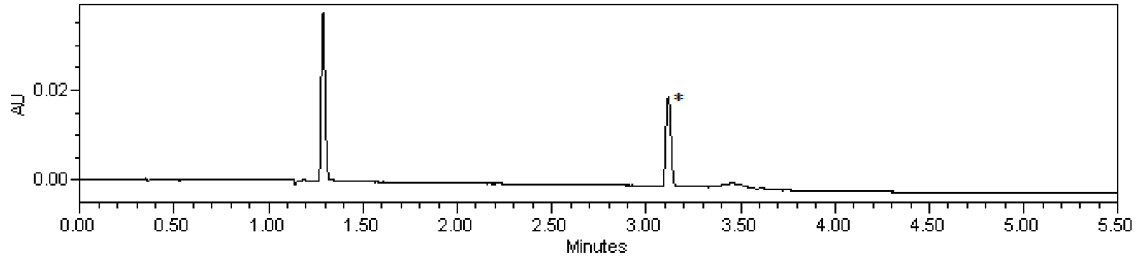
Histidine Aldehyde Reaction: Histidine reaction as predicted much like lysine reacts with itself to produce a large amount of side products.



Leucine THI: Product of the reaction with dopamine and leucine aldehyde is shown with a star.



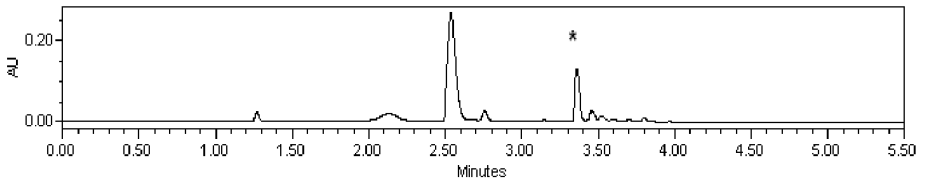
Lysine Aldehyde Reaction: Significant side reactions were present in this reaction thus it was not used further.



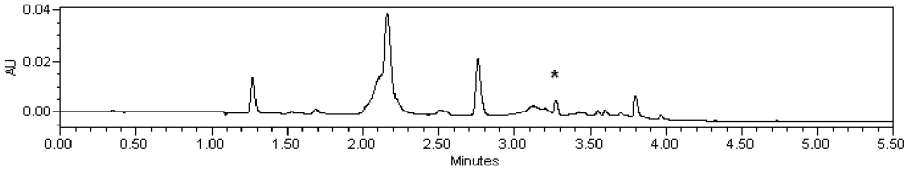
***Phenacetaldehyde THI*:** The reaction product from phenacetaldehyde with dopamine.

Dopamine THI analogs

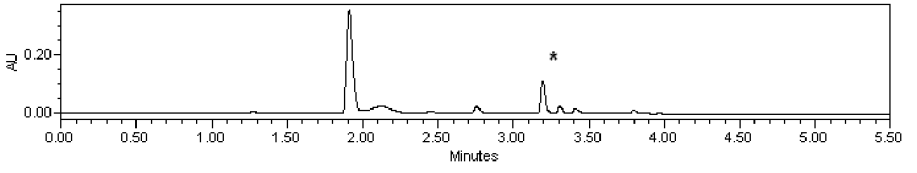
The extracted traces from HPLC were observed at 280 nm.



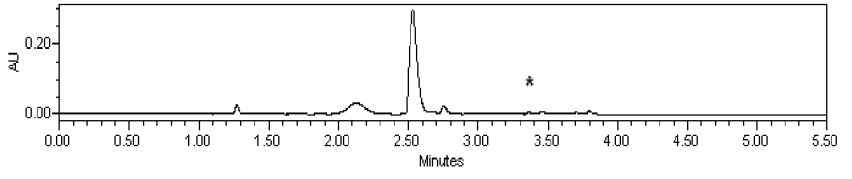
5-iodo dopamine THI analog: Produced by reaction with 4-HPAA in 100 mM phosphate buffer pH 7.0.



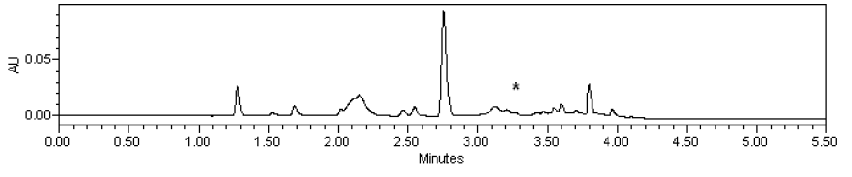
5-bromo dopamine THI analog: Produced by reaction with 4-HPAA in 100 mM phosphate buffer pH 7.0.



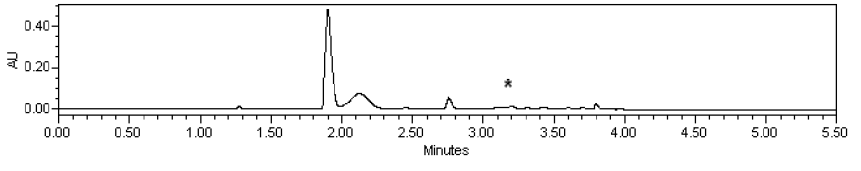
5-chloro dopamine THI analog: Produced by reaction with 4-HPAA in 100 mM phosphate buffer pH 7.0.



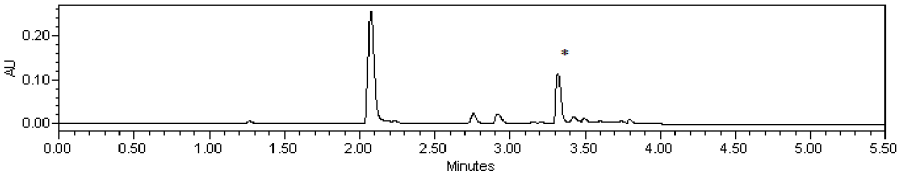
5-iodo dopamine Enzymatic THI analog: Produced by reaction with 4-HPAA and NCS 100 mM BES pH 7.0.



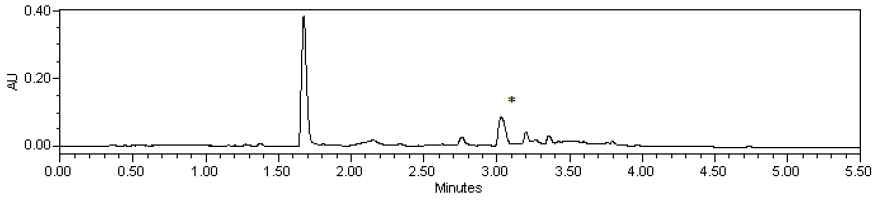
5-iodo dopamine Enzymatic THI analog: Produced by reaction with 4-HPAA and NCS 100 mM BES pH 7.0



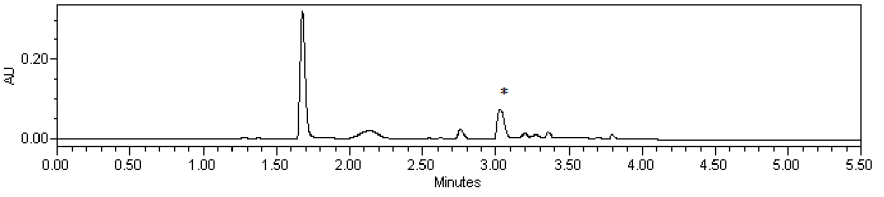
5-iodo dopamine Enzymatic THI analog: Produced by reaction with 4-HPAA and NCS 100 mM BES pH 7.0



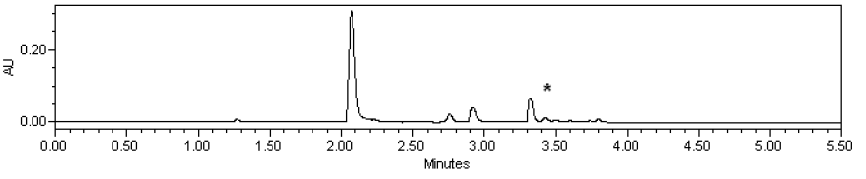
2-iodo dopamine THI analog: Produced by reaction with 4-HPAA in 100 mM phosphate buffer pH 7.0.



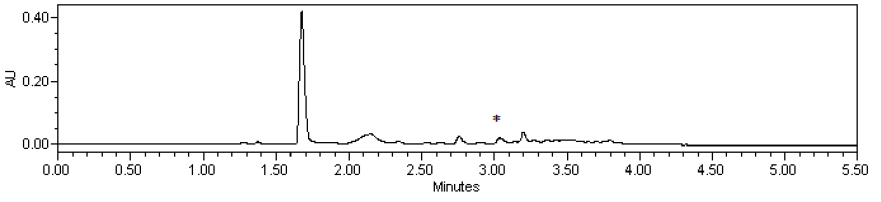
2-iodo dopamine THI analog: Produced by reaction with 4-HPAA in 100 mM phosphate buffer pH 7.0.



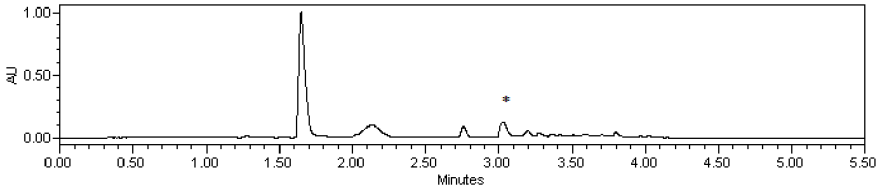
2-iodo dopamine THI analog: Produced by reaction with 4-HPAA in 100 mM phosphate buffer pH 7.0.



2-iodo dopamine Enzymatic THI analog: Produced by reaction with 4-HPAA and NCS 100 mM BES pH 7.0



2-iodo dopamine Enzymatic THI analog: Produced by reaction with 4-HPAA and NCS 100 mM BES pH 7.0



2-iodo dopamine Enzymatic THI analog: Produced by reaction with 4-HPAA and NCS 100 mM BES pH 7.0

Appendix C - References

1. Patrick G (2003) *An Introduction to Medicinal Chemistry* (Oxford University Press, United States) 4th Ed p 749.
2. Paddon CJ*, et al.* (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* 496(7446):528-532.
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