6-2011

Precursor-directed biosynthesis of non-natural berberine and galanthamine analogues

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PRECURSOR-DIRECTED BIOSYNTHESIS OF NON-NATURAL BERBERINE AND GALANTHAMINE ANALOGUES

A Thesis

Presented in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

June, 2011

BY

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I dedicate this work to my parents. Mom and Dad you have always supported my curiosity of science and for as long as I can remember have answered all of my questions of "why." Hopefully now I can answer some of yours.

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Chapter 1
Precursor-directed Biosynthesis

Over the past 30 years natural products have contributed immensely to the discovery of new drugs. Of the 1184 new medicinal chemical entities reported from 1981-2006, 70% have been based on natural products, and over 60% of all available anticancer drugs since the 1940s are naturally derived.[1] In the approach to new drug discovery the development of high throughput screens has increased the demand for vast libraries of compounds. These libraries often contain a random assortment of synthetic compounds that are screened for binding to a target. This approach to drug discovery is kind of like playing the lottery. A new approach focused on small libraries that resemble natural products may actually be more beneficial. This is because naturally inspired drugs greatly outnumber synthetic discoveries in many important areas of medicine, such as cancer treatment (**Figure 1.1).** Nevertheless, libraries of natural product analogues remain scarce because these complex molecules can be overwhelmingly challenging to synthesize. In this regard precursor-directed biosynthesis (PDB) and mutasynthesis (MBS) are gaining popularity as novel ways to generate complex natural product analogues.[1-5]

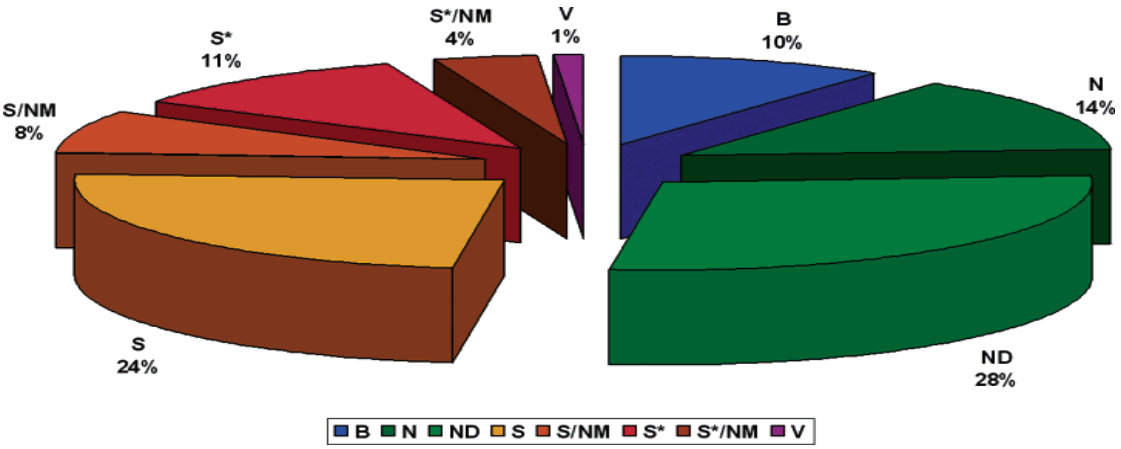


Figure 1.1: All available anticancer drugs, 1940's - 2006 (*N* = 175). The naturally inspired categories include N – Natural, ND - Naturally derived, S/NM – Synthetic natural product mimic, S* - Synthetic, natural product is/was pharmacaphore , and S*/NM - Synthetic natural product mimic based on natural product pharmacaphore. (This figure was reproduced without permission from: Newman, D. J; Cragg, G. M. *J. Nat. Prod.* 2007, 70, 461-467.)

Precursor directed biosynthesis and mutasynthesis encompass the same principle, which is to use nature's machinery to produce "non-natural" natural products (**Figure 1.2**).[2] While natural products constitute an important role in drug discovery, the derivatives of these compounds are often more effective and exhibit fewer side-effects. The most basic example of this is the conversion of salicylic acid into aspirin. One challenge in drug design is synthesizing analogues of complex natural products that resemble medicinal leads. This is because natural products often contain a large number of stereogenic centers and hetero atoms. While the total synthesis of natural products continues to be challenging in the lab, plants possess the indispensable enzymes to produce these products on demand.

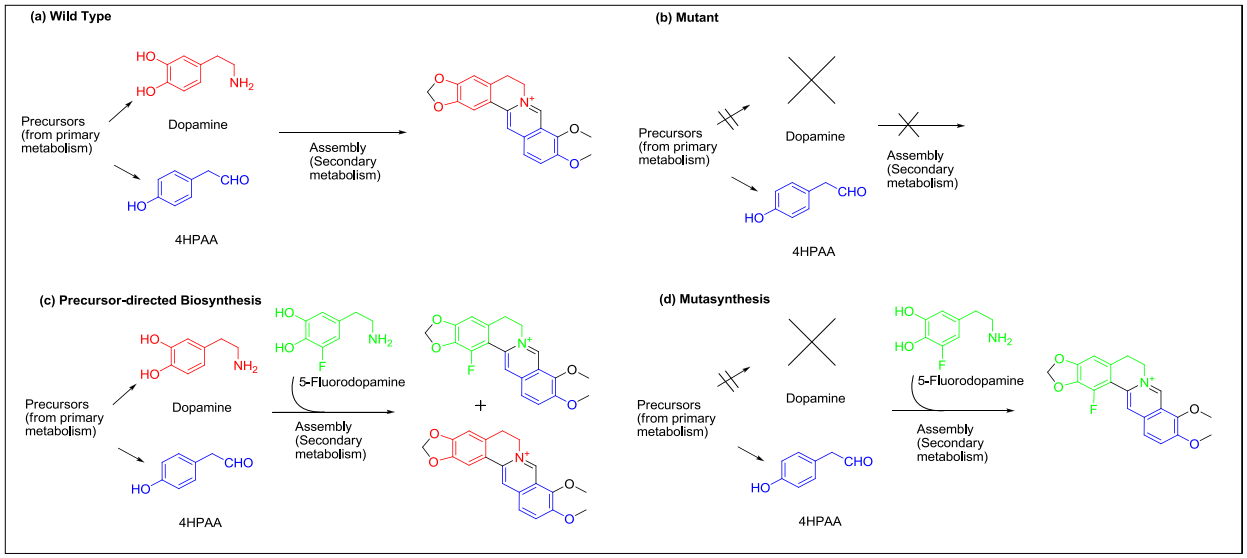


Figure 1.2: Precursor-directed Biosynthesis (PDB) and Mutasynthesis (MBS).[2] (a) The wild type cell assembles a secondary metabolite from primary precursors. (b) A mutant cell no longer expresses the gene coding for the enzyme that generates one of the primary precursors. The secondary metabolite is no longer produced. (c) Precursor-directed biosynthesis: A cell is supplemented with a precursor analogue in order to produce both a natural product and a natural product analogue. (d) MBS: A cell is supplemented with a precursor analogue in order to only produce a natural product analogue.

PDB and MBS provide the opportunity to assemble complex natural products that wouldn't otherwise be accessible. MBS was recently used to generate variants of the polyketide anti-cancer compounds rapamycin and borrelidin (**Figure 1.3**).[2] These compounds are both isolated from bacteria of the Steptomyces genus and are structurally challenging to synthesize. Using MBS seven novel rapamycin analogues and six novel borrelidin analogues were synthesized and isolated, one of which shows a decrease in cytotoxicity when compared to borrelidin. Further advances in MBS include introducing external genes into the biosynthetic pathway of secondary metabolites.[3] The halogenase gene *prnA* was introduced into *Streptomyces coeruleorubis* which resulted in efficient *in situ* chlorination of the uridyl peptide antibiotic pacidamycin. The chlorinated pacidamycin analogue was then functionalized using mild cross-coupling to yield a number of post biosynthetic derivatives that were previously inaccessible (**Figure 1.4**). This research exploits the potential of chlorine as a modification handle on natural product analogues.

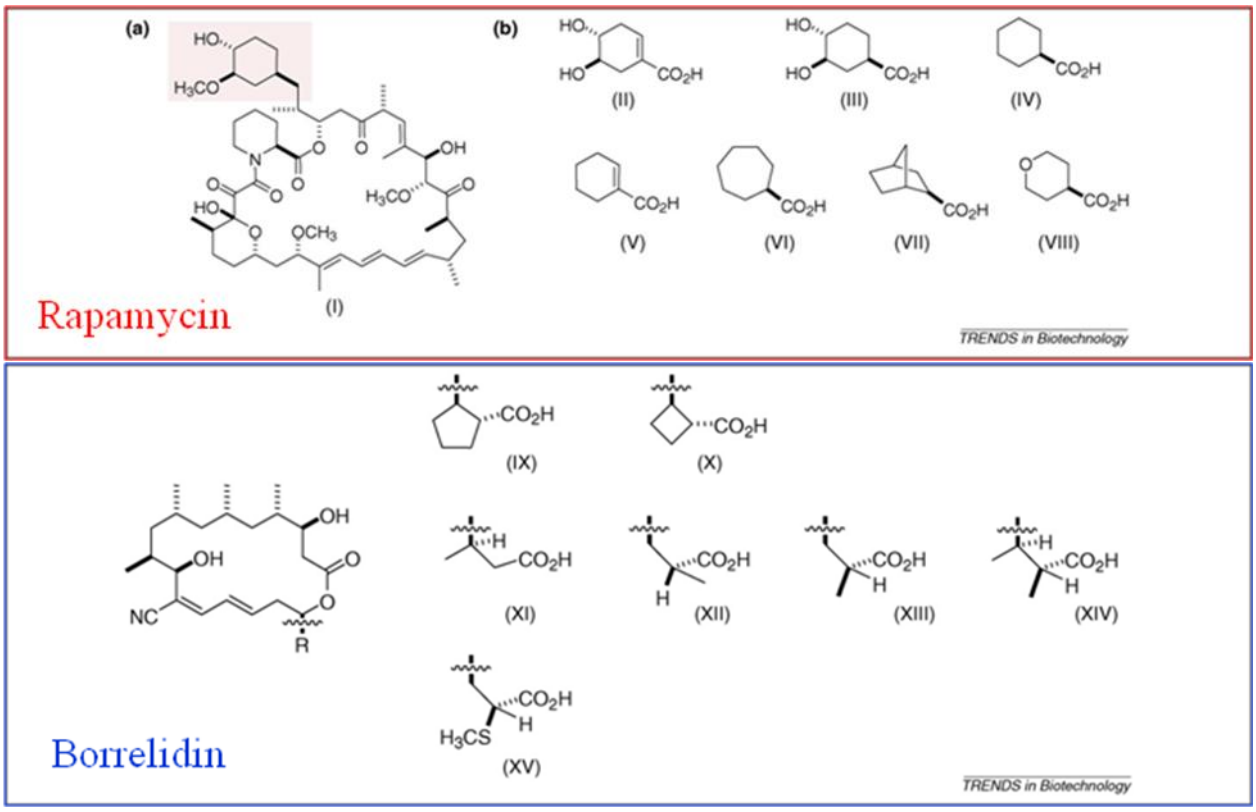


Figure 1.3: Natural Product Analogues of Potential Anit-Cancer Drugs Rapamycin and Borrelidin. (This figure was reproduced without permission from: Weissman, K. J. *Trends Biotechnol.* 2007, 25, 139-142.)

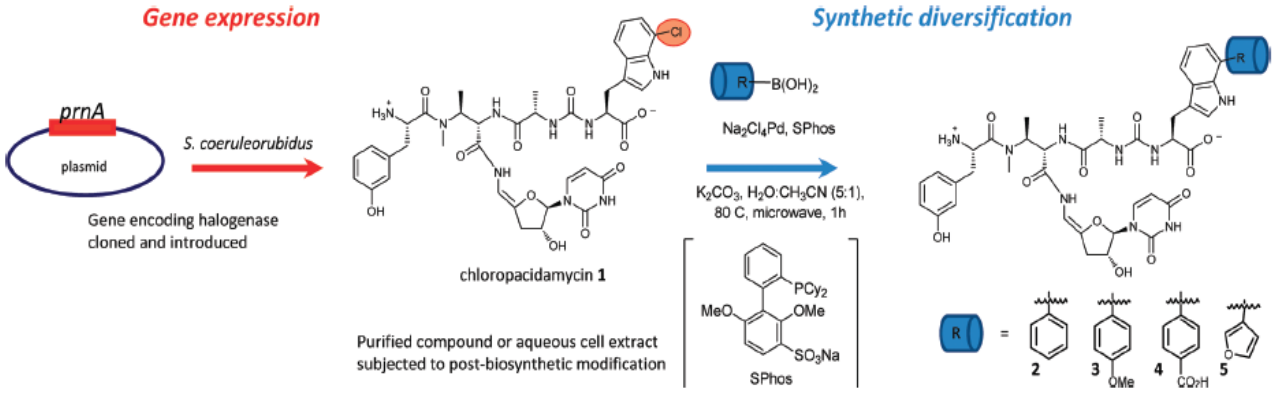


Figure 1.4:Chlorine as a Synthetic Handle. The bacteria *S. coeruleorubidus* was genetically engineered to produce the non-natural, natural product chloropacidamycin which was further modified by a cross-coupling reaction. (This figure was reproduced without permission from: Goss, J. M. R; Roy, A. D; Gruschow, S. and Cairns, N. *J. Am. Chem. Soc.* 2010. 132, 12243-12245.)

Although MBS has been implemented in bacteria biosynthesis for over 40 years, the technique has not been fully explored in complex lengthy plant pathways. Recent evidence, however, has shown that MBS can also be used to generate natural product analogues in eukaryotic cells. The suppression of tryptamine biosynthesis in *Catharanthus roseus* cells resulted in a viable hairy root culture that no longer produced monoterpene indole alakloids, their natural secondary metabolites.[4] However, when supplemented with fluorinated tryptamine analogues *C. roseus* produced multiple fluorinated monoterpene indole alkaloids (**Figure 1.5)**. In a separate project a halogenase was engineered into *C. roseus* in order to biosynthetically produce site selective chlorinated and brominated tryptamine analogues.[5] These halogenated precursors were generated *in vivo* and then accepted into the plants enzymatic pathway to produce halogenated monoterpene indole alkaloid analogues. This evidence shows that MBS provides a feasible method of using plants to synthesize natural product analogues in complex eukaryotic cells.

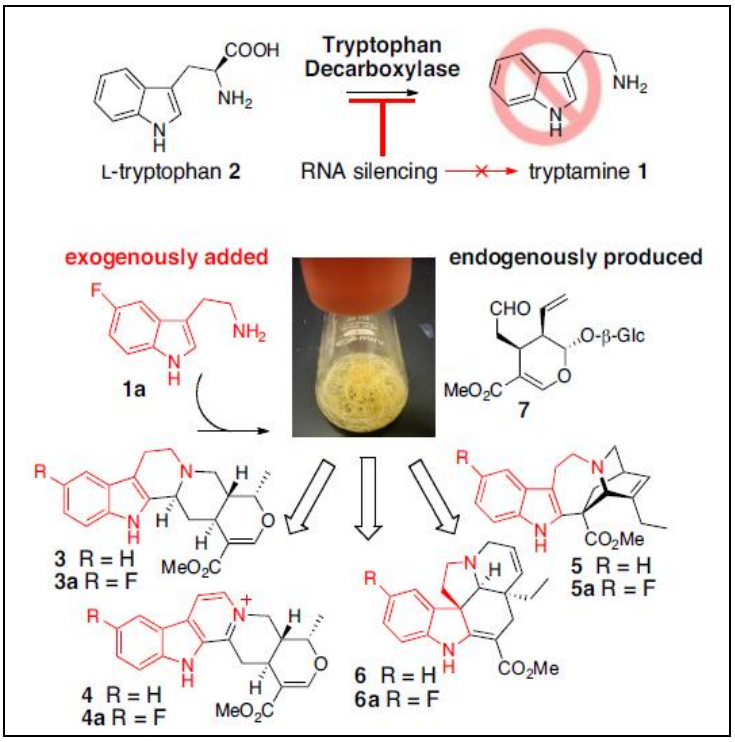


Figure 1.5: Natural Product Analogues Produced in Eukaryotic Cells. RNA silencing was used to prevent production of the natural primary metaboltite tryptamine. Natural product analogues were produced upon the addition of the primary metabolite analogue fluoro-tryptamine. (This figure was reproduced without permission from: Maresh, J. M; O'Connor S. E; Runguphan, W. *Proc. Natl. Acad. Sci. USA*. 2009. 106, 13673-13678.)

PDB involves a much simpler approach than MBS in that no genetic engineering is required. The purpose of PDB is often to establish that a specific enzymatic pathway is flexible enough to produce evidence that an unnatural precursor has been accepted. Once proof of natural product analogues has been established, metabolic engineering can be used to increase yields and diversify precursor selectivity. In this sense PBD can be thought of as pioneering the way for MBS.

The first step in PDB is to select an organism and secondary metabolite targets. The organism *Berberis vulgaris L.*, which produces an array of alkaloids[6,7] including berberine, palmatine and oxyacanthine, was chosen for this project. *B. vulgaris* is a promising organism to work with because cell cultures are readily available from the DSMZ (German Collection of Microorganisms and Cell Cultures) and not only is it known to produce large amounts of berberine,

but the enzymatic pathway to the production of berberine is well established (**Figure 1.6**). A well established metabolic pathway can prove useful in identifying enzymes that are not flexible enough to handle unnatural analogues; as is evident by the build-up of intermediates. For example, if HPLC-MS analysis showed a large amount of halogenated (S)-reticuline, the BBE (berberine bridge enzyme) may not be flexible enough to accept halogenated (S)-reticuline.

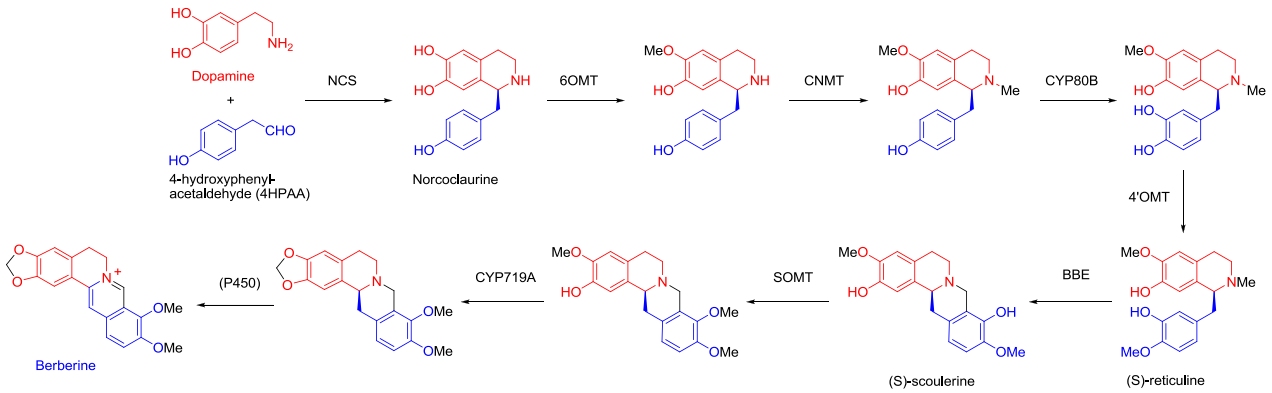


Figure 1.6: The Metabolic Pathway of Berberine Synthesis. The primary metabolites dopamine and 4HPAA undergo a series of enzyme catalyzed reactions which ultimately generate the secondary metabolite berberine.

The primary metabolite precursors of many alkaloids that *B. vulgaris* produces are dopamine and 4-hydroxyphenylacetaldehyde (4HPAA). Dopamine and 4-HPAA are also primary metabolites used in the production of alkaloids in many related species (**Figure 1.9**). In this regard, fluorinated dopamine analogues were chosen as target precursor analogues for the production of fluorinated berberine (**Figure 1.7**), as well as any other alkaloids produced by *B. vulgaris*. The use of fluorine in PDB has many benefits. Fluorine can help identify products by its signature +19 *m/z* in mass spectrometry and its unique NMR activity.[8] Fluorine is relatively small (when compared to chlorine and bromine) which may make it more likely to be accepted by enzymes in metabolic pathways. Finally fluorine exhibits molecular properties that have proven to be pharmalogically useful, as is evident by the fact that fluorine is present in 15% of approved drugs.[8] The medicinal use of fluorine in drugs can be broken down both

pharmacokinetically and pharmacodynamically. Kinetically the fluorine-carbon bond is strong which increases the half-life of drugs. This can decrease the dose and frequency of drug use. Dynamically fluorine has electronic effects that alter a drugs interaction with target enzymes. This effect can result in more successful drugs

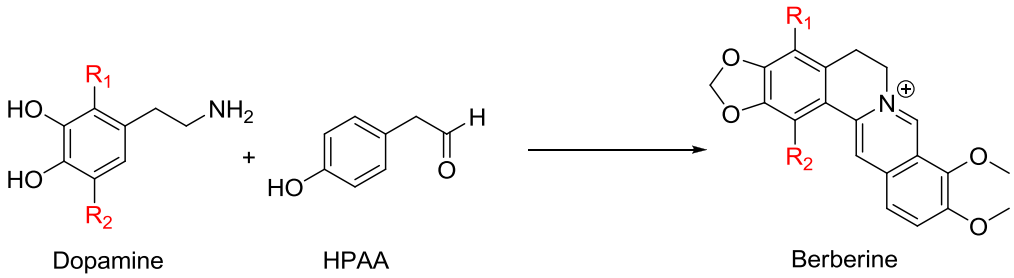


Figure 1.7: Dopamine Analogues as Target Precursors. If accepted by the plant, dopamine analogues 5-fluorodopamine (R_2=F, R_1=H) and 2-fluorodopamine (R_1=F, R_2=H) will combine with 4HPAA to produce fluorinated berberine analogues.

The synthesis of fluorinated dopamamine has been reported in the literature.[9] The reported synthetic approach from Kirk incorporates the use of the Balz-Schiemann reaction and a high pressure catalytic hydrogenation. After repeated failed attempts of reproducing these methods, a new scheme was devised. The new approach can be broken down into two pathways, both of which proceed through a fluorinated vanillin intermediate (**Figure 1.8**). In pathway 1, 3-fluoroanisole is converted to 2-fluoroisovanillin[10] (**3)** which is then used to synthesize 2-fluorodopamine (**6**). In pathway 2, 3-fluoroanisole is converted to 5-fluorovanillin[10] (**9**) which is used to synthesize 5-fluorodopamine (**12**). Chapter 2 describes the chemistry used to synthesize the vanillin intermediates, and the chemistry used to convert the vanillin intermediates to dopamine analogues is highlighted in chapter 3.

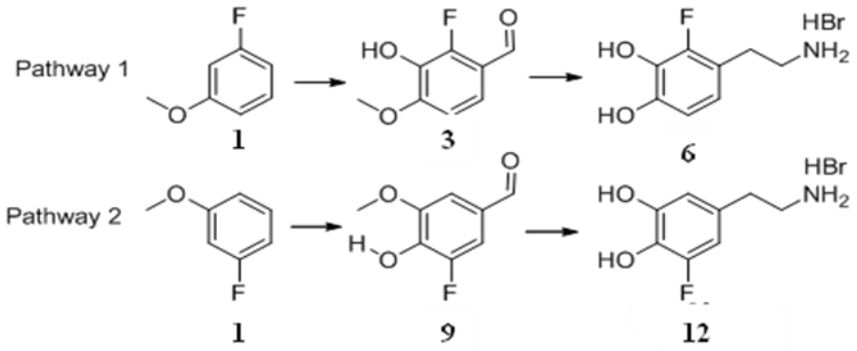


Figure 1.8: Synthesis of Fluorinated Dopamine Analogues. In pathway 1, 3-fluoroanisole is converted to 2-fluoroisovanillin (**3**) which is then used to synthesize 2-fluorodopamine (**6**). In pathway 2, 3-fluoroanisole is converted to 5-fluorovanillin (**9**) which is used to synthesize 5-fluorodopamine (**12**).

Dopamine represents an opportunistic primary metabolite because it is used by diverse families of plants to generate many secondary metabolites (**Fig 1.9)**. The chemistry used to convert vanillin to dopamine provides a chemist with an arsenal of potential primary metabolite analogues. These analogues, in theory, can be used to generate a variety of natural product analogues that could serve a better medicinal purpose than their natural product counterparts.

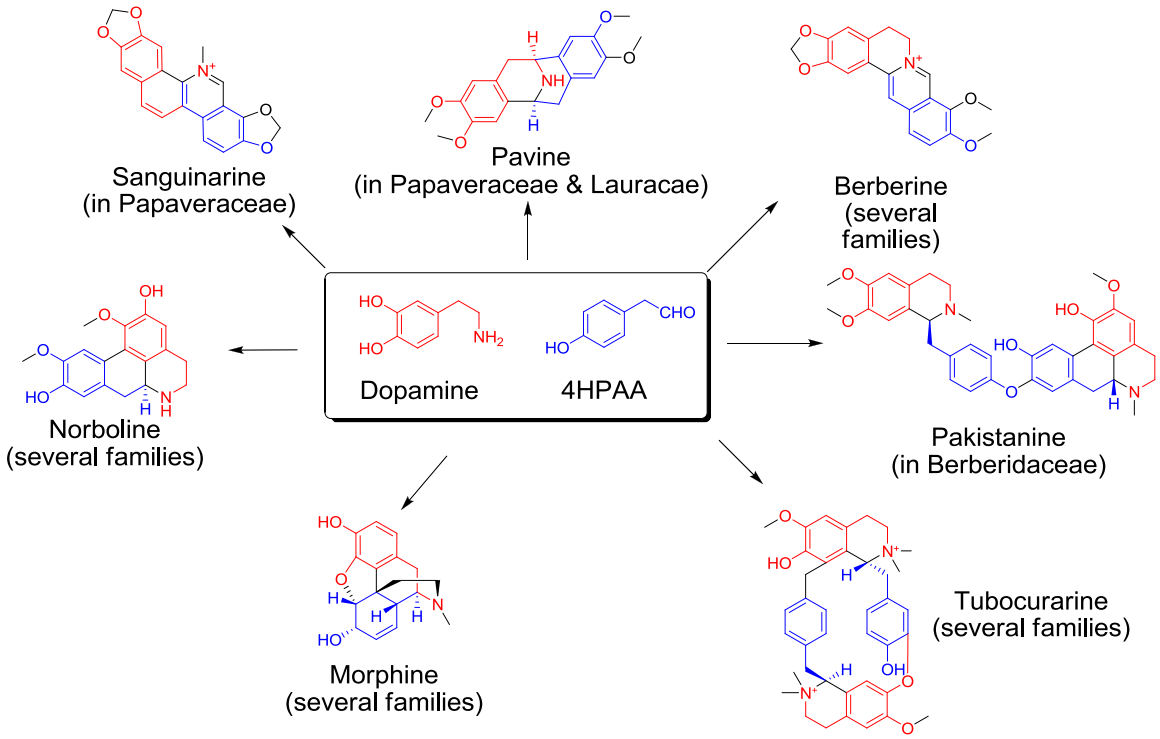


Figure 1.9: Alkaloids Constructed with Dopamine and 4-Hydroxyphenelacetaldehyde (4HPAA). The primary metabolites dopamine and 4HPAA are used by a variety of organisms to produce a range of different alkaloids.

Chapter 2
Fluorinated Vanillin Analogues

Compounds **3** (2-fluoroisovanillin) and **9** (5-fluorovanillin) (**Figure 2.1**) were chosen as precursors for 2-fluorodopamine (**6,** R_1=F, R_2=H) and 5-fluorodopamine (**12,** R_1=H, R_2=F). The first attempt at synthesizing these fluorinated vanillin analogues incorporated the use of the Balz-Schiemann reaction.[11] The Balz-Schiemann reaction (**Figure 2.2**) involves the conversion of an aniline to an aryl fluoride via a diazonium tetrafluoroborate intermediate. The availability of 2-aminovanillin and the literature[9] precedent for its conversion to 2-fluorovanillin made this approach appear convenient.

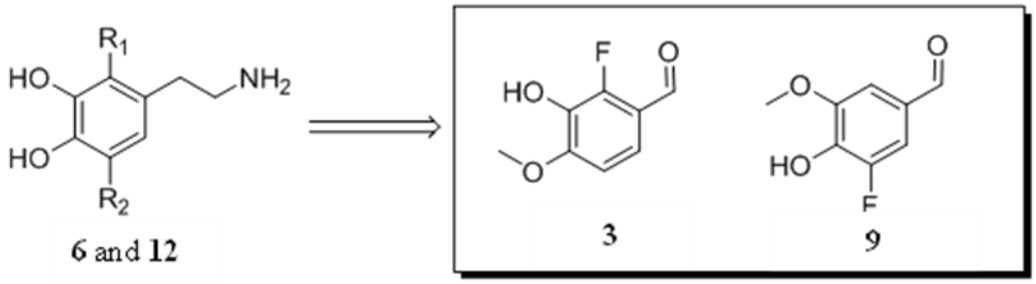
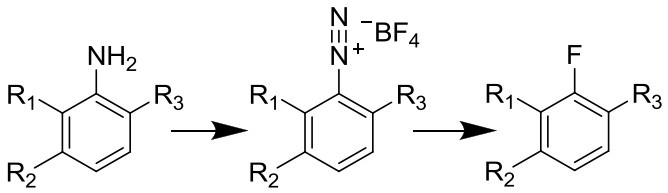


Figure 2.1: Target fluorinated vanillin isomers. Compound **3**; 2-fluoro-3-hydroxy-4-methoxybenzaldehyde (2-fluoroisovanillin). Compound **9**; 3-methoxy-4-hydroxy-5-fluorobenzaldehyde (5-fluorovanillin).

The conversion of aniline to fluorobenzene (reaction ***A*, Figure 2.2**) was attempted in order to test its viability and become familiar with the Balz-Schiemann reaction. When following the experimental procedure of ***A***, the conversion of aniline to the diazonium tetrafluoroborate salt went smoothly; however, the subsequent reaction of the salt was not as easily accomplished. These results were expected, as the diazonium salt is a very reactive intermediate. GC-MS analysis of the reaction ***A*** product suggested a product mixture of

fluorobenzene and phenylhydrazine. While GC-MS analysis was optimistic, the product itself was an oily tar that was difficult to handle. Due to the volatility of fluorobenzene and phenol (another likely side-product), and in order to test an aromatic compound with ring substituents, dimethyl analine was chosen as a better suited test reaction.



Reaction	Compound	R_1	R_2	R_3
A	Analine	H	H	H
B	2,6-dimethylanaline	CH_3	H	CH_3
C	2-aminovanillin	OCH_3	OH	COH

Figure 2.2: Balz-Schiemann Reaction Schemes. Three compounds, analine, 2,6-dimethylanaline, and 2-aminovanillin, were used to test the Balz-Schiemann reaction.

Reaction ***B*** was carried out in a similar fashion as ***A*** and once again the diazonium salt was easily isolated. The decomposition product of ***B*** was not purified, but GC-MS analysis showed that 2,6-dimethylfluorobenzene was the major product. The results of reactions ***A*** and ***B*** suggested that the diazonium salt could be generated in high yield, but that the decomposition step would need optimization. The decision was made to forgo optimizing the test reactions and to attempt reaction ***C***.

Reaction ***C*** was attempted three times with minimal success. The desired product was neither verified nor isolated. One major synthetic problem encountered was that the literature reference used a "home-made" apparatus to pyrolyze the diazonium salt by irradiation.[9] This apparatus was not available for the present work. In order to overcome this issue, two distinctive procedural changes were made. The first change was that $NOBF_4$ was used in place of aqueous $NaNO_2$ and HBF_4. This was done in an attempt to exclude water from the reaction. As a result

of generating the aryl cation, water can react competitively with fluorine during decomposition to generate phenols instead of fluorinated compounds. The second procedural change was that the diazonium salt was decomposed in xylene in order to control the temperature during decomposition. This was done because there is an increased risk of side products if the diazonium salt decomposes too aggressively. After three attempts of reaction **3** failing to indicate any sign of the desired product, a new synthetic approach was devised.

The second approach to fluorinated vanillin analogues is based on 3-fluoroanisole (**1**) as the starting compound (**Figure 2.3**). This starting compound is inexpensive and commercially available, but more importantly it already contains a fluorine atom which obviates the difficulties associated with uncontrollable (i.e. F_2) direct fluorination. From 3-fluoroanisole (**1**), the desired products 2-fluoro-isovanillin (**3**) and 5-fluorovanillin (**9**) were synthesized in 45% and 55% yields' respectively.

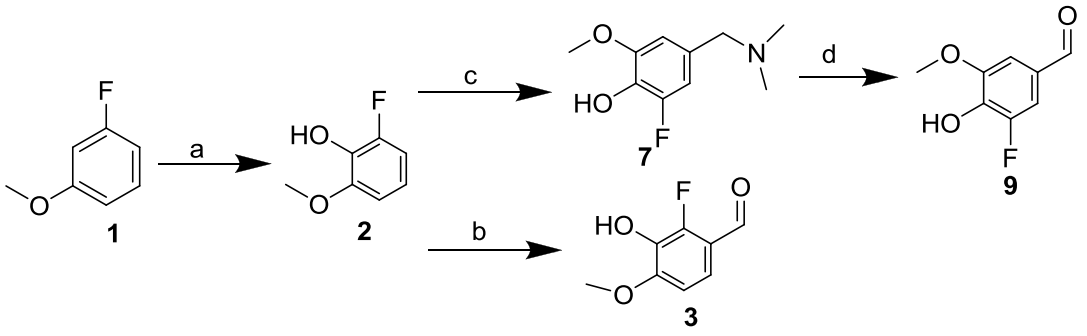


Figure 2.3: Direct Routes to Fluorinated Vanillin Analogues. Reaction conditions: (a) n-butyl lithium (-78^{o}C), $B(OMe)_3$, AcOH (0^{o}C), 30% H_2O_2, (b) refluxing HMTA in TFA, (c) 40% dimethylamine, 37% formaldehyde in ethanol reflux, and (d) Iodomethane followed by HMTA reflux in acetic acid.

3-Fluoroanisole (**1**) was converted to **2 (**80%**)** using n-butyllithium and $B(OCH_3)_3$ at -78^{o}C followed by treatment with acetic acid and hydrogen peroxide at 0^{o}C. This reaction was 100% selective for the desired position under the conditions chosen. This was confirmed by comparing the ^{1}H-NMR and ^{19}F-NMR spectra of **2** to a standard purchased from Sigma Aldrich.

The Duff reaction, which employs hexamethylenetetramine in refluxing trifluoroacetic acid, was used to generate **3** (56%) from **2**. This reaction was regioselective on the 1-5 mmol scale, however, when scaled up (>50 mmol) the reaction lost regioselectivity. The presence of two isomers was easily observed by different retention times on GC-MS and HPLC, however, the formyl position of each product was difficult to assign by NMR. In order to correctly assign the formyl position, the product of a small scale reaction producing only one isomer was sublimed, re-crystallized, and the X-ray structure was determined (**Figure 2.4)**. This crystal structure verified GC-MS, UPLC, HNMR and FNMR spectra for 2-fluoroisovanillin (**3**). The second major product was thought to be 5-fluorovanillin (**9**), a conclusion that was verified by the more selective reaction scheme 2.

A synthetic route to 5-fluorovanillin (**Figure 2.3)** was necessary in order to confirm the characterization of the second product from the Duff reaction on **2**, and to establish a reliable synthetic route to the desired fluorodopamine analogue **12.** A regioselective reaction using dimethylamine and formaldehyde in absolute ethanol generated the benzylamine **7** (95%) from **2**. Benzylamine **7** was converted to the quaternary amine **8** (100%) using methyl iodide. This was done in order to generate a better leaving group for conversion to the aldehyde. Upon treatment with hexamethylenetetramine in refluxing acetic acid the quaternary amine was converted to 5-fluorovanillin **9** (72%). Spectral analysis of **9** confirmed that it was the other major product in the treatment of **2** with hexamethylenetetramine in TFA.

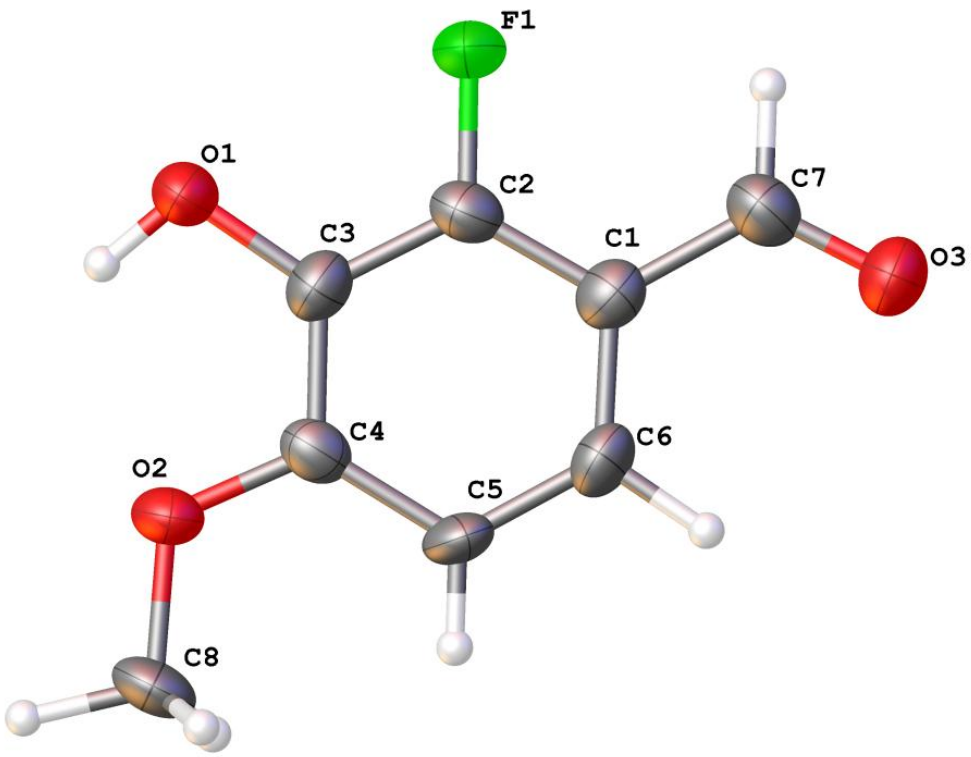


Figure 2.4 X-ray Structure of 2-Fluoroisovanillin.

Table 2.2: Crystal and Structure Refinement Data for 2-fluoroisovanillin. The .res file is included in appendix B.

Compound	**2-Fluoroisovanillin**
Formula	$C_8H_7O_3F$
Fw	170.14
space group	Pc
a, Å	6.360(4)
b, Å	6.857(4)
c, Å	8.497(5)
α, deg	90
β, deg	98.169(19)
γ, deg	90
V, Å^3	366.8(4)
Z	2
ρ_{calc}, g/cm^{-3}	1.604
F_{000}	1976
μ(Mo Kα), mm^{-1}	0.139
λ, Å	0.71073
temp, K	300
R; R_w	0.0776; 0.1729
GOOF	1.059

The reactions starting with 3-fluoroanisole afforded the fluorinated vanillin analogues in a much more reliable fashion than the Balz-Schiemann approach. While the Balz-Schiemann reaction was untamable in the present work, one approach in the future may be to attempt the decomposition step in a microwave reactor. The controlled input of energy in the form of microwaves could excite the diazonium intermediate to the appropriate state for conversion to the aryl fluoride. This would be a worthwhile endeavor because the best attribute of the Balz-Schiemann approach is its selectivity. One drawback of the hexamethylenetetramine approach is the unpredictability when formylating **2** directly. Each attempt yielded the products in different proportions and large scale reactions gave poor yields. A convenient method using prep-HPLC is currently being developed in order to separate the regio-isomers of fluorinated vanillin, however this does not resolve the issue of low yields on large scale reactions. An interesting project would be to tailor the reaction conditions to selectively produce each isomer in high yield.

The versatility of the fluorovanillin products is two-fold. First, it can be used to synthesize fluorinated dopamine analogues for the incorporation into the berberine pathway. The synthetic pathway to fluorinated dopamine analogues is discussed in the chapter 3 and the preliminary feeding experiments are described in chapter 4. The secondary purpose of fluorovanillin is to be converted into fluorinated protocatechauldehyde analogues for incorporation into the galanthamine pathway. The galanthamine project is further elucidated in chapter 5.

Chapter 3
Fluorinated Dopamine Analogues

5-Fluorodopamine (**12**) was synthesized from 5-fluorovanillin (**9**) in 79% yield (**Figure 3.1**). While this scheme may seem straightforward, no available***** route to the synthesis of **12** had been previously reported in the literature**.** The greatest challenge encountered in this scheme was working with and isolating the charged products of the amine reductions; the pKa values for the amine and phenol are within ~1 unit making extractions into organic solvents challenging and impractical. In an attempt to synthesize **12** a variety of reducing agents were used in combination with protective and de-protective chemistry. This chapter outlines the various approaches that resulted in the selected pathway.

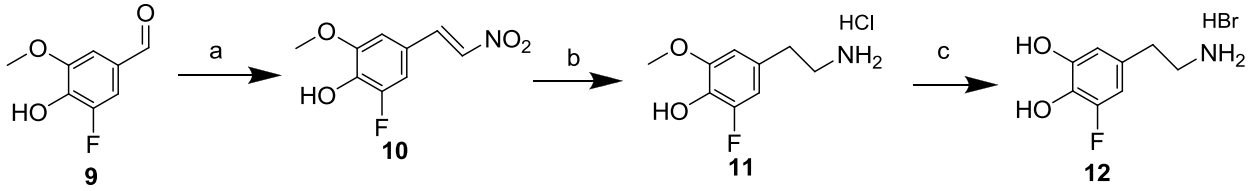


Figure 3.1: Synthetic Scheme for 5-Fluorodopamine. 5-fluorovanillin (**9**) was converted into 5-fluorodopamine (**12**) in three steps.[9,12,13] Reaction conditions: (a) nitromethane and ammonium acetate in acetic acid reflux, (b) Pd/C HCl under atmospheric H_2, and (c) concentrated HBr reflux.

The condensation reaction of vanillin and nitromethane in refluxing acetic acid has been extensively reported in the literature.[12] This reaction was carried out on large scale (50 mmol, 72% re-crystallized yield) in order to generate material for test reactions. The condensation reaction that generated **10** was optimized to 97% crude yield. The fluorinated compounds required ~2x as long of reflux to go to completion and ethyl acetate was found to be the best solvent for extraction.

*****The only previously reported synthesis of **12** required a high pressure (40 psi) catalytic hydrogenation step. The required reaction conditions were not reproducible, so a new scheme had to be developed.

The previously reported synthesis of **12** involved reducing the alkene using sodium borohydride and the nitro group was reduced under high pressure H_2 over a platinum oxide catalyst.[8] This procedure was attempted, but no pure product was isolated. The alkene was easily reduced using sodium borohydride (90%), however, the reduction of the nitro group at atmospheric pressure using platinum oxide resulted in numerous side products and an unrecoverable desired product. The failure of this reaction is probably two-fold. The obvious problem is that the reducing pressure of H_2 was not achieved (no high pressure hydrogenator was available for use) and the secondary concern is that the phenol (**11**) was protected (with a secondary methyl group) in the reported synthesis. Due to the fact that methylating the phenol would not guarantee success, alternative means of reduction were explored.

Catalytic hydrogenation was originally ruled out because limited success was observed with PtO_2/C, Pt/C, and Rainey-nickel catalysts. HPLC analysis of product from these metal catalysts revealed numerous side products and unreacted starting material. The first major lead was observed when Zn dust in methanol with concentrated HCl resulted in complete conversion of **4** to one product by HPLC. The product obtained was an oil which displayed the predicted ^{1}H- NMR peaks for the desired product, but also had an un-identifiable multiplet at δ 7.11 ppm (**Figure 3.2**). The desired product could not be easily separated from the product resulting in the multiplet because both were aqueous soluble and organic insoluble. The side product was thought to some sort of Zn complex. The procedure to remove Zn from the reaction involved making the solution slightly basic after the reaction. This was done because $ZnCl_4^{2+}$ (product at low pH) is soluble and $Zn(OH)_4^{2-}$ (product at high pH) is also soluble, but $Zn(OH)_2$ (product at intermediate pH) is insoluble in aqueous solution. This approach was attempted using both NaOH and NH_4OH, but in both cases the product still could not be isolated. In another

procedure Na_2CO_3 was added in an attempt to precipitate $ZnCO_3$, but this also failed to yield a purified product. H_2S was also used in an attempt to precipitate the insoluble compound ZnS. In a final attempt column chromatography was used in four approaches where both silica and alumina under acidic and basic work-up were implemented. After these columns failed to result in a purified product, a new approach using protective group chemistry was devised.

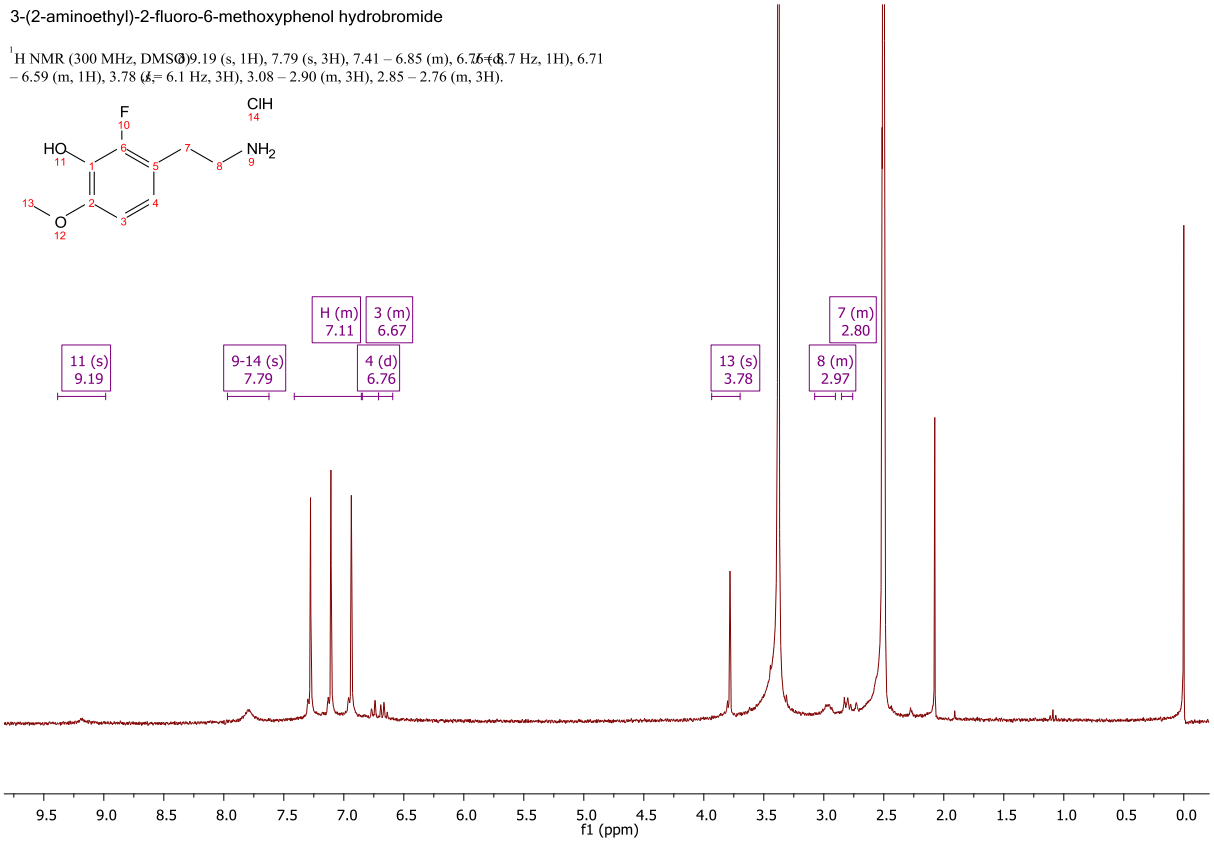


Figure 3.2 : ^{1}H NMR Displaying Side Product 3-Peak. The product **5** of the zinc reduction was difficult to purify and exhibited an unidentifiable multiplet at 7.11 Hz.

Protective group chemistry was used in an attempt to make the reduction product soluble (extractable) in organic solvent. The phenol of vanillin was protected with a benzyl group.[14] The benzyl group was chosen because high yields were previously reported for benzylating vanillin (as opposed to methylating) and the large hydrophobic group would likely increase organic solubility (**Figure 3.3)**. The synthesis of benzyl vanillin **20** was easily accomplished

(91%), and the nitro-aldol condensation to produce **21** was also relatively high yield (93%). The zinc catalyzed reduction of **21** did not go as smoothly as anticipated. The reduction was attempted five times, but **22** was never isolated. The analysis of the product by HPLC showed multiple peaks, so the success of the reaction remained in question. After the desired product could not be extracted with either ether or dichloromethane, the protective approach was put on hold. In the future, another solvent might be better for extraction such as ethyl acetate or benzene, however, the actual success of the reduction remains in question.

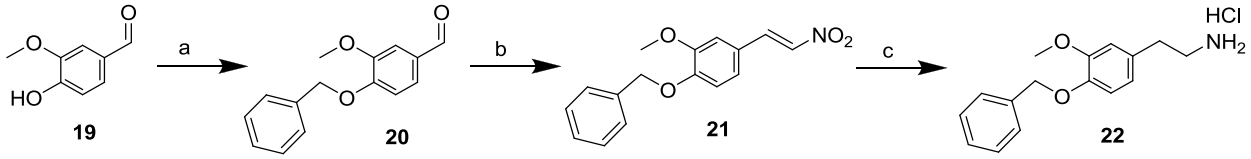


Figure 3.3: Benzyl Protection Scheme. The phenol of vanillin was protected with a benzyl group in an attempt to generate the phenol protected amine **22**. Reaction conditions: (a) benzyl chloride, KI and $NaHCO_3$ in acetonitrile, (b) nitromethane and ammonium acetate in acetic acid reflux, and (c) Zn/HCl in methanol (unsuccessful).

Consultation of the literature turned up a reference in which nitrovinyl groups were reduced to ethanamines using Pd/C with concentrated HCl and H_2 at atmospheric pressure.[13] After a successful test reaction, this method proved to be worth pursuing. A method was developed in which **10** was reduced to **11** (90%) overnight at 1^oC. An unexpected observation of the 3-peak multiplet (**Figure 3.2**) was encountered in the ^{1}H-NMR spectrum of this product. Interestingly a wash with acetonitrile removed the byproduct at the cost of 10-20% of the yield. The filtrate containing the byproduct was not analyzed further. This reduction has yet to be used on the **4** because this isomer has to be isolated or synthesized. A method is currently being developed to separate the nitrovynl compounds **4** and **10** by prep-HPLC.

The final step in the synthesis of dopamine analogues from vanillin involves de-methylating **11**. The demethylating methods tested were HBr and $AlCl_3$.[9] The $AlCl_3$ method was ideal for reactions in which the desired product was organic soluble and could be extracted

from the aqueous reaction mixture. This reaction was found to be successful in de-protecting vanillin and vanillin nitrostyrene. However, the de-protected catechol nitrovynl product was not easily reduced. The second method, reflux in concentrated HBr, worked well only under specific conditions. The product of the reaction, dopamine, is a catechol and under the harsh reaction conditions can decompose to benzoquinone and subsequently polymerize via Diels Alder reactions or Michael addition (**Figure 3.4**).[14] In order to prevent the oxidation and decomposition of the dopamine as it formed, H_2 gas was bubbled through the refluxing reaction. 5-Fluorodopamine was synthesized in 90% to quantitative yields using this method.

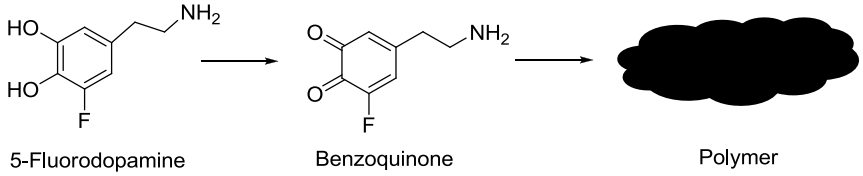


Figure 3.4: The decomposition of Catechols. When exposed to oxygen catrechols auto-oxidize to benzoquinones. This process is facilitated by an aqueous environment. The benzoquinone is a reactive intermediate that can easily polymerize.

The crystal structure of 5-Fluorodopamine was solved (**Figure 3.5)**. 5-Fluorodopamine crystallized in the $P2_{1/C}$ space group with a maximum resolution 0.84 Å. The extended structure was made up of layers of hydrophobic ring stacking interactions and hydrogen bonding networks between the amine, phenols, and the bromide ion. In combination with ^{1}H-NMR, ^{19}F-NMR and ^{13}C-NMR, this crystal structure provided proof of structure for the final product.

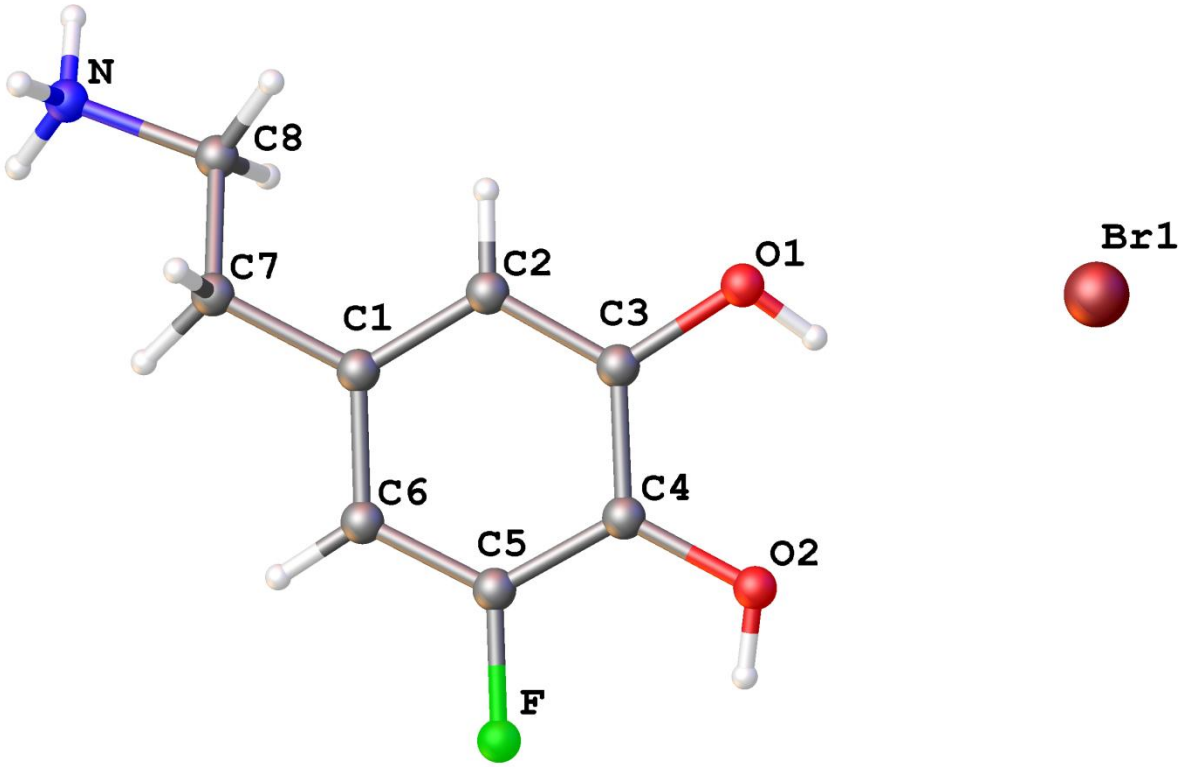


Figure 3.5: The crystal Structure of 5-Fluorodopamine.

Table 3.1: Crystal and Structure Refinement Data for 5-fluorodopamine. The .res file is included in appendix B.

Compound	**5-Fluorodopamine**
Formula	$C_8H_6BrFNO_2$
Fw	252
space group	$P2_{1/C}$
a, Å	10.822 (3)
b, Å	12.884 (3)
c, Å	6.845 (2)
α, deg	90
β, deg	92.384 (10)
γ, deg	90
V, Å^3	953.5 (5)
Z	4
ρ_{calc}, g/cm^{-3}	1.721
F_{000}	484
μ(Mo Kα), mm^{-1}	0.139
λ, Å	0.71073
temp, K	300
R; R_w	0.092; 0.258
GOOF	1.05

Chapter 4
Feeding Experiments and Initial Results

The first step in setting up feeding experiments was to pick an ideal organism. For the purposes of this project, a producer of the alkaloid berberine was targeted. The organism chosen was *Berberis stenophyllia* from the Berberidaceae family. This species is a known producer of Berberine as well as a well documented trove of other alkaloids. A cell culture of *Berberis stenophyllia* was purchased from Deutche Sammlung von Mikro-organismen und Zellkulturen (DSMZ) and these cell cultures are continuously re-plated in-house on LS media. A typical feeding experiment involves transferring cells from a plated cell culture to a liquid media in which the precursor compound is dissolved. Another feeding option is to pre-cultivate friable cell cultures in liquid media. Friable cell cultures consist of numerous small cell colonies that posses increased surface area as opposed to plate colonies which tend to be large and globular. Upon completing the synthesis of 5-fluorodopamine (**12**), initial feeding experiments were set underway.

The first feeding experiment was designed as a test reaction to see how the cells would respond to an environment of exogenous 5-fluorodopamine. The contents of one plate of cell culture was transferred to a sterilized 1 L Erlenmeyer flask which was filled with 100 mL of liquid LS media supplemented with 1.0 mM 5-fluorodopamine. After three days of incubation, the media had become visibly darkened. The results were not ideal as it was thought that the fluorinated dopamine had oxidized and polymerized after being dissolved in the media (**Figure 3.4**). In order to test this hypothesis, a sample of 5-fluorodopamine was incubated with liquid LS

media and a black substance precipitated overnight. In order to give the cells a chance to absorb the precursor, a new strategy had to be developed.

A procedure to acetylate the phenols in order to prevent the auto-decomposition of dopamine analogues is currently being developed (**Figure 4.1**). Acetylation of the phenols was chosen as an ideal protective group because plant cells contain a large number of deacetylating enzymes. In theory, the plant should absorb the acetylated precursor and deacetylate it *in vivo*. Both phenols of dopamine were selectively acetlyated (avoiding acetylation of the amine) using acetyl chloride in trifluoroacetic acid to produce diacetyl dopamine in 60% yield.[15] An experiment comparing the tendency of acetyl dopamine and dopamine to decompose was conducted. The rate of decomposition of acetylated dopamine visually appeared to be much slower. In comparison to dopamine the half life of acetyl dopamine is estimated to be roughly 2-5 times as long. This amount of time will hopefully be long enough for the culture to absorb the precursor before it oxidizes. The next steps in this project are to acetylate the fluorinated dopamine analogues, as well as examine the first feeding experiment to determine if any 5-fluorodopamine was incorporated into alkaloid synthesis before it decomposed.

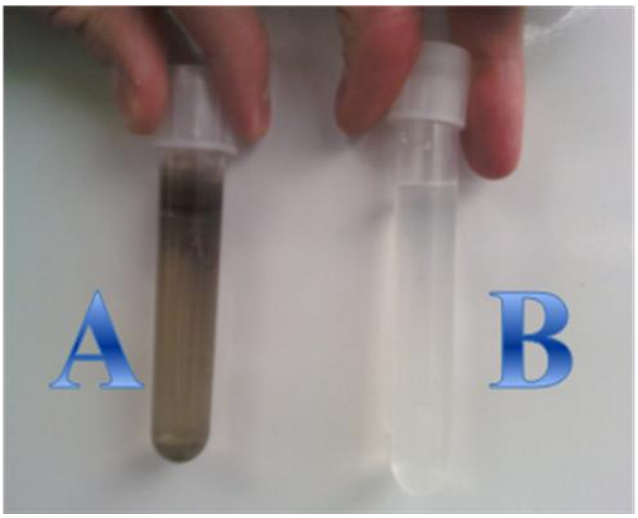


Figure 4.1: Auto-Decomposition of Dopamine. After 24 hrs dopamine visibly polymerizes in liquid LS media (A), however, acetylated dopamine remains unchanged (B).

UPLC-MSMS is needed in order to analyze the cell extracts for natural product analogues. Our research group is currently working on collaborating with the University of Illinois, Chicago in order use their instruments for analysis of our cellular extracts. In the mean time the cellular extracts containing alkaloids can be frozen for preservation.

Chapter 5

Galanthamine Project

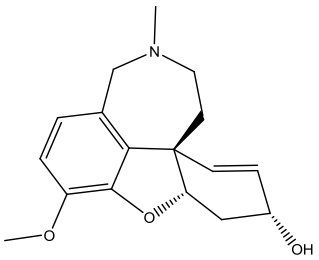


Figure 5.1: Galanthamine

Galantamine (GAL; **Figure 5.1**) is an alkaloid found in the bulbs and flowers of the common snowdrop and several other members of the Amaryllidaceae family. The structure is composed of four ring systems that form a rigid structure with minimal conformational mobility. In the early 1950s the extracted compound was used to treat nerve pain and was later found to be useful in treating many other ailments including facial nerve paralysis and schizophrenia.[17] The therapeutic effects of GAL are thought to be derived from its interaction with acetylcholinesterases (AChEs) and nicotinic acetylcholine receptors (nAChRs).[16-19] GAL binds AChE in human erythrocytes with an IC_{50} of 0.35 μM and neuronal AChE at a 10-fold lower potency.[16] GAL also sensitizes nAChRs of muscles and the brain. After FDA approval, the hydrobromide salt of GAL became marketed under the name Razadyne® ER as a prescription treatment for the symptoms of Alzheimer's disease (AD). GAL is currently being tested in clinical trials for the treatment of schizophrenia, Parkinson's Disease, and other neurological disorders. Clinical trials exhibit a minor improvement for treating the symptoms of AD, but GAL has not been proven to cure the underlying causes of AD.

The crystal structure of GAL bound to *Torpedo californica* acetylcholinesterase (TcAChE) has been reported.[16] GAL binds to this receptor with an IC_{50} of 0.652 μM. The relatively tight binding of GAL to TcAChE (**Figure 5.2**) is reversible and does not include any covalent linkage. GAL is not a transition state analogue, however, it does block the active site pocket and catalytic residues. The crystal structure shows that that GAL interacts with TcAChE via two hydrogen bond moieties and numerous hydrophobic interactions. The hydroxyl group of GAL forms a hydrogen bond with the carboxylic acid of Glu199 and the o-methoxy group of the phenyl ring acts as a hydrogen bond acceptor for the hydroxyl group on Ser200. The most prominent hydrophobic interactions seem to arise from pi-stacking interactions of the inhibitor with Phe288, Phe330, Phe331, and Trp84. The rigid geometry of the inhibitor ring systems allows GAL to conveniently fit in the active site pocket without the need to bind to the enzyme. Thus, the tertiary amine of GAL does not covalently interact with the catalytic residue that bind the amine of choline (Trp84), but rather blocks access to these sites.

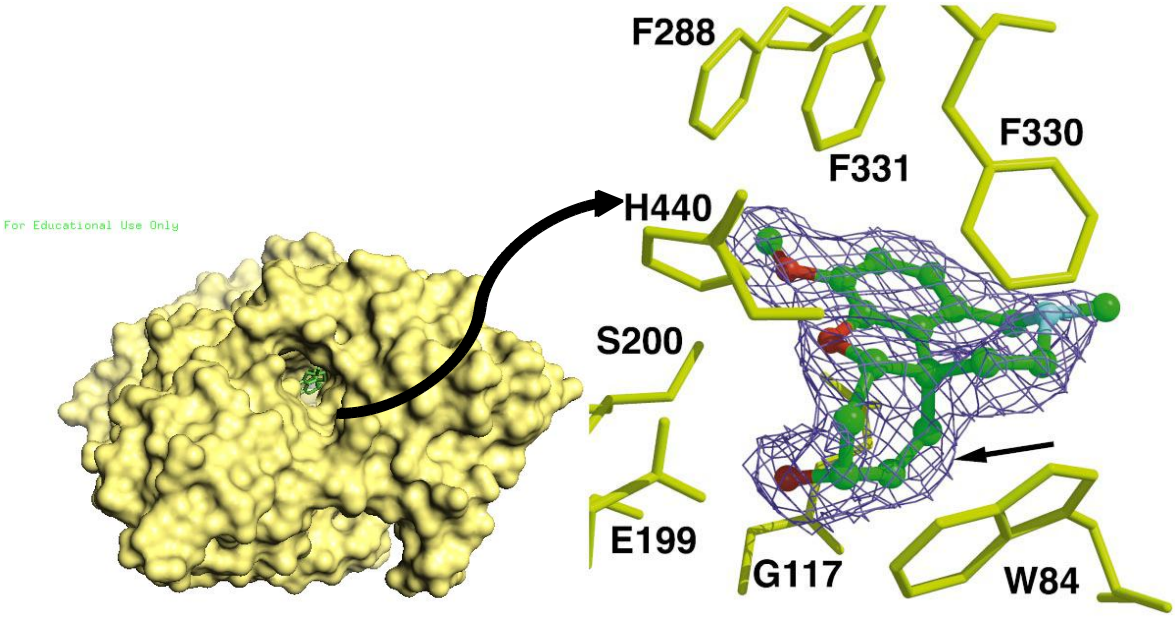


Figure 5.2: **Galanthamine and Acetylcholinesterase Crystal Structure.** Galanthamine bound in the active site pocket of *Torpedo californica* acetylcholinesterase (TcAChE).

Galanthamine represents an interesting target compound for PDB because of its well documented medicinal pharmacology.[16-19] The resolved crystal structure of bound GAL provides a platform to generate hypotheses about the pharmalogical activity of analogues. For example, a galanthamine analogue fluorinated at either X or Y (**Figure 5.3**) would have an altered hydrogen bonding network with the active site of its target enzyme. The two precursor metabolites for the biosynthesis of galanthamine are tyramine and protocatechauldehyde PCA (**Figure 5.4**). A chemical library of these analogues could potentially generate 100s of different galanthamine analogues. These analogues would have significantly different properties than galanthamine and could potentially be better drug candidates.

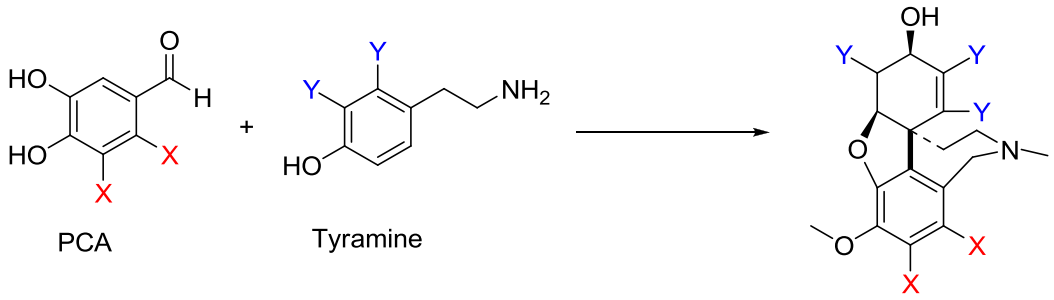


Figure 5.3: Primary Metabolites for the Biosynthesis of Galanthamine. PCA and Tyramine analogues can be used to generate natural product analogues of galanthamine.

The synthesis of fluorinated PCA and tyramine analogues is currently underway. The tyramine analogue 3-fluorotyramine (**15)** was synthesized from tyramine in 1 step using selectflour in 38% yield. The fluorinated product, however, remains as a mixture with some un-reacted tyramine. A prep-HPLC method is currently being developed in order to isolate pure 5-fluorotyramine. The PCA analogue 2-fluoro-3,4-dihydroxy benzaldehyde (**23**) can be easily generated in one step from 5-fluorovanillin. This reaction has not yet been performed because the product, a catechol, is sensitive to auto-oxidation. The product will be synthesized when daffodil cell cultures are cultivated.

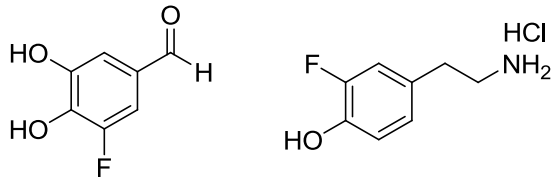


Figure 5.4: Analogue Precursor Targets for Galanthamine Project. Compound **23**; The PCA analogue 2-fluoro-3,4-dihydroxy benzaldehyde. Compound **15**; The tyramine analogue 3-fluorotyramine.

In order to initiate PDB experiments viable cell cultures need to be cultivated. Our group has been attempting to generate these cultures for the previous six months with little success. Upon completing the synthesis of precursor analogues for this project, a new focus will be to generate viable culture. The daffodil bulb extracts show that the species our group is working with produce large amounts of galanthamine. The most promising aspect of galanthamine project is that the secondary metabolites can be analyzed in-house by GC-MS (**Figure 5.5**).

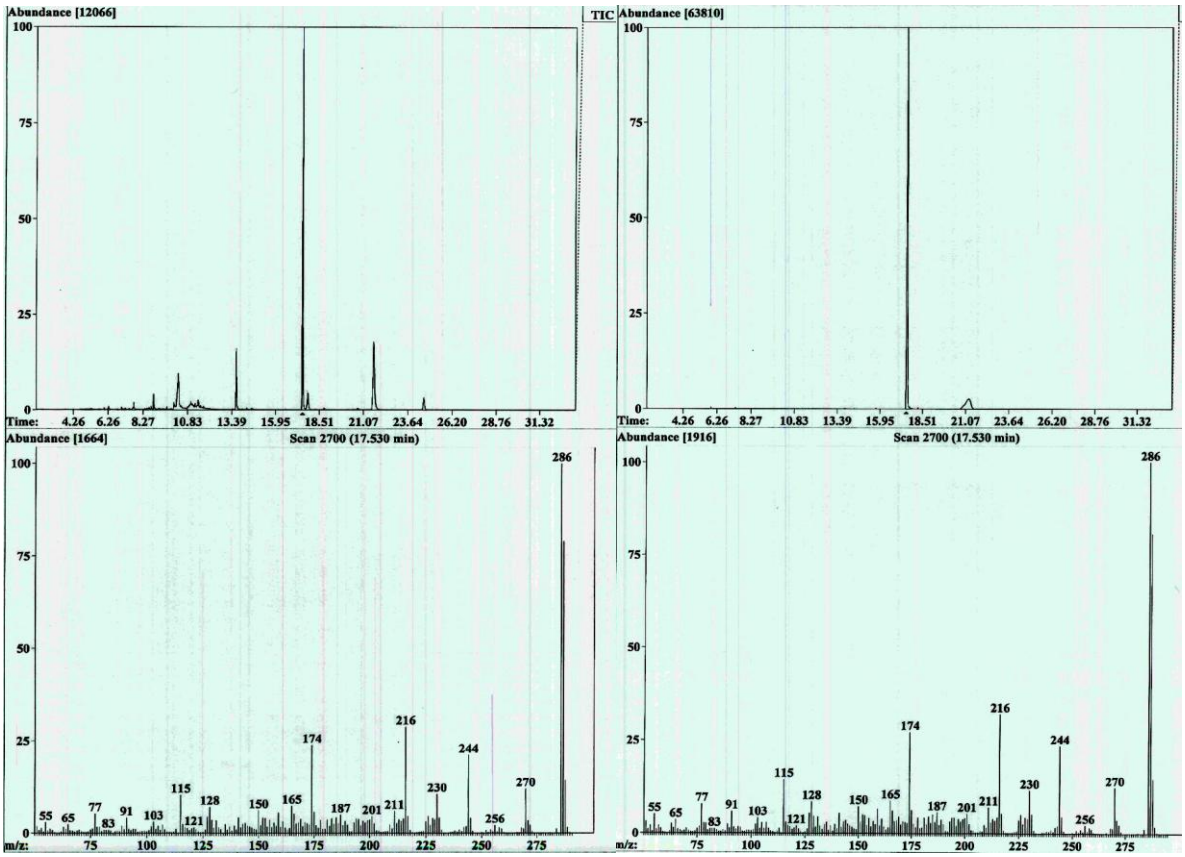
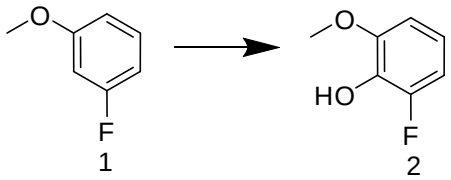


Figure 5.5: Galanthamine GC-MS. The mass spectrum on the left is daffodil bulb extract and the mass spectrum on the right is a galanthamine standard.

Chapter 6
Synthetic Procedures

Methods. All reagents used were purchased from Sigma-Aldrich. GC-MS analysis was performed using a Hewlett Packard HP6890 GC System with an Agilent Technologies 5975 inert mass selective detector. HPLC analysis was performed using a Waters Aquity Ultra Performance liquid chromatography instrument equipped with an Aquity UPLC BEH C18 (1.7 µm) 2.1x100 mm column. Using a Bruker Avance 300 MHz instrument ^{1}H-NMR, ^{13}C-NMR and ^{19}F-NMR spectra were acquired at 300 MHz, 100 MHz and 282 MHz respectively. ^{1}H-NMR chemical shifts and ^{13}C-NMR chemical shifts are relative to $(CH_3)_4Si$, and ^{19}F-NMR chemical shifts are referenced to an internal standard of $CFCl_3$.



2-Fluoro-6-methoxyphenol (2). 4.0 mL (9.4 mmol) of 2.36 M n-butyl lithium was added dropwise over 1 hr to a solution of 1.28 g (10 mmol) fluoroanisole (**1**) in 10.0 mL dry THF kept below -65 ^{o}C by a dry ice/acetone bath. The solution was stirred for 2 hrs at -78 ^{o}C under nitrogen. 1.17 g (11 mmol) $B(OMe)_3$ in 2.00 mL dry THF was added dropwise over 1 hr at -78 ^{o}C and the solution was stirred for an additional 0.5 hr at -78 ^{o}C. The solution was warmed to 0 ^{o}C and 0.88 mL (15 mmol) AcOH was added followed by the dropwise addition of 1 mL (10 mmol) of 30% H_2O_2. The solution was stirred overnight at 25 ^{o}C. The solution was diluted with H_2O (10 mL) and extracted with THF (2 X 10 mL). The combined ether extracts were washed

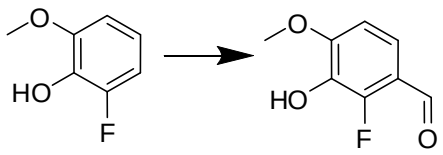
with H_2O (2 X 6.66 mL) and 10% Mohrs salt (2 X 3.33 mL). The ether phase was dried with $MgSO_4$ and evaporated under reduced pressure to yield 1.16 g (**2**) (80%). Reaction scale, (yield): 174 mmol, (63%); 261 mmol, (80%). ^{1}H NMR (300 MHz, *CDCL$_3$*) δ 6.82 – 6.63 (m, 1H), 5.51 (s, 1H), 3.90 (s, 1H). ^{19}F NMR (282 MHz, *CDCl$_3$*) ppm -137.61 (m, 1F). *GCMS-EI*: m^+ 142, 127.

Observations/Notes

The nmr/gc/uplc suggest that the reaction was site specific adding solely at the specified position. A good deal of product (roughly 20-30% was lost during vacuum distillation). This was a result of ungreased joints and a relatively weak house vacuum. I believe that these losses could be reduced if the experiment is repeated. A slower addition would also be beneficial; however, the experiment already takes a long time. There was no way to cool the liquid being added because it was in a syringe. If the n-butyl lithium could have been cooled before it was added the yield may be improved.

Reference

Ladd, D. L.; Weinstock, J. *J. Org. Chem.* **1981**, 46, 203.



2 3

2-Fluoro-3-hydroxy-4-methoxybenzaldehyde (3). A solution of 0.500 g (3.5 mmol) 2-Fluoro-6-methoxyphenol (**2**) in 5 mL trifluoroacetic acid was added dropwise over 1 hr to a refluxing solution (80 ^{o}C) of 0.986 g (7.0 mmol) hexamethylenetetramine in 5 mL trifluoroacetic acid.

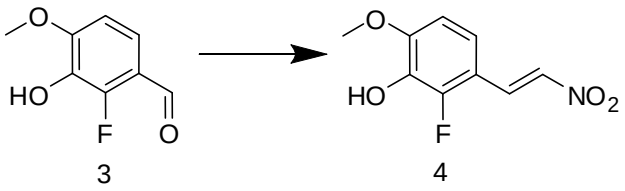
The reaction was refluxed for 1 hr, allowed to cool to room temp and concentrated under vacuum. 25 mL of ice water was added and the reaction was stirred for 15 min. The pH of the reaction mixture was neutralized by the addition of solid potassium carbonate. The reaction slurry was stirred for 20 min, extracted with ether (3 X 25 mL), washed with water (3 X 25), dried with $MgSO_4$ and evaporated under vacuum to yield 0.3378 g (56%) of 2-fluoro-3-hydroxy-4-methoxybenzaldehyde (**3**). Scale, % yield: 3.5 mmol, 40%; 3.5 mmol, 34%; 56 mmol, 14%; 100 mmol, 28%. ^{1}H NMR (300 MHz, DMSO) δ 10.03 (s, 1H), 9.65 (s, 1H), 7.32 (dd, *J* = 8.7, 7.5 Hz, 1H), 6.99 (dd, *J* = 8.8, 1.2 Hz, 1H), 3.91 (s, 2H). ^{19}F NMR (282 MHz, DMSO) δ -135.60 (ddd, *J* = 9.4, 2.3, 1.1 Hz). *GCMS-EI*: m^+ 142; b.p. 127.

Observation/Notes

This reaction worked a lot better on a smaller scale. The small scale reaction resulted in mainly one isomer while scaled up reactions resulted in formulation at all three positions (the isomer with F para to the aldehyde was only ~1%) with differnet ratios each time. On a large scale the desired isomer (**3**) ultmately became the minor product to 3-fluoro-4-hydroxy-5-methoxybenzaldehyde (**5**) which was also a desired product. GCMS and UPLC showed that the ratio between **(3)** and **(5)** was 40:60 on the largest scale reaction. The lower yield on larger scale reactions may have resulted from an inability to remove trifluoroacetic acid which led to difficult extraction. For an unknown reason the trifluoroacetic acid was very difficult to evaporate, even with heat. The *J* coupling of 9.4 Hz for the fluorine was interesting because no proton exhibited direct coupling of 9.4 Hz. This may be a result of the F NMR being aquired in D-acetone, or a complex splitting system.

Reference

Clark, M. T.; Miller, D. D. *J. Org. Chem.* **1986**, 51, 4072.



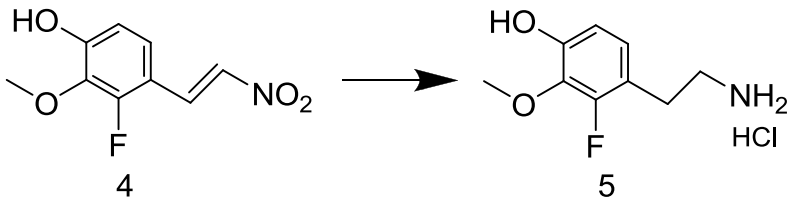
(E)-2-fluoro-6-methoxy-3-(2-nitrovinyl)phenol (4). A reaction mixture of 0.800 g (4.7 mmol) 2-Fluoro-3-hydroxy-4-methoxybenzaldehyde (3), 1.44 g (24 mmol) nitromethane, 0.363 g (4.7 mmol) ammonium acetate, and 5.0 mL acetic acid was refluxed for 6 hours. After cooling, the reaction mixture was diluted with water (15 mL) and extracted with ether (3 X 25 mL). The combined ether extracts were washed with water (1 X 25 mL), dried with $MgSO_4$, and evaporated under reduced pressure. 0.65 g (65%) of (E)-2-fluoro-6-methoxy-3-(2-nitrovinyl)phenol was collected without further purification. ^{1}H NMR (300 MHz, DMSO) δ 9.68 (s, 1H), 8.02 (q, *J* = 13.7 Hz, 1H), 7.41 (t, *J* = 8.3 Hz, 1H), 6.95 (d, *J* = 8.9 Hz, 1H), 3.89 (s, 1H). ^{19}F NMR (282 MHz, DMSO) δ -134.04 (dd, *J* = 7.8, 1.6 Hz).

Observation/Notes

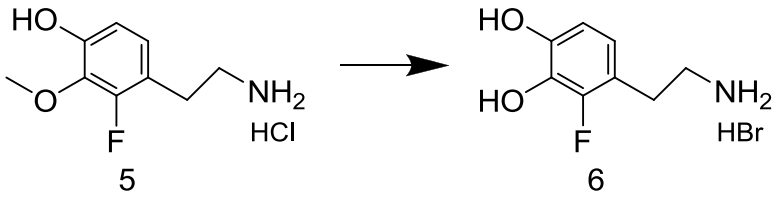
The fluorinated analog of vanillin requires much more time to react than vanillin. The reflux should be done for at least 7 hours (or until complete by TLC). Ethyl acetate was also found to be a much more effective solvent for extraction. The extraction process may require up to 5 extractions with ethyl acetate. As long as the reaction goes to completion the only impurities should be acetic acid and nitromethane. These impurities can be mostly washed away and the product can be recrystallized from a 75:25 water/methanol solution.

References

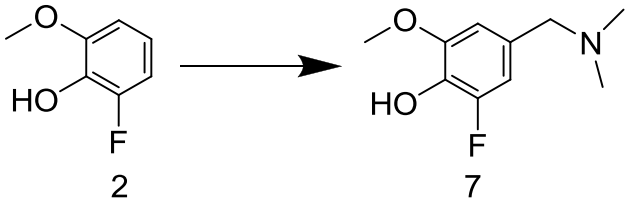
J. Org. Chem., Vol. 41, No. 14, 2372 ***1976***



3-(2-aminoethyl)-2-fluoro-6-methoxyphenol hydrobromide (5). This compound will be made using the same method as 4-(2-aminoethyl)-2-fluoro-6-methoxyphenol hydrochloride (**11**). The starting compound needs to be isolated from an isomeric mixture with (E)-2-fluoro-6-methoxy-4-(2-nitrovinyl)phenol (**10**).



3-(2-aminoethyl)-2-fluoro-6-methoxyphenol hydrobromide (2-Fluorodopamine) (6). This compound will be made using the same method as 2-Fluoro-3,4-dihydroxyphenethylamine Hydrobromide (5-Flurodopamine HBr) (**12**).



***N,N*-Dimethyl-3-hydroxy-4-methoxy-5-fluorobenzylamine (7).** 2-Fluoro-6-methoxyphenol 13.0 g (91 mmol) was added to a solution of 19.5 g (160 mmol) 40% dimethylamine and 11.7 mL (160 mmol) 37% formaldehyde in 91 mL of absolute ethanol. The reaction mixture was heated at reflux for 2 hours, cooled to room temperature, and concentrated under vacuum to a white solid. The solid was triturated with ether to give 17.25 g (95%). Scale, % yield: 8.5

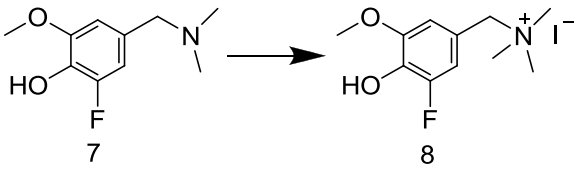
mmol, 72%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.67 (t, *J* = 4.6 Hz, 1H), 3.90 (s, 1H), 3.32 (s, 1H), 2.23 (s, 3H). ^{19}F NMR (282 MHz, $CDCL_3$) δ -138.08 (dd, *J* = 11.1, 1.7 Hz).

Observation/Notes

This reaction can easily be scaled up with high yields. Also, the product does not need to be purified before continuing with next step.

Reference

Ladd, D. L.; Weinstock, J. *J. Org. Chem.* **1981**, 46, 203.

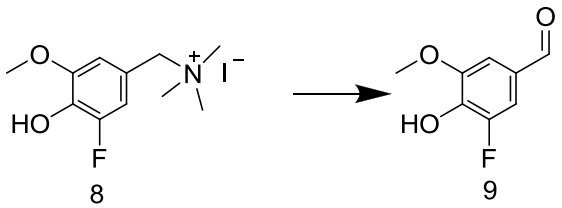


7 8

1-(3-fluoro-4-hydroxy-5-methoxyphenyl)-N,N,N-trimethylmethanaminium iodide (8). Iodomethane (170 mL) was added to a solution of *N*,*N*-Dimethyl-3-hydroxy-4-methoxy-5-fluorobenzylamine 17.0 g (85 mmol) in $CHCl_3$ (850 mL). The mixture was stirred overnight at room temperature. The solution was filtered and an off-white solid was collected 25.6 g (78%). Scale, % yield: 5 mmol, 100%. ^{1}H NMR (300 MHz, DMSO) δ 9.78 (d, *J* = 1.8 Hz, 1H), 7.14 – 6.86 (m, 2H), 4.41 (d, *J* = 7.0 Hz, 2H), 3.85 (d, *J* = 2.7 Hz, 3H), 3.13 – 2.84 (m, 9H). ^{19}F NMR (282 MHz, DMSO) δ -135.24 (dd, *J* = 25.8, 10.4 Hz).

Reference

Ladd, D. L.; Weinstock, J. *J. Org. Chem.* **1981**, 46, 203.



8 9

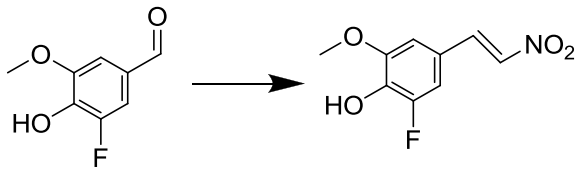
3-Methoxy-4-hydroxy-5-fluorobenzaldehyde (9). A solution of (3-fluoro-4-hydroxy-5-methoxyphenyl)-N,N,N-trimethylmethanaminium iodide 25.4 g (74 mmol) in acetic acid (65 mL) and H_2O (65 mL) was heated to reflux. Hexamethylenetetramine 40 g (285 mmol) was added to the refluxing solution in one portion. The mixture was stirred at reflux for 2 hrs and then concentrated HCl (16.4 mL) was added. The solution was stirred an addition 5 minutes, cooled, and extracted with ether (3 X 175 mL). The organic layer was washed with H_2O (2 X 175 mL), dried with $MgSO_4$ and concentrated under vacuum to give (9.00 g, 72%) of a white powder. Scale, % yield: 5 mmol, 60%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.75 (s, 1H), 7.35 (d, *J* = 10.3 Hz, 1H), 7.31 (s, 1H), 3.88 (s, 3H). ^{19}F NMR (282 MHz, C_6D_6) δ -134.71 (d, *J* = 10.2 Hz).

Observation/Notes

The product of this reaction smelled like ammonia (or some other amine). The product can be purified by sublimation or should be dissolved in an organic solvent and washed with water until amines are no longer present.

Reference

Ladd, D. L.; Weinstock, J. *J. Org. Chem.* **1981**, 46, 203.



9 10

(E)-2-fluoro-6-methoxy-4-(2-nitrovinyl)phenol (10). A reaction mixture of 3-Methoxy-4-hydroxy-5-fluorobenzaldehyde 2.55 g (15 mmol), nitromethane 4.58 g (75 mmol), ammonium acetate 1.18 g (15 mmol), and acetic acid (15 mL) was refluxed for 7 hours under nitrogen. After cooling the reaction mixture was diluted with water (30 mL) and extracted with ethyl acetate (5 X 50 mL). The combined organic extracts were washed with water (3 X 75 mL), dried

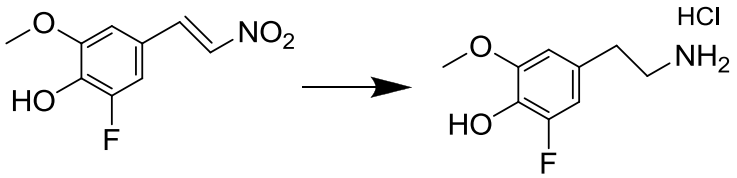
with $MgSO_4$, and evaporated under reduced pressure. (E)-2-fluoro-6-methoxy-4-(2-nitrovinyl)phenol 3.10 g (97%) was collected as a red powder without further purification. ^1H NMR (300 MHz, DMSO) δ 8.09 (dd, *J* = 45.6, 13.3 Hz, 2H), 7.41 (d, *J* = 11.6 Hz, 1H), 7.35 (s, *J* = 11.8 Hz, 1H), 3.85 (s, 3H). ^{19}F NMR (282 MHz, DMSO) δ -135.35 (d, *J* = 11.5 Hz).

Observation/Notes

The reaction should be allowed ample time to complete as side products were generally not formed and nearly quantitative yields of the product can be achieved. The extraction process may require that the aqueous washed be re-extracted with ethyl acetate. As long as the reaction goes to completion the only impurities should be ethyl acetate, acetic acid, and nitromethane. These impurities can be mostly washed away and the product can be recrystallized from a 75:25 water/methanol solution. When a 50:50 water/methanol solution was used only 57% of the product was collected. Due to limited solubility of the product in water, increasing the water ratio during recrystalization was found to improve recovery.

References

J. Org. Chem., Vol. 41, No. 14, 2372 ***1976***



10 11

4-(2-aminoethyl)-2-fluoro-6-methoxyphenol hydrochloride (11). A 25 mL round bottom flask was charged with 200 mg 5% palladium on carbon, 1 mL concentrated HCl, and 10 mL MeOH. The flask was sealed, placed in an ice bath and equilibrated under a hydrogen filled

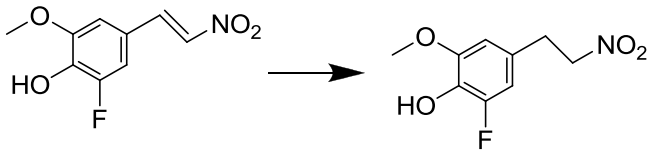
balloon for 30 minutes. E-2-fluoro-6-methoxy-4-(2-nitrovinyl)phenol (**10**) 0.213 g (1 mmol) was added and the reaction mixture was stirred vigorously over night in a refrigerator (2^{o} C) under a hydrogen filled balloon. The next day the reaction was stirred under a new hydrogen filled balloon for 5 hours at room temperature. The reaction mixture was then filtered through celite and the celite (containing catalyst) was washed with 40 mL of additional methanol. The methanol was evaporated under reduced pressure and the residue was dried overnight in a vacuum desicator to yield 0.200 g (90%) (**11**) as an off white powder. ^{1}H NMR (300 MHz, DMSO) δ 9.06 (s, 1H), 7.89 (s, 3H), 6.69 (dd, *J* = 9.6, 1.9 Hz, 2H), 3.80 (s, 3H), 3.01 (s, 2H), 2.77 (t, *J* = 7.6 Hz, 2H). ^{19}F NMR (282 MHz, DMSO) δ -135.70 (d, *J* = 10.2 Hz).

Observation/Notes

The reaction was also completed using 2X the mass of 5% palladium on carbon catalyst. This reaction should not produce any side products visualized by UPLC, however, a strange "3-peak" is normally observed on the proton NMR with a chemical shift of 7.20 (t, *J* = 51.7 Hz). This "3-peak" involves exchangeable protons (D_2O) and can be removed by washing the product with dried ($MgSO_4$) ice-cold acetone and acetonitrile separately, followed by dry ether. The reaction has been scaled up to 3 mmol, however, the yield was reduced to 50% after washings. If the product is an oil, it can be dissolved in THF and re-crystalized by dripping into ether. The resulting solid should be centrifuged in order to concentrate for collection. If the product has "3-peak" impurities it will be hygroscopic and oil up during vacuum filtration. Also, it may be helpful to sonicate the crude product of the reaction in acetonitrile, or a mixture of acetonitrile and ether.

Reference

Bull. Chem. Soc. Jpn., **63**, 1252-1254 (1990)



10 13

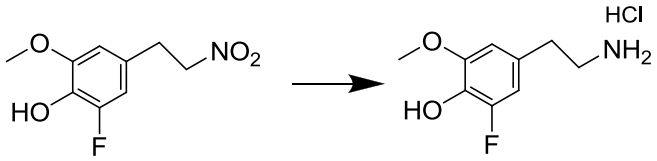
2-fluoro-6-methoxy-4-(2-nitroethyl)phenol (13). Sodium borohydride, 0.210 g (5.6 mmol) was dissolved in 7 mL of ethanol and cooled to 0^{o} C under nitrogen. E-2-fluoro-6-methoxy-4-(2-nitrovinyl)phenol (**12**), 0.570 g (2.5 mmol) was dissolved in 20 mL of ethanol and added dropwise to the sodium borohydride solution over 1.66 hours. Following the addition, the reaction mixture was stirred in a refrigerator for 26 hours. After storage, the solution was made slightly acidic with 4 M HCl and diluted with H_2O (20 mL). The solution was extracted with ether (3 X 20 mL) and the organic phase was washed with brine (1 X 20 mL), dried with $MgSO_4$, and evaporated under reduced pressure to obtain ~1.0 g of oil. The oil was extracted with ether (leaving behind a slightly orange solid) and evaporated under reduced pressure. The residue was sonicated and extracted into toluene and the solvent was removed under reduced pressure to yield 0.5359 g (99%) of an orange oil. The product was verified by UPLC.

Observation

The work-up of this reaction can be difficult when it comes to removing the borohydride salts, which seem to be somewhat organic soluble. Toluene was used in order to extract product from an impure mixture, however, any method that isolates product from borohydride salts should be sufficient. Also, the product of this reaction and of test reactions involving non-halogenated vanillin nitrostyrenes were generally oils.

References

J. Org. Chem., Vol. 41, No. 14, 1976



13 11

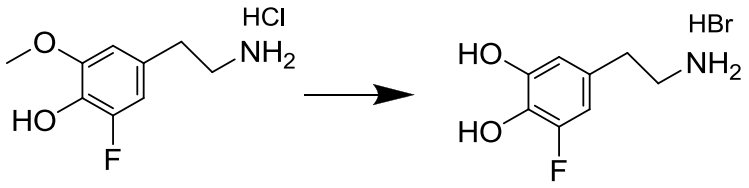
2-Fluoro-3-hydroxy-4-methoxyphenethylamine hydrochloride (11). A 50 mL round bottom flask was charged with 2-fluoro-6-methoxy-4-(2-nitroethyl)phenol (**13**) 0.536 g (2.5 mmol), 10% Pt/C 0.125 g, and 20 mL of ethanol. The reaction mixture was stirred under a hydrogen balloon for 3 days and then 0.125 g of 10% Pt/C was added and the reaction was stirred for an additional day. The mixture was filtered through celite using an additional 20 mL of ethanol and 0.5 mL of concentrated HBr was added to make solution acidic. The solvent was removed by vacuum and a brown oil was collected. The oil was diluted with 10% Acetonitrile/Ether and sonicated to give a white solid. The NMR results were inconclusive.

Observations/Notes

This reaction was monitored by UPLC and after 24 hrs the reaction appeared to be about 50% complete. Over the course of the next few days side products built up and the starting material remained present. The NMR results suggest that the desired product was made, however, the product was never separated from the other species present in the NMR so it remains uncertain whether the final product contained two different compounds or one compound that was a side product which formed during the reduction. This reaction was based off a procedure in which a catalytic hydrogenator was used in order to generate a reducing environment at 40 psi whereas this reaction proceeded at roughly 14.7 psi of H_2.

References

J. Org. Chem., Vol. 41, No. 14, 1976



11 12

2-Fluoro-3,4-dihydroxyphenethylamine Hydrobromide (5-Flurodopamine HBr) (12). Hydrogen gas was bubbled through 13 mL of refluxing concentrated hydrobromic acid which was closed off from the atmosphere. After 20 minutes, 2-Fluoro-3-hydroxy-4-methoxyphenethylamine hydrochloride (**11**) 0.290 g (1.3 mmol) was added and the mixture was refluxed for 3 hours while hydrogen gas was continually bubbled through. The excess hydrobromic acid was removed by vacuum and the resulting product was dissolved in methanol (15 mL) and evaporated to dryness under vacuum. This step was repeated again using methanol (15 mL), followed by H_2O (15 mL), and finally methanol (15 mL). After the final evaporation the product was placed in a vacuum desicator overnight and 0.367 g (>100%) of the hydrobromide salt was collected. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87 (s, 5H), 6.52 (dd, *J* = 11.3, 2.0 Hz, 1H), 6.48 (d, *J* = 1.4 Hz, 1H), 3.02 – 2.90 (m, 2H), 2.74 – 2.62 (m, 2H). ^{13}C NMR (75 MHz, DMSO) δ 152.41 (d, *J* = 237.2 Hz), 148.10 (d, *J* = 6.3 Hz), 132.34 (d, *J* = 14.3 Hz), 127.92 (d, *J* = 8.8 Hz), 112.31 (s), 107.17 (d, *J* = 19.3 Hz), 40.43 (s), 32.74 (s). ^{19}F NMR (282 MHz, DMSO) δ -135.23 (dd, *J* = 11.2, 1.2 Hz).

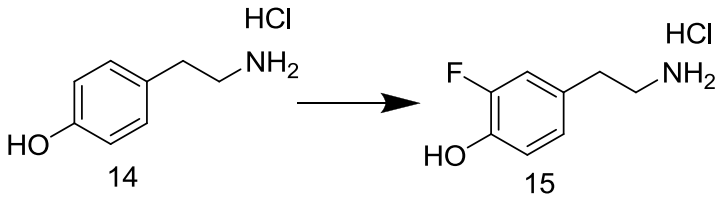
Notes/Observations

The starting material for this reaction needs to be pure. The reaction mixture turns black if the starting material is impure and the product is a black tar. The product can be purified by

dissolving it in hot ethanol and adding ether as the solution cools. The best results were obtained when the reaction was kept under a hydrogen atmosphere. For the best results an apparatus should be created to bubble hydrogen through the refluxing reaction. The slow diffusion of ether into isopropanol works well to re-crystallize the product.

References

J. Org. Chem., Vol. 41, No. 14, 1976



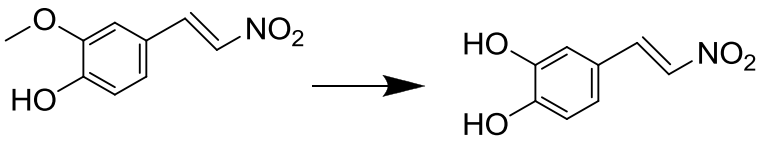
4-(aminomethyl)-2-fluorophenol hydrochloride (3-Fluorotyramine Hydrochloride) (15). Tyramine Hydrochloride (4.00 g, 23 mmol) and selectfluor (1-Chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate)) (8.24 g, 23.2 mmol) were dissolved in 104 mL of 50/50 methanol:water and stirred at room temperature for 72 hrs. The solvent was removed from the reaction mixture under vacuum, and the crude product was dissolved in 80 mL methanol and filtered. The solvent was removed from the filtrate and the product was precipitated from acetonitrile (40 mL) to give 1.61 grams (37%). UPLC, NMR, and GCMS analysis showed a mixture of 3-Fluorotyramine hydrochloride and tyramine Hydrochloride. ^{1}H NMR (300 MHz, DMSO) δ 9.36 (s, 1H), 8.04 (s, 2H), 7.12 – 6.98 (m, 1H), 6.98 – 6.81 (m, 1H), 3.06 – 2.87 (m, 2H), 2.86 – 2.67 (m, 2H). ^{19}F NMR (282 MHz, DMSO) δ -136.31 (dd, *J* = 12.3, 8.9 Hz).

Observations/Notes

The product of this reaction can be purified by dissolving in hot ethanol and precipitating with ether. The product appears to contain a 60:40 mixture of fluorinated to non-fluorinated tyramine. A more accurate analysis of the desired product is pending on separation of the mixture by HPLC. I think that a majority of the product may have been lost because it was not protonated and remained soluble in acetonitrile. If this reaction is perfomed again it might be a good idea to acidify the solution with concentrated HCl before precipitating with acetonitrile.

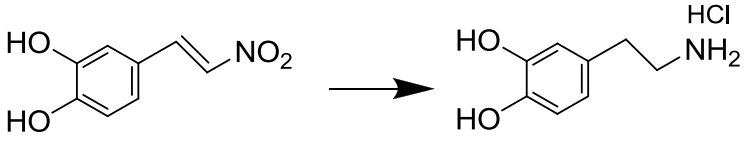
Reference

Anal. Chem. **1998,** 70, 2676-2684



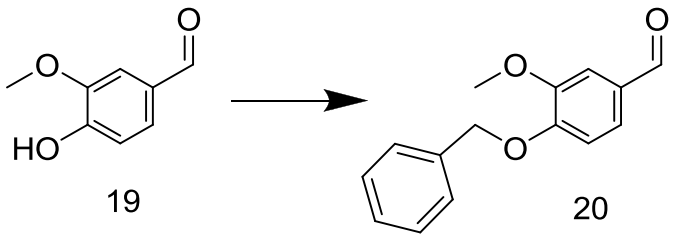
16 17

(E)-4-(2-nitrovinyl)benzene-1,2-diol (17). The success of this reaction is questionable. **16** (0.250 g), $AlCl_3$ (0.180 g) and 0.5 mL of pyridine were refluxed for 18 hours. The reaction mixture was extracted with $CHCl_3$, washed with H_2O, dired with $MgSO_4$ and evaporated to yield 0.200 g of a red/brown powder.^{13}C-NMR (75 MHz, DMSO) δ 150.85 (s, *J* = 7.7 Hz), 146.29 (s, *J* = 82.2 Hz), 140.82 (s, *J* = 55.6 Hz), 135.11 (s, *J* = 29.3 Hz), 124.20 (s, *J* = 56.9 Hz), 121.97 (s, *J* = 62.4 Hz), 116.89 (s, *J* = 10.2 Hz), 116.46 (s, *J* = 9.2 Hz).

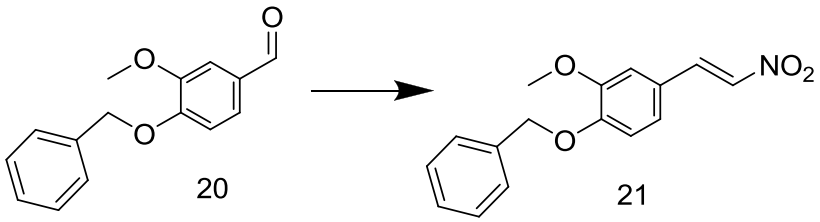


17 18

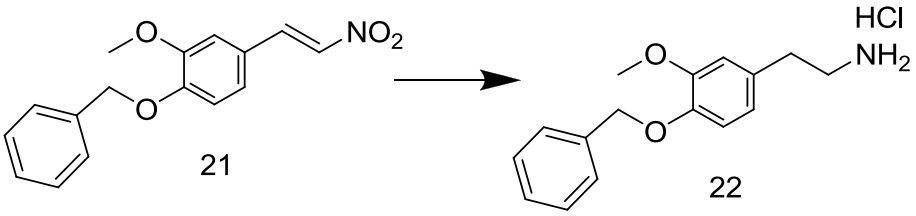
1,2-Dihydroxyphenethylamine hydrochloride (Dopamine) (18). Doapmine was unable to be synthesized from the same reaction conditions that generated 4-(2-aminoethyl)-2-fluoro-6-methoxyphenol hydrochloride (**11**).



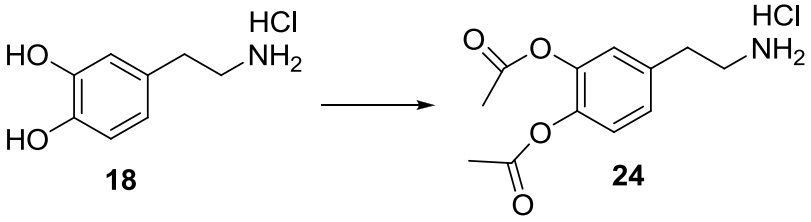
4-(benzyloxy)-3-methoxybenzaldehyde (20). Vanillin (1.52 g, 10 mmol), benzyl chloride (1.39 g, 11 mmol), KI (0.166 g, 1 mmol), and $NaHCO_3$ (0.958 g, 11.4 mmol) were refluxed in 10 mL acetonitrile for 17 hours. The solvent was removed by vacuum and **20** was extracted into ether. The organic extract was dried using $MgSO_4$ and evaporated to yield 2.215 g (91.5%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.91 – 9.76 (m, 1H), 7.47 – 7.32 (m, 7H), 6.99 (d, *J* = 8.2 Hz, 1H), 5.25 (s, 2H), 3.95 (s, 3H).



(E)-1-(benzyloxy)-2-methoxy-4-(2-nitrovinyl)benzene (21). The same procedure used to generate (E)-2-fluoro-6-methoxy-4-(2-nitrovinyl)phenol (**10**) resulted in 92% yield of **21** on an 8 mmol scale**.** ^{1}H NMR (300 MHz, DMSO) δ 8.23 (d, *J* = 13.5 Hz, 1H), 8.07 (d, *J* = 13.5 Hz, 1H), 7.52 (d, *J* = 1.9 Hz, 1H), 7.49 – 7.34 (m, 6H), 7.15 (d, *J* = 8.4 Hz, 1H), 5.18 (s, 2H), 3.83 (s, 3H).



2-(4-(benzyloxy)-3-methoxyphenyl)ethanamine hydrochloride (22). 22 was unable to be synthesized from the same reaction conditions that generated 4-(2-aminoethyl)-2-fluoro-6-methoxyphenol hydrochloride (**11).**



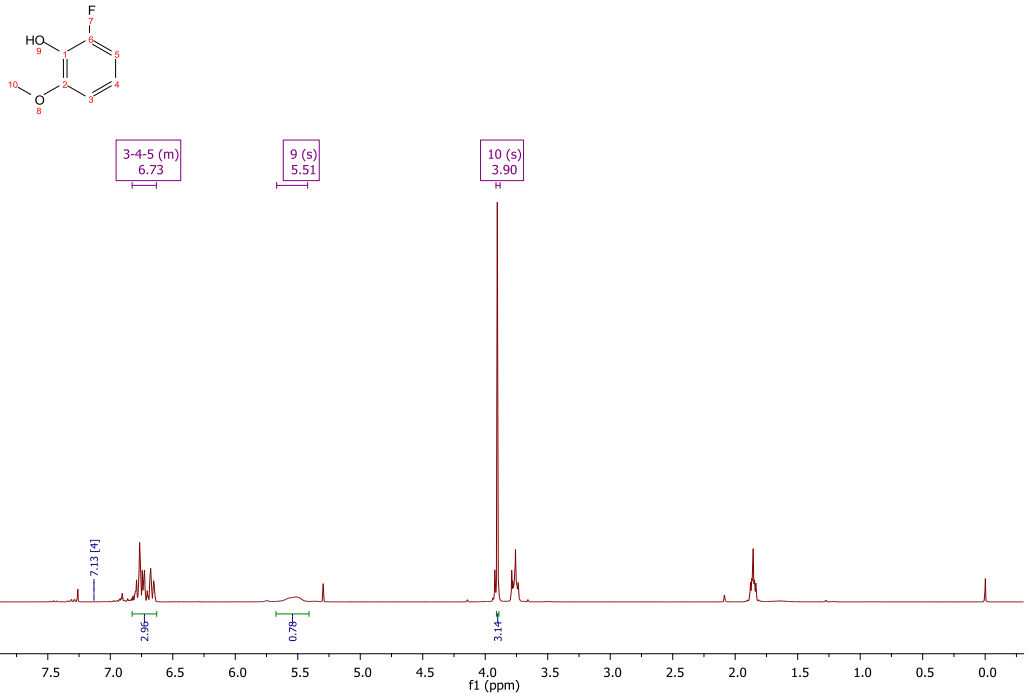
4-(2-aminoethyl)-1,2-phenylene diacetate hydrochloride (24). Dopamine (**18**) (0.379 g, 2 mmol) and acetyl chloride (0.628 g, 8 mmol) were stirred in 8 mL TFA under nitrogen for 4.5 hours at room temperature. 1 mL of H_2O was added and the volatiles were evaporated under vacuum. The product was triturated with ether and collected on filter paper to yield 0.325 g (60%). ^{1}H NMR (300 MHz, DMSO) δ 8.18 (s, 1H), 7.25 – 7.16 (m, 1H), 3.03 (dt, *J* = 7.7, 4.4 Hz, 1H), 2.92 (dd, *J* = 8.8, 5.7 Hz, 1H), 2.27 (s, 1H), 2.26 (s, 1H).

Appendix A

NMR SPECTRA

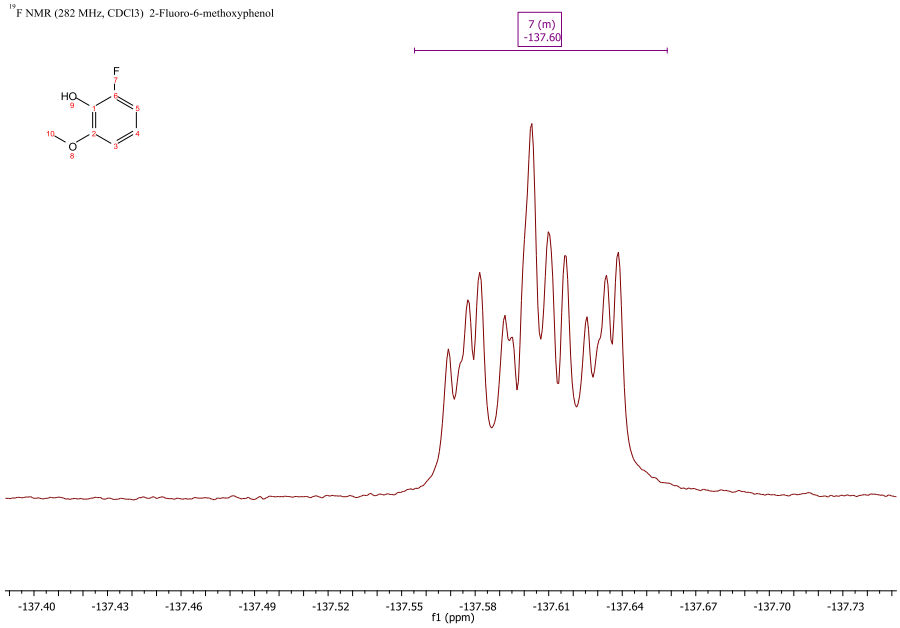
2-Fluoro-6-methoxyphenol

^{1}H NMR (300 MHz, CDCl3) 2-Fluoro-6-methoxyphenol



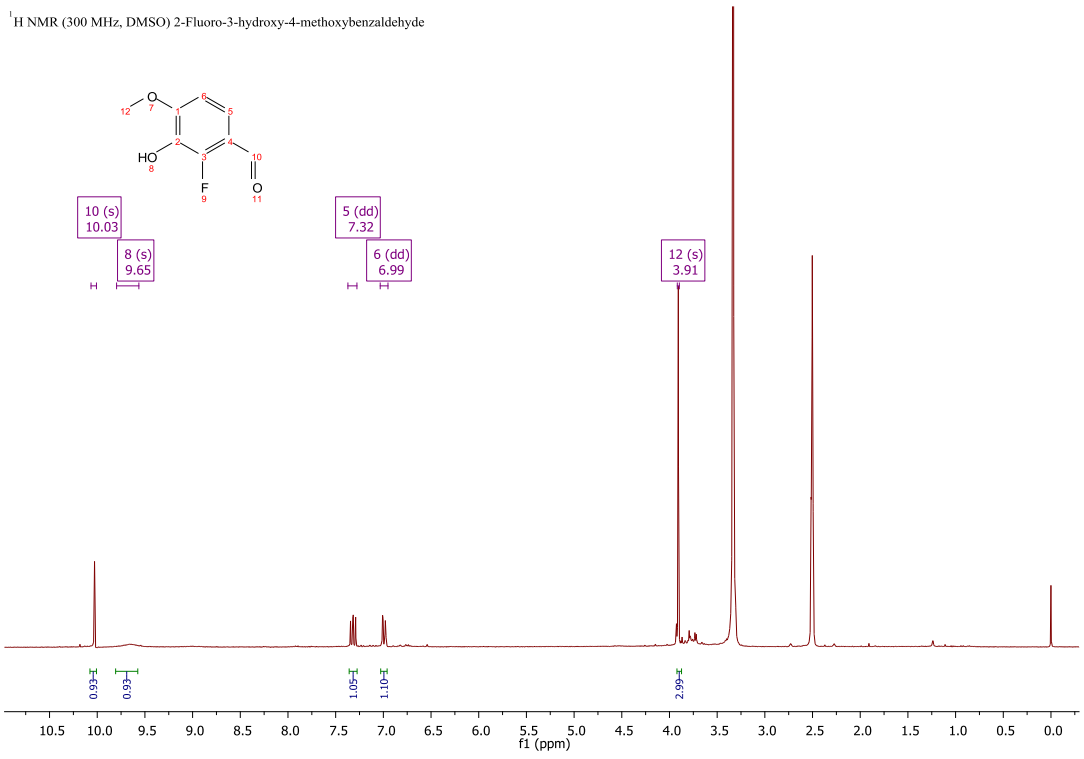
(2)

^{19}F NMR (282 MHz, CDCl3) 2-Fluoro-6-methoxyphenol



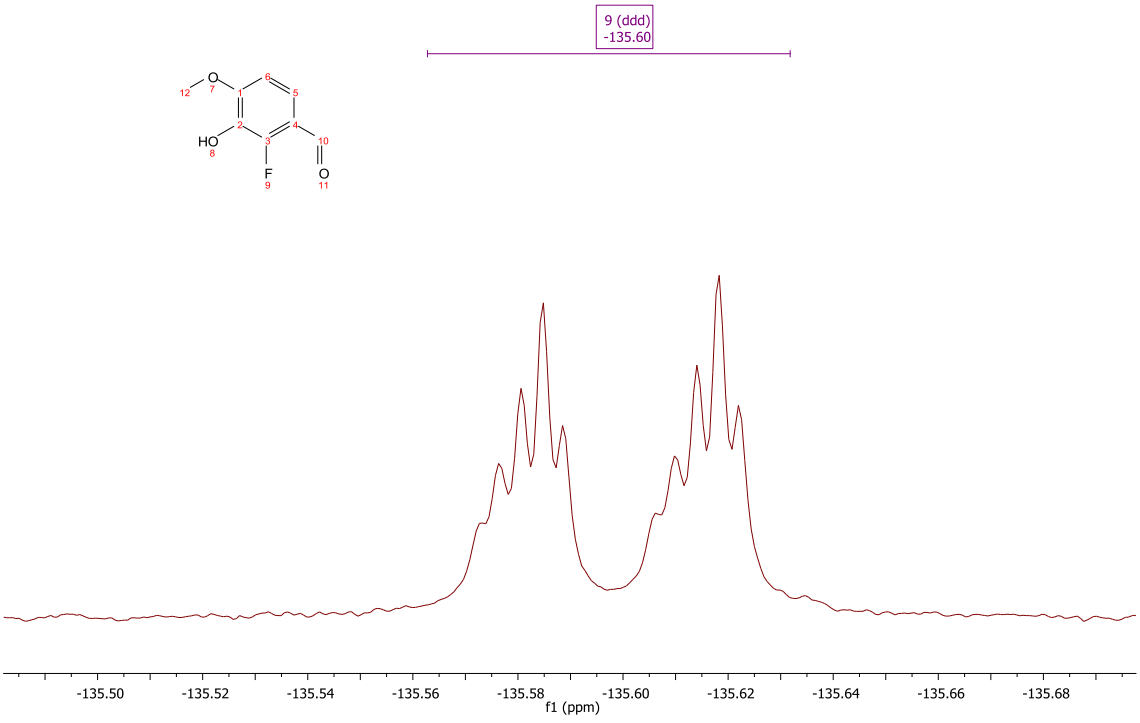
2-Fluoro-3-hydroxy-4-methoxybenzaldehyde

^{1}H NMR (300 MHz, DMSO) 2-Fluoro-3-hydroxy-4-methoxybenzaldehyde



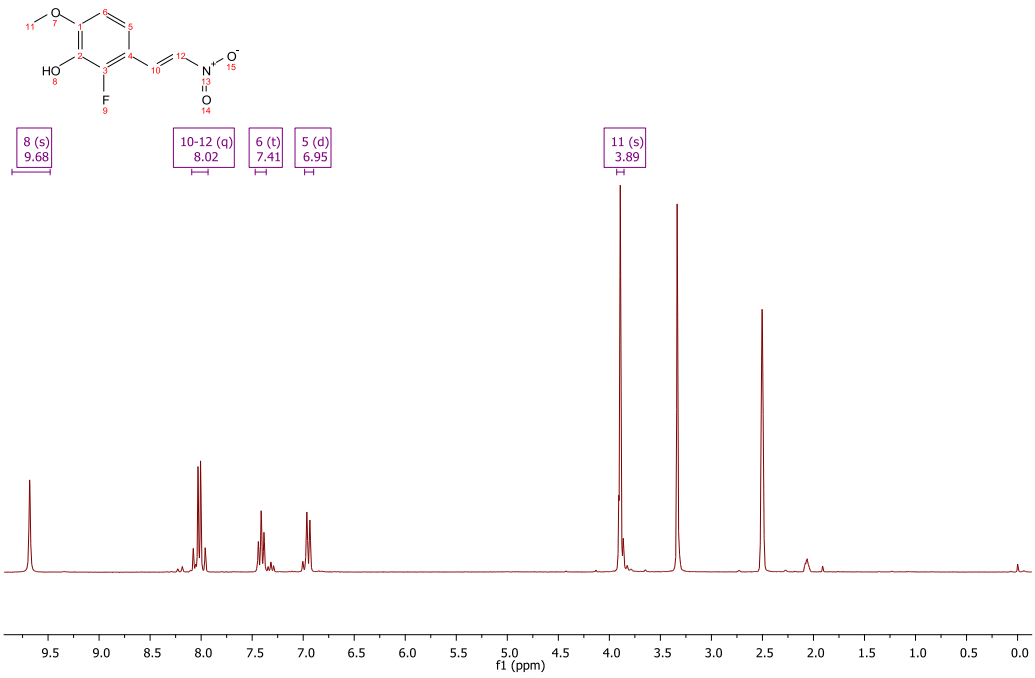
(3)

^{19}F NMR (282 MHz, Acetone) 2-fluoro-3-hydroxy-4-methoxybenzaldehyde



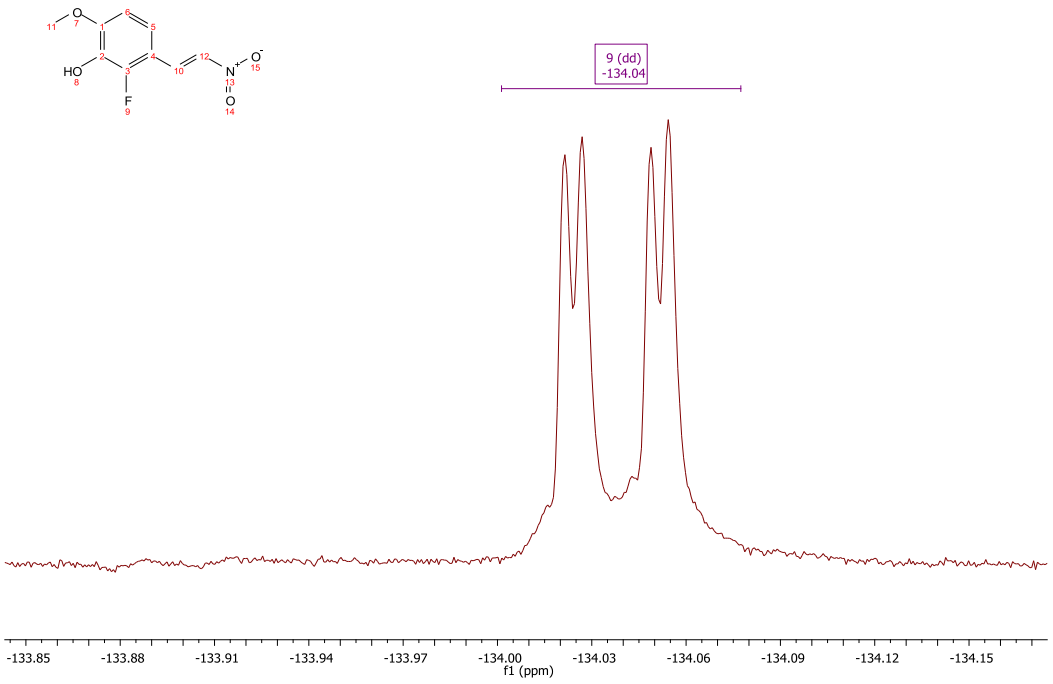
(E)-2-fluoro-6-methoxy-3-(2-nitrovinyl)phenol

^{1}H NMR (300 MHz, DMSO)

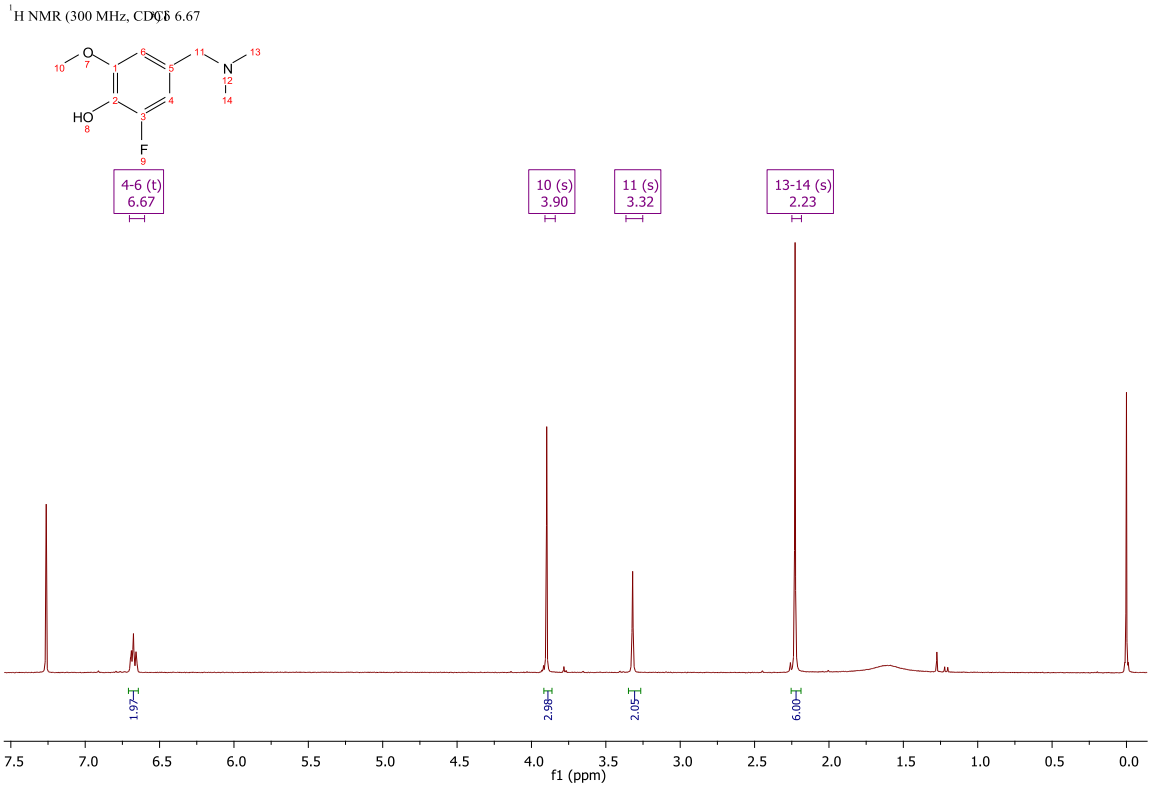


(4)

^{19}F NMR (282 MHz, DMSO)



N,N-Dimethyl-3-hydroxy-4-methoxy-5-fluorobenzylamine



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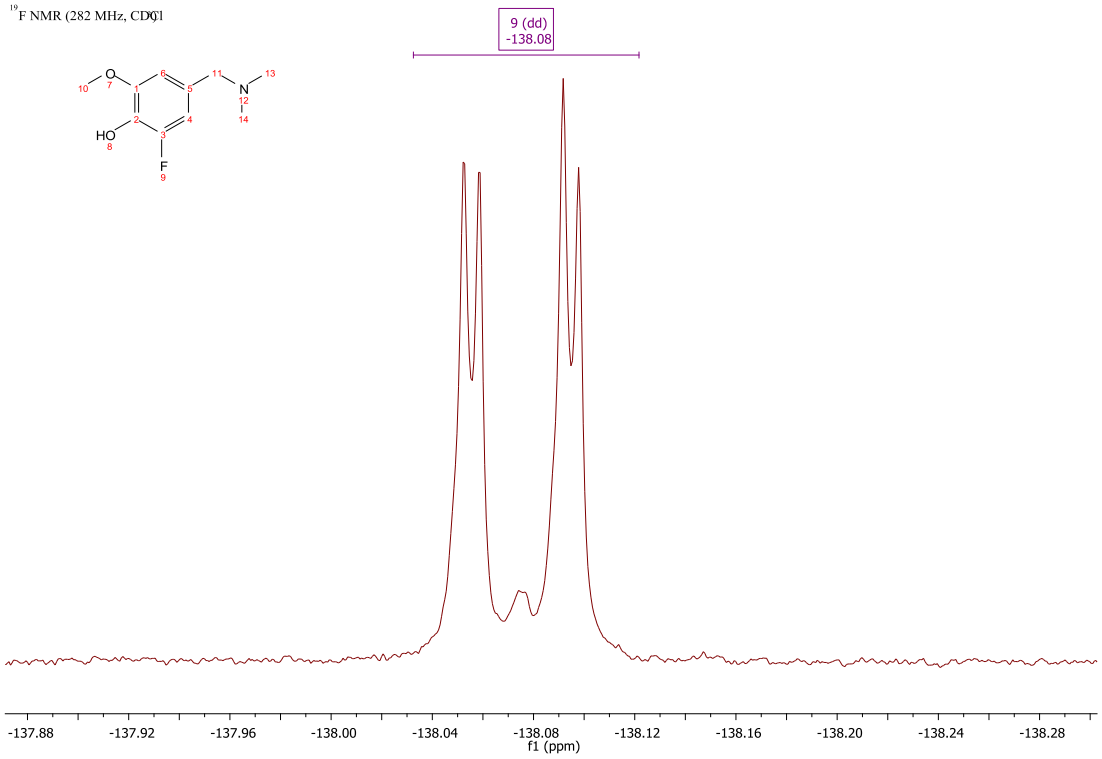
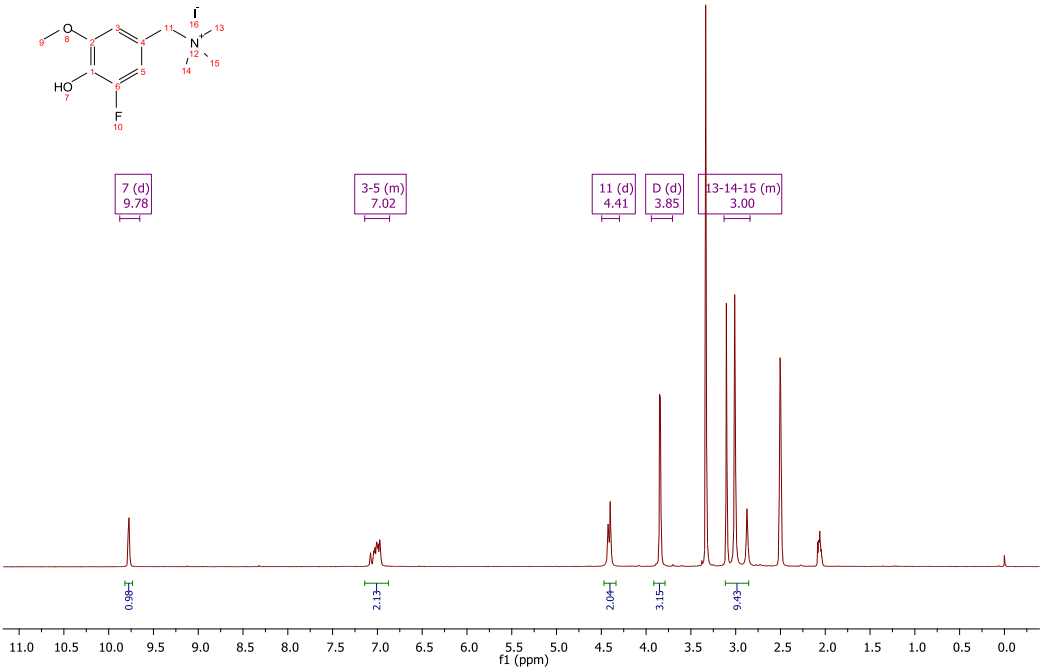


1-(3-fluoro-4-hydroxy-5-methoxyphenyl)-N,N,N-trimethylmethanaminium iodide



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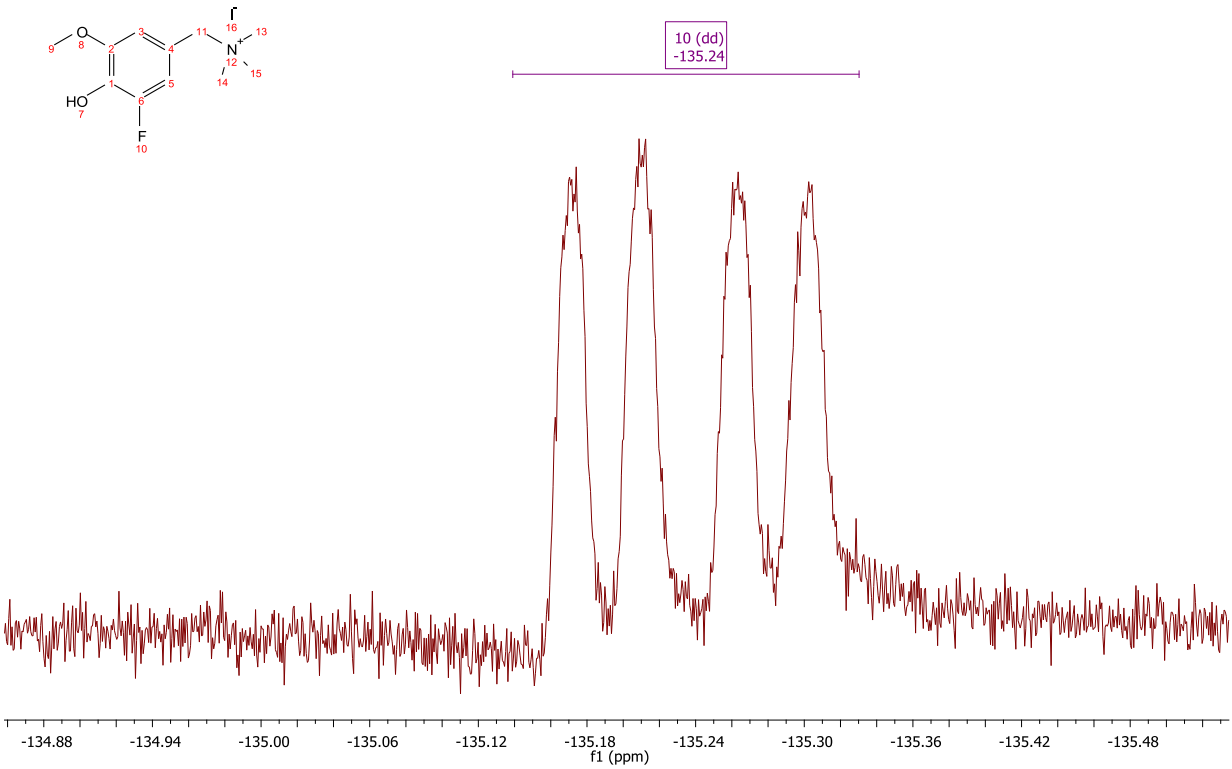
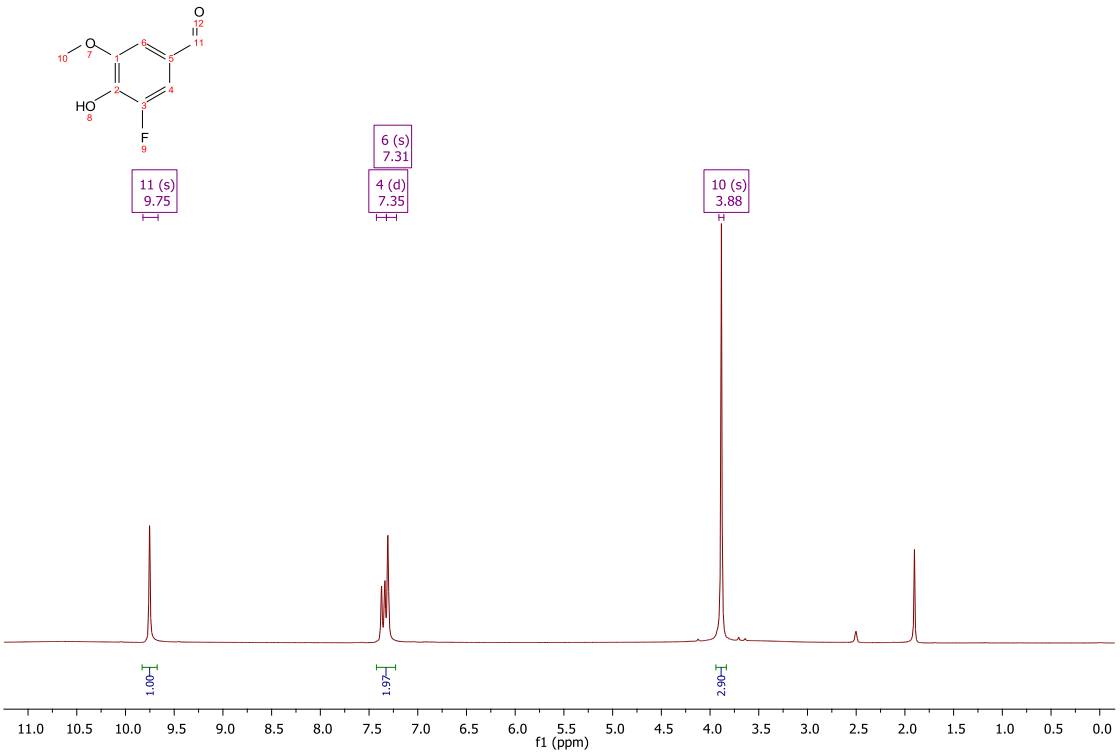


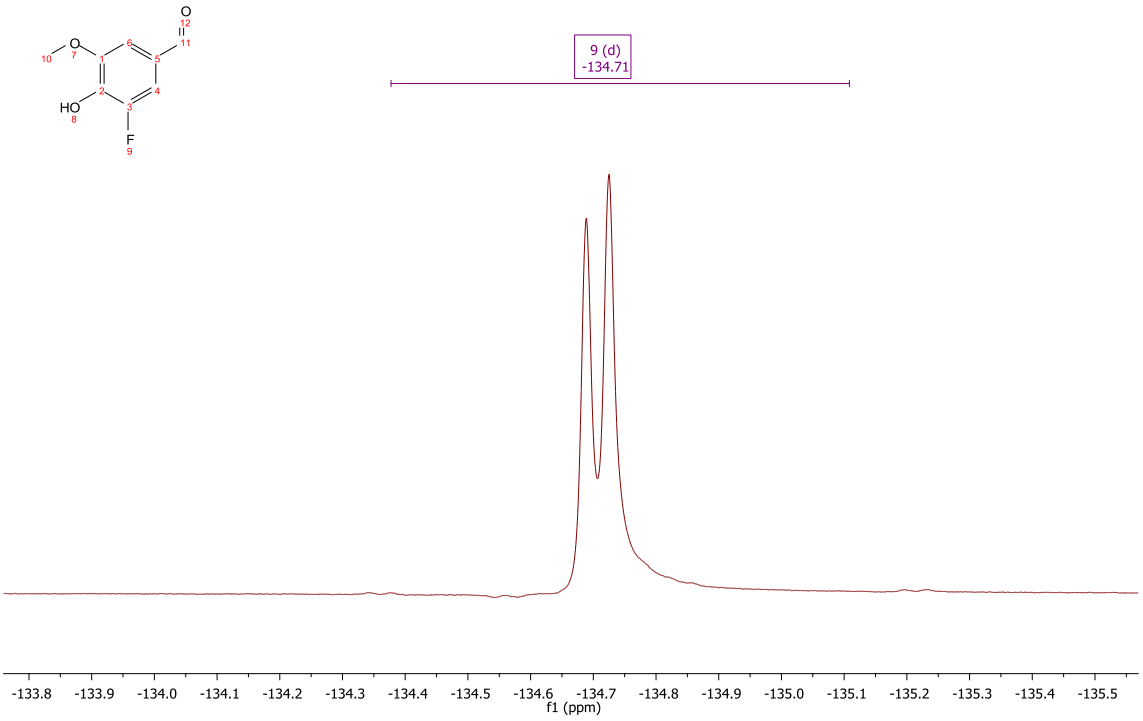
3-Methoxy-4-hydroxy-5-fluorobenzaldehyde

^{1}H NMR (300 MHz, DMSO)



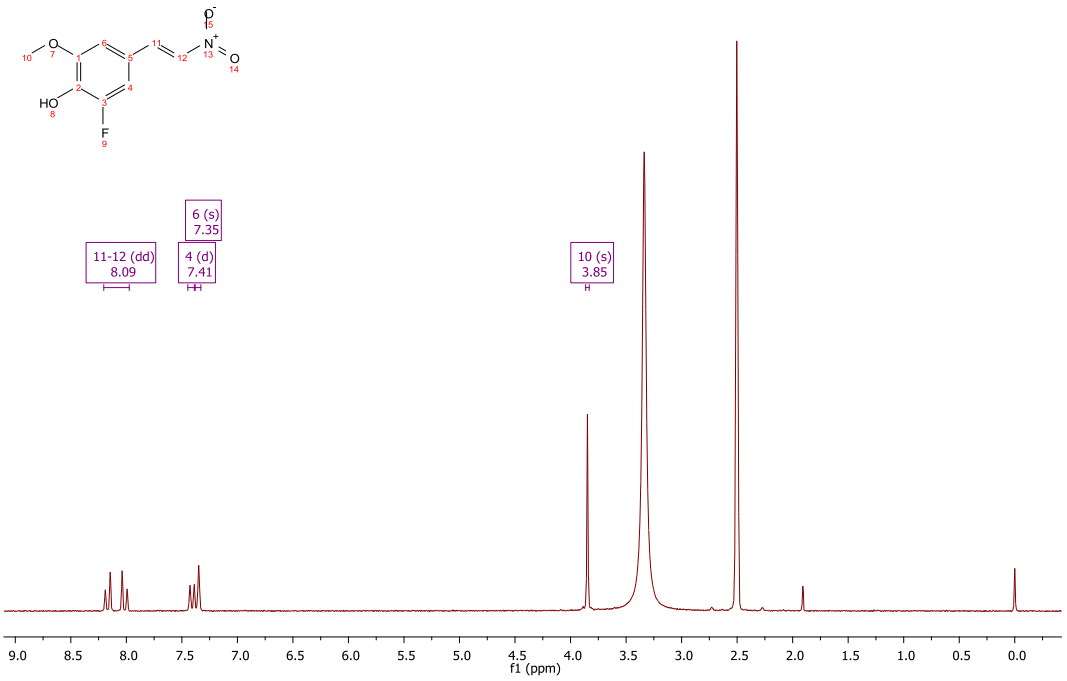
(9)

^{19}F NMR (282 MHz, DMSO)

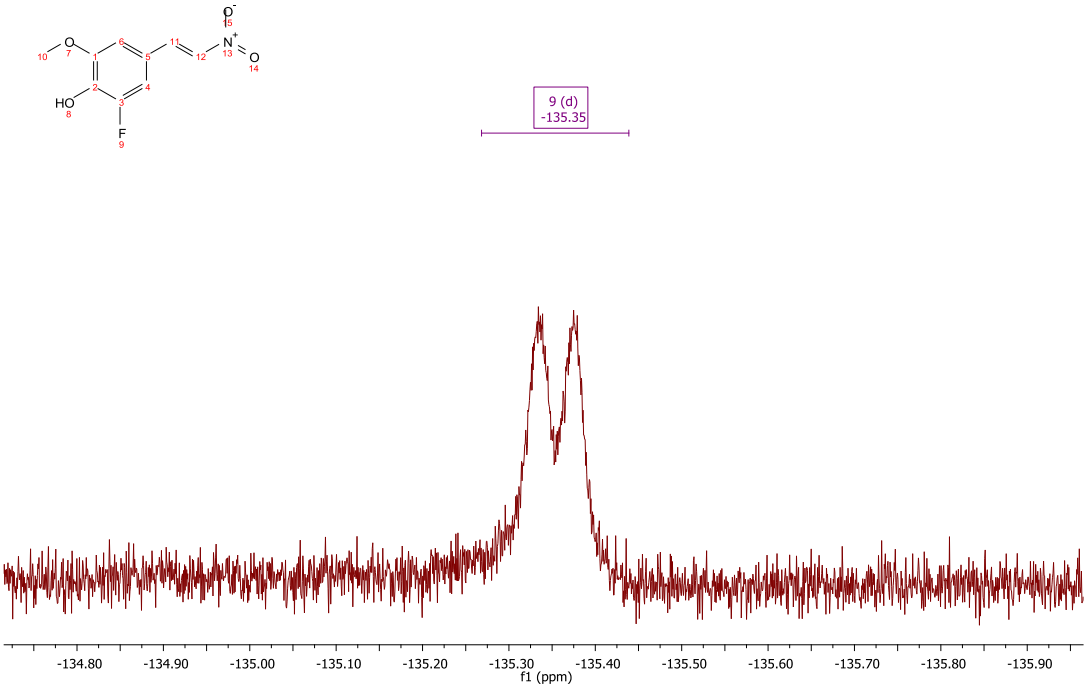


(E)-2-fluoro-6-methoxy-4-(2-nitrovinyl)phenol (10)

^{1}H NMR (300 MHz, DMSO)

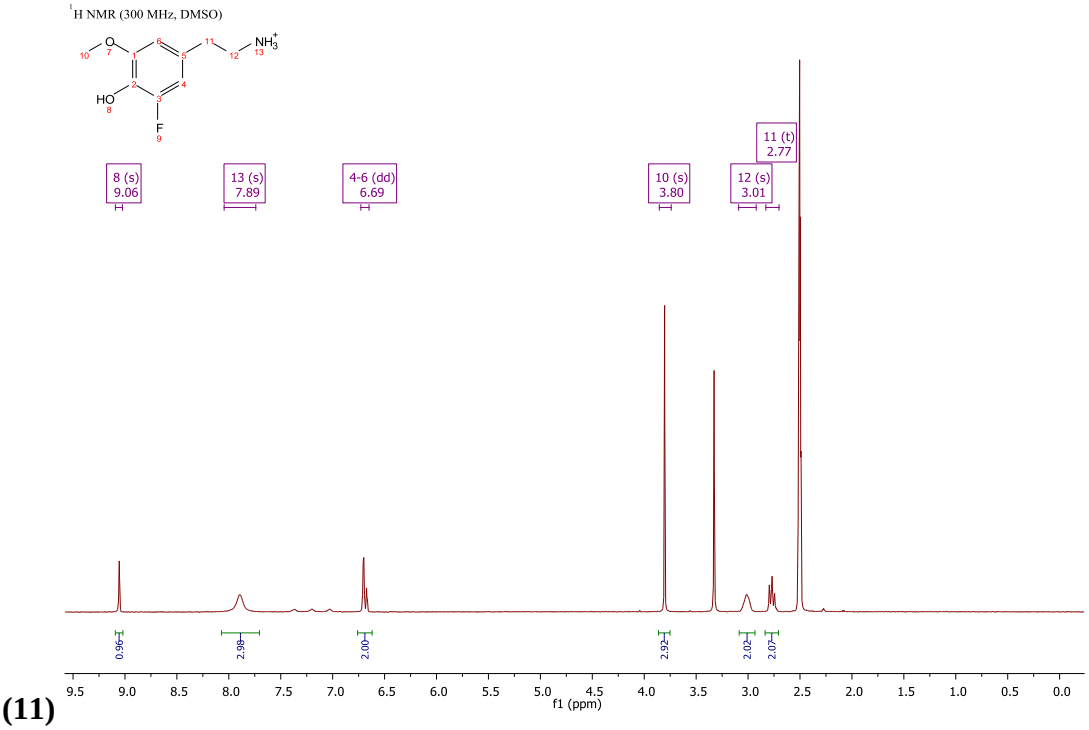


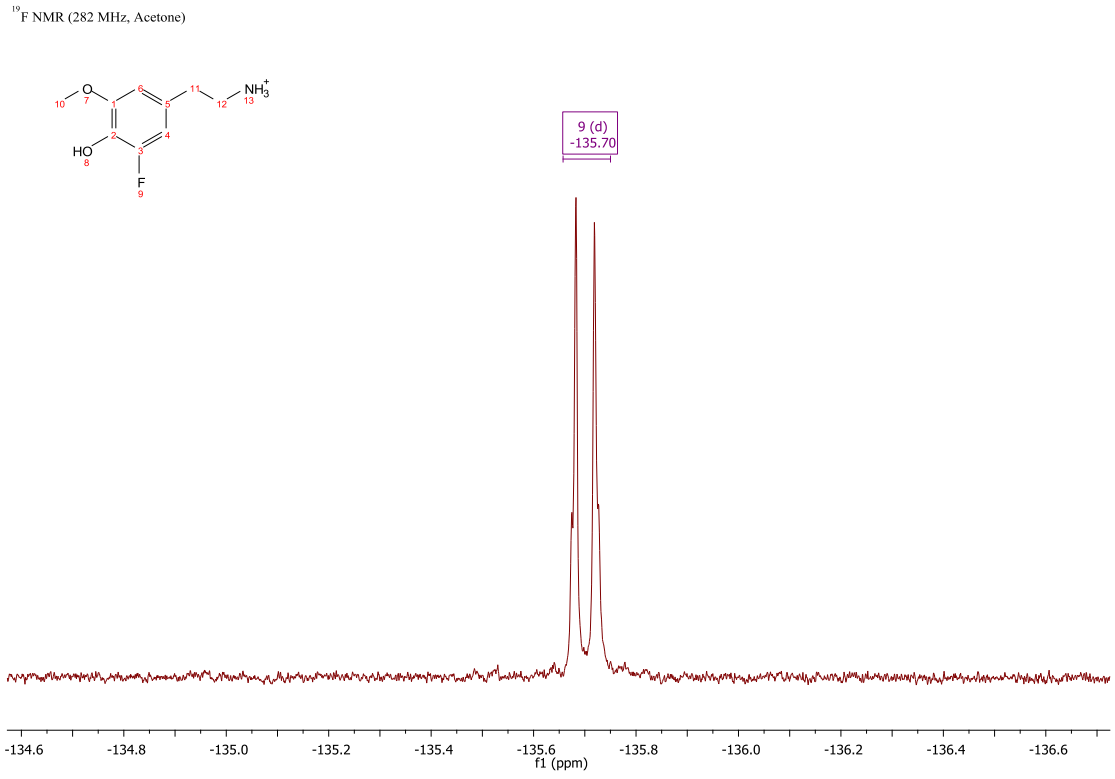
^{19}F NMR (282 MHz, DMSO)



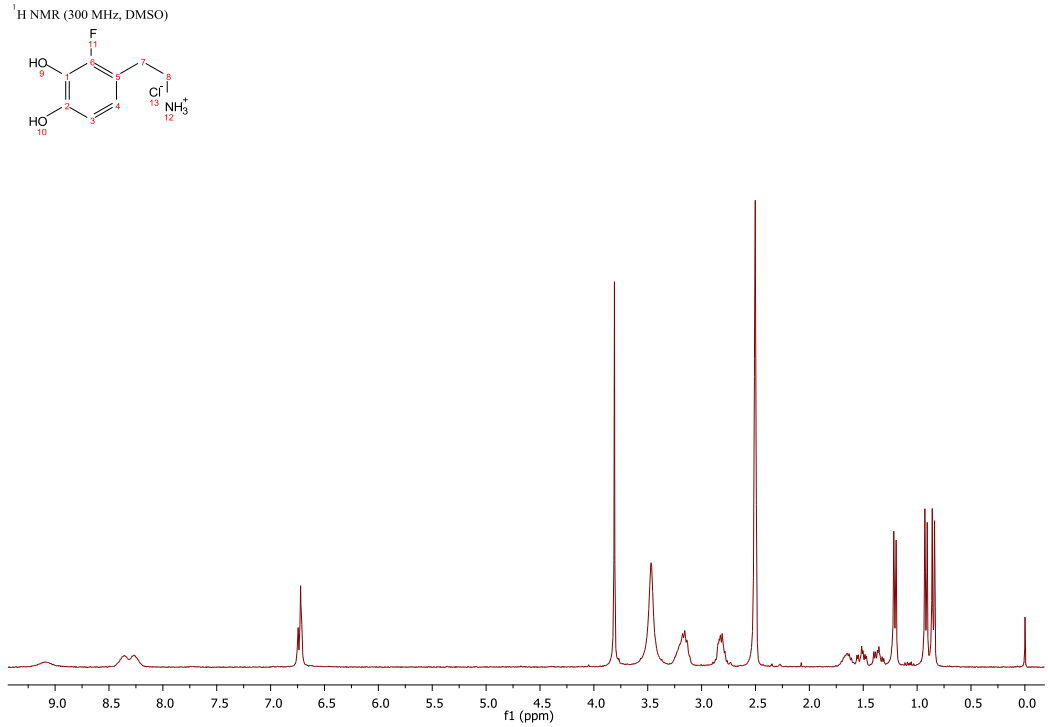
4-(2-aminoethyl)-2-fluoro-6-methoxyphenol hydrochloride

(11)



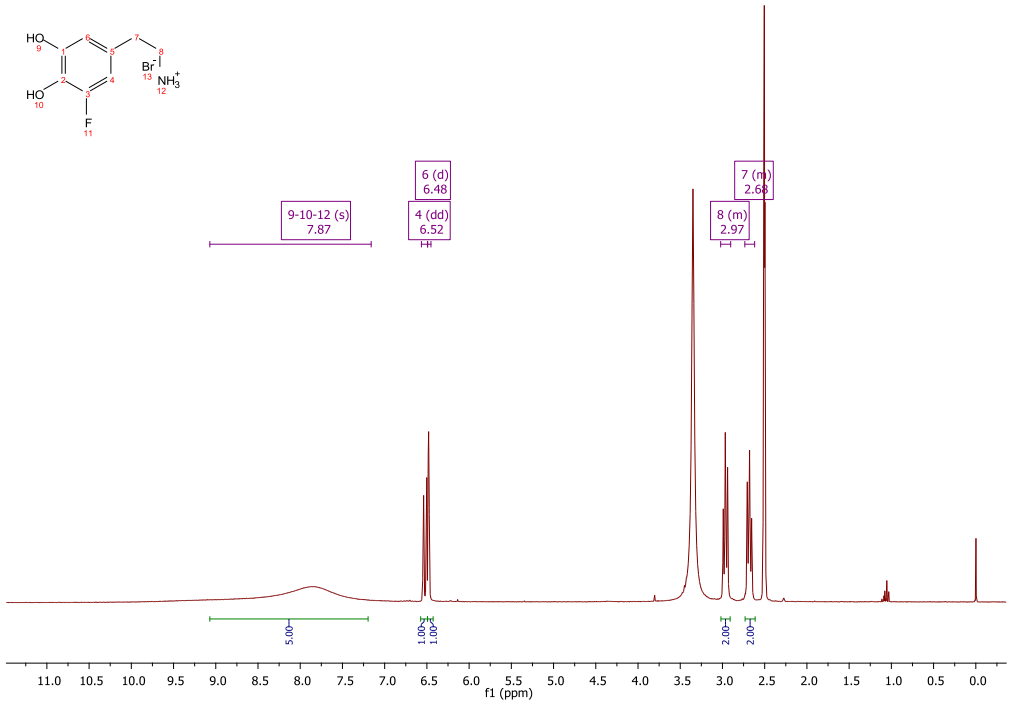


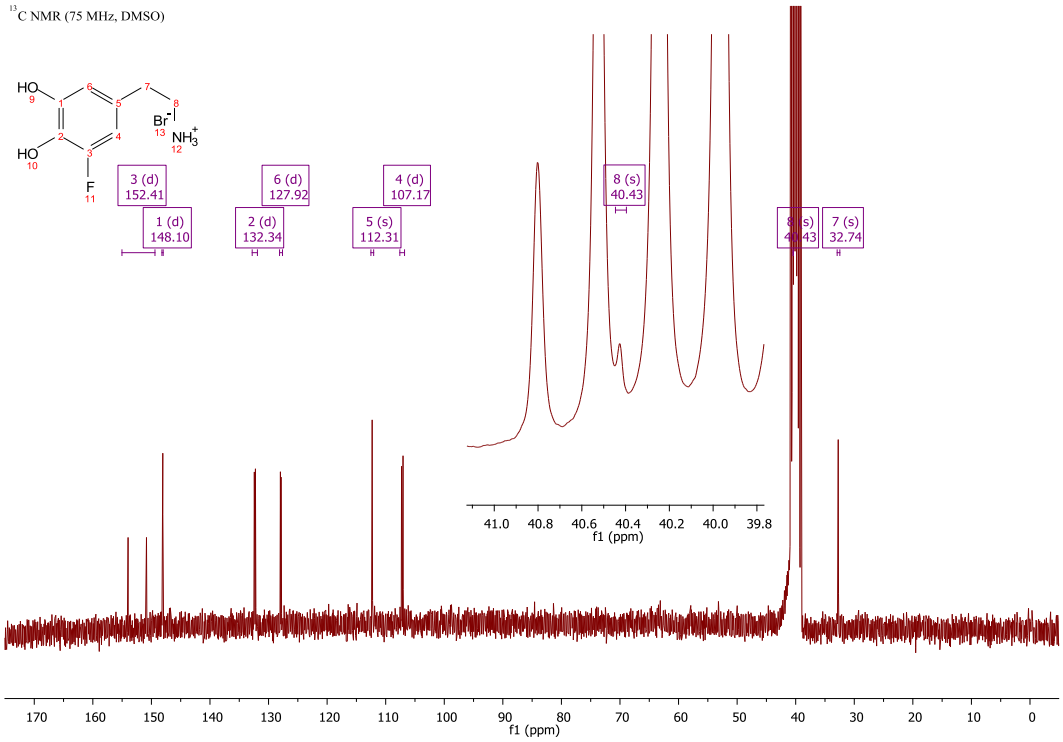
3-(2-aminoethyl)-2-fluoro-6-methoxyphenol hydrobromide (2-Fluorodopamine)

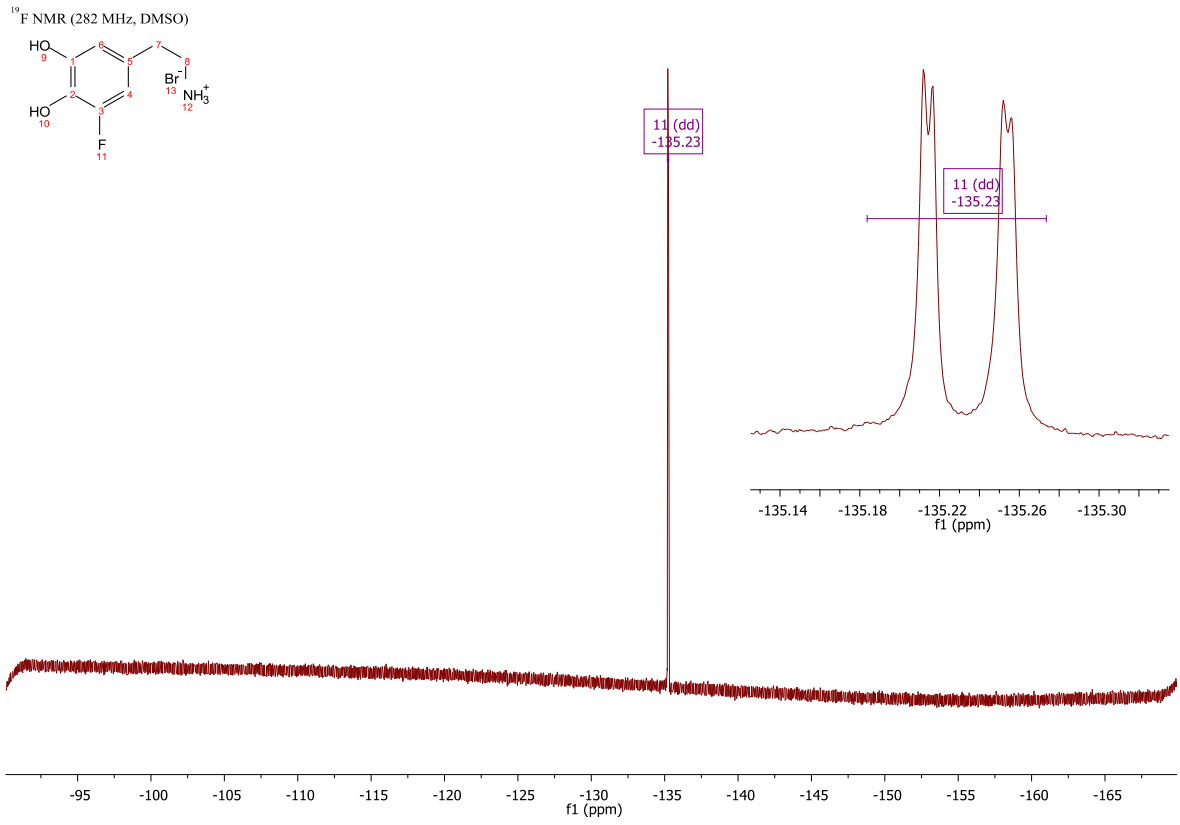


(6)

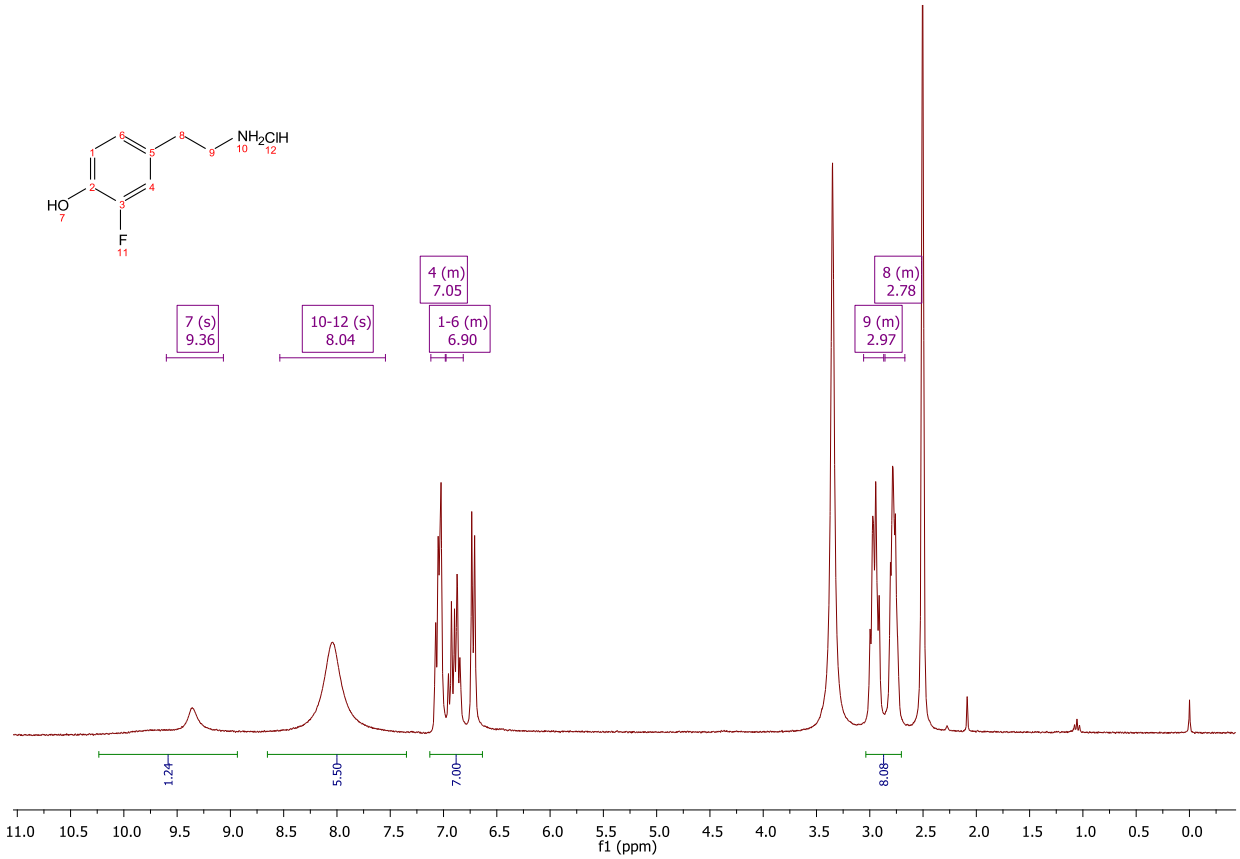
2-Fluoro-3,4-dihydroxyphenethylamine Hydrobromide (5-Flurodopamine HBr) (12)



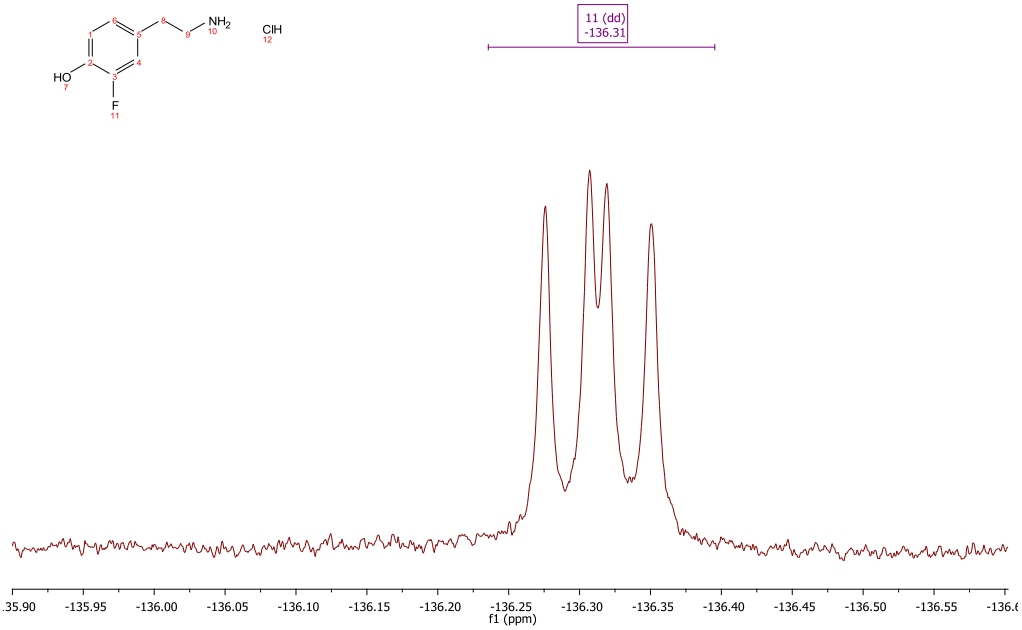

13C NMR (75 MHz, DMSO)
3 (d) 152.41
6 (d) 127.92
4 (d) 107.17
1 (d) 148.10
2 (d) 132.34
5 (s) 112.31
8 (s) 40.43
7 (s) 32.74
f1 (ppm)


19F NMR (282 MHz, DMSO)
11 (dd) -135.23
f1 (ppm)

4-(aminomethyl)-2-fluorophenol hydrochloride (3-Fluorotyramine Hydrochloride) (15)

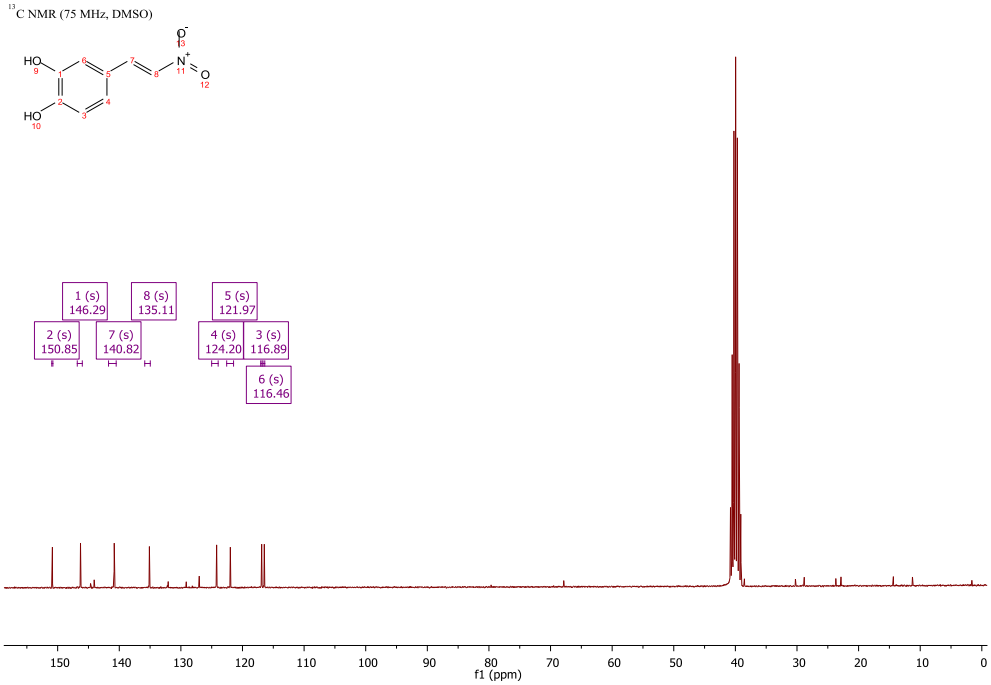


^{19}F NMR (282 MHz, DMSO)



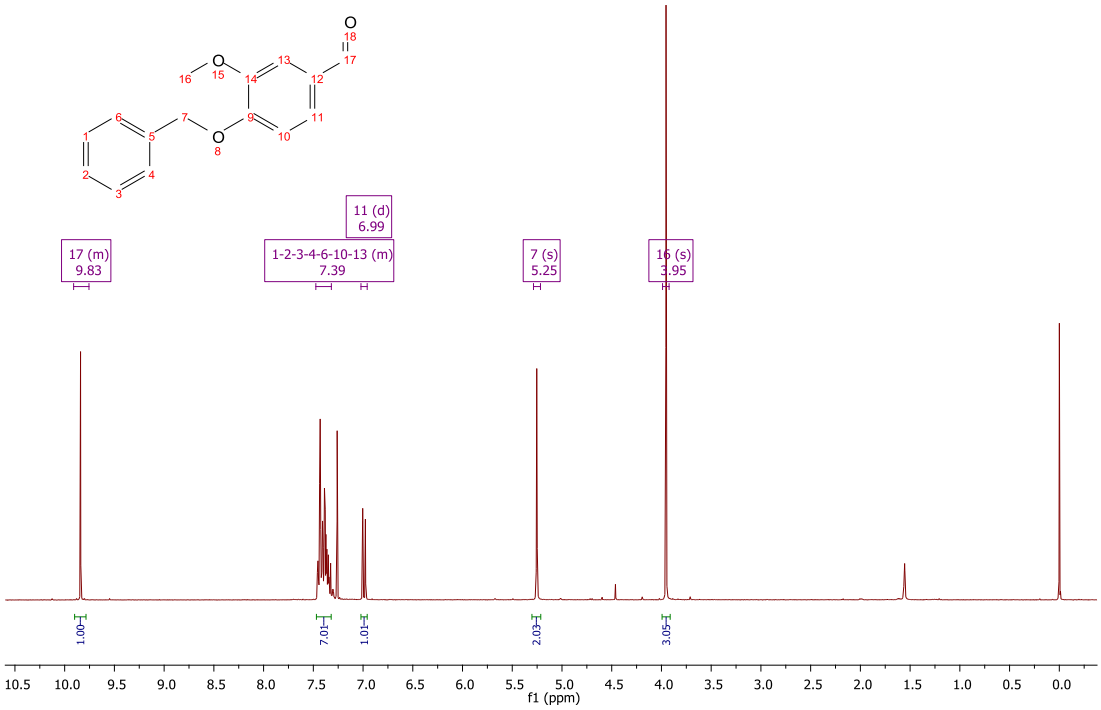
(E)-4-(2-nitrovinyl)benzene-1,2-diol (17)

^{13}C NMR (75 MHz, DMSO)

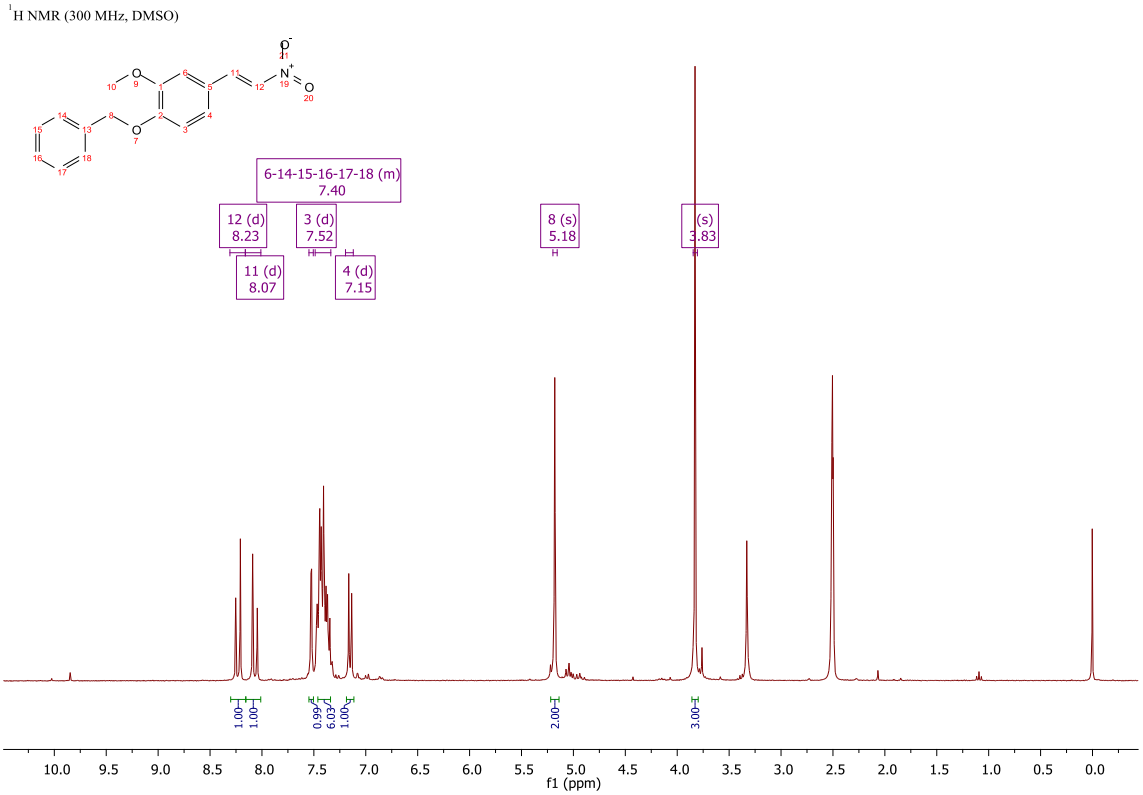


4-(benzyloxy)-3-methoxybenzaldehyde (20)

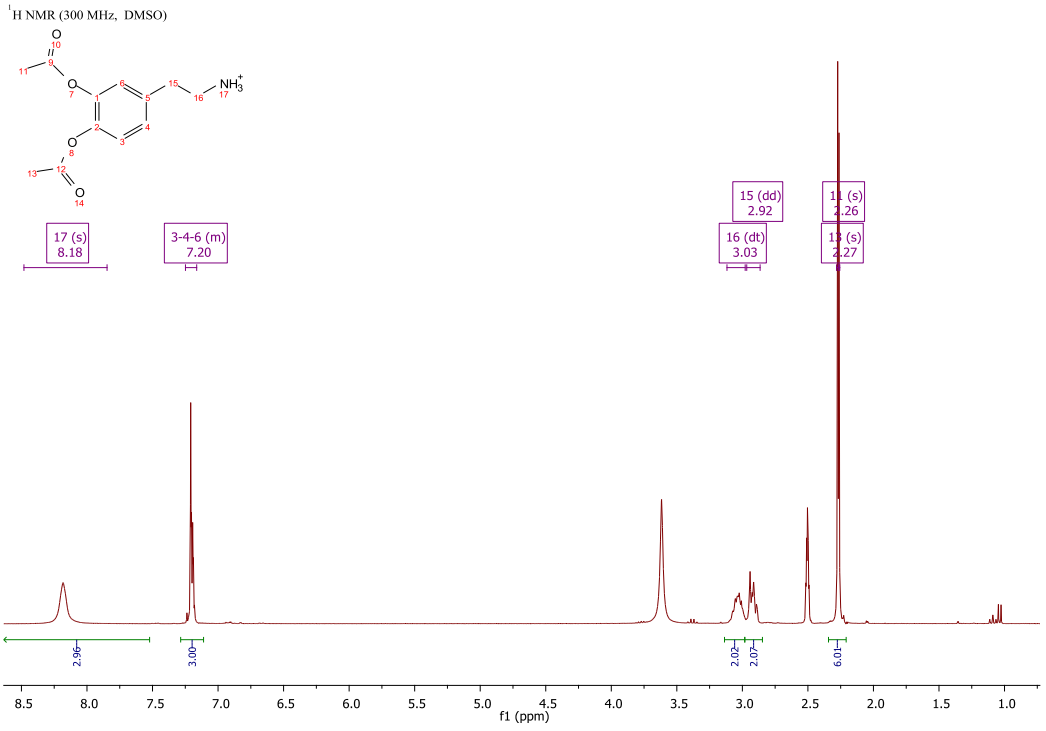
^{1}H NMR (300 MHz, CDCl3)



(E)-1-(benzyloxy)-2-methoxy-4-(2-nitrovinyl)benzene (21)



4-(2-aminoethyl)-1,2-phenylene diacetate hydrochloride (23)



Appendix B

X-ray Structure Files

2-Fluoroisovanillin .res

TITL scan_0m_Pc in Pc #7

CELL 0.71073 6.36 6.8574 8.497 90 98.169 90

ZERR 2 0.0036 0.0035 0.0051 0 0.019 0

LATT -1

SYMM +X,-Y,0.5+Z

SFAC C H O F

UNIT 16 14 6 2

L.S. 100

PLAN 10 0 0

SIZE 0.19 0.2 0.2

TEMP 27

REM reset to Pc #7

BOND

fmap 2

acta

REM <HKL>C:/Users/Tom/Documents/Grad%20School/Classes/crystal%20class/work%20f

REM %20iso%20van%20the%20copy/scan_0m_Pc.hkl</HKL>

WGHT 0.059100

FVAR 0.29183

O1 3 0.079423 0.200885 0.545022 11.00000 0.05204 0.03147 =

0.07534 -0.00923 0.03118 0.00000

C2 1 0.291142 -0.010556 0.426422 11.00000 0.05036 0.03337 =

```
      0.05346    0.00202    0.02045   -0.00686
O3    3    0.179643   -0.166551    0.467885    11.00000    0.06527    0.02936 =
      0.08105   -0.00746    0.04567   -0.00366
AFIX 147
H3    2    0.096600   -0.131088    0.527671    11.00000   -1.50000
AFIX   0
C4    1    0.245724    0.179331    0.463481    11.00000    0.03916    0.03630 =
      0.04068   -0.00237    0.01363    0.00841
C5    1    0.461030   -0.042868    0.345805    11.00000    0.04447    0.03180 =
      0.04641   -0.00444    0.01452    0.00461
F     4    0.507846   -0.229445    0.313492    11.00000    0.06706    0.02588 =
      0.08786   -0.00417    0.04283   -0.00020
C8    1    0.364416    0.333471    0.414082    11.00000    0.04650    0.01942 =
      0.06798   -0.00200    0.01762   -0.00567
AFIX  43
H8    2    0.331576    0.461348    0.437733    11.00000   -1.20000
AFIX   0
C12   1    0.583153    0.105417    0.294325    11.00000    0.04145    0.03306 =
      0.04959   -0.00121    0.00890   -0.00226
C14   1    0.529854    0.295763    0.330443    11.00000    0.04580    0.03303 =
      0.05487    0.00623    0.01956   -0.00744
AFIX  43
H14   2    0.607548    0.399130    0.297395    11.00000   -1.20000
AFIX   0
O     3    0.867962    0.189154    0.155311    11.00000    0.06142    0.04110 =
      0.08468    0.00188    0.03622   -0.01058
C     1    0.755812    0.065877    0.203889    11.00000    0.05123    0.04234 =
      0.06161   -0.00125    0.02301    0.00555
AFIX  43
H     2    0.782622   -0.063748    0.181421    11.00000   -1.20000
```

AFIX 0

C0AA 1 0.017013 0.394399 0.582324 11.00000 0.06375 0.03763 =

0.08051 -0.01199 0.03617 0.00978

AFIX 137

H0AA 2 -0.018934 0.467346 0.485821 11.00000 -1.50000

H0AC 2 0.132301 0.457431 0.648261 11.00000 -1.50000

H0AB 2 -0.104123 0.388022 0.637873 11.00000 -1.50000

HKLF 4

REM scan_0m_Pc in Pc #7

REM R1 = 0.0622 for 835 Fo > 4sig(Fo) and 0.0791 for all 1054 data

REM 111 parameters refined using 2 restraints

END

WGHT 0.0589 0.0000

REM Highest difference peak 0.245, deepest hole -0.234, 1-sigma level 0.058

Q1 1 1.0536 0.2157 0.2846 11.00000 0.05 0.25

Q2 1 0.3814 -0.2044 0.1691 11.00000 0.05 0.23

Q3 1 0.7308 0.1796 -0.0124 11.00000 0.05 0.22

Q4 1 0.7534 0.2133 0.0324 11.00000 0.05 0.22

Q5 1 0.6518 -0.2037 0.4636 11.00000 0.05 0.19

Q6 1 0.2894 -0.1640 0.6133 11.00000 0.05 0.19

Q7 1 0.1759 0.2047 0.6631 11.00000 0.05 0.18

Q8 1 0.1276 -0.2134 0.3168 11.00000 0.05 0.18

Q9 1 0.4971 0.0252 0.1237 11.00000 0.05 0.17

Q10 1 0.4721 0.1502 0.1736 11.00000 0.05 0.16

5-Fluorodopamine.res

```
TITL scan_0m_p21_c in P21/c #14
CELL 0.71073 10.8217 12.8844 6.8447 90 92.384 90
ZERR 4 0.0032 0.0033 0.0023 0 0.01 0
LATT 1
SYMM -X,0.5+Y,0.5-Z

SFAC C H N O F Br
UNIT 32 24 4 8 4 4

L.S. 20
SIZE 0.08 0.48 0.65
TEMP 27
REM reset to P21/c #14
BOND
fmap 2
acta
OMIT -5 5 7
OMIT 4 2 7
OMIT 3 2 7
OMIT 5 2 7
OMIT -4 1 7
REM <HKL>C:/Users/Tom/Desktop/crystal%20structure/Fluoro%20dopamine%20crystal/
REM scan_0m_P21_c.hkl</HKL>

WGHT    0.196300
FVAR       0.10641
BR1   6    0.472600    0.120548    0.237025    11.00000    0.03365    0.02904 =
      0.05684    0.00030    0.00420    0.00002
```

F 5 0.192245 -0.363433 0.293668 11.00000 0.04002 0.02918 =
0.10399 -0.00466 0.00484 0.01172
O0AA 4 0.322844 -0.181782 0.247179 11.00000 0.02560 0.03389 =
0.10142 -0.00558 0.00429 0.00082
AFIX 147
H0A 2 0.347079 -0.241617 0.234624 11.00000 -1.50000
AFIX 0
O 4 0.192770 0.002580 0.247882 11.00000 0.03276 0.02324 =
0.14008 -0.00149 0.01123 -0.00739
AFIX 147
H 2 0.259763 -0.008462 0.200124 11.00000 -1.50000
AFIX 0
C4 1 -0.058910 -0.179691 0.325150 11.00000 0.03847 0.03306 =
0.04045 0.00235 0.00487 -0.00274
N 3 -0.406029 -0.117787 0.277495 11.00000 0.02607 0.04082 =
0.06601 0.00537 0.00536 0.00462
AFIX 137
HA 2 -0.448280 -0.068955 0.211446 11.00000 -1.50000
HC 2 -0.422494 -0.113884 0.403599 11.00000 -1.50000
HB 2 -0.427923 -0.180013 0.231438 11.00000 -1.50000
AFIX 0
C6 1 0.129128 -0.270515 0.296916 11.00000 0.04374 0.02933 =
0.04644 -0.00064 0.00604 0.00604
C7 1 0.003986 -0.272919 0.322137 11.00000 0.03884 0.02574 =
0.05358 0.00139 0.00030 -0.00404
AFIX 43
H7 2 -0.037295 -0.335568 0.336786 11.00000 -1.20000
AFIX 0
C8 1 0.132118 -0.089614 0.271942 11.00000 0.03882 0.03211 =
0.05491 -0.00472 0.00184 -0.00811

C9 1 0.006020 -0.087615 0.298486 11.00000 0.03722 0.02620 =

0.07481 -0.00065 0.00338 0.00157

AFIX 43

H9 2 -0.035650 -0.024488 0.298538 11.00000 -1.20000

AFIX 0

C10 1 0.197402 -0.181978 0.273390 11.00000 0.02151 0.02972 =

0.05289 0.00055 0.00189 0.00209

C11 1 -0.270647 -0.101847 0.253984 11.00000 0.02899 0.04701 =

0.05546 0.01188 0.00546 -0.00746

AFIX 23

H11B 2 -0.247981 -0.032561 0.297795 11.00000 -1.20000

H11A 2 -0.252847 -0.107243 0.116629 11.00000 -1.20000

AFIX 0

C12 1 -0.194433 -0.179853 0.368024 11.00000 0.03277 0.04025 =

0.05367 0.00507 0.00428 -0.00129

AFIX 23

H12B 2 -0.227641 -0.248491 0.340584 11.00000 -1.20000

H12A 2 -0.202644 -0.166549 0.506365 11.00000 -1.20000

HKLF 4

REM scan_0m_p21_c in P21/c #14

REM R1 = 0.0922 for 1321 Fo > 4sig(Fo) and 0.1067 for all 1684 data

REM 121 parameters refined using 0 restraints

END

WGHT 0.1963 0.0000

REM Highest difference peak 4.397, deepest hole -0.931, 1-sigma level 0.242

Q1 1 0.4742 0.1221 0.3887 11.00000 0.05 4.40

Q2 1 0.4701 0.1226 0.0951 11.00000 0.05 3.14

Q3	1	0.0054	-0.2658	0.1556	11.00000	0.05	0.94
Q4	1	-0.2002	-0.1830	0.1516	11.00000	0.05	0.94
Q5	1	-0.0562	-0.1805	0.1763	11.00000	0.05	0.91
Q6	1	-0.4153	-0.1209	0.1340	11.00000	0.05	0.83
Q7	1	-0.3838	-0.1166	0.0278	11.00000	0.05	0.82
Q8	1	0.1911	-0.3608	0.1366	11.00000	0.05	0.81
Q9	1	-0.2004	-0.1820	0.0130	11.00000	0.05	0.75
Q10	1	0.3187	-0.1838	0.3857	11.00000	0.05	0.65
Q11	1	0.1337	-0.2653	0.1471	11.00000	0.05	0.64
Q12	1	0.5599	0.1211	0.2043	11.00000	0.05	0.60
Q13	1	0.1908	-0.1871	0.1282	11.00000	0.05	0.58
Q14	1	0.1841	0.0014	0.4088	11.00000	0.05	0.57
Q15	1	0.0023	-0.0837	0.1349	11.00000	0.05	0.57
Q16	1	0.1779	0.0086	0.0635	11.00000	0.05	0.46
Q17	1	0.3169	0.1209	0.2386	11.00000	0.05	0.46
Q18	1	0.0681	-0.2587	0.3701	11.00000	0.05	0.45
Q19	1	-0.2549	-0.3676	0.3632	11.00000	0.05	0.45
Q20	1	-0.5951	-0.0730	0.1606	11.00000	0.05	0.43

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