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N-Acyltyramines as Substrates for Tyrosinase: Enzymatic Lag and Dopamine Precursor

Jacob A. Shafer

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N-Acyltyramines as Substrates for Tyrosinase:
Enzymatic Lag and Dopamine Precursor

by

Jacob A. Shafer

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Chemistry
College of Arts and Sciences
University of South Florida

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Keywords: Kinetics, Enzyme, Quinone, Mushroom, Oxygen Electrode

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Dedication

To my family and friends - thanks.

“What do you say to taking chances
What do you say to jumping off the edge
Never knowing if there’s solid ground below
Or hand to hold
Or hell to pay
What do you say
What do you say”

Celine Dion

Written by Kara DioGuardi and David A. Stewart
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Tyrosinase is a widespread, highly studied and important enzyme involved in processes ranging from the browning of mushrooms to roles in mammalian cancer. The enzyme suffers from a noticeable lag phase while the enzyme generates all necessary cofactors from available substrates. There have not been significant studies of the effect on lag from moving through a family of substituted substrates. This thesis reports the results of one such study using a family of N-acyltyramines.

The selection of N-acyltyramines was ideal because the substrates in this reaction may be related to synthesis of N-acyldopamines, which serve many important physiological functions. It was concluded that the product formed from N-acyltyramine is 1-acetyl-2,3-dihydro-1H-indole-6,7-dione, a quinone.
Chapter 1

Introduction

Tyrosinase

Tyrosinase (E.C. 1.14.18.1) is an enzyme which is distributed widely throughout all life forms [1]. The primary biological role is to produce melanin, which causes a darkening of tissues [2]. Due to its presence in mushrooms and plants, tyrosinase has been studied from the mid 1800s, as the related browning of harvested mushrooms was, and remains to be, a chief agricultural concern [3, 4]. In mammals, it was thought to be involved in cancer [5]. More recently, there has been interest from the cosmetic industry for finding inhibitors to act as skin whiteners [6] and it is being explored for its role in many types of albinism [7]. Tyrosinase has been considered as a tumor suppressor; however, it might have a role in mutagenicity as a free radical producer [8, 9]. One of the primary products of tyrosinase is melanin. This is a family of pigments found in life ranging from higher mammals down to insects and arthropods. Its role in higher organisms relates to coloration, such as hair and skin, as well as protection for solar UV radiation [10, 11]. In lower organisms, the role served by tyrosinase is more expansive, ranging from exoskeleton coloration to immune system response and wound healing [12, 13].

Scheme 1: General reaction catalyzed by tyrosinase. R≠H.
The enzyme is a heterotetramer, with two heavy and two light chains for a combined molecular mass of 120 kDa (Figure 1) [14]. The central domain, containing the active site, is well conserved across the various organisms [15]. The active site is composed of six histidines binding two copper ions, and has binding pockets for both the substrate and a reductant (Figure 2).

Figure 1. Structure of tyrosinase. Coloring scheme: Cu (brown), O$_2$ (red), NO$_3$$^-$ (blue, red) with no bound substrate [16].

The enzyme’s primary mechanism occurs in two steps using molecular oxygen both to hydroxylate monophenols to o-diphenols and to oxidize diphenols to o-quinones (Scheme 1) [10, 11]. Tyrosinase expresses stereospecificity with regard to binding affinity, favoring D-isomers, but does not show any specificity towards the reaction rate [17]. There is ongoing research as to the exact mechanism used by tyrosinase. Scheme 2 shows one proposal where substrates are M, a monophenol, and D, an o-diphenol, and the active site states are $E_{ox}$-oxytyrosinase, $E_m$-mettyrosinase and $E_d$-deoxytyrosinase. In $E_{ox}$, there is O$_2$ bound to the Cu ions and both are in +2 oxidation state, while in $E_d$ there is no oxygen moiety bound and the Cu ions are both in the +1 oxidation state, and the $E_m$ has a hydroxyl bound and both Cu ions are in +2 oxidation state. In this proposal, $E_{ox}$ can react
with either D or M. In the case of M, D is formed which can be released leaving the active site as E_m or the final product, a quinone, can be evolved leaving tyrosinase as E_d. If E_{ox} reacts with D, then the quinone is made and tyrosinase ends in E_m. E_m binding with M is a dead-end because the hydroxyl is unable to form a new C-O bond. In order to have meaningful forward movement of this reaction scheme, the enzyme needs to be available in the E_{ox} state or the substrate must be a diphenol. In most cases, however, the substrate is a monophenol and tyrosinase is predominately found in the E_m state [18-26].

![Active site of tyrosinase. Color scheme: Cu (orange), O_2 (red) and histidines with no bound substrate (generated from [16]).](image)

These two factors set-up what is known as the lag (τ) phase of the reaction. During this time, a small amount of naturally occurring E_{ox} enzyme (2-30%) is converting M to D where the reaction can move forward readily. This unusual phenomenon has been studied to some extent and it is known that increasing the enzyme concentration will reduce τ, due to the increased amount of enzyme present in E_{ox}. Additionally, by increasing the substrate (M) concentration, τ will increase. This is because k_{2} is larger than k_{3} (Scheme 2) and E_m is generated along with D, leaving enzyme inactive until enough D is accumulated to make k_{3} favorable, and M out competes D at the active site of remaining E_{ox} [18, 20, 22, 23].
Scheme 2. Proposed tyrosinase mechanism. $E_{m}$ mettyrosinase, $E_{ox}$ oxytyrosinase, $E_{d}$ deoxytryrosinase, $M$ monophenol, $D$ o-diphenol, and the final product of a quinone (adapted from [21]).
N-Acyl dopamine

Dopamines have many important biological roles. Dopamine is most widely known for its role as a neurotransmitter. Also it has a primary role as a precursor in the formation of melanin, which is involved in pigmentation in many species and sclerotization, the hardening and coloring of exoskeletons, in insects [24, 26-28]. More recently the role of N-acyldopamine in brain and central nervous system has been explored. N-Arachidonoyl-, N-palmitoyl-, N-oleoyl- and N-stearoyldopamine are all known to present in mammals. In the case of N-arachidonoyl- and N-oleoyldopamine, they bind to TRPV1 and CB1 receptors which experimentally cause hyperalgesia. Also, N-oleoyldopamine has been shown to reduce risk of ischemia and reperfusion injury by action on the TRPV1 receptor in the heart [29]. N-Palmitoyl- and N-stearoyldopamine have an unknown biological role [30-33]. There is also interest in N-acyldopamines as drugs for both their physical and inhibitory properties. N-Acyl dopamines has been shown to self-aggregate into micelle supramolecules and then hydrolyze slowly, gradually releasing dopamine to receptors [35]. In rats, there is evidence that N-acetyldopamine inhibits malondialdehyde production, which is tied to oxidative stress, better than the natural agent, melatonin [27, 36]. N-Acetyledopamine has been shown to decrease superoxide production in THP-1 derived human monocytes [38]. It has been discovered that N-linoleoyldopamine is produced in plants and acts as a strong inhibitor to arachidonate 5-lipoxygenase, a good drug target in humans for asthma [37].

N-Acyl dopamine biosynthesis is not completely understood, and two competing pathways have been proposed (Scheme 3). There is evidence that tyrosine can be directly acylated via the enzyme N-acetytyrosine synthase followed by oxidation by tyrosinase to N-acyldopamine (pathway A). An analogous pathway involves acylation of tyramine by an unknown enzyme. Alternatively, acyl groups may be transferred to tyramine or tyrosine via acyl-coenzyme A; the resulting species would then be oxidized by tyrosinase (pathway B) [32, 39, 40].
Tyrosine (Figure 3) has been explored to some extent in the literature, mostly as the primary substrate for tyrosinase with a reported $K_M$ of 1.25 mM [41]. It has been shown that tyrosinase can catalyze the oxidation of $N$-acetyltyrosine to the corresponding quinone [42]. Additionally, the similar family of compounds of $N$-acetyl-4-S-cysteaminylphenol (Figure 3) and $N$-propanoyl-4-S-cysteaminylphenol have been shown to be substrates for tyrosinase [43]. There has also been some work suggesting that $N$-methyl-, $N$-methoxy- and $N$-formyltyrosine as well as the methyl ester (Figure 3) are substrates for tyrosinase [19, 44-46]. On the other hand, $N$-acyltarmines (Figure 3) are not widely studied. They have been found in trace amounts throughout the central nervous system of insects and mammals [47, 48]. In honeybees, there is evidence that the ratio of tyramine and $N$-acetyltyramine is dependent on the presence of a queen – causing workers to convert to queens as needed. $N$-acetyltyramine may be a viable drug in the conversion of resistant state to sensitive state leukemia [49].

Scheme 3: Proposed biosynthetic pathways for $N$-acyldopamine. $A$ represents an acyl group. Pathway A is on the left, Pathway B is on the right.
Figure 3: Structures of tyramine, tyrosine and similar compounds.

Even though most of the literature indicates that the precursors for N-acyldopamines are N-actyltyrosines, the recent discovery by a Russian group [39] that N-acyltymaramines are preferred to N-acytyrosines in vivo drew our attention. As mentioned above, N-acyltymaramines are found in tissues where N-acyldopamines are located, but it is not known what, if any, role they serve there. In order to further investigate the possibility of N-acyltymaramines acting as precursors to N-acyldopamines, a series of N-acyltymaramines, with varying acyl chain lengths, were synthesized and evaluated as tyrosinase substrates. This also gave us the opportunity to study the effect acyl chain length has on $\tau$, the enzymatic lag tyrosinase exhibits. Ideally, the data will show that N-acyltymaramines have superior kinetic parameters to tyramine. Additionally, characterization should indicate the product to be N-acyldopamine or its derivatives.
Chapter 2

Materials and Methods

*Mushroom Tyrosinase*

Mushroom tyrosinase (EC 1.14.18.1) was acquired from Sigma and used without further purification. The enzyme was dissolved in 100% water and stored at 5 °C. Initially, the concentration of protein in solution was determined by Bradford assay using bovine serum albumin as a standard [50]. A base-line rate was established as described later in the Material and Methods chapter under “Kinetic Assays of N-Acyltyramines Substrate Activity with Tyrosinase” where there was no EtOH added and 2 mM tyramine was used. Then to account for change in enzyme activity from day-to-day, and to account for the effect of 20% EtOH, a standard reaction was performed each day in the same manner as the baseline where tyramine was always 2 mM, this stock was dissolved in 100% water. The average of two correction activities was compared to the standards and a correction was multiplied into each reaction thereafter.

*Synthesis and Purification of N-Acyltyramines*

All substrates were synthesized using a modified approach published by Johnson et al (Scheme 4) [51, 52]. In general, tyramine was dissolved in acetonitrile and triethylamine, to which a 1:1 ratio of acylchloride was added drop wise, while vigorously stirred at room temperature. After addition, the solution was allowed to stir for another 90 minutes before being evaporated under a stream of air. Purification was accomplished by dissolving the product in methanol and running on a silica gel column in a gradient solvent system starting at 1% methanol in dichloromethane and progressing to 20%
methanol. The final spot to elute, as checked on TLC run in dichlormethane:methanol (4:1) and visualized under UV light, was the desired product. Yields for each are below:

- N-acetyltymine – 40%
- N-propionyltyramine – 45%
- N-butyryltyramine – 80%
- N-pentanoyltyramine – 50%
- N-hexanoyltyramine – 50%

N-Octanoyltyramine and N-decanoyltyramine were also synthesized, but due to extremely low water solubility (<< 1 nM in 20% EtOH), enzymatic activity assays were not possible, and these were not further characterized.

Identity of synthesized substrates was confirmed and purity assessed by $^1$H and $^{13}$C NMR on iNOVA 400 MHz and processed on MestReNova (Appendix A).

Scheme 4. Synthesis of N-acyltyramines. $R=CH_3, C_2H_5, C_3H_7, C_4H_9, C_5H_{11}, C_7H_{15}, C_9H_{19}, Ph.$

Synthesis of N-benzoyltyramine

Synthesis was identical to the N-acyltyramines. It was not possible to find a solvent which would dissolve the crude product and allow for enzymatic assays; this was not characterized.
**Kinetic Assay of L-3,4-Dihydroxyphenylalanine**

The reactions were conducted on a Yellow Springs Instrument Model 53 oxygen monitor which is outfitted to digitally output the O₂ reading into Excel every second. The oxygen electrode chamber was always prepared in the following order after cleaning and the positioning of the stir bar: water, phosphate buffer, ethanol and substrate. The solution was capped with the oxygen electrode and allowed to stir while coming to equilibrium with the water bath at 37 °C. Oxygen bubbles were aggregated and removed at least once and until there were no noticeable bubbles left. Reaction conditions were 100 mM phosphate, pH=7.0, 0.025 mg/mL tyrosinase, and the indicated amount of substrate with a final reaction volume of 2 mL. Background rates were determined with all components except enzyme present for 30 seconds followed by addition of tyrosinase over less than 10 seconds. Due to the small change in volume, data was not collected during this small window. The reaction was then followed for two minutes. All calculated slopes from Excel (Figure 4) were corrected for the amount of enzyme (0.05 mg for tyramine and 0.025 mg for other substrates) and converted from %O₂ to µmol/min/mg. SigmaPlot was used to fit a Michaelis-Menten curve (Figure 5, Appendix B).

**Kinetic Assays of N-Acyltyramines Substrate Activity with Tyrosinase**

The general reaction conditions are identical to the L-DOPA assays with the following modifications: 20% ethanol, 100 mM phosphate, pH=7.0, 0.0125-0.025 mg/mL tyrosinase (all N-acyltyramines were dissolved in 100% EtOH and were conducted at 0.0125 mg/mL tyrosinase, tyramine was dissolved in 100% water and was conducted at 0.025 mg/mL tyrosinase) and the indicated amount of substrate with a final reaction volume of 2 mL (Appendix C). Attempts were made to take substrate concentration from .1 to 10 times than K_M, however, solubility constraints on N-pentanoyltyramine and N-hexanoyltyramine made this impossible.
**Product Determination**

A reaction mixture was allowed to react for 24 hours at room temperature containing 100 mM phosphate, pH=7.0, 0.05 mg/mL tyrosinase, 20% ethanol and ~500mM N-acetyltyramine. The reaction was dried under a stream of air and the resulting brown solid vortexed exhaustively in 100% MeOD-d$_4$. $^1$H and $^{13}$C NMR were done on iNOVA 400 MHz and processed on MestReNova (Figure 8, 10). The sample was dried under air and dissolved in 100% acetone-d$_6$. $^1$H NMR was done on iNOVA 400 MHz and processed on MestReNova (Figure 11).

**Exploration of Enzymatic Lag**

Reactions were carried out as above in “Kinetic Assay of L-3,4-dihydroxyphenylalanine.” The substrate level was held at $K_M$ for each substrate and enzyme concentration was increased to the indicated amount (Appendix D).

**Modeling Experiments**

The crystal structure of mushroom tyrosinase (PDB ID 1WX2 [16]) was used for grid based ligand docking. All co-crystallized ligands deemed superfluous for enzyme function were removed from the crystal structure and polar hydrogens were added using AutoDockTools. Charges were then corrected for the requisite copper ions. The receptor grid was prepared with a grid point spacing of 0.2 Å using AutoGrid. The substrates of interest were then prepared using AutoDockTools to define torsions, rotamers and polar hydrogens. The ligands were then docked into the active site of mushroom tyrosinase using AutoDock 4.0 [53, 54]. All default settings were utilized with the exception of increasing the number of energy evaluations from $2.5 \times 10^4$ to $2.5 \times 10^7$. 

---

11
Chapter 3

Results and Discussion

*Kinetic Evaluation of L-DOPA and N-Acyltyramines*

Results for the kinetic constants for the six compounds studied are shown in Table 1. To verify the method, this process was done with the substrate L-DOPA, which is well studied in the literature.

![Processed oxygen electrode data for 50 mM tyramine with .05 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.](image)

Figure 4: Processed oxygen electrode data for 50 mM tyramine with .05 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure 5. Michaelis-Menten plot of tyrosinase activity with tyramine.

Table 1. Kinetic constants for \( N \)-acyltyramines. L-DOPA (C\(_{9}\)H\(_{11}\)NO\(_{4}\)), was used as a validation, table values are experimental but match literature values [19, 55].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_M ) (mM)</th>
<th>( V_{\text{max}} ) (( \mu \text{mol/min/mg} ))</th>
<th>Relative ( V_{\text{max}}/K_M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-DOPA</td>
<td>1.4 ± .38</td>
<td>140 ± 12</td>
<td>100</td>
</tr>
<tr>
<td>Tyramine</td>
<td>6.6 ± .88</td>
<td>12 ± .48</td>
<td>1</td>
</tr>
<tr>
<td>( N )-acetyltyramine</td>
<td>1.2 ± .27</td>
<td>37 ± 2.6</td>
<td>16.4</td>
</tr>
<tr>
<td>( N )-propanoyltymine</td>
<td>1.3 ± .13</td>
<td>19 ± .63</td>
<td>7.7</td>
</tr>
<tr>
<td>( N )-butyryltymine</td>
<td>2.3 ± .20</td>
<td>36 ± 1.2</td>
<td>8.2</td>
</tr>
<tr>
<td>( N )-pentanoyltymine</td>
<td>1.7 ± .28</td>
<td>19 ± 1.8</td>
<td>5.9</td>
</tr>
<tr>
<td>( N )-hexanoyltymine</td>
<td>1.9 ± .30</td>
<td>31 ± 2.7</td>
<td>8.6</td>
</tr>
</tbody>
</table>

Results show that \( N \)-acyltyramines are substrates for tyrosinase. It is clear that all the \( N \)-acyltyramines had higher binding affinity and turnover values than tyramine. There is little variation in \( V_{\text{max}}/K_M \) values; however, \( N \)-acetyltyramine exhibited the highest turnover and tightest binding values of the substituted tyramines. The acyl chain length does not appear to have an effect on the binding properties, as all \( K_M \)s are in the same range.
Docking Experiments

Tyramine and \( N \)-acytytyramines with chain lengths between two and four carbons were docked into the mushroom tyrosinase crystal structure using AutoDock 4.0 (Figure 6) in order to evaluate their relative binding energies (Table 2). The logarithmic nature of calculated binding energies indicates all \( K_M \) values should be of the same magnitude, which is in agreement with experimental values.

Table 2. Kinetic constants and calculated free energy of binding for \( N \)-acyltyramines.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_M ) (mM)</th>
<th>Calculated Free Energy of Binding (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyramine</td>
<td>6.6 ± .9</td>
<td>-4.55 ± -2.5</td>
</tr>
<tr>
<td>( N )-acetyltyramine</td>
<td>1.2 ± .3</td>
<td>-4.28 ± -2.5</td>
</tr>
<tr>
<td>( N )-propanoyltyramine</td>
<td>1.3 ± .1</td>
<td>-4.43 ± -2.5</td>
</tr>
<tr>
<td>( N )-butyryltyramine</td>
<td>2.3 ± .2</td>
<td>-4.29 ± -2.5</td>
</tr>
<tr>
<td>( N )-pentanoyltyramine</td>
<td>1.7 ± .3</td>
<td>-4.37 ± -2.5</td>
</tr>
<tr>
<td>( N )-hexanoyltyramine</td>
<td>1.9 ± .3</td>
<td>-4.72 ± -2.5</td>
</tr>
</tbody>
</table>

Figure 6. Active site of tyrosinase with \( N \)-acetyltyramine bound. Color scheme: Cu (orange), \( O_2 \) (red), hisitidines and bound \( N \)-acetyltyramine (generated from [16]).
Product Determination

A reaction was conducted as described in “Product Determination” in the Materials and Methods section, where the solution was a dark brown color at the end. The sample was dried under air leaving a brown solid which consisted of the product, unreacted N-acetyltyramine, enzyme and buffer. Only N-acetyltyramine and product would be expected to dissolve in MeOH; the solid was exhaustively vortexed in MeOD-d₄ in ¹H and ¹³C NMR taken on iNOVA 400 and processed with MestReNova (Figure 8, 10). The NMR appears to be of a pure compound and the peaks can be assigned to the expected product as indicated for ¹H (Figure 7, 8), and ¹³C (Figure 9, 10). Note that in MeOD-d₄, there is a pentet overlapping the expected triplet at 3.3 ppm, which makes integration unsuccessful. Based on the ¹H and ¹³C spectra, it was determined that the product was 1-acetyl-2,3-dihydro-1H-indole-6,7-dione. In order to verify that the product was a quinone rather than a diphenol, the sample was dried as before and dissolved in acetone-d₆; NMR was run on an iNOVA 400 and processed with MestReNova (Figure 11). The resulting spectrum showed no evidence of an OH band suggesting that the compound is a quinone. This is not an unreasonable outcome, as Borovanksy et al proposed cyclization after N-acetyldopamine is oxidized [28].
Figure 7. Product structure: 1-acetyl-2,3-dihydro-1H-indole-6,7-dione with \(^1\)H predictions.

Figure 8. \(^1\)H NMR spectrum in MeOD-d₄ of tyrosinase reaction product where N-acetyltaramine was the substrate.
Figure 9. Product structure: 1-acetyl-2,3-dihydro-1H-indole-6,7-dione with $^{13}$C predictions.

Figure 10. $^{13}$C NMR spectrum in MeOD-d$_4$ of ryrosinase reaction product where $N$-acetyltyramine was the substrate in MeOD-d$_4$. 
Figure 11. $^1$H NMR spectrum in acetone-$d_6$ of tyrosinase reaction product where $N$-acetyltyramine was the substrate in acetone-$d_6$.

**Enzymatic Lag**

The reaction was conducted as described above in “Exploration of Enzymatic Lag” in Materials and Methods section as well as “Kinetic Assays.” Each substrate was held at $K_M$ and tyrosinase concentration was increased. This was not feasible with $N$-pentanoyltyramine and $N$-hexanoyltyramine because their $K_M$ was roughly at the limit of solubility. The oxygen electrode data is in Appendix D. A graph of the lag times is shown in Figure 12. Lag decreases as enzyme concentration increases, in agreement with the literature. This is due to the low natural abundance of tyrosinase in the $E_{ox}$ state increasing. Lag is shorter for aclytyramines over tyramine, but $\tau$ is unaffected by the change in chain length. The decrease in lag is likely caused by the larger $V_{max}/K_M$
leading to faster accumulation of diphenol which can then generate $E_{ox}$, the active form of the enzyme.

![Figure 12. Lag times for tyramine and $N$-acyltamines with increasing tyrosinase.](image)

**Conclusion**

$N$-acyltamines were shown to be substrates for tyrosinase, suggesting that they could act as the precursors to $N$-acyldopamines. Furthermore, $N$-acyltamines have substantially higher $V_{max}/K_M$ than tyramine; even though, changing the length of the substituent does not have a significant effect on $K_M$ or $V_{max}/K_M$ values. While $\tau$ is shorter for $N$-acyltamines than for tyramine, changing chain lengths has no effect. The observed decrease in lag can be attributed to higher $V_{max}/K_M$ values for $N$-acyltamines than tyramine. The product is exactly what is expected after formation of $N$-acyldopamine, which further supports $N$-acyltamines as a precursor for $N$-acyldopamines. In order to get a more definitive answer, a study of $N$-acyltyrosines as precursors for $N$-acyldopamines should be conducted.


Appendix A: NMR Spectra

Figure A-1. $^1$H NMR spectrum of N-acetyltartramine in MeOD-d$_4$. 
Figure A-2. $^{13}$C NMR spectrum of N-acetyltyramine in MeOD-d$_4$. 
Figure A-3. $^1$H NMR spectrum of N-propanoyltyramine in MeOD-d$_4$. 
Figure A-4. $^{13}$C NMR spectrum of N-propanoyltetramine in MeOD-d$_4$. 
Figure A-5. $^1$H NMR spectrum of N-butyryltyramine in MeOD-d$_4$. 
Figure A-6. $^{13}$C NMR spectrum of N-butyryltyramine in MeOD-$d_4$. 

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Figure A-7. $^1$H NMR spectrum of N-pentanoyltyramine in MeOD-$d_4$. 
Figure A-8. $^{13}$C NMR spectrum of $N$-pentanoyltyramine in MeOD-d$_4$. 
Figure A-9. $^1$H NMR spectrum of $N$-hexanoyltyramine in MeOD-$d_4$. 
Figure A-10. $^{13}$C NMR spectrum of N-hexanoyltyramine in MeOD-d$_4$. 

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Appendix B: Michaelis-Menten Graphs

Figure B-1. Michaelis-Menten plot of tyrosinase activity with N-acetytyramine.
Figure B-2. Michaelis-Menten plot of tyrosinase activity with N-propanoyltyramine.

Figure B-3. Michaelis-Menten plot of tyrosinase activity with N-butyryltyramine.
Figure B-4. Michaelis-Menten plot of tyrosinase activity with \(N\)-pentanoyltyramine.

Figure B-5. Michaelis-Menten plot of tyrosinase activity with \(N\)-hexanoyltyramine.
Appendix C: Oxygen Electrode Traces for Kinetic Parameters

Figure C-1. Processed oxygen electrode data for 1 mM tyramine with .05 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-2. Processed oxygen electrode data for 2 mM tyramine with .05 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= 0.0051x + 94.645 & y &= -0.1183x + 103.35 \\
y &= 0.0005x + 84.068 & y &= -0.0938x + 90.414
\end{align*}
\]

Figure C-3. Processed oxygen electrode data for 5 mM tyramine with .05 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= 0.0042x + 82.637 & y &= -0.1954x + 97.12 \\
y &= -0.0062x + 83.342 & y &= -0.2238x + 99.036
\end{align*}
\]
Figure C-4. Processed oxygen electrode data for 15 mM tyramine with .05 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= 0.0052x + 77.839 \\
y &= -0.0084x + 83.606 \\
15 \text{ mM Tyramine} & y = -0.3112x + 101.13 \\
y &= -0.2912x + 107.93
\end{align*}
\]

Figure C-5. Processed oxygen electrode data for 25 mM tyramine with .05 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= -0.004x + 78.126 \\
y &= 0.0061x + 76.412 \\
25 \text{ mM Tyramine} & y = -0.3508x + 107.24 \\
y &= -0.3805x + 99.406
\end{align*}
\]
Figure C-6. Processed oxygen electrode data for .15 mM N-acetyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-7. Processed oxygen electrode data for .31 mM N-acetyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-8. Processed oxygen electrode data for .625 mM N-acetyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-9. Processed oxygen electrode data for 1.25 mM N-acetyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-10. Processed oxygen electrode data for 2.5 mM $N$-acetyltyramine with .025 mg tyrosinase.
Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-11. Processed oxygen electrode data for 5 mM $N$-acetyltyramine with .025 mg tyrosinase.
Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-12. Processed oxygen electrode data for 10 mM N-acetyltamrine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-13. Processed oxygen electrode data for .31 mM N-propanoyltamrine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-14. Processed oxygen electrode data for .625 mM N-propanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= -7 \times 10^{-15}x + 85.086 \\
y &= -0.0141x + 88.104 \\
y &= -0.0044x + 83.693 \\
y &= -0.0045x + 84.581
\end{align*}
\]

Figure C-15. Processed oxygen electrode data for 1.25 mM N-propanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= -0.2459x + 104.48 \\
y &= -0.2337x + 105.12 \\
y &= -0.3565x + 115.83 \\
y &= -0.3624x + 115.89
\end{align*}
\]
Figure C-16. Processed oxygen electrode data for 2.5 mM N-propanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-17. Processed oxygen electrode data for 5 mM N-propanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-18. Processed oxygen electrode data for 10 mM N-propanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-19. Processed oxygen electrode data for .31 mM N-butyryltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-20. Processed oxygen electrode data for .625 mM N-Butyryltymamine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-21. Processed oxygen electrode data for 1.25 mM N-Butyryltymamine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-22. Processed oxygen electrode data for 2.5 mM $\text{N}$-butyryltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-23. Processed oxygen electrode data for 5 mM $\text{N}$-butyryltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-24. Processed oxygen electrode data for 10 mM N-butyryltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-25. Processed oxygen electrode data for .15 mM N-pentanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-26. Processed oxygen electrode data for .31 mM N-pentanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-27. Processed oxygen electrode data for .625 mM N-pentanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-28. Processed oxygen electrode data for 1.25 mM N-pentanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-29. Processed oxygen electrode data for 2 mM N-pentanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-30. Processed oxygen electrode data for .15 mM N-hexanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

Figure C-31. Processed oxygen electrode data for .31 mM N-hexanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.
Figure C-32. Processed oxygen electrode data for .625 mM \( N \)-hexanoyltiramime with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= -0.0068x + 98.033 \\
y &= -0.0023x + 96.664 \\
y &= -0.0043x + 103.05
\end{align*}
\]

Figure C-33. Processed oxygen electrode data for 1.25 mM \( N \)-hexanoyltiramime with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= -0.1334x + 106.19 \\
y &= -0.1204x + 104.92 \\
y &= -0.217x + 117.87 \\
y &= -0.2231x + 115.04
\end{align*}
\]
Figure C-34. Processed oxygen electrode data for 1.75 mM \(N\)-hexanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= 4E-15x + 96.041 & y &= -0.2739x + 119.49 \\
y &= -0.0065x + 96.869 & y &= -0.2887x + 119.69 \
\end{align*}
\]

Figure C-35. Processed oxygen electrode data for 2.5 mM \(N\)-hexanoyltyramine with .025 mg tyrosinase. Best-fit line formulas are shown which form the basis for determining reaction and background rates.

\[
\begin{align*}
y &= -0.0477x + 109.84 & y &= -0.3547x + 140.84 \\
y &= -0.0094x + 101.76 & y &= -0.2977x + 129.40 \
\end{align*}
\]
Appendix D: Oxygen Electrode Traces for Lag Determination

Figure D-1. Processed oxygen electrode data for 6.6 mM tyramine with .025 mg tyrosinase.

Figure D-1. Processed oxygen electrode data for 6.6 mM tyramine with .025 mg tyrosinase.
Figure D-2. Processed oxygen electrode data for 6.6 mM tyramine with .05 mg tyrosinase.

Figure D-3. Processed oxygen electrode data for 6.6 mM tyramine with .075 mg tyrosinase.
Figure D-4. Processed oxygen electrode data for 6.6 mM tyramine with .1 mg tyrosinase.

Figure D-5. Processed oxygen electrode data for 6.6 mM tyramine with .15 mg tyrosinase.
Figure D-6. Processed oxygen electrode data for 6.6 mM tyramine with .2 mg tyrosinase.

Figure D-7. Processed oxygen electrode data for 1.2 mM N-acetyltyramine with .00625 mg tyrosinase.
Figure D-8. Processed oxygen electrode data for 1.2 mM N-acetyltyramine with .0125 mg tyrosinase.

Figure D-9. Processed oxygen electrode data for 1.2 mM N-acetyltyramine with .025 mg tyrosinase.
Figure D-10. Processed oxygen electrode data for 1.2 mM N-acetyltyramine with .05 mg tyrosinase.

Figure D-11. Processed oxygen electrode data for 1.2 mM N-acetyltyramine with .075 mg tyrosinase.
Figure D-12. Processed oxygen electrode data for 1.2 mM N-acetyltyramine with .1 mg tyrosinase.

Figure D-13. Processed oxygen electrode data for 1.2 mM N-propanoyltyramine with .00625 mg tyrosinase.
Figure D-14. Processed oxygen electrode data for 1.2 mM N-propanoyltysamine with .0125 mg tyrosinase.

Figure D-15. Processed oxygen electrode data for 1.2 mM N-propanoyltysamine with .025 mg tyrosinase.
Figure D-16. Processed oxygen electrode data for 1.2 mM N-propanoyltyramine with .05 mg tyrosinase.

Figure D-17. Processed oxygen electrode data for 1.2 mM N-propanoyltyramine with .075 mg tyrosinase.
Figure D-18. Processed oxygen electrode data for 1.2 mM N-propanoyltyramine with .15 mg tyrosinase.

Figure D-19. Processed oxygen electrode data for 2.3 mM N-butyryltyramine with .0031 mg tyrosinase.
Figure D-20. Processed oxygen electrode data for 2.3 mM N-butyrylttyramine with .0062 mg tyrosinase.

Figure D-21. Processed oxygen electrode data for 2.3 mM N-butyrylttyramine with .0125 mg tyrosinase.
Figure D-22. Processed oxygen electrode data for 2.3 mM N-butyryltyramine with .05 mg tyrosinase.

Figure D-23. Processed oxygen electrode data for 2.3 mM N-butyryltiramine with .075 mg tyrosinase.
Figure D-24. Processed oxygen electrode data for 2.3 mM N-butyryltyramine with .1 mg tyrosinase.