SYNTHESIS & EVALUATION OF SUBSTRATE ANALOGS FOR HUMAN & BACTERIAL KYNURENINASE AND SYNTHESIS & STABILITY STUDIES OF CAGED KYNURENINE

by

CHANDAN MAITRANI

(Under the Direction of ROBERT S PHILLIPS)

ABSTRACT

The present dissertation includes five chapters: Chapter 1 includes the introduction to tryptophan and the enzyme kynureninase, along with literature review.

Chapter 2 includes the synthesis of the various substrate analogs of the enzyme kynureninase. A detailed synthetic method for the preparation of the racemic 3-chloro, 3-fluoro, 3-methyl, 5-bromo, and 5-chloro kynurenines has been described in this chapter. The racemic 3-chloro, 3-fluoro, and 3-methyl kynurenines have been prepared starting from the corresponding o-substituted anilines. A diazotization of these anilines followed by a stannous chloride reduction gives the corresponding 2-substituted phenylhydrazines. Reaction of the phenylhydrazines with the Michael adduct of diethyl acetamidomalonate and acrolein give the corresponding 2-substituted phenylhydrazone derivatives. These phenylhydrazone derivatives are then subjected to a Fischer indole cyclization to give the 3,7-disubstituted indoles. Ozonolysis of the indoles followed by acid hydrolysis affords the racemic kynurenines. The 5-bromo-L-kynurenine and 5-chloro-L-kynurenine have been prepared from L-tryptophan.
Chapter 3 includes the results and discussion of the steady state kinetic studies of the synthesized substrate analogs.

Chapter 4 includes the synthesis of a caged kynurenines and its stability studies using HPLC.

Chapter 5 includes Conclusions.

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by

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009
SYNTHESIS & EVALUATION OF SUBSTRATE ANALOGS FOR HUMAN & BACTERIAL KYNURENINASE AND SYNTHESIS AND STABILITY STUDIES OF CAGED KYNURENINE

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Dean of the Graduate School
The University of Georgia
August 2009
DEDICATION

To my parents, sisters, all friends and well wishers back home and at the UGA for their constant blessings, encouragement and trust in me over the years
ACKNOWLEDGEMENTS

First of all I would like to thank Prof. Dr. Robert Phillips for his extreme patience while providing me valuable guidance over the years of working in his research lab. He is one of the most uniquely warm personalities I have known. From the very time I have met Dr. Phillips I have always seen him as a ‘Speaking Tree’ that bends down to earth as it bears the fruits of knowledge. He always was extremely helpful in giving me valuable suggestions, but at the same time also challenged me to think and try to figure out things myself, which turned out to be very beneficial in the long run. I have always seen him as a great instructor who is very friendly with the students and very approachable. I very much appreciate his treating each of us students as equals and never making a student feel silly for asking a question. Also, he is very knowledgeable about the research subjects involved. I always had the impression that he genuinely wanted to help us. Above all the most positive trait that I have learned from Dr. Phillips is his kindness. There were several occasions when I would need some help with the instruments in the biochemistry lab, and I had no hesitation to walk up to his office and ask for guidance. Without any exception he would always find time to help me with whatever I needed. He has been my most ideal boss ever. If I am to become a boss in future I will never forget the attributes that Dr. Phillips possesses as a boss.

Secondly, I would like to thank my advisory committee members Prof. Dr. George Majetich, and Prof. Dr. Jonathan Amster for willing to serve on the committee and provide valuable suggestions and constant encouragement over the years. My special thanks to Dr. Majetich for giving me valuable lessons in the Organic Reaction Mechanisms class. Also, my special thanks to Dr. Amster for the valuable lessons in the Mass Spectrometry class.
I would also like to thank the Department Head, Prof. Dr. John Stickney, as well as all other faculty members for contributing toward my educational and career goals in some way or the other.

I also owe huge gratitude to all my group members Austin, Bryan, Chris, Johnny, Kyle, Nathan, Phanneth, and Sunil for their unforgettable company, and help while working in the lab. Also, I would like to thank my past group members Jalandhar Borra, Dr. Santiago Lima, Dr. Vijay Gawandi, and Dr. Bhaktavatsalam Sundararaju for their help and valuable suggestions. Apart from all these I owe thanks to Dr. Majetich, Dr. Popik, Dr. Geng, Dr. Dore, and the research group members of all these groups for allowing me to use chemicals from their labs whenever I needed. I owe special thanks to Dr. Popik and his research group members for helping me with the GCMS of my samples on their instrument.

Also, thanks to the Department of Chemistry, and the Graduate School for continuously supporting me on an assistantship over the years.

And last but not the least thanks to my wonderful parents, sisters, and all my friends for their non-stop blessings, encouragement, and trust in me over the years.
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<tr>
<td>Ac</td>
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<td>bs</td>
<td>broad singlet</td>
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<td>CNS</td>
<td>central Nervous System</td>
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<td>d</td>
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<tr>
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<tr>
<td>m</td>
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</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>rac. or DL</td>
<td>racemic mixture</td>
</tr>
<tr>
<td>RT</td>
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<tr>
<td>TEA</td>
<td>triethanolamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<td>TRIS</td>
<td>tris (hydroxymethyl) amino methane</td>
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Tryptophan

Tryptophan (1)\(^1\) is an essential amino acid required by humans for protein anabolism. The term ‘essential amino acid’ refers to the fact that the body cannot synthesize the amino acid but has to depend on the external dietary sources. These dietary sources include meat, poultry, eggs, turkey, fish, milk, yogurt, cheese, sesame seeds, garbanzo beans, and peanuts. In the catabolic pathway tryptophan is involved in the biosynthesis of several biologically active compounds\(^2,3\) in the central nervous system. These biologically active compounds include the neurotransmitter serotonin\(^4-6\) (5-hydroxytryptamine - 2), neurohormone melatonin\(^7,8\) (N-acetyl-5-methoxytryptamine - 3), kynuramine metabolites of melatonin, and the products of kynurenine pathway of tryptophan catabolism including 3-hydroxykynurenine (4), 3-hydroxyanthranilic acid (5), quinolinic acid\(^9-13\) (6) and kynurenic acid\(^14\) (7). Apart from this tryptophan is also involved in the biosynthesis of niacin via the kynurenine catabolic pathway. The neurotransmitter serotonin is

![Chemical structures of tryptophan-related compounds](image-url)
involved in the modulation of mood, anger, emotion and appetite, and is implicated in the control of several behavioral and physiological functions. The neurohormone melatonin serves as a biological clock that controls the sleep patterns of the individual. The metabolite quinolinic acid has been found to have agonist effects on the N-methyl-D-aspartate receptors in the central nervous system and thus acts as a potent neurotoxin. Thus, different metabolites of tryptophan play an important role in the central nervous system and in the overall physiology and behavioral patterns of the organism.

**Catabolism of tryptophan**

The kynurenine pathway is the primary pathway for the catabolism of the essential amino acid tryptophan. Out of the different catabolic breakdown pathways for tryptophan leading to the formation of the bioactive compounds, 99% of the dietary tryptophan that is not used in protein synthesis is catabolised by the kynurenine pathway\(^\text{15}\). In the central nervous system before crossing the blood-brain barrier, approximately 90% of the tryptophan is complexed with plasma albumin\(^\text{16}\) and this complex cannot cross the blood brain barrier. However, the free tryptophan\(^\text{17}\) can cross blood-brain barrier where it is then available for further metabolism in the brain.

In the serotonergic neurons and mast cells of the CNS (Scheme 1) the free tryptophan is acted upon by the enzyme tryptophan hydroxylase-2 also called tryptophan-5-monooxygenase (EC: 1.14.16.4). This enzyme uses molecular oxygen and catalyzes the hydroxylation of tryptophan to 5-hydroxy-L-tryptophan in the presence of the cofactor tetrahydrobiopterin. The 5-hydroxy-L-tryptophan is then rapidly decarboxylated to serotonin\(^\text{18-20}\) in the presence of the PLP
dependent enzyme dopa decarboxylase, also called aromatic-L-amino acid decarboxylase (EC: 4.1.1.28). The serotonin is then converted into N-acetyl serotonin by the action of arylalkyl amine-N-acetyl transferase (EC: 2.3.1.87). Finally melatonin\textsuperscript{21} is obtained by the action of acetylserotonin-O-methyl transferase (EC: 2.1.1.4) on N-acetyl serotonin.

In the CNS (including the astrocytes, microglia, macrophages, and dendritic cells) and in the hepatic and non-hepatic tissues (including the lungs, small intestine, and placenta of mammals such as rabbits, rats, mice, and humans) L-tryptophan is catabolised by the kynurenine pathway\textsuperscript{15}. The first step in this pathway (Scheme 2) is the oxidative cleavage of the pyrrole ring of tryptophan by the action of the hemeprotein indoleamine-2,3-dioxygenase (EC: 1.13.11.52) in the presence of molecular oxygen to give N-formyl-L-kynurenine. However, in the mammalian liver, the major site for L-tryptophan catabolism, the same reaction is carried out by another
heme protein tryptophan-2,3-dioxygenase (EC: 1.13.11.11). Despite both the enzymes catalyzing the same reaction using a heme cofactor, there is no significant sequence similarity between IDO and TDO. Furthermore, it has been found that TDO stereospecifically catabolises only L-tryptophan, but IDO can catabolise the oxidative cleavage of D-tryptophan, L-tryptophan, as well as the various indoleamines such as melatonin, serotonin, hence the name IDO\textsuperscript{22} for the latter. The N-formyl-L-kynurenine so formed in the first step is then deformylated to L-kynurenine by an aryl formamidase (EC: 3.5.1.9). L-kynurenine is then hydroxylated by a flavoenzyme kynurenine-3-monooxygenase (EC: 1.14.13.9) to give 3-hydroxy-L-kynurenine. Subsequent action of the PLP dependent enzyme kynureninase (EC: 3.7.1.3) on 3-hydroxy-L-kynurenine results in the cleavage of the $\beta,\gamma$ C-C bond to give 3-hydroxyanthranilate and L-
alanine. 3-Hydroxyanthranilate is then converted by a non heme 3-hydroxyanthranilate-3,4-dioxygenase (EC: 1.13.11.6) to 2-amino-3-carboxymuconate semialdehyde which spontaneously cyclizes to form quinolinate\(^{23}\). Alternatively, the 2-amino-3-carboxymuconate semialdehyde is enzymatically decarboxylated by aminocarboxymuconate semialdehyde decarboxylase (EC: 4.1.1.45) and then oxidized to 2-aminomuconic acid finally resulting in acetylCoA in the ‘Glycolysis’ pathway. In the liver, further metabolism of the quinolinate serves as the de novo pathway to NAD(P)\(^+\). This finally leads to nicotinamide (niacinamide; vitamin B\(_3\)) which can thus be biosynthesized in mammals at times of dietary shortage. However, quinolinic acid if produced extrahepatically in excess of biosynthetic requirements, acts as a potent neurotoxin with agonist effects on the NMDA receptors\(^{24}\) in the CNS.

![Scheme 3](image-url)
In another side biochemical reaction (Scheme 3) of the kynurenine pathway, L-kynurenine is acted upon by kynurenine-oxoglutarate transaminase to give 4-(2-aminophenyl)-2,4-dioxobutanoate which spontaneously dehydrates to produce kynurenate. The neuroactive metabolite kynurenic acid has been found to have antagonist effects\textsuperscript{14} on the NMDA and α7 nicotinic acetyl choline receptors. Similarly, 3-hydroxy-L-kynurenine is acted upon by kynurenine-oxoglutarate transaminase to give 4-(2-amino-3-hydroxyphenyl)-2,4-dioxobutanoate which spontaneously dehydrates to produce xanthurenicate.

The enzyme kynureninase

Kynureninase\textsuperscript{25} or L-kynurenine hydrolase (EC: 3.7.1.3) is a pyridoxal-5'-phosphate (PLP) dependent enzyme that catalyzes the hydrolytic cleavage of L-kynurenine (Scheme 4) to

\[
\begin{align*}
\text{L-Kynurenine} & \Rightarrow \text{Anthranilate} + \text{L-Alanine} \\
\text{3-hydroxy-L-Kynurenine} & \Rightarrow \text{3-hydroxyanthranilate} + \text{L-Alanine}
\end{align*}
\]

Scheme 4
give anthranilic acid and L-alanine. This is a key enzyme in the kynurenine pathway in the tryptophan catabolism and catalyzes the unique $\beta,\gamma$-cleavage of aryl substituted $\gamma$-keto-\(\alpha\)-amino acids\(^{26}\). The enzyme has been isolated from \textit{Pseudomonas fluorescens}\(^{27}\), \textit{Neurospora crassa}\(^{28,29}\), rat liver\(^{30}\) and porcine liver\(^{31}\). It has been found that the mammalian liver kynureninase cleaves 3-hydroxy-L-kynurenine about twice as rapidly as it does L-kynurenine\(^{26,30}\) while the bacterial kynureninase from \textit{Pseudomonas fluorescens} cleaves L-kynurenine about five times\(^{32}\) as rapidly as it does 3-hydroxy-L-kynurenine. Thus, L-kynurenine is the preferred substrate for the pseudomonad enzyme while 3-hydroxy-L-kynurenine is the preferred substrate for the mammalian liver kynureninase.

The two distinct types of kynureninases\(^{33}\) that have been shown to occur differ in terms of their kinetic and chemical properties toward kynurenine and 3-hydroxykynurenine and in terms of their response to PLP. Of these two types, the inducible enzyme is termed kynureninase and is involved in preferential reaction with L-kynurenine in the aromatic and the quinoline pathway of tryptophan catabolism. The specific activity of the inducible enzyme depends on the concentration of tryptophan in the medium to such an extent that almost no inducible activity\(^{34}\) is observed in the cells not supplemented with L-tryptophan. Thus, the cells utilize L-tryptophan as the sole source of carbon, nitrogen, and energy for growth. On the other hand, the non-inducible or the constitutive enzyme is termed 3-hydroxykynureninase and is mainly involved in the biosynthesis of NAD i.e. the NAD pathway of tryptophan catabolism. The specific activity of the non-inducible enzyme is independent of the concentration of tryptophan in the growth medium\(^{34}\). The inducible enzyme has low $K_m$ for L-kynurenine while the non-inducible enzyme has low $K_m$ for 3-hydroxy-L-kynurenine. Furthermore, it has been found that the inducible
enzyme is reversibly inactivated\textsuperscript{9} by L-alanine resulting in a transamination reaction to give pyridoxamine-5'-phosphate (Scheme 5) and pyruvate from L-alanine. However, the enzyme activity is restored either by addition of PLP or pyruvic acid in the latter case there being a reverse transamination between pyridoxamine-5'-phosphate and the added pyruvate to give back PLP and alanine.

On the other hand the non inducible (or constitutive) enzyme is little or not at all affected by the presence of L-alanine or other amino acids\textsuperscript{35a}. Even then the rate of the hydrolytic cleavage reduces with time indicating that the product 3-hydroxyanthranilate inhibits the non inducible enzyme thereby regulating the enzyme action in the NAD biosynthetic pathway\textsuperscript{35b}.

Scheme 5
The bacterial cultures that possess the inducible enzyme include *Pseudomonas fluorescens* and *Bacillus cereus*\(^{36}\), *Bacillus megaterium*\(^{37,38}\) *Acinetobacter calcoaceticus*\(^{39}\) and *Xanthomonas pruni*\(^{40}\). Among the fungal species, *Neurospora crassa*, *Aspergillus niger*, and *Penicillium roqueforti* possess both the inducible as well as the constitutive kynureninases while *Rhizopus stolonifer*\(^ {34}\) possesses only the constitutive enzyme. The kynureninases obtained from yeast and the livers of mammals like dog, mouse, guinea pig, beef, and human are the constitutive enzyme\(^ {26}\).

**Mechanism of kynureninase action**

The enzyme kynureninase catalyzes the unique \(\beta,\gamma\)-cleavage of aryl substituted \(\gamma\)-keto-\(\alpha\)-amino acids in the kynurenine pathway of tryptophan catabolism. The mechanism of kynureninase has been the subject of considerable interest due to the unique nature of this PLP dependent reaction. In one of the mechanisms by Dalgliesh *et al* it was proposed that kynureninase catalyzes the transamination of kynurenine\(^ {41}\) by PLP to give the \(\beta\)-anthraniloyl pyruvic acid (Scheme 6) which is then hydrolyzed to anthranilic acid and pyruvate or partly undergoes a spontaneous dehydrative cyclization to give kynurenic acid. The pyruvate in turn recycles with PMP to give back PLP and alanine. But later another enzyme kynurenine amino transferase\(^ {42-44}\) was shown to be involved in the formation of kynurenic acid.

In another mechanism proposed by Braunstein *et al*\(^ {45-46}\) (Scheme 7) the initially formed Schiff’s base between PLP and kynurenine undergoes a tautomerization followed by hydrolysis at the \(\gamma\)-carbonyl carbon. This cleaves the \(\beta,\gamma\)-carbon bond in a way that the \(\beta\)-carbon takes up
Scheme 6
Kynurenine

PLP bound to lysine

Kynurenine aldime

Kynurenine ketimine intermediate

Alanine

Pyravate ketimine

Anthranilic acid

Scheme 7
Scheme 8
the \(\sigma\)-electrons of the \(\beta,\gamma\)-carbon bond to give anthranilic acid and the pyruvate ketimine. The pyruvate ketimine is then converted into alanine and PLP after tautomeration.

Longenecker et al\(^{47}\) however proposed a slightly different mechanism than the Braunstein group based on their study of mechanisms of enzymes including serine dehydrase, tryptophanase, and cysteine desulphhydrase. In this mechanism (Scheme 8) after the initial formation of the kynurenine ketimine, instead of the \(\beta\)-carbon keeping the electrons of the \(\beta,\gamma\)-carbon bond, the \(\gamma\)-carbonyl carbon takes up the electron pair as shown by the tautomerization process to give the anthranyl anion and the aldimine of \(\alpha\)-amino acrylate. The anthranyl anion can then either before or after stabilization (as anthraldehyde) undergoes a non-enzymatic redox reaction to give anthranilic acid, and the Schiff’s base of alanine, the latter being eventually hydrolyzed to alanine with the regeneration of PLP.

In their mechanistic studies on kynureninase from *Pseudomonas marginalis* Bild and Morris\(^{48}\) suggested that the \(\beta\)-carbon of kynurenine must be serving as carbanion. This was based on the formation of 2-amino-4-hydroxy-4-phenyl butanoic acid (Scheme 9) via an aldol type reaction\(^{49}\) between the incipient alanine and benzaldehyde.

![Scheme 9](image_url)
Evidence for the existence of a $\beta$ carbanion was also indicated by Tanizawa and Soda$^{50}$ who reported the formation of anthraldehyde in a retro aldol reaction (Scheme 10) from the reduced form of kynurenine viz. dihydro kynurenine. If the $\gamma$ carbanion were formed as proposed by Longenecker et al then the product would have been o-amino benzyl alcohol which would not undergo oxidation to anthraldehyde under the experimental conditions.

![Scheme 10](image)

Phillips and Dua$^{49}$ also confirmed the formation the aldol from the reaction of kynurenine with benzaldehyde, which gave a 2:3 mixture of the 4S:4R diastereomers of 2-amino-4-hydroxy-4-phenyl butanoic acid. Based on their findings they concluded the formation of a gem-diolate intermediate in the cleavage mechanism (Scheme 11). It was found by Palcic et al $^{51}$ that the $\varepsilon$-amino group of a lysine residue is involved in the $\alpha$-proton abstraction which produces the kynurenine ketimine that subsequently serves as a sink for the electrons from the $\beta,\gamma$- carbon-carbon bond cleavage. Thus there are two bases involved in the mechanism of the cleavage. The first in the $\alpha$-proton abstraction to give the ketimine and the second in the removal of a 4-hydroxy proton from the dihydrokynurenine OR the hydration of the carbonyl carbon in kynurenine (to give the gem-diolate intermediate). In the subsequent mechanism the tetrahedral gem-diolate intermediate rapidly collapses to give out anthranilic acid and the PLP enamine of
Scheme 11
alanine. This enamine first takes up a proton at the β-carbon to give the pyruvate ketimine that accepts a second proton at the α-carbon to give alanine aldimine which finally releases alanine and the cofactor recycles in the process.

Later Dua and Phillips\textsuperscript{52} also showed that the sulfone analog of kynurenine viz. S-(2-aminophenyl)-L-cysteine-S,S-dioxide was a potent inhibitor of kynureninase with a $K_i$ value of 70 nM which is about 300-fold lower than the $K_m$ for L-kynurenine. This further supports the gem-diolate hypothesis. Kinetic isotope effect studies\textsuperscript{53} by the Phillips group led to the conclusion that the rate determining step is the deprotonation of the aldehydic carbon of PLP in the pyruvate ketamine intermediate to give the alanine quinonoid intermediate. Using rapid-scanning stopped-flow spectrophotometry and rapid chemical quench methods\textsuperscript{54} the L-kynurenine quinonoid intermediate, and the pyruvate ketimine intermediate were detected with L-kynurenine as the substrate. Thus, the mechanism for kynureninase proposed by Phillips \textit{et al} is shown in Scheme 12.
Scheme 12
Kynurenines

Kynurenine or β-(2-aminobenzoyl)alanine was first discovered by Matsuoka and Yoshimatsu\textsuperscript{55} in the urine of rabbits fed large quantities of the amino acid tryptophan. About two decades later the structure was determined by Butenandt \textit{et al.}\textsuperscript{56} In \textit{Pseudomonas fluorescens} and some other bacteria kynurenine is a substrate in its reaction with the enzyme kynureninase to give anthranilic acid and L-alanine in the tryptophan catabolic pathway. As described above, in eukaryotes a similar substrate viz. 3-hydroxy-L-kynurenine is involved in a similar reaction to produce L-alanine and 3-hydroxyanthranilic acid (Scheme 4). In animals including humans 3-hydroxyanthranilic acid serves as a precursor for the biosynthesis of quinolinic acid (Scheme 2). Excessive levels of quinolinic acid have been implicated in a range of neurological disorders\textsuperscript{57-58} such as Huntington’s chorea, Lou Gehrig’s disease\textsuperscript{59}, epilepsy\textsuperscript{60}, and AIDS related dementia. An excessive level of quinolinate has been shown to be present after a stroke and is responsible for further damage\textsuperscript{61}. Furthermore, the brains of Alzheimer’s patients have also been shown to have high levels of quinolinate which may be responsible for the progression of the disease\textsuperscript{62}. It has also been shown that spontaneously hypertensive rats have a significantly higher kynureninase activity in tissues\textsuperscript{63} and recently it has been established that there is close link between hypertension and an allele of the human kynureninase viz. K412E\textsuperscript{64}. Selective inhibitors of 3-hydroxykynureninase could thus be used as drugs for the treatment of these diseases. Several structural analogs of kynurenine have been synthesized in the past to check for their inhibitory activity. The most potent inhibitor of kynureninase reported the date is the S-(2-aminophenyl)-L-cysteine-S,S-dioxide, \textbf{8} with a $K_i$ of 27 nM, some 925-fold lower than the $K_m$ of L-kynurenine ($\sim 25 \mu\text{M}$)\textsuperscript{52,65}. The 4-bromo, \textbf{9}, and the 5-bromo, \textbf{10} analogs of S-(2-aminophenyl)-L-cysteine-
S,S-dioxide were also found to be potent inhibitors\textsuperscript{66} with $K_i$ values of 300 nM and 400 nM respectively. The other less potent inhibitors in this category were the non-sulfone analog viz. S-(2-aminophenyl)-L-cysteine, \textbf{11} with a $K_i$ of 2.5 µM and the des-amino analog viz. S-phenyl-L-cysteine-S,S-dioxide, \textbf{12} with a $K_i$ of 3.9 µM.
Also, the 4-amino and the 4-nitro sulfone analogs viz. S-(4-aminophenyl)-L-cysteine-S,S-dioxide, 13 and S-(4-nitrophenyl)-L-cysteine-S,S-dioxide, 14 were shown to have competitive inhibitory activity with $K_i$ values of 8.5 µM, and 12 µM respectively. The diastereomeric 4R, 15 and 4S, 16 dihydro kynurenines, have also been shown by Phillips et al. 49 to be potent inhibitors of kynureninase with $K_i$ values of 1.4 µM, and 0.3 µM respectively. The 5-bromo analogs of the dihydrokynurenines have also been shown by Heiss et al. 66 to possess good inhibitory activity with $K_i$ values of 55 nM and 170 nM respectively for the 4R, 17 and the 4S, 18 diastereomers.

In chapter 2 of this dissertation the synthesis of a new class of substrate analogs of kynurenines has been described.
References


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CHAPTER 2

SYNTHESIS OF SUBSTRATE ANALOGS OF KYNURENINE

Abstract

The DL-3-bromo, DL-3-chloro, DL-3-fluoro, DL-3-methyl, L-5-bromo, and L-5-chloro kynurenines have been synthesized. The DL analogs have been synthesized starting from acrolein. Reaction of acrolein with the diethyl acetamidomalonate anion gives the Michael adduct\(^1\)\(^-\)\(^4\) which on treatment with the corresponding 2-halosubstituted phenylhydrazine\(^5\)\(^-\)\(^11\) yields a phenylhydrazone derivative\(^2\). The different 2-halosubstituted phenylhydrazones are then subjected to a Fischer indole cyclization to give the 7-halosubstituted indolylmethylacetamido malonates\(^12\)\(^-\)\(^16\). An ozonolysis of these indole compounds give the respective diethyl-2-amino-3-halobenzoylmethylacetamidomalonates which upon acid hydrolysis produce the racemic 3-halo substituted kynurenines. The 5-substituted kynurenines\(^17\) have been synthesized from L-tryptophan via first the ozonolysis of the methyl ester of N\(^\alpha\)-acetyl-L-tryptophan followed by TFA hydrolysis and acylation of the intermediate to give the methyl ester of N\(^\alpha\),N-diacytelykynurenine. This intermediate on bromination or chlorination, followed by acid hydrolysis produces the respective 5-halosubstituted-L-kynurenines.
Experimental methods

Instrumentation

$^1\text{HNMR}$, $^{13}\text{CNMR}$, and $^{19}\text{FNMR}$ spectra were recorded on a Varian 400MHz instrument. Two different deuterated solvents were used for the different products. Of these, the 2-substituted phenylhydrazine, the phenylhydrazone derivative, the diethyl-7-substituted indolylmethylacetamidomalonate, and the 2-amino3-substituted benzoylmethylacetamidomalonate intermediates were tested in deuterated methanol, while the ultimate substituted kynurenines were tested in deuterated water containing 1 – 2 % of DCl. HPLC measurements were carried out on a Spectrasystem P 2000 instrument connected to a UV 6000 detector and controlled by a Dell PC using Chromquest software. A gradient elution was used consisting of 5 % MeOH, and 95 % 0.1 % aq. acetic acid from 0 – 5 mins. followed by a programmed increase of MeOH percentage from 5% to 70% over 5 – 20 mins. with a corresponding decrease of the percentage of 0.1 % aq. acetic acid from 95% to 30% over the same time period. This is followed by an increase of MeOH percentage to 100% with the corresponding decrease of the percentage of 0.1% aq. acetic acid to 0% over 20 – 25 mins. And finally, a programmed return back of the elution system to 5% MeOH, and 95% of 0.1% aq. acetic acid over the period from 25 – 30 mins. A 100 µM solution of the individual substituted kynurenines in 1 mM HCl was used for injection. Chiral HPLC of the DL-3-methylkynurenine was done using a chiral Pro-Cu column (5µ, 4.5 x 250 mm) and a 1 mM aq. CuSO$_4$ solution was used as the eluant. Elution for both columns was done with a flow rate of 1ml/min. with detection by absorbance at 254 nm and 370 nm. GCMS of the intermediate compounds was done on a Shimadzu instrument in Prof. Dr. V. Popik’s lab in the Chemistry Department.
Synthesis of 2-chlorophenyl hydrazine\textsuperscript{6,18-20}

Take 10 g of 2-chloroaniline hydrochloride (prepared by dissolving 10 ml of 2-chloroaniline in 100 ml acetone and adding 14 ml conc. HCl with stirring. Chill the resulting suspension, filter and wash the white solid with about 15 ml acetone) in 200 ml conc. HCl, stir at RT for about 15 mins. when a white suspension results. Cool the soln. to -20 °C, in a dry ice-acetone bath, add to it an aq. soln. of 5.05 gm of sodium nitrite in 25 ml d/w. (Addition of sodium nitrite solution is done in such a way that the tip of the dropping funnel is dipping into the RM via a small tube attached to the dripping tip of the dropping funnel) Complete the addition in about 15 mins. and then continue stirring at -20 °C for about 10 - 15 mins. Then to the same RM while maintaining the temp. at -20 to -25°C add a soln. of 27.52 gm of stannous chloride dihydrate in 25 ml of conc. HCl. Complete the addition in about 45 mins when a thick precipitate of the hydrazine hydrochloride salt is formed. Allow the RM to stir at 0 to -10°C for about 45 mins. Check TLC (Fig. 1) Cool the suspension to -45 to -50 °C, for about 15 mins. then filter. Spread the solid on a petri dish to let it air dry overnight to give 24 g of a crude solid from which the free base is obtained.

The free base of the 2-chlorophenylhydrazine is released by treatment of the hydrochloride salt with 2.7 equivalents of NaOH and the free base extracted with ether.

Yield of the free base = 7 g, 81 % , m.p. = 45-46°C
$^1$HNMR of free base: $d$-MeOH $\delta$ 6.65 (d, 1H), 6.75 (t, 1H), 7.3 (t, 1H), 7.6 (d, 1H) the NH protons exchanged with the solvent and merged around $\delta$ 4.5

$^{13}$CNMR of free base: $d$-MeOH $\delta$ 108.5, 118.2, 121, 126, 123.2, 140.5

**Synthesis of 2-chlorophenylhydrazone derivative**

To a suspension of 9.71 g of diethyl acetamidomalonate in 20 ml benzene add 97 mg MeONa, with stirring. Stir the RM at RT for about 5 mins. Then cool the suspension in an ice-water bath and add 3.6 ml of acrolein dropwise in about 20 - 25 mins. while maintaining the temp. of the RM below 5°C. After completion of addition, warm the RM to RT and stir at RT for about 2 hrs. when a clear pale yellow solution results. At the end of 2 hrs. of stirring, add 2.7 ml of AcOH, and then add a solution of 7 g of 2-chlorophenylhydrazine in 14 ml benzene, when a clear orange colored solution results. Warm the resulting RM to 55-60°C, for about 30 mins. and then leave the RM stirring to gradually attain RT. Stir for 2.5 days at RT. Check TLC (Fig. 2) by quenching a small portion of the RM in water, extract with a few drops of ethyl acetate and spot the top ethyl acetate layer.

Concentrate the RM under vacuum, to give a reddish brown oil which is used as it is for the Fischer indole cyclization.

Yield = 14 g, 72%
1H NMR: $d$ MeOH $\delta$ 7.4 (d, 1H), 7.3 (t, 1H), 7.2 (d, 1H), 7.1 (t, 1H), 6.7 (t, 1H), 4.2 (q, 4H), 2.2 (t, 1H), 2.1 (s, 3H), 1.9 (q, 2H), 1.2 (t, 6H)


**Synthesis of diethyl 7-chloroindolylmethylacetamidomalonate**

Take 14 g of the 2-chlorophenylhydrazone derivative (obtained as reddish brown oil) in 85 ml 10 % aq. sulfuric acid. Heat the RM on a boiling water bath for about 2 hrs. with vigorous stirring when a dark brown RM results. Check TLC. (Fig. 3) Cool the RM to 55 -60˚C. Add 100 ml EtOAc, to dissolve the dark brown semisolid that is found sticking to the inner walls of the flask. Stir for about 10 mins. to dissolve the semisolid completely. Then cool the RM to RT. Add 21 g NaCl, 50 ml d/w, stir at RT for about 10 mins. Separate the top organic layer. Extract the lower aq. layer with 75 ml more of ethyl acetate (EtOAc). Wash the combined organic layers once with 75 ml of saturated brine soln. then dry the organic layer over anhydrous sodium sulfate; concentrate the solvent under vacuum to give a brown semisolid.

Yield = 11 g, 82 %

1H NMR: $d$ MeOH $\delta$ 7.3 (d, 1H), 7.1 (d, 1H), 7.05 (s, 2H), 6.9 (t, 1H), 4.1 (q, 4H), 3.7 (s, 2H), 1.9 (s, 3H), 1.2 (t, 6H)
13CNMR: \(d\) MeOH \(\delta\) 13.3, 19.9, 28.2, 62.5, 68.1, 109.4, 116.7, 117.1, 119.7, 120.8, 125.1, 130.2, 133.4, 167.8, 171.5

Dissolve the resulting brown semisolid in 50 ml MeOH, add 1 g activated charcoal, stir at RT for about 10 - 15 mins. Filter through Celite, and wash the Celite bed with about 50 ml MeOH. The dark brown filtrate is used as is for the ozonolysis step.

**Synthesis of diethyl 2-amino-3-chlorobenzoylmethylacetamidomalonate and its acid hydrolysis to give DL-3-chlorokynurenine**

Cool the soln. of diethyl 7-chloroindolylmethylacetamidomalonate (11 g in 100 ml MeOH) to below -70°C using a dry ice – acetone bath. Bubble ozone gas (at 0.5 psi) through the RM for about 90 mins. Check TLC. (Fig. 4) Quench the RM with an aq. soln. of sodium bisulfite (44 g in 200 ml d/w), when a yellow suspension results. Stir for about 10 – 15 mins. to allow the RM to attain RT. Concentrate the solvent MeOH under vacuum, Add 100 ml distilled water (d/w), extract with two 75 ml portions of EtOAc. Wash the combined organic layers with 75 ml saturated brine solution. Charcoalize the organic layer, filter over Celite, dry the filtrate over anhydrous sodium sulfate, concentrate to remove the solvent and give the product as a semisolid.

Yield = 5.5 g, 50 %
Recrystallization from 30 ml of 2-propanol gives 4 g of the product as a pale yellow solid with m.p. of 177 -178ºC.

$^1$HNMR: $d$-MeOH $\delta$ 7.4 (d, 1H), 7.2 (d, 1H), 7.05 (t, 1H), 6.8 (s, 1H), 4.4 (q, 4H), 3.4 (s, 2H), 2.1 (s, 3H), 1.2 (t, 6H)

$^{13}$CNMR: $d$-MeOH $\delta$ 13.5, 20.1, 36.5, 62.2, 70.1, 116.2, 118.6, 122.1, 124.3, 130.6, 142.1, 170.4, 173.1, 205.3

Take the solid from the previous step in 40 ml of 6N HCl. Reflux on an oil bath for about 4 hrs. Then cool the RM to RT, concentrate to remove water under vacuum. Take the resulting semisolid in 20 ml d/w, charcoaloize at RT for about 15 mins. Filter through celite, wash the bed with 5ml d/w. Basify the filtrate to approx. pH 6.5 using 2N NaOH, when a solid precipitates. Filter the solid racemic 3-chlorokynurenine; wash with about 5 ml d/w. Allow to air dry overnight.

Weight of product = 1.2 g, 48 %, m.p. = 216 - 218ºC

$^1$HNMR: (1 – 2% DCl – D$_2$O) $\delta$ 7.7 (d, 1H), 7.6 (d, 1H), 6.7 (t, 1H), 4.2 (t, 1H), 3.7 (d, 2H)

$^{13}$CNMR: (1 – 2% DCl – D$_2$O) $\delta$ 43.2, 55.4, 119.1, 121.8, 123.3, 125.2, 131.4, 143.6, 172.1, 204.2

**Synthesis of 2-fluorophenylhydrazine**

Take 10 g of 2-fluoroaniline hydrochloride (prepared by dissolving 10 ml of 2-fluoroaniline in 100ml acetone and adding 13.5 ml conc. HCl with stirring. Chill the resulting suspension, filter and wash the white solid with about 15ml acetone) in 200 ml conc. HCl, stir at
RT for about 15 mins. when an almost clear solution results. Cool the soln. to -20 ºC, in a dry ice-acetone bath, add to it an aq. soln. of 5.61 g of sodium nitrite in 28 ml d/w. (Addition of sodium nitrite solution is done in such a way that the tip of the dropping funnel is dipping into the RM via a small tube attached to the dripping tip of the dropping funnel) Complete the addition in about 15 mins. and then continue stirring at -20 ºC for about 10 - 15 mins. Then to the same RM while maintaining the temp. at -20 to -25ºC add a soln. of 31 gm of stannous chloride dihydrate in 28 ml of conc. HCl. Complete the addition in about 45 mins. when a thick precipitate of the hydrazine hydrochloride salt is formed. Allow the RM to stir at 0 to -10 ºC for about 45 mins. Check TLC (Fig. 1 above) Cool the suspension to -45 to -50 ºC, for about 15 mins. then filter. Spread the solid on a petri dish to let it air dry overnight to give 20 g of a crude solid from which the free base is obtained.

The free base of 2-fluorophenylhydrazine is released by treating the hydrochloride salt with 2.7 equivalents of NaOH and the free base extracted with ether.

Yield of free base = 6 g, 71 %,  m.p. = 44 – 45ºC.

$^1$HNMR of free base: $d$-MeOH $\delta$ 6.55 (d, 1H), 6.67 (t, 1H), 7.2 (t, 1H), 7.5 (d, 1H) the NH protons exchanged with the solvent and merged around $\delta$ 4.5

$^{13}$CNMR of free base: $d$-MeOH $\delta$ 111.5, 118.2, 123.5, 128.2, 138.4, 146.1

$^{19}$FNMR of free base: $d$-MeOH $\delta$ -137.5
Synthesis of 2-fluorophenylhydrazone derivative

To a suspension of 9.4 g of diethyl acetamidomalonate in 20 ml benzene add 94 mg MeONa, with stirring. Stir the RM at RT for about 5 mins. Then cool the suspension in an ice-water bath and add 3.46 ml of acrolein dropwise over 20 - 25 mins, while maintaining the temp. of the RM below 5˚C. After completion of addition, warm the RM to RT and stir at RT for about 2 hrs. when a clear pale yellow solution results. At the end of 2 hrs. of stirring, add 2.5 ml of AcOH, and then add a solution of 6 g of 2-fluorophenylhydrazine in 12 ml benzene, when a clear orange colored solution results. Warm the resulting RM to 55-60˚C, for about 30 mins. and then leave the RM stirring to gradually attain RT. Stir for about 2.5 days at RT. Check TLC (Fig. 2 above) For TLC check quench a small portion of the RM in water, extract with a few drops of ethyl acetate and spot the top ethyl acetate layer.

Concentrate the RM under vacuum, to give a reddish brown oil which is used as it is for the Fischer indole cyclization.

Yield = 11 g, 61 %

\[ ^1\text{HNMR: } d-\text{MeOH } \delta \text{ 7.39 (t, 1H), 7.25 (t, 1H), 6.96 (d, 1H), 7.01 (d, 1H), 6.92 (s, 1H), 6.68 (m, 1H), 4.21 (q, 4H), 2.54 (t, 2H), 2.21 (q, 2H), 2.03 (s, 3H), 1.21 (t, 6H) } \]

\[ ^{13}\text{CNMR: } d -\text{MeOH } \delta \text{ 13.3, 21.5, 26.8, 29.9, 62.5, 66.5, 118.4, 118.5, 124.6, 134.3, 141.2, 150.1, 168, 171.3 } \]

\[ ^{19}\text{FNMR: } d-\text{MeOH } \delta \text{ -137.9 } \]
**Synthesis of diethyl 7-fluoroindolylmethy acetamidomalonate**

Take 11 g of the 2-fluorophenylhydrazone derivative (obtained as reddish brown oil) in 66 ml 10 % aq. sulfuric acid. Heat the RM on a boiling water bath for about 2 hrs. with vigorous stirring when a dark brown RM results. Check TLC. (Fig. 3 above) Cool the RM to 55 - 60°C. Add 75 ml EtOAc, to dissolve the dark brown semisolid that is found sticking to the inner walls of the flask. Stir for about 10 mins. to dissolve the semisolid completely. Then cool the RM to RT. Add 16.5 g NaCl, 40 ml d/w, stir at RT for about 10 mins. Separate the top organic layer. Extract the lower aq. layer with 50 ml more of ethyl acetate (EtOAc). Wash the combined organic layers once with 50 ml of saturated brine soln. then dry the organic layer over anhydrous sodium sulfate; concentrate the solvent under vacuum to give a brown semisolid.

Yield = 7.8 g, 74 %

$^1$HNMR: $d$ MeOH $\delta$ 7.12 (d, 1H), 7.04 (s, 1H), 6.93 (d, 1H), 6.81 (t, 1H), 4.18 (q, 4H), 3.76 (s, 2H), 1.97 (s, 3H), 1.2 (t, 6H)

$^{13}$CNMR: $d$ MeOH $\delta$ 13.3, 21.6, 28.2, 62.5, 68.1, 105.9, 109.1, 114.2, 119.1, 125.1, 132.4, 142.8, 167.9, 172.4

$^{19}$FNMR: $d$-MeOH $\delta$ -137.1

Dissolve the resulting brown semisolid in 40 ml MeOH, add activated charcoal, stir at RT for about 10 - 15 mins. Filter through Celite, and wash the Celite bed with about 24 ml MeOH. The dark brown filtrate is used as is for the ozonolysis step.
Synthesis of diethyl 2-amino-3-fluorobenzoylmethylacetamidomalonate and its acid hydrolysis to give DL-3-fluorokynurenine

Cool the soln. of diethyl 7-fluoroindolylmethylacetamidomalonate (7.8 g in 64 ml MeOH) to below -70°C using a dry ice – acetone bath. Bubble ozone gas (at 0.5 psi) through the RM for about 90 mins. Check TLC. (Fig. 4 above) Quench the RM with an aq. soln. of sodium bisulfite (31.2 g in 156 ml d/w), when a yellow suspension results. Stir for about 10 – 15 mins. to allow the RM attain RT. Concentrate the solvent MeOH under vacuum, Add 70 ml distilled water (d/w), extract with two 60 ml portions of EtOAc. Wash the combined organic layers with 50 ml saturated brine solution. Charcoalize the organic layer, filter over Celite, dry the filtrate over anhydrous sodium sulfate, concentrate to remove the solvent and give the product as a brown oil.

Yield = 3.5 g, 45 %

\(^1\)HNMR: \textit{d-MeOH} \(\delta\) 7.62 (d, 1H), 7.25 (d, 1H), 7.05 (t, 1H), 6.4 (s, 1H), 4.25 (q, 4H), 4.1 (s, 2H), 1.97 (s, 3H), 1.25 (t, 6H)

\(^13\)CNMR: \textit{d-MeOH} \(\delta\) 13.8, 24.2, 36.5, 66.5, 72.5, 119.3, 121.4, 123.2, 127.6, 138.4, 162.5, 169.5, 172.2, 204.1

\(^19\)FNMR: \textit{d-MeOH} \(\delta\) -137.6

Take the oil from the previous step in 32 ml of 6N HCl. Reflux on an oil bath for about 4 hrs. Then cool the RM to RT, concentrate to remove water under vacuum. Take the resulting semisolid in 7 ml d/w, charcoalize at RT for about 15 mins. Filter through Celite, wash the bed with 3 ml d/w. Basify the filtrate to approx. pH 6.5 using 2N NaOH, when a brown solid
precipitates. Filter the solid racemic 3-fluorokynurenine; wash with about 2 ml d/w. Allow to air dry overnight.

Weight of product = 0.65 g, 31 %, m.p. = 205 - 210°C

$^1$HNMR: (1 – 2% DCl – D$_2$O) $\delta$ 7.5 (d, 1H), 7.2 (d, 1H), 7.1 (t, 1H), 4.1 (t, 1H), 3.5 (d, 2H)

$^{13}$CNMR: (1 – 2% DCl – D$_2$O) $\delta$ 46.5, 53.2, 119.2, 121.6, 123.1, 128.2, 140.6, 159.2, 176.5, 204.2

$^{19}$FNMR: d-MeOH $\delta$ -126

**Synthesis of 2-methylphenylhydrazine**$^{20,24-30}$

Add 10 ml of predistilled 2-methylaniline drop wise to 200 ml of conc. HCl, over 20 mins. with stirring. Then stir at RT for about 15 mins. when an almost clear yellow solution results. Cool the soln. to -20 ºC, in a dry ice-acetone bath, add to it an aq. soln. of 7.73 g of sodium nitrite in 39 ml d/w. (Addition of sodium nitrite solution done in such a way that the tip of the dropping funnel is dipping into the RM via a small tube attached to the dripping tip of the dropping funnel) Complete the addition in about 15 mins. and then continue stirring at -20 ºC for about 10 - 15 mins. Then to the same RM while maintaining the temp. at -20 to -25 ºC add a soln. of 42.1 g of stannous chloride dihydrate in 38 ml of conc. HCl. Complete the addition in about 45 mins. when a thick precipitate of the hydrazine hydrochloride salt is formed. Allow the RM to stir at 0 to -10 ºC for about 45 mins. Check TLC (Fig. 1 above). Cool the suspension to -45 to -50 ºC, for about 15 mins. then filter. Spread the solid on a petri dish to let it air dry overnight to give 22 g of a crude solid from which the free base is obtained.
The free base of the 2-methylphenylhydrazine is released only when needed, by treatment of the hydrochloride salt with 2.7 equivalents of NaOH and the free base extracted with ether.

Yield of free base = 7.5 g, 65 %,  m.p. = 45°C.

$^1$HNMR of free base: $d$-MeOH $\delta$ 7.1 (t, 1H), 6.9 (d, 2H), 6.7 (t, 1H), 2.1 (s, 3H), the NH protons exchanged with the solvent and merged around $\delta$ 4.9

$^{13}$CNMR of free base: $d$-MeOH $\delta$ 16.1, 110.3, 118.8, 122.3, 126.8, 129.8, 149.2

**Synthesis of 2-methylphenylhydrazone derivative**

To a suspension of 12.13 g of diethylacetamidomalonate in 24 ml benzene add 121 mg MeONa, with stirring. Stir the RM at RT for about 5 mins. Then cool the suspension in an ice-water bath and add 4.5 ml of acrolein dropwise in about 20 - 25 mins. while maintaining the temp. of the RM below 5°C. After completion of addition, warm the RM to RT and stir at RT for about 2 hrs. when a clear pale yellow solution results. At the end of 2 hrs. of stirring, add 3.6 ml of AcOH, and then add a solution of 7.5 g of 2-methylphenylhydrazine in 15 ml benzene, when a clear orange colored solution results. Warm the resulting RM to 55-60 °C, for about 30 mins. and then leave the RM stirring to gradually attain RT. Stir for about 2.5 days at RT. Check TLC (Fig. 2 above) For TLC check quench a small portion of the RM in water, extract with a few drops of ethyl acetate and spot the top ethyl acetate layer.

Concentrate the RM under vacuum, to give a reddish brown oil which is used as it is for the Fischer indole cyclization.

Yield = 16 g, 69 %
\[^{1}\text{HNMR: } d\text{-MeOH } \delta \ 7.31 \ \text{(d, 1H), 7.23(t, 1H), 7.05 (t, 1H), 6.98 (d, 1H), 6.67 (t, 1H), 4.21 (q, 4H), 2.54 (t, 2H), 2.23 (q, 2H), 2.02 (s, 3H), 1.98(s, 3H), 1.2 (t, 6H)}\]

\[^{13}\text{CNMR: } d\text{-MeOH } \delta \ 13.4, 16.6, 21.5, 26.8, 30.1, 62.5, 66.6, 112.5, 118.9, 120.8, 126.7, 130.2, 140.7, 143.9, 168, 171.3\]

**Synthesis of diethyl 7-methylindolylmethylacetamidomalonate\[^{31}\]**

Take 16 g of the 2-methylphenylhydrazone derivative (obtained as reddish brown oil) in 96 ml 10 % aq. sulfuric acid. Heat the RM on a boiling water bath for about 2 hrs. with vigorous stirring when a dark brown RM results. Check TLC. (Fig. 3 above) Cool the RM to 55 – 60 °C. Add 100 ml EtOAc, to dissolve the dark brown semisolid that is found sticking to the inner walls of the flask. Stir for about 10 mins. to dissolve the semisolid completely. Then cool the RM to RT. Add 24 g NaCl, 60 ml d/w, stir at RT for about 10 mins. Separate the top organic layer. Extract the lower aq. layer with 75 ml more of EtOAc. Wash the combined organic layers once with 75 ml of saturated brine soln. then dry the organic layer over anhydrous sodium sulfate; concentrate the solvent under vacuum to give the product as a brown semisolid.

**Yield = 11 g, 72 %**

\[^{1}\text{HNMR: } d\text{-MeOH } \delta \ 7.22 \ \text{(d, 1H), 6.97 (s, 1H), 6.91 (d, 1H), 6.87 (t, 1H), 4.18 (q, 4H), 3.76 (s, 2H), 2.45 (s, 3H), 1.95 (s, 3H), 1.22 (t, 6H)}\]

\[^{13}\text{CNMR: } d\text{-MeOH } \delta \ 13.3, 15.9, 21.6, 28.3, 62.4, 68.2, 108.4, 115.6, 119, 120.8, 121.8, 123.7, 128.1, 135.9, 167.9, 171.3\]
Dissolve the resulting brown semisolid in 70 ml MeOH, add activated charcoal, stir at RT for about 10 - 15 mins. Filter through Celite, and wash the Celite bed with about 40 ml MeOH. The dark brown filtrate is used as is for the ozonolysis step.

**Synthesis of diethyl 2-amino-3-methylbenzoylmethylacetamidomalonate and its acid hydrolysis to give DL-3-methylkynurenine**

Cool the soln. of diethyl 7-methylindolylmethylacetamidomalonate (11gm in 110 ml MeOH) to below -70°C using a dry ice – acetone bath. Bubble ozone gas (at 0.5 psi) through the RM for about 90 mins. Check TLC. (Fig. 4 above) Quench the RM with an aq. soln. of sodium bisulfite (44 g in 220 ml d/w), when a yellow suspension results. Stir for about 10 – 15 mins. to allow the RM to attain RT. Concentrate the solvent MeOH under vacuum, Add 70 ml distilled water (d/w), extract with two 75 ml portions of EtOAc. Wash the combined organic layers with 50 ml saturated brine solution. Charcoalize the organic layer, filter over Celite, dry the filtrate over anhydrous sodium sulfate, concentrate to remove the solvent and give the product as a semisolid.

Yield = 6.1 g, 55 %

Recrystallization from 42 ml of 2-propanol gives 4.5 g of the product as a pale yellow solid with m.p. of 183 -185°C.

\[^1\text{HNMR:} \text{d-MeOH} \delta \text{7.71 (d, 1H), 7.46 (d, 1H), 7.28 (t, 1H), 4.28 (s, 2H), 4.26 (q, 4H), 2.28 (s, 3H), 1.96 (s, 3H), 1.25 (t, 6H)}\]

\[^13\text{CNMR:} \text{d-MeOH} \delta \text{13.9, 19.5, 22.9, 36.7, 43.9, 62.9, 63.9, 112, 126.2, 127.9, 136.2, 158.9, 167.2, 169.7, 200.1}\]
Take the solid from previous step in 54 ml of 6N HCl. Reflux on an oil bath for about 4 hrs. Then cool the RM to RT, concentrate to remove water under vacuum. Take the resulting semisolid in 12 ml d/w, charcoalize at RT for about 15 mins. Filter through Celite, wash the bed with 8 ml d/w. Basify the filtrate to approx. pH 6.5 using 2N NaOH, when a pale yellow solid precipitates. Filter the solid racemic 3-methylkynurenine; wash with about 5 ml d/w. Allow to air dry overnight.

Yield = 2.1 g, 75 %, m.p. = 215 – 217 ºC

¹H NMR: (1 – 2% DCl – D₂O) δ 7.46 (d, 1H), 7.11 (d, 1H), 6.97 (t, 1H), 4.01 (t, 1H), 3.43 (d, 2H), 1.81 (s, 3H)

¹³C NMR: (1 – 2% DCl – D₂O) δ 16.2, 39.1, 47.2, 126.6, 129.3, 130, 134.3, 138, 142.4, 170.6, 201

**Synthesis of 2-bromophenyl hydrazine**

Take 10 g of 2-bromoaniline hydrochloride (prepared by dissolving 10 g of 2-bromoaniline in 100 ml acetone and adding 7.6 ml conc. HCl with stirring. Chill the resulting suspension, filter and wash the white solid with about 15 ml acetone) in 200 ml conc. HCl, stir at RT for about 15 mins. when a white suspension results. Cool the RM to -20 ºC, in a dry ice-acetone bath, add to it an aq. soln. of 4.81 g of sodium nitrite in 24 ml d/w. (Addition of sodium nitrite solution done in such a way that the tip of the dropping funnel is dipping into the RM via a small tube attached to the dripping tip of the dropping funnel). Complete the addition in about 15 mins. and then continue stirring at -20 ºC for about 10 - 15 mins. Then to the same RM while maintaining the temp. at -20 to -25ºC add a soln. of 26.3 g of stannous chloride dihydrate in 24
ml of conc. HCl. Complete the addition in about 45 mins. when a thick precipitate of the
hydrazine hydrochloride salt is formed. Allow the RM to stir at 0 to -10 ºC for about 45 mins.
Check TLC (Fig. 1 above) Cool the suspension to -45 to -50 ºC, for about 15 mins. then filter.
Spread the solid on a petri dish to let it air dry overnight to give 27 g of a crude solid from which
the free base is obtained.

The free base of the 2-bromophenylhydrazine is released by treatment of the hydrochloride salt
with 2.7 equivalents of NaOH and the free base extracted with ether.

Yield of free base = 8 g, 89 %, m.p = 44 – 45 ºC

\(^1\)HNMR of free base: \(d\)-MeOH \(\delta 7.33\) (d, 1H), 7.19 (t, 1H), 7.01 (d, 1H), 6.61 (t, 1H), the NH
protons exchanged with the solvent and merged around \(\delta 4.9\)

\(^1^3\)CNMR of free base: \(d\)-MeOH \(\delta 107.7, 112.6, 119.4, 128.4, 131.2, 148.1\)

**Synthesis of 2-bromophenylhydrazone derivative**

To a suspension of 8.46 g of diethyl acetamidomalonate in 17 ml benzene add 84 mg
MeONa, with stirring. Stir the RM at RT for about 5 mins. Then cool the suspension in an ice-
water bath and add 3.2 ml of acrolein drop wise in about 20 - 25 mins. while maintaining the
temp. of the RM below 5°C. After completion of addition, warm the RM to RT and stir at RT for
about 2 hrs. when a clear pale yellow solution results. At the end of 2 hrs. of stirring, add 2.4 ml
of AcOH, and then add a solution of 8 g of 2-bromophenylhydrazine in 16 ml benzene, when a
clear orange colored solution results. Warm the resulting RM to 55-60°C, for about 30 mins. and
then leave the RM stirring to gradually attain RT. Stir for 2.5 days at RT. Check TLC (Fig. 2
above) For TLC check quench a small portion of the RM in water, extract with a few drops of ethyl acetate and spot the top ethyl acetate layer.

Concentrate the RM under vacuum, to give a reddish brown oil which is used as it is for the Fischer indole cyclization.

Yield = 16.5 g, 87 %

$^1$HNMR: $d$- MeOH $\delta$ 7.39 (t, 1H), 7.3(dd, 1H), 7.18 (dd, 1H), 6.65 (t, 1H), 4.21 (q, 4H), 2.61 (t, 2H), 2.21 (q, 2H), 2.03 (s, 3H), 1.21 (t, 6H)

$^{13}$CNMR: $d$-MeOH $\delta$ 13.4, 21.5, 26.8, 29.7, 62.5, 66.5, 106.1, 114.3, 119.9, 128.3, 132.2, 132.5, 142.5, 167.9, 171.3

**Synthesis of diethyl 7-bromoindolylmethylacetamidomalonate**

Take 16.5 g of the 2-bromophenylhydrazone derivative (obtained as reddish brown oil) in 99 ml 10 % aq. sulfuric acid. Heat the RM on a boiling water bath for about 2 hrs. with vigorous stirring when a dark brown RM results. Check TLC. (Fig. 3 above) Cool the RM to 55 - 60°C. Add 100 ml EtOAc, to dissolve the dark brown semisolid that is found sticking to the inner walls of the flask. Stir for about 10 mins. to dissolve the semisolid completely. Then cool the RM to RT. Add 32 g NaCl, 100 ml d/w, stir at RT for about 10 mins. Separate the top organic layer. Extract the lower aq. layer with 100 ml more of EtOAc. Wash the combined organic layers once with 100 ml of saturated brine soln. then dry the organic layer over anhydrous sodium sulfate; concentrate the solvent under vacuum to give the product as a brown semisolid.

Yield = 14 g, 88 %
\(^1\)HNMR: \(d\)-MeOH \(\delta\) 7.38 (d, 1H), 7.23 (d, 1H), 7.01 (s, 1H), 6.9 (t, 1H), 4.16 (q, 4H), 3.77 (s, 2H), 1.96 (s, 3H), 1.19 (t, 6H)

\(^1\)\(^3\)CNMR: \(d\)-MeOH \(\delta\) 13.5, 20.1, 28.4, 62.6, 68, 104.7, 109.5, 117.7, 120.2, 124, 125.2, 129.9, 134.9, 167.9, 171.5

Dissolve the resulting brown semisolid in 100 ml MeOH, add activated charcoal, stir at RT for about 10 - 15 mins. Filter through celite, and wash the celite bed with about 60 ml MeOH. The dark brown filtrate is used as is for the ozonolysis step.

**Synthesis of diethyl 2-amino-3-bromobenzoylmethylacetamidomalonate and its acid hydrolysis to give DL-3-bromokynurenine**

Cool the soln. of diethyl 7-bromoindolylmethylacetamidomalonate (14gm in 140 ml MeOH) to below -70°C using a dry ice – acetone bath. Bubble ozone gas (at 0.5 psi) through the RM for about 90 mins. Check TLC. (Fig. 4 above) Quench the RM with an aq. soln. of sodium bisulfite (84 g in 420 ml d/w), when a yellow suspension results. Stir for about 10 – 15 mins. to allow the RM attain RT. Concentrate the solvent MeOH under vacuum, Add 100 ml distilled water (d/w), extract with two 100 ml portions of EtOAc. Wash the combined organic layers with 75 ml saturated brine solution. Charcolize the organic layer, filter over Celite, dry the filtrate over anhydrous sodium sulfate, concentrate to remove the solvent and give the product as a semisolid.

Yield = 7.2 g, 51 %

Recrystallize the semisolid from 50 ml of 2-propanol to give 4.8 g of the product as a pale yellow solid.
\(^1\)HNMR: \(d\)-MeOH \(\delta\) 8.21 (d, 1H), 7.77 (d, 1H), 7.21 (t, 1H), 4.26 (q, 4H), 4.21 (s, 2H), 2.01 (s, 3H), 1.25 (t, 6H)

\(^13\)CNMR: \(d\)-MeOH \(\delta\) 13.9, 22.9, 36.7, 42.9, 63, 63.9, 110.1, 126.4, 132.1, 136.4, 158.5, 167.1, 169.9, 201

Take the solid from previous step in 45 ml of 6N HCl. Reflux on an oil bath for about 4 hrs. Then cool the RM to RT, concentrate to remove water under vacuum. Take the resulting semisolid in 12 ml d/w, charcolize at RT for about 15 mins. Filter through Celite, wash the bed with 8 ml d/w. Basify the filtrate to approx. pH 6.5 using 2N NaOH, when a pale yellow solid precipitates. Filter the solid racemic 3-bromokynurenine; wash with about 5 ml d/w. Allow to air dry overnight.

Yield = 1.1 g, 34 %, m.p. = 200 – 205 ºC

\(^1\)HNMR: (1 – 2% DCl – D\(_2\)O) \(\delta\) 7.43 (d, 1H), 7.28 (d, 1H), 6.41 (t, 1H), 4.18 (t, 1H), 3.51 (d, 2H) The compound being impure there are other peaks also seen in the \(^1\)HNMR.

**Synthesis of the methyl ester of L-tryptophan**

Suspend 10 g of L-tryptophan in 100 ml of methanol, add to this suspension, dropwise and with stirring 10 ml of sulfuric acid over about 10 - 15 minutes. After completion of addition, stir the RM for about 18 hrs. at RT. Concentrate the MeOH under vacuum, add 100 ml water, extract with one 50 ml portions of EtOAc. Basify the aq. layer to pH 8 with 6N NaOH, extract with two 50 ml portions of EtOAc. Wash the combined organic layers with two 75 ml portions of water, then with one 75 ml portions of satd. aq. sodium bicarbonate soln. Finally wash the
organic layer with 75 ml brine, then dry over anhydrous sodium sulphate, concentrate under vacuum to give a yellow oil.

**Yield = 9 g, 84 %**

$^1$HNMR: CDCl$_3$ $\delta$ 8.92 (s, 1H), 7.85 (d, 1H), 7.54 (d, 1H), 7.32 (s, 1H), 7.28 (t, 2H), 4.53 (t, 1H), 4.05 (s, 3H), 3.85 (d, 2H)

$^{13}$CNMR: CDCl$_3$ $\delta$ 29.2, 49.5, 52.2, 108.4, 110.5, 116.2, 117.5, 119.7, 121.2, 126.2, 135.4, 175.3

**Synthesis of methyl ester of N$^{\alpha}$-acetyl-L-tryptophan**

Dissolve 9 g of the methyl ester of L-tryptophan (yellow oil) in about 45 ml of THF. Add 8 ml of triethylamine, and 5 ml of acetic anhydride. Continue stirring the RM at RT for about 2 hrs. Check TLC. (Fig. 5) Concentrate the THF under vacuum, add about 50 ml water, stir at RT. A solid product precipitates; allow the suspension to stir for about 2 hrs. at RT. Filter the solid, wash with about 50 ml water, suck dry. Allow to air dry overnight.

**Yield = 10 g, 93 %, m.p. = 147 -149 °C**

$^1$HNMR: CDCl$_3$ $\delta$ 8.93 (s, 1H), 7.83 (d, 1H), 7.56 (d, 1H), 7.38 (s, 1H), 7.31 (t, 2H), 6.67 (d, 1H), 4.62 (t, 1H), 4.12 (s, 3H), 3.86 (d, 2H), 1.96 (s, 3H)

$^{13}$CNMR: CDCl$_3$ $\delta$ 22.1, 30.8, 50.1, 55.2, 109.1, 111.2, 116.9, 118.3, 120.2, 122.5, 127.9, 136.8, 169.3, 171.5
**Synthesis of methyl ester of Nα,N-diacetyl-L-kynurenine**

Take 10 g of Nα-acetyltryptophan methyl ester in 150 ml methanol, stir to dissolve, cool to -78 °C, using a dry ice - acetone bath. Bubble ozone (at 0.5 psi) through the cold RM, for about 2 hrs. maintaining temperature below at -70 °C. Check TLC. (Fig. 6) Quench the RM with an aq. sodium bisulphite solution (prepared by dissolving 40 g of sodium bisulfite in 120 ml water). Stir for about 10 -15 mins as the RM attains RT. Concentrate the methanol, and add about 100 ml water. Extract the RM with two 75 ml portions of EtOAc, wash the combined EtOAc layers with about 75 ml water, followed by 75 ml brine. Dry the organic layer over anhydrous sodium sulfate; concentrate under vacuum to give a yellow oil (9 g) which is used as is for the next TFA hydrolysis step.

Take the oil from the previous step in 180 ml MeOH, add 18 ml trifluoroacetic acid (TFA), stir overnight at RT. Check TLC (Fig. 7) Concentrate the RM under vacuum to remove all the solvent MeOH, to give 12 g of a reddish brown oil. Take the oil in 240 ml chloroform, add 10.5 ml acetic anhydride. Stir the RM at RT for about 3 hrs. Check TLC (Fig. 7). Wash the RM with two 75 ml portions of aq. saturated sodium bicarbonate solution followed by 75 ml brine. Dry the organic layer over anhydrous

[Detection: uv 254 nm Solvent system: CHCl₃ / MeOH (3 ml) / (9 drops) SM = Starting material Co = Mixture spot]
sodium sulfate, concentrate under vacuum to remove the solvent completely. Take the resulting oil in about 40 ml n-hexane, scratch the inner walls of the flask with a spatula to induce crystallization. Filter the solid and wash with about 10 – 15 ml n-hexane, allow to air dry.

Yield = 9.1 g, 78 %,  m.p. = 161 - 163ºC

$^1$H NMR: CDCl$_3$  $\delta$ 11.45 (s, 1H), 8.71 (d, 1H), 7.91 (d, 1H), 7.45 (t, 1H), 7.25 (t, 1H), 6.54 (d, 1H), 4.75 (t, 1H), 4.02 (s, 3H), 3.95 (m, 2H), 2.12 (s, 3H), 1.97 (s, 3H)

$^{13}$C NMR: CDCl$_3$  $\delta$ 22.1, 24.2, 41.2, 49.4, 53.5, 119.6, 122.3, 123.2, 127.5, 131.2, 135.4, 169.5, 170.2, 171.8, 201.2

Fig. 7
Synthesis of methyl ester of 5-bromo-\(N^\alpha,\alpha\)-diacetyl-L-kynurenine\(^{17}\)

Dissolve 5 g of the methyl ester of \(N^\alpha,\alpha\)-diacetyl-kynurenine in 100 ml acetic acid. Add 7.5 g of anhydrous sodium acetate with stirring and then add dropwise 1.26 ml of liquid bromine in about 10 mins. After completion of addition a dark brown RM results but the color of the RM fades after stirring for about 1 hr. at RT. Check TLC (Fig 8) at this point, by quenching a small portion of the RM in an aq. solution of sodium bisulfite, and extract with a few drops of EtOAc. Spot the EtOAc layer. Quench the RM with an aq. solution of sodium bisulfite (10 gm sodium bisulfite dissolved in 40 ml water). Stir for about 5 mins. and extract with two 50 ml portions of chloroform. Wash the combined organic layers with 50 ml water, followed by 50 ml of brine. Dry the organic layer over anhydrous sodium sulfate, concentrate to give 6 g of a semisolid. Recrystallization from 42 ml of MeOH to give the product as pale yellow needles.

Yield = 4.5 g, 72 %,  m.p. = 187-189\(^\circ\)C

\(^1\)HNMR: CDCl\(_3\) \(\delta\) 11.35 (s, 1H), 8.62 (s, 1H), 7.95 (d, 1H), 7.52 (dd, 1H), 6.49 (d, 1H), 4.62 (m, 1H), 4.12 (s, 3H), 3.65 (m, 2H), 2.15 (s, 3H), 1.98 (s, 3H)

\(^13\)CNR: CDCl\(_3\) \(\delta\) 22.5, 25.2, 41.3, 52.5, 53.2, 116.3, 117.8, 122.1, 131.3, 136.5, 137.9, 167.7, 168.5, 169.5, 201.6
Synthesis of 5-bromo-L-kynurenine$^{17}$

Reflux 4.5 g of the methyl ester of 5-bromo-N$^\alpha$,N-diacetyl-L-kynurenine in 41 ml of 6N HCl for about 4 hrs. Concentrate the RM and take the resulting semisolid in 15 ml water, charcoalize at RT for about 20 mins. Filter through Celite, and wash the Celite with 10 ml water. Basify the filtrate with 6N NaOH to pH 6.5 when the product precipitates as a pale yellow solid. Filter, wash the solid with 10 ml water, and allow to air dry overnight.

Yield = 1.9 g, 57 %, m.p. = 225 – 227 ºC. Sp. rotation = -14.6º (c = 0.28 in 1:1 dioxane : water)

$^1$HNMR: (1 – 2% DCI – D$_2$O) $\delta$ 8.12 (s, 1H), 7.79 (dd, 1H), 7.22 (d, 1H), 4.28 (t, 1H), 3.65 (d, 2H)

$^{13}$CNMR: (1 – 2% DCI – D$_2$O) $\delta$ 42.3, 54.2, 117.3, 119.8, 121, 131.5, 139.5, 151.2, 171.6, 202.1

Synthesis of methyl ester of 5-chloro-N$^\alpha$,N-diacetyl-L-kynurenine

Dissolve 2 g of the methyl ester of N,N$^\alpha$-diacetylkynurenine in 40 ml acetic acid and stir at RT until a clear solution results. In a separate Erlenmeyer, dissolve 2 g of N-chloro succinimide in 12 ml of AcOH (which has been pre-bubbled and saturated with dry HCl gas), and add the resulting yellowish green solution to the above acetic acid solution of the methyl ester of N,N$^\alpha$-diacetylkynurenine. After completion of addition, stir at RT for about 1 hr. Check TLC (Fig. 9). For checking the TLC, quench a small portion of the RM in aq. sodium bisulfite, extract with a few drops of EtOAc, shake well, and spot the EtOAc layer.

![Diagram](image-url)
Quench the RM with an aq. solution of sodium bisulfite (6 g dissolved in 24 ml water). Extract with two 40 ml portions of chloroform. Wash the combined organic layers with 40 ml water and then with 40 ml brine. Dry the organic layer over anhydrous sodium sulfate; concentrate under vacuum to give 2.2 g of a semisolid. Recrystallize the semisolid from 20 ml of 2-propanol to give the product as pale yellow needles.

Yield = 1.6 g, 72 %, m.p = 185- 187ºC

\[ ^1 \text{HNMR: } \text{CDCl}_3 \delta 11.32 \text{ (s, 1H)}, 8.12 \text{ (d, 1H)}, 7.98 \text{ (s, 1H)}, 7.78 \text{ (dd, 1H)}, 6.52 \text{ (d, 1H)}, 4.65 \text{ (m, 1H)}, 4.21 \text{ (s, 3H)}, 3.58 \text{ (m, 2H)}, 2.21 \text{ (s, 3H)}, 2.01 \text{ (s, 3H)} \]

\[ ^{13} \text{CNMR: } \text{CDCl}_3 \delta 22.8, 24.8, 40.5, 51.3, 54.6, 121.2, 122.3, 128.5, 129.8, 134.3, 135.6, 168.5, 169.3, 170.1, 200.3 \]

**Synthesis of 5-chloro-L-kynurenine**

Reflux 1.6 g of the methyl ester of 5-chloro-N\(^\alpha\),N-diacetyl-L-kynurenine in 15 ml of 6N HCl for about 4 hrs. Concentrate the RM and take the resulting semisolid in 12 ml water, charcoalize at RT for about 10 mins. Filter through Celite, and wash the Celite with 10 ml water. Basify the filtrate with 6N NaOH to pH 6.5 when the product precipitates as a pale yellow solid. Filter, wash the solid with 10 ml water, and allow to air dry overnight.

Yield = 0.7 gm, 61 %, m.p. = 216 – 218 ºC. Sp. rotation= -15.7º (c = 0.28 in 1:1 dioxane : water)

\[ ^1 \text{HNMR: } (1 – 2\% \text{ DCl – D}_2\text{O}) \delta 7.75 \text{ (s, 1H)}, 7.35 \text{ (dd, 1H)}, 7.25 \text{ (d, 1H)}, 4.31 \text{ (t, 1H)}, 3.58 \text{ (d, 2H)} \]
Results and Discussion

The 3-substituted DL kynurenines have been synthesized starting from the corresponding 2-substituted anilines (Scheme 13). These aniline compounds are first diazotized by the regular procedures using sodium nitrite in a conc. HCl system to give the corresponding diazonium salts. Reduction of the diazonium salts with stannous chloride pulls the 2-substituted phenylhydrazines. The 2-substituted phenylhydrazines are stable as hydrochloride salts, and the free bases are generated only when they are to be used immediately in the respective further reactions. Furthermore, isolation of the free base of the 2-substituted phenylhydrazines is done by a solvent extraction method using ether as the extracting solvent. Use of ethyl acetate should be avoided owing to the enhanced potential of the substituted phenylhydrazines to attack nucleophilically (the α-effect) on the carbonyl carbon of ethyl acetate to produce a hydrazide impurity. The free bases forms of the 2-substituted phenylhydrazines are generated by treating the hydrochloride salt with a strong base like NaOH or KOH. GCMS of the free base forms of the 2-substituted phenylhydrazines shows about 98% purity and are used as such for the next step without further purification.

In order to synthesize the phenylhydrazone derivatives from the substituted phenylhydrazines, the carbonyl compound required for the reaction is first synthesized by a
Scheme 13
Michael addition reaction\(^3\) between acrolein and the diethylacetamidomalonate anion. The intermediate aldehyde of this reaction is not isolated and is used as such for its reaction with the 2-substituted phenylhydrazine to give the corresponding phenylhydrazone derivative. GCMS of the substituted phenylhydrazones shows about 97% purity and are used as such for the next step without further purification.

The phenylhydrazone derivative is then subjected to an acid catalyzed Fischer indole cyclization to give the corresponding 7-substituted indolylmethylacetamidomalonates\(^{12-16}\) as gummy semisolids. Purity check by GCMS show the 7-substituted indolylmethylacetamidomalonates to be about 95% pure and are used as such for the next reaction.

Ozonolysis of the 7-substituted indolylmethylacetamidomalonate intermediate in methanol produces the diethyl 2-amino-3-substitutedbenzoylmethylacetamidomalonate intermediate as either a brown oil or a semisolid which is crystallized from 2-propanol to give a yellow solid. Finally, hydrolysis of the diethyl 2-amino-3-substituted benzoymethylacetamidomalonate intermediate with 6N HCl produces the corresponding 3-substituted DL-kynurenine. The racemic compound is isolated from an aq. solution, as a pale yellow solid by adjusting the pH to approximately 6.5. In the case of 3-fluoro kynurenine the racemic compound was obtained as a brown solid.

Purity of the racemic 3-chloro (\(\lambda_{\text{max}}\) at 262 nm and 365 nm), 3-fluoro (\(\lambda_{\text{max}}\) at 258 nm and 360 nm), and the 3-methyl (\(\lambda_{\text{max}}\) at 259 nm and 362 nm) kynurenines is about 99.5% by HPLC. The retention times for these three compounds are 14.7 mins., 8.7 mins., and 13.5 mins. respectively. The racemic 3-methylkynurenine when run on the Pro-Cu chiral column separated
the two enantiomers with the retention times for the D and the L enantiomers being 26 mins. and 31 mins. respectively. The racemic 3-bromokynurenine ($\lambda_{\text{max}}$ at 263 nm and 370 nm) isolated after hydrolysis step was found to be about 85% pure with about 10% of unsubstituted kynurenine impurity ($\lambda_{\text{max}}$ at 255 nm and 360 nm), and 5% of possible another substituted kynurenine type impurity ($\lambda_{\text{max}}$ at 258 nm and 383 nm). The retention times for these three peaks are 6.4 mins., 15.5 mins., and 20.5 mins. respectively. We suspect the impurities to have possibly formed by partly removal of the bromo group and partly its migration to some other carbon on the aromatic ring. Recrystallization of this crude 3-bromokynurenine from methanol partially removed these impurities and raised the HPLC purity of the sample to about 92%.
For synthesizing the 5-bromo, and 5-chloro L-kynurenines, the intermediate methyl ester of Nα,N-diacetylkynurenine is first synthesized (Scheme 14). Thus, Fischer esterification of L-tryptophan with methanol in the presence of sulfuric acid gives the methyl ester of L-tryptophan, which is about 99% pure by GCMS and used as such for the acylation step. Acylation with acetic anhydride in THF produces the methyl ester of Nα-acetyl-L-tryptophan which is about 99% pure by GCMS and used as such for the ozonolysis step. Ozonolysis of the methyl ester of Nα-acetyl-L-tryptophan, followed by hydrolysis with trifluoroacetic acid, and then acylation with acetic anhydride, gives the methyl ester of Nα,N-diacetylkynurenine as a white solid with 99% purity by GCMS. Having prepared the methyl ester of Nα,N-diacetylkynurenine, either bromination or chlorination gives the methyl ester of the corresponding 5-halosubstituted-Nα,N-diacetylkynurenine which finally upon acid hydrolysis give the 5-halosubstituted-L-kynurenines. (Scheme 15)

Bromination of the methyl ester of Nα,N-diacetylkynurenine is done with liquid bromine, and gives regioselectively the methyl ester of 5-bromo-Nα,N-diacetylkynurenine. This compound upon crystallization from methanol gives the product as pale yellow needles with 99.5% purity by GCMS. Finally hydrolysis of the 5-bromo intermediate with 6N HCl gives the 5-bromo-L-kynurenine as a pale yellow solid. The reported melting point for this solid is 213-217 ºC. However, we got a much higher melting point of 225-227 ºC which could be because of the greater purity of our sample. The greater purity of our sample could be because of the different isolation method used by us. The HPLC purity was found to be about 99.8%, with the retention time being 14.4 mins. and λ\text{max} of 265 nm and 370 nm.
Chlorination of the methyl ester of Nα,N-diacetyl-L-kynurenine was done with ‘in situ’ generated chlorine gas (by the reaction between NCS and acetic acid pre-bubbled with dry HCl gas) and the reaction regioselectively gives the methyl ester of 5-chloro-Nα,N-diacetyl-L-kynurenine. This compound upon crystallization from 2-propanol gives the product as pale yellow needles with 99.5% purity by GCMS. Finally hydrolysis of the 5-chloro intermediate with 6N HCl gives the 5-chloro-L-kynurenine as a pale yellow solid. The reported melting point
for this solid is 208-211 ºC\textsuperscript{21}. However, we got a much higher melting point of 216-218 ºC which could be because of the greater purity of our sample. The HPLC purity was found to be about 99.8\%, with the retention time being 12.8 mins. and $\lambda_{\text{max}}$ of 260 nm and 370 nm.
References


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CHAPTER 3

STEADY STATE KINETICS OF SUBSTRATE ANALOGS FOR HUMAN AND BACTERIAL KYNURENINASE

Abstract

Different substituted kynurenines have been tested for their substrate activity with human as well as *Pseudomonas fluorescens* kynureninase. All the synthesized compounds viz. the 3-chloro-DL-kynurenine, 3-fluoro-DL-kynurenine, 3-methyl-DL-kynurenine, 5-bromo-L-kynurenine, and the 5-chloro-L-kynurenine have good substrate activity for both human as well as *Pseudomonas fluorescens* kynureninase. For the human enzyme, 3-chloro-DL-kynurenine, 5-bromo-L-kynurenine, and the 5-chloro-L-kynurenine have closely comparable k\text{cat} and k\text{cat}/K_m value to that of the natural substrate 3-hydroxykynurenine. And for the bacterial enzyme, 3-fluoro-DL-kynurenine, 5-bromo-L-kynurenine, and the 5-chloro-L-kynurenine have closely comparable k\text{cat} and k\text{cat}/K_m value to that of the natural substrate L-kynurenine. Thus, 5-bromo-L-kynurenine, and the 5-chloro-L-kynurenine seem to be good substrates for both human as well as bacterial enzyme.
Experimental Methods

General

The steady state kinetic measurements were performed on a Varian Cary 1E UV/Visible spectrophotometer equipped with a Peltier-type 6 x 6 thermoelectric cell block for temperature control. The instrument was controlled by a PC using software provided by Varian Instruments.

Enzyme assay

Kynureninase activity was measured from the decrease in absorbance at 360 nm (\(\Delta \varepsilon = 4500 \text{ M}^{-1}.\text{cm}^{-1}\)) upon conversion of kynurenine to anthranilic acid. Similarly the human enzyme activity was measured from the decrease in absorbance at 370 nm upon conversion of DL-3-hydroxykynurenine to 3-hydroxyanthranilic acid. The reaction mixtures for these measurements contained 100 µM of the substrate L-kynurenine or 3-hydroxy-DL-kynurenine in 30 mM of potassium phosphate buffer pH 8, containing 40 µM of pyridoxal-5'-phosphate at 25 ºC. Kinetic and scanning kinetic measurements of the L-compounds was done in a similar manner but for the DL compounds a final concentration of 200 µM of each compound was used for the individual assay, while keeping the other conditions same.

Kinetic measurements

The scanning kinetic measurements to determine the absorbance change for the 3-substituted substrate analogs was done using the human enzyme since 3-hydroxykynurenine is the natural substrate for the human enzyme. The scanning kinetic measurements to determine the absorbance change for the 5-substituted substrate analogs was done using the Pseudomonas fluorescens enzyme since 5-substitued kynurenines have been shown to be good substrates\(^2\) for
the *P. fluorescens* enzyme. The wavelength range used for the scanning kinetic measurements was 450 nm – 220 nm and the scan was performed at the rate of 200 nm/min. As shown in Table 3.1 below, different quantities of the human and *P. fluorescens* enzyme were used for different substrate analogs with the final volume of 600 µL for each assay solution. These same quantities of enzyme were used for determining the initial rates of the respective substrate analogs with the two different enzymes.

**Table 3.1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human enzyme mg</th>
<th><em>P. fluorescens</em> enzyme mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-chloro-DL-kynurenine</td>
<td>3.29 x 10^{-3}</td>
<td>1.33 x 10^{-3}</td>
</tr>
<tr>
<td>3-fluoro-DL-kynurenine</td>
<td>6.58 x 10^{-3}</td>
<td>3.33 x 10^{-4}</td>
</tr>
<tr>
<td>3-methyl-DL-kynurenine</td>
<td>6.58 x 10^{-3}</td>
<td>6.66 x 10^{-4}</td>
</tr>
<tr>
<td>5-bromo-L-kynurenine</td>
<td>1.32 x 10^{-3}</td>
<td>3.33 x 10^{-5}</td>
</tr>
<tr>
<td>5-chloro-L-kynurenine</td>
<td>2.63 x 10^{-3}</td>
<td>3.33 x 10^{-5}</td>
</tr>
</tbody>
</table>

The initial rates of reaction of 3-chloro-DL-kynurenine were measured at its absorption maximum, 365 nm (\(\Delta \varepsilon = 4648 \text{ M}^{-1}\cdot\text{cm}^{-1}\)). The initial rates of reaction of 3-fluoro-DL-kynurenine were measured at its absorption maximum, 360 nm (\(\Delta \varepsilon = 3053 \text{ M}^{-1}\cdot\text{cm}^{-1}\)). The initial rates of reaction of 3-methyl-DL-kynurenine were measured at its absorption maximum, 362 nm (\(\Delta \varepsilon = 3440 \text{ M}^{-1}\cdot\text{cm}^{-1}\)). The initial rates of reaction of 5-bromo-L-kynurenine were measured at its absorption maximum, 370 nm (\(\Delta \varepsilon = 4006 \text{ M}^{-1}\cdot\text{cm}^{-1}\)). And the initial rates of reaction of 5-chloro-L-kynurenine were measured at its absorption maximum, 370 nm (\(\Delta \varepsilon = 4330 \text{ M}^{-1}\cdot\text{cm}^{-1}\)). The \(K_m\),
\[ v = \frac{V_{\text{max}} [S]}{(K_m + [S])} \]  

The values of \( V_{\text{max}} \), and \( V_{\text{max}}/K_m \) were divided by the respective extinction coefficients and by the enzyme concentration to give the \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values (Tables 3.2 & 3.3)

**Results and discussion**

A representative scan graph for 5-bromo-L-kynurenine is shown below in Fig. 10 (X-axis shows the wavelength in nm, Y-axis shows the absorbance) which shows the \( \lambda_{\text{max}} \) of 370 nm for this compound. The other scan graphs are shown in the Appendix section. The reaction shows clear isosbestic points at 245 nm, 258 nm, 280 nm, and 338 nm indicating that there are only two species in the reaction viz. 5-bromo-L-kynurenine and the product, 5-bromoanthranilate as shown below.
As seen from tables 3.2 and 3.3 the human as well as the *P. fluorescens* enzyme have about the same catalytic turnover number for 3-chloro-DL-kynurenine with $k_{\text{cat}}$ values of 0.67 sec$^{-1}$ and 0.71 sec$^{-1}$ respectively. When compared with the natural substrate (3-hydroxykynurenine$^5$) 3-chloro-DL-kynurenine is about 5-fold less efficiently cleaved by the human enzyme but has a 3-fold higher $k_{\text{cat}}$ than L-kynurenine for the same enzyme. For the bacterial enzyme it is found that 3-chloro-DL-kynurenine has a $k_{\text{cat}}$ value that is 22-fold lower than the natural substrate, L-kynurenine, 17-fold lower than 5-bromo-L-kynurenine, 13-fold lower than 5-chloro-L-kynurenine, and 15-fold lower than 3-bromo-L-kynurenine$^2$ for the same enzyme. Thus the turnover number with the 3-Cl substituent is much less affected with the
human enzyme than with the bacterial enzyme. This is consistent with the preference of the human enzyme for 3-hydroxy-L-kynurenine.

The $k_{cat}/K_m$ for 3-chloro-DL-kynurenine is also about the same i.e. $(8.16 \pm 1.34) \times 10^3$ and $(1.05 \pm 0.075) \times 10^4$ for the human and the bacterial enzyme respectively. Considering no interference by the D enantiomer the value of $k_{cat}/K_m$ for 3-chloro-L-kynurenine would be $(1.63 \pm 0.27) \times 10^4$ and $(2.1 \pm 0.15) \times 10^4$ for the human and the bacterial enzyme, respectively. For the human

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ $\mu$M</th>
<th>$V_{max}$</th>
<th>$k_{cat}$ sec$^{-1}$</th>
<th>$k_{cat}/K_m$ M$^{-1}$ sec$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-chloro-DL-kynurenine</td>
<td>83.4 ± 21</td>
<td>0.018 ± 0.0017</td>
<td>0.67 ± 0.063</td>
<td>$(8.16 \pm 1.34) \times 10^3$</td>
</tr>
<tr>
<td>3-fluoro-DL-kynurenine</td>
<td>85.8 ± 21.6</td>
<td>0.0080 ± 0.00079</td>
<td>0.23 ± 0.022</td>
<td>$(2.66 \pm 0.45) \times 10^3$</td>
</tr>
<tr>
<td>3-methyl-DL-kynurenine</td>
<td>179 ± 28.3</td>
<td>0.013 ± 0.0010</td>
<td>0.33 ± 0.025</td>
<td>$(1.81 \pm 0.15) \times 10^3$</td>
</tr>
<tr>
<td>5-bromo-L-kynurenine</td>
<td>42.2 ± 4.7</td>
<td>0.0058 ± 0.00020</td>
<td>0.63 ± 0.022</td>
<td>$(1.51 \pm 0.11) \times 10^4$</td>
</tr>
<tr>
<td>5-chloro-L-kynurenine</td>
<td>43.5 ± 6.3</td>
<td>0.0094 ± 0.00044</td>
<td>0.47 ± 0.022</td>
<td>$(1.097 \pm 0.12) \times 10^4$</td>
</tr>
<tr>
<td>L-kynurenine$^5$</td>
<td>495</td>
<td>-</td>
<td>0.23</td>
<td>465</td>
</tr>
<tr>
<td>3-hydroxy-DL-kynurenine$^5$</td>
<td>28.3</td>
<td>-</td>
<td>3.5</td>
<td>$1.23 \times 10^5$</td>
</tr>
</tbody>
</table>

enzyme this $k_{cat}/K_m$ value of $(1.63 \pm 0.27) \times 10^4$ is about 15-fold lower that for the natural substrate 3-hydroxy-L-kynurenine$^5$ $(2.46 \times 10^5)$ but about 35-fold higher than L-kynurenine for
the same enzyme. Thus, having a Cl substituent at the 3-position of L-kynurenine increases the substrate activity for the human enzyme but not as much as that of the natural substrate. For the bacterial enzyme the $k_{\text{cat}}/K_m$ value of $(2.1 \pm 0.15) \times 10^4$ is about 29-fold lower than that of the natural substrate L-kynurenine, but about 85-fold lower than that of 5-chloro-L-kynurenine, and a huge 156-fold lower than that of 5-bromo-L-kynurenine, and about 4-fold higher than that of 3-bromo-L-kynurenine for the same enzyme. Thus, a Cl substituent at 3-position of L-kynurenine seems to decrease the catalytic efficiency while a substituent at 5-position actually increases the catalytic efficiency for the bacterial enzyme and even more so with the increased size of the substituent at the 5-position. To conclude, 3-chloro-DL-kynurenine has about the same substrate

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ $\mu$M</th>
<th>$V_{\text{max}}$</th>
<th>$k_{\text{cat}}$ sec$^{-1}$</th>
<th>$k_{\text{cat}}/K_m$ M$^{-1}$ sec$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-chloro-DL-kynurenine</td>
<td>70.8 ± 9.2</td>
<td>0.0095 ± 0.00056</td>
<td>0.71 ± 0.042</td>
<td>$(1.05 \pm 0.075) \times 10^4$</td>
</tr>
<tr>
<td>3-fluoro-DL-kynurenine</td>
<td>75.5 ± 11.2</td>
<td>0.015 ± 0.0010</td>
<td>6.88 ± 0.46</td>
<td>$(8.99 \pm 0.78) \times 10^4$</td>
</tr>
<tr>
<td>3-methyl-DL-kynurenine</td>
<td>69.4 ± 14.8</td>
<td>0.0074 ± 0.00057</td>
<td>1.51 ± 0.12</td>
<td>$(2.24 \pm 0.33) \times 10^4$</td>
</tr>
<tr>
<td>5-bromo-L-kynurenine</td>
<td>3.58 ± 1.11</td>
<td>0.0034 ± 0.000198</td>
<td>11.89 ± 0.69</td>
<td>$(3.29 \pm 0.87) \times 10^6$</td>
</tr>
<tr>
<td>5-chloro-L-kynurenine</td>
<td>4.90 ± 0.96</td>
<td>0.0027 ± 0.00012</td>
<td>8.73 ± 0.39</td>
<td>$(1.78 \pm 0.29) \times 10^6$</td>
</tr>
<tr>
<td>L-kynurenine</td>
<td>25</td>
<td>-</td>
<td>16</td>
<td>$6 \times 10^5$</td>
</tr>
</tbody>
</table>
activity for both the human as well as the bacterial enzyme but not better than the natural substrate for each of these enzymes.

In the case of 3-fluoro-DL-kynurenine the $k_{\text{cat}}$ for the bacterial enzyme is about 34-fold greater (6.88 sec$^{-1}$) than that for the human enzyme (0.23 sec$^{-1}$). For the human enzyme this $k_{\text{cat}}$ value of 0.23 sec$^{-1}$ is about the same as that of L-kynurenine but about 15-fold lower than that of the natural substrate 3-hydroxy-L-kynurenine$^5$. Thus, the human enzyme seems to be very specific about the type of substituent at the 3-position, a larger and polar substituent being the best. On the other hand, for the bacterial enzyme the $k_{\text{cat}}$ value of 6.88 sec$^{-1}$ is about half that of the natural substrate L-kynurenine$^4$ but about the same as that of 5-bromo, 3-bromo, and 5-chloro-L-kynurenines. Thus, the catalytic turnover number of this substrate with the bacterial enzyme does not seem to be affected as much as with the human enzyme by the presence of a 3-F substituent. The $k_{\text{cat}}/K_m$ values for 3-fluoro-DL-kynurenine are (2.66 ± 0.45) x 10$^3$ and (8.99 ± 0.78) x 10$^4$ respectively for the human and bacterial enzyme. Assuming that the D enantiomer does not affect the enzyme activity the value of $k_{\text{cat}}/K_m$ for 3-fluoro-L-kynurenine would be (5.32 ± 0.9) x 10$^3$ and (1.80 ± 0.16) x 10$^5$ for the human and the bacterial enzyme respectively. Apparently, the $k_{\text{cat}}/K_m$ value for this substrate is about 34-fold higher with the bacterial enzyme than with the human enzyme. For the human enzyme this $k_{\text{cat}}/K_m$ value of (5.32 ± 0.9) x 10$^3$ is about 46-fold lower that for the natural substrate 3-hydroxy-L-kynurenine$^5$ (2.46 x 10$^5$) but about 11-fold higher than L-kynurenine for the same enzyme.$^5$ Thus, based on the discussion in the previous paragraph, it can be said that the human enzyme prefers a 3-Cl over 3-F, both of which are obviously better than having no substituent on the aromatic ring, but at the same time both of them are not as good as having a 3-OH substituent because of the capability of the –OH group to undergo H-bonding as indicated by the crystal structure studies$^5$. For the bacterial enzyme,
however, the $k_{cat}/K_m$ value of $(1.80 \pm 0.16) \times 10^5$ is just about 3-fold lower that of the natural substrate L-kynurenine$^4$ ($6 \times 10^5$) but about 18-fold lower than that of 5-bromo-L-kynurenine, 10-fold lower than that of 5-chloro-L-kynurenine and actually about 33-fold higher than that of 3-bromo-L-kynurenine$^2$. Thus, based on the previous discussion, it can be said that unlike the human enzyme, the catalytic efficiency of the bacterial enzyme is not affected much by introducing a 3-F substituent but this catalytic efficiency is not better than having a 5-Br or 5-Cl substituent although it is better than having a 3-Br substituent. Thus, only a small substituent such as H or F is optimal for bacterial kynureninase, which is consistent with our recently proposed model of substrate specificity$^5$.

In the case of 3-methyl-DL-kynurenine, the turnover number with the bacterial enzyme is 5-fold better than with the human enzyme, as seen from the $k_{cat}$ values of 0.33 sec$^{-1}$ and 1.51 sec$^{-1}$ for the human and bacterial enzyme, respectively. For the human enzyme, this $k_{cat}$ value of 0.33 sec$^{-1}$ is about the same as that of L-kynurenine but about 11-fold lower than that of the natural substrate 3-hydroxy-L-kynurenine$^5$. Thus, based on the previous discussion, it seems like the turnover ability of the human enzyme is better when having a 3-methyl rather than a 3-F, but not as good as having a 3-Cl though all three have a lower turnover number than the natural substrate 3-hydroxykynurenine$^5$. On the other hand, with the bacterial enzyme the $k_{cat}$ value of 1.51 sec$^{-1}$ is about 11-fold lower than that of the natural substrate L-kynurenine$^4$ and about 8-fold lower that of 5-bromo-L- & 3-bromo-L-kynurenine$^2$, and about 5-fold lower than that of 3-fluoro-DL- & 5-chloro-L- kynurenines. Thus the turnover number of the bacterial enzyme is less affected with having a 3-Me than having a 3-Cl, although both of these lower the turnover number than for the substrate with a 3-F substituent. Considering the $k_{cat}/K_m$ values for this substrate we found these to be $(1.81 \pm 0.15) \times 10^3$ and $(2.24 \pm 0.33) \times 10^4$ for the human and the
bacterial enzyme respectively. Thus, the catalytic efficiency of the bacterial enzyme is about 12-fold better than the human enzyme for this substrate. Assuming that the D enantiomer does not affect the enzyme activity the value of $k_{cat}/K_m$ for 3-methyl-L-kynurenine would be $(3.62 \pm 0.3) \times 10^3$ and $(4.48 \pm 0.65) \times 10^4$ for the human and the bacterial enzyme, respectively. For the human enzyme, this $k_{cat}/K_m$ value of $(3.62 \pm 0.3) \times 10^3$ is about 68-fold lower that for the natural substrate 3-hydroxy-L-kynurenine$^5$ $(2.46 \times 10^5)$ but about 8-fold higher than L-kynurenine for the same enzyme.$^5$ Thus, the catalytic efficiency of the human enzyme is reduced to a greater extent by having a 3-methyl substituent rather than having a 3-Cl or a 3-F substituent, all of the three being better than having no substituent on the aromatic ring of kynurenine but not as good as having a 3-OH substituent, probably because the 3-OH forms an H-bond with Asn-333.$^5$ For the bacterial enzyme the $k_{cat}/K_m$ value of $(4.48 \pm 0.65) \times 10^4$ is about 14-fold lower than that of the natural substrate L-kynurenine$^4$ $(6 \times 10^5)$ but about 73-fold lower than that of 5-bromo-L-kynurenine, 40-fold lower than that of 5-chloro-L-kynurenine and actually about 8-fold higher than that of 3-bromo-L-kynurenine$^2$. Thus, the catalytic efficiency of the bacterial enzyme is better when having a 3-methyl rather than a 3-Cl, both of them not better than having 3-F, 5-Br or 5-Cl substituent.

Considering the activity of 5-bromo-L-kynurenine, we have found the $k_{cat}$ values for this substrate to be 0.63 sec$^{-1}$ and 11.9 sec$^{-1}$ for the human and bacterial enzyme respectively. Although Heiss et al.$^2$ have reported a $k_{cat}$ value of 2.1 sec$^{-1}$ for the bacterial enzyme we got about 6-fold higher $k_{cat}$ of 11.9 sec$^{-1}$. This could most probably be due to the greater purity of our substrate as indicated by its much higher melting point than the one reported previously.$^2$ For the human enzyme this $k_{cat}$ value of 0.63 sec$^{-1}$ is about the 3-fold higher than that of L-kynurenine but about 6-fold lower than that of the natural substrate 3-hydroxykynurenine$^5$. Thus the catalytic
turnover number of the human enzyme is almost equal when having a 5-Br or a 3-Cl substituent but lowered to a greater extent when having a 3-F or 3-methyl substituent although all of the four being less active than the natural substrate 3-hydroxykynurenine. With the bacterial enzyme the $k_{\text{cat}}$ value of 11.9 sec$^{-1}$ is about the same as that of 3-bromo-L-kynurenine$^2$, 5-chloro-L-kynurenine and the natural substrate L-kynurenine$^4$ but about 8-fold higher than 3-methyl and 16-fold higher than 3-Cl. Thus, the turnover number of the bacterial enzyme does not seem to lowered as much as when having a 3-methyl or a 3-Cl substituent. Considering the catalytic efficiency of this substrate for both enzymes we got the $k_{\text{cat}}/K_m$ values of $(1.51 \pm 0.11) \times 10^4$ and $(3.29 \pm 0.87) \times 10^6$ for the human and the bacterial enzyme respectively. For the bacterial enzyme, we got an 18-fold higher $k_{\text{cat}}/K_m$ value than the value of $1.8 \times 10^5$ reported earlier$^2$ which could again be due to the greater purity of our substrate and the enzyme sample. For the human enzyme this $k_{\text{cat}}/K_m$ value of $(1.51 \pm 0.11) \times 10^4$ is about 16-fold lower than for the natural substrate 3-hydroxy-L-kynurenine$^5$ ($2.46 \times 10^5$) but about 32-fold higher than L-kynurenine for the same enzyme.$^5$ Thus, the catalytic efficiency with the human enzyme is about the same when having a 5-Br or a 3-Cl substituent which is better than having a 3-methyl or a 3-fluoro substituent but all four are not better than having a 3-OH substituent. For the bacterial enzyme the $k_{\text{cat}}/K_m$ value of $(3.29 \pm 0.87) \times 10^6$ is about 6-fold higher than that of the natural substrate L-kynurenine$^4$ ($6 \times 10^5$), 600-fold higher than that of 3-bromo-L-kynurenine, and about twice that of 5-chloro-L-kynurenine. Thus, the catalytic efficiency for the bacterial enzyme is improved by introducing a 5-Br or 5-Cl on the aromatic ring of kynurenine, and these substituents are hugely better than having a 3-Br substituent.

For our newly synthesized substrate 5-chloro-L-kynurenine the $k_{\text{cat}}$ values are 0.47 sec$^{-1}$ and 8.73 sec$^{-1}$ for the human and the bacterial enzyme respectively. Thus, the turnover number
for this substrate with the bacterial enzyme is about 19-fold higher than with the human enzyme. For the human enzyme this $k_{cat}$ of 0.47 sec$^{-1}$ is about the same as that of 5-bromo-L-kynurenine but about twice that of L-kynurenine and actually 7-fold lower than that of the natural substrate 3-hydroxy-L-kynurenine. Thus, having a 5-Cl substituent has a similar effect on the turnover number as having a 5-Br or a 3-Cl substituent, and this turnover number is higher than when having a 3-F or a 3-methyl substituent, but all the five compounds are not better than having a 3-OH substituent as in the natural substrate. With the bacterial enzyme, the $k_{cat}$ value of 8.73 sec$^{-1}$ is about the same as that for 3-F, 3-Br or 5-Br substituent, and about half that of the natural substrate L-kynurenine (16 sec$^{-1}$), but based on the previous discussion this turnover number is better than when having a 3-Cl or a 3-methyl substituent. Considering the catalytic efficiency with this substrate we have got $k_{cat}/K_m$ values of $(1.097 \pm 0.12) \times 10^4$ and $(1.78 \pm 0.29) \times 10^6$ for the human and the bacterial enzyme, respectively. For the human enzyme, this $k_{cat}/K_m$ value of $(1.10 \pm 0.12) \times 10^4$ is about 22-fold lower than for the natural substrate 3-hydroxy-L-kynurenine (2.46 x 10$^5$) but about 24-fold higher than L-kynurenine for the same enzyme. Thus, based on this and the previous discussion the catalytic efficiency of the human enzyme with 5-chloro-L-kynurenine is lesser than when having a 3-Cl or a 5-Br substituent but better than when having a 3-F or a 3-methyl substituent although all five being not as better as having a 3-OH substituent. For the bacterial enzyme the $k_{cat}/K_m$ value of $(1.78 \pm 0.29) \times 10^6$ is about 3-fold higher than that of the natural substrate L-kynurenine (6 x 10$^5$), 324-fold higher than that of 3-bromo-L-kynurenine, and about half that of 5-bromo-L-kynurenine. Based on this and the previous discussion a 5-Br or 5-Cl actually has a positive effect on the catalytic efficiency of the bacterial enzyme, but with the exception of 3-F, the other 3-substituents in the group seem to have a negative effect on the catalytic efficiency of the enzyme.
To conclude, these results are useful in drug design. Thus, substituents in the 5-position are well tolerated by both enzymes, which is consistent with the X-ray structures\textsuperscript{5}. Furthermore, a hydroxyl group at 3-position is not absolutely necessary for good activity, as seen for the human enzyme that the halogens in place of hydroxyl group also work fine.
References


CHAPTER 4

SYNTHESIS AND STABILITY STUDIES OF CAGED KYNURENE

Abstract

Kynureninase or L-kynurenine hydrolase, EC 3.7.1.3 is a pyridoxal-5'-phosphate (PLP) dependent enzyme catalyzing the hydrolytic cleavage of kynurenine to anthranilic acid and L-alanine. This is the key step in the catabolism of tryptophan in *Pseudomonas fluorescens*, and some other bacteria. In eukaryotes a similar enzyme catalyzes the hydrolytic cleavage of 3-hydroxykynurenine to 3-hydroxanthranilic acid and L-alanine. Earlier, the mechanism for the hydrolytic cleavage of kynurenine has been proposed\(^1\). The external aldimine and quinonoid intermediate are formed too rapidly within the dead time of the stopped-flow instrument. We therefore synthesized a novel caged kynurenine which would release kynurenine ‘in situ’ thereby allowing the detection of formation and decay of the external aldimine intermediate. This chapter provides a detailed synthesis of the caged compound, along with its stability studies.
Introduction

Caged biomolecules have been known for about three decades now\textsuperscript{2,3} and have become increasingly important by virtue of the light initiated release of the biologically active molecule. In the broader sense, caged compounds are protected photo-labile bioactive substrates that release the desired substrate upon irradiation with light of a suitable wavelength (> 300 nm). Some of the caged compounds include protected phosphates, carboxylates, amines, alcohols, and phenols. Caged compounds find extensive application in investigating molecular processes in biochemistry and biophysics. Some bioorganic reactions are too rapid to allow for detection of the intermediate or measure the rate of such reactions. However, by using a caged biomolecule the bioactive substrate can be released ‘in situ’ only when desired by means of a light flash for a few nanoseconds duration. Caged compounds thus allow chemists, biologists, and molecular physiologists to examine the rates of even the fastest biological reactions known. At the same time, light initiated substrate release also gives good insight into the mechanism of enzyme catalyzed biological reactions.

Caging chromophores need to satisfy several key properties or attributes\textsuperscript{4}. Among these are a reasonable absorption in the uv-visible region (> 300nm). This is important as most of the enzymes are destroyed at shorter wavelengths. Also, there should be a hypsochromic shift of the absorption spectrum due to the photoprodut so that this absorption does not interfere with the absorptions of any intermediates in the biological processes being studied. It should be easy to attach the chromophore to the substrate, and without the introduction of any new stereocenters. The caged compound as well as the photoprodut should be biologically inert, and should also be inert or at least benign with respect to other reagents or products. Furthermore, the caged compound should have good aqueous solubility for biological studies. Also, the photochemical
release must be efficient, and the departure of the substrate from the protecting group should be the primary photochemical process occurring directly from the excited state of the caged chromophore. Apart from all these the caging chromophore should preserve the chiral integrity of the substrate (i.e. not convert the substrate into a racemate or other enantiomer) during the protection or the photolytic deprotection cycle. Some of the chromophores used for caging bioactive substrates include, 2-nitrobenzyl$^{5-10}$ and 7-nitroindoline$^{11}$ derivatives, coumarin-4-ylmethyl$^{12-19}$ phototriggers, and the p-hydroxyphenacyl group$^{20-24}$. Among these the p-hydroxyphenacyl group is a versatile photoremovable protective group with wide range of applications in mechanistic bioorganic chemistry. However, this group can only be used to protect conjugate bases of acids$^4$ such as carboxylic acids, thiols, and phosphates.

In the catabolism of tryptophan in *Pseudomonas fluorescens*, and some other bacteria, anthranilic acid and alanine are produced by the hydrolytic cleavage of kynurenine in the presence of the enzyme kynureninase. The mechanism of the cleavage has been proved by Phillips and Dua$^1$ and proceeds through the initial formation of an external aldimine and quinonoid intermediate as shown in Scheme 16. However, the reaction involving the formation of these intermediates is too rapid for the rate to be measured, and is over within the dead time of the stopped-flow instrument (ca. 2 milliseconds). Often detection of an intermediate is essential in proving the mechanism that an enzyme follows. In our attempt to prepare a caged kynurenine, we hope to release the substrate kynurenine ‘in situ’ which would then undergo the reaction with kynureninase thereby allowing us to detect the external aldimine intermediate. Future experiment would involve obtaining a structure of kynureninase “in action” with kynurenine bound in the active site. For this experiment the caged substrate will be soaked into the crystals of either human or *Pseudomonas fluorescens* kynureninase, then the reaction initiated by flash photolysis.
followed by rapid cooling in a cold nitrogen stream, and immediate collection of the X-ray diffraction data. Since kynureninase is a drug target enzyme, these structures will be invaluable in the design of the next generation of more potent and selective inhibitors of kynureninase.

Scheme 16
Experimental Methods

General

$^1$HNMR and $^{13}$CNMR spectra were recorded on a Varian 400MHz instrument in deuterated DMSO. HPLC measurements were carried out on a Spectrasystem P 2000 instrument connected to a UV 6000 detector and controlled by a Dell PC using Chromquest software. A gradient elution was used consisting of 5 % MeOH, and 95 % 0.1 % aq. acetic acid from 0 – 5 mins. followed by a programmed increase of MeOH percentage from 5% to 70% over 5 – 20 mins. with a corresponding decrease of the percentage of 0.1 % aq. acetic acid from 95% to 30% over the same time period. This is followed by an increase of MeOH percentage to 100% with the corresponding decrease of the percentage of 0.1% aq. acetic acid to 0% over 20 – 25 mins. And finally, a programmed return back of the elution system to 5% MeOH, and 95% of 0.1% aq. acetic acid over the period from 25 – 30 mins. A 100 µM solution of the caged compound in 1 mM HCl was used for injection. The flow rate for the elution was 1 ml/min. with detection by absorbance at 254 nm and 370 nm.

Synthesis of 2-bromo-4'-hydroxyacetophenone

To a solution of 5 g of 4-hydroxyacetophenone in 50 ml methanol, add portion wise 30 g copper (II) bromide while maintaining temperature of the reaction mixture below 25ºC. After completion of addition, stir the reaction mass between 20 -25ºC, for 4 hrs. Check TLC. (Fig. 10) Concentrate the methanol under vacuum below 25 ºC. Add 100 ml d/w to the residual semisolid, extract with two 50 ml portions of EtOAc. Wash the combined EtOAc layers first with two 50 ml satd. NaHCO$_3$ solution, then with two 50 ml water, dry the EtOAc layer over anhydrous sodium sulfate, and concentrate under vacuum. Take the resulting semisolid in about 25 ml toluene. Stir
the suspension at 5 -10 °C for about 15 minutes, filter the product, and wash with 15 ml of chilled toluene. Allow to air dry overnight.

Yield = 7.5 g, 96 %, m.p. = 124 -127 °C

$^1$HNMR: $d$-MeOH δ 7.81 (d, 2H), 6.78 (d, 2H), 4.42 (s, 2H)

$^{13}$CNMR: $d$-MeOH δ 35.3, 111.2, 126.3, 131.2, 159.3, 195.6

**Synthesis of 4-hydroxyphenacyl ester of N$^{\alpha}$-Boc-L-tryptophan**

Add 5 g of p-hydroxyphenacyl bromide to a solution containing 3.5 gm of K$_2$CO$_3$, and 7.1 g of Boc-tryptophan, in 50 ml dry DMF. Stir the resulting suspension at RT for about 3 hrs. Check TLC (Fig.11). For checking the TLC quench a small portion of the RM in water, extract with a few drops of EtOAc, and spot the EtOAc layer. Add 500 ml water to the RM, extract with two 100 ml portions of EtOAc. Dry the combined organic layer over anhydrous sodium sulfate; concentrate under vacuum to give 12 gm of pale yellow oil. Take the oil in about 75 ml of toluene, scratch the inner walls of the flask to induce crystallization. Chill the resulting suspension in an ice-water bath for about 10 mins. Filter, and wash the product with about 35 ml of chilled toluene.

Yield = 10 g, 98 %, m.p = 195 – 198 ºC

$^1$HNMR: CDCl$_3$ δ 7.85 (d, 2H), 7.75 (d, 1H), 7.54 (d, 1H), 7.32 (t, 1H), 7.25 (t, 1H), 7.1 (d, 2H), 7.05 (s, 1H), 5.62 (s, 2H), 4.57 (m, 1H), 3.57 (dd, 2H), 2.95 (s, 9H)
$^{13}$CNMR: CDCl$_3$ $\delta$ 27.6, 28.9, 55.3, 67.1, 78.9, 110.8, 112.2, 116.2, 118.7, 119.2, 121.7, 124.6, 126.2, 127.8, 131.1, 136.9, 156.2, 163.4, 172.9, 191.3

**Synthesis of 4-hydroxyphenacyl ester of kynurenine (Caged kynurenine)**

Dissolve 1 g of the pure ester in about 30 ml of methanol by warming on a water bath. Cool the clear solution in a dry ice-acetone bath to about -15 to -20 °C. Bubble ozone gas through the cold reaction mass at pressure of 0.5 psi. Check TLC after about 90 mins. (Fig. 12) Quench the reaction mass with an aq. solution of sodium bisulfite (prepared by dissolving 4 gm in 20 ml d/w). Concentrate the RM under vacuum, and remove methanol below 30 °C. Add about 20 ml water to the resulting reaction mass, and extract it with two 30 ml portions of dichloromethane. Dry the combined organic layers over anhydrous sodium sulfate. Decant the organic layer into a clean dry reaction flask. Add 3 ml of TFA when a clear yellow solution results. Stir the reaction mass at RT overnight. Check TLC (Fig. 12). Concentrate the RM under vacuum below 25 °C. Add 15 ml water, and extract with two 15 ml portions of EtOAc, keep
aside the aqueous layer. Wash the combined organic layer with 15 ml of approx. 2N HCl, separate the aqueous layer, and charcoalize it with a pinch of activated charcoal, for about 5 mins. at RT, filter through celite, lyophilize the aqueous layer.

Yield: 0.2 g, 26 %

$^1$HNMR: $d$-MeOH $\delta$ 7.82 (d, 2H), 7.75 (d, 1H), 7.25 (t, 1H), 6.92 (d, 2H), 6.89 (d, 2H), 6.58 (t, 1H), 5.52 (d, 2H), 4.51 (t, 1H), 3.73 (dd, 2H),

$^{13}$CNMR: $d$-MeOH $\delta$ 38.3, 48.2, 68.1, 115.7, 119.7, 120.6, 121.2, 124.9, 131.1, 131.6, 136, 142.8, 162.1, 169.8, 194.2, 198.5

$[\alpha]_D = -14.2^\circ$ (c = 1.3 in water)

HRMS analysis MW = 342.1294, Calculated MW = 342.1216

Results and Discussion

In order to synthesize the caged compound, 2-bromo-4'-hydroxyacetophenone is first synthesized from 4-hydroxyacetophenone (Scheme 17). The published procedures$^{25-28}$ for the synthesis of 2-bromo-4'-hydroxyacetophenone use a variety of reagents like NBS, liquid bromine, pyridinium hydrobromide perbromide, and copper (II) bromide, with yields of the product ranging from 58-94%. We however, have an even more efficient procedure (96%) without the formation of the undesirable side products viz. the 2,2-dibromo-4'-hydroxyacetophenone compound. The aromatic ring in our method is also less likely to be brominated due the presence of the deactivating acetyl group as well as the fact that the bromonium electrophile is weak enough and even more so because we carry out the reaction
below 25°C. The protocol using CuBr$_2$ in a refluxing mixture of CHCl$_3$/EtOAc$^4$ was especially giving us a mixture of the mono and the dibromo compounds. Using our method the reaction was done conveniently in MeOH at RT using copper (II) bromide as the brominating agent. An SN$^2$ reaction between the conjugate base of N$^\alpha$-Boc tryptophan and 2-bromo-4'-hydroxyacetophenone in a polar aprotic solvent like DMF is carried out to give the 4-hydroxyphenacyl ester of N$^\alpha$-Boc tryptophan. This ester upon ozonolysis followed by TFA hydrolysis$^{29}$ produces the desired caged kynurenine. The caged compound is isolated by lyophilization as a pale yellow hygroscopic solid hydrochloride salt.

A photo-Favorskii rearrangement has been proposed for the release of the substrates from caged biomolecules$^{30,31}$. According to the mechanism proposed by Givens et al (Scheme 18) initially the chromophore gets excited to the singlet excited state, followed by a rapid intersystem crossing$^4$ ($k_{ST} = 2.7 \times 10^{11}$ s$^{-1}$) to quantitatively generate the excited triplet state. In the excited triplet state the phenolic group then adiabatically loses a proton to the solvent to give phenoxide anion which then releases the substrate in a rate-limiting step, via the intermediacy of the spirodienedione. The caged kynurenine has a retention time of about 18 mins. using the above mentioned gradient elution system. HPLC analysis shows the compound to be about 99% pure. HRMS analysis shows the molecular weight of 342.1294 with the calculated value being 342.1216. The uv absorption pattern shows three distinct $\lambda_{\text{max}}$ values at 263, 283 and 365 nm.
Scheme 17
Stability studies for caged kynurenine

The caged compound is stable as a solid hydrochloride salt for a long period at -78 °C in the dark. We have used HPLC analysis to determine the stability of solutions of the caged compound under different conditions. As a solution in dry DMSO it is stable at RT in the dark for a period of about 15 days. As a solution in 10 mM aq. HCl the caged compound is stable for about 3 hours at 20 °C in the dark. However, later on hydrolysis products become apparent and after about 8 hrs. the HPLC shows three peaks corresponding to kynurenine (retention time 6.4
mins.), 2,4’-dihydroxyacetophenone (retention time 15 mins., $\lambda_{\text{max}}$ 273 nm), and the caged compound in an approximate ratio of 2:3:5. Based on a similar stability in 1 mM aq. HCl solution, we irradiated a 100µM aq. acidic solution of the caged compound with a Xenon flash lamp using a 330 nm filter to check if the photocleavage is initiated. However, HPLC analysis showed no cleavage of the cage.

The caged compound is found to be extremely unstable in pH 8 phosphate and TEA buffers and gets almost completely hydrolyzed in about 15 mins. The half life for the caged compound in these buffer systems was 3.75 mins. and 4.65 mins. respectively. We were expecting the stability of the caged compound and hence its half life to grow 10-fold in going from pH 8 to pH 7 and 100-fold in going from pH 8 to pH 6. However, the half life of the caged compound in pH 7 and pH 6 phosphate buffer was found to be 9.75 mins. and 17.1 mins. respectively. Thus, the stability did not raise considerably in going from pH 8 to pH 6 buffer system. Nevertheless, the caged compound is stable for up to 3 hrs. in a 1 mM aq. HCl (pH 3) solution. We then checked the stability of the caged compound in pH 7 Tris buffer containing 55 mM MgCl$_2$ and 25% w/v polyethylene glycol (PEG), the solution used for crystallization of human kynureninase. We have found that the caged compound is fairly stable in this buffer system for about 30 mins. but later on hydrolysis products are seen in the HPLC analysis. Based on this stability we irradiated a 200µM solution of the caged compound with one hundred 1 ns laser pulses and found by HPLC that the cage has been removed completely. In future experiments this would be the buffer system of choice to study the reaction involving the release of kynurenine in situ. We were however not able to perform the experiment due to instrumental problems wherein the enzyme fluorescence skewed the observance of the desired intermediates.
References


CHAPTER 5

SUMMARY AND CONCLUSIONS

In conclusion, we have prepared some novel substrates and determined their activity for human as well as *Pseudomonas fluorescens* kynureninase. We synthesized the 3-chloro-DL-kynurenine, 3-fluoro-DL-kynurenine, 3-methyl-DL-kynurenine, 5-bromo-L-kynurenine, and 5-chloro-L-kynurenine and compared the turnover number as well as the catalytic efficiency of each of these substrates as compared to L-kynurenine and 3-hydroxykynurenine for both the human and the bacterial enzyme. Thus, for the human enzyme a comparison of the turnover number for each of these substrates with the natural substrate 3-hydroxykynurenine, shows that all of them have a lower turnover number than the natural substrate and the turnover number decreases in the order: 3-OH > 3-Cl > 5-Br > 5-Cl > 3-Me > 3-F. And for the human enzyme a comparison of the turnover number for each of these substrates with L-kynurenine shows the turnover number to decrease in the order: 5-Br = 3-Cl > 5-Cl > 3-F = 3-Me = L-kynurenine. Thus, for the human enzyme having a substituent on the aromatic ring of kynurenine increases the turnover number but not as much as that of the 3-OH substituent. For the bacterial enzyme a comparison of the turnover number for each of these substrates with the natural substrate L-kynurenine the turnover number decreases in the order: L-kynurenine = 3-Br = 5-Br > 3-F = 5-Cl > 3-Me > 3-Cl. Thus, the turnover number with the bacterial enzyme seems to be unaffected by the presence of a larger substituent but other substituents have a reduced turnover number than the natural substrate. From the foregoing discussion it can be concluded that while the bacterial enzyme seems to be bothered by the presence of a substituent other than 3-Br or 5-Br, the human enzyme on the other hand prefers to have a substituent on the aromatic ring, as seen by an improved turnover number than when there is no substituent (L-kynurenine).
For the human enzyme a comparison of the catalytic efficiency for each of these substrates with the natural substrate 3-hydroxykynurenine, shows that the catalytic efficiency is much lower than that for the natural substrate and it decreases in the order: 3-OH > 3-Cl = 5-Br > 5-Cl > 3-F > 3-Me. Thus, for the human enzyme having a substituent other than –OH seems to actually reduce the catalytic efficiency of the enzyme for that substrate as compared to the natural substrate. For the human enzyme a comparison of the catalytic efficiency for each of these substrates with L-kynurenine shows the decreasing order to be: 5-Br = 3-Cl > 5-Cl > 3-F > 3-Me > L-kynurenine. Thus, for the human enzyme having a substituent on the aromatic ring actually increases the catalytic efficiency than when there is no substituent (L-kynurenine) but this improvement in catalytic efficiency is not better than having a 3-OH substituent as in the natural substrate, presumably because these other groups are not capable of H-bonding like –OH, which has been recently proved from the crystal structure of the enzyme (Lima. S. et al 2009). For the bacterial enzyme a comparison of the catalytic efficiency for each of these substrates with the natural substrate L-kynurenine shows the decreasing order to be: 5-Br > 5-Cl > L-kynurenine ~ 3-F > 3-Me > 3-Cl > 3-Br. Thus, the catalytic efficiency of the bacterial enzyme is actually better than the natural substrate when there is a 5-Br or a 5-Cl substituent, but the catalytic efficiency is lowered by the other substituents. And from the foregoing discussion it can be concluded that while the catalytic efficiency of the bacterial enzyme seems to be bothered by the presence of a substituent other than 5-Br or 5-Cl, the human enzyme on the other hand actually prefers to have a substituent on the aromatic ring, as seen by an improved catalytic efficiency than when there is no substituent (L-kynurenine).

Also, to conclude we have synthesized a novel caged kynurenine which in future will be used for the generation of kynurenine ‘in situ’ by a flash photolysis thereby allowing the
detection of formation and decay of the external aldimine intermediate in the reaction of kynureninase. This detection will further prove the mechanism of action followed by the enzyme kynureninase.
APPENDIX
Scanning kinetic spectrum for 3-chloro-DL-kynurenine
Scanning kinetic spectrum for 3-fluoro-DL-kynurenine
Scanning kinetic spectrum for 3-methyl-DL-kynurenine
Scanning kinetic spectrum for 5-chloro-L-kynurenine
Rate kinetics data for 3-chloro-DL-kynurenine with human enzyme

<table>
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<th>Sr. No.</th>
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<th>1M pH8 KP_{i} (µL)</th>
<th>3mM 3-Cl-DL-KYNU (µL)</th>
<th>Enzyme Solution (µL)</th>
<th>Ionized water (µL)</th>
<th>Final concn. of 3-Cl-DL-KYNU (µM)</th>
<th>Final Vol. of assay soln. (µL)</th>
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**Notes:**

1. Stock solution of human enzyme used was about 7.9 mg/ml by assay. 5µL of this solution was diluted to 600µL using ionized water and used the above mentioned volumes of this enzyme solution for the assay.

2. Wavelength used: 365nm, Cycle repeated every minute for 60 mins., Block temp.: 37°C
Rate kinetics data for 3-fluoro-DL-kynurenine with human enzyme

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**Notes:**

1. Stock solution of human enzyme used was about 7.9 mg/ml by assay. 5µL of this solution was diluted to 600µL using ionized water and used the above mentioned volumes of this enzyme solution for the assay.

2. Wavelength used: 360nm, Cycle repeated every 2 mins. for 120 mins., Block temp.: 37°C
Rate kinetics data for 3-methyl-DL-kynurenine with human enzyme

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Notes:

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2. Wavelength used: 362nm, Cycle repeated every 1.5 mins. for 90 mins., Block temp: 37ºC
Rate kinetics data for 5-bromo-L-kynurenine with human enzyme

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Notes:

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2. Wavelength used: 370 nm, Cycle repeated every 0.5 mins. for 30 mins., Block temp: 37°C
Rate kinetics data for 5-chloro-L-kynurenine with human enzyme

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<th>Final concn. of 5-Cl-L-KYNU (µM)</th>
<th>Final Vol. of assay soln. (µL)</th>
<th>Rate set 1</th>
<th>Rate set 2</th>
</tr>
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</table>

Notes:

1. Stock solution of human enzyme used was about 7.9 mg/ml by assay. 5µL of this solution was diluted to 600µL using ionized water and used the above mentioned volumes of this enzyme solution for the assay.

2. Wavelength used: 370 nm, Cycle repeated every 0.5 mins. for 30 mins., Block temp: 37ºC
Rate kinetics data for 3-chloro-DL-kynurenine with bacterial enzyme

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>2mM PLP (µL)</th>
<th>1M pH8 KP (µL)</th>
<th>3mM 3-Cl-DL-KYNU (µL)</th>
<th>Enzyme Solution (µL)</th>
<th>Ionized water (µL)</th>
<th>Final concn. of 3-Cl-DL-KYNU (µM)</th>
<th>Final Vol. of assay soln. (µL)</th>
<th>Rate set 1</th>
<th>Rate set 2</th>
</tr>
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</tr>
</tbody>
</table>

Notes:

1. Stock solution of bacterial enzyme used was about 19 mg/ml by assay. 2.1µL of this solution was diluted to 600µL using ionized water. A further ten times dilution of this solution was done and used the above mentioned volumes of this enzyme solution for the assay.

2. Wavelength used: 365nm, Cycle repeated every minute for 60 mins., Block temp.: 37°C
Rate kinetics data for 3-fluoro-DL-kynurenine with bacterial enzyme

<table>
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<th>1M pH8 KP, (µL)</th>
<th>3mM 3-F-DL-KYNU (µL)</th>
<th>Enzyme Solution (µL)</th>
<th>Ionized water (µL)</th>
<th>Final concn. of 3-F-DL-KYNU (µM)</th>
<th>Final Vol. of assay soln. (µL)</th>
<th>Rate set 1</th>
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Notes:

1. Stock solution of bacterial enzyme used was about 19 mg/ml by assay. 2.1µL of this solution was diluted to 600µL using ionized water. A further ten times dilution of this solution was done and used the above mentioned volumes of this enzyme solution for the assay.

2. Wavelength used: 360nm, Cycle repeated every minute for 60 mins., Block temp.: 37°C
Rate kinetics data for 3-methyl-DL-kynurenine with bacterial enzyme

<table>
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<th>3mM 3-Me-DL-KYNU (µL)</th>
<th>Enzyme Solution (µL)</th>
<th>Ionized water (µL)</th>
<th>Final concn. of 3-Me-DL-KYNU (µM)</th>
<th>Final Vol. of assay soln. (µL)</th>
<th>Rate set 1</th>
<th>Rate set 2</th>
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Notes:

1. Stock solution of bacterial enzyme used was about 19 mg/ml by assay. 2.1µL of this solution was diluted to 600µL using ionized water and used the above mentioned volumes of this enzyme solution for the assay.

2. Wavelength used: 362nm, Cycle repeated every minute for 60 mins., Block temp: 37ºC
Rate kinetics data for 5-bromo-L-kynurenine with bacterial enzyme

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<th>3mM 5-Br-L-KYNU (µL)</th>
<th>Enzyme Solution (µL)</th>
<th>Ionized water (µL)</th>
<th>Final concn. of 5-Br-L-KYNU (µM)</th>
<th>Final Vol. of assay soln. (µL)</th>
<th>Rate set 1</th>
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Notes:

1. Stock solution of bacterial enzyme used was about 19 mg/ml by assay. 2.1µL of this solution was diluted to 600µL using ionized water. A further hundred times dilution of this solution was done and used the above mentioned volume of this enzyme solution for the assay.

2. Wavelength used: 370 nm, Cycle repeated every 0.33 mins. for 30 mins., Block temp: 37ºC
Rate kinetics data for 5-chloro-L-kynurenine with bacterial enzyme

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>2mM PLP (µL)</th>
<th>1M pH8 KP, (µL)</th>
<th>3mM 5-Cl-L-KYNU (µL)</th>
<th>Enzyme Solution (µL)</th>
<th>Ionized water (µL)</th>
<th>Final concn. of 5-Cl-L-KYNU (µM)</th>
<th>Final Vol. of assay soln. (µL)</th>
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</table>

Notes:

1. Stock solution of bacterial enzyme used was about 19 mg/ml by assay. 2.1 µL of this solution was diluted to 600 µL using ionized water. A further hundred times dilution of this solution was done and used the above mentioned volume of this enzyme solution for the assay.

2. Wavelength used: 370 nm, Cycle repeated every 0.33 mins. for 30 mins., Block temp: 37°C.