

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

INDEX WORDS: Kynureninase, *Pseudomonas fluorescens*, kynurenine, enzymatic resolution, *Aspergillus acylase*, substituted phenylhydrazine, substituted phenylhydrazones, Fischer indole cyclization, ozonolysis, hydrolysis

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

by

CHANDAN L MAITRANI

BS, University of Mumbai, India, 1994

MS, University of Mumbai, India, 1996

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

© 2009

CHANDAN L MAITRANI

All Rights Reserved

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

by

CHANDAN L MAITRANI

Major Professor: Robert S Phillips

Committee: George F Majetich
Jonathan Amster

Electronic Version Approved:

Maureen Grasso
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.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	v
LIST OF ABBREVIATIONS.....	x
CHAPTER	
1 INTRODUCTION AND LITERATURE REVIEW.....	1
Tryptophan.....	1
Catabolism of tryptophan.....	2
The enzyme kynureninase.....	6
Mechanism of kynureninase action.....	9
Kynurenines.....	18
References.....	21
2 SYNTHESIS OF SUBSTRATE ANALOGS FOR HUMAN AND BACTERIAL KYNURENINASE.....	25
Abstract.....	25
Experimental methods.....	26
Instrumentation.....	26

Results and Discussion.....	51
References.....	58
3 STEADY STATE KINETIC STUDIES OF THE SUBSTRATE ANALOGS FOR HUMAN AND BACTERIAL KYNURENINASE.....	60
Abstract.....	60
Experimental methods.....	61
Results and Discussion.....	63
References.....	73
4 SYNTHESIS AND STABILITY STUDIES OF CAGED KYNURENINE.....	74
Abstract.....	74
Introduction.....	75
Experimental methods.....	78
Results and Discussion.....	81
Stability studies for caged kynurenine.....	84
References.....	86
5. SUMMARY AND CONCLUSIONS.....	89

6. APPENDIX.....	92
Scanning kinetic spectrum for 3-chloro-DL-kynurenine.....	93
Scanning kinetic spectrum for 3-fluoro-DL-kynurenine.....	94
Scanning kinetic spectrum for 3-methyl-DL-kynurenine.....	95
Scanning kinetic spectrum for 5-chloro-L-kynurenine.....	96

LIST OF ABBREVIATIONS

AA	<i>Aspergillus acylase</i>
Ac	acetyl
AcOH	acetic acid
Ac ₂ O	acetic anhydride
Boc	t-butoxy carbonyl
bs	broad singlet
CNS	central Nervous System
d	doublet
dd	doublet of doublet
DMF	N,N-dimethyl formamide
dt	doublet of triplet
d/w	distilled water
ee	enantiomeric excess
Et	ethyl
EtOAc	ethyl acetate
g	gram
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
IDO	indoleamine-2,3-dioxygenase
m	multiplet
Me	methyl

mg	milligram
mM	millimolar
μ M	micromolar
mol	number of moles
MeOH	methanol
mins.	minutes
ml	milliliter
NAD ⁺	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP ⁺	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBS	N-bromosuccinimide
NCS	N-chlorosuccinimide
nm	nanometer
NMDA	N-methyl-D-aspartate
ns	nanosecond
PMP	pyridoxamine-5'-phosphate
Ph	phenyl
PLP	pyridoxal-5'-phosphate
psi	pounds per square inch
rac. or DL	racemic mixture
RT	room temperature

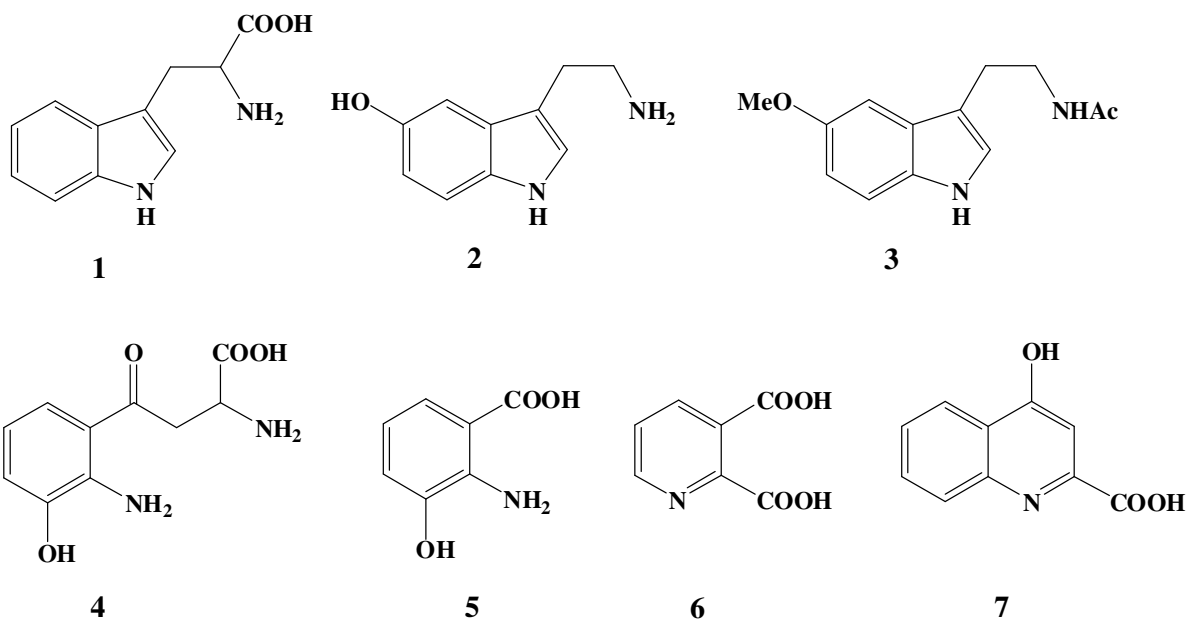
RM	reaction mixture
t	triplet
TDO	tryptophan-2,3-dioxygenase
TEA	triethanolamine
TFA	trifluoroacetic acid
TRIS	tris (hydroxymethyl) amino methane

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 serotonin⁴⁻⁶ (5-hydroxytryptamine - **2**), neurohormone melatonin^{7,8} (N-acetyl-5-methoxy tryptamine - **3**), kynuramine metabolites of melatonin, and the products of kynurenine pathway of tryptophan catabolism including 3-hydroxykynurenine (**4**), 3-hydroxyanthranilic acid (**5**), quinolinic acid⁹⁻¹³ (**6**) and kynurenic acid¹⁴ (**7**). Apart from this tryptophan is also involved in the biosynthesis of niacin via the kynurenine catabolic pathway. The neurotransmitter serotonin is



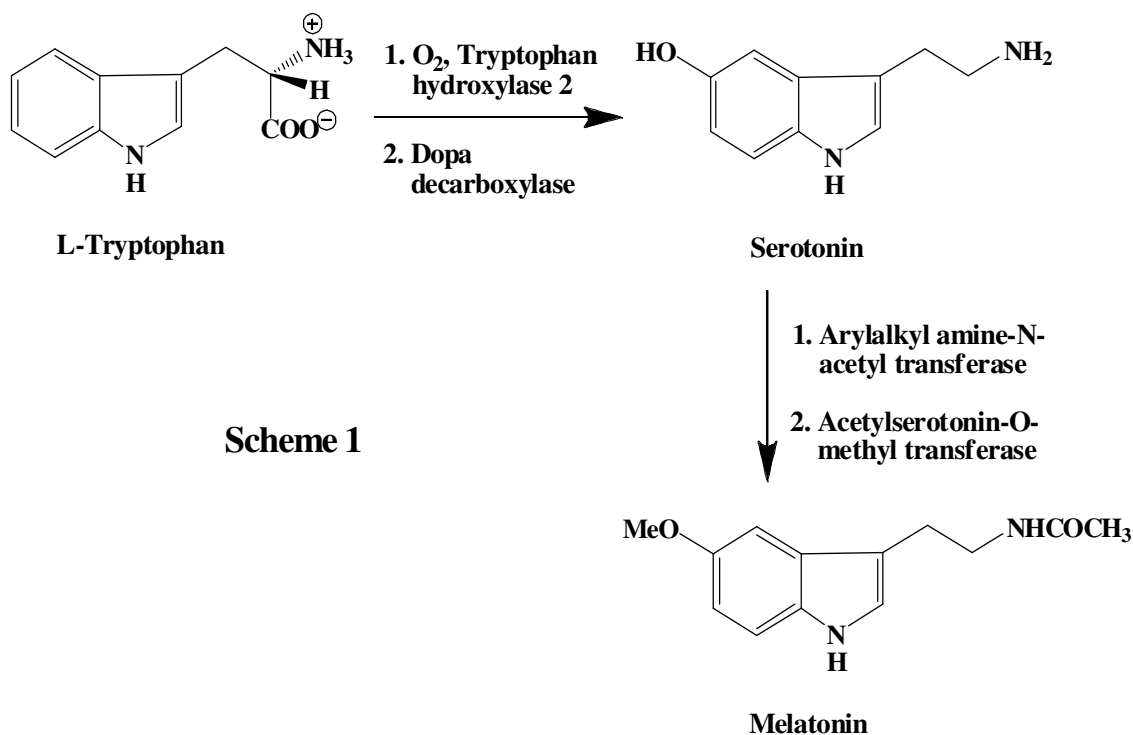
involved in the modulation of mood, anger, emotion and appetite, and is implicated in the control of several behavioral and physiological functions. The neurohormone melatonin serves as a biological clock that controls the sleep patterns of the individual. The metabolite quinolinic acid has been found 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 pathway¹⁵. In the central nervous system before crossing the blood-brain barrier, approximately 90% of the tryptophan is complexed with plasma albumin¹⁶ and this complex cannot cross the blood brain barrier. However, the free tryptophan¹⁷ can cross blood-brain barrier where it is then available for further metabolism in the brain.

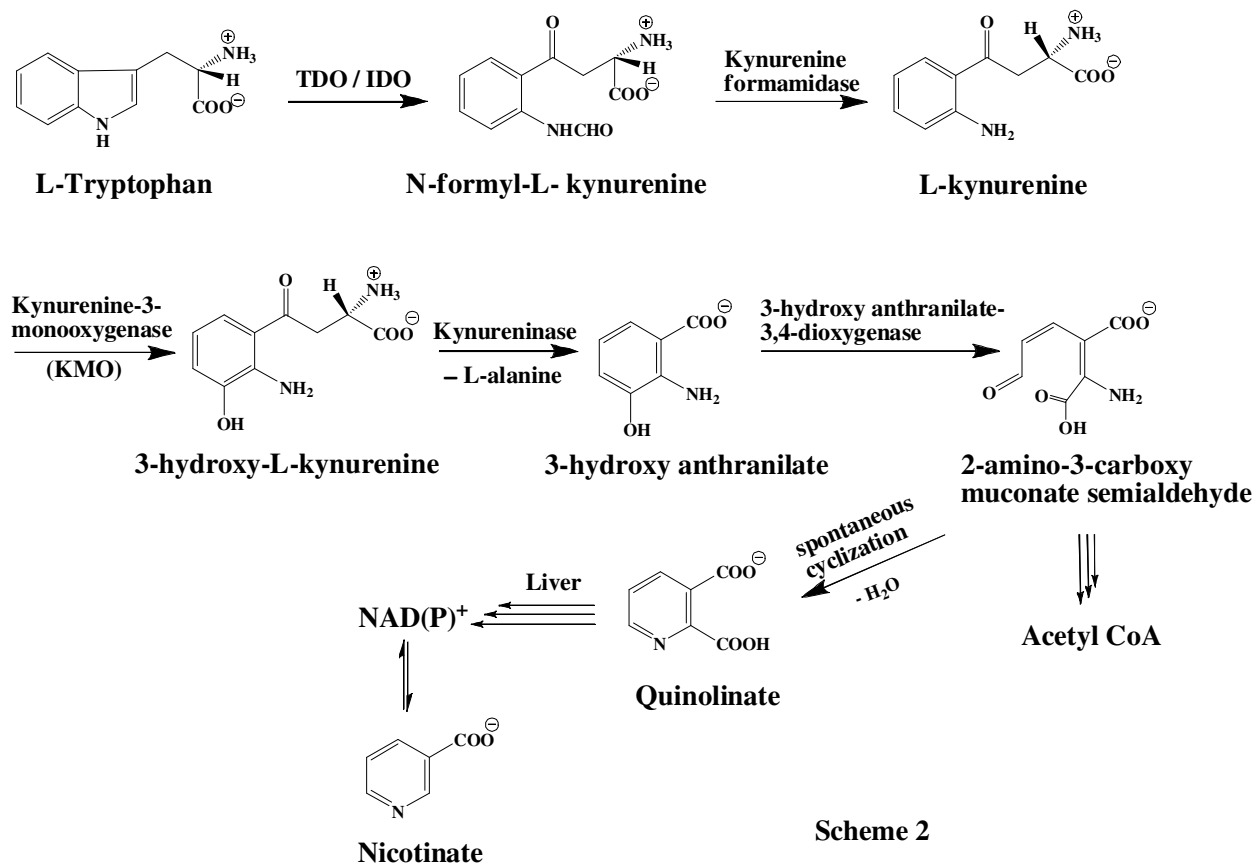
In the serotonergic neurons and mast cells of the CNS (Scheme 1) the free tryptophan is acted upon by the enzyme tryptophan hydroxylase-2 also called tryptophan-5-monoxygenase (EC: 1.14.16.4). This enzyme uses molecular oxygen and catalyzes the hydroxylation of tryptophan to 5-hydroxy-L-tryptophan in the presence of the cofactor tetrahydrobiopterin. The 5-hydroxy-L-tryptophan is then rapidly decarboxylated to serotonin¹⁸⁻²⁰ 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 melatonin²¹ is obtained by the action of acetylserotonin-O-methyl transferase (EC: 2.1.1.4) on N-acetyl serotonin.



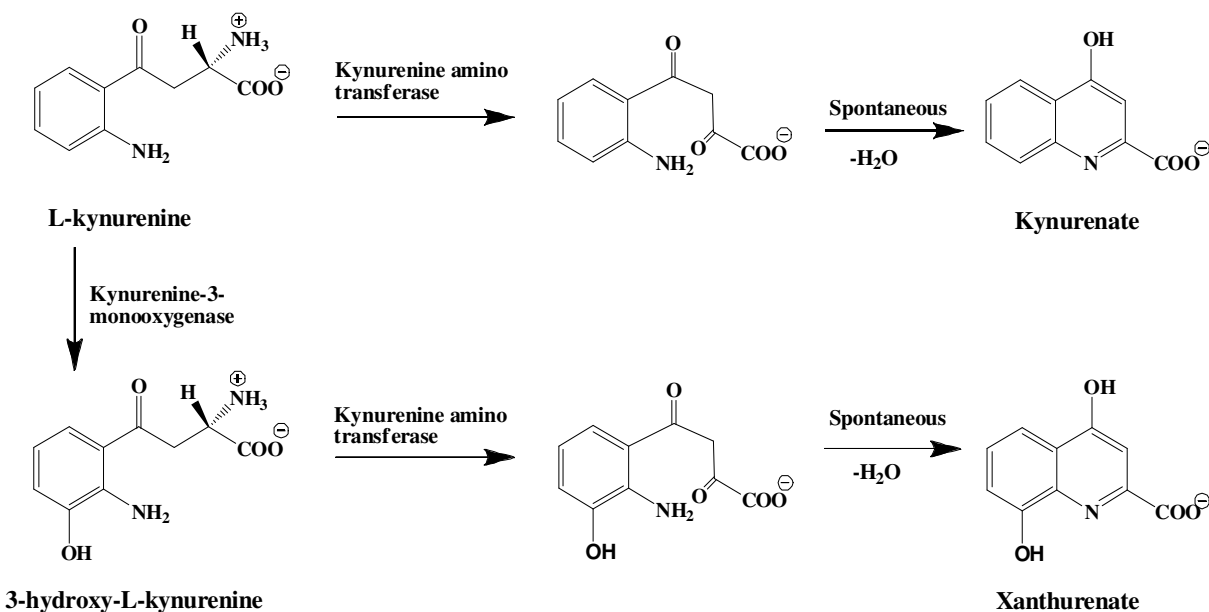
In the CNS (including the astrocytes, microglia, macrophages, and dendritic cells) and in the hepatic and non-hepatic tissues (including the lungs, small intestine, and placenta of mammals such as rabbits, rats, mice, and humans) L-tryptophan is catabolised by the kynurenine pathway¹⁵. The first step in this pathway (Scheme 2) is the oxidative cleavage of the pyrrole ring of tryptophan by the action of the heme protein 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

hemeprotein 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 catalyse the oxidative cleavage of D-tryptophan, L-tryptophan, as well as the various indoleamines such as melatonin, serotonin, hence the name IDO²² 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 β,γ 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 quinolinate²³. 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 B₃) 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 receptors²⁴ in the CNS.

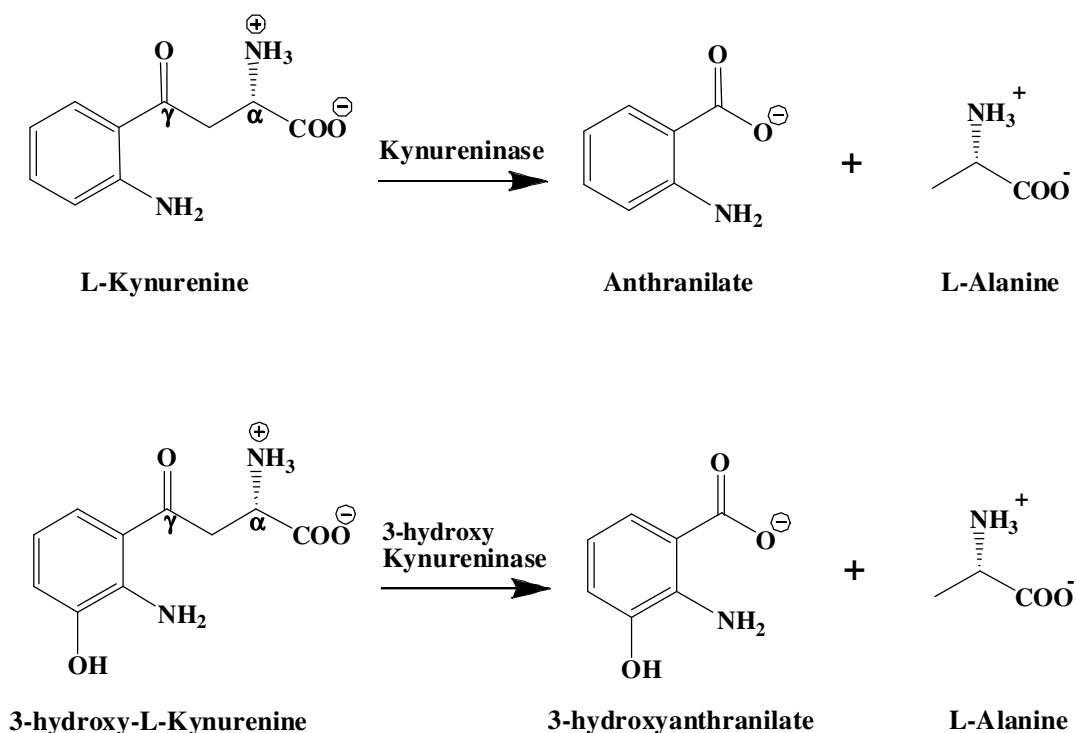


Scheme 3

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 effects¹⁴ on the NMDA and $\alpha 7$ 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-dioxo butanoate which spontaneously dehydrates to produce xanthurenate.

The enzyme kynureninase

Kynureninase²⁵ 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

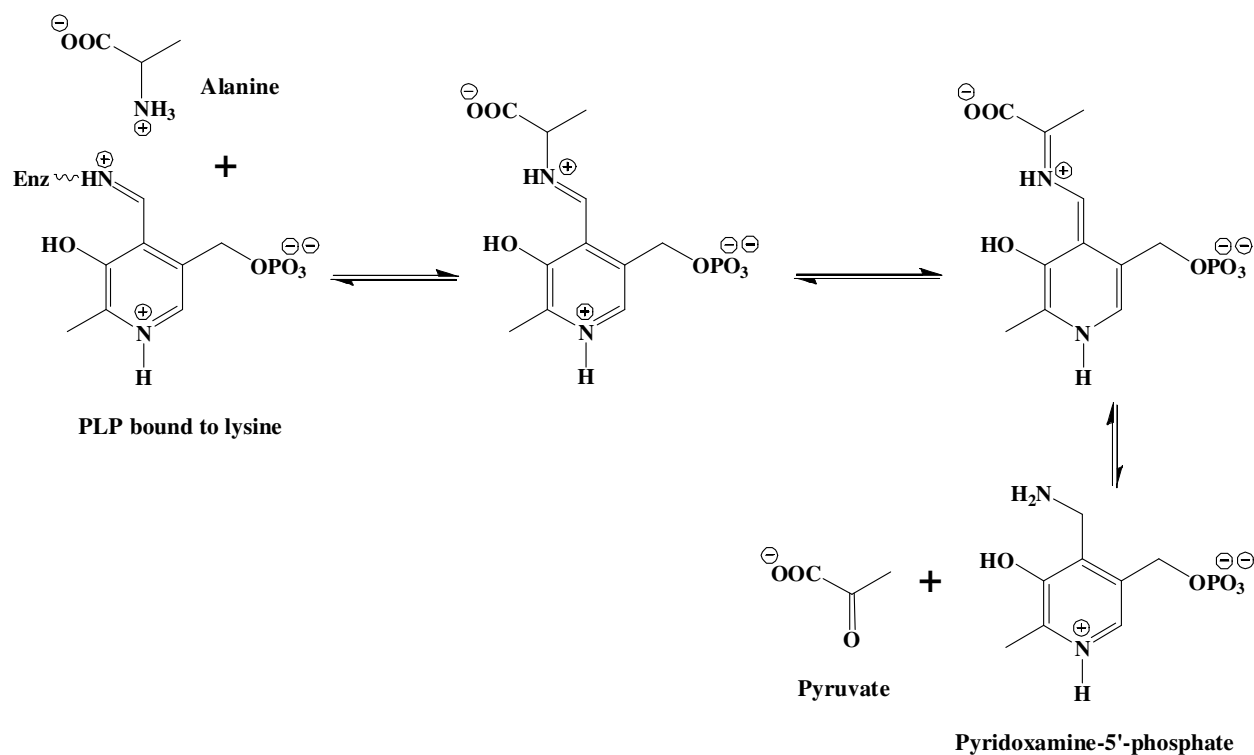


Scheme 4

give anthranilic acid and L-alanine. This is a key enzyme in the kynurenine pathway in the tryptophan catabolism and catalyzes the unique β,γ -cleavage of aryl substituted γ -keto- α - amino acids²⁶. The enzyme has been isolated from *Pseudomonas fluorescens*²⁷, *Neurospora crassa*^{28,29}, rat liver³⁰ and porcine liver³¹. 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 *Pseudomonas fluorescens* cleaves L-kynurenine about five times³² 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 kynureninases³³ 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 activity³⁴ 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 medium³⁴. 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 inactivated⁹ 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.



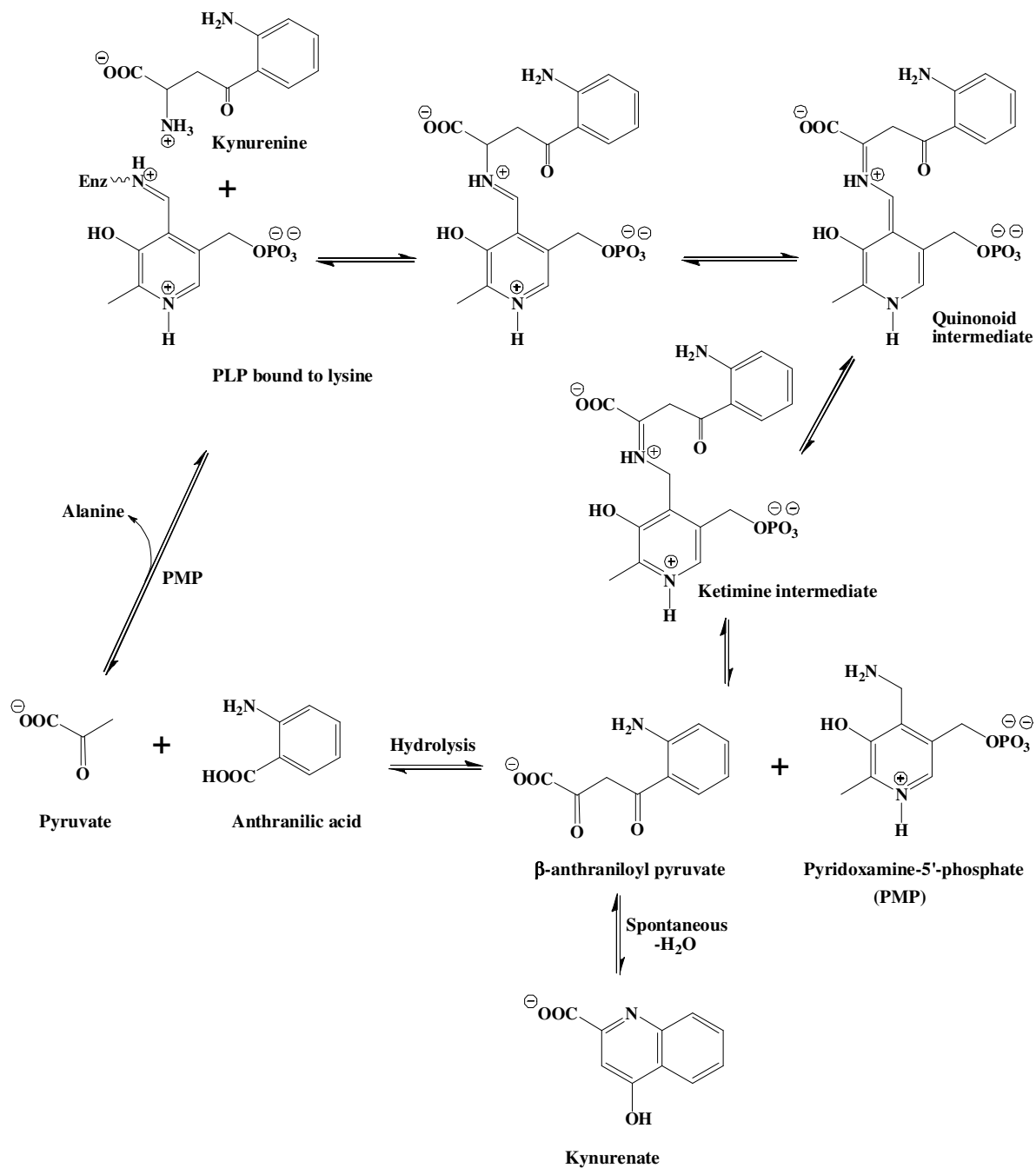
On the other hand the non inducible (or constitutive) enzyme is little or not at all affected by the presence of L-alanine or other amino acids^{35a}. Even then the rate of the hydrolytic cleavage reduces with time indicating that the product 3-hydroxyanthranilate inhibits the non inducible enzyme thereby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

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 enzyme²⁶.

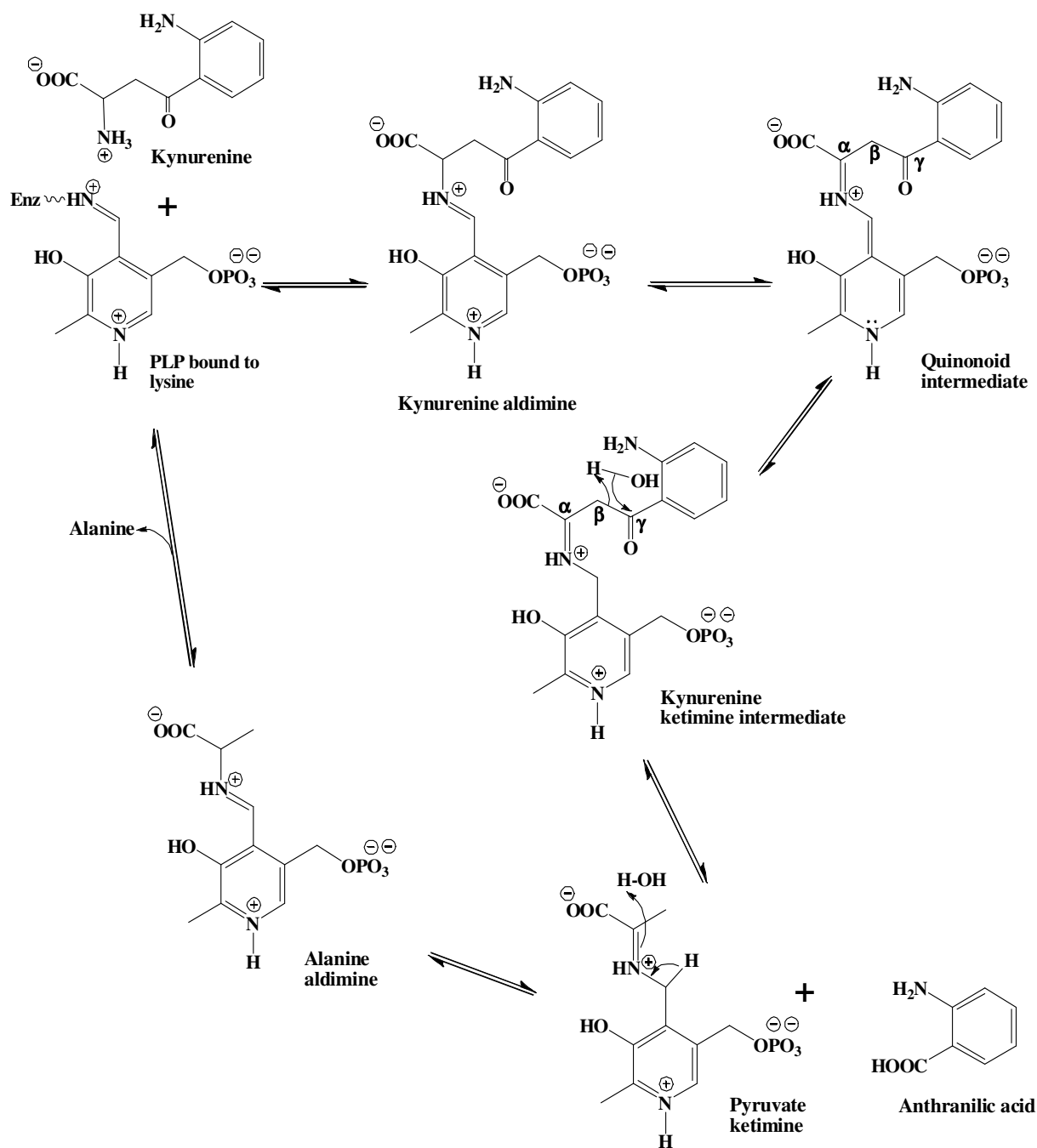
Mechanism of kynureninase action

The enzyme kynureninase catalyzes the unique β,γ -cleavage of aryl substituted γ -keto- α -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 Dalglish *et al* it was proposed that kynureninase catalyzes the transamination of kynurenine⁴¹ by PLP to give the β -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 transferase⁴²⁻⁴⁴ was shown to be involved in the formation of kynurenic acid.

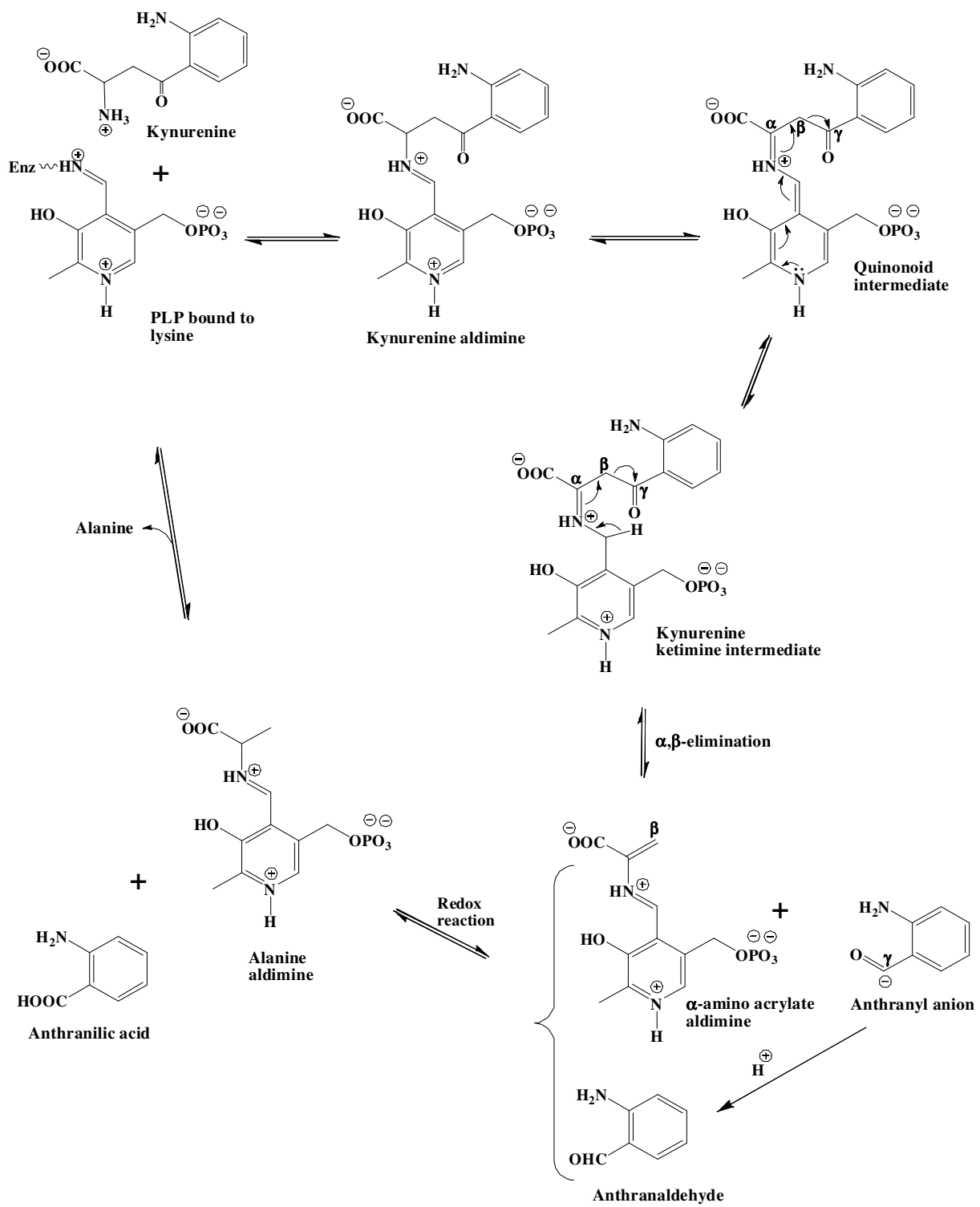
In another mechanism proposed by Braunstein *et al*⁴⁵⁻⁴⁶ (Scheme 7) the initially formed Schiff's base between PLP and kynurenine undergoes a tautomerization followed by hydrolysis at the γ -carbonyl carbon. This cleaves the β,γ -carbon bond in a way that the β -carbon takes up



Scheme 6



Scheme 7

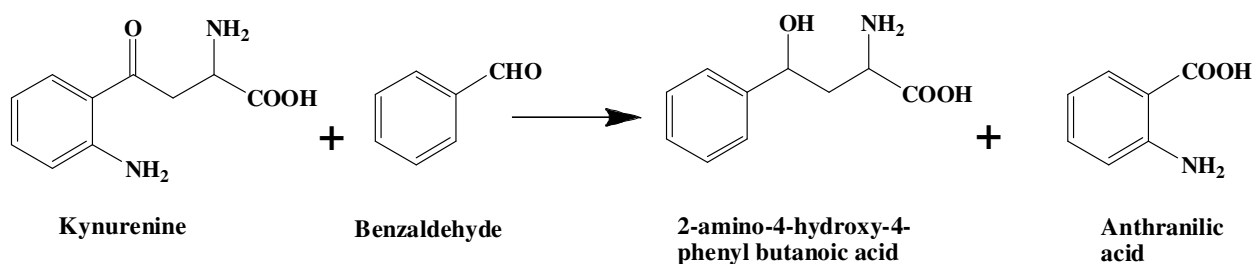


Scheme 8

the σ -electrons of the β,γ -carbon bond to give anthranilic acid and the pyruvate ketimine. The pyruvate ketimine is then converted into alanine and PLP after tautomerization.

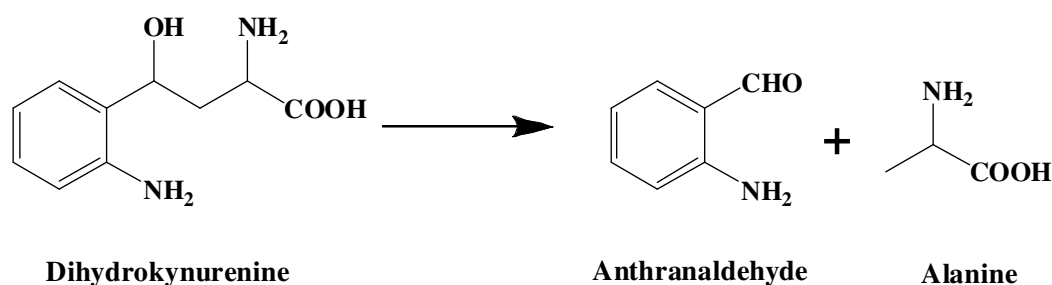
Longenecker *et al*⁴⁷ however proposed a slightly different mechanism than the Braunstein group based on their study of mechanisms of enzymes including serine dehydrase, tryptophanase, and cysteine desulfhydrase. In this mechanism (Scheme 8) after the initial formation of the kynurenine ketimine, instead of the β -carbon keeping the electrons of the β,γ -carbon bond, the γ -carbonyl carbon takes up the electron pair as shown by the tautomerization process to give the anthranyl anion and the aldimine of α -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 Morris⁴⁸ suggested that the β -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 reaction⁴⁹ between the incipient alanine and benzaldehyde.



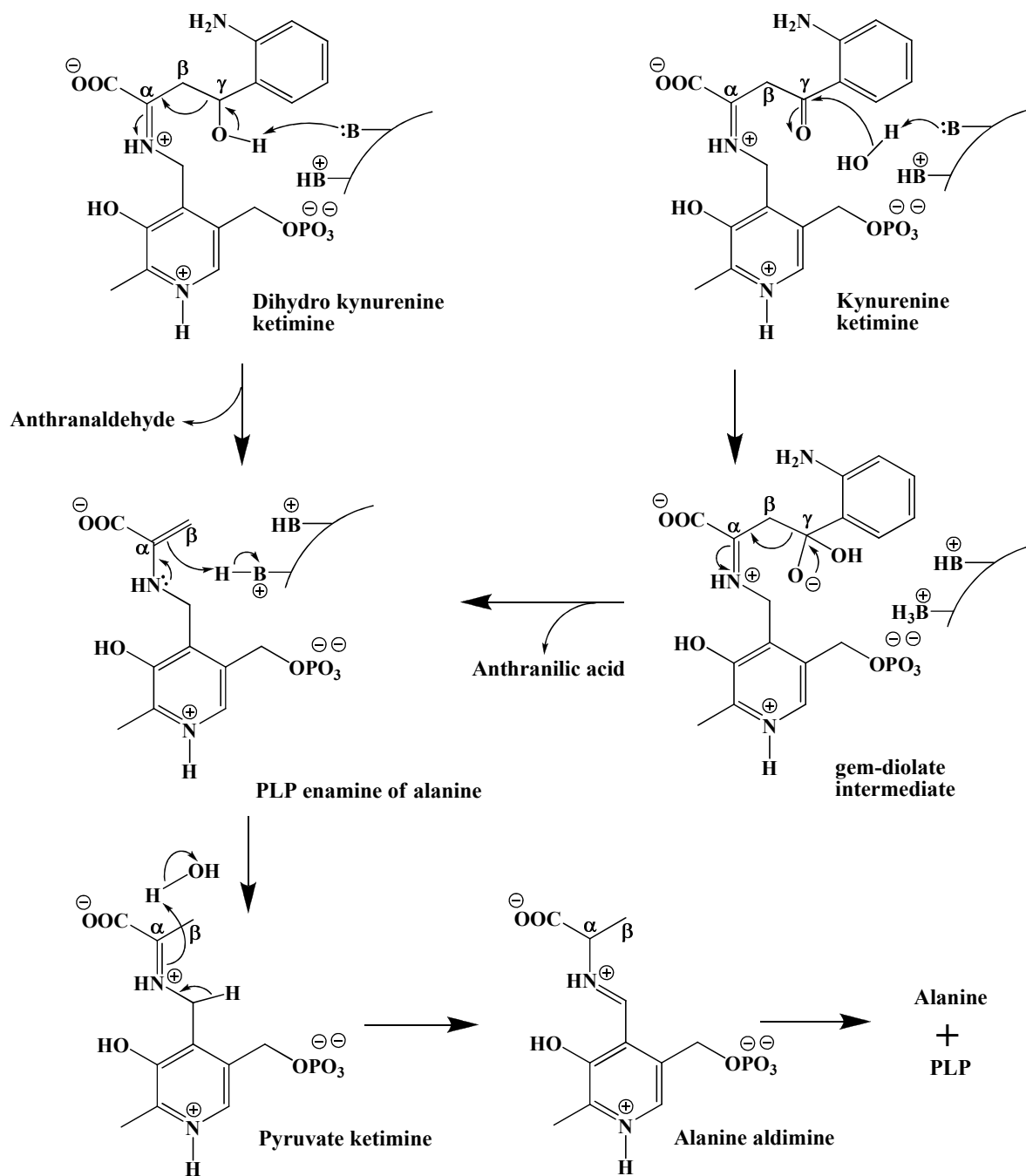
Scheme 9

Evidence for the existence of a β carbanion was also indicated by Tanizawa and Soda⁵⁰ who reported the formation of anthraldehyde in a retro aldol reaction (Scheme 10) from the reduced form of kynurenine viz. dihydro kynurenine. If the γ 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

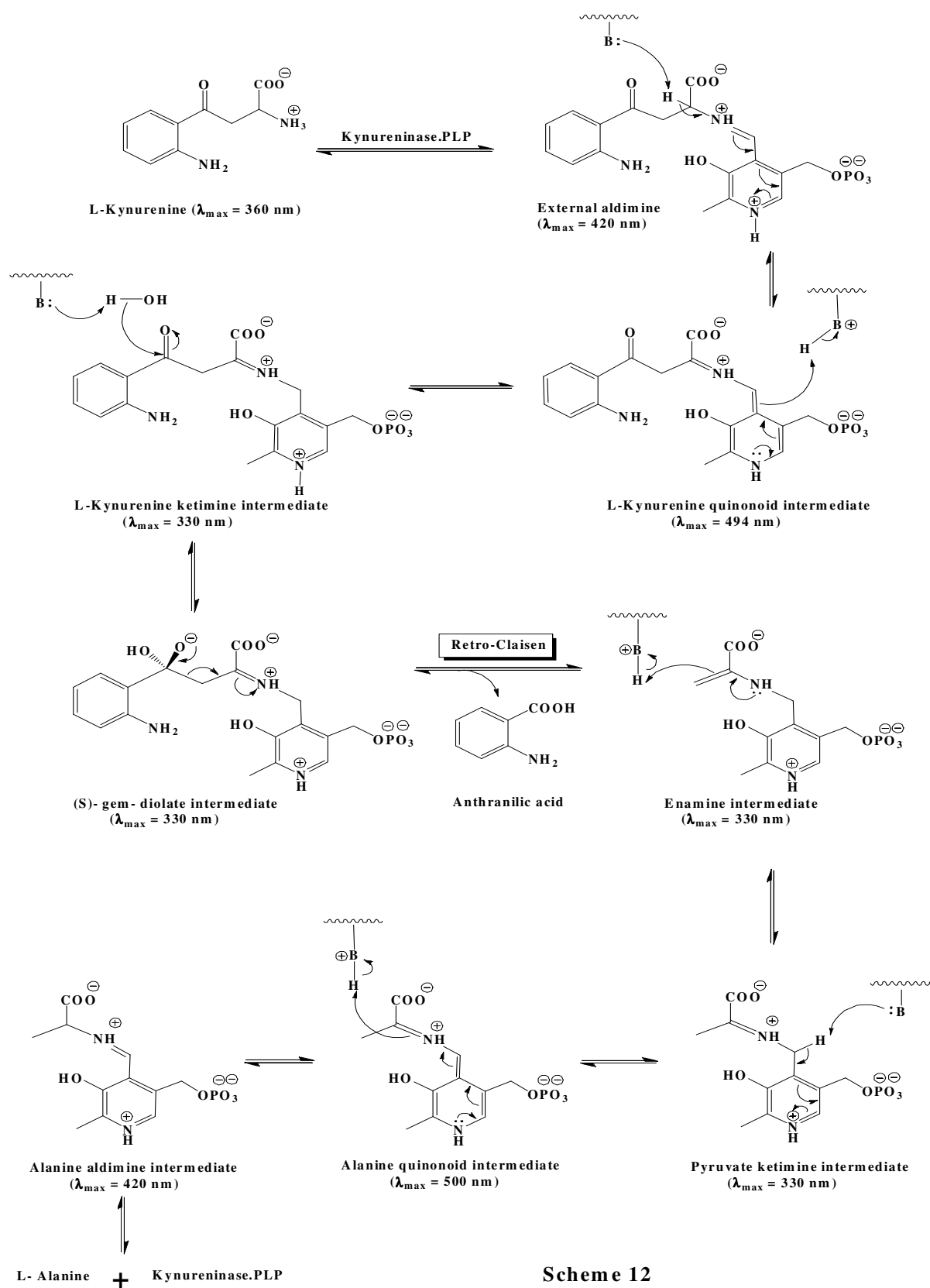
Phillips and Dua⁴⁹ also confirmed the formation of the aldol from the reaction of kynurenine with benzaldehyde, which gave a 2:3 mixture of the 4S:4R diastereomers of 2-amino-4-hydroxy-4-phenyl butanoic acid. Based on their findings they concluded the formation of a gem-diolate intermediate in the cleavage mechanism (Scheme 11). It was found by Palcic *et al*⁵¹ that the ϵ -amino group of a lysine residue is involved in the α -proton abstraction which produces the kynurenine ketimine that subsequently serves as a sink for the electrons from the β,γ -carbon-carbon bond cleavage. Thus there are two bases involved in the mechanism of the cleavage. The first is in the α -proton abstraction to give the ketimine and the second is 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.

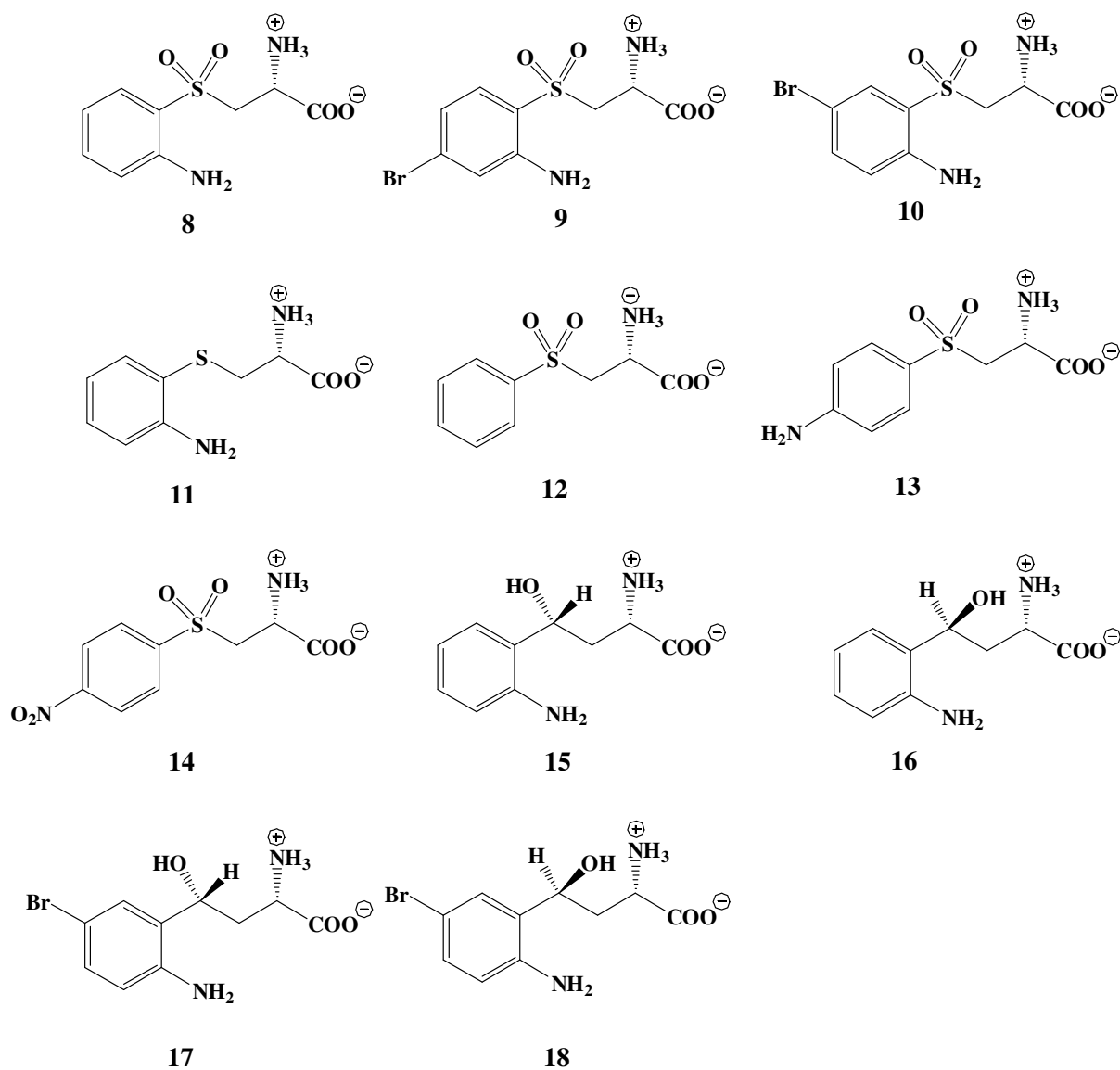
Later Dua and Phillips⁵² also showed that the sulfone analog of kynurenine viz. S-(2-aminophenyl)-L-cysteine-S,S-dioxide was a potent inhibitor of kynureninase with a K_i value of 70 nM which is about 300-fold lower than the K_m for L-kynurenine. This further supports the *gem*-diolate hypothesis. Kinetic isotope effect studies⁵³ 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 ketimine intermediate to give the alanine quinonoid intermediate. Using rapid-scanning stopped-flow spectrophotometry and rapid chemical quench methods⁵⁴ 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 *et al* is shown in Scheme 12.



Kynurenines

Kynurenine or β -(2-aminobenzoyl)alanine was first discovered by Matsuoka and Yoshimatsu⁵⁵ in the urine of rabbits fed large quantities of the amino acid tryptophan. About two decades later the structure was determined by Butenandt *et al.*⁵⁶ In *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 disorders⁵⁷⁻⁵⁸ such as Huntington's chorea, Lou Gehrig's disease⁵⁹, epilepsy⁶⁰, and AIDS related dementia. An excessive level of quinolinate has been shown to be present after a stroke and is responsible for further damage⁶¹. 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 disease⁶². It has also been shown that spontaneously hypertensive rats have a significantly higher kynureninase activity in tissues⁶³ and recently it has been established that there is close link between hypertension and an allele of the human kynureninase viz. K412E⁶⁴. 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, **8** with a K_i of 27 nM, some 925-fold lower than the K_m of L-kynurenine ($\sim 25 \mu\text{M}$)^{52, 65}. The 4-bromo, **9**, and the 5-bromo, **10** analogs of S-(2-aminophenyl)-L-cysteine-

S,S-dioxide were also found to be potent inhibitors⁶⁶ 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, **11** with a K_i of 2.5 μ M and the des-amino analog viz. S-phenyl-L-cysteine-S,S-dioxide, **12** with a K_i of 3.9 μ M.



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

1. Matarese, V.; Bernlohr, D. A. *J. Biol. Chem.* **1988**, *263*, 14544-14551.
2. Zubay, G.; in *Biochemistry*, **1988** pp 630-631, MacMillan, New York
3. The Chemical Society, *The Alkaloids*, **1971** London, Specialist Periodical Report
4. Pihel, K.; Hsieh, S. C.; Jorgenson, J. W.; Wightman, R. M. *Biochemistry* **1998**, *37*, 1046-1052.
5. Purcell, W. M.; Atterwill, C. K. *Neurochem. Res.* **1995**, *20*, 521-32.
6. Niacaris, T.; Avery, L. *J. Exp. Biol.* **2003**, *206*, 223-231.
7. Altun, A.; Ugur-Altun, B. *Int. J. Clin. Pract.* **2007**, *61*, 835-845.
8. Hardeland, R. *Endocrine* **2005**, *27*, 119-130.
9. Beal, M. F.; Kowall, N. W.; Ellison, D. W.; Mazurek, M. F.; Swartz, K. J.; Martin, J. B. *Nature* **1986**, *321*, 168-171.
10. Heyes, M. P.; Brew, B. J.; Martin, A.; Price, R. W.; Salazar, A. M.; Sidtis, J. J.; Yergey, J. A.; Mouradian, M. M.; Sadler, A. E.; Keilp, J.; Rubinow, D.; Markey, S. P. *Ann. Neurol.* **1991**, *29*, 202-209.
11. Saito, K.; Crowley, J. S.; Markey, S. P.; Heyes, M. P. *J. Biol. Chem.* **1993**, *268*, 15496-15503.
12. Carpenedo, R.; Chiarugi, A.; Russi, P.; Lombardi, G.; Carla, V.; Pellicciari, R.; Moroni, F.; Mattoli, L. *Neuroscience* **1994**, *61*, 237-244.
13. Stone, T. W.; Perkins, M. N. *Eur. J. Pharmacol.* **1981**, *72*, 411-412.
14. Perkins, M. N.; Stone, T. W. *Brain Res.* **1982**, *247*, 184-187.
15. Peters, J. C. *Adv. Exp. Med. Biol.* **1991**, *294*, 345-58.
16. McMenamy, R. H. *J. Biol. Chem.* **1965**, *240*, 4235-4243.

17. Madras, B. K.; Cohen, E. L.; Messing, R.; Munro, H. N.; Wurtman, R. J. *Metab. Clin. Exp.* **1974**, *23*, 1107-1116.
18. Wang, L.; Erlandsen, H.; Haavik, J.; Knappskog, P. M.; Stevens, R. C. *Biochemistry* **2002**, *41*, 12569-12574.
19. Martinez, A.; Knappskog, P. M.; Haavik, J. *Curr. Med. Chem.* **2001**, *8*, 1077-1091.
20. Donaldson, R. M., Jr.; Gray, S. J.; Letsou, V. G. *Lancet* **1959**, *1959-II*, 1002-3.
21. Kopp, N.; Claustrat, B.; Tappaz, M. *Neurosci. Lett.* **1980**, *19*, 237-242.
22. Yoshida, R.; Hayaishi, O. *Meth. Enzymol.* **1987**, *142*, 188-195.
23. Colabroy, K. L.; Begley, T. P. *J. Am. Chem. Soc.* **2005**, *127*, 840-841.
24. Schwarcz, R.; Whetsell, W. O.; Mangano, R. M. *Science* **1983**, *219*, 316-318.
25. Braunstein, A. E.; Goryachenkova, E. V.; Paskhina, T. S. *Biokhimiya (Moscow)* **1949**, *14*, 163-79.
26. Soda, K.; Tanizawa, K. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1979**, *49*, 1-40.
27. Hayaishi, O.; Stanier, R. Y. *J. Biol. Chem.* **1952**, *195*, 735-740.
28. Jakoby, W. B.; Bonner, D. M. *J. Biol. Chem.* **1953**, *205*, 699-707.
29. Jakoby, W. B.; Bonner, D. M. *J. Biol. Chem.* **1953**, *205*, 709-715.
30. Knox, W. G. *Biochem. J.* **1953**, *53*, 379.
31. Wiss, O.; Weber, F. *Hoppe-Seyler's Z. Physiol. Chem.* **1956**, *304*, 232-40.
32. Hayaishi, O. in *A Symposium on Amino Acid Metabolism*, W. D. McElroy and H. B. Glass, Eds. Johns Hopkins Press, Baltimore, **1955**, pp. 914-929
33. Turner, J. R.; Drucker, H. *Biochem. Biophys. Res. Commun.* **1971**, *42*, 698
34. Shetty, A. S.; Gaertner, F. H. *J. Bacteriol.* **1975**, *122*, 235-244.
35. a) Tanizawa, K.; Soda, K. *J. Biochem.* **1979**, *86*, 499-508.

- b) McDermott, C. E.; Casciano, D. A.; Gaertner, F. H. *Biochem. Biophys. Res. Commun.* **1973**, *51*, 813-818.
36. Prasad, C.; Srinivas.Vr *Biochem. J.* **1970**, *119*, 343-&.
37. Tabone, J.; Robert, D. *Bull. Soc. Chim. Biol.* **1952**, *34*, 1102-1105.
38. Bouknight, R. R.; Sadoff, H. L. *J. Bacteriol.* **1975**, *121*, 70-76.
39. Wheelis, M. L. *Arch. Mikrobiol.* **1972**, *87*, 1-&.
40. Brown, A. T.; Wagner, C. *J. Bacteriol.* **1970**, *101*, 456-&.
41. Dalglish, C. E.; Knox, W. E.; Neuberger, A. *Nature* **1951**, *168*, 20-22.
42. Miller, I. L.; Tsuchida, M.; Adelberg, E. A. *J. Biol. Chem.* **1953**, *203*, 205-211.
43. Mason, M. *J. Biol. Chem.* **1954**, *211*, 839-844.
44. Jakoby, W. B.; Bonner, D. M. *J. Biol. Chem.* **1956**, *221*, 689-695.
45. Braunstein, A. E.; Shemyakin, M. M. *Biokhimiya* **1953**, *18*, 393-411.
46. Braunstein, A. E. in *The Enzymes*, Vol. II, 2nd ed. P. D. Boyer, Ed., Academic Press, New York, **1960**, p. 170
47. Longenecker, J. B.; Snell, E. E. *J. Biol. Chem.* **1955**, *213*, 229-235.
48. Bild, G. S.; Morris, J. C. *Arch. Biochem. Biophys.* **1984**, *235*, 41-47.
49. Phillips, R. S.; Dua, R. K. *J. Am. Chem. Soc.* **1991**, *113*, 7385-7388.
50. Tanizawa, K.; Soda, K. *J. Biochem.* **1979**, *86*, 1199-1209.
51. Palcic, M. M.; Antoun, M.; Tanizawa, K.; Soda, K.; Floss, H. G. *J. Biol. Chem.* **1985**, *260*, 5248-5251.
52. Dua, R. K.; Taylor, E. W.; Phillips, R. S. *J. Am. Chem. Soc.* **1993**, *115*, 1264-1270.
53. Koushik, S. V.; Moore, J. A.; Sundararaju, B.; Phillips, R. S. *Biochemistry* **1998**, *37*, 1376-1382.

54. Phillips, R. S.; Sundararaju, B.; Koushik, S. V. *Biochemistry* **1998**, *37*, 8783-8789.
55. Matsuoka, Z.; Yoshimatsu, N. *Z. Physiol. Chem.* **1925**, *143*, 206-10.
56. Butenandt, A.; Weidel, W.; Weichert, R.; von Derjugin, W. *Hoppe-Seyler's Z. Physiol. Chem.* **1943**, *279*, 27-43.
57. Schwarcz, R.; Okuno, E.; White, R. J.; Bird, E. D.; Whetsell, W. O. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4079-4081.
58. Mazzari, S.; Aldinio, C.; Beccaro, M.; Toffano, G.; Schwarcz, R. *Brain Res.* **1986**, *380*, 309-316.
59. Guillemin, G. J.; Meininger, V.; Brew, B. J. *Neurodegener. Dis.* **2005**, *2*, 166-176.
60. Kaminski, R. M.; Zielinska, E. B.; Dekundy, A.; van Luijtelaar, G.; Turski, W. A. *Pol. J. Pharmacol.* **2003**, *55*, 741-746.
61. Stone, T. W. *Expert Opin. Investig. Drugs* **2001**, *10*, 633-645.
62. Guillemin, G. J.; Brew, B. J.; Noonan, C. E.; Takikawa, O.; Cullen, K. M. *Neuropathol. Appl. Neurobiol.* **2005**, *31*, 395-404.
63. Mizutani, K.; Sugimoto, K.; Okuda, T.; Katsuya, T.; Miyata, T.; Tanabe, T.; Higaki, J.; Ogihara, T.; Yamori, Y.; Tsujita, Y.; Tago, N.; Iwai, N. *Hypertens. Res.* **2002**, *25*, 135-140.
64. Zhang, Y.; Zhang, K. X.; He, X.; Yuan, W. T.; Wang, G. L.; Mao, S. Y.; Gao, P. J.; Huang, W.; Zhu, D. L. *Zhonghua Xin Xue Guan Bing Za Zhi* **2005**, *33*, 588-591.
65. Drysdale, M. J.; Reinhard, J. F. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 133-138.
66. Heiss, C.; Anderson, J.; Phillips, R. S. *Org. Biomol. Chem.* **2003**, *1*, 288-295.

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 adduct¹⁻⁴ which on treatment with the corresponding 2-halosubstituted phenylhydrazine⁵⁻¹¹ yields a phenylhydrazone derivative². The different 2-halosubstituted phenylhydrazones are then subjected to a Fischer indole cyclization to give the 7-halosubstituted indolymethylacetamidomalonates¹²⁻¹⁶. 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 kynurenines¹⁷ have been synthesized from L-tryptophan via first the ozonolysis of the methyl ester of N^α-acetyl-L-tryptophan followed by TFA hydrolysis and acylation of the intermediate to give the methyl ester of N^α,N-diacetylkynurenine. This intermediate on bromination or chlorination, followed by acid hydrolysis produces the respective 5-halosubstituted-L-kynurenines.

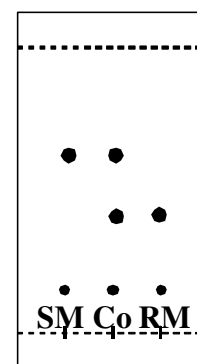
Experimental methods

Instrumentation

^1H NMR, ^{13}C NMR, and ^{19}F NMR 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-amino-3-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 (5 μ , 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^{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.



System: Hexane: EtOAc
2ml : 1ml
Detection: uv 254 nm
or I₂ vapors
SM = Starting material
Co = Mixture spot

Fig. 1

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.

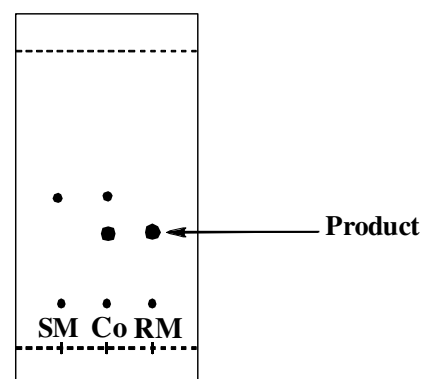
Yield of the free base = 7 g, 81 % , m.p. = 45-46°C

^1H NMR of free base: *d*-MeOH δ 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 δ 4.5

^{13}C NMR of free base: *d*-MeOH δ 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.



System: Hexane: EtOAc
1 : 1
Detection: uv 254 nm
SM = Starting material
Co = Mixture spot

Fig. 2

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 %

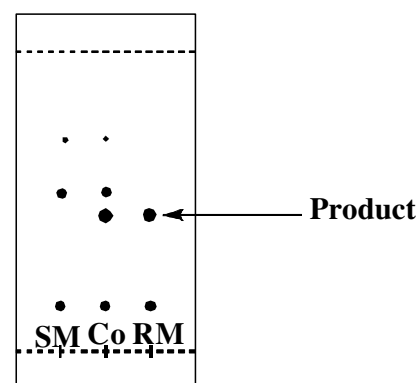
$^1\text{H NMR}$: *d* MeOH δ 7.4 (d, 1H), 7.3 (t, 1H), 7.2 (d, 1H), 7.1 (t, 1H), 6.7 (t, 1H), 4.2 (q, 4H), 2.2 (t, 1H), 2.1 (s, 3H), 1.9 (q, 2H), 1.2 (t, 6H)

$^{13}\text{C NMR}$: *d* MeOH δ 13.4, 20.2, 21.6, 26.9, 62.5, 62.7, 66.5, 116.5, 119.3, 127.8, 129.1, 129.2, 142.4, 168, 171.3

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 %



System : Hexane : EtOAc
1 : 1
Detection: uv 254 nm
SM = Starting material
Co = Mixture spot

Fig. 3

$^1\text{H NMR}$: *d* MeOH δ 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)

^{13}C NMR: *d* MeOH δ 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

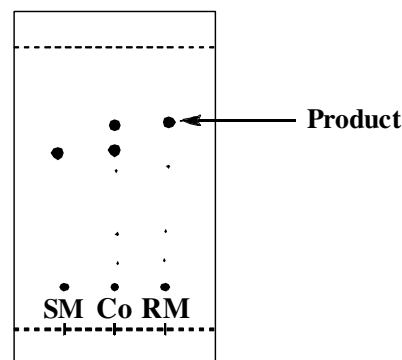
Dissolve the resulting brown semisolid in 50 ml MeOH, add 1 g activated charcoal, stir at RT for about 10 - 15 mins. Filter through Celite, and wash the Celite bed with about 50 ml MeOH. The dark brown filtrate is used as is for the ozonolysis step.

Synthesis of diethyl 2-amino-3-chlorobenzoylmethylacetamidomalonate and its acid hydrolysis to give DL-3-chlorokynurenine²¹

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.



System: Hexane : EtOAc
3ml : 2ml
Detection: uv 254 nm
SM = Starting material
Co = Mixture spot

Fig. 4

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 -178°C.

$^1\text{HNMR}$: *d*-MeOH δ 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)

$^{13}\text{CNMR}$: *d*-MeOH δ 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, charcoalize 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 - 218°C

$^1\text{HNMR}$: (1 – 2% DCl – D₂O) δ 7.7 (d, 1H), 7.6 (d, 1H), 6.7 (t, 1H), 4.2 (t, 1H), 3.7 (d, 2H)

$^{13}\text{CNMR}$: (1 – 2% DCl – D₂O) δ 43.2, 55.4, 119.1, 121.8, 123.3, 125.2, 131.4, 143.6, 172.1, 204.2

Synthesis of 2-fluorophenylhydrazine^{22,23}

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 $-20\text{ }^{\circ}\text{C}$, 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 $-20\text{ }^{\circ}\text{C}$ for about 10 - 15 mins. Then to the same RM while maintaining the temp. at -20 to -25°C 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 $-10\text{ }^{\circ}\text{C}$ for about 45 mins. Check TLC (Fig. 1 above) Cool the suspension to -45 to $-50\text{ }^{\circ}\text{C}$, 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. = $44 - 45^{\circ}\text{C}$.

^1H NMR of free base: *d*-MeOH δ 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 δ 4.5

^{13}C NMR of free base: *d*-MeOH δ 111.5, 118.2, 123.5, 128.2, 138.4, 146.1

^{19}F NMR of free base: *d*-MeOH δ -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 %

¹HNMR: *d*- MeOH δ 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)

¹³CNMR: *d*-MeOH δ 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

¹⁹FNMR: *d*-MeOH δ -137.9

Synthesis of diethyl 7-fluoroindolylmethylacetamidomalonate

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 %

¹HNMR: *d* MeOH δ 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)

¹³CNMR: *d* MeOH δ 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

¹⁹FNMR: *d*-MeOH δ -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 %

$^1\text{HNMR}$: *d*-MeOH δ 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)

$^{13}\text{CNMR}$: *d*-MeOH δ 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

$^{19}\text{FNMR}$: *d*-MeOH δ -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

^1H NMR: (1 – 2% DCl – D₂O) δ 7.5 (d, 1H), 7.2 (d, 1H), 7.1 (t, 1H), 4.1 (t, 1H), 3.5 (d, 2H)

^{13}C NMR: (1 – 2% DCl – D₂O) δ 46.5, 53.2, 119.2, 121.6, 123.1, 128.2, 140.6, 159.2, 176.5, 204.2

^{19}F NMR: *d*-MeOH δ -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 °C, 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 °C for about 10 - 15 mins. Then to the same RM while maintaining the temp. at -20 to -25 °C 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 °C for about 45 mins. Check TLC (Fig. 1 above). 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 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.

¹HNMR of free base: *d*-MeOH δ 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 δ 4.9

¹³CNMR of free base: *d*-MeOH δ 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 %

$^1\text{H NMR}$: *d*-MeOH δ 7.31 (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)

$^{13}\text{C NMR}$: *d*-MeOH δ 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-methylindolylmethylacetamidomalonate³¹

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 %

$^1\text{H NMR}$: *d*-MeOH δ 7.22 (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)

$^{13}\text{C NMR}$: *d*-MeOH δ 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 $183 - 185^{\circ}\text{C}$.

$^1\text{HNMR}$: *d*-MeOH δ 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)

$^{13}\text{CNMR}$: *d*-MeOH δ 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 °C

¹HNMR: (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)

¹³CNMR: (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 °C, 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 °C for about 10 - 15 mins. Then to the same RM while maintaining the temp. at -20 to -25°C 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 °C for about 45 mins. Check TLC (Fig. 1 above) 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 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 °C

¹HNMR of free base: *d*-MeOH δ 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 δ 4.9

¹³CNMR of free base: *d*-MeOH δ 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 %

¹HNMR: *d*- MeOH δ 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)

¹³CNMR: *d*-MeOH δ 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 %

^1H NMR: *d*-MeOH δ 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)

^{13}C NMR: *d*-MeOH δ 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.

$^1\text{HNMR}$: *d*-MeOH δ 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)

$^{13}\text{CNMR}$: *d*-MeOH δ 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, 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-bromokynurenine; wash with about 5 ml d/w. Allow to air dry overnight.

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

$^1\text{HNMR}$: (1 – 2% DCl – D₂O) δ 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 $^1\text{HNMR}$.

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.

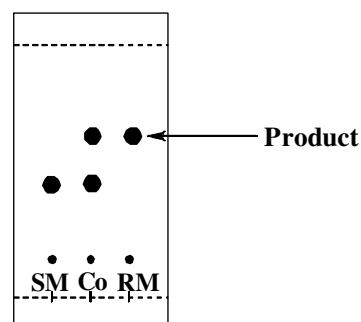
Yield = 9 g, 84 %

^1H NMR: CDCl_3 δ 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}C NMR: CDCl_3 δ 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^a -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.



Solvent system: EtOAc
 Detection: uv 254 nm
 SM = Starting material
 Co = Mixture spot

Fig. 5

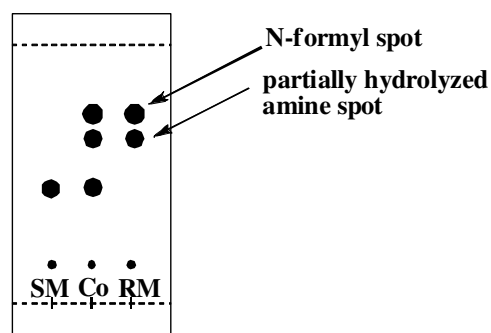
Yield = 10 g, 93 %, m.p. = 147 -149 °C

^1H NMR: CDCl_3 δ 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}C NMR: CDCl_3 δ 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 N^α,N-diacetyl-L-kynurenine

Take 10 g of N^α-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.



Detection: uv 254 nm
 Solvent system: CHCl₃ / MeOH
 (3 ml) / (9 drops)
 SM = Starting material
 Co = Mixture spot

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)

Fig. 6

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

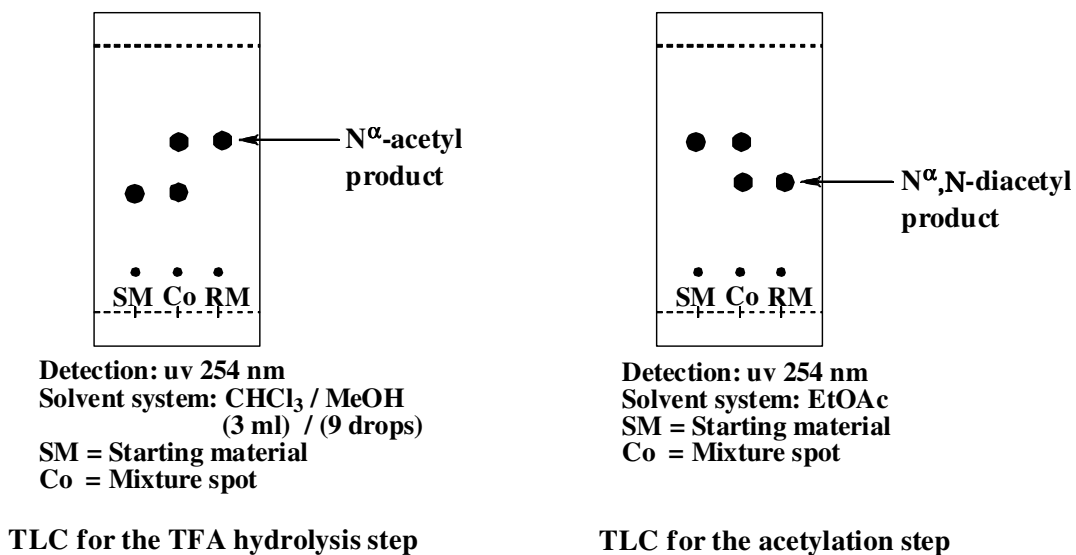


Fig. 7

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 - 163°C

¹HNMR: CDCl₃ δ 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)

¹³CNMR: CDCl₃ δ 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

Synthesis of methyl ester of 5-bromo-N^α,N-diacetyl-L-kynurenine¹⁷

Dissolve 5 g of the methyl ester of N^α,N-diacetylkynurenine 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.

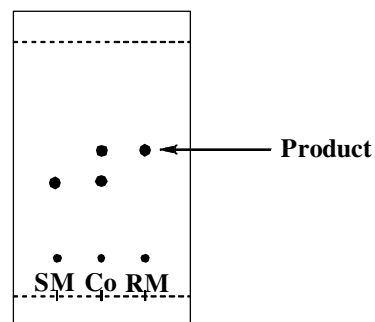
Quench the RM with an aq. solution of sodium bisulfite (10 gm sodium bisulfite dissolved in 40 ml water). Stir for about 5 mins. and extract with two 50 ml portions of chloroform. Wash

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°C

¹HNMR: CDCl₃ δ 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)

¹³CNMR: CDCl₃ δ 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



System: Ethyl acetate
 Detection: uv 254 nm
 SM= Starting material
 Co = Mixture spot

Fig. 8

Synthesis of 5-bromo-L-kynurenine¹⁷

Reflux 4.5 g of the methyl ester of 5-bromo-N^α,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.

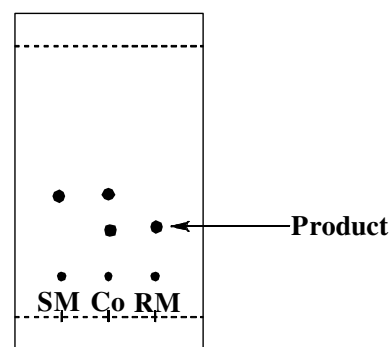
Yield = 1.9 g, 57 %, m.p. = 225 – 227 °C. Sp. rotation = -14.6° (c = 0.28 in 1:1 dioxane : water)

¹HNMR: (1 – 2% DCl – D₂O) δ 8.12 (s, 1H), 7.79 (dd, 1H), 7.22 (d, 1H), 4.28 (t, 1H), 3.65 (d, 2H)

¹³CNMR: (1 – 2% DCl – D₂O) δ 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^α,N-diacetyl-L-kynurenine

Dissolve 2 g of the methyl ester of N,N^α-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^α-diacetylkynurenine. After completion of addition, stir at



System: Ethyl acetate
 Detection: uv 254 nm
 SM= Starting material
 Co = Mixture spot

Fig. 9

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.

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- 187°C

$^1\text{HNMR}$: CDCl_3 δ 11.32 (s, 1H), 8.12 (d, 1H), 7.98 (s, 1H), 7.78 (dd, 1H), 6.52 (d, 1H), 4.65 (m, 1H), 4.21 (s, 3H), 3.58 (m, 2H), 2.21 (s, 3H), 2.01 (s, 3H)

$^{13}\text{CNMR}$: CDCl_3 δ 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^{α} , 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 °C. Sp. rotation= -15.7° (c = 0.28 in 1:1 dioxane : water)

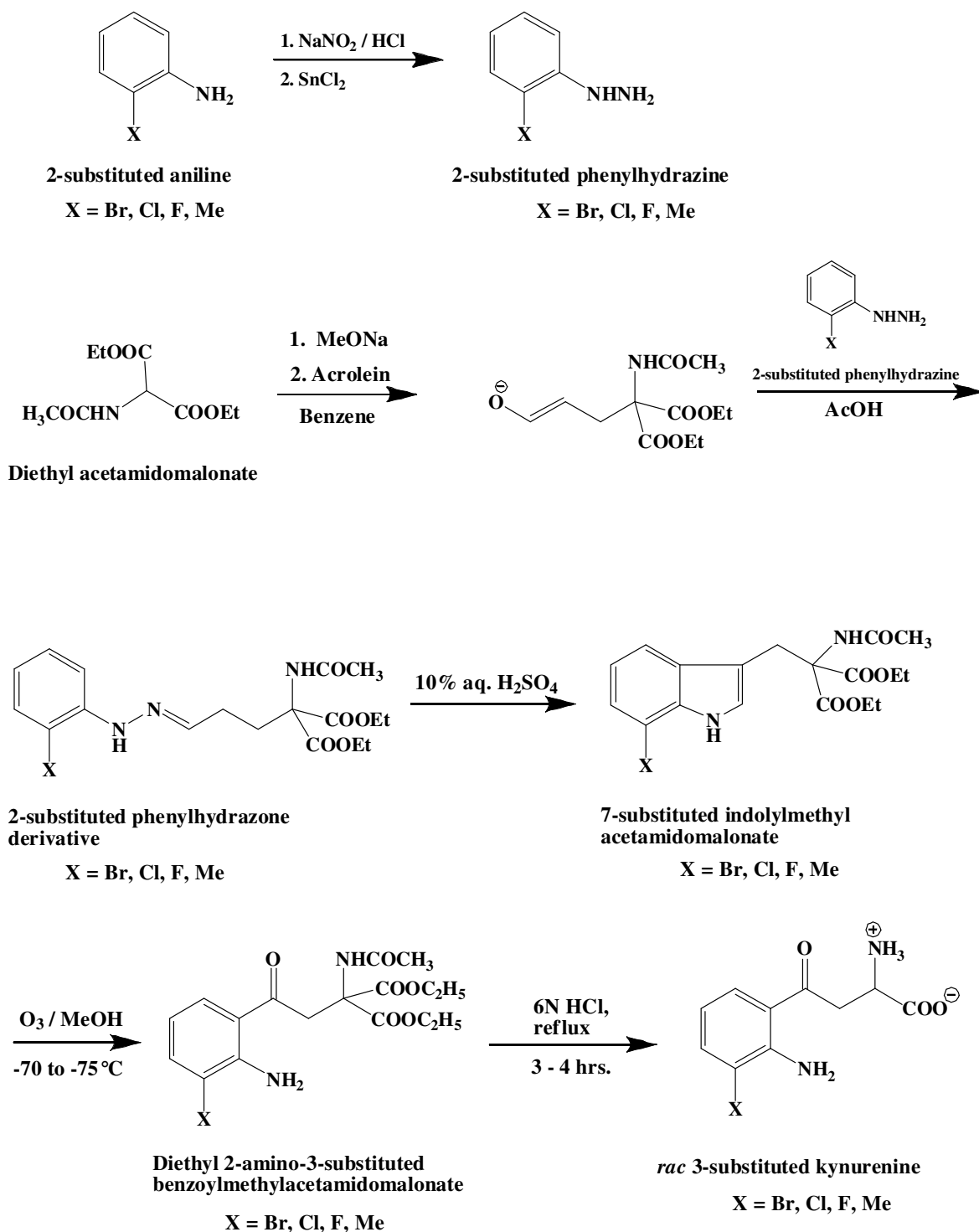
$^1\text{HNMR}$: (1 – 2% DCl – D_2O) δ 7.75 (s, 1H), 7.35 (dd, 1H), 7.25 (d, 1H), 4.31 (t, 1H), 3.58 (d, 2H)

^{13}C NMR: (1 – 2% DCl – D₂O) δ 41.8, 53.6, 118.2, 120.1, 125.3, 130.5, 137.8, 150.1, 170.5, 201.8

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 chloride¹¹ gives the 2-substituted phenylhydrazines^{6,18-20, 22-30}. 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

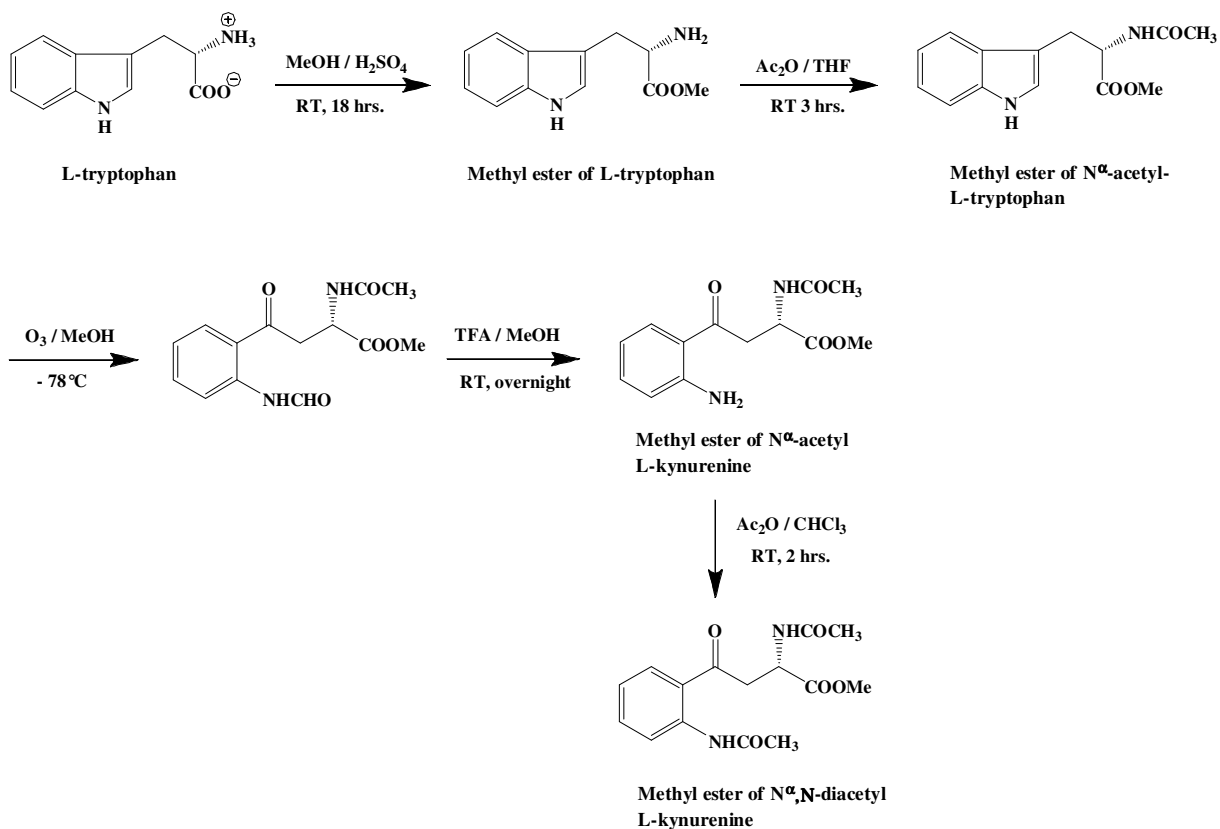
Michael addition reaction³ between acrolein and the diethylacetamidomalonate anion. The intermediate aldehyde of this reaction is not isolated and is used as such for its reaction with the 2-substituted phenylhydrazine to give the corresponding phenylhydrazone derivative. GCMS of the substituted phenylhydrazones 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 indolylmethylacetamidomalonates¹²⁻¹⁶ 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 benzoylmethylacetamidomalonate 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 (λ_{\max} at 262 nm and 365 nm), 3-fluoro (λ_{\max} at 258 nm and 360 nm), and the 3-methyl (λ_{\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 (λ_{\max} at 263 nm and 370 nm) isolated after hydrolysis step was found to be about 85 % pure with about 10% of unsubstituted

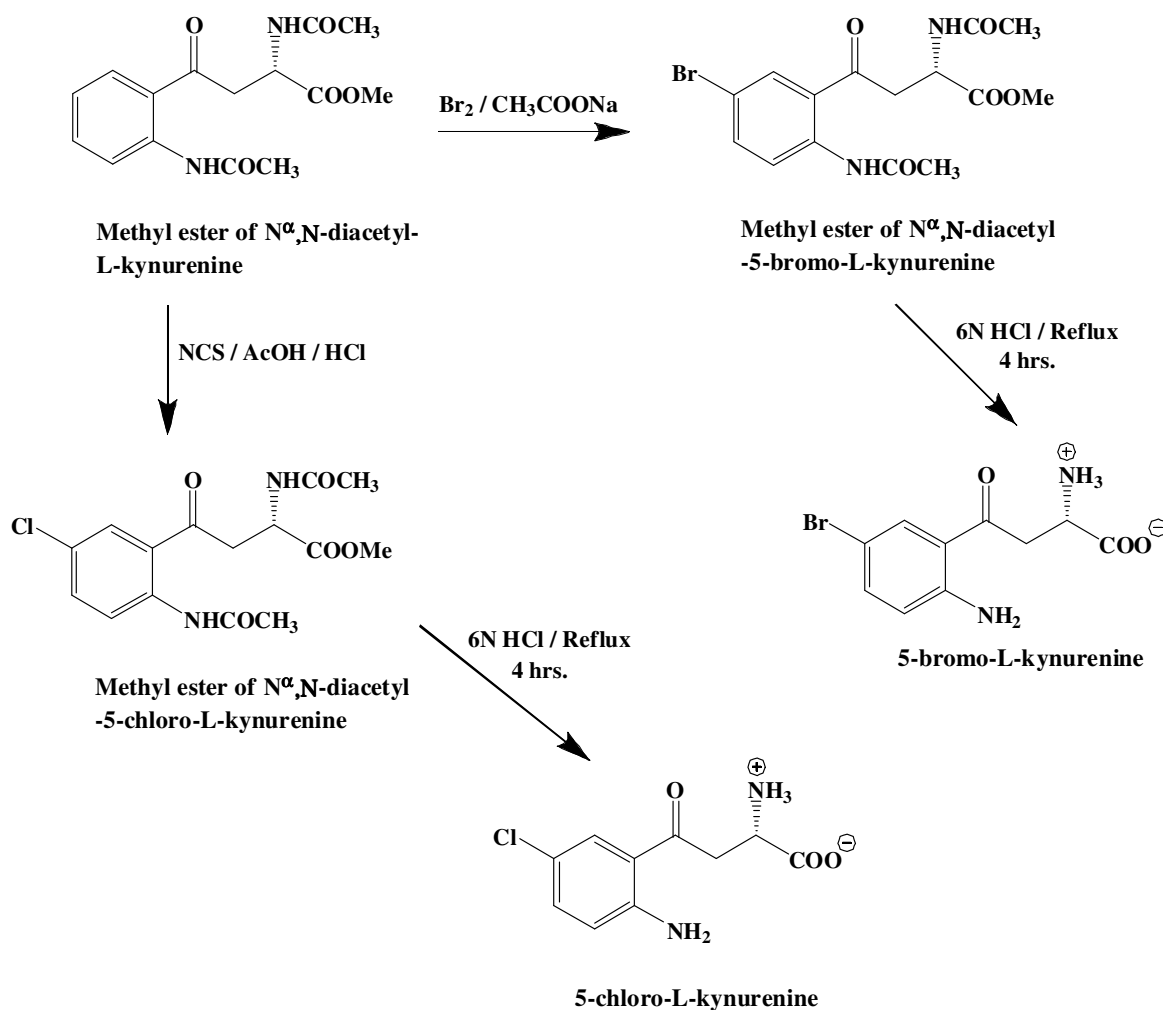


Scheme 14

kynurenine impurity (λ_{\max} at 255 nm and 360 nm), and 5% of possible another substituted kynurenine type impurity (λ_{\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 N^α,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 N^α-acetyl-L-tryptophan which is about 99% pure by GCMS and used as such for the ozonolysis step. Ozonolysis of the methyl ester of N^α-acetyl-L-tryptophan, followed by hydrolysis with trifluoroacetic acid, and then acylation with acetic anhydride, gives the methyl ester of N^α,N-diacetylkynurenine¹⁷ as a white solid with 99% purity by GCMS. Having prepared the methyl ester of N^α,N-diacetylkynurenine, either bromination or chlorination gives the methyl ester of the corresponding 5-halosubstituted-N^α,N-diacetylkynurenine which finally upon acid hydrolysis give the 5-halosubstituted-L-kynurenines. (Scheme 15)

Bromination of the methyl ester of N^α,N-diacetylkynurenine is done with liquid bromine, and gives regioselectively the methyl ester of 5-bromo-N^α,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 °C¹⁷. However, we got a much higher melting point of 225-227 °C 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_{\max} of 265 nm and 370 nm.



Scheme 15

Chlorination of the methyl ester of N^α,N-diacetylkynurenine 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-N^α,N-diacetylkynurenine. 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 °C²¹. However, we got a much higher melting point of 216-218 °C 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 λ_{max} of 260 nm and 370 nm.

References

1. Furniss, B. S.; Hannaford, A.J.; Smith Peter, W. G.; Tatchell, A. R. in *Vogel's Textbook of Practical Organic Chemistry*, 5th ed. Longman Scientific & Technical, England, **1989**, pp 757-759.
2. Moe, O. A.; Warner, D. T. *J. Am. Chem. Soc.* **1948**, *70*, 2763-2765.
3. Warner, D. T.; Moe, O. A. *J. Am. Chem. Soc.* **1948**, *70*, 2765-2767.
4. Warner, D. T.; Moe, O. A. *J. Am. Chem. Soc.* **1948**, *70*, 3470-3472.
5. Stevens, F. J.; Higginbotham, D. H. *J. Am. Chem. Soc.* **1954**, *76*, 2206-2207.
6. Harden, F. A.; Quinn, R. J.; Scammells, P. J. *J. Med. Chem.* **1991**, *34*, 2892-2898.
7. Fugger, J.; Tien, J. M.; Hunsberger, I. M. *J. Am. Chem. Soc.* **1955**, *77*, 1843-8.
8. Tien, J. M.; Hunsberger, I. M. *J. Am. Chem. Soc.* **1955**, *77*, 6696-8.
9. Tien, J. M.; Hunsberger, I. M. *J. Am. Chem. Soc.* **1955**, *77*, 6604-7.
10. Tien, J. M.; Hunsberger, I. M. *Chem. Ind. (London)* **1955**, 119-119.
11. Hunsberger, I. M.; Shaw, E. R.; Fugger, J.; Ketcham, R.; Lednicer, D. *J. Org. Chem.* **1956**, *21*, 394-399.
12. Thiruvikraman, S. V.; Sakagami, Y.; Katayama, M.; Marumo, S. *Tetrahedron Lett.* **1988**, *29*, 2339-2342.
13. Shiba, T.; Mukunoki, Y.; Akiyama, H. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 1902-1906.
14. Vanpee, K. H.; Salcher, O.; Lingens, F. *Liebigs Ann. Chem.* **1981**, 233-239.
15. Rydon, H. N.; Tweddle, J. C. *J. Chem. Soc.* **1955**, 3499-3503.
16. Porter, J.; Dykert, J.; Rivier, J. *Int. J. Pept. Protein Res.* **1987**, *30*, 13-21.
17. Heiss, C.; Anderson, J.; Phillips, R. S. *Org. Biomol. Chem.* **2003**, *1*, 288-295.
18. Wang, Y.; Liu, Q. *Pige Huagong* **2002**, *19*, 23-27.

19. Bandgar, B. P.; Uppalla, L. S. *J. Chem. Res., Synop.* **1999**, 714-715.
20. Nagarajan, K.; Talwalker, P. K.; Kulkarni, C. L.; Venkateswarlu, A.; Prabhu, S. S.; Nayak, G. V. *Indian J. Chem. Sect. B* **1984**, 23, 1243-1257.
21. Varasi, M.; Della Torre, A.; Heidempergher, F.; Pevarello, P.; Speciale, C.; Guidetti, P.; Wells, D. R.; Schwarcz, R. *Eur. J. Med. Chem.* **1996**, 31, 11-21.
22. Pegurier, C.; Collart, P.; Danhaive, P.; Defays, S.; Gillard, M.; Gilson, F.; Kogej, T.; Pasau, P.; Van Houtvin, N.; Van Thuyne, M.; van Keulen, B. *Bioorg. Med. Chem. Lett.* **2007**, 17, 4228-4231.
23. Suschitzky, H. *J. Chem. Soc.* **1953**, 3326-7.
24. Carlin, R. B.; Odioso, R. C. *J. Am. Chem. Soc.* **1954**, 76, 100-104.
25. Bullock, M. W.; Hand, J. J. *J. Am. Chem. Soc.* **1956**, 78, 5852-5854.
26. Bullock, M. W.; Hand, J. J. *J. Am. Chem. Soc.* **1956**, 78, 5854-5857.
27. Lee, An-Rong; Huang, Wen-Hsin; Lin, Tung-Liang; Shih, Kun-Min; Lee, Hsiao-Feng; Lin, Cheng-I. *J. Heterocycl. Chem.* **1995**, 1, 1-12.
28. Bloss; Timberlake *J. Org. Chem.* **1963**, 28, 267-268.
29. Cook; France *J. Am. Chem. Soc.* **1934**, 56, 2225
30. Burkhard.W; Kauffman.T *Angew. Chem. Int. Ed.* **1967**, 6, 84.
31. Bajwa, G. S.; Brown, R. K. *Can. J. Chem.* **1968**, 46, 1827.

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_{cat} and k_{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_{cat} and k_{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 ($\Delta\epsilon = 4500 \text{ M}^{-1} \cdot \text{cm}^{-1}$)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 °C. 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-substituted kynurenines have been shown to be good substrates² 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 μ L 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

Compound	Human enzyme mg	<i>P. fluorescens</i> enzyme mg
3-chloro-DL-kynurenine	3.29×10^{-3}	1.33×10^{-3}
3-fluoro-DL-kynurenine	6.58×10^{-3}	3.33×10^{-4}
3-methyl-DL-kynurenine	6.58×10^{-3}	6.66×10^{-4}
5-bromo-L-kynurenine	1.32×10^{-3}	3.33×10^{-5}
5-chloro-L-kynurenine	2.63×10^{-3}	3.33×10^{-5}

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

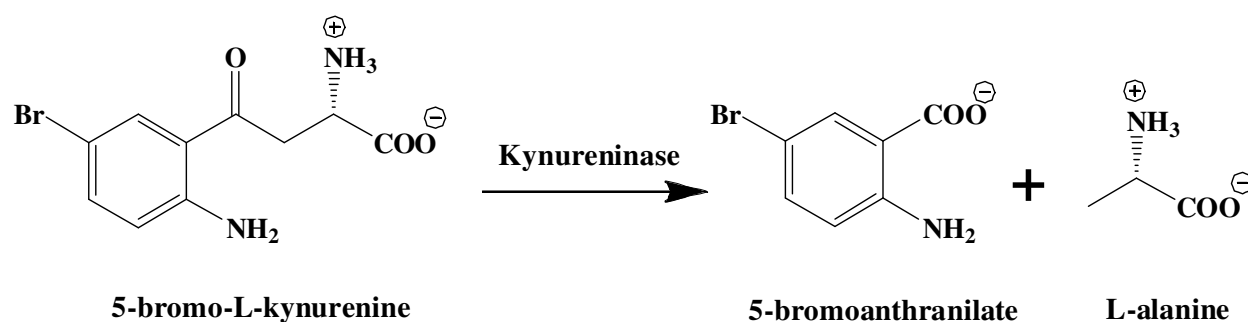
V_{\max} , and V_{\max}/K_m were determined by fitting of the initial rate data (See Appendix 1 for tables of raw data) into the equation 1 using the compiled FORTRAN program HYPER of Cleland³ on a Dell personal computer.

$$v = V_{\max} [S]/(K_m + [S]) \quad (1)$$

The values of V_{\max} , and V_{\max}/K_m were divided by the respective extinction coefficients and by the enzyme concentration to give the k_{cat} and k_{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 λ_{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_{cat} values of 0.67 sec^{-1} and 0.71 sec^{-1} respectively. When compared with the natural substrate (3-hydroxykynurenine⁵) 3-chloro-DL-kynurenine is about 5-fold less efficiently cleaved by the human enzyme but has a 3-fold higher k_{cat} than L-kynurenine for the same enzyme. For the bacterial enzyme it is found that 3-chloro-DL-kynurenine has a k_{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-kynurenine² for the same enzyme. Thus the turnover number with the 3-Cl substituent is much less affected with the

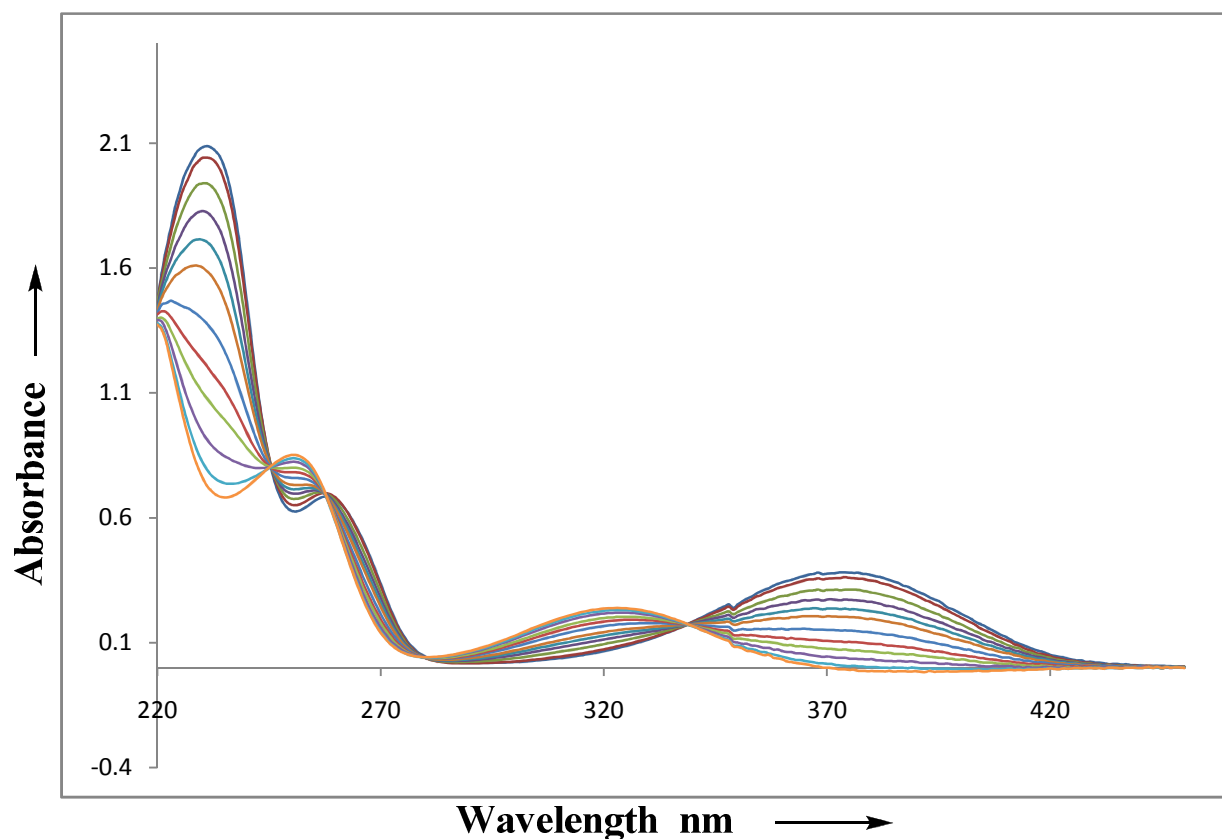


Fig. 10

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 3.2 Substrate analogs for human enzyme

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

enzyme this k_{cat}/K_m value of $(1.63 \pm 0.27) \times 10^4$ is about 15-fold lower than that for the natural substrate 3-hydroxy-L-kynurenine⁵ (2.46×10^5) 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_{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-kynurenine⁴, 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

Table 3.3 Substrate analogs for bacterial enzyme

Compound	K_m μM	V_{max}	k_{cat} sec^{-1}	$k_{\text{cat}} / K_m \text{ M}^{-1} \cdot \text{sec}^{-1}$
3-chloro-DL-kynurenine	70.8 ± 9.2	0.0095 ± 0.00056	0.71 ± 0.042	$(1.05 \pm 0.075) \times 10^4$
3-fluoro-DL-kynurenine	75.5 ± 11.2	0.015 ± 0.0010	6.88 ± 0.46	$(8.99 \pm 0.78) \times 10^4$
3-methyl-DL-kynurenine	69.4 ± 14.8	0.0074 ± 0.00057	1.51 ± 0.12	$(2.24 \pm 0.33) \times 10^4$
5-bromo-L-kynurenine	3.58 ± 1.11	0.0034 ± 0.000198	11.89 ± 0.69	$(3.29 \pm 0.87) \times 10^6$
5-chloro-L-kynurenine	4.90 ± 0.96	0.0027 ± 0.00012	8.73 ± 0.39	$(1.78 \pm 0.29) \times 10^6$
L-kynurenine	25	-	16	6×10^5

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

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_{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_{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-kynurenine⁵. 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_{cat} value of 6.88 sec^{-1} is about half that of the natural substrate L-kynurenine⁴ 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 $k_{\text{cat}}/K_{\text{m}}$ values for 3-fluoro-DL-kynurenine are $(2.66 \pm 0.45) \times 10^3$ and $(8.99 \pm 0.78) \times 10^4$ respectively for the human and bacterial enzyme. Assuming that the D enantiomer does not affect the enzyme activity the value of $k_{\text{cat}}/K_{\text{m}}$ for 3-fluoro-L-kynurenine would be $(5.32 \pm 0.9) \times 10^3$ and $(1.80 \pm 0.16) \times 10^5$ for the human and the bacterial enzyme respectively. Apparently, the $k_{\text{cat}}/K_{\text{m}}$ value for this substrate is about 34-fold higher with the bacterial enzyme than with the human enzyme. For the human enzyme this $k_{\text{cat}}/K_{\text{m}}$ value of $(5.32 \pm 0.9) \times 10^3$ is about 46-fold lower than for the natural substrate 3-hydroxy-L-kynurenine⁵ (2.46×10^5) 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 studies⁵. 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 than that of the natural substrate L-kynurenine⁴ (6×10^5) 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-kynurenine². 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 specificity⁵.

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-kynurenine⁵. 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-hydroxy-L-kynurenine⁵. 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-kynurenine⁴ and about 8-fold lower than that of 5-bromo-L- & 3-bromo-L-kynurenine², 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 than that for the natural substrate 3-hydroxy-L-kynurenine⁵ (2.46×10^5) 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-kynurenine⁴ (6×10^5) 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-kynurenine². 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 previously². 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-hydroxykynurenine⁵. 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_{cat} value of 11.9 sec^{-1} is about the same as that of 3-bromo-L-kynurenine², 5-chloro-L-kynurenine and the natural substrate L-kynurenine⁴ 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 be 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 $k_{\text{cat}}/K_{\text{m}}$ 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 $k_{\text{cat}}/K_{\text{m}}$ value than the value of 1.8×10^5 reported earlier² which could again be due to the greater purity of our substrate and the enzyme sample. For the human enzyme this $k_{\text{cat}}/K_{\text{m}}$ value of $(1.51 \pm 0.11) \times 10^4$ is about 16-fold lower than for the natural substrate 3-hydroxy-L-kynurenine⁵ (2.46×10^5) 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 $k_{\text{cat}}/K_{\text{m}}$ value of $(3.29 \pm 0.87) \times 10^6$ is about 6-fold higher than that of the natural substrate L-kynurenine⁴ (6×10^5), 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_{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-kynurenine⁵. 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-kynurenine⁴ (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 $k_{\text{cat}}/K_{\text{m}}$ 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 $k_{\text{cat}}/K_{\text{m}}$ value of $(1.10 \pm 0.12) \times 10^4$ is about 22-fold lower than that for the natural substrate 3-hydroxy-L-kynurenine⁵ (2.46×10^5) but about 24-fold higher than L-kynurenine for the same enzyme.⁵ 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 $k_{\text{cat}}/K_{\text{m}}$ value of $(1.78 \pm 0.29) \times 10^6$ is about 3-fold higher than that of the natural substrate L-kynurenine⁴ (6×10^5), 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 structures⁵. 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

1. Kishore, G. M. *J. Biol. Chem.* **1984**, *259*, 10669-74.
2. Heiss, C.; Anderson, J.; Phillips, R. S. *Org. Biomol. Chem.* **2003**, *1*, 288-295.
3. Cleland, W. W. *Meth. Enzymol.* **1979**, *63*, 103-38.
4. Gawandi, V. B.; Liskey, D.; Lima, S.; Phillips, R. S. *Biochemistry* **2004**, *43*, 3230-3237.
5. Lima, S.; Kumar, S.; Gawandi, V.; Momany, C.; Phillips, R. S. *J. Med. Chem.* **2009**, *52*, 389-396.

CHAPTER 4

SYNTHESIS AND STABILITY STUDIES OF CAGED KYNURENINE

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-hydroxyanthranilic acid and L-alanine. Earlier, the mechanism for the hydrolytic cleavage of kynurenine has been proposed¹. 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

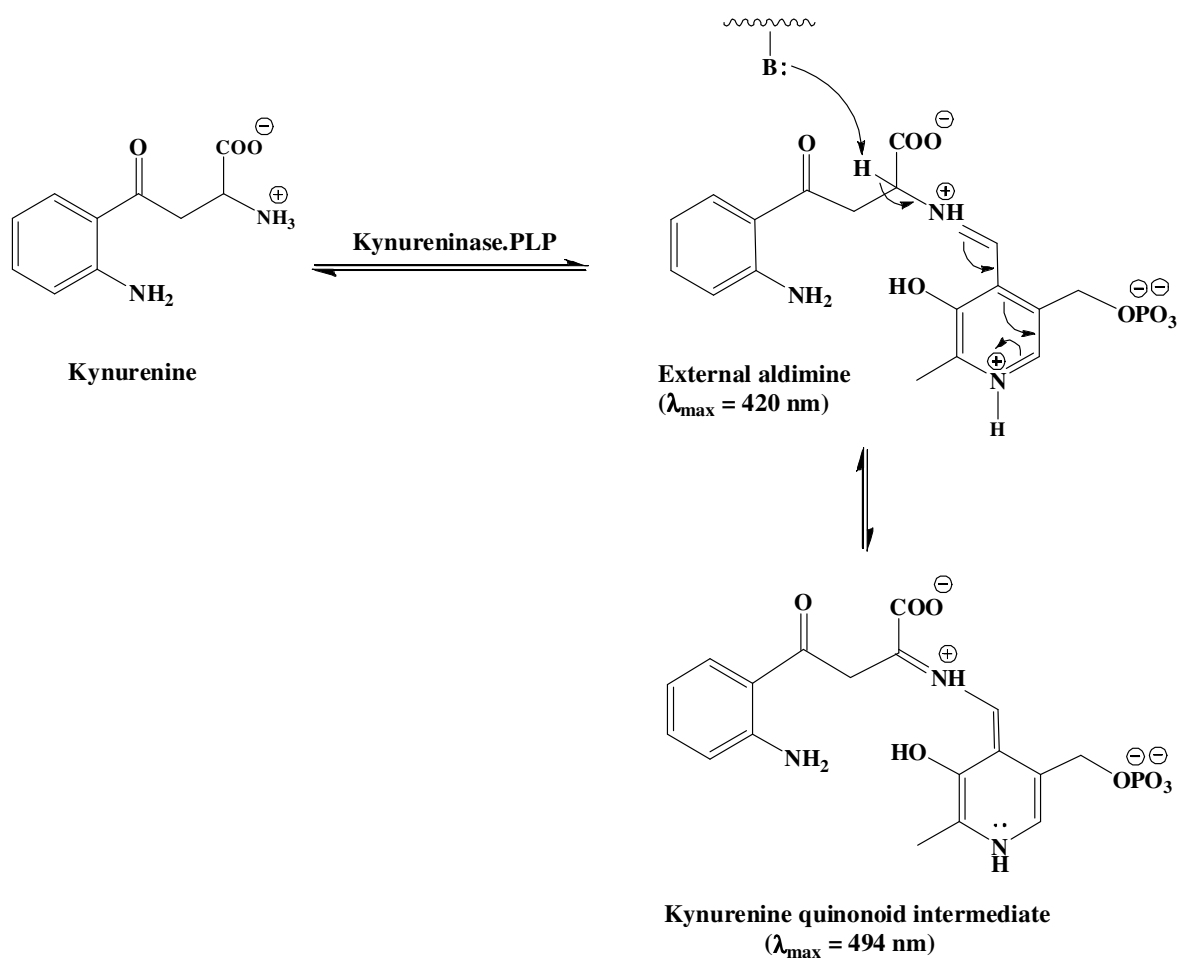
Caged biomolecules have been known for about three decades now^{2,3} and have become increasingly important by virtue of the light initiated release of the biologically active molecule. In the broader sense, caged compounds are protected photo-labile bioactive substrates that release the desired substrate 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 attributes⁴. 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 photoproduct 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 photoproduct 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-nitrobenzyl⁵⁻¹⁰ and 7-nitroindoline¹¹ derivatives, coumarin-4-ylmethyl¹²⁻¹⁹ phototriggers, and the p-hydroxyphenacyl group²⁰⁻²⁴. 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 acids⁴ 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 Dua¹ 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

¹HNMR and ¹³CNMR 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 25°C. After completion of addition, stir the reaction mass between 20 -25°C, for 4 hrs. Check TLC. (Fig. 10) Concentrate the methanol under vacuum below 25 °C. 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. NaHCO₃ 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

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

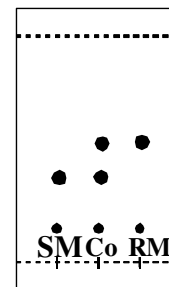
^{13}C NMR: *d*-MeOH δ 35.3, 111.2, 126.3, 131.2, 159.3, 195.6

Synthesis of 4-hydroxyphenacyl ester of N^{α} -Boc-L-tryptophan

Add 5 g of *p*-hydroxyphenacyl bromide to a solution containing 3.5 gm of K_2CO_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.

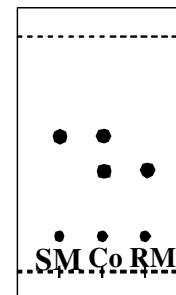
Yield = 10 g, 98 %, m.p = 195 – 198 °C

^1H NMR: 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)



System: Toluene : EtOAc
8 : 2
Detection: uv 254 nm
SM = starting material
Co = mixture spot

Fig. 10



System: CHCl_3 : MeOH
9 : 1
Detection: uv 254 nm
SM = Starting material
Co = Mixture spot

Fig. 11

^{13}C NMR: CDCl_3 δ 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 $^{\circ}\text{C}$. 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

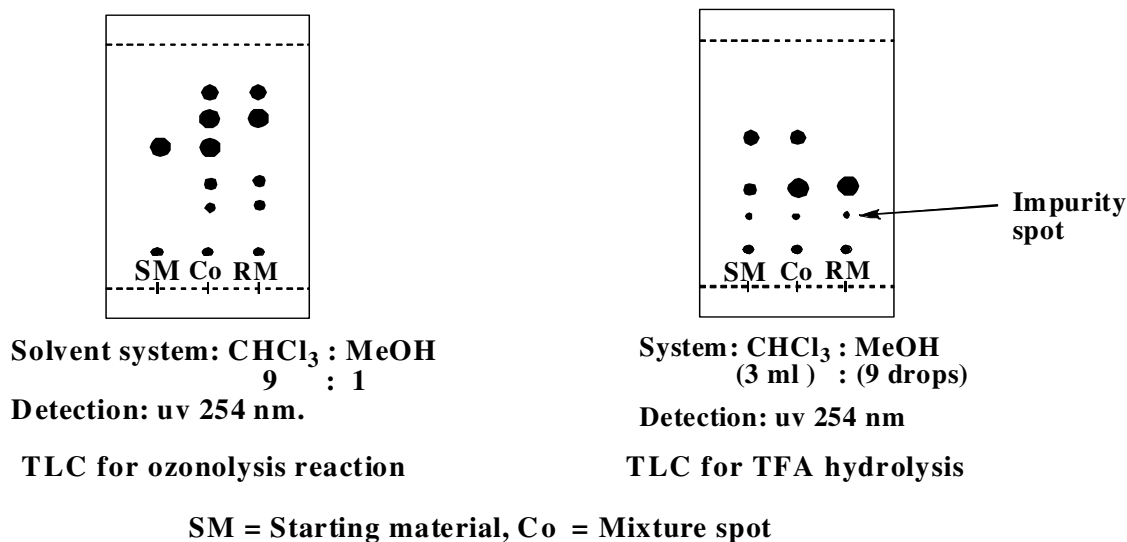


Fig. 12

in 20 ml d/w). Concentrate the RM under vacuum, and remove methanol below 30 $^{\circ}\text{C}$. 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 $^{\circ}\text{C}$. 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 %

$^1\text{H NMR}$: *d*-MeOH δ 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),

$^{13}\text{C NMR}$: *d*-MeOH δ 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

$[\alpha]_{\text{D}} = -14.2^\circ$ ($c = 1.3$ in water)

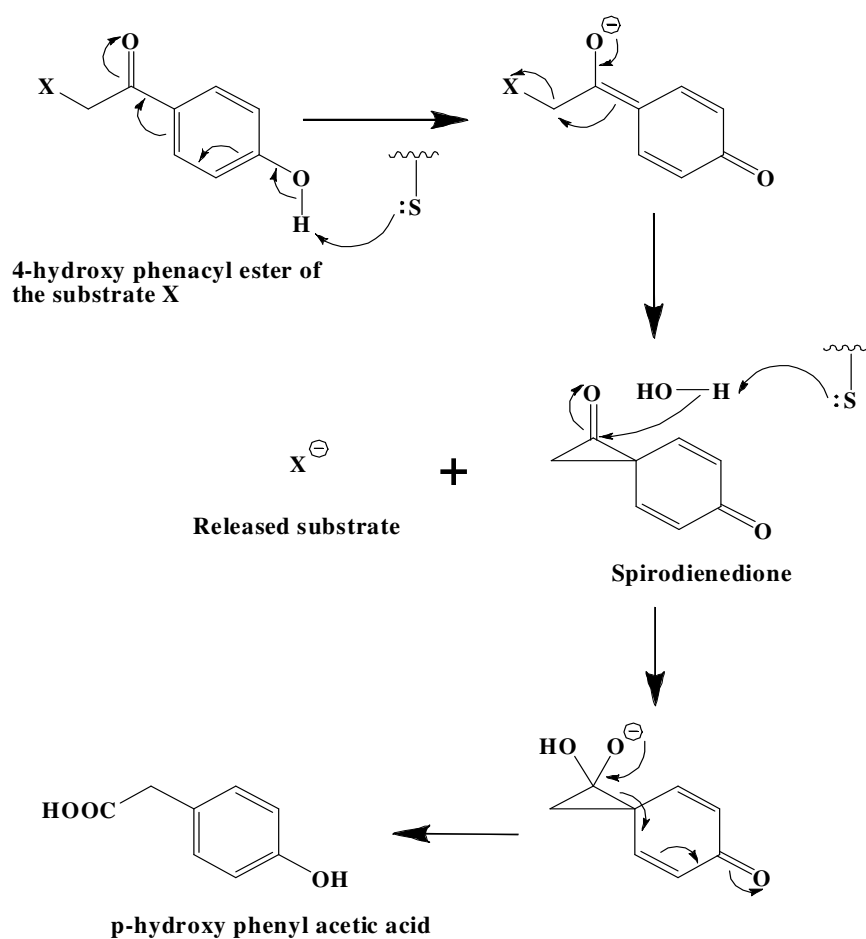
HRMS analysis MW = 342.1294, Calculated MW = 342.1216

Results and Discussion

In order to synthesize the caged compound, 2-bromo-4'-hydroxyacetophenone is first synthesized from 4-hydroxyacetophenone (Scheme 17). The published procedures²⁵⁻²⁸ 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 CuBr₂ in a refluxing mixture of CHCl₃/EtOAc⁴ 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 SN² reaction between the conjugate base of N^α-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^α-Boc tryptophan. This ester upon ozonolysis followed by TFA hydrolysis²⁹ 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 crossing⁴ ($k_{ST} = 2.7 \times 10^{11} \text{ s}^{-1}$) 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 λ_{max} values at 263, 283 and 365 nm.



Scheme 18

Stability studies for caged kynurenine

The caged compound is stable as a solid hydrochloride salt for a long period at $-78\text{ }^\circ\text{C}$ 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 $20\text{ }^\circ\text{C}$ 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., λ_{\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 100 μ M 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 17.1mins. 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 MgCl₂ 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 200 μ M solution of the caged compound with one hundred 1ns 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

1. Dua, R. K.; Taylor, E. W.; Phillips, R. S. *J. Am. Chem. Soc.* **1993**, *115*, 1264-1270.
2. Kaplan, J. H.; Forbush, B.; Hoffman, J. F. *Biochemistry* **1978**, *17*, 1929-1935.
3. Engels, J.; Schlaeger, E. J. *J. Med. Chem.* **1977**, *20*, 907-911.
4. Givens, R. S.; Yousef, A. L. in *Dynamic Studies in Biology* Goeldner, Maurice; Givens, R. S. Ed., Wiley-VCH, **2005**.
5. Lester, H. A.; Nerbonne, J. M. *Annu. Rev. Biophys. Bioeng.* **1982**, *11*, 151-175.
6. Rothman, D. M.; Vazquez, E. M.; Vogel, E. M.; Imperiali, B. *Org. Lett.* **2002**, *4*, 2865-2868.
7. Dinkel, C.; Wichmann, O.; Schultz, C. *Tetrahedron Lett.* **2003**, *44*, 1153-1155.
8. Shigeri, Y.; Tatsu, Y.; Yumoto, N. *Pharmacol. Therapeutics* **2001**, *91*, 85-92.
9. Marriott, G.; Roy, P.; Jacobson, K. In *Biophotonics, Pt A* 2003; Vol. 360, p 274-288.
10. Brubaker, M. J.; Dyer, D. H.; Stoddard, B.; Koshland, D. E. *Biochemistry* **1996**, *35*, 2854-2864.
11. Canepari, M.; Nelson, L.; Papageorgiou, G.; Corrie, J. E. T.; Ogden, D. J. *Neurosci. Methods* **2001**, *112*, 29-42.
12. Furuta, T.; Torigai, H.; Osawa, T.; Iwamura, M. *Chem. Lett.* **1993**, 1179-1182.
13. Takaoka, K.; Tatsu, Y.; Yumoto, N.; Nakajima, T.; Shimamoto, K. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 965-970.
14. Schoenleber, R. O.; Giese, B. *Synlett* **2003**, 501-504.
15. Suzuki, A. Z.; Watanabe, T.; Kawamoto, M.; Nishiyama, K.; Yamashita, H.; Ishii, M.; Iwamura, M.; Furuta, T. *Org. Lett.* **2003**, *5*, 4867-4870.

16. Kaupp, U. B.; Solzin, J.; Hildebrand, E.; Brown, J. E.; Helbig, A.; Hagen, V.; Beyermann, M.; Pampaloni, F.; Weyand, I. *Nat. Cell Biol.* **2003**, *5*, 109-117.
17. Furuta, T.; Takeuchi, H.; Isozaki, M.; Takahashi, Y.; Kanehara, M.; Sugimoto, M.; Watanabe, T.; Noguchi, K.; Dore, T. M.; Kurahashi, T.; Iwamura, M.; Tsien, R. Y. *Chembiochem* **2004**, *5*, 1119-1128.
18. Nishigaki, T.; Wood, C. D.; Tatsu, Y.; Yumoto, N.; Furuta, T.; Elias, D.; Shiba, K.; Baba, S. A.; Darszon, A. *Dev. Biol.* **2004**, *272*, 376-388.
19. Hagen, V.; Frings, S.; Wiesner, B.; Helm, S.; Kaupp, U. B.; Bendig, J. *Chembiochem* **2003**, *4*, 434-442.
20. Givens, R. S.; Park, C. H. *Tetrahedron Lett.* **1996**, *37*, 6259-6262.
21. Givens, R. S.; Weber, J. F. W.; Conrad, P. G.; Orosz, G.; Donahue, S. L.; Thayer, S. A. *J. Am. Chem. Soc.* **2000**, *122*, 2687-2697.
22. Evanko, D. S.; Sul, J.-Y.; Zhang, Q.; Haydon, P. G. *Glial & Neuronal Signaling* **2004**, 397-416.
23. Specht, A.; Ludwig, S.; Peng, L.; Goeldner, M. *Tetrahedron Lett.* **2002**, *43*, 8947-8950.
24. Geibel, S.; Barth, A.; Amslinger, S.; Jung, A. H.; Burzik, C.; Clarke, R. J.; Givens, R. S.; Fendler, K. *Biophys. J.* **2000**, *79*, 1346-1357.
25. Gupta, R.; Gupta, M.; Paul, S.; Gupta, R.; Loupy, A. *Lett. Org. Chem.* **2008**, *5*, 153-157.
26. Shang, G.; Liu, D.; Allen, S. E.; Yang, Q.; Zhang, X. *Chem. Eur. J.* **2007**, *13*, 7780-7784.

27. Shen, Y.; Sheng, R.; Zhang, J.; He, Q.; Yang, B.; Hu, Y. *Bioorg. Med. Chem.* **2008**, *16*, 7646-7653.
28. Ueda, S.; Fujita, M.; Tamamura, H.; Fujii, N.; Otaka, A. *Chembiochem* **2005**, *6*, 1983-1986.
29. Heiss, C.; Anderson, J.; Phillips, R. S. *Org. Biomol. Chem.* **2003**, *1*, 288-295.
30. Conrad, P. G.; Givens, R. S.; Hellrung, B.; Rajesh, C. S.; Ramseier, M.; Wirz, J. *J. Am. Chem. Soc.* **2000**, *122*, 9346-9347.
31. Givens, R. S.; Lee, J.-I. *J. Photosci.* **2003**, *10*, 37-48.

CHAPTER 5

SUMMARY AND CONCLUSIONS

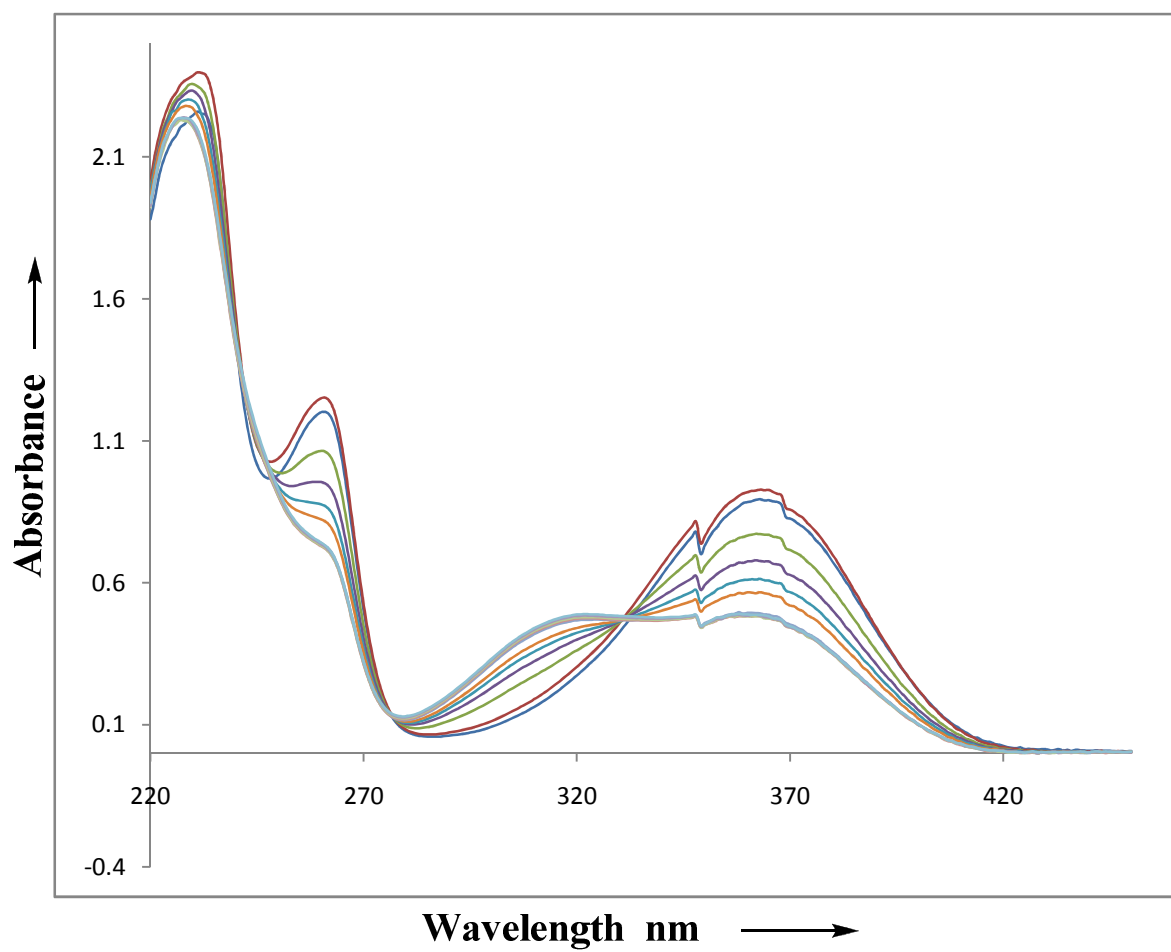
In conclusion, we have prepared some novel substrates and determined their activity for human as well as *Pseudomonas fluorescens* kynureninase. We synthesized the 3-chloro-DL-kynurenine, 3-fluoro-DL-kynurenine, 3-methyl-DL-kynurenine, 5-bromo-L-kynurenine, and 5-chloro-L-kynurenine and compared the 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).

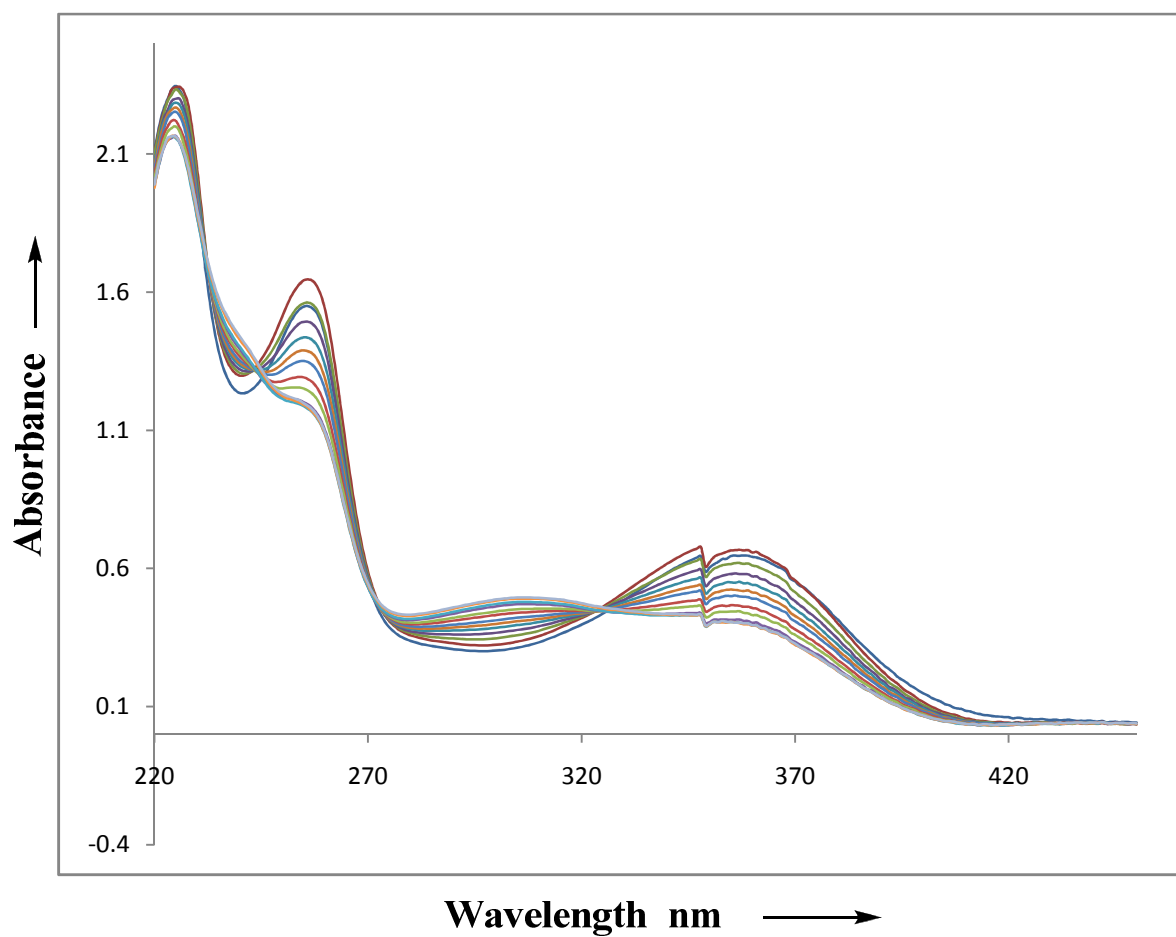
For the human enzyme a comparison of the catalytic efficiency for each of these substrates with the natural substrate 3-hydroxykynurenine, shows that the catalytic efficiency is much lower than that for the natural substrate and it decreases in the order: 3-OH > 3-Cl = 5-Br > 5-Cl > 3-F > 3-Me. Thus, 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).

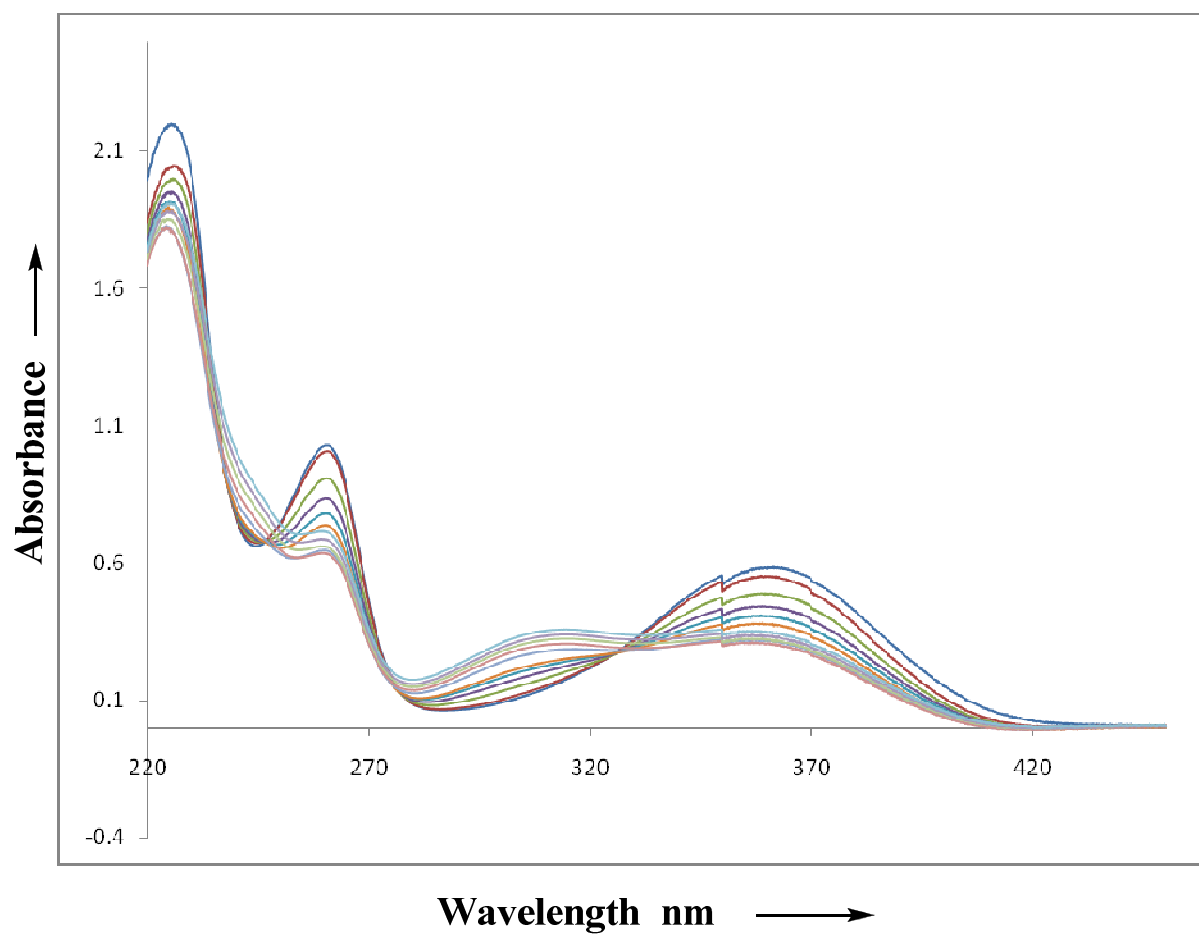
Also, to conclude we have synthesized a novel caged kynurenine which in future will be used for the generation of kynurenine ‘in situ’ by a flash photolysis thereby allowing the

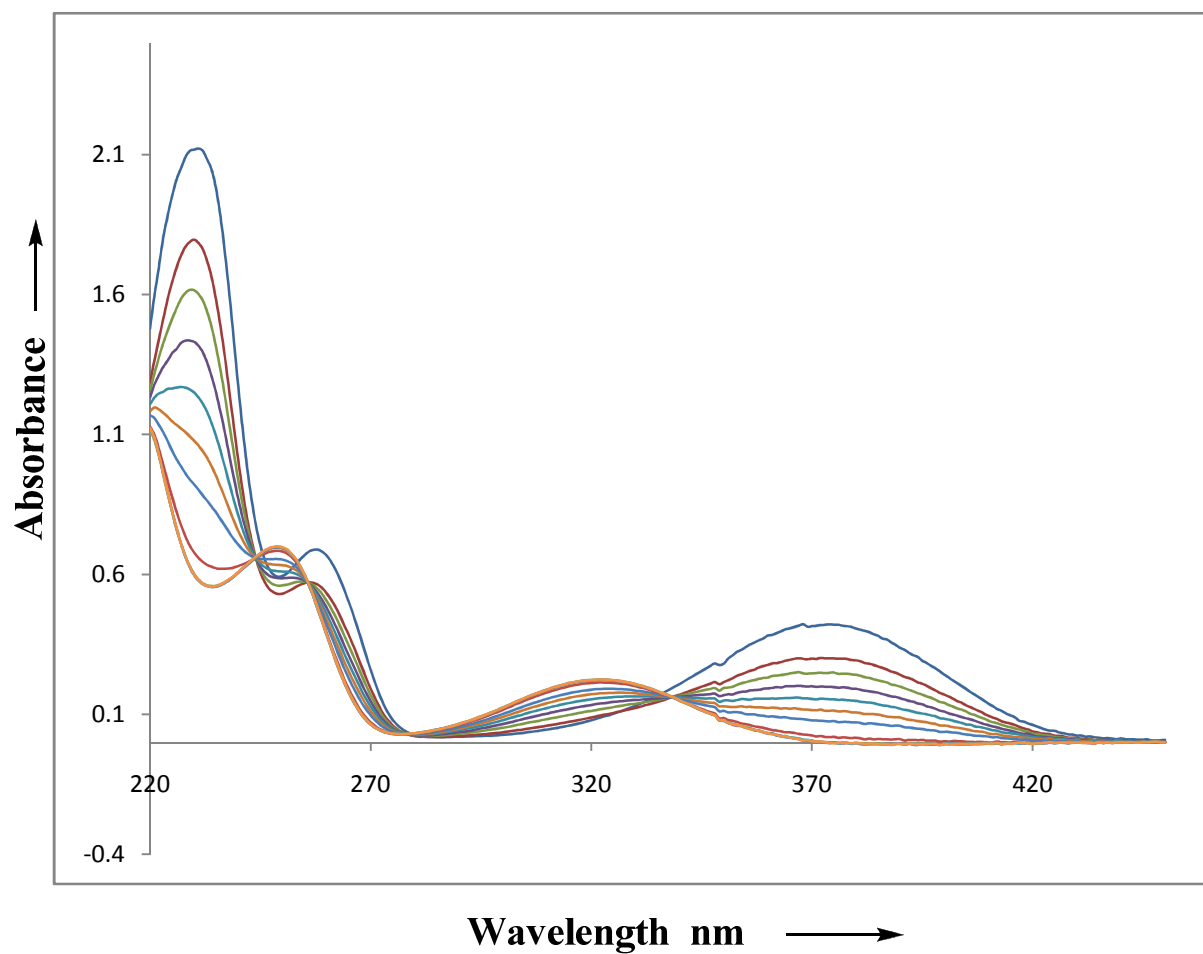
detection of formation and decay of the external aldimine intermediate in the reaction of kynureninase. This detection will further prove the mechanism of action followed by the enzyme kynureninase.

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

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 3-Cl-DL-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 3-Cl-DL-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	4	50	516	20	600	-0.0026	-0.0027
2	12	18	8	50	512	40	600	-0.0048	-0.0054
3	12	18	16	50	504	80	600	-0.0100	-0.0092
4	12	18	32	50	488	160	600	-0.0117	-0.0139
5	12	18	64	50	456	320	600	-0.0123	-0.0146

Notes:

1. Stock solution of human enzyme used was about 7.9 mg/ml by assay. 5 μL of this solution was diluted to 600 μL 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

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 3-F-DL-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 3-F-DL-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	4	100	466	20	600	-0.0012	-0.0012
2	12	18	8	100	462	40	600	-0.0026	-0.0019
3	12	18	16	100	454	80	600	-0.0045	-0.0037
4	12	18	32	100	438	160	600	-0.0062	-0.0054
5	12	18	64	100	406	320	600	-0.0062	-0.0057

Notes:

1. Stock solution of human enzyme used was about 7.9 mg/ml by assay. 5 μL of this solution was diluted to 600 μL 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

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 3-Me-DL-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 3-Me-DL-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	4	100	466	20	600	-0.0014	-0.0015
2	12	18	8	100	462	40	600	-0.0024	-0.0019
3	12	18	16	100	454	80	600	-0.0044	-0.0039
4	12	18	32	100	438	160	600	-0.0064	-0.0056
5	12	18	64	100	406	320	600	-0.0079	-0.0087

Notes:

1. Stock solution of human enzyme used was about 7.9 mg/ml by assay. 5 μL of this solution was diluted to 600 μL using ionized water and used the above mentioned volumes of this enzyme solution for the assay.
2. Wavelength used: 362nm, Cycle repeated every 1.5 mins. for 90 mins., Block temp: 37°C

Rate kinetics data for 5-bromo-L-kynurenine with human enzyme

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 5-Br-L-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 5-Br-L-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	2	20	548	10	600	-0.0013	-0.0013
2	12	18	4	20	546	20	600	-0.0019	-0.0018
3	12	18	8	20	542	40	600	-0.0028	-0.0026
4	12	18	16	20	534	80	600	-0.0039	-0.0038
5	12	18	32	20	518	160	600	-0.0041	-0.0046
6	12	18	64	20	486	320	600	-0.0050	-0.0055

Notes:

1. Stock solution of human enzyme used was about 7.9 mg/ml by assay. 5 μL of this solution was diluted to 600 μL 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: 37°C

Rate kinetics data for 5-chloro-L-kynurenine with human enzyme

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 5-Cl-L-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 5-Cl-L-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	2	40	528	10	600	-0.0018	-0.0014
2	12	18	4	40	526	20	600	-0.0031	-0.0028
3	12	18	8	40	522	40	600	-0.0048	-0.0043
4	12	18	16	40	514	80	600	-0.0064	-0.0056
5	12	18	32	40	498	160	600	-0.0083	-0.0068
6	12	18	64	40	466	320	600	-0.0086	-0.0076

Notes:

1. Stock solution of human enzyme used was about 7.9 mg/ml by assay. 5 μL of this solution was diluted to 600 μL 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: 37°C

Rate kinetics data for 3-chloro-DL-kynurenine with bacterial enzyme

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 3-Cl-DL-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 3-Cl-DL-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	4	20	546	20	600	-0.0023	-0.0023
2	12	18	8	20	542	40	600	-0.0031	-0.0035
3	12	18	16	20	534	80	600	-0.0049	-0.0051
4	12	18	32	20	518	160	600	-0.0069	-0.0064
5	12	18	64	20	486	320	600	-0.0060	-0.0053

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 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

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 3-F-DL-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 3-F-DL-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	4	50	518	10	600	-0.0015	-0.0016
2	12	18	8	50	516	20	600	-0.0030	-0.0028
3	12	18	16	50	512	40	600	-0.0047	-0.0053
4	12	18	32	50	504	80	600	-0.0082	-0.0081
5	12	18	64	50	488	160	600	-0.0103	-0.0093

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 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

Sr. No.	2mM PLP (μ L)	1M pH8 KP_i (μ L)	3mM 3-Me-DL-KYNU (μ L)	Enzyme Solution (μ L)	Ionized water (μ L)	Final concn. of 3-Me-DL-KYNU (μ M)	Final Vol. of assay soln. (μ L)	Rate set 1	Rate set 2
1	12	18	4	10	556	20	600	-0.0016	-0.0015
2	12	18	8	10	552	40	600	-0.0025	-0.0027
3	12	18	16	10	544	80	600	-0.0041	-0.0042
4	12	18	32	10	528	160	600	-0.0059	-0.0046
5	12	18	64	10	496	320	600	-0.0066	-0.0054

Notes:

1. Stock solution of bacterial enzyme used was about 19 mg/ml by assay. 2.1 μ L of this solution was diluted to 600 μ L 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: 37°C

Rate kinetics data for 5-bromo-L-kynurenine with bacterial enzyme

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 5-Br-L-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 5-Br-L-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	1	50	519	5	600	-0.0022	-0.0017
2	12	18	2	50	518	10	600	-0.0024	-0.0025
3	12	18	4	50	516	20	600	-0.0026	-0.0035
4	12	18	8	50	512	40	600	-0.0030	-0.0028
5	12	18	16	50	504	80	600	-0.0031	-0.0035

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

Rate kinetics data for 5-chloro-L-kynurenine with bacterial enzyme

Sr. No.	2mM PLP (μL)	1M pH8 KPi (μL)	3mM 5-Cl-L-KYNU (μL)	Enzyme Solution (μL)	Ionized water (μL)	Final concn. of 5-Cl-L-KYNU (μM)	Final Vol. of assay soln. (μL)	Rate set 1	Rate set 2
1	12	18	1	50	519	5	600	-0.0013	-0.0014
2	12	18	2	50	518	10	600	-0.0016	-0.0020
3	12	18	4	50	516	20	600	-0.0021	-0.0021
4	12	18	8	50	512	40	600	-0.0026	-0.0026
5	12	18	16	50	504	80	600	-0.0023	-0.0025

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.