Synthesis and Biological Evaluation of Indoleamine 2, 3-Dioxygenase Inhibitors and Induction, Inhibition Studies of Tryptophanase

By

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(Under the Direction of Robert S. Phillips)

ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is responsible for degradation of L-tryptophan. The metabolites from this pathway lead to certain neurological disorders like, AIDS dementia complex, etc. Thus, IDO inhibitors have significant therapeutic value and the development of potent IDO inhibitors is a major goal of my research.

Potent competitive inhibitors of IDO were reported and were distinguished by replacement of the N-H function of the indole ring of the tryptophan. A series of N-Alkylated L-and D-tryptophan derivatives were synthesized using alky halides as alkylating agents. These compounds are being tested later *in vivo* on IDO.

Tryptophanase is a pyridoxal 5'-phosphate dependent tetrameric enzyme. Tryptophanase is responsible for Tryptophan degradation into Indole, Pyruvate, and Ammonia. We examined the induction of Tryptophanase in *E. coli* with N-alkyl derivatives of tryptophan as inducers. We have performed the inhibitory studies on pure Tryptophanase enzyme. Allyltryptophan showed maximum inhibition.

INDEX WORDS: IDO, Tryptophan derivatives, Kynurenine pathway, Tryptophanase, Tryptophan synthase, Alkylation, *E.Coli*.

SYNTHESIS AND BIOLOGICAL EVALUATION OF INDOLEAMINE 2,3-DIOXYGENASE INHIBITORS AND INDUCTION, INHIBITION STUDIES OF TRYPTOPHANASE

By

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DEDICATION

I dedicate this thesis to my brother, Jagadeesh Borra, and my parents,

B.V. Rao and B.S. Veni

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CHAPTER 1 INTRODUCTION & LITERATURE

Enzymes and Life Processes

The living cell is the site of tremendous biochemical activity called metabolism. This is the process of chemical and physical changes that goes on continually in the living organism. Build-up of new tissue, replacement of old tissue, conversion of food to energy, disposal of waste materials, and reproduction are all the activities that we characterize as "life."

This building up and tearing down takes place in the face of an apparent paradox. The majority of these biochemical reactions do not take place spontaneously. The phenomenon of catalysis makes possible biochemical reactions necessary for all life processes. Catalysis is defined as the acceleration of a chemical reaction by some substance which itself undergoes no permanent chemical change. The catalysts of biochemical reactions are enzymes and are responsible for bringing about almost all of the chemical reactions in living organisms.[1] Without enzymes, these reactions take place at a rate far too slow for the pace of metabolism.

The oxidation of a fatty acid to carbon dioxide and water is not a gentle process in a test tube extremes of pH, high temperatures and corrosive chemicals are required. Yet, in a cell, such reactions take place smoothly and rapidly within a narrow range of pH and temperature. In the laboratory, the average protein must be boiled for about 24 hours in a 20% HCl solution to achieve a complete breakdown. In the body, the breakdown takes place in four hours or less under conditions of mild physiological temperature and pH.

It is through attempts at understanding more about enzyme catalysts - what they are, what they do, and how they do it, and how to control their activity, that many advances in medicine and the life sciences have been brought about.

Early Enzyme Discoveries

The existence of enzymes has been known for well over a century. Some of the earliest studies were performed in 1835 by the Swedish chemist Jon Jakob Berzelius who termed their chemical action "catalytic". It was not until 1926, however, that the first enzyme was obtained in pure form, a feat accomplished by James B. Sumner at Cornell University[2]. Sumner was able to isolate and crystallize the enzyme urease from the jackbean. His research earned him the 1947 Nobel Prize.

Chemical Nature of enzymes

All known enzymes are proteins. They are high molecular weight compounds made up principally of chains of amino acids linked together by peptide bonds. (See Figure # 1.1) Enzymes can be denatured and precipitated with salts,[3] organic solvents and other reagents. They have molecular weights ranging from 10,000 to 2,000,000. Many enzymes require the presence of other compounds - cofactors - before their catalytic activity can be exerted. This entire active complex is referred to as the holoenzyme; i.e., apoenzyme (protein portion) plus the cofactor (coenzyme, prosthetic group or metal-ion-activator) is called the holoenzyme. Thus, Apoenzyme + Cofactor = Holoenzyme.

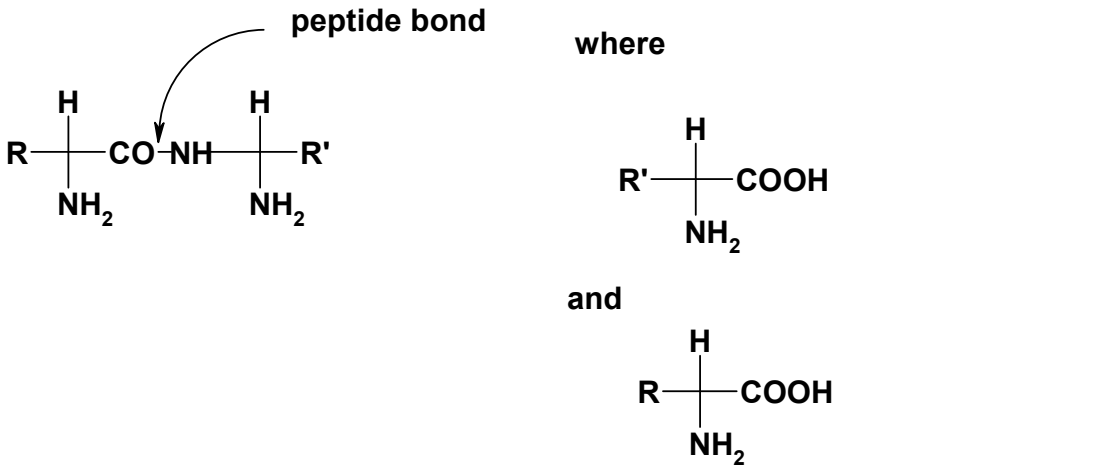


Fig. 1.1. Typical protein structure, two or more amino acids joined through peptide bonds

Specificity of Enzymes

One of the properties of enzymes that makes them so important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular reaction.[4] Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificity:

1) Absolute specificity - the enzyme will catalyze only one reaction.
2) Group specificity - the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
3) Linkage specificity - the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
4) Stereochemical specificity - the enzyme will act on a particular stereoisomer or optical isomer.

Except for some of the early studied enzymes such as pepsin, rennin, and trypsin, most enzyme names end in "ase". The International Union of Biochemistry (I.U.B.) initiated standards of enzyme nomenclature that recommend enzyme names indicate both the substrate acted upon and the type of reaction catalyzed. Under this system, the enzyme uricase is called urate: O_2 oxidoreductase, while the enzyme glutamic oxaloacetic transaminase (GOT) is called L-aspartate: 2-oxoglutarate aminotransferase.

Enzymes allow many chemical reactions to occur within the homeostasis constraints of a living system. Enzymes function as organic catalysts. All enzymes function by lowering the

activation energy of reactions. By bringing the reactants closer together, chemical bonds may be weakened and reactions will proceed faster than without the catalyst.

Mechanism of enzyme action

The basic mechanism by which enzymes catalyze chemical reactions begins with the binding of the substrate (or substrates) to the active site on the enzyme. The active site is the region of the enzyme that combines with the substrate. The binding of the substrate to the enzyme causes changes in the distribution of electrons in the chemical bonds of the substrate and ultimately causes the reactions that lead to the formation of products.[5a] The products are released from the enzyme's active site to regenerate the enzyme for another reaction cycle.

The active site has a unique geometric shape that is complementary to the geometric shape of a substrate molecule, similar to the fit of puzzle pieces. This means that enzymes specifically react with only one or very few similar compounds.

Lock and Key Theory

The specific action of an enzyme with a single substrate can be explained using a Lock and Key analogy first postulated in 1894 by Emil Fischer.[5b] In this analogy, the lock is the enzyme and the key is the substrate. Only the correctly sized key (substrate) fits into the key hole (active site) of the lock (enzyme). Smaller keys, larger keys, or incorrectly positioned teeth on keys (incorrectly shaped or sized substrate molecules) do not fit into the lock (enzyme). Only the correctly shaped key opens a particular lock.

Induced Fit Theory

Not all experimental evidence can be adequately explained by using the so-called rigid enzyme model assumed by the lock and key theory. For this reason, a modification called the induced-fit theory has been proposed by Koshland.[5c] The induced-fit theory assumes that the substrate plays a role in determining the final shape of the enzyme and that the enzyme is partially flexible. This explains why certain compounds can bind to the enzyme but do not react because the enzyme has been distorted too much. Other molecules may be too small to induce the proper alignment and therefore cannot react. Only the proper substrate is capable of inducing the proper alignment of the active site.

Transition-state theory

According to this hypothesis, the substrate does not bind most effectively in the enzyme-substrate complex (E.S) complex; as the reaction proceeds, the enzyme conforms to the transition-state structure, leading to highest interactions (increased binding energy).[5d] This increased binding energy, known as transition-state stabilization, results in rate enhancement.

There are over 2000 known enzymes, each of which is involved with one specific chemical reaction. There are twenty different amino acids. Out of these, mammalians can synthesize twelve amino acids. The others must be obtained from diet. These are called essential amino acids.

Tryptophan

L-Tryptophan is an essential amino acid for humankind, but it is a rare constituent of mammalian proteins. The amino acid was isolated from casein (milk protein) in 1901, and its

structure was established in 1907 (fig.1.2). Tryptophan metabolism plays a central role in physiology. Nicotinic acid (niacin), a vitamin of the B complex, can be made from tryptophan. Deficiency of tryptophan in the diet enhances the progress of the vitamin-deficiency disease pellagra, which is treated by restoring nicotinic acid to the diet, usually supplemented with tryptophan.

Tryptophan achieves its effects by way of serotonin, one of the key brain chemicals involved in regulating mood. Among other functions, serotonin promotes feelings of calm, relaxation, and sleepiness. Lack of serotonin is associated with depression. Many of today's powerful antidepressant drugs work as serotonin reuptake inhibitors to increase the level of available serotonin in the brain. Tryptophan is the key ingredient in making serotonin; without it, serotonin will not be produced.

Tryptophan Biosynthesis

Tryptophan biosynthesis in microbes involves five enzymes, encoded by 7 genes. The genes (trp A,B,C,D,E,F and G) are usually arranged in a single cluster,[6] and form an operon whose transcription is negatively regulated by tryptophan.[7] Additional regulation mechanisms exist at the translational and post-translational levels, presumably because tryptophan synthesis is very expensive (78 mol ATP for each mol of tryptophan produced).

Tryptophan is an amino acid required by all forms of life for protein synthesis and other important metabolic functions, but animals do not possess the enzymatic machinery to synthesize it from simpler molecules. At the level of primary producers, tryptophan is synthesized from molecules such as phosphoenolpyruvate in bacteria, fungi and plants, and these organisms fuel the tryptophan flux through the food chain. Because animals are incapable of synthesizing

tryptophan, they must ingest it in the form of proteins, which are then hydrolyzed into the constituent amino acids in the digestive system. Dietary tryptophan is delivered to the liver through the hepatic portal system, and that portion which is not used for protein synthesis in the liver can then follow one of two basic metabolic fates. First, it can be distributed to the blood stream to be used for protein synthesis and other functions by cells throughout the body. Second, it can be degraded in the liver through a series of metabolic steps known collectively as the kynurenine pathway. In addition to being one of the building blocks for protein synthesis in humans and animals, tryptophan is also the only source of substrate for the production of several very important molecules. In the nervous system and gut, tryptophan is a required substrate for the synthesis of serotonin, whereas in the pineal gland, it is required for the synthesis of melatonin. Also, when niacin content in the diet is insufficient for metabolic requirements, tryptophan is necessary for the synthesis of the essential cellular cofactor, nicotinamide adenine dinucleotide (NAD^+). The synthesis of NAD^+ from tryptophan has long been thought to be a reaction that takes place solely in the liver.

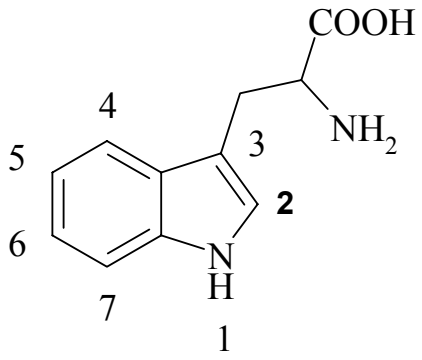


Fig. 1.2 Structure of Tryptophan.

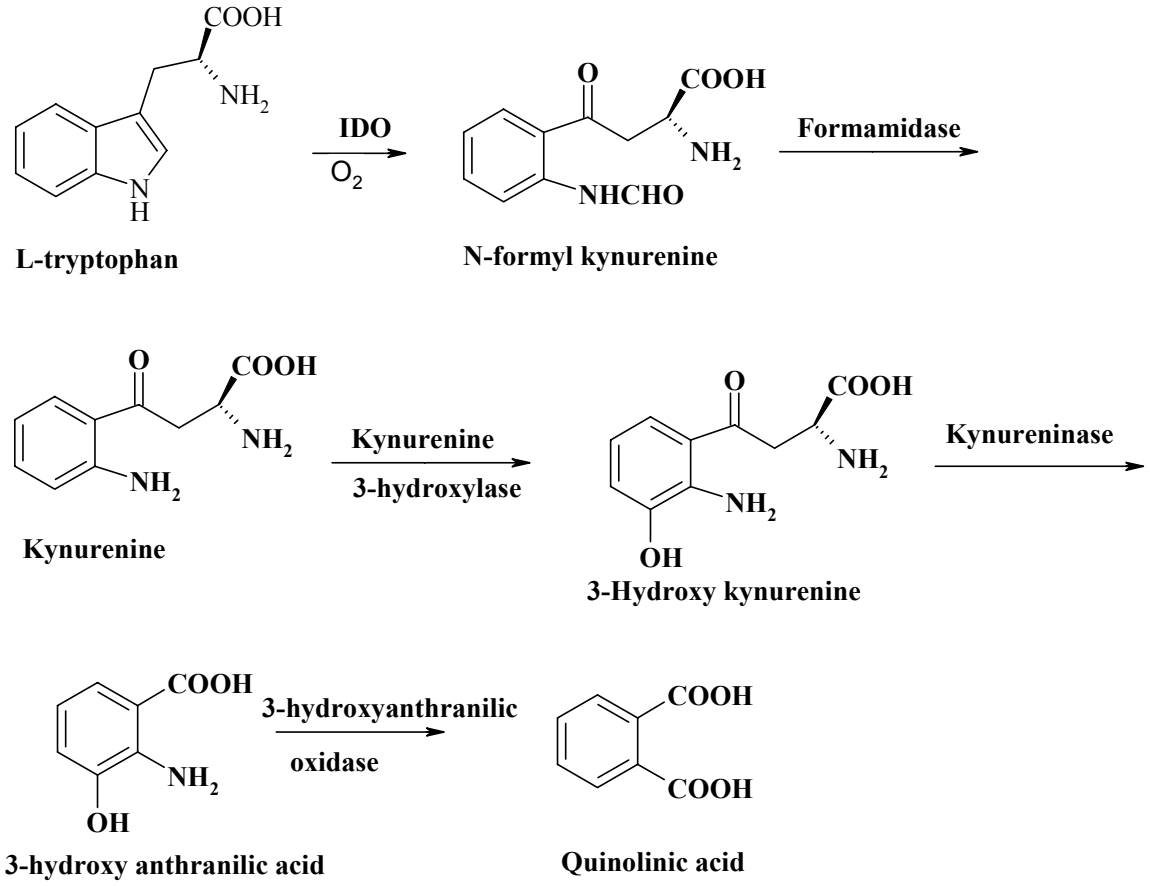
The kynurenine pathway

The kynurenine pathway is responsible for 90% of human tryptophan catabolism. The first and rate limiting step of the kynurenine pathway (Scheme 1.1) is the oxidative cleavage of the 2, 3-double bond by molecular oxygen or superoxide anion.[8]

The hemoprotein, indoleamine 2,3-dioxygenase (IDO), is the first and rate-limiting enzyme in mammalian tryptophan metabolism. It has received considerable attention in recent years, particularly due to its role in the pathogenesis of many diseases.

Discovery of IDO

In 1927 Kotake isolated the major intermediate of this pathway, kynurenine, and the structure of this compound was established by Butenandt et al. in 1940.[9] The first enzymatic step, the formation of formylkynurenine, is the rate-limiting step in degradation of tryptophan. In 1936 Kotake and Masayama[10] isolated an enzyme that catalyzed the conversion of tryptophan to formylkynurenine, and called it tryptophan pyrrolase. This enzyme was later named tryptophan 2, 3-dioxygenase (TDO) (EC 1.13.11.11). The enzyme is found only in the liver, and is induced by administration of tryptophan. The TDO-catalyzed oxidation reaction is the limiting step in tryptophan catabolism, as the formamidase activity was found to be in 600-fold excess. TDO increases within several hours after administration of an active inducer, and returns to normal within 15-20 hours after removal of the substance. TDO is induced by histidine and kynurenine and to a lesser extent by tyrosine and phenylalanine. When large amounts of tryptophan, or tryptophan and hydrocortisone or hydrocortisone alone, are given to rats, the liver TDO activity increases from 5- to 10-fold over control levels. TDO was the first inducible enzyme system discovered in mammals.[11]



Scheme. 1.1. Tryptophan metabolism by the kynurenine pathway

It has been recognized for some time that elevated levels of various tryptophan metabolites can be found in the urine of patients suffering from a variety of diseases, such as rheumatoid arthritis, tuberculosis, leukemia, Hodgkins disease, bladder cancer, and prostate disorders. However, TDO is not elevated in the liver in these patients.[12, 13] These findings suggested that TDO may not be the only enzyme initiating the catabolism of tryptophan.

In 1963, Hayaishi and colleagues[14, 15] isolated a second enzyme that catalyzed conversion of tryptophan to kynurenine. Unlike TDO, this enzyme is not found predominantly in the liver, but is distributed ubiquitously in nonhepatic organs of mammals, with the lung and placenta having the highest activities.[15, 16] It has a wider spectrum of substrates than TDO and can utilize many indoleamine derivatives, including L- and D-tryptophan, tryptamine, 5-hydroxytryptophan, and serotonin, and is therefore named indoleamine 2,3-dioxygenase (IDO: indole:oxygen 2,3-oxidoreductase (decyclizing). Indoleamine 2,3-dioxygenase uses superoxide, a univalently reduced form of molecular oxygen for activity, and differs from TDO not only in organ distribution but in molecular weight (approximately, 45,000 for IDO and 320,000 for TDO). IDO is a heme containing protein, the enzyme from the rabbit intestine having protoheme IX as a catalytic center, and it has absolute requirement in vitro for methylene blue and ascorbate to maintain an active ferrous form during catalysis. In vitro, catalase is required to protect the enzyme from H_2O_2, which is generated by the reducing system.[15, 16] The native IDO has a relative abundance of hydrophobic amino acids and contains approximately 5% carbohydrate by weight.[15] IDO is evolutionarily related to the myoglobin family of enzymes and[16] oxygen-transporter proteins; it is not related to TDO. Genes encoding TDO and IDO have distinct patterns of expression. IDO is expressed at basal levels in epididymis, thymus, gut, lung, and the

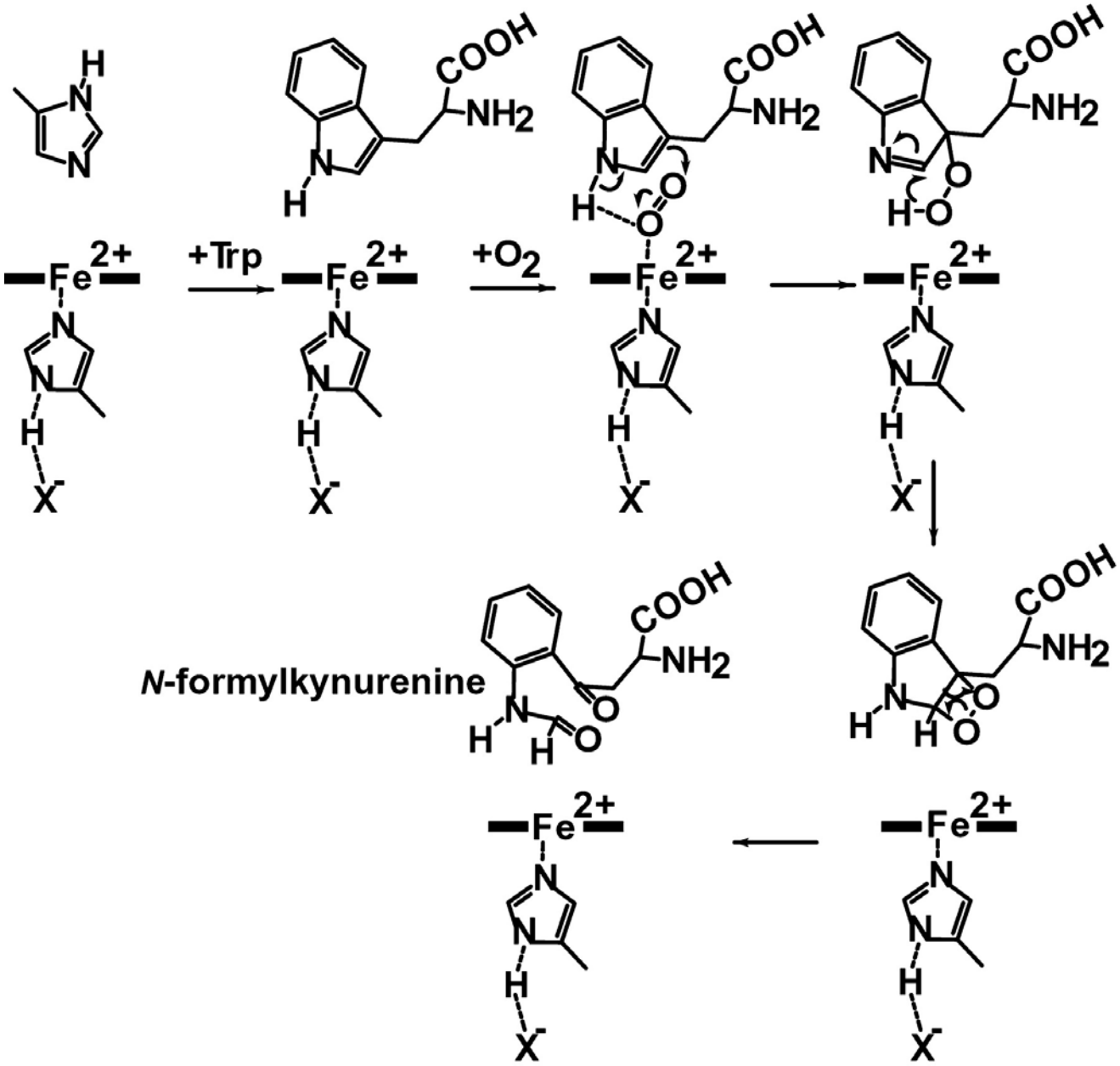
maternal-fetal interface during gestation and is up-regulated in response to infection and tissue inflammation.

IDO can also be distinguished from TDO on the basis of antigenicity.[17] Unlike TDO, IDO is not induced either by tryptophan or glucocorticoid. IDO has been shown to be induced in vivo in lungs of mice by lipopolysaccharide (LPS). [18] or virus [19], by interferon (IFN-γ), [20] and to a lesser extent by rHu-IFN-α A/D and by tumor necrosis factor (TNF).[21] *In vitro* studies with human peripheral blood mononuclear cells have shown that all three classes of IFN (α, β, and γ) can induce IDO.[20, 22] Although TNF-α had no effect on IDO induction in vitro in human macrophages [22] or in human ME-180 cells [23], induction by IFNs could be potentiated by TNF.[22] IDO can also be induced to a limited extent by LPS in the absence of interferon or interferon-producing cells in a cell line THP-1 (a human myelomonocytic cell line), suggesting that there may be other ways of inducing IDO. *In vivo* induction of IDO occurs in certain pathological conditions, including virus infections [19] and endotoxic shock [23], which again may reflect interferon induction. Native human IDO is 403 amino acids long, [24, 25] with a molecular weight of 45,326 Da and a p*I* of 7.1. The sequence of amino acids is as follows.[26, 27]

MAHAMENSWT ISKEY**H**IDEE VGFALPNPQE NLPDFYNDWM FIAKHLPDLI 50
ESGQLRERVE KLNMLSIDHL TDHKSQRLAR LVLGCITMAY VWGKGHGDVR 100
KVLPRNIAVP YCQLSKKLEL PPILVYADCV LANWKKKDPNK PLTYENMDV 150
LFSFRDGDCS KGFFLVSLLV EIAAASAIKV IPTVFKAMQM QERDTLLKAL 200
LEIASCLEKA LQVFHQIHDH VNPKAFFSVL RIYLSGWKGN PQLSDGLVYE 250
GFWEDPKEFA GGSAGQSSVF QCFDVLLGIQ QTAGGGHAAQ FLQDMRRYMP 300
PA**H**RNFLCSL ESNPSVREFV LSKGDAGLRE AYDACVKALV SLRSY**H**LQIV 350
TKYILIPASQ QPKENKTSED PSKLEAKGTG GTDLMNFLKT VRSTTEKSLL KEG
403

All heme proteins carry iron coordinated to protoporphyrin IX.[27] The four coordination sites provided by the porphyrin ring are not sufficient to satisfy the coordination requirements of the iron. Normally, groups from the heme-binding protein occupy the remaining coordination sites. The common coordinating functional groups are the imidazole nitrogen of histidine, the phenoxide group of tyrosine, the sulfur of methionine and cysteine, and the carboxylate group of aspartic acid and glutamic acid. In both myoglobin and hemoglobin, the heme is attached to the protein through a histidine residue to the 5th coordination position on the iron (the proximal ligand). A second histidine (the distal ligand) occupies space immediately above the 6th coordination position of the iron, and coordination is through an oxygen molecule, not directly to the iron.

Dioxygenases, such as IDO, catalyze the oxidative cleavage of a substrate and, as such, both oxygen and the substrate need to bind to the enzyme. A common property of oxygenases is that the binding of the substrate precedes that of dioxygen, and this has been shown to be the case with tryptophan 2,3-dioxygenase.[28] By contrast, Hirata et al.[29] found that IDO could bind oxygen to form a relatively stable complex that has catalytic activity. In later investigations it was determined that ferrous IDO binds L-tryptophan, followed by molecular oxygen.[30] The following mechanism (scheme1.2) was proposed for the IDO catalyzed conversion of tryptophan to N-formylkynurenine.[27] (1) IDO-Fe^{2+} binds the substrate. (2) The IDO-Fe^{2+}-tryptophan complex then binds oxygen to form an IDO-$Fe^{3+}O$ complex (ternary complex); and (3) this complex is converted to N- formylkynurenine, releasing IDO-Fe^{2+}.



Scheme. 1.2 Proposed Mechanism of IDO on Tryptophan (Adapted from Reference 27)

The amino acid sequence showed that there are three conserved histidines, namely His^{16}, His^{303}, and His^{346} (in bold). The conserved nature of these residues is indicative of an important role in the function of the enzyme, such as a coordinating residue involved in heme binding. Suzuki *et al* [31] proposed that His^{303} and His^{346}, two histidines close to the C terminus, were likely to be either the proximal or distal heme-coordinating histidines because of their proximity to each other.

Conversion of His^{346} to Ala resulted in a total loss of heme content in IDO, although the secondary structure of the protein was maintained: therefore, this provides strong evidence for the proposal that this histidine is the proximal heme ligand in IDO. Site-directed mutagenesis showed that Asp^{274} is also required for maintaining heme binding in IDO. The role of His^{346} and Asp^{274} in the binding of heme in IDO may be direct; i.e. they may act as the proximal and distal heme ligands. The mutation of His^{303} produced a protein that retained significant heme binding ability, which suggest that His^{303} is not the proximal ligand.

The role and biological significance of IDO

IDO (EC1.13.11.17) is a cytosolic hemoprotein distributed in many tissues of mammals, including primates.[32, 33] IDO is systemically as well as locally induced under a variety of pathological conditions including viral, [34–37] bacterial, [33, 38] and protozoan infections, [39] allograft rejection,[40, 41,] and tumor regression.[42– 44] The primary role of IDO induction in infectious disease appears to be to suppress the growth of pathogens by removing tryptophan from tissue microenvironments.[45–51]

Host defense and tissue inflammation of IDO

Historically, studies on IDO have focused on the notion that IDO expression is a host defense mechanism against infectious pathogens such as HIV and HTLV, which works by

depriving infected cells of access to tryptophan, an essential amino acid degraded by IDO. [52, -55,] Chronic infections enhance IDO activity, most likely due to IFNγ production. However, the notion that IDO is an inducible host defense mechanism is incompatible with observations of basal IDO activity in lymphoid tissues, mucosal surfaces and at the maternal–fetal interface during mammalian pregnancies. [56, 57]

Aberrant tryptophan catabolism has been linked to some inflammatory and neurodegenerative diseases.[58] Several studies show that tryptophan catabolism occurs at sites of tissue inflammation and IDO expression may be anti-inflammatory by ameliorating tissue damage at these sites.[57] For example, IFNγ-mediated inhibition of matrix-metalloproteinase (MMP) gene expression by human synovial fibroblasts depends on tryptophan catabolism.[59] Moreover, transient IDO expression by embryonal carcinoma cells inhibits MMP gene expression and allows formation of multicellular aggregates.[60]

These observations hint that IDO expression inhibits processes mediating tissue damage during chronic inflammation. Beneficial effects of IDO expression might accrue from local removal of reactive oxygen, enhanced inhibition of immune or stromal cell proliferation and function or suppression of tumor growth. However, detrimental effects of IDO expression may accrue from the production of toxic metabolites, as when HIV-induced IDO expression and production of quinolinate damage neurologic functions.

IDO role in allograft transplantation

Liver allografts are unusual because they can induce donor-specific tolerance without the use of immunosuppressive drugs. The underlying tolerogenic mechanisms remain elusive but are

associated with hepatocytes.[61] A role for cells expressing IDO in this phenomenon is suggested by recent studies in which orthotopic murine liver allografts were rejected when recipient mice were exposed to 1-methyl-tryptophan at the time of engraftment.[62]

These data implicate IDO activity in the suppression of liver allograft rejection, since 1-methyl-tryptophan specifically inhibits IDO but not hepatic tryptophan dioxygenase (TDO) activity the only other mammalian enzyme that degrades tryptophan.[63] Support for the notion that genetic manipulation of IDO expression may protect cell and tissue allografts is provided by a recent study in which prolonged survival of transplanted allogeneic pancreatic islet cells was observed when adenoviral gene transfer was used to drive IDO expression in donor islets.[64] These outcomes suggest that local modulation of IDO activity may facilitate islet cell transplantation as a therapy for type 1 diabetes.

Anti-tumor immunity suppression

Research on tumor-associated IDO expression has focused previously on the notion that IFNγ-dependent inhibition of tumor growth is mediated by tryptophan catabolism[56] As for host defense, the hypothesis is that endogenous IDO activity deprives growing tumor cells by reducing access to tryptophan. Although many cultured tumor cells express IDO when treated with IFNγ, experimental support for this concept is controversial. One complication is that cellular infiltrates are attracted to sites of tumor growth by chemokine cues and are intimately associated with tumor masses. Cellular components of these infiltrates may also express IDO activity.

Another possible hypothesis is that tumors attract cells expressing IDO to protect themselves from T-cell immunity targeting tumor-specific antigens.[65] This concept is analogous to the situation of allogeneic fetal tissues implanted in the maternal uterus. If verified, this hypothesis could explain why tumor cells resist T-cell immunity that targets rejection of non-tumor tissues following transplantation. Potentially, cells expressing IDO may be effective antigen-presenting cells (APCs) that promote T-cell entry into cell cycle while blocking cell cycle progression. Support for this notion emerges from recent studies in which murine tumor cells are transfected to express IDO-promoted T-cell activation, as assessed by the expression of T-cell activation markers such as CD69, while inhibiting T-cell proliferation.[66] In principle, APCs expressing IDO such as macrophages[67] and dendritic cells (DCs)[68] may suppress anti-tumor responses in vivo, as suggested by recent studies in mice.[69,70] This predicts that it may be possible to enhance anti-tumor immunity by treating cancer patients with IDO inhibitors which would allow T cells to make more potent contributions to tumor-specific immune responses.

Suppression of T-cell responses

The concept that cells expressing IDO afford immunological protection to allogeneic cells and tissues suggests that IDO expression is a natural immunosuppressive mechanism. Evidence hinting at a general immunosuppressive role for cells expressing IDO arose initially from studies with human macrophages that blocked in vitro T-cell proliferation by catabolizing tryptophan.[67] Based on these experiments, a conclusive hypothesis is that cells expressing IDO suppress T-cell responses by limiting the availability of tryptophan in local tissue microenvironments.[65] Subsequently, human DCs (Dendritic Cells) were shown to express IDO and suppressed in vitro T-cell proliferation under certain culture conditions.[68]

Recently, Mellor *et al* proposed that cells expressing IDO inhibit T-cell proliferation in vitro.[66] Interestingly, activated T cells are exquisitely sensitive to the amount of free tryptophan available to them during T-cell cycle progression following T-cell receptor (TCR) signaling.[67] Thus, activated human and mouse T cells were arrested at a specific point midway through the G1 phase of the cell cycle when access to free tryptophan was limited. In contrast, human T cells activated in medium containing neither isoleucine nor leucine progressed through the first cell cycle before being affected by the limited supply of these two essential amino acids. These data hint that tryptophan availability in tissue microenvironments may be a critical factor determining whether T cells activate and proliferate in response to encounters with Antigen presenting cells (APCs) displaying antigen.

In pregnancy

Pregnant mice exposed to an IDO inhibitor exhibited dramatically increased tendencies to lose conceptuses in allogeneic but not syngeneic mating combinations.[71, 72] These pharmacologic effects of an IDO inhibitor on pregnancy outcomes suggested that cells expressing IDO provide an immunosuppressive barrier that protects allogeneic conceptuses from maternal T-cell immunity. These data suggest that T-cell-dependent, antibody-independent activation of maternal complement is a risk factor in allogeneic pregnancies and that IDO activity minimizes this risk.

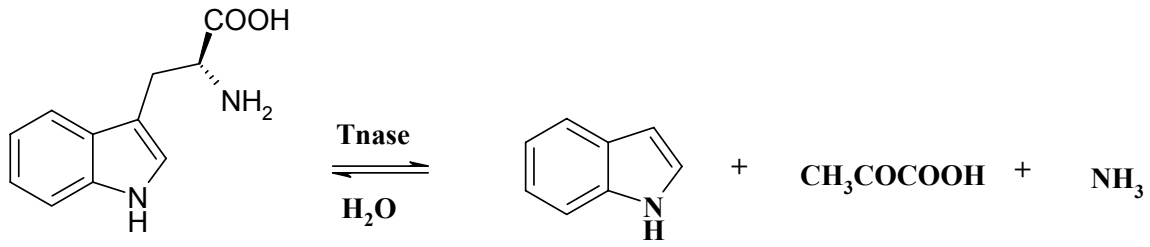
Tryptophanase

In 1875, Nencki and Kuhne found that Indole was produced during putrefaction of proteins.[73] Hopkins *et al* in 1903 showed that it was formed by bacterial decomposition of the

newly isolated amino acid, tryptophan.[74] In later years, indole production in appropriate growth media became a standard diagnostic test in identifying different enteric bacterial strains and species (IMViC test), which stands for 1st letter of the following compounds, Indole, Methyl red, Voges-Proskauer, and Citrate.

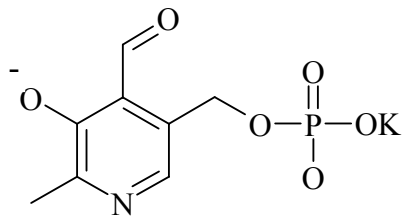
In the 1950's Happold and Wada discovered a series of intermediates in tryptophan metabolism and found Tryptophanase,[75] later named as Tryptophan Indole–lyase (Tna). Tryptophanase (EC 4.1.99.1) is a pyridoxal 5'- phosphate (PLP) dependent enzyme. (Fig.1.3) This tryptophanase is responsible for tryptophan degradation into indole, pyruvate, and ammonia[73] (stoichiometric amount of ammonia and pyruvate formed). Pyruvate and ammonia can be used as carbon and nitrogen sources.

See equation 1.1



(1.1)

Cofactor



Pyridoxal-phosphate

Fig: 1.3 (Vitamin B $_6$)

Structure and properties

Native tryptophanase is a tetrameric enzyme. Its dissociation into subunits and the reverse process are highly dependent on concentration, temperature, ionic environment, pH, and the presence or absence of the coenzyme, pyridoxal phosphate.[73] Equilibrium studies showed that apotryptophanase, complete enzyme without coenzyme, binds one mole of pyridoxal-P per 57,500 grams of protein,[76] that is, 4 pyridoxal-P per molecule of native tetrameric enzyme. The resulting holoenzyme shows characteristic pH dependent absorption maxima at 420 and 337 nm in the presence of catalytically essentially K^+ or NH_4^+ ions. Several findings in the early days thought that each subunit was composed of two identical peptide chains connected through one disulfide bond, with only single binding site for pyridoxal phosphate. Snell *et al* later proved that there is only one peptide chain per tryptophanase subunit of molecular weight 55,000.

In addition to the physiological reaction of L-tryptophan, it catalyzes α, β elimination reactions, β-replacement reactions, and α-hydrogen exchange reactions with a variety of L-amino acids as substrates, including substituted tryptophans, serine, cysteine, S-methylcysteine and many other amino acids carrying appropriate electronegative groups in the β- position.[77, 78] Over the past several years, the mechanism of this enzyme and its physical properties have been studied extensively. Snell,[73] proposed a mechanism of tryptophanase which is composed of several steps: 1. inter-conversion of inactive and active forms of the enzyme; 2. formation of the enzyme-substrate complex; 3. labilization of the α-hydrogen; 4. elimination of the β-substitutent with formation of enzyme bound α-aminoacrylate; and 5. decomposition of enzyme bound α-aminoacrylate to pyruvate and ammonia. Among these steps, the β-elimination of indole is mechanistically interesting, since the indole ring must tautomerize in order for the elimination reaction to proceed.[79-81] Phillips and coworkers proposed a new mechanism for the enzyme based

on the results of the reaction of the enzyme with analogues of the proposed reaction intermediate, oxindolyl-L-tryptophan.[82] The results of the steady-state and presteady-state kinetics of the reaction of the enzyme with L-tryptophan and other substrates support this mechanism.[82]

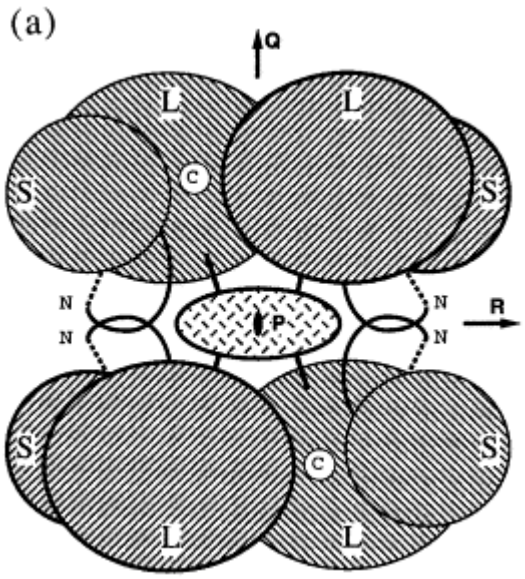


Figure1.4. The Tnase tetramer, Schematic representation of the domain architecture and inter subunit contacts in Tnase, viewed along the molecular dyad *P* with the molecular dyad *Q* vertical. Large domains are marked as L, small domains as S, the catalytic cleft is marked as C and the N termini are marked N. The hydrophobic cluster is shown as an ellipsoid in the centre of the molecule. Figure adapted from reference 93.

Tryptophan synthase

Tryptophan synthase (EC 4.2.1.20) is a pyridoxal- 5-phosphate (PLP) based enzyme with multiple activities. It is found in bacteria, yeast, molds, and plants. The enzyme of *Escherichia coli* is composed of 2α and β_2 subunits. [83] The three-dimensional structure of the α_2 β_2 complex of tryptophan synthase from *Salmonella typhimurium* has been determined by x-ray crystallography at 2.5 Å resolution. The four polypeptide chains are arranged nearly linearly in an alpha beta beta alpha order forming a complex 150 Å long. The overall polypeptide fold of the smaller alpha subunit, which cleaves indole-3-glycerol phosphate, is that of an 8-fold alpha/beta barrel, and pyridoxal-5-phosphate is bound to the β subunit.[83] The β_2 subunit normally exists as a dimer with a molecular weight of about 90,000 and can be reversibly dissociated into monomers by urea.[84] The α subunit catalyses the cleavage of indole-3-glycerol phosphate to indole and glyceraldehyde 3-phosphate, whereas the β subunit catalyses the condensation of indole with serine in a reaction mediated by pyridoxal phosphate.[85] In chapter 2 of this thesis, this reaction is used to synthesize 5-methyltryptophan, as shown in the synthetic part. Although tryptophan synthase from bacteria and plants is a two component heterotetramer, [86] the enzyme from *Neurospora crassa* has been shown to be a homodimer, with α and β activities in a single polypeptide chain.[87-88] Some of the reactions catalyzed by the tryptophan synthase are:

1. β-Elimination or β- replacement reactions:

Most of the reactions catalyzed by the β_2 subunit of the tryptophan synthase can be classified as β-elimination reactions (Equation 2) or β-replacement reactions.[89]

(Equation 3)

$$RCH_2CHNH_2COOH \rightarrow CH_3COCOOH + RH + NH_3 \text{ (Eq 2)}$$

$$RCH_2CHNH_2COOH + RH \rightarrow R'CH_2CHNH_2COOH + RH \text{ (Eq 3)}$$

Four of these amino acids have been found to be substrates for β-elimination reactions catalyzed by the β_2 subunit: L-serine (Equation 4), S- methyl-L-cysteine, O-methyl-L-serine and β –chloro-L-alanine.

$$\text{L-serine} \rightarrow \text{Pyruvate} + \text{Ammonia (Eq 4)}$$

Each of these amino acids is also a substrate for tryptophanase as earlier discussed. The basic difference between the β-elimination reaction specificity of the subunit of the tryptophan synthase and tryptophanase is that the β_2 subunit of the latter does not carry out β-elimination of indole from tryptophan.

2. β- Addition reactions:

All the above mentioned substrates of β-elimination reactions also undergo β- addition reactions according to equation 2, where R'H is indole, to yield tryptophan (Equation 5)

$$\text{Indole} + \text{L-serine} \rightarrow \text{L-tryptophan} + H_2O \text{ (Eq 5)}$$

L-serine also undergoes β-addition reactions where R'H is a thiol compound, methylmercaptan or β–mercaptoethanol, to yield S-methyl - L- cysteine or S-hydroxyethyl - L-cysteine. (equation 6) [90] Use of β – addition to synthesize 5-methyltryptophan is shown in a later chapter of this thesis.

$$\beta\text{-mercaptoethanol} + \text{L- serine} \rightarrow \text{S- hydroxyethyl –L-cysteine} + H_2O \text{ (Eq 6)}$$

3. Thiol dependent transamination reaction:

An unusual thiol – dependent transamination reaction is catalyzed by the β_2 subunit of tryptophan synthase in the presence of β- mercaptoethanol and L- serine. (Eq 7) [91]

β-Mercaptoethanol + L- Serine + PLP → S- hydroxyethyl thiopyruvate +H_2O + pyridoxamine 5'-phosphate (PMP) (Eq 7)

Under these conditions, the predominant reaction is the synthesis of S-hydroxyethyl-L-cysteine.

4. Conversion of β, γ- unsaturated amino acids to saturated α- keto acids:

A new type of pyridoxal-dependent reaction catalyzed by both the β_2 subunit and $\alpha_2\beta_2$ complex is the conversion of 2-amino-3-butenoic acid (vinyl glycine) to α-ketobutyric acid and ammonia (Equation 8).[92]

$$CH_2{=}CHCHNH_2COOH \rightarrow \quad CH_3CH_2COCOOH + NH_3 \text{ (Eq 8)}$$

Tryptophan synthase is one of the pyridoxal phosphate based enzymes which is present only in bacteria, plants, fungi and molds, and it is not present in human beings and animals. Therefore, inhibitors of this enzyme could have possible non-toxic herbicidal properties.

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CHAPTER 2 SYNTHESiS OF TRYPTOPHAN DERIVATIVES

Negative role of IDO and synthesis of IDO inhibitors

Indoleamine-2,3-dioxygenase (IDO) plays a role in the defense mechanism against various pathogens. However, IDO expression may also have adverse effect, and, for example, induction in the brain may cause an increase in production of the neurotoxic tryptophan metabolites, quinolinic acid and 3-hydroxykynurenine.

The kynurenine pathway is responsible for 90% of human tryptophan catabolism.[1] In healthy humans, IDO activation remains low, with kynurenine pathway metabolites existing in sub-nanomolar concentrations apparently exerting little or no physiological effects.[2] As an immune response to a variety of pathological conditions, such as viral, [3-6] bacterial,[7,8] and protozoan infections, IDO is over-expressed and tryptophan depletion ensues.[9,10] Induction of IDO within the central nervous system leads to increased concentrations of kynurenine pathway metabolites, most notably, two neurotoxic compounds, 3-hydroxykynurenine and quinolinic acid. Increased levels of quinolinic acid are implicated in neurological disorders, such as AIDS dementia complex, [11-13] cerebral malaria, [14] and ischemic brain injury.[15, 16]

Quinolinic acid as a neurotoxin

Glutamate receptors in the brain are thought to play a dominant role in the neuronal damage occurring in some clinical neurodegenerative disorders. Of the several receptor subtypes which can be activated by glutamate, those sensitive to *N*-methyl-D-aspartate (NMDA) have received most attention since their activation induces a substantial increase of calcium influx into

neurons which can trigger a series of events, such as the activation of proteases, leading to neuronal damage.[17-20]

Quinolinic acid was recognized as an intermediate of the kynurenine pathway leading to the synthesis of the essential co-factors nicotinic acid and Nicotinamide Adenine Dinucleotide (NAD), but the potential for a novel role for this compound came with the discovery that it could activate selectively the NMDA-sensitive subpopulation of glutamate receptors.[21] This allowed quinolinic acid not only to excite neurons,[22] but also to cause neuronal damage when injected directly into areas of the brain.[23,24] Subsequent study with analogues of quinolinic acid[18] and of differences in the sensitivity of neurons to quinolinic acid in different regions of the brain[25,26] led to one of the earliest proposals for the existence of NMDA receptor subtypes.[27] A large volume of evidence has been accumulated since 1981, suggesting that quinolinic acid may play an important pathological role in the acquired immunodeficiency syndrome (AIDS)-dementia complex, and in a number of other CNS disorders.

There has been much debate on whether the amounts of quinolinic acid encountered in the brain or the cerebrospinal fluid (CSF) would be sufficient to produce neuronal damage. The amounts of quinolinate in the brain rarely exceeds 1 μ*M*. However, these levels would be sufficient to cause significant neuronal damage either by direct activation of NMDA receptors or via the release of endogenous glutamate.[28,29] Micromolar concentrations of quinolinic acid are toxic when cells are exposed to those concentrations for several hours[20,30,31] and submicromolar concentrations can produce neurotoxicity in culture if maintained for several weeks,[32] with some neurones being killed on exposure to only 100 nM quinolinic acid,[33-36, 11] Giulian *et al* have examined the effects of quinolinic acid on human central neurons in culture.[33] In concentrations

of 350 nM for 5 weeks, quinolinic acid caused a loss of cell density and microtubule-associated protein.[33]

Many cells were found to be swollen with dendritic varicosities and damaged microtubular assemblies. As these concentrations are comparable with those found in AIDS patients, the potential importance of this neurotoxin to brain damage associated with this condition is clear. According to Power *et al.*[37] almost 20% of patients with AIDS develop marked CNS involvement, with cognitive decline, motor dysfunction and behavioral abnormalities. The evidence for a role of quinolinic acid in this AIDS-dementia complex involves work on infected humans in addition to animal models.

In patients with the AIDS-dementia complex, the levels of quinolinate in the CSF are increased up to 20-fold, and correlate with the cognitive and motor dysfunction in the affected patients.[38, 39, 40] The amount of quinolinic acid in the brain of human immunodeficiency virus (HIV)-infected patients can increase up to levels 300 times of those measured in the CSF.[41] One of the reasons for the high quinolinic acid content in AIDS brain is likely to be the increased activity of the normally rate-limiting enzyme IDO which was demonstrated by Hayes.[42] Enzyme activity was greater in subjects exhibiting dementia than in those not so affected.

The chronic infusion of quinolinic acid into the rat striatum induces deficits of spatial learning in a radial arm water maze, leading to the proposal that chronically raised quinolinic acid could induce the behavioural and motor deficits seen in Huntington's disease.[43-45] Quinolinic acid lesions of the striatum in monkeys produce dystonia and dyskinesia closely resembling those of human Huntington's disease[46-48] and those effects can be suppressed by lesions of the pallidum.[49] The importance of this observation is that pallidal lesions in humans

can abolish some of the motor abnormalities of Huntington's disease and Parkinson's disease, by disrupting the neuronal output pathway from the basal ganglia to the thalamus, motor cortex and brainstem. The similar effects on disease symptoms and quinolinic acid-induced symptoms, therefore, could support the view that quinolinic acid could contribute to the disease progress in humans.

In addition to the toxicity of quinolinic acid, mediated by the NMDA receptor, the kynurenine pathway includes another compound with significant neurotoxic potential, 3-hydroxykynurenine. This substance is a less potent toxin than quinolinic acid, and the neuronal damage produced seems to be mediated by free radicals and not glutamate receptors.[50-53] 3-Hydroxykynurenine can be converted to quinonimines with the accompanying generation of reactive oxygen species.[54] Levels of 3-hydroxykynurenine are also elevated in cases of HIV infections, especially in those cases associated with dementia,[55-57] infantile spasms,[58] and hepatic encephalopathy.[59] It is possible that some of the deleterious actions attributed to 3-hydroxykynurenine are actually due to its metabolite 3-hydroxyanthranilic acid,[60,61] since the latter readily undergoes auto-oxidation with the formation of superoxide anion. This metabolite, 3-hydroxykynurenine, has also been shown to react with lens proteins (crystalline), producing a tanned/yellow product resembling nuclear cataractous materials.[62] The activity of the first enzyme in the synthetic pathway from tryptophan to quinolinate, IDO, has been measured in the post-mortem brains of AIDS patients.[42] Enzyme activity was increased significantly in tissue from those patients with dementia compared with tissue from controls or non-demented AIDS patients. The increased enzyme would lead to elevations both in quinolinate and 3-hydroxykynurenine.

Poliovirus infection raises the activity of IDO and consequently the concentration of quinolinic acid in the spinal cord.[63] The increases were in proportion to the degree of inflammation, neurological damage and the severity of motor paralysis. The levels of quinolinic acid are raised significantly in the CSF of Lyme disease patients with CNS involvement, and those levels correlate strongly with the invasion of the CNS by leucocytes.[64]

Other proposed involvement of kynurenines in neurodegenerative disorders are cerebral malaria,[65] ischemic brain injury,[66, 67] Parkinson's disease, Alzheimer's disease, multiple sclerosis, and Systemic Lupus Erythematosus (SLE).

Recently, Uyttenhove *et al.* [68] explored the role of IDO in tumors by using the P815 mouse mastocytoma cell line. Most human tumors constitutively express IDO and they observed that expression of IDO by immunogenic mouse tumor cells prevents their rejection by preimmunized mice. This effect is accompanied by a lack of accumulation of specific T cells at the tumor site and can be partly reverted by systemic treatment of mice with an inhibitor of IDO, in the absence of noticeable toxicity. Transfection of IDO renders tumor cell lines immunosuppressive *in vitro,* [69] and 1-methyl tryptophan (1MT) significantly delays tumor outgrowth in a model of Lewis Lung carcinoma.[70] Many human tumor cell lines and primary human tumors, comprising various histologies, were found to contain a significant proportion of tumor cells expressing IDO.[68] Taken together, these data suggest that IDO might have a role in enabling tumors to escape the immune system. These results suggest that the efficacy of therapeutic vaccination of cancer patients might be improved by concomitant administration of an IDO inhibitor.

Inhibitors of IDO

Enzyme inhibitors act by interfering with the action of the enzymes at active sites and by decreasing the rates of their catalysis. Inhibitors can be either reversible or irreversible. Irreversible inhibitors bind to enzymes by covalent bonds and modify or alter the enzyme conformation. This type of inhibition cannot be regenerated. Reversible inhibitors bind to enzymes and are subsequently released to leave the enzymes in their original condition. There are three subclasses in this class of inhibitors.

1. Competitive Inhibitors
2. Noncompetitive Inhibitors.
3. Uncompetitive Inhibitors.

Classically, a competitive inhibitor is a compound which bears a close structural and chemical similarity to the substrate of the enzyme. Because of this similarity, the inhibitor binds to the active site in place of the substrate as a sort of molecular mistake. However, because the substrate and inhibitor are not identical the enzyme is unable to convert the inhibitor into a product. The inhibitor simply blocks the active site. While it is there, the substrate cannot enter and consequently the enzyme can not be converted it to a product. If an inhibitor binds to a site of an enzyme distinct from the site that binds substrate the inhibition cannot be overcome by increasing the concentration of substrate, and they are called noncompetitive inhibitors. Uncompetitive inhibitors bind to a site other than the active site, but only when substrate is bound.

Two types of inhibitors of IDO have been reported, competitive and noncompetitive. The first competitive IDO inhibitor, 2, 5-dihydro-L-phenylalanine (K_i = 230 μ*M*), was reported by

Watanabe and coworkers in 1978.[71] Since then Cady and Sono have also reported examples of potent competitive inhibitors of IDO.[72] Their compounds were distinguished by replacement of the N-H function of the indole ring of the tryptophan with N-Me, O, and S functions. These inhibitors exhibited activity towards rabbit small intestine IDO with a K_i values of 7-70 μ*M* and competed with tryptophan for the active site of the ferrous-O_2 enzymatic form. The (K_m) of L-tryptophan for IDO is 13 μM.[73] In contrast to indole modified inhibitors, modification of the amino acid function of the tryptophan has been shown to produce compounds which are neither substrates nor inhibitors of IDO. Hayaishi and coworkers first reported that β-carbolines were inhibitors of IDO.[74] They found that norharman inhibited IDO with K_i value of 120 μ*M*. A noncompetitive mechanism of inhibition for norharman was later established based on kinetic and spectroscopic data.[75] Peterson *et al.* reported that 3-n-butyl-β-carboline is a very potent noncompetitive inhibitor (K_i = 3.3 μ*M*) in the human macrophage IDO system.[7]

The existence of an allosteric site on IDO has been proposed. High levels of L-tryptophan and other compounds inhibit IDO by binding to this allosteric site.[77] Moreover, binding of 3-indolethanol (tryptophol) to rabbit IDO via this allosteric site resulted in a 61% decrease in IDO towards tryptophan metabolism.[71] This allosteric site may be involved in the regulation of IDO or may be a site incidental to the principal function of IDO.

Structure activity relationship (SAR) studies

Although both L and D- tryptophan have been shown to be metabolized by rabbit small intestine IDO, the L isomer is metabolized 131 times faster than D isomer at pH 7.[78] This suggests that the active site of IDO differentiated between the two stereoisomers. Optically pure L-1-methyltryptophan (fig. 2.1a) and D-1-methyl tryptophan (fig. 2.1b) were tested for inhibitor

activity, and the L isomer was found to be a stronger competitive inhibitor of IDO *in vitro* with tryptophan as substrate than the D form (fig. 2.1b). The activity of 1-ethyl analogs (fig. 2.2a) and (fig. 2.2b) was greatly reduced from L-1-methyl tryptophan. This result defines the approximate size of the substituent on the indole nitrogen of active ligands to a methyl group and further defines potent activity to the L-amino acid analogs. The weak inhibition observed for Na-sulfonamidotryptophan (fig. 2.3) further reinforces this hypothesis (size) however; the electron withdrawing ability of the sulfonamide function may contribute to the weak activity of sulfonamide. Further definition of tryptophan binding site was accomplished with β-methyl analogs (fig. 2.5 and fig. 2.6) which exhibited almost no inhibition of IDO. The β-position of tryptophan can tolerate a group no larger than a methyl group for competitive inhibition. Modification of the indole 2, 3-double bond provides derivatives

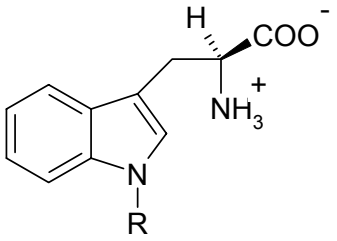


Fig. 2.1a R = CH_3

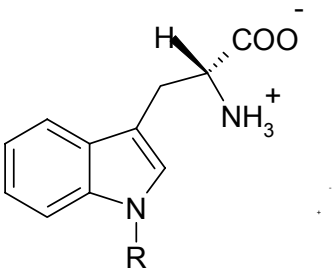


Fig. 2.1b R = CH_3

Fig. 2.2a R = CH_2 CH_3

Fig. 2.2a R = CH2 CH_3

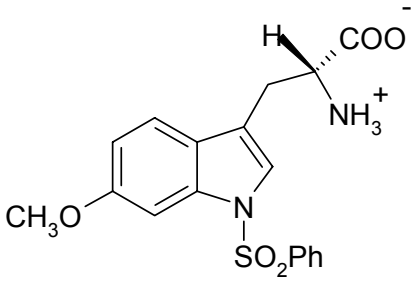


Fig. 2.3

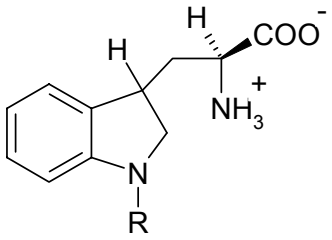


Fig. 2.4

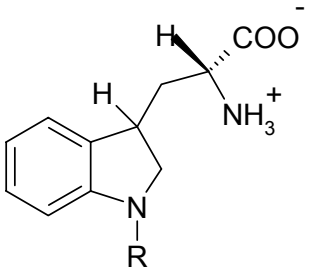


Fig. 2.5

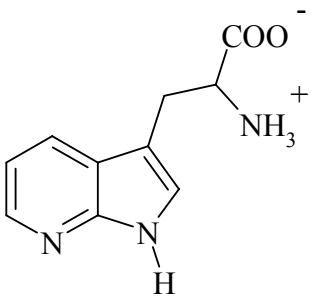


Fig. 2.6

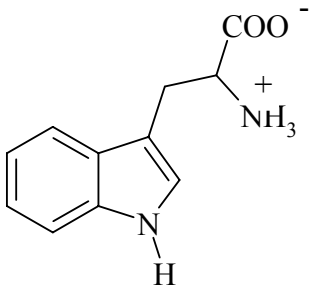


Fig. 2.7

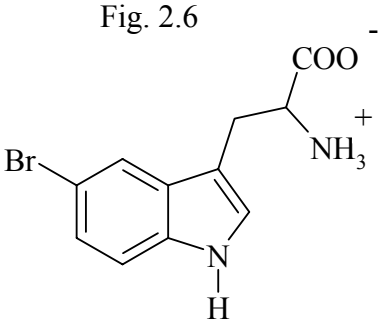


Fig. 2.8

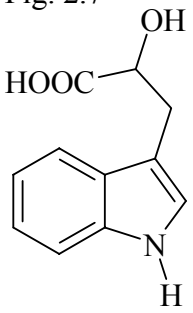


Fig. 2.9

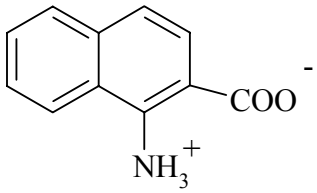


Fig. 2.10

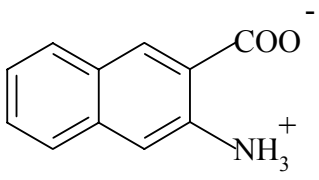


Fig. 2.11

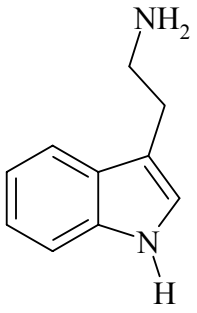


Fig. 2.12

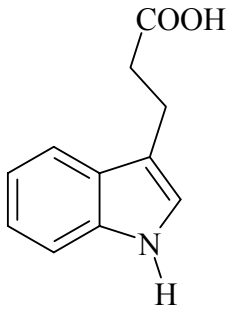


Fig. 2.13

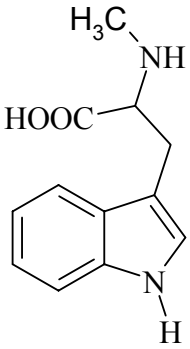


Fig. 2.14

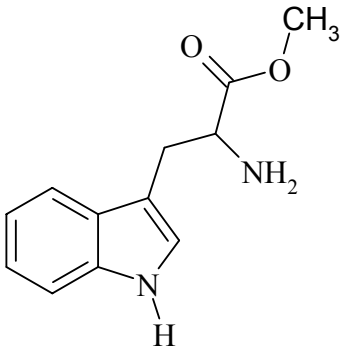


Fig. 2.15

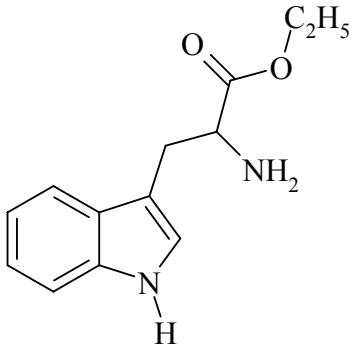


Fig. 2.16

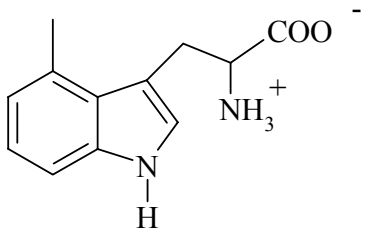


Fig. 2.17

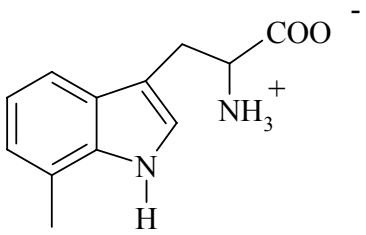


Fig. 2.18

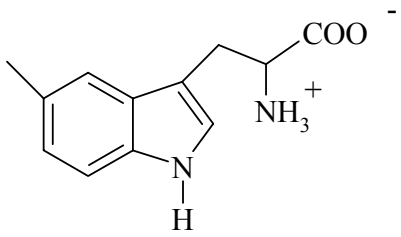


Fig. 2.19

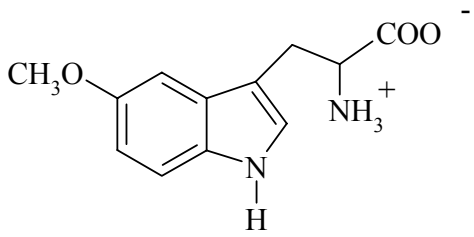


Fig. 2.20

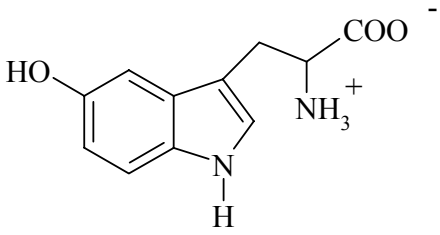


Fig. 2.21

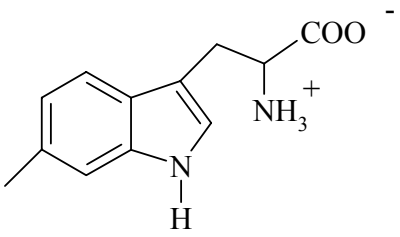


Fig. 2.22

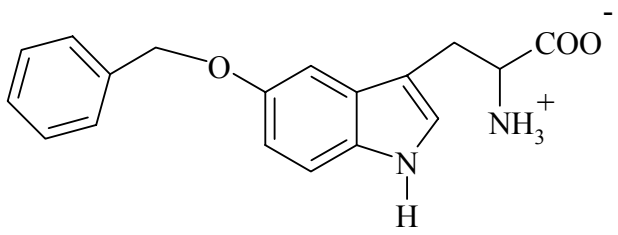


Fig. 2.23

which cannot be oxidized by IDO. Reduction of the indole 2, 3-double bond of both L- and D-tryptophan gave indolines (fig. 2.4 and fig. 2.5), which were devoid of activity. Based on this limited amount of evidence, this result suggests that the binding site requires a substrate with planarity at the 2, 3 and 4 positions of tryptophan. Addition of a chlorine, bromine or hydroxyl at C-2 resulted in compounds with low activity as both inhibitors and substrates. A substituent at C-2 may prevent binding of the compound to the active site by steric hindrance or by changing the electronic configuration around the indole ring. This supports the planarity at 2, 3 position of substrate for the binding activity to the enzyme.

Azatryptophans (fig. 2.6 and fig. 2.7) which have a nitrogen atom substituted for a CH group at the 2 or 7 position were inactive.[78] This result suggests that the indole portion of tryptophan is binding in a largely lipophilic cleft. The weak inhibition of 5-bromotryptophan (fig. 2.8) also supports this hypothesis. Modification of the amino acid side chain reduced the ability of the compound to inhibit IDO [79] the indole lactic acid (fig. 2.9) exhibited poor inhibition with rabbit small intestine IDO.[76] The aromatic β-amino acids (fig. 2.10 and fig. 2.11) were also evaluated as inhibitors. Amino acid (fig. 2.11) was found to be a potent inhibitor of IDO, while isomer (fig. 2.10) was a weak inhibitor of IDO. Examination of this result further demonstrates that nonindolic amino acids are capable of inhibition of IDO and that the indole N-H function is not absolutely necessary for binding at the active site.

The requirements of the recombinant human IDO for the α-amino acid portion of the molecule are relatively specific. Modification of the amino, carboxyl, α-carbon or β-carbon groups resulted in compounds with low substrate and inhibitor activities, indicating that the free amino acid portion of the tryptophan is important for binding to the active site. For example,

tryptamine (fig. 2.12) was a weak inhibitor and substrate. Indole-3-propionic acid (fig. 2.13) was inactive, whereas addition of a methyl group to the α or β carbon, resulted in a compound that has low activity, but was more effective as a substrate than an inhibitor. A methyl group added to the α- amino function fig. 2.14 resulted in a compound that was more effective as an inhibitor of IDO than as a substrate.[80] The methyl ester (fig. 2.15) binds to the active site and was more effective as an inhibitor than as a substrate, whereas the larger ethyl ester (fig. 2.16) appears to be excluded from the active site, because it has low substrate and inhibitor activity. These modifications of the aliphatic portion of tryptophan indicate that integrity of the amino acid group is required for binding of the active site of IDO.

Methyl substituents added to positions 4 or 7 (fig. 2.17, fig. 2.18) resulted in low activity as either substrates or inhibitors. Steric hindrance at these positions may prevent tryptophan derivative binding.[81] Tryptophan analogues with a substituent at positions 5 or 6 have higher activities as either substrates, inhibitors or both. Tryptophan derivatives with electron-donating groups at the 5 or 6 position, e.g., 5-methyl-DL-tryptophan (fig. 2.19), 5-methoxy-DL-tryptophan (fig. 2.20), 5-hydroxy-L-tryptophan (fig. 2.21) and 6-methyl-DL-tryptophan (fig. 2.22) were better substrates than inhibitors. Interestingly, 5-methyl-DL-tryptophan was a better substrate than L-tryptophan. Surprisingly, 5-methyl-DL-tryptophan (fig. 2.19) had a greater V_{max} than L-tryptophan but binding affinity for IDO was less as indicated by the larger K_m value. 5-methoxytryptophan was found to be more substrate than inhibitor. When 5-methoxytryptophan was replaced by the larger 5-benzyloxy-DL-tryptophan (fig. 2.23) there was no substrate or inhibitor activity, indicating that the size of the active site 'pocket' that accommodates 5-substituted tryptophans is limited.[80] Tryptophan derivatives with electron withdrawing groups at 5 or 6 position were generally better inhibitors than substrates.

The compound 1-methyltryptophan has been reported to be a potent competitive inhibitor of IDO when tested *in vitro* using purified enzyme.[18, 1, 80] 5-methyl-DL- tryptophan was shown to be the better substrate than tryptophan. Hence the compound which has methyl substitution at the both 1 and 5 positions would be predicted to be a better inhibitor for IDO.

The aim of this research was to design and synthesize tryptophan derivatives with better IDO inhibitor activity than other derivatives *in vivo,* and to test the induction activity of tryptophanase in *E. coli* by these tryptophan derivatives.

Results and Discussion

As with other derivatization reagents, alkylation reagents reduce molecular polarity by replacing active hydrogens with an alkyl group. These reagents are used to modify compounds having acidic hydrogens, such as carboxylic acids and phenols. Alkylation reagents can be used alone to form esters, ethers and amides or they can be used in conjunction with acylation or silylation reagents. A two-step approach is commonly used in the derivatization of amino acids, where multiple functional groups of these compounds may necessitate protection during derivatization.

In general alkyl halides (RX) are employed for the alkylation reactions, as they undergo S_N2 type reactions with ease. Since the reaction is an S_N2 reaction, methyl and primary halides are more suitable for the alkylation reactions. Alkylation of tryptophan at position 1 is carried out by different methods. In the early 1950's, Snyder *et al.* reported the synthesis of 1-methyl tryptophan by using alkylating properties of 1-methylgramine methiodide.[82] (Fig. 2.24) A synthesis of 1-methyltryptophan has also been reported in connection with investigations into the chemical nature of a certain toad poison.

Edward Leete used ethyl-α-acetylamino-α-carbethoxy-β-(3-indole) propionate as the starting material for the alkyl derivatives.[83] This indole derivative was alkylated on refluxing in an inert solvent with alkyl p-toluenesulfonates in the presence of potassium carbonate. However this process led to both 1- and 3-alkyl derivatives.

The 1-substituted alkyltryptophans were synthesized using L or D- tryptophan as starting material (unless specified) following a procedure by Yamada *et al.*[84] The general method employed consisted of preparing the disodium salt of tryptophan in liquid ammonia and then allowing it to react with alkyl halides. Tryptophan was selectively alkylated at the indole-N position under this condition. This procedure is essentially analogous to that of Potts and Saxton.[85] But in this case, one equivalent of sodium was not enough to conduct the reaction and led to the recovery of the starting material.

The compound 1,5-dimethyltryptophan, was synthesized enzymatically following a procedure by Soda *et al.* [86] incubating 5-methylindole in toluene for seven days with L- serine in the presence of tryptophan synthase (scheme 2.1). In the first attempt, the enzyme was denatured after two days, and TLC showed more than 70% of starting material in the reaction mixture. In the next attempt, in place of toluene, 5-methylindole was dissolved in methylene chloride. The % yield was poor and more starting material was recovered than product.

To test enzyme activity, this enzymatic reaction was carried out with only indole in chloroform, not 5-methylindole. After two days of incubation at 37^{o} C, TLC showed more than 50% tryptophan in the reaction medium. This indicated that tryptophan synthase was working and had good activity.

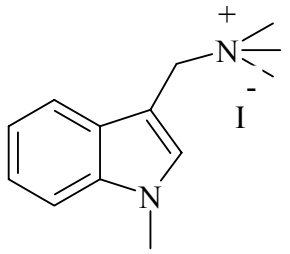
Next, 5-methyl-L-tryptophan (2.1) was synthesized using tryptophan synthase by adding it to the reaction mixture of 5-methylindole in portions over a 6 day period, so that a steady supply of active enzyme was available in the reaction medium. The resultant 5-methyltryptophan was alkylated by using the alkylating agent, methyl iodide, by dissolving 5-methyltryptophan in Dimethyl formamide (DMF), to give 1, 5-dimethyltryptophan. Due to the poor yield, it was necessary to find out an alternate method.

In Scheme 2.2, 1,5-dimethyl-L-tryptophan (2.2) was synthesized by dissolving the resulting 5-methyltryptophan from step 1 in liquid ammonia in the presence of excess metallic sodium with methyl iodide. In this procedure, some starting material was recovered, but the yield was better than the earlier one (Scheme 2.1).

To make 1-ethyl L and D-tryptophan derivatives (2.4 and 2.5), the method of Cook *et al.* was tried [79] using ethyl bromide, but starting material was recovered. However, replacing ethyl bromide by ethyl iodide provides a good yield of 1-ethyl-D-tryptophan, because iodine is a better leaving group than bromide. In the preparation of 1-ethyl-L-tryptophan(2.4), the reaction was performed by adding ethyl iodide drop wise over a 3 hour period after formation of anion due to the slow reaction rate.

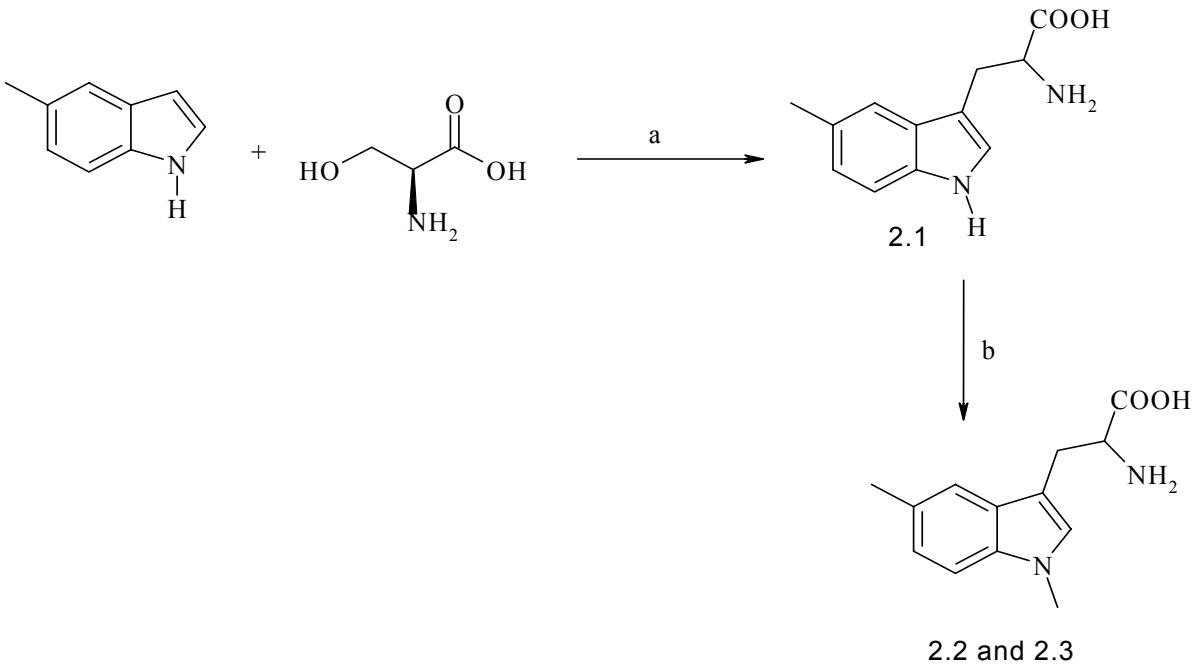
Higher 1-alkyl D and L-derivatives (propyl, allyl) were also synthesized using the same method, except for a few changes, such as using the alkyl bromide and higher reaction temperatures, -40 ^{o} C, Scheme 2.3 The final product was separated by using reverse-phase column chromatography. The details are discussed in the experimental part of this thesis.

In Scheme 2.4, the synthesis of both D-and L-N-carboxymethyltryptophan (2.10 and 2.11) is shown. The first step is similar to the Yamada *et al.* procedure using liquid ammonia. In the second step, the resulting ester, on hydrolysis with NaOH, gives N-carboxymethyltryptophan.



1-methylgramine methiodide

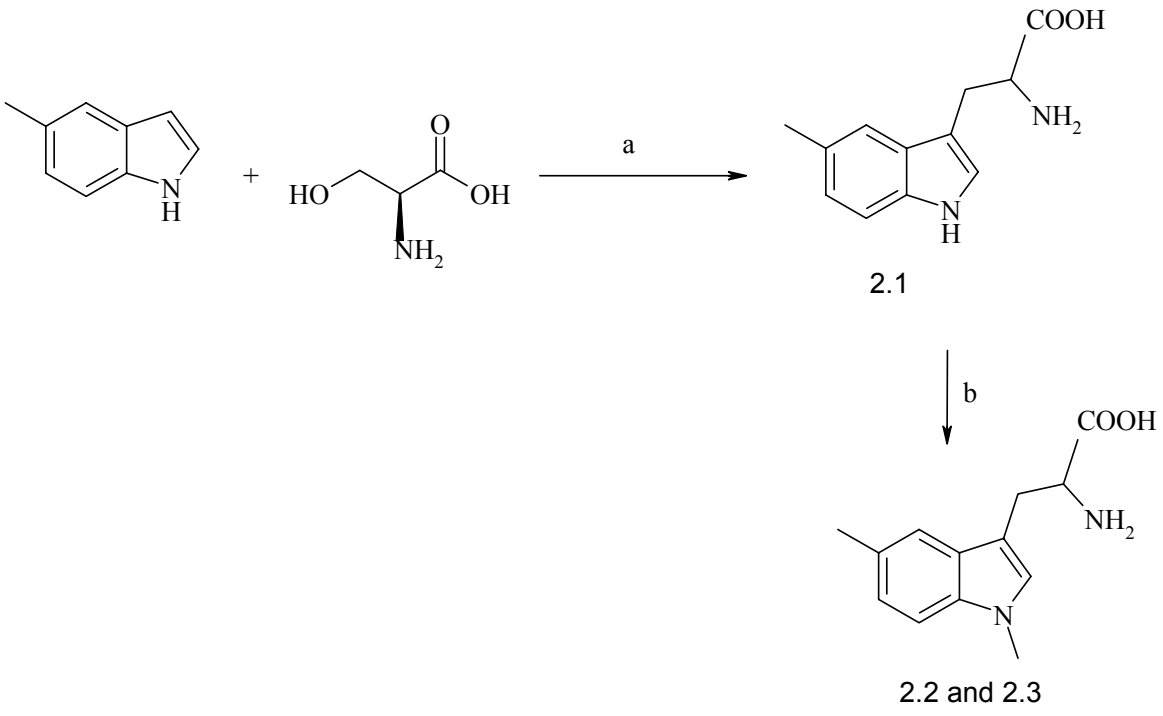
Fig. 2.24



a : Potassium Phosphate, PLP, NaCl, NaN_3, Tryptophan Synthase, incubate in water bath at 37^0C, 7 days.

b : CH_3I, DMF, NaH.

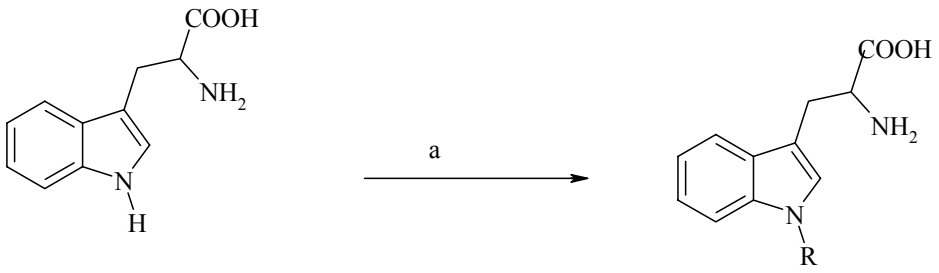
Scheme 2.1



a : Potassium Phosphate, PLP, NaCl, NaN_3, Tryptophan Synthase, incubate in water bath at 37° C 7 days;

b : CH_3I, Ferric Nitrate, Liq NH_3, Metalic Sodium, Stirr overnight;

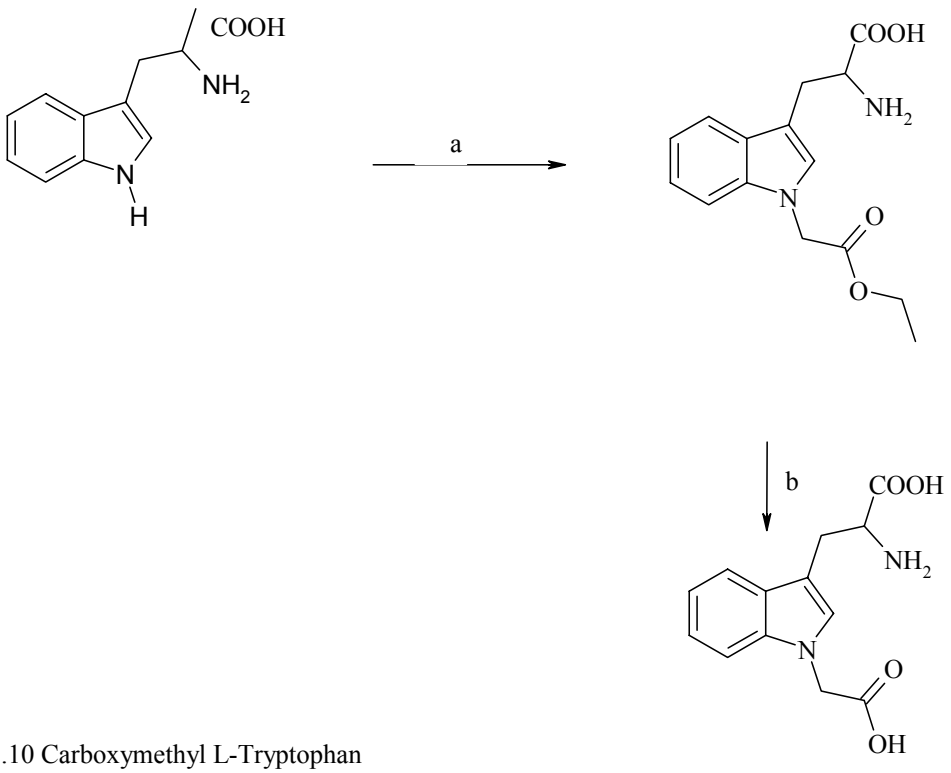
Scheme 2.2



a : RI/ RBr, Ferric Nitrate, Liq NH_3, Metallic Sodium, -78 ^{O}c, no reflux/ -40^{O}C,

	R
2.4 Ethyl L-Tryptophan	CH_3CH_2-
2.5 Ethyl D-Tryptophan	CH_3CH_2-
2.6 Propyl L-Tryptophan	$CH_3CH_2CH_2$-
2.7 Propyl D-Tryptophan	$CH_3CH_2CH_2$-
2.8 Allyl L-Tryptophan	CH_2CHCH_2-
2.9 Allyl D-Tryptophan	CH_2CHCH_2-

Scheme 2.3



2.10 Carboxymethyl L-Tryptophan

2.11 Carboxymethyl D-Tryptophan

a. Ethyl bromoacetate, Ferric Nitrate, Liq NH_3, Metalic Sodium, -40^{O}C, reflux 2-3 hours;

b : 25% NaOH, H_2O, reflux 2 hours:

Scheme: 2.4

Experimental

Melting points were determined on an Electrotherm apparatus and are uncorrected. NMR spectra were obtained in $CDCl_3$ or D_2O with tetramethylsilane as an internal standard on a Varian (400 MHz) instrument. The chemical shifts (δ) are given in parts per million (ppm). Splitting patterns are designated as follows: s, singlet; bs, broad singlet; t, triplet; q, quadruplet; and m, multiplet. Mass spectra analysis by ESI and molecular weights were shown with few important breakdown pattern of molecule. Optical rotation was determined by using a Rudolph Autopol IV in 1 M HCl.

2.1. 5-Methyl-L-tryptophan. A solution of 0.1 *M* Potassium Phosphate (pH 8, 100 mL) and 1 mg pyridoxal phosphate, 2*M* NaCl (0.5 ml), 150 mg L-serine, 10.45 mg sodium azide were mixed in a 250 ml Erlenmeyer flask. After mixing was complete, tryptophan synthase (240 units/ml), 125 µl, was added in small aliquots (50, 50, 25, µl) on the first, fourth, and seventh day of incubation to supply a steady active enzyme. 5-Methylindole, from Aldrich Chem. Co., 100 mg was dissolved in 10 ml toluene, added slowly to the above mixture, was heated and incubated for one week at 37^o C in water bath with shaking. After 7 days, the mixture was heated in a water bath at 80^o C for 20 min, extracted with 2 X 20 ml water and filtered. The filtrate was concentrated under reduced pressure until dry and kept at -20^o C to get crystals. Crystals were collected by filtration. The filtrate contained 5-methyl-L-tryptophan, which was first separated with a Dowex cation exchange column to separate salts, and finally pure 5-methyltryptophan was separated with a C-18 reverse-phase column using 10% ethanol as eluent. m.p. 292-295^o C, yield 95 mg. (95.9%). ^{1}H NMR (400MHz, D_2O) δ (ppm) : 1.625-1.682 (s, 3H), 2.681-2.815 (t, 2H),

3.625-3.712 (d, 1H), 6.315-6.375 (d, 1H), 6.565-6.61 (s, 1H), 6.681-6.723 (d,1H), 6.795-6.815 (s, 1H), MS: ESI = 219 m/z.

2.2. 1,5-Dimethyl-L-tryptophan. A 250 ml three neck flask equipped with a mechanical stirrer and dry ice condenser was cooled in a dry ice/EtOAc bath and filled with liquid ammonia (4.5 ml), which is produced by condensation of vapors produced from the reaction between NH_4OH and NaOH, at -78^o C with dry ice/EtOAc. Metallic Na (0.032 g) was added with stirring in small pieces to liquid ammonia containing ferric nitrate nonhydrate (2 mg). After dissolution was complete, 5-methyltryptophan (80 mg.) suspended in anhydrous Et_2O (appropriate amount) was added to the stirred mixture. After 30 min., iodomethane (114 mg, 0.8 *M*mole) was added dropwise over 15 min period, and the resultant mixture was stirred for 1 hour. The cooling bath was removed, and the ammonia was allowed to evaporate in the fume hood for 12 h. Water (3.5 ml) was added to the residue and the mixture was heated to dissolve, filtered and adjusted to pH 5.0 with glacial acetic acid (0.6 ml) while hot, followed by the addition of EtOH (0.35 ml). The mixture was allowed to stand in a refrigerator overnight. The resultant white precipitates were collected and washed successively with H_2O (0.4 ml), 50% aq. EtOH (0.4 ml) EtOH (0.4 ml), and Et_2O (0.4 ml.) TLC showed some starting material in the filtrate. The final product was then purified using reverse phase column with ethanol concentration ranging from 10-20%. m.p 290-300^o C yield 50 mg (97%), ^{1}H NMR (400MHz, D_2O) δ (ppm): 1.82-1.85 (s, 3H), 2.835-2.915 (d, 2H), 3.321-3.351 (s, 3 H), 3.825-3.875 (t, 1H), 6.621-6.735 (m, 2H), 6.82-6.882 (d, 1H), 6.925-6.946 (d, 1H), MS: (ESI) = 233 m/z, $[\alpha]^{20}_D$ = +11.6 (c = 0.25 1*M* HCl)

2.3. 1,5-Dimethyl-D-tryptophan. The procedure for the preparation of 1,5-dimethyl-D-tryptophan was similar to second step as above, except starting compound was 5-methyl D-

tryptophan. The physical and spectral properties of this amino acid were identical to its L-amino acid except for the optical rotation. $[\alpha]^{20}_{D}$ = -12.8 (c= 0.25), yield 45mg (90%).

2.4. 1-Ethyl-L-tryptophan. L-Tryptophan was alkylated with ethyl iodide under same conditions as above, except ethyl iodide was added over a period of 3 hours after anion formation. mp 201-203^{o} C, Yield 65 mg (88 %). ^{1}H NMR(D_2O/DCl) δ (ppm):0.756-0.792 (t, 3H), 2.835-2.915 (d, 2H), 3.625-3.691 (q, 2H), 3.825-3.875 (t, 1H), 6.621-6.681 (t, 1H), 6.721-6.785 (m, 2H), 6.923-6.958 (d, 1H), 7.028-7.123 (d,1H), MS: (ESI) = 233 m/z, $[\alpha]^{20}_{D}$ = +2.04 (c = 0.83, 1*M* HCL)

2.5. 1-Ethyl-D-tryptophan. D-Tryptophan was alkylated with ethyl iodide under the same conditions as above, yield 65 mg. (82 %). All properties are the same except optical rotation $[\alpha]^{20}_{D}$ = -2.24 (c = 0.83)

2.6. 1-Propyl-L-tryptophan. L-Tryptophan was alkylated with propyl bromide, like the earlier reaction conditions, except the reaction temperature was kept at -40^{o} C by dry ice/acetonitrile. The pure compound was separated with C-18 reverse column with EtOH, up to 45%, as eluent. mp. 225-230^{o} C, yield 65 mg (71%). ^{1}H NMR (D_2O) δ (ppm): 0.545—0.568 (t, 3H), 1.523-1.608 (m, 2H), 4.175-4.2.5 (t, 1H), 6.826-6.912 (t, 1H), 6.927-7.172 (m, 2H), 7.285-7.327 (d, 1H), 7.418-7.48 (d, 1H), MS (ESI) (m+ H) = 247, optical rotation $[\alpha]^{20}_{D}$ = +2.76 (c = 1.25)

2.7. 1-Propyl-D-tryptophan. The reaction was carried with propyl bromide with D-tryptophan under the above reaction conditions. The physical and spectral properties are identical with its L-isomer except optical rotation $[\alpha]^{20}_{D}$ = -2.98 (c = 1.25), yield 60 mg, (67 %)

2.8. 1-Allyl-L-Tryptophan. Allyl bromide was used under same conditions as described above. Yield, 80 mg (86 %). m.p 196-198 ^{0}, ^{1}H NMR(D_2O/DCl) δ (ppm):3.285-3.315 (d, 2H), 4.215-4.232 (t, 1H), 4.615-4.625 (d, 2H), 4.755-4.825(d, 1H), 4.925-5.05 (d, 1H), 5.875-5.955 (m, 1H),7.05-7.075 (t, 1H), 7.156-7.185 (m, 2H), 7.356-7.385 (d, 1H), 7.525-7.546 (d, 1H), MS (ESI) = 245 m/z, mp. optical rotation $[\alpha]^{20}_D$ = +1.807 (c = 0.833)

2.9. 1-Allyl-D-tryptophan. The amino acid, D-tryptophan was used under the conditions above and reacted with allyl bromide. The physical and spectral properties are identical with the L isomer, except the optical rotation $[\alpha]^{20}_D$ = -1.807 (c = 0.833), yield 85 mg (91 %).

2.10. 1-Carboxymethyl-L-tryptophan. L-Tryptophan was used in this reaction with ethylbromoacetate at -40^o C as above. The resulting ester was hydrolyzed with NaOH (25%) and water (2 ml) by refluxing for 2 hours to get 1-carboxymethyl-L-tryptophan. TLC showed two indistinct spots with 5% ethanol as solvent, then using only water, two separate spots were found. After separating with a C-18 reverse phase column, in a few fractions two compounds came together. Finally, the unreacted starting reagents were separated with reverse phase column again. mp. 220-225^{o}C, yield 45 mg (64 %). ^{1}H NMR (D_2O) δ (ppm): 3.185-3.195 (d, 2H), 4.185-4.212 (t, 1H), 4.849 (s, 2H), 7.035-7.039 (m, 2H), 7.108-7.115 (t,1H), 7.208- 7.215 (D, 1H), 7.478-7.49 (d, 1H), MS: (ESI) = 263 m/z, optical rotation $[\alpha]^{20}_D$ = +1.04 (c = 1.25)

2.11. 1-Carboxymethyl-D-tryptophan. The same reaction was performed as above to get this final product, except D-tryptophan was used as starting material. The physical and spectral properties were identical to the L-isomer except for optical rotation $[\alpha]^{20}_D$ = -1.04 (c=1.25), yield 50 mg, (71.4%).

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CHAPTER 3 TRYPTOPHANASE INDUCTION AND INHIBITION STUDIES

Introduction

Escherichia coli (*E. coli*) uses several mechanisms to regulate the expression of its tryptophan (*trp*) operon and control the rate of tryptophan biosynthesis. The most important of these are repression, transcription attenuation, and feedback inhibition.[1] Their combined action permits the bacterium to vary the rate of tryptophan production over a several thousand-fold range. Since tryptophan is costly to produce, efficient shutdown of synthesis is advantageous to the bacterium whenever the amino acid is present in its environment. Consistent with this conclusion, most organisms that feed on other organisms have lost the capacity to synthesize tryptophan.

E. coli is capable of efficiently transporting tryptophan from its environment. It can use tryptophan as a sole source of carbon or nitrogen because of the action of the inducible enzyme tryptophanase, (Tnase) which degrades tryptophan to indole, pyruvate, and ammonia. Tryptophanase can also catalyze the synthesis of tryptophan from indole and serine or cysteine.[2] The *tna* operon of Escherichia coli has been cloned and sequenced[3] and found to contain two major structural genes, *tnaA* and *tnaB*. The promoter proximal gene, *tnaA*, encodes Tnase, while *tnaB* encodes a low-affinity, high-capacity tryptophan permease[4]. To achieve its tryptophan uptake objectives, *E. coli* synthesizes three tryptophan permeases, designated *mtr, tnaB,* and AroP.[5-10] *mtr* and *tnaB* are tryptophan specific, whereas *aroP* also transports phenylalanine and tyrosine. The genes for these permeases have been cloned and sequenced [9-13] and the sequences of their predicted polypeptide products suggest that each functions as a transmembrane protein.[9] Comparison of the amino acid sequences of *mtr* and *tnaB* revealed that they are members of the same protein family, [9] and this family also includes the tyrosine-specific permease *tyrP*.[7,9] In a

medium containing all of the amino acids, the *tnaB* permease was most important for tryptophan uptake. However, the *mtr* permease also contributed to tryptophan transport. In a medium lacking phenylalanine and tyrosine, all three permeases were active in transporting tryptophan. The *mtr* permease was found to be principally responsible for transporting indole, the degradation product of tryptophanase action. The *tnaB* permease was shown to be essential for growth on tryptophan as the sole carbon source.[4]

Results and discussions

Studies on the mechanism of tryptophan induction of the *tna* operon have established that induced expression is due to a transcription antitermination mechanism that allows RNA polymerase to bypass Rho factor dependent transcription termination sites located in the leader region of the operon.[14, 15] Recently, it has been demonstrated that transcription attenuation is the sole mechanism of specific regulation of the *tna* operon. [14,-16]

One of the principal advantages of regulating gene expression by transcription termination/antitermination is that short, unique, RNA sequences and structures can mediate crucial regulatory decisions. Most bacteria use two very different mechanisms of transcription termination, designated intrinsic and factor-dependent termination. During intrinsic termination, a segment of the transcript being synthesized by RNA polymerase forms a stable hairpin, followed by a series of U residues. This hairpin initially serves as a transcription pause signal, and then, upon addition of the string of U residues, the transcribing polymerase responds by terminating transcription and releasing both transcript and DNA template.[16] In factor dependent termination, on the other hand, Rho protein binds as a hexamer to specific recognition sequences in an unstructured transcript segment and progresses in a 3' direction on the transcript. If Rho contacts an RNA polymerase molecule paused at a transcription pause site, it directs that

polymerase to release the transcript and abort transcription. Rho-dependent termination can be modulated by controlling access of Rho protein to the transcript or to RNA polymerase, or by altering the sensitivity of RNA polymerase to pause signals.

The tryptophanase (*tna*) operon of *E. coli* is involved in utilization of tryptophan as a carbon and nitrogen source. Transcription of the *tna* operon structural genes is subject to Rho-mediated transcription termination (Fig. 3.1)[16] Initiation of transcription of this operon is regulated by catabolite repression; continued transcription beyond its 300 +bp leader region is regulated by tryptophan-induced transcription antitermination. This antitermination mechanism results from blockage of Rho access to the nascent transcript by the ribosome engaged in translation of a segment of the *tna* operon leader RNA. The *tna* operon leader transcript includes a 24-residue coding region, *tnaC*, which specifies a leader peptide, *TnaC*, that contains a single crucial tryptophan residue. Synthesis of *tnaC* in the presence of inducing levels of tryptophan results in transcription antitermination. Cleavage of the nascent *tnaC-peptidyl-tRNA* is inhibited by the presence of excess tryptophan. The translating ribosome, therefore, stalls at the *tnaC* stop codon. Since the Rho factor binding sites in the leader transcript are immediately adjacent to the *tnaC* stop codon, the stalled *tnaC-peptidyl-tRNA-ribosome* complex blocks binding of Rho to the leader transcript, thereby preventing Rho binding and Rho-dependent termination. In the absence of high levels of tryptophan, the *tnaC-peptidyl-tRNA* is cleaved, the translating ribosome dissociates at the leader peptide stop codon, and Rho factor binds to the leader RNA, activating transcription termination. Some feature of *tnaC-peptidyl-tRNA* appears to create a specific tryptophan binding site in the ribosome. When tryptophan is bound, it prevents the appropriate ribosome release factor from activating cleavage of the peptidyl-tRNA ester linkage. This is another example where the sequence of the leader transcript is sufficient to direct efficient,

amino acid-specific regulation of transcription termination, in this case by directing synthesis of the appropriate leader peptide and by placement of the Rho binding sites in the necessary position to allow interference by the stalled ribosome.

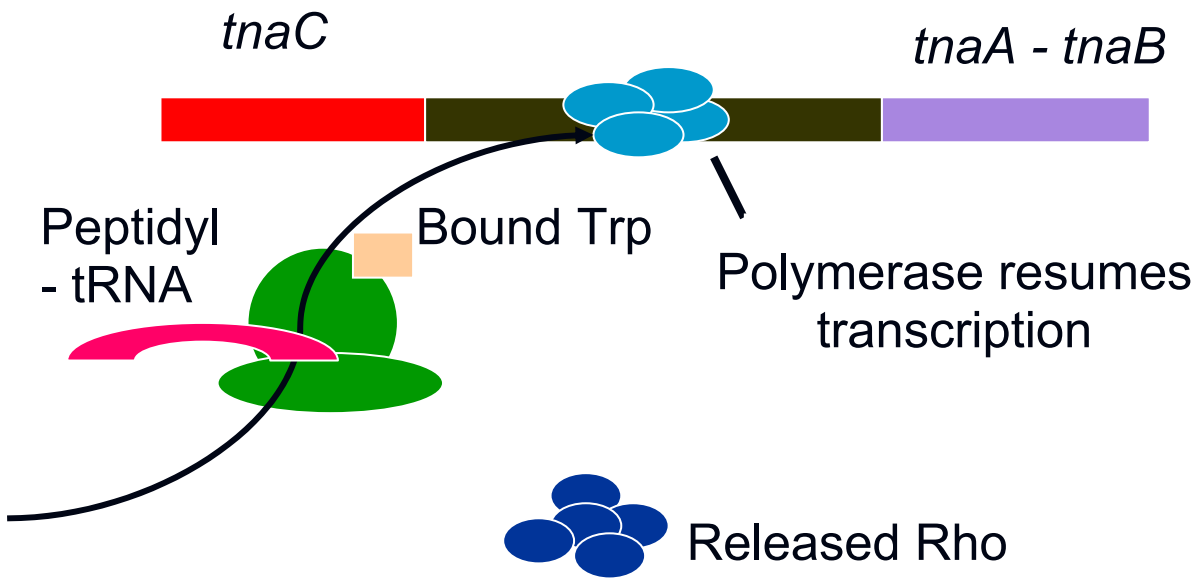

Fig. 3.1 *E. coli tna* operon. Termination: In the absence of excess tryptophan, the ribosome translating *tnaC* releases the leaderRNA at the *tnaC* stop codon. This allows Rho factor to bind to the transcript, contact a paused RNA polymerase, and terminate transcription. Antitermination: In the presence of excess tryptophan, the newly synthesized *tnaC-peptidyl-tRNA* cannot be cleaved, hence the translating ribosome remains stalled at the *tnaC* stop codon. The stalled ribosome blocks Rho binding and prevents transcription termination, allowing transcription of the downstream coding regions.

Figure modified from Ref 16.

Yanofsky *et al.* showed that maximal induction of the *tna* operon in media that do not contain a catabolite-repressing carbon source requires high concentrations of tryptophan.[4] Acid-hydrolyzed casein, an amino acid mixture lacking tryptophan, is a nonrepressing carbon source, as is succinate plus the five nonaromatic amino acids (glycine, serine, threonine, aspartate, and glutamate). The addition of tryptophan to media containing either of these carbon source mixtures leads to a rapid increase in the expression of the *tna* operon.

The high levels of tryptophan required for maximum induction of the *tna* operon could be due to limited tryptophan uptake and/or rapid degradation of intracellular tryptophan by tryptophanase, the product of *tna*A of the *tna* operon. *Tna*B permease is required in this medium for full induction by tryptophan.[4] The requirement for high levels of tryptophan for maximal induction in the *tna*+ strain can be attributed to tryptophanase-mediated degradation of tryptophan.

In acid-hydrolyzed casein medium, 1-methyltryptophan was equally effective as an inducer in the *tna*A+ strain and the *tna* mutant strain. This is to be expected, since 1-methyltryptophan is hydrolyzed very slowly by tryptophanase. Approximately 1 μg of 1-methyltryptophan per ml was sufficient for half-maximum induction as compared to 1g/L for tryptophan.

Vogel and Bonner (VB) minimal medium[17] and morpholinepropanesulfonic acid (MOPS) minimal medium[15] were employed in this study to test which medium was supporting cell growth and induction of tryptophanase. The media preparations were described in materials and methods. In VB medium, we observed very low growth and low induction. This could be due to high concentration of salts and undissolved acid-hydrolysed casein, but in MOPS medium, cell growth was good and induction of tryptophanase was 100-fold that of the VB medium.

The maximum induction for 1-methyltryptophan was observed at 10 μg/ml.[4] We used 10 μg/ml of each tryptophan derivatives to check which derivatives have good induction. Of all the derivatives 1,5-dimethyl-L-tryptophan showed 9-fold relative specific activity over the control, (fig 3.2) since both 1-methyltryptophan and 5-methyltryptophan were active inducers of the tryptophanase,[4] and expecting that both are hydrolyzed very slowly by tryptophanase. The allyl derivative of tryptophan was the next best inducer, having 8-fold higher relative specific activity than the control. Surprisingly, 1-propyltryptophan, which has almost the same molecular weight and steric size as allyltryptophan, showed poor induction (3-fold over the control). 1-Ethyl-L-tryptophan, which showed 7-fold relative specific activity, is in third rank for induction for tryptophanase. Carboxymethyltryptophan had the second lowest induction of all compounds. This could be due to poor transport or poor binding to the translation complex because of the polar substituent.

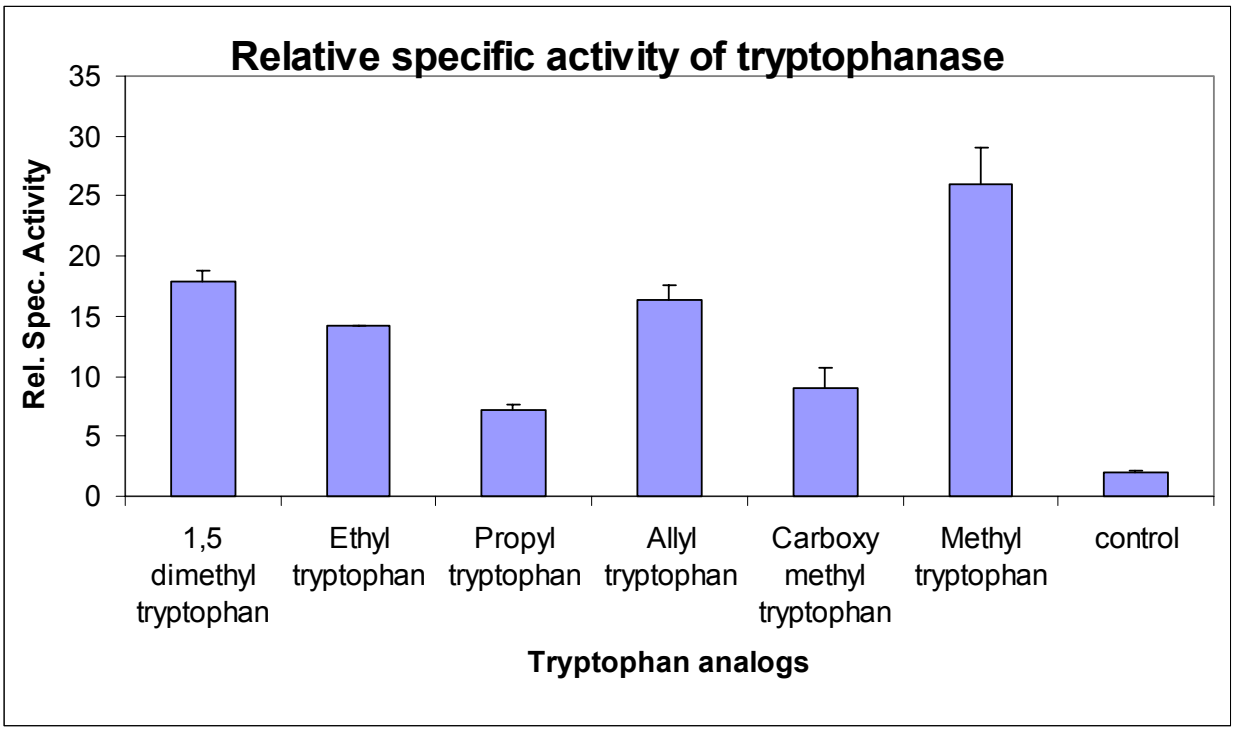
Relative specific activity of tryptophanase
Rel. Spec. Activity
35
30
25
20
15
10
5
0
1,5 dimethyl tryptophan
Ethyl tryptophan
Propyl tryptophan
Allyl tryptophan
Carboxy methyl tryptophan
Methyl tryptophan
control
Tryptophan analogs

Fig. 3.2

From the data in (Fig. 3.3) for 1,5-dimethyltryptophan, induction increased as the concentration was increased, up to 15 μg/ml. We interpret these results to indicate that in this medium tryptophan was efficiently transported into the cell. *TnaA* was maximally induced up to 38 units, but induction was weaker than methyltryptophan, since half maximal induction was 1 μg/ml, and for 1, 5-dimethyltryptophan, it was approximately 8 μg/ml.

Allyl-L-tryptophan showed maximum induction at a concentration of 25 μg/ml, and its half maximal induction is similar to methyltryptophan, 1 μg/ml, but maximal induction was less than that of 1,5-dimethyl tryptophan. For ethyl-L-tryptophan, maximal induction was at 15 μg/ml, like 1,5-dimethyltryptophan, but induction was poor as compared to it. The addition of carboxymethyl-L-tryptophan to MOPS media containing acid hydrolyzed casein, leads to a slow increase in the expression of Tnase (Fig. 3.3). Induction increased as the concentration was increased, up to 50 μg/ml. Enzyme levels did not reached a plateau at the highest tryptophan concentration tested, as it did for the other like all the above compounds. This is consistent with a transport problem because of the polar substituent.

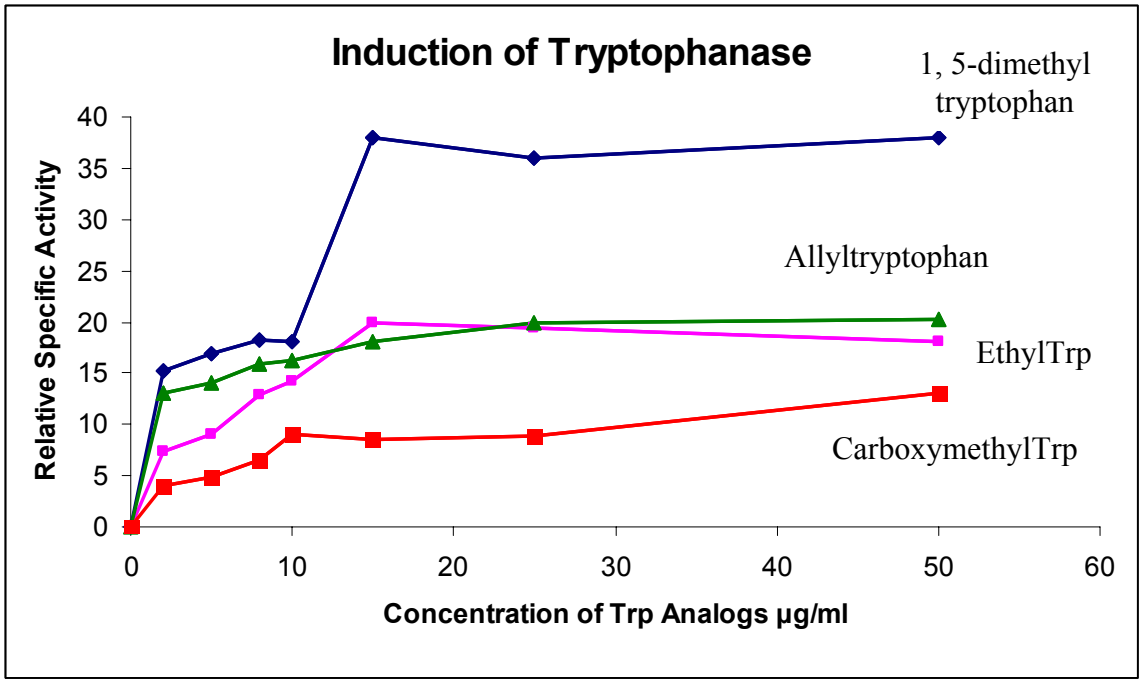
Induction of Tryptophanase
1, 5-dimethyl tryptophan
Allyltryptophan
EthylTrp
CarboxymethylTrp
Relative Specific Activity
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
Concentration of Trp Analogs µg/ml

Fig. 3.3

Materials and Methods

Bacterial strain The *E. coli* strain employed in this study is wild type *JM*101 *tna*$^{+}$A strain which has *tna*B in the genome and plasmid pMD6, with *tna*A and *tna*C, which gives very high expression.

Media Vogel and Bonner (VB) minimal medium[17] and morpholinepropanesulfonic acid (MOPS) minimal medium[15] were employed in this study. These media are prepared as follows: The "MOPS medium" of Neidhardt *et al.*[18] is ideal for enterobacteria. We have used the simplified formula of Bruce *et al,* [19] a 10-fold concentrate of the medium is prepared, sterilized by filtration through a 0.45 pm Millipore filter, and stored in the dark at 4^{o}C. The 10-fold concentrate contains 400 mM MOPS, 40 mM Tricine buffer, pH adjusted to 7.2 with KOH, 500 mM KCl, 100 mM NH_4C1, 13.2 mM K_2HPO_4, modified[18] 5 mM $MgSO_4$, and 0.1 mM $FeCl_3$. This medium (diluted 10-fold) is supplemented with 1% acid hydrolyzed casein (BMP Pharmaceuticals, Inc) as a source of carbon energy and autoclaved.

Culture conditions Cultures for measurements of enzyme specific activity were inoculated by diluting 0.05 ml of an overnight MOPS-AHC culture into 5 ml of fresh medium. Cultures contained in 20 ml test tubes were incubated at 37°C with vigorous shaking in gyratory water baths. After 5 hours of incubation, 10 μg/ml of all 5 different L-tryptophan derivatives starting from 1,5-dimethyltryptophan, 1-ethyltryptophan, 1-propyltryptophan, 1-allyltryptophan and carboxymethyltryptophan, 10 μg/ml were added and incubated for induction together with one control without any inducer. Culture tubes were shaken at 37°C in water bath until they reached the mid-exponential growth phase. Each time culture densities were monitored with a Cary 1 UV-spectrophotometer at 600 nm.

After they reached mid-exponential phase (optical density 0.4- 0.8) the culture tubes were centrifuged at 10,000 rpm for 10 min. The resultant cell pellets were resuspended in 1 ml cold lysis buffer (0.1*M* potassium phosphate pH 7, 5 m*M* 2-mercaptoethanol, and 50 μ*M* PLP). Cells were sonicated for 20 s x 3 on ice (Sonicator W-225R; Heat Systems Ultrasonics), and the cell debris was removed by centrifugation in 1.5-mL eppendorf tubes in a Beckman Microspin centrifuge for 10 min at 12,000 x g. The supernatant liquid was removed with gentle suction and stored at 4^{o} C for assay.

Tna assay Tryptophanase assays were performed with extracts of sonically disrupted cells and measured using S-o-nitrophenyl-L-cysteine (SOPC) as the substrate by the absorbance decrease at 370 nm using the molar extinction coefficient 1860 $M^{-1}*cm^{-1}$ [20] with the Cary 1 spectrophotometer. Cell extracts were mixed with assay buffer (50 mM potassium phosphate (pH 8), 50, μ*M* pyridoxal phosphate, 5 m*M* 2-mercaptoethanol, 0.6 m*M* SOPC) in a final volume of 1 ml. The amount of enzyme catalyzing the conversion of 1 μmol SOPC per 1 min was taken as the activity unit of tryptophanase. The specific activity was expressed as U/μg protein. The assay procedure was adapted from that of Phillips *et al*.[20] All assays were performed in triplicate. Errors in mean specific activities in individual cultures were estimated from standard deviations of enzyme assays and of protein determinations by use of standard methods of error propagation.

Protein determinations Protein concentration was determined by coomassie blue dye (Bio-Rad Corporation) binding technique (Bradford method) and bovine serum albumin as standards.[21]

Enzyme assay for inhibition studies Tryptophanase was a generous gift from Candace Corn, a student who purified it in Dr Phillips lab. The activity was measured using SOPC as the substrate by the optical density decrease at 370 nm using the molar extinction coefficient

1860 $M^{-1}*cm^{-1}$ [20] using a Cary1 spectrophotometer. Cell extracts were mixed with assay buffer (50 mM potassium phosphate (pH 8), 50 μM pyridoxal phosphate, 5 mM β-mercaptoethanol, and different concentrations of SOPC in a final volume of 1 ml at 25 ^{o} C, similar to one described above. First different concentrations of inhibitors were tested (0.5mM- 4mM) at 96 μM of SOPC (K_m= 96 μm). The maximum inhibition of compounds is different for each compound. At the maximum inhibition concentration of the substrate, inhibition tests were performed at different concentrations of SOPC (48 μM, 96 μM, 192 μM, 288 μM, 386 μM) to get inhibition data to calculate percentage of inhibition. Taking control (only SOPC) value as 0% inhibition, we calculated the percentage of inhibition by the compounds.

Tryptophanase inhibition studies

Demidkina *et al.* proved that tryptophanase was inhibited competitively by certain amino acids.[22] L-Phenylalanine and L-methionine, are competitive inhibitors of the wild-type enzyme. [22] For tryptophanase from *E. coli*, the relation between the structural features of amino acid inhibitors and effective inhibition constant was quantitatively analyzed using correlation methods.[23] The competitive inhibition constant of SOPC decomposition of L-tryptophan was 0.278 m*M.* K_i values of amino acids depend on hydrophobicity.[23] The affinity of tryptophanase enzymes, from *Proteus vulgaris,* and *E. coli,* to phenylalanine is significantly lower than could be expected accounting for its hydrophobicity. This fact can be rationalized by the impossibility of the "proper" orientation of the phenyl ring (nearly parallel to the cofactor plane). The inhibition also depends on the existence of a specific interaction with the electronegative protein moiety.[22] According to the X-ray diffraction data, [24] this moiety may be the Asp133 residue, which is positioned at the distance of a hydrogen bond from the nitrogen atom of the indole ring. Due to the wide number of possible substrates, the enzyme can be competitively inhibited by a

large number of compounds. The most effective inhibitors have a skeleton identical or closely related in structure or size to that of tryptophan.[2] The aim of this part of thesis is how tryptophan derivatives inhibit the tryptophanase.

L-Ornithine and L-arginine are anomalously potent inhibitors taking into account the low hydrophobicity of their side chains. This can be explained by an interaction between a positively charged group of the side chain of L-arginine or L-ornithine and an anionic group of the active site.[25] The affinity of tryptophanase for L-phenylalanine and L-homophenylalanine indicates that there is a special locus in the active site where aromatic groups are bound and oriented approximately parallel to the cofactor plane experiencing no steric hindrance.

Results and discussions

The interaction of amino acid inhibitors (tryptophan derivatives) occurs at the active site and hence these are competitive inhibitors of tryptophanase.[2] Decreasing polarity of the –OH or –SH group by substitution with methyl, ethyl, or benzyl greatly increases the affinity to the enzyme.[2] This leads to competition between substrate and inhibitor. Oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan are potent competitive inhibitors of tryptophanase. Since these analogues have tetrahedral geometry at carbon-3 of the heterocyclic ring, they are structurally similar to the indolenine tautomer of L-tryptophan, a proposed intermediate in reactions of L-tryptophan.[26]

All five tryptophan derivatives were tested for inhibition of pure tryptophanase. 1,5-Dimethyl tryptophan showed no inhibitory effect at concentrations tested ranging from 0.5 m*M* to 3 m*M* at K_m value of SOPC. It may be due to methyl substitution at position 5, which was having not much difference in hydrophobicity of compound. Ethyl, propyl and allyl compounds showed good inhibition of tryptophanase. (Table 3.1) From ethyl to propyl there was increase in

inhibition, which may be due to increased hydrophobicity of the derivatives. Ethyltryptophan showed 57% inhibition at 2 mM concentration as compared to the control (SOPC only, no inhibitor). Propyltryptophan had 62% inhibition at 1 mM concentration, this could be explained due to decreased polarity of the compound, making it hold at enzyme active site. Allyltrptophan has almost the same size as the propyl derivative but exhibits better inhibition than propyltryptophan, 61.8% at 0.5 m*M* concentration. This may be due to steric factors favoring more inhibition of tryptophanase. Carboxymethyltryptophan had no inhibitory effect on Tryptophanase. One reason for this result might be increased polarity and electrostatic repulsion with Asp 133.

Inhibition of tryptophanase by tryptophan analogs

	Tryptophan analog	**% of inhibition**	**Concentration**
1	Ethyltryptophan	57	2mM
2	Propyltryptophan	62	1mM
3	Allyltryptophan	61.8	0.5mM

Table 3.1

Conclusions

Induction of tryptophan and cell growth of *E. coli* are better in MOPS + AHC medium than VB medium, where 100-fold induction and much better growth were observed. At 10 μg/ml concentration, maximum induction was observed with 1,5-dimethyltryptophan, probably because of slow hydrolysis by tryptophanase. Carboxymethyltryptophan had the second lowest induction of all compounds. This could be due to poor transport or poor binding to the translation complex. Allyl-L-tryptophan showed maximum induction at a concentration of 25μg/ml, and its half maximal induction is similar to methyltryptophan, 1 μg/ml, but maximal induction was less than 1,5-dimethyl tryptophan. The addition of carboxymethyl-L-tryptophan to MOPS media, leads to a gradual increase in the expression of the Tnase induction. Induction increased as the concentration was increased, up to 50 μg/ml. Enzyme levels had not reached a plateau at the highest tryptophan concentration tested, unlike all other compounds examined. This is consistent with a transport problem because of the polar substituent.

Inhibition of tryptophanase increases from ethyl, propyl, to allyltryptophan derivatives, and is likely due to increased hydrophobicity. The better inhibition of the allyl compound may be due to proper orientation of allyl compound parallel to the cofactor plane.

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Table of Abbreviations for the Dissertation

I.U.B	International Union of Biochemistry
GOT	Glutamic Oxaloacetic Transaminase
ATP	Adenosine Triphosphate
NAD^+	Nicotinamide adenine dinucleotide
IDO	Indoleamine 2,3-dioxygenase
TDO	Tryptophan 2,3-dioxygenase
LPS	Lipopolysaccharide
IFNγ	Interferon γ
TNF	Tumor Necrosis Factor
HTLV	Human T-cell Lymphotropic Virus
HIV	Human immunodeficiency virus
MMP	Matrix-Metalloproteinase
APCs	Antigen-Presenting cells
DC	Dendritic Cells
TCR	T-Cell Receptor
IMViC	Indole, Methyl red, Voges-Proskauer, and Citrate
Tna	Tryptophanase
PLP	Pyridoxal 5'- phosphate
NMDA	N-methyl-D-aspartate
CSF	Cerebrospinal fluid
CNS	Central Nervouse System

SLE	Systemic Lupus Erythematosus
1MT	1-Methyl Tryptophan
TLC	Thin Layer Chromatography
DMF	Dimethyl formamide
ESI	Electro Spray Ionization
Trp	Tryptophan
VB Medium	Vogel and Bonner minimal medium
MOPS	Morpholine propanesulfonic acid
AHC	Acid hydrolyzed Casein
SOPC	S-o-nitrophenyl-L-cysteine