Synthesis of mechanism probes and potential Inhibitors for Tryptophan 2, 3-dioxygenase and Indoleamine 2, 3-dioxygenase enzymes

Thesis submitted for the degree of
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at the University of Leicester

by

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Statement

The concomitant thesis submitted for the degree of PhD under the title 'Synthesis of mechanism probes and potential Inhibitors for Tryptophan 2,3-dioxygenase and Indoleamine 2,3-dioxygenase enzymes' constitutes work conducted by the author in the Department of Chemistry at the University of Leicester fundamentally during the period of time from January 2013 to October 2016. The work involved here is original otherwise indicated in the text or references. To the best of my knowledge, no one has been submitted this work for another degree in this or any other universities.

Signed…Fouad Alamee… Date…03-10-2016………..

"Anyone who has never made a mistake has never tried anything new."

Albert Einstein (1879-1955)
Abstract

Synthesis of mechanism probes and potential Inhibitors for Tryptophan 2,3-dioxygenase and Indoleamine 2,3-dioxygenase enzymes

Fouad E. A. Alameer, MSc (University of Sebha)

In biological terms, the kynurenine pathway is considered as a one of the major degradation pathway of tryptophan (L-Trp) in which the first and rate-limiting step in this pathway is the oxidation of L-Trp to N-formylkynurenin (NFK). In spite of the fact that many mechanism have been proposed to explain how the transformation occurs, the mechanism of this oxygen-dependent reaction has not yet been determined. Nonetheless, this type of unique reaction is catalysed by two heme-containing dioxygenase enzymes: tryptophan 2,3-dioxygenase (TDO), and indoleamine 2,3-dioxygenase (IDO), and due to the fact that the overexpression of these enzymes leads to degradation of this amino acid to NFK. From a clinical point view, the breakdown of L-Trp through this pathway leads to the production of a variety of secondary metabolites, such as quinolic acid, anthranilic acid (AA), and 3-hydroxyanthranilic acid (3-HAA). These compounds, amongst others are implicated in a broad range of diseases such as cataract formation, neurological disorders and suppression of T-cells proliferation. In this context, both enzymes apparently present as significant targets for drug intervention. As a result, developing new inhibitors for these enzymes is ongoing. The current work has included the synthesis, purification and characterisation of some heterocyclic compounds. Generating these compounds has relied on indole derivatives as a core component; other species such as alkenes have been used as precursors for generating a cyclopropane ring after the amine groups of the main indole derivatives had been protected by various protection groups. However, a number of difficulties were encountered when trying to remove some of these groups. In application terms, some of these species have been employed as potential inhibitors for both TDO and IDO, in presence of tryptophan as a substrate. The kinetic assays carried out here have shown that there is a slight change in the slope (velocity) for some of these compounds with the enzyme IDO in the presence of L-Trp. as a substrate. This might imply a
reduction in enzyme activity, which could mean some of our compounds were slightly effective, at least to some degree, as inhibitors, whilst others had no effect on the same enzyme. In case of TDO, due to the fact that the amount of protein obtained after purification was insufficient, just a very few of these compounds were tested with this enzyme, however no action was observed.
Acknowledgements

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I would also like to offer my enormous thanks to postdoctoral researcher Dr. Sofia Kapetanaki in the biochemistry lab for her patient and excellent help during the expression and purification of the protein. I would also like to thank Dr. Jaswir Basran for her help and advice with kinetic assays and interpretation of my results, and Dr. Elizabeth Booth for offering some of her pure protein and for using her protocols.

Carrying out this project would not have been possible of course, without the support and patience of my parents, my family and relatives. Lastly, I would like to acknowledge all PhD students in both labs for their help and their friendship.
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<tr>
<td>1-MT</td>
<td>1-Methyl-DL-tryptophan</td>
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<td>3-HAA</td>
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<td>Blood-brain barrier</td>
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<td>DmTDO</td>
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</tr>
<tr>
<td>Abbreviation</td>
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<td>Deoxyribonuclease</td>
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<td>Electron withdrawing group</td>
</tr>
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<td>Electrospray ionization</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>hIDO</td>
<td>Human Indole amine 2,3 dioxygenase</td>
</tr>
<tr>
<td>hTDO</td>
<td>Human Tryptophan 2,3 dioxygenase</td>
</tr>
<tr>
<td>Ki</td>
<td>Inhibitory constant</td>
</tr>
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<td>KYNA</td>
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<td>Kynureninase</td>
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<tr>
<td>KYN</td>
<td>Kynurenine</td>
</tr>
<tr>
<td>KMO</td>
<td>Kynurenine monooxygenase</td>
</tr>
<tr>
<td>KAT</td>
<td>Kynurenine aminotransferase</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Lethal dose</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>MIRC</td>
<td>Michael-initiated ring-closing</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>MTH-Trp</td>
<td>Methylthiohydantoin-DL-tryptophan</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NFK</td>
<td>N-Formylkynurenin</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser effect</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PMB</td>
<td>p-Methoxybenzyl</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>QA</td>
<td>Quinolinic acid</td>
</tr>
<tr>
<td>QPRT</td>
<td>Quinolinic acid phosphoribosyltransferase</td>
</tr>
<tr>
<td>Rm TDO</td>
<td>Ralstonia metallidurans tryptophan2,3 dioxygenase</td>
</tr>
<tr>
<td>S</td>
<td>Seconds</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure activity relationship</td>
</tr>
<tr>
<td>BOC</td>
<td><em>Tert</em>-Butyloxycarbonyl protecting group</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
<tr>
<td>Tos</td>
<td>Tosyl</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>Trp. w</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>T-Cell</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>λ</td>
<td>Wavelength</td>
</tr>
<tr>
<td>Xc TDO</td>
<td>Xanthomonase campestris tryptophan2,3 dioxygenase</td>
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</tbody>
</table>
Chapter 1: Introduction
1. Introduction

1.1. L-Tryptophan (Trp.)

Scheme 1, L-Trp, most animals eat a wide variety of plant foods, which in turn provides them with a full set of essential amino acids. In this context, animal sources such as poultry, eggs meat, fish, milk and cheese provide all the essential amino acids. One of these essential amino acids is tryptophan, which was discovered over 100 years ago when Hopkins and Cole separated it from the pancreatic digest of casein in 1901. Six years later, in 1907, the structure of this compound was elucidated by Ellinger and Flamand, who generated an analogous compound to the one separated by Hopkins and Cole. Moreover, tryptophan is one of the natural amino acids that contains an indole side chain; indole itself can be subdivided into two rings, benzene (a six-membered ring) and pyrrole (a five-membered ring). Furthermore, due to the fact that this compound has a nitrogen atom, the associated lone pair makes the reactivity of this molecule much more sensitive to electrophilic aromatic substitution compared to aromatic systems such as benzene. In mammals, tryptophan is the least abundant of the essential amino acids with a concentration in human plasma of ~40 to 80 µM and in mice ~ 60 to 100 µM. Tryptophan is one out of nine of essential amino acids for the whole mammalian systems; the human body has no capability to produce it, thus this amino acid must be obtained from external sources such as eggs, fish, spinach and soybeans. It is used not only for protein synthesis, but also for the biosynthesis of melatonin and serotonin through the serotonin pathway. In addition, it helps with the formation of NAD$^+$ via the kynurenine pathway; this pathway is responsible for the breakdown of more than 90% of this amino acid, inside and outside cells, whereas just a small amount of Trp, about 1%, is transformed to serotonin. This essential amino acid is generated by bacterial tryptophanase activity, which is responsible for catalysing the condensation reaction between L-serine and indole. The resultant product of this reaction is tryptophan.

Likewise, tryptophan is involved in several metabolic pathways and its metabolites are responsible for regulating neurobehavioral effects like pain perception, appetite, and the
sleep-wake-cycle. In addition, it is the only amino acid that binds to serum albumin at high point.\textsuperscript{6} The majority of dietary tryptophan goes into the synthesis of proteins in living organisms. L-Tryptophan is necessary for T-lymphocyte effector functions, in which the mechanism of breakdown of this amino acid is selective for cancers cells which resist immune destruction.\textsuperscript{11}

10\% of the essential amino acid Trp is transported in its free form around the periphery of blood and this is the only form that can cross the blood-brain barrier (BBB) by means of the competitive and nonspecific L-type amino acid transporter, whereas, the remaining 90\% is transported by binding to albumin.\textsuperscript{12}

1.2. Hemoproteins

Several biochemical functions are achieved by hemoproteins such as electron transfer, gas sensing, oxygen transport, chemical catalysis and storage. For activating both oxygen atoms in the oxygen molecule and their insertion into organic compounds (substrates), the haem iron can be used, and this is commonly found in nature.\textsuperscript{9}

1.3. Discovery of Tryptophan 2,3-dioxygenases (TDO) and Indoleamine 2,3-dioxygenases

In the 1930s, tryptophan 2,3-dioxygenase was initially discovered "and described as being both eukaryotic and prokaryotic".\textsuperscript{13} Tryptophan pyrrolase was initially detected in 1936 in rat livers, and was later renamed as tryptophan 2,3-dioxygenase. In 1955, it was first described in the rat liver and then was characterized in many other organisms. In 1963, Hayaishi and co-workers discovered IDO in the small intestine of rabbits, and this group were able to show this enzyme can catalyse the oxidation of L-tryptophan in order to convert it to kynurenine in the same manner as TDO.\textsuperscript{14} In contrast, indoleamine 2,3-dioxygenase was first isolated in 1967 from rabbit intestines.\textsuperscript{6}

The initial account of haem dioxygenase enzyme was written over 70 years ago.\textsuperscript{15} However, during the period from the 1970s to the 1980s an enormous number of studies have reported the kinetic and spectroscopic properties of these enzymes.\textsuperscript{15} Brief information about this earlier haem-containing dioxygenase literature was published in
1996. At that time, there was insufficient information regarding “recombinant expression systems for dioxygenases” and, as a consequence, no informative X-ray crystallographic structure has been published to date.

1.3.1 Tryptophan 2,3 dioxygenases (TDO)

Figure 1 shows the crystal structure of hTDO lacking the haem cofactor. Sequence alignment points out that human tryptophan 2,3-dioxygenase (TDO) shares about 60% sequence identity with TDO from Drosophila melanogaster Dm TDO, and about 30% identity with Ralstonia metallidurans tryptophan2,3 dioxygenase Rm TDO and Xanthomonas campestris tryptophan 2,3 dioxygenase Xc TDO. Generally, tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11), is found in several bacterial species, mosquitoes and almost all animals; however, it is not found in fungi. TDO is expressed predominantly in mammalian livers and skin. However, when it is stimulated, it can also be detected in many other tissues such as the brain, epididymis, testis, placenta, the uterus during pregnancy and in rat skin, which contributes to the generation of NAD$^+$ through the kynurenine pathway by breaking down dietary tryptophan and also keeping the level of tryptophan in blood stable. In the mammalian livers, tryptophan 2,3-dioxygenase is fundamentally expressed for regulation of the level of tryptophan supply, in which more than 90% of the total L-tryptophan is catabolized. The most important feature of this enzyme is that it is a homotetramer of ~190 kDa in eukaryotes and ~120 kDa in prokaryotes. It is exclusively found in the liver in mammals, the same place where it was characterised for the first time. It is highly specific for L-tryptophan and a few of its analogues, especially those which have substituents in the 5- and 6- positions of the indole ring. TDO is induced by glucocorticoids, tryptophan, and kynurenine.
Tryptophan 2,3-dioxygenase is present in mouse and rat in addition to humans and other mammal species. TDO is also found in bacteria and mosquitoes. Moreover, one of TDO functions, is the regulation of systemic tryptophan levels in the liver.

1.3.2 Indoleamine 2,3-dioxygenases (IDO)

Figure 2 illustrates an X-ray crystal structure of human IDO1 with the complete protein, in which the large (red to green) and small (blue to violet) α-helical domains are shown. Also shown is demonstrated the haem prosthetic group complexed to the ligand inhibitor 4-phenylimidazole. In 2006, Sugimoto et al., elucidated the first crystal structure of human IDO1, which contains 4-phenylimidazole PI; this acts as a competitive inhibitor and is interconnected with the haem iron as a distal ligand. Furthermore, this enzyme includes a large and a small α-helical domains; the large domain helix forms a cavity for the active-site haem, whilst the top of this cavity is covered by the small domain. Indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.52) is an extrahepatic enzyme and monomeric at ∼42-45 kDa; it is inducible by interferon-γ through inflammatory stimuli. IDO is omnipresent throughout the human body, except for the liver. A broad range of cells express IDO, such as dendritic cells, endothelial cells, macrophages, B-cells and tumour cells. The activity of this enzyme; has recently been reported in fungi and vertebrates. This enzyme displays the activity toward a large number of substrates such as melatonin, tryptamine, serotonin and 5-hydroxytryptophan. Physiologically, indoleamine 2,3-dioxygenase is significant in terms of maintaining tissue integrity. Despite the fact that both haem dioxygenase enzymes are catalysing the same reaction and have a
significant amount of their amino acid sequences in common; however, their sequence identity is just about 10%.\textsuperscript{13} During gestation, IDO is supposed to have the responsibility for supplying immune protection in order to develop mammalian fetal tissue by lowering the availability of local tryptophan and then suppressing the T-Cell-based immune response.\textsuperscript{30}

1.4. Activation of IDO1’s dioxygenase

To activate this enzyme, an electron is required to reduce the ferric form (Fe$^{\text{III}}$) of haem to an active ferrous (Fe$^{\text{II}}$) form, which leads to the facilitation of the binding of oxygen and L-tryptophan to shape an active ternary complex. Based on equilibrium binding studies, Sono \textit{et al.} suggested that the ferrous enzyme binds first with L-Trp, followed by O$_2$. On the other hand, recent studies involving the combination of electrochemical and kinetic measurements have illustrated that "at low concentrations of L-Trp, O$_2$ binds first followed by L-Trp binding, but, at higher L-Trp concentrations, the binding order is reversed".\textsuperscript{6,31,32}

1.5. Comparison between three haem-containing dioxygenase enzymes involved in tryptophan catabolism

Tryptophan is catabolised in the first and rate-limiting step of the kynurenine pathway by cleaving the carbon-carbon double bond across the 2- and 3- positions of the indole ring as shown in scheme 2.\textsuperscript{33} This step can be done by one of the three dioxygenase enzymes called indoleamine 2,3-dioxygenase 1 (IDO1), tryptophan 2,3-dioxygenase 2 (TDO2) and indoleamine 2,3-dioxygenase 2 (IDO2) respectively, all of which have a prosthetic group (haem), which has to be reduced in order to obtain optimal enzymatic activity.\textsuperscript{34}

Comparing IDO1 and IDO2, both species can be seen to have an approximately 43% similarity in terms of amino acid level; whereas TDO2 is not homologous to the IDO enzymes in terms of its protein sequence.\textsuperscript{34} In mammalian systems, IDO1 and IDO2 are isoform and probably grow during gene duplication. Conversely, other vertebrates such as amphibians, birds and fish, seem to have just one IDO whose sequence is more comparable to mammalian IDO2.\textsuperscript{34} Despite these three forms catalysing the same
reaction, biochemical characterisation and expression style suggests that the biological function for each enzyme is distinct from the others.\textsuperscript{34}

Tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) are heme-containing proteins, both of which are responsible for cleaving the pyrrole ring of L-tryptophan (L-Trp) by inserting both the oxygen atoms from the dioxygen molecule into the organic substrate to give N-formyl kynurenine as shown in scheme 2. The oxidative cleavage of tryptophan is the first and rate-limiting step in L-Trp catabolism via the kynurenine pathway.\textsuperscript{35}

\begin{center}
\includegraphics[width=0.5\textwidth]{scheme2}
\end{center}

\textbf{Scheme 2}: The oxidation of L-tryptophan through the kynurenine pathway by IDO and TDO

The N-formyl-kynurenine thus formed is a secondary metabolic route for the formation of bioactive metabolites, such as kynurenine, kynurenic acid, 3-hydroxy-kynurenine, quinolinic acid, and others, as in scheme 3, which are involved in several number of neurological disorders like Parkinson's disease, cerebral ischemia and Alzheimer's disease.\textsuperscript{36,37}
1.6. Clinical perspective

From a clinical point view, the mechanism of oxidation of L-tryptophan via the kynurenine pathway is of fundamental interest due to degradation of this amino acid leading to the generation of a number of secondary metabolites such as anthranilic acid, 3-hydroxanthranilic acid, quinolinic acid and 3-hydroxykynurenine. These are implicated in a broad range of diseases such as suppression of T-cell proliferation, neurological disorders such as Alzheimer’s disease that are caused by great production of quinolic acid, age-related cataract formation “due to kynurenilation” and...
neurotoxins,\textsuperscript{5,39} and, also, IDO is appearing as significant modern therapeutic target for the treatment of illnesses such as tumours and viral infections.\textsuperscript{40} Furthermore, IDO supports a wide range of chronic infections, involving parasitic, viral and bacterial infections, such as the human immunodeficiency virus (HIV), malaria, \textit{Toxoplasma gondii} and chlamydia hepatitis C.\textsuperscript{41} Not only these, but recent, studies \textit{in vivo} have shown an important role for IDO in immune regulation via promotion of immune tolerance by suppressing local T-cell responses under different physiological and pathophysiological conditions, involving autoimmunity, mammalian pregnancy, chronic infections, tumour resistance and chronic inflammation.\textsuperscript{42}

1.7. “Escape of tumour cells from immune surveillance by IDO expression Immune”

A crucial feature of cancer progression is evasion of the immune system, as the majority of human malignant cancer cells express IDO. This was discovered by Uytterhove \textit{et al}, in 2003.\textsuperscript{5} This research group showed that localised degradation of tryptophan in host animals, occurs due to tumour cells expressing a high degree of indoleamine 2,3-dioxygenase, which in turn effectively facilitates the cancer escaping immune surveillance (when a mouse model system was used),\textsuperscript{5} whereas the same tumour cells expressing a small amount or no IDO can be easily distinguished by the host animal’s immune system and then rejected.\textsuperscript{5} This was shown by employment of siRNA to knock out IDO expression in tumour cells which re-established the antitumor immunity.\textsuperscript{5} The above discovery showed that extension of expression of IDO in tumour cells can be utilized for prediction of such cancers. Recently, some studies have displayed that the expression of high level of IDO in cancers cells can be used as positive sign that advance prediction of colorectal cancer,\textsuperscript{43} endometrial cancer\textsuperscript{44} and ovarian cancer\textsuperscript{45} will be poor.

1.8. \textbf{The relation between induction of IDO, accumulation of quinolinic acid (QA) and Alzheimer’s disease (AD)}

Globally, millions of people are affected by one of the major widespread forms of mental disease called Alzheimer's.\textsuperscript{5} Guillemin \textit{et al.} reported that increased level of the neurotoxin quinolinic acid occurs due to upregulation of the kynurenine pathway in an
afflicted person’s brain when immunostaining was used, in spite of the fact that the aetiology for AD is still unknown. Both QA and expression of IDO are revealed in different types of cells such as neurons, astrocytes and cortical microglia. Rogers et al. took into account that progression of AD being related to inducing IDO in different kinds of brain inflammation, brain and in others cases of complex and multiple inflammations.

1.8.1 Some chemical properties of dioxygen

In aerobic life, dioxygen serves two fundamental roles, in that it can act as both a biosynthetic reagent and a terminal electron acceptor, these lead to the breakdown of organic compounds (substrates) and result in the incorporation of one or two oxygen atoms into the substrate; this process includes the splitting of the oxygen-oxygen bond, which is energetically favourable. In other words, it is exothermic. Although this is a strong thermodynamic driving force, the chemical reactivity of dioxygen with organic compounds at standard temperatures is very low. If this was not the case, dioxygen would spontaneously oxidize organic molecules and should therefore be damaging or even cause death rather than being useful to living organisms.

1.8.2 Inhibition of TDO and IDO enzymes

Presumably, inhibition of the IDO enzyme may enhance the efficacy of cancer treatments, due to several human cancers expressing this enzyme, in which a rising level of human indoleamine 2,3-dioxygenase in cancerous cells is connected to a poor prognosis for survival in many tumour types. The discovery of IDO inhibitors presents certain challenges, such as their active site topology being responsible for their resistance to a high degree of inhibition processes. The small size of IDO leads to the prevention of large inhibitor compounds from binding. Whilst the kinetics of the inhibition by IDO are not completely understood, many IDO inhibitors have been reported to bind IDO in a non-competitive manner, whilst others are competitive. There is considerable interest in the identification of low molecular mass chemical inhibitors of the IDO enzyme, and in this context the ability of certain tumours to escape the immune response. This is due to IDO, when expressed by tumour cells or tolerogenic dendritic cells and tumour-associated macrophages and has the ability to
contribute to a pathological state of immune tolerance toward tumour-associated antigens. Based on this, inhibition of IDO restores the antitumor immune response and represents an efficient adjuvant for chemotherapeutic and immuno-therapeutic antitumor strategies.\textsuperscript{51,42}

Predominantly; the kinetic experiments of IDO inhibitors have yielded non-competitive or uncompetitive modes of inhibition, even though there has been, in one instance strong evidence appearing in the case of 4-phenylimidazole (PIM),\textsuperscript{52} that they bind directly to the active site of the enzyme and should thus act as competitive inhibitors with respect to the substrate L-Trp.\textsuperscript{52,53,54,55,56} On the other hand, in terms of interpretation of the kinetics, inhibition of IDO might be complex via preferential binding of some inhibitors to the inactive ferric form of IDO1 and by the presence of dioxygen, as a second substrate.\textsuperscript{56}

Frédérick \textit{et al.} proposed that tryptophan 2,3-dioxygenase will also be expressed in several types of cancer cells such as hepatic, breast, colorectal, melanoma, bladder and lung. The expression of TDO in cancerous cells has a similar impact as the expression of IDO; both of have the capability to prevent the rejection of tumour cells due to local degradation of tryptophan when a murine model of cancer is used.\textsuperscript{13}

In this context, TDO and IDO have emerged as significant medicinal targets. As a result, the evolution of new inhibitors for these enzymes is still ongoing,\textsuperscript{39} such as 1-methyl-DL-tryptophan(1-MT) \textbf{3} and INCB024360 namely (E)-4-amino-N’-(3-chloro-4-fluorophenyl)-N-hydroxy-1,2,5-oxadiazole-3-carboximidamide \textbf{6}.\textsuperscript{57} There are also other more potent inhibitors which are used to inhibit IDO, such as brassinin \textbf{4}, methylthiohydantoin-DL-tryptophan (MTH-Trp) \textbf{5}, an analogue of a natural product of marine sponges \textbf{7} and 3-butyl-β-carboline \textbf{8}.\textsuperscript{57} Scheme \textbf{4} shows the chemical structure of these inhibitors.\textsuperscript{57}
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The tryptophan derivatives which have the substituents on the benzene ring are able to act as inhibitors for IDO. The reaction between serine and indole in the presence of acetic anhydride was the first workable synthesis of tryptophan derivatives that contained substituents on the benzene ring to be reported. Through this study, the researchers had developed a workable synthesis of benzo tryptophan, and tried to synthesize analogues in order to discover new inhibitors for indoleamine 2,3-dioxygenase. As result, tryptoline derivatives (MTH-benz[e]tryptoline, 9, MTH-benz[f]tryptoline, 10, and MTH-benz[g]tryptoline, 11, have been discovered to act as novel inhibitors for the IDO enzyme. Scheme 5 shows the chemical structure of these novel inhibitors for IDO.

Scheme 4: The six IDO inhibitors that have been reported

Scheme 5: Chemical structure for some novel IDO inhibitors
Table 1: Shows some of IDO inhibitor properties

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name and numbers</th>
<th>Ki (µM)</th>
<th>Mod of inhibition</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure" /></td>
<td>Annulin A 12</td>
<td>0.12</td>
<td>Unknown</td>
<td>58</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure" /></td>
<td>Necrostatin-1 13</td>
<td>11.4</td>
<td>Competitive &amp; Noncompetitive</td>
<td>59</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure" /></td>
<td>Norharman 14</td>
<td>120.0</td>
<td>Noncompetitive</td>
<td>5</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure" /></td>
<td>NSC401366 15</td>
<td>1.5</td>
<td>Competitive</td>
<td>59</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure" /></td>
<td>Exiguamine A 16</td>
<td>0.20</td>
<td>Unknown</td>
<td>30</td>
</tr>
</tbody>
</table>
Thomas et al. reported that Ebselen drug (2-phenyl-benzo[d]isoselenazol-3-one) changes the active heme site of recombinant human rhIDO by inducing a transition of ferric heme iron, from its mainly high spin to a low spin form and by reducing the vibrational frequency of the Fe-CO stretch of the CO complex; which indicated an opening of the distal haem pocket, as elucidated by resonance Raman spectroscopy.

The valuable structural information elucidating the co-crystal structure of IDO presented by Yang et al. found that 1H-1,2,3-triazole may be a new key pharmacophore of potent IDO inhibitors due to this compound having a similar structure to 4-phenylimidazole (4-PI), with only a small difference which involved replacing the imidazole ring by a triazole ring. This in turn could have a wide range of applications in agrochemicals and pharmaceuticals. For these reasons, researchers have sought to synthesize different series of 1,2,3-triazoles, and also to screen for high efficacy IDO inhibitors. Recently, studies on the relationship between IDO inhibitory activity and the structure activity relationship (SAR) of 4-aryl-1H-1,2,3-triazole derivatives was carried out.

Table 2: Inhibitory activity of triazole derivatives

<table>
<thead>
<tr>
<th>Structures</th>
<th>Compounds</th>
<th>IC50(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Structure 1]</td>
<td>17</td>
<td>143</td>
</tr>
<tr>
<td>![Structure 2]</td>
<td>18</td>
<td>N.I</td>
</tr>
<tr>
<td>![Structure 3]</td>
<td>19</td>
<td>6000</td>
</tr>
<tr>
<td>![Structure 4]</td>
<td>20</td>
<td>1028</td>
</tr>
</tbody>
</table>
Table 2 shows sixteen compounds used to examine IDO inhibition alongside their IC$_{50}$ values. As can clearly be seen, some of the compounds displayed strong inhibitory activity for IDO, namely the 4-aryl-1H-1,2,3-triazoles compounds 17, 22 and 24, which showed stronger IDO inhibitory activity compared with 1-Me-L-Trp. The IC$_{50}$ of 1-Me-L-Trp is 380 µM, whilst the IC$_{50}$s for the three mentioned 4-aryl-1H-1,2,3-triazole compounds in the this study showed values of 143, 148 and 86 µM, respectively.$^{55}$ The position of the substituent on the 4-aryl-1H-1,2,3-triazole compounds 17-27 and their electronegativity seem to be a very important factor in the IDO inhibitory activity, in which “an electron withdrawing group with low steric hindrance near the free NH of triazoles was essential for IDO inhibition”. Conversely, compounds 28–30 that have substituent on the triazole demonstrated no, or entirely fortuitous, activity towards the enzyme, while compounds 31 and 32 showed improved inhibitory potency even without considering the substituent on the triazole ring.$^{55}$

In 2011, Frédérick et al. reported on structural activation studies on a chain of 3-(2-(pyridyl)-ethenyl)-indoles, for which over seventy new derivatives were generated and their TDO inhibition potency was estimated, leading to the discovery of the very promising molecule 33, which exhibited a good inhibitory potency towards TDO with Ki = 5.5 µM; also, this compound showed good oral bioavailability. As consequence, 33 was selected for preclinical estimation.$^{13}$
Compound 34 was discovered and characterized by Frédérick et al. and can be described by an indole nucleus substituted in the 2-position by a 2-pyridinyl-ethanone functional group, which gave the best result (IDO IC$_{50}$ of 65±7) compared with the inhibitory potency of 1MT, which gave an IC$_{50}$ of 100±20 with the same enzyme. An automated GOLD program was carried out for an initial docking study of 34 within the IDO binding cleft, which showed the necessary fundamental features for stabilizing 34 in the IDO binding cleft as follows; (i) the indole ring is stabilized in the lipophilic A-pocket of IDO; and (ii) the oxygen atom of the ketone of 34 is located ~2 Å over the plane of the heme group which coordinates to the haem iron. The stability of the indole ring in the pocket A has been already proposed by another group. “In this position, the 3-pyridyl group is projected towards the entrance of the active site and is stabilized in the aromatic Pocket B through T-shape interactions with Phe163 and Phe226.” As can be seen in Fig.3 structural adjustments of 34 were concentrated on the exploitation of three particular binding interactions with IDO: (i) the haem iron binding group, (ii) Pocket B occupied by an N-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer molecule in the crystal structure; and. (iii) via particular interactions with C129 and S167 in Pocket A of the interior of the active site.
In 2014, Pantouris and Mowat screened ~2800 compounds at the National Cancer Institute, USA, in which seven potent reversible competitive inhibitors for TDO were identified with inhibition constants ranging between the nanomolar and low micromolar. All of which appear to have antitumour properties, which have the capability to kill several cancer cell lines. In comparative terms, the inhibition potencies for these seven compounds were tested against IDO, and their inhibition constants have been reported in the literature.

In this context, scheme 7 shows these seven reversible competitive inhibitors for TDO. To begin with, the class of compound to which they belong is that of flavonoids. NSC 36398 (35) (dihydroquercetin, taxifolin) is considered one of most potent inhibitors of TDO of the many flavonoids that were tested, which gave a Ki = 16.3±3.8 µM. In comparison with IDO, NSC 36398 (35) did not show any inhibition activity, even when its concentration was increased up to 100 µM. Due to the lower toxicity of flavonoids, and because of their previously reported anticancer role, NSC 36398 (35) is thus an attractive therapeutic target for cancer intervention. NSC 36398 (35) or (dihydroquercetin, taxifolin), is the first TDO-selective inhibitor reported with an in vitro Ki of~16 µM.
Also, in addition to NSC 36398 (35), β-lapachone or NSC 26326 (36) is a natural occurring quinone that was isolated from the lapacho tree (Tabebuia avellanedae). A number of studies have shown that this compound has an effect on survival rate of tumours cell types such as colonic, retinoblastoma, breast, leukaemia, pancreatic and non-small cell lung tumours, with IC\textsubscript{50} and LD\textsubscript{50} values ranging from 1–4 µM depending on cell type.\textsuperscript{19} Amongst all inhibitors presented in scheme 7, NSC 26326 (36) was found to be the strongest inhibitor of both TDO and IDO, with inhibition constants Ki =\textasciitilde30–70 and 97±14 nM respectively. 3-deazaguanine, or NSC 261726 (37), showed inhibitory activity towards IDO and TDO in the lower micromolar ranges with Ki values of 5.6±0.4 and 21.4±2.4 µM for TDO and IDO, respectively; this type of inhibitor is active in colonic and leukemic tumour cell lines.\textsuperscript{19}

Nanaomycin, NSC 267461 (38) is used as an antibiotic for animals. It is a naphthoquinone-based inhibitor, which inhibited TDO and IDO with Ki values of 360±30 and 950±270 nM, respectively. Nanaomycin is active in 59 tumour cell lines, killing several types of cancer cells with IC\textsubscript{50} values between 400 nM to 4 µM depending on tumour cell type.\textsuperscript{19} Furthermore, NSC 111041 (39), examined regarding colonic and breast cancer cell lines showed inhibitory activity for TDO and IDO with inhibition constants of 4.3±0.9 and 1.1±0.3 µM, respectively.\textsuperscript{19}

17-aminodimethoxygeldanamycin, NSC 255109 (40), is also a strong inhibitor for both TDO and IDO, showing low inhibition constants ranging between the nanomolar and micromolar levels (600±70 nM, and 1.4±0.5 µM, respectively).\textsuperscript{19} Examination of cancer cell lines displayed that NSC 255109 (40) is active in 65 cell lines, with IC\textsubscript{50} values between ~200 nM and 8 µM in breast cancer cell lines, and the types of tumour cells that are affected by this compound are leukemic, non-small cell lung, prostate, melanoma, central nervous system, colonic, breast, renal and ovarian cancers.\textsuperscript{19}

Mitomycin C (41) is one of the compounds identified through screening, as having a confirmed oncological affect with action in 74 cancer lines. Mitomycin C (41) has been identified as an IDO inhibitor with a Ki = 24.2±1.2 µM. Noticeably, Mitomycin C is an approximately 8-fold more potent inhibitor of TDO than IDO, with an inhibition constant for the latter of Ki = 2.86±0.03 µM.\textsuperscript{19} Scheme 7 gives the chemical structures of for these seven potent inhibitors.
A new type of phenyl-benzene sulfonyl hydrazide has been identified by Shau-Hua Ueng et al. as a potent indole amine 2,3-dioxygenase (IDO) inhibitor whose activity relationship has been determined. The appropriate reactions to make these were carried out between phenyl-hydrazides and benzenesulfonyl chlorides fructification on several substituents. One of the most potent inhibitors found was compound 42, which showed an IC$_{50}$ of 61 nM in enzymatic assay and 172 nM in HeLa cell. Computational studies proposed that the major interaction between this molecule (inhibitor) 42 and the IDO enzyme occurred via coordination between haem iron of IDO and one of the oxygen atoms of the sulfone group of 42, with a distance of 2.04 Å. The other oxygen atom of the sulfone group established with chain group of Ala264. Compound 42 occupied both pocket A and pocket B, so the binding conformation adopts a V-shape, binds to the enzyme as shown in figure 4. In addition, a hydrogen bond is formed between the benzoic acid group of 42 and Cys 129, and hydrophobic contacts with the surrounding residues in pocket A, involving Try126, Cys129, Phe164, Gly262, Ser 263 and Ala 264 occur. The bromide group of phenyl-hydrazine extends into the hydrophobic cavity of pocket B, which is formed by Phe163, Phe226, Arg231, Leu234, Ile354 and the heme ring.
In 2014 Moody et al. also, reported that collections of benzofuranquinones, analogues of the marine metabolite annulin A were generated and their inhibitory activity were evaluated against human indoleamine 2,3-dioxygenase (IDO). 5-methoxy-2-methylbenzofuranquinones containing a CH$_2$OR group at position-3 were the most potent compounds with IC$_{50}$ ~ 0.2 µM, were discovered. In addition, to their discovery they found out also that the compounds which relied on the benzimidazolequinone structures also acted as inhibitors for IDO. Scheme 8 shows the chemical structure for 5-methoxy-2-methylbenzofuranquinones containing a CH$_2$OR group at position-3.
1.9. Previously proposed mechanisms for Heme Dioxygenase

The initial proposal for the mechanism of tryptophan oxidation shown in scheme 9a was suggested over 40 years ago, and has been found to be broadly reproducible.

Scheme 9: Several initial steps from different proposed reaction mechanisms of oxidation of tryptophan for heme dioxygenases

Scheme 9a shows the mechanistic abstraction via the base catalyst. Alternatively, 9b shows the abstraction of protons through binding with O₂ instead of the base-catalysed mechanism. Pathway 9c shows electrophilic addition reaction. Whilst, 9d gives a possible radical addition reaction. The initial mechanisms in the scheme 9a and b have been suggested based on computational work, mass spectrometry, crystallography and mutagenesis and it is unknown yet if the addition takes place at C3 or C2, most likely be processes related to mechanisms a and b in scheme 9, both of which have been suggested in the literature.

Furthermore, the formation of an epoxide, as shown in scheme 10, has been envisaged via two possible routes; (i) following route 2a as an electrophilic reaction mechanism or (ii) through route 2b as a radical addition reaction, both of which would result in the formation of an epoxide. However, neither mechanism has been absolutely confirmed.
Both the steps 2a or 2b are followed by ring opening in the step 3 of the reaction, though ring opening can be concurrent with step 4, the cleavage of the C2 and C3 bond, which leads ultimately and straightforwardly to formation of NFK.\textsuperscript{39}

Scheme 10: Proposed mechanism for the formation of NFK heme dioxygenase\textsuperscript{39,74}

1.10. Overview on cyclopropane amino acids and some methods for preparation of cyclopropane, its derivatives and their roles in biological and medicinal chemistry

Amino acids that contain a cyclopropane ring are an important class in terms of biologically active compounds owning to their unique properties.\textsuperscript{75} Recently, cyclopropane amino acids have shown promising effect in therapeutic terms, due to their intervention to treat several types of neurological diseases and disorders. Therefore, considerable research efforts have been invested in order to identify new drug candidate within this scope.\textsuperscript{75}

The synthesis of cyclopropane-containing analogues of L-tryptophan such as 2,3-methano-L-tryptophan has attracted much attention by many research groups, with difficulty usually being encountered when trying to remove protecting groups without causing vinylogous opening of the cyclopropane ring.\textsuperscript{76}
The angle strain in cyclopropane is due to the compression of the usual 109.5° tetrahedral bond angle of \( sp^3 \)-hybridized carbon atoms to that of planar bonding at 60°. This “severe angle strain leads to nonlinear overlap of the orbitals and bent bonds”.\(^ {77} \)

Bonding of strained cyclic compounds is now well understood and the release strain of about 110 kJ/mol in cyclobutanes and 115 kJ/mol in cyclopropanes has been exploited in various synthetic endeavours.\(^ {78} \)

The cyclopropane unit has been found to be a fundamental structural element in several important natural products\(^ {79} \) and non-natural compounds \(^ {80} \) and this component is a common feature in medicinal chemistry.\(^ {81} \) Due to its unique reactivity, the three-membered ring can be employed as an intermediate in the synthesis of molecules with increased complexity or as a diverse building block.\(^ {82,83} \) In 1885, Adolf von Baeyer, introduced the concept of ring strain, which reflects the higher reactivity of cyclopropane compared with its unstrained congeners.\(^ {78} \) In other words, some molecules such as cyclopropane are forced by geometry to have non-ideal bond angles i.e. are strained, which means that the bonds are weaker compared with the molecules that have ideal bond angles. This leads to increased reactivity and decreased thermodynamic stability.\(^ {84} \)

Furthermore, cyclopropane derivatives are found to occur naturally, being central to certain biological activity and having extensive biological utility.\(^ {85} \) Consequently, synthetic studies of the cyclopropane ring remain important. A number of workable methods to construct cyclopropanes from olefin groups have been achieved in the presence of free carbene generated from haloforms using appropriate bases.\(^ {86} \) Amongst these cyclopropanation methods is the Simmons-Smith reaction,\(^ {87} \) which is considered
as the most common useful reaction of this type, with diazo-olefins utilizing an Rh-catalyst.88 On the other hand, broadly speaking, cyclopropane formation requires electron-rich alkenes, due to the fact that these types of cyclisation reactions occur through electrophilic means. However, it is both interesting and challenging, to try to form cyclopropanes via radical pathways because they are thermodynamically disfavoured due to carbon-centred radicals being nucleophilic and due to the associated ring strain.89 Thus, electron-deficient alkenes are required.90

Due to the fact that a variety of the compounds that contain cyclopropanes are biologically active, chemists have frequently attempted to discover new and simple methods to synthesise these classes of compounds.91 Therefore, the methodology for the synthesis of cyclopropane derivatives is a chemical challenge that has stimulated much activity, with the ultimate goal of establishing a synthetic route to a basic structural unit that is present in a broad range of naturally occurring compounds.92 Furthermore, these intermediates are very useful due to their unusual strain and bonding. The general methodology required to create three-membered ring construction supply relatively few highly-substituted cyclopropanes with high stereo- and regio-selectivity.92 One well-known method to synthesise cyclopropanes is the Corey-Chaykovsky reaction,91 which relies on the abstraction of a single proton from trimethylsulfoxonium iodide by bases such as NaH or KOH to form dimethylsulfoxonium methylides, which lead ultimately to addition CH₂ to the α,β-carbons of the olefin resulting in cyclopropane, as shown in scheme 11.

![Scheme 11: The Corey-Chaykovsky reaction](image)

Furthermore, Michael-initiated ring-closing (MIRC) reactions have been successfully applied to obtain cyclopropanes,93 as shown in scheme 12.
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Scheme 12: MIRC path to obtain intensively functionalised three-membered ring (cyclopropane)

In spite of the fact that the approaches require [2+1] cycloaddition, which involves the addition of carbenoid or carbene to an alkene (decomposition of diazo compounds, and Simmons-Smith reaction) are broadly employed to synthesise cyclopropane products in these types of reactions.\(^{92}\) The last reaction involves the treatment of alkenes with a zinc-copper couple and methylene iodide.\(^ {87}\)

In addition, powerful tools for assessing reaction mechanisms are radical clock reactions.\(^ {94}\) In the recent decades, interest in organic radical chemistry has increased tremendously, synthetic methods for employing radicals in chemical synthesis have been considerably refined and the occurrences of radical intermediates which occur in biologically productive operations have become better understood. Interestingly, and an over the past several years, the use of radical reactions in organic chemistry has rapidly increased. It has become increasingly clear that the presence of radical intermediates can be established for a number of important biological reactions.\(^ {95,96}\)

Scheme 13 shows that cyclopropylmethyl radical ring opening to afford the but-3-enyl radical with a rate constant at 37 °C of $1.2 \times 10^8 \text{ s}^{-1}$; the rapidity with which this class of ring opening reaction occurs has resulted in its use in a wide range of cyclopropane rings as a probe reaction mechanism in both chemical and enzymatic catalytic field reactions.\(^ {89}\)

EWG = electron withdrawing group as COAr, CN, R\(_1\) = aryl, R\(_2\) = alkyl, aryl, OMe,

Scheme 13: The ring opening of the cyclopropylmethyl radical
1.11. Enzyme and Enzyme inhibition

An enzyme is a protein that acts as a catalyst to accelerate enzymatic reactions. These are fundamental for the appropriate propagation and preservation of any organism where a necessary reaction product could only otherwise be produced at too great an energetic penalty or timescale. These characteristics make enzymes excellent candidates in the resistance to illnesses of both pathogenic and genetic origin. In this context, the single target of molecular medicine is that of developing and implementing efficient means to adjust the activity of different enzymes involved in fundamental biological pathways. Enzyme inhibition processes require knowledge of the enzyme-substrate reactions affected by any inorganic metal, organic chemical compounds or biosynthetic compounds due to the fact that their interaction is through covalent or non-covalent bonds within the active sites of enzymes.

1.11.1 Enzyme inhibitors and their classifications

Enzyme inhibitors are chemical compounds of low molecular weight that have the capability to reduce or completely inhibit enzymatic activity either irreversibly (i.e. permanently) or reversibly. Inhibitors can be designed to modify one or more amino acid side chains, which are required for the catalytic effect of enzyme activity. In addition, enzyme inhibitors have many forms: A) inhibitors of a specific ion cofactor, such as for enolase enzyme which uses fluoride for chelation; B) coenzyme inhibitors, such as hydroxylamine, cyanide and hydrazine which inhibit dicumarol which in turn is a competitive antagonist for K vitamins and pyridoxal; C) physiological modulators of reactions, including temperature and pH, that denature the enzyme catalytic site; D) prosthetic group inhibitors, such as cyanide, that are used to inhibit the heme prosthetic group of cytochrome oxidase; and E) apoenzyme inhibitors: “that attack the apoenzyme component of the holoenzyme”.

Apoenzyme inhibitors, (E), are divided into two classes; 1) Irreversible inhibitors; due to the fact that these have the capability to make inactivation irreversible via covalent modification of an essential residue of the enzyme, and 2) reversible inhibitors whose action reversible when associated with the enzyme. Furthermore, apoenzyme inhibitors
have an impact on $V_{\text{max}}$ and $K_m$. One class of reversible apoenzyme inhibitors are metabolic antagonists, which can be divided into three unique subtypes:

i) competitive, ii) non-competitive iii) uncompetitive and/or vi) the associated mixed type.\(^{97}\)

1.12. Aims of the project

1- Synthesis of a set of small organic compounds in order to test them as potential inhibitors for two limited enzymes (TDO and IDO). All synthesised compounds will be based on an indole ring as their core structural feature.

2- Expression and purification of the enzymes: (human tryptophan 2,3-dioxygenase, TDO, and indoleamine 2,3-dioxygenase, IDO).

3- Running kinetic assays for each one of these enzymes with L-tryptophan as substrate as well as some of the synthesised molecules (potential inhibitors), in order to measure enzyme activity with and without these compounds.

Scheme 14, shows the proposal as to how the potential cyclopropane compounds may inhibit the TDO and IDO enzymes, based primarily on the mechanism reported by Raven et al. in 2011.\(^{39,74}\) This explains how these enzymes break down L-Trp based on the free radical mechanism pathway, as shown in scheme 10, and secondly would also provide some additional proof for the radical-based proposal,\(^{89}\) as shown in scheme 13.

![Scheme 14](image-url)  

**Scheme 14:** proposed inhibition mechanism by our potential inhibitor for both dioxygenase enzymes
Chapter 2: Results and discussion

*Synthesis of α,β-unsaturated ethyl esters attached to the C3 position of the indole ring and their use in generating cyclopropane derivatives*
2. Synthesis of α,β-unsaturated ethyl esters attached to the C3 position of the indole ring and their use in generating cyclopropane derivatives

2.1. Introduction

2.1.1 Alkenes

Alkenes are a class of hydrocarbon that contains one or more carbon–carbon double bonds, sometimes referred to as olefins. The overlapping of a \( sp^2 \) hybrid orbital on carbon with the 1s orbital of a hydrogen atom leads to the formation of carbon–hydrogen σ-bonds. The π-bond in ethylene, for instance, is formed from through the overlap of two hybridized \( p \) orbitals on two \( sp^2 \) hybrid carbon atoms; the two ends of the molecule are coplanar, which is required to achieve best overlap. Figure 6 demonstrates how the π-bonding orbital of ethylene is divided into two halves; the first half of this orbital is over the carbon–carbon σ-bond, whereas the second half is under the same sigma bond.

![Figure 6: The \( p \)-orbital of the \( \pi \)-bond of ethylene](image)

There are several methods for the preparation of alkenes which include the Wittig reaction, which involves a nucleophilic attack at the carbonyl group. The substitution in this reaction involves converting the C=O of a ketone or aldehyde to a C=C group by reaction with a phosphonium ylid species. This reaction is shown in scheme 16.
Chapter 2: Results and discussion

Scheme 15: Synthetic route to alken using phosphonium ylid species

\[
\begin{align*}
\text{Ph}_3\text{P} + \text{Ph}_{nA} & \xrightarrow{\text{Mel, NaH}} \text{Ph}_3\text{P} - \text{CH}_2
\end{align*}
\]

Scheme 16: Synthesis of alkenes through the Wittig reaction

The Peterson reaction is a stereospecific elimination reaction. In many organic chemical reactions a group SiMe\textsubscript{3} acts in a similar way of a proton.\textsuperscript{99} The elimination reaction in scheme 17 has taken place through \textit{syn-periplanar} transition state due to the silicon and the oxygen being bonded with each other and because of the strength of this bond leading to the elimination forward.\textsuperscript{99}

Scheme 17: Generating alkenes via the Peterson reaction

Alkenes can also be prepared by elimination reactions in two ways:

1. Dehydration can be achieved when treating an alcohol with a strong acid, which results in loss of a water molecule and, for instance in scheme 18, the formation of 1-methylcyclohexene.\textsuperscript{100}
2- A dehydrohalogenation reaction usually occurs when reacting a strong base, such as potassium hydroxide, with an alkyl halide to, for instance, yield cyclohexene by treating bromocyclohexane with KOH in presence of ethanol as a solvent, as in scheme 19.101

2.1.2 Cyclopropane Synthesis

Generally, cyclopropanes are synthesised from alkenes.102 Organic chemists have always been fascinated by the subunit of cyclopropane,103 in spite of the fact that the cyclopropane ring is a highly strained entity. Nevertheless, it is found in a large number of naturally occurring compounds, such as fatty acid metabolites and unusual amino acids, terpenes and pheromones.103,104 In addition, analogues of cyclopropane from amino acids and other biomolecules are very useful derivatives for studying the pharmacological properties of these molecules. However, organic chemists can be faced with some considerable difficulty carrying out synthetic procedures without causing the three-membered cyclopropane ring to open.76

There are several synthesis methods that have been applied in order to generate cyclopropane and one of these methods is Simmons and Smith cyclopropanation that
was discovered over 40 years ago.\textsuperscript{104} This reaction occurred when treating diiodomethane with alkenes in the presence of activated zinc to afford a high yield of the cyclopropane.\textsuperscript{104,105} There are other ways to prepare cyclopropanes which involve using Zn (Cu) or Zn (Ag) or ethyl zinc iodide / I\textsubscript{2}.\textsuperscript{106} or diethyl zinc iodide,\textsuperscript{104} as in scheme 20.

![Scheme 20: Synthetic route to generate cyclopropane by Simmons Smith reaction, and others](image)

A second method that can be used employs carbenes which are of the form R\textsubscript{2}C: this is a neutral molecule that contains a divalent carbon. Thus, they have only six electrons in their valence shell. Carbenes acts in a similar manner to electrophiles, which are electron-deficient, as the reaction occurs in just a single step without the formation of intermediates.\textsuperscript{107} The three-membered rings (i.e; the cyclopropane rings) are formed by addition of carbene to an olefin, as shown in scheme 21.\textsuperscript{108}

![Scheme 21: General route for synthesis of cyclopropane from a carbene R and R' = Cl, H](image)

Thirdly, cyclopropane can also be made by conversion of electron-deficient alkenes when its reacted with a mixture of Me\textsubscript{3}S(O)I and NaH or KO\textsubscript{t}-Bu or KOH at 50 – 60 °C in THF or DMSO.\textsuperscript{108}
Recently, cyclopropane amino acids (Δ-AA) have attracted a great deal of interest due to many types of molecules being identified as naturally occurring, which has afforded structural modifications aimed at improving their potency and selectivity. Increasingly, the cyclopropyl group is introduced into the side chain of proteinogenic amino acids, frequently in the C2-C3 position. Currently, one of the more active research areas is conformationally restricted amino acids, which have the ability to be incorporated in peptides in place of the corresponding amino acid. Therefore, they become stereochemical probes for selective interactions or bioactive conformations and, also, they are able to increase binding stability to show enzyme or receptor inhibiting properties, or grant improved metabolic stability.

Scheme 23 shows the exception for 2,3-methanol analogues of valine, proline and pyroglutamic acid, in which each protein amino acid cyclopropane analogue exists in diastereomeric Z- and E-forms, where the functional characteristic at the β-carbon atom of the specific amino acid is cis to the carboxyl, or to the amino function, respectively.

\[
\begin{align*}
\text{Z-Isomer} & & \text{E-Isomer} \\
\text{H} & & \text{H} \\
\text{COOH} & & \text{COOH} \\
\text{NH}_2 & & \text{NH}_2 \\
R & & R
\end{align*}
\]

Scheme 23: Diastereomeric Z and E, geometric isomers of cyclopropane amino acid

2.2. Synthesis of (Z)-Δ–tryptophan

A synthetic route to (±)-(Z)-Δ–Trp. 79, is depicted in scheme 25. Firstly, hippuric acid was prepared according to the conditions shown in scheme 24.
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Scheme 24: Addition elimination reaction pathway for generating 71

Sodium hydroxide was employed as a reagent for converting glycine from an insoluble amino acid compound in water to its (soluble) sodium salt. This kind of reaction proceeds via an addition elimination reaction mechanism, in which the reaction starts with nucleophilic attack at the C=O group of benzoyl chloride by the amine lone pair of glycine, followed by elimination of the good leaving group to afford an 84\% yield of product 71, which was somewhat lower than that reported in the literature,\textsuperscript{113} which was 96\%. Figure 7 shows the X-ray crystallography for compound 71, which was crystallised by dissolving it in acetonitrile with a few drops of chloroform, which was then left to evaporate slowly.

As has already been mentioned, the goal beyond synthesis of 71 was to use it as a starting material with the commercial 1H-Indole-3-carbaldehyde, 72, in order to generate 73, which was to be used as a precursor to synthesise 74, as per the reactions presented in scheme 25.
Pellicciari et al., in 1996 \(^{109}\) reported that scheme 25 was synthesised by reacting 3-formyl indole 2 with hippuric acid in the presence of in Ac\(_2\)O /AcONa at 80 °C for 40 min to give 2-phenyl-4-(N-acetyl-3-indolyl methyl)-oxazol-5-one in a 65% yield and 4:1 (Z/E) stereoselectivity, which allowed recovery of 46% of Z-azalactone 73. The major abundant isomer was assigned by an NOE of 74 through a reciprocal interaction found between 3-CH and NH. \(^{109}\) In this research project, in order to generate the product 73 we employed the same procedures with some minor modifications, which involved increasing reaction time by 50 minutes followed by filtration and drying, to afford a 58% yield of 73 as a pure solid. In comparison, it can be clearly seen that the yield achieved here was higher than that reported in the literature, the possible reason for which might be due to the increased reaction time. In addition, the NMR data are consistent with NMR reported in the literature.\(^{12}\)
Also, we managed to crystallise this product by dissolving it in ethyl acetate and adding three drops of chloroform; subsequent recrystallization afforded the crystal structure for this product as shown in scheme \textbf{27}. The stereochemistry of the product was determined from crystal structure instead of NOE. Broadly speaking, no difficulties were encountered regarding the synthesis of product \textbf{73}.

\textbf{Scheme 27}: The X-ray crystallography for product \textbf{73}
On the other hand, this was not the case when attempting to synthesise product 74 using the reaction shown in scheme 25 to generate the cyclopropane ring. In practice, some modification to the literature procedures\textsuperscript{109} was required since the original reports had used diazomethane (which is a highly toxic and dangerous precursor) to prepare the cyclopropane.

Instead, sodium hydride was used as the base with dry DMF as solvent with the reagent (trimethylsulfoxonium iodide); the reaction was performed at room temperature for 3.5 hours under inert gas (N\textsubscript{2}) followed by work up. Analysis of the crude product from this reaction by NMR showed that just 10\% of 73 had converted to 74. A second attempt was made using the same conditions but stirring the reaction mixture for a longer period of time (overnight). However, this just resulted in starting material with some impurities. Despite repeated attempts, however, the reaction did not work again under these conditions, one possible reason for this may have been moisture in the NaH or DMF, otherwise, the reason for this failure is unclear.

As a consequence, other modifications were investigated including the use of KOH as the base and dry DMSO as solvent. The reaction was performed at 80 °C overnight but again failed. In this case, this may have been due to a hydroxyl anion reacting with the protecting group of 73 (presumably via nucleophilic addition) instead of deprotonating the trimethylsulfoxonium iodide.

Ultimately, the reaction had to be performed as shown in scheme 25. Potassium tertiary butoxide was utilized as a base and toluene as a solvent with trimethylsulfoxonium iodide at 110 °C for 5 hours to give the cyclopropane product 74. This was purified using a flash column chromatography using 90:10 and 80:20 petroleum ether: ethyl acetate, respectively, as eluting solvents to afford a 27\% yield of 74 as a pure, sticky brown gum. The 1H NMR spectrum of 74 showed that there were multiple peaks at $\delta = 2.18$–2.22, 2.27–2.30 and 3.16–3.20 ppm, which originate from the protons on the cyclopropane.

An attempt to generate product 75 was made based on the reaction scheme reported in the literature,\textsuperscript{109} which involved opening the 5-membered ring of 74 by adding methanol in the presence of DMAP to form 75. However, the NMR data for the crude material isolated from this reaction showed that the precursor of 74 had decomposed and no evidence of 75 could be found. Subsequently, a considerable number of potential
methods / variations were attempted to form 75 but all of these met with failure. As a result of these problems of synthesis of the initial target 79 was abandoned and we turned our attention to producing compounds that have some similar features to 79. The majority of these were accessed through the key intermediate 81 and its derivatives, as discussed below.

2.3. Synthesis of indolyl α,β-unsaturated ethyl esters and their uses for generating cyclopropane based inhibitors

This reaction is started by addition of sodium hydride to triethyl phosphonoacetate, which results in deprotonation of triethyl phosphonoacetate by the hydride base. This results in evolution of hydrogen gas and the formation of oxosulfonium ylide, as shown in the related mechanism in scheme 29. On addition of indole-3-carboxaldehyde, a nucleophilic reaction takes place which involves attack of the aldehyde group of indole-3-carboxaldehyde, which carries a $\delta^+$ charge by the phosphonate carbanion, followed by elimination according to the mechanism shown in scheme 29.

The main $^1$H NMR features that were looked for in the crude product before purification were the signals for the alkene protons in 81. This should have two doublets at different chemical shifts, which would give good evidence for the formation of 81. These features were, in fact, seen in the associated spectrum. As a result purification was undertaken. Petroleum ether (40-60, 300 ml) was added to wash the crude product, resulting in a 65% yield of pure 81 obtained as pale brown solid. However, this yield is considerably less than the 81% yield reported in the literature, which used silica gel chromatography as the purification method and a mixture of hexane:ethyl acetate in a 6:1 ratio as an eluting solvent. Although, exactly the same purification method reported in literature was tried, the product 81 retained some of the ethyl acetate solvent even when purified.
after being subjected to the high vacuum for three days. However, all of the petroleum ether could be removed hence the reason for washing the crude product in petroleum ether instead of purification by flash column. The NMR data after purification was identical with that reported in literature\textsuperscript{114} when using CDCl\textsubscript{3} as the NMR solvent (as per ref. \textsuperscript{114}). For instance, the NMR data showed two doublets at $\delta = 6.46$ and 7.93 ppm; these originate from the alkene protons 5 and 4, respectively, both of which have the same coupling constant, $J = 16$ Hz. These results are consistent to those in the literature\textsuperscript{114} with regard to these same protons, which had $\delta = 6.47$ and 7.98 ppm, respectively and $J = 16$ Hz, splitting. It should be noted that the large value of the coupling between the two alkene protons provides evidence for the $E$-alkene geometry.

\textbf{Scheme 29:} Synthetic route to product 81

\textbf{Scheme 30:} Synthesis of 82
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After the reaction in scheme 30 was performed and followed by extraction and evaporation the product 82 was obtained. Subsequent NMR data showed 82 as a pure product and no further purification was required. Formation of product 82 was straightforward; the pair of electrons on the nitrogen atom of indole 1H-indole-3-carbaldehyde attacked the methyl group of methyl iodide, which in turn, left a positive charge on nitrogen, which was then deprotonated by the carbonate ion.

This kind of reaction proceeds via an SN2 mechanism. ¹H NMR of this product shows a singlet at δ = 3.76 and, once the integral of this peak was determined, it can be clearly seen that it originates from three identical protons, or in other words the methyl group at position 12, which is evidence for the formation of the required product with a 91% yield.

Scheme 31: Mechanism of formation of product 82

Scheme 32: Synthetic route to generation of product 83

Scheme 33: Synthetic pathway to cyclopropane product 84
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The reaction for preparing 84 starts with the dissociation of potassium hydroxide to generate the hydroxyl ion, which in turn, is responsible for the abstraction of a proton from trimethylsulfoxonium iodide, as result the negative charge on CH₂ of this compound was formed and then it was responsible for attacking the alkene group of 83 and then, by rearrangement, product 84 is formed as per the mechanism below (Scheme 34). The main aspect that should be focused on in the crude ¹H NMR data is formation of a cyclopropane ring and therefore, the disappearance of the two doublets, originating from the alkene group. Afterwards, the flash column was used to purify the crude product, with a 29% yield of pure 84 obtained. NMR data showed multiple signals at δ = 1.27-1.31, 1.54-1.58, 1.84-1.88 and 2.57-2.62ppm, which originate from the protons directly attached to the cyclopropane ring, which gave clear evidence for the successful synthesis of 84. The ¹H NMR spectrum shows evidence for all the 17 protons of product 84, and that there are 4 cyclopropane protons among these can be clearly seen in the NMR spectrum shown in figure 8.

![Figure 8: The ¹H NMR spectrum showing evidence for the formation of product 84](image-url)
The reaction of synthesis the compound 85 as in scheme 35 was achieved with dissociation of the potassium hydroxide, liberating a hydroxyl anion that then attacks carbonyl of the ester, followed by loss of ethoxide to form product 85, as per the mechanism in scheme 36. From NMR data we can clearly see that triplet and quartet originating from the CH₃ and CH₂ group protons, respectively, disappear in the course of our reaction, whilst a broad singlet originating from proton 1₂ appears at δ = 11.73, (using CDCl₃ as the NMR solvent).
The addition-elimination reaction is the type of the synthetic reaction used to produce 87, which begins by nucleophilic attack by the lone pair of electrons on the nitrogen on the sulphur atom of the tosyl (Ts) group, which leads to temporary transfer of one of double bonds of the S = O group above the plane of this group. When this bond returns to its original position, it forces the chlorine (a good leaving group) to leave. The main reason behind protecting the amine group is to prevent any unfavourable reaction from taking place on the amine atom during the conversion of the alkene of 87 to a cyclopropane group in 88. There are two main features expected in the $^1$H NMR, before and after purification, in order to determine if formation of product 87 has occurred. The first of these is a singlet at $\delta = 2.37$, which originates from methyl group 18; the second an increasing multiplicity in the chemical shift ranging approximately over 6–8 which originate from the two benzene rings. $^1$H NMR clearly showed this to be the case with further supporting evidence from exact mass spectrometry, of a parent mass of 370.1113 corresponding to the expected formula C$_{20}$H$_{19}$NO$_4$S. The species with a molecular weight identical to 87 with one extra proton has been observed.
The mechanism of formation of 88 occurs in the same way as the synthesis of 84. NMR data demonstrated that there are multiple peaks at $\delta = 1.25–1.29$ and another at $\delta = 1.56–1.60$, originating from protons on carbon 21 as shown in scheme 39. This was clear evidence for generating the cyclopropane product. Furthermore, other multiple peaks appeared at $\delta = 1.85–1.90$ and $\delta = 2.46–2.52$, corresponding to protons 10 and 9, respectively, their multiplicity due to coupling between these protons and protons on carbon 21 as shown in scheme 39. In addition, exact mass spectrometry showed a
product with an identical molecular weight to the target species of 384.1276 (MH⁺)

Scheme 40: Synthetic route to 89 through deprotecting the Ts group

In 2006, Bajwa et al. reported that N-tosylated indoles can be deprotected when caesium or potassium carbonate is used in presence of methanol and tetrahydrofuran as solvents. These conditions would be used in order to deprotect the tosyl group of products 87 and 88, though with some modification to the conditions. NMR analysis of the attempted reactions illustrated that deprotection was occurring for product 87, where a broad singlet at δ = 8.91 ppm could clearly be observed, which proved the formation of an NH group. Whilst the same conditions were then applied in order to deprotect the Ts group from 88, after the reaction had been run and the product extracted, evaporation followed by the ¹H NMR did not show similar clear singlet related to NH formation.

Nonetheless, flash column chromatography was used in order to achieve separation, in which the segregate fractions involved pure starting material and a decomposing mixture which had starting material peaks with many extra new signals. These may be due to both the product and the liberated protection group Ts–OH containing OH, which would make both polar, hence our choice of eluting solvents of EtOAc : MeOH, that are polar, and used in the ratios 97.5:2.5, 95:5 and lastly just pure MeOH. Another attempt to
separate the same product 88 from another preparation used preparative TLC and eluting solvents of EtOAc: MeOH; 29.25:0.75, respectively.

The preparative TLC showed three clear spots. The first and second materials to be separated were found to be impurities, whereas the main spot that was concentrated on the base line. Therefore, scratching and collecting took place and this was followed by extraction using 20 ml ethyl acetate and 3 ml methanol, filtering and evaporating, then recording the $^1$H NMR with CDCl$_3$ as the solvent showed the right product with the Ts group still present, thought with other extra signals appearing. The same occurred for a second attempted preparation, whilst mass spectrometry showed a highest mass peak with a molecular weight [MH$^+$] of 171, originating from toluenesulfonic acid. Consequently, HPLC was used to separate the product; the eluting solvents utilized were H$_2$O:CH$_3$CN. Although, the product obtained was pure, the amount recovered was just 5 mg out of 168 mg submitted to the column, which means the isolated yield was extremely poor. Figure 9 confirms that this was the target product 89 without the singlet expected for Me attached to Ts. The NMR solvent employed here was CD$_3$OD because the product did not dissolve in CDCl$_3$, possibly because it is acidic. Further support for having obtained on the product 89 came from the exact mass, which was found as 202.09 [MH$^+$], compared to that can be calculated for C$_{12}$H$_{12}$NO$_2$ of 202.09. However, the isolated yield was extremely poor, at 6%. 

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Figure 9: 1H NMR using CD$_3$OD as solvent showing pure product 89 after separation by HPLC

Scheme 41: Synthesis route to compounds 90 and 91

Scheme 42: Mechanism for formation of product 90
Formation of product 90 was achieved by protecting the amine group of indole-3-carboxaldehyde with a benzyl protection group; this type of process is an $S_N2$ reaction. $^1H$ NMR data showed there to be a singlet at $\delta = 5.23$, which originates from the CH$_2$ of the benzyl group, showing that the target compound had been obtained. Mass spectrometry also showed the anticipated molecular weight of 236.1070 for the product. Product 90 was then used as a precursor for synthesizing product 91. This compound was synthesised since it might be used as a potential inhibitor having different substituents for inhibiting the enzymes in this study after the protection group is removed. The synthetic route applied for 91, was the same as for compound 81. The $^1H$ NMR data for pure product 91 showed a triplet and a quartet at $\delta = 1.34$ and 4.26, respectively, originating from protons on carbons 13 and 12, respectively. Further evidence from the NMR data showing the required product had been obtained, was from two doublets which appeared at $\delta = 6.43$ and 7.90, originating from $\alpha,\beta$-protons 10 and 9, respectively, both of which have the same $J$ splitting value ~16 Hz. The yield for this compound was just 51% due to some of this product being lost during the washing process, which was done in order to purify it.

Scheme 43: Synthesis of product 92

It can be clearly seen in the mechanism below in scheme 44 that the reaction starts with nucleophilic attack on the ester carbonyl of 91 by the methoxy anion, followed by loss of ethoxide leading to product 92, as confirmed by NMR. The NMR spectrum of the pure product shows that the triplet and quartet, which belong to ethyl group, no longer appear in the spectrum, whilst a singlet emerges at $\delta = 3.72$, which can be attributed to the CH$_3$ group in position 12 (see the exp. chapter 5).
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Scheme 44: Synthetic route to product 92

Scheme 45: Synthesis of compound 93 occurs by the same route to synthesis of compound 85

Scheme 46: Synthesis of product 94

The mechanism for formation of 94 proceeds in the same manner as for products 84, and 88. The NMR spectrum of 94 exhibited four multiple peaks emerging at $\delta = 1.18–1.26, 1.47–1.53, 1.78–1.84$ and $2.51–2.58$, which can be attributed to the protons on carbon 21, 10 and 9, respectively. When CDCl$_3$ was used as the NMR solvent, two doublets attributed to the starting material at $\delta = 6.43$ and 7.90, disappeared, giving further evidence for the formation of the cyclopropane group associated with product 94. The main reason behind protecting the nitrogen of 91 via the benzyl protection group is that creating the cyclopropane from alkenes forms product 94; a number of experiments were used in order to subsequently deprotect the benzyl protecting group.
Firstly, palladium carbon and hydrogen gas in methanol was utilized to deprotect the benzyl group. However, NMR showed that deprotection had not occurred, and that instead the cyclopropane ring of 94 had opened due to a hydrogenation reaction (reduction) occurring. This lead to the breakdown of the cyclopropane ring via the generating of two free radical electrons on the carbon atoms in positions 1 and 3 in the scheme 47; followed by addition of two hydrogens atoms in same positions, giving a 57% yield of by-product 95 in scheme 47. Indeed, the associated NMR demonstrates that there are multiple peaks at δ = 1.94–2.0, two triplets at δ = 2.30 and 2.73; these chemical shifts can be attributed to protons in the 2, 1 and 3 positions of byproduct 95, respectively. The rest of the mixture, the remaining 43%, was starting material; these ratios were determined through NMR. In addition, the exact mass spectroscopy for this mixture has shown that there are two main peaks found (MH\(^+\)) at 322.2 and 320.2; molecular weights can be calculated as 321.2 C\(_{21}\)H\(_{23}\)NO\(_2\) and 319.2 for C\(_{21}\)H\(_{21}\)NO\(_2\), i.e; by-product 95 and the starting material, respectively, as in figure 10 and scheme 47.

![Figure 10: The 1H NMR of the mixture of 95 and its starting material](image-url)
Scheme 47: Attempts to deprotect the benzyl protecting group

The opening of the three-membered ring in the reaction in scheme 47 is consistent with the hydrogenation reaction of an alkene, which occurs rapidly at room temperature in presence of the same catalyst as in scheme 47, as shown in scheme 48.\(^{116}\)

\[
\text{H} \quad \text{H} \\
\text{H} \\
\text{H}
\]

96

\[
\text{H} \quad \text{H} \\
\text{H} \\
\text{H}
\]

97

Scheme 48: Hydrogenation reaction of alkenes

Another attempt was made to deprotect the benzyl group by reacting the starting material 94 with RuCl\(_3\).H\(_2\)O and NaIO\(_4\) at room temperature for three hours in the presence of H\(_2\)O, DCM and CH\(_3\)CN as solvents,\(^{117}\) followed by working up the reaction and extraction and evaporation. \(^1\)H NMR of the crude product showed that deprotection did indeed occur, due to the singlet of the CH\(_2\) bound to the benzyl group of starting material at \(\delta = 5.17\) being shifted upfield to \(\delta = 4.79\); this may be evidence of formation of toluene, whilst the second piece of evidence suggesting deprotection of this group is the singlet observed at \(\delta = 8.20\) associated with the formation of NH (on the indole group). Although column chromatography was employed to purify the product, no
separation occurred. This might be because both compounds have a similar polarity, and indeed TLC did show that the eluted spots were very close to each other.

In 2011, Dallavalle et al. reported that the benzyl protecting group could be removed through a suspension of anhydrous AlCl₃ in a suitable amount of anisole with their compounds (which contained the benzyl protection group). The mixture was refluxed for five hours followed by the addition of an appropriate amount of MeOH, followed by evaporation of the solvent¹¹⁸. The same procedures were applied in order to deprotect the benzyl group in compound 94. However, the NMR spectrum has showed that no change occurred to the starting material.

![Scheme 49: Reaction to synthesise product 98](image)

Potassium hydroxide and ethanol are considered to be as rich sources of the hydroxide anion after their dissociation thus promote nucleophilic attack by OH⁻ targeting the ester carbonyl group of compound 94, leading to removal of CH₃CH₂O⁻ (as a leaving group) as shown in the mechanism below. In this way, product 98 can be straightforwardly synthesised. ¹H NMR data using CDCl₃ as solvent shows triplet and quartet features which originated from the ethyl group have disappeared, whilst at the same time there is a broad singlet that appears at δ = 11.45, which is associated with proton 12. In addition, exact mass spectrometry showed the same molecular weight as expected for the product. Additionally, the product was crystallised by dissolving it in ethyl acetate with a few drops of chloroform. Recrystallisation then allowed for its X-ray crystallography to be recorded as shown in figure 11, which also demonstrated that 98 has the trans form of cyclopropane.
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Scheme 50: Mechanism for creation of product 98

Figure 11: X-Ray crystallography of product 98 showing the trans form of cyclopropane (three-membered ring)

Scheme 51: Synthesis of product 99
Methoxybenzylbromide was used to protect the secondary amine of the indole ring; the reaction mechanism is $S_N2$, which begins with the amine lone pair attacking the $\delta^+$ of CH$_2$ that bound to the bromine, ultimately replacing the bromide anion as a good leaving group. The carbonate then abstracts the proton from the secondary amine of indole ring. The main purpose behind protecting the amine of the indole ring is to synthesise the cyclopropane group from the alkene in product 99. From the NMR data of pure product 99, employing CDCl$_3$ as the NMR a solvent, it is clear that there are two singlets that have emerged with chemical shifts of $\delta = 3.81$ and 5.27, associated with protons in positions 21 and 14, respectively as showed in scheme 51 product 99, these features are the main evidence for having synthesised product 99.

![Scheme 52: The synthetic pathway for product 99](image)

![Figure 12: The crystal structure of 99](image)
Formation of the cyclopropane compound was via the same synthetic pathway of compounds 84, 88 and 94. This reaction is an addition reaction to the alkene, which begins with the base (OH\(^-\)) deprotonating the trimethyl-sulfoxonium iodide, leaving it negatively charged, followed by nucleophilic attack allowing easy addition to the α,β-unsaturated carbonyl compound 99 followed by rearrangement resulting product 100 with a yield of 29%. The NMR data shows are multiple peaks at δ = 1.14–1.22, 1.44–1.50, 1.76–1.8 and 2.49–2.55, associated with cyclopropane protons in the 2×14, 10 and 9 positions, respectively, which affords clear evidence for formation of product 100. Figure 13 shows the NMR spectrum for compound 100.
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Figure 13: 1H NMR, showing chemical shifts and integrations for product 100

In addition to the proton NMR for compound 100 in figure 13, its stereochemistry was determined by nOe, as shown in figure 14. In this figure the chemical structure for compound 100 is included and as can be seen there was correlation between H9, H10 and one of the protons in location 14 with H7. In spite of the fact that H10 is quite distant from proton 7, they still showed correlation between each other. However, the nOe spectrum in figure 14 was not easy to interpret due to the fact that it contained many correlations and these may be due to the rotation of the cyclopropane and ester group part for this product.
Two experiments were attempted in order to deprotect the $p$-methoxybenzyl (PMB) protecting group to obtain the compound in the step 1 of scheme 54. Both these reactions are shown in scheme 54.
Scheme 54: Reactions conditions 2 & 3 were attempts at achieving the conversion shown in reaction 1 in this scheme

$^1$H NMR data for the product in reaction 2 (AlCl$_3$ in toluene at room temperature) in scheme 54 shows that there was only starting material mixed with some impurity peaks. Accordingly, it was obvious that no reaction had occurred. By contrast, the NMR data for reaction 3 (TFA and H$_2$SO$_4$ at room temperature) in the same scheme showed two triplets at $\delta = 2.39$ and 2.52, these may associated with the protons 1 and 2 in positions for the product in step 3, respectively. In order to prove that these protons are coupling to each other, the associated $J$ coupling constant have to be similar to each other, these were calculated as $J = 7.1$ and 7.4 Hz for protons 1 and 2, respectively. It is obviously that as these values are essentially equal to each other, which means these two protons are coupled. It should also be noted that multiple peaks associated with the cyclopropane group in the staring material had vanished.$^{119,120,121}$
4-Dimethylaminopyridine (DMAP) in the scheme 55 initiated the nucleophilic reaction attacking of one of carbonyl groups of di-tert-butyl dicarbonate (BOC₂O), as can be seen from the mechanism in scheme 56. The resulting product was attacked by the nitrogen lone pair of 1H-indole-3-carbaldehyde followed by liberating the catalyst (DMAP), deprotonation and affording a 87% yield of the product 103. The main feature evidencing the formation 103 from the ¹H NMR data was the broad singlet of the amine proton at δ = 8.64 disappearing and the emergence of the singlet peak at δ = 1.74, associated with the nine protons in positions 12, 13 and 14 as showed in the scheme 55 product 103. These are clear evidence for synthesis of 103; further support was offered through favorable comparison of theoretical mass and that determined by mass spectroscopy.

Scheme 55: Synthesis of product 103

Scheme 56: The mechanism of formation of the BOC₂O group of compound 103
Chapter 2: Results and discussion

Scheme 57: Synthetic route to compound 104

The mechanism of formation of the product 104 is likely to be the same as seen for the synthesis of products 81 and 103.

Scheme 58: All attempts to synthesise product 105

Several experiments to synthesise 105 were attempted, the first reaction being that of the reacting 104, trimethylsulfoxonium iodide with a base (potassium t-butoxide) in toluene. The second attempt used sodium hydride as a base, trimethylsulfoxonium iodide and 104 in DMSO; a third attempt used identical conditions, except this time used the DMF as solvent. Finally, the experiment was conducted using DMSO, employing potassium hydroxide as a base, with trimethylsulfoxonium iodide with 104. All NMR spectra obtained after the appropriate extractions have demonstrated that none of these reactions gave the desired product, which might have been due to breakdown of the BOC protecting group. If this indeed the case, it might be suggest that nucleophilic
attack proceeds against the carbonate protection group, which leads to NH formation after rearrangement instead of synthesising the cyclopropane product. It can be clearly seen from the $^1$H NMR data that there is a broad singlet at $\delta = 9.04$ that can be associated with indole NH.

2.4. Conclusions

The first three steps of the pathway to generate the tryptophan analogue (Z)-$\Delta$−Trp have been successfully completed. However, this route had to be changed due to the difficulties faced when attempting to synthesise product 75. Alternatively, several α,β-unsaturated alkene compounds have been successfully synthesised, and their amine groups were protected through different protection groups as follows: Me, BOC, Bz1,Ts and PMB. The protected products were use as precursors for successfully generating cyclopropane products, in which trimethylsulfoxonium iodide and potassium hydroxide were utilized to form the anions, used to initiate nucleophilic attack on the α, β-unsaturated bond of the starting materials, forming three-membered rings as pre-final products. As can be seen in table 3, alkene products as precursors for synthesising three-membered ring products are summarised.
Table 3: Shows alkene products as precursors for synthesising three-membered ring products with mentioning to the novelty and obtained yields

<table>
<thead>
<tr>
<th>Alkene products</th>
<th>The same products but with three-membered ring</th>
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However, there were many difficulties to overcome when attempting to deprotect some of these groups in order to obtain the final desired products, which have cyclopropane rings and no protection groups, (which themselves have been replaced with a proton). These difficulties included opening the three-membered ring of cyclopropane products due to a hydrogenation reaction (reduction), failure of the protection group leading to no reaction occurring, and the cyclopropane product described in table 3 not being formed; (which may have been on account of an interaction between this group and the anions). Also, observed was decomposition of the product material during the purification process by the flash column, which might have been due to the Tos protecting group product being sensitive to acid. Product 89 was only obtained in a poor yield of only 6% once its separation had been achieved by HPLC.
Chapter 3: Results and discussion

Synthesis of α,β-unsaturated carbonyl core compound derivatives that contain an indole rings and are terminated with two aryl ring systems and their uses for generating potential inhibitors
3. Synthesis of α,β-unsaturated carbonyl core compound derivatives that contain an indole rings and are terminated with two aryl ring systems and their uses for generating potential inhibitors

3.1. Introduction

3.1.1 Indole

Heterocyclic compounds are compounds that have had one or more of the carbon atoms in their rings replaced by other atoms. These usually are electronegative atoms, such as the nitrogen atom in indole; the word indole is a combination of indigo and oleum, derived from the fact that it was initially isolated when indigo was treated with oleum. The indole ring typifies a class of molecules that is one of the most important and abundant heterocyclic products in nature. It is generally found in a variety of natural products and is also, along with its derivatives such as tryptophan, tryptamine and serotonin, a species of particular biological importance. Indole containing compounds often display biological activity due to their capability for binding reversibly to proteins and mimicking the structure of peptides. For instance, one of the most important compounds in this area is naturally occurring indole. Furthermore, medicinal chemistry is concerned with determination of the impact of chemical structure on biological activity; in practical terms, medicinal chemistry relies considerably on the adjustment of chemical structure through advanced methods of organic synthesis followed by identification of their biological activity. In this context, there is an important aspect that has been established between chemical structure and pharmacological activity; approximately half of therapeutic agents contain heterocyclic compounds.

The hetero ring can play two very fundamental roles, those of the pharmacophore and the active moiety. As mentioned above, one of the most frequently used heterocyclic compound is indole, which is a fused aromatic heterocyclic ring, which includes a six-membered aromatic ring fused to a five-membered nitrogen–containing pyrrole ring.
Dissimilar to most amines, indole is not basic, and a very strong acid, such as HCl, is required to protonate indole.\textsuperscript{129}

The protonated form of indole has a $pK_a = -3.6$. This explains the sensitivity of several compounds that contain an indole ring such as tryptamine under acidic conditions which is due to protonation. In pharmacological terms, indole forms the core of several important drugs for the treatment of various diseases, as shown in figure 15.\textsuperscript{129}

![Figure 15: Indole ring derivatives in components of medicine used in treatment of a broad range of diseases](image)

In addition, in spite of the fact that indole is a very small moiety, it fascinates scientists due to its range of biological activity that is limited not only to indole but is also seen over a diverse range of its substituted derivatives.\textsuperscript{128}

### 3.2. Pyridine

Pyridine is another type of heterocyclic compound. The word pyridine is the combinations of two words derived from the Greek words "pyr," meaning fire and "idine," used for aromatic bases. Pyridine was first synthesised through bone pyrolysis via condensation of simple ketones and aldehydes with ammonia, which was thought to be generated through decomposition of the nitrogen-containing materials and glycerol in bone oil under these conditions.\textsuperscript{130}
Pyridine is an analogue of the benzene ring, which contains a six-membered heterocyclic ring and has a six π-electrons. In terms of structure; there is only one difference between benzene and pyridine, in that pyridine contains a nitrogen atom which takes the place one of the CH groups of the benzene ring. This analogue has a nonbonding pair of electrons on the nitrogen atom that are in an $sp^2$ hybrid orbital in the plane of the ring. These lie perpendicular to π-system and hence do not interact with the π-electron system of the ring as in figure 16.131

![Figure 16: Pyridine has six delocalized electrons in its cyclic π-system. The π-bonding structure of pyridine and the two nonbonding electrons on nitrogen are in sp2 orbital](image)

### 3.3. Synthesising products that contain indole, pyridine rings and aryl groups, as well as other features such as cyclopropanes and alkenes

![Scheme 59: Synthetic route to product 107](image)

According to Raphaël Frédérick et al.13 compound 107 can be synthesised using the reactants and conditions shown in scheme 59. Based on this literature method, the synthesis of the product 107 was carried out, using some modifications including an increased period of time under standard reflux conditions instead of using a microwave as in literature. However, after purification using the same procedures described in the
literature, the isolated yield of 107 was 62%, which is less than the yield of 86% reported in the reference.\textsuperscript{13,132} The reason behind this difference might be due to the switch from microwave to normal thermal heating conditions or is perhaps due to the sample of pyridin-3-yl acetic acid hydrochloride not being 100% pure. Regarding the NMR data when DMSO-d\textsubscript{6} was used as a solvent, the data recorded for 107 was identical to that in with reference\textsuperscript{13} and the product obtained was crystalline, and crystals could be grown that allowed the X-ray crystal structure to be determined as shown in figure 17.

![Figure 17: X-ray crystal structure of 107](image)

\textbf{Figure 17:} X-ray crystal structure of 107

\textbf{Scheme 60:} The mechanism formation of 107

As can be seen, the reaction starts with deprotonation of carboxylic acid of pyridin-3-yl acetic acid hydrochloride by the lone pair of piperidine, leaving a negative charge on the O\textsuperscript{−} followed by decarboxylation and concomitant nucleophilic attack on the aldehyde group of 1 H-indole-3-carbaldehyde due to the δ\textsuperscript{+} on the carbonyl carbon atom. By
decarboxylative dehydration as it has been shown in the scheme 60; product 107 was formed.

![Scheme 61: Using 107 as the precursor to attempt to synthesis of 113]

Product 109 was not the product that we had set out to prepare. The target was to protect the NH group of the starting material with Me. However, due to both N atoms having lone pairs of electrons, this makes both moieties reactive with the methylating agent. The evidence for the formation of product 109 was confirmed by NMR when DMSO was used as a solvent, with the \(^1\)H NMR spectrum clearly showing that there are two singlets at δ values of 3.86 and 4.34 ppm, originating from the methyl groups in positions 18 and 19. Furthermore, the exact mass of the product was determined, and found to be identical to the correct molecular weight for product 109.

As an alternative strategy to obtain the desired mono-protected product 113, the amine group of 1 H-indole-3-carbaldehyde was first protected to give 111. This product was then reacted with 112 using the standard conditions (discussed above) to produce an alkene containing compound that we initially assigned as structure 113. Indeed, further reaction of this product with potassium hydroxide / trimethylsulfoxonium iodide generated a product that was confirmed to contain cyclopropane hydrogens (by NMR analysis) and that was initially assigned the structure 114. As discussed below these initial structural assignments were incorrect and the actual structures for these products are given below as 113a and 114b.
An attempt to purify expected product 114 was carried out using flash column chromatography with a 3:7 mix of ethyl acetate: hexane as eluting solvent, from which (0.035 g, 0.14 mmol) of expected 114 was obtained as pure product. The calibrated $^1$H NMR of 114 showed that there were just 12 proton environments instead of the 16 protons expected for 114, which prompted further characterisation and to request the mass spectrum for 113 alongside the mass spectrum and a full NMR characterisation of 114. From this, the following results were obtained: the molecular mass of unknown 113a [MH$^+$] was 341.17; this was greater than the expected molecular mass of 113 which is 234.1. Furthermore, $^{13}$C NMR for 114a showed that there was peak at 208.2, implying the existence of a C=O group in the unknown product 113a. The information regarding 113a and 114a gained from mass spectrometry and NMR spectroscopy showed both of them were not our desired products. However, we failed to identify these products due to 114a being a sticky compound, whereas 113a is solid to some extent. Ultimately we crystallised 113a in order to determine its crystal structure and from this, the structures of both 113a and 114a could be determined. A sample of [solid] 113a was dissolved in ethyl acetate to which a few drops of chloroform were added. The sample container was then covered with a membrane in which small holes were made, and which was left for two days to crystallise. The crystal structure for 113a was subsequently determined, and is shown in figure 18.
113a

In terms of the mechanism for 113a, it is assumed that this occurred in the similar mechanism by which product 107 is nominally synthesised.

From the crystal structure of 113a it can be clearly seen that no reaction had occurred between 1-methyl-1H-indole-3-carbaldehyde and pyrid-3-ylacetic acid hydrochloride, but instead between two molecules of 1-methyl-1H-indole-3-carbaldehyde and one molecule of acetone, which explains larger molecular weight of 113a. The acetone required for this reaction had presumably been brought through from the proceeding reaction where it was used as a solvent. Once the structure of 113a had been confirmed we could now propose a chemical structure for cyclopropane 114a (Scheme 63) that explain the fact that ¹H NMR showed just 12 proton environments as well as the mass spectral data we had obtained. The more precise ¹H NMR features for 114a were existence of multiplets at δ = 1.32–1.41 and δ = 1.65–1.73 which can be assigned to protons in position 12 as well as there two multiplets at δ = 2.21–2.29 and δ = 2.57–2.67 which can be assigned to coupling of α and β hydrogens in positions 10 and 9 respectively with the hydrogen in position 12. In addition, as can be obviously seen, there is carbonyl group which is consistent with the ¹³C NMR for 114a and due to the fact that the ¹H and ¹³C of 114a compound were not complex which may gave clue it is meso. Scheme 63 shows its chemical structure.
Scheme 63: The chemical structure of 114a

The lesson gained from the unwanted formation of products 113a and 114a is that cyclopropane products cannot be synthesised without the presence of a carbonyl group or an ester group by the olefin, the evidence for which is the failure encountered when trying to convert alkenes in compounds 107 and 109 to cyclopropane products 108 and 110. As a consequence, the attempt to synthesise 108 and 110 was not possible, and synthesis of similar compounds that involve the ketone group had begun, as per scheme 64 and 65.

Scheme 64: Synthesis of product 116

This reaction was conducted in order to find out whether there a reaction will take place when compound 111 reacts with just acetone 115 under refluxing conditions for 2 days. The NMR data for 116 showed that rather than getting the same condensation to give 113a (as discussed above), here the reaction occurred, with 1:1 ratio giving the product shown. Further proof for this came from the exact mass, which showed the expected molecule weight 200.1077; calculated for C_{13}H_{14}NO; 200.1075.

In 2012 Maltese et al.\textsuperscript{133} reported that several compounds containing on α,β-unsaturated ketone core can be synthesised through a Claisen–Schmidt condensation between indole-3-carboxaldehyde and aryl ketones, as well as the condensation of acetophenone (or other acetyl-pyridines) with indole-3-carboxaldehyde.
According to Maltese et al.\textsuperscript{133} 118 was prepared by the reaction of 3-acetyl-pyridine with indole-3-carboxaldehyde in the presence of piperidine and methanol as solvent. Work up the reaction and purification by flash column chromatography gave a 19\% yield of 118 as a pure yellow solid.\textsuperscript{133} The same procedures were employed here to synthesise product 118. However, some modifications were made in order to improve the yield, which involved washing the crude product with 300 ml of cold methanol followed by filtration, to give a yield of 66\% as pure yellow product 118. This gives an approximately 3.5 fold improvement in yield compared with that reported in the literature.\textsuperscript{133} $^{1}$H NMR data when DMSO–d$_6$ was used as solvent showed that there are two doublets at $\delta = 7.66$ and 8.13; these originate from protons 11 and 10, respectively as shown in the scheme 65. Not only this, but additionally both of these two doublets have approximately the same $J$ coupling values 15.4 and 15.6 MHz, respectively; this is considered clear evidence for formation of product 118, which is $\alpha,\beta$-unsaturated ketone. In addition, other supporting data come from mass spectrometry, and elemental analysis, and a determination of the product melting point (see experimental part 5; are consistent with literature).\textsuperscript{133}
Scheme 66: Mechanism to generate 118, according to Claisen–Schmidt Condensation

Scheme 67: Synthetic pathway of 119

The same procedures used for the synthesis of 118, were applied to generate the novel product 119, which was recrystallized from methanol giving a 70% yield of very pure yellow 119. This was characterised through various methods, such as X-ray analysis after 119 crystallised from ethyl acetate and chloroform afforded the crystal structure seen in figure 19. From this X-ray structure, it can be easily deduced that 119 has a trans alkene structure. Not only this, but $^1$H NMR data also helped further elucidate the chemical structure of 119. For instance, there is singlet at $\delta = 3.85$ originating from a methyl group; there are also two other doublets at $\delta = 7.49$ and 8. 11, which show that an $\alpha,\beta$-unsaturated ketone, molecule 119 was synthesised. Furthermore, mass spectrometry, elemental analysis and IR data support the above characterisation of 119. The synthetic mechanism of 119 is the same as 118.
Figure 19: X-ray crystallography for structure 119

![X-ray crystallography](image)

Pure product 118 was used as a precursor for synthesising alcohol products 120 by the reduction of the carbonyl group on 118, for which the reagent sodium borohydride was used as a source of the hydride anion. The borohydride attacks the carbon atom of the carbonyl group. NMR data obtained for 120 when CD$_3$OD was employed as an NMR solvent. There are doublets at $\delta = 5.38$, a doublet of doublets centred on $\delta = 6.29$ and a further doublet at $\delta = 6.87$ originating from to protons 12, 20 and 21 respectively, as shown in the scheme 68 product 120.

These signals are consistent with expectations for the formation of product 120. In addition, $^{13}$C NMR showed that the C=O signal for 118 at $\delta = 187.9$ had disappeared, but the NMR solvent in this case was DMSO-d6. Nonetheless, it does give further evidence for the formation of product 120. Although the NMR data demonstrated that

$X = \text{H, Me for 120 & 120a respectively}$

Scheme 68: Synthesis of alcohols produced from 118 and 119: 120 and 120a

![Scheme 68](image)
120 is almost pure, an attempt was made to purify it further was undertaken through 2D TLC, with the best selective eluting solvents DCM: MeOH in a 9:1 ratio, respectively. However, this process showed product decomposition. This may be because 120 contained hydroxyl group, which bound tightly to silica, and can result in the removal of this group from 120 as a first step. As a consequence, the crude product 120 was dissolved in 2 ml ethyl acetate followed by 5ml hexane and was left for 30 minutes at room temperature until the solid emerged. This was followed by filtration, transferral to a round bottom flask and final exposure to high vacuum to afford a 46% yield as pure yellow product 120.

In the same manner that 120 was prepared, 120a was prepared from 119. 1H NMR data with CDCl3 as solvent showed that the crude 120a was over 95% pure, but again several potential methods have been explored that might have allowed for further purification; for instance, flash column chromatography was employed with the chosen selective solvents DCM: MeOH; in the ratio of 9.7:0.3, respectively. However, 120a decomposed under these conditions, which may have been due to the acidity of the silica. Another purification attempt was made through crystallization from hexane and ethyl acetate; however, this resulted in an increase in impurities, instead of their removal. 120a was re-prepared and used without further purification since it was shown to be generally reasonably pure, as shown in figure 20. As can be seen from the 1H NMR spectrum in figure 20, there is a broad singlet at δ = 4.44, a doublet at δ = 5.33, and, a doublet of doublets at δ = 6.25, which originate from the protons in positions 14, 12 and 11 respectively as shown in the chemical structure for 120a in figure 20. Again, this gave clear evidence for having generated the desired product.
The compound 121 was prepared in the same manner by generating the anion from trimethylsulfoxonium iodide by using a different base. So instead of using KOH as base sodium hydride was used because of KOH was not as successful for forming the cyclopropane product 121 when it was tested. This may be due to high temperature that was used or the fact that KOH is less basic than NaH. So, we found that when sodium hydride was employed as a base instead of KOH product 121 was produced in excellent yield.\textsuperscript{102,134} NMR data showed that there are four multiple peaks, which originate from
the three-membered ring protons of 121. In addition, it can be deduced from NMR spectra that the number of carbons and hydrogens in 121 are identical to those in the chemical formula; for $^1$H NMR, see the integral for the product in figure 21. The stereochemistry of the compound 121, was identified by NOESY (NOE); in which correlations have been found between protons in position 9 and one of the protons in position 18 with H in position 7 as can be seen in figure 22. However, when attempting to purify this product via the flash column, it converted to 122 through ring-opening of the three-membered ring and formation of a five membered dihydrofuran. The NMR data for 122 exhibits multiple peaks at $\delta = 3.08$–3.31 and these originated from protons in position 10 as shown in the chemical structure of 122 in figure 23. Surprisingly this converted 122 product was found to have exactly the same molecular mass as 121. A $^1$H NMR (CDCl$_3$) spectrum was recorded for the converted to known product 122 is shown in figure 23.

In addition, other methods were tried in order to obtain product 121 in its completely pure form, which involved mixing it with alumina in the presence of hexane and ethyl acetate to find out if there is any the decomposition occurred or not. However, the impurities again started to increase so, therefore, efforts were directed towards crystallizing product 121 from diethyl ether and ethyl acetate. All these potential purification methods met with failure due to total or partial conversion of 121 into a totally different product, or due to an increase in impurities. Furthermore, to test whether this species is stable at different conditions, $^1$H NMR was recorded using CDCl$_3$ as solvent. The sample was left overnight in the same solvent at room temperature; again $^1$H NMR was recorded, which showed no change had occurred. The crude product was placed in the fridge overnight, again followed by NMR, which demonstrated that there are no changes to be observed.
Figure 21: The 1H NMR spectrum of 121
Figure 22: Shows NOESY for the compound 121
Chapter 3: Results and Discussion

Figure 23: Conversion of product 121 to 122 showed in the figure

\[ \text{Synthesis of 124 and 124a relied on the same procedures reported by Maltese and co-workers in 2012. The compound 124 was obtained with a 36% yield, which approached the 39% yield reported in the literature. Purification of 124 was through crystallisation from dichloromethane and petroleum ether 40–60°C followed by drying to prove 124 was a pure product. NMR data recorded for 124 are consistent with that reported in the literature when DMSO was used as NMR solvent. The literature method for purification was through recrystallizing product 124 from 100% EtOH, attempts to reproduce this was unsuccessful. Pure 124a was obtained after} \]

\[ \text{Scheme 70: Synthetic route of alkene molecules 124 and 124a} \]

\[ \times = \text{H, Me for 124 & 124a respectively.} \]
recrystallization from hot methanol to afford just an 18\% isolated yield of 124a. \textsuperscript{1}H NMR and \textsuperscript{13}C NMR exhibited singlet peaks at $\delta_H = 3.74$ and $\delta_C = 30.9$, which originate from methyl group at position 10 (see exp. Chapter 5); the X-ray crystallography shown in figure 24 shows beyond doubt in that the required product 124a had been generated.

![Figure 24: X–Ray structure of 124a](image)

The compound 124a was used as precursor for generating cyclopropane 125 and alcohol 126 products. In 2002 Ila et al.\textsuperscript{134} reported that 125 can be made (according to the conditions on the arrow in scheme 71 below). The reaction had been performed for 15 minutes at room temperature under inert N\textsubscript{2} gas to ultimately give a 98\% yield of crude cyclopropyl ketone 125 that was of a high enough purity to characterise by NMR spectroscopy.\textsuperscript{134} The same procedures reported therein was followed with some modifications, such as leaving the reaction to stir overnight at room temperature and employed by N\textsubscript{2}(g) according to scheme 71. The crude NMR for 125 seems to be pure enough for characterisation by \textsuperscript{1}H NMR and \textsuperscript{13}C NMR, to give a 100\% obtained yield. The NMR data for forming cyclopropyl ketone are consistent with reference\textsuperscript{134} and are also supported by mass spectrometry.
Figure 25 shows NOE spectrum for 125, as can be clearly observed that there are no correlations between protons H9, H10 and one of H in position 12 as showed in the chemical structure for compound 125 in the figure 25; this may be due to their locations are remote from H8. Whilst there is one correlation has been confirmed by this spectrum for one H in the location 12, with H8. According to that, the stereochemistry for this product is predicted, as has shown in the structure 125 in scheme 71.
The ketone group of 124a was converted to the alcohol group (hydroxyl) in 126 using sodium borohydride as a source of H\(^-\) which attacked the ketone group while the other hydrogen atom was deprotonated from the MeOH solvent (reduced reaction) to afford product 126 in a high enough purity to characterise by NMR without requiring any further purification. \(^1\)H NMR data for product 126 shows a singlet at \(\delta = 2.11\), a doublet \(\delta = 5.26\) and a doublet of doublets at \(\delta = 6.23\) which originate from protons in positions 15, 13 and 12, respectively as showed in scheme 72 product 126, which gives clear evidence for formation of product 126. Additionally, \(^{13}\)C NMR data recorded for this product showed that the characteristic C=O signal of the precursors at \(\delta = 188.4\) had disappeared in the NMR spectrum of 126.

\[X = H, \text{ Me. } Y = CH, N\]

**Scheme 73**: General mechanism for generating alcohols from ketone products
3.4. Conclusions

In this chapter, 14 compounds have been successfully generated, the common aspects of which is the presence of an indole group and its derivatives. According to the practical experiments which have been carried out in this work with these kinds of compounds, which have an olefin group, it has been shown that three-membered rings cannot be formed without the presence of a carbonyl group (ketone or ester).

The three member ring compounds have not been formed when there was no carbonyl group neighbouring the alkene group in the starting compounds, for instance, the cyclopropane compound 108 was not synthesised from compound 107 due to above mentioned reason. Also, in this chapter despite the fact different purifications procedures were attempted to purify these compounds such as flash column chromatography, only extraction and recrystallization were successful. However, all our efforts to purify products that contained carbonyl or hydroxyl groups met with failure due to the products decomposing when flash column or recrystallization were employed, as a consequence of which several extra signals peaks were observed in the associated $^1$H NMR spectra.

Further reasons for this particular failure might be due to these compounds not being resistant to acidic media such silica gel, or they may have been affected by polar solvents, such as ethyl acetate, during the recrystallization process. Other factors that might have affected their synthesis are that some of these species may have decomposed when, for instance, they were stirred with alumina or when dissolved in polar solvents, such as ethyl acetate.

For instance, the cyclopropane product 121 was converted to unknown pure product 122 merely submitting it to flash column chromatography when using an ethyl acetate: hexane mix was employed as the eluting solvent. Surprisingly, the unknown compound 122 had the same molecular weight as 121; on the other hand, it can be clearly observed from figure 23; that three-membered ring of compound 121 was opened and an olefin was made 122. Some of products that were synthesised in this chapter have never, to the best of our knowledge, been previously reported in the literature, and hence are assumed to be new compounds, which are reported in table 4.
Table 4: A summary of novel species obtained in the current study

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<th>Novel compound</th>
<th>Novel compound</th>
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<td>with 97% yield</td>
</tr>
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<td>with 100% yield</td>
<td>with 59% yield</td>
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<tr>
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<td><img src="image6.png" alt="Image 6" /></td>
</tr>
<tr>
<td>with 6% yield</td>
<td>with 63% yield</td>
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Chapter 4: Results and discussion

Kinetic assay of enzymes (IDO and TDO) with Tryptophan (as a substrate) and various synthesised compounds (as potential inhibitors)
4. Kinetic assay of enzymes (IDO and TDO) with Tryptophan (as a substrate) and various synthesised compounds (as potential inhibitors)

4.1. Introduction

4.1.1 Enzymes and their kinetic parameter assays

In 1877, the German physiologist Wilhelm Kühne was first person to use the word ‘enzyme’, which was in his description of the capacity of yeast to produce alcohols from sugars. The word itself is derived from the Greek words *en* (meaning ‘within’) and *zume* (meaning ‘yeast’).  

Significant improvements were made in connection with the characterization, extraction and commercial exploitation of several types of enzymes in the late nineteenth and early twentieth century. However, the crystallization of enzymes was only later achieved in 1920s, which showed that their catalytic activity is related to protein molecules.

In biological terms, enzymes are catalysts that are responsible for accelerating biochemical reactions in living organisms. These catalysts can be extracted from a variety of cells and employed in the catalysis of a broad range of commercially important processes.

Nowadays, enzymes play a vital function in various manufacturing processes, such as beverages and foodstuffs, and are ingredients in number of consumer goods such as washing detergents. Furthermore, enzymes are also essential elements in the health sciences due to the fact that several diseases can be related to enzyme activity. Therefore, very low concentrations of enzymes are required for catalysing processes without being consumed during the reaction. These enzymes have the capability to convert the substrate to a product, as follows:

\[
\text{Substrate} \xrightarrow{\text{enzyme}} \text{Product}
\]
For any enzymatic assay, several factors have to be taken into consideration. These are ionic strength (type of buffer), pH, temperature, and suitable concentrations of the essential components such as enzymes and substrates.\textsuperscript{137,138,139} However, there are some features that have the capability to prevent unification of assay conditions, such as the existence of a variety of different enzymes in the same sample. The typical optimal temperature and pH for enzymes that are obtained from mammalian sources are pH 7.5 and a temperature of 37°C, which, unsurprisingly, are consist with the prevailing conditions in the mammalian body.\textsuperscript{137}

Enzyme assays are standard procedures used to measure the specific amount of the enzyme in a sample, whilst the activity of an enzyme can be used to illustrate its general catalytic properties.\textsuperscript{140}

### 4.1.2 Enzyme inhibition

The rates of enzyme-catalysed reactions can be influenced by a class of compounds variously named moderators, modulators or modifiers. Commonly, their impact is one of rate reduction; this is called inhibition. Conversely, where the effect of a compound on enzymatic action is to increase the reaction rate, would be termed activation; in this same sense, any compounds responsible for such effects would be termed inhibitors and activators,\textsuperscript{98} respectively.

As mentioned above, the process of blocking or reducing the activity of the active site of an enzyme through specific substrates or their analogues is called enzymatic inhibition (the substrate in this case is named as an enzyme inhibitor). Commercially, nutraceutical and pharmaceutical enzyme inhibitors show their precise interior action in the cells of animals, plants, viruses, bacteria, and the human body. In terms of drug discovery, the action of enzymatic inhibition has become an essential approach in drug research centres, university research laboratories and pharmaceutical industries.\textsuperscript{141} From this point view, several compounds have been prepared to employ as potential inhibitors for human tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase in the presence of L-tryptophan as the substrate.
4.2. Types of inhibitors

4.2.1 Reversible enzyme inhibition

A reversible enzyme inhibitor is a compound that reversibly binds to an enzyme, decelerating or preventing the enzyme from turning over substrate to product. Many metabolic processes are controlled by naturally-occurring inhibitors, though synthetic inhibitors have also been employed for agricultural reasons, such as with weed-killers, insecticides and fungicides, or as drugs. This type of inhibitor can be classified as either competitive, non-competitive/mixed, and uncompetitive inhibition.

1) Competitive inhibitor is a type of inhibitor that binds directly to the active site of the free enzyme, blocking the substrate from doing so (and vice versa).

2) Non-competitive inhibitor is an inhibitor that has the ability to bind to both the free enzyme, E, and the enzyme-substrate complex, E • S.

3) Uncompetitive inhibition, which has some similarity to the non-competitive inhibition, is where the inhibitor binds to the E • S complex to produces an inactive E • S • I complex.

Irreversible enzyme inhibitors are compound that interact with the enzyme in such a manner as to cause permanent loss of enzyme activity; this class of compounds are also called catalytic poisons. Reversible inhibitors are considered to be the much more important class due to the fact that they shape dynamic complexes with enzymes, which have various catalytic properties compared to those of the free enzymes. The inhibition process of the enzyme might be responsible for increasing the $K_m$ value (competitive inhibition), decreasing the $V$ value (pure non-competitive inhibition), reducing both $V$ and $K_m$ in a constant ratio (uncompetitive inhibition), or some combination of these impacts thereof (mixed inhibition).

4.2.2 Enzyme Kinetics

Enzyme kinetics can be described in two ways:

1) Chemical mechanism of action of enzyme
Chapter 4: Results and Discussion

This deals with four fundamental aspects of enzyme action, as follows: a) the functions of any cofactors; b) the determination of any intermediates; c) determination of any transition state to the chemical reaction catalysed by the enzyme; and d) determination of any groups of the enzymes that act as acid-base catalysts.

2) Kinetic mechanism of enzyme reactions

This includes: a) a qualitative description of the arrangement of substrate combination and product liberation from the enzyme; and b) is also responsible for determining the rate-limiting step(s) from quantitative analysis of the kinetic mechanism.\[^{145}\]

4.2.3 The Michaelis-Menten Equation 1

The Michaelis-Menten equation is an essential equation of enzyme kinetics which was originally derived for the simple case of an irreversible enzyme reaction that involves converting a single substrate to a product. However, the equation can also be applied to several enzyme catalysed reactions such as proteases, peptidases, nucleases, and so on or isomerisations “considering only the forward reaction”.\[^{146}\]

Equation 1

\[ V = V_{\text{max}} \frac{[S]}{[S] + K_m} \]

4.2.4 Identification of some important enzyme assay parameters

\( K_s \) is also known as \( K_m \) and is referred to as the dissociation constant, \( (K_s) \) and the Michaelis–Menten constant \( (K_m) \). \( K_m \) is the concentration of substrate that is required to obtain \( 1/2 V_{\text{max}} \), where \( V_{\text{max}} \) is referred to maximum velocity. Figure 26 shows these parameters.\[^{142,98}\]

\( k_{cat} \) is known as the catalyst constant or turnover number of an enzyme, which represents the maximum number of substrate molecules that can be converted to products per active site of the enzyme per unit of time. At \( 10^{-3} \) s\(^{-1} \), approximately 1000 molecules of the substrate are converted to the product every second; this is a typical value.\[^{142}\]
Equation 2

\[ k_{cat} = \frac{V_{max}}{[E]} \]

Here, [E] is the total concentration of enzyme used in the reaction.

**Figure 26:** Representation of the initial reaction velocities against substrate concentration, [S]. [S] ranges from 5 to 300 mM. The symbol (●) represents experimental initial velocity data obtained at the specified [S]. According to the plot, \( V_{max} = 4.2 \text{ nM s}^{-1} \) and \( K_m = 20 \text{ mM} \).  

4.2.5 **Experimental Measurement of \( V_{max}, k_{cat}, \) and \( K_m \)**

\( V_{max} \) and \( K_m \) are kinetic constants, and are determined graphically by measuring the initial velocity, \( V_0 \), as obtained previously from different substrate concentrations. The most straightforward manner in which to show the related data is to directly plot the reaction rates as a function of substrate concentration, providing that Michaelis–Menten kinetics is obeyed, which can be achieved using various graphical software programs.

4.3. **Some kinetic assays have been conducted to obtain the best optimum conditions to measure the change in enzyme hIDO velocity with some potential inhibitors**
Figure 27: A graph to illustrate Michaelis–Menten initial velocities of hIDO versus substrate concentration, [S], of tryptophan, where [S] ranges from 5 to 200 mM. The symbol (●) represents experimental initial velocity data obtained at the specified [S] of tryptophan in the presence of 200 nM hIDO. According to the plot, $V_{\text{max}} = 0.18 \mu M \text{s}^{-1}$ and $K_m = 52 \mu M$.

Figure 27 shows the velocity at which L-tryptophan is converted to NFK in the presence of hIDO, when the absorbance measurements were taken place by monitoring the wavelength at 321 nm which corresponds to the product NFK. Various kinetic constants can be determined from this figure, such as the Michaelis constant, $K_m$, and the maximum velocity, $V_{\text{max}}$, purely by knowing the total concentration of enzyme used (in this case human indoleamin 2,3-dioxygenase, hIDO). The turnover number, $k_{\text{cat}}$, can be measured using equation 2. Furthermore, $\frac{1}{2} V_{\text{max}}$ is 0.09, which can be used to determine $K_m$ by projecting from 0.09 on the ordinate to the corresponding point on the abscissa, giving 52 µM.
Figure 28: The kinetic assay of tryptophan and 200 nM hIDO, with and without potential inhibitor 84, which was dissolved in acetonitrile and Tris at pH = 8.
Table 5: Shows the parameters determined from kinetic assays for the potential compound 84, which is presented in figure 28.

<table>
<thead>
<tr>
<th>Compounds with 200 nM hIDO</th>
<th>Average of slope A/min</th>
<th>Time second</th>
<th>Solvent used in assays</th>
<th>Number of repeat measurements</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Just 100 µM Trp</td>
<td>0.0415</td>
<td>10-50</td>
<td>tris</td>
<td>3</td>
<td>1.5×10⁻⁶</td>
</tr>
<tr>
<td>100 µM Trp</td>
<td>0.073</td>
<td>10-50</td>
<td>20 µl CH₃CN blank and tris</td>
<td>4</td>
<td>7×10⁻³</td>
</tr>
<tr>
<td>Potential inhibitor 84 100 µM &amp; Trp. 100 µM</td>
<td>0.076</td>
<td>10-50</td>
<td>20 µl CH₃CN and tris</td>
<td>5</td>
<td>6×10⁻³</td>
</tr>
</tbody>
</table>

As mentioned in the experimental section in chapter 5, all assays were conducted in the presence of ascorbic acid, catalyst and methylene blue. As can be seen in table 5, there was almost no difference in terms of the average slope (velocity) between the kinetic assay of tryptophan and hIDO with just 20 µl acetonitrile and Tris compared with the average slope of a similar assay that included 100 µM of potential inhibitor 84. This further suggests that potential inhibitor 84 has no effect on decreasing the enzyme activity that leads to the conversion of tryptophan to NFK at 321 nm which might be leaving no room for doubt that bending in end of the curves in figure 28 were occurred due to the effect of acetonitrile. Previous potential inhibitors had been dissolved in DMSO, instead of acetonitrile and Tris, and then assayed under the same circumstances and conditions as acetonitrile. However, using this solvent did not show any decrease in the slope, which gave clear evidence that the above compounds 84 did not work as inhibitors for hIDO.
Figure 29: The kinetic assay of tryptophan and 200 nM hIDO, with and without potential inhibitor 84, which was dissolved in acetonitrile and Tris at pH = 8.

The assay in the figure 29 was conducted in order to determine the effect of time on the straightening out in the assay curves as following the blue curve for 100 µM tryptophan with 200 nM hIDO dissolved in Tris at pH = 8 can be seen that at 8.52 minutes the curve starts to level out giving evidence that all of the substrate has been converted to NFK. This can be compared to the curve shown in red for the assay containing 100 µM of potential inhibitor 84 and 100 µM tryptophan with 200 nM hIDO. For this case the levelling off of the red curve starts at 4.88 minutes, which is approximately half the time for levelling off with the control containing just tryptophan. However, this can be explained by the affect of acetonitrile in increasing the rate of the enzyme reaction as discussed above.
Figure 30: The kinetic assay for potential inhibitors 81 and 87 in the presence and absence of tryptophan, with hIDO.

From figure 30, it can be clearly seen that both compounds 81 and 87 are not substrates (see the control) and also they do not reduce the enzyme activity i.e. are not inhibitors when the slopes of the rate data were measured and compared with the slope for just tryptophan with the same enzyme.
The results of kinetic assays with the potential inhibitor 109 dissolved in DMSO and Tris in a 0.2:0.8 ratio, respectively, and including all assay components mentioned above with tryptophan and at a concentration of 200 nM hIDO are shown in table 6.

Table 6: Kinetic assays parameters of potential inhibitor 109 with hIDO as an enzyme and tryptophan as substrate; assays were run at $\lambda = 321$ nm.

<table>
<thead>
<tr>
<th>Compounds and their concentrations.</th>
<th>Average of slope</th>
<th>Number of repeat measurements</th>
<th>Time (seconds)</th>
<th>$\pm$SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µM just Trp</td>
<td>0.026</td>
<td>3</td>
<td>10-50</td>
<td>$6 \times 10^{-3}$</td>
</tr>
<tr>
<td>100 µM Trp and 50 µM PI 109</td>
<td>0.021</td>
<td>3</td>
<td>10-50</td>
<td>$1 \times 10^{-3}$</td>
</tr>
<tr>
<td>100 µM Trp and 80 µM PI 109</td>
<td>0.021</td>
<td>3</td>
<td>10-50</td>
<td>$2 \times 10^{-3}$</td>
</tr>
<tr>
<td>100 µM Trp and 100 µM PI 109</td>
<td>0.017</td>
<td>3</td>
<td>10-50</td>
<td>$1 \times 10^{-3}$</td>
</tr>
<tr>
<td>100 µM Trp and 150 µM PI 109</td>
<td>decomposed</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>100 µM Trp and 200 µM PI 109</td>
<td>decomposed</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

PI refers to potential inhibitor 109.

From table 6, it can be seen that the gradients decreased with increasing concentration of inhibitor 109, from 50 to 100 µM, in the presence of a constant 100 µM concentration of tryptophan as substrate. The decrease in the enzyme activity (gradient)
might be due to inhibition processes taking place, or due to the effect(s) of other element(s) such as solvent or any other the assay components. Furthermore, decomposition of 109 had taken place when its the concentration was subsequently increased, first to 150 µM, and then further to 200 µM. This decomposition was observed when their curves were drawn.

![Graph showing kinetics](image)

**Figure 31:** Kinetic assay at constant concentration of compound 124a with L-tryptophan and 200 nM hIDO over 5 minutes.

Figure 31 shows the activity of hIDO with tryptophan dissolved in Tris at pH = 8. The slope was calculated for this graph (red line, figure 31); this was followed by measuring the activity of the above compound 124a in which its stock solution 2 mg was dissolved in a total of 1 ml of solution made up from 0.8 ml Tris 50 mM and 0.2 ml acetonitrile, also at pH=8. A suitable aliquot of this solution was taken and run with the same concentration 100 µM of tryptophan and 200 nM of hIDO, which showed an increase in the absorbance at a limited time at 321 nm. This was evidence on the formation of NFK. However, in case of just 100 µM 124a and 200 nM hIDO, without tryptophan, an
increase in the initial absorbance was observed (control the purple line) so at 0 second the absorbance was 0.17, this may be due to the fact that the absorbance is directly proportional with the increase of the concentration; but there was not any an increase in absorbance at 321 nm. This may give evidence that 124a was not acting as a substrate or activator. Furthermore, another assay was conducted using 100 µM of 124a under exactly the same conditions but without either the tryptophan or hIDO (control line green), which showed no increase in the absorbance. Therefore, the assay of 100 µM tryptophan and 100 µM 124a with 200 nM hIDO, and the rest of assay conditions such as ascorbic acid, methylene blue and catalyst was conducted and its result are shown in figure 31. Obviously, there was an increase in both the absorbance and slope and also the end of the blue curve levelled out after 5 minutes. The reason for these changes might be due to the fact that the activity of the enzyme has been affected by the solvent (acetonitrile), rather than due to 124a acting as an activator, because we did not observe any increase in absorbance when the 124a compound in figure 31 was tested with just the hIDO and without Trp.
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Figure 32: A study of the effect of acetonitrile as solvent on the activity of hIDO with tryptophan, as well as the effect of two potential inhibitors, compounds 120a and 126, on hIDO in the presence of tryptophan.

All assay measurements were carried out on the same day in Tris solution at pH= 8, ascorbic acid, methylene blue, catalyst and tryptophan using inhibitors 120a and 126 in respective solutions in the presence of hIDO. As can be seen in figure 32, the assays measured absorbance at $\lambda = 321$ nm.

The assay with just 100 µM tryptophan and no inhibitors present resulted in the dark blue line shown in Figure 32. Noticeably, there was an increase in the absorbance during 5 minutes, and no levelling off was observed. However, when 20 µl of acetonitrile was added to this assay (red line) we did observe the characteristic levelling off after 200 sec. The explanation for this is that there was some effect of the acetonitrile on the conversion of tryptophan to NFK, or on the activity of hIDO. Similar observations were made using the same components with the addition of 100 µM potential inhibitor 120a; as can been seen in the orange curve. Furthermore, figure 32 shows the control assays for both potential inhibitors 120a and 126 without 1-
tryptophan, from which it is obvious that the activities of these compounds are approximately zero in the absence of tryptophan and in the presence of 200 nM hIDO.

At the same time, and due to the fact that the absorbance measured is directly proportional to the concentrations of the substances in solution, it can be observed that the absorbance of the potential inhibitors 120a and 126 with tryptophan, 200 nM hIDO, and other assay components has increased. However, no decrease was observed with either of these two compounds when the slopes were measured before and after added 120a and 126 in present of tryptophan and hIDO.

4.4. Human tryptophan 2,3-dioxygenase hTDO

After expression of hTDO, purification achieved by using Ni-NTA metal–affinity chromatography, followed by detection of separate proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the presence of the required protein, or otherwise, by comparison of the molecular weight of hTDO to a standard protein marker.
Figure 33: The SDS-PAGE gel of hTDO purified by nickel column. Lane A: molecular markers (masses shown next to lane A) and gels 1, 2 and 3 from different sample collection fractions for same column for hTDO and others enzymes.

In figure 33, G1, G2 and G3 refer to gels 1, 2 and 3. Samples of gels 1 and 2 were concentrated utilizing a Millipore Amicon Ultra centrifugal filter with a molecular weight cut-off of 30 kDa to remove as much eluting solvent as possible (which contains amidazole) and from the same figure it can be observed that G1 and G2 contained fewer impurities than G3. Therefore, the original proteins in sample tubes related to G1 and G2 were combined and injected into the gel filtration column (Bio-Rad 10 DG) for purification. This gave pure hTDO, as shown in figure 34.
The SDS-PAGE gel, for hTDO in figure 34 shows pure protein bands with a molecular weight of 47.872 KDa. Compared with protein marker A, this molecular weight seems to be identical to the molecular weight of the human tryptophan 2,3-dioxygenase which was obtained after purification of hTDO enzymes by gel filtration column (Bio-Rad 10 DG). The above gel represents a pure, though not yet concentrated by centrifuge, sample of hTDO, which was collected by passing this enzyme through a gel filtration column (Bio-Rad 10 DG), from which the purified sample was received in different sample collection tubes.
Chapter 4: Results and Discussion

Figure 35: SDS-PAGE gel of hTDO purified by gel filtration followed by concentration and combination of B9 and run it again on both sides right and left of protein marker A and carried out symbols B9

The SDS-PAGE gel in figure 35 shows the pure concentrated and combined hTDO enzyme which is referred to as B9 in the same figure. This was presented in the figure 34, were run against a protein marker to exhibit the expected hTDO, with a few impurities due to other proteins. This purified protein was concentrated utilizing a Millipore Amicon Ultra centrifugal filter with a molecular weight cut-off of 30 kDa and kept in the fridge at -80°C as separate samples. Each sample of pure hTDO was treated separately with determined amount of hemin in order to make sure of equilibrium of hemin in each part of enzyme. Therefore, after each addition of hemin the absorbance was measured at 408 nm until the absorbance of this peak reached or equalled the absorbance peak at 280 nm. At this point the concentration of pure hTDO with additional hemin was determined using the known absorbance of hTDO peak at 408 nm, absorbance coefficient 196 mM⁻¹ cm⁻¹ of this enzyme at 408 nm and length of cuvette 1 cm, as shown in figure 36
A certain concentration 60 µM of prepared hemin was added in different amounts to pure hTDO, as can be seen in the figure 36. The reason behind these additions is that there was some parts of hTDO that contained relatively low amounts of hemin compared to other parts. As a consequence of these additions, the absorbance peaks at 408 nm increased until they reached approximately the same intensity as their neighbouring peaks at 280 nm, as shown in figure 36; at this point, the concentration of pure protein was measured using the Beer-Lambert law, as shown below:

\[ \varepsilon_{408} = 196 \text{ mM}^{-1} \text{ cm}^{-1} = \frac{0.79}{C \times 1 \text{ cm}} \]
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\[ C = \frac{0.79}{196 \text{ mM}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}} \times 1000 = 4.03 \mu\text{M} \]

It can be clearly seen that the concentration obtained for hTDO, after purification and addition of hemin, is still insufficient.

0.5 μM of fresh hTDO treated with hemin was used for each kinetic assay with some of our synthesised compounds as potential inhibitors in the presence of 100 μM of tryptophan as substrate and catalyst, with methylene blue and ascorbic acid in Tris as a buffer solution at pH =8. Compounds that were examined as potential inhibitors of hTDO are 84, 85, and 119, in the presence of tryptophan as substrate. However, no decrease in the rate of the assay were observed in the presence of these species compared with a tryptophan only assay (i.e., in the absence of these compounds).

4.5. Conclusion

The majority of our synthesised compounds have no apparent inhibition effects on IDO and TDO enzyme that lead to turnover of tryptophan to NFK. The potential inhibitor 109 showed a decrease in hIDO velocity at concentrations of 100 μM and less, which might be due to inhibition of the enzyme; however, decomposition of 109 was observed for kinetic assay components at concentrations of 150 and 200 μM of potential inhibitor 109, which means at these concentrations, there was great impact on enzyme activity and the rest of assays components.

In terms of expression and purification of hTDO and in spite of the fact that we have started using large amount of media for feeding cells as described in experimental chapter 5. Although, the expression and purification of hTDO was performed twice, the concentrations of hTDO were 5.6 μM at the first time and 4 μM at the second, were obtained at 280 nm after the final purification using the gel filtration column.

One might consider that 0.5 μM pure hTDO, after adding hemin, is required for each assay. This suggests that the total amount of pure hTDO is not sufficient to run assays with our various potential inhibitors; we note this with due regard to the fact that the expression and purification of this protein takes long period of time (approximately a month in order to gain the pure form). As a consequence, only a very few of the synthesised products were assayed in the presence of tryptophan as a substrate. However, these experiments did not show any decrease in velocity (gradient) during
kinetic assay once 84, 85 and 119 as potential inhibitors, as compared to the assay of tryptophan only as a substrate (with the same hTDO enzyme and other assay components.
Chapter 5: Experimental
5. Experimental

5.1. General Experimental section

Generally, all the commercial materials (chemicals) that were used were obtained from commercial sources such as Sigma Aldrich and Fisher. These were used without further purification unless otherwise stated.

The melting point measurements for the solid compounds were conducted using a Stuart Scientific SMP3 apparatus.

Different diameters of column have employed for the flash chromatography purification of some of these compounds; in all such purifications the column was loaded with silica gel 60 from Merck (40–60 µm).

A variety of deuterated NMR solvents were used to dissolve the samples, which included CDCl₃, CD₃OD, DMSO-d6 and D₂O. Bruker DXR500 (500 MHz), Bruker DRX400 (400 MHz) and Bruker DRX300 (300 MHz) instruments were used for ¹H, ¹³C, DEPT, COSY, NOE and HMQC analysis.

IR spectra for liquids and solids were recorded for the neat sample using a Perkin Elmer FT-IR spectrometer.

The dry solvents were taken from the solvent purification system Pure Solve™.

Compounds were analysed by LC-MS using a Devo Stove mass spectrometer (Waters) coupled to an Acquit LC system (Waters) using an Acquit UPLC BEH C18 column (2.1 x 50 mm, Waters). The flow rate was 0.6 ml min⁻¹ and the gradient was as follows: 95% Solvent A (0.1% formic acid in water) with 5% solvent B (0.1% formic acid in acetonitrile) was held constant for 0.5 min, followed by a linear gradient to 100% B over the next 2.1 min. After 1 min at 100% solvent B, the gradient was returned to 95% solvent A and 5% solvent B over 0.2 min. The ESI capillary voltage was 3 kV, cone voltage 30 V and collision energy 4 eV. The MS acquisition rate was 10 spectra per second and m/z data ranging from 50 to 2000 Da was collected. Mass accuracy was achieved using a reference lock mass scan, once every 10 seconds.
Whilst there were some samples analysed using ASAP (Atmospheric Solids Analysis Probe), the corona discharge pin current was 5 µA, cone voltage 30 V and collision energy 4 eV. The MS acquisition rate was 5 spectra per second and m/z data ranging from 50 to 1000 Da was collected. Mass accuracy was achieved using a reference lock mass scan, once every 10 seconds in ESI mode.

Data for X-ray structures were collected on a Bruker Apex 2000 CCD diffractometer using graphite monochromated Mo-Kα radiation, λ = 0.7107 Å. The data were corrected for Lorentz and polarisation effects and empirical absorption corrections were applied. The structure was solved by direct methods and with structure refinement on $F^2$ employed SHELXTL version 6.10. Hydrogen atoms were included in calculated positions (C—H = 0.95–0.99 Å) riding on the bonded atom with isotropic displacement parameters set to 1.5U$_{eq}$ (C) for methyl hydrogen atoms and 1.2U$_{eq}$ (C) for all other H atoms. All non-hydrogen atoms were refined with anisotropic displacement parameters

5.1.1 Protein purification and Kinetic assays

Perkin-Elmer Lambda40, Lambda35 and Lambda25 spectrometers have been used for UV/Vis analysis as well as a JENWAY 6305 UV/Vis Spectrophotometer.

Two different types of gel electrophoresis cell connected with a BIO-RAD power PAC 300, were employed.

Three different types of centrifuge apparatus were employed, which included an Allegra X-30R Beckman Coulter, max. load 4×100 g, Eppendorf F45-12-11, max.load12×4g, and a SORVALL® Evolution, SLC-6000 and SS-34.

Gel filtration column used superdex™ 200

All solutions, media and buffers were prepared employing ultra-pure, doubly-deionised water from an Elga PureLab water purifier.
5.2. Synthetic section

N-Benzoylglycine 71

Sodium hydroxide (10 g) was dissolved in 100 ml deionized water. A 50 ml aliquot of this solution was used to dissolve glycine (5 g, 67 mmol, 0.72 eq.) followed by adding benzoyl chloride (13 g, 93 mmol) in portions. The reaction mixture was stirred vigorously after each addition. 50 g of crushed-ice was then added to the reaction mixture, which was then stirred for an hour and a half. HCl was then added to the solution dropwise until the recorded pH had reached 2. The suspension thus formed was filtered from the solution to give hippuric acid (2-benzamidoacetic acid) 71 as a pure white powder (10.5 g, 84%), mp: -180–185°C.

δ_H (400 MHz, CD_3 OD), 4.1 (2H, s, 2 × H9), 7.44–7.49 (2H, m, H3 & H5), 7.52–7.60 (1H, m, H4), 8.88 (2H, dd, J = 8.6, 1.4 Hz, H2 & H6), 7.87–7.89 (1H, dd, J = 8.1, 1.4 Hz, H8), 8.83 (1H, s, H11). δ_C (100 MHz, CD_3 OD), 42.5 (CH_2, C9), 128.5 (2 × CH_3 & CH5), 129.7 (2 × CH2 & CH6), 132.6 (CH4), 135.1 (C1), 170.6 (C7), 172.2 (C10). MS (ESI) m/z found 180.0663 (MH^+) calculated for C_9H_10NO_3, 180.0661. IR, ν_max (neat), 3338w, 3064w, 1740m, 1679m, 1599s, 1554s, 1292s, 1178s, 999.0m, 942m, 720.4s, 658.2s, cm^{-1}.

Synthesis of (Z)-4-((1-acetyl – 1H-indole -3-yl) methylene)-2-phenyloxazol-5(4H)-one 73

Hippuric acid 71 (1.825 g, 10 mmol, 1 eq.), sodium acetate (0.868 g, 10 mmol, 1eq) and indole-3-carboxyaldehyde (1.522 g, 10 mmol, 1 eq.) were combined and acetic anhydride (25 ml, 260 mmol, 26 eq.) added as solvent. The reaction mixture was stirred magnetically for 3.5 hours at 80°C, and then the warm solution was filtered directly and washed with10 ml acetic anhydride, followed by evaporation in a rotary evaporator to give dried semi solid orange mass still containing residual solvent. This was ultimately submitted to **high vacuum** for one hour to give (Z)-4-((1-acetyl-1H-indol-3-yl) methylene)-2-phenyloxazol-5(4H)-one 73 (1.92 g, 58%) as a pure orange solid, mp: - 193–195°C compared with melting point for the same product (203–205°C) that was reported by literature.
δ_H (400 MHz, CDCl₃), 2.79 (3H, s, 3 × H 21), 7.38–7.45 (2H, m, H1 & H6), 7.47 (1H, s, H11), 7.53 (2H, t, J = 7.5 Hz, H16 & H18), 7.62 (1H, tt, J = 14.8, 6.1 Hz, H17), 7.84 (1H, dd, J = 6.7, 1.1 Hz, H2), 8.12 (2H, d, J = 7.2 Hz, H15 & H19), 8.47 (1H, d, J = 7.1 Hz, H3), 8.73 (1H, s, H7) δ_C (100 MHz, CDCl₃), 23.9 (CH₃), 116.6 (C8), 116.9 (CH₃), 118.8 (CH2), 121.8 (CH1), 124.7 (CH6), 125.6 (C5), 126.2 (CH7), 128.2 (2 × CH16 & CH18), 129.0 (2 × CH15 & CH19), 129.1 (C4), 131.2 (CH17), 132.6 (C14), 133.4 (CH9), 135.7 (C10), 163.0 (C12), 166.8 (C13), 168.7 (C20). MS (ASAP) m/z found 331.1075 (MH⁺), calculated for C₂₀H₁₅N₂O₃: 331.1083. IR, 𝜈_{max} (neat), 1789m, 1717m, 1641m, 1267m, 1017m, 863.6m, 753.2s, 700.4s, cm⁻¹.

1-(1-acetyl-1H-indol-3-yl)-5-phenyl-6-oxa-4-azaspiro[2.4]-hept-4-en-7-one 74

This reaction was conducted as follows: (Z)-4-((1-acetyl-1H-indol-3-yl) methyl)-2-phenyloxazol-5-(4H)-one 73 (0.11 g, 0.33 mmol) was mixed with trimethylsulfoxonium iodide (0.09 g, 0.41 mmol, 1.24 eq.) and potassium t-butoxide (0.046 g, 0.41 mmol, 1.24 eq.). The reaction mixture was suspended in 4 ml toluene and stirred for 5 hours at 110°C. 1 ml brine was added, followed by extraction by ethyl acetate (3×10 ml) and washing with water (3×10 ml). To the combined organic layer, MgSO₄ as a drying agent was added, followed by filtration and evaporation to give the crude product 1-(1-acetyl-1H-indol-3-yl)-5-phenyl-6-oxa-4-azaspiro[2.4]hept-4-en-7-one 74 (0.046 g). This was ultimately submitted into a flash column and eluted by 90:10 and 80:20 petroleum ether:ethyl acetate, respectively, to afford sticky compound 74 (0.03 g, 27%), which was pure enough to characterise by NMR and mass spectrometer.

δ_H (400 MHz, CDCl₃): 2.18–2.22 (2H, m, 1 × H22), 2.26–2.30 (1H, m, 1 × H22), 2.60 (3H, s, 1 × H21), 3.16–3.20 (1H, m, H11) 7.16–7.51 and 7.82–8.36 (10 H, 2 m, 2 aromatics). δ_C (100 MHz, CDCl₃), 23.1 (CH₃), 23.98 (CH2, C22), 27.6 (CH9), 52.1 (C10), 117.6 (CH3), 122.4 (CH6), 122.8 (C8), 123.1 (CH7), 124.6 (CH1), 125.8 (CH2), 126.4 (2 × CH15 & CH19), 126.8 (C14), 127.8 (2 × CH16 & CH18), 127.9 (C5), 131.5 (CH17), 136.9 (C4), 161.9 (C12), 168.1 (C20), 176.1 (C13). MS (ESI) m/z found 345.1217 (MH⁺), calculated for C₂₁H₁₇N₂O₃: 345.1239. IR, 𝜈_{max} (neat) 1789m, 1717s, 1650s, 1529 m, 1149s, 1087w, 999.1m, 700.8m, cm⁻¹.
(E)-ethyl-3-(1H-indol-3-yl) acrylate compound 81

This was synthesised according to Sheu et al.\textsuperscript{114} triethyl phosphonoacetate (3.5 g, 15.5 mmol, 2.25 eq.) in 17 ml dry THF was added to a reaction vessel and stirred with sodium hydride 60\% dispersion in mineral oil (0.4 g, 10 mmol, 1.45 eq.) in 7 ml dry THF; which was added dropwise over 15 minutes. The reaction mixture was stirred for 2 hours at room temperature and then a solution of indole-3-carboxaldehyde (1 g, 6.9 mmol) in 17 ml of dry THF was added dropwise over 10 minutes. The reaction mixture was heated under reflux conditions overnight, followed by treatment with 5 ml brine, extraction using ether (3×10 ml) and then drying by MgSO\textsubscript{4}. The resultant solution was evaporated to give a mixture of (E)-ethyl-3-(1H-indol-3-yl)-acrylate 81 with some residue of the starting material triethyl-phosphonoacetate. The crude product was washed with petroleum ether at 40–60°C ten times to give (E)-ethyl 3-(1H-indol-3-yl) acrylate 81 (0.933 g, 65\%) as a pure brown solid, m.p.: 116–118°C compared with the melting point for the same product (121°C) that was reported by literature.\textsuperscript{114}

\[\delta_H (400 \text{ MHz, CDCl}_3): 1.34 (3H, t, J = 7.2 \text{ Hz, } 3 \times \text{H14}), 4.28 (2H, q, J = 7.2 \text{ Hz, } 2 \times \text{H13}), 6.46 (1H, d, J = 16 \text{ Hz, } \alpha\text{-H11}), 7.19–7.26 (2H, m, H1 & H2), 7.34–7.39 (2H, m, H3 & H8), 7.85–7.87 (1H, m, H6), 7.93 (1H, d, J = 16 \text{ Hz, } \beta\text{-H10}), 9.28 (1H, s, NH1).\]

\[\delta_C (100 \text{ MHz, CDCl}_3): 14.5 (\text{CH}_3, C14), 60.3 (\text{CH}_2, C13), 112.1 (\text{CH3}), 112.9 (\text{CH10}), 113.3 (C9), 120.4 (\text{CH6}), 121.5 (\text{CH1}), 123.3 (\text{CH2}), 125.3 (C5), 129.5 (\text{CH8}), 137.4 (C4), 138.8 (C11), 168.8 (C12).\]

MS (ASAP) m/z found 216.1023 (MH\textsuperscript{+}), calculated for C\textsubscript{13}H\textsubscript{14}NO\textsubscript{2}: 216.1025. IR, \(\nu_{\text{max}}\) (neat), 3276m, 2983w, 1666s, 1616s, 1240 s, 747.7s cm\textsuperscript{-1}. Elemental analysis: found: C: 72.64, H: 6.14, N: 6.60. Calculated for C\textsubscript{13}H\textsubscript{13}NO\textsubscript{2}: C: 72.54, H: 6.09, N: 6.59.

1-methyl-1H-indole-3-carbaldehyde 82

A mixture of K\textsubscript{2}CO\textsubscript{3} (2.38 g, 17 mmol, 4.93 eq.) and 1H-indole-3-carbaldehyde (0.5 g, 3.45 mmol) was suspended in 15 ml dry acetone and stirred at room temperature for 1 hour, after which methyl iodide (1.14 g, 8.0 mmol, 2.32 eq.) was added.\textsuperscript{147} This reaction mixture was stirred at 30°C in the presence of a condenser and under a nitrogen atmosphere overnight. The product was filtered in order to remove
the remaining K$_2$CO$_3$, followed by removal of the solvent by rotary evaporation. 20 ml water was then added to the crude product, followed by extraction (2×15 ml) in DCM. The organic layers were combined and dried using MgSO$_4$. Afterwards, this was filtered and evaporated to give 1-methyl-1H-indole-3-carbaldehyde 82 (0.501 g, 91%) as pure brown product, m.p.: 70–73 °C.

δ$_H$ (400 MHz, CDCl$_3$): 3.76 (3H, s, 3 × H10), 7.18–7.28 (1H, m, H2), 7.56 (1H, s, H7), 8.17–8.24 (1H, m, H6), 9.89 (1H, s, H9). δ$_C$ (75 MHz, CDCl$_3$): 33.7 (CH$_3$, C10), 109.9 (CH$_3$), 118.1 (C8), 122.0 (CH1), 122.9 (2 × CH6 & CH2), 124.0 (C5), 137.9 (C4), 139.3 (CH7), 184.5 (CH9). MS (ASAP) m/z found 160.0762 (MH$^+$), calculated for C$_{10}$H$_{10}$NO: 160.0762. IR, $\nu_{\max}$ (neat), 3108w, 2918w, 1655s, 1536m, 786.6m, 1399m, 745.21 m, cm$^{-1}$.

**Ethyl (E)-3-(1-methyl-1H-indol-3-yl) acrylate 83**

To a stirred solution of sodium hydride 60% dispersion in mineral oil (0.18 g, 4.5 mmol, 1.49 eq.) in 3 ml dry THF, triethylphosphonoacetate (1.53 g, 6.04 mmol, 2 eq.) in 7.6 ml dry THF was added dropwise over 10 minutes at room temperature. The reaction was left to stir for a further 30 minutes; subsequently a solution of 1-methyl-1H-indole-3-carbaldehyde (0.482 g, 3.03 mmol) 82 in 7.6 ml dry THF was added dropwise over a 10 minute period. The whole reaction mixture was then stirred and refluxed overnight. 30 ml of water was then added, followed by extraction by diethyl ether (3×20 ml) and evaporation, to give a crude mixture of 83 and a residue of the starting material, triethyl phosphonoacetate. The crude product was washed four times with 100 ml cool petroleum ether (40–60) in order to remove of starting material as possible. After these procedures, just (0.14 g) of product was obtained as pure. An attempt was made to purify the remaining impure of 83 by a crystallization process, where impure crude was dissolved in ethyl acetate and a few drops of chloroform were added. The sample container lid was left on for two days to give Ethyl (E)-3-(1-methyl-1H-indol-3-yl) acrylate 83 (0.3402 g) as pure crystalline. In order to gain the actual weight and percentage yield of this product both pure products were combined to give the total pure product 83 (0.4802 g, 70%), m.p.: 104–105°C.
δ_H (300 MHz, CDCl₃): 1.38 (3H, t, J = 7.1 Hz, 3 × H13), 3.84 (3H, s, 3 × H14), 4.30 (2H, q, J = 7.1 Hz, 2 × H12), 6.44 (1H, d, J = 16 Hz, α-H10), 7.26–7.40 (4H, m, H1, H2, H3 & H7), 7.91 (1H, d, J = 16 Hz, β-H9), 7.96 (1H, t, J = 2.1 Hz, H6). δ_C (75 MHz, CDCl₃): 14.5 (CH₃, C13), 33.2 (CH₃, C14), 60.0 (CH₂, C12), 109.9 (CH₃), 112.1 (C8), 112.6 (CH6), 120.6 (CH1), 122.9 (CH10), 126.1 (C5), 133.1 (CH7), 137.9 (CH9), 138.1 (C4), 168.4 (C11). MS (ASAP) m/z found 230.1182 (MH⁺) requires 230.28.

Ethyl 2-(1-methyl-1H-indol-3-yl) cyclopropane-1-carboxylate) 84

To the ethyl (E)-3-(1-methyl-1H-indol-3-yl)-acrylate (0.295 g, 1.29 mmol) 83 in 5 ml dry DMSO, potassium hydroxide (0.06 g, 1.07 mmol, 0.83 eq.) and trimethylsulfoxonium iodide (0.3 g, 1.36 mmol, 1.05 eq.) were added. The reaction mixture was then left to stir under a nitrogen atmosphere at 80°C for 48 hours. Then the crude product was worked up by adding 30 ml water followed by liquid-liquid extraction using ethyl acetate (3×20 ml). 2 ml of brine was then added to the combination of the organic layer followed by drying over by MgSO₄. The mixture was evaporated down in order to remove solvent then submitted to high vacuum to give (0.31 g) as the crude mixture of product 84 and starting material. As a consequence, the crude mixture was purified in the flash column, using hexane: ethyl acetate in an 80:20, ratio respectively, as eluting solvent. This gave ethyl 2-(1-methyl-1H-indol-3-yl) cyclopropane-1-carboxylate) 84 (0.088 g, 29%) as pure white solid, m.p: 70–72°C.

δ_H (400 MHz, CDCl₃): 1.27–1.31 (1H, m, 1 × H15), 1.29 (3H, t, J = 7.1 Hz, 3 × H13), 1.54–1.58 (1H, m, 1 × H15), 1.84–1.88 (1H, m, α-H10), 2.57–2.62 (1H, m, β-H9) 3.67 (3H, s, 3 × H14), 4.19 (2H, q, J = 7.1 Hz, 2 × H12), 6.76 (1H, br, H7), 7.01–7.13 (1H, m, H1), 7.19–7.26 (2H, m, H2 & H3), 7.64 (1H, br d, J = 7.9 Hz, H6). δ_C (75 MHz, CDCl₃): 14.4 (CH₃, C13), 15.7 (CH₂, C15), 18.1 (CH9), 22.2 (CH10), 32.7 (CH₃, C14), 60.6 (CH₂, C12) 109.4 (CH3), 114.2 (C8), 119.1 (CH6), 119.1 (CH1), 121.9 (CH2) 125.9 (CH7), 127.9 (C4) 137.1 (C5), 174.1 (C11). MS (ASAP) m/z found 244.1332 (MH⁺), calculated for C₁₅H₁₈NO₂: 244.1338. IR, ν_max (neat), 3055w, 2925m, 2854w,
1721s, 1371m, 1178s, 1044m, 737.7m, cm\(^{-1}\). Elemental analysis: found: C: 74.17, H: 7.15, N: 5.77. Calculated for C\(_{13}\)H\(_{17}\)NO\(_2\): C: 74.05, H: 7.04, N: 5.76.

2-(1-methyl-1H-indol-3-yl) cyclopropane-1-carboxylic acid 85

Potassium hydroxide (0.029 g, 0.52 mmol, 1.49 eq.) in 0.425 ml of water was added to ethyl 2-(1-methyl-1H-indol-3-yl)-cyclopropane-1-carboxylate 84 (0.085 g, 0.35 mmol) in 4.25 ml ethanol.\(^{148}\) The reaction mixture was left to stir for 24 hours. 20 ml of water was added, followed by 40 ml ethyl acetate; it could be clearly observed the most of the yellow product remains in the aqueous layer. Consequently, this layer was acidified using 1N HCl, until pH = 2 was achieved; however, no precipitation of the product occurred. Therefore, re-extraction of the aqueous layer was undertaken using the same organic solvent. Afterward, the organic layer was evaporated and then submitted to high vacuum overnight to give a pure brown sticky product of 2-(1-methyl-1H-indol-3-yl) cyclopropane-1-carboxylic acid 85 (0.073 g, 97%).

\[
\delta_H (300 \text{ MHz, CDCl}_3): 1.30–1.37 (1H, m, 1 \times H14), 1.55–1.61 (1H, m, 1 \times H14), 1.78–1.83 (1H, m, \alpha-H10), 2.60–2.66 (1H, m, \beta-H9), 3.67 (3H, s, 3 \times H13), 6.75 (1H, s, H7), 7.04–7.09 (1H, m, H1), 7.24–7.146 (2H, m, H2 & H6), 7.61 (1H, d, J = 6 Hz, H3), 11.73 (1H, s, H12). \delta_C (100 \text{ MHz, CDCl}_3): 16.2 (CH, C14), 19.1 (CH9), 22.2 (CH10), 32.6 (C13), 109.3 (CH3), 113.7 (C8), 118.9 (CH6), 119.2 (CH1), 121.9 (CH2), 125.9 (CH7), 127.8 (C5), 136.7 (C4), 180.7 (C11). MS (ASAP) m/z found 216.1025 (MH\(^+\)), calculated for C\(_{13}\)H\(_{14}\)NO\(_2\): 216.1023. IR, \(v_{\text{max}}\) (neat), 3051w, 2937w, 1690s, 1332m, 1229m, 922.7m, 737.7m, cm\(^{-1}\). Elemental analysis: found: C: 72.58, H: 6.19, N: 6.6. Calculated for C\(_{13}\)H\(_{13}\)NO\(_2\): C: 72.54, H: 6.09, N: 6.61.

(E)-3-(1-methyl-1H-indol-3-yl) acrylic acid 85a

Ethyl (E)-3-(1-methyl-1H-indol-3-yl) acrylate 83 (0.053 g, 0.23 mmol) in 3 ml ethanol was added to KOH (0.018 g, 0.32 mmol, 1.39 eq.) in 0.3 ml H\(_2\)O. The whole reaction mixture was left to stir for 24 hours at 50°C. Afterwards, 20 ml water was added followed by extraction with ethyl acetate (2×20 ml). It can be clearly seen that most of the product remained in the aqueous layer; consequently, the aqueous layer was acidified by 1N
HCl until pH = 2 was achieved and the pure, white product precipitated out. The product was kept in the fridge for 30 minutes before filtration through a funnel; thereafter, the filter paper with the product was put into the oven for 30 minutes to dry then transferred to a round-bottomed flask and submitted to high vacuum overnight. This gave (E)-3-(1-methyl-1H-indol-3-yl) acrylic acid 85a (0.022 g, 46.8%) as a pure product, m.p.: 173–175°C.

δ_H (300 MHz, CDCl_3): 3.82 (3H, s, 3 × H14), 6.31 (1H, d, J = 16 Hz, α-H10), 7.19–7.31 (2H, m, H2 & H3), 7.52 (1H, d, J = 7.9 Hz, H1), 7.79 (1H, d, J = 16 Hz, β-H9), 7.88 (2H, d, J = 9.8 Hz, H6 & H7), 11.92 (1H, s, H13). δ_C (100 MHz, CDCl_3): 32.8 (CH3,C14), 110.7 (CH3), 112.2 (C9), 119.9 (CH1), 121.0 (CH2), 122.5 (C6), 125.4 (C5), 134.7 (CH7), 137.8 (C10), 137.9 (C4), 168.5 (C12). MS (ESI) m/z found 202.0858 (MH⁺), calculated for C_{12}H_{12}NO_2: 202.0868. IR, ν_max (neat), 3111w, 2823w, 1651s, 1208m, 919.7m, 730.1m, 642.9m, cm⁻¹.

**Ethyl (E)-3-(1-tosyl-1H-indol-3-yl) acrylate 87**

P-toluene sulfonyl chloride (2.73 g, 14 mmol, 1.54 eq.) was added to ethyl (E)-3-(1H-indol-3-yl) acrylate 81 (1.95 g, 9.1 mmol) in 22 ml of trimethylamine, Et₃N. This solution was left to stir for 1 an hour at 90°C, and the resulting product was poured into 50 ml ice water and then kept in the fridge for 1 an hour. Afterwards, the product was filtrated, followed by drying on the filter paper in the oven for 1 hour in order to remove the Et₃N. The solid product was transferred to round-bottomed flask, followed by submission to high vacuum overnight to give ethyl (E)-3-(1-tosyl-1H-indol-3-yl) acrylate 87 (2.84 g, 86%) as pure product, m.p.: 131–133°C.

δ_H (300 MHz, CDCl_3): 1.34 (3H, t, J = 7.1 Hz, 3 × H13), 2.37 (3H, s, 3 × H18), 6.43 (2H, q, J = 7.1 Hz, 2 × H12), 6.51 (1H, d, J = 16 Hz, α-H10), 7.22–7.39 (4H, m, Ph), 7.76–7.84 (5H, m, 3 × Ph+2 × H7& H9), 8.0 (1H, d, J = 8.1 Hz, H20). δ_C (75 MHz, CDCl_3): 14.4 (CH₃, 3 × H13), 21.6 (CH₃, C18), 29.7 (C8), 60.5 (CH₂, C12), 113.8 (CH6), 118.2 (C5), 118.4 (CH10), 120.7 (CH2), 124.1 (CH1), 125.5 (CH3), 127.0 (2 × CH 15 & H20), 128.1 (C4), 128.4 (CH7), 130.0 (2 × CH16 & CH19), 134.8 (C14), 119
135.6 (CH9), 145.6 (C17), 167.1 (C11). MS (ASAP) m/z found 370.1096 (MH⁺), calculated for C₂₀H₂₀NO₄S: 370.1113. IR, 𝜈max (neat), 3122w, 2980w, 1705s, 1174s, 1036m, 745.6m, 666.9m, cm⁻¹.

**Ethyl 2-(1-tosyl-1H-indol-3-yl) cyclopropane-1-carboxylate 88**

Potassium hydroxide (0.12 g, 2.14 mmol, 1.55 eq.) was added to ethyl (E)-3-(1-tosyl-1H-indol-3-yl) acrylate 87 (0.51 g, 1.38 mmol) in 6.1 ml DMSO and placed in a 10 ml round-bottomed flask, to which trimethylsulfoxonium iodide (0.33 g, 1.5 mmol, 1.09 eq.) was further added. Thereafter, the whole reaction mixture was left to stir for 72 hours at 80°C under a nitrogen atmosphere. 40 ml water was then added, followed by extraction using ethyl acetate (3×30 ml). The combined organic layers were treated by adding 5 ml brine and ultimately MgSO₄ was added as a drying agent, followed by filtration via funnel and subsequent evaporation. The final crude product was submitted to *high vacuum* overnight. The NMR spectrum of the resultant product has showed that some starting material, desired product and other impurities were present. The resultant was consequently submitted to the flash column for the purification, using 95:5, 90:10 and 85:15 ml ratios of hexane: ethyl acetate as eluting solvent to give ethyl 2-(1-tosyl-1H-indol-3-yl) cyclopropane-1-carboxylate 88 (0.178 g, 34%) as an oily pure compound.

δ_H (400 MHz, CDCl₃): 1.25–1.29 (1H, m, 1 × H21), 1.29 (3H, t, J = 7.1 Hz, 3 × H13), 1.56–1.60 (1H, m, 1 × H21), 1.85–1.90 (1H, m, α-H110), 2.31 (3H, s, 3 × H18), 2.46–2.52 (1H, m, β-H9), 4.20 (2H, q, J = 7.1 Hz, 2 × H12), 7.18–7.34 (2H, m, H1 & H7), 7.55 (3H, d, J = 7.8 Hz, H2, H16 & H19), 7.73 (3H, d, J = 8.4 Hz, H3, H15 & H20), 7.96 (1H, d, J = 8.2 Hz, H6). δ_C (75 MHz, CDCl₃): 14.3 (CH₃,C13), 15.3 (CH₂, C21), 16.9 (CH9), 21.6 (CH₃, C18), 21.9 (CH10), 60.9 (CH₂, C12), 113.8 (C8), 119.6 (CH6), 122.3 (CH1), 122.4 (CH7), 123.3 (CH3), 125.1 (CH2), 126.8 (2 × CH15 & CH20), 129.9 (2 × CH16 & CH19), 130.8 (C4), 135.2 (C14), 144.9 (C17), 173.4 (C11). MS (ASAP) m/z found 384.1276 (MH⁺), calculated for C₂₁H₂₂NO₄S: 384.1270. IR, 𝜈max (neat), 3110w, 2981w, 1721s, 1367m, 1178s, 670.8m, cm⁻¹.
Ethyl 2-(1H-indol-3-yl)-cyclopropanecarboxylate 89

The product 88 (0.165 g, 0.43 mmol) was treated with Cs$_2$CO$_3$ (0.42 g, 1.29 mmol, 3 eq.). This was dissolved in 6.3:3.1 ml THF:MeOH. The reaction mixture was left to stir overnight at 50 °C followed by evaporation of the solvent and the crude product was redissolved in 25 ml THF. In order to remove the insoluble Cs$_2$CO$_3$, the solution was filtered through filter paper. The filtrate was placed in a round-bottomed flask of known weight, followed by evaporation and submission to high vacuum for 4 hours. However, the weight of the crude product 89 obtained was 0.189 g, which was higher than original starting material. The weight of crude indicated that there was still a considerable amount of caesium carbonate remaining. The curde product 89 was redissolved in 20 ml DCM: D$_2$O in a 15:5 ratio, respectively, followed by drying over 89 to give a yield of 0.168 g. This was dissolved in an acetonitrile:water 0.8:0.2 ml respectively, and injected in portions into an HPLC instrument to give the product 89 with only (5 mg, 6%) as an isolated yield.

$\delta_H$ (300 MHz, CD$_3$OD) 1.36–1.42 (1H, m, 1 × H13), 1.50–1.56 (1H, m, 1 × H13), 1.75–1.80 (1H, m, H11), 2.52–2.58 (1H, m, H10), 7.01–7.15 (3H, m, H1, H2 & H8), 7.34 (1H, dt, $J = 8.2, 0.9$ Hz, H3), 7.63 (1H, dt, $J = 8.2, 0.9$ Hz, H6). MS (ASAP) m/z found 202.0869 (MH$^+$), calculated for C$_{12}$H$_{12}$NO$_2$: 202.0868.

1-Benzyl-1H-indole-3-carbaldehyde 90

A solution containing K$_2$CO$_3$ (4.76 g, 34 mmol, 4.93 eq.) and indole-3-carboxaldehyde (1 g, 6.9 mmol) in 30 ml dry acetone was stirred at room temperature for one an hour and then benzyl chloride (0.94 ml, 8 mmole, 1.16 eq.) was added. The reaction mixture was refluxed for 5 hours and filtered. The filtrate was evaporated, and the solid product extracted by adding 30 ml water and dichloromethane (3×60 ml). The combined organic layer was dried over MgSO$_4$ followed by filtration, and evaporation to remove the solvent, and then submitted to high vacuum overnight to provide 1-benzyl-1H-indole-3-carbaldehyde 90 (0.583 g, 36%) as a green pure solid, m.p.: 115–118°C.

$\delta_H$ (300 MHz, CDCl$_3$) 5.23 (2H, s, 2 × H10), 7.05–7.09 (2H, m, H12 & H16) 7.20–7.25 (4H, m, H1, H3, 13H & H14), 7.59 (1H, s, H7), 9.88 (1H, s, H9) $\delta_C$ (75 MHz, CDCl$_3$):
50.9 (CH₂, 10), 110.5 (CH₃), 118.5 (CH₂), 122.2 (CH₁), 123.1 (CH₆), 124.2 (CH₂), 125.5 (CH₁), 127.3 (2 × CH₁ & CH₁₀), 128.4 (CH₁₄), 129.2 (2 × CH₁₃ & CH₁₅), 135.6 (CH₁₁), 137.5 (CH₇), 138.7 (CH₉), 184.7 (CH₉). MS (ESI) m/z [MH⁺] found 236.1070 calculated for C₁₆H₁₄NO:236.28. IR, \( \nu_{\text{max}} \) (neat), 3028w, 2921w, 1670s, 1611s, 1280m, 1166s, 741.7m, cm⁻¹.

(E)-Ethyl 3-(1-benzyl-1H-indol-3-yl) acrylate 91

NaH 60% in mineral oil (0.29 g, 7.3 mmol, 4.9 eq.) was stirred in 5 ml dry THF under a nitrogen atmosphere; triethylphosphonoacetate (2.5 g, 11.2 mmol, 7.5 eq.) in 12 ml THF was added dropwise over 5 minutes at room temperature. The reaction mixture was left to stir for 2 hours. Thereafter, 1-benzyl-1H-indole-3-carbaldehyde 90 (0.35 g, 1.5 mmol) in 12 ml THF was added dropwise during 5 minutes, after which the mixture was heated under reflux for 8 hours. 3 ml brine was added to the reaction mixture, followed by quenching with 10 ml water. It was then extracted using diethyl ether (3 × 10 ml). The crude was concentrated by rotary evaporation. It was then submitted to high vacuum overnight to give (E)-ethyl 3-(1-benzyl-1H-indol-3-yl) acrylate 91 with some impurities. In order to gain pure compound 91, the crude product was washed for several times with approximately 100 ml cold petroleum ether at 40-60 °C to give (E)-ethyl 3-(1-benzyl-1H-indol-3-yl) acrylate 91 (0.234 g, 51%) as pure brown solid, m.p.: 106–107°C.

δ_H (300 MHz, CDCl₃): 1.34 (3H, t, J = 7.1 Hz, 3 × H₁₃), 4.26 (2H, q, J = 7.1 Hz, 2 × H₁₂), 5.3 (2H, s, 2 × H₁₄), 6.43 (1H, d, J = 16 Hz, α-H₁₀), 7.13–7.16 (2H, m, 1 × H₁₆ & 1 × H₂₀) 7.24–7.36 (6H, m, Ph, H₁, H₂, H₃, H₂₀, H₁₈ & H₁₉), 7.40 (1H, s, H₇) 7.9 (1H, d, J = 16 Hz, β-H₉), 7.93–7.96 (1H, m, H₆). δ_C (75 MHz, CDCl₃): 14.5 (CH₃, C₁₃), 50.4 (CH₂, C₁₄), 60.1 (CH₂, C₁₂), 110.5 (CH₃), 112.7 (C₮), 113.1 (CH₆), 120.7 (CH₁), 121.5 (CH₂), 123.15 (CH₁₈), 126.34 (2 × CH₁₆ & CH₂₀), 127.01 (C₅), 128.09 (2 × CH₁₇ & CH₁₉), 129.0 (CH₇), 132.4 (C₄), 136.2 (C₁₅), 137.9 (CH₉), 168.3 (C₁₁). MS (ASAP) m/z found 306.1480 (MH⁺), calculated for C₂₀H₂₀NO₂: 306.37. IR, \( \nu_{\text{max}} \) (neat), 3103w, 2977w, 2977w, 1702s, 1627s, 1249m, 1167s, 745.6m, cm⁻¹. Elemental analysis: found: C: 78.73, H: 6.25, N: 4.70. Calculated for C₂₀H₁₉NO₂: C: 78.66, H: 6.27, N: 4.59.
Methyl (E)-3-(1-benzyl-1H-indol-3-yl) acrylate 92

(E)-ethyl-3-(1-benzyl-1H-indol-3-yl) acrylate 91 (0.05 g, 0.16 mmol) was dissolved in 3 ml methanol; subsequently potassium carbonate (0.115 g, 0.84 mmol, 5.3 eq.) was added. The reaction mixture was left to stir for 7 hours at 50°C. Thereafter, the crude product was filtered and the filtrate was evaporated by rotary evaporation followed by submission to high vacuum. The $^1$HNMR spectrum obtained for the crude shows that the ethyl group had been removed, so the crude was extracted by adding 30 ml water followed by adding ethyl acetate (3×15 ml) in portions. Thereafter, 5 ml brine was added to the combined organic layer, followed by drying over MgSO$_4$, filtration, evaporation and, lastly submission to high vacuum overnight to give methyl (E)-3-(1-benzyl-1H-indol-3-yl) acrylate 92 (0.041 g, 85%) as pure brown solid, m.p.: 100–102°C.

δ$_H$ (400 MHz, CDCl$_3$): 3.72 (3H, s, 3 × H12), 5.22 (2H, s, 2 × H13), 6.35 (1H, d, J = 16 Hz, α-H10), 7.05–7.07 (1H, m, H2), 7.15–7.26 (5H, m, Ph), 7.31 (1H, s, H7), 7.57 (1H, d, J = 16 Hz, β-H9), 7.83–7.87 (1H, m, H6). δ$_C$ (75 MHz, CDCl$_3$): 49.4 (CH$_3$, C12), 50.4 (CH$_2$, C13), 119.5 (CH3), 111.6 (C8), 119.6 (CH10), 120.5 (CH6), 122.1 (CH1), 125.3 (CH2), 125.9 (CH17), 127.04 (C5, 2 × CH15 & CH19), 127.9 (2 × CH16 & CH18), 131.4 (CH7), 135.1 (CH9), 136.6 (C4), 137.11 (C14), 167.6 (C11). MS (ESI) m/z found 292.1337 (MH$^+$), calculated for C$_{19}$H$_{18}$NO$_2$: 292.1338. IR, $\nu_{max}$ (neat), 3107w, 2949w, 2917w, 2854w, 1702s, 1623s, 1249m, 1163s, 741.7m, cm$^{-1}$.

(E)-3-(1-benzyl-1H-indol-3-yl) acrylic acid 93

A mixture of (E)-ethyl 3-(1-benzyl-1H-indol-3-yl) acrylate 91 (0.05 g, 0.16 mmol) and KOH (0.014 g, 0.25 mmol, 1.56 eq.) were dissolved in 2.2 ml ethanol and 0.22 ml water. The whole reaction mixture was left to stir for 24 hours at 50°C and then 10 ml H$_2$O was added, followed by extraction with ethyl acetate. (3×10 ml) The aqueous layer was acidified with 1N HCl until pH= 7 was performed. As a result, a white solid compound was precipitated. The aqueous layer was filtered, and the filter paper with the product was then left in the oven for 30 mins at 60°C; thereafter, the solid product was transferred into round-bottomed
flask, which in turn was kept in the oven overnight. A white solid of pure acid 93 (0.038 g, 84%) was obtained from the aqueous layer. m.p.: 107–110°C.

δ\( _H \) (400 MHz, CDCl\( _3 \)): 5.36 (2H, s, 2 × H13), 6.46 (1H, d, \( J = 15.9 \) Hz, α-H10), 7.17–7.37 (8H, m, Ph), 7.47 (1H, s, H7), 7.97 (1H, dd, \( J = 1.8, 7.2 \) Hz, H6), 8.01 (1H, d, \( J = 15.9 \) Hz, β-H9), 11.88 (1H, s, H12). δ\( _C \) (100 MHz, CDCl\( _3 \)): 50.5 (CH\( _2 \), C13), 110.5 (CH\( _3 \)), 111.8 (C8), 112.6 (CH6), 120.7 (CH10), 121.7 (CH1), 123.3 (CH2), 126.3 (CH17), 127.0 (3 × C5, CH15 & CH19), 128.1 (C14), 129.01 (2 × CH16 & CH18), 132.9 (CH7), 137.7 (C4), 140.2 (CH9), 172.4 (CH11). MS (ASAP) \( m/z \) found 278.1167 (MH\(^+\)), calculated for C\( _{18} \)H\( _{16} \)NO\(_2\): 278.1181. IR, \( \nu_{\text{max}} \) (neat), 1689s, 1573s, 1454m, 1179m, 1107m, 1079m, 742.2m, cm\(^{-1}\).

Ethyl 2-(1-benzyl-1H-indol-3-yl) cyclopropanecarboxylate 94

(E)-ethyl 3-(1-benzyl-1H-indol-3-yl) acrylate 91 (0.21 g, 0.69 mmol) was dissolved in 3 ml DMSO. Trimethylsulfoxonium iodide (0.16 g, 0.73 mmol, 1.1 eq.) was added, followed by potassium hydroxide (0.03 g, 0.54 mmol, 0.78 eq.). The whole reaction mixture was left to stir for 48 hours at 80°C and then quenched in 30ml water followed by extraction with ethyl acetate (3×20 ml). This gave oily cyclopropane 94 with some starting material, as shown by NMR data. The flash column was used to purify the crude product with ethyl acetate: hexane chosen as the best eluting solvent through the column, used in ratios of 97:3 to 95:5. Thereafter, pure ethyl acetate was used to remove any polar material that might have accumulated in the column. The cyclopropane 94 was obtained (0.09 g, 40%) as a pure sticky oily product.

δ\( _H \) (300 MHz, CDCl\( _3 \)): 1.18–1.26 (1H, m, 1 × H21), 1.22 (3H, t, \( J = 7.1 \) Hz, 3× H13), 1.47–1.53 (1H, m, 1 × H21), 1.78–1.84 (1H, m, α-H10), 2.51–2.58 (1H, m, β-H9), 4.12 (2H, q, \( J = 7.1 \)Hz, 2 × H12), 5.17 (2H, s, 2 × H14), 6.79 (1H, s, H7), 7.0–7.22 (5H, m, Ph), 7.56–7.62 (4H, m, Ph). δ\( _C \) (100 MHz, CDCl\( _3 \)): 14.4 (CH3, C13), 15.7 (CH2, C21), 18.2 (CH10), 22.3 (CH11), 49.9 (CH2, C14), 60.6 (CH2, 12), 109.9 (CH3), 115.1 (2 × C5 & C8), 119.2 (CH6), 119.5 (CH1), 122.2 (CH2), 125.1 (CH18), 126.9 (CH7), 127.7 (CH16), 127.9 (CH20), 128.3 (CH17), 128.8 (CH19), 136.8 (C4), 137.5 (CH15), 174.1
Chapter 5: Experimental

(C11). MS (ASAP) m/z C_{21}H_{21}NO_2 [MH^+] requires 320.40; observed 320.1642. IR, \( \nu_{\text{max}} \) (neat), 2980w, 2925w, 1720s, 1467m, 1178s, 738.5s, cm\(^{-1} \).

(2S)-2-(1-benzyl-1H-indol-3-yl)-cyclopropanecarboxylic acid 98

Ethyl-2-(1-benzyl-1H-indol-3-yl)-cyclopropanecarboxylic acid 94 (0.05 g, 0.16 mmol) was dissolved in 2.17 ml ethanol and KOH (0.014 g, 0.25 mmol, 1.6 eq.) in 0.22 ml H_2O. The reaction mixture was left to stir for 24 hours at 50°C, and the ethanol was then evaporated off by rotary evaporation; thereafter, 10 ml water was added, followed by extraction with ethyl acetate (3×10 ml). 20 ml water was added to the organic layer for the same purpose. The combined aqueous layer was acidified by 1N HCl in order to obtain pH=5, afterwards, the reaction mixture was left in the fridge for 1 an hour in order to give the compound more time to precipitate. The mix was filtered; and the pallid white compound in the filter paper was left in the oven for 30 mins at 60°C in order to remove the residual water. This product was transferred to the round-bottomed flask in whose weight was known in advance; once again, this was left overnight in the oven at 60°C. Compound 98 was obtained; however, the amount was small, so the organic layer was evaporated; as most of the compound 98 was actually contained in this layer. The total amount of cyclopropane carboxylic acid 98 obtained from both layers was (0.018 g, 39%). m.p: -140–145°C.

\[ \delta_H (300 \text{ MHz, CDCl}_3): 1.31–1.37 (1H, m, 1 × H21), 1.55–1.61 (1H, m, 1 × H21), 1.79–1.85 (1H, m, \alpha-H10), 2.61–2.68 (1H, m, \beta-H9), 5.18 (2H, s, 2 × H13), 6.81 (1H, s, H7), 7.02–7.28 (8H, m, Ph), 7.6 (1H, d, \( J = 7.4 \text{ Hz, H6} \)), 11.45 (1H, s, H12). \delta_C (75 \text{ MHz, CDCl}_3): 16.2 (CH\text{2}, C21), 19.2 (CH9), 22.1 (CH10), 49.9 (CH\text{2}, C13), 109.8 (CH3), 114.5 (2 × C8 & C5), 119.2 (CH6), 119.5 (CH1), 122.3 (CH2), 125.2 (CH17), 126.9 (2 × CH15 & CH19), 127.7 (CH7), 128.8 (2 × CH16 & CH18), 136.7 (C4), 137.4 (C14), 180.1 (C11). \] MS(ASAP) m/z found 292.1324 (MH^+), calculated for C_{19}H_{18}NO_2: 292.1338. IR, \( \nu_{\text{max}} \) (neat), 3028w, 2926w, 1690s, 1470m, 1230m, 694.4m, cm\(^{-1} \).
Potassium carbonate (1.7 g, 12 mmol, 5.2 eq.) was added to ethyl (E)-3-(1H-indol-3-yl) acrylate (0.5 g, 2.32 mmol); the mixture was stirred for 1 hour in 10.7 ml dry acetone at room temperature, after which 4-methoxybenzyl bromide (0.421 ml, 4.1 mmol, 1.8 eq.) was added. The reaction was stirred overnight under reflux and a nitrogen atmosphere. The reaction mix was then filtered in order to remove the potassium carbonate, followed by evaporation to remove the acetone residue. The extraction process was performed using 50 ml DCM and 25 ml H2O; the organic layer was washed with 10 ml of brine followed by addition of MgSO4 drying agent in order to remove the remaining water and then evaporated. The resultant crude product was submitted to high vacuum to give the above compound with some impurities. The flash column was used to purify the compound, using hexane: acetate ethyl as eluting solvent in the ratio 8:2 respectively, (found as the best ratio for these solvents for the separation) to give (E)-3-(1-(4-methoxybenzyl)-1H-indol-3-yl) acrylate 99 (0.331 g, 42%) pure yellow solid, m.p.: 110–112°C.

δH (300 MHz, CDCl3): 1.36 (3H, t, J = 7.1 Hz, 3 × H 13), 3.81 (3H, s, 3 × H21), 4.29 (2H, q, J = 7.1 Hz, 2 × H 12), 5.27 (2H, s, 2 × H 14), 6.44 (1H, d, J = 16 Hz, α-H10), 6.9 (2H, d, J = 8.7 Hz, H 17 & H19), 7.14 (2H, d, J = 8.7 Hz, H16 & H20), 7.24–7.32 (1H, m, H2), 7.33–7.39 (1 H, m, H1), 7.91 (1H, d, J = 16 Hz, β-H9), 7.95–7.99 (1H, m, H6). δC (75 MHz, CDCl3): 14.5 (CH3, C13), 49.9 (CH2, C14), 55.3 (CH3, C21), 60.1 (CH2, C12), 110.5 (CH3), 112.9 (C8), 114.4 (2 × CH17 & CH19), 120.7 (CH6), 121.4 (CH1), 123.1 (2 × CH2 & C11), 126.4 (C5), 128.1 (C18), 128.57 (2 × CH16 & CH20), 132.31 (CH7), 137.63 (C4), 137.98 (CH9), 159.45 (C18), 168.26 (C11). MS (ESI) m/z found 336.1607 (MH+), calculated for C21H22NO3: 336.1600. IR, νmax (neat), 2840w, 2841w, 1696s, 1619s, 975.1s, 823.9s, 797.7s, 742.2s, cm−1. Elemental analysis: found: C: 75.34, H: 6.46, N: 4.24. Calculated for C21H21NO3; C: 75.20, H: 6.31, N: 4.18.
Ethyl 2-(1-(4-methoxybenzyl)-1H-indol-3-yl) cyclopropane-1-carboxylate 100

A mixture of ethyl (E)-3-(1-(4-methoxybenzyl)-1H-indol-3-yl) acrylate 99 (0.254 g, 0.76 mmol), potassium hydroxide (0.052 g, 0.93 mmol, 1.22 eq.) and trimethylsulfoxonium iodide (0.274 g, 1.25 mmol, 1.6 eq.) in 5.3 ml dry DMSO was stirred under a nitrogen atmosphere for 72 hours at 80°C. Afterwards the extraction procedure for this mixture was performed by adding ethyl acetate (3×30 ml) and water (3×15 ml); thereafter, the organic layers were combined and 5 ml brine was added. This layer was dried using MgSO₄, followed by evaporation and submission to high vacuum overnight. The NMR data showed the required product with some starting material and some other impurities; as a consequence, the flash column was used in order to obtain the required product in its pure form. Ethyl acetate and hexane, in a 2:8 ml ratio, respectively, were used to give ethyl-2-(1-(4-methoxybenzyl)-1H-indol-3-yl) cyclopropane-1-carboxylate 100 (0.075 g, 29%) as a pure oily product.

δ_H (300 MHz, CDCl₃): 1.14–1.22 (1H, m, 1 × H14), 1.21 (3H, t, J = 7.2 Hz, 3 × H13), 1.44–1.50 (1H, m, 1 × H14), 1.76–1.8 (1H, m, H10), 2.49–2.55 (1H, m, H9), 3.66 (3H, s, 3 × H22), 4.10 (2H, q, J = 7.2 Hz, 2 × H12), 5.05 (2H, s, 2 × H15), 6.71–6.74 (2H, m, H18 & H20), 6.93–6.96 (1H, m, H1), 6.97–7.0 (2H, m, H17 & H21), 7.04–7.07 (1H, m, H2), 7.16–7.18 (1H, m, H6). δ_C (75 MHz, CDCl₃): 14.4 (CH₃, C13), 15.7 (CH₂, C14), 18.2 (CH9), 22.3 (CH10), 49.5 (CH₂, C15), 55.3 (CH₃, C22), 60.6 (CH₂, C12), 109.8 (CH3), 114.2 (2 × CH18 & CH20), 114.9 (2 × C5 & C8), 119.2 (CH₆), 119.4 (CH1), 122.1 (CH2), 124.9 (CH7), 128.2 (C16), 129.4 (2 × CH17 & CH21), 136.7 (C4), 159.2 (C19), 174.1 (C11). MS (ASAP) m/z found 350.1754 (MH⁺), calculated for C₂₂H₂₄NO₃: 350.1756. IR, ν_{max} (neat), 2982w, 2840w, 1695 s, 1619 s, 1378m, 1302m, cm⁻¹.

Tert-butyl 3-formyl-1H-indole-1-carboxylate 103

4-Dimethylaminopyridine DMAP (2.5 mg, 0.18 mmol, cat.), di-tert-butyl dicarbonate (BOC₂O) (0.5 g, 2.3 mmol, 1.28 eq.), Et₃N (0.32 ml, 2.3 mmol, 1.28 eq.) were added to a stirred solution of 1H-indole-3-carbaldehyde (0.225 g, 1.8 mmol). The resultant mixture was stirred overnight at room temperature followed by addition of 10 ml of brine. Thereafter,
40 ml CH₂Cl₂ was added for extraction purposes, after which MgSO₄ was added to dry the solution, followed by filtration, evaporation and submission to high vacuum overnight to give tert-butyl 3-formyl-1H-indole-1-carboxylate 103 (0.382 g, 87%), m.p.: 120–124°C.

δ_H (400 MHz, CDCl₃): 1.74 (9H, s, H₁₂, H₁₃ & H₁₄), 7.37–7.46 (2H, m, H₁ & H₂), 8.17 (1H, d, J = 7.8 Hz, H₃), 8.25 (1H, s, H₇), 8.30–8.33 (1H, m, H₆), 10.13 (1H, s, H₉). δ_C (100 MHz, CDCl₃): 28.0 (3 × CH₃, C₁₂, C₁₃ & C₁₄), 85.6 (C₁₀), 115.2 (CH₃), 121.5 (C₈), 122.1 (CH₁), 124.5 (CH₆) 125.8 (CH₂), 126.0 (CH₇), 135.9 (C₅), 136.8 (C₄), 148.7 (C₁₁), 185.7 (CH₉). MS (ESI) m/z found 246.1101(MH⁺), calculated for C₁₄H₁₆NO₃: 246.1130. IR, ν_max (neat): 2981w, 2816w, 1741s, 1678s, 1241m, 761.3s, cm⁻¹.

(E)-tert-butyl 3-(3-ethoxy-3-oxoprop-1-en-1-yl)-1H-indole-1-carboxylate compound 104

Camp et al.¹²³ reported that the BOC protection group on a pyrrole ring can be achieved as follows: (E)-ethyl 3-(1H-indol-3-yl) acrylate 81 (0.244 g, 1.1 mmol) was stirred in 17 ml dry (DCM) at room temperature dimethylamineopyridine (DMAP) (0.023 g, 0.19 mmol, 0.17 eq.) was added as catalyst followed by an equivalent amount of both di-tert-butyldicarbonate (BOC₂O) and triethylamine (Et₃N) (0.25 g, 1.1 mmol; 0.115 g, 1.1 mmol, respectively). The reaction mixture was left to stir overnight at room temperature. Thereafter, 10 ml brine and 17 ml of DCM were added, followed by 10 ml of water to achieve this extraction. Afterwards, the organic layer was dried over MgSO₄ followed by filtration via filter paper; subsequently the solvent was removed by rotary evaporation submission to high vacuum to afford (E)-tert-butyl 3-(3-ethoxy-3-oxoprop-1-en-1-yl)-1H-indole-1-carboxylate compound 104 (0.349 g, 100%), m.p.: 140–143°C.

δ_H (400 MHz,CDCl₃): 1.28 (3H, t, J = 7.1 Hz, 3× H₁₃), 1.60 (9H, s, H₁₆, H₁₇ & H₁₈), 4.20 (2H, q, J = 7.2 Hz, 2 × H₁₂), 6.46 (1H, d, J = 16 Hz, α-H₁₀), 7.23–7.33 (2H, m, H₁ & H₂), 7.66–7.67 (1H, m, H₃), 7.6 (1H, s, H₇), 7.75 (1H, d, J = 16 Hz, β-H₉), 7.76–7.78 (2H, m, H₇ & H₆) 8.12 (1H, d, J = 7.7 Hz, H₃). δ_C (100 MHz,CDCl₃): 14.4 (CH₃, C₁₃), 28.2 (3 × CH₃, C₁₆, C₁₇ & C₁₈), 60.4 (CH₂, C₁₂), 84.7 (C₁₅), 115.6 (CH₁₀),
116.8 (C8), 117.5 (CH3), 120.3 (CH6), 123.6 (CH1), 125.3 (CH2), 127.9 (CH7), 128.6 (C5), 136.2 (CH9) 136.4 (C4) 167.4 (C11 & C14) MS (ASAP) m/z found 316.1550 (MH⁺), calculated for C18H22NO4 316.1549.

(E)-3-(2-(pyridin-3-yl)-vinyl)-1H-indole 107

Pyrid-3-ylacetic hydrochloride (1.302 g, 7.5 mmol, 1.5 eq.) was added to Et₃N (2.68 ml) and the reaction mixture stirred at room temperature for 20 min. Afterwards 1H-indole-3-carbaldehyde (0.726 g, 5 mmol) and piperidine (1.1 ml, 11 mmol, 2.2 eq.) were added. This mixture was stirred under reflux overnight. Thereafter, the reaction mixture was evaporated in order to remove the trimethylamine solvent. NMR data showed a mix of product and starting material so ethyl acetate was added and evaporated on silica. The product was submitted to the flash column for purification in a hexane: ethyl acetate 3:6 mix, respectively, were used as eluting solvent to give (E)-3-(2-(pyridin-3-yl)-vinyl)-1H-indole product 107 (0.678 g, 62%) as a pure yellow solid, m.p.: 192–193 °C.

δ_H (400 MHz, DMSO-d6): 7.11 (1H, d, J = 17 Hz, H11), 7.11–7.20 (3H, m, H1, H2 &H3), 7.36 ( 1H, dd, J = 7.9, 4.7 Hz, H6), 7.44 (1H, d, J = 7.5 Hz, Ph), 7.56 (1H, d, J = 17 Hz, 1 × H10), 7.68 (1H, s, H8), 8.01 (1H, d, J = 6.2 Hz, H17), 8.04 (1H, d, J = 7.4 Hz, H15), 8.37 (1H, dd, J = 4.7, 1.5 Hz, H13), 8.76 (1H, d, J = 2.1 Hz, Ph), 11.38 (1H, s, H7). δ_C (100 MHz, DMSO-d6) 111.9 (CH3), 113.5 (C9), 119.4 (CH6), 119.8 (CH1), 119.9 (CH2) 121.9 (CH16), 123.6 (C5), 124.7 (CH8), 125.1 (CH11), 126.7 (C12), 131.4 (CH17), 134.4 (CH10), 137.1 (C4), 146.9 (CH13), 147.5 (CH15). MS (ESI) m/z found 221.1070 (MH⁺), calculated for C15H12N2: 221.1079. IR, ν_max (neat), 1630m, 1459m, 951.8s, 737.4s, 697.4s, cm⁻¹. Elemental analysis: found: C: 81.66, H: 5.57, N: 12.65. Calculated for C15H12N2; C: 81.79, H: 5.49, N: 12.72.
(E)-1-methyl-3-(2-(1-methyl-1H-indol-3-yl)-vinyl) pyridin-1-ium 109

(E)-3-(2-(pyridin-3-yl) vinyl)-1H-indole (0.202 g, 0.92 mmol) 107 was added to potassium carbonate (0.64 g, 4.6 mmol, 5 eq.). The resulting mixture was left to stir for 1 an hour at room temperature in 4.1 ml dry acetone. Methyl iodide (0.14 ml, 2.3 mmol, 2.5 eq.) was then added. The reaction was stirred overnight under a nitrogen atmosphere at 30°C. The reaction was worked up by filtering through filter paper to remove the potassium carbonate than evaporated to eliminate residues of methyl iodide and acetone. Thereafter, the crude product was dissolved in DCM (3×20 ml) and H₂O (3×20 ml) for extraction, with the combined organic layer dried over MgSO₄ followed by evaporation and then dried under high vacuum to give (E)-1-methyl-3-(2-(1-methyl-1H-indol-3-yl) vinyl) pyridin-1-ium 109 (0.12g, 52%) as a pure yellow solid, m.p.: 197–200°C.

δ_H (400 MHz, DMSO-d6): 3.86 (3H, s, 3 × H18), 4.34 (3H, s, 3 × H19), 7.19 (1H, d, J = 17Hz,α-H11), 7.24 (1H, t, J = 7.9 Hz, H2), 7.30 (1H, t, J = 7.6 Hz, H1), 7.5 (1H, d, J = 8.1 Hz, H3), 7.78 (1H, s, H8), 7.84 (1H, d, J = 17Hz, β-H10), 8.03 (1H, t, J = 3.2 Hz , H16), 8.09 (1H, d, J = 7.8Hz, H6), 8.59–8.72 (1H, m, H17), 9.21 (1 H, s, H13). δ_c (100 MHz, DMSO-d6): 32.8 (CH₃, C18), 47.9 (CH₃, C19), 110.6 (CH3), 111.9 (C9), 115.4 (CH6), 120.1 (CH1), 120.6 (CH2), 122.4 (C5), 125.2 (CH16), 127.3 (CH8), 129.3 (CH11), 132.6 (CH10), 137.7 (C4), 138.9 (C12), 139.1 (CH15), 141.3 (CH17), 142.1 (CH13). MS (ESI) m/z found 249.1395 (MH⁺), calculated for C₁₇H₁₇N: 249.1392. IR, ν_max (neat), 3450w, 3037w, 2924w, 2854w, 1637s, 1466m, 1376m, 748.2m, 666.3m, cm⁻¹.

Bis-[2-(1-methyl-1H-indol-3-yl)-cyclopropyl]-methanone 114a

1,5-Bis-(1-methyl-1H-indol-3-yl)-penta-1,4-dien-3-one (0.052 g, 0.15 mmol) 113a was dissolved in 1 ml DMSO, about 2 ml acetone and 1 ml of Et₃N were added, followed by adding resulting mixture of potassium hydroxide (0.0098 g, 0.18 mmol, 1.2eq) and trimethyl sulfoxonium iodide (0.052 g 0.24 mmol, 1.6eq). The reaction was stirred for 48 an hours at 80°C, followed by purification using ethyl acetate: hexane with ratio
3:7 respectively to give bis-[2-(1-methyl-1H-indol-3-yl)-cyclopropyl]-methanone 114a (0.035 g, 63%) as an oily isolated yield.

δ\(H\) (300 MHz, CDCl\(_3\)): 1.32–1.41 (1H, m, 1×H12), 1.65–1.73 (1H, m, 1×H12), 2.21–2.29 (1H, m, α–H10), 2.57–2.67 (1H, m, β–H9), 3.62 (3H, s, 3×H11), 6.72 (1H, s, H7), 6.93–6.99 (1H, m, H1), 7.01–7.07 (1H, m, H2), 7.11–7.20 (1H, m, H3), 7.56–7.62 (1H, m, H6). δ\(H\) (75 MHz, CDCl\(_3\)): 17.4, 17.6 (2×CH), 21.4, 21.5 (2×CH), 31.6 (CH), 32.7 (CH\(_3\)), 109.3, 109.4 (2×CH), 114.8 (C), 119.1 (CH), 119.2, 119.2 (2×CH), 122.0 (CH), 125.6 (C), 125.9 (CH), 127.9 (C), 128.0 (C), 137.1 (C), 208. (C). MS (ASAP) m/z found 369.1966 (MH\(^+\)), calculated for C\(_{25}\)H\(_{25}\)N\(_2\)O: 369.1967.

(E)-4-(1-methyl-1H-indol-3-yl) but-3-en-2-one 116

Acetone (7 ml, 95 mmol, 32 eq.) was added to 1-methyl-1H-indole-3-carbaldehyde (0.5 g, 3 mmol). The reaction was left to stir for two days heating under reflux. The crude product was mixed with silica in order to obtain it dry and then submitted to the flash column using an ethyl acetate: hexane as eluting solvent in a 6:3 ratio, respectively. This gave (E)-4-(1-methyl-1H-indol-3-yl) but-3-en-2-one 116 (0.206 g, 33%) as a pure yellow solid, m.p: 105–107 °C.

δ\(H\) (300 MHz, CDCl\(_3\)): 2.37 (3H, s, 3 × H14), 3.82 (3H, s, 3 × H12), 6.76 (1H, d, J = 16Hz, α-H11), 6.73–7.39 (4H, m, Ph), 7.76 ( 1H, d, J = 16Hz, β-H10), 7.91–7.94 ( 1H, m, H6). δ\(C\) (75 MHz, CDCl\(_3\)): 27.4 (CH\(_3\), C14), 33.3 (CH\(_3\), C12), 110.1 (CH3), 112.1 (C9), 120.6 (CH6), 121.5 (CH1), 122.5 (CH2), 123.1 (C5), 126.1 (CH8), 133.6 (C4), 137.0 (CH11), 138.2 (CH10), 198.5 (13). MS (ASAP) m/z found 200.1077(MH\(^+\)), calculated for C\(_{13}\)H\(_{14}\)NO: 200.1075. IR, \(\nu_{\text{max}}\) (neat), 1671m, 1589m, 1527m, 1277m, 1237m, 1157m, 1087s, 964.5m, 817.9m,733.0s, cm\(^{-1}\).

(E)-3-(1H-indol-3-yl)-1-(pyridin-3-yl)-prop-2-en-1-one 118

Piperidine (0.59 g, 6.9 mmol, 1 eq.) was added to the combination of 1H-indole-3-carbaldehyde (1 g, 6.9 mmol) and 1-(pyridin-3-yl)ethan-1-one (1.25 g, 10.4 mmol, 1.5 eq.) in 20 ml anhydrous methanol; the suspended mixture was stirred and refluxed overnight under a nitrogen atmosphere. The product obtained
by evaporation of the solvent was followed by washing the solid material with 300 ml cool methanol to give (E)-3-(1H-indol-3-yl)-1-(pyridin-3-yl)-prop-2-en-1-one 118 (1.123 g, 66%) as a pure yellow solid. m.p: -190–192°C.

δ_H (400 MHz, DMSO-d_6): 7.23–7.28 (2H, m, H1 & H2), 7.50–7.53 (1H, m, H3), 7.57–7.60 (1H, m, H16), 7.66 (1H, d, J = 15Hz, α-H11) 8.13 (1H, d, J = 15 β-H10) 8.13–8.17 (2H, m, H6 & H8), 8.5 (1H, d, J = 7.8 Hz, H15), 8.80 (1H, d, J = 3.7 Hz, H17), 9.30 (1H, s, H19), 11.99 (1H, s, H7). δ_c (100 MHz, DMSO-d_6): 112.5 (CH_3), 122.8 (C9), 115.1 (CH6), 120.5 (CH1), 121.3 (CH2), 122.8 (CH11), 123.8 (CH16), 125.1 (C5), 133.7 (C13), 133.8 (CH8), 135.6 (CH15), 137.6 (C4), 139.6 (CH10), 149.3 (CH19), 152.6 (CH17), 187.9 (C12). MS (ESI) m/z found 249. 1022 (MH^+) calculated for C_{16}H_{13}N_2O: 249.1028. IR, ν_{max} (neat), 2982w, 1652m, 1559m, 1370m, 9780m, 810.2 m, 740.7s cm^{-1}. Elemental analysis: found: C: 77.28, H: 4.62, N: 11.4. Calculated for C_{16}H_{12}N_2O; C: 77.40, H: 4.87, N: 11.28.

(E)-3-(1-methyl-1H-indol-3-yl)-1-(pyridin-3-yl) prop-2-en-1-one 119

![E-3-(1-methyl-1H-indol-3-yl)-1-(pyridin-3-yl) prop-2-en-1-one](image)

1-Methyl-1H-indole-3-carbaldehyde (0.99 g, 6.21 mmol) in a 50 ml round-bottomed flask was dissolved in 20 ml anhydrous methanol, followed by addition 1-(pyridin-3-yl)-ethan-1-one (1.0 ml, 9.27 mmol, 1.5 eq.) and piperidine (0.62 ml, 6.3 mmol, 1 eq). This reaction mixture was stirred under reflux and a nitrogen atmosphere for 24 hours, after which 10 ml cool methanol was added. The crude product of this reaction was left in the fridge for 20 minutes. The concentrated solid was filtered and washed with 130 ml cool methanol, and the very pure yellow solid on filter paper was left in a fume cupboard for 30 minutes in order to remove solvent residue.

This was followed by transferring the pure product to a round-bottomed flask, then submitting to high vacuum overnight which giving (E)-3-(1-methyl-1H-indol-3-yl)-1-(pyridin-3-yl)-prop-2-en-1-one 119 (1.144 g, 70%) as a pure yellow solid. m.p: 161–163°C.

δ_H (400 MHz, CDCl_3): 3.85 (3H, s, 3 × H20), 7.31–7.40 (3H, m, H1, H2 & H3), 7.42–7.46 (1H, m, H16), 7.49 (1H, d, J = 15Hz, α-H11), 7.49 (1H, s, H8), 7.99–8.01 (1H, m, H6), 8.11 (1H, d, J = 15Hz, β-H10), 8.31 (1H, dt, J = 7.92, 1.92Hz, H15), 8.78 (1H, dd, J = 4.8, 1.72Hz, H17), 9.27 (1H, m, H19). δ_c (100 MHz, CDCl_3): 33.4 (CH_3, C20),
Chapter 5: Experimental

110.3 (CH3), 112.9 (C9), 116.3 (CH2), 120.8 (CH6), 121.9 (CH1), 123.4 (CH11), 123.6 (CH16), 126.1 (C5), 134.4 (C13), 135.2 (CH8), 135.7 (CH15), 138.4 (C4), 139.8 (CH10), 149.6 (CH19), 152.6 (CH17), 189.1 (C12).

MS (ASAP) m/z found 263.1177 (MH+), calculated for C17H15N2O: 263.1184. IR, \( \nu_{\text{max}} \) (neat), 1651 m, 1583 s, 1371 s, 1279 s, 1007 s, 747.9 s, 695.9 s, cm\(^{-1}\). Elemental analysis: found: C: 77.62, H: 5.48, N: 10.52, calculated for C17H14N2O: C: 77.84, H: 5.38, N: 10.68.

(E)-3-(1H-indol-3-yl)-1-(pyridin-3-yl) prop-2-en-1-ol 120

(2E)-3-(1H-indol-3-yl)-1-(pyridin-3-yl)-prop-2-en-1-one (0.2 g, 0.81 mmol) was placed in 25 ml round-bottomed flask containing 10 ml methanol. This solution was treated with sodium borohydride (0.062 g, 1.64 mmol). The mixture left to stir overnight at room temperature, followed by evaporating the methanol and dissolving the solid material in ethyl acetate and hexane. Afterwards, the solid was removed by the filtration through filter paper and allowed to dry. The resultant solid yellow material was transferred to a round-bottomed flask and then submitted to high vacuum overnight to afford (2E)-3-(1H-indol-3-yl)-1-(pyridin-3-yl) prop-2-en-1-ol 120 (0.097 g, 46%) as a pure yellow solid.

\( \delta_H \) (400 MHz, CD3OD): 5.38 (1H, d, \( J = 7.2 \) Hz, H12), 6.31 (1H, dd, \( J = 7.9, 3.6 \) Hz, H20), 6.87 (1H, d, \( J = 16 \) Hz, H21), 7.05–7.16 (2H, m, H1 & H2), 7.31–7.39 (3H, m, H3, H8 & H16), 7.78–7.82 (1H, m, H15), 7.89 (1H, d, \( J = 5.9 \)Hz, H6), 8.39 (1H, d, \( J = 4.3 \) Hz, H17), 8.65 (1H, s, H19). \( \delta_C \) (100 MHz, CD3OD): 74.7 (CH12), 112.7 (CH3), 114.6 (C9), 120.6 (CH1), 120.9 (CH6), 123.0 (CH2), 125.2 (CH16), 126.3 (CH10), 126.5 (CH11), 126.9 (C5), 127.6 (CH8), 136.4 (CH15), 138.8 (C4), 142.1 (C13), 148.5 (CH17), 148.7 (CH19). IR, \( \nu_{\text{max}} \) (neat), 3214 s, 1696 w, 1574 s, 1454 w, 1340 m, 1101 m, 749 s, 629.2 w, cm\(^{-1}\).

(E)-3-(1-methyl-1H-indol-3-yl)-1-(pyridin-3-yl) prop-2-en-1-ol 120a.

(E)-3-(1-methyl-1H-indol-3-yl)-1-(pyridin-3-yl)prop-2-en-1-one 119 (0.100 g, 0.38 mmol) was dissolved in 10 ml methanol, then sodium borohydride (0.029 g, 0.77 mmol, 2 eq.) was added. The reaction was stirred for 3 hours; in the meantime, the reaction was monitored by TLC against the
starting material. Afterwards, the reaction was extracted by DCM: H2O (60:40 ml) respectively. The combined organic layer was dried over MgSO4 and then transferred to a dry round-bottomed flask in order to evaporate the solvent. The product was submitted to high vacuum to give (E)-3-(1-methyl-1H-indol-3-yl)-1-(pyridin-3-yl)-prop-2-en-1-ol 120a (0.101 g, 100%) almost pure orange solid, m.p.: 90–95°C.

δH (300 MHz, CDCl3): 3.63 (3H, s, 3 × H20), 4.44 (1H, br s, H14), 5.33 (1H, d, J = 7.1 Hz, H12), 6.25 (1H, dd, J = 16, 7.1Hz, α-H11), 6.75 (1H, d, J = 16 Hz, β-H10), 6.98 (1H, s, H19), 7.09–7.26 (3H, m, H1, H2 & H3), 7.74–7.7 9 (1H, m, H15), 8.39 (1H, d, J = 4.4Hz, H6), 8.61 (1H, d, J = 1.4 Hz, H17). δc (75 MHz, CDCl3): 32.8 (CH3, C20), 73.6 (CH12), 109.6 (CH3),112.6 (C9), 120.1 (CH6), 122.2 (CH1), 123.6 (CH2), 124.4 (CH16), 127.1 (C5), 128.9 (CH10), 134.4 (3 × CH8, CH11 & C13), 137.6 (CH15), 139.7 (C4), 147.9 (CH17), 148.2 (CH19). MS (ASAP) m/z found 265.1349 (MH+), calculated for C17H17N2O: 265.1341. IR, 𝒫max (neat), 2921w, 1580m, 1426m, 1245m, 737.7s, cm⁻¹.

2-(1-methyl-1H-indol-3-yl)-cyclopropyl-(pyridin-3-yl)-methanone 121

A mixture of trimethylsulfoxonium iodide (0.052 g, 0.24 mmol, 1.2 eq.) and (60%) sodium hydride (0.0096 g, 0.24 mmol, 1.2 eq.) were stirred for 25 minutes in a dry 2:2 ml THF:DMF solution under a nitrogen atmosphere, after which (E)-3-(1-methyl-1H-indol-3-yl)-1-(pyridin-3-yl)-prop-2-en-1-one 119 (0.052 g, 0.2 mmol) was added. The reaction solution was stirred at room temperature overnight; afterwards, 30 ml ethyl acetate and 60 ml water were added to the separation funnel, followed by 3 ml brine, after which the aqueous layer was removed. The organic layer was dried over MgSO4, thereafter, the solvent was removed by rotary evaporation and the product submitted to high vacuum to give 2-(1-methyl-1H-indol-3-yl)-cyclopropyl-(pyridin-3-yl)-meth-anone 121 (97%) as a pure sticky brown, which was assessed by NMR by measuring the ratio of one proton of the original product to the ratio of impurities or secondary products.

δH (400 MHz, CDCl3): 1.64–1.69 (1H, m, 1 × H18), 1.96–2.0 (1H, m, 1 × H18), 2.80–2.88 (2H, m, H9 & H10), 3.74 (3H, s, 3 × H17), 6.88 (1H, s, H7), 7.07–7.11 (1H, m, H1), 7.21–7.30 (1H, m, H3), 7.36 (2H, ddd, J = 7.2, 4.0, 0.8Hz, H6 & H14), 7.58 (1H,
dt, J = 7.1, 0.8 Hz, H13), 8.25 (1H, dt, J = 8, 2 Hz, H2), 8.76 (1H, dd, J = 4.8, 1.7 Hz, H15), 9.24 (1H, d, J = 3 Hz, H16). δc (100 MHz, CDCl3) 18.3 (CH2, C18), 23.4 (CH10), 28.4 (CH9), 32.7 (CH16), 109.5 (CH3), 114.3 (C8), 118.8 (CH6), 119.4 (CH1), 122.1 (CH2), 123.5 (C14), 125.9 (CH7), 127.8 (C5), 133.1 (C12), 135.3 (CH13), 137.1 (C4), 149.5 (CH3, C17), 153.2 (C15), 197.9 (C11). MS (ASAP) m/z found 277.1350 (MH+), calculated for C18H17N2O: 277.1341. IR, νmax (neat), 3051 w, 2926 w, 1665 s, 1585 s, 1417 s, 1240 s, 741.8 s, cm⁻¹.

(E)-3-(1H-indol-3-yl)-1-phenylprop-2-en-1-one 124

Acetophenone (480 µl, 4.14 mmol, 1 eq.) and piperidine (200 µl, 2 mmol, 0.48 eq.) were added to 1H-indole-3-carbaldehyde (0.6 g, 4.14 mmol, 1 eq.) in 25 ml anhydrous methanol in a 100 ml dry round-bottomed flask under a nitrogen atmosphere. The reaction mixture was stirred overnight under reflux, followed by evaporation of the solvent. Subsequently, dichloromethane and petroleum ether at 40–60°C were added to the crude product in order to crystallize; as a result, some of the brown impurities were kept in the round-bottomed flask and the yellow product was dissolved in the solvents, which were then filtered. The product was left in a fume cupboard for 30 minutes at room temperature to give the pure, solid product; this was followed by a second filtration. The yellow solution was evaporated to remove the remaining solvent and then submitted to high vacuum to give (E)-3-(1H-indol-3-yl)-1-phenylprop-2-en-1-one 124 (0.366 g, 36%) as a pure yellow solid, m.p: 185–190 °C compared with melting point for the same product (167–170 °C) that was reported by literature.

δH (400 MHz, DMSO-d6): 7.21–7.29 (4H, m, H 11, H16, H17 & H18), 7.53 (2H, br d, J = 8.0 Hz, H3 & H6), 8.13 (2H, br d, J = 8.0 Hz, H15 & H19), 8.28 (2 H, s, H2 & H17), 9.96 (2H, s, H), 12.12 (1H, br s, H7). δc (100 MHz, DMSO-d6): 112.36 (CH3 & C9), 115.5 (CH6), 118.2 (CH1), 120.8 (CH2), 122.1 (CH11), 122.7 (C5), 123.4 (2 × CH15 & CH19), 124.1 (2 × CH16 & CH18), 128.0 (CH8), 128.1 (CH17), 132.3 (C4), 133.1 (C13), 136.9 (C5), 138.3 (CH10), 184.9 (C 12). MS (ESI) m/z found 248.1066 (MH+), calculated for C17H14NO: 248.1075. IR, νmax (neat), 2929 w, 2819 w, 1631 s, 1446 s, 1241 m, 788.9 m, cm⁻¹.
1-methyl-1H-indole-3-carbaldehyde (0.33 g, 2.07 mmol, 1 eq.) was dissolved in 8 ml anhydrous methanol and kept in 25 ml round-bottomed flask under a nitrogen atmosphere. Then piperidine (100 µl, 1.00 mmol, 0.48 eq.) and acetophenone (204 µl, 2.07 mmol, 1 eq.) were added. The whole mixture was stirred and heated under reflux overnight; thereafter, 20 ml cool methanol was added in order to recrystallize the product. The solution was filtered through filter paper to separate the pure yellow solid compound which gave (2E)-3-(1-methyl-1H-indol-3-yl)-1-phenylprop-2-en-1-one 124a (0.096 g, 18%) as a pure yellow solid, m p: -145–147 °C.

\[ \delta \text{H} (400 \text{ MHz, CDCl}_3): 3.74 (3\text{H}, \text{s}, 3\times \text{H}10), 7.27–7.31 (3\text{H}, \text{m}, \text{H}1, \text{H}2 \& \text{H}3), 7.38 (1\text{H}, \text{s}, \text{H}8), 7.46–7.54 (3\text{H}, \text{m}, \text{H}12, \text{H}16, \text{H}18), 7.97–8.07 (4\text{H}, \text{m}, \text{H}17, \text{H}11, \text{H}6 \& \text{H}8). \delta \text{C} (100 \text{ MHz, CDCl}_3): 30.9 (\text{CH}_3, \text{C}10), 107.9 (\text{CH}3, \text{C}10), 110.7 (\text{C}9), 114.7 (\text{CH}6), 118.5 (\text{CH}1), 119.3 (\text{CH}2), 120.9 (\text{CH}12), 123.9 (\text{C}5), 126.0 (2 \times \text{CH}16 \& \text{CH}20), 126.2 (2 \times \text{CH}17 \& \text{CH}19), 129.9 (\text{CH}8), 132.4 (\text{CH}18), 136.0 (\text{C}4), 136.4 (\text{C}14), 136.9 (\text{CH}11), 188.4 (\text{C}13). \]  
MS (ASAP) m/z found 262.1245 (MH^+), calculated for C_{18}H_{16}NO: 262.1232. IR, v_{max} (neat), 2906w, 1646s, 1564s, 1217m, 690.5m, 729.9m, cm^{-1}. Elemental Analysis: found: C: 82.62, H: 5.68, N: 5.45. Calculated for C_{18}H_{16}NO; C: 82.73, H: 5.79, N: 5.36.

(E)-3-(1-methyl-1H-indol-3-yl)-1-phenylprop-2-en-1-one (0.052 g, 0.2 mmol), sodium hydride (60%) (9.6 mg, 0.24 mmole, 1.2 eq.) and trimethylsulfoxonium iodide (0.052 g, 0.24 mmol, 1.2 eq.) were dissolved in 5 ml 3:2 DMF:THF, respectively. The reaction was stirred overnight at room temperature under a nitrogen atmosphere, followed by extraction using ethyl acetate and water, evaporation and submission to high vacuum to give (2-(1-methyl-1H-indol-3-yl) cyclopropyl)-(phenyl)methanone 125 (0.055 g, 100%) almost pure sticky brown.

\[ \delta \text{H} (400 \text{ MHz, CDCl}_3): 1.56–1.60 (1\text{H}, \text{m}, 1 \times \text{H}12), 1.91–1.95 (1\text{H}, \text{m}, 1 \times \text{H}12), 2.76–2.82 (1\text{H}, \text{m}, \text{H}9), 2.83–2.89 (1\text{H}, \text{m}, \text{H}10), 3.71 (3\text{H}, \text{s}, 3 \times \text{H}20), 6.84 (1\text{H}, \text{s}, \text{H}8), \]
7.06–7.10 (1H, m, H1), 7.20–7.29 (1H, m, H2), 7.40–7.44 (1H, m, H3), 7.52 (2H, tt, J = 7.4, 1.28 Hz, H16 & H18), 7.59 ((1H, dt, J = 7.9, 1 Hz, H17), 7.99–8.03 (2H, m, H15 & H19). δc (100 MHz, CDCl3): 14.2 (CH2, C12), 17.8 (CH10), 22.7 (CH9), 32.7 (CH3, C21), 109.5 (C3), 114.8 (C7), 119.1 (C6), 119.3 (C1), 122.1 (C2), 125.9 (C8), 127.9 (C5), 128.1 (2 × CH16 & CH18), 128.3 (2 × CH15 & CH19), 132.8 (CH17), 137.4 (C14), 138.0 (C4), 199.3 (C13). MS (ESI) m/z found 276.1401 (MH+), calculated for C19H18N: 276.1388.

IR, νmax (neat), 3053 w, 2925 w, 1661 s, 1371 s, 1222 s, 741 s, 705.1 m, cm−1.

(E)-3-(1-methyl-1H-indol-3-yl)-1-phenylprop-2-en-1-ol 126

(E)-3-(1-methyl-1H-indol-3-yl)-1-phenylprop-2-en-1-one 124a (0.088 g, 0.34 mmol) was transferred to a 25 ml round-bottomed flask and sodium borohydride (0.025 g, 0.66 mmol, 1.9 eq.) was added. The reaction mixture was stirred overnight under reflux in 9 ml MeOH; thereafter, the solvent was evaporated, followed by extraction using 50 ml DCM with 50 ml H2O. Afterwards, the organic layer was dried over MgSO4 and then submitted to high vacuum to give (E)-3-(1-methyl-1H-indol-3-yl)-1-phenylprop-2-en-1-one 126 (0.053 g, 59%) almost pure solid.

δH (400 MHz, CDCl3): 2.11 (1H, s, H15), 3.76 (3H, s, 3 × H10), 5.26 (1H, d, J = 7 Hz, H13), 6.23 (1H, dd, J = 16, 7 Hz, H12), 6.71 (1H, d, J = 16 Hz, H11), 7.03–7.07 (1H, m, H2), 7.11–7.19 (1H, m, H1), 7.23–7.28 (1H, m, H3), 7.25–7.38 (4H, m, H17, H19, H16 & H20), 7.71–7.73 (1H, m, H6). δc (100 MHz, CDCl3): 31.7 (CH3, C10), 74.9 (C13), 108.4 (CH3), 111.8 (9), 118.9 (CH6), 119.1 (CH2), 121.1 (CH1), 122.8 (2 × CH16 & CH20), 124.9 (CH18), 125.1 (C5) 125.3 (2 × CH17 & CH19), 126.4 (CH8), 126.6 (CH11), 127.5 (CH12), 136.5 (C4), 142.5 (C14). MS (ESI) m/z found: 246.1273 [M–OH], calculated for C19H17NO: 246.1283.

5.3. Prepared Solution for Kinetic Assay of Human IDO with L-tryptophan and some of the above compounds purposed as potential inhibitors.

5.3.1 Buffer solution
Tris(hydroxymethyl)aminomethane (Tris) (3.029 g, 50 Mm) was dissolved in 495 ml double deionized water, a few drops of conc. HCl added until pH = 8 was achieved, and the volume made up to 500 ml. This buffer solution was used to prepare the following solutions that have each been used for hIDO kinetic assay.

5.3.2 Ascorbic Acid

1 ml buffer solution was added to (0.72 g, 1000 mM) of ascorbic acid. From this solution, 20 µl aliquots were added to each assay run, which gave 20 mM in the total volume of each enzymatic assay cuvette.

5.3.3 Methylene Blue

Methylene blue (0.16 g, 100 mM) was dissolved in 5 ml buffer solution at pH = 8; a 10 µl aliquots was taken from this solution and transferred to a sample container with 990 µl buffer solution to give 1000 µl total volume with a concentration equal to (1 mM) methylene blue. 10 µl aliquots were added to every kinetic assay, which is equal to a concentration of 10 µM in the cuvette.

5.3.4 Bovine liver catalase

Bovine liver catalase 10 mg was dissolved in 1 ml buffer solution; 10 µl of this solution was transferred into a cuvette; the same amount was taken for each kinetic assay.

5.3.5 L–tryptophan

L-Tryptophan (0.204 g, 10 mmol) was dissolved in 1 ml (Tris) buffer; thereafter, different concentrations of this solution were prepared at (5, 10, 20, 30, 40, 50 80 and 100 µM)

5.3.6 Pure human Indoleamine 2,3 dioxygenase (hIDO)

4 µl of pure stock hIDO was transferred to a cuvette containing 996 µl of Tris buffer solution, at pH = 8, which had already been autozeroed in advance; the total volume in
the cuvette was 1000 μl. The cuvette was transferred to the UV\VIS spectrophotometer and scanned, giving a highest absorbance of 0.088 at 404 nm. The absorbance coefficient, ε, of hIDO is 172 mM\(^{-1}\) cm\(^{-1}\) at wavelength (λ) = 404; by applying the Beer-Lambert law, the concentration of the stock hIDO protein was determined as follows:

\[ A = \varepsilon \cdot c \cdot l \]

Where \(l\) is the cuvette path length, (1 cm), \(c\) is the protein concentration, \(\varepsilon\) is the molar absorbance coefficient, and \(A\) is the absorbance of the stock solution.

Since \(\varepsilon = A/c/l\)

If \(C = A/\varepsilon/l\)

\[ c = \frac{0.0877662/172 \text{ mm}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}^{-1}}{5.097 \times 10^{4} \text{ 1000 } \mu\text{M} \times 250} = 127.4 \mu\text{M} \]

which refer to the concentration of the stock; by using the dilution low \(c1\times v1 = c2\times v2\)

\[ 127.4 \mu\text{M} \times v1 = 0.1 \mu\text{M} \times 1000 \mu\text{M}, \text{ if } v1 = 0.1 \mu\text{M} \times 1000 \mu\text{L} / 127.4 \mu\text{M} = 0.78 \mu\text{L} \]

this amount of protein was put into every single cuvette assay with other compounds that were referred above.

5.3.7 UV-visible spectroscopy

The absorbance spectra of all assays experiments were measured in 50 mM Tris-HCl buffer, pH 8.0 at 25 °C using a Perkin Elmer lambda 25, 35 or 40 UV–visible spectrophotometer. There were two different of measurements were conducted by these instruments: 1- scanning the absorbance against time 5 and 10 minutes at 321 nm, which refers to the wavelength of the product NFK and then slopes were determined at fixed time. 2- Typical a 200-700 nm wavelength was obtained with employing 1 ml quartz cuvette. Baseline corrections were made against all buffers and solvents.

5.3.8 Protein expression (hTDO)

Human tryptophan 2,3-dioxygenase (hTDO) was over-expressed in \textit{E.coli} cells. The starter (7.75 g) of 2×YT–media in 250 ml water with (30 mg/ml, 250 μl) of antibiotic
kanamycin was vaccinated from an LB-agar plate. This mixture was incubated at 37 °C overnight with shaking at 120 rpm. The incubating starter culture used to vaccinate 12 × 600 ml (18.6 g) 2×YT–media. Form this, 600 µl antibiotic kanamycin and 20 ml starter culture was added to each conical flask. The large culture was incubated at 37°C for 90 minutes with shaking at 140 rpm until the absorbance reached approximately 0.8 at 600 nm (OD₆₀₀). 120 µl of IPTG was then added to each conical flask that contained large culture. Afterwards, the cells were incubated for 24 hours with shaking at 140 rpm and at a temperature of 25°C. Cells were harvested by centrifuging at 18000 rpm for 50 minutes at 4 °C, with the resultant pellets stored at -80 °C.

5.3.9 Protein purification

All the buffer solution was purification as listed in Table 7.

5.3.10 Bacterial cell lysis

50 ml from the lysis buffer table 7 was used for suspension of cell pellets, followed by addition of two full protease inhibitor tablets (Roche), which was then lysed by adding 5 mg of lysozyme and subsequent sonication (6×30 seconds pulses at one minute intervals, utilizing an MSE Soniprep 150 sonicator). After this step, 20 mM of both MgCl₂ and DNase I were added, and the mixture was stirred for 30 minutes at 4°C. The lysate was centrifuged for 50 minutes at 18000 rpm.
Table 7: Shows the buffer that was utilized for the purification of hTDO

<table>
<thead>
<tr>
<th>Types</th>
<th>Types</th>
<th>Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein hTDO</td>
<td><strong>Lysis buffer</strong></td>
<td>Solvent</td>
</tr>
<tr>
<td></td>
<td>300 mM NaCl</td>
<td>50 ml Tris buffer</td>
</tr>
<tr>
<td></td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>pH = 8.0</td>
</tr>
<tr>
<td></td>
<td>pH = 8</td>
<td></td>
</tr>
<tr>
<td>Protein hTDO</td>
<td><strong>Wash buffer</strong></td>
<td>Solvent</td>
</tr>
<tr>
<td></td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>1000 ml Tris buffer</td>
</tr>
<tr>
<td></td>
<td>300 mM NaCl</td>
<td>pH = 8.0</td>
</tr>
<tr>
<td></td>
<td>20 mM imidazole</td>
<td></td>
</tr>
<tr>
<td>Protein hTDO</td>
<td><strong>Elution buffer</strong></td>
<td>Solvent</td>
</tr>
<tr>
<td></td>
<td>250 mM imidazole</td>
<td>200 ml Tris buffer</td>
</tr>
<tr>
<td></td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>pH = 8.0</td>
</tr>
<tr>
<td></td>
<td>300 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>Last step for Protein hTDO</td>
<td><strong>Wash and Elution buffer</strong></td>
<td>for gel filtration column</td>
</tr>
<tr>
<td>purification</td>
<td></td>
<td>in 300 ml deionised water</td>
</tr>
<tr>
<td></td>
<td>50 mM Tris</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mM NaCl</td>
<td></td>
</tr>
</tbody>
</table>
5.4. Ni-NTA metal–affinity Chromatography

Ni-NTA superflow resin (Qiagen) was introduced into a 20 ml column, which was washed with 500 ml deionised water followed by washing the resin (3×15 ml) with washing buffer in table 7. Protein cells were then submitted into the column followed by washing the column with 500 ml washing buffer. hTDO protein was then eluted using the linear gradient of imidazole ranging from 20 to 250 mM by mixing equal amounts of washing and eluting buffer solvents.150

Table 8: Shows solutions utilized for preparation of SDS-PAGE

<table>
<thead>
<tr>
<th>Resolving gel master mix</th>
<th>Stacking gel master mix</th>
<th>Pouring resolving gel</th>
<th>Pouring Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ml 10% SDS</td>
<td>62.5 ml 1.0 M Tris pH = 6.8</td>
<td>3.96 ml resolving gel master mix</td>
<td>2.51 ml Stacking gel master mix</td>
</tr>
<tr>
<td>400 ml H2O</td>
<td>340 ml H2O</td>
<td>1.98 ml of 40% acrylamide</td>
<td>3 µl of TEMED</td>
</tr>
<tr>
<td>250 ml 1.5 M Tris pH= 8</td>
<td>5 ml 10 % SDS</td>
<td>5 µl TEMED</td>
<td>0.5 ml acrylamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 µl of 10 % APS</td>
<td>30 µl of 10 % APS</td>
</tr>
</tbody>
</table>

5.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

10 µl protein samples (hTDO) in elution buffer were mixed with equal amounts of Coomassie Blue then boiled for 5 minutes at 90 to 100 °C. These were loaded into the gel along with (New England BioLabs) a pre-satined protein marker. The gels were run at 200 V for 55 minutes; by the end of this time, the dye-front had to reach to the end of the gels. Afterwards, the gel layers in each experiment were run, were taken out
electrophoresis cell followed by washing the gels three times with water and microwaved for 1 minute per each wash and finally the gels were stained by Coomassie Blue Staining solution followed by microwaved for 45 seconds in order to visualising the proteins places and compared with protein markers.  

5.6 Collecting of hTDO after passed through Ni-column and calculated the concentrations for the more pure protein at both peaks 280 and 408 nm

Followed by using SDS-PAGE to separate based on its electrophoretic mobility and distinguish it from the others protein using protein marker with knowing the molecular weight of hTDO. After hTDO was determined, collected samples were concentrated by centrifuge using special centrifuge tube has filter to allow eluting solvent that containing imidazole to separate from hTDO based on the size of materials and then concentrated proteins were submitted again to SDS-PAGE. The gels 2 and 3 were seemed to be more pure UV spectroscopy was used to measured absorbance for this protein at 280 nm and 408 nm after minimised the absorbance of baseline at 700 nm from each absorbance at mentioned wavelength to providing concentrations as following: since

\[
\varepsilon = \frac{A}{cl}
\]

The concentration of hTDO after purified by Ni-column at 280 nm, \(\varepsilon = 57180\,M^{-1}\,cm^{-1}\)

\[
c = \frac{1.9250922}{57180\,M^{-1}\,cm^{-1} \times 1\,cm} \times 1000 \times 1000 = 33.67\,\mu M
\]

Whilst the concentration to the same protein sample after purified by Ni-column at 408 nm at \(\varepsilon = 196\,mM^{-1}\,cm^{-1}\)

\[
c = \frac{0.7617422}{196\,mM^{-1}\,cm^{-1} \times 1\,cm} \times 1000 = 3.89\,\mu M
\]

Above concentration of protein was submitted to the gel filtration column for further purification followed by identified hTDO using electrophoresis then concentrated and measured the concentration at 280 nm, using Beer-lambert law

\[
c = \frac{0.816297}{57180\,M^{-1}\,cm^{-1} \times 1\,cm} \times 1000 \times 1000 = 14.28\,\mu M
\]
As pure hTDO, this kept as pure protein in the fridge at – 80°C.

### 5.6.1 Preparation of hemin

Approximately, 5 to 10 mg of hemin chloride was dissolved in 120 ml NaOH. This solution has been taken to micro-tube (centrifuge tube), which contained on 600 µl Tris buffer with pH = 8, followed by centrifuged for 1 minutes, followed by transferring the supernatant to another centrifuge tube and centrifuging had taken place again for 1 mins and then supernatant had transferred to sample container followed by taken 250 µl of this solution to cuvette had contained 750 µl Tris buffer. The cuvette analysed by UV spectroscopy in order to measure the absorbance, which can clearly be seen that the highest peak at 384 nm, the absorbance was 0.87. This data had used to determine the concentration of stock hemin chloride; if we know that absorbance coefficient of hemin chloride at 384 nm is 58.44 mM⁻¹ cm⁻¹. Since Beer-Lambert law, \( \varepsilon = \frac{A}{c_1} \),

\[
c = \frac{0.87}{58.44 \text{ mM}^{-1} \text{ cm}^{-1}} = 0.015 \text{ mM}
\]

Since, the law of diluting is: \( c_1 \times v_1 = c_2 \times v_2 \)

\( c_1 \times 250 \mu l = 15 \mu M \times 1000 \mu l = 60 \mu M \), which represent concentration of stock hemin. This stock has used to equilibrate pure hTDO peak at 408 nm with 280 nm followed by measuring the concentration of pure protein hTDO at this point as described in chapter 4.

### 5.7. Steady-state kinetics

Enzyme activity measurements were aimed at monitoring the formation of NFK at 321 nm. The reactions were conducted at ambient temperature, approximately 25°C, and pH = 8 in 50 mM Tris-HCl buffer that included 100 µg catalase, 10 µg methylene blue, 20 mM L-ascorbate, and suitable concentrations of hTDO and hIDO, ranging from 100 to 200 nM. All reactions conducted were started by adding L-tryptophan, with or without potential inhibitors and the initial rate calculated from the absorbance at 321 nM (\( \varepsilon_{321} = 3750 \text{ M}^{-1} \text{ cm}^{-1} \)). The \( K_m \) and \( k_{cat} \) values were determined by different of concentrations.
of substrate and a fit of the data to the Michaelis–Menten equation (Equation 1). Data was fitted using the GraphPad Prism software package (version 6).

**Equation 1**

\[
V = V_{\text{max}} \frac{[S]}{[S] + K_m}
\]

In related to crystals of compounds 71, 73, 81, 91, 93, 98, 99, 107, 113a, 119 and 124a suitable for X-ray analysis were grown from (ethyl acetate and chloroform) except 71 that was grown from (acetonitrile and chloroform); the data for this analysis is given in the appendix I.
Chapter 6: References
6. References

Chapter 6: References


Chapter 6: References

4968–4977.


97 R. Sharma, Enzyme Inhibition and Bioapplications, InTech, Rijeka, Croatia, 1st edn., 2012.
141 R. Sharma, Enzyme Inhibition and Bioapplications, InTech, Rijeka, 1st edn., 2012.
148 Wan K. Chan, F.-C. Huang, Matthew M. Morrissette, J. D. Warus, K. J.


Appendix I: Crystal structure data
7. Appendix I

7.1. Crystal structure data

Crystal data and structural refinement for 71

Figures show 50% displacement ellipsoids. There is intermolecular hydrogen bonding:

Hydrogen bonds with \( H.A < r(A) + 2.000 \) Angstroms and \( <DHA > 110 \) deg.

\[
\begin{array}{cccccc}
D-H & d(D-H) & d(H..A) & \langle DHA \rangle & d(D..A) & A \\
O3-H3 & 0.840 & 1.841 & 165.12 & 2.661 & O1 [ x-1/2, -y+1/2, -z ] \\
N1-H1 & 0.850 & 2.144 & 164.74 & 2.972 & O1 [ -x, y-1/2, -z+1/2 ] \\
\end{array}
\]

\( R1 = 0.0411, wR2 = 0.0781. \)

Empirical formula \( \text{C9 H9 N O3} \)
Formula weight 179.17
Temperature 150(2) K
Wavelength 0.71073 Å
Crystal system Orthorhombic
Space group \( \text{P2(1)2(1)2(1)} \)
Unit cell dimensions \( a = 8.7227(12) \) Å \( \alpha = 90^\circ. \)
Appendix I

Volume $837.2(2)$ Å$^3$

$Z$ 4

Density (calculated) 1.422 Mg/m$^3$

Absorption coefficient 0.108 mm$^{-1}$

F(000) 376

Crystal size 0.23 x 0.18 x 0.13 mm$^3$

Theta range for data collection 2.96 to 25.00°.

Index ranges $-10\leq h\leq 10$, $-10\leq k\leq 10$, $-12\leq l\leq 12$

Reflections collected 6112

Independent reflections 880 [R(int) = 0.0965]

Completeness to theta = 25.00° 100.0 %

Absorption correction Empirical

Max. and min. transmission 0.981 and 0.400

Refinement method Full-matrix least-squares on F$^2$

Data / restraints / parameters 880 / 0 / 118

Goodness-of-fit on F$^2$ 0.954

Final R indices [I>2sigma(I)] R1 = 0.0411, wR2 = 0.0781

R indices (all data) R1 = 0.0541, wR2 = 0.0816

Absolute structure parameter ?

Largest diff. peak and hole 0.159 and -0.182 e.Å$^{-3}$
Appendix I

Crystal data and structural refinement for 73

Figures show 50% displacement ellipsoids. R1 = 0.0766, wR2 = 0.1589.

Empirical formula \( \text{C}_{20} \text{H}_{14} \text{N}_{2} \text{O}_{3} \)
Formula weight \( 330.33 \)
Temperature \( 150(2) \text{ K} \)
Wavelength \( 0.71073 \text{ Å} \)
Crystal system Triclinic
Space group P-1
Unit cell dimensions
\[
\begin{align*}
a &= 8.687(2) \text{ Å} & \alpha &= 95.091(5)^\circ \\
b &= 9.774(2) \text{ Å} & \beta &= 104.828(5)^\circ \\
c &= 10.389(3) \text{ Å} & \gamma &= 112.105(5)^\circ \\
\end{align*}
\]
Volume \( 772.9(3) \text{ Å}^3 \)
Appendix I

Z 2
Density (calculated) 1.419 Mg/m³
Absorption coefficient 0.097 mm⁻¹
F(000) 344
Crystal size 0.29 x 0.14 x 0.05 mm³
Theta range for data collection 2.07 to 25.00°.
Index ranges -10<=h<=9, -11<=k<=11, 0<=l<=12
Reflections collected 2709
Independent reflections 2709 [R(int) = 0.0000]
Completeness to theta = 25.00° 99.4 %
Absorption correction Empirical
Max. and min. transmission 0.981 and 0.529
Refinement method Full-matrix least-squares on F²
Data / restraints / parameters 2709 / 0 / 227
Goodness-of-fit on F² 0.908
Final R indices [I>2sigma(I)] R1 = 0.0766, wR2 = 0.1589
R indices (all data) R1 = 0.1437, wR2 = 0.1850
Largest diff. peak and hole 0.321 and -0.335 e.Å⁻³

Crystal data and structural refinement for 81

![Crystal structure image]
Figures show 50% displacement ellipsoids. There is intermolecular hydrogen bonding; Hydrogen bonds with H..A < r(A) + 2.000 Angstroms and <DHA > 110 deg.

D-H     d(D-H) d(H..A) <DHA d(D..A) A
N1-H1A  0.880  2.002  161.91  2.852  O2 [ x, y, z-1 ]

R1 = 0.0656, wR2 = 0.1243.

Empirical formula            C_{13} H_{13} N O_{2}
Formula weight               215.24
Temperature                 150(2) K
Wavelength                  0.71073 Å
Crystal system               Monoclinic
Space group                  P2(1)/n
Unit cell dimensions        a = 9.115(2) Å α= 90°.
b = 12.811(3) Å β= 107.681(5)°.  
c = 9.902(2) Å γ = 90°.

Volume                      1101.7(4) Å³
Z                            4
Density (calculated)        1.298 Mg/m³
Absorption coefficient      0.088 mm⁻¹
F(000)                      456
Crystal size                0.22 x 0.20 x 0.13 mm³
Theta range for data collection    2.66 to 25.99°.
Index ranges               -11<=h<=11, -15<=k<=15, -12<=l<=12
Reflections collected       8417
Independent reflections     2154 [R(int) = 0.1401]
Completeness to theta = 25.99° 99.8 %
Absorption correction Empirical
Max. and min. transmission  0.981 and 0.403
Refinement method         Full-matrix least-squares on F²
Data / restraints / parameters 2154 / 0 / 146
Goodness-of-fit on F²     0.899
Final R indices [I>2sigma(I)]
R1 = 0.0656, wR2 = 0.1243
R indices (all data)       R1 = 0.1284, wR2 = 0.1436
Largest diff. peak and hole 0.290 and -0.232 e.Å⁻³
Crystal data and structural refinement for 91

Figures show 50% displacement ellipsoids. One of the rings is disordered and has been split.

R1 = 0.0848, wR2 = 0.1418.

- **Empirical formula**: C20 H19 N O2
- **Formula weight**: 305.36
- **Temperature**: 150(2) K
- **Wavelength**: 0.71073 Å
- **Crystal system**: Monoclinic
- **Space group**: P2(1)/n
- **Unit cell dimensions**:
  - a = 16.883(15) Å \(\alpha = 90^\circ\)
  - b = 4.959(4) Å \(\beta = 99.942(18)^\circ\)
  - c = 19.860(16) Å \(\gamma = 90^\circ\)
- **Volume**: 1638(2) Å³
- **Z**: 4
Density (calculated) 1.238 Mg/m$^3$
Absorption coefficient 0.080 mm$^{-1}$
F(000) 648
Crystal size 0.35 x 0.14 x 0.03 mm$^3$
Theta range for data collection 1.74 to 25.00°
Index ranges -20<=h<=19, -5<=k<=5, -23<=l<=23
Reflections collected 11228
Independent reflections 2874 [R(int) = 0.3310]
Completeness to theta = 25.00° 100.0 %
Absorption correction Empirical
Max. and min. transmission 0.969 and 0.283
Refinement method Full-matrix least-squares on F$^2$
Data / restraints / parameters 2874 / 0 / 246
Goodness-of-fit on F$^2$ 0.793
Final R indices [I>2sigma(I)] R1 = 0.0848, wR2 = 0.1418
R indices (all data) R1 = 0.2823, wR2 = 0.2041
Extinction coefficient 0.0038(12)
Largest diff. peak and hole 0.229 and -0.263 e.Å$^{-3}$

Crystal data and structural refinement for 93
Figures show 50% displacement ellipsoids. There is intermolecular hydrogen bonding; Hydrogen bonds with H..A < r(A) + 2.000 Angstroms and <DHA > 110 deg.

D-H           d(D-H)    d(H..A)    <DHA    d(D..A)    A
O2-H2          0.840      1.777     170.71      2.610    O1 [ -x, -y, -z+2 ]

R1 = 0.0573, wR2 = 0.1093.

Empirical formula          C_{18} H_{15} N O_{2}
Formula weight            277.31
Temperature              150(2) K
Wavelength               0.71073 Å
Crystal system           Monoclinic
Space group              P2(1)/n
Unit cell dimensions
  a = 12.840(5) Å       α = 90°.
  b = 8.018(3) Å         β = 103.450(7)°.
  c = 14.232(5) Å       γ = 90°.
Volume                   1425.0(9) Å^{3}
Z                        4
Density (calculated)      1.293 Mg/m^{3}
Absorption coefficient   0.084 mm^{-1}
F(000)                   584
Crystal size              0.41 x 0.20 x 0.06 mm^{3}
Theta range for data collection 1.93 to 25.00°.
Index ranges             -15<=h<=15, -9<=k<=9, -16<=l<=16
Reflections collected    9980
Independent reflections  2507 [R(int) = 0.1137]
Appendix I

Completeness to theta = 25.00° 100.0 %
Absorption correction Empirical
Max. and min. transmission 0.981 and 0.515
Refinement method Full-matrix least-squares on F^2
Data / restraints / parameters 2507 / 0 / 190
Goodness-of-fit on F^2 0.913
Final R indices [I>2sigma(I)] R1 = 0.0573, wR2 = 0.1093
R indices (all data) R1 = 0.1054, wR2 = 0.1240
Largest diff. peak and hole 0.195 and -0.195 e.Å^-3

Crystal data and structural refinement for 98

Figures show 50% displacement ellipsoids. There is intermolecular hydrogen bonding; Hydrogen bonds with H.A < r(A) + 2.000 Angstroms and <DHA > 110 deg.
Appendix I

\begin{verbatim}
D-H   d(D-H)   d(H..A)  \langle DHA  d(D..A)  A
O2-H2  0.840    1.831   176.91   2.670  O1  [-x, -y+1, -z+2 ]

R1 = 0.0708, wR2 = 0.1515.

Empirical formula  C19 H17 N O2
Formula weight      291.34
Temperature         150(2) K
Wavelength          0.71073 Å
Crystal system      Triclinic
Space group         P-1
Unit cell dimensions
a = 5.2311(13) Å   \alpha= 77.394(4)°.
b = 9.465(2) Å   \beta= 88.305(5)°.
c = 15.366(4) Å   \gamma = 87.735(5)°.
Volume              741.7(3) Å³
Z                    2
Density (calculated) 1.304 Mg/m³
Absorption coefficient 0.085 mm⁻¹
F(000)               308
Crystal size         0.28 x 0.25 x 0.10 mm³
Theta range for data collection  2.21 to 25.00°.
Index ranges         -6\leq h \leq 6, -11 \leq k \leq 11, -18 \leq l \leq 18
Reflections collected 5417
Independent reflections 2595 [R(int) = 0.0746]
Completeness to theta = 25.00°  99.0 %
Absorption correction  Empirical
Max. and min. transmission  0.969 and 0.547
Refinement method    Full-matrix least-squares on F²
Data / restraints / parameters  2595 / 0 / 199
Goodness-of-fit on F² 0.956
Final R indices [I>2sigma(I)]
R1 = 0.0708, wR2 = 0.1515
R indices (all data)
R1 = 0.1139, wR2 = 0.1677
Largest diff. peak and hole 0.234 and -0.262 e.A⁻³
\end{verbatim}
Crystal data and structural refinement for 99

Figures show 50% displacement ellipsoids. R1 = 0.0663, wR2 = 0.1215.

Empirical formula C21 H21 N O3
Formula weight 335.39
Temperature 150(2) K
Wavelength 0.71073 Å
Crystal system Monoclinic
Space group P2(1)/n
Unit cell dimensions 
\[ a = 14.843(7) \text{ Å}, \quad \alpha = 90^\circ. \]
\[ b = 4.992(2) \text{ Å}, \quad \beta = 103.158(10)^\circ. \]
\[ c = 24.611(11) \text{ Å}, \quad \gamma = 90^\circ. \]
Volume \(1775.9(14) \text{ Å}^3\)
Z 4
Density (calculated) 1.254 Mg/m\(^3\)
Absorption coefficient 0.084 mm\(^-1\)
F(000) 712
Crystal size 0.38 x 0.11 x 0.05 mm\(^3\)
Theta range for data collection 1.47 to 25.00°
Index ranges \(-17 \leq h \leq 17, -5 \leq k \leq 5, -28 \leq l \leq 29\)
Appendix I

Reflections collected 12113
Independent reflections 3114 [R(int) = 0.2205]
Completeness to theta = 25.00° 100.0 %
Absorption correction Empirical
Max. and min. transmission 0.981 and 0.503
Refinement method Full-matrix least-squares on F^2
Data / restraints / parameters 3114 / 0 / 228
Goodness-of-fit on F^2 0.768
Final R indices [I>2sigma(I)] R1 = 0.0663, wR2 = 0.1215
R indices (all data) R1 = 0.1941, wR2 = 0.1589
Largest diff. peak and hole 0.248 and -0.327 e.Å^{-3}

Crystal data and structural refinement for 107

Figures show 50% displacement ellipsoids. There is intermolecular hydrogen bonding:
Hydrogen bonds with H.A < r(A) + 2.000 Angstroms and <DHA > 110 deg.

\[
\begin{align*}
&D-H & d(D-H) & d(H..A) & <DHA & d(D..A) & A \\
&N1-H1A & 0.973 & 1.949 & 168.59 & 2.909 & N2 [ x, y, z-1 ] \\
R1 &= 0.0907, wR2 = 0.2261.
\end{align*}
\]
<table>
<thead>
<tr>
<th>Property</th>
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</tr>
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<tbody>
<tr>
<td>Empirical formula</td>
<td>C15 H12 N2</td>
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<tr>
<td>Formula weight</td>
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<tr>
<td>Temperature</td>
<td>150(2) K</td>
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<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Triclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P-1</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a = 7.528(3) Å</td>
<td>α = 88.305(8)°</td>
</tr>
<tr>
<td>b = 7.870(4) Å</td>
<td>β = 77.286(8)°</td>
</tr>
<tr>
<td>c = 11.134(5) Å</td>
<td>γ = 62.248(7)°</td>
</tr>
<tr>
<td>Volume</td>
<td>567.5(4) Å</td>
</tr>
<tr>
<td>Z</td>
<td>2</td>
</tr>
<tr>
<td>Density (calculated)</td>
<td>1.289 Mg/m³</td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>0.077 mm⁻¹</td>
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<td>F(000)</td>
<td>232</td>
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<tr>
<td>Crystal size</td>
<td>0.42 x 0.25 x 0.03 mm³</td>
</tr>
<tr>
<td>Theta range for data collection</td>
<td>1.88 to 25.00°</td>
</tr>
<tr>
<td>Index ranges</td>
<td>-8&lt;=h&lt;=8, -9&lt;=k&lt;=9, -13&lt;=l&lt;=13</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>4089</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>1980 [R(int) = 0.0809]</td>
</tr>
<tr>
<td>Completeness to theta = 25.00°</td>
<td>99.1 %</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>Empirical</td>
</tr>
<tr>
<td>Max. and min. transmission</td>
<td>0.969 and 0.068</td>
</tr>
<tr>
<td>Refinement method</td>
<td>Full-matrix least-squares on F²</td>
</tr>
<tr>
<td>Data / restraints / parameters</td>
<td>1980 / 0 / 154</td>
</tr>
<tr>
<td>Goodness-of-fit on F²</td>
<td>0.995</td>
</tr>
<tr>
<td>Final R indices [I&gt;2sigma(I)]</td>
<td>R1 = 0.0907, wR2 = 0.2261</td>
</tr>
<tr>
<td>R indices (all data)</td>
<td>R1 = 0.1213, wR2 = 0.2484</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>0.368 and -0.355 e.Å⁻³</td>
</tr>
</tbody>
</table>
Appendix I

Crystal data and structural refinement for 113a

Figures show 50% displacement ellipsoids. The ‘A’ atoms have been generated by symmetry, symmetry operation -x, y, -z+5/2.

R1 = 0.0678, wR2 = 0.1550.

- Empirical formula: C23 H20 N2 O
- Formula weight: 340.41
- Temperature: 150(2) K
- Wavelength: 0.71073 Å
- Crystal system: Monoclinic
- Space group: C2/c
- Unit cell dimensions:
  - a = 13.094(3) Å  
  - b = 10.849(3) Å          α= 90°.
- Volume: 1765.2(8) Å³
- Z: 4
- Density (calculated): 1.281 Mg/m³
- Absorption coefficient: 0.079 mm⁻¹
- F(000): 720
- Crystal size: 0.17 x 0.11 x 0.07 mm³
- Theta range for data collection: 2.50 to 25.00°.
- Index ranges: -15<=h<=15, -12<=k<=12, -15<=l<=15
Appendix I

Reflections collected 6275
Independent reflections 1561 [R(int) = 0.1306]
Completeness to theta = 25.00° 99.9 %
Absorption correction Empirical
Max. and min. transmission 0.969 and 0.091
Refinement method Full-matrix least-squares on F²
Data / restraints / parameters 1561 / 0 / 120
Goodness-of-fit on F² 0.959
Final R indices [I>2sigma(I)] R1 = 0.0678, wR2 = 0.1550
R indices (all data) R1 = 0.0885, wR2 = 0.1666
Largest diff. peak and hole 0.285 and -0.319 e.Å⁻³

Crystal data and structural refinement for 119
Figure show X-ray structures with thermal ellipsoids drawn at 50% probability level.

R1 = 0.0459, wR2 = 0.1085.

Empirical formula C17 H14 N2 O
Formula weight 262.30
Temperature 150(2) K
Wavelength 0.71073 Å
Crystal system Monoclinic
Space group P2(1)/c
Unit cell dimensions
\[ a = 5.1840(11) \text{ Å} \quad \alpha = 90°. \]
\[ b = 13.602(3) \text{ Å} \quad \beta = 97.281(4)°. \]
\[ c = 18.418(4) \text{ Å} \quad \gamma = 90°. \]
Volume 1288.2(5) Å³
Z 4
Density (calculated) 1.352 Mg/m³
Absorption coefficient 0.086 mm⁻¹
F(000) 552
Crystal size 0.37 x 0.16 x 0.10 mm³
Theta range for data collection 1.87 to 25.00°.
Index ranges -6≤h≤6, -16≤k≤15, -21≤l≤21
Reflections collected 9131
Independent reflections 2265 [R(int) = 0.0521]
Completeness to theta = 25.00° 100.0 %
Absorption correction Empirical
Max. and min. transmission 0.981 and 0.685
Refinement method Full-matrix least-squares on F²
Data / restraints / parameters 2265 / 0 / 182
Goodness-of-fit on F² 1.007
Final R indices [I>2sigma(I)] R1 = 0.0459, wR2 = 0.1085
R indices (all data) R1 = 0.0578, wR2 = 0.1132
Largest diff. peak and hole 0.227 and -0.191 e.Å⁻³
Crystal data and structural refinement for 124a

Figures show 50% displacement ellipsoids. R1 = 0.0713, wR2 = 0.1497.

- **Empirical formula**: C18 H15 N O
- **Formula weight**: 261.31
- **Temperature**: 150(2) K
- **Wavelength**: 0.71073 Å
- **Crystal system**: Monoclinic
- **Space group**: P2(1)/n
- **Unit cell dimensions**: a = 5.6340(13) Å \( \alpha = 90^\circ \).
<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>9.380(2) Å</td>
</tr>
<tr>
<td>$\beta$</td>
<td>92.854(5)$^\circ$</td>
</tr>
<tr>
<td>c</td>
<td>25.356(6) Å</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>90$^\circ$</td>
</tr>
<tr>
<td>Volume</td>
<td>1338.3(5) Å$^3$</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
</tr>
<tr>
<td>Density (calculated)</td>
<td>1.297 Mg/m$^3$</td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>0.080 mm$^{-1}$</td>
</tr>
<tr>
<td>F(000)</td>
<td>552</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.19 x 0.15 x 0.03 mm$^3$</td>
</tr>
<tr>
<td>Theta range for data collection</td>
<td>1.61 to 25.00$^\circ$.</td>
</tr>
<tr>
<td>Index ranges</td>
<td>-6$\leq$h$\leq$6, -11$\leq$k$\leq$11, -29$\leq$l$\leq$30</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>9341</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>2345 [R(int) = 0.1277]</td>
</tr>
<tr>
<td>Completeness to theta = 25.00$^\circ$</td>
<td>99.9%</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>Empirical</td>
</tr>
<tr>
<td>Max. and min. transmission</td>
<td>0.969 and 0.038</td>
</tr>
<tr>
<td>Refinement method</td>
<td>Full-matrix least-squares on F2</td>
</tr>
<tr>
<td>Data / restraints / parameters</td>
<td>2345 / 0 / 182</td>
</tr>
<tr>
<td>Goodness-of-fit on F2</td>
<td>0.900</td>
</tr>
<tr>
<td>Final R indices [I$&gt;2\sigma$(I)]</td>
<td>R1 = 0.0713, wR2 = 0.1497</td>
</tr>
<tr>
<td>R indices (all data)</td>
<td>R1 = 0.1294, wR2 = 0.1684</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>0.305 and -0.290 e.Å$^{-3}$</td>
</tr>
</tbody>
</table>