Hybrid molecules as inhibitors of the monoamine oxidases and caspase 3 for neurodegenerative disorders

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To my sisters, Shahin and Fariba, and my brothers, Mohammad and Masoud

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“My success is in the hands of God”, praise be to Him.

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Abstract

Neurodegenerative diseases are multifactorial in nature, and taking the complex nature of these disorders into consideration, multi-target directed ligands may present as better options to treat these disorders than the classic 'one molecule, one target' approach.

Neurotransmitter amines are catabolized by monoamine oxidase A and B (MAO-A and MAO-B), therefore they have been targeted for the treatment of neuropsychiatric and neurodegenerative diseases. Besides offering a potential antidepressant action in PD, MAO-A inhibitors may also provide a symptomatic benefit by reducing MAO-A-catalysed oxidation of dopamine. The oxidative deamination reaction catalyzed by MAO-B is one of the major catabolic pathways of dopamine in the brain. Inhibition of this enzyme leads to enhanced dopaminergic neurotransmission and are currently used in the symptomatic treatment of PD. Furthermore, MAO-B inhibitors may also exert neuroprotective effects by reducing the concentration of potentially hazardous by-products produced by MAO-B-catalysed dopamine oxidation. Apoptosis or programmed cell death occurs in a number of neurodegenerative disorders and it has been proven that inhibition of the executing enzyme, caspases-3, slows down or even stops apoptosis. Having this in mind we focused on the propargylamine function of selegiline and the fluorophenyl function of safinamide, because of their inherent monoamine oxidase inhibitory activities; and isatin as a non-peptide inhibitor of caspase-3. Therefore we attempted to design and synthesize multifunctional hybrid molecules acting simultaneously to halt the apoptotic neuronal breakdown process and eliminate the signs and symptoms of diseases such as PD.

Seven novel compounds were successfully synthesized utilizing multistep processes. The synthesis of 5-chlorosulfonyl isatin was accomplished starting from the commercially available isatin in two steps, which were, sulfonation using tetramethylene sulfone and chlorination with $POCl_3$. Next 5-chlorosulfonyl isatin was conjugated to the fluorophenylamine derivative with the fluoro-function at either the ortho, meta or para position through a nucleophilic substitution reaction on the chlorosulfonyl position. The resultant compounds were coupled on the *N* position of the isatin function with propargyl bromide, using a microwave synthesis procedure, in a nucleophilic substitution reaction. The structures and purity were confirmed by nuclear magnetic resonance (NMR) and mass spectrometry (MS).

In the biological evaluations recombinant human MAO-A, MAO-B and caspase 3 enzymatic assays were performed to determine the activity of the novel compounds at an enzymatic level. The inhibition percentages for these compounds were calculated and plotted against the logarithm

of the inhibitor concentrations to obtain a sigmoidal dose-response curve and consequently the IC_{50} value.

The synthesized compounds showed inhibition of the MAO-A, MAO-B and caspase-3 enzymes at low to high micro molar concentrations. The role of the fluorophenylamine moiety in the synthesized compounds was significant for their multifunctional activity as shown by compounds **3** and **4** having good inhibitory activity towards MAO-A, MAO-B and also excellent inhibitory activity against caspase 3, making them ideal candidates for further lead compound development and multifunctional drug design. The introduction of the propargylamine moiety only increased the MAO-A inhibitory activity; this was shown by compounds **7**, **8** and **9** which exhibit good MAO-A selectivity with low MAO-B and caspase-3 inhibitory activities.

Chapter 1
Introduction

1.1 BACKGROUND

It has been discovered that neurodegeneration occurs in which neurons in the central nervous system (CNS) and the brain in particular are destroyed by several mechanisms (Araki et al. 2001). These neurodegenerative disorders are progressive and irreversible, and a loss of neurons from specific regions of the brain is characteristic of these disorders (Alexi et al. 2000). The quality of life of patients that suffer from Huntington's disease (HD), Alzheimer's disease (AD) and Parkinson's disease (PD) are significantly diminished, especially among the global aging population and it is a huge problem worldwide (Rang 2007). A vast amount of time and research has gone into this area of identifying what the causes are for these disorders. The current drugs that are available are used more for the management of these disorders rather than for treatment of these diseases. These disorders have a profound impact on the life of the affected patients and their families. Therefore there is a very strong motivation to search for the best treatment possible (Tarrants et al. 2010).

Currently there are nearly 36 million people in the world that suffer from dementia and the numbers of people living with this disease worldwide is expected to double every 20 years. The estimated total cost of dementia worldwide is $604 billion dollars. A report done by the World Health Organization in 2012, called on all countries to recognize that dementia would be a global health challenge and include dementia in public health planning. (Alzheimer's Society UK 2014). PD is the second most common neurodegenerative disorder after AD and affects nearly 0.3% of the general population (de Lau, Breteler 2006). In the United States, medication costs for an individual person with PD is estimated to be nearly $2,500 dollars a year, and therapeutic surgery can cost up to $100,000 per patient (Parkinson's Disease Foundation 2016).

Not a single cause or process leads to a neurodegenerative disorder; instead it is the result of several factors which most likely include a variety of mechanistic pathways (Dauer, Przedborski 2003). It is postulated that genetic, environmental and endogenous factors play an important part in neurodegenerative disorders such as AD and PD and a number of general pathways have been discovered that may be among the factors which are involved in different pathogenic cascades. These include excitotoxicity, oxidative stress and free radical formation, protein misfolding and aggregation, phosphorylation impairment, metal dyshomeostasis and

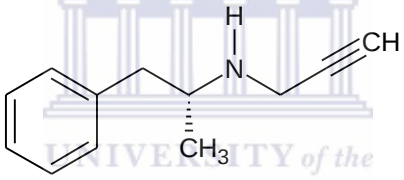
mitochondrial dysfunction. Some of these seem to happen simultaneously, resulting in the death of essential neuronal cells (Jellinger 2003a).

Apoptosis or programmed type I cell death is a complicated constant mechanism to dispose of surplus, potentially dangerous and damaged cells resulting in apoptotic bodies which get cleared without activation of the immune system (Hengartner 2000). Apoptosis as seen in physiological cellular processes such as control of size and shape of tissue, fetal development, maintenance of the immune system, and cell homeostasis, all play a key role. In the case of dysregulation, apoptosis plays a vital role in a variety of diseases such as neurodegenerative and developmental deficiencies, cardiovascular and autoimmune diseases, cancer and atherosclerosis (Limpachayaporn et al. 2013b). Apoptotic cell death is generally characterized by several subcellular alterations winding up in a specific cell death program (Martins, Earnshaw 1997). The programmed type I cell death has been identified by three stages which are initiation, effector and degradation (Kroemer et al. 1995), in PD the apoptotic process most probably appears as an end-stage process (Anglade et al. 1997). In the case of PD, upregulation of the antiapoptotic protein Bcl-2 (Marshall et al. 1997) and augmented levels of the proapoptotic protein Bax (Hartmann et al. 2001) have been seen in the substantia nigra of the affected patients. In addition, caspase 3 and caspase 8 may act as effectors of apoptotic cell death in the brain of PD patients (Mogi et al. 2000, Hartmann et al. 2001).

1.2 ENZYME INHIBITION

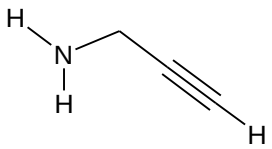
Neurotransmitter amines are catabolized by MAO-A and -B, these enzymes have been targeted for the treatment of neuropsychiatric and neurodegenerative diseases (Youdim, Edmondson & Tipton 2006a, Youdim, Weinstock 2004, Riederer, Lachenmayer & Laux 2004). MAO-A metabolizes both serotonin and norepinephrine, two monoamines implicated in depressive illness and anxiety disorders (Youdim, Edmondson & Tipton 2006b, ZISOOK, BRAFF & CLICK 1985). The antidepressive effect of MAO-A inhibitors is associated with blocking the central breakdown of these monoamines extending their action. In PD patients, the inhibition of MAO-A may be beneficial since a significant proportion of these patients exhibit signs of depression (Di Monte et al. 1996a). These inhibitors may also provide a symptomatic benefit by reducing MAO-A-catalysed oxidation of dopamine (Youdim, Edmondson & Tipton 2006b, Di Monte et al. 1996a) and consequently enhancing dopamine level in the human basal ganglia (Kalaria, Mitchell & Harik 1988a, Collins et al. 1970a).

MAO-B inhibitors are effectively used in treatment of PD (Fernandez, Chen 2007), they act by blocking the MAO-B-catalysed metabolism of dopamine and as a result extend the physiological action of dopamine in the basal ganglia. They may also boost dopamine levels acquired from levodopa, the metabolic precursor of dopamine. As an example, selegiline which inhibits MAO-B irreversibly can be administered as adjunct to levodopa or as independent therapy, it is approved therapeutically for PD worldwide and increases the elevation of dopamine levels after levodopa therapy (Di Monte et al. 1996b, Chen, Swope 2005, Finberg et al. 1998). MAO-B inhibitors are administered mainly to treat PD, but they may also protect against the neurodegenerative processes of this disease. They block the formation of dopanal and H_2O_2, the byproducts of the metabolism of dopamine by MAO-B (Gesi et al. 2001, Lamensdorf et al. 2000). These by-products are neurotoxic and may speed up the neurodegenerative process (Kalaria, Mitchell & Harik 1988b, Nicotra et al. 2004, Di Monte et al. 1996b, Fowler et al. 1997), therefore MAO-B inhibitors may bring about a neuroprotective effect by decreasing MAO-B catalysed aldehyde and H_2O_2 production.



Selegiline

Anti-apoptotic pathways are activated by compounds containing the propargylamine function and this gives the opportunity to have a single compound combining an anti-apoptotic effect with a MAO inhibitory effect (Maruyama et al. 2002, Blandini 2005). Selegiline and rasagiline, irreversible inhibitors of MAO-B, are examples of such compounds (Edmondson et al. 2003, Binda et al. 2005). As an example, rasagiline was shown to provide protection against apoptotic cell death in neuroblastoma caused by the pro-apoptotic toxin *N*-methyl-R-salsolinol. The mechanism of action by which these compounds show their anti-apoptotic effect is still not clearly defined (Maruyama et al. 2000).

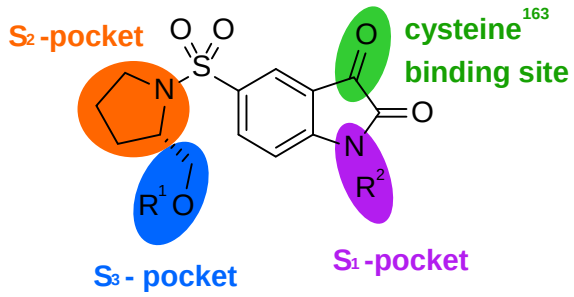


N-propargylamine

Intracellular enzymes strictly controlling the apoptotic pathway are called caspases. Over a dozen caspases have been discovered so far and identified as initiating (e.g. caspases 8, 9 and 10) and executing caspases (e.g. caspases 3, 6 and 7). Activation of caspase 3 has been observed in all kinds of apoptosis (Porter, Jänicke 1999) including neurodegenerative disorders (O'Brien, Lee 2004). Among other caspases, caspase 3 is principal in the cleavage of the amyloid-precursor protein (APP) alongside its distinct ability to increase neuronal cell death of AD affected brains and colocalization of the product of its APP cleavage with amyloid-ß present in senile plaques (Albrecht et al. 2007). Polyglutamine causes HD by expanding the *N*-terminus of protein huntingtin, which is a caspase substrate (Chu et al. 2009). To execute the programmed type I cell death (apoptosis) caspases 3 is crucial (Limpachayaporn et al. 2013a).

Nearly all known caspase inhibitors have a common structural characteristic which is an electrophilic group that can make a reversible or irreversible bond with the active site cysteine of the enzyme and making it inactive (Lee et al. 2001). Synthetic peptide inhibitors including Z-DEVD-fmk and Z-VAD-fmk have been broadly applied to block type I cell death (Lopez-Hernandez et al. 2003). Due to the moderate caspase selectivities associated with these reagents it is difficult to assess the importance of a specific caspase in apoptosis and this often makes the analysis of results ambiguous (Cryns, Yuan 1998, Schotte et al. 1999). To overcome this problem, more recently the identification of selective, non-peptidic caspase inhibitors has been a center of attention (Deckwerth et al. 2001, Han et al. 2002). Numerous research groups have reported their attempt toward small molecule selective caspase 3 inhibitors (Lee et al. 2001, Abbavaram, Reddyvari 2013), a selective inhibitor of caspase 3 may give the opportunity to evaluate the inhibition of apoptosis potentially at the level of an executioner caspase (Porter, Jänicke 1999).

Isatin sulfonamides were discovered by screening methods and introduced as potent, non-peptide, selective inhibitors of caspases 3 and 7. In contrast to previously studied peptide-based caspase inhibitors, the selectivity of isatin sulfonamides for caspases 3 and 7 results primarily from interacting with the S_2 subsite, they also do not always bind in primary aspartic acid binding pocket (S_1) of the caspase (Lee et al. 2000a) (Figure 1.1).

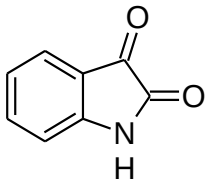


1 (R^1 = Ph, R^2 = Me)

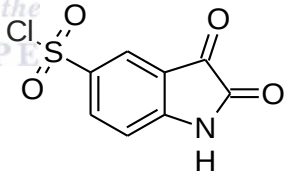
2 (R^1 = Me, R^2 = H)

Figure 1.1: Designated interactions of the general structure of isatin sulfonamides with caspase 3 and 7 enzyme's sub-pockets (Limpachayaporn et al. 2013a).

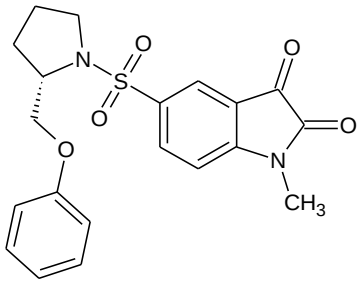
These group of compounds derived from isatin have been shown to reduce tissue damage in an isolated rabbit heart model of ischemia injury and also to block apoptosis in murine bone marrow neutrophils and human chondrocytes (Lee et al. 2000a, Chapman et al. 2002). This isatin derived compounds (represented by compounds A – D, Figure 1.2) have a higher cell permeability, metabolic stability and capability to differentiate apoptosis from necrosis. This may make these molecules superior compared to peptide-based inhibitors (Lee et al. 2000a).



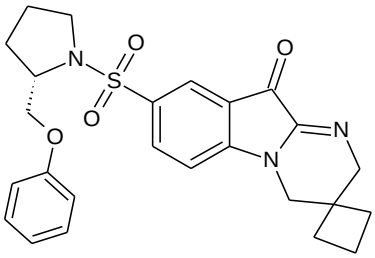
Isatin



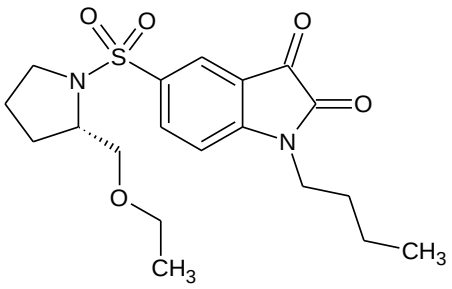
5-chlorosulfonyl isatin



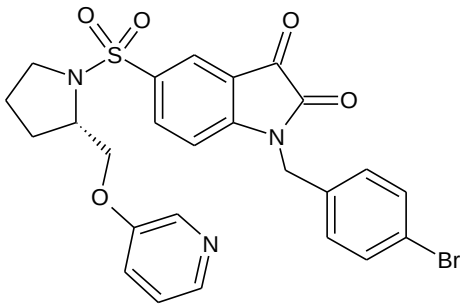
Compound A, IC_{50} = 15 nM (Lee et al. 2000b)



Compound B, IC_{50} = 1.29 nM (Havran et al. 2009)



Compound C, IC_{50} = 16.9 nM (Limpachayaporn et al. 2013a)



Compound D, IC_{50} = 3.6 ± 0.5 nM (Chu et al. 2007)

Figure 1.2: The structure of isatin and the isatin derived caspase 3 inhibitors

1.3 RATIONAL OF THIS STUDY

So far there has not been any treatment proven which can simultaneously eliminate the signs and symptoms of diseases such as AD and PD and also to stop cell death or bring back damaged neurons to a state of normality (Joubert et al. 2011, Zhou, Zhong & Silverman 1996, Ramlawi et al. 2006). Disease-modifying approaches have turned into an area of focus in research since these strategies interrupt the initial pathologic events associated with the onset of the disease; hindering the neurotoxic cascade in the case of neurodegenerative diseases is an example of these approaches (McLean et al. 1999, Näslund et al. 2000). Neurodegenerative disorders are known to be multifactorial in nature, bearing this in mind, it is evident that the pattern of targeting a single disease factor might not be an efficacious strategy for treatment of neurodegenerative diseases.

In clinical practice, multiple targeting has proceeded through the polypharmaceutical approach by using a combination of therapeutic agents functioning on different etiological targets independently. This approach has been proved to be effective in treatment of diseases with similar complexity such as hypertension, cancer and HIV (Jellinger 2003b, Smid et al. 2005). It has been proposed that treatment for neurodegenerative diseases will be a blend of drugs having different mechanisms of action while acting on more than one related neurotherapeutic target (Van der Schyf, Cornelis J, Geldenhuys & Youdim 2006). Treatment with combination preparations is, however, a challenging and complex field for drug development due to the likelihood of drug–drug interactions and further complications met while combining drug entities that have potentially various degrees of bioavailability, metabolism, pharmacokinetic profiles and

toxicity (Smid et al. 2005). The design of single drug molecules for a particular disease acting on multiple specific etiological targets can be worthwhile especially in neurodegenerative diseases. Numerous studies have reported the possibility of having a single drug which acts at several sites in the neurotoxic cascade (Smid et al. 2005, Morphy, Rankovic 2005). Taking the complex nature of neurodegenerative disorders into consideration, multi-target directed ligands may present as a better option to treat neurodegenerative diseases than the classic 'one molecule, one target' approach (Cavalli et al. 2008, Marlatt et al. 2005).

1.4 AIM OF STUDY

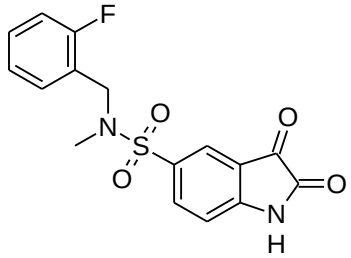
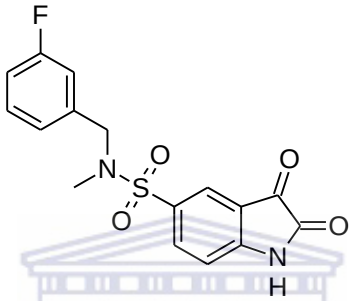
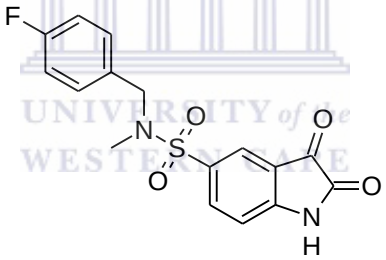
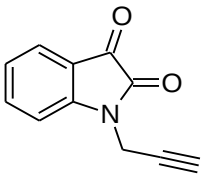
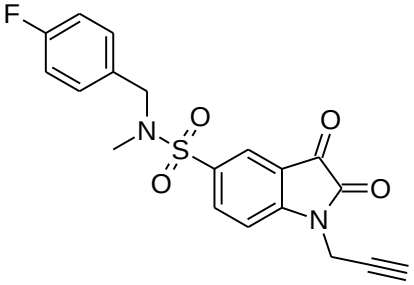
From the information above, current research focus moved from a 'one-drug-one-target approach' to that of drug which is able to act at various relevant biological targets. It was decided to design and synthesize multifunctional agents which might halt the apoptotic neuronal breakdown process and eliminate the signs and symptoms of diseases such as AD and PD. This would happen by: (a) Inhibiting the MAO-A and MAO-B enzymes thus allowing increase in dopamine levels in the CNS and reducing the levels of the highly oxidative products produced by the activity of this enzyme, and (b) inhibiting apoptotic processes to halt neuronal cell death. With this in the mind, the propargylamine portion of selegiline, the isatin moiety and a fluorophenylamine function was used as pharmacophoric groups to design potent, non-peptide, selective inhibitors of caspases-3 with MAO-A and/or MAO-B inhibitory properties. Therefore, the aim of this project was:

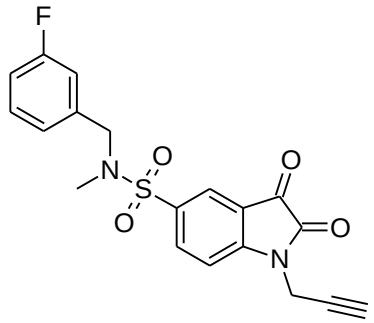
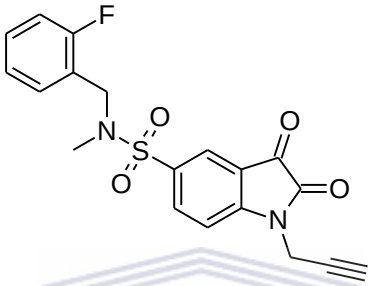
- To provide promising strategies in the development of a multifunctional neuroprotective therapeutic single agent for the treatment/prevention of neurodegenerative disorders.

To reach the aim the following was done:

- Synthesis of drug-like molecules that comprise of features of both selegiline and isatin.
- In vitro evaluation of caspase-3, MAO-A and MAO-B inhibition by these compounds.

Table 1.1: Assigned names and structures of final compounds selected for synthesis.

Assigned number	Structure and the Name of Compounds
2	 ***N*-[(2-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*- indole-5-sulfonamide**
3	 ***N*-[(3-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide**
4	 ***N*-[(4-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide**
5	 **1-(Prop-2-yn-1-yl)-1*H*-indole-2,3-dione**
7	 ***N*-[(4-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-indole-5-sulfonamide**

8	 ***N*-[(3-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-indole-5-sulfonamide**
9	 ***N*-[(2-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-indole-5-sulfonamide**

1.5 CONCLUDING REMARKS

It is expected that the compounds **7**, **8** and **9** will have moderate to high caspase-3, MAO-A and MAO-B inhibitory activity since the propargylamine portion of selegiline, the isatin moiety and a fluorophenylamine function are used as pharmacophoric groups to design and synthesize these compounds therefore they may exhibit the biological properties of the components that they are composed of.

CHAPTER 2
LITERATURE REVIEW

2.1 NEURODEGENERATIVE DISORDERS

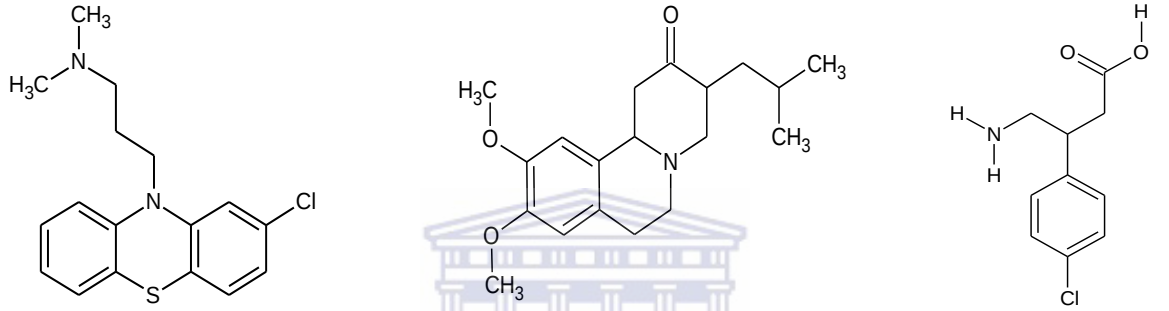
Huntington's, Alzheimer's and Parkinson's disease are progressive neurodegenerative disorders that reduce the quality of life of patients who suffer from these conditions and it is growing into a serious problem for the world's ageing population (Rang 2007). A lot of time and effort have been put into identifying the markers and causes of these disorders. Neurodegeneration is the process in which neurons in the central nervous system (CNS), and the brain in particular, are damaged by several mechanisms (Araki et al. 2001). Progressive and irreversible loss of neurons from specific regions of the brain characterizes these disorders (Alexi et al. 2000).

Not a single cause or process alone can lead to neurodegenerative disorders; instead it is the result of several factors which most likely include a variety of mechanistic pathways (Dauer, Przedborski 2003). It is postulated that genetic, environmental and endogenous factors are probably involved in neurodegenerative disorders such as, Alzheimer's disease (AD) and Parkinson's disease (PD) and a number of general pathways have been discovered that may be among the factors which are involved in different pathogenic cascades. These include excitotoxicity, oxidative stress and free radical formation, protein misfolding and aggregation, phosphorylation impairment, metal dyshomeostasis and mitochondrial dysfunction. Some of these seem to happen simultaneously, resulting in the death of essential neuronal cells (Jellinger 2003a).

2.2 HUNTINGTON'S DISEASE

Huntington's disease (HD) is an inherited autosomal dominant disorder which reveals itself in adulthood with symptoms such as clumsiness, temperament and personality changes (Kremer et al. 1994) leading to progressive brain degeneration and consequently sharp mental deterioration and death (Voisine et al. 2007). HD is the most common disorder among a group of so-called trinucleotide repeat neurodegenerative diseases which is characterized by the expansion of the number of repeats of the CAG sequence in specific genes and subsequently the number, 40 or more, of consecutive glutamine residues at the *N*-terminal of the expressed protein. (Voisine et al. 2007). The protein coded by the HD gene, huntingtin, which normally contains a chain of less than 30 glutamine residues, is a soluble cytosolic protein with unknown function found in all cells.

When the mutant protein contains 40 or more repeats, HD starts to develop. The expanded polyglutamine changes protein folding resulting in the generation of aggregates in neurons which appear to be essential for the neurodegenerative process (Goldberg et al. 1996, Bano et al. 2011). There is no cure for HD and the treatment is only supportive. Dopamine receptor antagonists, such as chlorpromazine, can reduce the involuntary movements and ease the motor symptoms (Figure 2.1). Tetrabenazine, an inhibitor of the vesicular monoamine transporter, decreases dopamine storage and is used to treat HD symptomatically. The GABA agonist baclofen is also another option for the symptomatic treatment of HD. These drugs are not effective against dementia and do not delay the progress of the disease. (Rang et al. 2014).



Chlorpromazine Tetrabenazine Baclofen

Figure 2.1: The structures of symptomatic therapeutic options for HD.

2.3 ALZHEIMER'S DISEASE

Degeneration of cognitive ability due to ageing is a normal process whose rate and extent fluctuates from one person to another. Alzheimer's disease (AD) was originally described as *presenile dementia,* but now it seems that the same pathology is involved in the dementia regardless of the age of onset. AD is a progressive, degenerative disease of the brain and its presence becomes more prevalent with age, it refers to dementia that does not have a previous cause, such as brain trauma, stroke or alcohol abuse. Until recently dementia caused by aging was thought to be the result of the constant loss of neurons that normally continues throughout life and this process is probably hastened by a failure in blood supply connected to atherosclerosis. However, studies have shown that certain genetic and molecular mechanisms underlie AD (Selkoe 1997, Bossy-Wetzel, Schwarzenbacher & Lipton 2004, Yamada, Nabeshima 2000). AD is accompanied by localized loss of neurons and shrinkage of brain, mainly in the hippocampus and basal forebrain. The loss of cholinergic neurons in the frontal cortex and hippocampus is a characteristic hallmark of the disease and is believed to underline the cognitive inability and loss of short-term memory that takes place in AD. Decoding the mechanism of

neurodegeneration in AD has yet to lead to therapies able to delay the degenerative process. Presently, the *N*-methyl-D-aspartate receptor antagonist, memantine, and cholinesterase inhibitors such as donepezil, tacrine and rivastigmine are the only drugs approved for treatment of the symptoms accompanying AD (Citron 2004).

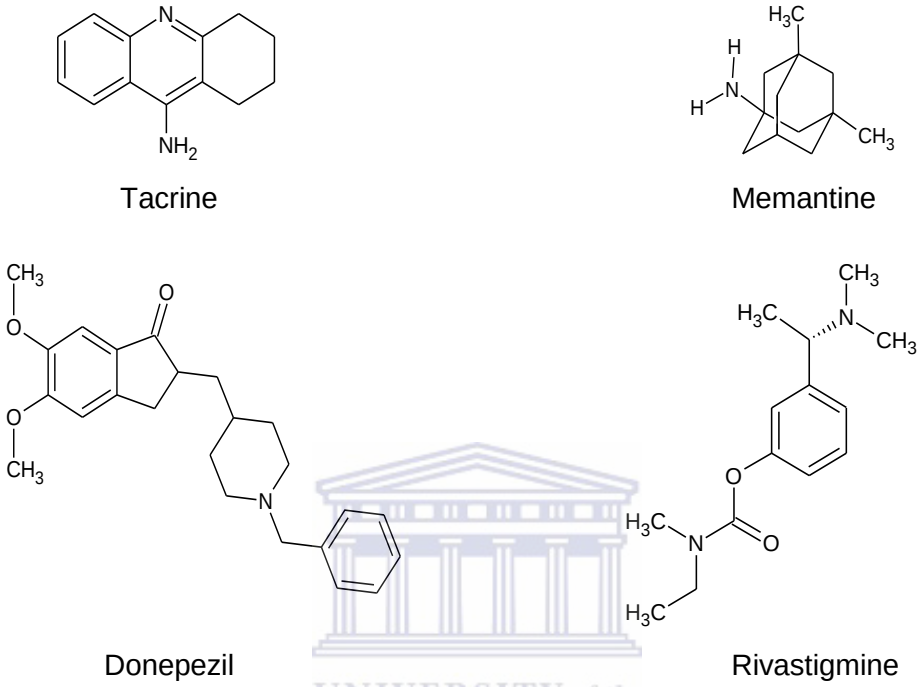


Figure 2.2: The structures of symptomatic therapeutic options for AD.

2.4 PARKINSON'S DISEASE

PD is the second most common neurodegenerative disorders after AD (de Lau, Breteler 2006) and affects nearly 0.3% of the general population. The disorder is clinically characterized by a classic triad of signs; including resting tremor, bradykinesia and rigidity (Foye, Lemke & Williams 2008). It is identified by a specific loss of dopaminergic neurons in the substantia nigra region of the brain (Favaloro et al. 2012a) resulting in decreased levels of dopamine in the nerve terminals of the striatum in the forebrain (Foye, Lemke & Williams 2008). PD is generally accompanied with dementia, depression and autonomic dysfunction. These clinical features of PD arises because the degenerative process probably is not only limited to the basal ganglia but also influences other parts of the brain. The non-motor symptoms often predominate in the later stages of the disease (Rang et al. 2014).

PD disease is believed to be caused mainly by a combination of genetic and environmental factors, but genetic predisposition, predominantly in early-onset PD, has been increasingly seen

as the main cause (Schapira, Jenner 2011, Warner, Schapira 2003). Like other neurodegenerative disorders, the damage is inflicted by protein misfolding and aggregation, while being assisted by other factors namely mitochondrial dysfunction, inflammation and apoptosis, oxidative stress and excitotoxicity. (Lotharius, Brundin 2002, Schapira 2008).

It often takes place with no clear cause underlying it, but it may be due to viral encephalitis, cerebral ischaemia or other types of pathological damage. The symptoms can also be attributed to therapeutic drug use, the main drugs involved are those which decrease the amount of dopamine in the brain (e.g. reserpine) or the ones blocking dopamine receptors (e.g. antipsychotic drugs such as chlorpromazine). There are few cases of familial early-onset PD, and several gene mutations have been associated, such as those encoding *synuclein* and *parkin*. The study of these gene mutations has provided some hints into the mechanism which underlies the neurodegenerative process (Rang et al. 2014).

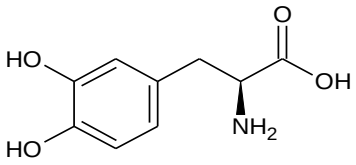
Since current treatments can neither stop the progression of the neurodegenerative process nor reverse it, there is no certain cure for PD. However, the symptoms can be brought under control with several types of drugs. The drugs used for treatment of PD can either increase the levels of DA in the brain or imitate the effects presented by DA (Singh, Pillay & Choonara 2007b). The present treatments try to improve the patients' functional capability for as long as possible. The side effects of these agents make limited options available for patients with pre-existing cardiovascular complications. Adverse effects such as orthostatic hypotension put the patients at the risk of bone fractures (Mukai, Lipsitz 2002). Therefore, newer and more effective agents are needed and subject to intense and comprehensive research. Significant research has also concentrated on developing enhanced systems for the delivery of existing treatments and agents able to halt or reverse the neurodegenerative process.

2.4.1 Current drugs used for treatment of PD

2.4.1.1 Levodopa

This is the main compound for treating PD, used as a precursor of dopamine. When levodopa is taken orally it is rapidly decarboxylated and only a small amount of the dose reaches the CNS without being changed. Therefore, a large dose is needed for the optimum effect which causes nausea and vomiting in patients. In order to tackle this problem levodopa and a peripheral dopa-decarboxylase inhibitor such as benserazide or carbidopa which cannot penetrate the blood-brain barrier are administered in combination (Aminoff 2007). Cardiac arrhythmias is a minor side effect for patients who suffer from heart disease. Administration of levodopa has also been attributed to

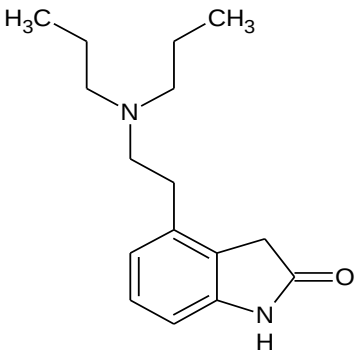
insomnia, depression, agitation and anxiety (Singh, Pillay & Choonara 2007b). Long term levodopa therapy is also characterized by several negative motor effects that restrict its use including wearing off, the 'on–off' phenomena, dose failure, akinesia and dyskinesias (Olanow et al. 2004, Jankovic 2005).



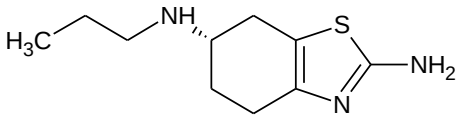
Levodopa

2.4.1.2 Dopamine receptor agonists

Dopamine receptor agonists can be classified into two categories; the first class is the ergot derivatives such as lisuride, pergolide, bromocriptine, cabergoline and the second class is non-ergot derivatives such as pramipexole, ropinirole, piribedil or apomorphine. They can be used alone to retard the need for levodopa, or may be used with levodopa to raise their efficacy (Schapira 2002b, Schapira 2002a, Schapira, Olanow 2003, Schapira 2003). The ergot derivatives have the potential to create psychiatric disturbances and cardiovascular disorders that can develop into myocardial infarctions and following death. Patients even at lower doses of these agents experience dyskinesias, constipation, orthostatic hypotension, confusion and insomnia (Katzung 2001a, Wong et al. 2003).



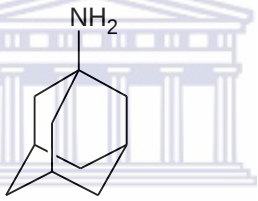
Ropinirole



Pramipexole

2.4.1.3 Amantidine

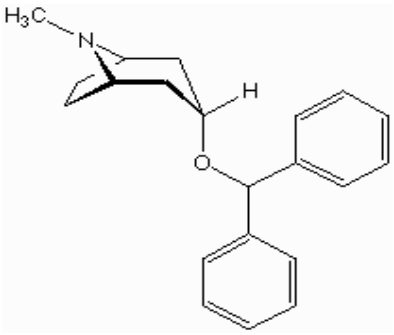
It was originally administered as an antiviral for treating influenza, but accidently discovered to improve the symptoms of PD. Amantadine is found to act as a non-competitive antagonist at the phencyclidine (PCP) site in the NMDA-receptor at therapeutic doses (Danysz et al. 1997). Amantadine is also known to increase the release of dopamine from nerve terminals and retard its re-uptake (TAKAHASHI et al. 1996, Mizoguchi et al. 1994). Early clinical experiments illustrated the antiparkinsonian activity of amantadine either alone or as an adjuvant with levodopa (Fahn, Isgreen 1975, Butzer, Silver & Sahs 1975). Unfortunately, all patients do not respond to amantadine and tolerance to its effects can occur, although it has been proposed that by combining amantadine with levodopa the tolerance is less pronounced (Zeldowicz, Hubermann 1973). It can potentially have worsening effects on cardiovascular disease and even cause seizures in vulnerable patients. Depression, restlessness, confusion and hallucinations are considered some of its CNS effects (Singh, Pillay & Choonara 2007a).



Amantadine

2.4.1.4 Anticholinergics

Anticholinergics such as benztropine or trihexyphenidyl are especially effective for controlling tremor. In a recent review by the Cochrane database it is mentioned that anticholinergics do not potentially have better effects on tremor than on other outcome measures (Katzenschlager et al. 2002). In addition, these agents do not have much effect on decreasing bradykinesia or akinesia (Comella, Tanner 1995). Anticholinergic dosing is also often limited because of their side effects, such as drowsiness, agitation, confusion and hallucinations (Katzung 2001b, de Smet et al. 1982).



Benztropine

2.5 THE ROLE OF APOPTOSIS IN NEURODEGENERATION

Apoptosis or programmed cell death is a complicated constant mechanism to dispose of surplus, potentially dangerous and damaged cells resulting in apoptotic bodies which get cleared without activation of the immune system (Hengartner 2000). Apoptosis in physiological cellular processes such as control of size and shape of tissue, fetal development, maintenance of the immune system, and cell homeostasis, plays a key role. Dysregulation of apoptosis plays a vital role in a vast variety of diseases, such as cancer, atherosclerosis, neurodegenerative and developmental deficiencies, autoimmune and cardiovascular diseases. (Limpachayaporn et al. 2013b)

Apoptotic cell death is generally characterized by several subcellular alterations winding up in a specific cell death program (Martins, Earnshaw 1997). Three apoptotic stages have been identified namely the initiation, effector and degradation stage. In the first stage, the cell encounters the death stimulus which may be given by exogenous physical, chemical or biological agents or by a shortage of intracellular survival factors. In the next effector phase a number of reactions cause cell death triggering. The degradation phase comes after this regulatory step which is irreversible and associated with typical morphological and histochemical markers of apoptosis such as DNA fragmentation (Kroemer et al. 1995). It has recently been suggested that mitochondrial membrane potential is likely to play a vital role in the early events of the apoptotic cascade (Kroemer, Zamzami & Susin 1997)

Regarding PD, the apoptotic process appears most probably as an end-stage process (Anglade et al. 1997). For instance, signs of apoptosis, upregulation of the antiapoptotic protein Bcl-2 (Marshall et al. 1997) and augmented levels of the proapoptotic protein Bax (Hartmann et al. 2001) have been seen in the substantia nigra of PD patients. In addition, caspase 3 and caspase 8 may act as effectors of apoptotic cell death in the brain of patients affected with PD (Mogi et al. 2000, Hartmann et al. 2001). Monoamine oxidase B has also been connected with apoptotic cell

death through generating toxic by-products in the aged parkinsonian brain (Kalaria, Mitchell & Harik 1988a, Nicotra et al. 2004, Di Monte et al. 1996a, Fowler et al. 1997).

2.6 MONOAMINE OXIDASES AND ITS ROLE IN APOPTOSIS

Although the activity of monoamine oxidase (MAO) exists in most mammalian tissues, the two isoforms, MAO-A and MAO-B, are presented in a tissue-specific manner. MAO-B is the main form found in human liver tissue (Inoue et al. 1999) whereas MAO-A is the major form in human placental (Weyler, Salach 1985) and intestinal tissues (Saura et al. 1996). Both isoforms exist in the human brain, but they are distributed differently (Westlund et al. 1985, Thorpe et al. 1987). MAO-B exists in larger concentrations in both human and subhuman primate brains, and demonstrates a higher degree of activity (Fowler et al. 1980, Riachi, Harik 1992). It is particularly important to observe that MAO-B is the dominant isoform in the basal ganglia of the human brain (Kalaria, Mitchell & Harik 1988a, Collins et al. 1970a) and that the activity and concentration of MAO-B increases with age in most brain regions (Kalaria, Mitchell & Harik 1988a, Nicotra et al. 2004, Fowler et al. 1997). Since MAO-B lies in the glial cells, this heightened activity may be due to glial cell proliferation, a process related to increasing age (Westlund et al. 1985, Levitt, Pintar & Breakefield 1982, Fowler et al. 2002). On the other hand, MAO-A activity undergoes no changes as age increases (Fowler et al. 1980). Neurotransmitter amines are catabolized by MAO-A and –B therefore they have been targeted for the treatment of neuropsychiatric and neurodegenerative diseases (Youdim, Edmondson & Tipton 2006a, Youdim, Weinstock 2004, Riederer, Lachenmayer & Laux 2004).

MAO-A metabolises both serotonin and norepinephrine, two monoamines implicated in depressive illness and anxiety disorders (Youdim, Edmondson & Tipton 2006b, ZISOOK, BRAFF & CLICK 1985) and the antidepressive effect of MAO-A inhibitors is associated with blocking the central breakdown of these monoamines extending their action. Since a significant proportion of patients with PD exhibit signs of depression, the inhibition of MAO-A in these patients may be beneficial (Di Monte et al. 1996b). Besides offering a potential antidepressant action in PD, MAO-A inhibitors may also provide a symptomatic benefit by reducing MAO-A-catalysed oxidation of dopamine (Youdim, Edmondson & Tipton 2006b, Di Monte et al. 1996b). Although MAO-B is present in higher concentrations than MAO-A in the human basal ganglia (Kalaria, Mitchell & Harik 1988b, Collins et al. 1970b), MAO-A inhibitors have also been shown to enhance dopamine level in this region. For example, clorgyline, a selective irreversible inhibitor of MAO-A, elevates dopamine levels in the striatum of primates treated with levodopa to a similar degree than the

elevation obtained with selegiline, a selective irreversible inhibitor of MAO-B (Shoulson et al. 2002a). In order to conserve dopamine in the basal ganglia, mixed MAO-A/B inhibitors may therefore be more efficacious than selective inhibitors (Youdim, Bakhle 2006a).

MAO-B inhibitors are used to treat the symptoms of PD (Fernandez, Chen 2007). The effectiveness of MAO-B inhibitors against PD is believed to depend on blocking the MAO-B-catalysed metabolism of dopamine in the basal ganglia. This preserves exhaustion of dopamine stores and extends the physiological action of dopamine. MAO-B inhibitors also may boost dopamine levels acquired from levodopa, the metabolic precursor of dopamine. For example it has been observed that selegiline, an inhibitor of MAO-B, increases the elevation of dopamine levels in the striatum of primates after levodopa therapy (Di Monte et al. 1996a, Chen, Swope 2005, Finberg et al. 1998). This elevation is characterized by a decline in the oxidative metabolism of dopamine (Di Monte et al. 1996a). MAO-B inhibitors are thus suggested as adjuvant treatment for levodopa in PD (Fernandez, Chen 2007) and in the early phases of PD, combination therapy with MAO-B inhibitors may allow decreasing the doses of levodopa and dopamine agonists administered. Moreover, MAO-B inhibitors may also retard the rise of disabilities requiring the initiation of levodopa treatment (Shoulson et al. 2002b, Palhagen et al. 1998). MAO-B inhibitors modulate central β-phenylethylamine levels and this may also contribute to their beneficial effects in PD. Since β-phenylethylamine is both an inhibitor of active dopamine uptake and also a releaser of dopamine (Finberg, Lamensdorf & Armoni 2000), blocking its metabolism leads to an escalation in striatal extracellular dopamine levels. The central levels of β-phenylethylamine, usually only in trace amounts in the CNS, may be enhanced several thousand times by the administration of MAO-B inhibitors.

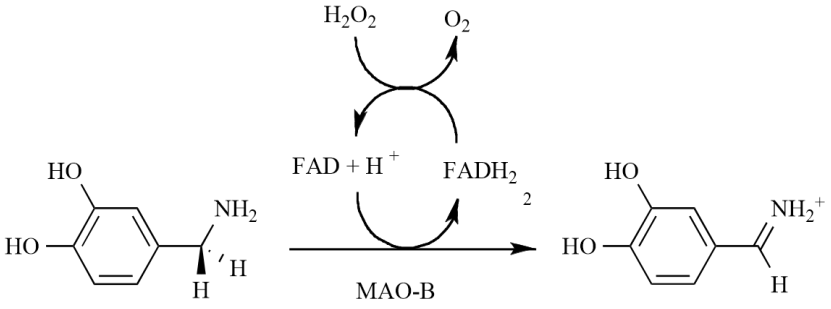


Figure 2.3: MAO-B oxidises dopamine and simultaneously reduction of the FAD cofactor of MAO takes place. Reoxidation of the FAD through reaction with O_2 and ultimately formation of H_2O_2 (Joubert et al. 2014a).

MAO-B inhibitors are administered clinically mainly to treat PD symptomatically, but they may also protect against the neurodegenerative processes of this disease. The mechanism is believed to block the formation of dopanal and H_2O_2, the byproducts of the metabolism of dopamine by MAO-B (Gesi et al. 2001, Lamensdorf et al. 2000). The hydrolysis of the iminiumyl intermediate produces an aldehyde product in the catalytic cycle of MAO-B, whereas reoxidation of the FAD cofactor necessitates the reduction of O_2 to H_2O_2 (Figure 2.3). For each mole of monoamine substrate oxidized one mole of these species is produced. Under certain conditions these by-products are neurotoxic and may speed up the neurodegenerative process. As an example, dopanal has been connected with the aggregation of α-synuclein, a process involved in the pathological development of PD (Burke et al. 2008). Moreover, the reaction of aldehydic products, including dopanal, with *N*-terminal and lysine ε-amino groups of proteins and exocyclic amino groups of nucleosides may occur (Youdim, Bakhle 2006b). Generally aldehydes are quickly deactivated by the enzyme aldehyde dehydrogenase (ADH) (Gesi et al. 2001, Marchitti, Deitrich & Vasiliou 2007), however in the substantia nigra of patients suffering from PD, the expression of ADH maybe decreased. This implies that reduced levels of ADH in the CNS may bring about the buildup of aldehydic species resulting from the action of MAO-B (Fowler et al. 1997, Grünblatt et al. 2004). H_2O_2, consequently, may cause oxidative damage by reacting with ferrous ion in the Fenton reaction to produce the extremely reactive hydroxyl radical (Youdim, Bakhle 2006b). The hydroxyl radical inflicts a lot of damage almost on all types of biomolecules such as proteins, lipids, DNA, amino acids and carbohydrates. This reaction may be particularly relevant with this knowledge that the iron content of the brain increases with aging. Glutathione peroxidase generally inactivates H_2O_2 in the brain. In the brain affected by PD, the amount of glutathione acting as an electron donor for reduction of H_2O_2 by glutathione peroxidase, may be decreased (Riederer et al. 1989). The other mechanism in which H_2O_2 may aid neurodegeneration is by enhancing apoptotic signaling events (Grünblatt et al. 2004, Mallajosyula et al. 2008). Since the activity of MAO-B in the CNS rises with age, inhibition of formation of toxic by-products derived from the MAO-B catalysis in the aged brain affected by PD is particularly significant (Kalaria, Mitchell & Harik 1988a, Nicotra et al. 2004, Di Monte et al. 1996a, Fowler et al. 1997). Therefore, MAO-B inhibitors may bring about a neuroprotective effect by stoichiometrically decreasing MAO-B catalysed aldehyde and H_2O_2 production.

Selegiline and rasagiline are approved therapeutically for PD worldwide, they can be administered as adjunct to levodopa or as independent therapy. They are both mechanism-based inhibitors and inhibit MAO-B irreversibly (Fernandez, Chen 2007). Lazabemide, a reversible MAO-B

inhibitor, also appears to delay the requirement for levodopa in untreated patients in the early phase of PD (Kieburtz et al. 1996). The benefits presented by lazabemide bore resemblance to those observed after 1 year of selegiline therapy (Palhagen et al. 2006, Kieburtz et al. 1996). Phase III trials have demonstrated that safinamide, also a reversible MOA-B inhibitor, improves motor scores significantly when administered along with dopamine agonist drugs (Stocchi et al. 2006).

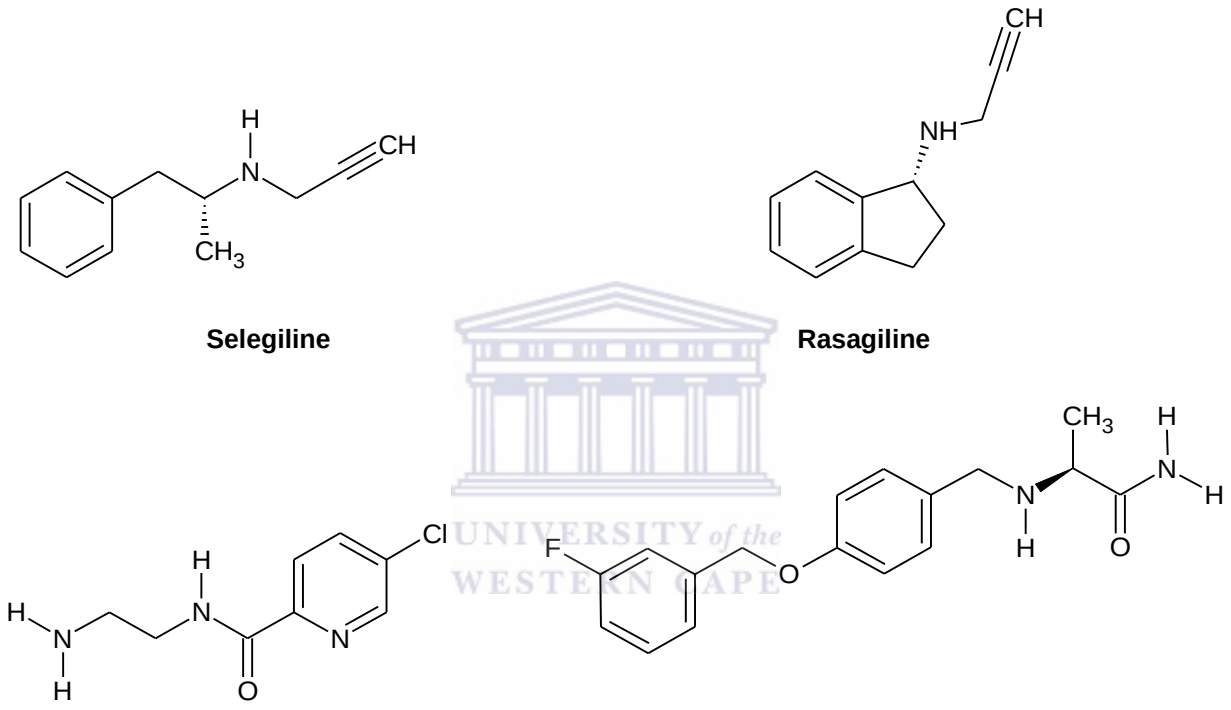


Figure 2.4: The structure of some MAO inhibitors.

2.7 CASPASES AND THE MODULATION OF APOPTOSIS

Caspases are a set of intracellular enzymes strictly controlling the apoptotic pathway (cysteinyl aspartate-specific proteinases). Over a dozen caspases have been discovered so far and identified as initiating (e.g. caspases 8, 9 and 10) and executing caspases (e.g. caspases 3, 6 and 7). Basically two biological pathways are known for the initiation of apoptosis, either by a particular protein which binds to the death receptor (death receptor pathway) or by damaging DNA and subsequently mitochondrial induction of the cell death program (mitochondrial pathway, Figure 2.5). The apoptotic process is driven by signals from both paths cumulating in the activation of executing caspases (Limpachayaporn et al. 2013b). Activation of caspase 3 has been observed

in all kinds of apoptosis (Porter, Jänicke 1999) including neurodegenerative apoptotic processes involved in disorders such as PD, AD, and HD (O'Brien, Lee 2004). Numerous proteins involved in cell maintenance and/or repair are natural substrates of caspase 3 (Nicholson, Thornberry 1997). Caspase 3 is the main caspase participating in the cleavage of amyloid-precursor protein (APP) alongside its distinct increase in neuronal cell death of AD brains and colocalization of the product of its APP cleavage with amyloid-ß present in senile plaques (Albrecht et al. 2007). Polyglutamine causes HD by expanding the *N-terminus* of protein huntingtin, which is a caspase substrate (Chu et al. 2009).

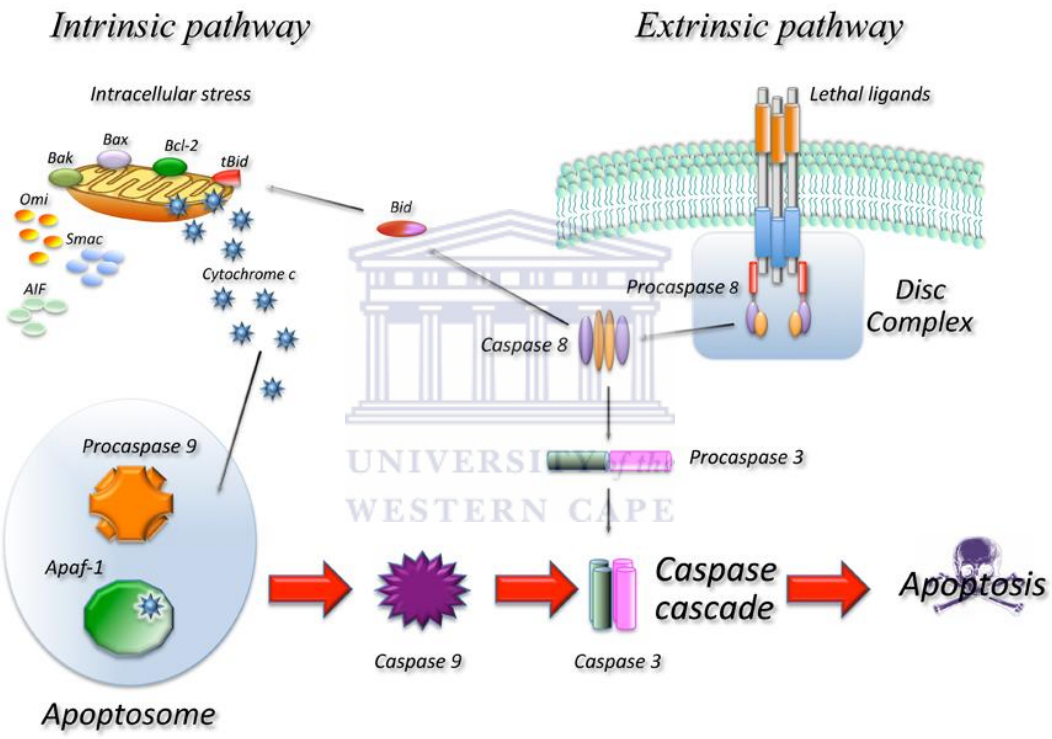
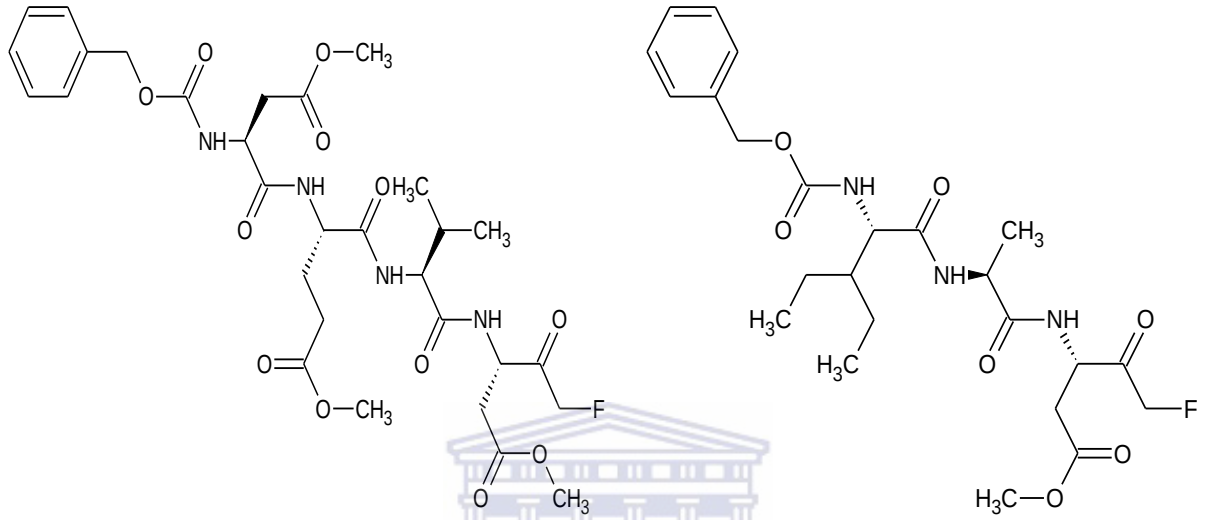


Figure 2.5: Diagram of the dominant molecular pathways resulting in apoptosis. In the extrinsic pathway upon ligand binding to specific receptors the formation of DISC complex and activation of caspase 8 take place. Cyt c is released from the mitochondria in the intrinsic pathway resulting in the formation of the apoptosome and consequently caspase 9 activation. The activation of downstream caspases such as caspase 3 by caspase 8 and 9 causes cell death (Favaloro et al. 2012b).

To execute the programmed type I cell death (apoptosis) caspases 3 and 7 are crucial (Limpachayaporn et al. 2013a). A significant structural characteristic of nearly all known caspase inhibitors is an electrophilic group that can make a reversible or irreversible bond with the active

site cysteine making the enzyme inactive (Lee et al. 2001). Synthetic peptide inhibitors including Z-DEVD-fmk and Z-VAD-fmk have been broadly applied to block type I cell death. (Lopez-Hernandez et al. 2003).



Z-DEVD-fmk, IC_{50} = 18 µM

Z-VAD-fmk, IC_{50} = 40 µM

Figure 2.6: The structures of two peptide-based caspase inhibitors

The involvement of caspases in the process of cell death is more regularly characterized in vitro by measuring the activity of the enzyme and evaluation of the inhibitors' effects in tissue extracts and cell lysates. Cell-based studies have been done by using irreversible peptide inhibitors, peptide inhibitors having limited cell permeability and pro-drug peptide inhibitors. To assess the importance of a specific caspase in apoptosis is difficult, due to the moderate caspase selectivities associated with these reagents and this often makes the analysis of results ambiguous (Cryns, Yuan 1998, Schotte et al. 1999).

More recently the identification of selective, non-peptidic caspase inhibitors has been a center of attention (Deckwerth et al. 2001, Han et al. 2002). Numerous research groups have reported their attempt toward small molecule selective caspase 3 inhibitors (Lee et al. 2001, Abbavaram, Reddyvari 2013). Having a selective inhibitor of caspase 3 gives the opportunity to evaluate the inhibition of apoptosis potentially at the level of an *executioner* caspase (Porter, Jänicke 1999).

Screening methods were used to discover isatin sulfonamides and these molecules were introduced as potent, non-peptide, selective inhibitors of caspases 3 and 7 (Figure 7 and 8).

An x-ray co-crystal structure was solved to 2.8 Å resolution to identify the molecular interaction of the complex between recombinant human caspase 3 and an isatin sulfonamide inhibitor (Figure 2.7). In contrast to previously studied peptide-based caspase inhibitors, the selectivity of isatin sulfonamides for caspases 3 and 7 results primarily from interacting with the S_2 subsite, they also do not bind in primary aspartic acid binding pocket (S_1) of the caspase. The cysteine of enzyme covalently binds at the 3-carbonyl group of isatin leading to formation of a tetrahedral thiohemiacetal intermediate (Lee et al. 2000a).

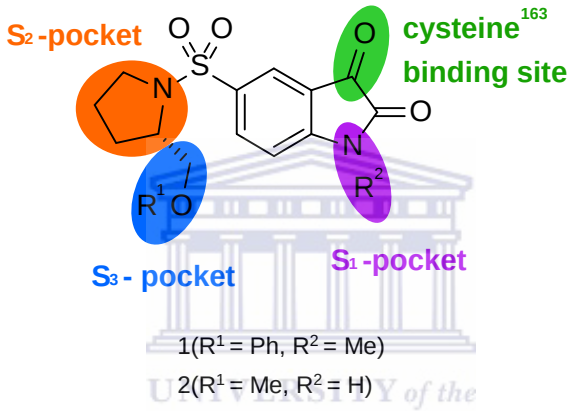
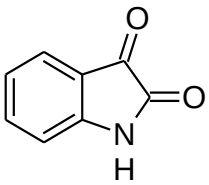
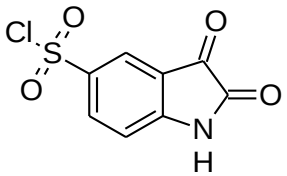


Figure 2.7: Designated interactions of general structure of isatin sulfonamides with caspase 3 and 7 enzyme's sub-pockets (Limpachayaporn et al. 2013a).

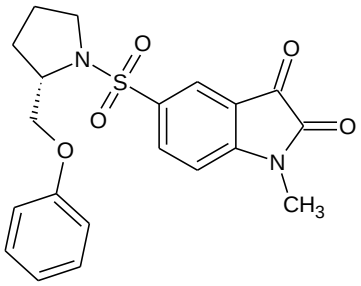
These isatin derived compounds have been shown to reduce tissue damage in an isolated rabbit heart model of ischemia injury and also to block apoptosis in murine bone marrow neutrophils and human chondrocytes (Lee et al. 2000a, Chapman et al. 2002). Knowing that caspase 3 is specifically involved in apoptosis, this group of compounds (represented by compounds A – D, Figure 2.8) presenting with higher cell permeability, metabolic stability and their capability to differentiate apoptosis from necrosis may make them superior compared to peptide-based inhibitors (Lee et al. 2000a).



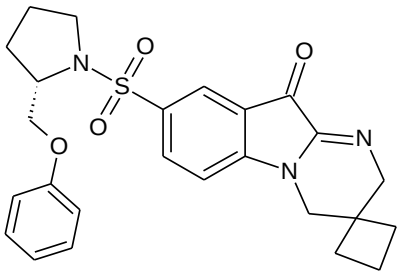
Isatin



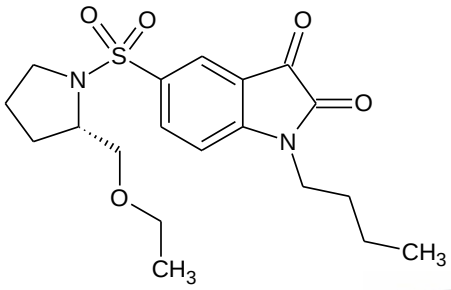
5-chlorosulfonyl isatin



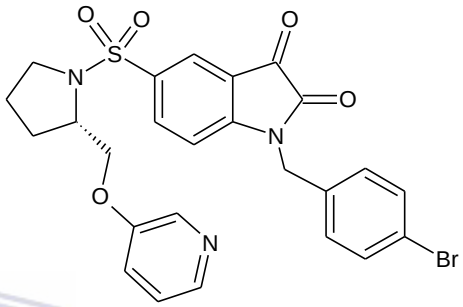
Compound A, IC_{50} = 15 nM (Lee et al. 2000b)



Compound B, IC_{50} = 1.29 nM (Havran et al. 2009)



Compound C, IC_{50} = 16.9 nM (Limpachayaporn et al. 2013a)

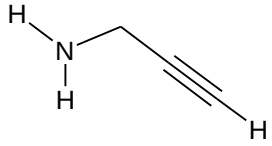


Compound D, IC_{50} = 3.6 ± 0.5 nM (Chu et al. 2007)

Figure 2.8: The structure of isatin and the isatin derived caspase 3 inhibitors.

2.8 ANTI-APOPTOTIC ACTIVITY AND THE PROPARGYLAMINE MOIETY

Anti-apoptotic pathways are activated by compounds containing the propargylamine function and this gives the opportunity to have a single compound combining an anti-apoptotic effect with a MAO inhibitory effect (Maruyama et al. 2002, Blandini 2005), selegiline and rasagiline are examples of such compounds (see section 2.6). The mechanism-based inhibition demonstrated by these compounds is derived from their propargylamine moiety and, after being oxidized by MAO, this moiety binds covalently to the FAD cofactor of MAO-B to produce a flavocyanine adduct (Edmondson et al. 2003, Binda et al. 2005). The propargylamine moiety is also believed to be responsible for the anti-apoptotic effects of this group of compounds. The mechanism of action by which these compounds show their anti-apoptotic effect is still not clearly defined.



N-propargylamine

As an example, rasagiline was shown to provide protection against apoptotic cell death in neuroblastoma caused by the pro-apoptotic toxin *N*-methyl-R-salsolinol (Maruyama et al. 2000). It is thought that rasagiline exerts this effect, while downregulating pro-apoptotic proteins such as Bax and Bad, by inducing the expression anti-apoptotic proteins such as Bcl-2 and Bcl-xL (Blandini 2005). This results in the stability of the mitochondrial permeability transition pore, consequent reduction in inflammation of the mitochondria, also the decline in mitochondrial membrane potential as well as the released cytochrome c (Lemasters et al. 1998).

The mechanism of anti-apoptotic activity of propargylamines includes several stages of the apoptotic pathway and also involves the reduction in caspase 3 activation and polymerase-1 and the inhibition of the nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (Blandini 2005, Maruyama et al. 2000, Bar-Am et al. 2004, Youdim, Weinstock 2001). Propargylamines such as rasagiline might perform their neuroprotective activities through the activation of pathways of protein kinase C (PKC)-dependent and mitogen-activated protein kinase as well as by inducing glial-derived neurotrophic factor and the expression of brain-derived neurotrophic factor ((Youdim, Edmondson & Tipton 2006c, Mandel et al. 2005). It is necessary to bear in mind that propargylamine containing compounds do not exert their anti-apoptotic effects by inhibition of MAO, because even the propargylamines not exhibiting MAO inhibitory activity, such as *N*-propargylamine and the S enantiomer of rasagiline, also show these effects (Binda et al. 2005, Youdim et al. 2001). Therefore, the anti-apoptotic properties of this group of compounds appear to be an intrinsic characteristic of the propargylamine moiety (Joubert et al. 2014b).

2.9 MULTI-TARGET-DIRECTED LIGANDS

So far there has not been any treatment proven to stop cell death or bring back damaged neurons to a state of normality (Joubert et al. 2011). Disease-modifying approaches have turned into an area of focus in research since these strategies interrupt the initial pathologic events associated with the onset of the disease; hindering the neurotoxic cascade in the case of neurodegenerative diseases is an example of these approaches (McLean et al. 1999, Näslund et al. 2000). Bearing this in mind, it is evident that the pattern of targeting a single disease factor might not be an efficacious strategy for treatment of neurodegenerative diseases.

So far in clinical practice, multiple targeting has proceeded through the polypharmaceutical approach by using a combination of therapeutic agents functioning on different etiological targets independently. This approach has been proved to be effective in treatment of diseases with similar complexity such as hypertension, cancer and HIV (Jellinger 2003b, Smid et al. 2005). It has been

proposed that treatment for neurodegenerative diseases will be a blend of drugs having different mechanisms of action while acting on more than one related neurotherapeutic target (Van der Schyf, Geldenhuys & Youdim 2006). Treatment with combination preparations is, however, a challenging and complex field for drug development due to the likelihood of drug–drug interactions and further complications met while combining drug entities that have potentially various degrees of bioavailability, metabolism, pharmacokinetic profiles and toxicity (Smid et al. 2005). The design of single drug molecules for a particular disease acting on multiple specific etiological targets can be worthwhile especially in neurodegenerative diseases. Numerous studies have reported the possibility of having a single drug which acts at several sites in the neurotoxic cascade (Smid et al. 2005, Morphy, Rankovic 2005).

This strategy is accompanied with advantages including a lower probability of experiencing unwanted side effects, the likelihood to 'design out' any side-effects and having pharmacokinetic considerations simplified (Van der Schyf, Geldenhuys & Youdim 2006). There has been a shift from single to multi-target-directed ligands resulting from the fact that a single compound might be capable of hitting multiple targets (Van der Schyf, Cornelis J, Geldenhuys & Youdim 2006, Cavalli et al. 2008). Taking the complex nature of neurodegenerative disorders into consideration, the multi-target directed ligands may present as a better option to treat neurodegenerative diseases than the classic 'one molecule, one target' approach (Cavalli et al. 2008, Marlatt et al. 2005).The requirements for structural activity of an agent at a particular target might become very rigorous and structural modification to improve activity at another related target repeatedly may result in a pharmacological loss of the original activity, therefore this makes the design of multifunctional agents a major challenge (Van der Schyf, Geldenhuys & Youdim 2006, Cavalli et al. 2008).

2.10 CONCLUDING REMARKS

Design and synthesis of multifunctional drugs which would halt the apoptotic neuronal breakdown process and eliminate the signs and symptoms of diseases such as AD and PD may be an effective therapeutic option. This can happen by: (a) Inhibiting the MAO-A and/or MAO-B enzymes thus allowing increase in dopamine levels in the CNS and reducing the levels of the highly oxidative products produced by the activity of this enzyme, and (b) inhibiting apoptotic processes to halt neuronal cell death. With this in mind, the propargylamine portion of selegiline and isatin can be used as pharmacophoric groups to design potent and selective non-peptide inhibitors of caspases-3 with MAO-A and/or MAO-B inhibitory properties. The objective of this project is to

design drug-like molecules that comprise of features of both selegiline and isatin, and in so doing develop a potential multifunctional single agent to treat neurodegenerative disorders.

CHAPTER 3
SYNTHETIC PROCEDURES

3.1. STANDARD EXPERIMENTAL PROCEDURE

3.1.1. Instrumentation

Nuclear magnetic resonance spectroscopy (NMR): ^{1}H and ^{13}C NMR spectra were determined using Bruker Avance III HD spectrometer at a frequency of 400 MHz and 100 MHz, respectively. Tetramethylsilane (TMS) was used as internal standard. All chemical shifts are reported in parts per million (ppm) relative to the signal from TMS ($\delta = 0$) added to an appropriate deuterated solvent. The following abbreviations are used to describe the multiplicity of the respective signals: s - singulet, bs - broad singulet, d - doublet, dd - doublet of doublets, t - triplet, q - quartet and m - multiplet. Spectra of selected compounds are included in annexure A.

Infrared spectroscopy (IR): The IR spectra were recorded on a Perkin Elmer Spectrum 400 spectrometer, fitted with a diamond attenuated total reflectance (ATR) attachment. Relevant spectra are included in annexure A.

Mass spectroscopy (MS): The MS spectra were recorded on a Perkin Elmer Flexar SQ 300 mass spectrometer by means of direct injection with a syringe pump. Relevant spectra are included in annexure A.

Melting point determination (MP): Melting points were determined using a Stuart SMP-10 melting point apparatus and capillary tubes. The melting points are uncorrected.

Microwave reactor: Microwave synthetic procedures were performed utilising a CEM Discover™ focused closed vessel reactor.

3.1.2. Chromatographic techniques

Thin layer chromatography (TLC): TLC was performed on 0.20 mm thick aluminium silica gel sheets (Alugram® SIL G/UV254, Kieselgel 60, Macherey-Nagel, Düren, Germany). Visualisation was achieved using UV light (254 nm and 366 nm), an ethanol solution of ninhydrin or iodine vapours, with mobile phases prepared on a volume-to-volume basis. Chromatographic purifications were performed by flash chromatography using silica gel (pore size 60 Å, 230-400 mesh particle size, 40-63 µm particle size) purchased from Sigma-Aldrich® as the stationary phase and appropriate mobile phases.

3.1.3. Materials

Unless otherwise stated, all reagents were obtained from Sigma Aldrich and were used without further purification.

3.2. SYNTHESIS OF SELECTED MOLECULES

The general synthesis reaction is shown below in figure 3.1. The preparation of 5-chlorosulfonyl isatin was accomplished starting from the commercially available isatin in two steps, that is, sulfonation using tetramethylene sulfone and chlorination with $POCl_3$ (Figure 3.1, step 1). 5-Chlorosulfonyl isatin was added to the aromatic amine with the fluoro-function in either the ortho, meta or para position through a nucleophilic substitution reaction. The resultant compounds were coupled with propargyl bromide, using a microwave synthesis procedure, in a nucleophilic substitution reaction (Figure 3.1, step 2).

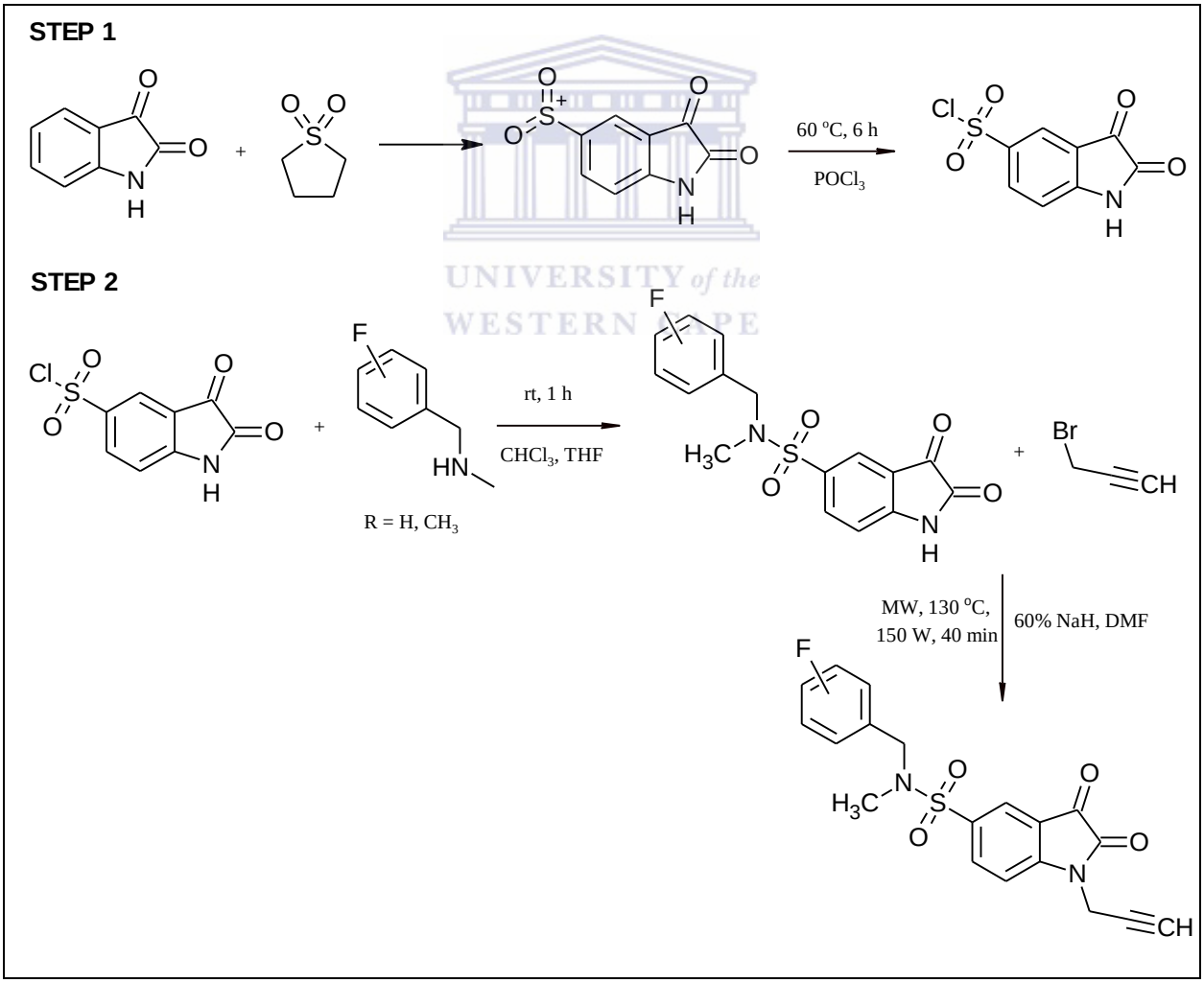
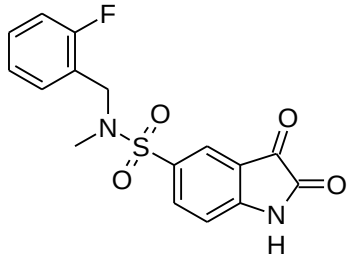
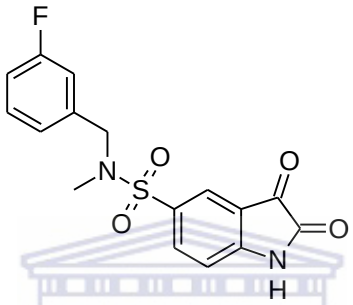
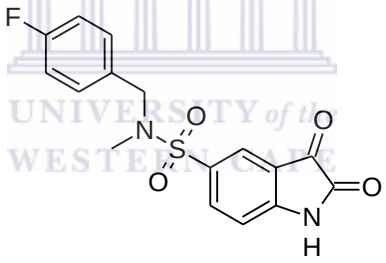
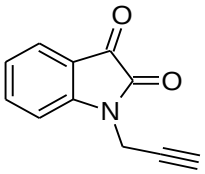
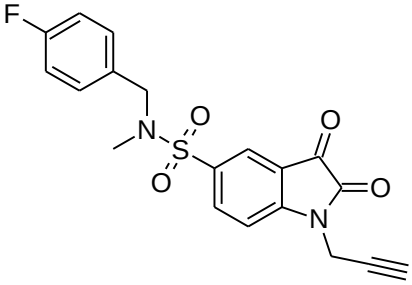
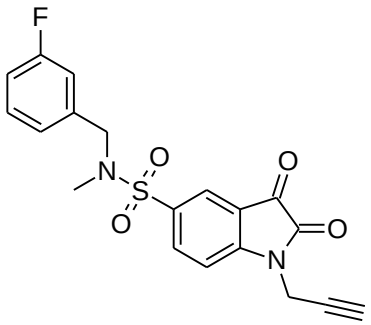
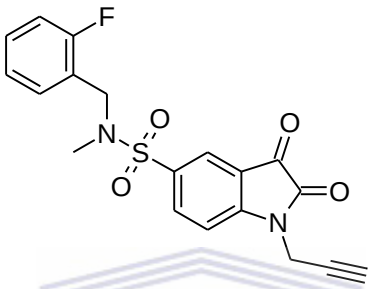


Figure 3.1: The general synthesis of the novel isatin compounds.

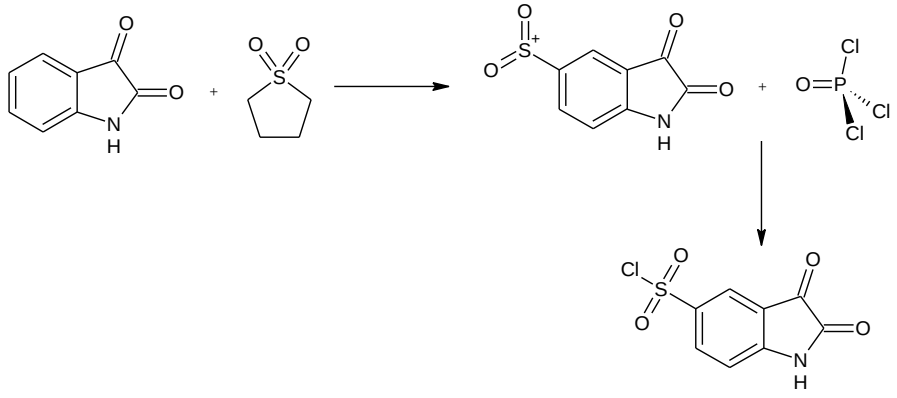
Table 3.1: Assigned names and structures of final compounds selected for synthesis**.**

Assigned number	Structure and the Name of Compounds
2	 ***N*-[(2-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*- indole-5-sulfonamide**
3	 ***N*-[(3-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide**
4	 ***N*-[(4-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide**
5	 **1-(Prop-2-yn-1-yl)-1*H*-indole-2,3-dione**
7	 ***N*-[(4-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-indole-5-sulfonamide**

8	 ***N*-[(3-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-indole-5-sulfonamide**
9	 ***N*-[(2-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-indole-5-sulfonamide**

3.2.1. Synthetic procedures

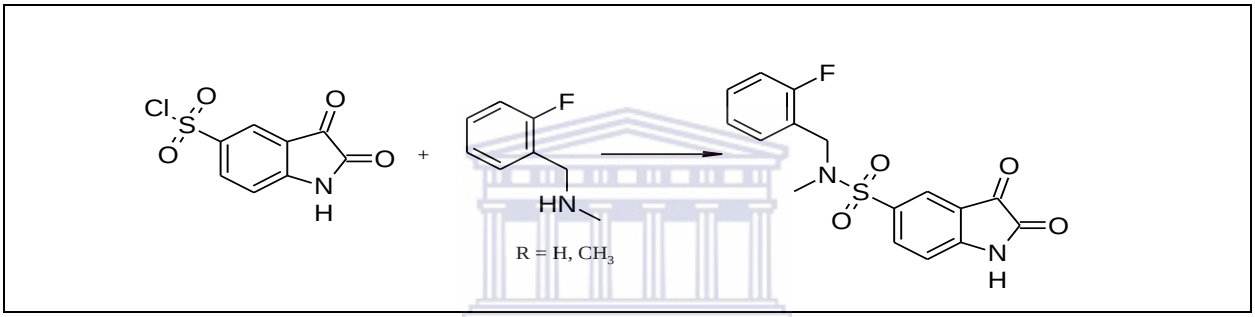
3.2.1.1. 2,3-Dioxoindoline-5-sulfonyl chloride (compound 1)



Synthesis: 2,3-Dioxoindoline-5-sulfonyl chloride was synthesised using a modified procedure from the literature (Havran et al. 2009, Martinez, Naarmann 1990). A mixture of isatin-5-sulfonic acid sodium salt dihydrate (4 g, 14.04 mmol, 1 eq.) was dissolved in tetramethylene sulfone (20 mL) and phosphorous oxychloride (7.4 mL, 79.2 mmol, 5.6 eq.) was added dropwise. The mixture

was heated at 60 °C for 3 h. The reaction was allowed to cool to room temperature and was added dropwise to a stirring slurry of crushed ice/CH_2Cl_2 (200 g/200 g). After the ice had melted, the mixture was separated and the organic layer retained, from which the CH_2Cl_2 removed was by evaporation, diluted with ethyl acetate (200 mL), washed with H_2O (10 × 80 mL) and evaporated to dryness. The crude product was purified by flash column chromatography using hexane: ethyl acetate in a ratio of 1:1 as eluent to yield the title compound, 2,3-dioxoindoline-5-sulfonyl chloride (**1**) as a bright yellow solid. The physical properties of compound **1** was similar to that reported in the literature (Havran et al. 2009, Martinez, Naarmann 1990).

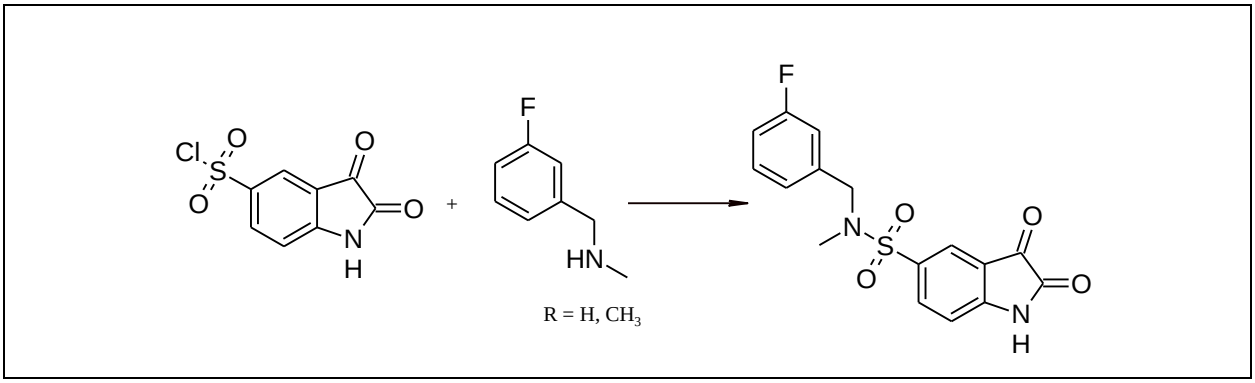
3.2.1.2. *N*-[(2-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide (Compound 2)



Synthesis: A solution of 1-(2-fluorophenyl)-*N*-methylmethanamine (0.139 g, 1 mmol) and diisopropylethylamine (0.172 g, 1.332 mmol) in $CHCl_3$ (0.733 mL) was added dropwise to a stirred solution of 2,3-dioxoindoline-5-sulfonyl chloride (250 mg, 1 mmol) in $CHCl_3$/THF (1:1, 12.65 mL) at room temperature. The resulting solution was stirred at room temperature for 1 h and the solvent was completely removed *in vacuo*. The crude product was purified by flash column chromatography using hexane: ethyl acetate in a ratio of 1:1 as eluent to obtain *N*-[(2-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide as a brown wax.

Physical data: $C_{16}H_{13}FN_2O_4S$; **mp:** wax; **^{1}H NMR** (200 MHz, CD_3OD) (Spectrum 1) δ_H: 7.83 (d, 1H), 7.43 (t, 1H), 7.34 (dd, 1H), 7.2 (m, 1H), 7.21-7.1 (m, 3H), 4.25 (s, 2H), 2.64 (s, 3H); **^{13}C NMR** (50 MHz, CD_3OD) (Spectrum 2) δ_C: 162.34, 159.89, 145.52, 136.87, 133.00, 132.22, 130.89, 130.58, 130.53, 124.23, 124.19, 123.68, 123.19, 112.54, 46.99, 33.81; **MS** (DI-ESI-MS) *m/z* (Spectrum 3): 349.11 $[M+H]^+$; IR (ATR, cm^{-1}) v_{max} (Spectrum 4): 3352.00, 2924.27, 1747.87, 1614.86, 1333.82, 1151.89 cm^{-1}.

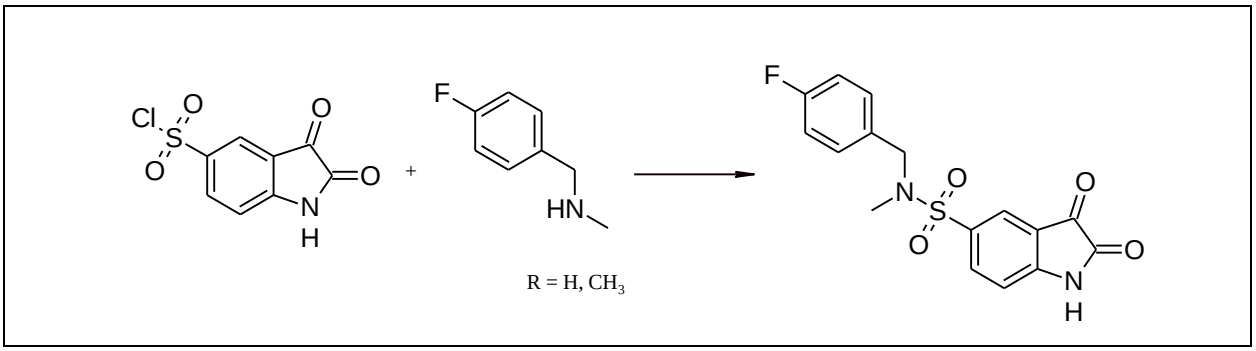
3.2.1.3. *N*-[(3-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide (Compound 3)



Synthesis: A solution of 1-(3-fluorophenyl)-*N*-methylmethanamine (0.280 g, 2 mmol) and diisopropylethylamine (0.350 g, 2.664 mmol) in $CHCl_3$ (1.5 mL) was added dropwise to a stirred solution of 2,3-dioxoindoline-5-sulfonyl chloride (0.5 g, 2 mmol) in $CHCl_3$/THF (1:1, 25.3 mL) at room temperature. The resulting solution was stirred at room temperature for 1 h and the solvent was completely removed *in vacuo*. The crude product was purified by flash column chromatography using hexane: ethyl acetate in a ratio of 1:1 as eluent to obtain *N*-[(3-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide as a light yellow powder.

Physical data: $C_{16}H_{13}FN_2O_4S$; **mp:** 153-155 °C; **^{1}H NMR** (200 MHz, CD_3OD) (Spectrum 5) δ_H: 7.83 (s, 1H), 7.35 (2 x dd, 2H), 7.14 (dd, 1H), 7.09-7.06 (m, 2H), 7.04 (t, 1H), 4.17 (s, 2H), 2.61 (s, 3H); **^{13}C NMR** (50 MHz, CD_3OD) (Spectrum 6) δ_C: 164.27, 161.83, 145.53, 139.08, 138.92, 132.23, 130.16, 130.13, 130.08, 130.05, 129.00, 123.77, 123.66, 110.49, 53.17, 33.70; **MS** (DI-ESI-MS) *m/z* (Spectrum 7): 349.02 $[M+H]^+$; IR (ATR, cm^{-1}) ν_{max} (Spectrum 8): 3280.90, 2924.97, 1750.43, 1614.86, 1336.01, 1147.72 cm^{-1}.

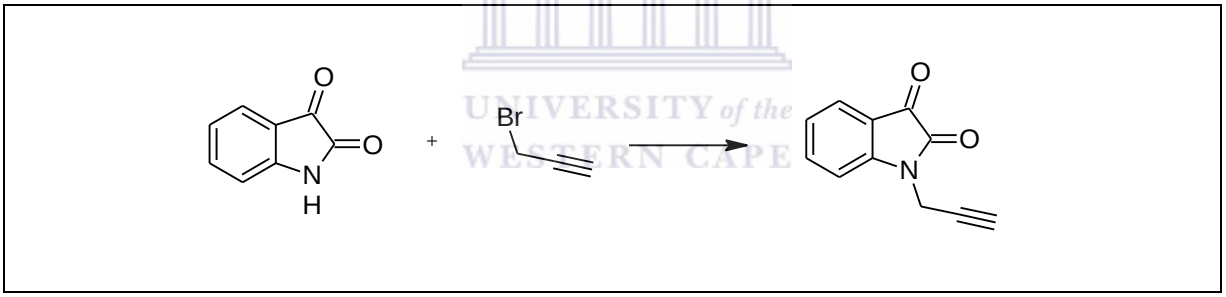
3.2.1.4. *N*-[(4-Fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide (Compound 4)



Synthesis: A solution of 1-(4-fluorophenyl)-*N*-methylmethanamine (0.280 g, 2 mmol) and diisopropylethylamine (0.350 g, 2.664 mmol) in $CHCl_3$ (1.5 mL) was added dropwise to a stirred solution of 2,3-dioxoindoline-5-sulfonyl chloride (0.5 g, 2 mmol) in $CHCl_3$/THF (1:1, 25.3 mL) at room temperature. The resulting solution was stirred at room temperature for 1 h and the solvent was completely removed *in vacuo*. The crude product was purified by flash column chromatography using Hexane: EtOAc in a ratio of 1:1 as eluent to obtain *N*-[(4-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1*H*-indole-5-sulfonamide as a strong yellow powder.

Physical data: $C_{16}H_{13}FN_2O_4S$; **mp:** 150-152 °C; **^{1}H NMR** (200 MHz, CD_3OD) (Spectrum 9) $\delta_{H:}$ 7.82 (s, 1H), 7.35 (dd, 1H), 7.32 (d, 1H), 7.08-7.05 (m, 4H), 4.13 (s, 2H), 2.55 (s, 3H). **^{13}C NMR** (50 MHz, CD_3OD) (Spectrum 10) δ_C: 175.87, 165.25, 147.00, 138.40, 133.55, 133.52, 132.48, 132.07, 131.60, 131.52, 130.52, 125.17, 124.67, 111.99, 54.50, 34.98; **MS** (DI-ESI-MS) *m/z* (Spectrum 11): 349.01 $[M+H]^+$; IR (ATR, cm^{-1}) ν_{max} (Spectrum 12): 3291.61, 2926.54, 1752.12, 1614.86, 1334.42, 1146.03 cm^{-1}.

3.2.1.5. 1-(Prop-2-yn-1-yl)-1*H*-indole-2,3-dione (Compound 5)

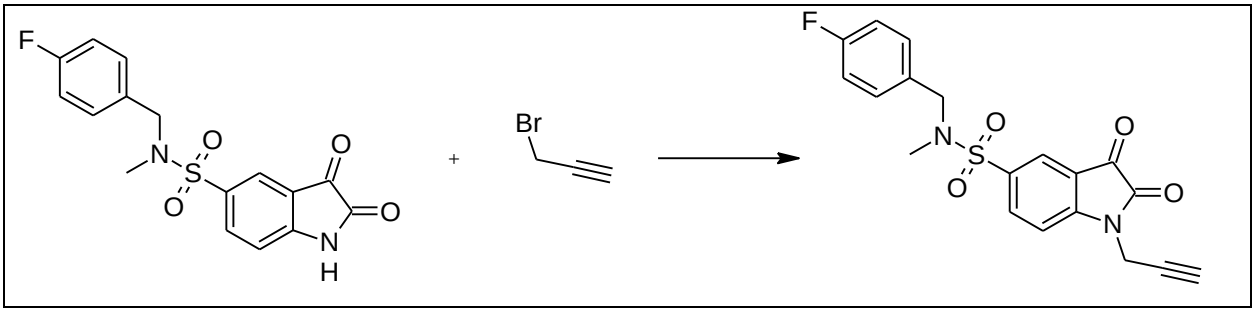


Synthesis: In a microwave compatible glass-vessel, a stirred solution of isatin (186 mg, 1 mmol) dissolved in DMF (6 ml) was treated with NaH (60%, 40 mg, 1 mmol) at room temperature. The mixture was stirred for 10 minutes and then propargyl bromide (0.215 ml, 2 mmol) was slowly added while stirring. Subsequently the vessel was sealed and the mixture was stirred at 95 °C for 10 minutes using microwave irradiation (maximum power = 150 W). Thereafter the mixture was added to 60 ml of water, extracted with 100 ml of ethyl acetate, washed with 60 ml of saturated NaCl and then washed with 60 ml water and dried *in vacuo*. After solvent removal, the crude product was purified by column chromatography using hexane: ethyl acetate in a ratio of 1:1 as eluent to obtain the 1-(prop-2-yn-1-yl)-1*H*-indole-2,3-dione as an orange powder.

Physical data: $C_{11}H_7NO_2$; **mp:** 159-161 °C; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 13) $\delta_{H:}$ 7.63 (m, 1H), 7.25 (m, 1 H), 7.17 (t, 1H), 7.12 (d, 1H), 4.55 (s, 2H), 2.30 (s, 1H). **^{13}C NMR** (50 MHz,

$CDCl_3$) (Spectrum 14) δ_C: 182.50, 157.13, 149.59, 138.38, 125.46, 124.18, 117.68, 111.05, 75.64, 73.31, 29.43; **MS** (DI-ESI-MS) *m/z* (Spectrum 15): 186.05 $[M+H]^+$; IR (ATR, cm^{-1}) ν_{max} (Spectrum 16): 3263.86, 2964.14, 1737.80, 1614.86 cm^{-1}.

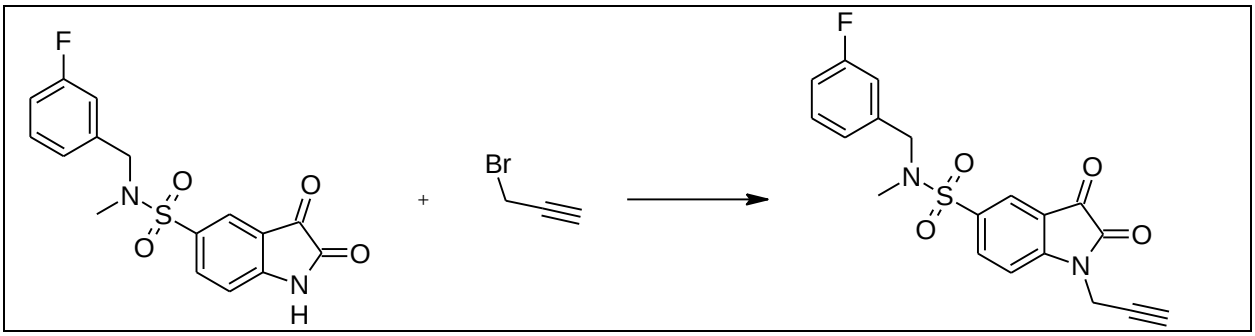
3.2.1.6. *N*-[(4-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-di-hydro-1*H*-indole-5-sulfonamide (Compound 7)



Synthesis: In a microwave compatible glass-vessel, a stirred solution of *N*-[(4-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1H-indole-5-sulfonamide (200 mg, 0.57 mmol) dissolved in DMF (3.42 ml) was treated with NaH (60%, 11.5 mg, 0.29 mmol) at room temperature. The mixture was stirred for 10 minutes and then propargyl bromide (11.5 mg, 0.123 ml) was slowly added while stirring. Subsequently the vessel was sealed and the mixture was stirred at 130 °C for 40 minutes using microwave irradiation (maximum power = 150 W). Thereafter the mixture was added to 35 ml of water, extracted with 57 ml of ethyl acetate, washed with 35 ml of saturated NaCl and dried *in vacuo*. After solvent removal, the crude product was purified by column chromatography using hexane: ethyl acetate in a ratio of 70:30 as eluent to obtain *N*-[(4-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-di-hydro-1*H*-indole-5-sulfonamide as a yellow wax.

Physical data: $C_{19}H_{15}FN_2O_4S$; **mp:** wax; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 17) $\delta_{H:}$ 8.37 (s, 1H), 7.68 (d, 1H), 7.28 (d, 2H), 7.03 (d, 2H), 6.75 (d, 1H), 4.91 (s, 2H), 4.10 (s, 2H), 2.58 (s, 3H), 2.52 (s, 1H). **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 18) δ_C: 166.29, 161.25, 153.52, 132.95, 132.29, 131.53, 131.50, 130.13, 124.10, 116.88, 115.67, 115.45, 109.12, 76.71, 75.30, 53.46, 34.36, 29.70; **MS** (DI-ESI-MS) *m/z* (Spectrum 19): 378.20 $[M+H]^+$; IR (ATR, cm^{-1}) ν_{max} (Spectrum 20): 3289.30, 2921.50, 1695.22, 1621.10, 1334.17, 1152.49 cm^{-1}.

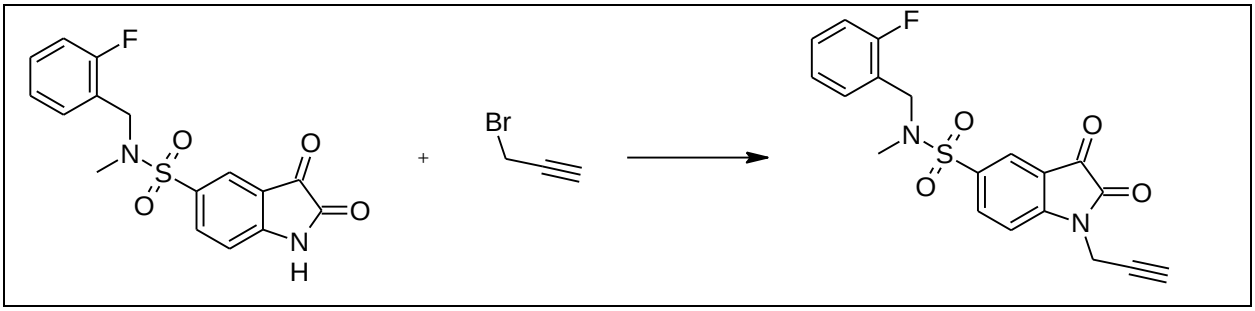
3.2.1.7. *N*-[(3-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-di-hydro-1*H*-indole-5-sulfonamide (Compound 8)



Synthesis: In a microwave compatible glass-vessel, a stirred solution of *N*-[(3-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1H-indole-5-sulfonamide (200 mg, 0.57 mmol) dissolved in DMF (3.42 ml) was treated with NaH (60%, 11.5 mg, 0.29 mmol) at room temperature. The mixture was stirred for 10 minutes and then propargyl bromide (11.5 mg, 0.123 ml) was slowly added while stirring. Subsequently the vessel was sealed and the mixture was stirred at 130 °C for 40 minutes using microwave irradiation (maximum power = 150 W). Thereafter the mixture was added to 35 ml of water, extracted with 57 ml of ethyl acetate, washed with 35 ml of saturated NaCl and dried *in vacuo*. After solvent removal, the crude product was purified by column chromatography using hexane: ethyl acetate in a ratio of 70:30 as eluent to obtain *N*-[(3-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-di-hydro-1*H*-indole-5-sulfonamide as a yellow orange powder.

Physical data: $C_{19}H_{15}FN_2O_4S$; **mp:** 149-153 °C; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 21) δ_H: 8.12 (dd, 1H), 8.06 (dd, 1H), 7.32-7.29 (m, 2H), 7.08 (d, 1H), 7.04 (dd, 1H), 7.01 (s, 1H), 4.61 (s, 2H), 4.20 (s, 2H), 2.68 (s, 3H), 2.39 (s, 1H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 22) δ_C: 164.00, 161.80, 156.55, 152.29, 137.73, 137.37, 134.22, 130.45, 124.44, 123.83, 117.65, 115.32, 115.26, 111.65, 74.86, 74.25, 53.67, 34.59, 29.87; **MS** (DI-ESI-MS) *m/z* (Spectrum 23): 387.15 $[M+H]^+$; IR (ATR, cm^{-1}) ν_{max} (Spectrum 24): 3304.55, 2918.37, 1737.30, 1612.86, 1345.08, 1150.80 cm^{-1}.

3.2.1.8. *N*-[(2-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-indole-5-sulfonamide (Compound 9)



Synthesis: In a microwave compatible glass-vessel, a stirred solution of N-[(2-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-2,3-dihydro-1H-indole-5-sulfonamide (30 mg, 0.09 mmol) dissolved in DMF (0.6 ml) was treated with NaH (60%, 2 mg, 0.05 mmol) at room temperature. The mixture was stirred for 10 minutes and then propargyl bromide (0.02 ml) was slowly added while stirring. Subsequently the vessel was sealed and the mixture was stirred at 130 °C for 40 minutes using microwave irradiation (maximum power = 150 W). Thereafter the mixture was added to 6 ml of water, extracted with 10 ml of ethyl acetate, washed with 6 ml of saturated NaCl and dried *in vacuo*. After solvent removal, the crude product was purified by TLC using hexane: ethyl acetate in a ratio of 40:60 as eluent to obtain *N*-[(2-fluorophenyl)methyl]-*N*-methyl-2,3-dioxo-1-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-indole-5-sulfonamide as a yellowish brown wax.

Physical data: $C_{19}H_{15}FN_2O_4S$; **mp:** wax; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 25) $\delta_{H:}$ 8.12-8.09 (dd, 1H), 8.03 (d, 1H), 7.45 (d, 1H), 7.30-7.25 (m, 2H), 7.16 (t, 1H), 7.02 (m, 1H), 4.60 (s, 2H), 4.29 (s, 2H), 2.72 (s, 3H), 2.37 (s, 1H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 26) δ_C: 162.23, 159.77, 156.59, 152.23, 137.36, 134.39, 130.79, 129.99, 124.67, 122.21, 117.59, 115.62, 115.40, 111.59, 74.89, 74.22, 47.24, 34.76, 29.86; **MS** (DI-ESI-MS) *m/z* (Spectrum 27): 387.15 $[M+H]^+$; IR (ATR, cm^{-1}) ν_{max} (Spectrum 28): 3275.75, 2917.70, 1744.44, 1610.19, 1323.93, 1152.49 cm^{-1}.

3.3 CONCLUSION

Seven novel compounds were successfully synthesized, the synthesis of all compounds resulted in lower yields that could be attributed to the formation of various unidentified impurities. Varying success has thus been achieved with the synthesis of these compounds and its purification. Solubility and multiple reactions taking place made it difficult to obtain some of the compounds in absolutely pure form and optimization of these reactions for further studies are thus essential.

CHAPTER 4
BIOLOGICAL EVALUATION AND RESULTS

The aim of this study was to design and synthesize a series of hybrid molecules and to evaluate these compounds for neuroprotective activity. It is hypothesized that these compounds may have the ability to inhibit monoamine oxidases (MAO) and caspase 3, as these drug-like molecules comprise of features of both selegiline and isatin which inhibit these two enzymes respectively.

4. IC_{50} DETERMINATION FOR THE INHIBITION OF HUMAN MAO AND CASPASE 3

4.1 IC_{50} DETERMINATION FOR THE INHIBITION OF HUMAN MAO

4.1.1 Introduction

MAO catalyze the oxidative deamination of both endogenous and exogenous molecules, affecting the concentration of monoamine neurotransmitters, dietary amines and hormones. This results in formation of monoamine aldehydes, which are consequently oxidized to acids by aldehyde dehydrogenase or turned into alcohols or glycols by aldehyde reductase. Inhibition of MAO increases dopamine levels in the basal ganglia of brain and decreases the formation of oxidative by-products (Youdim, Bakhle 2006, Kuhn, Murphy & Youdim 1985, GREEN et al. 1977).

4.1.2 Assay procedure

This method is based on the fact that certain MAO substrates are oxidized to fluorescent products. The substrate used in this study was kynuramine which is oxidized to the fluorescent 4-hydroxyquinoline. The generation of this product can subsequently be measured with a fluorescence spectrophotometer (Zhou, Zhong & Silverman 1996). This fluorometric method is frequently used to determine the activities of recombinant human MAO-A and MAO-B. It has the advantage that it is more sensitive than spectrophotometric methods (Matsumoto et al. 1985) and is therefore more suitable to measure activities of recombinant human MAO-A and MAO-B (Strydom et al. 2010). Inhibitor potencies are expressed as IC_{50} values for each inhibitor.

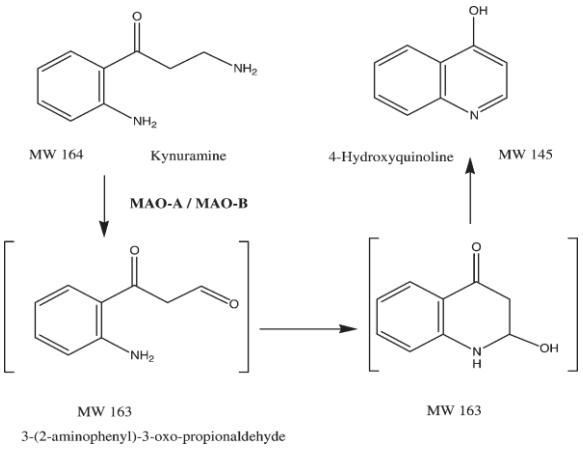


Figure 4.1: Conversion of kynuramine to 4-hydroxyquinoline catalyzed by monoamine oxidases (Yan et al. 2004).

4.1.3 Materials and Methods

4.1.3.1 Chemicals and Reagents

Isatin (compound **0**)**,** potassium dihydrogen orthophosphate (KH_2PO_4), potassium chloride (KCl), dimethyl sulfoxide (DMSO) and recombinant human MAO-A and MAO-B were purchased from Sigma Aldrich. The potassium phosphate buffer consisted of 100 mM KH_2PO_4, the pH was adjusted to 7.4 and it was made isotonic with NaCl. Recombinant human MAO-A and MAO-B were dissolved in potassium phosphate buffer (0.075 mg/ml) and pre-aliquoted and stored at -70 ºC before use. The test compounds (including isatin) were first dissolved in DMSO and serially diluted to the desired concentration range. Clorgyline and selegiline were used as control compounds for MAO-A and MAO-B respectively and inhibition percentages were measured at four or five different concentrations (0.1 µM, 1 µM, 10 µM, 100 µM and 1000 µM), allowing IC_{50} values to be calculated.

4.1.3.2 Instrumentation

A Fluorescent Microplate Reader (Biotek Instruments, Inc) and Gen 5® Software were used to determine the fluorescence at an excitation wavelength of 310 nm and an emission wavelength of 400 nm.

4.1.3.3 Materials and Method

All enzymatic reactions were carried out in potassium phosphate buffer to a final volume of 450 µL. DMSO, as co-solvent was added to each reaction. The reactions contained the different test inhibitor concentrations. The enzyme reactions were initiated with the addition of MAO-A or MAO-B and incubated for 10 min at 37 °C. The MAO-A/B substrate kynuramine (45 µM for MAO-A and 30 µM for MAO-B) was added and further incubated for 20 minutes at 37 °C. Thereafter the

reaction was terminated with the addition of 200 µl NaOH (2 N) and the mixture was centrifuged by vortex mixer. The fluorescence of the MAO generated 4-hydroxyquinoline in the fractions were measured at an excitation wavelength of 310 nm and an emission wavelength of 400 nm.

4.1.4 Results

The MAO-A and MAO-B inhibition percentages were calculated and plotted against the logarithm of the inhibitor concentrations to obtain a sigmoidal dose-response curve (See. Figures 4.2 - 4.17). Each curve was constructed from four or five different inhibitor concentrations spanning a least 3 orders of magnitude. This data was fitted to the one site competition model incorporated into GraphPad Prism software and the percentage inhibition at each concentration was determined in triplicate and are expressed as mean ± standard deviation. The IC_{50} value is obtained by calculating the concentration of the inhibitor which results in a reduction of 50 % of the rate obtained in the absence of inhibitor.

In order to compare the MAO inhibitory activity of the synthesized compounds they were tested in an identical procedure. With regards to MAO-A inhibition, all compounds tested showed significant IC_{50} values. Regarding to MAO-B inhibition, compounds **0**, **3**, **4**, **5**, **8** and **9** showed significant IC_{50} values, but compounds **2** and **7** showed low or no inhibition. Compound **1** and **6** were not evaluated as they were only intermediates and not part of the final series of compounds to be evaluated. All the IC_{50} values obtained from the test compounds are listed in table 4.1 and are expressed in µM. Synthesized compounds exhibiting very weak inhibition are expressed in the maximum percentage inhibition at the highest possible concentration. The most potent value (lowest IC_{50}) for each enzyme is highlighted in grey (Table 4.1).

4.1.4.1 Dose response curves for MAO-A inhibition studies.

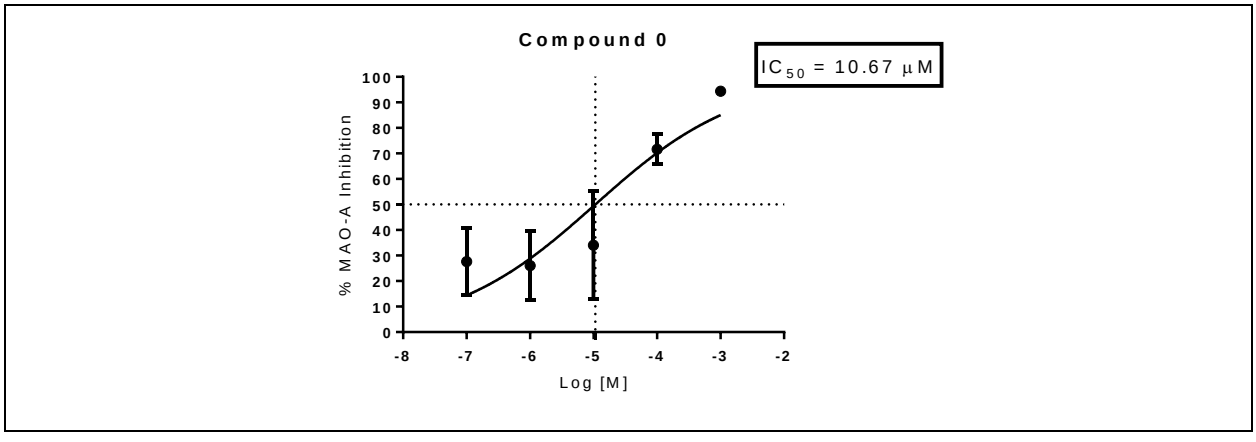


Figure 4.2: Inhibition curve of compound **0** on MAO-A activity.

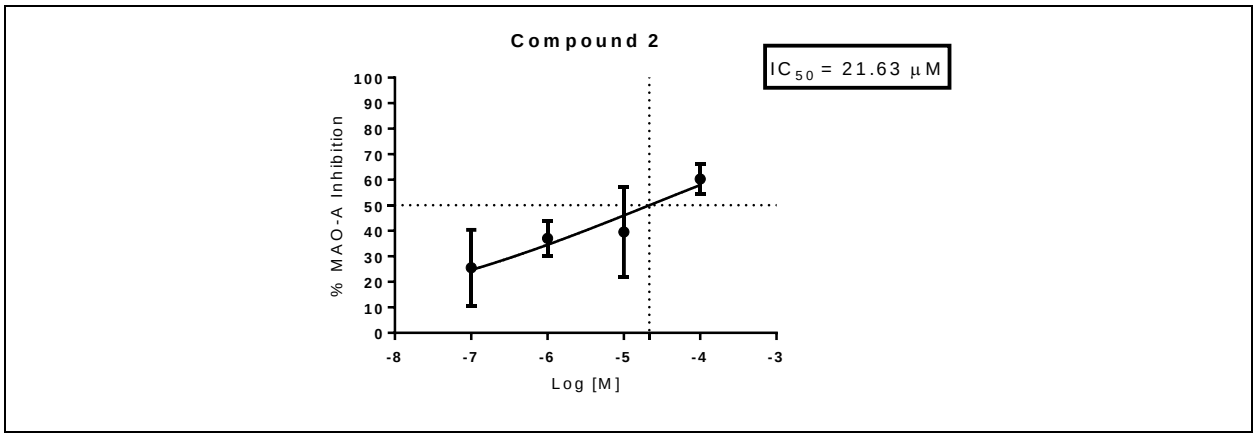


Figure 4.3: Inhibition curve of compound **2** on MAO-A activity.

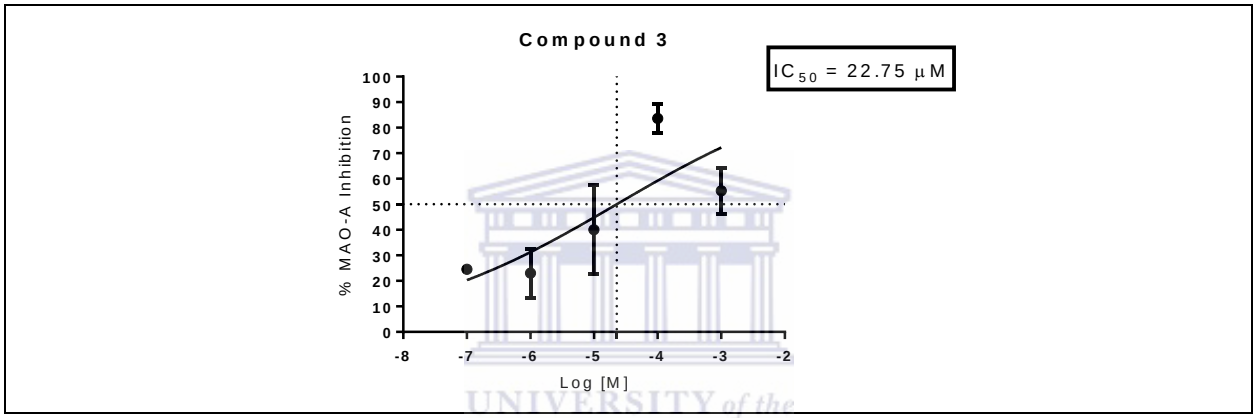


Figure 4.4: Inhibition curve of compound **3** on MAO-A activity.

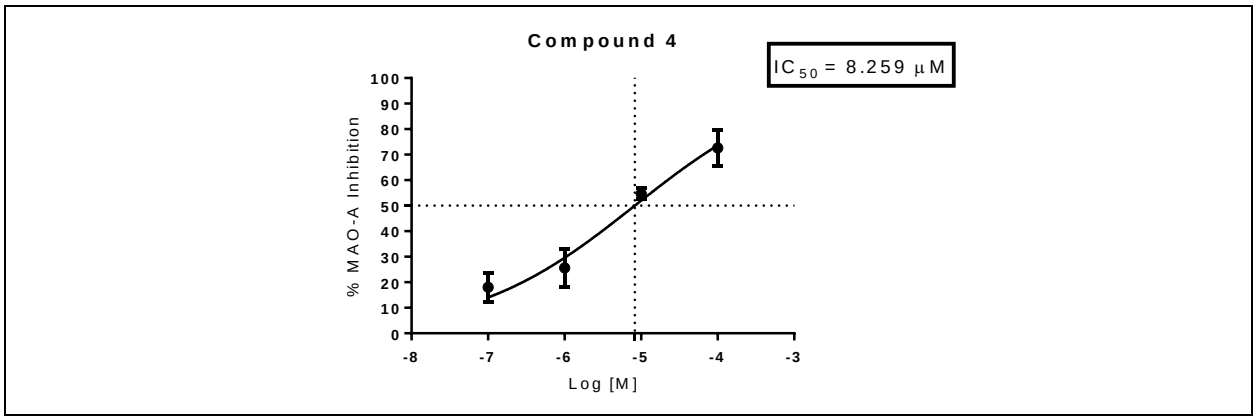


Figure 4.5: Inhibition curve of compound **4** on MAO-A activity.

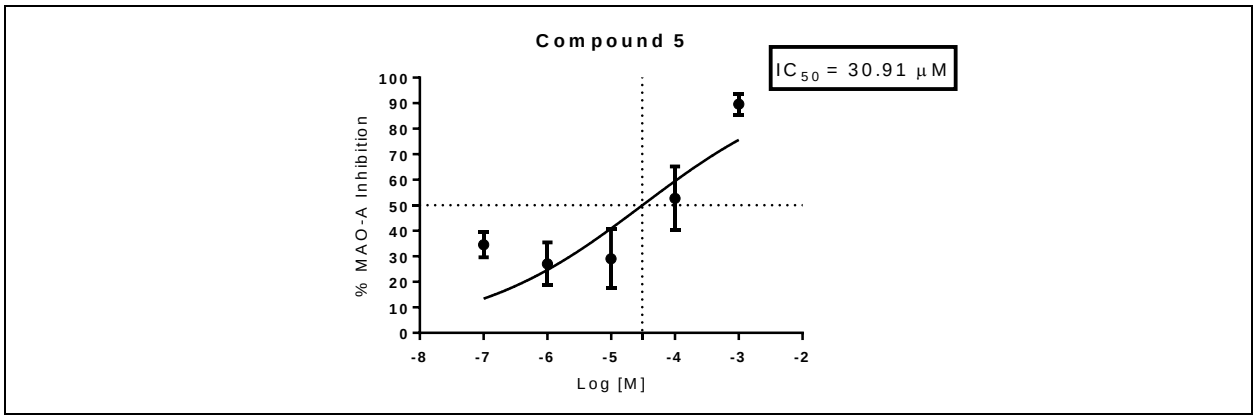


Figure 4.6: Inhibition curve of compound **5** on MAO-A activity.

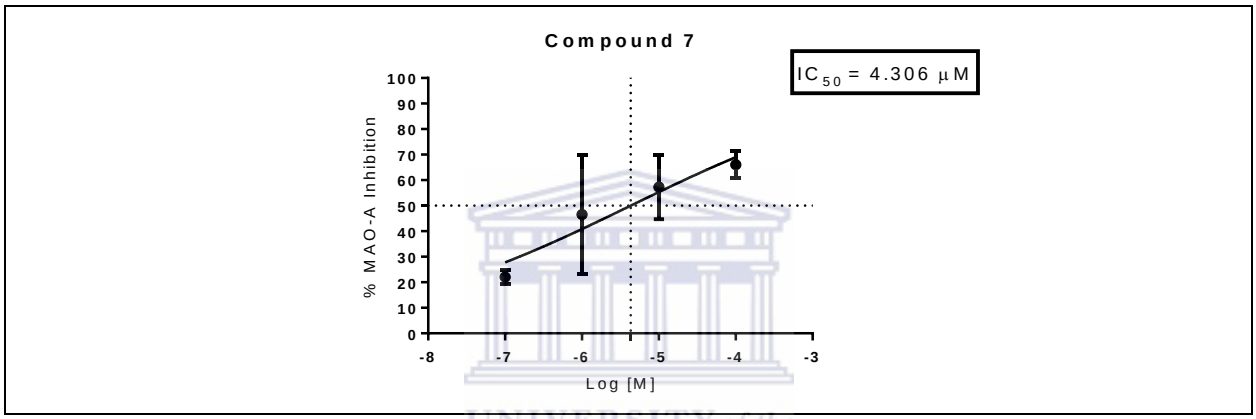


Figure 4.7: Inhibition curve of compound **7** on MAO-A activity.

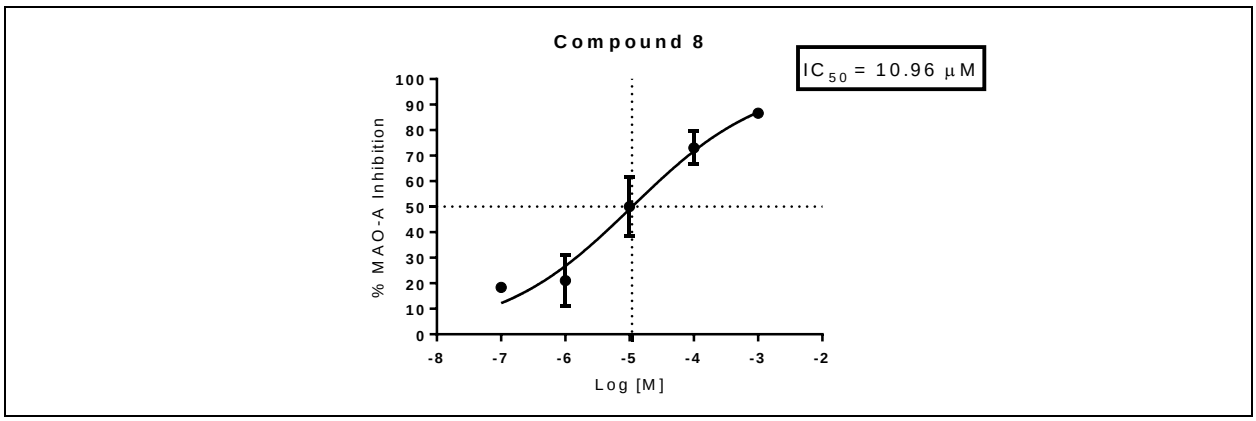


Figure 4.8: Inhibition curve of compound **8** on MAO-A activity.

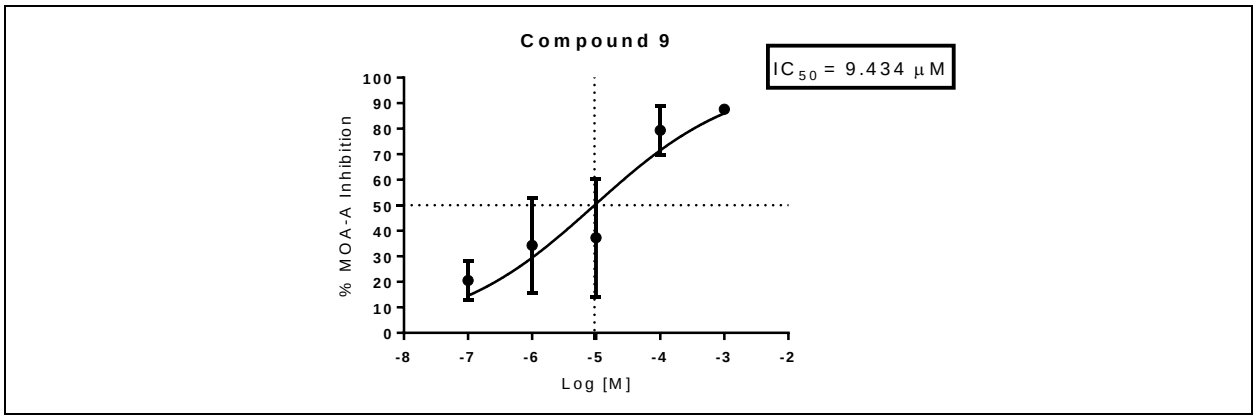


Figure 4.9: Inhibition curve of compound **9** on MAO-A activity.

4.1.4.2 Dose response curves for MAO-B inhibition studies.

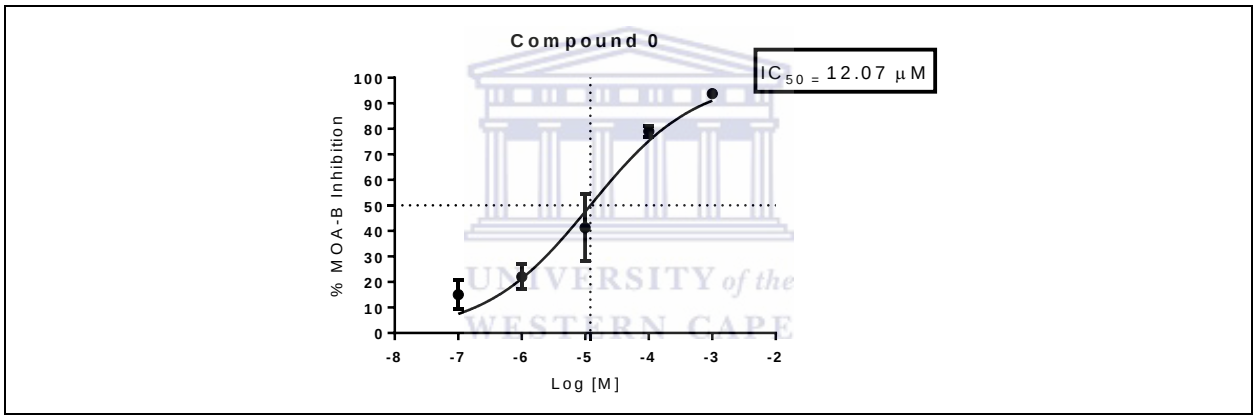


Figure 4.10: Inhibition curve of compound **0** on MAO-B activity.

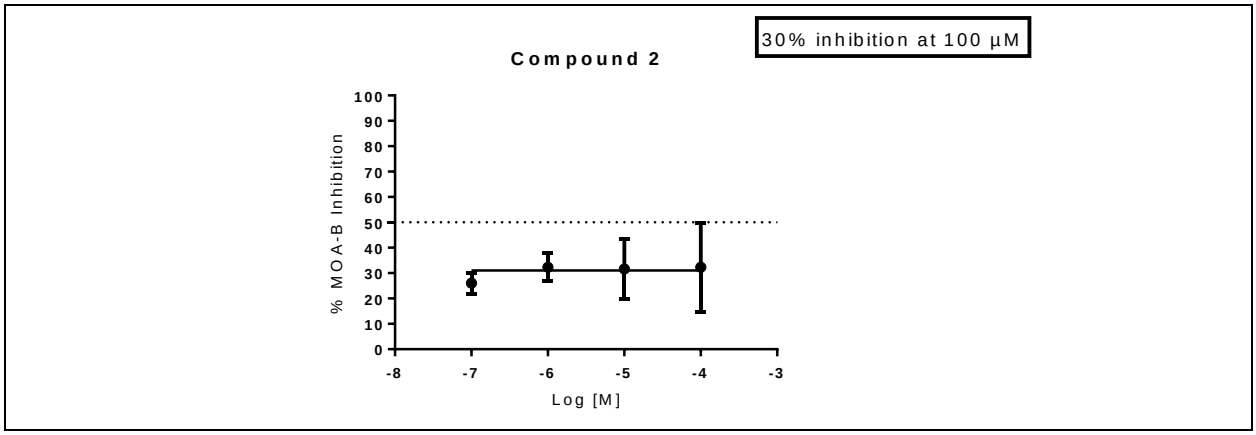


Figure 4.11: Inhibition curve of compound **2** on MAO-B activity.

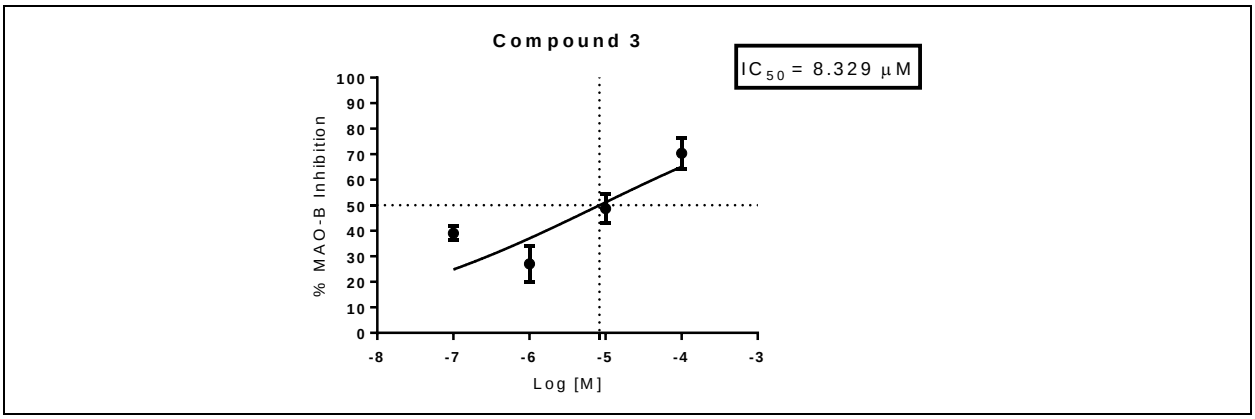


Figure 4.12: Inhibition curve of compound **3** on MAO-B activity.

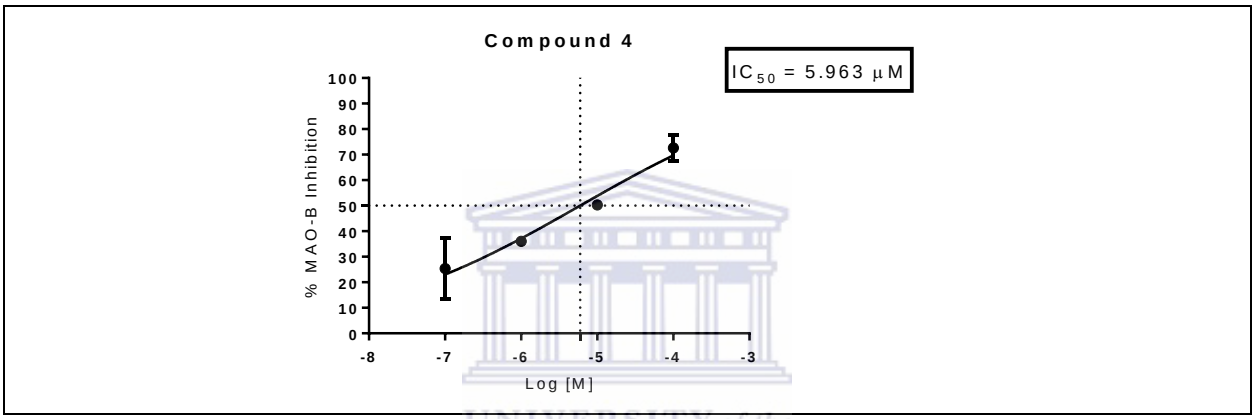


Figure 4.13: Inhibition curve of compound **4** on MAO-B activity.

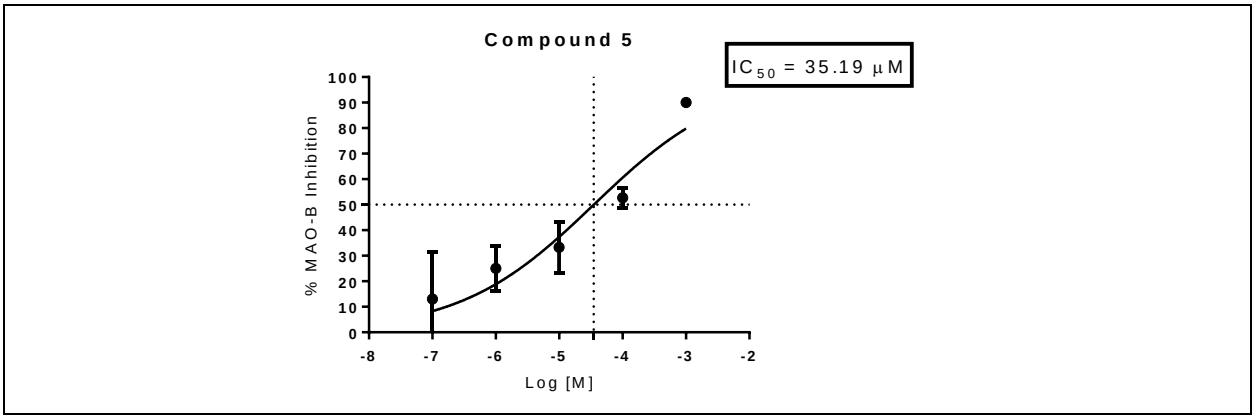


Figure 4.14: Inhibition curve of compound **5** on MAO-B activity.

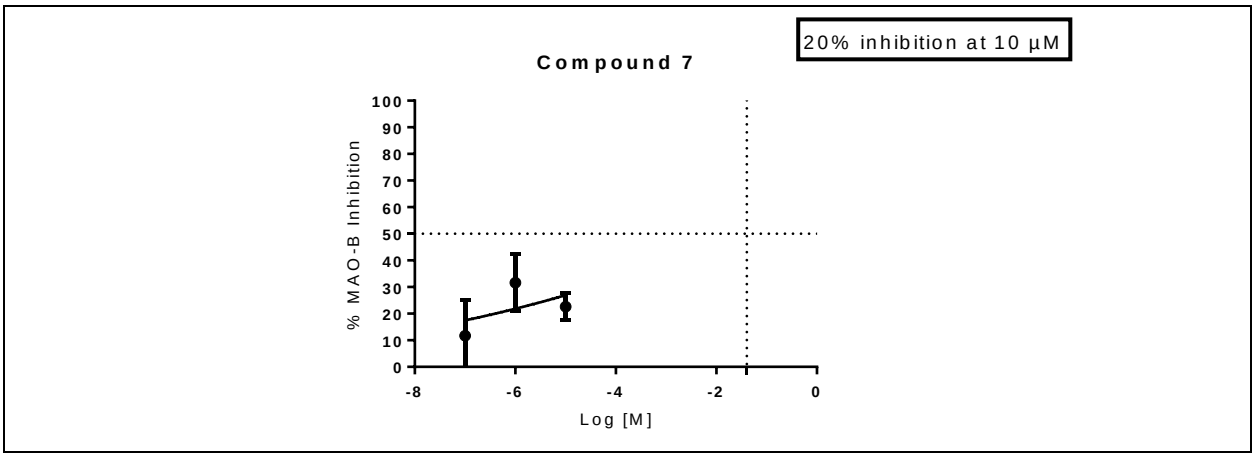


Figure 4.15: Inhibition curve of compound **7** on MAO-B activity.

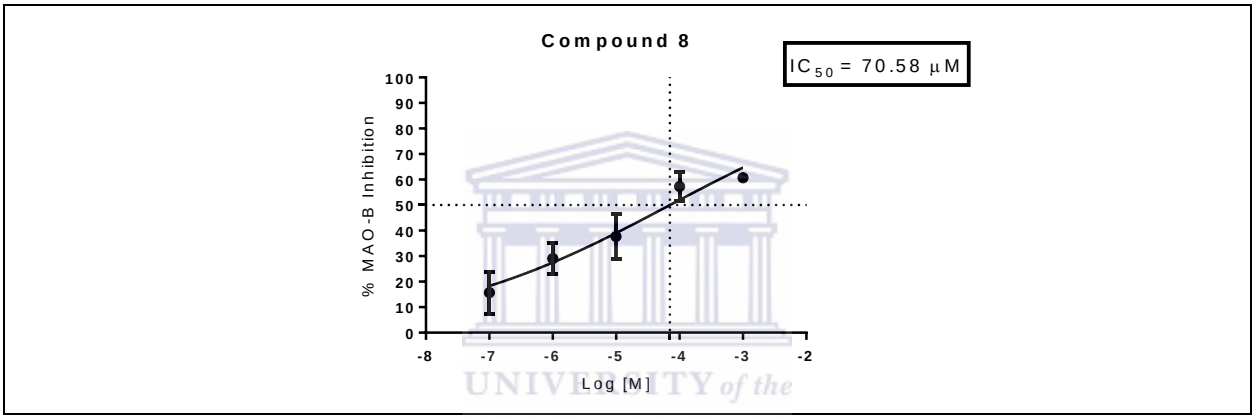


Figure 4.16: Inhibition curve of compound **8** on MAO-B activity.

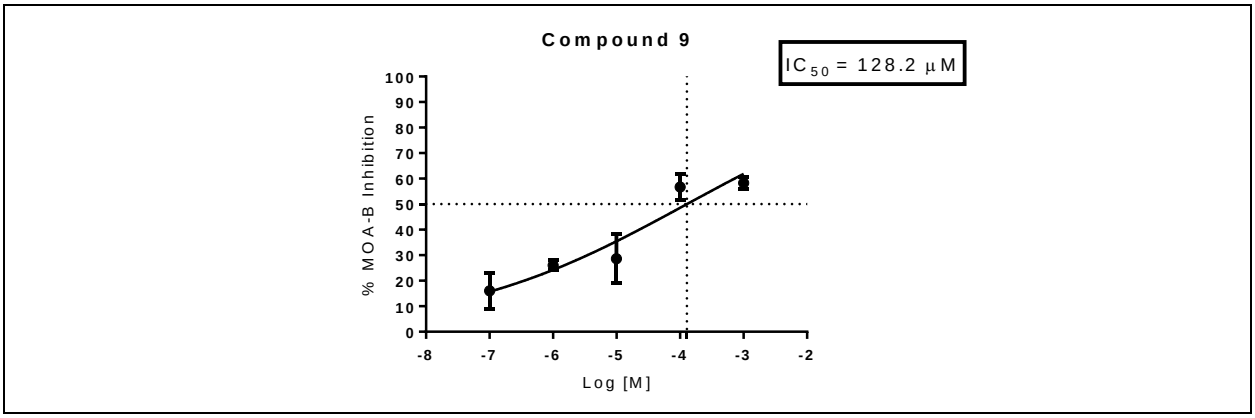


Figure 4.17: Inhibition curve of compound **9** on MAO-B activity.

4.1.5 Discussion

Compound **0**, isatin, showed some inhibitory activity for MAO-A (IC_{50} = 10.67 µM). The introduction of the aromatic amine with the fluorine in the ortho or meta position (compounds **2** and **3**), resulted in decreased activity by almost twofold. Compound **4** which has the fluorine in the para position

showed increased activity compared to isatin. In compound **5** the propargylamine functional group was added to the isatin scaffold, which decreased the MAO-A activity. However the introduction of this moiety in compounds **7**, **8** and **9** improved the activity when compared to isatin and compounds **2**, **3** and **4** (Figures 4.1-4.9 and Table 4.1).

Compound **0**, displayed some inhibitory activity for MAO-B (IC_{50} = 12.07 µM). In compound **2**, by introducing the aromatic amine with the fluorine in the ortho position, the activity was drastically reduced (30% inhibition at 100 µM). However, addition of this moiety at the meta or para position in compounds **3** and **4** respectively resulted in increased activity. Propargylamine has a significant role in MAO-B inhibitory activity of selegiline and rasagiline, but importantly it seems in the synthesized compounds that this moiety does not bind to the MAO-B enzyme in a similar manner as selegiline and rasagiline. The reduction in MAO-B activity of compounds **5**, **7** (20% inhibition at 10 µM), **8** and **9** confirms this observation. Therefore it can be concluded that compounds **3** and **4** are the best candidates for MAO-B inhibitory activity (Figures 4.10-4.17 and Table 4.1).

Compounds **7**, **8** and **9** containing the propargylamine functional group are thus selective inhibitors of the MAO-A enzyme. These compounds might not find application for neurodegenerative disorders but they may be potential candidates as antidepressant agents whereas compounds **3** and **4** are dually active inhibitors of MAO-A and MAO-B. Compound **4** is the best dually active inhibitor of MAO-A (IC_{50} = 8.26 µM) and MAO-B (IC_{50} = 5.96 µM) in this series and a novel lead compound for further development.

4.2 IC_{50} DETERMINATION FOR THE INHIBITION OF HUMAN CASPASE 3

4.2.1 Introduction

One of the earliest and most regularly observed attributes that play a role in apoptosis is a family of cytosolic proteases called caspases which cleave substrates resulting in apoptotic morphology (Keppler-Hafkemeyer, Brinkmann & Pastan 1998, Stennicke, Salvesen 1997). Caspase 3, also known as apopain, YAMA, and CPP32, has been identified to be a significant member of this caspase family of proteases (Kumar 1999). It is believed that activation of the ICE-family proteases and caspase-3 activity are essential for many phenotypes involved in apoptosis in mammalian cells. A significant structural characteristic of nearly all known caspase inhibitors is an electrophilic group that can make a reversible or irreversible bond with the active site cysteine, making the enzyme inactive (Lee et al. 2001). Inhibition of caspases can delay apoptosis, implicating a potential drug target against neurodegenerative disorders (Ramlawi et al. 2006, Wei et al. 2006).

4.2.2 Assay procedure

This assay effectively uses fluorometric methods for screening caspase 3 inhibitors by utilizing the synthetic peptide substrate DEVD-AFC (AFC, 7-amino-4-trifluoromethyl coumarin). The cleavage of the synthetic substrate to active caspase 3 releases free AFC, which is quantifiable by fluorometry. To screen a compound, it can be added to the reaction directly and the level to which caspase 3 activity is inhibited can be determined by comparing the fluorescence intensity in samples with and without the test inhibitors (Ramlawi et al. 2006, Wei et al. 2006).

4.2.3 Materials and Methods

4.2.3.1 Chemicals and Reagents

The caspase 3 inhibitor drug detection kit (ab102491) including 2X reaction buffer 10 mL, caspase substrate Ac-DEVD-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC,1 mM), 0.5 mL dithiothreitol (DTT, 1 M), 100 µL active caspase 3 (lyophilized, 100 units), and 10 µL of the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(O-Me)fluoromethyl ketone (Z-VAD-FMK, 2 mM) was purchased from Abcam. The reaction buffer was prepared immediately before use and enough 2X reaction buffer was aliquoted for the number of assays to be performed. 10 µl DTT stock solution was added to 1 ml of 2X reaction buffer, to reach a 10 mM final concentration. To prepare the active caspase 3, 100 units of active caspase 3 was reconstituted in 550 µl 2X reaction buffer then aliquoted and immediately stored at -80°C. The test compounds, including purchased isatin (compound **0**), were first dissolved in dimethyl sulfoxide (DMSO) and serially diluted to the desired concentration range. Z-VAD-FMK was used as the control compound and inhibition percentages of the test compounds were measured at four different concentrations (1 µM, 10 µM, 100 µM and 1000 µM) in duplicate, allowing IC_{50} values to be calculated.

4.2.3.2 Instrumentation

A Fluorescent Microplate Reader (Biotek Instruments, Inc) and Gen 5® Software were used to determine the fluorescence at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

4.2.3.3 Assay procedure

The test samples were prepared in DMSO up to a final volume of 50 µl per well, 5 µl of active caspase 3 was added and then mixed. A negative and positive inhibition control was prepared by adding 1 µl of DMSO and the caspase 3 inhibitor (Z-VAD-FMK) respectively instead of the test inhibitor. A 50 µM master mix for each assay containing 45 µl reaction buffer and 5 µl DEVD-AFC

substrate was prepared and mixed. The reaction was started by addition of 50 µl of the master mix to each well and it was incubated at 37 °C for 1 hour. The samples were read in a fluorescence plate reader equipped with a 400 nm excitation filter and 505 nm emission filter. Finally the fluorescence intensity of the test samples was compared with samples containing no inhibitors to determine the inhibition efficiency of the testing inhibitors.

4.2.4 Results

The caspase 3 inhibition percentages were calculated and plotted against the logarithm of the inhibitor concentrations to obtain a sigmoidal dose-response curve. Each curve was constructed from 4 different inhibitor concentrations spanning a least 3 orders of magnitude. This data was fitted to the one site competition model incorporated into GraphPad Prism software and the percentage inhibition at each concentration was determined in duplicate are expressed as mean ± standard deviation. The IC_{50} value is obtained by calculating the concentration of the inhibitor which results in a reduction of 50 % of the fluorescence obtained in the absence of inhibitor.

In order to compare the caspase 3 inhibitory activity of the synthesized compounds they were tested in an identical procedure. Compounds **3** and **4** showed excellent inhibitory activity, compound **9** showed very good inhibitory activity, compounds **2** and **8** showed good inhibitory activity and compounds **0**, **5** and **7** showed low or no inhibition. All the IC_{50} values obtained from the caspase 3 inhibitors are shown in figures 4.18 – 4.21 and are listed in table 4.1. Synthesized compounds exhibiting weak inhibition (compounds **0**, **5** and **7**) are expressed in the maximum percentage inhibition at the highest concentration. The most potent value (lowest IC_{50}) for each enzyme is highlighted in grey (Table 4.1).

4.2.4.1 Dose response curves for caspase 3 inhibition studies.

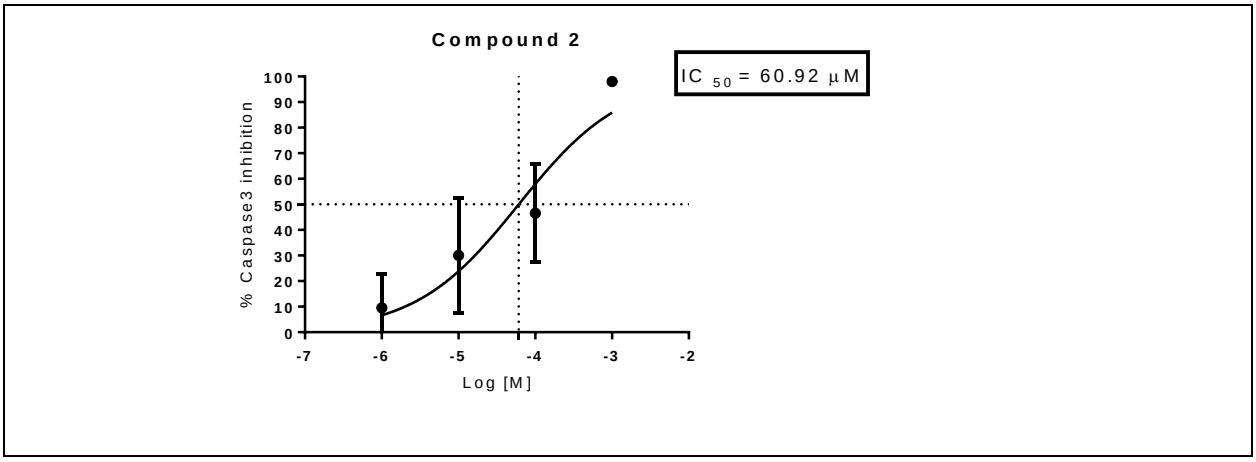


Figure 4.18: Inhibition curve of compound **2** on caspase 3 activity.

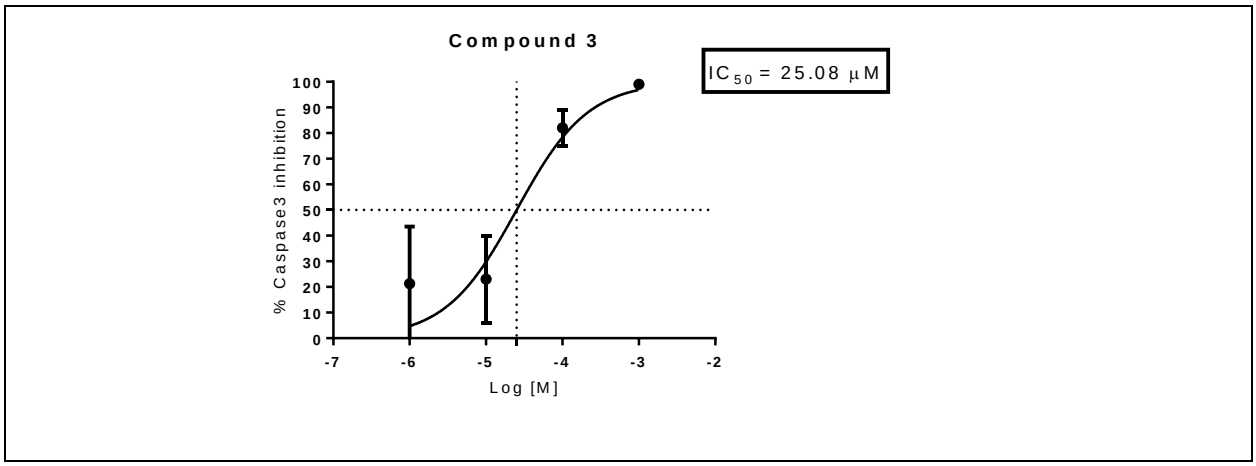


Figure 4.19: Inhibition curve of compound **3** on caspase 3 activity.

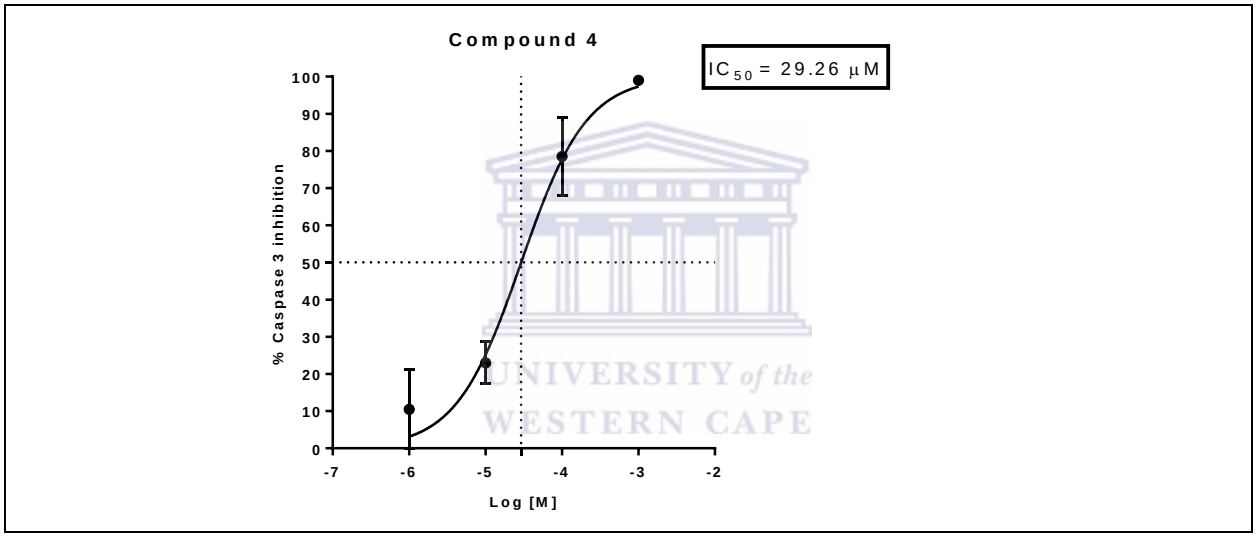


Figure 4.20: Inhibition curve of compound **4** on caspase 3 activity.

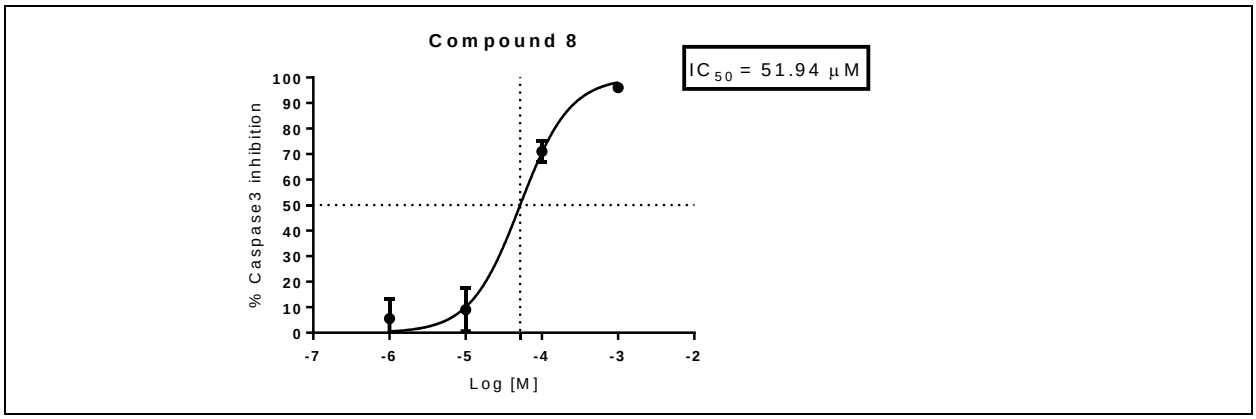


Figure 4.21: Inhibition curve of compound **8** on caspase 3 activity.

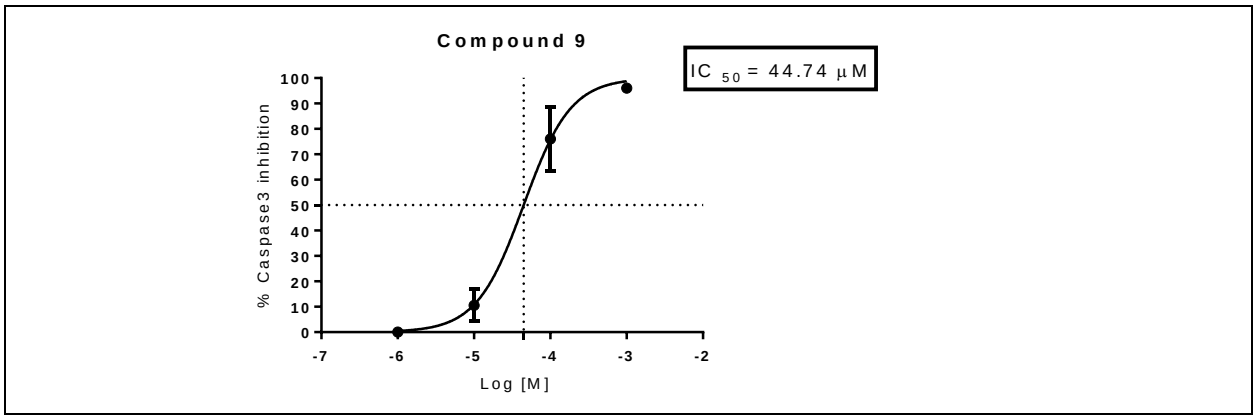


Figure 4.22: Inhibition curve of compound **9** on caspase 3 activity.

4.2.5 Discussion

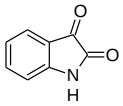
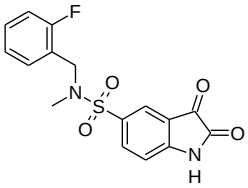
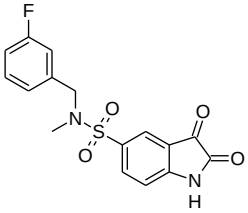
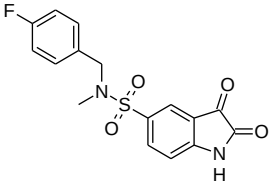
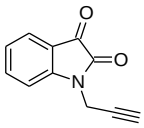
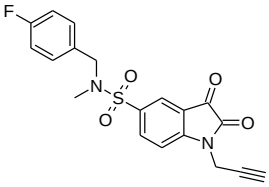
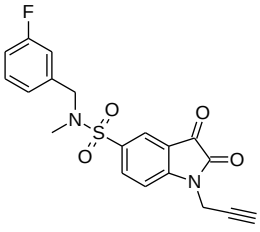
Compound **0**, isatin, did not display good caspase 3 inhibitory activity (24.5% inhibition at 100 µM). As it was mentioned, one of the main reasons of the isatin sulfonamides' selectivity for caspases 3 was derived by interacting primarily with the S_2 subsite, in other words their sulfonamide moiety is responsible for binding to S_2 pocket and in compound **0** this moiety does not exist, which may be the reason why it does not have caspase 3 inhibitory activity.

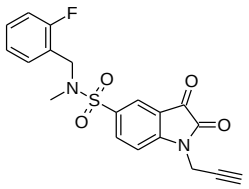
Compounds **2**, **3** and **4** has the sulfonamide function conjugated to an aromatic amine with a fluorine-moiety substituted at their ortho, meta and para positions respectively. This improved their inhibitory activity giving compounds **3** (IC_{50} = 25.08 µM) and **4** (IC_{50} = 29.26 µM) potent caspase 3 inhibitory activity. In compound **5** the propargylamine functional group was introduced to the isatin scaffold and this decreased its activity (18% inhibition at 100 µM), putting it in a similar inhibitory activity range as isatin.

Compound **7**, **8** and **9** were synthesized by conjugating the propargylamine moiety to compounds **2**, **3** and **4** respectively. This reduced the inhibitory activity of these compounds compared to their counterparts **2**, **3** and **4**. When comparing compound **8** with compound **3** a twofold reduction was observed. Compound **9** in comparison with compound **2** had a slight increase in the activity although it is not as active as compounds **3** and **4**. The activity of compound **7** (26% inhibition at 100 µM) compared to compound **4** was drastically reduced. Therefore, based on what was observed, the introduction of the propargylamine functional group in general decreased the

caspase 3 inhibitory activity. This may indicate that the propargylamine moiety does not fit optimally into the S_1 pocket of caspase 3 resulting in decreased inhibitory activity.

Table 4.1: Test compounds with their IC_{50} values (μM) and maximum percentage MAO- and Caspase 3 inhibition.

Compounds		**IC_{50} for MAO-A**	**IC_{50} for MAO-B**	**IC_{50} for Caspase 3**
No	Controls	0.001 (Clorgyline)	0.36 (Selegiline)	40 (Z-VAD-FMK)
0		11	12.07	24.5% inhibition at 100 μM
2		21.63	30% inhibition at 100 μM	60.92
3		22.75	8.329	25.08
4		8.259	5.963	29.26
5		30.91	35.19	18% inhibition at 100 μM
7		4.306	20% inhibition at 10 μM	26% inhibition at 100μM
8		10.96	70.58	51.94

9		9.434	128.2	44.74

4.3 CONCLUSION

Compounds **3** and **4** are new lead compounds to design multifunctional neuroprotective agents because they have good inhibitory activity towards MAO-A, MAO-B and also excellent inhibitory activity against caspase 3. Compounds **7**, **8** and **9** showed good MAO-A selectivity and do not exhibit good MAO-B and caspase 3 inhibitory activities. The role of the aromatic amine functional group in the synthesized compounds was significant for their multifunctional activity. The introduction of the propargylamine moiety only increased the MAO-A inhibitory activity. For future studies it can be recommended to test other functional groups for binding activity in the caspase primary aspartic acid binding pocket (S_1) to consider improvement in the multifunctional activity. In the process of apoptosis there are different apoptotic mechanisms involved. Although compounds **7**, **8** and **9** did not show good caspase 3 activity, they can be tested for general anti-apoptotic activity because the propargylamine functional group in these compounds might function in a completely different manner than what was tested for in this assay.

CHAPTER 5

SUMMARY AND CONCLUSION

5.1 INTRODUCTION

Neurodegeneration is the process in which neurons in the central nervous system (CNS), and the brain in particular, are damaged by several mechanisms (Araki et al. 2001). Progressive and irreversible loss of neurons from specific regions of the brain characterize these disorders (Alexi et al. 2000). Huntington's disease (HD), Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive neurodegenerative disorders that reduce the quality of life of patients who suffer from these conditions and it is growing into a serious problem for the world's ageing population. Not a single cause or process alone can lead to neurodegenerative disorders; instead it is the result of several factors which most likely include a variety of mechanistic pathways (Dauer, Przedborski 2003).

Neurotransmitter amines are catabolized by monoamine oxidase A and B (MAO-A and MAO-B), therefore they have been targeted for the treatment of neuropsychiatric and neurodegenerative diseases (Youdim, Edmondson & Tipton 2006a, Youdim, Weinstock 2004, Riederer, Lachenmayer & Laux 2004). MAO-A metabolizes both serotonin and norepinephrine, two monoamines implicated in depressive illness and anxiety disorders (Youdim, Edmondson & Tipton 2006b, ZISOOK, BRAFF & CLICK 1985) and the antidepressive effect of MAO-A inhibitors is associated with blocking the central breakdown of these monoamines extending their action. Since a significant proportion of patients with Parkinson's disease (PD) exhibit signs of depression, the inhibition of MAO-A in these patients may be beneficial (Di Monte et al. 1996a). Besides offering a potential antidepressant action in PD, MAO-A inhibitors may also provide a symptomatic benefit by reducing MAO-A-catalysed oxidation of dopamine (Youdim, Edmondson & Tipton 2006b, Di Monte et al. 1996a). The oxidative deamination reaction catalyzed by MAO-B is one of the major catabolic pathways of dopamine in the brain. Inhibition of this enzyme leads to enhanced dopaminergic neurotransmission and is currently used in the symptomatic treatment of PD (Di Monte et al. 1996a). Furthermore, MAO-B inhibitors may also exert neuroprotective effects by reducing the concentration of potentially hazardous by-products produced by MAO-B-catalysed dopamine oxidation (Kalaria, Mitchell & Harik 1988a, Nicotra et al. 2004, Di Monte et al. 1996a, Fowler et al. 1997). Apoptosis or programmed cell death occurs in a number of

neurodegenerative disorders. It has been proven that inhibition of the executing enzymes, caspases-3 and -7, slows down or even prevent apoptosis (Lee et al. 2000b).

Disease-modifying approaches have turned into an area of focus in research since these strategies interrupt the initial pathologic events associated with the onset of the disease; hindering the neurotoxic cascade in the case of neurodegenerative diseases is an example of one of these approaches (McLean et al. 1999, Näslund et al. 2000). In clinical practice, multiple targeting has proceeded through the polypharmaceutical approach by using a combination of therapeutic agents functioning on different etiological targets independently. This approach has been proven to be effective in treatment of diseases with similar complexity such as hypertension, cancer and HIV (Jellinger 2003b, Smid et al. 2005). Bearing this in mind, it is evident that the pattern of targeting a single disease factor might not be an efficacious strategy for treatment of neurodegenerative diseases.

So far there has not been any treatment proven which can stop cell death or bring back damaged neurons to a state of normality. We therefore decided to synthesise multifunctional drugs which may halt the apoptotic neuronal breakdown process and eliminate the signs and symptoms of diseases such as AD and PD. This would happen by: (a) Inhibiting the MAO-A and/or MAO-B enzymes thus allowing increase in dopamine levels in the CNS and reducing the levels of the highly oxidative products produced by the activity of these enzymes, and (b) Inhibiting apoptotic processes to halt neuronal cell death. With this in the mind, the propargylamine portion of selegiline, the fluorophenyl function of safinamide, and isatin were used as pharmacophoric groups and incorporated into one structure in an attempt to design potent, non-peptide, selective inhibitors of caspases-3 with MAO-A and/or MAO-B inhibitory properties.

Synthesis and enzymatic assays on recombinant human MAO-A, MAO-B and caspase 3 enzymes were performed to determine the potential of the designed compounds as multifunctional neuroprotective therapeutic agents for the treatment/prevention of neurodegenerative disorders.

5.2 SYNTHESIS AND CHARACTERISATION

Seven novel compounds were successfully synthesised utilising multistep processes. The synthesis of 5-chlorosulfonyl isatin was accomplished starting from the commercially available isatin in two steps, which were, sulfonation using tetramethylene sulfone and chlorination with

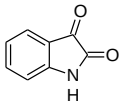
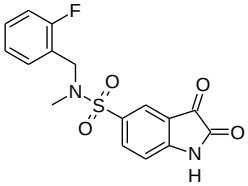
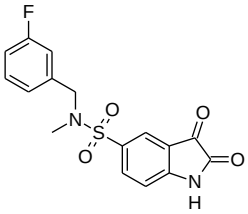
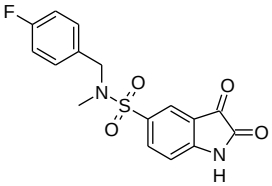
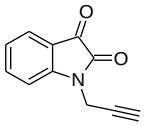
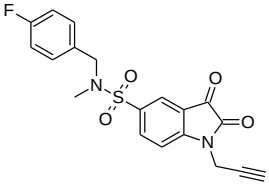
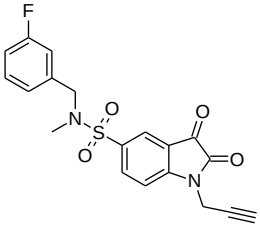
$POCl_3$. Next 5-chlorosulfonyl isatin was conjugated to the fluorophenylamine derivative with the fluoro-function at either the ortho, meta or para position through a nucleophilic substitution reaction on the chlorosulfonyl position. The resultant compounds were coupled on the *N* position of the isatin function with propargyl bromide, using a microwave synthesis procedure, in a nucleophilic substitution reaction.

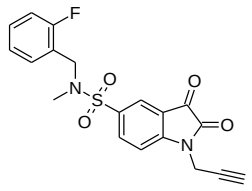
The synthesis of all compounds resulted in moderate to low yields that could be attributed to the formation of various unidentified impurities. Varying success has thus been achieved with the synthesis of these compounds and its purification. Solubility and multiple reactions taking place made it difficult to obtain the compounds in absolutely pure form and optimization of these reactions for further use are thus essential. Flash column chromatography was mostly used in the purification of the compounds. To confirm the chemical structure of the compounds NMR was used and the ^{13}C NMR showed characteristic signals including the signals of the two carbonyls on isatin which appeared between 160 -190 ppm thus indicating the presence of the isatin moiety. The propargyl moiety which was present in compounds **5**, **7**, **8** and **9** was identified with the signals for its CH and CH_2 groups which appeared at around 4.10 and 2.50 ppm respectively. The fluorophenylamine function in the synthesized compounds which played a significant role in their multifunctional activity was identified in the aromatic region between 6.5 ppm and 8.5 ppm for all the novel compounds The IR spectra was determined and showed the characteristic signals and MS confirmed the molecular masses of the compounds.

5.3 BIOLOGICAL EVALUATION

In the biological evaluation, recombinant human MAO-A, MAO-B and caspase 3 enzymatic assays were performed to determine the activity of the novel compounds at an enzymatic level. The activity of test compounds was measured against various controls, clorgyline and selegiline for inhibition of MAO-A and MAO-B respectively, and Z-VAD-FMK for caspase 3 inhibition. The inhibition percentages for these compounds were calculated and plotted against the logarithm of the inhibitor concentrations to obtain a sigmoidal dose-response curve and consequently the IC_{50} value was obtained by calculating the concentration of the inhibitor which resulted in a 50 % inhibition of the enzyme. In order to compare the MAO and caspase 3 inhibitory activity of the synthesized compounds they were tested in identical procedures.

Table 5.1: Summary of compounds synthesised and tested with their IC_{50} values (μM) and maximum percentage MAO and Caspase 3 inhibition.

Compounds		IC_{50} for MAO-A	IC_{50} for MAO-B	IC_{50} for Caspase 3
No	Controls	0.001 (Clorgyline)	0.36 (Selegiline)	40 (Z-VAD-FMK)
0		11	12.07	24.5% inhibition at 100 μM
2		21.63	30% inhibition at 100 μM	60.92
3		22.75	8.329	25.08
4		8.259	5.963	29.26
5		30.91	35.19	18% inhibition at 100 μM
7		4.306	20% inhibition at 10 μM	26% inhibition at 100 μM
8		10.96	70.58	51.94

9		9.434	128.2	44.74

With regards to MAO-A inhibition, all compounds tested showed meaningful IC_{50} values (IC_{50} = 4 – 30 µM). Regarding MAO-B inhibition, compounds **0**, **3**, **4**, **5**, **8** and **9** showed significant IC_{50} values (IC_{50} = 5 – 35 µM), with compounds **2** and **7** showing low or no inhibition (IC_{50} > 100 µM). Compound **1** and **6** were not evaluated as they were only intermediates and not part of the final series of compounds to be evaluated. With regards to caspase 3 inhibition, compounds **3** and **4** showed excellent inhibitory activity (IC_{50} = 25 – 29 µM), compounds **2**, **8** and **9** showed good inhibitory activity (IC_{50} = 44 – 60 µM) and compounds **0**, **5** and **7** exhibited low or no inhibition (IC_{50} > 100 µM).

5.4 CONCLUSION

Neurodegenerative diseases are considered to be multifactorial in nature and taking the complex nature of these disorders into consideration, multi-target directed ligands may present as a better option to treat these disorders than the classic 'one molecule, one target' approach. The combination of inhibition properties, selective caspases-3 inhibition with MAO-A and MAO-B inhibition, makes the novel structures excellent drug candidates for possible treatment/prevention of neurodegenerative disorders.

The synthesized compounds showed inhibition of the MAO-A, MAO-B and caspase 3 enzymes in low to high micro molar concentration. Compounds **3** and **4** are new lead compounds to design multifunctional neuroprotective agents because they have good inhibitory activity towards MAO-A, MAO-B and also excellent inhibitory activity against caspase 3. Compounds **7**, **8** and **9** showed good MAO-A selectivity and do not exhibit good MAO-B and caspase 3 inhibitory activities. The role of the fluorophenylamine in the synthesized compounds was significant for their multifunctional activity. The introduction of the propargylamine moiety only increased the MAO-A inhibitory activity. For future studies it is recommended to test other functional groups for binding activity in the caspase primary aspartic acid binding pocket (S_1) to consider improvement in the multifunctional activity. In the process of apoptosis there are different apoptotic mechanisms involved. Although compounds **7**, **8** and **9** did not show good caspase 3 activity, they can be tested for general anti-apoptotic activity because the propargylamine functional group in these

compounds might function in a completely different manner than what was tested for in this assay (Zindo, Joubert & Malan 2015).

These compounds thus have potential as novel multifunctional neuroprotective agents and further investigation is necessary to determine their maximum benefit in the treatment of neurodegenerative disorders.

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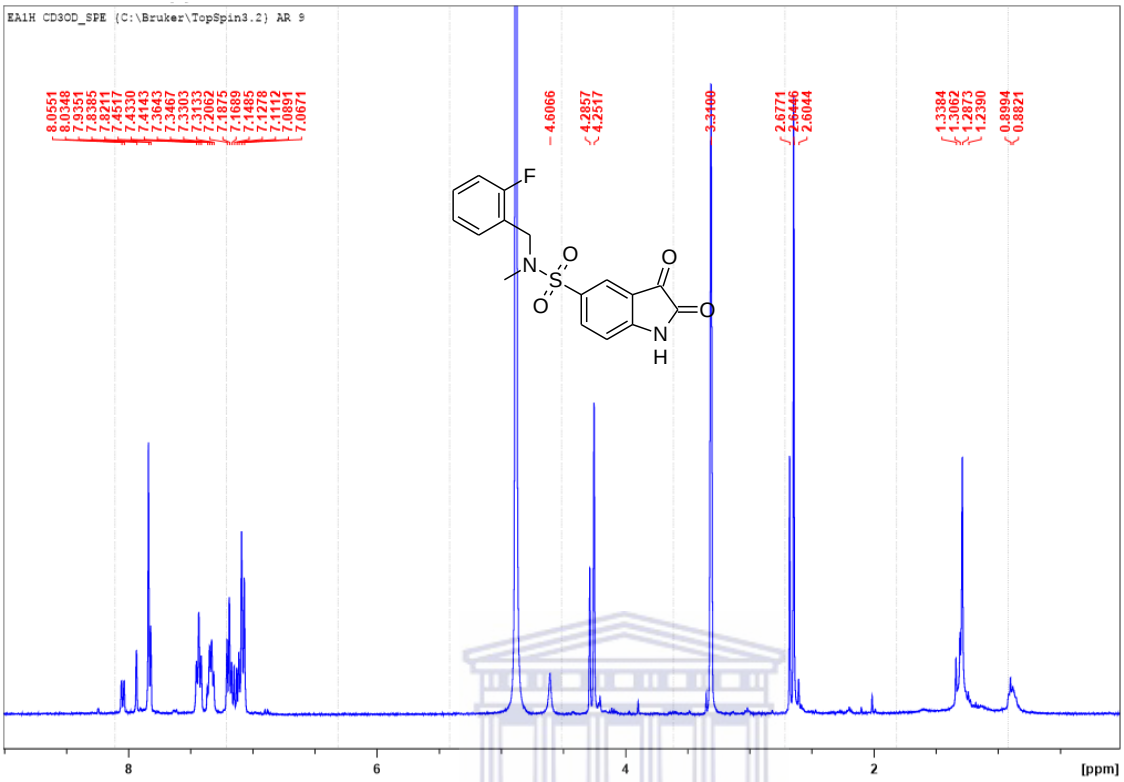
Appendix

SPECTRAL DATA:

^{1}H-NMR, ^{13}C-NMR, MS and IR

Spectrum 1:



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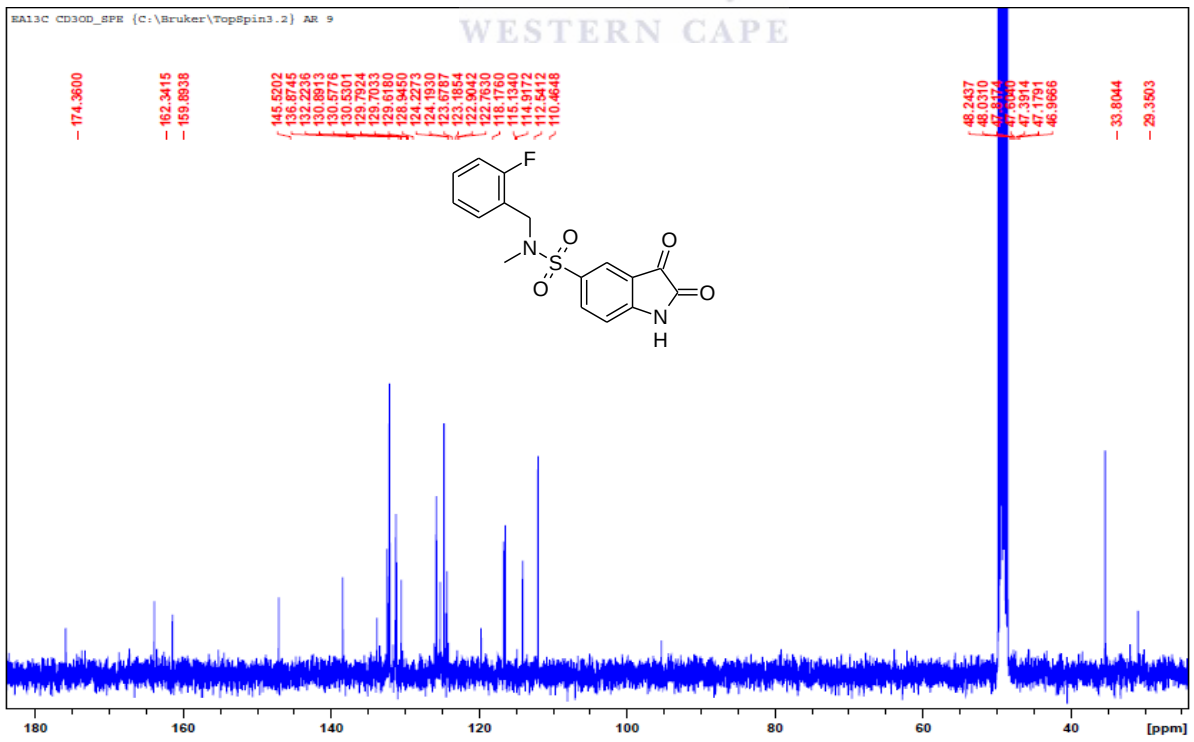

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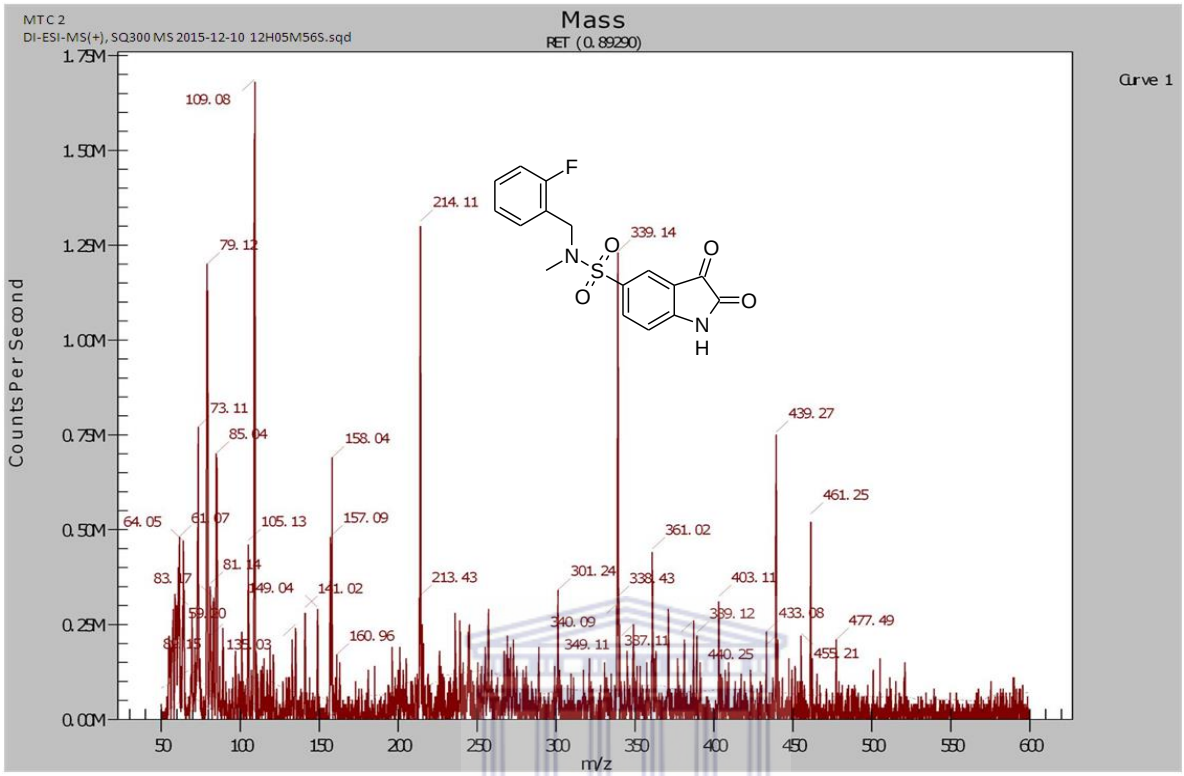

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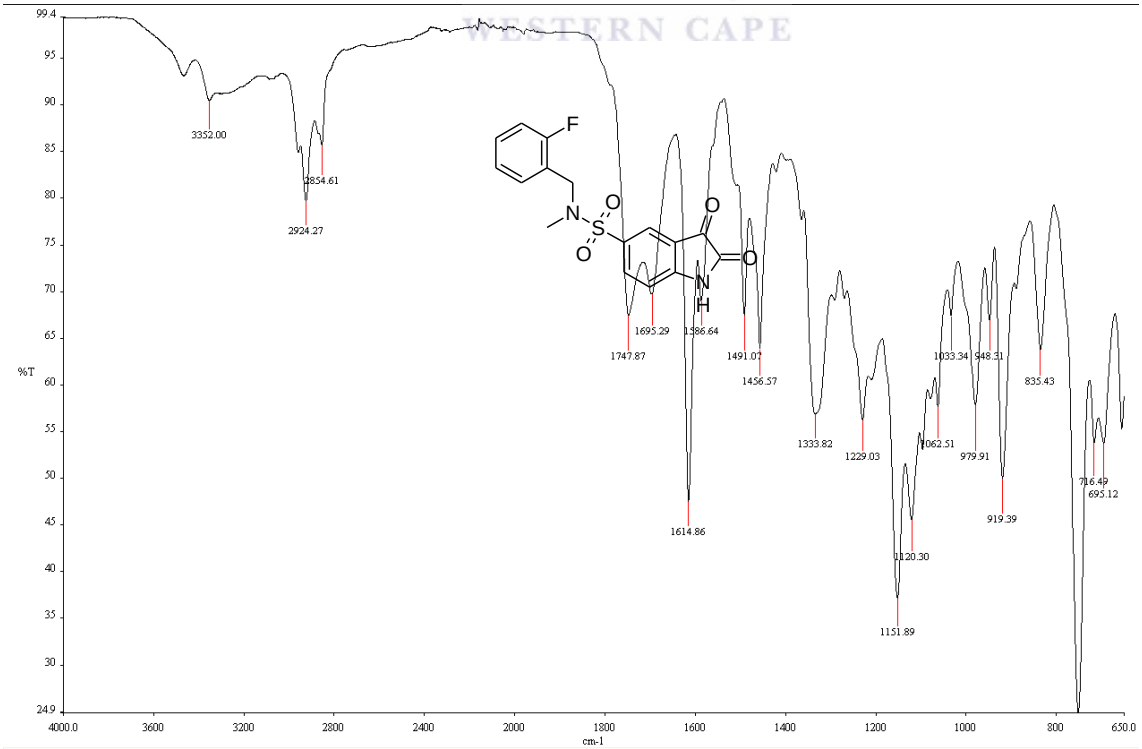

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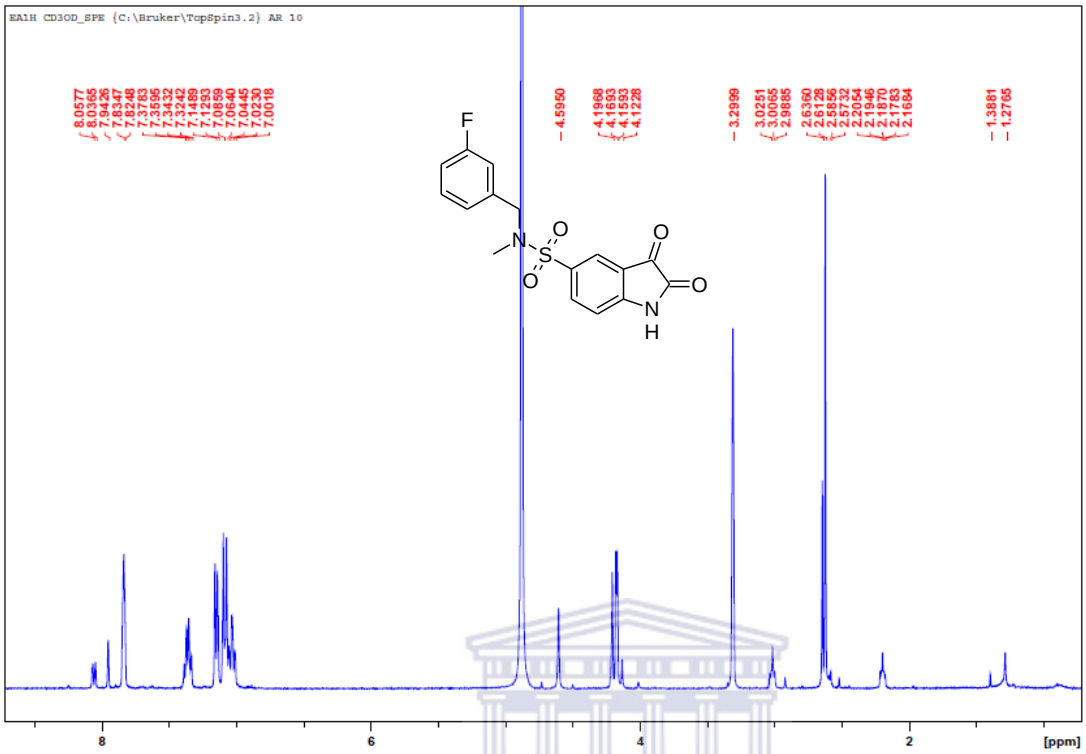

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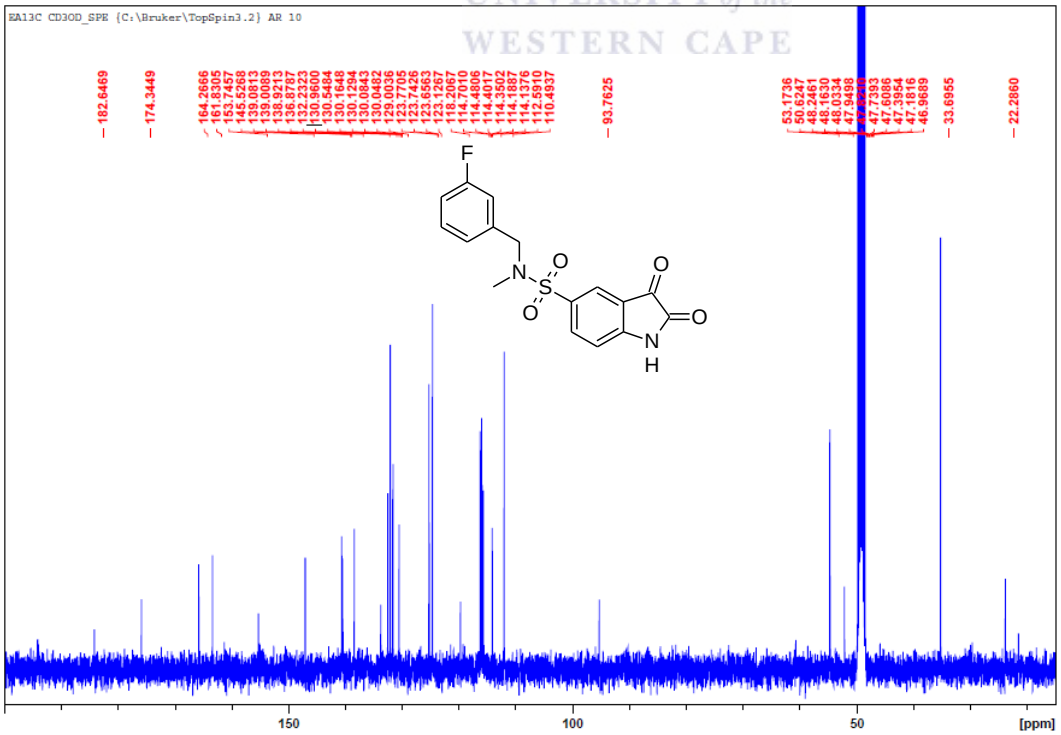

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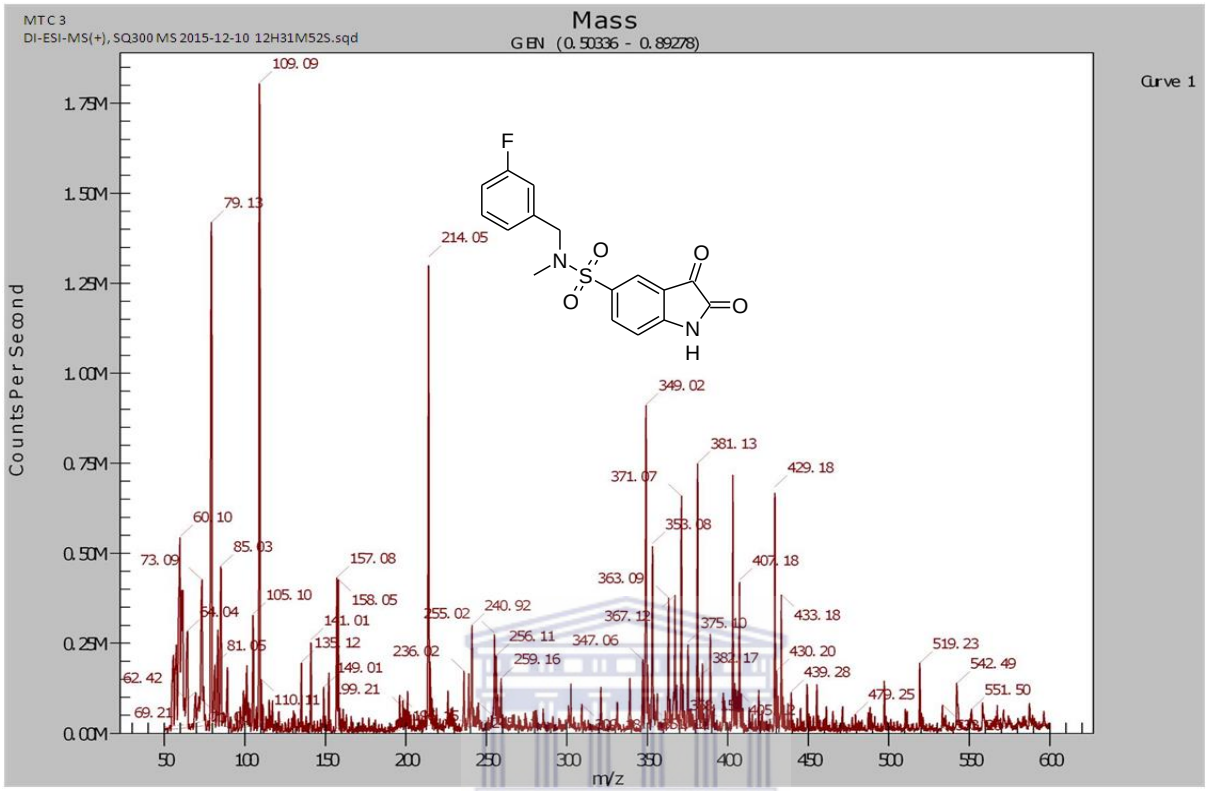

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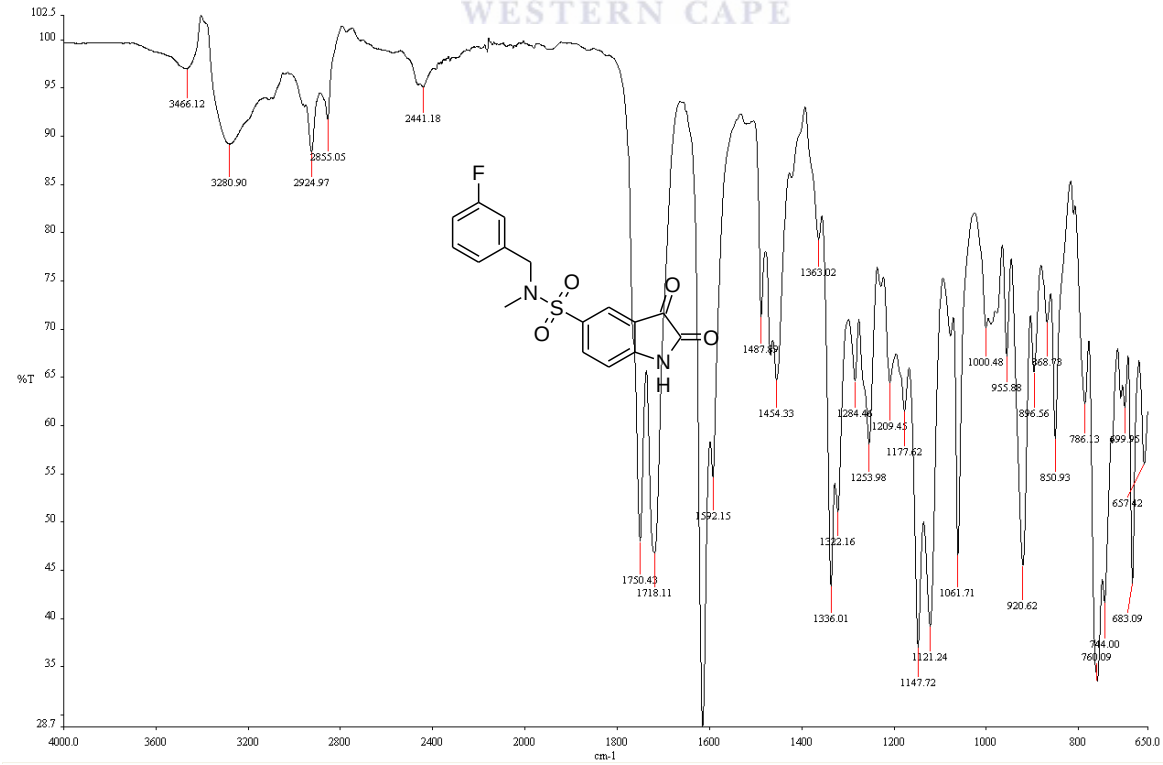

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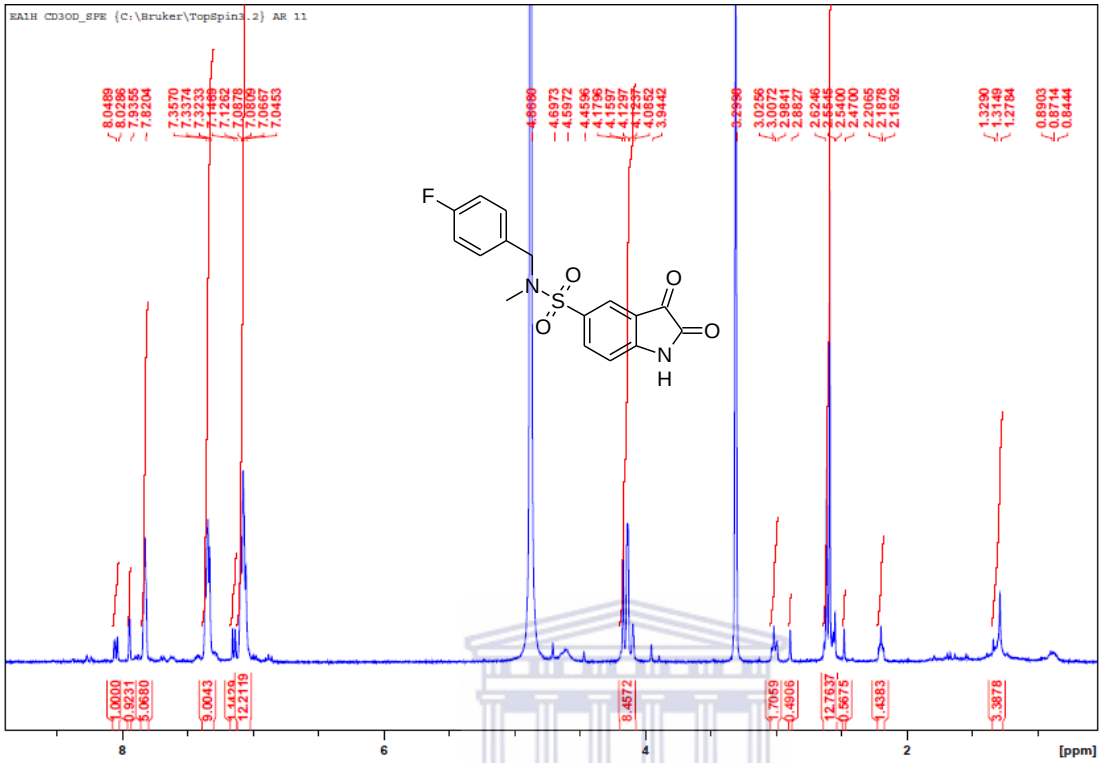

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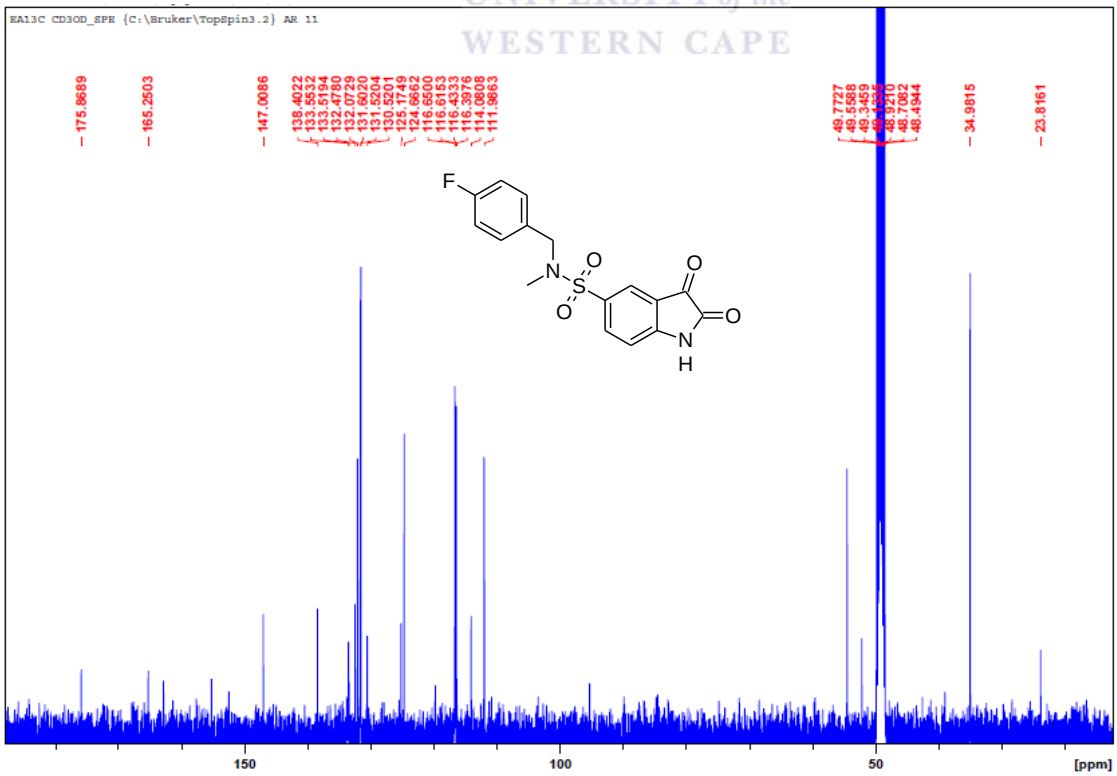

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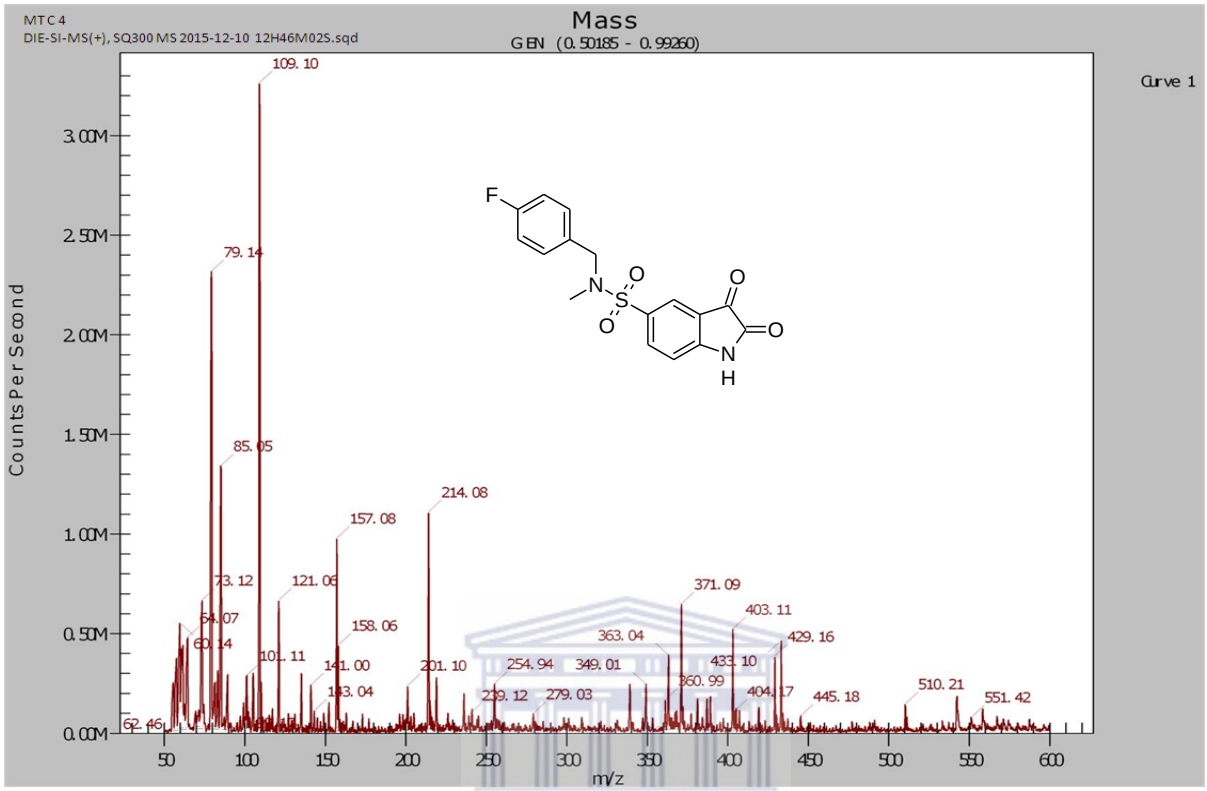

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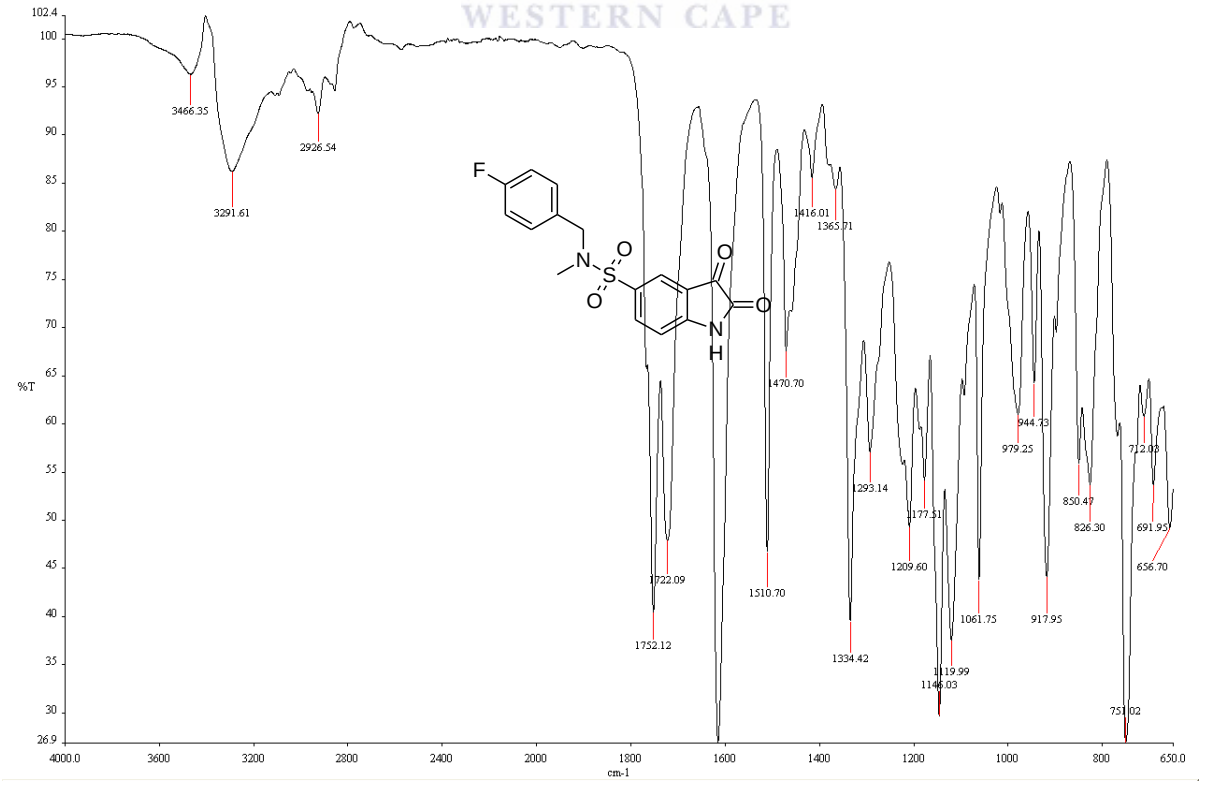

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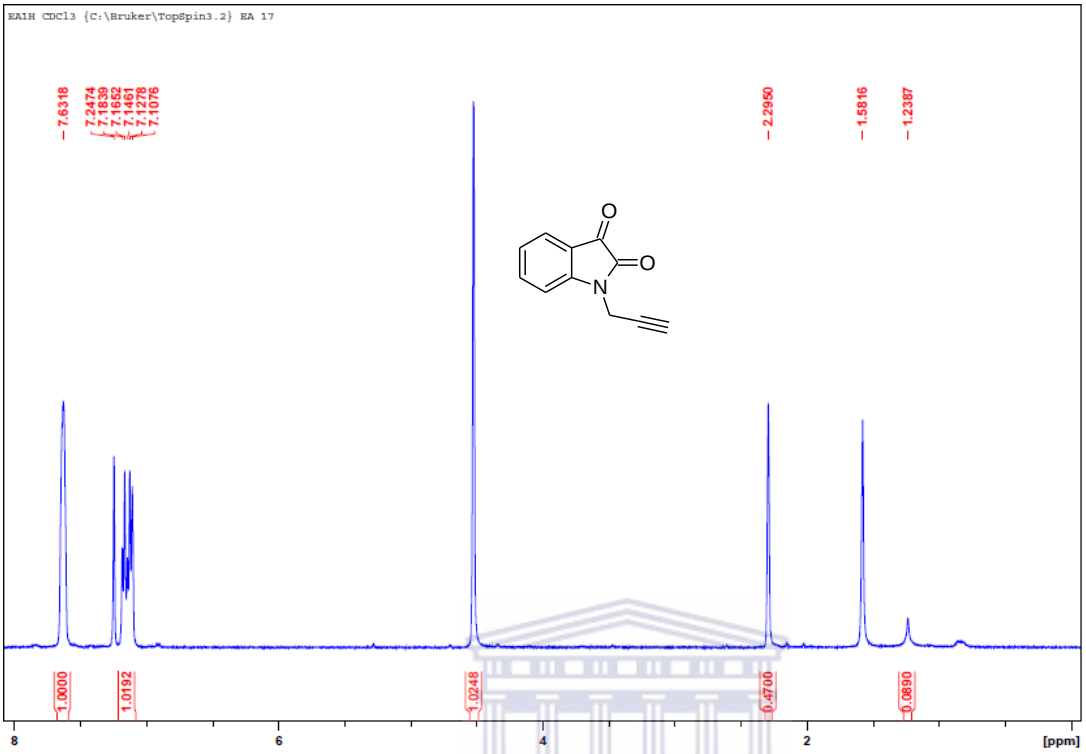

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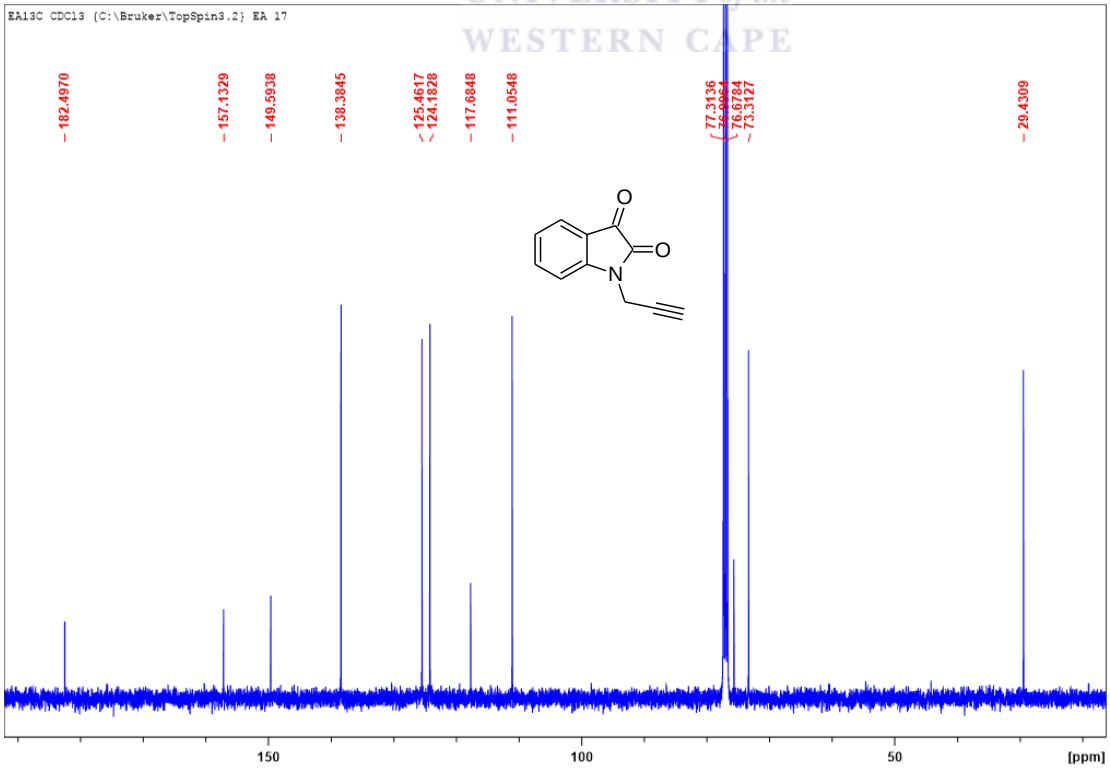

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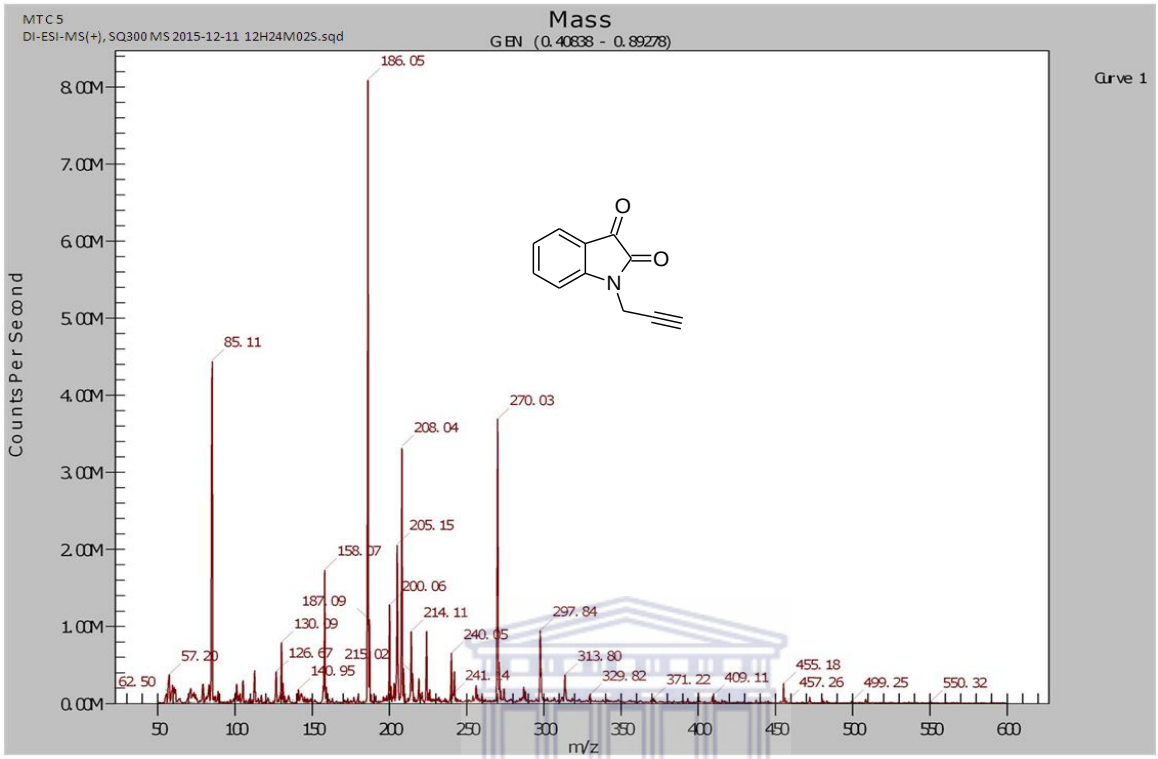

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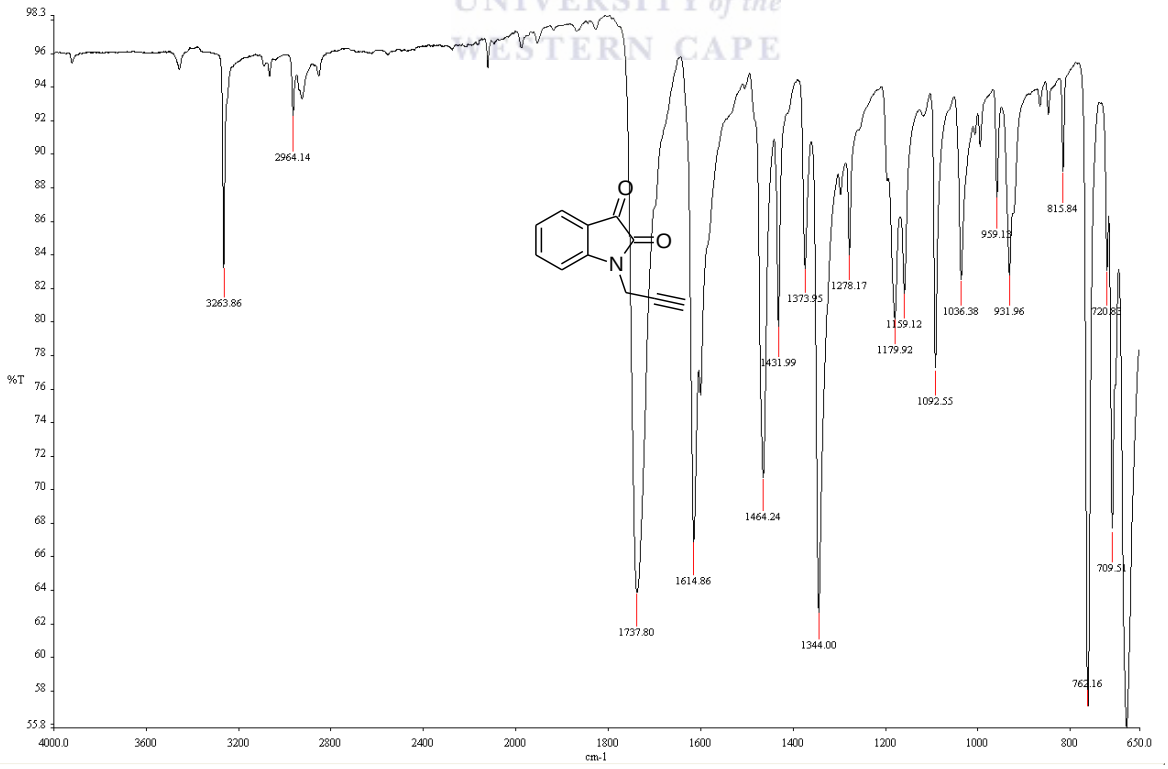

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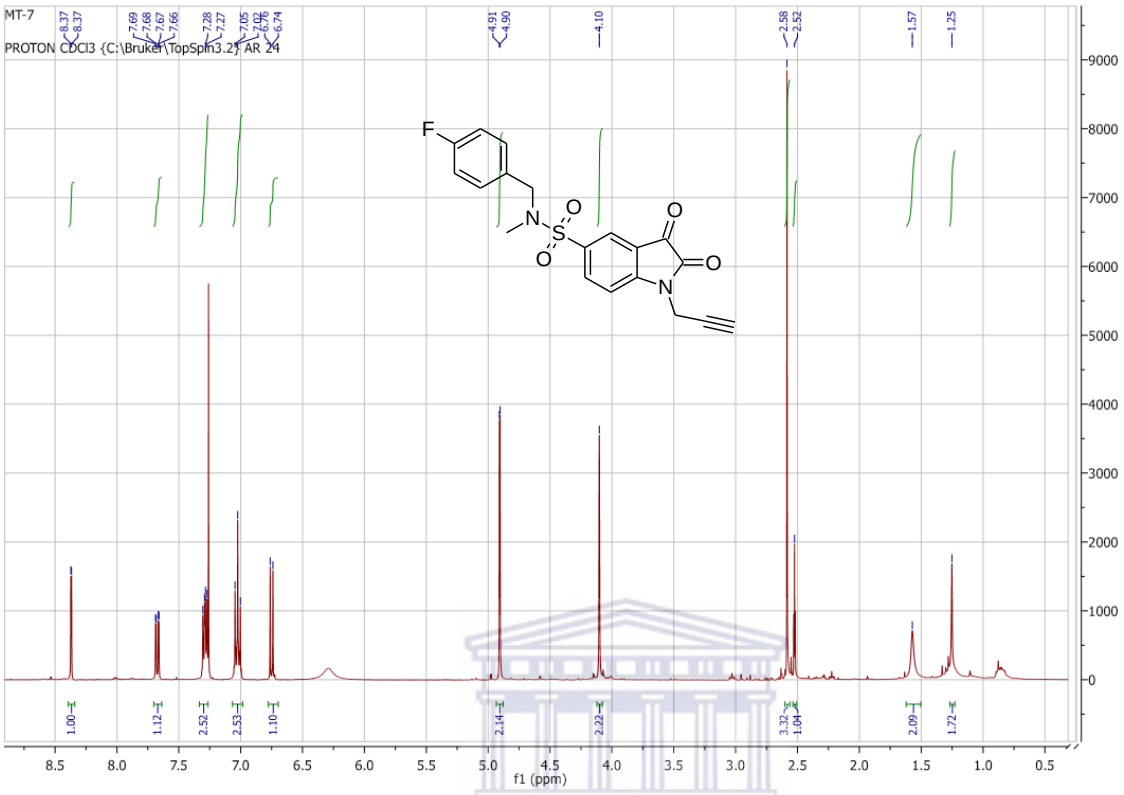

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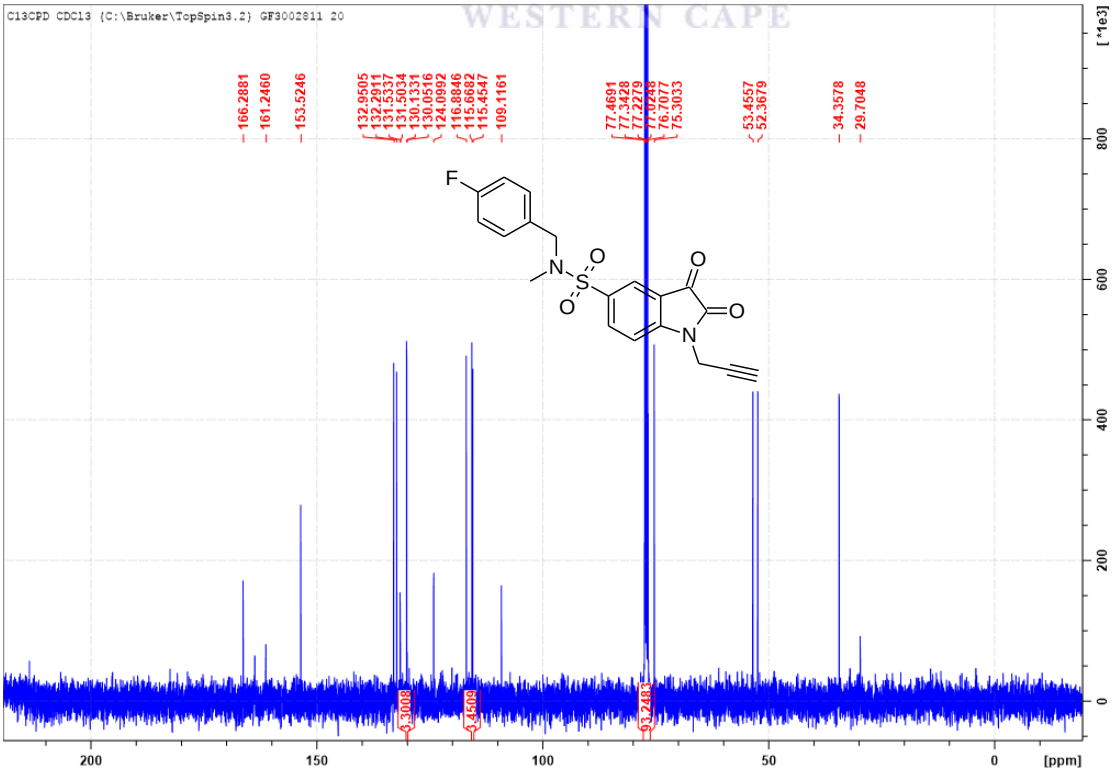

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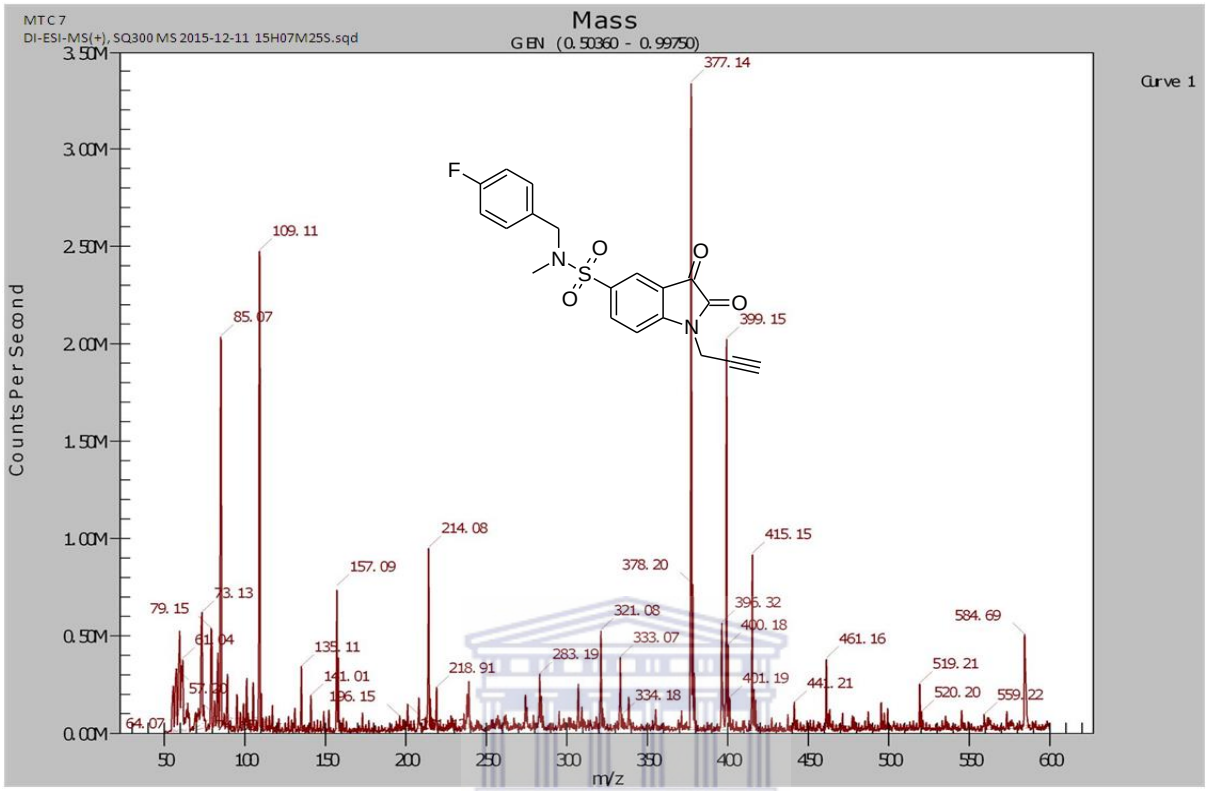

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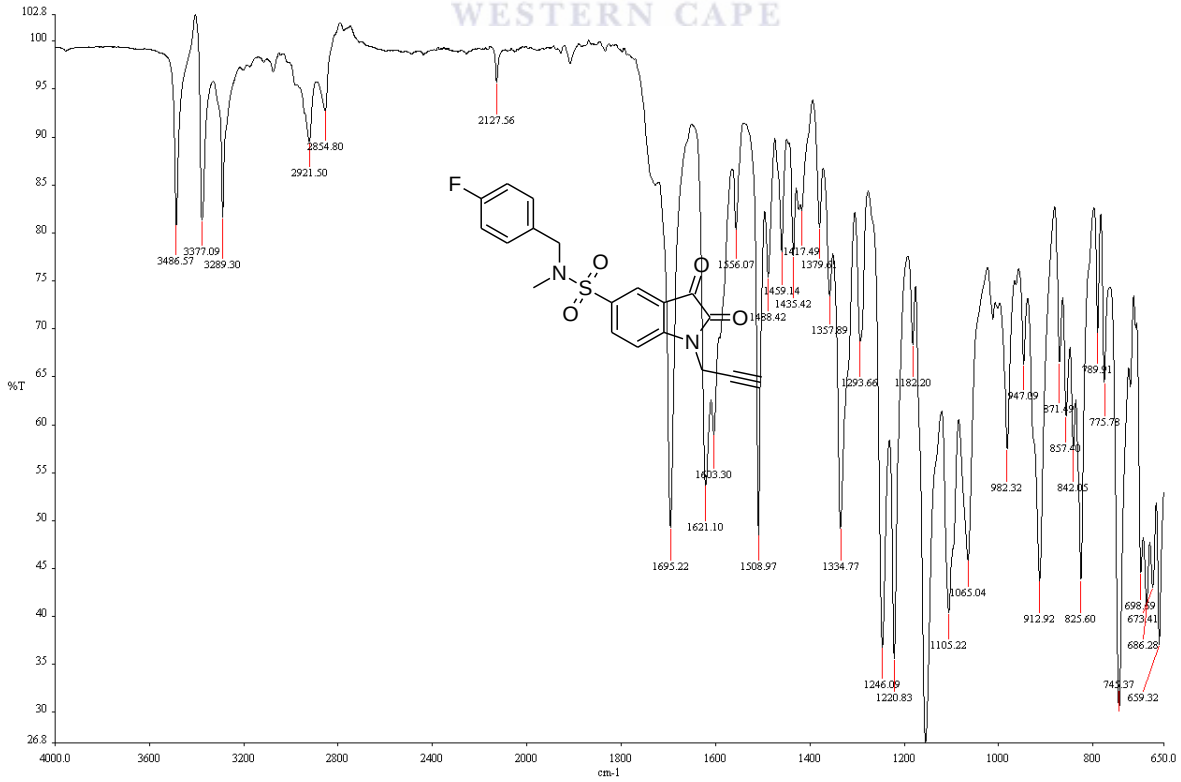

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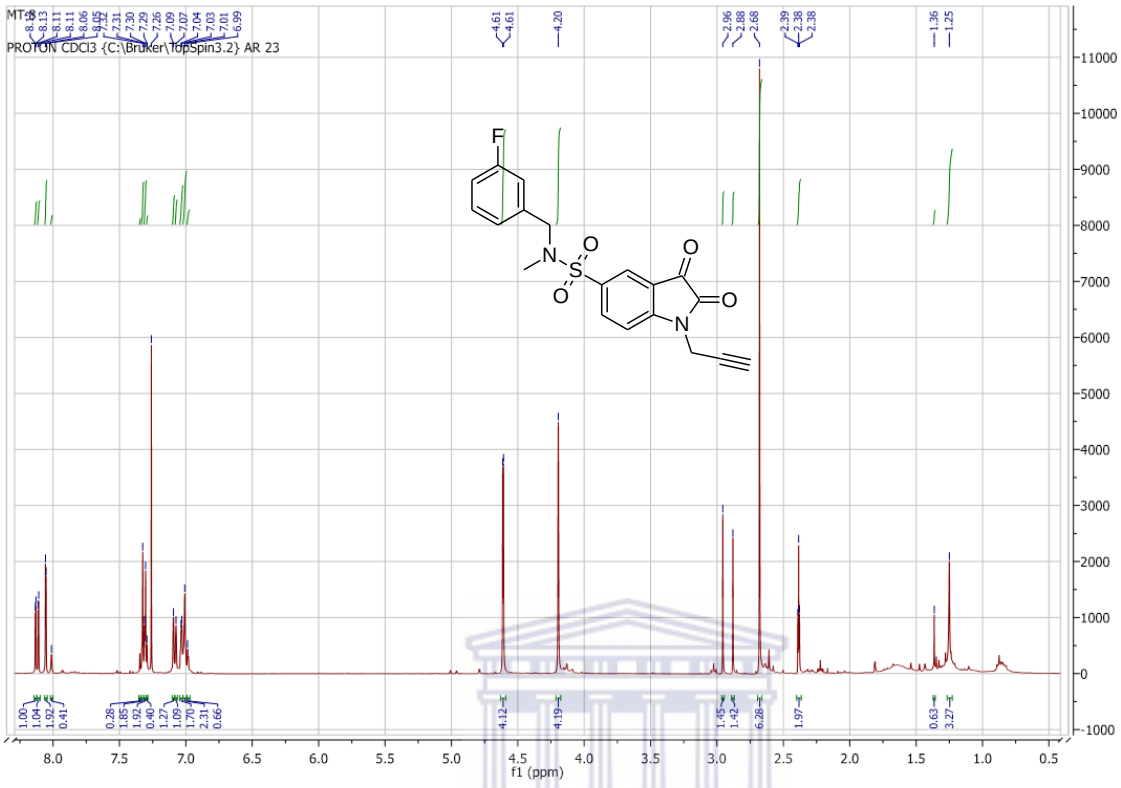

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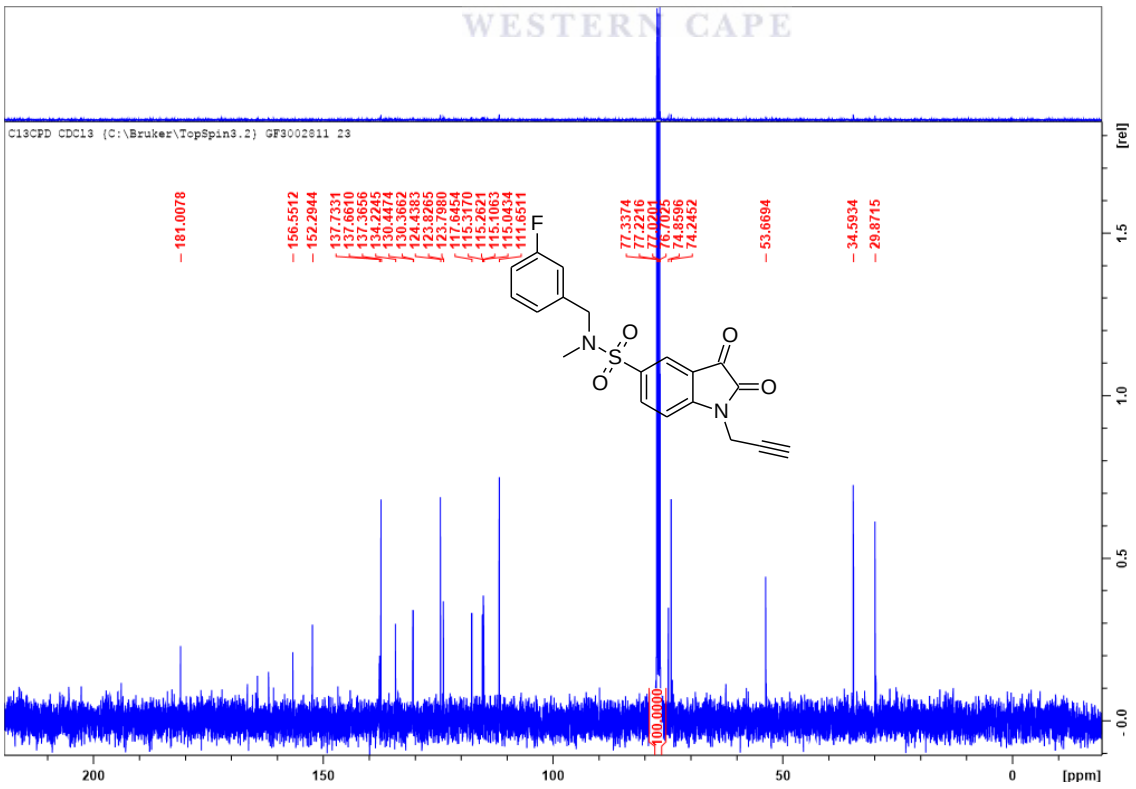

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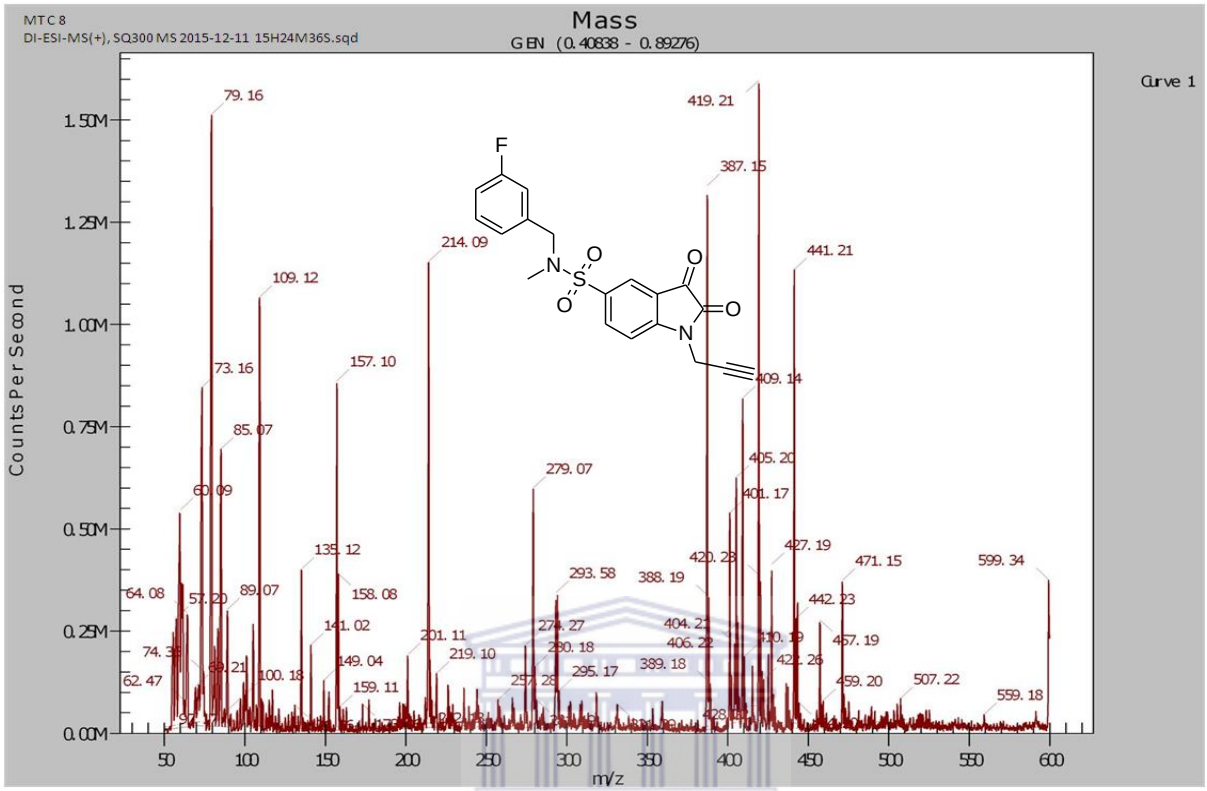

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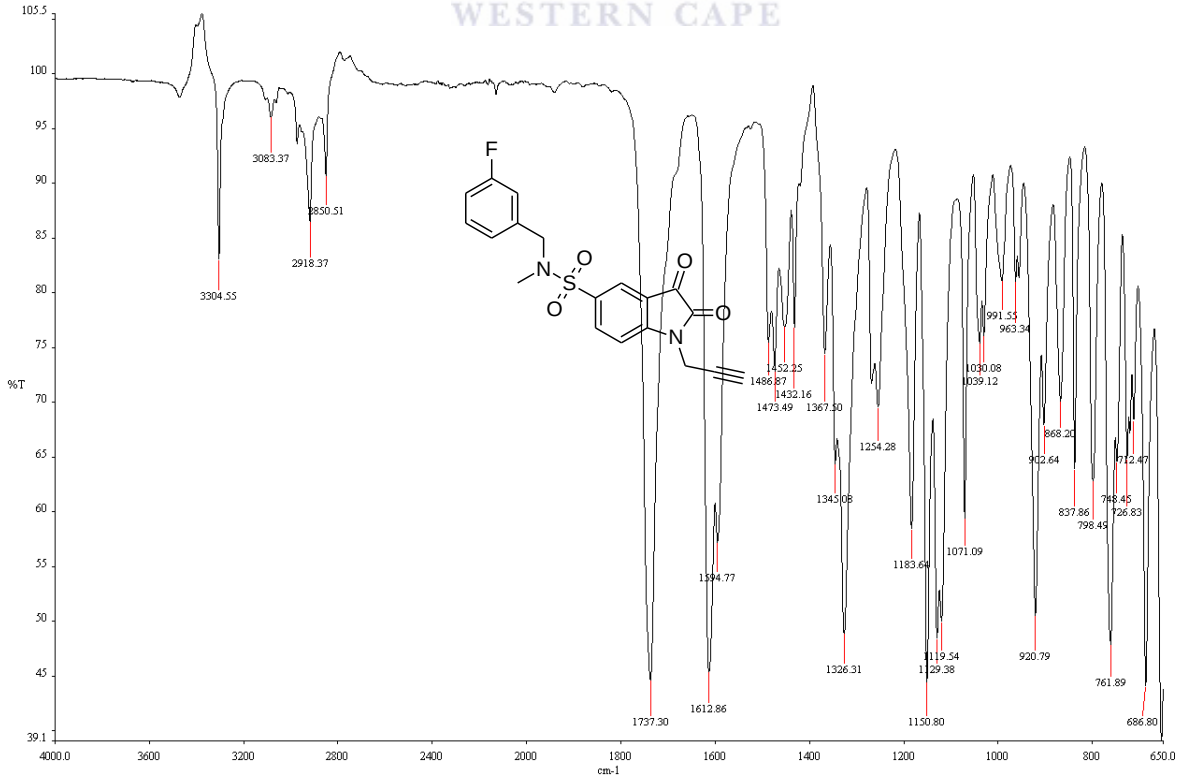

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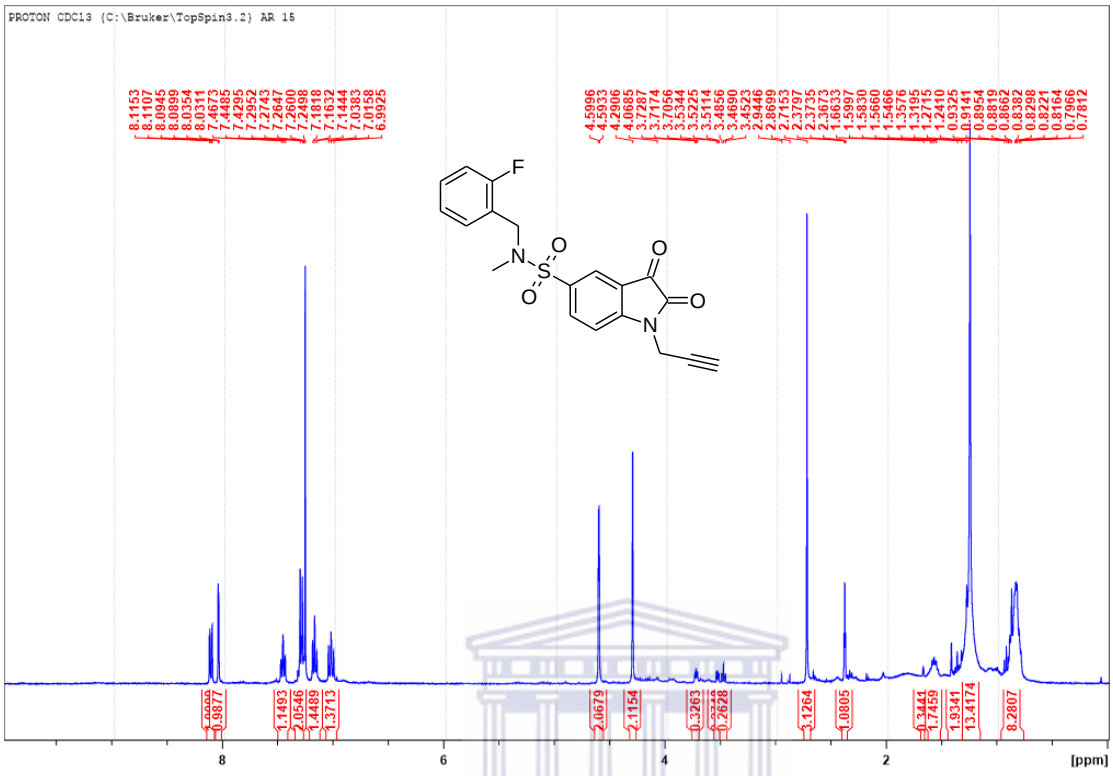

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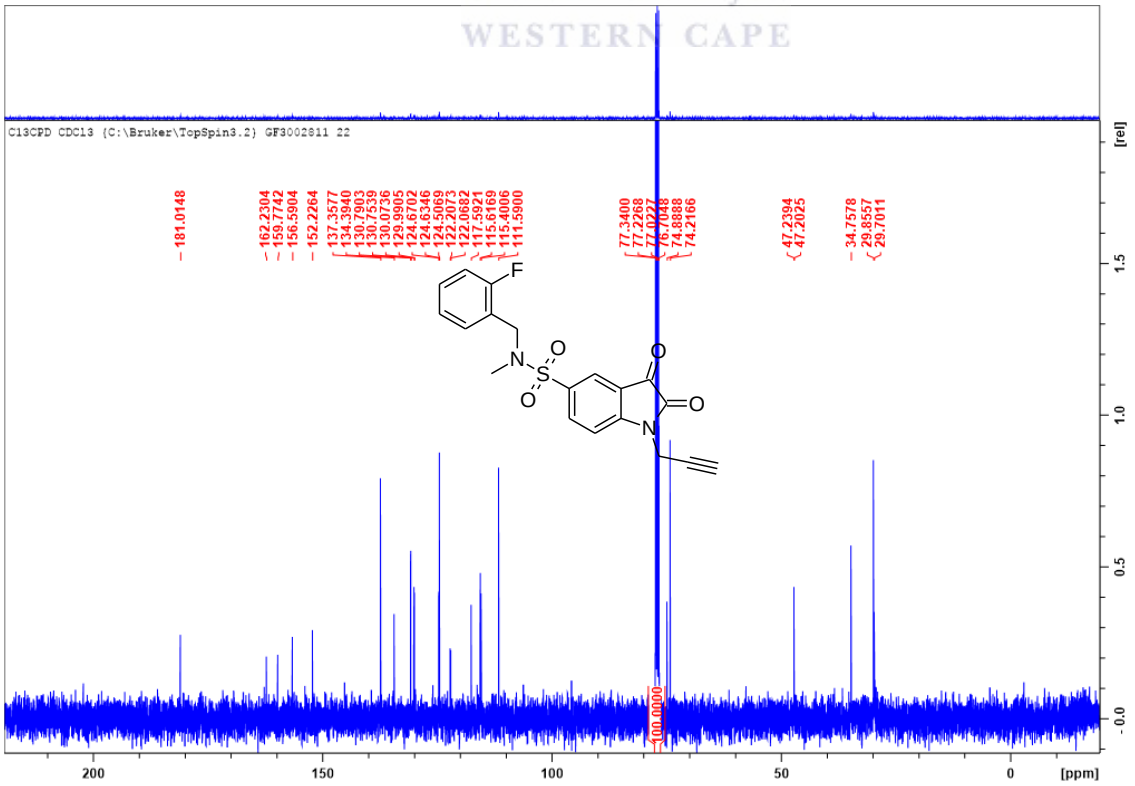

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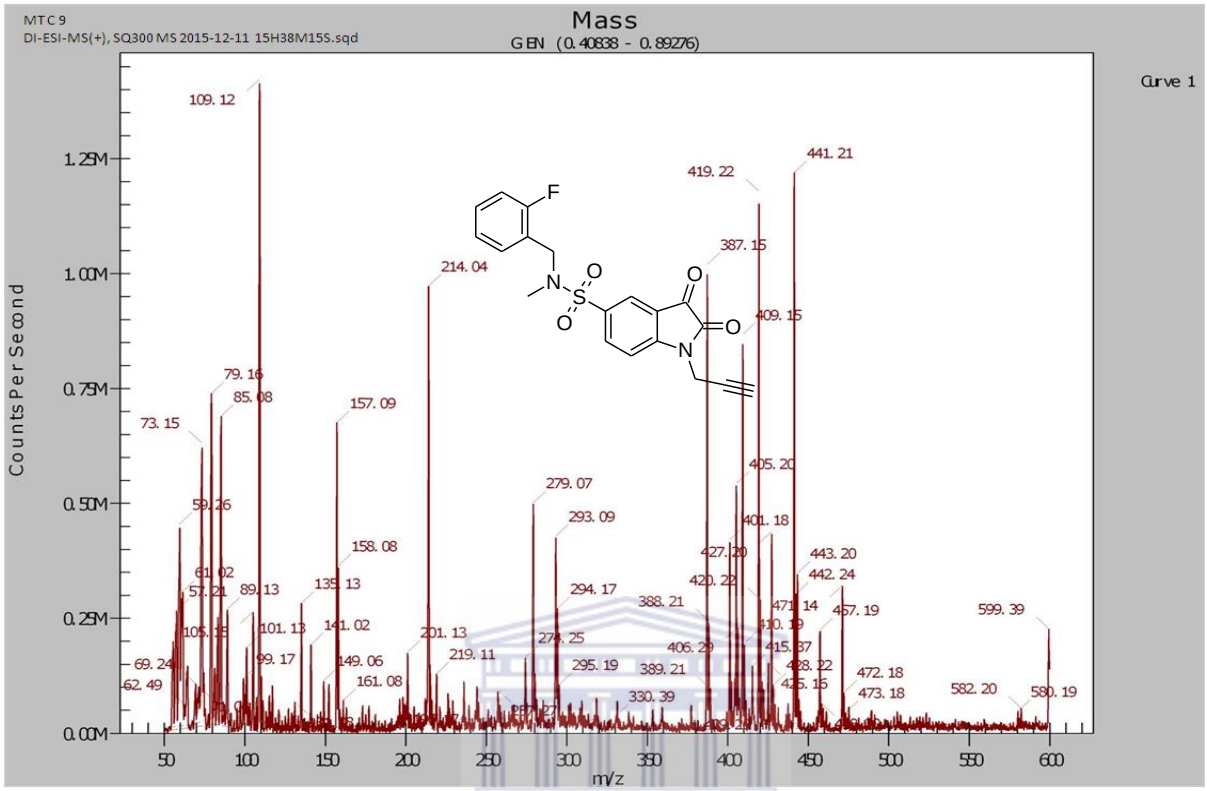

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