Design, Synthesis and Evaluation of Indole Derivatives as Multifunctional Agents against Alzheimer's disease.

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in

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To my parents Peter and Theodora Denya,

And my sisters Ivy, Idah and Tracy Denya.

ABSTRACT

Alzheimer's disease (AD) is an age related neurodegenerative disorder characterised by progressive memory loss and cognitive impairment. It is one of several neurodegenerative disorders occurring as a result of a process of programmed cell death known as apoptosis. The process is set off by various stimuli in numerous pathways that ultimately lead to apoptosis. AD stands out as one of the leading causes of death in the elderly in today's society.

The cholinergic hypothesis of AD states that there is an extensive loss of cholinergic neurons in the central nervous system that contributes to cognitive impairment. Acetylcholinesterase inhibitors decrease the breakdown of the neurotransmitter acetylcholine to maintain its post synaptic levels and compensate for the loss of functional brain cells. Amyloid β plaque formation has been shown to be extensively involved in the pathogenesis of AD. The abnormal regulation of the amyloid precursor protein gives rise to plaques that impair neuronal homeostasis and eventually lead to apoptosis. The monoamine oxidase enzymes catalyse the hydrolysis of amine neurotransmitters such as dopamine. The process produces peroxides that cause oxidative stress alongside the depletion of neurotransmitters. Inhibition of monoamine oxidase allows for accumulation of neurotransmitters and reduces the formation of oxidative free radicals to confer neuroprotection.

Recent observations have suggested that dual cholinesterarase (ChE) and monoamine oxidase (MAO) inhibition is likely to be more effective against AD as observed in ladostigil. Herein we designed a series of compounds structurally similar to ladostigil, possessing an indole ring, propagylamine, carbamate and urea moieties. The main objective was to improve the inhibitory profile of existing molecules as well as eliminate the stereochemistry associated with rasagline and more recently, ladostigil. We also sought to improve the metabolic stability of carbamate derivatives.

The synthesis of the compounds was carried out by nucleophilic substitution of an acyl chloride to the indole ring followed by the substitution of propargylamine moiety at position 1. Eight compounds were successfully synthesised. For structural characterisation purposes; NMR, MS, and IR were determined. Thereafter *in vitro*

assays were carried out on four enzymes (MAO-A, MAO-B, acetyl- and butyrylcholinesterase) to ascertain the inhibitory capacity of the compounds. Molecular modelling was carried out using MOE to visualise binding interactions in the enzymes. The results showed that the compounds occupied the active sites of the enzymes and had interactions with significant amino acids.

These assay results indicate that the compounds could act as dual non-selective cholinesterase inhibitors to slow down the hydrolysis of acetylcholine and hence increase its post synaptic levels. The cholinesterase assays provided a proof of concept regarding the ability of the carbamoyl moiety to inhibit the cholinesterase class of enzymes. We have identified the urea moiety as an improvement to the carbamoyl portion. The presence of the propargylamine moiety did not influence ChE activity negatively.

The compounds were able to inhibit both isoforms of MAO. The results agree with the reported potential of the propargylamine moiety against MAO. . Compounds **2B** and **4B** had good IC_{50} values. Comparison with ladostigil proves that some of our compounds may serve as lead compounds for further development. Compound **2B** was particularly a good inhibitor of all 4 enzymes (IC_{50} AChE = 3.70 µM, BuChE = 2.82 µM, MAO-A = 4.31 µM and MAO-B = 2.62 µM). The results of a chemical stability test showed that **2B** is more stable than the carbamate linked compound **4B**. Compound **2B** would probably prove more chemically stable than ladostigil. This compound can thus be considered for optimisation and perhaps development as a multipotent drug molecule against AD.

This study set out to demonstrate that the carbamoyl/urea substituted indole propargylamine derivatives have great potential in alleviating the cholinergic symptoms of AD. They have the potential to reduce the rate of amyloid plaque formation in the brain, confer neuroprotection by inhibiting the MAO catalysed breakdown of amine neurotransmitters and reduce the progression of AD.

DECLARATION

I declare that *Design, synthesis and evaluation of indole derivatives as multifunctional agents against Alzheimer's disease* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Student: Ireen Denya Date: 20/07/2017

Signed: 

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to my parents, Peter and Theodora Denya for allowing me the opportunity to further my studies, for moral and financial support, for believing in me and mostly for letting me find my way, I love you.

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DESIGN, SYNTHESIS AND EVALUATION OF INDOLE DERIVATIVES AS MULTIFUNCTIONAL AGENTS AGAINST ALZHEIMER'S DISEASE.

Ireen Denya

Keywords

1. Alzheimer's disease
2. Indole
3. Propargylamine
4. Acetylcholinesterase
5. Butyrylcholinesterase
6. Monoamine oxidase
7. Ladostigil
8. Multi-target directed ligands

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CHAPTER 1

1. INTRODUCTION

1.1 BACKGROUND

Alzheimer's disease (AD) is an age related neurodegenerative disorder characterised by progressive memory loss and cognitive impairment (Goedert and Spillantini, 2006). Only 1 in 4 of the almost 47 million cases of AD worldwide have been diagnosed. Although highly prevalent in Western Europe, the disease is essentially a global problem incurring an estimated cost of $818 billion in 2015 and expected to reach $1 trillion in 2018 (Prince, *et al*., 2015). As witnessed by the statistics, AD poses a great burden both to healthcare professionals and the economy with foreseen exponential growth.

AD is one of several neurodegenerative disorders occurring as a result of a process of programmed cell death known as apoptosis. The process is set off by various stimuli in numerous pathways that ultimately lead to apoptosis (Spencer and Schaumberg, 2000). Since its discovery in the early 1900s, extensive research has been carried out to identify and understand the complex pathophysiology of the disease. Risk factors that increase the likelihood of developing AD have been identified; these include age, genetics and family history (Prince *et al*., 2015). As a result disease progression varies widely among individuals.

Over the years several pathways have been indicated in the pathology of the disease. One of the most consistent indicators of AD is a loss of the cholinergic enzymes acetyltransferase and acetylcholinesterase (AChE) that serve as distinct markers of cholinergic progression (Perry *et al*., 1977). This loss of neurons together with a decline in mental test scores led to the cholinergic hypothesis of cognitive impairment (Perry *et al*., 1978a; Perry *et al*., 1978b). This states that there is an extensive loss of cholinergic neurons in the central nervous system that contributes to impairment in the cognitive and memory symptoms of the affected person (Bartus, 2000).

The amyloid hypothesis of AD has been the dominating model of pathogenesis in AD and significant focus has been applied on the reduction and/or

modulation of amyloid β (Hardy, 2006). Neurodegeneration is linked with the production of Aβ from the amyloid precursor protein (APP). This gives rise to Aβ plaques whose removal does not reverse the damage or slow the progression of AD(Morris and Mucke, 2006).Recent reports have suggested that Aβ is a physiological antioxidant whose property is modified with age (Atwood *et al*., 2003).

Reactive oxygen species seem to play an important role in the pathophysiological cascade leading to AD (Christen, 2000). Oxygen radicals are chemically unstable and highly reactive compounds formed during normal cellular metabolism. Due to their high reactivity, they may result in cellular and tissue damage when they reach an imbalance with their indigenous species (Christen, 2000; Halliwell and Gutteridge, 2007). Neurons are vulnerable to attack by free radicals for several reasons. These include a low glutathione content [glutathione is a natural antioxidant] (Cooper, 1997), the presence of polyunsaturated fatty acids in high quantities (Hazel and Williams, 1990) and the substantial quantities of oxygen needed for brain metabolism (Smith *et al*., 2002). Free radicals produced by reactive oxygen species i.e. peroxides, superoxide, hydroxyl radicals and singlet oxygen accumulate over an individual's lifetime and hence supports the age factor in risk of AD. The free radicals hypothesis is able to account for the vastly heterogeneous nature of AD and the pathophysiology of other neurodegenerative disorders (Christen, 2000).

Indirect evidence implicates nitric oxide in the mechanisms underlying cell death. The toxicity associated with nitric oxide is due to its reactivity to form peroxynitrite, a highly oxidising agent that also degrades to form hydroxyl radicals (Kirkinezos and Morales, 2001; Poderoso *et al*., 1999). The consequent oxidative stress significantly contributes to neuronal cell death in neurodegenerative diseases. Antioxidant therapy prevents neuronal cell death by either inhibiting free radical formation or preventing Aβ toxicity (Uttara *et al*., 2009).

The monoamine oxidase (MAO) enzyme catalyses the oxidative deamination of neurotransmitters and neuromodulators such as dopamine, noradrenaline and 5-hydroxytryptamine as well as some exogenous bioactive monoamines (Youdim and Bakhle, 2006). The enzyme, occurring in two isoforms, produces peroxides that cause oxidative stress alongside depletion of neurotransmitters. Inhibition of MAO permits

accumulation of neurotransmitters and reduces the formation of oxidative free radicals toconfer neuroprotection (Burke *et al*., 2004; Nicotra *et al*., 2004).

With such a broad spectrum of causative pathways, the treatment of AD has proved difficult and complicated. Registered drugs offer only a palliative care by interfering one pathway at a time. The approved treatment options are:

- Anti-cholinesterases or acetylcholinesterase inhibitors decrease the breakdown of the neurotransmitter acetylcholine to maintain its levels. They therefore boost cholinergic transmission in the basal forebrain and compensate for the loss of functional brain cells (Stahl, 2000). Medications currently approved by regulatory agencies to treat the cognitive manifestations of AD and improve quality of life are donepezil (Aricept®), rivastigmine (Exelon®) and galantamine (Razadyne®, Nivalin®). These drugs are reversible inhibitors of the enzyme. Tacrine, the first inhibitor to be approved in1993 was discontinued due to high incidence of side effects, including hepatotoxicity (Bond *et al*., 2012; Watkins *et al*., 1994). Generally the disadvantage of this class of drugs is the modest and temporary benefits and failure to reduce the rate of decline in cognitive or functional capacities over long term (Courtney *et al*., 2004; Giacobini, 2000). Regardless, reversible anti-cholinesterases have significant benefits with plenty of evidence to support their use (Colovic *et al*., 2013).
- Memantine is a moderate-affinity, voltage dependant, uncompetitive NMDA receptor antagonist approved for moderate to severe dementia of the Alzheimer's type. It inhibits excessive calcium influx induced by over-excitation of the NMDA receptor. Memantine, marketed as Ebixa®, Axura®, Namenda® and Memor®, is associated with less serious side-effects such as appetite loss and nausea (Robinson and Keating, 2006).

All of the drugs approved for AD offer a non-permanent relief option towards the disease, as discussed above. With time it has turned out to be reasonable to include multiple therapies to address the varied pathological aspects of AD. The multi-target-directed ligand (MTDL) approach is a promising strategy that yielded ladostigil. Sterling, *et al*., 2002 and Yogev-Falach *et al*., 2006 managed to synthesise and evaluate neuroprotective bifunctional analogue with the aminoindan structure of rasagiline and carbamate cholinesterase inhibitory moiety of rivastigmine. Ladostigil (TV3326) [*N*-

propargyl-(3*R*) aminoindan-5yl)-ethyl-methyl-carbamate] was initially designed for AD in high doses but failed to meet its endpoint in a Phase 2b clinical trial in 2012. It is currently under investigation for mild cognitive impairment due to its neuroprotective, anti-oxidant and microglial activation abilities (Avraham Pharma, 2016).

So far research has managed to produce various combinations in this category featuring two or more of the following properties; monoamine oxidase-, cholinesterase- and amyloid β inhibition, anti-inflammatory activity, metal and iron homeostasis and anti-apoptotic activity (Samadi *et al*., 2012; Silva *et al*., 2011).

1.2 RATIONALE

Currently the only drugs that have shown clinically relevant improvement in AD symptoms are AChE inhibitors such as tacrine and rivastigmine (Weinstock, 1999). These therapeutic agents act by increasing cholinergic transmission. However they do not posses neuroprotective activity, hence the use of MAO inhibitors like rasagiline, selegiline & ladostigil to combat oxidative stress (Youdim, 2013; Harkins *et al*., 1997). These MAO inhibitors have been found to confer neuprotection and studies have gone on to prove that this is independant of enzyme inhibition. For instance the *S*-isomer of rasagiline, TVP1022, does not inhibit MAO-B and yet is neuroprotective (Youdim, 2013). Neuroprotective ability of these molecules was found to be brought about by the propargylamine moiety which posseses neurorescue activity *via* down regulation of the anti-apoptotic B-cell lympoma 2 (BCL-2) family of proteins that control apoptosis. Furthermore propargylamine reduces the expression of APP that contributes to the production of amyloid plaques in the brain (Bar-Am *et al*., 2005).

1.2.1 MULTI-TARGET DIRECTED LIGANDS

Neurodegenerative disorders can be treated in one of two possible ways (**Figure 1.1**). The first approach is the use of more than one drug to treat a particular condition (polypharmacy). Another approach would be the combination of drugs into a single dosage form as opposed to taking them separately, probably as a way of improving patient compliance and / or targeting multiple pathways and finally, through a single drug that may act at more than one site/receptor/system in order to have a synergistic effect (Mdzinarishvili *et al.*, 2005; Youdim, 2010).Research in medicinal chemistry is moving from compounds with single mechanisms to multifunctional compounds in

order to have a multi-target effect and minimise side effects and the need of polypharmacy (Geldenhuys *et al*., 2011).

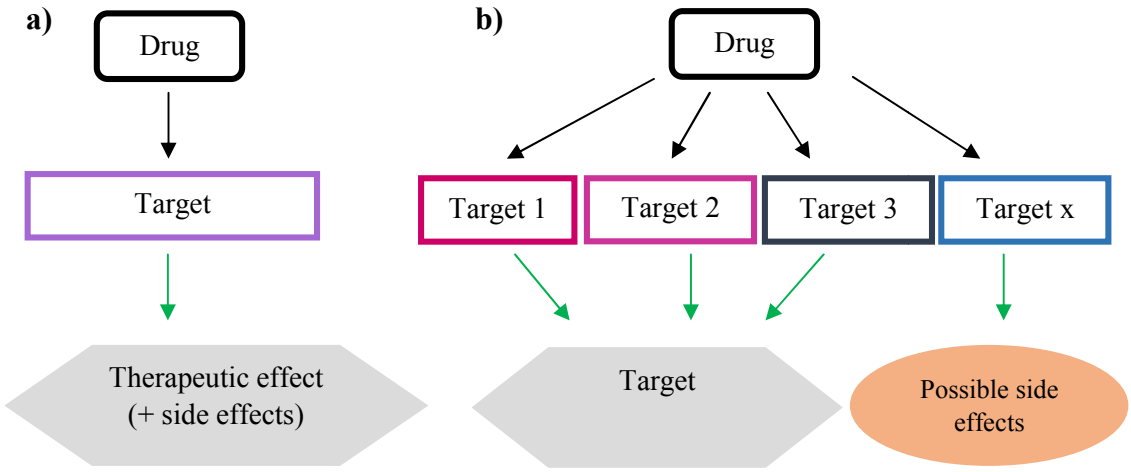


Figure 1.1: a) Target driven drug discovery. **b)** MTDL approach to drug discovery.

MAO has been widely used as a target in the design ofMDTLs for the purpose of treating AD even though many more targets exist. The most popular of strategies is the dual MAO/ChE inhibition which is illustrated in **Figure 1.2** below.

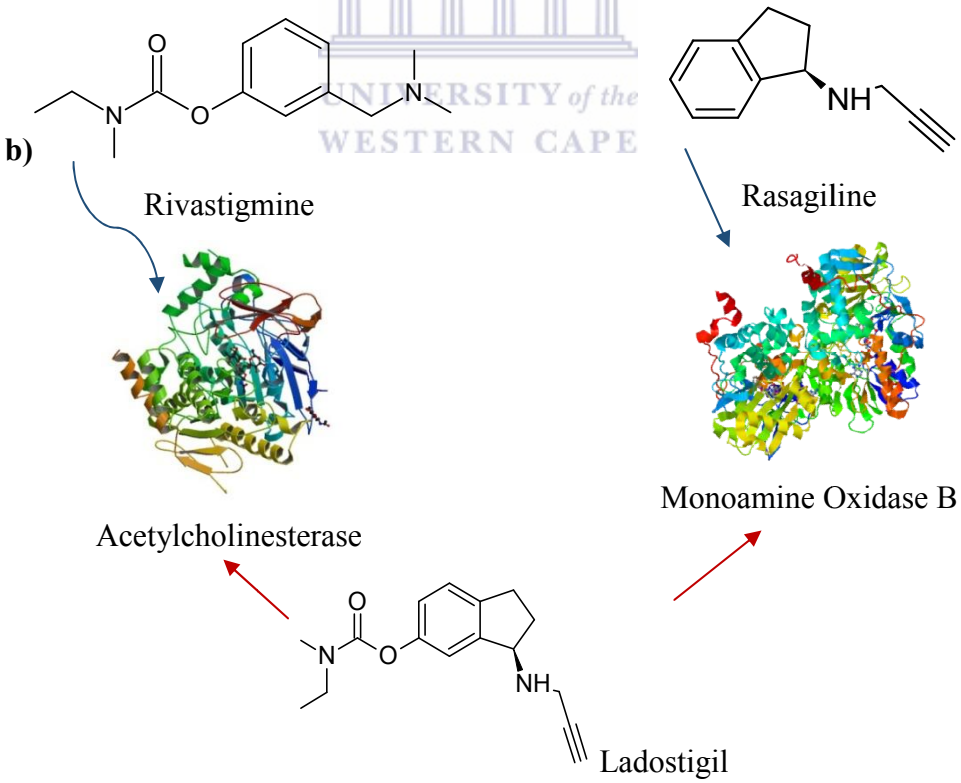


Figure 1.2: Design strategy of the reversible AChE and BuChE and irreversible MAO-B inhibitor ladostigil from parent molecules rivastigmine and rasagiline (Geldenhuys *et al*., 2011; Sterling, *et al*., 2002)

This approach is evident from the development of drugs such as ladostigil, a reversible acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitor and an irreversible MAO-B inhibitor, which was derived from two known neuroactive drugs, namely rasagiline and rivastigmine. The design of ladostigil enables it to block the activity of MAOs and subsequent hydrogen peroxide generation, thus preventing the Fenton reaction and the formation of neurotoxic free radical species. In addition, MAOs' inhibition confers potential antidepressant activity by increasing the levels of dopamine, noradrenaline, and serotonin in the central nervous system (Youdim and Buccafusco, 2005). In addition to this, ladostigil also showed APP processing regulation and mitochondrial membrane potential stabilisation (Bar-Am et al., 2008; Weinreb, 2008). Furthermore, ladostigil proved neuroprotective against oxidative stress-induced apoptosis (Van der Schyf et al., 2007).

1.2.2 INDOLE POLYCYCLIC RING

In the context of the discovery of neuroprotective agents an indole scaffold possesses efficiency in scavenging free radicals and as an antioxidant (Buemi et al., 2013). The indole polycyclic ring has been investigated in the design of potential agents for neurodegenerative disorders. The indole moiety was used to produce AChE-induced Aβ aggregation inhibitors (**Figure 1.3**). The indole ring (as a part of a heterodimer in which tacrine was attached via a suitable linker) was shown to occupy the entrance of the cationic gorge of the AChE enzyme and interact with Trp 286 (Cavalli, *et al*., 2008). This dual action of the compound allowed for the inhibition of major pathways that lead to neuronal death.

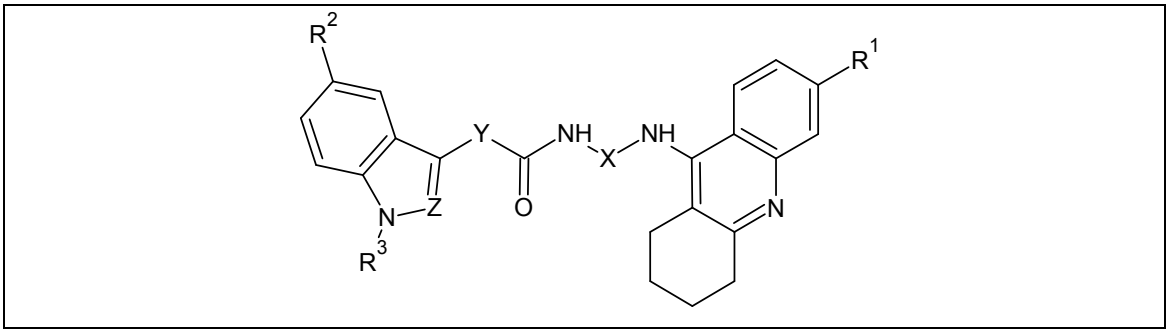


Figure 1.3: Structure of indole based dual AChE / Aβ aggregation inhibitors (Muñoz-Ruiz *et al.*, 2005)

The indole structure was also used in the design and synthesis of a series of 2,5 disubstituted indole derivatives that showed to be selective inhibitors of human

neuronal nitric oxide synthase (nNOS) and MAO-B. In this seriesPF9601N (**Figure 1.4**) showed the best dual MAO-B and nNOS inhibition (Fernández García, *et al*., 1992). Structure activity relationships of the series indicated that an aromatic substitution at the 5 position shifted the selectivity of the compounds towards MAO-B. PF9601N and its metabolite has demonstrated neuroprotective effect in models of PD. The synergism stemming from MAO and nNOS inhibition showed considerable antioxidant activity (Bellik et al., 2010).

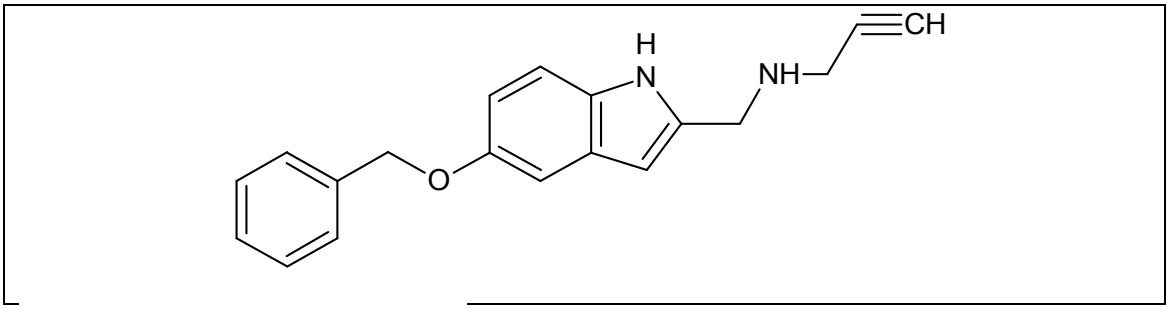


Figure 1.4: Structure of PF9601N.

Tzvetkov *et al*., 2014, investigated indazole and indole carboxamides for selective and reversible MAO-B inhibition (figure 1.5). The most potent compound was an indole-5-carboxamide analogue. The results suggested that the nitrogen in position 1 of the indole is important for MAO-B binding and compound potency.

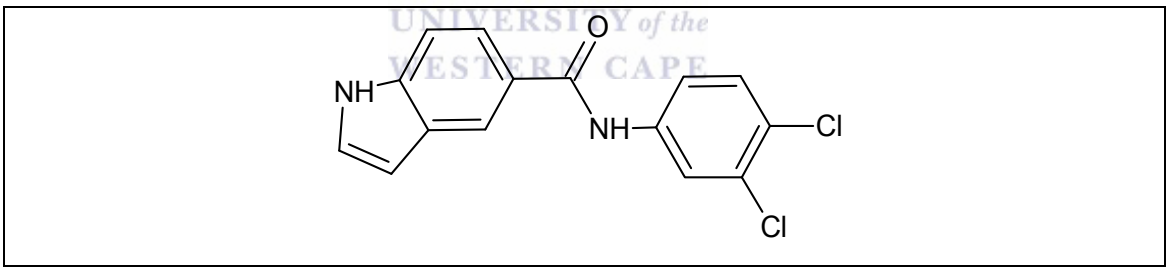


Figure 1.5: Structure of *N*-(3,4-dichlorophenyl)-indole-5-carboxamide. [IC_{50}hMAO – A: 1.30 µM, hMAO – B: 0.227 µM] (Tzvetkov *et al*., 2014)

From the above examples it is clear that the indole scaffold possesses great potential in neurodegeneration to design compounds able to inhibit MAO, nNOS and possess anti-apoptotic activity.

1.2.3 CHOLINESTERASE INHIBITION

Regulation of Ach turnover and levels in the neurons and synaptic junction plays an important role in neurodegenerative diseases, particularly AD. The use of AChE inhibitors increases the post synaptic levels of Ach by inhibiting the catalytic activity of the enzyme (Fifer, 2012).AChE inhibitors inhibit AChE in either of two ways; those that react with the enzyme to form a more stable acylated enzyme and those that bind to

AChE with greater affinity than Ach without reacting with the enzyme (e.g. carbachol an alkyl carbamate). The former include arylcarbamates, such as physostigmine and its analogues, which bind to the catalytic site of AChE. The carbamate is hydrolysed via esterificaion of the carbamoyl group to the serine residue forming a carbamylated enzyme. Aryl carbamates have better affinity for AChE than their alkyl counterparts hence they possess superior activity (Tarihi, Çokuğraş, and Adresi, 2003; Giacobini, 2003; Fifer, 2012). The activity of this moiety (**Figure 1.6**) was first observed in the extracted alkaloid physostigmine and this led to efforts to improve AChE inhibition by using the carbamate scaffold in drug design efforts.

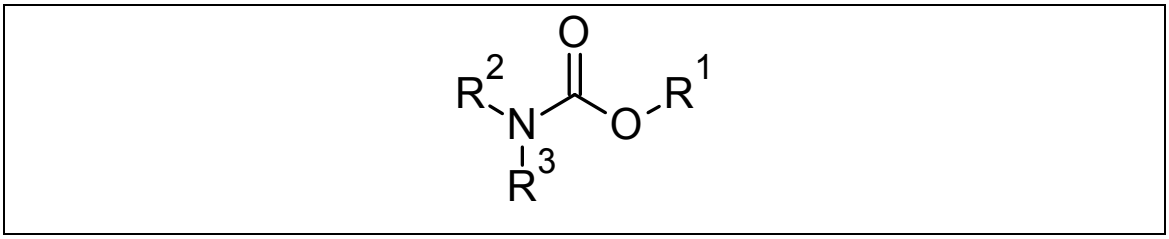


Figure 1.6: Structure of the carbamate moiety

1.2.4 MONOAMINE OXIDASE INHIBITION

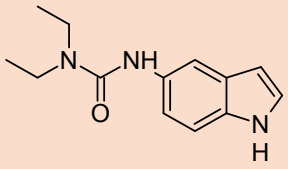
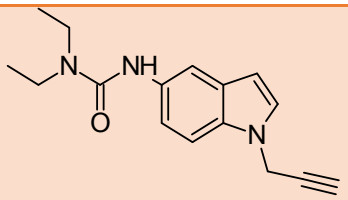
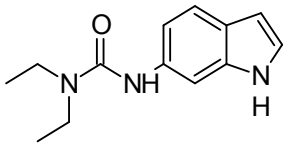
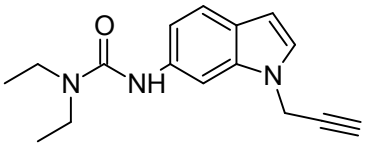
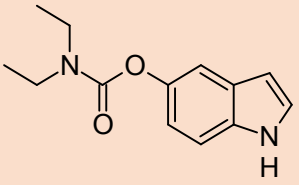
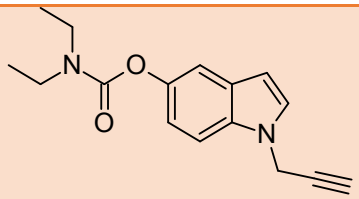
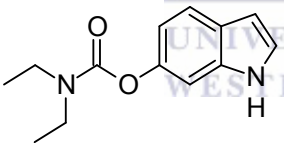
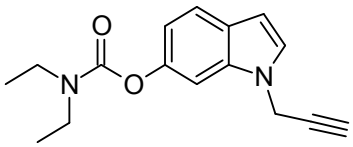
The MAO enzymes (A and B isoforms) natively metabolise amine neurotransmitters and have been identified as attractive agents for the treatment of neurological disorders (Youdim, Edmondson, and Tipton, 2006). Inhibitors of the enzyme are expected to protect form neurodegeneration due to their ability to reduce the formation of peroxides and radical species from amine catalysis, this independent of their inhibitory function.MAO inhibitors are currently registered for depression and Parkinson's disease and widely investigated for AD. The propargylamine moiety of selegiline was identified to possess MAO inhibitory, neuroprotective and neurorescue capacity, the latter via at least seven accepted mechanisms (Riederer, Danielczy, and Grunblatt, 2004). Propargylamine derived neuroprotective compounds such as ladostigil are generally anti-apoptotic (Youdim, Edmondson, and Tipton, 2006). The moiety has been widely applied in the design of multi potent ligands for the design of potential agents effective against AD.

1.3 AIM

Recent observations have suggested that dual ChE and MAO inhibition is likely to be more effective against AD (Zhang, H.-Y. 2005) as indicated by ladostigil a novel dual ChE/MAO inhibitor that is currently in Phase 2b clinical trials for mild cognitive

impairement. However only the *R*-enantiomer of ladostigil is active, raising issues of synthesis and *in vitro* stability. The carbamate bond on the compound is also open to acid hydrolysis and once this metabolism takes place, it leaves a non-inhibiting neuroprotective molecule TV3279(Avraham Pharma, 2016; Sterling, *et al*., 2002).This led us to the new series of proposed compounds (**Table 1.1**and **Figure 1.7**).

Table 1.1: Proposed series of compounds

1A		**1B**	
2A		**2B**	
3A		**3B**	
4A		**4B**	

We set out todesigna series of compounds structurally similar to ladostigil, but based on an indole structure, featuring the propagylamine moiety, with a carbamate or urea at the 5 or 6position (**Table 1.1**).Our objectivewas to improve the inhibitory profile of existing inhibitors as well as eliminate the stereochemistry associated with rasagline and more recently, ladostigil.It was hypothesised that these compounds will serve as dual AChE/BuChE and MAO-A or B inhibitors and possess superior activity and pharmacokinetic profiles compared to ladostigil and other anti-AD drugs (**Figure 1.7)**.

To achieve the above mentioned aim, the following objectives were set out to be met:

- Design a series of compounds based on literature and currently available treatment options.

- Explore the potential binding interactions for these proposed compounds at the MAO and cholinesterase (ChE) enzyme active sites, respectively, using molecular modelling software.
- Synthesise the compounds using conventional and microwave synthetic techniques.
- Perform structural elucidation by means of ^{1}H and ^{13}C NMR, mass and infrared spectroscopy on the synthesised compounds.
- Evaluate the compounds *in vitro* for possible AChE, BuChE, MAO-A and MAO- B inhibitory activity.
- Assess the chemical stability of the compounds.

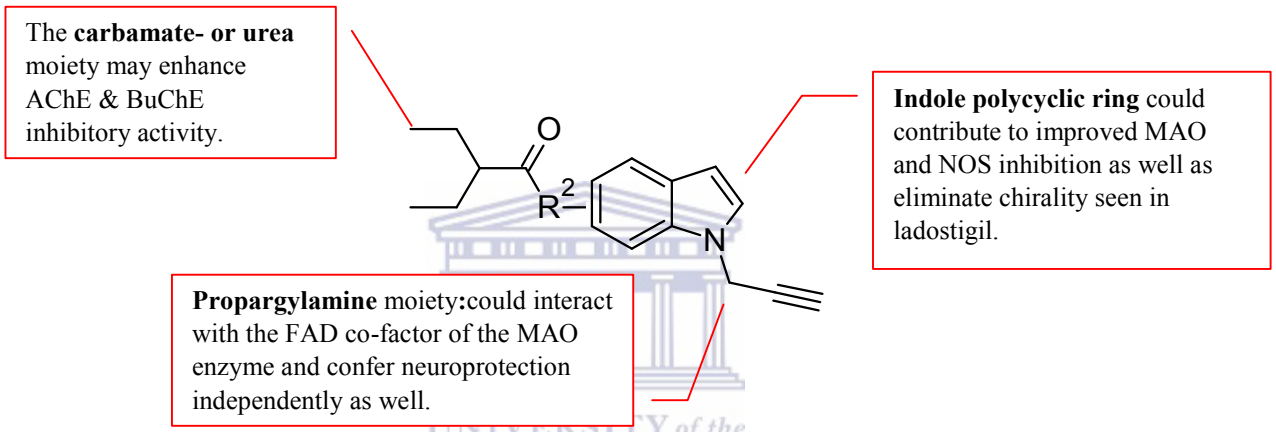


Figure 1.7: The rationale used to conjugate different functional groups to indole structure as well as the expected structure activity relationships (SAR) of the compounds

1.4 CONCLUSION

We expect the compounds to display the ability to act upon multiple pathways against AD namely acetylcholine hydrolysis, MAO activity and apoptosis. The expected structure activity relationships are summarised in **Figure 1.7**. We hypothesized that the compounds would possess superior activity compared to ladostigil and other known inhibitors of the enzymes. This study could thus identify new potential MTDLs that may reduce the progression of AD and relieve the burden of the disease

CHAPTER 2

2. LITERATURE REVIEW

2.1 INTRODUCTION

Neurodegenerative disorders affect a wide number of the elderly population as well as the family members and caretakers of the affected patients. The discovery of improved treatments is of critical importance in the prevention and symptomatic relief of these conditions of which AD is one. This second chapter will address AD as well as various neuroprotective strategies in depth.

2.2 NEURODEGENERATIVE DISORDERS

Acute and chronic neurodegenerative diseases are illnesses of cell death associated with high morbidity and mortality rates with few to no effective options of treatment available. They are mainly characterised by neuronal cell death (Yuan and Yankner, 2000). Neurodegenerative diseases include stroke, brain trauma, amyotrophic lateral sclerosis (ALS), Huntington's disease, Parkinson's disease and Alzheimer's disease. Central nervous system (CNS) tissue has very limited degenerative capacity, if any, so it is of the utmost importance to limit the damage caused by neuronal death (Eriksson *et al*., 1998). Cell death occurs by either of two ways: necrosis or apoptosis.

Necrosis is the death of cells after acute ischaemia or trauma to the brain and/ or spinal cord. It follows a sudden biochemical collapse, which leads to the generation of free radicals and excitotoxins and in turn cell death. Cell necrosis occurs in such a complex and rapid manner that is not easy to treat or prevent (Martin, 2001; Kerr, Wyllie and Currie, 1972).

Apoptotic cell death, also known as programmed cell death, is the predominant form of cell death in chronic neurodegenerative disorders. During apoptosis, a biochemical cascade activates protease enzymes that destroy molecules that are required for cell survival and others that mediate a programmed cell suicide (LeBlanc, 2005). Various kinds of stimuli have been proposed by *in vivo* and *in vitro* experiments to induce apoptosis of dopaminergic neurons (Ziv, *et al*., 1994).

2.3 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of subtle onset, characterised by memory loss and cognitive impairment (Khachaturian, 1985). Dr Alois Alzheimer, a German psychiatrist, first observed it in a patient presenting with senile dementia. The patient was under observation from 1901 till 1906 upon her death (Ashford, Schmitt and Kumar, 1996; Stelzmann, *et al*., 1995).

The symptoms of AD can be divided into four stages, with a progressive pattern of functional and cognitive impairment. It starts with the mild effects of ageing on memory such as an occasional forgetfulness of exact details and minor short term memory loss (Backman *et al*., 2004). In early stage Alzheimer's an increasing impairment of cognition and memory including not remembering the episodes of forgetfulness is observed. At this point one may forget the names of friends and family and have some confusion in situations outside the familiar. These changes may only be noticed by those close to the patient (Black, 2012). In the moderate stage of the disease the subjects are not able to perform the common activities of daily living hindering their independence. Motor activities become less coordinated, the memory worsens and insomnia sets in (Frank, 1994). At this point it becomes very difficult for the patient to remember recently acquired information or to know where they are at a given moment. The late stage, which is the last and dementia stage of the disease, involves the complete dependence and mental incapacity of the patient (Förstl and Kurz, 1999). Language is reduced to simple phrases and single words, eventually leading to loss of speech (Frank, 1994). People with severe AD will ultimately not be able to perform any task on their own and will get to a point where they are bedridden and unable to feed themselves. Death eventually occurs due to infection or pneumonia and not the disease itself (Förstl and Kurz, 1999; Black, 2012).

2.4 AETIOLOGY AND PATHOPHYSIOLOGY

The major pathological hallmarks of AD include neurofibrillary tangles (NFTs), amyloid (senile or neuritic) plaques and synaptic loss (Katzman and Saitoh, 1991). The amyloid peptide is the principal component of the senile plaque and is a large contributor to AD (Selkoe, 1996). Besides the amyloid β (Aβ) plaques and the NFTs, the brains of AD patients exhibit evidence of reactive oxygen species and reactive nitrogen species (ROS and RNS, respectively) mediated injury (Praticò and Sung,

2004). **Figure 2.1** below illustrates how these pathways correlate and ultimately lead to AD.

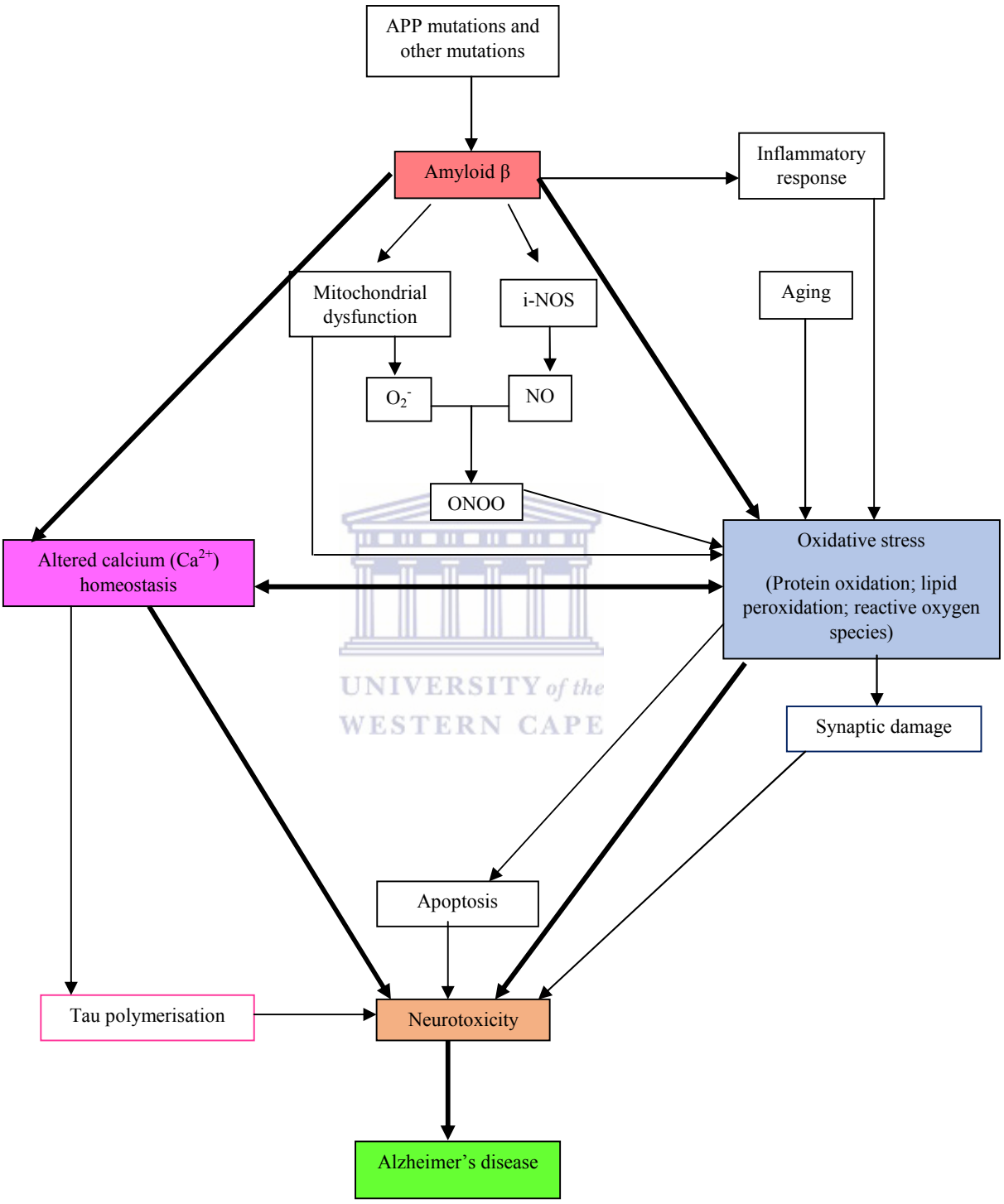


Figure 2.1: Various pathways leading to AD

2.4.1 AMYLOID HYPOTHESIS

The amyloid hypothesis is a prevailing pathological model of AD that has recently gained acceptance. According to the hypothesis, the overproduction and accumulation of Aβ peptides in plaques represents an early and central event in the pathophysiology of AD leading to the formation of neuritic or senile plaques. The cytoskeletal changes that arise from the hyperphosphorylation and intracellular aggregation of microtubule-associated tau protein form neurofibrillary tangles that are regarded as downstream phenomena. Both pathological cascades impair neuronal homeostasis and eventually result in cell death and neurodegeneration. These changes manifest clinically with slowly progressive cognitive decline (evolving from mild cognitive impairment to complete dementia), behavioural symptoms and functional impairment (Hardy, 2006; Hardy, 2009; Morris and Mucke, 2006). Amyloid β originates from the cleavage of the amyloid precursor protein (APP) into smaller peptides by non-amyloiodogenic α-secretase and amyloiodogenic β- and γ-secretases. In AD the abnormal regulation of the APP secretases, leading to the activation of the amyloiodogenic pathways, is associated with the overproduction of toxic amyloid species. Aβ oligomers and plaques exert neurotoxic effects (Morris and Mucke, 2006).

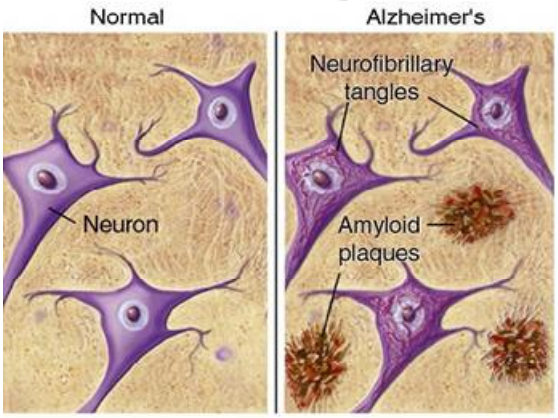


Figure 2.1: Abnormal pathology characteristic of Alzheimer's disease. Illustration from http://www.ahaf.org/alzheimers

Neurofibrillary tangles (NFTs), another pathological hallmark of the disease, are largely constituted by intracellular aggregates of paired helical filaments, which arise from the collapse of the neuronal cytoskeleton. The structure and function of microtubules are impaired as a consequence of the abnormal hyperphosphorylation of the tau protein (Morris and Mucke, 2006). Hyperphosphorylated tau aggregates into oligomers to form

helical filaments which in turn form fibrillary tangles (**Figure 2.2**) (Meraz-Ríos *et al*., 2010).

2.4.2 CHOLINERGIC HYPOTHESIS

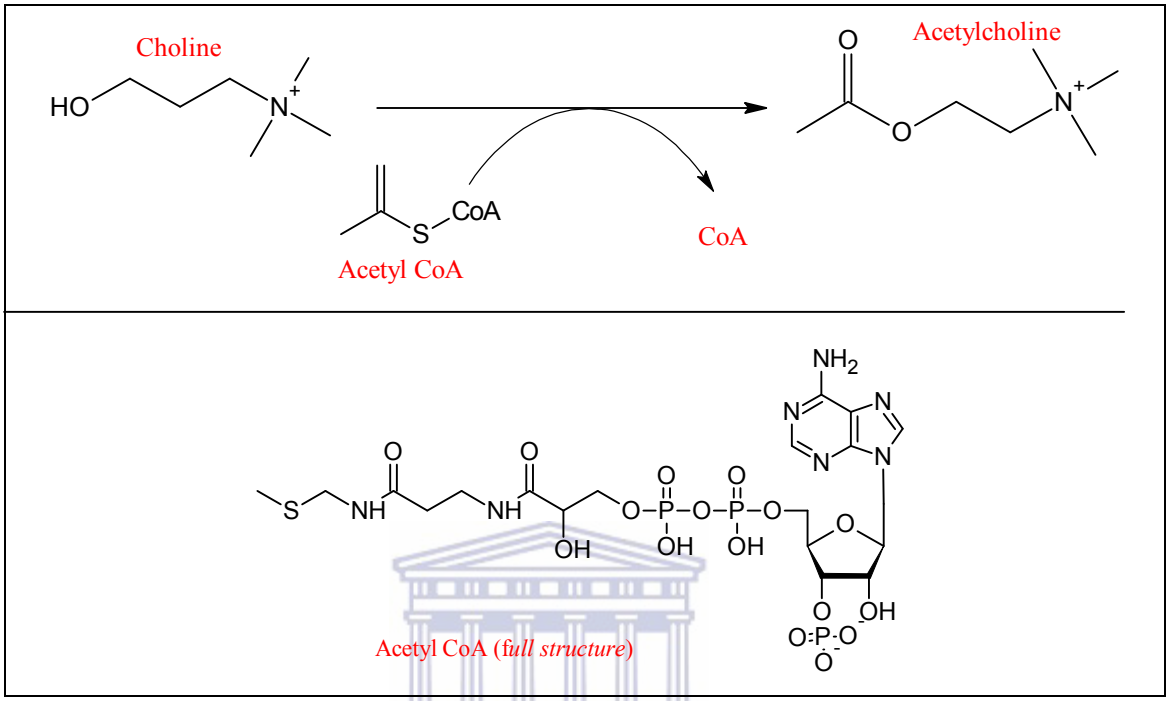


Figure 2.3: Synthesis of acetylcholine

Choline acetyltransferase (CAT) is an enzyme that catalyses the acetylation of choline by acetyl coenzyme A to produce acetylcholine and coenzyme A. The original building blocks, acetate and choline, enter the nerve cell via specific transporters. After CAT intervention, the resultant acetylcholine is stored in presynaptic neuronal vesicles until it is released into the synapse during neurotransmission. In the synapse acetylcholine binds to nicotinic and muscarinic (cholinergic) receptors of the postsynaptic neuron resulting in a transfer between neurons (**Figure 2.4**). CAT is produced mainly by the nucleus basalis of Meynert in the basal forebrain (Hebb and Whittaker, 1958).

Memory impairment and dementia are primary symptoms of AD and this is supported by Gibson and Peterson (1981) who observed that aged cholinergic neurons were unable to synthesize acetylcholine without potassium stimulation. This impairment results in a consistent reduction in CAT and thus acetylcholine levels. Excess acetylcholine post synapse is hydrolysed by the cholinesterase enzyme to recyclable choline and acetate and terminates transmission of the impulse. In the brain of an AD

patient; production of acetylcholine is lower than its hydrolysis resulting in idle nerve cells that eventually die and waste away (Francis *et al*., 1999).

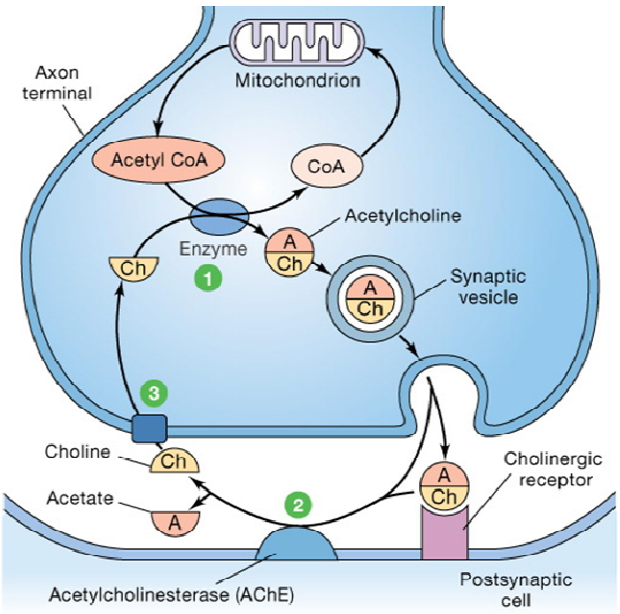


Figure 2.4: The cycle of events of acetylcholine synthesis and breakdown.

Multiple human studies have indicated that forebrain cholinergic pathways to the thalamus serve important functional roles in conscious awareness, attention, working memory and other cognitive processes (Perry *et al*., 1999). The activity of CAT, the enzyme responsible for the synthesis of acetylcholine was found to be significantly reduced in pathological samples from the cortex and hippocampus in Alzheimer's patients (Davies and Maloney, 1976; Perry *et al*., 1977). A study by Bartus, (2000) stated the cholinergic hypothesis to be essentially a loss of cholinergic function in the CNS that contributes to the cognitive decline associated with advanced age and AD. The cortex and hippocampus have also exhibited a depolarisation-induced acetylcholine release and choline uptake in nerve terminals to replenish the acetylcholine (Rylett, Ball, and Colhoun, 1983; Nilsson *et al*., 1986). At the same time, a reduced number of cholinergic projection neurons in the nucleus basalis of Meynert, led to the conclusion that the pathogenesis of AD was mostly represented by the degeneration of the cholinergic connection from the nucleus basalis of Meynert to the cortex and hippocampus. There exists ample evidence to conclude that assault to the forebrain cholinergic neurons could result in interference with acetylcholine release for normal brain function (Burk *et al*., 2002).

2.5 CHOLINESTERASES

Cholinesterases are widespread enzymes found in both cholinergic and non-cholinergic tissues as well as in plasma and other bodily fluids (Massoulié *et al*., 1993). They are classified into two forms according to their substrate specificity, behaviour in excess substrate and susceptibility to inhibitors (Tarihi, Çokuğraş and Adresi, 2003).

Acetylcholinesterase (AChE) also referred to as 'true cholinesterase' is encoded by a gene on chromosome 7 (Aldridge, 1953). It is the more prominent of the cholinesterases and the only one consistently associated with cholinergic pathways. It was found that cholinergic neurons display an AChE rich enzyme activity in the perikaryon, proximal dendrite and axon (Mesulam and Geula, 1991). AChE is known to be abundantly available in the brain, muscle and erythrocyte membrane (Dave, Syal, and Katyare, 2000).

The second cholinesterase, found mainly in the liver, heart and lungs is called butyrylcholinesterase (BuChE). It is also known as pseudocholinesterase or non specific cholinesterase and is present in much lower concentrations than AChE with restricted distribution in the central nervous system (Dave, Syal, and Katyare, 2000; Mesulam, 2000).

The two cholinesterases present similar soluble molecular forms in tissues and body fluids with differing tissue distribution. These oligomeric forms are summarised as follows:

1. **Type I amphiphilic dimers:** These are dimers anchored to plasma membranes by glycophosphotidyl inositol in the muscles, erythrocytes and lymphocytes. This is the more abundant form of AChE.
2. **Type II amphiphilic monomers and dimers:** These lack a glycolipid anchor. They are solubilised by salt solutions and abundant in the brain, muscles and intestine for both cholinesterases.
3. **Hydrophobic-tailed tetramers:** This form is anchored to the plasma membrane by a hydrophobic polypeptide subunit. Hydrophobic tailed tetramers are abundant in the central nervous system.
4. **Asymetric forms:** This form of the enzymes is characterised by the presence of a collagen-like tail for support to the basal lamina. It is formed by the triple

helical structure of three collagenic units each associated with either one, two or three tetramers of cholinesterases. It is more abundant for AChE than BuChE in nervous muscle junctions.

5. **Soluble tetrameric form:** This is composed of four identical monomers and stabilised by hydrophobic interactions of hydrophobic amino acids at the C terminal of the monomers. This form is most abundant for BuChE in body fluids (Tarihi, Çokuğraş, and Adresi, 2003b; (Massoulié, Sussman, *et al*., 1993; Altamirano and Lockridge, 1999).

AChE and BuChE both have the capacity to hydrolyse acetylcholine (Ach), be it in different ratios. Excess Ach levels in the brain cause saturation of AChE and in turn increase the activity of BuChE towards the neurotransmitter (Giacobini, 2004). In as much as AChE hydrolyses more Ach than BuChE, the latter contributes more to AD because of the decreased levels of the true cholinesterase (Mesulam *et al*., 2002).

AChE and BuChE share a 65% amino acid sequence homology and have similar molecular forms and active site structure (Allderdice *et al*., 1991). Early kinetic studies indicate that the active site of AChE contains two subsites, the esteratic and anionic subsites, corresponding to the catalytic and choline binding pockets respectively. The anionic active subsite interacts with the charged quaternary group of the choline moiety of acetylcholine (Augustinsson *et al*., 1950; Rosenberry, 2006). It contains the amino acids Trp 86, Tyr 133, Tyr 337 and Phe 338 and these bind the quaternary trimethylammonium choline moiety of the substrate mainly through π-cation interactions (Harel *et al*., 1993) positioning the ester optimally at the acylation site. The acyl pocket, responsible for substrate selectivity by preventing access of the larger choline esters is composed of Phe 295 and Phe 297. The oxyanion hole, Gly 121, Gly 122 and Ala 204, provides hydrogen bond donors that stabilise the tetrahedral transition state of the substrate (Ordentlich *et al*., 1998). Cationic substrates are not bound by a negatively charged amino acid in the anionic site, but by interaction of 14 aromatic residues that line the gorge leading to the active site (Colovic *et al*., 2013). Among the aromatic amino acids, Trp 84 is reported to critical and not substitutable (Tougu, 2001). The esteratic subsite or CAS where Ach is hydrolysed contains a catalytic triad of three amino acids namely Ser 203, His 447 and Glu 334 (**Figure 2.5**) (Colovic *et al*., 2013).

In addition to these two subsites of the catalytic centre, AChE also possesses one or more additional binding sites for its substrates. This 'peripheral' anionic site (PAS), very different to the choline binding pocket of the active site, has been implicated in the inhibitory characteristic of acetylcholinesterase (Mooser and Sigman, 1974; Reiner *et al*., 1991).The PAS lies essentially on the surface of AChE, and it binds acetylcholine as the first step in the catalytic pathway (Sussman *et al*., 1991; Hosea *et al*., 1996). It is also responsible for modulating the catalysis as well as the binding of specific inhibitory compounds (Johnson and Moore, 2006). The PAS has also been identified as a site promoting non-cholinergic functions: cell adhesion and neurite outgrowth in developing and transformed neural cells and amyloiodosis through an interaction with the Aβ in AD (Hosea *et al*., 1996; Inestrosa *et al*., 1996; Inestrosa and Alarcón, 1998).

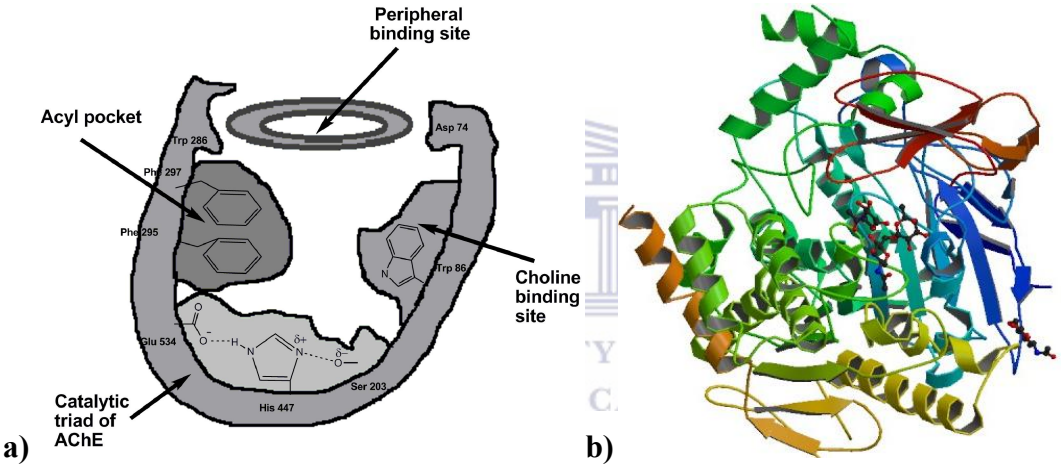
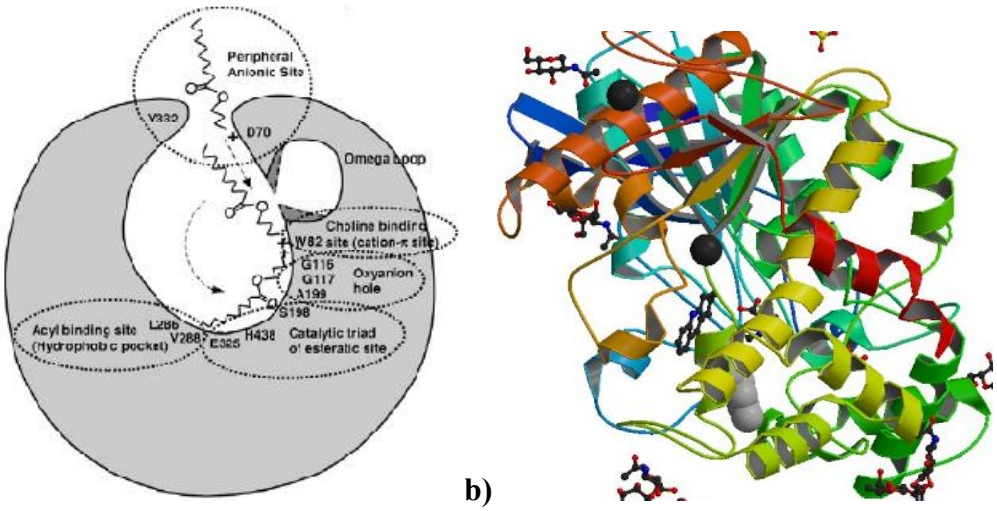


Figure 2.5: a) Schematic representation of AChE binding sites (Colovic *et al*., 2013). **b)**Ribbon structure of the AChE protein in complex with fasciculin-2 (RCSB Protein Data Bank).

The PAS consists of 5 residues namely Tyr 72, Asp 74, Tyr 124, Trp 286 and Tyr 341 clustered around the entrance of the active site gorge (Harel *et al*., 1993). The site has also been shown to have interaction with a large omega loop that incorporates Tyr 72 and Asp 74. It has been proposed that this loop may be involved in the accessibility of small molecules to the active site as well as allosterism (Shi *et al*., 2003). There are ten acidic residues in the area surrounding the PAS. This concentration of negative charge, the 'annular electrostatic motif' is shared by AChE and homologous signalling molecules but not by BuChE (Botti *et al*., 1998). The aromatic PAS residues, with Trp 286 at their core, have been shown to act synergistically due to the very large increases in inhibition constants induced by multiple mutants (Bourne *et al*., 1999). The aromatic rings of Tyr 72 and Tyr 124 flank the indole ring of Trp 286 and together interact with

charged groups of ligands (Johnson and Moore, 2006b). The indole ring of Trp 286 exhibits a variety of interactions including aromatic-aromatic, stacking and π-cation (Kreienkamp et al., 1991) depending on the nature of the ligand. Significant shifting of the indole is also observed when comparing crystal structures of apo- and complexed AChE (Bourne, Taylor, and Marchot, 1995) indicating its importance in ligand binding. Asp 74, like Trp 286, is also able to utilise several different interaction modes, operating by charge-charge or hydrogen bond interactions mediated by its carboxylated moiety. The residues also appears to act as a trap for charged substrate (Mallender, *et al.,* 2000; Hosea, *et al*., 1996b)

Regarding the structure of BuChE, each monomer has a narrow 20Å deep active site gorge lined by approximately 55 amino acid residues. **Figure 2.6** show a schematic representation of this active site. The PAS is found at the mouth of the gorge with Asp 70 and Tyr 332 involved in the initial binding of positively charged substrates and in activation control. BuChE contains a hydrogen bond between these two residues which controls the functional design of the active site gorge. When a positively charged substrate is bound to the enzyme by a cation-π interaction with the aromatic ring of Tyr 332, the substrate interacts with the negatively charged Asp 70 and this process triggers a change in the conformation of the monomer. Thereafter the two flexible arm of the omega loop come close to each other and the substrate moves down to the Trp 82 residue of the choline binding site. Trp 82 also forms a cationic-π complex with the substrate (Masson *et al*., 1997; Masson *et al*., 1999; Masson *et al*., 2001).



a) **b)**

Figure 2.6a: Schematic representation of BuChE binding sites. (Tarihi, Çokuğraş, and Adresi, 2003b); **b:** Ribbon structure of the BuChE protein (RCSB Protein Data Bank).

The oxyanion hole, found near the choline binding site includes Gly 116, Gly 117 and Ala 199, helps to rotate the substrate to a position where it can be hydrolysed by Ser 198 (Ekholm and Konschin, 1999). When the substrate rotates horizontally, the substrate binds to the acyl binding pocket of the enzyme active site. Leu 286 and Val 288 are found in the acyl binding pocket of BuChE (Phe 295 and Phe 297 in AChE) and allow for catalysis of larger acyl group containing substrates such as butyrylcholine (Radic *et al*., 1993). Stabilised substrate is hydrolysed by the catalytic triad, composed of Ser 198, His 438 and Glu 325, in the esteratic site of the active centre (Masson *et al*., 1997). It is important to note that six of the 14 aromatic amino acid residues lining the active site gorge of AChE are replaced by aliphatic residues in BuChE. This explains the larger volume of the BuChE active site gorge in comparison. The replacement is also responsible for selective sensitivity against different inhibitors of the enzymes (Saxena *et al*., 1999).

2.6 MONOAMINE OXIDASE

Monoamine oxidase (MAO) is an outer mitochondrial membrane bound flavin containing enzyme with a molecular weight of about 60 kDa, it plays an important role in the metabolism of endogenous and exogenous amines (Weyler, Hsu, and Breakafield, 1990; Shih, Chen, and Ridd, 1999). Intraneuronal MAO terminates the action of endogenous amine neurotransmitters, regulates intraneuronal amine stores and protects cells from dietary amine (false transmitters) (Youdim, Edmondson, and Tipton, 2006).

The enzyme exists in two isoforms MAO-A and MAO-B, these isoforms have different substrates specificities in that MAO-A catalyses the oxidation of serotonin whilst MAO-B catalyses the oxidation of both benzylamine and 2-phenylethylamine. Other neurotransmitters like dopamine, norepinephrine, epinephrine, tryptamine and tyramine are oxidised by both isoforms of the enzyme (Youdim, Finberg, and Tipton, 1988). MAO enzyme activity is different in various regions of the brain, where the hypothalamus and basal ganglia show the highest activity while the cerebellum and neocortex show the lowest activity (O'Carroll *et al*., 1983). Fowler and company mapped the human brain MAO in 1987 to identify the distribution of the MAO isozymes throughout the brain and it was found that basal ganglia contain mainly MAO-B.

2.6.1 STRUCTURE OF MONOAMINE OXIDASE

a) Monoamine oxidase A

Human MAO-A contains 527 residues and is specific to serotonin, dopamine and norepinephrine. Its active site is formed by a single cavity that extends from the flavin ring to the cavity shaping loop of residues 210-216. The volume of this cavity is estimated to be approximately 550Å and is lined by 11 aliphatic and 5 aromatic residues, which demonstrate that it is hydrophobic (De Colibus *et al*., 2005). Two cysteine residues (Cys 321 and Cys 323) are located near the entry of the catalytic site.

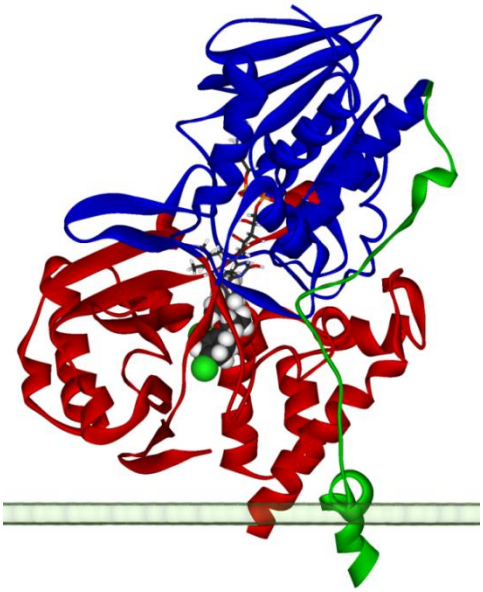


Figure 2.7: Ribbon diagram of a monomer of human monoamine oxidase A with FAD (blue) and clorgyline bound oriented as if attached to the outer membrane of mitochondrion (RCSB Protein Data Bank).

The 'face' of the covalent FAD is situated at one end of the active site opposite to the entrance. It also has two parallel tyrosyl 407 and 444 residues forming an aromatic cage in front of the FAD (Edmondson, Binda, and Mattevi, 2007).

b) Monoamine oxidase B

Monomaine oxidase B (MAO-B) is made up of 520 amino acids and FAD cofactor. The monomer involves a membrane binding domain, a substrate domain and a flavin binding domain (Kearney *et al*., 1971; Binda *et al*., 2001) MAO-B **(Figure 2.8)** undergoes modification post translation i.e. acetylation where the amino terminal methionine is cleaved and the resultant *N*-terminal serine is acetylated (Li *et al*., 2002; Newton-Vinson, Hubalek, and Edmondson, 2000). It is tightly bound to the outer membrane of the mitochondria by the C-terminal amino acids 461-520. Analysis of the

enzyme residue sequence revealed that residues 489-515 form a transmembrane helix 27 amino acids long (Binda et al., 2001). The substrate binding site is formed by a flat cavity of 420Å. This cavity is lined by a number of aromatic and aliphatic amino acids, providing a highly hydrophobic environment (Walker and Edmondson, 1994). Adjoining the substrate cavity is a separate, smaller hydrophobic cavity lined by residues Phe 103, Pro 104, Trp 119, Leu 164, Leu 167, Phe 168, Leu 171, Ile 199, Ile 316 and Tyr 326. This cavity is situated between the active site and the protein surface, and is shielded from solvent by loop 99-112 to open access to the smaller entrance cavity. The substrate and hydrophobic entrance cavity are separated by residues Trp 119, Leu 171, Ile 199 and Tyr 326. When a substrate reaches the entrance cavity, a brief movement of the four separatory residues must occur to allow its diffusion into the active site. Loop 99-112 functions as a sort of gating switch to the entrance cavity (Walker and Edmondson, 1994; Binda et al., 2001).

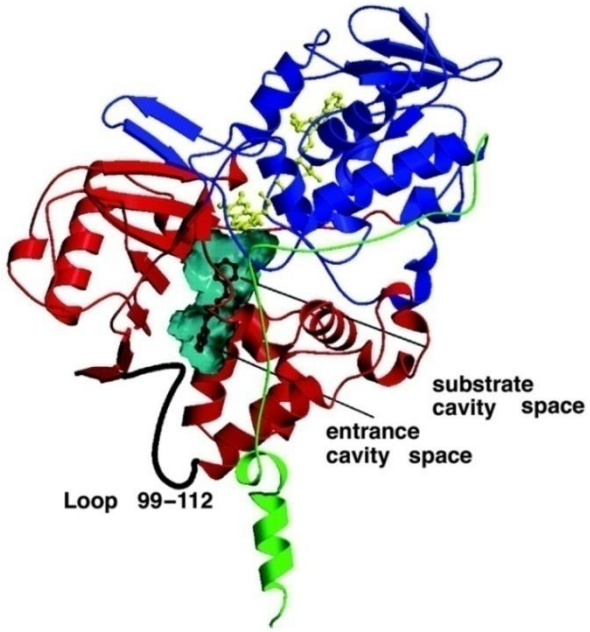


Figure 2.8: Three dimensional structure of human MAO-B monomeric unit in complex with 1,4-diphenyl-2-butene. The FAD binding domain (blue), the substrate binding domain (red) and the C-terminal membrane binding region is in green. The FAD cofactor and the inhibitor are shown as yellow and black ball-stick models, respectively (Binda *et al*., 2003).

In order to covalently bind with the FAD cofactor and undergo metabolism the substrate must pass through two hydrophobic cavities, an entrance cavity and a larger substrate cavity (Binda *et al*., 2001). The outer mitochondrial membrane is covered with negative charges and these attract amine substrates that are positively charged into

the enzyme (Edmondson *et al*., 2004). The FAD cofactor is located at the end of the substrate cavity, covalently bound to the apo-enzyme and considered an important component for MAO catalytic activity (Edmondson, 2004). It is of significance to note that the amino acids that interact with FAD in the MAO-B enzyme are conserved in MAO-A suggesting that FAD is identical in both enzymes (Edmondson, 2004; Kirksey, Kwan, and Abell, 1998)

c) Structural comparison between human MAO-A and MAO-B

MAO-B has a bipartite elongated cavity that occupies a volume of approximately 700Å when the side chain of Ileu199 is in the 'open' conformation. MAO-A has a single cavity that exhibits a rounder shape compared to that of MAO-B and is larger in volume (550 Å) to the substrate cavity of MAO-B which is about 390 Å. QSAR comparison on the catalytic properties of the two enzymes showed that the substrate has less freedom for rotation in the MAO-B site than the MAO-A (De Colibus *et al*., 2005; Edmondson, Binda, and Mattevi, 2007). Mass spectrometry data have conclusively shown the absence of any disulfide bridges in either isozyme (Binda *et al*., 2003). Although the overall chain-folds of the two isozymes are quite similar, there are similarities and differences in the respective active sites. The structure of the covalent FAD coenzymes and the two tyrosines constituting the aromatic cage in the active sites are identical (De Colibus *et al*., 2005). Since this region is directly involved with substrate oxidation, this close similarity supports the notion that both enzymes follow the same catalytic mechanisms (Edmondson *et al*., 2004). However, alterations in the active site residues constituting the active site of MAO-A (7 residues are changed in total) and the conformation of loop 20-216 cause the shape and sizes of the active sites to differ significantly and are the basis for the specificity of the isozymes (De Colibus *et al*., 2005). The entrances to the active sites of either enzyme have been proposed to be at the surface of the negatively charged outer membrane which could function to increase the effective substrate concentration by electrostatic attraction (Edmondson, Binda, and Mattevi, 2007).

d) Reaction mechanism of MAO

The MAO enzyme catalyzes the oxidative deamination of primary, secondary and some tertiary amines resulting in the formation of hydrogen peroxide, an aldehyde and either ammonia (if the substrate in primary amine) or substituted amine in the case of a secondary amine(Youdim, Edmondson, and Tipton, 2006; Edmondson *et al*., 2004).

The catalytic mechanism (**Figure 2.9**) consists of a reductive half reaction where the C_α-H bond of the amine is cleaved with the transfer of two reducing equivalents to the flavin to form the imine and flavin hydroquinone respectively (Edmondson, Bhattacharyya, and Walker, 1993).

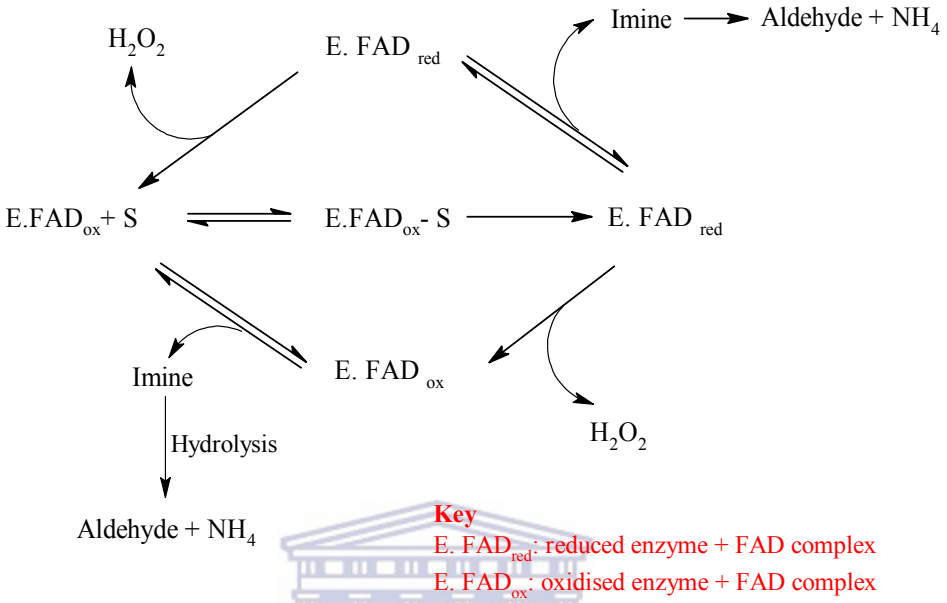


Figure 2.9: The catalytic mechanism of MAO

2.7 TREATMENT OF AD

The goals of treatment in patients with AD are to improve or slow down the progression of the disease, i.e. loss of memory and cognition and to maintain independent function (Mayeux and Sano, 1999). Effective treatments are urgently needed. Current drug treatment for AD targets cholinergic and glutamatergic transmission to improve disease symptoms. In addition, neuroprotective and neurorescue treatment is being investigated.

2.7.1 ACETYLCHOLINESTERASE INHIBITORS

Identification of the cholinergic component of AD as a major causative pathway focused research to the development of inhibitors of the cholinesterase class of enzymes. AChE inhibitors in AD act by boosting the endogenous levels of acetylcholine in the brain and thereby enhance cholinergic transmission (Talesa, 2001). Early research into AChE inhibitors included tetrahydroaminoacridine (tacrine), physostigmine and velnacrine. Of the three only tacrine made it to commercial launch, followed more recently by donepezil, rivastigmine and metrifonate (B. M. McGleenon,

1999). Presently in South Africa registered treatment includes donepezil, rivastigmine and galantamine (**Figure 2.10**) (Rossiter, 2016).

Tacrine has many actions but prominently acts as a potent reversible cholinesterase inhibitor. It is rapidly absorbed and cleared by first pass metabolism in the liver and results in hepatotoxicity (McNally *et al*., 1988; Selen, Balogh, and Siedlik, 1988). The bioavailability of tacrine is low upon oral administration and slightly improved with high and multiple doses (McNally *et al*., 1988). It is rapidly absorbed into the brain with higher concentrations there than in plasma (Nielsen *et al*., 1989).

Taking into account the pharmacokinetic inadequacies of tacrine, donepezil became the second AChE inhibitor to be registered with the FDA. It is a rationally designed piperadine derivative able to reversibly inhibit AChE with increased specificity than tacrine (Sugimoto *et al*., 1992).

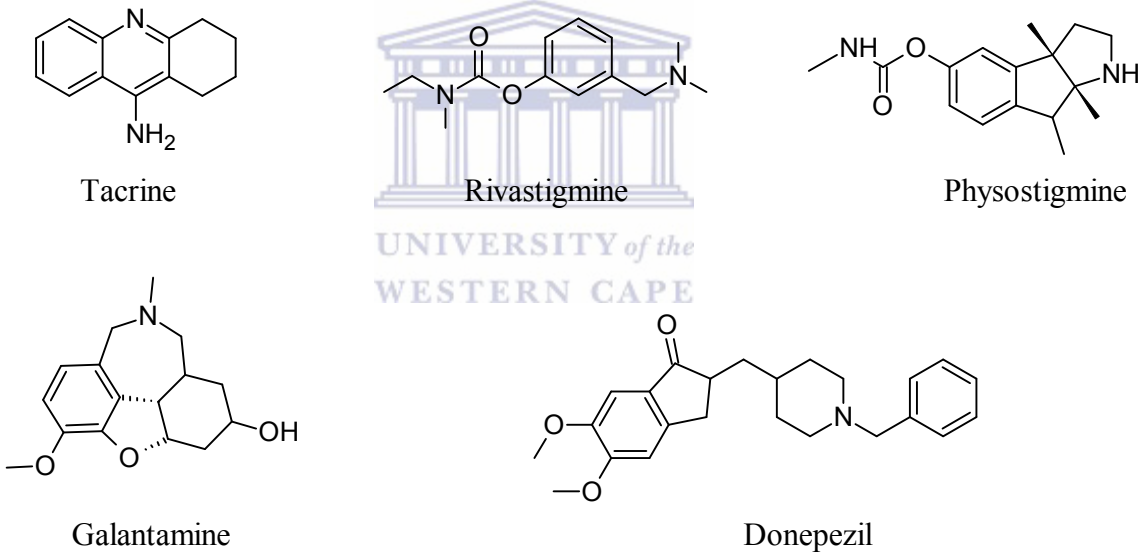


Figure 2.10: Structure of AChE inhibitors

Moreover donepezil lacks activity in peripheral, cardiac and smooth muscle tissue making it even more patient friendly. The next drug to highlight is rivastigmine, a brain selective carbamate inhibitor of the enzyme. It is known as a pseudo-irreversible inhibitor because it mimics acetylcholine in binding with the enzyme and forming a carbamylated complex (B. M. McGleenon 1999). This complex formation prevents the enzymatic hydrolysis of rivastigmine for several hours post plasma elimination. This, in addition, results in an increased duration of action of about 10 hours and consequently more side-effects. Physostigmine and galantamine are other known researched inhibitors of acetylcholinesterase (B. M. McGleenon, 1999). Unfortunately due to

undesirable side effects in small scale clinical trials they were not registered with the FDA. However the two serve as scaffolds and lead compounds for continued research on the treatment of AD. Treatment with AChE inhibitors has generally been beneficial in meliorating a global cognitive dysfunction and, more specifically, are most effective in improving attention (Parnetti *et al*., 1997). AChE inhibitors serve as one aspect of the treatment package for AD; they however do not halt the process of apoptosis nor improve the depressive symptoms of the disease warranting added therapy.

2.7.2 NMDA RECEPTOR ANTAGONISTS

Neuronal death may also be due to over-activation of the *N*-methyl-D-aspartate receptor (NMDAR) that allows influx of calcium ions for normal neuronal function. The receptor requires glutamate and glycine as agonists for the activation of ion influx. As AD progresses pyramidal neurons of the glutamitergic system diminish hence reducing glutamate concentrations in the brain leading to impaired cognition (Wenk, 2006). Over excitation of the NMDA receptors ultimately leads to neuronal loss and apoptosis which may cause and/or worsen AD (Greenmyre, *et al*. 1988). Blockers of this receptor have been shown to possess neuroprotective capacity and thus serve as therapy and lead compounds in the management of AD (Bezprozvanny, 2009). However the blockers of this receptor including adamantine and NGP1-01 are associated with adverse central nervous effects and this limits their use (Lipton, 2004). Preclinical evidence investigating the safety and clinical efficacy of memantine (**Figure 2.11**) show that it is effective for moderate to severe AD and also reverses cognitive impairment (Danysz and Parsons, 2003). Memantine is still under further study and is approved in the United States, several European countries and South Africa (Memor®) for dementia (Parsons, Stöffler, and Danysz, 2007, Rossiter, 2016).

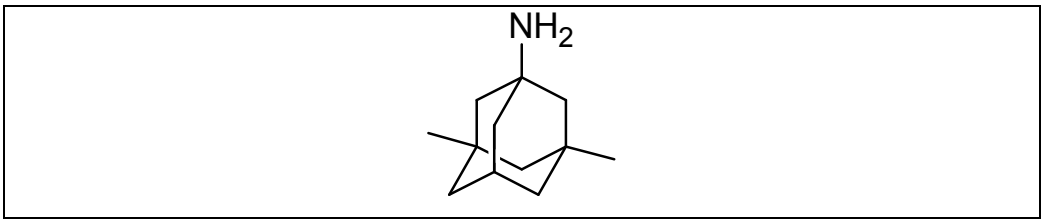


Figure 2.11: Structure of NMDAR blocker memantine

2.7.3 OTHER TREATMENT OPTIONS

The above discussed classes of drugs are currently registered for the treatment AD and as mentioned, they provide only a palliative solution in the management of the disease. This however does not mean that research has not produced some potentially successful compounds. Due to the multi-factorial nature of AD other targets are currently being investigated to combat the disease from all angles. Discussed below are approaches under exploration for the treatment of AD.

2.7.3.1 MONOAMINE OXIDASE INHIBITORS

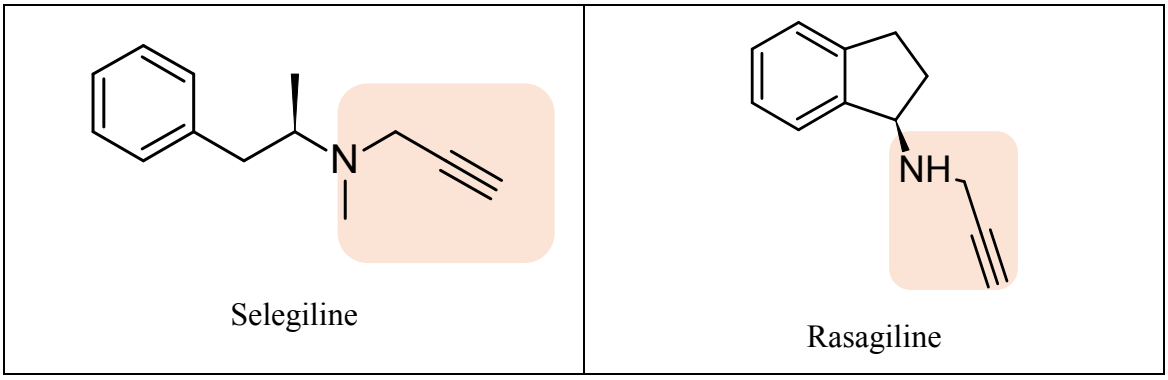
There exists a wide range of reversible and irreversible inhibitors of MAO- A and B (**Table 2.1**). They act by inhibiting the activity of the MAO family of enzymes. This in turn prevents the breakdown of monoamine neurotransmitters and thereby increasing their availability. They have been proven to have therapeutic value in diverse conditions including those of neurodegenerative origin (Youdim and Weistock, 2004).

Table 2.1: Inhibitors of monoamine oxidase and their applications.

Compound	Compound	Binding	Applications
Befloxatone	A	Reversible	Antidepressant
Brofaromine	A	Reversible	Antidepressant
Clorgyline	A	Irreversible	Antidepressant
Selegiline	B	Irreversible	Antiparkinsonian
Iproniazid	A and B	Irreversible	Antidepressant and anti-TB *
Isocarboxazid	A and B	Irreversible	Antidepressant
Lazabemide	B	Reversible	Antiparkinsonian **
M30	A and B	Irreversible	Antidepressant, antiparkinsonian and anti-Alzheimer
Moclobemide	A	Reversible	Antidepressant and anxiolytic
Nialamide	A and B	Irreversible	Antidepressant*
PF9601N	B	Irreversible	Antiparkinsonian
Phenelzine	A and B	Irreversible	Antidepressant
Rasagiline	B	Irreversible	Antiparkinsonian
Safinamide	B	Reversible	Antiparkinsonian
Toloxatone	A	Reversible	Antidepressant
Tranylcypromine	A and B	Irreversible	Antidepressant and anxiolytic

*discontinued due to toxicity

Rasagiline is currently marketed as Azilect® for Parkinson's disease. In early literature they were reported to have potentially lethal dietary and drug interactions (Blackwell and Marley, 1966). Regardless, the propargylamine pharmacophore of this class has opened a path to drug design for the treatment of AD. It possesses unique neuroprotective ability independent of MAO inhibitory function (Bar-Am *et al*., 2005).



Selegiline Rasagiline

Figure 2.12: Structure of selegiline and rasagiline, propargylamine highlighted.

2.7.3.1 ANTI-INFLAMMATORY AGENTS

Cerebral deposits of Aβ peptides and NFTs are surrounded by inflammatory cells. Epidemiological studies have shown that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD and delays the onset of the disease (Zandi, 2001). This stems from evidence that NSAIDs preferentially reduce the secretion of highly amyloiodogenic Aβ peptide (Zhou *et al*., 2003). It has been postulated that some NSAIDs, such as indomethacin, naproxen and aspirin, target pathological hallmarks of AD by interacting with inflammatory pathways such as inhibiting cyclooxegenases and activating peroxisome proliferator-activated receptor γ (Gasparini, Ongini, and Wenk, 2004). Assessment of the benefit versus side-effects of this class of drugs is still ongoing.

2.7.3.2 ANTI-AMYLOID β THERAPY

In AD, pathophysiological mechanisms change soluble Aβ peptides into fibrillary oligomers and insoluble plaques, which accumulate in the neural tissue and sometimes systemic vessels (Solomon, 2009). Toxic Aβ species also trigger secondary pathological mechanisms, such as oxidative stress and inflammation, which speed up neuronal dysfunction and eventual death (Mathew *et al*., 2012). Pharmaceuticals that target the clearance of Aβ from the brain, inhibit the production, or prevent its aggregation, may

represent a strategy to delay the progression of the pathological process of AD. In view of this, there exist ongoing clinical trials with humanised monoclonal antibodies like bapineuzumab and gantenerumab that target the reduction of Aβ and facilitate its clearance from the brain by immune mediated mechanisms (Panza *et al*., 2011; Burstein *et al*., 2013; Hardy and Selkoe, 2002).

2.7.3.3 ANTI-OXIDANTS

With oxidative stress playing a major role in the causation of AD, free radical scavenging and antioxidation may prove useful in combating the progression of the disease. Ordinarily, one would get sufficient antioxidants from the diet and naturally occurring sources or metabolism but when these defences are low, free radicals will damage the cell constituents (Coyle and Puttfarcken, 1993).Antioxidant therapy has been studied for years as one of the promising therapeutic strategies for AD. Reports state that antioxidants such as lipoic acid, vitamin E, vitamin C and β-carotene may help break down intra- and extracellular superoxide radicals and H_2O_2 cell damaging compounds (Staehelin, 2005; Grundman, 2000). In 1997 Sano *et al*. reported that in patients with moderately severe AD, treatment with α-tocopherol reduces neuronal damage and slows the progression of the disease. Melatonin, an antiamyloiodogenic antioxidant that passes the blood brain barrier, is also a direct scavenger on top of its ability to protect neurons (Pappolla *et al*., 2000).

2.8 MULTITARGET DIRECTED LIGAND

Over a hundred years since the discovery of the disease by Dr Alois Alzheimer, the scientific community still does not fully understand the pathophysiology of AD. It is a multifactorial disease caused by various factors including protein misfolding, aggregation, oxidative stress, free radical formation and neuro-inflammatoryprocesses. These insights together with ongoing research have provided the rationale for therapies directly targeting AD molecular causes (Cavalli et al., 2008; Munoz-Torrero and Camps, 2006). In the context of such a complex disease it is not insignificant to state that these hypotheses are not mutually exclusive but rather complimentary. The cholinergic hypothesis has so far produced the majority of drugs approved for treating AD together with calcium modulatory compounds (Cavalli et al., 2008).

Multiple medication therapy or polypharmacy is a successful concept for diseases like cancer where it achieves maximum efficacy by attacking several targets simultaneously, exploiting synergy and minimising individual toxicity. Clinically, memantine and an acetylcholinesterase inhibitor (rivastigmine, donepezil or galantamine) appear to produce an additional effect (Bolognesi *et al*., 2006; Grossberg, Edwards, and Zhao, 2006). However it comes with issues of patient adherence and high cost. Natural evolution of the polypharmacy system takes us to the emergence of the multitarget directed ligand (MTDL), a relatively novel drug design approach (Zhang, 2005).

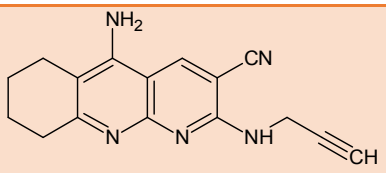
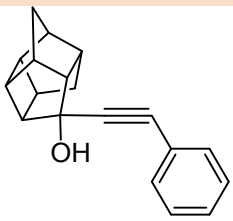
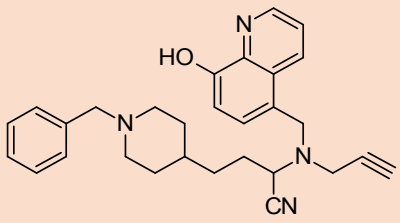
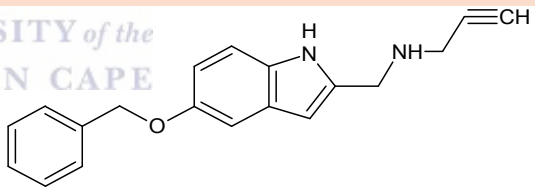
The MTDL strategy is simply a combination of distinct pharmacophores of different drugs in the same structure to obtain hybrid molecules. Principally each pharmacophore should retain the ability to interact with its specific sites on the target and, consequently, produce specific pharmacological responses that slow down or block a neurodegenerative process (Pisani *et al*., 2011). These combinations occur in various ways but the most widely adopted is the modification of the molecular structure of an AChE inhibitor so as to provide it with additional properties useful for the treatment of AD.

The table below shows results from recent studies on the application of the MTDL design strategy. In 2010 Samadi and colleagues successfully synthesised a range of tacrine based propargylamine derivatives (**Table 2.2**). These were then assessed for neuroprotective ability against an oxidative stress model. The results showed compounds with modest antioxidant activity in the same range as the well-known reference *N*-acetylcysteine. The pharmacophoric group resembling tacrine was able to retain the ability to inhibit acetyl- and butyrylcholinesterase. Their results concluded that tacrine is a considerable candidate for structural modification for activity on two, or possibly more, pharmacological targets that play a role in the progression Alzheimer's disease.

Memantine is the only polycyclic cage compound currently registered for AD and possesses great potential to explore structural modifications. In that line of thought, polycyclic cage compounds such as pentacycloundecanes are proven NMDA receptor antagonists as well as voltage calcium gated channel blockers that may serve as therapeutic agents in neurodegeneration (Geldenhuys *et al*., 2007). In 2014, Zindo and colleagues rationally incorporated these cage moieties and the propargylamine

functional group into novel structures. The resultant compounds portrayed the ability to inhibit MAO, block NMDA and calcium channels as well as confer a degree of anti-apoptotic activity.

Table 2.2: MTDLs in neurodegeneration

Dual inhibition strategy	**Example**
ChE + anti-apotosis (Samadi *et al*., 2010)	
MAO + Ca^{2+} homeostasis (Zindo *et al*., 2014)	
MAO + ChE + metal homeostasis (Wang *et al*., 2014)	
Antioxidant + MAO + free radical scavenging (Perez and Unzeta, 2003)	

PF9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] was the first non-amphetamine MAO-B inhibitor to be synthesised and has been shown to posses neuroprotective properties in models of Parkinson's disease (Perez and Unzeta, 2003). It was also demonstrated to be a good candidate for the treatment of excitotoxicity mediated neurodegeneration (Bolea *et al*., 2014) by its ability to bestow neuroprotective ability. Moreover evidence suggests that PF9601N and its metabolites serve as exceptional antioxidants (Bellik *et al*., 2010).

A large body of evidence exists to support the feasibility of the MTDL paradigm. Furthermore one of the most exploited ways to obtain a MTDL is the combination of the pharmacophoric features of an acetylcholinesterase inhibitor and a monoamine oxidase inhibitor. Pioneering work in the field of hybrid multitarget anti-AD drugs was

carried out by Youdim and colleagues (Sterling *et al*., 2002). They synthesised a large series of *N*-propargylaminoindans and *N*-propargyl-phenylethylamines as dual MAO/AChE inhibitors. The combination of the carbamate moiety of rivastigmine with rasagiline led to the compound ladostigil (Sterling *et al*., 2002).

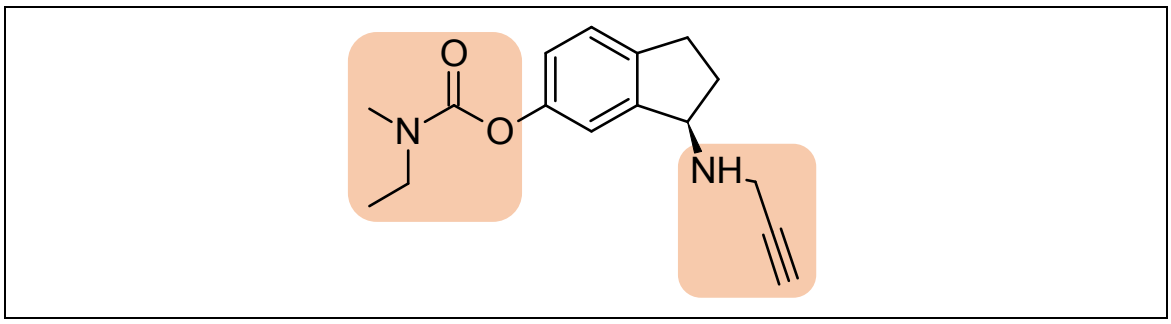


Figure 2.13: Structure of ladostigil, pharmacophores highlighted.

Ladostigil shown in **Figure 2.13** above is able to inhibit both AChE and BuChE for a longer duration than the parent compound rivastigmine. This is particularly important due to the significance of butrylcholinesterase induced metabolism of acetylcholine (Greig *et al*., 2005). Ladostigil also blocks the activity of brain MAO-A and B resulting in an increase in the levels of neurotransmitters like dopamine and thus exerting an antidepressant action. It has also been shown to retain the neuroprotective and anti-apoptotic ability observed in the propargylamine derived parent compound rasagiline (Weinstock *et al*., 2003; Yogev-Falach, 2002; Sagi, Weinstock, and Youdim, 2004). Moreover ladostigil also possesses cognition enhancing activity, being the most advanced MTDL of this category with promising results obtained from phase 2 clinical trials (Youdim *et al*., 2006).

2.9 CONCLUSION

Alzheimer's disease is a leading causation of morbidity and mortality in today's society. Breakthroughs have been obtained towards its treatment with moderate success in the understanding of the pathophysiology and halting the progression of the AD. Pathways leading to AD are numerous and varied (multifactorial) and include cascades of enzymatic events, genetic mutations and excitotoxicity amongst others. This poses an obstacle in drug design and development for management of the disease. Fortunately the MTDL paradigm has been established as a promising approach in tackling this complex disease. The compounds produced in this project serve as an addition, if not

improvement, to the ever growing arsenal of potential multifunctional agents against AD.

CHAPTER 3

3. SYNTHETIC PROCEDURES

3.1 STANDARD EXPERIMENTAL PROCEDURES

3.1.1 REAGENTS AND CHEMICALS

All reagents used in the synthesis of compounds were obtained from Sigma-Aldrich® (UK) and used without further purification. Solvents used for reactions and chromatography were purchased from various commercial sources.

3.1.2 INSTRUMENTATION

Nuclear magnetic resonance spectroscopy (NMR): Proton (^{1}H) and carbon (^{13}C) spectra were obtained on a 400 MHz Bruker Avance IIIHD Nanobay instrument equipped with a 5 mm BBO probe. Tetramethylsilane was used as internal standard, with deuterated solvent. All chemical shifts are reported in parts per million using the internal standard ($\delta = 0$) and the solvent peaks as reference. The following abbreviations are used to indicate the multiplicity of respective signals:

- s - singlet
- d - doublet
- dd - doublet of doublets
- t – triplet
- dt – doublet of triplet
- m - multiplet.

Infrared absorption spectroscopy (IR): Infrared spectra were obtained on a Perkin Elmer Spectrum 400 spectrometer, fitted with a diamond attenuated total reflectance attachment.

Mass spectroscopy (MS): The MS of the samples were obtained with the use of a Flexar Single Quad 300 LCMS detector made by Perkin Elmer.

Melting point determination (MP): MPs were determined by using a Lasec Melting Point SMP 10 apparatus and capillary tubes. MPs were determined for the solid forms of the final compounds and are specified for each test compound.

Microwave synthesis: Microwave synthesis was performed using a CEM Discover™ focused closed vessel microwave synthesis system

3.1.3 CHROMATOGRAPHIC TECHNIQUES

THIN LAYER CHROMATOGRAPHY (TLC): Analytical TLC was performed on 0.20 mm thick aluminium silica gel sheets (TLC Silica gel 60 F245 Merck KGaA). Visualisation was achieved using UV light (254 nm and 366 nm), with mobile phases prepared on a volume-to-volume basis and are specified for each compound

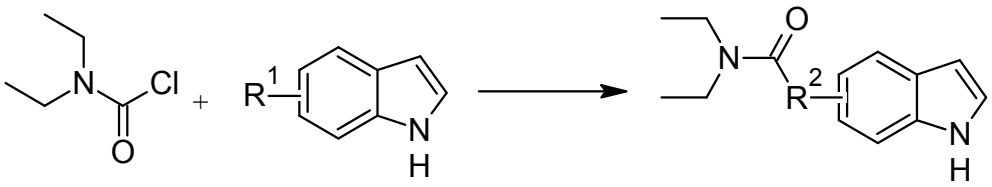
COLUMN CHROMATOGRAPHY: Impure product was purified using standard glass columns varying in size. The stationary phase used was silica gel (0.063 - 0.200 mm/70 - 230 mesh ASTM, Macherey - Nagel, Duren, Germany) with mobile phases as indicated for each compound.

3.2 SYNTHESIS OF COMPOUNDS

The synthesis of the proposed compounds was carried out by nucleophilic substitution (S_N2) of an acyl chloride to the indole polycyclic ring to form the amide and carbamate derivatives. This will occur by conjugation of the carbamoyl moiety at either position 5 or 6 via amidation or esterification followed by the substitution of the propargylamine moiety at position 1. Eight compounds were successfully synthesised. The compounds were separated into 2 series according to their respective substitutions

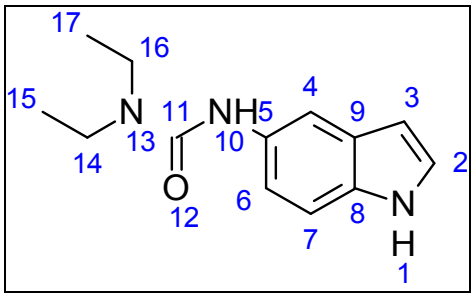
3.3 SYNTHESIS OF COMPOUNDS 1A TO 4A

The synthetic pathway followed for compounds **1A** to **4A** is shown in **Scheme 1** below. The indole was conjugated to the carbamoyl group at position 5 or 6 in a 1:1 ratio, using appropriate solvent and 1.2 mmol of potassium carbonate (K_2CO_3). The mixture was stirred for 2 hours at room temperature and monitored using TLC (1 hexane: 2 ethyl acetate). Once the reaction was complete the mixture was filtered to remove the K_2CO_3, solvent removed *in vacou* and the compound extracted at least 3 times with a DCM:water mixture. The organic extract was then evaporated and allowed to dry rendering the desired compounds.



Scheme 3.1: Synthetic route for the synthesis of Compounds **1A** to **4A**

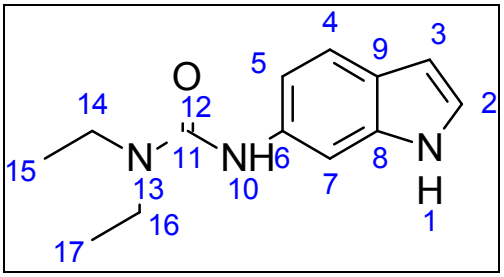
3.3.1 *N,N*-diethyl-*N*'-1*H*-indol-5-ylurea (1A)



Synthesis: In 5 ml acetonitrile, 132.20 mg of 5-aminoindole was dissolved. To the mixture 277.5 mg K_2CO_3 was added. Thereafter 127 µL (135.60 mg) of diethylcarbamoyl chloride in 3 ml acetonitrile was added dropwise over 10 minutes. The mixture was stirred at room temperature for 2 hours and monitored using TLC (1 hexane: 2 ethyl acetate).. Once the reaction was complete, K_2CO_3 was filtered out and the acetonitrile removed *in vacou*. An extraction was carried three times in water:DCM (ratio 1:1). The organic phase was washed with a saturated solution on NaCl then again with water. The DCM was removed *in vacou* to render a blackish amorphous product which was purified via column chromatography.

Physical data: $C_{13}H_{17}N_3O$; **mass**: 198.96 mg; **yield**: 74.2%; **mp**: 143 ºC;**^{1}H NMR**: (400 MHz, $CDCl_3$) δ_H (Spectrum 1): 7.89 (s, 1 H, H – 4), 7.49 - 7.47 (d, 1 H, J = 8.4 Hz, H – 7), 7.11 – 7.10 (t, 1 H, J = 5.6; 2.8 Hz, H – 2), 6.77 – 6.75 (dd, 1 H, J = 4.8; 2.4 Hz, H – 6), 6.45 – 6.34 (m, 1 H, H – 3), 3.42 – 3.37 (m, 4 H, H – 14; 16), 1.26 – 1.24 (t, 6 H, J = 8.0; 4.0 Hz, H – 15, 17); **^{13}C NMR** (100 MHz, CD_3OD) (Spectrum 2): 158.15, 137.74, 134.54, 126.26, 125.37, 120.74, 116.81, 106.73, 102.10, 42.43, 14.12;**IR** (FT – IR, cm^{-1}) (Spectrum 3): 3247.42, 2978.13, 1220.22; **MS** (DI – ESI – MS), m/z, SQ 300 (Spectrum 4): 232.10 (M+), 233.14, 133.11.

3.3.2 *N,N*-diethyl-*N*'-1*H*-indol-6-ylurea (2A)

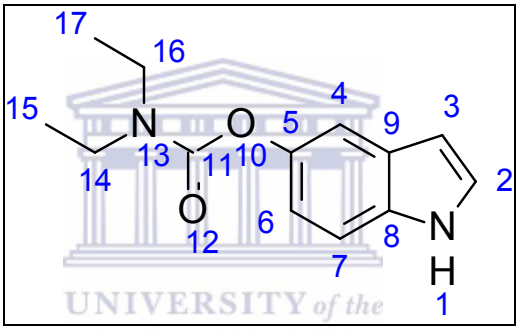


Synthesis: For the synthesis of **2A**, 154.10 mg of 6-aminoindole was reacted with 325.71 mg K_2CO_3 and 149 µL (159.08 mg) diethylcarbamoyl chloride under the same

conditions as compound **1A.** The compound was a brown and oily product which was purified via column chromatography.

Physical data: $C_{13}H_{17}N_3O$; **mass**: 164.42 mg; **yield**: 52.5 %; **mp**: 141 ºC;**^{1}H NMR**: (400 MHz, $CDCl_3$) δ_H (Spectrum 5): 7.63 (s, 1 H,H – 7), 7.28 - 7.26 (d, 1 H, J = 8.0 Hz, H – 5), 7.16 – 7.15 (d, 1 H, H – 4), 6.47 – 6.46 (t, 1 H, J = 4.8; 2.4 Hz, H – 2), 6.26 (m, 1 H, H – 3), 3.42 – 3.37 (m, 4 H, H – 14; 16), 1.26 – 1.22 (t, 6 H, J = 14.4; 7.2 Hz, H – 15, 17); **^{13}C NMR** (400 MHz, CD_3OD) (Spectrum 6):153.69, 126.35, 123.97, 121.48, 121.23, 114.60, 106.23, 97.42, 79.24, 36.97, 13.41;**IR** (FT – IR, cm^{-1}) (Spectrum 7): 3288.30, 2922.65, 1760.52, 1217.09; **MS**(DI – ESI – MS), m/z, SQ 300 (Spectrum 8): 232.15 (M+), 226.08, 204.10, 169.08.

3.3.3 1*H*-indol-5-yl diethylcarbamate (3A)

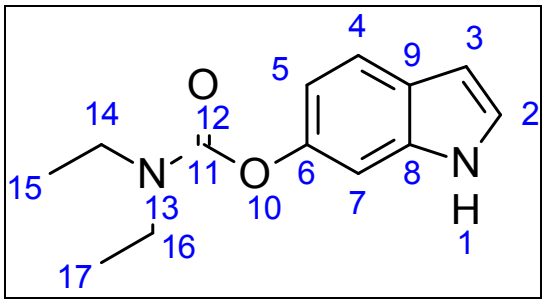


Synthesis: In a round bottomed flask, 60.8 mg of 5-hydroxyindole was dissolved in $CHCl_3$/THF (1:1, 10ml). Thereafter 58 µL (61.93 mg) of diethylcarbamoyl chloride in 3 ml $CHCl_3$/THF was added dropwise over 10 minutes. The mixture was stirred at room temperature for 2 hours and monitored using TLC. Once the reaction was complete the solvent removed *in vacou*. An extraction was carried three times in water: DCM (1:1). The organic phase was washed with a saturated solution on NaCl then again with water. The DCM was removed *in vacou* to render an orange waxy product.

Physical data: $C_{13}H_{16}N_2O_2$; **mass**: 100.73 mg; **yield**: 81.4 %; **mp**: 126 ºC;**^{1}H NMR**: (400 MHz, CD_3OD) δ_H (Spectrum 9): 7.19 – 7.8 (d, 1 H, J = 8.8 Hz, H – 7), 7.14 - 7.13 (d, 1 H, J = 2.8 Hz, H – 4), 6.92 – 6.91 (d, 1 H, J = 4.0 Hz, H – 2), 6.60 – 6.40 (dd, 1 H, J = 8.8; 2.4 Hz, H – 6), 6.27 – 6.25 (dd, 1 H, J = 3.2; 0.8 Hz; H – 3), 3.21 – 3.16 (m, 4 H, H – 14; 16), 1.13 – 1.09 (t, 6 H, J =14.4; 6.8 Hz, H – 15, 17); **^{13}C** NMR (400 MHz, CD_3OD) (Spectrum 10):158.38, 137.79, 134.52, 126.28, 125.43, 120.71, 116.83, 106.66, 102.06, 42.47, 14.13;**IR** (FT – IR, cm^{-1}) (Spectrum 11):3298.82, 2971.20,

1210.75; **MS**(DI – ESI – MS), m/z, SQ 300 (Spectrum 12): 233.11 (M+), 189.14, 152.17, 137.40.

3.3.4 1*H*-indol-6-yl diethylcarbamate (4A)

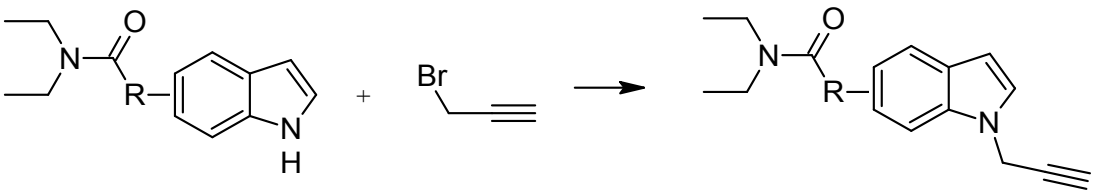


Synthesis: 183.00 mg of 6-hydroxyindole was dissolved in $CHCl_3$/THF (1:1, 10ml). Thereafter 116 µL (123.86 mg) of diethylcarbamoyl chloride in 3 ml $CHCl_3$/THF was added dropwise over 10 minutes and treated under the same conditions as **3A**

Physical data: $C_{13}H_{17}N_3O$; **mass**: 245.92 mg; **yield**: 80.10 %; **mp**: 159 ºC;**^{1}H NMR**: (400 MHz, $CDCl_3$) δ_H (Spectrum 13): 7.63 – 7.26 (d, 1 H, J = 1.6 Hz, H – 7), 6.95 – 6.94 (d, 1 H, J = 2.0 Hz, H – 4), 6.68 – 6.65 (dd, 1 H, J = 8.4; 2.0 Hz, H – 5), 6.48 – 6.46 (t, 1 H, J = 6.4; 3.2 Hz, H – 2), 6.38 – 6.36 (m, 1 H, H – 3), 3.43 – 3.37 (m, 4 H, H – 14; 16), 1.26 – 1.22 (t, 6 H, J =14.4; 6.8 Hz, H – 15, 17); **^{13}C NMR** (400 MHz, CD_3OD) (Spectrum 14): 158.52, 137.78, 134.55, 125.36, 122.75, 120.76, 116.79, 106.73, 101.26, 42.39, 14.08;**IR** (FT – IR, cm^{-1}) (Spectrum 15): 3253.61, 1683.13, 1215.52; **MS**(DI – ESI – MS), m/z, SQ 300 (Spectrum 16): 233.13 (M+), 152.15, 135.15.

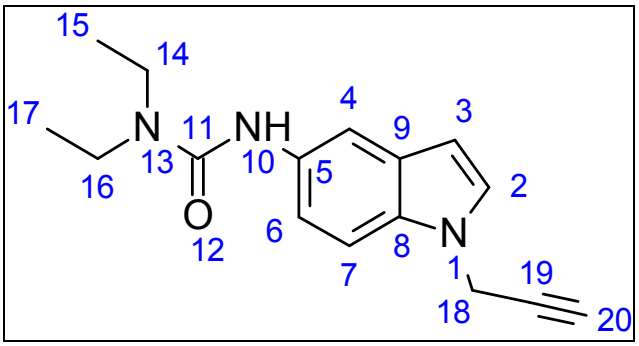
3.4 SYNTHESIS OF COMPOUNDS 1B TO 4B

The second series of compounds (**1B** to **4B**) involved the conjugation of the propargylamine moiety to position 1 of the already synthesised **1A** to **4A**. The reactants were mixed in dimethylformamide and left to stir in the microwave for 2 to 4 hours with TLC monitoring(1 hexane: 2 ethyl acetate: 1 petroleum ether). Once the reaction was complete ice water was added followed by at least 5 extractions with ethyl acetate. Thereafter the organic phase was washed with a saturated sodium chloride solution and distilled water again. The ethyl acetate was removed *in vacou* to render pure product. The reaction is summarised in **Scheme 3.2** below.



Scheme 3.2: Synthetic route for the synthesis of compounds **1B** to **4B.** R = O/NH

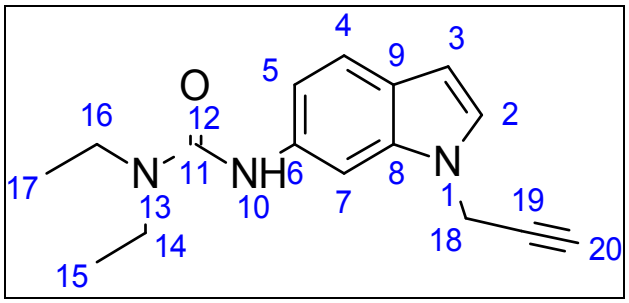
3.4.1 *N,N*-diethyl-*N'*-[1-(prop-2-yn-1-yl)-1*H*-indol-5-yl]urea (1B)



Synthesis: In a microwave compatible glass-vessel, 50.00 mg of **1A**was dissolved in DMF (6 ml) and treated with NaH (60 %, 40 mg, 1 mmol). The mixture was stirred at room temperature for 10 minutes followed by the addition of excess propargyl bromide solution (35.36 µL, 28.29 mg) while stirring. Subsequently the vessel was sealed and the mixture was stirred at 95 ºC for 2.5 hours using microwave irradiation (maximum power = 150W). Thereafter the mixture was added to 60 ml water, extracted with 100 ml ethyl acetate, washed with saturated NaCl solution and then washed 3 times again with water and dried *in vacou* to render a black amorphous powder.

Physical data: $C_{16}H_{19}N_3O$; **mass**: 74.39 mg; **yield**: 95.02 %; **mp**:158 ºC;**^{1}H NMR**: (400 MHz, $CDCl_3$) δ_H (Spectrum 17): 7.64 – 7.63 (d, 1 H, J = 1.6 Hz, H – 4), 7.29 – 7.27 (d, 1 H, J = 8.8 Hz, H – 7), 7.15 – 7.14 (m, 2 H, J = 10.4 Hz, H – 2, 6), 6.47 – 6.46 (t, 1 H, J = 1.0, 2.0 Hz, H – 3), 4.12 (s, 2 H, H – 18), 3.42 – 3.37 (m, 4 H, H – 14; 16),2.22 (s, 1 H, H – 20), 1.26 – 1.23 (t, 6 H, J =14.0; 6.8 Hz, H – 15, 17); **^{13}C NMR** (400 MHz, CD_3OD) (Spectrum 18):157.03, 153.96, 147.94, 139.35, 126.21, 120.56, 111.42, 114.78, 110.24, 109.63, 105.20, 97.47, 43. 55, 11.50;**IR** (FT – IR, cm^{-1}) (Spectrum 19): 3293.69, 2922.34, 1761.13, 1217.23; **MS** (DI – ESI – MS), m/z, SQ 300 (Spectrum 20): 270.94, (M+), 236.89, 228.97, 214.00.

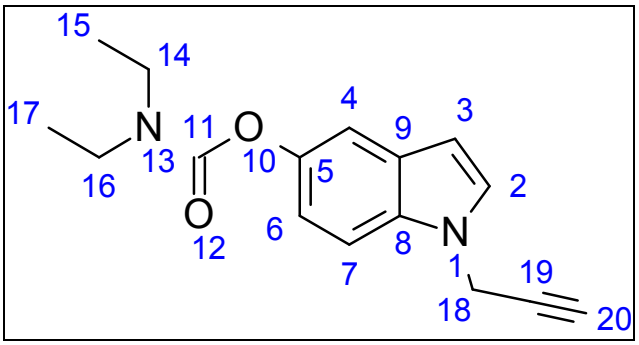
3.4.2 *N*,*N*-diethyl-*N*'-[1-(prop-2-yn-1-yl)-1*H*-indol-6-yl]urea (2B)



Synthesis: For the synthesis of **2B**, 65.20 mg of **2A** (*N*,*N*-diethyl-*N*'-1*H*-indol-6-ylurea) was reacted with 46.11 µL (36.89 mg) propargyl bromide under the same conditions as compound **1B.**

Physical data: $C_{13}H_{17}N_3O$; **mass**: 89.77 mg; **yield**: 87.93 %; **mp**: 166 ºC; **^{1}H NMR**: (400 MHz, CD_3OD) δ_H (Spectrum 21): 7.42 – 7.40 (m, 2 H, H – 7,4), 7.15 – 7.14 (d, 1 H, J = 3.8 Hz, H – 3), 6.94 – 6.92 (d, 1 H, J = 8.4 Hz, H – 5), 6.37 – 6.36 (d, 1 H, J = 3.2 Hz, H – 2), 4.61 (s, 2 H, H – 18), 3.46 – 3.41 (m, 4 H, H – 14, 16), 2.16 (s, 1 H, H – 20); 1.23 – 1.19 (t, 6 H, J =14.4; 6.8 Hz, H – 15, 17); **^{13}C NMR** (400 MHz, CD_3OD) (Spectrum 22): 157.13, 139.29, 127.18, 126.16, 120.56, 114.69, 110.23, 109.85, 105.26, 102.33, 97.49, 43.46, 11.61;**IR** (FT – IR, cm^{-1})(Spectrum 23): 3300.95, 3194.06, 2927.43, 1210.50; **MS** (DI – ESI – MS), m/z, SQ 300 (Spectrum 24): 271.15 (M+), 255.10, 233.11, 114.66.

3.4.3 1-(prop-2-yn-1-yl)-1*H*-indol-5-yl diethylcarbamate (3B)

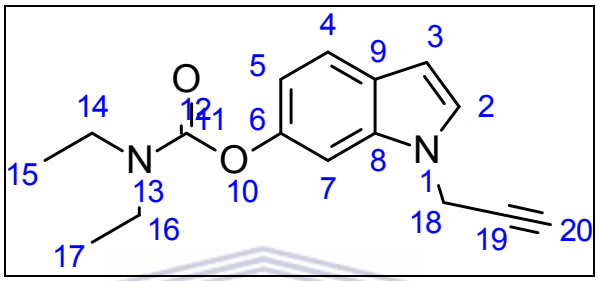


Synthesis: For the synthesis of **3B**, 71.66 mg of **3A** (1*H*-indol-5-yl diethylcarbamate) was reacted with 43.37 µL (34.69 mg) propargyl bromide under the same conditions as compound **1B.**

Physical data: $C_{16}H_{18}N_2O_2$; **mass**: 100.39 mg; **yield**: 94.4 %; **mp**: 187 ºC; **^{1}H NMR**: (400 MHz, CD_3OD) δ_H (Spectrum 25): 7.50 – 7.46 (d, 1 H, J = 16.0 Hz, H – 7), 7.24 –

7.19 (dd, 2 H, J = 19.2; 3.2 Hz, H – 4, 6), 7.16 – 7.13 (dd, 1 H, J = 10.4; 1.6 Hz, H – 2), 6.41 – 6.37 (dd, 1 H, J = 15.6; 2.8 Hz, H – 3),4.96 – 4.95 (d, 2 H, H – 18); 3.45 – 3.40 (m, 4 H, H – 14; 16), 2.79 – 2.78 (t, 1 H, J = 4.8; 2.4 Hz, H – 20), 1.23 – 1.19 (t, 6 H, J =14.4; 7.2 Hz, H – 15, 17);**^{13}C NMR** (400 MHz, CD_3OD) (Spectrum 26): 158.52, 135.13, 132.06, 129.38, 125.73, 120.36, 116.34, 114.23, 111.79, 107.90, 102.34, 101.38, 42.32, 14.13;**IR** (FT – IR, cm^{-1}) (Spectrum 27): 3299.52, 3195.74, 1210.83; **MS** (DI – ESI – MS), m/z, SQ 300 (Spectrum 28): 271.11 (M+), 236.02, 202.03 115.05.

3.4.4 1-(prop-2-yn-1-yl)-1*H*-indol-6-yl diethylcarbamate (4B)



Synthesis: For the synthesis of **4B**, 63.95 mg of **4A** (1*H*-indol-6-yl diethylcarbamate) was reacted with 38.69 µL (30.95 mg) propargyl bromide under the same conditions as compound **1B.**

Physical data: $C_{16}H_{18}N_2O_2$; **mass**: 90.48 mg; **yield**: 95.34 %; **mp**: 168 ºC;**^{1}H NMR**: (400 MHz, CD_3OD) δ_H (Spectrum 29): 7.47 – 7.46 (dd, 1 H, J = 4.0, 2.0 Hz, H – 7), 7.39 – 7.36 (d, 1 H, J = 12.0 Hz, H – 4), 7.24 – 7.23 (d, 1 H, J = 4.0 Hz, H – 5), 7.20 – 7.19 (d, 1 H, J = 4.0 Hz, H – 2), 6.43 – 6.33 (m, 1 H, H – 3), 4.96 – 4.95 (d, 2 H, J = 4.0 Hz, H – 18); 3.46 – 3.40 (m, 4 H, H – 14,16), 2.79 – 2.70 (t, 1 H, J = 8.0; 4.0 Hz, H – 20), 1.23 – 1.21 (t, 6 H, J = 8.0; 4.0 Hz, H – 15, 17); **^{13}C NMR** (400 MHz, CD_3OD) (Spectrum 30):158.76, 135.15, 132.05, 129.32, 126.16, 126.08, 124.58, 119.97, 116.27, 111.79, 102.32, 101.29, 42.52, 14.14; **IR** (FT – IR, cm^{-1}) (Spectrum 31): 3291.83, 2922.52, 1761.08, 1217.03; **MS** (DI – ESI – MS), m/z, SQ 300 (Spectrum 32): 271.59 (M+), 236.01, 228.96, 115.06.

3.5 CONCLUSION

Eight compounds were successfully synthesised. The compounds in the first series i.e. carbamate derivatives (**1A**, **2A**, **3A**, **4A**) were mostly impure with percentage yields ranging between 50 and 80 %. The second series (**1B**, **2B**, **3B**, **4B**) was synthesised under microwave irradiation conditions that improved yield and efficiency. All

compounds were successfully elucidated via NMR, IR and the molecular masses confirmed using MS. The compounds were then analysed for biological activity *in vitro* and the results are discussed in Chapter 4.The compounds were also susceptible to light degradation hence required storage in dark, cool environments.

CHAPTER 4

4. MOLECULAR MODELLING AND BIOLOGICAL EVALUATION

4.1 INTRODUCTION

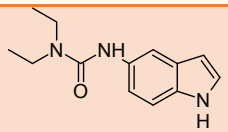
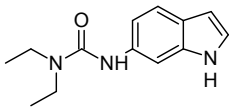
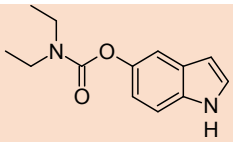
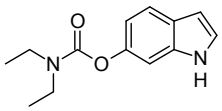
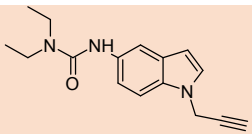
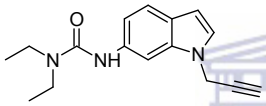
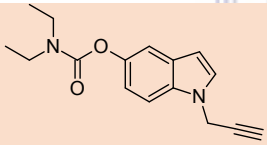
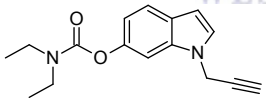
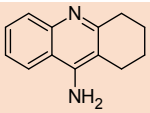
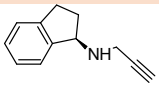
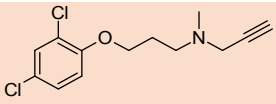
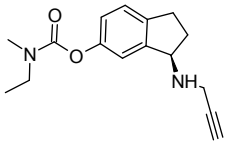
The medicinal chemistry and therapeutic potential of MTDL has been extensively explored by a number of research groups. This has led to the development of a variety of potential multifunctional neuroprotective compounds. Studies have shown the significance of the carbamoyl moiety of rivastigmine for cholinesterase inhibition (Gavrilova *et al*., 1999; Enz *et al*., 1993), the propargylamine group of selegiline and rasagiline for MAO inhibitory activity and neuroprotection (Zindo, *et al*., 2015) and the indole heterocyclic moiety for MAO and NOS inhibition and neuroprotection (Stolc *et al*., 2006; Buemi *et al*., 2013). As discussed earlier the MTDL strategy has become popular in designing compounds able to treat the multifactorial nature of Alzheimer's disease. This approach also avoids the application of polypharmacy which is costly both to the patient and the economy and comes with compliance issues (Cavalli, *et al*., 2008).

The aim of this study was to design and synthesise a series of novel indole derivatives and to evaluate them for AChE, BuChE, MAO-A and MOA-B enzyme inhibition. Molecular modelling and four assays were conducted to achieve this aim. We hypothesised that the 8 compounds, successfully synthesised (**Table 4.1**), would exert promising results on all relevant assays.

4.2 MOLECULAR MODELLING

Molecular modelling is a frequently used tool in structure based rational drug design. Docking programs such as AutoDock, Molecular Operating Environment (MOE) and SURFLEX – DOCK simulate how a target macro-molecule such as a receptor or enzyme interacts with small molecule ligands (substrates, inhibitors etc.). To model the binding between the ligand and the target molecule, the known three dimensional structures are super-imposed (docking) and the interactions between the two is then analysed. In this study we utilised MOE 2015 to model the binding of our potential inhibitors to co-crystallised forms of AChE, MAO-A and MAO-B.

Table 4.1: Successfully synthesised compounds assayed for activity and the controls

1A		*N*,*N*-diethyl-*N*'-1*H*-indol-5-ylurea
2A		*N*,*N*-diethyl-*N*'-1*H*-indol-6-ylurea
3A		1*H*-indol-5-yl-diethylcarbamate
4A		1*H*-indol-6-yl-diethylcarbamate
1B		*N*,*N*-diethyl-*N*'-[1-(prop-2-yn-1-yl)-1*H*-indol-5-yl]urea
2B		*N*,*N*-diethyl-*N*'-[1-(prop-2-yn-1-yl)-1*H*-indol-6-yl]urea
3B		1-(prop-2-yn-1-yl)-1*H*-indol-5-yl-diethylcarbamate
4B		1-(prop-2-yn-1-yl)-1*H*-indol-6-yl-diethylcarbamate
Tacrine		1,2,3,4-tetrahydroacridin-9-amine
Rasagiline		(1*R*)-*N*-(prop-2-yn-1-yl)-2,3-dihydro-1*H*-inden-1-amine
Clorgyline		*N*-[3-(2,4-dichlorophenoxy)propyl]-*N*-methylprop-2-yn-1-amine
Ladostigil		(3*R*)-3-[(prop-2-yn-1-yl)amino]-2,3-dihydro-1*H*-inden-5-yl ethyl(methyl)carbamate

4.2.1 MOLECULAR MODELLING METHODS

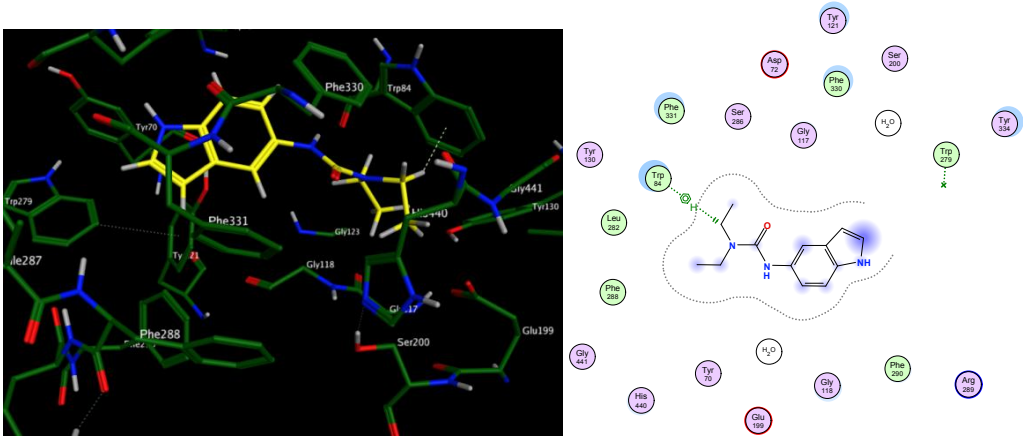
The necessary enzyme complexes were obtained from the PSLIO / PDB databank. The codes for the enzymes used were 1EVE for AChE co-crystallised with donepezil, 2v5z for MAO-B co-crystallised with safinamide and 2BXR for MAO-A co-crystallised with clorgyline. Prior to docking, the enzymes were protonated and cleaned up for any unnecessary amino acids surrounding the binding sites, while maintaining the integrity of the enzyme backbone. This helps to make the viewing of the interactions easier. The existing ligand, was then selected in order to specify the binding pocket of the enzyme. The energy minimised compounds (V3000 MDL molfiles drawn using Chemsketch) were then docked using the induced fit protocol. We discuss below the results that of selected compounds that showed the lowest binding energy and best fit during molecular docking.

4.2.2 ACHE DOCKING RESULTS

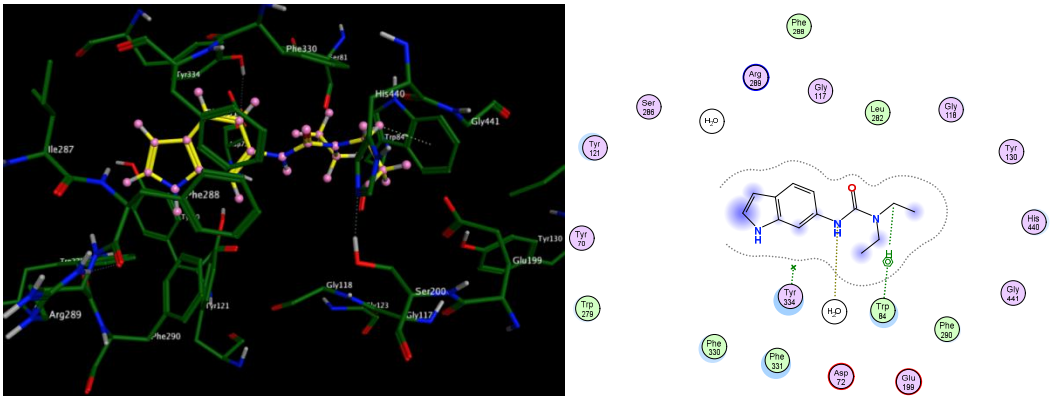
AChE contains two subsites; the PAS and CAS sites as well as a gorge lined with aromatic amino acids connecting the two (Colovic *et al.*, 2013). It was expected that the carbamate/urea component of the molecule will possess interactions in the CAS and the rest of the molecule with the PAS (Jampilek et al., 2012).

The results showed that the compounds interacted with the whole cholinesterase active site. The alkyl component of the carbamate moiety of the compounds (**1A**, **2A** and **4B**) showed π – H interactions with Trp 84 in the CAS as can be seen in **Figure 4.1** below. This interaction has been reported to be very crucial in AChE inhibitory activity (Tougu, 2001).

1A



2A



4B

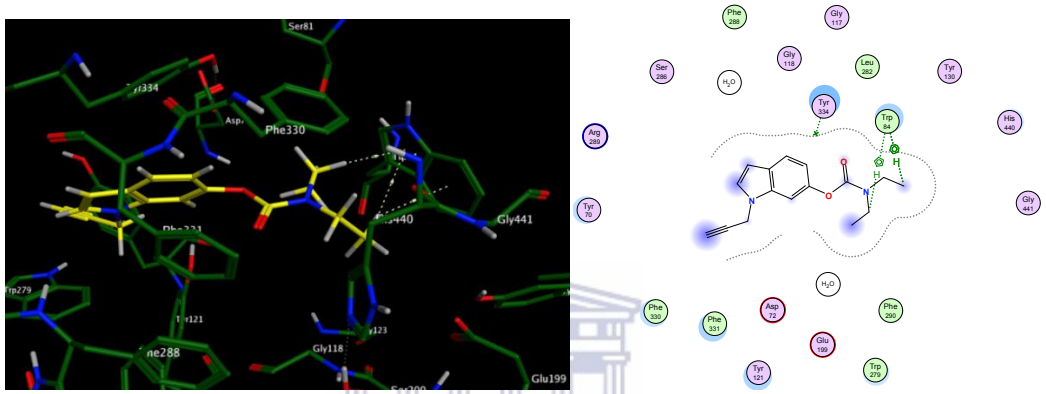
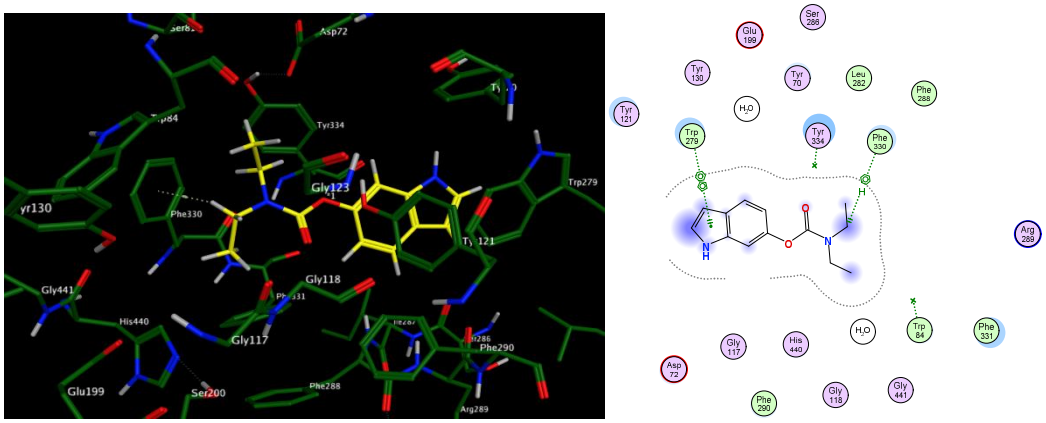


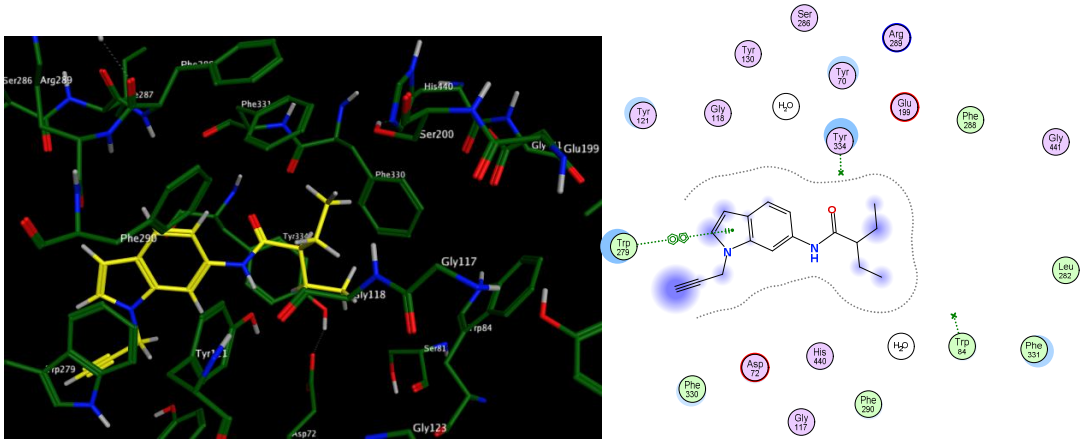
Figure 4.1: Docking pose of compounds **1A**, **2A** and **4B** in AChE active site. The ligand is shown in yellow and AChE residues in green.

Compounds **4A**, **2B** and **3B** possessed π – π interactions between the indole polycyclic ring and Trp 279 in the PAS site **(Figure 4.2)**. Moreover compound **4A** possessed π – H interactions with Phe 330, a key residue involved in ligand binding in the active gorge. We can surmise that compounds **4A**, **2B** and **4B** would possess good AChE inhibitory activity. It is important to note that the propargylamine moiety did not show any significant interaction with amino acids in the active site. The moiety occupied the gorge (**4B**) facing the PAS

4A



2B



3B

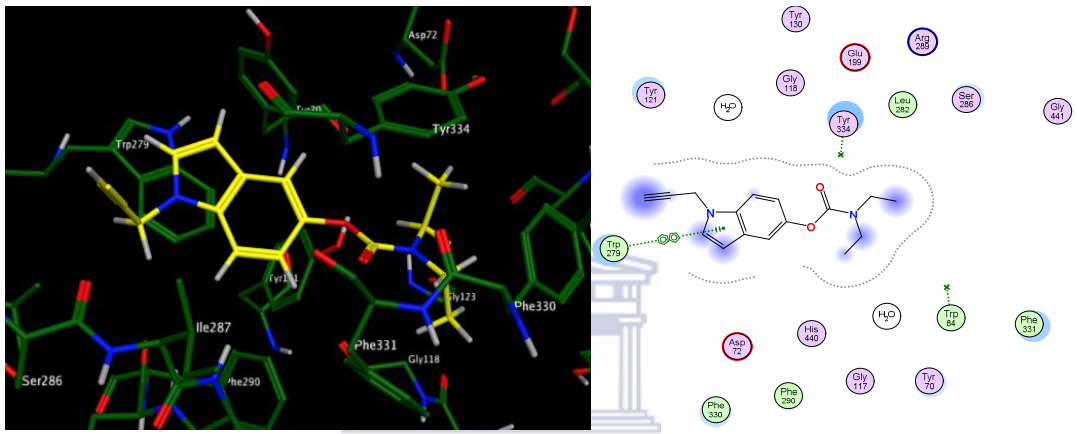


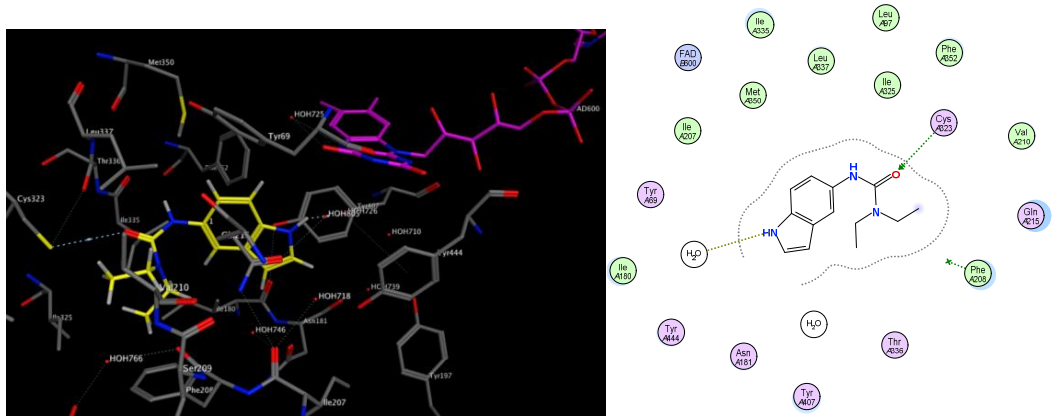
Figure 4.2: Docking pose of compounds **4A**, **2B** and **3B** in the AChE active site.

4.2.3 MAO DOCKING RESULTS

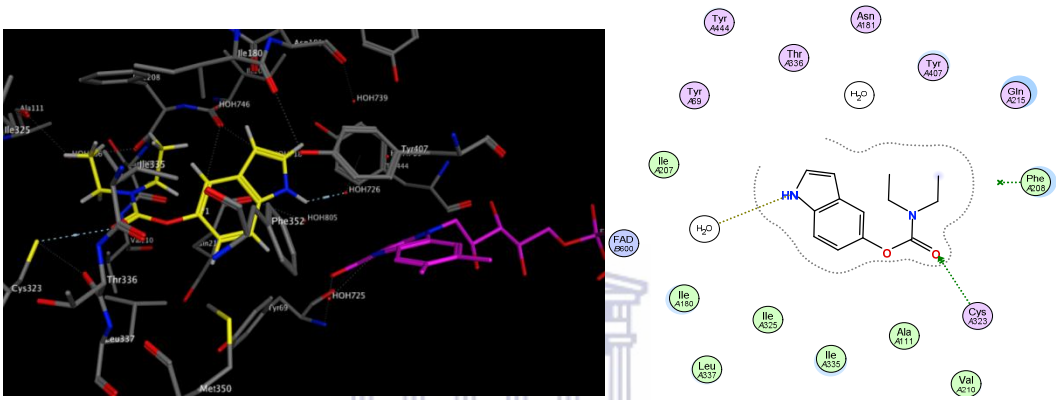
A review of the MAO-A enzyme showed that it exists as a monomer that is highly inhibited by clorgyline (De Colibus *et al.*, 2005).

Docking studies showed that the compounds possessed little interaction with the amino acids in the MAO-A active site. The results showed that the carbamylated compounds **1A- 4A** lacked interactions with vital residues in the active site such as Phe 208 and the FAD cofactor. We observed that for these compounds the indole polycyclic ring was best orientated towards the FAD cofactor in the substrate cavity (**Figure 4.5**) except for **2A**.They also had hydrogen bonds with the water molecules in the substrate cavity. From these observations these compounds did not appear to be good inhibitors of the enzyme. We observed that the urea component of compound **2A** was oriented towards the substrate cavity. This allowed it to have non – covalent interactions with Tyr 407 and Gln 215 in the active site unlike the other compounds.

1A



3A



4A

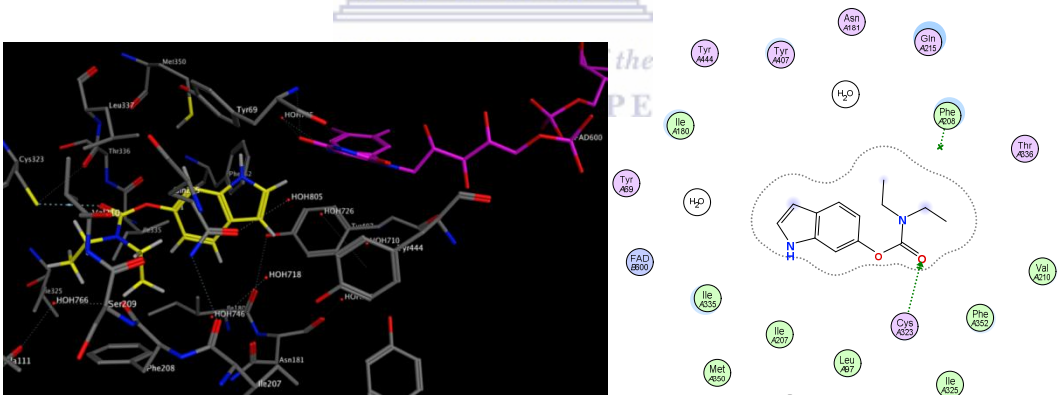


Figure 4.5: Docking pose of compounds **1A**, **3A** and **4A** in the MAO-A active site. The ligand is shown in yellow, the FAD in purple and the rest of the molecule in grey.

The compounds that contained the propargylamine moiety were not aligned in a manner that we expected i.e. with the moiety in close proximity to the FAD cofactor with the rest of the molecule occupying the active site. Compound **2B** was the only compound that had the propargylamine oriented in the direction of the FAD as seen in **Figure 4.6**. This has been reported to be essential for MAO inhibitory capacity (Edmondson, 2004), thus the compound may prove to be a good inhibitor of the enzyme. The indole polycyclic ring did not portray any significant interactions with the MAO-A active site, but the compounds seem to sit well in the active site.

2B

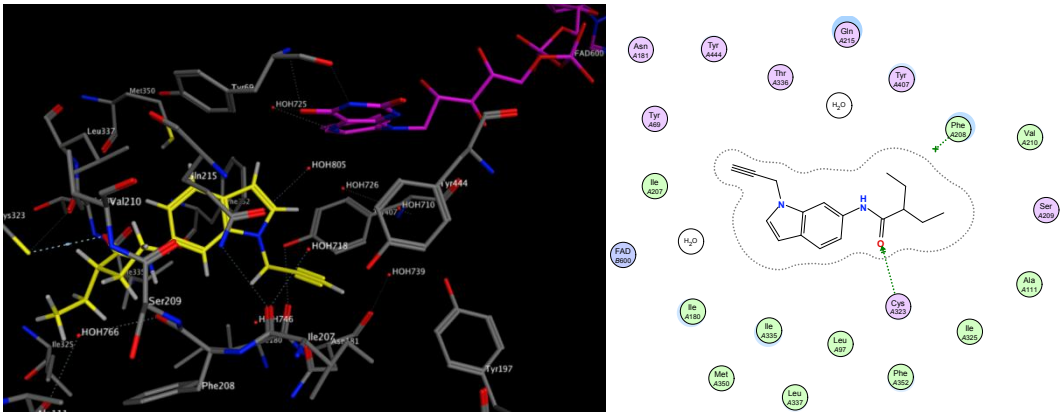
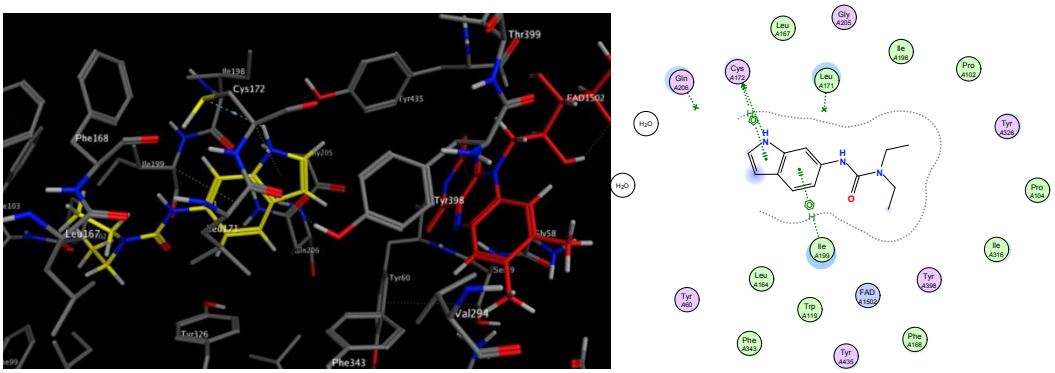


Figure 4.6: Docking pose of compounds 2B in the MAO-A active site.

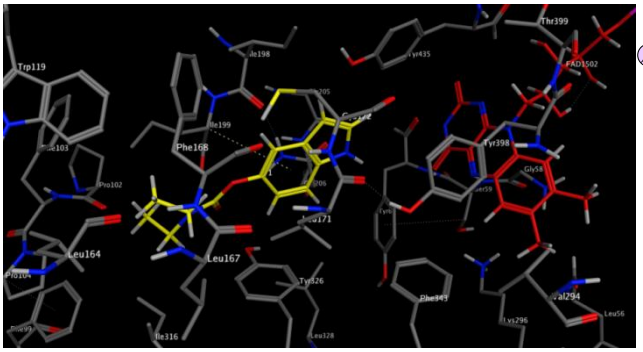
All the above mentioned compounds had interactions with Cys 323 via the carbonyl group of the carbamate component of the molecule. This is one of the residues that line the entrance to the catalytic site of the MAO-A enzyme (De Colibus *et al.*, 2005). This indicates that these compounds enter the active site even though they do not have the desired interactions to portray good activity. The rest of the compounds had little to no interactions in the active site and so may not be good inhibitors of the MAO-A enzyme.

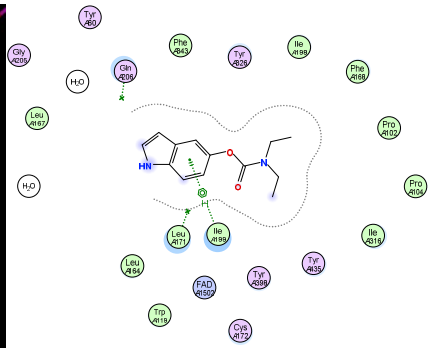
With regards to MAO-B molecular docking experiments we expected specific interactions between the compounds and the FAD co-factor, rather we observed that compounds **1A**, **2A**, **3A** and **4A** had π – H interactions with the gating residue Ile 199 meaning they do enter the active site to the hydrophobic cavity but perhaps do not reach the substrate cavity. The compounds were not in close proximity to the FAD cofactor which is essential for good activity (**Figure 4.7**). The indole polycyclic ring of these compounds did not show any interactions, except in the case of compound **2A** that interacted with Cys 172. This may indicate that that **2A** could possess better MAO-B inhibitory activity compared to the other three.

2A

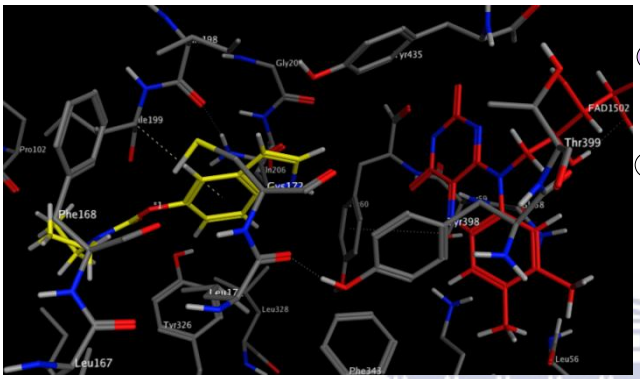


3A





4A



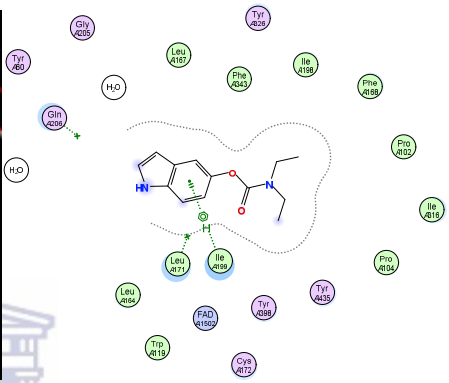
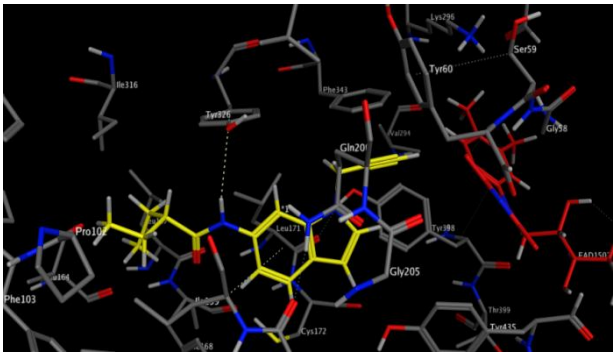


Figure 4.7: Docking pose of compounds 2A, 3A and 4A in the MAO-B active site. The ligand is shown in yellow, the FAD in red and the rest of the molecule in grey

The propargylamine containing compounds showed better interactions. The moiety was aligned much closer to the FAD with different compounds showing various interactions with Leu 171 (**1B**), Tyr 326 (**2B**) and Tyr 398 (**3B**). Compound **2B** was aligned closest to the FAD as seen in **Figure 4.8** and so may prove to have good inhibitory activity. It also showed interactions with Tyr 326, a residue that contributes to substrate specificity. This leads us to ascertain that the compound may be more selective to MAO–B.

2B



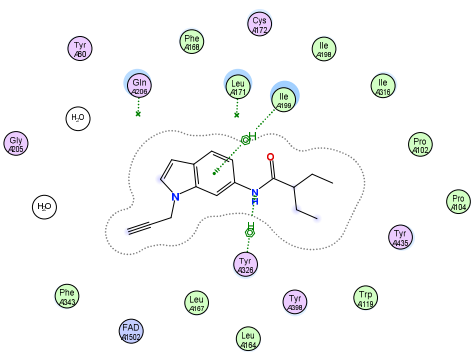


Figure 4.8: Docking pose of compounds **2B** in the MAO-B active site

4.3 CHOLINESTERASE ASSAY

AChE and BuChE efficiently catalyse the hydrolysis of acetylthiocholine, a sulphur analog of the natural substrate (acetylcholine) of these enzymes. Upon hydrolysis, the acetylthiocholine breaks down into acetate and thiocholine. Thiocholine, in the presence of the highly reactive dithiobisnitro-benzoate (DTNB) ion generates a yellow colour, which can be quantitatively monitored by spectrophotometric absorption at a wavelength of 405 nm. In this assay, acetylthiocholine and butyrylthiocholine were used as substrates for AChE and BuChE respectively. This method was first described by Ellman and colleagues (1961) and has been optimised over the years (Riddles, Blakeley, and Zerner, 1979). AChE form electric eel and BuChE of equine origin were used.

4.3.1 CONSUMABLES AND INSTRUMENTATION

Unless otherwise stated, all chemicals and reagents used for this assay were purchased from SigmaAldrich. A Rayto 6500 microplate reader was used to read the intensity of colour change at a wavelength of 405 nm. A pH meter was used to determine the pH of the assay buffer. Finally data analysis was carried out using Graph Pad Prism 7.

4.3.2 EXPERIMENTAL PROCEDURES

Stock solutions of test compounds and controls (tacrine for both cholinesterase enzymes) were prepared by dissolving the test compounds in dimethyl sulfoxide (DMSO) and were kept under refrigeration until use. Stock solutions were made at 10 mM concentrations and further serial diluted to achieve final concentrations of 100µM, 10 µM, 1 µM, and 0.1 µM. The buffer, trizma hydrochloride, was prepared in water at a concentration of 50 mM with the pH adjusted to 8 using diluted 1N sodium hydroxide. It was also refrigerated until use. The trizma hydrochloride was used to prepare 1.5 mM DTNB, 15 mM acetylthiocholine iodide, 15 mM butyrylthiocholine iodide, 22 units/ml *electric eel* AChE stock solution and 12 units/ml equine BuChE stock solution. The enzyme stock solutions were stabilised with 1% bovine serum albumin and stored in aliquots at -80 ºC.

A clear, flat base 200 µL 96 well plate was used. Prior to the experiment the enzyme stock solution was diluted to 0.22 units/ml AChE and 0.12 units/ml BuChE. Each plate row consisted of a background control (no compound), a positive control (tacrine) and 8

wells of test compounds. DTNB (148 µL) and 50 µL enzyme solution was added into the required well on the plate. DMSO (2 µL) was added to the background control well, 2 µL tacrine for positive control followed by 2 µL test compound in consecutive wells. The plate was then incubated at 25 ºC for 10 minutes. Using a micropipette, 30 µL of the substrate was added simultaneously to all wells. The well plate was further incubated for another 10 minutes at 25 ºC. Thereafter absorbance was read at 405 nm using a Rayto 6500 spectrophotometer. The data obtained was used to calculate percentage cholinesterase inhibition and plot dose-response curves using Graph Pad Prism 7 software which was then used to extrapolate IC_{50} values. All experiments were done in triplicate. It is important to note that DMSO may influence assay readings hence the necessity to keep it constant and less than 1 %. At a 1 % concentration the DMSO did not influence the assay results.

4.3.3 RESULTS AND DISCUSSION

Previous studies have shown that the carbamoyl moiety of FDA approved dual cholinesterase inhibitor rivastigmine (**Figure 2.7**) is important in its action against cholinesterase enzymes. We applied this logic by attaching the carbamoyl moiety via an S_N2 nucleophilic reaction to the 5 or 6 position of the indole polycyclic ring to render compounds **3A, 4A, 3B, 4B**.We also explored a series of urea based compounds that have an amide linkage at position 5 or 6 (**1A, 2A, 1B, 2B**) to observe how this change would affect activity. We expected all the compounds to have some degree of inhibitory activity towards the enzymes. **Figure 4.3** below highlights the results of the AChE assay with a p-value below 0.05.The p-value expresses the statistical validity of the data used to plot the curves. A desirable p-value is below 0.05 which means the data is statistically significant (Nuzzo, 2014). A one-way ANOVA statistical analysis was done using Graphpad Prism 7 to calculate the p values.

As can be observed all the compounds were able to inhibit AChE with IC_{50} values between 1 µM and4 µM. This illustrates that the diethylcarbamoyl moiety both as a carbamate or urea bond contributes to inhibition of the AChE enzyme. These results also support the docking observations. Literature reports indolyl alkaloids and their metabolites to exert good cholinesterase inhibitory capacity amongst other functions (Ismail *et al*., 2012; Passos *et al*., 2013). The docking results showed some interactions between the indole polycyclic ring and the amino acids lining the entrance of the active

site. As a result we also speculate that the indole polycyclic ring may have contributed to some inhibitory capacity of the compounds. Also, the compounds exerted good activity regardless of the position of the carbamoyl moiety, whether it's a carbamate or urea based compound or whether it contains the propargylamine moiety or not. Compound **1A** had the lowest IC_{50} of 1.97 µM. These results agree with some of the modelling results where we observed that the carbamoyl and urea moieties interact with important residues in the CAS and the rest of the molecule fits in the (PAS) similar to rivastigmine (Greenblatt et al., 2003).Addition of the propargylamine moiety did not affect the inhibitory capacity of the compounds showing again that the carbamate and urea moieties are highly involved in activity.

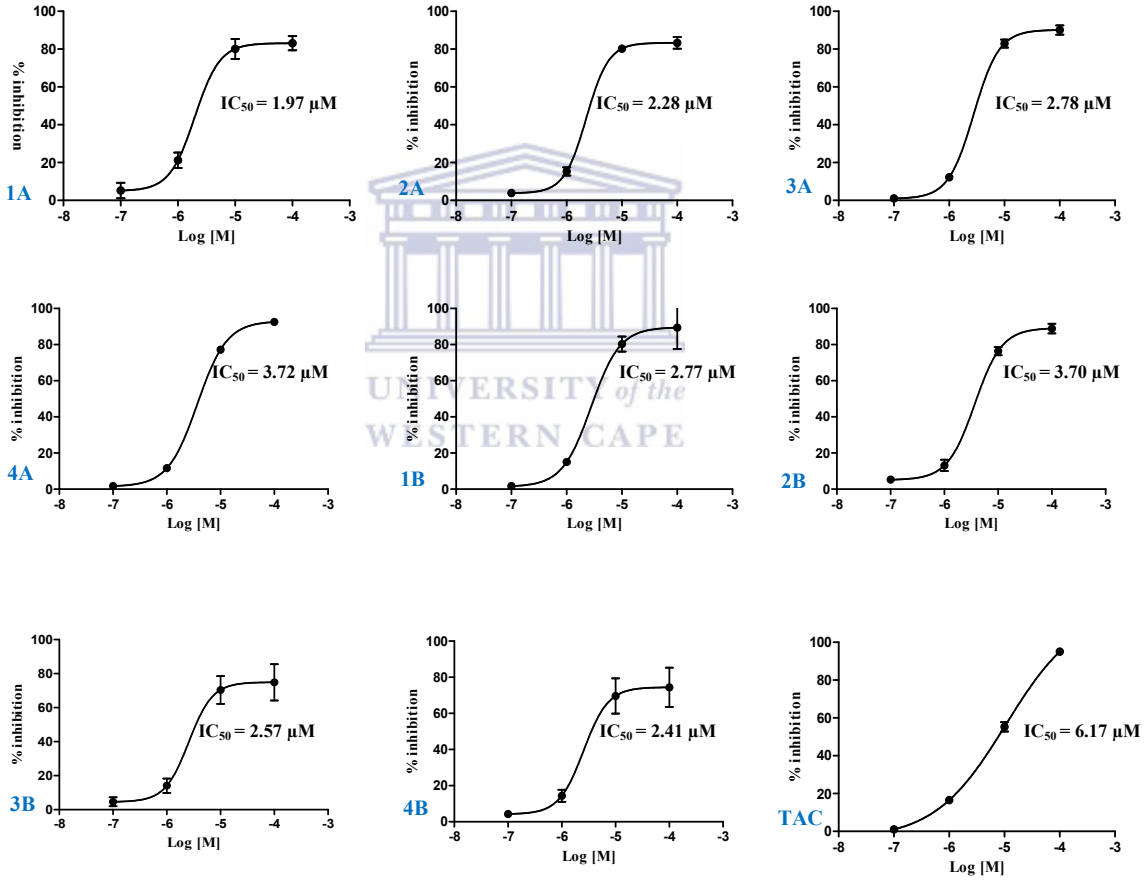


Figure 4.3: Non-linear regression curves for AChE activity for the tested compounds (**1A – 4B**) and tacrine. IC_{50} values are shown.

Comparing the IC_{50} values of the test compounds to that of tacrine (6.17 µM) shows that the test compounds are good inhibitors of the enzyme and are equally as potent as tacrine. The IC_{50} of tacrine was in the same range as reported by Chao et al., 2012. Literature reports the IC_{50} of rivastigmineas501µMon *electric eel* AChE (Imramovsky

et al., 2012). These studies suggest that the synthesised compounds are almost 100 times more potent than rivastigmine on the same species of AChE.

The role that BuChE plays in the progression of AD is its ability to hydrolyse acetylcholine in the event of AChE saturation (Giacobini, 2004). Accordingly a compound that inhibits this enzyme, in addition to AChE, would prove beneficial for the treatment of AD. We therefore went on to assay our compounds for BuChE inhibitory activity and the results are summarised in **Figure 4.4**.The IC_{50} values of the compounds were also compared to tacrine.

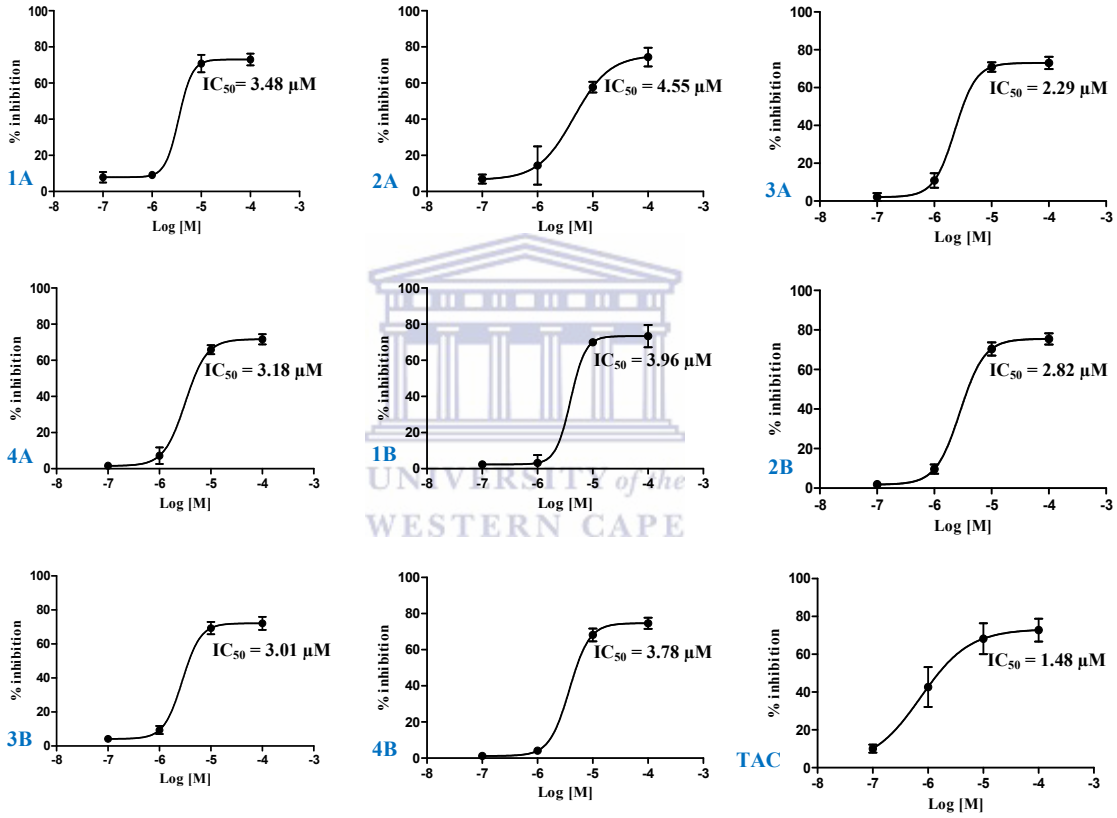


Figure 4.4: Non-linear regression curves for BuChE activity for the tested compounds and tacrine as control. IC_{50}values are shown.

These results showed that the synthesised compounds had good activity in inhibiting this enzyme. The IC_{50} values, as can be seen from **Figure 4.4** above, ranged between 2 and 5 µM. The compounds also showed activity in the same range as tacrine. Carbamoyl containing compounds have also been shown to be BuChE enzyme substrates (Colovic *et al*., 2013) so we postulate that the diethylcarbamoyl moiety interacts with the enzyme structure to block its catalytic activity. Molecular modelling was not carried out on this enzyme; hence we can only hypothesize that the carbamoyl

and perhaps the indole polycyclic ring might have an interaction in the active site to inhibit ACh hydrolysis.

Rivastigmine was reported to inhibit 50 % of the enzyme at a concentration of 19.95 µM and 37 nM in equine and human BuChE respectively (Krátký *et al*., 2016; Luo *et al*., 2006).We can see that all the synthesised compounds have higher activity than rivastigmine in equine BuChE and evaluation in human BuChE is thus essential.

When we evaluate the different substitutions of the test compounds, we see that the 5 substituted compounds yielded similar results compared to the 6 substituted compounds. Compounds **1A**, **2A**, **1B** and **4B** showed a slight selectivity towards AChE and the other compounds exhibited an equivalent activity for BuChE and to AChE. Selectivity indices were close enough for the compounds to be classified as non-selective ChE inhibitors *in vitro*.

Table 4.2:$IC_{50}$and selectivity indices of the compounds towards ChE[1]

Compound ID	AChE (µM)	BuChE (µM)	SI $\{\frac{AChE}{BuChE}\}$
1A	1,97	3,48	0.57
2A	2,28	4,55	0.50
3A	2,77	2,29	1.21
4A	3,72	3,18	1.17
1B	2,77	3,96	0.70
2B	3,70	2,82	1.31
3B	2,57	3,01	0.85
4B	2,41	5,01	1.48
Ladostigil	31.80	1.98	16.06
Rivastigmine*	56.10	19.95	2.81

*ChE inhibitory activity of rivastigmine as reported by Krátký, *et al*., 2016b.

We can conclude that the compounds are good dual ChE inhibitors able to reduce the hydrolysis of AChE. They also show a good balance between AChE and BuChE activities. Importantly, the compounds portray non-selective inhibitory activities compared to ladostigil which is 16 times more selective towards AChE. Moreover literature has reported that AChE promotes the assembly of Aβ into amyloid fibrils and that compounds that interact well with the PAS may inhibit this effect (Inestrosa et al.,

[1] The selectivity index (SI) of the compound is a ration of the compounds selectivity to one site compared to the other, and is calculated by dividing the IC_{50} values.

1996, Reyes et al., 1997). These compounds may also prove to reduce the conglomeration of amyloid fibrils into plaques in addition to reducing Ach hydrolysis.

4.4 MAO ASSAY

The MAO assay measures the ability of the compounds to inhibit the isozymes MAO-A and MAO-B and reduce the metabolism of amine neurotransmitters. The concept of this assay (**Figure 4.9**) involves the ability of MAO to convert the non-fluorescent and non-selective substrate, kynuramine, into fluorescent 4-hydroxyquinoline (Krajl, 1965).Kynuramine is the substrate in place of naturally occurring neurotransmitters such as dopamine. In the presence of an inhibitor, there is reduced interaction of kynuramine with the enzyme thus reduction in the fluorescence level (in comparison to a blank) indicates activity. **Figure 4.9** highlights the basic principle of this assay (Yan *et al*., 2004).

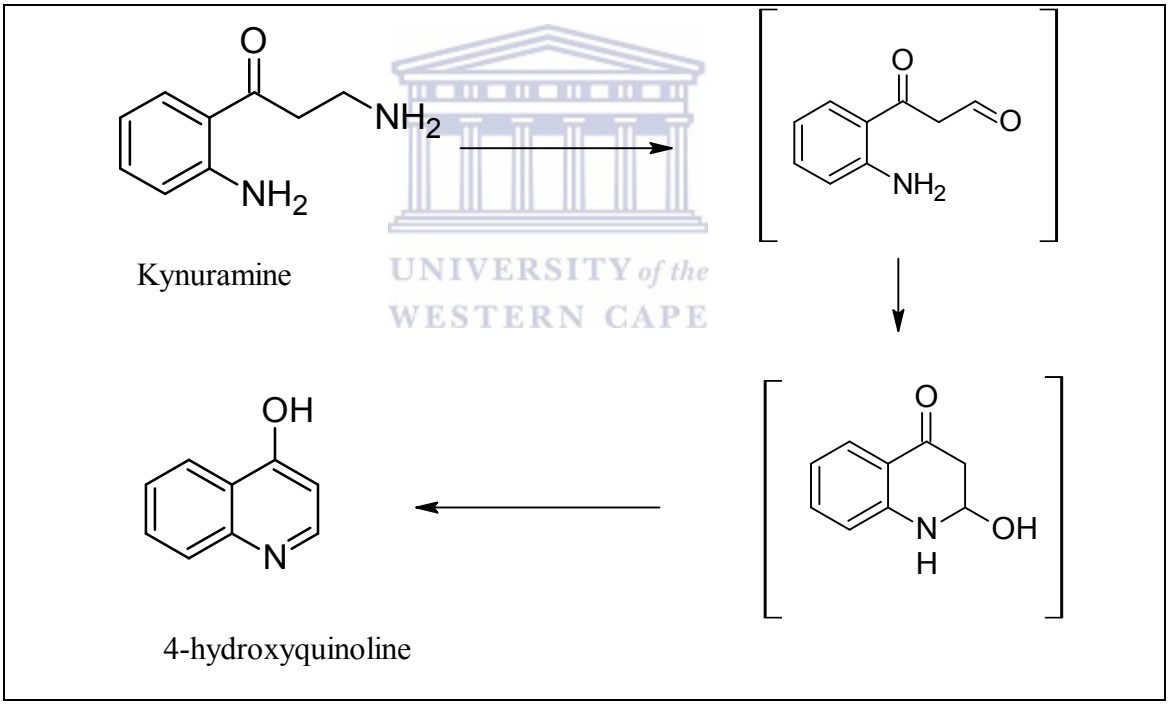


Figure 4.9: Principle of MAO catalysed conversion of kynuramine.

The fluorescence values obtained were used to calculate the percentage enzyme inhibition, plot dose-response curves and to extrapolate IC_{50} values. As with the ChE assay a p-value below 0.05 is desirable implying statistical significance and reproducibility of the results.

4.4.1 CONSUMABLES AND INSTRUMENTATION

Unless otherwise stated, all chemicals and reagents used for this assay were purchased from Sigma Aldrich. A SynergyMx Biotek spectrophotometer was used to read the fluorescence intensity at an excitation wavelength of 310 nm and emission of 410 nm. A pH meter was used to determine the pH of the assay buffer. Finally data analysis was carried out using Graph Pad Prism 7.

4.4.2 EXPERIMENTAL PROCEDURES

Stock solutions of the test compounds and controls (clorgyline for MAO-A and rasagiline for MAO-B) were prepared by dissolving the test compounds in DMSO and it was kept under refrigeration until use. The solutions were made at 10 mM concentrations and further serial diluted to achieve final concentrations of 100 µM, 10 µM, 1 µM, 0.1 µM and 0.01 µM. The potassium phosphate buffer was prepared in water at a concentration of 100 mM (adjusted to pH 7.4 by 2N NaOH). It was also refrigerated until use. The stock solution of the substrate kynuramine was made at 750 µM (45 µM final assay concentration) for MAO-A and 500 µM(30 µM final assay concentration) for MAO-B. Fresh substrate was prepared for each day to ensure that the activity was sustained. Using the phosphate buffer, MAO enzyme stock solution was prepared and stored in aliquots at -80 ºC until use to avoid thermal induced deactivation.

The assay was carried out in eppendorf vials and needed an incubator oven set at 37 ºC. To each vial 207.5 µL of phosphate buffer and 2.5 µL of corresponding test compound or control compound was added. This was followed by 25 µL of the enzyme at 10 second intervals. The mixture was then incubated for 10 minutes at 37 ºC. Thereafter 15 µL of kynuramine (45 µM for MAO-A and 30 µM for MAO-B) was added also at 10 second intervals and it was then further incubated for 20 minutes. 150 µL of 2 N NaOH were used to stop the reaction. The mixture was well shaken after each addition. Finally 200 µL of the mixture was added to each well of a black 96 well plate, placed in the fluorescent plate reader and read at excitation/emission wavelength of 310 nm/400 nm. Each assay was run in triplicate and the data was analysed with Graph Pad Prism 7 software.

4.4.3 RESULTS AND DISCUSSSION

The results (**Figure 4.10**) show that all the compounds but **1A** had the ability to inhibit the MAO-A enzyme at concentrations below 100 µM. As observed from the IC_{50} values the compounds that did not possess the propargylamine moiety had, in general, lower activity compared to the propargylamine. Compounds **2A** and **3A** were the exceptions, showing significantly low IC_{50} values. The modelling results showed that the compounds' indole ring had interactions with some water molecules in the substrate cavity and this may contribute to activity. Compound **2A** had a different orientation in the active site of MAO–A but it portrayed a relatively low IC_{50}.

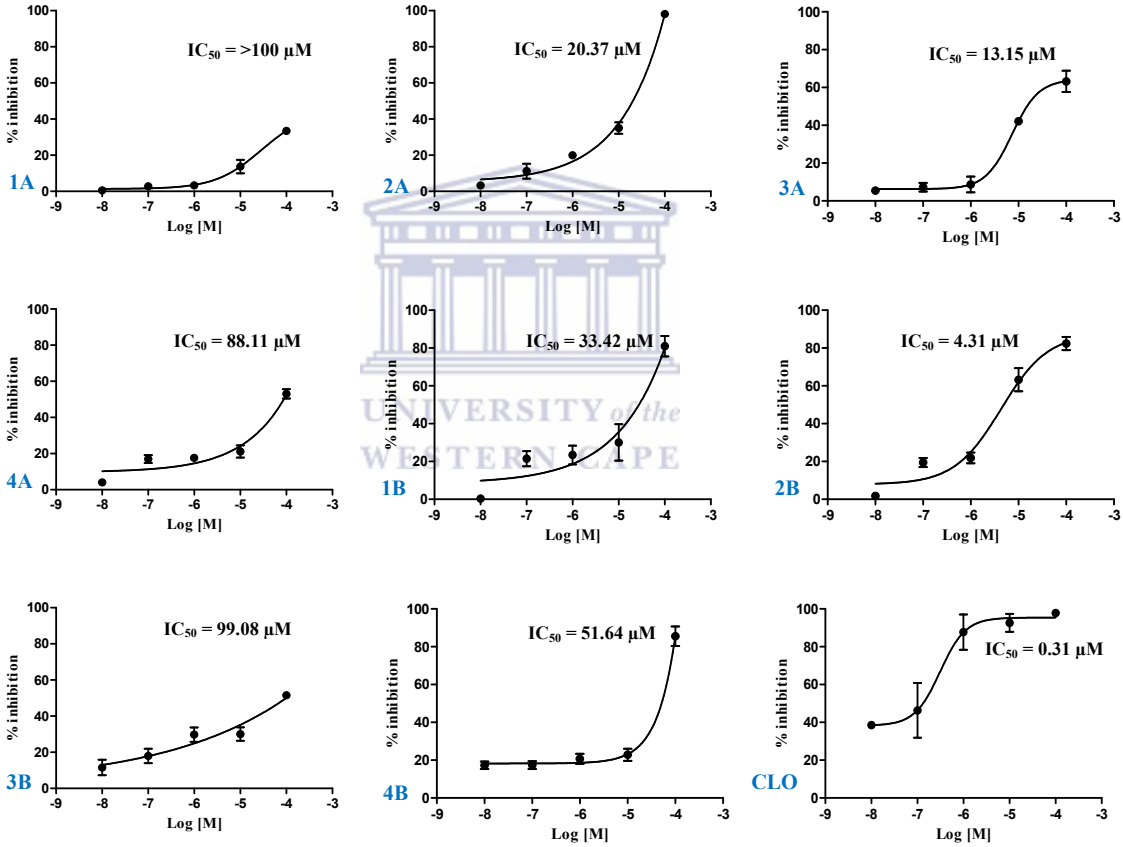


Figure 4.10: Non-linear regression curves for MAO-A activity for the tested compounds and clorgyline as control. IC_{50}values are shown

Literature suggests that the propargylamine moiety is responsible for the MAO inhibitory properties of compounds containing this moiety (Bar-Am *et al*., 2005).The results obtained agree with this, as can be seen by the lowering of the IC_{50} value (i.e. increase in potency) upon conjugation of the moiety. The presence of a urea moiety (compounds **1B** and **2B**) resulted in compounds that have comparably better inhibitory abilities than the carbamate linked compounds **3B** and **4B**. This permits us to assume

that the hydrogen donating ability of the secondary amine in the urea moieties may contribute to important interactions with amino acids in the active site of the enzyme. Looking at the results overall the compounds were moderately good inhibitors of the MAO-A enzyme. None of the compounds showed activity in the same range as clorgyline. Clorgyline was used as the control for MAO-A due to its high potency inhibitory capacity

Compound **2B** had the lowest IC_{50}within the series (4.31 µM) towards MAO-A. It possesses the carbamoyl substitution at position 6 and the propargylamine at the N1 position. The docking results support this activity where **2B** was observed to have the best interactions with the enzyme compared to the other compounds. The comparable compound **4B** with similar orientation except for a carbamate bond instead of the urea conjugation is 12 times less potent towards the same enzyme.

With regards to MAO – B inhibition, rasagiline was as positive control for comparison. It was observed that compounds **1A**, **2A**, **3A** and **4A** had IC_{50} values above 100 µM (**Figure 4.11**). These were the compounds that did not possess the propargylamine substitution at position 1 and we may surmise that they lacked vital interactions with the FAD co-factor. The docking results did show such lack of interactions within the active site. With further conjugation of the propargylamine moiety, a significant increase in the potency of these compounds against the enzyme was observed. This is in line with literature that suggests that the propargylamine moiety is a good contributor to MAO inhibitory capacity (Bar-Am *et al*., 2005).

The compounds that had the carbamate moiety had better activity that their urea linked counterparts. We observe this when we compare **1B** with **3B** and **2B** with **4B**. We can go on to surmise that the electron accepting abilities of the oxygen molecule contributes to some interaction in the MAO-B active site. Compounds **2B** and **4B** had the lowest IC_{50} values of 2.62 and 1.84 µM respectively. It is noted that these compounds had the carbamate moiety at position 6 and the position 1 propargylamine moiety similar to ladostigil. Our deduction is that this orientation of the propargylamine to the carbamoyl moiety is favours good inhibitory activity of the compounds.

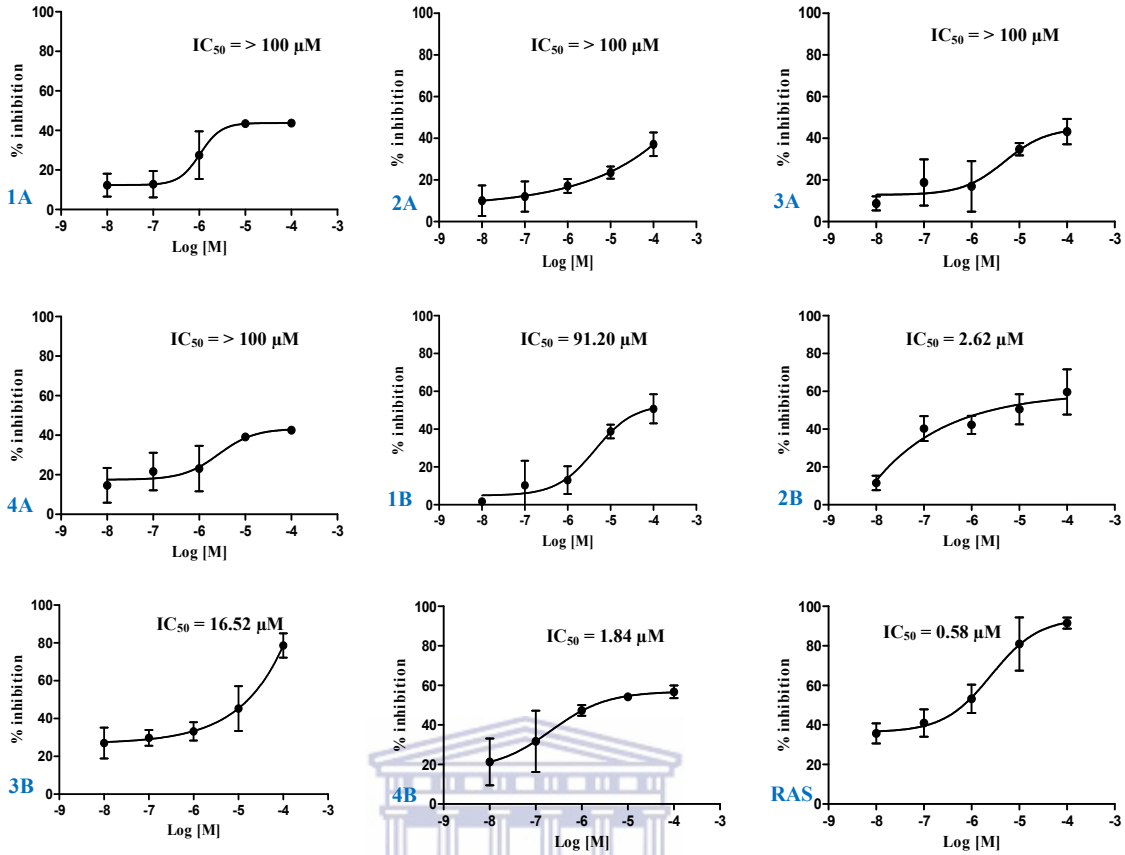


Figure 4.11: Non-linear regression curves for MAO-B activity for the tested compounds and rasagiline as control. IC_{50} values are shown ($p < 0.05$)

Comparison of the four most active compounds to rasagiline (IC_{50} = 0.58 µM) demonstrates that our compounds **2B** and **4B** are good inhibitors of MAO-B. Earlier we hypothesised that the indole polycyclic ring contributes to MAO inhibitory capacity and it is in this case that its abilities can be highlighted. Also, these compounds are considerably longer in length compared to rasagiline and could possibly fit better into the elongated active site with better orientation and interactions.

Table 4.3: IC_{50} and selectivity indices of the compounds towards MAO

Compound ID	MAO-A	MAO-B	SI $\{\frac{MAO-A}{MAO-B}\}$
1A	>100	>100	--
2A	20,37	>100	--
3A	13,15	>100	--
4A	88,11	>100	--
1B	33,42	28,84	1.16
2B	4,31	2,62	1.64
3B	99,08	16,52	6
4B	51,64	1,84	28.11

As can be seen from the table above compounds **1A**, **2A**, **3A** and **4A** which inhibit the MAO-A isozyme at lower concentrations compared to MAO-B inhibition may prove selective for it and so may contribute to peripheral potentiation of tyramine levels (a consequence of high peripheral MAO-A inhibition). However if the compounds prove to be reversible inhibitors then this shortcoming becomes inconsequential as tyramine cannot cross the blood brain barrier (Haefely *et al*., 1992). If a compound is reversible in its inhibition of the MAO-A enzyme tyramine will eventually be metabolised peripherally and will not accumulate as happens in the case of non-reversibility (Youdim and Weinstock, 2004).The rest of the compounds are close to non-selective inhibitors of the enzyme with very low SI values. This means that the compounds would inhibit both enzymes in equal capacity except perhaps compound **4B**that may prove more selective for MAO-B.

4.5 IN VITRO INHIBITORY DATA OF LADOSTIGIL AND 2B

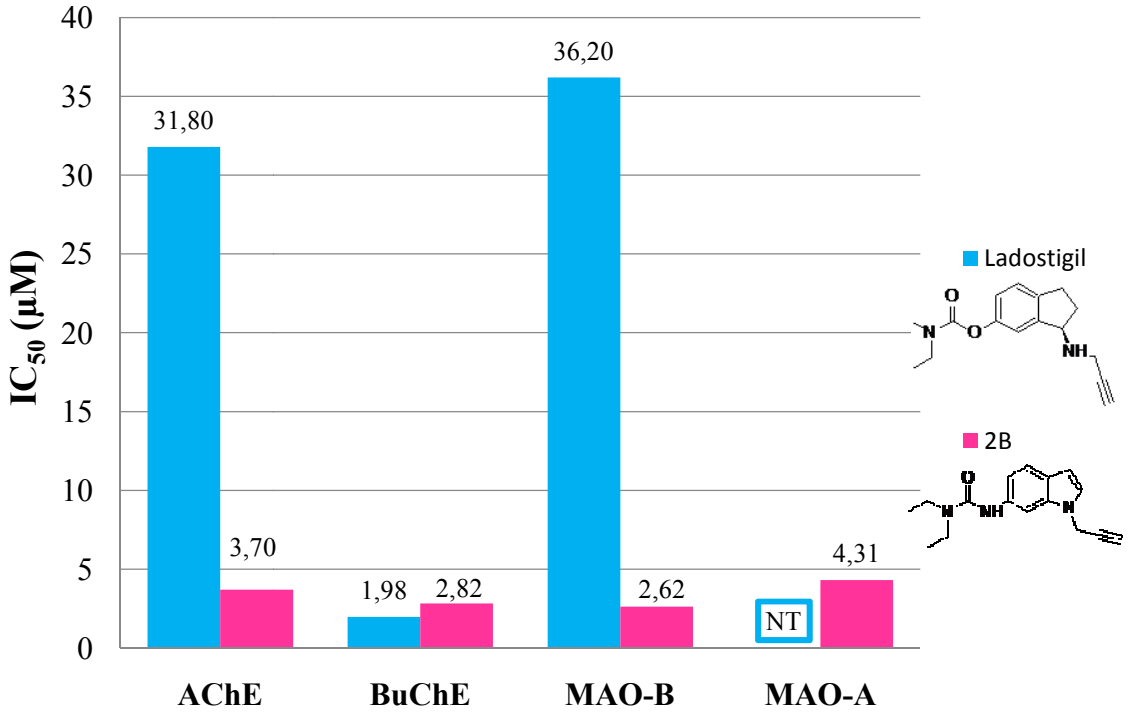
The design objectives and strategy of this study were based on the MTDL paradigm of drug discovery as well as the dual ChE-MAO inhibitor ladostigil currently in clinical trials (Avraham Pharma, 2016). Hence before concluding this chapter it is necessary to compare the results of the compounds in this study to the ChE and MAO inhibitory activity of ladostigil.

Compound **2B** showed the best overall activity with good IC_{50} values for all the tested enzymes. As a result we compared ladostigil to compound **2B** (**Graph 4.1**). The ChE and MAO studies for ladostigil were carried out under similar assay conditions and the same enzyme species as those used in this study (Sterling *et al*., 2002).

As can be seen from **Graph 5.1**[2] compound **2B** had almost 9-fold increase in activity on AChE. We ascribe this to the indole ring that we used as compared to the indane moeity that ladostigil and rasagiline possess. Notably compound **2B** possesses a urea compared to the carbamate on ladostigil, which may portray superior interactions The two compounds are more or less equipotent BuChE inhibitors indicating that compound **2B**could actively prevent BuChE induced hydrolysis of acetylcholine.

[2] NT: Not tested, ladostigil was not tested *in vitro* for MAO inhibitory activity.

Graph 4.1: Bar graph comparing compound **2B** to ladostigil



As earlier mentioned, MAO-B is the more abundant isoform hence its potent inhibition is of great significance. Compound **2B** is over 13 times more potent an inhibitor of the MAO-B enzyme compared to ladostigil. This, together with the AChE abilities of the compound would mean a reduction in consumed concentrations, reduced side effects and a more effective treatment option. Moreover we may attribute the good activity of the compound **2B** towards MAO being due to the presence of the indole polycyclic ring compared to the indane of ladostigil.

The propargylamine is conjugated directly to the polycyclic ring and this eliminates (1) the chiral centre present in ladostigil and (2) the secondary amine versus a tertiary amine with possibly better interactions with the enzyme. Ladostigil was not tested on human MAO-A *in vitro* and so we cannot quantifiably compare it to our compounds. One study by Weinstock, *et al*., 2000, states that ladostigil did not portray MAO-A activity *in vitro* but only *in vivo* and after chronic treatment in rats. We do, however, highlight that compound **2B** had an excellent *in vitro* MAO-A IC_{50} value comparable to that of the MAO-B IC_{50} value.

4.6 STABILITY TESTING

Chemical stability of pharmaceutical molecules is a matter of great importance as it ultimately affects the stability and efficacy of the final drug product. One of the drawbacks of the carbamate linkage of ladostigil is its ability to be rapidly hydrolysed in acidic conditions. Considering that the compounds in our study contained a carbamate or urea linkage that may affect stability, we decided to employ a forced chemical degradation test to study the stability of these moieties.

4.6.1 METHODS

Blessy et al., 2014, recommended a range of conditions mostly used for forced degradation studies and we modified our method accordingly. Compounds **2B** and **4B** were selected for the testing because they contained both the carbamoyl and propargylamine moiety and portrayed good activity as either a carbamate (**2B**) or urea (**4B**) derivative. Test compound (1 mg) was dissolved per ml water with DMF as co-solvent. Hydrochloric acid (HCl, 0.1 M) or 0.1 M NaOH were used to adjust the pH of the solutions with water as control. The solutions were kept at 37ºC in an incubator for 7 days and sampled on days 0, 1, 3, 4 and 7. UV/vis absorbance of the sample was measured at maximal wavelengths of 232 nm for **2B** and 234 nm for **4B.**

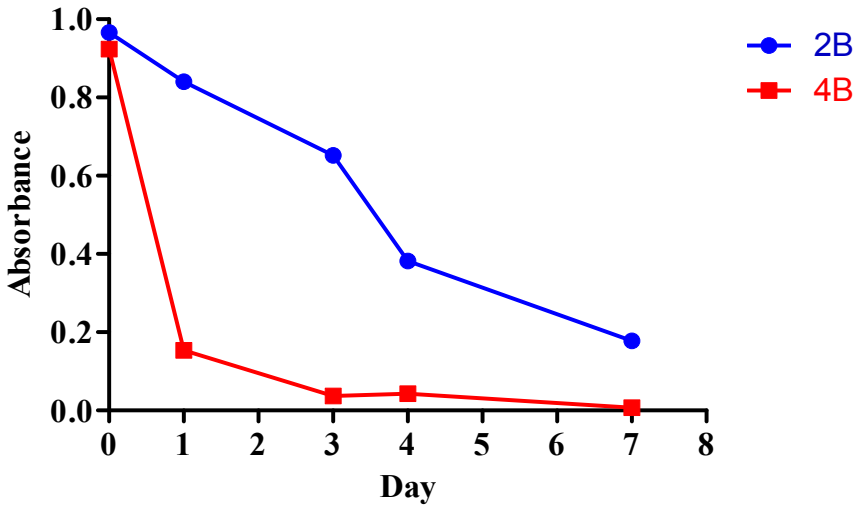
4.6.2 RESULTS AND DISCUSSION

It is important to note that both urea and carbamate functional groups undergo hydrolysis but at different rates. Amides undergo hydrolysis at a slower rate than the structurally related esters under comparable reaction conditions(Armstrong, Farlow and Moodie, 1968).Hence our aim in this study was to produce compounds that are more stable in acidic and basic conditions. **Graph 4.2** shows the results of the compounds after 7 days in 0.1 M HCl.

Compound **2B**displayed an almost linear reduction in intact compound compared to **4B**that portrayed a more rapid decay. From these result we can postulate that the carbamates are less stable than their urea containing counterparts. Ladostigil is a carbamate linked compound and so could act similarly to **4B**in acid conditions. Following this logic, urea linked **2B** should prove to be more stable than the carbamate derivative ladostigil. This should minimise the metabolic effects that induce rapid

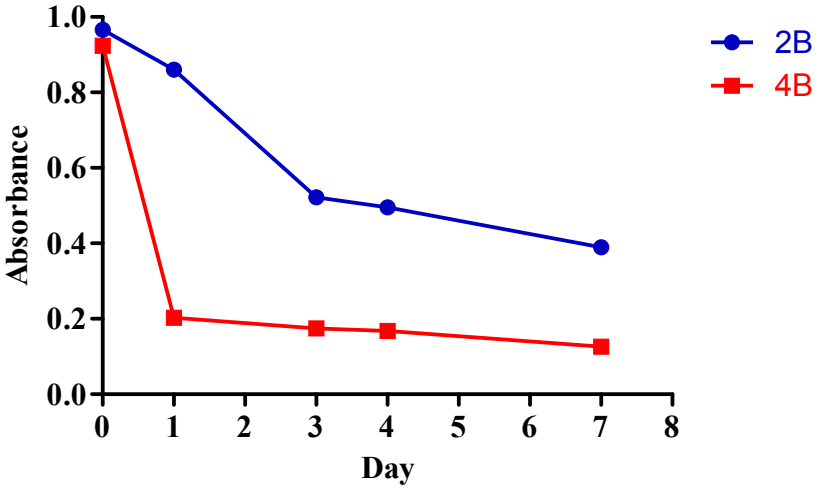
hydrolysis to prevent limiting factors such as a short half life. The compounds behaved in a similar manner in alkaline conditions (**Graph 4.3**).

Graph 4.2: Chemical stability in 0.1 M HCl



We can see that **4B** acts poorly in both conditions resulting in a situation where the whole molecule is degraded or broken down at a rapid rate as compared to **2B**. At this stage we are not informed as to the activity of the metabolites of the compounds but like rasagiline and ladostigil whose metabolites portray neuroprotective activity; our compounds may act in a similar manner.

Graph 4.3: Chemical stability in 0.1 M NaOH



4.7 CONCLUSION

The main objective of this study was to design a series of chemically stable novel multifunctional compounds able to inhibit AChE, BuChE, MAO-A and MAO-B. We

managed to synthesise 8 compounds with the desired pharmacophoric groups. The assay results are summarised in the **Table 4.4**.

Table 4.4: Summary of the IC_{50} values of all novel compounds

Compound ID	AChE (µM)	BuChE (µM)	MAO-A (µM)	MAO-B (µM)
1A	**1,97**	3,48	>100	>100
2A	2,28	4,55	20,37	>100
3A	2,78	**2,29**	13,15	>100
4A	3,72	3,18	88,11	>100
1B	2,77	3,96	33,42	28,84
2B	3,70	2,82	**4,31**	2,62
3B	2,57	3,01	99,08	16,52
4B	2,41	5,01	51,64	**1,84**

These results indicate that the compounds could act as dual non-selective cholinesterase inhibitors to inhibit the hydrolysis of acetylcholine and hence increase its levels post-synapse. The cholinesterase assays have given us a proof of concept regarding the ability of the carbamoyl moiety to inhibit the cholinesterase class of enzymes. We have identified the urea moiety as an improvement to the carbamoyl portion especially in the propargylated compounds. Addition of the propargylamine group did not influence ChE activity. The compounds have been shown to be more potent than the FDA approved AChE inhibitor rivastigmine (U.S. Food and Drug Administration).

Addition of the propargylamine moiety yielded compounds that were active towards the MAO enzymes. Again here we have proof of concept of the moiety's inhibitory properties. Comparison with rasagiline or ladostigil proves that some of our compounds may serve as lead compounds for optimisation. Compound **2B** was particularly (on average) a good inhibitor of all 4 enzymes (IC_{50} AChE = 3.70 µM, BuChE = 2.82µM, MAO-A = 4.31 µM and MAO-B = 2.62 µM). The results of the chemical stability tests showed that it (**2B**) is also more stable than the carbamate linked compound **4B** by virtue of possessing the urea. We also managed to eliminate the stereochemistry associated with ladostigil and rasagiline. Compound **2B** would thus probably prove more chemically stable than ladostigil. This compound can thus be considered for optimisation and perhaps development as a multipotent drug molecule against Alzheimer's disease.

CHAPTER 5

5. CONCLUSION

5.1 INTRODUCTION

Our understanding of AD has advanced enormously over the past few years, with much effort being devoted into research ligands that portray excellent *in vivo* activity. However these highly selective ligands do not necessarily result into clinically effective drugs due to the multifactorial nature of the disease (Cavalli *et al*., 2008). In response, different pharmacological approaches, such as polypharmacy, have offered possible means of overcoming the drawbacks of single target therapies. Recent research has introduced the concept of MTDLs which is simply a molecule that can target multiple pathways that contribute to disease pathogenesis (Bolognesi *et al*., 2006, Zhang, 2005). We capitalised on the structural relevance of single target molecules to design a series of compounds where pharmacophoric moieties of rivastigmine and rasagiline were employed to obtain compounds proposed to inhibit both cholinesterase and MAO enzymes respectively. We further incorporated an indole polycyclic nucleus for which anti-oxidant and NOS inhibitory capacity has been reported (Buemi *et al*., 2013,Fernández García, *et al*., 1992).

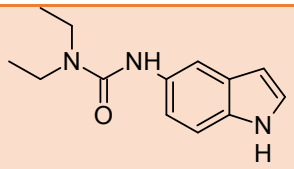
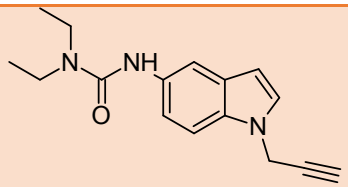
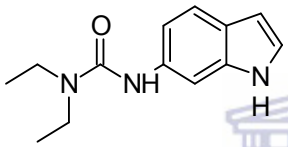
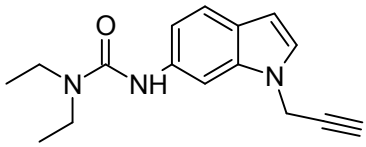
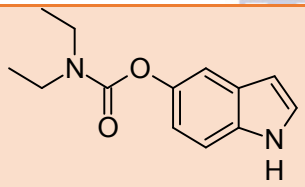
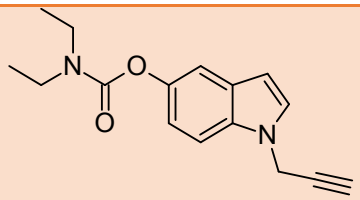
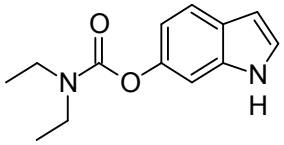
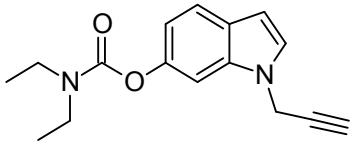
5.2 SYNTHESIS

In order to obtain the desired series of compounds, S_N2 nucleophilic substitution reactions were employed to conjugate the carbamoyl and propargyl moieties to the indole polycyclic ring. The first series of compounds had the carbamoyl moiety at either the 5 or 6 position of the indole polycyclic ring. Of the 4 synthesised compounds 2 possessed the carbamate bond and 2 the urea. The synthesis of these compounds **1A**, **2A**, **3A** and **4A** (**Table 5.1)** was carried out under conventional conditions (room temperature and stirring for 2 hours). The yields ranged from 50 to 80 %.These compounds were then used to further synthesise a series of 4 propargylated compounds **1B**, **2B**, **3B** and **4B**. The propargylamine moiety was conjugated to the N1 position to yield final compounds similar to ladostigil. These reactions were carried out in the microwave to provide sufficient energy for the propargylamine moiety to react. The

final products were obtained in yields of over 75 %.NMR and IR spectra showed characteristic signals and MS confirmed the compounds.

Within the series, the compounds were differentiated by the type of linkage of the carbamoyl moiety (carbamate or urea) and the position of this conjugation (5 or 6). A total of 8 compounds were successfully synthesised and characterised as shown in **Table 5.1**.

Table 5.1: Successfully synthesised series of compounds.

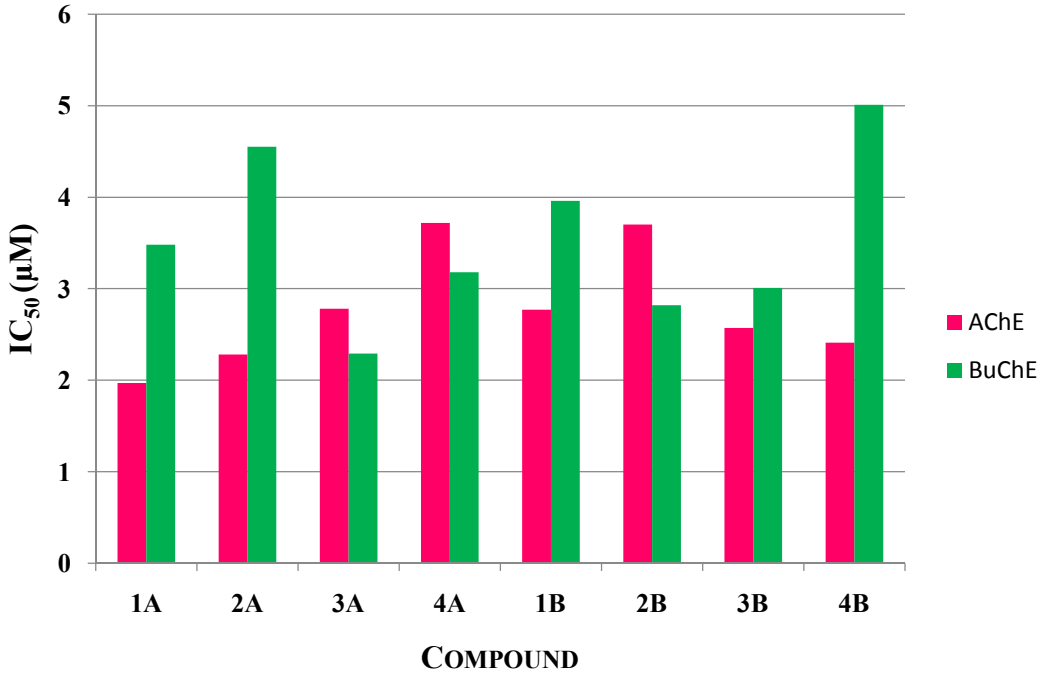
1A		**1B**	
2A		**2B**	
3A		**3B**	
4A		**4B**	

5.3 MOLECULAR MODELLING AND BIOLOGICAL EVALUATION

Docking studies of the novel compounds on the AChE, MAO–A and MAO–B enzymes were performed to elucidate possible binding interactions in the active sites. The AChE modelling results showed that the compounds possessed vital interactions with the cholinesterase active site. Both series of compounds showed interaction with Trp84, a key residue in the CAS. The propargylamine moieties of the structures were oriented towards the FAD cofactor of the MAO isozymes and hence should have good inhibitory activity. Some interactions were also observed between the indole ring with Trp 279 in AChE and Ille 199 in MAO which showed that it plays a role in the inhibitory capacity of the compounds.

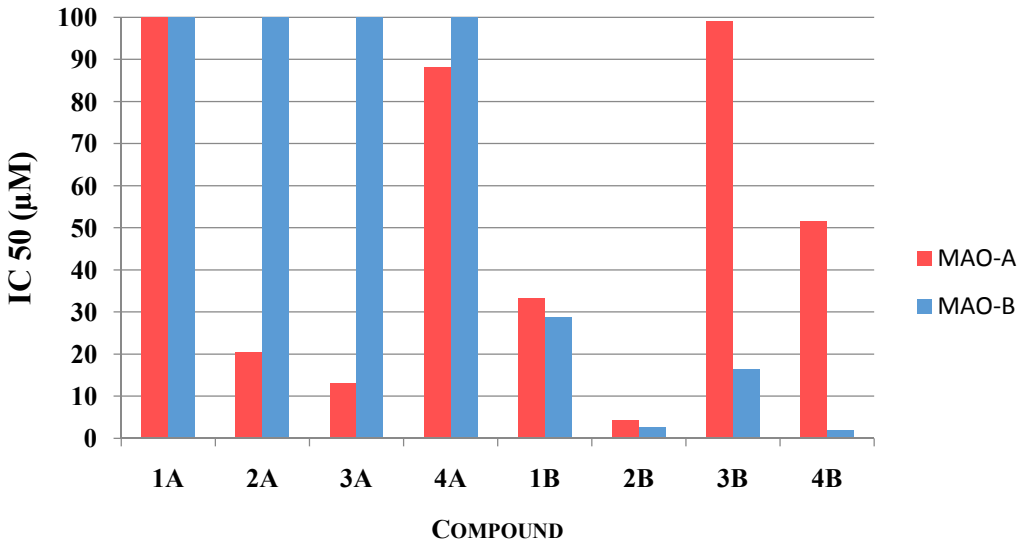
The *in vitro* biological testing of all the synthesised compounds for cholinesterase and MAO inhibitory activity was carried out using *electrophorus electricus* AChE, equine BuChE and recombinant human forms of the MAO–A and -B enzymes. The assay results showed that the compounds are dual and non-selective inhibitors of the cholinesterase enzymes *in vitro*. They all possessed IC_{50} values lower than 5 µM. Addition of the propargylamine substitution on position 1 did not influence ChE inhibitory activity significantly.

Graph 5.1: Cholinesterase inhibitory activity



The compounds possessed good activity towards MAO – A and B and the presence of the urea moiety resulted in compounds that have comparably better inhibitory capacities than the carbamate linked compounds. Some of the compounds (**2A** and **3A**) showed uncharacteristically good activity on the MAO–A enzyme compared to their propargylated counterparts. The compounds that had the propargylamine moiety had good inhibitory activity except **3B** with **2B**and **4B** portraying the lowest IC_{50} values. The compounds were overall more selective towards MAO – B.

Graph 5.2: Monoamine oxidase inhibitory capacity



Compound **2B** portrayed the best overall activity of all the tested compounds. When compared to ladostigil, compound **2B** demonstrated an AChE inhibitory capacity 9 times that of ladostigil and a 13 fold increase in MAO–B inhibition. The two are more or less equipotent BuChE inhibitors. Moreover compound **2B** also possesses superior MAO–A inhibitory capacity *in vitro*. Compound**2B** is also more chemically stable compared to the carbamate linked compound **4B** based on a 7 day forced degradation study. These results indicate that the compound may prove more stable than ladostigil. This compound can thus be considered for optimisation and perhaps development as a multipotent drug molecule against Alzheimer's disease.

5.4 CONCLUSION

Research in the area of AD has been ongoing for many years and has not resulted in a set treatment option for the disease. Current therapies, rivastigmine and memantine affect the symptomatic stages to provide palliative care. Over the last 2 decades research trends have shifted from the one drug–one target paradigm to multifunctional molecules. In this light, numerous targets have been explored to achieve an ultimate multi–potent molecule against the ChE and MAO enzymes, apoptosis as well as amyloid plaque formation.

The objective of this study was to synthesise and evaluate a series of compounds with the indole ring, propargylamine and carbamate/urea moieties to inhibit AChE, BuChE and MAO. Eight novel compounds were successfully synthesised and evaluated and

they all proved to be good inhibitors of the cholinesterase enzymes. They also proved to inhibit MAO enzymes especially with the propargylamine moiety added. This proves that the novel compounds have great potential in alleviating the cholinergic symptoms of AD, reducing the rate of amyloid plaque formation in the brain, confer neuroprotection by inhibiting the MAO catalysed breakdown of amine neurotransmitters and in total reduce the progression of AD.

With regards to future studies, the anti–apoptotic potential and ability of the compounds to cross the blood brain barrier is the next step to ascertain the full capacity of the compounds to affect a significant change in all stages of Alzheimer's disease.

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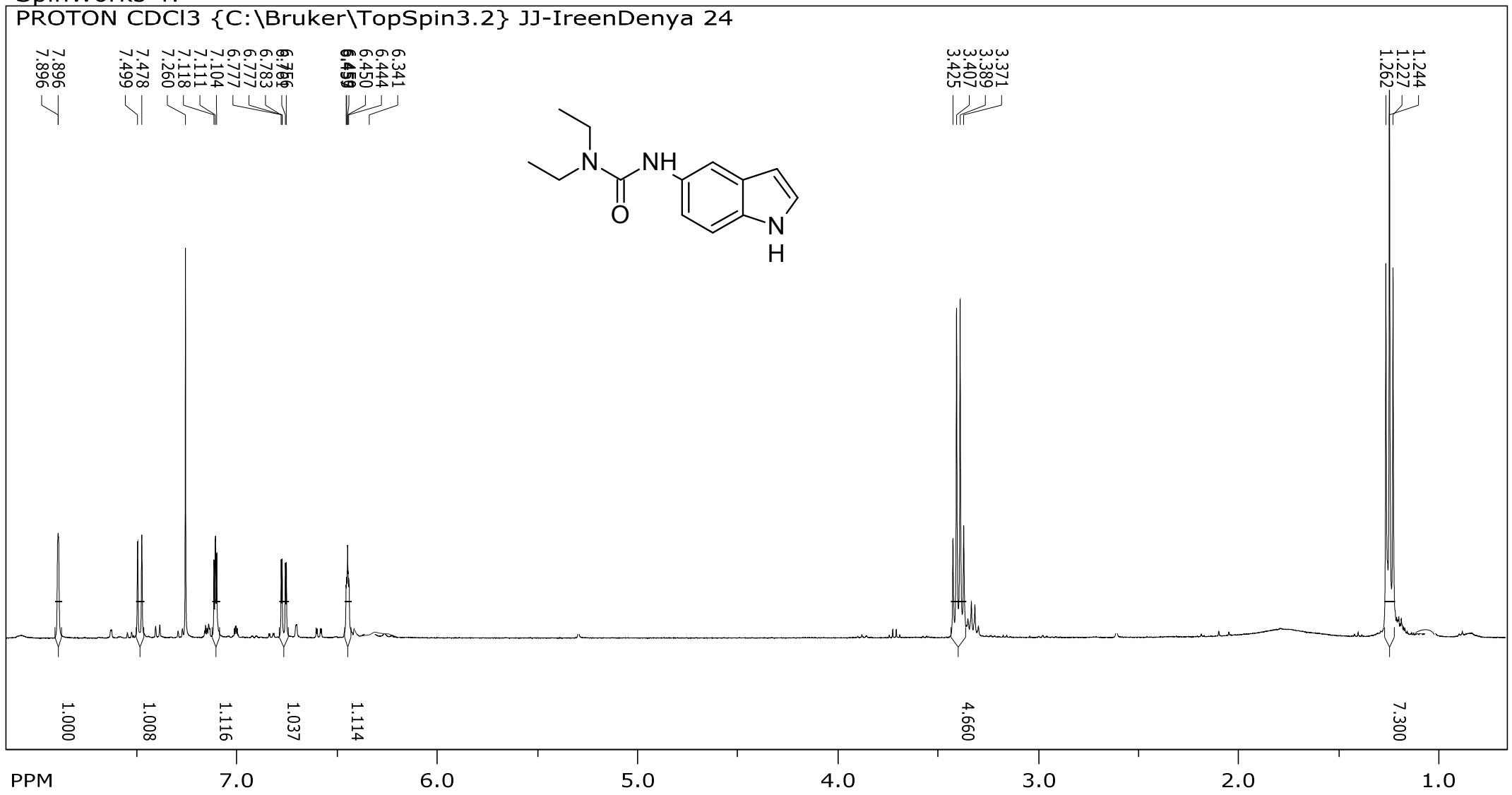
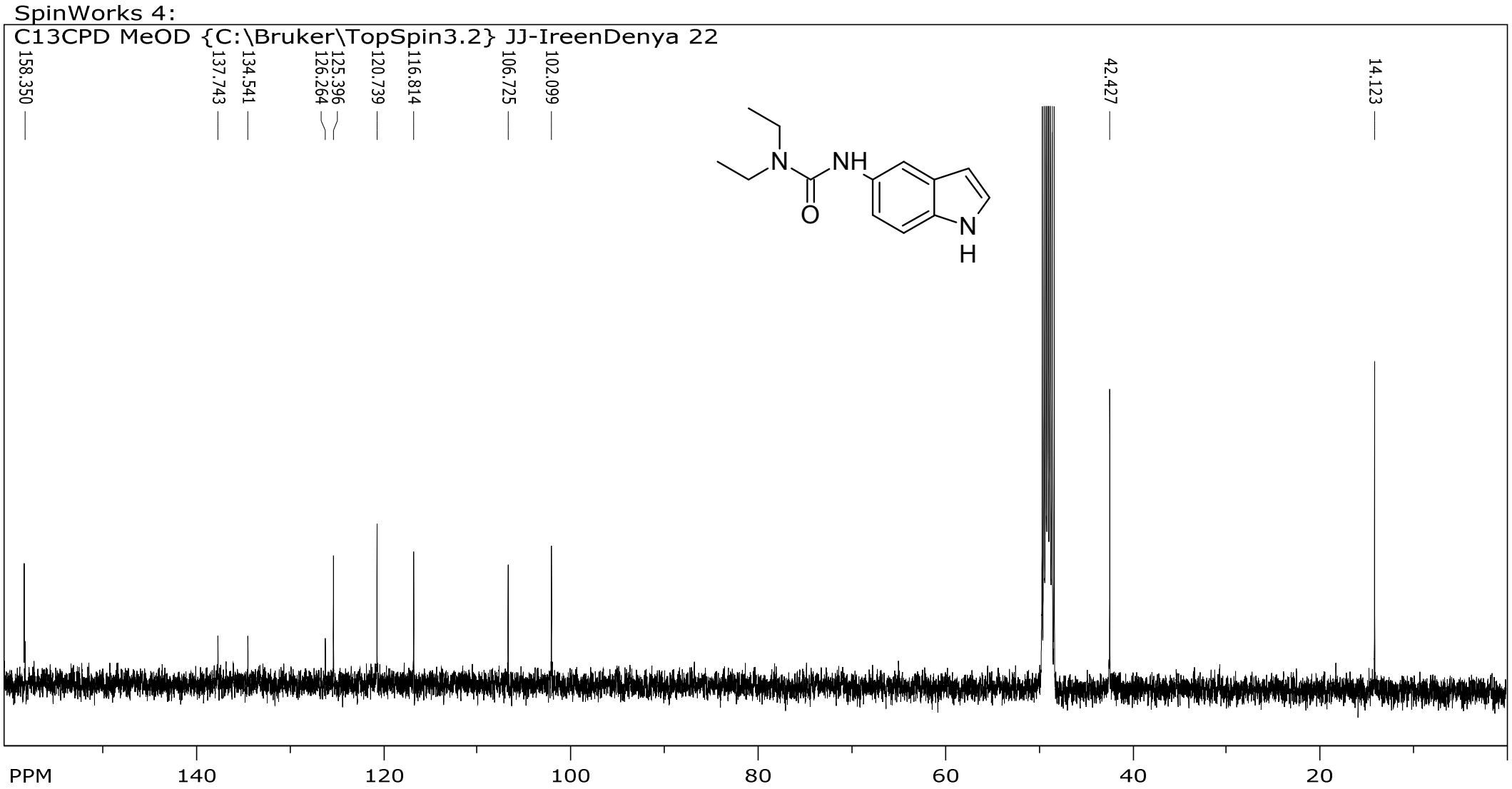
ANNEXURE: SPECTRAL DATA

^{1}HNMR, ^{13}CNMR IR, MS

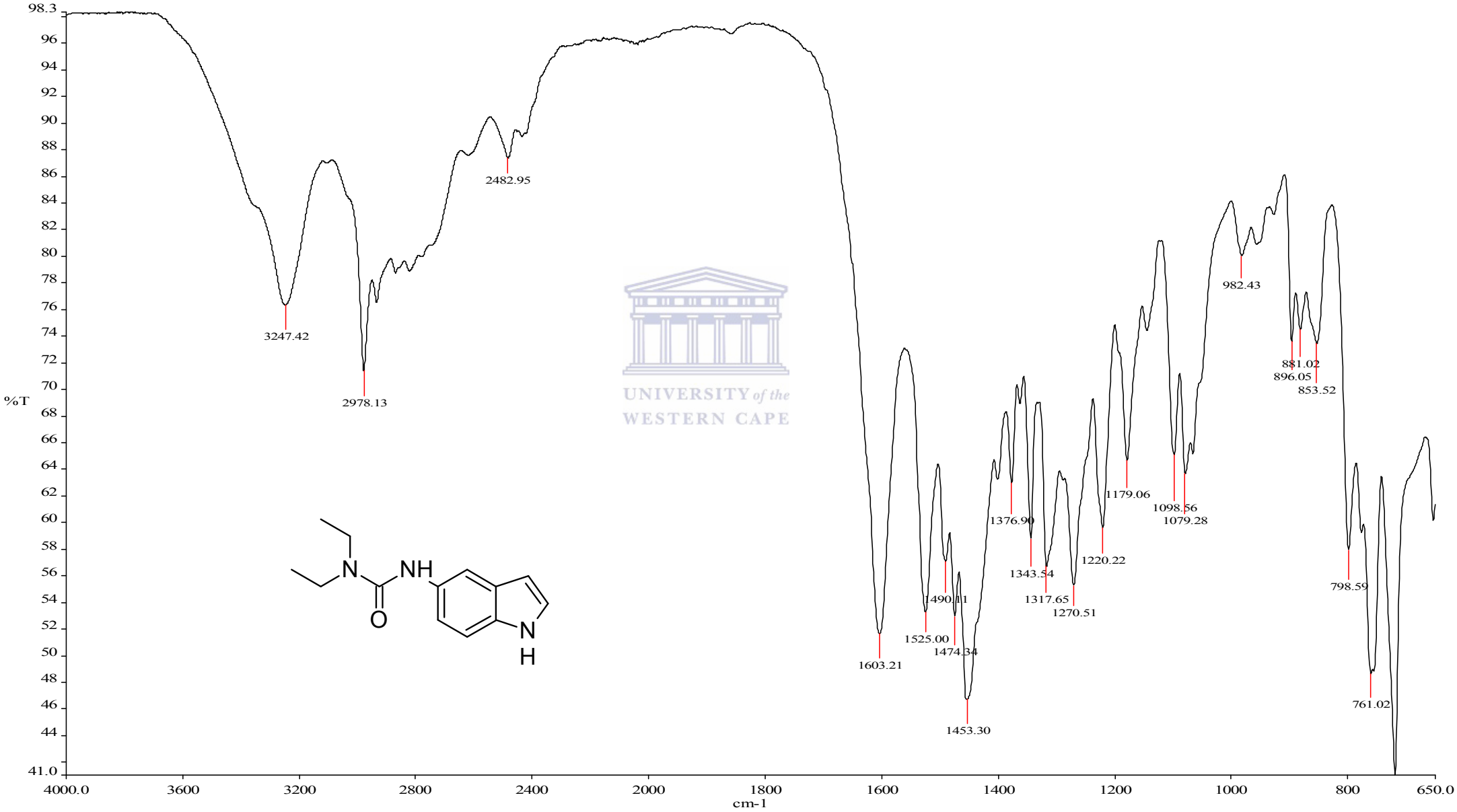
Spectrum 1: HNMR COMPOUND 1A



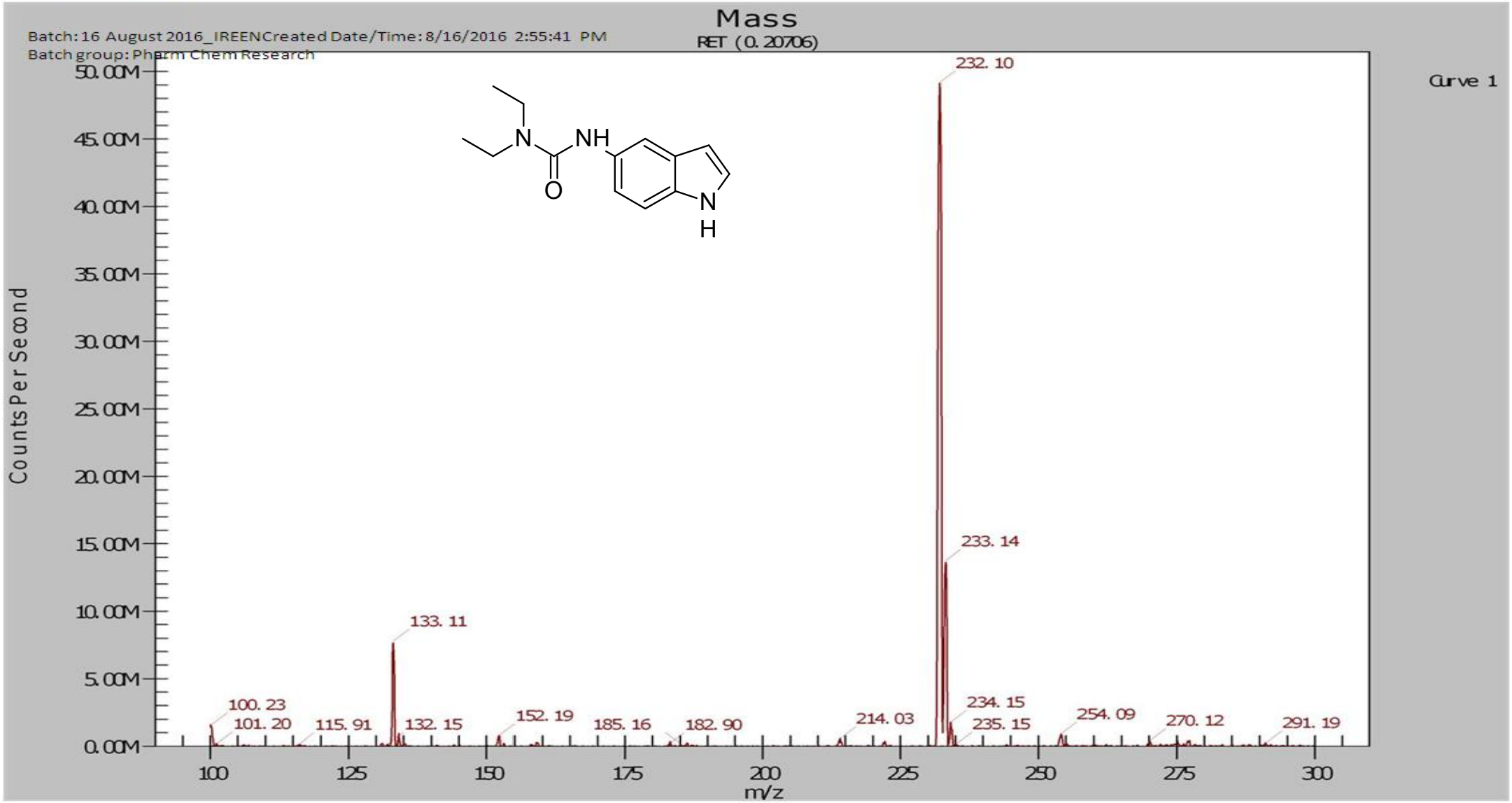
Spectrum 2: ^{13}C NMR COMPOUND 1A



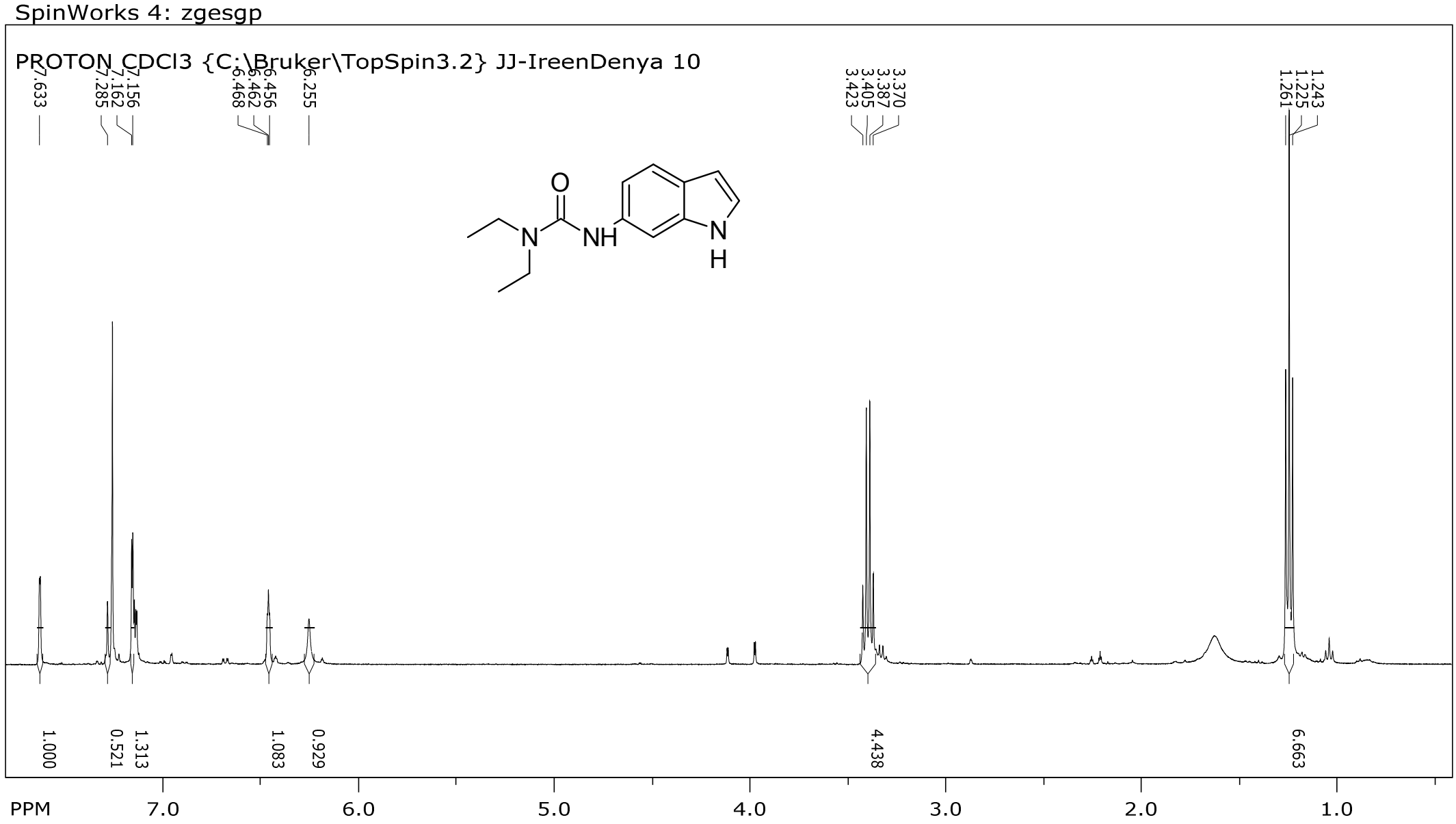
Spectrum 3: IR COMPOUND 1A



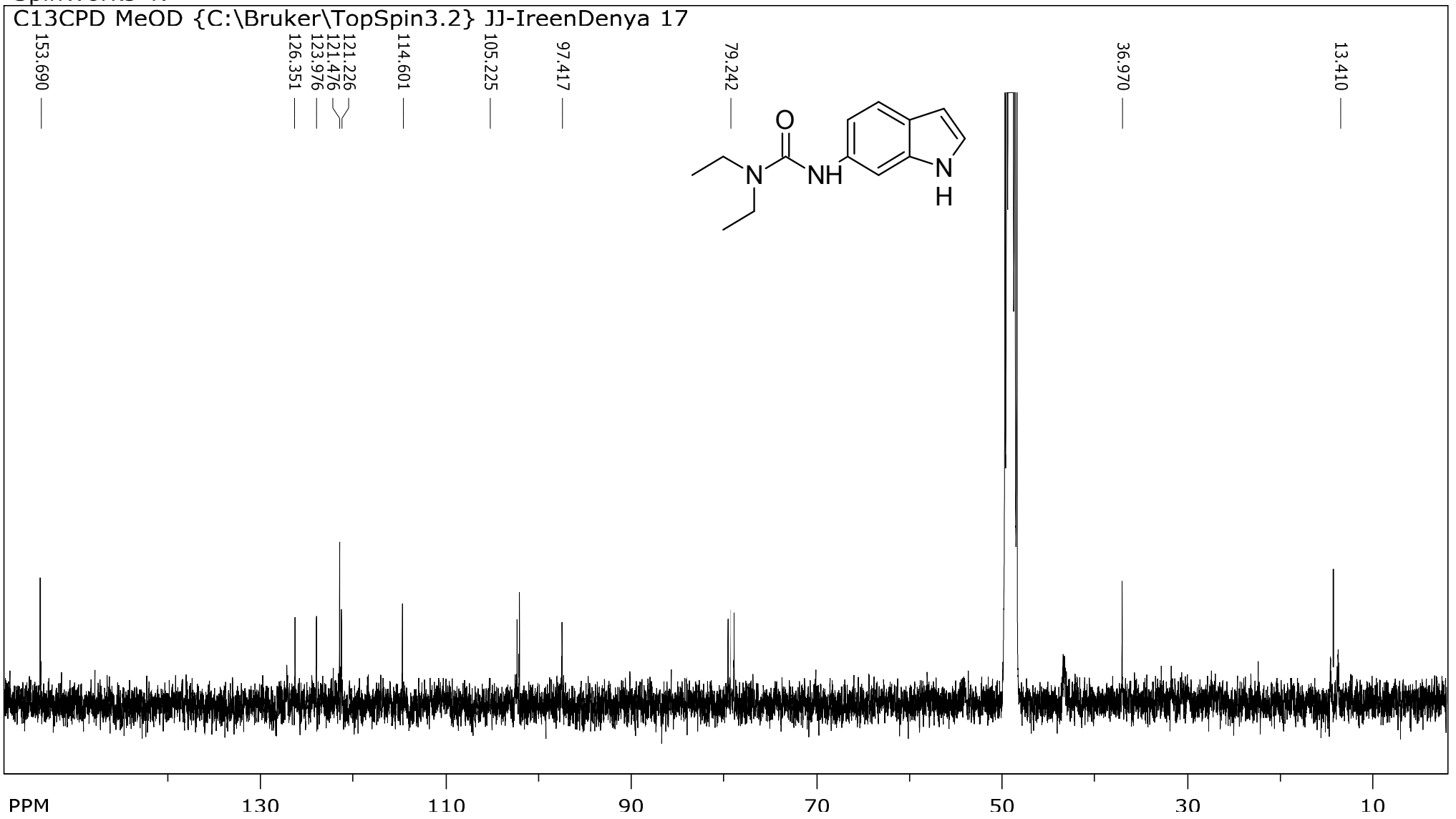
Spectrum 4: MS COMPOUND 1A



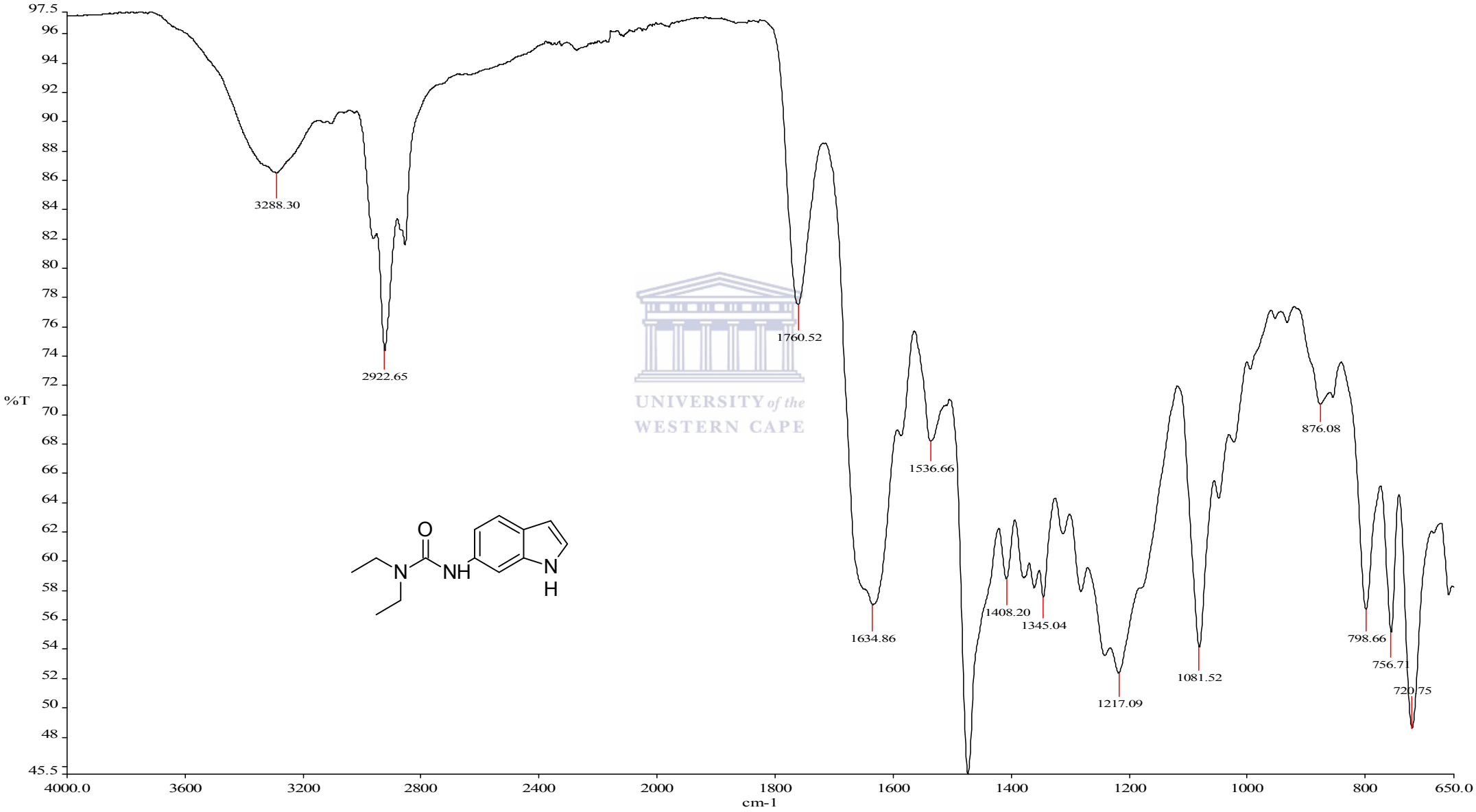
Spectrum 5: HNMR COMPOUND 2A



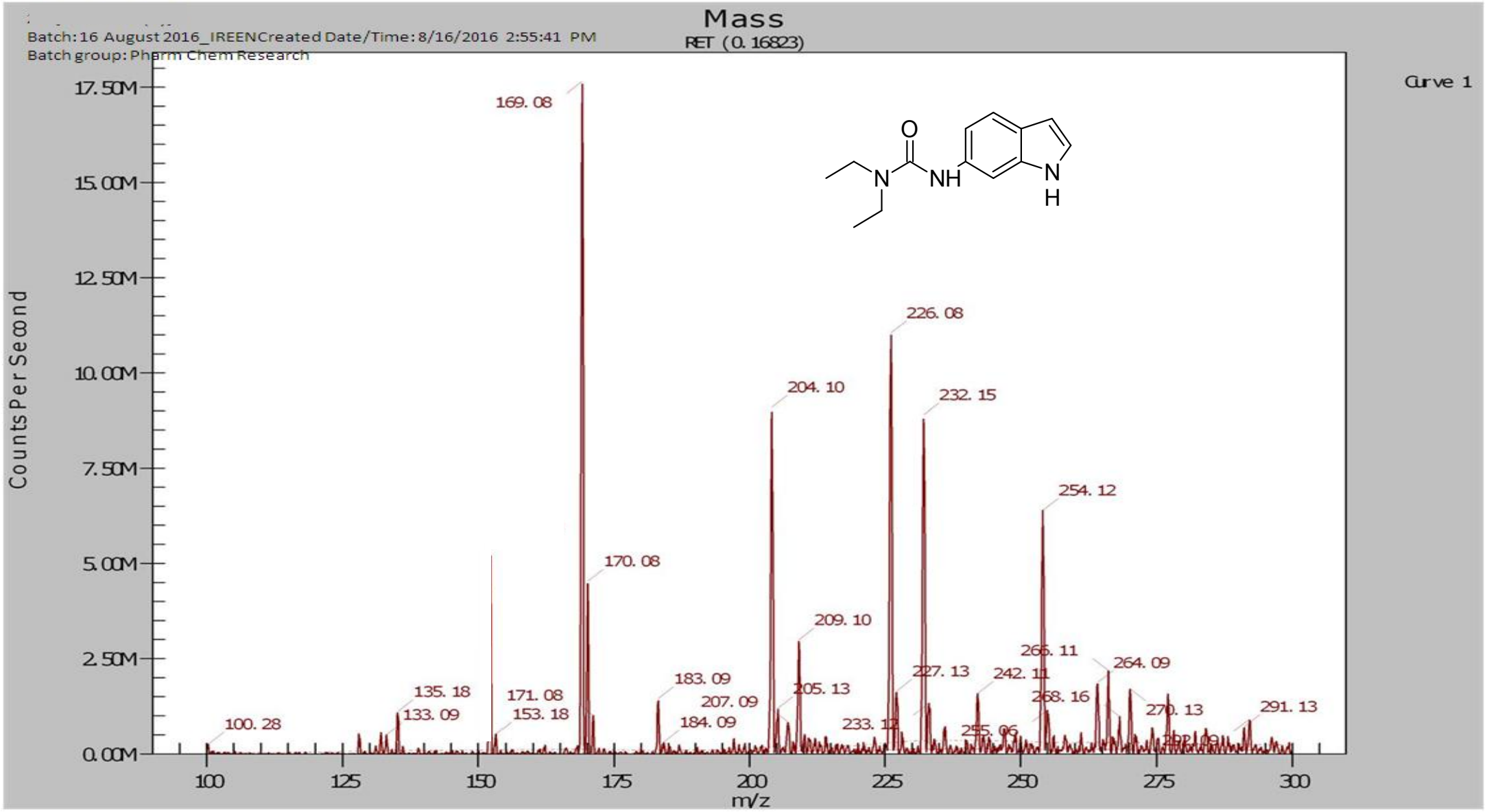
Spectrum 6: ^{13}C NMR COMPOUND 2A



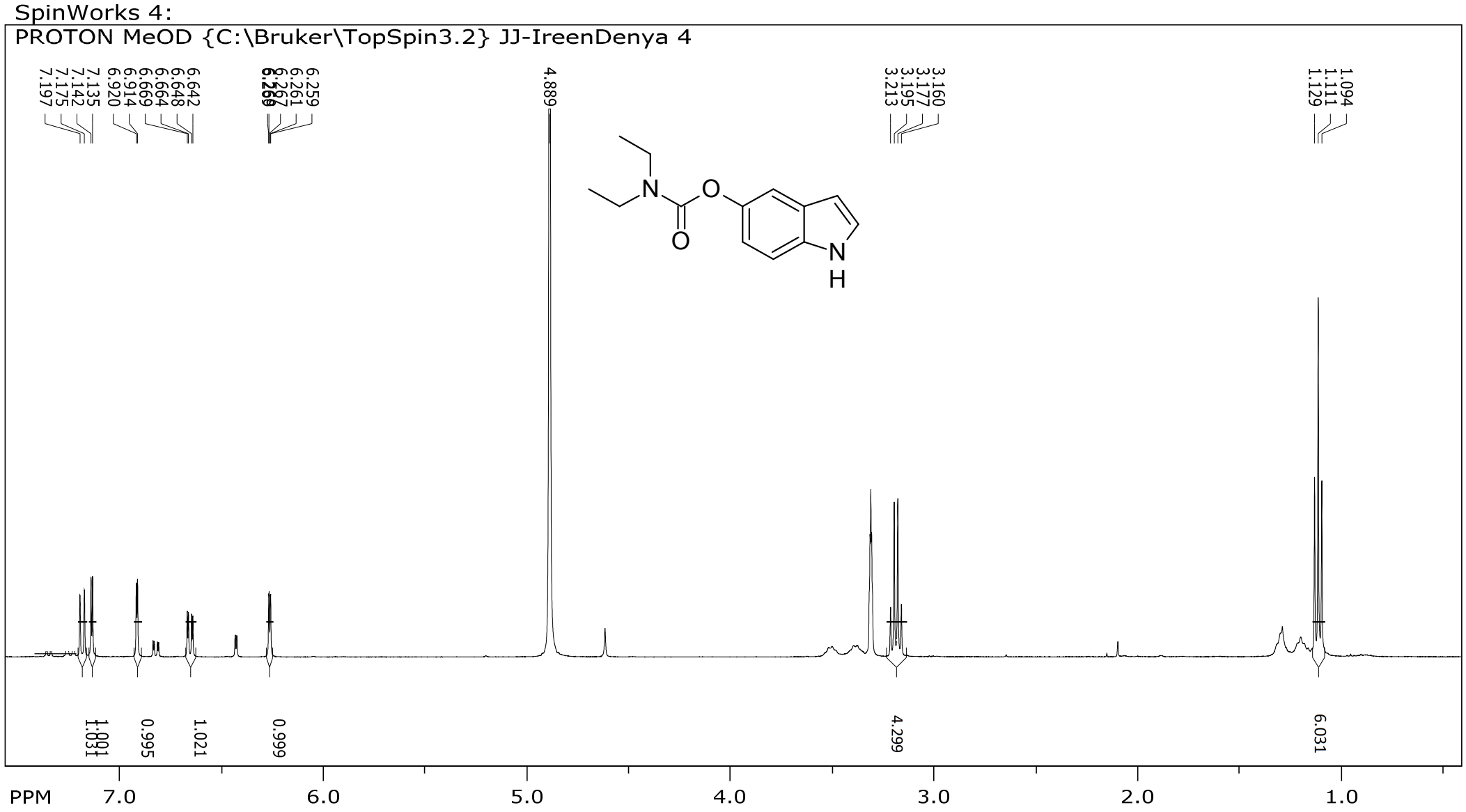
Spectrum 7: IR COMPOUND 2A



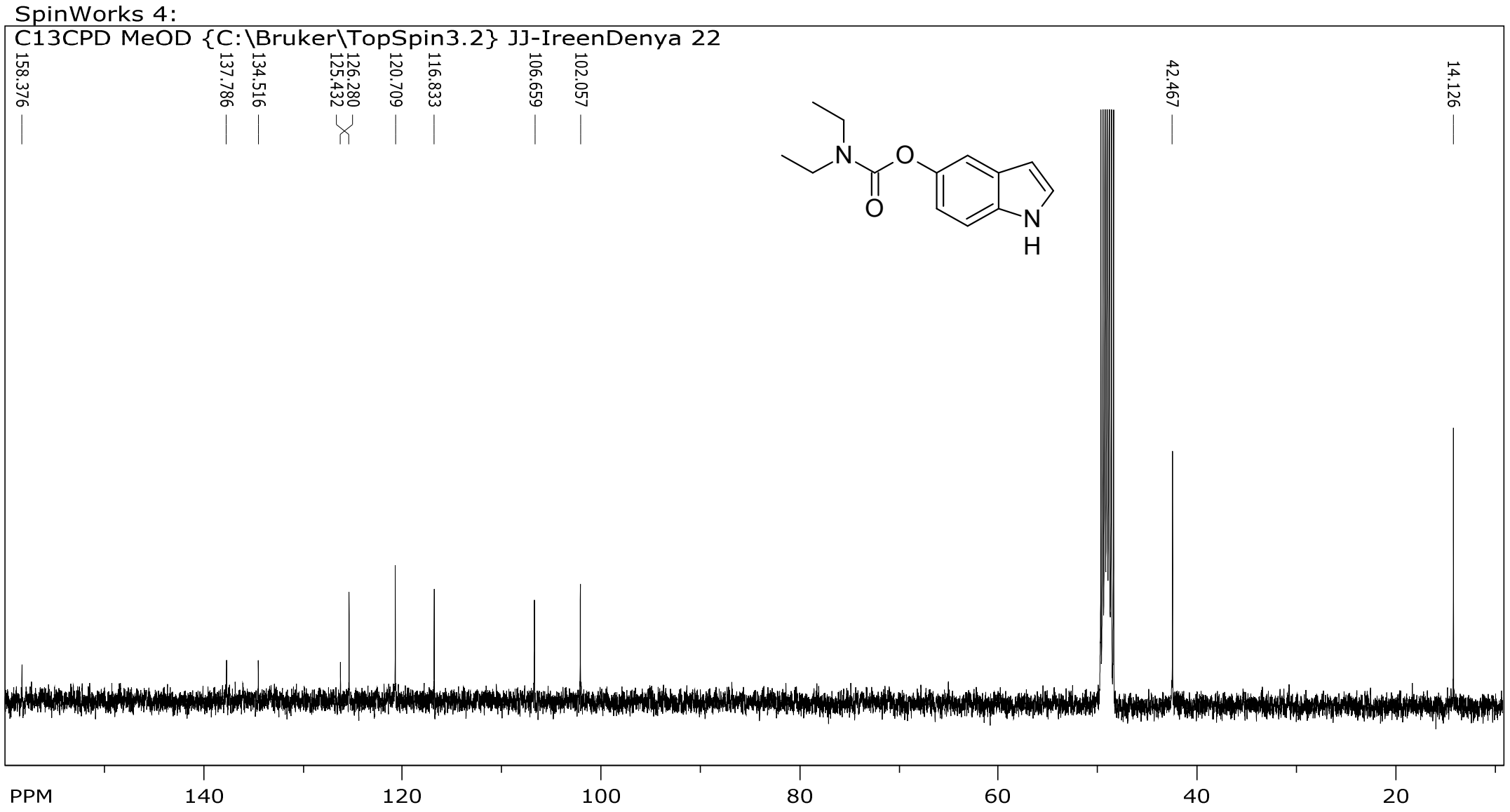
Spectrum 8: MS COMPOUND 2A



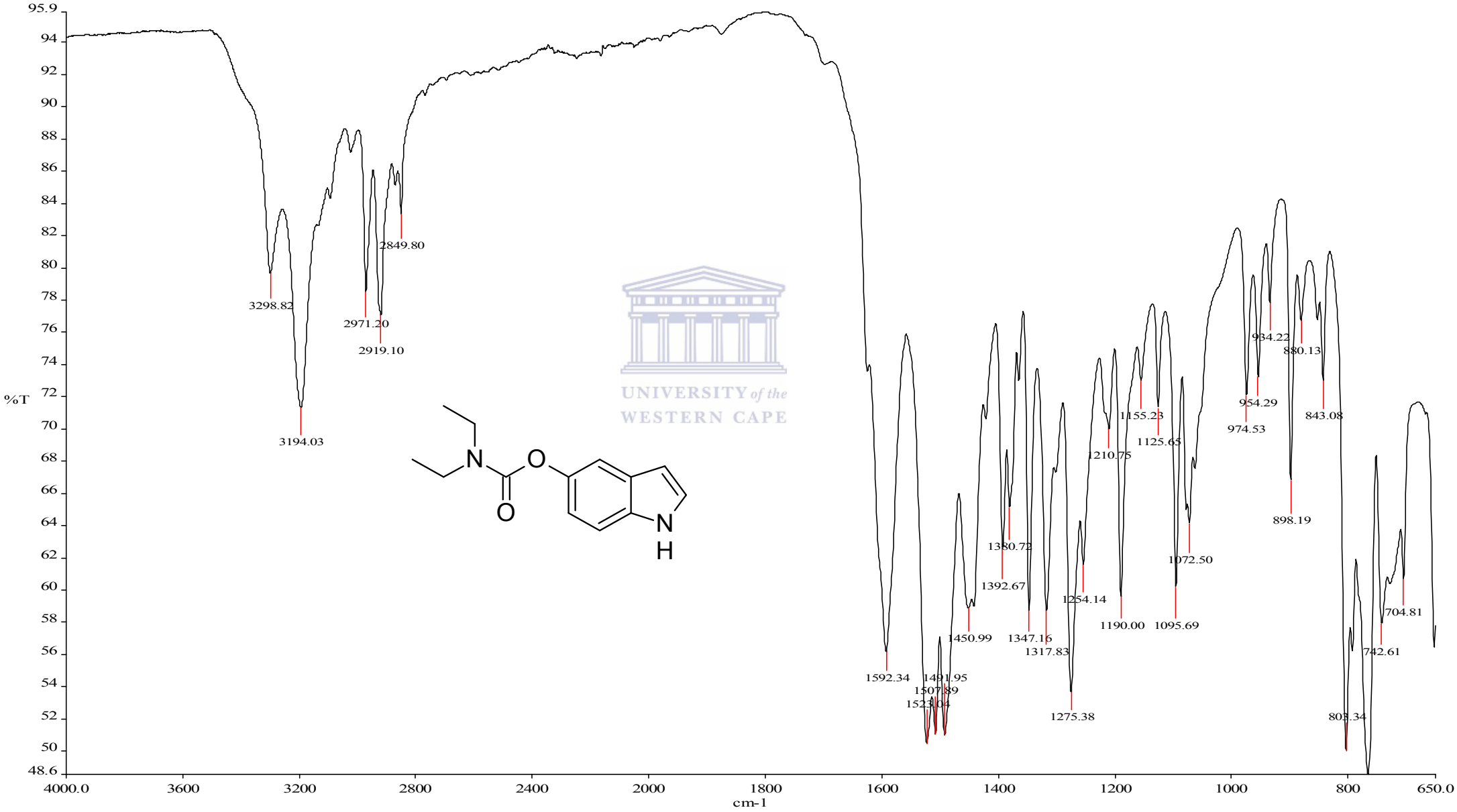
Spectrum 9: HNMR COMPOUND 3A



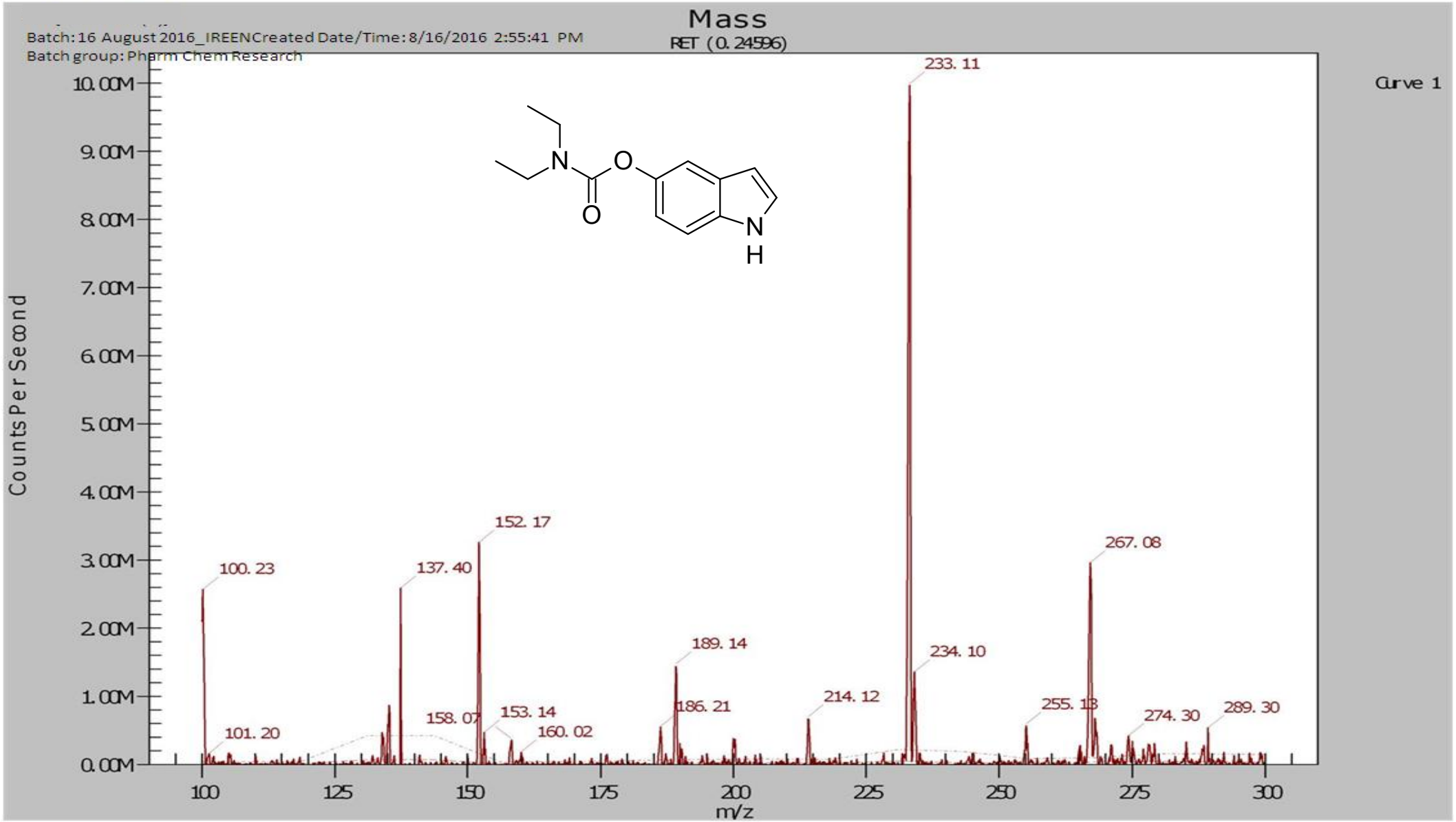
Spectrum 10: ^{13}C NMR COMPOUND 3A



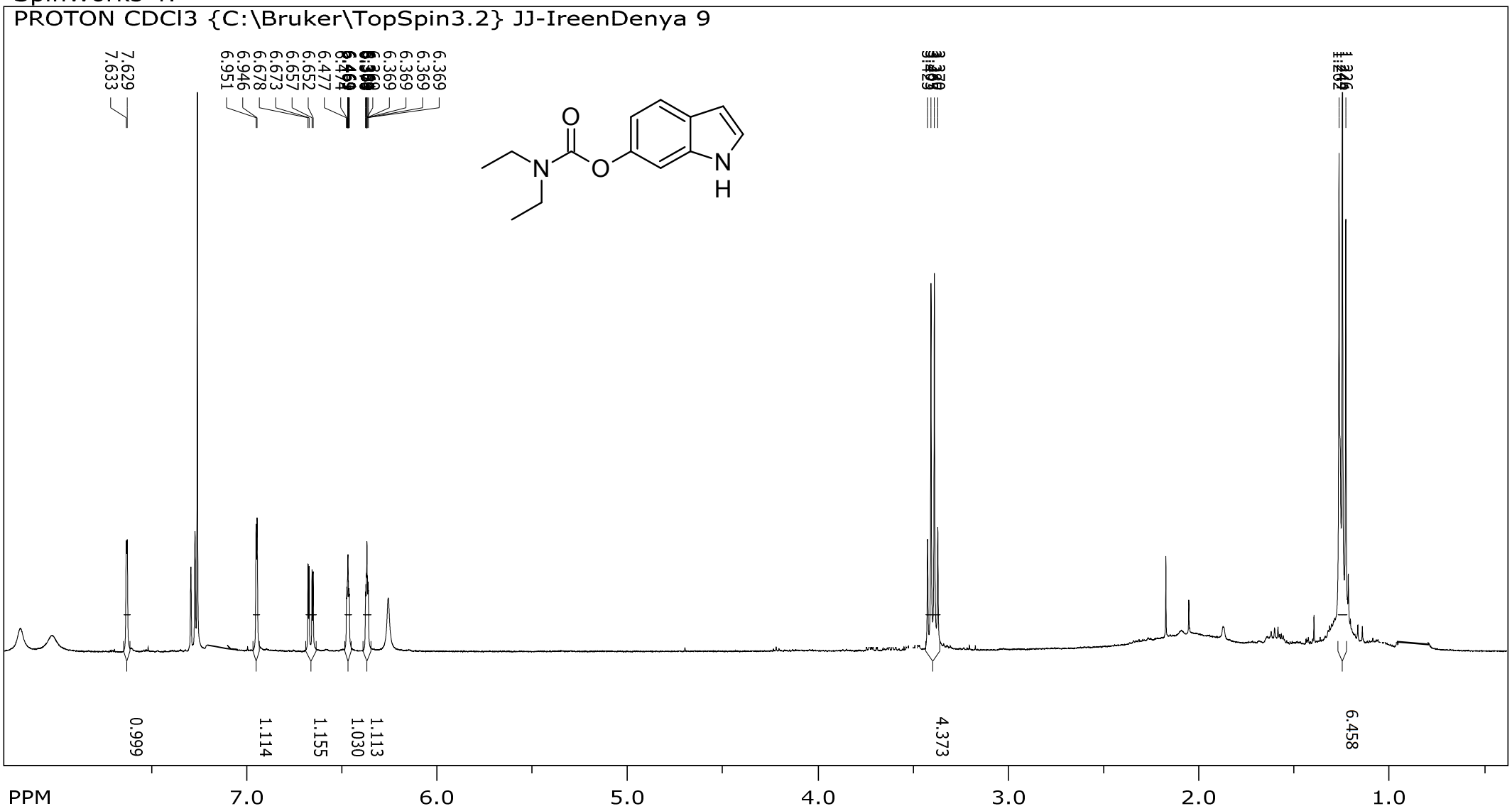
Spectrum 11: IR COMPOUND 3A



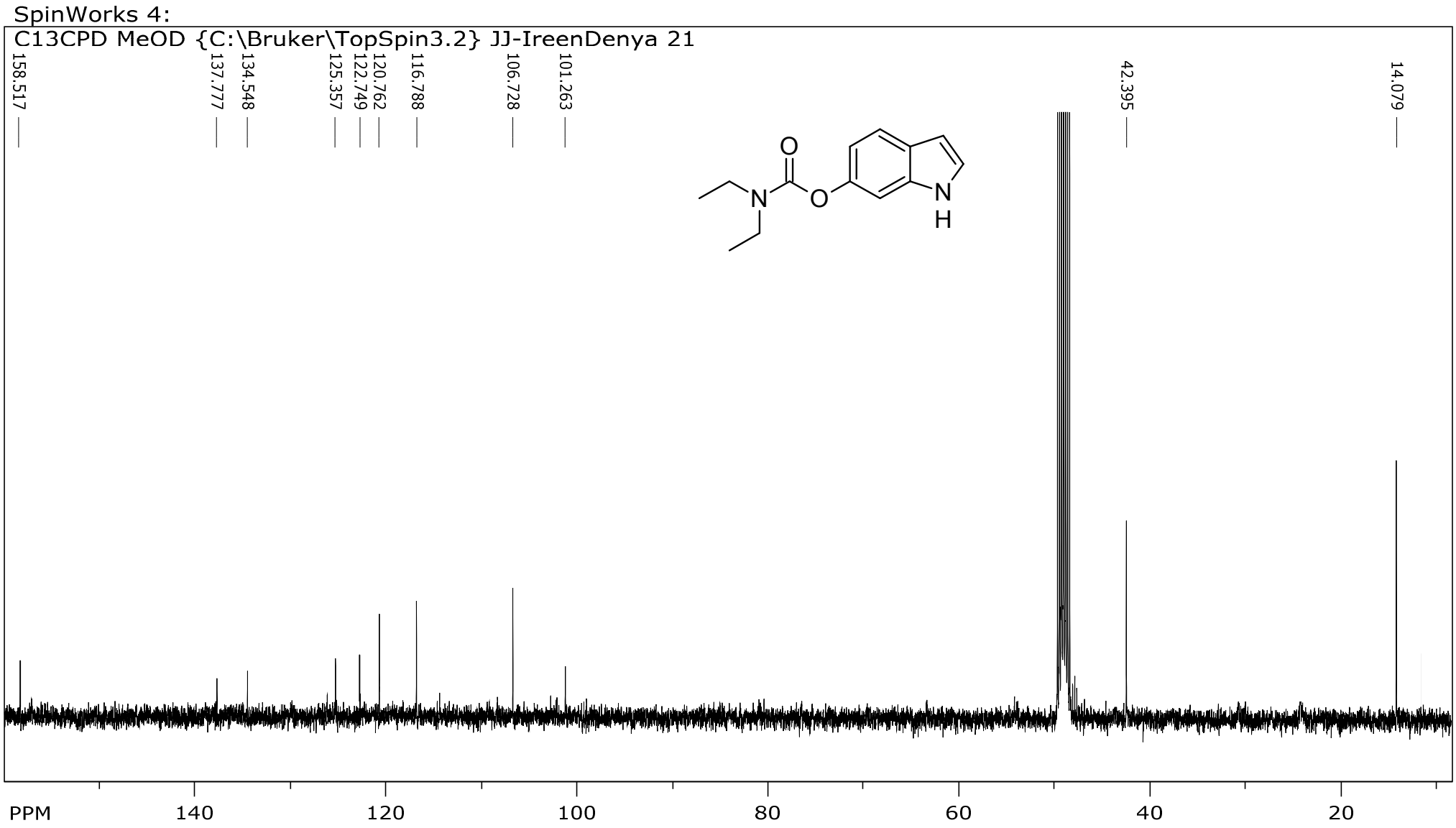
Spectrum 12: MS COMPOUND 3A



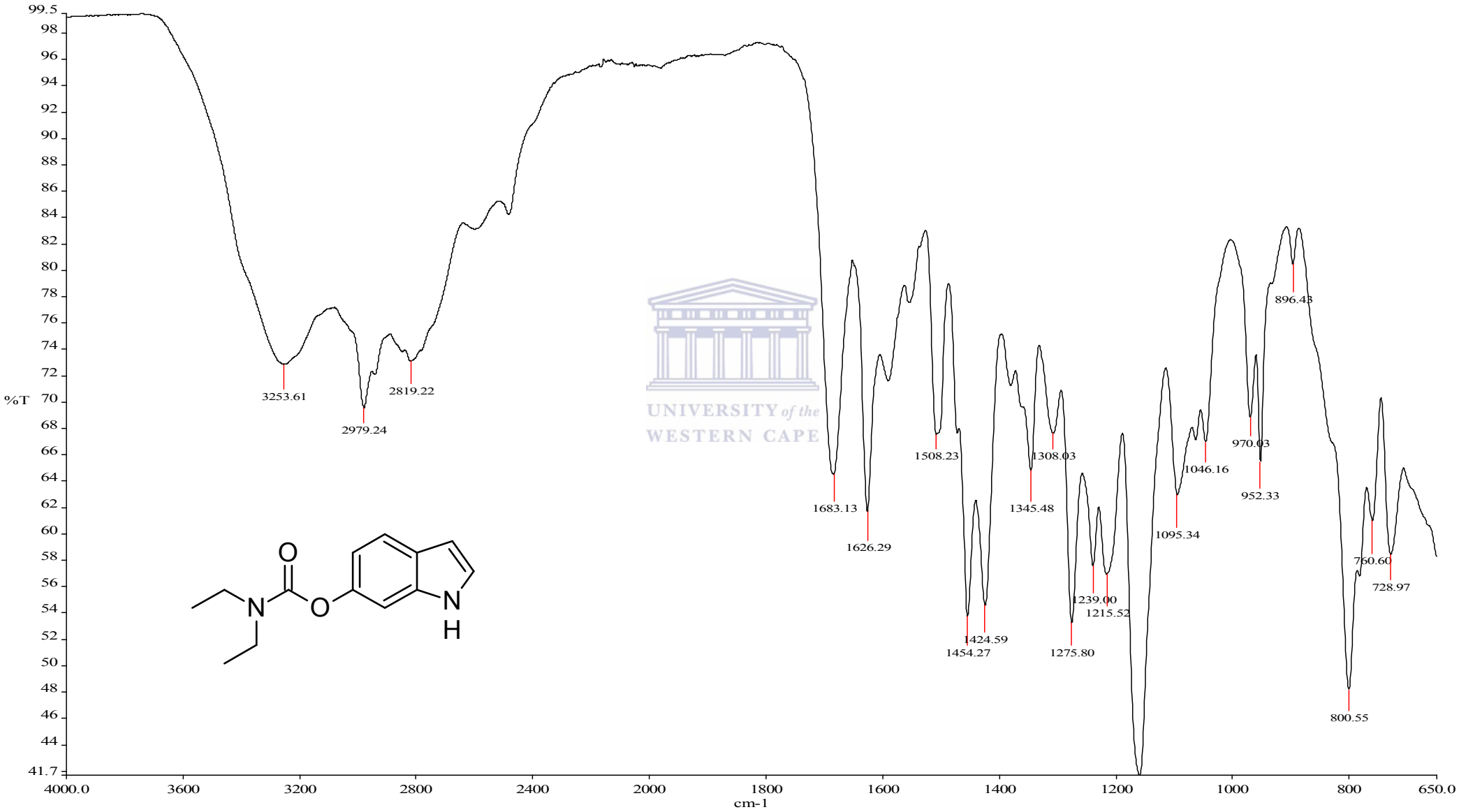
Spectrum 13: HNMR COMPOUND 4A



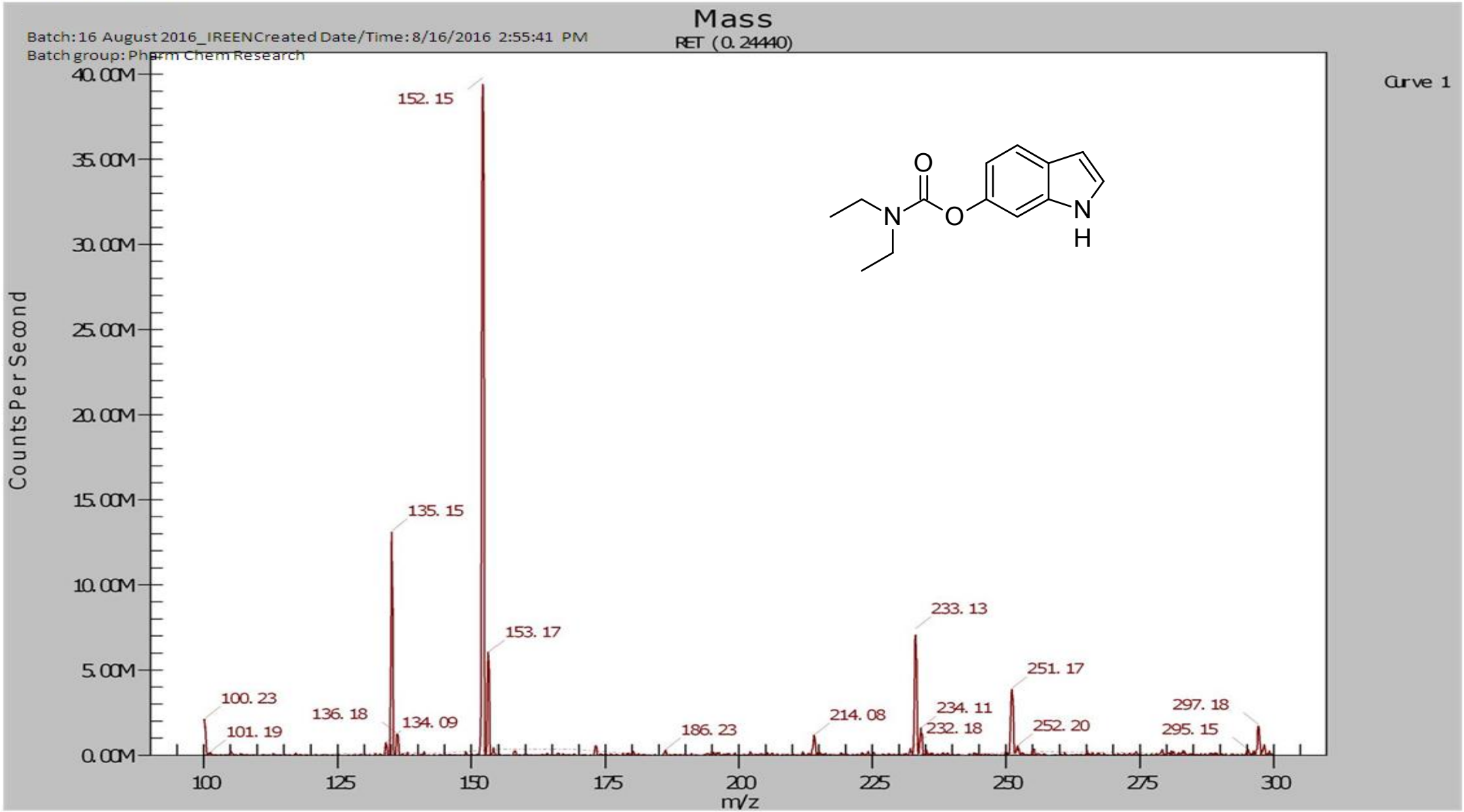
Spectrum 14: ^{13}C NMR COMPOUND 4A



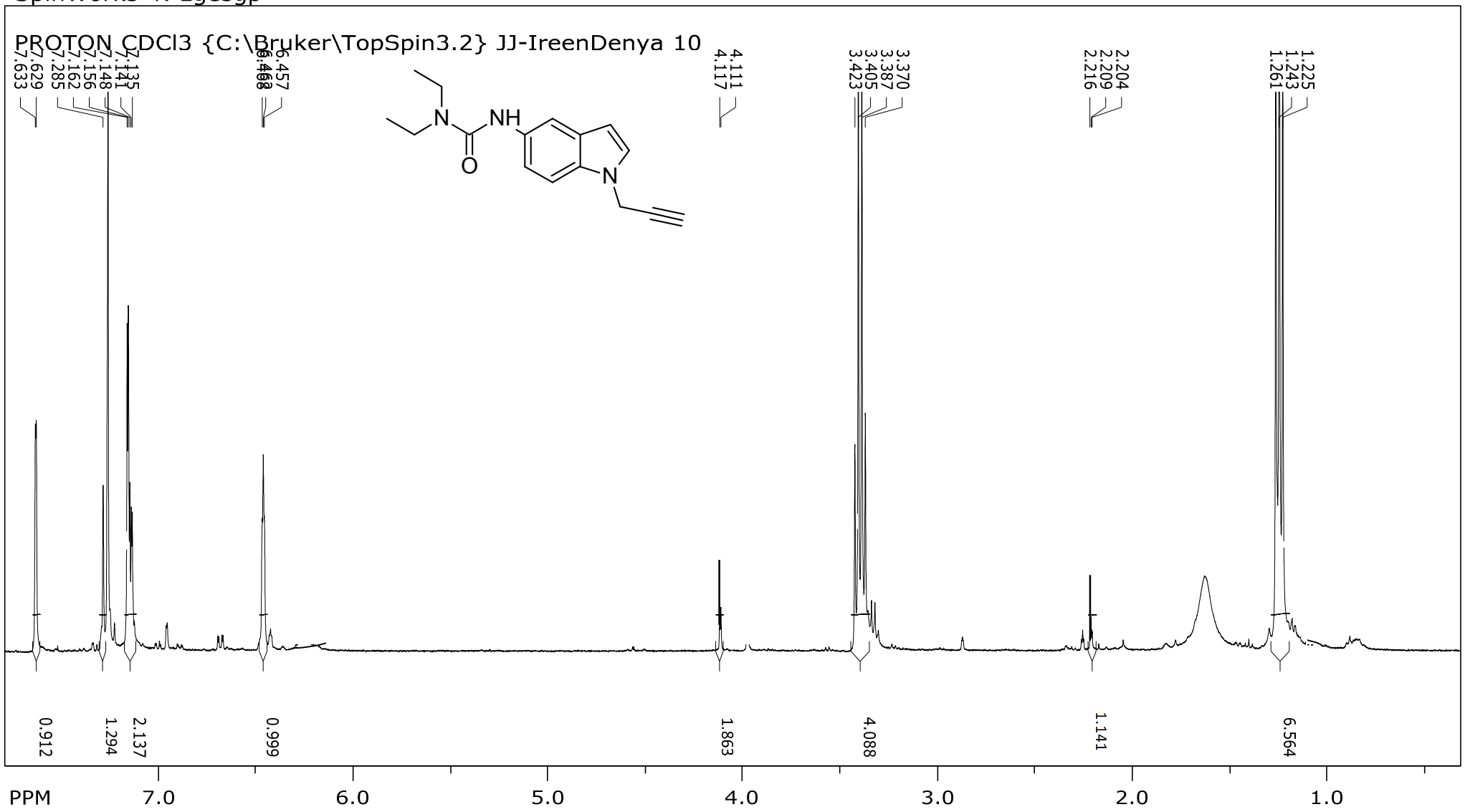
Spectrum 15: IR COMPOUND 4A



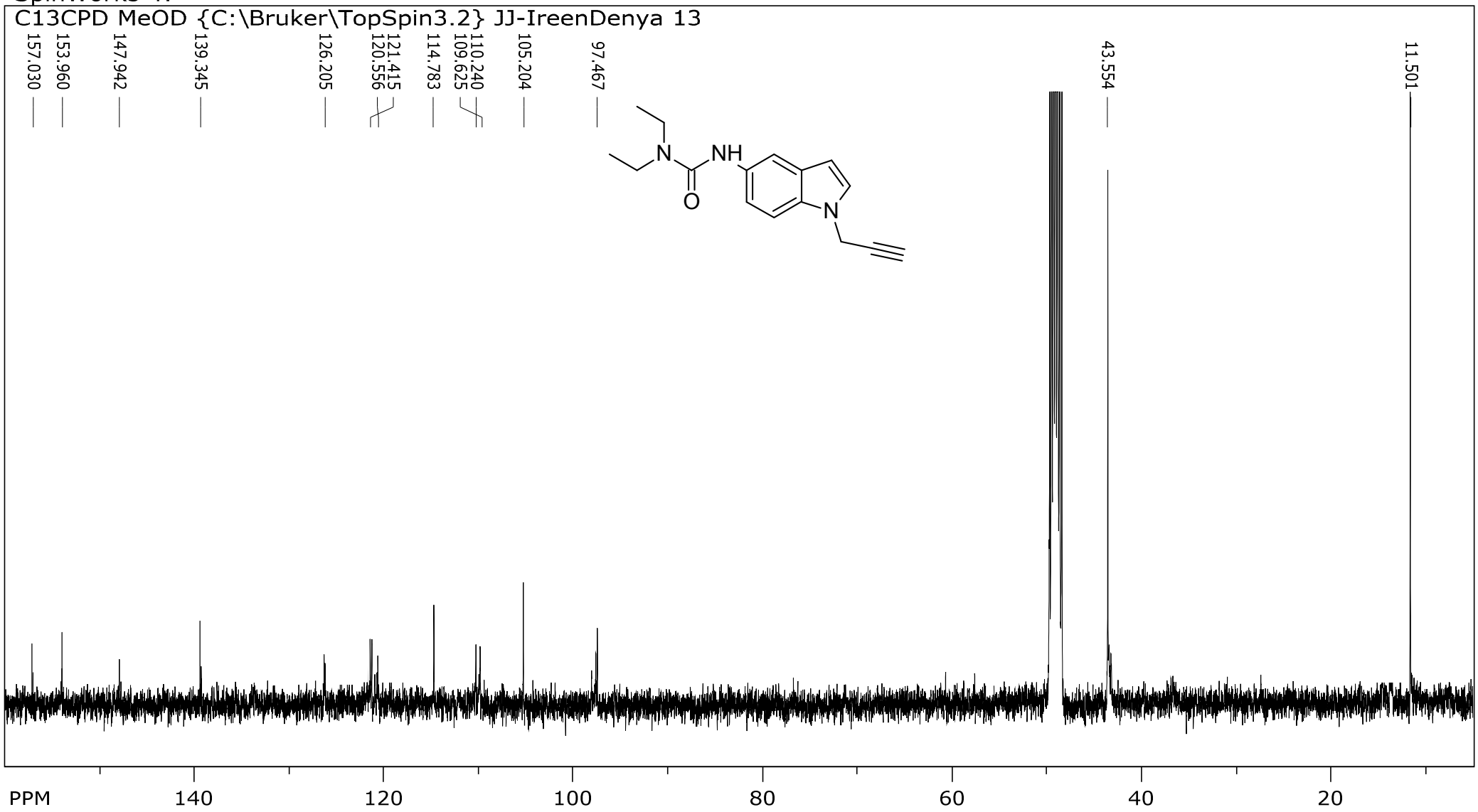
Spectrum 16: MS COMPOUND 4A



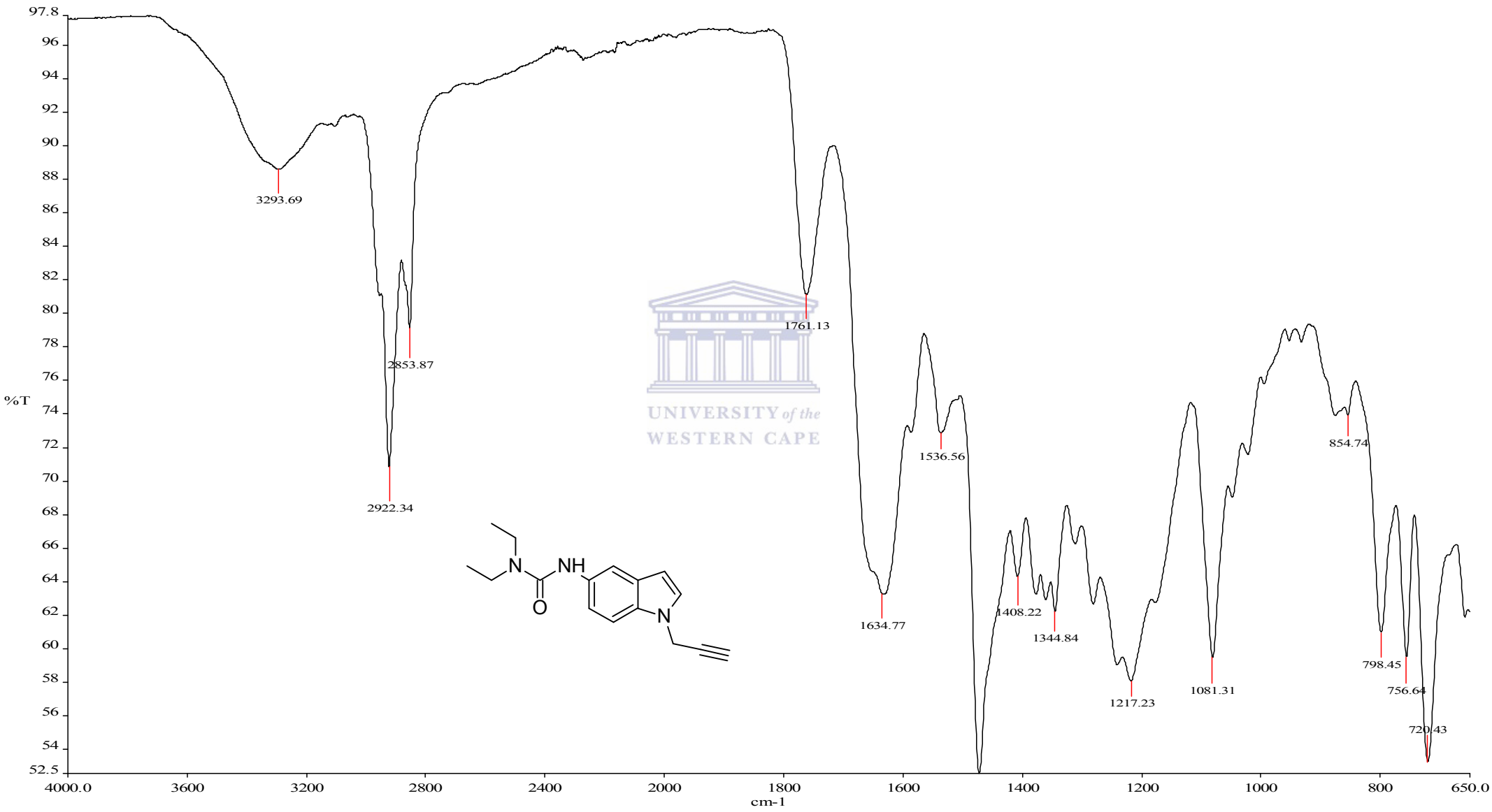
Spectrum 17: HNMR COMPOUND 1B



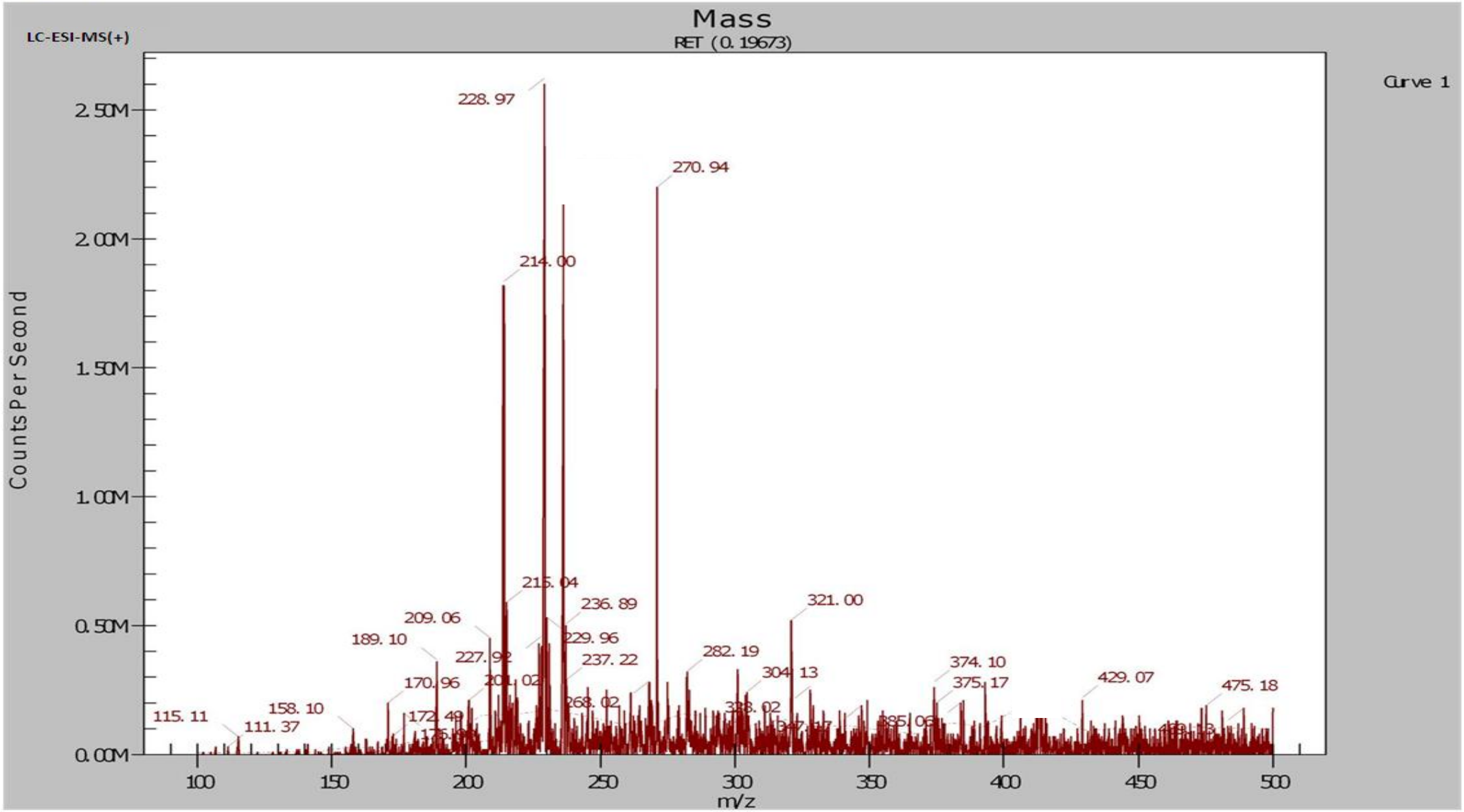
Spectrum 18: ^{13}C NMR COMPOUND 1B



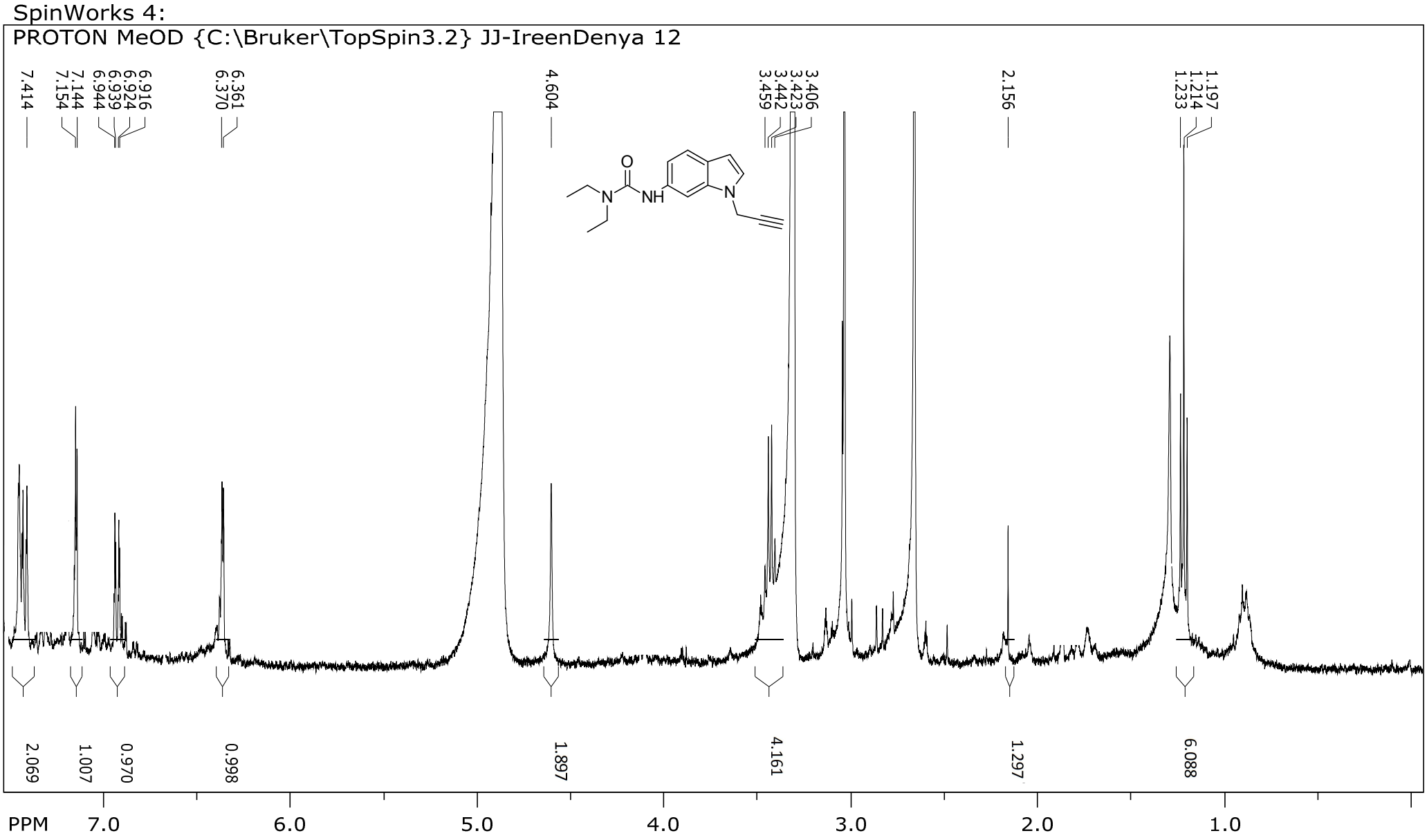
Spectrum 19: IR COMPOUND 1B



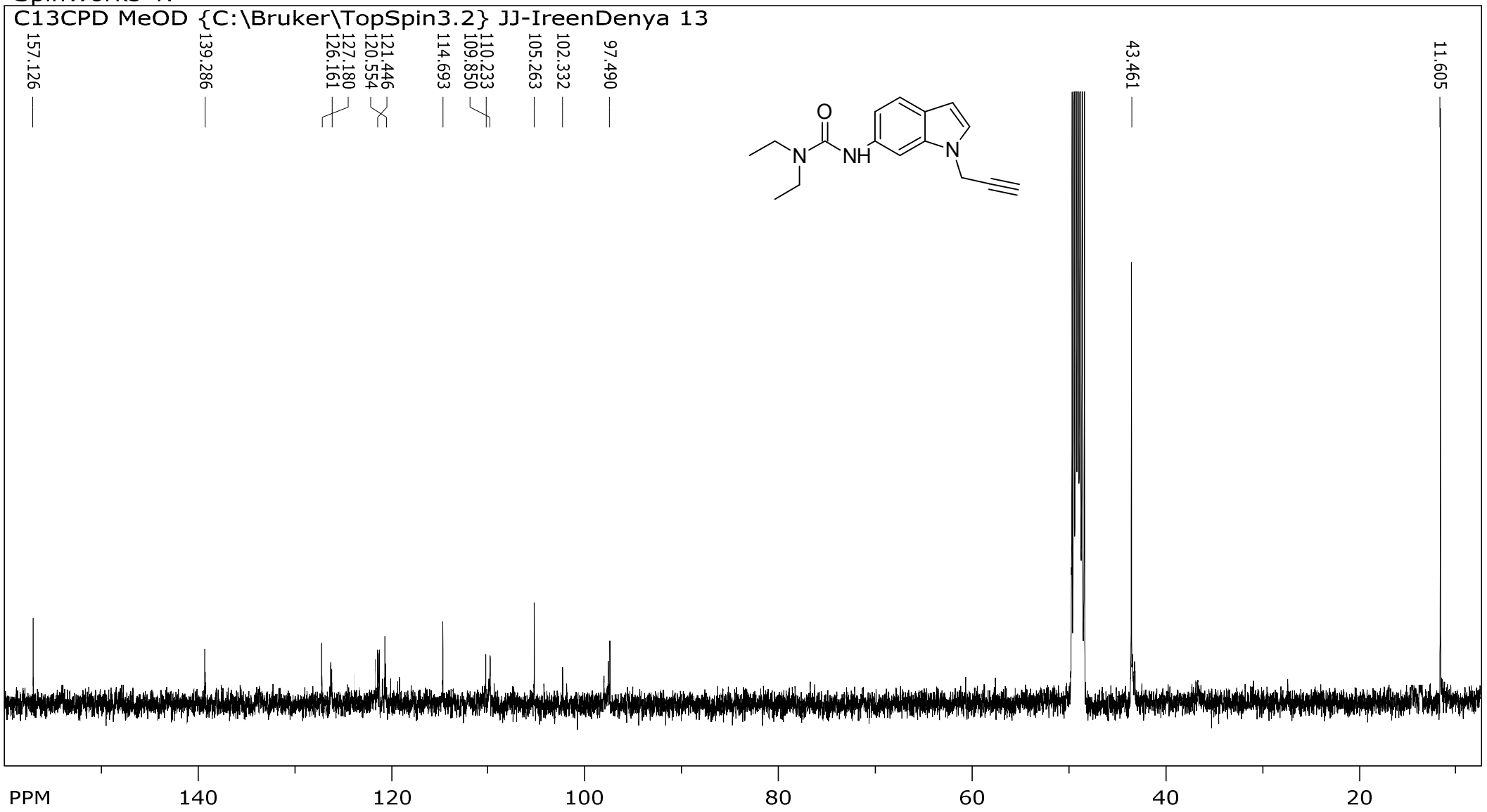
Spectrum 20: MS COMPOUND 1B



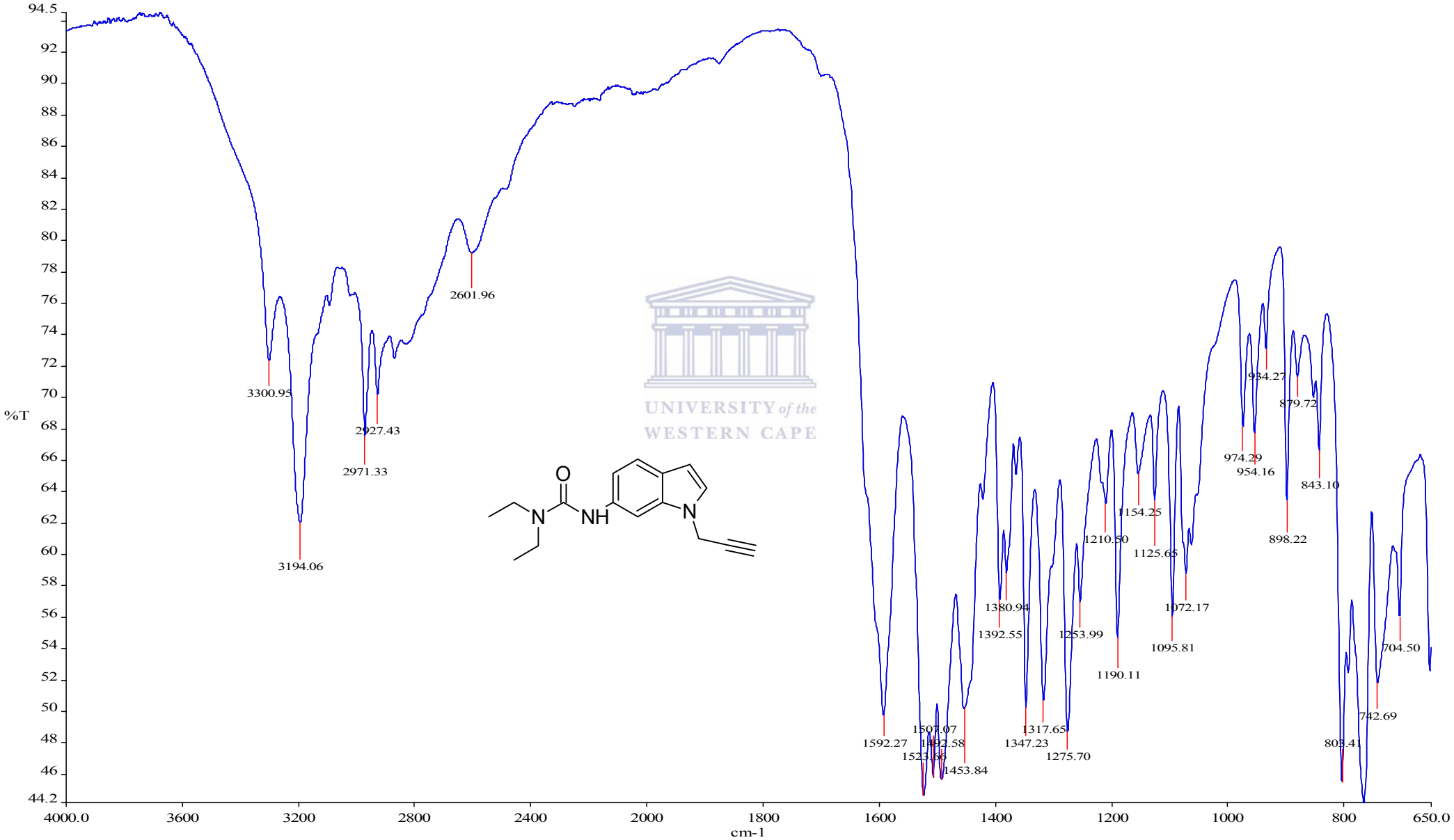
Spectrum 21: HNMR COMPOUND 2B



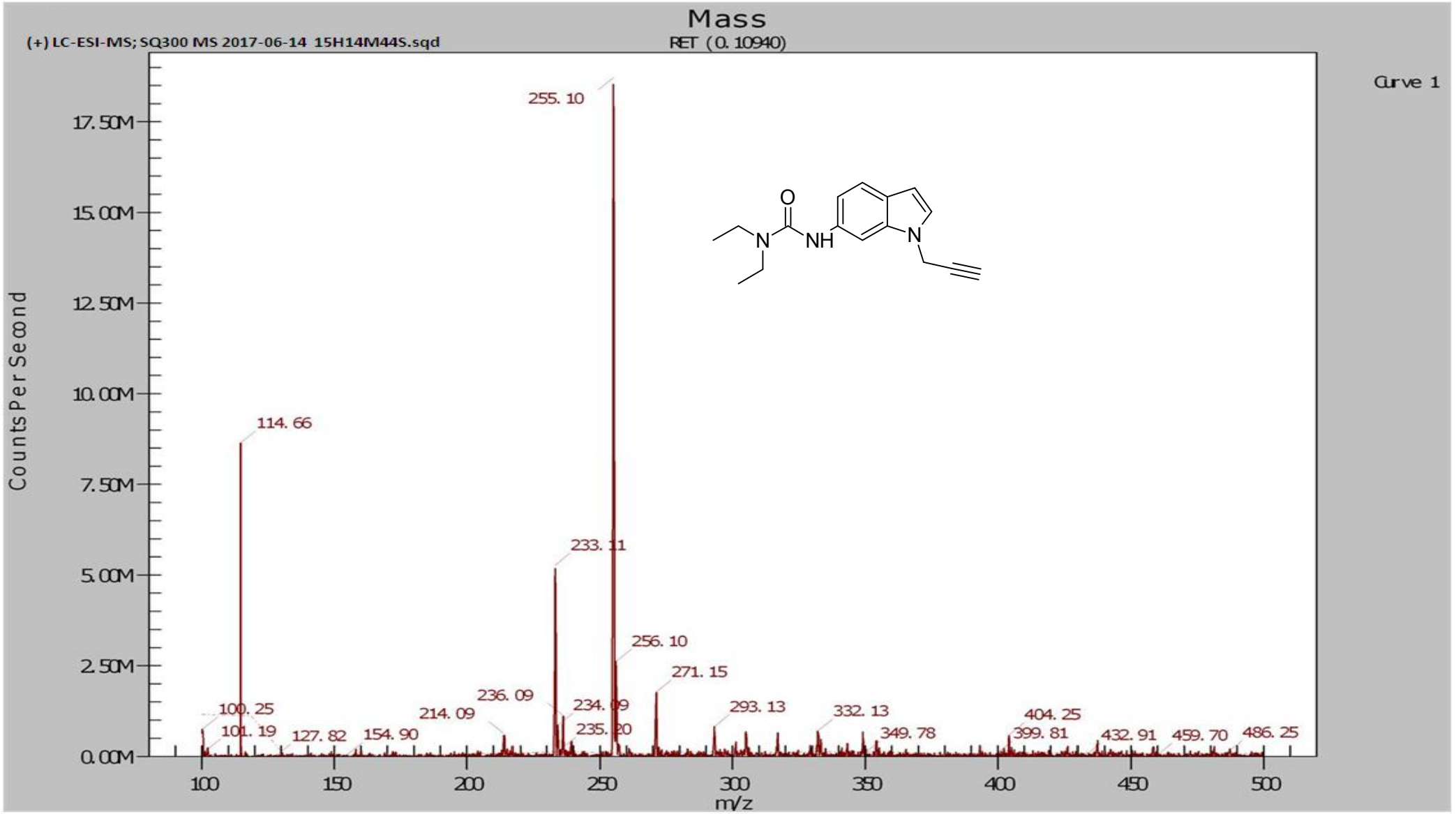
Spectrum 22: ^{13}C NMR COMPOUND 2B



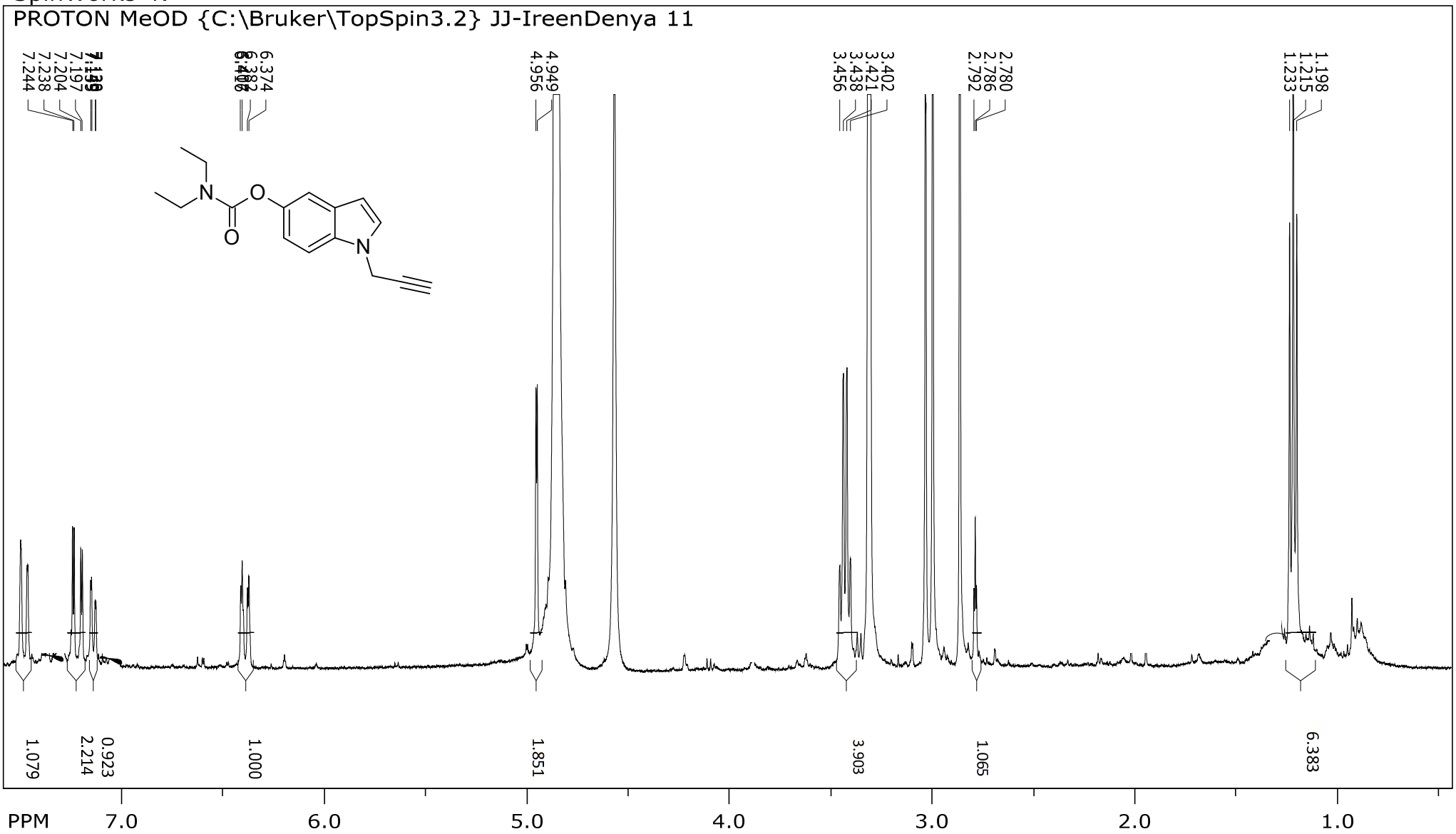
Spectrum 23: IR COMPOUND 2B



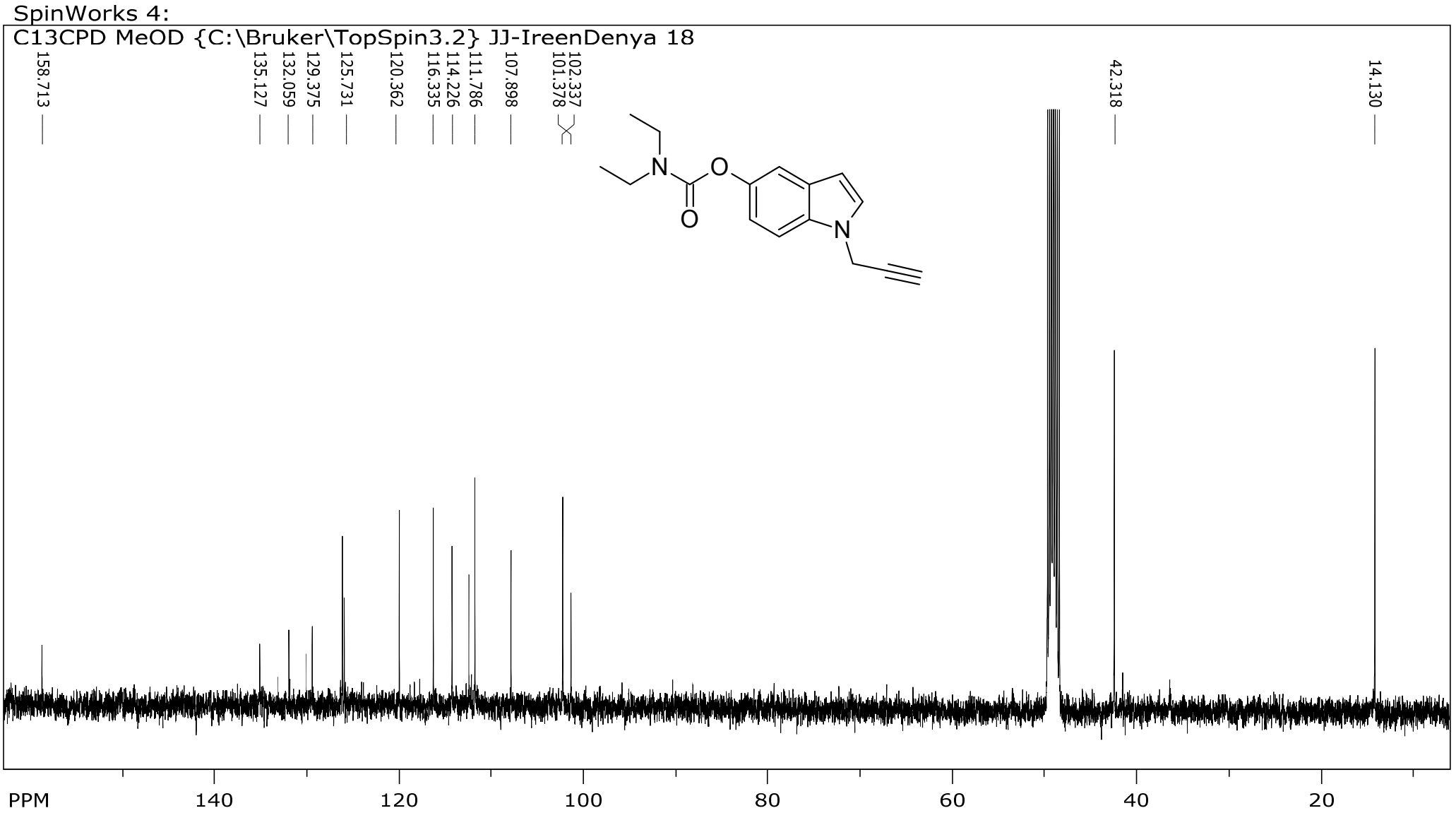
Spectrum 24: MS COMPOUND 2B



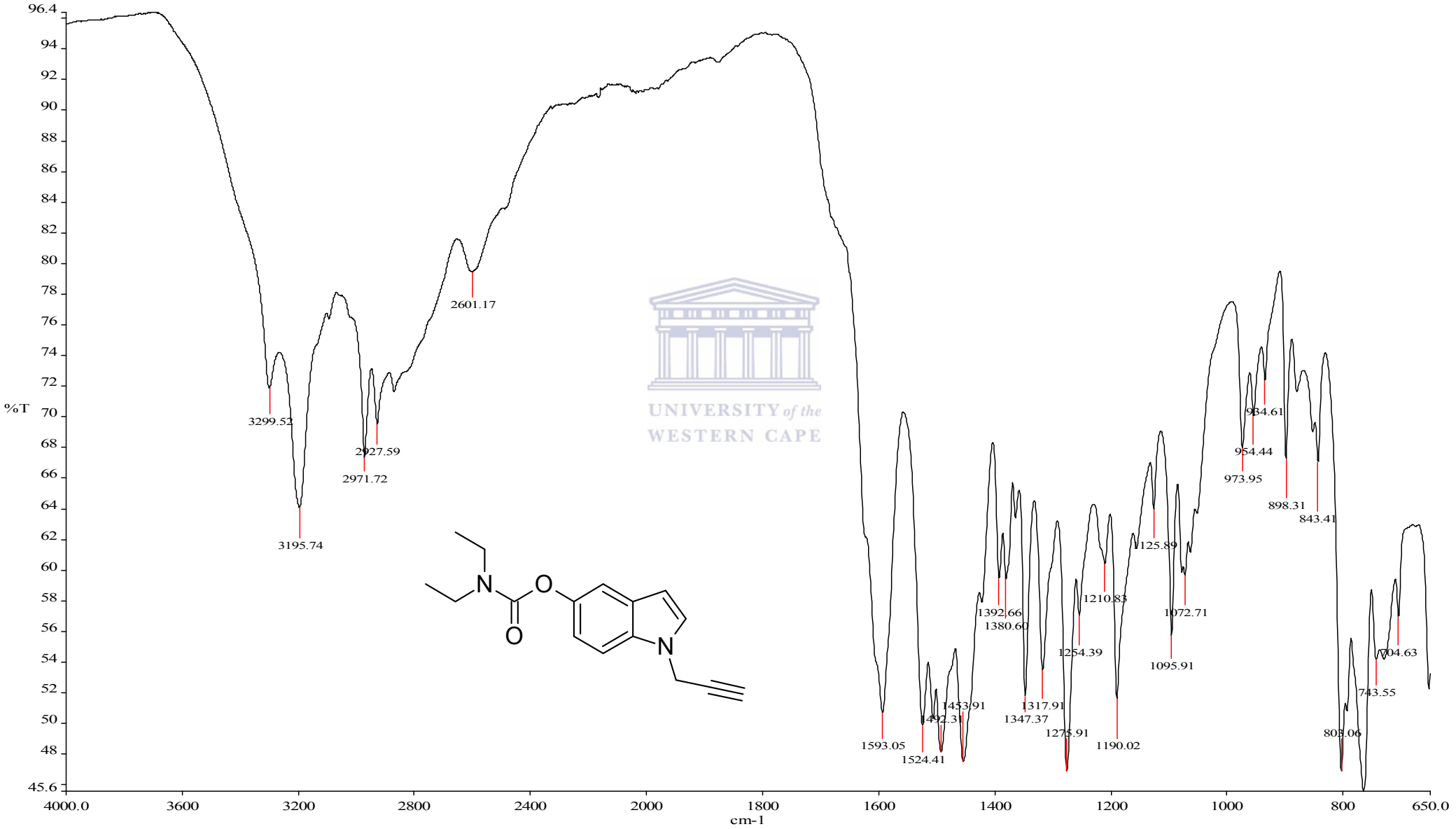
Spectrum 25: HNMR COMPOUND 3B



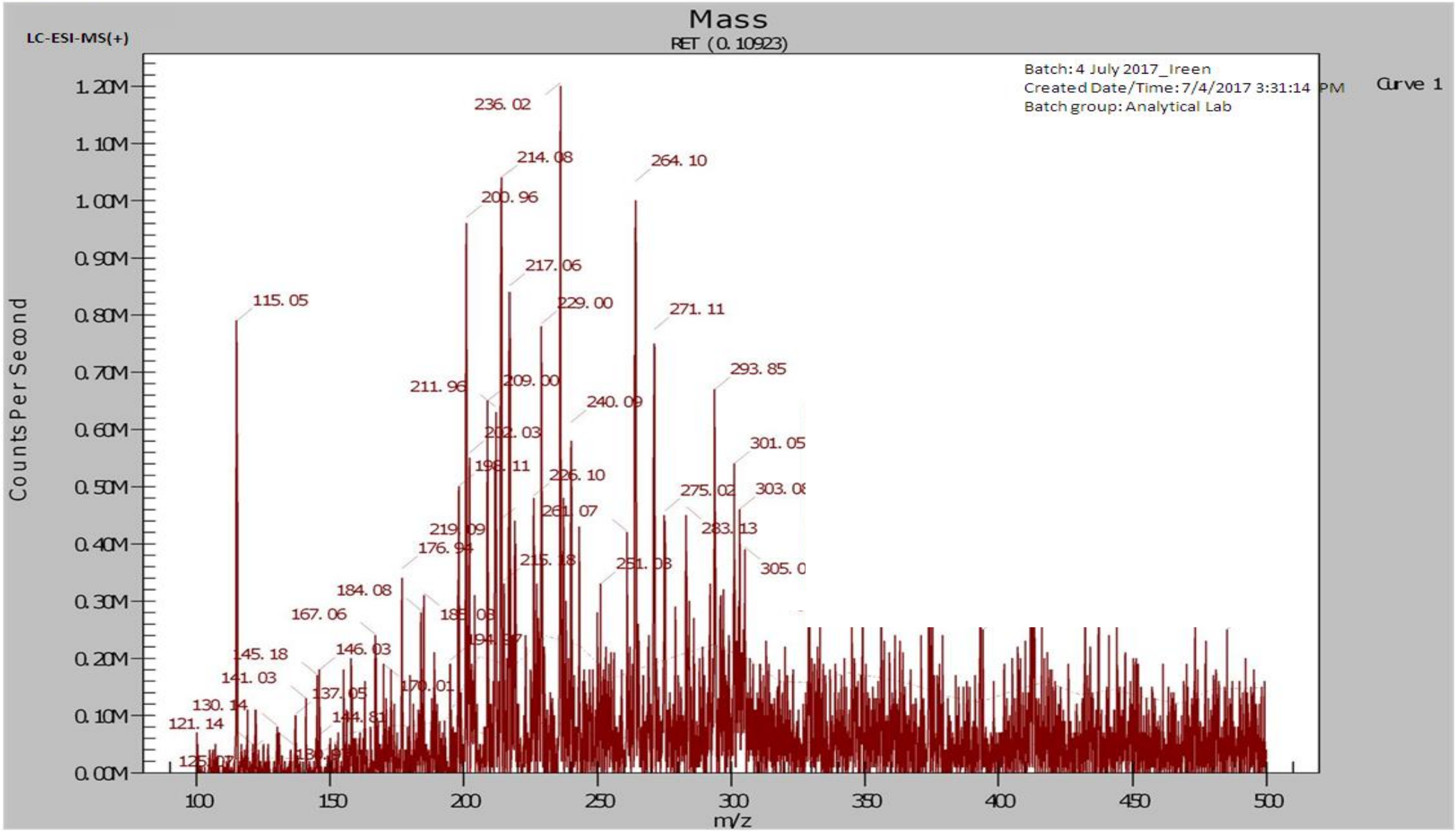
Spectrum 26: ^{13}C NMR COMPOUND 3B



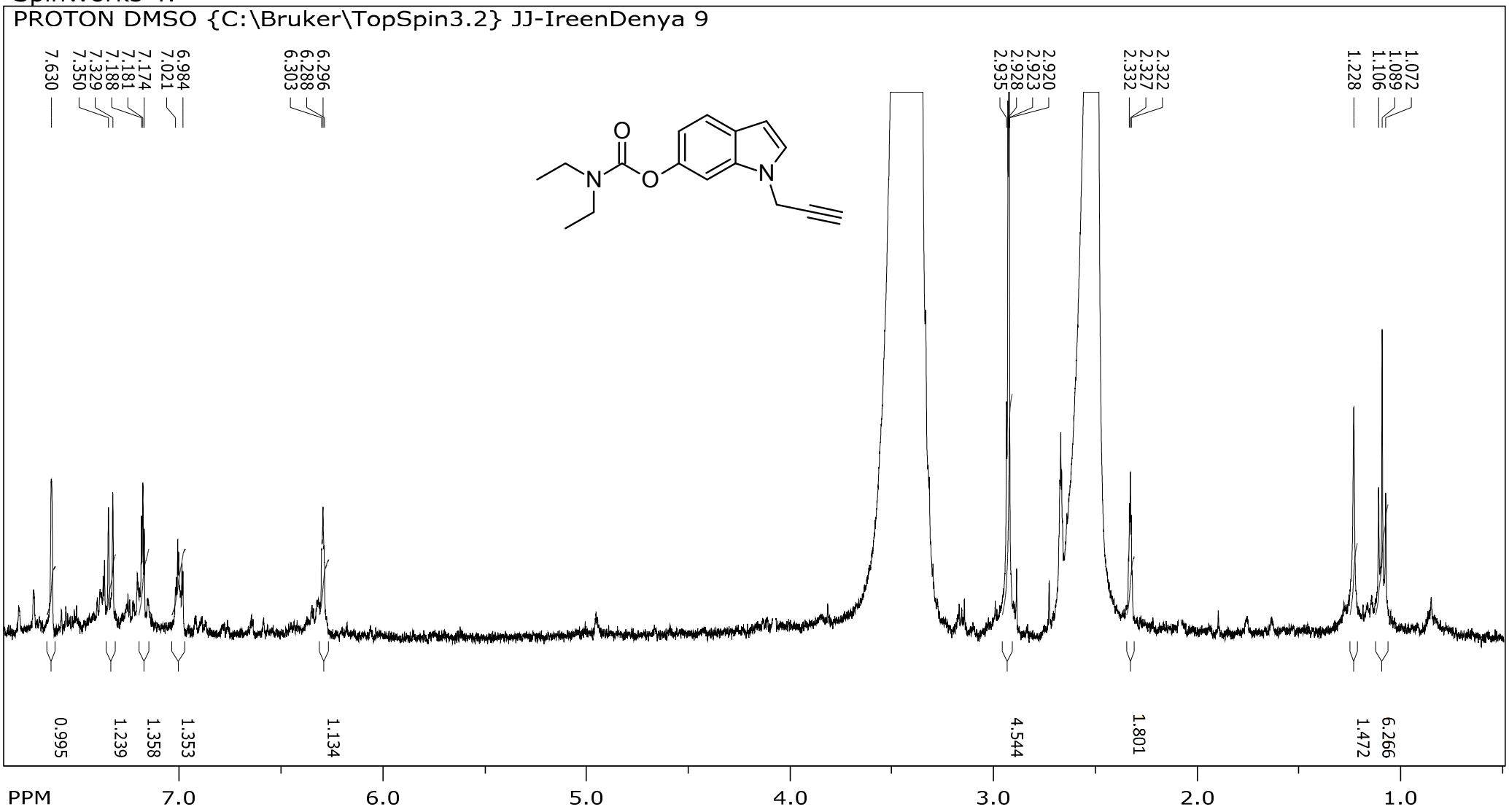
Spectrum 27: IR COMPOUND 3B



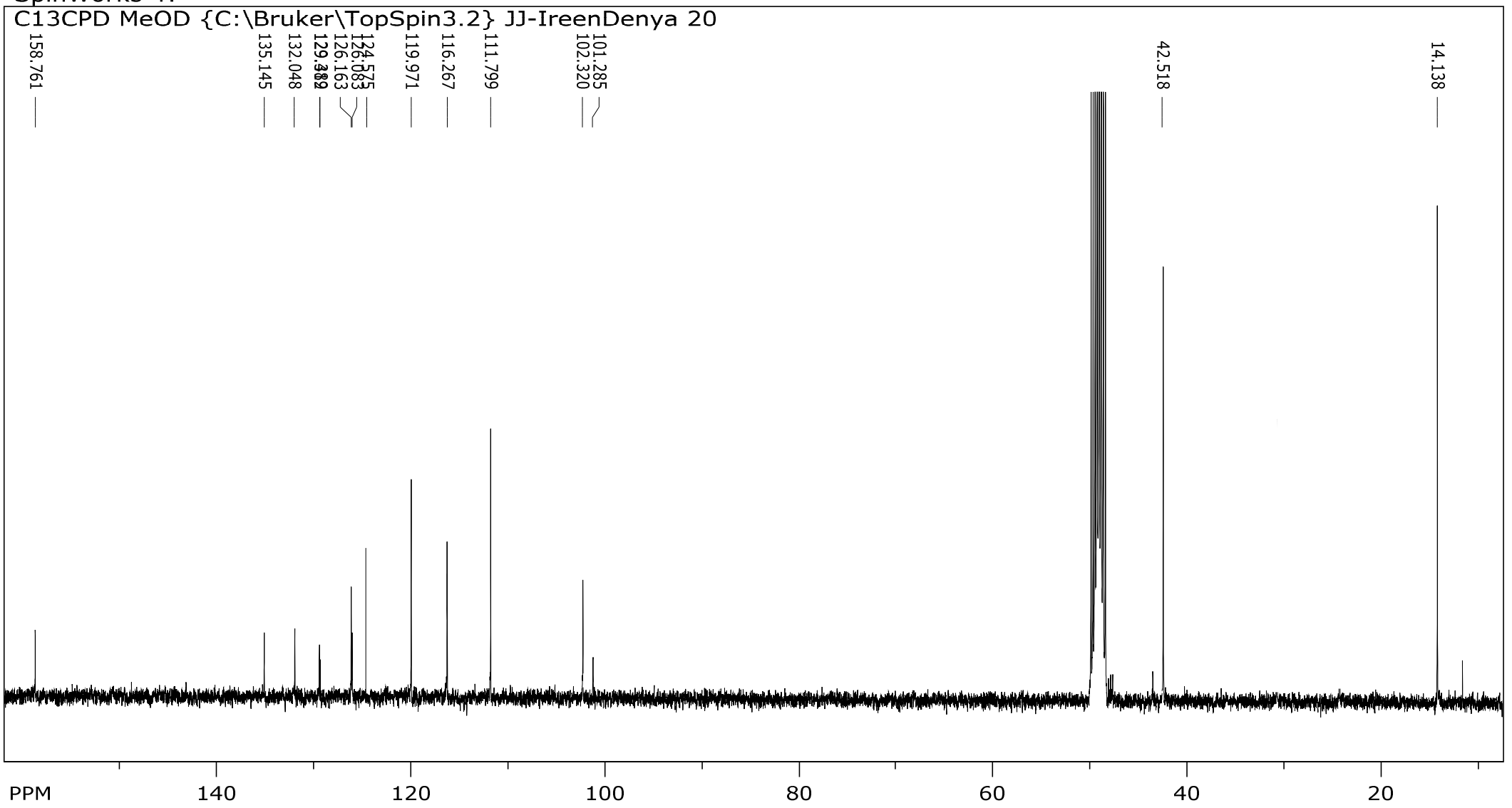
Spectrum 28: MS COMPOUND 3B



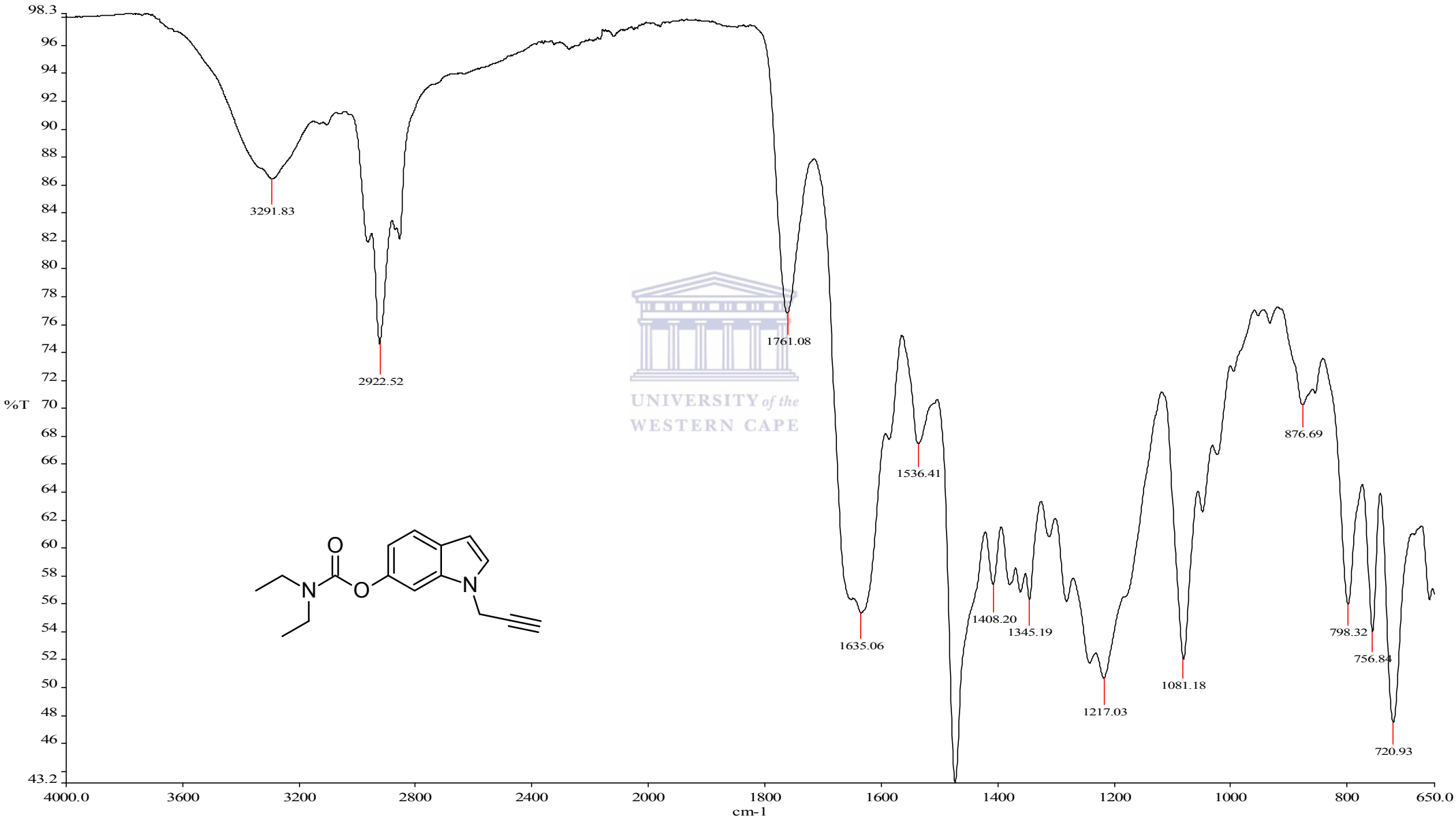
Spectrum 29: HNMR COMPOUND 4B



Spectrum 26: ^{13}C NMR COMPOUND 4B



Spectrum 31: IR COMPOUND 4B



Spectrum 32: MS COMPOUND 4B