Novel adamantane derivatives as multifunctional neuroprotective agents

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To my parents, Salma and Esmail. I am where I am today because of you. Thank you for everything. I love you

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ABSTRACT

The pathology of neurodegenerative disorders involves multiple steps, and it is probably for this reason that targeting one particular step in a multi-step process has only yielded limited results. Nitric oxide (NO) is synthesised from *L*-Arginine by an enzyme known as nitric oxide synthase (NOS). Three isoforms of NOS exist, including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). In the central nervous system (CNS), nNOS is involved in the synthesis of NO, which is involved in various neurological functions. NO is a free radical and this probably explains why an excess amount of it has been implicated in the development of neurodegenerative disorders. In the CNS, the *N*-methyl-D-aspartate (NMDA) receptor in its active state allows the influx of calcium ions which activate nNOS thus increasing the amount of NO and other detrimental reactive nitrogen species within neuronal cells. Calcium entry through voltage-gated calcium channels (VGCC) may also contribute to this. Although calcium ions are important for physiological functioning, an excess is responsible for excitotoxicity, which can ultimately lead to neurodegeneration.

Our aim was to synthesise a series of adamantane-derived compounds that act at multiple target sites in the neurodegenerative pathway. By conjugating benzyl and phenylethyl moieties with different functional groups (-H, -NO_2, -NH_2, -$NHC(NH)NH_2$, -OCH_3) to the amantadine structure, we aimed to synthesise compounds that display calcium channel and NMDA receptor (NMDAR) channel inhibition, as well as selective inhibition of nNOS.

A series of compounds (-H, -NO_2, -NH_2, -OCH_3) were obtained in yields that varied between 16.5 % and 90.25 %. These novel compounds were tested for calcium influx through VGCC and NMDAR inhibition using synaptoneurosomes isolated from rat brain homogenate against the reference compounds MK-801, NGP1-01, amantadine, memantine and nimodipine. A lack of success with the synthesis of the guanidine compounds prevented the use of the oxyhemoglobin capture assay for the determination of nNOS inhibitory activity of these compounds.

The novel synthesised compounds display inhibitory activity towards VGCC and the NMDAR in the micromolar range. Further tests are recommended on compounds SE-1, SE-4, SE-11 and SE-12 as they displayed good inhibitory activity against both NMDAR- as well as

KCl-mediated calcium influx. These novel compounds may be better therapeutic options than amantadine and memantine as they inhibit both NMDAR and VGCC-mediated calcium influx, whereas amantadine and memantine only inhibit NMDA-mediated calcium influx. These novel adamantane derived compounds may possibly serve as novel leads or potential therapeutic agents for the treatment of neurodegenerative disorders.

CHAPTER 1

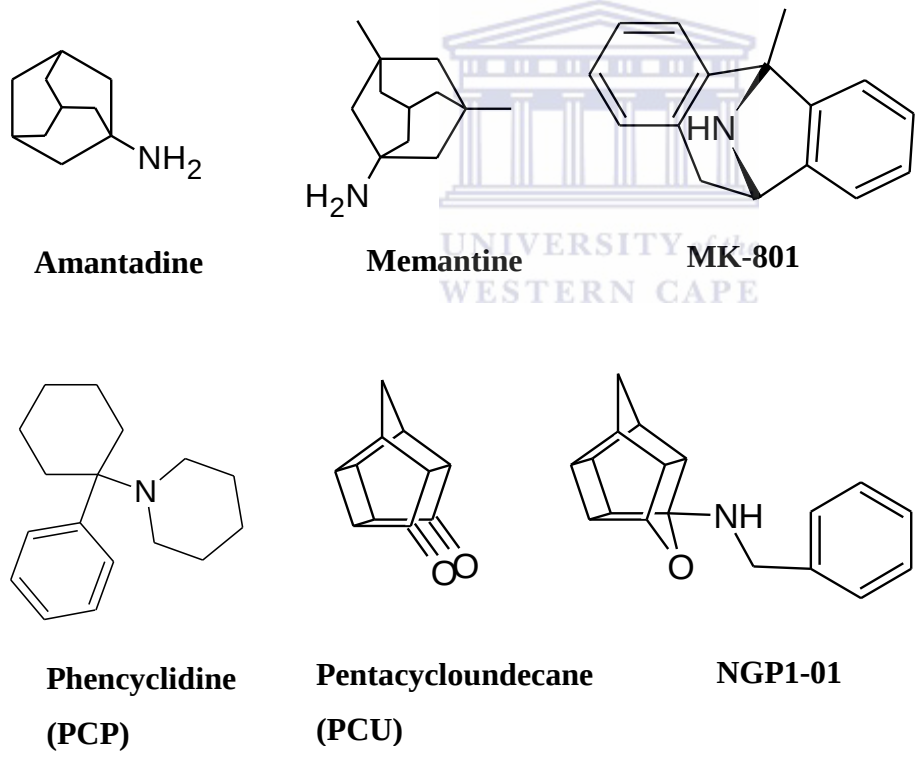
INTRODUCTION

1.1 Background

Neurodegeneration is defined as the progressive loss of structure and functions of neurons. In an aging population, neurodegenerative disorders are becoming increasingly rife. Neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease have been the focus of many different research groups over the past years, be it chemistry, neuroscience or pharmacology and they are the leading causes of loss of normal functioning in the elderly population (Geldenhuys *et al.*, 2004; Van der Schyf & Geldenhuys, 2009). The ability of these disorders to impact not only on the life of the affected person, but also their loved ones, provides a strong motivation to search for better treatment options. Current drugs available are used more for management of these disorders rather than treatment (Tarrants *et al.*, 2010; Youdim, 2010). The two most common neurodegenerative disorders are AD and PD. PD affects approximately 1 % of the global population over the age of 50 years (Singh & Dikshit, 2007). It is expected that approximately 34 million people will be suffering from AD worldwide by the year 2025 (Hynd *et al.*, 2004).

Various etiologies have been proposed for neurodegenerative disorders, and it is evident that a number of processes, rather than any single one are responsible. One such collection of events, which may act separately or collectively, is known as the lethal triplet and consists of excitotoxicity, mitochondrial dysfunction and oxidative stress. Excitotoxicity occurs as a result of an overstimulation of the *N*-methyl-D-aspartate (NMDA) receptor during pathological conditions. It can result from the presence of an excess amount of glutamate in the synapse, due to excessive release and inadequate uptake (Aarts & Tymianski, 2003; Van der Schyf & Geldenhuys, 2009). Oxidative stress is the damage that occurs to cellular structures and organelles, including the mitochondria (mitochondrial dysfunction), caused by the formation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) (Emerit *et al*., 2004).

Nitric oxide (NO) is a free radical synthesised from *L*-Arginine by the enzyme nitric oxide synthase (NOS). It is an important signalling molecule that is involved in a variety of physiological processes such as vasodilation, immune response and neurotransmission. Due to its free radical properties, an excess of NO has been associated with neurodegeneration and subsequently the development of neurodegenerative disorders (Low, 2005). There are three isoforms of NOS that exist, including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). While nNOS and eNOS are activated through a calcium dependent process, iNOS is activated independently of calcium (Erdal *et al.*, 2005). In the mitochondria, NO reacts with the superoxide anion to form peroxynitrite, which has a pro-apoptotic effect and is damaging to tissues and may lead to the development of neurodegenerative disorders (Low, 2005; Singh & Dikshit, 2007).



Amantadine **Memantine** **MK-801**

Phencyclidine (PCP) **Pentacycloundecane (PCU)** **NGP1-01**

Figure 1.1: Chemical structures of NMDA antagonists.

The NMDA receptor is unique, in that it requires the binding of two agonists, namely glutamate and glycine for activation. Both these agonists must bind to their respective binding sites for the receptor to be activated (Scatton, 1993; Danysz & Parsons, 1998; Klein

& Castellino, 2001). The NMDA receptor in its active state allows the influx of sodium and calcium ions which activates nNOS, leading to an increased amount of NO within the neuronal cells. Although calcium ions are important for cell growth, survival and physiological functioning, an excess is responsible for excitotoxicity, which can ultimately lead to neurodegeneration (Lynch & Guttman, 2002).

There are four different types of antagonists that can affect the activity of the NMDA receptor, namely:

1. Competitive antagonists would prevent the binding of glutamate to its binding site
2. Glycine antagonists would specifically prevent the binding of glycine to its binding site
3. Non-competitive antagonists would bind to an allosteric site separate from that of glutamate and glycine, and subsequently modulate the channel
4. Uncompetitive antagonists (or channel blockers) would prevent the influx of ions by blocking the channel in its open or closed state (Wong & Kemp, 2001, Geldenhuys, 2004).

The amino-adamantane derivatives amantadine and memantine (fig. 1.1) are low affinity uncompetitive antagonists which display fast blocking or unblocking effects at NMDA receptor channels and bind to the channel when it is in an open state. These agents are therefore better tolerated than high affinity channel blockers such as MK-801 (dizocilpine) and phencyclidine (PCP). These compounds (amino-adamantanes, MK-801 and PCP) bind to the PCP binding site located in the NMDA receptor/ion complex (Dingledine *et al*., 1999; Parsons *et al.*, 1998). The low affinity amino-adamantane uncompetitive antagonists leave the receptor site before the channel closes and thus allow neurons to function normally (Parsons *et al.*, 1998; Geldenhuys *et al.*, 2004; Geldenhuys *et al.*, 2005; Chen & Lipton, 2006; Joubert *et al.*, 2011).

1.2 Multifunctional neuroprotective agents

Current 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 (Geldenhuys *et al.*, 2005). Neurodegenerative disorders can be treated in one of three possible ways. 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 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). The latter approach is the one that is being adopted by a number of research groups and pharmaceutical companies as is evident from the development of drugs such as ladostigil (fig. 1.2), 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 (Youdim & Buccafusco, 2005a; Van der Schyf & Geldenhuys, 2009; Geldenhuys *et al.*, 2011; Weinreb *et al.*, 2012).

Although the initial multiple action drugs were discovered accidentally, medicinal chemists are now involved in the deliberate synthesis of such ligands. A number of therapeutic areas have witnessed this shift in ideology and approach. Such compounds are designed rationally with the intention of modifying a disease at various targets while ensuring safety by minimising side effects, as well as improving patient compliance (Morphy *et al.*, 2004).

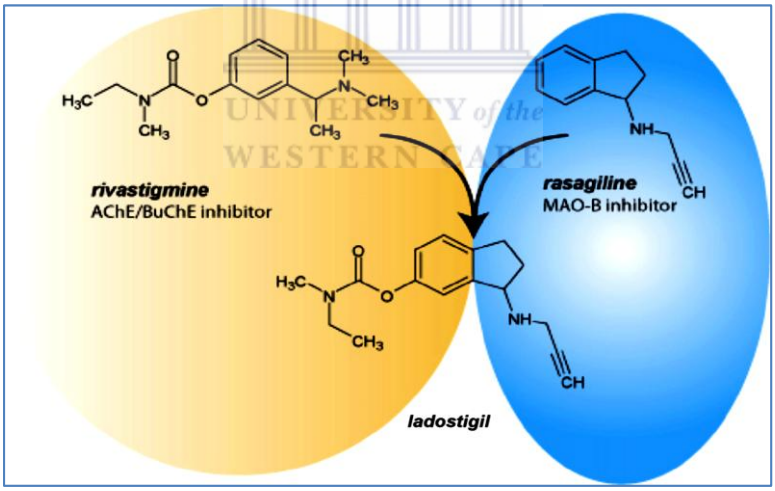


Figure 1.2: The design of ladostigil, a reversible AChE and BuChE and irreversible MAO-B inhibitor from its parent molecules rivastigmine and rasagiline (Geldenhuys *et al.*, 2011).

Polycyclic cage derivatives such as pentacycloundecane (PCU, fig. 1.1) and amantadine are useful as drug scaffolds and also improve the pharmacokinetics as well as pharmacodynamics of privileged moieties connected to it. The lipophilicity of these privileged moieties is also improved, which enables them to cross the blood brain barrier and have secondary

neuroprotective effects in the CNS (Geldenhuys *et al.*, 2005). The actions of one such neuroprotective polycyclic cage compound, NGP1-01 (fig. 1.1), include voltage-gated calcium channel (VGCC) blockade and NMDA receptor inhibition. Calcium influx is regulated through a combination of VGCC as well as the NMDA receptors and both these channels are recognised as potential targets to curb the neurodegenerative process. A distinct structural similarity exists between NGP1-01 and the amino-adamantanes (Geldenhuys *et al.*, 2003; Geldenhuys *et al.*, 2005). Memantine is the 3,5-dimethyl derivative of amantadine and is currently approved for the treatment of moderate to severe AD (Danysz & Parsons, 2003; Geldenhuys *et al.*, 2005). Amantadine itself is approved by the FDA for the treatment of PD. NGP1-01, amantadine and memantine are believed to have neuroprotective effects through the modulation of voltage-gated sodium, potassium and calcium channels as well as NMDA receptor ion channels (Geldenhuys *et al.*, 2003; Grobler *et al.*, 2005, Van der Schyf & Geldenhuys, 2009).

1.3 Rationale of this study

The drugs that are currently available for the management of neurodegenerative disorders target only one pathway or have just one mechanism of action (Geldenhuys *et al.*, 2004). It is therefore necessary to develop and synthesise compounds that function through different mechanisms so as to act on as many pathways as possible. Drugs need to be developed that can modify neurodegenerative disorders at various targets whilst ensuring safety by minimising side effects, as well as improving patient compliance.

The first NOS inhibitors were structurally similar to *L*-arginine and are thought to bind competitively at the *L*-arginine binding site. Due to the active site of nNOS being similar to that of iNOS and eNOS, the early NOS inhibitors lacked selectivity for one isoform over the others (Collins *et al.*, 1998; Alderton *et al.*, 2001). Since NO is involved in regulatory processes in various tissues in the body, selective inhibition of one particular isoform over the others is of extreme importance in order to avoid complications. If an inhibitor is not selective for nNOS and inhibits eNOS as well, it will cause a change in the blood pressure homeostasis of the body and could end up being harmful to the patient (Collins *et al.*, 1998; Li & Poulos, 2005; Lawton *et al.*, 2009). The challenge for medicinal chemists is to design compounds with a balance between good potency and selectivity (Masic *et al.*, 2006).

In order to develop isoform-selective NOS inhibitors, there are three requirements:

1. A structural scaffold that provides a hydrogen bond donating group to the glutamate residue (such as a guanidino group) in the NOS active site and a small hydrophobic group, such as an alkyl group that would be responsible for non-polar interactions with proteins adjacent to the glutamate residue.
2. A functional group with hydrogen bonding capability that would confer isoform selectivity to the compound. This would enable the differentiation between amino acid residues of different isoforms. The functional group in question should reach into the substrate-access channel from the active site.
3. An appropriate linker between the scaffold and the functional group. The linker should be of appropriate length as well as flexibility in order for it to reach isoform-specific regions (De Vries, 2006).

It was thus speculated that novel nNOS selective inhibitors could be synthesised by attaching different functional groups to an amantadine moiety *via* a benzyl linker (fig. 1.3) as this would significantly improve their blood brain barrier (BBB) permeability since amantadine is known to have high permeability across the BBB. The compounds may also show NMDA and calcium channel inhibitory activity due to the amantadine moiety and the structural similarity thereof towards NGP1-01, thereby making them potential multifunctional neuroprotective agents.

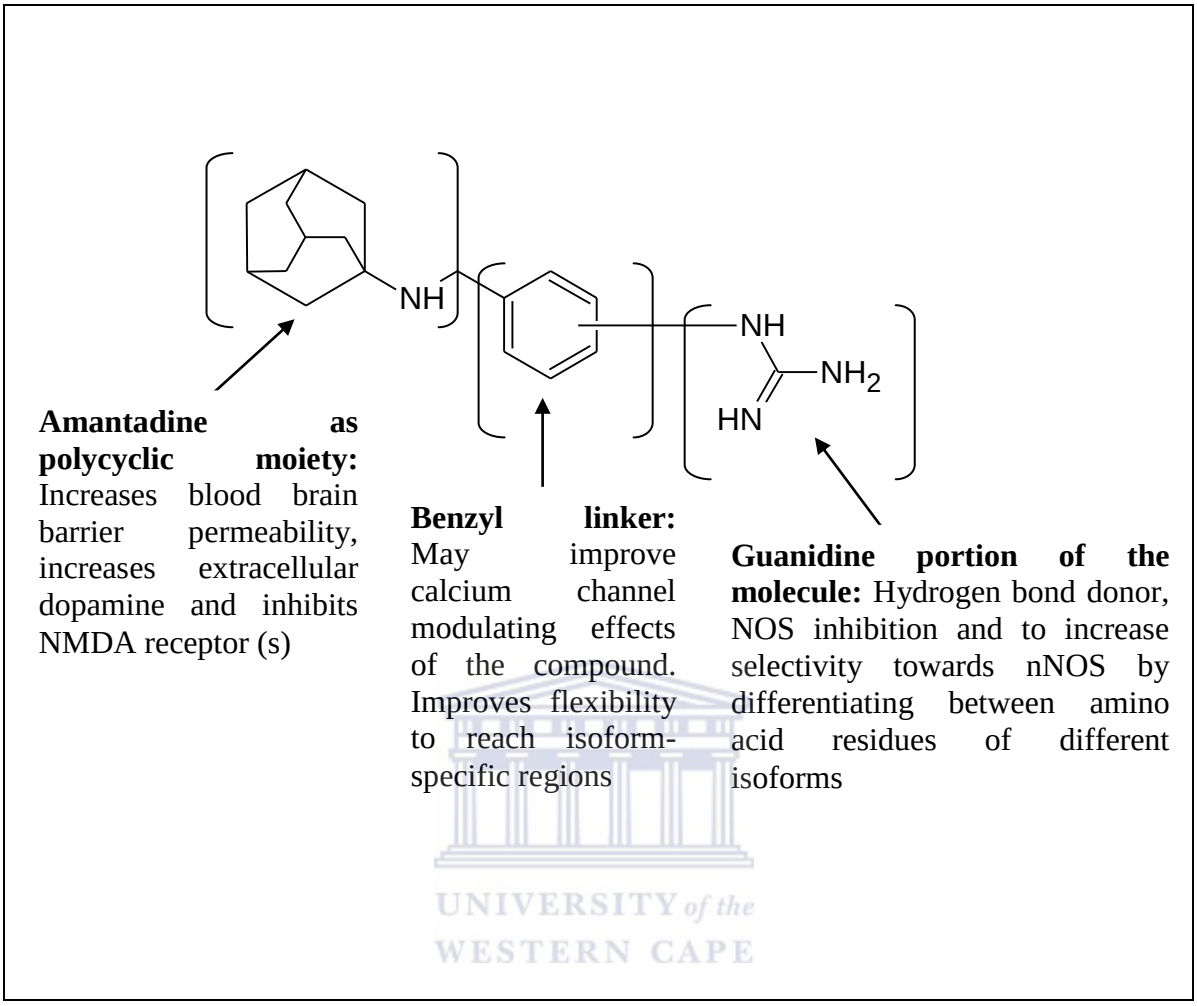


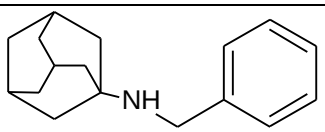
Figure 1.3: An example of the rationale used to conjugate different functional groups (in this case a guanidino group) to an amantadine moiety *via* a benzene linker, as well as the expected structure activity relationships (SAR) of the compounds.

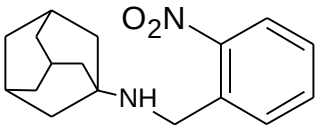
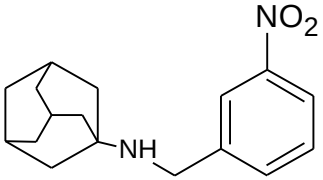
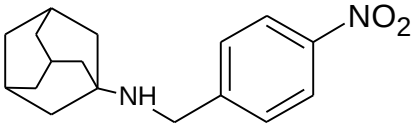
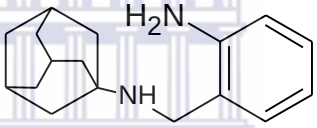
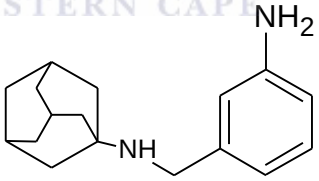
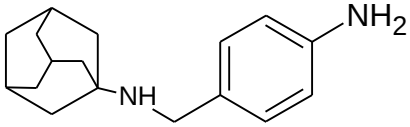
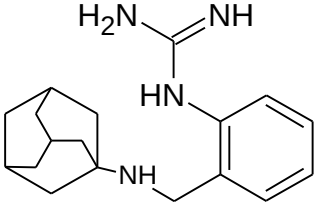
1.4 Aim of study

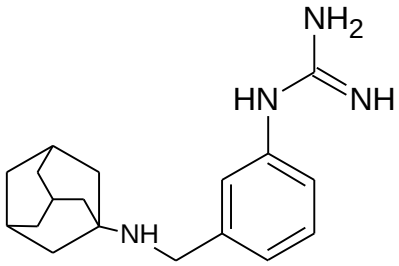
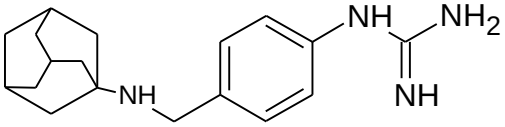
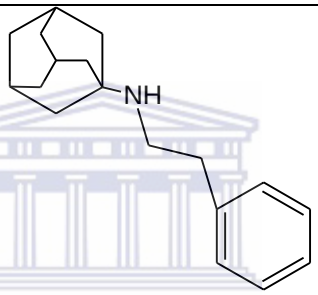
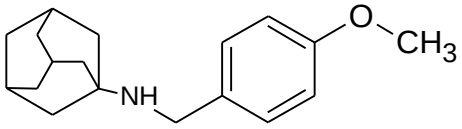
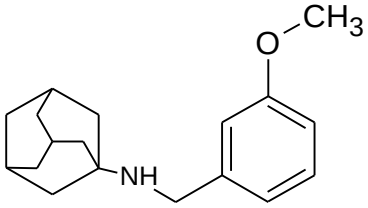
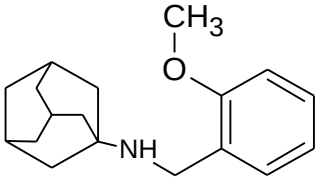
A series of adamantane derivatives bearing a structural similarity to the lead compound NGP1-01 will be synthesised. A benzyl linker with different functional groups (nitro, amine, guanidine and methoxy) attached will be conjugated to the amantadine moiety and the subsequent compounds chemically manipulated in order to obtain the desired products. Compounds containing nitro or methoxy substituent's have displayed free radical scavenging activity (Rice-Evans, 1997) as well as NMDAR and VGCC inhibitory activity. It is thus postulated that the free radical scavenging effect along with nNOS inhibition as well as NMDA receptor and VGCC inhibition will enable these compounds to act at multiple

pathways of the neurodegenerative cascade and thus have neuroprotective effects. Geldenhuys and colleagues (2005, 2009) found that compounds with *ortho* and *meta* substitution of nitro and methoxy groups on the aromatic ring of structurally similar compounds were more potent inhibitors of calcium channels than the *para* substituents (Geldenhuys *et al.*, 2005; Van der Schyf & Geldenhuys, 2009). The nitro compounds will be synthesised first. These will then be selectively reduced to the respective amines, which in turn will be converted to the respective guanidines (table 1.1). As NGP1-01 has shown to display neuroprotective activity, substituting the PCU polycyclic cage of NGP1-01 with the amantadine moiety (SE-1) will result in a molecule with structural similarity to NGP1-01. Such a compound should display the inherent properties of both NGP1-01, as well as the amantadine moiety. In a separate study, while carrying out experiments on NGP1-01 and its substituents, Geldenhuys and colleagues (2007) found that increasing the chain length between the PCU moiety and the aromatic ring from a methyl to ethyl linker resulted in an eight-fold increase in potency (Geldenhuys *et al.*, 2007). This led to the speculation that a similar effect would be observed if the chain length between the amantadine moiety and aromatic ring in SE-1 is increased to yield SE-11. All the novel synthesised compounds were evaluated *in vitro* for activity against the NMDA receptor, as well as voltage gated calcium channels using synaptoneurosomes obtained from rat brain homogenate and the ratiometric fluorescent calcium indicator, FURA 2-AM. Amantadine itself has also shown neuroprotective activity and will be used as a reference compound along with other NMDA/calcium channel inhibitors such as memantine, nimodipine, NGP1-01 and MK-801. The compounds could also be evaluated for nNOS inhibitory activity using the oxyhemoglobin capture assay.

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

ASSIGNED NAME	STRUCTURE & NAME
SE-1	 *N*-benzyltricyclo[3.3.1.1^{3,7}]decan-1-amine

SE-2	 *N*-(2-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-3	 *N*-(3-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-4	 *N*-(4-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-5	 *N*-(2-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-6	 *N*-(3-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-7	 *N*-(4-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-8	 1-{2-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine

SE-9	 1-{3-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine
SE-10	 1-{4-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine
SE-11	 *N*-(2-phenylethyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-12	 *N*-(4-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-13	 *N*-(3-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-14	 *N*-(2-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine

1.5 Conclusion

It is hypothesised that the compounds will display NMDA receptor- and calcium channel inhibitory activity in the micromolar range due to the presence of the amantadine moiety and the structural similarity thereof towards NGP1-01. Compounds SE-8, SE-9 and SE-10 are expected to have, together with NMDA receptor- and calcium channel inhibitory activity, good potency against NOS as well as good selectivity for nNOS due to the presence of the guanidine group. These compounds may then serve as novel leads or potential therapeutic agents for the treatment of neurodegenerative disorders.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Neurodegenerative disorders affect a vast number of the elderly as well as their family members. It is thus imperative that better treatments be discovered in order to prevent a higher number of people from being affected.

In this chapter, the two most common neurodegenerative disorders, the relevant contributors of neurodegeneration such as excitotoxicity, mitochondrial dysfunction and oxidative stress, as well as certain neuroprotective strategies will be discussed.

2.2 Alzheimer's Disease (AD)

AD is a progressive neurodegenerative disorder characterised by the irreversible loss of memory and dementia (Filley, 1995). The disease was first reported in 1906 by a German psychiatrist named Alois Alzheimer when one of his patients (whom he called Auguste D) presented with dementia. He followed her case from 1901 up to her death in 1906 (Alzheimer *et al.,* 1995). This neurodegenerative disorder, affects memory, behaviour, speech, cognition, as well as the ability to perform day to day activities (Khachaturian, 1985).

2.2.1 Etiology

A number of hypotheses have been proposed for the development of AD. One of these, the cholinergic hypothesis, states that cognitive impairment in AD results from the death of cholinergic neurons in the basal forebrain area, which causes a deficit of acetylcholine (ACh) in the brain (Sonkusare *et al.*, 2005; Contestabile, 2011). This hypothesis led to the development and use of the cholinesterase inhibitors such as tacrine, donepezil and galantamine to treat AD.

Another hypothesis is that AD is caused by accumulation of the amyloid-β protein which leads to cell death (Cummings, 2001). There is also a genetic hypothesis that accounts for early-onset familial cases of AD. The identified genes mutated in familial cases of AD are the

amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) (Blennow *et al.*, 2006). These familial cases are, however, quite rare with sporadic AD being more common. The mutated gene identified in sporadic cases of AD is the apolipoprotein E (APOE) (Qiu *et al.*, 2009; Blennow *et al.*, 2006). APOE is required for the deposition of amyloid-β and may promote amyloid-β fibrillisation and plaque formation (Blennow *et al.*, 2006). The two hallmarks of the AD brain are amyloid-β aggregation and deposition, with the development of senile plaques and tau hyperphosphorylation with neurofibrillary tangles (NFT) (Blennow *et al.*, 2006; Alzheimer, 1995).

There has also been some evidence of vascular risk factors in the development of AD. Risks included here are smoking, obesity, hyperlipidemia and even dietary factors, whereas vascular morbidity factors such as hypertension and diabetes may also play a role (Qiu *et al.*, 2009).

Psychosocial factors have also been implicated in the development of AD and a higher risk of AD has been associated with patients of lower education as well as a poor social network, lack of mental activities and low levels of physical activity (Qiu *et al.*, 2009).

2.2.2 Treatment

The goal of treatment in AD is to improve, or at least slow down memory and cognitive loss, and to maintain independent function (Mayeux & Sano, 1999). Drugs used to treat AD include tacrine, donepezil, rivastigmine, galantamine and memantine. While memantine is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor inhibitor, the rest are acetylcholinesterase (AChE) inhibitors. Although the cholinesterase inhibitors improve memory in mild dementia, they have no neuroprotective ability and do not halt the progression of AD (Hake, 2001).

2.2.2.1 Acetylcholinesterase inhibitors

AChE is an enzyme responsible for the hydrolysis of the neurotransmitter ACh, thereby preventing it from binding to post-synaptic ACh receptors (Katzung, 2001). AChE inhibitors thus increase the levels of ACh in the synapses due to decreased hydrolysis of ACh released by the synaptic neurons (Mayeux & Sano, 1999). Tacrine and donepezil are reversible AChE inhibitors while rivastigmine is irreversible. Some side effects associated with these drugs include gastrointestinal disturbances such as nausea, vomiting, cramps and diarrhoea (SAMF,

2012). Tacrine is associated with hepatotoxicity and therefore requires hepatic monitoring (Cummings, 2001). Galantamine is another AChE inhibitor, however, as AD worsens and the amount of ACh available decreases, the effect of galantamine declines (MayoClinic, 2011).

2.2.2.2 Memantine

Glutaminergic overstimulation may contribute to neuronal damage through a process known as excitotoxicity. This process eventually leads to neuronal calcium overload and has been implicated in neurodegenerative diseases. Glutamate stimulates the postsynaptic NMDA receptor, which has been implicated in dementia and pathogenesis of AD (Reisberg *et al.*, 2003). Memantine, a non-competitive NMDA receptor antagonist, is useful in the treatment of moderate to severe AD (SAMF, 2012; Reisberg *et al.*, 2003). Some noted side effects of memantine include hallucinations, confusion, dizziness, headache and constipation (SAMF, 2012).

2.3 Parkinson's Disease (PD)

PD, as described by Dr. James Parkinson in 1817 (Hou & Lai, 2007), is a progressive movement disorder of the central nervous system (CNS). It involves the degeneration of dopamine (DA) producing neurons in the substantia nigra of the brain, which leads to an imbalance between dopaminergic and cholinergic systems. The hallmark motor symptoms of PD include tremor, rigidity, bradykinesia and postural instability (Wood *et al.*, 2010). PD is the second most common neurodegenerative disorder in the United States, behind AD (Dewey, 2004). The mainstay of PD management is drug treatment focused on increasing dopaminergic activity (Tarrants *et al.*, 2010), as well as supportive care. The aim of treatment is to increase dopaminergic activity in the affected areas of the brain.

2.3.1 Etiology

The etiology of PD is thought to be a combination of environmental and genetic factors, but genetic predisposition, particularly in early-onset PD, is increasingly seen as the main cause (Schapira & Jenner, 2011; Nelson *et al.*, 2005; Warner & Schapira, 2003). The mutated genes identified in familial PD includes genes encoding for mitochondrial proteins such as Parkin, PTEN-induced putative kinase 1 (PINK-1), DJ-1, mitochondrial polymerase gamma 1

(POLG1) and genes coding for non-mitochondrial proteins such as α-synuclein and leucine-rich repeat kinase 2 (LRRK2) (Aquilano et al., 2008; Zhang et al., 2006). The strongest risk factor for the development of PD, however, remains aging (Schapira & Jenner, 2011). Some environmental factors such as industrialisation, rural areas and plant derived toxins as well as exposure to carbon disulphide and organic solvents have also been implicated in the development of PD (Schapira & Jenner, 2011).

2.3.2 Treatment

There are certain objectives to be achieved in order to provide PD patients with effective treatment. These include efficacy in reducing PD symptoms and slowing down the disease progression, ensuring safety of the treatment, or at least decrease the risk of adverse effects and reducing the costs associated with therapy (Rascol *et al.*, 2003). The drug classes currently in use include (structures shown in fig. 2.1):

1. DA precursor (levodopa) with or without a dopa-decarboxylase inhibitor (carbidopa, benserazide)
2. DA agonists (pramipexole, ropinirole)
3. Monoamine oxidase B (MAO-B) inhibitors (rasagiline, selegiline)
4. Catechol-*o*-methyltransferase (COMT) inhibitors (entacapone) (Tarrants *et al.*, 2010)
5. Amantadine and derivatives
6. Anticholinergic agents (biperiden, orphenadrine and trihexyphenydyl) (Katzung, 2001).

2.3.2.1 Levodopa

DA cannot cross the blood brain barrier (BBB), therefore levodopa is used. Levodopa is the immediate metabolic precursor of DA. It undergoes decarboxylation in the brain by the enzyme dopa-decarboxylase to produce DA. When administered alone, only about 1 % to 3 % of the total levodopa dose crosses the BBB to enter the brain. The remainder is metabolised by peripheral dopa-decarboxylase to produce DA, which cannot cross the BBB (Singh *et al.*, 2007). In order to overcome this problem, levodopa is administered in combination with a peripheral dopa-decarboxylase inhibitor such as carbidopa or benserazide which cannot cross the BBB. This results in decreased peripheral metabolism of levodopa and larger amounts are available to cross the BBB (Aminoff, 2007). Levodopa has a half-life

of approximately 45 to 90 minutes with the peak therapeutic response expected after two to three weeks of therapy (Halkias *et al.*, 2007). It is able to alleviate all of the cardinal motor symptoms of Parkinson's disease. Although it does not stop the progression of PD, it does lower the mortality rate. Levodopa can cause gastrointestinal disturbances such as nausea and vomiting. This occurs in 80 % of patients when levodopa is administered alone, but in only 20 % when administered in combination with carbidopa. There are cardiovascular effects such as arrhythmias, (Singh *et al.*, 2007) although the incidence is low. The incidence of arrhythmia is further reduced when levodopa is taken in combination with carbidopa. The patient may experience behavioural effects such as depression, anxiety, insomnia, confusion, delusions, hallucinations, nightmares and euphoria (Aminoff, 2007).

2.3.2.2 DA agonists

DA agonists are divided into two groups, the ergot derivatives such as bromocriptine and the non-ergot derivatives such as ropinirole and pramipexole. DA agonists may not be as effective as levodopa in treating the bradykinesia, gait disturbances and other symptoms of advanced PD, but they are useful when managing the mild disabilities that are associated with early PD (Nelson *et al.*, 2005). These agents are generally used as monotherapy to delay the initiation of levodopa therapy, or they are used in combination with levodopa in order to decrease the total levodopa requirement by enhancing the antiparkinsonian effects of the drug (Singh *et al.*, 2007). The DA agonists are not dependent on dopa-decarboxylase which is needed by levodopa for conversion to DA. These drugs have a longer half-life than levodopa and may provide longer periods of symptomatic relief (Stern, 2001).

Some common adverse effects associated with the use of pramipexole and ropinirole are confusion, insomnia, hallucinations, dizziness, dyskinesias, somnolence, nausea constipation, peripheral oedema and postural hypotension (SAMF, 2012). They are also associated with sudden sleep episodes or somnolence. Patients may fall asleep without any warning which compromises their ability to operate machinery (SAMF, 2012).

2.3.2.3 MAO-B inhibitors

Selegiline and rasagiline act by irreversibly inhibiting the breakdown of DA by MAO-B, thus increasing its levels. Therefore, when used in combination with levodopa, they may allow the total dose of levodopa to be reduced (Singh *et al.*, 2007). Some common adverse effects

associated with selegiline include insomnia, hallucinations, confusion, dyskinesias, postural hypotension, dry mouth and vertigo (SAMF, 2012). Some commonly experienced adverse effects with rasagiline include headache, malaise, neck pain, fever, angina, rhinitis and vertigo (SAMF, 2012).

2.3.2.4 COMT-inhibitors

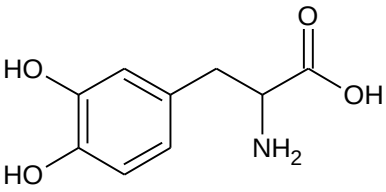
Entacapone acts to inhibit the peripheral metabolism of levodopa by the enzyme catechol-*o*-methyltransferase (COMT), thus increasing the plasma levels of levodopa. COMT-inhibitors are used to extend the effects of levodopa and are generally used in combination with other antiparkinsonian drugs (Nelson *et al.,* 2005). There are no side effects commonly associated with entacapone. However, it has shown to be associated with hepatotoxicity, although rarely (SAMF, 2012).

2.3.2.5 Amantadine

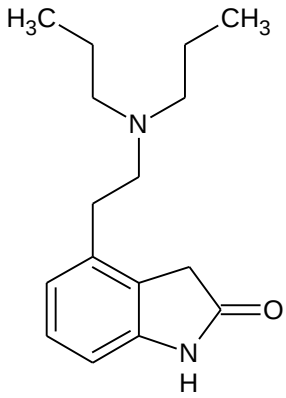
Amantadine is an antiviral agent that showed potential in reducing the symptoms of PD. Amantadine and its derivative memantine, act as an NMDA receptor antagonist and may decrease tremor by increasing the release and decreasing the uptake of DA (Marjama-Lyons & Koller, 2000). It may also possess neuroprotective activity *via* the inhibition of excess Ca^{2+} entry through the NMDA receptor ion channel (Geldenhuys *et al.,* 2005). Some adverse effects experienced with amantadine include insomnia, anxiety, nightmares and livedo reticularis (Fernandez, 2012; Marjama-Lyons & Koller, 2000; MayoClinic, 2011).

2.3.2.6 Anticholinergic agents

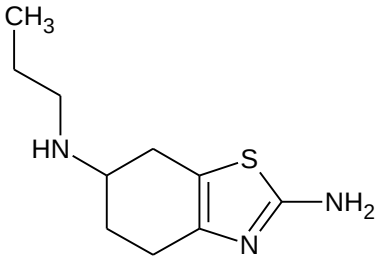
Anticholinergic agents such as biperidin, orphenadrine and trihexyphenidyl were amongst the first agents used for the management PD (Brocks, 1999). However, they are prone to cause adverse effects such as dry mouth, dry eyes, urinary retention and constipation. The most disturbing of the adverse effects include confusion, sedation and hallucinations (Katzenschlager *et al.*, 2009).



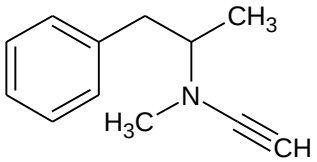
Levodopa



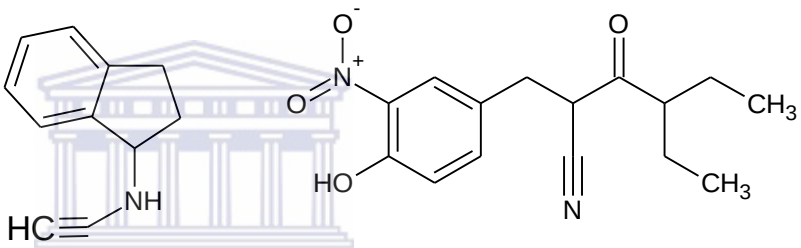
Ropinirole



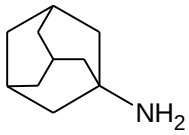
Pramipexole



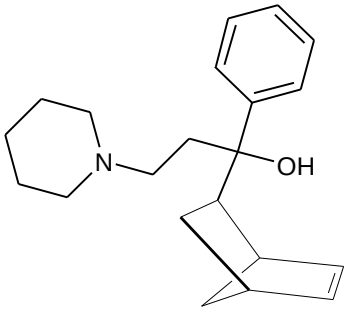
Selegiline



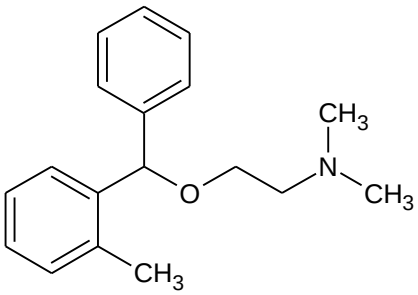
Rasagiline **Entacapone**



Amantadine



Biperidin



Orphenadrine

Figure 2.1: Chemical structures of drugs currently used to treat PD.

2.3.3 Non-motor symptoms

Although classified as a movement disorder, PD presents with a variety of non-motor symptoms, some of which may have a more negative impact on the patient's life than the motor symptoms. These non-motor symptoms may appear before the motor symptoms are even recognised (Park and Stacy, 2009), and may also be present at more advanced stages of the disease (Chaudhuri and Schapira, 2009). Non-motor symptoms experienced by PD patients include disorders of mood and affect causing apathy, anhedonia, depression, cognitive dysfunction, hallucinations as well as complex behavioural disorders including impulse-control disorders (Poewe, 2008; Ceravolo *et al.*, 2010; Eng & Welty, 2010). Sensory dysfunction with pain is experienced by almost all patients. Sleep-wake cycle disturbances are also commonly experienced. Autonomic dysfunction resulting in orthostatic hypotension, urogenital dysfunction as well as constipation is also present in a large number of patients (Hou & Lai, 2007; Poewe, 2008; Ceravolo *et al.*, 2010).

2.4 The NMDA receptor

L-glutamate is the most common neurotransmitter in the CNS and is involved in intracellular communication as well as growth and differentiation within the brain (Aarts & Tymianski, 2003; Chaffey & Chazot, 2008). There are two types of post-synaptic receptors that bind glutamate namely, metabotropic glutamate receptors (mGluRs), which are guanosine triphosphate (GTP) dependent and ionotropic glutamate receptors (iGluRs) which are ligand-gated receptors. iGluRs are classified based on their pharmacological properties into the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtype, the kainate subtype and the *N*-methyl-D-aspartate (NMDA) subtype (Yamakura & Shimoji, 1999; Chaffey & Chazot, 2008). The three subunits that make up NMDA receptors are NR1, NR2 and NR3. Functional NMDA receptors are tetrameric structures (fig. 2.2), which are made up of two NR1 subunits and at least two NR2 subunits.

The NMDAR channels are highly permeable to calcium ions and are co-activated by glutamate and glycine, which bind to NR2 and NR1 respectively (Scatton, 1993; Danysz & Parsons, 1998; Klein & Castellino, 2001; Fan & Raymond, 2007). The two characteristic special features of NMDA receptors are thus voltage-dependant magnesium ion blockade at physiological concentration (Nowak *et al.*, 1984; Ascher & Nowak, 1988) and the

requirement of co-agonist glycine, along with glutamate for activation (Meguro *et al.*, 1992; Meldrum, 2000). Once the NMDA receptor is activated, it allows the influx of sodium and calcium ions. The calcium ions can activate NOS, which leads to an increased amount of NO within the cell (Gorman *et al.*, 1996).

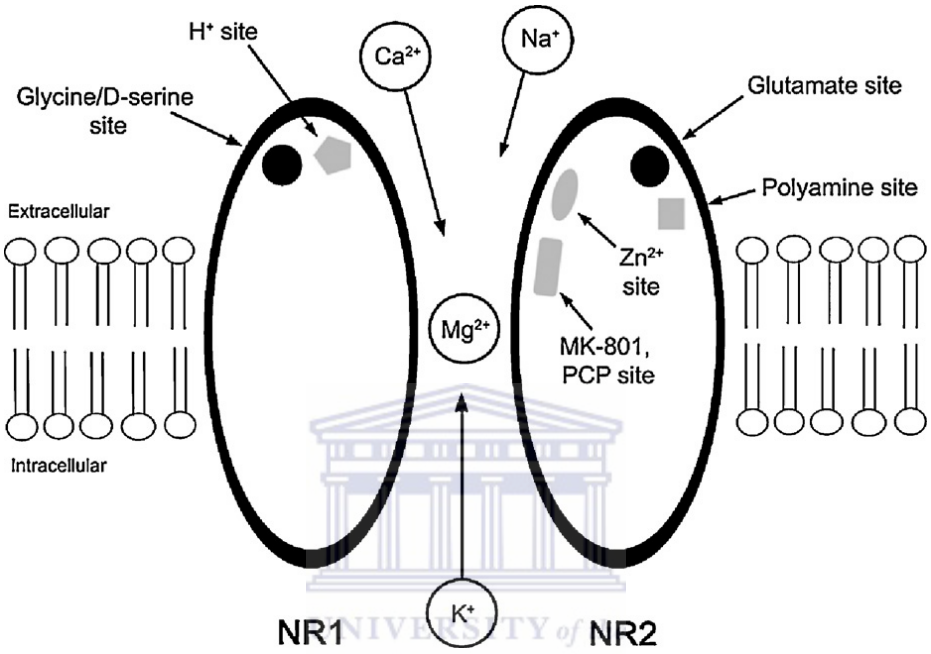


Figure 2.2: Cross-sectional structure of the NMDA receptor showing one NR1 and one NR2 subunit, as well as binding sites for a variety of mediators (Labrie & Roder, 2010). Activation of the receptor requires membrane depolarisation, as well as the binding of glutamate and glycine to their respective binding sites. Once the membrane is depolarised, the voltage-dependant magnesium ion block is displaced. On activation of the channel, sodium and calcium ions permeate into the channel while potassium ions permeate out. The open NMDA channel may be blocked by magnesium ions, uncompetitive NMDA antagonists such as memantine, as well as non-competitive antagonists such as MK-801 (Parsons *et al.*, 2007; Labrie & Roder, 2010; Cioffi, 2013).

2.5 The lethal triplet

Various etiologies have been proposed for neurodegenerative disorders and it is evident that a number of processes, rather than any single one are responsible. One such collection of processes is known as the lethal triplet and it consists of excitotoxicity, oxidative stress and

mitochondrial dysfunction (fig. 2.3). It is believed that each of these three aspects either individually or collectively contribute to neurodegeneration (Greene & Greenamyre, 1996a; Alexi *et al.*, 1998).

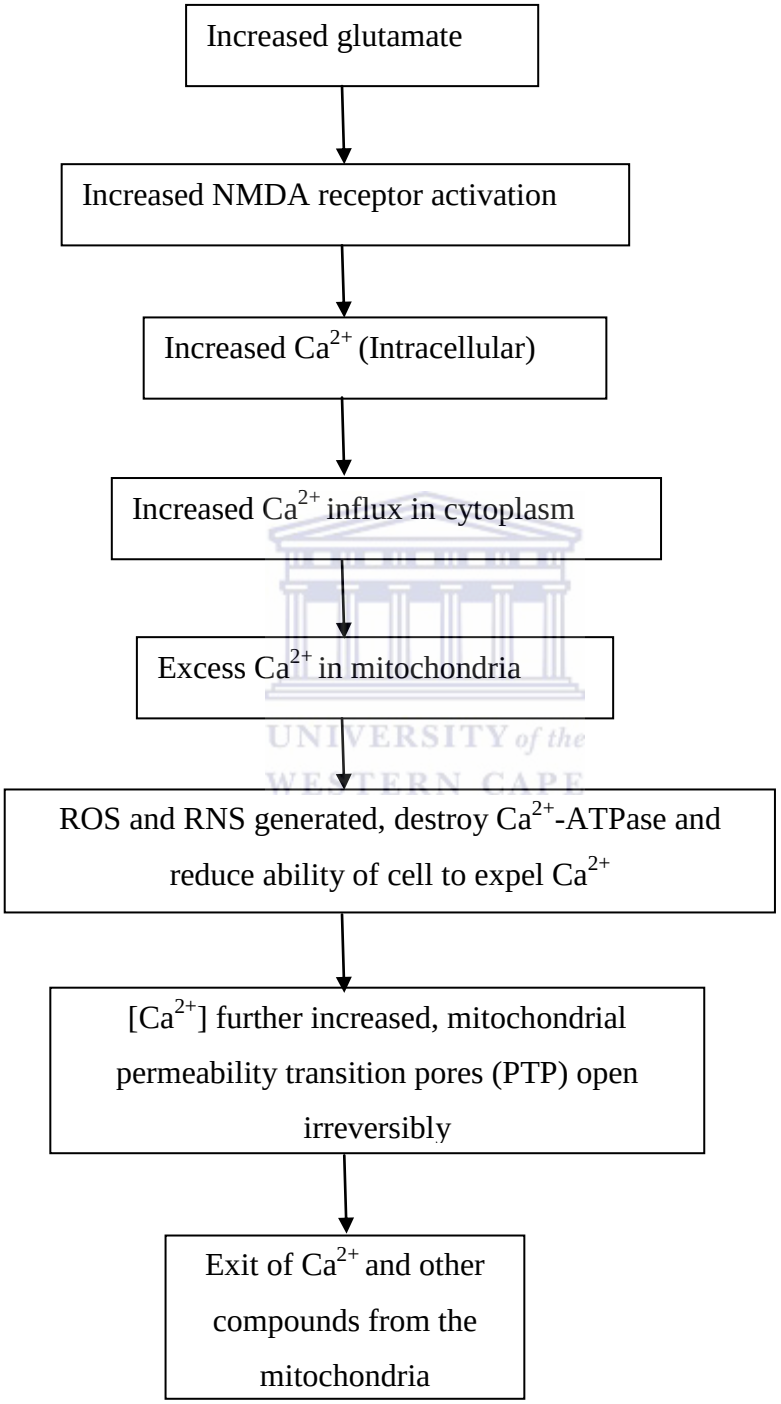


Figure 2.3: Relation of excitotoxicity, mitochondrial dysfunction and oxidative stress. Adapted from Green & Reed, 1998; Montal, 1998; Nicholls & Ward, 2000.

2.5.1 Excitotoxicity

Although calcium ions are important for cell growth, survival and physiological functioning, an excess is responsible for excitotoxicity, which can ultimately lead to neurodegeneration (Lynch & Guttman, 2002). Due to the involvement of NMDA receptors in physiological as well as pathological processes, it is of utmost importance that more selective drugs are developed (Yamakura & Shimoji, 1999).

Non-selective NMDAR channel blockers such as phencyclidine (PCP), MK-801 and ketamine (fig. 2.4) were amongst the first generation of NMDA receptor antagonists developed for the treatment of stroke and trauma, but were unsuccessful in clinical trials due to neurotoxicity and severe side effects (Woodruff *et al.*, 1987; Williams *et al.*, 2001; Wang & Shuaib, 2005; Chaffey & Chazot, 2008). MK-801 and PCP bind to the PCP binding site, which is located in the ion-channel pore at the NMDA receptor (Dingledine *et al.*, 1999).

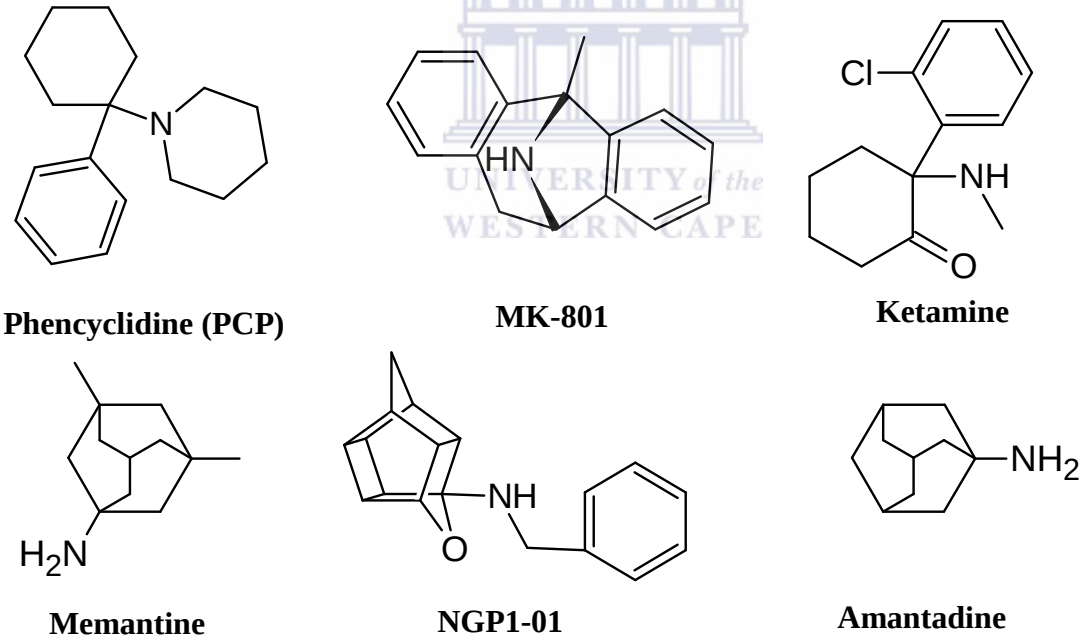


Figure 2.4: Chemical structures of NMDA receptor antagonists (channel blockers).

The blockade by amino-adamantanes is use-dependent, with a preference for the channel in the open state (Geldenhuys *et al.*, 2007). The amino-adamantane derivatives memantine and amantadine (fig. 2.4) display fast blocking or unblocking effects at NMDA receptor channels (fig. 2.5) and the block is only strong when there is sustained stimulation of the receptor, for

example during brain trauma or stroke. This is why they are better tolerated than MK-801 and phencyclidine, and are effective in the treatment of neurodegenerative disorders (Kornhuber *et al.*, 1994; Kornhuber & Weller, 1997).

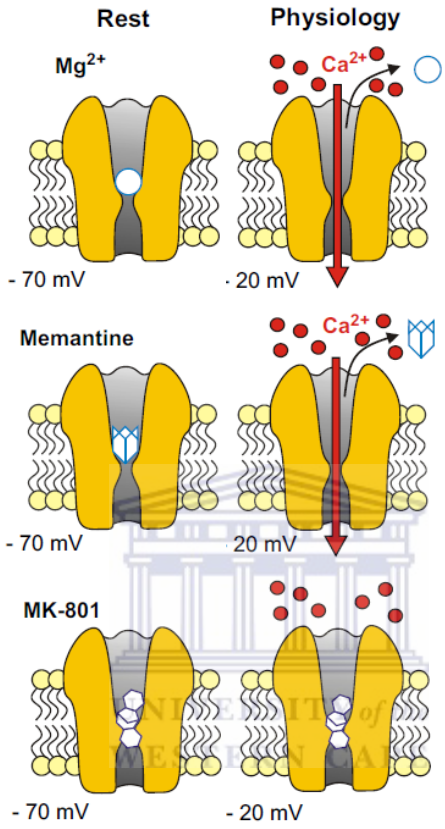


Figure 2.5: Diagram showing the fast unblocking kinetics of magnesium and memantine. Under resting therapeutic conditions (left), magnesium (top), memantine (centre) and MK-801 (bottom) all occupy the NMDA receptor. Under normal physiological circumstances, when depolarisation of the membrane occurs, both magnesium and memantine are able to leave the NMDA receptor channel due to their strong voltage-dependency and fast unblocking kinetics whereas MK-801 remains in the channel due to its slow unblocking kinetics (Kornhuber & Weller, 1997; Parsons *et al.*, 1999b; Parsons *et al.*, 2007).

2.5.2 Oxidative stress

Under normal conditions, a clear balance exists between generation of reactive oxygen species (ROS) such as hydroxide (OH^-), superoxide (O_2^-) and hydrogen peroxide (H_2O_2) as

by-products of cellular metabolism and their detoxification. However, under certain conditions, the production of these ROS exceeds their detoxification (fig. 2.3) thus leading to oxidative stress (Mamelak, 2007). Naarala and colleagues found that glutamate-induced oxidative stress takes place when cellular glutathione (GSH) levels are depleted. They also found that protein kinase C (PKC) may be involved in glutamate-induced production of ROS (Naarala *et al.*, 1995). While the brain contributes to a very low percentage of the total body weight, it is an organ that requires a very high supply of oxygen, approximately 25 % of the total inhaled oxygen, and is thus severely affected under conditions of oxidative stress (Perry *et al.*, 2002). Oxidative stress has been implicated in the development of all neurodegenerative disorders. Mismanagement of iron in the brain is responsible for neurodegenerative disorders through the generation of free radicals and ROS, which lead to the death of neurons. Iron chelators have shown neuroprotective effects both *in vivo* and *in vitro*. Neuroprotection due to iron chelation may be due to the reduction of iron related oxidative stress. The scavenging effect against the free radicals involved in neurodegeneration is one of the strategies for making compounds more neuroprotective (Xue *et al.*, 2011).

2.5.3 Mitochondrial dysfunction/metabolic compromise

Mitochondria are responsible for adenosine triphosphate (ATP) production as well as its supply to cells *via* oxidative phosphorylation (OXPHOS). Since brain tissue has a high energy demand, normal functioning is dependent on adequate ATP supply from the mitochondria. Inadequate supply may alter energy metabolism and this may lead to neurodegeneration (Federico *et al.*, 2012). Mutations in mitochondrial DNA (mtDNA) and pathological free radical reactions may impair the electron transport chain (ETC). ETC defects would lead to a decrease in ATP production and an increase in production of free radicals by blocking the transfer of electrons down the chain and, subsequently, their reduction to molecular oxygen and water (Cassarino & Bennett Jr., 1999). In order to enter the mitochondrial matrix, calcium ions are required to cross two boundaries, the outer mitochondrial membrane (OMM) which is permeable to ions and proteins with a molecular weight less than 10 KDa due to the voltage-gated anion channel, and the inner mitochondrial membrane (IMM) which is impermeable to ions, but the activity of respiratory chain complexes generates an electrochemical gradient (known as the mitochondrial membrane

potential, -180mV), which provides a driving force for calcium ion entry. Excess calcium ion entry (due to excitotoxicity or any other factor) causes a collapse in the mitochondrial membrane potential which leads to a decrease in cellular ATP and release of apoptosis inducing factors. These factors lead to an increase in intracellular calcium concentration, resulting in irreversible opening of the mitochondrial permeability transition pores (PTP). The mitochondrial PTP is a non-selective ion channel that is dependent on intracellular calcium concentration, and inhibited by cyclosporine A. While brief opening of this channel allows rapid calcium ion release, prolonged openings (caused by apoptotic factors) lead to structural alterations and subsequent release of caspase co-factors such as cytochrome C, which lead to cell death (Montal, 1998; Celsi *et al.*, 2009).

N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a powerful neurotoxin that causes neuronal degeneration in the substantia nigra pars compacta of mammals (Langston *et al.*, 1984) and it is used to induce an experimental form of PD in laboratory animals. MPTP binds to MAO-B with a high affinity and is then converted to *N*-methyl-4-phenylpyridine (MPP^+). MPP^+ is transported into DA neurons *via* the DA transporter. It accumulates in DA neurons, thereby leading to their destruction (Javitch *et al.*, 1985). MPP^+ inhibits the mitochondrial electron transport chain *via complex 1* inhibition (Obata, 2006).

2.6 Nitric oxide and NOS

Nitric oxide (NO) is an endogenous enzyme which is involved in a variety of physiological processes aimed at maintaining homeostasis. NO is a highly permeable gas which is able to easily diffuse across biological membranes (Cavas & Navarro, 2006). It is synthesised from *L*-arginine (fig. 2.6) by an enzyme known as nitric oxide synthase (NOS). Three different isoforms of mammalian NOS have been cloned, namely: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS is involved in the formation of NO in blood vessels, which leads to vasodilation. nNOS is involved in the synthesis of NO in the central nervous system, while iNOS promotes NO synthesis in lymphatic tissue, which leads to immune responses (Schumann *et al.*, 2001). Although NO is produced by nearly all human tissues, physiologically its concentration is highest in the CNS (Koppenol & Traynham, 1996).

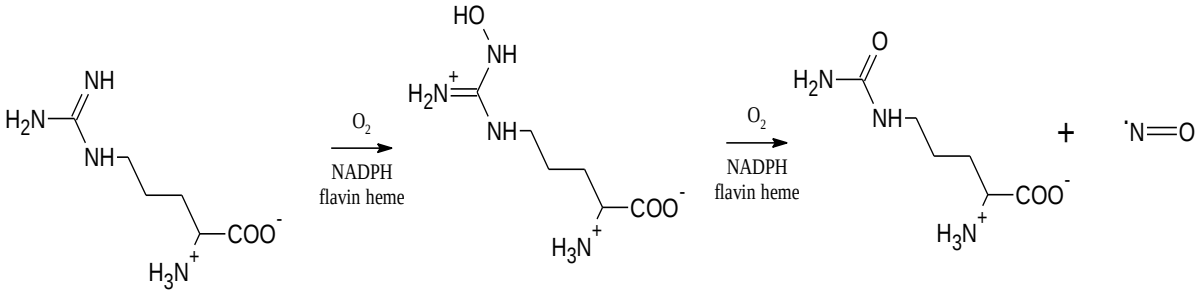


Figure 2.6: Synthesis of nitric oxide (NO) from *L*-arginine. Adapted from Erdal *et al.*, 2005

The neuronal and endothelial isoforms of NOS are expressed constitutively and depend on calcium ion concentration, whereas the inducible isoform (iNOS) is independent of calcium concentration (Collins *et al.*, 1998). Once iNOS is induced, it remains active for several hours to days and produces nitric oxide in quantities 1000 times greater than nNOS (Singh & Dikshit, 2007).

Each subunit of NOS contains three distinct domains (fig. 2.7): a c-terminal reductase domain, a calmodulin binding domain, and an n-terminal oxygenase domain. The reductase domain binds flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADPH) and flavin mononucleotide (FMN). The calmodulin binding domain binds the calcium calmodulin, and the oxygenase domain binds tetrahydrobiopterin (BH_4), heme and *L*-arginine. The oxygenase domain catalyses the conversion of *L*-arginine to *L*-citrulline and NO (Conti *et al.*, 2007). The calmodulin binding site connects the reductase and oxygenase domains (Kavya *et al.*, 2006).

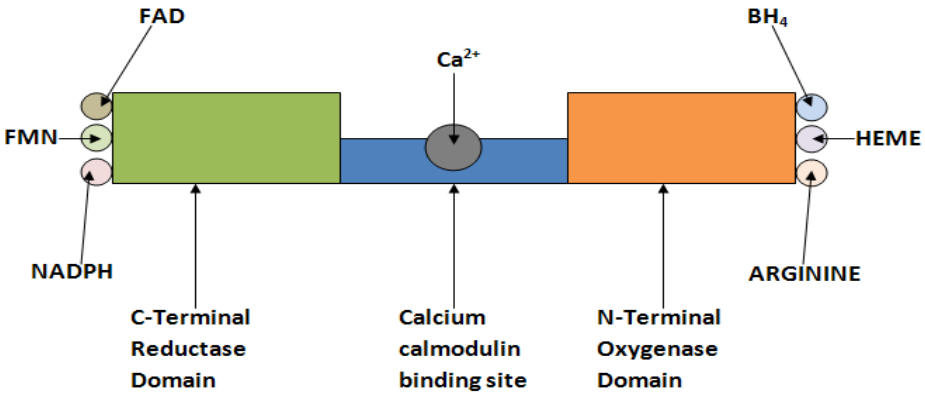


Figure 2.7: NOS homodimer showing the various co-factors and their respective binding sites. The conversion of *L*-arginine to *L*-citrulline and NO occurs at the oxygenase domain.

The NOS isoforms belong to the cysteine-coordinated heme protein(s) family. In this family, the proximal ligand to the heme-iron (heme-Fe^{2+}) complex is the sulfur atom of an intrinsic cysteine residue (White & Marletta, 1992; McMillan & Masters, 1995; Richards *et al.*, 1996; McMillan et al., 1996). *L*-arginine binds above the heme-Fe^{2+} atom, while BH_4 binds from the side of heme (fig. 2.8). The *L*-arginine and BH_4 are linked through inter-connected hydrogen bonds *via* one of the two heme-propionate groups (Rousseau *et al.*, 2005).

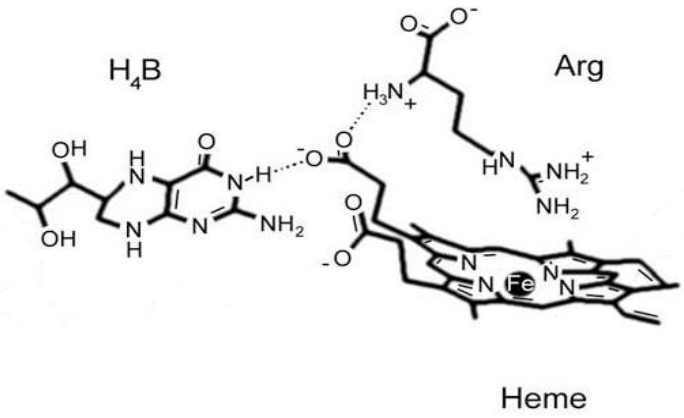


Figure 2.8: Linking of *L*-arginine and BH_4 through hydrogen bonds *via* the heme-propionate group (Daff, 2010). *L*-arginine (right) and BH4 (left) are linked through inter-connected hydrogen bonds *via* a heme-propionate group. *L*-arginine binds in such a way, so as to allow the guanidine group to be adjacent to the ferric iron of heme.

NOS catalyses production of NO *via* the oxidation of one of the guanidine groups of *L*-arginine. This occurs through the oxidation of NADPH and the reduction of molecular oxygen. The process takes place at a catalytic site which is adjacent to the *L*-arginine binding site. The catalytic site which is on the oxygenase domain, also has an iron-containing heme group. FAD and FMN assist in the transfer of electrons from NADPH to the catalytic site (fig. 2.9). The binding of *L*-arginine occurs in such a way that the guanidine group is adjacent to the ferric iron of heme (fig. 2.8). The iron is reduced by an electron from NADPH and binds molecular oxygen. The molecular oxygen is cleaved, releasing one atom as water while the other is incorporated in the terminal guanidino nitrogen of *L*-arginine to yield hydroxyarginine. Activation of another oxygen molecule causes further oxidation of hydroxyarginine to produce water, NO and *L*-citrulline. Calmodulin binding is thought to be essential as it controls the transfer of electrons from flavin to heme, possibly by causing a re-orientation of the reductase and oxygenase domains (thus making electron transfer between them possible). For nNOS and eNOS, calmodulin activity is dependent on calcium entry. In iNOS however, calmodulin is bound as a prosthetic group and activation is thus independent of calcium (Marletta, 1988; Marletta, 1993; Southan & Szabó, 1996).

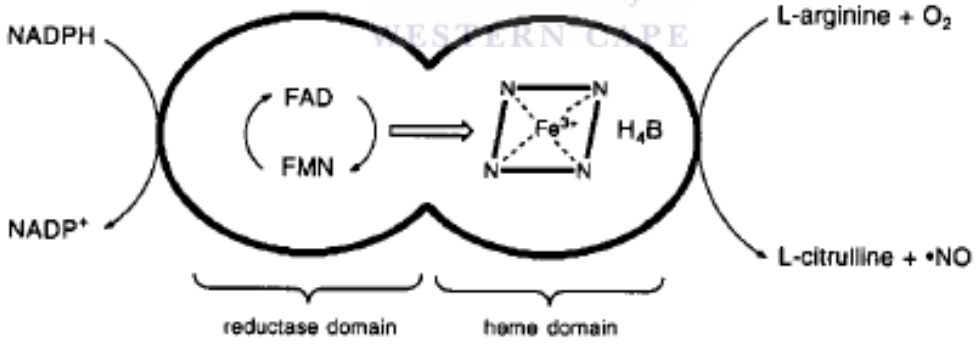


Figure 2.9: Electron transfer pathway in the synthesis of NO (Marletta, 1993). FAD and FMN assist in the transfer of electrons from NADPH to the catalytic site (heme domain).

The biological effects of NO were first described in 1977 (SoRelle, 1998). In the CNS, NO is involved in brain development, memory, learning and modification of pain perception (Erdal *et al.*, 2005) and is present in the cerebellum, hypothalamus, striatum, hippocampus and medulla oblongata (Zhang *et al*, 2006). Due to the free radical properties of NO, an excess amount has been implicated in the development of stroke and neurodegenerative disorders

such as AD, PD and Huntington's disease. Selective inhibition of nNOS (over iNOS and eNOS) may be beneficial in the treatment of neurodegenerative disorders (Lawton *et al.*, 2009). Both excess and deficient tissue NO concentrations have been implicated in the development of neurodegenerative disorders (Low, 2005) and several other disease states (Erdal *et al.*, 2005). At higher concentrations, NO has a pro-apoptotic effect whereas, at physiological concentrations, it has anti-apoptotic effects. Peroxynitrite ($ONOO^-$) has a pro-apoptotic effect and is produced in greater quantities when there is more NO available (Singh & Dikshit, 2007).

Przedborski and colleagues found that 7-nitroindazole (7-NI, a selective nNOS inhibitor, table 2.1) protected mice from the neurotoxic effects of MPTP. Through their experiments on mice lacking the nNOS gene, they found that nNOS may be responsible (at least in part) for MPTP-induced neurotoxicity (Przedborski *et al.*, 1996). Rose and colleagues also found similar results (Rose *et al.*, 1999).

Hicks and colleagues (1999) carried out experiments to evaluate the effect of administering MK-801 (fig. 2.4) in combination with NOS inhibitors 7-NI or ARL17477 (table 2.1) in the gerbil model of cerebral ischemia. They found that administration of MK-801 with 7-NI provided a greater degree of neuroprotection (44.5 %) compared to administering either of them alone (20 % and 10 %, respectively). The degree of neuroprotection was also greater when MK-801 was administered together with ARL17477 (78 %) as opposed to administering the inhibitors individually (26 % and 8 %, respectively). The combination also enabled them to administer lower doses of the inhibitors. The enhanced neuroprotection was due to a synergistic effect, where the NMDA receptor antagonist would inhibit calcium ion entry and thus reduce NOS production (fig. 2.10). The NOS inhibitor will also reduce the synthesis of NOS and thus NO (Hicks *et al.*, 1999).

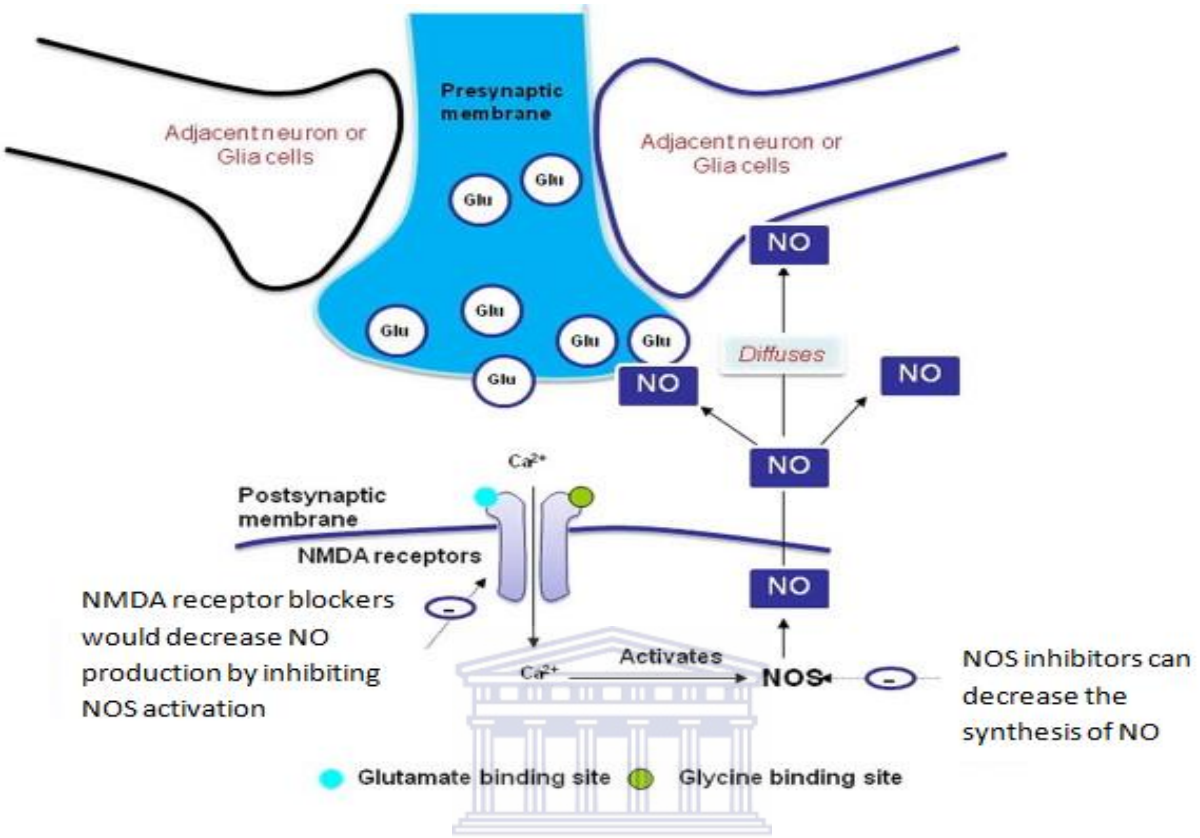


Figure 2.10: Role of glutamate, NMDA receptors and calcium in NOS activation. Adapted from Dhir & Kulkarni, 2011.

The cell death that results from neurodegenerative disorders may be difficult to prevent or treat with the drugs currently available, as they target one specific pathway or have just one mechanism of action (Van der Schyf & Geldenhuys, 2009). It is therefore logical to develop compounds that act through different mechanisms so as to act on more than one pathway to have a synergistic effect.

2.7 NOS inhibitors

NOS inhibitors can be divided into three broad categories:

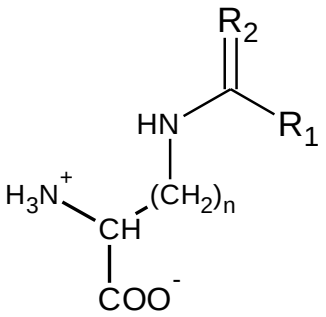
1. *L*-arginine based inhibitors
2. Other amino acid-based inhibitors of NOS (*L*-citrulline and *L*-lysine derivatives)
3. Non-amino acid-based inhibitors of NOS (amidines, guanidines, isothioureas, imidazoles and indazoles) (Southan & Szabó, 1996)

NOS inhibitors with less than ten-fold selectivity should be regarded as non-selective because they may still affect another isoform to a large extent. Those with ten- to fifty-fold selectivity can be regarded as partially selective, while those with greater than fifty-fold selectivity can be regarded as selective, as they are less likely to affect other isoforms (Alderton *et al.*, 2001).

Since the discovery that *L*-arginine was a substrate for NOS, there has been a gradual transformation in the NOS inhibitors synthesised over the years (Erdal *et al.*, 2005). The first reported NOS inhibitors were analogs of *L*-arginine (fig. 2.11) including, but not limited to N^G-methyl-*L*-arginine (*L*-NMMA) and N^G-nitro-*L*-arginine (*L*-NNA) (Collins *et al.*, 1998). *L*-arginine inhibitors were designed to mimic the endogenous binding of *L*-arginine to its active site. The problem is that crystal structures of iNOS and eNOS bound to inhibitors revealed highly similar active sites (Fischmann *et al.*, 1999). Although the inhibitors had IC_{50} values in the low micro molar range, they displayed very little or no selectivity for one isoform over the other. However, data showed that elongated *L*-arginine analogs would offer good selectivity by protruding out of the *L*-arginine binding pocket and interacting with other conserved residues (Paige & Jaffrey, 2007). Erdal and colleagues (2005) found that *L*-NMMA and *L*-NNA were more potent inhibitors of nNOS and eNOS compared to iNOS.

Most of the early NOS inhibitors (analogs of *L*-arginine) showed moderate potency, but very poor selectivity for various isoforms of NOS and are not suitable where selective inhibition of a particular isoform is required (Yoon *et al.*, 2011).

While many different NOS inhibitors have been synthesised, the main problems still appear to be selectivity for one isoform over the others as well as crossing the blood brain barrier (BBB). This impacts on the pharmacokinetic properties of the drug, thereby reducing its activity in the CNS (Prins *et al.*, 2009). Since NO is involved in regulatory processes in various tissues in the body, selective inhibition of one particular isoform over the others is of extreme importance in order to avoid complications (Collins *et al.*, 1998). If for example an inhibitor is not selective for nNOS and inhibits eNOS as well, it will cause a change in the blood pressure homeostasis of the body and could end up being harmful to the patient (Erdal *et al.*, 2005). The challenge for medicinal chemists is to design compounds with a balance between good potency and good pharmacokinetic parameters (Masic, 2006).



	R_1	R_2	n
***L*-arginine**	NH_2	NH	3
***L*-NMA**	NH_2	NCH_3	3
***L*-NA**	NH_2	NNO_2	3
***L*-NAME**	NH_2	NNO_2	3
***L*-citrulline**	NH_2	O	3
***L*-thiocitrulline**	NH_2	S	3
***L*-hydroxyarginine**	NH_2	NOH	3

Figure 2.11: General structure of the *L*-arginine based inhibitors. (Southan & Szabó, 1996).

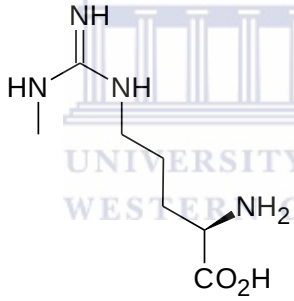
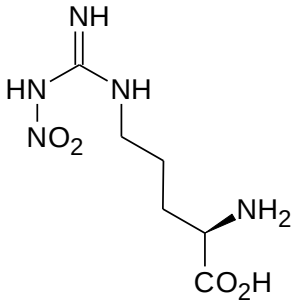
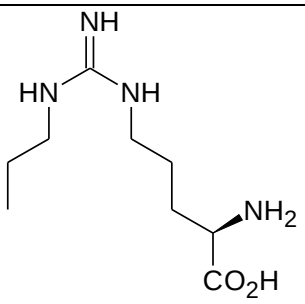
L-N^G-nitro-arginine methyl ester (L-NAME) is a non-selective inhibitor of NO, as it has activity at all three NOS isoforms (Resink *et al.*, 1996). It also has activity at other sites and has been reported to cause an increase in blood pressure (Umans & Levi, 1995) as well as anti-muscarinic effects (Buxton *et al.*, 1993). *L*-NMMA has been shown to cause dose-dependent bradycardia and hypertension, although it has no effect on muscarinic receptors (Gardiner *et al.*, 1990). *L*-NMMA can be converted to *L*-citrulline and subsequently to *L*-arginine (Hecker *et al.*, 1990). $N^{(\omega)}$-Propyl-*L*-arginine (*L*-NPA, **1c**) is an *L*-arginine-related molecule, but differs from other such inhibitors due to substitution of the guanidine group with a short hydrocarbon chain. This improves its selectivity for nNOS, since *L*-NPA displays 149-fold and 3158-fold selectivity over eNOS and iNOS, respectively (Zhang *et al.*, 1997; Huang *et al.*, 1999).

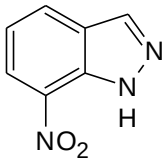
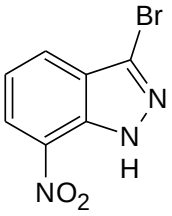
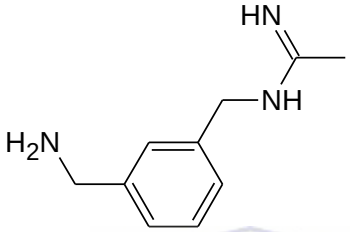
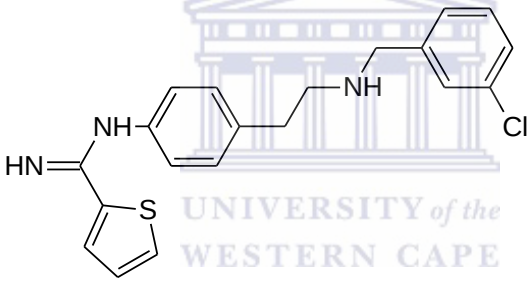
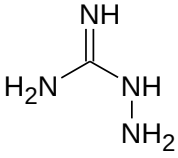
Generally, the indazole derivatives display good selectivity towards nNOS and cause minimum cardiovascular effects as a result of this selectivity (Moore *et al.*, 1993). 7-NI however, has been shown to have cardiovascular effects by decreasing heart rate (Kelly *et al.*, 1995). 3-Bromo-7-Nitroindazole has shown a twenty-fold higher selectivity for nNOS when compared to 7-NI (Bland-Ward *et al.*, 1994; Bland-Ward & Moore; 1995).

1400W, a highly selective iNOS inhibitor displays approximately 32-fold and 4000-fold selectivity over nNOS and eNOS, respectively. It binds in the *L*-arginine binding pocket and

interacts with Glu-371 and two heme-propionate moieties. Although it has the same interactions with nNOS, it seems to bind more tightly to iNOS as a result of the conformational restriction of glutamate residues in nNOS (Raman *et al.*, 2001; Fedorov *et al.*, 2003; Paige & Jaffrey; 2007). ARL-17477 (also known as AR-R17477) is an isothiourea derivative which shows good selectivity for nNOS over other isoforms. This selectivity is apparently due to a single residue difference, Leu-337 (which corresponds to Asn-115 in iNOS) that stabilizes the chlorophenyl moiety of ARL-17477 (Federov *et al.*, 2004; Paige & Jaffrey, 2007).

Table 2.1: List of some NOS inhibitors and their IC_{50} values (Alderton *et al.*, 2001; Bland-Ward & Moore, 1995; Zhang *et al.*, 1997; Valance & Leiper, 2002).

Inhibitor	Structure	IC_{50} values (μM)		
		iNOS	nNOS	eNOS
L-NMMA		6.6	4.9	3.5
L-NNA		3.1	0.29	0.35
N^{ω}-Propyl-*L*-arginine (*L*-NPA)		180 (K_i)	0.06 (K_i)	8.5 (K_i)

7-Nitroindazole		9.7	8.3	11.8
3-Bromo-7-nitroindazole		0.29	0.17	0.05
1400W		0.23	7.3	1000
ARL 17477		0.33	0.07	1.6
Aminoguanidine		31	170	330

2.8 Polycyclic amines

The first polycyclic cage compound discovered was amantadine (fig. 2.4). A large amount of interest in the pharmacology of the polycyclic cage compounds was generated when scientists discovered that 1-amino-adamantane or amantadine exhibited anti-viral activity against influenza, hepatitis C and herpes zoster neuralgia (Geldenhuys *et al.*, 2005). While treating an influenza patient who coincidentally suffered from PD, it was found that amantadine showed anti-parkinsonian activity (Danysz *et al.*, 1997; Danysz & Parsons, 1998). Amantadine can be used for short term treatment of certain patients with PD (Danysz *et al.*,

1997). It showed a stimulating effect on the dopaminergic system and inhibitory effect on the NMDA receptor (Spasov *et al.*, 2000). Stoof and colleagues (1992) found that the concentration of amantadine required to block NMDA receptors was much lower than that required to stimulate DA release, indicating that low dose amantadine may be useful in combination with DA agonists for a synergistic effect.

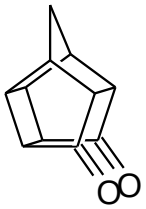


Figure 2.12: Chemical structure of pentacyloundecane (PCU), a useful drug scaffold that helps improve the lipophilicity of conjugated privileged molecules thereby enabling them to cross the BBB easily.

After the discovery of memantine (fig. 2.4), there was an increased interest regarding the role of cage compounds in neuroprotection (Parsons *et al.,* 1998). Memantine, an uncompetitive NMDA receptor antagonist, is the 3,5-dimethyl derivative of amantadine, and is used for the treatment of moderate to severe AD (Danysz & parsons, 2003; Geldenhuys *et al*, 2005). Memantine primarily blocks the NMDA glutamate receptor and reduces the calcium influx into cells thus reducing excitotoxicity and exerting a neuroprotective effect (Lockman *et al.*, 2012).

Research on the polycyclic cage compounds, pentacycloundecanes (PCU, fig. 2.12) as well as the adamantane group, revealed that these compounds are highly permeable across the BBB. The polycyclic cage is useful as a drug scaffold and also improves the lipophilicity of a conjugated privileged molecule, which enables it to cross the BBB easily (Geldenhuys *et al.*, 2005). Polycyclic compounds have been useful for the chemical and structural manipulation in the design of numerous multifunctional drugs (Van der Schyf & Geldenhuys, 2009).

NGP1-01 (8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane, fig. 2.4) was first prepared by Sasaki and colleagues (1971 & 1974). However, since no pharmacological activity of this compound was reported by the original authors, it was developed and its calcium antagonistic activity was first reported by Van der Schyf and colleagues (Van der Schyf *et al.*, 1986; Kiewert *et al.*, 2006). Although it was initially described as an *L*-type calcium channel antagonist (Van der Schyf *et al.*, 1986), Kiewert and colleagues (2006)

through their experiments found that NGP1-01 simultaneously blocks both the neuronal voltage-gated calcium channel and the NMDA receptor with potency slightly less than that of memantine. They also found, *via* the use of *in-vivo* microdialysis experiments, that NGP1-01 is significantly brain-permeable (Kiewert *et al.*, 2006). It may also prevent iron-induced oxidative damage by blocking excess iron uptake *via* the VGCC (Lockman *et al.*, 2012).

While carrying out experiments on NGP1-01, Geldenhuys and colleagues found that compounds with *meta*- substitution of nitro- and methoxybenzylamino moieties were more potent inhibitors of calcium channels than the *para*- substituents. They also found that the addition of bulky substituents to the nitrogen atom increases calcium channel blocking activity for polycyclic cage compounds (Geldenhuys *et al*, 2005; Van der Schyf & Geldenhuys, 2009). The polycyclic cage structures (NGP1-01 and memantine) are thought to have a dual mechanism of action, that include:

1. Antagonism at the NMDA receptor, thereby preventing a large influx of calcium ions
2. Direct blockade of *L*-type calcium ion channels (Geldenhuys et al., 2003).

The actions of NGP1-01 include voltage dependent calcium channel blockade, with a preference for the channel in the 'open state' (Van der Schyf & Geldenhuys, 2009). Calcium influx is regulated through a combination of voltage-gated calcium ion channels as well as the NMDA receptors. The pentacycloundecylamines are believed to have neuroprotective effects through the modulation of voltage-gated sodium, potassium and calcium ion channels, as well as NMDA receptor ion-channels (Grobler *et al.*, 2006). A single agent possessing both calcium channel inhibitory activity as well as NMDA receptor antagonism would be of great use for the treatment of neurodegenerative disorders (Hao *et al.*, 2007). NGP1-01 and other polycyclic amines seem to fit this description, and may serve as lead compounds for the synthesis of multifunctional neuroprotective agents.

2.9 Conclusion

As outlined in this chapter, multiple mechanisms are thought to play a part in the neurodegenerative cascade. Processes such as excitotoxicity, mitochondrial dysfunction, oxidative stress as well as an excess of nitric oxide act either individually or collectively to activate the neurodegenerative cascade, ultimately leading to the death of neuronal cells. While determining effective therapeutic strategies, it would be logical and effective to concentrate on the synthesis of compounds that will act at multiple points in the

neurodegenerative cascade and arrest the progression of these disorders or reverse the neuronal damage altogether.

One such group of compounds, the polycyclic amines such as amantadine, memantine and the PCU derivative, NGP1-01, have shown good inhibitory activity against NMDA-mediated calcium influx and NGP1-01 has also shown good inhibitory activity against VGCC. These agents are also highly lipophilic and enable effective passage of privileged moieties connected to the amantadine or PCU scaffolds across the blood brain barrier. This would thus improve the pharmacokinetic as well as pharmacodynamic characteristics of the resulting compounds.

Combining the structural features of the polycyclic cage structures with other functional groups may provide compounds that would indeed act *via* multiple modes of action thereby addressing the multifactorial nature of neurodegenerative disorders.

CHAPTER 3

GENERAL SYNTHETIC PROCEDURES

3.1 Introduction

A series of compounds were synthesised by conjugating the amantadine moiety to a phenyl linker with different functional groups (-NO_2, -NH_2, -$NHC(NH)NH_2$, -OCH_3) attached at the *ortho*, *meta* and *para* positions of the phenyl moiety. The compounds were characterised by nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared (IR) spectroscopy. The synthetic pathways, procedures, as well as the problems encountered are stated.

3.2 Instrumentation

3.2.1 Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) spectra were recorded on a Gemini Varian 200 instrument at 200 MHz. Deuterated chloroform was used as NMR solvent and tetramethylsilane (TMS) as internal standard. The chemical shifts were reported in (δ) parts per million (ppm). The following abbreviations are used to describe the multiplicity of the respective signals: s-singlet, bs-broad singlet, d-doublet, dd-doublet of doublets, t-triplet, q-quartet, m-multiplet and Ar-aromatic.

3.2.2 Mass Spectrometry (MS)

Mass spectra were recorded on a Perkin Elmer Flexar SQ 300 MS using direct infusion electro-spray ionisation mass spectrometry (DI-ESI-MS).

3.2.3 Infrared (IR) spectroscopy

Infrared spectra were recorded on a Perkin Elmer Spectrum 400 spectrophotometer fitted with a diamond attenuated total reflectance (ATR) attachment.

3.3 Chromatographic techniques

3.3.1 Thin Layer Chromatography (TLC)

All reactions were monitored by TLC on 0.20 mm thick aluminium silica gel sheets (Alugram® SIL G/UV254, Kieselgel 60, Macherey-Nagel, Düren, Germany). Mobile phases were prepared on a volume-to-volume (v/v) basis, using different ratios of ethyl acetate, dichloromethane (DCM), acetone and hexane. Where new mobile phases were required, the prism model of Nyiredy *et al.* (1985) was used. Visualization was achieved using ultraviolet (UV) light at 254 nm and 366 nm using a Chromato-vue® cabinet, iodine vapours and a 2 % ninhydrin solution.

3.3.2 Column chromatography

Separation and purification of mixtures was achieved using flash chromatography. Silica gel (0.063 – 0.2 mm) obtained from Merck® was used as the stationary phase.

3.4 Melting point determination

Melting points were determined using a Stuart SMP-300 melting point apparatus and capillary tubes. The melting points were uncorrected.

3.5 Microwave (MW) chemistry

A CEM Discover Labmate (model number 908010) fitted with the IntelliVent™ Pressure Control System and CEM's Synergy™ software was used for microwave (MW) synthesis.

Using MW chemistry, reactions are completed in minutes as opposed to hours and days. The reactions conducted *via* MW synthesis are reproducible because the heating is uniform, highly controlled and the temperature is also accurately controlled. Due to uniform heating throughout the reaction vessel, the synthesis proceeds uniformly and reaches completion simultaneously. The yields are generally higher and this method may provide a means for the synthesis of compounds which are not conventionally available. It is thus a method for the future and ideal for medicinal chemists, as it enables us to optimise reactions much faster than using conventional heating methods (England, 2003).

3.6 Synthesis of selected compounds

3.6.1 Reagents

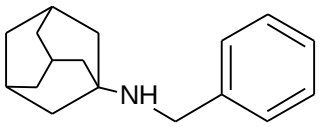
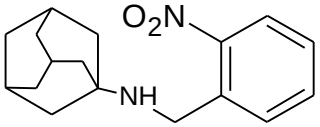
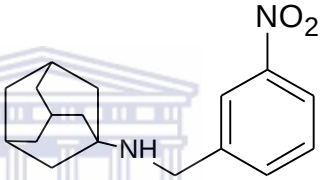
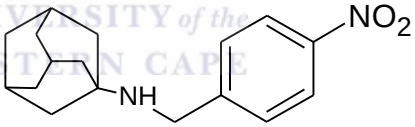
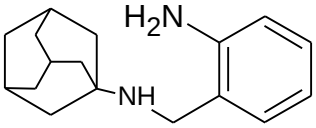
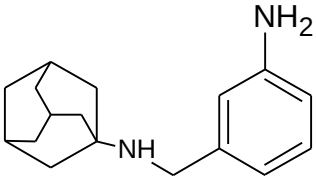
Unless otherwise specified, materials were obtained from Sigma Aldrich (South Africa) or Merck (Darmstadt, Germany) and were used without further purification. Solvents were dried using standard methods.

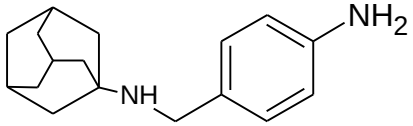
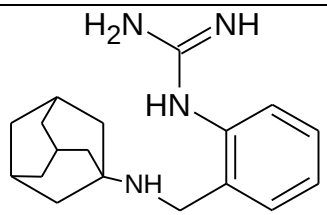
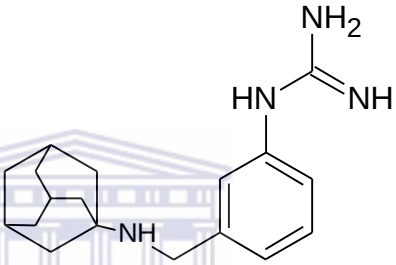
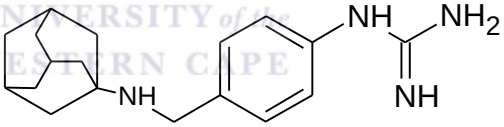
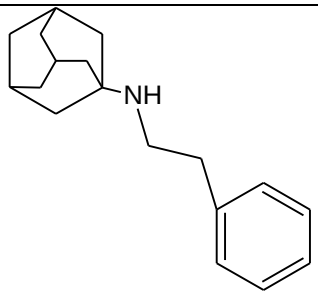
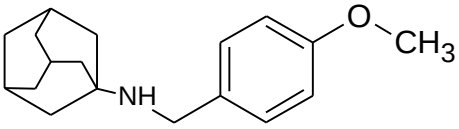
3.6.2 Structures proposed for this study

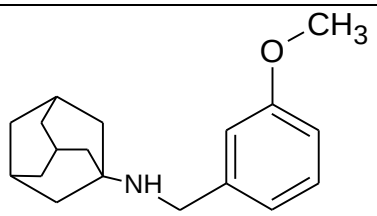
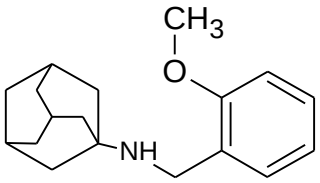
The first NOS inhibitors that were successfully synthesised were analogs of *L*-arginine (see section 2.6). The presence of the guanidine group in *L*-arginine, as well as inhibitors such as *L*-NMMA and *L*-NNA is thought to be responsible for the interactions with the *L*-arginine active site. However, since the active site of nNOS is similar to that of iNOS and eNOS, these inhibitors lacked selectivity for one isoform over the others. As was mentioned in the previous chapters, novel guanidines attached to an amantadine moiety *via* a benzene linker (fig. 3.1) would significantly improve their BBB permeability (since amantadine is known to be highly permeable across the BBB), as well as their selectivity for nNOS over eNOS and iNOS. The compounds are also speculated to have NMDA and calcium channel inhibitory activity, due to their structural similarity to NGP1-01 which blocks both NMDA receptor- and voltage-gated calcium channel (VGCC) mediated calcium influx. Compounds SE-1 and SE-11 (table 3.1) were to be synthesised in order to observe the effect of increasing chain length on the structure activity relationships of the new compounds. Geldenhuys and colleagues (2004, 2007) observed through their experiments on NGP1-01 and its phenethyl derivative that an increase in the chain length of the linker led to an improvement in NMDAR- and VGCC-mediated calcium influx. In order to observe the effect of an increase in chain length, SE-11 was included for synthesis and evaluation.

Compounds containing a methoxy and/ or nitro substituent(s) have also been shown to possess free radical scavenging activity (Rice-Evans et al., 1997). As a result, a group of methoxy compounds (SE-12, SE-13 and SE-14; table 3.1) were to be synthesised.

Table 3.1 Names and structures of compounds relevant to this study

ASSIGNED NAME	STRUCTURE & NAME
SE-1	 *N*-benzyltricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-2	 *N*-(2-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-3	 *N*-(3-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-4	 *N*-(4-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-5	 *N*-(2-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-6	 *N*-(3-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine

SE-7	 *N*-(4-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-8	 1-{2-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine
SE-9	 1-{3-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine
SE-10	 1-{4-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine
SE-11	 *N*-(2-phenylethyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-12	 *N*-(4-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine

SE-13	 *N*-(3-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine
SE-14	 *N*-(2-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine

3.6.3 Synthesis procedures and discussion

The general synthesis route followed was initiated through the conjugation of a nitrobenzyl bromide to the amantadine free base, followed by reduction of the nitro group to an amine, and subsequent conversion of the amine to a guanidine (fig. 3.1). In order to evaluate the effect of substitution at different positions on the aromatic ring, 2-, 3- and 4-nitrobenzyl bromide were used, to yield the *ortho*, *meta* and *para* products, respectively.

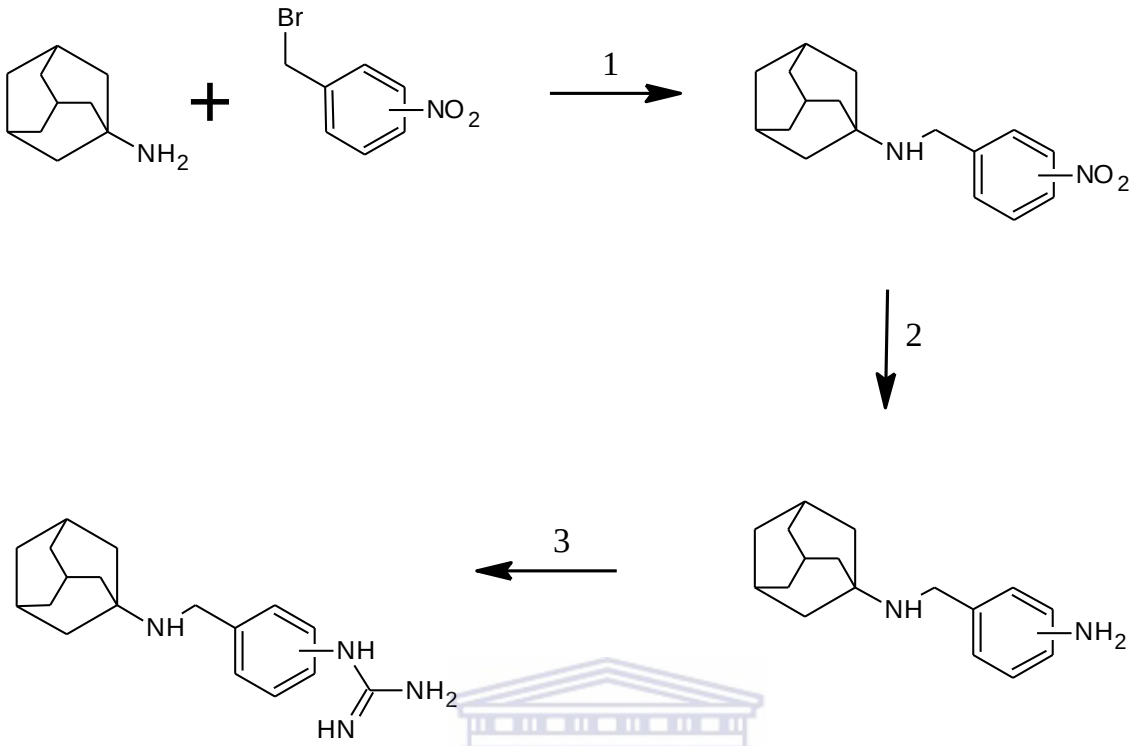


Figure 3.1: The general synthetic route that was followed. [1: K_2CO_3, Reflux; 2: Sn, HCl, EtOH; 3: S-methylisothiourea hemisulfate, 50 % EtOH, Reflux]

For the preparation of the amines (SE-5, SE-6 and SE-7), the respective nitro compounds had to be reduced (SE-2, SE-3 and SE-4, respectively). From literature (Annedi *et al.*, 2011), it was found that palladium on activated charcoal in the presence of ethanol and hydrogen gas can act as a suitable agent for the selective reduction of nitro compounds to amines. The reaction was thus conducted under these conditions (fig 3.2) and monitored *via* TLC (40 % EtOAc: 60 % Hexane as mobile phase).

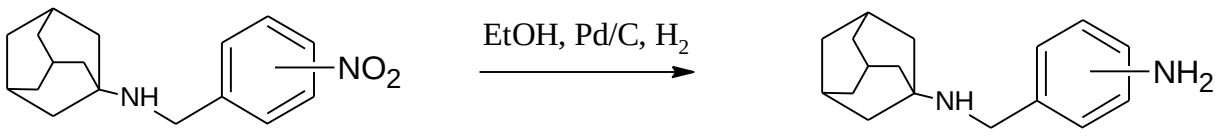


Figure 3.2: The initial conditions and reagents used to prepare SE-5, SE-6 and SE-7.

Although the reactant spot was disappearing (as per TLC analysis), the product spot (which was visible under ninhydrin) was forming at a higher R_f value than anticipated (above rather

than below the reactant spot). It is assumed that the reactant was breaking down, as the product is probably cleaved under these conditions (fig. 3.3).

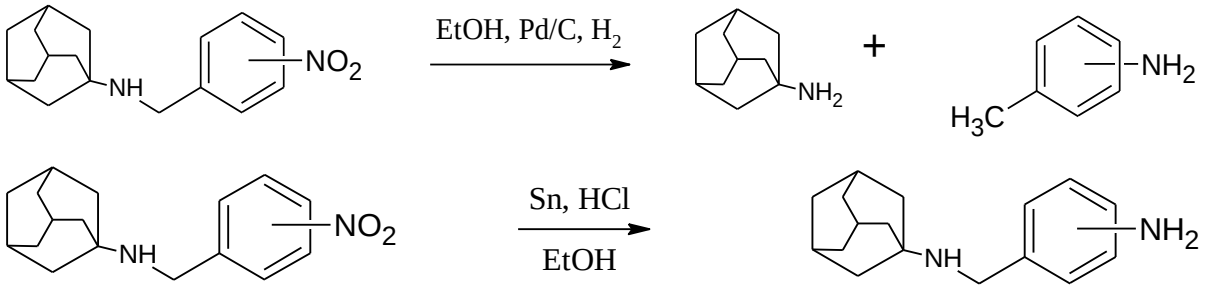


Figure 3.3: The reaction that is thought to actually occur under the proposed reductive conditions (top) and the reaction conditions that were subsequently adopted to perform the reaction (bottom).

The preparation of SE-8, SE-9 and SE-10 was unsuccessful. It is postulated that the guanidines (SE-8, SE-9 and SE-10) could not be prepared due to steric hindrance of the aromatic amines (SE5, SE-6 and SE-7), as well as the electronic deactivation of the aromatic amine by the amino-linker group which ultimately reduces the reactivity of the aromatic amine and thus prevent formation of the guanidine (Katritzky *et al.*, 2005).

SE-12 and SE-13 were synthesised using MW chemistry. However, the synthesis of SE-14 was unsuccessful. This could be as a result of steric hindrance (to the reaction of the amine group) due to the methoxy substituent being in the *ortho* position of the benzene ring. The starting compound used for the synthesis of SE-12 was 1-bromoadamantane.

SE-11 was synthesised using 2-bromoethylbenzene to provide the ethyl linker between the amantane amine and the benzene group as opposed to SE-1 which has a methyl linker (table 3.1).

3.7 Preparation and characterisation of compounds

3.7.1 Amantadine free base

Amantadine free base was prepared by adding 2 g of amantadine hydrochloride to water saturated with sodium bicarbonate. The precipitate was filtered and dried overnight at 40 ^{o}C, yielding the free base amantadine which was used in all further reactions.

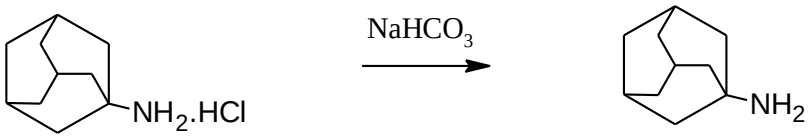


Figure 3.4: Preparation of amantadine free base from amantadine hydrochloride.

3.7.2 *N*-benzyltricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-1)

Amantadine free base (1.72 g, 11.37 mmol) and benzyl bromide (2.15 g, 10.34 mmol) was added to a round-bottomed flask followed by acetonitrile (10 ml) and potassium carbonate (2.15 g, 15.53 mmol). This mixture was refluxed for 24 hours. After 24 hours of refluxing, no compound was formed as per TLC analysis. The reaction was then placed in a microwave reactor under the following conditions; open vessel, 1 atm pressure, 110 ^{o}C, 250 W and 1 hour. After irradiating the mixture for 1 hour, it was filtered by vacuum filtration, to remove the potassium carbonate. The filtrate was collected and the excess solvent evaporated *in vacuo*. The reaction mixture was then acidified using 30 ml water which was made acidic with HCl until the pH was 3. This was followed by an extraction with DCM (3 x 30 ml). The combined aqueous layers were collected and made basic (pH = 12-14) using water saturated with NaOH and this was followed by a second extraction with DCM (3 x 30 ml). The combined organic layers were dried over $MgSO_4$, filtered through a sinter and the solvent evaporated *in vacuo* to yield the product as a light yellow oil. (Yield: 48.1 %, 0.241 g). ***Physical data:*** $C_{17}H_{23}N$; **mp:** oil; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 1) δ_H: 7.4-7.1 (m, 5H), 3.8-3.6 (s, 2H), 2.2-2.0 (m, 3H), 1.8-1.4 (m, 12H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 2) δ_C: 141.48, 128.37, 128.30, 126.69, 50.94, 45.09, 42.80, 36.73, 29.61; **MS** (DI-ESI-MS) (Spectrum 3) *m/z*: 242.12 (M^+), 243.15, 134.97, 78.94; **IR (ATR; cm^{-1})** (Spectrum 4)**:** 2900.94, 2845.96, 1451.37, 1146.48, 1097.40, 734.21, 694.60.

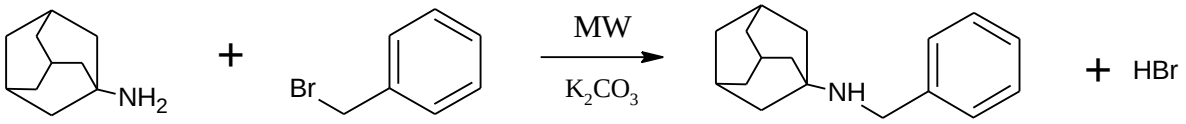
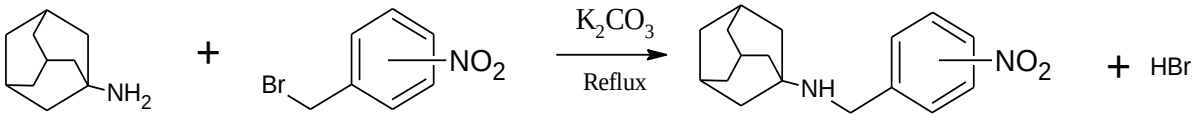


Figure 3.5: Preparation of SE-1.

3.7.3 *N*-(2-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-2)

Amantadine free base (1.72 g, 11.37 mmol) and 2-nitrobenzyl bromide (2.15 g, 10.34 mmol) was added to a round-bottomed flask followed by acetonitrile (10 ml) and potassium carbonate (2.15 g, 15.53 mmol). This mixture was refluxed for 24 hours. The reaction mixture was filtered by vacuum filtration to remove the potassium carbonate. The filtrate was collected and the excess solvent evaporated *in vacuo*. The mixture was purified *via* flash chromatography (using DCM: ethyl acetate: hexane in a ratio of 1:1:1 as eluent) to yield the product as a yellow solid. (Yield: 32.18 %, 0.161 g). ***Physical data:*** $C_{17}H_{22}N_2O_2$; **mp:** 94 ^oC; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 5) δ_H: 7.9-7.8 (dd, 1h), 7.7-7.5 (m, 2H), 7.4-7.3 (dd, 1H), 4.0-3.8 (s, 2H), 2.2-1.9 (m, 3H), 1.8-1.4 (m, 12H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 6) δ_C: 136.22, 133.29, 132.14, 127.84, 124.45, 51.14, 45.13, 42.40, 42.16, 36.56, 35.99, 29.62, 29.45; **MS** (DI-ESI-MS) (Spectrum 7) *m/z*: 287.54 (M^+), 288.56, 214.35, 135.15, 79.08; **IR (ATR; cm^{-1})** (Spectrum 8)**:** 2900.28, 2848.56, 1524.13, 1477.20, 1463.30, 1443.94, 1355.35, 1310.59, 1133.86, 1109.28, 1095.40, 867.22, 817.71, 792.16, 779.44, 739.59, 716.50.



NO_2 = *o* (SE-2), *m* (SE-3), *p* (SE-4)

Figure 3.6: Preparation of SE-2, SE-3 and SE-4.

3.7.4 *N*-(3-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-3)

Amantadine free base (1.72 g, 11.37 mmol) and 3-nitrobenzyl bromide (2.15 g, 10.34 mmol) was added to a round-bottomed flask followed by acetonitrile (10 ml) and potassium carbonate (2.15 g, 15.53 mmol). This mixture was refluxed for 24 hours. The reaction mixture was filtered by vacuum filtration to remove the potassium carbonate. The filtrate was

collected and the excess solvent evaporated *in vacuo*. The mixture was purified *via* flash chromatography (using DCM: ethyl acetate: hexane in a ratio of 1:1:1 as eluent) to yield the product as a yellow solid. (Yield: 54.48 %, 0.272 g). ***Physical data:*** $C_{17}H_{22}N_2O_2$; **mp:** 61 ^oC; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 9) δ_H: 8.3-8.2 (s, 1H), 8.1-8.0 (dd, 1H), 7.7-7.6 (dd, 1H), 7.5-7.4 (m, 1H), 3.9-3.8 (s, 2H), 2.2-2.0 (m, 3H), 1.8-1.4 (m, 12H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 10) δ_C: 144.28, 134.34, 129.08, 122.96, 121.71, 50.96, 45.06, 44.34, 42.93, 36.65, 36.04, 29.56; **MS** (DI-ESI-MS) (Spectrum 11) *m/z*: 287.55 (M^+), 288.56, 135.16, 79.08; **IR (ATR; cm^{-1})** (Spectrum 12)**:** 2898.34, 2847.10, 1524.46, 1476.32, 1463.34, 1359.83, 1309.98, 1133.50, 1095.21, 778.84, 738.99, 715.83.

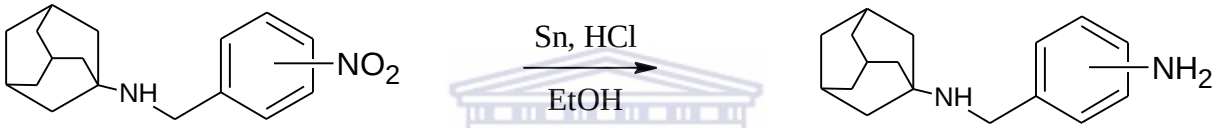
3.7.5 *N*-(4-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-4)

Amantadine free base (1.72 g, 11.37 mmol) and 4-nitrobenzyl bromide (2.15 g, 10.34 mmol) was added to a round-bottomed flask followed by acetonitrile (10 ml) and potassium carbonate (2.15 g, 15.53 mmol). This mixture was refluxed for 24 hours. The reaction mixture was filtered by vacuum filtration to remove the potassium carbonate. The filtrate was collected and the excess solvent evaporated *in vacuo*. The mixture was purified *via* flash chromatography (using DCM: ethyl acetate: hexane in a ratio of 1:1:1 as eluent) to yield the product as a yellow solid. (Yield: 56.7 %, 0.284 g). ***Physical data:*** $C_{17}H_{22}N_2O_2$; **mp:** 94 ^oC; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 13) δ_H: 8.1-8.0 (dd, 2H), 7.6-7.4 (dd, 2H), 4.0-3.8 (s, 2H), 2.2-2.0 (m, 3H), 2.6-2.4 (m, 12H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 14) δ_C: 149.95, 128.68, 123.48, 51.03, 44.47, 42.91, 36.62, 29.55, 29.84; **MS** (DI-ESI-MS) (Spectrum 15) *m/z*: 287.52 (M^+), 288.53, 248.53, 214.25, 135.11, 79.05; **IR (ATR; cm^{-1})** (Spectrum 16)**:** 2900.02, 2848.47, 1523.81, 1477.30, 1463.31, 1443.91, 1354.97, 1310.63, 1133.85, 716.57, 669.23.

3.7.6 *N*-(2-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-5)

Previously prepared *N*-(2-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-2, 0.15 g, 0.52 mmol) and tin powder (0.096 g, 0.79 mmol) was added to a round-bottomed flask and set up under reflux conditions. This was followed by addition of 10 % hydrochloric acid (1.5 ml) down the condenser with continuous stirring, while elevating the temperature to reflux conditions (75-85 ^oC). Ethanol (5 ml) was added as a solubilisation agent. Further HCl additions (2 x 2.3 ml) were made at 10 minute intervals. The reaction was refluxed overnight

followed by an aqueous extraction with DCM (3 x 20 ml). The water phase was collected and alkalinised using a 40 % sodium hydroxide solution just past the turning point, and then extracted with DCM (3 x 20 ml). The combined organic fractions were washed with brine (2 x 15 ml), dried with anhydrous $MgSO_4$ and the solvent evaporated *in vacuo* to yield the product as a dark brown solid. (Yield: 70 %, 0.096 g). ***Physical data:*** $C_{17}H_{24}N_2$; **mp:** 71 ^oC; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 17) δ_H: 6.9-6.8 (m, 2H), 6.5-6.3 (m, 2H), 3.8 (s, 2H), 3.5-3.4(d, 2H), 2.1-1.8 (m, 5H), 1.7-1.4 (m, 10H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 18) δ_C: 146.94, 129.55, 128.07, 125.34, 117.76, 115.75, 43.95, 42.73, 36.73, 29.54; **MS** (DI-ESI-MS) (Spectrum 19) *m/z*: 257.48 (M^+), 291.52, 258.49, 106.08, 79.04; **IR (ATR; cm^{-1})** (Spectrum 20)**:** 3402.23, 3023.54, 2903.16, 2849.39, 1616.94, 1494.42, 1456.77, 1357.87, 1343.14, 1308.70, 1276.40, 1072.38, 1035.59, 858.28, 816.52, 785.16, 730.60, 710.00.



NO_2 = *o* (SE-2), *m* (SE-3), *p* (SE-4) NH_2 = *o* (SE-5), *m* (SE-6), *p* (SE-7)

Figure 3.7: Preparation of SE-5, SE-6 and SE-7.

3.7.7 *N*-(3-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-6)

Previously prepared *N*-(3-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-3, 0.21 g, 0.698 mmol) and tin powder (0.12 g, 1.05 mmol) was added to a round-bottomed flask and set up under reflux conditions. This was followed by addition of 10 % hydrochloric acid (1.5 ml) down the condenser with continuous stirring, while elevating the temperature to reflux conditions (75-85 ^oC). Ethanol (5 ml) was added as a solubilisation agent. Further HCl additions (2 x 2.3 ml) were made at 10 minute intervals. The reaction was refluxed overnight followed by an aqueous extraction with DCM (3 x 20 ml). The water phase was collected and alkalinised using a 40 % sodium hydroxide solution just past the turning point, and then extracted with DCM (3 x 20 ml). The combined organic fractions were washed with brine (2 x 15 ml), dried with anhydrous $MgSO_4$ and the solvent evaporated *in vacuo* to yield the product as a light brown solid. (Yield: 75 %, 0.140 g). ***Physical data:*** $C_{17}H_{24}N_2$; **mp:** 79 ^oC; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 21) δ_H: 6.9 (m, 1H), 6.5-6.2 (m, 3H), 3.5-3.3 (d, 2H), 2.0-1.8 (m, 3H), 1.6-1.4 (m, 12H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 22) δ_C: 146.47,

129.24, 118.48, 115.01, 113.50, 50.78, 45.04, 42.77, 36.72, 29.59; **MS** (DI-ESI-MS) (Spectrum 23) *m/z*: 257.48 (M^+), 258.49; **IR (ATR; cm^{-1})** (Spectrum 24)**:** 3447.28, 3399.98, 3367.64, 3174.35, 2897.43, 2845.99, 1603.74, 1588.27, 1493.64, 1450.19, 1356.65, 1343.00, 1313.10, 1290.63, 1138.04, 1097.62, 1067.89, 1038.08, 933.97, 869.79, 846.66, 817.35, 773.51, 680.00, 642.36.

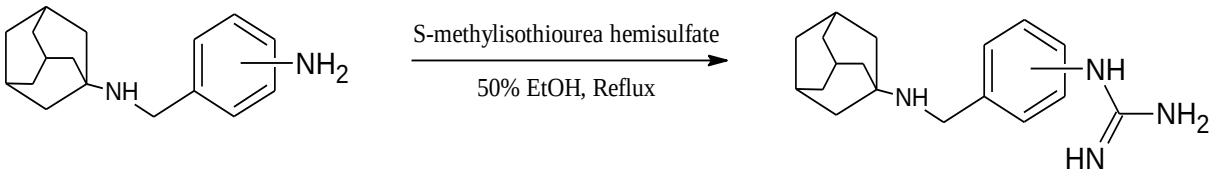
3.7.8 *N*-(4-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-7)

Previously prepared *N*-(4-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-4, 0.24 g, 0.844 mmol) and tin powder (0.15 g, 1.27 mmol) was added to a round-bottomed flask and set up under reflux conditions. This was followed by addition of 10 % hydrochloric acid (1.5 ml) down the condenser with continuous stirring, while elevating the temperature to reflux conditions (75-85 ^oC). Ethanol (5 ml) was added as a solubilisation agent. Further HCl additions (2 x 2.3 ml) were made at 10 minute intervals. The reaction was refluxed overnight followed by an aqueous extraction with DCM (3 x 20 ml). The water phase was collected and alkalinised using a 40 % sodium hydroxide solution just past the turning point, and then extracted with DCM (3 x 20 ml). The combined organic fractions were washed with brine (2 x 15 ml), dried with anhydrous $MgSO_4$ and the solvent evaporated *in vacuo* to yield the product as a light brown solid. (Yield: 90.25 %, 0.1953 g). ***Physical data:*** $C_{17}H_{24}N_2$; **mp:** 91 ^oC; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 25) δ_H: 6.9-6.8 (m, 2H), 6.4-6.3 (m, 2H), 3.4-3.3 (d, 2H), 1.9-1.8 (m, 3H), 1.5-1.4 (m, 12H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 26) δ_C: 145.05, 129.32, 128.81, 115.15, 50.73, 44.59, 42.76, 36.73, 29.59; **MS** (DI-ESI-MS) (Spectrum 27) *m/z*: 257.49 (M^+), 258.49, 106.07, 79.06; **IR (ATR; cm^{-1})** (Spectrum 28)**:** 3429.13, 3315.88, 3183.04, 2898.25, 2847.51, 1610.19, 1518.83, 1457.87, 1357.24, 1294.57, 1095.75, 1069.75, 1038.90, 973.42, 932.38, 818.28, 761.66, 708.40.

3.7.9 1-{2-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine (SE-8)

Previously prepared *N*-(2-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (0.05 g, 0.170 mmol) was added to a round-bottomed flask followed by S-methylisothiourea hemisulfate (0.040 g, 0.293 mmol) and 50 % ethanol (10 ml). The mixture was refluxed for 24 hours during which time a yellow precipitate formed. The solvent was then evaporated *in vacuo*.

The reaction mixture was washed with solvent followed by solvent evaporation *in vacuo.* The reaction was unsuccessful and did not yield a compound.



NH_2 = *o* (SE-5), *m* (SE-6), *p* (SE-7) $NHC(NH)NH_2$ = *o* (SE-8), *m* (SE-9), *p* (SE-10)

Figure 3.8: Preparation of SE-8, SE-9 and SE-10. Reactions were unsuccessful.

3.7.10 1-{3-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine (SE-9)

Previously prepared *N*-(3-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (0.1 g, 0.339 mmol) was added to a round-bottomed flask followed by S-methylisothiourea hemisulfate (0.081 g, 0.585 mmol) and 50 % ethanol (10 ml). The mixture was refluxed for 24 hours during which time a yellow precipitate formed. The solvent was then evaporated *in vacuo*. The reaction mixture was washed with solvent followed by solvent evaporation *in vacuo.* The reaction was unsuccessful and did not yield a compound.

3.7.11 1-{4-[(tricyclo[3.3.1.1^{3,7}]dec-1-ylamino)methyl]phenyl}guanidine (SE-10)

Previously prepared *N*-(4-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (0.1 g, 0.339 mmol) was added to a round-bottomed flask followed by S-methylisothiourea hemisulfate (0.081 g, 0.585 mmol) and 50 % ethanol (10 ml). The mixture was refluxed for 24 hours during which time a yellow precipitate formed. The solvent then evaporated *in vacuo*. The reaction mixture was washed with solvent followed by solvent evaporation *in vacuo.* The reaction was unsuccessful and did not yield a compound.

3.7.12 *N*-(2-phenylethyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-11)

Amantadine free base (0.5 g, 3.31 mmol) was added to a round-bottomed flask containing acetonitrile (15 ml), potassium carbonate (0.625 g, 4.515 mmol) and 2-bromoethylbenzene (0.557 g, 3.01 mmol). The mixture was placed in the microwave reactor under the following

conditions; open vessel, 100 ^{o}C, 100 W, 2 hours, 1 atm pressure. After irradiating the mixture for 2 hours, it was filtered by vacuum filtration, to remove the potassium carbonate. The filtrate was collected and the excess solvent evaporated *in vacuo*. The reaction mixture was then acidified using 30 ml water which was made acidic with HCl until the pH was 3. This was followed by an extraction with DCM (3 x 30 ml). The combined aqueous layers were collected and made basic (pH = 12-14) using water saturated with NaOH and this was followed by a second extraction with DCM (3 x 30 ml). The combined organic layers were dried over $MgSO_4$, filtered through a sinter and the solvent evaporated *in vacuo* to yield the product as a white wax. (Yield: 46.9 %, 0.235 g). ***Physical data:*** $C_{18}H_{25}N$; **mp:** wax; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 29) δ_H: 7.4-7.1 (m, 5H), 3.6-3.5 (t, 2H), 3.1-3.0 (t, 2H), 1.9-1.5 (m, 3H), 1.4-1.1 (m, 12H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 30) δ_C: 138.85, 137.08, 136.82, 136.50, 134.22, 130.95, 129.28, 129.05, 128.79, 128.76, 128.69, 128.62, 128.58, 127.69, 127.39, 126.97, 126.89, 125.57, 58.30, 50.00, 41.63, 39.38, 38.66, 38.49, 36.75, 35.44, 35.26, 32.92, 32.60, 32.48, 32.89, 31.89, 31.39, 30.13, 29.66, 29.46, 29.33, 29.21, 28.97, 22.66, 14.01; **MS** (DI-ESI-MS) (Spectrum 31) *m/z*: 256.48 (M^+), 257.51, 135.14, 79.06; **IR (ATR; cm^{-1})** (Spectrum 32)**:** 2912.23, 2891.95, 2852.00, 2802.56, 2755.00, 2632.46, 2436.52, 1603.36, 1500.06, 1455.19, 1362.77, 1309.95, 1074.96, 1028.47, 776.90, 716.92, 695.84.

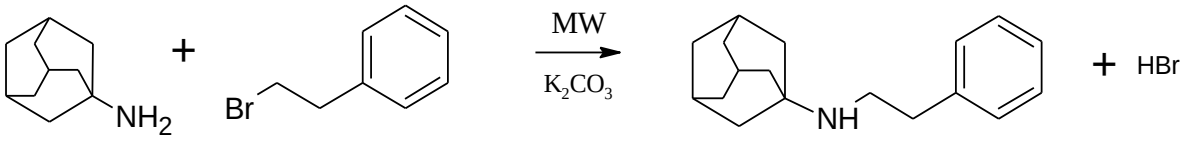


Figure 3.9: Preparation of SE-11.

3.7.13 *N*-(4-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-12)

1-Bromoadamantane (0.5 g, 2.32 mmol) was added to a reaction vessel containing acetonitrile (3 ml), potassium carbonate (0.4818 g, 3.49 mmol) and 4-methoxybenzylamine (0.3188 g, 2.32 mmol). The mixture was placed in the microwave reactor under the following conditions; closed vessel, 240 Psi, 300 W, 150 ^{o}C, 1 hour. After irradiating the mixture for 1 hour, it was filtered by vacuum filtration, to remove the potassium carbonate. The filtrate was collected and the excess solvent evaporated *in vacuo*. The reaction mixture was then acidified using 30 ml water which was made acidic with HCl until the pH was 3. This was followed by

an extraction with DCM (3 x 30 ml). The combined aqueous layers were collected and made basic (pH = 12-14) using water saturated with NaOH and this was followed by a second extraction with DCM (3 x 30 ml). The combined organic layers were dried over $MgSO_4$, filtered through a sinter and the solvent evaporated *in vacuo* to yield the product as a yellow amorphous powder. (Yield: 25.12 %, 0.126 g). ***Physical data:*** $C_{18}H_{25}NO$; **mp:** 225 ^oC; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 33) δ_H: 7.3-7.1 (dd, 2H), 6.9-6.7 (dd, 2H), 5.4-4.7 (bs, NH), 3.75 (s, OCH_3), 2.2-1.9 (m, 3H), 1.9-1.5 (m, 12H); **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 34) δ_C: 128.46, 114.48, 55.28, 51.56, 45.28, 42.48, 36.01, 35.57, 29.65, 29.17; **MS** (DI-ESI-MS) (Spectrum 35) *m/z*: 314.30, [M+ACN+H] 313.23, 299.19; **IR (ATR; cm^{-1})** (Spectrum 36)**:** 2905.95, 2849.65, 1627.72, 1512.97, 1453.77, 1368.72, 1304.02, 1248.01, 1176.72, 1087.85, 1031.99, 813.00.

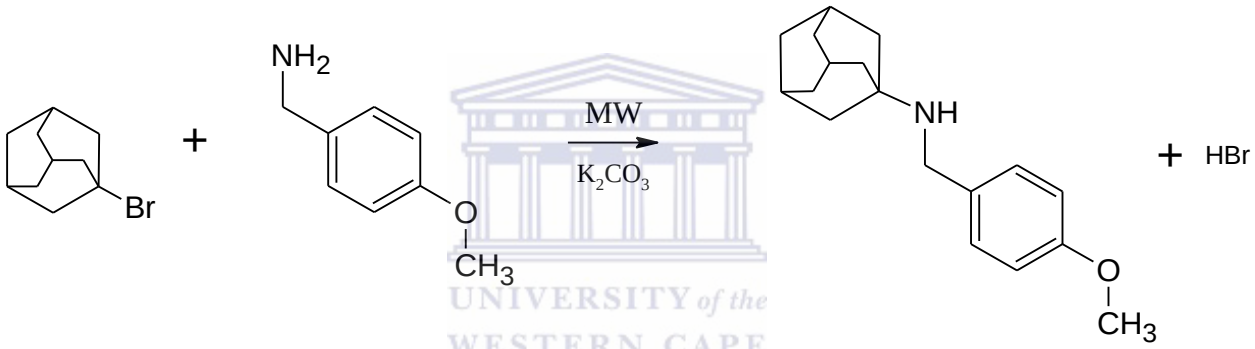


Figure 3.10: Preparation of SE-12.

3.7.14 *N*-(3-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-13)

Amantadine free base (0.5 g, 3.31 mmol) was added to a round-bottomed flask containing acetonitrile (15 ml), potassium carbonate (0.625 g, 4.515 mmol) and 3-methoxybenzylchloride (0.516 g, 3.29 mmol). The mixture was placed in the microwave reactor under the following conditions; open vessel, 80 ^oC, 100 W, 2 hours, 1 atm pressure. After irradiating the mixture for 2 hours, it was filtered by vacuum filtration, to remove the potassium carbonate. The filtrate was collected and the excess solvent evaporated *in vacuo*. The reaction mixture was then acidified using 30 ml water which was made acidic with HCl until the pH was 3. This was followed by an extraction with DCM (3 x 30 ml). The combined aqueous layers were collected and made basic (pH = 12-14) using water saturated with NaOH and this was followed by a second extraction with DCM (3 x 30 ml). The combined organic

layers were dried over $MgSO_4$, filtered through a sinter and the solvent evaporated *in vacuo* to yield the product as a white amorphous powder. (Yield: 18.32 %, 0.092 g). ***Physical data:*** $C_{18}H_{25}NO$; **mp:** 124 ^oC; **^{1}H NMR** (200 MHz, $CDCl_3$) (Spectrum 37) δ_H: 9.8-9.6 (s, 1H), 7.7-7.5 (dd, 1H), 7.4-6.7 (2 x m, 2H), 4.6-4.3 (bs, NH), 3.9-3.7 (OCH_3), 2.6-1.5 (m, 15H) ; **^{13}C NMR** (50 MHz, $CDCl_3$) (Spectrum 38) δ_C: 162.59, 130.14, 128.68, 121.76, 110.68, 110.20, 56.07, 55.27, 44.43, 42.57, 35.35, 29.28; **MS** (DI-ESI-MS) (Spectrum 39) *m/z*: 314.28, [M+ACN+H] 313.23, 299.17, 151.98, 78.91; **IR (ATR; cm^{-1})** (Spectrum 40)**:** 2934.65, 2864.29, 2750.97, 2643.30, 2436.30, 1586.15, 1454,84, 1265.05, 1174.73, 1075.22, 1032.70, 852.27, 792.04.

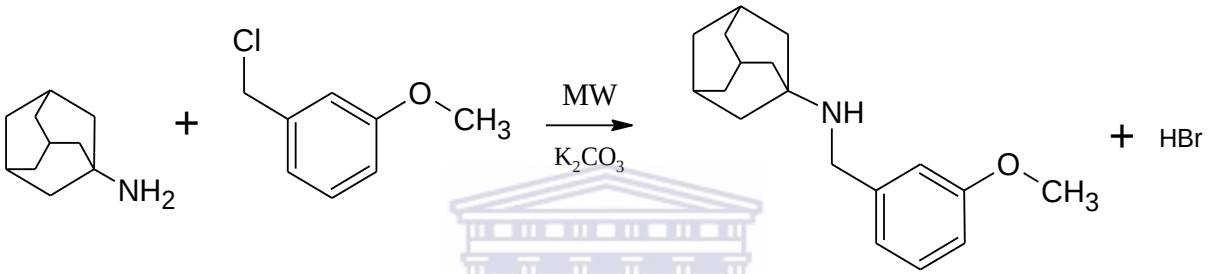


Figure 3.11: Preparation of SE-13.

3.7.15 *N*-(2-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine (SE-14)

Amantadine free base (0.5 g, 3.31 mmol) was added to a round-bottomed flask containing acetonitrile (15 ml), potassium carbonate (0.625 g, 4.515 mmol) and 2-methoxybenzylchloride (0.516 g, 3.29 mmol). The mixture was placed in the microwave reactor under the following conditions; open vessel, 80 ^oC, 100 W, 2 hours, 1 atm pressure. The reaction did not successfully yield a compound.

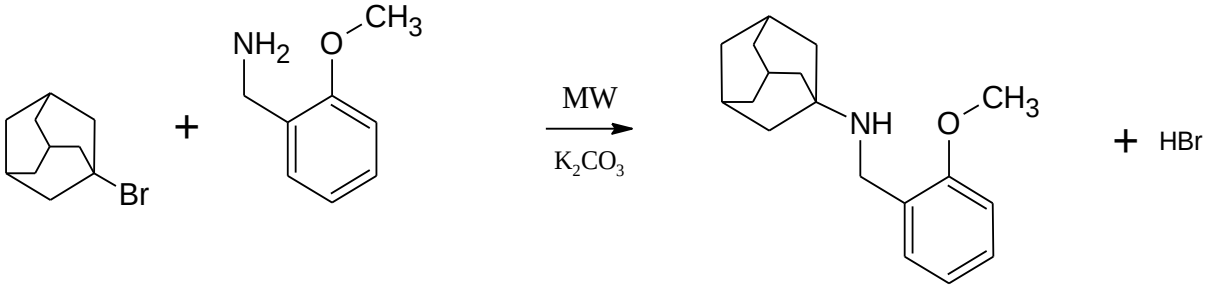


Figure 3.12: Preparation of SE-14.

3.8 Conclusion

Compounds SE-8, SE-9, SE-10 and SE-14 could not be synthesised due to the reasons stated above (see section 3.6.3). Due to time limitations, the reactions were not optimised and standardised further. However, it is speculated that these reactions may yet be possible under the right conditions. It is thus recommended that these reactions be standardised as these compounds may possess activity against nNOS as well as the NMDA receptor and voltage gated calcium channels.

The synthesis of the 10 successful compounds resulted in yields between 16.5 % and 90.25 %. The lower yields could be attributed to the formation of various side products as well as the choice of purification method (extraction as opposed to column chromatography). It is recommended that these reactions be further optimised for future use.

CHAPTER 4

BIOLOGICAL EVALUATION

In the following chapter, the biological evaluation of compounds that were synthesised in chapter 3 is discussed. The different assays employed, their significance, the problems encountered as well as the results obtained are discussed

4.1 Introduction

The fluorescent ratiometric indicator, FURA-2 AM, a Fluorescent Microplate Reader (Biotek Instruments, Inc) and Gen 5® Software were used to evaluate the influence of the test compounds on the NMDA receptor (NMDAR) as well as potassium chloride (KCl)-mediated calcium influx into murine synaptoneurosomes. All novel test compounds and controls were evaluated at 100 μM. The activity of test compounds was measured against reference compounds which are known inhibitors of the NMDAR and voltage gated calcium channels (VGCC). Amantadine, memantine and MK-801 were used as references due to their known affinity for the PCP binding site of NMDAR, while nimodipine is a known VGCC blocker. NGP1-01, a dual NMDAR and VGCC blocker (Van der Schyf *et al.*, 1986; Geldenhuys *et al.*, 2007) was also included as a reference. The amantadine moiety was selected as the scaffold for the newly synthesised compounds because of its known uncompetitive NMDAR antagonism which is involved in modulation of the channel and allows normal neuronal functioning as opposed to complete NMDAR channel blockage (Simon *et al.*, 1984; Choi, 1985; Garthwaite *et al.*, 1986; Parsons *et al.*, 1998; Joubert *et al.*, 2011).

4.2 NMDA receptor inhibition assay

4.2.1 Introduction

The fluorescent ratiometric indicator, FURA-2 AM, a Fluorescent Microplate Reader (Biotek Instruments, Inc) and Gen 5® Software were used to evaluate the influence of the test compounds on NMDA/Glycine mediated calcium influx *via* the NMDAR in murine synaptoneurosomes. All novel compounds and controls were tested at 100 μM. Procedures

similar to those of published studies (Benavide *et al.*, 1988; Stout & Reynolds, 1991; Takahashi *et al.*, 1999; Lambert, 1999; Crawley *et al.*, 2001; Geldenhuys *et al.*, 2007) were used to prepare the synaptoneurosomes and solutions, and to establish the techniques for experimental measurement of fluorescence.

4.2.2 Materials

Unless stated otherwise, all materials were purchased from Sigma-Aldrich (South Africa) and Merck (Darmstadt, Germany).

4.2.3 Animals

The study protocol was approved by the Ethics Committee for Research on Experimental Animals of the University of the Western Cape (SRIRC 2012/06/13). Male Wistar rats were sacrificed by decapitation and the brain tissue was removed and kept on ice for homogenation.

4.2.4 Data analysis

All data analysis, calculations and graphs were done using GraphPad Prism 6.03® (GraphPad, Sorrento valley, CA). Data analysis was carried out using the Student Newman Keuls multiple range of test compounds versus controls.

4.2.5 Preparation of buffers

4.2.5.1 Calcium free buffer

Sodium chloride (118 mM), potassium chloride (4.7 mM), HEPES (20 mM) and glucose monohydrate (30.9 mM) were added to a volumetric flask and made up to 1 L using distilled water. The pH was adjusted to 7.4 at room temperature using either HCl or NaOH.

4.2.5.2 Incubation buffer

Calcium chloride dihydrate (0.1 mM) was added to a volumetric flask and made up to 200 ml using previously prepared calcium free buffer.

4.2.5.3 Calcium containing buffer

Calcium chloride dihydrate (2 mM) was added to a volumetric flask and made up to 200 ml using previously prepared calcium free buffer.

4.2.5.4 Stimulation buffer

Calcium chloride dihydrate (0.1 mM), glycine (0.1 mM) and NMDA (0.1 mM) were added to a volumetric flask and made up to 200 ml using previously prepared calcium free buffer.

4.2.6 Assay procedure

4.2.6.1 Preparation of synaptoneurosomes

Fresh rat brain was weighed in a 50 ml pre-cooled Falcon tube and placed on ice. The rat brain was rinsed in 15 ml of ice cold incubation buffer and the buffer discarded. The sample was then homogenised with a Teflon® glass homogeniser in 20 ml of ice-cold incubation buffer (8 strokes). The tissue suspension was then transferred to two 15 ml Falcon tubes and centrifuged at 1,000 x *g* for 5 minutes at 4 °C. The supernatants were then divided into 2 ml aliquots and centrifuged at 15,000 x *g* for 20 min at 4 °C. The resulting pellets were re-suspended in calcium free buffer at a protein concentration of 3 mg/ml. The protein yield was about 10 mg/g of tissue (Joubert *et al.*, 2011). The suspension was allowed to reach room temperature following which FURA-2 AM (5 mM in dimethylsulfoxide [DMSO] – solution was protected from light at all times) was added to a final concentration of 5 μM and incubated for 30 minutes at 30 °C. The suspension was centrifuged at 12,500 x *g* for 5 minutes to remove the extra-synaptoneurosomal FURA-2 AM. The supernatant was then decanted and the resulting pellet re-suspended in calcium containing buffer to a final concentration of 0.6 mg/ml, and kept at room temperature until used. The suspension was protected from light.

4.2.6.2 Measurement of intracellular calcium

Stock solutions of test compounds (10 mM) were prepared by dissolving them in DMSO. DMSO without any test compound was used as a control. MK-801, NGP1-01, amantadine and memantine were used as the reference compounds. A small amount of stock solution (2 μl) was diluted with 0.2 ml synaptoneurosomal-FURA-2 AM solution in a 96 well-plate to give a 100 μM concentration of the compound. The mixture was then shaken and incubated at

37 °C for 5 minutes. Dual wavelength excitation at 340 and 380 nm was used and the resting fluorescence (emission) was measured at 510 nm. Stimulation buffer (10 µl) was introduced into the well plate by means of an auto-injector built into the plate reader to activate NMDAR mediated calcium influx. The effect of test compound(s) on calcium influx was measured by monitoring changes in fluorescence. Experiments were repeated three times on different tissue preparations with three determinations in each replicate.

4.2.7 Results and discussion

Synaptoneurosomes incubated with test compounds SE-2 (60.6 %), SE-4 (74.8 %), SE-1 (66.7 %), SE-11 (89.5 %), SE-13 (70.2 %) and SE-12 (79.2 %) showed good inhibitory activity (> 50 %) of NMDA/glycine-mediated calcium influx (fig. 4.1, table 4.1). SE-3 (47.2 %), SE-5 (31.3 %), SE-6 (48.3 %) and SE-7 (50.4 %) showed weaker inhibitory activity (< 50 %), although more significant than the reference compound NGP1-01 (13 %). Of the controls, MK-801 (100 %) was the most potent inhibitor followed by memantine (92.5 %) which showed higher inhibition than amantadine (84.6 %) and NGP1-01 (13.5 %). This was in agreement with previous studies (Danysz *et al.*, 1997; Geldenhuys, 2006; Joubert *et al.*, 2011). Although MK-801 is used as a control, there is no standard deviation because it caused a complete block of NMDA-mediated calcium entry. This is because it binds to the PCP-binding site within the NMDAR in a use-dependant manner and it can be trapped by channel closure since it displays slow unblocking kinetics (Parsons *et al.*, 1998; Geldenhuys *et al.*, 2007).

SE-11 (89.5 %) showed the highest percentage NMDAR inhibition of all synthesised compounds. We observed that an increase in chain length between the amantadine moiety and the aromatic group led to an increase in NMDAR inhibitory activity as can be seen from the activities of SE-1 (66.7 %) and SE-11 (fig. 4.1). Although both compounds are thought to fit into the channel in such a way as to allow free movement of the polycyclic (amantadine) structure to a certain angle, the ethyl linker is thought to enable the compound to penetrate deeper into the channel lumen thereby enabling more favourable interactions with the PCP-binding site (Geldenhuys, 2004). Geldenhuys and colleagues (2004, 2007) observed similar results through their experiments on NGP1-01 and its phenethyl derivative (Geldenhuys, 2004; Geldenhuys *et al.*, 2007).

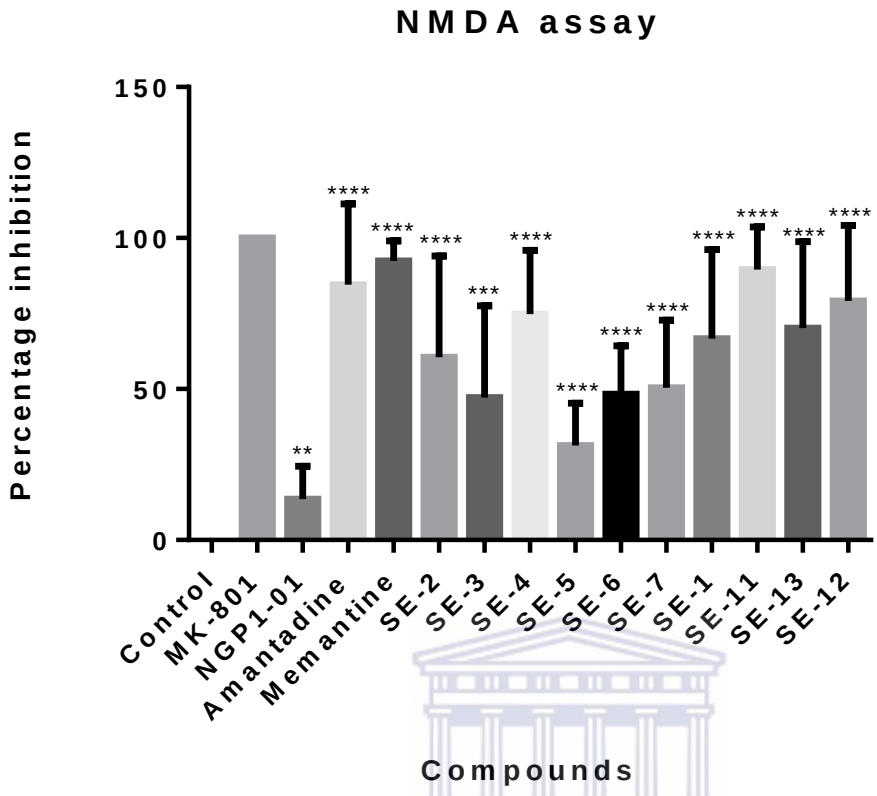


Figure 4.1: Screening of test compounds (100 μM) for antagonism of NMDAR-mediated calcium influx into murine synaptoneurosomes. Each bar represents mean percentage NMDAR channel inhibition ± SEM. Statistical analysis was performed on raw data, with asterisks signifying significant inhibitory effect [(*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$] when compared to the control (0 % inhibition).

The methoxy derivatives SE-13 and SE-12 both showed good inhibitory activity (70.2 % and 79.2 %, respectively). This may be due to the presence of an electron donating (methoxy) group whose electron donating effect may result in increased hydrophobic/π-π bonding stabilisation of the compounds, which are favourable for NMDAR channel inhibition.

Of the 3 nitro containing compounds, SE-3 showed the weakest (albeit still moderate) activity with 49 % inhibition. SE-4 showed the highest activity with 74.8 % inhibition and the rank order that is followed for these compounds is *para>ortho>meta*. The good inhibitory activity of the nitro compounds may be as a result of the nitro group contributing to the *S*-nitrosylation of cysteine residues in the NMDAR channels, thus increasing NMDAR activity (Lipton *et al.*, 2002; Joubert *et al.*, 2011; Lemmer *et al.*, 2012). The weaker activity of SE-2 (60.6 %) and

SE-3 (compared to SE-4) may be attributed to the electron withdrawing effects of the nitro group as well as steric hindrance of the aromatic ring due to the nitro group being in the *ortho* or *meta* position. As the nitro group is in the *para* position in SE-4, this may enable the compound to fit better at the site of interaction.

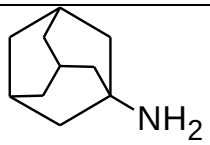
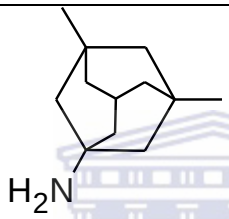
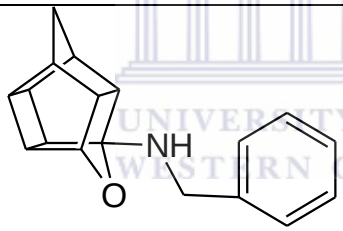
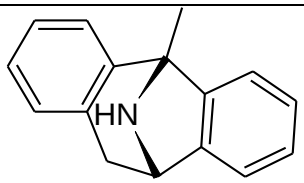
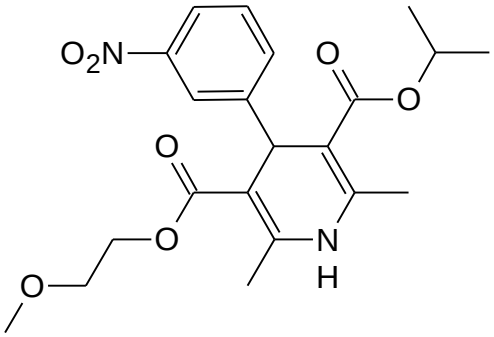
The amine compounds (SE-5, SE-6 and SE-7) show weaker activity (31-51 %) than their respective nitro counterparts (SE-2, SE-3 and SE-4), as well as the methoxy compounds (SE-13 and SE-12). This could indicate that the nitro group was involved in favourable binding interactions with NMDAR channels, and the reduction to the amines led to a decrease in activity. The rank order that follows for the amines is the same as that of the nitro compounds, *para*>*ortho*>*meta*.

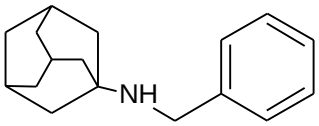
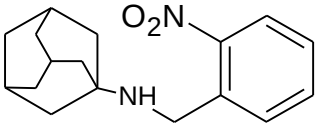
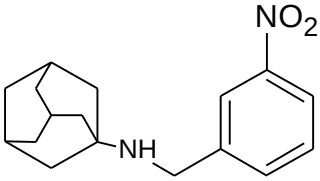
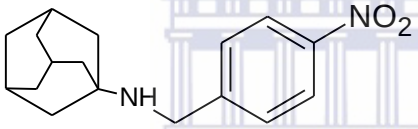
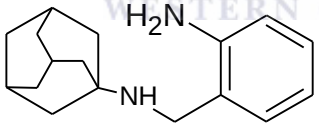
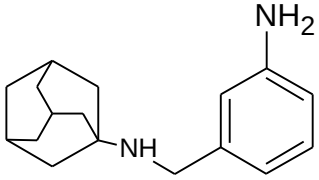
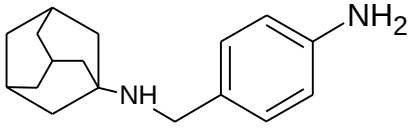
All the tested compounds showed better activity than NGP1-01 and none of them showed better NMDAR inhibitory activity than memantine. All the inhibitors and controls displayed statistically significant activity (fig. 4.1, $p < 0.05$).

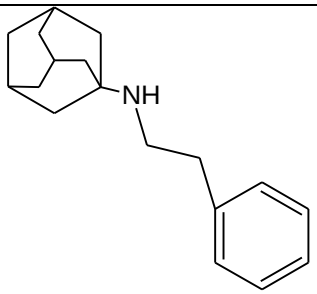
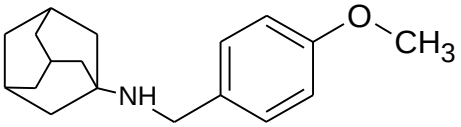
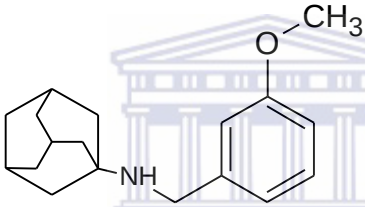
4.2.8 Conclusion

All test compounds were incubated with murine synaptoneurosomes and tested for NMDAR-mediated calcium influx against the reference compounds MK-801, NGP1-01, amantadine and memantine. All the reference compounds showed statistically significant ($p < 0.05$) inhibition of calcium influx. Test compounds SE-2 (60.6 %), SE-4 (74.8 %), SE-1 (66.7 %), SE-11 (89.5 %), SE-13 (70.2 %) and SE-12 (79.2 %) showed good inhibitory activity (> 50 %) while SE-3, SE-5, SE-6 and SE-7 showed weaker inhibitory activity (< 50 %).

Table 4.1: Summary of the percentage inhibition of test and reference compounds for NMDA- and VGCC-mediated calcium influx.

Name	**Structure**	**% NMDAR inhibitory activity [100 µM][a]**	**% VGCC inhibitory activity [100 µM][a]**
Amantadine		84.6****	7.7
Memantine		92.5****	21.9
NGP1-01		13.5**	13.4*
MK-801		100	n.d
Nimodipine		n.d	100

SE-1	 *N*-benzyltricyclo[3.3.1.1^{3,7}]decan-1-amine	66.7****	58.8****
SE-2	 *N*-(2-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	60.6****	49.2***
SE-3	 *N*-(3-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	47.2***	14.3**
SE-4	 *N*-(4-nitrobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	74.8****	85.7****
SE-5	 *N*-(2-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	31.3****	37.1****
SE-6	 *N*-(3-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	48.3****	30.0**
SE-7	 *N*-(4-aminobenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	50.4****	49.5****

SE-11	 *N*-(2-phenylethyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	89.5****	61.8****
SE-12	 *N*-(4-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	79.2****	71.2****
SE-13	 *N*-(3-methoxybenzyl)tricyclo[3.3.1.1^{3,7}]decan-1-amine	70.2****	44.7****

[a]Statistical analysis was performed on raw data, with asterisks signifying significant inhibitory effect [(*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$] when compared to the control (0 % inhibition).

n.d. = not determined

4.3 Voltage gated calcium channel inhibition assay

4.3.1 Introduction

The fluorescent ratiometric indicator, FURA-2 AM, a Fluorescent Microplate Reader (Biotek Instruments, Inc) and Gen 5® Software were used to evaluate the influence of the test compounds on KCl-mediated calcium influx *via* VGCC in murine synaptoneurosomes. All novel compounds and controls were tested at 100 μM. Procedures similar to those of published studies (Benavide *et al.*, 1988; Stout & Reynolds, 1991; Takahashi *et al.*, 1999; Lambert, 1999; Crawley *et al.*, 2001; Geldenhuys *et al.*, 2007) were used to prepare the

synaptoneurosomes and solutions, and to establish the techniques for experimental measurement of fluorescence.

Amantadine and memantine were selected as negative controls because of their known affinity for NMDAR as well as their structural similarity with the calcium channel inhibitor NGP1-01. NGP1-01 and nimodipine were used as positive controls because of their known affinity for VGCC. The application of depolarising buffer (containing 140 mM of potassium) resulted in an increase in the fluorescent ratio in the presence of extracellular calcium. This increase in fluorescence is due to calcium influx through VGCC.

4.3.2 Materials

Unless stated otherwise, all materials were purchased from Sigma-Aldrich (South Africa) and Merck (Darmstadt, Germany).

4.3.3 Animals

The study protocol was approved by the Ethics Committee for Research on Experimental Animals of the University of the Western Cape (SRIRC 2012/06/13). Male Wistar rats were sacrificed by decapitation and the brain tissue was removed and kept on ice for homogenation.

4.3.4 Data analysis

All data analysis, calculations and graphs were done using GraphPad Prism 6.03® (GraphPad, Sorrento valley, CA). Data analysis was carried out using the Student Newman Keuls multiple range of test compounds versus controls.

4.3.5 Preparation of buffers

4.3.5.1 Calcium free buffer

Sodium chloride (118 mM), potassium chloride (4.7 mM), magnesium chloride hexahydrate (1.18 mM), HEPES (20 mM) and glucose monohydrate (30.9 mM) were added to a volumetric flask and made up to 1 L using distilled water. The pH was adjusted to 7.4 at room temperature using either HCl or NaOH.

4.3.5.2 Incubation buffer

Calcium chloride dihydrate (0.1 mM) was added to a volumetric flask and made up to 200 ml using previously prepared calcium free buffer.

4.3.5.3 Calcium containing buffer

Calcium chloride dihydrate (2 mM) was added to a volumetric flask and made up to 200 ml using previously prepared calcium free buffer.

4.3.5.4 Depolarising buffer

Sodium chloride (5.4 mM), potassium chloride (140 mM), calcium chloride dihydrate (1.4 mM), HEPES (20 mM), glucose monohydrate (5.5 mM), sodium bicarbonate (10 mM), potassium hydrogen phosphate (0.6 mM), disodium hydrogen phosphate (0.6 mM) and magnesium sulphate (0.9 mM) were added to a volumetric flask and made up to 100 ml using distilled water. The pH was adjusted to 7.4 at room temperature using either HCl or NaOH.

4.3.6 Assay procedure

4.3.6.1 Preparation of synaptoneurosomes

Fresh rat brain was weighed in a 50 ml pre-cooled Falcon tube and placed on ice. The rat brain was rinsed in 15 ml of ice cold incubation buffer and the buffer discarded. The sample was then homogenised with a Teflon® glass homogeniser in 20 ml of ice-cold incubation buffer (8 strokes). The tissue suspension was then transferred to two 15 ml Falcon tubes and centrifuged at 1,000 x *g* for 5 min at 4 °C. The supernatants were then divided into 2 ml aliquots and centrifuged at 15,000 x *g* for 20 minutes at 4 °C. The resulting pellets were re-suspended in calcium free buffer at a protein concentration of 3 mg/ml. The protein yield was estimated about 10 mg/g of tissue (Joubert *et al.*, 2011). The suspension was allowed to reach room temperature following which FURA-2 AM (5 mM in dimethylsulfoxide [DMSO] – solution was protected from light at all times) was added to a final concentration of 5 μM and incubated for 30 minutes at 30 °C. The suspension was centrifuged at 12,500 x g for 5 minutes to remove the extra-synaptoneurosomal FURA-2 AM. The supernatant was then decanted and the resulting pellet re-suspended in calcium containing buffer to a final concentration of 0.6 mg/ml, and kept at room temperature until used. The suspension was protected from light.

4.3.6.2 Measurement of intracellular calcium

Stock solutions of test compounds (10 mM) were prepared by dissolving them in DMSO. DMSO without any test compound was used as a control. Nimodipine, NGP1-01, amantadine and memantine were used as the reference compounds. A small amount of stock solution (2 µl) was diluted with 0.2 ml synaptoneurosomal-FURA-2 AM solution in a 96 well-plate to give a 100 µM concentration of the compound. The mixture was then shaken and incubated at 37 °C for 5 minutes. Dual wavelength excitation at 340 and 380 nm was used and the resting fluorescence (emission) was measured at 510 nm. Depolarising buffer (10 µl) was introduced into the well plate by means of an auto-injector built into the plate in order to depolarise the cell membrane and allow VGCC-mediated calcium influx. The effect of test compound(s) on calcium influx was measured by monitoring changes in fluorescence. Experiments were repeated three times on different tissue preparations with three determinations in each replicate.

4.3.7 Results and discussion

The controls that were tested include nimodipine (100 %), NGP1-01 (13.4 %), amantadine (7.7 %) and memantine (21.9 %). Of the polycyclic structures, NGP1-01 was the only one that displayed statistically significant ($p < 0.05$) inhibition of calcium influx *via* VGCC. Both amantadine and memantine showed statistically insignificant inhibition ($p > 0.05$). There is no standard deviation for nimodipine as it caused a complete (100 %) block of calcium influx through VGCCs in all the experiments. Murine synaptoneurosomes incubated with the test compounds SE-4 (85.7 %), SE-1 (58.8 %), SE-11 (61.8 %) and SE-12 (71.2 %) all displayed good inhibitory activity (fig. 4.2, table 4.1) of calcium influx through VGCCs(> 50 %).

Test compound SE-4 (85.7 %) displayed the highest inhibition of calcium influx *via* VGCC. SE-2 (49.2 %) and especially SE-3 (14.3 %) display significantly weaker inhibitory activity than SE-4. This may be attributed to the electron withdrawing effects of the nitro group as well as steric hindrance of the aromatic ring due to the nitro group being in the *ortho* or *meta* position. The good inhibitory activity of SE-4 may be as a result of the nitro group being in the *para* position, which enables the compound to fit better in the binding pocket at the site of interaction. SE-11 (61.8 %) shows slightly better activity than SE-1 (58.8 %) and this may be attributed to the presence of an ethyl linker between the amantadine moiety and aromatic

group in SE-11, which enables the compound to penetrate deeper into the calcium channel and more favourable interactions with the putative binding site (Geldenhuys, 2004; Liebenberg *et al.*, 2000; Joubert *et al.*, 2011).

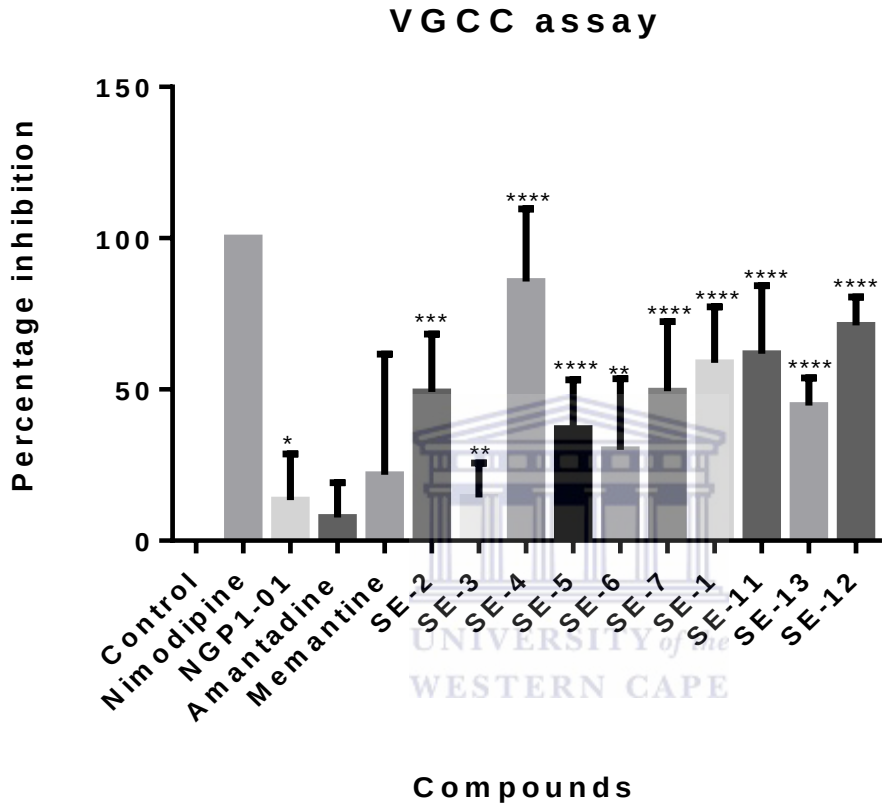


Figure 4.2: Screening of test compounds (100 μM) for antagonism of KCl-mediated calcium influx inhibition *via* VGCC into murine synaptoneurosomes. Each bar represents mean percentage inhibition ± SEM. Statistical analysis was performed on raw data, with asterisks signifying significant inhibitory effect [(*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$] when compared to the control (0 % inhibition).

The amine compounds (SE-5 and SE-7) show weaker activity (30-49.5 %) than their respective nitro counterparts (SE-2 and SE-4). This could be because of the protonation of the aniline moiety at the pH of the experiment. However, SE-6 (30.0 %) displays better inhibitory activity than SE-3 (14.3 %). The rank order that follows for the amines is the same as that of the nitro compounds, *para>ortho>meta*.

The methoxy derivative SE-12 showed better inhibitory activity than SE-13. This may be due to the presence of an electron donating (methoxy) group in the *para* position as opposed to the *meta* position whose electron donating effect may result in increased hydrophobic/π-π bonding stabilisation of the compounds, which are favourable for VGCC inhibition.

All the test compounds displayed better inhibitory activity ($p < 0.05$) than the reference compound NGP1-01.

4.3.8 Conclusion

All test compounds were incubated with murine synaptoneurosomes and tested for KCl-mediated calcium influx against the reference compounds nimodipine, NGP1-01, amantadine and memantine. Of the reference compounds, only nimodipine and NGP1-01 showed statistically significant ($p < 0.05$) inhibition of calcium influx. Test compounds SE-4 (85.7 %), SE-1 (58.8 %), SE-11 (61.8 %) and SE-12 (71.2 %) all displayed good inhibitory activity of calcium influx (> 50 %) while SE-2 (49.2 %), SE-5 (37.1 %), SE-6 (30.0 %), SE-7 (49.5 %) and SE-13 (44.7 %) showed weaker inhibitory activity (< 50 %). SE-3 (14.3 %) showed significantly weak inhibitory activity.

4.4 Summary

The NMDA and VGCC assays were conducted on synaptoneurosomes obtained from rat brain homogenate. These assays were used as a proof of concept to give an indication of the inhibitory activity of test compounds towards NMDA receptors and VGCC, respectively. Activity of test compounds (100 µM) was measured against various controls of known inhibitory activity. The assays were conducted using a fluorescent plate reader and the fluorescent ratiometric calcium indicator FURA-2 AM. In the NMDA assay, test compounds SE-1 (66.7 %), SE-2 (60.6 %), SE-4 (74.8 %), SE-11 (89.5 %), SE-12 (79.2 %) and SE-13 (70.2 %) all had inhibitory activity upwards of 50 % (fig. 4.1). For the VGCC, SE-4 (85.7 %) had a percentage inhibition of higher than 80 % (fig. 4.2), while SE-1 (58.8 %), SE-11 (61.8 %) and SE-12 (71.2 %) displayed good inhibitory activity (> 50 %). All compounds were tested in triplicate, with mean and standard deviation values calculated. All data of the novel synthesised compounds were statistically significant ($p < 0.05$) as shown by the student t-test. Further tests are recommended on SE-1, SE-4, SE-11 and SE-12 as they displayed good

inhibitory activity against both NMDAR- as well as KCl-mediated calcium influx. These novel compounds may be better therapeutic options than amantadine and memantine as they inhibit NMDAR and VGCC-mediated calcium influx, whereas amantadine and memantine only inhibit NMDA-mediated calcium influx.

Due to a lack of success in synthesising the guanidine compounds SE-8, SE-9 and SE-10, these compounds could not be tested for nNOS inhibitory activity using the oxyhemoglobin capture assay. It is recommended that the synthesis of compounds SE-8, SE-9 and SE-10 be optimised, and all test compounds be tested for nNOS inhibitory activity in future studies as these compounds are expected to display NOS inhibition. SE-8, SE-9 and SE-10 are expected to have good selectivity for nNOS due to the presence of the polycyclic amantadine scaffold as well as the guanidine functional group.

These novel adamantane derived compounds may possibly serve as lead compounds or potential therapeutic agents for the treatment of neurodegenerative disorders.

CHAPTER 5

CONCLUSION

5.1 Introduction

Neurodegeneration is defined as the progressive loss of structure and functions of neurons. In an aging population, neurodegenerative disorders are becoming increasingly rife. Neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease have been the focus of many different research groups over the past years, be it chemistry, neuroscience or pharmacology and they are the leading causes of loss of normal functioning in the elderly population (Geldenhuys *et al.*, 2004; Van der Schyf & Geldenhuys, 2009). The drugs that are currently available for the management of neurodegenerative disorders target only one pathway or have just one mechanism of action (Geldenhuys *et al.*, 2004). It is therefore necessary to develop and synthesise compounds that function through different mechanisms so as to act on as many pathways as possible.

The lethal triplet has been implicated in the development of neurodegeneration and consists of excitotoxicity, mitochondrial dysfunction and oxidative stress. Excitotoxicity occurs as a result of an overstimulation of the *N*-methyl-D-aspartate (NMDA) receptor during pathological conditions. It can result from the presence of an excess amount of glutamate in the synapse, due to excessive release and inadequate uptake (Aarts & Tymianski, 2003; Van der Schyf & Geldenhuys, 2009). Oxidative stress is the damage that occurs to cellular structures and organelles including the mitochondria (mitochondrial dysfunction) caused by the formation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) (Emerit *et al.*, 2004).

Nitric oxide (NO) is a free radical synthesised from *L*-Arginine by the enzyme nitric oxide synthase (NOS). It is an important signalling molecule that is involved in a variety of physiological processes such as vasodilation, immune response and neurotransmission. Due to its free radical properties, an excess of NO has been associated with neurodegeneration and subsequently the development of neurodegenerative disorders (Low, 2005). There are three

isoforms of NOS that exist including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Erdal *et al.*, 2005). In the mitochondria, NO reacts with the superoxide anion to form peroxynitrite, which has a pro-apoptotic effect and is damaging to tissues and may lead to the development of neurodegenerative disorders (Low, 2005; Singh & Dikshit, 2007).

The NMDA receptor is unique, in that it requires the binding of two agonists, namely glutamate and glycine for activation (Scatton, 1993; Danysz & Parsons, 1998; Klein & Castellino, 2001). The NMDA receptor in its active state allows the influx of sodium and calcium ions which activates calcium dependant nNOS, leading to an increased amount of NO within the neuronal cells. Although calcium ions are important for cell growth, survival and physiological functioning, an excess is responsible for excitotoxicity, which can ultimately lead to neurodegeneration (Lynch & Guttman, 2002).

The amino-adamantane derivatives amantadine and memantine are low affinity uncompetitive antagonists which display fast blocking or unblocking effects at NMDA receptor channels and bind to the channel when it is in an open state which makes them better tolerated than high affinity channel blockers such as MK-801 (dizocilpine) and phencyclidine (PCP). These compounds (amino-adamantanes, MK-801 and PCP) bind to the PCP binding site located in the NMDA receptor/ion complex (Dingledine *et al*., 1999; Parsons *et al.*, 1999).

Current 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 (Geldenhuys *et al.*, 2005). Neurodegenerative disorders can be treated in one of three possible ways. Firstly, through 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 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). Although the initial multiple action drugs were discovered accidentally, medicinal chemists are now involved in the deliberate synthesis of such ligands. Such compounds are designed rationally with the intention of modifying a disease at various targets while ensuring safety by minimising side effects, as well as improving patient compliance (Morphy *et al.*, 2004).

Polycyclic cage derivatives such as pentacycloundecane (PCU) and amantadine are useful as drug scaffolds and also improve the pharmacokinetics as well as pharmacodynamics of privileged moieties connected to it. The lipophilicity of these privileged moieties is also improved, which enables them to cross the blood brain barrier and have secondary neuroprotective effects in the CNS (Geldenhuys *et al.*, 2005). The actions of one such neuroprotective polycyclic cage compound, NGP1-01, include voltage-gated calcium channel (VGCC) blockage and NMDA receptor inhibition. A distinct structural similarity exists between NGP1-01 and the amino-adamantanes (Geldenhuys *et al.*, 2003; Geldenhuys *et al.*, 2005).

Since NO is involved in regulatory processes in various tissues in the body, selective inhibition of one particular isoform over the others is of extreme importance in order to avoid complications. If an inhibitor is not selective for nNOS and inhibits eNOS as well, it will cause a change in the blood pressure homeostasis of the body and could end up being harmful to the patient (Collins *et al*., 1998; Li & Poulos, 2005; Lawton *et al.*, 2009).

The rationale for this study was that novel nNOS selective inhibitors could be synthesised by attaching different functional groups to an amantadine moiety *via* a benzene linker (fig. 1.1) as this would significantly improve their blood brain barrier (BBB) permeability since amantadine is known to be highly permeable across the BBB. The compounds may also show NMDA and calcium channel inhibitory activity due to the amantadine moiety and the structural similarity thereof towards NGP1-01, thereby making them potential multifunctional neuroprotective agents. We therefore decided to synthesise a series of adamantane derivatives bearing a structural similarity to the lead compound NGP1-01.

The primary aim of the current study was thus to design novel structures with increased neuroprotective activity which would be effective through a multiple mechanism of action, owing to the potential NOS inhibitory effect of the structures, as well as the documented antagonistic action of the cage amines on NMDA receptors (NMDAR) and VGCC. These compounds would then be evaluated for biological activity against NOS, NMDAR and VGCC as a proof of concept for multifunctional neuroprotective activity.

5.2 Synthesis

A series of compounds were synthesised by conjugating the amantadine moiety to a phenyl linker with different functional groups (-NO_2, -NH_2, -$NHC(NH)NH_2$, -OCH_3) attached at the *ortho*, *meta* and *para* positions of the phenyl moiety. The compounds were characterised by nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared (IR) spectroscopy.

The general synthetic route followed was initiated through the conjugation of a nitrobenzyl bromide to the amantadine free base, followed by reduction of the nitro group to an amine, and subsequent conversion of the amine to a guanidine (fig. 3.2). In order to see the effect of substitution at different positions on the aromatic ring 2-, 3- and 4-nitrobenzyl bromide were used, to yield the *ortho*, *meta* and *para* products, respectively.

The preparation of the guanidines SE-8, SE-9 and SE-10, was unsuccessful. It is postulated that the guanidines (SE-8, SE-9 and SE-10) could not be prepared due to steric hindrance of the aromatic amines (SE5, SE-6 and SE-7), as well as the electronic deactivation of the aromatic amine by the amino-linker group which ultimately reduces the reactivity of the aromatic amine and thus prevent formation of the guanidine (Katritzky *et al.*, 2005).

Since compounds containing a methoxy and/or nitro substituent(s) have been shown to increase both NMDAR and VGCC activity (Geldenhuys *et al.*, 2007), a group of methoxy compounds (SE-12, SE-13 and SE-14; table 3.1) were to be synthesised. SE-12 and SE-13 were synthesised using MW chemistry. However, the synthesis of SE-14 was unsuccessful. This could be as a result of steric hindrance (to the reaction of the amine group) due to the methoxy substituent being in the *ortho* position of the benzene ring. The starting compound used for the synthesis of SE-12 was 1-bromoadamantane.

In order to observe the effect of an increase in chain length, SE-11 was synthesised. It has an ethyl linker between the amantadine amine and the benzene group as opposed to SE-1 which has a methyl linker.

Flash column chromatography was mostly used in the purification of the compounds. Other methods applied included acid-base extractions. The synthesis of the 10 successful compounds resulted in yields between 16.5 % and 90.25 %. The lower yields could be attributed to the formation of various side products as well as the choice of purification method (extraction as

opposed to column chromatography). These compounds were adequately purified and characterised for biological evaluation.

5.3 Biological evaluation

Due to a lack of success in synthesising the guanidine compounds SE-8, SE-9 and SE-10, these compounds could not be tested for nNOS inhibitory activity using the oxyhemoglobin capture assay.

The 10 successfully synthesised compounds were evaluated for NMDAR- and VGCC-mediated calcium influx inhibition. These assays were conducted on synaptoneurosomes obtained from rat brain homogenate. The activity of test compounds (100 µM) was measured against various controls (MK-801, NGP1-01, amantadine, memantine and nimodipine) of known inhibitory activity. The assays were conducted using a fluorescent plate reader and the fluorescent ratiometric calcium indicator FURA-2 AM. In the NMDA assay, test compounds SE-2 (60.6 %), SE-4 (74.8 %), SE-1 (66.7 %), SE-11 (89.5 %), SE-13 (70.2 %) and SE-12 (79.2 %) all had inhibitory activity upwards of 50 % (fig. 4.1). For the VGCC, SE-4 (85.7 %) had a percentage inhibition of higher than 80 % (fig. 4.2), while SE-1 (58.8 %), SE-11 (61.8 %) and SE-12 (71.2 %) displayed good inhibitory activity (> 50 %). All compounds were tested in triplicate, with mean and standard deviation values calculated and all data was statistically significant ($p < 0.05$) as shown by the student t-test.

5.4 Conclusion

There is a dire and urgent need of drugs possessing multifunctional neuroprotective activity. Drugs possessing nNOS selective inhibitory activity, as well as NMDAR- and VGCC-mediated calcium influx inhibition will be of great value in the treatment of neurodegenerative disorders due to their effectiveness in curbing neurodegenerative processes at multiple points in the neurodegenerative cascade.

With this aim in mind, we set out to synthesise novel amantadine-derived structures with potential nNOS, NMDA and VGCC inhibitory activity. Of the 14 structures that were originally selected to be synthesised, 10 were successfully synthesised and purified. The guanidine

compounds could not be synthesised and their NOS inhibitory activity could thus not be evaluated. Nonetheless, the 10 successfully synthesised compounds were evaluated for biological activity against NMDAR and VGCC. Further tests are recommended on SE-1, SE-4, SE-11 and SE-12 as they displayed good inhibitory activity against both NMDAR- as well as KCl-mediated calcium influx. These novel adamantane derived compounds may be better therapeutic options than amantadine and memantine as they inhibit NMDAR and VGCC-mediated calcium influx, whereas amantadine and memantine only inhibit NMDA-mediated calcium influx. These compounds may also serve as new lead structures or potential therapeutic agents for the treatment of neurodegenerative disorders.

Additional assays on the influence on dopamine transmission, apoptosis and blood brain barrier permeability will elaborate on the potential value of these compounds.

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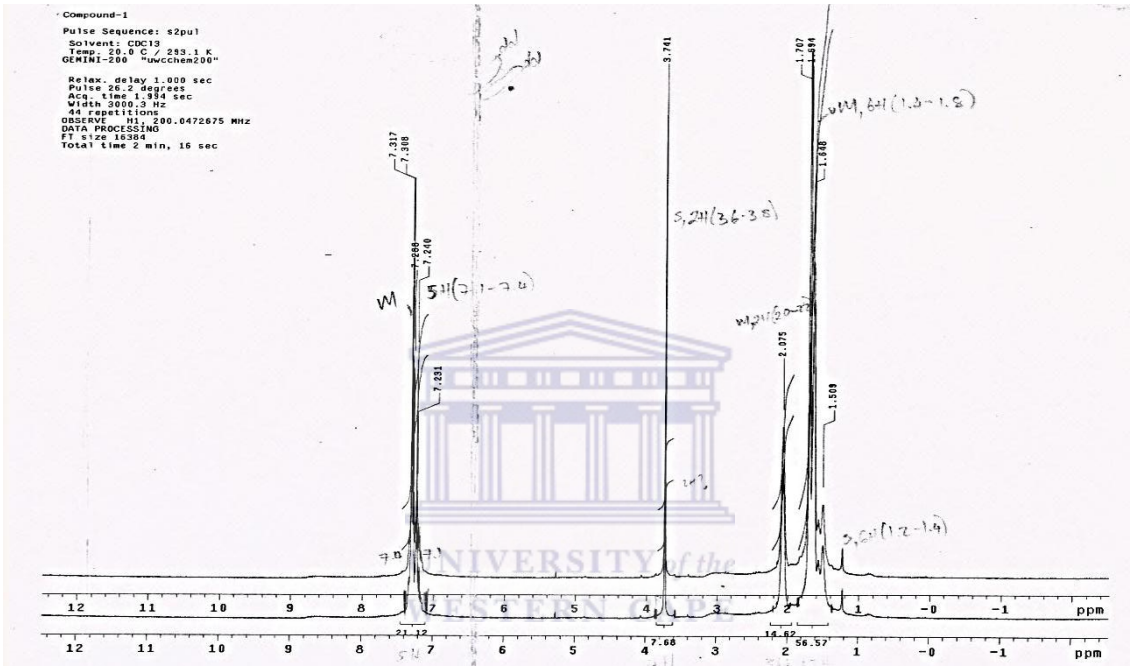
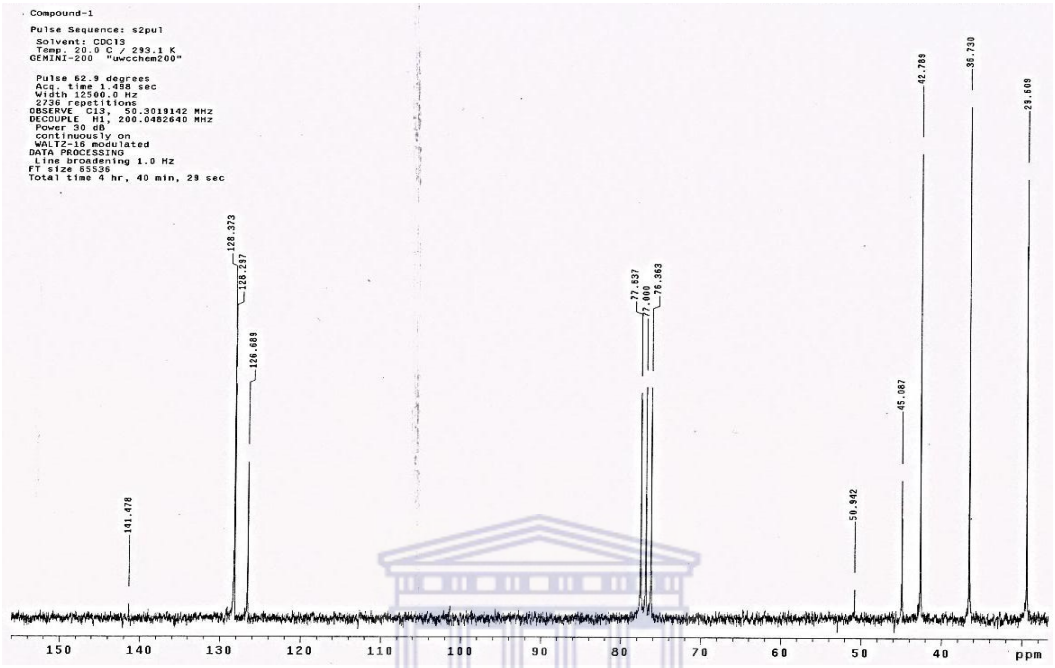
SPECTRAL DATA

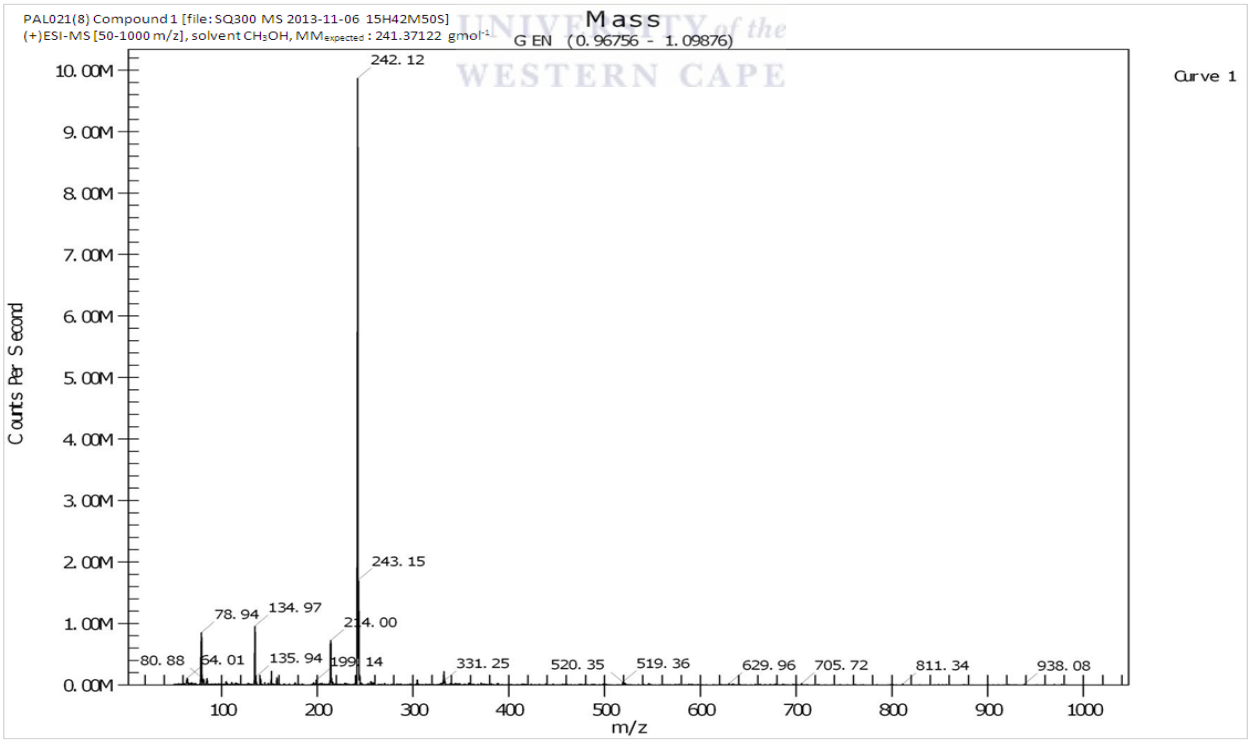
Spectrum 1: ^{1}H NMR spectrum of SE-1



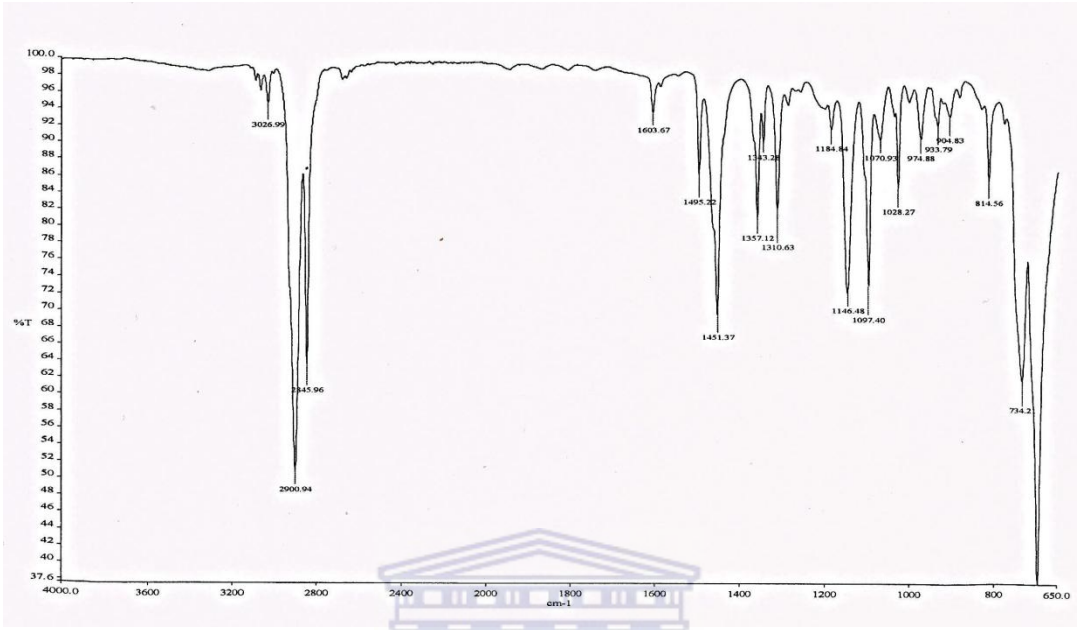
Spectrum 2: ^{13}C NMR spectrum of SE-1



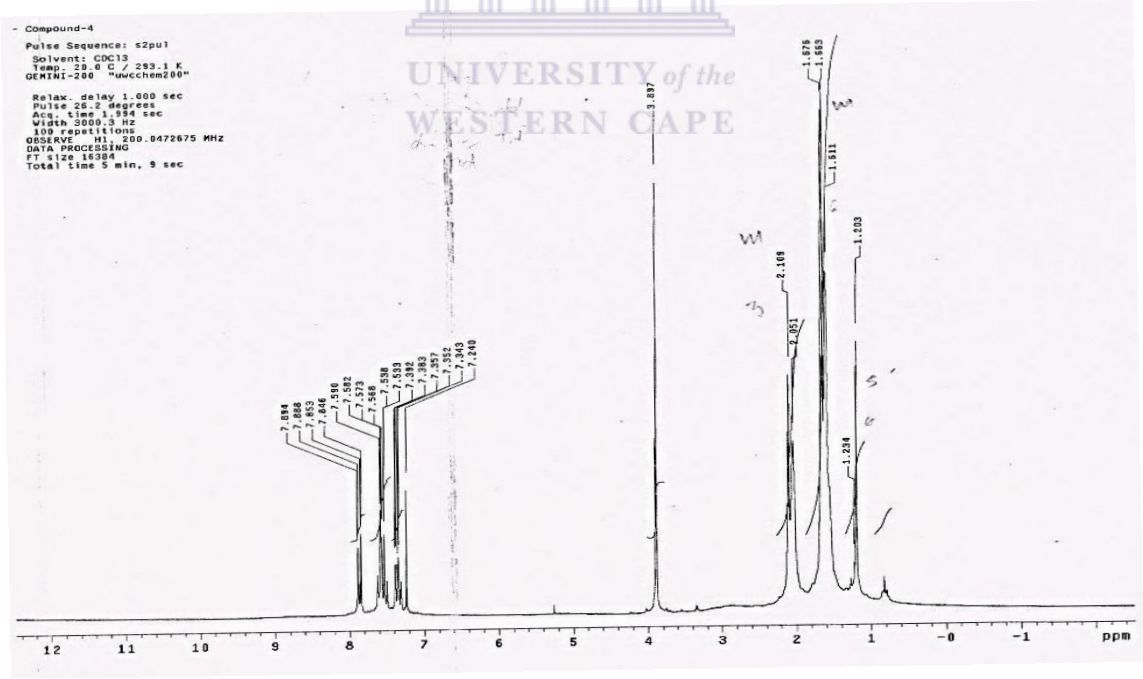
Spectrum 3: MS of SE-1



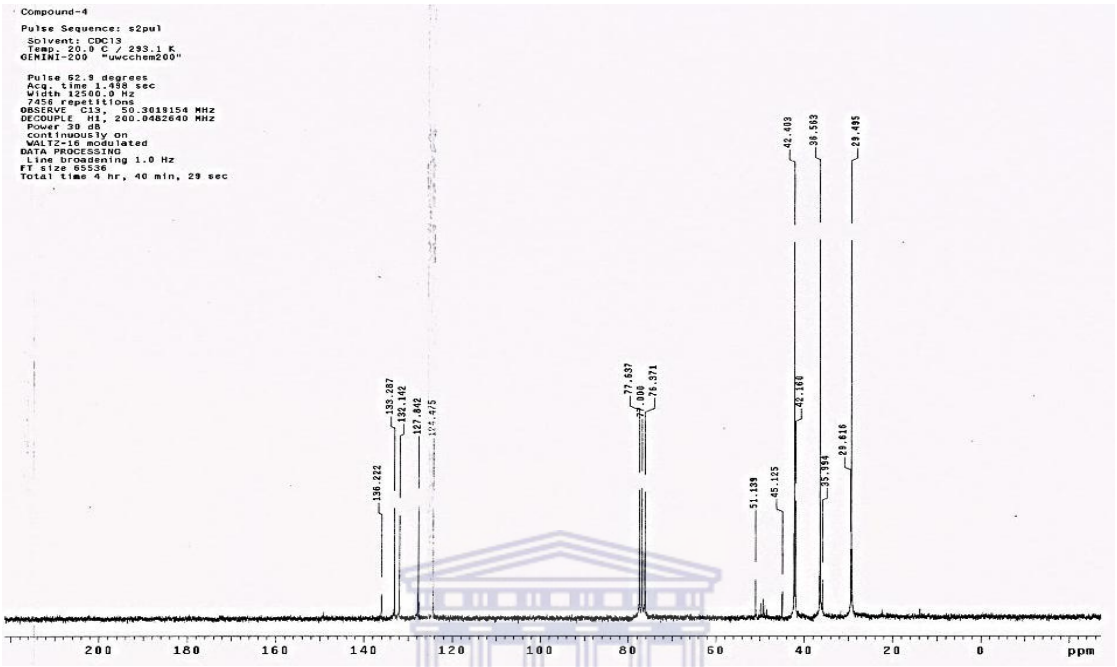
Spectrum 4: IR spectrum of SE-1



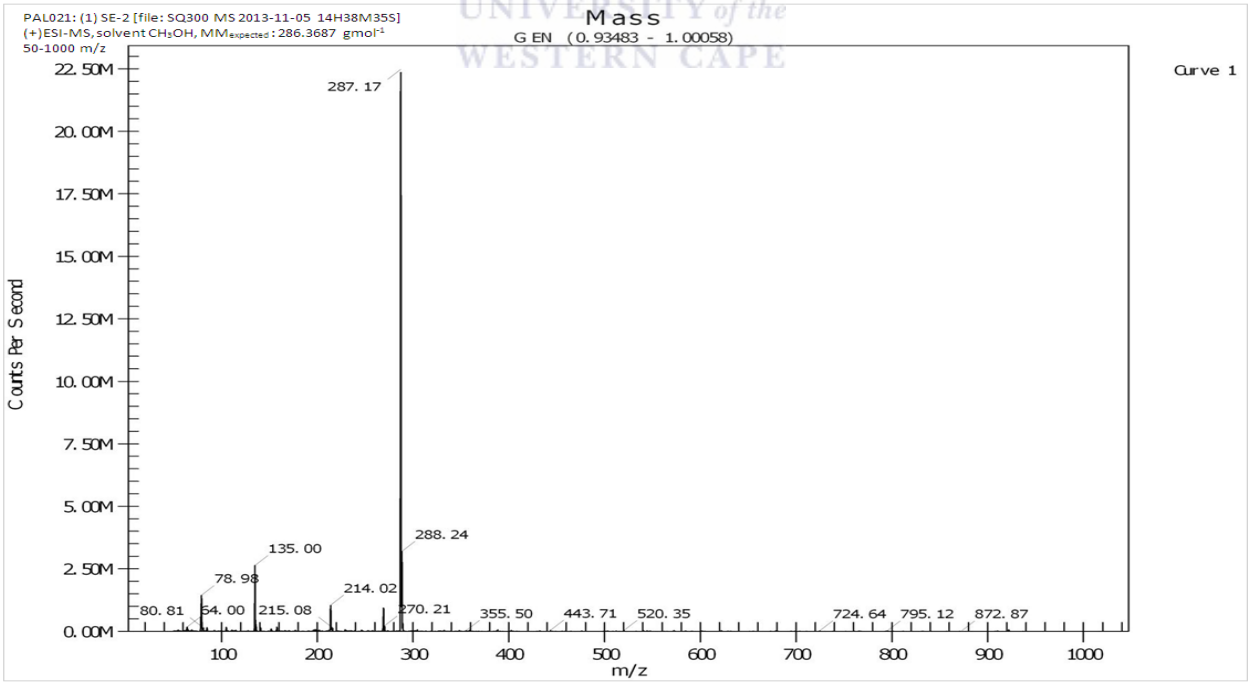
Spectrum 5: ^{1}H NMR spectrum of SE-2



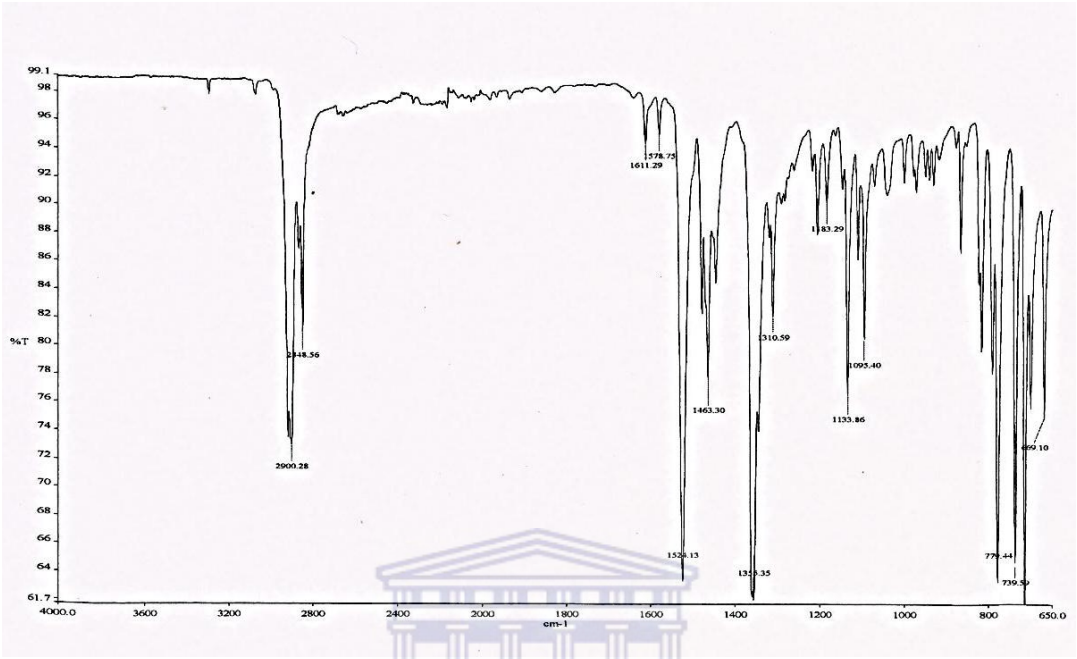
Spectrum 6: ^{13}C NMR spectrum of SE-2



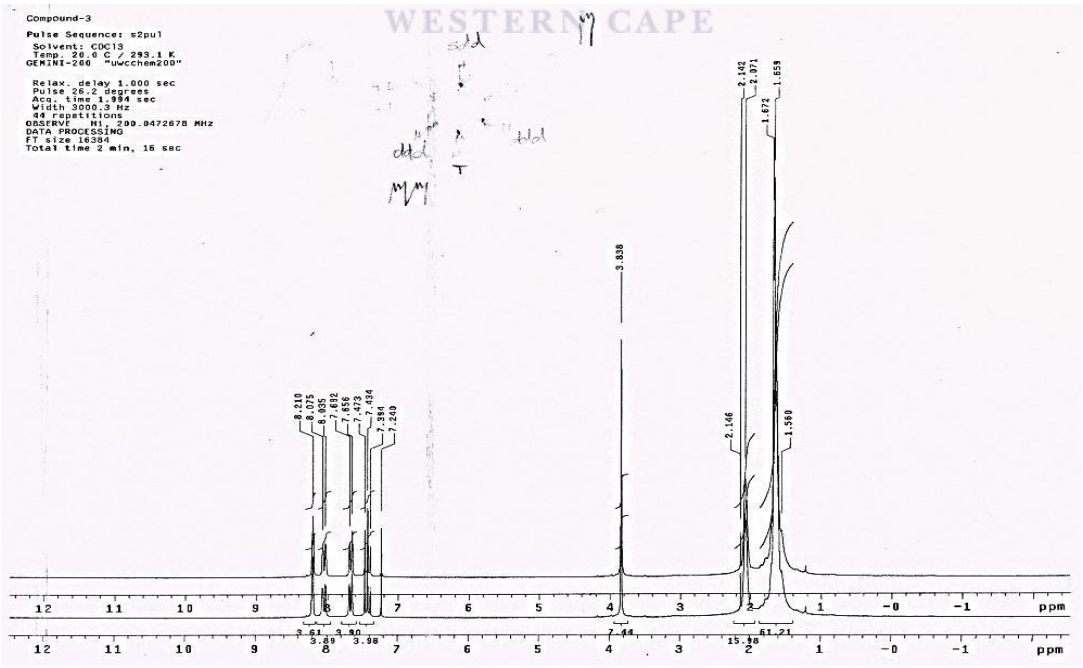
Spectrum 7: MS of SE-2



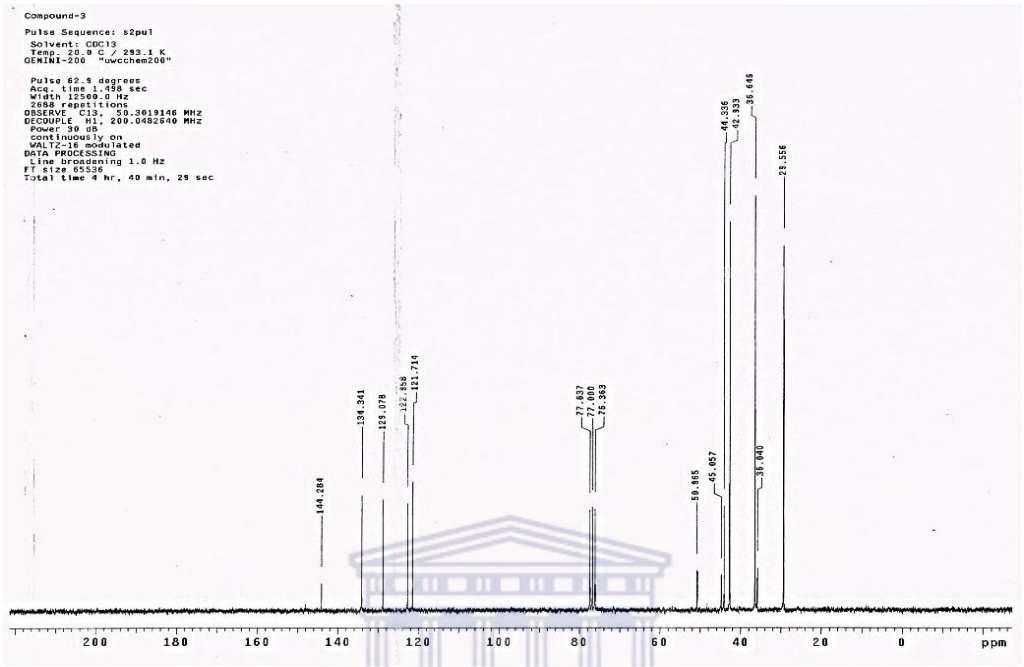
Spectrum 8: IR spectrum of SE-2



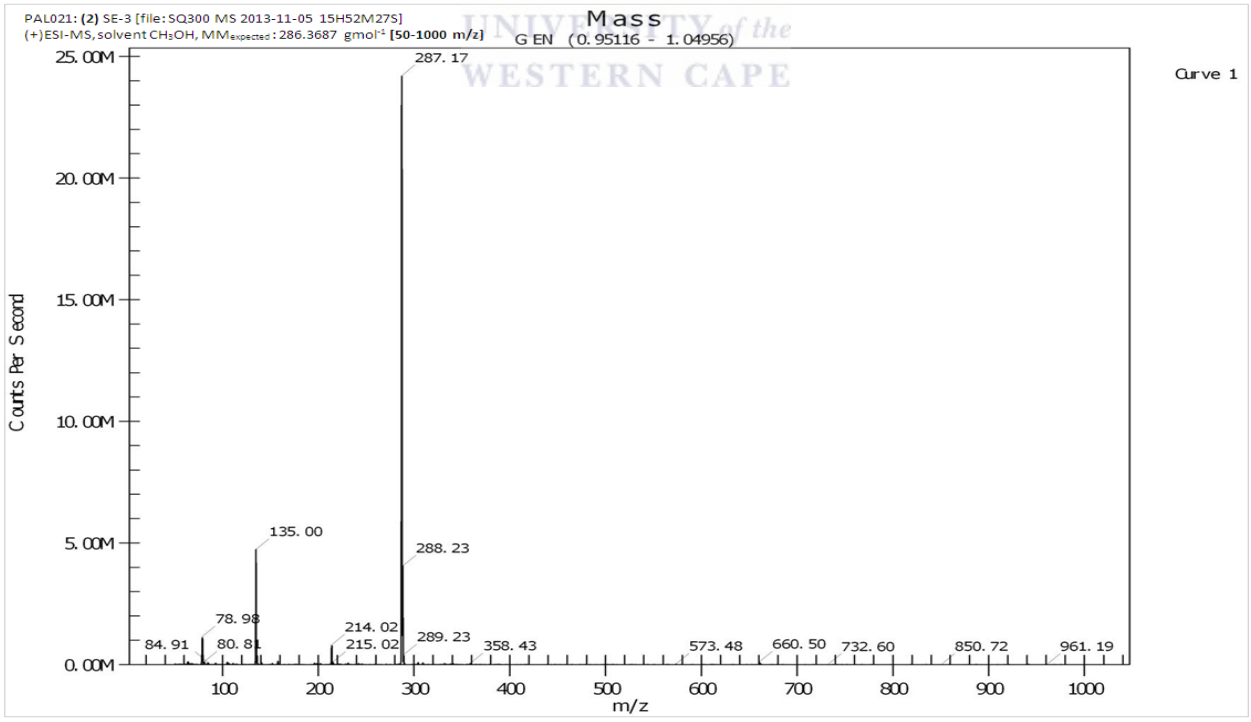
Spectrum 9: ^{1}H NMR spectrum of SE-3



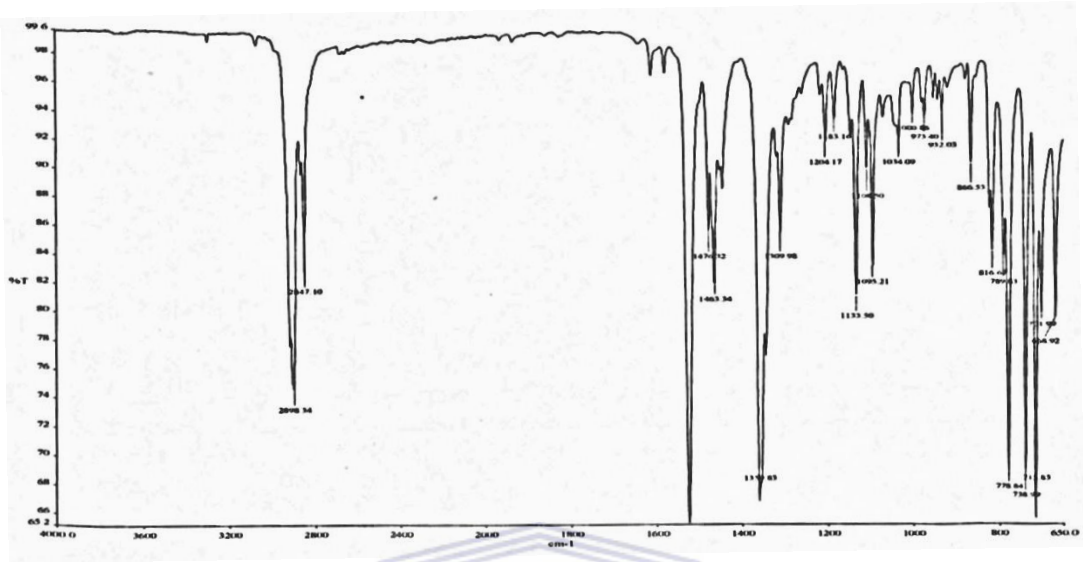
Spectrum 10: ^{13}C NMR spectrum of SE-3



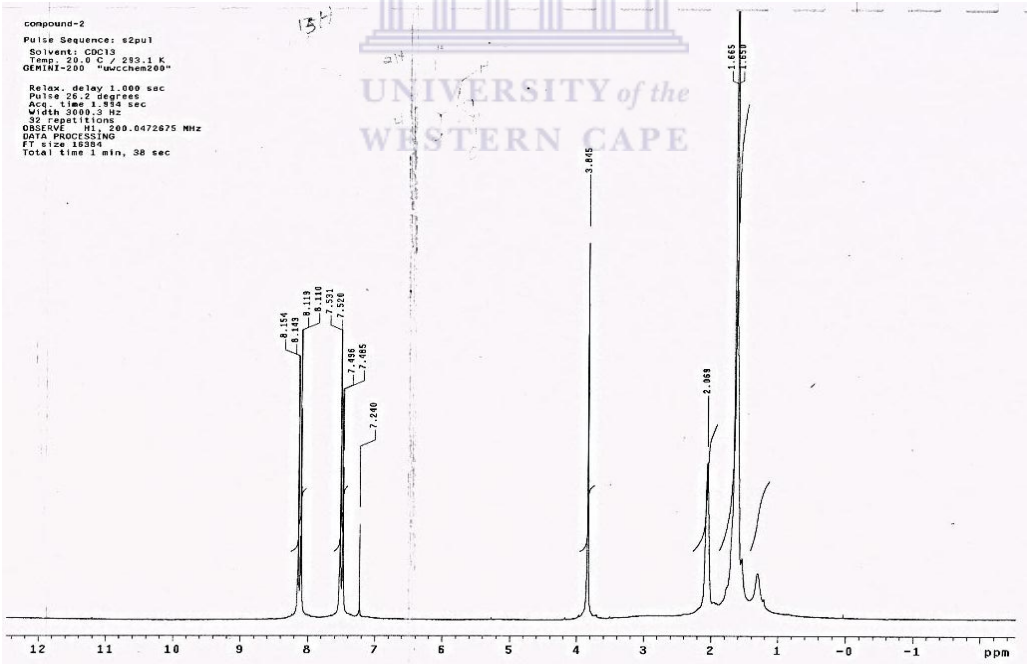
Spectrum 11: MS of SE-3



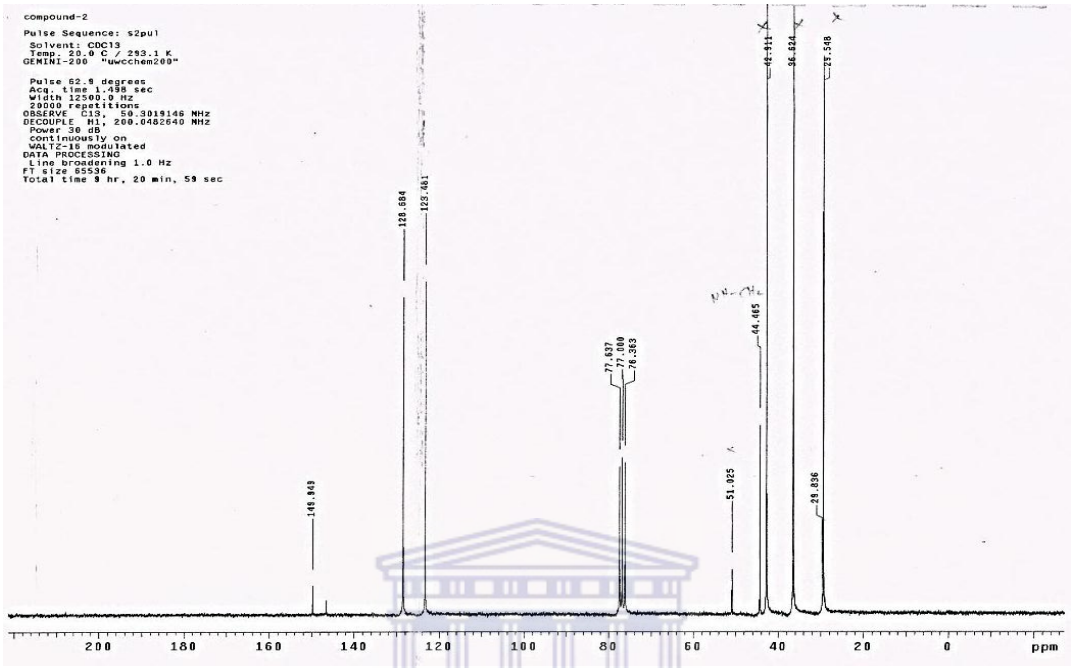
Spectrum 12: IR spectrum of SE-3



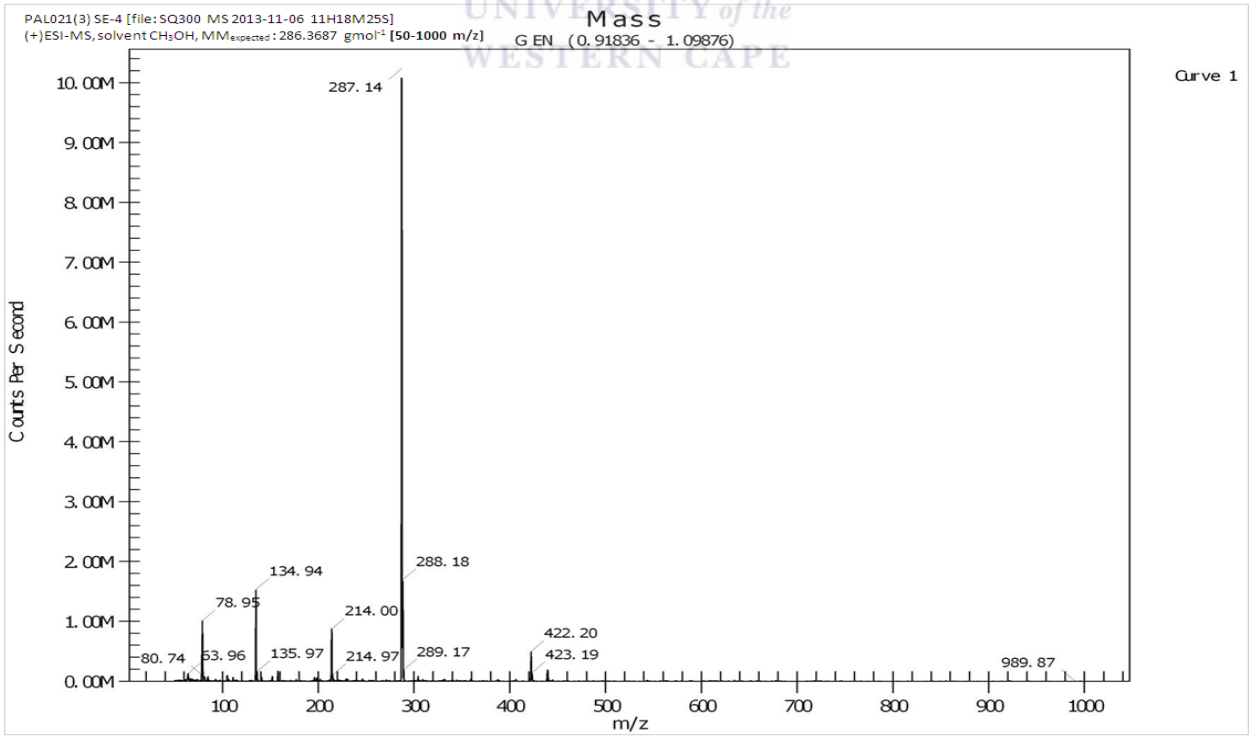
Spectrum 13: ^{1}H NMR spectrum of SE-4



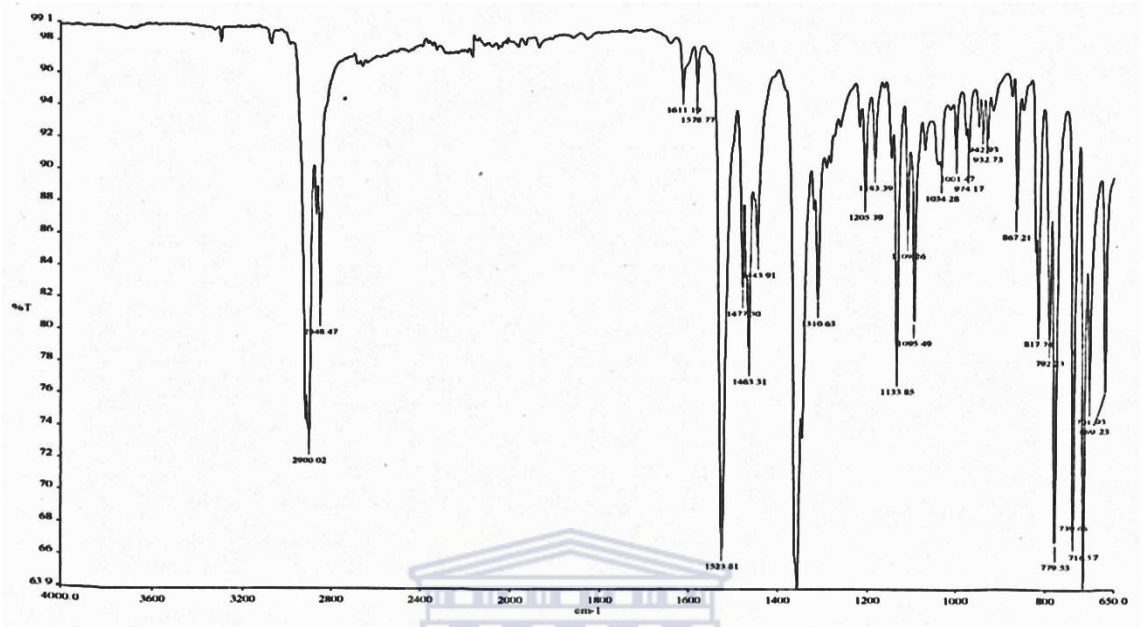
Spectrum 14: ^{13}C NMR spectrum of SE-4



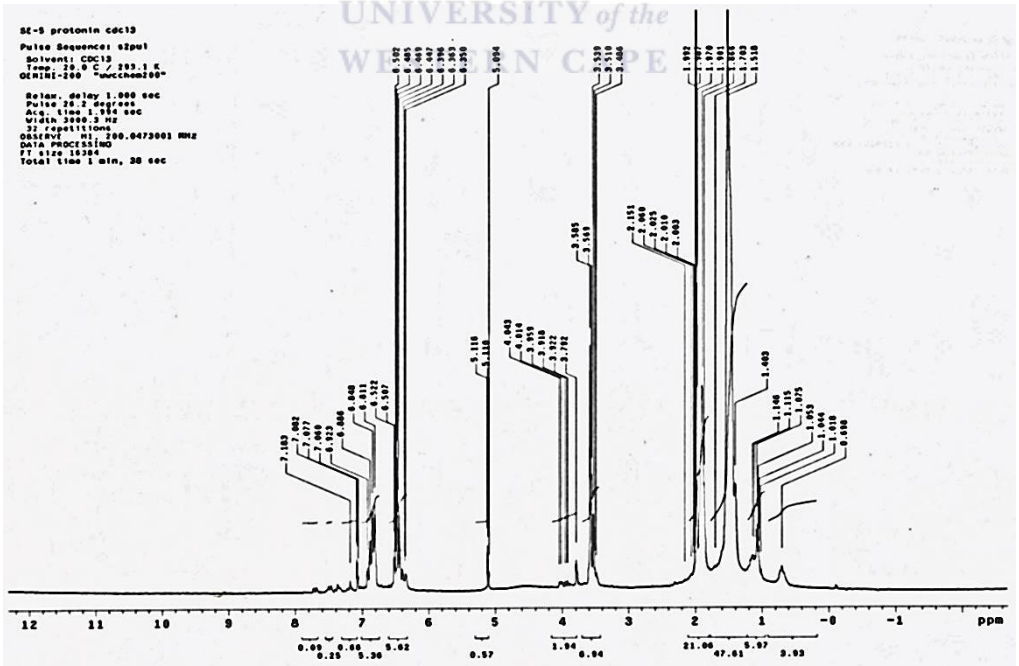
Spectrum 15: MS of SE-4



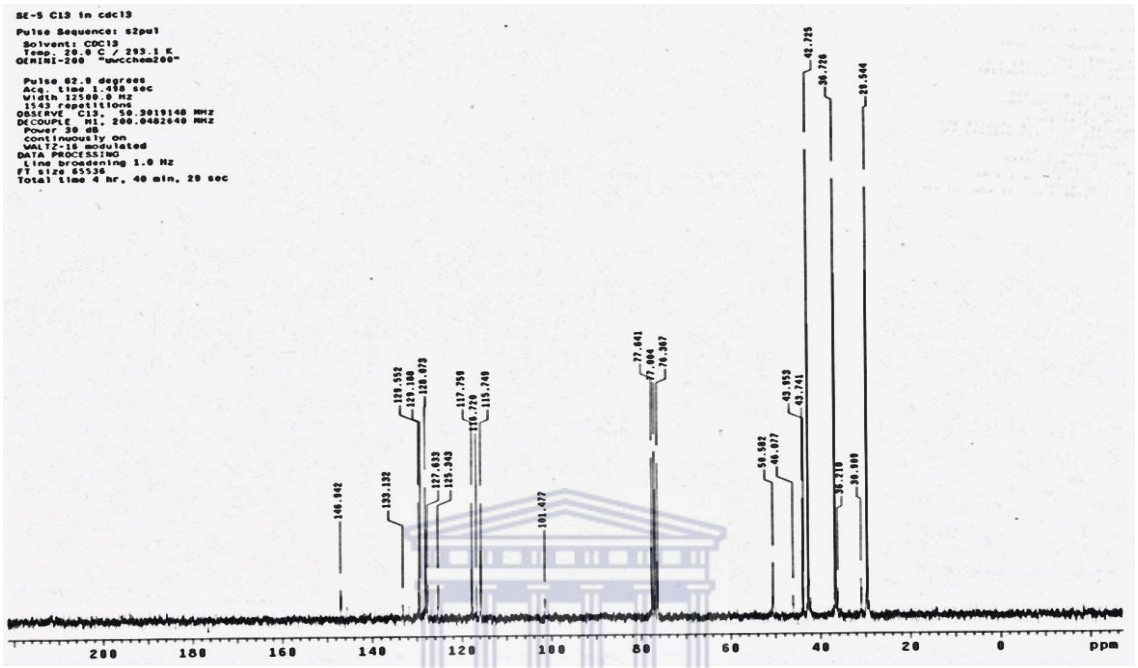
Spectrum 16: IR spectrum of SE-4



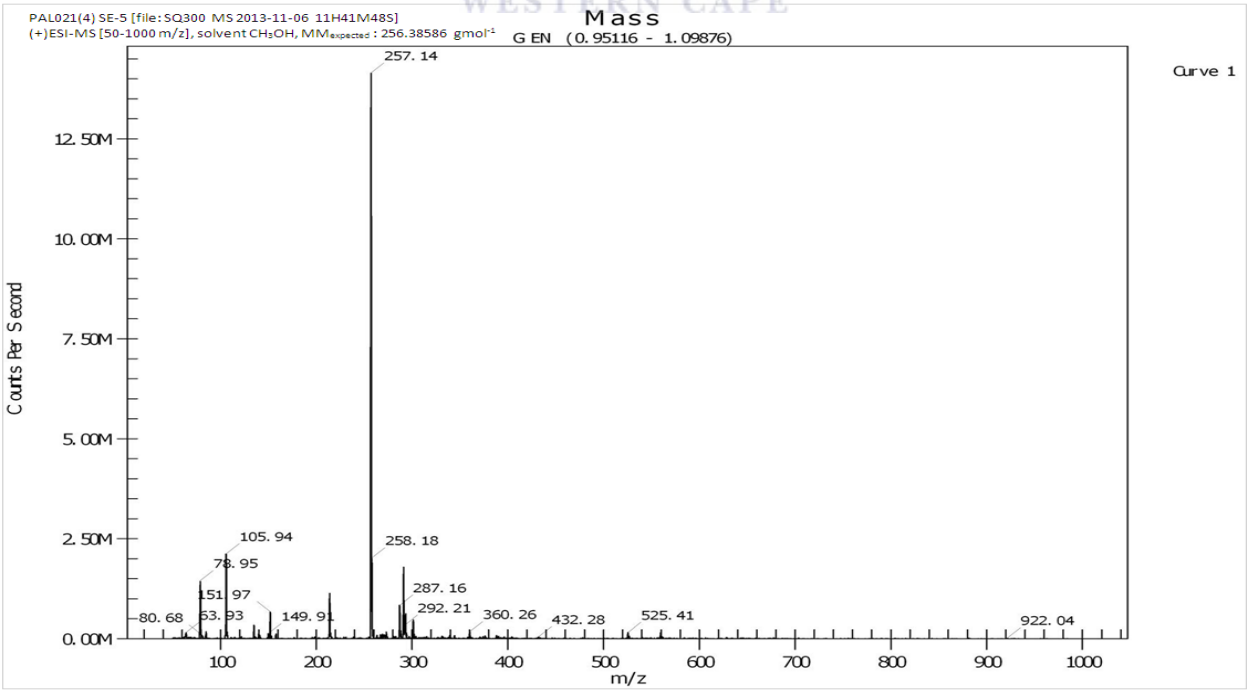
Spectrum 17: ^{1}H NMR spectrum of SE-5



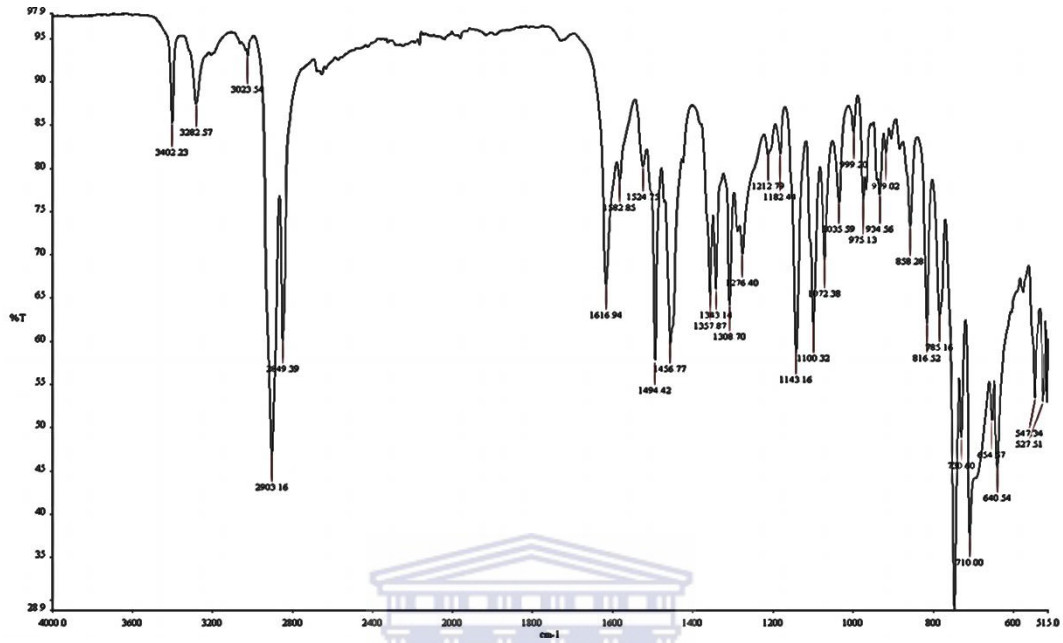
Spectrum 18: ^{13}C NMR spectrum of SE-5



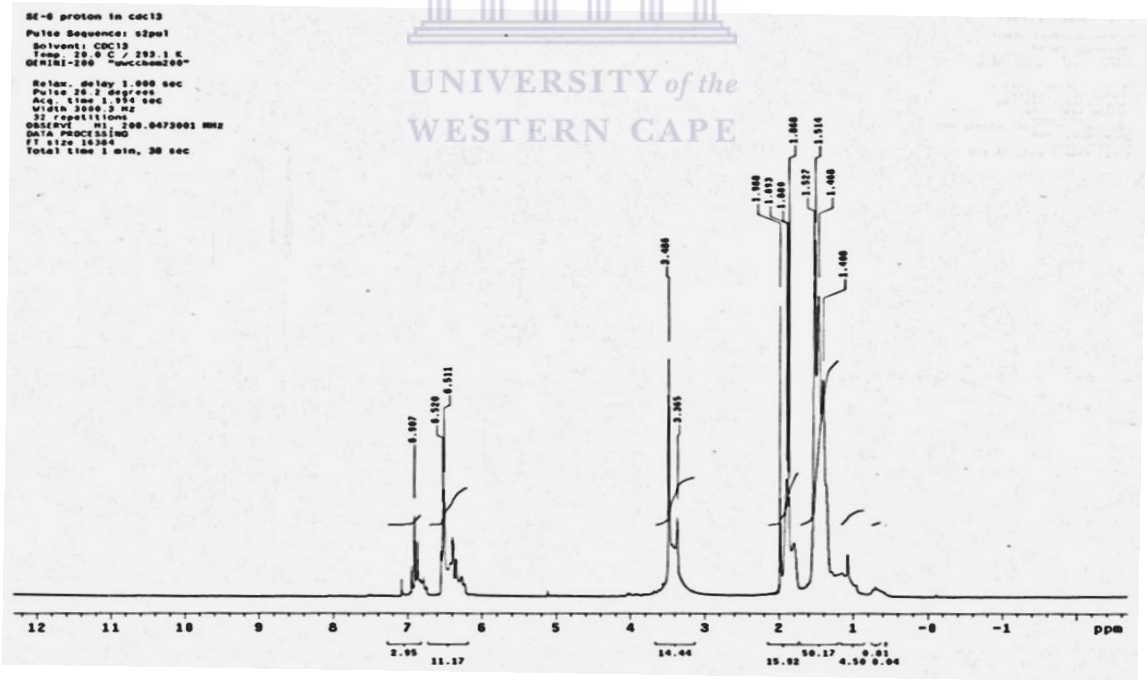
Spectrum 19: MS of SE-5



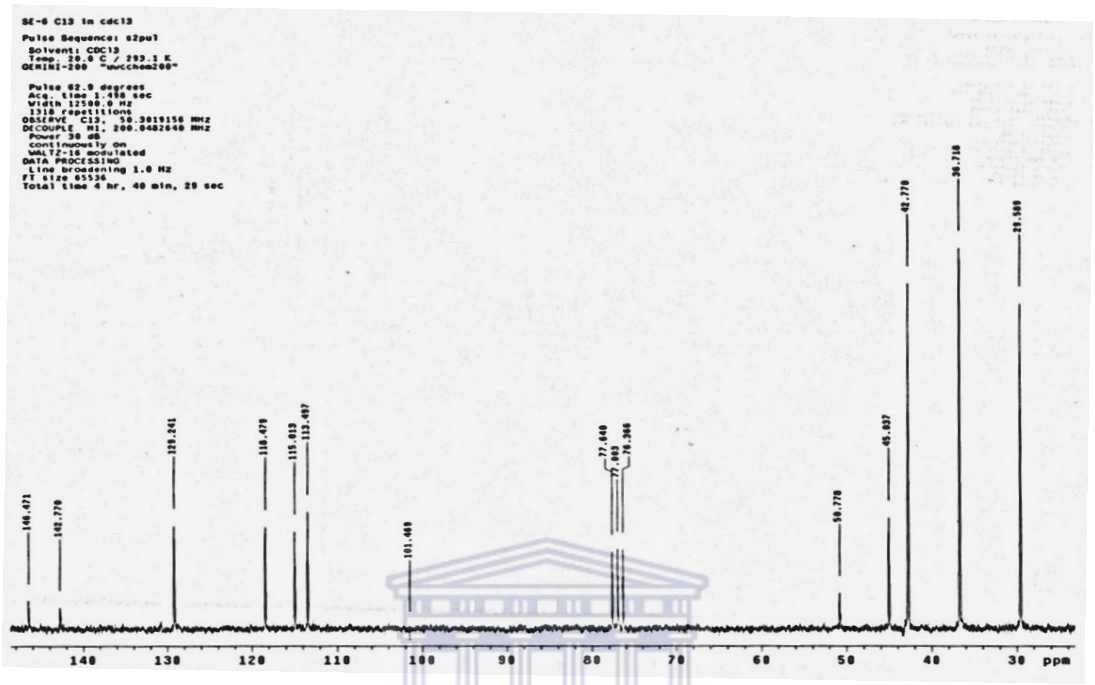
Spectrum 20: IR spectrum of SE-5



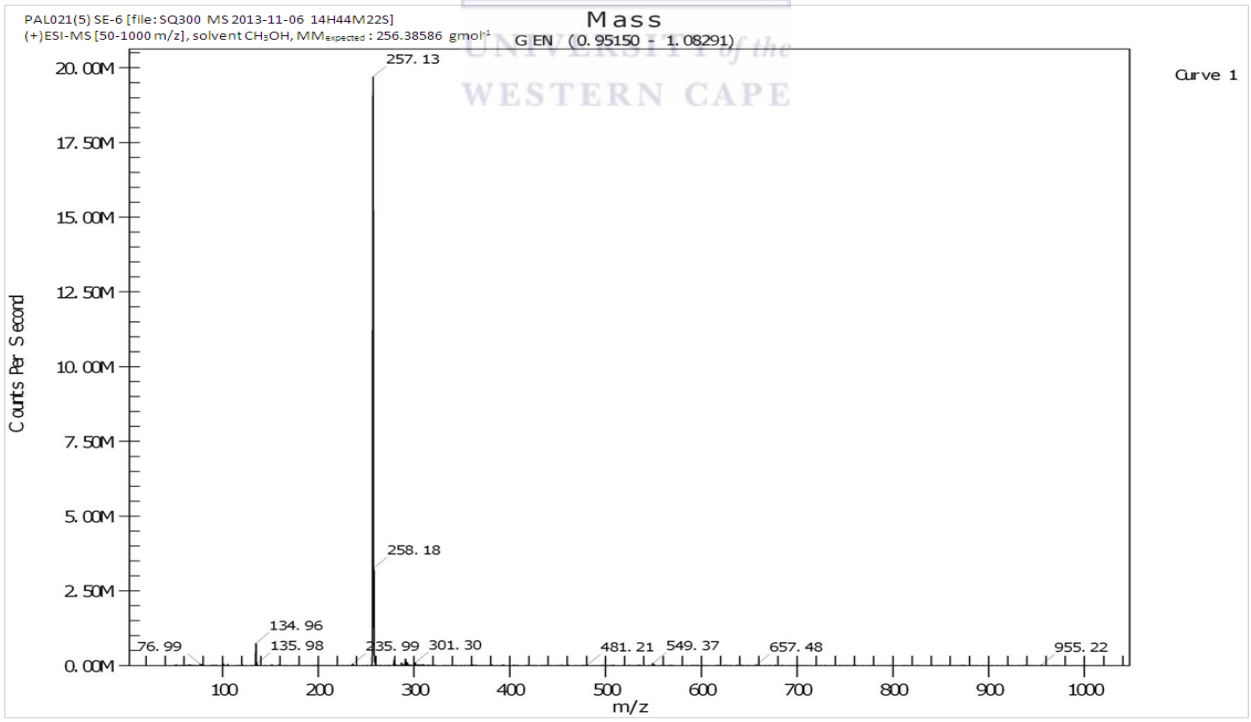
Spectrum 21: ^{1}H NMR spectrum of SE-6



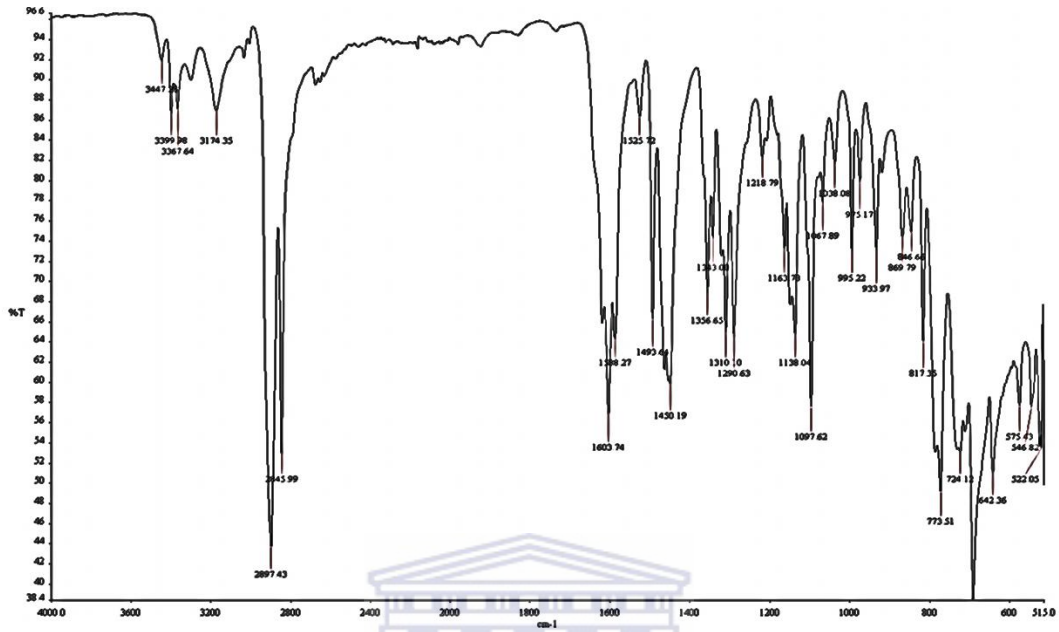
Spectrum 22: ^{13}C NMR spectrum of SE-6



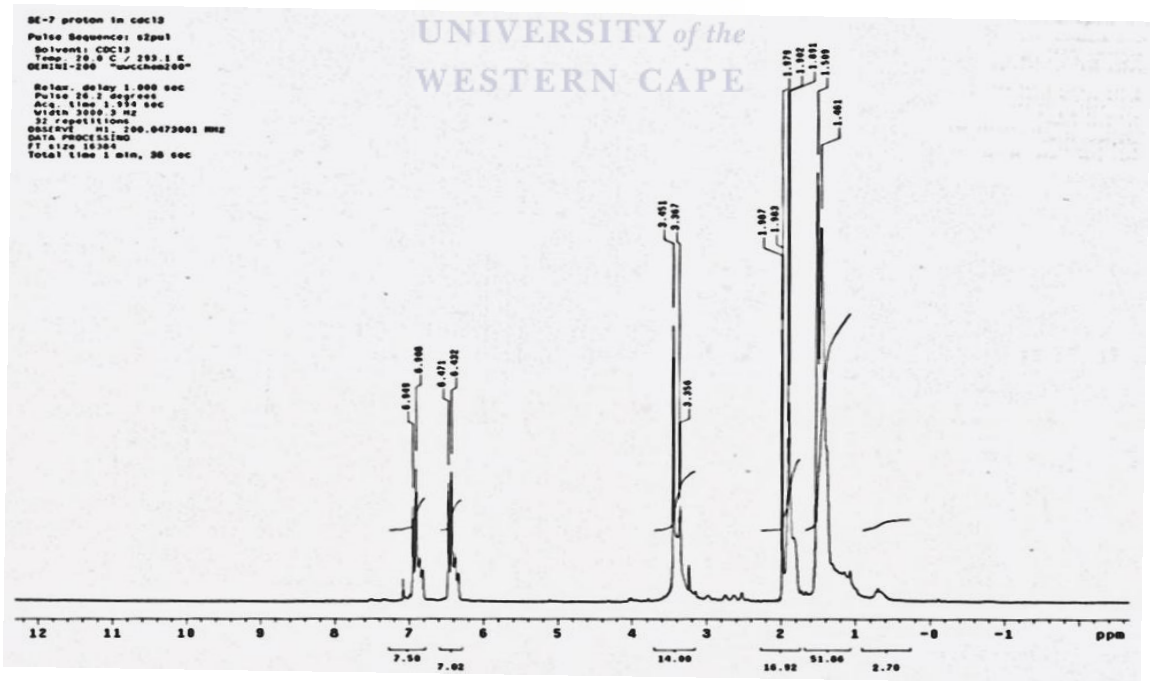
Spectrum 23: MS of SE-6



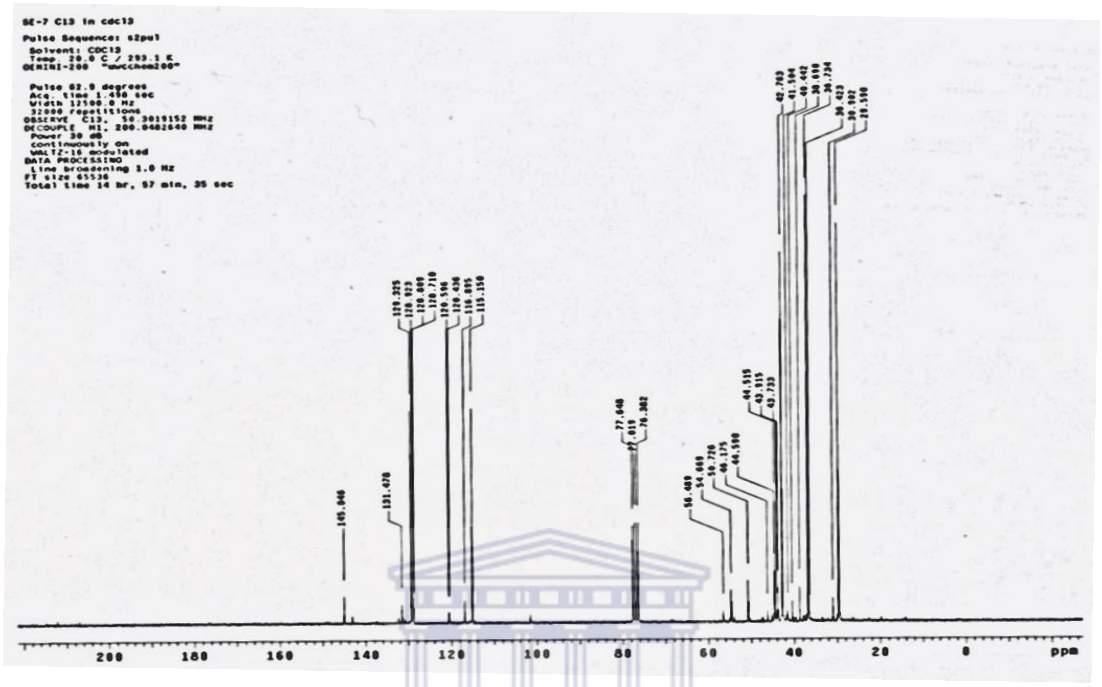
Spectrum 24: IR spectrum of SE-6



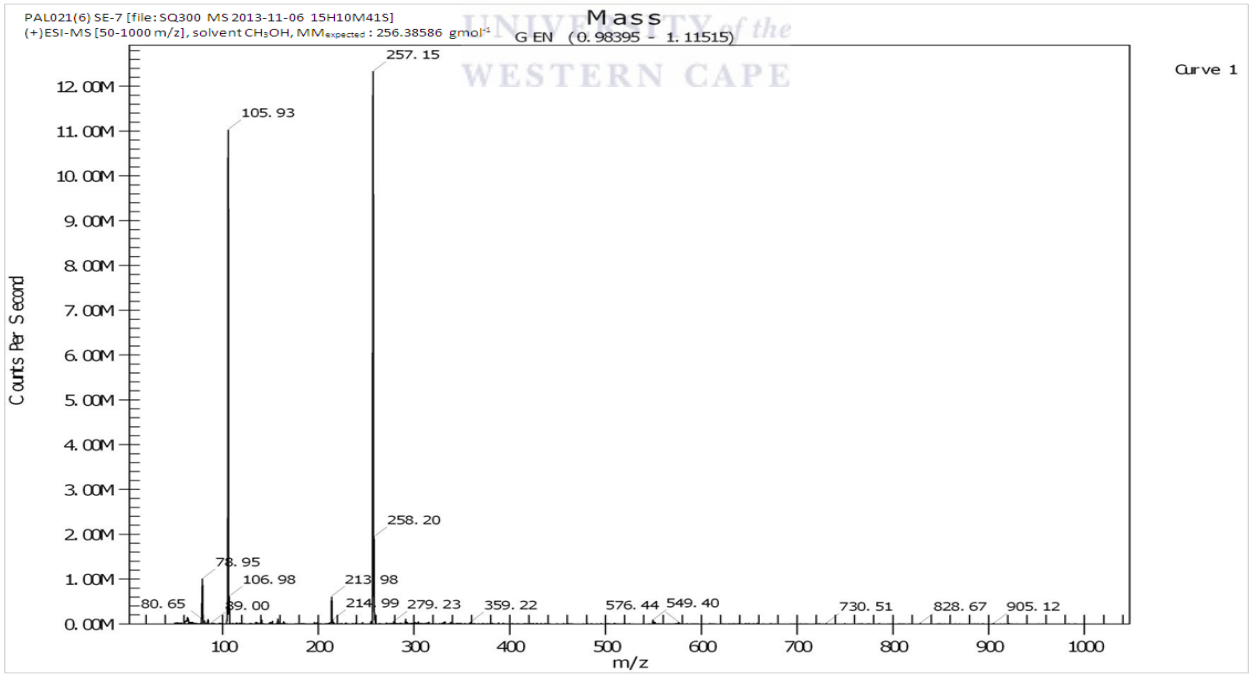
Spectrum 25: ^{1}H NMR spectrum of SE-7



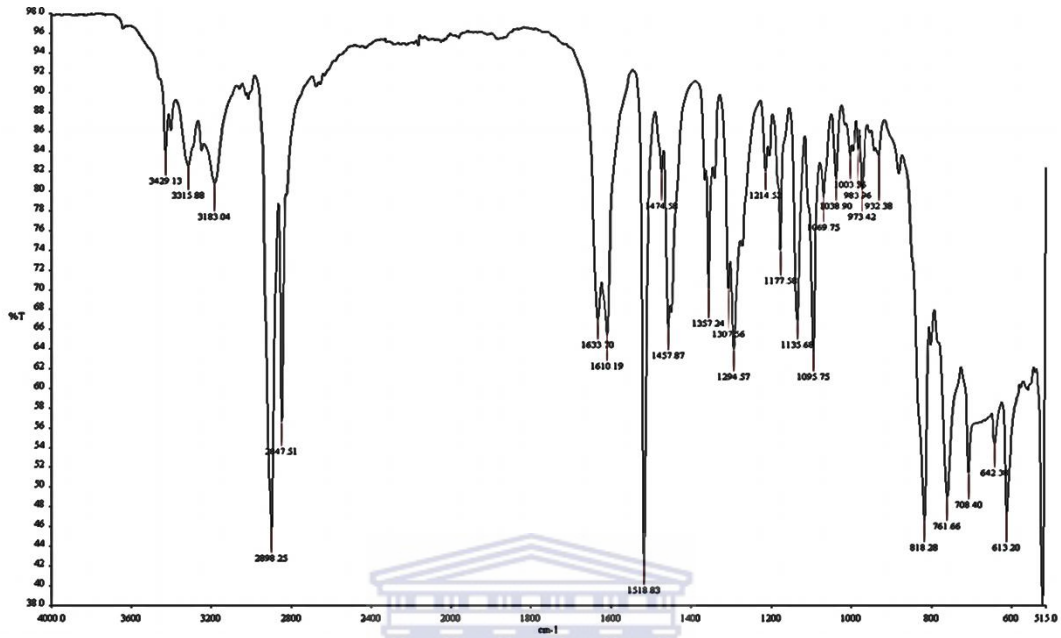
Spectrum 26: ^{13}C NMR spectrum of SE-7



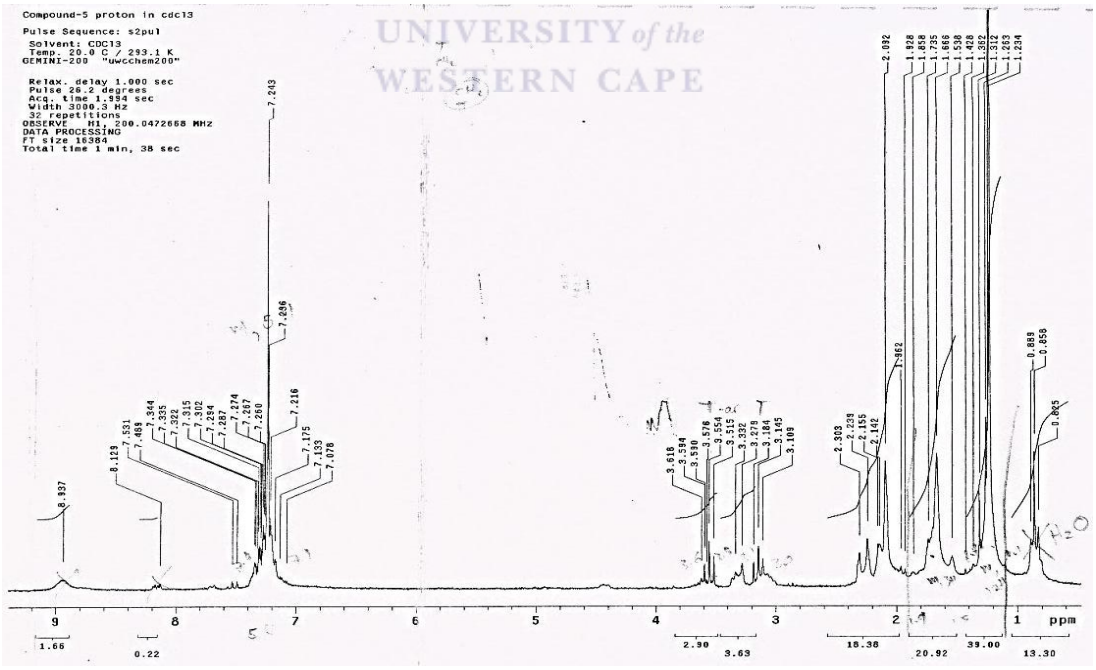
Spectrum 27: MS of SE-7



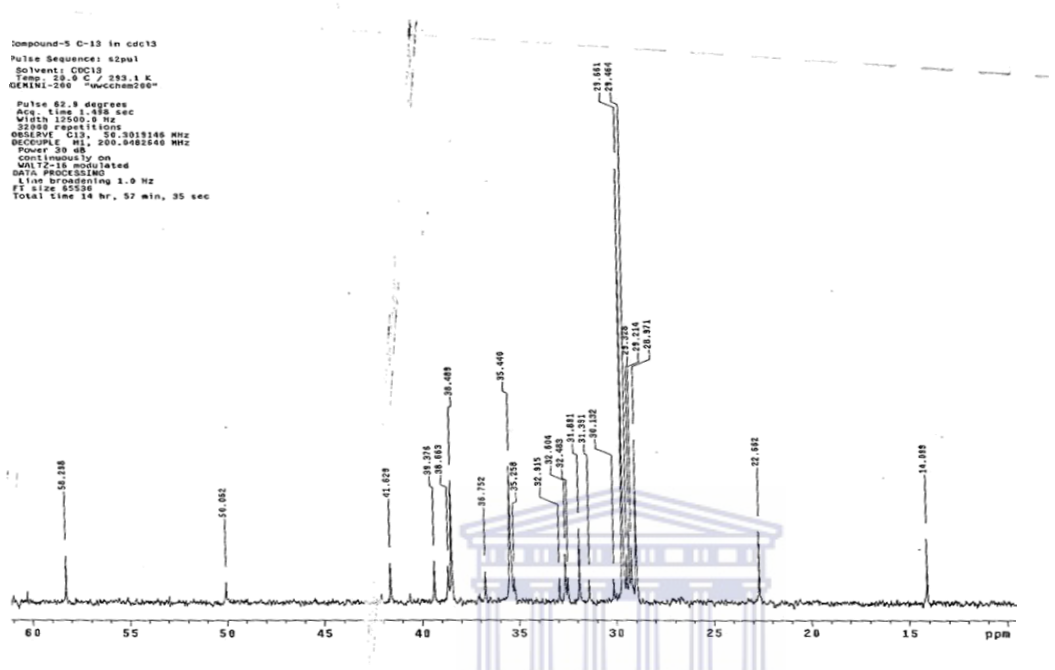
Spectrum 28: IR spectrum of SE-7



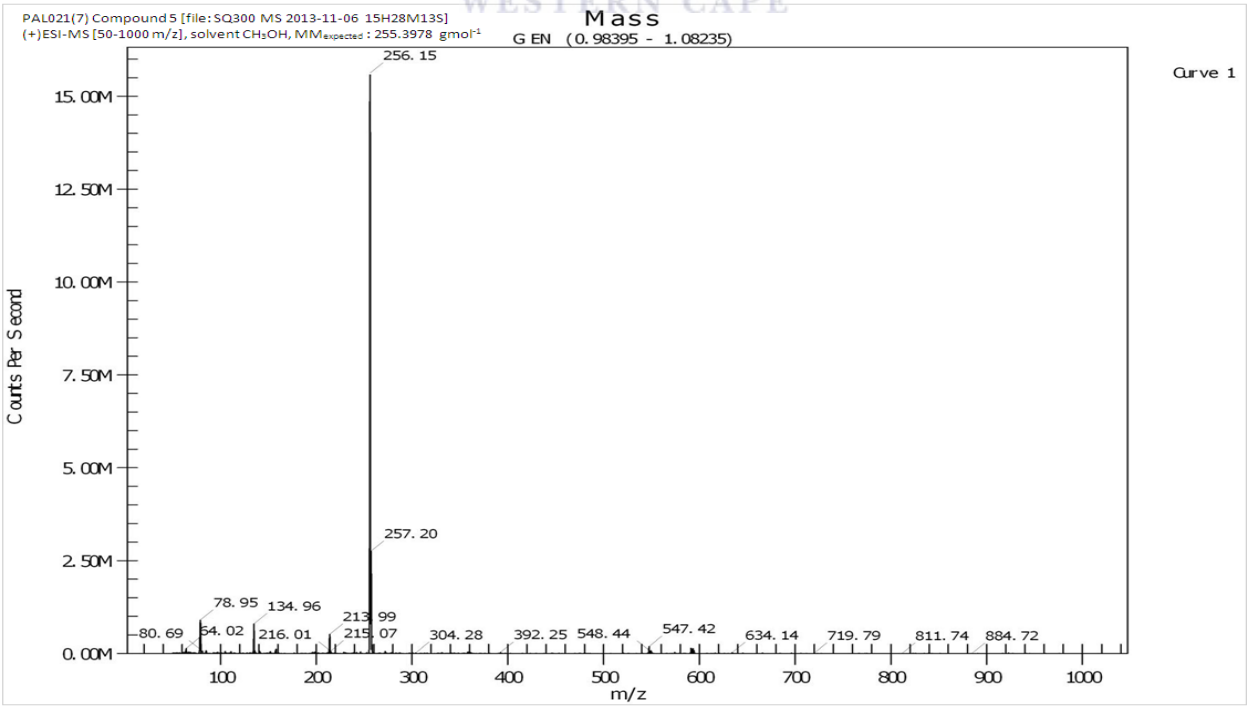
Spectrum 29: ^{1}H NMR spectrum of SE-11



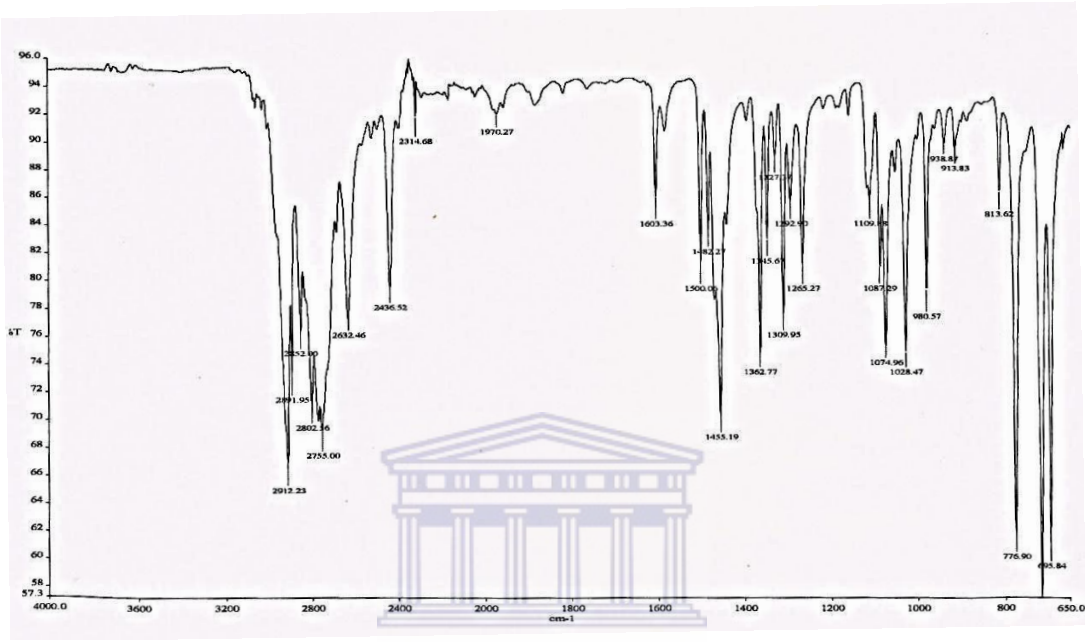
Spectrum 30: ^{13}C NMR spectrum of SE-11



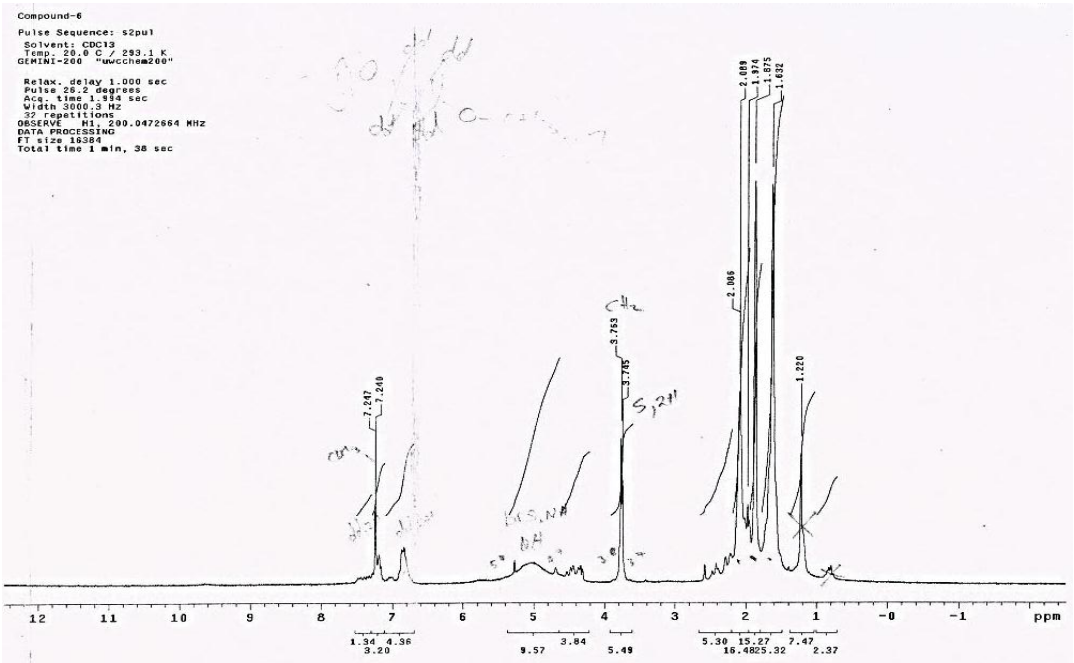
Spectrum 31: MS of SE-11



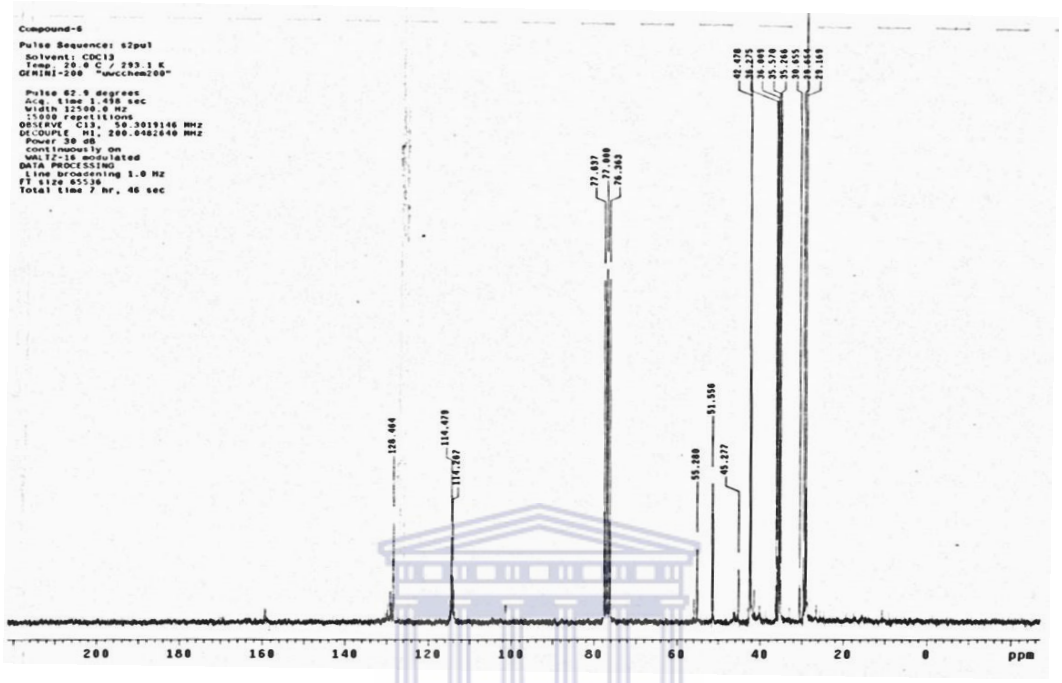
Spectrum 32: IR spectrum of SE-11



Spectrum 33: ^{1}H NMR spectrum of SE-12



Spectrum 34: ^{13}C NMR spectrum of SE-12



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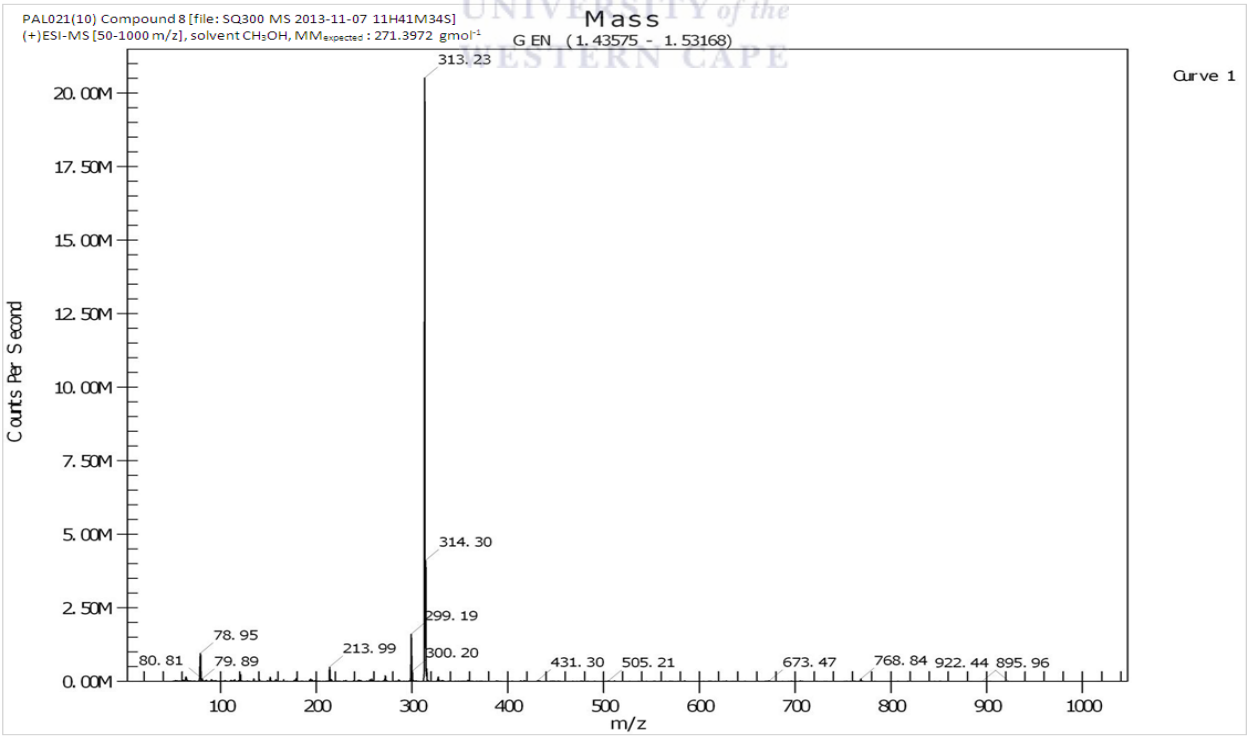
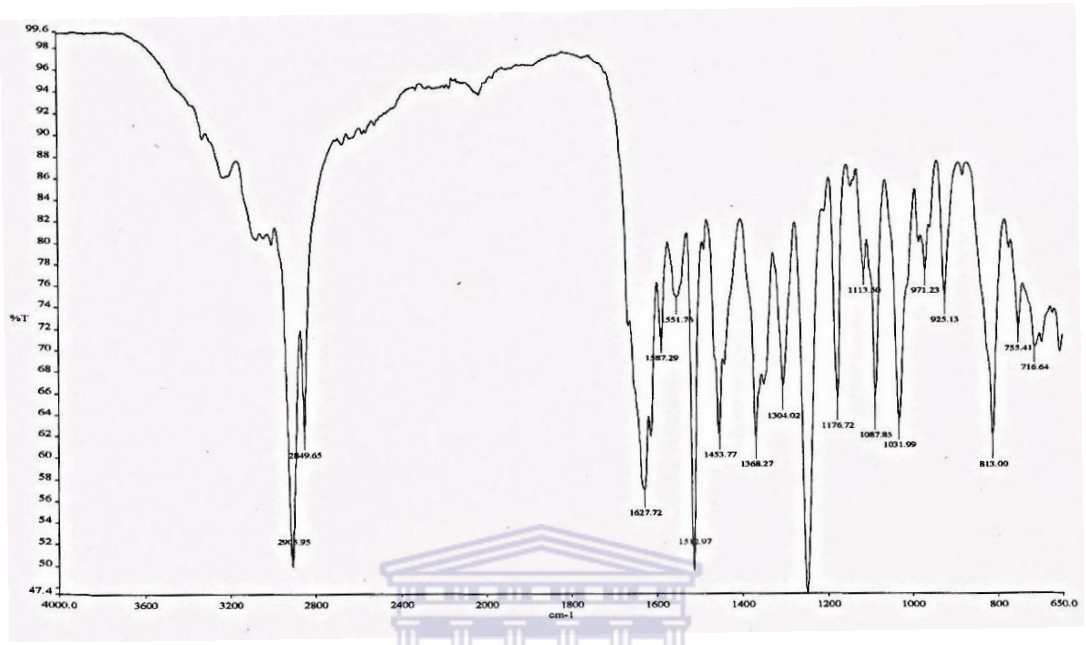
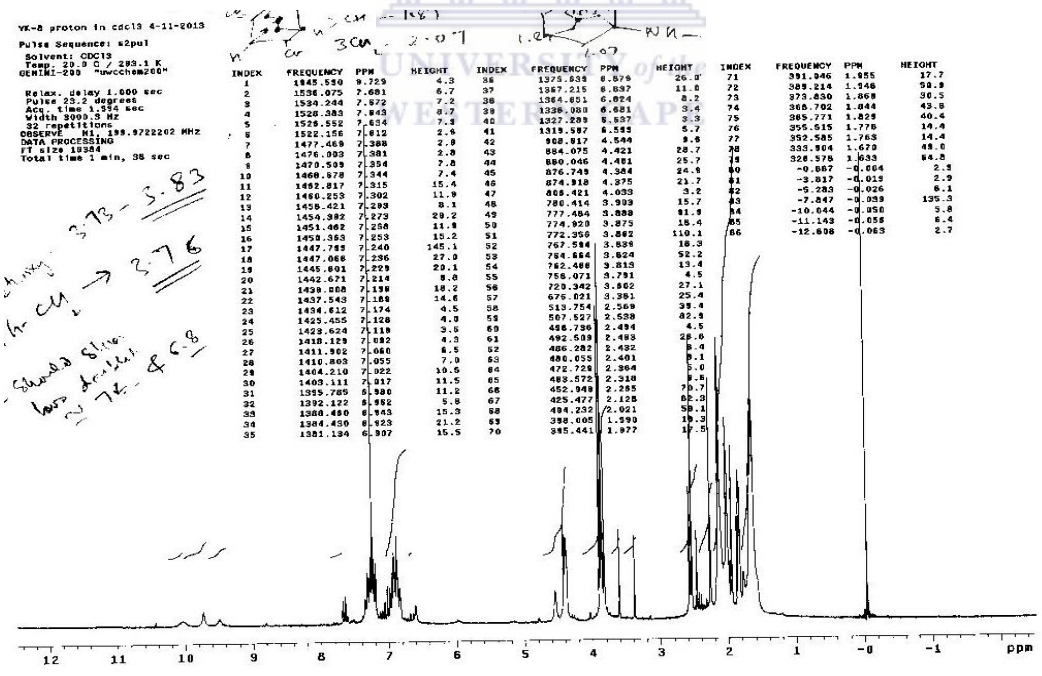


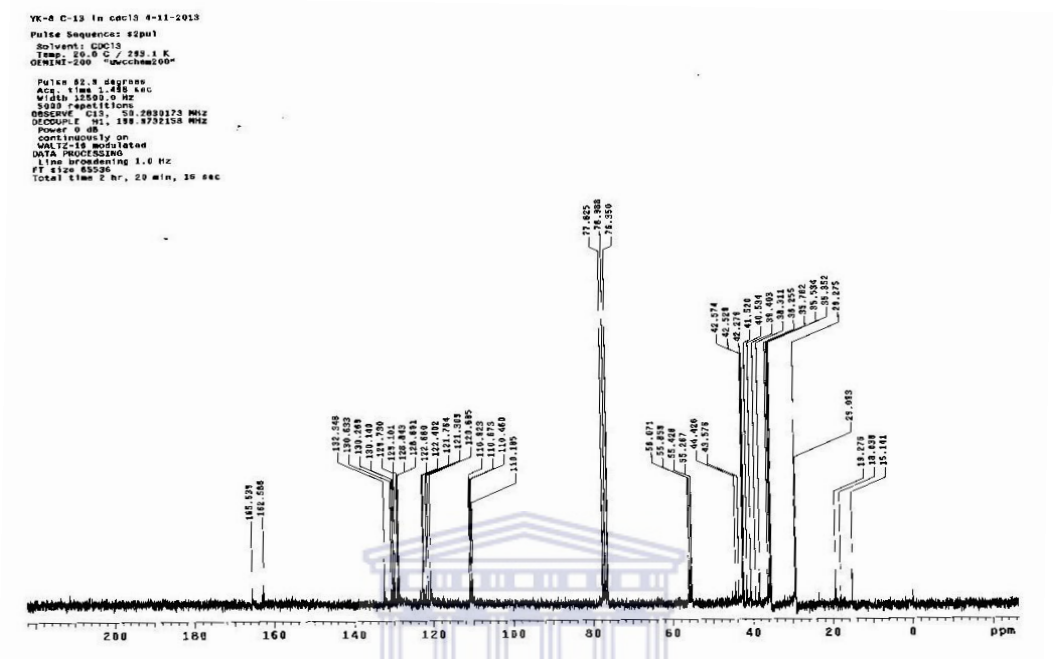
Spectrum 36: IR spectrum of SE-12



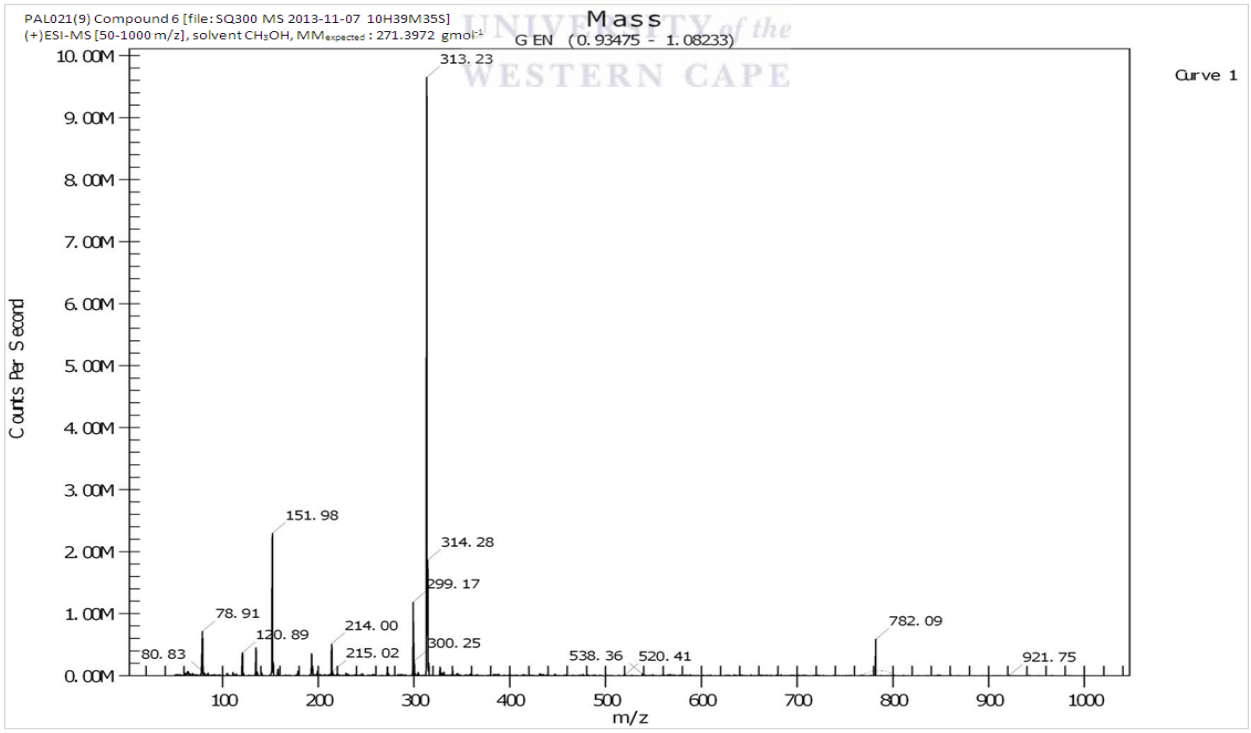
Spectrum 37: ^{1}H NMR spectrum of SE-13



Spectrum 38: ^{13}C NMR spectrum of SE-13



Spectrum 39: MS of SE-13



Spectrum 40: IR spectrum of SE-13