Polycyclic aminoguanidines: novel entities for neuroprotection

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To my parents, Derek and Lesia Wilkes

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Abstract

Neurodegenerative diseases such as Parkinson's and Alzheimer's disease as well as acute disorders for example cerebral ischemia are amongst others associated with an excess production of nitric oxide (NO) in the central nervous system. Identification of potent and selective inhibitors of the inducible and neuronal nitric oxide synthase (NOS) isoforms is therefore of great interest due to their therapeutic potential in the treatment of these diseases. Recent strategies in the development of neuroprotective agents for acute and chronic neurodegenerative disorders also focus on drugs that exhibit antioxidant properties and drugs that inhibit excitatory amino acid neurotransmission, for example N-methyl-D-aspartate (NMDA) receptor antagonists.

In this study novel compounds containing the guanidine and pentacyclic cage moieties were synthesised with the intention of simultaneously addressing NOS and NMDA receptors as possible target sites. Aminoguanidine (AG) and the pentacycloundecylamines have been associated with neuroprotection via their inhibitory and antagonistic effects on inducible NOS and NMDA receptor channels respectively. Both moieties were therefore included in new structures with an approach to obtain a dual mechanism for neuroprotection.

The oxyhemoglobin assay was employed to determine the NOS activity of the above guanylhydrazines and related structures using individual rat brain homogenate incubations. Although the selectivity of the test compounds were not accounted for, due to the presence of multiple NOS isoforms, promising NOS inhibition for the guanylhydrazine compounds was observed.

An increased potency for the novel guanylhydrazine compounds **2** (8-imino-N-guanidino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane) and **3** (8-imino-N-guanidino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecan-11-one) was observed when compared to

AG for *in vitro* NOS inhibition. The two terminal N-substituted aminoguanidines, compounds **2** and **3**, presented with IC_{50} values of $7.6x10^{-5}$ M and $2.8x10^{-4}$ M respectively. Compared to AG's IC_{50} value of $2.3x10^{-3}$, a 30 and 8 fold increase in potency was observed. None of the other test compounds showed any significant activity, suggesting the importance of the carbamidine moiety as a pharmacophore for effective NOS inhibition.

These results confirm that potent NOS inhibition is achievable by terminal N-substitution of aminoguanidine with the pentacycloundecyl cage structure. In view of the increase in lipophilicity originating from the pentacycloundecyl cage structure, it is expected that the new structures will display an increase in blood brain barrier permeability when compared to AG. The novel compounds represent a new class of NOS inhibitors and provide the foundation for potential therapeutic agents. Further analysis regarding the isoform selectivity, the central nervous system penetration and NMDA receptor activity of these compounds is important.

Uittreksel

Parkinson en Alzheimer se siektes sowel as ander akute neurodegeneratiewe toestande soos serebrale iskemie kan moontlik deur die oormatige produksie van stikstofoksied (NO) in die sentrale senuweestelsel veroorsaak word. In hierdie gevalle is die ontwikkeling van meer potente en selektiewe ensiemremmers van stikstofoksiedsintetase (NOS) uiters noodsaaklik. Ontwikkeling van nuwe remmers wat hoofsaaklik die induseerbare en neuronale isoforme van NOS sal teiken, is veral van belang weens die geneesmiddels se terapeutiese potensiaal in die behandeling van neurodegeneratiewe siektes. Die ontwerp van geneesmiddels vir die behandeling van akute en chroniese neurodegeneratiewe toestande is tans ook gemik op middels wat oor antioksidant eienskappe beskik sowel as middels soos N-metiel-D-aspartaat (NMDA) reseptorantagoniste wat die neurostimulasie van eksitatoriese aminosure sal rem.

In hierdie studie is nuwe guanielhidrasienverbindings gesintetiseer wat oor 'n guanidien en 'n pentasikliese hokstruktuur beskik. Die doel was om geneesmiddels met 'n tweeledige neurobeskermende effek te ontwerp wat beide NOS en NMDA-reseptore sal teiken. Aminoguanidien (AG) se remmende effek op induseerbare NOS sowel as die pentasikloundekielamiene se antagonistiese effek op NMDA-reseptore dui op 'n assosiasie met neurobeskerming. Gevolglik is beide entiteite in die nuwe verbindings geïnkorporeer.

Die NOS-aktiwiteit van die bogenoemde guanielhidrasien en verwante verbindings is met behulp van die oksihemoglobientoets bepaal deur gebruik te maak van individuele rotbreinhomogenaatinkubasies. Hoewel die geneesmiddels se selektiwiteit ten opsigte van die verskillende NOS-isoforme nie bepaal is nie, is daar belowende NOS-remming met die guanielhidrasien verbindings waargeneem.

In vergeleke met die *in vitro* NOS-remming van AG, is daar 'n toename in die vermoë van verbindings **2** (8-imino-N-guanidinopentasiklo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undekaan) en **3** (8-imino-N-guanidino-pentasiklo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undekaan-11-oon) om NOS te inhibeer waargeneem. IC_{50} waardes van $7.6x10^{-5}$ M en $2.8x10^{-4}$ M is onderskeidelik vir verbindings **2** en **3** bereken en teenoor AG se IC_{50} waarde van $2.3x10^{-3}$, dui dit op 'n 30- en 8-voudige verhoging in potensie. In die reeks toetsverbindings is slegs **2** en **3** aktief. Dit is 'n aanduiding van die noodsaaklikheid van die karbamidienstruktuur as 'n farmakofoor vir NOS-remming.

Hierdie resultate bevestig dat 'n verhoogde NOS-remming haalbaar is deur middel van terminale N-substitusie aan AG met pentasikloundekiel-strukture. Die gesintetiseerde verbindings verteenwoordig 'n nuwe klas NOS-remmers met 'n potensiële verbetering in die bloedbreinskansdeurlaatbaarheid weens die aanname van 'n verhoogde lipofiliteit, afkomstig van die pentasikloundekiel-strukture. Hierdie verbindings verskaf 'n grondslag vir moontlike terapeutiese geneesmiddels. Addisionele studies met betrekking tot die isoform-selektiwiteit en sentrale senuweestelselpenetrasie asook die NMDA-reseptor aktiwiteit van hierdie verbindings is dus belangrik.

Chapter 1

Introduction

In this chapter, the rationale and aim of the study as well as new initiatives for drug discovery are discussed proposing innovative ideas and novel compounds for the treatment of neurological disorders.

1.1 Background

Since the 1980's when endothelium derived relaxing factor was identified as nitric oxide (NO) (Palmer *et al.*, 1987), a surge of interest in the biochemistry and pharmacology of NO occurred (Moore & Handy, 1997). The reactive diatomic radical controls a variety of physiological processes ranging from the cardiovascular system to the regulation of memory function and is also the precursor of a number of chemical species, including nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$). These NO derivatives have their own significant effects, some beneficial and some detrimental. The recent suggestions that $ONOO^-$ may be responsible for some of the cytotoxic effects exerted by NO (Moncada *et al.*, 1991) led to the investigation of possible inhibitors of the enzyme responsible for NO biosynthesis (Moore & Handy, 1997).

An increase in the prevalence of neurological disorders such as Parkinson's (PD) and Alzheimer's disease (AD), along with the contribution of NO in these diseases makes NO of special interest as a new drug target. Diseases like these are a huge cost burden to health care and create a medicinal challenge where new therapeutic strategies and novel drugs are needed for long term solutions. Current long term treatment aggravates the disease, therefore, not only should

new drugs alleviate symptoms, but also modify the pathophysiology and outcome of the disease in such a way as to render neuroprotection (Lipton & Chen, 2004).

Hibbs *et al.* (1987) first reported on N^G-monomethyl-L-arginine (L-NMMA), the arginine-based nitric oxide synthase (NOS) inhibitor that along with similar NOS inhibitors provided invaluable tools for probing the biological roles of NO in health and disease. It has increased our understanding of NO's function, particularly the role of NO in the central nervous system (CNS) where enhanced NO formation following N-methyl-D-aspartate (NMDA) receptor activation or induction of inducible NOS plays a general role in neuronal injury. The respective blockade or inhibition of the particular receptors or enzymes may be of therapeutic benefit in CNS pathologies (Dawson *et al.*, 1991). However, due to the fact that excessive NOS inhibition not only disrupts normal physiological function, but also enhances NMDA receptor excitotoxicity (Connop *et al.*, 1995), further advances in this important area now require the development of potent and isoform selective NOS inhibitors (Moore & Handy, 1997).

The relationship between NMDA receptors and NOS in neurodegenerative disorders provides an excellent opportunity for treatment of these diseases with dual mechanism drugs acting on both targets. (Kemp & McKernan, 2002).

In view of this promising medicinal potential, neuroprotective drug development has undergone a tremendous paradigm shift that breaks the old rules of drug screening by high-affinity binding (Lipton & Chen, 2004). Dizocilpine (MK-801) and ketamine for example were discovered by high-affinity screening for their targets. The effects of these compounds however result in a total blockade of virtually all NMDA receptor activity and subsequently interfere with normal cellular functions that eventually lead to unacceptable side effects. Accordingly, this complexity of human physiology led to the formation of four basic concepts:

(1) The mechanism of open (selective) channel blockade, (2) binding kinetics, (3) dual mechanism agents and (4) pathologically activated therapeutics (Lipton & Chen, 2004).

1.1.1 Mechanism of open channel block

Open channel blockers can only enter receptor ion channels after activation of the receptor when channels are in an open conformation (Lipton & Chen, 2004). Due to the dependence on prior activation of receptors, this interaction between drug and receptor is termed non-competitive antagonism. During pathological conditions more channels are available for drug entry into the channel and pharmacological modulation of receptors is thus more profound as a result of the enhanced access to the binding site. Therefore a non-competitive antagonist will be more effective against excessive receptor activity than against the normal physiological activity of receptors. Memantine for example, acts as a non-competitive antagonist of the NMDA receptor to attenuate excitotoxic processes and to normalise synaptic transition. Other non-competitive drugs such as MK-801 and phencyclidine (PCP) however, still lead to excessive blockade of NMDA receptors rendering additional factors responsible for the high affinity blockade of these drugs and will be discussed in the following section.

In contrast to the above, competitive antagonists compete with endogenous agonists, glutamate and glycine, to block pathologically activated receptor channels. However, in addition to this effect, these drugs also block healthy areas of the brain (Lipton & Chen, 2004). Subsequently several CNS adverse events occur with the use of these drug treatments (Kemp & McKernan, 2002). Despite the preferential action of open channel blockers such as memantine, these drugs still cannot reverse neurological disease because neurons will already be lost (Lipton & Chen, 2004). For this reason additional approaches with respect to neuronal recovery should be considered.

1.1.2 Binding kinetics

According to Lipton & Chen (2004), when designing clinically effective channel blockers, drug-receptor kinetics is of significant importance. The drug's disassociation rate from the receptor (off-rate) is a key determinant of efficacy. A fast off-rate implies that the drug has a reduced affinity towards the receptor. This will lead to a transient blockade of the receptor and accordingly preserve normal function (Kemp & McKernan, 2002). A slower off-rate will lead to a more permanent blockade with a subsequent loss of normal physiological function due to the higher binding affinity between the drug and receptor. Important to note is that the off-rate is an intrinsic property of the drug-receptor complex.

A drug's association rate with the channel (on-rate) is also of importance when regarding the drug's apparent affinity (Lipton & Chen, 2004). Apparent affinity regards the relation between the off and on-rate that also determines the efficacy of the drug. In comparison to the off-rate that is an intrinsic property of the drug-receptor complex, the on-rate is mainly dependent on drug concentration.

The magnesium cation is an example of a blocker with a fast off-rate (Lipton & Chen, 2004). At a physiological resting membrane potential of -70 mV, magnesium effectively blocks ion fluxes through the NMDA receptor (Kornhuber & Weller, 1997). Magnesium is however easily dissociated from the channel by depolarisation, causing an excess calcium influx through the receptor channel and is thus an ineffective channel blocker. In contrast to magnesium, MK-801, a high-affinity non-competitive blocker with a slow off-rate disrupts normal physiological function by completely blocking calcium influx. Although complications resulting from excessive calcium influx such as excitotoxicity are prevented by MK-801, normal neurotransmission is affected leading to severe psychotomimetic side effects (Lipton & Chen, 2004).

Non-competitive NMDA receptor antagonists such as MK-801 and magnesium exhibit a spectrum of binding affinities for the PCP binding site and may respectively be divided into high affinity and low affinity channel blockers (Kornhuber & Weller, 1997). Knowing that the off-rate is an intrinsic property of the drug-receptor complex, it is hypothesised that there may be an optimal dissociation rate of the ligand from the receptor (Lipton & Chen, 2004). NMDA receptor antagonists such as memantine and amantadine with K_i values higher than 200 nM are considered most advantageous as they are clinically well tolerated and not associated with unacceptable side effects (Kornhuber & Weller, 1997). The latter is associated with clinical efficient drugs having an optimal binding affinity for the receptor and may represent very effective neuroprotective agents.

1.1.3 Dual mechanism agents

Using the same approach by which NO-donating aspirin was developed to overcome the limitations of traditional non-steroidal anti-inflammatory drugs (Rigas & Kashfi, 2004), additional protective properties of neuroprotective agents can be obtained by adding other substituents to existing drugs (Lipton & Chen, 2004). Diseased neurons which manifest with excessive channel activity and that are potentially susceptible, will be targeted by these dual acting drugs exerting their effect on different modulatory sites of receptors involved in neuropathology.

Since NOS readily affects nitrosation of thiols and amines (Stamler *et al.*, 1992), thiol groups on cysteine residues of the NMDA-receptor may act as additional modulatory sites for safe and effective intervention. S-nitrosylation, resulting from the transfer of NO to a thiol group, was shown to down regulate NMDA

receptor activity (Lei *et al.*, 1992). Although NO acting on NMDA thiol groups displays a neuroprotective effect, systemically administered NO may cause serious side effects and even neurodegeneration (Lipton & Chen, 2004). In order to overcome this phenomenon, NO's delivery can be targeted to specific effectors by the production of distinct NO containing compounds that will transport NO to elicit specific biological responses.

1.1.4 Pathologically activated therapeutics

The fourth concept proposes that pathologically activated therapeutics be designated to a newly recognised mode of action (Lipton & Chen, 2004). Owing to the preferential affinity of certain drugs for pathologically active receptors, additional protective moieties can be transported to other modulatory sites in already targeted areas (Lipton & Chen, 2004). This readily explains how pathologically activated therapeutics is possible.

It seems that affinity, binding kinetics and the voltage dependency of non-competitive NMDA receptor antagonists are correlated in that high affinity is linked to slow off-rates and low voltage dependency (Kornhuber & Weller, 1997). Ideally, the profile of these drugs should be intermediate between Mg^{2+} ions and high affinity antagonists such as MK-801. This will permit affinity low enough and binding kinetics rapid enough to allow clearance from the NMDA receptor channel under physiological activation while still retaining affinity high enough to permit receptor inhibition during pathological conditions.

Drugs possessing these qualities will thus effectively target susceptible pathological areas to reduce excitotoxicity without disrupting normal physiological function (Lipton & Chen, 2004).

1.2 Rationale

Neuroprotective drug development has reached a new frontier where the principles of pathologically activated therapeutics and multiple drug targets are in the spotlight (Lipton & Chen, 2004). Dual or even multiple modes of drug interactions with targeted sites will expand Paul Ehrlich's concept of a "magic bullet" to the "magic shotgun". Interventions at numerous drug targets already identified will therefore alleviate neurodegeneration by normalising neuronal function (Lipton & Chen, 2004). These targets, to name but a few, include the NMDA receptors, calcium channels and the nitric oxide synthase (NOS) enzymes.

Nitric oxide plays various roles in both normal and pathological physiologies (Garvey *et al.*, 1994) where it operates as an important signalling molecule that exerts a variety of regulatory and cytostatic functions (Boer *et al.*, 2000). Elevated levels of NO however, may contribute to neuronal destruction and have been implicated in diverse pathological conditions (Wolff *et al.*, 1997). Clearly, pathophysiological conditions associated with alterations in the body's NO homeostasis, makes NOS an attractive target for new drug development.

Various NOS isoforms catalyse the hydrolysis of L-arginine (L-Arg) to produce NO and are accountable for the specific role of NO (Garvey *et al.*, 1994). Selective inhibition of the appropriate isoforms could therefore avoid therapeutic complications. In addition, high intracellular L-Arg concentrations that are typically in excess of its K_m value of NOS (~1.6 μM for neuronal NOS; Furfine *et al.*, 1994), also calls for more potent inhibitors to therapeutically inhibit the required isoforms (Garvey *et al.*, 1994).

Non-amino acid analogues of L-Arg such as aminoguanidine (Misko *et al.*, 1993) and other alkylguanidines (Hasan *et al.*, 1993) were found to be inhibitors of

NOS. Moreover these compounds revealed some selectivity towards the inducible NOS isoform (Garvey *et al.*, 1994) and the neuroprotective properties of aminoguanidines in the rat brain were ascribed to their inhibitory action on inducible NOS (Cash *et al.*, 2001). In addition, Lu *et al.* (2003) found that compounds which selectively inhibited inducible NOS (iNOS), halted excessive NO formation and presented a potential strategy for treatment of neurological disorders pertaining to the over production of NO.

Aminoguanidine (AG) can also be viewed as a non-amino acid analogue of the potent but non selective NOS inhibitor, N^G-amino-L-arginine (L-NAA). Despite the lower potency, AG revealed a 10-fold selectivity for iNOS compared to the endothelial isoform (Moore *et al.*, 1996) and was 26-fold more potent on iNOS than neuronal NOS (Moore & Handy, 1997). The moderate iNOS selectivity of AG makes it an excellent lead compound to further explore structure activity relationships and to identify more potent inhibitors that retain their isoform selectivity (Wolff *et al.*, 1997). This will result in a selective decrease of NO production and can be of significant value in treatment of neuropathologies.

The possible involvement of pathologic glutamate receptor activity in neurological conditions motivates the better understanding of the NMDA receptor's involvement in neurodegeneration (Kornhuber & Weller, 1997). Glutamate is the most abundantly active neurotransmitter acting on probably more than 50% of all synapses in the brain. Over-stimulation of NMDA receptors by glutamate will result in a substantial rise of intracellular calcium levels that can further enhance NO production along with its toxic effects (Lipton & Chen, 2004). Glutamate receptor activity is also required for various neurological processes and the balance between pathological NMDA receptor activity and normal physiology is critical (Kemp & McKernan, 2002). The adamantanamines, amantadine and its dimethyl derivative memantine, were found to act as non-competitive antagonists for the NMDA receptor. These low affinity NMDA receptor channel blockers are

clinically approved for the respective treatment of Parkinson's and Alzheimer's disease (Kornhuber & Weller, 1997) and serves as lead compounds for further investigation.

Structural similarities between the pentacyclic cage compounds and the adamantanamines as well as the favourable comparison of their activities provide the cage structures with potential therapeutic value (Oliver *et al.*, 1991a,b,c).

The blood brain barrier (BBB) restricts the passive diffusion of ionised and hydrophilic molecules into the central nervous system and to date, no functional expression of organic cation transporters or guanidine transport proteins at the BBB are known (Mahar Doan *et al.*, 2000). Based on their data, these authors identified only a minor diffusal mechanism for AG and guanidine into the central nervous system. No saturable transport mechanism as for amino acids exists and AG is thus poorly available in the brain. Studies done by Zah *et al.* (2003) however, established that the polycyclic cage compounds have a high likelihood to cross the BBB. Therefore it is expected that the substitution of these compounds onto AG may improve the availability of AG in the brain.

Incorporation of the cage structure onto guanidino moieties may thus increase the BBB penetration of these novel compounds and in addition to the guanidine's selective inhibition of iNOS, antagonise NMDA receptors. This may be beneficial during the treatment of neurological disorders and it is also proposed that the presence of the pentacyclic group will increase the novel compounds' potency of NOS inhibition whilst retaining its isoform selectivity.

1.3 Aim of study

Some of the challenges associated with neurodegenerative disorders such as PD and AD are that presently available pharmacological therapy is limited to symptomatic treatment that only seems to delay disease progression with an effect that is often found to diminish with time (Standaert & Young, 1996). The goal of current research is to develop treatments that can prevent or reverse neuronal cell death. Promising areas for drug development are the mechanisms implicated in the etiology of neurodegenerative diseases: excitotoxicity, defects in energy metabolism and oxidative stress (Standaert & Young, 1996).

Guanidine and its derivatives together with the pentacyclic cage compounds have drawn attention as potential therapeutic agents in this regard. The unique basicity and cationic nature of the guanidine moiety in addition to the side chain diversity that can help develop unique pharmacological properties, led to the inclusion of the guanidine functionality into numerous pharmacologically active compounds. Furthermore, structural similarities of the pentacyclic cage compounds and the adamantanamines also warranted the inclusion of these moieties into new drugs.

In view of the promising medicinal potential of these structures, the primary objective of this study was to synthesise novel compounds containing both the guanidine and polycyclic cage moieties with the intention of simultaneously addressing NOS and NMDA receptors as possible target sites. It was also expected that the alkyl substitution on AG would increase the potency thereof and as a secondary objective, the activity of these newly synthesised structures were assessed for their potential NOS inhibition properties.

1.4 Study design

The well-known diketone, pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione along with its derived monoketone first described by Dekker & Oliver (1978) were synthesised and used as key intermediates for further synthetic reactions. Photocyclisation of the Diels-Alder adduct obtained from the reaction between *p*-benzoquinone and cyclopentadiene afforded the pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]-undecane-8,11-dione. Additional manipulations of the diketone via the ketol-pathway (Dekker & Oliver, 1978) yielded the desired monoketone, pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8-one.

Condensation of the key intermediate cage structures with AG and a series of other amines were conducted to afford the required guanylhydrazines and pentacyclic imine derivatives. Pentacyclic amine structures were obtained by reduction of the intermediate imine derivatives.

Biological evaluation of the newly synthesised compounds was conducted by employing an adapted oxyhemoglobin method described by Salter & Knowles (1998). The assay is based on the *in vitro* conversion of oxyhemoglobin (oxyHb) to methemoglobin (metHb) following the reaction of NO with oxyHb. Nitric oxide synthase activity can be expressed as the rate of metHb formation subsequent to the reaction between oxyHb and NO. Analysis was done by spectrophotometrically monitoring the absorption difference between 401 nm and 421 nm against time and gave an indication of NOS activity.

Chapter 2

Literature

This chapter presents a pertinent review on neurodegenerative diseases along with the influence of nitric oxide (NO) in the aetiology of Alzheimer's (AD) and Parkinson's disease (PD). Insight into other possible drug targets as well as their role in neuronal death will be given. A better understanding of the underlying mechanisms resulting in cell death will help to determine how specific drug targets pertain to symptomatic treatment or prevention of the disease. Such a perspective of the pathogenesis is essential for the development of new, safe and effective drugs.

2.1 Neuropathology

Although the molecular mechanisms of neuronal degeneration remain largely unknown (Rao & Balachandran, 2002), NO was found to play a central role in the pathogenesis of various neurological disorders including neurodegenerative diseases such as PD and AD (Law *et al.*, 2001). The highest prevalence of NO is found in neurons (De la Torre & Stefano, 2000) where high NO concentrations may contribute to selective vulnerability and neuronal cell death (Ischiropoulos & Beckman, 2003). It has been suggested that several of NO's cytotoxic effects are related to the production of peroxynitrite, derived from the rapid reaction between NO and superoxides (Moncada *et al.*, 1991). Other NO-derived species that may also contribute towards the cytotoxic effects includes nitrogen dioxide (NO_2) nitrite (NO_2^-) and dinitrogen trioxide (N_2O_3; Eiserich *et al.*, 1998). The resulting oxidative stress may cause cell death and tissue damage that characterise human disease states like stroke, acute reperfusion injuries, inflammatory conditions and neurological disorders (Rao & Balachandran, 2002).

Specific oxidative damage in many different types of neurodegeneration suggests a common underlying mechanism of cell death (Ischiropoulos & Beckman, 2003). This has been observed in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and other neurological disorders (Law *et al.*, 2001; Iravani *et al.*, 2002; Deckel, 2001) where the remarkable feature of this group of disorders is an exquisite and specific loss of particular neuron types (Standaert & Young, 1996). Anatomical and histological studies have also established the existence of selective regional susceptibility to neurodegeneration and cell death (Ischiropoulos & Beckman, 2003).

Neuronal injury in AD is most severe in the hippocampus, cortex and neo-cortex where a loss of cholinergic neurons in these areas leads to impairment of memory and cognitive ability (Standaert & Young, 1996). Despite the apparent involvement of the cholinergic system in AD pathogenesis, therapeutic effects of cholinergic agents have been modest and variable. Therefore further research regarding the preferential vulnerability of this system is warranted. Putative relationships between NO and AD led to the recognition of the possible significance of NO in AD as both neurotoxic and neuroprotective (Law *et al.*, 2001).

Parkinson's disease is distinguished by extensive destruction of dopaminergic neurons in the substantia nigra while neurons in the cortex and other areas are unaffected (Standaert & Young, 1996). This clinical syndrome is presented with akinesia, muscular rigidity, resting tremor and impairment of postural balance. Increased levels of nitrite (NO_2^-) in cerebrospinal fluid support the involvement of NO in PD (Iravani *et al.*, 2002). Further studies have also implicated a synergistic action of NO and dopamine (DA) in cell death (Brown & Borutaite, 2004). Accordingly it may explain why dopaminergic neurons are more susceptible to neurodegeneration during increased NO production.

Despite this regional sensitivity, oxidative processes may represent a specific and selective mechanism for neurodegeneration (Ischiropoulos & Beckman, 2003). The diversity of neuronal loss led to the proposal that neuronal injury must be viewed as an interaction of both genetic and environmental factors with the intrinsic physiological characteristics of affected neurons (Standaert & Young, 1996). These intrinsic factors may include susceptibility to (1) excitotoxicity, (2) oxidative stress and (3) metabolic compromise. These three intrinsic factors are well known as the so called "lethal triplet" and are both apoptotic as well as necrotic in nature. Apoptosis is generally viewed as a programmed cell death that forms part of a physiological cell death pathway and is mediated by active intrinsic mechanisms (Brune *et al.*, 1998). Necrosis on the other hand is regarded as a pathological cell death mechanism and results from extrinsic insults on the cell. Both necrosis and apoptosis are viewed as the main routes of cell death and it is difficult to attribute cell death exclusively to either one. It is generally accepted that these different forms of cell death vary in their contribution towards neuropathology (Brune *et al.*, 1998).

The contribution of the intrinsic factors towards cell death identified them as role players in the possible mechanisms of neurodegeneration (Standaert & Young, 1996). This study's main focus is the treatment of specific target sites involved in neuronal death. Therefore, the intrinsic factors will be further discussed as they contribute to selective vulnerability and might prove useful as potential drug targets to address in neurodegenerative diseases (Standaert & Young, 1996).

2.1.1 Excitotoxicity

Nitric oxide synthase has been implicated in the processes of excitotoxicity and is considered to make an important contribution to neuronal cell death that occurs in acute processes such as stroke and head trauma (Ayata *et al.*, 1997). Even

though the role of excitotoxicity in chronic neurodegenerative diseases is uncertain (Standaert & Young, 1996), a number of studies have demonstrated that the pharmacological inhibition of NOS resulted in a neuroprotective effect (Ayata *et al.*, 1997).

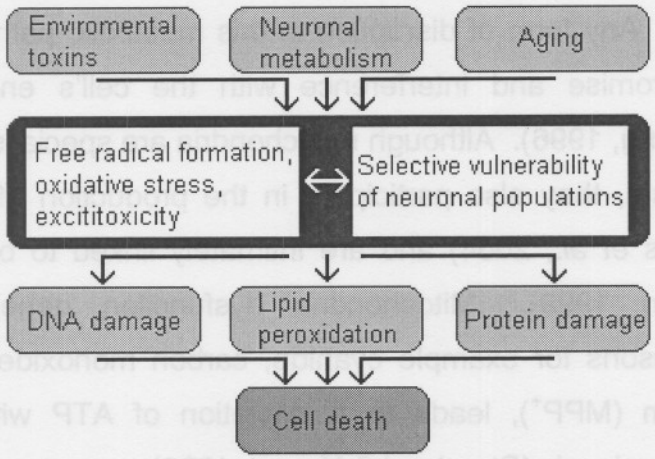


Figure 2.1: Selective vulnerability in neurodegenerative diseases (Standaert & Young, 1996).

Excitotoxicity is also thought to contribute to the selective vulnerability of neurons and results from the presence of excess glutamate concentrations in the brain (Standaert & Young, 1996). Although glutamate is a neurotransmitter essential for normal brain function, excessive amounts of glutamate can also lead to excitotoxic cell death. The destructive effects of glutamate are mediated by glutamate receptors especially that of the N-methyl-D-aspartate (NMDA) type. Unlike other glutamate gated channels that regulate Na^+ influx, overexcited NMDA receptors cause an increase in Ca^{2+} concentration (Standaert & Young, 1996). High Ca^{2+} concentrations can activate a variety of potential destructive processes such as the activation of nitric oxide synthase (NOS) and an increase in NO levels (Law *et al.*, 2001).

2.1.2 Metabolic compromise

Mitochondrial respiration is responsible for virtually all energy production in mammals and every other process in living organisms ultimately depends on this energy (Brown, 1999). Mitochondria generate cellular energy in the form of adenosine triphosphate (ATP) by the process of oxidative phosphorylation (Wallace, 1999). Any form of disruption in this metabolic pathway will result in metabolic compromise and interference with the cell's energy metabolism (Standaert & Young, 1996). Although mitochondria are specialised organelles for energy metabolism, they also participate in the production of reactive oxygen species (Carreras *et al.*, 2004) and are intimately linked to both necrosis and apoptosis (Brown, 1999). Mitochondrial dysfunction, generally induced by mitochondrial poisons for example cyanide, carbon monoxide and 1-methyl-4-phenyl pyridinium (MPP^+), leads to a depletion of ATP with a consequent reduction in energy levels (Standaert & Young, 1996).

Damage to the mitochondrial respiratory chain has been proposed to underlie the pathology of a number of neurodegenerative disorders (Heales *et al.*, 1999). Recent findings that NO inhibits mitochondrial respiration via distinct mechanisms (Brown, 1999) indicate that the mitochondrion is the target of NO and its free radical metabolites (Iravani *et al.*, 2002). Rapid inhibition of mitochondrial respiration at the level of cytochrome oxidase (complex IV) (Cleeter *et al.*, 1994) and NADH ubiquinone reductase (complex I) was reported after the brief exposure of isolated mitochondria to NO (Cassina & Radi, 1996). Decreased complex I activity in the substantia nigra (Schapira *et al.*, 1990) and diminished activity of complex IV in the cerebral cortex (Kish *et al.*, 1992) were also respectively reported in post mortem samples of patients with PD and AD. Since inhibition of oxygen consumption appeared to be reversible, it has been postulated that NO may be a physiological regulator of mitochondrial respiration (Brown & Cooper, 1994). However, increased generation of NO and

peroxynitrite ($ONOO^-$) in neurological disorders may be the direct cause of mitochondrial damage (Heales *et al.*, 1999).

The capacity of neurons to handle oxidative metabolism progressively decreases with age (Standaert & Young, 1996). Although this is a normal feature of aging, most people do not lose the 80% to 90% of dopaminergic neurons required to cause symptomatic PD. Patients with PD exhibit reduced energy metabolism that is much greater than that of healthy individuals of the same age. These observations suggest that disturbances in energy metabolism may underlie the selective pathology for neurodegenerative diseases (Standaert & Young, 1996).

2.1.3 Oxidative stress

Accumulation of oxidative damage in neurons may account for the increased incidence of neurological disorders in aged populations (Rao & Balachandran, 2002). A growing body of evidence implicates free radical toxicity, radical induced mutations and mitochondrial dysfunction in the clinical manifestations of neurodegenerative diseases (Rao & Balachandran, 2002). Oxidative metabolism is responsible for the production of partially reduced oxygen species such as hydrogen peroxide (H_2O_2) and oxyradicals (Standaert & Young, 1996). Although neurons depend on oxidative metabolism for normal functioning, their high susceptibility to oxidative stress can induce both neuronal necrosis and apoptosis (Ischiropoulos & Beckman, 2003).

Even though superoxide and hydrogen peroxide alone are non-toxic, two distinct pathways via peroxidases and NOS however may enhance the toxicity of these partially reduced oxygen species (Ischiropoulos & Beckman, 2003). Inflammatory cells produce a range of harmful hypohalous acids such

hypochlorous acid (HOCl) by means of peroxidases and thereby enhance the toxicity of hydrogen peroxide. The toxicity of superoxide is increased by the cell's ability to produce NO. Nitric oxide and oxyradicals react by the fastest known reaction in biology to form peroxynitrite ($ONOO^-$), a powerful oxidising and nitrating agent (Ischiropoulos & Beckman, 2003), that is implicated in lipid peroxidation, DNA damage and cytotoxicicty (Lipton *et al.*, 1993). Lipid peroxidation is thought to be especially damaging because it is a self propagating process, particularly in the brain, as this organ is enriched with poly unsaturated fatty acids, the substrate for lipid peroxidation (Neely *et al.*, 2000). Overproduction of the above mentioned reactive species during disease states is generally aggravated by the inability of compromised cells to cope with oxidative stress (Ischiropoulos & Beckman, 2003). Genetic and biochemical manipulations that enhance antioxidant effects provide sound support for the hypothesis that oxidative stress is a critical mechanism in neurodegeneration.

2.2 *Nitric oxide synthase*

Nitrogen oxides have found uses in food preservation, explosives and even in cardiovascular therapy (Law *et al.*, 2001). Since mammalian cells were thought incapable of synthesising such compounds, the biological relevance of NO was seen as insignificant. It was first noted in 1916 that the high concentrations of nitrites and nitrates in human urine could not be attributed to normal dietary intake alone. This sparked the further investigation into the possibility that nitrate biosynthesis was possible in mammals.

In 1990 the enzyme responsible for the synthesis of NO was isolated for the first time (Bredt & Snyder, 1990). Mammalian NO synthesis is catalyzed by nitric oxide synthase (NOS). Nitric oxide synthase oxidises one of the two terminal

guanidino nitrogens of L-arginine (L-Arg) to yield NO with a stoichiometric production of L-citrulline (Feldman *et* al., 1995). Another interesting finding was that endogenous L-Arg seemed to be the only substrate for all NOS isoforms and that there existed a balance between the recycling of L-citruline to L-Arg. It was proposed that an intercellular citrulline–NO cycle is operational in the brain to benefit cells in need of L-Arg for a proper synthesis of NO (Wiesinger, 2001).

Although NO alone is not exclusively accountable for the oxidative stress observed during disease states, its involvement appears to be quite significant (Law *et al.*, 2001). Recent data indicates that several chemical reactions involving NO-derived species such as nitrite, peroxynitrite, hypochlorous acid along with peroxidases may contribute to tissue damage (Eiserich *et al.*, 1998). Currently there is enough documentation to place oxidative and nitrative processes in the centre of the pathogenic mechanism that leads to neuronal loss and neurodegeneration (Ischiropoulos & Beckman, 2003). The role of NO in neuronal injury arose from the use of selective NOS inhibitors (Law *et al.*, 2001). Inhibition of specific NOS isoforms could lower NO synthesis in targeted areas and avoid therapeutic complications, whereas simultaneous inhibition of other NOS isoforms may result in severe side effects and even neurodegeneration (Boer *et al.*, 2000). It is therefore noteworthy to consider the various NOS isoforms when discussing the putative role of NO in neurodegenerative disorders (Law *et al.*, 2001).

2.2.1 Isoforms

According to Marletta (1993) the family of NOS isoforms can be classified in two main categories: (1) constitutive NOS (cNOS) that forms part of the normal cell metabolism and (2) inducible NOS (iNOS). Initially iNOS was thought to be provoked by endotoxins and cytokines, but it is now also known to be constitutively expressed in several cell lines (Kim *et al.*, 1997).

Under normal circumstances cNOS isoforms are present in brain and endothelial cells (Misko *et al.*, 1993). They produce small amounts of NO that participates in normal physiological neuronal transmission. Constitutive NOS is also involved in regulatory functions such as blood pressure regulation and memory formation (Boer *et al.*, 2000). Observations that cNOS isoforms in the brain were cytosolic while those in endothelial cells were membrane bound, led to a further subdivision in this class (Law *et al.*, 2001). The terms neuronal- (nNOS) and endothelial (eNOS) nitric oxide synthase were respectively attributed to these two isoforms. In the brain nNOS is expressed inside neurons at diverse locations including the neocortex, hippocampus and brainstem. Endothelial NOS has been identified not only in endothelial cells, but also in neurons. Both these isoforms are calcium dependent and their activity is regulated by the concentration of calcium available (Law *et al.*, 2001).

An additional expansion of this constitutive category came in the late 1990's when Ghafourifar *et al.* (1999) reported for the first time the presence of a constitutively expressed and continuously active NOS isoform in mitochondria (mtNOS). Their findings indicated that mtNOS was located at the inner mitochondrial membrane and that it also was calcium dependent. Given the diverse role of NO it could be hypothesised that NO utilised in the modulation of mitochondrial respiration might be derived from mtNOS (Giulivi *et al.*, 1998). This may represent a novel biochemical pathway that controls the supply of O_2 and energy to tissues under dynamic conditions.

Inducible NOS continuously produce high amounts of NO and is found in microglia as well as astrocytes (Misko *et al.*, 1993). The inducible isoform has evoked great attention as iNOS was found to be involved in a number of pathologies such as septic shock and inflammatory conditions of the central nervous system (Boer *et al.*, 2000). It is transcriptionally activated and once expressed, active for a long period of time without any major short term

regulation. This activity profile leads to high and cytotoxic levels of NO necessary for an effective immune defence against invasive pathogens (Boer *et al.*, 2000). While essential for survival, the overproduction of NO may be inappropriately activated leading to detrimental side effects and neurodegeneration (Ischiropoulos & Beckman, 2003). In contrast with cNOS isoforms, the iNOS isoforms appears to be independent of calcium concentrations as fluctuations in calcium levels does not appear to alter enzyme activity (Stuehr, 1999). This is possibly because of iNOS' higher affinity towards calmodulin; the calcium binding protein that plays a role in the activation of NOS.

2.2.2 Structure

Nitric oxide synthase is a family of dimeric enzymes where each monomer NOS polypeptide contains an N-terminal oxygenase domain and a C-terminal reductase domain (Stuehr, 1999). Located between these two domains is a recognition sequence for calmodulin (CaM). The N-terminal region that is located upstream from the oxygenase core varies in length for the different NOS isoforms and participates in the cellular targeting of NOS (figure 2.2).

Calmodulin is a calcium binding protein that plays a critical role in the dual mechanistic activation of NOS (Abu-Soud *et al.*, 1994). Binding of CaM increases the rate at which NADPH-derived electrons are transferred into the flavins and also enables electron transfer from the reductase domain to the heme iron in the oxygenase domain. This permits electrons to bind and activate O_2 and catalyse NO synthesis. The CaM inhibitory loop is a distinct structural element present in the reductase domain of constitutive isoforms that appears to negatively regulate CaM binding (Salerno *et al.*, 1997). Inducible NOS isoforms lack this structural feature which, along with the additional region essential for

high CaM affinity (Venema *et al.*, 1996), might explain the difference in calcium dependence between the inducible and constitutive isoforms of NOS.

The C-terminal reductase domain starts at the end of the CaM binding sequence and binds flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH (Masters *et al.*, 1996). During NO synthesis NADPH provides electrons to the reductase flavins where in the presence of CaM the flavins transfer them to the heme-containing subunit in the oxygenase domain. Activation is thought to be brought about by a conformational change in the reductase domain by CaM. This increases the transfer rate of electrons from NADPH into the flavins as well as the rate at which electrons are transferred from the reductase domain to other electron acceptors such as cytochrome *c* or ferricyanide. Calmodulin activates the reductase domain independent of the oxygenase domain, therefore the oxygenase domain's properties and reactivity remains unchanged (McMillan & Masters, 1995). It must however be emphasised that these structural changes are much more prevalent in nNOS than it is in eNOS (Chen *et al.*, 1996).

The N-terminal oxygenase domain's core structure is formed by continuous overlapping β-sheets where cofactors bind to form the active site where NO is synthesised (Stuehr, 1999). Both cNOS and iNOS are dependent on the cofactors tetrahydrobiopterin (BH_4), FAD and FMN along with the heme for enzyme activity. Although these cofactors are widely used by other enzymes to catalyse redox reactions, NOS is the only known mammalian enzyme to utilise all four groups (Feldman *et al.*, 1995). The requirement of all four cofactors along with the overlapping β-sheets distinguishes NOS from a similar enzyme group, cytochrome *P*-450.

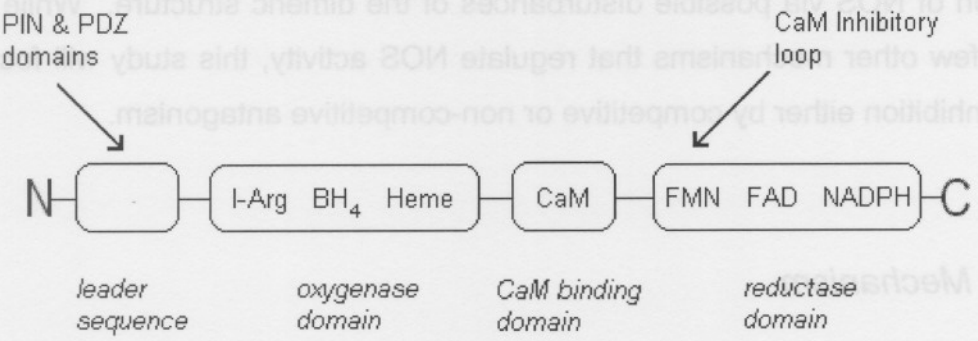


Figure 2.2: Domain arrangement in rat nNOS. The enzyme consists of a N-terminal leader sequence that targets the enzyme in cells, a core oxygenase domain which forms the active catalytic site, a CaM binding domain and a reductase domain responsible for electron import and transport to the oxygenase domain. Structural organization of iNOS and eNOS are similar to nNOS except that they do not contain an extensive leader sequence and the iNOS reductase domain is missing the CaM auto inhibitory loop (Stuehr, 1999).

Adjacent to the oxygenase domain is the amino acid N-terminal leader sequence containing the so-called PDZ binding motif that target NOS to specific areas within the cell and may alter its catalytic activity (Stricker *et al.*, 1997). The PDZ motif is a repetition of certain patterns in the N-terminal sequence that will result in a domain to facilitate binding with other complexes or with other protein's PDZ domains. One such protein is the brain protein that specifically binds to NMDA receptor channels (Hemmens & Mayer., 1998). The PDZ domains of NOS and the protein associate and in this way NOS is localised to the NMDA receptor. Since the N-terminal amino acid leader sequence is unique to nNOS and it is thereby targeted to NMDA receptors, NO derived from nNOS may play a key role in the regulation of the NMDA receptor activity. This interaction may be part of the functional linkage between NOS inhibitors and pathological NMDA receptor activity.

Tochio *et al.* (1998) reports on another domain that exists in the N-terminal leader sequence of NOS enzymes. This site binds to a highly conserved and widely expressed protein inhibitor of NOS (PIN). Binding of PIN inhibits the

function of NOS via possible disturbances of the dimeric structure. While there are a few other mechanisms that regulate NOS activity, this study will focus on NOS inhibition either by competitive or non-competitive antagonism.

2.2.3 Mechanism

Although the precise mechanism of NO formation is not fully understood, it is proposed to be very similar to that of the oxidizing *P*-450 enzymes where a resemblance exists in the heme based, step-wise activation of oxygen. Nitric oxide synthase however, generates reactive oxygen species at two steps in this reaction (Stuehr, 1999).

The first step utilises electrons from NADPH along with molecular oxygen and L-Arg as substrates. A guanidine nitrogen from L-Arg and the molecular oxygen respectively provides the nitrogen and oxygen atoms required for NO synthesis. An N-hydroxy-L-arginine intermediate is formed and then converted to the products L-citrulline and NO during the second step of the reaction (figure 2.3).

In addition to the cofactors, FMN, FAD and BH_4, two other functional proteins, the heme and CaM are also essential for enzyme activity (Law et al., 2000). If there is sufficient supply of L-Arg and all essential cofactors, NOS will synthesise NO (Bates *et al.*, 1996). Under L-Arg deprivation however, NOS is predisposed to accept substrates other than L-Arg as electron acceptors. The mitochondrial protein Cytochrome *c* and oxygen might act as such acceptors leading to the formation of oxygen radicals and other toxic NO species. This can be attributed to the similarity in the *C*-terminal domain of NOS and cytochrome *P*450 reductase (Giulivi, 2003).

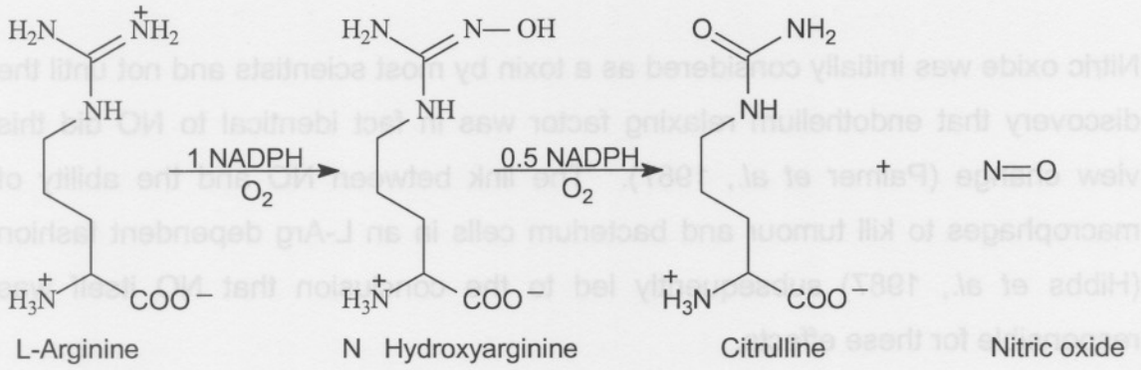


Figure 2.3: Enzyme catalyzed NO production. Hydroxylation of L-arginine (L-Arg) generates N-hydroxyl-l-arginine (NOH-ARG) as intermediate. The second step converts NOH-Arg to the products NO and L-citrulline (Stuehr, 1999).

The formation of toxic species such as $ONOO^-$ derived from NOS activity and their contribution towards cell death may however be insignificant (Bates *et al.*, 1996). Several cofactors needed for full NOS activity are tightly bound to NOS and the presence of a possible circulation of L-citrulline to L-Arg avoids substrate depletion (Wiesinger, 2001). According to Lafon-Cazal *et al.* (1993) NOS is not the main pathway of oxygen radical formation and other pathways such as the NMDA receptors are also implicated, especially under excitotoxic conditions. The NMDA-channels can generate oxygen radicals independent of NOS and it is considered to attribute to the increase of calcium levels and the release of arachidonic acid.

Exploitation of the cytotoxic effect of NO led to interesting models for proposed mechanisms of protection as well as destruction (Brune et al., 1998). This controversial issue of NO being neuroprotective or destructive will subsequently be discussed.

2.3 *Nitric oxide*

Nitric oxide was initially considered as a toxin by most scientists and not until the discovery that endothelium relaxing factor was in fact identical to NO did this view change (Palmer *et al.*, 1987). The link between NO and the ability of macrophages to kill tumour and bacterium cells in an L-Arg dependent fashion (Hibbs *et al.*, 1987) subsequently led to the conclusion that NO itself was responsible for these effects.

As a short lived free radical molecule, NO serves as an inter- and intra-cellular messenger (Murad, 1995). Peptides containing thiol groups for example glutathione (GSH) may possibly act as transporters or storage forms of NO and thereby prolongs its short half life and exerts its effects on distant targets (Kröncke *et al.*, 2001). This supports the possibility of NO acting as a classical humoral substance as suggested by Murad (1995).

The diverse action of NO is evident due to various localisations of NOS enzymes in a variety of systems such as the gastro intestinal tract, cardiovasculature and the central nervous system (Misko *et al.*, 1993). Despite the fact that NO functions as a regulatory molecule, the over production of NO has been implicated in neurotoxicity due to the formation of toxic intermediates at high NO concentrations (Dawson *et al.*, 1991).

In view of the above, the therapeutic potential of NOS inhibitors becomes apparent in situations where levels of NO are elevated above what is considered normal (Marletta, 1993). It was also found that NO donors may be of therapeutic benefit in circumstances where NO concentrations were below that which leads to the desired response (Marletta, 1993). Therefore, the effects of NO are not only considered cytotoxic, but also cell protective (Brune *et al.*, 1998).

2.3.1 Neurotoxicity of NO

Nitric oxide synthase does not only produce NO, but also those species resulting from oxidation, reduction or adduction of NO (Brune *et al.*, 1998). Depending on the physiological milieu, various nitrogen oxides, S-nitrosothiols, $ONOO^-$ and transition metal adducts are produced (Brune *et al.*, 1998). The generation of oxidants are often attributed to accidents of metabolism, but growing evidence however suggests that it is produced by an active process (Ischiropoulos & Beckman, 2003). It is estimated that up to 1% of all oxygen consumption is reduced to superoxide and hydrogen peroxide. Nitric oxide enhances the toxicity of these ROS by the formation of $ONOO^-$ which, on its own is a powerful oxidising agent, but also generates powerful nitrating agents via the catalytic reaction with carbon dioxide (Ischiropoulos & Beckman, 2003). During physiological conditions controlled formation of NO sets up a steady state concentration of $ONOO^-$ and the relatively low yields of $ONOO^-$ are detoxified by endogenous antioxidant mechanisms. Continuous long term production of NO however, limits the capacity of cells to deal with oxidative stress and may lead to cytotoxicity (Carreras *et al.*, 2004).

According to the excitotoxic theory, excessive glutaminergic activation of the NMDA receptors leads to a pathological Ca^{2+} influx (Choi, 1988). The resultant increase of intraneuronal Ca^{2+} stimulates the binding of CaM to NOS, thereby increasing its catalytic activity (Law *et al.*, 2001). Increased synthesis of NO by nNOS has been implicated in excitotoxicity and the role of nNOS in NMDA-mediated excitotoxicity confirmed by the neuroprotective effect observed during the inhibition of nNOS (Ayata *et al.*, 1997). In a different study, nNOS was also found to be the primary contributor towards ischemic neurodegeneration (Cash *et al.*, 2001).

The involvement of the NMDA receptor and the subsequent influx of Ca^{2+} are also implicated in neurodegenerative diseases and neuronal injuries resulting from acute brain insults (Lafon-Cazal *et al.*, 1993). Glutamate has emerged as an important mediator of ischemic brain injury and the latter is also evident for NO in view of the Ca^{2+} dependent increase of NOS activity (Iadecola, 1997). Increased activity of the glutamate receptors localised on dopaminergic neurons may enhance dopaminergic cell death and has been implicated in excitotoxicity and neurodegeneration (Connop *et al.*, 1995).

Nitric oxide may affect nitrosylation in a variety of proteins which are then preferentially targeted for fast proteolytic degradation (Law *et al.*, 2001). Protein nitration is a cumulative and damaging process in which proteins lose their activity leading to a loss of cell function and has been associated with certain neuronal disorders (Ischiropoulos & Beckman, 2003).

The proposed regulatory function of NO in oxidative phosphorylation is supported by the presence of mtNOS in many of the major tissue types (Bates *et al.*, 1996). However, mtNOS may also be of importance in pathological conditions. High Ca^{2+} levels following ischemia, led to the glutamate dependent increase of mitochondrial Ca^{2+} uptake that resulted in an enhanced activity of mtNOS (Bates *et al.*, 1996). The increased mtNOS activity contributed to apoptosis via increased intra-mitochondrial formation of $ONOO^-$ (Ghafourifar *et al.*, 1999) and was also deemed responsible for the decrease in mitochondrial respiration (Bates *et al.*, 1996). It was thus proposed that the Ca^{2+} dependent induction of mtNOS played a distinct role in the cytotoxic injury of cells (Ghafourifar *et al.*, 1999).

Oxygen radical production by glutamate receptors have long been presumed to function on the basis of two NMDA induced effects: first, the above mentioned increase in intracellular Ca^{2+} levels and secondly, the release of arachidonic acid

(Lafon-Cazal *et al.*, 1993). Arachidonic acid is normally stored within cell membranes and can be liberated by the hydrolysing action of phospholipase A_2 (Diasio & LoBuglio, 1996). Released arachidonic acid can be converted to highly reactive regulators of physiological and pathological processes. These regulators known as eicosanoids are involved in inflammatory conditions and produced by various cell types such as leucocytes, platelets and endothelial cells (Diasio & LoBuglio, 1996).

Inflammatory mechanisms play a significant role in for example ischemic brain injury where microvasculature endothelial cells initiate inflammation via their contact with infiltrating leukocytes (Diasio & LoBuglio, 1996). Cytokines produced by leucocytes form a group of distinct proteins that are known modulators of endothelial cell function. They are responsible for the massive synthesis of NO resulting from increased iNOS expression. The over production of iNOS derived NO seems to play an important role in the pathology of inflammatory processes and also appears to be cell specific (Bonmann *et al.*, 1997). Inhibition of the iNOS isoform has been effective in reducing tissue damage in several models of inflammation, rendering iNOS inhibition as a potential therapeutic treatment for ischemic brain injuries (Misko *et al.*, 1993).

Several lines of evidence thus indicate that NO mediates glutamate neurotoxicity via NMDA receptors (Dawson *et al.*, 1991) and it is also widely acknowledged that NO is produced in response to NMDA receptor activation (Connop *et al.*, 1995). Accordingly, the inhibition of NO synthesis may be beneficial in the treatment of neurological disorders resulting from excessive NMDA stimulation (Wolff *et al.*, 1997). In addition to its regulatory function during respiration (Carreras *et al.*, 2004), NO can also inhibit mitochondrial function (Brown, 1999) where the resultant inhibition of mitochondrial respiration relates to energy depletion and neurotoxicity (Brune *et al.*, 1998). Nitric oxide also stimulates mitochondrial production of reactive oxygen species (ROS) which may ultimately

lead to a diminished ability of the cells to deal with oxidative stress (Carreras *et al.*, 2004).

Elevated NO levels may therefore exert neurotoxic effects via several mechanisms (Brown, 1999). As stated previously, NO is a free radical that may combine with ROS to form highly reactive and destructive species that can induce oxidative stress and also disrupt normal mitochondrial function. It has also been observed that sensitivity to NO varies considerably from one cell type to another (Brune *et al.*, 1998) and that some neurons are more resistant to oxidative stress than others (Dawson *et al.*, 1991). Neuronal susceptibility to NO induced damage could depend on the physiological environment (Lafon-Cazal *et al.*, 1993) and therefore surviving neurons could be the cells with the most effective antioxidant capacities (Ischiropoulos & Beckman, 2003).

2.3.2 Neuroprotection of NO

Although NO has been reported to be an important mediator in neuronal degeneration, increasing evidence for protective mechanisms has also been obtained (Lautenschlager *et al.*, 2000). Association between NO and dementias must not be limited to NO mediated toxicity, but the potential neuroprotective properties of NO should also be considered (Law *et al.*, 2001). Nitric oxide's protective properties have been attributed to its ability to inhibit NMDA receptor mediated neurotoxicity (Connop *et al.*, 1995) along with its regulatory function in mitochondrial respiration (Bates *et al.*, 1996). It has even been described as an antioxidant. In essence, the above mentioned mechanisms may provide NO with a major cellular action against oxidative stress (de la Torre & Stefano, 2000).

While some studies have provided support for the intermediary role of NO in NMDA receptor excitotoxicity, others have provided evidence against this notion (Connop *et al.*, 1995). In view of NO's inhibitory action on voltage activated Ca^{2+}

channels, inhibition of NOS is thus expected to increase neuron excitability due to enhanced channel activity (Chaban *et al.*, 2001). Evidence has shown that dysfunction in Ca^{2+} homeostasis is associated with neurodegenerative diseases and in this case NO may exert its neuroprotectivity by means of inhibiting Ca^{2+} influx through NMDA receptors (Law *et al.*, 2001).

Other *in vivo* neuroprotective mechanisms of NO suggested by several studies include the inhibition of NMDA receptors through a negative feedback mechanism and the regulation of local cerebral blood flow (Law *et al.*, 2001). The former was demonstrated by the blockade of NMDA receptor function by NO donors. A proposed mechanism was that an oxidized form of NO inhibited the voltage dependent Ca^{2+} channels associated with NMDA receptor activation. It was therefore concluded that removal of basal NO levels would enhance NMDA toxicity (Law *et al.*, 2001).

Glutamate receptor agonists in the brain produce an activity-dependent vasodilation that is subsequently blocked by NOS inhibition, implicating NO as a role player during this process (Stuehr, 1999). It was found that the inhibition of nNOS or eNOS interfered with the regulation of regional cerebral blood flow (RCBF) and NO derived from these isoforms was therefore associated with neuroprotection (Connop *et al.*, 1995). It must however be considered that the nNOS selective inhibitor 7-nitro-indazole itself may decrease RCBF. In this case, nNOS may be excluded and eNOS may be the only isoform responsible for the protective effects of NO (Connop *et al.*, 1995). This is also supported by the difference in structural changes during activation of the individual enzymes (Stuehr, 1999).

In other studies, independent of glutamate excitotoxicity, NO again did not contribute to neuronal cell loss (Lautenschlager *et al.*, 2000). Non selective inhibition of NOS by L-NAME even resulted in an exacerbation of neuronal death.

Since the selective inhibition of nNOS along with iNOS did not potentiate neuronal destruction, the harmful effects of NOS inhibition were again mainly attributed to the inhibition of eNOS. The detrimental properties of eNOS inhibition during neurodegeneration are supported by the above mentioned influence on RCBF (Lautenschlager *et al.*, 2000). Reduction of RCBF is one of the common factors that may influence the excitotoxicity of NMDA receptors and complete NOS inhibition may thus attenuate the glutamate activity dependent vasodilatation and as a result metabolically compromise neurons (Connop *et al.*, 1995). The possibility that metabolic compromise may potentiate excitotoxicity is supported by findings that the inhibition of oxidative phosphorylation by various mitochondrial inhibitors can lead to excitotoxic lesions (Connop *et al.*, 1995).

During the last decade different investigators provided evidence of the existence of mtNOS (Carreras *et al.*, 2004). Nitric oxide derived from mtNOS has marked effects on mitochondrial functions such as, O_2 uptake, energy gain and cell signalling (Carreras *et al.*, 2004). Given the important role of endogenous NO in mitochondrial respiration, it is not surprising that the enzyme is localised close to its target site, cytochrome oxidase (Elfering *et al.*, 2002). The constitutive production of NO regulates the ratio of NO/O_2 and affects cytochrome oxidase activity (Carreras *et al.*, 2004). Studies indicate that the production of mitochondrial NO modulates the O_2 consumption of the organelle by competitively inhibiting cytochrome oxidase (Elfering *et al.*, 2002). This relationship links mitochondrial O_2 uptake and O_2 availability which are otherwise dependent on critical hypoxic conditions (Carreras *et al.*, 2004).

Based on these findings it can be proposed that mitochondrial production of NO assists in the O_2 utilisation amongst cells (Elfering *et al.*, 2002). This allows cells close to blood vessels to reduce their O_2 consumption resulting in a deeper penetration of O_2 to cells that are further away from blood vessels. Nitric oxide

may also facilitate the dilation of blood vessels and potentially increase O_2 delivery to borderline hypoxic cells (Elfering *et al.*, 2002).

Additionally, it has been reported that mitochondrial NO acts as an antioxidant by inhibiting the oxidation of mitochondrial lipids and proteins and thereby protects mitochondria from oxidative stress (Paxinou *et al.*, 2001). Nitric oxide has also been shown to be protective against an array of other agents that produce oxidative stress (Lautenschlager *et al.*, 2000). Heme proteins that react with H_2O_2 form toxic ferryl cations (Fe^{4+}=O) which are subsequently detoxified by their reaction with NO (de la Torre & Stefano, 2000).

Overall, these observations suggest that NO-mediated regulation of mitochondrial respiration, its antioxidant activity as well as its inhibitory action on NMDA receptors may represent a primary line of defence against oxidative stress (Paxinou *et al.*, 2001). The initial perception that the protective properties of NO are attributed to cNOS derived NO and the potential toxic properties to iNOS derived NO has therefore been incorrect and it is wrong to assume that a general inhibition of iNOS might be beneficial, as is to assume the opposite (Kröncke *et al.*, 2001). Future investigations will thus have to consider the need for targeted inhibition of specific NOS isoforms (Kröncke *et al.*, 2001) as the biological milieu may also modulate NO's toxicity and a wide range of NO's effects are achieved through its interaction with various target sites via redox and additive chemistry (Brune *et al.*, 1998).

2.3.3 Redox-potential

Neuronal vulnerability to NO induced damage could depend on the nature of the neurons and on the physiological conditions (Lafon-Cazal *et al.*, 1993). This is seen in cells containing high levels of NOS that are more resistant to NO-induced cell death than others. The apparent paradox of NO's neuroprotective and

neurodestructive role might be explained by its broader chemistry involving an array of interrelated redox forms (Stamler *et al.*, 1992).

Table 2.1: Functions and chemistry of redox-interrelated and other oxide forms of NO.

Name	Symbol	Known function
Nitric oxide	NO	Vasodilator, platelet inhibitor, immune regulator, neurotransmitter (Thomas & Ramwell, 2001). Primary reactions involve oxygen, super oxides and redox metals (Stamler *et al.*, 1992).
Nitrosonium	NO^+	Nitrosating agent, including electrophilic aromatic substitutions and additions to bases including peroxide (Stamler *et al.*, 1992).
Nitroxyl anion	NO^-	Smooth muscle relaxant (Thomas & Ramwell, 2001). Reactive with metals and sulfhydryls (Stamler *et al.*, 1992).
Nitrous oxide	N_2O	Anaesthetic (Thomas & Ramwell, 2001) .
Nitrite	NO_2^-	Produce NO at acidic pH (Thomas & Ramwell, 2001).
Nitrate	NO_3^-	Stable oxidation product of NO (Thomas & Ramwell, 2001).

The two moieties that have been focused on are NO and its reduced nitrosonium ion (NO^+) which, depending on the ambient milieu can act as either neuroprotective or neurotoxic (Lipton *et al.*, 1993). Nitric oxide's toxicity is in part prompted by the reaction with ROS leading to $ONOO^-$ formation that along with its decomposition products (equation 2.1) are implicated in lipid peroxidation and cytotoxicity.

Lipton *et al.* (1993) investigated several NO-generating drugs to assess the effect of NO's different redox forms. Morpholinosydnoimine, a NO-generating drug, was found to induce a dose-dependent neuronal cell death. Addition of super oxide dismutase (SOD) lowered the ROS that could interact with NO, thereby increasing NO concentrations and in effect halted the formatiom of $ONOO^-$. Even though NO generation was enhanced in the presence of SOD, no neurotoxicity was observed. This provided evidence that $ONOO^-$ was responsible for the observed toxicity (Lipton *et al.*, 1993).

$$NO^{\bullet} + O_2^{\bullet -} \xrightarrow{H^+} ONOO^- \leftrightarrow ONOOH \quad (2.1)$$

$$ONOOH \leftrightarrow O{=}NO^{\bullet} \cdots\cdots {}^{\bullet}OH \rightarrow NO_3^- + H^+$$

In contrast, other NO-generating compounds reduce NMDA receptor-mediated neurotoxicity even in the absence of SOD (Lipton *et al.*, 1993). This suggests that NO-generating drugs such as nitroglycerin (NTG) and sodium nitroprusside (SNP) possess protective properties even in the presence of $ONOO^-$. An important mechanistic implication however, is that both compounds required reductive activation to generate NO. Nitroglycerine and SNP does thus not spontaneously liberate NO, but requires the presence of a thiol group to release NO. This led to the conclusion that the redox-activated nitrosonium ion was responsible for the protective effect (Lipton *et al.*, 1993).

It is known that thiols at the NMDA receptor's redox modulatory site exert a neuroprotective action by means of inhibiting excessive calcium influx (Lei *et al.*, 1992). This mechanism involves S-nitrosylation of critical thiols at the NMDA receptor's redox modulatory site. In the appropriate redox form NO can therefore react with NMDA receptor protein thiols to down regulate channel activity (Lipton *et al.*, 1993). In addition, thiol groups may also act as potential transport or storage entities for NO which, upon further S-nitrosation of the complex may again yield NO (Kröncke *et al.*, 2001). These processes can considerably prolong the otherwise limited half life of NO and in effect exert NO's effects on distant targets. Thus, besides the modulating effect on NMDA receptors, the reaction of NO with thiol groups can also target NO to other specific areas (Kröncke *et al.*, 2001).

Nitric oxide's effect can therefore be influenced by the existing biological milieu, the relative rate of NO production (Brune *et al.*, 1998) and its different redox forms (Lipton *et al.*, 1993). The form in which NO is transported and delivered

may accordingly determine its toxic potential and resolve the apparent paradox of whether NO is cytotoxic or cytoprotective (Stamler *et al.*, 1992).

2.4 NOS inhibitors

The diverse role of NO necessitates the selective and targeted inhibition of specific NOS isoforms (Kröncke *et al.*, 2001). Drugs reduce or prevent the biological effects of NO in numerous ways and involves the reduction of cellular availability of substrate or co-factors as well as the scavenging of NO or the inhibition of enzyme activity (Moore & Handy, 1997). Inhibition of NOS activity has been the most thoroughly investigated approach and has provided the majority of useful compounds. Several of these new agents however, exhibit activity other than NOS inhibition and are thus unsuitable leads for the identification of selective NOS inhibitors (Moore & Handy, 1997).

Because individual isoforms produce NO for different purposes, selective inhibition of the appropriate enzyme is crucial to avoid complications during therapeutic regimes. Potent inhibitors are also needed, seeing that the high intracellular concentration of L-Arg is typically in excess of its K_m value (~ 1.6 μM for nNOS; Furfine *et al.*, 1994) for NOS (Garvey *et al.*, 1994).

Increasing evidence suggest that eNOS derived NO plays a protective role during neurodegenerative diseases (Lautenschlager *et al.*, 2000). It appears that a basal NO production may be sufficient to provide an endogenous neuroprotective system. Experiments with various NOS inhibitors indicate the important contribution of eNOS in this regard. Connop *et al.* (1995) referred to the negative feedback mechanism of NO on the NMDA receptor and the detrimental effect of eNOS inhibition on LCBF as possible indications of NO's protective mechanisms. As constitutive NOS isoforms are mostly involved in the

maintenance of blood pressure regulation and memory formation, it seems clear that inhibition of the constitutive eNOS isoform will result in adverse effects such as severe hypertension organ damage and memory loss (Boer *et al.*, 2000).

Nitric oxide synthesised by nNOS has been implicated in many pathophysiological processes including cerebral ischemia and excitotoxicity (Ayata *et al.*, 1997). Numerous *in vivo* and *in vitro* studies have demonstrated that the pharmacological inhibition or gene knock-out of nNOS, conferred resistance to cerebral ischemia and excitotoxicity (Ayata *et al.*, 1997). Conditions involving the neurotoxic mediated effect of glutamate receptors could also benefit from nNOS inhibition (Dawson *et al.*, 1991). Potent and selective inhibition of nNOS may thus be useful to treat stroke and other neurodegenerative diseases (Furfine *et al.*, 1994). Two challenging hurdles that hinder the clinical exploration of nNOS inhibitors however, involve an incomplete understanding of the precise role of nNOS in the central nervous system and the possible unwanted side effects. Impotence and the loss of memory would however not rule out the use of nNOS inhibitors during the treatment of stroke and related diseases (Moore & Handy, 1997).

Experiments furthermore proved that during inflammatory conditions, brain endothelial cells express iNOS that produce excessive amounts of NO (Bonmann *et al.*, 1997). Cytotoxicity as a result of massive NO formation has now been established to be initiated by apoptosis and the protective principles of iNOS inhibition become evident when cell destruction is redirected to cell protection (Brune *et al.*, 1998). Great effort has since been made to develop selective iNOS inhibitors (table 2.2), as this isoform is involved in a number of pathologies including septic shock and inflammatory conditions (Boer *et al.*, 2000). It seems clear that the selective inhibition of iNOS is an absolute necessity due to the severe side effects resulting from simultaneous inhibition of the constitutive NOS isoforms (Boer *et al.*, 2000).

Table 2.2: Potency and selectivity of NOS inhibitors at human isoenzymes (Boer *et al.*, 2000)

Compunds	Potency (log IC_{50} values)			Selectivity		
	iNOS	nNOS	eNOS	i/e NOS	i/n NOS	n/e NOS
Substrate analogues						
L-NA	5.8	7.5	7.1	0.05	0.02	3
L-NAME	4.3	5.7	5.6	0.05	0.05	1
L-PA	4.6	6.3	5.7	0.08	0.02	4
L-NMMA	5.9	6.0	6.5	0.3	0.8	0.3
L-NIO	6.6	6.3	6.2	3	2	1
L-VNIO	6.15	6.3	5.84	2	0.7	3
L-NIL	6.2	5.3	5.06	14	8	2
S-me-TC	7.2	8.1	7.27	0.9	0.1	7
Miscellaneous						
1400W	6.9	5.6	4.6	200	20	10
AMT	8.3	8.4	7.9	3	0.8	3
DPI	7.4	7.17	6.55	7	2	4
AG	4.5	4.3	3.57	9	2	5
2-AP	7.3	7.3	7.2	1	1	1

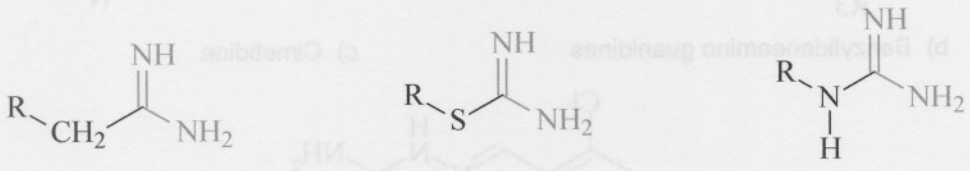
Abbreviations: **L-NA**, N^G-nitro-L-arginine; **L-NAME**, N^G-nitro-L-arginine methyl ester; **L-PA**, N-propyl-L-arginine; **L-NMMA**, N^G-monomethyl-L-arginine; **L-NIO**, L-N^5-(1-iminoethyl)-ornithine; **L-VNIO**, L-N^5-(1-imino-3-butenyl)-ornithine; **L-NIL**, L-N^6-(1-iminoethyl)lysine; **S-me-TC**, S-methyl-L-thiocitrulline; **1400W**, N-(3-aminoethyl)-benzyl-acetamide; **AMT**, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; **DPI**, diphenylene-iodonium; **AG**, aminoguanidine; **2-AP**, 2-amino-4-picoline.

The majority of NOS inhibitors described in literature have been amino acid analogues of the substrate L-Arg (Garvey *et al.*, 1994). Inhibition of NOS by N^G-nitro-L-arginine (L-NA) has demonstrated that inhibition could be both extremely potent and selective for individual isoforms (Garvey *et al.*, 1994). It has also been shown that the potency and selectivity of NOS inhibitors are solely determined by their affinity towards the different isoforms (Boer *et al.*, 2000).

Non amino acid analogues of L-Arg such as aminoguanidine (Misko *et al.*, 1993) and other alkylguanidines (Hassan *et al.*, 1993) have also come forth. These compounds prove to be more selective towards the iNOS isoform, but their potency however, is much weaker than compared to the amino acid analogues (Garvey *et al.*, 1994).

2.4.1 Guanidines

The comparison of potency and selectivity between amino acid and non amino acid based inhibitors of NOS, revealed the carbamidine moiety to be a common pharmacophore of NOS inhibitors (Moore *et al.*, 1996). Non amino acid based inhibitors of NOS include several classes of isoform selective inhibitors that has been identified as amidines, isothioureas and guanidines (figure 2.4; Wolff *et al.*, 1997).



a) Amidines b) Isothioureas c) Guanidines

Figure 2.4: General structures of amidine derivatives. The carbamidine moiety (blue) can be seen as a common structure in al three classes.

Guanidine and its derivatives have over the years been investigated for their pharmacological action and therapeutic application in diverse medicinal fields ranging from virology and cancer to cardio- and neuroprotection (Oliver *et al.*, 2004). Even the respective bradykinin (Dardonville *et al.*, 1998) and histamine H_2 receptor antagonists, martinelline (figure 2.5a) and cimetidine (figure 2.5c) contain guanidine-like structures and the Schiff base imidazoline I_2-receptor

antagonist, 1-(benzylidenamino)-3,3-dimethylguanidine (figure 2.5b), have also been reported on (Wikberg & Hudson, 1997).

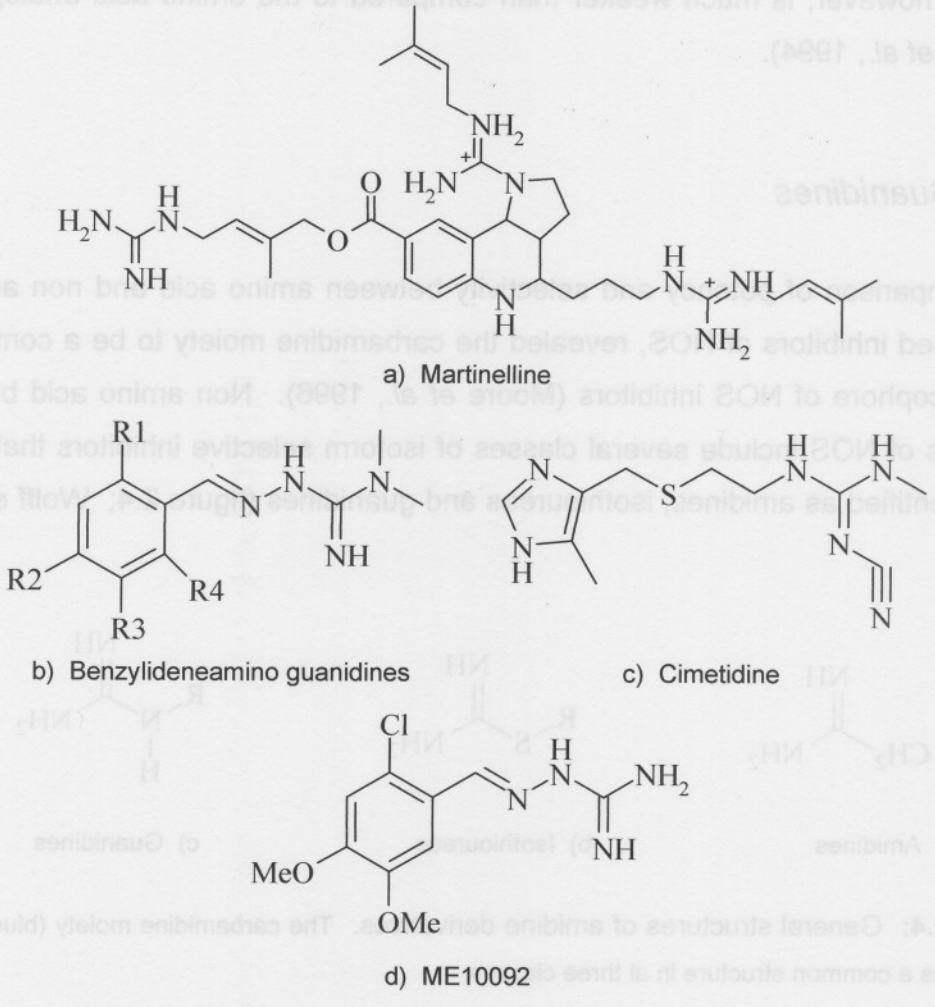


Figure 2.5: Guanidine-like compounds illustrating the diversity in structure and activity.

Hydroxyguanidines was described as antitumor agents (Adamson, 1972) and Schiff bases for example N-hydroxy-N^1-aminoguanidines have been explored for their antiviral activity (Doubell & Oliver, 1992). Pinacidil, a cyanoguanidine, is currently used as a potassium channel opener in the treatment of hypertension. Another guanidine compound, 1-(3,4-dimethoxy-2-chlorobenzylideneamino)-

guanidine (ME10092; figure 2.5d), also possesses strong cardioprotective effects that may be mediated through the modulation of NO production (Dambrova *et al.*, 2004). In view of guanidines interaction with enzyme systems such as nitric oxide synthase their potential to afford neuroprotection also came to light (Lu *et al.*, 2003).

Guanidines have since the initial observations significantly evolved into a variety of compounds with different therapeutic potentials (figure 2.5). The unique guanidine/guanidinium group (figure 2.6) that was found to be a pharmacophore in bradykinin antagonists may apply to other compounds with different pharmacological activities that also present the guanidine/guanidinium motif in their structures (Dardonville *et al.*, 1998). Interference of Ca^{2+} and Na^{+} conductances via the familiar inhibitory effect of guanidine on K^{+} channels as well as direct effects on voltage dependent Ca^{2+} channels have also been described (Cruz-Hofling & Rodrigues-Simioni, 1998). Guanidines' known ability to cause pressor responses and their similarity to L-Arg specifically led to their investigation as inhibitors of NOS (Southan & Szabo, 1996). Although at first, guanidine itself was not found to be an inhibitor, its derivatives ethylguanidine (figure 2.4c, R= $-CH_2CH_3$) and endogenous methylguanidine (figure 2.4c, R= $-CH_3$) did inhibit NOS. Sorrentino *et al.* (1997) however found, both guanidine and its methyl analogue to be inhibitors of NOS, but methylguanidine proved to be 8 and 3 times more potent than guanidine in inhibiting neuronal and inducible NOS respectively. The drawback of these compounds though, is the lower potency compared to the amino acid based inhibitors i.e. L-NMMA and that the majority of other alkyl substituted guanidines show negligible effects on NOS activity.

Aminoguanidine (figure 2.4c, R= $-NH_2$) on the other hand has received much attention as it is nearly equipotent to L-NMMA in its ability to inhibit iNOS and is 10 to 100-fold less potent as an inhibitor of the constitutive isoforms (Misko *et al.*,

1993). It also has low acute toxicity and has been found to enhance the survival of neurons and accelerate the pace of nerve recovery after injury (Gilad *et al.*, 1996). This beneficial action has been attributed to the inhibition of NO formation.

Present in a variety of drugs, aminoguanidine elicits different activities with it's most characteristic feature being the high basicity arising from its ability to delocalise positive charges on three guanidine nitrogen atoms. However, most of the pharmacologically active aminoguanidines are not strong bases, since electron-withdrawing groups bound to any of the nitrogen atoms dramatically decrease their basicity. The unique guanidine/guanidinium group along with guanidine's effect on voltage dependent Ca^{2+} channels may furthermore contribute to the diversity of AG's actions. Aminoguanidine itself also displays various pharmacological activities, where it not only inhibits nitric oxide synthase, but also histidine decarboxylase, catalase and diamine oxidase (Melero *et al.*, 2000).

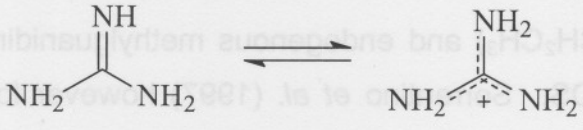
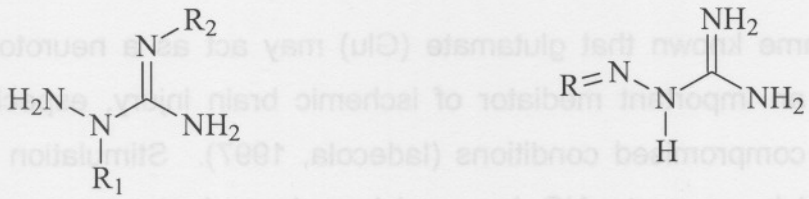


Figure 2.6: The unique guanidine/guanidinium cation (Dardonville *et al.*, 1998).

Observations confirmed AG's value as a selective inhibitor of the inducible isoform in the treatment of disease states characterised by the pathological over production of NO (Misko *et al.*, 1993). Wolff & Lubeskie (1995) found AG to be a mechanism based inactivator of NOS and also established that it exhibited a marked specificity for the inducible isoform. Despite AG's relative selectivity it was found to be an extremely weak inhibitor of iNOS both *in vivo* (Southan &

Szabo, 1996) and *in vitro* (Boer *et al.*, 2000), where complete inhibition of iNOS could only be observed at high concentrations of up to 15-45 mg/kg AG in rats or 400 mg/kg/day in mice (Southan & Szabo, 1996). Such an inefficient inhibitor of iNOS would probably be of little clinical use (Garvey *et al.*, 1994).

In their goal to identify more potent and isoform selective NOS inhibitors, Wolff *et al.* (1997) explored a series of substituted aminoguanidines. Aminoguanidines derivatised at the R_1 and R_2 positions were compared to the parent AG with respect to NOS inhibitory properties (figure 2.7). Based on their measurement of IC_{50} values, ethyl substitution at the R_2 position increased the potency and retained isoform selectivity. Previous observations on the vasculature effects of guanidino compounds also showed that guanidino moieties containing bulky substituents possessed a smooth muscle relaxing action, whereas smaller guanidines exhibited pressor effects that were partially related to NOS inhibition (Ozawa & Sugawara, 1968).



a) Aminoguanidine derivatised at the R_1 and R_2 positions

b) Alkyl substitution at the terminal N-position

Figure 2.7: Substituted aminoguanidines. Ethyl substitution in the R_2 position increased the potency and retained the selectivity of the guanidine compound. In contrast, methyl substitution at either R_1 or R_2 and hydroxyl or phenyl substitution at the R_2, reduced the potency and selectivity of compounds. In our investigational study, the effect on potency of alkyl substitution in the terminal N-position will be determined.

Another aspect that may contribute to AG's low potency is its poor blood brain barrier (BBB) permeability. At physiological pH, AG is positively charged and a significant transport through the BBB is not expected (Gilad *et al.*, 1996). The absence of a saturable transport mechanism for AG into the brain will result in poor availability of AG in the central nervous system (CNS; Mahar Doan *et al.*, 2000).

Therefore it seems that the requirement for an effective NOS inhibitor in the CNS is efficient BBB permeability and the presence of a carbamidine group. Alkyl substitution on AG and the assumed increase in lipophilicity might thus improve on its potency and selectivity profile and ultimately lead to a drug design that is more effective and clinically tolerable.

2.5 Polycyclic cage compounds

Since it became known that glutamate (Glu) may act as a neurotoxin, it has emerged as an important mediator of ischemic brain injury, especially under energetically compromised conditions (Iadecola, 1997). Stimulation of NMDA-receptors which, generate NO in a calcium dependent manner, raised the possibility that NO might participate in glutamate toxicity and gave rise to the proposal that neuronal injury in various neurodegenerative disorders is caused by the over stimulation of Glu receptors.

Glutamate is one of the main excitatory neurotransmitters of the CNS and along with other excitatory amino acids (EAA) operates through four different classes of receptors (Braune-Osborne *et al.*, 2000). In addition to the ionotropic EAA receptors (iGluR) namely NMDA, amino proionic acid (AMPA) and kianic (KA) receptors a fourth class of G-protein coupled EAA receptor (mGluR) have been shown to exert important functions in the neuronal signalling process. It is now

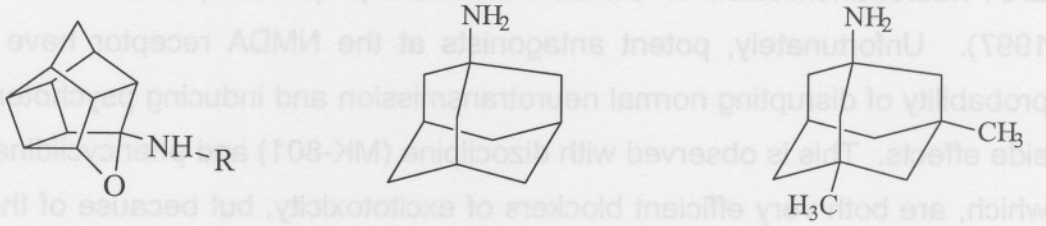
generally agreed that both iGluR and mGluR play important roles in the healthy as well as diseased CNS (Braune-Osborne *et al.*, 2000).

Earlier development of neuroprotective agents has focused on drugs that inhibit EAA neurotransmission or exhibit antioxidant properties (Kornhuber & Weller, 1997). Unfortunately, potent antagonists at the NMDA receptor have a high probability of disrupting normal neurotransmission and inducing psychotomimetic side effects. This is observed with dizocilpine (MK-801) and phencyclidine (PCP) which, are both very efficient blockers of excitotoxicity, but because of their high affinity, blocks critical normal functions. These drugs have further been associated with acute neurotoxicity which precluded their clinical use (Kornhuber & Weller, 1997).

Low affinity NMDA antagonists like amantadine and memantine however have been clinically approved for the respective treatment of PD and AD (Lipton & Chen, 2004). Moreover, memantine did not substantially affect normal synaptic activity and still prevented excessive NMDA receptor activation. Unlike other NMDA receptor antagonists, side effects are thus averted with memantine because of the preservation of physiological neurotransmission (Lipton & Chen, 2004).

Structural similarities and comparable activities between the pentacyclic cage compounds and adamantanamines indicate therapeutic potential for the former compounds (Oliver *et al.*, 1991a,b,c). These authors suggest that polycyclic amines may have potential as a new class of anti-parkinsonian agents due to their anticataleptic and anticholinergic activities. L-type calcium channel antagonism has also been described for pentacycloundecylamines, in particular for the prototypical compound 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane (NGP1-01; figure 2.8a, R= -

$NHCH_2Ph$) which was extensively studied in this regard (Malan *et al.*, 2000; Van der Schyf *et al.*, 1986).



a) Pentacycloundecylamine derivative b) Amantadine c) Memantine

Figure 2.8: Structural similarities between the pentacycloundecane derivatives with amantadine and memantine.

The structural resemblance between the polycyclic cage structure of memantine and NGP1-01 prompted the evaluation of these compounds and their derivatives for possible neuroprotective activity (Geldenhuys *et al.*, 2003b). Such putative protective activity can be hypothesised to be initiated by a dual mechanism of action including attenuation of NMDA receptor activity, thereby preventing excessive influx of Ca^{2+} into neuronal cells, as well as direct blockade of L-type Ca^{2+} channels. *In silico* results suggest a similar mechanism of interaction with the PCP binding site and polycyclic cage structures to that of MK-801 and memantine (Geldenhuys *et al.*, 2003a). A screening of novel pentacycloundecylamine derivatives for neuroprotection revealed that 8-phenylethylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (figure 2.8a, R= -$NHCH_2CH_2Ph$), protected neurons in the parkinsonian mouse model against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced neurotoxicity (Geldenhuys *et al.*, 2003b).

Another benefit of the pentacycloundecylamines is their ability to enter the CNS. For drugs to exert meaningful effects in the CNS they have to cross the BBB and it was found that the pentacycloundecylamines penetrated the CNS in sufficient amounts to exert pharmacological effects (Zah *et al.*, 2003). It was proposed that the main mode of entry into the CNS was by means of passive diffusion across the BBB.

2.6 *Conclusions*

Oxidation and nitration of proteins are markers of neurodegeneration and growing evidence suggests that the generation of oxidants does not simply result from accidental disruption of aerobic metabolism, but rather from active processes involved in the body's immune defences. As neurons are highly susceptible to oxidative stress the inappropriate activation of these processes may induce neurodegeneration (Ischiropoulos & Beckman, 2003).

The normal functionality of NO as a physiological neurotransmitter may be disturbed by the excessive production of NO in various tissues (Levecque *et al.*, 2003). Inappropriate induction of NOS in the brain may therefore result in glutamate neurotoxicity and be responsible for neuronal death. Nitric oxide is a free radical and its reaction with super oxides yields peroxinitrite which is an even more potent oxidant. These free radicals inherently react and mediate cellular toxicity by damaging critical metabolic enzymes. Therefore NO contributes to oxidative stress by inducing numerous cell changes including lipid peroxidation, nitrosylation, DNA damage and mitochondrial energy dysfunction. Through these mechanisms NO seems to exhibit a major contribution to neurodegenerative diseases (Lu *et al.*, 2002).

Nitric oxide's neurotoxic properties arose from the neuroprotective effects of NOS inhibitors and their ability to inhibit the formation of excessive NO. Recent development of isoform specific NOS inhibitors demonstrated that the selective blockade of specific isoforms offers a useful pharmacological strategy for the treatment of neurodegenerative disorders (Lu *et al.*, 2002).

The significance of iNOS expression in neuronal dysfunction has been demonstrated by the analysis of iNOS deficient mice (Iadecola *et al.*, 1997). While several studies have shown the detrimental effects of iNOS activity, others have indicated that in certain instances, iNOS expression could also be associated with neuroprotective mechanisms (Lu *et al.*, 2002).

Neuroprotective effects of NO have been attributed to the down regulation of NMDA receptor activity (Lipton *et al.*, 2002) and its regulatory role in both respiration (Bates *et al.*, 1996) and LCBF (Connop *et al.*, 1995). The biological responses to NO and the significance thereof are thus correlated by where, when and how much NO is present or being produced (Lautenschlager *et al.*, 2000).

Aminoguanidine demonstrated neuroprotective properties in several experimental models. More important was that this non amino acid analogue of L-NMMA had a relative specific affinity for iNOS in relation to its amino acid predecessor (Lu *et al.*, 2002). The higher selectivity for iNOS however, is hampered by the lower potency (Boer *et al.*, 2000) and lack of BBB permeability (Gilad *et al.*, 1996).

The close relation between the role of glutamate, NO and calcium homeostasis in neurodegeneration warrants the design of drugs that will modulate the effect of both neurotransmitters and regulate calcium homeostasis. Therefore, our objective was to explore the possibility of combining structures containing the guanidine moiety with the polycyclic cage intermediate and evaluating the effect

of these novel compounds on NOS activity. Aminoguanidine was selected due to its relative selectivity for the inhibition of iNOS as well as its attractive therapeutic potential and low toxicity. The polycyclic cage compounds were included because of their structural resemblance to the adamantanamines and their antagonistic effects on both NMDA receptors and L-type calcium channels.

Chapter 3

Synthesis

A series of guanylhydrazine and other polycyclic amine derivatives were synthesised for evaluation of their activity as nitric oxide synthase (NOS) inhibitors. Both monoketone and diketone pentacyclo-undecane structures were converted by condensation with aminoguanidine (AG) to the desired guanylhydrazines. Other pentacycloundecyl amines were obtained by the reductive amination of the intermediate cage structures with the respective amines. The compounds were characterised by nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared spectroscopy (IR).

3.1 Selection of compounds

A series of guanylhydrazine and other polycyclic amine derivatives (table 3.1) were synthesised. Both the guanidine and the pentacycloundecyl moieties were included to asses the effect that the combination of these two structures will have on the potency of NOS inhibition. Other structures related to guanidine and hydrazine were also of interest.

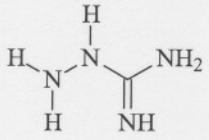
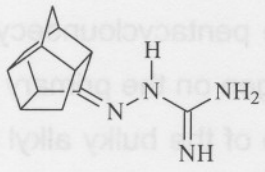
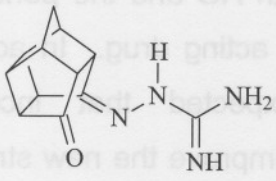
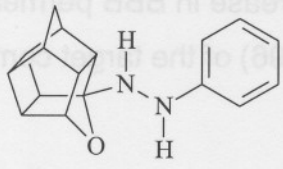
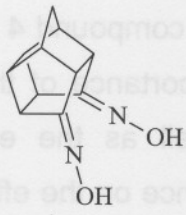
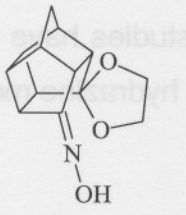
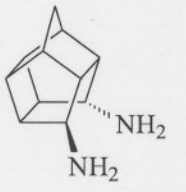
The moderate selectivity of AG (**1**) for inducible nitric oxide synthase (iNOS) made it an ideal compound to further explore structure activity relations regarding the potency of AG as an iNOS inhibitor. Previous studies revealed that alkyl substitution at the R_1 and R_2 positions of AG (figure 2.7; p. 43) had various outcomes with regard to its potency and selectivity (Wolff *et al.*, 1997). It was found that ethyl substitution at the R_2 position remarkably increased the potency of the compound while still preserving its isoform selectivity. Guanidine

compounds containing bulky substituents also show variable NOS inhibition (Ozawa & Sugawara, 1968). This directed our investigation towards the effect that bulky alkyl substitutions on AG's terminal N-position would have on its potency as a NOS inhibitor.

Owing to their neuroprotective effects similar to that of amantadine and memantine (Oliver *et al.*, 1991a,b,c), the pentacycloundecyl cage was selected as the alkyl group to substitute the hydrogen on the primary amine of AG. It was therefore hypothesised that the presence of the bulky alkyl group as well as the synergistic neuroprotective effects of both AG and the pentacycloundecyl cage would result in a more potent and dual acting drug. In addition to the above mentioned effects, it was also expected that incorporation of the pentacycloundecyl cage onto AG would improve the new structure's blood brain barrier (BBB) permeability. Such an increase in BBB permeability could enhance the therapeutic potential (Gilad *et al.*, 1996) of the target compounds.

Compounds **2** and **3** represent the novel guanylhydrazine compounds investigated in this study. The inclusion of compound **4** which contains a phenyl group, will allow the evaluation of the importance of the amidine moiety as a pharmacophore in NOS inhibitors as well as the effect of possible steric hindering. It will also provide further evidence on the effect of NOS inhibition by the hydrazine structure alone, since other studies have stressed the importance of a guanidino carbon being attached to the hydrazine moiety (Wolff *et al.*, 1997).

Table 3.1: Compounds evaluated and synthesised in this study.

Compound	Structure	Name
1		Aminoguanidine
2		8-imino-N-guanidino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane
3		8-imino-N-guanidinopentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecan-11-one
4		8-phenylhydrazine-8,11-oxapentacyclo [5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane
5		Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dioxime
6		Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane-8-ketal-11-oxime
7		Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-diamine

Compounds **5** and **6** were included in this series to assess the effect of the pentacycloundecyl cage that only contained an imine or oxime moiety. Pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-diamine **7** could provide evidence on the importance of the primary amine functionality on the pentacycloundecyl cage for NOS inhibition.

3.2 Experimental procedures

Reagents were purchased from Sigma-Aldrich (UK), Acros Organics (USA), Fluka (Switzerland) and reaction solvents from commercial sources. Melting points (mp) were determined in capillary tubes using the Gallenkamp melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on the Nicolet 470 FT-IR spectrophotometer and mass spectrometry (MS) was carried out on the VG 7070E mass spectrometer using electron ionization (EI) at 70 eV. A Varian Gemini 300 spectrometer was used to acquire ^{1}H spectra at a frequency of 300.075 MHz and ^{13}C spectra at a frequency of 75.462 MHz in a 7 Tesla magnetic field. Chemical shifts are reported in parts per million (ppm) relative to the internal standard tetramethylsilane (TMS). Abbreviations used to indicate the multiplicities of the respective signals are as follows: s, singulet; d, doublet; t, triplet; q, quartet and m, multiplet; bs, broad singlet.

3.3 Synthesis

The general route of synthesis (figure 3.1) included the preparation of the well known Cookson diketone (**D**), which was formed by the photocyclisation of the Diels-Alder adduct (**C**) resulting from the reaction between *p*-bensoquinone (**A**) and cyclopentadiene (**B**; Cookson *et al.*, 1964). Further manipulation of the diketone yielded the monoketone (Dekker & Oliver, 1978; Dekker *et al.*, 1982) which, like the diketone, was converted by condensation with AG to the desired guanylhydrazines. Pentacycloundecyl amines were obtained by reductive amination of the pentacycloundecane derivatives with the respective amines (Malan *et al.*, 2000). The resulting carbinolamines (**E**) were dehydrated under Dean Stark conditions and yielded imines (**F**), which were reduced to afford the final amines (**G**).

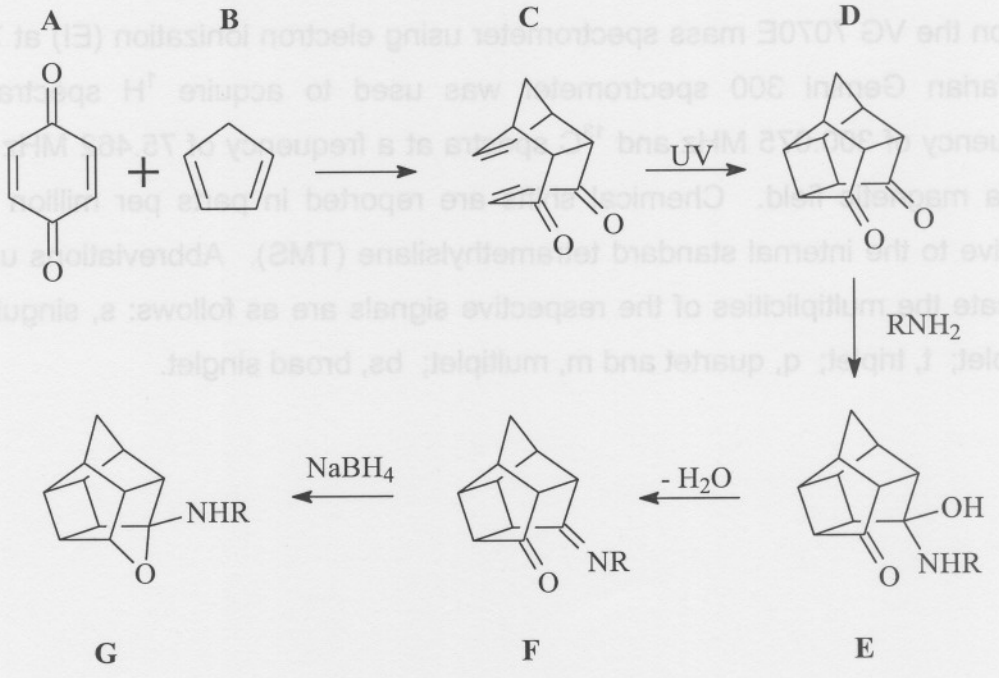


Figure 3.1: The basic synthesis of polycyclic amines (Malan *et al.*, 2000).

3.3.1 *Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**D**)*

p-Benzoquinone (50 g, 0.46 mol) was dissolved in dried benzene (400 ml) and oxidised with MnO_2 (30 mg) under reflux for 20 minutes. After this period activated charcoal was added and the mixture was refluxed for a further 5 minutes to absorb fine impurities present in the reaction mixture. The warm solution was then filtered to produce a clear yellow solution. Recrystallisation of the oxidised *p*-benzoquinone from this solution was prevented by the addition of dried methanol (100 ml). The mixture was then placed in an ice bath and allowed to cool down to 5°C. While the temperature remained at approximately 5°C, freshly monomerised cyclopentadiene (65 g, 0.5 mol) was added. This was done slowly and stoichiometrical to prevent the formation of the diadduct. After the addition of the cyclopentadiene the reaction mixture was removed from the ice bath, protected from light and stirred overnight at room temperature. The reaction was monitored by means of thin layer chromatography (TLC) and presumed complete as soon as the *p*-benzoquinone spot was no longer visible on the TLC plate. After completion, the excess solvents were removed *in vacuo* and allowed to fully evaporate in a dark fume cupboard to afford the yellow Diels-Alder adduct crystals. The Diels-Alder adduct was dissolved in acetone (3 g in 100 ml) and placed in a photochemical reactor where it was irradiated with UV light for approximately 6 hours. Decolouration of the solution confirmed that cyclisation of the adduct was complete and the solvent was subsequently removed *in vacuo*. The resulting light yellow residue was purified by Soxhlett extraction with cyclohexane to produce the diketone as fine white crystals (Yield 69.08 g, 0.397 mol, 80.934%). The physical characteristics of these crystals correlated with that in Cookson *et al.* (1964) and no spectral data is presented.

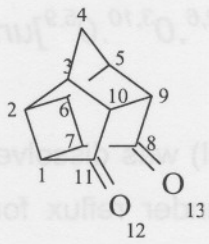


Figure 3.2: Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**D**).

3.3.2 Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-one (L)

A mixture of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**D**) (3 g, 17.22 mmol), ethylene glycol (1.2 g) and *p*-toluenesulfonic acid (20 mg, 0.01 mmol) in benzene (30 ml) was stirred under reflux for 5 hours (figure 3.3). The reaction mixture was cooled and treated with activated charcoal which was subsequently removed by filtration. *In vacuo* removal of benzene yielded the monoethylene-acetal (**H**) of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione as colourless crystals. A suspension of the oxo-acetal (**H**) was made in dry ether (10 ml) and over a period of 30 minutes gradually added to a suspension of lithium aluminium hydride (LAH; 300 mg) in dry ether (6 ml). The reaction mixture was refluxed for 2 hours and then cooled to 0°C. After reduction, excess LAH was deactivated with aqueous ammonium chloride and the organic phase concentrated *in vacuo* to afford clear homogenous oil (**I**). Hydrolysis of the acetal was done by stirring the oil in 6% hydrochloric acid (50 ml) for 2 hours at room temperature. Loss of the initial blue colour indicated complete hydrolysis upon which the reaction mixture was diluted with water (50 ml) and extracted with dichloromethane (5 x 20 ml). *In vacuo* removal of the organic solvent gave the hydroxy-ketone (**J**). A mixture of the hydroxy-ketone and hydrazine hydrate (5 ml) in diethylene glycol (60 ml) was kept at 120°C for 1.5 hours. Potassium hydroxide (2 g) was added

and the excess hydrazine and water distilled until the temperature reached 190°C, at which point the reaction mixture was refluxed for a further 3 hours. Dichloromethane extraction of the distillate yielded the pure alcohol (**K**) which was dissolved in 94% acetic acid (60 ml) and added to a mixture of chromium trioxide (4 g) in water (6 ml). The mixture was stirred at 90°C for 4 hours, then cooled, diluted with water (300 ml) and extracted with dichloromethane (4 X 40 ml). After successive washes with water (2 X 100 ml), saturated aqueous sodium hydrogen carbonate (2 X 100 ml) and again with water (100 ml), the organic phase was removed *in vacuo* to afford the desired monoketone (**L**).

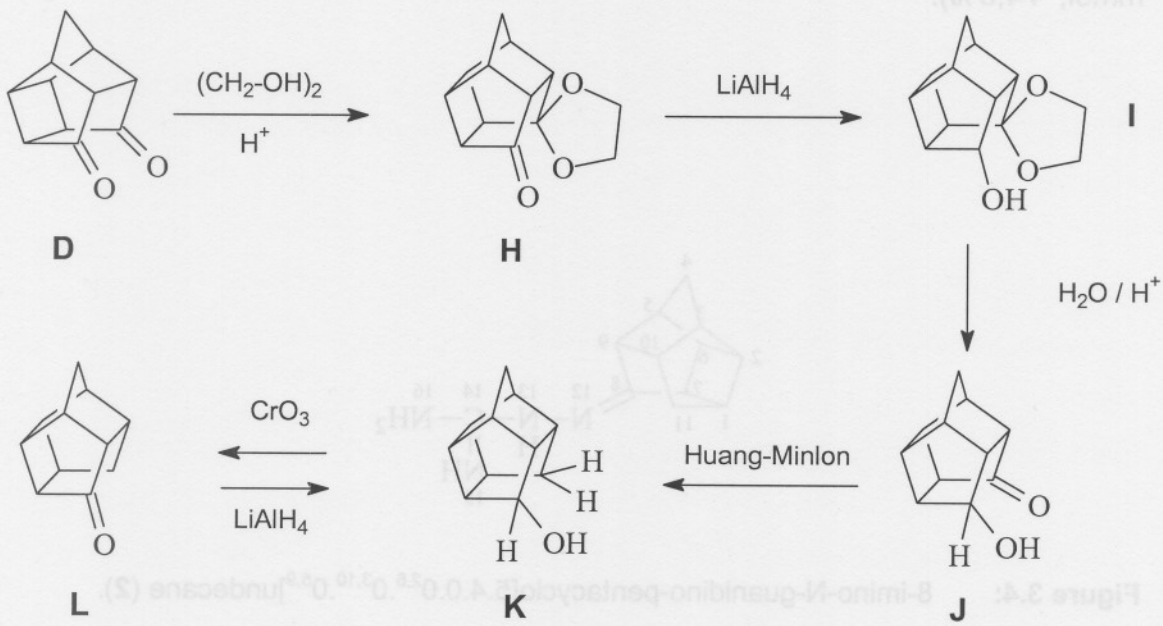


Figure 3.3: Synthesis of the monoketone (**L**) via the modified Huang-Minlon reduction of the hydroxy-ketone (**J**).

3.3.3 8-imino-N-guanidino pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane (2)

Aminoguanidine (HCl) (90 mg, 0.8 mmol) was dissolved in 2 ml ethanol and pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-one (200 mg, 1.25 mmol) was slowly added to the aminoguanidine solution while being stirred. The reaction mixture was placed under reflux for 40 hours where after the solvent was removed *in vacuo* to yield yellowish oil that was purified with column chromatography (8:2, ethanol:dichloromethane). After purification the product was dehydrated under Dean Stark conditions using dried benzene which was again removed *in vacuo* to produce the final product in the form of white crystals (Yield: 128.9 mg, 0.596 mmol, 74,5%).

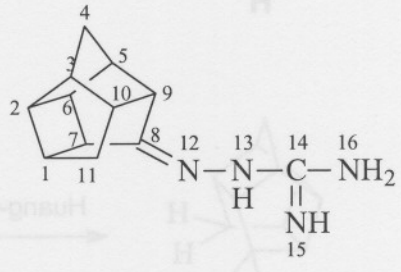


Figure 3.4: 8-imino-N-guanidino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**2**).

$C_{12}H_{16}N_4$; **mp**:>240°C; **IR (KBr)ν_{max}** (Spectrum 1): 3353, 3158, 2957, 2860, 1658, 1637 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 2): 216 (M^+), 158, 151, 137, 108, 91, 77, 66, 43, 28; **^{1}H NMR** δ_H (Spectrum3, $CDCl_3$): 10.7 (bs, 1H, H-13), 7.8, 7.45 & 6.25 (3 x s, 3H, H-15 &16), 2.3-3.7 (7 x m, 12H, H-1,2,3,4,5,6,7,9,10,11); **^{13}C NMR** δ_C (Spectrum 4, $CDCl_3$): 169.99 (s, C-14), 156.17 (s, C-8),47.55, 47.35, 46.10, 44.55, 42.91, 40.43, 39.80 & 36.72 (d, C-1,2,3,5,6,7,9,10), 36.13 (t, C-4/11), 31.12 (t, C-11/4)

*3.3.4 8-imino-N-guanidinopentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane-11-one (**3**)*

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**D**) (500 mg, 2.9 mmol) was dissolved in ethanol and aminoguanidine (HCl) (371 mg, 2.9 mmol) was slowly added while the mixture was stirred. The reaction mixture was then placed under reflux for approximately 15 minutes. Thin layer chromatography was used to monitor the reaction and although product formation seemed instantly, the reaction mixture was stirred overnight at room temperature to ensure a better yield. After *in* vacuo removal of the solvent, amber coloured oil was obtained which was subsequently dehydrated with dried benzene (50 ml) under Dean Stark conditions. The benzene was removed *in vacuo* and the product was purified by the addition of ethanol and the subsequent filtration of the residue. Fine white crystals were obtained as the final product (Yield: 276.32 mg, 1.2 mmol, 41.38%).

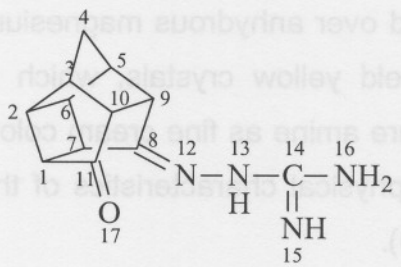


Figure 3.5: 8-imino-N-guanidinopentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-11-one (**3**).

$C_{12}H_{14}N_4O$; **mp**:113°C; **IR (KBr)ν_{max}** (Spectrum 5): 3378, 3173, 2978, 2931, 2859, 1740, 1673, 1627, 1113, 1067, 595 cm^{-1}; **MS** (FAB, 70 eV) *m/z* (Spectrum 6): 231 (M^+), 175, 154, 136, 107; **^{1}H NMR** δ_H (Spectrum 7, DMSO): 11.58 (s, 1H, H-13), 5.3 (s, 3H, H-15 &16), 2.4-4.5 (6 x m, 8H, H-1,2,3,5,6,7,9,10), 1.6-2.0 (2 x s, 2H, H-4); **^{13}C NMR** δ_C (Spectrum 8, DMSO): 213.99 (s, C-11), 161.39 (s, C-14), 156.06 (s, C-8), 53.39, 46.93, 46.22, 44.35, 43.16, 41.37, 40.81, 36.80 (d, C-1,2,3,5,6,7,9,10), 39.11, (t, C-4)

*3.3.5 8-phenylhydrazine-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}] undecane (**4**)*

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**D**) (5 g, 29 mmol) was dissolved in tetrahydrofurane (THF) (50 ml) and cooled to 5°C on an ice bath. Phenylhydrazine (3.1 g, 29 mmol) was then slowly added to the reaction mixture while being stirred on the ice bath for approximately one hour. The reaction was removed from the ice bath and stirred at room temperature for a further three hours. The THF was removed *in vacuo* and the resulting hydroxylamine in the form of fine yellow crystals was dehydrated under Dean-Stark conditions with dried benzene. *In vacuo* removal of benzene yielded the Schiff-base as a yellow oil which was subsequently reduced with sodium borohydride ($NaBH_4$) (1.5 g, 40 mmol) in dried methanol/THF (30 ml: 150 ml). Reduction continued overnight at room temperature where after the solvent was removed *in vacuo* and water added to the residue. The mixture was extracted with dichloromethane (4 x 50 ml) and the combined organic fractions washed with water (2 x 100 ml). The organic fraction was dried over anhydrous magnesium sulphate and the solvent removed *in vacuo* to yield yellow crystals, which was then recrystallised in acetone to afforde the pure amine as fine cream coloured crystals (Yield: 4.8 g, 18 mmol, 62.8%). The physical characteristics of these crystals correlate with that in Malan *et* al. (2000).

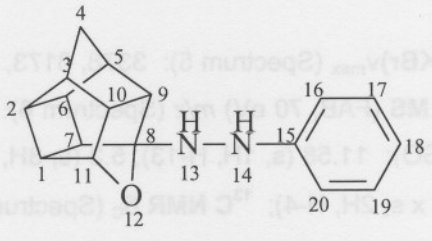


Figure 3.6: 8-phenylhydrasine-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**4**).

*3.3.6 Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dioxime (**5**)*

A mixture of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**D**) (1.74 g, 10.0 mmol), hydroxylamine hydrochloride (2.80 g, 40.0 mmol) and K_2CO_3 (2.76 g, 20.0 mmol) in ethanol/water (70 ml : 40 ml) was refluxed for approximately 3 hours. After the mixture was condensed to about 50 ml, the addition of water afforded a colourless precipitate (Yield: 1.4 g, 6.86 mmol, 68.6%).

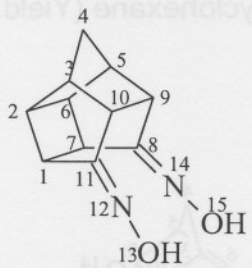


Figure 3.7: Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dioxime *(**5**)*.

$C_{11}H_{12}N_2O_2$; **mp**: 240°C; **IR (KBr)ν_{max}** (Spectrum 9): 3276, 3183, 2988, 2936, 2870, 1457, 928, 744 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 10): 204 (M^+), 169, 156, 91, 78, 65, 51, 39, 28; **^{1}H NMR** δ_H (Spectrum 11, DMSO): 10.15 (m, 2H, H-13,15), 2.4-3.7 (5 x m, 8H, H-1,2,3,5,6,7,9,10), 1.65 (ABq, 2H, J=10.77, H-4a,4b); **^{13}C NMR** δ_C (Spectrum 12, DMSO): 161.57 (s, C-8/11), 161.18 (s, C-11/8), 47.55, 45.46, 45.24, 44.79, 42.09, 40.28, 39.67, 34.61 (d, C-1,2,3,5,6,7,9,10), 37.45 (t, C-4)

3.3.7 *Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-ketal-11-oxime(**6**)*

A mixture of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**D**) (3 g, 17.22 mmol), ethylene glycol (1.2 g) and *p*-toluenesulfonic acid (20 mg, 0.01 mmol) in benzene (30 ml) was stirred under reflux for 5 hours. The reaction mixture was cooled and treated with activated charcoal which was subsequently removed by filtration. *In vacuo* removal of benzene yielded the monoethylene-acetal of the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}] undecane-8,11-dione as colourless crystals. The monoethylene-acetal (500 mg, 2.21 mmol) along with hydroxylamine hydrochloride (300 mg, 4.2 mmol) and K_2CO_3 (400 mg, 2.9 mmol) in ethanol/water (70 ml : 40 ml) was then refluxed overnight. The reaction was cooled and the addition of water yielded the end product as fine white crystals, which was recrystallised from cyclohexane (Yield: 300 mg, 56%).

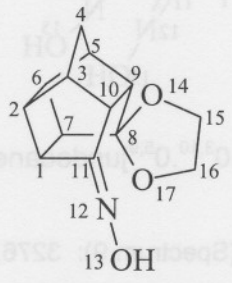


Figure 3.8: Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-ketal-11-oxime (**6**).

$C_{13}H_{15}NO_3$; **mp**: 73°C; **IR (KBr)ν_{max}** (Spectrum 13): 3455, 3296, 2978, 2891, 1335, 1107, 1037, 929 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 14): 233 (M^+), 217, 190, 159, 138, 66; **^{1}H NMR** δ_H (Spectrum 15, $CDCl_3$): 7.65 (bs, 1H, H-13), 3.7-3.95 (m, 4H, H-15,16), 3.6-3.7 (m, 1H, H-HCl), 2.3-2.85 (5 x m, 7H, H-1,2,3,5,6,7,10), 1.9 (bs, 1H, H-9), 1.65 (ABq, 2H, J=10.92, H-4a,4b); **^{13}C NMR** δ_C (Spectrum 16, $CDCl_3$): 165.28 (s, C-11), 115.46 (s, C-8), 65.87 (t, C-15/16), 63.75 (t, C-16/15), 50.56, 45.79, 45.23, 44.92, 42.36, 40.27, 40.00, 31.41 (d, C-1,2,3,5,6,7,9,10), 37.01 (t, C-4)

3.3.8 Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-diamine (7)

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**D**) (3 g, 17.22 mmol) was dissolved in THF (30 ml) and ammonia gas (NH_3) was bubbled through the solution while it was vigorously stirred. Within 15 minutes a fine white precipitate formed. This hydroxylamine which is insoluble in THF was filtered off and dehydrated under Dean-Stark conditions in dried benzene (50 ml) for one hour. After the *in vacuo* removal of the solvent the resulting imine was dissolved in MeOH/THF (15 ml: 75 ml), cooled on ice and reduced with lithium aluminium hydride ($LiAlH_4$) (1 g) for 5 hours at room temperature. The solution was then neutralised with the addition of 10% $HCl_{(aq)}$ and extracted with dichloromethane which was then dried over anhydrous magnesium sulphate. After the *in vacuo* removal of dichloromethane the crude product was purified with column chromatography (9:1, dichloromethane:ethanol) to afford fine white crystals (Yield: 1.87 g, 10.6 mmol, 68 %).

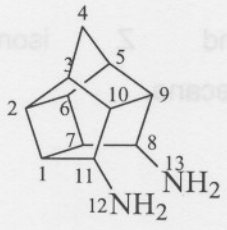
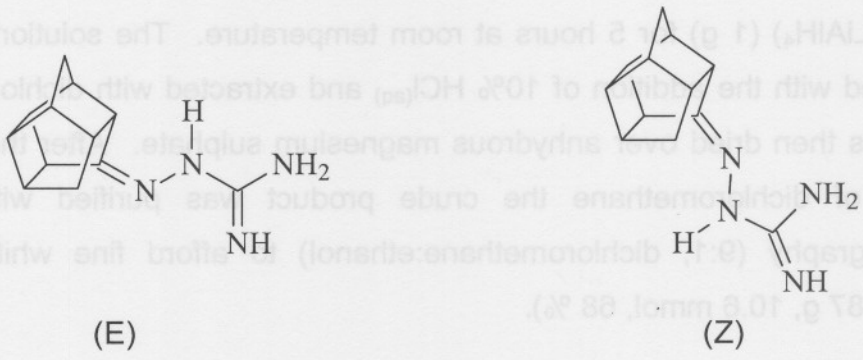


Figure 3.9: Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-diamine (**7**).

$C_{11}H_{16}N_2$; **mp**: 148°C; **IR (KBr)ν_{max}** (Spectrum 17): 3400, 3211, 2955, 2863, 1485, 1270, 1096, 1070 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 18): 178 (M^{+2}), 160, 145, 132, 117, 94, 91, 82, 78, 65, 55, 53, 39; **^{1}H NMR** δ_H (Spectrum 19, $CDCl_3$): 6.28 (d, 4H, H-12,13), 3.61 (s, 2H, H-8,11), 2.2-2.5 (s & m, 8H, H-1,2,3,5,6,7,9,10), 1.26 (ABq, 2H, J=10.58, H-4a,4b); **^{13}C NMR** δ_C (Spectrum 20, $CDCl_3$): 70.51 (d, C-8 & 11), 45.23, 42.37, 39.21, 38.12 (d, C-1 & 7, 2 & 6, 3 & 5, 10 & 9), 34.08 (t, C-4)

3.4 *Discussion*

The double set of NMR signals appearing in the ^{13}C spectra of compound **2** may be explained by the possible formation of distinct E and Z isomers (figure 3.10). This is also seen in the spectral data of compounds **3** and **5**. The E/Z isomerism may be viewed as diastereomers due to their non-superimposable, non-mirror image stereoisomers (McMurry, 1995).



(E) (Z)

Figure 3.10: The E and Z isomers of 8-imino-N-guanidino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane.

Another interesting form of stereoisomers is the formation of different enantiomers (figure 3.11). Although not directly affecting the spectral data, these enantiomers may play a significant role in the activity of the novel compounds. This stereoisomerism may come as a result of the nucleofilic addition reactions either occurring on the eleventh or eighth carbonyl carbon of the diketone cage structure as well as the presence of both monoketone enantiomers in the racemic mixture used during synthesis.

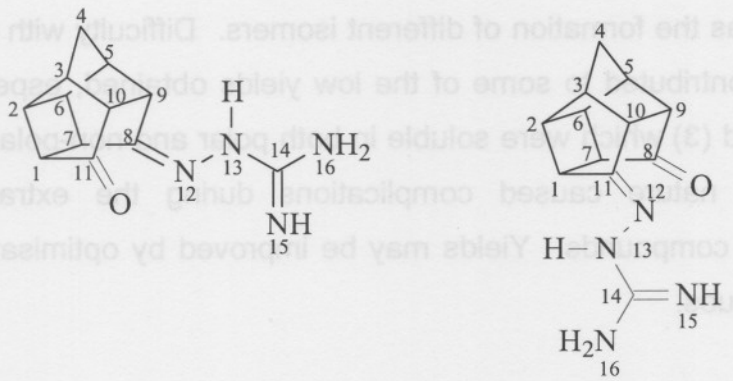


Figure 3.11: Enantiomers of 8-imino-N-guanidinopentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane-11-one.

In addition to the above mentioned isomerism, the possible formation of tautomers may also contribute to the double signals appearing on the ^{13}C spectra. Tautomers are different compounds with different structures due to the difference in atom arrangement and are also rapidly interconvertible (McMurray, 1995).

3.5 Conclusion

Different tautomers as well as the formation of E and Z isomers may be responsible for the double set of signals appearing in the ^{13}C spectra of compounds (**2**), (**3**) and (**5**). In view of possible racemic mixtures that may have formed during the synthesis of these compounds, it is also hypothesised that the various enantiomers may play a role in the activity of these new compounds.

The synthesis of all compounds resulted in yields ranging between 40% and 80% where the lower yields could be attributed to the formation of various unidentified

impurities as well as the formation of different isomers. Difficulty with regards to purification also contributed to some of the low yields obtained, especially with compounds (**2**) and (**3**) which were soluble in both polar and non-polar solvents. This amphipathic nature caused complications during the extraction and recrystallisation of compounds. Yields may be improved by optimisation of the purification techniques.

Chapter 4

Biological evaluation

In this chapter the oxyhemoglobin assay employed and the activity of the novel compounds at an enzymatic level are described. Nitric oxide synthase (NOS) activity of rat brain homogenate was measured utilising the conversion of oxyhemoglobin (oxyHb) to methemoglobin (metHb) by nitric oxide (NO). This *in vitro* screening was performed by spectrophotometrically monitoring the difference spectra between the wavelengths 390 nm and 430 nm against time. IC_{50} values were determined by plotting the percentage of NOS activity against increasing concentrations of novel inhibitors and the comparison of these IC_{50} values were used to relate structure with potency.

4.1 Introduction

Nitric oxide synthase catalyses the hydrolysis of L-Arginine (L-Arg) and molecular oxygen to L-citrulline and NO. Various NOS assays such as the widely used measurement of radiolabeled citrulline and the measurement of the stable end products of NO metabolism using the Griess reaction exist. The oxyhemoglobin assay is based on the reaction of NO with oxyHb which leads to the formation of metHb and nitrate (equation 4.1), a reaction that also largely accounts for the inhibitory effect of hemoglobin on the biological effects of NO (Feelisch *et al.*, 1996). Utilisation of this conversion of oxyHb to metHb provides an indication of NOS activity by monitoring the absorbance difference between these two species of hemoglobin.

The applicability of this method is primarily based on its sensitivity and specificity for NO under aerobic conditions, inexpensiveness, ease of implementation and for the rather modest technical requirements (Feelisch *et al.*, 1996).

$$\text{oxyHb} + \text{NO} \longrightarrow \text{metHb} + NO_3^- \qquad (4.1)$$

Under aerobic conditions in an aqueous solution, NO is highly unstable and prone to rapid reactions with oxygen, superoxide, thiols and a variety of metal containing proteins (Feelisch *et al.*,1996). As is true for any method of NO quantification, the reaction of NO with the detection molecule should be more rapid than that of the other competing species. In the oxyHb assay, this requirement is met by the high reaction rate between NO and oxyHb, which has been estimated to be 26 times faster than the auto oxidation of NO in aqueous solution (Hoshino *et al.*, 1993). The quenching effect of superoxides may be limited by the addition of superoxide dismutase (SOD) which will increase the trapping efficiency of oxyHb for NO and prevent the potential oxidation of oxyHb (Sutton *et al.*, 1976). Thiols will also not interfere with NO measurement as the reaction rate of NO/oxyHb still precedes that of the reaction rate between NO and thiols (Wink *et al.*, 1994). The rapid reaction between NO and oxyHb has the advantage of almost stoichiometrically trapping NO under most experimental conditions. Therefore, the oxyHb assay is somewhat unique among methods for NO determination as it is less subjected to constraints imposed by competing reactions with oxygen, superoxide or thiols.

The absolute spectra of oxyHb is characterised by an intense absorption Soret- or γ-band with a maximum at 415 nm and two weaker β- and α-bands with absorption maxima at 542 nm and 577 nm (Feelisch *et al.*, 1996). Methemoglobin's γ-band has an absorption maximum at 405 nm and the α- and

β-bands are only detectable under alkaline conditions indicating the marked pH sensitivity of metHb. At certain wavelengths the absorption of metHb is more intense than that of oxyHb and vice versa. Differences between metHb's and oxyHb's spectral characteristics become evident when both spectra are superimposed (figure 4.1).

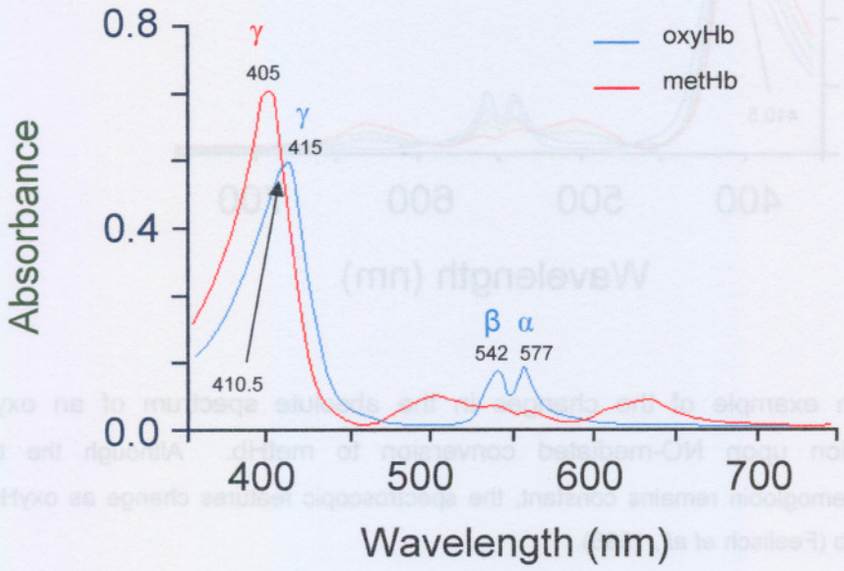


Figure 4.1: Superimposed absolute absorption spectra of oxyhemoglobin (oxyHb) and methemoglobin (metHb). Intersection points represent the *isosbestic* wavelengths at which the absolute absorbances of both species are identical (Feelisch *et al.*, 1996).

Only at a few discrete wavelengths are the absorbance intensities of both species equal. Under ideal conditions these so-called *isosbestic* points do not change during the conversion of oxyHb to metHb and the absorbance difference at a given *isosbestic* point will stay zero throughout the entire reaction.

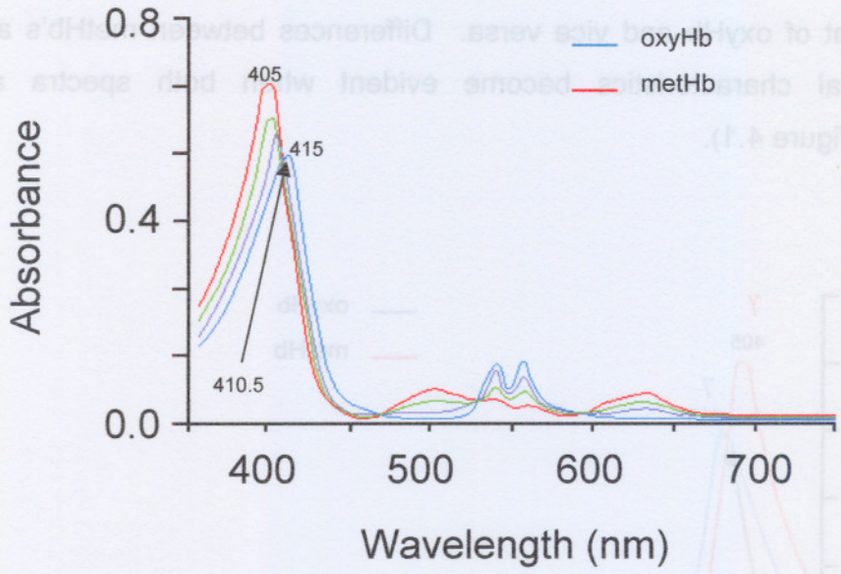


Figure 4.2: An example of the changes in the absolute spectrum of an oxyHb containing solution upon NO-mediated conversion to metHb. Although the total concentration of hemoglobin remains constant, the spectroscopic features change as oxyHb is converted to metHb (Feelisch *et al.*, 1996).

In this procedure, an absolute spectrum (figure 4.1) is recorded with the sample and reference cuvettes respectively containing oxyHb and vehicle. If NO now reacts with oxyHb to form metHb, the new spectra recorded over time will present the absolute spectra of varying mixtures of oxyHb and metHb (figure 4.2).

The disadvantage of this procedure however, is that the actual concentrations of the individual hemoglobin derivatives can only be read from the absolute absorbance when complete conversion of oxyHb to metHb has occurred (Feelisch *et al.*, 1996). In order to obtain information about the extent of

conversion and thus the amount of NO generated, the vehicle is no longer used as a reference, but only the oxyHb containing solution is employed. Instead of using the contents of a second cuvette, the absorbance of the same cuvette at a second wavelength is chosen as a reference. If the light absorption properties of the sample at a specific wavelength are recorded against the reference wavelength (difference spectra) in a repetitive manner, the changes in absorbance will reflect the loss of oxyHb and the simultaneous formation of metHb (figure 4.3). In addition to the *isosbestic* points there are certain regions corresponding to the formation of metHb where the absorbance increases (between 370 nm and 410.5 nm with a maximum at 401 nm) and regions corresponding to the loss of oxyHb where absorbance decreases (e.g. between 410.5 nm and 472 nm with a minimum at 421 nm). From the difference spectra, the wavelength at which maximum change in absorbance occur and a nearby *isosbestic* wavelength is usually subtracted from each other to determine metHb formation.

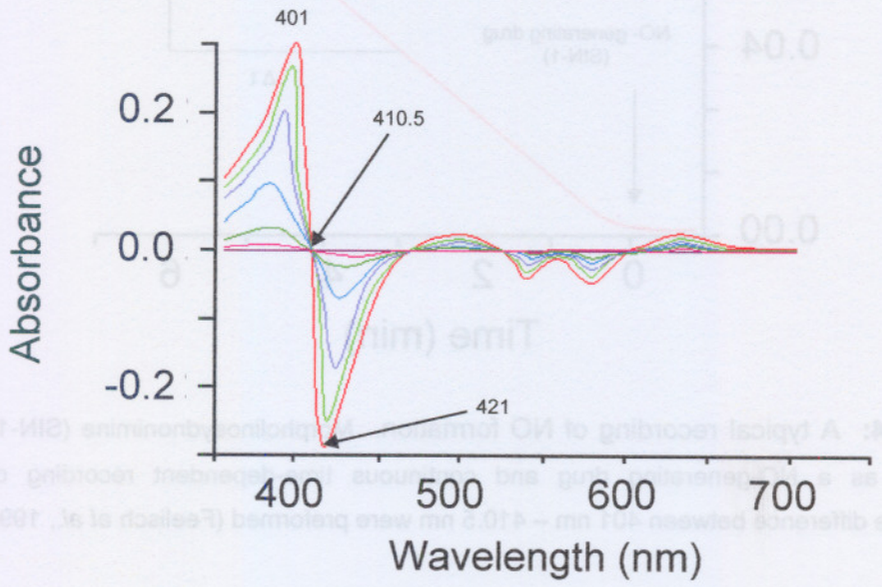


Figure 4.3: Repetitive scans of difference spectra. Spectra were recorded during conversion by NO of oxyHb to metHb illustrating maximum absorbance differences and *isosbestic* points (Feelisch *et al.*, 1996).

The absorbance difference (ΔA) will increase in relation to the amount of NO generated. This linear relationship between the absorbance difference and the formation of metHb is used to calculate the change in metHb concentration. In order to calculate the time dependent increase of metHb concentration, the absorbance difference between a wavelength of maximal absorbance change (e.g. 401 nm) and an internal reference wavelength (e.g. 410.5 nm or 421 nm) are measured as a function of time. The slope of the resulting curve is a measure of the increase in metHb concentration and thus NO formation or enzyme activity (figure 4.4).

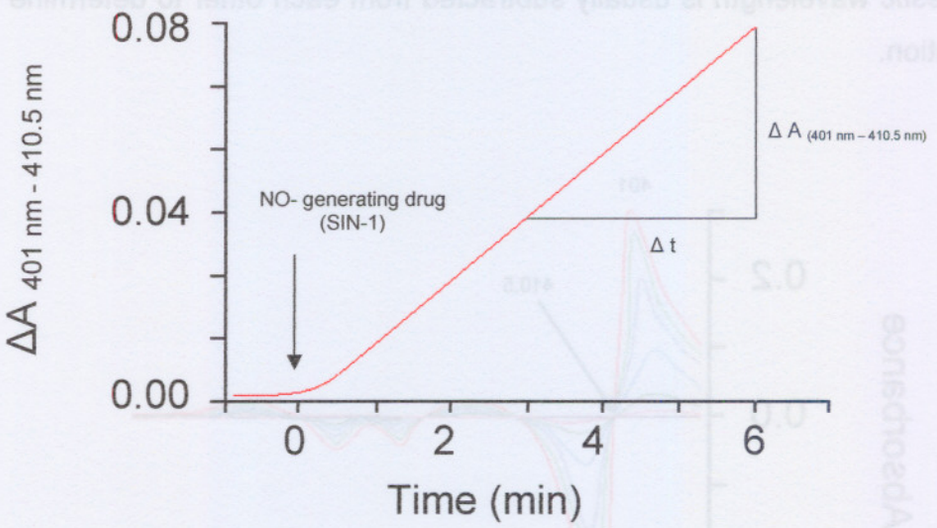


Figure 4.4: A typical recording of NO formation. Morpholinosydnonimine (SIN-1) was employed as a NO-generating drug and continuous time-dependent recording of the absorbance difference between 401 nm – 410.5 nm were preformed (Feelisch *et al.*, 1996).

4.2 Experimental procedures

The study protocol was approved by the Ethics Committee for Research on Experimental Animals of the North-West University (Potchefstroom campus). Male Sprague-Dawley rats were sacrificed by decapitation and the brain tissue was removed and preserved. The assay of NOS activity following the conversion of oxyHb to metHb was performed employing an adapted method as described by Salter & Knowles (1998). All materials were purchased from Sigma-Aldrich (UK) and Merck (St. Louis, MO, USA). Repetitive scans of spectra were recorded using a Varian Cary-50 UV-Visible spectrophotometer. The software package, Cary Win-UV (version 3.00) was used to calculate the slope values at certain wavelengths and IC_{50} values were subsequently calculated from enzyme inhibition data. All data processing, calculations and graphs were done using Prism 4.02 (GraphPad, Sorrento Valley, CA).

4.3 Materials

Hemoglobin (0.144 mM) which largely consists of metHb was converted to its oxygenated form (Feelisch *et al.*, 1996). This was achieved by gently dissolving the crystallised hemoglobin (20 mg) in distilled water (1.5 ml), followed by the addition of sodium dithionate (2-3 fold molar excess). Upon addition of the reductant the colour of the solution immediately changed from brownish red (mixture of oxyHb and metHb) to dark red (deoxyhemoglobin). Oxygen was then blown over the surface and the solution gently swirled. The gradual colour change from dark red to bright red was indicative of the oxygenation of hemoglobin. Purification and desalting was performed by passing the resulting oxyHb solution over a column of Sephadex G-25.

Potassium phosphate buffer (100 mM) was prepared at room temperature by mixing both the monobasic (KH_2PO_4, 300 ml; 100 mM) and the dibasic (K_2HPO_4, 1347 ml; 100 mM)) species and adjusting the pH to 7.4 by the subsequent addition of either KH_2PO_4 or K_2HPO_4.

Calcium chloride solution ($CaCl_2$; 12.5 mM) was prepared in the potassium phosphate buffer and l-arginine (1 mM) and NADPH (5 mM) in water (Salter & Knowles, 1998).

The test compounds were dissolved in potassium phosphate buffer or dimethylsulphoxide (DMSO; final DMSO concentration in the incubation < 0.5%) to give a series of final incubation concentrations ranging from 20 µM to 10 mM. Aminoguanidine (HCl) was dissolved in potassium phosphate buffer and was used as the positive control in final concentrations ranging from 100 µM to 10 mM.

The extraction buffer was prepared by dissolving sucrose (320 mM), HEPES (20 mM) and ethylenediaminetetra-acetic acid (EDTA; 1 mM) in distilled water and adjusting the pH to 7.2 at room temperature by the addition of 10% $HCl_{(aq)}$ (Knowles & Salter, 1998). The following constituents were then added to the final concentrations indicated: D/L-dithiothretiol (DTT; 1 mM), leupeptin (10 µg/ml), soybean-trypsin inhibitor (10 µg/ml) and aprotinin (2 µg/ml). The extraction buffer was then made up to its final volume with distilled water and 50 ml aliquots were stored at -20°C until required.

Phenylmethylsulphonyl fluoride (PMSF; 10 mg/ml) was prepared as a separate solution in absolute ethanol and stored at -20°C. Because PMSF is unstable in aqueous solution, it is not included in the buffer at this stage, but only added to the extraction buffer during the extraction procedure.

4.4 Methods

4.4.1 Sample preparation

Extraction and storage of tissue samples prior to the assay were carried out at 0°C - 4°C to avoid loss of enzyme activity. Fresh rat brain was weighed in 50 ml pre-cooled clear-plastic tubes and placed on ice. After being rinsed with ice cold extraction buffer, a measured volume of extraction buffer (5 ml/g tissue) was added to the tissue which was snipped into small fragments with cooled scissors. The sample was then homogenised with a mechanical homogeniser while the temperature was maintained at 4°C. After 10 seconds of homogenisation the PMSF (10 µl/ ml of extraction buffer) was added to the mixture and it was further homogenised for 30 seconds. The homogenate was then centrifuged at 12000 x g for 10 minutes to minimise turbidity. Once the supernatant was collected, it was divided into 2 ml aliquots which were either assayed immediately or rapidly frozen and stored at -70°C.

4.4.2 Assay procedure

Oxyhemoglobin and $CaCl_2$ were combined in the potassium phosphate buffer to give final concentrations of 1.44 µM and 250 µM respectively and to achieve at least 80% of the final reaction mixture volume. Tissue extract (100 µl) was added to the incubation and the reaction mixture prewarmed for two minutes to the required assay temperature of 37°C. After adding NADPH to a final concentration of 100 µM along with the test compound or control, the reaction was started by the addition of L-Arg to a final concentration of 100 µM.

After establishing the baseline, continuous scans (one scan every 12 seconds) between 390 nm and 430 nm monitored the conversion rate of oxyHb to metHb (figure 4.5).

4.5 Results

During the spectrophotometric scans where oxyHb was converted to metHb, the slope value of the difference spectra between 401 nm and 421 nm against time was utilised to determine NOS activity. Two of the spectrophotometric scans representing the control incubation and compound **3** at an inhibition concentration of 1 mM are respectively depicted in figure 4.5 and figure 4.6. Comparison of the spectra revealed a marked change in absorbance at 401 nm and 421 nm, indicating the inhibitory effect of compound **3** on NOS activity.

Reduction in the absorbance at 401 nm along with an increase at 421 nm is indicative of a decrease in metHb formation of and thus a subsequent decrease in enzyme activity. Enzyme activity is expressed as the rate at which NO (or metHb) is generated. The slope value of the difference in the change in absorbance between two specific wavelengths (e.g. 401 nm and 421 nm) over time is representative of this rate (figure 4.4).

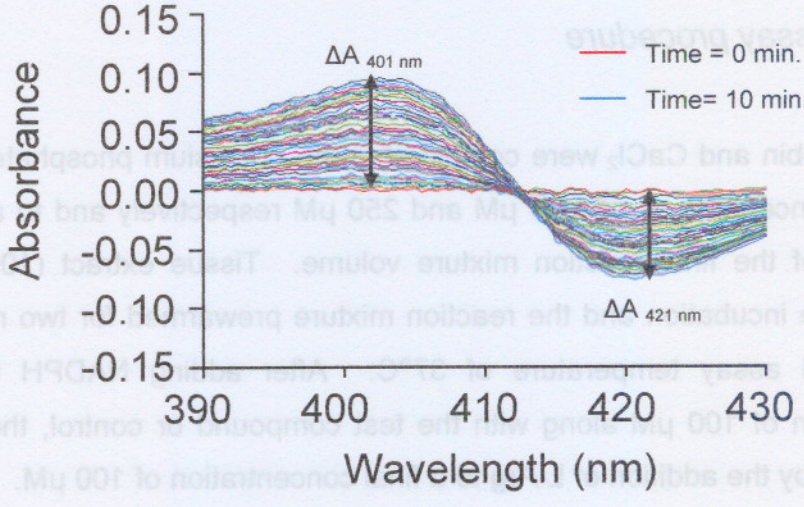


Figure 4.5: Original spectrophotometric recordings of the control incubation (0 mM inhibitor). Recordings were monitored over a period of 10 minutes between 390 nm and 430 nm. Arrows indicate the change in absorbance (ΔA) over time at 401 nm and 421 nm.

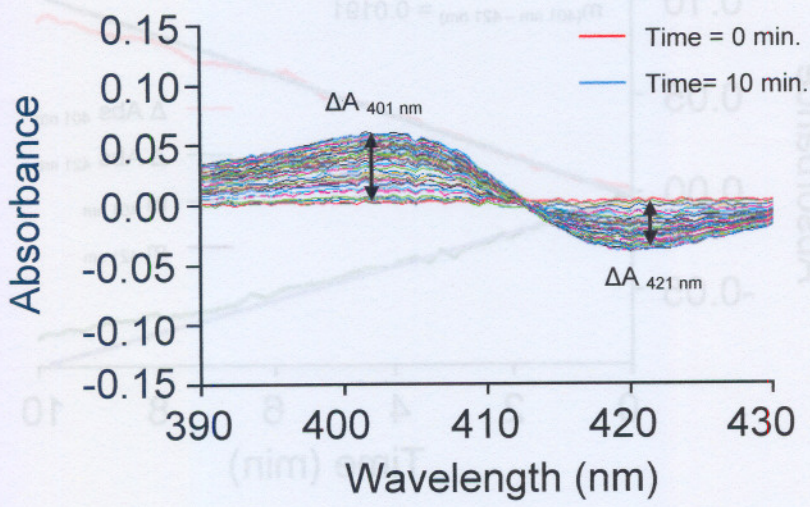


Figure 4.6: Original spectrophotometric recordings of NOS activity at a 1 mM concentration of compound **3**. Comparison with the control's difference spectra (figure 4.5) reveals a marked change in absorbance. Arrows indicate the change in absorbance (ΔA) over time at 401 nm and 421 nm.

From the resulting difference spectra (figures 4.5 & 4.6) the difference in the change of absorbance (ΔA) between 401 nm and 421 nm versus time were calculated by plotting the ΔA of the respective wavelengths against time (figures 4.7 & 4.8). Subtraction of the resulting curves' slope values ($m_{\Delta A(401\ nm)} - m_{\Delta A(421\ nm)}$) are indicative of the extent of oxyHb's conversion to metHb which is directly proportional to the amount of NO generated and enzyme activity.

Comparison of the control's slope value to that of a 1 mM inhibitor containing incubation reveals decrease in enzyme activity (table 4.1). Since the slope values of the curves in figures 4.7 and 4.8 are representative of NO's formation rate, the initial linear phase of the reaction was determined to be in the region between one and four minutes.

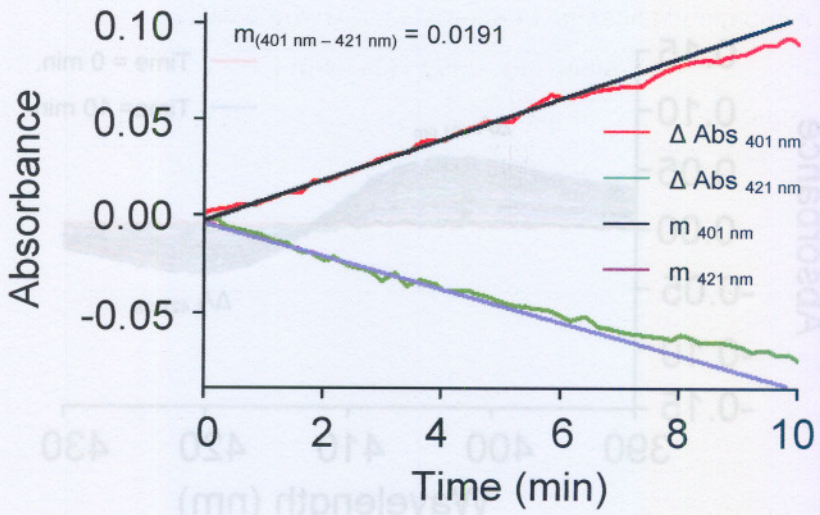


Figure 4.7: Determination of the change in absorbance of the control's difference spectra (figure 4.5). The change in absorbance at 401 nm and 421 nm versus time was calculated and the difference of the respective slope values ($m_{401\ nm} - m_{421\ nm}$) was indicative of enzyme activity.

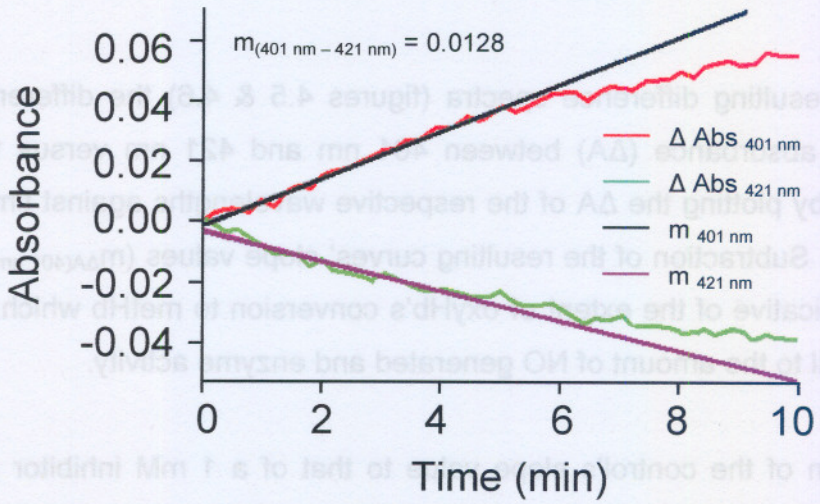


Figure 4.8: Change in absorbance of an incubation containing 1 mM of compound **3** (figure 4.6). The difference in slope values ($m_{401\ nm} - m_{421\ nm}$) is representative of enzyme activity and in comparison with the control (figure 4.7) indicates a decrease in enzyme activity with the addition of the novel inhibitor

Table 4.1: Comparison of the differences in slope (m) values during the initial linear phase of the incubation indicating a decrease in enzyme activity

Incubation	Slope (m) values (Abs/min)			% NOS activity
	$m_{401\ nm}$	$m_{421\ nm}$	$m_{(401\ nm - 421\ nm)}$	
Control 1* (figure 4.7)	0.0105	-0.0086	0.0191	~92
Control 2*	0.0129	-0.0067	0.0196	~95
Control 3*	0.0180	-0.0053	0.0233	~113
1 mM inhibitor 1*(figure 4.8)	0.0078	-0.0050	0.0128	~62
1 mM inhibitor 2*	0.0088	-0.0072	0.0160	~77
1 mM inhibitor 3*	0.0128	-0.0010	0.0138	~67

*Incubations were performed in triplicate and the data of Control 1 and one of the incubations containing 1 mM inhibitor are shown in figure 4.7 and figure 4.8.

Enzyme activity during the initial linear phase was expressed as a percentage of the control value (incubations containing 0 mM inhibitor represent ~100% activity) and the dose response curves were obtained by plotting inhibition data of enzyme activity against a series of logarithmic concentration of the novel compounds. Dose response curves were calculated as an average of two or three experiments where each experiment was conducted in triplicate. Data points are the mean values ± standard error of the mean.

The inhibition curves of selected compounds were superimposed on a single graph (figure 4.12) and compared by calculating the respective IC_{50} values (table 4.2). Of the newly synthesised structures only compounds **2** (figure 4.10) and **3** (figure 4.11) revealed promising results as possible NOS inhibitors. These two compounds are further discussed and compared to the inhibition data of aminoguanidine (figure 4.3).

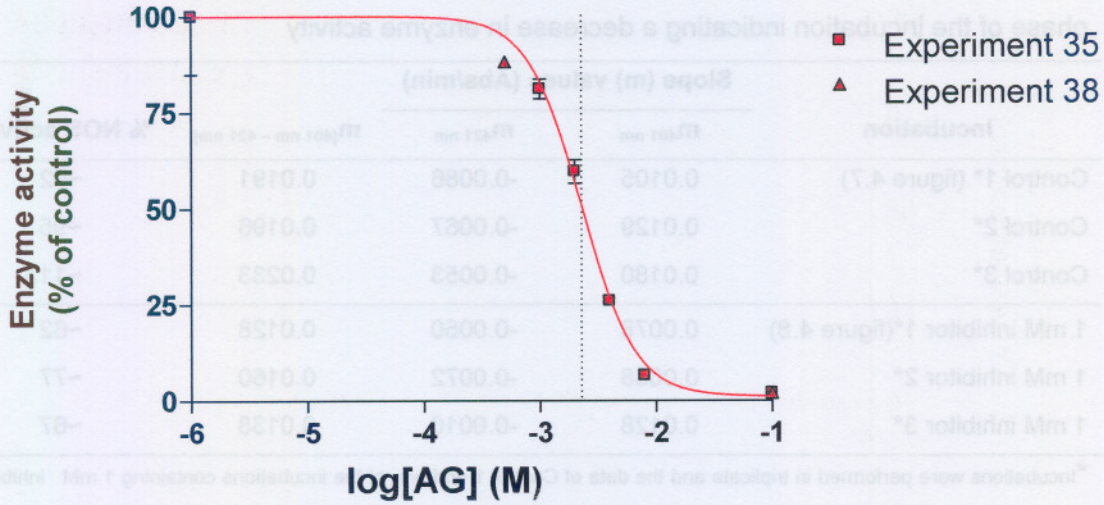


Figure 4.9: Inhibition curve of aminoguanidine on NOS activity. L-arginine (100 mM) was used as substrate. **log (IC_{50})**: -2.640; **IC_{50}**: $2.3x10^{-3}$ as indicated by dotted line.

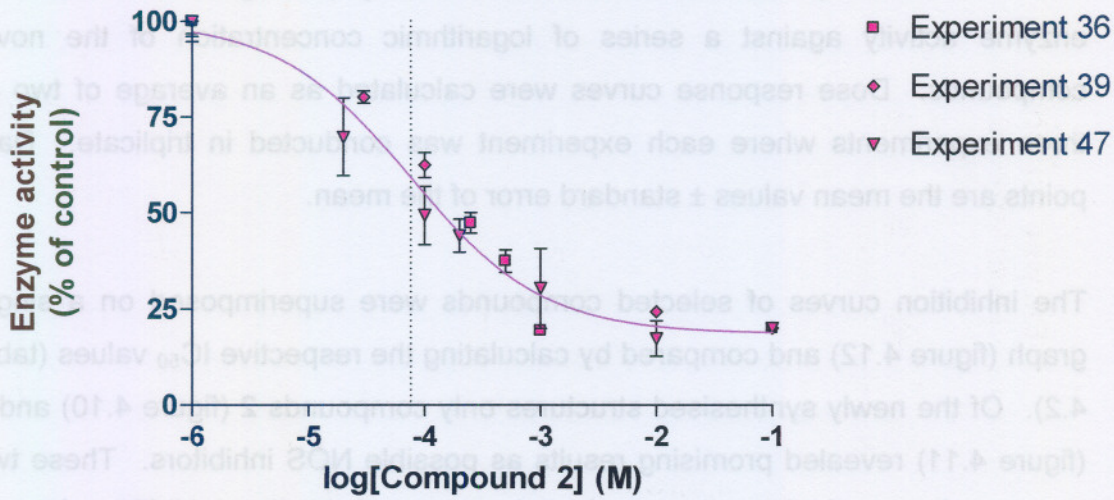


Figure 4.10: Inhibitory curve of compound **2** on NOS activity. L-arginine (100 mM) was used as substrate. **log (IC_{50})**: -4.119; **IC_{50}**: $7.6x10^{-5}$ as indicated by dotted line.

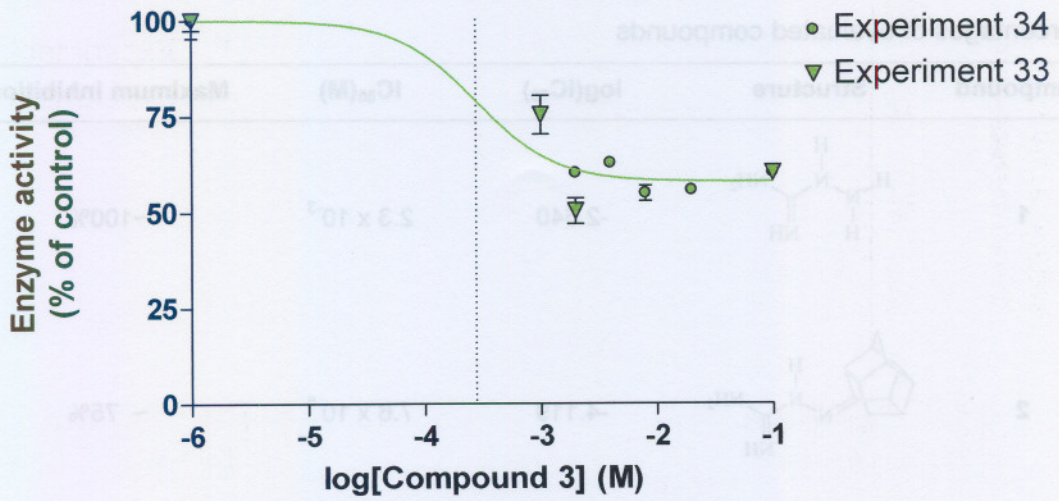


Figure 4.11: Inhibitory curve of compound **3** on NOS activity. L-arginine (100 mM) was used as substrate. **log (IC_{50})**: -3.556; **IC_{50}**: 2.8×10^{-4} as indicated by dotted line.

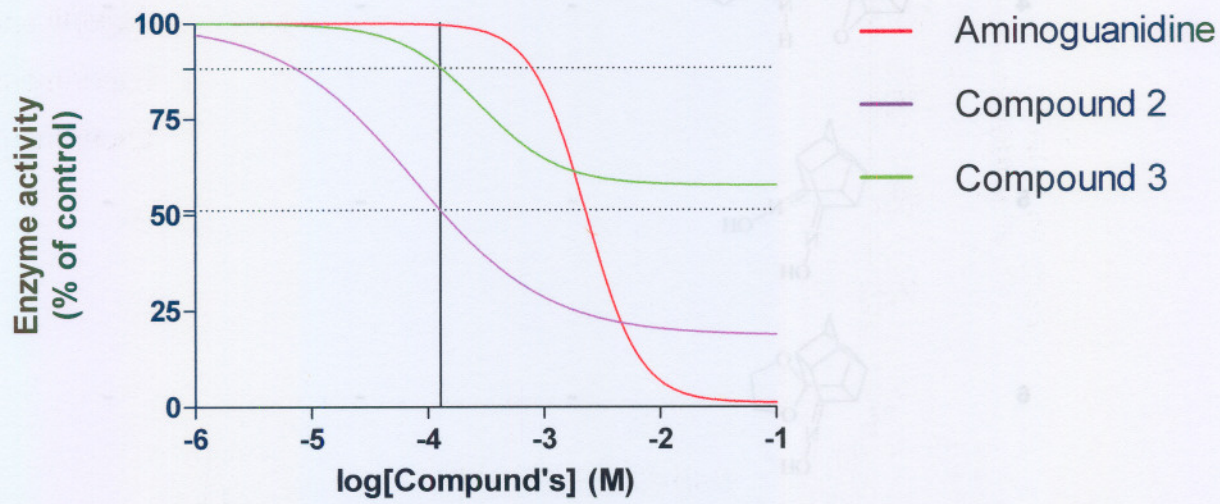
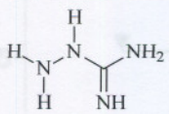
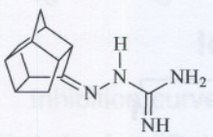
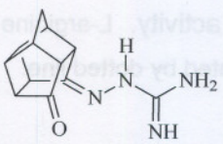
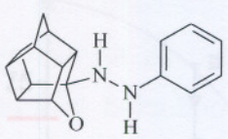
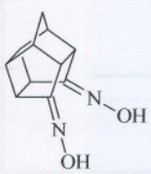
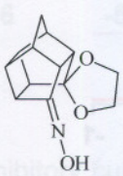
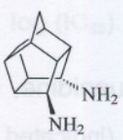


Figure 4.12: Superimposed inhibitory curves of Aminoguanidine, compound **2** and compound **3**. It is observed that at a concentration of 128 µM (indicated with the solid line) where no inhibition for aminoguanidine occurred, compound **3** revealed an ~12% inhibition and compound **2** an ~49% inhibition.

Table 4.2: Comparison of the log(IC_{50}) values, IC_{50} values and maximum inhibition percentages of evaluated compounds

Compound	Structure	log(IC_{50})	IC_{50}(M)	Maximum inhibition
1		-2.640	2.3×10^{-3}	~100%
2		-4.119	7.6×10^{-5}	~ 75%
3		-3.556	2.8×10^{-4}	~ 49%
4		-	-	-
5		-	-	-
6		-	-	-
7		-	-	-

- Compounds were inactive and no inhibition data were recorded.

4.6 Discussion

From the results it is clear that in the series of compounds evaluated, only structures containing the carbamidine moiety, i.e. **1**, **2** and **3**, show promising NOS inhibition. Although high inhibitor concentrations of the novel compounds failed to completely block NOS activity, these structures readily inhibited enzyme activity at lower drug concentrations where insignificant inhibition with aminoguanidine occurred. At an inhibitor concentration of 128 μM for example, compounds **2** and **3** respectively inhibited ~49% and ~12% of enzyme activity (figure 4.12) whereas the same concentration of AG proved to be ineffective. Comparison of the IC_{50} values of aminoguanidine and compound **2** revealed a more than 30 fold increase in potency.

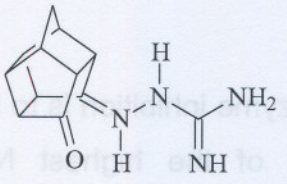
The apparent inability of compounds **2** and **3** to afford complete inhibition of NOS activity may perhaps be explained by various variables in the assay used, as well as the isomeric properties of the tested compounds and the possibility that these compounds may even act as potential substrates for NOS under saturated enzyme conditions.

An important part of the initial evaluation of enzyme inhibition is to have a source of known activity. Brain tissue has one of the highest NOS activities demonstrated in animals and therefore represents a simple and robust positive control. Biological tests were conducted on crude extracts of rat brain homogenate consisting of several NOS isoforms and accordingly, the selectivity of compounds was thus unaccounted for.

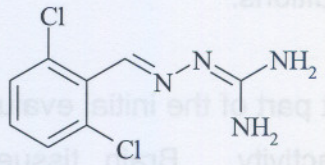
Regarding the effect of isomerism on NOS inhibition as discussed in chapter 3, racemic mixtures will have different outcomes on NOS activity when compared to the effects of pure isomers. As explained earlier, it was hypothesised that the

formation of different enantiomers may have significantly contributed towards the partial inhibition of NOS activity observed during the assays. Additional tautomerisation of compounds **2** and **3** and the formation of their respective E/Z isomers may also contribute to the incomplete inhibition of NOS activity.

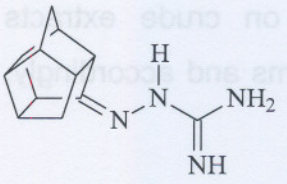
Furthermore, variable *in vitro* and *in vivo* effects must also be considered. Although *in vivo* effects were not evaluated in the present study, the structural similarities between ME1009 and compounds **2** and **3** propose this consideration (figure 4.13). In view of the bulky pentacyclic structure attached to the terminal N position of AG, the respective compounds resembles the structure of ME1009. Comparable *in vivo* inhibitory effects of ME1009 on NO synthesis in rat tissues to that of AG and guanabenz (Dambrova *et al.*, 2003) were contrasted by the *in vitro* inhibitory results of ME1009 (Dambrova *et al.*, 2004). *In vitro* findings indicated that ME1009 inhibited eNOS activity, but not that of iNOS. The apparent structural similarities of the individual compounds with ME1009 therefore warrants further exploration of the *in vivo* effects of these new structures.



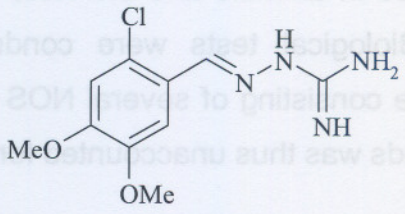
3



Gaunabenz



2



ME1009

Figure 4.13: Structural similarities of compounds **2** and **3** with that of ME1009 and guanabenz.

4.7 Conclusion

Although both cage compounds failed at high drug concentrations to completely inhibit enzyme activity, the respective compounds proved to be more promising inhibitors at lower concentrations than aminoguanidine.

The utilisation of crude enzyme extracts may explain the phenomenon of incomplete NOS inhibition. This may possibly be attributed to the presence of multiple NOS isoforms in the rat brain homogenate where the selectivity of these novel compounds may have caused the inhibition of specific isoforms only, while unaffected isoforms remained active.

Another explanation for the partial NOS inhibition of the new compounds considers the presence of different isomers, including enantiomers, tautomers and diastereomers. The potential of these new structures to act as substrates for NOS during saturating conditions as well as the possible differences between their *in vitro* and *in vivo* effects must also be held in perspective.

In view of the above, additional studies regarding isoform selectivity and both the *in vitro* and *in vivo* assessment of the individual configurational- and stereoisomers' effects are warranted.

Chapter 5

Summary, discussion and conclusion

Nitric oxide's (NO) role in neurodegenerative diseases is well known and although not the sole entity responsible for the aetiology of these diseases, it forms an integral part of the mechanisms involved in neurodegeneration. The rationale behind this study therefore led to the synthesis and *in vitro* evaluation of the effects of novel compounds on nitric oxide synthase (NOS) inhibition. The pentacyclic "cage" and guanidine structures were incorporated into the novel NOS inhibitors, which revealed promising *in vitro* activity. This study and further investigations of the novel inhibitors will contribute to the better understanding of the mechanisms involved in neurodegeneration and contribute to potential therapeutic compounds in this field.

An exploration of alkyl substitution on the terminal nitrogen of aminoguanidine (AG) was conducted by employing the pentacyclic cage structure as the substitution moiety for the synthesis of the respective guanylhydrazines (table 5.1). Pentacycloundecyl amines were prepared as additional analogues via the reductive amination of the intermediate cage structure with a series of selected amines. The primary aim of the current study was thus to design novel structures with increased neuroprotective activity, by means of a duel mechanism of action, owing to the guanidine's inhibitory effect on NOS and secondly, due to the antagonistic action of the cage amines on N-methyl-D-aspartate (NMDA) receptors. This study reports on the synthesis of the novel compounds and the inhibitory action of the newly synthesised compounds on NOS as a measure for neuroprotection.

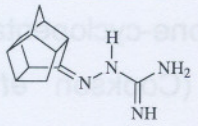
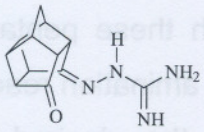
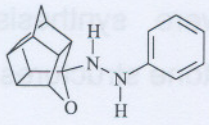
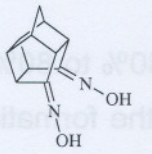
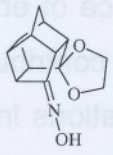
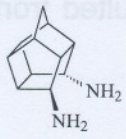
5.1 Synthesis

Ultraviolet irradiation of the *p*-benzoquinone-cyclopentadiene adduct afforded high yields of the Cookson diketone (Cookson *et al.*, 1964). Further manipulations of the diketone resulted in the formation of its monoketone derivative (Dekker & Oliver, 1978). Both these pentacycloundecyl structures were employed as intermediates in further amination reactions. Condensation of these intermediates with AG afforded the desired guanylhydrazines and additional pentacycloundecyl amines were synthesised via the reductive amination of the individual mono- and diketone structures (Malan *et al.*, 2000).

Although the percentage yield ranged from 40% to 80%, the purification of these structures proved to be a challenge due to the formation of various unidentified impurities and different isomers. The presence of enantiomers and subsequent formation of racemic mixtures may also have contributed to the inability of these compounds to recrystallise. Further complications in the extraction, purification and recrystallisation processes may have resulted from the unique amphipathic nature of some of these structures.

Tautomerisation of compounds **2**, **3** and **5** as well as the possible formation of E/Z isomers probably contributed to the observed double signals which appeared in the NMR spectra. Future projects could elucidate this phenomenon.

Table 5.1: Novel compounds used during the *in vitro* evaluation of NOS inhibition

Compound	Structure	Name
2		8-imino-N-guanidino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane
3		8-imino-N-guanidinopentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecan-11-one
4		8-phenylhydrazine-8,11-oxapentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane
5		Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dioxime
6		Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-ketal-11-oxime
7		Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-diamine

5.2 *Nitric oxide synthase inhibition*

An *in vitro* oxyhemoglobin (oxyHb) assay was employed to determine the NOS inhibitory activity of the newly synthesised compounds. This assay is based on the reaction between NO and oxyHb with the subsequent formation of methemoglobin (metHb). Measurement of the change in absorption (ΔA) during

the conversion of oxyHb to metHb is indicative of NO formation and thus NOS activity.

Of the newly synthesised compounds, only structures **2** and **3** demonstrated meaningful NOS inhibition in the micro molar range. This suggests that the presence of a carbamidine moiety is an important pharmacophore for effective NOS inhibition.

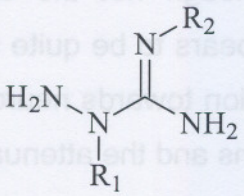
Comparison of the individual compounds' dose response curves with that of AG revealed interesting results. At high drug concentrations both compounds **2** and **3** failed to completely inhibit NOS activity, whereas at lower concentrations the novel structures proved to be more effective inhibitors than AG.

5.3 Discussion

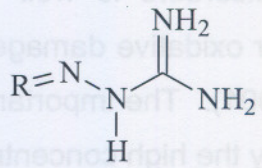
Despite NO's diverse physiological role, the participation thereof in various neurological disorders is well established and although not the sole entity responsible for oxidative damage, its involvement appears to be quite significant (Law *et al.*, 2001). The importance of NO's contribution towards neuronal injury is confirmed by the high concentration of NO in neurons and the attenuation of its detrimental effects by the use of NOS inhibitors.

It is well known that different NOS isoforms are responsible for the numerous effects exerted by NO. These isoforms are the source of NO in various tissue types and since these isoforms are limited to particular areas, they are accountable for the specific role of NO in that vicinity. This diverse role of NO thus calls for the selective and targeted inhibition of certain NOS isoforms.

The potential of aminoguanidine (AG) to selectively inhibit particular NOS isoforms prompted the further investigation into the structural requirements for these selective inhibitors. The enhanced selectivity of this non amino acid analogue of arginine was however at the expense of the inhibitor's potency (Boer *et al.*, 2000). Although AG was relatively iNOS selective, complete inhibition could only be observed at high concentrations and AG is therefore an extremely weak *in vitro* (Boer *et al.*, 2000) and *in vivo* (Southan & Szabo, 1996) inhibitor of the preferential isoform. In an attempt to identify a more potent and yet selective NOS inhibitor, Wolff *et al.* (1997) explored the activity of a series of substituted aminoguanidines. It was found that ethyl substitution at R_2 of AG increased selectivity while still retaining the potency of the inhibitor. Previous studies also established that guanidine moieties containing bulky substituents may influence NOS inhibition (Ozawa & Sugawara, 1968). Based on these results, the present study intended to explore the effect on potency of bulky alkyl substitutions at the terminal nitrogen position of AG by incorporating polycyclic cage moieties into the structure.



a) Aminoguanidine derivatised at R_1 and R_2



b) Alkyl substitution at the terminal N-position

Figure 5.1: Substituted aminoguanidines.

Considering that NOS is primarily activated by the NMDA receptor, the association between these two entities during neurodegenerative disorders make

them excellent targets for possible dual mechanistic drugs (Kemp & McKernan, 2002). In theory, any central nervous system (CNS) disorder in which neuronal loss is caused by glutamate induced excitotoxicity has the potential to be treated with NMDA inhibitors. This approach may also be applied to neurodegenerative disorders in which excess glutamate is not primarily a problem, but where compromised neurons become sensitised to excitotoxic damage.

Since the structural features of the pentacyclic cage compounds favourably compare to that of the adamantanamines, it was selected as the alkyl groups for substitution at the terminal nitrogen position of AG. Both amantadine and memantine have been approved for the clinical treatment of PD and AD respectively. The adamantanamines exert their uncompetitive antagonistic effects on the NMDA receptor in a more subtle way than other high affinity channel blockers (Lipton & Chen, 2004). These low affinity NMDA receptor channel blockers have limited CNS side effects when compared to other non-competitive antagonists such as phencyclidine (PCP) and dizocilpine (MK-801). The polycyclic cage moieties were therefore considered as candidate structures for alkyl substitution at the terminal nitrogen of AG and used as key intermediates during the synthesis of the new compounds.

Our inhibition studies reveal only compounds **2** and **3** to exhibit meaningful inhibition of NOS. An important observation in the experimental data is that both compounds were not able to fully inhibit NOS activity and that these compounds proved to be more potent inhibitors than AG at lower concentrations. The secondary objective of this study was therefore achieved to a certain degree in view of the structures ability to effectively inhibited enzyme activity, albeit that maximal inhibition could not be attained. Interpretation of the above mentioned results may be explained by different variables in the assay procedure as well as certain aspects regarding the chemistry of the novel compounds.

It is hypothesised that the formation of E and Z isomers (McMurry, 1995) may have contributed to the partial enzyme inhibition noticed during the experimental procedure and could furthermore explain the double signals observed in the ^{13}C spectra. Throughout the biological evaluation, the cage compounds were evaluated as racemic mixtures and the resultant enantiomeric composition could also be responsible for the incomplete inhibition of enzyme activity. Adding to the effect of stereoisomerism is the possible formation of tautomers which may once more contribute to the partial inhibition of these new compounds at high concentrations as well as the additional signals of the NMR spectra.

A crude enzyme extract of rat brain tissue was employed in the assay and the presence of multiple NOS isoforms may furthermore explain the threshold activity of NOS at high inhibitor concentrations. The possibility that different NOS isoforms may interfere with the outcome of the inhibition data is attributed to the selectivity of the test compounds. As a consequence, the presence of an isoform not inhibited by the compound might be accountable for the threshold activity observed during saturated conditions.

5.4 Conclusions

To conclude, the primary objective of the present study was achieved by the successful synthesis of a series of polycyclic guanidine and amine derivatives. The secondary aim was the evaluation of these compounds as possible NOS inhibitors with an expected improved potency and was also achieved to a certain degree. Although the assay employed in the biological evaluation did not differentiate between the respective NOS isoforms, results show that the test compounds have promising activity as NOS inhibitors and could be of potential value in the treatment and prevention of neurological disorders. It is

hypothesised that the inability of the active novel compounds to fully inhibit enzyme activity can primarily be attributed to the presence of multiple NOS isoforms that were present in the crude enzyme extract as well as the various stereo and conformational isomers of the newly synthesised structures.

Future studies to better understand the present results are therefore prompted by the above mentioned conclusions. In order to more accurately determine the selectivity of the novel inhibitors, inhibition studies with individual NOS isoforms along with a larger series of derivatives needs to be conducted. Additional assays on the NMDA receptor and blood brain barrier permeability will furthermore elaborate on these compounds' potential value. Separation and assessment of different isomers will also significantly contribute to the understanding of the role that isomerism plays in the interaction with NOS and subsequent activities. The application of computer assisted molecular modelling will aid in this regard and may be of value for the further exploration of other novel neuroprotective structures.

The results of this study may serve as guidelines for future investigations into the structural modifications of guanidine containing compounds for optimisation of their potential role as neuroprotective agents, not only as improved NOS inhibitors, but also for their ability to act on additional target sites.

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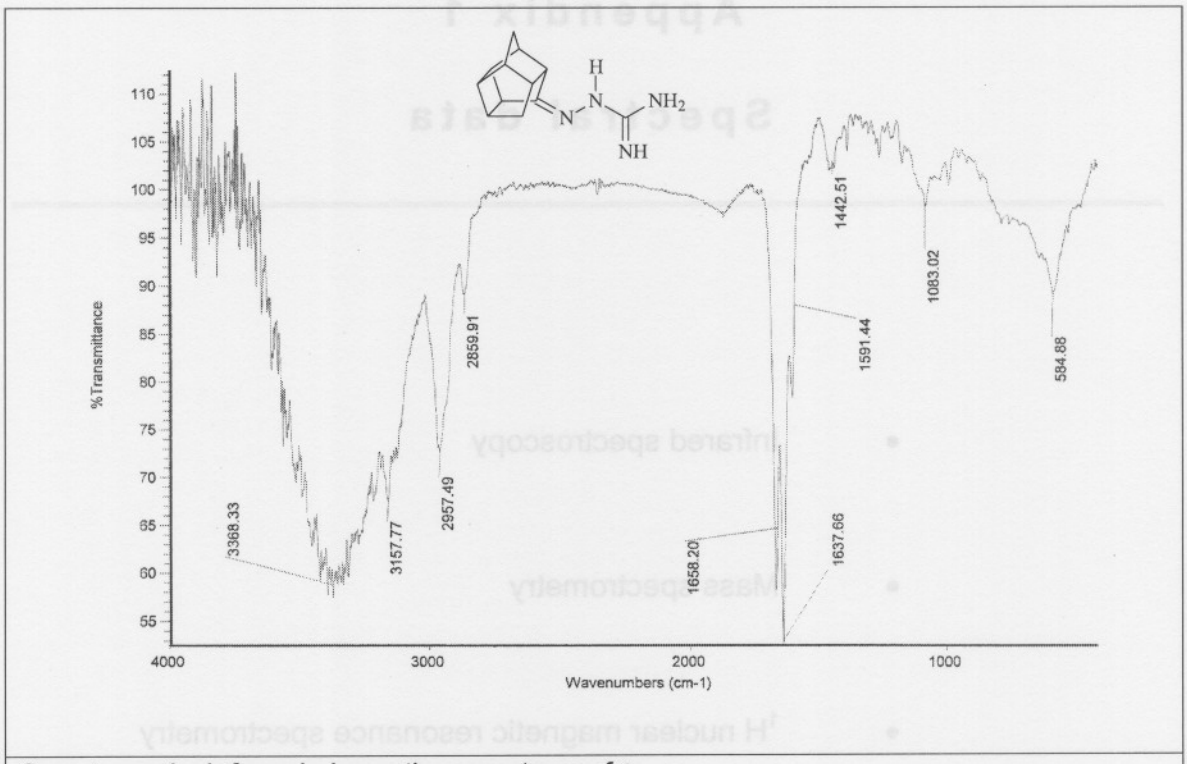
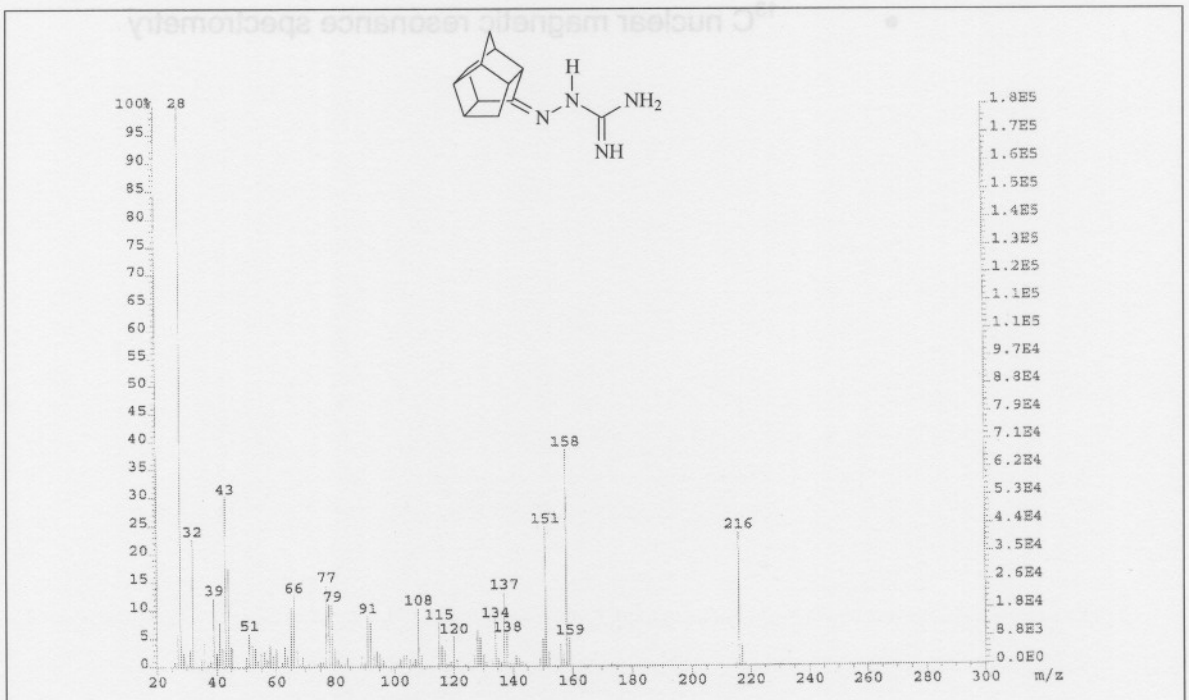
Appendix 1

Spectral data

- Infrared spectroscopy
- Mass spectrometry
- ^{1}H nuclear magnetic resonance spectrometry
- ^{13}C nuclear magnetic resonance spectrometry



Spectrum 1: Infrared absorption spectrum of **2**.



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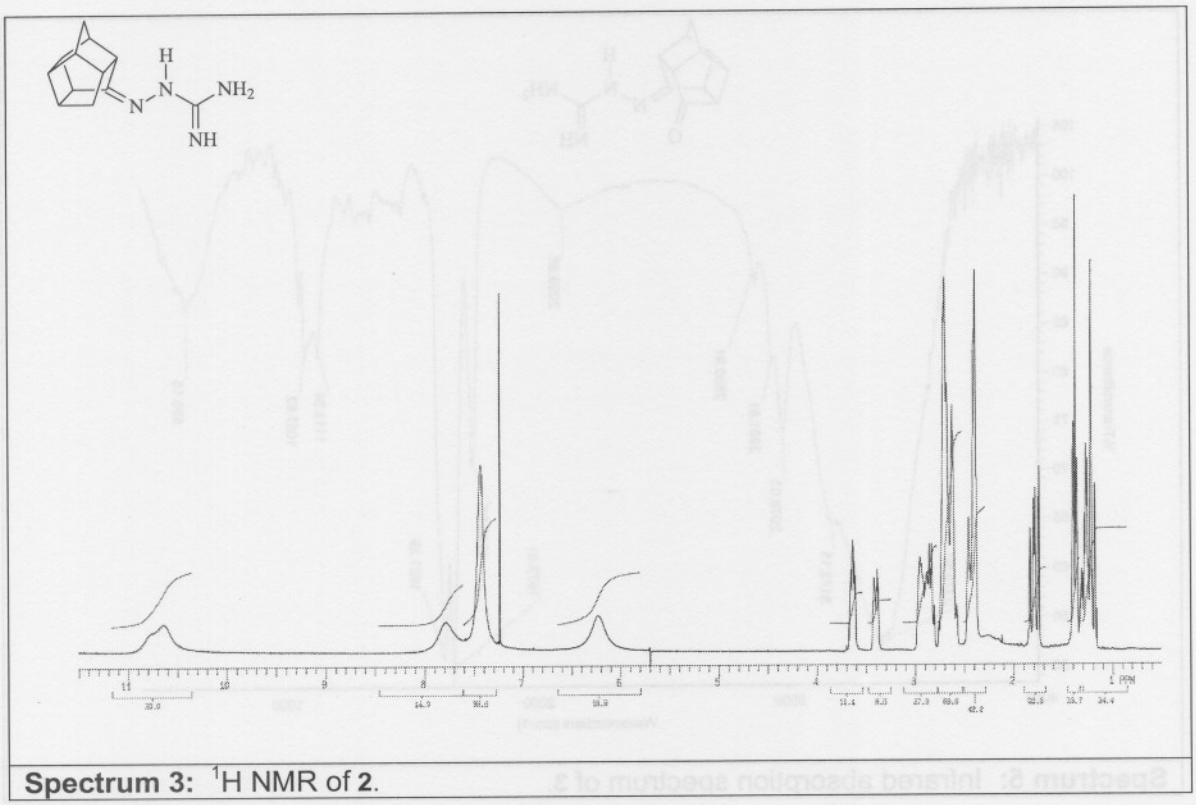
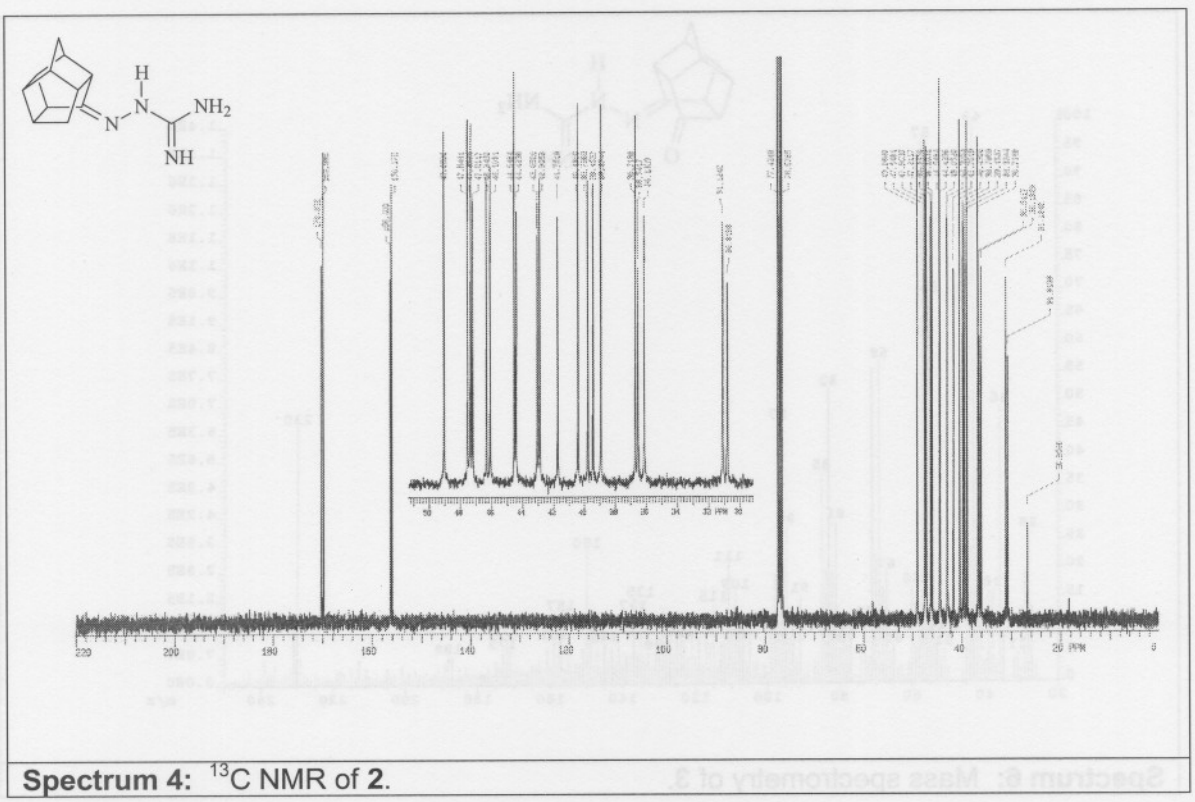


Spectrum 3: ^{1}H NMR of **2**.



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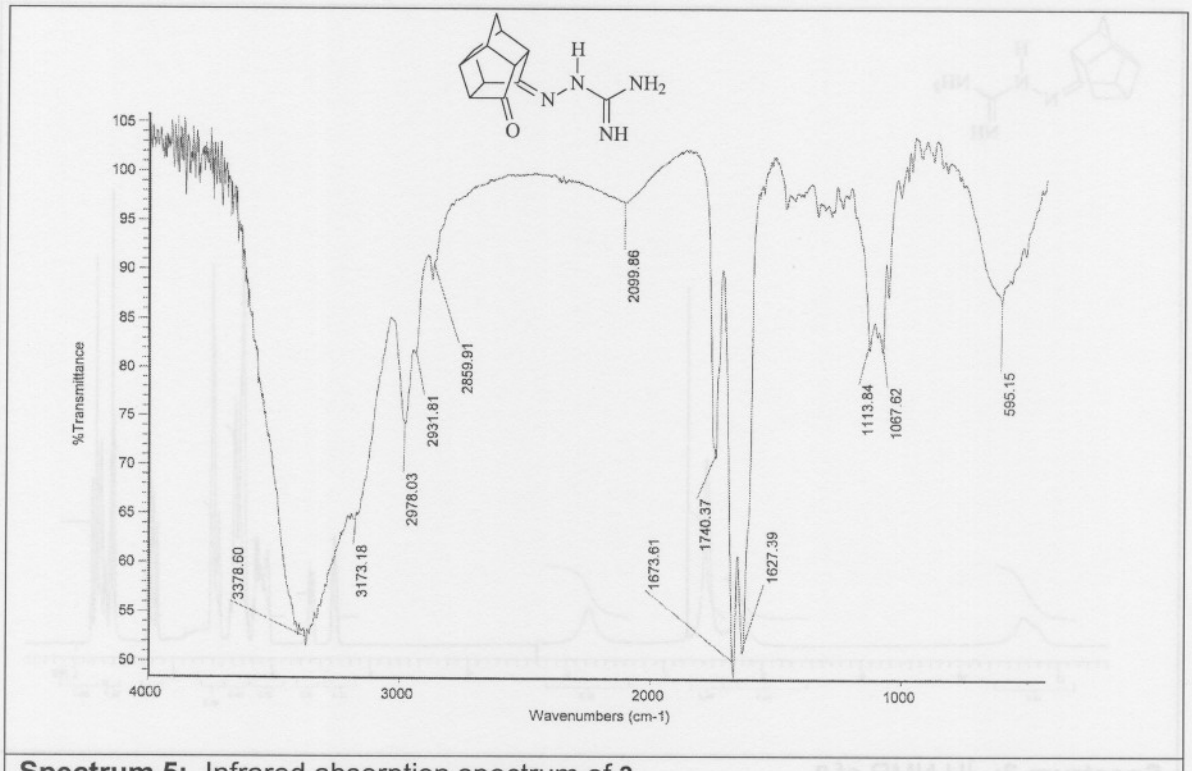
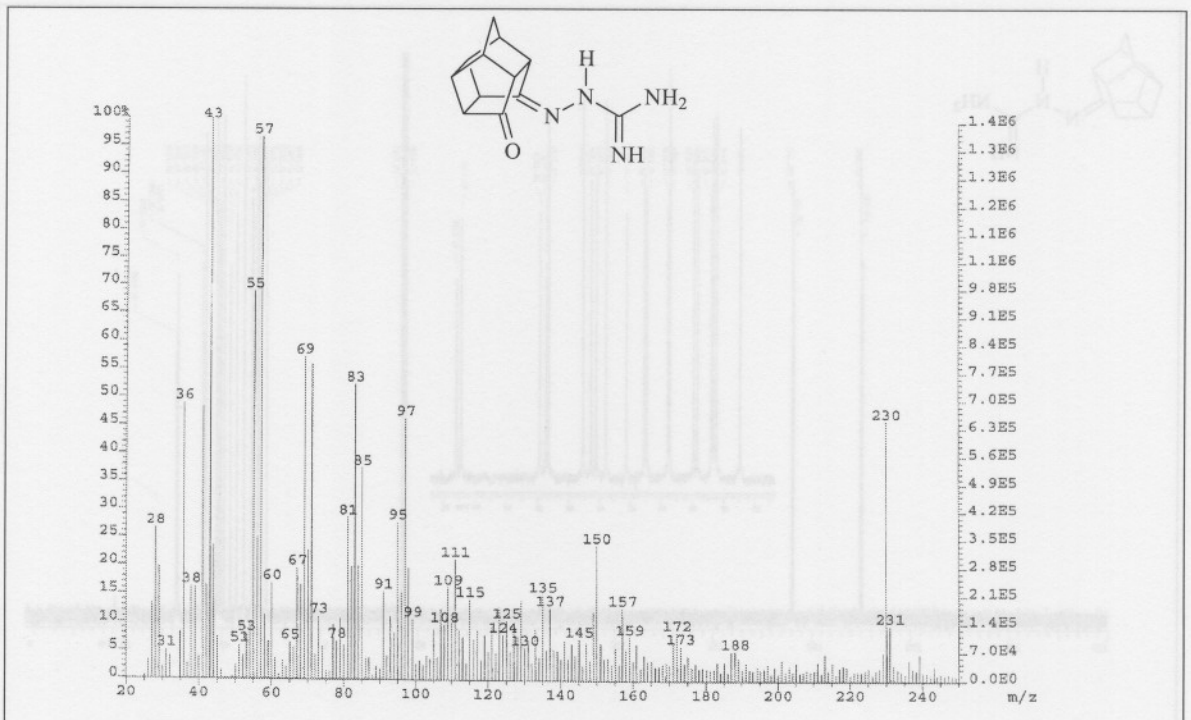
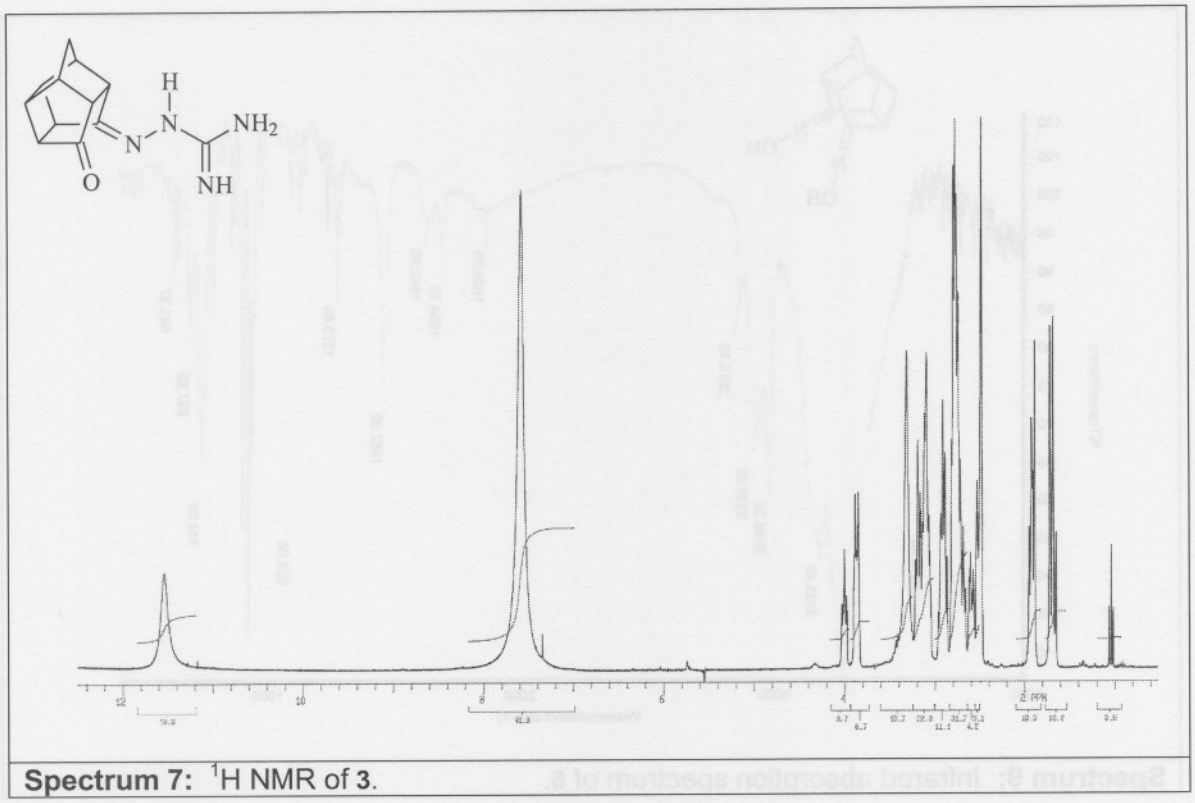


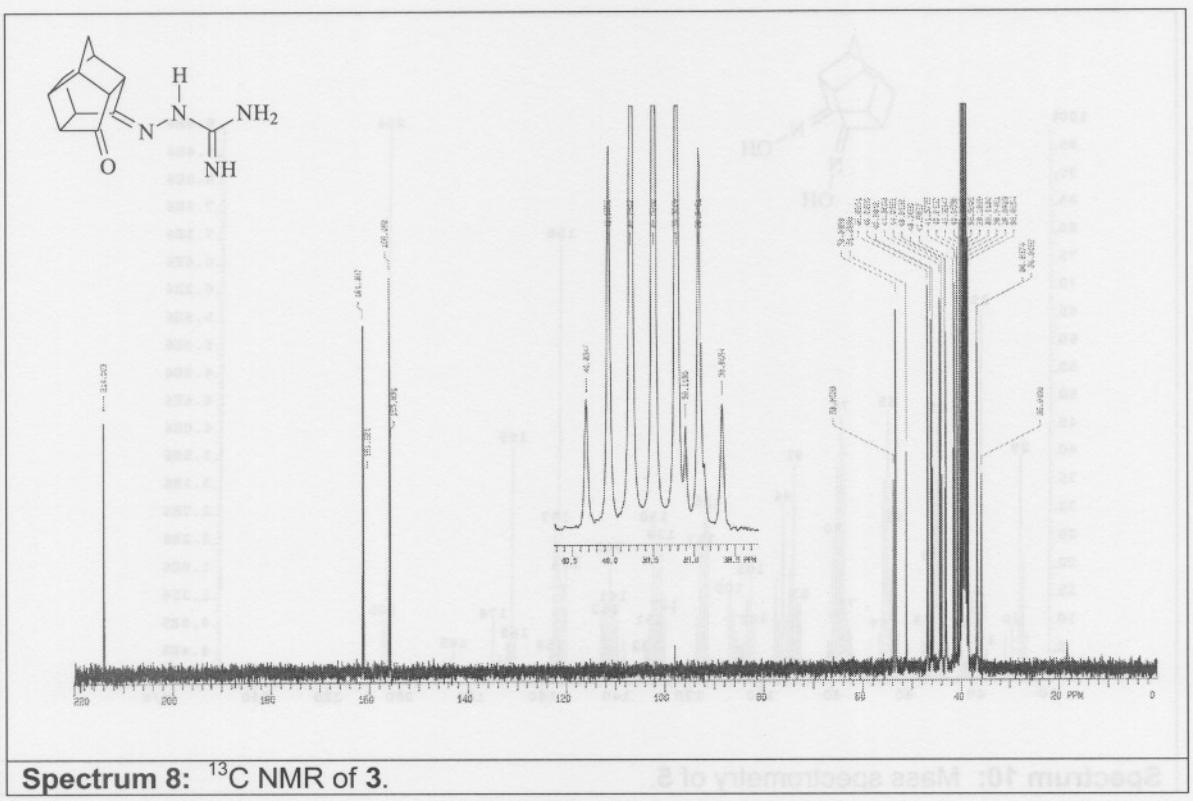
Spectrum 5: Infrared absorption spectrum of **3**.



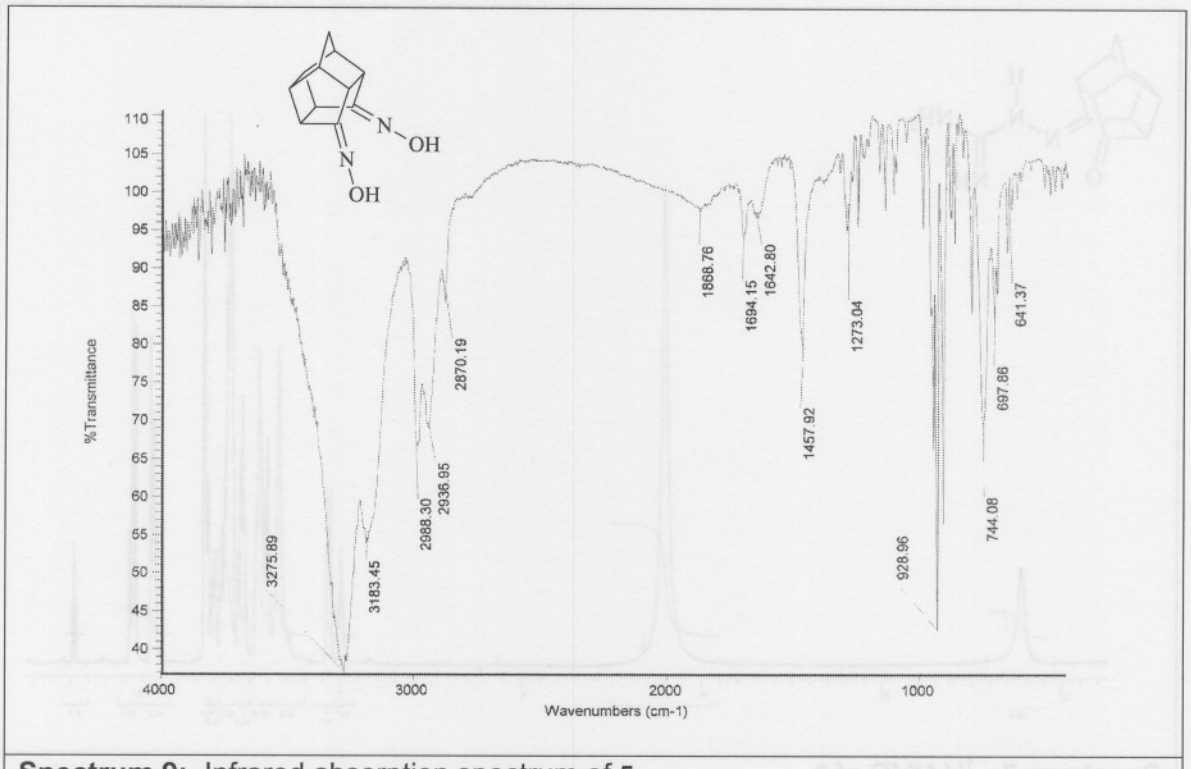
Spectrum 6: Mass spectrometry of **3**.



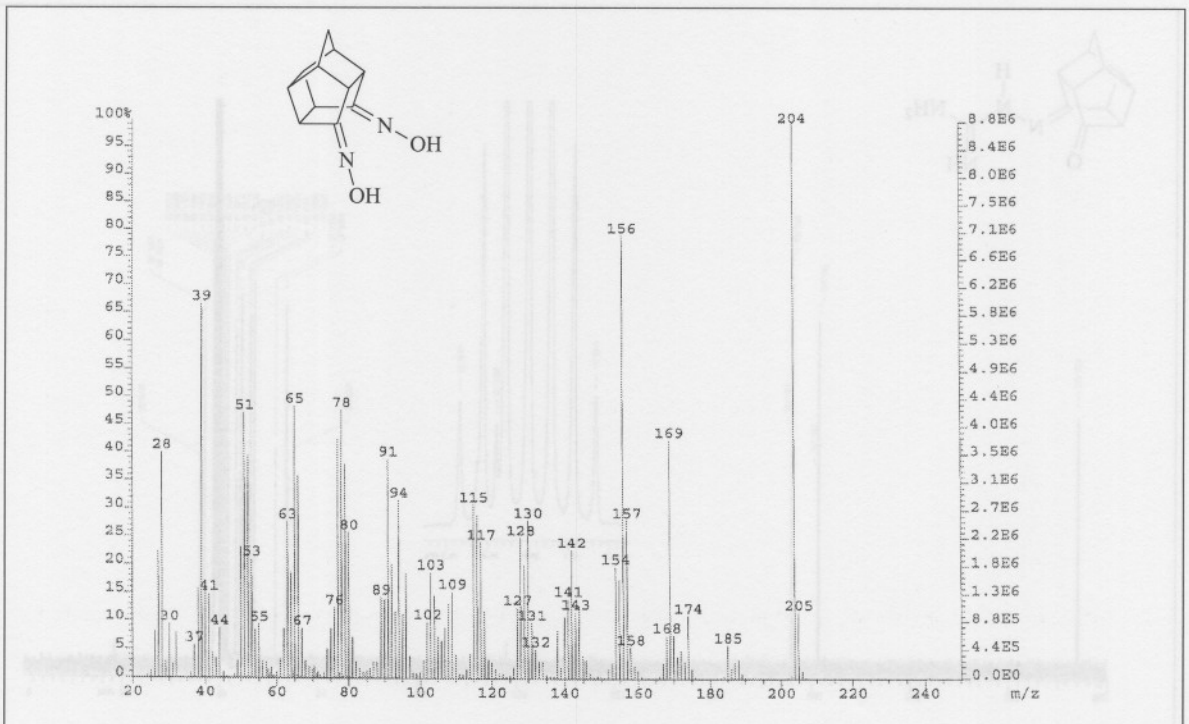
Spectrum 7: ^{1}H NMR of **3**.



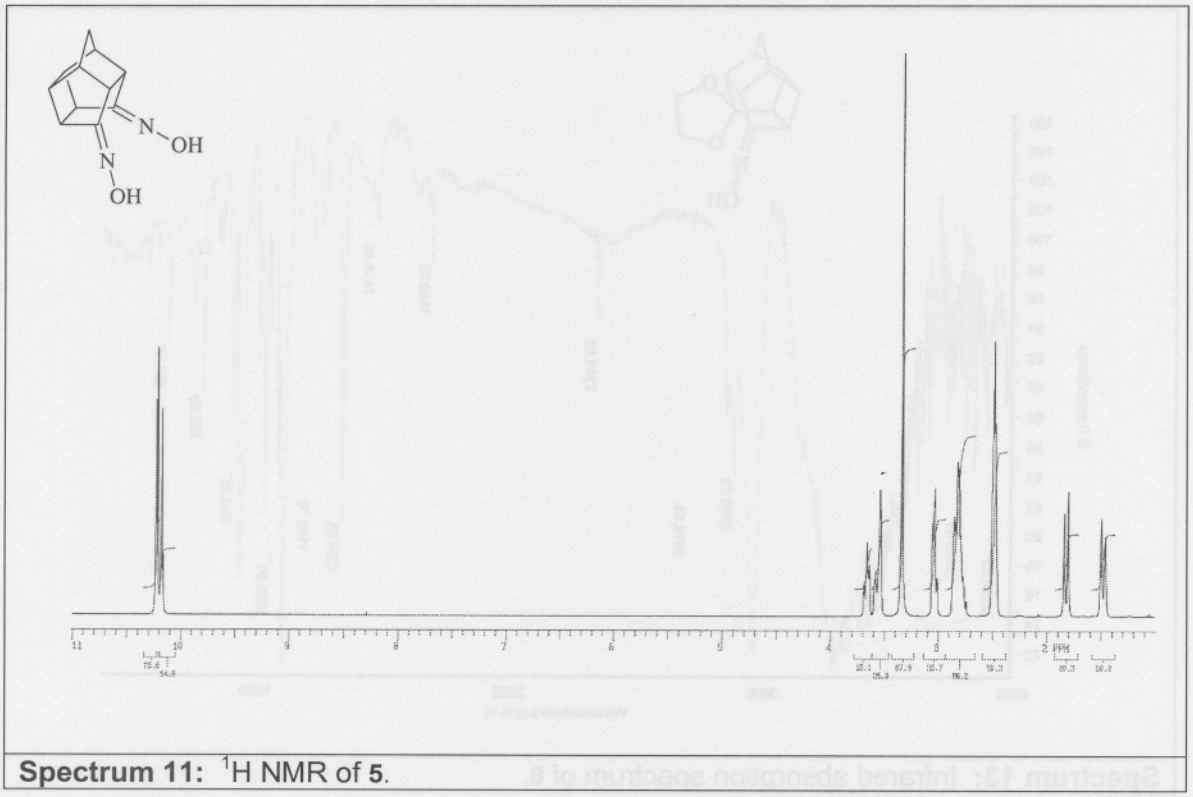
Spectrum 8: ^{13}C NMR of **3**.



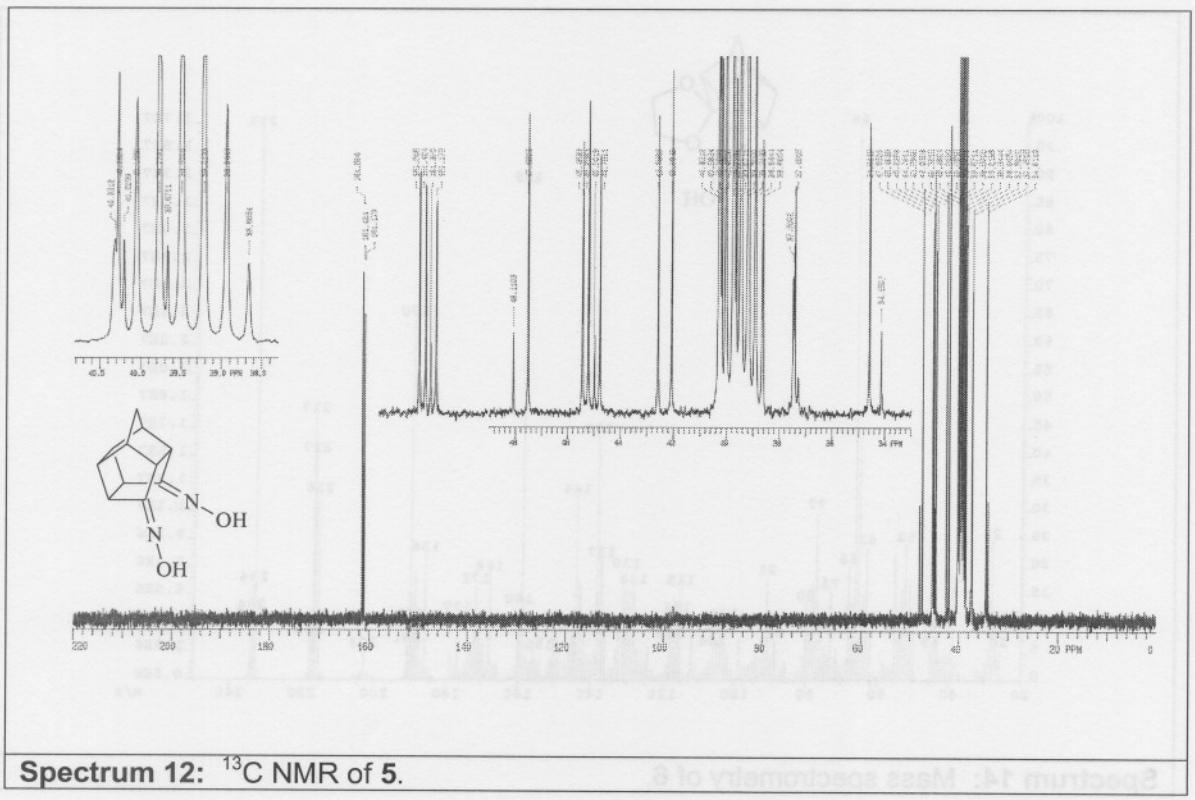
Spectrum 9: Infrared absorption spectrum of **5**.



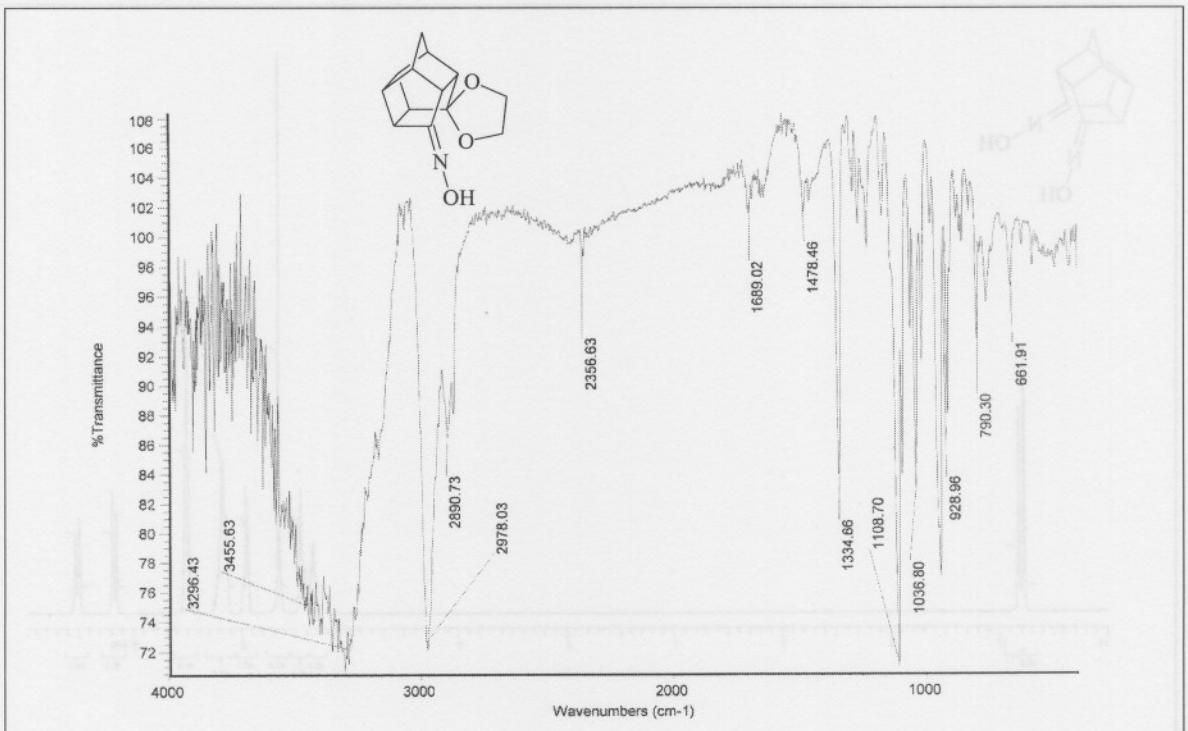
Spectrum 10: Mass spectrometry of **5**.



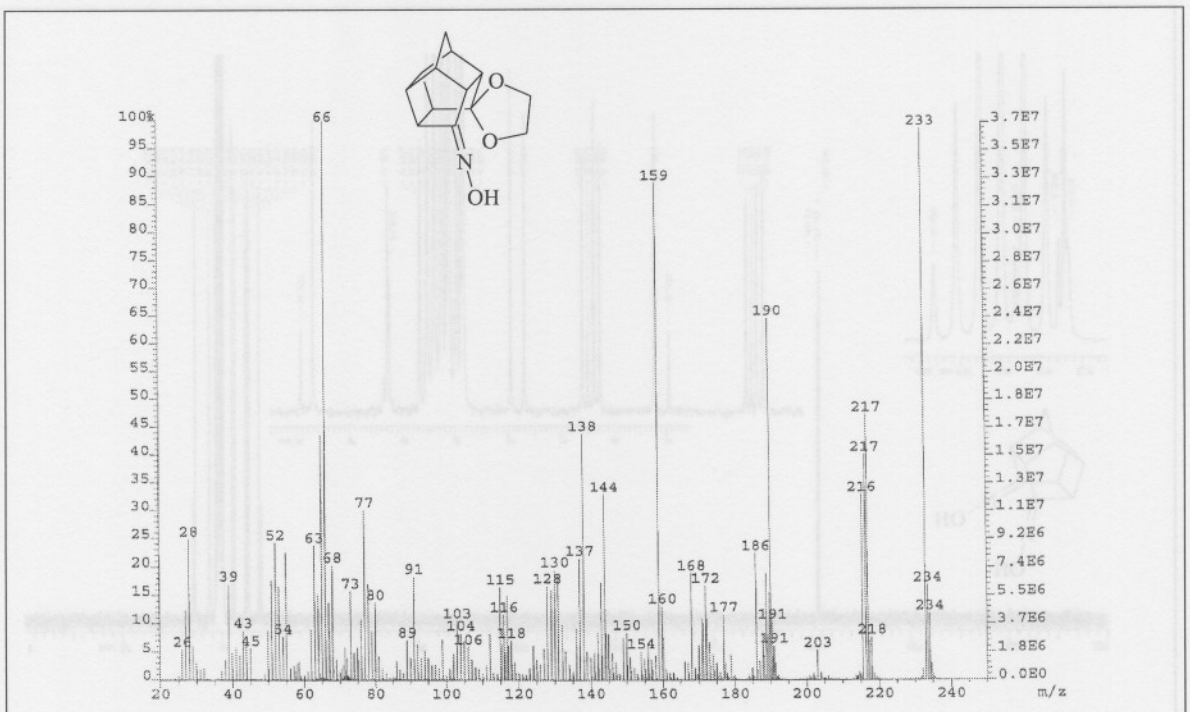
Spectrum 11: ^{1}H NMR of **5**.



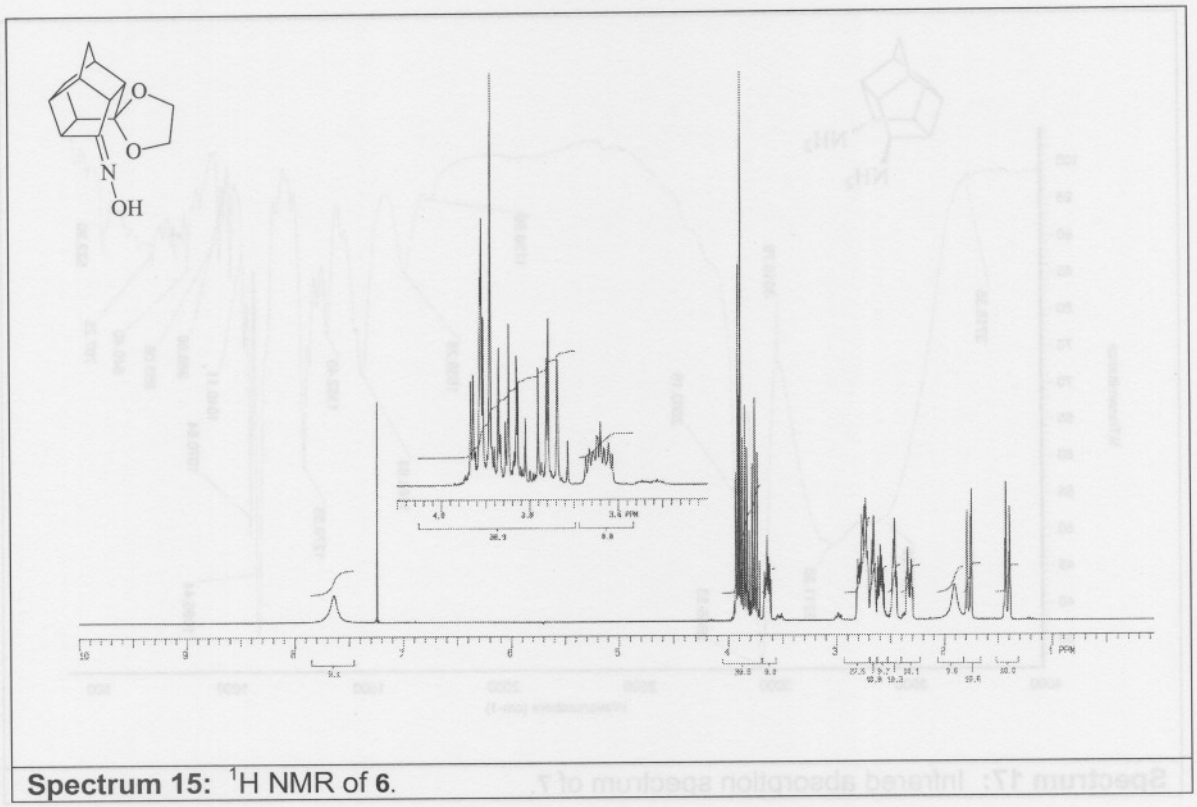
Spectrum 12: ^{13}C NMR of **5**.



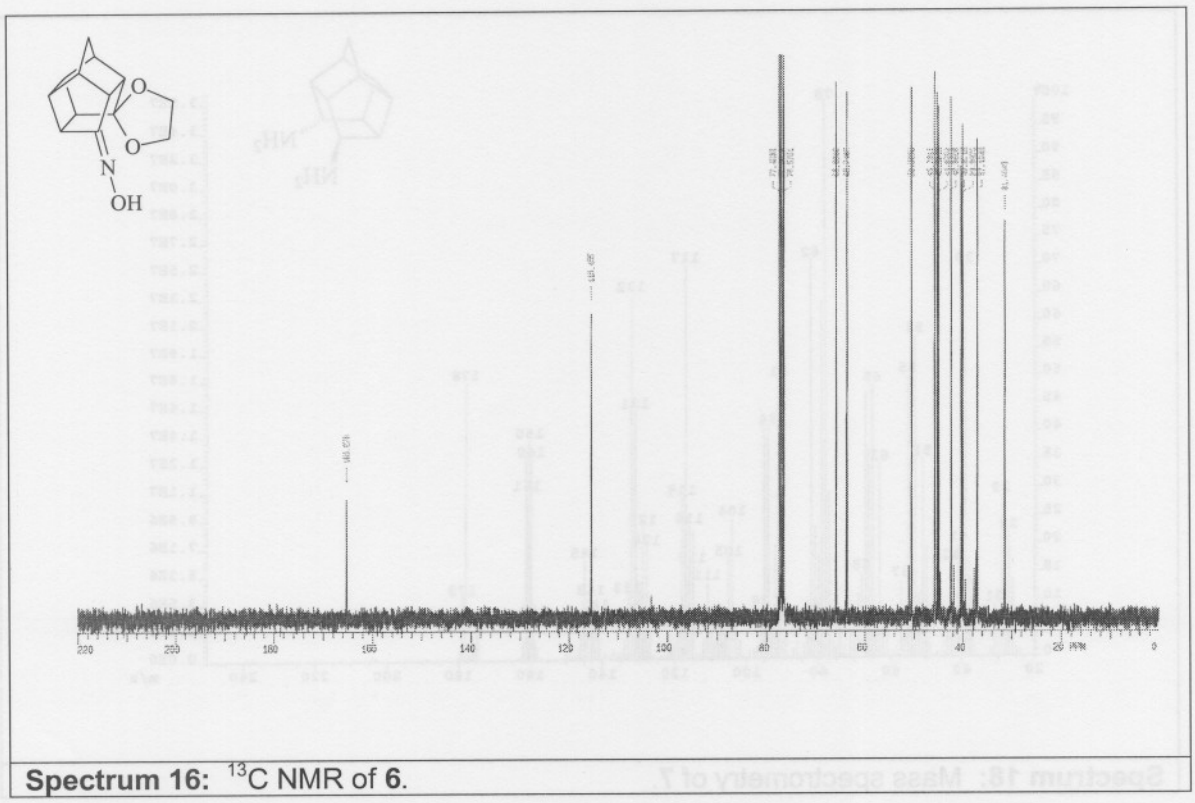
Spectrum 13: Infrared absorption spectrum of **6**.



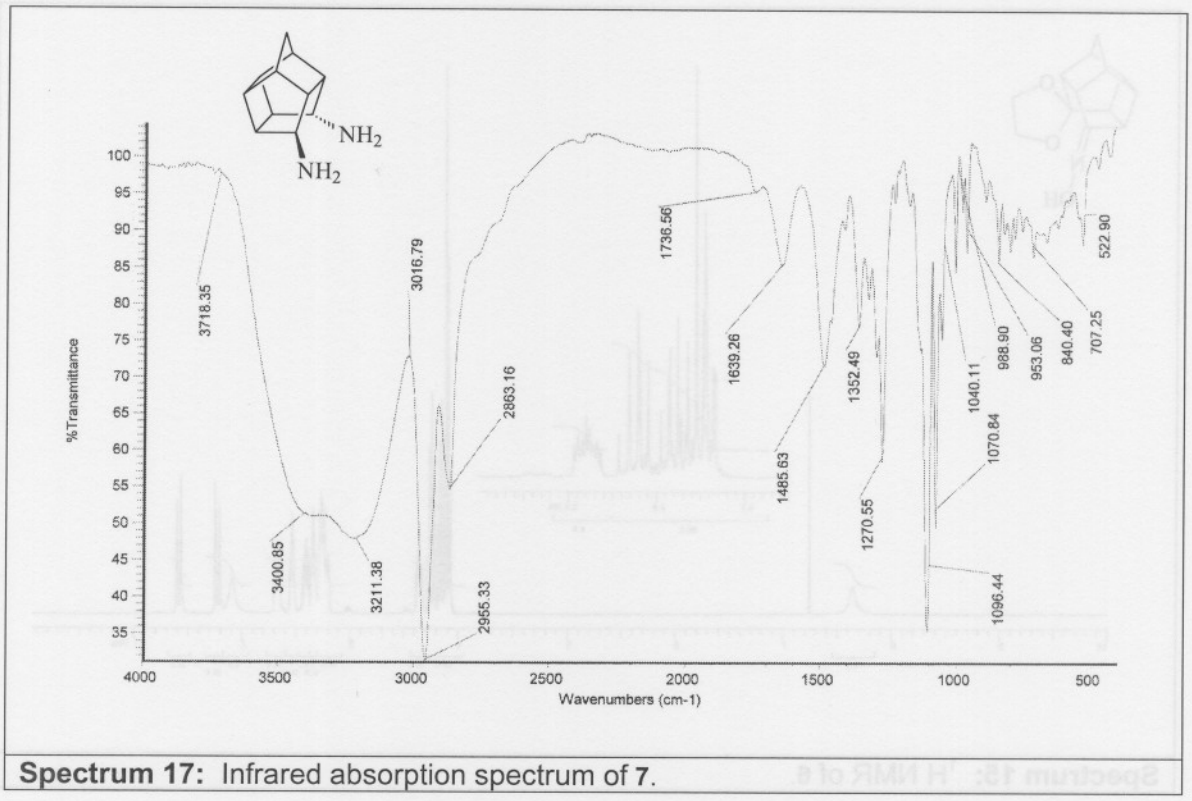
Spectrum 14: Mass spectrometry of **6**.



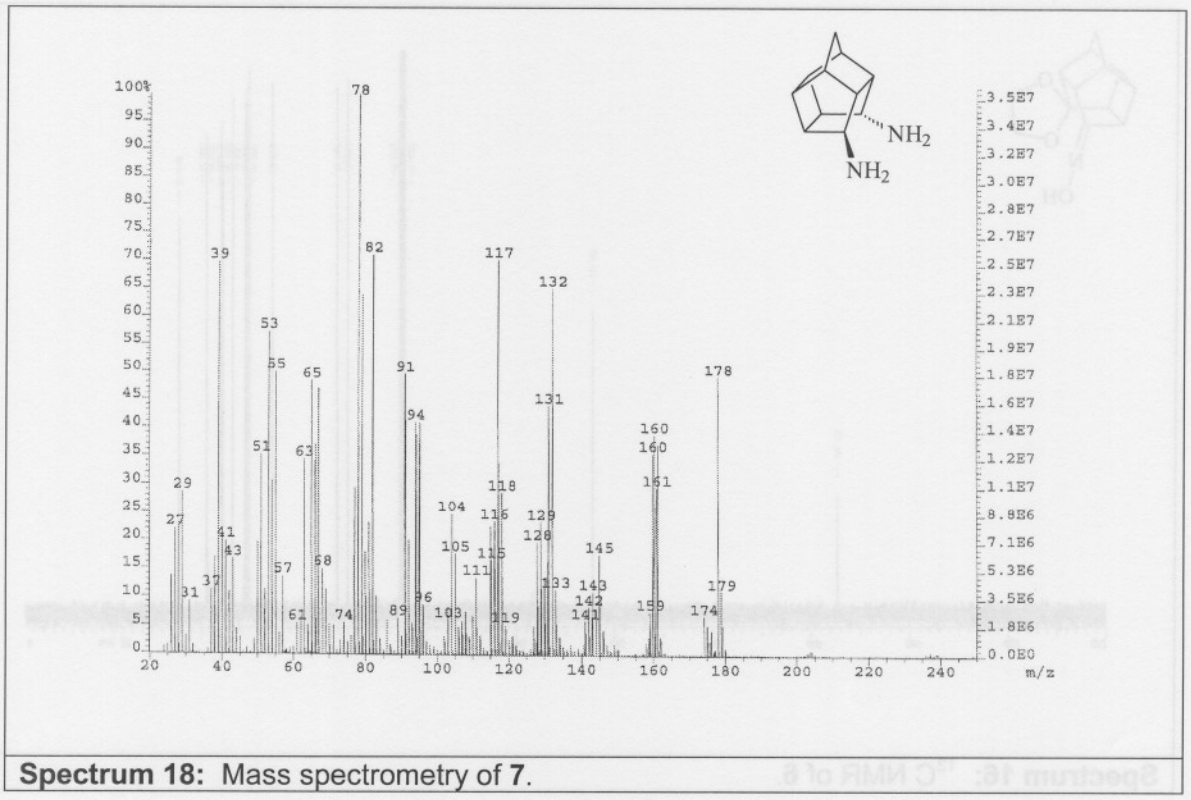
Spectrum 15: ^{1}H NMR of **6**.



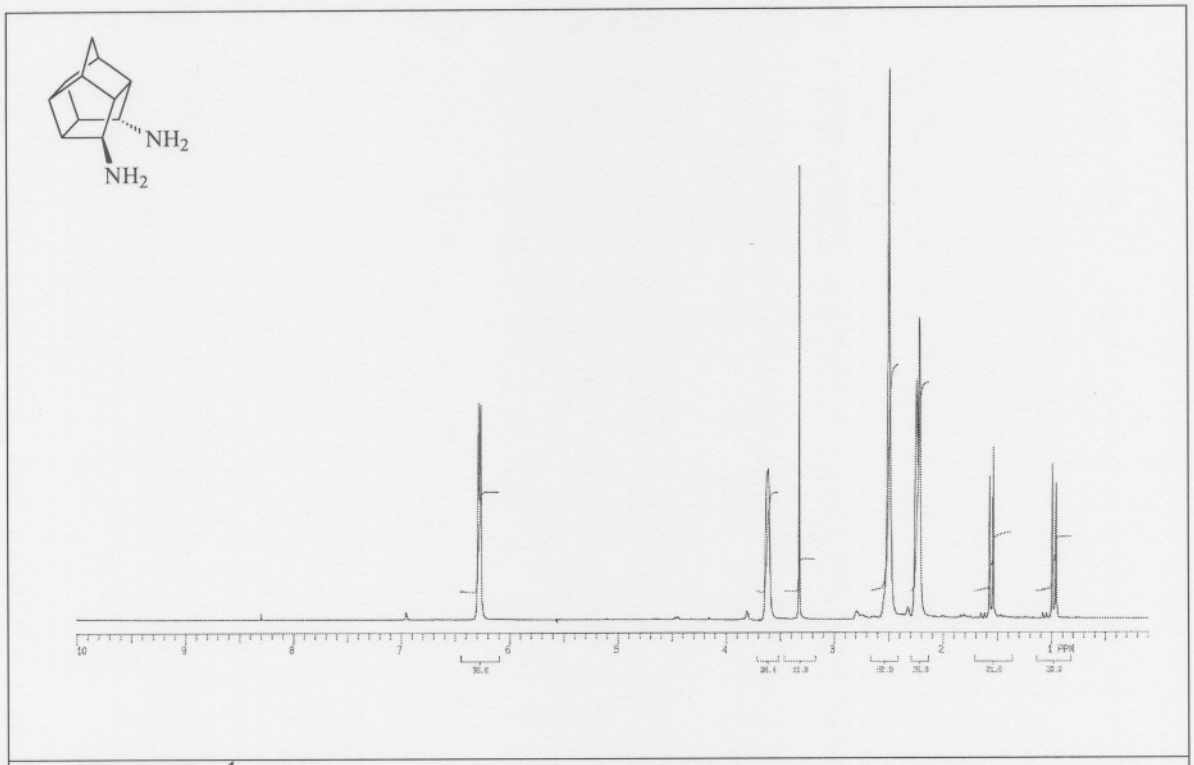
Spectrum 16: ^{13}C NMR of **6**.



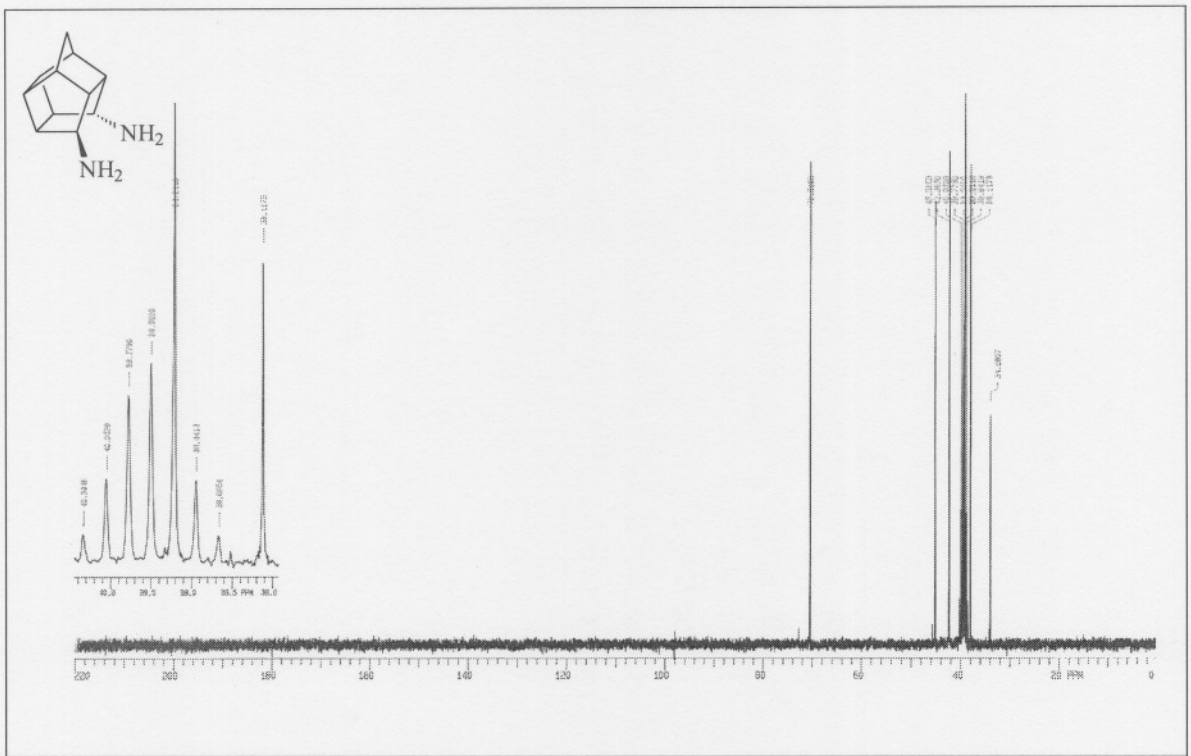
Spectrum 17: Infrared absorption spectrum of **7**.



Spectrum 18: Mass spectrometry of **7**.



Spectrum 19: ^{1}H NMR of **7**.



Spectrum 20: ^{13}C NMR of **7**.

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"Omnia ad majorem Dei gloriam" – All for the greater glorification of God