TRIQUINYLAMINES & AZA-CAGE COMPOUNDS AS NEURONAL CALCIUM FLUX MODULATORS

Olwen Domingo, B.Pharm

Dissertation submitted in partial fulfillment of the requirements for the degree
Magister Scientiæ in Pharmaceutical Chemistry at the
North-West University
Potchefstroom Campus

Supervisor:	Prof. S.F. Malan
Co-supervisor:	Prof. C.J. van der Schyf

2008
Potchefstroom

HABAKKUK 3:17 & 18

[17]Though the fig tree does not blossom and there is no fruit on the vines, [though] the product of the olive fails and the fields yield no food, though the flock is cut off from the fold and there are no cattle in the stalls,
[18]Yet I will rejoice in the Lord; I will exult in the [victorious] God of my salvation!

THE AMPLIFIED BIBLE

CONTENTS

ABSTRACT

The group of disorders that are generally characterised by changes in the normal function of neurons, also referred to as neurodegenerative conditions, are known to follow various underlying molecular pathways. With the pathogenesis of these molecular pathways gradually being elucidated, targets are being identified for the prevention of several diseases that involve the death of neurons. One of these molecular pathways involves the overload of neuronal cells with intracellular calcium ions. Since this may eventually lead to excitotoxicity, the block of calcium flux through the ion channels with the highest calcium ion permeability may thus serve as a major step towards the prevention of neurodegeneration.

In this study, the *N*-methyl-D-aspartate receptor (NMDAR) ion channel and voltage-gated calcium channels served as primary targets for an inhibition of the flux of calcium ions into neuronal cells. The triquinylamines share structural similarities with existing NMDAR antagonists. These similarities include the three linearly fused cyclopentane rings that serve as lipophilic moiety, as well as the nitrogen atom that forms a hydrogen bond with amino acids in the binding site. In addition to these characteristics that might define them as NMDAR antagonists, the triquinylamines also share structural similarities to the pentacycloundecane cage compounds. In view of previous findings on the NMDAR and calcium channel blocking activity of several pentacycloundecyl amines, it was thus envisaged that the triquinylamines would present similar results. The main aim of this investigation was thus to perform structure-activity analyses between the pentacycloundecylamines and triquinane derivatives with regard to their respective NMDAR and calcium channel blocking activities.

Flash vacuum pyrolysis of the cage dione and subsequent hydrogenation yielded the saturated form of the triquinane dione. Reductive amination of this dione, using sodium borohydride, yielded the aza-triquinylamines. Aza-cage compounds were also obtained by reductive amination, but by using sodium cyanoborohydride as reducing agent instead. An additional compound, the lactol derivative of the triquinanes, was synthesised by reducing the triquinane dione with sodiumborohydride. Purification of reaction mixtures was done by means of either open column chromatography or by using a Versaflash system. Identification of the compounds was achieved by means of Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS) and Infrared (IR) techniques.

Confocal laser scanning microscopy, together with Fluo-5N and the SHSY-5Y cell line to measure calcium flux, unfortunately did not deliver reproducible results. The ratiometric dye, Fura-2/AM, in combination with a microplate reader with undifferentiated PC-12 rat phaeochromocytoma cells proved to be more effective and adequate in measuring the extent of calcium flux into the cells after KCl-stimulated depolarisation.

An interesting observation made with all the aza-bridgehead compounds as well as with NGP1-01, was their tendency to increase intracellular calcium levels at low concentrations. This was accompanied by calcium flux suppression at higher concentrations. This dualistic feature of these compounds can be attributed to activity on both the sigma receptors and calcium channels, as the PC-12 cells distinctly express both these pharmacological entities. Another surprising observation was the increased calcium influx in the presence of increasing lactol triquinane derivative concentrations. These results suggest that the lactol / hemiacetal might have the ability to antagonise σ_1-receptors and / or stimulate σ_2-receptors, as both these effects could lead to calcium level increases.

In order to determine a more specific site of action for these compounds, a competition radioligand binding study was performed. The potent phencyclidine binding site blocker, [^{3}H]MK-801, served as reference compound in this study. The binding of [^{3}H]MK-801 remained fairly constant in the presence of increasing concentrations of the test compounds, indicating that they exert their calcium modulating effect by binding to a site other than the phencyclidine binding site of the NMDA receptor ion channels.

This study confirms the potential of the polycyclic structures as lead compounds in the search for molecules that prevent neuronal death due to apoptosis. Their dualistic effects, as well as the possibility of sigma receptor activity provide new avenues for investigation in the search for biological lead compounds amongst the polycyclic compounds. This places an emphasis on the necessity of further studies on these potential therapeutic agents as neuroprotectors. With the new observations in biological activity for the triquinanes and pentacycloundecylamines in the current study, the possibility of a diversion in structure-activity relationship studies can also be expected in future investigations on this group of molecules.

UITTREKSEL

Neurodegeneratiewe toestande volg normaalweg spesifiek gedefinieerde molekulêre weë. Soos wat die patogenese van hierdie molekulêre weë geleidelik deur navorsers blootgelê word, word meer en meer teikens geïdentifiseer vir die voorkoming van verskeie siektetoestande waarin die afsterf van neurone 'n belangrike rol speel. Een van die belangrikste faktore hier is die oorbelading van die neuronale selle met intrasellulêre kalsiumione. Aangesien hierdie kalsiumoorlading uiteindelik tot neuronale dood kan lei, kan die blokkering van die kalsiumfluks deur die reseptor-ioonkanale met die hoogste kalsiumioondeurlaatbaarheid 'n belangrike stap wees om uiteindelike neurodegenerasie te voorkom.

In hierdie studie is beide die *N*-metiel-D-aspartaatreseptor (NMDAR)- en kalsiumioonkanale as primêre teikens vir die antagonisme van kalsiumfluks tot in neurone gekies. Die trikwinielamienverbindings toon strukturele ooreenkomste met reeds bestaande NMDAR-antagoniste. Die trikwinielamienmolekuul besit onder andere drie siklopentaanringe as lipofiele entiteit, asook 'n stikstofatoom. Hierdie stikstofatoom is noodsaaklik vir die vorming van waterstofbindings tussen die ligand en die bindingsetel. Die trikwinaanderivate is egter ook struktureel aan die pentasikliese hokkieverbindings verwant. Hierdie feit versterk verder die hipotese dat hulle ook moontlik kalsiumkanaalblokkerende aktiwiteit kan toon, aangesien die pentasikloundekielamienverbindings welbekend is vir hul inhibisie van kalsiumfluks, deur sowel die L-tipe kalsiumkanale en NMDAR-ioonkanale te blokkeer. Daar bestaan dus geldige gronde om te vermoed dat die trikwinielamienderivate sowel NMDAR- as kalsiumkanaalblokkerende aktiwiteit besit. Die hoofdoel van hierdie studie was dus om 'n reeks polisikliese verbindings te sintetiseer en struktuuraktiwiteitsverwantskappe van hul NMDAR- en kalsiumkanaalblokkerende aktiwiteit vas te stel.

Die pirolise van die hokkieverbindings, gevolg deur die hidrogenering van die piroliseproduk met Pd/C as katalisator, lewer die versadigde vorm van die trikwinaanstruktuur. Hierdie trikwinaandione het verdere reduktiewe aminering ondergaan, om sodoende die verlangde asatrikwinielamienderivate te vorm. Die ooreenstemmende pentasikloundekielamiene is deur middel van soortgelyke reduktiewe aminering gesintetiseer. In die geval van die hokkieverbindings is daar egter tydens die reduksiestap van natriumsianoboorhidried in plaas van natriumboorhidried gebruik gemaak. Daarby het die natriumboorhidriedreduksie van die trikwinaandioon tot die vorming van die laktolderivaat van die trikwinane gelei. Alle gesintetiseerde verbindings is met oop kolomchromatografie of 'n Versaflash-sisteem gesuiwer. Kernmagnetieseresonans (KMR)-, massa- (MS) en infrarooi-absorpsiespektrometrie (IR) is gebruik vir die struktuurbevestiging van die gesintetiseerde verbindings.

Vir die biologiese evaluering van die gesintetiseerde verbindings is daar onder andere van fluoressensietegnieke gebruikgemaak. Die SH-SY5Y-sellyn en die ratiometriese kleurstof, Fluo-5N, is gekies vir die evaluering van die invloed van die depolariserende middel, KCl, op kalsiumfluks in die neuronale selle in. Hierdie metode het egter geen herhaalbare resultate gelewer nie. 'n Ander ratiometriese kleurstof, Fura-2, in kombinasie met 'n mikroplaatleser en ongedifferensïeerde feochromositoomselle is gebruik om 'n aanduiding te gee van die intrasellulêre kalsiumkonsentrasies ná KCl-geïnduseerde membraandepolarisasie.

'n Interessante waarneming met al die asa-verbindings was hul vermoë om by lae konsentrasies die kalsiumvlakke te laat toeneem, terwyl 'n afname in kalsiumfluks by hoër konsentrasies plaasgevind het. Hierdie dualsitiese werkingsmeganisme van die toetsverbindings kan moontlik toegeskryf word aan aktiwiteit op sowel die sigmareseptor en kalsiumkanale. Hierdie vermoede is in ooreenstemming daarmee dat beide hierdie farmakologiese entiteite in die PC-12 selle uitgedruk word. Die ander verrassende waarneming tydens hierdie toetse was die toename in kalsiumfluks wat met toenemende konsentrasies van die laktolverbinding voorgekom het. Hierdie resultate suggereer dat die laktolverbinding moontlik die vermoë besit om die σ_1-reseptore te blokkeer en / of die σ_2-reseptore te stimuleer, aangesien beide hierdie effekte 'n toename in kalsiumfluks tot gevolg kan hê.

Om 'n moontlike setel van interaksie aan hierdie verbindings toe te ken, is 'n verplasingsradioligandbindingstudie met die radioaktiefgemerkte [^{3}H]MK-801 gebruik. Die persentasie binding van die [^{3}H]MK-801 het egter relatief konstant gebly ten spyte van toenames in die toetsverbindingkonsentrasies. Hierdie waarneming dui daarop dat die toetsverbindings waarskynlik hul kalsiummodulerende effekte uitoefen deur op 'n ander setel as die fensiklidienbindingsetel van die NMDAR's te bind.

Hierdie studie bevestig weereens die potensiaal van die polisikliese strukture as leidraadverbindings in die soeke na molekules wat neuronale dood as gevolg van apoptose kan voorkom. Daar is egter steeds 'n gebrek aan kennis oor die presiese meganisme waarvolgens hierdie polisikliese verbindings hul effek op kalsiumfluks uitoefen. Dit is dus belangrik om verdere studies op hierdie verbindings te doen om sodoende meer inlgting oor hul potensiaal as terapeutiese neurobeskermende middels te verkry. Die dualistiese effekte van hierdie verbindings wat tydens hierdie studie waargeneem is, verskaf egter ook die moontlikheid dat toekomstige navorsing rakende hul biologiese akiwiteit 'n ander vorm sal kan aanneem.

CHAPTER 1

INTRODUCTION

1.1 Background

The human central nervous system is an important focus area for many scientists and academics. This statement remains true, despite the numerous contributions to the field of neuroscientific research from as early as 4000 B.C. up until the more recent discovery of the large olfactory receptor gene family by Linda Buck and Richard Axel (Buck and Axel, 1991). The pathology of the brain, in particular, has over the last few decades generated an upsurge in scientific research and due to the irreversibility and debilitating nature of most neurodegenerative disorders the development of a new generation of drug treatments for these conditions has become a necessity.

To date, various types of neurodegenerative disorders have been identified and investigated. One of the more commonly studied disorders in this category is the neuronal disorder that affects the basal ganglia in the brain. This condition, also known as Parkinson's disease, is a progressive movement disorder and occurs when dopaminergic nerve cells in the substantia nigra either become impaired, or die. Due to the resulting decrease in dopamine levels in the brain, current pharmacological treatments for this condition mainly involve an attempt to replenish the depleted neurotransmitter. Sadly, these treatments are mostly associated with complexity and numerous side effects. This, together with the enormous financial, physical and emotional strain on an individual affected by conditions like Parkinsonism, increases the urgency for new drug development in this field.

A promising observation was made with the serendipitous discovery of the antiparkinsonian properties of the substituted cage derivative, amantadine (Schwab *et al.*, 1972). This breakthrough led to renewed hope within the neuroscientific research community, bringing to attention the possibility of preventing the neuronal death that usually takes place during the early stages of Parkinson's disease. Due to the structural properties of amantadine, scientists had reason to suspect that other polycyclic cage compounds might also possess the ability to attenuate parkinsonian symptoms.

Together with some of its derivatives, the polycyclic cage compound known as NGP1-01, 8-Benzylamino-8,11-oxapentacyclo[5.4.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (Van der Schyf *et al.*, 1986), showed promising effects in calcium homeostasis studies. These results may be extrapolated to possible neuroprotective activity for these compounds and, in light of these findings, several other pentacyclic amines have since been tested for their calcium channel and neuroprotective activities (Geldenhuys *et al.*, 2004).

Following the studies on NGP-101, thermal ring-opened derivatives of this compound were synthesised by Liebenberg (1989). The intent of the so-called *cis, syn, cis* triquinylamine system that was obtained, was to further investigate a hypothesis that originated regarding the pentacycloundecane compounds. According to this hypothesis, the pentacycloundecane skeleton serves only as bulk contributor for the biological activity of NGP1-01 and its derivatives. It was determined that the triquinylamines exhibited Ca^{2+} channel activity similar to that of the lead structure NGP1-01 and their basic structure thus forms a central part of the ongoing investigation into the biological activity of the polycyclic cage amines.

1.2 Rationale

In addition to the structural similarities with the polycyclic cage compounds, the triquinanes share structural similarities with various *N*-methyl-D-aspartate (NMDA) receptor antagonists. The triquinanes consist of three linearly fused cyclopentane rings and, with the incorporation of a nitrogen atom into the structure, they could meet the basic requirements for activity in the central nervous system. These two requirements include the availability of an atom for hydrogen bonding with the receptor structure, as well as two lipophilic moieties (Leeson *et al.*, 1990). The former requirement is based on the findings of a number of geometric models that were proposed for compounds that might be active on the phencyclidine (PCP) binding site of the NMDA receptor (Andrews and Lloyd, 1986). Various polycyclic cage compounds have previously been tested as ligands on the sigma receptors (Kassiou *et al.*, 1996). Since this receptor subtype is known to play an important role in movement and posture (Walker *et al.*, 1990), the discovery of new sigma receptor ligands may also contribute to future neuroprotective strategies and the triquinylamines may have such activity in store.

The significance of the possible activity of the triquinylamines on Ca^{2+} channels and the NMDA and sigma receptors is evident from the role that these receptors play in apoptosis. The NMDA receptors, for one, are well known for their high permeability for calcium ions. The excessive and prolonged stimulation of these receptors may lead to an excessive influx of Ca^{2+} which, in turn, leads to several detrimental intracellular signals (Kaul and Lipton, 2002). Blocking the NMDA receptors can thus lead to a decrease in calcium flux into the cell and eventually prevent neuronal apoptosis. Several non-competitive NMDA receptor antagonists have been identified to date (Kornhuber and Weller, 1995). Unfortunately, despite and also due to their high affinity for the PCP binding site, these antagonists show a high side effect profile (Rückert and Schmidt, 1993).

The sigma receptors have also been indicated in calcium homeostasis. Stimulation of the sigma receptors (particularly sigma-1) lead to an inhibition of high-voltage-activated calcium channels (Zhang and Cuevas, 2002). Agonistic activity on these receptors may thus prevent neuronal apoptosis in the same way that NMDA receptor blockers do and a dualistic effect of the polycyclic compounds on these two receptor subtypes may hold promising results for the future of neuroprotection.

1.3 Aims & Objectives of this Investigation

With regard to the obvious need for novel preventative treatment for neurodegenerative disorders, the basic aim of this study was thus to synthesise a series of polycyclic compounds and evaluate their influence on Ca^{2+} homeostasis. Through the introduction of diverse substituents onto the basic triquinane and polycyclic cage structures, it was intended that structure-activity relationships for this activity be established. The triquinanes are closely related to the polycyclic cage compounds and the effect that ring opening of the cage moiety has on the activity thereof, has also been explored.

In order to determine to what extent the polycyclic compounds would modulate calcium flux, fluorescence techniques with a confocal microscope and microplate reader were used respectively. Radioligand binding studies with $[^3H]$MK-801 was used to determine the affinity of the triquinylamines and related compounds for the MK-801 or phencyclidine site.

CHAPTER 2
LITERATURE REVIEW

NEURODEGENERATION

2.1 Introduction

Neurodegenerative disorders, often referred to as tauopathies, describe a group of disorders characterised by changes in the normal function of neurons. In most cases, this leads to neuronal death, which is known to follow particular underlying molecular pathways. A few processes are known to represent unifying events in many of these slowly progressing disorders. At molecular level, the accumulation of aberrant or misfolded proteins, protofibril formation, ubiquitin-proteasome system dysfunction, synaptic failure and other cellular dysfunctions might contribute to the impairment of normal neuronal function and further lead to abnormal apoptosis - *i.e.* the process of programmed cell death. This cell death is known to take place rapidly and also usually in response to a toxic stimulus.

According to Kerr *et al.* (1972), apoptosis plays an important role in both pathological and physiological conditions and it is because of the disregulation of apoptosis that several diseases might occur (Macmanus *et al.*, 1993). Recent studies have furthermore proven that this programmed neuronal death could be the final cause of demise in the neurodegenerative processes that underlie conditions such as Alzheimer's disease, Huntington's chorea and Parkinson's disease (PD) (Linnik *et al.*, 1993).

Alzheimer's disease is an incurable condition, characterised by neurofibrillary tangles and senile plaques as some of the major microscopical features. It is the predominant cause of dementia and after cancer, stroke and heart disease Alzheimer's disease is amongst the leading causes of death in developed countries. A peptide known as amyloid beta (Aβ) seems to be the prevalent feature in this condition and its excessive production is what eventually gives rise to Alzheimer's disease (Selkoe, 1999). Although it is unclear exactly how Aβ causes the damage, a number of mechanisms have been proposed to date. One of these suggests that the peptides cluster into so-called amyloid plaques on the blood vessels and on the outside surface of neurons. It is these plaques that eventually lead to the death of neurons, resulting in the Alzheimer's disease symptoms. Some researchers have found evidence that amyloid-beta protofibrils are also responsible for the formation of pores in neurons, by triggering the excessive release of certain excitatory amino acids from glial cells (Lashuel *et al.,* 2002). Eventually, the overactivation of glutamate receptors results in an increase in the calcium flux and, finally, neuronal death associated with Alzheimer's disease occurs.

Huntington's chorea is caused by a faulty gene on chromosome four, which is responsible for the production of the protein Huntingtin. The defect in the gene can lead to damage of the nerve cells in certain areas of the brain. The generated damage eventually causes the gradual physical, mental and emotional changes that are associated with the condition.

The third neurodegenerative condition and also the focus of this study, Parkinson's disease (PD), is a common neurological disorder that is clinically characterised by slowly progressive akinesia, rigidity, tremor and postural abnormality. The area in the brain most affected by the condition is the substantia nigra, where the degeneration of neurons in the pars compacta is a common phenomenon during the development of the disease. Among the factors that have been implicated in neuronal degeneration in PD are mitochondrial dysfunction, oxidative stress, deficient neurotrophic support, immune mechanisms and the activities of excitotoxins (Limousine *et al.*, 1997). It has been established that a breakdown of the regulation of intracellular ionised calcium concentrations ($[Ca^{2+}]_i$) plays a major role in the development of these abnormal cellular processes (Siesjo and Bengtsson, 1989).

2.2 The Role of Calcium

The influx of calcium into the cell plays a major role in apoptosis. Intracellular calcium ion overload in neurons may lead to excitotoxicity and these divalent ions can therefore be seen as key mediators of excitotoxic damage. Apoptosis involves systemic signals that may instruct cells to undergo a complex process of self-digestion and packaging. Under normal physiological conditions, it usually occurs under circumstances in which a particular cell function is no longer needed. Although there is considerable evidence for a role of Ca^{2+} in these processes (Schwartzman and Cidlowski, 1993), it is, however important to first consider the general role of calcium in the human body, before examining its role in neuronal death.

2.2.1 Cellular calcium ion regulation

Calcium plays a major role in many regulated and also other more prolonged cellular events in the body. These ions are absolutely fundamental in the regulation of a number of cellular processes, such as chemical transmission, enzyme function and exocytosis. These processes normally do not occur unless excess calcium is present in the intracellular fluid and therefore strict regulation of the free intracellular calcium level is required (Holz and Fisher, 1999).

Normally, a large electrochemical gradient for Ca^{2+} exists across the plasma membrane. The cytoplasmic concentration of Ca^{2+} is less than one ten thousandth of that in the extracellular milieu and the interior of the cell is thus the more negative. In the resting state of neurons, free calcium levels are maintained at low concentrations (100 nM) (Putney, 1999).

There are a number of mechanisms in place in cells for the sequestering or buffering of Ca^{2+}. The purpose of these mechanisms is to prevent inappropriate raises in $[Ca^{2+}]_i$ in the long term and to ensure a tight control of cytosolic Ca^{2+} levels. In particular, two distinct mechanisms for controlling $[Ca^{2+}]_i$ at the plasma membrane are the Ca^{2+}-ATPase pump and Na^+/Ca^{2+} exchanger. It is the activity of these plasma membrane transport processes that mainly determine the steady-state level of the $[Ca^{2+}]_i$ (Catterall, 1996).

The combination of restricted calcium entry, efficient efflux and restricted intracellular mobility ensures the regulation of the intracellular calcium levels and thus permits calcium homeostasis (Pringle, 2004). Three fundamental mechanisms that regulate calcium ion entry across the plasma membrane include the activities of ligand-gated channels, the process of capacitative calcium ion entry and voltage-dependent Ca^{2+} channels (VDCC's). In neurons, the latter are predominantly responsible for the entry of Ca^{2+} into the cells. As the name indicates, these channels are subject to the complex combinations of voltage-dependent activation. The inactivation mechanisms can be either calcium-mediated or voltage-dependent (Putney, 1999).

There are at least five types of VDCC's in the central nervous system and they all regulate calcium entry into both pre and postsynaptic neurons. They differ with regard to their gating kinetics, modes of inactivation and regulation by Ca^{2+}, as well as their sensitivity to specific marine toxins (Dunlap *et al.*, 1995). It is important to distinguish between the types of channels, since different subtypes are believed to subserve different cellular functions (Putney, 1999). Calcium entry into the presynaptic compartment is essential for neurotransmitter release (Turner *et al.*, 1993). It is the VDCC's that provide activator Ca^{2+} for this discharge of neurotransmitters (Putney, 1999). In the postsynaptic neuron, L-type channels open as the membrane depolarises, which permits the initiation of calcium-dependent signalling.

The VDCC's are also responsible for the primary regulation of nitric oxide synthase (NOS) within the central nervous system. This enzyme is actively involved in the production of nitric oxide (NO) from L-arginine. NO mediates diverse physiological functions associated with neurons and may act as a neuromodulator to control behavioural activity, influence memory formation and intensify responses to painful stimuli. It is clear that the biosynthesis of NO in excitable tissues is regulated by increases in intracellular calcium, which activates NOS through the enzyme's dependence upon calmodulin (Bredt and Snyder, 1990).

2.2.2 Calcium in neuronal excitotoxicity

Although the exact mechanism by which calcium ions mediate excitotoxicity is not clear, it is believed that the mechanism involves the neurotransmitter and postsynaptic receptor activator, glutamate. Upon activation of the *N*-methyl-D-aspartate (NMDA), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)-proprionate (AMPA) and kainate (KA) receptors, their associated ion channels open to allow the influx of calcium and sodium ions. An excessive influx of Ca^{2+}, together with any Ca^{2+} release from intracellular compartments, may overwhelm Ca^{2+} regulatory mechanisms and eventually lead to metabolic derangements and cell death (Choi, 1998 and Tymianski and Tator, 1996). This cell death may be caused by the overactivation of enzymes, in addition to the overactivation of neighbouring ion channels. Examples of such enzyme systems include proteases, lipases, phosphatases and endonucleases. This can either cause direct damage to the cell structure, or induce the formation of oxidative free radicals that will eventually mediate the cell death (Lipton and Rosenberg, 1994, Lipton and Nicotera, 1998 and Lipton, 2006).

The importance of the Ca^{2+}-apoptosis link in disease pathogenesis is thus clearly evident. It should however be taken into account that cellular calcium overload is unlikely to be the sole mechanism mediating neuronal death. However, several lines of evidence still support a close relationship between excessive Ca^{2+} influx and neuronal injury (Tymianski and Tator, 1996).

2.3 The Ionotropic Receptors

The ionotropic receptors belong to one of two major subclasses of receptors in the central nervous system. In contrast to the other subtype (metabotropic receptors), where activation is coupled to an intracellular biochemical cascade, the ionotropic receptors' activation is linked directly to a membrane ion channel. These ion channels appear to be very critical components of cellular function in general. They play a major role in the translocation of ions across the cell membrane, usually in response to a variety of chemical or physical stimuli. These processes eventually lead to the transduction of the information that was received at the receptor, into responses. This makes ion channels a major class of cellular effectors in the human body.

2.3.1 Sigma Receptors (σ-Rs)

The sigma receptors are a group of excitatory, ionotropic receptors that are best known for their involvement in an indirect ligand-activated K^+ channel blockade (Polya, 2003). However, it has recently been proven that these receptors also play a significant role in Ca^{2+} channels. This observation was made with the application of sigma receptor agonists during whole cell patch-clamp recordings. During these experiments, it was determined that sigma receptor stimulation led to a depression of the peak Ca^{2+} channel currents, with the maximum inhibition of ≥ 95%. These high values suggest that all the calcium channel subtypes found on the cell body of the neurons used during these experiments, were blocked (N-, L-, P/Q- and R-type calcium channels) (Zhang and Cuevas, 2002).

In addition to the depressing peak calcium channel current, sigma receptors also altered the biophysical properties of these channels. The rate at which the calcium channels were inactivated after stimulation was accelerated and the voltage dependence of both steady-state activation and inactivation shifted toward more negative potentials. This suggests that stronger depolarisations would thus be necessary to activate these ion channels after they were stimulated (Zhang and Cuevas, 2002).

Based on the findings from biochemical and radioligand binding experiments, two sigma receptor subtypes have been identified to date, *i.e.* the σ_1- and σ_2-receptors (Hellewel and Bowen, 1990 and Quirion *et al.*, 1992). Although these two subclasses of receptors share many similarities, there are also several differences between them. Unlike the σ_1-subtype, the σ_2-receptor has the ability to evoke the release of calcium ions from intracellular stores

(Vilner and Bowen, 2000). Pharmacological experiments also suggest that these receptors (σ_2) are the ones that modulate the calcium channels in neurons and that they couple to these calcium channels *via* a signal transduction cascade (Zhang and Cuevas, 2001).

Another major difference between these two receptor subtypes is their distinct pharmacological profiles and binding characteristics. The σ_1-receptors, for example, have a much greater affinity for (+)-pentazocine (Quirion *et al.*, 1992), (+)-SKF-10,047 and other (+)-benzomorphans, whereas the σ_2-receptors show low binding affinities for these compounds. However, ibogaine is one of the sigma receptor ligands that bind very strongly to the σ_2-receptor subtype (Hellewel and Bowen, 1990, Hellewel *et al.*, 1994 and Bowen *et al.*, 1995). These and other common sigma receptor ligands are presented in figure 2.1.

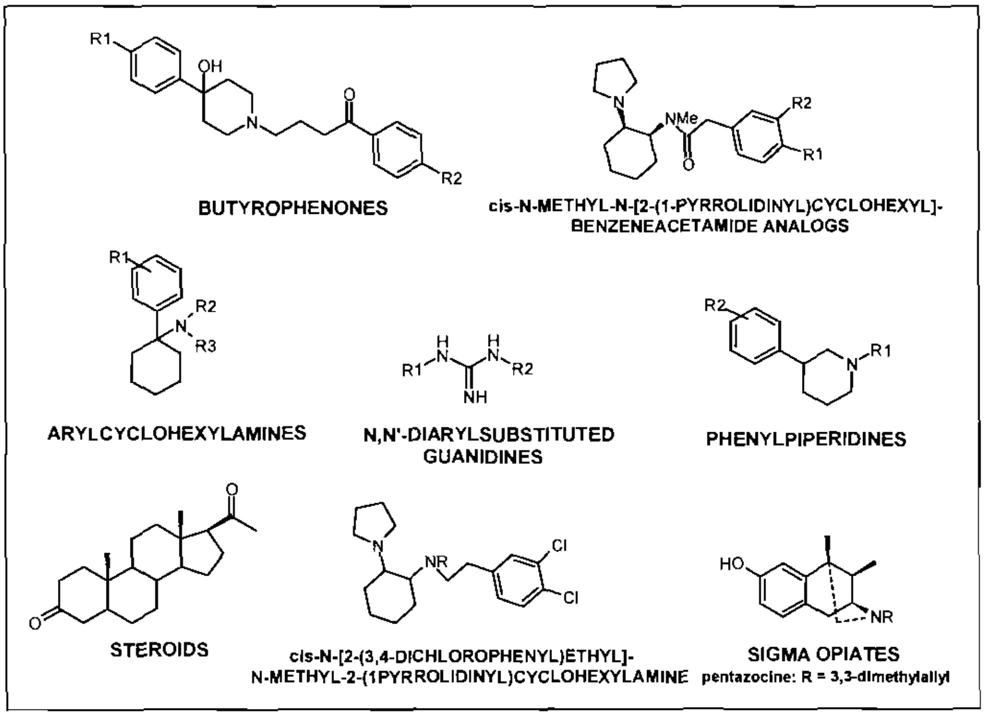


Figure 2.1. Selected sigma receptor ligands (adopted from Walker *et al.*, 1990).

The drugs that normally bind and are active at sigma receptors, encompass diverse classes of pharmacological agents. These active compounds include (i) neuroleptics, *e.g.* haloperidol; (ii) benzomorphans, *e.g.* pentazocine and SKF-10,047; (iii) antitussive agents, *e.g.* dextromorphan; (iv) dissociative anaesthetics, *e.g.* phencyclidine; (v) antihypertensives, *e.g.* propranolol; (vi) antidepressants, *e.g.* imipramine (Su, 1982) and (vii) steroids, *e.g.* progesterone (Su *et al.*, 1988).

Although the function of the sigma receptors is not yet well understood, they have been implicated in the modulation of various biochemical, behaviour and physiological processes. Because of the high density of these receptors in brain areas that are involved in movement (Gundlach *et al.*, 1986 and McLean and Weber, 1988), it is believed that sigma receptor binding should have a significant influence on the motor system. Behavioural experiments performed by Goldstein *et al.* (1989) showed that sigma ligands act in the substantia nigra to increase motor activity. Biochemical data also suggest that sigma ligands induce the release of dopamine from central nervous system neurons (Walker *et al.*, 1990). The σ_2-receptor subtype, in particular, has shown an enhancement in dopamine release from PC-12 rat phaeochromocytoma cells upon amphetamine stimulation (Hellewel and Bowen, 1990). Other studies performed by Patrick *et al.* (1993) also suggest that the sigma binding site and sigma ligands play a role in the modulation of nigrostriatal dopamine neurotransmission.

In addition to these significant roles that the sigma receptor system might play in movement disorders, other receptor systems have been discovered that have a definite influence on the activity of motor neurons. Amongst these, are the excitatory amino acid receptors, known as the glutamate ion channels.

2.3.2 The Glutamate Receptors

Aspartate and glutamate (as well as certain of their analogues) are the amino acids that mediate most of the excitatory synaptic transmission in the brain. The family of excitatory amino acid (EAA) receptors that act as targets for these amino acids, are usually named after their most prominent neurotransmitter agonist. This explains the use of "glutamate receptor" for reference to all EAA receptors (Dingledine and McBain, 1999).

The glutamate system occupies a key place in the functioning of the central nervous system. Glutamate receptors are present in all nuclei of the basal ganglia, with the highest densities within the striatum. The excitatory neurotransmitter, glutamate (Glu) with its large receptor family, is probably the most versatile and complex signalling system in the mammalian brain and possibly also the most susceptible to pathological disturbances. Therefore, knowledge of their structure at molecular level is extremely important to comprehend their function in humans, as well as for the understanding of the mechanism of their involvement in neurodegeneration (Baskin *et al.*, 2000).

At present, the ionotropic receptors are pharmacologically defined as N-methyl-D-aspartate (NMDA), kainate (KA) and 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)-proprionate (AMPA) receptors. As mentioned earlier, these three classes originally were named after compounds with reasonable preferential affinity for their respective binding sites (Dingledine and McBain, 1999). As the NMDA receptor (NMDAR) is the excitatory amino acid receptor subclass that is most studied, the AMPA and KA receptors are often referred to as non-NMDA receptors. This classification and identification of the ionotropic glutamate receptors has been the subject of intense study over several decades (table 2.1).

Table 2.1. Classification of the molecular families of the ionotropic glutamate receptors (Dingledine and McBain, 1999).

	FUNCTIONAL CLASS	GENE FAMILY/ COMPONENT
	NMDA	• NR1 • NR2 A – D • NR3 A
NON-NMDAR	**AMPA**	• GluR 1 • GluR 2 • GluR 3 • GluR 4
	KA	• GluR 5-7 • KA 1-2

Despite all the findings involving the non-NMDA receptors, the NMDA receptors still remain the receptor subclass of most interest to the majority of neuroscientists. The reason for this is that very little evidence exists to suggest that non-NMDA receptors play a role in neurodegeneration. Greene and Greenamyre (1996) reported that, whereas toxicity caused by a mild metabolic stress is mediated solely by the NMDA receptor, the non-NMDA receptors only mediate a portion of the stress once the condition has increased in severity. Also, as suggested by the so-called source-specificity hypothesis, the rate-limiting enzymes or substrates responsible for excitotoxicity must be co-localised with the NMDA receptors

(Tymianski and Tator, 1996). It is thus evident that the NMDA receptors play a particularly important role in the manifestation of neurotoxicity produced by metabolic inhibition and therefore one of the focus points of this study was this subtype of glutamate receptors.

2.3.2.1 The NMDA ionotropic receptors

The NMDA receptors are amongst the most tightly regulated neurotransmitter receptors (Dingledine and McBain, 1999) and because their permeability to Ca^{2+} is much higher than that of the non-NMDA receptors, they have attracted particular interest as a major role player in excitotoxicity.

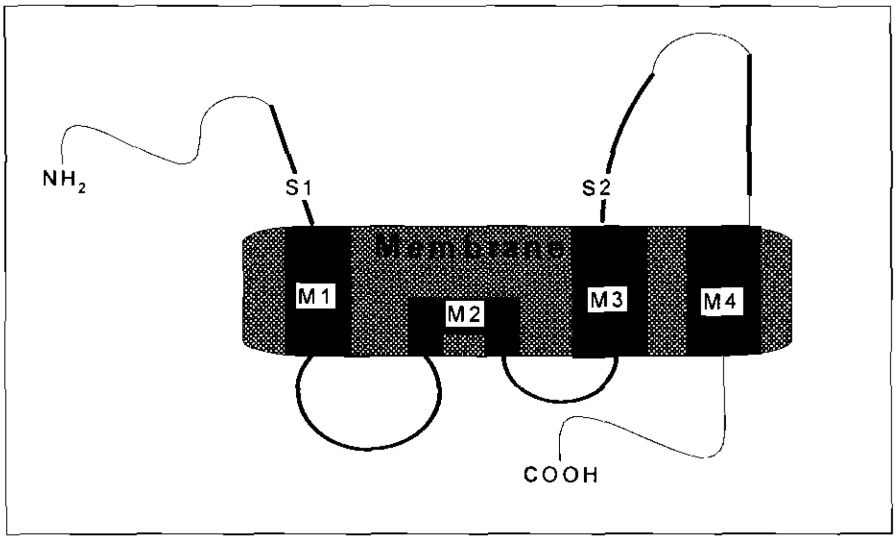


Figure 2.2. Transmembrane topology of the ionotropic glutamate receptor (Adopted from Bigge, 1999).

Three NMDA receptor subunit families have been identified to date and the receptor contains at least five subunits (Liu and Zhang, 2000). One of these subunit families is represented by a single gene *(NR1)*, whereas the rest are represented by multiple genes *(NR2A-NR2D, NR3A)* (Dingledine and McBain, 1999). Each of the NMDA receptor subunits is composed of four hydrophobic membrane domains, an extracellular N-terminal (amino terminal) domain and an intracellular C-terminal tail (figure 2.2). Three of the membrane domains appear to be transmembranal (M1, M3 & M4), whereas the M2-domain forms a re-entrant membrane loop, facing the cytoplasm. The latter segment seems to be an essential structural component of the ionic pore (Nakanishi and Masu, 1994). A 400-amino acid region, encoding the transmembrane domains, follows the N-terminal part.

Although the exact three-dimensional structure of the NMDA receptor has not yet been determined experimentally, Baskin *et al.* (2000) were successful in building a spatial model for one of the numerous subtypes of the NMDA receptor. This was achieved by means of homology studies of the amino acid sequences of its transmembrane domain. The results of X-ray diffraction analysis of these amino acid sequences have recently been published. The obtained model (figure 2.3) describes the four subunits of which the channel consists, as can be seen from different angles (Baskin *et al.*, 2000).

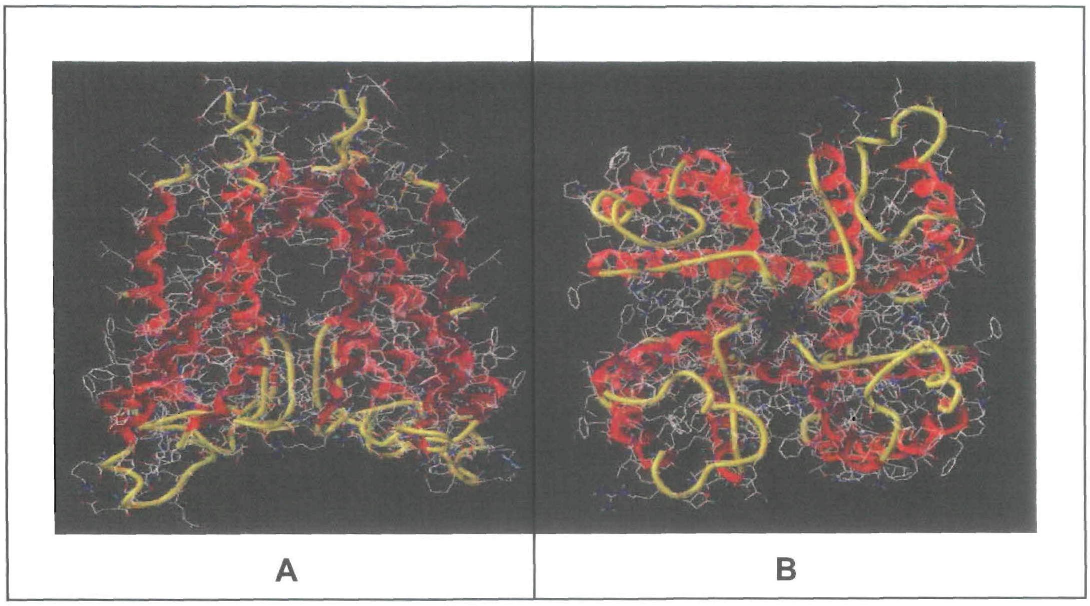


Figure 2.3. Ion channel of the NMDA receptor (Baskin *et al.*, 2000): **A** - From the side; **B** – From below.

An asparagine residue forms part of the amino terminal in the second transmembrane domain (TM2) of the NR1 subunit within the channel pore loop structure. This residue is responsible for controlling the receptor's Ca^{2+} permeability by controlling gating properties, potentiation and block by polyamines, inhibition by protons and affinity to the co-agonists, glutamate and glycine (Schneggenburger and Ascher, 1997).

As mentioned earlier, the NMDA receptors transmit their signals by changing membrane permeability to Na^{+} and Ca^{2+} upon activation. The binding of agonists to the ionotropic receptors leads to the opening of the ion channel, allowing the influx of the relevant ions. This takes place under the influence of the electrostatic potential gradient that exists within the ion channel, resulting in membrane depolarisation and the transference of a nerve impulse (Baskin *et al.*, 2000).

The NMDA receptor ion channel has no less than six distinct binding sites for endogenous ligands that influence the probability of ion channel opening. These binding sites (figure 2.4) include two different co-agonist recognition sites, the one for glutamate (NR2) and the other for glycine (NR1). In addition to these recognition sites, a polyamine regulatory binding site also exists. Together with the other two agonist recognition sites, the polyamine binding site promotes receptor activation. The inhibition of ion flux takes place through the binding of various cations. For this purpose, separate recognition sites exist for Zn^{2+}, H^+ and Mg^{2+} (Dingledine and McBain, 1999). The recognition site for the magnesium ions is located within the ion channel of the receptor.

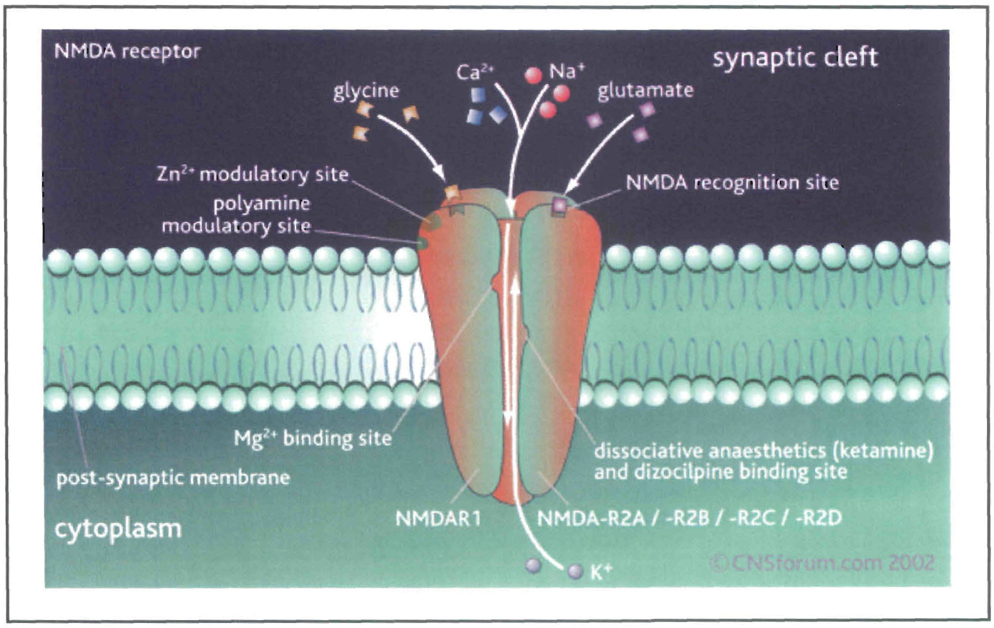


Figure 2.4. Basic structure of the NMDA receptor complex with its recognition sites (CNSforum.com, 2002).

An octahedral co-ordination of the magnesium ion is preferred and water molecules occupy the co-ordination spheres (figure 2.5). Carbonyl groups, which form part of the asparagine residues in the channel pore, are actively involved in the binding to cations (such as Mg^{2+}) which pass through the channel. In this process, the free electron pairs of the carbonyl oxygen and/or amide nitrogen of the asparagine side chain substitutes the water molecules. This eventually leads to the loss of water molecules from the hydrated shell of the cation (Frausto Da Silva and Williams, 1991).

This whole process is blocked when compounds like MK-801 bind to the non-competitive antagonistic site of the NMDA receptor, known as the phencyclidine (PCP) binding site.

During this interaction between antagonist and receptor, a hydrogen bond is formed with part of the asparagine residue. Due to its occupation by the non-competitive receptor antagonist, the amino acid can thus no longer participate in the dehydration of cations. As a result, the function of the ion channel is put on hold and antagonists with affinity for the PCP binding site thus imitate the natural channel blocker, namely Mg^{2+} (Baskin *et al.*, 2000).

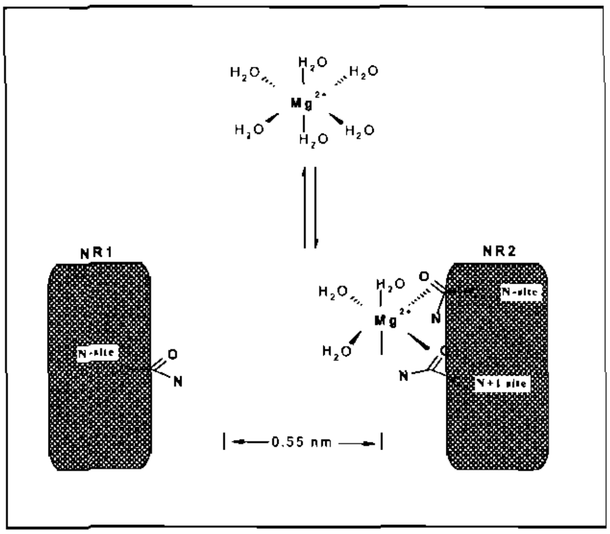


Figure 2.5. The adjacent asparagines in the NR2A-subunit of the NMDA receptor underlies the block of the receptor's ion channels by extracellular Mg^{2+} (Wollmuth *et al.*, 1998).

• NMDA Receptors in Neuronal Apoptosis

Although the mechanisms involved during the cause of many neurodegenerative diseases differ, they may still share a final common pathway to neuronal injury. This pathway necessarily involves calcium ions. Unfortunately, despite the important role of Ca^{2+} in the functioning of these receptors, the mechanism by which the NMDA receptor leads to neuronal apoptosis mainly involves its high permeability to calcium ions. The excessive and prolonged stimulation of the NMDA receptor is what might lead to several detrimental intracellular signals, which may eventually contribute to apoptosis or necrosis. With the NMDA receptors in particular, this overstimulation results in excessive Ca^{2+} influx, which, in turn, leads to an elevation of the $[Ca^{2+}]_i$. The levels elevate to a point where mitochondria are overloaded with Ca^{2+} and depolarisation of the mitochondrial membrane potential takes place. Consequently the synthesis of ATP decreases and the neurons die (Kaul and Lipton, 2002).

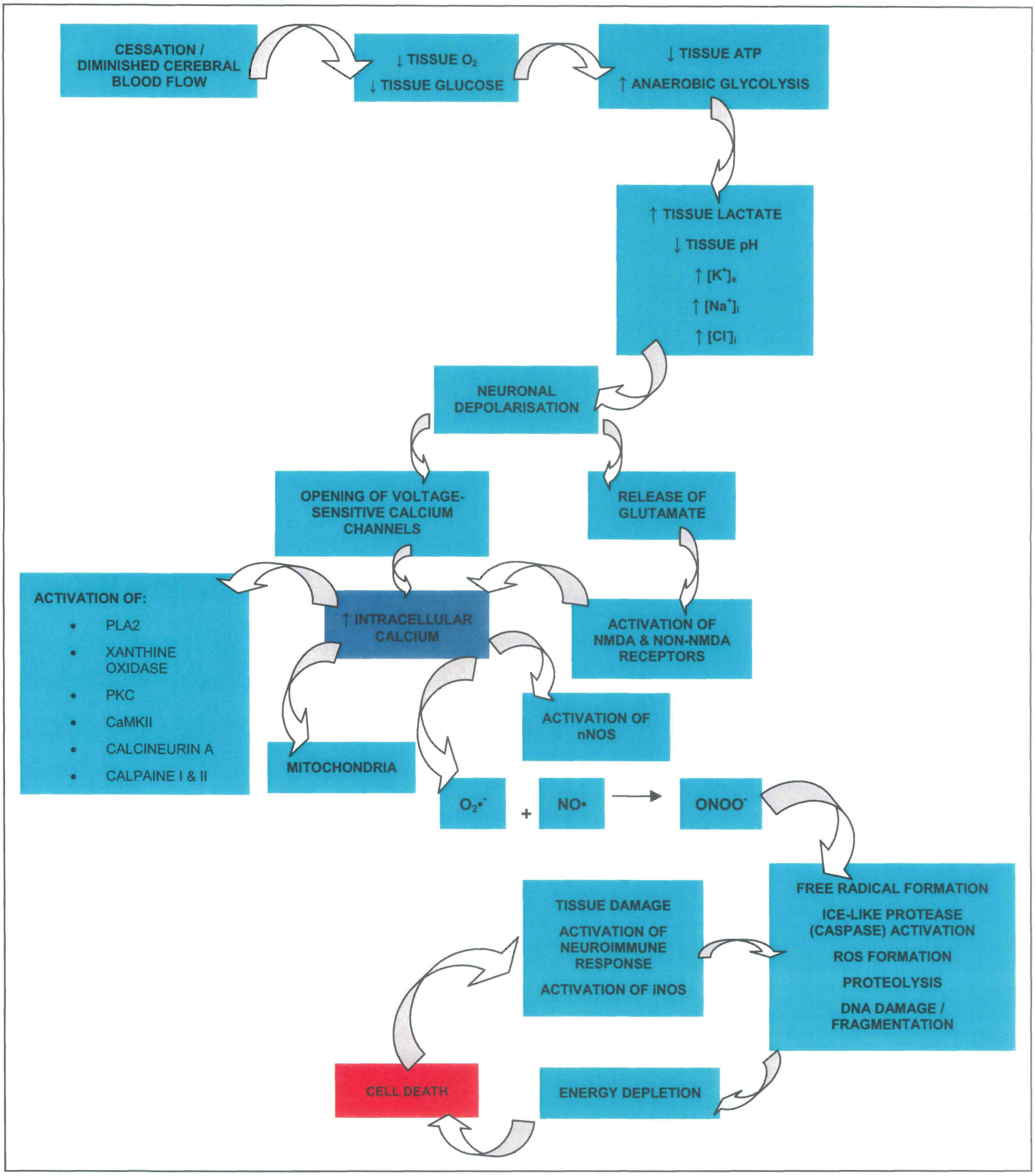


Figure 2.6. Postulated mechanisms of neuronal death after focal ischaemia (Samdani *et al.*, 1997). (**PLA2**, Phospholipases A2; **PKC**, Protein Kinase C; **CaMK II**, Calcium/Calmodulin Dependent Protein Kinase II; **ICE**, Interleukin Converting Enzyme; **PARS**, Proteinase Activated Receptors)

The excessive intracellular Ca^{2+} can also stimulate protein kinase cascades, as well as the generation of free radicals, which include both reactive oxygen species (ROS) and nitric oxide (Kaul and Lipton, 2002). After intensive research, the role that nitric oxide (NO) plays in the excitotoxic effects of calcium is also now quite clear and it is known that an abnormal increase in its production can mediate tissue injury. Neuronal nitric oxide synthase (nNOS) activity is primarily regulated by local increases in intracellular calcium, since distinct calcium influx pathways specifically regulate nNOS in various tissues (Garthwaite *et al.*, 1988). An increased calcium influx may lead to the elevation of NOS and thus also NO levels (Kohl and Dannhardt, 2001). NO can react with ROS to form cytotoxic peroxynitrite ($OONO^-$) and in alternative redox states, NO can also activate p21ras by S-nitrosylation. The NO group can also inhibit caspases in cerebrocortical neurons *via* S-nitrosylation (Kaul and Lipton, 2002). Ultimately, the inappropriate induction of NOS in brain tissues and the subsequent elevation of NO further mediate injury that is implicated in diverse disease states (Kohl and Dannhardt, 2001).

Due to the fact that the NMDA receptors are complex ion channels with various binding sites, several NMDA receptor modulators have been identified to date. The analysis of structure-activity relationships of these modulators provided a blueprint for the topology of the receptor's binding sites, in particular for that of the L-glutamate site (Jane *et al.*, 1994).

Although NMDA receptor agonists can be of clinical significance, it is mainly the antagonists of this receptor that receives attention. This is due to cumulative evidence that shows that antagonism of this receptor can notably attenuate neurodegeneration (Dingledine and McBain, 1999). Compounds with antagonistic activity on the NMDA receptor can be classified pharmacologically according to the site of action on the receptor-channel complex (figure 2.4). These include ligands that act at the (i) NMDA recognition site, (ii) glycine site, (iii) channel pore and (iv) other modulatory sites, such as the polyamine, S-nitrosylation and glycine binding sites (Wong and Kemp, 1991). Competitive glycine site antagonists were also developed with the hope that they would improve receptor selectivity. Examples of these antagonists include the partial antagonist, (R)-HA-966 and the full antagonist, kynurenic acid (figure 2.7).

- **Compounds with an affinity for the NMDA receptors**

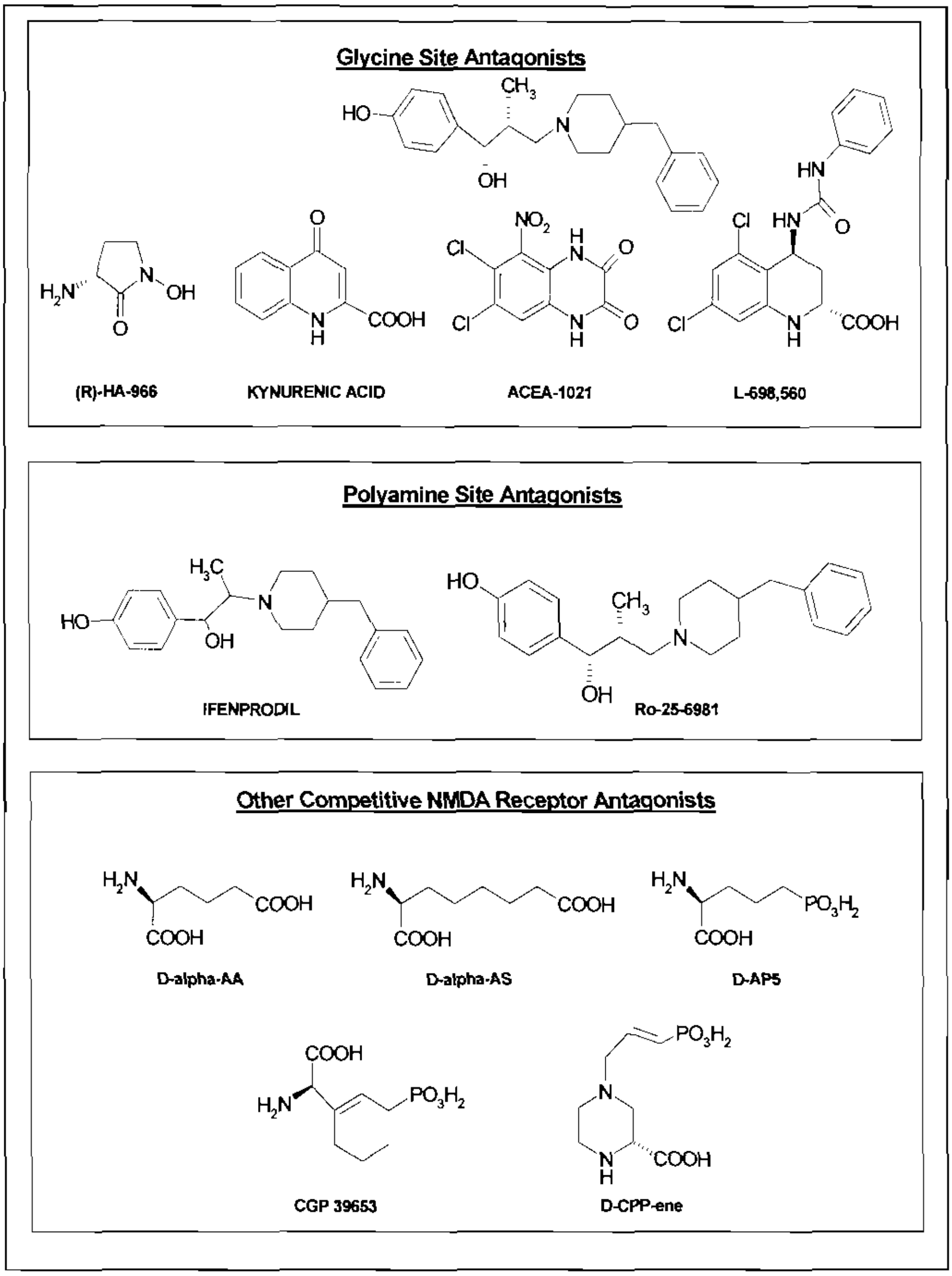


Figure 2.7. Competitive NMDA Receptor Antagonists (Watkins and Evans, 1981, Biscoe *et al.*, 1977, Evans *et al.*, 1978, Evans *et al.*, 1979 and Davies and Watkins, 1972).

Compounds that bind to the NMDA receptor in a voltage-dependent manner are known as non-competitive antagonists. The non-competitive antagonists interact with active receptors and they show a preferential blockade of highly activated receptors, while having minimal interaction with physiologically working channels. This characteristic makes them more preferable than competitive blockers, due to the side effect profile that is usually associated with the competitive block of these receptors. High affinity non-competitive NMDA antagonists include MK-801 and PCP (figure 2.8).

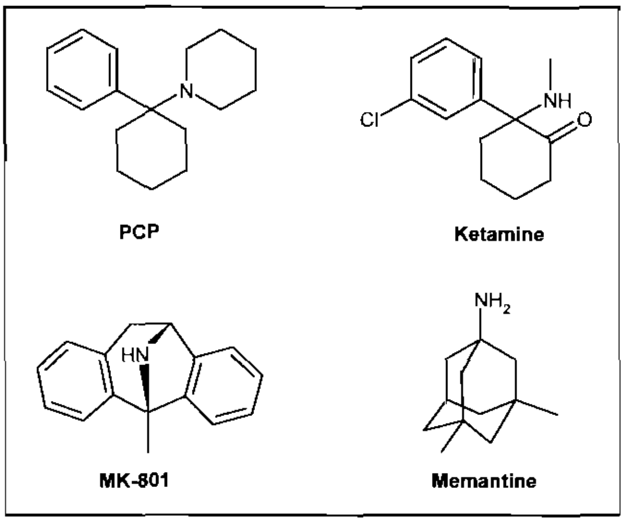


Figure 2.8. Non-competitive NMDA Receptor Antagonists (Lodge and Anis, 1982, Wong *et al.*, 1986 and Iversen and Kemp, 1994).

2.4 The L-type Voltage-Gated Calcium Channels

Although more energy and attention have been focused on the NMDA glutamate receptors in research that involves the calcium ion overload of cells, other sources of such calcium ion overload have also been considered (Small *et al.*, 1997 and Mason *et al.*, 1999). Amongst these are the high threshold voltage-gated calcium channels.

Based on their pharmacology and biophysiology, various voltage-gated calcium channel subtypes have been identified (Takahashi and Momiyama, 1993 and Olivera *et al.*, 1994). These subtypes are known to be differential in their regional expression of specific subunit isoforms as well as in their localisations in cells. These differences and the existence of multiple types of calcium channels in neurons seem to be important factors in controlling many calcium dependent processes such as neurotransmitter release, gene expression and neurite overgrowth (Neelands and Macdonald, 1999).

According to Catterall (1999), the different subtypes of calcium channels have different voltage ranges and their rates for activation and inactivation also differ. Enough evidence exists that suggests that L-type calcium channels participate during Ca^{2+} entry in conditions like ischaemia (Mason *et al.*, 1999). Also, in a study to determine which of the various subtypes of calcium channels predominantly contribute to necrotic and apoptotic cell death, Cano-Abad *et al.* (2001) came to the conclusion that it is mainly the L-channels that play a role in these processes.

Because of the relatively hyperpolarised potential at which they are activated, the L-type calcium channels are classified amongst the so-called high-voltage-activated subtypes. The high-voltage-activated calcium channels are distinguished mainly by their pharmacological sensitivity to specific blockers and toxins (Adams and Olivera, 1994). It is interesting to note that these channels share all the properties associated with conventional receptors and can thus be classified as such (Kwon and Triggle, 1991).

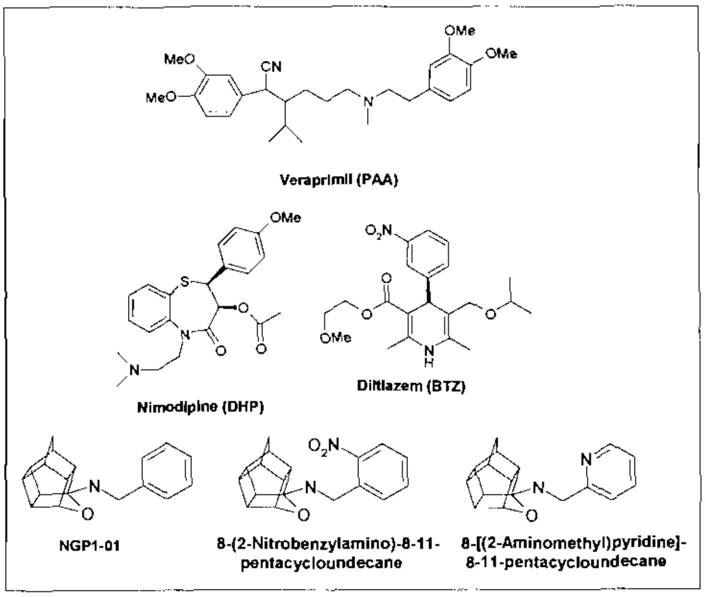


Figure 2.9. Calcium channel blockers (Van der Schyf *et al.*, 1986 and Malan *et al.*, 2000).

The L-type calcium channels are the only subtype that contains high-affinity binding domains for different chemical classes of drugs. These chemical classes for the channel blockers include the benzothiazepines (BTZs) and the dihydropyridines (DHPs), which both reach their receptors from the extracellular side, as well as the phenylalkylamines (PAAs). Radioligand binding studies have revealed that these different chemical classes of calcium channel blockers do not interact with the same binding site (Glossmann and Striessnig, 1990, Glossmann *et al.*, 1982 and Glossmann *et al.*, 1987). The phenylalkylamines and benzothiazepines are believed to block the channel by obstructing the pore. The location of the receptor site for the dihydropyridines is, however, a subject of controversy (Zhorov and Tikhonov, 2004). A few of these channel blockers, as well as some polycyclic cage compounds that have also been identified as calcium channel blockers by Van der Schyf *et al.* (1986) and Malan *et al.* (2000) are presented in figure 2.9 above.

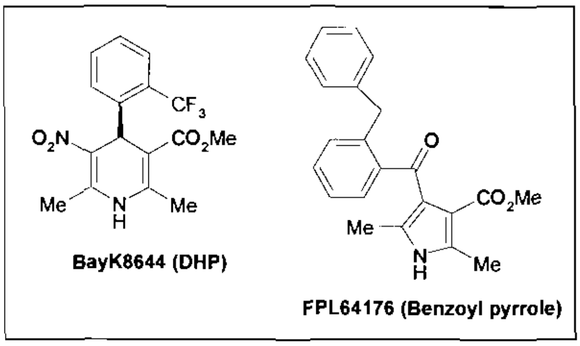


Figure 2.10. Calcium channel agonists (Kane *et al.*, 1995).

Although not used therapeutically, several L-type calcium channel agonists have also been described. Amongst these are the dihydropyridine, (-)-BayK8644 and the benzoyl pyrrole, FPL64176 (figure 2.10).

2.5 Conclusion

With the molecular basis underlying the pathogenesis of neurodegenerative disorders disclosed, new targets are emerging for the prevention of neuronal injury. It is also quite evident that the receptor system with the most prominent effect on excitotoxicity, is the NMDA receptor ion channels. This is mainly due to their high permeability for calcium ions, which might lead to an intracellular calcium overload. This calcium overload is normally the effect of an overstimulation of these receptor ion channels, eventually leading to the death of neurons. The L-type calcium channels also play a role in calcium overload, because of their voltage-dependent activation. As a result, this subtype of calcium ion channels will also receive attention during this study.

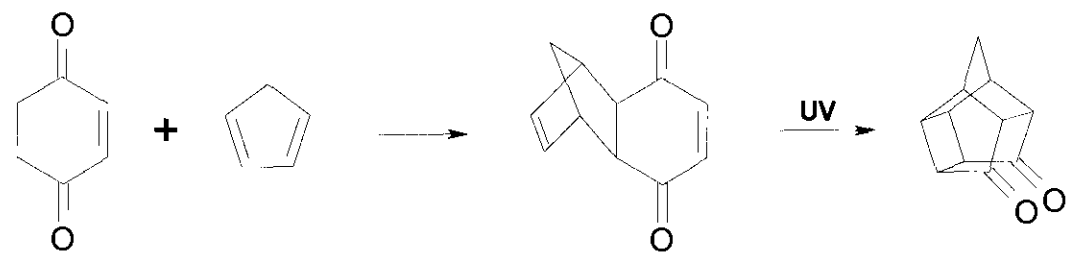
NMDA receptor and calcium channel antagonists could thus potentially be of therapeutic value in a number of acute and chronic neurological disorders (Koroshetz and Moskowitz, 1996 and Kemp and McKernan, 2002), where their antagonistic activity might lead to the deactivation of the ion channel and thus decrease the risk of a cellular Ca^{2+} overload. With the resulting decrease in unwanted processes like free radical formation and the activation of second messenger systems, neurodegeneration might become a phenomenon that occurs less often.

CHAPTER 3
LITERATURE REVIEW

CHEMISTRY & STRUCTURE-ACTIVITY RELATIONSHIPS (SAR'S)

3.1 The polycyclic cage compounds

Over the last few decades, the polycyclic compounds have attracted considerable interest. As early as 1958, Cookson *et al.* (1958) described that the pentacyclic dione - the so-called "bird cage" compound **(2)** - was formed during the intramolecular cyclisation of the Diels-Alder adduct **(1)**, which can be obtained from the reaction of *p*-benzoquinone with cyclopentadiene (Cookson *et al.*, 1958). This synthetic pathway to the cage-like structure formed the basis of numerous studies into the chemistry of the pentacycloundecyl compounds.

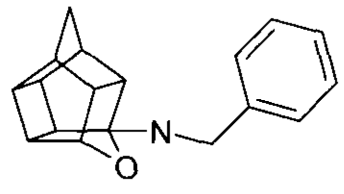


1 **2**

Scheme 3.1. Synthesis of Cookson's diketone **(2)** (Cookson *et al.*, 1958).

In particular, the benzylamine derivative of the cage structure **(3)** has become the focus of an increasing amount of attention. 8-Benzylamino-8,11-oxapentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane was first synthesised by Sasaki *et al.* (1971), but it was not until much later that its structure was assigned correctly (Marchand *et al.*, 1988 and Van der Schyf *et al.*, 1989). Even before the correct chemical nomenclature was assigned to this compound, it was characterised and patented by Van der Schyf *et al.* (1986) as NGP1-01 and described as a calcium channel antagonist. The initial investigations with

NGP1-01 were based on electrophysiological experiments on isolated guinea-pig papillary muscle and sheep purkinje fibres. It was later discovered that the mechanism of calcium current blockade by NGP1-01 involve both a frequency and a voltage-dependent block of the calcium channels (Van der Walt *et al.*, 1988).



3

Figure 3.1. 8-Benzylamino-8,11-oxapentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane (Marchand *et al.*, 1988 and Van der Schyf *et al.*, 1989).

NGP1-01 further served as model compound during a pilot *in vivo* neuroprotection study performed by Geldenhuys *et al.* (2003). During this study, a small series of pentacycloundecylamines with different side-chains were synthesised and evaluated for neuroprotective activity. It was theorised that these compounds could have a dual mechanism of action as neuroprotective agents. In addition to their antagonistic activity on the L-type calcium channels, the pentacycloundecylamines also have attenuated NMDA receptor activity.

These and other investigations into the pharmacological properties of the polycyclic compounds (including investigations by Oliver *et al.*, 1991 and Malan *et al.*, 2003), have led scientists to believe that some of the cage-like polycyclic amines might play a significant role in neuroprotection. The pentacycloundecylamines might thus be considered as potential lead compounds for Parkinson's disease therapy and possibly other neurodegenerative conditions as well.

3.1.1 The polycyclic cage amines as bioactive compounds

Serendipitous discoveries of amantadine's pharmacological profile led to the redirection of the research on other polycyclic compounds from antiviral investigations into a course of neuroprotection (Schwab *et al.*, 1972). After this discovery, amantadine was once again the cause of new explorations on the polycyclic cage amines (Kornhuber *et al.*, 1993). Only a few of the diverse pharmacological applications that have been explored for the polycyclic cage compounds of late are discussed here.

3.1.1.1 Calcium Channel Antagonism

The calcium channel blocking activity of the polycyclic cage amines was probably one of the first biological activities of these compounds to be investigated and NGP1-01 was initially identified as an L-type calcium channel antagonist by Van der Schyf *et al.* (1986). Not long after, this discovery led to structure-activity investigations of similar polycyclic aromatic amines as calcium channel blockers (Liebenberg *et al.*, 2000 and Malan *et al.*, 2000).

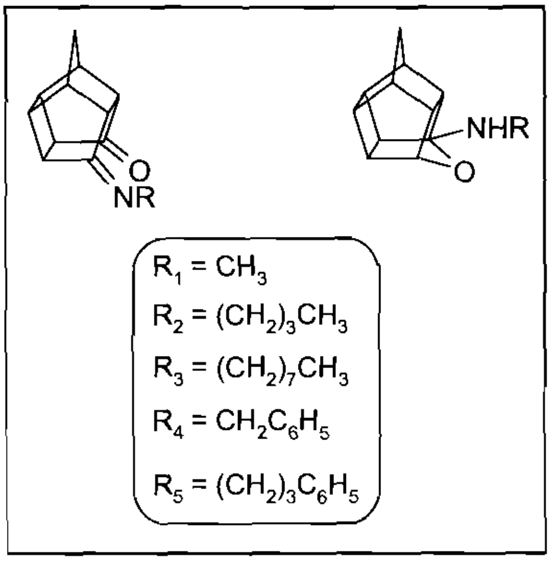


Figure 3.2. Some of the polycyclic cage compounds synthesised and tested for calcium channel blocking activity by Liebenberg *et al.* (2000).

Liebenberg *et al.* (2000) performed electrophysiological experiments on the synthesised compounds in order to determine the extent to which the respective compounds hinder the Ca^{2+} currents in L-type calcium channels. Calcium-mediated action potentials were recorded and the inhibitory effects of these compounds on the excitation and contraction of heart cells were thus evaluated. All compounds with aromatic side-chains were active in suppressing the action potentials. Compounds substituted with short aliphatic side chains were inactive, but an increase in this chain length resulted in biologically active compounds.

Malan *et al.* (2000) performed similar experiments on another series of polycyclic compounds (figure 3.3). Once again, it was established that structure-activity relationships for these compounds are dominated by geometric or steric factors.

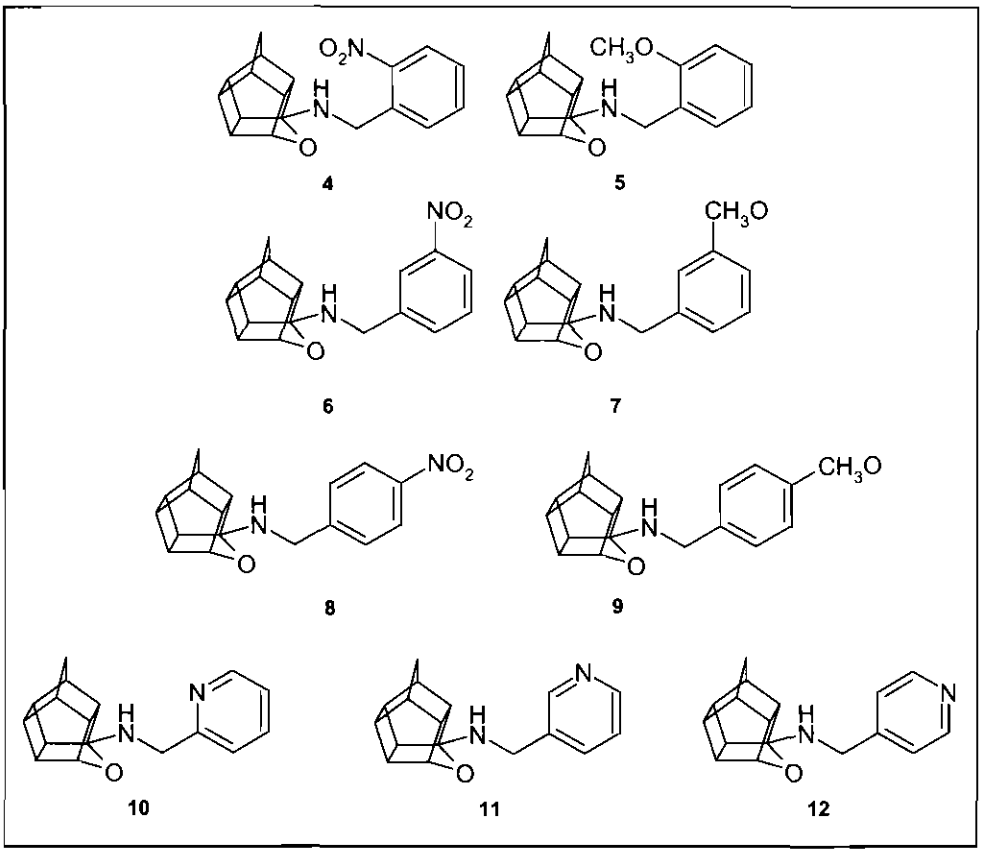


Figure 3.3. Some of the NGP1-01 derivatives synthesised by Malan *et al.* (2000) for evaluation of their calcium blocking capacity.

Basically, this study revealed that the benzylamino substituent is important in the activity of these compounds as calcium channel blockers. This can be seen from the major decrease in activity when this substituent was replaced with other groups (**10**–**12**). Also, substitution of the preferred aromatic group is mostly favoured in the *ortho* and *meta* positions. Finally, the methoxy substituted benzyl groups showed a slightly higher activity when compared to the benzylamines that were substituted with nitro groups.

In summary, it can be said that the basic requirement for activity of these compounds as L-type calcium channel blockers, is the pentacyclic cage amine moiety. It seems as if substituents that will lead to an increase in lipophilicity will also lead to an increase in such activity. Finally, when a substituted aromatic ring is present in the structure, such substitution is more favoured when it is present in the *ortho* and *meta* positions.

3.1.1.2 NMDA Receptor Activity

A series of compounds, amongst which NGP1-01 and its derivatives (**13-21**), were also evaluated for antagonism on the NMDA receptors. During this study, the selected compounds were tested for their ability to inhibit NMDA-mediated $^{45}Ca^{2+}$ influx into murine synaptoneurosomes. NGP1-01 proved to be the most potent compound in the series, with results similar to that of the reference compound, memantine (Geldenhuys *et al.*, 2007).

During this functional assay, a structure-activity relationship for NMDA receptor blocking was drawn up. As observed for Ca^{2+} channel activity, the structure-activity relationships of these compounds were determined primarily by geometric factors, with the polycyclic cage amine being the pharmacophoric element in such activity. The presence of a phenyl ring seems to add to the interaction of the compound with the NMDA receptor and therefore leads to an increase in activity. However, substitution on the aromatic ring resulted in the opposite effect and it might be argued that these moieties are too bulky and cause steric hindrances. Better tolerance was observed for compounds substituted at the *meta* position (**17** and **20**). In addition to this, the number of linker atoms between the cage moiety and the aromatic ring also played a significant role on NMDA receptor blocking activity (**14** and **15**) and an increase in potency was observed for compounds with longer alkyl chains between these groups, as can be seen for compound **15**.

Because of the obvious structural similarities between the pentacycloundecylamines and existing non-competitive NMDA receptor antagonists, it was believed that the cage compounds elicit their NMDA blocking effect by binding to the PCP binding site within the ion channel. However, results obtained from radioligand binding studies with [^{3}H]MK-801 and [^{3}H]TCP proved that the selected test compounds (**13-15**) were unable to displace the PCP blockers from their binding site. This implies that another mechanism of action is possibly involved in the block of the NMDA receptor by these compounds.

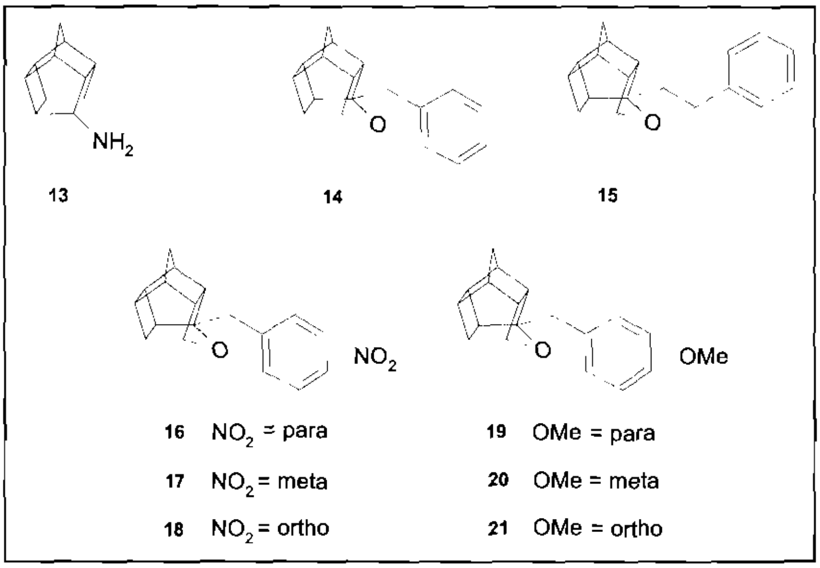


Figure 3.4. Some of the polycyclic cage compounds synthesised and tested for NMDA Receptor antagonism by Geldenhuys *et al.* (2007).

- **Conformational requirements for non-competitive NMDA Receptor blocking and Structure-Activity Relationships**

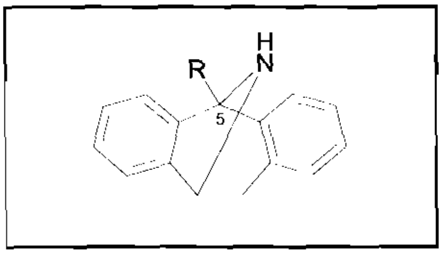


Figure 3.5. Basic structure for C5-substituted derivatives of desmethyl-MK-801 (Monn *et al.*, 1990).

There are various factors that determine the affinity of a molecule for the phencyclidine (PCP) receptor binding site. Firstly, for a compound to have any activity on this binding site whatsoever, it has to meet the two basic requirements of the common structure for PCP-like activity in the central nervous system. The structure requires an aromatic ring as well as an amino group for such activity. The aromatic ring is said to contribute to the stabilisation of the receptor structure, while the protonated nitrogen of the amino group forms a hydrogen bond with the receptor residue. Based on this, several researchers proposed a number of geometric models for compounds that might be active on the PCP binding site of the NMDA receptor.

In a structure-activity relationship binding study of certain analogues of MK-801, Monn *et al.* (1990) discovered that the presence of groups larger than ethyl in an alkoxy-alkyl series on C5 cause a decrease in NMDA receptor activity. A possible explanation for this diminished activity is that the binding site might not be able to accommodate groups larger than a two-membered carbon chain. As a result, decreased activity is observed with an increase in chain length of the substituent (Monn *et al.*, 1990).

After a systematic theoretical analysis of certain compounds, Kroemer et al. (1998) were successful in deriving common pharmacophore elements for the PCP binding site. In this study, it was concluded that steric interactions in certain areas of the ligand molecule with the receptor have a major influence on the degree of binding of the ligand to the receptor. This might be an explanation as to why antagonists such as PCP and MK-801 show an increased binding affinity when compared to other molecules that do contain substituents in the regions of interest. When these two potent NMDA receptor blockers are compared to one another, these results can be confirmed. It was further proven that substitutions in Region A (as referred to in figure 3.6) will lead to a repulsive interaction with the receptor and thus also cause a lower affinity. The absence of a substituent in this area in the MK-801 molecule may thus be what accounts for its higher affinity in comparison with the less potent blocker, PCP (Kroemer *et al.*, 1998).

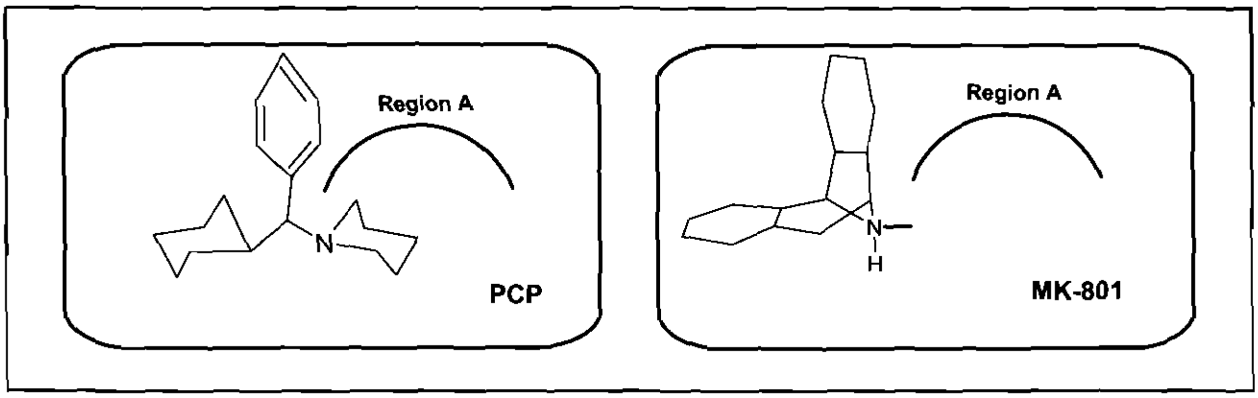


Figure 3.6. Sterically disfavoured areas (Region A) (Kroemer *et al.*, 1998).

Jirgensons *et al.* (2000) determined the antagonistic activity of 1-adamantane structural analogues on the phencyclidine binding site. The basic aim of this study was to determine the effect of the size of lipophilic moieties on the binding affinities of their compounds. It was established that, in the case of the more bulky compounds, the steric effect becomes of greater importance for activity. The performed experiments proved that an increase in the lipophilicity of the substituents is accompanied by a decline in activity. An opposite observation could be made for the less bulky compounds, suggesting that the steric factor in such compounds is negligible. The structural requirements for binding to the PCP site are thus well defined and, by keeping these descriptors in mind, new structures can be evaluated for such activity.

3.1.1.3 Activity on the sigma receptors

Kassiou *et al.* (1996) were the first to synthesise a series of pentacycloundecane derivatives for *in vitro* receptor binding experiments on the sigma binding site. During these experiments, two 4-aza-pentacyclo[5.4.1.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$.$0^{8,11}$]dodecane compounds (as this group of researchers refer to the pentacycloundecylamines) were synthesised amongst other polycyclic cage compounds. The neuroreceptor selectivity and affinity of these compounds were tested during their biological evaluation. Both compounds displayed selectivity and high affinity for the sigma binding site, with the higher binding affinity belonging to **22** (figure 3.7). Whereas other common sigma binding site ligands exhibit cross-reactivity with the NMDA receptor ion channel (Manallack *et al.*, 1986), it was not the case with the particular pentacyclic cage compounds tested.

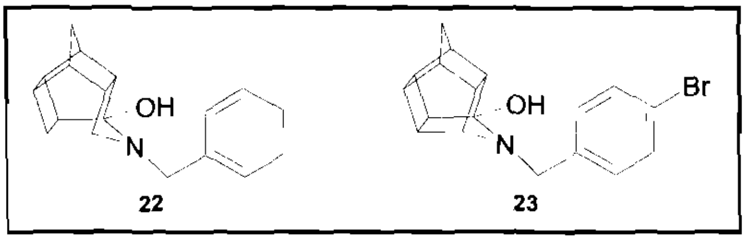


Figure 3.7. Polycyclic cage compounds synthesised and tested for sigma binding affinity by Kassiou *et al.* (1996).

Similar compounds were synthesised by Liu *et al.* (2001). Once again, it was confirmed that this class of compounds exhibit moderate to high affinity at the sigma receptors. A structure-activity relationship between these compounds show that the fluorine substituted benzylamine derivative **(27)** had the highest affinity and selectivity for the σ_2-receptor subtype. The data obtained suggest that there is a potential role for these compounds as σ_2-receptor agonists, making them a class of compounds that have calcium-releasing activity in neuronal cells.

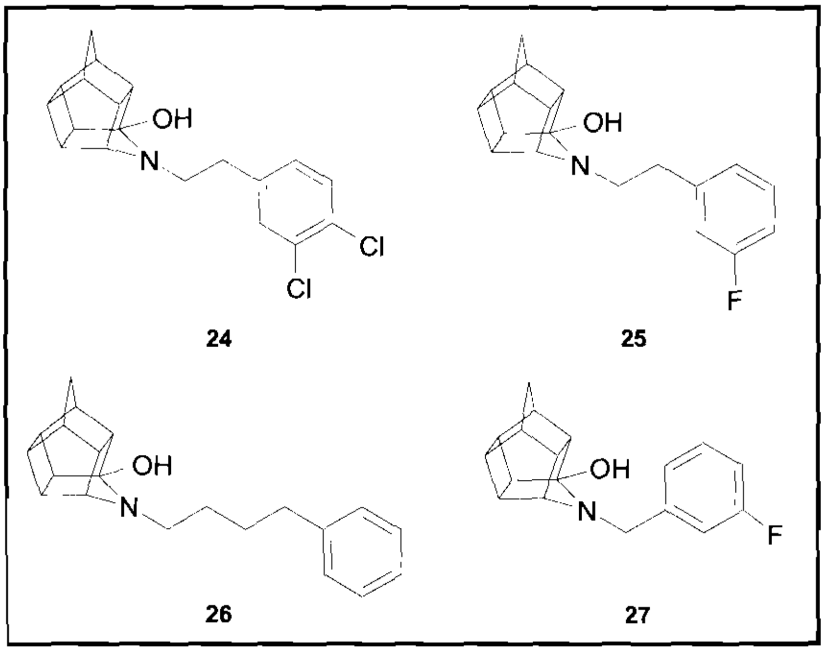
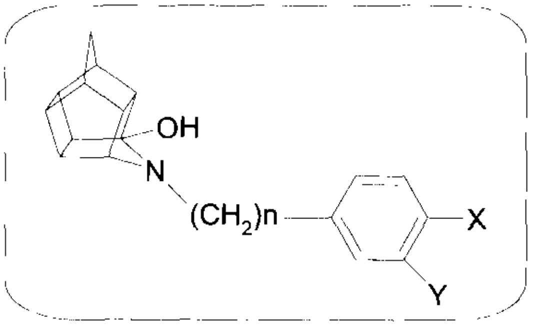


Figure 3.8. Polycyclic cage compounds synthesised and tested for sigma binding affinity by Liu *et al.* (2001).

By making use of the radioactive forms of the selective σ_1-agonist, (+)-pentazocine and the non-selective sigma agonist, DTG, Nguyen *et al.* (1996) tested novel pentacycloundecylamines for their affinities at the σ_1- and σ_2-binding sites, respectively. All the compounds tested displayed moderate to high affinity for both sigma binding sites. The highest affinity for the σ_1-site in this series was exhibited by N-(4-phenylbutyl)-3-hydroxy-4-azahexacyclo[5.4.1.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$.$0^{8,11}$]dodecane **(36)**. This higher affinity, compared to the rest of the synthesised molecules, is probably due to the increased alkyl chain length between the cubane moiety and the aromatic ring. N-(3'-fluorophenyl)methyl-3-hydroxy-4-azahexacyclo[5.4.1.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$.$0^{8,11}$]dodecane **(41)** proved to be the compound with the highest affinity for the σ_2-binding site. This higher affinity is a result of the fluoro substitution in the *meta* position of the aromatic ring and this is in agreement with the findings of Liu *et al.* (2001).



28 n = 1; X = H; Y = H
29 n = 1; X = H; Y = Br
30 n = 1; X = I; Y = H
31 n = 1; X = OCH$_3$; Y = H
32 n = 2; X = H; Y = H
33 n = 1; X = H; Y = H
34 n = 2; X = Br; Y = H
35 n = 2; X = H; Y = Cl
36 n = 3; X = H; Y = H
37 n = 4; X = H; Y = H
38 n = 1; X = H; Y = CH$_3$
39 n = 1; X = H; Y = I
40 n = 1; X = H; Y = Cl
41 n = 1; X = H; Y = F
42 n = 1; X = Br; Y = H

Figure 3.9. Polycyclic cage compounds synthesised and tested for sigma binding affinity by Nguyen *et al.* (1996).

- **Structure-Activity Relationships for sigma receptor binding**

Largent *et al.* (1987) proposed a basic pharmacophore for drugs to possess affinity at the sigma receptor binding site. According to this study, a phenylpiperidine with a lipophilic nitrogen substituent seems to be the molecular framework responsible for the biological activity of drugs on these receptors. Other researchers came to the same conclusion after molecular modelling analyses, *i.e.* that all sigma selective ligands consist of a substituted nitrogen and a phenyl centre, separated by an intermediate carbon chain (Manallack and Beart, 1987 and Manallack *et al.*, 1988).

The above description of the pharmacophore for the sigma binding site explains the activity of the aza-pentacyclic compounds on these receptors. Nguyen *et al.* (1996) confirmed that there are various structural features of these compounds that affect their selectivity and affinity for sigma binding. Affinity for the σ_1-receptor seems to be affected by the chain length between the cubane moiety and the aromatic ring in the series of compounds. Substitution of the aromatic ring in the *meta* position, is what influenced σ_2-receptor binding and the order of highest to lowest affinity was F > Cl > Br > I > H > CH_3.

Based on the amphetamine-stimulated release of dopamine upon sigma receptor stimulation, Liu *et al.* (2001) measured the [^{3}H]-dopamine release as an indication of the potency of a variety of compounds (figure 3.8) on the sigma receptors. All the compounds tested were identified as σ_2-receptor agonists, and as mentioned earlier, the benzyl substituted derivative **(27)** showed the greatest potency. This, once again, proves the importance of aromatic ring substitution for the activity of these ligands. The reversal of their effect by σ_2-receptor antagonists in these experiments further confirmed them as σ_2-agonists.

3.2 The Triquinanes

The triquinanes belong to a group known as the polyquinanes. This is the generic name for structures characterised by carboxylic frames of fused rings. In the case of the triquinanes, the structures consist of three linearly fused cyclopentane rings.

During the last few decades, the linearly fused triquinane sesquiterpenoids have been encountered in increasing numbers among diverse natural sources. After the discovery of so many new and challenging polycyclopentanoid carbon skeleta from plant (Corbett *et al.*, 1979), marine (Sheikh *et al.*, 1976) and fungal (Nozoe *et al.*, 1976) sources, further interest was directed at the more rapid and efficient acquisition of these compounds.

The groups of Mehta and Srikrishna are well known for their contributions to the synthesis of several triquinanes that occur naturally. The key in these syntheses was the flash vacuum pyrolysis (FVP) step. The basic triquinane skeleton was obtained after the fragmentation of the cyclobutane ring of the readily available pentacyclic caged dione **(2)** during FVP (Mehta and Srikrishna, 1997).

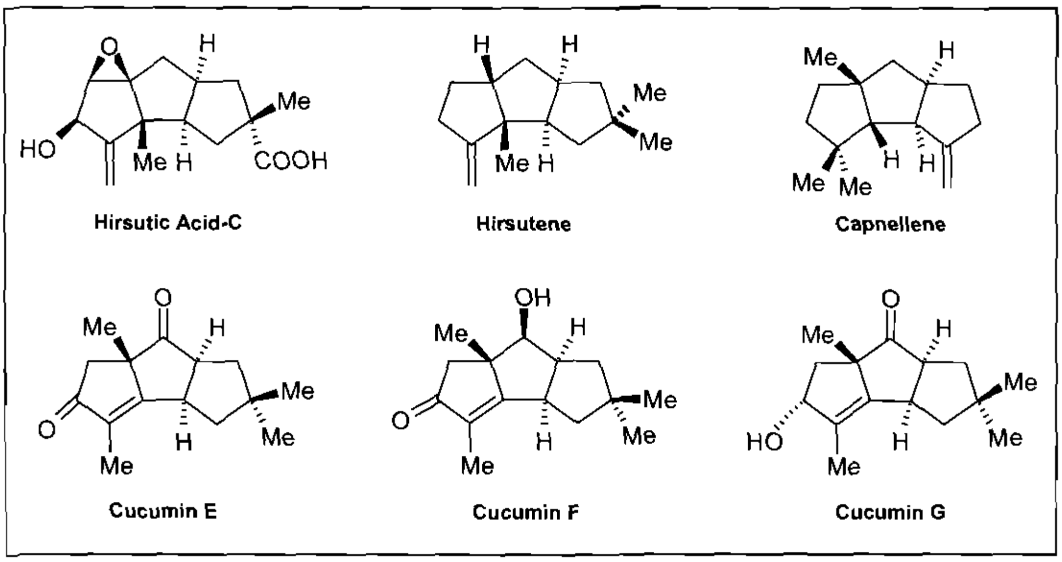


Figure 3.10. Natural triquinane products (Mehta and Umarye, 2001).

3.2.1 Synthetic pathways

During the flash vacuum pyrolysis of the caged dione, the compound is exposed to very high temperatures and the chemical decomposition of the carbonaceous material takes place in an oxygen-starved environment. The particle kinetic energy of the heat is what mainly contributes to the decomposition of the chemicals (Brown, 2001).

In a study intended to determine synthetic applications of flash vacuum pyrolysis, McNab (2004) made use of this vacuum distillation process, during which a substrate was transmitted through a hot tube. For the duration of this experiment, the empty pyrolysis tube and substrate were heated with an external tube furnace and a small Kugelrohr oven respectively, with the substrate being contained in a borosilicate glass test tube. The oven used for heating the substrate was connected *via* a B24 cone to the furnace tube (McNab, 2004). Instead of the Kugelrohr oven, an external air bath, heating tapes (Trahanovsky and Doyle, 1968), or a hot air blower (Bonnett *et al.*, 1973) can be used to bring about sublimation of the substrate in the glass test tube. The initial substrate undergoes sublimation before being exposed to the high temperature. In order to ensure the rapid sublimation of small samples, the compound is spread as a thin layer over the whole inner surface of the sublimation vessel – usually a round bottomed flask of about 50 ml in volume. This is achieved by rotary evaporation of a solution in a volatile solvent (Bonnett *et al.*, 1973).

Because of its low boiling point and also because many compounds are soluble in it, dichloromethane seems to be the solvent of choice in most cases.

A packed quartz tube is the ideal choice as oven tube, as it allows the heating of very specific areas in a short period of time. The temperature inside the tube is measured by using a thermocouple in an internal well. Finally, the produced vapour is collected in liquid nitrogen cold traps at a temperature of about -78 °C (McNab, 2004).

Due to the high temperatures involved in this reaction, any prolonged exposure of the compound molecules in the oven area might lead to their decomposition. During a typical throughput rate of 1-2 grams of substrate per hour, individual substrate molecules would spend only a fraction of a second in the hot zone (Brown, 1980). This throughput rate is however sufficient for the compound to react to form the required product and can be achieved by maintaining a pressure of less than 0.01 mmHg. In order to achieve the desired pressure, the whole apparatus is evacuated with a high-capacity vacuum pump. The pressure is usually measured either fairly close to the hot zone or beyond the nitrogen cold trap with a Pirani or McLeod gauge. Pressure differences across the length of the tube are usually not observed (Brown, 1980).

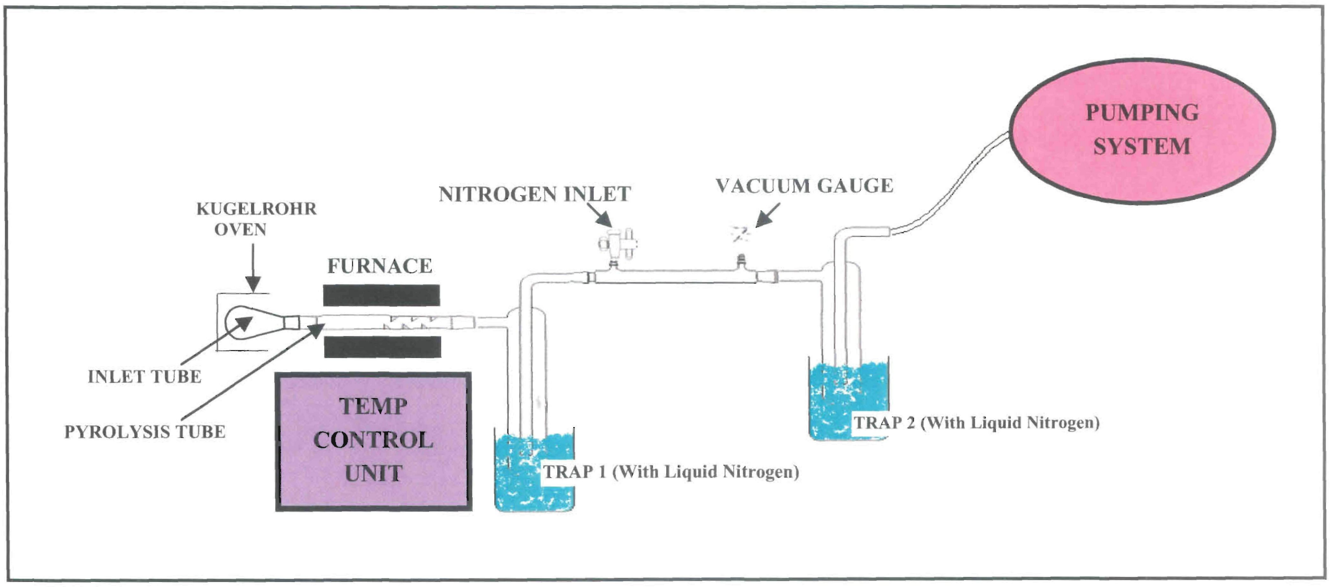
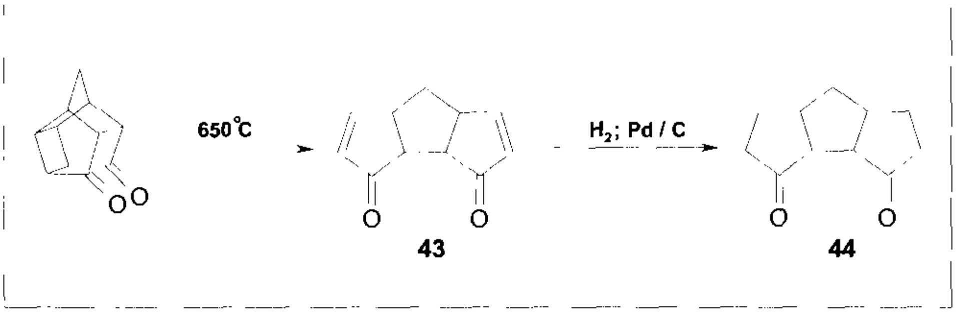


Figure 3.11. Apparatus for Flash Vacuum Pyrolysis (FVP) (McNab, 2004).

Liebenberg (1989) was the first to perform a synthesis of the triquinanes that was accompanied by a variety of biological tests on them. The activity of a series of these compounds were determined in order to compare it to the polycyclic amines on which Van der Schyf *et al.* (1989) worked in previous studies. By making use of pyrolysis reactions, as performed by Mehta *et al.* (1981), access to the triquinane system was achieved, with the polycyclic cage diketone as starting material. The catalytic hydrogenation of the triquinanediene **(43)** led to the formation of the required saturated triquinane diketone **(44)**. This compound served as basic structure for the synthesis of all the aminated triquinane compounds in this particular study (Liebenberg, 1989).



Scheme 3.2. Basic scheme for the synthesis of the triquinanes from the polycyclic diketone (Mehta *et al.*, 1981).

3.3 Concluding Remarks

There is thus clearly a variety of mechanisms through which the pentacycloundecyl compounds can play a role in conditions like Parkinson's disease. From various studies on neuroprotection in the past, it is evident that the NMDA receptor ion channels are one of the main drug targets for the prevention of such conditions. In particular, non- and uncompetitive NMDA receptor antagonists show promise as neuroprotective agents.

The following factors have been shown to influence the affinity of the non-competitive NMDA receptor antagonists for their receptor:

- the presence of substituents to form two hydrophobic interactions with the receptor (lipohilic groups);
- the presence of a protonated amine to form a hydrogen bond;
- the presence of steric interactions in certain areas of the ligand molecule;
- bulkiness of compound;

- the size of lipophilic moieties in lipophilic ligands and
- the position of the substituents in the ligand molecule.

By making use of flash vacuum pyrolysis and amination reactions, access can be gained to a polyquinane structure that theoretically meet the basic requirements for activity on the PCP binding site of the NMDA receptor – *i.e.* the triquinylamines. With manipulation of the functional groups attached to the triquinanes, structure-activity relationships can be drawn up for these compounds' activity on the phencyclidine binding site. The synthesis and biological evaluation of similar pentacyclic cage compounds can be used to draw a comparison between these two types of polycyclic compounds.

CHAPTER 4

EXPERIMENTAL PROCEDURES - SYNTHESES

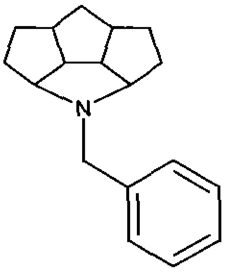
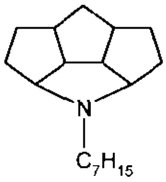
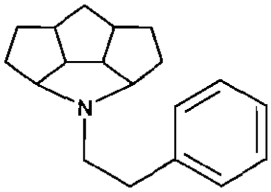
4.1 Selection of Relevant Compounds for Synthesis

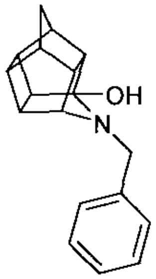
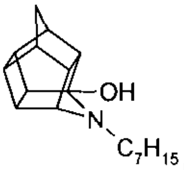
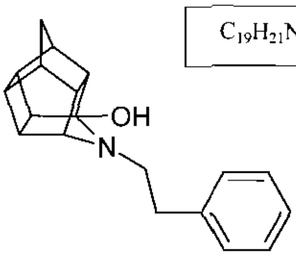
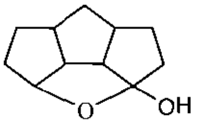
After the discovery of the calcium channel blocking capacity of NGP1-01, a postulation was made that the pentacycloundecane skeleton served only as a bulk contribution to its biological activity (Liebenberg *et al.*, 1996 and Malan *et al.*, 1998). To investigate this hypothesis, Liebenberg *et al.* (1996) synthesised the thermal ring-opened triquinanes. The biological results that were obtained, suggested that ring opening of the cage moiety of NGP1-01 did not at all diminish its calcium channel activity or chronotropy effects (Liebenberg *et al.*, 1996).

To expand on this speculation, a series of triquinylamines and similar pentacycloundecyl compounds were selected for synthesis and evaluation. Since previous comparison between the triquinanes and the cage-like compounds were conducted with the oxa form of the cage compounds, three aza-bridgehead structures were selected for comparison in this study. The selection of the relevant side chains was based on the findings of earlier studies into the structure-activity relationships of a pharmacophore on both the sigma receptors (Largent *et al.*, 1987) and the phencyclidine binding site of the NMDA receptor (Kroemer *et al.*, 1998). The substituents were selected specifically to evaluate the effect of the chain length between the two lipophilic moieties, the effect of steric interactions in certain areas of the ligand molecule, the effect of bulkiness and the effect of the size of lipophilic ligands, as described by Nguyen *et al.* (1996), Kroemer *et al.* (1998) and Jirgensons *et al.* (2000) respectively.

The benzylamino substituent of both the triquinylamine **(I)** and the aza-cage **(IV)** structures was selected to allow the comparison with the lead compound, NGP1-01. The side chain with the same amount of carbon atoms as the benzylamino substituent, namely the heptylamino side chain **(II; V)**, was selected to determine the effect of chain length on calcium blocking activity. This would thus allow the evaluation of the importance of a cyclic substituent in neuronal activity, in contrast with aliphatic substitution. In order to determine the effect of an increase in chain length between the polycyclic moiety and the aromatic ring, the phenethylamine side chain **(III; VI)** was synthesised to be compared to the compounds with the benzylamine side chain as substituent. The triquinylamine and cage compounds with this particular substituent would give an indication of the available space or volume for interaction within the receptor binding sites. It would also give an indication of the effect of increased flexibility of the aromatic moiety on the calcium blocking capacity of the molecule. In addition to the role that the different side chains might play in the binding to the calcium channel and NMDA receptor ion channel, the hydroxyl group on the cage compounds might also serve as an extra site for hydrogen bonding with the molecule.

Table 4.1. Compounds selected for synthesis and evaluation.

	COMPOUND	NOMENCLATURE
I	 $C_{18}H_{23}N$	***N*-Benzyl-3,11-azatricyclo[6.3.0.0^{2,6}]undecane**
II	 $C_{18}H_{31}N$	***N*-Heptyl-3,11-azatricyclo[6.3.0^{2,6}]undecane**
III	 $C_{19}H_{25}N$	***N*-(Phenylethyl)-3,11-azatricyclo[6.3.0^{2,6}]undecane**

IV	 $C_{18}H_{19}NO$	***N*-(4-Benzyl)-3-hydroxy-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}0^{5,9}.0^{8,11}]dodecane**
V	 $C_{18}H_{27}NO$	***N*-(4-Heptyl)-3-hydroxy-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}0^{5,9}.0^{8,11}]dodecane**
VI	$C_{19}H_{21}NO$ 	***N*-(4-Phenethyl)-3-hydroxy-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}0^{5,9}.0^{8,11}]dodecane**
VII	$C_{11}H_{16}O_2$ 	**3-Hydroxy-3,11-oxatricyclo[6.3.0.0^{2,6}]undecane**

Finally, in order to establish the necessity of both a nitrogen atom and substitution thereof for the neuronal activity of the triquinane structure, the hemiacetal derivative **(VII)** of the triquinanes was synthesised. This was achieved by the direct reduction of the triquinane diketone with sodium borohydride, followed by purification by means of open column chromatography.

4.2 Standard Experimental Procedures

4.2.1 Reagents and Chemicals

Reagents were obtained from Sigma-Aldrich (U.K. & U.S.A.) and Merck Chemicals (Germany). Reaction and elution solvents were purchased from various commercial sources in South Africa.

4.2.2 Instrumental Methods

Melting Point (MP) Determination: Melting Points were measured with Gallenkamp and Stuart SMP10 melting point apparatuses.

Infrared (IR) Absorption Spectrophotometry: Infrared spectra were recorded on a Nicolet 470 FT-IR spectrophotometer, using KBr disks. Oily products were applied on the disks as a thin film.

Mass Spectrometry (MS): Mass spectra were recorded on an analytical VG 7070E mass spectrometer. Ionisation was induced by means of electron impact at 70 eV.

Nuclear Magnetic Resonance (NMR) Spectrometry: ^{1}H, ^{13}C and Distortionless Enhancement by Polarisation Transfer (DEPT) NMR spectra were acquired on a Varian Gemini 300 spectrometer, with the ^{1}H spectra recorded at a frequency of 300,075 MHz and the ^{13}C spectra at 75,462 MHz. Tetramethylsilane (TMS) was used as internal standard, with $CDCl_3$ as solvent. All chemical shifts are reported in parts per million (ppm), relative to the internal standard. The following abbreviations are used to indicate the multiplicities of the respective signals: s, singulet; br s, broad singulet; d, doublet; t, triplet; m, multiplet.

4.2.3 Chromatographic Methods

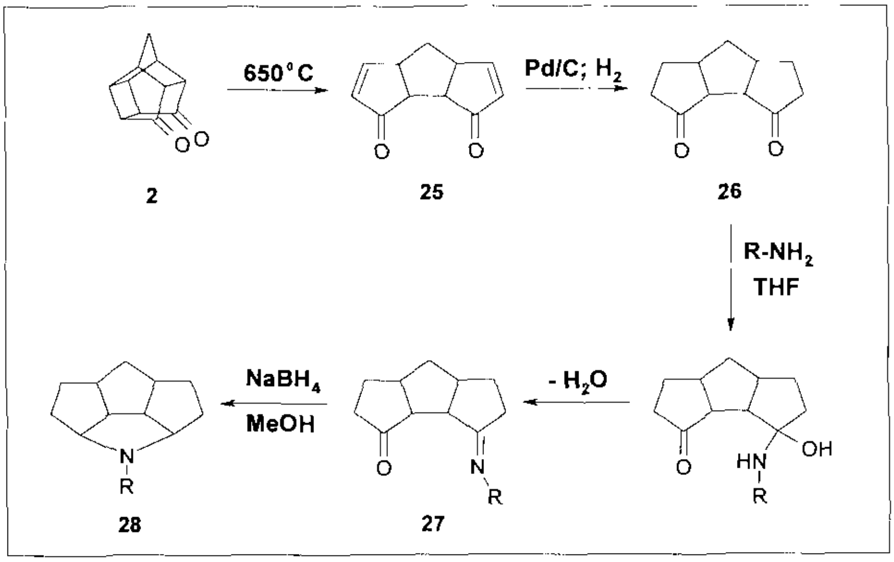
The mobile phases used for column and thin layer chromatography (TLC) were prepared on a volume-to-volume (v/v) basis, employing the Prism model that was introduced by Nyiredy *et al.* (1985).

Thin Layer Chromatography (TLC): Thin layer chromatographic methods were used to monitor the progress of all chemical reactions. Visualisation of thin layer chromatography was achieved by making use of UV light (254 nm), iodine vapours and heating the TLC plates after the application of Ninhydrin or a 10% H_2SO_4 solution.

Column Chromatography: Separation and purification of mixtures were done either by flash chromatography (Harwood *et al.*, 1999) with nitrogen gas on glass columns, packed with silica gel (0.063–0.200 mm), or by utilising the Versa Flash station with VersaPak columns (silica cartridge 40 x 75 mm).

4.3 Synthesis

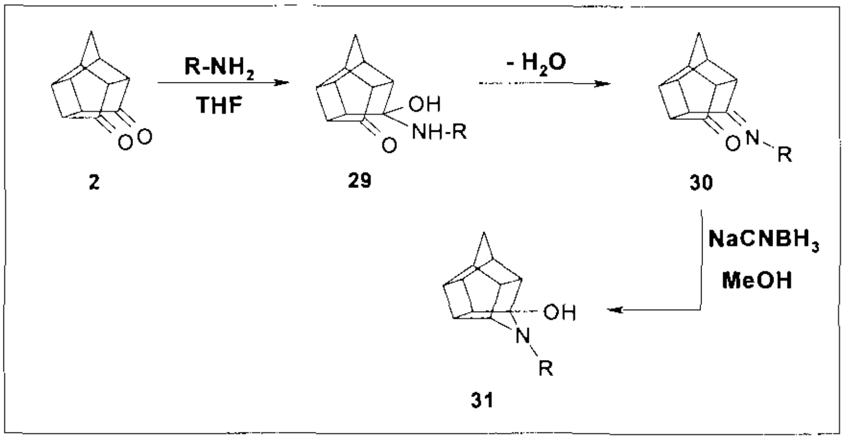
The diketone, pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione **(2)**, was used as starting material for the synthesis of both the triquinanes and the pentacycloundecylamines. The basic method for the synthesis of the caged dione, as described by Cookson *et al.* (1958), was utilised to obtain this starting material.



Scheme 4.1. Basic scheme for the synthesis of the triquinylamines (Liebenberg, 1986).

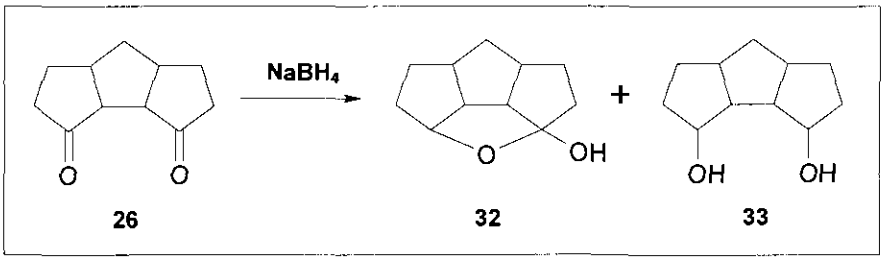
The pyrolysis of the cage diketone was performed to obtain the *cis, syn, cis* triquinane system (chapter 3). Due to the photolability of the required tricyclo[6.3.0.0^{2,6}]undecane-4,9-diene-3,11-dione **(25)**, hydrogenation of this compound followed immediately after its purification by recrystallisation from cyclohexane. This hydrogenation took place at atmospheric pressure, in the presence of 10% Pd/C as catalyst. In order to obtain the required triquinylamines, amination was performed with the respective primary amines to afford the corresponding Schiff bases **(27)**. Reduction of these compounds in the presence of sodium borohydride ultimately resulted in the formation of the required triquinylamines **(28)**.

Direct amination of the pentacyclic diketone resulted in the formation of the carbinol amine **(29)** which, upon dehydration by Dean-Stark, yielded the caged Schiff base **(30)**. The aza-cage compounds **(31)** were formed by means of reduction of the Schiff base with sodium cyanoborohydride.



Scheme 4.2. Basic scheme for the synthesis of the aza-pentacycloundecylamines (Marchand *et al.*, 1988).

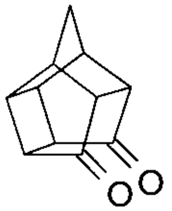
In addition to the aminated polycyclic compounds, the hemiacetal / lactol derivative was synthesised. Reduction of the diketone **(26)** resulted in the formation of a mixture of the lactol **(32)** and diol **(33)** derivatives.



Scheme 4.3. $NaBH_4$ reduction of tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (Mehta *et al.*, 1981).

4.3.1 Synthetic procedures

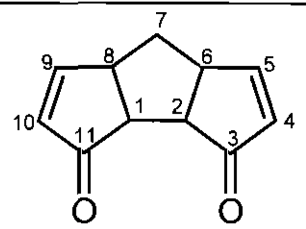
4.3.1.1 Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (2)



$C_{11}H_{10}O_2$

A stoichiometric volume of freshly monomerised cyclopentadiene (12,45 ml; 0,0925 mol) was added to a solution of *p*-benzoquinone (10 g; 0,0925 mol) in 100 ml dry benzene. In order to prevent the formation of the undesired Diels Alder di-adduct, the reaction mixture was maintained at a low temperature of ± 5 °C, while the cyclopentadiene was added slowly. While protected from light, the mixture was stirred for 1 hour to ensure the completion of the reaction, as was indicated by TLC. This was followed by the addition of three (3) spatula of activated charcoal and the reaction mixture was magnetically stirred for a further 30–60 min. After filtration through Celite®, the solvent was removed *in vacuo* and the formed syrupy yellow oil was allowed to remain overnight in a dark fume cupboard. This allowed the full evaporation of all benzene, affording the Diels Alder adduct as yellow crystals. The Diels Alder adduct was dissolved in ethyl acetate (± 4 g/100 ml) and irradiated for approximately 6 hours in a photochemical reactor (1000 W medium pressure UV lamp; Phillips HPA 1000/20). Decolouration of the solution confirmed completion of the cyclisation of the adduct. The solvent was subsequently removed *in vacuo* to afford the impure pentacyclic dione as a light yellow residue. This residue was purified by Soxhlett extraction in cyclohexane to produce the pure diketone as a waxy, off-white precipitate (Yield: 10,994 g; 0,063 mol; 68,2%). Since the data from the physical characterisation of the obtained compound correlates with that of the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (174,2 g/mol) as described by Cookson *et al.* (1958), no spectral data for this compound are presented in this dissertation.

4.3.1.2 Tricyclo[6.3.0.0^{2,6}]undecane-4,9-diene-3,11-dione (25)



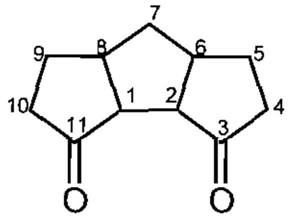
$C_{11}H_{10}O_2$

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (0,7 g; 0,004 mol) was dissolved in a minimal amount of dichloromethane and was spread over the inner surface of a tube-shaped vessel by means of evaporation with a heat gun at a temperature of ± 90 °C. A copper tube, heated up to 150 °C and isolated with glass wool to retain the temperature, was fitted around this sublimation vessel after it was attached to the rest of the pyrolysis apparatus. The sublimation vessel was directly attached to the vigruex quartz column inside the oven furnace (650 °C) by interspersing Teflon joint tape. The high-capacity, rotary vane oil pump that was attached to the system was switched on and gaseous nitrogen was flushed through the system. Upon closure of the nitrogen inlet, a vacuum of ±1 torr was obtained. This evacuation not only led to the sublimation of the caged diketone at the temperature of 150 °C, but also ensured the rapid migration of the compound through the vigruex column, where the actual pyrolysis took place. A typical throughput rate of 0,7 g of substrate every 15 minutes was maintained. The pyrolised form of the caged diketone was collected as a dark brown tar in a liquid nitrogen trap at ± -78 °C. The condensate from the nitrogen trap was purified by recrystallisation from cyclohexane, to afford the unsaturated triquinane as off-white crystals (Yield: 2,5 g; 0,024 mol for every 4,2 g caged diketone pyrolysed; 59,64%).

PHYSICAL DATA:

$C_{11}H_{10}O_2$; mp. 108 °C; **IR (KBr)** ν_{max} (Spectrum 1.1): 2930, 1725, 1347 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 2.1): 174 (M$^+$), 146, 131, 117, 91, 66, 55, 39, 27; ^{1}**H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 3.1): 7,51–7,32 (m, 2 H, H_5, H_9); 5,98–5,74 (d, 2H, H_4, H_{10}); 3,62–3,37 (m; 2H, H_6, H_8); 3,29–3,07 (m, 2H, H_1, H_2); 2,42–2,13 (m, 1H, H_{7b}); 2,00–1,69 (m, 1H, H_{7a}); ^{13}**C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 4.1): 207,355 (s, 2C, C_3, C_{11}); 166.032 (d, 2C, C_4, C_{10}); 133,201 (d, 2C, C_5, C_9); 52,970 (d, 2C, C_1, C_2); 50,235 (d, 2C, C_6, C_8); 31,231 (t, 1C, C_7).

4.3.1.3 Tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (26)



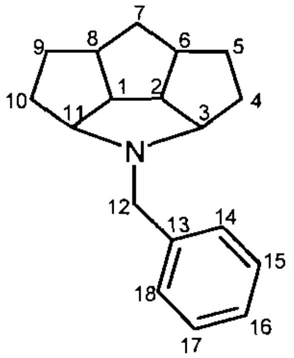
$C_{11}H_{14}O_2$

Tricyclo[6.3.0.0^{2,6}]undecane-4,9-diene-3,11-dione (5,414 g; 0,0088 mol) was dissolved in 200 ml dry ethyl acetate. The solution was added to 10% Pd/C (150 mg) in a hydrogenation flask. Reduction was performed at a pressure of 2 atm (H_2) for 30 min. After filtration of the catalyst with Celite®, the solvent was removed *in vacuo* and the product was recrystallised from cyclohexane. The obtained tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione appeared as white needle-like crystals (1,342 g; 0,0075 mol; 85,14%).

PHYSICAL DATA:

$C_{11}H_{14}O_2$; mp. 91 °C; **IR (KBr)** ν_{max} (Spectrum 1.2): 2956, 1738, 1402 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 2.2): 178 (M^+), 122, 96, 80, 66, 55, 39, 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 3.2): 2,9–2,7 (t, 2H, H_1, H_2); 2,5–2,3 (m, 1 H, H_{7a}); 2,3–2,1 (m, 4H, H_{4a}, H_{5a}, H_{9a}, H_{10a}); 2,1–1,9 (m, 2H, H_{4b}, H_{10b}); 1,7–1,5 (m, 1H, H_{7b}); 1,3–1,1 (m, 2H, H_{5b}, H_{9b}); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 4.2): 218,648 (s, 2C, C_3, C_{11}); 56,010 (d, 2C, C_1, C_2); 43,473 (t, 1C, C_7); 38,564 (t, 2C, C_4, C_{10}); 37,058 (d, 2C, C_6, C_8); 26,046 (t, 2C, C_5, C_9).

4.3.1.4 N-Benzyl-3,11-azatricyclo[6.3.0.0^{2,6}]undecane (I)



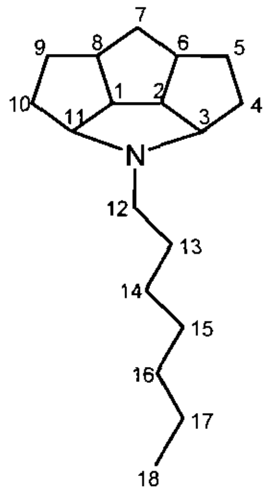
$C_{18}H_{23}N$

Tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (1,068 g; 0,006 mol) was dissolved in 50 ml dry tetrahydrofuran (THF) and stirred on an external ice bath. An equimolar amount of benzylamine (0,643 g; 0,006 mol) was added to the solution in a dropwise manner. The reaction was allowed to stir for 1 hour at room temperature, during which its progress was constantly monitored on TLC plates. Although the TLC did not indicate the completion of the reaction after 1 hour, the reaction was terminated, in fear of the formation of unwanted by-products. The THF was removed *in vacuo* and the obtained yellow oil was dissolved in ice cold methanol. The addition of $NaBH_4$ (0,272 g; 0,007 mol) initiated the reduction of the Schiff base that formed spontaneously from the carbinol amine in the previous step. The reaction mixture was allowed to stir at room temperature for 1 hour and the methanol was removed *in vacuo*. Distilled H_2O was added to the obtained viscous oil to inactivate any unreacted $NaBH_4$. Dichloromethane (DCM) (5 x 20 ml) was used for the extraction of the product. The combined DCM fractions were washed with distilled H_2O (2 x 50 ml) and the organic phase was dried with anhydrous $MgSO_4$. The subsequent *in vacuo* removal of the DCM resulted in the formation of a light orange oil. The oil was purified by means of a silica Versaflash column with the mobile phase combination of petroleum ether: ethyl acetate: ethanol in a 5:4:1 ratio. The triquinylamine was obtained as a light-yellow oil (0,225 g; 0,00009 mol; 14,81%).

PHYSICAL DATA:

$C_{18}H_{23}N$; **IR (KBr)** ν_{max} (Spectrum 3.1): 2924, 1460, 1261 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 3.2): 253 (M^+), 224, 170, 162, 91, 65, 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 3.3): 7,4–6,9 (m, 5H, H_{14}, H_{15}, H_{16}, H_{17}, H_{18}); 3,7–3,6 (s, 2H, H_{12a}, H_{12b}); 3,3–3,1 (m, 2H, H_3, H_{11}); 2,9–2,7 (m, 1H, H_{7a}); 2,6–2,4 (m, 1H, H_{7b}); 2,1–1,8 (m, 2H, H_{10a}, H_{4a}); 1,8–1,6 (m, 2H, H_{9a}, H_{5a}); 1,6–1,45 (m, 2H, H_1, H_2); 1,5–1,4 (m, 4H, H_{4b}, H_6, H_8, H_{10b}); 1,4–1,1 (m, 2H, H_{5b}, H_{9b}); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 3.4): 140,991 (s, 1C, C_{13}); 128,757 (d, 2C, C_{14}, C_{18}); 127,863 (d, 2C, C_{15}, C_{17}); 126,447 (d, 1C, C_{16}); 73,578 (d, 1C, C_3); 73,539 (d, 1C, C_{11}); 58,532 (t, 1C, C_{12}); 54,495 (d, 2C, C_1, C_2); 47,349 (d, 2C, C_6, C_8); 37,502 (t, 1C, C_7); 32,794 (t, 2C, C_5, C_9); 32,477 (t, 2C, C_4, C_{10}).

4.3.1.5 **N-Heptyl-3,11-azatricyclo[6.3.0.0^{2,6}]undecane (II)**



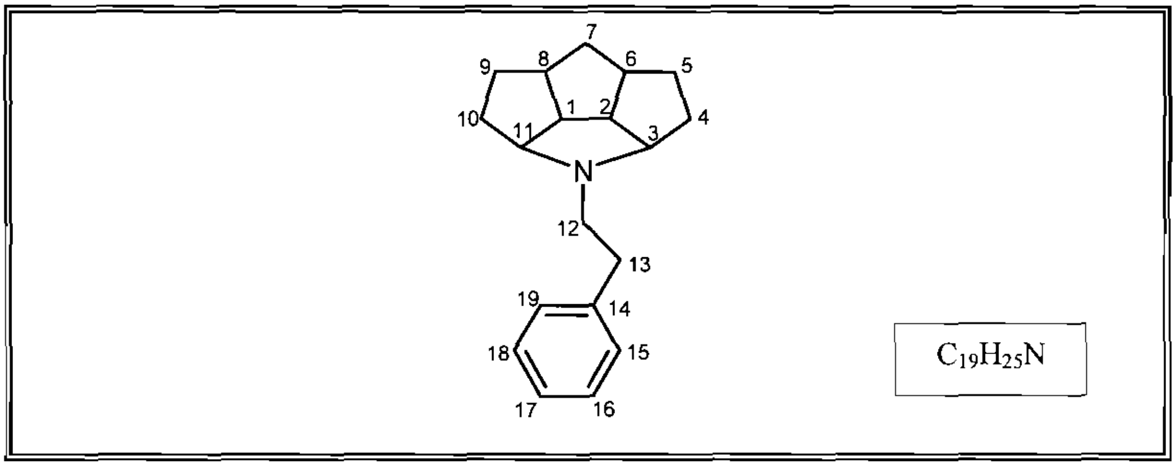
$C_{18}H_{31}N$

Tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (1,094 g; 0,006 mol) was dissolved in 50 ml dry tetrahydrofuran (THF) and stirred on an external ice bath. An equimolar amount of the heptylamine (0,71 g; 0,006 mol) was added to the solution in a dropwise manner. The reaction was allowed to stir for 1 hour at room temperature, during which its progress was constantly monitored by TLC. Although the TLC did not indicate the completion of the reaction after 1 hour, the reaction was terminated, in fear of the formation of unwanted by-products. The THF was removed *in vacuo* and the obtained yellow oil was dissolved in ice cold methanol. The addition of $NaBH_4$ (0,272 g; 0,007 mol) initiated the reduction of the Schiff base that formed spontaneously from the carbinol amine in the previous step. The reaction mixture was allowed to stir at room temperature for 1 hour and the methanol was removed *in vacuo*. Distilled H_2O was added to the obtained viscous oil to inactivate any unreacted $NaBH_4$. DCM (5 x 20 ml) was used for the extraction of the product. The combined DCM fractions were washed with distilled H_2O (2 x 50 ml) and the organic phase was dried with anhydrous $MgSO_4$. The subsequent *in vacuo* removal of the DCM resulted in the formation of a yellowish oil. The obtained oil was purified by means of a flash column, with petroleum ether and ethyl acetate as mobile phase, combined in a 4:1 ratio. This was followed by a silica Versaflash column of several of the obtained fractions from the previous column in the same mobile phase. The pure triquinylamine was obtained as a light-yellow oil (0,291 g; 0,00111 mol; 18,12%).

PHYSICAL DATA:

$C_{11}H_{10}O_2$; IR (KBr) ν_{max} (Spectrum 4.1): 2929, 1458, 1352, 1158 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 4.2): 261 (M^+); 176; 91; 69; 55; 43; 29; ^{1}**H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 4.3): 3,1–3,0 (t, 2H, H_{12a}, H_{12b}); 2,85–2,65 (m, 2H, H_3, H_{11}); 2,55–2,35 (t, 1H, H_{7a}); 1,9–1,8 (t, 1H, H_{7b}); 1,8–1,75 (m, 2H, H_{4a}, H_{10a}); 1,75–1,65 (2H, H_{5a}, H_{9a}); 1,65–1,50 (t, 2H, H_1, H_2); 1,50–1,45 (m, 4H, H_{4b}, H_6, H_8, H_{10b}); 1,4–1,3 (6H, H_{5b}, H_{9b}, H_{13a}, H_{13b}, H_{17a}, H_{17b}); 1,3–1,2 (6H, H_{14a}, H_{14b}, H_{15a}, H_{15b}, H_{16a}, H_{16b}); 0,95–0,8 (t, 3H, H_{18a}, H_{18b}, H_{18c}); ^{13}**C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 4.4): 73,554 (d, 2C, C_3, C_{11}); 54,955 (t, 1C, C_{12}); 54,713 (d, 2C, C_1, C_2); 47,381 (d, 2C, C_6, C_8); 37,522 (t, 1C, C_7); 35,220 (t, 1C, C_{16}); 32,735 (t, 2C, C_5, C_9); 31,963 (t, 2C, C_4, C_{10}); 29,314 (t, 1C, C_{15}); 28,589 (t, 1C, C_{13}); 27,756 (t, 1C, C_{14}); 22,673 (t, 1C, C_{17}); 14,092 (q, 1C, C_{18}).

4.3.1.6 N-Phenylethyl-3,11-azatricyclo[6.3.0.0^{2,6}]undecane (III)



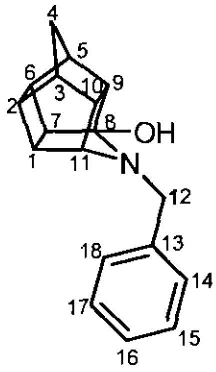
$C_{19}H_{25}N$

Tricyclo[6.3.0.0^{2,6}]undecane-3,11-dione (0,179 g; 0,001 mol) was dissolved in 20 ml dry tetrahydrofuran (THF) and stirred on an external ice bath. An equimolar amount of the phenethylamine (0,12 g; 0,001 mol) was added to the solution in a dropwise manner. The reaction was allowed to stir for 1 hour at room temperature, during which its progress was constantly monitored by TLC. Although the TLC did not indicate the completion of the reaction after 1 hour, the reaction was terminated, in fear of the formation of unwanted by-products. The THF was removed *in vacuo* and the obtained yellow oil was dissolved in ice cold methanol. The addition of $NaBH_4$ (0,048 g; 0,001 mol) initiated the reduction of the Schiff base that formed spontaneously from the carbinol amine in the previous step. The reaction mixture was allowed to stir at room temperature for 1 hour and the methanol was removed *in vacuo*. Distilled H_2O was added to the obtained viscous oil to inactivate any unreacted $NaBH_4$. Dichloromethane (DCM) (5 x 20 ml) was used for the extraction of the product. The combined DCM fractions were washed with distilled H_2O (2 x 50 ml) and the organic phase was dried with anhydrous $MgSO_4$. The subsequent *in vacuo* removal of the DCM resulted in the formation of a yellowish oil. The obtained oil was purified by means of a silica Versaflash column with the mobile phase combination of petroleum ether: ethyl acetate: ethanol in a 5:4:1 ratio. The triquinylamine was obtained as a light yellow oil (0,072 g; 0,00027 mol; 26,79%).

PHYSICAL DATA:

$C_{11}H_{10}O_2$; **IR (KBr)** ν_{max} (Spectrum 5.1): 2949, 1453 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 5.2): 267 (M^+); 190; 176; 162; 151; 91; 67; 27;**^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 5.3): 7,3–7,1 (m, 5H, H_{15}, H_{16}, H_{17}, H_{18}, H_{19}); 3,3–3,1 (t, 4H, H_{12a}, H_{12b}); H_{13a}, H_{13b}); 2,9–2,7 (m, 2H, H_3, H_{11}); 2,6–2,4 (t, 2H, H_{13a}, H_{13b}); 2,3–2,2 (m, 2H, H_6, H_8); 2,0–1,4 (m, 8H, H_{4a}, H_{4b}, H_{5a}, H_{5b}, H_{9a}, H_{9b}, H_{10a}, H_{10b}); 1,5–1,2 (m, 4H, H_1, H_2, H_{7a}, H_{7b}); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 5.4): 140,600 (s, 1C, C_{14}); 128,688 (d, 2C, C_{18}, C_{18}); 128,196 (d, 2C, C_{15}, C_{19}); 125,660 (d, 1C, C_{17}); 72,963 (d, 2C, C_3, C_{11}); 55,000 (t, 1C, C_{12}); 54,742 (d, 2C, C_1, C_2); 47,358 (d, 2C, C_6, C_8); 34,942 (t, 1C, C_{13}); 34,677 (t, 1C, C_7); 32,677 (t, 2C, C_5, C_9); 32,663 (t, 2C, C_4, C_{10}).

4.3.1.7 8-Hydroxy-N-benzylamino-8,11-azapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (IV)



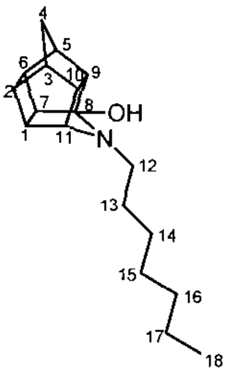
$C_{18}H_{19}NO$

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5 g; 0,029 mol) was dissolved in 50 ml dry THF and cooled on an external ice bath. Benzylamine (3,08 g; 0,029 mol) was added in a dropwise manner, while magnetically stirring the solution. The carbinol amine formed as a white precipitate, which was separated from the solution by filtration. Water was removed by means of Dean-Stark distillation, with dry benzene (100 ml) as solvent. The mixture was concentrated *in vacuo* to afford the Schiff base as a yellow oil, which was dissolved in a mixture of acetic acid (15 ml) and methanol (250 ml). The reducing agent, $NaCNBH_3$ (2 g; 0,032 mol), was slowly added over a period of 5 minutes. The reaction mixture was allowed to stir overnight at room temperature. The solvent was removed *in vacuo* and H_2O (100 ml) was added. While stirring, solid $NaHCO_3$ was added portion-wise until the evolution of carbon dioxide ceased. Excess $NaHCO_3$ (2 g) was added and the mixture was extracted with DCM (4 x 50 ml). The combined organic phase was washed with H_2O (2 x 100 ml), dried with anhydrous $MgSO_4$ and concentrated *in vacuo*. Recrystallisation from benzene yielded the aza-compound as white crystals (0,882 g; 0,003 mol; 11,58%).

PHYSICAL DATA:

$C_{18}H_{19}NO$; mp. 159 °C; **IR (KBr)** ν_{max} (Spectrum 6.1): 3130, 2834, 1454, 1322, 1275, 921, 729 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 6.2): 265 (M^+); 173; 131; 91; 77; 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 6.3): 7,7–7,0 (m, 5H, H_{14}, H_{15}, H_{16}, H_{17}, H_{18}); 5,1–4,3 (br s, 1H, OH); 4,0–3,9 (s, 1H, H_{12a}); 3,9–3,8 (s, 1H, H_{12b}); 3,5–3,2 (m, 1H, H_{11}); 2,9–2,2 (m, 8H, H_1, H_2, H_3, H_5, H_6, H_7, H_9, H_{10}); 1,9–1,3 (ABq, J_{AB} = 10,5 Hz, 2H, H_{4a}, H_{4b}); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 6.4): 138,870 (s, 1C, C_{13}); 128,608 (d, 2C, C_{14}, C_{18}); 128,359 (d, 1C, C_{15}, C_{17}); 128,261 (d, 1C, C_{16}); 126,886 (s, 1C, C_8); 64,776 (d, 1C, C_{11}); 54,950 (d, 2C, C_9, C_{10}); 51,629 (t, 1C, C_{12}); 50,721 (d, 1C, C_5); 45,581 (d, 1C, C_3); 44,599 (d, 1C, C_6); 42, 991 (d, 1C, C_2); 42,200 (d, 1C, C_7); 41,686 (d, 1C, C_1); 41,308 (t, 1C; C_4).

4.3.1.8 8-Hydroxy-N-heptylamino-8,11-azapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (V)



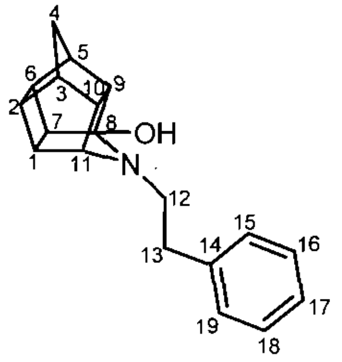
$C_{18}H_{27}NO$

Pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (3 g; 0,017 mol) was dissolved in 30 ml dry THF and cooled on an external ice bath. The heptylamine (1,98 g; 0,017 mol) was dissolved in 10 ml dry THF and was added dropwise, while magnetically stirring the solution. The carbinol amine formed as a white precipitate and was separated from the solution by filtration. Water was removed by means of Dean-Stark distillation with dry benzene (100 ml) as solvent. The mixture was concentrated *in vacuo* to afford the Schiff base as an orange oil. This was dissolved in a mixture of acetic acid (15 ml) and methanol (250 ml). The reducing agent, $NaCNBH_3$ (1,19 g; 0,019 mol), was slowly added over a period of 5 minutes. The reaction mixture was allowed to stir for 2 hours at room temperature. The solvent was removed *in vacuo* and H_2O (100 ml) was added. While stirring, solid $NaHCO_3$ was added portion-wise until the evolution of carbon dioxide ceased. Excess $NaHCO_3$ (2 g) was added and the mixture was extracted with DCM (4 x 50 ml). The combined organic phase was washed with H_2O (2 x 100 ml), dried with anhydrous $MgSO_4$ and was concentrated *in vacuo*. The compound was purified by means of a silica gel flash column, with the mobile phase petroleum ether: ethyl acetate: ethanol in a 5:4:1 ratio. The final product was obtained as white crystals (2,031 g; 0,007 mol; 43,13%).

PHYSICAL DATA:

$C_{18}H_{27}NO$, **mp.** 100 °C; **IR (KBr)** ν_{max} (Spectrum 7.1): 1333, 1296, 1213, 1149 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 7.2): 271 (M^+); 256; 242; 228; 213; 200; 156; 129; 91; 77; 29; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 7.3): 6,3–5,7 (br s, 1H, OH); 3,7–3,4 (m, 1H, H_{11}), 3,0–2,8 (t, 1H, H_{12a}); 2,8–2,7 (t, 1H, H_{12b}); 2,7–2,1 (m, 8H, H_1, H_2, H_3, H_5, H_6, H_7, H_9, H_{10}); 1,6–0,8 (ABq, J_{AB} = 10,5 Hz, 2H, H_{4a}, H_{4b}); 1,9–1,7 (m, 1H, H_{13}); 1,6–1,1 (6H, H_{14a}, H_{14b}, H_{15a}, H_{15b}, H_{16a}, H_{16b}); 0,7–1,0 (t, 3H, H_{18a}, H_{18b}, H_{18c}); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 7.4): 179,859 (s, 1C, C_8); 64,689 (d, 1C, C_{11}); 54,562 (d, 2C, C_9, C_{10}); 50,506 (t, 1C, C_{12}); 46,594 (d, 1C, C_5); 45,754 (d, 1C, C_3); 44,435 (d, 1C, C_6); 43,259 (d, 1C, C_2); 41,907 (d, 1C, C_7); 41,642 (d, 1C, C_1); 41,556 (t, 1C, C_4); 31,741 (t, 1C, C_{16}); 29,064 (t, 1C, C_{15}); 27,757 (t, 1C, C_{13}); 27,587 (t, 1C, C_{14}); 22,556 (t, 1C, C_{17}); 14,010 (q, 1C, C_{18}).

4.3.1.9 8-Hydroxy-N-phenethylamino-8,11-azapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (VI)



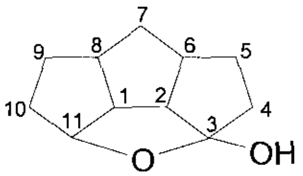
$C_{19}H_{27}NO$

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (4,065 g; 0,023 mol) was dissolved in 50 ml dry THF and cooled on an external ice bath. The phenethylamine (2,83 g; 0,023 mol) was dissolved in 10 ml dry THF and was added dropwise, while the solution was magnetically stirred. The carbinol amine formed as a white precipitate and was separated from the solution by filtration. Water was removed by means of Dean-Stark distillation, with dry benzene (100 ml) as solvent. The mixture was concentrated *in vacuo* to afford the Schiff base as an orange oil, which was then dissolved in a mixture of acetic acid (15 ml) and methanol (250 ml). The reducing agent, $NaCNBH_3$ (1,19 g; 0,019 mol), was slowly added over a period of 5 minutes. The reaction mixture was allowed to stir for 2 hours at room temperature. The solvent was removed *in vacuo* and H_2O (100 ml) was added. While stirring, solid $NaHCO_3$ was added portion-wise until the evolution of carbon dioxide ceased. Excess $NaHCO_3$ (2 g) was added and the mixture was extracted with DCM (4 x 50 ml). The combined organic phase was washed with H_2O (2 x 100 ml), dried with anhydrous $MgSO_4$ and concentrated *in vacuo.* The compound was purified by means of a flash column with silica gel and the mobile phase petroleum ether: ethyl acetate: dichloromethane: ethanol in a 1:1:1:2 ratio was used. The final product was obtained as white crystals (2,064 g; 0,007 mol; 32,13%).

PHYSICAL DATA:

$C_{19}H_{27}NO$; **mp.** 150 °C; **IR (KBr)** ν_{max} (Spectrum 8.1): 2954, 2880, 1453, 1325, 1275, 920 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 8.2): 279 (M^+); 246; 188; 131; 91; 77; 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 8.3) 7,4–7,1 (m, 5H, H_{15}, H_{16}, H_{17}, H_{18}, H_{19}); 5,9–4,6 (br s, 1H, OH); 3,7–3,4 (m, 1H, H_{11}); 3,0–2,7 (t, 4H, H_{12a}, H_{12b}, H_{13a}, H_{13b}); 2,7–2,3 (8H, H_1, H_2, H_3, H_5, H_6, H_7, H_9, H_{10}); 1,9–1,4 (ABq, J_{AB} = 10,5 Hz, 2H, H_{4a}, H_{4b}); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 8.4): 140,336 (d, 1C, C_{14}); 128,551 (d, 2C, C_{15}, C_{19}); 128,384 (d, 2C, C_{16}, C_{18}); 128,288 (d, 1C, C_{18}); 126,031 (s, 1C, C_8); 65,121 (d, 1C, C_{11}); 55,070 (d, 2C, C_9, C_{10}); 50,760 (t, 1C, C_{12}); 48,981 (t, 1C, C_{13}); 45,555 (d, 1C, C_5); 44,730 (d, 1C, C_3); 43,100 (d, 1C, C_6); 42,356 (d, 1C, C_2); 41,663 (d, 1C, C_7); 41,593 (d, 1C, C_1); 41,518 (t, 1C, C_4).

4.3.1.10 **3-Hydroxy-3,11-oxatricyclo[6.3.0.$0^{2,6}$]undecane (VII)**



$C_{11}H_{16}O_2$

The tricyclo-undecane-3,11-dione **(26)** (0,5 g; 0,0028 mol) was dissolved in 50 ml methanol on an external ice bath. $NaBH_4$ (0,425 g; 0,0112 mol) was slowly added, where after the cooling bath was removed. The reaction proceeded at room temperature for 1 hour. The methanol was removed *in vacuo* and the remaining contents of the flask were diluted with H_2O (20 ml). The products of the reaction were extracted with DCM (2 x 20 ml). Evaporation of the solvent furnished 0,475 g of the crude material, which was purified on a silica gel column with ethyl acetate: benzene (3:17) as eluent. The hemiacetal was obtained as white crystals (0,081 g; 0,0005 mol; 16,1%).

PHYSICAL DATA:

$C_{11}H_{16}O_2$; mp. 95 °C; **IR (KBr)** ν_{max} (Spectrum 9.1): 3382, 1463, 1013 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 9.2): 180 (M^+); 163; 120; 96; 91; 79; 67; 53; 41; 27; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 9.3): 4,9–4,7 (m, 1H, H_{11}); 3,6–3,1 (br s, 1H, OH); 3,1–2,4 (m, 4H, H_1, H_2, H_6, H_8); 2,2–1,4 (m, 10 H, H_{4a}, H_{4b}, H_{5a}, H_{5b}, H_{7a}, H_{7b}, H_{9a}, H_{9b}, H_{10a}, H_{10b}); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 9.4): 118,220 (s, 1C, C_3); 87,750 (d, 1C, C_{11}); 60,700 (d, 1C, C_1); 56,384 (d, 1C, C_2); 47,139 (d, 1C, C_6); 46,016 (d, 1C, C_8); 39,627 (t, 1C, C_7); 37,850 (t, 1C, C_4); 34,515 (t, 1C, C_{10}); 32,565 (t, 1C, C_5); 32,256 (t, 1C, C_9).

4.4 Concluding Remarks

The synthesis of the aminated compounds, particularly the triquinylamines, produced exceptionally low yields. In the case of the triquinylamines, these low yields can be partially attributed to the early termination of the reactions due to the fear of the formation of unwanted by-products. The low yields obtained for the hemiacetal triquinane can be ascribed to the small difference in R_f-values of the two products that formed during the chemical reaction.

With the synthesis and purification processes being especially difficult, there is a definite need for the optimisation of the synthetic procedures and purification techniques used for these compounds, in order to improve on their obtained yields.

CHAPTER 5

BIOLOGICAL EVALUATION

5.1 Introduction

The SHSY-5Y cell line was used to perform calcium flux studies on the synthesised polycyclic compounds. This cell line is a third generation neuroblastoma, cloned from SK-N-SH (SK-N-SH → SH-SY → SH-SY5), which was established in 1970 from a metastatic bone tumour. Due to their cancerous nature, the neuroblastoma cells duplicate at a faster rate than normal cells. In addition to this, the SH-SY5Y cells were also selected due to their profound expression of the glutamatergic receptors. In order to determine the effect of the polycyclic test compounds on calcium flux, a real-time confocal laser microscope (Nikon PCM 2000 Confocal Laser Scanning Microscope [He/Ne laser]) was used, in combination with the fluorescent ratiometric calcium indicator, Fluo-5N/AM. The non-competitive NMDA receptor blocker, Amantadine, as well as the NMDA receptor blocking cage compound, NGP1-01, were used as reference compounds in these calcium homeostasis experiments.

The other cell line that was selected for the biological evaluation of the test compounds is the undifferentiated PC-12 rat phaeochromocytoma cells. For calcium measurements, these cells were used on a microplate reader, in combination with the ratiometric calcium indicator, Fura-2/AM.

Keeping in mind that the synthesised triquinylamines show structural similarities to several existing phencyclidine binding site antagonists, a radioligand binding study was also performed, with the radiolabelled [^{3}H]MK-801 as reference. This was to investigate the possible site of action of the synthesised compounds.

5.2 Evaluation of Calcium Homeostasis – Confocal Laser Scanning Microscopy

5.2.1 Chemical Fluorescent Indicator (Fluo-5N)

Fluo-5N shows low calcium binding affinity, making it suitable for detecting high concentrations of calcium, as well as for tracking calcium flux kinetics (Muschol and Salzberg, 2000).

The selection of Fluo-5N for this calcium measurement was also dependent on the ability of the confocal microscope to excite the probe at a particular wavelength (494 nm), as well as to detect its emission at 516 nm. The fluorescence emitted by the probe after excitation is directly related to the calcium concentration. In contrast to the non-ratiometric calcium indicators, a calcium dependent fluorescence emission spectrum of the ratiometric molecular probe (figure 5.1) shows no emission in the absence of calcium ions. The choice of Fluo-5N as a ratiometric indicator in this study thus eliminated the problem of detecting fluorescence when no calcium ions were present.

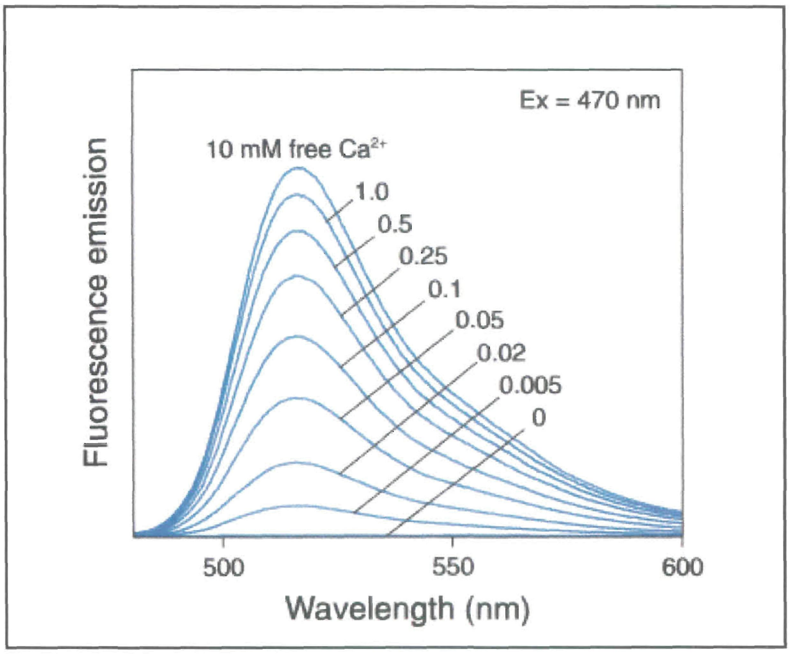


Figure 5.1. Ca^{2+}-dependent fluorescence emission spectra of Fluo–5N (Haugland, 2005).

Based on their chemical form, calcium indicators can be divided into salts (acids), esters and dextran conjugates. The chemical form of the indicator is an important factor when considering its incorporation into cells. The esters contain an acetoxymethyl (AM) group that makes the molecules cell permeable (Tsien, 1981 and Tsien *et al.*, 1982). This allows the probe to cross the cell membrane by means of passive diffusion. Once inside the cell, non-specific esterases cleave the AM group from the probe, leading to a cell-impermeant indicator (Takahashi *et al.*, 1999). Due to its high polarity, this polyanionic form of the probe that is obtained after esterase activity would thus leak out of the cell far slower than the parent compound.

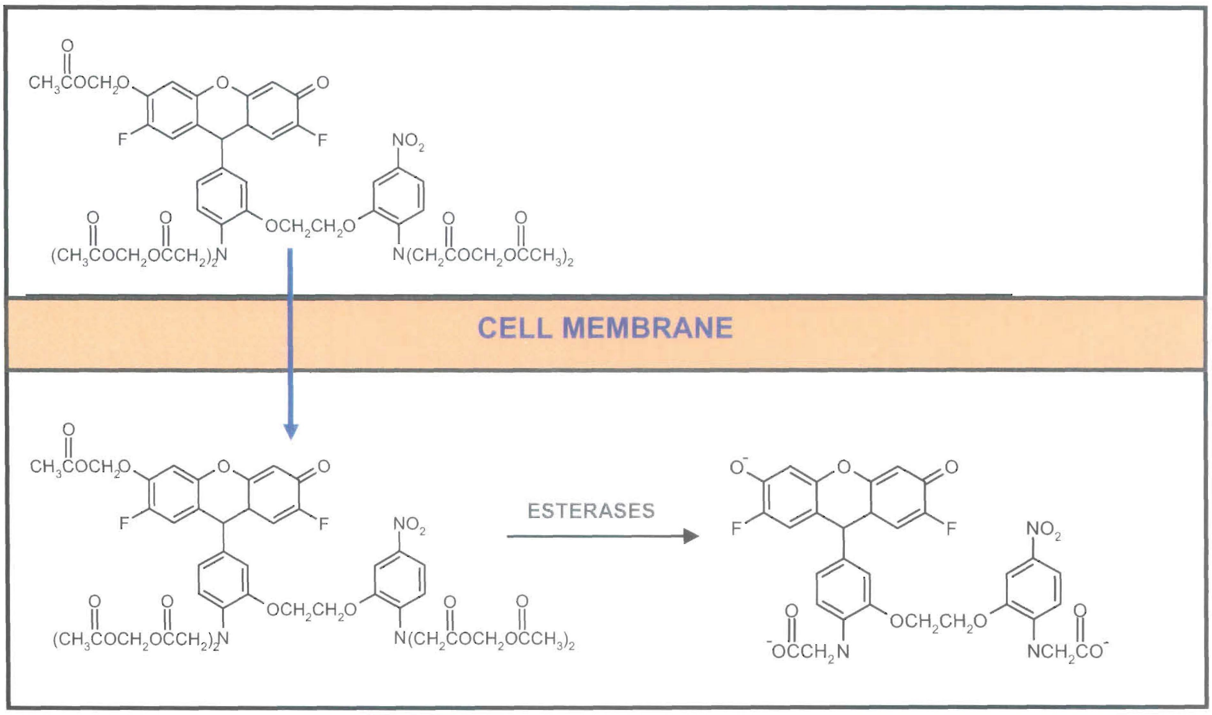


Figure 5.2. Conversion of the ester conjugate of Fluo-5N to the cell-impermeant form by non-specific esterase activity.

5.2.2 Confocal Laser Scanning Microscopy (CLSM)

The Confocal Laser Scanning Microscope produces images by moving a scanning point across the specimen and collecting the emitted fluorescence through a pinhole. This pinhole is located at the confocal point of the scanned focus. By excluding the out-of-focus fluorescence from the fluorophore, CLSM offers high vertical and horizontal spatial resolution (Dilberto *et al.*, 1994).

Since the obtained resolution is much less than the thickness of the cell, the fluorescence intensity of each pixel in the obtained image can show the concentration of the functional ion with precision (Takahashi *et al.*, 1999). In this study, we attempted to take advantage of this property of the CLSM to perform a quantitative analysis on the influence of various test molecules on calcium flux.

5.2.3 Cell Cultures

Human SH-SY5Y neuroblastoma cells (Obtained from American Type Culture Collection, Catalogue number CRL-2266) were used in this study. These cells are a clonal subline of the neuroepithelioma cell line SK-N-SH and are epithelial-like cells that grow as a monolayer and in cell clusters when cultured at high density. SH-SY5Y cells are known to express acetylcholinergic, adenosinergic and glutamatergic receptors and are also dopamine beta hydroxylase active.

Cells were maintained in a growing cocktail consisting of a 1:1 mixture of Earle's Minimum Essential Medium (EMEM) and Ham's F-12 medium, supplemented with 10% foetal calf serum, 1% 100 units penicillin/ml, 1% 100 µg streptomycin/ml and 1 mM l-Glutamine. Incubation of the cells took place at 37 °C in a 5% CO_2 and 95% O_2 humidified atmosphere.

The cells duplicated at a rate of approximately once every 48 hours and when 95% confluency was reached, they were detached from the flask bottom by means of trypsination. To ensure complete detachment of the cells, they were incubated with trypsine/versine for approximately 5-10 minutes or until all cells were visibly detached from the flask bottom. After trypsination, the cells were seeded in new flasks at a density of no less than 1/6 confluency.

5.2.4 Dye Loading

STANDARD RECORDING SOLUTION: *126 mM NaCl, 4 mM KCl, 2 mM* $CaCl_2$*, 1 mM* $MgCl_2$*, 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) and 15 mM glucose; pH adjusted to 7,3 with NaOH.*

To allow their adherence to coverslips before dye loading commenced, the cells were seeded onto 25 mm glass coverslips and incubated overnight in 2 cm^3 Petri dishes. The culture medium was replaced with EMEM:Ham's F-12 medium (1:1) containing 500 nM of the fluorescent calcium indicator, Fluo-5N (obtained from Molecular Probes). To enhance its solubility in the aqueous environment surrounding the cells, a stock solution of the probe was first prepared in a minimal amount of anhydrous dimethylsulphoxide (DMSO) before further dilution in serum-free culture medium took place. In order to allow complete dye equilibration, the cells were incubated for 40 minutes at 37 °C (5% CO_2 and 95% O_2, humidified atmosphere), after which the dye-containing medium was replaced with standard recording solution.

5.2.5 Imaging and Analysis

TEST COMPOUND LOADING SOLUTION: *Test compounds were dissolved in DMSO and diluted to a 100 μM concentration in standard recording solution. The final concentration of DMSO in the incubations did not exceed 0,1%.*

CONTROL SOLUTION: *Standard Recording Solution, with DMSO not exceeding 0,1% in the final incubations.*

DEPOLARISING SOLUTION: *100 mM KCl, 40 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 15 mM glucose; pH adjusted to 7,3 with NaOH.*

All experiments were conducted at room temperature. Glass coverslips were individually mounted in a recording chamber and the dye loading solution was replaced with standard recording solution. Cells were incubated with the respective polycyclic compounds for 2 minutes. In the case of control experiments, the cells were incubated with the control solution for the same amount of time. Dye loading solution (100 nM) was added to minimise dye leakage from cells.

A real-time CLSM (Nikon PCM 2000) was used to record the images. After the first image was taken, the high concentration potassium solution was added to initiate membrane depolarisation. Because a disturbance in the surface might lead to a disturbance in the optical effect, care had to be taken during the addition of the depolarising solution. A time series of 30 optical scans were recorded at 30 second intervals. Cells were exposed to the 505 nm line of a He/Ne laser and emissions were detected at 568 nm. A 60 x 1.4 NA (Nikon) oil immersion objective was used to view, and when necessary, further magnify the cells for imaging. A 5 μm pinhole was employed to minimise dye bleaching and light scattering. Image processing and analysis were performed using the supplied Nikon software and the NIH Image program (National Institutes of Health, 2008). Experiments were repeated at least three (3) times.

5.2.6 Results

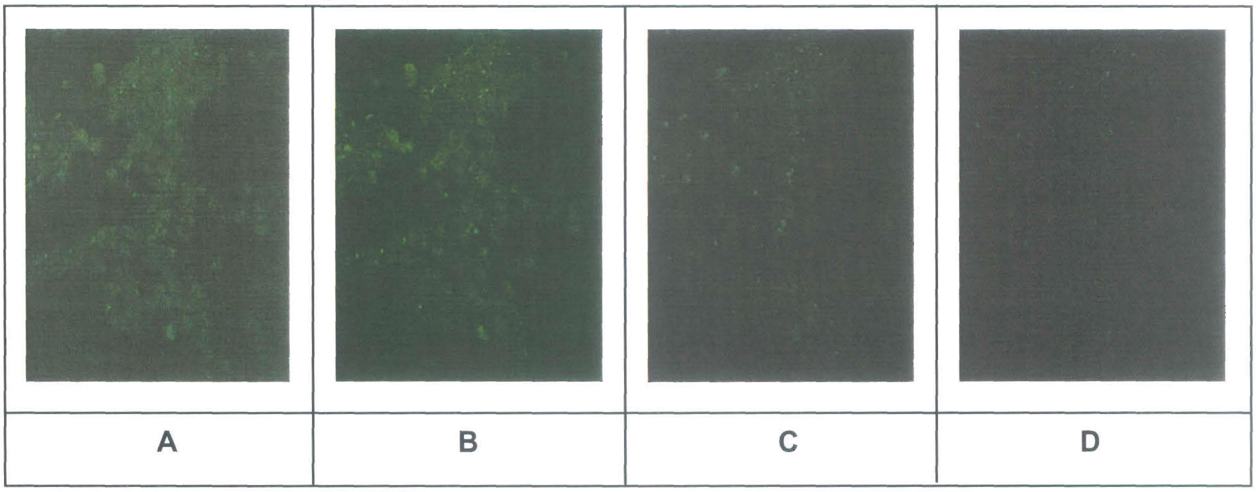


Figure 5.3. Representative frames for the time series of the control experiment in the absence of Nile Red at time points: **A** – t0; **B** – t1; **C** – t15; **D** – t29.

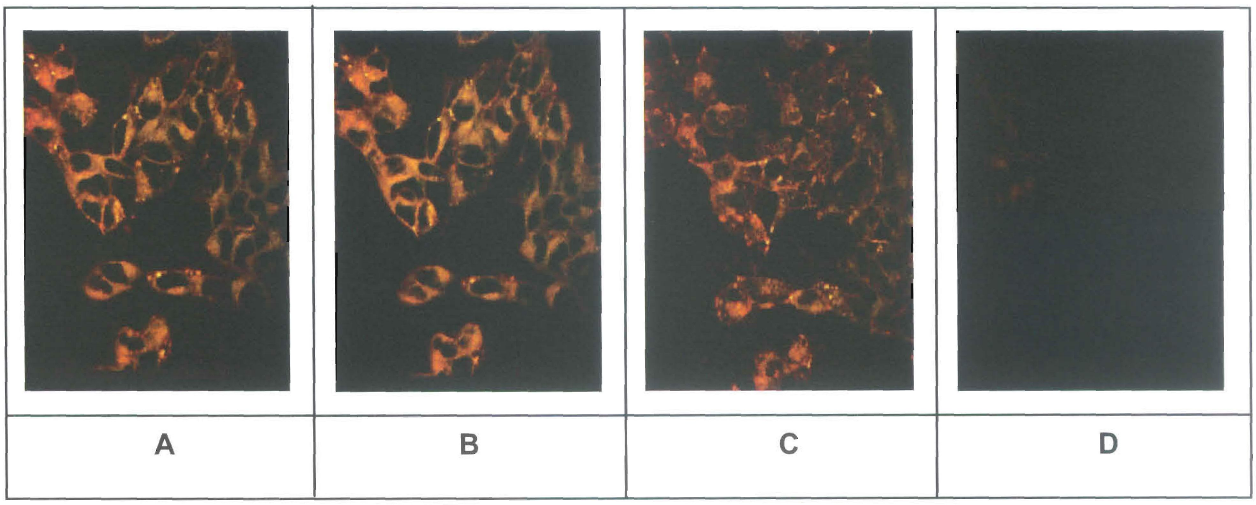


Figure 5.4. Representative frames for the time series of the control experiment in the presence of Nile Red, at time points: **A** – t0; **B** – t1; **C** – t15; **D** – t29.

The statistical analysis of these data was performed by using Microsoft Excel 2003 and Graphpad Prism, Version 4.03 of 2005.

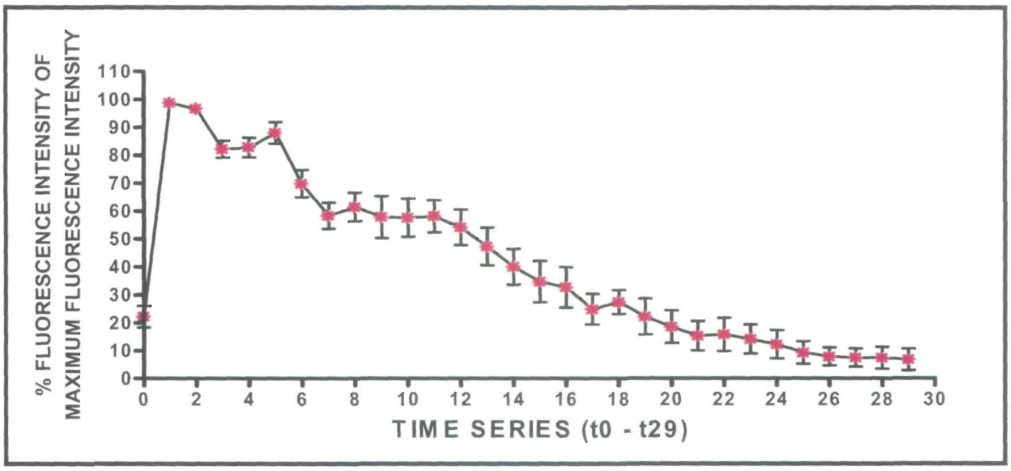


Figure 5.5. The effect of KCl-induced depolarisation on fluorescence intensity (CONTROL EXP).

A common region of interest (ROI) was selected for all the images that were obtained during each experiment. Analyses of the selected regions of interest were performed, using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). A mean gray value was calculated for each ROI, indicating the fluorescence intensity of the chosen area.

At least two runs of experiments were performed for each test compound, each trial repeated at least three times. As can be seen from the graphs (figure 5.6), it is clear that the results obtained during these experiments showed no reproducibility whatsoever.

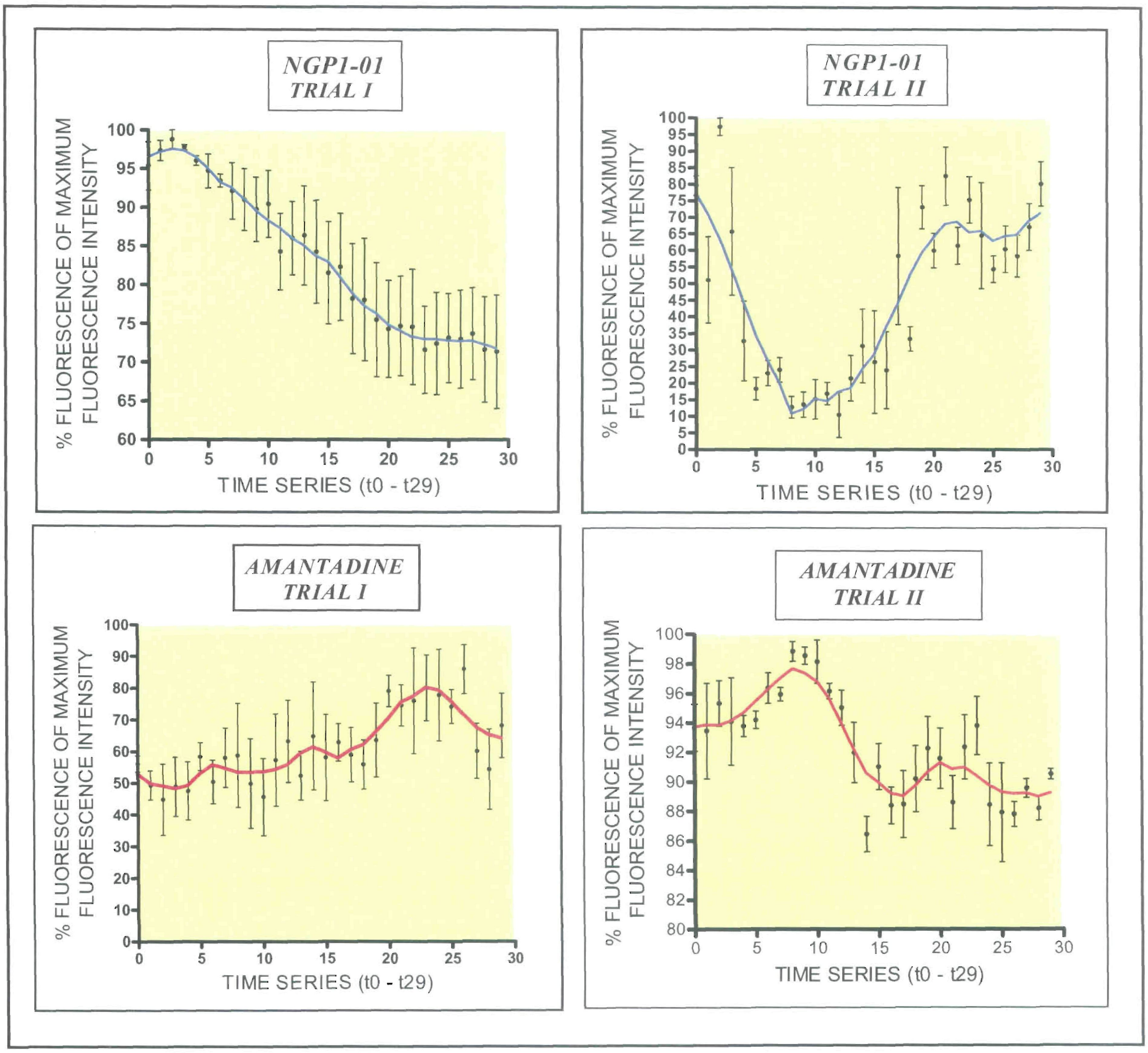


Figure 5.6. Graphs obtained during two separate experimental runs for the respective reference compounds, Amantadine and NGP1-01.

5.2.7 Discussion

The graph obtained for the control experiment (figure 5.5) gives a clear indication of the sudden raise in intracellular calcium ([Ca^{2+}]$_i$) directly after membrane depolarisation. In response to the KCl stimulus, a sudden increase in fluorescence intensity can be observed, with the percentage fluorescence reaching 100% directly after stimulation. This was followed by a gradual decrease in fluorescence with time, possibly due to the photochemical destruction of the fluorophore, which is also known as photobleaching.

The results obtained during the evaluation of the test compounds were not reproducible and hence the graphs associated with the synthesised compounds were not included in this report. This can be seen from the results obtained for the reference compounds, NGP1-01 and Amantadine (figure 5.6). This lack of reproducibility is possibly due to the movement of the cells during experimental recording, as well as a prolonged recording time. An alternative method had to be considered for the evaluation of the test compounds and a microplate reader with a different cell line was utilised for this purpose.

5.3 Evaluation of Calcium Homeostasis – Fluorometric Microplate Reader

5.3.1 Chemical Fluorescent Indicator (Fura-2)

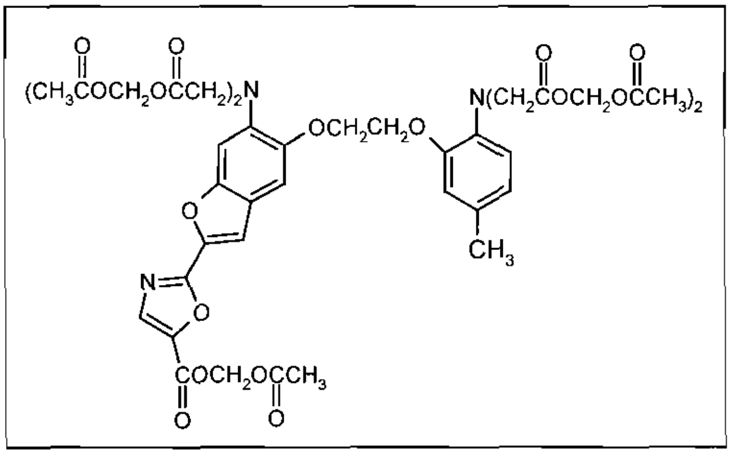


Figure 5.7. The ester conjugate of Fura-2 (Fura-2/AM) (Haugland, 2005).

Fura-2 was the first frequently used dye for calcium imaging and still remains the dye of choice for quantitative ratiometric measurements (Grynkiewicz *et al.*, 1985). Similar to Fluo-5N, Fura-2 is also a ratiometric fluorescent dye and upon binding calcium ions, it exhibits a shift in absorption (figure 5.8). Being excited at both 340 nm and 380 nm, Fura-2 thus shows dual-excitation ratiometry in the Ultraviolet-wavelength region (Grynkiewicz *et al.*, 1985). To facilitate passive diffusion across cell membranes, the acetoxymethyl ester of Fura-2 (Fura-2/AM) was used during these experiments and the use of invasive and biochemical loading techniques could thus be avoided (Haugland, 2005). Because the AM have low aqueous solubility, some dispersing agents are often used to facilitate cell loading. The non-ionic dispersing agent, Pluronic F-127, was used in these experiments, to help with the solubilisation of the large dye molecules in the physiological medium (Ozaki *et al.*, 1987).

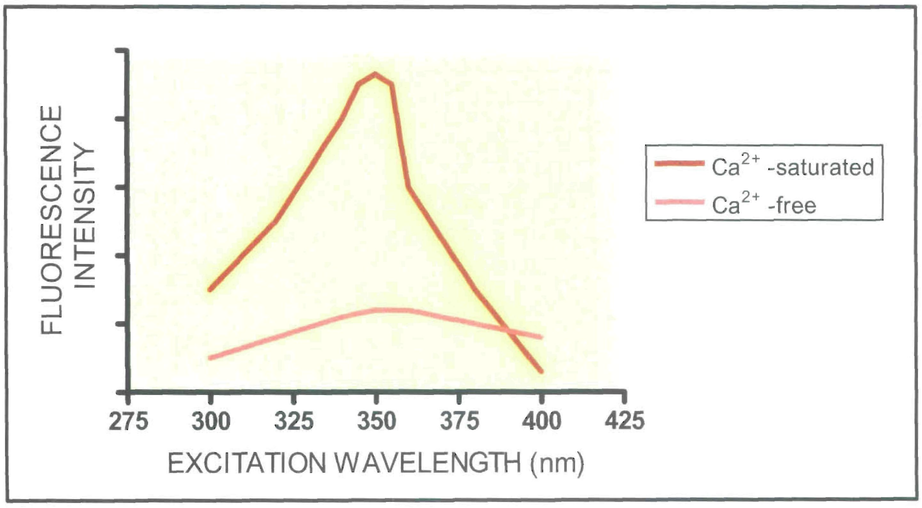


Figure 5.8. The Ca^{2+}-saturated and Ca^{2+}-free spectra of Fura-2 (Lambert, 2006).

5.3.2 Fluorometric Imaging Microplate Reader

Fluorometric Imaging Plate Readers (FLIPR) are used extensively to record dynamic intracellular processes, such as changes in intracellular calcium ion concentrations. One of the key advantages of this apparatus, is its ability to measure fluorescence emission from multiple wells (96-well or 384-well) simultaneously, while high temporal resolution is maintained (Lambert, 2006).

The Synergy™ 4 Multi-Mode Microplate Reader from BioTek® was used during these experiments. As part of its optical design, it consists of both a fluorescence filter- and a monochromator-based detection system. The filter-based system is usually selected when the particular test requires read speeds that should be both fast and of high sensitivity. Amongst its several detection technologies, the Synergy™ 4 Multi-Mode Microplate Reader offers UV-visible absorbance, making it suitable to use Fura-2 as the probe of choice. Other features include a precise temperature control system, plate shaking and advanced reader control and data analysis software (BioTek®, 2006).

A Tungsten Halogen or High Energy DPR Xenon Flash was used as light source to excite the dye at 340/380 nm. Emission was detected at 510 nm. Each experiment was run for 40 seconds, with a 150 millisecond interval in between each reading. A high concentration KCl-solution was injected into each well 10 seconds into the recording time to depolarise the cell membranes and thus initiate calcium flux into the cells.

5.3.3 Cell Cultures

Undifferentiated PC-12 phaeochromocytoma cells (Obtained from American Type Culture Collection, Catalogue number CRL-1721) were used in this study. The PC-12 cell line is a cancer cell line, derived from the adrenal medulla of rats. These cells are known to express particular types of ion channels in the membranes, amongst which are at least three types of Ca^{2+} channels (Streit and Lux, 1987, Streit and Lux, 1989, Garber *et al.*, 1989 and Plummer *et al.*, 1989). They also show a distinct expression of both subtypes of sigma receptors, but PCP binding site receptors are absent in these cells (Hellewel and Bowen, 1990 and Yang *et al.*, 1989).

The culture medium for these cells consisted of RPMI-1640 (Roswell Park Memorial Institute Medium), supplemented with 5% foetal bovine serum (FBS), 0,5% 1mM l-Glutamine and 0,5% penicillin/streptomycin Amphotericin B. The required subcultivation ratio of these cells is 1:3, twice weekly, with a required renewal of medium every 2-3 days. Cells duplicated at a rate of approximately once every 48 hours and subculturing was done by scraping the cells off the surface, after replacing the old culture medium with fresh medium. The cells were aspirated and appropriate aliquots of the cell suspension were added to new flasks with the appropriate amount of fresh growth medium. Incubation of the cells took place at 37 °C with an air:CO_2 atmosphere ratio of 95:5%.

5.3.4 Dye Loading

FLUORESCENT INDICATOR STOCK SOLUTION: *Fura-2/AM and Pluronic F-127 were purchased from Invitrogen (USA).The Pluronic F-127 is available as a 20% solution in anhydrous DMSO; 50 µl Pluronic F-127/anhydrous DMSO was added to 50 µg of Fura-2/AM. The solution was vortexed, in order to properly disperse the fluorescent dye and the container was covered with aluminium foil to protect from light.*

HANK'S BALANCED SALT SOLUTION (HBSS): *137 mM NaCl, 5 mM KCl, 10 mM* $NaHCO_3$*, 0,6 mM* KH_2PO_4*, 0,6 mM* Na_2HPO_4*, 1,4 mM* $CaCl_2$*, 0,9 mM* $MgSO_4$*, 5,5 mM glucose; pH adjusted to 7,4 with NaOH.*

PHOSPHATE BUFFERED SALINE (PBS): *137 mM NaCl, 10 mM Phosphate, 2,7 mM KCl; pH adjusted to 7,4 with HCl.*

The cell loading solution was prepared by dispersing 50 µl of the fluorescent indicator stock solution in 10 ml Hank's Balanced Salt Solution (obtained from Fisher Scientific; USA). Ten percent bovine serum albumin (BSA) was added and the solution was vortexed to ensure the proper dispersion of the indicator in the saline solution. The final concentration of the fluorescent indicator was 5 µM. The container was protected from light and only freshly prepared solution was used for cell loading purposes.

Cells were seeded as a monolayer onto black-walled, transparent bottomed 96-well tissue culture treated plates, at a confluency of less than 50% or 400 000 cells/100 µl. In order to ensure adherence to the wells, the plates were incubated overnight, before dye loading commenced. The growth medium and neurons were washed twice with HBSS and the cells were also maintained in this medium.

Directly before cell loading with the fluorescent indicator, the HBSS was aspirated and the neurons were washed twice with phosphate buffered saline (PBS). While working in a dark room, 50 µl of the dye solution was loaded into each well. The cells were incubated for 30–45 min at 37 °C, after which the dye containing medium was removed and the cells washed twice with HBSS. This last step is necessary to ensure the complete removal of the excess calcium indicator. The cells were then loaded with the test compounds and incubated in 100 µl of HBSS for 20–30 min. To prevent compartmentalisation and dye leakage, analysis of these cells had to be done within an hour after loading.

5.3.5 Test Compound Loading and Experimental Recording

TEST COMPOUND LOADING SOLUTION: *By dissolving the compounds to be tested in anhydrous DMSO, stock solutions of the test compound loading solution was prepared in the following concentrations: 0,1 M, 0,01 M and 0,001 M. The final concentrations were prepared by diluting 1 µl of the stock solutions in 1 ml HBSS to give a range of concentrations of 100 µM, 10 µM, 1 µM, 0,1 µM and 0,01 µM. The final concentration of DMSO in the incubations did not exceed 0,1%.*

REFERENCE COMPOUND SOLUTION: *NGP1-01 and Nimodipine were used as reference compounds during these experiments. The reference compounds were prepared in the same concentrations as the test compound loading solutions.*

CONTROL SOLUTION: *HBSS, with DMSO not exceeding 0,1% in the final incubations.*

DEPOLARISING SOLUTION: *100 mM KCl, 43 mM NaCl, 10 mM $NaHCO_3$, 1,4 mM $CaCl_2$, 0,9 mM $MgSO_4$, 20 mM HEPES and 5,5 mM glucose; pH adjusted to 7,4 with NaOH.*

The cells were loaded with the test or reference compounds about 20 minutes after the addition of the fluorescent dye. This delay is to allow enough time for the complete de-esterification of the probe. The HBSS on the cells was replaced with 100 µl of each dilution of the test compound containing solution or reference compound solution. Three (3) replicates were allowed per dilution and at least one (1) row contained control solution, thus excluding any test compound. The cells were incubated for 10 minutes directly before analysis.

The experiments were conducted at 37 °C and readings were taken at 150 millisecond intervals over a total runtime of 40 seconds. 100 µl of the depolarising solution was injected into each well at about 10 seconds into the recording time.

5.3.6 Results

Microsoft Excel 2003 and Graphpad Prism, Version 4.03 of 2005, were used to perform the statistical analyses of the obtained data for these experiments. In order to determine the statistical significance of all data, the two-way ANOVA analysis and the Bonferroni post-test of Graphpad Prism (version 4.03 of 2005) were used.

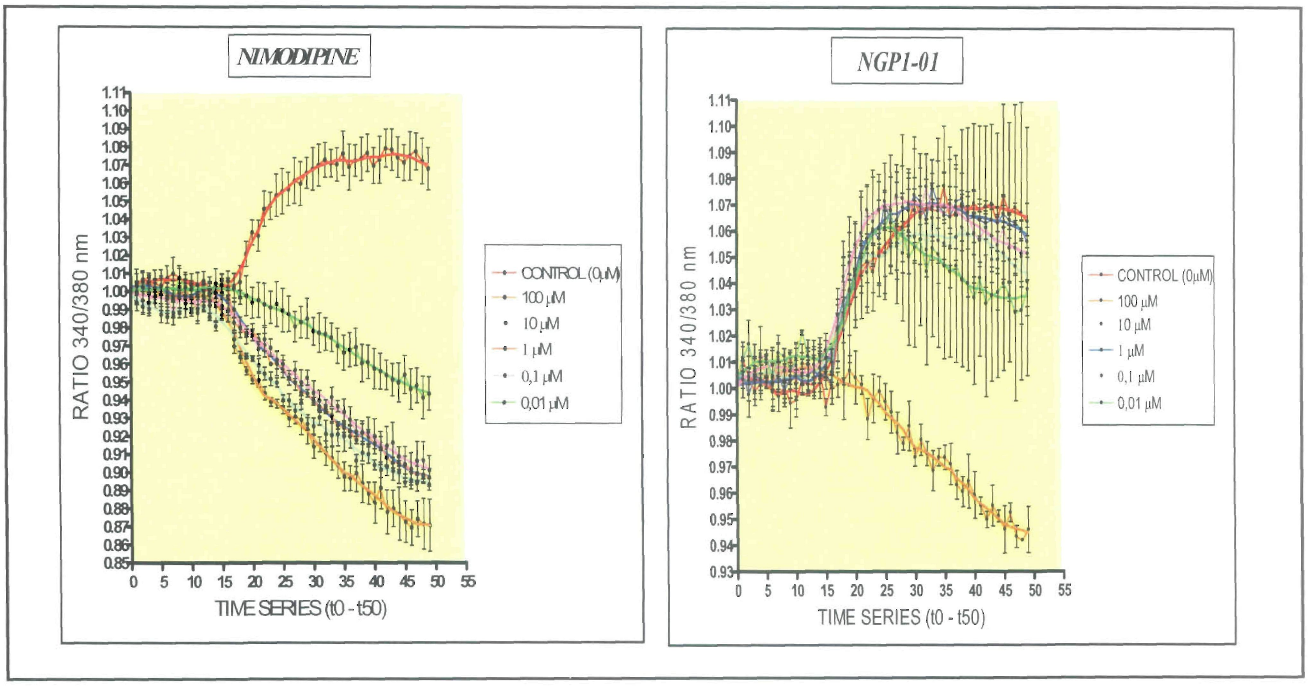


Figure 5.9. Graphs depicting the time-dependent effect of various concentrations of the reference compounds (Nimodipine and NGP1-01) on the emission ratio (340/380 nm) of the dye, after KCl-induced depolarisation.

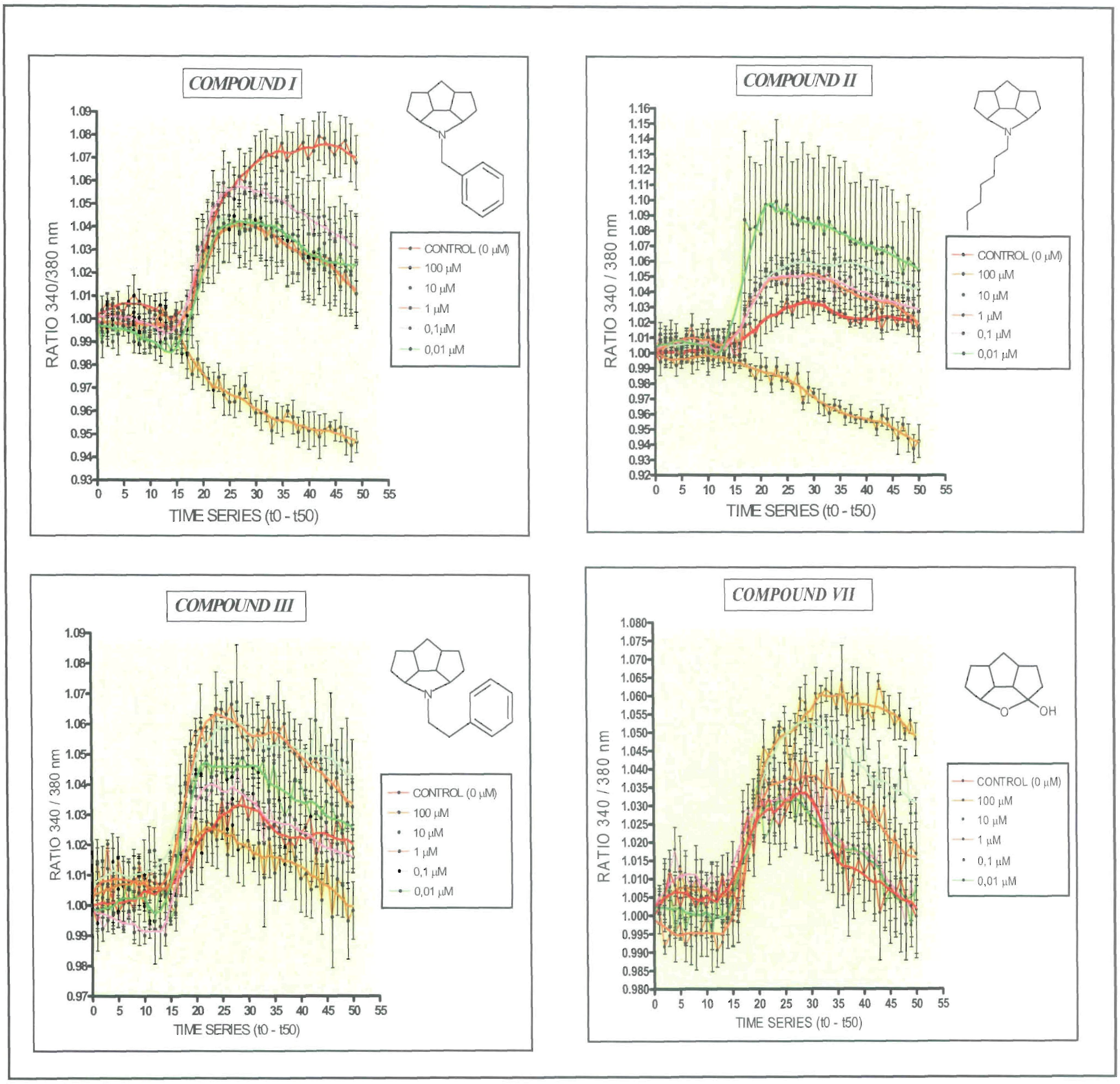


Figure 5.10. Graphs depicting the time-dependent effect of various concentrations of the triquinane derivatives on the emission ratio (340/380 nm) of the dye, after KCl-induced depolarisation.

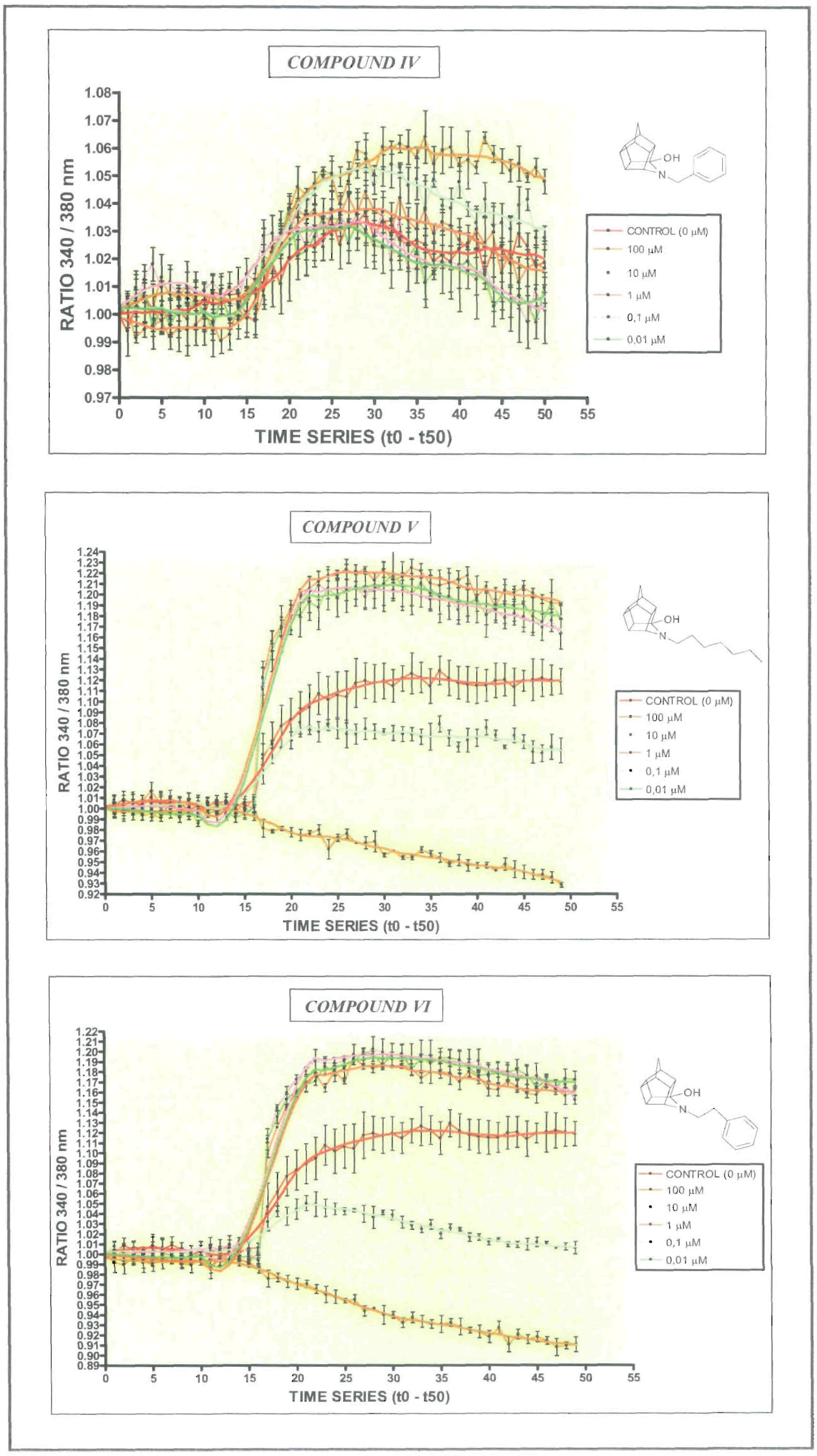


Figure 5.11. Graphs depicting the time-dependent effect of various concentrations of the cage derivatives on the emission ratio (340/380 nm) of the dye, after KCl-induced depolarisation.

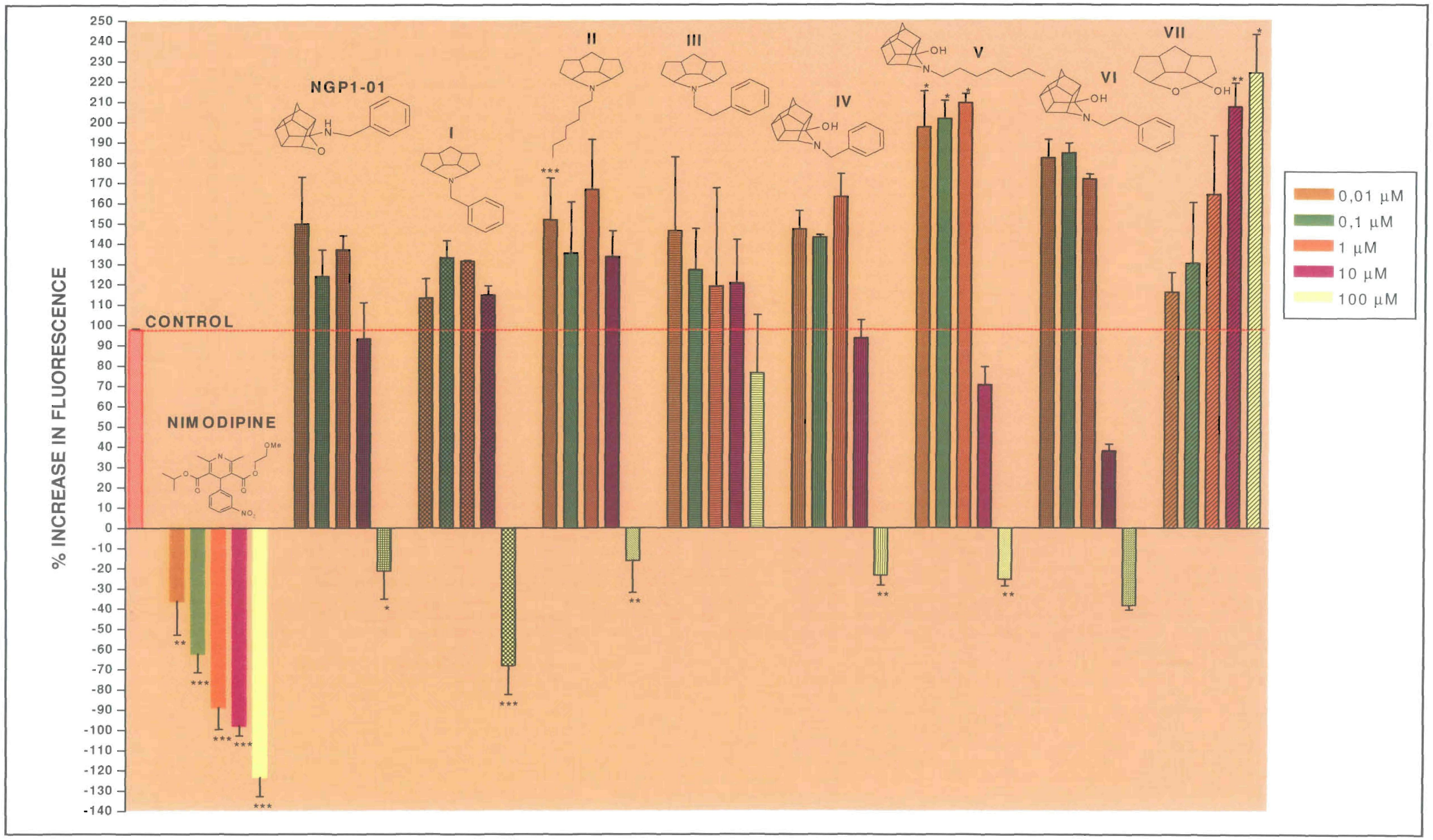


Figure 5.12. Summary of the effects of the respective test compounds on $[\mathrm{Ca}^{2+}]_i$, at different test compound concentrations 0,01–100 µM. $p < 0{,}001$ (***): extremely significant; $0{,}01 < p < 0{,}001$ (**): very significant; $0{,}01 < p < 0{,}05$ (*): significant; $p > 0{,}05$.

Two sets of graphs were fitted for each experiment – one giving an indication of the ratio of fluorescence at 340 nm and 380 nm (figures 5.9, 5.10 & 5.11) and the other depicting the percentage increase in fluorescence (figure 5.12) due to the KCl-induced depolarisation of the cell membrane. These graphs indicate the average decrease (or increase) in fluorescence in the presence of each of the derivatives at different compound concentrations, compared to fluorescent values obtained during control experiments. Baseline shifts of the fluorescence intensity ratios were taken into consideration when calculating the histograms for all the compounds (figure 5.12).

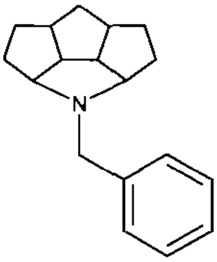
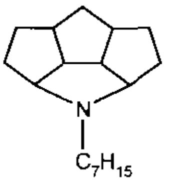
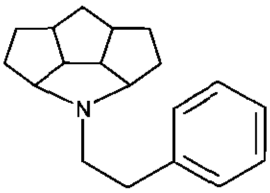
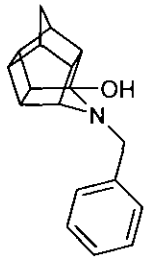
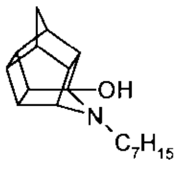
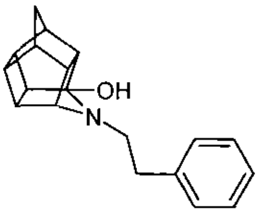
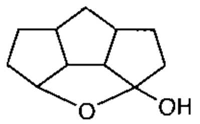
It is clear that an immediate increase in fluorescence intensity can be observed directly after the application of KCl (figures 5.9, 5.10 and 5.11) in the control experiments. This gives a definite indication of the depolarising effects of the added solution on the cell membranes, which in turn, leads to a flux of calcium into the cell.

The reference compound, Nimodipine, caused both a time and concentration-dependent suppression of the KCl-induced increase in calcium flux. Even after the addition of depolarising solution, no increase could be observed in the fluorescence intensity curves and baseline fluorescence was further suppressed (figures 5.9 and 5.12). The extent to which Nimodipine reduced the calcium influx was evident even in very low concentrations of the compound. The results for Nimodipine in these experiments are, once again, confirmation of the mechanism of action of this well known calcium channel blocker and thus also of the reliability of the results obtained during these fluorescence imaging experiments. NGP1-01, which served as the second reference compound, was effective in suppressing the calcium levels only at higher concentrations. Although not as drastic as Nimodipine, this compound also seems to suppress baseline calcium flux at its highest concentration, *i.e.* 100 µM (figures 5.9 and 5.12).

Of all the test compounds, the benzylamino substituted derivative of the triquinanes (compound **I**) proved to be the most effective in blocking cellular calcium influx at high concentrations. This suppression of calcium flux at 100 µM is however only comparable to that achieved by very low concentrations of Nimodipine (0,1–1 µM). Of the other triquinane structures, only compound **II** showed significant calcium flux suppression and then only at 100 µM. For the aza-cage compounds, the best calcium flux suppression was observed with compound **VI** (by ± 62% and

138% at 10 μM and 100 μM, respectively). Compounds **IV** and **V** also suppressed calcium flux at 100 μM and 10 and 100 μM respectively (by 124% and ± 29% and 125%).

Table 5.1. Summary of compounds selected for synthesis and evaluation.

Compound I	**Compound II**	**Compound III**	**Compound IV**
			
Compound V	**Compound VI**	**Compound VII**	
			

However, a very interesting observation was made with the compounds tested, especially with the nitrogen bridgehead pentacyclic compounds (compounds **IV** – **VI**). After causing a distinct suppression in intracellular calcium levels at high concentrations, lower concentrations of the test compounds brought about an increase in these levels, reaching values significantly above that of the control. Amongst these, the compound with the most significant effect on increasing intracellular calcium levels is compound **V**, at concentrations of 0,01-1 μM. The drastic difference in the concentration-related effects for compound **V** is evident from figures 5.11 and 5.12. After causing a significant suppression in the [Ca^{2+}]$_i$ at high concentrations (100 μM), an increase in Ca^{2+} flux is observed at concentrations below 10 μM. A similar effect is observed for the triquinane derivative with the heptylamino substituent (compound **II**). At 100 μM this compound caused a 117% decrease in calcium flux, while all test concentrations below this caused an increase in Ca^{2+} levels. The same trend is observed for the cage compounds **IV** and **VI**, where after showing suppression at 10 and 100 µM, significant increases in calcium flux were seen at lower concentrations of these compounds.

The effect of compound **VII** on Ca^{2+} flux was also surprising. As no nitrogen atom is included within its structure, it was expected that compound **VII** would show no effect on the measured fluorescence at all. However, a concentration-dependent increase in calcium levels was observed, with the most significant elevation in calcium levels at the highest compound concentrations (10 and 100 µM).

5.3.7 Discussion

According to literature, Nimodipine acts as an L-type calcium channel blocker (Hockerman *et al.*, 1997) and is used clinically in the treatment of cardiovascular abnormalities (Triggle, 1991). The dramatic decreases in calcium levels, as indicated by the results obtained during these experiments (figures 5.9 and 5.12), confirms the effectiveness of this compound to block especially voltage-dependent calcium channels after depolarisation.

With the triquinylamines, it seems as if the benzylamino substituent plays a key role in their ion channel blocking effects at higher concentrations. However, an increase in chain length between the aromatic ring and the polycyclic moiety increases the calcium stimulating effects at low concentrations. The higher flexibility of the aromatic substituent can serve as explanation for this.

In addition to suppressing calcium levels at high concentrations, most of the test compounds showed a surprising increase in calcium levels. Due to previous observations made of the pentacyclic cage compounds (Kassiou *et al.*, 1996), it is suspected that this calcium increase is caused by their activity on sigma receptors. Although the previous studies mainly describe the polycyclic cage compounds as either sigma receptor agonists or antagonists, the current study may act as proof that the test compounds may possess both agonistic and antagonistic activities on these receptors in a concentration dependent manner. Although it is generally accepted that sigma receptors inhibit high-voltage-activated calcium channels, there seems to be a difference in the effects caused by σ_1- and σ_2-receptor binding. Whereas antagonism of the σ_1-receptor leads to an increase in $[Ca^{2+}]_i$ (Zhang and Cuevas, 2002), the opposite is true for the σ_2-

receptors. The latter effect can be partially ascribed to the release of Ca^{2+} from the endoplasmic reticulum upon σ_2-receptor stimulation (Vilner and Bowen, 2000).

When compared to the other test compounds, the effect on calcium levels by compound **VII** is probably the result of a completely different mechanism of action. This suspicion is due to the absence of a nitrogen atom within its structure, contrary to the other test compounds. Unlike the rest of the polycyclic derivatives, compound **VII** caused no suppression in calcium ion levels. However, an increase in $[Ca^{2+}]_i$ was observed with higher compound concentrations. This observation might suggest selective binding to either one of the two sigma receptor subtypes. Both σ_1-receptor antagonism and σ_2-receptor stimulation might lead to the effects that are observed for the lactol derivative of the triquinanes.

The difference observed between the oxa and aza-bridgehead structures with regard to calcium stimulating effects probably indicates significant differences in the binding affinities of these compounds for the ion channels and the receptors affecting it.

5.4 [^{3}H]MK-801 Displacement Study

5.4.1 Introduction

Competition radioligand binding studies were performed, during which the competition of the test compounds with the radiolabelled form of the potent NMDA receptor blocker, MK-801 (dizocilpine) was measured. These studies were performed on the cortex of dissected rat brains. The [^{3}H]MK-801 binding was measured after stimulation with the co-agonists, Glycine (300 µM) and l-Glutamate (100 µM).

5.4.2 Radioligand Binding Assay

HTS– BUFFER: *4,5 mM Tris/HCl, 5 mM HEPES; pH adjusted to 7,4 with NaOH.*

TEST COMPOUND SOLUTION: *Test compounds were dissolved in DMSO and diluted to 2,2 x* 10^{-6} *M with HTS buffer; This parent solution was diluted into seven (7) different concentrations (*10^{-5} *M –*10^{-11} *M); Final DMSO concentration in the incubations did not exceed 0,1%.*

Whole brains of rats were dissected and the cortex of each was isolated and stored at -80 °C, until necessary for analysis. This tissue was homogenised in ice-cold HTS–buffer (4,5 mM Tris/HCl, 5 mM HEPES; pH adjusted to 7,4 with NaOH) using a Politron homogeniser. The obtained suspension was centrifuged for 15 minutes at 4 °C (48 000 x g). The pellet was resuspended in HTS buffer and centrifuged again. Resuspension of the obtained pellet in the buffer solution (1 g/100 ml) gave an adequate concentration of brain tissue for experimental use. The homogenate was kept on ice until use.

The reaction mixture consisted of 900 µl brain homogenate, 50 µl [^{3}H]MK-801, 50 µl test compound solution and 50 µl each of the Glycine and l-Glutamate solutions to stimulate the NMDA receptor channel. The test compound solution was substituted with 50 µl of unlabelled MK-801 (100 µM) as positive control. For the dose response curve, the test compound selected for the screening test was incubated at different concentrations. The experiment was performed in duplicate for each compound concentration in the concentration range 10^{-5}M to 10^{-11}M. This was repeated three (3) times for each compound.

Each test tube was vortexed and incubated in a water bath at 25 °C for 90 minutes. The reaction was terminated by the addition of ice cold buffer and filtration on Whatman GF/B filters on a Hoeffler apparatus. Each filter was rinsed twice with ice cold buffer, where after they were placed into scintillation vials. After the addition of 3 ml filter count scintillation cocktail to each scintillation vial, the radioactivity retained on the filters was determined with a Packard Tri-Carb 4660 scintillation counter.

5.4.3 Results

A dose-response curve for the reference compound, MK-801, was fitted in order to determine the reliability of the experimental method performed. A typical sigmoidal curve for the displacement of the radioactive compound by the unlabelled compound indicated that the results obtained during the experimental method for the test compounds were reliable.

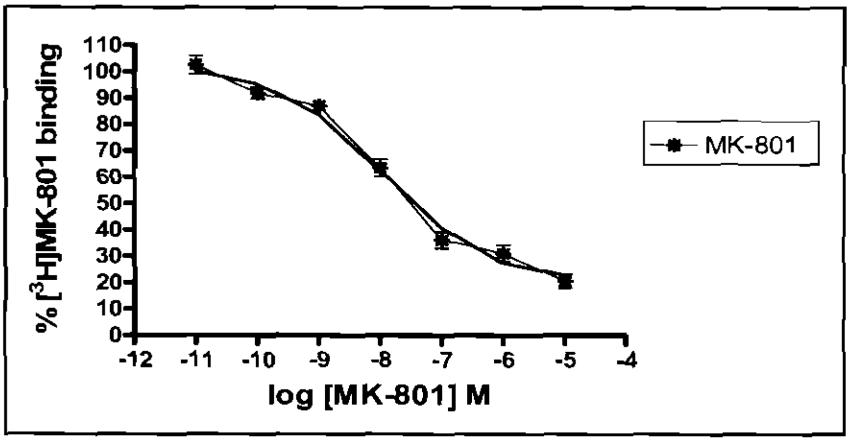


Figure 5.13. Dose-dependent displacement of the radiolabelled MK-801 ([^{3}H]MK-801) by the unlabelled compound, MK-801.

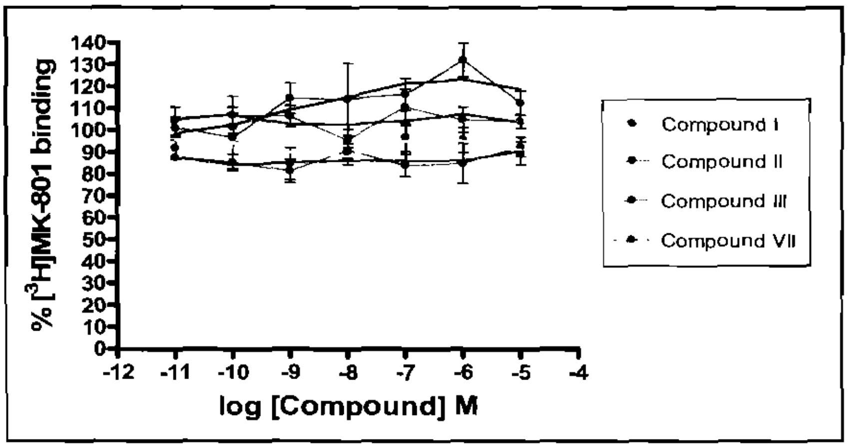


Figure 5.14. Dose-dependent effect of the triquinane derivatives on [^{3}H]MK-801 binding.

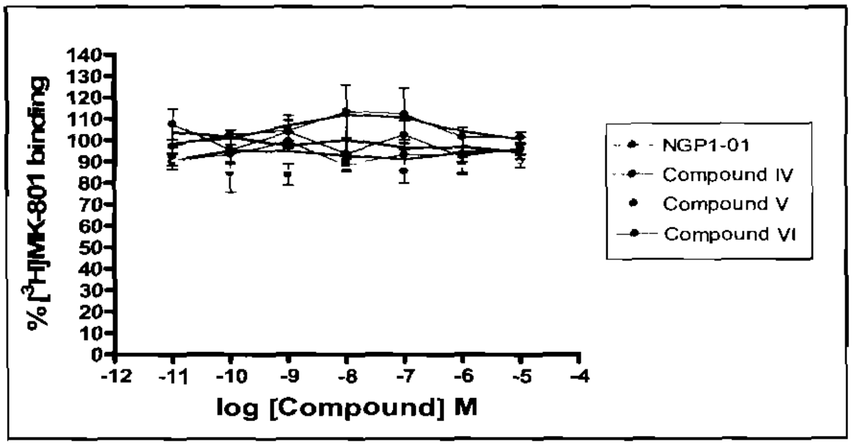


Figure 5.15. Dose-dependent effect of the pentacycloundecane derivatives on [^{3}H]MK-801 binding.

5.4.4 Discussion

When comparing the dose-response curve of the unlabelled MK-801 to those of the test compounds, it is evident that the test compounds show very little or no effect on [^{3}H]MK-801 displacement. Where the effect of an increase in the MK-801 concentration on percentage [^{3}H]MK-801 binding presents a sigmoidal curve (figure 5.13), which is typical of competitive displacement, the percentage binding of the radioligand remained fairly constant with increasing concentrations of the test compounds. These results are in accordance with previous findings by both Bezuidenhout (2007) and Geldenhuys *et al.* (2007) and suggest that neither the triquinane derivatives (figure 5.14), nor the polycyclic cage compounds (figure 5.15) accomplish significant displacement of [^{3}H]MK-801 from the phencyclidine binding site. However, when taking into consideration that MK-801 is one of the most potent PCP binding site blockers in use (Parsons *et al.*, 1995), this observation does not entirely exclude the synthesised compounds as phencyclidine binding site antagonists. It is however an indication that the calcium channel effects observed in our laboratory could probably be attributed to interaction at another site of the NMDA channel or on an alternative channel or system.

5.5 Concluding Remarks

Due to inadequate experimental conditions in the confocal microscope experiments, no significant data could be obtained during this evaluation of the influence of the test compounds on calcium homeostasis. The use of a dispersing agent (*e.g.* Pluronic F-127) to facilitate the solubilisation of the dye molecules in the physiological medium, as well as shorter recording times, are suggestions that could be utilised in future to improve on these experimental results. The cells should also be properly rinsed with phosphate buffered saline and Hank's Balanced Salt Solution before and after dye loading, to ensure the complete removal of foetal bovine serum from the cell surface, since the serum might contain esterase enzymes. The activity of these enzymes might lead to the hydrolysis of the acetoxymethyl form of the probe before its entrance into the cell.

Of the results obtained from the microplate reader experiments, the most interesting observation was the suppression in calcium levels at higher concentrations of the test compounds, whilst increased Ca^{2+} levels were observed for most compounds at lower concentrations. High concentrations of these test compounds are thus necessary to achieve calcium channel block. Examples of such concentration-dependent dualistic calcium channel ligands include FPL64176 and BayK8644 (García *et al.*, 1984 and Fonteríz *et al.*, 1987). The exact mechanism by which these compounds cause this unique effect in attenuating calcium levels is, however, still unknown.

It can thus be assumed that the effects of the individual polycyclic compounds are possibly the result of a combination of more than one pharmacological mechanism of action. It has also already been established in previous papers that certain polycyclic cage analogues have the ability to act as sigma receptor (Kassiou *et al.*, 1996, Kornhuber *et al.*, 1993 and Nguyen *et al.*, 1996), NMDA receptor (Geldenhuys *et al.*, 2007) and calcium channel ligands (Van der Schyf *et al.*, 1986, Malan *et al.*, 2000 and Kiewert *et al.*, 2006).

CHAPTER 6

CONCLUSION

Over the last five decades, the polycyclic compounds have attracted considerable interest from medicinal chemists. Ranging from their antiviral activity to their inhibiting potential in excitotoxicity, this group of compounds have been scrutinised as chemical entities to be used in the treatment, cure and prevention of various disease states (Geldenhuys *et al.*, 2005).

After intense research on the pentacycloundecane cage structures during the first era of these investigations, this study intended to divert the attention to their derivatives, the triquinane compounds. With a particular focus on NMDA receptor and calcium channel block, these compounds were synthesised for biological testing, to determine their effect on calcium flux.

The Diels Alder reaction for the synthesis of the polycyclic cage compounds was utilised to obtain these basic building blocks of the triquinanes. The thermal fragmentation of pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione during a pyrolysis process and subsequent hydrogenation, yielded the triquinanes. Reductive amination of the triquinanes gave lead to the formation of the required triquinylamine test compounds, although in exceptionally low yields. We ascribed these low yields to unoptimised reaction conditions, as well as the oily nature of the products, which complicated purification. An additional derivative of the triquinanes, the lactol derivative, was synthesised to evaluate the necessity of a nitrogen atom as part of the compound structure for calcium homeostasis activity.

The biological evaluation included the measurement of intracellular calcium ion concentrations by means of confocal microscopic techniques and fluorometric microplate reader measurements. Due to possible experimental inadequacies, prolonged recording times and possible cell movement during this time, the former technique yielded no statistically significant data. The microplate reader measurements yielded unexpected, but particularly interesting results. Measurements of the ratio of fluorescence at two excitation wavelengths of the molecular probe, Fura-2/AM, served as confirmation of previous results obtained for the pharmacological activity of this group of compounds.

According to various authors, the pentacycloundecylamine structure has the ability to attenuate intracellular calcium ion concentrations by acting on a variety of receptors (Geldenhuys *et al.*, 2004). However, the compounds synthesised in this study have never before been described as calcium channel agonists. According to the results obtained during the microplate reader analyses, all the aza-bridgehead test compounds increased the $[Ca^{2+}]_i$ at test compound concentrations below 10-100 µM. This observation, together with the observation that they inhibited calcium flux at high concentrations, can serve as proof that the nitrogen containing polycyclic compounds act *via* a dual mechanism in the homeostasis of calcium ions.

It has also previously been described that the antagonism of σ_1-receptors leads to an increase in $[Ca^{2+}]_i$ (Zhang and Cuevas, 2002) and that, due to the release of calcium ions from the endoplasmic reticulum after σ_2-receptor stimulation (Hellewel and Bowen, 1990), the opposite is true for this receptor subtype. The dualistic effect of especially the aza-bridgehead test compounds is thus probably the result of binding to sigma receptors and / or L-type calcium channels. The main reason for suspecting these two mechanisms of action is the profound expression of both types of sigma receptors (Hellewel and Bowen, 1990), as well as at least three types of calcium channels in the cells that were used during these experiments (Streit and Lux, 1987, Streit and Lux, 1989, Garber *et al.*, 1989 and Plummer *et al.*, 1989). In addition to this, it has been established that the PC-12 rat phaeochromocytoma cells lack the PCP binding site receptors that reside in the NMDA receptor ion channels (Yang *et al.*, 1989). The calcium channel blocking effects obtained at high concentrations in these experiments can thus not be attributed to non-competitive NMDA receptor block through interaction with these specific sites.

It is interesting to note that the test compound with the most profound calcium blocking activity at high concentrations was the benzylamino substituted triquinane derivative. This compound was previously included in a series of triquinylamine derivatives synthesised and tested by Bezuidenhout (2007) for calcium channel and NMDA receptor antagonistic activity, during which it was described as only a moderate blocker of the voltage-dependent calcium channels. The current study, however, suggests that sigma receptor effects may also play a role in the observed activity of these compounds.

With the exception of the results obtained for the phenethylamino substituted derivative, the calcium flux inhibition obtained for the triquinylamines were generally higher than that for the pentacycloundecylamines at 100 µM test concentrations. However, all the pentacycloundecylamines showed a suppression in calcium levels at 10 µM, which was not observed for the triquinylamines. The difference in activity between the aza-bridged pentacycloundecylamines and the triquinylamines can partially be attributed to the increased hydrophilicity of the cage compounds caused by the additional hydroxyl group. This structural difference is possibly also what accounts for a different pharmacological mechanism of action in modulating calcium flux. On the other hand, the aza-bridgehead cage compounds were generally more active as calcium ion channel stimulators, when compared to the triquinylamines. Once again, different mechanisms of action are possibly accountable for this effect.

The lactol triquinane derivative showed completely different results from the nitrogen containing compounds. An elevation in calcium flux was observed with an increase in the test compound concentrations. This might also be ascribed to binding to either σ_1- or σ_2-receptors, since antagonism of the σ_1-receptor and / or stimulation of the σ_2-receptors will result in such a concentration-dependent increase in calcium levels. It can, however, not be declared with any certainty which one of the two receptors play the bigger role in this mechanism of action. Clearly, more specific biological evaluations on these compounds are required to determine a more detailed description of their pharmacological action.

The radioligand binding evaluations that were performed in this study indicated that none of the test compounds displaced [^{3}H]MK-801 from the phencyclidine binding site. Despite this inability to displace MK-801 from its binding site, these compounds still show an inhibition of calcium flux into neuronal cells at high concentrations. This suggests that a mechanistic site(s) of action other than the phencyclidine binding site of the NMDA channel or effects on other calcium channels might play a role in the activity of these polycyclic derivatives.

Final Remarks

It has been repetitively proven over the years that excessive calcium influx is what eventually leads to neuronal death and apoptosis. Several compounds, amongst which the polycyclic amines, have been investigated and identified as inhibitors of such calcium flux and the current study is no exception. Amongst the pharmacological mechanisms involved in this calcium flux inhibition, is the possibility of these compounds acting as sigma receptor ligands. When taking into consideration the role that the sigma receptors play in the motor system (Goldstein *et al.*, 1989), the identification of the test compounds as sigma receptor ligands might thus automatically classify them as possible averters of conditions like Parkinson's disease. The sigma stimulated release of dopamine (Berkowitz, 1974), as well as the role of sigma receptors in idiopathic dystonia (Bowen *et al.*, 1988) further support this theory. However, the sigma receptor activity of these compounds should still be confirmed by means of further biological evaluations.

In addition to their possible role as sigma receptor ligands, the compounds that were tested in this study may also have a direct action on the L-type calcium channels, especially at higher concentrations. This conclusion is derived from results obtained in former studies on the pentacycloundecyl amines (Van der Schyf *et al.*, 1986, Liebenberg *et al.*, 2000 and Malan *et al.*, 2000), as well as from the distinct expression of these calcium channels in the cells that were used for this biological testing (Streit and Lux, 1987, Streit and Lux, 1989, Garber *et al.*, 1989 and Plummer *et al.*, 1989). As L-type calcium channel blockers, the test compounds may thus still play a role in neuroprotection.

From the results obtained it is thus clear that, despite the possibility of a new role for the test compounds as biological agents, the biological profile of both the cage compounds and triquinane derivatives remains to be clarified. More in depth structure-activity analyses of these potential multifunctional agents are thus required to enhance their current position in preventative drug treatment. Radioligand binding studies with radioactive sigma receptor ligands and calcium channel antagonists are warranted as the focal point of future ongoing studies on these compounds. In addition to this, cell viability studies may be used to exclude possible toxic effects of the test compounds in the results obtained on neuronal cells.

ADAMS, M.E. & OLIVERA, B.M. 1994. Neurotoxins: Overview of an emerging research technology. *Trends in Neuroscience,* 17:151-155.

ANDREWS, P.R. & LLOYD, E.J. 1986. Common structural features of drugs, transmitters and peptides in the central nervous system. *Progress in Medicinal Chemistry,* 23:91-119.

BASKIN, I.I., BELENIKIN, M.S., EKIMOVA, YE, V., PALYULIN, V.A. & ZEFIROV, N.S. 2000. Molecular modeling of glutamate receptors. [1st Russian Electronic conference on bioformatics (RECIB-2000), 15.03.2000-21.04.2000, Poster E02, Full display: http://www.ibmh.msk.su/recob/E/E02-poster-rus.htm] [Date of access: 13 March 2005].

BERKOWITZ, B.A. 1974. Effects of the optical isomers of the narcotic antagonist analgesic pentazocine on brain and heart biogenic amines. *European Journal of Pharmacology,* 26:359-365.

BEZUIDENHOUT, L. 2007. Triquinylamines as regulators of calcium homeostasis of neuronal cells. North-West University: Potchefstroom campus. (Dissertation – M.Sc.) 154 p.

BIGGE, C.F. 1999. Ionotropic glutamate receptors. *Current Opinion in Chemical Biology,* 3:441-447.

BIOTEK®. 2006. Synergy™ 4 Multi-Mode Microplate Reader with Hybrid Technology™. [Web:] http://www.biotek.com/products/ [Date of access: 17 August 2008].

BISCOE, T.J., EVANS, R.H., FRANCIS, A.A., MARTIN, M.R., WATKINS, J.C., DAVIES, J. & DRAY, A. 1977. D-α-aminoadipate as a selective antagonist of amino acid-induced and synaptic excitation of spinal neurons. *Nature,* 270:743-745.

BONNETT, R., BROWN, R.F.C. & SMITH, R.G. 1973. Isonindole. *Journal of the Chemical Society, Perkin Transactions 1,* 13:1432-1436.

BOWEN, W.D., VILNER, B.J., WILLIAMS, W., BERTHA, C.M., KUEHNE, M.E. & JACOBSON, A.E. 1995. Ibogaine and its congeners are sigma 2 receptor-selective ligands with moderate affinity. *European Journal of Pharmacology,* 279:R1-R3.

BOWEN, W.D., WALKER, J.M., YASHAR, A.G., MATSUMOTO, R.R., WALKER, F.O. & LORDEN, J.F. 1988. Altered haloperidol-sensitive sigma receptors in the genetically dystonic (dt) rat. *European Journal of Pharmacology,* 147:153-154.

BREDT, D.S. & SNYDER, S.H. 1990. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proceedings of the National Academy of Sciences U.S.A.,* 87:682-685.

BROWN, R.F.C. 1980. Pyrolytic methods in organic chemistry: application of flow and flash vacuum pyrolytic techniques. Vol. 41. New York: Academic Press. 374 p.

BROWN, R.F.C. 2001. Chemobiography. Royal Australian Chemical Institute: Melbourne . 137p.

BUCK, L. & AXEL, A. 1991. A novel multigene family may encode odorant receptors: A basis for odor recognition. *Cell*, 65:175–187.

CANO-ABAD, M.F., VILLARROYA, M., GARCÍA, A.G., GABILAN, N.H. & LÓPEZ, M.G. 2001. Calcium entry through L-type calcium channels causes mitochondrial disruption and chromaffin cell death. *The Journal of Biological Chemistry*, 276:39695-39704.

CATTERALL, W.A. 1996. Molecular properties of sodium and calcium channels. *Journal of Bioenergetic and Biomembranes*, 28:219-230.

CHOI D.W. 1998. Glutamate neurotoxicity and diseases of the nervous system. *Neuron*, 1:623–634.

CNSFORUM, LUNDBECK INSTITUTE. 2002. CNS Image bank: Dementia – NMDA receptor systems. NMDA receptor, showing different subtypes. [Web:] http://www.cnsforum.com/content/pictures/imageband/hirespng/hrl_rcpt_sys_NMDA.png [Date of access: 10 November 2008].

COOKSON, R.C., GRUNDWELL, E. & HUDEC, J. 1958. Synthesis of cage-like molecules by irradiation of Diels-Alder adducts. *Chemistry and Industry*, 1003-1004.

CORBETT, R.E., LAUREN, D.R. & WEAVERS, R.T. 1979. The structure of laurenene, a new diterpene from the essential oil of *Dacrydium cupressinum*. *Journal of the Chemical Society, Perkin Translations I*, 1774-1790.

DAVIES, J. & WATKINS, J.C. 1979. Selective antagonism of amino acid-induced and synaptic excitation in the cat spinal cord. *Journal of Physiology (London)*, 297:621–635.

DILBERTO, P.A., WANG, X.F. & HERMAN, B. 1994. Confocal imaging of Ca^{2+} in cells. *Methods in Cell Biology*, 40:243–262.

DINGLEDINE, R. & McBAIN, C.J. 1999. Glutamate and aspartate. (*In* Siegel, G.J., Agranoff, B.W., Albers, R.W., Fisher, S.K. & Uhler, M.D., *eds*. Basic neurochemistry. Philadelphia, N.Y. : Lippincot-Raven Publishers. p.316-333.)

DUNLAP, K., LUEBKE, J.I. & TURNER, T.J. 1995. Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends in Neurological Science,* 18:89-98.

EVANS, R.H., FRANCIS, A.A., HUNT, K., OAKES, D.J. & WATKINS, J.C. 1979. Antagonism of excitatory amino acid-induced responses and of synaptic excitation in the isolated spinal cord of the frog. *British Journal of Pharmacology,* 67:591–603.

EVANS, R.H., FRANCIS, A.A. & WATKINS, J.C. 1978. Mg^{2+}-like selective antagonism of excitatory amino acid-induced responses by α,ε–diaminopimelic acid, D-α-aminoadipate and HA-966 in isolated spinal cord of frog and immature rat. *Brain Research,* 148:536–542.

FONTERÍZ, R.I., GANDÍA, L., LOPÉZ, M.G., ARTALEJO, C.R. & GARCÍA, A.G. 1987. Dihydropyridine chirality at the chromaffin cell calcium channel. *Brain Research,* 408:359-363.

FRAUSTO DA SILVA, J.J.R. & WILLIAMS, R.J.P. 1991. The biological chemistry of the elements: The inorganic chemistry of life. 1st ed. Oxford : Clarendon Press. 557p.

GARBER, S.S., HOSHI, T. & ALDRICH, R.W. 1989. Regulation of ionic currents in pheochromocytoma cells by nerve growth factor and dexamethasone. *Journal of Neuroscience,* 9:3976–3987.

GARCÍA, A.G., SALA, F., REIG, J.A., VINIEGRA, S., FRAS, J., FONTERÍZ, R. & GANDÍA, L. 1984. Dihydropyridine BAY-K-8644 activates chromafin cell calcium channels. *Nature,* 309:69-71.

GARTHWAITE, J., CHARLES, S.L. & CHESS-WILLIAMS, R. 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature,* 336:385-388.

GELDENHUYS, W.J., MALAN, S.F., BLOOMQUIST, J.R., MARCHAND, A.P. & VAN DER SCHYF, C.J. 2005. Pharmacology and structure-activity relationships of bioactive polycyclic cage compounds: a focus on pentacycloundecane derivatives. *Medicinal Research Reviews,* 25:21-48.

GELDENHUYS, W.J., MALAN, S.F., BLOOMQUIST, J.R. & VAN DER SCHYF, C.J. 2007. Structure-activity relationships of pentacycloundecylamines at the *N*-methyl-D-aspartate receptor. *Bioorganic & Medicinal Chemistry,* 15:1525-1532.

GELDENHUYS, W.J., MALAN, S.F., MURUGENSAN, T., VAN DER SCHYF, C.J. & BLOOMQUIST, J.R. 2004. Synthesis and biological evaluation of pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane derivatives as potential therapeutic agents in Parkinson's disease. *Bioorganic and Medical Chemistry,* 12:1799-1806.

GELDENHUYS, W.J., TERRE'BLANCHE, G., VAN DER SCHYF, C.J. & MALAN, S.F. 2003. Screening of novel pentacyclo-undecylamines for neuroprotective activity. *European Journal of Pharmacology,* 458:73-79.

GLOSSMANN, H., FERRY, D.R., LÜBBECKE, F., MEWES, R. & HOFMANN, F. 1982. Calcium channels: Direct identification with radioligand binding studies. *Trends in Pharmacological Sciences,* 13:256-262.

GLOSSMAN, H., FERRY, D.R., STRIESSNIG, J., GOLL, A. & MOOSBURGER, K. 1987. Resolving the structure of the Ca^{2+} channel by photoaffinity labelling. *Trends in Pharmacological Sciences,* 8:95-100.

GLOSSMANN, H. & STRIESSNIG, J. 1990. Molecular properties of calcium channels. *Journal of Physiological and Biochemical Pharmacology,* 114:1-105.

GOLDSTEIN, S.R., MATSUMOTO, R.R., THOMPSON, T.L., PATRICK, R.L., BOWEN, W.D. & WALKER, J.M. 1989. Motor effects of two sigma ligands mediated by nigrostriatal dopamine neurons. *Synapse,* 4:254-258.

GREENE, J.G. & GREENAMYRE, J.T. 1996. Bioenergetics and glutamate excitotoxicity. *Progress in Neurobiology,* 48:613-634.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry,* 260: 3440–3450.

GUNDLACH, A.L., LARGENT, B.L. & SNYDER, S.H. 1986. Autoradiographic localization of σ-receptor binding sites in guinea pig and rat central nervous system with (+)-^{3}H-3-(3-hydroxyphenyl)-N-(1-propyl)-piperidine. *Journal of Neuroscience,* 6:1757-1770.

HARWOOD, L.M., MOODY, C.J. & PERRY, J.M. 1999. Experimental organic chemistry. 2nd ed. Oxford: Blackwell Science. 689 p.

HAUGLAND, R.P. 2005. The handbook: A guide to fluorescent probes and labelling technologies. 10th ed. Invitrogen Corp. 1126 p.

HELLEWEL, S.B. & BOWEN, W.D. 1990. A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. *Brain Research,* 527:244-253.

HELLEWEL, S.B., BRUCE, A., FEINSTEIN, G., ORRINGER, J., WILLIAMS, W. & BOWEN, W.D. 1994. Rat liver and kidney contain high densities of sigma 1 and sigma 2 receptors: characterization by ligand binding and photoaffinity labeling. *European Journal of Pharmacology,* 268:9-18.

HOCKERMAN, G.H., PETERSON, B.Z., JOHNSON, B.D. & CATTERALL, W.A. 1997. Molecular determinants of drug binding and action on L-type calcium channels. *Annual Reviews in Pharmacology and Toxicology,* 37:361-396.

HOLZ, R.W. & FISHER, S.K. 1999. Basic Neurochemistry. (*In* Siegel, J., Agranoff, B.W., Albers, R.W., Fisher, S.K. & Uhler, M.D., *eds.* Synaptic transmission and cellular signalling: an overview. Philadelphia, N.Y. : Lippincot-Raven Publishers. p. 192-212.)

IVERSEN, L.L. & KEMP, J.A. 1994. Non-competitve NMDA antagonists as drugs. (*In* Collingridge, G. & Watkins, J. *eds.* The NMDA Receptor. 2nd ed. Oxford University Press, Oxford. p. 469-486.)

JANE, D.E., JONES, P.L.S.J., POOK, P.C.-K., TSE, H.-W. & WATKINS, J.C. 1994. Actions of two new antagonists showing selectivity for different sub-types of metabotropic glutamate receptor in the neonatal rat spinal cord. *British Journal of Pharmacology,* 112:809-816.

JIRGENSONS, A., KAUSS, V., KALVINSH, I., GOLD, M.R., DANYSZ, W., PARSONS, C.G. & QUACK, G. 2000. Synthesis and structure-affinity relationships of 1,3,5-alkylsubstituted cyclohexylamines binding at NMDA receptor PCP site. *European Journal of Medicinal Chemistry,* 35:555-565.

KANE, J.M., DALTON, C.R., VELAYO, N.L. & RAMPE, D. 1995. fpl 64176: The effect of the chain length separating the two aryl groups on calcium agonist activity. *Bioorganic & Medicinal Chemistry Letters,* 5:873-876.

KASSIOU, M., NGUYEN, V.H., KNOTT, R., CHRISTIE, M.J. & HAMBLEY, T.W. 1996. Trishomocubanes, a new class of selective and high affinity ligands for the sigma binding site. *Bioorganic and Medicinal Chemistry Letters,* 6:595-600.

KAUL, M. & LIPTON, S.A. 2002. The NMDA receptor – Its role in neuronal apoptosis and HIV-associated dementia. (*In* Herman, B.H., Frankenheim, J., Utten, R., Sheridan, P., Weight, F.F., Zukin, S., *eds.* Glutamate and addiction. Totowa, N.J. : Humana p. 71-80.)

KEMP, J.A. & McKERNAN, R.M. 2002. NMDA receptor pathways as drug targets. *Nature Neuroscience,* 5:1039-1042.

KERR, J.F.R., WYLLIE, A.H. & CURRIE, A.R. 1972. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *British Journal of Cancer,* 26:239–257.

KIEWERT, C., HARTMANN, J., STOLL, J., THEKKUMKARA, T.J., VAN DER SCHYF, C.J. & KLEIN, J. 2006. NGP1-01 is a brain-permeable dual blocker of neuronal voltage- and ligand-operated calcium. *Neurochemical Research,* 31:395-399.

KOHL, B.K. & DANNHARDT, G. 2001. The NMDA receptor complex: a promising target for novel antiepileptic strategies. *Current Medicinal Chemistry,* 8:1275-1289.

KORNHUBER, J., SCHOPPMEYER, K. & REIDERER, P. 1993. Affinity of 1-aminoadamantanes for the σ binding site in post-mortem human frontal cortex. *Neuroscience Letters,* 163:129-131.

KORNHUBER, J. & WELLER, M. 1995. Predicting psychotomimetic properties of PCP-like NMDA receptor antagonists. (*In* Fog, R., Gerlach, J., Hemmingsen, R., Krogsgaard-Larsen, P. & Thaysen, J.H. *eds.* Schizophrenia - An Integrated View. Alfred Benzen Symposium 38. Copenhagen : Munksgaard p.314-325.)

KOROSHETZ, W.J. & MOSKOWITZ, M.A. 1996. Emerging treatments for stroke in humans. *Trends in Pharmacological Sciences,* 17:227-233.

KROEMER, R.T., KOUTSILIERI, E., HECHT, P., LIEDL, K.R., RIEDERER, P. & KORNHUBER, J. 1998. Quantitative analysis of the structural requirements for blockade of the *N*-methyl-D-aspartate receptor at the phencyclidine binding site. *Journal of Medicinal Chemistry,* 41:393-400.

KWON, Y.W. & TRIGGLE, D.J. 1991. Ligand gated and Voltage-Gated Ion Channels. *Annual Reports in Medicinal Chemistry,* 25:225-234.

LAMBERT, D.G. 2006. Calcium Signalling Protocols. 2^{nd} ed. Humana Press: Totowa, New Jersey. 359 p.

LARGENT, B.L., WIKSTROM, H., GUNDLACH, A.L. & SNYDER, S.H. 1987. Structural determinants of sigma-1 receptor affinity. *Molecular Pharmacology,* 32:772-784.

LASHUEL, H.A., HARTLEY, D., PETRE, B.M., WALZ, T. & LANSBURY P.T. 2002. Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature,* 418:291.

LEESON, P.D., CARLING, R.W., JAMES, K., SMITH, J.D., MOORE, K.W., WONG, E.H.F. & BAKER, R. 1990. Role of hydrogen bonding in ligand interaction with the N-methyl-D-aspartate receptor ion channel. *Journal of Medicinal Chemistry,* 33:1296–1305.

LIEBENBERG, W. 1986. Stikstofbevattende derivate van pentasiklo-undekaan – 'n Nuwe reeks kalsiumkanaalblokkers. North-West University (Potchefstroom University for CHE): Potchefstroom Campus. (Dissertation – M.Sc.) 100p.

LIEBENBERG, W. 1989. Die sintese, konformasie en biologiese aktiwiteit van trikwinielamiene en verwante verbindings. Potchefstroom: PU for CHE (Thesis – Ph.D.) 129p.

LIEBENBERG, W., VAN ROOYEN, P.H. & VAN DER SCHYF, C.J. 1996. The biological activity of two symmetric amine derivatives of the *cis-syn-cis* triquinane system. *Pharmazie,* 51:20-24.

LIEBENBERG, W., VAN DER WALT, J.J. & VAN DER SCHYF, C.J. 2000. Effects of derivatives of NGP1-01, a putative calcium channel antagonist, on electrically stimulated guinea-pig papillary muscle. *Pharmazie,* 55:833-836.

LIMOUSINE, P., GREENE, J., POLLAK, P., ROTHWELL, J., BENABID, A.L., FRACKOWIAK, R. 1997. Changes in cerebral activity pattern due to subthalamic nucleus or internal pallidum stimulation in Parkinson's disease. *Annals of Neurology,* 42: 283-291.

LINNIK, M.D., ZOBRIST, R.H. & HATFIELD, M.D. 1993. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke,* 24:2002-2008.

LIPTON, S.A. 2006. Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. *Nature Reviews: Drug Discovery,* 5:160-170.

LIPTON, S.A. & NICOTERA, P. 1998. Excitotoxicity, free radicals, necrosis, and apoptosis. *Neuroscientist,* 5:345-352.

LIPTON, S.A. & ROSENBERG, P.A. 1994. Excitatory amino acids as a final common pathway for neurologic disorders. *The New England Journal of Medicine,* 330:613-622.

LIU, X., NUWAYHID, S., CHRISTIE, M.J., KASSIOU, M. & WERLING, L.L. 2001. Trishomocubanes: novel σ-receptor ligands modulate amphetamine-stimulated [^{3}H]dopamine release. *European Journal of Pharmacology,* 422:39-45.

LIU, Y. & ZHANG, J. 2000. Recent development in NMDA receptors. *Chinese Medical Journal,* 113:948-956.

LODGE, D. & ANIS, N.A. 1982. Effects of phencyclidine on excitatory amino acid activation of spinal interneurones in the cat. *European Journal of Pharmacology,* 77:203-204.

MACMANUS, J.P., BUCHAN, A.M., HILL, I.E., RASQUINHA, I. & PRESTON, E. 1993. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neuroscience Letters,* 159:89–92.

MALAN, S.F., DOCKENDOLF, G., VAN DER WALT, J.J., VAN ROOYEN, J.M. & VAN DER SCHYF, C.J. 1998. Enantiomeric resolution of the calcium channel antagonist 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01). *Pharmazie,* 53:859-862.

MALAN, S.F., DYASON, K., WAGENAAR, B., VAN DER WALT, J.J. & VAN DER SCHYF, C.J. 2003. The structure and ion channel activity of 6-benzylamino-3-hydroxyhexacyclo[6.5.0.0^{3,7}.0^{4,12}.0^{5,10}.0^{9,13}]tridecane. *Archive der Pharmazie,* 2:127-133.

MALAN, S.F., VAN DER WALT, J.J. & VAN DER SCHYF, C.J. 2000. Structure-activity relationships of polycyclic aromatic amines with calcium channel blocking activity. *Archiv der Pharmazie – Pharmaceutical & Medicinal Chemistry,* 333:10-16.

MANALLACK, D.T. & BEART, P.M. 1987. Quantitative conformational analyses predict distinct receptor sites for PCP-like and sigma drug. *European Journal of Pharmacology,* 144:231-235.

MANALLACK, D.T., BEART, P.M. & GUNDLACH, A.L. 1986. Psychotomimetic sigma-opiates and PCP. *Trends in Pharmacological Sciences,* 7:448-451.

MANALLACK, D.T., WONG, M.G., COSTA, M., ANDREWS, P.R. & BEAN, P. 1988. Receptor site topographies for phencyclidine-like and sigma drugs: Predictions from quantitative conformational, electrostatic potential, and radioreceptor analyses. *Molecular Pharmacology,* 34:863-879.

MARCHAND, A.P., ARNEY, B.E., DAVE, P.R. & SATYANARAYANA, N. 1988. Transannular cyclization in the pentacyclo$[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]$undecane-8-11-dione system: a reinvestigation. *Journal of Organic Chemistry,* 53:2644-2647.

MASON, R.P., LEEDS, P.R., JACOB, R.F. HOUGH, C.J., ZHANG, K.-G., MASON, P.E. & CHUANG, D.-M. 1999. Inhibition of excessive neuronal apoptosis by the calcium antagonist Amlodipine and antioxidants in cerebellar granule cells. *Journal of Neurochemistry,* 72:1448-1456.

McLEAN, S. & WEBER, E. 1988. Autoradiographic visualisation of haloperidol-sensitive sigma receptors in guinea-pig brain. *Neuroscience,* 25:259-269.

McNAB, H. 2004. Chemistry without reagents: synthetic applications of flash vacuum pyrolysis. (*In* Firsan, S.J., *ed.* Aldrichimica Acta. Vol.37:No.1. Aldrich : Sigma-Aldrich Co. p. 13-28.)

MEHTA, G., SRIKRISHNA, A., REDDY, A.V. & NAIR, M.S. 1981. A novel, versatile synthetic approach to linearly fused tricyclopentanoids *via* photo-thermal olefin metathesis. *Tetrahedron,* 37:4543.

MEHTA, G. & SRIKRISHNA, A. 1997. Synthesis of polyquinane natural products: an update. *Chemistry Reviews,* 97:671-719.

MEHTA, G. & UMARYE, J.D. 2001. A stereoselective total synthesis of the novel triquinane sesquiterpene cucumin E. *Tetrahedron Letters,* 42:1991-1993.

MONN, J.A., THURKAUF, A., MATTSON, M.V., JACOBSON, A.E. & RICE, K.C. 1990. Synthesis and structure-activity relationship of C5-substituted analogues of (±)-10,11-Dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine [(±)-desmethyl-MK801]: ligands for the NMDA receptor-coupled phencyclidine binding site. *Journal of Medicinal Chemistry,* 33:1069-1076.

MUSCHOL, M. & SALZBERG, B.M. 2000. Dependence of transient and residual calcium dynamics on action-potential patterning during neuropeptide secretion. *Journal of Neuroscience,* 20:6773–6780.

NAKANISHI, S. & MASU, M. 1994. Molecular diversity and functions of glutamate receptors. *Annual Review of Biophysiology and Biomolecular Structure,* 23:319-348.

NATIONAL INSTITUTES OF HEALTH. 2008. NIH Image. [Web:] http://rsb.info.nih.gov/nih-image/ [Date of access: 15 March 2008].

NEELANDS, T.R. & MACDONALD, R.L. 1999. Voltage-gated calcium ion channels. (*In* Siegel, G.J., Agranoff, B.W., Albers, R.W., Fisher, S.K. & Uhler, M.D. *eds.* Basic Neurochemistry. Philadelphia, N.Y. : Lippincott-Raven Publishers. p. 460-461.)

NGUYEN, V.H., KASSIOU, M., JOHNSTON, G.A.R. & CHRISTIE, M.J. 1996. Comparison of binding parameters of sigma-1 and sigma-2 binding sites in rat and guinea pig brain membranes: novel subtypes-selective trishomocubanes. *European Journal of Pharmacology,* 311:233-240.

NOZOE, S., FURUKAWA, J., SANKAWA, U. & SHIBATA, S. 1976. Isolation, structure and synthesis of hirsutene, a precursor hydrocarbon of coriolin biosynthesis. *Tetrahedron Letters,* 17:195-198.

NYIREDY, S.Z., MEIER, B., ERDELMEIER, C.A.J. & STICHER, O. 1985. "PRISMA": A geometrical design for solvent optimization in HPLC. *HRC & CC. Journal of High Resolution Chromatography and Chromatography Communications,* 8:186-188.

OLIVER, D.W., DEKKER T.G. & SNYCKERS, F.O. 1991. Pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecylamines. Synthesis and pharmacology. *European Journal of Medicinal Chemistry,* 26:375-379.

OLIVERA, B.M., MILJANICH, G., RAMACHANDRAN, J. & ADAMS, M.E. 1994. Calcium Channel Diversity and Neurotransmitter Release: The ω-Conotoxins and ω-Agatoxins. *Annual Reviews in Biochemistry,* 63:823-867.

OZAKI, H., SATO, K., SATOH, T. & KARARI, H. 1987. Simultaneous recording of calcium signals and mechanical activity using fluorescent dye Fura-2 in isolated strips of vascular smooth muscle. *Japanese Journal of Pharmacology,* 45:429 – 433.

PARSONS, C.G., QUACK, G., BRESINK, I., BARAN, L., PRZEGALINSKI, E., KOSTOWSKI, W., KRZASCIK, P., HARTMANN, S. & DANYSZ, W. 1995. Comparison of the potency, kinetics and voltage-dependency of a series of uncompetitive NMDA receptor antagonists *in vitro* with anticonvulsive and motor impairment activity *in vivo*. *Neuropharmacology,* 34:1239-1258.

PATRICK, S.L., WALKER, J.M., PERKEL, J.M., LOCKWOOD, M. & PATRICK, R.L. 1993. Increases in rat striatal extracellular dopamine and vacuous chewing produced by two sigma receptor ligands. *European Journal of Pharmacology,* 231:243-249.

PLUMMER, M.R., LOGOTHETIS, D.E. & HESS, P. 1989. Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. *Neuron,* 2:1453–1463.

POLYA, G. 2003. Biochemical targets of plant bioactive compounds: A pharmacological reference guide to sites of action and biological effects. Taylor & Francis, Taylor & Francis Group, London and New York. 847 p.

PRINGLE, A. 2004. In, out, shake it all about: elevation of $[Ca^{2+}]_i$ during acute cerebral ischaemia. *Cell Calcium,* 36:235-245.

PUTNEY, J.W. 1999. Calcium. (*In* Siegel, G.J., Agranoff, B.W., Albers, R.W., Fisher, S.K. & Uhler, M.D., *eds.* Basic Neurochemistry. Philadelphia, N.Y. : Lippincott-Raven Publishers. p. 454-469.)

QUIRION, R., BOWEN, W.D., ITZHAK, Y., JUNIEN, J.L. MUSACCHIO, J.M. ROTHMAN, R.B., SU, T.P., TAM, S.W. & TAYLOR, D.P. 1992. A proposal for the classification of sigma binding sites. *Trends in Pharmacological Science,* 13:85-86.

RÜCKERT, N.G.H. & SCHMIDT, W.J. 1993. The receptor σ ligand 1,3-di-(2-tolyl)guanidine in animal models of schizophrenia. *European Journal of Pharmacology,* 233:261-267.

SAMDANI, A.F., DAWSON, T.M. & DAWSON, V.L. 1997. Nitric oxide synthase in models of focal ischemia. *Stroke,* 28:1283-1288.

SASAKI, T., EGUCHI, S. & KIRIYAMA, T. 1971. A facile synthesis of mono-oxa and -aza-bird-cage compounds via transannular cyclization. *Tetrahedron Letters,* 12:2651-2654.

SCHNEGGENBURGER, R. & ASCHER, P. 1997. Coupling of permeation and gating in an NMDA–channel pore mutant. *Neuron,* 18:167–177.

SCHWAB, R.S., POSKANZER, D.C., ENGLAND, A.C., Jr., YOUNG, R.R. 1972. Amantadine in the treatment of Parkinson's disease. Review of more than two years' experience. *Journal of the American Medical Association,* 222:792-795.

SCHWARTZMAN, R.A. & CIDLOWSKI, J.A. 1993. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocrine Reviews,* 14:133-151.

SELKOE, D.J. 1999. Translating cell biology into therapeutic advances in Alzheimer's disease. *Stroke,* 28:1283-1288.

SHEIKH, Y.M., SINGY, G., KAISIN, H., EGGERT, H., DJERASSI, C., TURSCH, B., DALOZE, D. & BRAECKMAN, J.C. 1976. Terpenoids LXXI. Chemical studies of marine invertebrates-XIV. Four representatives of a novel sesquiterpene class – the capnellane skeleton. *Tetrahedron,* 32:1171-1178.

SIESJO, B.K. & BENGTSSON, F. 1989. Calcium fluxes, calcium antagonists and calcium-related pathology in brain ischemia, hypoglycaemia spreading depression: A unifying hypothesis. *Journal of Cerebral Blood Flow Metabolism,* 9:127-140.

SMALL, D.L., MONETTE, R., BUCHAN, A.M. & MORLEY, P. 1997. Identification of calcium channels involved in neuronal injury in rat hippocampal slices subjected to oxygen and glucose deprivation. *Brain Research,* 753:209-218.

STREIT, J. & LUX, H.D. 1987. Voltage dependent calcium currents in PC12 growth cones and cells during NGF-induced cell growth. *Pflügers Archiv,* 408:634–641.

STREIT, J. & LUX, H.D. 1989. Distribution of calcium currents in sprouting PC12 cells. *Journal of Neuroscience,* 9:4190–4199.

SU, T.-P. 1982. Evidence for sigma opioid receptor: Binding of [^{3}H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain. *Journal of Pharmacology & Experimental Therapeutics,* 223:284-290.

SU, T.-P., LONDON, E.D. & JEFFE, J.H. 1988. Steroid Binding at σ Receptors Suggests a Link between Endocrine, Nervous, and Immune systems. *Science,* 240:219-221.

TAKAHASHI, A., CAMACHO, P., LECHLEITER, J.D. & HERMAN, B. 1999. Measurement of intracellular calcium. *Physiological Reviews,* 79:1089–1125.

TAKAHASHI, T. & MOMIYAMA, A. 1993. Different types of calcium channels mediate central synaptic transmission. *Nature,* 366:156-158.

TRAHANOVSKY, W.S. & DOYLE, M.P. 1968. Medium effects. II. Cyclization of 6-heptenyl p-nitrobenzenesulfonate during solvolysis in 2,2,2-trifluoroethanol. *Tetrahedron Letters,* 9:2155-2158.

TRIGGLE, D.J. 1991. Calcium channel drugs: structure-function relationships and selectivity of action. *Journal of Cardiovascular Pharmacology,* 18:S1-S6.

TSIEN, R.Y. 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature,* 290:527–528.

TSIEN, R.Y., POZZAN, T. & RINK, T.J. 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *Journal of Cellular Biology,* 94:325–334.

TURNER, T.J., ADAMS, M.E. & DUNLAP, K. 1993. Multiple calcium channel type coexist to regulate synaptosomal neurotransmitter release. *Proceedings of the National Academy of Sciences U.S.A.,* 90:9518-9522.

TYMIANSKI, M. & TATOR, C.H. 1996. Normal and abnormal calcium homeostasis in neurons: a basis for the pathophysiology of traumatic and ischemic central nervous system injury. *Neurosurgery,* 38:1176–1195.

VAN DER SCHYF, C.J., SQUIER, G.J. & COETZEE, W.A. 1986. Characterisation of NGP1-01, an aromatic polycyclic compound, as a calcium antagonist. *Pharmacological Research Communications,* 18:407-417.

VAN DER SCHYF, C.J., LIEBENBERG, W., BORNMAN, R., DEKKER, T.G., VAN ROOYEN, P.H., FOURIE, T.G., MATTHEE, E. & SNYCKERS, F.O. 1989. The polycyclic calcium antagonist, NGP1-01, has an oxa rather than aza bird-cage structure: evidence from n.m.r. spectroscopy and the X-ray crystal structure. *South African Journal of Chemistry,* 42:46-48.

VAN DER WALT, J.J., VAN DER SCHYF, C.J, VAN ROOYEN, J.M., DE JAGER, J. & VAN AARDE, M.N. 1988. Calcium current blockade in the cardiac myocytes by NGP1-01, an aromatic polycyclic amine. *South African Journal of Science,* 84:448-450.

VILNER, B.J. & BOWEN, W.D. 2000. Modulation of cellular calcium by sigma-2 receptors: release from intracellular stores in human SK-N-SH neuroblastoma cells. *Journal of Pharmacology & Experimental Therapeutics,* 292:900-911.

WALKER, J.M., WAYNE, D.B., WALKER, F.O., MATSUMOTO, R.R., DE COSTA, B. & RICE, K.C. 1990. Sigma receptors: Biology and function. *Pharmacological Reviews,* 42:355-402.

WATKINS, J.C. & EVANS, R.H. 1981. Excitatory amino acid transmitters. *Annual Review of Pharmacology and Toxicology,* 21:165-204.

WOLLMUTH, L.P., KUNER, T. & SAKMANN, B. 1998. Adjacent asparagines in the NR2-subunit of the NMDA receptor channel control the voltage-dependent block by extracellular Mg^{2+}. *The Journal of Physiology,* 506:13-32.

WONG, E.H.F. & KEMP, J.A. 1991. Sites for antagonism on the N-methyl-D-aspartate receptor channel complex. *Annual Review of Pharmacology and Toxicology,* 31:401-425.

WONG, E.H.F., KEMP, J.A., PRIESTLY, T., KNIGHT, A.R., WOODRUFF, G.N. & IVERSEN, L.L. 1986. The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist. *Proceedings of the National Academy of Sciences U.S.A.,* 83:7104-7108.

YANG, Z.W., PALEOS, G.A. & BYRD, J.C. 1989. Expression of (+)-3-PPP binding sites in the PC12 pheochromocytoma cell line. *European Journal of Pharmacology,* 164:607-610.

ZHANG, H. & CUEVAS, J. 2001. Sigma receptors modulate electrical properties of autonomic neurons. *Federation of American Societies for Experimental Biology Journal,* 15:202-221.

ZHANG, H. & CUEVAS, J. 2002. Sigma receptors inhibit high-voltage-activated calcium channels in rat sympathetic and parasympathetic neurons. *Journal of Neurophysiology,* 87:2867-2879.

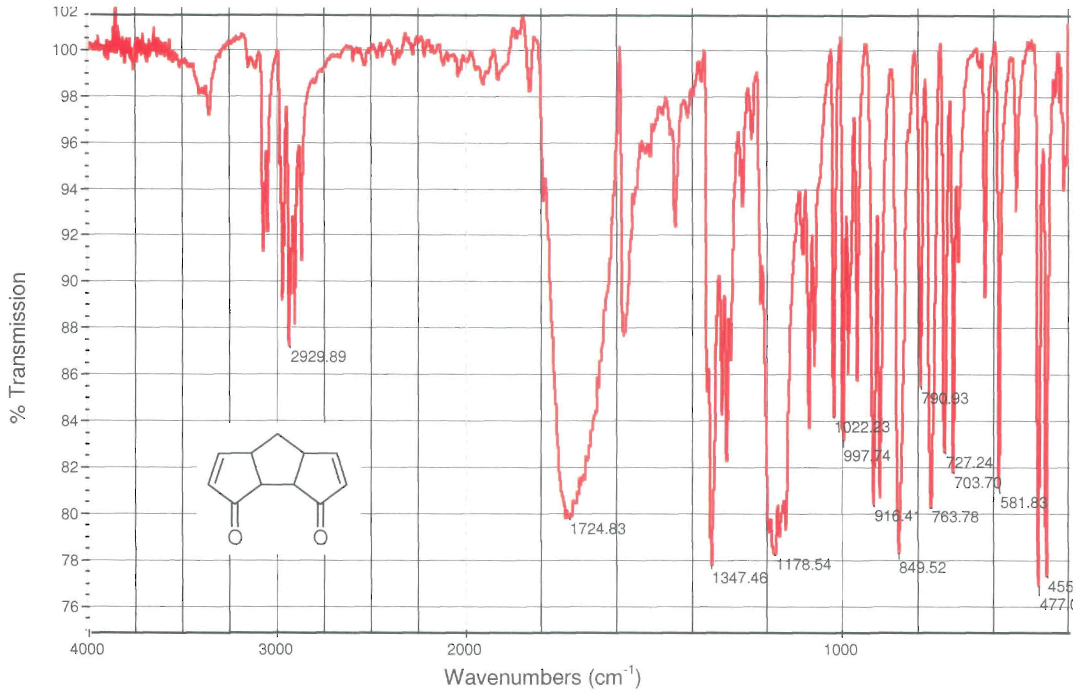
ZHOROV, B.S. & TIKHONOV, D.B. 2004. Potassium, sodium, calcium and glutamate-gated channels: pore architecture and ligand action. *Journal of Neurochemistry,* 88:782-799.

APPENDIX I

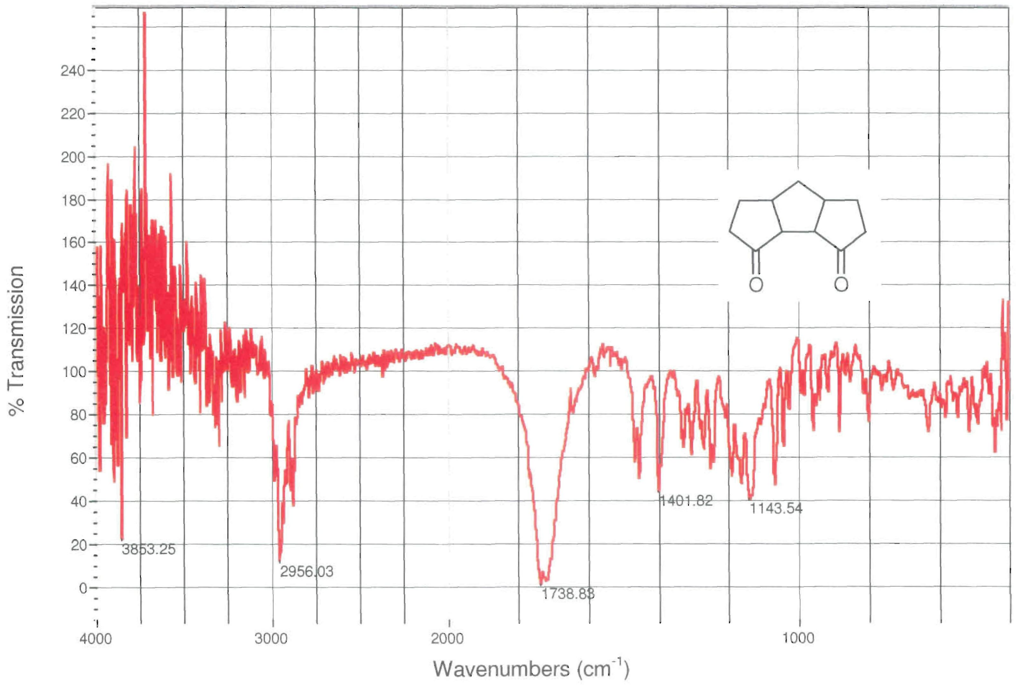
SPECTRAL DATA

1. INFRARED SPECTROSCOPY
2. MASS SPECTROMETRY
3. ^{1}H NUCLEAR MAGNETIC RESONANCE SPECTROMETRY
4. ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

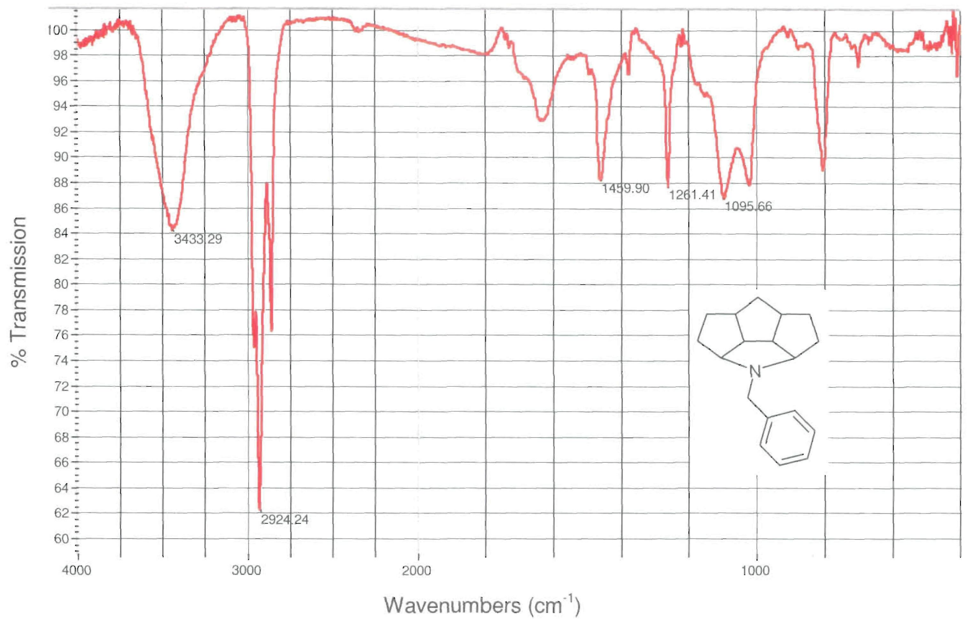
1. INFRARED SPECTROSCOPY



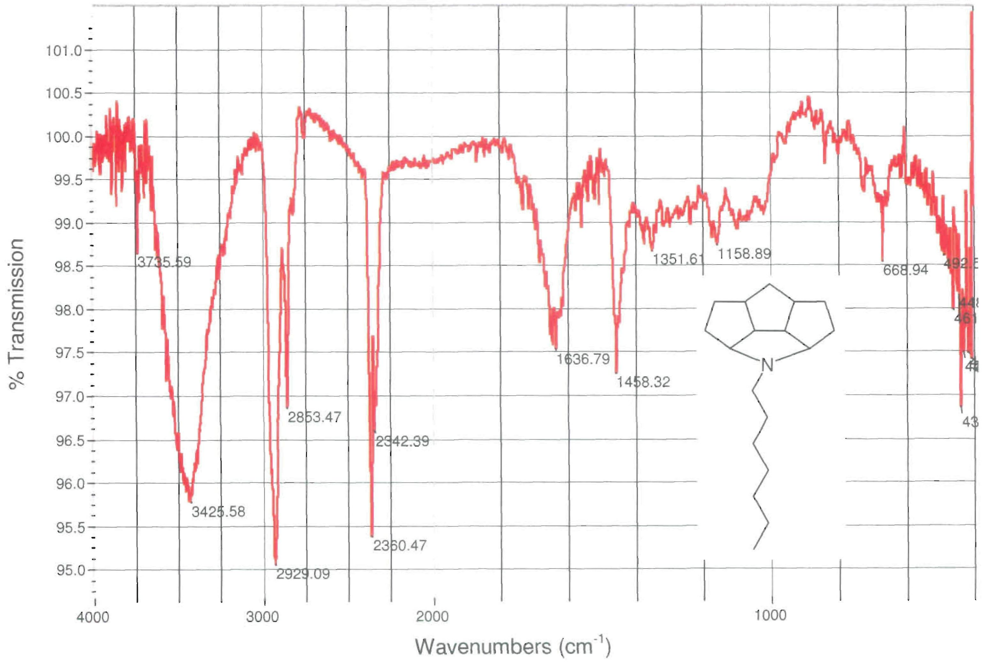
SPECTRUM 1.1



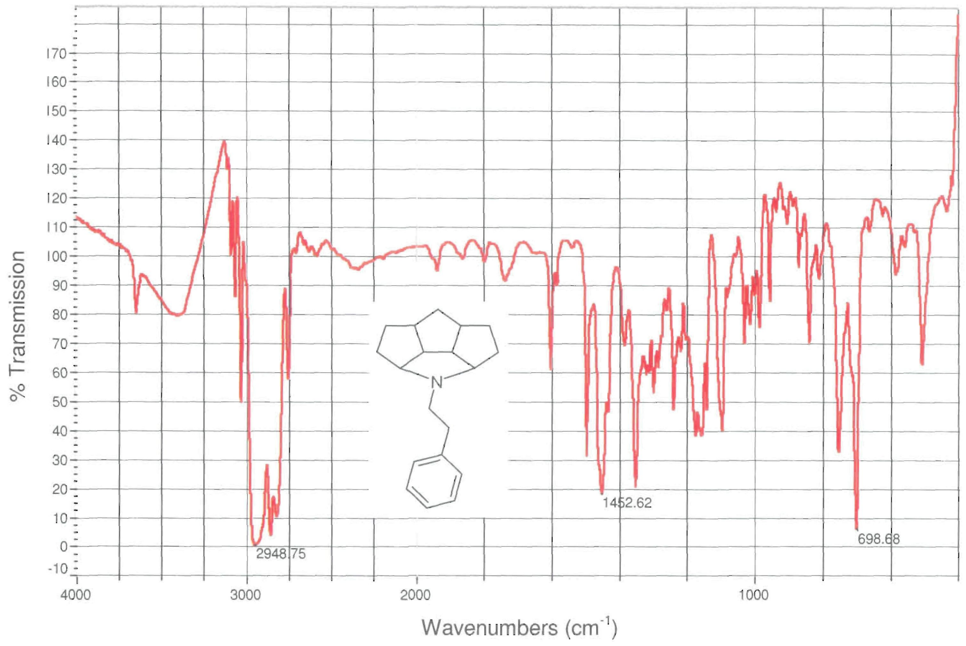
SPECTRUM 1.2



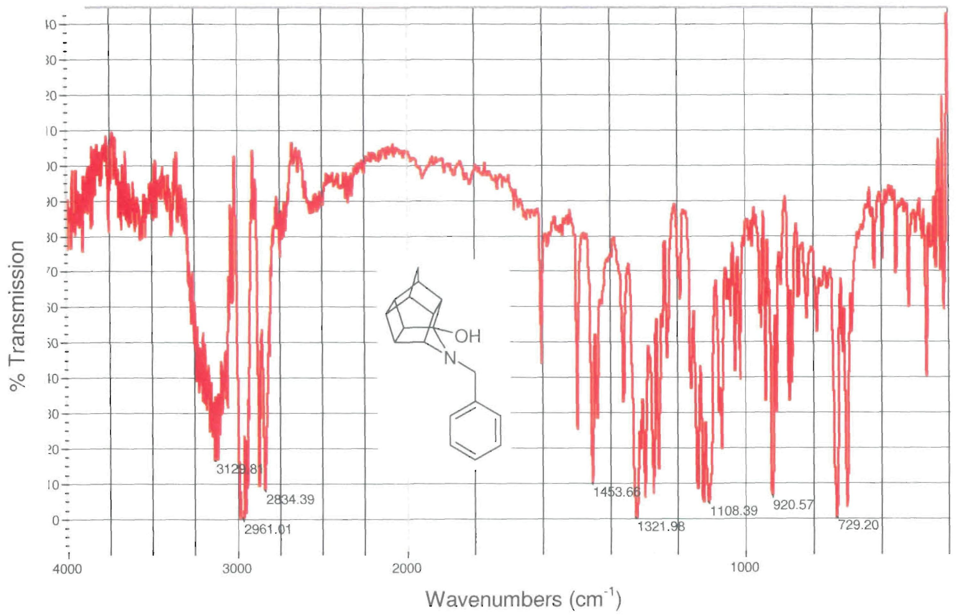
SPECTRUM 1.3



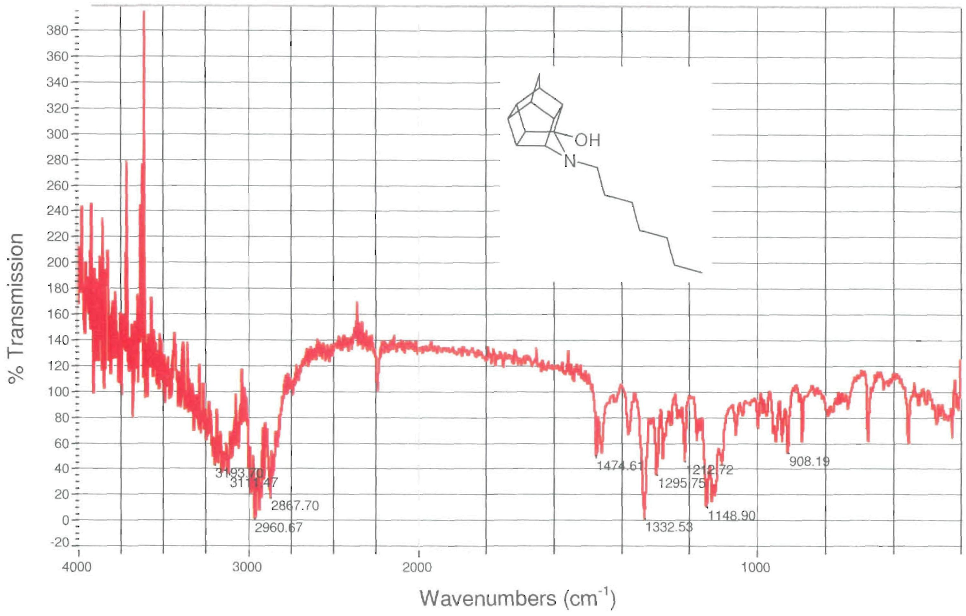
SPECTRUM 1.4



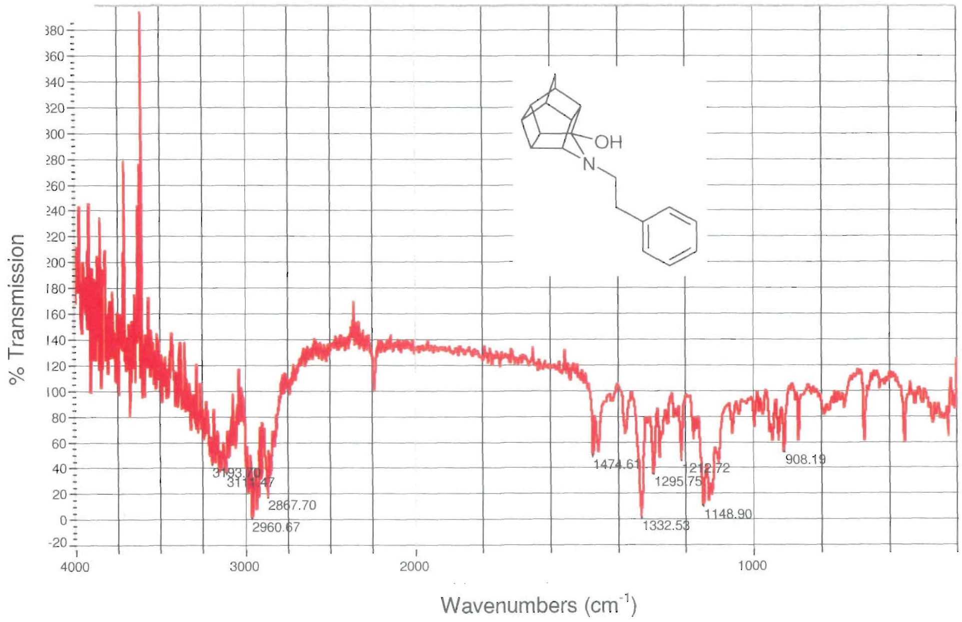
SPECTRUM 1.5



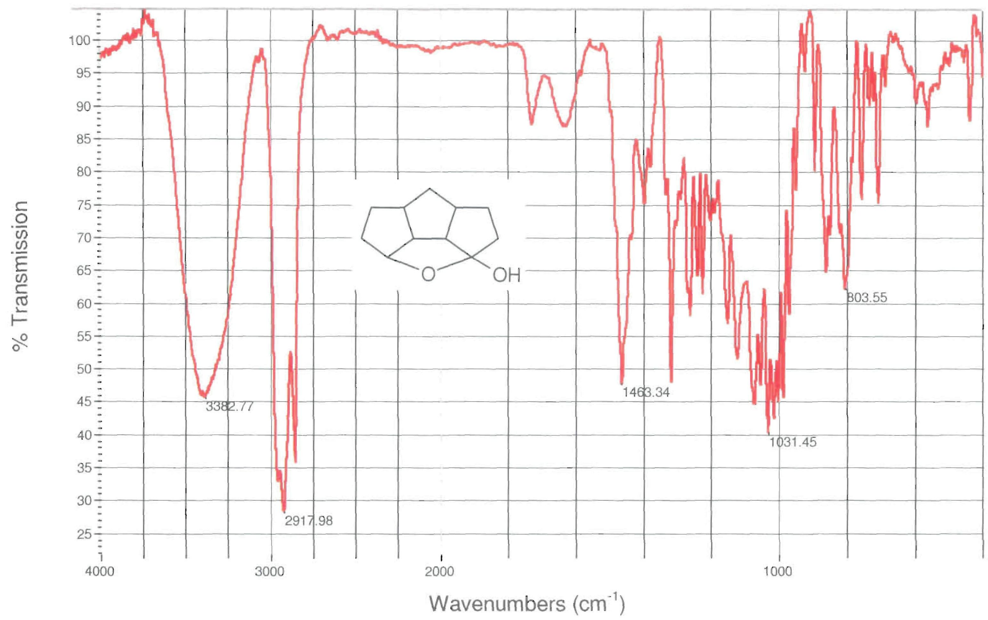
SPECTRUM 1.6



SPECTRUM 1.7

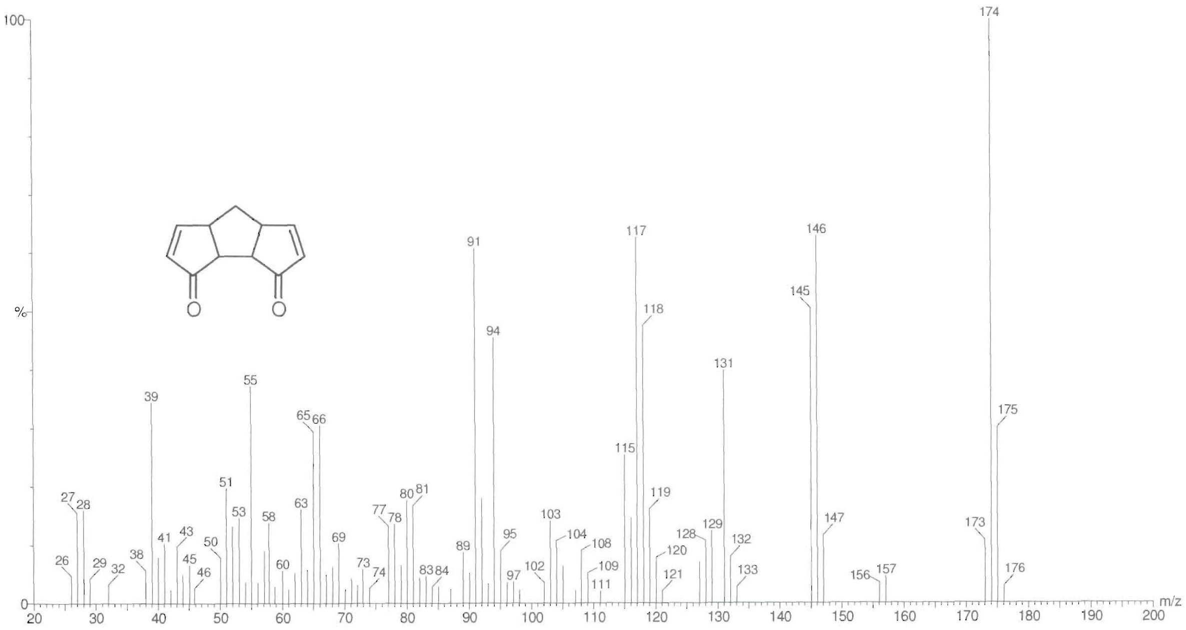


SPECTRUM 1.8

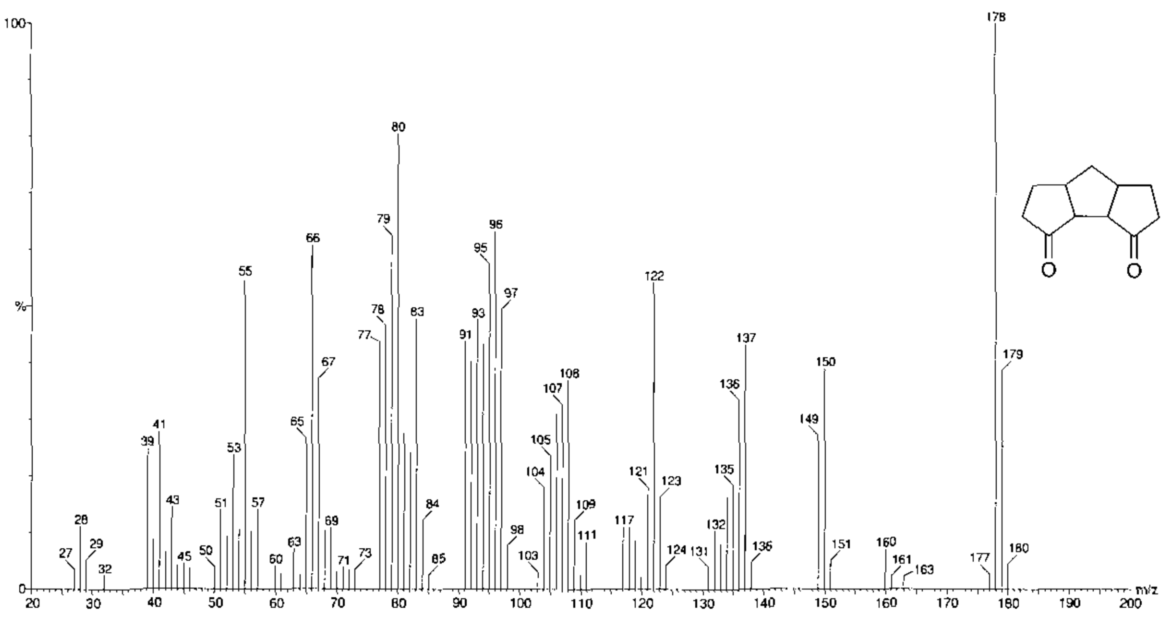


SPECTRUM 1.9

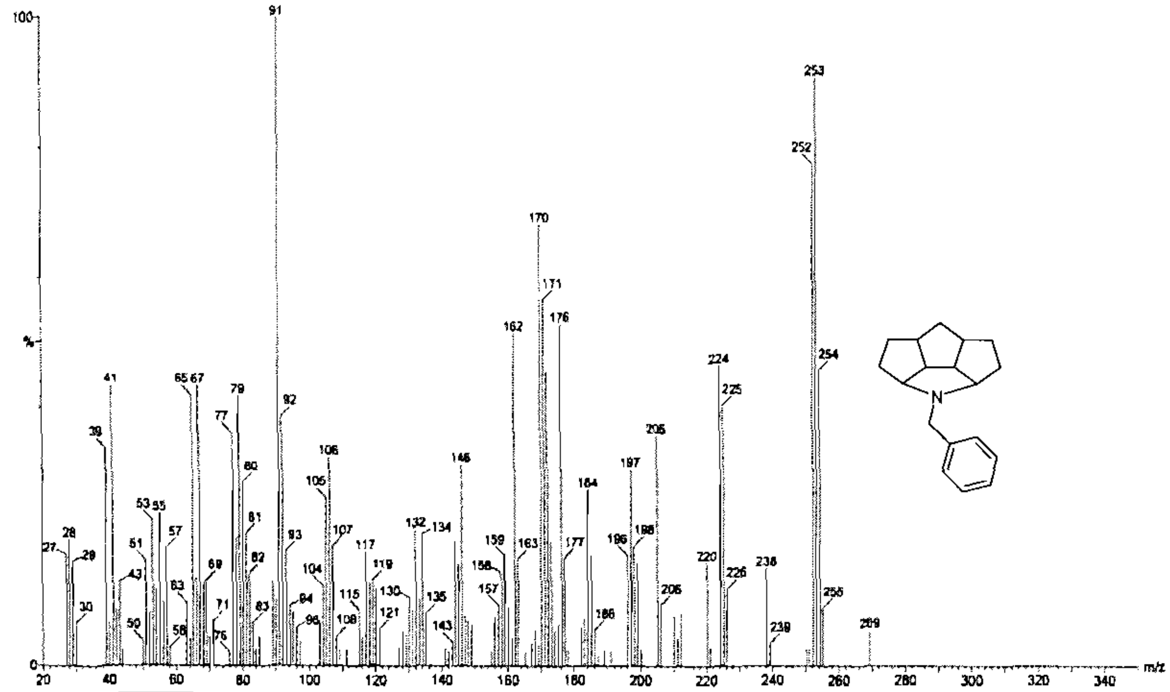
2. MASS SPECTROMETRY



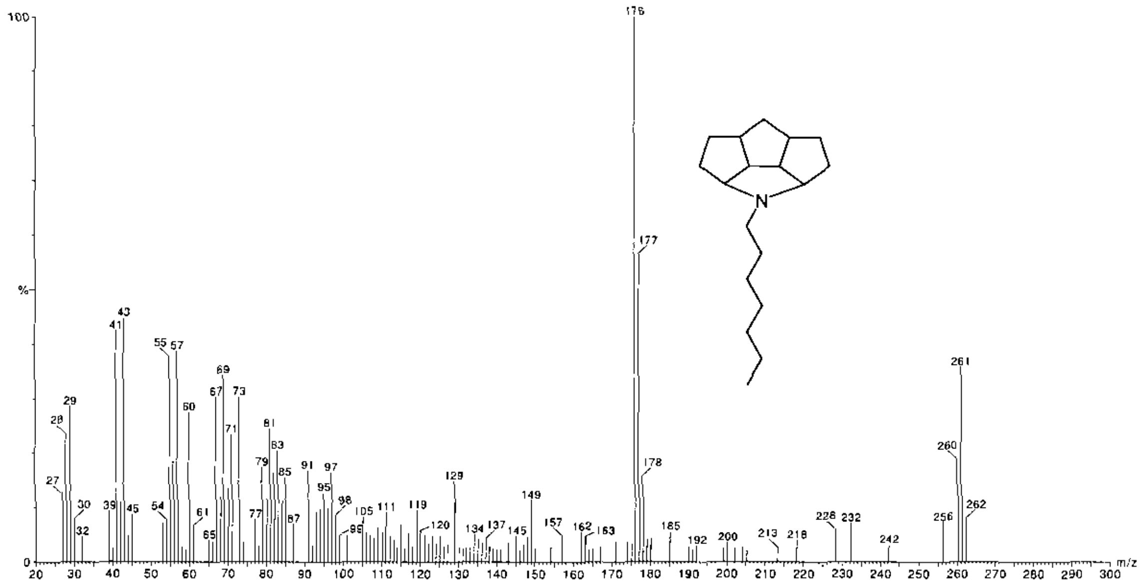
SPECTRUM 2.1



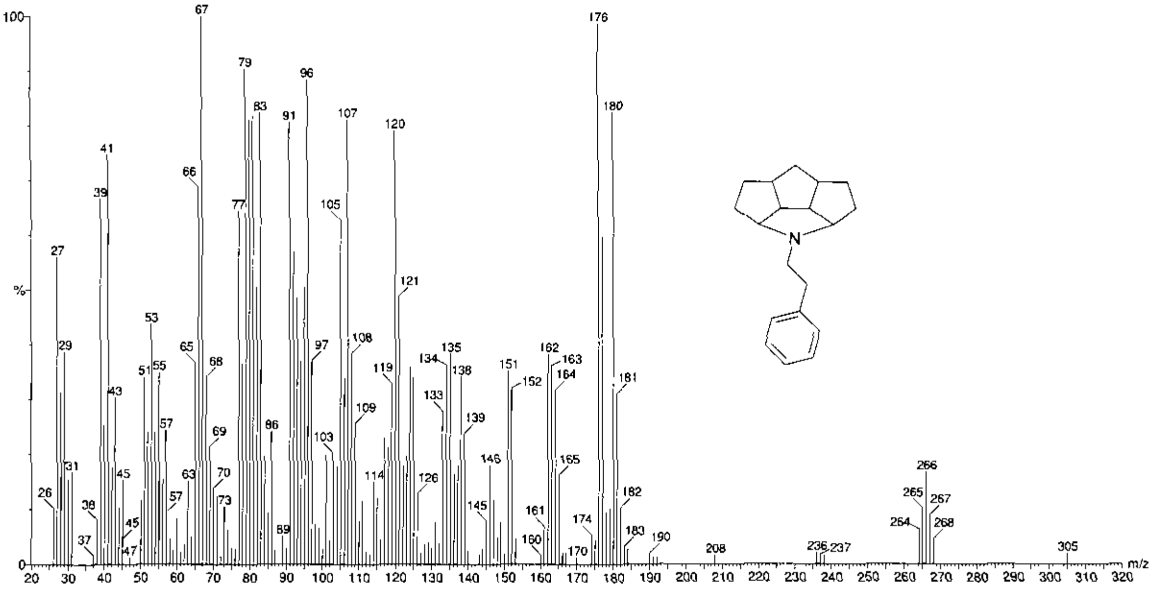
SPECTRUM 2.2



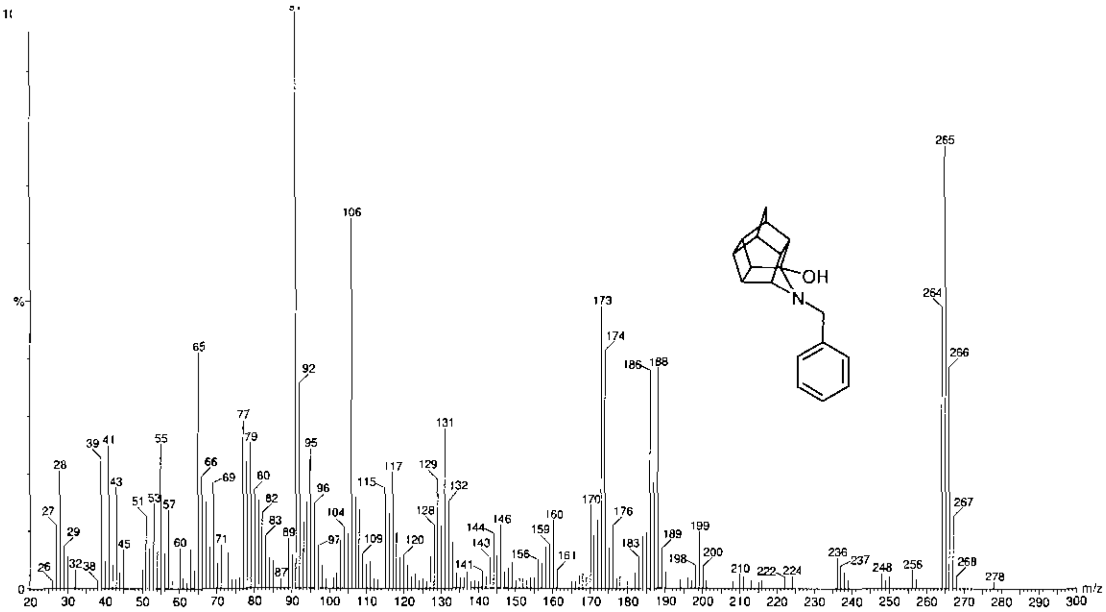
SPECTRUM 2.3



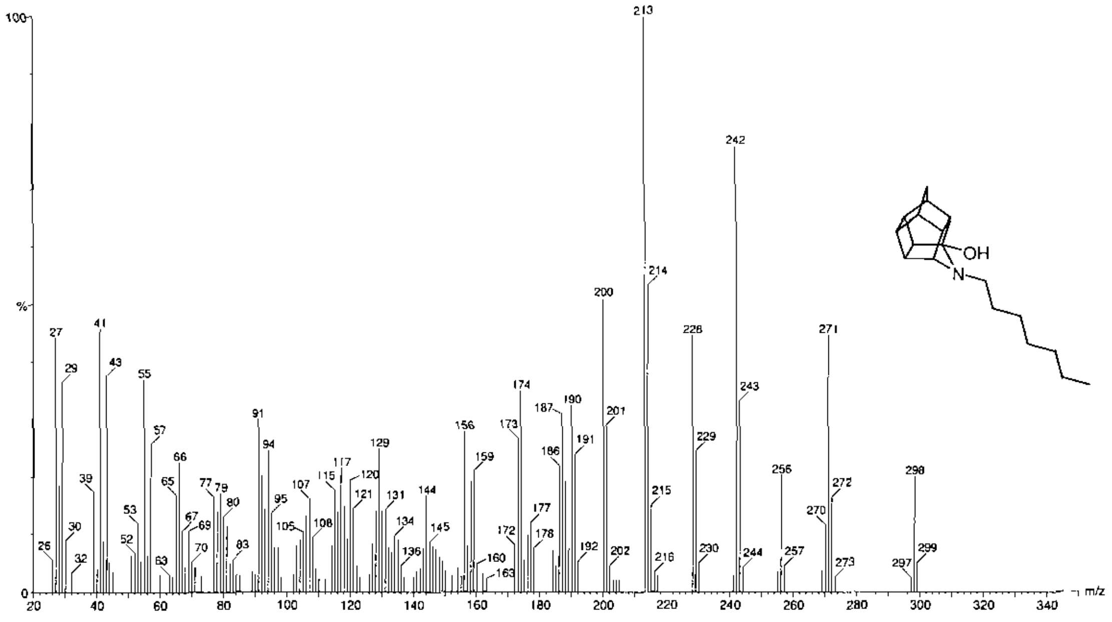
SPECTRUM 2.4



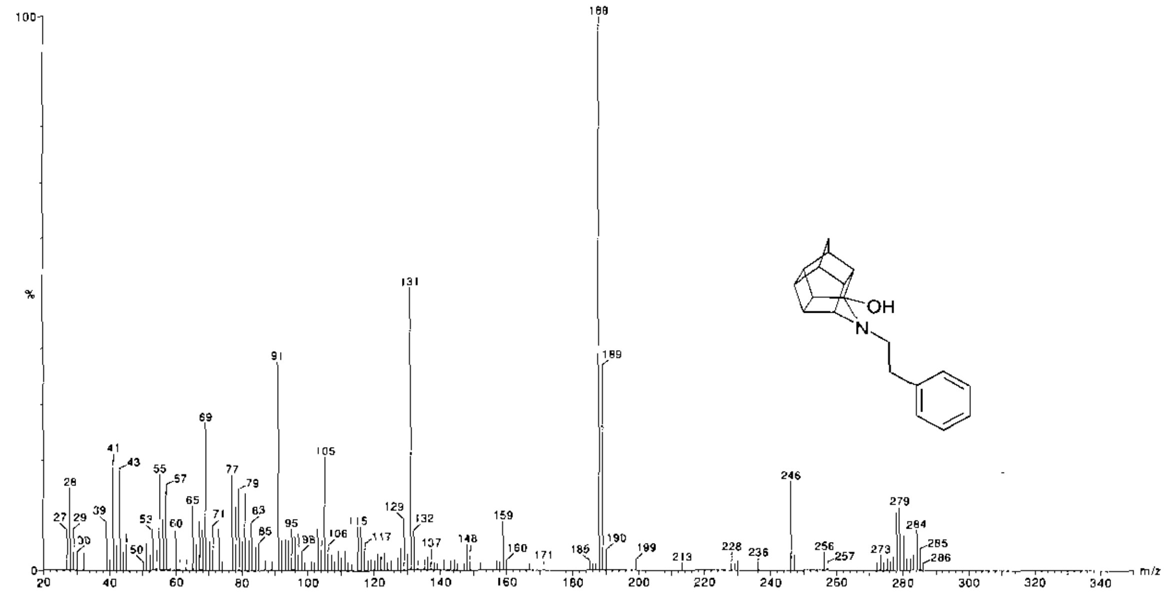
SPECTRUM 2.5



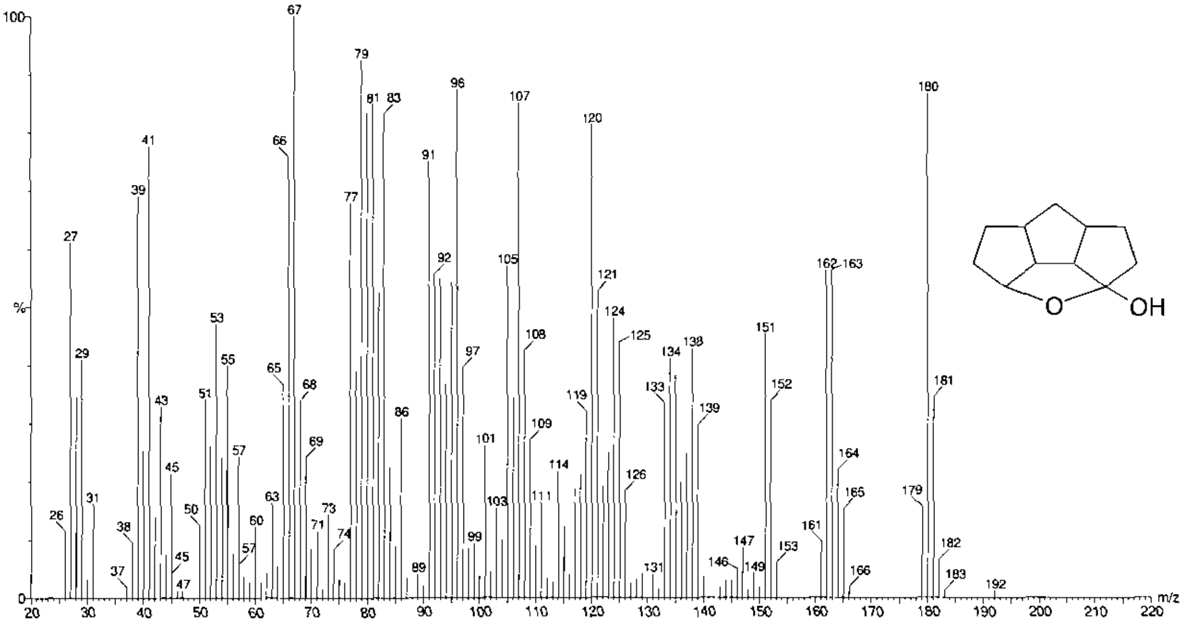
SPECTRUM 2.6



SPECTRUM 2.7

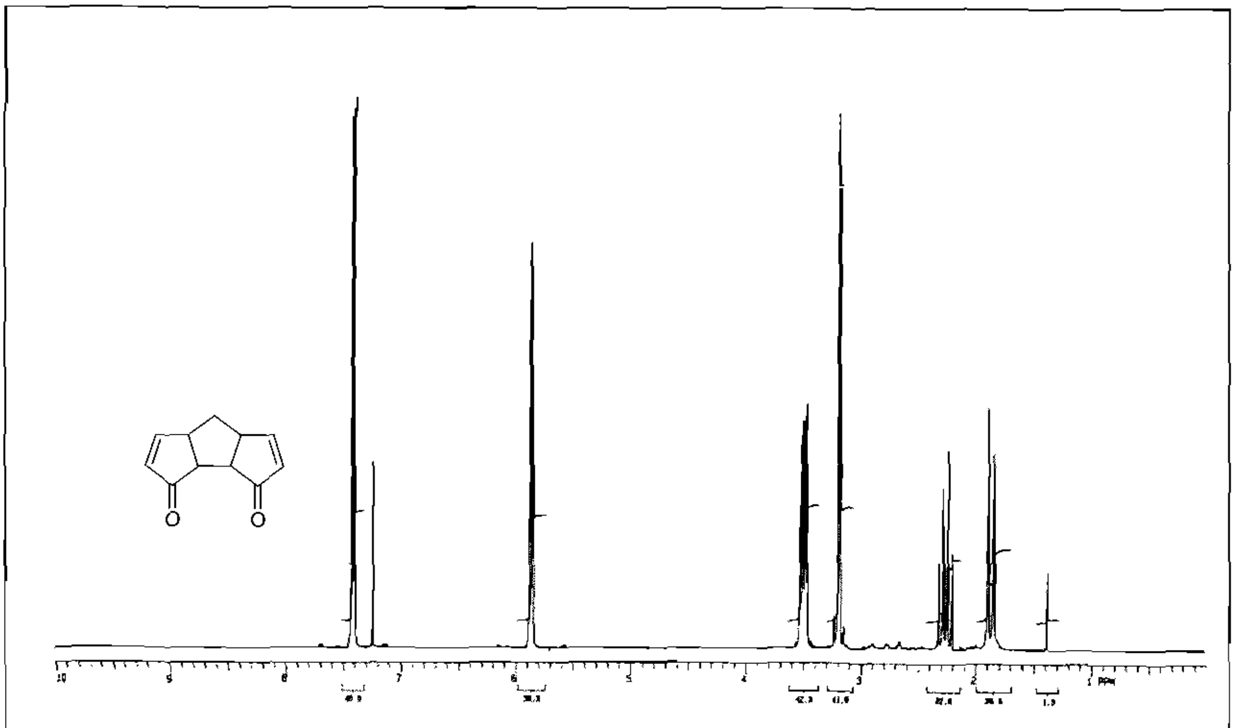


SPECTRUM 2.8

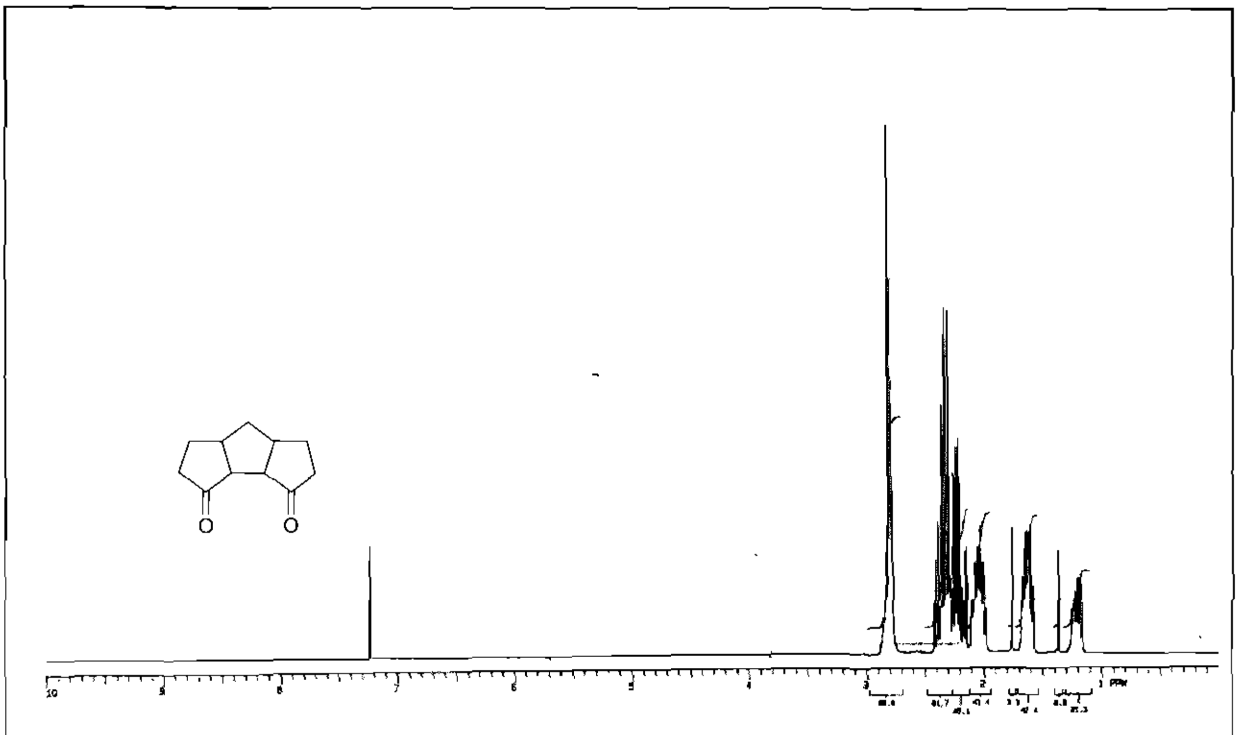


SPECTRUM 2.9

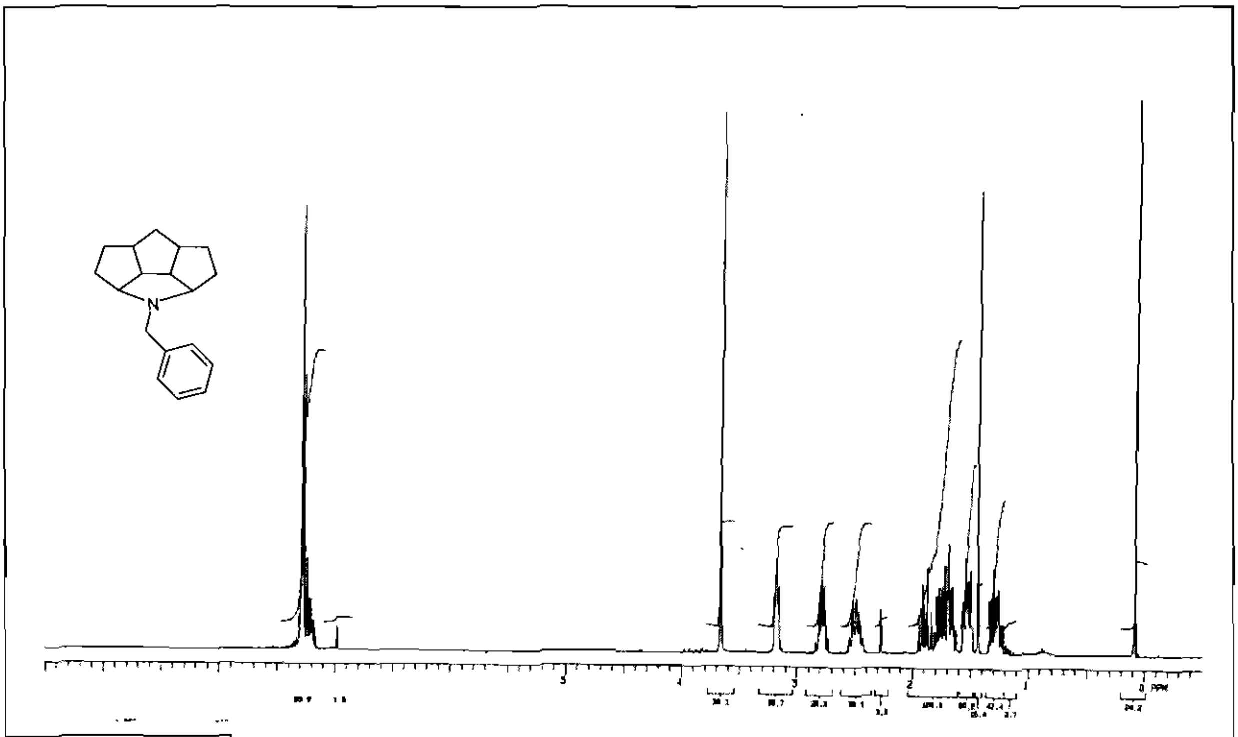
3. ^{1}H NUCLEAR MAGNETIC RESONANCE SPECTROMETRY



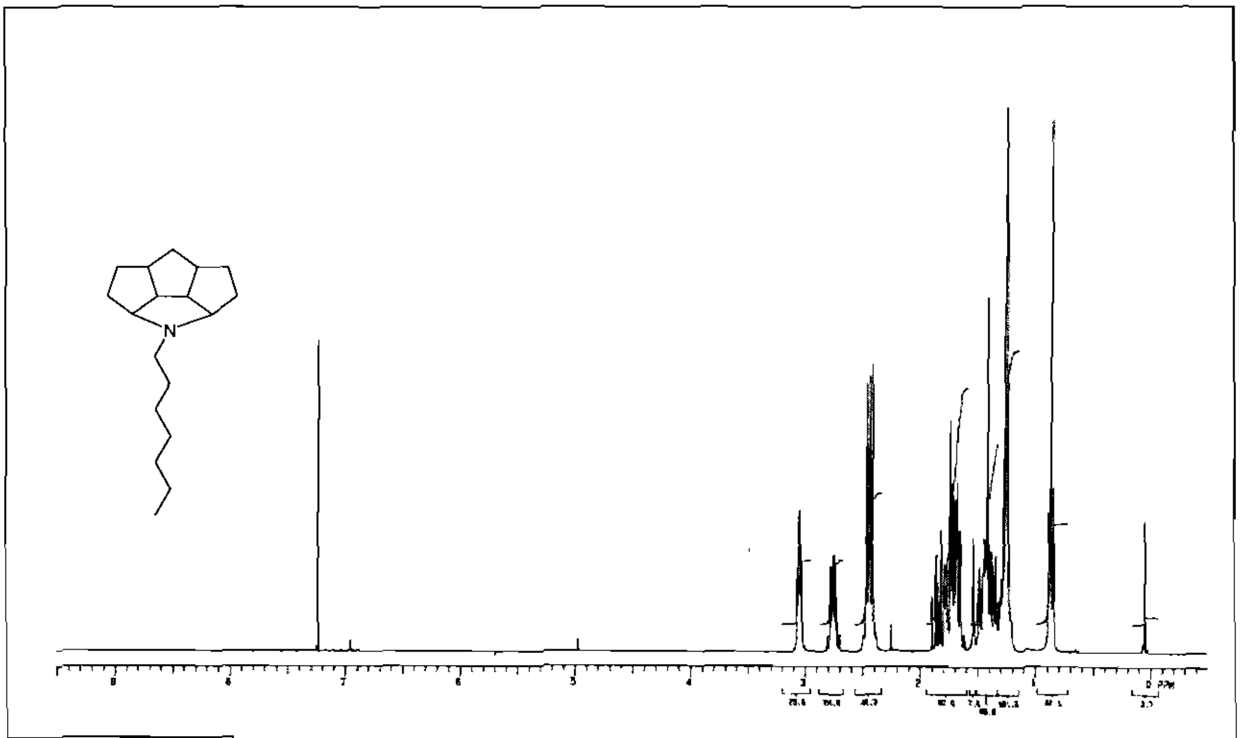
SPECTRUM 3.1



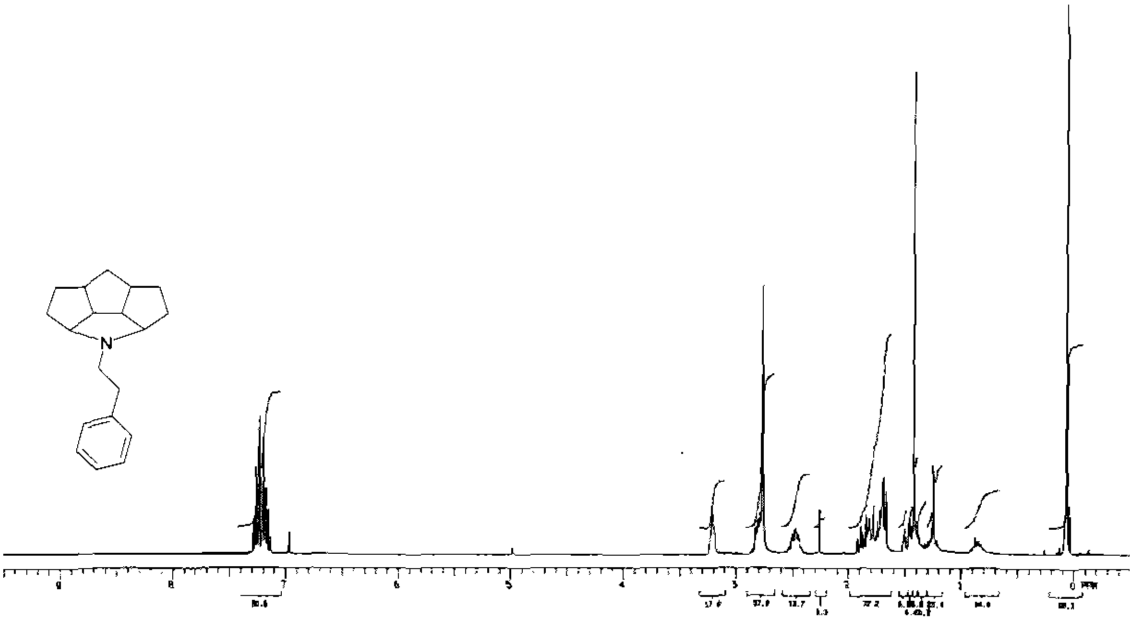
SPECTRUM 3.2



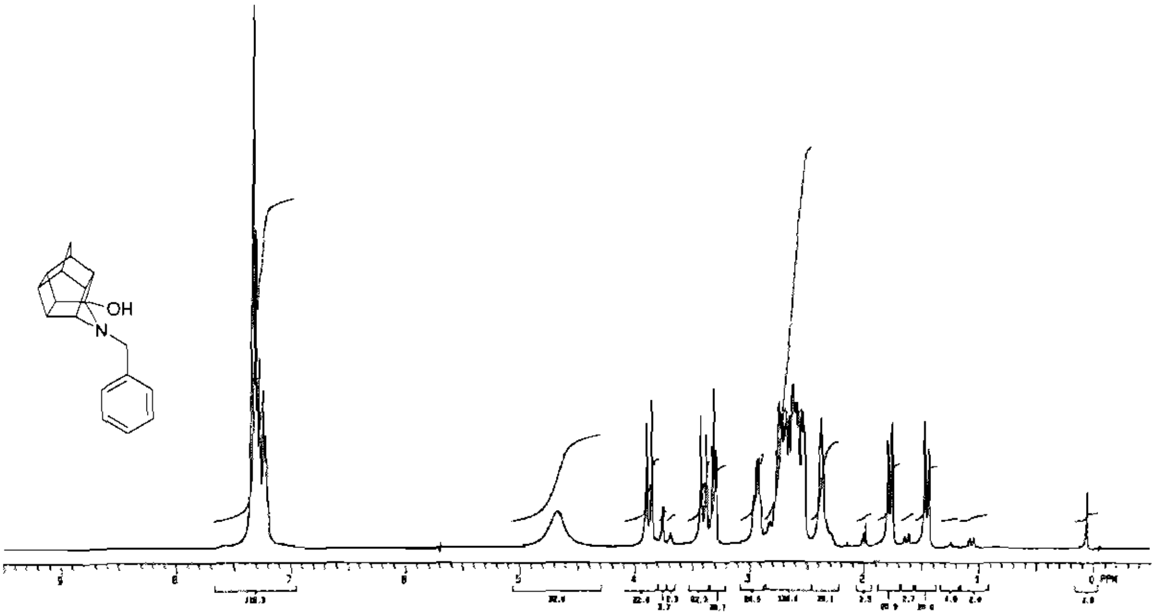
SPECTRUM 3.3



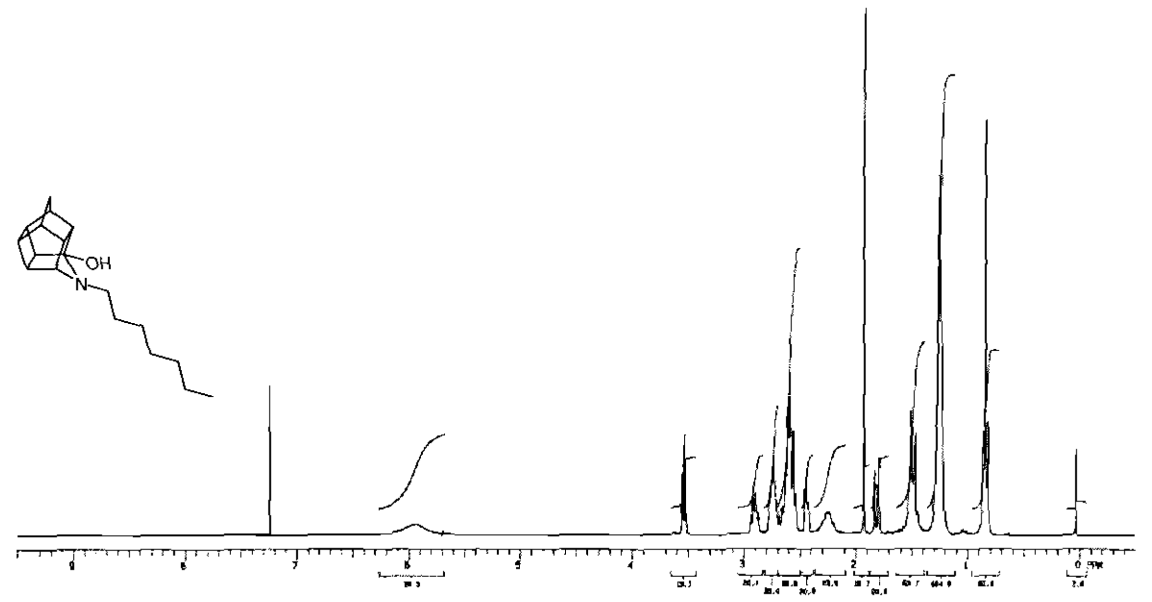
SPECTRUM 3.4



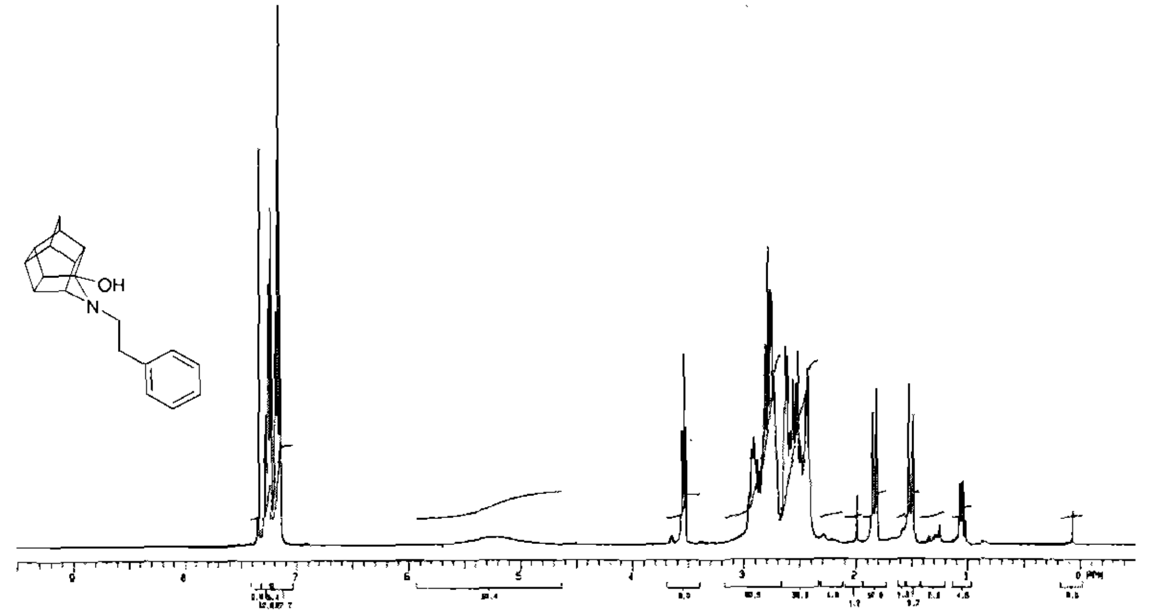
SPECTRUM 3.5



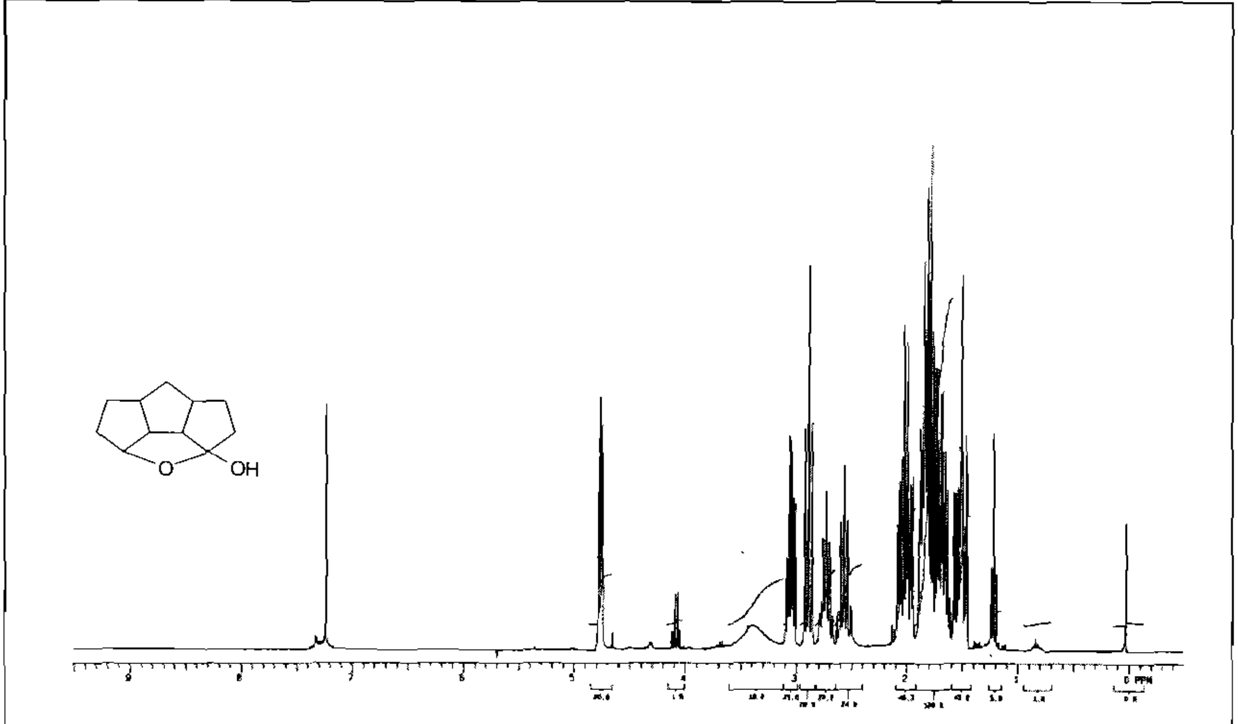
SPECTRUM 3.6



SPECTRUM 3.7

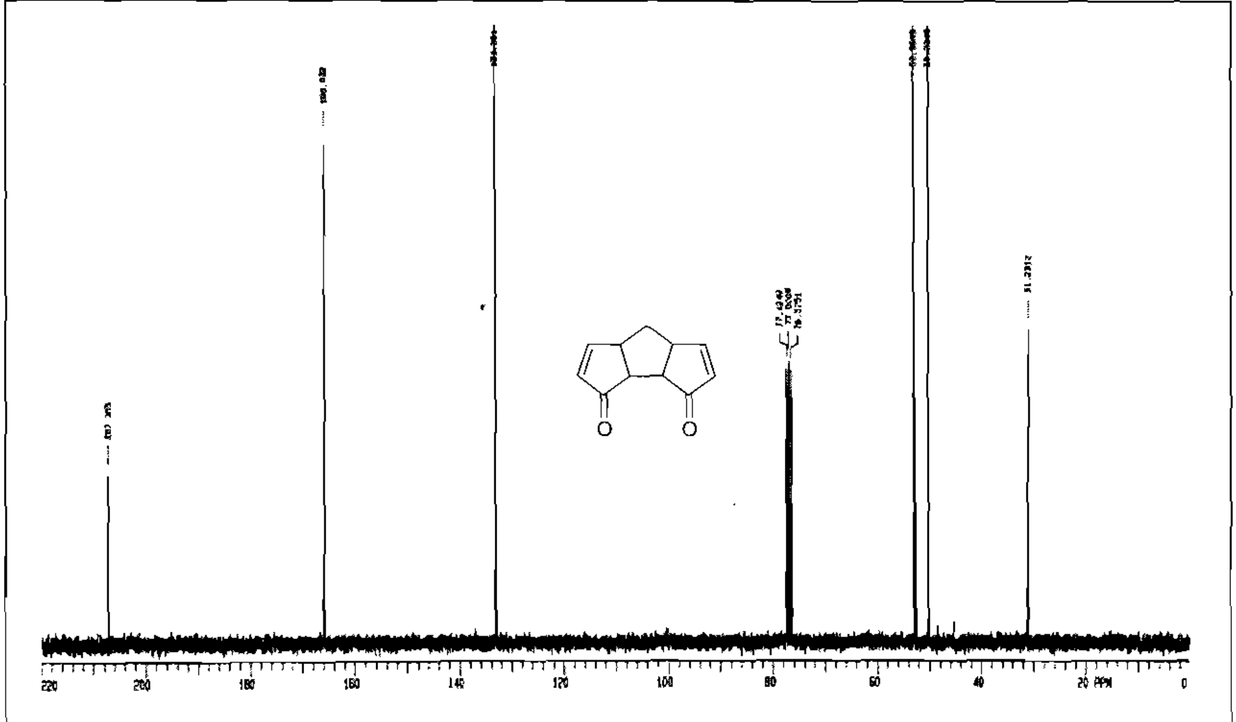


SPECTRUM 3.8

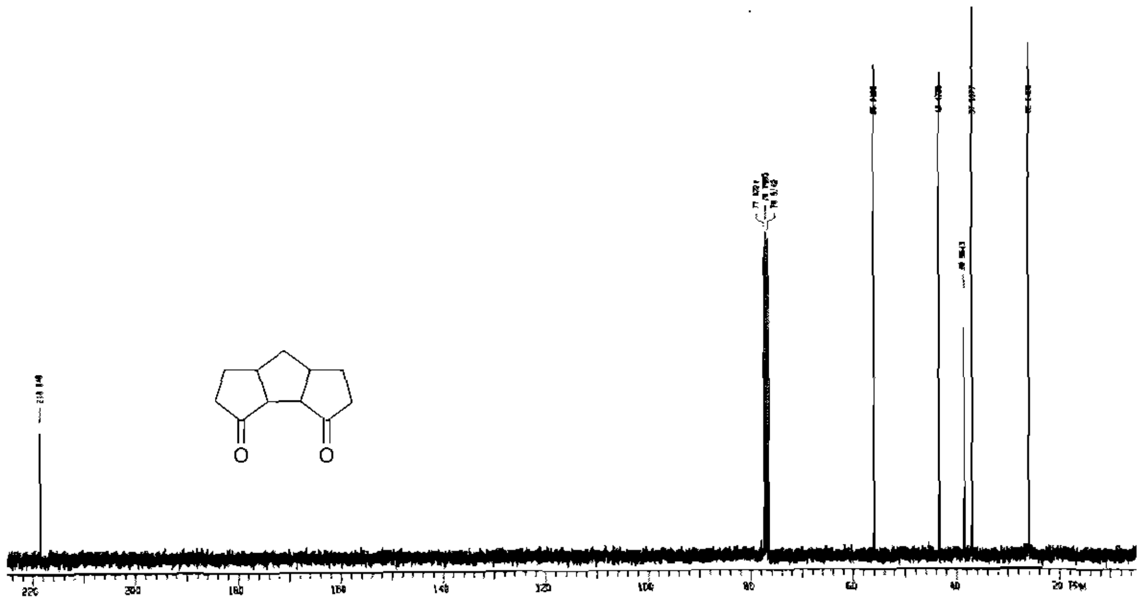


SPECTRUM 3.9

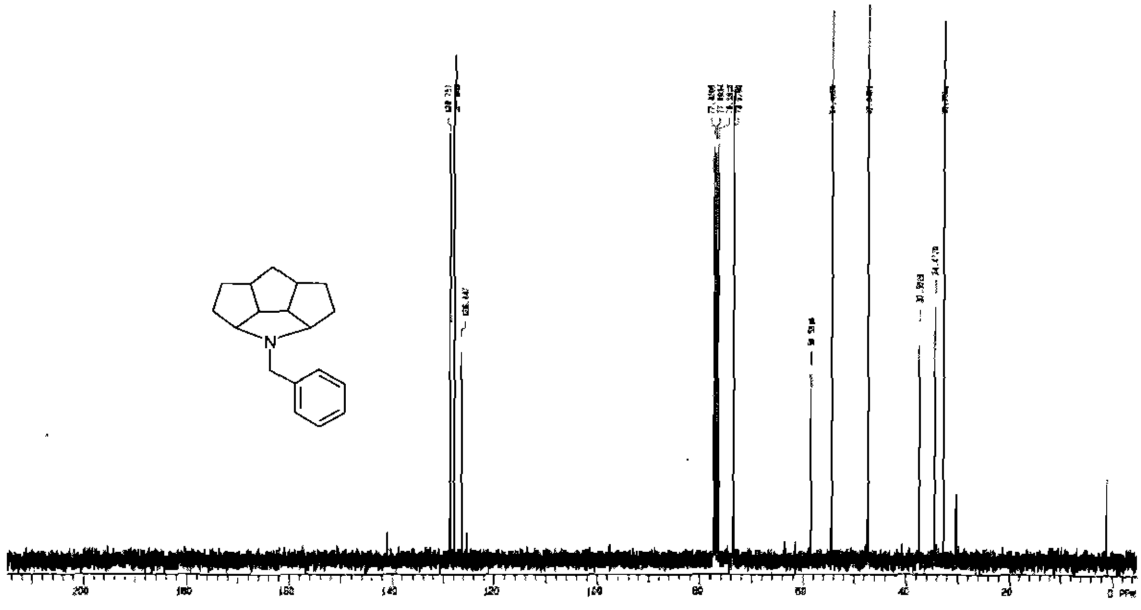
4. ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTROMETRY



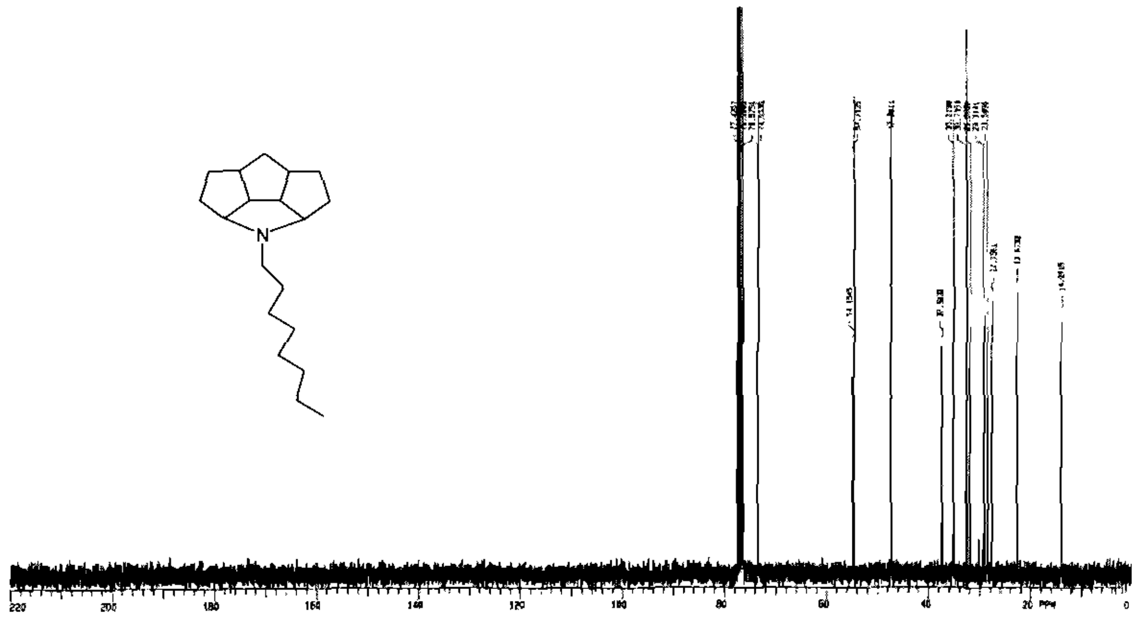
SPECTRUM 4.1



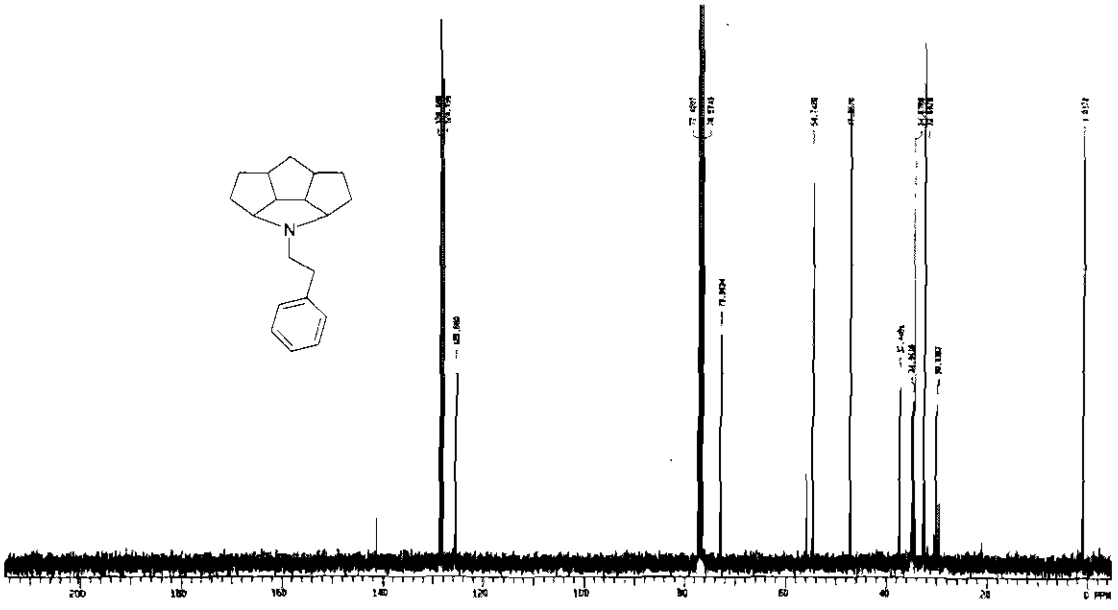
SPECTRUM 4.2



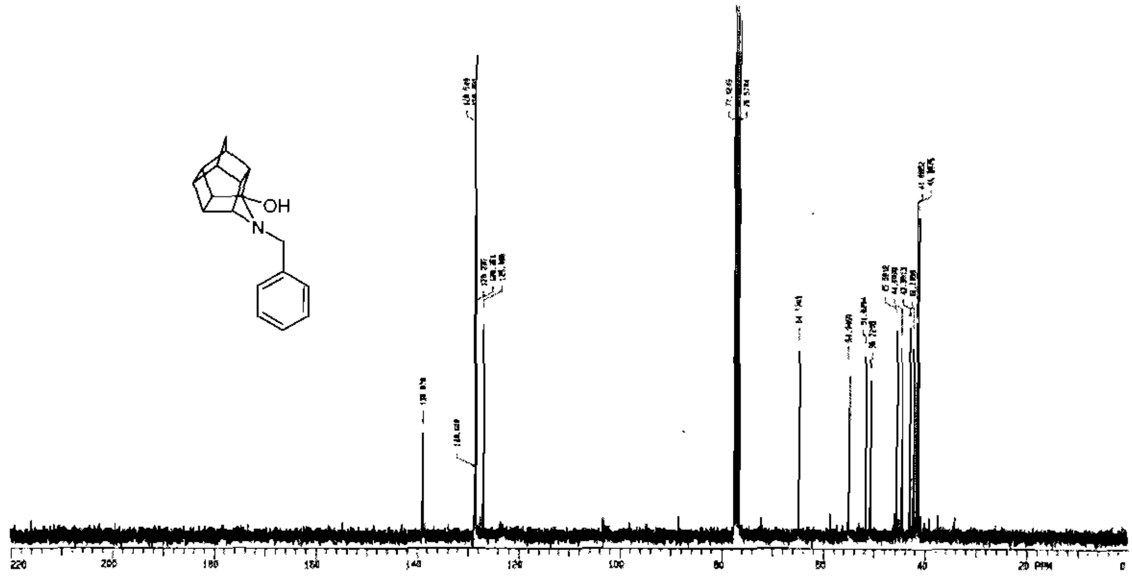
SPECTRUM 4.3



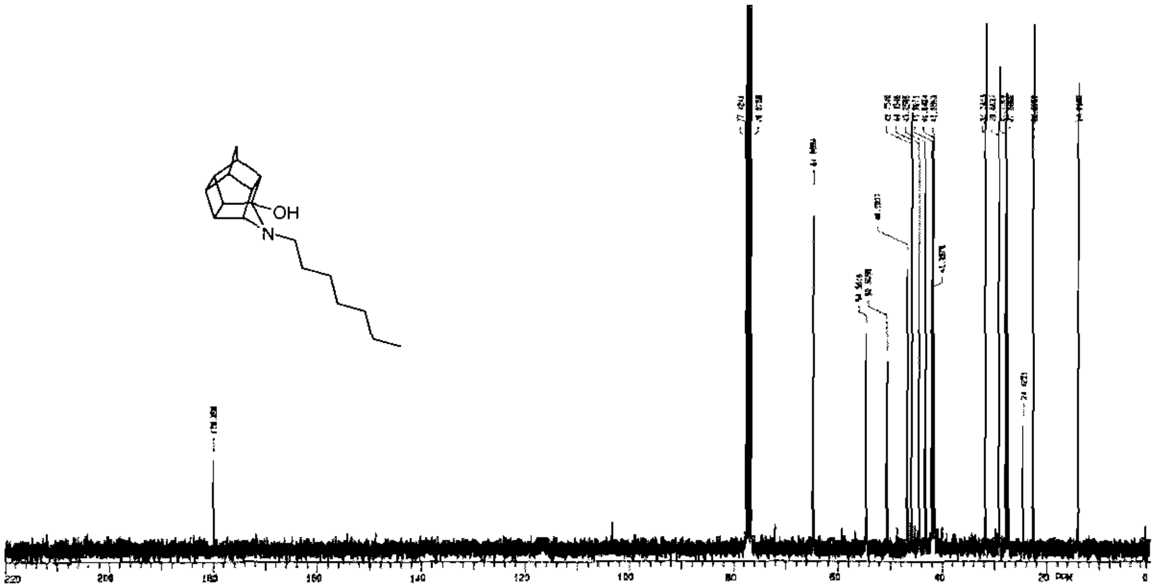
SPECTRUM 4.4



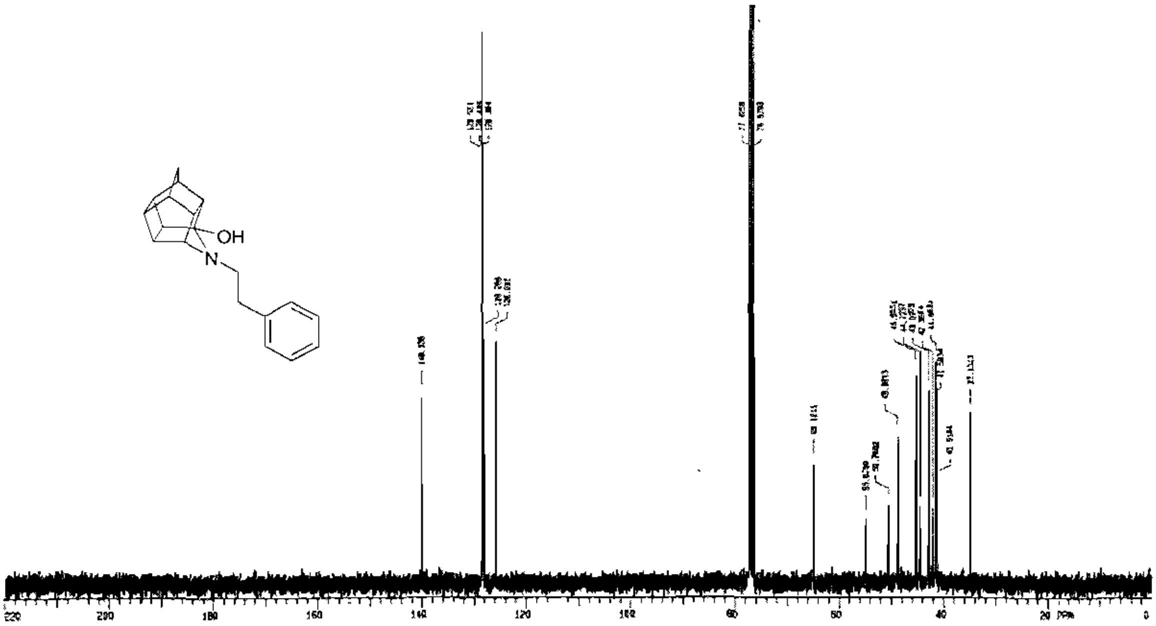
SPECTRUM 4.5



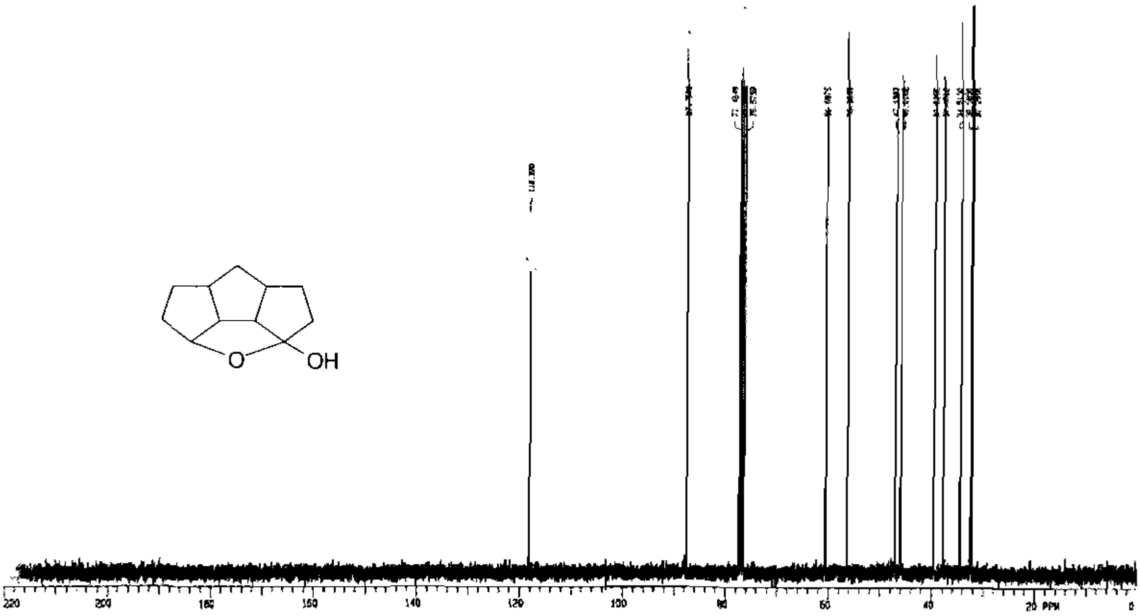
SPECTRUM 4.6



SPECTRUM 4.7

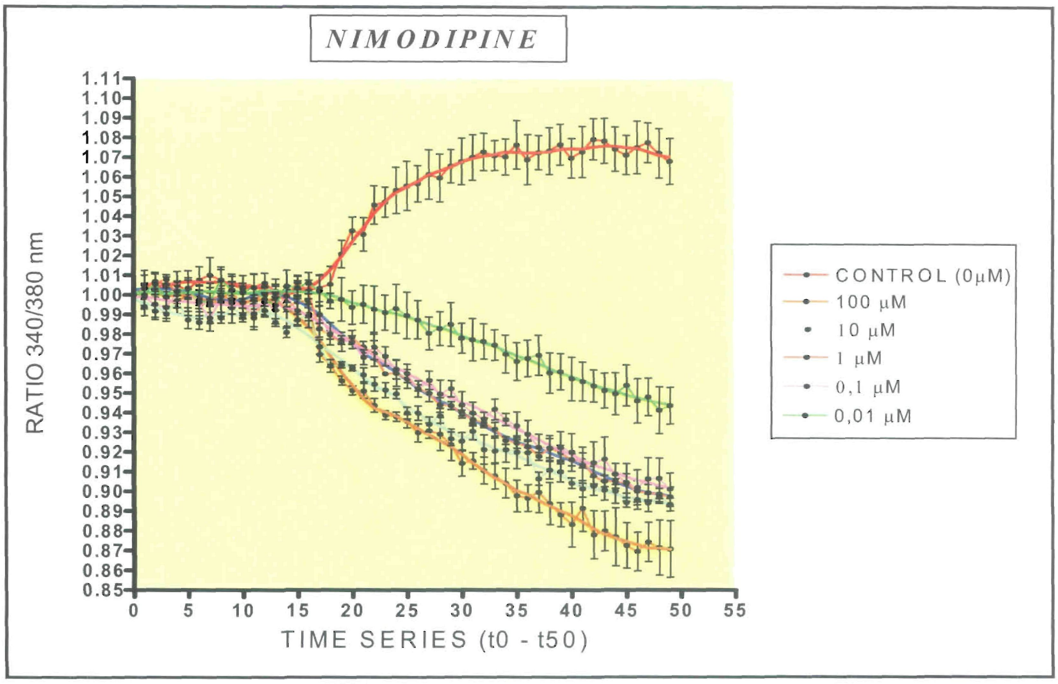


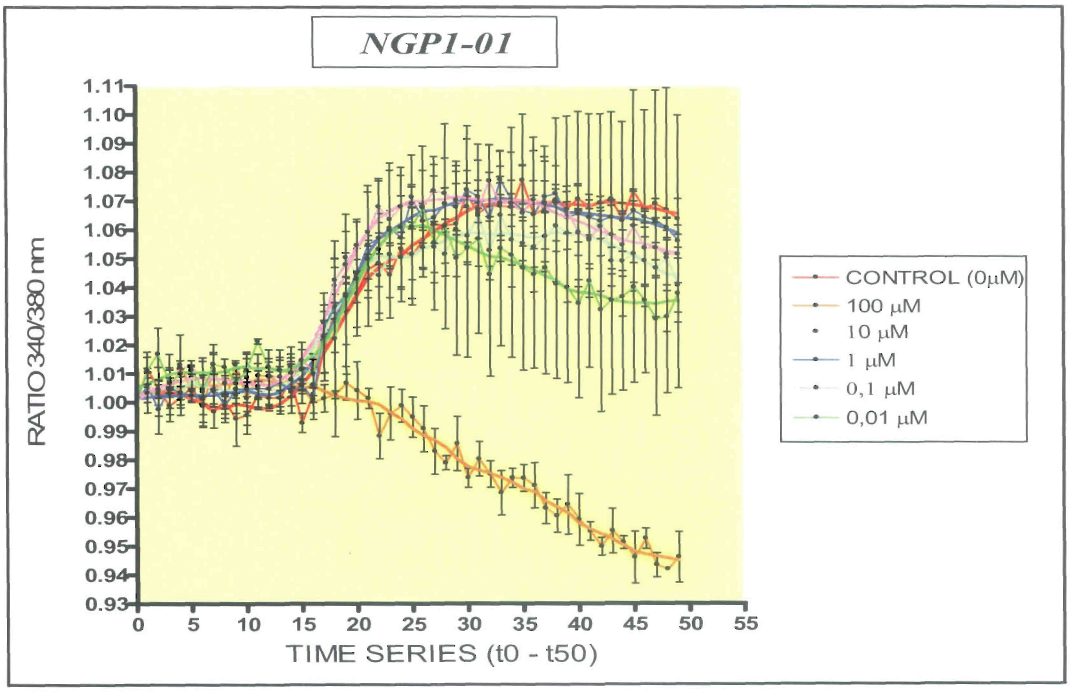
SPECTRUM 4.8

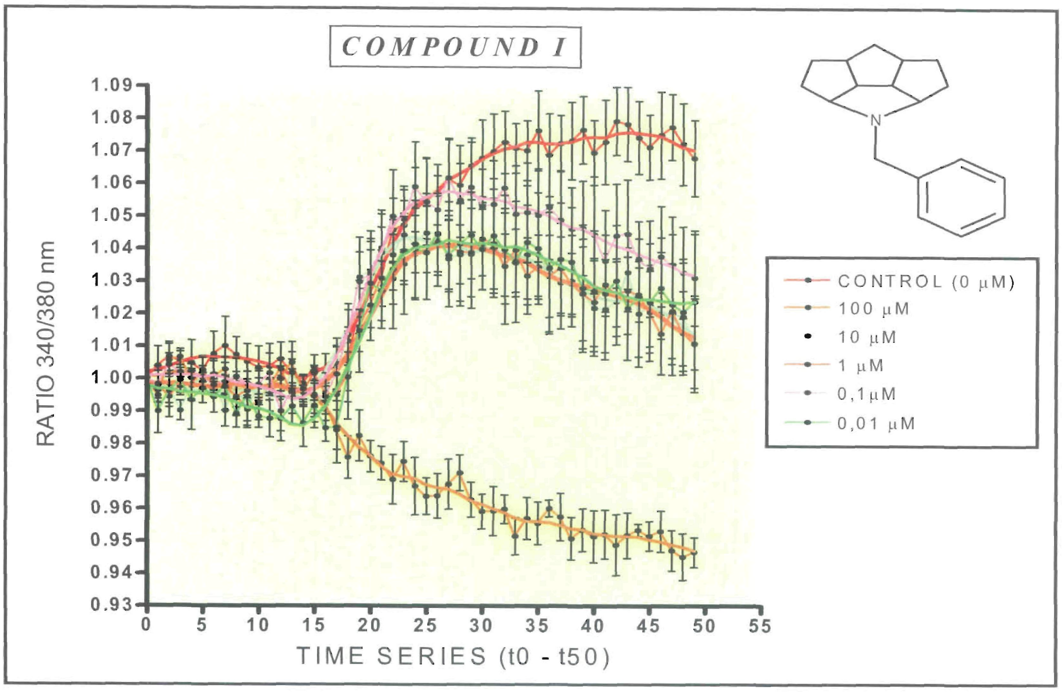


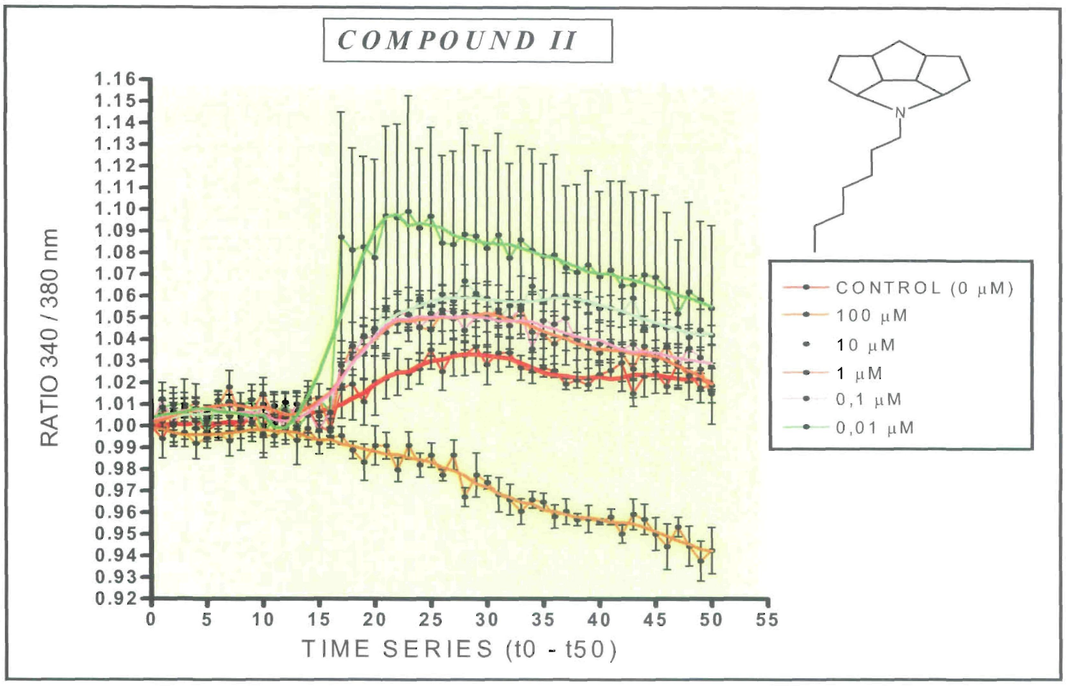
SPECTRUM 4.9

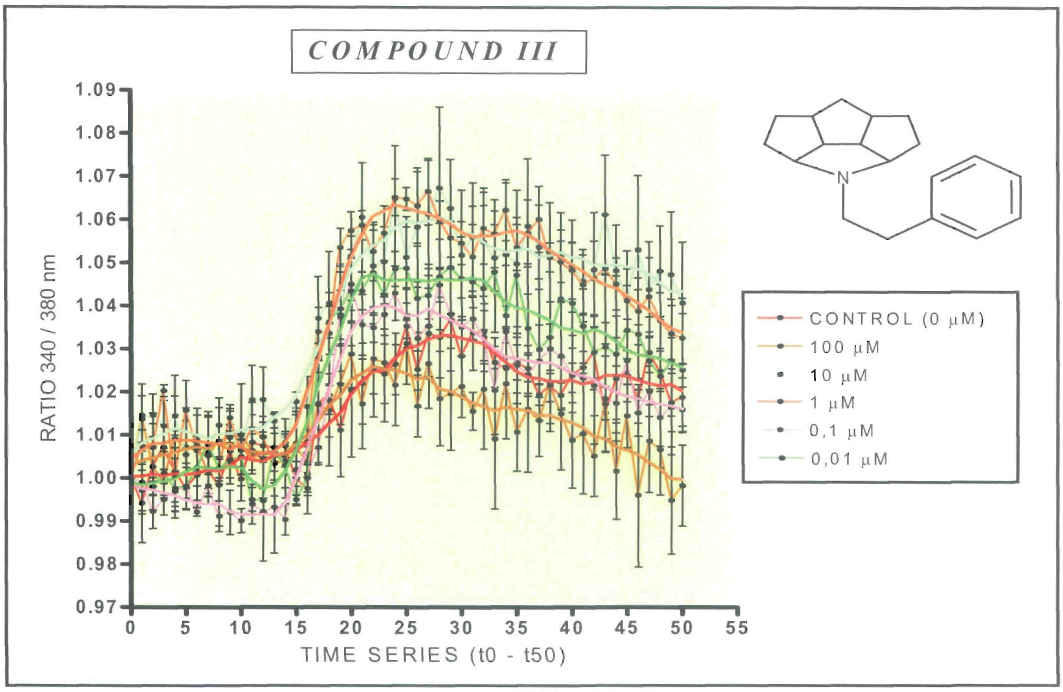
APPENDIX II

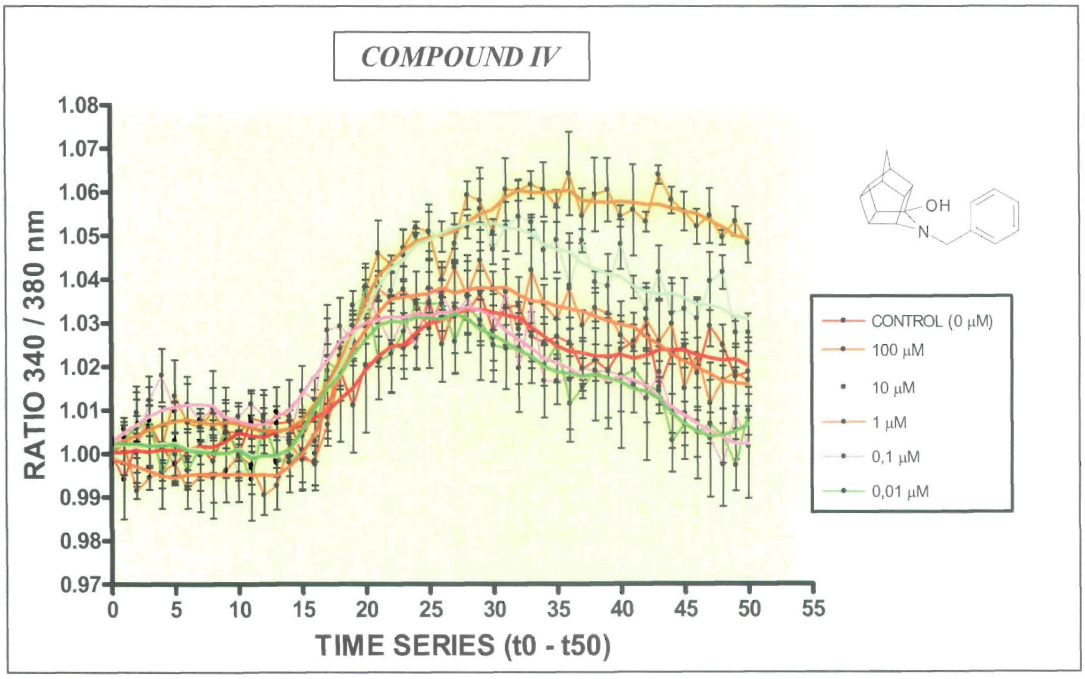

NIMODIPINE

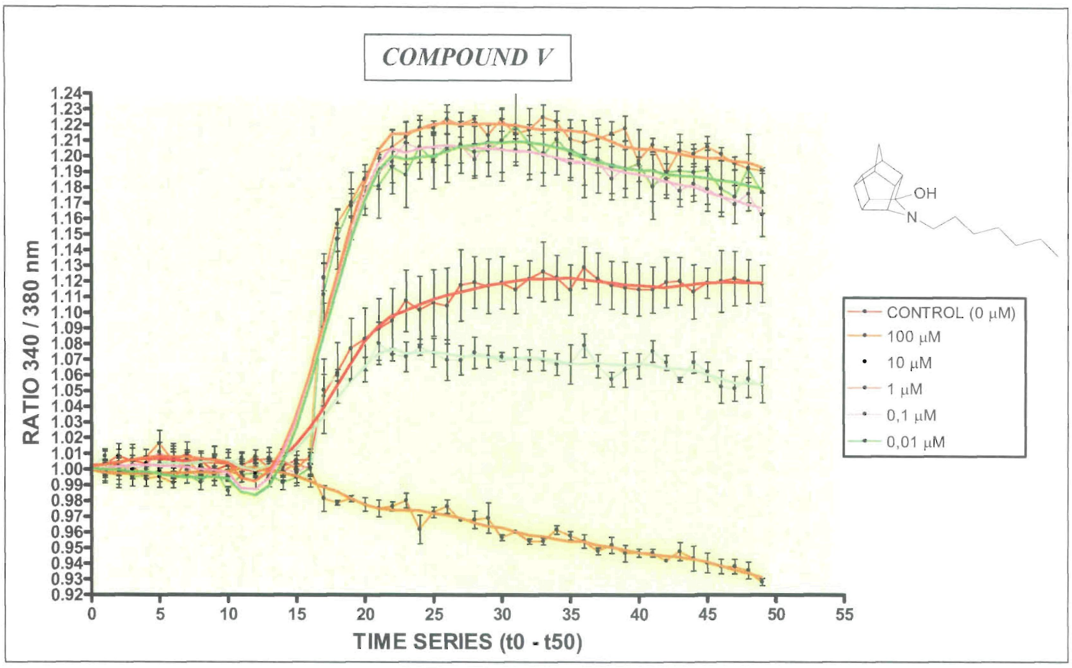

NGP1-01

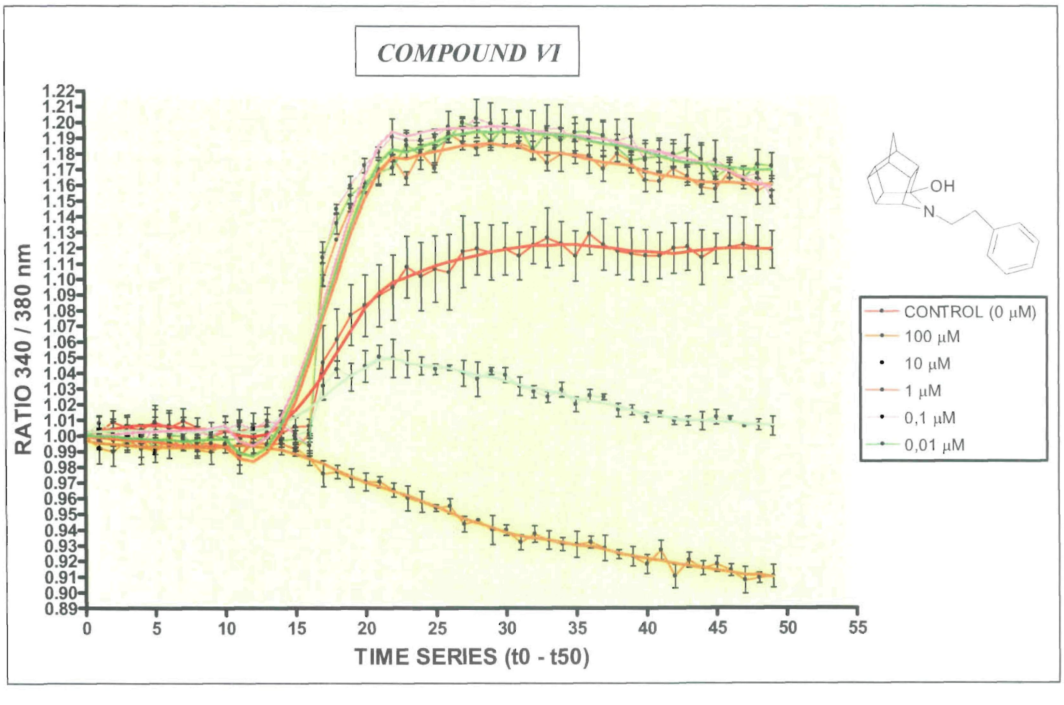

COMPOUND I

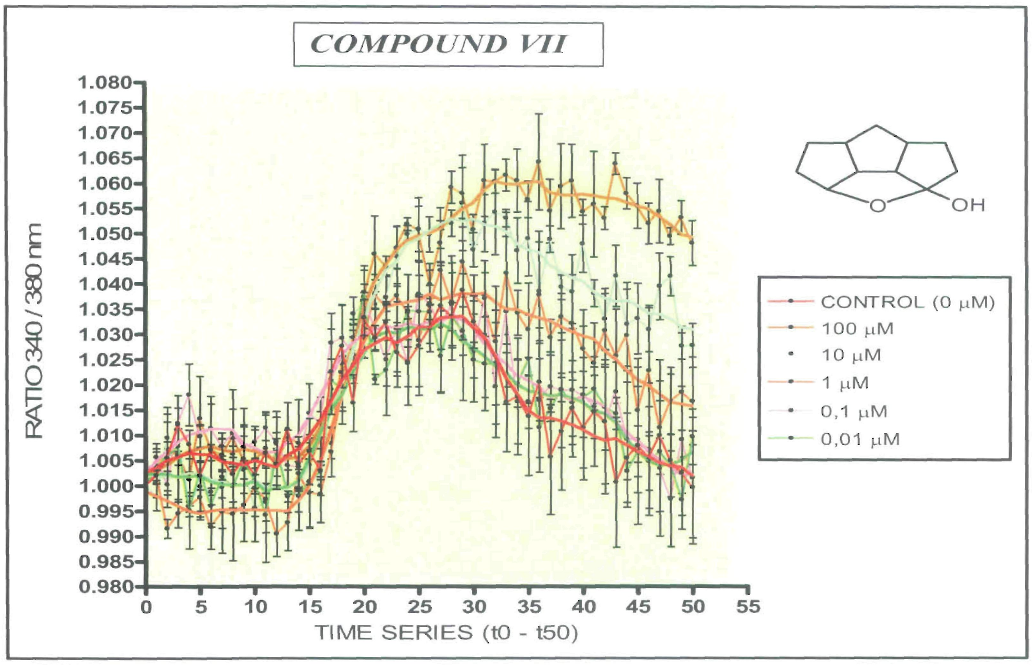

COMPOUND II


COMPOUND III
RATIO 340 / 380 nm
1.09
1.08
1.07
1.06
1.05
1.04
1.03
1.02
1.01
1.00
0.99
0.98
0.97
0
5
10
15
20
25
30
35
40
45
50
55
TIME SERIES (t0 - t50)
N
CONTROL (0 µM)
100 µM
10 µM
1 µM
0,1 µM
0,01 µM


COMPOUND IV
RATIO 340 / 380 nm
1.08
1.07
1.06
1.05
1.04
1.03
1.02
1.01
1.00
0.99
0.98
0.97
0
5
10
15
20
25
30
35
40
45
50
55
TIME SERIES (t0 - t50)
OH
N
CONTROL (0 µM)
100 µM
10 µM
1 µM
0,1 µM
0,01 µM


COMPOUND V
RATIO 340 / 380 nm
0.92
0.93
0.94
0.95
0.96
0.97
0.98
0.99
1.00
1.01
1.02
1.03
1.04
1.05
1.06
1.07
1.08
1.09
1.10
1.11
1.12
1.13
1.14
1.15
1.16
1.17
1.18
1.19
1.20
1.21
1.22
1.23
1.24
0
5
10
15
20
25
30
35
40
45
50
55
TIME SERIES (t0 - t50)
OH
N
CONTROL (0 μM)
100 μM
10 μM
1 μM
0,1 μM
0,01 μM


COMPOUND VI
RATIO 340 / 380 nm
0.89
0.90
0.91
0.92
0.93
0.94
0.95
0.96
0.97
0.98
0.99
1.00
1.01
1.02
1.03
1.04
1.05
1.06
1.07
1.08
1.09
1.10
1.11
1.12
1.13
1.14
1.15
1.16
1.17
1.18
1.19
1.20
1.21
1.22
0
5
10
15
20
25
30
35
40
45
50
55
TIME SERIES (t0 - t50)
OH
N
CONTROL (0 μM)
100 μM
10 μM
1 μM
0,1 μM
0,01 μM


COMPOUND VII
RATIO 340 / 380 nm
1.080
1.075
1.070
1.065
1.060
1.055
1.050
1.045
1.040
1.035
1.030
1.025
1.020
1.015
1.010
1.005
1.000
0.995
0.990
0.985
0.980
0
5
10
15
20
25
30
35
40
45
50
55
TIME SERIES (t0 - t50)
O
OH
CONTROL (0 μM)
100 μM
10 μM
1 μM
0,1 μM
0,01 μM

ACKNOWLEDGEMENTS

I dedicate this dissertation to my late father, Owen and my mother, Sarah Domingo. Thank you for your patience and priceless support over the years. Thank you for teaching me to pray unceasingly. You have taught me to praise God and trust in Him in every circumstance and in everything. I will carry that with me for the rest of my life, wherever I may go. I love you.
Your loving daughter, Olwen.

- To my heavenly Father: I believe and know that You have a plan for all Your children. Thank you for carrying me through my Master's degree, even when I failed to believe. May Your Holy Name be glorified through the research I have done and am still to do.

- To Sharon and my brothers, their spouses and children (Mouse, I love you): Thank you for all your support (especially financially☺). May God bless you in manifold more than you have blessed me.

- Shawn, there is a special place in my heart that will be occupied by you always. I'm at a lack of words to describe what a major role you have played in my life since the day we met. Thank you for loving me.

- There were many times I was on the verge of giving up. Aunty Maya, thank you for always having valuable advice for me regarding anything. Thank you for opening up your house to me and for keeping me on the right track.

- To my closest friends (in no particular order): Quinton; Thomas; Louis; Lois; Johan; Dewald; Nico; Corlea; Sonja. Each one of you has played a very special and unique part in my life over the last few years. Buff, you are God sent. Just trust in Him in everything. I love you so so much. Thomas, vielen Dank für Deine Geduld, Liebe und Unterstützung. Ich liebe Dich.

- Prof. Malan: Baie dankie vir Prof. se geduld, advies en ondersteuning. Dankie dat ek met enige probleem na Prof. toe kon kom. Ek waardeer dit ontsettend baie.

- To everybody involved with my biological tests: Professor Elizabeth van der Merwe at UCT; Anne Grobler; Liezl-Mare Niewoudt; Tannie Maureen Steyn; Sharleen Lowe; Professor Linda Brand; Professor van der Westhuizen and everybody at the department of biochemistry that were helpful to me. Thank you for helping me during a time I had very few options. I have learned a lot from you and I truly appreciate everything. I would like to extend a special thanks to Lois Bezuidenhout, Dr. Geldenhuys and Professor van der Schyf at the NEOUCOM. Thank you so much for agreeing to perform a large portion of my biological evaluations at your institution. Lois, ek skuld jou. ☺ Baie dankie!

- To everyone at the department of Pharmaceutical Chemistry. I would like to make use of this opportunity to particularly thank Professor Breytenbach. Thank you so so much for such valuable advice regarding the chemistry component of my research. Professor Liebenberg at the department of Pharmaceutics and Industrial Pharmacy, thank you for advising me on the synthesis of the triquinane compounds. I really appreciate your help. I would also like to give a special thanks to Nellie, Zelda and William for everything. God bless.