THE INFLUENCE OF POLYCYCLIC AMINES ON CALCIUM HOMEOSTASIS OF NEURONAL CELLS

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ABSTRACT

Ineffective regulation of calcium homeostasis within the central nervous system has been implicated as one of the main factors in the pathology underlying neurodegeneration. Calcium influx is mainly gated through voltage dependent calcium channels (VDCC) as well as N-Methyl-D-Aspartate (NMDA) receptor operated channels. Although the NMDA receptor complex can be modulated by many endogenous compounds, the activation state of both aforementioned channels is regulated through changes in membrane potential. Biological activity attributed to a class of polycyclic amine compounds, the pentacycloundecylamine derivatives, suggest possible intervention in the neurodegenerative cascade through modulation of membrane potential as well as a direct interaction with both voltage dependent and NMDA receptor operated channels.

The aim of this study was to synthesise a series of pentacycloundecylamine derivatives and evaluate the effect of these compounds on neuronal membrane potential and calcium homeostasis within the central nervous system. A method was developed to assess the influence of these derivatives on membrane potential of neuroblastoma cells using confocal microscopy. The effects of the test compounds on influx of $^{45}Ca^{2+}$ into murine synaptosomes were also evaluated.

Compounds were synthesised by reductive amination of the polycyclic "cage", pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione, and characterised using NMR, MS and IR techniques.

A real-time confocal laser microscope (Nikon PCM 2000 Confocal Laser Scanning Microscope [He/Ne laser]) and fluorescent potentiometric indicator, tetramethylrhodamine methyl ester (TMRM, Molecular Probes, the Netherlands), were used to evaluate the influence of the test compounds on the membrane potential of human SH-SY5Y neuroblastoma cells. Cells were loaded with 500nM TMRM, in HEPES-buffered recording solution and changes in membrane potential, in response to stimulation by high concentration potassium (KCl) solution, were measured in the presence and absence of the pentacycloundecylamine derivatives.

Results indicate that 8-benzylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**1**) and derivatives thereof influence the profile of KCl-induced membrane depolarisation. Test compounds caused an overall reduction in membrane depolarisation with compound **1**, compound **5** (8-[(4-aminomethyl)pyridine]-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]-undecane) and compound **6** (8-methylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]-undecane) causing the more prevalent reductions in depolarisation. Compound **3** (8-hepthylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane) caused initial stabilisation of membrane potential but failed to provide significant protection over the 870 sec period. Best overall results and significant inhibition of KCl-induced depolarisation were obtained with compound **1**. These results indicate that cyclic unsaturated substituents on the pentacycloundecane template are important for the activity of these compounds. Introduction of hydrogen bonding (**5**) and decreased volume could also be significant.

Test compounds were evaluated for antagonism of uptake of $^{45}Ca^{2+}$ in murine synaptosomes. Synaptosomes were stimulated with high concentration potassium solution in the presence of $^{45}Ca^{2+}$. The radioactivity of the absorbed $^{45}Ca^{2+}$ was measured by means of liquid scintillation counting. All the compounds within this series of derivatives significantly antagonise absorption of calcium into neuronal cells. 8-Hepthylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**3**) most extensively antagonised the $^{45}Ca^{2+}$ uptake with compound **6** showing poor inhibition. From the results it is suggested that side chain substitution with moieties of larger volume might increase the activity of these derivatives. Hydrogen bonding seems to play a role in activity and compounds with cyclic groups in their side chain also present good activity. Peripheral calcium channel activity described for these derivatives, to a large extent correlate with the central activity indicated in this study.

It is suggested that 8-benzylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane and derivatives thereof could influence membrane potential of cells within the central nervous system through modulation of voltage activated sodium channels, NMDA receptor operated calcium channels and to some extent VDCC. Screening tests evaluating the antagonism of $^{45}Ca^{2+}$ uptake in neuronal cells indicated that all test compounds inhibit influx of calcium into neuronal cells.

Taking into consideration the activity indicated for the series of derivatives in this study, it is postulated that these compounds could reduce calcium influx into neuronal cells through modulating membrane depolarisation, and in event of an action potential, inhibiting calcium influx through both VDCC and NMDA calcium channels.

UITTREKSEL

Wanregulasie van normale kalsiumhomeostase in die sentraal senuwee stelsel vorm 'n baie belangrike deel van die onderliggende patologie van neurodegenerasie. Kalsium influks word hoofsaaklik beheer deur spanningsafhanklike- en N-metiel-D-Aspartaat (NMDA) reseptorbeheerde kalsiumkanale. Alhoewel die NMDA-kanaal se werking deur verskeie endogene verbindings gemoduleer kan word, word beide die kanale se aktiwiteit deur die membraanpotensiaal van die sel gereguleer. Vorige studies aangaande die biologiese aktiwiteit van 'n reeks polisikliese amienderivate, die pentasiklo-undekielamien-verbindings, toon dat die verbindings moontlik neurobeskermende aktiwiteit kan openbaar – of deur die sel se membraanpotensiaal te verander, en/of deur die spanningsafhanklike en NMDA-kalsium kanale direk te beïnvloed.

Die doel van die studie was om 'n reeks pentasiklo-undekielamien derivate te sintetiseer en hul invloed op membraanpotensiaal sowel as kalsiumhomeostase in die sentrale senuwee stelsel te evalueer. 'n Metode is ontwerp om die invloed van die reeks verbindings op die membraanpotensiaal van neuronale selle te ondersoek deur gebruik te maak van konfokale mikroskopie. Die effek van die reeks verbindings op die absorpsie van $^{45}Ca^{2+}$ in neuronale selle is ook ondersoek.

Die verbindings is gesintetiseer deur reduktiewe aminering van pentasiklo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undekaan-8,11-dioon en gekarakteriseer deur gebruik te maak van KMR, MS en IR.

'n Konfokale lasermikroskoop (Nikon PCM 2000 laserskanderingsmikroskoop [He/Ne laser]) en fluoresserende potensiometriese indikator, tetrametielrhodamien metiel ester (TMRM; Molecular Probes, Nederland), is gebruik om die gesintetiseerde verbindings se invloed op membraanpotensiaal te evalueer. Die SH-SY5Y-neuroblastoom selle is vooraf gelaai met 500nM fluoresserende indikator in 'n HEPES-gebufferde oplossing. Veranderinge in membraanpotensiaal, in reaksie op 'n stimulus deur 'n hoë konsentrasie kalium oplossing, is gemeet in die teenwoordigheid en afwesigheid van die pentasiklo-undekielamien derivate

In die studie is daar gevind dat hierdie reeks verbindings die profiel van KCl-geïnduseerde depolarisasie in neuronale selle beïnvloed. Die geëvalueerde verbindings het oor die algemeen die mate van membraandepolarisasie verminder met verbindings **1** (8-bensielamino-8,11-oksapentasiklo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undekaan), **5** (8-[(4-aminometiel)-piridien]-8,11-oksapentasiklo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undekaan), en **6** (8-metielamino-8,11-oksapentasiklo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undekaan) wat die grootste veranderinge tot gevolg gehad het. Verbinding **3** (8-hepthielamino-8,11-oxapentasiklo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undekaan) het aanvanklik die membraanpotensiaal gestabiliseer maar kon egter nie beskerming tot aan die einde van die evaluasie tydperk verleen nie. Betekenisvolle inhibisie van KCl-geïnduseerde membraandepolarisasie is met verbinding **1** waargeneem. Uit die resultate kan daar afgelei word dat sikliese onversadigde substituente belangrik is vir goeie aktiwiteit van die reeks verbindings. Verbindings waar waterstofbinding in die interaksie geïnkorporeer is het verhoogde aktiwiteit getoon. Dit wil ook voorkom asof verbindings met 'n kleiner volume dalk verhoogde aktiwiteit kan openbaar.

Studies wat die mate van antagonisme van die absorpsie van $^{45}Ca^{2+}$ in neuronale selle ondersoek, is uitgevoer om die invloed van die verbindings op kalsiumhomeostase in die sentrale senuwee stelsel te evalueer. Al die geëvalueerde verbindings het die absorpsie van $^{45}Ca2^{+}$ in neuronale selle inhibeer. Verbinding **3** het die grootste mate van antagonisme getoon met verbinding **6** wat die absorpsie van $^{45}Ca^{2+}$ die minste beïnvloed het. Uit die resultate word daar voorgestel dat substitusie op die pentasiklo-undekaan templaat deur groepe met 'n groter volume die aktiwiteit van die reeks verbindings kan verhoog. Waterstof binding blyk ook 'n rol te speel in die interaksie van die verbindings. Dit wil voorkom asof die perifere kalsium kanaal aktiwiteit van die verbindings tot 'n groot mate met hul sentrale senuwee stelsel aktiwiteit korreleer.

Waarnemings uit die studie bevestig dat 8-bensielamino-8,11-oxapentasiklo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undekaan en derivate van hierdie verbinding, die membraanpotensiaal van selle in die sentrale senuwee stelsel kan beïnvloed deur inhibisie van spanningsafhanklike natriumkanale, interaksie met NMDA-kanale asook, tot 'n mate, deur inhibisie van spanningsafhanklike kalsium kanale.

Uit die bogenoemde bevindings word daar voorgestel dat die verbindings kalsiuminfluks in neuronale selle op 'n tweesydige wyse kan inhibeer. Eerstens deur membraandepolarisasie van die sel te onderdruk en tweedens deur direkte inhibisie van spanningsafhanklike- en NMDA-kalsiumkanale tydens 'n aksie potensiaal.

CHAPTER 1

INTRODUCTION

SUMMARY

Neurodegeneration and the development of neuroprotective agents have in recent years become an increasingly important focus of research. Much time and effort have gone into establishing the exact causes of disorders like Parkinson's disease, Alzheimer's disease and Huntington's disorder and ultimately developing agents to successfully prevent resulting neuronal loss. Intracellular calcium homeostasis, or rather the lack thereof, have in many studies been implicated in neuronal degeneration and is currently believed to be one of its main causes. At this stage, treatments available for neurodegenerative disorders are at best symptomatic, rather than focussing on cure or prevention.

Previous investigations into the biological activity of a series of polycyclic compounds, structurally related to the therapeutic agent amantadine and its dimethyl derivative memantine, have aroused interest as to their possible use in the prevention of neurodegeneration. Studies conducted on the pentacycloundecylamine derivatives strongly indicate that these compounds could yet be of therapeutic value in the treatment of neurodegenerative disorders.

ABBREVIATIONS

CNS	Central nervous system
VDCC	Voltage dependent calcium channels
NMDA	N-Methyl-D-Aspartate

1.1 RATIONALE

Calcium plays an important part in the functioning of the central nervous system (CNS). Influx of calcium ions initiate a cascade of events that ultimately leads to the activation of vital physiological functions including cell growth, release of neurotransmitters from nerve terminals and memory formation (Ashcroft, 2000; Slish *et al.*, 1992).

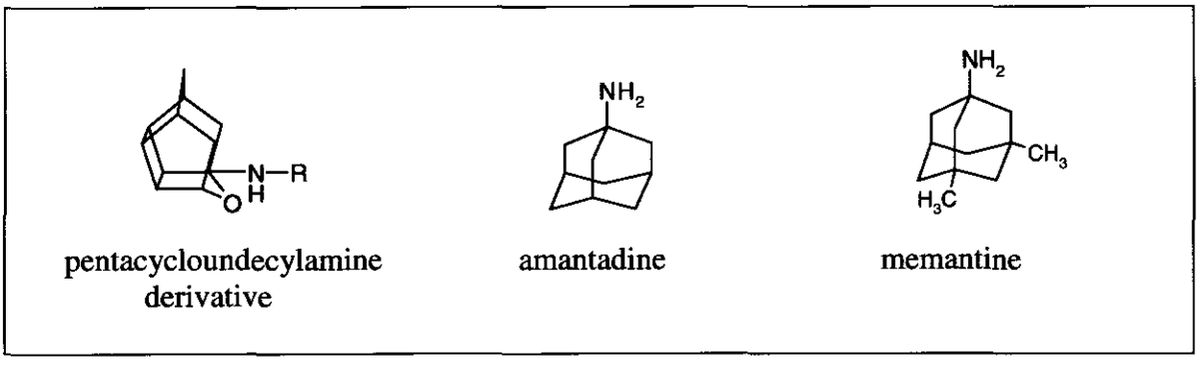
Adequate neuronal calcium is essential for normal CNS function and yet excessive calcium influx into neuronal cells is pathogenic and can cause serious neuronal damage. An intracellular calcium overload leads to the activation of proteases, nucleases, and phospholipases (Suzuki *et al.*, 2003), as well as accumulation of free radicals intracellularly. These and other calcium related processes ultimately lead to cell death and neurodegeneration.

As calcium influxes occur mainly through voltage dependent calcium channels (VDCC), or through ion channels integral to the N-Methyl-D-Aspartate (NMDA) receptor, prevention of calcium overload could be achieved by blocking either the NMDA receptor operated channels, or by blocking the VDCC directly. The activation state of both these channels can also be modulated through changes in membrane potential.

Because the NMDA receptor channel has a very high calcium conductance, overstimulation by glutamate causes massive calcium influxes and consequently calcium-induced neurodegeneration termed excitotoxicity (Choi *et al.*, 1988; Marini *et al.*, 1997). The NMDA receptor complex is both ligand and voltage gated and changes in membrane potential can accordingly have significant effects on its activation. Excitotoxic receptor activation resulting from membrane depolarisation is therefore termed weak excitotoxicity (Green & Greenamyre, 1996). Many NMDA receptor antagonists have been described in literature. Potent compounds, antagonising this specific complex, was developed and evaluated but unfortunately exhibit many adverse pschycotomimetic side effects. Non-competitive NMDA receptor channel antagonists for example amantadine and its dimethyl derivative, memantine present a more attractive pharmacological profile. These compounds permit a level of physiological NMDA receptor activation and therefore show much less

side effects than the potent NMDA antagonists (Kemp & Kew, 1988; Kemp & McKernan, 2002).

Structural similarities between these two compounds and the polycyclic cage compounds were what firstly prompted further studies regarding the neuroprotective abilities of the cage compounds (Fig. 1.1).



pentacycloundecylamine derivative | amantadine | memantine

Figure 1.1: Structural similarities between the pentacycloundecylamine derivatives and the non-competitive NMDA antagonists amantadine and memantine.

Studies evaluating the biological activity of the pentacycloundecylamine derivatives indicate activity that could be useful in the treatment of neurodegenerative disorders. Activities reported include anti-parkinsonian properties (Oliver *et al.*, 1991b) and selectivity and high affinity for the sigma binding site (Kassiou *et al.*, 1996). In addition to this, L-type calcium channel activity has also been described for the pentacycloundecylamine derivatives (Van der Schyf *et al.*, 1986; Van der Walt *et al.*, 1988). Studies done by Zah *et al.* (2003) established that the polycyclic cage compounds show a high likelihood to cross the blood-brain barrier and can therefore effectively exert their needed pharmacological effects in the central nervous system. Recently Malan *et al.* (2003) found that 6-benzylamino-3-hydroxyhexacyclo[6.5.0.0^{3,7}.0^{4,12}.0^{5,10}.0^{9,13}]tridecane inhibits L-type calcium channels, sodium channels as well as the fast component of the delayed rectifier potassium channels.

Bearing in mind that the polycyclic cage compounds show structural similarities to NMDA receptor antagonists, and that L-type calcium channel antagonism has also been indicated for these compounds, further studies regarding the neuroprotective abilities of the polycyclic amine derivatives are definitely warranted.

1.2 AIM AND OBJECTIVES OF THIS INVESTIGATION

The aim of this study was to synthesise and evaluate the influence of a series of pentacycloundecylamine derivatives on membrane potential and calcium homeostasis within the central nervous system. A method employing fluorescent confocal microscopy was developed to evaluate the effect of the pentacycloundecylamine derivatives on the transmembrane potential of SH-SY5Y neuroblastoma cells. The effect of the test compounds on absorption of $^{45}Ca^{2+}$ into murine synaptosomes was also evaluated. From these results, basic structure activity relationships for the series of derivatives were proposed.

CHAPTER 2

BACKGROUND

SUMMARY

Although not the sole mechanism mediating neuronal death, calcium plays an integral role in the pathology underlying neurodegeneration. Disregulation of calcium influx through voltage dependent calcium channels (VDCC) as well as N-methyl-D-aspartate (NMDA) receptor operated channels is a key contributor to accumulation of excessive calcium intracellularly. Biological activity attributed to a novel class of polycyclic amine derivatives, the pentacycloundecylamines, suggest possible neuroprotective abilities for these compounds through modulation of voltage activated sodium, potassium and calcium channels as well as interaction with NMDA receptor operated channels.

ABBREVIATIONS

AMPA	Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate;
CNS	Central nervous system
ER	Endoplasmic reticulum
NMDA	N-Methyl-D-Aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
$ONOO^-$	Peroxynitrite
PCP	Phencyclidine
ROS	Reactive oxygen species
Log BB	$\text{Log}\left(\frac{[\text{Brain}]}{[\text{Blood}]}\right)$
TMRE	Tetramethylrhodamine ethyl ester
TMRM	Tetramethylrhodamine methyl ester
VDCC	Voltage dependent calcium channels

2.1 THE ROLE OF CALCIUM HOMEOSTASIS IN NEURODEGENERATION

Calcium plays a fundamental role regulating a variety of cellular functions. Calcium ions acts as intracellular messengers controlling cellular functions such as differentiation and growth, membrane excitability, synaptic activity, muscle contraction, release of hormones from secretory cells and gene expression. In the central nervous system calcium plays an important part in neurotransmitter release, regulation of synaptic plasticity and memory formation. The proper function of most cellular processes is dependent on the maintenance of intra- and extracellular calcium concentrations. In the CNS in particular, these concentrations are strictly controlled by specialised homeostatic mechanisms. Calcium channels (VDCC and NMDA receptor operated channels) embedded in the plasmamembranes of cells forms a crucial part of this specialised system (Ashcroft, 2000; Arundine & Tymianski, 2003; Slish *et al.*, 1992).

The presence of calcium ions is for obvious reasons an absolute necessity for maintenance of all physical functions and yet, excessive calcium influx is pathogenic and large increases in calcium concentrations can cause extensive cellular damage (Greene & Greenamyre, 1996).

Neuronal calcium concentrations are maintained through a multifaceted process consisting of Ca^{2+} influx and efflux, intracellular Ca^{2+} storage and an intracellular Ca^{2+} buffering system (fig. 2.1). Calcium influx is gated by voltage dependent calcium channels as well as glutamate-controlled NMDA receptor operated channels. Efflux is controlled through calcium/sodium exchanger pumps as well as energy dependent calcium-ATPase pumps. Intracellular calcium buffering is achieved by pumping calcium into organelles such as the mitochondria and the endoplasmic reticulum (ER) and releasing it from the ER upon metabotropic receptor activation. Intracellular calcium can also be buffered through binding to calcium-binding proteins.

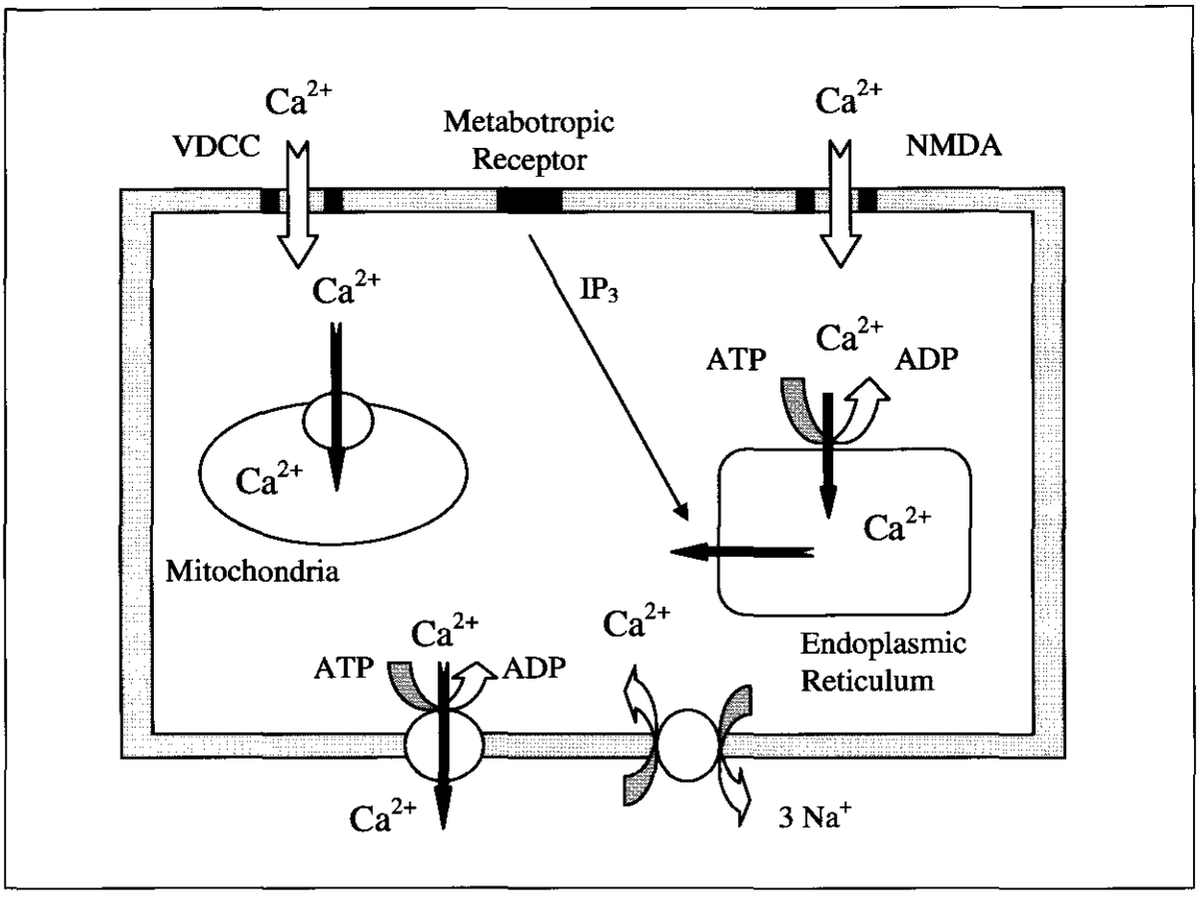


Figure 2.1: Schematic presentation of main mechanisms controlling intra- and extracellular calcium homeostasis in cells.

Despite existing homeostatic mechanisms, pathological elevations in intracellular Ca^{2+} do occur and leads to the inapt activation of normally dormant (or low level) calcium dependent processes (Arundine & Tymianski, 2003). These processes include amongst others, the activation of calcium dependent nucleases, phospholipases and proteases as well as the activation of many nonspecific enzymes. Proteases convert xantine dehydrogenase to xantine oxidase, the latter generating toxic level free radicals and other reactive oxygen species (ROS). ROS damages macromolecules and cell membranes and can cause lipid peroxidation.

Elevated levels of intracellular calcium result in the excessive activation of nitric oxide synthase (NOS), the enzyme converting L-arginine to nitric oxide (NO), and accordingly causes the accumulation of toxic levels of NO in the cell (fig. 2.2). At physiological levels NO acts as a neuromodulator influencing brain development, memory formation, pain

perception and behaviour but, when overproduced, can act as a mediator in the neurodegenerative process (Christopherson & Bredt, 1997). The free radical character of NO enables it to interact with prosthetic groups of many proteins and also facilitates the interaction with superoxide ($O_2^{\cdot-}$) to produce peroxynitrite ($ONOO^-$) (Christopherson & Bredt, 1997). Peroxynitrite oxidates lipids, protein and DNA and may mediate much of the NO neurotoxicity. Overproduction of NO resulting from the excessive or inappropriate activation of NOS plays a foremost part in calcium related neurodegeneration.

In addition to this, accumulation of intracellular calcium causes impairment of mitochondrial calcium buffering, a mitochondrial calcium overload and cessation of ATP production. This results in a decreased energy production, local free radical formation and an inability to handle free calcium intracellularly (Greene & Greenamyre, 1996).

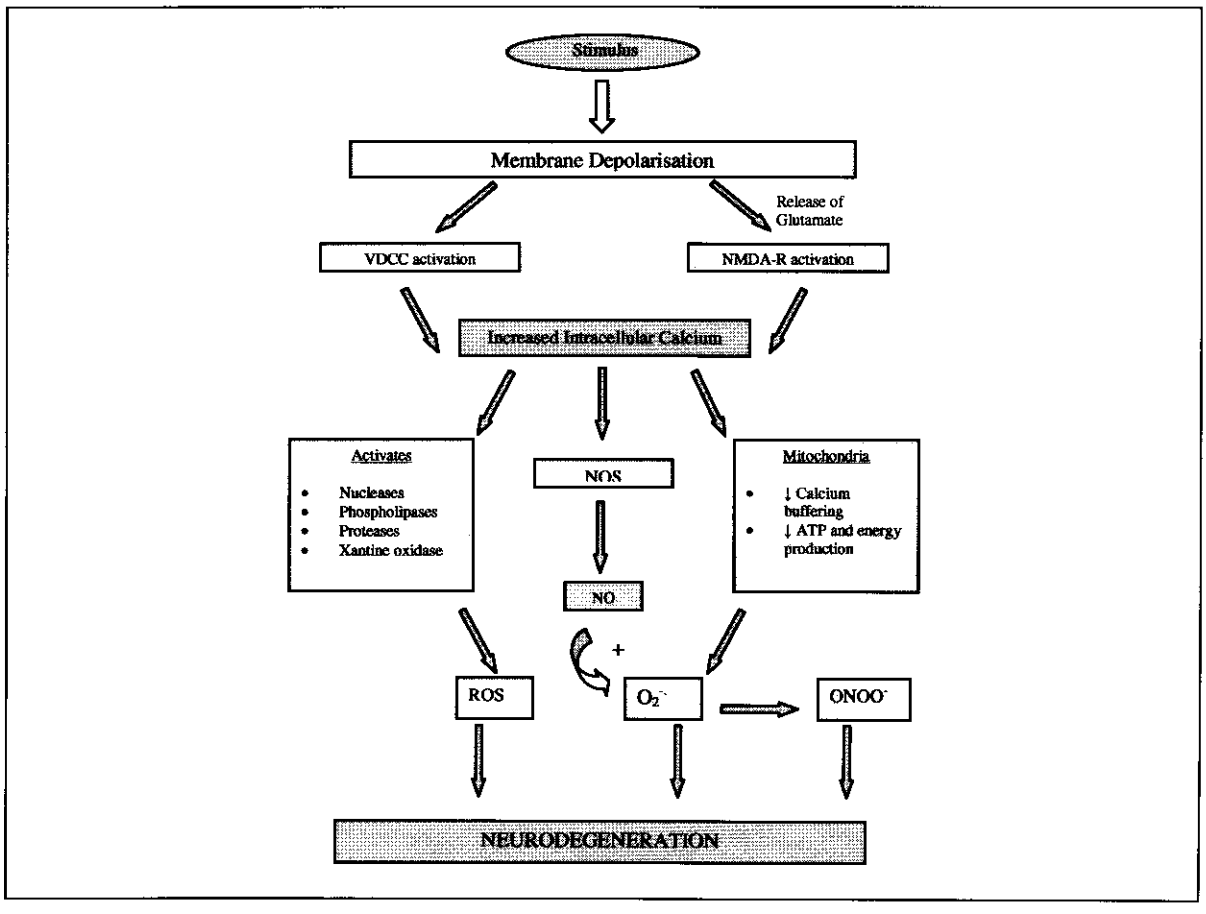


Figure 2.2: Schematic presentation of basic mechanisms of neurodegeneration resulting from elevated intracellular calcium.

An intracellular calcium overload can be caused by one or a combination of a few processes. Increased calcium influx through voltage dependent calcium channels, influx through NMDA receptor operated channels, enhanced calcium mobilisation from intracellular stores and impairment of the calcium buffering / extrusion mechanisms can all lead to excessive calcium intracellularly (Shubert *et al.*, 1997). Pathologically increased calcium influx through VDCC, AMPA- and NMDA receptor operated channels has been implicated in neuronal death in many studies (Shubert *et al.*, 1997).

2.1.1 VOLTAGE DEPENDENT CALCIUM CHANNELS

Under normal physiological conditions intracellular calcium levels are maintained in the vicinity of 100 nM opposed to 1 mM calcium in extracellular media. This gives rise to a considerable concentration gradient causing a flow of calcium over the cell membrane into the intracellular media. A rise in intracellular calcium plays a second messenger role, coupling nerve signals to cellular processes. Voltage dependent calcium channels (VDCC) serve as the only link to transduce depolarisation into excitation controlled cellular activities, such as neurosecretion. VDCC are found in virtually all areas of synaptic activity and control calcium entry into both pre- and post synaptic neurones (Ashcroft, 2000; Kobayashi & Mori, 1998).

The existence of multiple types of VDCC have been recognised, i.e. L, N, P, Q, R and T-types. These channels differ in both biological and pharmacological properties (Ashcroft, 2000; Kobayashi & Mori, 1998). On the basis of their voltage dependence for activation, VDCC are classified into two groups: Low threshold activated channels, and high threshold activated channels.

T-type calcium channels are low threshold channels, and can therefore be opened by a small depolarisation. L, N, P, Q and R-type calcium channels require stronger depolarisations for activation (Ashcroft, 2000).

N and P-type calcium channels are largely restricted to neurons and influx through these channels control neurotransmitter release. L-type VDCC are found in virtually all excitable

tissues, and many non-excitable cells and are very sensitive to antagonism by calcium channel blockers. The L-type calcium channel has a high threshold for activation but inactivation occurs slowly. In contrast to N and P-type calcium channels that exist mainly on the pre-synaptic terminal, the majority of VDCC on post synaptic terminals are of the L-type (Kobayashi & Mori, 1998; Slish *et al.*, 1992).

Voltage dependent calcium channels comprise of 5 subunit proteins (fig. 2.3). The α_1-subunit forms the channel pore and regulates channel activation kinetics. The α_2-subunit consist of 2 proteins bound via a disulphide bridge and associates with the α_1-subunit to increase current amplitude while the β-subunit regulates activation, inactivation, current and voltage dependence. The γ-subunit controls channel activation at more hyperpolarised membrane potentials and causes an increased peak Ca^{2+} current and activation rate (Catterall, 1998).

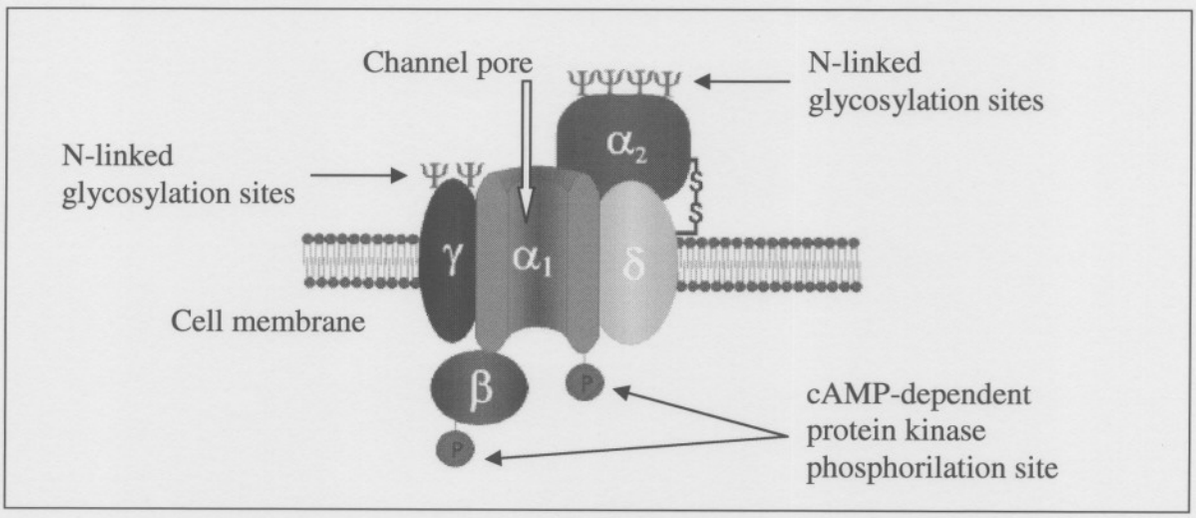


Figure 2.3: Substructure of the voltage dependent calcium channel (Catterall, 1998)

It is evident that any abnormal influx through VDCC would have a pronounced effect on the intracellular calcium homeostasis in the CNS and many selective and non-selective derivatives of classical calcium channel antagonists have been evaluated for neuroprotective abilities. Two mechanisms were proposed for the possible neuroprotection provided by these compounds. Firstly, an improvement of cerebral blood circulation and secondly a direct inhibition of neuronal calcium channels (Kobayashi & Mori, 1998).

The existence of three major types of channel blockers has been recognised: Inorganic compounds that include metals such as nickel and lanthanum; peptides or venoms and organic compounds. Organic compounds can be divided into 4 classes on the basis of their chemical structure. These include benzothiazepines (diltiazem); dihydropyridines (nicardipine, nifedepine and nimodipine); phenylalkylamines (verapamil) and diphenylpiperazines (Kobayashi & Mori, 1998).

Numerous studies have demonstrated organic L-type calcium channel antagonists to be neuroprotective. Pizzi *et al.* (2002) found that the dihydropyridine derivative, nifedipine, significantly reduce neuronal damage induced by excess glutamate. Isradipine and also the phenylalkylamine, verapamil, show the same neuroprotective abilities. Kobayashi & Mori (1998) investigated the neuroprotective abilities of nimodipine, nicardipine, isradipine and other dihydropyridines. Although these compounds exhibit significant neuroprotection, systemic hypotension limits the utility of these compounds in the central nervous system.

2.1.2 NMDA RECEPTOR OPERATED CALCIUM CHANNELS

The NMDA receptor channel has a very high calcium conductance and calcium influx through this complex is thought to be the primary mediator for both the physiological as well as the toxic responses of the NMDA receptor complex. NMDA receptor activation is crucial in the proper function of the CNS and plays an important part in the mechanism underlying learning and memory formation.

NMDA receptor pharmacology is exceedingly complex. The receptor has binding sites for glycine and glutamate and binding of both these substances is required for activation of the receptor operated channel. Glycine and glutamate are thus termed co-agonists for the NMDA receptor complex. (Greene & Greenamyre, 1996 ; Kemp & McKernan, 2002). The ion channel integral to the NMDA receptor is voltage dependently blocked by magnesium and membrane depolarisation beyond a critical point removes this magnesium block allowing influx of calcium into intracellular media. The NMDA receptor accordingly links neurotransmitter activation with the electrical state of the neuron (Kemp & McKernan, 2002; Shubert *et al.*, 1997).

The NMDA receptor complex also has a number of other modulary sites (fig 2.4) and is thus subject to modulation by many endogenous and exogenous compounds. These include zinc and protons as well as polyamines. Many of these sites have attracted attention as possible pharmacological targets to prevent NMDA receptor mediated neurotoxicity (Kemp & Kew, 1998).

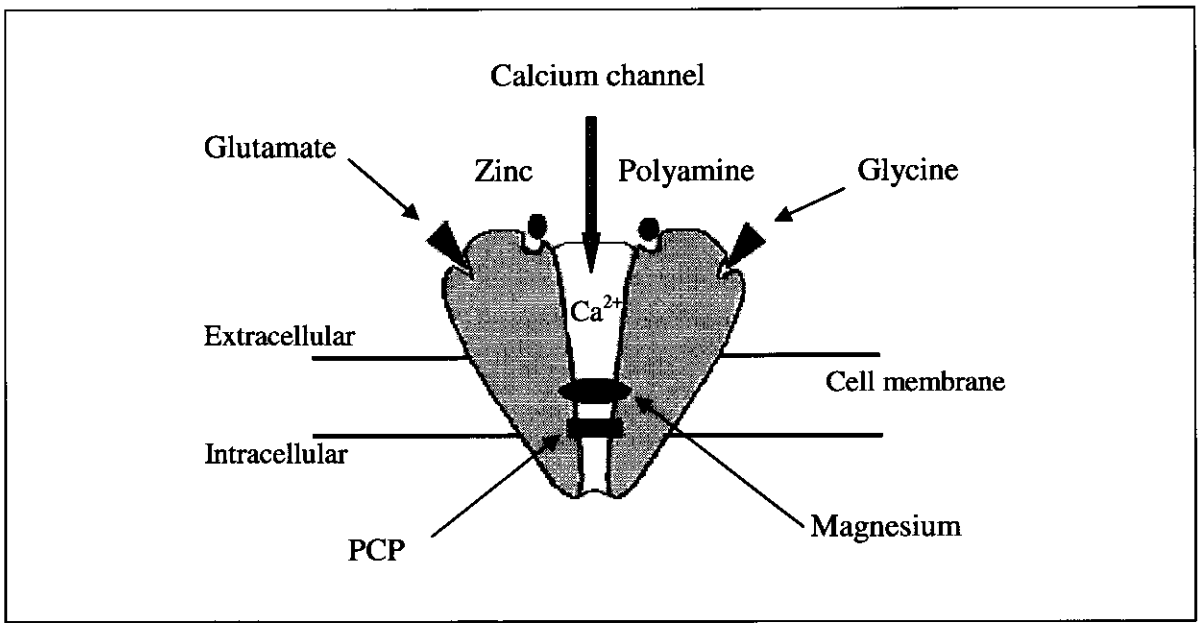


Figure 2.4: Schematic representation of modulary sites on the NMDA receptor

Chronic over stimulation of the NMDA reseptor channel results in accumulation of excess intracellular calcium which results in a neurodegenerative process termed, excitotoxicity. Excitotoxicity is a paradoxical phenomenon where neuroexcitation by glutamate results in a cascade of events that is ultimately neuropathological. An event termed weak excitotoxicity is caused by excessive depolarisation of the cell membrane resulting in excitotoxic receptor activation by normal levels of extracellular glutamate (Green & Greenamyre, 1996). Although excitotoxicity can be mediated by various excitatory amino acid receptors, the overstimulation of the NMDA complex (and the subsequent loss of calcium homeostasis) has been implicated in this type of neuronal damage (Choi *et al.*, 1988; Marini *et al.*, 1997).

Given the fact that the NMDA receptor appears to mediate a sequence of biochemical events that ultimately leads to neuronal cell degeneration, it seems that antagonising it, would be a positive step towards providing neuroprotective therapy. In theory, any CNS

disorder in which neuronal damage is caused by glutamate induced excitotoxicity, has the potential to be treated by blocking the NMDA receptor. This approach can also be applied to neurodegenerative disorders in which glutamate excess is not the primary problem, but rather neurons that become over sensitised to excitotoxic damage (for example Parkinson's and Huntington's diseases) (Kemp & Kew, 1998).

A number of competitive agonists and non-competitive antagonists acting on the NMDA receptor complex have been developed. Many of these compounds exhibit significant neuroprotection, but unfortunately, produce a number of adverse CNS effects. In humans these effects range from light-headedness, dizziness and paresthesia at low doses, to hallucinations and centrally mediated blood pressure increases at moderate doses. Dissociative anaesthesia and catatonia have been indicated at high doses (Kemp & Kew, 1998; Muir & Lees, 1995).

An alternative approach to avoid these side effects is to only partially block the NMDA receptor. This should allow adequate calcium influx not to disturb normal physiological processes but still prevent excessive calcium entry. A partial block can be achieved by blocking the glycine site on the NMDA receptor as glycine appears to play a modulary role in receptor activation. When the glycine site is blocked, the receptor – even at high glutamate concentrations – cannot be fully active. Blockade of the glycine site should thus permit a level of physiological NMDA receptor activation, while at the same time preventing excessive calcium entry (Kemp & Kew, 1998; Kemp & McKernan, 2002).

Non-competitive antagonists i.e. channel blockers could present an even more attractive pharmacological profile. Non-competitive NMDA inhibitors are known to bind at the so called Phencyclidine (PCP) binding site, located in the ion channel pore of the receptor (Kroemer *et al.*, 1998). Non-competitive antagonists include: Phencyclidine, ketamine, MK-801 (dizolcipine), dexoxadrol, amantadine and its dimethyl derivative memantine. The more potent the antagonists on the PCP-site, the higher their probability to induce psychomimetic side effects. MK-801 and phencyclidine (high affinity channel blockers) have, apart from their psychomimetic side effects, also been associated with acute neurotoxicity (Kornhuber & Weller, 1997).

Lower affinity channel blockers i.e. amantadine and memantine as well as ketamine seem to have a better therapeutic window. This could possibly be due to their more transient block of the activated receptor channels. Amantadine and memantine have been clinically administered for the management of Parkinson's disease, dementia, neuroleptic drug-induced side effects and spasticity. These agents only rarely induce significant psychomimetic side effects (Kemp & McKernan, 2002; Kornhuber & Weller, 1997).

It was found that memantine's mechanism of action is similar to that of MK-801 i.e. an open channel block of the NMDA receptor complex. Both these compounds are thus activity dependent and require channel opening to bind and block the receptor. Such activity dependency can, in contrast to competitive antagonists, be seen as a potential desirable feature for possible therapeutic calcium channel blockers. This type of binding would result in a reversible channel block during periods of high activity while leaving resting channels relatively unaffected. Memantine's safety at low micromolar concentrations may be related to its low affinity and fast voltage dependent channel blocking kinetics. At low concentrations memantine acts as an uncompetative antagonist and should thus theoretically allow near to normal NMDA activity – even in the face of pathologically high glutamate concentrations (Kemp & McKernan, 2002; Konhuber & Weller, 1997; Chen *et al.*, 1992).

Given the structural resemblance between the adamantanamines and the pentacyclo-undecylamine derivatives, it is postulated that these compounds could interact with the NMDA receptor complex in the same way, as do amantadine and memantine.

2.1.3 THE INFLUENCE OF MEMBRANE POTENTIAL ON INTRACELLULAR CALCIUM HOMEOSTASIS

The basis for the ability of excitable cells to respond to any external signal is the membrane potential (Messer, 2000). This electric potential that exists over the cell membrane is established through intracellular-extracellular concentration differences of positively and negatively charged ions. The concentration gradient is maintained through the selective permeability of the cell membrane to specific ions as well as the utilization of ionic pumps

(e.g. Na^+, K^+-ATPase) that drive ions against the concentration gradient in an energy dependent manner. The regulated permeability of the cell membrane to various ions is an important factor in a number of crucial cellular mechanisms. The resting membrane potential of neurons vary from -65 to -90 mV and represents the sum of the equilibrium potentials for each of the contributing ions (i.e. Na^+, K^+, Ca^{2+}, Cl^-). The equilibrium potential is the voltage relating the current and conductance for an individual ion and is described by the Nernst equation (Messer, 2000).

$$\text{Equilibrium potential} = 2.303 * (RT/zF) * \log \frac{[\text{ion}]\text{out}}{[\text{ion}]\text{in}}$$

Equation 2.1 Nernst Equation (Messer, 2000).

When the intracellular/extracellular concentration difference of a specific ion is known, the equilibrium potential can be calculated by substituting R (gas constant, 8.3143 joules/mole-degree); T (absolute temperature in degrees Kelvin); z (ionic valence of the ion) and F Faraday's constant (96.487 coulombs/mole) into the Nernst equation.

The contribution of a specific ion depends on the relative permeability of the ion through the cell membrane. At rest the membrane is permeable to both Na^+ and K^+ but as the relative permeability of potassium is much higher than that of sodium, the resting membrane potential of a cell is much closer to the equilibrium potential of potassium (-88.3 mV) (Messer, 2000).

Neuronal cells utilise specific changes in membrane potential termed action potentials to communicate signals along the axon to neurons in close proximity. An action potential consists of depolarisation of the cell membrane, repolarisation and finally hyperpolarisation (fig. 2.5). Initial depolarisation can be activated by a number of stimuli, but ultimately results in the opening of sodium channels causing the generator potential. Sodium channels are low voltage activated channels and thus has an increased probability for activation at low levels of depolarisation. When the generator potential reaches a critical level, or the

threshold potential (generally about 15 mV above the resting potential), an action potential is triggered (Messer, 2000). Transmembrane potential changes that occur during an action potential are a result of changes in the permeability of the membrane to the ions that maintain the resting membrane potential.

The depolarisation phase of an action potential is due to the opening of voltage gated ion channels, either sodium or calcium or a combination thereof. In an axon's presynaptic terminus as well as in some dendrites, influx of mostly calcium ions is responsible for membrane depolarisation. Voltage gated sodium channels closes after about 1 millisecond whereas the calcium mediated action potential can be much longer in duration. Opening of delayed rectifying voltage gated potassium channels and the outflow of K^+ trigger the repolarisation phase of the action potential. The opening of potassium channels has a slow onset but channels stay open for longer resulting in the consistent outflow of positive potassium ions and the hyperpolarisation of the membrane potential (Messer, 2000).

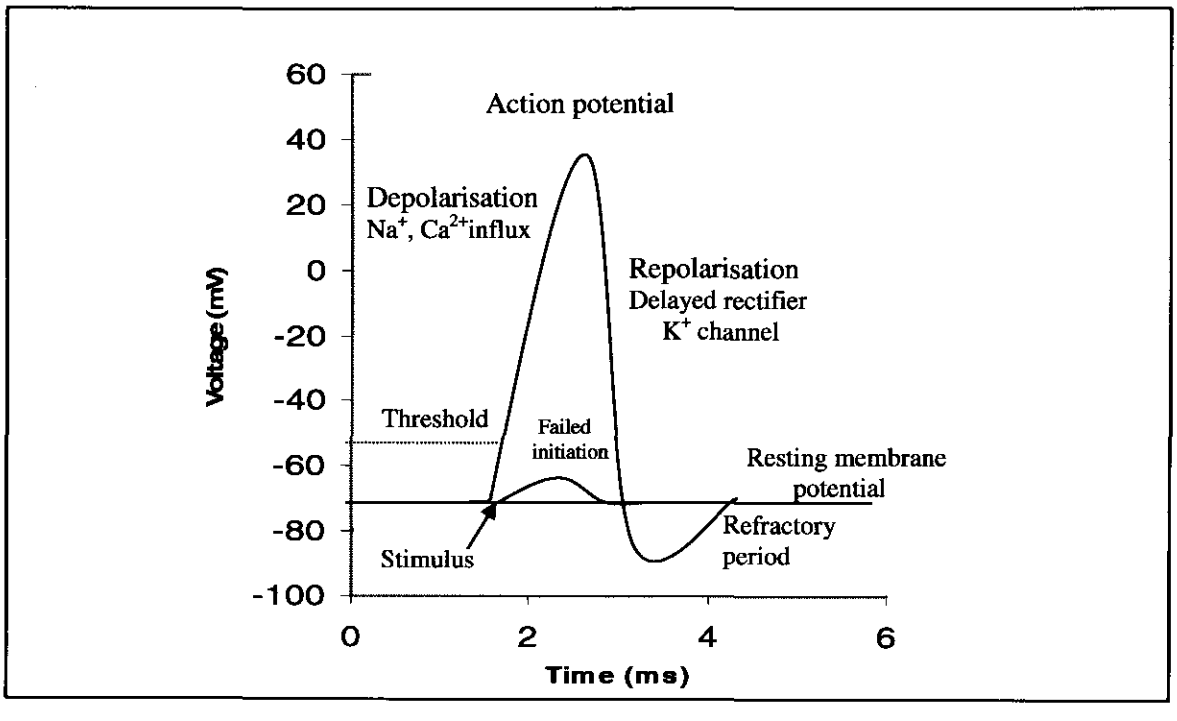


Figure 2.5: Schematic representation of events during an action potential.

During an action potential the absolute amounts of sodium and potassium that cross the cell membrane are low compared to the quantities in intracellular and extracellular media. Accordingly a single action potential has very little effect on the concentration gradient of these ions. This is however not the case with calcium. As the intracellular-extracellular concentration of calcium differs with several orders of magnitude, each action potential significantly alters the free calcium concentration within the cell.

Neuronal cells respond to an action potential through the opening of voltage dependent calcium channels as well as the release of glutamate and activation of NMDA receptor operated calcium channels. (Messer, 2000; Samdani *et al.*, 1997). A Change in membrane potential also removes the voltage dependent magnesium block from the NMDA receptor and accordingly facilitates the influx of calcium ions through this complex (Greene & Greenamyre, 1996).

VDCC activation as well as inactivation varies with changes in action potential waveform. The rate and amplitude of calcium influx as well as the time course of channel activation significantly influences the manner and amount of calcium entry into the cell. Alterations in the profile of calcium entry considerably influence the magnitude of calcium dependent responses in neuronal cells. Aforementioned processes are primarily controlled by the membrane potential but can be modulated through activation of G-protein-coupled signalling pathways. It was found that increases in the duration of an action potential augments total calcium influx but prolongs and also blunts Ca^{2+} signals by slowing influx rate and reducing peak amplitude (Park & Dunlap, 1998).

Van Goor *et al.* (1999) evaluated the relationship between electrical membrane activity and Ca^{2+} influx on GnRH-secreting neurons of the hypothalamus. Cells exhibited spontaneous extracellular Ca^{2+} dependent action potentials that changed in waveform in response to changes in resting membrane potential. More hyperpolarised cells fired sharp action potentials (resulting in decreased Ca^{2+} influx) whereas more depolarised cells fired broad action potentials with enhanced capacity for Ca^{2+} influx. Cells exhibited low voltage-activated Na^{+} and T-type Ca^{2+} which was dependent on more hyperpolarized membrane potentials for activation as well as high voltage-activated L-type VDCC. Accordingly all three channels were involved in the generation of sharp action potentials but only the high

voltage-activated L-type calcium channels participated in the generation of the broad action potentials. Consequently more depolarised resting membrane potentials limited the participation of Na^+ and low voltage activated calcium channels but facilitated action potential driven calcium influx through L-type VDCC (Van Goor *et al.*, 1999).

Compounds that influence the resting membrane potential of neuronal cells can accordingly manipulate the extent of participation of different types of voltage activated ion channels as well as influence the waveform of the action potential. Through alteration of the membrane potential or manipulation of the action potential waveform, compounds could significantly alter the calcium homeostasis within the cell and accordingly modify calcium dependent physiological processes.

2.2 BIOLOGICAL ACTIVITY OF THE PENTACYCLO-UNDECYL AND RELATED AMINE DERIVATIVES

Numerous studies have evaluated the biological activity of polycyclic amine compounds. As early as 1964 Davies *et al.* found that amantadine (1-amino-adamantane) exhibit anti-viral activity. It was later observed that amantadine is beneficial in the treatment of patients with Parkinson's disease. Amantadine is currently used in the prophylaxis and treatment of influenza A, as well as in treatment of Parkinson's disease and dementia (Standeart & Young, 2001).

The structural similarities between these compounds and the pentacycloundecylamine derivatives prompted further studies into the biological activity of the cage compounds.

Apart from the L-type calcium channel activity that was indicated mainly for the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecylamine derivatives (Van der Schyf *et al., 1986*; Van der Walt *et al.*, 1988), Oliver *et al.* (1991a) concluded that the D3-Trishomocubyl-4-amines show antiviral activity comparable to that of amantadine and acyclovir. It was also indicated that the pentacycloundecylamines antagonise reserpine-induced catatonia and show weak to mild anticholinergic properties. The pentacycloundecylamines also show the ability to

reduce oxotremorine-induced tremors and salivation in mice. These properties indicate potential value for therapeutic use of the polycyclic cage compounds as anti-parkinsonian agents (Oliver *et al.*, 1991b).

Liebenberg (1986) studied the structure activity relationships of the polycyclic amine derivatives on calcium channels using an electrically-stimulated guinea-pig papillary muscle. It was found that the antagonistic activity of these compounds improve with increased chain length between the polycyclic cage and the aromatic moiety. Studies by Dockendolf (1992) contradicted these findings, as it was found that the phenylethyl derivative had lower activity than the lead compound, NGP1-0l (**1**) (fig. 2.6). Liebenberg (1986) also indicated that channel activity improves with increased compound lipophilicity.

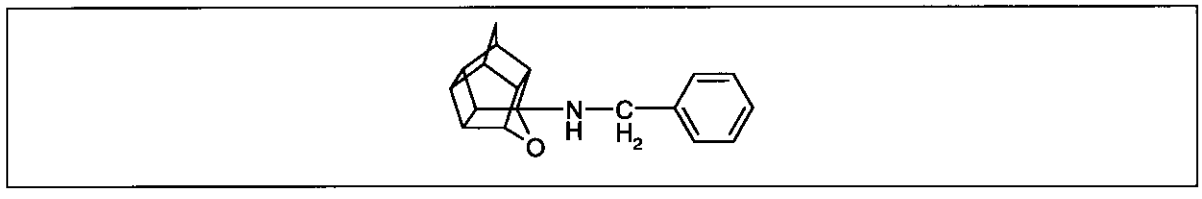


Figure 2.6: 8-Benzylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (NGP1-01)

Studies by Kassiou *et al.* (1996) revealed that the cage compounds, specifically the pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecylamines, as well as the azahexacyclo [5.4.1.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$.$0^{8,11}$]dodecanes, exhibit selectivity, and high affinity for the sigma (σ) binding site. As many studies have indicated that σ-binding could play a role in the modulation of striatal dopamine neurotransmission, it was suggested that this mechanism could account for the anti-parkinsonian properties of the cage compounds (Kassiou *et al.*, 1996).

The polycyclic cage compounds show calcium channel antagonism that relates predominantly to that of the 1,4-dihydropyridines. Similar active binding sites also seem to be involved. At lower membrane potentials however, the cage compounds show better activity on calcium channels than do the 1,4-dihydropyridines (Dockendolf, 1992).

Very little stereoselectivity was indicated for the interaction of NGP1-01 with VDCC (Malan *et al.*, 1998) and the structure activity relationships for calcium channel activity of the polycyclic compounds on cardiac myocytes was found to be more steric and geometric, than electronic, in nature (Malan *et al.*, 2000). This conclusion was drawn from the findings that both the methoxy and the nitrobenzyl derivatives of NGPl-0l show more pronounced calcium channel activity than do the lead compound. Aromatic side chain substitution predominantly showed an increase in calcium channel activity. Increased antagonism was found especially with orto and meta substitution. As it was found that the methoxybenzylamine derivatives show better activity than do nitrobenzylamine derivatives, electronic properties of the ring can however not be deemed irrelevant. Lower calcium channel activity was indicated for compounds in which the aromatic side chain tends to fold back onto the polycyclic cage i.e. the aminomethylpyridine derivatives as well as the phenylhydrazine derivative.

Malan *et al.* (2003) established that 6-benzylamino-3-hydroxyhexacyclo-[6.5.0.0^{3,7}.0^{4,12}.0^{5,10}.0^{9,13}]tridecane mainly interact with the inactivated state of the L-type VDCC, but show no effect on the T -type calcium channels. These compounds inhibited L-type calcium channels, sodium channels as well as the fast component of the delayed rectifier potassium channels.

Geldenhuys *et al.* (2002) further investigated the neuroprotective abilities of the pentacycloundecylamines. NMDA receptor antagonism and/or a direct block of neuronal VDCC were proposed as mechanisms for prevention of neurodegeneration. Structural similarities between the pentacycloundecylamines and known NMDA antagonists were compared using computer modelling. Favourable root-mean-square fittings were found when fitting the cage compounds, MK-801 and memantine onto the PCP-ligand. These findings suggest that the pentacycloundecylamines might have a similar mechanism of interaction with the PCP binding site to that of MK-801 and memantine (fig. 2.7).

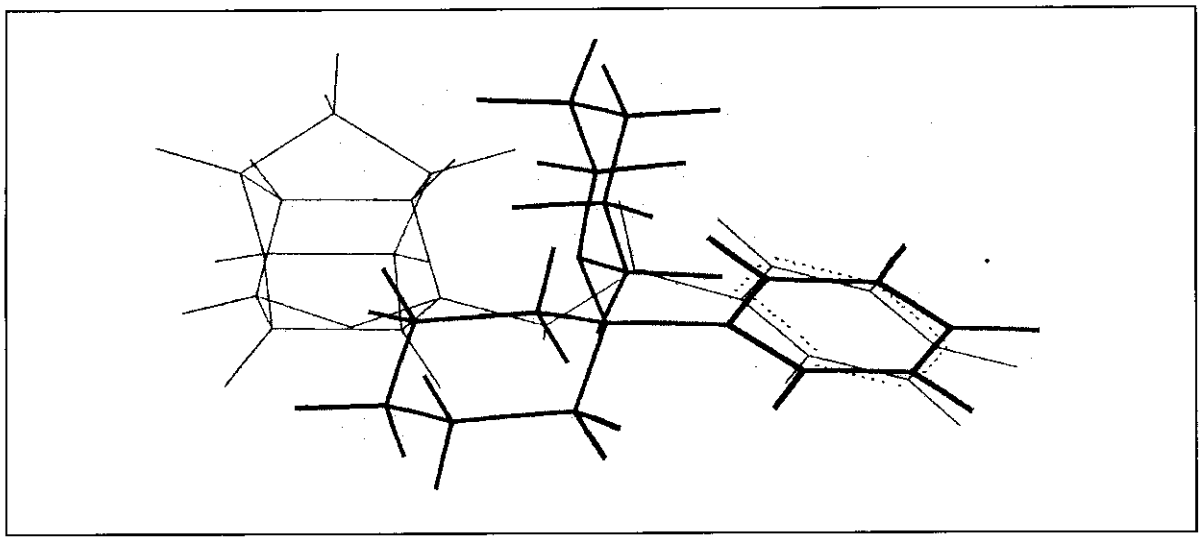


Figure 2.7: Rms fit of NGP1-01 (thin line) on PCP (thick line) (Geldenhuys *et al.*, 2002).

The MPTP-parkinsonian mouse model was used to evaluate the protection of the chosen polycyclic amine derivatives against MPTP-induced striatal dopamine depletion. It was found that, with the exception of the phenylethyl derivative of NGP1-0l, these compounds did not provide meaningful protection against striatal neurodegeneration. As the two known NMDA antagonists, MK-801 and memantine, also failed to provide significant neuroprotection under these experimental conditions, it was postulated that the MPTP parkinsonian mouse model is inadequate for screening of neuroprotection, mechanistically based on NMDA receptor antagonism. In this regard, the cage compounds couldn't be dismissed as being totally devoid of any neuroprotective activity and the apparent failure of NGP1-01 and derivatives thus needs to be re-evaluated (Geldenhuys *et al.*, 2002).

Zah *et al.* (2003) investigated the ability of the pentacycloundecylamines to cross the blood-brain barrier. Optimal permeability is usually found for compounds with a log BB value in the range of 0.3. Larger values indicate highly permeable drugs, and values smaller than -1.0 indicate poor permeability. It was found that all studied polycyclic cage compounds exhibit log BB values that range between -0.5 and 0.43 and accordingly, penetrate the CNS in sufficient amounts to exert a meaningful pharmacological effect. A model, by which the blood-brain permeability of the cage compounds could be predicted, was also developed. In accordance to the data obtained, Zah *et al.* (2003) suggested that the polycyclic compounds

show a high likelihood to penetrate the CNS and that the main mode of entry into the CNS, is by passive diffusion.

2.3 USE OF FLUORESCENT PROBES IN VISUALISATION OF REAL-TIME CELLULAR PHYSIOLOGY

The use of fluorescent probes in visualising cellular physiology enables the specific targeting of cellular components and dynamic processes in living cells. Fluorescent probes are designed to localise in specific regions of the cell (i.e. mitochondria, endoplasmic reticulum, nucleus or membranes) or can be utilised to monitor dynamic cellular processes for example membrane potential, changes in pH and concentrations of inorganic ions.

2.3.1 FLUORESCENT POTENTIOMETRIC INDICATORS

The transmembrane potential over the plasma membrane of resting cells is normally maintained in the vicinity of -70 mV and originates from ion concentrations gradients upheld by the active transport systems in the plasma membrane. The activation of voltage dependent ion channels embedded in the plasma membrane of a specific cell is modulated by changes in its transmembrane potential (Bezanilla, 2000).

Voltage sensitive (potentiometric) dyes allows for the visualisation of changes in the membrane potential in a more convenient and less invasive way than conventional microelectrode-based methods (Loew, 1998). Potentiometric probes are divided into two classes based on their response to variations in transmembrane potential. Fast response probes operate via changes in their electronic structure and subsequently changes in the emitted fluorescence whilst slow response probes show potential dependent transmembrane redistribution, concentrating in areas with higher anion concentrations. Fast response probes display optical responses sufficiently fast to detect millisecond potential changes but often only show small changes in potential-dependent fluorescence (typically 2-10% fluorescence change per 100 mV). Slow response probes exhibit much larger potential

dependent changes in fluorescence (1% fluorescence change per mV) and are thus suitable for the measurement of much smaller changes in transmembrane potential (Haugland, 2002).

Tetramethylrhodamine methyl ester (TMRM) and Tetramethylrhodamine ethyl ester (TMRE) are slow response dyes that are commonly used for the quantitative measurement of membrane potentials (Loew, 1998). Because TMRM and TMRE display strong fluorescence it can be used in lower concentrations, thus preventing aggregate formation in the cell membranes. These dyes rapidly cross the plasma membrane and only minimally interact with membrane proteins. As their fluorescence is relatively insensitive to environmental changes, the transmembrane distribution of these dyes can be directly related to the plasma membrane potential using the Nernst equation (eq. 2.2) (Haugland, 2002).

$$\Delta V = -60\ \text{mV} \log \frac{[D]_{in}}{[D]_{out}} = -60\ \text{mV} \log \frac{F_{in}}{F_{out}}$$

Equation 2.2: Relation of dye distribution to fluorescence by means of the Nernst equation (Loew, 1998).

The emitted fluorescence [F] in a specific area should be proportional to the concentration of the dye [D] in that area and the ratio of the fluorescence intracellularly (F_{in}) to extracellularly (F_{out}) should be logarithmically related to the potential over the cell membrane (Loew, 1998).

2.3.2 CONFOCAL MICROSCOPY IN COMBINATION WITH FLUORESCENT INDICATORS

In recent years the popularity of confocal microscopy has increased with leaps and bounds and applications for this technique is without end. Confocal imaging presents extreme high-quality images and can be used independently, or in combination with classical fluorescence techniques. This technique permits precise, non-destructive optical sectioning in planes

either perpendicular or parallel to the axis of the microscope and offers controllable depth of field as well as elimination of background haze. Confocal imaging ensures better resolution both vertically and horizontally and allows the assemblence of consecutive optical images from larger specimens (Laurent *et al.*, 1994).

The major optical difference between a conventional microscope and a confocal microscope is the presence of the confocal pinholes. A pinhole positioned relative to the focal point blocks most of the fluorescence light originating from planes other than the plane of focus. This results in images with exceptional resolution and clarity and significantly less fluorescence blur. In a confocal microscope, the excitation laser is scanned across the specimen in a series of directly adjacent parallel lines. Emitted light that passes through the pinhole is collected in a photomultiplier allowing electronic amplification of the received signal. The signal is then processed using specialised computer software. As the confocal microscope in effect only sees one plane of focus for any given image, it is possible to collect a series of optical sections by scanning a sequence of focal planes at different depths within the specimen. These sections can then be recombined to produce 3-dimentional representations of the scanned specimen (Laurent *et al.*, 1994).

The confocal technique holds a great deal of potential for allowing the visualisation of the cellular world and is rapidly becoming the technique of choice in many areas of research.

CHAPTER 3

SELECTION AND SYNTHESIS OF RELEVENT COMPOUNDS

SUMMARY

Structures were selected in order to attain a meaningful series of polycyclic amine derivatives from which structure activity relationships could be proposed. Compounds were synthesised by reductive amination of the polycyclic "cage", pentacyclo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione and characterised using NMR, MS and IR techniques.

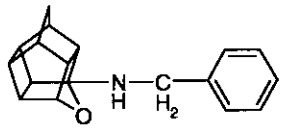
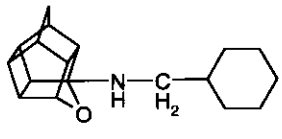
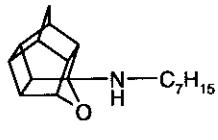
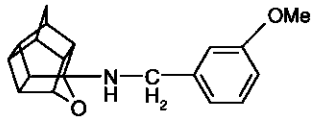
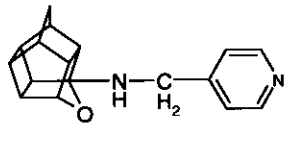
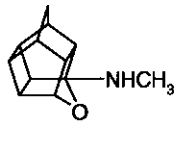
ABBREVIATIONS

mp	Melting point
HR-MS	High resolution Mass spectrometry
MS	Mass spectrometry
IR	Infrared spectroscopy
NMR	Nuclear magnetic resonance spectroscopy

3.1 SELECTION OF COMPOUNDS FOR SYNTHESIS

A series of derivatives (Table 3.1) were carefully compiled to facilitate evaluation of a wide variety of chemical features. The influence of an unsaturated system, cyclic and aliphatic substituents, hydrogen bonding and side chain length, volume and flexibility were assessed. 8-benzylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane was chosen as lead compound as it has thus far been extensively studied within this class of compounds.

Table 3.1: Structures selected for synthesis and evaluation

COMPOUND	STRUCTURE	
1		8-Benzylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane
2		8-Cyclohexylmethylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane
3		8-Hepthylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane
4		8-(3-Methoxybenzylamino)-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane
5		8-[(4-Aminomethyl)pyridine]-8,11-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane
6		8-Methylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane

8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane or NGP1-01 (**1**), was identified as the near optimal structure for peripheral calcium channel activity. Evaluating the central activity of this compound would provide the opportunity to correlate peripheral activity of the cage compounds to activity within the CNS. With the inclusion of compound 2 the role of the unsaturated system of NGP1-01 can be compared to that of a related, but saturated cyclohexyl moiety.

By incorporating compound 3 in the study, the importance of a cyclic substituent in neuronal activity can be evaluated with aliphatic substitution on the pentacycloundecane template. The hepthylamino substituent shows increased flexibility but the volume of the substituent is still comparable to that of the cyclohexylmethylamino derivative.

By including 8-(3-methoxybenzylamino)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**4**), the nature of the structure activity relationships on neuronal voltage activated ion channels can be evaluated. The heteroatom within the pyridine ring in compound **5** introduces hydrogen bonding to receptor interaction without a change in the molecular volume. By evaluating this compound the effects of hydrogen bonding can be compared to the normal hydrophobic interactions of the phenyl ring. It was also observed that the pyridine ring tends to fold back onto the polycyclic cage and influences thereof can accordingly be evaluated.

By comparing 8-methylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**6**) to compound **3**, the effect of increased chain length and N-substitution on the activity of the polycyclic cage compounds can be evaluated. These compounds would also give an indication of the available space or volume for interaction within the channel or receptor.

3.2 GENERAL PROCEDURES

Reagents were obtained from D.H. chemicals (U.K.), Sigma-Aldrich (U.K.), Acros Organics (U.S.A.) and Fluka (Switzerland) and reaction and elution solvents purchased from commercial sources. Melting points were measured using a Gallenkamp melting point apparatus and IR spectra recorded on a Nicolet 470 FT-IR spectrophotometer. Mass spectra and HR-MS were recorded using a VG 7070E mass spectrometer. NMR spectra were acquired on a Varian Gemini 300 with ^{1}H spectra recorded at a frequency of 300.075 MHz and ^{13}C spectra at 75.462 MHz. Chemical shifts are reported in parts per million (ppm) relative to the internal standard, tetramethylsilane (TMS). The following abbreviations indicate multiplicities of the respective signals: s, singulet; bs, broad singulet; d, doublet; t, triplet; q, quartet; m, multiplet.

3.3 SYNTHESIS OF COMPOUNDS

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane–8,11-dione (**A**) was formed by photocyclisation of the Diels-Alder adduct, resulting from the reaction between p-bensoquinone and cyclopentadiene. Reductive amination of the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane–8,11-diones, with the respective amines, resulted in the formation of the corresponding carbinolamine (**B**). This product was dehydrated under Dean Stark dehydrating conditions and the resulting intermediate imine was then reduced to the final polycyclic amine (**C**), using $NaBH_4$ in anhydrous methanol and THF (fig 3.1)

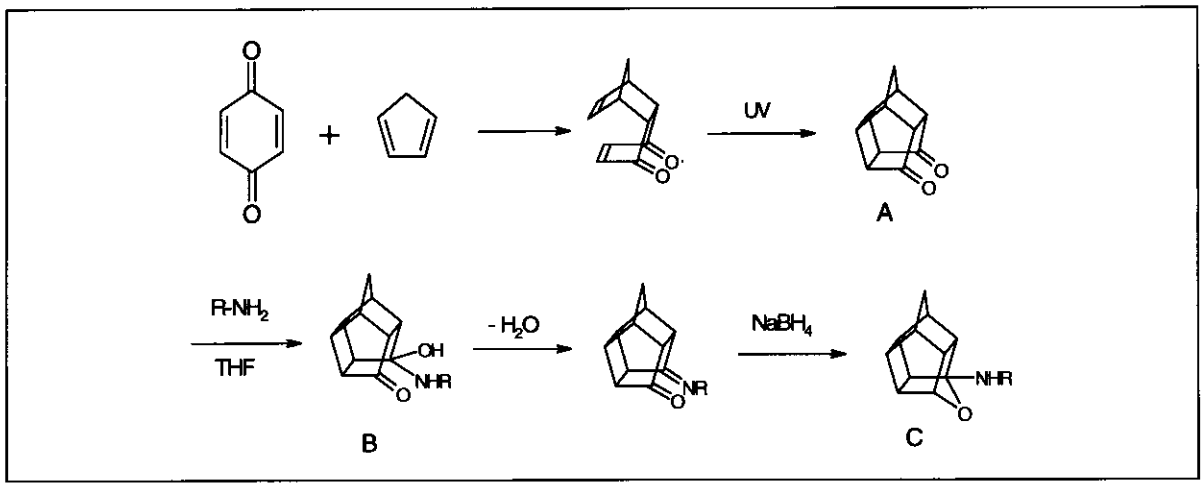
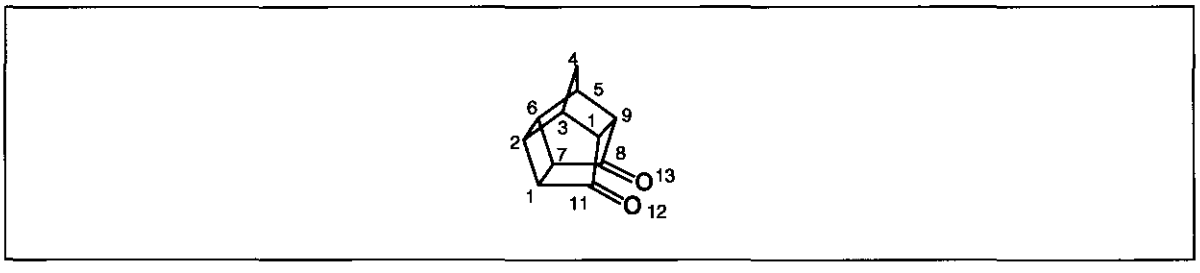


Figure 3.1: Basic synthesis of the polycyclic amines (Malan *et al.*, 2000).

3.3.1 PENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE-8,11-DIONE

p-Benzoquinone (50.00 g, 0.46 mol) was dissolved in dried Benzene (400 ml) and oxidized with MnO_2 (± 30 mg) under reflux for 20 min. 2 Spatula measures of activated charcoal were added and the mixture was refluxed for an additional 5 min. The warm solution was vacuum-filtered through celite® to produce a clear dark yellow solution. Recrystallisation of the oxidized 1,4-benzoquinone was prevented by immediately filtering 100 ml of dried methanol through the same system. The mixture was placed in an external ice bath and allowed to cool down to ± 5°C before stoichiometrically adding the freshly monomerised cyclopentadiene (65 g, 0.5 mol). The mixture was removed from the ice bath, protected from light and stirred overnight at room temperature. The reaction was monitored on TLC plates and presumed complete when all p-benzoquinone had been used. The reaction mixture was treated with activated charcoal, filtered through celite®, excess solvents removed *in vacuo* and allowed to fully evaporate in a dark fume cupboard to afford the Diels-Alder adduct as yellow crystals. Recrystallisation from petroleum ether resulted in the formation of bright yellow needle-like crystals. The Diels-Alder adduct was dissolved in acetone (± 3 g in 100 ml) and exposed to UV light from a Phillips HPA 1000-20 lamp for approximately six hours. Decolouration of the solution confirmed that cyclisation of the adduct was completed and the solvent was subsequently removed *in vacuo*. The resulting

light yellow residue was purified by Soxhlett extraction in *n*-hexane to produce the diketone as fine white crystals (Yield: 69.08 g, 0.397 mol, 80.934%).

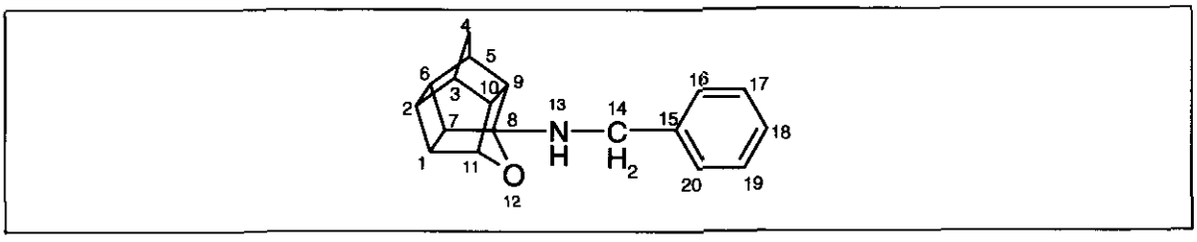


Physical characterisation of these crystals correlate with that in Cookson *et al.*, 1964 and no spectral data is presented in this dissertation.

3.3.2 8-BENZYLAMINO-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE (**1**; NGP1-01)

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5.01 g, 0.029 mol) was dissolved in anhydrous tetrahydrofuran (THF; 50 ml) and cooled down to 5°C in an external ice bath. Benzylamine (3.21 g, 0.030 mol) was slowly added while the mixture was stirred for approximately 30 min at lowered temperatures. The carbinolamine which started precipitating after ± 15 min was isolated by filtration and washed with cold THF (2 x 30 ml) producing the carbinolamine as a white paste. This product was dehydrated under Dean Stark conditions for approximately 1 hour using 50 ml dried benzene. Benzene was removed *in vacuo* to produce the Schiff base (8-benzyliminopentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}] undecane-11-one) in the form of a yellowish oil. The resulting oil was dissolved in anhydrous methanol (30 ml) and anhydrous THF (150 ml). Reduction was carried out by adding $NaBH_4$ (1.5 g, 39.5 mmol) and stirring overnight at room temperature. Solvents were removed and the resulting white residue was suspended in approximately 100 ml of distilled water. The product was extracted with dichloromethane (4 x 50 ml) and the combined dichloromethane fractions were washed with water (2 x 100 ml). The mixture was dried over anhydrous $MgSO_4$ and filtered. Subsequent *in vacuo* removal of the solvent resulted in the formation of a yellow oil which was purified with column chromatography

(1:1:1 petroleum ether: dichloromethane: ethyl acetate) to produce a colourless wax. Recrystallisation from anhydrous ethanol resulted in the formation of pure white crystals (Yield: 3.00 g, 0.03 mol, 39.02%).

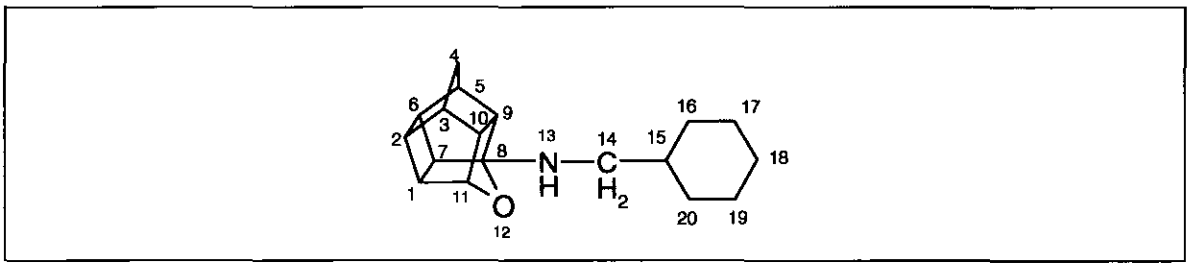


$C_{18}H_{19}NO$; **mp** 78 °C; **HR-MS:** calc. 265.147, exp. 265.146; **IR (KBr)**ν_{max} (Spectrum 1): 3313, 2970, 1352, 1009 cm^{-1}; **MS** (EI, 70 eV) *m/z* (Spectrum 7): 265 (M^+), 237, 186, 131, 91, 65, 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (spectrum 13): 7.42 – 7.09 (m, 5H, H-16,17,18,19,20), 4.74 (t, 1H, J = 5.33 Hz, H-11), 4.11 (ABq, 2H, J_{AB} = 13.46, H-14a,14b), 2.98 – 2.47 (3 x m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 2.27 (bs, 1H, NH), 1.95-1.62 (ABq, 2H, J = 10.11 Hz, H-4a,b); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 19): 140.85 (s, C-15), 128.40 (d, C-16,20), 127.84 (d, C-17,19), 126.78 (d, C-18), 109.60 (s, C-8), 82.48 (d, C-11), 55.27 (d, C-7/9), 54.78 (d, C-7/9), 47.80 (t, C-14), 44.90 (d, 1C), 44.86 (d, 1C), 44.57 (d, 1C), 43.25 (t, C-4), 43.14 (d, 1C), 42.00 (d, 1C), 41.54 (d, 1C).

3.3.3 8-CYCLOHEXYLMETHYLAMINO-8,11-OXAPENTACYCLO-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE (2)

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5.08 g, 0.029 mol) was dissolved in anhydrous tetrahydrofuran (THF; 50 ml) and cooled down to 5°C in an external ice bath. Cyclohexylmethylamine (3.51 g, 0.031 mol) was slowly added while the mixture was stirred for approximately 30 min at lowered temperatures. The carbinolamine which started precipitating after ± 15 min was isolated by filtration and washed with cold THF (2 x 30 ml) producing the isolated carbinolamine as a white paste. This product was dehydrated under Dean Stark conditions for approximately 1 hour using 50 ml dried benzene. Benzene was

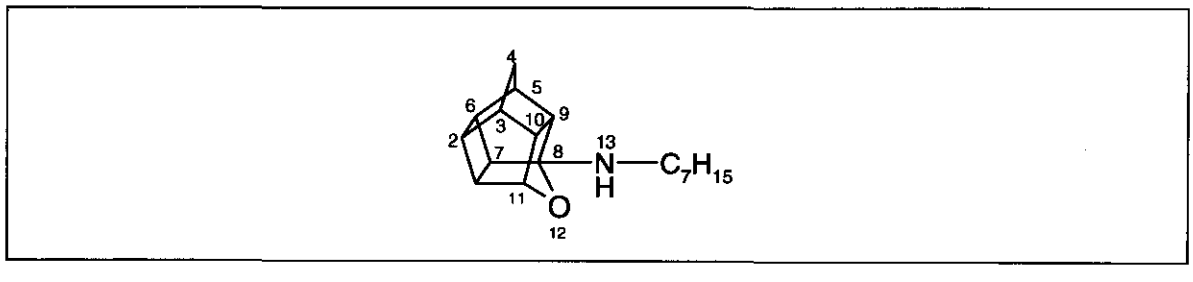
removed *in vacuo* to produce the Schiff base (8-cyclohexylmethyliminopentacyclo [5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-11-one) in the form of a yellow oil. The resulting oil was dissolved in anhydrous methanol (30 ml) and anhydrous THF (150 ml). Reduction was carried out by adding $NaBH_4$ (1.5 g, 39.5 mmol) and stirring overnight at room temperature. Solvents were removed and the resulting white residue was suspended in approximately 100 ml of distilled water. The product was extracted with dichloromethane (4 x 50 ml) and the combined dichloromethane fractions were washed with water (2 x 100 ml). The mixture was dried over anhydrous $MgSO_4$ and filtered. Subsequent *in vacuo* removal of the solvent resulted in the formation of a yellow oil which was purified with column chromatography (1:9 ethanol: ethyl acetate) to produce a yellowish oily residue (Yield: 2.07 g, 0.0077 mol, 24.69%)



$C_{18}H_{25}NO$; **HR-MS** calc. 271.193, exp. 273.193; **IR** (KBr)ν_{max} (Spectrum 2): 3324, 2914, 1449, 1357, 1014; **MS** (EI, 70 eV) *m/z* (Spectrum 8): 271 (M^+), 188, 176, 159, 131, 91, 56, 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 14): 4.67 (t, 1H, J = 5.22 Hz, H-11), 2.51-2.89 (3 x m, 9H, H-1, 2, 3, 5, 6, 7/9, 10, 14a, 14b), 2.39 (m, 1H, H-7/9), 1.93 (bs, 1H, NH), 1.89 : 1.53 (ABq, 2H, J = 10.3 Hz, H-4a, 4b), 1.78-0.97 (4 x m, 11H, H-15, 16, 17, 18, 19, 20); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 20): 109.77 (s, C-8), 82.34 (d, C-11), 55.04 (d, C-7/9), 54.73 (d, C-7/9), 50.24 (t C-14), 44.84 (d, 2C), 44.48 (d, 1C), 43.23 (d, 1C), 41.88(d, 1C), 41.51 (d, 1C), 38.29 (d, C-15), 26.64 (t, C-16,20), 25.99 (t, C-17,19).

3.3.4 8-HEPTHYLAMINO-8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE (3)

Pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (5.12 g, 0.029 mol) was dissolved in anhydrous tetrahydrofuran (THF; 50 ml) and cooled down to 5°C in an external ice bath. Hepthylamine (3.46 g, 0.03 mol) was slowly added while the mixture was stirred for approximately 30 min at lowered temperatures. The carbinolamine which started precipitating after ± 10 min was isolated by filtration and washed with cold THF (2 x 30 ml) producing the carbinolamine as a thick white paste. This product was dehydrated under Dean Stark conditions for approximately 1 hour using 50 ml dried benzene. Benzene was removed *in vacuo* to produce the Schiff base (8-hepthyliminopentacyclo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-11-one) in the form of a yellow oil. The resulting oil was dissolved in anhydrous methanol (30 ml) and anhydrous THF (150 ml). Reduction was carried out by adding $NaBH_4$ (1.5 g, 39.5 mmol) and stirring overnight at room temperature. Solvents were removed and the resulting white residue was suspended in approximately 100 ml of distilled water. The product was extracted with dichloromethane (4 x 50 ml) and the combined dichloromethane fractions were washed with water (2 x 100 ml). The mixture was dried over anhydrous $MgSO_4$ and filtered. Subsequent *in vacuo* removal of the solvent resulted in the formation of a dark yellow oil that was purified with column chromatography (1:1:8 ethyl acetate: dichloromethane: petroleum ether) to produce a yellow oil (Yield: 2.39 g, 0.0087 mol, 29.121%).

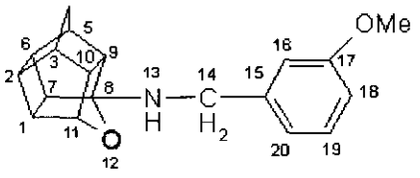


$C_{18}H_{27}NO$; **HR-MS**: calc. 273.413, exp. 273.412 **IR** (KBr)ν_{max} (Spectrum 3): 3308, 2924, 1465, 1357, 1019; **MS** (EI, 70 eV) *m/z* (Spectrum 9): 273 (M^+), 188, 131, 91, 43, 29; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 15): 4.69 (t, 1H, J = 10.43 Hz, H-11),

2.60-2.87 (3 x m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 2.41 (m, 1H, NH), 2.00-1.56 (ABq, 2H, J = 10.53 Hz, H-4a,4b), 1.35 (3 x m, 12H, H-14a,b, 15, 16, 17, 18, 19), 0.92 (t, 3H, J = 7.0 Hz CH_3); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 21): 109.73 (s, C-8), 82.36 (d, C-11), 55.21 (d, C-7/9), 54.75 (d, C-7/9), 44.81(t, C-14), 44.66 (d, 1C), 44.48 (d, 1C) 43.75 (d, 1C) 43.24 (t C-4) 43.04 (d, 1C), 41.71 (d, 1C), 41.51 (d, 1C), 31.77 (t, C-15), 31.74 (t, C-16), 31.03 (t, C-17), 29.10 (t, C-18), 27.22 (t, C-19).

3.3.5 8-(3-METHOXYBENZYLAMINO)-8,11-OXAPENTACYCLO [5.4.0.0^{2,6}.0^{3,10}.0^{5,9}] UNDECANE (**4**)

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5.04 g, 0.029 mol) was dissolved in anhydrous tetrahydrofuran (THF; 50 ml) and cooled down to 5°C in an external ice bath. Methoxybenzylamine (4.05 g, 0.029 mol) was slowly added while the mixture was stirred for approximately 30 min at lowered temperatures. The carbinolamine which started precipitating after ± 15 min was isolated by filtration and washed with cold THF (2 x 30 ml) producing the carbinolamine as a white paste. This product was dehydrated under Dean Stark conditions for approximately 1 hour using 50 ml dried benzene. Benzene was removed *in vacuo* to produce the Schiff base (8-methoxybenzylimino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-11-one) in the form of a yellow oil. The resulting oil was dissolved in anhydrous methanol (30 ml) and anhydrous THF (150 ml). Reduction was carried out by adding $NaBH_4$ (1.5 g, 39.5 mmol) and stirring overnight at room temperature. Solvents were removed and the resulting white residue was suspended in approximately 100 ml of distilled water. The product was extracted with dichloromethane (4 x 50 ml) and the combined dichloromethane fractions were washed with water (2 x 100 ml). The mixture was dried over anhydrous $MgSO_4$ and filtered. *In vacuo* removal of the solvent resulted in the formation of a dark yellow oil which was purified with column chromatography (1:3 ethyl acetate: petroleum ether) to produce a yellow oil (Yield: 2.83 g, 0.0096 mol, 32.98%).

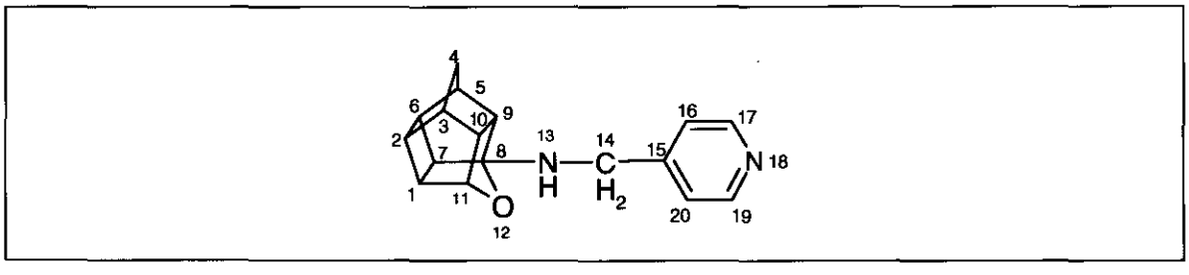


$C_{19}H_{21}NO_2$; HR-MS: calc. 295.157, exp. 295.158; **IR** (KBr)ν_{max} (Spectrum 4): 3324, 2965, 1603,1490, 1260; **MS** (EI, 70 eV) *m/z* (Spectrum 10): 295 (M+), 136, 121, 91, 77, 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 16): 7.23 (t, 1H, J = 5.23 Hz, H-19), 6.97 (m, 2H, H-16,20), 6.81 (m, 1H, H-18), 4.78 (t, 1H, J = 5.25 Hz, H-11), 4.07 (ABq, 2H, J = 13.73 Hz, H-14a,14b), 3.85 (s, 3H, OCH_3), 2.44-2.99 (3 x m, 8H, H-1, 2, 3,5,6,7,9,10), 2.26 (bs, 1H, NH), 1.96 : 1.61 (ABq, 2H, J = 10.34 Hz, H-4a,4b); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 22): 159,71 (s, C-17), 142.58 (s, C-15), 129.28 (d, C-19), 120.28 (d, C-20), 113.29 (d, C-16), 112.32 (d, C-18), 109.57 (s, C-8), 82.47 (d, C-11), 55.72 (d, C-7/9), 55.28 (k, CH_3), 54.75 (d, C-7/9), 47.71 (t, C-14), 44.87 (d, 1C), 44.84 (d, 1C), 44.55 (d, 1C), 43.23 (t, C-4), 43.14 (d, 1C), 42.00 (d, 1C), 41.53 (d, 1C).

3.3.6 8-[(4-AMINOMETHYL)PYRIDINE]-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE (**5**)

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5.02 g, 0.029 mol) was dissolved in anhydrous tetrahydrofuran (THF; 50 ml) and cooled down to 5°C in an external ice bath. 4-(Aminomethyl)pyridine (3.65 g, 0.032 mol) was slowly added while the mixture was stirred for approximately 30 min at lowered temperatures. The carbinolamine that started precipitating after ± 15 min was isolated by filtration and washed with cold THF (2 x 30 ml) producing the carbinolamine as a white paste. This product was dehydrated under Dean Stark conditions for approximately 1 hour using 50 ml dried benzene. Benzene was removed *in vacuo* to produce the Schiff base (8-[(4-iminomethyl)pyridine]-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-11-one) in the form of a yellow oil. The resulting oil was dissolved in anhydrous methanol (30 ml) and anhydrous THF (150 ml). Reduction was carried out by adding $NaBH_4$ (1.5 g, 39.5 mmol) and stirring overnight at room

temperature. Solvents were removed and the resulting white residue was suspended in approximately 100 ml of distilled water. The product was extracted with dichloromethane (4 x 50 ml) and the combined dichloromethane fractions were washed with water (2 x 100 ml). The mixture was dried over anhydrous $MgSO_4$ and filtered. *In vacuo* removal of the solvent resulted in the formation of a yellow oil with signs of crystallisation. The product was purified with column chromatography (9:1 ethyl acetate: ethanol) to produce white crystals (Yield: 3.424 g, 0.0129 mol, 42.85%).

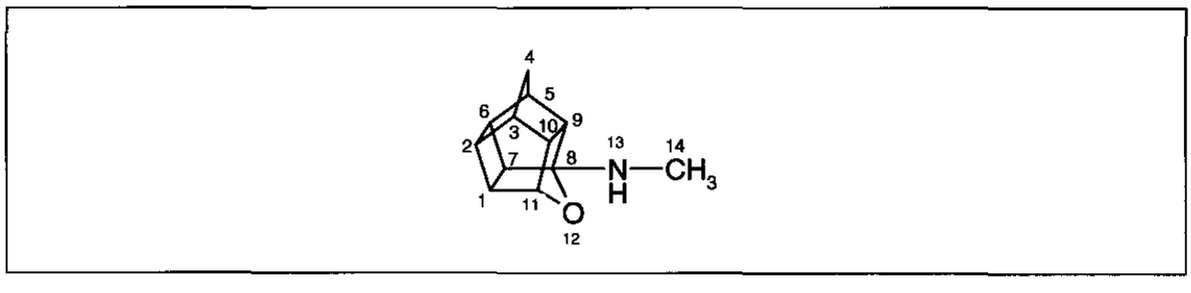


$C_{17}H_{18}N_2O$; **mp** 111.3; **HR-MS**: calc. 266.142, exp. 266.142; **IR** (KBr)ν_{max} (Spectrum 5): 3293, 2970, 1598, 1367, 1004, 845; **MS** (EI, 70 eV) *m/z* (Spectrum 11): 266(M^+), 187, 174, 131, 92, 65, 39, 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 17): 8.63 (d, 2H, J = 4.42 Hz, H-17,19), 7.38 (d, 2H, J = 4.67 Hz, H-16,20), 4.73 (t, 1H, J = 5.22 Hz, H-11), 4.18 (ABq, 2H, J = 15.39 Hz, H-14a,14b), 2.59-2.97 (3 x m, 9H, H-1, 2, 3, 5, 6, 7, 9, 10, 13), 2.03 : 1.67 (ABq, 2H, J = 10.44 Hz, H-4a,4b); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 23): 150.29 (s, C-15), 149.64 (d, C-17,19), 122.46 (d, C-16,20), 109.39 (s, C-8), 82.48 (d, C-11), 55.32 (d, C-7/9), 54.70 (d, C-7/9), 46.51 (t, C-14), 44.86 (d, 1C), 44.79 (d, 1C), 44.52 (d, 1C), 43.21 (t, C-4), 43.10 (d, 1C), 41.91 (d, 1C), 41.48 (d, 1C).

3.3.7 8-METHYLAMINO-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE (6)

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5.07 g, 0.029 mol) was dissolved in anhydrous tetrahydrofuran (THF; 50 ml) and cooled down to 5°C in an external ice bath. Methylamine (1.0 g, 0.032 mol) was slowly added while the mixture was stirred for

approximately 45 min at lowered temperatures. The reaction was monitored on TLC plates to ensure that all the Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione had been used. The carbinolamine which started precipitating after ± 25 min was isolated by filtration and washed with cold THF (2 x 20 ml) producing the carbinolamine as a white paste. This product was dehydrated under Dean Stark conditions for approximately 1 hour using 50 ml dried benzene. Benzene was removed *in vacuo* to produce the Schiff base (8-methyliminopentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-11-one) in the form of a yellow oil. The resulting oil was dissolved in anhydrous methanol (30 ml) and anhydrous THF (150 ml). Reduction was carried out by adding $NaBH_4$ (1.5 g, 39.5 mmol) and stirring overnight at room temperature. Solvents were removed and the resulting white residue was suspended in approximately 100 ml of distilled water. The product was extracted with dichloromethane (4 x 50 ml) and the combined dichloromethane fractions were washed with water (2 x 100 ml). The mixture was dried over anhydrous $MgSO_4$ and filtered. *In vacuo* removal of the solvent resulted in the formation of a yellow oil. The product was purified with column chromatography (9:1 ethyl acetate: ethanol) to produce a yellow oil (Yield: 1.60 g, 0.0084 mol, 28.13%)



$C_{12}H_{15}NO$; HR-MS: calc. 189.254, exp. 189.254; **IR** (KBr)ν_{max} (Spectrum 6): 3324, 2965, 1367, 999, 855; **MS** (EI, 70 eV) *m/z* (Spectrum 12): 189 (M^+), 131, 91, 28; **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H (Spectrum 18): 4.69 (t, 1H, J = 5.30 Hz, H-11), 2.40-2.89 (4 x m, 11H, H-1, 2, 3, 5. 6. 7. 9. 10. CH_3), 2.06 (s, 1H, NH), 2.62 : 2.01 (ABq, 2H, J = 10.44 Hz, H-4a,4b); **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C (Spectrum 24): 109.84 (s, C-8), 82.45 (d, C-11), 55.05 (d, C-7/9), 54.79 (d, C-7/9), 44.78 (d, 1C), 44.76 (d, 1C), 44.53 (d, 1C), 43.25 (t, C-4), 42.96 (d, 1C), 41.87 (d, 1C), 41.52 (d, 1C), 30.01 (q, CH_3).

3.4 CONCLUSION

Except for the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (yield 80.934%), synthesis of all other compounds resulted in yields between 24% and 43%. Synthesis of 8-[(4-aminomethyl)pyridine]-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**5**) yielded 42.85% of the pure product with 8-cyclohexylmethylamino-8,11-oxapentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**2**) presenting the lowest yield (24.69%). Low yields could result from formation of various possible by-products during the synthetic procedure as well as inadequate purification of product mixture during column chromatography. Yields could be improved through optimisation of synthetic procedures and purification techniques as well as further purification of remaining fractions of product mixture after completion of column chromatography.

CHAPTER 4

BIOLOGICAL EVALUATION AND RESULTS

SUMMARY

A real-time confocal laser microscope and fluorescent potentiometric indicator was used to evaluate the influence of the test compounds on the transmembrane potential of the SH-SY5Y human neuroblastoma cell line. *In vitro* studies were also performed to evaluate test compounds for antagonism of $^{45}Ca^{2+}$ uptake in murine synaptosomes. Changes in membrane potential and $^{45}Ca^{2+}$ uptake in response to a high concentration potassium (KCl) solution were measured in the presence and absence of the pentacyclo-undecane derivatives.

ABBREVIATIONS

CNS	Central nervous system
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulphoxide
HEPES	*N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
TMRM	Tetramethylrhodamine methyl ester
ROI	Region of interest
SEM	Standard error of mean

4.1 EVALUATION OF MEMBRANE POTENTIAL

4.1.1 INTRODUCTION

The potentiometric fluorescent probe, TMRM, was selected for this study as fluorescence is not quenched by uptake of dye into mitochondria and the cytoplasmic and mitochondrial fluorescence of the cell is therefore readily discernible. TMRM also exhibits minimal non-specific binding and displays reversible potential dependent redistribution and accordingly allows visualisation of depolarisation and repolarisation kinetics in living cells. Cells, pre-treated with TMRM and respective test compounds, were exposed to a 100μM KCl solution in order to induce membrane depolarisation and changes visualised employing confocal microscopy.

4.1.2 CELL CULTURES

Human SH-SY5Y neuroblastoma cells (Obtained from American Type Culture Collection (ATCC), catalogue number CRL-2266) were used in this study. The SH-SY5Y cell line is a thrise cloned (SK-N-SH → SH-SY → SH-SY5 → SH-SY5Y) subline of the neuroblastoma line SK-N-SH and was established in 1970 from a metastatic bone tumour. Cells were maintained in normal DMEM containing 10% foetal calf serum, 100 units penicillin/ml, 100 μg streptomycin/ml and 0.25 μg fungizone/ml and incubated at 37°C in a 5% CO_2 and 95% O_2 humidified atmosphere.

Cells duplicated at a rate of approximately once every 48 hours and, when 95% confluent, were detached from the flask bottom by means of trypsination (incubation with trypsin/versine for approximately 10 minutes) and seeded in new flasks at a density of no less than $^1/_6$ confluency.

Cells were seeded onto 25 mm diameter glass coverslips and incubated in covered Petri dishes for 5 hours to allow adherence to coverslips before dye loading commenced. When

not used immediately sufficient culture medium was added to Petri dishes and cells incubated overnight.

4.1.3 DYE LOADING

Culture medium was replaced with normal DMEM containing 500nM of the the fluorescent potentiometric indicator, tetramethylrhodamine methyl ester (TMRM, Molecular Probes, the Netherlands), and incubated for 40 minutes to allow complete dye equilibration. Dye containing medium was replaced with standard recording solution containing 126mM NaCl, 4mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES and 15mM glucose (pH was adjusted to 7.3 with NaOH and osmolarity adjusted to 320 mosmol/l with sucrose). 100nM TMRM was added to minimise dye leakage from cells.

4.1.4 IMAGING AND ANALYSIS

All experiments were conducted at room temperature. Pentacycloundecane derivatives were dissolved in dimethylsulphoxide (DMSO) and diluted to a 100μM concentration in normal recording solution (final concentration DMSO in incubations = 0.1%). Glass coverslips were mounted in a recording chamber during optical imaging and cells were incubated with the selected pentacycloundecane derivative for 2 minutes. Application of a high concentration potassium solution (containing KCl 100mM, NaCl 40mM, $CaCl_2$ 2mM, $MgCl_2$ 1mM, HEPES 10mM and Glucose 15mM [pH was adjusted to 7.3 with NaOH and osmolarity adjusted to 320 mosmol/ℓ with sucrose]) was used to initiate membrane depolarisation. Control experiments, conducted in the absence of test compounds, comprised of a 2 minute incubation in normal recording solution followed by the application of high concentration potassium solution.

A time series of 30 optical scans were recorded at 30 second intervals on a real time confocal laser scanning microscope (Nikon PCM 2000). Cells were exposed to the 505 nm line of a He/Ne laser and emission was detected at 586 nm. A 60 x 1.4 NA (Nikon) oil immersion objective was used to view, and when necessary to further magnify the cells for

imaging. A 5 μm pinhole was employed to prevent dye bleaching as well as light scattering. Image processing and analysis were performed using supplied Nikon software.

4.1.5 RESULTS

A region of interest (ROI) was defined in the cytoplasm of a cell in an area devoid of mitochondria. Fluorescence intensity histograms were generated for the selected ROI over a 15 minute time series. Histograms were analysed and the average decrease in fluorescence in the presence of each of the derivatives was calculated.

Figures 4.1-4.6 present examples of experiments in presence and absence of test compounds. Cells were incubated with the respective compound for 2 minutes before recording commenced. High concentration potassium solution was applied approximately 10 seconds after the first image was recorded. Every second image in the time series of 30 slides is presented.

All data are presented as % values of initial fluorescence. A one way analysis of variance was performed on all data. Student t-tests was performed to indicate significant differences between treatment and control groups ($p \leq 0.05$ indicates significance). .

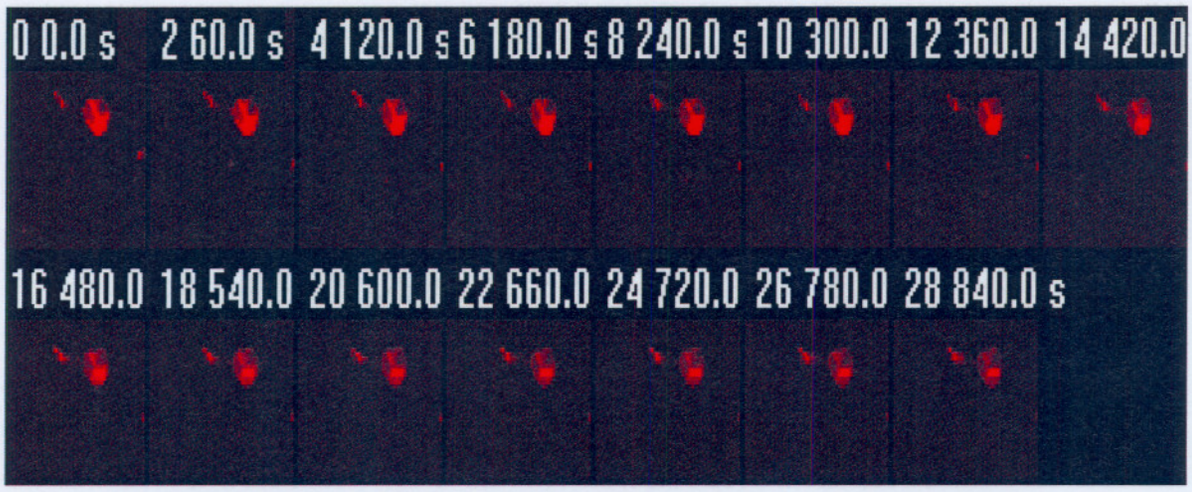


Figure 4.1: Time series of KCl-control experiment

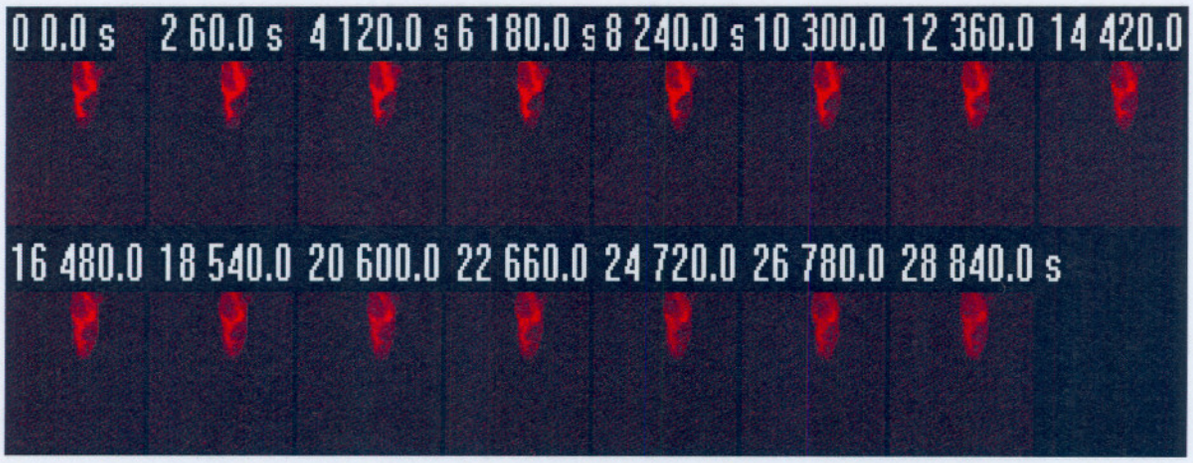


Figure 4.2: Time series of cells incubated with compound **1**

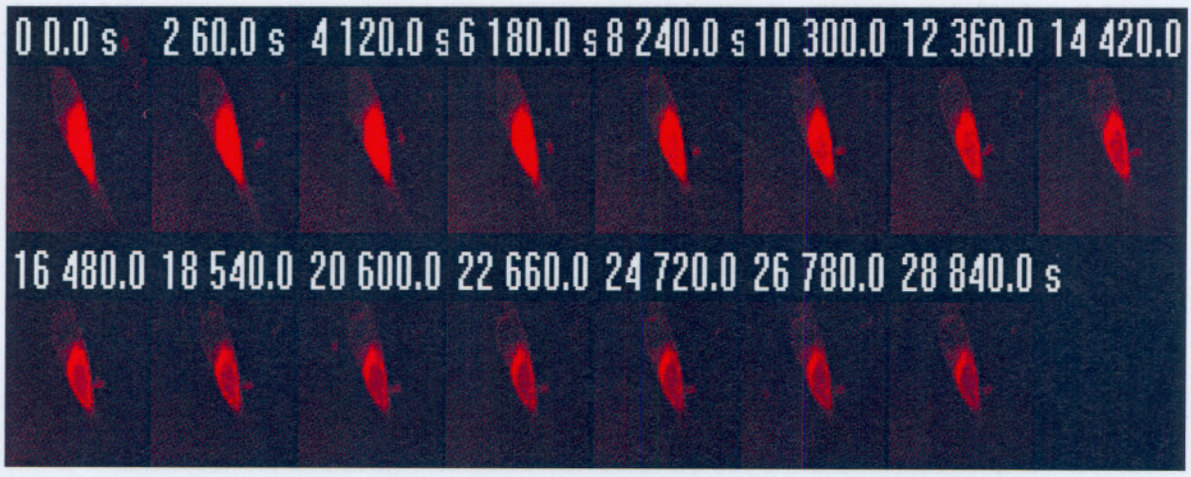


Figure 4.3: Time series of cells incubated with compound **3**

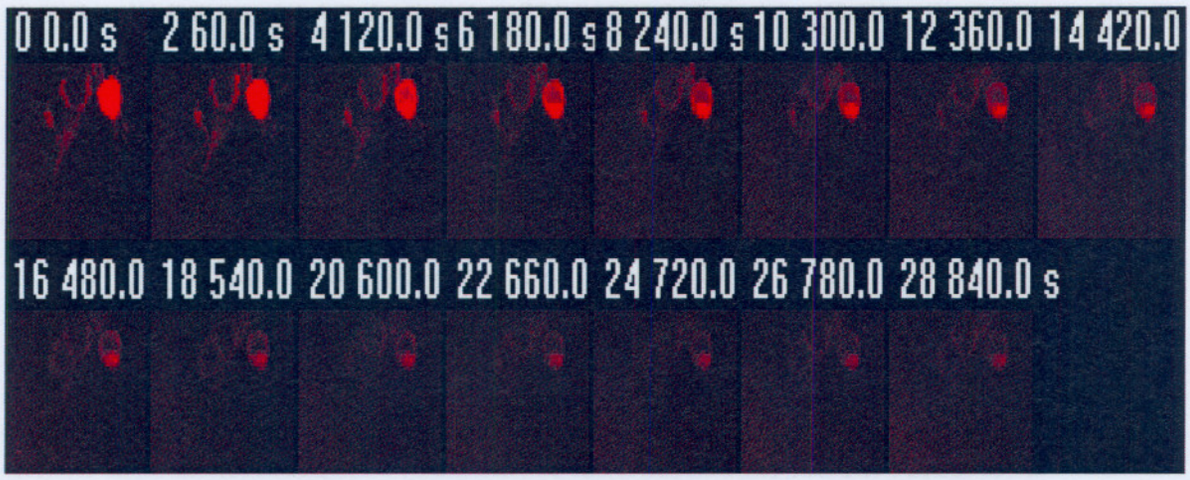


Figure 4.4: Time series of cells incubated with compound **4**

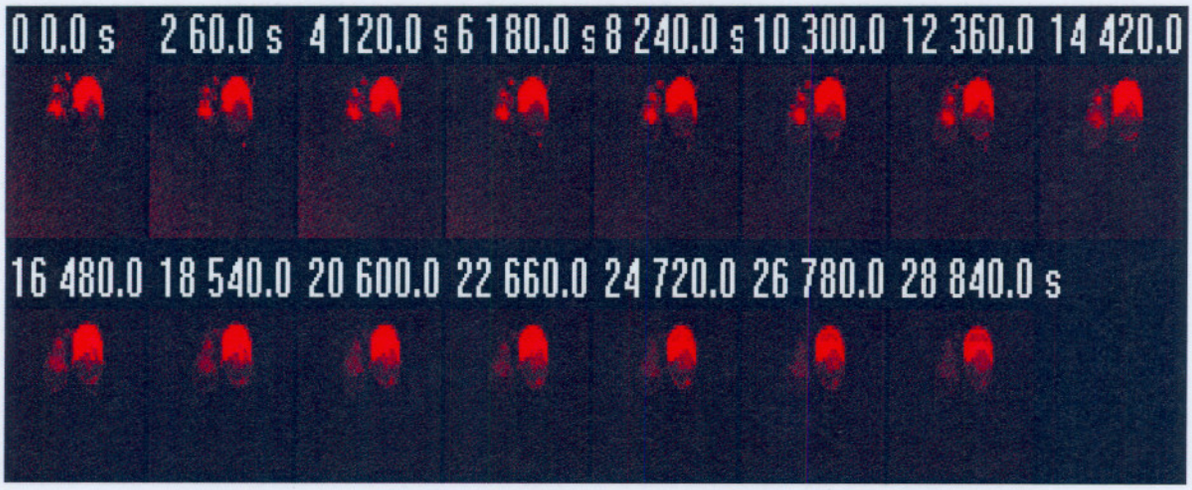


Figure 4.5: Time series of cells incubated with compound **5**

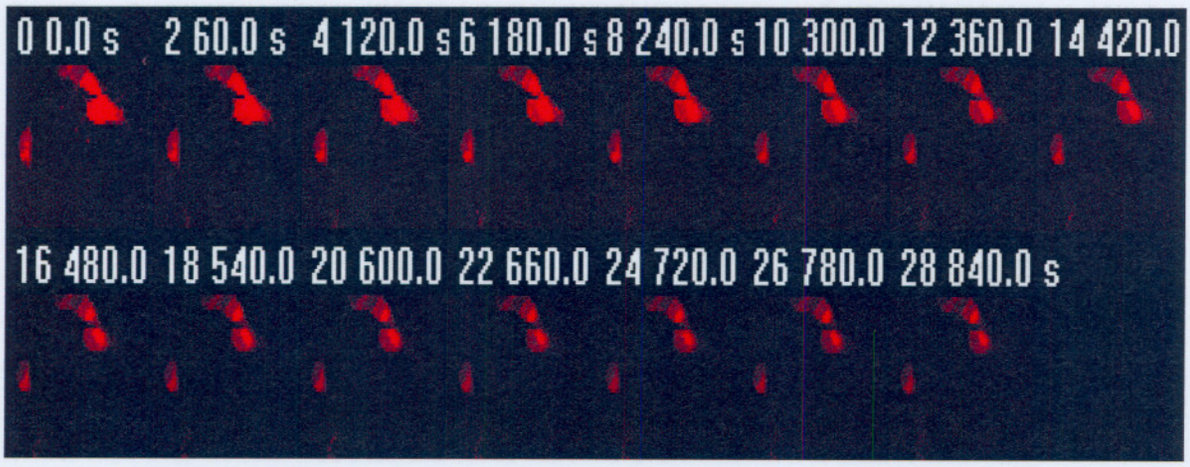


Figure 4.6: Time series of cells incubated with compound **6**

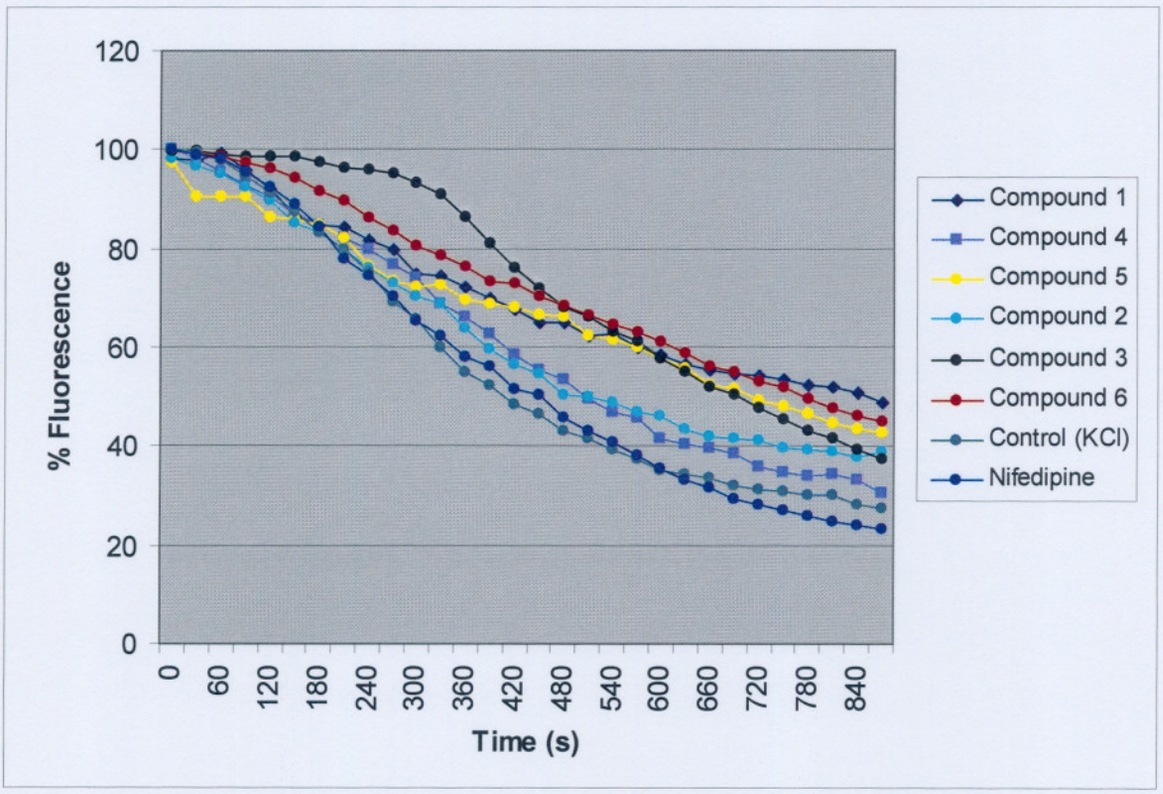


Figure 4.7: Summary of the average KCl-induced decrease in % fluorescence in the presence and absence of the test compounds.

KCl-control experiments showed an average of 72.32% decrease in fluorescence after 870 seconds while cells incubated with compounds **1**, **5** and **6** only showed a 49.30%, 54.74% and 55.07% decrease respectively. Compounds **2**, **3** and **4** showed fluorescence decreases of 59.27%, 62.33% and 69.61% respectively. The therapeutic calcium channel blocker, nifedipine, showed a decrease of 76.50% in fluorescence.

To gain a more accurate reflection of results, average fluorescence values of cells incubated with test compounds were plotted against average values of control experiment with standard error values incorporated as error bars. Compounds **1**, **3**, **5** and **6** presented the best profiles (fig. 4.8).

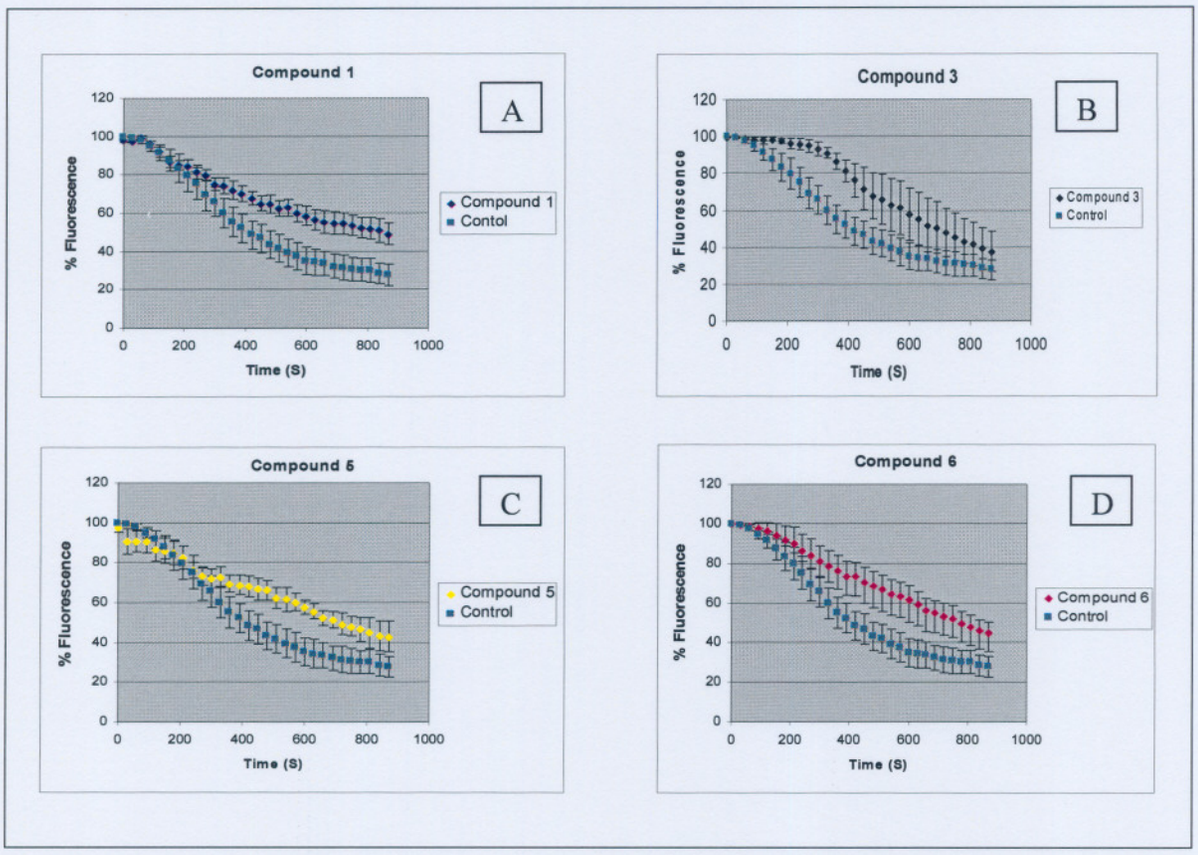


Figure 4.8: Fluorescence values of cells incubated with compounds **1** (A; n = 5), **3** (B; n = 3), **5** (C; n = 5) and **6** (D; n = 6) plotted with average values of KCl-control experiments (average ± SEM).

The average fluorescence values of the KCl-control experiments as well as cells incubated with the respective test compounds at 0 seconds (blue), 420 seconds (red) and 870 seconds (yellow) were calculated to evaluate significance of the observed differences at specific time points (fig. 4.9). Fluorescent values of control experiments (KCl), conducted in the absence of test compounds, decreased to 48.44% in the first 420 seconds followed by a further decrease of 20.75% to a fluorescence value of 27.68%. Compounds **1**, **3**, **5** and **6** showed a 30.71%, 23.51%, 29.68% and 26.88% decrease in fluorescence in the first 420 seconds respectively with compounds **1**, **5** and **6** showing a final decrease of less than 60% in fluorescence (Table 5.1).

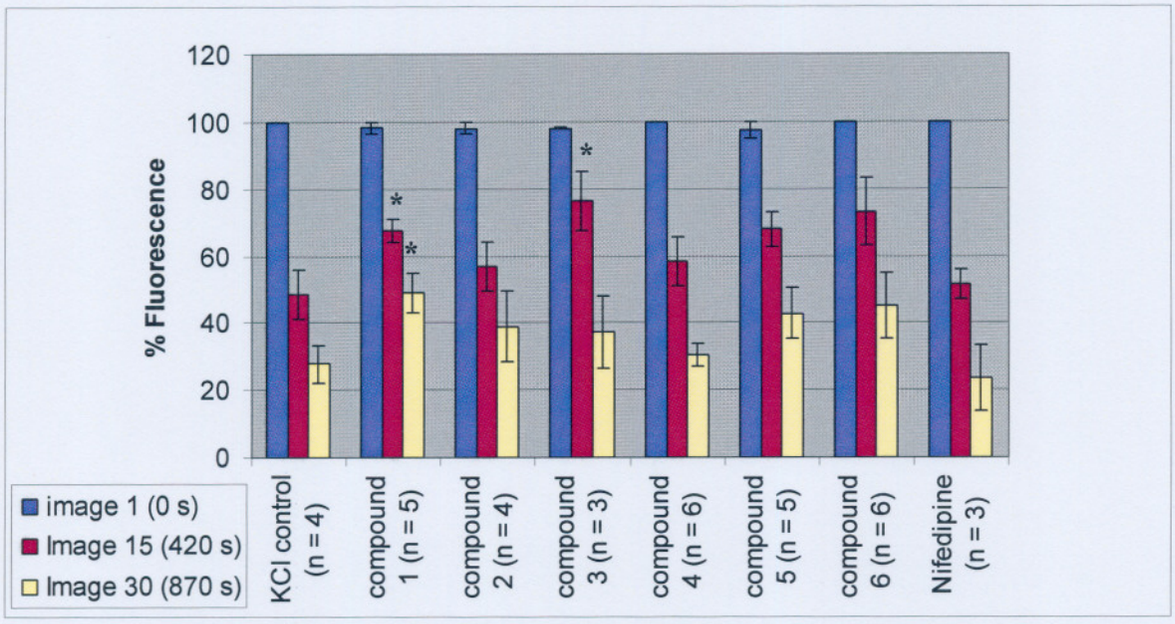


Figure 4.9: Representation of average fluorescence (± SEM) values for respective experiments at images 0, 15 and 30 (* indicate statistically significant differences from control values; $p \leq 0.05$).

4.1.6 DISCUSSION

From the results it can be ascertained that compounds **1**, **5** and **6** prevent membrane depolarisation to a greater extend than other evaluated compounds. Compound **3** initially prevented a decrease in fluorescence but failed to provide significant protection over the 870 sec period. Nifedipine also failed to provide protection against the KCl-induced depolarisation. Although statistically significant differences in fluorescence were only observed with compound **1** (t = 420 sec and t = 870 sec) and compound **3** (t = 420 sec), the overall tendency of all test compounds is to reduce the extent of KCl-induced depolarisation.

4.2 SCREENING OF ANTAGONISM OF $^{45}Ca^{2+}$ UPTAKE

4.2.1 INTRODUCTION

Murine synaptosomes isolated from mice striata were used for this experiment. $^{45}Ca^{2+}$-influx was initiated by application of a high concentration potassium solution (69.5mM KCl) in buffer solution containing $^{45}Ca^{2+}$. Synaptosomes were washed and the radioactivity of the intracellular $^{45}Ca^{2+}$measured with liquid scintillation counting.

4.2.2 MATERIALS

$^{45}CaCl_2$ was purchased from Perkin Elmer (USA) and buffer constituents were obtained from other commercial sources.

4.2.3 ANIMALS

ICR male mice (20 – 26 g, 6 – 8 weeks old) were obtained from Harlan, Dublin, VA. All animal care and experimental protocols were approved by the Texas Tech HSC Institutional Animal Care and Use Committee in accordance with guidelines established by the United States Public Health Service and Texas Tech.

4.2.4 PREPARATION OF SYNAPTOSOMES

The uptake experiments were performed employing a method described by Leong *et al.*, 2001. Male ICR mice were sacrificed by cervical dislocation and the striata and hippocampi were rapidly removed. Striatal tissue was homogenized in 4 ml of sucrose buffer (0.32 M sucrose, 2 mM HEPES, pH 7.4) and the homogenate was then centrifuged for 10 min at

1000 × g. The supernatant was removed and centrifuged for 15 min at 10,000 × g and the resulting pellets were washed once and resuspended in incubation buffer (125 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 10 mM glucose and 5 mM HEPES, pH 7.4).

The synaptosomes were aliquoted into tubes, treated with drug, and incubated for 10 min at 37°C. Cells were treated with a 100μM concentration of test compounds dissolved in DMSO (final DMSO concentration in the incubations ≤ 0.1%). Control experiments received 0.1% DMSO to compensate for possible alterations caused by addition of DMSO in experiments. $^{45}Ca^{2+}$ uptake was initiated by adding 0.1 ml of KCl (69.5mM) enriched buffer containing the $^{45}Ca^{2+}$. After an incubation period of 5 sec, the synaptosomes received 3 ml of wash buffer maintained at 37°C and were then filtered through 1 micron glass microfiber filters (Whatman GF/B) under vacuum. Synaptosomes were then washed an additional 3 times with 37°C buffer, followed by liquid scintillation counting to measure the radioactivity of $^{45}Ca^{2+}$ within the synaptosomes. For each compound screened, fresh striatal tissue was used.

4.2.5 RESULTS

Statistical significance for $^{45}Ca^{2+}$ uptake and release studies was analyzed using Student's t-test (InStat™, GraphPad Software, San Diego, CA) in order to identify statistically significant differences between test compounds and control groups.

The results of the screening assay are presented in figure 4.10 and table 4.1. The addition of 69.5 mM KCl led to depolarisation of the cell membrane and resulted in an increase of $^{45}Ca^{2+}$ flux into the murine synaptosomes. This increase was significant ($p \leq 0.05$) compared to DMSO only control. Inhibition of the $^{45}Ca^{2+}$ uptake was observed for all the compounds within the test series. When compared to KCl-control values, significant differences were found for each of the evaluated compounds ($p \leq 0.05$).

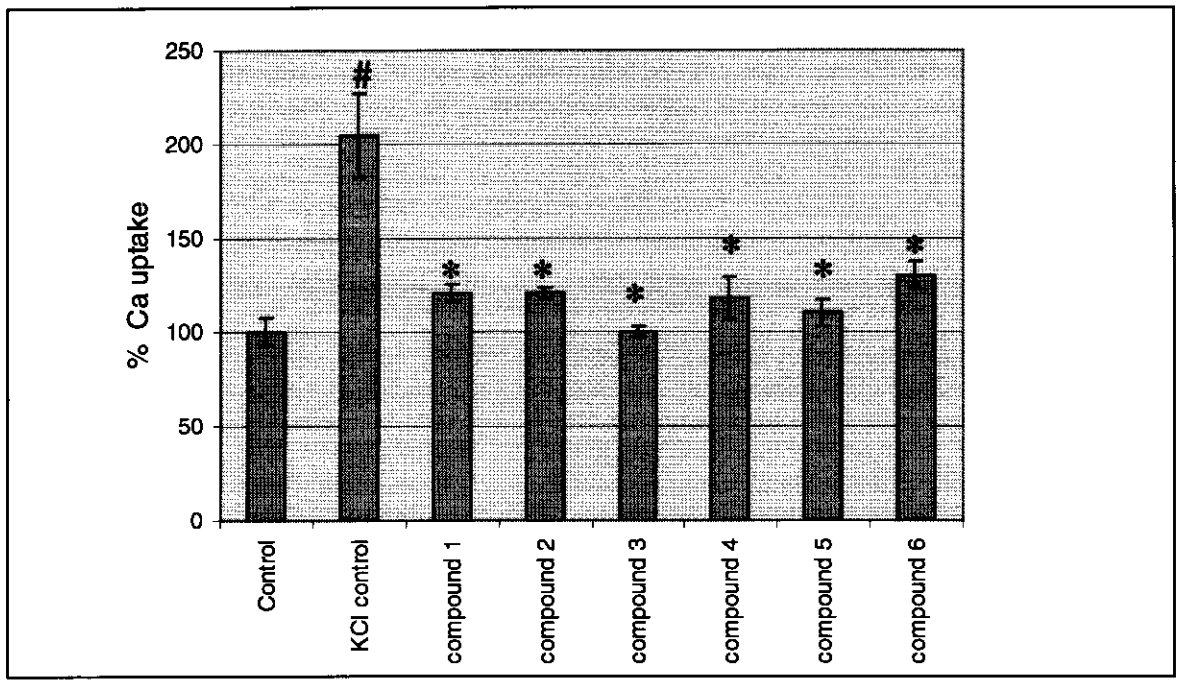


Figure 4.10: Inhibition of KCl-stimulated uptake of $^{45}Ca^{2+}$ into murine striatal synaptosomes by test compounds (**1-6**). Compounds were screened at a concentration of 100 μM. Each bar represents the mean ± S.E.M. of three experiments done in triplicate. Statistical significance ($p < 0.05$) indicated by #, when compared to vehicle control, and * when compared to KCl-control.

Table 4.1 Inhibition of $^{45}Ca^{2+}$ uptake inhibition by respective test compounds

Group	**% $^{45}Ca^{2+}$ uptake compared to control (mean ± S.E.M.)**
Control	100.33 ± 7.21
KCl-control	205.00 ± 22.06 #
Compound **1**	120.66 ± 4.70 *
Compound **2**	121.00 ± 3.00 *
Compound **3**	100.00 ± 3.05 *
Compound **4**	118.00 ± 11.00 *
Compound **5**	110.00 ± 7.37 *
Compound **6**	130.33 ± 7.85 *

4.2.6 DISCUSSION

All test compounds evaluated antagonised $^{45}Ca^{2+}$ uptake into murine synaptosomes. Compound **3** most extensively reduced this calcium uptake with a reduction to 100% uptake (vehicle control = 100%) compared to KCl-control uptake of 205.00%. Compound **6** provided the least inhibition but still caused a reduction to 130.33% in uptake of $^{45}Ca^{2+}$.

CHAPTER 5

DISCUSSION AND CONCLUSION

SUMMARY

Although not the sole mechanism mediating neuronal death, disruption of specialised homeostatic mechanisms controlling calcium concentrations within neuronal cells, plays an integral role in the pathology underlying neurodegeneration. Calcium influx can to a large extend be modulated through manipulation of VDCC as well as NMDA receptor operated calcium channels. Changes in the membrane potential modulates cellular calcium homeostasis by influencing both voltage dependent calcium and NMDA channels as well as altering the extent of participation of other types of voltage activated ion channels (i.e. Na^+ and K^+ channels).

Biological activity attributed to a class of polycyclic amine derivatives, the pentacycloundecylamines, suggest possible neuroprotective abilities through interaction with both voltage dependent and NMDA receptor operated channels. Derivatives of these compounds have also been shown to modulate L-type calcium channels, Na^+ channels as well as the fast component of the delayed rectifier K^+ channels. Although not enough is known about the mechanisms of CNS activity of the compounds, it is postulated that these derivatives could yet be of therapeutic value in the treatment of neurodegenerative disorders.

ABBREVIATIONS

CNS	Central nervous system
NMDA	N-Methyl-D-Aspartate
VDCC	Voltage dependent calcium channels

5.1 SUMMARY OF RESULTS

5.1.1 SYNTHESIS

Synthesis of the pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione resulted in a yield of 80.934%. Yields between 24% and 43%was obtained in synthesis of the respective polycyclic amine derivatives. Synthesis of 8-[(4-aminomethyl)pyridine]-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**5**) yielded 42.85% of the pure product with 8-cyclohexylmethylamino-8,11-oxapentacyclo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**2**) presenting the lowest yield (24.69%).

5.1.2 INHIBITION OF MEMBRANE POTENTIAL

In this study, a series of pentacycloundecylamine derivatives as well as the commercially available therapeutic calcium channel blocker, nifedipine were evaluated to determine possible effects on the membrane depolarisation of neuronal cells. Human SH-SY5Y neuroblastoma cells were used and changes in membrane potential was measured using confocal microscopy and the fluorescent potentiometric probe, TMRM.

Despite experimental variation in data, the results clearly demonstrate differences between control experiments and respective test compounds. Fluorescence in experiments conducted in the absence of test compounds, in response to KCl stimulus, decreased to 48.44 % after 420 sec with a total decrease of 72.32% to a final value of 27.68% over 870 sec (table 5.1). As aforementioned experiments analysed the response of cells to the KCl stimulus without any further external modulation, these values served as control values.

Compounds **2**, **3** and **4** provided the least protection against KCl-induced depolarisation with fluorescence decreasing to 38.89%, 37.40% and 30.39% respectively. Compound **3** however provided a good initial protection with a decrease of only 23.78% (t = 420 sec) compared to decreases of 51.56% in control experiments and 43.26% and 41.62% for compounds **2** and **4** after 420 sec respectively.

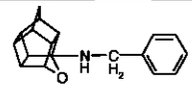
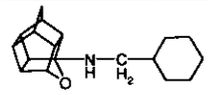
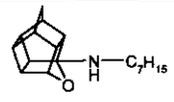
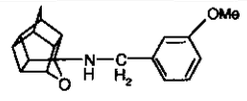
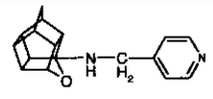
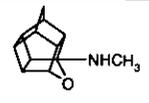
The best overall protection was provided by compounds **1**, **5** and **6** with fluorescence decreases of 32.44%, 32.13% and 26.88% (t = 420 sec) and final decreases of 51.03%, 57.19% and 55.07% after 870 sec. respectively.

Nifedipine failed to inhibit KCl-induced membrane depolarisation.

Table 5.1: Average decrease in % fluorescence (± SEM) at t = 0 sec, 420 sec and 870 sec.

	% Fluorescence		
	time = 0 sec	t = 420 sec	t = 870 sec
control (n = 4)	100	48.44 ± 7.32	27.68 ± 3.54
compound **1** (n = 5)	98.27	67.56 ± 3.45	48.97 ± 7.76
compound **2** (n = 4)	98.16	56.74 ± 7.22	38.89 ± 10.38
compound **3** (n = 3)	99.74	76.22 ± 8.81	37.40 ± 10.82
compound **4** (n = 6)	100	58.38 ± 7.26	30.39 ± 9.69
compound **5** (n = 5)	97.55	67.87 ± 5.17	42.81 ± 5.72
compound **6** (n = 6)	100	73.12 ± 9.95	44.93 ± 5.42
Nifedipine (n = 3)	100	51.43 ± 6.46	23.50 ± 2.55

Table 5.2: Structures of test compounds.

	8-Benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**1**)
	8-Cyclohexylmethylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**2**)
	8-Hepthylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**3**)
	8-(3-Methoxybenzylamino)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**4**)
	8-[(4-Aminomethyl)pyridine]-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**5**)
	8-Methylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**6**)

5.1.3 ANTAGONISM OF $^{45}Ca^{2+}$ UPTAKE

Murine synaptosomes were incubated for a period of 5 sec in a buffer containing $^{45}Ca^{2+}$ as well as 69.5mM KCl to initiate uptake of the radioactive calcium into cells and radioactivity of the absorbed $^{45}Ca^{2+}$measured with liquid scintillation counting.

Best inhibition of $^{45}Ca^{2+}$ uptake was obtained with compound **3** with total uptake of 100.00% (vehicle control = 100%) compared to 205.00% in KCl control experiments. Compounds **1, 2** and **4** exhibited comparable uptake inhibition with 120.66%, 121.00% and 118.00% respectively. Compound **5** resulted in a decrease in $^{45}Ca^{2+}$ uptake of 110.00% whilst compound **6** caused least inhibition with an uptake of 130.33%

5.2 DISCUSSION

5.2.1 MEMBRANE POTENTIAL EVALUATION

Normal changes in membrane potential can easily be altered through small interventions in the normal cellular physiology. Modulation of calcium, sodium or potassium voltage activated channels as well as changes in normal functioning of the NMDA receptor operated calcium channels can significantly alter the membrane potential of an excitable cell.

Compound **1** showed best overall inhibition of KCl-induced membrane depolarisation with a significant difference ($p \leq 0.05$) of 21.29% in fluorescence after 870 seconds in comparison to control values. Compared to KCl control, compounds **5** and **6** exhibited an overall difference of 15.13% and 17.25% in final fluorescence while compounds **2**, **3** and **4** showed less inhibition.

8-Benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**1**), which was previously identified as near optimal structure for peripheral calcium channel activity, showed best overall results. Replacement of the benzylamino side chain with a related but saturated cyclohexyl moiety (compound **2**) resulted in decreased protection and accordingly indicates the importance of the unsaturated system in the activity of these derivatives. Compound **3** (8-hepthylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane), which altered the profile of KCl-induced depolarisation but did not significantly protect against depolarisation, might indicate a transitory interaction with voltage activated channels. Aliphatic substitution on the pentacycloundecane template (with a substituent volume still comparable to that of the cyclohexylmethylamino derivative) accordingly resulted in decreased activity and thus confirms the importance of an unsaturated cyclic substituent. Good results found with compound **6** (8-methylamino-8,11-oxapentacyclo-[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane) might indicate better interaction through prevention of steric and geometric restrictions. This postulation is confirmed by the failure of 8-(3-methoxybenzylamino)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**4**), the largest molecule within the range, to prevent significant inhibition of depolarisation. Inhibition

found with 8-[(4-aminomethyl)pyridine]-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**5**) might indicate the importance of hydrogen bonding for activity of these derivatives.

5.2.2 ANTAGONISM OF $^{45}Ca^{2+}$ UPTAKE

Peripheral calcium channel antagonism have been indicated for compounds **1**, **2**, **4** and **5** (Malan *et al.*, 2000) while compounds **3** and **6** have not previously been evaluated.

The results from the screening assays indicated that compound **3** showed the best inhibition of KCl-stimulated $^{45}Ca^{2+}$ uptake into murine synaptosomes, while compound **6** showed the least inhibition.

The alkyl chain of compound **3** leads to an increased Log P value (7.21 through Hyperchem 7) compared to the log P value (2.41) of compound **1.** In addition to this, the flexibility of the side chain may result in folded conformers when in biological fluid. The high Log P (> 5) of compound **3**, violates the "rule of 5" (Lipinski et al., 1997), suggesting that although it exhibited the best inhibition of $^{45}Ca^{2+}$ uptake, it is probably unsuitable for future drug development studies. Compound **6** gave the least inhibition of $^{45}Ca^{2+}$ uptake and it accordingly seems as if side chain substitution with moieties of larger volume might increase antagonism of $^{45}Ca^{2+}$ uptake in murine synaptosomes.

Within the group of compounds with aromatic side chains, compound **5** showed slightly more inhibition than compound **1**. This might suggest that additional hydrogen bonding of the nitrogen within the pyridine ring will increase activity of these derivatives.

5.3 CONCLUSION

The maintenance of calcium homeostasis within the central nervous system is of immense importance to the survival of neuronal cells. Although neuronal calcium homeostasis is maintained through multifaceted processes of influx, outflux as well as release from, and incorporation into intracellular organelles, pathological increases in intracellular calcium do occur. Excessive calcium influx into neuronal cells can to some extent be prevented through modulation of voltage dependent calcium channels, NMDA channels and membrane potential of the cell (Greene & Greenamyre, 1996; Park & Dunlap, 1998; Van Goor *et al.*, 1999).

Results found in this study indicate that 8-benzylamino-8,11-oxapentacyclo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**1**) and derivatives thereof influence the profile of KCl-induced membrane depolarisation and reduces the extend to which the cell membrane depolarises.

Changes in membrane depolarisation results from modulation of voltage activated ion channels within the cell membrane. Activation of channels can be influenced by either channel blocking or modulation of channel kinetics (Wagenaar, 1996). Compounds that influence voltage dependent activation or inactivation of ion channels within the cell membrane can significantly change the waveform of occurring action potentials and accordingly to a large extent influence influx and outflux of ions (Park & Dunlap, 1998; Van Goor *et al.*, 1999). The large concentration gradient of calcium ions over the cell membrane enables small modulations in action potentials to cause large differences in final [Ca^{2+}].

It has been described that 8-benzylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**1**) influences voltage activated sodium channels through channel blocking rather than by modulation of activation state of the channel (Wagenaar, 1996) and it is therefore postulated that modulation of membrane depolarisation observed in this study initially results from partial blockade of the voltage activated sodium channel. The sodium channel is responsible for generating the initial threshold potential that activates the action potential (Messer, 2000) and inhibition of this channel would increase the difficulty of triggering an action potential.

The modulation of potassium channels by a derivative of compound **1**, 6-benzylamino-3-hydroxyhexacyclo[6.5.0.0^{3,7}.0^{4,12}.0^{5,10}.0^{9,13}]tridecane (Malan *et al.*, 2003), leads to the postulation that compounds evaluated in this study could also to some extent influence these channels. Potassium channels influence the repolarisation and hyperpolarisation phase of the action potential and accordingly controls the duration of the depolarisation phase of an action potential. It was found that 6-benzylamino-3-hydroxyhexacyclo-[6.5.0.0^{3,7}.0^{4,12}.0^{5,10}.0^{9,13}]tridecane had a prominent effect on the delayed rectifier potassium currents (Malan *et al.*, 2003). An increase in the duration of an action potential augments total calcium influx but prolongs and also blunts Ca^{2+} signals by slowing influx rate and reducing peak amplitude (Park & Dunlap, 1998). Increases in total calcium influx would however cause more extensive depolarisation of the cell membrane. As the test compounds evaluated in this study prevented KCl-induced depolarisation, it is assumed that these derivatives of 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**1**) do not significantly influence the delayed rectifier potassium currents. It should however be noted that the high concentration potassium in extracelluar media influences the potassium concentration gradient over the cell membrane. This in theory could result in a decreased outflux of potassium ions and thus decreased repolarisation of the cell membrane.

Compound **1** and derivatives thereof have been identified as peripheral VDCC blockers (Van der Schyf *et al.*, 1986; Van der Walt *et al.*, 1988). Opening of voltage activated sodium and calcium channels controls the depolarisation phase of an action potential and in many areas of the neuronal cell, influx of mostly calcium ions is responsible for membrane depolarisation (Messer, 2000). Inhibition of voltage activated calcium channels can accordingly largely influence the profile of membrane depolarisation and decrease the extent to which the membrane depolarises.

The failure of the therapeutic calcium channel blocker, nifedipine to reduce the extent of KCl-induced membrane depolarisation however leads to the postulation that the changes in membrane potential in the presence of the test compounds may not result from their expected VDCC block, but possibly (taking into consideration the structural resemblance to recognised NMDA channel blockers), through an interaction with the NMDA receptor. Membrane depolarisation results in the release of glutamate and accordingly activation of

the NMDA receptor complex (Messer, 2000; Samdani *et al.*, 1997). A change in membrane potential also removes the voltage dependent magnesium block from the NMDA receptor and therefore facilitates the influx of calcium ions through this complex (Greene & Greenamyre, 1996). These processes result in the massive influx of calcium ions into the neuronal cells and antagonism of the NMDA receptor during membrane depolarisation would reduce the extent to which the membrane depolarises. It should also be taken into consideration that membrane depolarisation was KCl-induced which might lead to a smaller contribution of Ca^{2+} ions in the depolarisation of the cell.

It is postulated that 8-benzylamino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**1**) and derivatives thereof might modulate membrane depolarisation and change the waveform of an action potential through blockade of voltage activated sodium channels, antagonism of NMDA receptor operated calcium channels and, to some extent, inhibition of VDCC.

Results obtained in the *in vitro* screening of the test compounds for antagonism of $^{45}Ca^{2+}$ uptake into murine synaptosomes indicate that these derivatives without doubt inhibit the influx of calcium into neuronal cells as expected. This screening however did not differentiate between VDCC and NMDA calcium channels and resulting inhibition is therefore accepted to be a combination of both.

Compounds with side chain substitution with groups of larger volume generally seem to have better activity. The additional substitution to the aromatic ring in compound **4** resulted in increased activity thus confirming the importance of steric and geometric interactions. Incorporation of hydrogen bonding in interaction (compound **5**) also seems to improve activity.

As interaction of these derivatives on calcium channels in cardiac myocytes was also influenced by the steric and geometric nature of the compounds (with compound **4** also showing more pronounced calcium channel activity than compound **1**) (Malan *et al.*, 2000) it can be postulated that some correlation exists between peripheral and neuronal calcium channel activity of these compounds. Lower activity was however indicated for the pyridine

derivatives of compound **1** where the chain tends to fold back onto the polycyclic cage (Malan *et al.*, 2000).

Modulation by the aforementioned processes would significantly alter the influx of calcium ions into the neuronal cell and it is therefore suggested that these derivatives could prevent neurodegeneration by influencing the calcium homeostasis within neuronal cells and accordingly modifying calcium dependent physiological processes in the central nervous system.

5.4 FINAL REMARKS

Results found in this study indicate that the evaluated test compounds show activity that could be of value in the prevention of neurodegeneration. Variation in data, found when evaluating effects of the pentacycloundecylamine derivatives on the membrane potential of neuronal cells, indicate that more trials are necessary to confirm these results. The effect of the respective test compounds on the membrane potential of neuronal cells, in the absence of KCl, should also be evaluated to determine possible modulation of membrane potential before an action potential is triggered. A known NMDA antagonist could also be included in the biological evaluations in order to verify the specific mechanism of membrane potential modulation.

In order to more accurately compare the potency of antagonism of $^{45}Ca^{2+}$ uptake IC_{50} values of respective compounds will have to be calculated and to facilitate a more in depth study of the structure activity relationships of these compounds on neuronal calcium channels, a larger series of derivatives needs to be tested on the current model.

Further tests regarding the specific mechanism of action as well as an in depth structure activity analysis of these derivatives in the central nervous system is definitely warranted as this would aid further, more specific, development of compounds within this series of derivatives.

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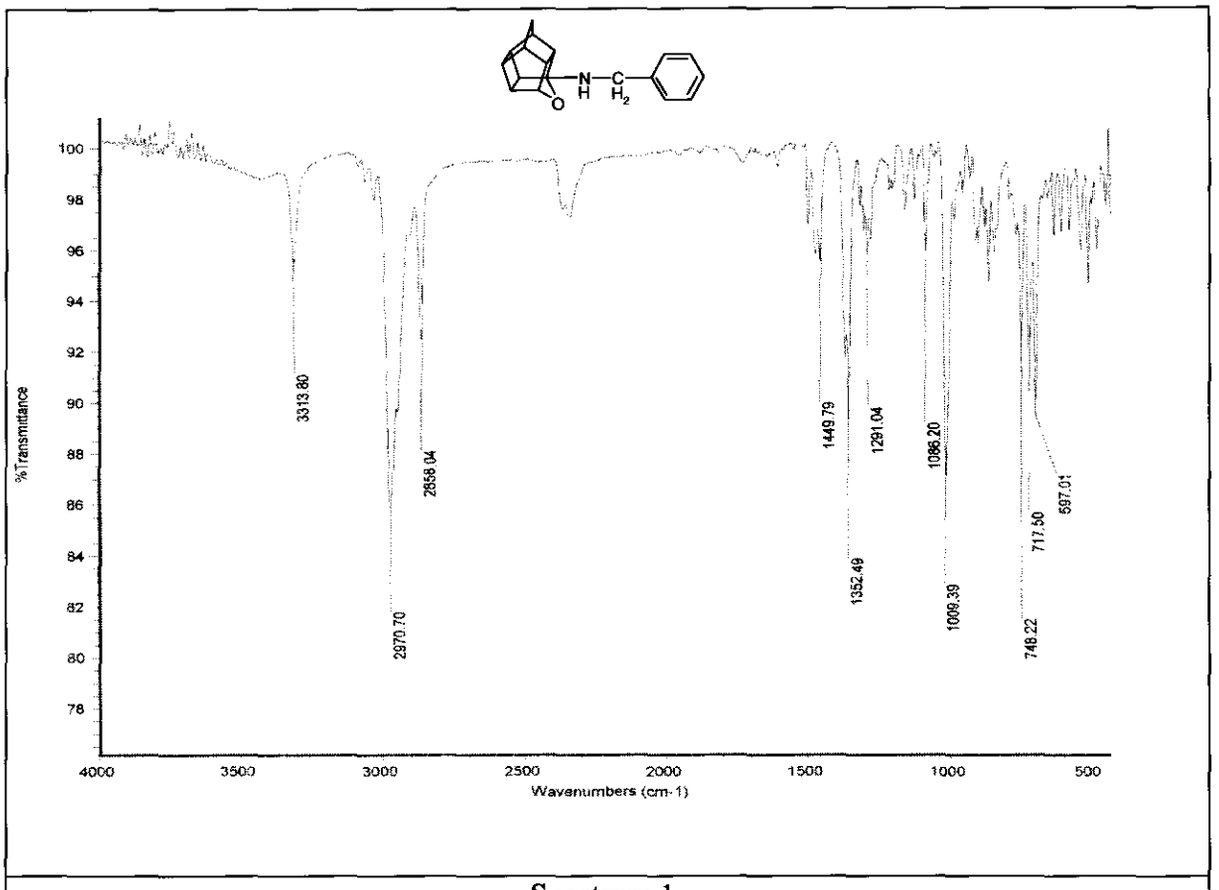
APPENDIX 1

SPECTRAL DATA

- INFRARED SPECTROSCOPY
- MASS SPECTROMETRY
- ^{1}H NUCLEAR RESONANCE SPECTROMETRY
- ^{13}C NUCLEAR RESONANCE SPECTROSCOPY



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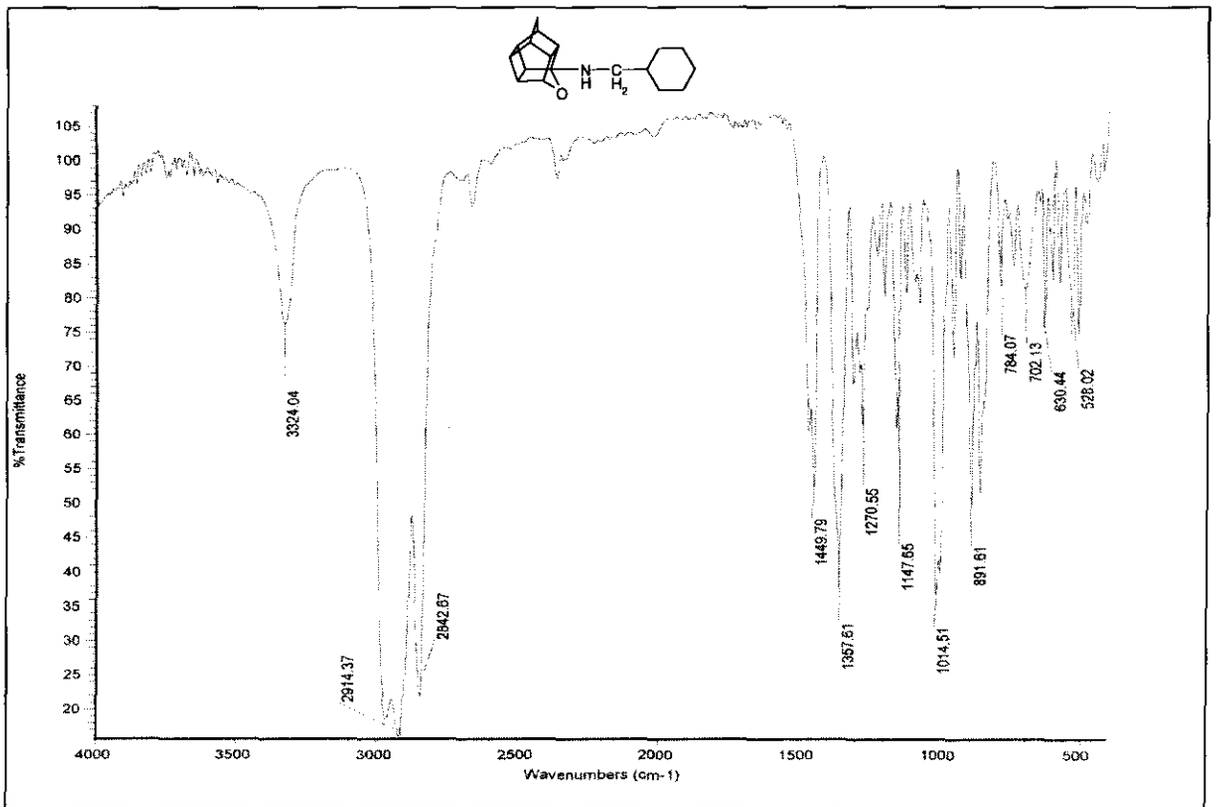

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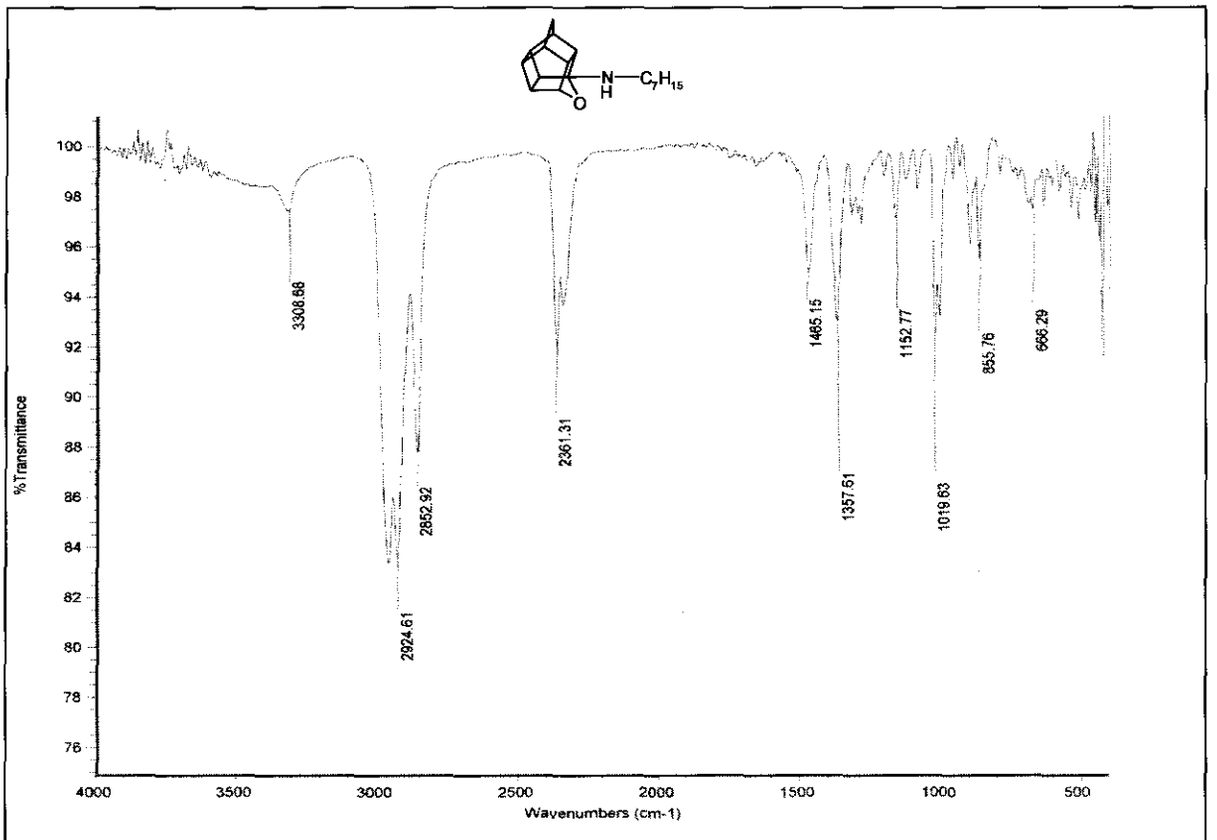

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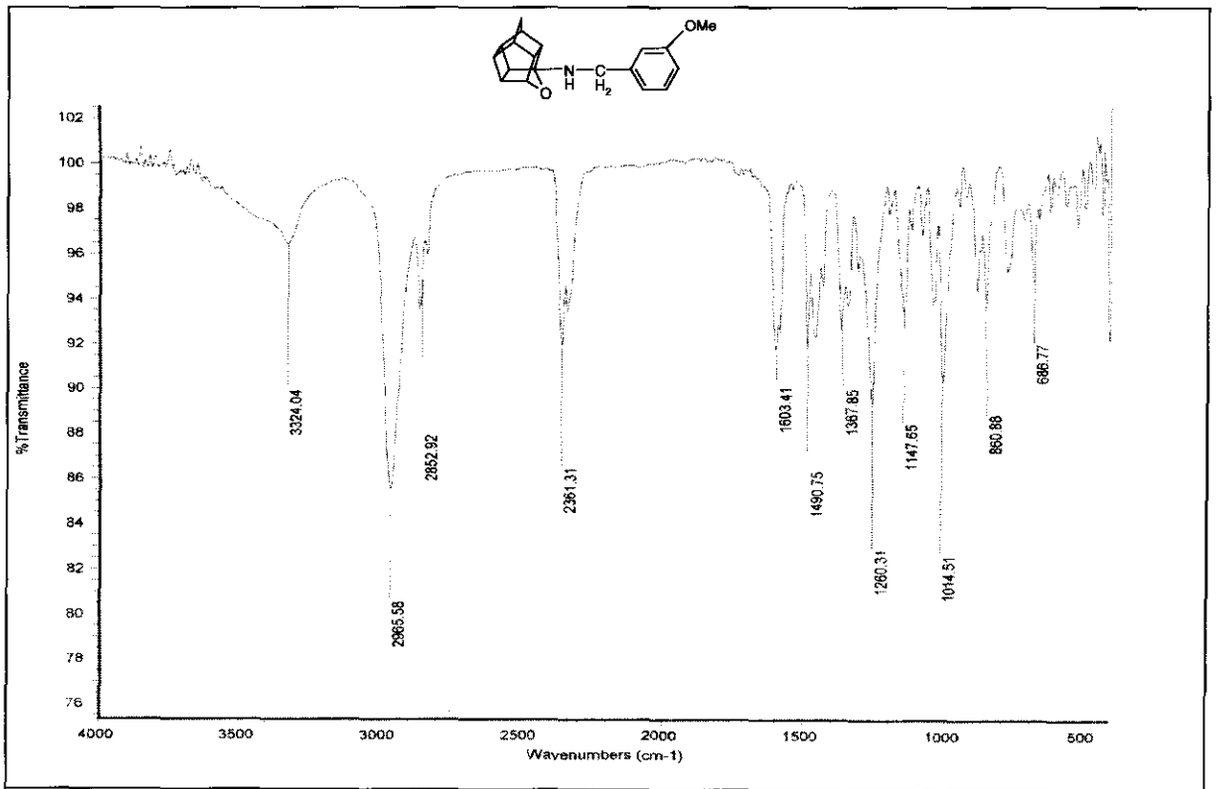

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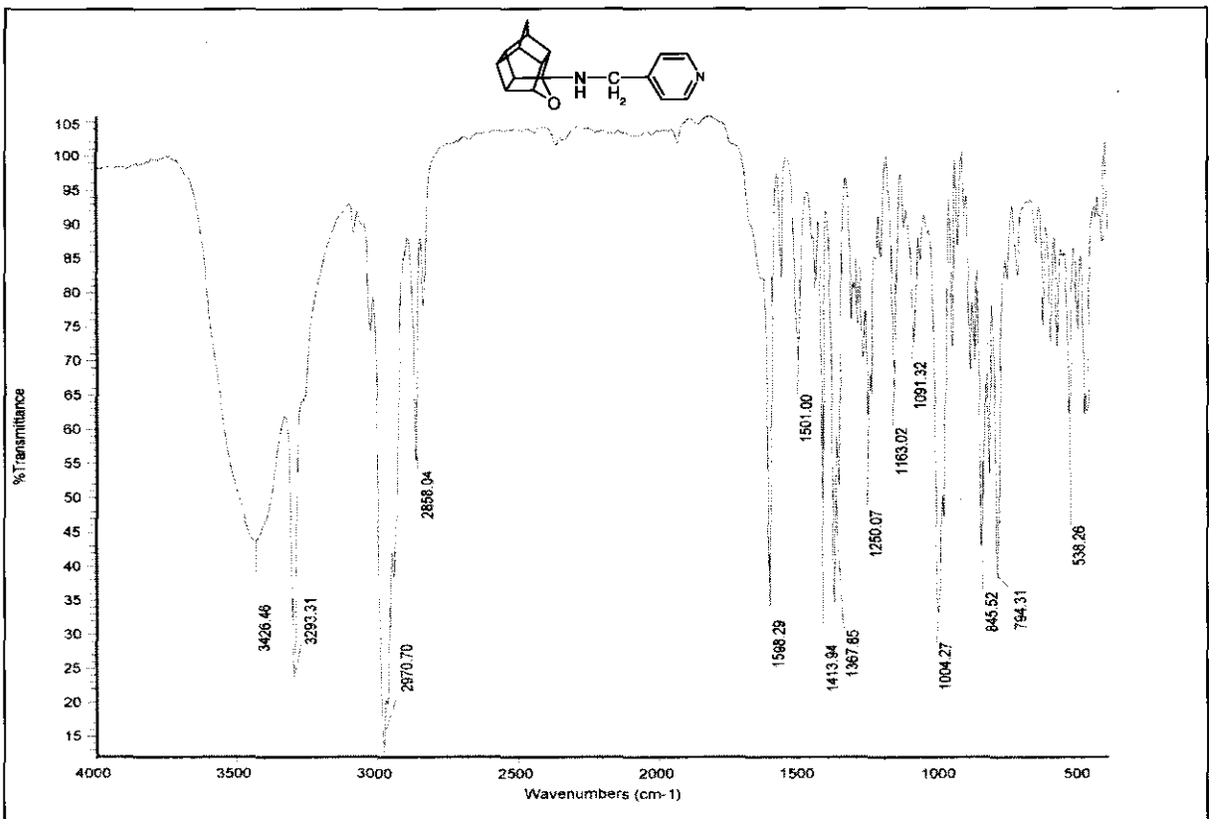

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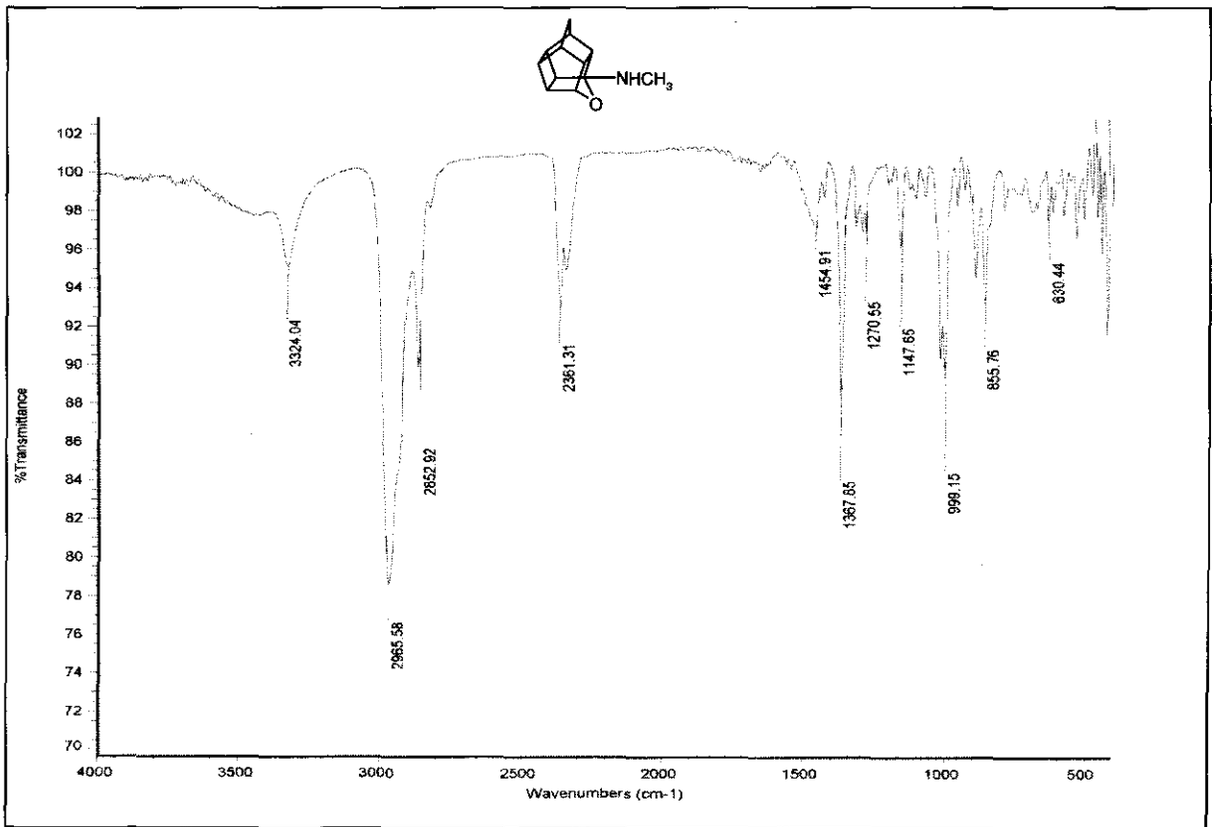

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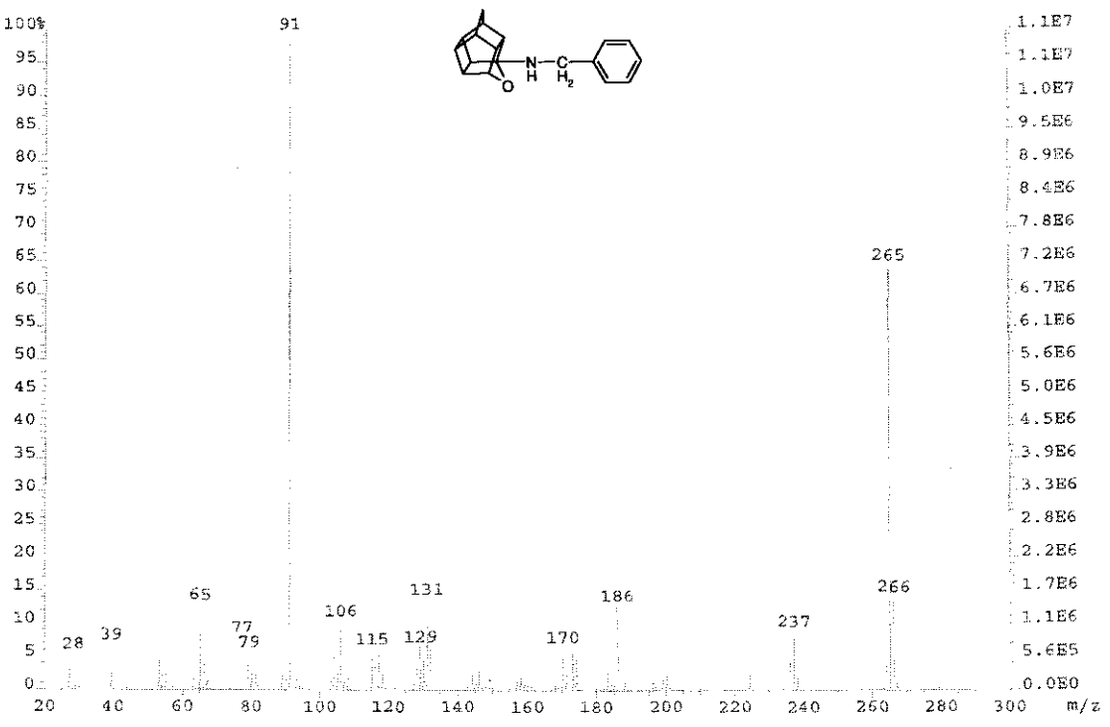

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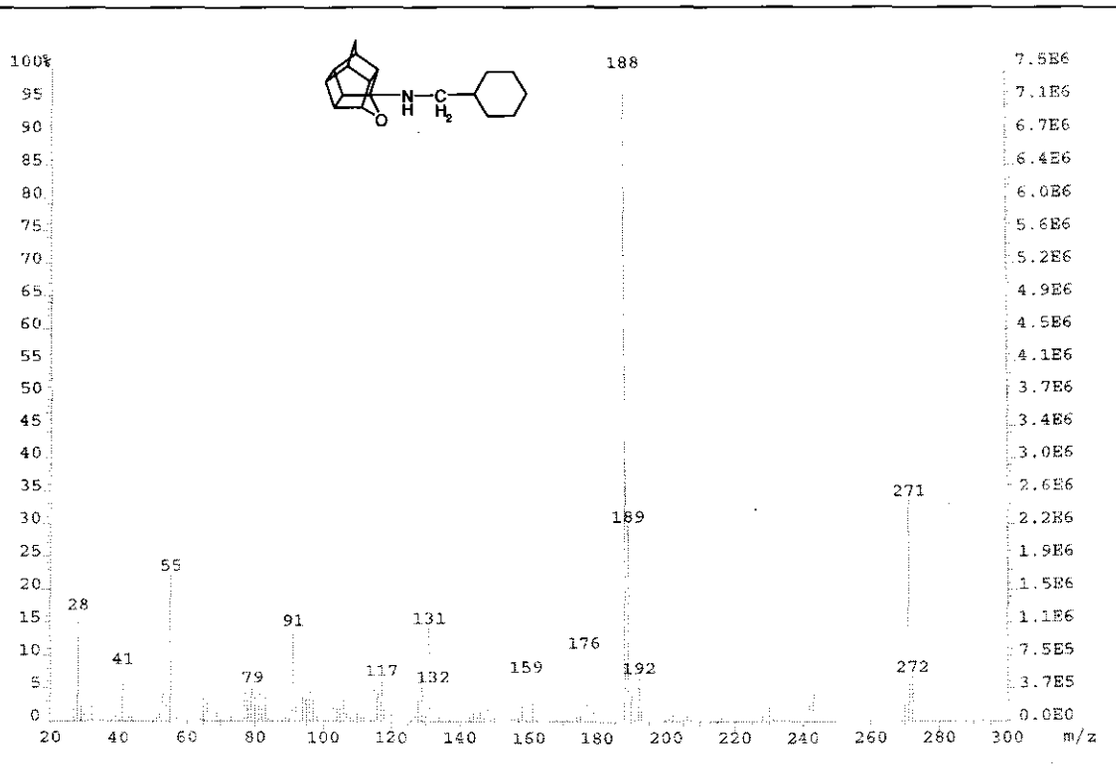

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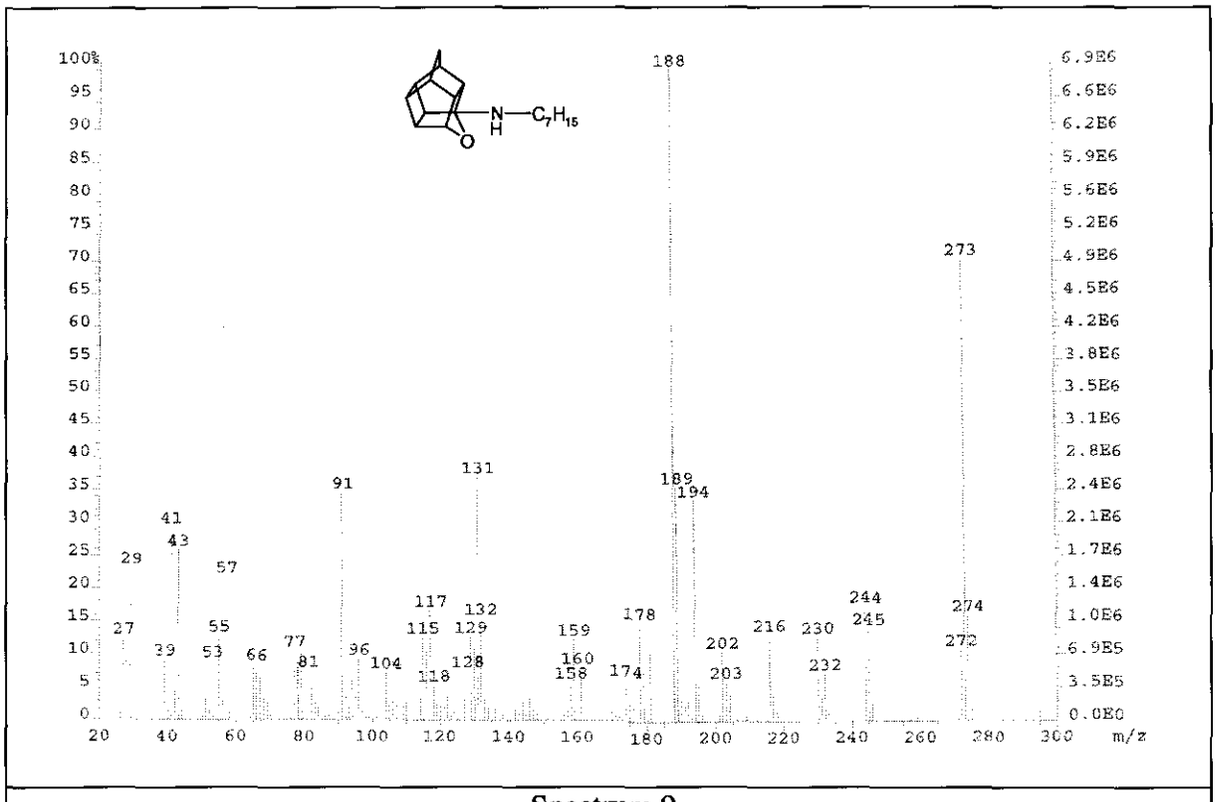

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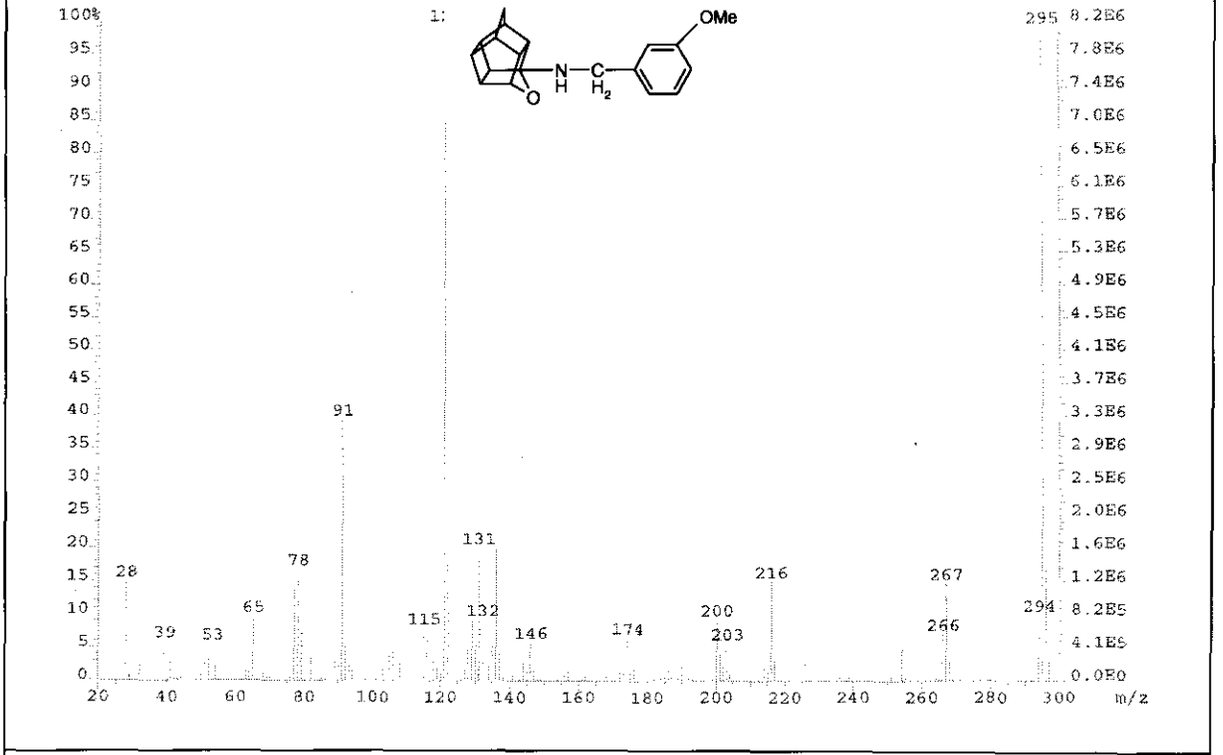

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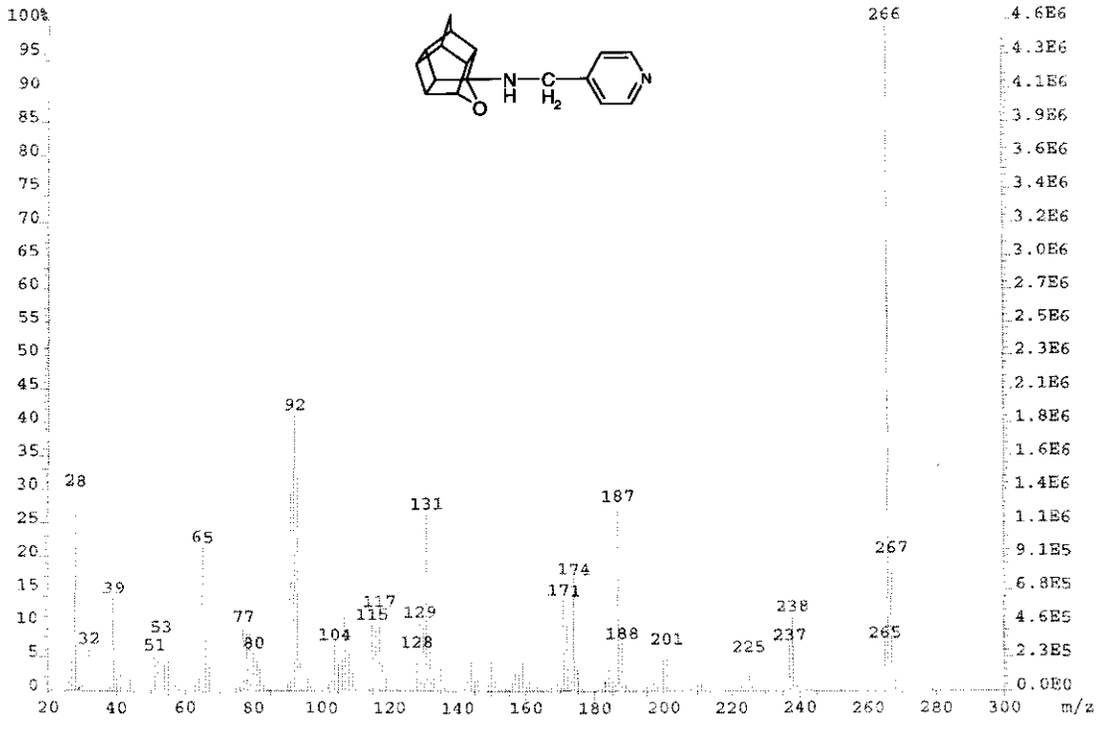

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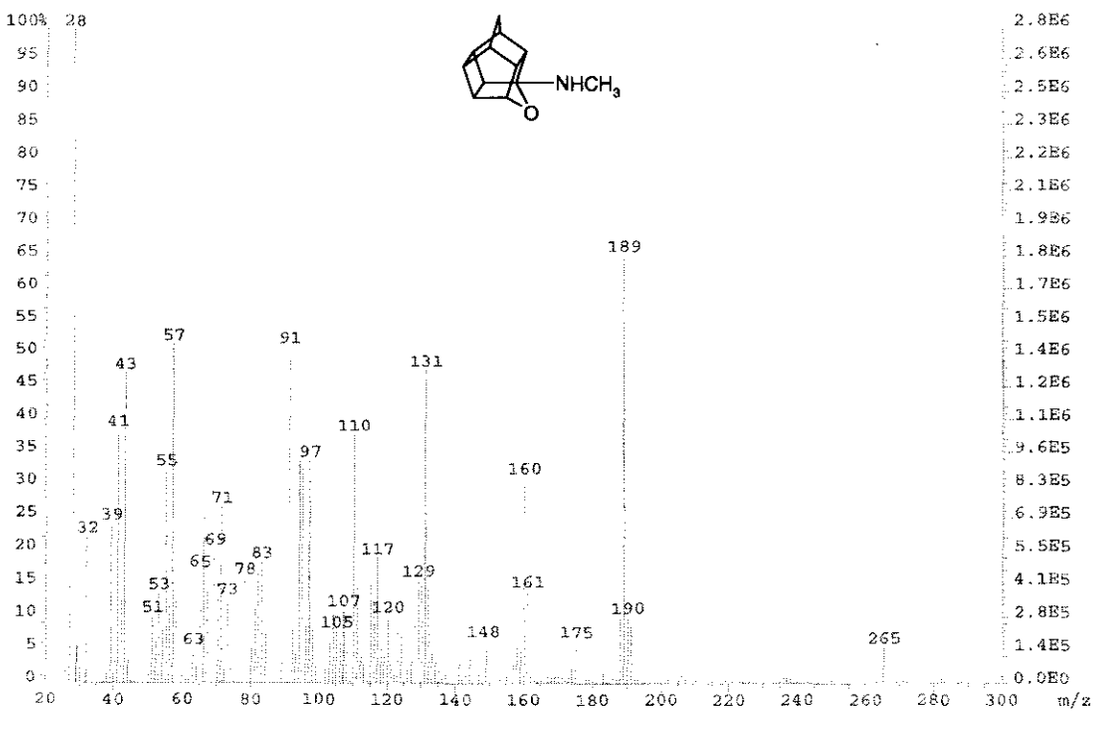

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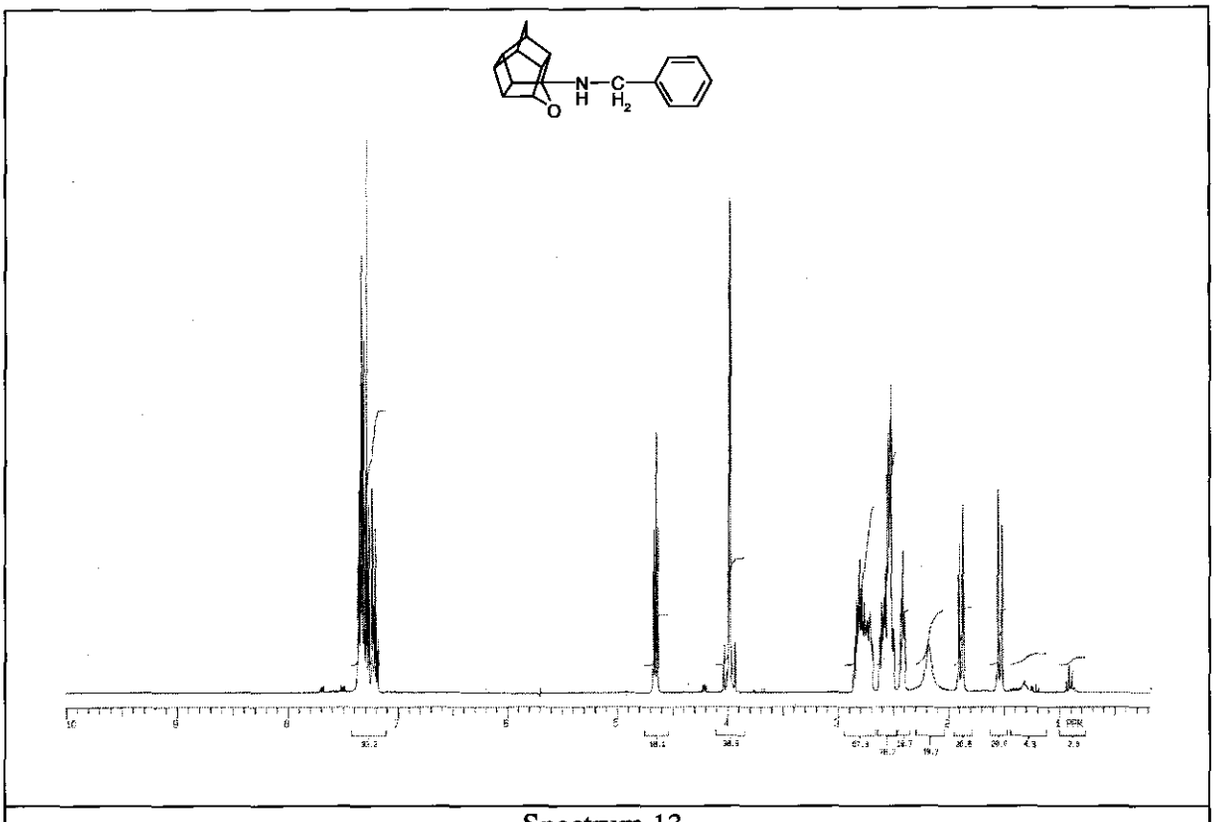

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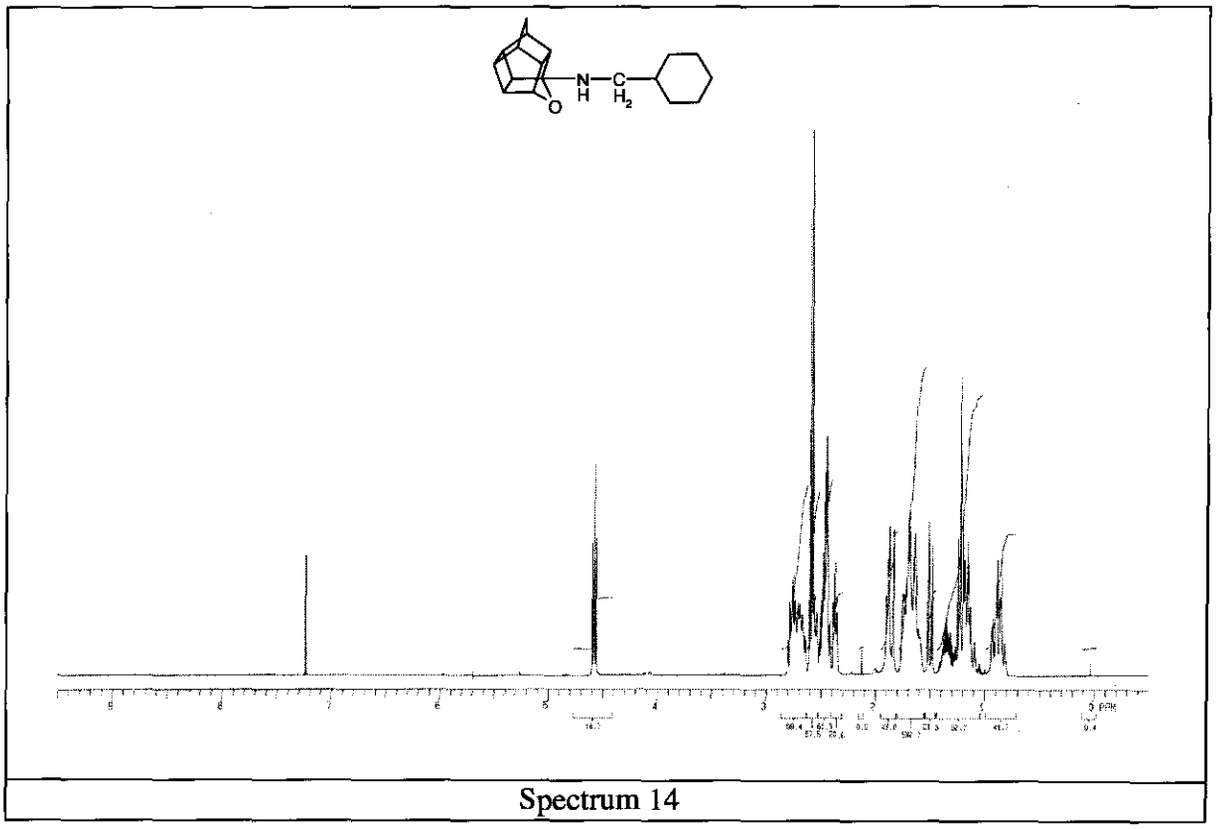

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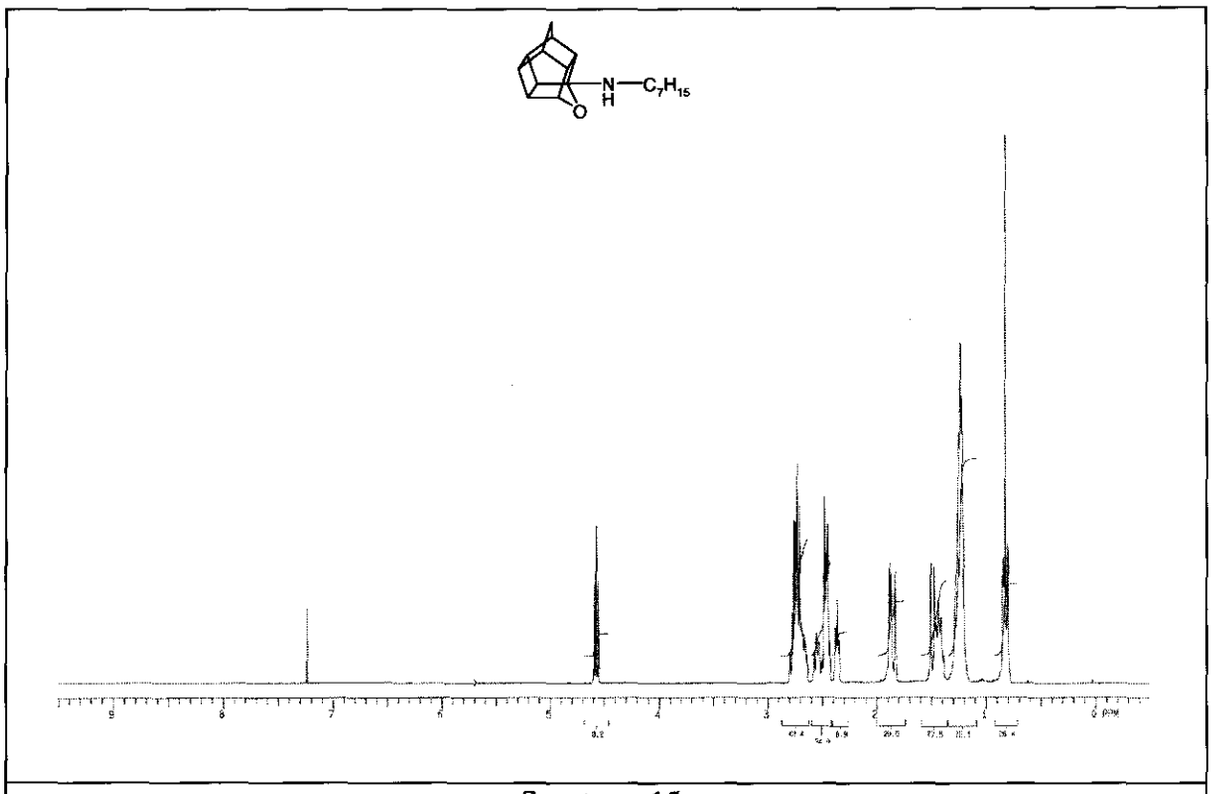

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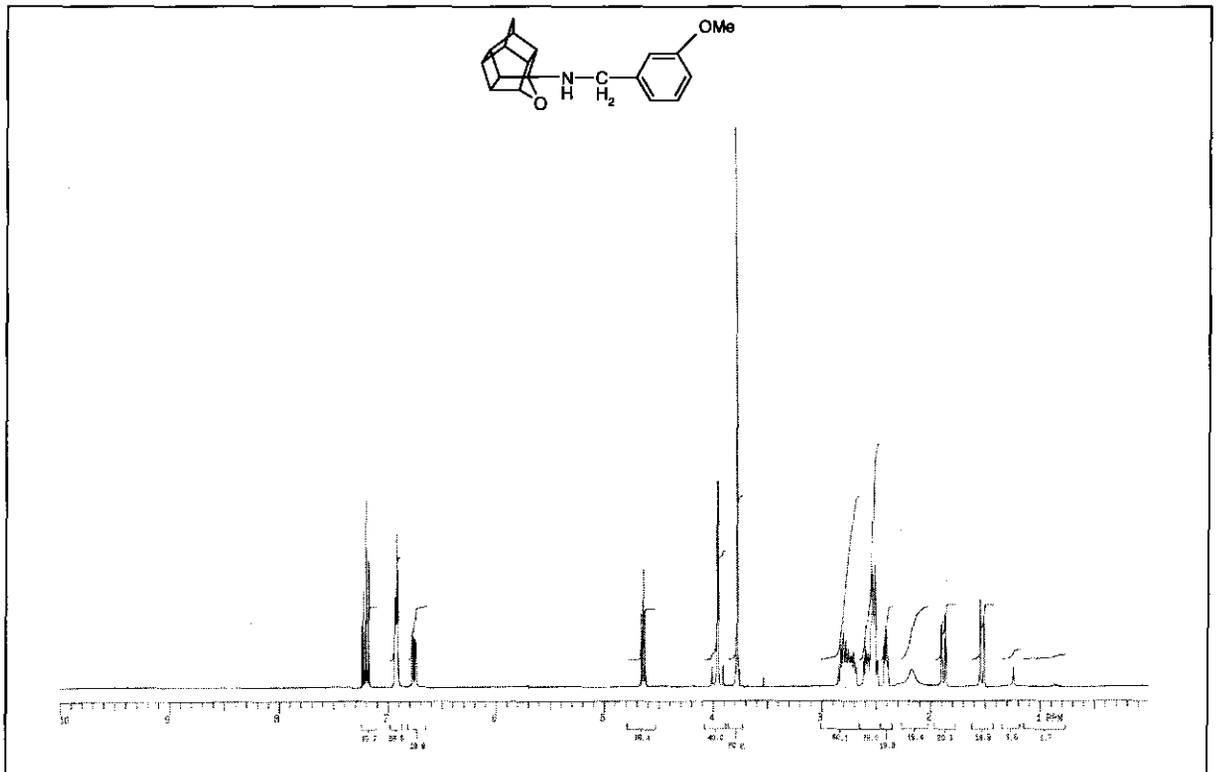

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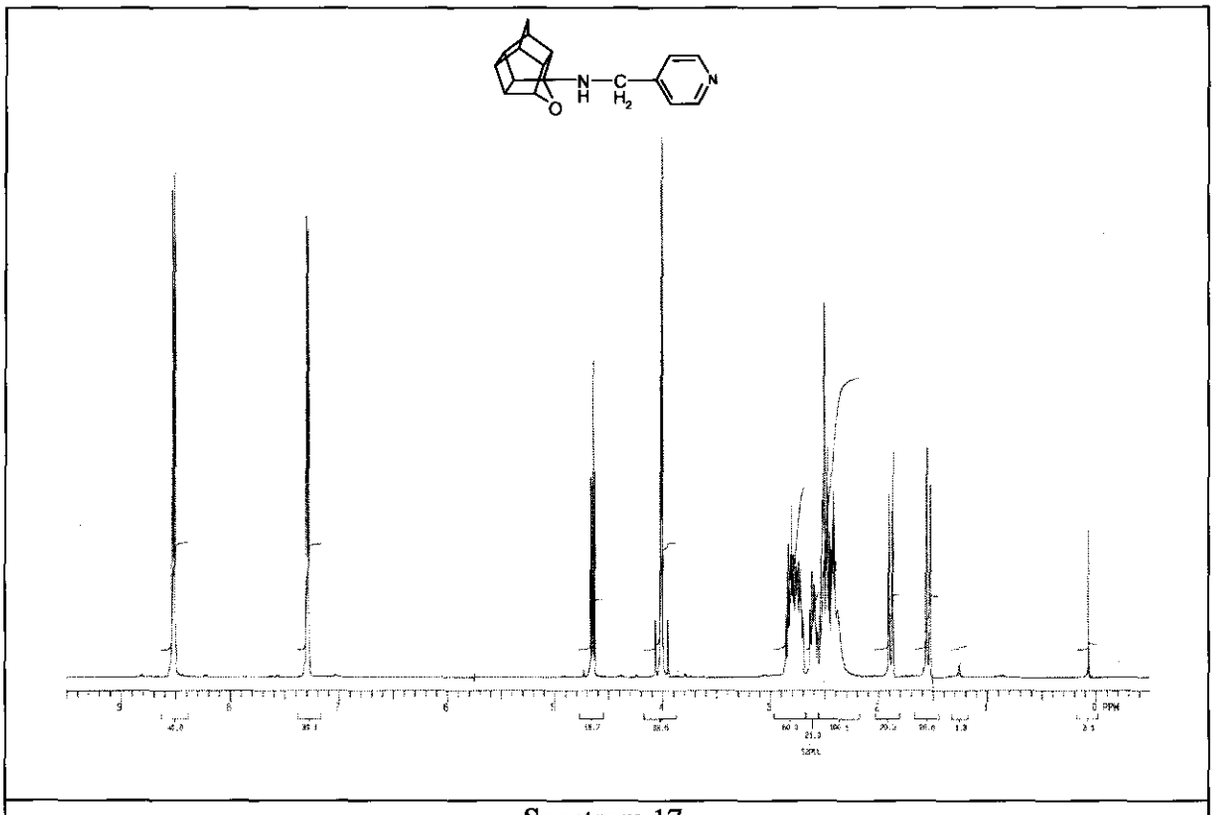

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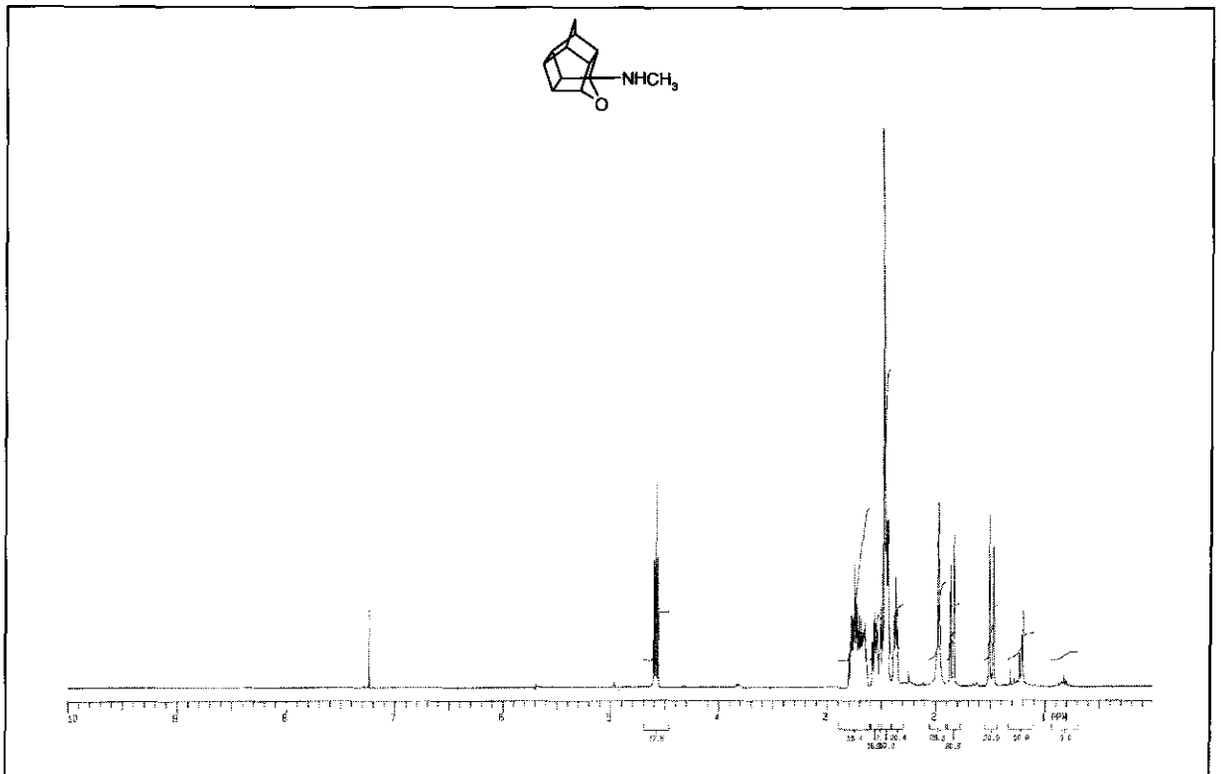

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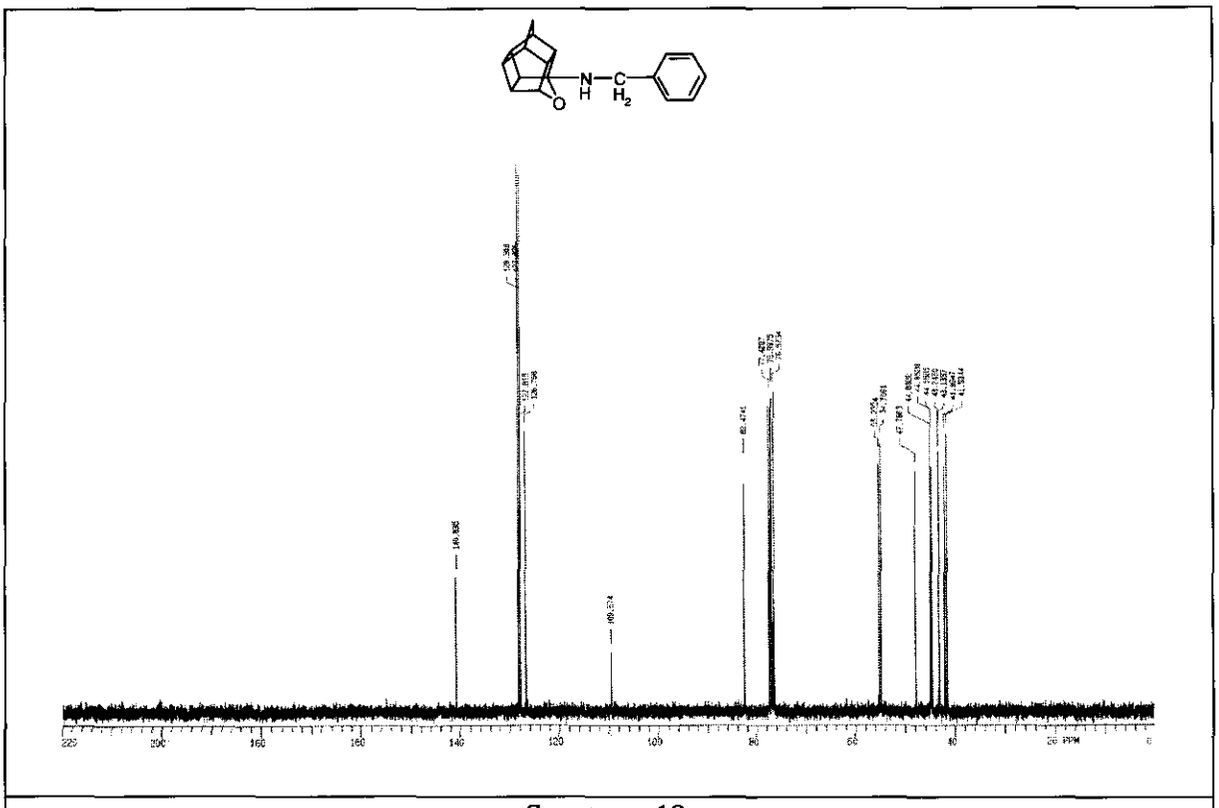

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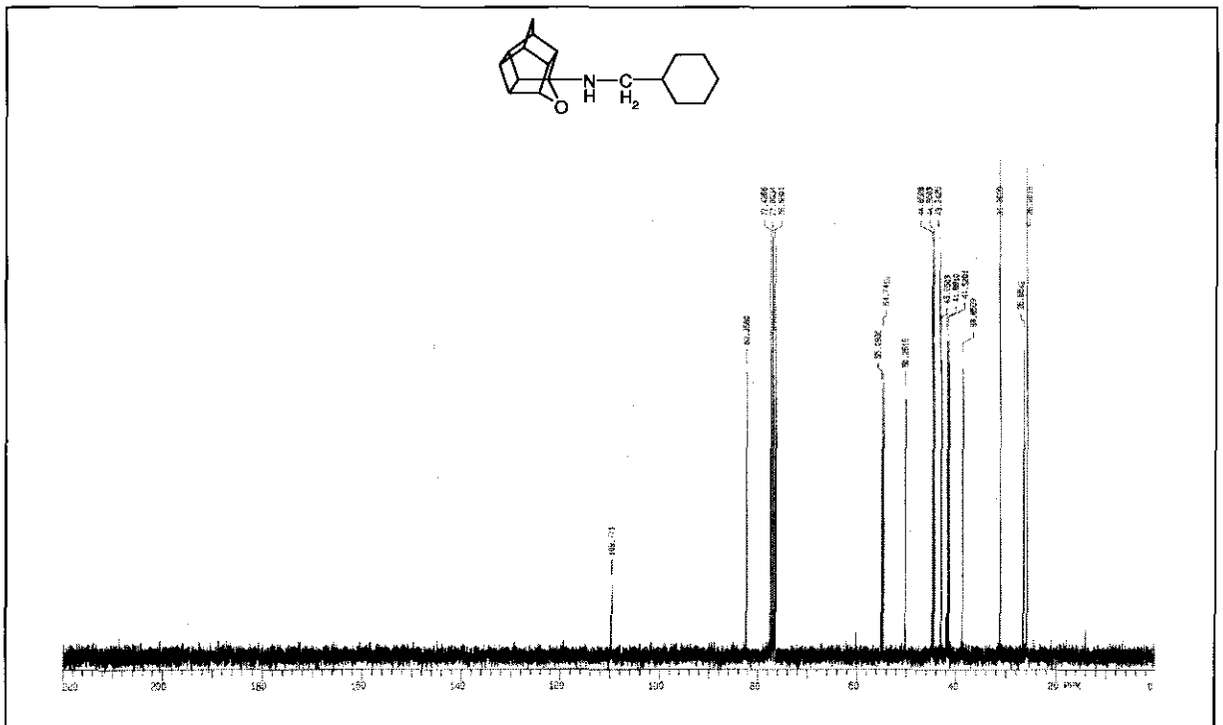

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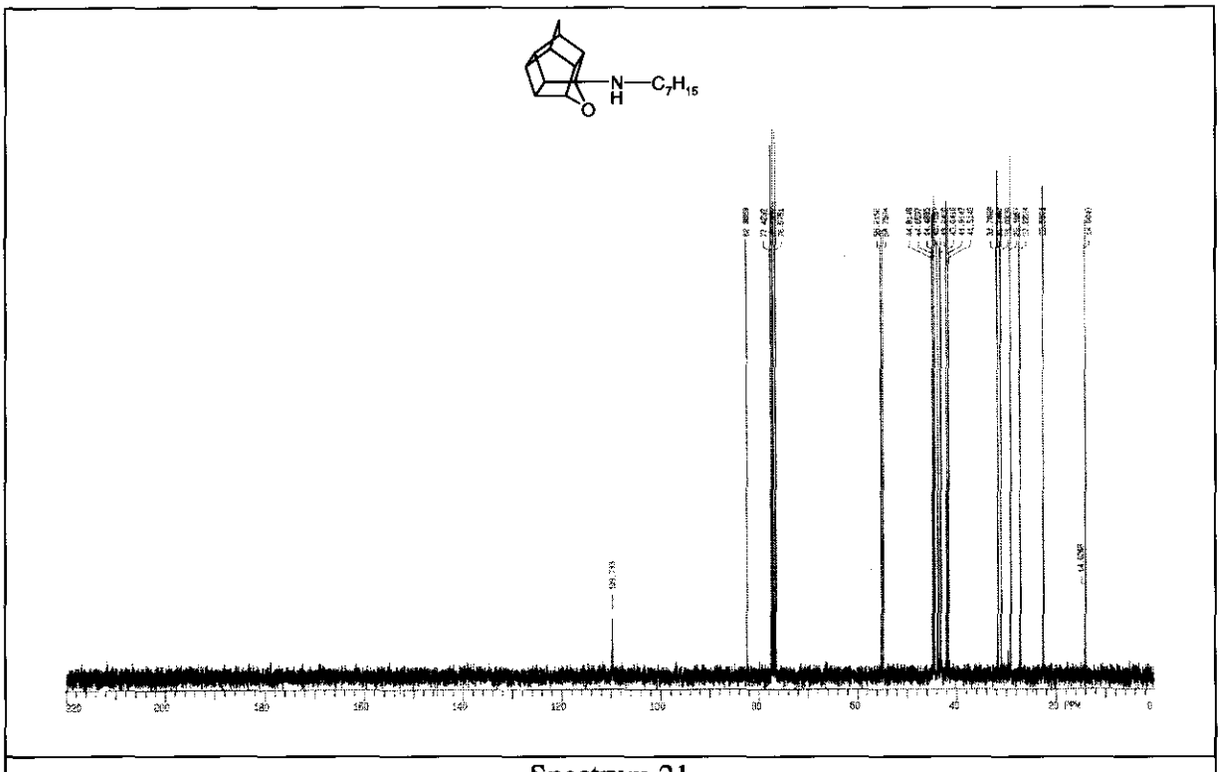

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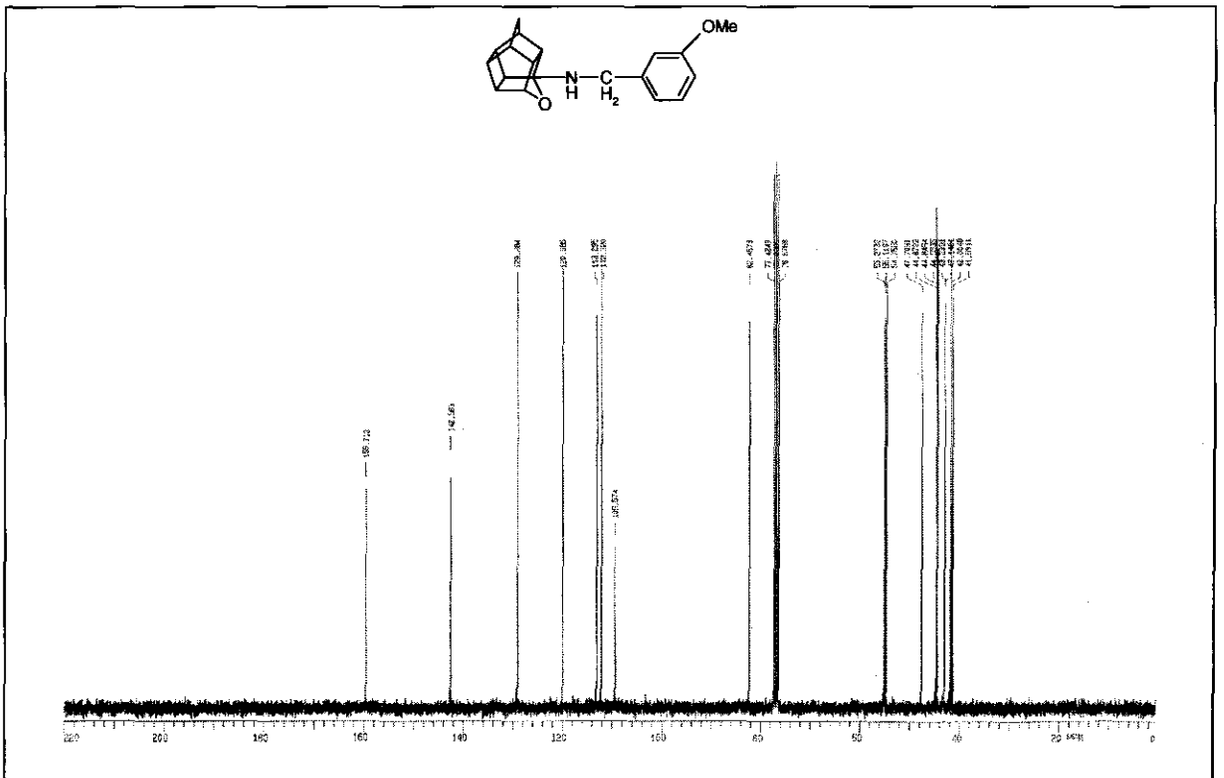

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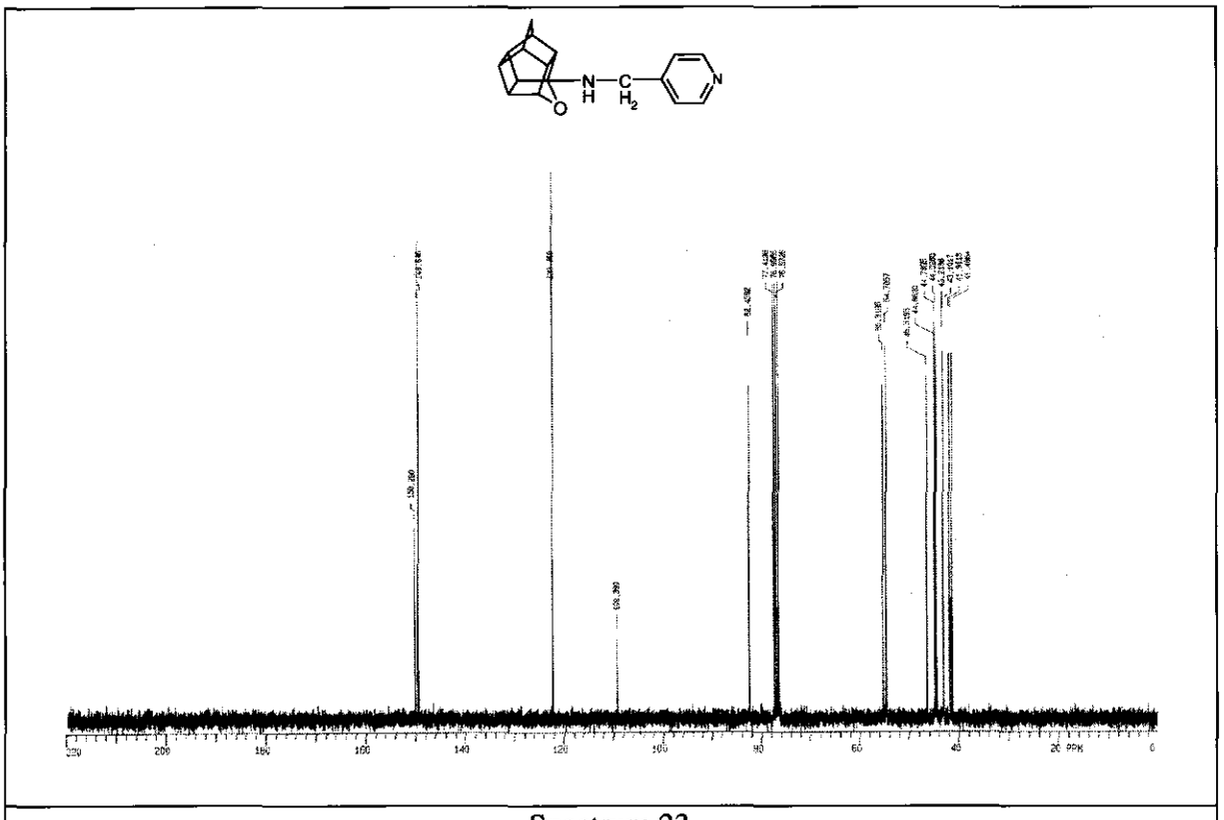

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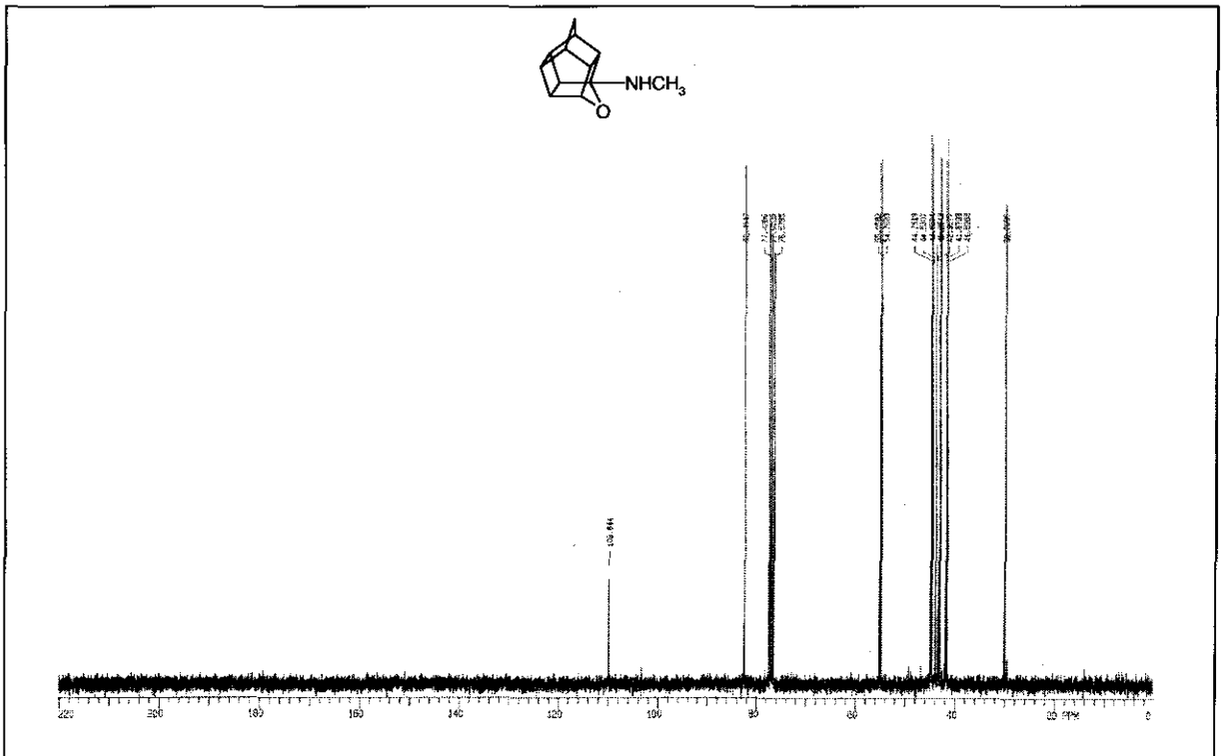

Spectrum 24

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