# N-methyl- D-aspartate (NMDA) and sigma receptor antagonism as neuroprotective strategy for polycyclic amines

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B.Pharm

Dissertation submitted in partial fulfilment of the requirements for the degree *Magister Scientiae* in Pharmaceutical Chemistry at the North-West University (Potchefstroom Campus)

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Potchefstroom

2008

Dedicated to my parents, Jan and Meisie Greyling

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# Abbreviations

| | |
|---|---|
| AD | Alzheimers' disease |
| ALS | Amyotrophic lateral sclerosis |
| AMPA | α-amino-3-hydroxy-5-methylisoxazole-4-propionate |
| ATP | Adenosine Triphosphate |
| BSA | Bovine serum albumin |
| CGRP | Calcitonin gene-related peptide |
| CPMA | Counts per minute average |
| DA | Dopamine |
| DCM | Dichloromethane |
| DD | Death domain |
| DED | Death effector domain |
| DMF | Dimethylformamide |
| DMSO | Dimethylsulfoxide |
| DTG | 1,3-Di-*o*-tolylguanidine, [p-ring-$^{3}$H]-), |
| ER | Endoplasmic reticulum |
| HD | Huntington's Disease |
| InsP3 | Inositol triphosphate |
| KA | Kaïnate |
| NMDA | *N*-methyl-*D*-aspartic acid |

| | |
|---|---|
| NMDR | N-methyl-D-aspartic acid receptor |
| nNOS | Oxide synthase |
| NO | Nitric oxide |
| NPY | Neuropeptide Y |
| P75NGFR | P75 nerve growth factor receptor |
| PCD | Programmed cell death |
| PCP | Phencyclidine |
| PD | Parkinsons' disease |
| ROS | Reactive oxygen species |
| TG-2 | Transglutaminase 2 |
| THF | Tetrahydrofurane |
| TNF | Tumor necrosis factor |
| VDCCs | Voltage dependant calcium channels |

# Abstract

Polycyclic cage compounds and their effects on different receptors and receptor channels have been studied extensively. It is evident that these compounds may prove to be of great value in future treatment of neurodegenerative diseases. Although many of the mechanisms involved in the process of neurodegeneration are still not fully elucidated, researchers are getting closer to identifying more and new possible targets for drug treatment.

In this study the focus was mainly on the effect of polycyclic cage compounds on calcium homeostasis, a key process in neurodegeneration. The role of sigma receptors in calcium homeostasis was also evaluated. As can be seen in the literature, these receptors are an exciting new prospect for drug targeting and treatment of not only neurodegenerative diseases but tumor related illnesses as well.

A series of pentacycloundecane derivatives containing sigma bias substituents were selected and synthesised using reductive amination. Their effect on intracellular calcium in synaptoneursomes, were evaluated using fluorescent techniques and their affinity for sigma receptors was determined through a radio ligand binding study on Sprague-Dawley rat liver membranes. The difference between the oxa-and aza derivatives as well as the effect of chain length between the cage and the piperidine moiety on calcium influx and binding affinity were evaluated.

8-{1-(2-Aminoethyl)piperidine}-8-11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane **(C1)** and 3-(1-piperidinemethyl) **(A1)** had the highest affinity for the sigma receptors. This implicated that compounds with a shorter chain length (C = 2), N-substituent and hydrophobic moiety with limited volume might be more favourable for binding to sigma receptors. They also had a significant effect on intracellular calcium concentration in the synaptoneurosomes and might be good lead compounds for future investigations. The structural features of these two compounds, according to literature indicate that they might have greater affinity for σ1 receptors and this together with their effect on intracellular calcium might implicate antagonism but further investigation will have to be conducted to confirm this. The oxa derivatives in general exhibited better inhibition of $Ca^{2+}$ flux, especially at higher concentrations with 8-{N-[3-(3-piperidin-1-ylmethylphenoxy)propyl]amine}-8-11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane **(A3)** showing the best inhibition at 10 μM.

The synthesised pentacycloundecane derivatives had an inhibitory effect on $Ca^{2+}$ flux and thus decreased intracellular calcium. This effect might also be linked to their interaction with sigma receptors. Selectivity for the different sigma receptors and affinity for other receptors needs to be explored to fully evaluate the potential neuroprotective effects of these structures.

# Opsomming

Studies oor die afgelope paar jaar toon duidelik dat polisikliese hokkie verbindings van groot belang kan wees in die toekomstige behandeling van neurodegeneratiewe siektes. Hierdie verbindings se invloed op verskeie reseptore en reseptorkanale is al deeglik ondersoek en al is daar aspekte van die meganismes wat betrokke is by die neurodegeneratiewe proses, wat nog nie ten volle verstaan word nie, kom navorsers al nader daaraan om nuwe teikens vir geneesmiddelbehandeling te identifiseer.

Kalsiumhomeostase is 'n sleutel proses in neurodegenerasie en hierdie studie het dan hoofsaaklik gefokus op die invloed van polisikliese verbindings op hierdie proses. Daar is ook gekyk na die rol wat sigma reseptore in kalsium homeastase speel. Soos gesien kan word uit die literatuur, toon hierdie verbindings baie potensiaal, nie net vir die behandeling van neurodegeneratiewe siektes nie, maar selfs ook vir tumorverwante siektes.

'n Reeks pentasikloundekaanderivate is gesintetiseer. Hierdie derivate is van so aard dat dit substituente bevat wat binding aan sigma reseptore begunstig. Die invloed van hierdie verbindings op intrasellulêre kalsiumkonsentrasies in sinaptoneurosome is ondersoek deur gebruik te maak van fluoressensietegnieke. Affiniteit vir binding aan die sigma reseptore is ook bepaal deur radioligandbindingstudies op die lewerweefsel van manlike Sprague Dawley-rotte te doen. Verskille tussen die verbindings ten opsigte van die invloed van die tipe derivaat (nl. oksa- of aza- derivate) en die lengte van die ketting tussen die hokkie en die piperidiengedeelte op kalsiumfluks, is geëvalueer.

Die resultate het getoon dat 8-{1-(2-aminoetiel)piperidien}-8-11-oksapentasiklo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undekaan **(C1)** en 3-(1-piperidienmetiel) **(A1)** die hoogste affiniteit vir die sigma reseptore en ook 'n noemenswaardige invloed op intrasellulêre kalsiumkonsentrasie in die sinaptoneurosome gehad het. Hierdie effekte dui daarop dat verbindings met 'n korter kettinglengte (C = 2), 'n N-substituent en 'n hidrofobiese of aromatiese gedeelte, met beperkte volume waarskynlik groter voorkeur het vir binding aan die sigma reseptore. Die strukturele samestelling van hierdie twee verbindings is dan ook van so aard dat, volgens die literatuur, hulle meer affiniteit vir die σ1 respeptor behoort te hê en dat hul invloed op intrasellulêre kalsium moontlik toegeskryf kan word aan antagonisme. Hierdie feit sal egter met verdere eksperimentele ondersoek bevestig moet word. In die algemeen het die oksaderivate kalsiumfluks beter onderdruk, veral by hoër konsentrasies. Hier het 8-{N-[3-(3-Piperidien-1-ylmetielfenoksie)propiel]amien}-8-11-oksapentasiklo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undekaan die beste gevaar by 'n konsentrasie van 10 µM.

Die gesintetiseerde reeks pentasikloundekaan derivate het 'n afname in intrasellulêre kalsiumkonsentrasie tot gevolg gehad deur hul onderdrukking van kalsiumfluks, wat moontlik gekoppel kan word aan hul interaksie met die sigma reseptore. In verdere studies om die volle neurobeskermende effek van hierdie strukture te ondersoek, sal die selektiwiteit vir die verskillende sigma reseptore asook ander reseptore, geëvalueer moet word.

# Chapter 1

# Introduction

## 1 Neurodegeneration

Although many investigations have been conducted on neurodegeneration in recent years, there are still aspects that are not fully understood. The discovery of the neuroprotective activity of polycyclic compounds, however lead to major breakthroughs in the development of new drugs for the treatment of neurodegenerative diseases.

The etiology of neuronal death in neurodegenerative diseases is still a mystery. These illnesses such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegia and cerebellar degeneration progress slowly and run an inevitable course. Research and advances in both molecular genetics and neurochemistry have improved our knowledge of the fundamental processes involved in cell death, including oxidative stress and mitochondrial dysfunction.

### 1.1 Oxidative stress and neurodegeneration

According to Sian *et al.* (1999), oxidative stress is a condition in which reactive oxygen-derived free radical species comprise the main factor leading to cell degeneration. Oxidative stress can also be described as an imbalance between oxidants and antioxidants (in favour of the former).

The nigral depletion of the antioxidant glutathione in Parkinson's disease provided the first evidence of oxidative stress in this condition. The catabolism of dopamine (DA), either through enzymatic deamination and/or auto-oxidation, has the reputation of generating toxic superoxide and hydroxy radicals triggering a self amplifying cell-destruction cycle (Sian *et al.*, 1999).

The normal energy metabolism of the brain has a few unusual features of which a high metabolic rate, limited intrinsic energy stores and critical dependence on aerobic metabolism of glucose are the most important, making it more vulnerable to ischemic injury than other tissue (Dugan and Choi, 1999). During hypoxia or ischemia, the energy demands of the brain tissue cannot be met and adenosine triphosphate (ATP) levels fall. This loss of ATP leads to a decrease in the function of active ion pumps of which the most important one is the Na, K-ATPase pump. This is the main transporter for maintaining high *intracellular concentrations*

of $K^+$ (~155 m*M*) and low concentrations of $Na^+$ (~12 m*M*). Loss in pump activity results in a rundown of trans-membrane ion gradients that leads to membrane depolarization, the opening of voltage gated ion channels and a cascade of events, which in a constant state eventually leads to cell death (Dugan and Choi, 1999).

Hypoxia also influences the extracellular concentrations of many other neurotransmitters, causing an increase in their concentrations. Depolarisation-induced entry of $Ca^{2+}$ *via* voltage sensitive $Ca^{2+}$ channels further stimulates the release of vesicular neurotransmitter pools, including the excitatory amino acid neurotransmitter glutamate. Simultaneously, $Na^+$-dependant uptake of certain neurotransmitters such as glutamate, is also impaired (Trotti *et al.*, 1997). This imbalance in especially glutamate regulation plays a key role in the events leading to neuronal cell death.

## 1.2 Glutamate, an excitatory amino acid neurotransmitter and its role in cell death.

Glutamate is a major excitatory transmitter in the brain and it participates mainly in synaptic interactions, as glutamatergic release sites are located predominantly within the synapses (Kiss *et al.*, 2004). Glutamatergic pathways originating from the sensomotor cortex and the subthalmic nucleus are the major routes of excitatory input to the corpus striatum. Not only do they play a crucial role in cognitive and motor coordination functions but they also participate in the pathogenesis of neurodegenerative diseases (Dohovics *et al.*, 2003). Within these pathways, glutamate regulates its own release *via* stimulation of ionotropic glutamate receptors, which are cationic-specific ion channels and mediate fast action (Varju *et al.*, 2001). Over exposure to glutamate or over stimulation of its membrane receptors lead to neuronal injury or death. This is called excitotoxicity (Lipton and Nicotera, 1998).

Glutamate initiated excitotoxicity is due to a sustained increase in intracellular $[Ca^{2+}]_i$. This increase activates $Ca^{2+}$-calmodulin dependant and protein kinase C regulated neuronal nitric oxide synthase (nNOS), which in turn produces nitric oxide (NO) (Patel and Li, 2003). NO is a highly reactive signal molecule that plays an important role in the regulation of neurotransmission in the central and peripheral nervous system but is also a reactive free radical with many potential targets which may initiate neurotoxic cascades and oxidative damage when present in excessive amounts (Gunasekar *et al.*, 1995). There are many triggers for the excessive release of glutamate within the nervous system. Severe mechanical injury like head or spinal cord injury are examples of the acute form and cell death occurs by necrosis in these cases (Lipton, 1993a; Lipton and Rosenberg, 1994). In chronic neurodegenerative diseases such as AD, PD and ALS the degeneration progress is

slow and more subtle. In this instance excitotoxicity causes cell death through apoptosis (Quigley *et al.*, 1995).

## 1.3 Apoptosis

The processes of necrosis and apoptosis are quite different. Apoptosis is a process of programmed cell death (PCD) where the cell basically commits suicide. Necrosis is a type of degeneration which results in cell death due to cellular trauma such as chemical trauma or tissue injuries. In the case of necrosis the cell ruptures and releases its contents, which could be toxic, to the surrounding tissue and can provoke an inflammatory reaction that leads to further cell death. Apoptosis is an active process which requires ATP where as necrosis is known to be a passive process (Wyllie *et al.*, 1984).

Degeneration of one or more nerve cell populations is a major feature in many acute and chronic neurological diseases. It is thus important to have an understanding of the molecular mechanisms underlying neuronal apoptosis. Apoptosis has extrinsic and intrinsic pathways. Whereas the extrinsic pathway is initiated by cell surface activation of cytokine receptors of the tumor necrosis factor (TNF) family, the intrinsic pathway depends on the integrity and function of mitochondria within the cell (Reed, 2000).

The most important cell organelle involved in apoptosis is the mitochondria. It regulates apoptosis by controlling the release of cytochrome c and other pro-apoptotic factors through a mechanism that is still not fully understood (Jurgensmeier *et al.*, 1998). Apoptosis is also characterised by the loss of mitochondrial membrane potential which in itself can result in cytochrome c release (Marchetti *et al.*, 1996).

The pathological rise in intracellular $Ca^{2+}$, as a result of toxic levels of glutamate and other neurotransmitters, is an important cause of mitochondrial dysfunction. The mitochondria actively accumulate calcium, via the $Ca^{2+}$ uniporter located in the inner mitochondrial membrane, to try to attenuate this increase in $Ca^{2+}$. This uniporter however is driven by the same electrochemical gradient used by mitochondrial ATPase, thus a part of the respiratory capacity of the electron transport chain is uncoupled from oxidative phosphorylation leading to a decline in ATP production. Furthermore, the increase in calcium increases the formation of reactive oxygen species (ROS) affecting the energy metabolism dually: directly by inhibiting complex I of the electron transport chain, or indirectly by producing oxidative membrane alterations that result in further increase of intracellular calcium (Fiskum, 2000).

## 1.4 The role of NMDA, AMPA/KA and sigma receptors in neurodegeneration.

*In vitro* studies on excitotoxicity, suggests that the *N*-methyl-D-aspartic acid receptor (NMDAR) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate and kainate receptors (AMPA/KA) - different classes of glutamate receptors - both mediate excitotoxicity but not equally. Some of the results of experiments done with hippocampal or cortical cell cultures suggest that neuronal death associated with brief, intense glutamate exposure is largely mediated by NMDA receptor stimulation as this activation can induce lethal amounts of $Ca^{2+}$ influx at a much quicker rate than the stimulation of the AMPA/KA receptor. Nevertheless, over activation of AMPA/KA receptors also leads to toxic intracellular $Ca^{2+}$ concentrations and eventually to neurodegeneration (Frederickson *et al.*, 1989).

The role of sigma (σ) receptors in mediating neuroprotection/degeneration has been studied in several *in vitro* models of central nervous system (CNS) injury. A consistent positive correlation between sigma neuroprotective potency and σ1 binding site affinity has been demonstrated, suggesting that the functional neuroprotective effect of sigma ligands may be mediated by binding at the σ1 site (antagonism) (De Coster *et al*, 1995). Activation of σ1 receptors results in a complex, bipolar modulation of calcium homeostasis. It facilitates the mobilisation of inositol triphosphate (InsP3 ) receptor-gated intracellular calcium pools at the endoplasmic reticulum (ER) level and modulates extracellular calcium influx through voltage-dependent calcium channels at the plasma membrane level (Bowen, 2000; Hayashi *et al.*, 2000). Activation of σ2 receptors was also shown to induce changes in cell morphology and apoptosis and causes a sustained increase in calcium ions (Bowen, 2000). The sigma receptors are thus potential targets for calcium modulation.

## 1.5 Role of polycylic compounds in neuroprotection

Screening studies on the pentacycloundecylamines indicated that these compounds are effective antagonists of NMDA-mediated $Ca^{2+}$ influx into synaptoneurosomes. The polycyclic cage amine seems to be the most important pharmacophoric element contained within the pentacycloundecylamine structure required to interact with the NMDA receptor (Geldenhuys *et al.,* 2004). Their uncompetitive nature of NMDA receptor antagonism, in addition to their use-dependent L-type calcium channel blocking effects, suggests that these compounds may be useful as dual mechanism neuroprotective agents in neurodegenerative disorders. Anti-parkinsonian properties of these pentacycloundecylamines were also attributed to their possible effects on dopaminergic systems (Geldenhuys *et al.,* 2004). Binding of a series pentacycloundecylamine derivative to both σ - sites was also explored by Nguyen *et al.*

(1996) but their influence on intracellular calcium concentrations and neuronal cell death has not been described.

## 1.6 Rationale and aim of study

High concentrations of intracellular calcium activate a cascade of events that ends in neurodegeneration. Over stimulation of sigma ($\sigma$) receptors contribute to the toxic intracellular calcium concentrations and are thus a target for neuroprotective drugs. Cage compounds such as the pentacycloundecanes have been shown to have neuroprotective activity through modulation of the NMDA receptor complex and binding studies confirmed that they also have affinity for sigma receptors. The purpose of this study was thus to synthesise and test a series of pentacycloundecane derivatives for modulating effect on intracellular calcium concentrations and thus to confirm their neuroprotective activity.

# Chapter 2

# Literature

## 2 Introduction

The role of calcium as a transmembrane charge carrier and critical second messenger is well established and an increase in intracellular calcium is a major consequence of exposure of neuronal cells to excitation amino acids such as glutamate (Choi, 1987; Garthwaite and Garthwaite, 1986). This increase in $[Ca^{2+}]_i$ results from a number of glutamate-induced processes including calcium influx through: voltage-gated calcium channels , receptor-gated NMDA calcium channels, and activation of the metabotropic receptor and subsequent release of calcium from intra-cellular calcium stores (Glaum, 1990). The result of sustained activation of calcium dependent processes is considered to be an important initiator of neuronal excitotoxicity.

### 2.1 Voltage-gated calcium channels

Voltage-gated calcium channels mediate calcium influx in response to membrane depolarisation and regulate intracellular processes such as contraction, secretion, neurotransmission, and gene expression. Their activity is essential to couple electrical signals in the cell surface to physiological events in cells. They are members of a gene superfamily of transmembrane ion channel proteins that includes voltage-gated potassium and sodium channels (European Research Network, 2008).

Calcium currents in different cell types have different physiological and pharmacological properties. An alphabetical nomenclature has evolved for the distinct classes of calcium currents. L-type calcium currents require strong depolarisation for activation and are long-lasting. They are blocked by the organic L-type calcium channel antagonists, including dihydropyridines, phenylalkylamines and benzothiazepines. They are also the main calcium currents recorded in muscle and endocrine cells, where they initiate contraction and secretion. N-type, P/Q-type, and R-type calcium currents also require strong depolarisation for activation, are relatively unaffected by L-type calcium channel antagonist drugs, but are blocked by specific polypeptide toxins from snail and spider venoms. They are primarily expressed in neurons, where they initiate neurotransmission at most fast synapses and also mediates calcium entry into cell bodies and dendrites. T-type calcium currents are activated by weak depolarisation and are transient. They are resistant to both organic antagonists and

to the snake and spider toxins used to define the N- and IP/Q-type calcium currents. They are expressed in a wide variety of cell types, where they are involved in shaping the action potential and controlling patterns of repetitive firing (European Research Network, 2000).

### 2.1.1 L-type calcium channels: structure and function

The L-type $Ca^{2+}$ channel has been studied in skeletal muscle because of the high concentration of L-type $Ca^{2+}$ channels in this tissue (Catterall, 1988; Fosset *el al.*, 1983). This $Ca^{2+}$ channel is composed of five different polypeptide subunits, having different molecular masses (Takahashi *et al.*, 1987): The $\alpha_1$ subunit (175 kD), which forms the ion channel and contains $Ca^{2+}$ antagonist binding sites (Tanabe *et al.*, 1987; Ellis *et al.*, 1988); the $\alpha_2$ subunit (143 kD), which is associated with $\alpha_1$ and does not contain any high-affinity binding site and three low-molecular- weight subunits, ß (54 kD), γ (30 kD), and δ (27 kD) (Ruth *et al.*, 1989; Jay *et al.*, 1990). The $\alpha_1$ and ß subunits contain phosphorylation sites for cAMP-dependent protein kinase. The $\alpha_2$, γ and δ subunits are heavily glycosylated, indicating that they have an extracellular face (Takanashi *et al.*, 1987). Four of the five subunits of the skeletal muscle channel have been independently cloned and sequenced: $\alpha_1$ (Tanabe *et al.*, 1987; Ellis *et al.*, 1988), $\alpha_2$ (Ellis *et al.*, 1988), ß (Ruth *et al.*, 1989) and γ (Jay *et al.*, 1990).

The $\alpha_1$ subunit is considered to be the principal structural component of the $Ca^{2+}$ channel. As with the $Na^+$ channel a subunit, the $Ca^{2+}$ channel $\alpha_1$ subunit possesses four homologous domains that are predicted to span the cell membrane and to contribute to the outer vestibule of the channel pore. Each domain has six recognised transmembrane regions (S1, S2...S6) (Catterall, 1988; Tanabe *et al.*, 1987; Jan & Jan, 1989). Both the short amino-terminal segment and the long carboxy- terminal segment of the $\alpha_1$ subunit are positioned intracellularly. Studies of mutated $Na^+$ channels have revealed that one of the transmembrane segments, S4, serves as the voltage sensor of the channel and is present in all voltage-gated channels (Stümer *et al.*, 1989).The single transmembrane segment of S4 in each motif is distinguished by a collection of repeating positively charged amino acids (arginine or lysine), which are located in every third or fourth position. It is these four positively charged transmembrane segments that are believed to comprise the voltage sensor of voltage dependant calcium channels (VDCCs) and $Na^+$ and $K^+$ channels (McCleskey et al., 1993).

The functions of the L-type $Ca^{2+}$ channel are related to the generation of action potentials and to signal transduction events at the cell membrane (Kostyuk, 1989). L-type VDCCs are expressed in neuronal, endocrine, cardiac, smooth, and skeletal muscle, as well as in fibroblasts and kidney cells. Recent reports suggest a role for L-type VDCCs in the process

of neurotransmitter secretion of the central nervous system (Banci *et al.*, 1998; Protti and Llano, 1998).

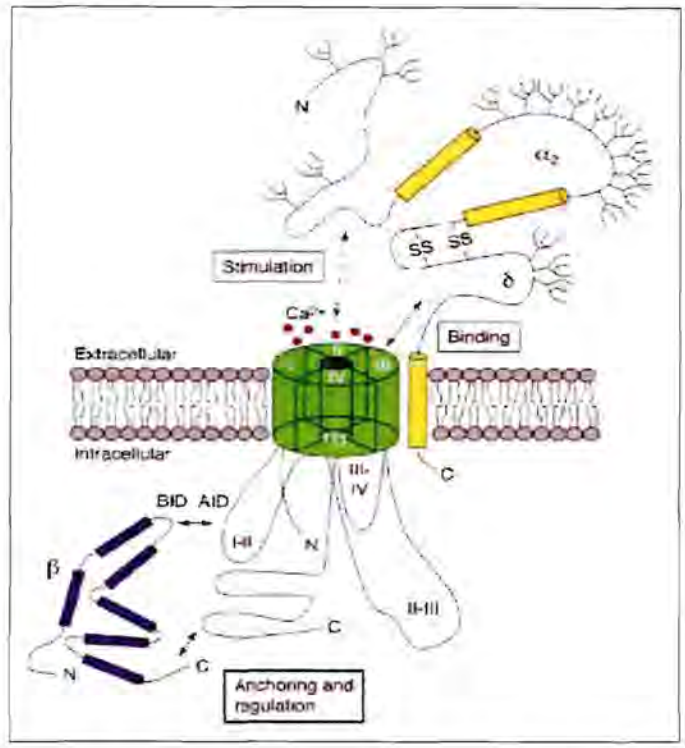



**Figure 1: Calcium channel heteromeric complex (redrawn from Walker & De Waard, 1998) taken from 'Calcium channel diversity' by ACD in Encyclopaedia of Life Sciences 2003**

## 2.2 NMDA, AMPA and KA receptors

NMDA receptors comprises of different subunits with a tetrameric composition. The NR1 subunit is mandatory while the other three subunits are made up from the NR2A-D and sometimes NR3A or B subunits, thus presenting a heteromolecule in contrast to AMPA/KA receptors. The pharmacology and other parameters of the receptor-ion channel complex are determined by the composition of the subunits (Wollmuth and Sobolevesky, 2004). Expression of these subunits differs both regionally in the brain and temporally during development. It is clear that physiological activity of the NMDA receptors is necessary for normal neuronal function (Cull-candy *et al.*, 2001) and is implicated in neuronal survival and maturation (Simon *et al.*, 1984; Balazes *et al.*, 1989), neuronal migration (Komuro and Rakic, 1993), induction of long-term potentiation (Bliss and Collingridge 1993), formation of sensory maps (Cline *et al.*, 1987) and neurodegeneration (Meldrum and Garthwaite, 1990).

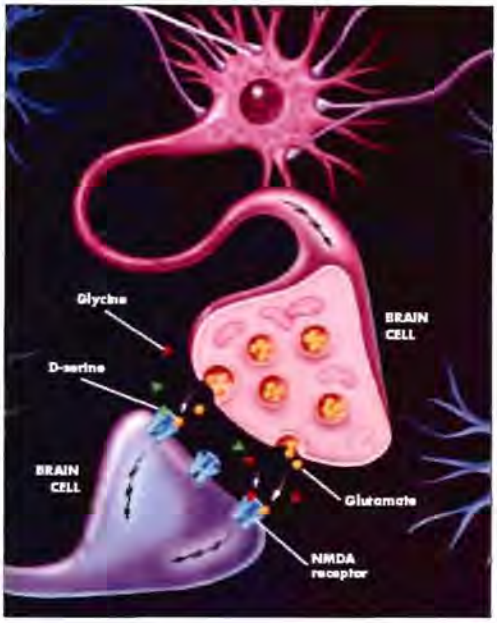



**Figure 2: Location and function of the NMDA receptor(www.sfn.org/index.cfm?pagename=brainbriefings)**

During normal synaptic transmission, the NMDAR-channel is gated by extracellular $Mg^{2+}$ positioned in the channel and is only activated for a short periods of time. When activated, $Ca^{2+}$ and other cations necessary for normal physiological function can move into the cell, during the brief opening of the channel. Under pathological conditions, however, over activation of the receptor relieves this $Mg^{2+}$ block and leads to the influx of excessive amounts of $Ca^{2+}$ into the nerve cell, triggering a variety of processes leading to necrosis and apoptosis (Lipton *et al.*, 1993; Bonfoco *et al.*, 1995). One of these processes is the formation of nitric oxide.

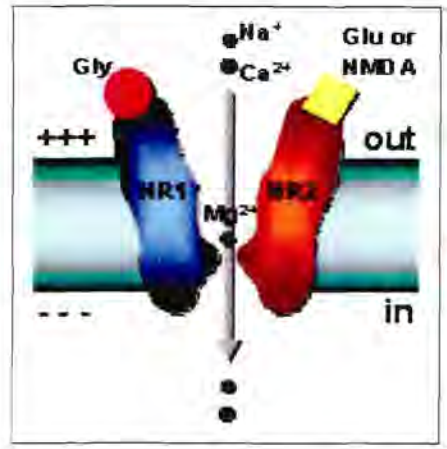



**Figure 3: Activation of the NMDA channel. (www.neuromolecular.com/science/index.html)**

The neural form of nitric oxide synthase, nNOS, produces NO primarily in response to the activation of NMDA receptors stimulated by glutamate (Kosenko *et al.*, 2003).

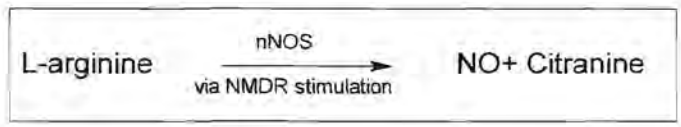



**Figure 4: Syntheses of nitric oxide**

An excessive increase in extracellular glutamate is however not necessary to activate an excitotoxic mechanism as this process can be invoked at normal levels of glutamate if NMDA receptor activity is abnormally increased. This happens when neurons are injured and become depolarised which results in relieve of the normal block of the ion channel by $Mg^{2+}$ (Mullins *et al.*, 1996).

AMPA/KA receptors are generally $Ca^{2+}$ impermeable but can still trigger neuronal injury, more slowly because prolonged periods of activation are needed for neurotoxicity to develop (Koh *et al.*, 1990). KA receptor stimulation also leads to neuronal NO production, which modulates glutamate transmission (Albadi *et al.*, 1999; Nakaki *et al.*, 2000) thus playing an important role in the normal physiological function of the brain. Over stimulation of KA receptors under pathological conditions also results in excitotoxicity and neurodegeneration (Borson *et al.*, 1999).

Though NMDA receptors are more likely to critically contribute to neurodegeneration during acute conditions, there are several studies implying that AMPA/KA receptors might be of

greater importance in the neurodegenerative process than currently thought (Carriedo *et al.*, 2000; Carriedo *et al.*, 1998).

## 2.3 Sigma receptors

The sigma1 (σ1) receptor has a long history, since its initial denomination proposed in the pioneering work of Martin *et al.* (1976) using the morphine-dependent chronic spinal dog model. The protein was first identified as an opiate receptor, and then closely related to the phencyclidine (PCP) binding site associated with the NMDA type of glutamate receptor. Eventually, the σ site was considered to be clearly distinct from any other receptor (Quirion *et al.*, 1987), but accepted to be of similar nature as membrane-bound receptors. It still, however remains of enigmatic nature.

Pharmacological studies revealed the existence of at least two subtypes of σ sites, named σ1 and σ2 (Quirion *et al.*, 1992). For each type of σ site, high affinity and often very selective compounds were described. The neuromodulatory effect of σ1 ligands, especially on NMDA responses, was shown and analysed (Monnet *et al.*, 1990, 1992). Behavioral effects of σ1 ligands, in learning and memory, depression, anxiety, stress, addiction or psychoses were also described (Walker *et al.*, 1990). Activation of $\sigma_2$ receptors has been shown to induce changes in cell morphology and apoptosis, and causes a sustained increase in intracellular calcium ions (Bowen, 2000).

### 2.3.1 Localisation and pharmacology of the σ receptors

The σ sites are found both centrally and peripherally but are mostly concentrated in the hippocampal formation and other limbic areas (Walker *et al.*, 1990 ; Debonnel *et al.*, 1996). It is expressed in the heart, lung, kidney, liver, intestines and sexual and immune glands, peripherally and is expressed in neurons, ependymocytes, oligodendrocytes and Schwann cells in the CNS (Palacios *et al.*, 2003, 2004). At the subcellular level, both receptors were found to be associated with microsomal, plasmic, nuclear or ER membranes (Bowen, 2000; Phan *et al.*, 2003) When stimulated by agonists, σ1 receptors translocate from the ER lipid droplets to plasmalemma or nuclear membranes. This translocation of σ1 receptors, associated with the ankyrin B protein, affects $Ca^{2+}$ mobilisation at the ER (Hayashi and Su, 2000). Activation of the σ1 receptor thus results in a complex, bipolar modulation of calcium homeostasis. Activation of the σ1 receptor facilitates the mobilisation of InsP3 receptor-gated intracellular calcium pools at the ER level and modulates extracellular calcium influx through voltage-dependent calcium channels at the plasma membrane level (Hayashi and Su, 2000). Monnet and Debonnel proposed an *in vivo* electrophysiological model to study the pharmacological activity of selective σ receptor ligands (Monnet *et al.*, 1990). Results from

this laboratory demonstrated that ligands, applied by microiontophoresis or administered i.v. at low doses, potentiated the neuronal response to NMDA in the CA3 region of the rat dorsal hippocampus, but did not modify kainate- nor quisqualate-induced activations.

Sigma1 (σ1) sites were pharmacologically identified by the binding ability of several chemically unrelated drugs with high affinity, including psychotomimetic benzomorphans, e.g. (+)-SKF-10,047 or (+)-pentazocine, the psychotomimetic drug phencyclidine, the psycho-stimulants cocaine, amphetamine and derivatives, certain neuroleptics, e.g. haloperidol, many new atypical antipsychotic agents, anticonvulsants, cytochrome P450 inhibitors, monoamine oxidase inhibitors, histaminergic receptor ligands, peptides from the neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) families, substance P and several neuroactive steroids (Walker *et al.*,1990; Maurice *et al.*, 1999, 2001). Initially the σ1/σ2 subtype classification was mostly based on radio ligand binding characteristics. Sigma1 (σ1) sites exhibited a stereoselectivity for dextrorotatory isomers of benzomorphans, whereas the levorotatory isomers as well as haloperidol or 1, 3-di-o-tolylguanidine (DTG) also bind to the σ2 sites (Hellewell *et al.*, 1994; Quirion *et al.*, 1992).

Distinction of the two σ sites was made possible by structure-activity relationship studies (Quirion *et al.,* 1992) and differences between the two sites are based on their different drug selectivity patterns and molecular weights. The σ1 receptor represents an identified protein, which was characterised after its cloning and a series of cellular biology studies (Su and Hayashi, 2003). It is a single polypeptide with a low molecular weight of 29 kDa (Hanner *et al.,* 1996; Kekuda *et al.,* 1996).

The receptor is a unique protein composed of 223 amino acids, highly conserved, with 87–92% identity and 90–93% homology among tissues and animal species. The protein sequence does not show homology with any classical neurotransmitter or neuropeptide receptor sequences, and its limited homology, with only a small number of proteins present in mammalian brain, outlines the uniqueness of the σ1 receptor when compared with any other known protein (Moebius *et al.,* 1993, Seth *et al.,* 1998).

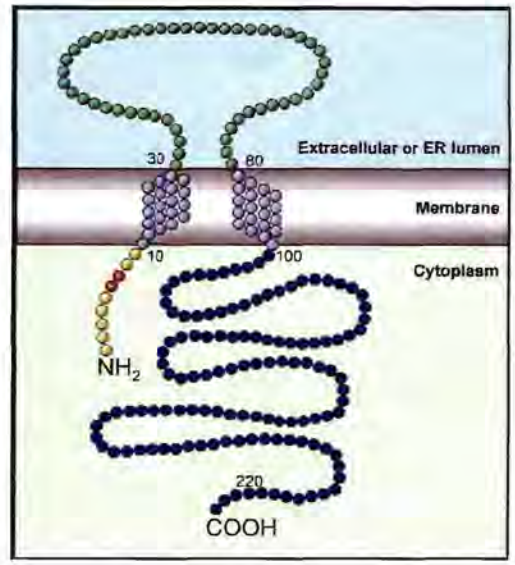



**Figure 5: Putative structure of the Sigma1 receptor (Aydar et al., 2004).**

According to Bowen (2000) σ2 receptors may be a part of a novel apoptotic pathway which could play a role in regulation of cell proliferation or cell development. This pathway consists of intracellular membrane bound σ2 receptors, localised on the endoplasmic reticulum and mitochondria, organelles known to store calcium and with the ability to cause release of calcium from these stores. Calcium signals may be utilised in normal cell signalling and /or for the induction of apoptosis.

Sigma 2 (σ2) receptor antagonists may be useful agents to lessen tardive dyskinesia which can result from chronic treatment of psychoses with typical antipsychotic drugs such as haloperidol and σ2 agonists may be useful as anti-neoplastic agents because they induced apoptosis in breast tumor cell lines which were resistant to the common DNA-damaging anti-neoplastics. Furthermore, σ2 receptor agonists potentiated the cytotoxic effects of these compounds at concentrations were the σ2 agonist was not cytotoxic. This, together with the fact that σ2 agonists appear to down-regulate expression of p-glycoprotein mRNA, suggests that activation of the σ2 receptor could have chemo sensitising effects. If considered in light of the use of sigma receptor ligands for non-invasive tumor imaging, it is clear that sigma receptors offer potential as targets for tools with which to fight cancer (Bowen, 2000).

Results from a study done by Prezzavento *et al.* (2006) suggests that activation of the apoptotic pathway, of which transglutaminase (TG-2) is part, are prevalently related to σ2 agonists. An increase in calcium ion influx activates several calcium-dependant proteins of

which tissue transglutaminase (TG-2) is one. This isoform of a family of transglutaminases catalyses the formation of ε-(γ-glutamyl)lysine cross links between polypeptide chains which results in polymerisation, the cross-linking of dissimilar proteins and the incorporation of diamines and polyamines into proteins (Lesort *et al.*, 2000). It is also part of cell processes such as cell differentiation, signal transduction, cell survival and wound healing. TG-2 is also expressed in the brain and is part of a variety of processes of the central and peripheral nervous systems (Lesort *et al.*, 2000). There are several lines of evidence suggesting that TG-2 activity may contribute to neurodegenerative diseases such as Huntington's, Alzheimer's and Parkinson's disease (Gentile and Cooper, 2004). Furthermore, TG-2 has a modulatory effect on apoptosis and cell response stressors, depending on the type op stimuli provoking an increase in transamidating activity (Tucholski and Johnson, 2002). Results further suggest that selective sigma ligands modulate intracellular calcium levels and eventually the up-regulation of TG-2 that is typical of several neurodegenerative diseases (Prezzavento *et al.*, 2006).

### 2.3.2 Structure - activity relationships

Compounds with 4-phenylpiperidine-4-ol and 4-benzylpiperidine moieties showed high affinity for σ-receptors. Structures can be viewed in figure 6. A N-substituent on the compound is also important for it to be an amino pharmacophore on σ-receptors, especially on σ1-receptors, and prevent interaction with other receptors.

Electron deficient systems such as 1-(pyridine-2-yl)piperazine and 1-phenyl-cyclopropyl methyl carboxylate , when linked, appear to be an unfavourable combination for σ1 and σ2 receptors (Prezzevanto *et al.*, 2006). In the case of butryphenone, the presence of both a 4-linked phenyl and an electron negative moiety, at position 1 on the butyl chain, is required for high affinity binding to σ1-receptors (Shetz *et al.*, 2007).

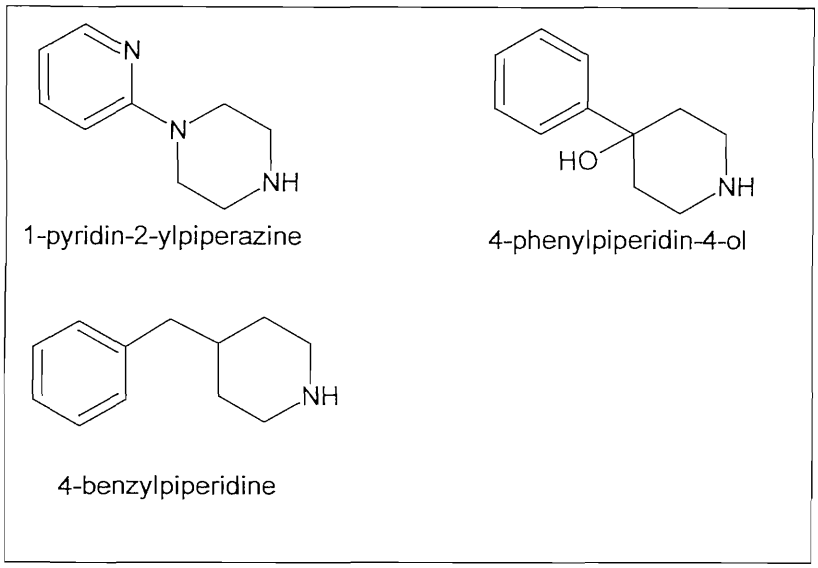
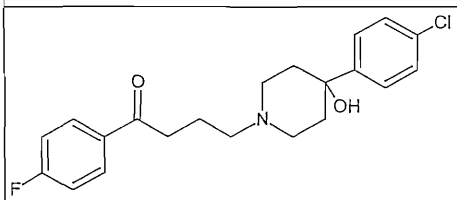
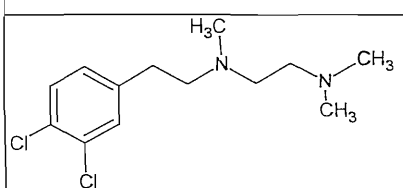
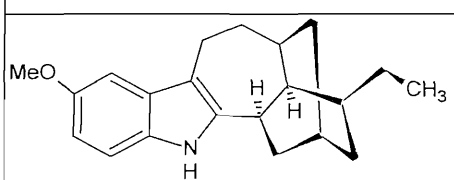
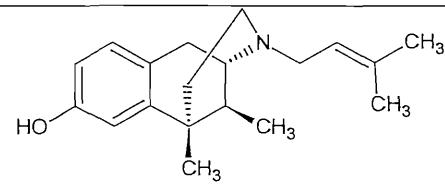



**Figure 6: Structures of 1-pyridin-2-ylpiperazine, 4-phenylpiperidin-4-ol and 4-benzylpiperidine**

**Table 1:** Structures with binding affinity to σ-receptors (Shetz *et al.*, 2007).

| Structure | Compound | $\sigma_1$ activity | $\sigma_2$ activity |
|---|---|---|---|
|  | Haloperidol | Antagonist | Agonist |
|  | BD1047 | Antagonist/partial agonist | Agonist |
|  | Ibogaine | None | agonist |

| | | | |
|---|---|---|---|
|  | (+)pentazocine | Agonist | None |

Nguyen *et al.* (1996) reported a small series of pentacycloundecane derivatives, as seen in figure 7, which showed high affinity for σ - binding sites and no cross-reactivity with other binding sites and receptors such as dopamine, opoid, phencyclidine, NMDA and serotonin. They determined the selectivity of these compounds for σ1 and σ2 receptors, but did not look into the possible neuroprotective activity they may have by modulating $Ca^{2+}$ influx.

Their data revealed that all the compounds displayed moderate to high affinity for σ1 and σ2 binding sites. Selectivity and affinity for the two subtypes were affected by various structural features. All the pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecylamines screened (compounds ANSTO 1-5), contain a secondary amine and ketal functionality and displayed preferential selectivity for the σ1 sites. The most potent binding was exhibited by ANSTO-2 which is substituted in the *meta* position of the aromatic ring with bromine ($K_i$ = 17.0 nM) while ANSTO-5 resulting from extension of the linker between the unsubstituted aromatic ring and the amine functionality by one carbon displayed equal affinity ($K_i$ = 15.0 nM). The σ1/σ2 ratios for ANSTO-2 and ANSTO-5 were 12 and 40, respectively (Nguyen *et al.* 1996). The 4-azahexacyclo[5.4.1.$0^{2,6}$.$0^{03,10}$.$0^{5,9}$.$0^{8,11}$]dodecane derivatives contain a tertiary amine and hydroxyl functionality. This series displayed variable binding to the σ1 and σ2 sites. Compound ANSTO-14 displayed the highest affinity for the σ1 site ($K_i$ = 9.4 nM). This structure was a result of increasing the alkyl chain between the cubane moiety and the aromatic ring in the series of compounds ANSTO-6, 10, 13, 14. Compounds (ANSTO-6, 7, 16-19) were potent at the σ2 site with affinity in the range of 107.6-19.6 nM. All compounds were substituted in the *meta* position of the aromatic ring, as this appears important for σ2 binding, with differences in the type of substitution. The order of highest to lowest affinity was F > Cl > Br > I > H > $CH_3$ (Nguyen *et al.* 1996).

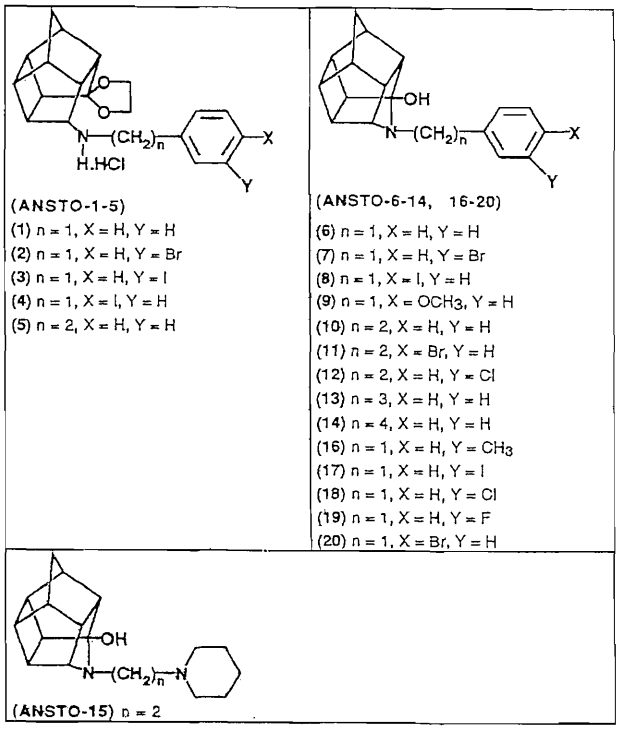
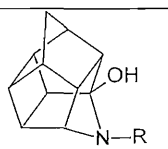
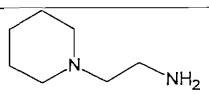
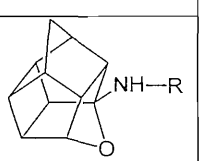
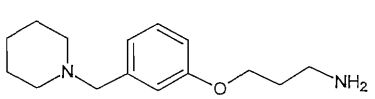



Figure 7: Series of pentacycloundecane derivatives tested for sigma binding activity by Nguyen *et al.* (1996)

### 2.3.3 Conclusion and selection of compounds for synthesis

After consideration of the structural requirements for sigma receptor binding, the following compounds were selected for synthesis in this study.

**Table 2:** Proposed series to be synthesised

| Compounds | R= |
|---|---|
|  |  |
|  |  |

The compounds differ in that one series is the aza- and the other the oxa- form of the pentacycloundecylamine. The linkers both contain the N-substituent but have different chain lengths and one also has an aromatic component. The reason for this was to compare the effect of the aza vs. oxa-structure, chain length and the presence of the aromatic moiety on sigma binding and intracellular calcium flux.

# Chapter 3

# Experimental: Synthesis

## 3 Introduction

The well known Cooksen's diketone was synthesised and linked to selected side chains through reductive amination. The side chains had different chain lengths and structural moieties which were selected to be sigma receptor specific.

### 3.1 Standard Experimental Procedures: Instrumentation and techniques

#### 3.1.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

$^{1}H$ and $^{13}C$ NMR spectra were obtained using a Varian Gemini 300 spectrometer at frequency of 300.075 MHz and 75.462 MHz, respectively. This was done in a 7 Tesla magnetic field and tetramethylsilane (TMS) was used as internal standard. A bandwidth of 1000 MHz at 24 kG was applied for $^{1}H$ and $^{13}C$ decoupling. All chemical shifts are reported in parts per million (ppm) relative to the signal from TMS ($\delta$ = 0), added to an appropriate deuterated solvent. The following abbreviations are used to describe the multiplicity of the respective signals: s-singulet, bs-broad singulet, d-doublet, dd-doublets of doublets, t-triplet, q-quintetand m-multiplet.

#### 3.1.2 Mass Spectroscopy (MS)

An analytical VG 7070E mass spectrometer was used to record mass spectra with fast atom bombardment (FAB) or electron impact (EI) at 70 eV as ionization techniques.

#### 3.1.3 Infrared Spectroscopy (IR)

IR spectra were recorded on Nicolet Magna- IR 550 spectrometer. Samples were applied either as film or incorporated in KBr pellets.

#### 3.1.4 Melting Point (MP) Determination

Melting points were determined using Gallenkamp-melting point apparatus in capillary tubes.

#### 3.1.5 Thin Layer Chromatography (TLC)

Analytical TLC was performed on 0.20 mm thick aluminium silica gel sheets (Alugram SIL $G/UV_{254}$, Kiesegel 60, Macherey-Nagel, Düren, Germany). Visualisation was achieved using

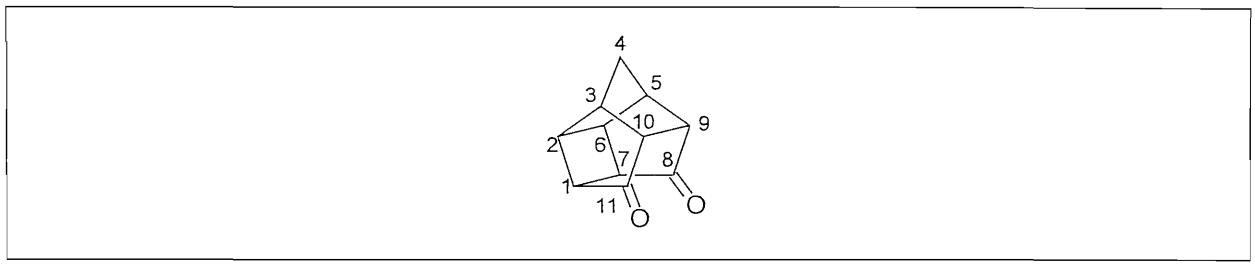
UV light (254nm), a spray agent (containing ninhydrin in ethanol) or iodine vapours, with mobile phases indicated for each compound.

### 3.1.6 Column Chromatography

Compounds were purified using a standard glass column. The stationary phase used was silica gel (0.063-0.00 mm/70-230 mesh ASTM, Macherey-Nagel, Düren, Germany) with mobile phases as indicated for each compound.

## 3.2 Synthesis of Selected compounds

### 3.2.1 Synthesis of Cooksons' diketone




This well described pentacyclo[5.4.0.$0^{2,6}$.$0^{3,1}$.$0^{5,9}$]undecane-8-11-dione was synthesised according to the published method (Cooksen *et al.*, 1958,1964). The photocyclisation of the endo conformation Diels-Alder adduct (3) of *p*-benzoquinone (1) and cyclopentadiene (2) yielded the pentacylic cage compound derivative pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (4). This was the starting material for the syntheses of the proposed series of compounds. A schematic representation of the synthesis is given in figure 8.

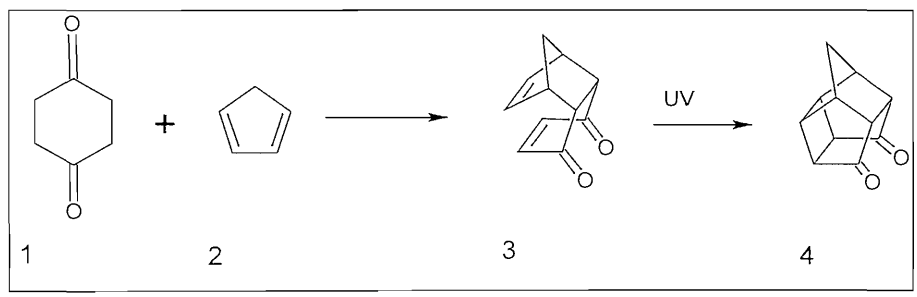



Figure 8: Syntheses of pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione

### 3.2.2 Synthesis of N-[3-(3-piperidin-1-ylmethylphenoxy)propyl]amine (Buschauer *et al.*, 1985) (A2.2)

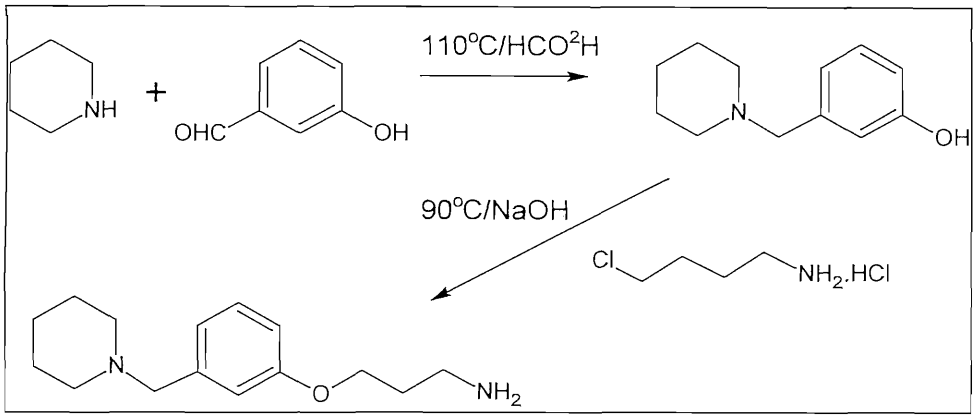
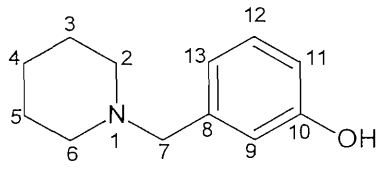



Figure 9: Synthesis of N-[3-(3-piperidin-1-ylmethylphenoxy)propyl]amine (Buschauer *et al.*, 1985) (A2.2)

This was a two step synthesis. 3-(1-Piperidinemethyl) phenol **(A1)**, was synthesised by adding 40 ml piperidine to 24 g of 3-hydroxybenzaldehyde. The reaction was stirred in ice-bath and the temperature was kept under 60 °C while 10 ml formic acid was added drop-wise with great caution. The reaction was then stirred for 2 hours at 110 °C. After cooling the mixture to 15 °C, 100 ml distilled water was added and the mixture was stirred vigorously. The solution was made alkaline with 32 % ammonium solution. On standing, the product crystallises, is filtered and washed with water. (Yield: 27.8 g, 145, 3 mmol, 73.96 %). Its physical data corresponds to literature (Buschauer *et al.*, 1985)

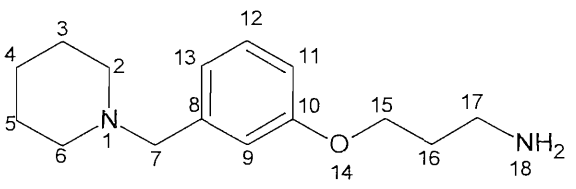



***Physical data***: $C_{12}H_{17}NO$; **mp**: 136-138 °C; MS (EI, 70 eV) *m/z* (Spectrum 1): 192.2 ($M^+$), 107.0, 94.1, 77.3, 53.1; **IR** (KBr) $\nu_{max}$ (Spectrum 7) 3019-2342.6, 3019.7, 2954.2, 2360.2, 2342.6, 1581.6, 1481.8, 1285.4, 1247.2 $cm^{-1}$; **$^1$H NMR** (300 MHz, $CDCl_3$) $\delta_H$ (Spectrum 14): 7.27 (s, 1H, H-9), 7.12 (t, 1H, *J* = 7.751 Hz, H-12), 6.70 (dd, 2H, *J* = 7.917 Hz, H-11,13), 2.4:2.5 (bs, OH), 1.57 (q, 2H, *J* = 5.705 Hz, H-4a, 4b), 1.4 (m, 4H, H-3a, 3b, 5a, 5b); **$^{13}$C NMR** (75 MHz, $CDCl_3$) δc (Spectrum 13): 160 (d, 1C), 138,5 (d, 1C), 129 (d, 1C), 121 (d, 1C), 115 (s, 1C), 63 (t, C-7), 54 (t, C-2/6), 25 (t, C-3/5), 23 (C-4).

***Structure elucidation***

Distinctive signals of this compound were the doublets of doublets of H-11, 13 ($\delta_H$ 6.70) and the triplet of H-12 ($\delta_H$ 7.12) in the aromatic region. These protons had the same coupling constant indicating that they were on adjacent carbons in the aromatic ring. The broad singlet of the hydroxyl group at $\delta_H$ 2.4 were also easily observed. Another distinctive signal was that of the quintet of protons 4a and 4b. This signal was observed up field ($\delta_H$ 1.57) due to its electron rich, saturated environment.

N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine was synthesised by adding 50 ml of dimethylformamide (DMF) to 5 g of 3-(1-iperidinemethyl)phenol, 4.27 g 3-chloropropylamine and 14.5 g sodium hydroxide pellets. The reaction was stirred at 80-90 °C for 2 hours after which it was cooled to 15 °C and the mixture filtered. DMF was evaporated under vacuum and the residue diluted with 100 ml distilled water. The solution was extracted four times with 25 ml dichloromethane, dried with magnesium sulphate and filtered. The product is obtained as an oily substance after evaporation of the solvent. (Yield: 4.5 g, 18.10 mmol, 69.08 %). Its physical data corresponds to the literature (Buschauer *et al.*, 1985)




***Physical data***: $C_{15}H_{24}N_2O$, **MS** (EI, 70 eV) *m/z* (Spectrum 2) 249.0 ($M^+$), 189.9, 147.2, 120.9, 107.0, 98.2, 91.0, 84.1, 65.2, 56.1; **IR** (KBr) $\nu_{max}$ (Spectrum 8) 3036.2-2932.2, 2852.4, 27951.1, 2754.3, 1584.3 1487.0, 1259.2, 1156.3 $cm^{-1}$; **$^1$H NMR** (300 MHz, $CDCl_3$) $\delta_H$ (Spectrum 16): 8.0 (s, 1H, H-9), 7.16 (t, 1H, *J* = 7.811 Hz , H-12), 6.86 (m, 2H, H-17a, 17b), 6.76 (dd, 1H, *J* = 8.02 Hz, H-13/11), 4.0 (t, 2H, *J* = 6.1 Hz , H-15a, 15b), 3.5 (s, 2H, H-7a, 7b), 2.85 (m, 4H, H-3a, 3b, 5a, 5b), 2.2-2.4(2 x bs, $NH_2$), 1.9 (q, 2H, *J* = 6.413 Hz, H-4a, 4b), 1.5 (q, 4H, *J* = 5.643 Hz , H-2a, 2b, 6a, 6b); **$^{13}$C NMR** (75 MHz, $CDCl_3$) $\delta_C$ (Spectrum 15): 160 (d,1C), 140 (d, 1C), 129 (d, 1C), 121 (d, 1C), 115 (s, 1C), 113 (s, 1C), 66 (t, C-15), 63.5 (t, C-7), 54 (t, C-17), 39 (t, C2/6), 36.5 (t, C6/12), 33 (t, 1C), 31.5 (t, 1C), 26 (t, 1C), 24 (t, 1C).

*Structure elucidation*

The same distinctive proton signals as for **A1**, except for the hydroxyl group, were observed. What discerned this structure from **A1** was the $NH_2$ signal at $\delta_H$ 2.2 and the absence of a broad OH-signal on the IR spectrum.

### 3.2.3 Synthesis of pentacycloundecane derivatives

#### 3.2.3.1 General approach

Reductive amination was used to respectively link 1-(2-Aminoethyl)piperidine and N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine to pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione. The reaction was done in tetrahydrofurane (THF) at -10 °C. The carbinolamine that formed was filtered of and refluxed under dehydrating conditions (Dean-Stark) for 1 hour in benzene. The benzene was evaporated under vacuum and the residue was dissolved in dry methanol and THF. $NaBH_4$ or $NaBH_3CN$ was added as reducing agent. The desired product was acquired after an extraction with dichloromethane (DCM) and column chromatography.

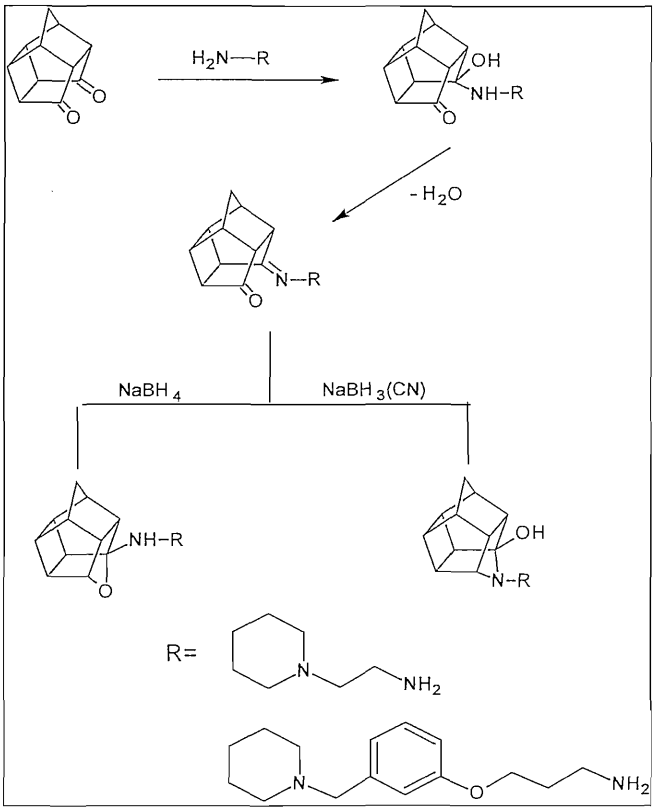
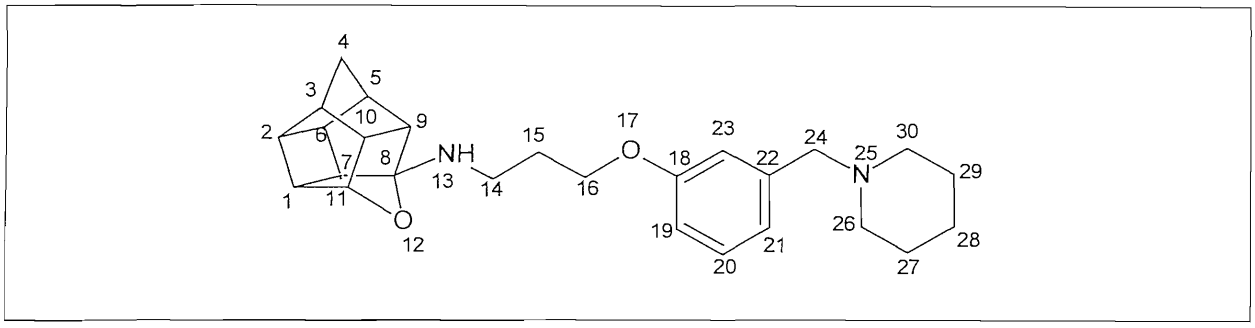



Figure 10: General synthesis route of pentacycloundecane derivatives

### 3.2.3.2. Synthesis of 8-{N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine}-8-11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane. (A3)



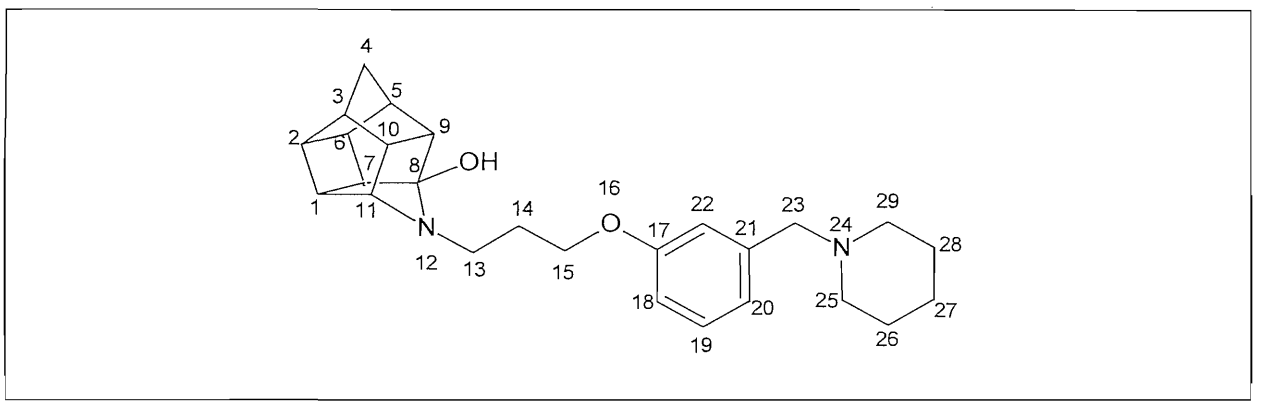

1.4 g (8.036 mmol) pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione was reacted with 1.7 g (8.036 mmol) N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine in 10 ml dry THF at -10 °C. The carbinolamine that formed after 10 min was filtered off and refluxed under dehydrating conditions (Dean-Stark) for 1 hour in benzene. The benzene was evaporated under vacuum and the residue was dissolved in 6 ml dry methanol and 30 ml dry THF. To this 0.3 g $NaBH_4$ was added as reducing agent. The mixture was stirred overnight at room temperature. The methanol and THF were evaporated under vacuum and the residue was extracted with 4 x 25 ml dichloromethane (DCM) and the organic fraction was dried with magnesium sulphate, filtered and evaporated. The desired product was obtained as an oily substance after column chromatography. (Yield: 1.313 g, 2.7957 mmol, 34.90 %).

***Physical data***: $C_{26}H_{32}N_2O_2$,, **MS** (EI, 70 eV) *m/z* (Spectrum 3) 405.2 ($M^+$), 373.2, 353.3, 249.0, 229.1, 204.1,198.9, 192.2, 177.2, 131.1, 91.2; **IR** (KBr) $\nu_{max}$ (spectrum 9) 3357.2, 2964.7, 2863.9, 1706.0, 1211.2, 1131.8 $cm^{-1}$; **$^1$H NMR** (300 MHz, $CDCl_3$) $\delta_H$ (Spectrum 18): 7.26 (s, 1H, H-23), 7.20 (t, 1H, *J* = 7.809 Hz, H-20), 6.74 (dd, 2H, *J* = 8.057 Hz, H-19, 21), 4.02 (q, 4H, *J* = 5.605 Hz, H-26a, 26b 30a, 30b,), 3.9 (t, 1H, H-11), 3.7 (t, 2H, *J* = 5.066 Hz, H-16a, 16b), 2.55-3.42 (m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 2.3 (bs, 1H, NH, H-13), 2.1 (q, 2H, *J* = 6.31 Hz, H-28a, 28b), 1.94 (s, 1H, H-24), 1.87 ( AB-q, 2H, *J* = 11.467 Hz, H-4a, 4b), 1.53 (m, 4H, H-27a, 27b, 29a, 29b); **$^{13}$C NMR** (75 MHz, $CDCl_3$) $\delta_C$ (Spectrum 17): 159 (s, 1C), 140 (d, 1C), 128 (d,1C), 122 (d,1C), 115 (d,1C), 112 (s,C-18), 65.2 (d, C-11), 64.9 (s, C-8), 64 (t, C-16), 54.9 (t, C-14), 54.8 (d, C-24), 54.6 (t, C-26/30), 54.0 (t, C-30/26), 46.0 (d, 1C), 44.5 (d, C-3), 43.9 (d, 1C), 43.5 (t, C-4), 43 (d, 1C) 42.5 (d, 1C), 42.2 (d, 1C), 38.8 (d, C-7/9), 41.5 (d, C-9/7), 26.8 (t, 1C), 26.2 (t, 1C), 26.2 (t, 1C), 26 (t, 1C), 24.2 (t, 1C).

***Structure elucidation***

This compound had distinctive proton signals. The doublet of doublets were observed for H-19, 21 ($\delta_H$ 6.74) and the triplet of H-20 ($\delta_H$ 7.20) in the aromatic region. The broad singlet of the NH proton at $\delta_H$ 2.3 were observed in the midfield as a result of the electron withdrawing effect of the neighbouring nitrogen atom. The quintets of H-26, 30 and 28 were observed up field as they are linked to saturated carbons. These protons showed mutual scalar coupling with H-16 implicating steric freedom around the oxygen at position 17 bringing the protons of H-26, 30 and 28 in close proximity to the protons of H-16. Obligatory signals for the cage such as the AB-q of H-4 ($\delta_H$ 1.87) and the triplet of H-11($\delta_H$ 3.9) and the prominent NH-signal on the IR spectrum were also observed.

### 3.2.3.3. Synthesis of 8-{N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine}-8-11-azapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane. (A4)




The same synthesis route as described above was used except that 0.3 g $NaBH_3CN$ was used as reducing agent to yield the aza-derivative. (Yield: 0.432 g, 1.0679 mmol, 13.28 %).

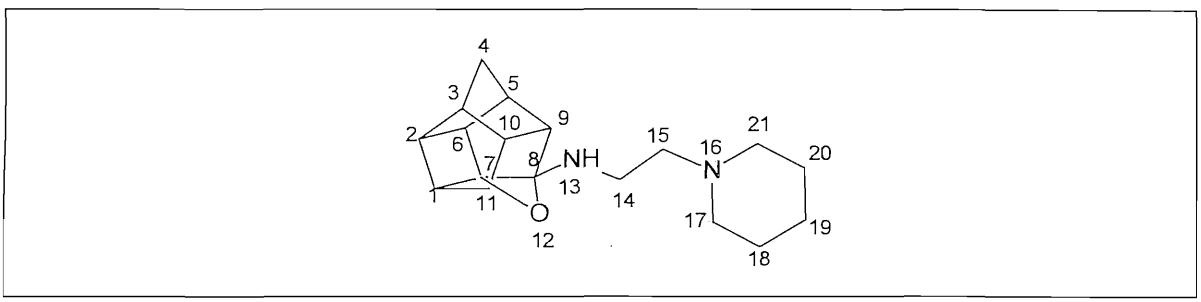
***Physical data***: $C_{26}H_{32}N_2O_2$,, oily substance, **MS** (EI, 70 eV) *m/z* (Spectrum 4) 407.2 ($M^+$) 405.2, 379.2, 373.0, 249.1, 229.0 204.1, 198.7 192.4, 177.3, 131.2, 91.3 ; **IR** (KBr) $\nu_{max}$ (Spectrum 10) 3342.8, 2969.1, 2865.4, 1731.0, 1337.7, 1274.1, 1098.0, 1557.9 cm-$^1$; **$^1$H NMR** (300 MHz, $CDCl_3$) $\delta_H$ (Spectrum 20): 7.26 (s, 1H, H-22), 7.20 (t, 1H, *J* = 7.877 Hz, H-19), 6.8 (dd, 2H, *J* = 8.21 Hz, H-18, 20), 4.02 (q, 4H, *J* = 5.481, H-25a, 25b, 29a, 29b), 3.9 (t, 1H, H-11), 3.7 (t, 2H, *J* = 5.052 Hz, H-15a, 15b), 2.55-3.42 (m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 2.2 (bs, 1H, OH), 2.1 (q, 2H, *J* = 6.414 Hz, H-27a, 27b), 1.94 (s, 1H, J =, H-23), 1.6 ( AB-q, 2H, *J* = 11.319 Hz, H-4a, 4b), 1.53 (m, 4H, H-26a, 26b 28a, 28b,); **$^{13}$C NMR** (75 MHz, $CDCl_3$) $\delta_C$ (Spectrum 19): 159 (s, 1C), 140 (d, 1C), 128 (d,1C), 122 (d,1C), 115 (d, 1C), 112 (s, C-17), 65.2 (d, C-11), 64.9 (s, C-8), 64 (t, C-13), 54.9 (t, C-14), 54.9 (d, C-23), 54.6 (t, C-25/29), 54.0 (t, C-29/25), 46.0 (d, 1C), 44.5 (d, C-3), 43.9 (d, 1C), 43.5 (t, C-4), 43 (d, 1C)

42.5 (d, 1C), 42 (d, 1C), 41.5 (d, C-9/7), 38.8 (d, C-7/9), 26.8 (t, 1C), 26.2 (t, 1C), 26.2 (t, 1C), 26 (t, 1C), 24.2 (t, 1C).

***Structure elucidation***

Proton signals similar to that of **A3** were observed for H18, 20 and 19 with similar scalar coupling. The same scalar coupling and steric freedom as with **A4** were also observed for protons H-25, 29 and 15 together with the obligatory cage signals. What discerned this compound from **A4** was the broad OH singlet at $\delta_H$ 2.2 and the broad OH-signal on the IR spectrum.

### 3.2.3.4. Synthesis of 8-{1-(2-Aminoethyl)piperidine}-8-11-oxapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane. (C1)




1.0 g (8.036 mmol) pentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane-8,11-dione was reacted with 1 ml 1-(2-aminoethyl)piperidine (5.740 mmol) in 10 ml dry THF at -10 °C. The carbinolamine that formed was filtered off and refluxed under dehydrating conditions (Dean-Stark) for 1 hour in benzene. The benzene was evaporated under vacuum and the residue was dissolved in 6 ml dry methanol, 30 ml dry THF and 0.3 g $NaBH_4$ was added as reducing agent and the mixture was stirred overnight at room temperature. The methanol and THF were evaporated under vacuum and the residue was extracted with 4 x 25 ml dichloromethane (DCM), dried with magnesium sulphate, filtered and evaporated. The desired product was obtained as an oily substance after column chromatography. (Yield: 0.7 g, 2.33 mmol, 40.60 %).

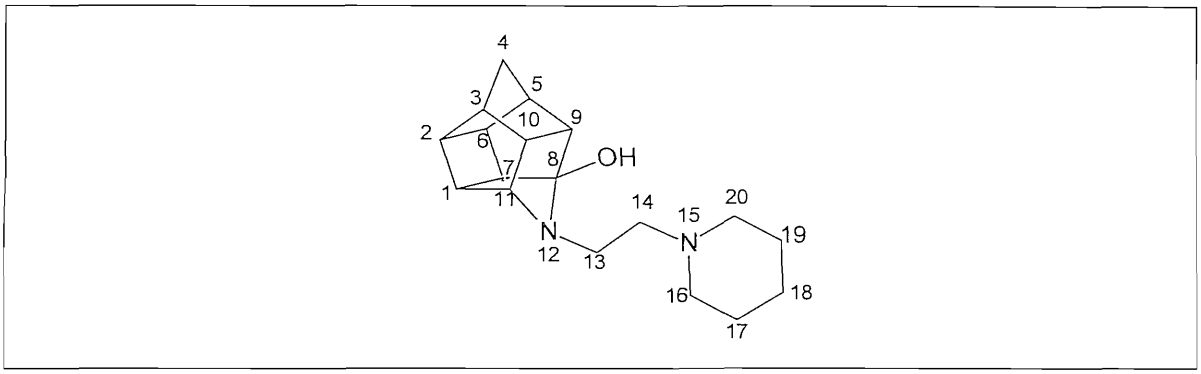
***Physical data***: $C_{18}H_{24}N_2O$, **MS** (EI, 70 eV) *m/z*( Spectrum 5) 287.2 ($M^+$), 269.0, 227.3, 216.1, 204.1, 201.9, 198.9, 186.9, 186.1, 112.2, 69.1, 56.1 **IR** (KBr) $\nu_{max}$ (Spectrum 11) 3317.7, 2930.7, 2856.4, 2803.4, 1731.8, 1667.4 $cm^{-1}$; $^1$**H NMR** (600 MHz, $CDCl_3$) $\delta_H$ (Spectrum 22): 4.61 (t, 1H, *J* = 5.224 Hz, H-11), 3.8 (bs, 1H, NH, H-13), 2.50-2.90 (m, 10H, H-1, 2, 3, 5 ,6, 7, 9, 10, 14a, 14b), 2.45 (t, 2H, *J* = 6.355 Hz , H-15), 1.87 (AB-q, 2H, *J* = 10.407 Hz, H- 4a, 4b), 1.5 (m, 4H, H-18a, 18b, 20a, 20b), 1.21 (q, 2H, *J* = 7.02 Hz , H-19a, 19b); $^{13}$**C NMR** (75 MHz, $CDCl_3$) $\delta_C$ (Spectrum 21): 71.9 (d, C-11), 71.8 (s, C-8), 59.1(t, C-

14), 58.1 (t, C-15), 54.9 (t, C-17/21), 54.8 (t, C-21/17), 45 (d, C-3), 44.8 (t, C-4), 43 (d, C-7), 42.6 (d, C-9), 40 (d, 1C), 35.2 (d, 1C), 34.4 (d, 1C), 30.5 (d, 1C), 26 (t, 1C), 24.6 (t, 1C), 18.6 (t, 1C).

***Structure elucidation***

The obligatory signals of the AB-q of H-4 and the triplet of H-11 were observed on the $^1H$ NMR. Distinct signals for this compound were the broad NH singlet at $\delta_H$ 3.8, the triplet of H-15 ($\delta_H$ 2.45), the quintet of H-19 and the NH-signal on the IR spectrum.

### 3.2.3.5. Synthesis of 8-{1-(2-Aminoethyl)piperidine}-8-11-azapenta-cyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane. (C2)




The same synthesis route as described above was used except that 0.3g $NaBH_3CN$ was used as reducing agent to yield the aza-derivative (Yield: 0,3 g, 1.00 mmol, 17.42 %).

***Physical data:*** $C_{18}H_{24}N_2O$, yellow oily substance **HR- MS** calc.284.39596; **MS** (EI, 70 eV) *m/z* (Spectrum 6) 287.2 ($M^+$), 269.0, 255.0, 249.0, 233.0, 216.9, 204.1, 199.1, 201.0, 192.2, 187.0112.3, 87.0, 72.9, 55.4; **IR** (KBr) $\nu_{max}$ (Spectrum 12) >3000, 2938.1, 2860.3, 2324.8, 2168.4 $cm^{-1}$; **$^1$H NMR** (600 MHz, $CDCl_3$) $\delta_H$ (Spectrum 24): 5.2 (bs, 1H, OH), 4.10 (t, 1H, *J* = 5.251 Hz, H-11), 1.08:1.60 (AB-q, 2H, *J* = 10.40 Hz, H-4a, 4b), 2.10-2.80 (m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 0.80-1.40 (m, 6H, H-18a, 18b, 19a, 19b, 20a, 20b);**$^{13}$C NMR** (75 MHz, $CDCl_3$) $\delta_C$ (Spectrum 23): 72 (s, C-8), 71.8 (d, C-11), 69 (t, C13), 59 (t, C-14), 55 (t, C-16/20), 47 (d, C-3), 46 (t, C-4), 26.5 (d, 1C), 26 (d, 1C), 24 (t, 1C), 23.5 (t, 1C) 23 (t, 1C).

*Structure elucidation*

Compound **C2** was discerned from **C1** on the 1H NMR spectrum with the following: A broad OH singlet was observed at $\delta_H$ 5.2. The rest of the signals were similar to that of **C1.**

## 3.3 Conclusion

Reductive amination was used to respectively link 1-(2-Aminoethyl)piperidine and N-{3-[3-(1-piperidinylmethyl)phenoxylpropyl}amine to pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]-undecane-8,11-dione to form oxa- and aza derivatives. The structures of the compounds were confirmed by $^{1}H$ and $^{13}C$ NMR, MS and IR and the spectral data can be viewed in Annexure A. The yields varied from 13 % - 73 %. Low yields may be optimised by improving experimental conditions and purification techniques.

# Chapter 4:

# Experimental: Biological evaluation

## 4 Introduction

As described in chapter two, sigma receptors are seen as good targets for neuroprotection through their involvement in calcium regulation. A calcium fluorescence assay using Fura-2/AM was used to determine the effect of the compounds on calcium flux and a radio ligand binding study with [$^3$H]-DTG was utilised to evaluate whether the test compounds had affinity for sigma receptors.

### 4.1 Standard Experimental Procedures

#### 4.1.1 Instrumentation

##### 4.1.1.1 Spectrofluorometry

A Varian Cary Eclipse Fluorescence spectrophotometer was used to record the percentage fluorescence during the calium flourescence assay.

##### 4.1.1.2 Radioactive scintillation count

Tri-Carb TR2100 Scintillation counter was used for scintillation count of radio ligand binding studies.

#### 4.1.2 Bradford protein concentration determination

Bradford reagent (purchased from Sigma-Aldrich) was withdrawn (8 ml) in a dark room, and allowed to reach 37°C in a water bath. The reagent was protected from light at all times. A BSA stock solution of 2 mg/ml was prepared by dissolving 2 mg BSA in 1ml reaction buffer. Protein standards were prepared as described in table 3. Protein standard (2 x 5 µl) and 3 x 5 µl of membrane suspension were added to separate wells of a 96- well plate. 250 µl Bradford reagent was then added to each well. The well was placed on the shaking facility of the plate reader for 30 seconds and incubated for 15 min at room temperature. The absorbance of the samples at 560 nm was determined and the protein concentration of the samples calculated.

**Table 3:** Preparation of protein standards

| Protein concentration | Dilution in test tubes | |
|---|---|---|
| mg/ml | Volume of BSA 2mg/ml<br><br>µl | Volume Buffer<br><br>µl |
| 0 | 0 | 100 |
| 0.1 | 5 | 95 |
| 0.4 | 20 | 80 |
| 0.7 | 35 | 65 |
| 1.0 | 50 | 50 |
| 1.4 | 70 | 30 |

## 4.2 $Ca^{2+}$ Measurement with Fura-2/AM using synaptoneurosomes

### 4.2.1 Background

Methods for the absolute measurement of total cellular $Ca^{2+}$ generally involve the destruction of tissue and liberation of bound $Ca^{2+}$ (Campbell, 1983). Tissue can be ashed or extracted with acid and the $Ca^{2+}$ in the ash or extract determined by atomic absorption spectrophotometry, which measures the characteristic absorptions of vaporised calcium ions at extremely high temperatures in a flame or graphite furnace (Sansui and Ruben, 1982). Another method is the incubation of tissue with radioactive $Ca^{2+}$ until tracer equilibrium is reached. The total cellular $Ca^{2+}$ is then determined with scintillation counting of the total radioactivity and specific activity. These methods are not frequently used anymore, perhaps because cells contain large quantities of statically bound $Ca^{2+}$. Therefore, the changes in total $Ca^{2+}$ that accompany signal transduction are usually buried in the experimental error, which includes variations in the amount of tissue in each successive sample.

More practical and common methods are those that measure the changes in total $Ca^{2+}$ as influx or efflux of $Ca^{2+}$ across the plasma membrane. When $^{45}Ca^{2+}$ is included in the bathing medium, unidirectional influxes can be measured as initial rates of uptake of the isotropic tracer. Sequential samples of tissue must be taken and subjected to scintillation counting. The main technical problem however, is how to rapidly wash away the large amount of radioactivity bound to the exterior of the cells without letting a significant amount of

intracellular calcium escape. Unidirectional effluxes may be measured by pre-labelling the tissue to isotopic equilibrium, then counting the radioactivity released back into successive samples of supernatant medium. These techniques were popular because they require no specialised equipment or reagents but are less used today because they demand skilled repetitive manipulation of samples containing a short lived hazardous isotope and their spatial and temporal resolution is poor (Miller and Korenbrot, 1987; Tepikin *et al.*, 1994; Belan *et al.*, 1994).

Calcium that flows through channels with known ionic selectivity can be measured in terms of the electrical current flowing through the channel. This method measures netto fluxes of $Ca^{2+}$. In small non excitable cells however, the currents associated with important $Ca^{2+}$ influxes and effluxes are often diminutive and difficult to measure (Miller and Korenbrot, 1987; Tepikin *et al.*, 1994; Belan *et al.*, 1994).

In other methods, the changes in extracellular free $Ca^{2+}$ outside the cell are measured. Increases and decreases in this $[Ca^{2+}]$ show a netto cellular extrusion and uptake respectively. Extracellular concentration changes can be detected by low affinity $Ca^{2+}$ indicators or $Ca^{2+}$ selective electrodes. The fractional changes in extracellular free $Ca^{2+}$ concentration is small, but it can be increased by lowering the background level of $Ca^{2+}$ so that the cellular fluxes cause bigger percentage changes, though such low $Ca^{2+}$ media are likely to depress $Ca^{2+}$ influxes. Decreasing the volume of extracellular medium being sampled by pressing cells against a $Ca^{2+}$ selective electrode or dispersing them in aqueous micro droplets under oil, is also necessary (Miller and Korenbrot, 1987; Tepikin *et al.*, 1994; Belan *et al.*, 1994).

Loading the cell with a fluorescent indicator at a concentration high enough for it to become the dominant $Ca^{2+}$ buffer and to keep the intracellular free $Ca^{2+}$ nearly constant, is a complementary method. Most of the $Ca^{2+}$ entering or leaving the cell binds to or comes from the dominant buffer, which optically reports the amount of $Ca^{2+}$ it has bound (Tsien *et al.*, 1982; Tsien and Rink, 1983). This is also the method used in this study. The fluorescent indicator used was fura-2/AM.

Fura-2 is one of the most popular $Ca^{2+}$ indicators (Grynkiewicz et al., 1985; Tsien, 1989a) because it combines convenient excitation rationing, fairly good photostabilty, and relatively easy loading *via* its acetoxymethyl ester, fura-2/AM. This made it the first indicator that could be readily imaged at the single cell level (Tsien and Poenie, 1986). The name "fura-2" reflects it origin as the second member of a family of indicators containing benzofuran groups. Free fura-2 has an excitation peak at 362 nm which shifts to 335 nm and increases

in amplitude upon binding to $Ca^{2+}$. The $K_d$ for $Ca^{2+}$ is 135 nM in 100 mM KCl at 20 °C *vs* 224 nM in buffer-simulating mammalian cytoplasm at 37 °C. The emission peak, at 518 nm for free dye, shifts to 510 when $Ca^{2+}$ binds (Grynkiewicz *et al.*, 1985), probably because in the excited state the amino group disengages from the $Ca^{2+}$.

### 4.2.2 Materials and Methods

Procedures similar to those of published studies were used to prepare the synaptoneurosomes and solutions for experimental measurement of fluorescence (Bezuidenhout, 2000). Assay protocols were approved by the Ethics and Research Committee of the North-West University.

#### 4.2.2.1 Preparation of synaptoneurosomes

Eight day old Sprague-Dawley rats of either sex were used. Rats were sacrificed by decapitation, and whole brains were removed. Whole-brain synaptoneurosomes were prepared by the techniques of Bloomquist *et al* (1995), slightly modified.

The brains from 4 rats were homogenised in 30 ml of ice-cold Krebs-bicarbonate buffer (NaCl, 118 mM; KCl, 4.7 mM; $MgCl_2$, 1.18 mM; $CaCl_2$, 1.2 mM; $NaHCO_3$, 24.9 mM; $KH_2PO_4$, 1.2 mM; and glucose, 10 mM). The homogenate was kept ice-cold at all times to minimize proteolysis throughout the isolation procedure. The tissue suspension was centrifuged at 0 °C for 15 min at 1100 g. The pellet was resuspended in 30 ml fresh incubation buffer and then centrifuged again for 15 min at 1100 g and 0 °C. The final pellet was gently resuspended in Krebs-bicarbonate buffer to a protein concentration of 3 mg/ml (measured with Bradford method spectrofluorimetrically; Bradford, 1976).

#### 4.2.2.2 Loading synaptoneurosomes with calcium-sensitive Fluorescent indicator (Fura-2 AM)

The suspension prepared in 4.2.2.1 was allowed to reach room temperature, where after Fura-2 AM (2.5 ml of a 5 mM in dimethylsulfoxide – protect solution from light) was added to a final concentration of 5 µM. Synaptoneurosomes were then incubated at 37 °C for 10 min, diluted with Krebs-bicarbonate buffer (room temperature) to a final concentration of 0.6 mg/ml, and kept at room temperature until used – from this stage protect from light, preferably work in dark room.

#### 4.2.2.3 Incubating synaptoneurosomes with compounds

Immediately before the experiment, 1 ml of the synaptoneurosomal suspension was centrifuged for 10 sec in a desk Eppendorf Microfuge and the pellet resuspended in 1 ml of

Krebs-HEPES buffer (20 mM HEPES substituting for NaHCO, and adjusted with NaOH to pH 7.4). HEPES buffer was used instead of bicarbonate buffer because the latter causes the appearance of bubbles in the cuvette and thus increased the "noise."

Test compounds were prepared as stock solutions of 10 mM by dissolving compounds in 100% DMSO. Stock solutions were diluted with Krebs-Hepes buffer solution, as prepared above, to give 100 µM, 10 µM and 0.1 µM concentrations of the compound (Final concentration DMSO in incubations = 0.1 %). The synaptoneurosmal suspension was incubated with the test compounds in the cuvette for 5 min before reading fluorescence. Readings were done in triplicate for each concentration. A control experiment comprising of synaptoneurosamal suspension without test compound was always included.

#### 4.2.2.4 Experimental recording and parameters

Experiments were carried out at 37 °C and fluorescence was measured with a spectrofluorimeter. Selected wavelengths were 340 nm, 380 nm (excitation) and 500 nm (emission). The procedure was run, recording a time series of 40 sec with 150 ms intervals. At about 10 sec into the recording 300 µl of KCl (100 mM) was added.

**Table 4:** Recording parameters.

| **Fure-2/AM** | |
|---|---|
| Excitation | 340/380 nm |
| Emission | 510 nm |
| Excitation filter sets | Set 1 – 340/11<br>Set 2 – 380/20 |
| Emmision filter set | 580/20 |
| Optics position | Bottom |
| Sensitivity | 100 |
| Runtime | 40 sec |
| Interval (msec) | 150 msec |

### 4.2.3 Results and discussion

The control experiment, containing no test compound, was set as 100 % fluorescence against which the test compounds were compared and its effect expressed as percentage fluorescence. A decrease in fluorescence is thus an indication of a decrease in $Ca^{2+}$-ion flux into the synaptoneurosomal cell. NGP1-01, a compound known for its ability to decrease intracellular calcium was used as positive control.

The series of compounds as described in chapter 2 together with 3-(1-piperidinemethyl) phenol and N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine were evaluated. Results are represented in graphs and p-values were calculated for each compound at different concentrations. Data tables can be viewed in Annexure B.

Compound **A1**, 3-(1-piperidinemethyl)phenol, is the precursor for N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine and contains a N-substituent and aromatic moiety substituted with a hydroxyl group in the meta position at C10. It showed suppression of calcium fluorescence at all three concentrations, indicating a decrease in calcium influx into the synaptoneurosomal cell similar to that obtained with NGP1-01 (figure 11). All three concentrations had a p-value of $p < 0.01$ indicating a significant difference from the control.

N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine (**A2.2**) was tested as a separate compound to get an indication of the effect this compound would have on calcium influx and compare it to when it is linked to the polycyclic cage. The p-value at a concentration of 0.1 µM was $p < 0.05$ while the two other concentrations (1 µM and 10 µM) had a p-value of $p < 0.01$. In higher concentrations this structure showed a significantly greater decrease in fluorescence and thus $Ca^{2+}$ inhibition than NGP1-01. The profile of this compound is similar when it is conjugated to the cage (**A3** and **A4**). It showed better inhibition than **A1** in the micro molar range and this might be attributed to the side chain and terminal amino group of this compound.

Compound **A3** and **A4** are the oxa and aza derivatives, respectively formed as products of reductive amination of N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine with pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione using $NaBH_4$ and $NaBH_3CN$ as reducing agents. As expected the compounds showed the same type of effect on the calcium influx as **A2.2.** Significant inhibition of $Ca^{2+}$ influx was observed with all concentrations of these compounds. Especially at higher concentrations the effects obtained compared favourably with that of NGP1-01.

Compounds **C1** and **C2** are also oxa and aza derivites of the pentacycloundecane and includes the 1-(2-Aminoethyl)piperidin moiety but not the aromatic ring and extended chain linker. At lower concentrations no effect on calcium influx was observed for **C1** but a decrease at micro molar concentration ($p < 0.01$ at 1 µM and 10 µm) is evident. Compound **C2** showed significant decrease in fluorescence at all three concentrations ($p < 0.01$). These two compounds also had a similar effect on calcium influx to that of **A3** and **A4** at lower concentrations. In general, the oxa compounds exibited better inhibition of $Ca^{2+}$ flux, especially at higher concentrations with compound **A3** showing the best inhibition at 10 µM.

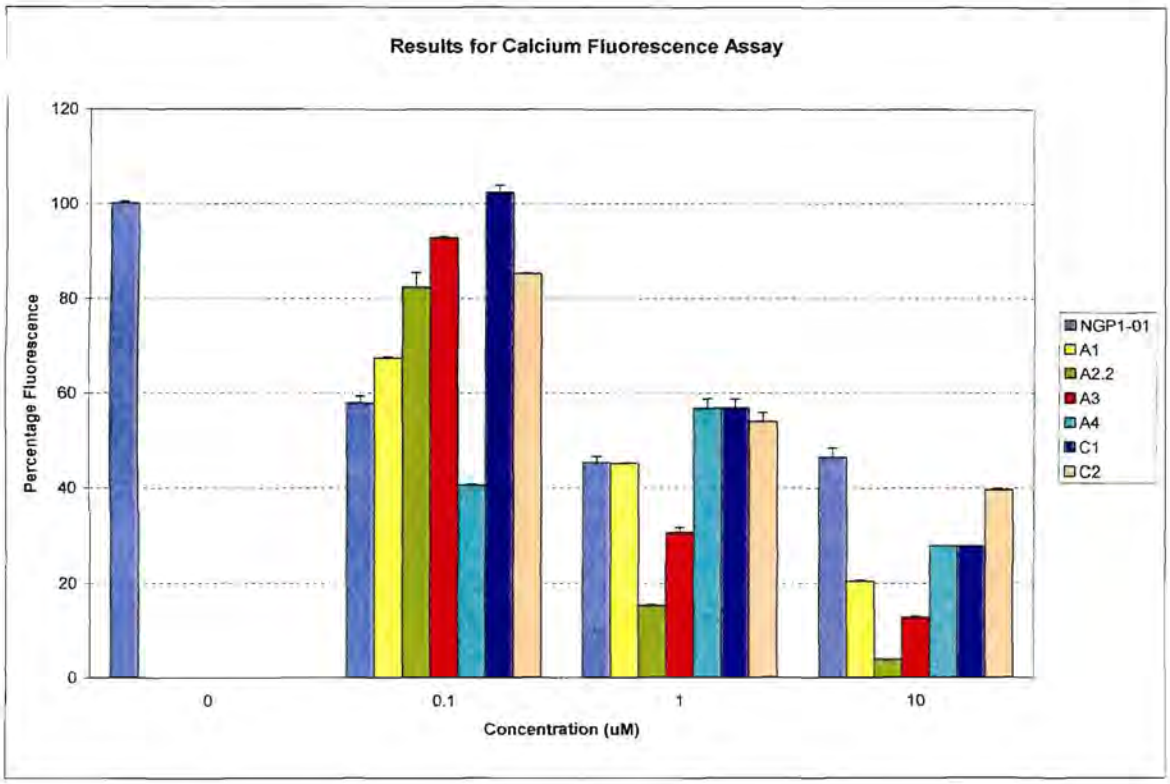



**Figure 11:** Results for calcium fluorescence assay.

## 4.3 Radio ligand binding assay with pentacycloundecane derivatives for sigma receptors

### 4.3.1 Materials and methods

Procedures similar to those of published studies were used to prepare cell cultures and perform binding studies (Matsumoto *et al.*, 1995).

### 4.3.2 Animals

The livers of Male Sprague-Dawley rats (250-300 g) from the animal testing facility on the Potchefstroom campus of the North-West University were used in study. Assay protocols were approved by the Ethics and Research Committee of the North-West University.

#### 4.3.2.1 Rat liver membranes

Membranes were prepared as previously described with minor adjustments (Matsumoto *et al.*, 1995). Membranes were prepared from the livers of male Sprague-Dawley rats (250–300 g). Animals were sacrificed by decapitation, and the livers were removed and minced before homogenisation. The liver tissue was homogenised in 10 volumes of ice-cold 0.32 M sucrose. The crude homogenate was centrifuged at 1000 g for 10 min at 4 °C. The supernatant was further centrifuged at 31000 g for 15 min at 4 °C. The pellet was resuspended in 3 volumes of ice cold 50 mM Tris-HCl / 0.32 M sucrose (pH 7.8) by vortexing and left to incubate for 30 min at 25 °C. The suspension was recentrifuged at 31000 g for 15 min at 4 °C and pellet was resuspended in a final volume of 1.53 ml/g ice-cold 50 mM Tris-HCl / 0.32 M sucrose (pH 7.8) (Matsumoto *et al.*, 1995). The protein concentrations were determined with the Bradford method (Bradford, 1976).

### 4.3.3 Binding assay for σ receptors

Membranes (160-200 µg membrane protein) were incubated in triplicate for 120 min at room temperature with 3 nM [3H]- DTG (PerkinElmer, specific activity 58.1 Ci/mmol) and various concentrations of the test compounds in 50 mM Tris-HCl (pH 8.0) in a final volume of 0.5 ml. The reaction was stopped by vacuum filtration through Whatman GF/B glass-fiber filters presoaked for 60 min with 0.5 % polyethylenimine, followed by rapid washing with 5 ml ice-cold buffer (3x). Filters were placed in 3 ml scintillation cocktail and the radioactivity determined by liquid scintillation counting (Matsumoto *et al.*, 1995).

As internal controls, three increasing concentrations of unlabelled DTG were included. The compounds were prepared as 10 mM stock solutions in 100 % DMSO and diluted with Tris-HCl buffer on the day of the experiment. The final DMSO concentration in the incubation tubes was maintained at 0.1 % (Shiba *et al.*, 2002).

### 4.3.4 Results and discussion

A homologous competitive study was done and the same compound was used as hot and cold ligand. Only three concentrations were tested for each compound as very small quantities of test compound and limited radio ligand were available. The amount of radio ligand left on the filter was counted after filtration and the CPMA-values converted to percentage. The control experiment contained only [$^3$H]-DTG and membrane protein, this value was taken as 100 % to which the test compounds were compared. A decrease in percentage is thus an indication of the displacement of radio ligand by the test compound implicating affinity for the receptor. This affinity is non-selective, representing affinity for σ1 and σ2 receptors. Unlabelled DTG, a known non-selective sigma ligand, was used as a positive control. Results obtained were expressed as percentage of radio ligand counted *vs* concentration of the test compounds in a bar chart and should only be interpreted as a rough indication of affinity.

Compound **A1** and **A2.2** showed significant affinity for the σ receptor at micro- and nano molar concentrations ($p < 0.01$) (figure 12).The affinity for the receptors seemed to be affected by the chain length and when compared to one another, **A1** had a higher affinity than **A2.2.** The oxa derivative, 8-{N-[3-(3-Piperidin-1-ylmethylphenoxy)propyl]amine}-8-11-oxapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane (**A3**) , showed no significant affinity at any of the concentrations, but the aza-derivative (**A4**) had affinity at micro molar concentrations ($p < 0.01$) comparable to **A2.2.** No value could be calculated at nano molar concentrations of **A4** because the radio ligand was depleted. 8-{1-(2-Aminoethyl)piperidine}-8-11-oxapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane (**C1**), showed affinity for the σ receptor in the micro and nano molar range but **C2** (aza derivative) had no significant affinity ($p > 0.05$) at any of the concentrations. The affinity for the receptors appeared to be affected by the chain length as **C1**; with the shorter chain length had better affinity than compound **A3.** This corresponded to the affinity observed for **A1**.

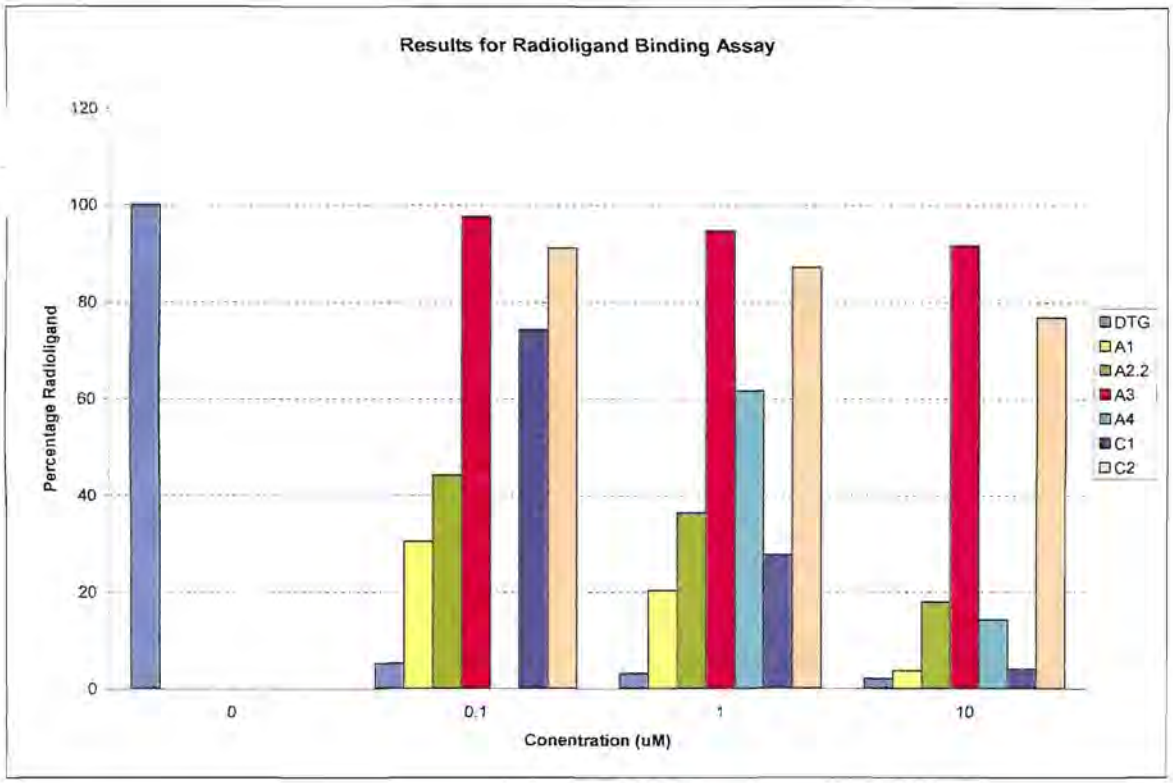



**Figure 12:** Results for radio ligand binding assay ( A4 not evaluated at 0.1 μM)

## 4.4 Conclusion

The oxa cage derivatives in general exhibited better inhibition of $Ca^{2+}$ flux, especially at higher concentrations with compound **A3** showing the best inhibition at 10 μM. Compound **C1** and **A1** had the highest affinity for the sigma receptors. This would implicate that compounds with a shorter chain length (C = 2), N-substituent and hydrophobic moiety with limited volume might be more favourable for binding to sigma receptors. These two compounds also had a significant effect on intracellular calcium concentration in the synaptoneurosomes and might be good lead compounds for future investigations. The structural features of these two compounds, when compared to the literature (chapter 2), indicate that they should have greater affinity for σ1 receptors and this together with their effect on intracellular calcium might implicate antagonism. This however will have to be confirmed by more extensive studies in future. Selectivity for the different classes of sigma receptors were unfortunately not explored and would also be important in future investigations. Problems experienced during the assays included inability to inject KCl at consistent force and time during the calcium fluorescence assay and limited radio ligand for the binding study which affected the results obtained.

# Chapter 5

# Conclusion

## 5 Introduction

It is evident that polycyclic cage compounds may prove to be of great value in future treatment of neurodegenerative diseases. There is still however mechanisms involved in the process of neurodegeneration that are not fully understood, but researchers are getting closer to identifying more and new possible targets for drug treatment.

This study focussed mainly on the effect of polycyclic cage compounds on calcium homeostasis, a key process in neurodegeneration. A closer look was taken at receptors and channels involved in this process. The involvement of sigma receptors in calcium regulation was also discussed. These receptors are an exciting new prospect for drug targeting and treatment of not only neurodegenerative diseases but tumour related illnesses such as cancer as well.

### 5.1 Chemistry

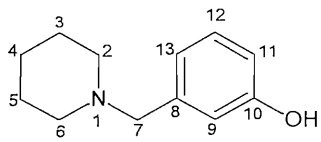
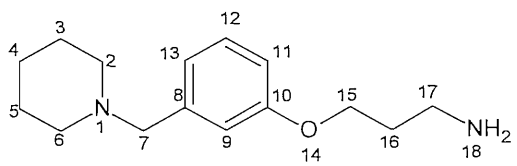
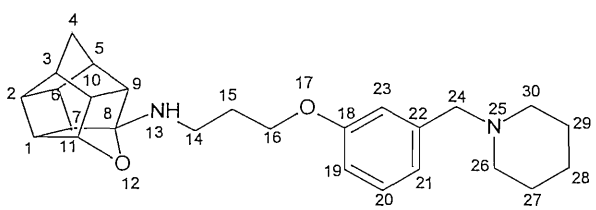
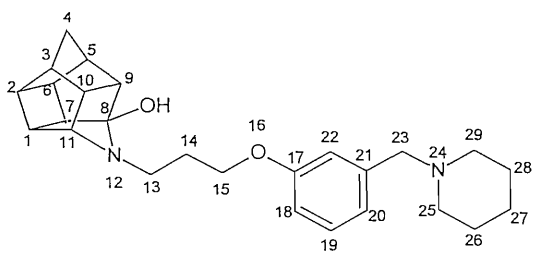
A series of pentacycloundecane derivatives with sigma receptor bias substituents were selected and synthesised through reductive amination. Reductive amination was used to link to 1-(2-Aminoethyl)piperidine and N-{3-[3-(1-piperidinylmethyl)phenoxylpropyl}amine to the pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione to form oxa- and aza derivatives and the structures of the compounds were confirmed by $^{1}$H NMR, $^{13}$C NMR, MS and IR. The yields of the compounds varied from 13 % - 73 %. Low yields obtained may be optimised by improving experimental conditions and purification techniques. A more extensive series of compounds may be considered for future investigation.

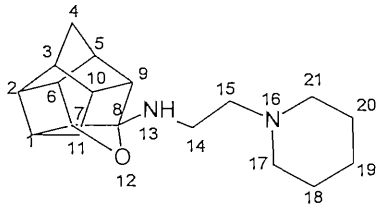
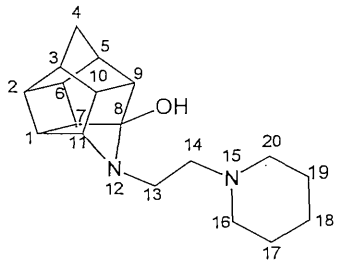
### 5.2 Biological screening

The effect of the test compounds on intracellular calcium in synaptoneursomes, were evaluated using fluorescent techniques and their affinity for sigma receptors determined through a radio ligand binding study on Sprague-Dawley rat liver membranes. The compounds had different structural properties which were compared to each other. The difference between the oxa-and aza derivatives as well as the effect of chain length between the cage and the piperidine moiety on calcium influx and binding affinity were evaluated. Compound **C1** and **A1** had the highest affinity for the sigma receptors. These compounds

had a shorter chain length, N-substituent and hydrophobic moiety with limited volume which proved to be more favourable for binding to sigma receptors. The structural components of these two compounds indicate that they might have greater affinity for σ1 receptors and this together with their inhibitory effect on intracellular calcium might implicate σ1 antagonism. This however will need further experimental confirmation. The oxa derivatives, in general exhibited better inhibition of $Ca^{2+}$ flux, especially at higher concentrations with compound **A3** showing the best inhibition at 10 μM. Selectivity for the different classes of sigma receptors were unfortunately not explored and would be important to investigate in future. Problems experienced during the assays may be overcome in future by improving techniques and biological models.

**Table 5:** Summary of synthesised test compounds

| Synthesised test compounds | |
|---|---|
| **A1** |  |
| **A2.2** |  |
| **A3** |  |
| **A4** |  |

| | |
|---|---|
| C1 |  |
| C2 |  |

## 5.3 Final remarks

The synthesised pentacycloundecane derivatives proved to have an inhibitory effect on $Ca^{2+}$ flux and decreased intracellular calcium. The $Ca^{2+}$ effects could also be linked to their interaction with sigma receptors. This does not exclude interaction with other receptors such as the NMDA and AMPA/KA receptors and further investigation to characterise the neuroprotective effects of these compounds needs to follow.

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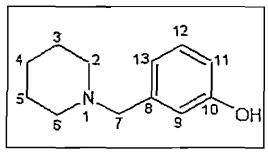
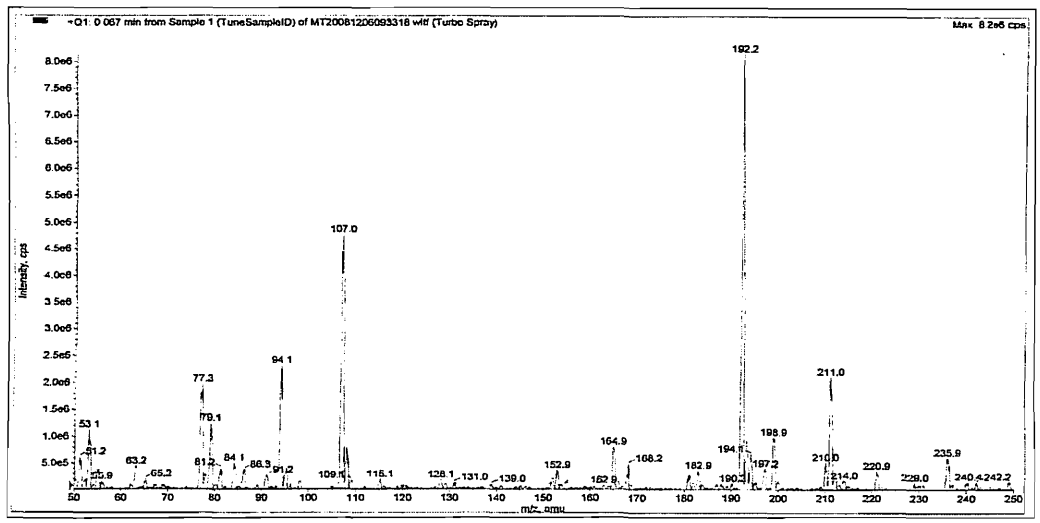
# Annexure A

## $^{1}H$ NMR, $^{13}C$ NMR MS and IR spectra

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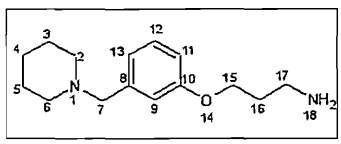


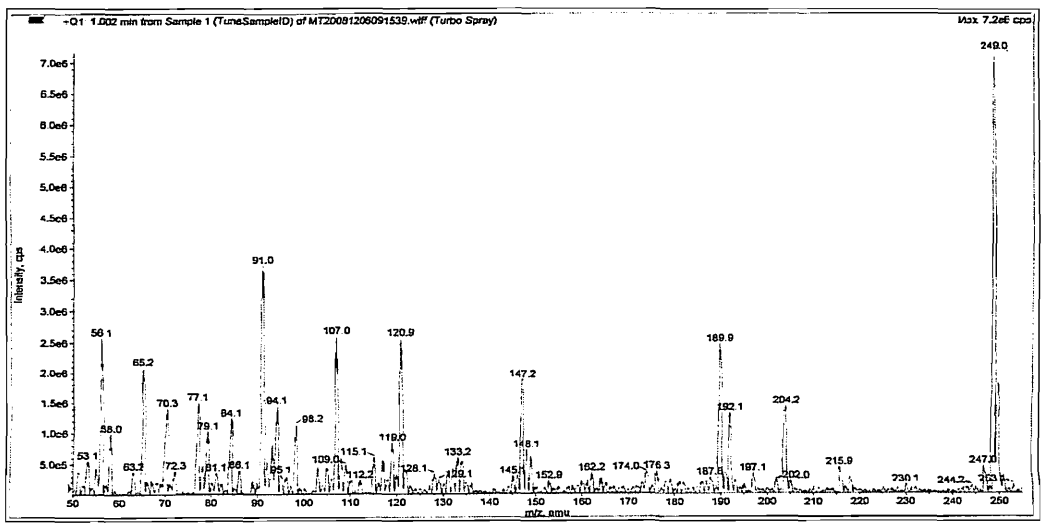


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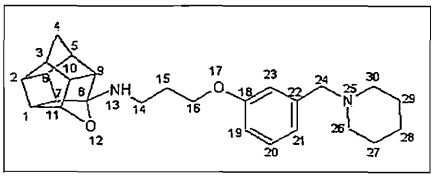

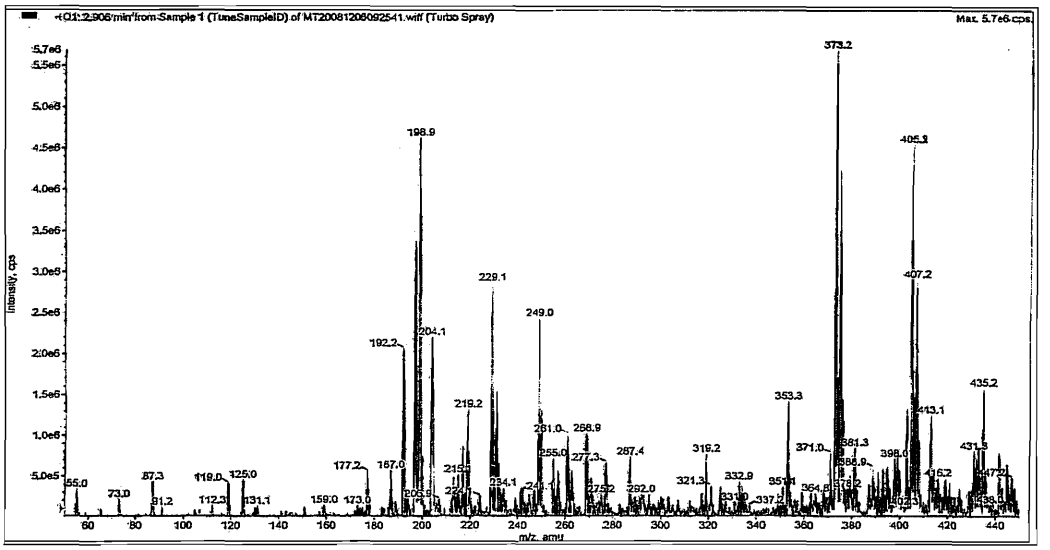


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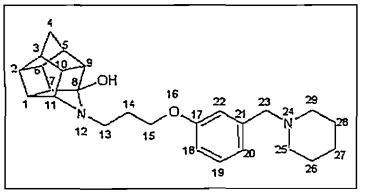

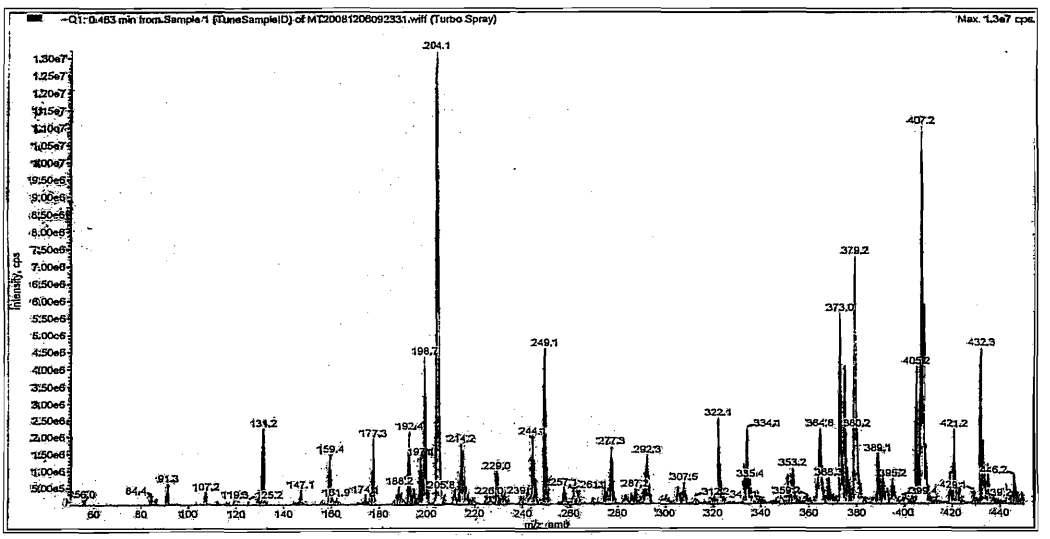


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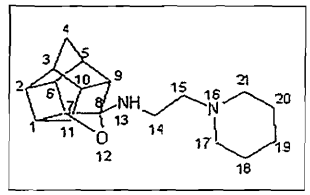


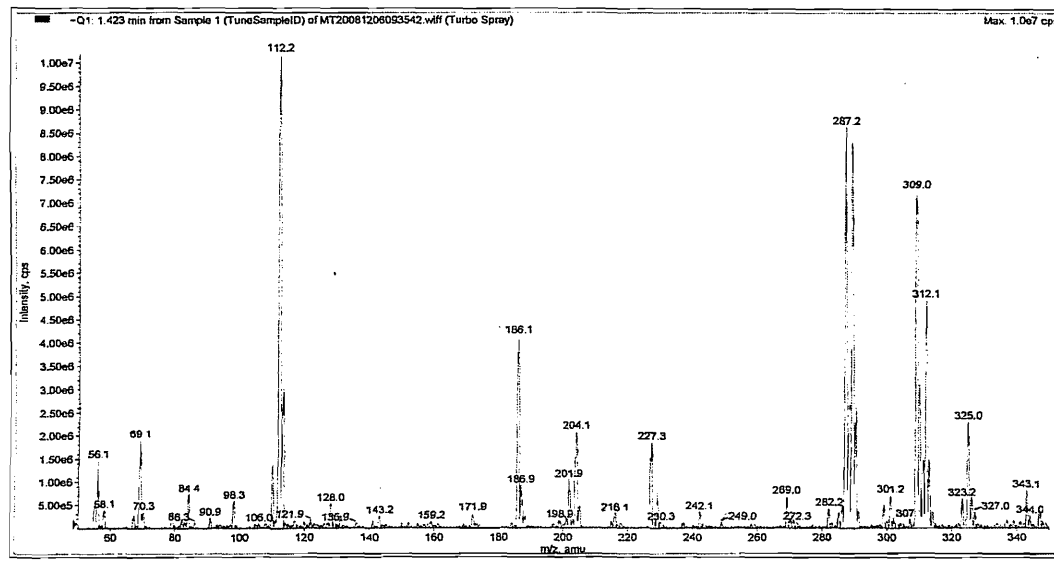


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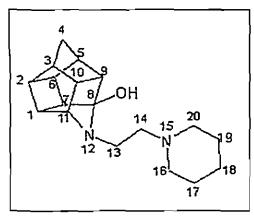


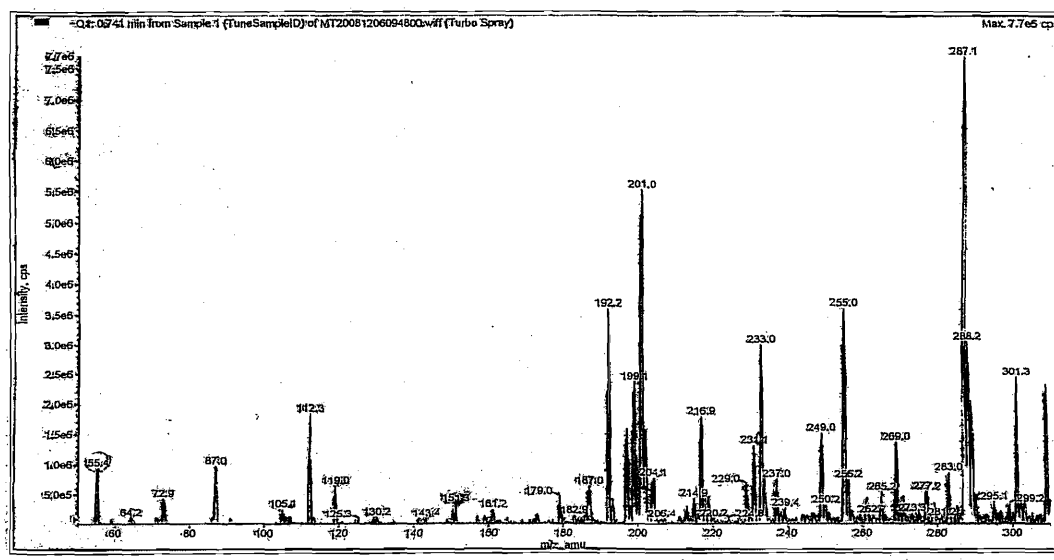


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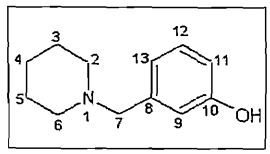


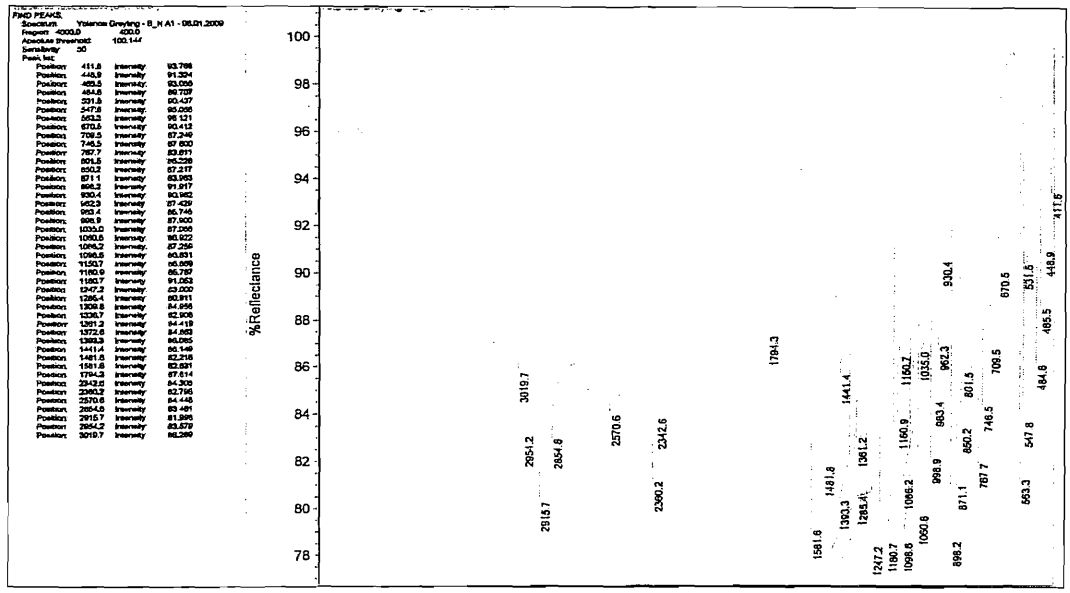


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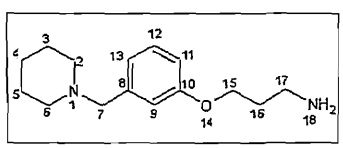


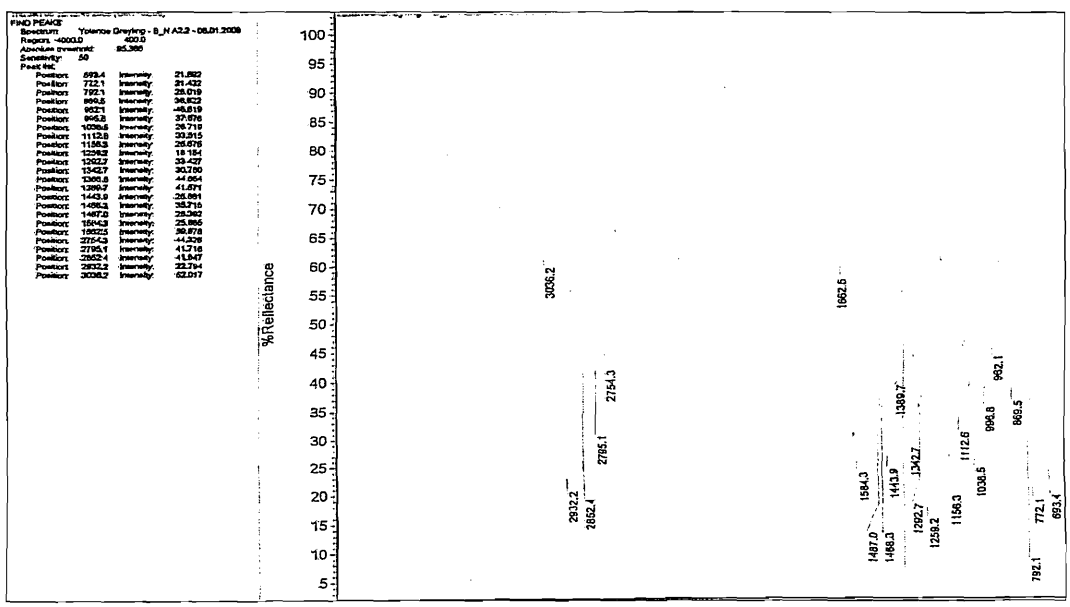


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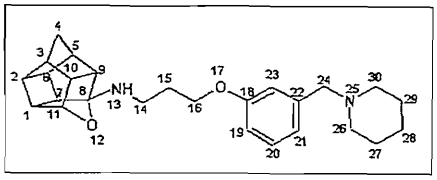

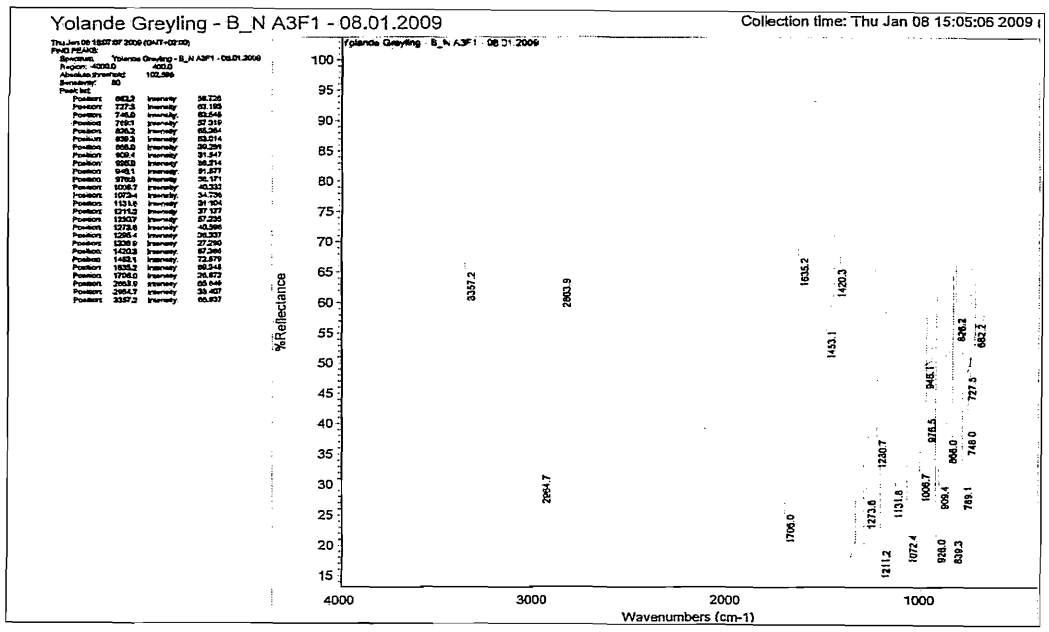


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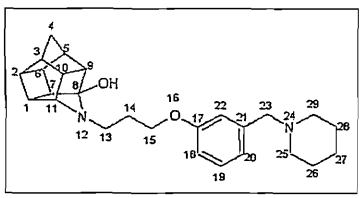

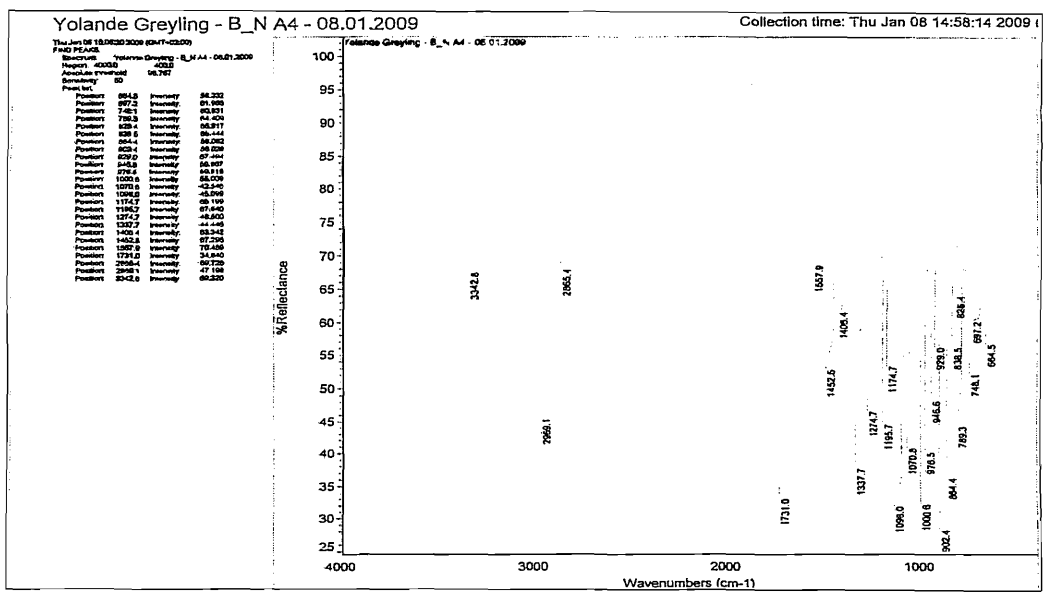


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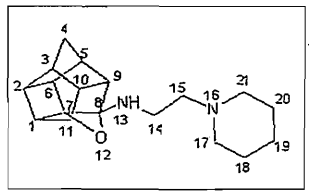


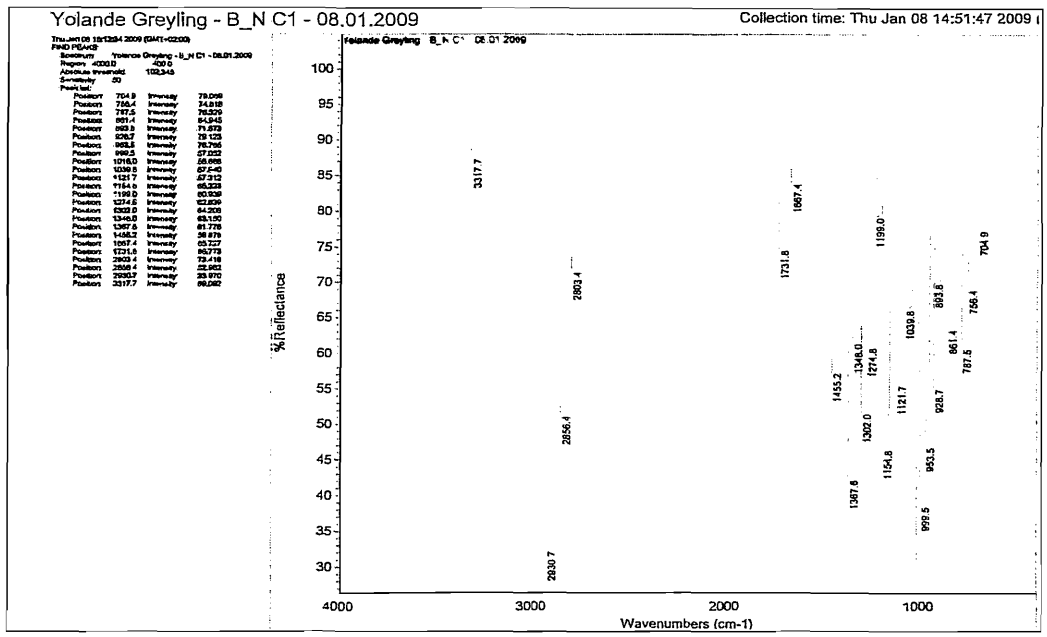


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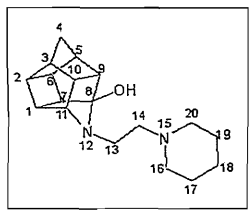


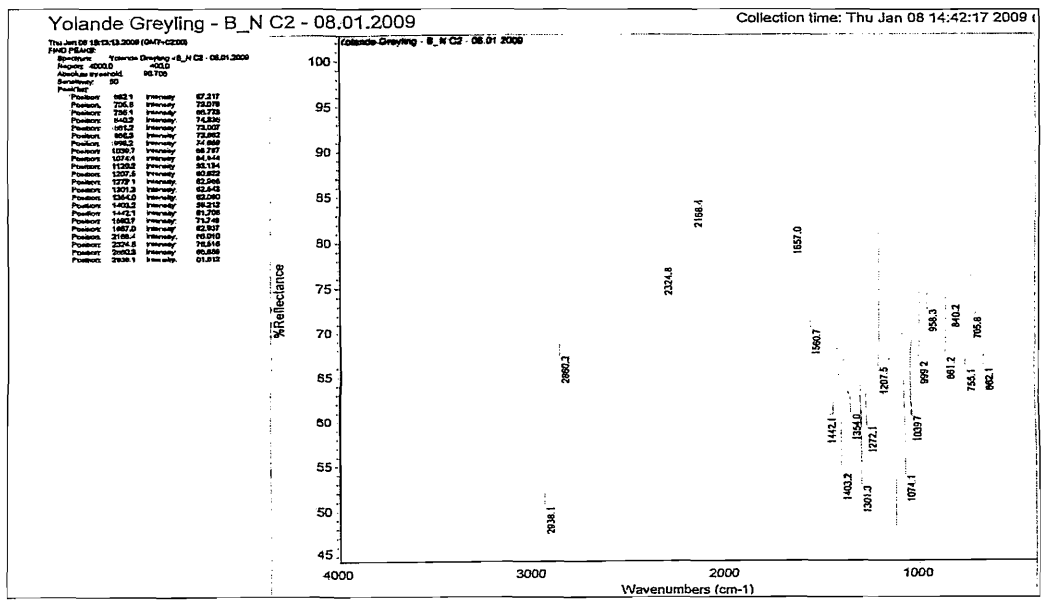


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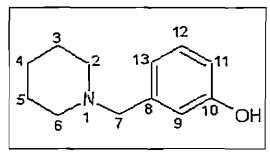


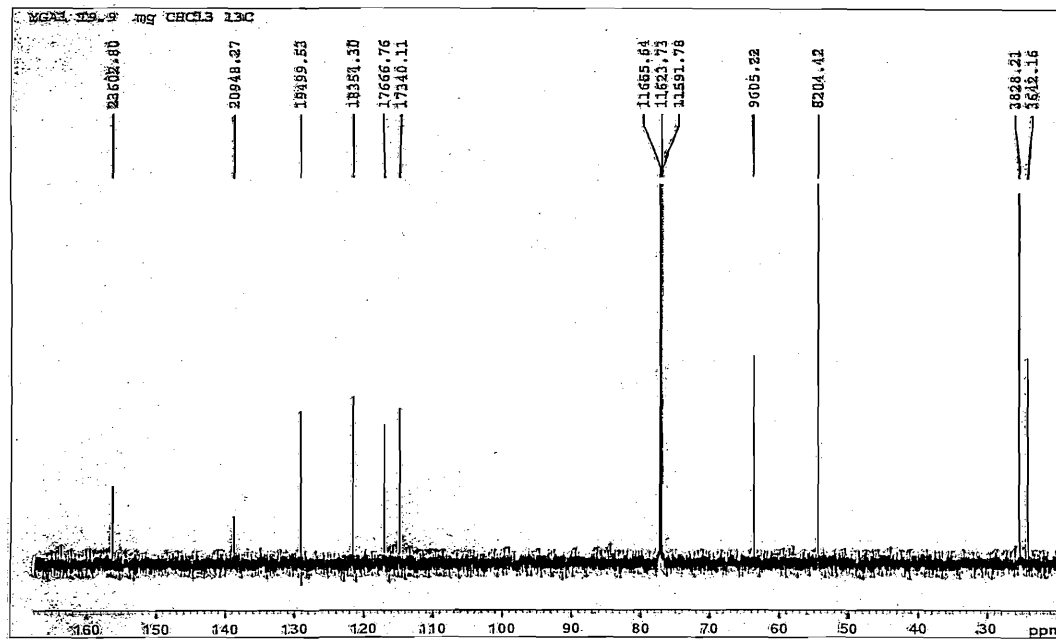


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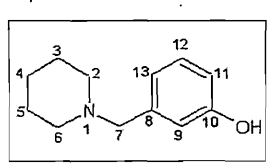


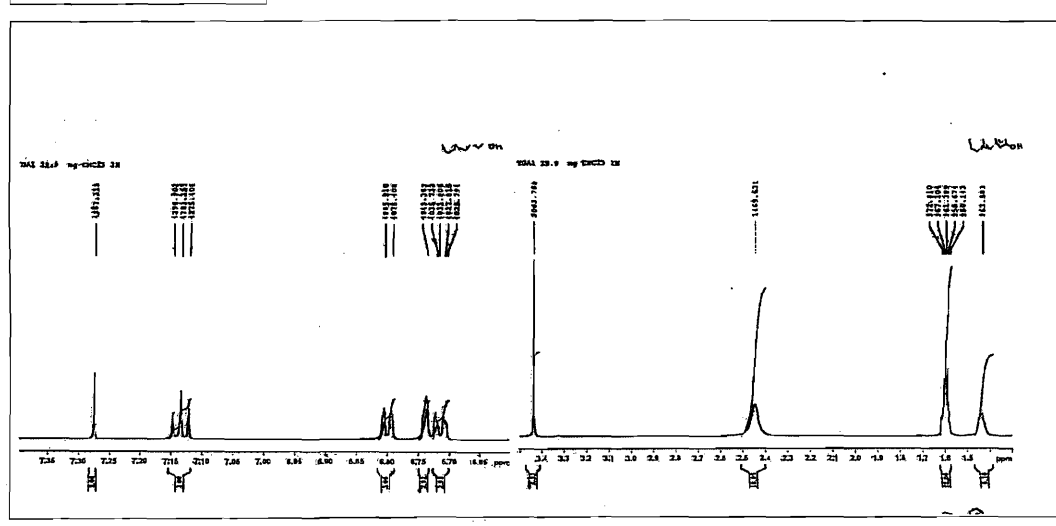

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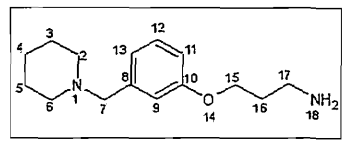


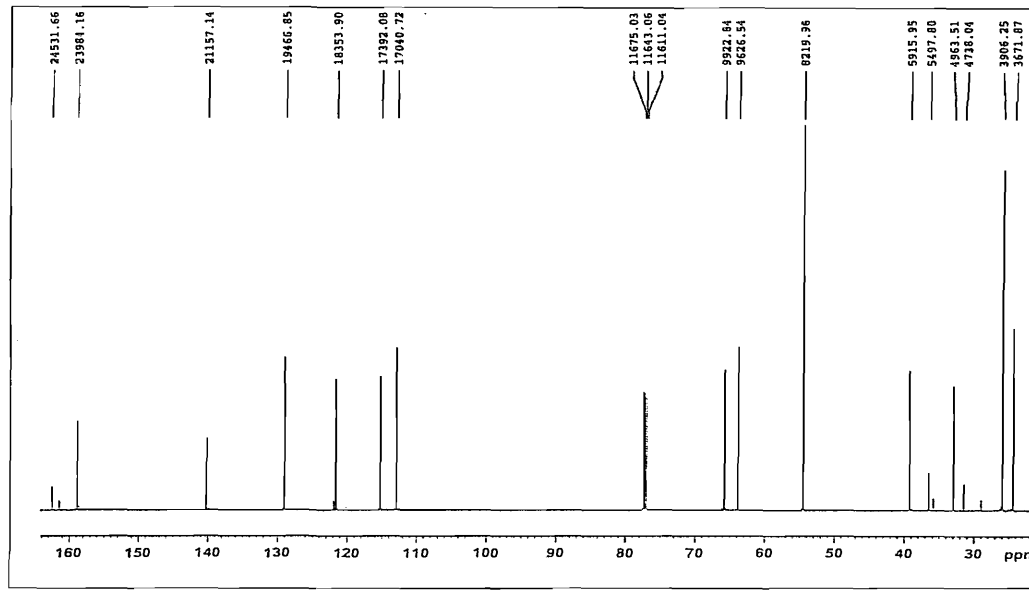


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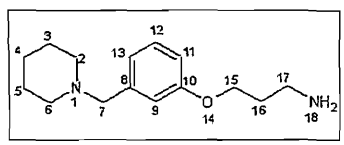


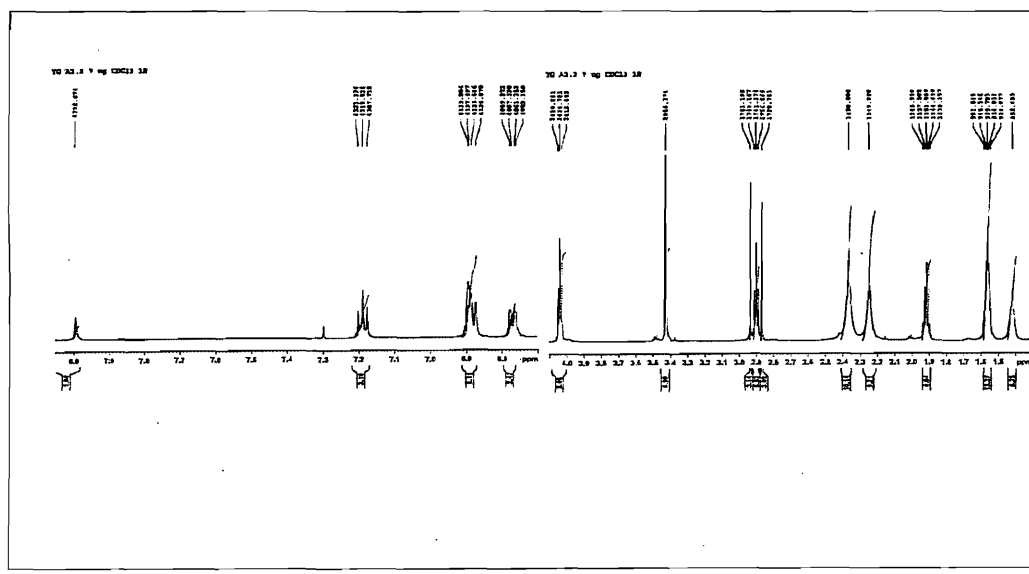

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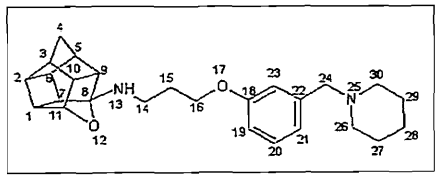

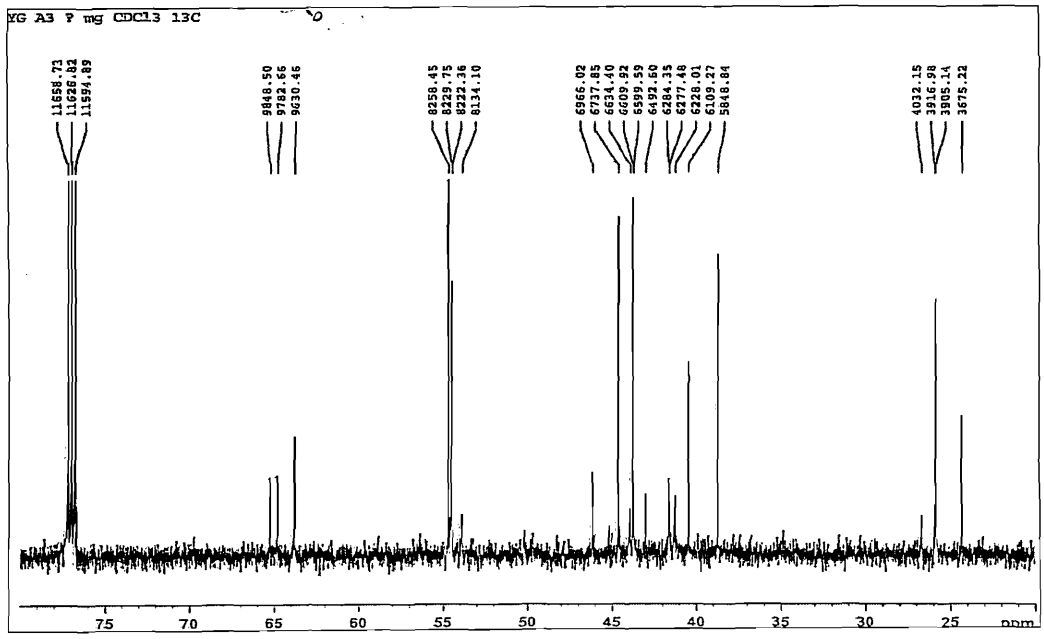


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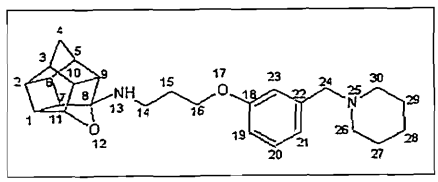

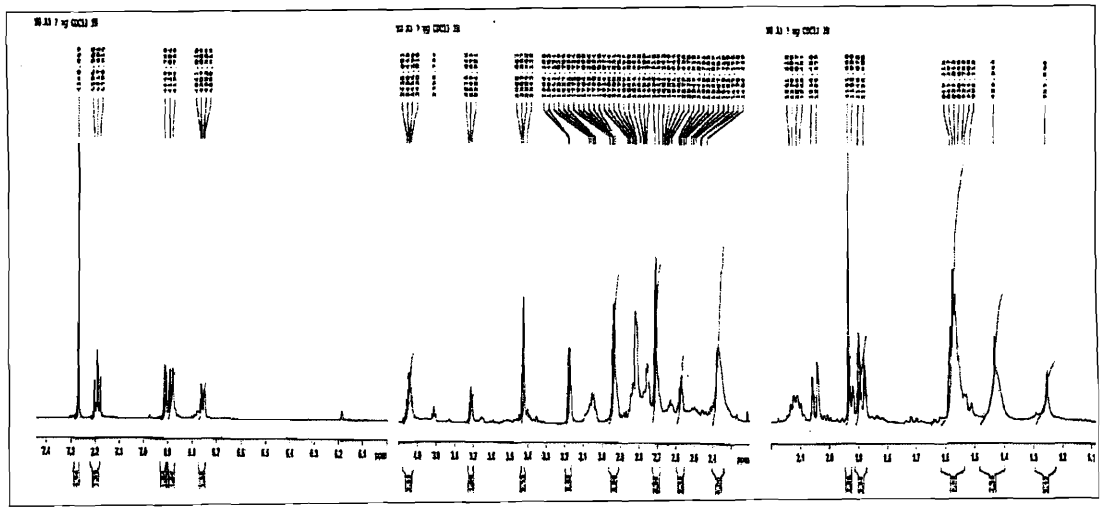

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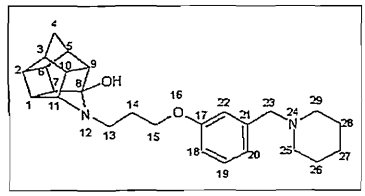

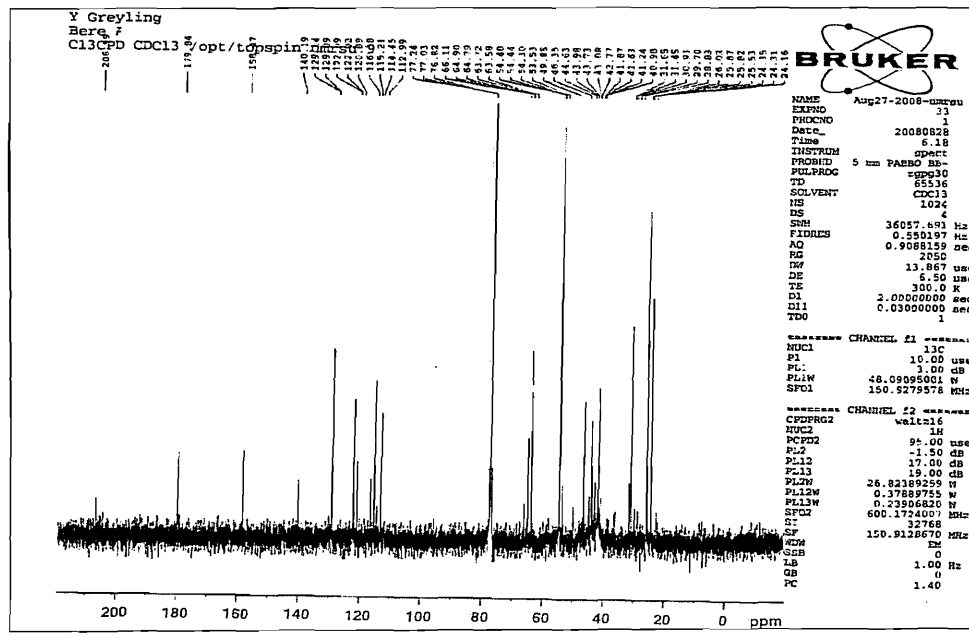


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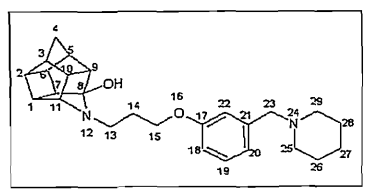

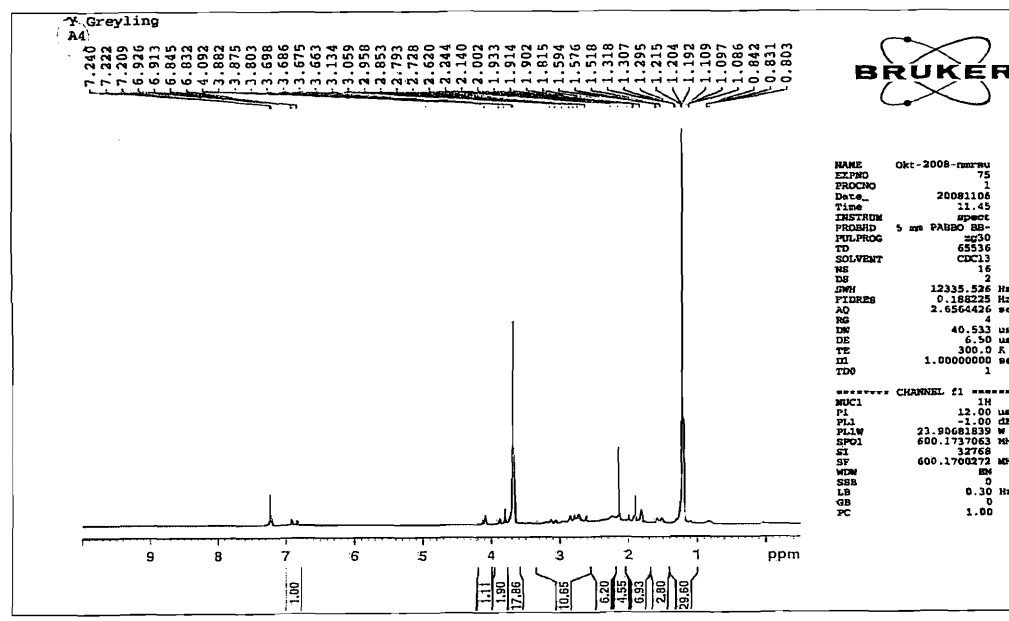


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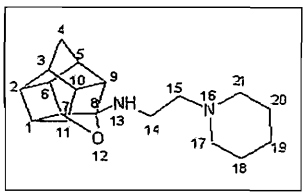


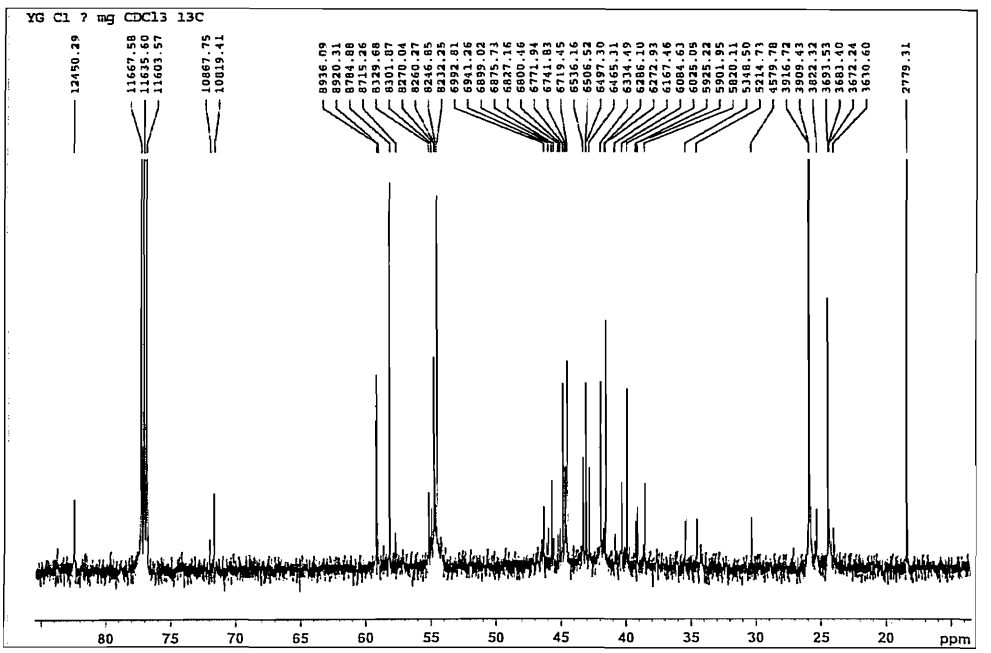


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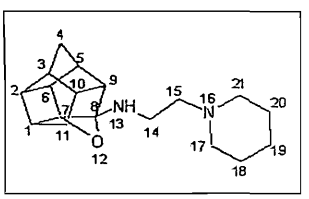


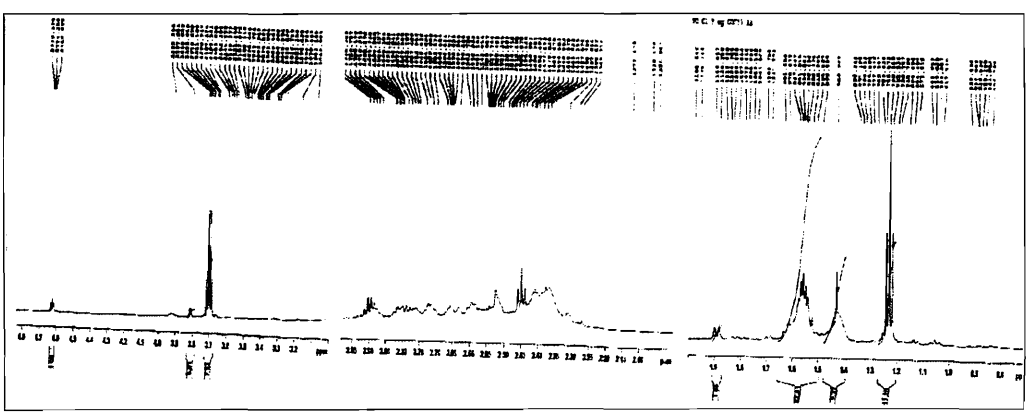

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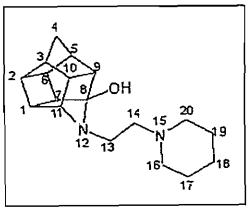
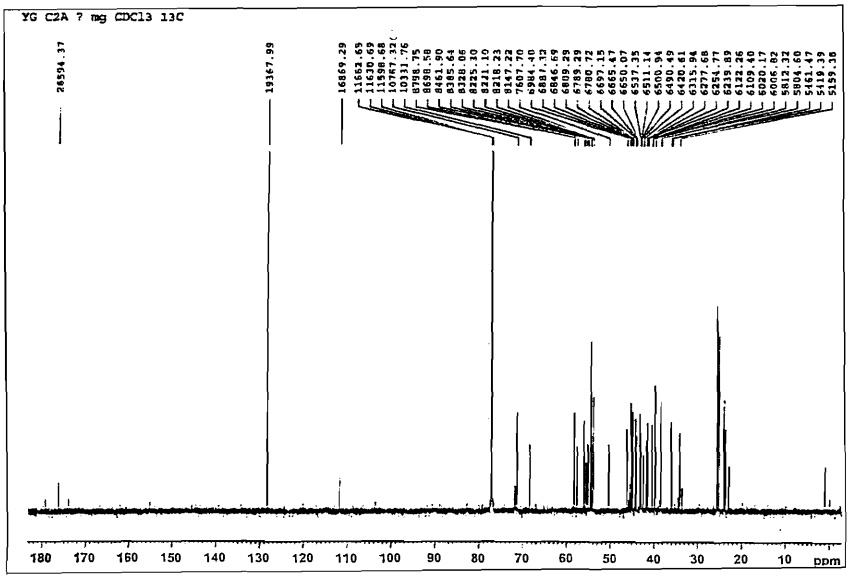
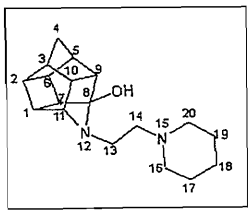
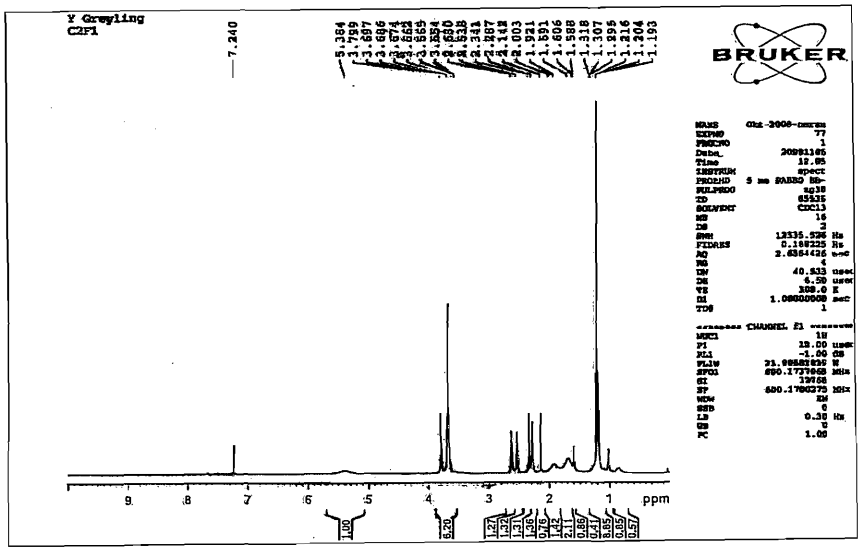



Spectrum 23




Spectrum 24

# Annexure B

## Data tables for Calcium Fluorescence Assay and Radio ligand binding assay

## Data tables for Calcium Fluorescence Assay

| Concentration µM | Percentage Fuorescence | | | | | | |
|---|---|---|---|---|---|---|---|
| | A1 | A2.2 | A3 | A4 | C1 | C2 | NGP1-01 |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.1 | 67.31 | 82.32653 | 92.8508 | 40.52892 | 102.2871 | 85.19515 | 57.76892 |
| 1 | 45.0575 | 15.27659 | 30.52107 | 56.73507 | 56.73507 | 53.88324 | 45.27217 |
| 10 | 20.32168 | 3.790756 | 12.71998 | 27.68614 | 27.68614 | 39.57327 | 46.35852 |

| Concentration µM | Standard error of means | | | | | | |
|---|---|---|---|---|---|---|---|
| | A1 | A2.2 | A3 | A4 | C1 | C2 | NGP1-01 |
| 0 | 0.5064 | 0.5064 | 0.5064 | 0.5064 | 0.5064 | 0.5064 | 0.5064 |
| 0.1 | 0.3598 | 3.206 | 0.3285 | 0.3657 | 1.631 | 0.2406 | 1.534 |
| 1 | 0.2088 | 0.3022 | 1.112 | 2.032 | 1.959 | 1.982 | 1.366 |
| 10 | 0.3623 | 0.07503 | 0.3321 | 0.1022 | 0.1415 | 0.3661 | 2.078 |

| Dunnett's Multiple Comparison Test | Mean Diff. | q | P value | 95% CI of diff |
|---|---|---|---|---|
| Controle vs NGP 0.1 | 16.73 | 10.54 | P < 0.01 | 12.83 to 20.64 |
| Controle vs NGP 1 | 23.18 | 14.6 | P < 0.01 | 19.27 to 27.09 |
| Controle vs NGP 10 | 24.32 | 15.32 | P < 0.01 | 20.41 to 28.22 |
| | | | | |
| Controle vs A1 0.1 | 16.27 | 34.07 | P < 0.01 | 15.10 to 17.45 |
| Controle vs A1 1 | 21.77 | 45.58 | P < 0.01 | 20.59 to 22.94 |
| Controle vs A1 10 | 31.57 | 66.1 | P < 0.01 | 30.40 to 32.75 |
| | | | | |
| Controle vs A2.2 0.1 | 7 | 3.091 | P < 0.05 | 1.427 to 12.57 |
| Controle vs A2.2 1 | 33.57 | 14.82 | P < 0.01 | 28.00 to 39.14 |
| Controle vs A2.2 10 | 38.12 | 16.83 | P < 0.01 | 32.55 to 43.69 |
| | | | | |

| | | | | |
|---|---|---|---|---|
| Controle vs A3 0.1 | 2.834 | 3.061 | P < 0.05 | 0.5557 to 5.113 |
| Controle vs A3 1 | 28.54 | 30.82 | P < 0.01 | 26.26 to 30.82 |
| Controle vs A3 10 | 34.58 | 37.35 | P < 0.01 | 32.30 to 36.86 |
| | | | | |
| Controle vs A4 0.1 | 11.9 | 8.05 | P < 0.01 | 8.265 to 15.54 |
| Controle vs A4 1 | 18.39 | 12.44 | P < 0.01 | 14.75 to 22.03 |
| Controle vs A4 10 | 37.44 | 25.32 | P < 0.01 | 33.80 to 41.08 |
| Controle vs C1 0.1 | -0.9062 | 0.5455 | P > 0.05 | -4.995 to 3.182 |
| Controle vs C1 1 | 19.02 | 11.45 | P < 0.01 | 14.93 to 23.10 |
| Controle vs C1 10 | 28.65 | 17.24 | P < 0.01 | 24.56 to 32.74 |
| | | | | |
| Controle vs C2 0.1 | 5.866 | 4.166 | P < 0.01 | 2.401 to 9.331 |
| Controle vs C2 1 | 18.27 | 12.97 | P < 0.01 | 14.80 to 21.73 |
| Controle vs C2 10 | 23.94 | 17 | P < 0.01 | 20.48 to 27.41 |

## Data tables for Radio ligand binding assay

| [ ] µM | A1 | A2.2 | A3 | A4 | C1 | C2 | DTG cold | [$^3$H]-DTG |
|---|---|---|---|---|---|---|---|---|
| 10 | 1882.42 | 7397.44 | 37986.6 | 5695.89 | 1942.31 | 32377.8 | 865.04 | 42451.45 |
| | 1619.31 | 7504.6 | 39857 | 6212.28 | 1534.42 | 32888.7 | 721.58 | |
| | 1236.84 | 7961.38 | 38917.5 | 6257.99 | 1612.53 | 32718.5 | 1034.45 | |
| | | | | | | | | |
| 1 | 8816.48 | 14469.8 | 40339.7 | 24829.9 | 11831.4 | 40286.5 | 1499.28 | |
| | 8858.8 | 17669.6 | 39259.2 | 27886.9 | 12234.9 | 34436.1 | 1171.52 | |
| | 8077.06 | 14137.7 | 41002.9 | 25754.7 | 11071.8 | 36317.1 | 1280.68 | |
| | | | | | | | | |
| 0.1 | 13416.6 | 19494.1 | 40177.3 | | 30602.2 | 37649.2 | 1984.86 | |
| | 9556.67 | 17772.1 | 40774 | | 31563.7 | 39315.6 | 2364.35 | |
| | 15654.5 | 18971.9 | 43257.4 | | 32462.9 | 39098.6 | 2383.14 | |
| | | | | | | | | |
| 10 | 1579.523 | 7621.14 | 38920.37 | 6055.387 | 1696.42 | 32661.67 | 873.69 | |
| 1 | 8584.113 | 15425.7 | 40200.6 | 26157.17 | 11712.7 | 37013.23 | 1317.16 | |
| 0.1 | 12875.92 | 18746.03 | 41402.9 | | 31542.93 | 38687.8 | 2244.117 | |

| [ ] µM | A1% | A2.2% | A3% | A4% | C1% | C1% | DTG% |
|---|---|---|---|---|---|---|---|
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.1 | 30.33094 | 44.15876 | 97.53001 | | 74.30355 | 91.13423 | 5.286314 |
| 1 | 20.22102 | 36.33728 | 94.69783 | 61.61667 | 27.59082 | 87.18957 | 3.102745 |
| 10 | 3.720776 | 17.9526 | 91.68208 | 14.26426 | 3.996142 | 76.93888 | 2.058092 |

| Dunnett's Multiple Comparison Test | Mean Diff. | q | P value | 95% CI of diff |
|---|---|---|---|---|
| Controle vs A1 10 | 40870 | 30.51 | P < 0.01 | 36720 to 45020 |
| Controle vs A1 1 | 33870 | 25.28 | P < 0.01 | 29710 to 38020 |
| Controle vs A1 0.1 | 29580 | 22.08 | P < 0.01 | 25420 to 33730 |
| | | | | |
| Controle vs A2.2 10 | 34830 | 35.15 | P < 0.01 | 31760 to 37900 |
| Controle vs A2.2 1 | 27030 | 27.27 | P < 0.01 | 23950 to 30100 |
| Controle vs A2.2 0.1 | 23710 | 23.92 | P < 0.01 | 20630 to 26780 |
| | | | | |
| Controle vs A3 10 | 3531 | 4.416 | P < 0.05 | 1052 to 6010 |
| Controle vs A3 1 | 2251 | 2.815 | P > 0.05 | -227.8 to 4729 |
| Kontrole vs A3 0.1 | 1049 | 1.311 | P > 0.05 | -1430 to 3527 |
| | | | | |
| Controle vs C1 10 | 40760 | 79.02 | P < 0.01 | 39160 to 42350 |
| Controle vs C1 1 | 30740 | 59.6 | P < 0.01 | 29140 to 32340 |
| Controle vs C1 0.1 | 10910 | 21.15 | P < 0.01 | 9310 to 12510 |

| | | | | |
|---|---|---|---|---|
| Controle vs C2 10 | 9790 | 6.94 | P < 0.01 | 5417 to 14160 |
| Controle vs C2 1 | 5438 | 3.855 | P < 0.05 | 1065 to 9811 |
| Controle vs C2 0.1 | 3764 | 2.668 | P > 0.05 | -609.6 to 8137 |
| | | | | |
| Controle vs DTG 10 | 41580 | 295.3 | P < 0.01 | 41140 to 42010 |
| Controle vs DTG 1 | 41130 | 292.1 | P < 0.01 | 40700 to 41570 |
| Controle vs DTG 0.1 | 40210 | 285.5 | P < 0.01 | 39770 to 40640 |

# Acknowledgements


- Firstly to my Creator, for bringing this life changing experience on my path, giving me the strength, courage and endurance to persist in succeeding.
- My parents, without you as my rock steady foundation none of this would have been possible. Thank you from the deepest of my heart, I love you.
- To Prof. Sarel Malan and Prof Sandra van Dyk for believing in me, giving me this opportunity and guiding me along the way.
- Mr Cor Bester, Mrs Antoinette Fick and Mr Petri Bronkorst for being there to help me with the decapitation of the rats, sometimes at the most impossible time, thanks for always being friendly and mindful.
- To Mrs Nellie Scheepers, for being an assistant in al my experiments, for guidance, encouragement and most of all for being a dear friend.
- To Prof Linda Brand, Mrs Sharlene Lowe and Mr Nico Liebenberg for your patience and guidance with the radio ligand study.
- Mr Andre Joubert and Mr Andre (Jouba) Joubert for your assistance with the NMR and IR spectra.
- Prof Jan du Preez, thank you for helping me with my MS-spectra on such short notice and always having a smile for me.
- Thank you to the NRF for my bursaries.
- Prof Jan Gerber, for being a friend, a mentor and an ear for all my hardships
- Miss Lize van Niekerk, my friend, my shoulder and colleague. Thank you for never giving up on me, for crying and laughing with me and putting up with me for 3 years in the same house. I love you lots.
- To my friends, Louis, Dennis, Jacques Petzer, Gizella, Petro you have each played a special part in my life and in this project. Thank you.

- Mr Rudolph van Deventer, Miss Zerilda van Deventer, Mr Tony van Deventer and Mrs Lográsia van Deventer, thank you for your love, for letting me in your home, being a family for me and all your encouragement.

- Last but not least my lab partner, Righard Lemmer, thank you for helping me, for laughing and swearing when times got rough, you go labbie!