THE INFLUENCE OF S-NITROSYLATION ON THE CHANNEL ACTVITY OF POLYCYCLIC AMINES

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"The opposite of a correct statement is a false statement.
The opposite of a profound truth may well be another profound truth."

Niels Bohr – 20th century physicist

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ABSTRACT

A novel series of polycyclic amines, containing nitrogen monoxide donating moieties, were synthesised and tested for possible neuroprotective activity. The premise for this study was the recent development of a series of nitro containing memantine derivatives, called the nitromemantines. These nitrogen monoxide donating moieties transfer a nitrogen monoxide to the thiol group of certain crucial cysteine residues in the receptor channel, a process known as S-nitrosylation, which then leads to disulfide bond formation, allosteric modification and desensitisation of the receptor. It was argued that these nitromemantines show better Ca^{2+} channel activity, and neuroprotective promise, than memantine alone. Because of the structural similarity of the pentacycloundecylamines and memantine, it was thus decided to investigate the influence of nitrogen monoxide donating moieties on the channel activity of the pentacycloundecylamines.

After a thorough structure-activity relationship overview and planning, a series of pentacycloundecylamines were synthesised, each containing a nitrogen monoxide donating group. Sufficient steric freedom was provided by the incorporation of a linker between the cage structure and the aromatic moiety. Esterification was done using the activating agent, 1-ethyl-3-(3'-dimethylamino)carbodiimide (EDC). The synthesised compounds could be classified into two groups, based on their nitrogen monoxide donating moieties: the unsaturated nitro compounds (**3.1**, **3.2** and **3.3**) and the nitro esters, or nitrates (**3.4**, **3.5** and **3.6**). The nitrates were obtained *via* the reaction of hydroxyl groups with thionylchloride nitrate. Structure elucidation was done using one and two-dimensional NMR spectroscopy, as well as IR absorption spectrophotometry and mass spectrometry.

The S-nitrosylation assay was performed by allowing the synthesised compounds to react with cysteine in a controlled environment. S-nitrosylation took place based on the compounds' ability to donate nitrogen monoxide and the unreacted cysteine residues were methylated using MMTS. Biotin-HPDP was added and the nitrosylated cysteines formed disulfide bonds with it, splitting off pyridine-2-thione, and absorbing UV-radiation at a wavelength of 343 nm. The intensity of this absorption was used as an indication of the extent to which S-nitrosylation took place. All of the compounds synthesised exhibited very

significant ($p < 0.01$) S-nitrosylation capacity, with the nitrate compounds showing better S-nitrosylation than the unsaturated nitro compounds.

The channel activity of the polycyclic amines was evaluated using a Ca^{2+} flux assay. Fresh synaptoneurosomes were prepared from rat brain homogenate and incubated with the fluorescent ratiometric indicator, Fura-2/AM. The synaptoneurosomes were incubated with the synthesised compounds after which 100 mM KCl solution was added to depolarise the cell membranes and allow Ca^{2+} to enter. The decrease in fluorescent intensity relative to a (100 %) control was related to the ability of the test compounds to block the Ca^{2+} channels. Two positive controls were introduced; NGP1-01, the lead pentacycloundecylamine, and the dihydropyridine L-type Ca^{2+} channel antagonist, nimodipine. At concentrations of 10 μM, all the compounds exhibited better Ca^{2+} channel antagonism than NGP1-01, with compounds **3.1** (96.3 %) and **3.3** (88 %) showing a very significant inhibition ($p < 0.01$) and **3.4** (92.2 %) showing significant inhibition ($p < 0.05$) when compared to the control. Compounds **3.1** and **3.3** also exhibited significant inhibition ($p < 0.01$) at concentrations of 1 and 0.1 μM. At concentrations of 10 and 1 μM, compound **3.1** exhibited better Ca^{2+} channel blockade then the commercially available nimodipine. Compounds **3.3** and **3.4** also showed comparable results to that of nimodipine at higher concentrations.

Although no clear correlation was observed between the S-nitrosylation capabilities of the compounds and their Ca^{2+} channel activity, it is possible that other factors might play a more decisive role in the mechanism of pentacycloundecylamine channel antagonism. This could include the geometric and steric bulk considerations that have been proven to contribute to the Ca^{2+} channel activity of the pentacycloundecylamines (Malan *et al.*, 1996:125 & 2000:10). The steric freedom brought on by the linker also seemed to have an influence on the channel activity. All the compounds synthesised exhibited promising Ca^{2+} activity and therefore show promise as potential agents against neurodegeneration. The compounds were also proven to be S-nitrosylators and can, therefore, have possible implications in other studies and/or fields of science.

UITTREKSEL

'n Nuwe reeks polisikliese amiene, wat stikstofmonoksiedskenkende groepe bevat, is gesintetiseer en getoets vir neuronbeskermende aktiwiteit. Hierdie studie is gegrond op die onlangse ontwikkeling van 'n reeks nitromemantiene. Die stikstofmonoksiedskenkende groepe in hierdie verbindings skenk 'n stikstofmonoksied aan die tiolgroepe van sekere kritiese sisteïenresiduë in die reseptorkanaal, 'n proses bekend as S-nitrosilering. Laasgenoemde lei dan tot die vorming van disulfiedbindings, allosteriese veranderinge en desensitisering van die spesifieke reseptor. Die hipotese is dat die nitromemantiene beter Ca^{2+} kanaal aktiwiteit en dus neurobeskermende aktiwiteit as memantien alleen sal toon. Na aanleiding van die strukturele ooreenkomste tussen die pentasikloundekielamiene en memantien, is daar besluit om die invloed van stikstofmonoksiedskenkende groepe op die kanaalaktiwiteit van pentasikloundekielamiene te ondersoek.

Na 'n deeglike struktuur-aktiwiteitsverwantskapstudie en beplanning, is 'n reeks pentasikloundekielamiene gesintetiseer. Hierdie reeks verbindings het ook elkeen beskik oor 'n stikstofmonoksiedskenkende groep. Verbeterde steriese vryheid is aan die verbindings verskaf deur die inkorporering van 'n koolstofketting tussen die hokkiestruktuur en die aromatiese groep. Esterifikasie is bewerkstellig deur gebruik te maak van 1-etiel-3-(3'-dimetielamino)karbodiïmiede (EDC), 'n aktiverende groep. Die gesintetiseerde verbindings kan in twee groepe geklassifiseer word op grond van hul stikstofmonoksiedskenkende groepe, naamlik die onversadigde nitroverbindings (**3.1**, **3.2** en **3.3**) en die nitrate (**3.4**, **3.5** en **3.6**). Die nitrate is verkry deur hidroksielgroepe te laat reageer met tionielchloriednitraat. Struktuuropklaring is gedoen deur gebruik te maak van een- en tweedimensionele kernmagnetieseresonans (KMR) spektroskopie, infrarooi (IR) absorpsie spektrofotometrie en massa spektrometrie (MS).

Evaluering van S-nitrosilerings is uitgevoer deur die gesintetiseerde verbindings toe te laat om met sisteïen te reageer, in 'n gekontroleerde sisteem. S-nitrosilering vind dan plaas op grond van die verbindings se vermoë om stikstofmonoksied te skenk. Die ongereageerde sisteïen is gemetileer deur die byvoeging van metielmetaanetiosulfonaat (MMTS) en sodoende uit die res van die reaksie gehaal. Biotin-HPDP is bygevoeg, waarna die genitrosileerde sisteïenresiduë disulfiedbindings daarmee vorm en piridien-2-tioon afsplit.

Piridien-2-tioon absorbeer ultraviolet (UV) straling by 'n golflengte van 343 nm. Die intensiteit van hierdie absorpsie is direk eweredig aan die mate waartoe S-nitrosilering plaasgevind het. Al die gesintetiseerde verbindings het baie betekenisvolle ($p < 0.01$) S-nitrosileringskapasiteit getoon. Die nitraatverbindings het beter S-nitrosilering getoon as die onversadigde nitroverbindings.

Die kanaalaktiwiteit van die pentasikloundekielamiene is bepaal deur gebruik te maak van 'n fluoresserende kalsiumfluksevaluering. Vars sinaptoneurosome is berei vanaf rotbreinhomogenaat en is geïnkubeer met die fluoresserende indikator, Fura-2/AM. Die sinaptoneurosome is daarna geïnkubeer met die gesintetiseerde verbindings, waarna 100 mM KCl-oplossing bygevoeg is om die selmembrane te depolariseer en kalsium in staat te stel om die sel binne te gaan. Die afname in fluoressensie intensiteit relatief tot die (100 %) kontrole is 'n aanduiding van die vermoë van die verbindings om kalsium kanale te blokkeer. Twee positiewe kontroles is gebruik; NGP1-01, die leidraad pentasikloundekielamien en die dihidropiridien L-tipe kalsiumkanaalantagonis, nimodipien. By konsentrasies van 10 µM het al die gesintetiseerde verbindings beter antagonisme as NGP1-01 getoon, met verbindings **3.1** (96.3 %) en **3.3** (88 %) wat 'n baie betekenisvolle inhibisie ($p < 0.01$) en **3.4** (92.2 %) wat 'n betekenisvolle inhibisie ($p < 0.05$) relatief tot die kontrole getoon het. Verbindings 3.1 en 3.3 het ook baie betekenisvolle inhibisies getoon by laer konsentrasies. Verbinding **3.1** het selfs beter inhibisie getoon as nimodipien by konsentrasies van 10 en 1 µM. Verbindings **3.3** en **3.4** het ook inhibisie vergelykbaar met nimodipien gelewer by hoër konsentrasies.

Geen ooglopende korrelasie tussen die S-nitrosilering van die verbindings en hul kalsiumkanaalaktiwiteit is waargeneem nie. Dit is egter moontlik dat ander faktore 'n groter rol kan speel in die meganisme van die kalsiumkanaalantagonisme van die pentasikloundekielamiene. Dit kan die geometriese en steriese grootte oorwegings wees wat bekend is om 'n invloed te hê op die kalsiumkanaalaktiwiteit van die pentasikloundekielamiene (Malan *et al.*, 1996:125 & 2000:10). Die steriese vryheid wat verkry is deur die insluiting van 'n langer koolstofketting tussen die hokstruktuur en die aromatiese groep blyk wel 'n invloed te hê op die kanaalaktiwiteit. Al die verbindings wat gesintetiseer is het belowende kalsiumkanaalaktiwiteit getoon en beskik steeds oor die potensiaal as toekomstige geneesmiddels wat teen neurodegenerasie ingespan kan word. Die reeks is ook potente S-nitrosileerders en mag moontlike toepassings vind in ander studies en/of velde van die wetenskap.

LIST OF ABBREVIATIONS

3D-QSAR	Three-dimensional quantitative structure-activity relationship
AM	Acetoxymethyl ester
AV	Atrioventricular
Aβ	β-amyloid peptide
Biotin-HPDP	N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide
CNS	Central nervous system
CoMFA	Comparative molecular field analysis
Cys	Cysteine
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization
DMAP	4-(dimethylamino)-pyridine
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EDC	1-ethyl-3-(3'-dimethylamino)carbodiimide
EtOAc	Ethyl acetate
Fura-2/AM	2-[5-[2-[(acetyloxy)methoxy]-2-oxoethoxy]-6-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-2-benzofuranyl]-(acetyloxy)methyl ester
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glu	Glutamate
Gly	Glycine
GTN	Glyceryl trinitrite
HEK	Human Embryonic Kidney 293
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
(H,H)-COSY	(proton, proton) – correlated spectroscopy
HMBC	Heteronuclear multiple quantum coherence
HNO	Nitroxyl
HSQC	Heteronuclear single quantum coherence
IC_{50}	The concentration of an inhibitor that is required for 50- percent inhibition of a specific reaction

IR	Infrared
ISDN	Isosorbide dinitrite
MK-801	Dizocilpine
MMTS	Methyl methanethiosulfonate
MP	Melting point
MS	Mass spectrometry
NO	Nitrogen monoxide
N_2O	Nitrous oxide
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
nNOS	Neuronal nitric oxide synthase
$ONOO^-$	Peroxynitrite
p38 MAPK	p38 mitogen-activated protein kinase
PA	Pharmacophore analysis
PCP	Phencyclidine
PE	Petroleum ether
SAR	Structure-activity relationship
SCAM	Substituted cysteine accessibility methods
SEM	Standard error of means
Std*coeff	Standard-deviation-times-coefficient
STDEV	Standard deviation
TCP	Tranylcypromine
THF	Tetrahydrofurane
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

SUMMARY

Over the past two decades polycyclic cage amines have received intense scrutiny as possible neuroprotective drugs. Amongst the aminoadamantanes, memantine (Namenda®) is already in use as an anti-Parkinsonian drug. Another class of polycyclic cage amines is the pentacycloundecylamines. Their structural similarity to memantine and calcium channel activity is well documented, making them excellent leads for novel drugs. Over activation of NMDA receptors, which is in itself a calcium channel, has been proven to play a role in most major neurodegenerative disorders. The affinity of the pentacycloundecylamines for NMDA receptors mean that they can also serve as a scaffold for target specific delivery. Conjugating a NO donating moiety to the pentacycloundecylamine cage structure to nitrosylate crucial cysteine residues on the NMDA receptor is thus possible. S-nitrosylation causes disulfide bond formation and the subsequent allosteric modification desensitises the receptor, thereby providing an alternative mechanism of modulating over stimulated NMDA receptors.

1.1 BACKGROUND

Dementia is a leading cause of death and disability worldwide. According to Lipton (2006:160) Alzheimer's disease, which is the leading cause of dementia, ranks fourth in mortality in the United States, followed closely by vascular dementia (multi-infarct dementia).

Glutamate-related excitotoxic neuronal cell injury and death has been reported to contribute to almost every major neurodegenerative disorder. This mechanism can also harm oligodentrocytes, which provides the myelin sheaths of axons. This means that excitotoxicity can cause white matter (glial) as well as grey matter (neuronal) disorders. One of the ways excitotoxicity can occur is by over activation of NMDA-sensitive glutamate receptors and the subsequent excessive Ca^{2+} influx into the associated cell.

1.2 EXCITOTOXICITY AND NEURODEGENERATIVE DISORDERS

Glutamate is a powerful neurotransmitter on which the nervous system's capacity to rapidly convey sensory information and its ability to form thoughts and memories is dependant. Because glutamate is so powerful, its presence in large amounts, or for long periods, can literally excite cells to death. This was termed, excitotoxicity (Lipton, 2006:162).

There are numerous insults that can lead to excessive glutamate release and stimulation of glutamate receptors, with subsequent excitotoxicity and cell death. These insults include, amongst others, head or spinal injury and stroke. All of these share the same pathway in that large amounts of glutamate is released from the injured cells which then reach the thousands of nearby cells, causing them to depolarise, swell, lyse and die by necrosis. The lysed cells release more glutamate, leading to a cascade of autodestructive events and progressive cell death that can continue for hours to days after the original injury. There are also subtler forms of excitotoxicity apparent in the penumbra of acute stroke damage, vascular dementia and Alzheimer's disease (*via* familial mutated $A\beta_{1-42}$ and hyperphosphorylated tau proteins). In these chronic diseases, it is proposed that modest amounts of glutamate over an extended period are to blame for the excitotoxic damage (Lipton, 2006:162).

1.2.1 THE MECHANISM OF NMDA RECEPTOR MEDIATED EXCITOTOXICITY

Over activation of the NMDA receptor causes excessive Ca^{2+} influx into the nerve cell, leading to cellular damage and death. In chronic diseases, this excessive influx of Ca^{2+} triggers a signalling cascade that leads to synaptic damage and eventually apoptotic-like cell death (figure 1.1).

These injurious processes include Ca^{2+} overload of the mitochondria with subsequent free-radical formation, activation of caspases and release of apoptosis-inducing factors. Furthermore, it produces nitric oxide *via* Ca^{2+} dependent activation of neuronal nitric oxide synthase (nNOS). The produced nitric oxide reacts with superoxide to produce toxic peroxynitrite ($ONOO^-$) and S-nitrosylated glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Stimulation of the p38 mitogen-activated protein kinase (p38 MAPK), which activates transcription factors that enter the nucleus, also lead to apoptosis (Lipton, 2006:162).

1.3 RATIONALE AND AIM OF STUDY

The aim of this study was to evaluate the influence of nitrosylation on the channel activity of polycyclic cage amines as a means of attenuating excitotoxicity. This was achieved by the synthesis of various novel pentacycloundecylamines, because of their well documented

calcium channel activity and the fact that they are open channel blockers (Van der Schyf *et al.*, 1988:448).

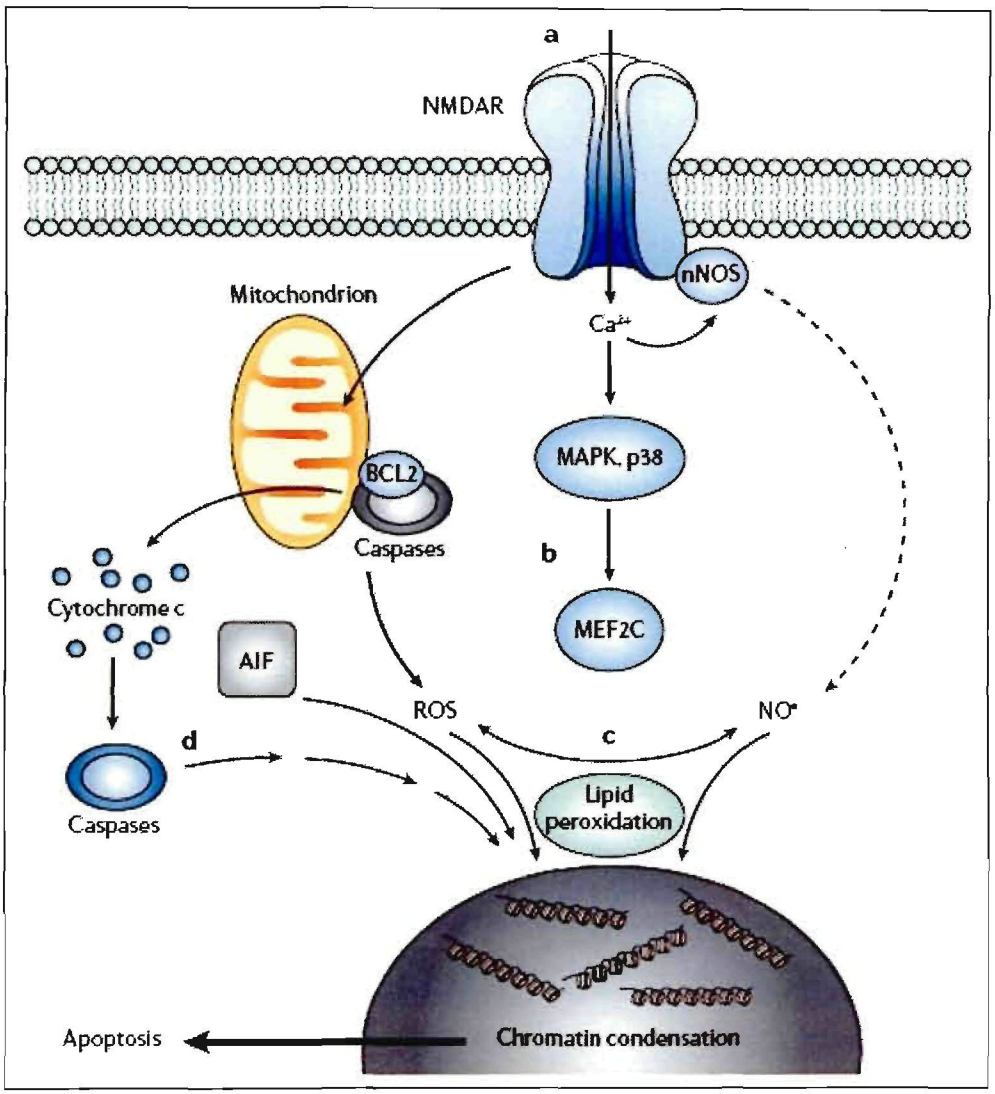


Figure 1.1: A schematic representation of how excessive NMDA receptor activation would lead to apoptotic-like cell injury and death (Lipton, 2006:162).

To augment the potential NMDA receptor antagonistic effects of the pentacycloundecylamines, each compound was conjugated to a nitrogen monoxide donating moiety, to S-nitrosylate crucial cysteine residues in the NMDA receptor, leading to receptor desensitisation. The pentacycloundecylamines thus served as not only calcium channel antagonists, but also as a scaffold to optimise NO delivery to the target receptor's cysteine residues, as systemic NO delivery will cause adverse effects associated with vasodilatation.

The effects of these compounds on both calcium channels and cysteine residues were tested by means of biological assays and the results evaluated to see if S-nitrosylation does in fact augment the calcium channel activity of polycyclic cage amines.

Based on the literature, it was decided to synthesise two different types of nitrogen monoxide donating compounds. The first three compounds were unsaturated nitro compounds (figure 1.2), and the second group consisted of three nitro esters, or nitrates (figure 1.3). All of the compounds complied with the structural requirements necessary for NMDA receptor antagonism.

To evaluate the biological effects of the synthesised compounds, two biological assays were performed. The first was used to detect whether the compounds cause S-nitrosylation and to determine their nitrosylation efficiency, while the second assay was used to evaluate the changes in calcium flux brought on by the synthesised compounds.

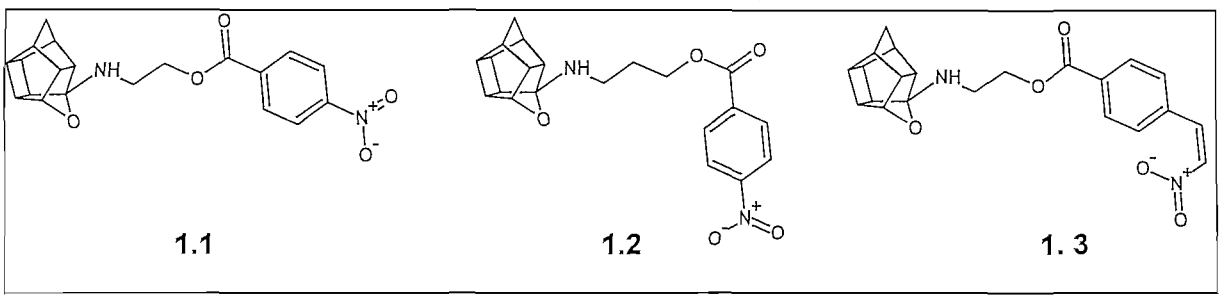


Figure 1.2: The unsaturated nitro compounds to be synthesised in this study.

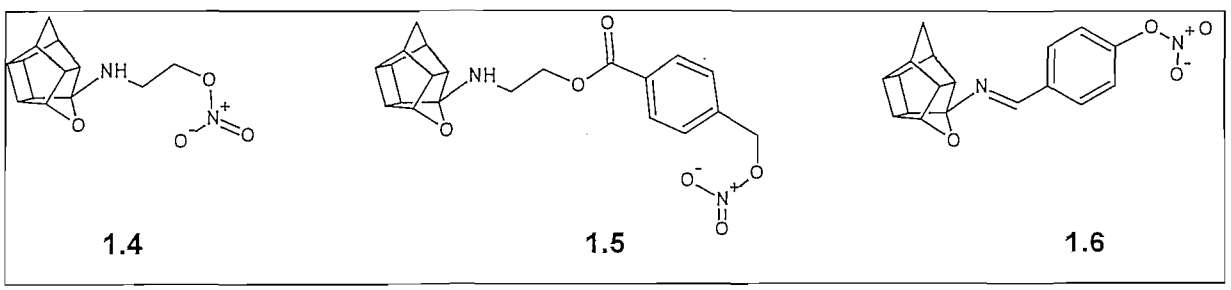


Figure 1.3: The nitrate containing compounds to be synthesised for this study.

Jaffrey *et al.* (2001:193) developed a system to detect S-nitrosylated proteins by converting nitrosylated cysteines into biotinylated cysteines. In the first step, free thiols were blocked with the thiol-specific methylthiolating agent MMTS. MMTS doesn't react with pre-existing disulphide bonds or nitrosothiols. The second step involved the reaction of the newly formed

thiols with biotin-HPDP, a sulfhydryl-specific biotinylating reagent and measuring the formed pyridine-2-thione by means of ultraviolet (UV) absorbance.

For the calcium flux assay spectrophotometry was once again employed. Synaptoneurosomes prepared from rat brain homogenate and incubated with the fluorescent indicator, Fura-2/AM (**1.7**), were used to determine the effects of the novel compounds on KCl-induced depolarisation (Geldenhuys *et al.*, 2007:1531). Comparing the calcium channel data with that of the S-nitrosylation assay gave an indication as to the influence of nitrosylation on the channel activity of polycyclic cage amines.

CHAPTER 2

LITERATURE

SUMMARY

Neurodegenerative disorders have a huge impact on not only the people suffering from the disorder, but also in their families. With its prevalence increasing, the world is eagerly searching for new drugs to help improve the quality of life for these patients. The treatment of excitotoxicity is imperative in order to combat major neurodegenerative disorders. By combining an open channel blocker, such as the pentacycloundecylamines, with the ability to nitrosylate crucial cysteine residues in the target NMDA receptor, a novel NMDA antagonist can be proposed. This chapter includes the literature used to plan the study and make decisions on the synthesis of the compounds as well as the biological tests that were performed to test the influence of nitrosylation on the calcium channel activity of polycyclic cage amines.

2.1 THE N-METHYL-D-ASPARTATE (NMDA) RECEPTOR

2.1.1 BACKGROUND

Under normal physiological conditions, glutamate receptors mediate excitatory synaptic transmission in the brain and is therefore crucial for normal brain functioning. The glutamate-gated ion channels, or ionotropic channels, can be divided into three classes, the AMPA receptors, the kianate receptors and the NMDA receptors (Lipton, 2006:160).

Of these three receptors, the NMDA receptor is the most permeable to Ca^{2+} and excessive activation of this receptor will lead to increased intracellular Ca^{2+} and the production of damaging free radicals as well as the activation of proteolytic processes, all of which contribute to cell injury or death.

The NMDA receptor is an unusual ligand-gated ion channel, because binding of the endogenous agonists, glutamate and glycine, needs to be accompanied by Mg^{2+} relief from the channel itself before activation can take place.

2.1.2 THE STRUCTURE OF THE NMDA RECEPTOR

The NMDA receptors are heteromeric ligand-gated ion channels, comprising of four subunits, of which the NR1, NR2 and NR3 subunits have been defined. In functional NMDA receptors, only the NR1 and NR2 subunits are obligatory; the NR3 subunit merely modulates NMDA receptor properties. For the channel to be activated, the binding of the two endogenous agonists to their binding sites is necessary, glutamate (Glu) at the NR2 subunit and glycine (Gly) at the NR1 subunit, as shown in figure 2.1 (Johnson & Kotermanski, 2006:62).

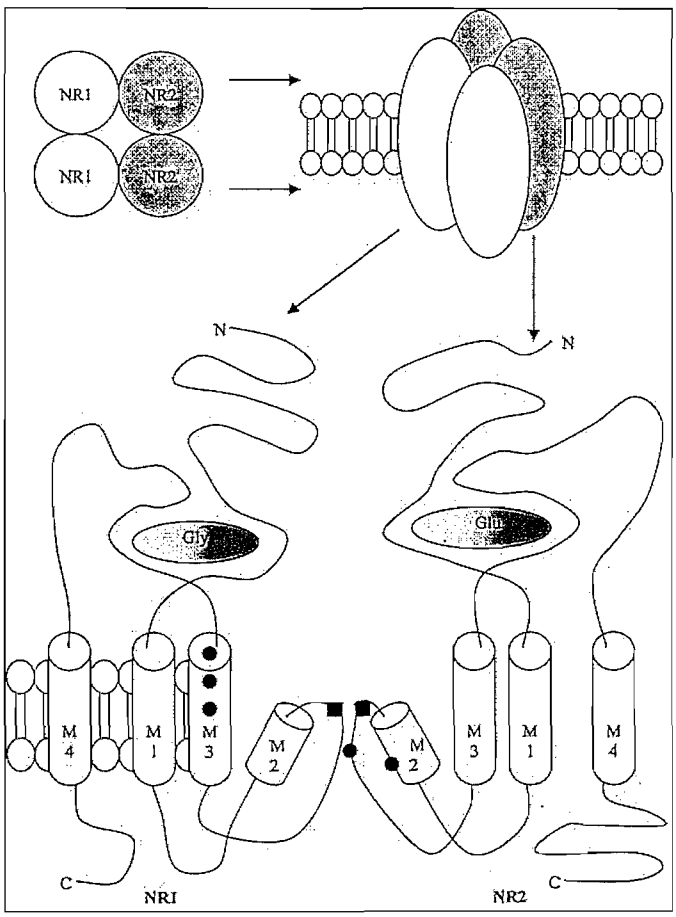


Figure 2.1: A model diagram of the structure of the NMDA receptor, adapted from Johnson & Kotermanski (2006:62).

In figure 2.1, the locations of asparagine residues which are critical for channel blockade (referred to as the "N-sites") are shown as blocks. The circles indicate the locations where mutations of the amino acids cause a greater than threefold change in memantine IC_{50} values (Johnson & Kotermanski, 2006:62).

2.1.3 ANTAGONISTS ON THE NMDA RECEPTOR

Wong and Kemp (1991:401) stated that antagonists of the NMDA receptor can be categorised into four groups. These groups are determined by the site of action on the receptor-channel complex, which include the NMDA agonist (glutamate) site, the co-agonist (glycine) site, the channel pore and the modulatory sites. The modulatory sites include the redox modulatory site, the proton-sensitive site, the high-affinity Zn^{2+} site and the polyamine site (figure 2.2).

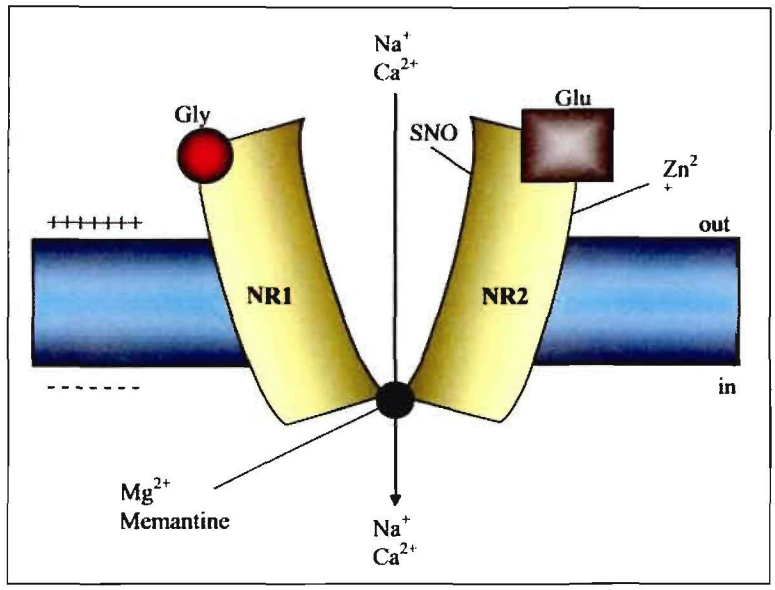


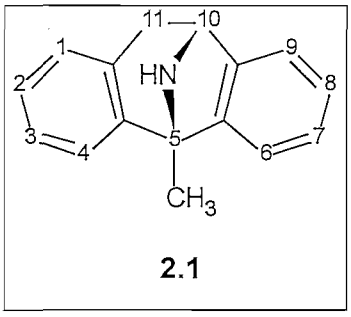
Figure 2.2: A model showing the important binding and modulatory sites on the NMDA receptor (Chen and Lipton, 2006:1615).

The two most important antagonist sites for this study are the open channel blocking sites within the receptor channel, and the S-nitrosylation site(s), indicated as (SNO) in figure 2.2, located toward the N-terminus (extracellular) region of the receptor.

2.1.4 STRUCTURE ACTIVITY RELATIONSHIPS OF NMDA RECEPTOR ANTAGONISTS

Since the mid 1980's it was known that there were certain common features shared by many of the central nervous system (CNS)-active drugs, such as a hydrophobic moiety, mostly an aromatic ring, and a basic nitrogen atom. This nitrogen atom may be charged either by protonation or quaternisation. In their model of the CNS-active pharmacophore, Lloyd and Andrews (1986:453) concluded that the optimal distance between the centre of the aromatic ring and the nitrogen atom is 5.0 Å. The central part of their model, however, was the requirement for a hydrogen bond to be formed between the protonated nitrogen atom and a receptor site situated tetrahedrally 2.8 Å away from the nitrogen atom.

One of the problems associated with these studies was the use of conformationally flexible ligands, which were good for molecular matching, but gave a poor indication of the critical spatial relationships between the nitrogen atoms and the aromatic rings. It was this problem that Leeson *et al.* (1990:1296) sought to overcome, by using a series of structurally rigid congeneric derivatives of dizocilpine (MK-801) (**2.1**). The reason for selecting MK-801 derivatives was because it is a conformationally rigid, potent, selective and uncompetitive antagonist on the NMDA receptor, which could give an unambiguous evaluation of the specific nitrogen-aryl ring geometry associated with ligand recognition. Both aryl- and alkyl-substituted cycloheptenimines served as bulk tolerance indictors in their study.



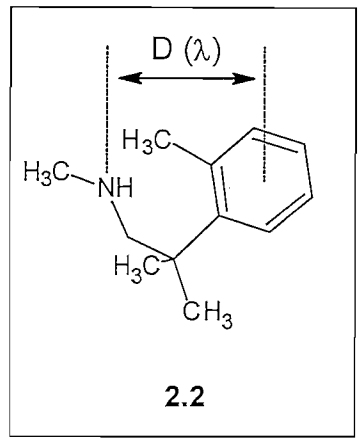
2.1

Figure 2.3: The cycloheptenimine structure of MK-801 (**2.1**) which served as the reference ligand (Leeson *et al.*, 1990:1296).

The study of Leeson *et al.* (1990:1299), found that methyl substitution at positions 1, 2, 8 and 9 reduced the affinity by at least one order of magnitude. The same were observed for benzo-substitution at positions 2, 3 and 7, 8. However, methyl substitution in positions 3, 7 and 10 were tolerated. The 3 and 7 positions also tolerate hydroxyl, halogen and methoxy substitution. Size-limitations for the hydrophobic bonding area of the aromatic rings are thus an essential requirement for NMDA receptor activity. The other essential requirement being the formation of a hydrogen bond between the protonated ligand and an acceptor group in the active site. They also found the direction of the hydrogen-bonding interaction to be important, since the imines and aziridines that were tested showed no activity. These two types of compounds share one difference from the other amines, the directionality of the lone pair of electrons on their nitrogens relative to the aromatic rings. Another discovery was that the distance between the nitrogen and the centre of the aromatic ring in the α-methylbenzylamine moiety i.e., their geometrical pharmacophore, showed significant statistical correlation with binding affinity, through equation 2.1:

$$pIC_{50} = -3{,}57D + 19{,}49 \qquad (2.1)$$

Where *D* is the distance between the nitrogen and the centre of the aromatic ring (figure 2.4).



2.2

Figure 2.4: The N-aryl distance (D) as depicted for α-methylbenzylamine (**2.2**), adopted from Leeson et al. (1990:1301).

The most potent compounds had a *D* value in the region of 3.4 Å (Leeson *et al.*, 1990:1301), which is significantly less than the 5.0 Å suggested by Lloyd and Andrews (1986:453). Furthermore, Leeson *et al.* (1990:1301) developed a model for the hydrogen bonding of PCP-binding site compounds. By examining the angle between the nitrogen of MK-801 (**2.1**) (N), a putative hydrogen bond acceptor (O) and the nitrogen of the other NMDA antagonist (N) the resulting N-O-N angle, θ, can be correlated with binding affinity through equation 2.2. The smaller the angle between the two nitrogens, the higher its binding affinity was found to be.

$$pIC_{50} = -0{,}088\theta + 7{,}63 \qquad (2.2)$$

Leeson *et al.* (1990:1296) thus developed two excellent models for the correlation of binding affinity with the two (then main) structure activity descriptors of NMDA receptor antagonists, the hydrophobic moiety and the hydrogen bonding nitrogen atom.

Although the exact three-dimensional configuration of the NMDA receptor, and hence the antagonist binding site, is still unknown, it is possible to apply other techniques such as the above mathematical theories and three-dimensional quantitative structure-activity relationship (3D-QSAR) to determine a good pharmacophore for NMDA receptor activity.

This was exactly what Kroemer *et al.* (1998:393) did when they decided to try and find structural requirements for NMDA receptor blockade. They knew that uncompetitive NMDA receptor inhibitors bind to the phencyclidine (PCP) binding site which is located inside the receptor channel. They also knew to search for antagonists with a K_i value of above 200 nM, because strong psychotomimetic side effects are common with drugs in which the affinity for the receptor is too high. It was also known that previous studies on the structural

requirements for PCP binding activity incorporated compounds that where known for their activity, but where all structurally similar, such as PCP (**2.3**) derivatives and MK-801-like molecules.

An experiment measuring the activities of several structurally diverse compounds, ranging from ketamine (**2.6**) and MK-801 (**2.1**) to adamantane amines, procyclidine (**2.5**) and alaproclate (**2.4**) was done. After determination of these compound's inhibition constants they employed a mathematical method called graph theory, in which each molecule is represented by points of pharmacological interest, for example, "positively charged atom", "centre of hydrophobic moiety" or "hydrogen bond acceptor/donor". Basically each molecule is described as a number of these points as well as the distances between them, in mathematical terms, a graph. One of the molecules was then selected to serve as reference, normally the one with the highest affinity, and all the structures that did not bind to the NMDA receptor were left out. Using maximum deviation point distances, the remaining compounds where "matched" to the reference compound, PCP (**2.3**), and divided into a subgraph. Finally they applied the 3D-QSAR technique called comparative molecular field analysis (CoMFA), made easy because of the subgraph's ability to be superimposed, to calculate the steric and electrostatic interaction energies between a probe atom and a set of superimposed molecules (figure 2.5).

The results of their experiment showed early on that two features were shared by all the compounds. The first being a protonated amine represented by three points of pharmacological interest, namely: a nitrogen "donor atom", a partial positive charge at the hydrogen and the putative interaction site, such as a suitable amino acid residue, at the receptor. The distance between this interaction site and the hydrogen is approximately 2 Å. The second feature of the pharmacophore was that it contained a hydrophobic moiety, for example the cyclohexyl ring of PCP.

While performing the QSAR model a very high degree of internal consistency appeared between the measured inhibition constants and the corresponding structural data. Very useful results of such a QSAR analysis are the standard-deviation-times-coefficient ("std*coeff") fields. These steric "std*coeff" fields combine information about the variance of interaction energies and the impact of putative modifications on biological activity and can therefore reveal features that influence the binding abilities of molecules.

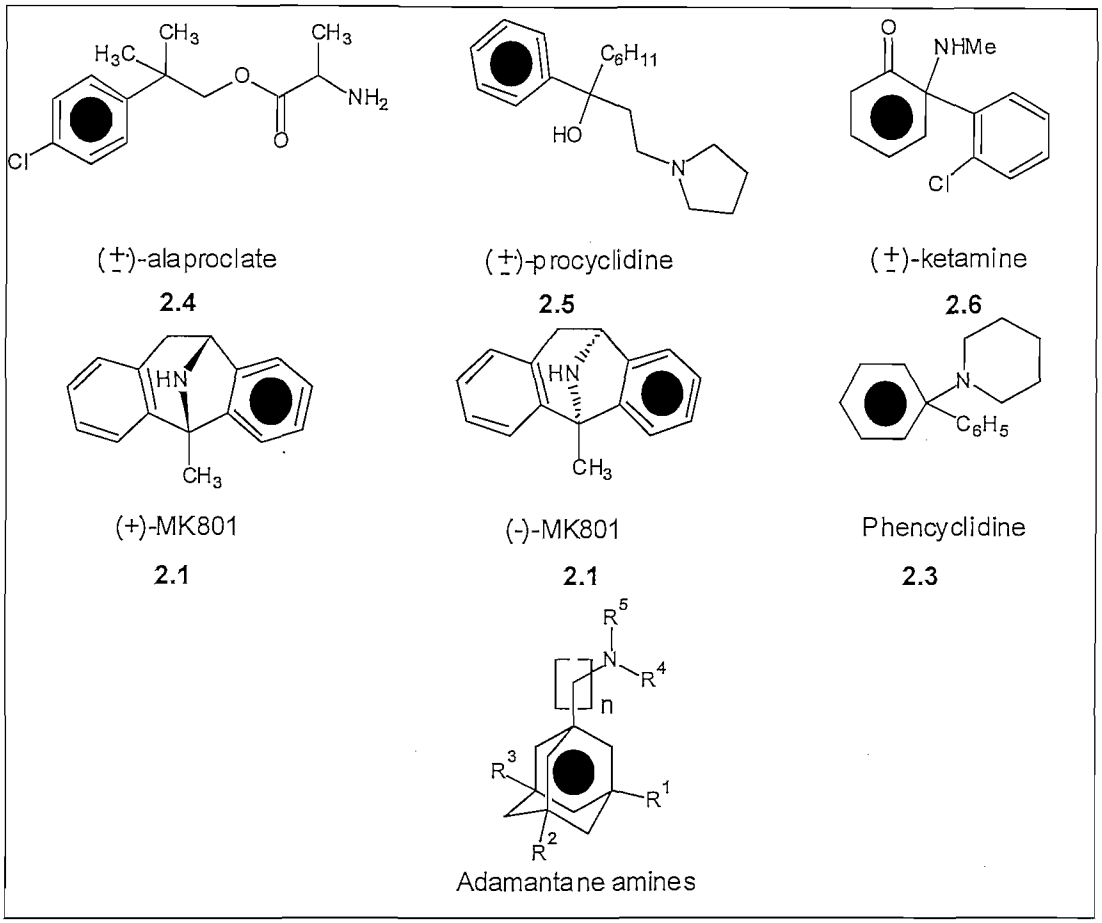


Figure 2.5: Some of the compounds used by Kroemer *et al.* (1998:395). The location of the hydrophobic pharmacophore point is illustrated by black-filled circles.

These steric fields brought new information to the forefront concerning the hydrophobic moiety of the pharmacophore analysis (PA). It showed that the hydrophobic moiety's interaction with the receptor is largely dependent on steric factors. Compounds like the adamantane amines, with a more bulky hydrophobic moiety, were less active than PCP (**2.3**). The polycyclic cage structure is simply too bulky for the receptor and doesn't count as a hydrophobic point of pharmacological interest any more. The 3D-QSAR analysis also indicated a third interaction between the molecule and the receptor: All the compounds with substituents in the same position as the aromatic rings of PCP (**2.3**) and MK-801 (**2.1**) have increased activity. This hydrophobic interaction with the receptor could be required for high-affinity binding. A summary of all these analytical results can be found in figure 2.6.

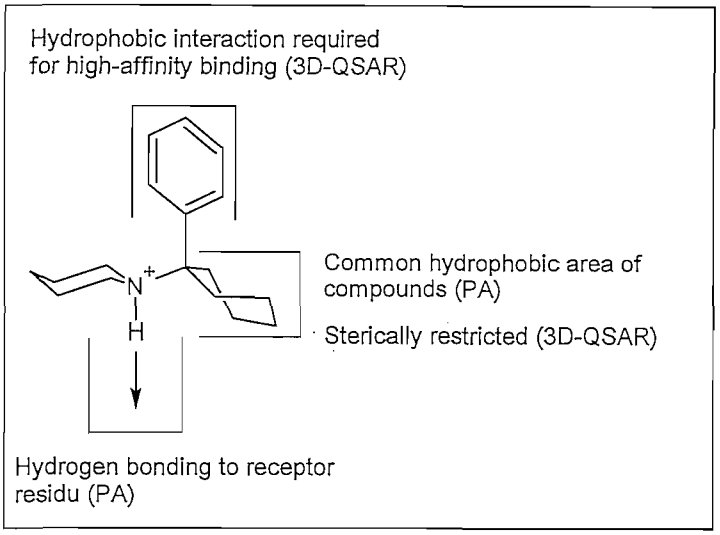


Figure 2.6: A combination of the results of the pharmacophore analysis (PA) and the 3D-QSAR, showing the structural requirements for PCP binding site blockers, using PCP as the reference compound, adapted from Kroemer *et al.* (1998:395).

Kroemer *et al.* (1998:398) also speculated that the more rigid structures did not loose as much entropy upon binding to the receptor and that this could be why MK-801 (**2.1**) had higher activity than PCP (**2.3**), which is less rigid.

In a follow-up study done by Jirgensons *et al.* (2000:555) the search for structure-activity relationships between NMDA receptor antagonists was continued. This team was also aware of the psychotomimetic side effects of drugs with a too high affinity for the NMDA receptor and indicated that the modest affinity of the two clinically used polycyclic drugs, amantadine (**2.7**) and memantine (**2.8**), was the reason why structure-activity relationship information on the adamantane class of compounds was limited.

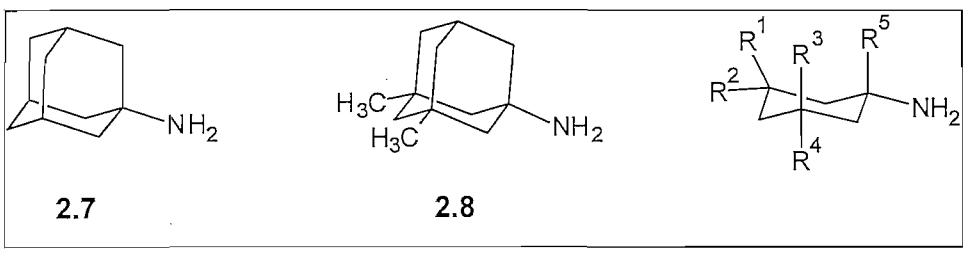


Figure 2.7: The chemical structures of amantadine (2.7), memantine (2.8) and the 1,3,5-substituted cyclohexylamines.

A series of 1,3,5-substituted cyclohexylamines were synthesised, to serve as structural analogs for amantadine (**2.7**) (figure 2.7). The variation of the substituents on these cyclohexylamines would then yield an estimation of the dependence of the size of the hydrophobic globule on the binding affinity. These 1,3,5-substituted cyclohexylamines differ in two aspects: their lipophilicity and steric requirements. Based on these differences the

Hansch analysis was selected for the structure-activity relationship (SAR) evaluation, because it can incorporate not only lipophilicity, but also steric effects. What their Hansch analysis showed was that for the compounds with little to no steric interference, there was a linear function between their affinity (log 1/Ki) and their lipophilicity (log P). This indicated that they fit properly on the PCP binding site, where as the compounds with a large steric factor showed a clear deviation from linearity, indicating a poor fit (figure 2.8).

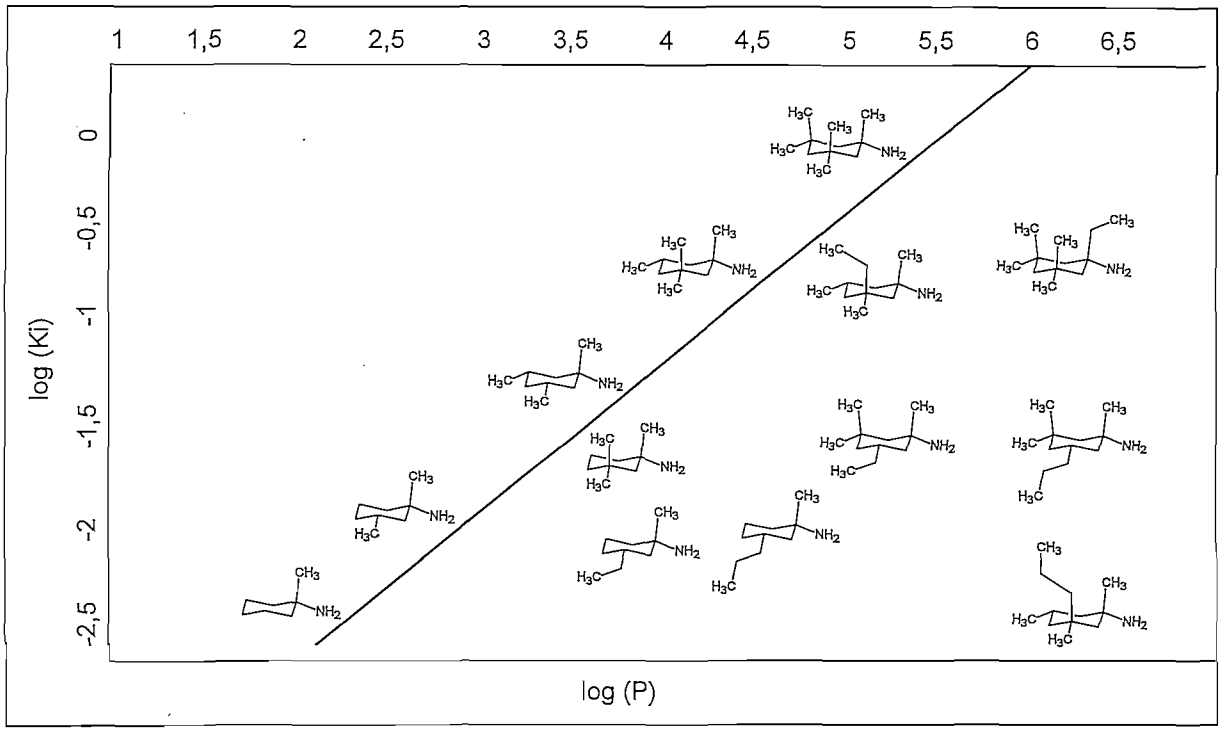


Figure 2.8: The Hansch analysis of some of the cyclohexylamines, adapted from Jirgensons *et al.* (2000:560).

With this taken into account, it is clear that polycyclic cage compounds, such as amantadine, memantine and the pentacycloundecylamines are simply too bulky to fit at the PCP binding site.

This was also realised by Geldenhuys *et al.* (2007:1530), when their group attempted to identify the binding site of pentacycloundecylamines using binding studies with [^{3}H]MK-801 and [^{3}H]TCP. They discovered that little to no displacement of the radio ligands took place, meaning that the pentacycloundecylamines have a different binding site, than the PCP binding site earlier speculated. Their group came up with the hypothesis that the phenyl ring, substituted onto the pentacycloundecylamine, can undergo a π-π type aromatic interaction with (an) aromatic amino acid(s) located at the entrance of the NMDA receptor. This interaction anchors the compound and allows the cage moiety to descend into the channel pore. The depth of this descend is determined by the length of the linker between the

aromatic moiety and the nitrogen of the pentacycloundecylamine. This descending mechanism could then bring the cage moiety with its protonated amino-group to close proximity with the PCP-binding site. This hypothesis however, is still under investigation.

2.2 S-NITROSYLATION

2.2.1 S-NITROSYLATION AND ITS ROLE IN RECEPTOR REGULATION

S-nitrosylation is the transfer of a nitrogen monoxide (NO) to a thiol (sulfhydryl) group (-SH) on a crucial cysteine residue (Lipton *et al.*, 2002:474) as shown in equation 2.3.

$$RS\text{-}H + NO \rightleftharpoons RS\text{-}NO + H \qquad (2.3)$$

Lipton *et al.* (2002:474) further stated that sulfhydryl reactions of cysteine residues with redox agents, transition metals or NO regulated molecules are responsible for modulation of protein activity. At least seven cysteine residues on NMDA receptor subunits are involved in regulation by redox agents, NO or Zn^{2+}. Certain cysteine residues are capable of reacting with an oxidising agent, NO or Zn^{2+} and these thiols are then said to be able to multi-task (figure 2.9).

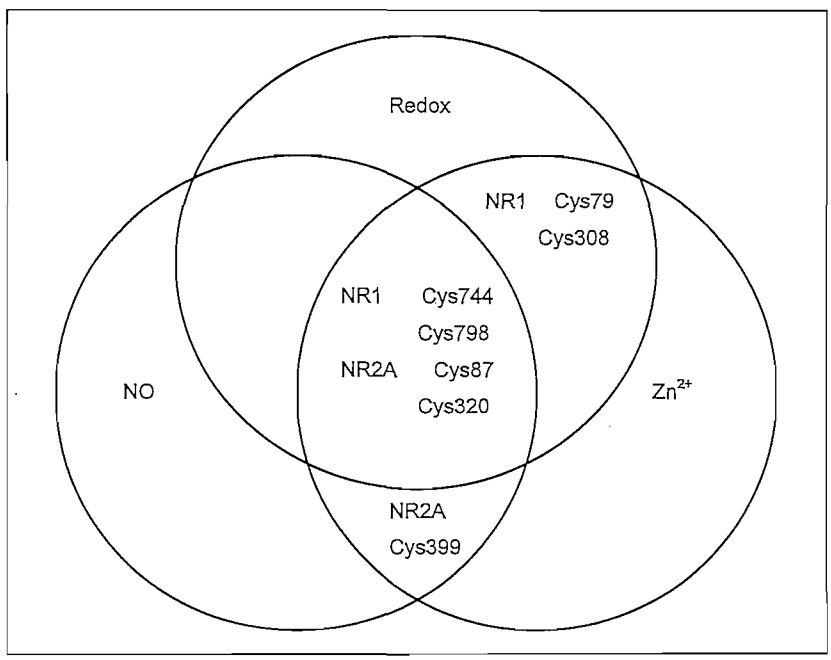


Figure 2.9: The multi-tasking of seven crucial cysteine residues important for modulation of the NMDA receptor as well as the subunits in which they can be found (Lipton *et al.*, 2002:474).

These potential competing reactions of the thiol groups of cysteine residues with nitric oxide and Zn^{2+} that regulate protein function can be found in the equations below (note that they are unbalanced, as far as charge and electron transfer goes, for simplicity).

$$RS\text{-}H + NO \rightleftharpoons RS\text{-}NO + H \quad (2.4)$$

$$RS\text{-}H + Zn \rightleftharpoons RS\text{-}Zn + H \quad (2.5)$$

$$RS\text{-}Zn + NO \rightleftharpoons RS\text{-}NO + Zn \quad (2.6)$$

Zn^{2+} has been shown to inhibit NMDA receptor neurotransmission at physiological levels, but only in NMDA receptors containing the NR2A subunit.

The five cysteine thiol groups that are involved in S-nitrosylation are Cys744 and Cys798 in the NR1 subunit, as well as Cys87, Cys320 and Cys399 in the NR2A subunit. Four of these are also involved in Zn^{2+} mediated inhibition of the NMDA receptor (figure 2.9).

These protein nitrosothiols (RS-NO), that are formed after nitrosylation, can react with adjacent thiol groups to form a disulfide bond.

2.2.1.1 DISULFIDE BOND FORMATION AS A RESULT OF S-NITROSYLATION

The chemistry of NO seems to favour disulfide-bond formation of thiol groups in the following, simplified, way.

$$RS\text{-}NO + RS\text{-}H \rightleftharpoons RS\text{-}SR + NO + H \quad (2.7)$$

The four cysteine residues that appear to favour this disulfide-bond formation through S-nitrosylation are Cys744 and Cys798 of NR1 and Cys87 and Cys320 of NR2A (figure 2.9). This disulfide bond formation following S-nitrosylation not only decreases NMDA-evoked responses, but also enhances the effect of Zn^{2+} (Lipton *et al.*, 2002:476), and cause an allosteric modification in the receptor protein structure.

2.2.1.2 ALLOSTERIC MODIFICATION AS A RESULT OF S-NITROSYLATION

According to Lipton *et al.* (2002:478) the predominant inhibitory effect of NO is mediated through a single crucial thiol group, that of Cys399 in the NR2A subunit. Enhancement of Zn^{2+} mediated inhibition of NMDA receptors via modification of Cys399 is the proposed mechanism of NO mediated NMDA inhibition. Zheng *et al.* (2001:894) recently demonstrated that receptor desensitisation resulted from enhanced glutamate affinity, which is caused by the inhibitory effect of Zn^{2+}. A model can now be postulated in which S-nitrosylation of Cys399 on NR2A allosterically modulates the quaternary organisation of the receptor, leading to enhanced glutamate binding and receptor desensitisation (figure 2.10).

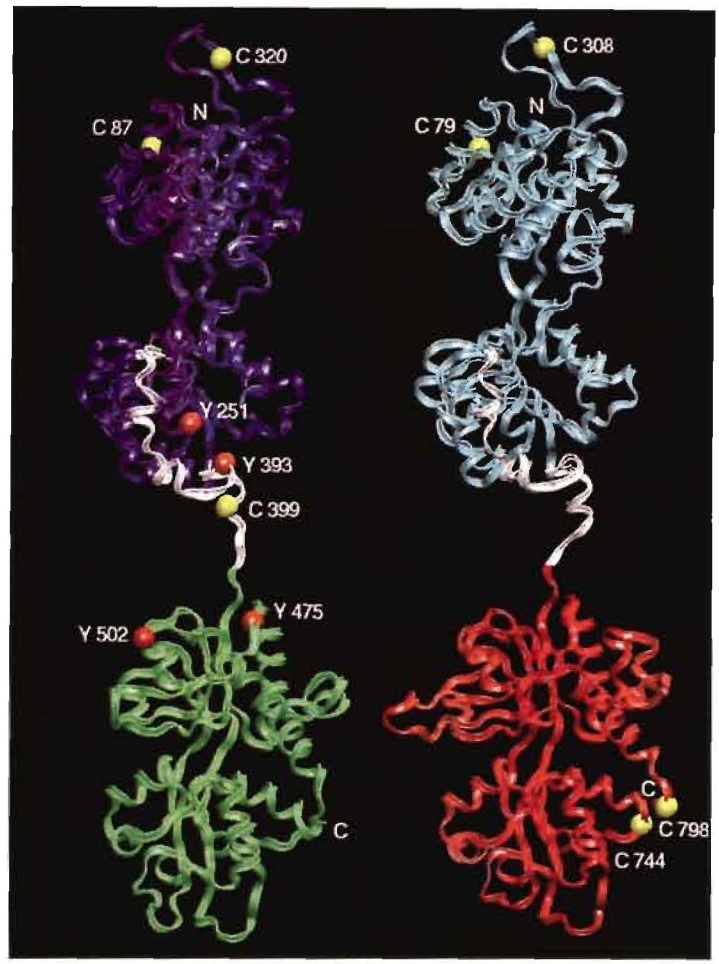


Figure 2.10: Predicted atomic structure of the extracellular domains of NR1 and NR2A, and the functional implications of the NMDA receptor. Cys399 (C399) can be found on the linker region separating the agonist-binding domain and the Zn^{2+}-binding domain of the NR2A subunit. Glutamate binds in the lower region of the NR2A subunit, while Zn^{2+} binds in the cavity of the upper region. Glycine's binding site is the cleft of the lower region of the NR1 subunit. The pale yellow balls represent cysteine residues involved in S-nitrosylation of the NMDA receptor, Cys744 and Cys798 of NR1 and Cys87, Cys320 and Cys399 of NR2A. Note that none of these residues can be found in the Zn^{2+}- or agonist-binding cavities, which is consistent with their allosteric mechanism of action. The red balls represent tyrosine residues (Y) presumably involved in the stabilization of π electrons of the NO group nitrosylating Cys399 (Lipton *et al.*, 2002:478).

Conversely, figure 2.11 gives a representation of the three quaternary states of the NMDA receptor as well as the inhibitory effect of S-nitrosylation. In state (i) there are two NR1 subunits and two NR2 subunits with open agonist binding sites. In states (ii) and (iii) glutamate (red ball) is bound to its binding site in NR2A subunit and glycine (dark-blue ball) is bound to its site in the NR1 subunit. In state (ii) the channel is open after the agonist binding and the linker separating the two extracellular domains is relaxed. In state (iii) Zn^{2+} (red square) is bound to its regulatory domain on the NR2A subunit and the linker region is tensed. This bending of the linker region stabilises the glutamate-bound state, slows the off-rate, increases glutamate affinity and eventually leads to receptor desensitisation. The yellow balls represent crucial cysteine residues that play a role in S-nitrosylation of the linker region

and potential disulfide bond formation due to redox modulation. The little red balls represent tyrosine residues that are possibly involved in stabilisation of the nitrosylating group (Lipton *et al.*, 2002:478).

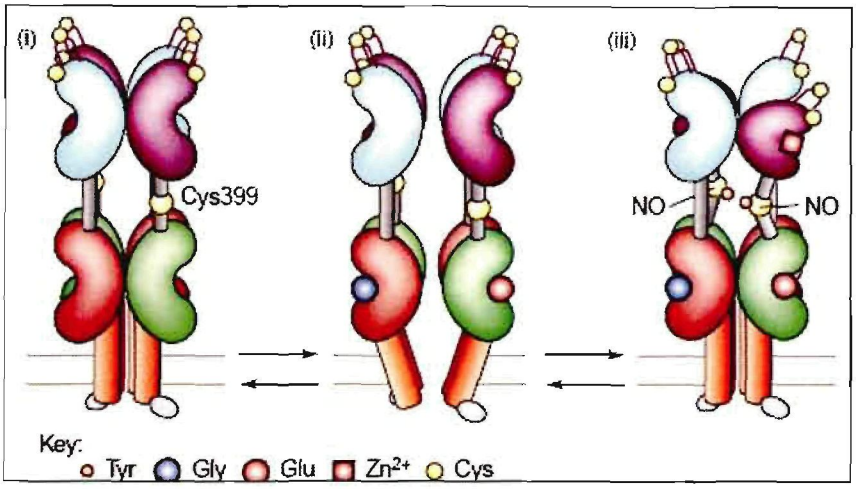


Figure 2.11: The three quaternary states of the NMDA receptor, Lipton *et al.* (2002:478).

2.2.1.3 NITROSYLATION OF CALCIUM CHANNELS IN GENERAL

As describe above, S-nitrosylation is the name used to denote the reversible transfer of NO to crucial cysteine residues in a target protein that has been deemed cytoprotective. Although the effect of nitrosylation on the NMDA receptor has been explained above, other calcium channels can also be S-nitrosylated, with protective consequences.

Eu *et al.* (2000:499) proved that the ryanodine receptor can be S-nitrosylated and Sun *et al.* (2006:403) also proved that L-type calcium channels can be S-nitrosylated. S-nitrosylation has also been discovered in Human Embryonic Kidney 293 (HEK) cells that express recombinant human cardiac calcium channel subunits (Carnes *et al.*, 2007:28063).

2.2.2 NITROGEN MONOXIDE DONATING MOIETIES

Organic nitrates and nitrites are the most commonly used NO donors (Chiroli *et al.*, 2003:442). As early as the nineteenth century, glyceryl trinitrite GTN (**2.9**) and amyl nitrite (**2.10**) were proposed as antianginal drugs. Isosorbide dinitrite (ISDN) (**2.11**) was discovered in the 1950's and had a longer duration of action than that of GTN (figure 2.12).

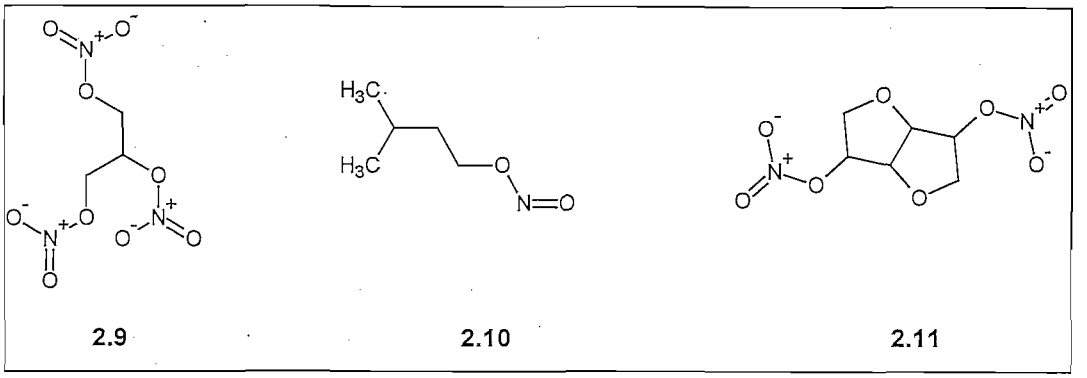


Figure 2.12: Examples of traditional NO donors, Chiroli *et al.* (2003:442).

Gorczynski *et al.* (2007:2013) suggested nitrated unsaturated fatty acids, as a unique class of NO-donating agents. Given the intense interest in new nitric oxide releasing agents Gorczynski *et al.*, suggested simple nitroalkenes with the same functional groups as the nitrated unsaturated fatty acids (figure 2.13).

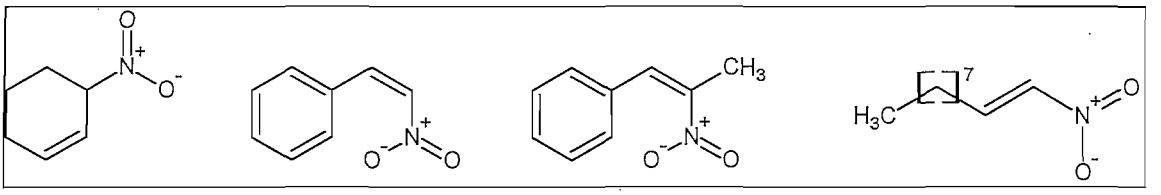


Figure 2.13: The structures of the nitroalkenes, adapted from Gorczynski *et al.* (2007:2014).

Another novel series of neuroprotective agents, currently being tested in the United States, are the nitromemantines. These compounds all contain a nitro group conjugated to memantine. Their effects are analogous to that of nitroglycerine, but by conjugating the nitro group to memantine a more site specific mechanism of NO-donation (and subsequent nitrosylation) can be achieved. This is important because systemic administration of nitroglycerine would lead to dangerous hypotension. This gives these compounds two possible mechanisms of modulating excessive receptor activity. Preliminary studies have indicated that nitromemantines are more effective neuroprotective agents than memantine in both the *in vitro* and *in vivo* animal models without displaying serious drops in blood pressure (Lipton, 2006:168).

2.3 POLYCYCLIC CAGE AMINES

2.3.1 BACKGROUND ON POLYCYCLIC NMDA ANTAGONISM

2.3.1.1 OPEN CHANNEL BLOCKADE

The term "open-channel" blockade defines a type of ion channel block that is only exerted when the channel is open. Open channel blockers are therefore dependent on prior activation (and opening) of the associated channel by its agonist before it can get to work. Statistically, the amount of open channels is more in the presence of an excessive pathological activation of the receptor, as is the case with excitotoxicity. An open channel blocker would be an obvious choice in the treatment of excitotoxicity (Chen & Lipton, 2006:1616) because the probability of it blocking excessive neurotransmission is much higher than the probability of it blocking normal physiological neurotransmission.

These antagonists are also called "uncompetitive" antagonists, since they block the receptor without competing with the agonist for its binding site. Based on their mechanism of action, it is argued that these antagonists would prevent more severe excitotoxic processes better than it would if the excitotoxicity is mild, because of the higher probability for it to enter the channel pore. It was therefore proposed by Chen & Lipton (2006:1616) that a clinically tolerated neuroprotective drug must be a low-affinity, open channel blocker with a relatively fast off-rate. An example of such a drug is memantine (**2.8**).

MK-801 (**2.1**) is an example of a drug with a high affinity and slow off-rate, which causes it to build up in the ion channels and interfere with normal neurological function. It can eventually produce coma and is therefore not clinically acceptable. Other drugs that also have a long dwell time (high affinity and slow off-rate), include PCP (**2.3**) (Angel Dust) which causes hallucinations and ketamine (**2.6**) which is used as an anaesthetic.

2.3.1.2 UNCOMPETITIVE ANTAGONISM *VERSUS* NON-COMPETITIVE ANTAGONISM

Both uncompetitive and non-competitive antagonism requires prior activation of the ion channel to exert their effects (Johnson & Kotermanski, 2006:63). During non-competitive antagonism (sometimes called partial-trapping), the receptor, in this case the NMDA receptor, must first be activated by its agonist (figure 2.14). When the agonist (A) binds to the receptor (R), the agonist, receptor complex reaches an activated state, A_nR^*. In this state, multiple rearrangements lead to channel opening. The blocker molecule (B) can now enter

the opened channel and bind to its binding site (A_nR^*B). The channel then closes around the blocker (A_nRB) and the agonist (A) leaves the receptor, trapping the blocker inside the channel pore (RB).

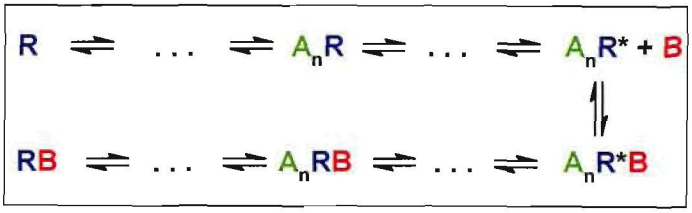


Figure 2.14: Schematic model of non-competitive antagonism (Johnson & Kotermanski, 2006:63).

Examples of such non-competitive antagonists include MK-801 (**2.1**), PCP (**2.3**), ketamine (**2.6**) and amantadine (**2.7**) and memantine (**2.8**).

Uncompetitive antagonism on the other hand is also called "sequential blocking" or the "foot-in-the-door" model of blocking. Here, binding of the blocker prevents the channel from closing and the states (A_nRB) and (RB) are thus not present in this type of blocking (figure 2.15).

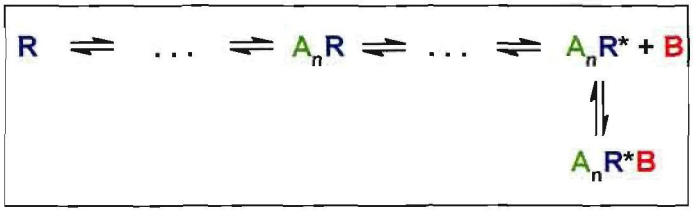


Figure 2.15: Schematic model of uncompetitive antagonism (Johnson & Kotermanski, 2006:63).

An example of an uncompetitive antagonist is memantine. In fact, it was shown by Chen & Lipton (1997:27) that memantine shows uncompetitive antagonism at low micromolar concentrations and non-competitive antagonism at higher concentrations. In 2006, Chen & Lipton (2006:1620) related this non-competitive antagonism to a second binding site, which has lower affinity and is located to the outer vestibule of the channel pore (figure 2.17). This superficial site has such a low affinity that it does not even show relevance at clinical dosing concentrations.

The reason for distinguishing between uncompetitive and non-competitive antagonism is that uncompetitive antagonism can block excessive activation of NMDA receptors, while sparing normal neurotransmission. Non-competitive antagonism on the other hand is equally up to

the task of blocking excessive NMDA activation, but leads to more interference with normal neurotransmission (Chen & Lipton, 2006:1620).

2.3.2 AMINO ADAMANTANE DERIVATIVES

Amino adamantane derivatives are polycyclic cage amines and include amantadine (**2.7**) and memantine (**2.8**), amongst others. Of the two compounds, memantine is the most widely used in the treatment of neurodegenerative disorders.

2.3.2.1 THE CHEMISTRY OF MEMANTINE

Eli Lilly and Company were the first to synthesize memantine and it was patented in 1968. Memantine (**2.8**) itself is an amantadine derivative. The structure of memantine shows a three-ring, or adamantane, structure with a bridge-head amine ($-NH_2$) group which is protonated ($-NH_3^+$) at physiological pH representing the region of memantine that binds at or near the Mg^{2+} binding site in the NMDA receptor, as well as the two methyl ($-CH_3$) side chains that prolongs its dwell time in the channel and slows its off-rate, relative to amantadine (Lipton 2006:164-165).

2.3.2.2 MECHANISM OF ACTION OF MEMANTINE

Memantine (**2.8**) exerts its NMDA receptor antagonism through open channel blockage (Chen & Lipton, 2006:1617). Since the early 90's it was established through a series of experiments that memantine's binding site must be located at, or near, the Mg^{2+} binding site (Chen *et al.*, 1992:4427; Chen & Lipton, 1997:27). This was confirmed by Kashiwagi *et al.*, (2002:533) during a mutational analysis of the NMDA receptor and has lead to memantine being represented as a "better magnesium".

This memantine site is located near the external Mg^{2+} site, is called the selectivity filter region of the NMDA receptor. This region is formed by asparagine (N) residues at the "N-site" of NR1 and "N + 1 site" of NR2A subunit. The N-site asparagine of the NR1 subunit represents the dominant blocking site for intracellular Mg^{2+}, while the N + 1 site asparagines of the NR2A subunit form the critical blocking site for extracellular Mg^{2+} (figure 2.17). The N and N + 1 sites provide the major electrostatic interaction with memantine when binding to this specific region.

Using point mutations and substituted cysteine accessibility methods (SCAM), Chen and Lipton (2005:961) found a second memantine blocking site. This one is more superficial and located at the outer vestibule pore of the channel. This site is also non-specific and overlaps with hexamethonium, a non-specific pore blocker's binding site.

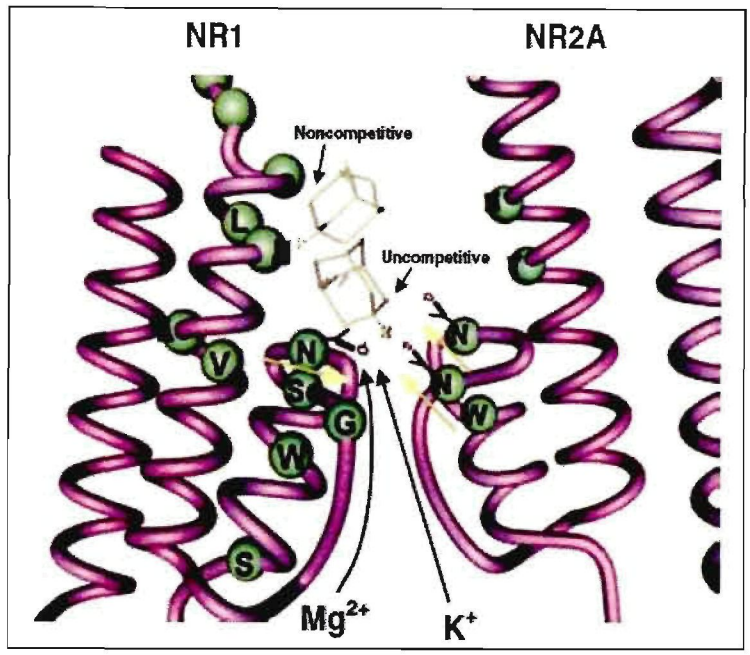
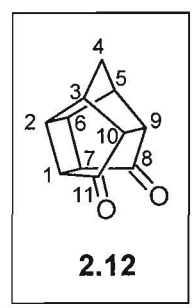


Figure 2.17: Atomic model showing the two proposed binding sites of memantine in the NMDA receptor channel pore (Chen & Lipton, 2006:1621).

2.3.3 PENTACYCLOUNDECANE DERIVATIVES

2.3.3.1 BACKGROUND ON THE PENTACYCLOUNDECANE CAGE COMPOUND

The pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (**2.12**), or the Cookson's diketone (figure 2.18) as it is also known, was reported as early as 1958 by Cookson *et al.*, (1958:1003).



2.12

Figure 2.18: The Cookson's diketone.

The synthesis consists of a Diels-Alder cycloaddition of cyclopentadiene and p-benzoquinone, followed by intramolecular [2 + 2] photocyclisation of the resulting endo adduct (figure 2.19) (Marchand & Suri, 1984:672).

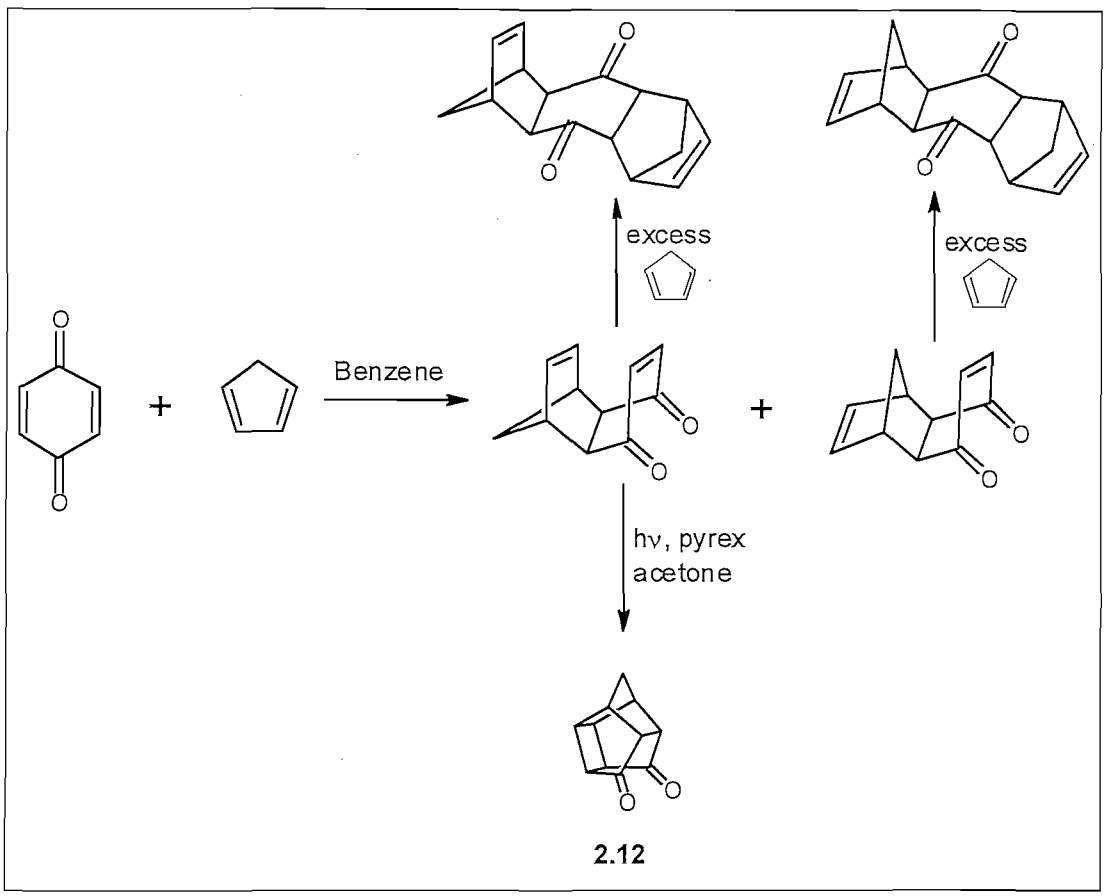


Figure 2.19: The synthesis of the pentacycloundecane dione.

These pentacycloundecane diones can be readily converted to pentacycloundecylamines by means of reductive amination. One of the ketone groups of the pentacycloundecane dione is allowed to react with an amine, yielding the corresponding carbinolamine. The carbinolamine is then dehydrated under Dean-Stark conditions to give the corresponding imine. This imine can then be reduced to the amine. Depending on the type of reducing agent used, the imine can be reduced to either the aza (**2.14**) or oxa (**2.13**) cage compound (figure 2.20).

2.3.3.2 STRUCTURE ACTIVITY RELATIONSHIPS FOR PENTACYCLOUNDECYLAMINE NMDA RECEPTOR ANTAGONISM

As stated earlier in the section about structure activity relationships of NMDA receptor antagonists, the structure activity relationships of pentacycloundecylamines are primarily determined by geometric factors, with electronic effects playing only a small part. This was in fact confirmed by Geldenhuys *et al.* (2007:1529), during their analysis of the structure activity relationships of the pentacycloundecylamines for the NMDA receptor in which they found that a polycyclic cage moiety with an attached amine seems to be the most important part of the pharmacophore. Phenyl substitution onto the pentacycloundecylamine increases the potency

and therefore the NMDA receptor antagonism. The bridgehead atom linking C-8 to C-11 doesn't seem to have an influence on NMDA receptor antagonism. The number of linker atoms between the phenyl ring and the amino group is important because an 8-fold decrease in activity is observed when this linker length increases from 1 carbon to 2 carbons. Substitution onto the phenyl ring influences potency, with substitution in the ortho or meta positions giving higher potency than substitution in the para-position. Electron-withdrawing groups on the phenyl ring decreases potency while electron-donating groups on the phenyl ring increases potency.

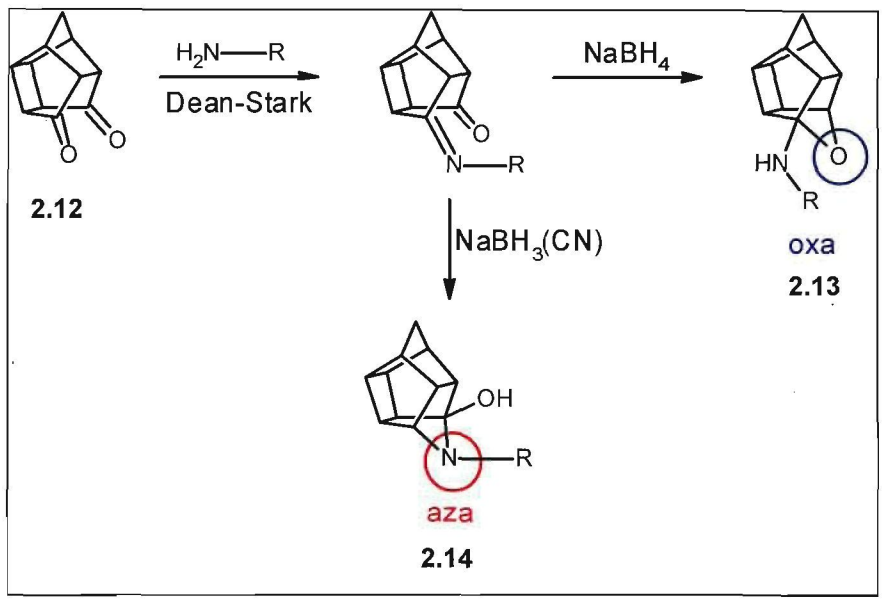


Figure 2.20: Reductive amination of the pentacycloundecane dione to its corresponding pentacycloundecylamine.

This corresponds with the findings of Liebenberg *et al.* (1986:46) when they studied nitrogen-containing derivatives of pentacycloundecane as a new series of calcium channel blockers in cardiac myocytes. The only exception is that the group of Liebenberg *et al.* (1986:46) found no correlation between the length of the linker and the calcium channel activity. Their findings are summarised in figure 2.21.

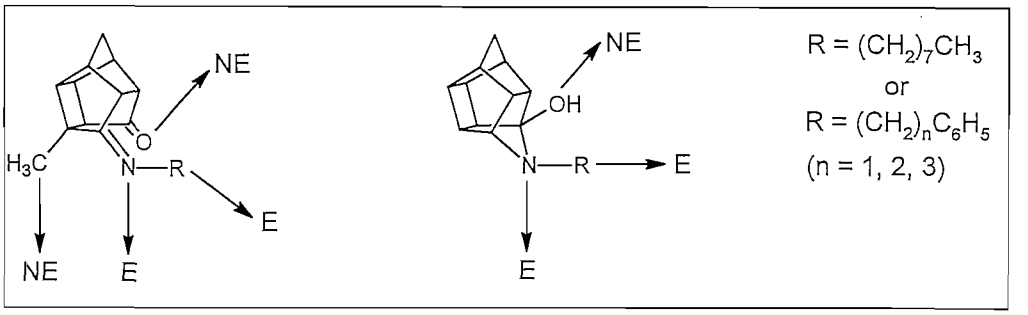
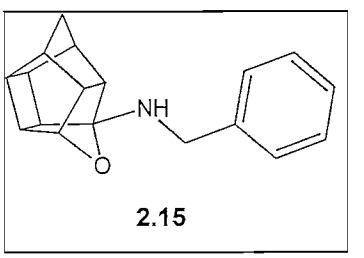


Figure 2.21: A diagram showing the structure activity relationships for calcium channel block by the nitrogen containing pentacycloundecane derivatives, adapted from Liebenberg *et al.* (1986:46). NE indicates groups that are not essential for calcium channel blockage and E indicates groups that are essential.

2.3.3.3 CALCIUM CHANNEL ACTIVITY OF PENTACYCLOUNDECYLAMINES

Of interest to this study, as per its title, was not only the influence of nitrosylation on the activity of polycyclic amines on NMDA receptors, but their calcium channel activity in general. For this reason, their calcium channel activity was tested not only on NMDA receptors, but on all the calcium channels in synaptoneurosomes. Van der Schyf *et al.* (1988:448) investigated the mechanism of calcium channel blockade in cardiac myocytes and found that, NGP1-01 (**2.15**) (figure 2.22) had frequency- and voltage-dependant calcium channel blocking activity. This suggest that pentacycloundecylamines, such as NGP1-01 (**2.15**), are open channel blockers and would be of excellent use in attenuating excitotoxicity, because of its higher affinity for over stimulated (more frequently open) NMDA receptors.



2.15

Figure 2.22: NGP1-01, a well documented pentacycloundecylamine and open channel blocker.

In the late 1990's, a series of azapentacycloundecylamines and pentacycloundecanes were tested for sigma receptor (a calcium channel modulator) activity (Kassiou *et al.,* 1996:595 and Marrazzo *et al.,* 2001:181). This might further contribute to the calcium channel activity of the pentacycloundecylamines.

2.3.3.4 GENERAL BIOLOGICAL ACTIVITIES OF PENTACYCLOUNDECYLAMINES

In a review article on the pharmacology and structure-activity relationships of bioactive polycyclic cage compounds, Geldenhuys *et al.* (2005:21) gave a good description of the biological activities of pentacycloundecane derivatives. These activities include:

- **ANTIVIRAL ACTIVITY**

The antiviral activity of 4-amino-(D_3)-trishomocubanes (the $C_{11}H_{14}$ stabilomer) was tested against Herpes simplex I and II, Influenza A2/Taiwan and Rhinovirus 1A by Oliver *et al.* (1991a:549). Two of the compounds (figure 2.23) tested showed *in vivo* activity, comparable to acyclovir and amantadine, against Herpes simplex II and Influenza A2/Taiwan. No in vitro activity against Herpes simplex I and II, or Rhinovirus 1A was observed.

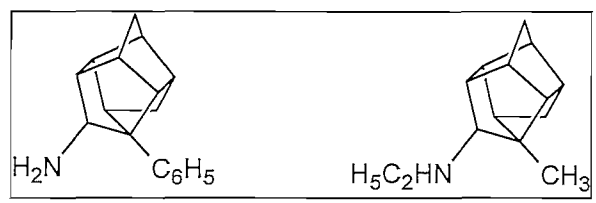


Figure 2.23: The 4-amino-(D_3)-trishomocubanes that showed promising antiviral activity.

- **ANTI-PARKINSONIAN ACTIVITY**

In another study done by Oliver *et al.* (1991b:375) pentacycloundecylamines were tested for anti-Parkinsonian activity. It was found that these compounds antagonise reserpine-induced catatonia. In a mouse model, the pentacycloundecylamines were also found to reduce oxotremorine tremor and salivation. Although these compounds' anti-cataleptic properties were comparable to that of amantadine, their therapeutic indices were less than favourable in comparison with that of amantadine. The anti-Parkinsonian properties of these compounds were attributed to their effects on the dopaminergic system.

- **ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY**

The analgesic and anti-inflammatory activities of two novel pentacycloundecylamines (figure 2.24) also were evaluated (Van der Schyf *et al.*, 1986a:409). The methylated pentacycloundecylamine showed significant and dose-related anti-inflammatory effect in normal rats. The effect was markedly reduced in adrenalectomised animals. This lead to the conclusion that these drugs stimulate the synthesis of adrenocortical hormones, instead of inhibiting cyclo-oxygenase.

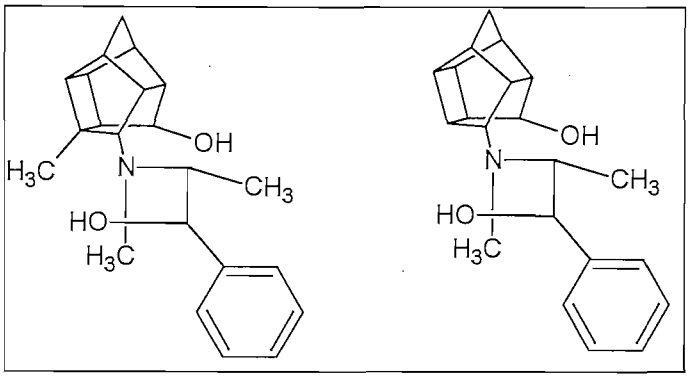


Figure 2.24: The pentacycloundecylamine derivatives synthesized by Van der Schyf *et al.* (1986a:409) and tested for analgesic and anti-inflammatory activity.

- **CARDIAC EFFECTS**

Van der Schyf *et al.* (1986b:407) confirmed the L-type calcium channel blocking activity of NGP1-01. In a follow-up study, Van der Schyf *et al.* (1988:448) discovered that NGP1-01 displayed activity similar to that of dihydropyridine calcium channel blockers. It also prolonged the PQ interval and therefore increased AV-conduction in the heart.

2.4 CONCLUDING REMARKS

From the literature, it is clear that the role of excitotoxicity in neurodegenerative disorders is undeniable and several strategies for the treatment of excitotoxicity have become apparent. For this study it was decided to use pentacycloundecylamines as modulators of (excessive) calcium channel activity. To exhibit channel activity the compounds had to contain a large hydrophobic component, the cage moiety, an amine group to be protonated at physiological pH and an aromatic moiety necessary for high affinity binding. A NO-donating moiety was also conjugated to the pentacycloundecylamines to give them the capacity to S-nitrosylate crucial cysteine residues and further modulate the channel activity of polycyclic amines.

CHAPTER 3

SYNTHESIS AND STRUCTURE ELUCIDATION

SUMMARY

To synthesise the various novel pentacycloundecylamines necessary for NMDA receptor blockade and NO donation, a series of synthesis were performed. Starting with the pentacycloundecane cage moiety, a linker was introduced using reductive amination. To the linker was conjugated the aromatic moiety and/or the NO donating moiety via esterification. The structures of the synthesised compounds were determined by one- and two-dimensional NMR spectroscopy. Characterisation of functional groups, in particular the NO donating moiety, was done by using IR spectrophotometry. In this chapter, the synthesis of each compound, as well as its structural elucidation are described.

3.1 PLANNING OF SYNTHESISED COMPOUNDS

The pentacycloundecylamines synthesised had to comply with the structural requirements necessary for NMDA receptor and calcium channel antagonism. The bulky cage structure served as the hydrophobic moiety necessary for binding to the PCP or memantine binding site (Jirgensons *et al.*, 2000:555). An amine, necessary for hydrogen bonding to a receptor residue, was conjugated to the cage structure (Kroemer *et al.*, 1998:395). An aromatic moiety was also conjugated to the final structure, since it is necessary for high-affinity binding. The absence of the aromatic moiety in compound **3.4** was to test the influence of its absence relative to the other compounds. Based on the hypothesis of Geldenhuys *et al.* (2007:1530), the length of the side chain was increased (**3.6** *versus* **3.1** *versus* **3.2**) to sterically free the movement between the phenyl group and the cage structure, and possibly bring the latter into closer proximity with its interaction site in the NMDA receptor channel pore. The esters in certain of the compounds also offered a chance to evaluate the effect of an electron withdrawing moiety conjugated to the NO donating moiety. The synthesised compounds also had to contain a nitrogen monoxide (NO) donating moiety and could be divided into two groups (figure 3.1). The first are the unsaturated nitro compounds, described by Gorczynski *et al.* (2007:2014) and the second are the nitrates, described by Chiroli *et al.* (2003:442).

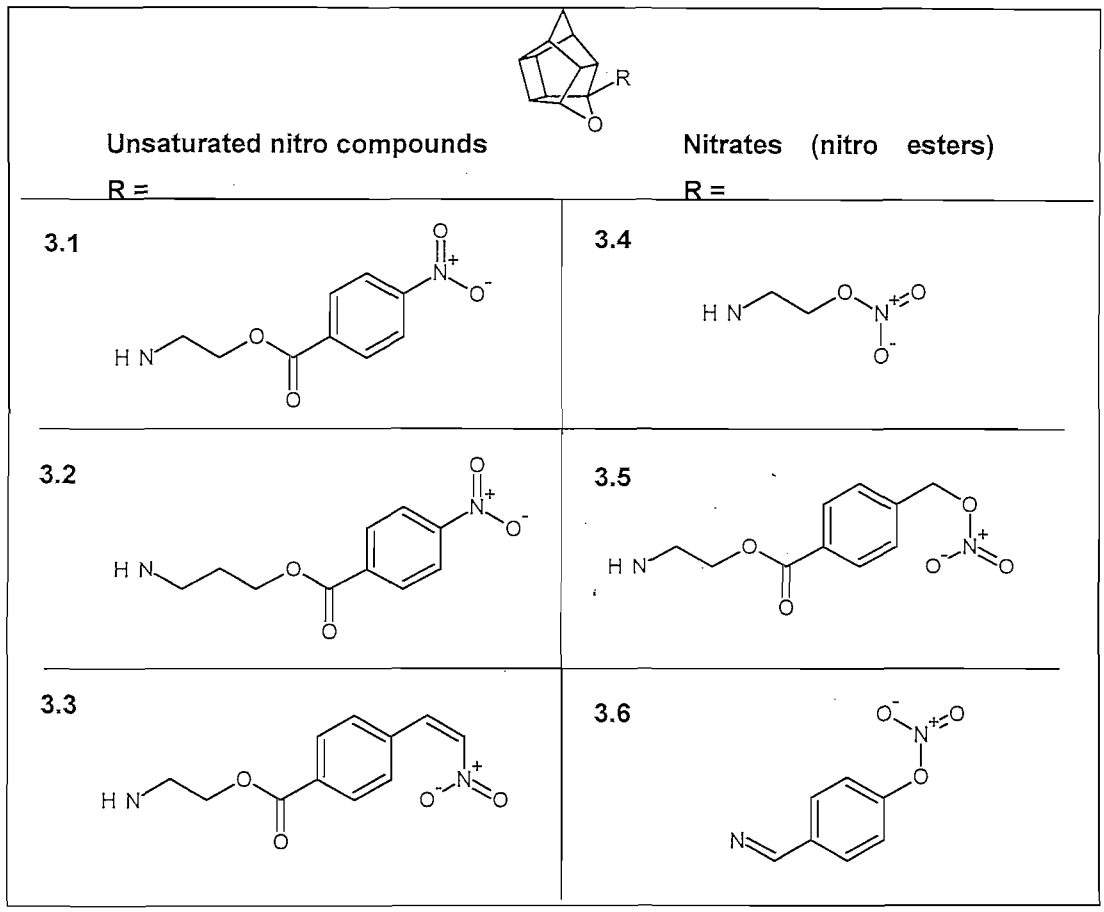


Figure 3.1: The compounds synthesised in this study.

Since all the compounds synthesised are reported for the first time, thorough structural elucidation was necessary. For this purpose, two dimensional nuclear magnetic resonance (NMR) spectroscopy was utilised. To correlate the couplings between the different atoms in a compound, (H,H)-correlated spectroscopy (COSY) NMR, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple quantum coherence (HMBC) was used. The NO donating moieties were characterised using infrared (IR) absorption spectrophotometry. Mass spectrometry was also employed to confirm the structures.

3.2 STANDARD EXPERIMENTAL PROCEDURES

3.2.1 CHEMICALS

1-Ethyl-3-(3'-dimethylamino)carbodiimide (EDC), 4-(dimethylamino)-pyridine (DMAP), benzylamine, 4-nitrobenzoic acid, 4-hydroxybenzaldehyde, 4-formylbenzoic acid, silver nitrate and thionylchloride were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium borohydride and magnesium sulphate were purchased from Saarchem (Krugersdorp, S.A.).

3.2.2 INSTRUMENTAL METHODS

3.2.2.1 MELTING POINT (MP) DETERMINATION

Melting points were determined by utilising a Lasec Stuart® SMP 10 instrument.

3.2.2.2 INFRARED (IR) ABSORPTION SPECTROPHOTOMETRY

IR spectra were recorded using a Shimadzu, IRPrestige®-21 spectrophotometer.

3.2.2.3 MASS SPECTROMETRY (MS)

Mass spectra were obtained using an Applied Bioscience, MDS SCIEX, API 2000® LC/MS/MS.

3.2.2.4 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

The ^{1}H, ^{13}C and DEPT spectra of compounds **3.2**, **3.10** and **3.11** were obtained on a Varian Gemini 300 spectrometer. ^{1}H spectra were recorded at a frequency of 300.075 MHz and ^{13}C spectra at 75.462 MHz, in a 7 Tesla magnetic field. The ^{1}H, ^{13}C, COSY, HSQC and HMBC spectra of compounds **3.1**, **3.3**, **3.4**, **3.5** and **3.6** were obtained on a Bruker Advance 600 spectrometer. ^{1}H spectra were recorded at a frequency of 600.14 MHz and ^{13}C at 150.92 MHz, in an ultra-stabilised 14.1 Tesla magnet and a temperature of 4.2 K. A xyz-axis pulsed field gradient (PFG) of 35 Gauss/cm was utilised. Tetramethylsilane (TMS) was used as a standard with a bandwidth of 1000 MHz at 24 kG applied for ^{1}H-^{13}C-decoupling. All chemical shifts were recorded in parts per million (ppm) relative to standard TMS ($\delta = 0$). Abbreviations used to indicate multiplicities in NMR spectra include: s – singlet, d – doublet, dd – doublet of doublets, t – triplet, m – multiplet.

3.2.3 CHROMATOGRAPHIC METHODS

Mobile phases were prepared by mixing solvents on a volume-to-volume base. The appropriate mobile phase was determined by using ethyl acetate (EtOAc) as starting mobile phase and adjusting the polarity accordingly. For visualisation, ultraviolet (UV) light (254, 366 nm), iodine vapours and ninhydrine were used.

3.2.3.1 THIN LAYER CHROMATOGRAPHY (TLC)

Analytical thin layer chromatography was done on aluminium silica gel sheets (*Alufolien, Kieselgel 60 F_{254}, Merck, Darmstadt, Germany*) to monitor the reaction progress.

3.2.3.2 COLUMN CHROMATOGRAPHY

Product purification was done on glass columns filled with silica gel (*MN Kieselgel 60, 0.063 – 0.2 mm, 70 – 230 mesh ASTM, Separations, Randburg, South Africa*).

3.3 SYNTHESIS AND STRUCTURE ELUCIDATION

As starting material for the synthesis of the compounds tested in this study, the well documented Cookson's diketone (**3.7**) was used. Diels-Alder cycloaddition of cyclopentadiene to p-benzoquinone, followed by intramolecular [2 + 2] photocyclization of the resulting endo cycloadduct, yielded the diketone (figure 3.2) (Marchand & Suri, 1984:672).

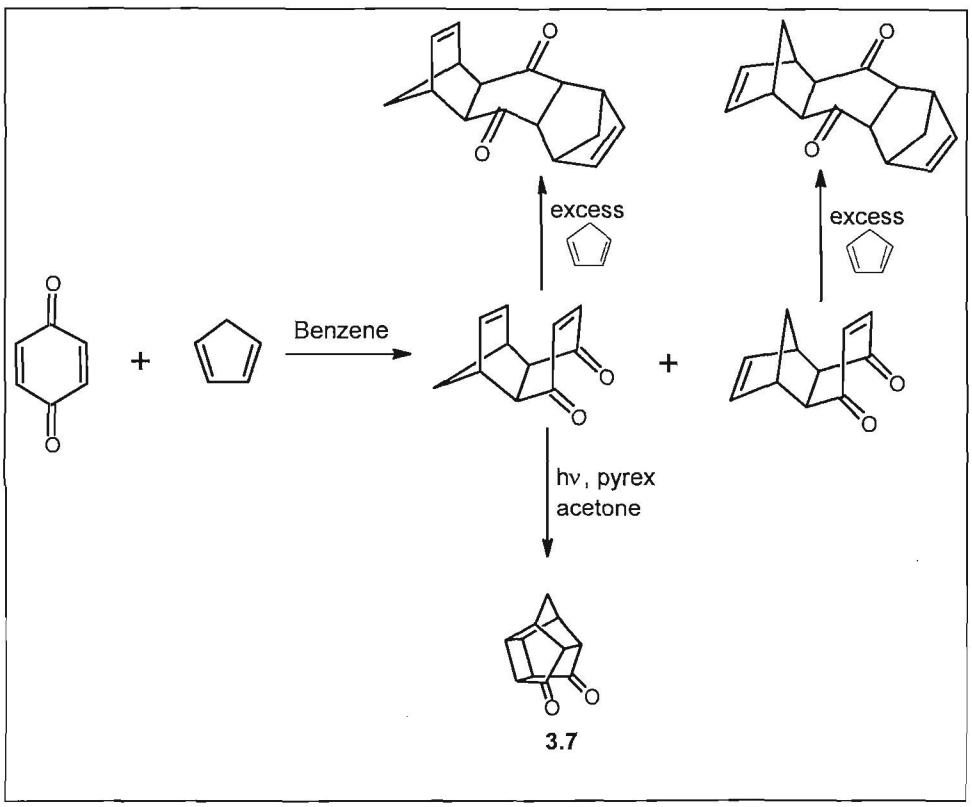


Figure 3.2: The synthesis of the pentacycloundecane dione (Marchand & Suri, 1984:672).

The pentacycloundecane dione (**3.7**) was reductively aminated to give the corresponding pentacycloundecylamine (figure 3.3). The reduction was carried out using $NaBH_4$ to give the oxa-amine.

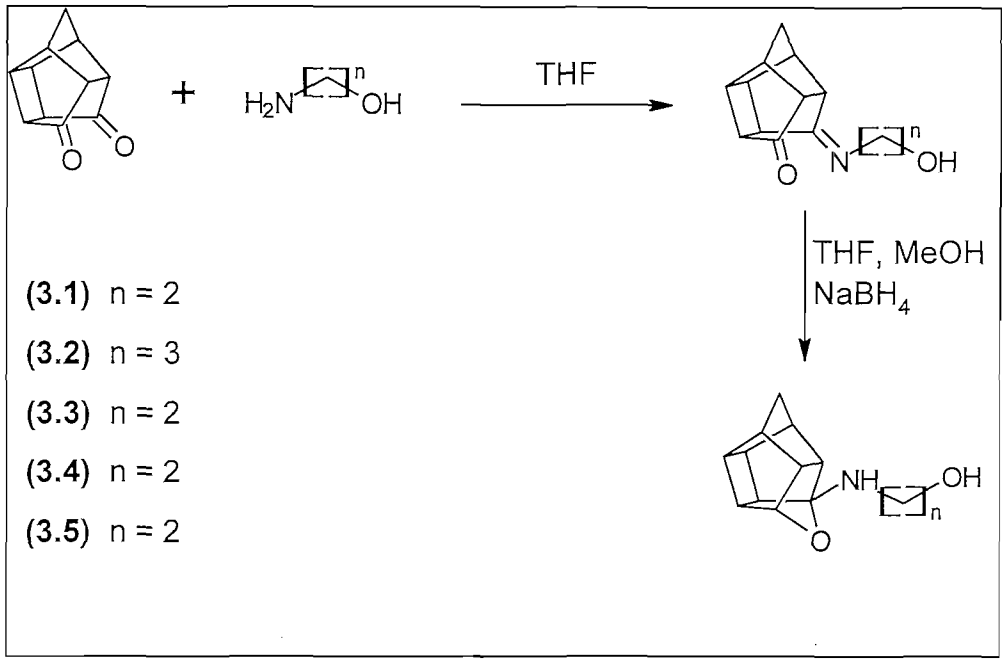


Figure 3.3: The reductive amination of the Cookson's diketone to yield the corresponding pentacycloundecylamines.

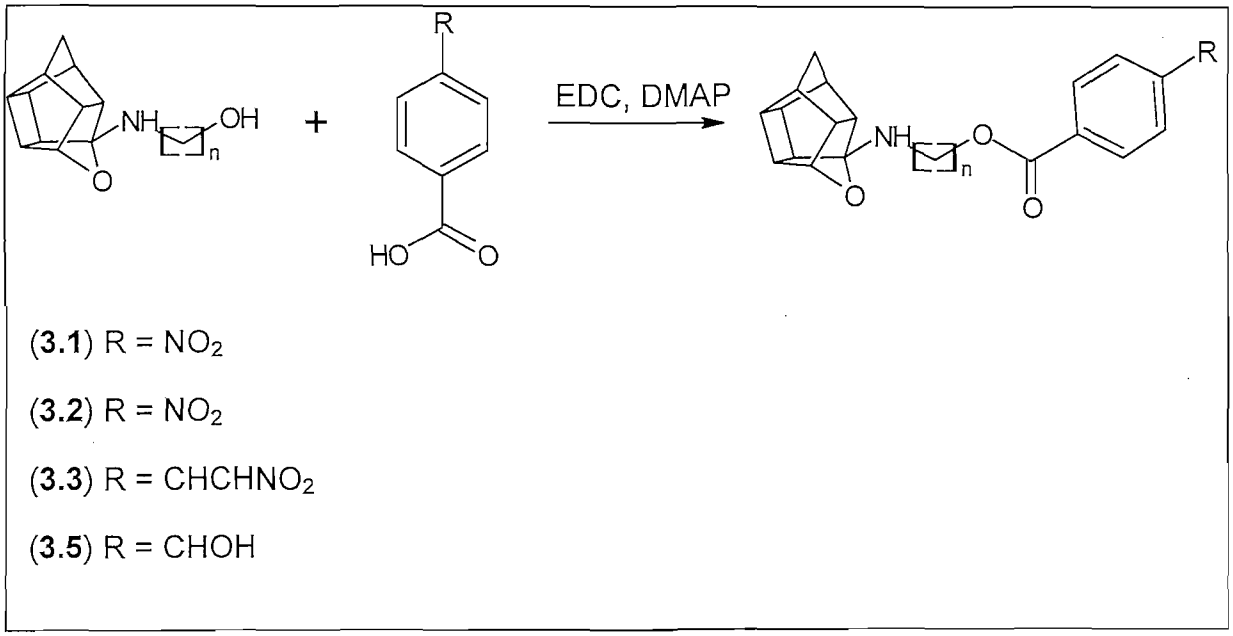


Figure 3.4: The synthesis of the ester compounds using activation chemistry.

The next step in the synthesis consisted of the formation of esters utilising activation chemistry with 1-ethyl-3-(3'-dimethylamino)carbodiimide (EDC) (figure 3.4). The 4-nitroethenylbenzoic acid, conjugated to yield compound 3.3, was prepared using a solventless microwave synthesis according to the method described by Varma *et al.* (1997:5133).

For the final step in the synthesis of compounds **3.4**, **3.5** and **3.6,** their respective hydroxyl groups were nitrated using thionylchloride nitrate (equation 3.2). The thionylchloride nitrate was prepared according to equation 3.1 below (Hakimelahi *et al.,* 1984:907).

$$AgNO_3 + SOCl_2 \rightleftharpoons AgCl + SOClNO_3 \quad (3.1)$$

$$SOClNO_3 + ROH \rightleftharpoons RONO_2 + SOCl(OH) \quad (3.2)$$

The synthesis of compound **3.6** was carried out by means of the catalytic reduction of NGP1-01 (**3.8**) with paladised carbon. The resulting pentacycloundecylamine was then reacted with 4-hydroxybenzaldehyde and dehydrated under Dean-Stark conditions to give the intermediate alcohol compound (**3.15**) (figure 3.5). This intermediate alcohol was then nitrated, as described above, to afford compound **3.6** (see page 54 for structure).

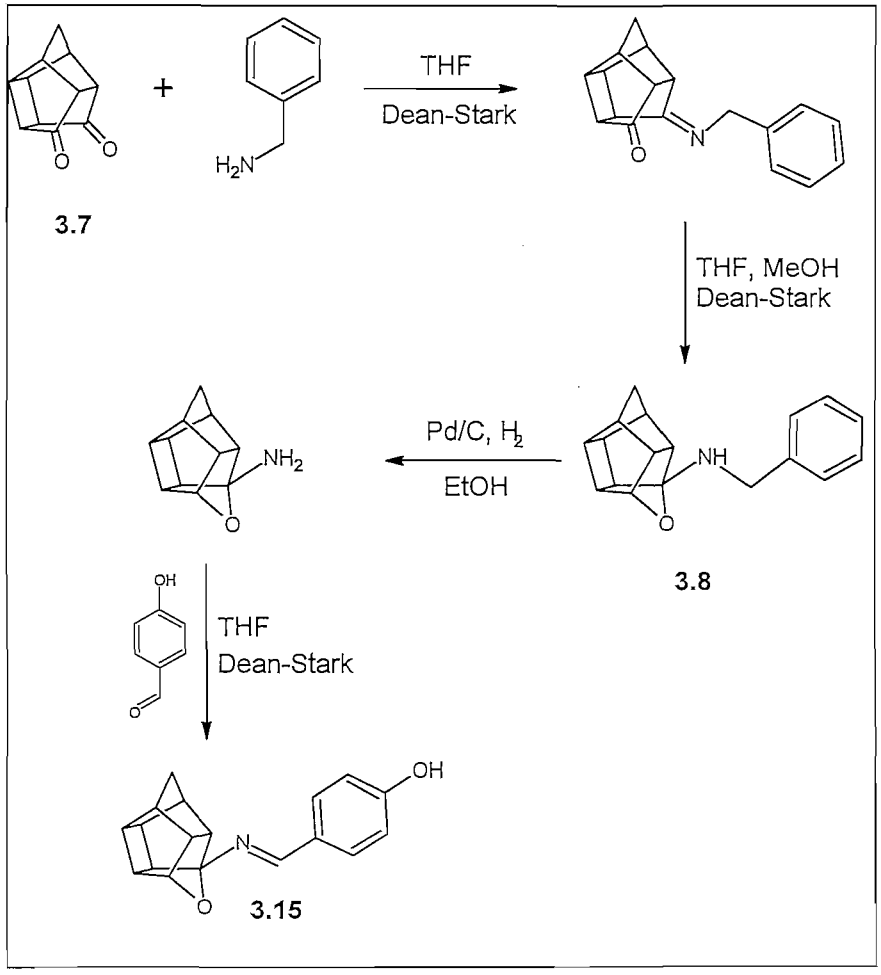
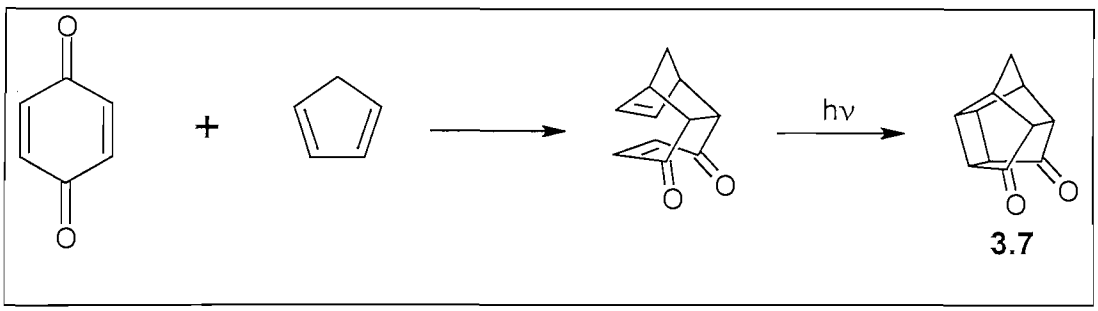


Figure 3.5: The synthetic route leading to compound 3.6.

3.3.1 THIONYLCHLORIDE NITRATE

In dry tetrahydrofurane, *n*-mol-equivalents of thionylchloride and silver nitrate (*n* = the number of alcoholic or phenolic H-atoms of the substrate) was mixed and stirred at room temperature. Quantitative precipitation of silver chloride indicated that the reaction was nearing completion. After 1 to 2 hours the mixture was filtered and the filtrate, containing the thionylchloride nitrate, was removed and added to the substrate.

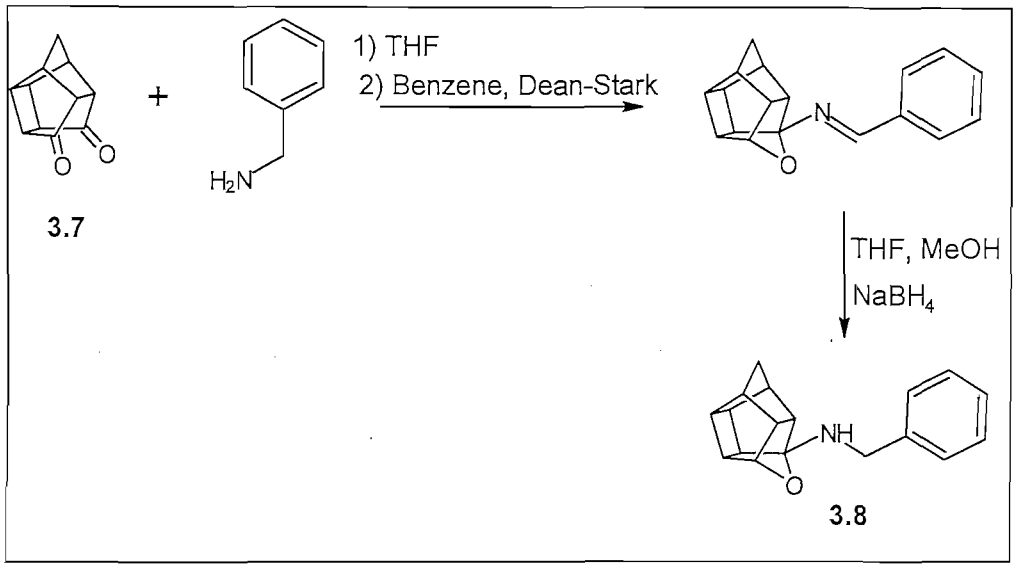
3.3.2 PENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE-8,11-DIONE (3.7)



3.7

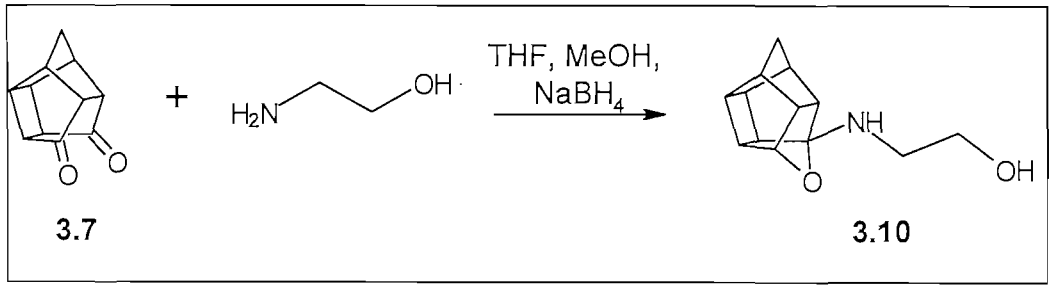
The *p*-benzoquinone (25 g, 0.23 mol) was dissolved in dry benzene (100 ml) and cooled down to 5 °C on an external ice bath. Freshly monomerized cyclopentadiene (15.2 g, 0.23 mol) was added stoichiometrically in 5 ml aliquots, keeping the reaction temperature between 5 and 18 °C. The photo labile reaction mixture was removed from the ice bath and stirred for 2 hours at room temperature. Two large spatula points (± 3 g) of activated charcoal was added and the mixture was stirred for another 30 minutes at room temperature. The mixture was then filtered through Celite® and concentrated in vacuo. The concentrate was left to evaporate overnight in a fume hood to yield the Diels-Alder adduct as yellow crystals. The adduct was then dissolved in acetone (± 4 g per 100 ml) and irradiated with a 1000 W medium pressure mercury UV lamp for 4 hours. Subsequent removal of the solvent gave a yellow residue, which was purified by Soxhlett extraction in cyclohexane to produce the cage compound as fine, waxy, colourless crystals (32.6 g, 0.187 mol, 77.3 %). The physical and spectral data of the product corresponds to that described in the literature (Cookson *et al.*, 1958:1003).

3.3.3 8-BENZYLAMINO-8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE (NGP1-01) (3.8)



The pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (5.3 g, 30 mmol) was dissolved in dry tetrahydrofurane (50 ml) and cooled down to 5 °C while stirring on an external ice bath. Benzylamine (3.3 g, 30 mmol) was added slowly, with continuous stirring at lowered temperature. The carbinolamine which started to precipitate out after 10 minutes was isolated by filtration after 30 minutes. It was then dehydrated under Dean-Stark conditions in benzene (60 ml) for 1 hour or until no more water was collected in the trap. The mixture was then concentrated in vacuo and the Schiff-base (8-benzylimino pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-11-one) was obtained as a yellow oil. The Schiff-base was then dissolved in a mixture of dry methanol (30 ml) and dry tetrahydrofurane (150 ml). Reduction was carried out by adding sodium borohydrite (1.6 g, 41 mmol) proportion wise to the reaction mixture and stirring overnight. The reaction mixture was concentrated *in vacuo,* water (100 ml) was added and this was followed by extraction with dichloromethane (4 x 50 ml). The combined organic fractions were combined and washed with water (2 x 100 ml), dried over anhydrous $MgSO_4$ and evaporated to give a yellow oil. Purification of the mixture was done by column chromatography (mobile phase, dichloromethane: petroleum ether: ethyl acetate (1:1:1) and the desired amine (**3.8**) was isolated as a colourless wax. Crystallization from ethyl acetate gave the final compound (**3.8**) as fine white needles (2.95 g, 11.12 mmol, 37.01 %). The physical and spectral data corresponds to that described in the literature, (Van der Schyf *et al.*, 1989:48), and is not given in this dissertation.

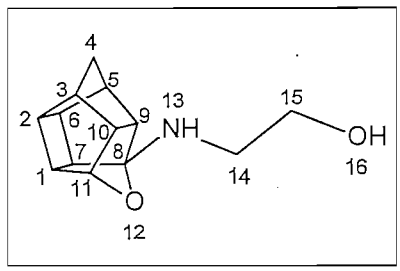
3.3.4 8-(2-AMINOETHANOL)-8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE (3.10)



3.7 3.10

The pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (5.1 g, 29 mmol) was dissolved in dry tetrahydrofurane (50 ml) and cooled down to 5 °C, while stirring on an external ice bath. 2-Aminoethanol (1.8 g, 29 mmol) was added drop wise, while stirring at lowered temperature (± 5 °C). The carbinolamine started to precipitate out after 10 minutes and the mixture was stirred for another 20 minutes, after which dry methanol (30 ml) was added and the mixture removed from the ice bath. Once all the carbinolamine was dissolved, a spatula point (± 1.5 g) of sodium borohydride was slowly added and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo*, water (100 ml, pH 2 with hydrochloric acid) was added and followed by extraction with dichloromethane (4 x 50 ml). The water fraction was alkalinised with sodium bicarbonate to a pH of 11 and extracted again with dichloromethane (4 x 50 ml). The second organic fractions were combined and washed with water (2 x 100 ml), dried overnight with anhydrous $MgSO_4$ and evaporated to give a light yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate: ethanol (2:1)) and the desired amine (**3.10**) was obtained as a light yellow oil (1.5 g, 6.85 mmol, 30 %).

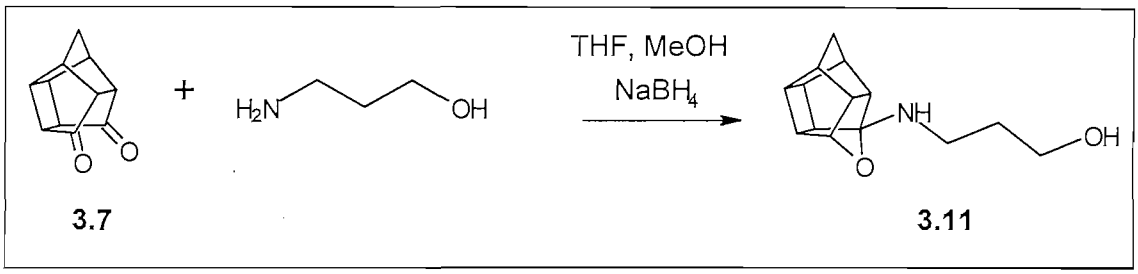
- **PHYSICAL DATA**



$C_{13}H_{17}NO_2$; TLC (2EtOAc:EtOH): R_f 0.40; **^{1}H NMR (300 MHz, $CDCl_3$)** δ_H (spectrum 1): 4.6 (t, 1H, J = 5.22 Hz, H-11), 3.6 (m, 2H, H-15a,b), 3.067 (m, 2H, H-14a,b), 2.911 – 2.630 (m, 4H, H-1,7,9,10), 2.619 – 2.425 (m, 4H, H-2,3,5,6), 1.949 (AB-q, 2H, J = 10.5 Hz, H-4a,4b); **^{13}C NMR (75 MHz, $CDCl_3$)** δ_C (spectrum 2): 82.335 (t, C-11), 77.427 (t, C-14), 76.580 (s, C-8),

63.332 (d, C-15), 55.092 (d, C-10), 54.653 (d, C-9), 46.275 (d, C-7), 44.797 (d, C-5), 44.483 (d, C-3), 44.295 (d, C-1), 43.006 (t, C-4), 41.824 (d, C-6), 41.429 (d, C-2).

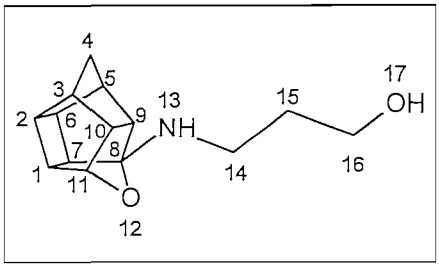
3.3.5 8-(3-AMINOPROPANOL)-8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE (3.11)



3.7 **3.11**

The pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (**3.7**) (5.4 g, 31 mmol) was dissolved in dry tetrahydrofurane (50 ml) and cooled down to 5 °C, while stirring on an external ice bath. 3-Aminopropanol (2.3 g, 31 mmol) was dissolved in dry methanol (30 ml), to which was added a spatula point (± 1.5 g) of sodium borohydrite. This mixture was then added dropwise to the pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione mixture over a period of 30 minutes and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo*, water (100 ml, pH 2 with hydrochloric acid) was added and followed by extraction with dichloromethane (4 x 50 ml). The water fraction was alkalised with sodium bicarbonate to a pH of 11 and extracted with dichloromethane (4 x 50 ml). The second organic fractions were combined and washed with water (2 x 100 ml), dried overnight with anhydrous $MgSO_4$ and evaporated to give a light yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate: ethanol (1:1)) and the desired amine (**3.11**) was obtained as a light yellow oil (0.481 g, 2.1 mmol, 24.42 %).

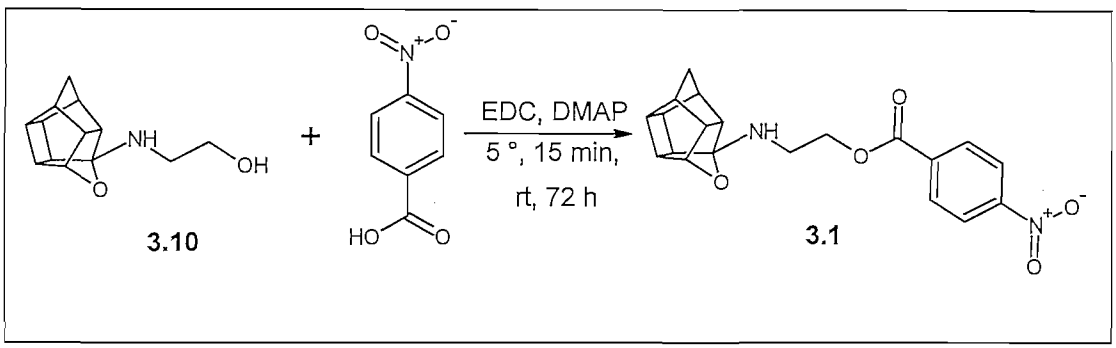
- **PHYSICAL DATA**



$C_{13}H_{17}NO_2$; TLC (EtOAc:EtOH): R_f 0.22; **^{1}H NMR (300 MHz, $CDCl_3$)** δ_H (spectrum 3): 4.705 (t, 1H, J = 5.22 Hz, H-11), 3.857 (quartet, 2H, J = 2.72 Hz, H-16a,b), 3.060 – 2.885 (m, 2H, H-14a,b), 2.866 – 2.622 (m, 4H, H-2,3,5,6), 2.618 – 2.423 (m, 4H, H-1,7,9,10), 1.995 – 1.575

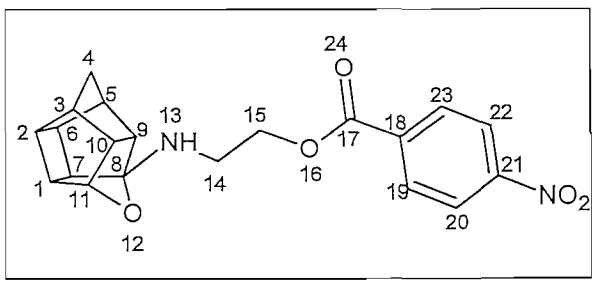
(AB-q, 2H, J = 10.4 Hz, H-4a,4b), 1.758 (m, 2H, H-15a,b); **^{13}C NMR (75 MHz, $CDCl_3$)** δ_C (spectrum 4): 82.601 (t, C-11), 77.424 (t, C-14), 76.576 (s, C-8), 62.897 (d, C-16), 55.322 (d, C-10), 54.740 (d, C-9), 44.813 (d, C-5), 44.531 (d, C-3), 43.278 (d, C-7), 42.982 (d, C-1), 42.645 (t, C-4), 41.794 (d, C-6), 41.517 (d, C-2), 32.391 (t, C-15).

3.3.6 8-AMINOETHYL-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE-2-(4-NITROBENZOATE) (3.1)



8-(2-aminoethanol)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**3.10**) (1.043 g, 4.7 mmol) was dissolved in dichloromethane (40 ml) and cooled down to 5 °C on an external ice bath. 1-Ethyl-3-(3'-dimethylamino)carbodiimide (1.003 g, 5.2 mmol), 4-nitrobenzoic acid (0.874 g, 5.2 mmol) and 4-(dimethylamino)-pyridine (0.057 g, 0.52 mmol) were added while stirring on an ice bath. After 15 minutes the reaction mixture was removed from the ice bath and stirred for a further 72 hours at room temperature. The mixture was then concentrated *in vacuo*. A saturated sodium bicarbonate solution (30 ml) was added and followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight with anhydrous $MgSO_4$ and evaporated to give a light yellow oil. The product was purified using column chromatography (mobile phase, ethanol: ethyl acetate (1:2)) to give the desired amine (**3.1**) as a light yellow oil (0.526 g, 1.43 mmol, 30.38 %).

- **PHYSICAL DATA**

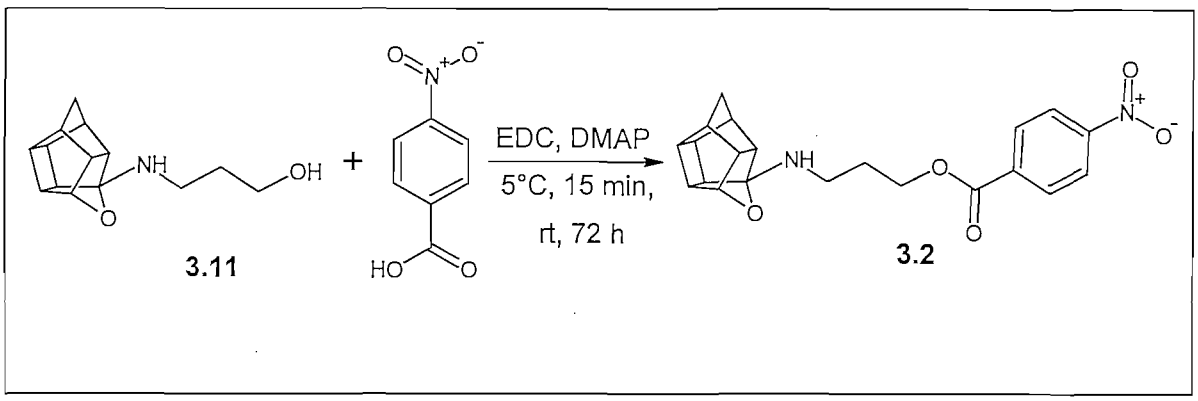


$C_{20}H_{20}N_2O_5$; TLC (2DCM:EtOAc): R_f 0.48; **IR (KBr)** ν_{max} (spectrum 5): 3412.08, 1709.65, 1527.62, 1490.97, 1352.10, 1240.70; **LC-MS (EI, 70 eV)** ***m/z*** (spectrum 6): 369.2(M^+), 353.4, 242.2, 230.9, 186.9, 118.8; **^{1}H NMR (600 MHz, $CDCl_3$) δ_H** (spectrum 7): 8.367 – 7.85 (m, 4H, H-19,20,22,23), 4.6 (t, 1H, J = 5.28 Hz, H-11), 4.407 (t, 2H, J = 5.58 Hz, H-15), 3.16 (sextet, 2H, J = 5.89 Hz, H-14), 2.78 – 2.36 (m, 8H, H-1,2,3,5,6,7,9,10), 1.842 (AB-q, 2H, J = 10.5 Hz, H-4a,4b). **^{13}C NMR (150 MHz, $CDCl_3$) δ_C** (spectrum 8): 163.65 (s, C-17), 149.52 (s, C-18), 134.58 (s, C-21), 129.81 (d, C-20/C-22), 129.65 (d, C-19/C-23), 108.21 (s, C-8), 81.59 (t, C-11) 65.45 (d, C-15), 54.38 (d, C-14), 53.74 (d, C-9), 43.83 (d, C-10), 43.70 (d, C-7), 43.55 (d, C-1), 42.31 (d, C-5), 42.05 (d, C-3), 41.43 (t, C-4), 40.9 (d, C-2), 40.53 (d, C-6).

- **STRUCTURE ELUCIDATION**

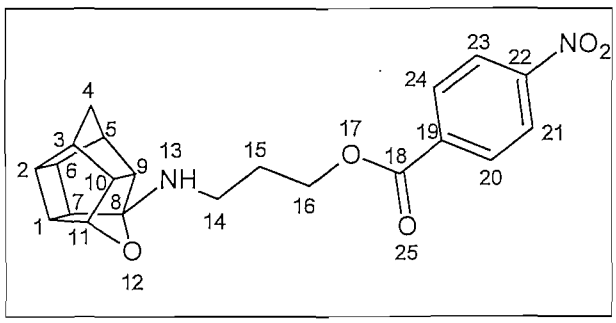
The two-dimensional homonuclear (H,H)-COSY NMR of compound **3.1** (spectrum 9) showed the obligatory geminal pentacycloundecylamine bridge protons (H-4a and H-4b), coupled to each other with resonances at δ_H 1.842 and 1.487 (d, J = 10.5 Hz). This can be attributed to H-3/H-5 at δ_H 2.368. The complex correlations between H-9/10 at δ_H 2.766, H-1/7 at δ_H 2.644, H-2/H-6 at δ_H 2.42 were also present. The triplet at δ_H 4.6 was assigned as H-11 since it showed mutual scalar coupling with protons in the cage moiety. A residual chloroform peak was found with a resonance at δ_H 7,19. The peaks at δ_H 8.255 and δ_H 8.148 were assigned to H-19/H-23 and H-20/H-22, respectively, and only showed coupling with each other. After preliminary ^{13}C elucidation using HSQC (spectrum 10) the remaining peaks were elucidated using HMBC (spectrum 11). The HMBC analysis indicated that H-15 showed vicinal coupling with the ^{13}C peak at δ_C 163.65, which was assigned as C-17. The ^{13}C peak at δ_C 134.58 only showed coupling with protons H-20/H-22 and was therefore assigned C-21. By elimination, the peak at δ_C 149.53 was assigned C-18, corresponding to its position further down field. The IR absorption spectrophotometry gave a sharp secondary amine peak at 3412.08 cm^{-1} as well as the carbonyl peak at 1709.65 cm^{-1}. The aromatic moiety was assigned to the peak at 1490.97 cm^{-1}. Nitro peaks at 1527.62 and 1352.10 cm^{-1} were observed. A C-O peak at 1240.70 cm^{-1} was also observed. The LC-MS gave the molecular ion (M^+) at m/z = 369.2, which correlates well with the predicted mass of 368.38 g/mol.

3.3.7 8-AMINOPROPYL-8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE-2-(4-NITROBENZOATE) (3.2)



8-(3-Aminopropanol)-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**3.11**) (0.481 g, 2.1 mmol) was dissolved in dichloromethane (40 ml) and cooled down to 5 °C on an external ice bath. 1-Ethyl-3-(3'-dimethylamino)carbodiimide (0.435 g, 2.2 mmol), 4-nitrobenzoic acid (0.379 g, 2.2 mmol) and 4-(dimethylamino)-pyridine (0.026 g, 0.2 mmol) were added while stirring on an ice bath. After 15 minutes the reaction mixture was removed from the ice bath and stirred for another 72 hours at room temperature. The mixture was then concentrated in vacuo. A saturated sodium bicarbonate solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight with anhydrous $MgSO_4$ and evaporated to give a light yellow oil. The mixture was purified using column chromatography (mobile phase, ethanol: ethyl acetate (1:1)) to give the desired amine (**3.2**) as a dark yellow oil.

- **PHYSICAL DATA**



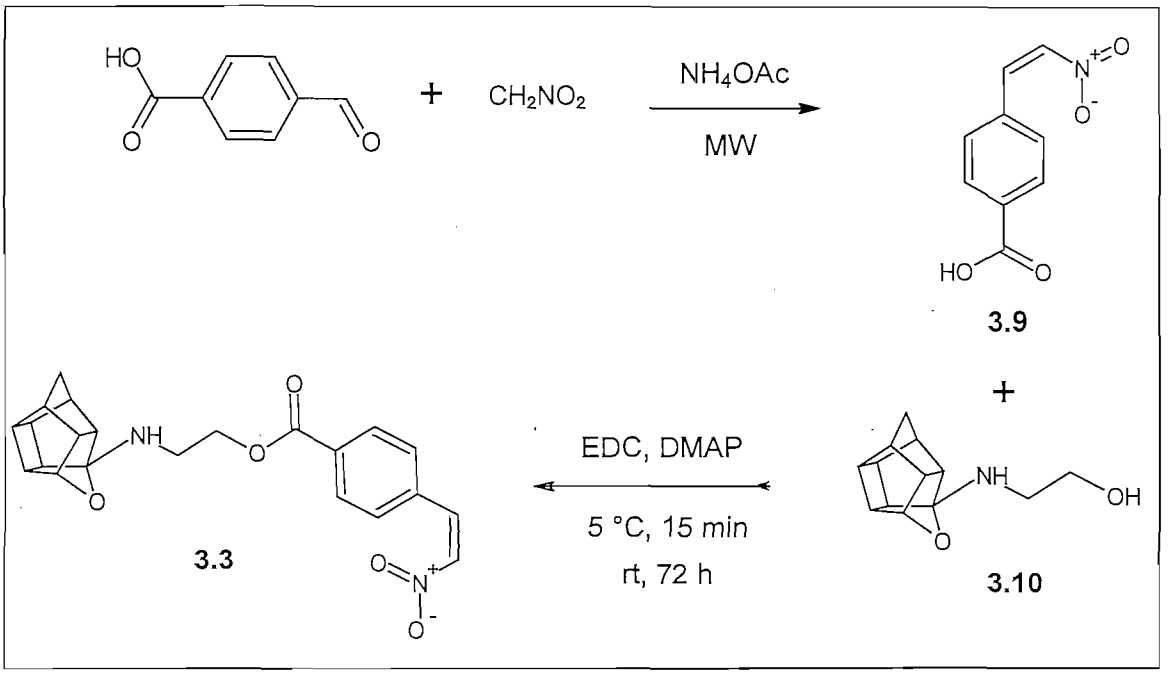
$C_{21}H_{22}N_2O_5$; TLC (EtOAc:EtOH): R_f 0.76; **IR (KBr)** ν_{max} (spectrum 12): 3403.12, 1722.43, 1606.70, 1523.76, 1352.10, 1105.21; **LC-MS (EI, 70 eV)** ***m/z*** (spectrum 13): 383.2(M^+), 256.3, 234.1; **^{1}H NMR (300 MHz, $CDCl_3$)** δ_H (spectrum 14): 8.312 – 8.136 (m, 4H, H-20,21,23,24), 4.725 (t, 1H, J = 5.32 Hz, H-11), 4.502 (t, 2H, J = 6.32 Hz, H-16), 3.077 (sextet, 2H, J = 5.08 Hz, H-14), 2.867 – 2.258 (m, 9H, H-1,2,3,5,6,7,9,10,11), 2.001 (sextet, 2H, J = 3.36 Hz, H-15), 1.889 (AB-q, 2H, J = 10.6 Hz, H-4a,4b). **^{13}C NMR (75 MHz, $CDCl_3$)** δ_C

(spectrum 15): 164.644 (s, C-18), 135.721 (s, C-19), 130.641 (d, C-20/24), 123.516 (s, C-22), 123.475 (d, C-23/21), 82.470 (t, C-11), 77.424 (s, C-8), 76.577 (d, C-14), 64.122 (d, C-16), 55.311 (d, C-10), 44.817 (d, C-9), 44.689 (d, C-5), 44.516 (d, C-3), 43.259 (d, C-7), 43.052 (d, C-1), 41.889 (t, C-4), 41.532 (d, C-6), 40.403 (d, C-2), 30.194 (d, C-15).

- **STRUCTURE ELUCIDATION**

The structural elucidation of compound **3.2** was confirmed using distortionless enhancement by polarization (DEPT) NMR spectroscopy (spectrum 16). The spectrum recorded a total of 13 CH carbons, 4 CH_2 carbons and zero CH_3 carbons. The four CH_2 carbons were assigned to C-4 with its H-4a/H-4b protons, C-14, C-15 and C-16. The 13 CH protonated carbons were assigned to carbons 1, 2, 3, 5, 6, 7, 9, 10, 11, 20, 21, 23 and 24. Assignments were done as above. The IR absorption spectrophotometry spectrum for compound **3.2** was elucidated as described above for compound **3.1**. The LC-MS gave the molecular ion (M^+) at m/z = 383.2, which correlates well with the predicted mass of 382.41 g/mol.

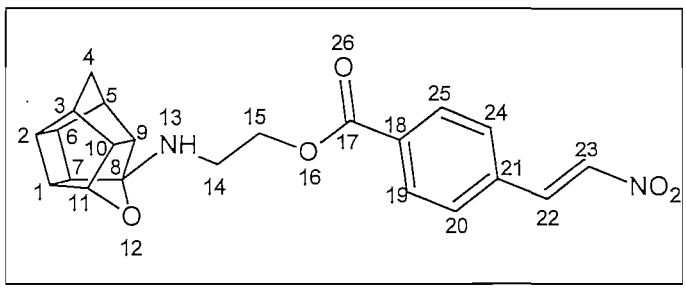
3.3.8 8-AMINOETHYL-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE-2-[4-(2-NITROETHENYL)BENZOATE] (3.3)



In a microwave tube, 4-formylbenzoic acid (1.126 g, 7.5 mmol) was added to nitromethane (0.4 ml, 7.5 mmol) and a catalytic amount of ammonium acetate (0.145 g, 0.19 mmol). The mixture was irradiated in a microwave station for 2 minutes and 280 W at 25 °C. The mixture was then removed and allowed to cool down. It was then irradiated twice more, for 2 minutes each and with cooling in between. Nitromethane (0.4 ml, 7.5 mmol) was added to the mixture again and the procedure was repeated as before. The 4-nitroethenylbenzoic acid was

purified using column chromatography (mobile phase, ethyl acetate). The 4-nitroethenylbenzoic acid (**3.9**) was obtained as a light yellow microcrystalline solid (yield: 0.545 g, 2.8 mmol, 37.33 %). In a one pot synthesis, 8-(2-aminoethanol)-8,11-oxa pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**3.10**) (0.236 g, 1.1 mmol), 1.1 equivalent of 4-nitroethenylbenzoic acid (**3.9**) (0.229 g, 1.2 mmol), 1.1 equivalent of 1-ethyl-3-(3'-dimethylamino)carbodiimide (0.227 g, 1.2 mmol) and 0.1 equivalent of 4-(dimethylamino)-pyridine (0.013 g, 0.1 mmol) was dissolved in dichloromethane (20 ml) and cooled down to 5 °C on an external ice bath. The reaction mixture was stirred for 15 minutes on the ice bath, removed and stirred for a further 72 hours at room temperature. The mixture was then removed and concentrated *in vacuo*. A saturated $NaHCO_3$ solution (30 ml) was added and followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight over anhydrous $MgSO_4$ and evaporated to yield a dark yellow oil. The crude compound was purified using column chromatography (mobile phase, ethyl acetate: dichloromethane (1:1)) and the desired amine **3.3** was isolated as a yellow oil which crystallized overnight to yield light yellow crystals (0.109 g, 0.3 mmol, 27.27 %).

- **PHYSICAL DATA**

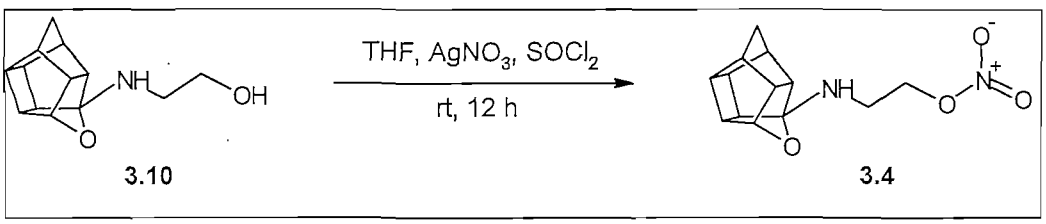


$C_{22}H_{22}N_2O_5$; TLC (EtOAc:DCM): R_f 0.64; **m.p.** 185 °C; **IR (KBr)** ν_{max} (spectrum 17): 3292.49, 1722.90, 1666.50, 1610.56, 1502.55, 1367.53, 1282.30; **LC-MS (EI, 70 eV)** ***m/z*** (spectrum 18): 406.2(M^+), 384.3, 374.0, 352.2, 242.3, 220.2, 177.1; **^{1}H NMR (600 MHz, $CDCl_3$)** δ_H (spectrum 19): 10.035 (s, 1H, H-23), 8.133 (dd, 2H, J = 4.92 Hz, H-19,25), 7.888 (dd, 2H, J = 4.52 Hz, H-20,24), 7.201 (t, 1H, H-22), 4.7 (t, 1H, J = 5.32 Hz, H-11), 4.507 (t, 2H, J = 6.32 Hz, H-14), 3.149 (t, 2H, J = 3.48 Hz, H-15), 2.759 – 2.24 (m, 8H, H-1,2,3,5,6,7,9,10), 1.839 (AB-q, 2H, J = 9.42 Hz, H-4a,4b). **^{13}C NMR (150 MHz, $CDCl_3$)** δ_C (spectrum 20): 191.88 (s, C-17), 191.87 (s, C-23), 165.49 (s, C-22), 139.11 (s, C-18), 135.16 (s, C-21), 130.2 (d, C-19/C-25), 129.47 (d, C-20/C-24), 109.26 (d, C-15), 82.56 (t, C-11), 66.21 (s, C-8), 55.36 (d, C-14), 54.73 (d, C-9), 44.82 (d, C-7), 44.70 (d, C-1), 44.53 (d, C-10), 43.29 (d, C-3), 43.05 (d, C-5), 42.47 (t, C-4), 41.89 (d, C-6), 41.51 (d, C-2).

- **STRUCTURE ELUCIDATION**

The NMR and two-dimensional homonuclear (H,H)-COSY NMR of compound **3.3** (spectrum 21) showed the obligatory signals for the pentacycloundecylamine cage structure (elucidated as described above with compound **3.1**). H-15 was detected at δ_H 3.149 and showed coupling with H-14 at δ_H 4.507. H-11 was detected at δ_H 4.7 and showed coupling with the down field protons on the cage moiety. The peak at δ 10.035 was assigned to H-23 since it would be the least shielded of the protons. The doublet of doublets at δ_H 7.888 and δ_H 8.133 were assigned to H-20/H-24 and H-19/H-25 respectively and only showed coupling with each other. The preliminary ^{13}C elucidation was done using HSQC (spectrum 22) and the remaining peaks were elucidated using HMBC (spectrum 23). The peak at δ_C 191.88 was assigned to C-17 since it showed no coupling with other protons. This made it possible to assign peaks to C-22 and C-23 (δ_C 165.49 and δ_C 191.87 respectively) since both of them were sufficiently down field and showed coupling with the aromatic protons. The IR absorption spectrophotometry spectrum for compound **3.3** was elucidated as described above for compound **3.1**, with the exception of the alkene peak at 1666.50 cm^{-1}. The LC-MS gave the molecular ion (M^+) at m/z = 406.2, which correlates with the predicted mass of 394.42 g/mol.

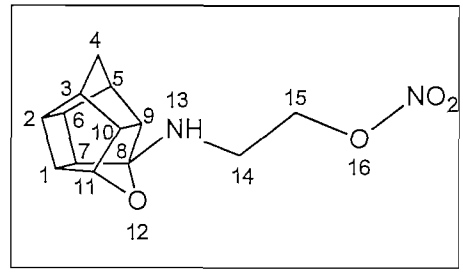
3.3.9 8-(2-AMINOETHYL NITRATE)-8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$] UNDECANE (3.4)



3.10 3.4

8-(2-Aminoethanol)-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**3.10**) (0.197 g, 0.9 mmol) was dissolved in dry THF (5 ml) at room temperature. Silver nitrate (0.153 g, 0.9 mmol) was added. An equimolar amount of thionylchloride (0.07 ml, 0.9 mmol) was added drop wise, while stirring at room temperature. The reaction mixture was allowed to stir for 12 hours. Quantitative precipitation of silver chloride started to occur as the reaction neared completion. After 12 hours, water (30 ml) was added to the mixture followed by extraction with ethyl acetate (4 x 20 ml). The combined organic fractions were dried overnight over anhydrous $MgSO_4$ and evaporated in vacuo to yield a yellow oil. The crude compound was purified using column chromatography (mobile phase, ethyl acetate) and the desired amine (**3.4**) was isolated as a light yellow oil (0.072 g, 0.03 mmol, 33.33%).

- **PHYSICAL DATA**

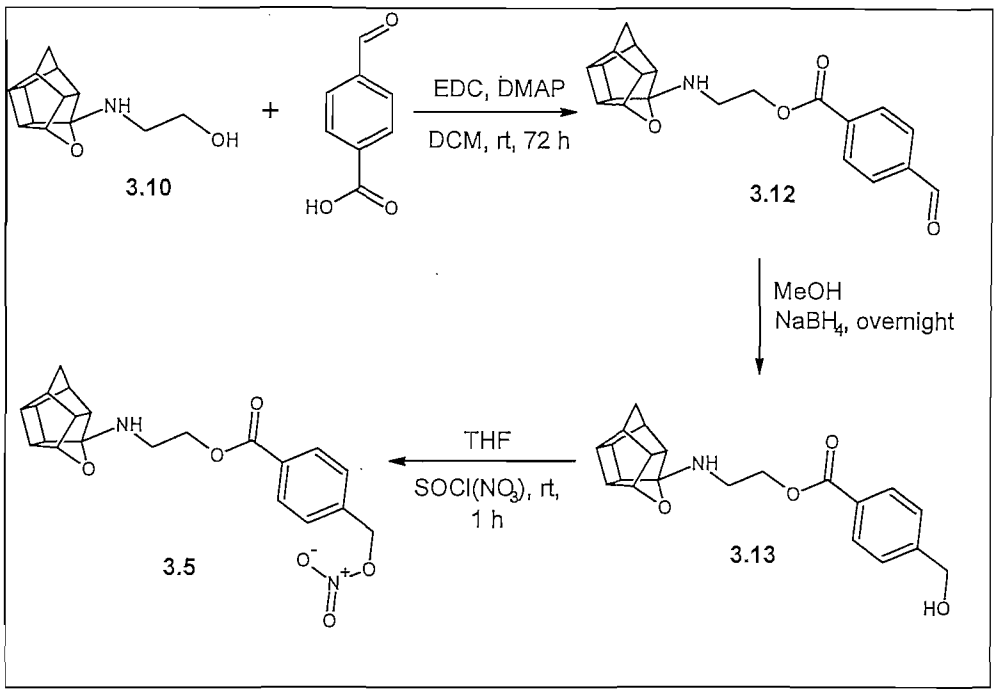


$C_{13}H_{16}N_2O_4$; TLC (EtOAc): R_f 0.86; **IR (KBr)** ν_{max} (spectrum 24): 2978.09, 1699.29, 1475.54, 1348.24, 1255.66; **LC-MS (EI, 70 eV)** ***m/z*** (spectrum 25): 264.1(M^+), 242.2, 220.1, 202.2, 183.1, 170.9; **^{1}H NMR (600 MHz, $CDCl_3$)** δ_H (spectrum 26): 4.59 (t, 1H, J = 5.18 Hz, H-11), 4.08 (m, 2H, H-15a,b), 3.65 (m, 2H, H-14a,b), 2.75 – 2.35 (m, 8H, H-1,2,3,5,6,7,9,10), 1.81 (AB-q, 2H, J = 10.5 Hz, H-4a,4b); **^{13}C NMR (150 MHz, $CDCl_3$)** δ_C (spectrum 27): 85.1 (t, C-11), 83.2 (s, C-8), 66.580 (d, C-14), 63.3 (d, C-15), 57.292 (d, C-10), 55.65 (d, C-9), 46.25 (d, C-7), 45.897 (d, C-5), 45.83 (d, C-3), 43.82 (d, C-1), 42.9 (t, C-4), 42.14 (d, C-6), 41.6 (d, C-2).

- **STRUCTURE ELUCIDATION**

The NMR and two-dimensional homonuclear (H,H)-COSY NMR of compound **3.4** (spectrum 28) showed the obligatory signals for the pentacycloundecylamine cage structure (elucidated as described above with compound **3.1**). H-11 was detected at δ_H 4.59 and showed coupling with the protons on the cage moiety. Due to technical problems with the NMR machine, no HSQC and HMBC spectra could be obtained for compound **3.4**, instead, the ^{13}C spectrum was correlated to that of **3.10**, since the two compounds are structurally the most similar. The IR absorption spectrophotometry spectrum for compound **3.4** was elucidated as described above for compound **3.1**, with the exception of the absence of the aromatic peak at 1623 – 1496 cm^{-1}. The LC-MS gave the molecular ion (M^+) at m/z = 264.1, which correlates well with the predicted mass of 264.28 g/mol.

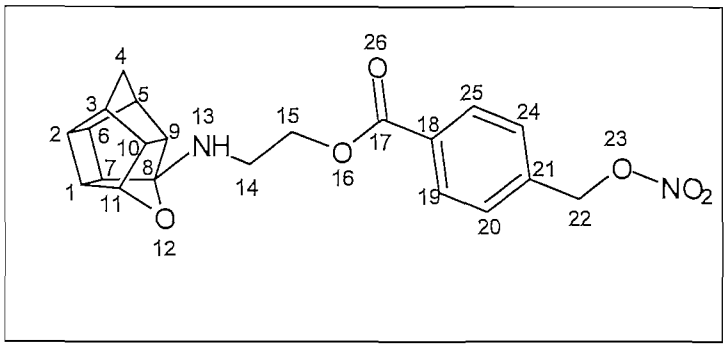
3.3.10 8-AMINOETHYL-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE-4-(2-[(NITROOXY)METHYL]BENZOATE) (3.5)



In a one pot synthesis, 8-(2-aminoethanol)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**3.10**) (1.535 g, 7.0 mmol), 1.1 equivalents of 4-formylbenzoic acid (1.156 g, 7.7 mmol), 1.1 equivalent of 1-ethyl-3-(3'-dimethylamino)carbodiimide (1.476 g, 7.7 mmol) and 0.1 equivalent of 4-(dimethylamino)-pyridine (0.086 g, 0.7 mmol) was dissolved in dichloromethane (20 ml) and cooled to 5 °C on an external ice bath. The reaction mixture was stirred for 15 minutes and removed from the ice bath, after which it was stirred for another 72 hours at room temperature. The mixture was concentrated *in vacuo*. A saturated sodium bicarbonate solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight with anhydrous $MgSO_4$ and evaporated to give a bright yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate: dichloromethane (1:3)) and the desired amine (**3.12**) was isolated as pure white crystals (0.698 g, 2.0 mmol, 28.57 %). Compound **3.12** was then dissolved in dry methanol (30 ml), cooled down to 5 °C on an external ice bath. A small spatula point (± 0.2 g) of sodium borohydrite was slowly added to the stirred mixture on the ice bath. After 30 minutes the reaction mixture was removed from the ice bath and stirred at room temperature overnight. The reaction mixture was concentrated in vacuo. A saturated sodium bicarbonate solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight with $MgSO_4$ and evaporated to give a light yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate) to yield the desired amine (**3.13**) as a murky oil which

crystallized overnight to give colourless crystals (0.128 g, 0.4 mmol, 20 %). Compound **3.13** (0.128 g, 0.4 mmol) was dissolved in dry THF (10 ml) and stirred at room temperature. Thionylchloride nitrate (0.3 ml, 0.4 mmol) was added dropwise to the reaction mixture and it was stirred for 120 minutes at room temperature. Diethyl ether (30 ml) was added to the mixture and it was washed with water (2 x 40 ml). The organic fraction was dried overnight with anhydrous $MgSO_4$ and evaporated to give a dark yellow oil. The crude compound was purified using column chromatography (mobile phase, ethyl acetate: dichloromethane (1:2)) to give the desired amine (**3.5**) as a dark orange oil (0.024 g, 0.06 mmol, 15 %).

- PHYSICAL DATA



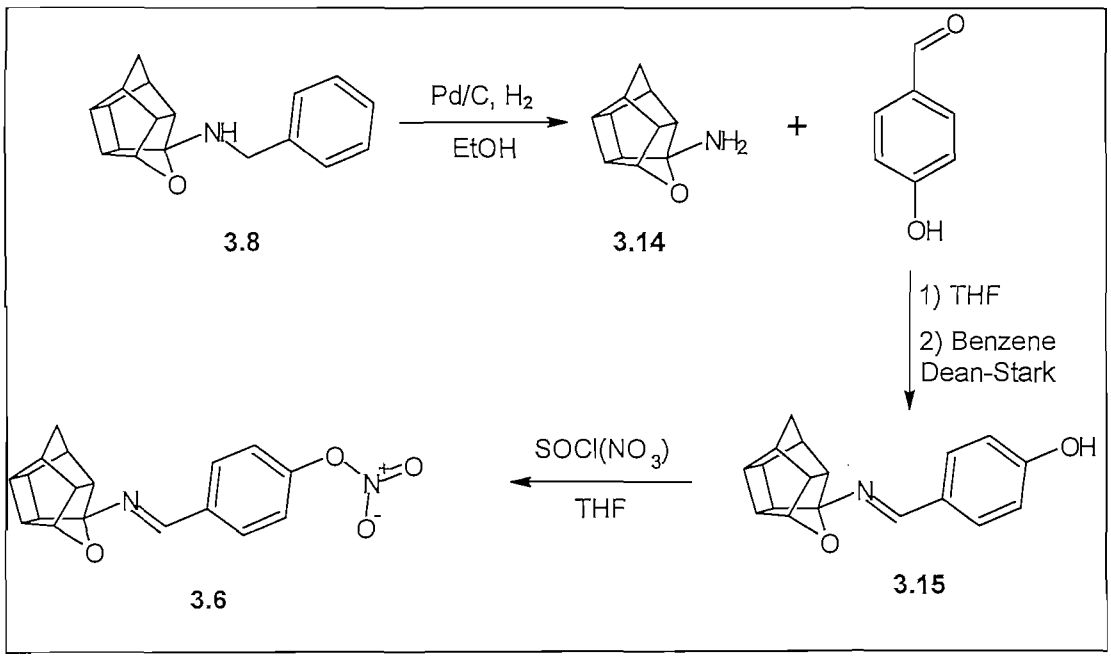
$C_{21}H_{22}N_2O_6$; TLC: (EtOAc:2DCM): R_f 0.90; **IR (KBr)** ν_{max} (spectrum 29): 2956.87, 1720.5, 1633.71, 1612.49, 1313.52, 1163.08; **LC-MS (EI, 70 eV) *m/z*** (spectrum 30): 393.1(M^+), 381.3, 372.2, 353.3, 283.1, 246.9, 220.1, 210.1; **^{1}H NMR (600 MHz, $CDCl_3$) δ_H** (spectrum 31): 7.926 (d, 2H, H-19.25), 7.375 (d, 2H, J = 8.1 Hz, H-20,24), 4.749 (t, 1H, J = 5.2 Hz, H-11), 4.52 (s, 2H, H-22), 2.923 – 2.451 (m, 4H, H-14a,H-14b,H-15a,H-15b), 3.164 – 3.076 (m, 8H, H-1,2,3,5,6,7,9,10), 1.924 (AB-q, 2H, J = 10.6 Hz, H-4a,4b). **^{13}C NMR (150 MHz, $CDCl_3$) δ_C** (spectrum 32): 165.65 (s, C-17), 143.03 (s, C-18), 143.36 (s, C-21), 130.09 (d, C-19/C-25), 128.29 (d, C-20/24), 84.03 (t, C-11), 60.95 (s, C-8), 56.51 (d, C-9), 54.90 (d, C-7), 45.85 (d, C-1), 45.19 (s, C-22), 45.05 (d, C-5), 44.87 (d, C-3), 43.5 (t, C-4), 43.41 (d, C-14), 41.95 (d, C-15), 41.84 (d, C-6), 41.54 (d, C-2).

- STRUCTURE ELUCIDATION

The NMR and two-dimensional homonuclear (H,H)-COSY NMR of compound **3.5** (spectrum 33) showed the obligatory signals for the pentacycloundecylamine cage structure. The peak at δ_H 4.749 was assigned to H-11 since it showed mutual scalar coupling with the protons in the cage compound. H-14 at δ_H 2.923 and H-15 at δ_H 2.451 showed mutual scalar coupling with each other and also with the singlet at δ_H 4.52, which was assigned to H-22. This suggest that the steric freedom brought on by the linker and ester group causes the protons

of H-22 to come in close proximity to the protons of H-14 and H-15. The singlet at δ_H 4.523 (H-22) also showed through space coupling with H-20/H-24, its position down field corresponds with its protons being less shielded due to the neighbouring nitrate group. The peaks at δ_H 7.93 were assigned as H-19/H-25 since it is conjugated to a carbonyl carbon (and would therefore be furthest down field) and showed coupling with the peaks assigned to H-20/H-24. Following preliminary structure elucidation using HSQC (spectrum 34), the remaining peaks were elucidated using HMBC (spectrum 35). The HMBC spectrum showed a coupling between H-22 and C-20/C-24 as well as with C-19/C-25, asserting the assignment of that proton. The HSQC showed the proton, H-22, to be coupled to the carbon at δ_C 45.19, thereby confirming this atom's position on the ^{13}C NMR as well. The IR absorption spectrophotometry spectrum for compound **3.5** was elucidated as described above for compound **3.1**. The LC-MS gave the molecular ion (M^+) at m/z = 393.1, which correlates with the predicted mass of 398.41 g/mol.

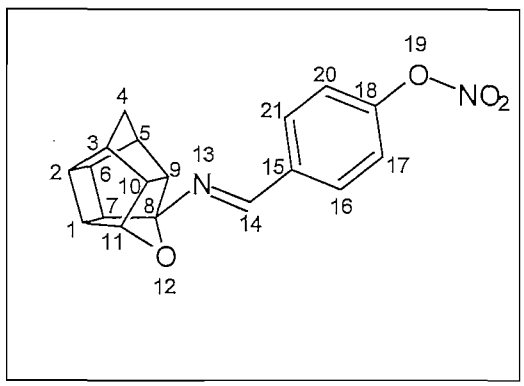
3.3.11 8-[4-(IMINOMETHYL)PHENYL NITRATE]-8,11-OXAPENTACYCLO [5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE (3.6)



NGP1-01 (**3.8**) (2.95 g, 11.12 mmol) was dissolved in ethanol (30 ml) and reacted with hydrogen over 10 % palladised charcoal (0.15 g) for 12 hours at atmospheric pressure and at room temperature. The reaction mixture was concentrated in vacuo and a saturated sodium bicarbonate solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight over anhydrous $MgSO_4$ and evaporated to give a dark yellow oil, which solidified overnight to give a waxy semisolid. Recrystallisation from dichloromethane: hexane (1:1) afforded the desired amine (**3.14**) as

colourless platelets (1.4 g, 7.99 mmol, 71 %). Compound **3.14** (1.4 g, 7.99 mmol) was then dissolved in tetrahydrofurane (20 ml) and cooled down to 5 °C on an external ice bath. 4-hydroxybenzaldehyde (1 g, 7.99 mmol) was slowly added and the reaction mixture was allowed to stir for 2 hours. The mixture was concentrated *in vacuo*, water (50 ml) was added, followed by extraction with dichloromethane (4 x 30 ml). The combined organic fractions were dried overnight with anhydrous $MgSO_4$, filtered and evaporated to give a dark yellow oil. The crude compound was purified using column chromatography, which yielded the desired amine (**3.15**) as a light yellow oil (1.5 g, 5.4 mmol, 67.58 %). Compound **3.15** (1.5 g, 5.4 mmol) was then dissolved in tetrahydrofurane (10 ml) and thionylchloride nitrate (0.4 ml, 5.4 mmol) was added dropwise. The reaction mixture was allowed to stir for 2 hours at room temperature. Diethyl ether (30 ml) was added and it was washed with water (2 x 20 ml). The organic fraction was dried overnight with anhydrous $MgSO_4$, filtered and evaporated to give a dark yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate: dichloromethane (1:2)) to yield the desired amine (**3.6**) as light yellow crystals (0.198 g, 0.6 mmol, 11.31 %).

- **PHYSICAL DATA**



$C_{18}H_{16}N_2O_4$; TLC: (EtOAc:2DCM): R_f 0.95; **m.p.** 95 °C; **IR (KBr)** ν_{max} (spectrum 36): 1726.29, 1598.99, 1556.55, 1373.32, 1101.35; **LC-MS (EI, 70 eV)** ***m/z*** (spectrum 37): 333.0(M^+), 317.0, 287.1, 265.9, 259.2; **^{1}H NMR (600 MHz, $CDCl_3$)** δ_H (spectrum 38): 7.294 – 7.157 (m, 4H, H-16,17,20,21), 4.883 (t, 1H, J = 15.16 Hz, H-11), 3.238 – 2.711 (m, 9H, H-1,2,3,5,6,7,9,10,11), 2.02 (d, 1H, J = 10.68 Hz, H-4b), 1.648 (d, 1H, J = 10.68 Hz, H-4a). **^{13}C NMR (150 MHz, $CDCl_3$)** δ_C (spectrum 39): 135.45 (s, C-18), 128.43 (d, C-16/C-21), 127.53 (d, C-17/C-20), 127.21 (s, C-15), 110.15 (s, C-8), 84.18 (t, C-11), 56.48 (d, C-9), 54.95 (s, C-14), 46.81 (d, C-7), 45.84 (d, C-1), 45.12 (d, C-10), 44.88 (d, C-5), 43.56 (d, C-3), 43.49 (d, C-4), 42.01 (d, C-6), 41.65 (d, C-2).

- **STRUCTURE ELUCIDATION**

The NMR and two-dimensional homonuclear (H,H)-COSY NMR of compound **3.6** (spectrum 40) showed all the obligatory signals for the pentacycloundecylamines. Ethanol peaks at δ_H 3.753 coupled with peaks at δ_H 1.3 suggest ethanol entrapment within the microcrystalline structure of the compound. H-11 with resonance at δ_H 4.883, showed through space coupling with aromatic protons. H-11 also showed coupling with the protons in the cage moiety. After the preliminary assignment of the ^{13}C spectrum using HSQC (spectrum 41), HMBC (spectrum 42) was used to elucidate the remaining peaks. Analysis of the HMBC spectrum also showed coupling of H-11 to carbons in the aromatic moiety, correlating with the data found in its (H,H)-COSY. H-14 also showed coupling with carbons in the cage structure, possibly C-10 and/or C-1. The IR absorption spectrophotometry spectrum for compound **3.6** was elucidated as described above for compound **3.1**, with the exception of the absence of the secondary amine peak at ~3403 cm^{-1}, confirming that the compound exists in the imine form. The LC-MS gave the molecular ion (M^+) at m/z = 333.0, which correlates with the predicted mass of 324.33 g/mol.

3.4 CONCLUDING REMARKS

The synthesised compounds all contain a pentacycloundecylamine moiety, necessary for the hydrophobic interaction as well as an amine group necessary for hydrogen bond formation with a hydrogen bond acceptor. They also contain aromatic moieties, with the exception of **3.4**, necessary for high affinity bonding. Increased steric freedom, relative to NGP1-01, was brought on by lengthening the chain between the cage structure and the aromatic moiety. Yields during the esterification were generally low because of the electron withdrawing group conjugated to the benzoic acid. Structure elucidation with one and two-dimensional NMR was invaluable in giving unambiguous assigning of the peaks.

CHAPTER 4

BIOLOGICAL EVALUATION

SUMMARY

The hypothesis that nitrosylation augments the channel activity of polycyclic cage amines was investigated by means of two biological assays. In the first assay the capability of each compound to nitrosylate cysteine residues was investigated. In the second assay, the ability of each compound to block voltage gated calcium channels was assessed. The inhibition of calcium flux and the nitrosylation potency was then correlated with each other to determine if nitrosylation augmented the channel activity of the polycyclic amines.

4.1 S-NITROSYLATION ASSAY

Jaffrey *et al.* (2001:193) developed a system to detect S-nitrosylated proteins in brain homogenate by converting nitrosylated cysteines into biotinylated cysteines. In the first step, free thiols were blocked with the thiol-specific methylthiolating agent, methyl methanethiosulfonate (MMTS). MMTS doesn't react with pre-existing disulphide bonds or nitrosothiols. The second step involves the reaction of the newly formed thiols with N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP), a sulfhydryl-specific biotinylating reagent.

Biotin-HPDP is a membrane-permeable biotin labelling agent that reacts with sulfhydryl (-SH) groups. The disulfide bond that forms can be cleaved by means of a reducing agent to yield the biotin group and the original protein or peptide. The main use of biotin-HPDP is the labelling of target molecules for reducing SDS-PAGE or mass analysis when purification is done with immobilised avidin, streptavidin or NeutrAvidin™ Protein. The high affinity interaction between the avidin and the biotin usually has to be cleaved with a strong acid or denaturant, but with biotin-HPDP the captured biotinylated molecules can be efficiently eluted from the support by cleaving the disulfide bond with dithiothreitol (DTT), as seen in figure 4.1. This reaction system was implemented, with a few modifications, to detect the extent of S-nitrosylation attained by the test compounds. The modifications include the substitution of rat brain homogenate with pure cysteine. The other modification is the use of ultraviolet (UV) absorbance, not only to detect nitrosylation, but to measure its extent. The

pyridine-2-thione that was used by Jaffrey *et al.* (2001:193) to detect nitrosylation, now served as an indicator of nitrosylation potency. The UV absorbance was measured at different points in time over 20 minutes, and the intensity of the absorbance was used as an indication of how much biotin-HPDP had reacted. The amount of biotin that reacted was directly proportional to the extent of nitrosylation that took place.

The extinction coefficient (ε) for pyridine-2-thione at 343 nm is 8.08 x 10^3 M^{-1} cm^{-1}. This extinction coefficient, or molar absorptivity, is a measure of how strongly the specific chemical species absorbs light at a given wavelength. The extinction coefficient is an intrinsic property of the specific chemical species. According to the Beer-Lambert law (equation 4.1), the actual absorbance, A, of a sample is dependent on the pathlength *l* and the concentration of the species, *c*.

$$A = \varepsilon c l \qquad (4.1)$$

If the extinction coefficient (molar absorbance) is known, the Beer-Lambert law can be applied to spectrophotometric analysis, without the need of extensive pre-processing of the sample. Therefore, if the pathlength is kept constant, and the extinction coefficient is known, the concentration of the chemical species for a specific absorbance value can be determined.

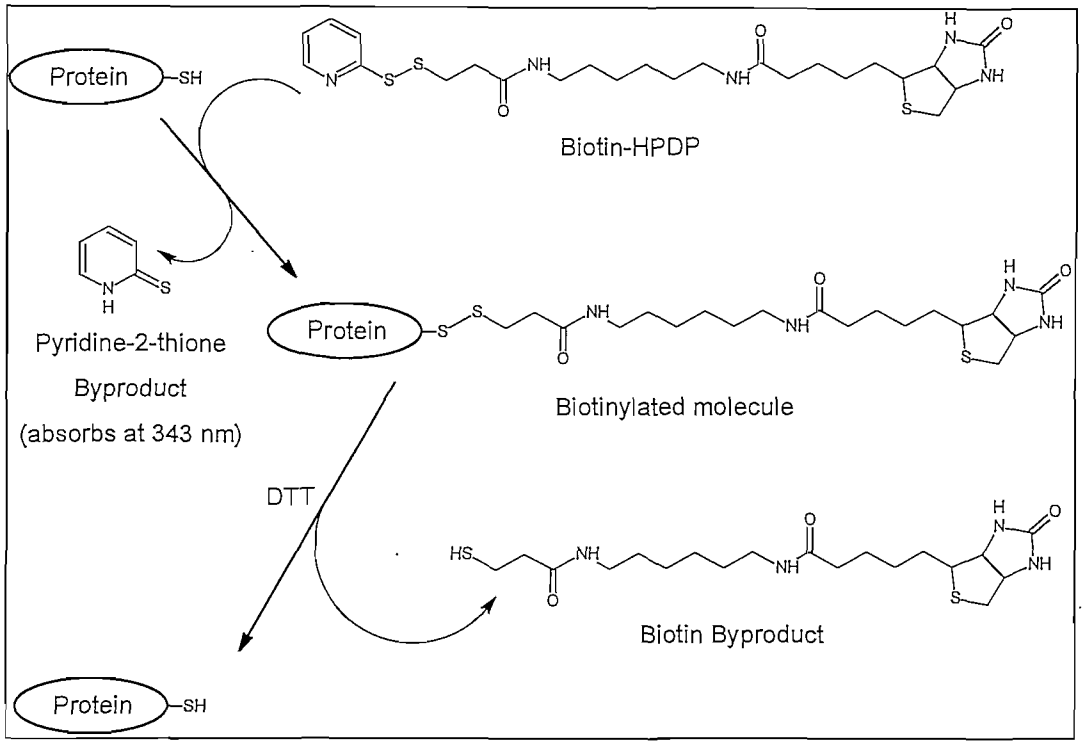


Figure 4.1: Reaction scheme for biotinylation of sulfhydryl molecules with Biotin-HPDP.

4.1.1 CHEMICALS

For the nitrosylation assay, cysteine-glycine, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), methyl methanethiolsulfonate (97 %) and neocuproine free base were purchased from Sigma-Aldrich (Steinheim, Germany). EZ-Link® Biotin-HPDP was purchased from Separations (Randburg, South Africa).

4.1.2 INSTRUMENTS

Measurements were taken using a Varian Cary Eclipse® Fluorescence Spectrophotometer, set to measure UV absorbance at 343 nm.

4.1.3 ASSAY PROCEDURE

The cysteine-glycine dipeptide (4 mM) was dissolved in HEN buffer (250 mM HEPES, 1 mM EDTA and 0.1 mM neocuproine, adjusted to pH 7.7 with NaOH). To the solution was added an equimolar concentration of the NO donor and the mixture was stirred at room temperature and in the dark for 1 hour. MMTS (200 µl, 20 mM, dissolved in dimethylformamide) was then added and the mixture was incubated at 50 °C for 20 minutes with frequent vortexing. Acetone (200 µl) was added and the mixture was vortexed for 10 minutes while the unreacted MMTS precipitated. 500 µl of the solution was removed using a micro pipette and

placed in a quarts cuvette in the spectrophotometer. Biotin-HPDP was added and the UV absorbance was measured over 20 minutes with 5 minute intervals (figure 4.2).

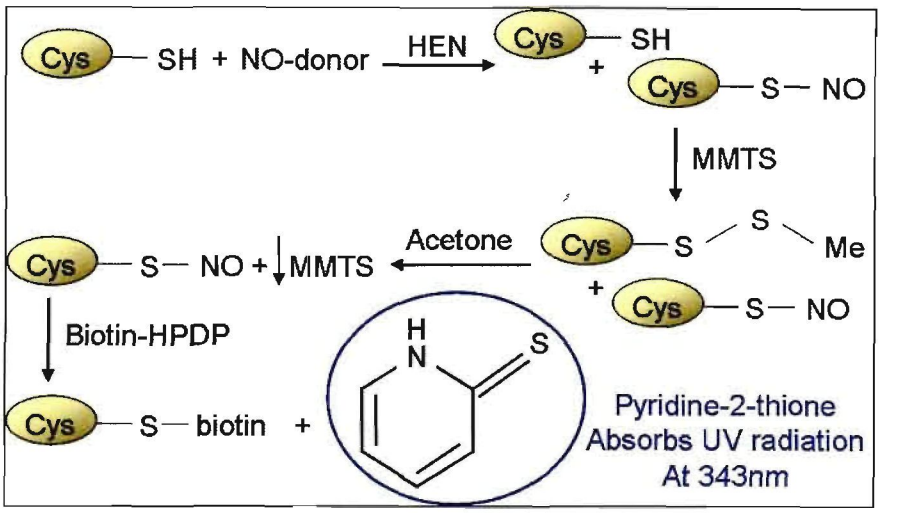


Figure 4.2: A schematic representation of the S-nitrosylation assay.

Before each measurement the spectrophotometer was auto-zeroed with a blank solution. For the blank solution the procedure was followed as above, except for the absence of the cysteine-glycine dipeptide, as it was the only compound in the assay without UV absorbance capability. Each concentration was tested in triplicate.

As a control, the procedure was carried out as above, but without the addition of the NO donor and MMTS, to allow the cysteine to react completely with the biotin-HPDP and give a representation of 100 % nitrosylation. The control was also auto-zeroed with its own blank solution containing only Hen buffer and Biotin-HPDP.

4.1.4 RESULTS AND DISCUSSION

Statistical analysis was performed using GraphPad InStat 2.0 (GraphPad Software, San Diego, CA, USA) and Microsoft Office Excel 2003, SP3. Dunnett's one-way analysis of variance (ANOVA) with post-test was performed on all the tested concentrations, compared to the standard (without test compound) to indicate significant differences (* $p < 0.05$ were considered to be statistically significant and ** $p < 0.01$ were considered to be statistically very significant). Data on the histogram are presented as % values of the reference standard fluorescence with each bar representing the mean ± S.E.M.

The average UV absorbance at 20 minutes was taken and the data expressed as a percentage relative to the control of each experimental run (table 4.1). The small S.E.M. can

be related to the experiment being a controlled chemical reaction as opposed to a reaction in a biological system.

Table 4.1: Data obtained from the S-nitrosylation assay.

	3.1		**3.2**		**3.3**	
Control	0.2787	100%	0.2371	100%	0.2883	100%
AVE	0.0425	15%	0.0259	11%	0.1121	39%
STDEV	0.0079	3%	0.0129	5%	0.0017	1%
SEM	0.0045	1.63%	0.0074	3.13%	0.0010	0.33%
	3.4		**3.5**		**3.6**	
Control	0.2582	100%	0.2045	100%	0.2868	100%
AVE	0.0514	20%	0.0611	30%	0.1826	64%
STDEV	0.0018	1%	0.0014	1%	0.0265	9%
SEM	0.0010	0.40%	0.0008	0.40%	0.0153	5.33%

Better visualisation of the experimental data can be obtained by exporting the data in table 4.1 to a histogram (figure 4.3). The compounds all exhibited the ability to S-nitrosylate cysteine residues, with the weakest one (**3.2**) nitrosylating 11 % relative to the control (100 %). In general, the nitrate compounds **3.4**, **3.5** and **3.6** gave larger percentages of absorbance (and therefore S-nitrosylation) than the unsaturated nitro compounds **3.1** and **3.2**, while **3.4** exhibited S-nitrosylation comparable to that of the nitrate compounds. It is known from the literature that the most common non-enzymatic pathway of organic nitrates to donate nitrogen monoxide is *via* interaction with thiol groups, while carbon-nitro compounds donate NO because of heat or light (Cai *et al.,* 2005:8). It was therefore postulated that the nitrate compounds would be better S-nitrosylators, and this was observed for the compounds tested. Compounds **3.4** and **3.5** would be expected to be better nitrogen monoxide donors then compound **3.3**, yet they show less nitrosylation than **3.3**. The only deduction that can be made is that they show less nitrosylation than expected because their nitrogen monoxide donating moieties are not directly conjugated to an electron withdrawing or unsaturated group. The nitrobenzoic compounds **3.1** and **3.2** showed the least S-nitrosylation, as expected since many of the commercial compounds containing this functional group are not known for their nitrosylation activity.

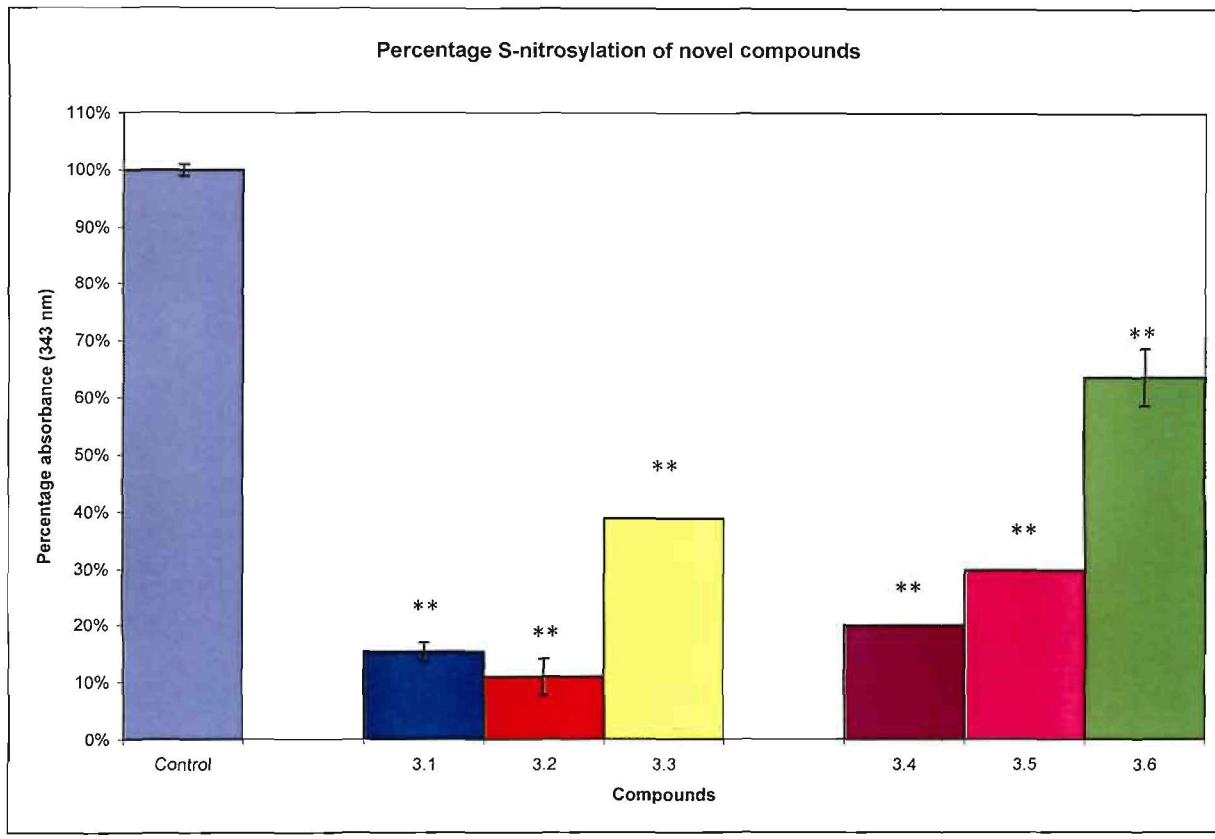


Figure 4.3: Histogram displaying the percentage S-nitrosylation brought on by the tested compounds relative to the standard. ** indicates very significant ($p < 0.01$) change from the (100 %) control.

4.2 CALCIUM FLUX ASSAY

To evaluate the calcium channel activity of the synthesised compounds, fluorescent techniques were employed to measure Ca^{2+} influx into synaptoneurosomes, a mixture of sealed presynaptic structures (synaptosomes) with attached postsynaptic structures (neurosomes). Synaptoneurosomes were obtained from rat brain homogenate and incubated with the fluorescent indicator, Fura-2/AM.

The Fura-2 was administered as the AM ester to facilitate membrane permeability. Once inside the cell the ester is cleaved by esterase enzymes and the resulting free Fura-2 (**4.1**) (figure 4.4) is less membrane permeable, trapping it inside the cell. The membranes of the synaptoneurosomes were then depolarised using a high concentration KCl solution, causing Ca^{2+} influx into the cells. The Fura-2 then binds the Ca^{2+} and undergoes a shift in absorption from 335 and 363 nm to 340 and 380 nm. Excitation at these wavelengths increases the emission at 500 nm (figure 4.5). This served as a control for the experiment.

In the assay of the test compounds, the synaptoneurosomes were first incubated with the compounds synthesised before depolarisation. Calcium channel block is indicated by a decrease in fluorescence intensity from that of the control.

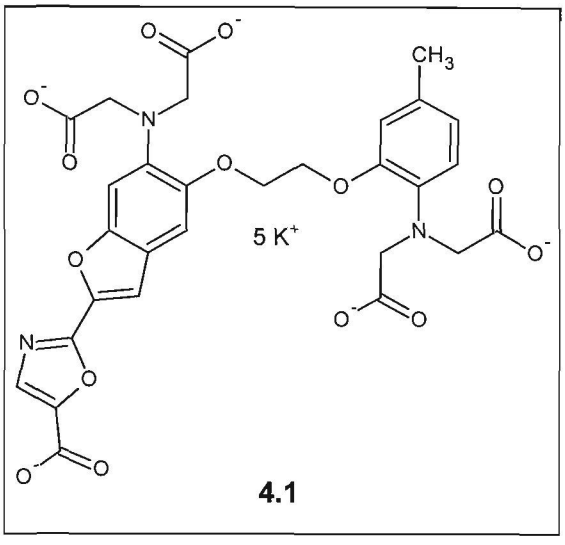


Figure 4.4: The fluorescent indicator, Fura-2.

To assess the calcium channel activity of the pentacycloundecylamines synthesised in this study, their activity was compared to that of NGP1-01, a well described calcium channel blocker, as well as nimodipine as a positive controls.

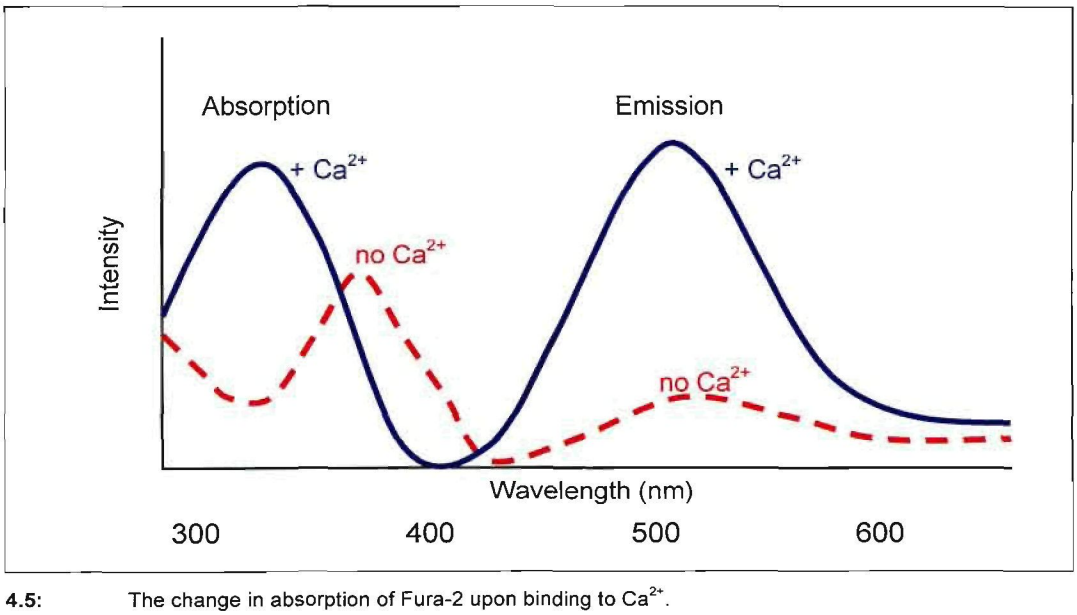


Figure 4.5: The change in absorption of Fura-2 upon binding to Ca^{2+}.

4.2.1 CHEMICALS

For the buffer solutions, D(+)glucose, sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), potassium chloride (KCl) and potassium hydrogen

phosphate (KH_2PO_4) were obtained from Saarchem (Krugersdorp, S.A). HEPES, anhydrous magnesium chloride, nimodipine and Fura-2/AM were obtained from Sigma-Aldrich (Steinheim, Germany).

4.2.2 INSTRUMENTS

Measurements were taken using a Varian Cary Eclipse® Fluorescence Spectrophotometer, set to the recording parameters in table 4.2.

4.2.3 ANIMALS

Eight day old Sprague-Dawley rats of either sex were used for the calcium flux assay. The experiment was carried out under the approval and in accordance with the guidelines as laid down by the Ethics Committee of the North West University.

Table 4.2: Recording parameters.

Fura-2/AM	Recording parameters
Excitation	340 / 380 nm
Emission	500 nm
Optics position	Bottom
Sensitivity	100
Runtime	40 seconds
Interval	150 milliseconds

4.2.4 PREPARATION OF SYNAPTONEUROSOMES

Eight day old Sprague-Dawley rats of either sex were sacrificed by decapitation and whole brains were removed. The brains from four rats were removed and homogenized (four strokes by hand using a Dounce homogenizer) in 30 ml of ice cold Krebs-bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgCl_2$, 1.2 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM glucose). To minimize proteolysis through the isolation procedure, the homogenate was kept ice cold from this step onward. The tissue suspension was placed in a 50 ml polycarbonate tube and centrifuged for 15 minutes at 1 100 x g and 0°C. After centrifugation the pellet was resuspended by hand in 30 ml fresh Krebs-bicarbonate buffer and centrifuged again as described above. The protein concentration was measured

spectrofluorimetrically using the Bradford method and Krebs-bicarbonate buffer was added to yield a final concentration of 1.9 mg/ml.

4.2.5 ASSAY PROCEDURE

The suspension described above was allowed to reach room temperature, after which Fura-2/AM was added to a final concentration of 5 µM. The 5 mM Fura-2/AM stock solution was prepared by dissolving 1 mg Fura-2/AM in 2.5 ml dimethylsulfoxide (DMSO). The synaptoneurosomes were diluted to a final concentration of 0.6 mg/ml using Krebs-bicarbonate buffer (22 °C) and incubated at 37 °C for 10 minutes. From this step onward the suspension was kept at room temperature and protected from light (working mostly in a dark room).

Immediately before the experiment, 1 ml of the synaptoneurosomal suspension was centrifuged for 10 seconds in a desk Eppendorf Microfuge, resuspended in 3 ml of 37 °C Krebs-HEPES buffer (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgCl_2$, 1.2 mM $CaCl_2$, 20 mM HEPES, 1.2 mM KH_2PO_4 and 10 mM glucose adjusted to pH 7.4 with NaOH). Here HEPES substitutes for $NaHCO_3$ since the latter causes the appearance of bubbles in the cuvette and increases the "noise".

0.01 M, 0.001 M and 0.0001 M concentrations of test and control compound were prepared in DMSO. 1 µl of the stock solution was diluted in 1 ml buffer solution to give 10 µM, 1 µM and 0.1 µM concentrations of the compound. The final concentration of DMSO in the incubation was 0.1 %. The different concentrations were incubated for 5 minutes with the synaptoneurosomes and used immediately thereafter.

All the experiments were carried out at 37 °C and fluorescence was measured as a kinetics run in a spectrofluorimeter equipped with a water-jacketed cuvette holder. The spectrofluorimetric parameters are given in table 4.1. The reference experiment (with the absence of test compound), comprised of 5 minutes incubation at 37 °C in Krebs-HEPES buffer containing 0.1 % DMSO, followed by the addition of 300 µl depolarisation solution. This served as the standard reference, representing 100% calcium influx.

Each experiment was carried out with two different sets of synaptoneurosomes and three replications of three different concentrations (10 µM, 1 µM and 0.1 µM) of each compound.

4.2.6 RESULTS AND DISCUSSION

Statistical analysis was performed using GraphPad InStat 2.0 (GraphPad Software, San Diego, CA, USA) and Microsoft Office Excel 2003, SP3. Dunnett's one-way analysis of

variance (ANOVA) with post-test was performed on all the tested concentrations, compared to the standard (without test compound) to indicate significant differences (* $p < 0.05$ were considered to be statistically significant and ** $p < 0.01$ were considered to be statistically very significant). Data on the histogram are presented as % values of the reference standard fluorescence with each bar representing the mean ± S.E.M (figure 3.6).

The average decrease in fluorescence intensity was expressed as a percentage value relative to the specific compound's (100 %) standard (table 4.3). For better visualisation, the data was expressed is a histogram (figure 4.6).

Table 4.3: Data obtained from the Ca^{2+}-flux assay, expressed in percentages relative to each compound's (100 %) standard.

Compound	**Nimodipine**			**NGP1-01**		
Concentration	0,1 µM	1 µM	10 µM	0,1 µM	1 µM	10 µM
Ave %	20.8116	14.5017	5.7122	81.6136	71.4744	68.0771
STDEV %	18.9820	18.3809	11.3433	34.9958	27.8436	25.8781
SEM %	2.8297	2.7401	1.6910	6.1864	4.9221	4.5746
Compound	**1**			**4**		
Concentration	0,1 µM	1 µM	10 µM	0,1 µM	1 µM	10 µM
Ave %	35.2206	5.3541	3.4485	107.0181	70.9555	7.5040
STDEV %	35.6959	5.2686	6.6826	101.1378	58.8279	53.1601
SEM %	43.6959	13.2686	14.6826	12.7422	7.4116	7.9373
Compound	**2**			**5**		
Concentration	0,1 µM	1 µM	10 µM	0,1 µM	1 µM	10 µM
Ave %	108.2385	52.3208	43.1154	110.5198	51.0891	36.0623
STDEV %	177.2335	60.8453	114.1916	158.0129	73.3956	52.3298
SEM %	22.5087	7.7274	14.5023	19.9078	9.2470	6.5929
Compound	**3**			**6**		
Concentration	0,1 µM	1 µM	10 µM	0,1 µM	1 µM	10 µM
Ave %	64.7582	63.0614	12.1022	158.8398	74.3280	68.4442
STDEV %	26.9398	19.4475	12.7540	111.5280	35.5986	54.7664
SEM %	4.1569	3.0008	1.9680	14.3982	4.5958	7.0703

Two control compounds, namely the dihydropyridine L-type Ca^{2+} channel blocker nimodipine and the well documented pentacycloundecylamine NGP1-01, were added to the series of compounds to be tested. Nimodipine served as a positive control to give a representation of what near complete inhibition of L-type Ca^{2+} channels would look like, where as the NGP1-01 gave a representation of what the expected inhibition with pentacycloundecylamines would be.

The compounds were tested in the selected concentration range since it was known from the literature (Johnson & Kotermanski, 2006:61) that other open channel blockers, such as memantine, have an IC_{50} of ±1 µM. In fact, when memantine's concentration in the

cerebrospinal fluid exceeds 10 µM, serious adverse effects can occur since it then affects numerous central nervous system targets such as serotonin and dopamine uptake, serotonin receptors, sigma-1 receptors and voltage gated Na^+ channels.

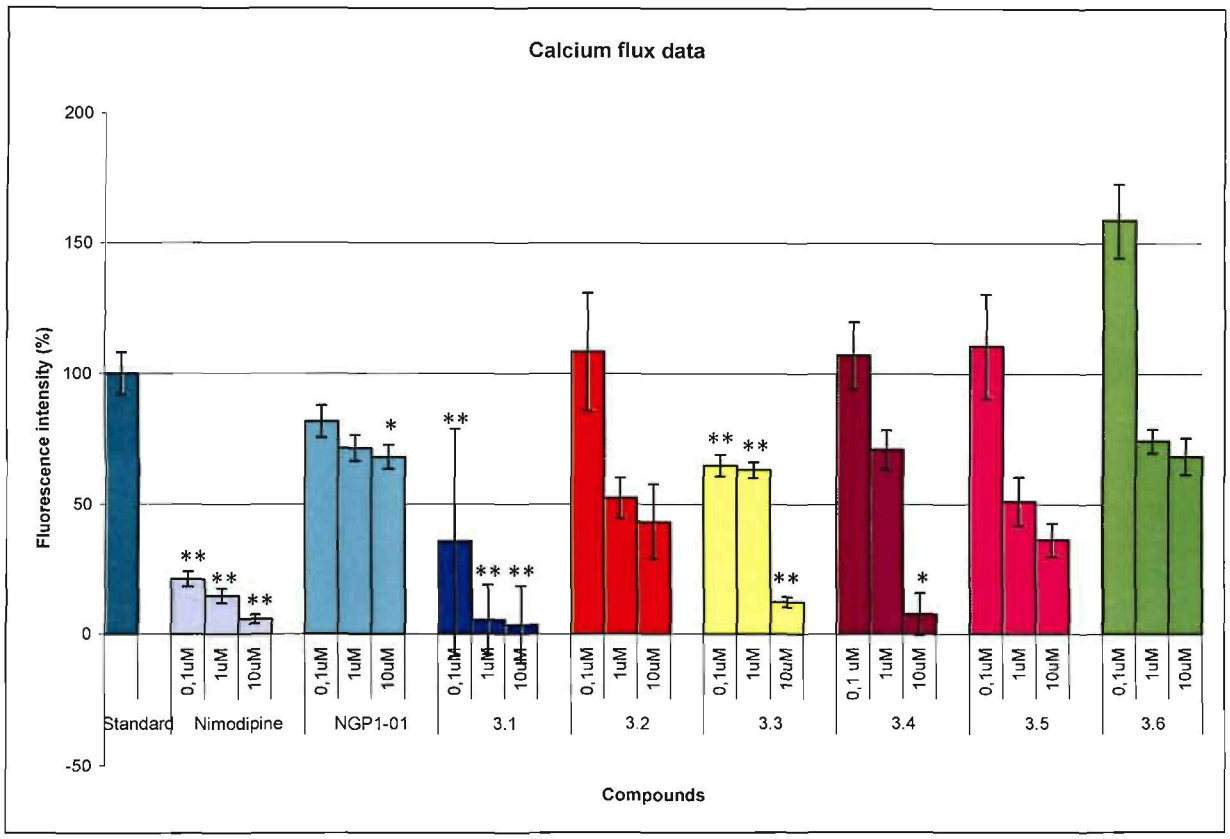


Figure 4.6: The results of the Ca^{2+} flux assay. The Dunnett t-test was applied to the data, ** indicates a very significant inhibition ($p < 0.01$) and * indicates a significant inhibition ($p < 0.05$).

From the Ca^{2+} flux data it was observed that nimodipine gave very significant inhibition of calcium influx into the synaptoneurosomes ($p < 0.01$) at all three concentrations. Compound **3.1** showed activity comparable to that of nimodipine, while compounds **3.3** and **3.4** exhibited inhibition of calcium flux at these levels only at higher (10 µM) concentrations. Compound **3.4** give significant inhibition ($p < 0.05$) at 10 µM. The data obtained from compound **3.6** correlated well with that of NGP1-01, possibly because the compounds were structurally the most similar. Although, at first glance, the data from **3.2**, **3.5** and **3.6** appeared to differ significantly from the (100 %) standard, no statistically significant difference was calculated. This could be attributed to the amount of data points used, which were 5 for this study. The decrease in calcium flux for these structures was dose dependant.

The general equation relating receptor occupancy to agonist concentration *A* in the presence of an antagonist (or the reduction of total receptor concentration R_t by an irreversible competitive blocker) is written as:

$$AR = R_t / [1 + u + (K_a/A)(1 + c)] \quad (4.1)$$

$$= R_t / (1 + u) \cdot 1 / [1 + (K_a/A)(1 + c)/(1 + u)] \quad (4.2)$$

Where *AR* is the activated agonist-receptor complex, *u* is the sum of the normalised concentrations of uncompetitive antagonists and *c* is the sum of the normalised concentrations of competitive antagonists. All the italicised letters except *K* represent concentrations. The agonist concentration at which *AR* is one half its maximum is therefore influenced by *u* and *c*, but only *u* determines the extent to which *AR* (determined by R_t) can be reduced (Pennefather & Quastel, 1982:371). Since the Ca^{2+} flux assay was carried out through depolarisation of the receptor membrane, the measured response has to be expressed as a change in membrane potential (*v*), altered by the conductance of a certain amount (*g*) of ions (eq. 4.5).

$$g = g' AR \quad (4.3)$$

$$v = v_{max} / (1 + G / g) = v_{max} / [1 + (G / g') / AR] \quad (4.4)$$

$$= v_{max} (g' R_t / G) / [g' R_t / G + R_t / AR] \quad (4.5)$$

This expression can be written

$$E = e' / [e + R_t / AR] \quad (4.6)$$

Where *E* is the effect and *e* is the effector system which is independent of the agonist concentration. Combining equation 4.6 and 4.1 gives the most representative equation (4.7):

$$E = e' / [e + 1 + u + (1 + c)K_a/A] \quad (4.7)$$

Assigning E_{max} as the maximal response that can be obtained by raising *A*, and that $u \neq 0$, equation 4.7 can be written as

$$E_{max} = e' / (1 + e + u) \quad (4.8)$$

All of the compounds synthesised were hypothesised to be uncompetitive open channel blockers, like memantine. To investigate this matter further, molecular pharmacological methods were implemented. Since it is known that the concentration of the uncompetitive

antagonist (u) determines the concentration of activated agonist-receptor complex (AR), due to a decrease in the total receptor concentration (R_t) according to equation 4.2, any reduction in R_t will reduce E_{max} when the concentration of the uncompetitive antagonist is appreciable relative to e. Equation 4.7 describes the sigmoidal relationship between the response and log A, which implicates that the maximal response becomes reduced by u when u is appreciable relative to e. Since the EC_{50} is directly proportional to $(1 + c)$ it will be reduced by exactly the same factor as the maximal response.

It was therefore postulated that for the compounds synthesised to be classified as uncompetitive antagonist, the maximal response should decrease proportional to the test compound's concentration, when stimulated with a fixed stimulation (KCl) concentration. The best way to visualise this is by extrapolating the EC_{50} value of each maximal response to the x-axes (agonist concentration) of the theoretical uncompetitive antagonist dose-response curve (figure 4.7a). This was exactly the trend noticed by the compounds tested in this study. The same trend is not possible with competitive antagonism, as seen by the same extrapolation in figure 4.7b.

From the theoretical dose-response curve it is thus apparent that at a fixed agonist concentration, higher concentrations of uncompetitive antagonist would decrease the maximum response. In this assay, the concentration of the depolarisation solution was kept constant and therefore it is safe to assume, for the purposes of this discussion, that the amount of opened Ca^{2+} channels are the same for each experiment. All the compounds tested in this study exhibited this behaviour. This is an important consideration, since correlation of the data from the S-nitrosylation assay with that of the Ca^{2+} flux assay indicated no direct relationship between the nitrosylation capability of the compounds and its Ca^{2+} channel activity. Compound **3.6**, which gave the best nitrosylation, did not give the best Ca^{2+} channel blockade, while compound **3.1**, which gave poor nitrosylation exhibited the best Ca^{2+} channel blockade. This can possibly be attributed to the compounds being uncompetitive antagonists. Johnson & Kotermanski (2006:63) describe uncompetitive antagonism as a "foot in the door" mechanism of blockade. Once the uncompetitive antagonist enters and blocks the receptor, it prevents the receptor from closing. This effect lasts until the uncompetitive antagonist leaves the receptor, a duration of time determined by the specific antagonist's off-rate. Even if S-nitrosylation took place, the allosteric modification brought on by the disulfide bond formation, which is known to slow the off-rates of glycine and glutamate, could also possibly further trap the test compounds inside the receptor channel. Another possibility is that the binding sites of the pentacycloundecylamines and the S-nitrosylation sites overlap. The presence of the test compound could possibly prevent the S-nitrosothiol from coming

into contact with the adjacent thiol, which was meant to form the disulfide bond, to such an extent that the disulfide bond couldn't form. It would seem that the structural properties, such as the steric effects and bulk (Malan *et al.*, 1996:125 & 2000:10), superseded the effects of S-nitrosylation and once again proved to be the most decisive contributors to pentacycloundecylamine Ca^{2+} channel antagonism.

As for the structure-activity relationships of the test compounds, it would appear that an increase in chain length between the cage compound and the aromatic moiety did in fact increase the Ca^{2+} channel activity. Compound **3.6** showed the closest structural similarity to NGP1-01 and its Ca^{2+} channel activity also corresponded to that of NGP1-01. This corresponds to the hypothesis of Geldenhuys *et al.* (2007:1530), which postulates that the phenyl ring, substituted unto the pentacycloundecylamine, undergoes a π-π type aromatic interaction with (an) aromatic amino acid(s) located at the entrance of the NMDA receptor. This interaction anchors the compound and allows the cage compartment to descend into the channel pore. The depth of this descend is determined by the length of the linker between the aromatic moiety and the nitrogen of the pentacycloundecylamine. This descending mechanism could then bring the cage moiety with its protonated amino-group to close proximity with the PCP-binding site. This hypothesis is however still under investigation. As usual, when it comes to the channel activity of pentacycloundecylamines, the steric effects seem to play the most important role, with the smallest of the longer linker compounds, **3.1**, exhibiting the best channel antagonism.

In an attempt to assay specifically the NMDA receptor's effect, the synaptoneurosomes were initially incubated with 10 μM nimodipine to minimize Ca^{2+} flux through L-type Ca^{2+} channels, a method described by Geldenhuys *et al.* (2007:1531). To stimulate Ca^{2+} influx into the cells, a 100 μM glycine/glutamate solution was added during the kinetics run. Unfortunately this method yielded fluorescent values too small for quantification. A series of experiments were performed and it was confirmed that the nimodipine inhibited ±95% of the possible fluorescence intensity at a concentration of 10 μM (figure 4.6). That explained the poor fluorescence and also indicated that at least 95% of the fluorescence intensity could be attributed to L-type Ca^{2+} channels, which explained why the glycine/glutamate solution gave such weak activation. The ratio of NMDA receptor effect to L-type Ca^{2+} channel effect was simply too small to give results that can be quantified using fluorescence spectroscopy under the conditions used in our laboratory.

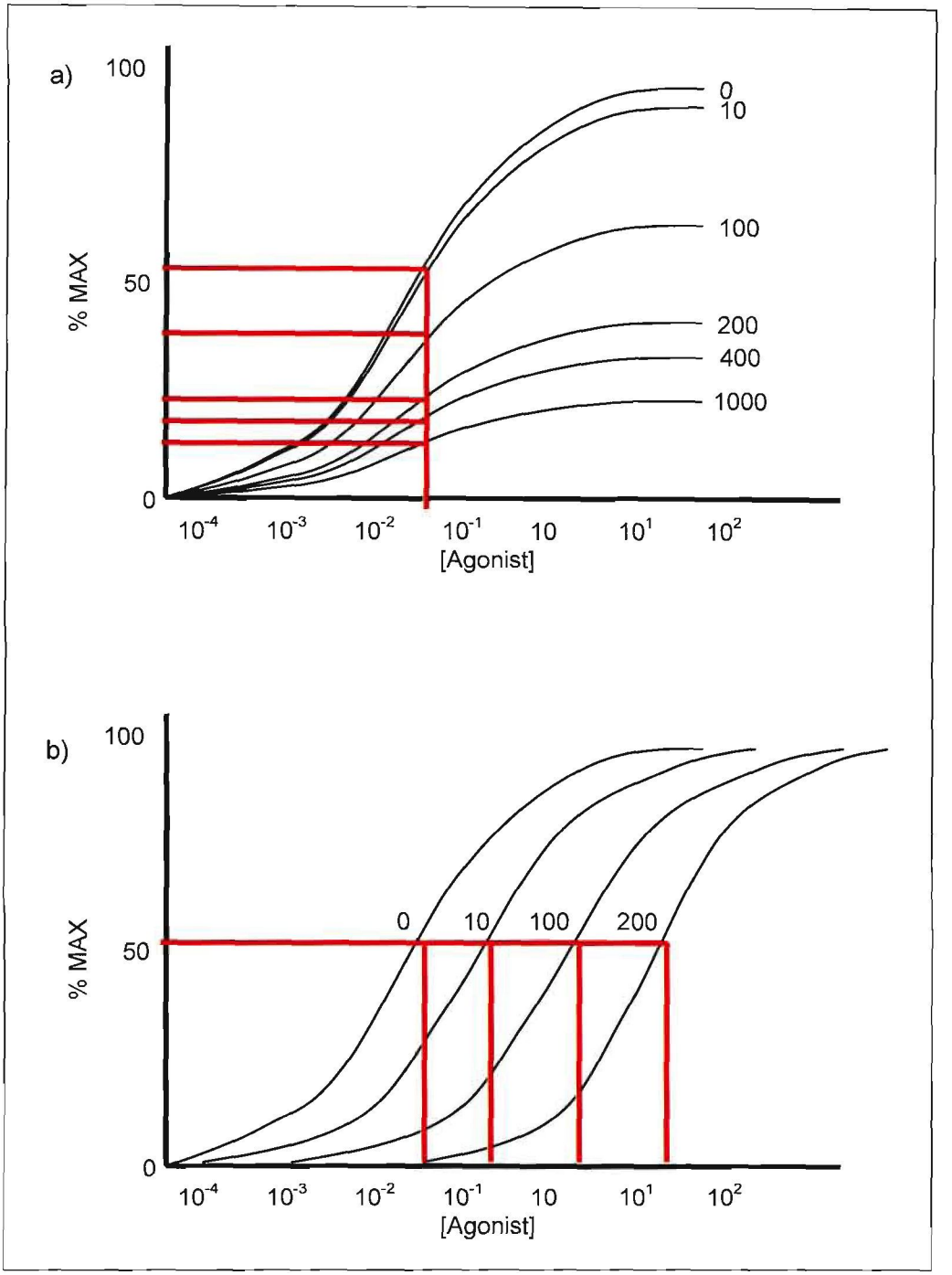


Figure 4.7: Theoretical response-log agonist concentration curve for uncompetitive antagonism (a) where, $u = I/K_I$ and $c = 0$, and competitive antagonism (b) where $u = 0$ and $c = I/K_I$. The uncompetitive antagonist only binds to receptors that have been activated by the agonist, while the competitive antagonist only binds to free receptors, adapted from Pennefather & Quastel, (1982:372).

4.3 CONCLUDING REMARKS

The biological evaluation of the synthesised compounds indicated that all of the test compounds were capable of S-nitrosylation. The compounds were also potent inhibitors of KCl induced Ca^{2+} flux, especially at higher concentrations. Although no clear direct correlation between their NO donating capability and their Ca^{2+} channel antagonism were

observed, the compounds were still effective Ca^{2+} channel blockers, in certain cases even reaching levels of antagonism equivalent to that of nimodipine. The test compounds thus conform to the requirements for being effective inhibitors of excitotoxicity, with them being uncompetitive open channel blockers and S-nitrosylators.

CHAPTER 5

CONCLUSION

5.1 BACKGROUND

Excitotoxicity is described as the excessive stimulation of NMDA receptors by its endogenous agonist, glutamate. This causes abnormal amounts of Ca^{2+} to enter the neuronal cell and triggers a cascade of events, eventually leading to apoptosis and neuronal cell death (Lipton, 2006:162). Therefore, the rational behind this study was to synthesise compounds capable of attenuating excitotoxicity. Selection of these novel neuroprotective agents was done by first doing a thorough study on the methods of antagonism, the structure-activity relationships (SAR) of known NMDA receptor antagonists and the different modulatory sites in the NMDA receptor.

Open channel blockers were selected as the type of antagonist to be used, since this mechanism of antagonism doesn't interfere with normal neuronal function (Chen & Lipton, 2006:1616). These blockers can only exert their antagonistic effects after the specific receptor has been activated by an agonist. Open channel blockers with a low affinity and fast off-rate are ideal because they selectively block receptors that are excessively stimulated (and therefore more frequently open). Another important consideration was that the synthesised compounds should preferably be uncompetitive antagonists, like memantine (Johnson & Kotermanski, 2006:63). Uncompetitive antagonists are open channel blockers that enter the activated receptor's channel pore and binds to a specific binding site. The antagonist then prevents the channel from closing until it again leaves the channel pore. The rate by which this takes place is determined by the specific antagonist's off-rate.

The pentacycloundecylamines are known open channel blockers (Van der Schyf *et al.*, 1988:448) and NMDA receptor antagonists (Geldenhuys *et al.*, 2007:1530). These bulky cage-like compounds incorporate the hydrophobic area and hydrogen bond donating group (amine) necessary for NMDA antagonism according to the NMDA antagonist SAR described by Kroemer *et al.* (1998:395). Conjugating an aromatic moiety to the pentacycloundecylamine structure provides high-affinity binding and increases its channel activity. A linker region, incorporated between the cage structure and the aromatic moiety,

provided steric freedom for the compound. This freedom was deemed important because of the recent hypothesis of Geldenhuys *et al.*, (2007:1530) in which the aromatic moiety "anchors" the compound at the entrance of the receptor channel, allowing the cage compound to descend into the channel pore where it comes into close proximity with the PCP-binding site and exerts its antagonistic effects.

On the modulatory sites of the NMDA receptor (and other Ca^{2+} channels) certain crucial cysteine residues are present. Transfer of a nitrogen monoxide (NO) to the thiol groups in these cysteine residues is called S-nitrosylation (Lipton *et al.*, 2002:474). The resulting nitrosothiol (RS-NO) then releases the NO group again to form a disulfide bond with an adjacent thiol group. The formation of such a disulfide bond causes an allosteric modification of the receptor's protein structure and in the case of the NMDA receptor, this conformational change increases the affinity of glycine and glutamate binding sites for their endogenous agonists, desensitising the receptor (Zheng *et al.*, 2001:894). It was thus decided to include a NO-donating moiety onto the pentacycloundecylamines, to evaluate the effect of possible S-nitrosylation on the channel activity of the polycyclic cage amines. This also gave another mechanism by which Ca^{2+} flux through the channel could be modulated.

5.2 SYNTHESIS

All of the compounds synthesised in this study started off as amine derivatives of the pentacycloundecane-dione. The first step was to conjugate an amine to the cage moiety by means of reductive amination. This gave a pentacycloundecylamine with a sterically free linker section. In the relevant compounds, the hydroxyl group on the linker was conjugated to a benzoic acid to yield an ester. This esterification was done using the activating agent, 1-ethyl-3-(3'-dimethylamino)carbodiimide (EDC). Yields were generally low, which could be attributed to the electron withdrawing groups (such as the nitro groups) conjugated to the benzoic acid. All of the compounds containing nitrate groups initially contained a hydroxyl group which were nitrated. This was achieved by adding thionylchloride nitrate, prepared from equimolar amounts of silver nitrate and thionylchloride, to the hydroxyl containing moiety (Hakimelahi *et al.*, 1984:907). Since all of the compounds were reported for the first time, thorough structure elucidation was necessary. This was done using one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) absorption spectrophotometry and mass spectrometry (MS).

5.3 BIOLOGICAL EVALUATION

To confirm the nitrosylation capabilities of the synthesised compounds, ultraviolet (UV) absorption was used in the modified method of Jaffrey *et al.* (2001:193). During the assay,

pyridine-2-thione was split off biotin-HPDP and absorbed UV-radiation was measured at 343 nm. The intensity of this UV-absorption was relative to a (100 %) control. The assay showed that all of the synthesised compounds were S-nitrosylators and exhibited a very significant change ($p < 0.01$) in UV-absorption relative to the control. The nitrates showed the best nitrosylation. A Ca^{2+} flux assay was performed to evaluate the channel activity of the polycyclic amines. As positive controls, the dihydropyridine L-type Ca^{2+} channel antagonist, nimodipine as well as NGP1-01, the lead pentacycloundecylamine, was used. All of the synthesised compounds contained the necessary structural requirements for Ca^{2+} channel activity as well as (now confirmed) NO-donating moieties. At concentrations of 10 µM, all of the synthesised compounds exhibited better Ca^{2+} channel antagonism than NGP1-01. At 10 µM, **3.1** and **3.3** exhibited very significant ($p < 0.01$) inhibition and **3.4** exhibited significant ($p < 0.05$) inhibition relative to the control, with values comparable to that of nimodipine at the same concentration. Compounds **3.1** and **3.3** also exhibited very significant inhibition at concentrations of 1 and 0.1 µM. Compound **3.6** has the closest structural similarity to NGP1-01 and also gave inhibition comparable to it. The steric freedom brought on by the increased linker length had a positive effect on the Ca^{2+} channel activity. Despite the good channel activity of the synthesised compounds, no clear correlation between the channel activity and S-nitrosylation could be made. The channel activity could more easily be related to the size of the compound tested. This could possibly mean that the steric effects of the pentacycloundecylamines (Malan *et al.*, 1996:125 & 2000:10) were once again the main determinants in their Ca^{2+} channel activity.

5.4 CONCLUDING REMARKS

Although S-nitrosylation showed little to no influence on the channel activity of the pentacycloundecylamines, some positive results can be taken from this study. Firstly, a novel series of S-nitrosylators were synthesised that could find application in other fields of health and sciences. Secondly, the steric freedom brought on by the linker in the synthesised compounds lead to an increase in channel activity and seems to confirm the hypothesis by Geldenhuys *et al.* (2007:1530) on the mechanism of pentacycloundecylamine Ca^{2+} channel antagonism. All of the compounds showed Ca^{2+} antagonistic activity, at concentrations equivalent to that of commercially available neuroprotective drugs.

Excitotoxicity still remains a big problem in neuroscience and with no clear cut solution on the horizon. The research done in this study gave a better insight into the attenuation of excitotoxicity and neurodegenerative disorders and compounds synthesised could be useful leads in further drug design.

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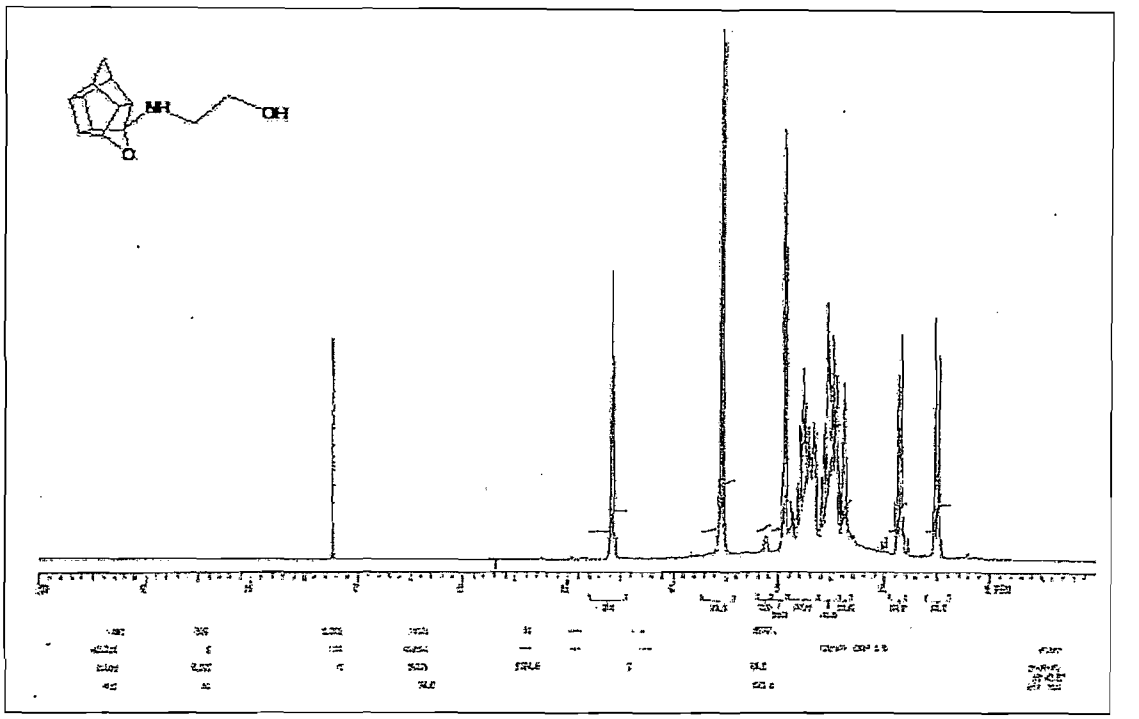
APPENDIX A

SPECTRAL DATA
IR, MS, ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC

SPECTRUM 1



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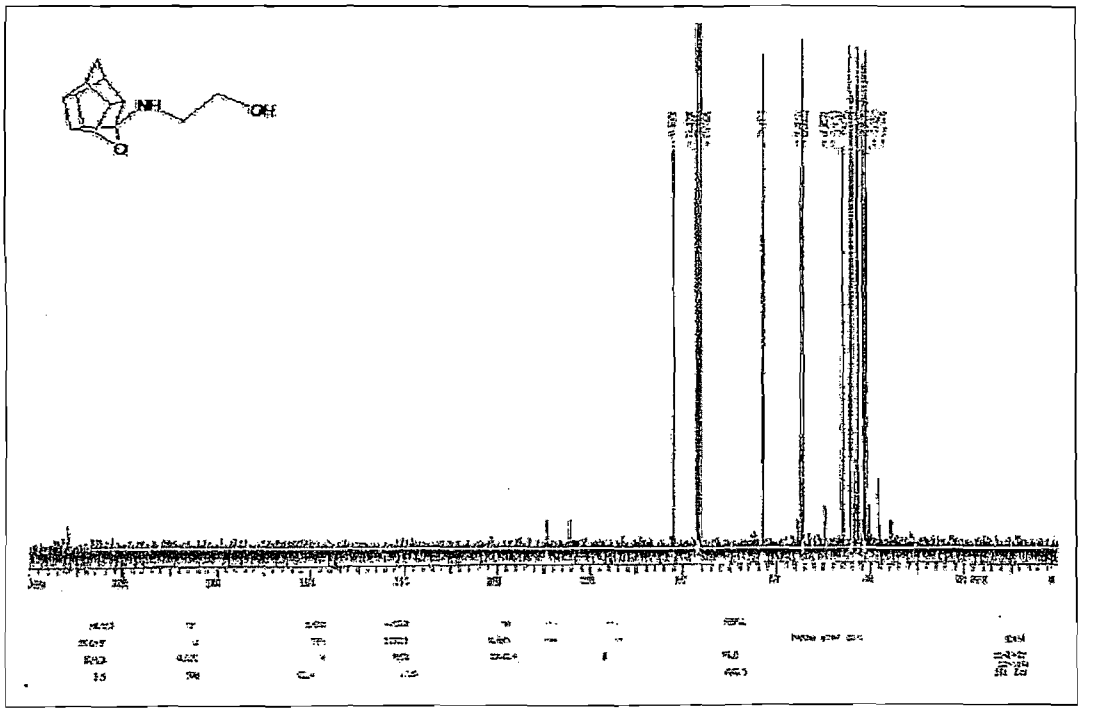

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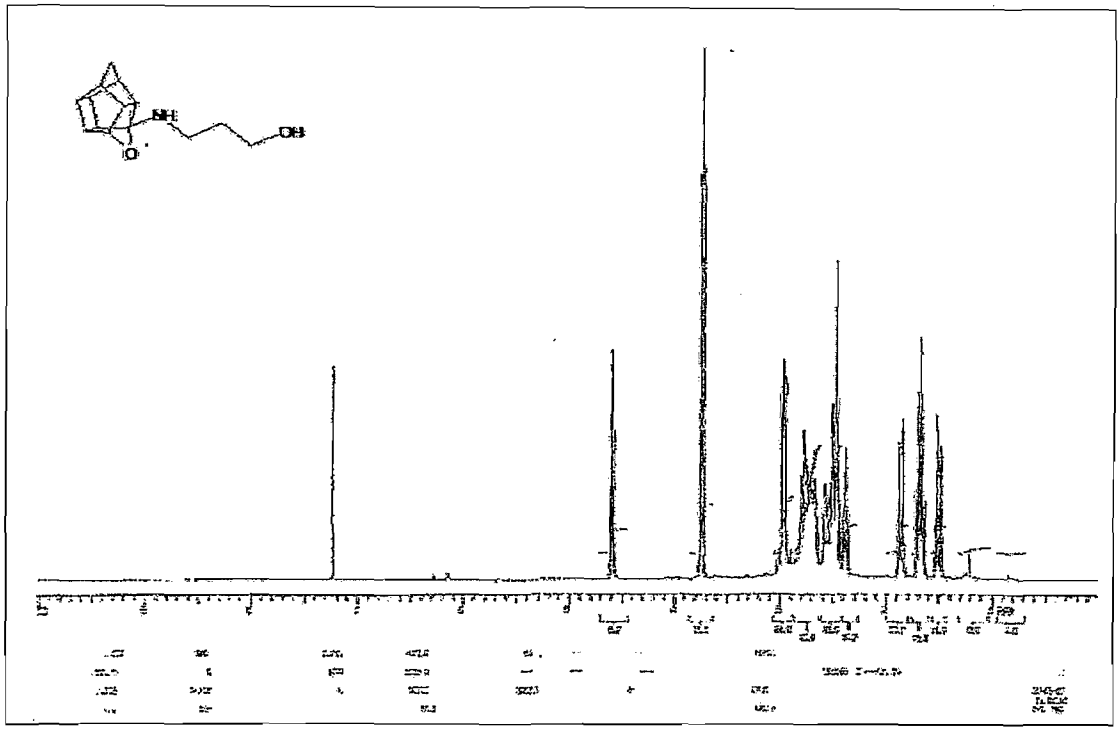

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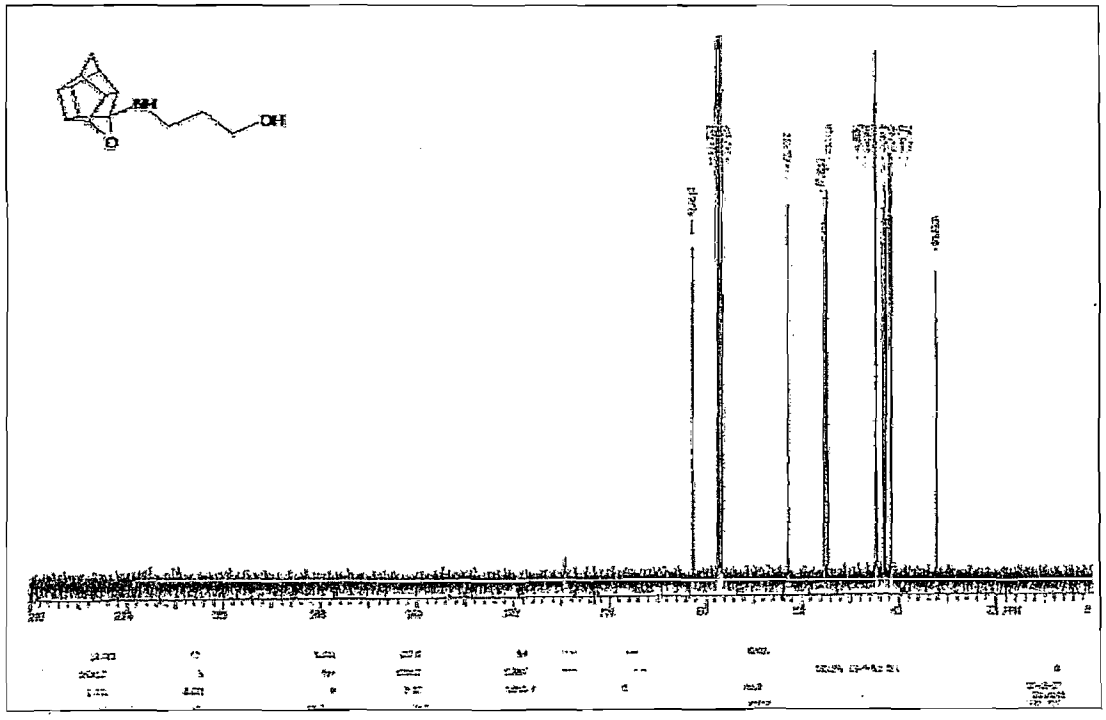

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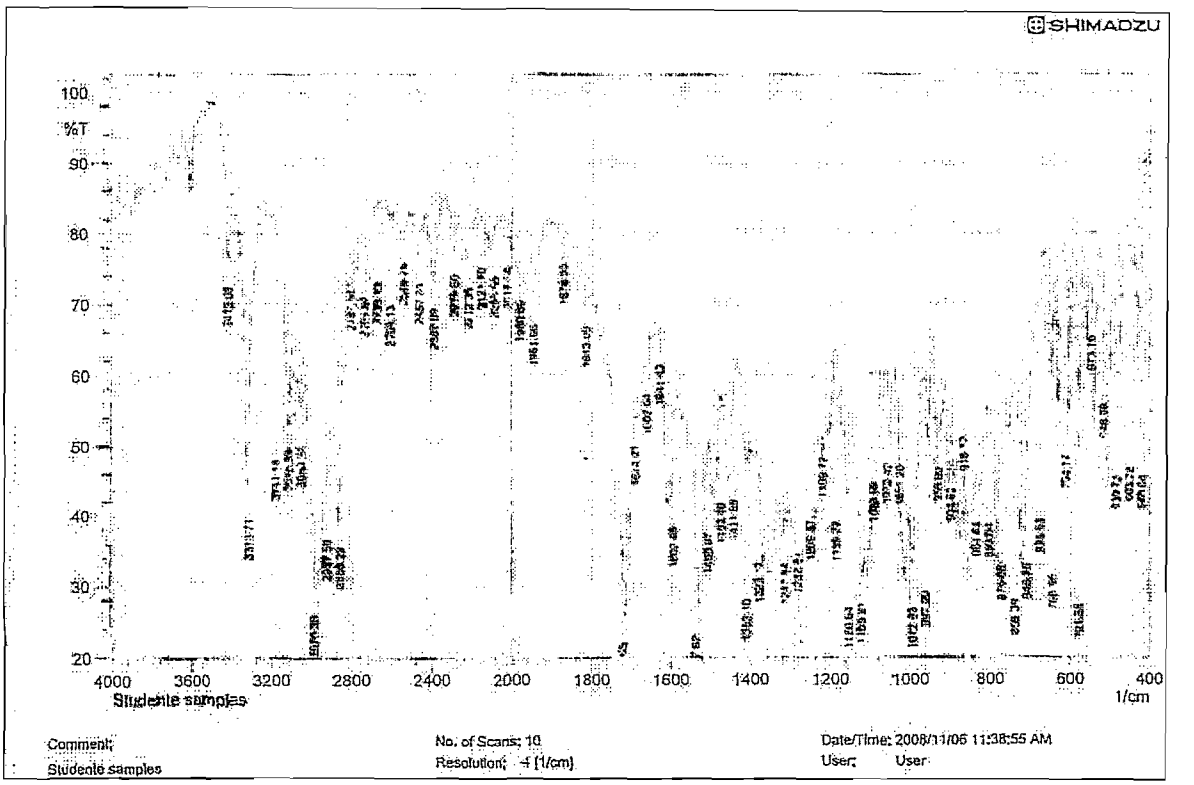

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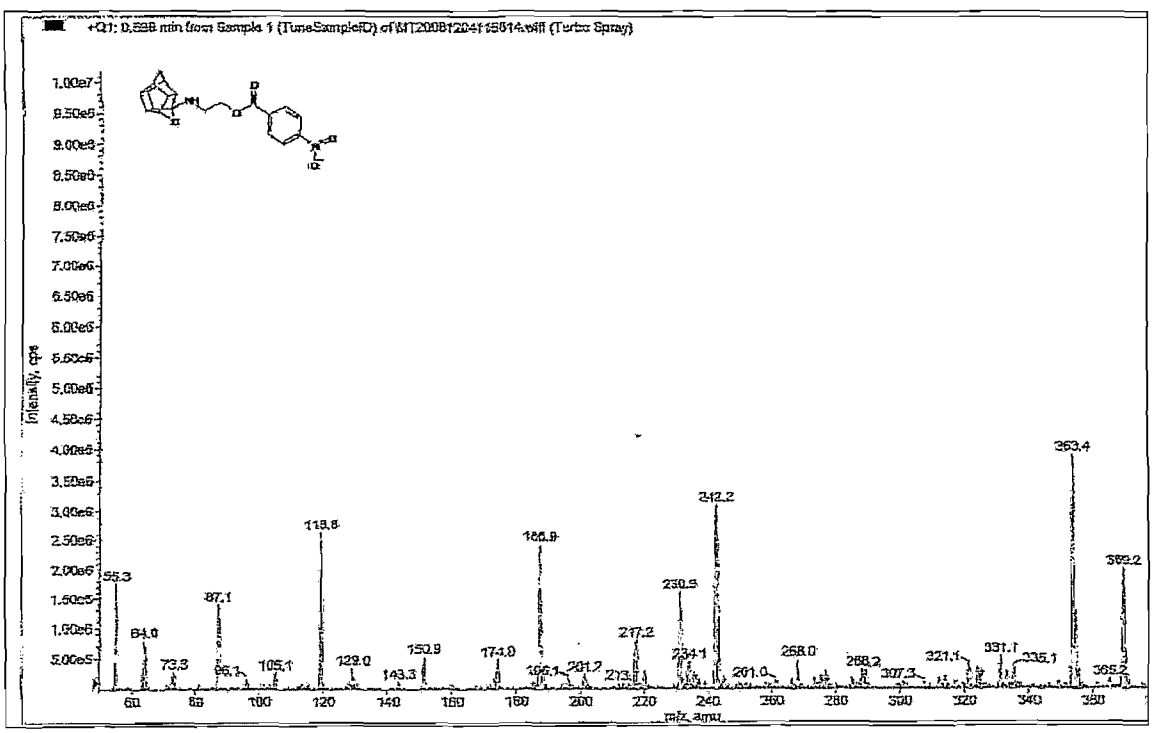

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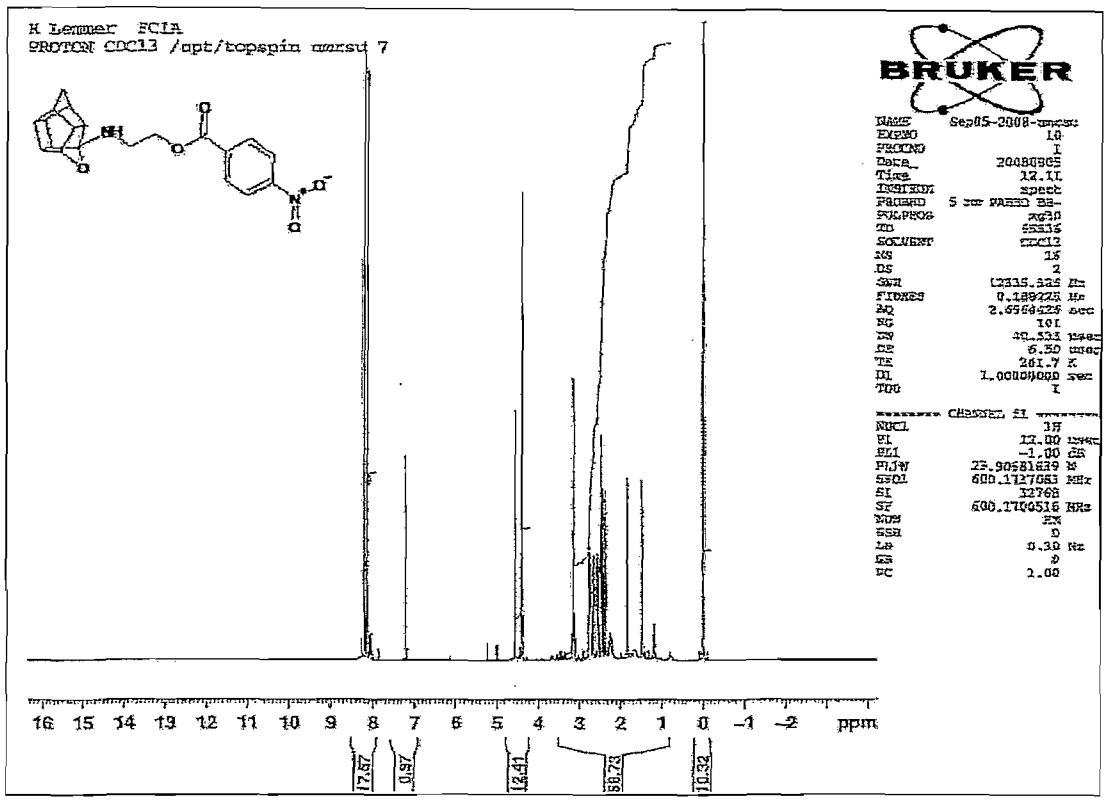

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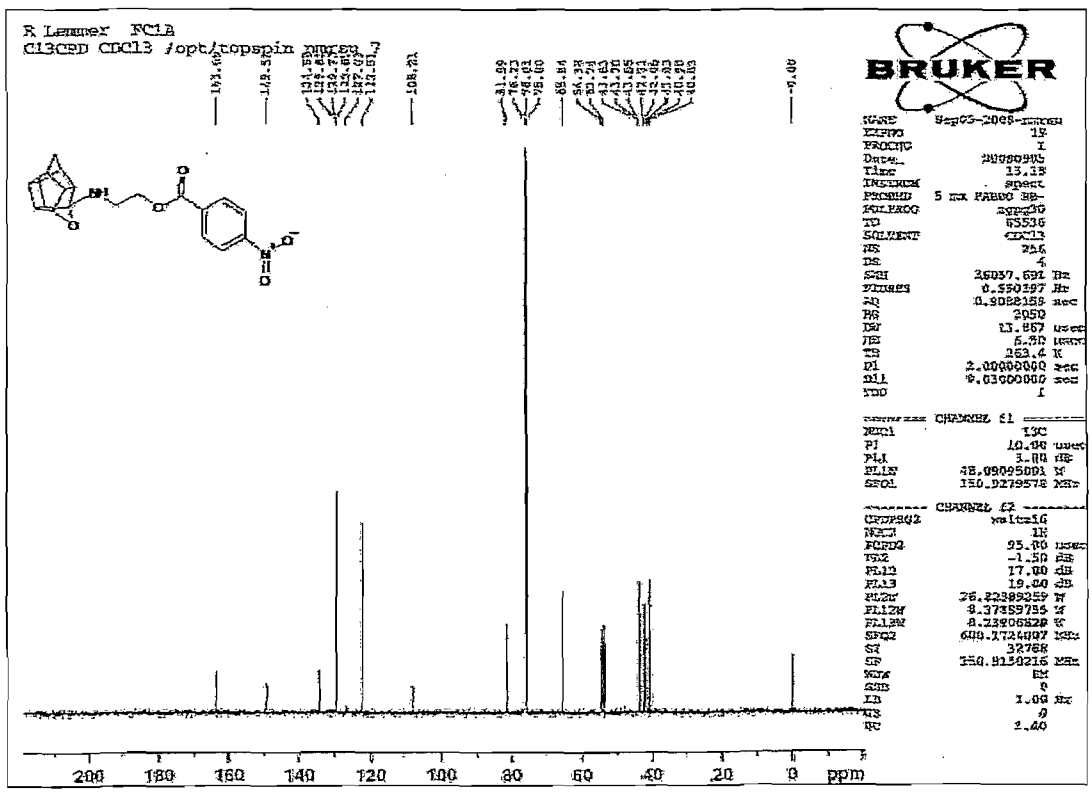

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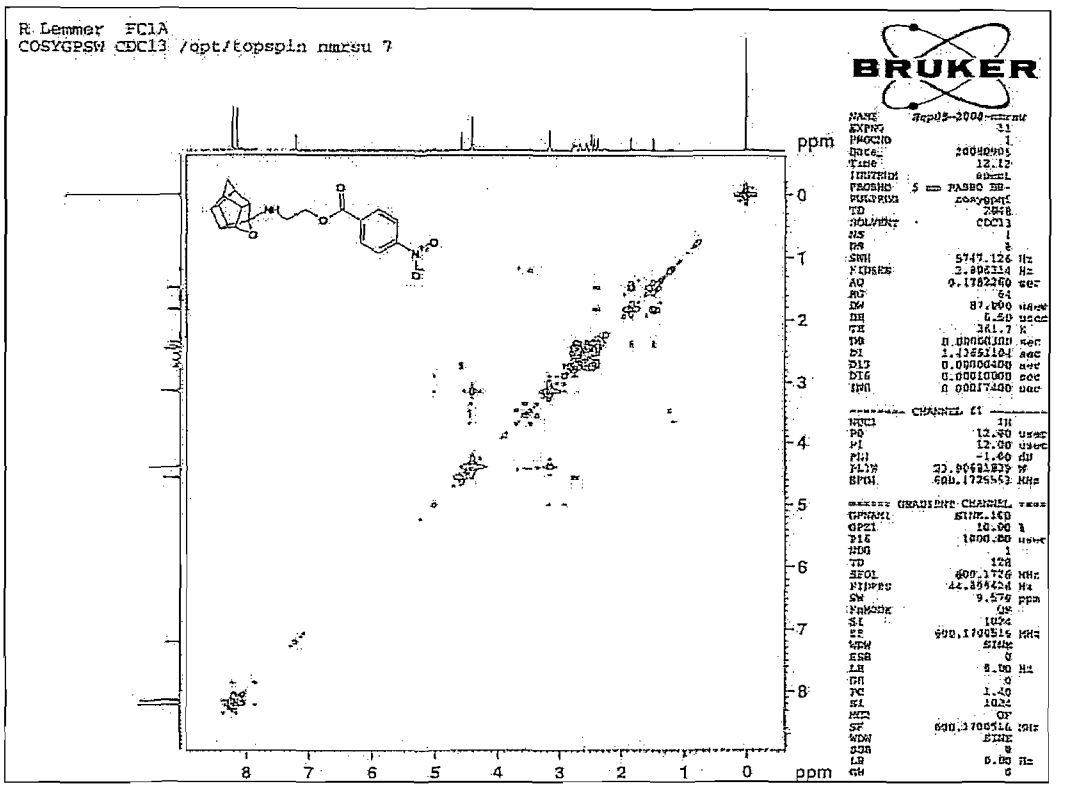

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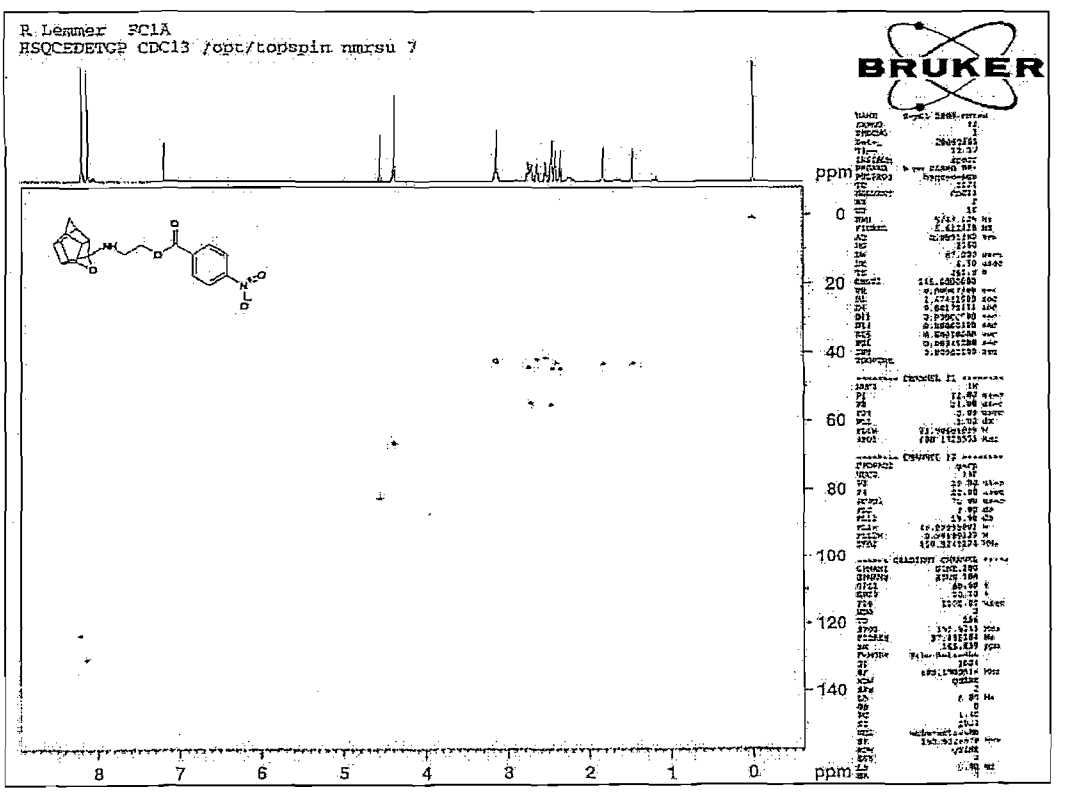

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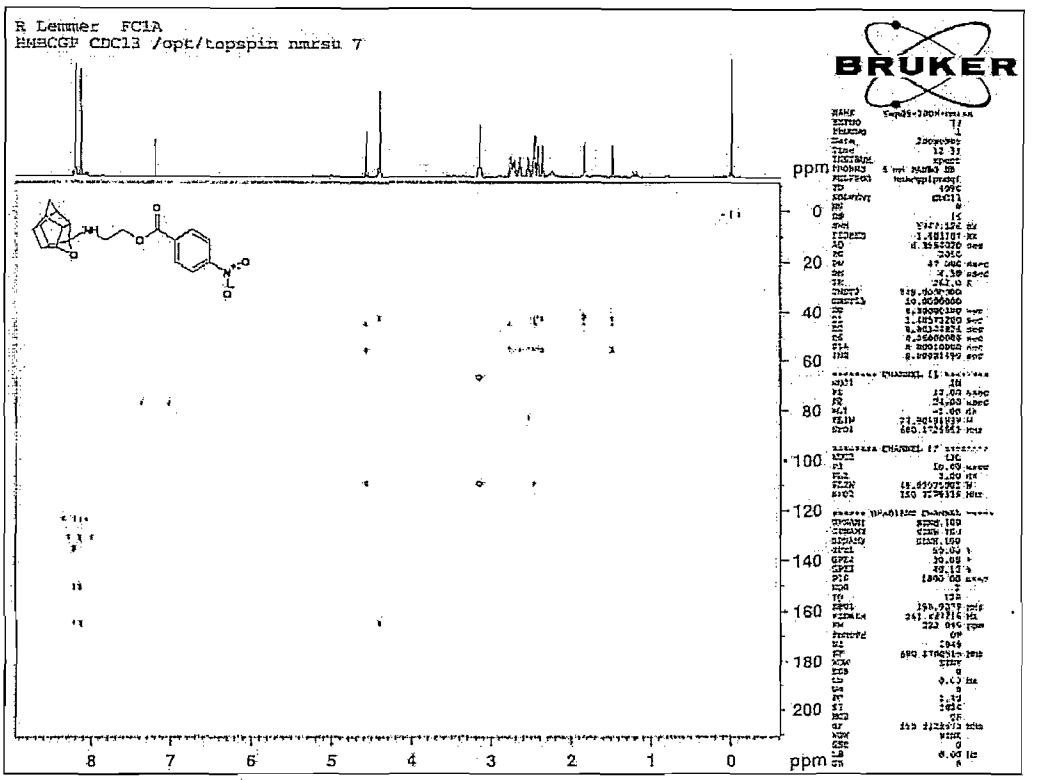

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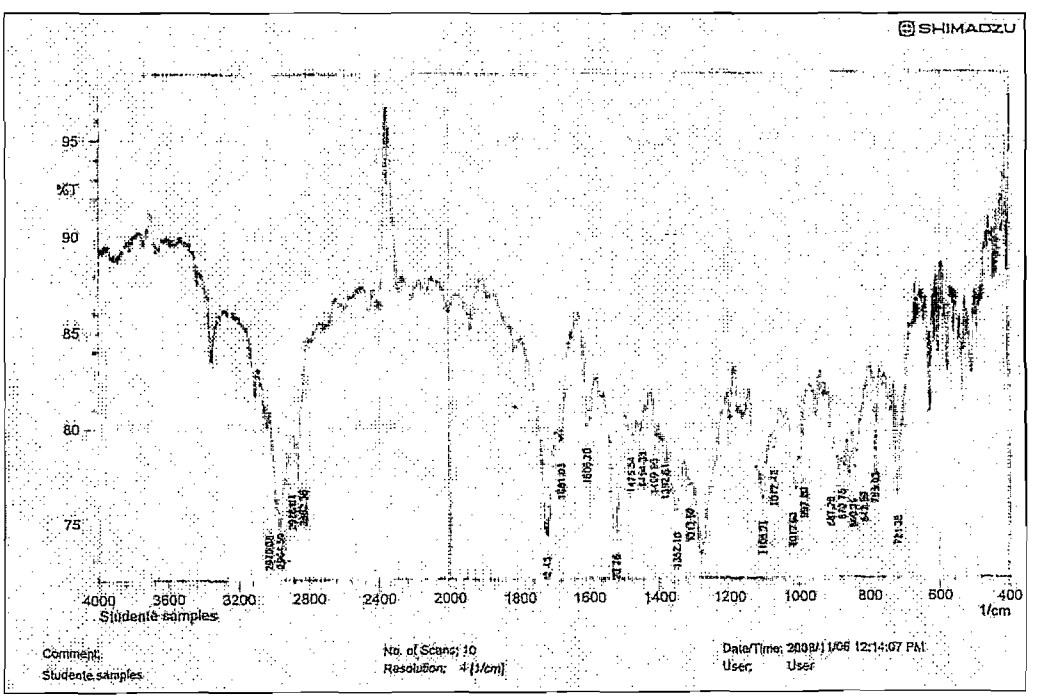

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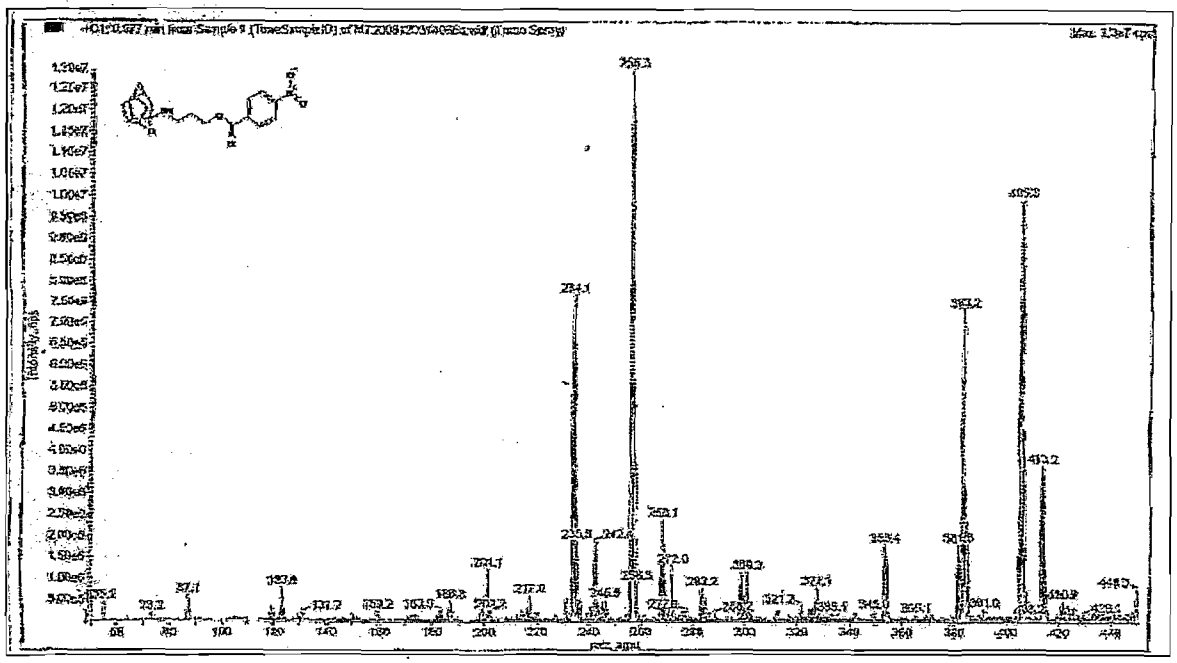

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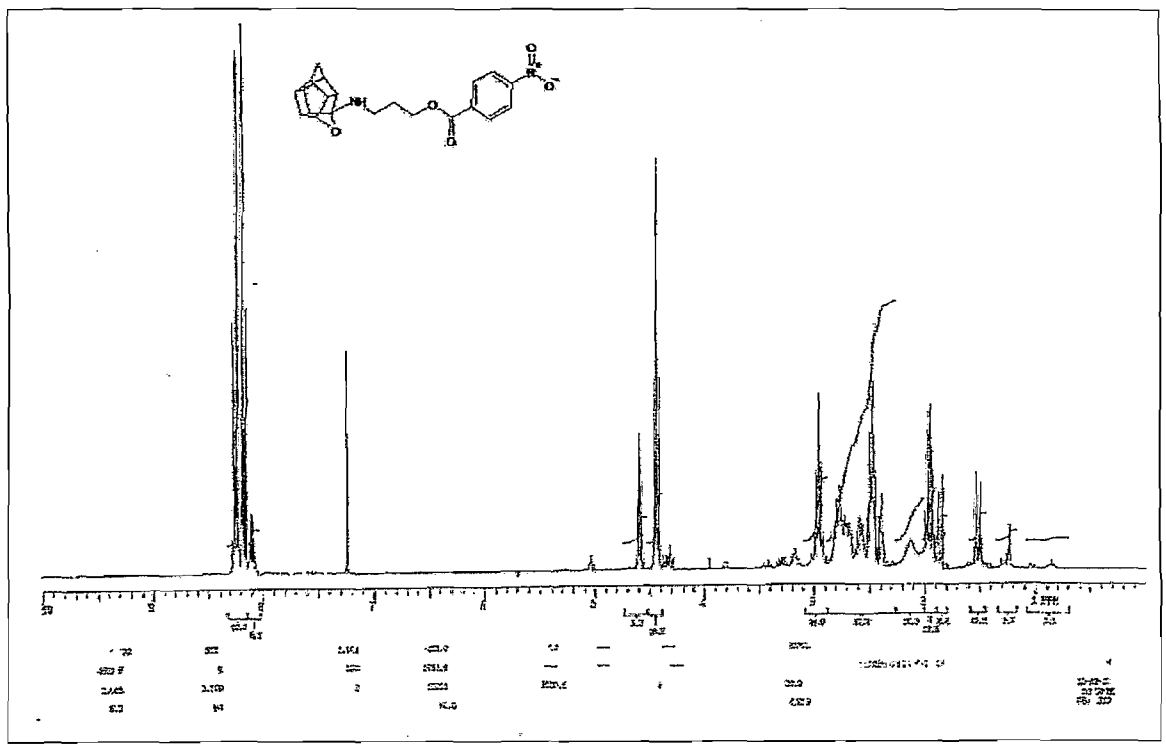

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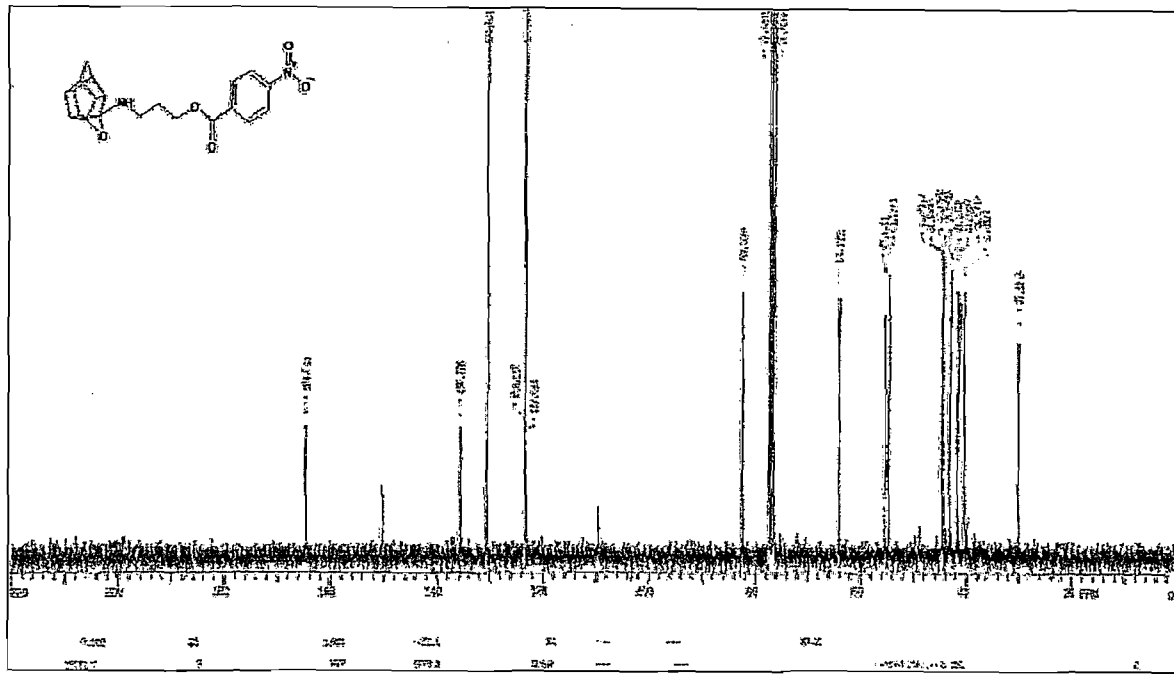

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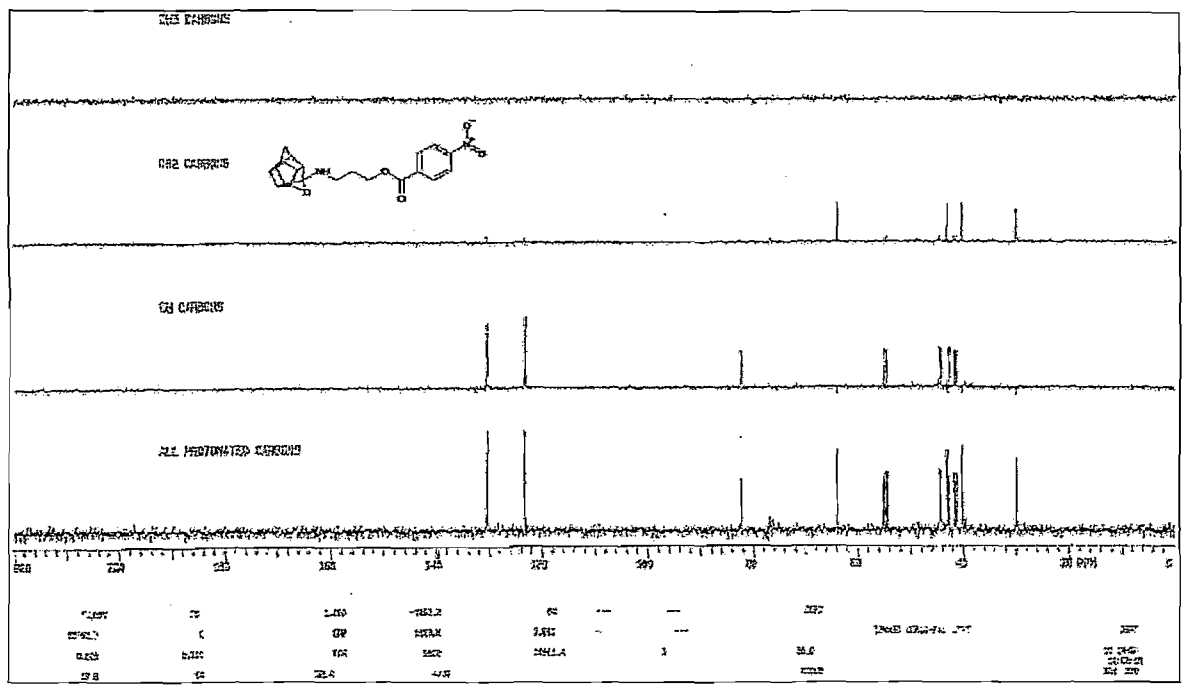

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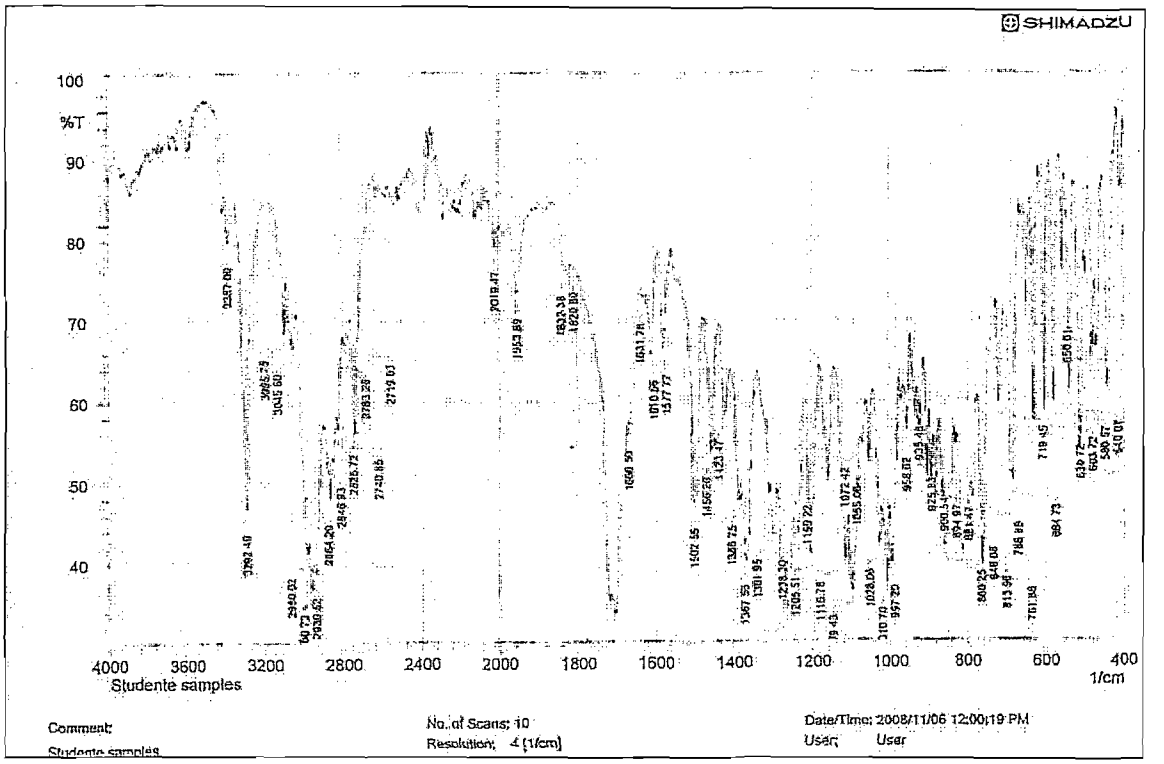

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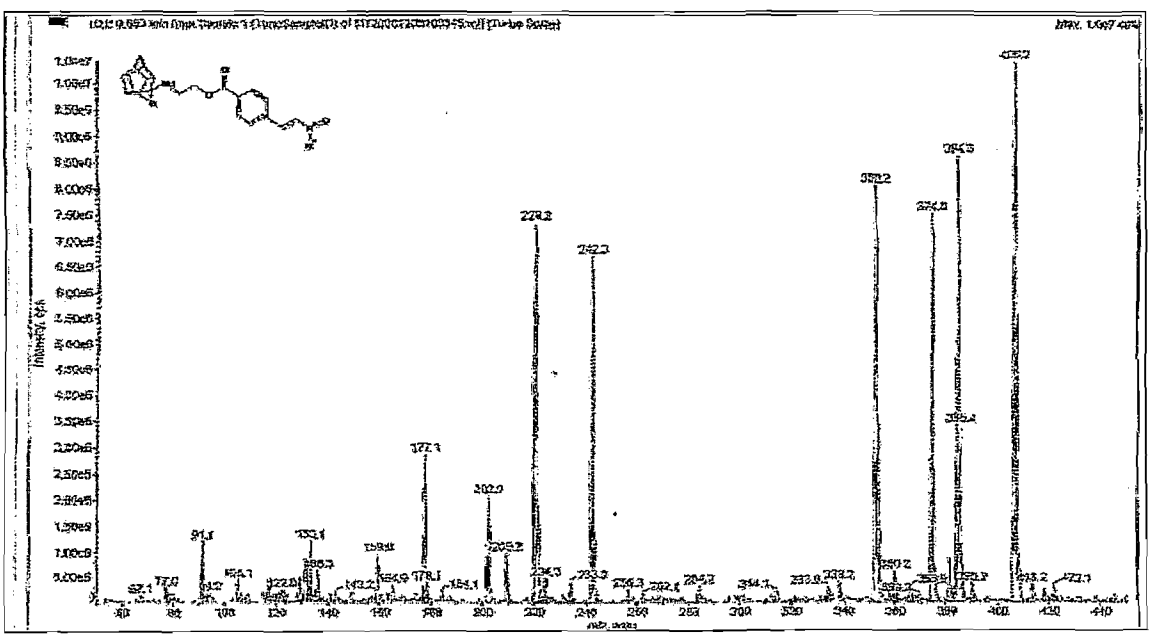

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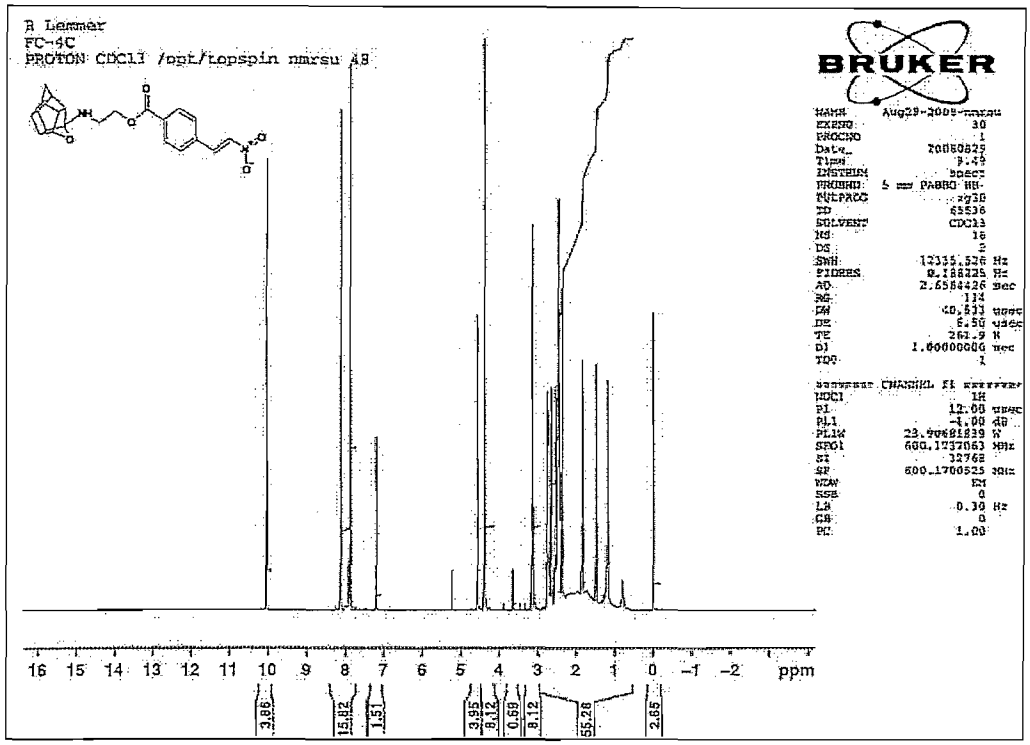

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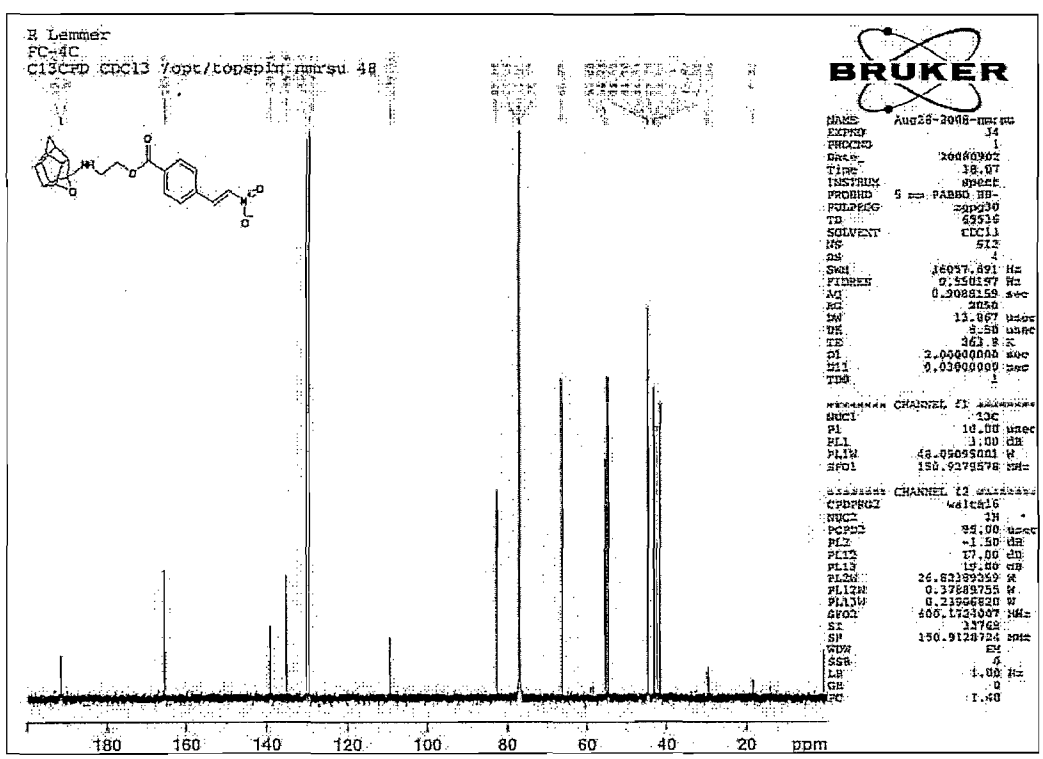

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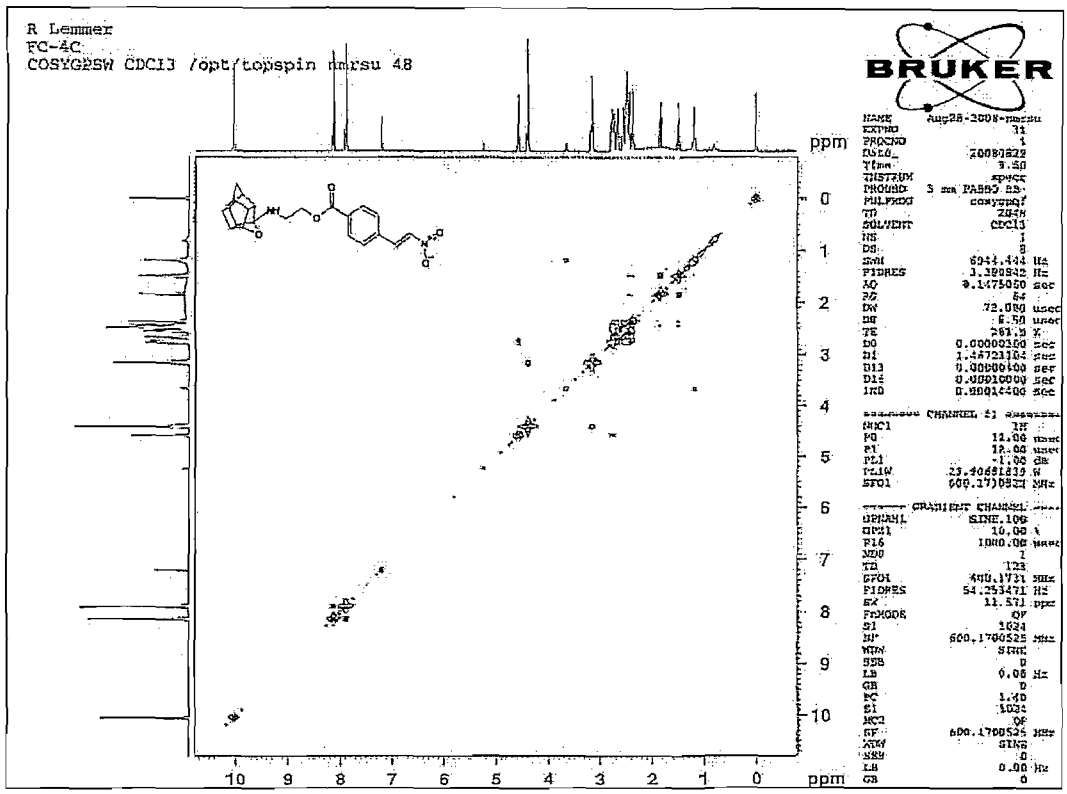

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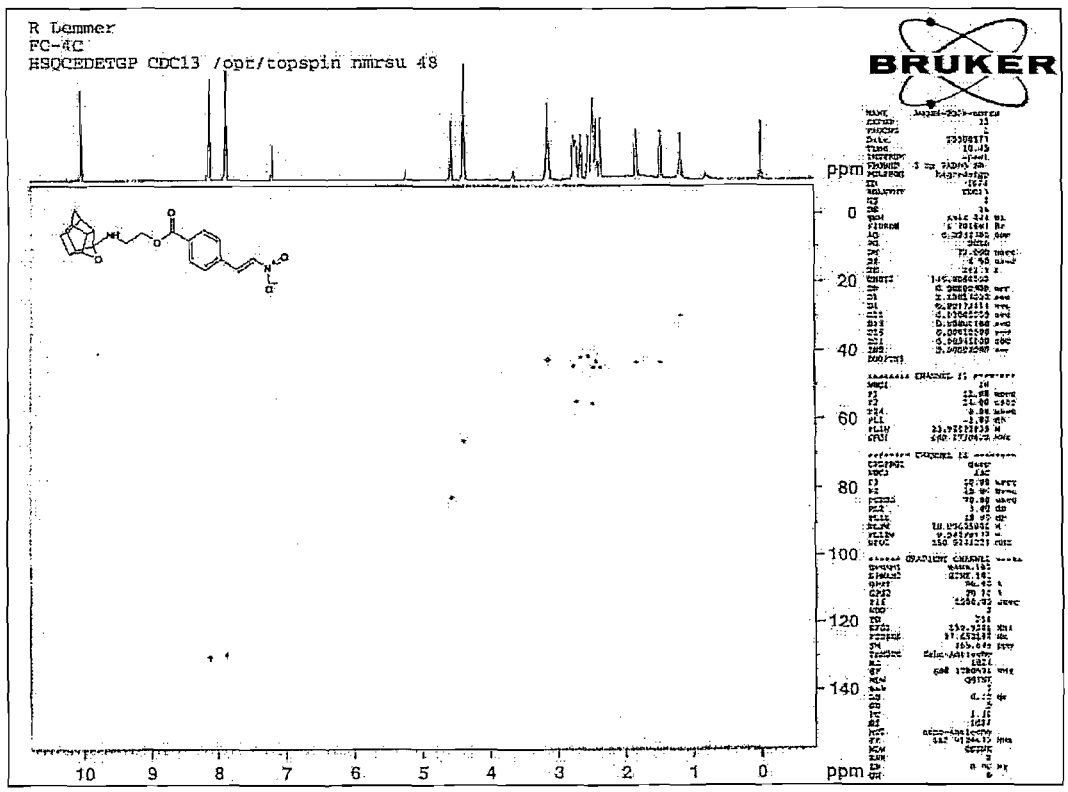

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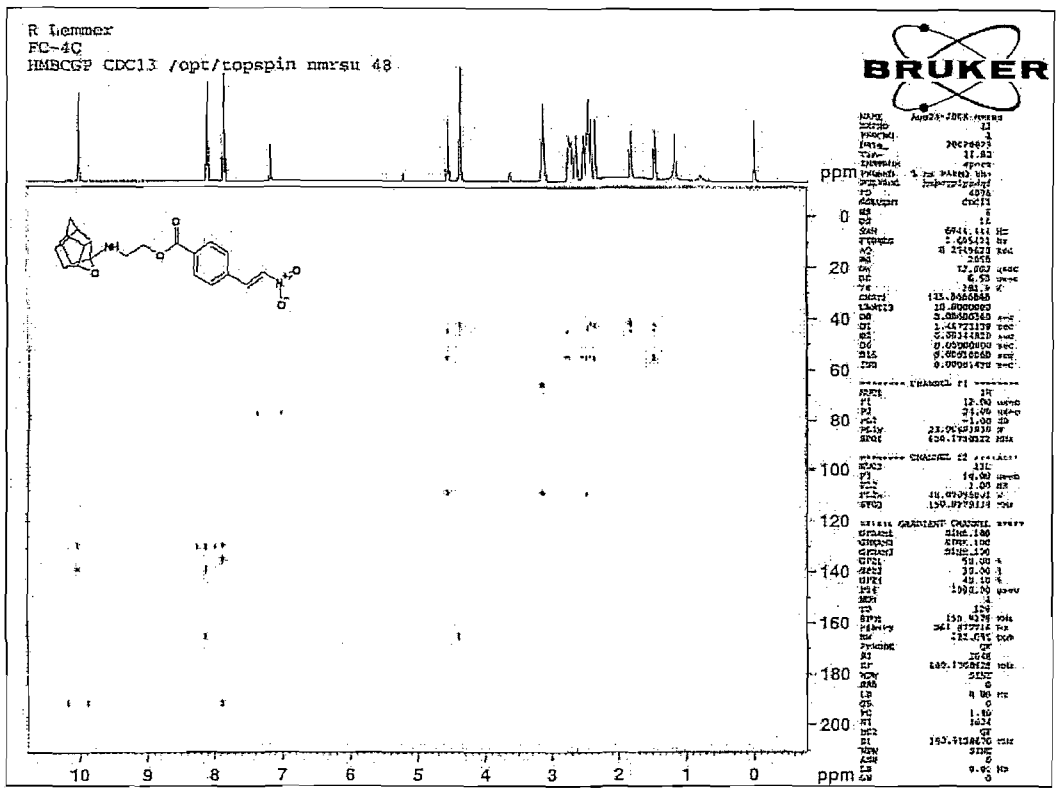

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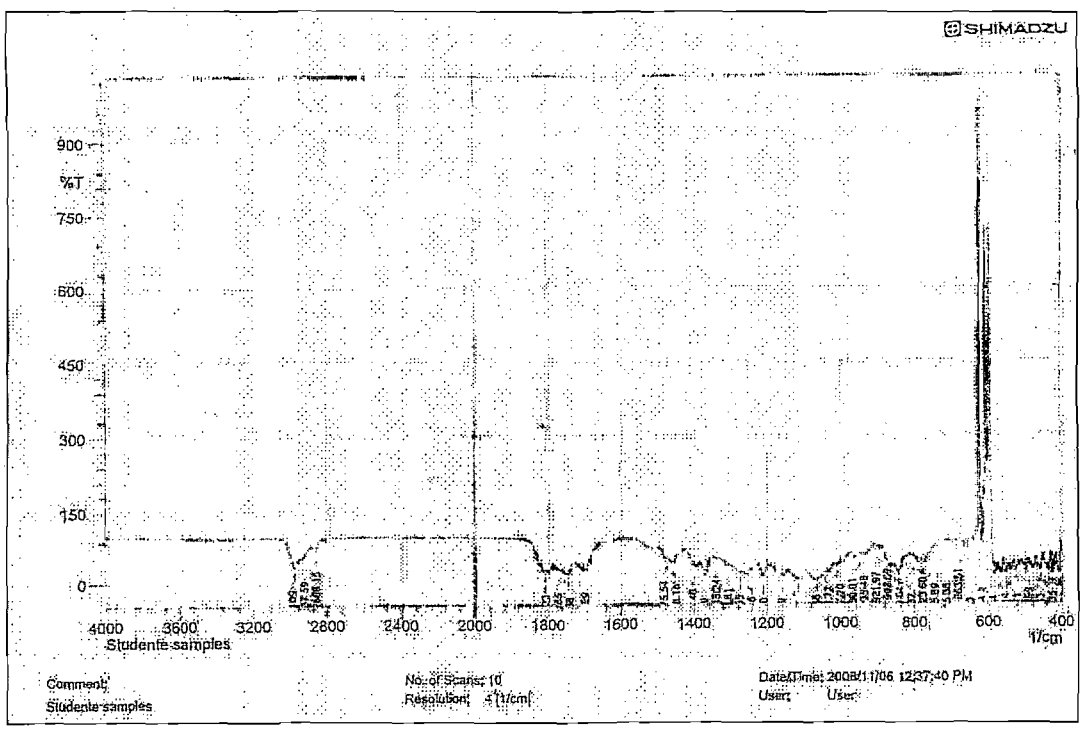

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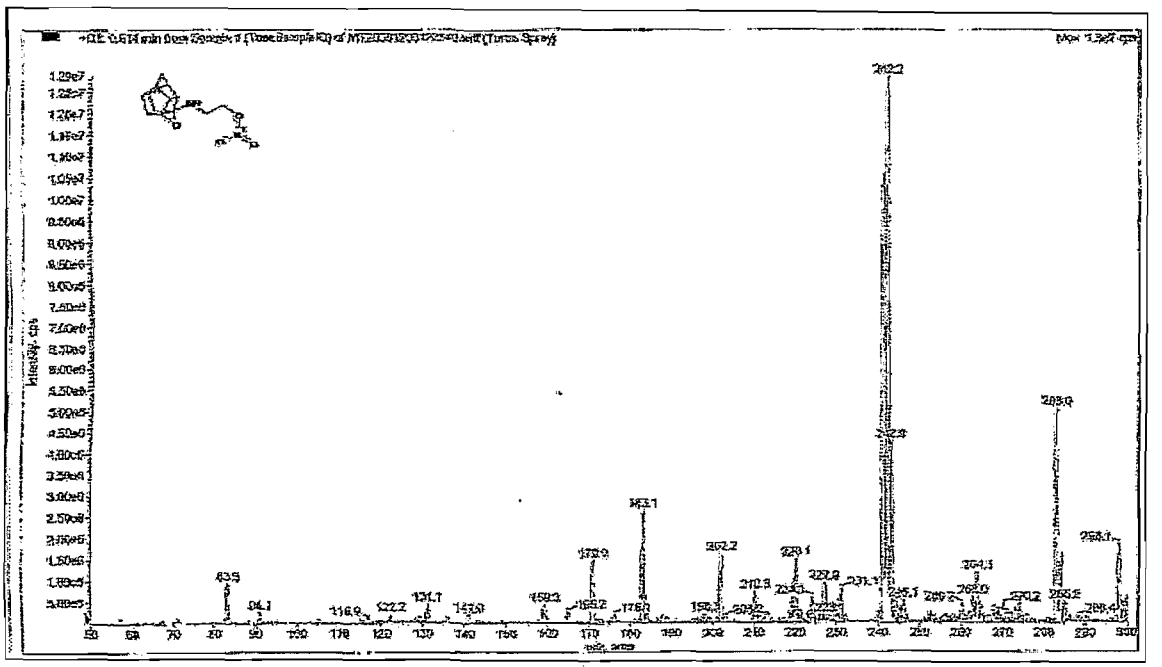

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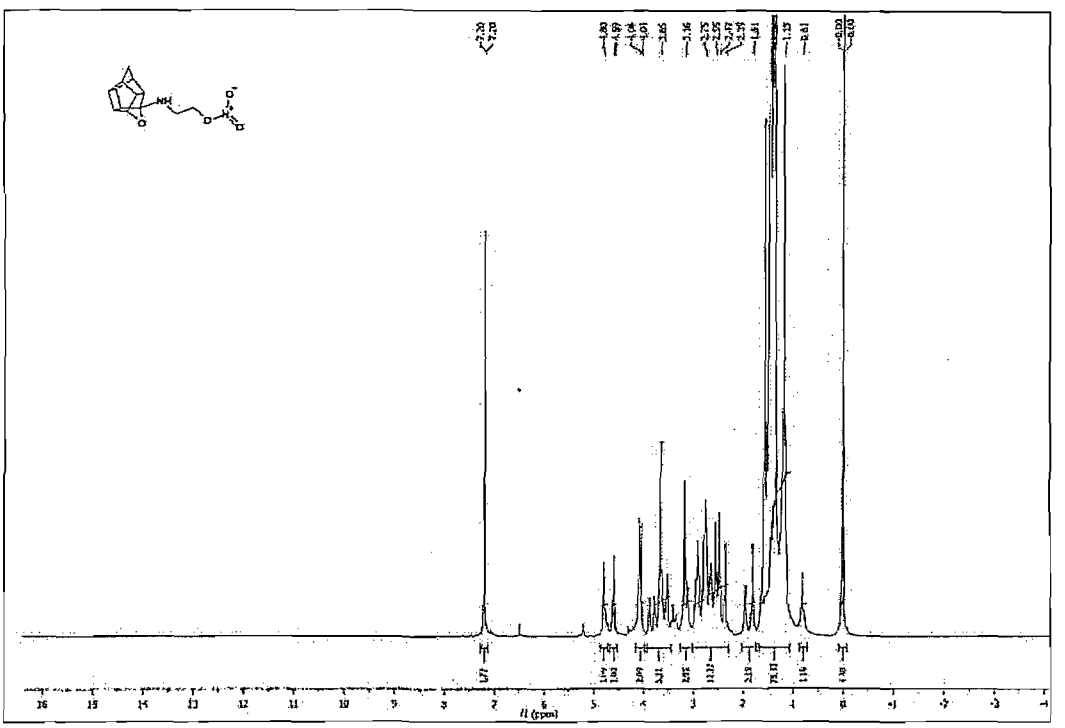

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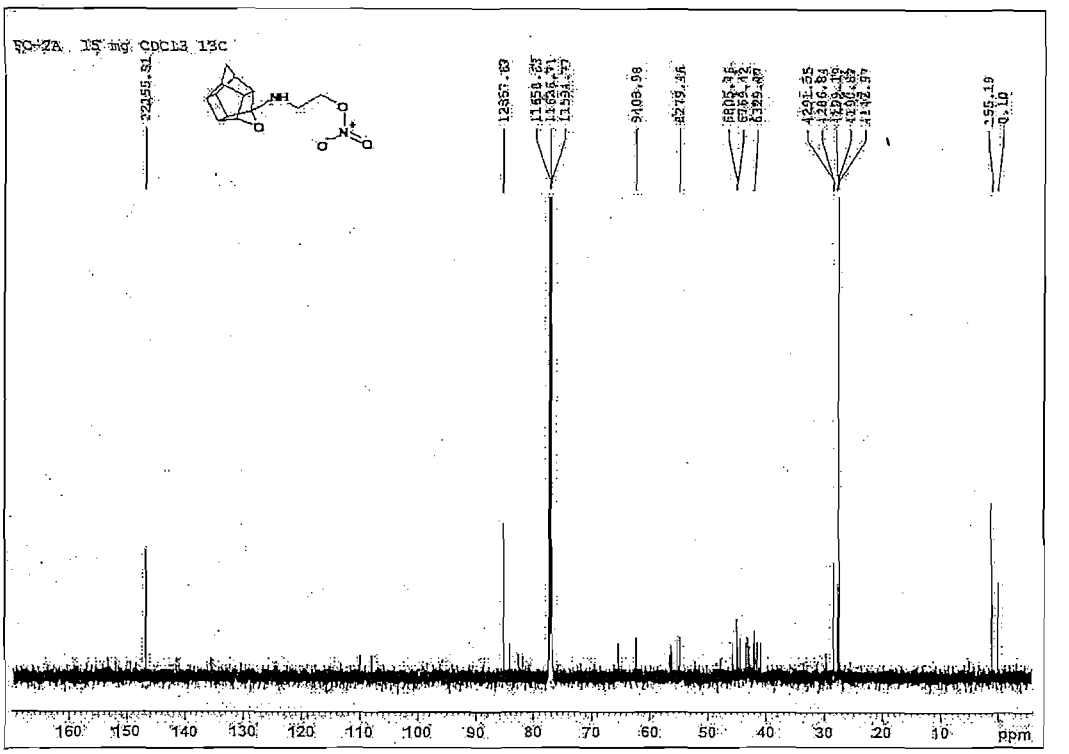

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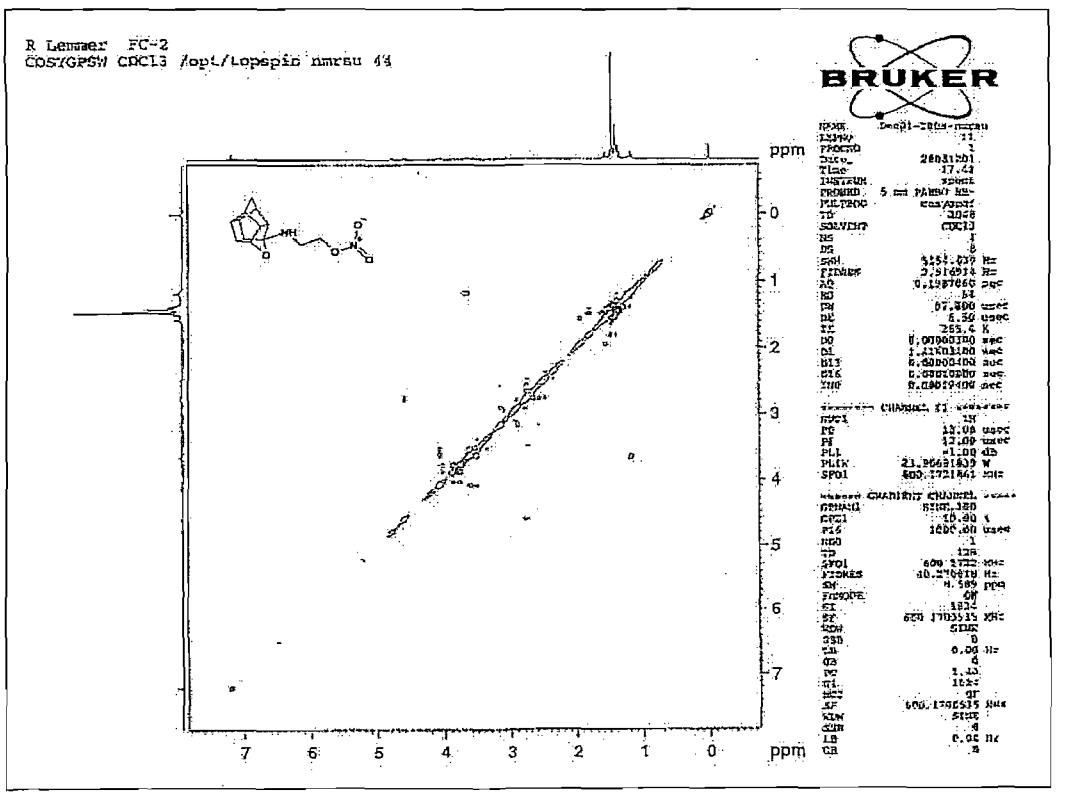

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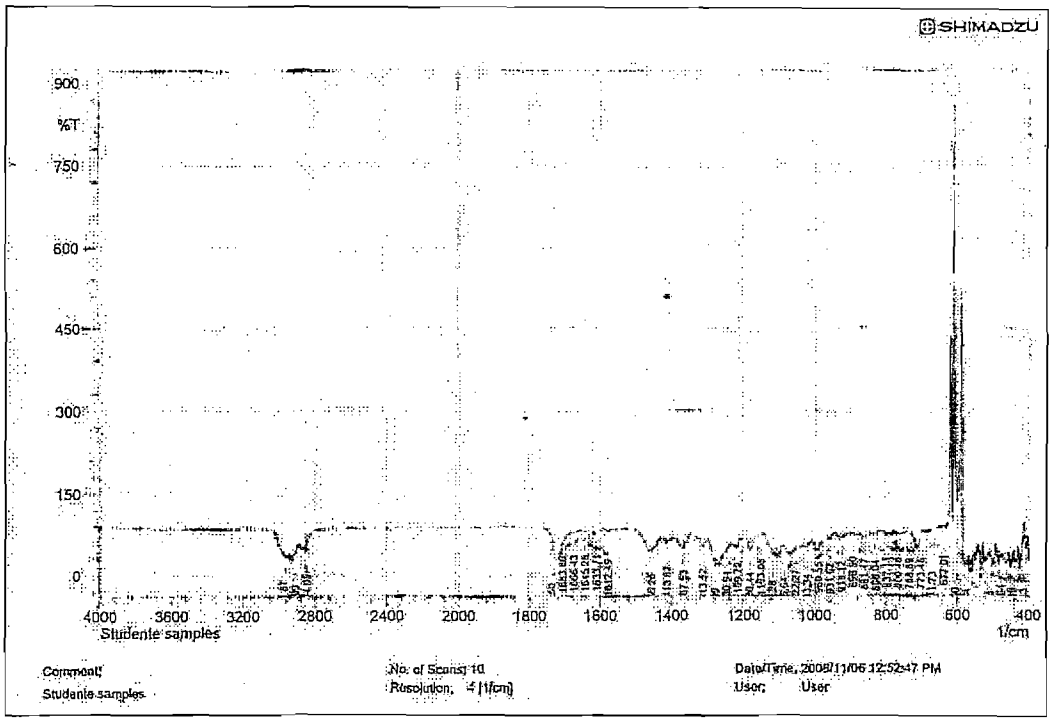

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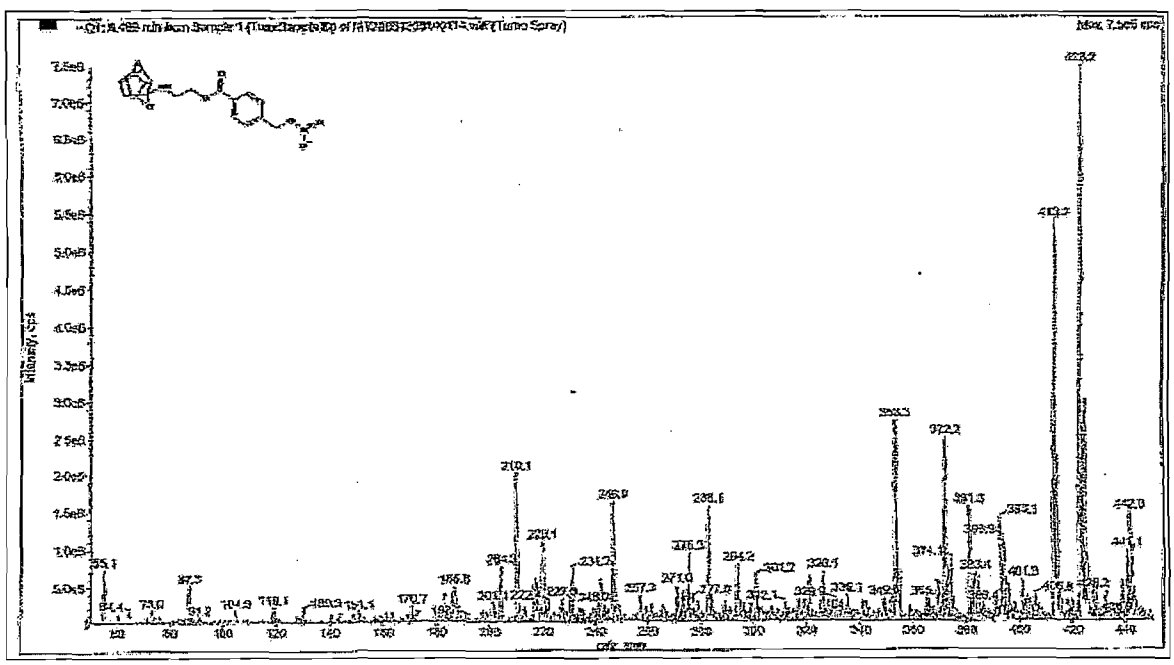

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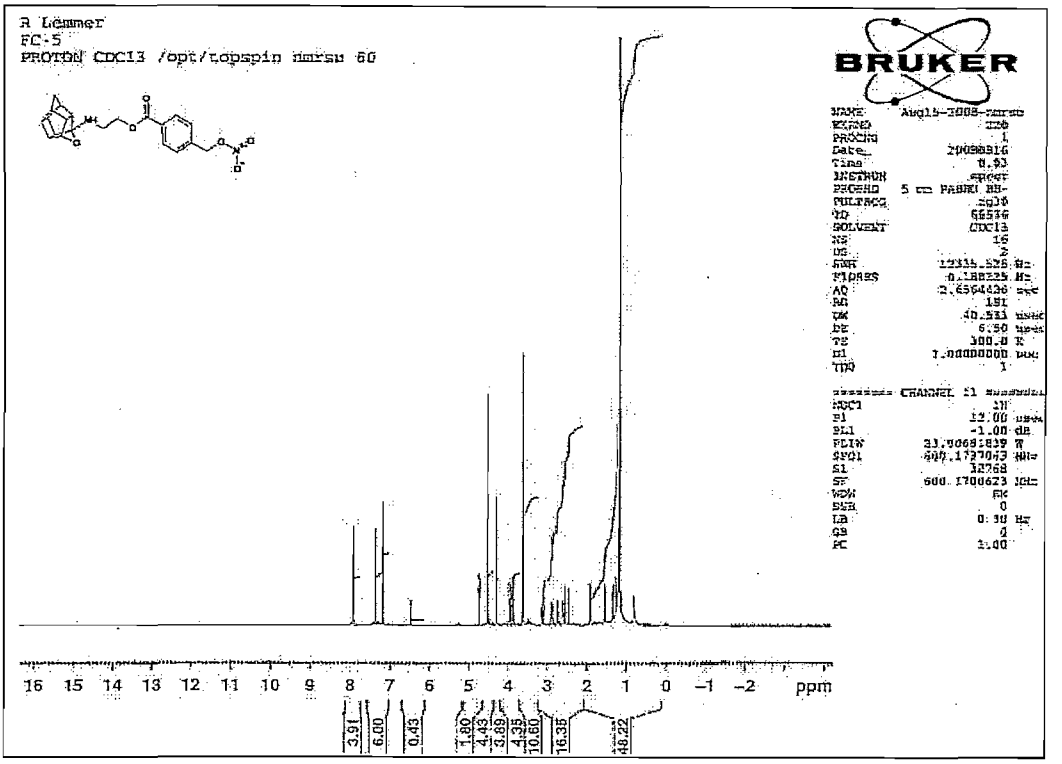

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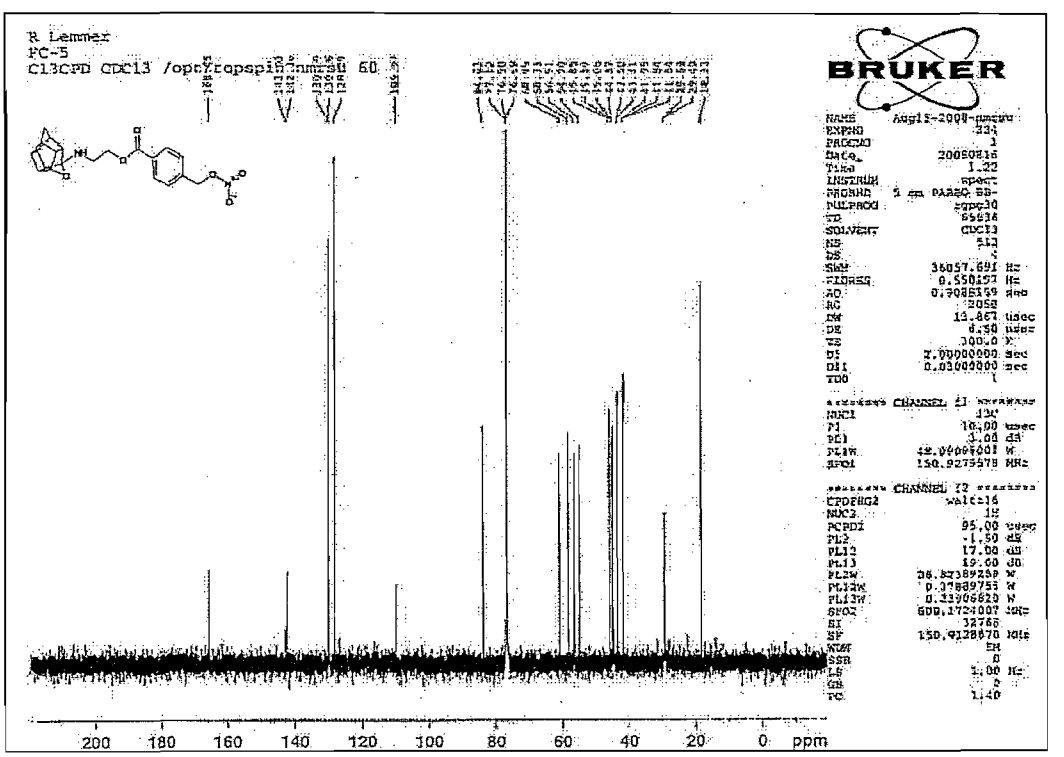

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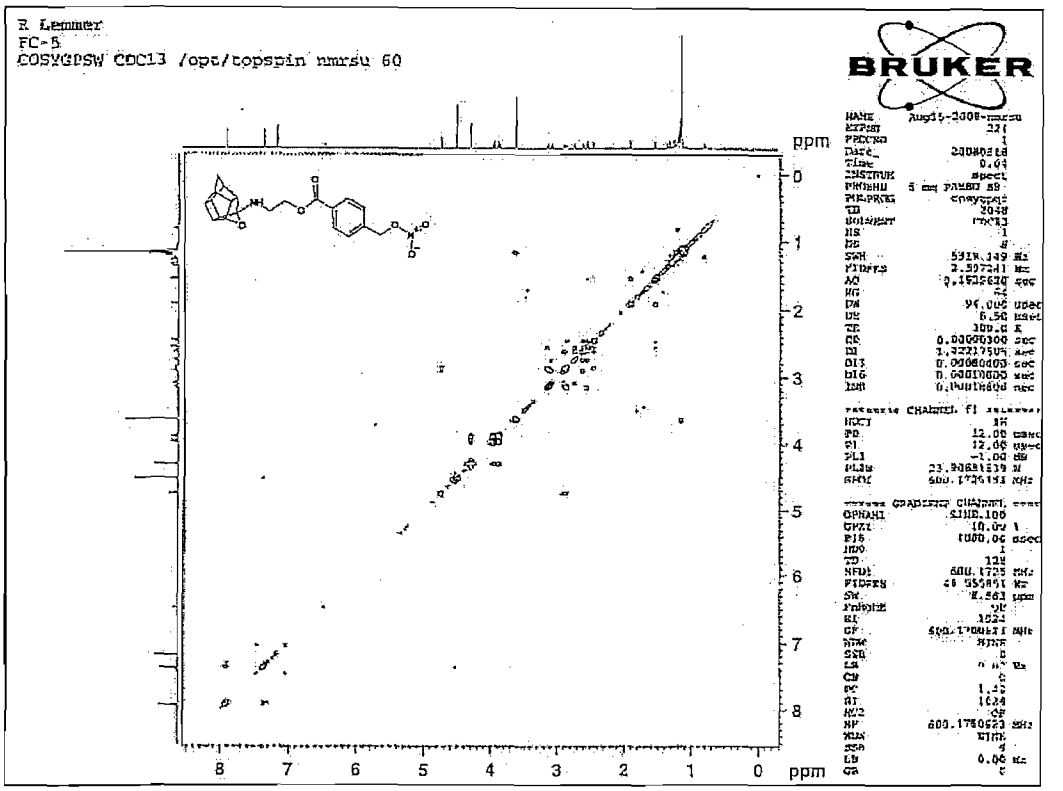

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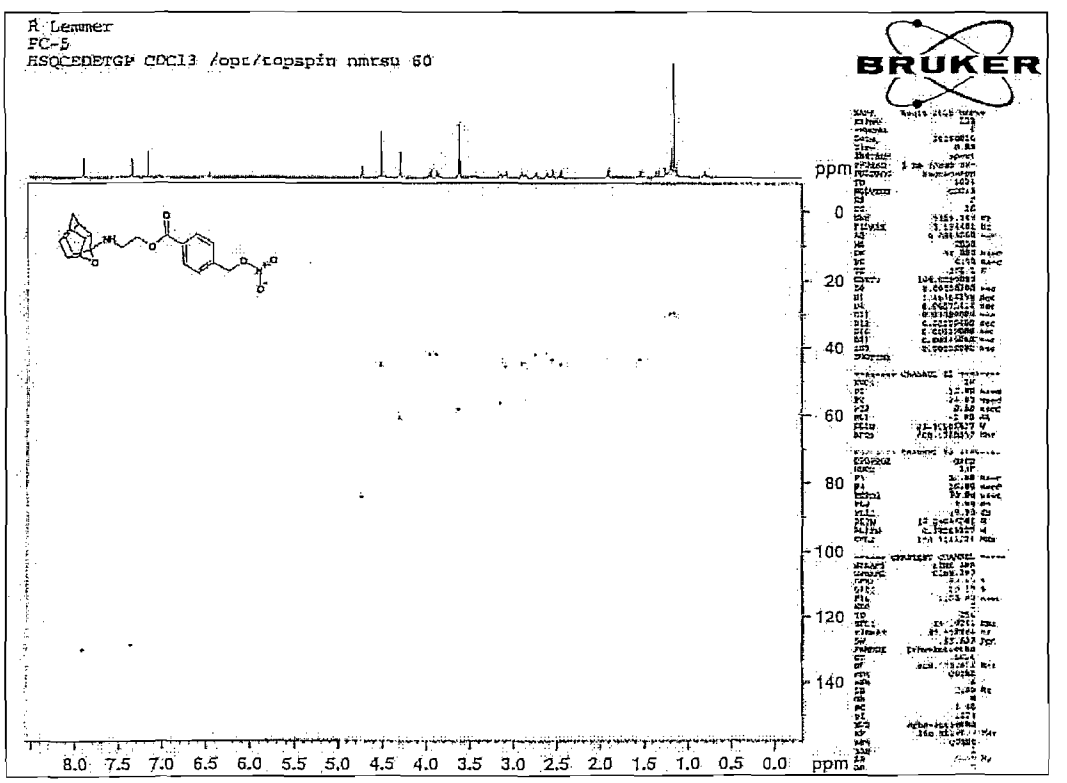

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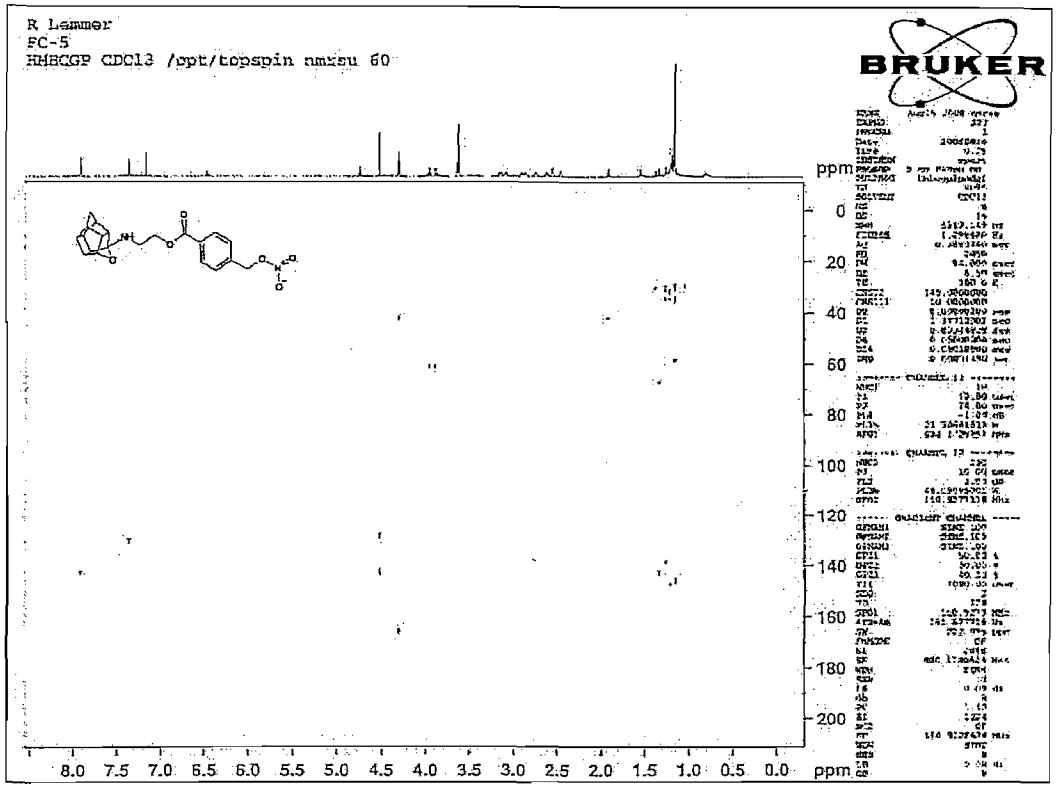

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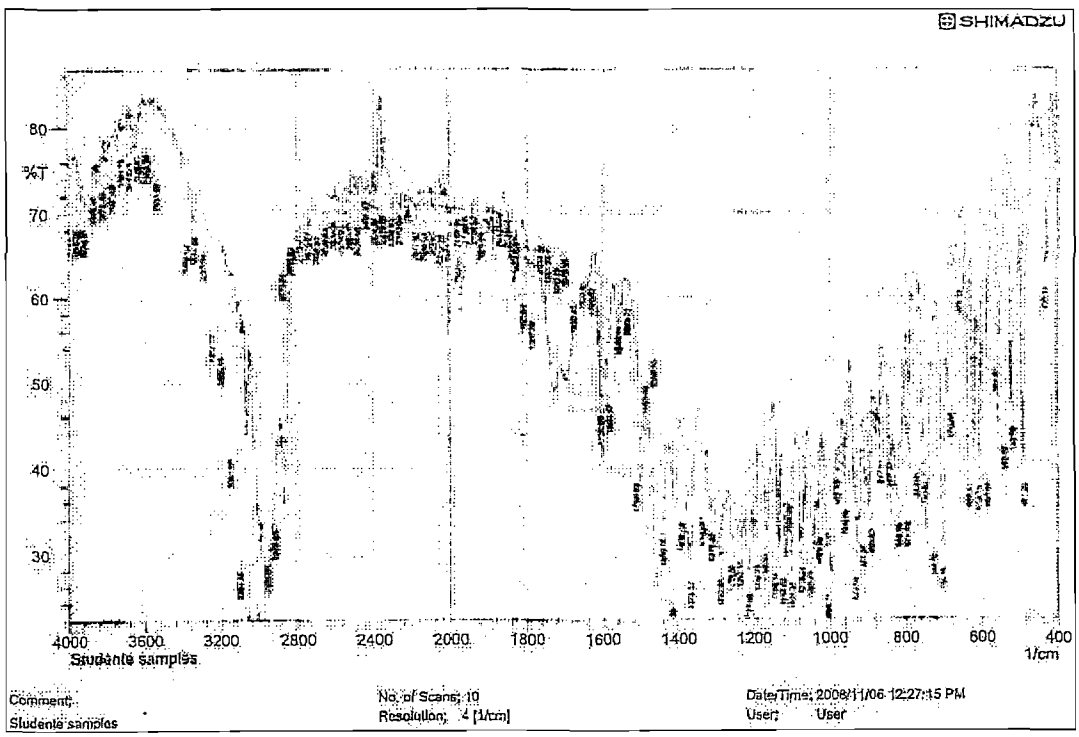

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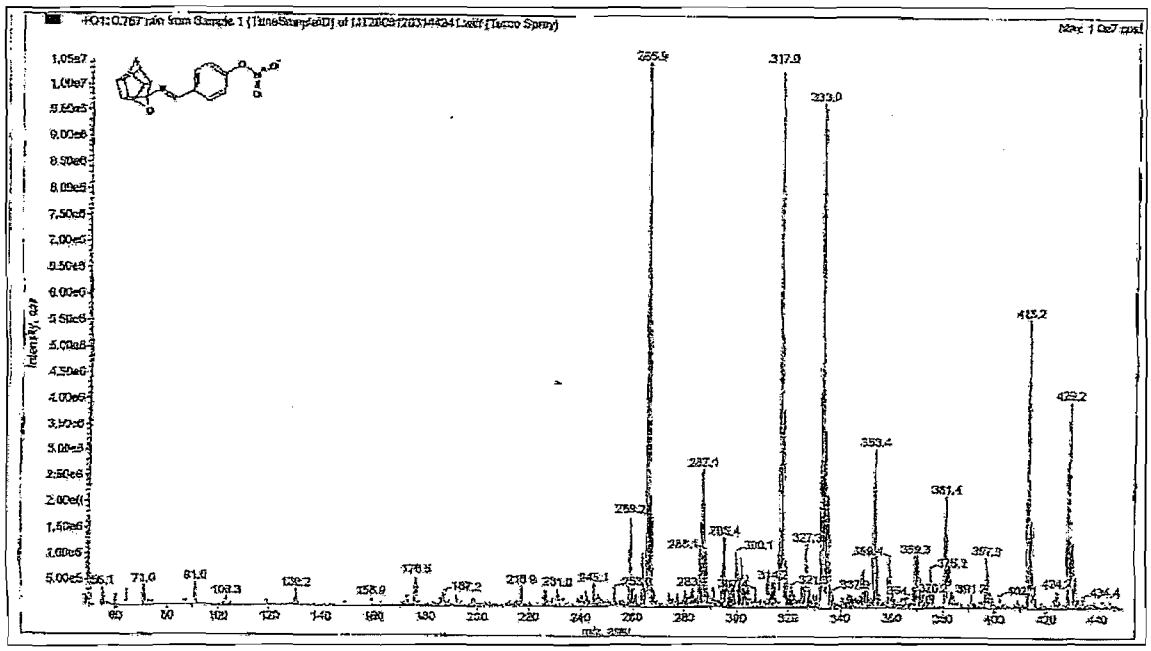

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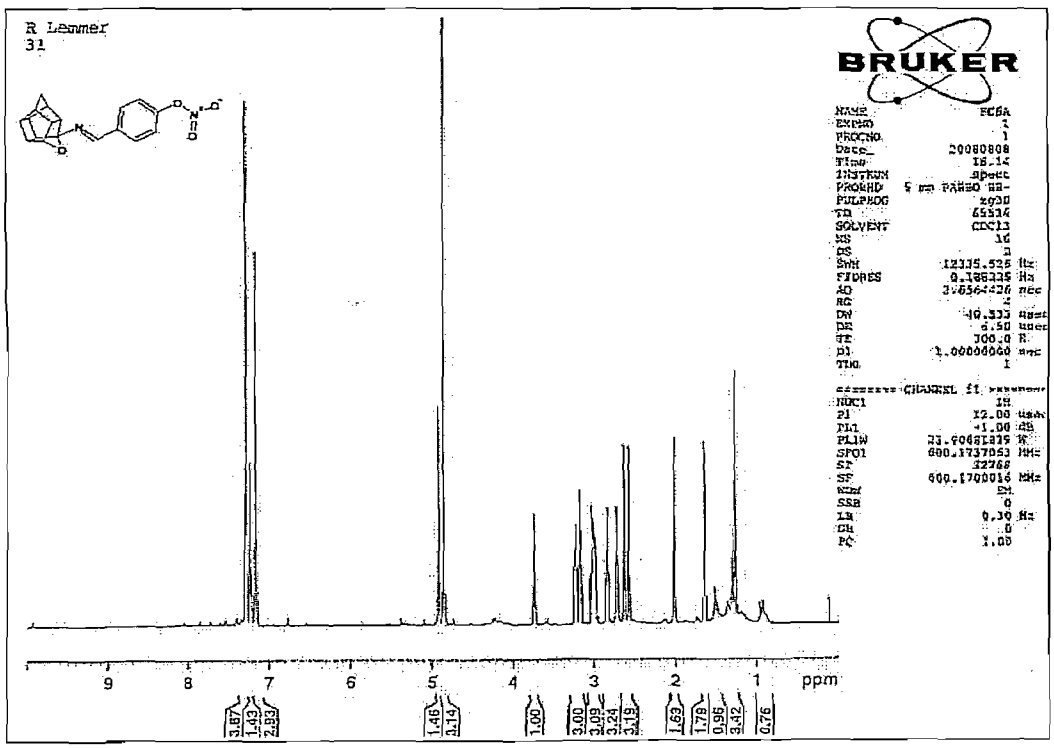

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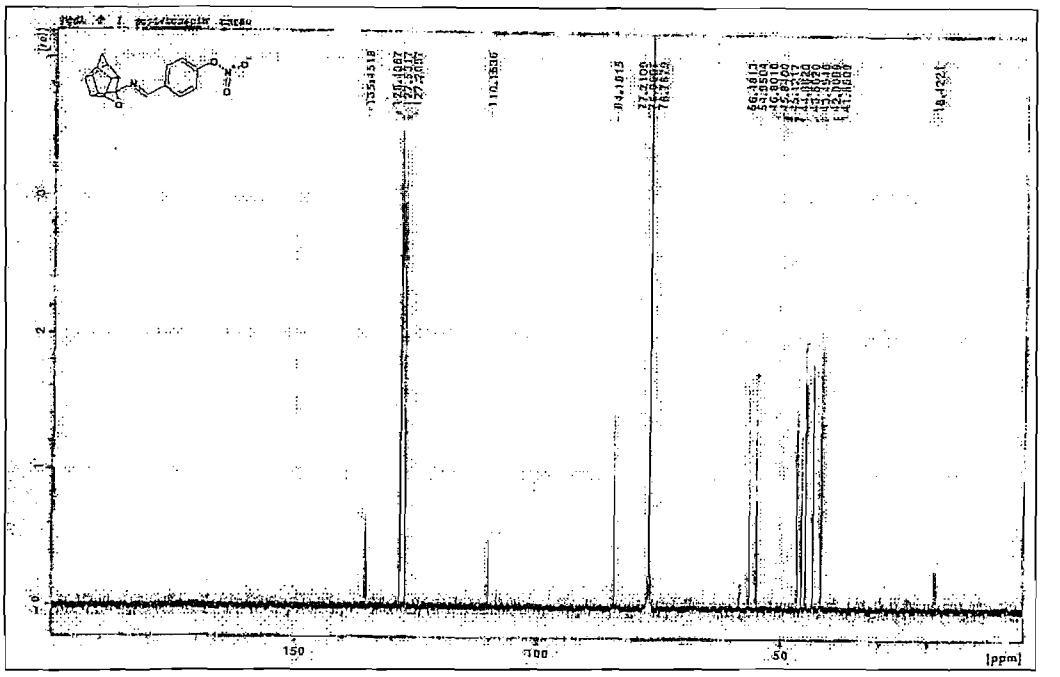

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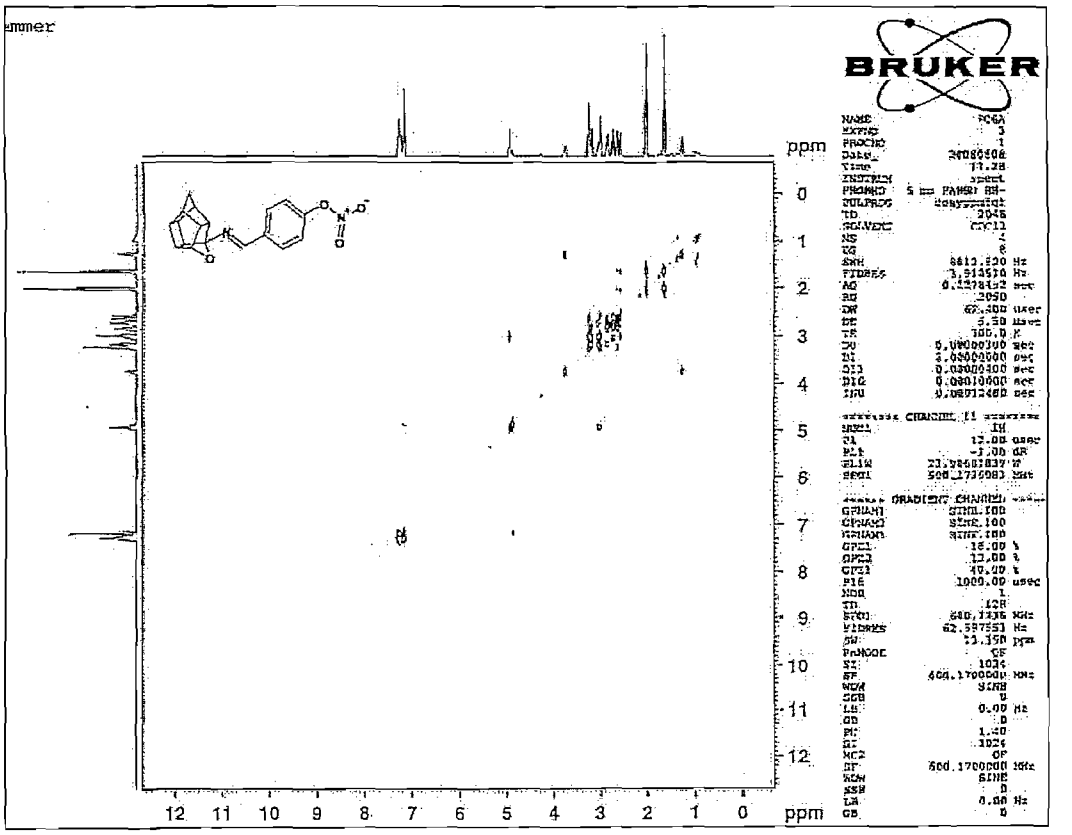

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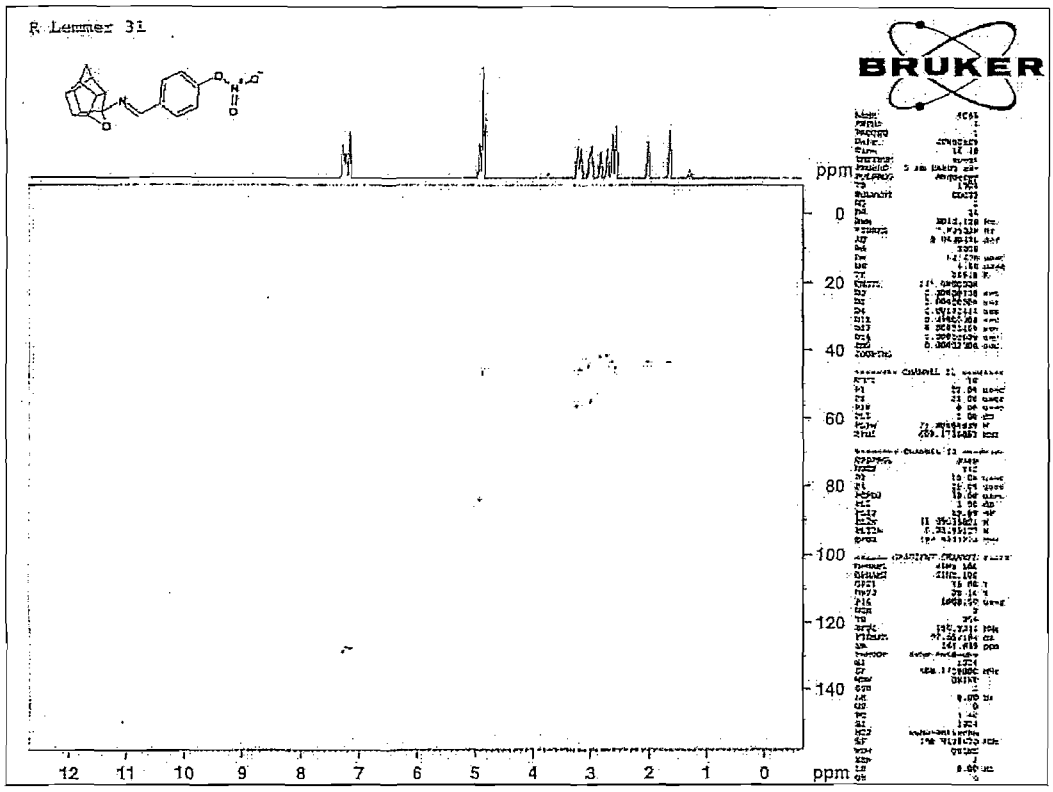

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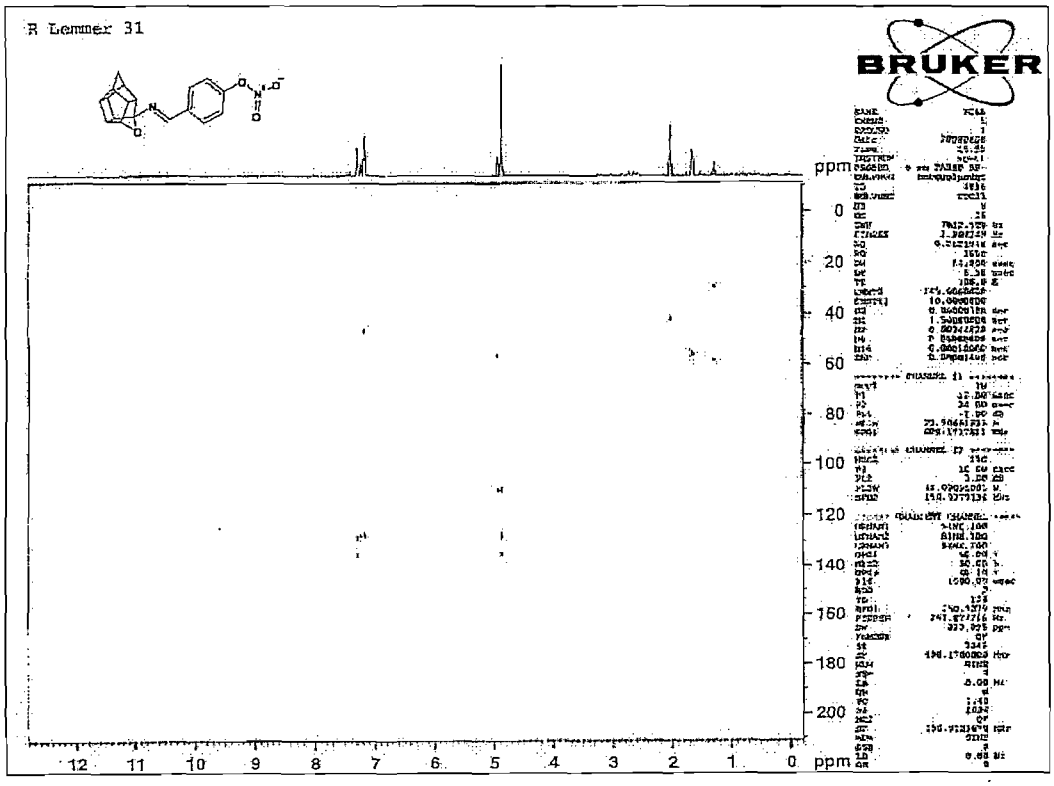

APPENDIX B

CONCEPT ARTICLE

S-NITROSYLATION AND ATTENUATION OF EXCESSIVE CALCIUM FLUX BY PENTACYCLOUNDECANE DERIVATIVES

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ABSTACT

A novel series of polycyclic amines, containing nitrogen monoxide donating moieties, were synthesised and tested for calcium channel modulating activity. The synthesised compounds were classified into two groups, based on their nitrogen monoxide donating moieties: unsaturated nitro compounds (**1**, **2** and **3**) and nitro esters, or nitrates (**4**, **5** and **6**). The nitrates were obtained *via* the reaction of hydroxyl functionalities with thionylchloride nitrate. All of the compounds synthesised exhibited very significant ($p < 0.01$) S-nitrosylation capacity. The channel activity of the polycyclic amines was evaluated using a fluorescent Ca^{2+} flux assay. At concentrations of 10 µM, all the compounds exhibited better Ca^{2+} channel antagonism than NGP1-01, with compounds **1** (96.3 %), **3** (88 %) gave a very significant inhibition ($p < 0.01$) and **4** (92.2 %) a significant inhibition ($p < 0.05$) when compared to the control. At concentrations of 10 and 1 µM, compound **1** exhibited Ca^{2+} channel blockade comparable to the commercially available nimodipine. Compounds **3** and **4** also showed comparable results to that of nimodipine at higher concentrations. No clear correlation was observed between the S-nitrosylation capabilities of the compounds and their Ca^{2+} channel activity, indicating that other factors might play a more decisive role in the mechanism of pentacycloundecylamine channel modulation. This could include the geometric and steric bulk considerations that have been proven to contribute to the Ca^{2+} channel activity of the pentacycloundecylamines. All the compounds synthesised exhibited promising Ca^{2+} channel activity and therefore show promise as potential agents against neurodegeneration.

1 MAIN TEXT

1.1 INTRODUCTION

Dementia is a leading cause of death and disability worldwide. Alzheimer's disease, which is the leading cause of dementia, is ranked fourth in mortality in the United States, followed closely by vascular dementia (multi-infarct dementia).[4] Glutamate-related excitotoxic neuronal cell injury and death has been reported to contribute to almost every major neurodegenerative disorder. This mechanism can also harm oligodentrocytes, which provides the myelin sheaths of axons. This means that excitotoxicity can cause white matter (glial) as well as grey matter (neuronal) disorders. One of the ways excitotoxicity can occur is by over activation of NMDA-sensitive glutamate receptors and the subsequent excessive Ca^{2+} influx into the associated cell. There are numerous insults that can lead to excessive glutamate release and stimulation of glutamate receptors, with subsequent excitotoxicity and cell death. These insults include, amongst others, head or spinal injury and stroke. All of these share the same pathway in that large amounts of glutamate is released from the injured cells which then reach the thousands of nearby cells, causing them to depolarise, swell, lyse and die by necrosis. The lysed cells release more glutamate, leading to a cascade of autodestructive events and progressive cell death that can continue for hours to days after the original injury. In these chronic diseases, it is proposed that modest amounts of glutamate over an extended period are to blame for the excitotoxic damage.[4] To attenuate the effects of excitotoxicity, two mechanisms of action were proposed for this study. The first was S-nitrosylation of crucial cysteine residues in the NMDA receptor channel and the second was the blockage of excessively stimulated Ca^{2+} channels by polycyclic amines.

S-nitrosylation is the transfer of a nitrogen monoxide (NO) to a thiol (sulfhydryl) group (-SH) on a crucial cysteine residue, forming a nitrosothiol.[5] This nitrosothiol donates its nitrogen monoxide group and forms a disulfide bond with an adjacent thiol group. The formation of this disulfide bond causes an allosteric modification in the receptor protein structure. The binding of Zn^{2+} on the NR2A subunit is increased and the linker region in the NMDA receptor is tensed. This bending of the linker region stabilises the glutamate-bound state, slowing glutamate's off-rate, increasing its affinity and eventually resulting in receptor desensitisation.[5] Coupling this mechanism with known Ca^{2+} channel antagonists may afford a unique method of attenuating excitotoxicity. Conjugating this NO donor to a known NMDA receptor antagonist will afford a site specific mechanism of action and eliminate the side effects, like vasodilatation and hypotension, associated with systemic NO administration.

Over the past two decades polycyclic cage amines have received intense scrutiny as possible neuroprotective drugs. Amongst the aminoadamantanes, memantine (Namenda®)

is already in use as an anti-Parkinsonian drug. The pentacycloundecylamines are structurally similar to memantine and their calcium channel activity has been described.[1,6-9] As for the structure-activity relationships for NMDA antagonism, the presence of an amine is compulsory. An aromatic moiety is also necessary for high affinity binding. Geldenhuys *et al.* (2007) developed the hypothesis that the phenyl ring, substituted onto the pentacycloundecylamine, can undergo a π-π type aromatic interaction with (an) aromatic amino acid(s) located at the entrance of the NMDA receptor.[1] This interaction anchors the compound and allows the cage moiety to descend into the channel pore. The depth of this descend is determined by the length of the linker between the aromatic moiety and the nitrogen of the pentacycloundecylamine. This descending mechanism could then bring the cage moiety with its protonated amino-group to close proximity with the PCP-binding site. A series of pentacycloundecylamines containing an aromatic moiety, a linker region for sufficient steric freedom, and a NO donating moiety were proposed as potential agents against neurodegeneration.

1.2 SYNTHESIS

The general synthetic route for the compounds used in this study (Figure 2) encompassed the reductive amination of the pentacycloundecane dione (**7**) with 2-aminoethanol or 3-aminopropanol and reduction with sodium borohydride to yield the oxa compounds **8** and **9**. Esterification was done using the activating agent, 1-Ethyl-3-(3'-dimethylamino)carbodiimide and a catalytic amount of 4-(dimethylamino)-pyridine (DMAP) (Figure 3). The reaction mixture was stirred on ice for 15 minutes and then at room temperature until completion. The 4-nitroethenylbenzoic acid, conjugated to yield compound **3**, was prepared using a microwave synthesis.[10] For the final step in the synthesis of compounds **4**, **5** and **6**, their respective hydroxyl groups were nitrated using thionylchloride nitrate. The thionylchloride nitrate was prepared using the method described by Hakimelahi *et al.* [2] Compound **6** was synthesised using an alternative method (Figure 4) in which NGP1-01 (**10**) was catalytically reduced using paladised carbon to afford the free pentacycloundecylamine **11**. This free amine group was allowed to react with the aldehyde group of 4-hydroxybensaldehyde and the free hydroxyl group of the intermediary alcohol (**12**) was nitrated to yield compound **6**.

1.3 BIOLOGICAL EVALUATION

To evaluate the S-nitrosylation capability of the synthesised compounds a modified S-nitrosylation assay was employed.[3] The intensity of the UV-absorbance caused by the pyridine-2-thione was used to measure the extent of S-nitrosylation. The results (Figure 5) indicated that all the synthesised compounds were potent S-nitrosylators ($p < 0.01$) with the nitrate compounds exhibiting better nitrosylation than the nitro compounds. This corresponds

well with the literature, since it is known that the most common way for nitrates to donate NO is by interaction with thiol groups, while carbon nitro compounds primarily donate NO as a cause of heat or light.

To evaluate the Ca^{2+} channel activity of the compounds, freshly prepared synaptoneurosomes were incubated with the ratiometric fluorescent calcium indicator, Fura-2/AM. The synthesised compounds were added and the cell membranes depolarised with a KCl-solution. Ca^{2+} influx into the cells was then monitored based on the fluorescence intensity relative to that of a (100 %) control. Two positive controls were also included: nimodipine, a commercially available L-type Ca^{2+} channel blocker and NGP1-01, the lead pentacycloundecylamine. The results (Figure 6) indicated that at concentrations of 10 and 1 μM all of the compounds attenuated Ca^{2+} flux into the cells. Compound **1** showed activity comparable to that of nimodipine, while compounds **3** and **4** inhibited calcium flux to these levels only at 10 μM concentrations. As observed for NGP1-01, compounds **2**, **5** and **6** exhibited their optimal calcium flux inhibition at 10 and 1 μM concentrations. The decrease in calcium flux observed for these structures was dose dependant.

1.4 CONCLUSION

The synthesised compounds were all potent S-nitrosylators and exhibited good Ca^{2+} channel activity at high concentrations. No direct correlation could however be drawn between the S-nitrosylation capability and the Ca^{2+} channel activity, suggesting that other factors might play a more significant role in the channel activity of the pentacycloundecylamines. As described previously, the steric and geometric properties of the pentacycloundecylamines tend to be the most important considerations when dealing with their channel activity.[6,7] Indeed, compound **6** had the closest structural similarity to NGP1-01 and its Ca^{2+} channel activity also correlated with that of NGP1-01. The compounds containing the longer linker exhibited better Ca^{2+} channel antagonism than NGP1-01, which seems to confirm the hypothesis by Geldenhuys *et al.* concerning the fold back mechanism of pentacycloundecylamine antagonism.[1] It is postulated that the reason for the poor correlation between the S-nitrosylation and the Ca^{2+} channel activity, is that the synthesised compounds' aromatic moieties underwent a π-π type aromatic interaction with (an) aromatic amino acid(s), located at or close to the entrance of the channel pore, folding back and blocking the receptor pore itself. This would prohibit the NO donating moiety from coming into contact with the crucial cysteine residues necessary for S-nitrosylation. All of the compounds however, exhibited good Ca^{2+} channel antagonism and S-nitrosylation capability, making them promising leads in neuroprotective drug discovery.

2 EXPERIMENTAL SECTION

2.1 MATERIALS

1-Ethyl-3-(3'-dimethylamino)carbodiimide (EDC), 4-(dimethylamino)-pyridine (DMAP), benzylamine, 4-nitrobenzoic acid, 4-hydroxybenzaldehyde, 4-formylbenzoic acid, silver nitrate and thionylchloride were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium borohydrite and magnesium sulphate were purchased from Saarchem (Krugersdorp, S.A.).

2.2 SYNTHESIS OF COMPOUNDS

2.2.1 8-BENZYLAMINO-8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE (NGP1-01) (10)

The pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (**7**) (5.3 g, 30 mmol) was dissolved in dry tetrahydrofurane (50 ml) and cooled down to 5 °C while stirring on an external ice bath. Benzylamine (3.3 g, 30 mmol) was added slowly, with continuous stirring at lowered temperature. The carbinolamine which started to precipitate out after 10 minutes was isolated by filtration after 30 minutes. It was then dehydrated under Dean-Stark conditions in benzene (60 ml) for 1 hour or until no more water was collected in the trap. The mixture was then concentrated in vacuo and the Schiff-base (8-benzylimino pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-11-one) was obtained as a yellow oil. The Schiff-base was then dissolved in a mixture of dry methanol (30 ml) and dry tetrahydrofurane (150 ml). Reduction was carried out by adding sodium borohydride (1.6 g, 41 mmol) proportion wise to the reaction mixture and stirring overnight. The reaction mixture was concentrated *in vacuo,* water (100 ml) was added, followed by extraction with dichloromethane (4 x 50 ml). The combined organic fractions were combined and washed with water (2 x 100 ml), dried over anhydrous $MgSO_4$ and evaporated to give a yellow oil. Purification of the mixture was done by column chromatography (mobile phase, dichloromethane: petroleum ether: ethyl acetate (1:1:1) and the desired amine (**10**) was isolated as a colourless wax. Crystallization from ethyl acetate gave the final compound (**10**) as fine white needles (2.95 g, 11.12 mmol, 37.01 %). The physical and spectral data corresponds to that described in the literature, (Van der Schyf *et al.*, 1989:48), and is not given in this article.

2.2.2 8-(2-AMINOETHANOL)-8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE (8)

The pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (**7**) (5.1 g, 29 mmol) was dissolved in dry tetrahydrofurane (50 ml) and cooled down to 5 °C, while stirring on an external ice bath. 2-Aminoethanol (1.8 g, 29 mmol) was added drop wise, while stirring at lowered temperature. The carbinolamine started to precipitate out after 10 minutes and the mixture was stirred for

another 20 minutes, after which dry methanol (30 ml) was added and the mixture removed from the ice bath. Once all the carbinolamine was dissolved, a spatula point (± 1.5 g) of sodium borohydrite was slowly added and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo*, water (100 ml, pH 2 with hydrochloric acid) was added, followed by extraction with dichloromethane (4 x 50 ml). The water fraction was alkalised with sodium bicarbonate to a pH of 11 and extracted again with dichloromethane (4 x 50 ml). The second organic fractions were combined and washed with water (2 x 100 ml), dried overnight with anhydrous $MgSO_4$ and evaporated to give a light yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate: ethanol (2:1)) and the desired amine (**8**) was obtained as a light yellow oil (1.5 g, 6.85 mmol, 30 %).

$C_{13}H_{17}NO_2$; TLC (2EtOAc:EtOH): R_f 0.40; **^{1}H NMR (300 MHz, $CDCl_3$) δ_H**: 4.6 (t, 1H, J = 5.22 Hz, H-11), 3.6 (m, 2H, H-15a,b), 3.067 (m, 2H, H-14a,b), 2.911 – 2.630 (m, 4H, H-1,7,9,10), 2.619 – 2.425 (m, 4H, H-2,3,5,6), 1.949 (AB-q, 2H, J = 10.5 Hz, H-4a,4b); **^{13}C NMR (75 MHz, $CDCl_3$) δ_C**: 82.335 (t, C-11), 77.427 (t, C-14), 76.580 (s, C-8), 63.332 (d, C-15), 55.092 (d, C-10), 54.653 (d, C-9), 46.275 (d, C-7), 44.797 (d, C-5), 44.483 (d, C-3), 44.295 (d, C-1), 43.006 (t, C-4), 41.824 (d, C-6), 41.429 (d, C-2).

2.2.3 8-(3-AMINOPROPANOL)-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE (9)

The pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**8**) (5.4 g, 31 mmol) was dissolved in dry tetrahydrofurane (50 ml) and cooled down to 5 °C, while stirring on an external ice bath. 3-Aminopropanol (2.3 g, 31 mmol) was dissolved in dry methanol (30 ml), to which was added a spatula point (± 1.5 g) of sodium borohydrite. This mixture was then added drop wise to the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione mixture over a period of 30 minutes and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo*, water (100 ml, pH 2 with hydrochloric acid) was added, followed by extraction with dichloromethane (4 x 50 ml). The water fraction was alkalised with sodium bicarbonate to a pH of 11 and extracted with dichloromethane (4 x 50 ml). The second organic fractions were combined and washed with water (2 x 100 ml), dried overnight with anhydrous $MgSO_4$ and evaporated to give a light yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate: ethanol (1:1)) and the desired amine (**9**) was obtained as a light yellow oil (0.481 g, 2.1 mmol, 24.42 %).

$C_{13}H_{17}NO_2$; TLC (EtOAc:EtOH): R_f 0.22; **^{1}H NMR (300 MHz, $CDCl_3$) δ_H**: 4.705 (t, 1H, J = 5.22 Hz, H-11), 3.857 (quartet, 2H, J = 2.72 Hz, H-16a,b), 3.060 – 2.885 (m, 2H, H-14a,b), 2.866 – 2.622 (m, 4H, H-2,3,5,6), 2.618 – 2.423 (m, 4H, H-1,7,9,10), 1.995 – 1.575 (AB-q, 2H, J = 10.4 Hz, H-4a,4b), 1.758 (m, 2H, H-15a,b); **^{13}C NMR (75 MHz, $CDCl_3$) δ_C**: 82.601 (t, C-11), 77.424 (t, C-14), 76.576 (s, C-8), 62.897 (d, C-16), 55.322 (d, C-10), 54.740 (d, C-9), 44.813 (d, C-5), 44.531 (d, C-3), 43.278 (d, C-7), 42.982 (d, C-1), 42.645 (t, C-4), 41.794 (d, C-6), 41.517 (d, C-2), 32.391 (t, C-15).

2.2.4 8-AMINOETHYL-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE-2-(4-NITROBENZOATE) (1)

8-(2-aminoethanol)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**7**) (1.043 g, 4.7 mmol) was dissolved in dichloromethane (40 ml) and cooled down to 5 °C on an external ice bath. 1-Ethyl-3-(3'-dimethylamino)carbodiimide (1.003 g, 5.2 mmol), 4-nitrobenzoic acid (0.874 g, 5.2 mmol) and 4-(dimethylamino)-pyridine (0.057 g, 0.52 mmol) were added while stirring on an ice bath. After 15 minutes the reaction mixture was removed from the ice bath and stirred for a further 72 hours at room temperature. The mixture was then concentrated *in vacuo*. A saturated sodium bicarbonate solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight with anhydrous $MgSO_4$ and evaporated to give a light yellow oil. The product was purified using column chromatography (mobile phase, ethanol: ethyl acetate (1:2)) to give the desired amine (**1**) as a light yellow oil (0.526 g, 1.43 mmol, 30.38 %).

$C_{20}H_{20}N_2O_5$; TLC (2DCM:EtOAc): R_f 0.48; **IR (KBr) ν_{max}**: 3412.08, 1709.65, 1527.62, 1490.97, 1352.10, 1240.70; **LC-MS (EI, 70 eV) *m/z***: 369.2(M^+), 353.4, 242.2, 230.9, 186.9, 118.8; **^{1}H NMR (600 MHz, $CDCl_3$) δ_H**: 8.367 – 7.85 (m, 4H, H-19,20,22,23), 4.6 (t, 1H, J = 5.28 Hz, H-11), 4.407 (t, 2H, J = 5.58 Hz, H-15), 3.16 (sextet, 2H, J = 5.89 Hz, H-14), 2.78 – 2.36 (m, 8H, H-1,2,3,5,6,7,9,10), 1.842 (AB-q, 2H, J = 10.5 Hz, H-4a,4b). **^{13}C NMR (150 MHz, $CDCl_3$) δ_C**: 163.65 (s, C-17), 149.52 (s, C-18), 134.58 (s, C-21), 129.81 (d, C-20/C-22), 129.65 (d, C-19/C-23), 108.21 (s, C-8), 81.59 (t, C-11) 65.45 (d, C-15), 54.38 (d, C-14), 53.74 (d, C-9), 43.83 (d, C-10), 43.70 (d, C-7), 43.55 (d, C-1), 42.31 (d, C-5), 42.05 (d, C-3), 41.43 (t, C-4), 40.9 (d, C-2), 40.53 (d, C-6).

2.2.5 8-AMINOPROPYL-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE-2-(4-NITROBENZOATE) (2)

8-(3-Aminopropanol)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**9**) (0.481 g, 2.1 mmol) was dissolved in dichloromethane (40 ml) and cooled down to 5 °C on an external ice bath. 1-Ethyl-3-(3'-dimethylamino)carbodiimide (0.435 g, 2.2 mmol), 4-nitrobenzoic acid (0.379 g, 2.2 mmol) and 4-(dimethylamino)-pyridine (0.026 g, 0.2 mmol) were added while

stirring on an ice bath. After 15 minutes the reaction mixture was removed from the ice bath and stirred for another 72 hours at room temperature. The mixture was then concentrated in vacuo. A saturated sodium bicarbonate solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried over night with anhydrous $MgSO_4$ and evaporated to give a light yellow oil. The mixture was purified using column chromatography (mobile phase, ethanol: ethyl acetate (1:1)) to give the desired amine (**2**) as a dark yellow oil.

$C_{21}H_{22}N_2O_5$; TLC (EtOAc:EtOH): R_f 0.76; **IR (KBr)** ν_{max}: 3403.12, 1722.43, 1606.70, 1523.76, 1352.10, 1105.21; **LC-MS (EI, 70 eV)** ***m/z***: 383.2(M^+), 256.3, 234.1; **^{1}H NMR (300 MHz, $CDCl_3$)** δ_H: 8.312 – 8.136 (m, 4H, H-20,21,23,24), 4.725 (t, 1H, J = 5.32, H-11), 4.502 (t, 2H, J = 6.32, H-16), 3.077 (sextet, 2H, J = 5.08, H-14), 2.867 – 2.258 (m, 9H, H-1,2,3,5,6,7,9,10,11), 2.001 (sextet, 2H, J = 3.36, H-15), 1.889 (AB-q, 2H, J = 10.6, H-4a,4b). **^{13}C NMR (75 MHz, $CDCl_3$)** δ_C: 164.644 (s, C-18), 135.721 (s, C-19), 130.641 (d, C-20/24), 123.516 (s, C-22), 123.475 (d, C-23/21), 82.470 (t, C-11), 77.424 (s, C-8), 76.577 (d, C-14), 64.122 (d, C-16), 55.311 (d, C-10), 44, 817 (d, C-9), 44.689 (d, C-5), 44.516 (d, C-3), 43.259 (d, C-7), 43.052 (d, C-1), 41.889 (t, C-4), 41.532 (d, C-6), 40.403 (d, C-2), 30.194 (d, C-15).

2.2.6 8-AMINOETHYL-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE-2-[4-(2-NITROETHENYL)BENZOATE] (3)

In a microwave tube, 4-formylbenzoic acid (1.126 g, 7.5 mmol) was added to nitromethane (0.4 ml, 7.5 mmol) and a catalytic amount of ammonium acetate (0.145 g, 0.19 mmol). The mixture was irradiated in a microwave station for 2 minutes and 280 W at 25 °C. The mixture was then removed and allowed to cool down. It was then irradiated twice more, for 2 minutes each with cooling in between. Nitromethane (0.4 ml, 7.5 mmol) was added to the mixture again and the procedure was repeated as before. The 4-nitroethenylbenzoic acid was purified using column chromatography (mobile phase, ethyl acetate). The 4-nitroethenylbenzoic acid was obtained as a light yellow microcrystalline solid (yield: 0.545 g, 2.8 mmol, 37.33 %). In a one pot synthesis, 8-(2-aminoethanol)-8,11-oxa pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**8**) (0.236 g, 1.1 mmol), 1.1 equivalent of 4-nitroethenylbenzoic acid (0.229 g, 1.2 mmol), 1.1 equivalent of 1-ethyl-3-(3'-dimethylamino)carbodiimide (0.227 g, 1.2 mmol) and 0.1 equivalent of 4-(dimethylamino)-pyridine (0.013 g, 0.1 mmol) was dissolved in dichloromethane (20 ml) and cooled down to 5 °C on an external ice bath. The reaction mixture was stirred for 15 minutes on the ice bath, removed and stirred for a further 72 hours at room temperature. The mixture was then removed and concentrated *in vacuo*. A saturated $NaHCO_3$ solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight over anhydrous $MgSO_4$ and evaporated to yield a dark yellow oil. The

crude compound was purified using column chromatography (mobile phase, ethyl acetate: dichloromethane (1:1)) and the desired amine **3** was isolated as a yellow oil which crystallized over night to yield light yellow crystals (0.109 g, 0.3 mmol, 27.27 %).

$C_{22}H_{22}N_2O_5$; TLC (EtOAc:DCM): R_f 0.64; **m.p.** 185 °C; **IR (KBr)** ν_{max}: 3292.49, 1722.90, 1666.50, 1610.56, 1502.55, 1367.53, 1282.30; **LC-MS (EI, 70 eV)** ***m/z***: 406.2(M^+), 384.3, 374.0, 352.2, 242.3, 220.2, 177.1; **^{1}H NMR (600 MHz, $CDCl_3$)** δ_H: 10.035 (s, 1H, H-23), 8.133 (dd, 2H, J = 4.92 Hz, H-19,25), 7.888 (dd, 2H, J = 4.52 Hz, H-20,24), 7.201 (t, 1H, H-22), 4.7 (t, 1H, J = 5.32 Hz, H-11), 4.507 (t, 2H, J = 6.32 Hz, H-14), 3.149 (t, 2H, J = 3.48 Hz, H-15), 2.759 – 2.24 (m, 8H, H-1,2,3,5,6,7,9,10), 1.839 (AB-q, 2H, J = 9.42 Hz, H-4a,4b). **^{13}C NMR (150 MHz, $CDCl_3$)** δ_C: 191.88 (s, C-17), 191.87 (s, C-23), 165.49 (s, C-22), 139.11 (s, C-18), 135.16 (s, C-21), 130.2 (d, C-19/C-25), 129.47 (d, C-20/C-24), 109.26 (d, C-15), 82.56 (t, C-11), 66.21 (s, C-8), 55.36 (d, C-14), 54.73 (d, C-9), 44.82 (d, C-7), 44.70 (d, C-1), 44.53 (d, C-10), 43.29 (d, C-3), 43.05 (d, C-5), 42.47 (t, C-4), 41.89 (d, C-6), 41.51 (d, C-2).

2.2.7 8-(2-AMINOETHYLNITRATE)-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}] UNDECANE (4)

8-(2-Aminoethanol)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**8**) (0.197 g, 0.9 mmol) was dissolved in dry THF (5 ml) at room temperature. Silver nitrate (0.153 g, 0.9 mmol) was added. An equimolar amount of thionylchloride (0.07 ml, 0.9 mmol) was added drop wise, while stirring at room temperature. The reaction mixture was allowed to stir for 12 hours. Quantitative precipitation of silver chloride started to occur as the reaction neared completion. After 12 hours, water (30 ml) was added to the mixture and it was extracted with ethyl acetate (4 x 20 ml). The combined organic fractions were dried overnight over anhydrous $MgSO_4$ and evaporated in vacuo to yield a yellow oil. The mixture of compounds was purified using column chromatography (mobile phase, ethyl acetate) and the desired amine (**4**) was isolated as a light yellow oil (0.072 g, 0.03 mmol, 33.33%).

$C_{13}H_{16}N_2O_4$; TLC (EtOAc): R_f 0.86; **IR (KBr)** ν_{max}: 2978.09, 1699.29, 1475.54, 1348.24, 1255.66; **LC-MS (EI, 70 eV)** ***m/z***: 264.1(M^+), 242.2, 220.1, 202.2, 183.1, 170.9; **^{1}H NMR (600 MHz, $CDCl_3$)** δ_H: 4.59 (t, 1H, J = 5.18 Hz, H-11), 4.08 (m, 2H, H-15a,b), 3.65 (m, 2H, H-14a,b), 2.75 – 2.35 (m, 8H, H-1,2,3,5,6,7,9,10), 1.81 (AB-q, 2H, J = 10.5 Hz, H-4a,4b); **^{13}C NMR (150 MHz, $CDCl_3$)** δ_C: 85.1 (t, C-11), 83.2 (s, C-8), 66.580 (d, C-14), 63.3 (d, C-15), 57.292 (d, C-10), 55.65 (d, C-9), 46.25 (d, C-7), 45.897 (d, C-5), 45.83 (d, C-3), 43.82 (d, C-1), 42.9 (t, C-4), 42.14 (d, C-6), 41.6 (d, C-2).

2.2.8 8-AMINOETHYL-8,11-OXAPENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE-4-(2-[(NITROOXY)METHYL]BENZOATE) (5)

In a one pot synthesis, 8-(2-aminoethanol)-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**8**) (1.535 g, 7.0 mmol), 1.1 equivalent of 4-formylbenzoic acid (1.156 g, 7.7 mmol), 1.1 equivalent of 1-ethyl-3-(3'-dimethylamino)carbodiimide (1.476 g, 7.7 mmol) and 0.1 equivalent of 4-(dimethylamino)-pyridine (0.086 g, 0.7 mmol) was dissolved in dichloromethane (20 ml) and cooled to 5 °C on an external ice bath. The reaction mixture was stirred for 15 minutes and removed from the ice bath, after which it was stirred for another 72 hours at room temperature. The mixture was concentrated *in vacuo*. A saturated sodium bicarbonate solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight with anhydrous $MgSO_4$ and evaporated to give a bright yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate: dichloromethane (1:3)) and the desired amine was isolated as pure white crystals (0.698 g, 2.0 mmol, 28.57 %) and it was then dissolved in dry methanol (30 ml), cooled down to 5 °C on an external ice bath. A small spatula point (± 0.2 g) of sodium borohydrite was slowly added to the stirred mixture on the ice bath. After 30 minutes the reaction mixture was removed from the ice bath and stirred at room temperature overnight. The reaction mixture was concentrated in vacuo. A saturated sodium bicarbonate solution (30 ml) was added and it was extracted with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight with $MgSO_4$ and evaporated to give a light yellow oil. The crude compound was purified using column chromatography (mobile phase, ethyl acetate) to yield the desired amine as a murky oil which crystallized overnight to give colourless crystals (0.128 g, 0.4 mmol, 20 %). The amine obtained (0.128 g, 0.4 mmol) was dissolved in dry THF (10 ml) and stirred at room temperature. Thionylchloride nitrate (0.3 ml, 0.4 mmol) was added drop wise to the reaction mixture and it was stirred for 120 minutes at room temperature. Diethyl ether (30 ml) was added to the mixture and it was washed with water (2 x 40 ml). The organic fraction was dried overnight with anhydrous $MgSO_4$ and evaporated to give a dark yellow oil. The compound was purified using column chromatography (mobile phase, ethyl acetate: dichloromethane (1:2)) to give the desired amine (**5**) as a dark orange oil (0.024 g, 0.06 mmol, 15 %).

$C_{21}H_{22}N_2O_6$; TLC: (EtOAc:2DCM): R_f 0.90; **IR (KBr)** ν_{max}: 2956.87, 1720.5, 1633.71, 1612.49, 1313.52, 1163.08; **LC-MS (EI, 70 eV) *m/z***: 393.1(M^+), 381.3, 372.2, 353.3, 283.1, 246.9, 220.1, 210.1; **^{1}H NMR (600 MHz, $CDCl_3$) δ_H**: 7.926 (d, 2H, H-19.25), 7.375 (d, 2H, J = 8.1 Hz, H-20,24), 4.749 (t, 1H, J = 5.2 Hz, H-11), 4.52 (s, 2H, H-22), 2.923 – 2.451 (m, 4H, H-14a,H-14b,H-15a,H-15b), 3.164 – 3.076 (m, 8H, H-1,2,3,5,6,7,9,10), 1.924 (AB-q, 2H, J = 10.6 Hz, H-4a,4b). **^{13}C NMR (150 MHz, $CDCl_3$) δ_C**: 165.65 (s, C-17), 143.03 (s, C-18),

143.36 (s, C-21), 130.09 (d, C-19/C-25), 128.29 (d, C-20/24), 84.03 (t, C-11), 60.95 (s, C-8), 56.51 (d, C-9), 54.90 (d, C-7), 45.85 (d, C-1), 45.19 (s, C-22), 45.05 (d, C-5), 44.87 (d, C-3), 43.5 (t, C-4), 43.41 (d, C-14), 41.95 (d, C-15), 41.84 (d, C-6), 41.54 (d, C-2).

2.2.9 8-[4-(IMINOMETHYL)PHENYL NITRATE]-8,11-OXAPENTACYCLO [5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE (6)

NGP1-01 (**10**) (2.95 g, 11.12 mmol) was dissolved in ethanol (30 ml) and reacted with hydrogen over 10 % paladised charcoal (0.15 g) for 12 hours at atmospheric pressure and at room temperature. The reaction mixture was concentrated in vacuo and a saturated sodium bicarbonate solution (30 ml) was added, followed by extraction with dichloromethane (4 x 20 ml). The combined organic fractions were dried overnight over anhydrous $MgSO_4$ and evaporated to give a dark yellow oil, which solidified overnight to give a waxy semisolid. Recrystallisation from dichloromethane: hexane (1:1) afforded the desired amine as colourless platelets (1.4 g, 7.99 mmol, 71 %) and it was then dissolved in tetrahydrofurane (20 ml) and cooled down to 5 °C on an external ice bath. 4-hydroxybenzaldehyde (1 g, 7.99 mmol) was slowly added and the reaction mixture was allowed to stir for 2 hours. The mixture was concentrated *in vacuo*, water (50 ml) was added, followed by extraction with dichloromethane (4 x 30 ml). The combined organic fractions were dried overnight with anhydrous $MgSO_4$, filtered and evaporated to give a dark yellow oil. The mixture was purified using column chromatography which yielded the desired amine as a light yellow oil (1.5 g, 5.4 mmol, 67.58 %). The amine obtained (1.5 g, 5.4 mmol) was then dissolved in tetrahydrofurane (10 ml) and thionylchloride nitrate (0.4 ml, 5.4 mmol) was added drop wise. The reaction mixture was allowed to stir for 2 hours at room temperature. Diethyl ether (30 ml) was added and it was washed with water (2 x 20 ml). The organic fraction was dried overnight with anhydrous $MgSO_4$, filtered and evaporated to give a dark yellow oil. The mixture was purified using column chromatography (mobile phase, ethyl acetate: dichloromethane (1:2)) to yield the desired amine (**6**) as light yellow crystals (0.198 g, 0.6 mmol, 11.31 %).

$C_{18}H_{16}N_2O_4$; TLC: (EtOAc:2DCM): R_f 0.95; **m.p.** 95 °C; **IR (KBr)** ν_{max}: 1726.29, 1598.99, 1556.55, 1373.32, 1101.35; **LC-MS (EI, 70 eV) *m/z***: 333.0(M^+), 317.0, 287.1, 265.9, 259.2; **^{1}H NMR (600 MHz, $CDCl_3$) δ_H**: 7.294 – 7.157 (m, 4H, H-16,17,20,21), 4.883 (t, 1H, J = 15.16 Hz, H-11), 3.238 – 2.711 (m, 9H, H-1,2,3,5,6,7,9,10,11), 2.02 (d, 1H, J = 10.68 Hz, H-4b), 1.648 (d, 1H, J = 10.68 Hz, H-4a). **^{13}C NMR (150 MHz, $CDCl_3$) δ_C**: 135.45 (s, C-18), 128.43 (d, C-16/C-21), 127.53 (d, C-17/C-20), 127.21 (s, C-15), 110.15 (s, C-8), 84.18 (t, C-11), 56.48 (d, C-9), 54.95 (s, C-14), 46.81 (d, C-7), 45.84 (d, C-1), 45.12 (d, C-10), 44.88 (d, C-5), 43.56 (d, C-3), 43.49 (d, C-4), 42.01 (d, C-6), 41.65 (d, C-2).

BIOLOGICAL EVALUATION

2.2.10 STATISTICS

Statistical analysis was performed using GraphPad InStat 2.0 (GraphPad Software, San Diego, CA, USA) and Microsoft Office Excel 2003, SP3. Dunnett's one-way analysis of variance (ANOVA) with post-test was performed on all the tested concentrations, compared to the standard (without test compound) to indicate significant differences (* $p < 0.05$ were considered to be statistically significant and ** $p < 0.01$ were considered to be statistically very significant). Data on the histogram are presented as % values of the reference standard with each bar representing the mean ± S.E.M.

2.2.11 S-NITROSYLATION ASSAY

2.2.11.1 MATERIALS

Cysteine-glycine, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), methyl methanethiolsulfonate (MMTS) (97 %) and neocuproine free base were purchased from Sigma-Aldrich (Steinheim, Germany). EZ-Link® Biotin-HPDP was purchased from Separations (Randburg, South Africa).

2.2.11.2 PROCEDURE

The cysteine-glycine dipeptide (4 mM) was dissolved in HEN buffer (250 mM HEPES, 1 mM EDTA and 0.1 mM neocuproine, adjusted to pH 7.7 with NaOH). To the solution was added an equimolar concentration of the NO donor and the mixture was stirred at room temperature and in the dark for 1 hour. Methyl methanethiosulfonate (MMTS) (200 µl, 20 mM, dissolved in dimethylformamide) was then added and the mixture was incubated at 50°C for 20 minutes with frequent vortexing. Acetone (200 µl) was added and the mixture was vortexed for 10 minutes while the unreacted MMTS precipitated. 500 µl of the solution was removed using a micro pipette and placed in a quarts cuvette in the spectrophotometer. Biotin-HPDP was added and the UV absorbance was measured over 20 minutes with 5 minute intervals.

Before each measurement the spectrophotometer was auto-zeroed with a blank solution. For the blank solution the procedure was followed as above, except for the absence of the cysteine-glycine dipeptide, as it was the only compound in the assay without UV absorbance capability. Each concentration was tested in triplicate. As control, the procedure was carried out as above, but without the addition of the NO donor and MMTS, to allow the cysteine to react completely with the biotin-HPDP and give a representation of 100% nitrosylation. The

control was also auto-zeroed with its own blank solution containing only Hen buffer and Biotin-HPDP.

2.2.12 Ca^{2+} FLUX ASSAY

2.2.12.1 MATERIALS

For the buffer solutions, D(+)glucose, sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), potassium chloride (KCl) and potassium hydrogen phosphate (KH_2PO_4) were obtained from Saarchem (Krugersdorp, S.A). HEPES, anhydrous magnesium chloride, nimodipine and Fura-2/AM were obtained from Sigma-Aldrich (Steinheim, Germany).

2.2.12.2 ANIMALS

Eight day old Sprague-Dawley rats of either sex were used for the calcium flux assay.

2.2.12.3 PREPARATION OF SYNAPTONEUROSOMES

Eight day old Sprague-Dawley rats of either sex were sacrificed by decapitation and whole brains were removed. The brains from four rats were removed and homogenised (four strokes by hand using a Dounce homogenizer) in 30 ml of ice cold Krebs-bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgCl_2$, 1.2 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM glucose). To minimize proteolysis through the isolation procedure, the homogenate was kept ice cold from this step forward. The tissue suspension was placed in a 50 ml polycarbonate tube and centrifuged for 15 minutes at 1 100 x g and 0°C. After centrifugation the pellet was resuspended by hand in 30 ml fresh Krebs-bicarbonate buffer and centrifuged again as described above. The protein concentration was measured spectrofluorimetrically using the Bradford method and Krebs-bicarbonate buffer was added to yield a final concentration of 1.9 mg/ml.

2.2.12.4 PROCEDURE

The suspension described above was allowed to reach room temperature, after which Fura-2/AM was added to a final concentration of 5 µM. The 5 mM Fura-2/AM stock solution was prepared by dissolving 1 mg Fura-2/AM in 2.5 ml dimethyl sulfoxide (DMSO). The synaptoneurosomes were diluted to a final concentration of 0.6 mg/ml using Krebs-bicarbonate buffer (22°C) and incubated at 37°C for 10 minutes. From this step the suspension was kept at room temperature and protected from light (working mostly in a dark room). Immediately before the experiment, 1 ml of the synaptoneurosomal suspension was centrifuged for 10 seconds in a desk Eppendorf Microfuge, resuspended in 3 ml of 37°C Krebs-HEPES buffer (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgCl_2$, 1.2 mM $CaCl_2$, 20 mM

HEPES, 1.2 mM KH_2PO_4 and 10 mM glucose adjusted to pH 7.4 with NaOH). Here HEPES substitutes for $NaHCO_3$ since the latter causes the appearance of bubbles in the cuvette and increases the "noise".

0.01 M, 0.001 M and 0.0001 M concentrations of test and control compound were prepared in DMSO. 1 µl of the stock solution was diluted in 1 ml buffer solution to give 10 µM, 1 µM and 0.1 µM concentrations of the compound. The final concentration DMSO in the incubation was 0.1 %. The different concentrations were incubated for 5 minutes with the synaptoneurosomes and used immediately thereafter.

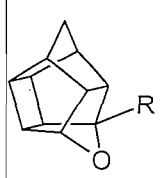
All the experiments were carried out at 37 °C and fluorescence was measured as a kinetics run in a spectrofluorimeter equipped with a water jacked cuvette holder. The spectrofluorimetric parameters are given in table 1.

The reference experiment (with the absence of test compound), comprising of 5 minutes incubation at 37 °C in Krebs-HEPES buffer containing 0.1 % DMSO, followed by the addition of 300 µl depolarisation solution. This served as the standard reference, representing 100% calcium influx.

Each experiment was carried out with two different sets of synaptoneurosomes and three replications of three different concentrations (10 µM, 1 µM and 0.1 µM) of each compound.

3 GRAPHICAL ABSTRACT

A novel series of nitrogen monoxide donating pentacycloundecylamines were synthesised and tested for S-nitrosylation and Ca^{2+} channel activity.



1) R = $NHCH_2CH_2OCOC_6H_4NO_2$

2) R = $NHCH_2CH_2CH_2OCOC_6H_4NO_2$

3) R = $NHCH_2CH_2OCOC_6H_4CHCHNO_2$

4) R = $NHCH_2CH_2ONO_2$

5) R = $NHCH_2CH_2OCOC_6H_4CH_2ONO_2$

6) R = $NCHC_6H_4ONO_2$

4 ACKNOWLAGEMENTS

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5 KEY WORDS

S-nitrosylation, pentacycloundecylamine, nitrogen monoxide, neuroprotective, calcium channel antagonism, excitotoxicity.

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7 LEGENDS FOR FIGURES AND SCHEMES

Table 1: Recording parameters.

Figure 1: The compounds synthesised for this study.

Figure 2: The reductive amination of the Cookson's diketone to yield the corresponding pentacycloundecylamines.

Figure 3: Esterification of the compounds using activation chemistry.

Figure 4: The synthesis of compound **6**.

Figure 5: Histogram displaying the percentage S-nitrosylation brought on by the tested compounds relative to the standard. ** indicates very significant ($p < 0.01$) change from the (100 %) control.

Figure 6: The results of the Ca^{2+} flux assay. The Dunnett t-test was applied to the data, ** indicates a very significant inhibition ($p < 0.01$) and * indicates a significant inhibition ($p < 0.05$).

8 TABLES

TABLE 1

Fura-2/AM	**Recording parameters**
Excitation	340 / 380 nm
Emission	500 nm
Optics position	Bottom
Sensitivity	100
Runtime	40 seconds
Interval	150 milliseconds

9 GRAPHICAL MATERIAL

FIGURE 1

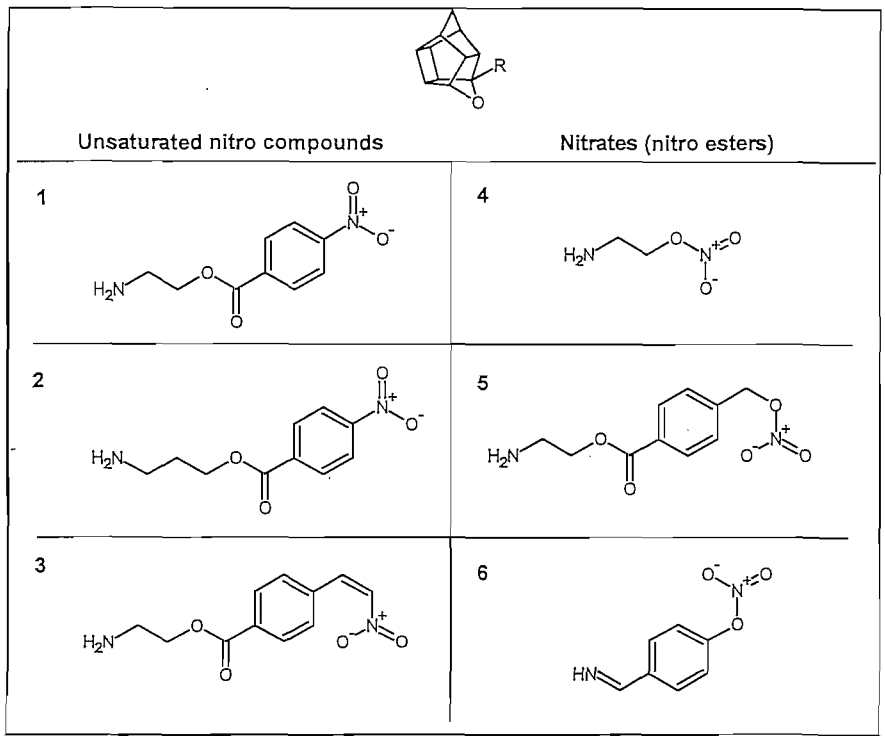
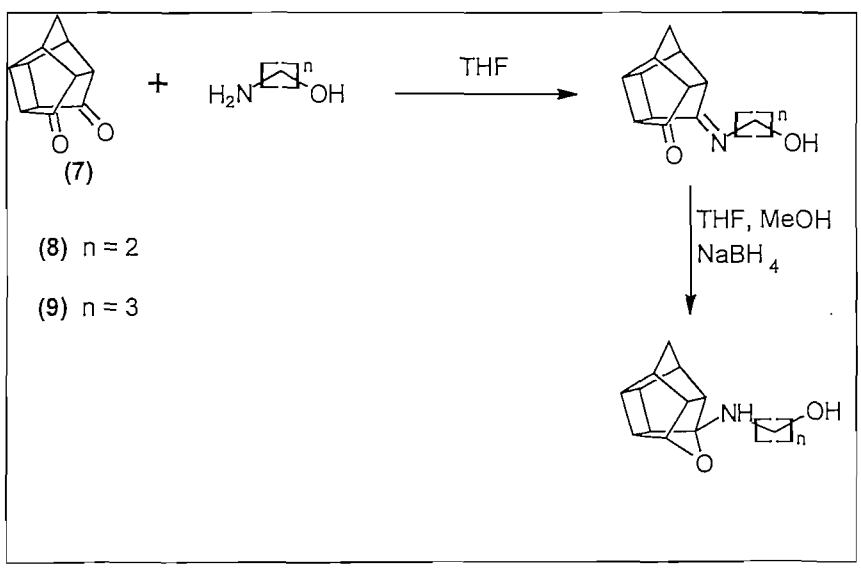


FIGURE 2



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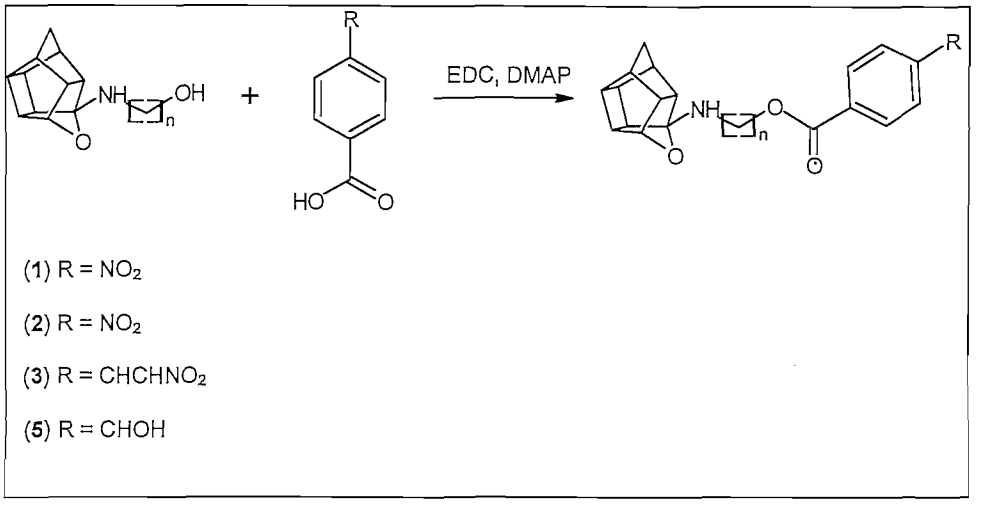


(**1**) R = NO_2

(**2**) R = NO_2

(**3**) R = $CHCHNO_2$

(**5**) R = CHOH

FIGURE 4

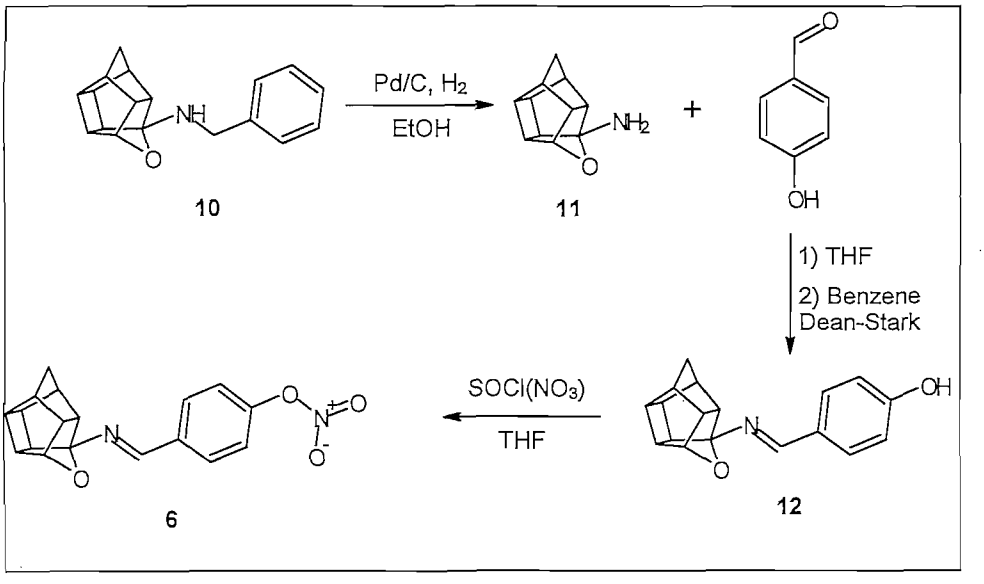


FIGURE 5

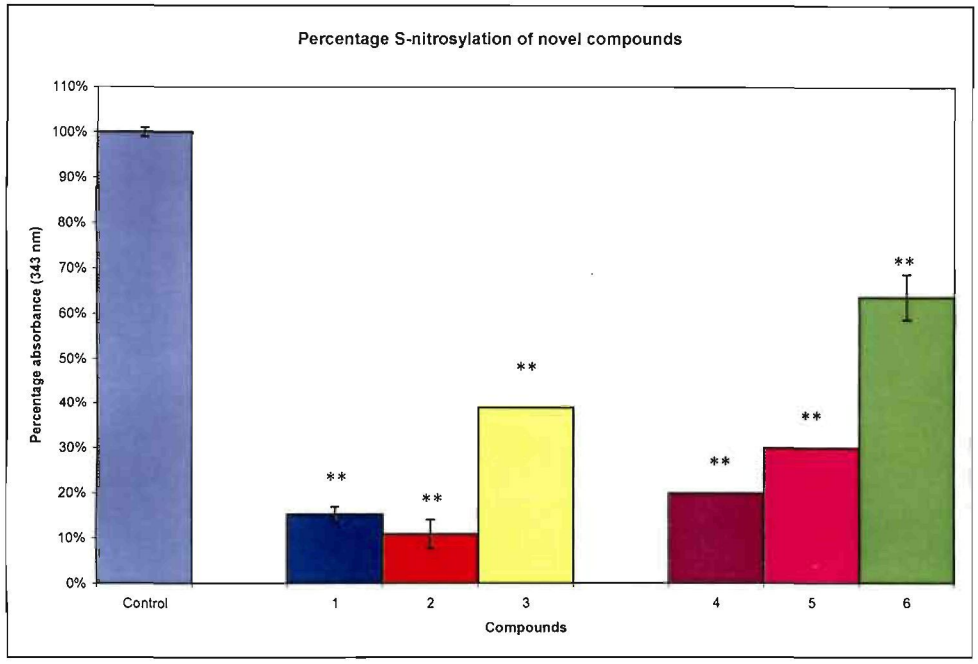
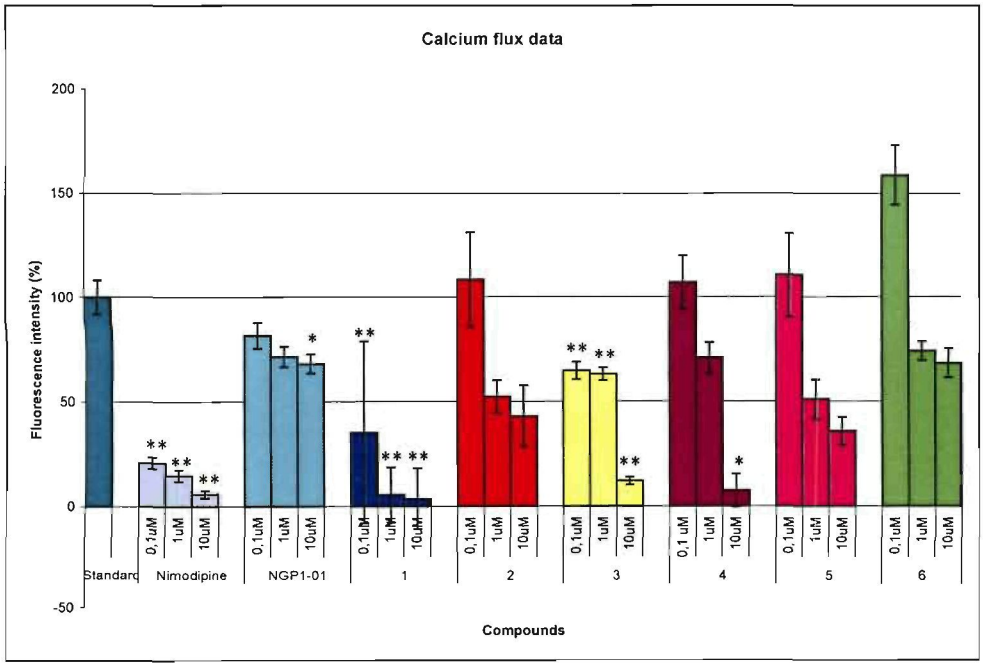


FIGURE 6



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