

**POLYCYCLIC PROPARGYLAMINES
AS
MULTI-FUNCTIONAL
NEURO-PROTECTIVE
AGENTS**



POLYCYCLIC PROPARGYLAMINES AS MULTI-FUNCTIONAL NEURO-PROTECTIVE AGENTS

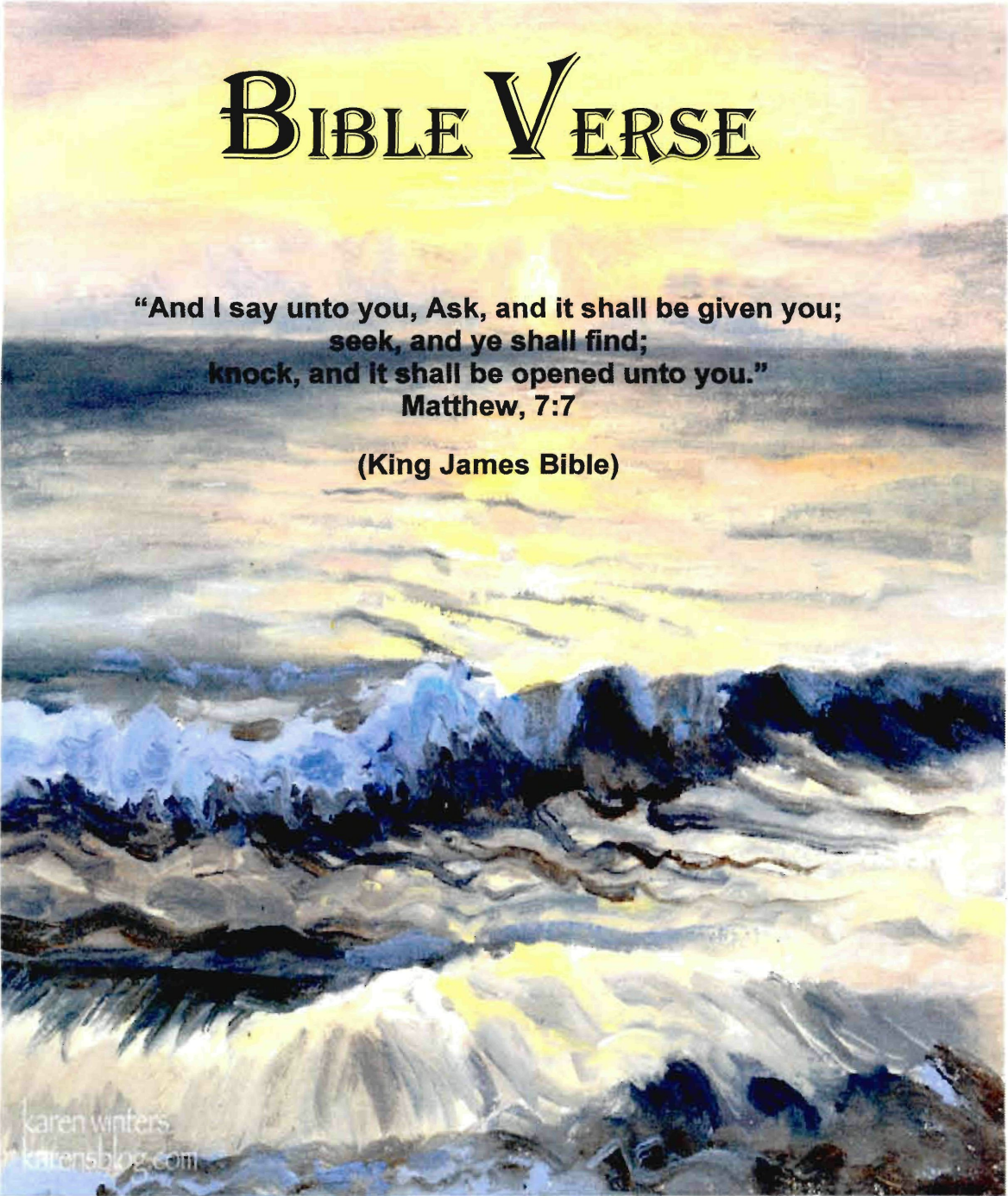
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BIBLE VERSE

**“And I say unto you, Ask, and it shall be given you;
seek, and ye shall find;
knock, and it shall be opened unto you.”
Matthew, 7:7**

(King James Bible)

TABLE OF CONTENTS

ABSTRACT.....	v
UITTREKSEL.....	vii
LIST OF ABBREVIATIONS.....	ix
CHAPTER 1 –	
NEURODEGENERATION: APOPTOSIS AND MONOAMINE OXIDASE B.....	1
1.1 Neurodegenerative diseases.....	1
1.1.1 Parkinson's disease (PD).....	2
i. Definition.....	2
ii. Epidemiology.....	2
iii. Aetiology.....	2
iv. Pathogenesis.....	3
1.1.2 Alzheimer's disease (AD).....	4
i. Definition.....	4
ii. Epidemiology.....	5
iii. Aetiology.....	5
iv. Pathogenesis.....	5
1.2 Apoptosis in Neurodegenerative diseases.....	8
1.2.1 Overview of apoptosis.....	8
i. Growth factors (brain-derived neurotrophic factor and glial cell-line derived neurotrophic factor).....	10
ii. p-53 dependent apoptosis.....	13
iii. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).....	15

iv.	Caspase-3 and poly(ADP-ribose) polymerase (PARP).....	16
v.	Bcl-2 family proteins.....	19
vi.	Protein kinase C (PKC)-pathway/amyloid precursor protein.....	21
vii.	Mitogen-activated protein kinase (MAPK)-pathway.....	24
1.3	Monoamine oxidase B (MAO-B) in neurodegenerative diseases.....	26
1.3.1	Mechanism of action of monoamine oxidase B (MAO-B).....	27
1.3.2	Protective strategies.....	27
1.4	Concluding remarks.....	29
2	CHAPTER 2 - RATIONALE AND SELECTION OF COMPOUNDS.....	30
2.1	Propargylamine.....	30
2.1.1	Introduction.....	30
2.1.2	Neuroprotective properties of propargylamine.....	31
2.2	Polycyclic cage structure compounds.....	32
2.2.1	Introduction.....	32
2.2.2	Biological activity.....	33
2.3	Relevant compounds to be synthesised.....	34
2.3.1	Rationale of compounds to be synthesised.....	37
2.4	Concluding remarks.....	37
3	CHAPTER 3 - SYNTHESIS OF RELEVANT COMPOUNDS.....	38
3.1	Standard experimental procedures.....	38
3.1.1	Reagents and chemicals.....	38
3.1.2	Instrumental methods.....	38
3.1.3	Chromatographic methods.....	39

3.2	Synthetic procedures	39
3.2.1	Synthesis of precursor compounds	39
i.	Pentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane-8-11-dione (1a)	39
ii.	1-Methyl-pentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane-8-11-dione (1b)	40
iii.	Pentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane-8-one (1c)	41
3.2.2	Synthesis of test compounds	42
i.	1-Methyl-8-ethynyl-11-hydroxy-8,11-oxapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane (2)	42
ii.	8-Benzylethynyl-8-hydroxy-pentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane (3)	44
iii.	8-(N)-Propargylamino-8,11-oxapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane (4a)	45
iv.	1-Methyl-8-(N)-propargylamino-8,11-oxapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane (4b)	47
v.	8-Hydroxy-(N)-propargyl-8,11-azapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane (5a)	48
vi.	1-Methyl-11-hydroxy-(N)-propargyl-8,11-azapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane (5b)	50
vii.	N,N-Propargyl-adamantan-1-amine (6)	52
viii.	N-Propargyl-N-benzylmethyl-adamantan-1-amine (7)	53
3.3	Concluding remarks	55
4	CHAPTER 4 –	
	BIOLOGICAL EVALUATION: APOPTOSIS AND MONOAMINE OXIDASE B	56
4.1	Apoptosis detection – DePsipher™ assay	56
4.1.1	Introduction	56
4.1.2	Principle by which DePsipher™ works	57
4.1.3	Biological material	57
i.	Neuroblastoma cells used – SK-N-BE(2)	57
ii.	Cell cultivation	57
iii.	Induction of apoptosis	58

iv.	Preparation of cells to be analysed by flow cytometry.....	58
a)	Cells used to evaluate anti-apoptotic activity of compounds.....	58
b)	Cells used for negative control samples.....	59
v.	Assay procedure.....	60
4.1.4	Data analysis.....	60
i.	Results.....	64
4.1.5	Discussion.....	65
4.2	Monoamine oxidase B inhibition assay.....	67
4.2.1	Introduction.....	67
4.2.2	Preparation of biological material.....	68
4.2.3	Assay procedure.....	68
4.2.4	Data analysis.....	69
i.	Results.....	69
4.2.5	Discussion.....	70
4.3	Concluding remarks.....	71
5	CONCLUSION.....	72
	BIBLIOGRAPHY.....	75
	APPENDIX I.....	104
	ACKNOWLEDGEMENTS.....	125

ABSTRACT

The pathology of neurodegenerative disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD), is caused by the abnormal loss of neuronal cells in certain areas of the brain. It consequently causes an imbalance of certain neurotransmitter levels in the brain, giving rise to the characteristic signs and symptoms of these diseases. Ultimately it compromises the normal functionality and well being of the individual suffering from the disease, thus making it an absolute necessity to create compounds which would halt this neuronal breakdown process, but will also aid in treating the signs and symptoms.

The abnormal death of neurons in the central nervous system of individuals suffering from neurodegenerative diseases, takes place by an intrinsic cell suicide program known as apoptosis. This process is triggered by several stimuli, and consists of numerous pathways and cascades, each one having an influence on the other, ultimately leading to the death of the cells. In PD and AD it has been shown that there are elevated levels of monoamine oxidase B (MAO-B), which not only acts indirectly as a trigger to the apoptotic process, but also gives rise to some of the signs and symptoms of the diseases.

In the current study the approach was to develop multifunctional drugs, which would halt the neuronal breakdown process, but will also eliminate some of the signs and symptoms of diseases such as AD and PD. Keeping this in mind we focused on the structures of rasagiline, pentacyclo-undecane and amantadine. Rasagiline is a well known MAO-B inhibitor with promising neuroprotective activity, which can be attributed to its propargylamine moiety. The pentacyclo-undecane and amantadine polycyclic structures are highly non-polar compounds, with high potential to contribute in the transport of drugs across the blood-brain barrier and across cell membranes into cells. It was thus a rational decision to incorporate the structures of propargylamine and the polycyclic cages into the structures of the synthesised compounds.

In synthesising the test compounds, several experimental procedures and methods, both conventional and modern, were utilised. In most of the synthetic routes either propargylamine, propargylbromide or ethynyl magnesium bromide were utilised together with pentacyclo-undecane or amantadine as reagents. The reaction of these substances afforded the target compounds. Most of the reactions took place without the necessity of a catalysing agent, even though an external source of energy was necessary to provide heat for the reactions to take place. Each compound was synthesised to evaluate the activity and benefit

of the presence of a certain group of atoms in the molecule. Some of these groups include a terminal acetylene group, an acetylene group between two non-polar groups, a secondary propargylamine connected to a polycyclic cage and a tertiary propargylamine in an aza polycyclic structure.

These synthesised compounds were evaluated for anti-apoptotic as well as MAO-B inhibiting activity. The anti-apoptotic activity were evaluated *in vitro* using the Desphiper™ kit, which marks changes in the mitochondrial membrane potential, that takes place during apoptosis. The quantitative and qualitative detection of these processes were done by means of flow cytometry, which made it possible to determine what percentage of the cells in the samples were still viable after treatment with the synthesised compounds. For this purpose SK-N-BE(2) neuroblastoma cells were used, and apoptosis was induced using a serum-deprivation model.

Besides their anti-apoptotic activity, the synthesised compounds were also evaluated *in vitro* as competitive inhibitors of MAO-B using a spectrophotometric assay that utilised MMTP, an analogue of the neurotoxin MPTP as substrate, with baboon liver mitochondria serving as enzyme source. The potency of MAO-B inhibition was expressed as percentage inhibition of the enzyme.

In evaluating the activity of the synthesised compounds, there was one compound, 8-phenylethynyl-8-hydroxy-pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane, that had significant MAO-B inhibiting activity as well as significant anti-apoptotic activity. The other compounds of the series all had moderate to weak anti-apoptotic activity, and showed limited MAO-B inhibition. Having moderate to weak anti-apoptotic activity, these compounds can be used as lead-compounds in the development and design of more potent inhibitors. It was confirmed that when linked to a polycyclic cage structure, propargylamine still displayed potent anti-apoptotic activity. It was also determined that a terminal acetylene group, as well as an acetylene group between two non-polar groups had comparable anti-apoptotic activity. The polycyclic propargylamines thus have potential as neuroprotective agents and further investigation is necessary to determine what their maximum benefit can be in the treatment of neurodegenerative diseases.

UITTREKSEL

Die patologie van neurodegeneratiewe versteurings, soos Parkinson se siekte (PS) en Alzheimer se siekte (AS), word deur abnormale verlies aan neurone in sekere dele van die brein veroorsaak. Dit veroorsaak 'n wanbalans in die vlakke van sekere neurotransmitters in die brein wat oorsprong gee aan die karakteristieke tekens en simptome van hierdie siektes. Uiteindelik tas dit die normale funksionering en welsyn van die individu wat aan die siekte ly aan, en daarom is dit absoluut noodsaaklik om verbindings te ontwikkel wat hierdie afbraakproses van neurone sal stop, asook sal help om die tekens en simptome te behandel.

Die abnormale afsterf van neurone in die sentrale senuwee stelsel van individue wat aan neurodegeneratiewe siektes ly, geskied volgens 'n intrinsieke program van selfmoord van selle, bekend as apoptose. Hierdie proses word deur verskeie stimuli afgesit en bestaan uit talle weë en kaskades wat mekaar beïnvloed en uiteindelik tot die dood van die sel lei. Dit is aangetoon dat daar met PS en AS hoër vlakke monoamienoksidase B (MAO-B) is, wat nie net indirek as sneller vir die proses van apoptose optree nie, maar ook oorsprong gee aan sommige van die tekens en simptome van hierdie siektes.

In die betrokke studie was die benadering om multifunksionele geneesmiddels te ontwikkel wat die neuronale afbraakproses sal stop asook sekere van die tekens en simptome van siektes soos PS en AS sal elimineer. Met dit ingedagte is daar op die strukture van rasagilien, pentasiklo-undekaan en amantadien gefokus. Rasagilien is 'n bekende MAO-B-remmer met belowende neurobeskermende aktiwiteit wat aan die propargielamiendeel daarvan toegeskryf kan word. Die polisikliese pentasiklo-undekaan- en amantadienstrukture is hoogs nie-polêre verbindings, met groot potensiaal om by te dra tot die transport van geneesmiddels oor die bloedsreinskans en oor selmembrane, in selle in. Dit was dus 'n rasonale besluit om die strukture van beide propargielamien en die polisikliese hokkies in die strukture van die gesintetiseerde verbindings te inkorporeer.

Verskeie eksperimentele prosedures en metodes, sowel konvensioneel as modern, is vir die sintese van die teikenverbindings gebruik. In die meeste sinteseroetes is óf propargielamien, óf propargielbromied óf etynielmagnesiumbromied saam met pentasiklo-undekaan of amantadien as reagense geselekteer. Die reaksie van hierdie stowwe het die geselekteerde verbindings gelewer. Die meeste reaksies het sonder 'n katalisator verloop, hoewel 'n eksterne bron van energie nodig was om hitte te verskaf sodat die reaksies kon plaasvind. Die verbindings is gesintetiseer om die aktiwiteit en voordeel van sekere groepe in die

molekuul te beoordeel. Sommige van hierdie groepe behels 'n terminale asetileengroep, 'n asetileengroep tussen twee nie-polêre groepe, 'n sekondêre propargielamien verbind aan 'n polisikliese hokkie en 'n tersiêre propargielamien in 'n asapolisikliese struktuur.

Hierdie gesintetiseerde verbindings is getoets vir hulle aktiwiteit teen apoptose asook remming van MAO-B. Die anti-apoptotiese aktiwiteit is *in vitro* bepaal deur die DesphiperTM-stel te gebruik, wat veranderinge in die mitochondriële membraanpotensiaal wat tydens apoptose voorkom merk. Die kwantitatiewe en kwalitatiewe opsporing van hierdie prosesse is deur middel van vloeisitometrie gedoen, wat kon bepaal watter persentasie selle in die monsters steeds lewendig was na behandeling met die gesintetiseerde verbindings. Vir hierdie doel was SK-N-BE(2)-neuroblastoomselle gebruik, waarin apoptose geïnduseer is deur gebruik te maak van die serumdeprivasiemodel.

Benewens hulle anti-apoptotiese aktiwiteit is die gesintetiseerde verbindings ook *in vitro* getoets as kompeterende remmers van MAO-B, deur gebruik te maak van 'n spektrofotometriese bepaling met MMTP, 'n analoog van die neurotoksien MPTP, as substraat, en lewerweefsel van bobbejane as bron van ensiem. Die mate van MAO-B-remming is uitgedruk as persentasie inhibisie van die ensiem.

Een van die gesintetiseerde verbindings, 8-fenieletyniel-8-hidroksie-pentasiklo [5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undekaan het beduidende aktiwiteit getoon as MAO-B-remmer asook anti-apoptotiese middel. Die ander verbindings in die reeks het almal matig tot swak anti-apoptotiese aktiwiteit getoon, met beperkte MAO-B-remming. Hierdie verbindings kan egter verder gebruik word as leidraad verbindings in die ontwikkeling en ontwerp van meer potente inhibeerders. Dit is bevestig dat as dit aan 'n polisikliese hokkiestruktuur gekoppel is, propargielamien steeds sterk anti-apoptotiese aktiwiteit uitoefen. Dit is ook vasgestel dat 'n terminale asetileengroep, asook 'n asetileengroep tussen twee nie-polêre groepe, vergelykbare anti-apoptotiese aktiwiteit het. Die polisikliese propargielamiene het dus potensiaal as neurobeskermende middels en verdere ondersoek is nodig om te bepaal wat hulle maksimum voordeel vir die behandeling van neurodegeneratiewe siektes kan wees.

LIST OF ABBREVIATIONS

6-OHDA	-	6-hydroxydopamine
AD	-	Alzheimer's disease
apo	-	apo-lipoprotein
APP	-	amyloid precursor protein
ATP	-	adenosine triphosphate
Aβ	-	β -Amyloid
BACE	-	beta-site APP-cleaving enzyme
Bak	-	bcl-2 homologous antagonist/killer
Bax	-	bcl-2-associated X protein
Bcl-2	-	basal cell lymphoma-2
Bcl-xL	-	basal cell lymphoma-extra large
BDNF	-	brain-derived neurotrophic factor
CAD	-	caspase activated DNase
CAG	-	cytosine adenine guanine
CNS	-	central nervous system
DA	-	dopamine
DFF-40	-	DNA Fragmentation Factor-40
DFF-45	-	DNA Fragmentation Factor-45
DNA	-	deoxyribonucleic acid
ER	-	endoplasmic reticulum
ERK	-	extra cellular signal-regulated kinases
FAS	-	apoptosis stimulating fragment
FBS	-	foetal bovine serum
FZ	-	fungizone
GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase
GDNF	-	glial cell line-derived neurotrophic factor
GFR	-	growth factor receptor
GMP	-	cyclic guanosine 5'-monophosphate
GRB	-	growth factor receptor-bound
H₂O₂	-	Hydrogen peroxide
ICAD	-	inhibitor of caspase activated DNase
JNK	-	c-Jun amino-terminal kinases
L-DOPA	-	levodopa
MAO	-	monoamine oxidase

MAO-A	-	monoamine oxidase A
MAO-B	-	monoamine oxidase B
MAPK	-	mitogen-activated protein kinase
MFI	-	mean fluorescence intensity
MP	-	mononuclear phagocytes
MPP⁺	-	1-methyl-4-phenylpyridinium
NADPH	-	nicotinamide adenosine dinucleotide phosphate
NF	-	neurotrophic factor/neurotrophin
NFT	-	neurofibrillary tangles
NF-κB	-	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	-	N-methyl-D-aspartic acid
NO	-	nitric oxide
NOS	-	nitric oxide synthase
OXPHOS	-	oxidative phosphorylation
p75	-	low affinity neurotrophin receptor
PARP	-	poly(ADP-ribose) polymerase
PC12	-	pheochromocytoma cell line 12 culture cells
PCP	-	phencyclidine
PD	-	Parkinson's disease
PEA	-	phenylethylamine
Pen/Strep	-	penicillin/streptomycin
PKC	-	protein kinase C
PTP	-	permeability transition pores
ROS	-	reactive oxygen species
RT	-	room temperature
sAPPα	-	secreted amyloid precursor protein alpha
SEM	-	standard error of mean
SOD	-	super oxide dismutase
SOS	-	Son of Sevenless
TLC	-	thin layer chromatography
Trk	-	tyrosine kinase receptor
UV	-	ultraviolet
$\Delta\Psi_m$	-	electrochemical gradient across the mitochondrial membrane

CHAPTER 1

NEURODEGENERATION: APOPTOSIS AND MONOAMINE OXIDASE B

1 Neurodegenerative diseases

Neuronal loss plays an important role in the normal development of a functional integrated nervous system (Oppenheim, 1991). Initially there is an excess of neuronal cells in the nervous system, which take part in a competitive survival process, with only those neurons that are functionally, temporally, and spatially correct surviving (Cowan *et al.*, 1984). Under normal conditions most of the surviving neurons stay viable and functional throughout the lifetime of an individual (Mattson, 2000), whereas the neurons that do not survive the competition die through apoptosis, an intrinsic cell suicide program (Holbrook *et al.*, 1996).

This intrinsic cell suicide program involves the induction of specific proteins, such as p53, and activation of degrading enzymes, such as endonucleases. What follows is a characteristic pattern that involves rapid nuclear collapse and DNA destruction. Although apoptosis during development is a beneficial process, its occurrence in the mature brain is harmful, and leads to a decrease in the number of functional neurons, which can not be replenished by cell division (Holbrook *et al.*, 1996).

The inappropriate activation of apoptosis in neuronal cells can be a result of their high metabolic rates and free radicals that are produced as a normal part of cell metabolism. As the processes of aging or disease impair mitochondrial activity, modify DNA and induce protein modifications, the equilibrium between survival and apoptosis shifts toward apoptosis. Neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, may mimic an accelerated aging process by increasing the levels of free radicals or disruption of calcium homeostasis (Holbrook *et al.*, 1996), and thereby lead to neuronal cell death through apoptosis.

Death of neurons in different anatomic parts of the brain give rise to the particular symptoms associated with these neurodegenerative diseases, with hippocampal and cortical neurons dying in Alzheimer's disease (AD) and midbrain dopaminergic neurons dying in Parkinson's disease (PD). The prevalence of neurodegenerative disorders is rapidly increasing as average lifespan increases (Mattson, 2000).

1.1 Parkinson's disease (PD)

1.1.1 Definition

Parkinson's disease (PD) is an idiopathic, slowly progressive, degenerative central nervous system (CNS) disorder, characterised by slow and decreased movement, muscular rigidity, resting tremor, and postural instability. These symptoms are a result of pigmented neuronal loss of the substantia nigra, locus ceruleus, and other brain stem dopaminergic cell groups (Beers *et al.*, 2006), which give rise to dopamine level depletion in the caudate nucleus and putamen, and results in reduction of cortical activation (Wells *et al.*, 2003). Contributing to the tremor of PD, there is also a relative elevation of striatal cholinergic interneuron activity (Wells *et al.*, 2003), which makes it clear that there is an imbalance of dopamine and acetylcholine neurotransmitter levels in the corpus striatum (Tierney *et al.*, 2006). Apart from dopaminergic neuronal loss in PD brains, development of neurofibrillary structures, termed Lewy bodies, also occur (Holbrook *et al.*, 1996).

1.1.2 Epidemiology

PD is the second most common neurodegenerative disease, with an overall prevalence of 0.1% in the total population (Bahr, 2004). It affects about 1% of people over 65 years of age and 0.4% of those above 40 years. PD rarely begins in childhood or adolescence (juvenile Parkinsonism), with a mean age of onset being 57 years (Beers *et al.*, 2006).

1.1.3 Aetiology

Until recently, primary Parkinsonism was described as an idiopathic disease, and although the cause of the disease still remains largely unidentified, emerging evidence suggests that multiple factors, both genetic and acquired (exposure to environmental factors) contribute to the neurodegeneration of the dopaminergic cells (Bahr, 2004). Evidence that dopaminergic neurons die by an apoptotic process in PD is accumulating, with analysis of post mortem brain tissue from PD patients revealing evidence for neuronal apoptosis. This evidence includes nuclear condensation, chromatin fragmentation and formation of apoptotic bodies (Mochizuki *et al.*, 1996; Tompkins *et al.*, 1997; Tatton *et al.*, 1998). The causes of the neuronal death are likely to involve age-related increased oxidative stress and mitochondrial dysfunction in dopaminergic neurons, as a result of sensitisation by environmental and genetic factors (Jenner & Olanow, 1998; Polymeropoulos, 1998).

Secondary Parkinsonism results from loss of or interference with dopamine's action in the basal ganglia due to other degenerative disorders, drugs or exogenous toxins (Beers *et al.*, 2006).

1.1.4 Pathogenesis

The pathological hallmarks of Parkinson's disease (PD) are a loss of dopaminergic neurons in the mesencephalon and the presence of Lewy bodies in altered neurons.

Genetic factors playing a role in the pathogenesis include Par-4 and α -synuclein. Par-4 levels are selectively increased in the substantia nigra dopaminergic neurons prior to their death, with suppression of Par-4 expression protecting dopaminergic neurons against death (Duan *et al.*, 1999a). The protein, α -synuclein, is a major component of the PD brain lesions called Lewy bodies, and mutations in α -synuclein are responsible for a small percentage of PD cases, with the expression of mutant α -synuclein in cultured cells promoting apoptosis (el-Agnaf *et al.*, 1998).

Biochemical abnormalities in PD substantia nigra include:

- (a) Abnormal iron accumulation, and alteration in the concentration of iron-binding proteins (Mattson, 2001);

Iron is capable of functioning as a catalysing agent in oxidative reactions that may generate hydrogen peroxide as well as hydroxyl ions. Thus, if iron is available in a free and reactive form, it has the potential for exacerbating oxidative stress and damage (Mattson, 2001). There is a 35% increase in substantia nigra iron levels in PD (Dexter *et al.*, 1987; Sofic *et al.*, 1991), which is a reflection of neuronal cell loss rather than any specific pathogenetic factor (Mattson, 2001). Abnormally high concentrations of iron can also be found in macrophages, astrocytes, and reactive microglia in the PD substantia nigra (Jellinger *et al.*, 1990).

- (b) Increased oxidative stress and oxidative damage (Mattson, 2001);

Based on recent evidence it appears that there are increased levels of oxidative stress and oxidative damage to bio-molecules in PD substantia nigra. These results suggests:

- ❖ Enhanced free-radical generation in the PD nigra;
- ❖ Increased super oxide generation in PD nigral neurons, with the super oxide dismutase (SOD) levels being the indicative factor of super oxide generation. In PD there appears to be increased levels of copper/zinc and manganese SOD (Marttila *et al.*, 1988a; Saggi *et al.*, 1989);
- ❖ Increased levels of poly-unsaturated fatty acids (Dexter *et al.*, 1986), malondialdehyde and hydro-peroxides (Jenner, 1991) in PD substantia nigra, which are the products of free-radical damage to lipid membranes (Mattson, 2001).

(c) Mitochondrial complex I deficiency (Mattson, 2001);

Through oxidative phosphorylation (OXPHOS) the mitochondria is responsible for producing adenosine triphosphate (ATP). OXPHOS also produces 95% of the cell's super oxide ions during aerobic metabolism (Mattson, 2001). In the PD nigra there is approximately a 35% deficiency in complex I (Schapira *et al.*, 1990). Defects in complex I activity may generate increased super oxide ions that may enhance oxidative stress and oxidative damage in the presence of elevated concentrations of iron (Mattson, 2001).

(d) Increased nitric oxide (NO) formation (Mattson, 2001) and

The free radical gas NO•, which is generated by the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS), is present in many tissues, including the CNS. It acts as an atypical molecular messenger, which may have a toxic role, and has been implicated in the neurodegeneration that occurs in PD. As a free radical, NO• could potentially contribute to dopaminergic neuronal death by mechanisms such as increased lipid per-oxidation, release of iron (II), and damage to DNA. It is also an inhibitor of numerous enzymes such as cytochrome c oxidase and SOD (Itzhak & Ali, 1996). In non-human primates and humans, NOS activity is at its highest in the nigrostriatal system (Kuiper *et al.*, 1994; Molina *et al.*, 1994, 1996; Qureshi *et al.*, 1995).

(e) The generation of nitro tyrosine residues within PD substantia nigra (Mattson, 2001).

The PD brains that become available for analysis are inevitably at the end stage of the disease process when the majority of the nigral neurons have already disappeared and gliosis is often widespread. For this reason it is important to distinguish between biochemical abnormalities and those that might be a consequence of post-mortem changes (Mattson, 2001).

1.2 Alzheimer's disease (AD)

1.2.1 Definition

Alzheimer's disease is a progressive dementia affecting both cognition and behaviour with no known cause or cure (Wells *et al.*, 2003), and is characterised by senile plaques, β -amyloid deposits, and neurofibrillary tangles in the cerebral cortex and sub cortical grey matter (Beers *et al.*, 2006).

1.2.2 Epidemiology

AD is the most common cause of dementia, and accounts for more than 65% of dementias in the elderly. The disease is twice as common amongst women as amongst men, partly because women have a longer life expectancy. It affects about 4% of people aged 65 to 74 and 30% of those older than 85. Prevalence in industrialised countries is expected to increase as the proportion of the elderly increases (Beers *et al.*, 2006).

1.2.3 Aetiology

Most cases of AD are sporadic, with late onset (over 60 years) and unclear aetiology. About 5-15% of AD cases are familial, with half of these having an early onset (younger than 60 years), and is typically related to specific genetic mutations (Beers *et al.*, 2006).

Dementia associated with the more advanced stages of AD is believed to be caused by neuronal degeneration in cognition-related brain regions. In the AD brain, large-scale cell death of mature neurons is a pathologic process that remains unsolved. Apoptosis and cell cycle re-entry are amongst the new types of cell death processes that have been proposed for the neuronal loss, wherein β -amyloid ($A\beta$) can be a driving force. The amyloid protein induces apoptosis through oxidative stress while also driving cell division and cell death in cultured neurons (Copani *et al.*, 1999). Evidence for DNA fragmentation, expression of apoptosis-related genes, and caspase activation support an apoptotic mechanism in AD neurodegeneration (Olariu *et al.*, 2005).

In PD there is a subset of patients who develop a dementia, termed sub-cortical dementia, which shows cortical pathology in the form of neuritic plaques (Holbrook *et al.*, 1996).

1.2.4 Pathogenesis

In AD the neurons in the cortex and limbic structures of the brain responsible for higher learning, memory, reasoning, behaviour and emotional control are degenerated (Wells *et al.*, 2003). Synapses and neurons in brain regions that serve learning and memory functions include the hippocampus, entorhinal cortex, basal forebrain and neocortical association cortices (DeKosky *et al.*, 1996). Cholinergic pathways, especially a large system of neurons located at the base of the forebrain in the nucleus basalis of Meynert as well as serotonergic neurons of the raphe nuclei and noradrenergic cells of the locus ceruleus are profoundly damaged and monoamine oxidase type B (MAO-B) activity is increased (Wells *et al.*, 2003).

The extra cellular β -amyloid deposits, intracellular neurofibrillary tangles, and extra cellular senile plaques/neuritic plaques that develop in AD, lead to neuronal loss (Beers *et al.*, 2006), and their presence is necessary for AD to occur (Wells *et al.*, 2003).

The progressive impairment of cognition and emotional disturbances that occur in AD result from degeneration of synapses and death of neurons in limbic structures such as the hippocampus and amygdale, and associated regions of the cerebral cortex. The damaged neurons exhibit aggregates of hyperphosphorylated tau protein and evidence of excessive Ca^{2+} -mediated proteolysis and oxidative stress (Yankner, 1996; Mattson, 1997). In AD there is abnormally increased levels of the tau protein (a component of neurofibrillary tangles and β -amyloid) in the brain and cerebrospinal fluid (CSF), and reduced levels of choline acetyltransferase. The latter plays an important role in the synthesis of acetylcholine (Beers *et al.*, 2006).

The mitochondrial function of brain cells in AD patients is compromised (Mattson, 2001) with increased levels of cellular oxidative stress in vulnerable regions of the AD brain (Bruce *et al.*, 1997; Moccoci *et al.*, 1994; Smith *et al.*, 1991). There is increased protein oxidation, protein nitration, and lipid per-oxidation in neurofibrillary tangles (NFT's) and neuritic plaques (NP's) (Good *et al.*, 1996; Smith *et al.*, 1997). Other alterations include membrane depolarisation, increased levels of mitochondrial oxyradicals, and membrane permeability transition that are commonly present in cells undergoing apoptosis (Mattson, 2001).

Expression of brain-derived neurotrophic factor (BDNF) and its high-affinity receptor, tyrosine kinase receptor B (trkB), are selectively decreased in the frontal cortex and hippocampus of AD patients (Ferrer *et al.*, 1999), and could possibly contribute to the neurodegenerative process in AD (Mattson, 2001).

Exposure of synaptosomes or intact synaptically connected neurons to $\text{A}\beta$ and related oxidative insults, result in caspase activation (which is increased in degenerating neurons and neuritis (Chan *et al.*, 1999)), loss of plasma membrane phospholipid asymmetry, increased Par-4 levels, mitochondrial calcium uptake, and release of factors capable of inducing nuclear chromatin condensation and fragmentation into the cytosol (Mattson *et al.*, 1998b, 1998c; Duan *et al.*, 1999b). Intraneuronal accumulation of $\text{A}\beta$ occurs in normal aging without deposition of $\text{A}\beta$ in amyloid plaques (Naslund *et al.*, 1994), whereas it is deposited extra cellular in the AD brain (Olariu *et al.*, 2005). Increased $\text{A}\beta$ levels lead to deposition and fibrillary aggregation thereof (Beers *et al.*, 2006). It can induce apoptosis directly (Loo *et al.*, 1993; Mark *et al.*, 1995) and can greatly increase neuronal vulnerability to death (Mattson, 1997) and excitotoxicity (Mattson *et al.*, 1992). It may lead to neuronal death and formation

of the neurofibrillary tangles and senile plaques, which consist of degenerated axonal or dendritic processes, astrocytes, and glial cells around an amyloid core (Beers *et al.*, 2006). Disordered glial immunity also plays a key role in the pathogenesis of Alzheimer's disease (AD) (McGeer & McGeer, 1999), with A β being able to activate super oxide forming nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase in mononuclear phagocytes (MP), resulting in H₂O₂ production (Bianca *et al.*, 1999). sAPP α and A β synergistically activate MP, in the absence of pro-inflammatory cytokines, with glutamate secretion and reactive oxygen species (ROS) production as a result (Ikezu *et al.*, 2003).

Experiments on isolated neurons have provided evidence that alterations in proteolytic processing of amyloid precursor protein (APP) may play a major role in the increased levels of oxidative stress in neurons in AD (Mattson, 1997). APP mutations may cause AD by altering proteolytic processing of APP, such that levels of A β are increased and levels of the secreted form of APP (sAPP α) are decreased (Mattson, 1997; Lannfelt *et al.*, 1995; Furukawa *et al.*, 1996b). Due to APP mutations in AD, soluble/secreted Amyloid Precursor Protein alpha (sAPP α) levels are decreased with the neuroprotective actions thereof not being reduced. sAPP α increases the resistance of neurons to oxidative injury induced by Fe²⁺ and A β (Furukawa *et al.*, 1996b). It also induces an increase in the basal level of glucose transport, and attenuates oxidative impairment of glucose transport in cortical synaptosomes (Mattson *et al.*, 1999a). A signal transduction pathway that mediates the neuroprotective effects of sAPP α has been elucidated and involves cyclic guanosine-5-monophosphate (GMP) production, activation of potassium channels (Furukawa *et al.*, 1996a), and activation of the transcription factor NF- κ B (Barger & Mattson, 1996), which has been shown to protect neurons against apoptosis and excitotoxicity in several different cell culture and *in vivo* models (Barger *et al.*, 1995; Mattson *et al.*, 1997; Yu *et al.*, 1999).

In addition to APP, two proteins called presenilin-1 and presenilin-2, can harbour mutations that cause early onset AD (Hardy, 1997; Mattson *et al.*, 1998a), by altering Ca²⁺ homeostasis in such a way that A β production is increased and neurons are made vulnerable to apoptosis and excitotoxicity (Mattson, 2001). Some of these mutations lead to increased production of β -amyloid peptides, which contribute to the pathology of AD (Olariu *et al.*, 2005). Other genetic determinants which influence β -amyloid deposition, cytoskeletal integrity, and efficiency of neuronal repair include the apo-lipoprotein (apo) E alleles (ϵ) (Beers *et al.*, 2006).

Protein kinase C (PKC) is a key signal transduction system that plays an important role in the production of A β and generally declines with aging. Not all aged individuals develop AD

although aging seems to be a prerequisite of AD. It can be hypothesised that in aging in the presence of high risk AD factors, PKC deficiency would imbalance the APP α -processing towards a β - and/or γ -processing with generation of soluble A β . Gradual elevation of soluble A β will initially activate PKC and related downstream pathways, while constant high levels of A β , as in the late stage of AD, will down regulate PKC and dampen the PKC-related intracellular pathways (Olariu *et al.*, 2005). In AD brain tissue, there also exists increased mitogen-activated protein kinase (MAPK) activity, an intracellular enzyme located downstream to PKC that could be partly responsible for the generation of neurofibrillary tangles (NFT) (Swatton *et al.*, 2004).

2 Apoptosis in neurodegenerative diseases

2.1 Overview of apoptosis

Apoptosis, otherwise known as type I cell death (Schweichel & Merker, 1973), is a genetically encoded, ubiquitous pathway enabling cells to undergo highly regulated death in response to pro-death signalling (Wyllie *et al.*, 1980). It describes the morphology of cells disappearing in a non-inflammatory manner (Kerr *et al.*, 1972), and is a form of programmed cell death that involves a stereotyped sequence of biochemical and morphological changes (Mattson, 2001). Death by apoptosis often occurs as part of normal development of homeostasis (Holbrook *et al.*, 1996).

Apoptosis is characterised by a number of unique distinguishing features, including cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, intra-nucleosomal DNA fragmentation, phosphatidylserine exposure and, finally, fragmentation into membrane-enclosed apoptotic bodies sequestered by macrophages or other engulfing cells (Wyllie *et al.*, 1980). These cellular remains that are removed by phagocytosis do not invoke an inflammatory response (Holbrook *et al.*, 1996).

It is triggered by a variety of stimuli that cause susceptible cells to execute the apoptotic program. Such triggers include:

(a) Neurotrophic factor deprivation:

Neurotrophic factor support is an intensively studied neuronal death signal, and lack thereof may trigger apoptosis during development of the nervous system and in neurodegenerative disorders (Mattson & Lindvall, 1997).

(b) Excitotoxicity:

The second most prominent trigger of neuronal apoptosis is the activation of glutamate receptors, of which glutamate is an excitatory amino acid neurotransmitter. Calcium influx through ionotropic glutamate receptor channels and voltage-dependent calcium channels mediates glutamate-induced neuronal apoptosis and necrosis (Ankarcrona *et al.*, 1995; Glazner *et al.*, 2000). Such “excitotoxicity” may occur in AD, PD, HD and ALS (Wong *et al.*, 1998; Mattson *et al.*, 1999b). Over activation of glutamate receptors under conditions of reduced energy availability or increased oxidative stress, results in Ca^{2+} influx into postsynaptic regions of dendrites. Ca^{2+} entering the cytoplasm through plasma membrane channels and endoplasmic reticulum (ER) channels induces apoptotic cascades that involve Par-4, pro-apoptotic Bcl-2 family members (Bax and Bad), and/or p53. These factors act on mitochondria to induce Ca^{2+} influx, oxidative stress, opening of permeability transition pores (PTP), and release of cytochrome c. This results in caspase activation and execution of the cell-death process (Mattson, 2001).

(c) Oxidative stress:

Oxidative stress (in which free radicals such as super oxide anion radicals and hydroxyl radicals damage cellular lipids, proteins, and nucleic acids by attacking chemical bonds in those molecules) is a very important trigger of neuronal death in neurodegenerative disorders (Mattson, 1998; Mattson, 2001; Sastry & Rao, 2000). Because the signals for apoptosis involve the same molecules that are produced during oxidative stress, it is increasingly evident that oxidative stress is a common inducer of apoptosis. Many of the agents that can induce apoptosis are oxidants or stimulate the production of free radicals through cellular metabolism (Holbrook *et al.*, 1996). Calcium and free radicals are able to induce proteins that are involved in apoptotic pathways, such as p53 (Holbrook *et al.*, 1996). Free radical-induced apoptosis especially, is dependent upon expression of functional p53 protein (Holbrook *et al.*, 1996). Even though p53 functions primarily as a trigger for apoptosis (e.g. Yonish-Rouach *et al.*, 1991), it does not participate in the execution phase of apoptosis, with apoptosis sometimes occurring in the absence of p53 (Holbrook *et al.*, 1996).

(d) Reduced energy availability & DNA damage:

Reduced energy availability to neurons (Beal, 1995; Bruce-Keller *et al.*, 1999; Duan *et al.*, 1999c), as well as DNA damage (Holbrook *et al.*, 1996) may also initiate neuronal apoptosis.

After being triggered, the process of apoptosis is mediated by specific biochemical cascades involving mitochondrial changes and activation of proteases called caspases. It provides a mechanism for cells to die without adversely affecting their neighbours (Wyllie, 1997). The process of apoptosis is very complex and involves several pathways, of which only two have been identified: A pathway that is directly activated by death receptors and a pathway that involves the mitochondria (Lei *et al.*, 2003). In the current study the focus will be on the mitochondrial pathway.

The different triggers explained give rise to certain events, which eventually lead to mitochondrial changes, which are central to the apoptotic process and ultimately leads to cell death, with the mitochondrion being the final controller of the cell death decision (Kroemer *et al.*, 1998). These changes that occur in mitochondria of cells undergoing apoptosis include increased oxyradical production, opening of pores in their membranes, and release of cytochrome c (Keller *et al.*, 1998; Matsumoto *et al.*, 1999). The events that occur upstream of the mitochondrial changes are complex and involve interaction of several types of proteins, such as Bcl-2, Par-4, caspases and telomerase. The signalling pathways that initiate or prevent apoptosis are highly concentrated in synaptic terminals, which are major sites of intercellular communication between neurons (Mattson, 2001).

The neuroprotective activity of the compounds studied, may contribute to inhibition of the mitochondrial pathway leading to apoptosis. For this purpose it is necessary to have a basic understanding of the different components, which form part of this pathway. These components will be explained in the following sections.

2.1.1 Growth factors

Neurotrophins (also called "neurotrophic factors") are a family of protein growth factors that control the survival of neurons. They are secreted proteins, usually found in the blood stream, that signal particular cells to survive, or differentiate, or grow. Neurotrophic factors are secreted by target tissue, and act by prohibiting the neurons from initiating apoptosis - thus signalling the neurons to survive. Neurotrophins also induce differentiation of progenitor cells, which act as precursor cells, to form neurons (Hempstead, 2006; Reichardt, 2006; Allen & Dawbarn, 2006).

There are two classes of growth factor receptors (GFR) namely nerve growth factor receptor/low-affinity neurotrophin receptor (p75) and the tyrosine kinase receptor ("Trk") family. p75 is a low affinity neurotrophin receptor, to which all neurotrophins bind, whereas the Trk family include TrkA, TrkB, and TrkC, and will only bind with specific neurotrophins,

but with a much higher affinity. The Trks mediate the functional signals of the neurotrophins (Arevalo & Wu, 2006).

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor found in the brain, but also in the periphery. More specifically, it is a protein that has activity on certain neurons of the central nervous system and the peripheral nervous system - it helps to support the survival of existing neurons, and encourage the growth and differentiation of new neurons and synapses through axonal and dendritic sprouting (Acheson *et al.*, 1995; Huang & Reichardt, 2001). In the brain, it is active in the hippocampus, cortex, cerebellum, and basal forebrain areas vital to learning, memory, and higher thinking (Yamada & Nabeshima, 2003), which makes it a neurotrophin that plays a vital role in the pathogenesis of AD. BDNF stimulates production of antioxidant enzymes, which may account, in part, for its ability to protect neurons against oxidative and metabolic insults relevant to the pathogenesis of AD and PD (Cheng & Mattson, 1994; Frim *et al.*, 1994). In addition to suppressing oxidative stress, it can enhance neuronal calcium homeostasis by modulating the expression and/or function of glutamate receptors, ion-motive ATPases, and calcium-binding proteins. BDNF may also induce production of anti-apoptotic proteins such as Bcl-2 (Allsopp *et al.*, 1995; Furukawa *et al.*, 1997), and binds to the TrkB receptor, with TrkB mediating the multiple effects, which includes neuronal differentiation and survival (Huang & Reichardt, 2001; Patapoutian & Reichardt, 2001).

Glial cell-line derived neurotrophic factor (GDNF) is a small protein that potently promotes the survival of many types of neurons. The most prominent feature of GDNF is its ability to support the survival of dopaminergic and motor neurons, which are of therapeutic importance in Parkinson's disease (Carnicella *et al.*, 2008; Arevalo & Wu, 2006). GDNF also signals through the tyrosine kinase receptor, with GFR α 1 being the GDNF-family-receptor (Arevalo & Wu, 2006).

The pathway, by which GDNF and BDNF act, is explained in figure 1.1. The specific neurotrophic factor (NF), binds to a growth factor receptor (GFR), which transmits activating signals to the Raf/MEK/ERK cascade through Ras. After binding to the GFR, the Growth factor receptor-bound (GRB) complex activates the Ras-activation guanine nucleotide exchange factor, Son of Sevenless (SOS), which activates Ras. This gives way to a cascade of phosphorylation and activation reactions, ultimately leading to the raf/MEK/ERK pathway and finally the functional neuroprotective effects of the neurotrophic factors (fig. 1.1). In section 2.1.7 a detailed explanation of the mitogen-activated protein kinase (MAPK) – pathway is given.

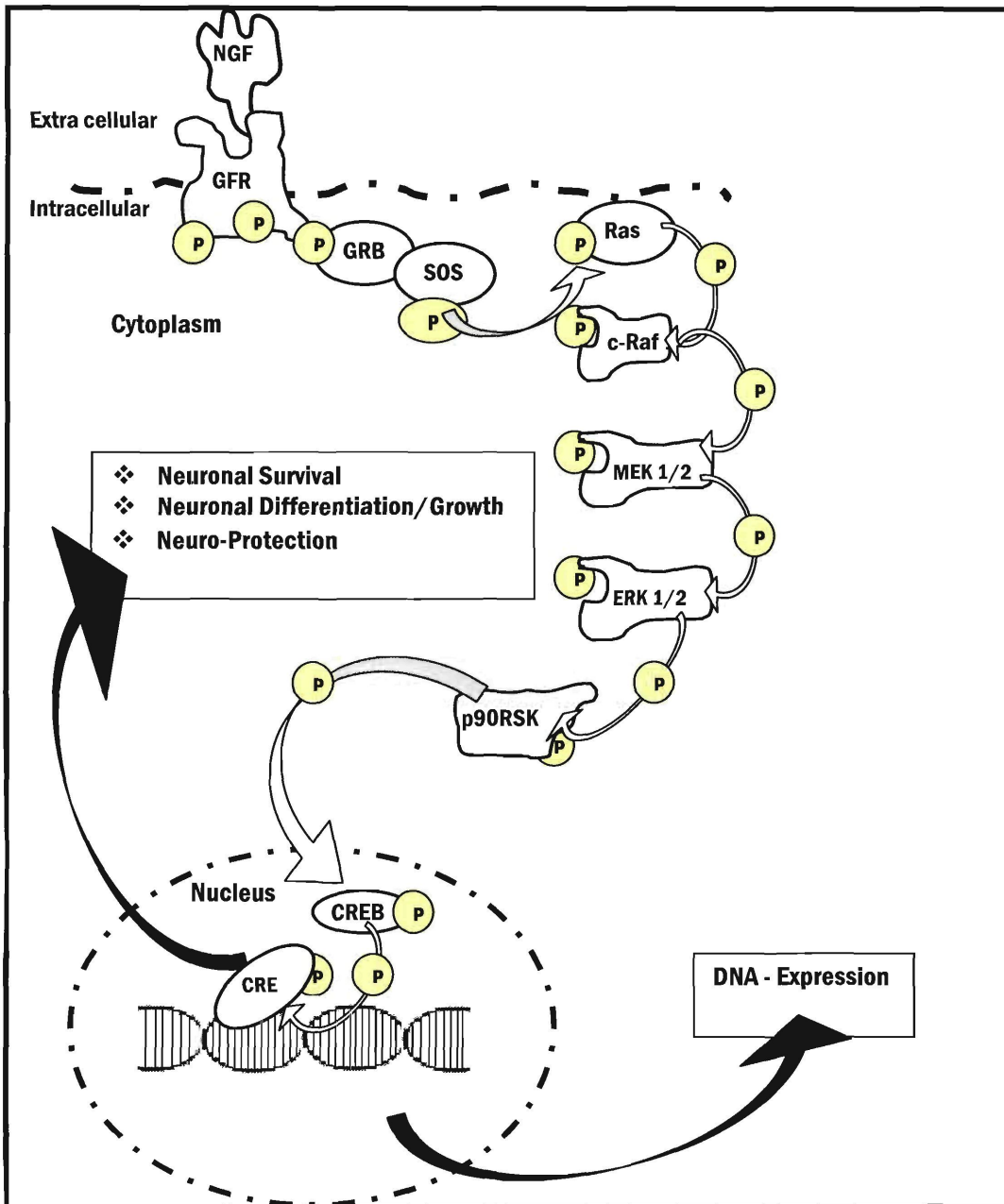


Figure 1.1: Pathway by which neurotrophic growth factors contribute to cell survival

Activation of nuclear factor kappa B (NF- κ B) can protect cultured neurons against death induced by trophic factor withdrawal and exposure to excitotoxic, oxidative, and metabolic insults (Yu *et al.*, 2000). Gene targets that mediate the survival-promoting action of NF- κ B may include manganese super oxide dismutase, Bcl-2, and apoptosis inhibitor proteins (Mattson, 2001). Increased Ca^{2+} levels or activation of membrane receptors (such as the receptor for secreted amyloid precursor protein α (sAPP α)) can stimulate cyclic guanosine 5'-monophosphate (GMP) production via a nitric oxide (NO)-mediated pathway, and cyclic

GMP can induce activation of K^+ channels and the transcription factor NF- κ B and thereby increase resistance of neurons to excitotoxic apoptosis (Furukawa *et al.*, 1996a) (fig. 1.2).

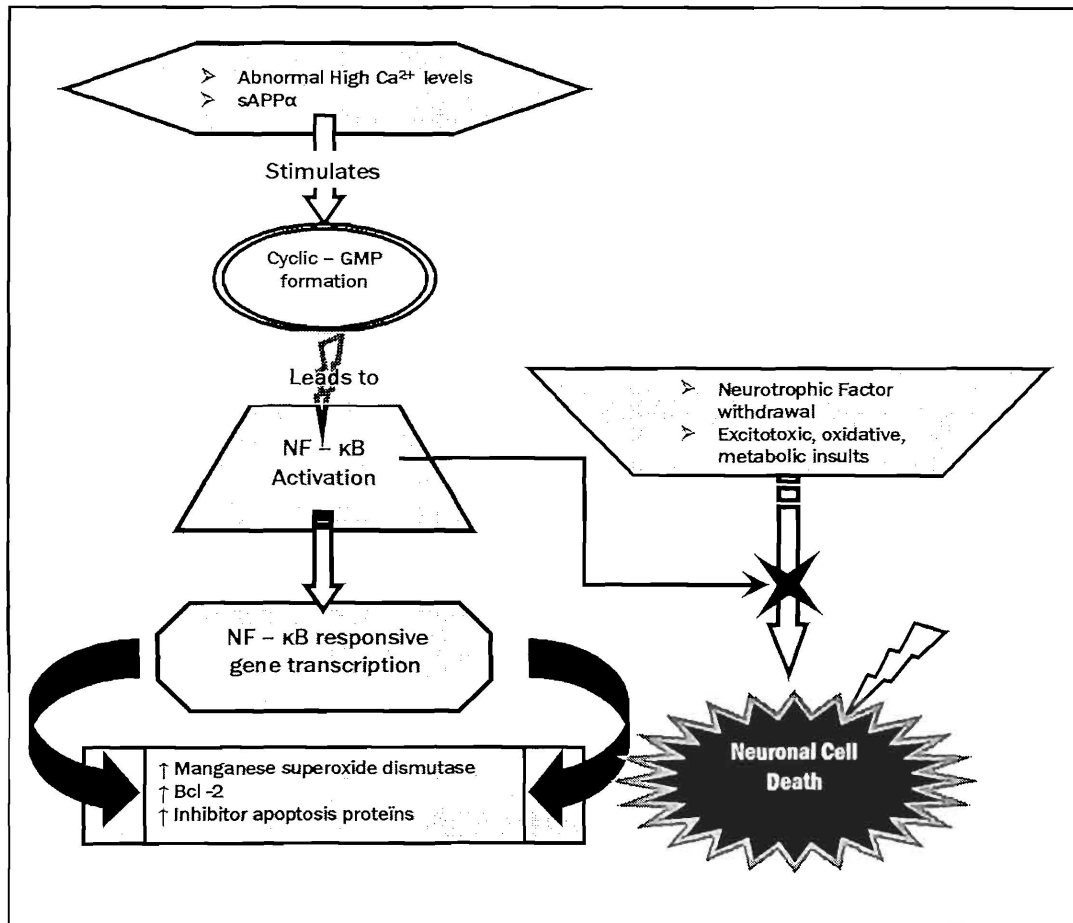


Figure 1.2: Neuroprotection by NF- κ B

2.1.2 p53 dependent apoptosis

p53 is a sequence-specific DNA-binding protein (e.g., El Dēiry *et al.*, 1992) that functions as a transcription factor (Farmer *et al.*, 1992; Kern *et al.*, 1992; Seto *et al.*, 1992) and interacts directly with proteins involved in both DNA replication (Dutta *et al.*, 1993) and DNA repair (Wang *et al.*, 1994). It can induce programmed cell death, suppress normal cell growth, or facilitate DNA repair (Oren, 1994). Endogenous p53 has been implicated in the cellular response to DNA damage (Kastan *et al.*, 1992), although p53-dependent apoptosis has previously occurred, in several instances, under circumstances not known to damage DNA (Holbrook *et al.*, 1996).

p53 presumably acts on an early event in the apoptotic pathway (Baffy *et al.*, 1993; Kane *et al.*, 1993), and can be induced by several factors. One of these factors include $A\beta$, which induces oxidative injury (Shearman *et al.*, 1994; Hensley *et al.*, 1994; Behl *et al.*, 1994) that

is an established inducer of p53 (Tishler *et al.*, 1993) (fig. 1.3). It activates the p53 dependent apoptotic pathway, with extra cellular deposition of $A\beta$ occurring secondarily to the neuronal cell death. For $A\beta$ to be able to activate the p53 dependent apoptotic pathway, it needs to accumulate to a threshold level (LaFerla *et al.*, 1996).

The downstream effectors of p53 in the apoptotic program are presently unknown. In principle, p53 may interact with the apoptotic machinery directly, transcriptionally regulating apoptotic genes, or indirectly by producing a cellular environment that facilitates apoptosis. Intriguing candidates for regulation by p53 during apoptosis are the products of the bcl-2 gene family (Holbrook *et al.*, 1996). Decreases in bcl-2 and increases in bax mRNA levels are associated with apoptosis induced by p53 over expression, suggesting that p53 regulates apoptosis by influencing the Bcl-2/Bax ratio (Miyashita *et al.*, 1994a, 1994b; Selvakumaran *et al.*, 1994). p53 also participates in a cell cycle checkpoint that arrests cell growth in response to DNA damage (Kastan *et al.*, 1992) (fig. 1.3).

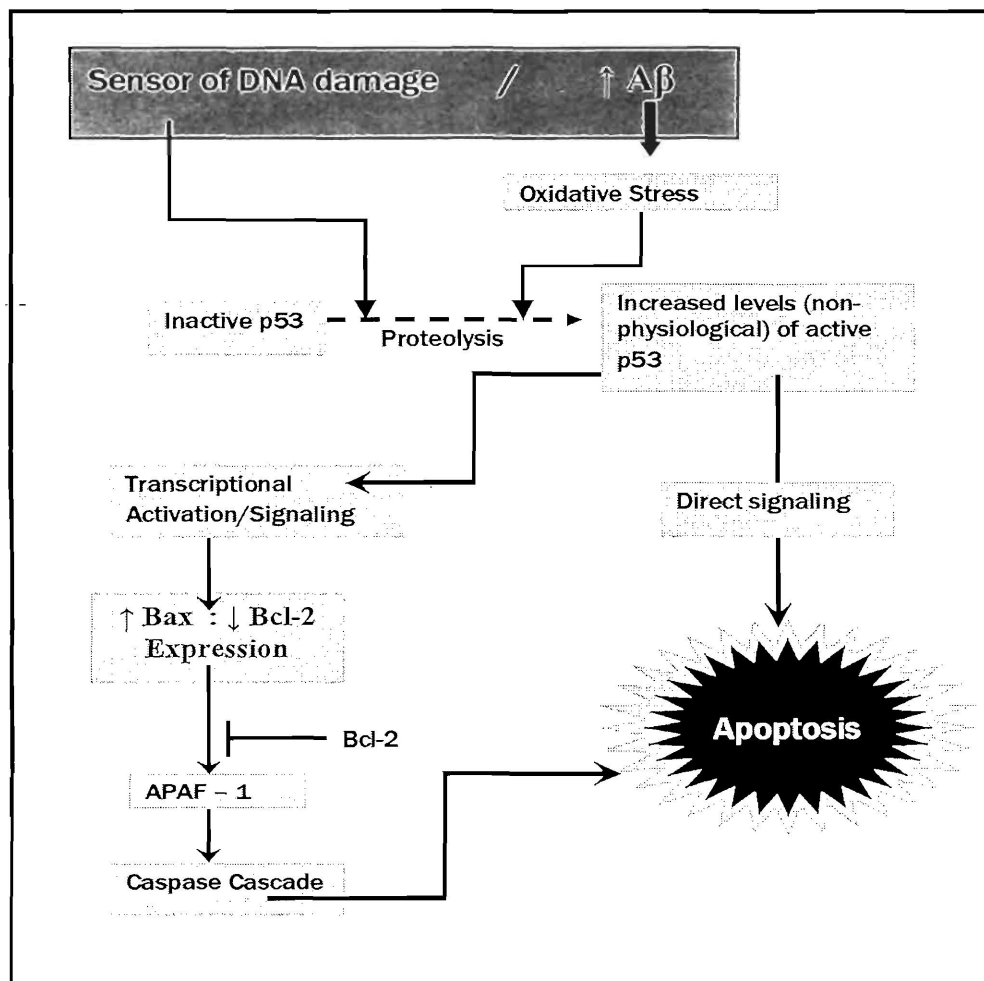


Figure 1.3: Apoptosis through the p53-pathway

2.1.3 Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a central glycolytic protein with a pivotal role in energy production, acting as a cellular kinase (Kawamoto & Caswell, 1986). It has a role in DNA replication and repair and possibly functions as a molecular chaperone. It acts as a pro-apoptotic enzyme downstream of Bcl-2 in the cell signal transduction cascade of apoptosis (Schulze *et al.*, 1993). In cells undergoing apoptosis, GAPDH is localised in the nuclear compartment, whereas this accumulation of GAPDH is normally suppressed in cells not undergoing apoptosis (Ishitani *et al.*, 1998; Saunders *et al.*, 1999; Sawa *et al.*, 1997). Its nuclear translocation in combination with other factors may be a key event during apoptosis and could have an influence in several neurodegenerative diseases (Schulze *et al.*, 1993). It is also important to note that the sub-cellular localisation and expression levels of endogenous GAPDH are different between neuronal and non-neuronal cell lines (Dastoor & Dreyer, 2001).

GAPDH exerts a role in neurodegenerative diseases that are characterised by the expansion of cytosine adenine guanine (CAG) repeats, in the genes that cause them. Some of these gene products include huntingtin and atrophin (Burke *et al.*, 1996), ataxin (Koshy *et al.*, 1996), androgen receptor and the β -amyloid precursor protein (Schulze *et al.*, 1993). They result in protein-protein interaction of GAPDH with the mentioned gene products of the different neurodegenerative diseases. When binding to GAPDH, these extended CAG repeats lead to inhibition of GAPDH activity, rendering it inactive, so that it can not play a role in DNA replication and repair (Burke *et al.*, 1996). GAPDH possibly serves as a carrier to mediate the translocation of these gene products into the nucleus (Dastoor & Dreyer, 2001).

Despite increasing evidence that GAPDH is involved in apoptosis, its role is unclear. Even though most cells that display nuclear GAPDH translocation do not have fragmented nuclei or show membrane blebbing (Dastoor & Dreyer, 2001), it is still possible that there is a correlation between its translocation into the nucleus and programmed cell death, since increased GAPDH expression, observed in apoptotic cells, is accompanied with nuclear fragmentation (Schulze *et al.*, 1993). Thus it can be concluded that the presence of GAPDH in the nucleus does not induce apoptosis *per se*, although nuclear localisation thereof might be an early indication of apoptotic cells or might even be responsible for apoptosis (Epner *et al.*, 1999; Ishitani *et al.*, 1998; Saunders *et al.*, 1997). GAPDH sometimes being present in the nucleus, without inducing or enhancing apoptosis, suggests that the nuclear localisation thereof is not responsible for the apoptotic action (Schulze *et al.*, 1993), but can be seen as an early event in the apoptotic cascade, most probably before the point of no return, where the cell death program can no longer be stopped (Dastoor & Dreyer, 2001). It is rather an

event coupled to the import of GAPDH into the nucleus that is responsible for the apoptotic action that follows (Schulze *et al.*, 1993).

2.1.4 Caspase-3 and poly(ADP-ribose)polymerase (PARP)

The presence of many different caspase substrates in synapses (Chan & Mattson, 1999), suggests that caspase-mediated cleavage of synaptic proteins may control the process of neuronal apoptosis (Mattson, 2001).

The caspases are a large family of cysteine proteases, which are responsible for the execution of apoptosis (Cryns & Yuan, 1998). They can act during the pre-mitochondrial phase (e.g., caspases 2 and 8) or post-mitochondrial phase (e.g., caspases 3 & 9) of apoptosis (Mattson, 2001).

Initially caspases are synthesised in inactive forms, called caspase zymogens (Degterev *et al.*, 2003). They can be activated in a reversible manner after trophic factor withdrawal or activation of glutamate receptors (Glazner *et al.*, 2000; Mattson, 2001). The apoptotic signalling pathways that lead to caspase zymogen processing can be subdivided into two major categories: cell surface sensor-mediated and intracellular sensor-mediated pathways. The cell surface sensor-mediated pathway is activated in response to extra cellular signals, indicating that the cell's existence is no longer needed for the well-being of the organism. These cell surface sensor-directed apoptotic signals are initiated by ligands binding to cell surface death-mediating receptors, and are exemplified by the signalling of the death receptor family. The other major category of apoptotic signalling, the intracellular sensor-mediated pathway, is activated by stimuli such as DNA damage and catatonic drugs, which act inside the cell. Cells possess multiple means of targeting mitochondria, which play an important role in these pathways, but most of these signals integrate at the level of the Bax/Bak gateway (Degterev *et al.*, 2003).

The caspases can be classified into two groups according to their function. These two groups are initiator (upstream) and execution (downstream) caspases. The initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11 and -12) are activated upon apoptotic signals. When activated this group of caspases activate the execution caspases (caspase-3, -6, -7), which perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates (Degterev *et al.*, 2003).

In the present study the focus will be on caspase-3 and caspase-9, since they are of significance in the mechanism of action of the compounds studied.

The release of cytochrome c, induced by a variety of death stimuli, results in the activation of a complex, which consists of apoptosis-activating factor 1 (Apaf-1) and caspase-9. This complex leads to the cytochrome c dependent processing of pro-caspase-3 to active caspase-3, and ultimately to apoptosis (Kluck *et al.*, 1997; Reed, 1997; Zou *et al.*, 1997). Apart from caspase activation, cytochrome c release causes slow irreversible loss of mitochondrial function and respiration, leading to the death of the cell. Therefore, the mitochondrial apoptotic pathway appears to result in a bipartite 'point-of-no-return' event, consisting of fast caspase activation and slow caspase-independent death through mitochondrial dysfunction (Degterev *et al.*, 2003).

Downstream from the mitochondria, caspase-9 is an important intracellular amplifier of caspase signalling (Degterev *et al.*, 2003), and acts as a critical upstream activator of caspase-3 (Porter & Janicke, 1999). It forms part of the apoptosome complex, and is activated through an apoptosome-induced conformational change. This forms active caspase-9, which processes the executioner caspase-3 to enable the initiation of the execution phase of apoptosis (Li *et al.*, 1997; Slee *et al.*, 1999). It is thus a critical upstream activator of caspase-3 (Porter & Janicke, 1999) (fig. 1.4).

Caspase-3 is a frequently activated death protease (Porter & Janicke, 1999), which plays a major role in the executive phase of apoptosis (Nicholson *et al.*, 1995; Tewari *et al.*, 1995), mediating nuclear apoptosis (Hirata *et al.*, 1998). Caspase-3 is important for cell death in a remarkable tissue-, cell type- or death stimulus-specific manner, and is essential for some of the characteristic changes in cell morphology to take place as well as certain biochemical events associated with the execution and completion of apoptosis (Porter & Janicke, 1999).

Caspase-3 activation in neurons possibly marks the beginning of the effector (downstream) phase of apoptosis (Hartmann *et al.*, 2000). Once it has been activated, downstream death substrates (Nicholson & Thornberry, 1997; Cryns & Yuan, 1998; Reed, 1997), such as Poly(ADP-ribose) polymerase (PARP), a protein that repairs damaged DNA and regulates chromatin structure, are cleaved and inactivated, irrespective of the involvement of cytochrome c (Nicholson & Thornberry, 1997; Cryns & Yuan, 1998; Reed, 1997). This proteolytic cleavage and inactivation of PARP by caspases is an early indicator of apoptosis, and is a process which consumes large amounts of NADP, thereby indirectly depleting the cellular ATP store (Kaufmann *et al.*, 1993). As a result of the inactivation of PARP, DNA fragmentation takes place.

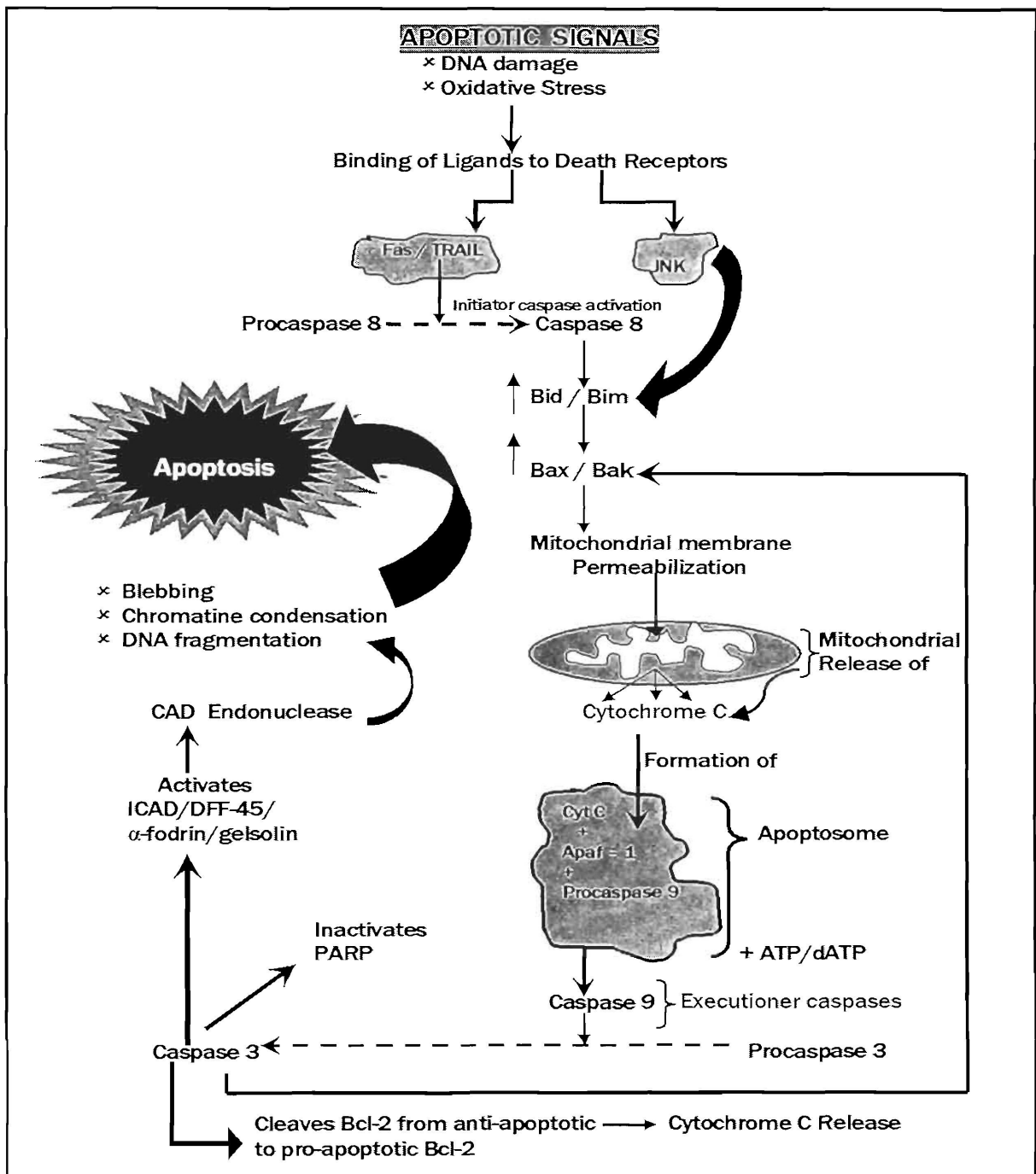


Figure 1.4: Bcl-2 regulated mitochondrial apoptosis

Caspase-3 might also amplify the upstream death cascade, including cytochrome c release from mitochondria, by cleaving Bcl-2 and converting it from an anti-apoptotic to a pro-apoptotic protein (Cheng *et al.*, 1997). Caspase-3 can exert multiple effects of which some are critical for cell death to occur. Other effects of caspase-3 may ensure the efficient completion of the apoptotic process once the cell has been committed to die (Porter & Janicke, 1999) (fig 1.4). Once the program has been completed, activated caspase-3 can no longer be detected, possibly because of degradation of the protease itself (Hartmann *et al.*,

2000). The cleavage of inhibitor of caspase activated DNase/DNA fragmentation factor-45 (ICAD/DFF-45), that results in the induction of the caspase activated DNase (CAD) endonuclease, is a caspase-3-dependent step in a major pathway to DNA fragmentation, by inter-nucleosomal degradation (Porter & Janicke, 1999). The DNA fragmentation mentioned forms part of the final steps of apoptosis.

The activation of caspase-3 has been associated with neuronal death in several experimental models of acute and chronic neurodegenerative disorders (Bergeron & Yuan, 1998; Pettmann & Henderson, 1998; Schulz *et al.*, 1999). Neurotoxins commonly used to induce experimental parkinsonian syndromes, e.g., 1-methyl-4-phenylpyridinium (MPP⁺) and 6-hydroxydopamine (6-OHDA), have been shown to exert their pro-apoptotic actions via activation of caspase-3-like proteases in neuronal *in vitro* models (Dodel *et al.*, 1998; Dodel *et al.*, 1999; Lotharius *et al.*, 1999). The increased expression of caspase-3 in Lewy body-containing neurons is in line with the hypothesis that caspase-3 is a probable effector of apoptotic cell demise (Hartmann *et al.*, 2000). Furthermore, it has been shown that the oligomerisation of A β 1-42 and A β 1-40 peptides, which arise from the β and γ -secretase cleavage of the trans-membrane portion of the amyloid precursor protein (APP), is critical to the progressive dysfunction and loss of cholinergic neurons in Alzheimer's disease (Selkoe, 2000, 2001). Caspase-3 has also been found to cleave the cytosolic tail of APP, stimulating the subsequent γ -secretase cleavage step approximately five-fold (Gervais *et al.*, 1999). The released cytosolic portion of APP has been suggested to play a separate role in the disease pathophysiology (Nishimura *et al.*, 2002).

2.1.5 Bcl-2 family proteins

The Bcl-2 family proteins are key regulators of apoptosis (Eisenmann *et al.*, 2003). They are integral membrane-bound proteins (Tsujimoto *et al.*, 1984) that are concentrated at the mitochondrial, nuclear, and endoplasmic reticular membranes, which are all sites of free radical production (Krajewski *et al.*, 1993).

Normal cellular homeostasis requires the suppression of pro-apoptotic players by various mechanisms, including phosphorylation, intracellular localisation, and heterodimerisation with pro-survival Bcl-2 family proteins. Disruption of the balance between pro- and anti-apoptotic Bcl-2 family members is suggested to be fundamental to the development of many diseases (Eisenmann *et al.*, 2003). These proteins may control the cell-death process by interacting with mitochondrial membranes in a manner that either promotes or prevents ion movements across mitochondrial membranes (Green & Reed, 1998).

Three sub-families have been identified: (a) the pro-survival Bcl-2 (e.g., Bcl-2 and Bcl-XL) proteins, which are anti-apoptotic; (b) the pro-apoptotic Bax (e.g., Bax and Bak) proteins and (c) the BH3 domain-only (e.g., Bad, Bim, and Bid) proteins, with Bad being pro-apoptotic (Adams & Cory, 1998; Cory & Adams, 2002; Gross *et al.*, 1999). Structurally, the BH3-only proteins are divergent from other Bcl-2 family members (Eisenmann *et al.*, 2003) (fig. 1.5).

Bcl-2 and Bax form homodimeric complexes or can heterodimerise with each other, and the ratio of Bcl-2 to Bax expression may ultimately determine cell survival following an apoptotic stimulus. Bcl-2 acts downstream of p53 to inhibit apoptosis (Oltvai *et al.*, 1993) by increasing resistance of neurons to death induced by excitotoxic, metabolic, and oxidative insults relevant to AD, stroke, and other disorders (Martinou *et al.*, 1994; Guo *et al.*, 1998). It also protects neurons against hydrogen peroxide and other agents that cause free radical damage, such as radiation. Bcl-2 prevents apoptosis by blocking the redistribution of calcium (Lam *et al.*, 1994; Holbrook *et al.*, 1996). Bax is structurally related to Bcl-2, but functionally promotes apoptosis (Oltvai *et al.*, 1993). In addition to direct p53 regulation (Miyashita *et al.*, 1994b), Bcl-2 appears to be regulated at the protein level by Bax (Oltvai *et al.*, 1993), which is a p53 immediate early response gene. Bcl-2 and Bax proteins appear to compete with one another to control the relative susceptibility of cells to p53-mediated apoptosis (Selvakumaran *et al.*, 1994). Another mechanism, by which Bcl-2 possibly protects cells from apoptosis, is its efficient prevention of translocation of endogenous GAPDH into the nucleus (Dastoor & Dreyer, 2001) (see section 2.1.3).

Bad is regulated through its phosphorylation and cytosolic sequestration. Dephosphorylated Bad promotes apoptosis, by binding to either Bcl-2 or Bcl-XL, and titrating them away from pro-apoptotic Bax/Bak proteins. Unbound Bax oligomerises which disrupts mitochondrial integrity, causing cytochrome c release and initiating the caspase cascade (Cory & Adams, 2002; Downward, 1999). Phosphorylated Bad is however bound and sequestered in the cytosol by the chaperone protein 14-3-3. Phosphorylation of one of at least three serine residues inactivates Bad by regulating interactions with either 14-3-3 or Bcl-2 family members. Several survival kinases are implicated in the direct phosphorylation of Ser⁷⁵, Ser⁹⁹ and Ser¹¹⁸, including RSKs, Akt or p70S6K and PKA, indicating that Bad functions as an important convergence point in signal transduction pathways affecting cell survival (Bonni *et al.*, 1999; Shimamura *et al.*, 2000; Fang *et al.*, 1999) (fig. 1.5).

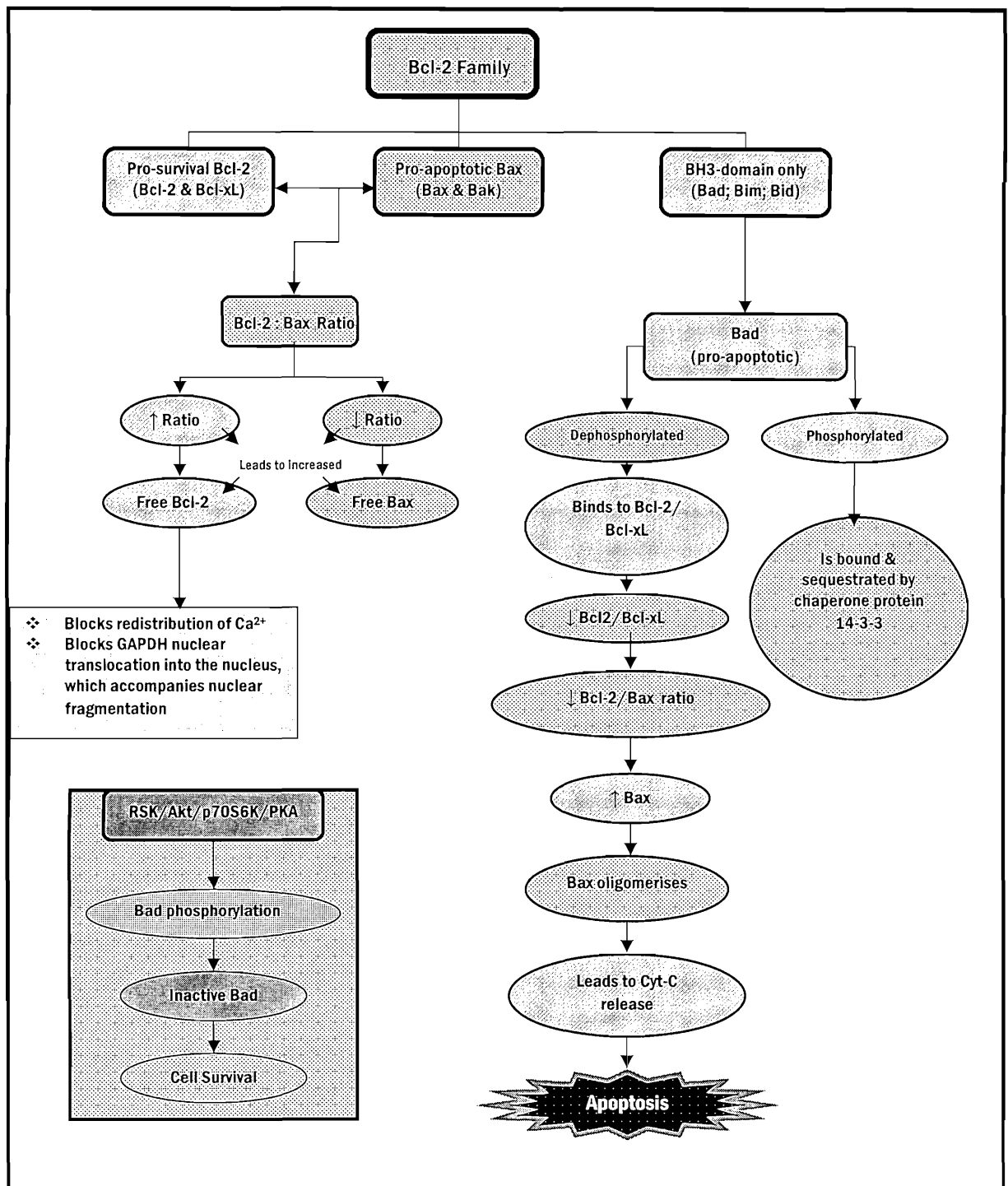


Figure 1.5: Bcl-2 family of proteins

2.1.6 Protein kinase C (PKC)-pathway/amyloid precursor protein (APP)

The β -amyloid protein and amyloid precursor protein (APP), are critical components of Alzheimer's' disease pathology and are greatly controlled and regulated by the PKC pathway. PKC is a phospholipid-dependent protein kinase that plays a crucial role in various cellular functions in neuronal and non-neuronal cells. In neurons it is a key enzyme in

neurotransmission, synaptic plasticity, learning and memory. Certain PKC isoforms are intimately involved in cell survival by suppressing apoptosis induced by A β (Weinreb *et al.*, 2004).

With regard to AD, PKC is linked to amyloid precursor protein (APP) processing (Fluhrer *et al.*, 2004). Non-amyloidogenic sAPP α is released by PKC- and MAPK-dependent pathways, with the activation of MAPK being dependent on PKC signalling pathway activity (Yogev-Falach *et al.*, 2003).

Proteolytic processing of the amyloid precursor protein (APP) can proceed via two opposing paths, with vastly different outcomes (Wilquet *et al.*, 2004): The amyloidogenic path, which produces β -amyloid (A β) fragments, the etiological agents of AD pathology, and the non-amyloidogenic path, which produces neuroprotective sAPP α fragments (Mattson, 1994; Meziane *et al.*, 1998).

The amyloidogenic path involves sequential cleavages of APP, by β -secretase/beta-site APP-Cleaving Enzyme (BACE) and γ -secretases, with the generation of β -amyloid (A β) fragments, which are the etiological agents of AD pathology (Allinson *et al.*, 2003). When A β is in an aggregated form, it can induce membrane lipid peroxidation in hippocampal and cortical neurons (Mark *et al.*, 1995; 1997a) as well as in cortical synaptosomes (Keller *et al.*, 1997). Lipid peroxidation promotes neuronal death in part by impairing the function of membrane ion-motive ATPases (Na⁺/K⁺-ATPase and Ca²⁺-ATPase) and glucose transporters (Mark *et al.*, 1995, 1997b), decreasing ATP levels, which promotes membrane depolarisation, energy depletion, and disruption of cellular Ca²⁺ homeostasis. Membrane lipid per-oxidation also impairs glutamate transport in astrocytes and synaptosomes (Keller *et al.*, 1997; Blanc *et al.*, 1998), which would be expected to further promote excitotoxic injury (Mattson, 2001). The oxidative stress induced by A β can also render neurons vulnerable to excitotoxicity and apoptosis (Mattson *et al.*, 1992; Mark *et al.*, 1995, 1997a; Kruman *et al.*, 1997). A β also induces time- and dose-dependent decreases in catalase activity and increases in Cu/Zn- and Mn-superoxide dismutase (SOD) activities (Bruce *et al.*, 1997). Besides the mentioned toxic effects of β -amyloid, it also appears to stimulate calcium release directly and hydrogen peroxide/super oxide production, which again, could lead to apoptosis (Holbrook *et al.*, 1996). Although A β is secreted throughout life, it begins to accumulate in old age (Olariu *et al.*, 2005). It is now understood that neurons are the main source of A β in AD and it has been hypothesised that A β gradually accumulates in the extra cellular space due to excess secretion and/or deficient clearance (Chaney *et al.*, 2003).

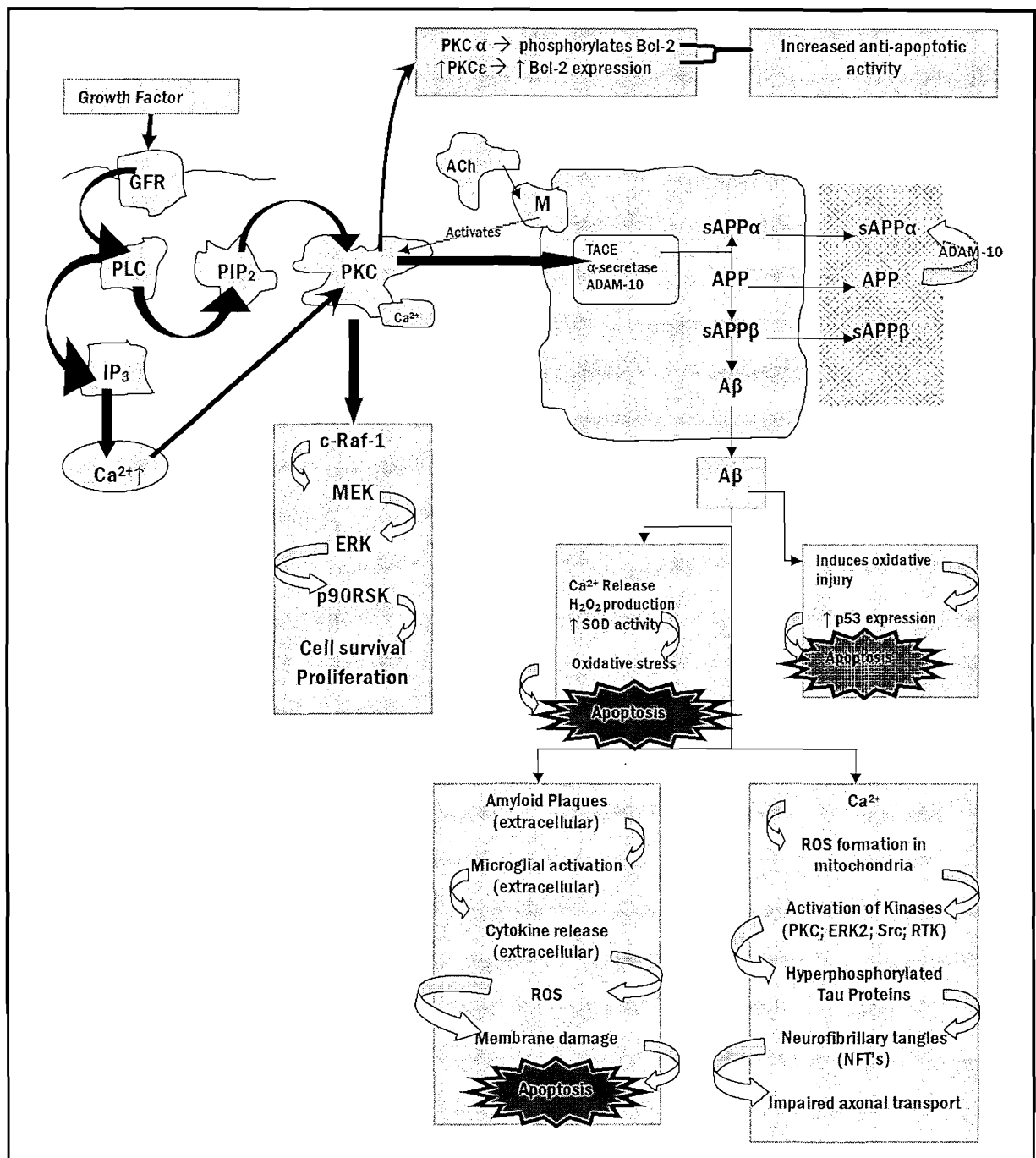


Figure 1.6: β -amyloid peptide and PKC in apoptosis

The non-amyloidogenic path, controlled by PKC (Takahashi *et al.*, 2002), involves APP cleavage by α -secretases (Allinson *et al.*, 2003), at a site which will preclude BACE cleavage, and release a neuroprotective sAPP α fragment (Mattson, 1994; Meziane *et al.*, 1998) (fig. 1.6). PKC α and - β are key regulators of α -secretory APP processing (Rossner *et al.*, 2001), with PKC α being specifically involved in sAPP α release. PKC ϵ is involved in coupling cholinergic receptors with APP metabolism (Lanni *et al.*, 2004), with stimulation of muscarinic receptors, increasing cleavage of APP through the α -secretase pathway, and

inhibiting A β production (Muller *et al.*, 1997). Increasing the α -secretase processing pathway could be beneficial for the treatment of AD, by shifting the balance of APP processing toward a presumably non-pathogenic pathway (Yogev-Falach *et al.*, 2003). Even though it does not necessarily affect A β generation or slow amyloid plaque formation (Olariu *et al.*, 2005) (fig. 1.6), the non-amyloidogenic sAPP α has potent neurotrophic and neuroprotective activities against excitotoxic and oxidative insults in various cellular models and can serve as a neuroprotective agent against the toxic activity of A β (Yogev-Falach *et al.*, 2003).

2.1.7 Mitogen-activated protein kinase (MAPK)-pathway

Cells recognise and respond to extracellular stimuli by engaging specific intracellular programs, such as the signalling cascade that leads to activation of the mitogen-activated protein kinases (MAPKs) (Roux & Blenis, 2004). These intracellular signalling pathways strictly control cell function and fate, i.e. differentiation, death and survival. The Ras/Raf/MEK/ERK pathway can regulate cell cycle progression and apoptosis, and plays a fundamental role in Bad inactivation (Chang *et al.*, 2003).

Each family of MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (Dan *et al.*, 2001; Kolch, 2000).

Five distinct groups of MAPKs have been characterised in mammals: Extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 isoforms α , β , and δ , ERK's 3 and 4 and ERK5. ERK1/2 is preferentially activated in response to growth factors and phorbol esters, while the JNK and p38 kinases are more responsive to stress stimuli (Roux & Blenis, 2004). A decrease in neurotrophic growth factors is shown to be part of the pathology of numerous neurodegenerative disorders (Mattson & Lindvall, 1997). For this reason the focus will be on the ERK1/2 pathway in this study. This mammalian ERK1/2 module, is also known as the classical mitogen kinase cascade, consisting of the MAPKKKs, A-Raf, B-Raf and Raf-1, the MAPKKs, MEK1/2, and the MAPKs, ERK1/2 (Roux & Blenis, 2004). B-Raf and Raf-1 presumably participates in neuronal apoptosis, with A-Raf mainly presiding in urogenital tissues (Wellbrock *et al.*, 2004). Apart from activating MEK1/2, B-Raf and Raf-1 also inactivates Bad indirectly, by increasing the activity of PKC θ (Hindley & Kolch, 2007).

Cell surface receptors such as tyrosine kinases (RTK) and G protein-coupled receptors transmit activating signals to the Raf/MEK/ERK cascade through Ras (Campbell *et al.*, 1998; Wood *et al.*, 1992). Activation of Ras is achieved through recruitment of Son of Sevenless (SOS), a Ras-activation guanine nucleotide exchange factor. SOS stimulates Ras to change

GDP to GTP, allowing it to interact with and activate Raf (Geyer & Wittinghoffer, 1997), a small GTP-binding protein of the Ras/Rho family. The activated Raf gives way to the activation of a MAPKK, e.g. MEK1/2, through phosphorylation in response to extra cellular stimuli (Dan *et al.*, 2001; Kolch, 2000). MAPKKK activation leads to the phosphorylation and activation of a MAPK, e.g. ERK1/2 (Hallberg *et al.*, 1994), which then stimulates MAPK activity through dual phosphorylation (Roux & Blenis, 2004), and acts as a key regulator of cell proliferation (Kohno & Pouyssegur, 2003; Roux & Blenis, 2004).

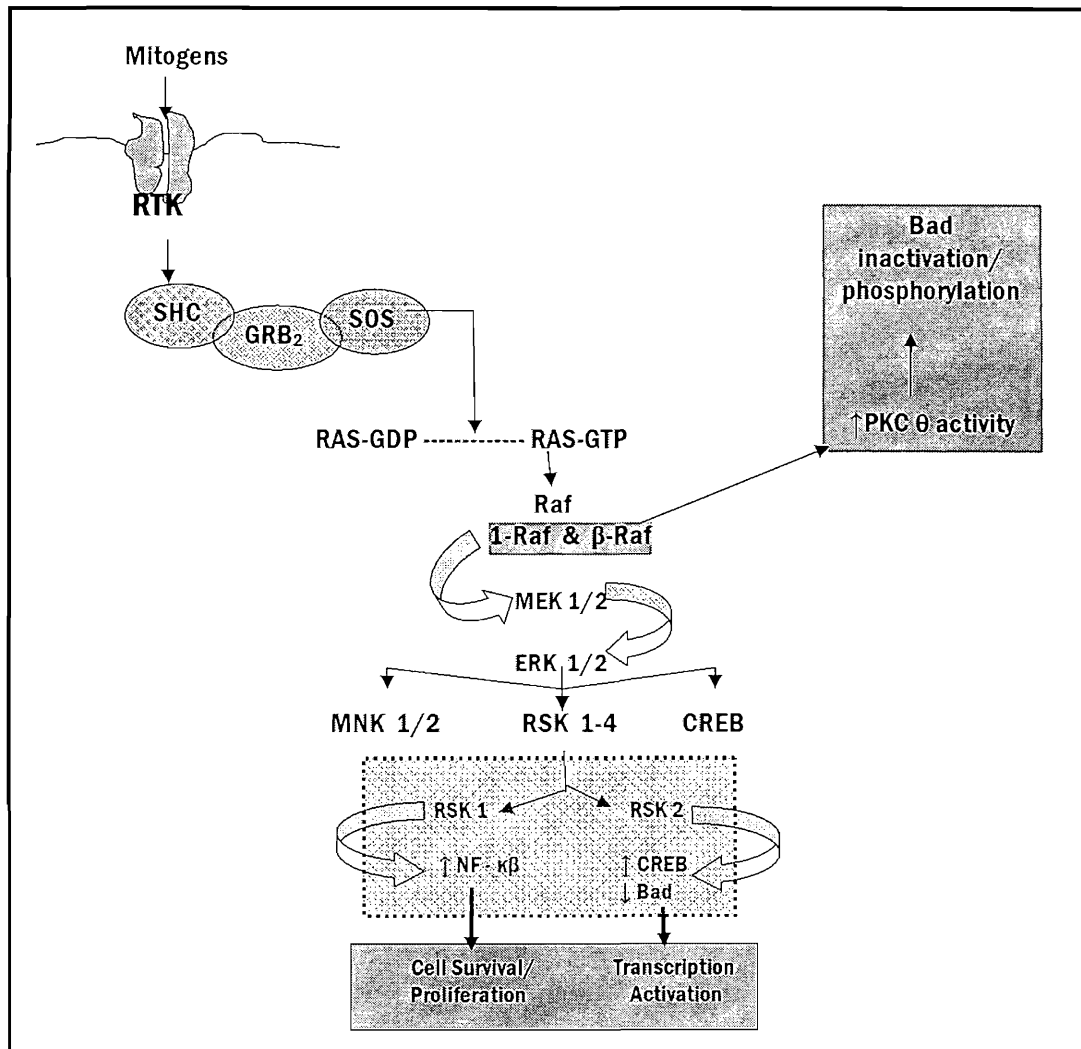


Figure 1.7: Ras/Raf/MEK/ERK pathway to cell survival

Once activated, MAPKs phosphorylate target substrates such as MAPK-activated protein kinases (MKs), of which the ribosomal S6 kinases (RSKs) are relevant. These RSKs form part of the MK (MAPK-activated protein kinases) family (Roux & Blenis, 2004), and are exclusively activated by the ERK's (Frodin & Gammeltoft, 1999; Roux & Blenis, 2004). Activated RSK phosphorylates multiple transcription factors (Thomson *et al.*, 1999). It has

been shown that the neurotrophic factor-stimulated RSK2 isoform promotes cortical neuron survival by phosphorylating Bad and CREB (Bonni *et al.*, 1999; Ginty, *et al.*, 1994; Xing *et al.*, 1996), whereas RSK1 can promote survival by activating NF- κ B (Ghoda *et al.*, 1997; Schouten *et al.*, 1997; Roux & Blenis, 2004). RSK2 phosphorylation of Bad facilitates Bad's inactivation through binding to 14-3-3 and sequestration from heterodimerising with mitochondria-bound Bcl-2 or Bcl-XL (Tan *et al.*, 1999; Lzcano *et al.*, 2000; Harada *et al.*, 2001; Datta *et al.*, 1997). Bad inactivation occurs through hyper-activation of the MEK/ERK/RSK signalling module, with B-Raf being an upstream activator of the MEK/ERK signalling pathway (Brose *et al.*, 2002; Davies *et al.*, 2002; Satyamoorthy *et al.*, 2003; Pollock *et al.*, 2003). Activation of the MEK/ERK pathway inhibits apoptotic cell death (Eisenmann *et al.*, 2003) (fig. 1.7).

3 Monoamine oxidase B (MAO-B) in neurodegenerative diseases

Monoamine oxidases are enzymes that catalyse the oxidation of monoamines. They are bound to the outer membrane of mitochondria in most cell types in the body, and belong to a protein family of flavin containing amine oxidoreductases (Kearney *et al.*, 1971).

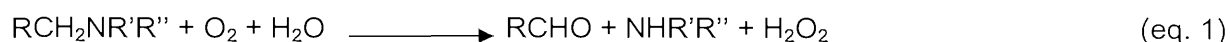
In mammals, MAO is present as two isoforms (MAO-A and MAO-B), which are separate gene products and separate enzymes, that exhibit over 70% sequence identity and distinct but overlapping substrate specificities in the catabolism of neurotransmitters such as dopamine and serotonin (Weyler *et al.*, 1990; Shih *et al.*, 1999). They are both implicated in a large number of neurological disorders and are targets for drugs against Parkinson's disease and depression (Cesura & Pletscher, 1992). Mammalian MAOs are bound to the outer mitochondrial membrane and have a FAD molecule as the co-factor, which is covalently bound to the protein (Kearney *et al.*, 1971). The FAD site is the site at which irreversible inhibitors of MAO are covalently linked (Youdim *et al.*, 2005). The enzymes are expressed in both a tissue-dependent and an age-dependent manner (Binda *et al.*, 2002) and have different patterns of tissue distribution and different substrate and inhibitor specificity (Johnston, 1968).

These enzymes catalyse the oxidative deamination of monoamine neurotransmitters and neuromodulators such as dopamine, noradrenalin, adrenaline, serotonin (5-hydroxytryptamine) and β -phenylethylamine (PEA), as well as some exogenous bioactive monoamines (Johnston, 1968). Serotonin, noradrenalin and adrenalin are mainly deaminated by MAO-A, whereas PEA is preferentially deaminated by MAO-B. Dopamine on the other hand is metabolised by both isoforms, MAO-A and MAO-B (Riederer *et al.*, 2004).

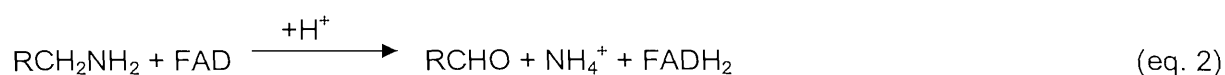
MAO-B constitutes about 80% of the total MAO activity in the human brain (Riederer *et al.*, 1978; Sonsalla & Golbe, 1988) and is the predominant form of the enzyme in the striatum (Riederer *et al.*, 1989, 2004). For this reason the focus will be mainly, but not exclusively, on the development of MAO-B specific inhibitors.

3.1 Mechanism of action of monoamine oxidase B (MAO-B)

Monoamine oxidase oxidises primary aliphatic amines as well as some secondary and tertiary amines according to equation 1 (Tipton *et al.*, 2004):



Kinetic studies have shown the reaction to involve the binding of the amine substrate to the enzyme before oxygen. The reaction proceeds in two steps: In the first of these, reduction of the enzyme-bound FAD results in the formation of the aldehyde product and ammonia, whereas the second step entails the re-oxidation of the enzyme-bound FAD by O_2 with the formation of hydrogen peroxide (Tipton *et al.*, 2004) (eq. 2 and eq. 3):



With primary amines the first partial reaction is believed to proceed *via* an imine intermediate, which is then hydrolysed by water to the final product (Tipton *et al.*, 2004) (eq. 4):



The hydrolysis of this type of intermediate does not occur in the case of some irreversible inhibitors or with the neurotoxin MPP⁺ (Tipton *et al.*, 2004).

3.2 Protective strategies

Ontogenetic studies have demonstrated that MAO-B activity stays unchanged until about the 60th year of life, after which it increases non-linearly (Delumeau *et al.*, 1994; Strolin & Dostert, 1989), with the expression levels increasing ~4-fold with age (Fowler *et al.*, 2003). Because MAO-B is predominantly located in glial cells, the increase of this enzyme with age may be attributed to glial cell proliferation associated with neuronal loss (Shih *et al.*, 1999; Mellick *et al.*, 1999). This age-related increase of brain MAO B activity is believed to cause an augmentation in oxidative stress (Barnham *et al.*, 2004), by the production of increased levels of hydrogen peroxide, and an increased level of dopamine metabolism. This is

thought to play a role in the etiology of neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Kumar *et al.*, 2003), where there is indeed an increased level of MAO activity (Mandel *et al.*, 2005). In conjunction with this, studies have demonstrated that elevated MAO-B levels induce apoptosis in neuronal cells (Boulton *et al.*, 1998).

The development of specific, reversible MAO-B inhibitors can lead to clinically useful neuroprotective agents (Hubalek *et al.*, 2005). The hydrogen peroxide (H_2O_2) produced during the mentioned amine oxidation can accumulate in PD patients, making it available for the Fenton reaction, wherein a highly active free radical, the hydroxyl radical, is formed and can damage nucleic acids, proteins, and membrane lipids, leading to neuronal degeneration (Nicotra *et al.*, 2004). MAO-B inhibitors, which decrease the rate of MAO-B catalysed oxidative deamination and consequently, the production of reactive oxygen species (ROS), might thus beneficially contribute to the treatment of Alzheimer's and Parkinson's diseases through neuroprotection (Youdim *et al.*, 2004). Moreover, in the context of Parkinson's disease, MAO-B inhibitors present a second therapeutic application, due to MAO being one of dopamine's major metabolising enzymes. As MAO-B is present in excess in the tissue in which metabolism occurs, the inhibition of the iso-enzyme B blocks the metabolism of dopamine, enhancing both the endogenous dopamine level and dopamine produced from exogenously administered precursor levodopa (L-DOPA) (Foley *et al.*, 2000; Yamada & Yasuhara, 2004). The inhibition of dopamine degradation by MAO-B inhibitors combined with supplementation of dopamine by L-DOPA has been shown to be successful in the treatment of PD patients (Palhagen *et al.*, 2006).

It is important to note that selective inhibition of MAO-A or -B, will not change the levels of dopamine drastically in the human striatum (Riederer & Youdim, 1986). This is in contrast with those monoamines that are substrates for only one isoform. Although selective inhibition of MAO-A or -B do not affect the steady state level of dopamine in the brain, such inhibition did affect its release, which would explain the anti-symptomatic effects observed in PD patients, with some of these drugs (Youdim & Bakhle, 2006). Non-selective MAO inhibitors, such as ladostigil, increase levels of all three monoamines, noradrenaline, 5-HT and dopamine in the hippo-campus and striatum of rats and mice. It also shows anti-depressant activity in animal models (Sagi *et al.*, 2005), which makes it a useful and beneficial side effect in PD patients, as a significant proportion (40-60%) of patients exhibit signs of depression (Youdim & Bakhle, 2006). Inhibitors of MAO-A have been proved to be effective anti-depressants, while MAO-B blockers have been emphasised in the treatment of Parkinson's disease (Riederer *et al.*, 2004).

4 Concluding remarks

In focusing on the neurodegenerative disorders, Alzheimer's disease and Parkinson's disease, it is clear that an intervention is necessary to slow down the progression of the neuronal breakdown process, which takes place as part of the pathophysiology of these diseases. The neurodegeneration in PD and AD takes place by an intrinsic cell suicide program known as apoptosis, which consists of several pathways and cascades, which ultimately lead to the death of neuronal cells in certain areas of the brain, depending on the disorder. Monoamine oxidase B also has a role in this cell death process, but also contributes to the signs and symptoms presented by PD patients.

RATIONALE AND SELECTION OF COMPOUNDS

Considering the need for improved strategies for the treatment of neurodegenerative diseases, the development of new neuroprotective agents is an active field of research. Alzheimer's disease (AD) and Parkinson's disease (PD) are two of many devastating neuronal breakdown diseases, with a curative strategy not known, although symptomatic treatment regimes are in practice. Since the underlying mechanisms of neurodegeneration are largely unknown, creating effective multifunctional neuroprotective drugs, is not an easily attainable goal. This study focused on the development of multifunctional drugs, which firstly may halt the neuronal breakdown process, and secondly may also be functional in treating the symptoms. The drugs should therefore act as symptomatic as well as curative treatment.

1 Propargylamine

1.1 Introduction

Rasagiline [N-propargyl-(R)-aminoindan] is a second generation propargylamine that irreversibly inhibits brain MAO-B, and has promising neuroprotective activity. Currently it is registered in several countries as monotherapy and adjunctive therapy for the management of PD (Chen *et al.*, 2007). After several years of study and research, it has been established that the neuroprotective effects of rasagiline [N-propargyl-(R)-aminoindan] can be attributed to its propargyl moiety. The propargylamine functional group is therefore the pharmacophore responsible for the neuroprotective activity. This observation was made on the grounds that propargylamine itself exerts the same neuroprotective effects as offered by rasagiline. It has also been established that the MAO-B inhibiting activity is not a prerequisite for the neuroprotection provided by rasagiline (Bar-Am *et al.*, 2005).

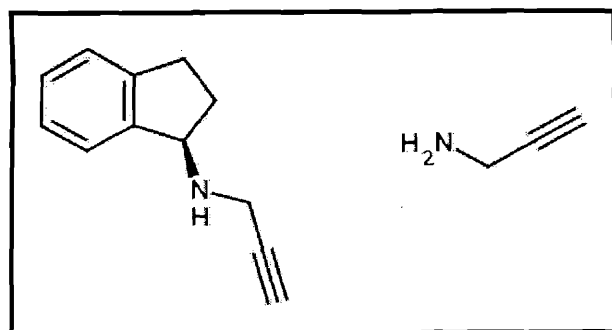


Fig. 2.1 The structures of rasagiline (left) and propargylamine (right)

Propargylamines have been reported to inhibit apoptosis (Bar-Am *et al.*, 2005). As described in the previous section, apoptosis is a cell death process by which nerve cells in certain areas of the brain are destroyed. This leads to the characteristic signs and symptoms of neurodegenerative diseases as seen in Parkinson's disease (PD) and Alzheimer's disease (AD). A well-known hypothesis suggests that this process is the predominant mode of neuronal death in neurodegenerative disorders (Bar-Am *et al.*, 2005). For this reason it is of significant importance to develop and study compounds, such as propargylamine derivatives (propargylamines), that may possibly attenuate the progressive neurodegeneration found in these diseases.

Also of significance is the observation that propargylamine is a MAO-inhibitor. Although this inhibition is very poor (Bar-Am *et al.*, 2005), it may contribute to its observed neuroprotective activity (Yu *et al.*, 1992). Other propargylamine derivatives are reported to act as more potent MAO-inhibitors. Since MAO activity is increased in both Alzheimer's and Parkinson's disease (Mandel *et al.*, 2005) MAO-inhibitors may be of further therapeutic benefit.

1.2 Neuroprotective properties of propargylamine

The neuroprotective properties of propargylamine (PA) are very diverse and include the following:

- ❖ Propargylamine significantly increases mRNA expression levels of glial cell-line derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF). GDNF's increased level is mediated through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Maruyama *et al.*, 2004).
- ❖ Propargylamine may inhibit p53 dependent apoptosis, which is mediated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Berry *et al.*, 1998; Berry, 1999; Zhang *et al.*, 1999).
- ❖ Propargylamine prevents the activation and translocation of GAPDH from the cytoplasm to the nucleus (Mandel *et al.*, 2005), possibly through binding to GAPDH (Kragten *et al.*, 1998), and thereby immobilising it.
- ❖ Propargylamine reverses the apoptotic effect, by preventing the activation of caspase-3, and the cleavage of poly(ADP-ribose)polymerase (PARP) (Bar-Am *et al.*, 2005).
- ❖ Propargylamine significantly increases Bcl-2 mRNA levels, while markedly reducing Bax gene expression. It increases the Bcl-2/Bax mRNA ratio, with an increase in Bcl-2 levels and a decrease in Bax levels (Bar-Am *et al.*, 2005; Mandel *et al.*, 2005). Bad- and Bim-protein (BH3-only proteins

of the Bcl-2 family) levels (Bar-Am *et al.*, 2005; Weinreb *et al.*, 2004; Youdim *et al.*, 2003) are reduced, while the anti-apoptotic Bcl-2 family member, Bcl-xL is induced (Weinreb *et al.*, 2004; Youdim *et al.*, 2003).

- ❖ Even though propargylamine does not affect holo-APP mRNA levels, it significantly down-regulates holo-APP protein levels. This indicates a post-transcriptional regulatory mechanism (Rodgers *et al.*, 2002; Shaw *et al.*, 2001).
- ❖ Propargylamine increases sAPP α levels (Bar-Am *et al.*, 2005).
- ❖ Propargylamine stimulates sAPP α release via the protein kinase C/mitogen-activated protein kinase (PKC/MAPK) signalling pathway (Yogev-Falach *et al.*, 2002, 2003).
- ❖ Propargylamine down-regulates PKC γ levels, whilst increasing PKC α and PKC ϵ levels (Weinreb *et al.*, 2004). It stimulates PKC phosphorylation and induces translocation of the isoforms PKC α and PKC ϵ to the hippocampal membrane department, and down-regulates PKC δ levels (Mandel *et al.*, 2005).
- ❖ Propargylamine dose dependently induces MAPK phosphorylation, with MEK and PKC-signalling being of significant importance (Yogev-Falach *et al.*, 2003). It activates ERK1/2 pathways resulting in increased processing of APP via activation of zinc-dependent metalloprotease (super oxide dismutase, SOD) and α -secretase, to release sAPP α (Mandel *et al.*, 2005).
- ❖ Propargylamine activates the PKC-MAPK pathway (Bar-Am *et al.*, 2005).
- ❖ Propargylamine inhibits monoamine oxidase, even though its neuroprotective activity is independent of its MAO-inhibitory activity (Maruyama *et al.*, 2004).

2 Polycyclic cage structure compounds

2.1 Introduction

Polycyclic cage compounds, such as amantadine and memantine, have various pharmaceutical applications, with special interest in the symptomatic and proposed curative treatment of neurodegenerative diseases such as Parkinson's and Alzheimer's disease. The polycyclic cage can be used to modify and improve the pharmacokinetic and pharmacodynamic properties of drugs, and from literature it is apparent that the polycyclic cage is useful as both a scaffold for side-chain attachment as well as for improving a drug's lipophilicity. This lipophilicity enhances a drug's transport across cellular membranes, and

increases its affinity for lipophilic regions in receptors (Zah *et al.*, 2003). In addition, these polycyclic moieties afford metabolic stability, thereby prolonging the pharmacological effect of a drug, leading to a reduction of dosing frequency of medicine and helping to improve patient compliance (Brookes *et al.*, 1992). The novel NMDA channel antagonism of these compounds, combined with DA uptake inhibition and L-type calcium channel blocking activity, suggests that pentacyclo-undecane derived compounds may have exciting potential as therapeutic agents for neurodegenerative diseases, such as traumatic brain injury, stroke, Parkinson's disease and Alzheimer's disease (Geldenhuys *et al.*, 2004).

With the focus being on the development of multifunctional drugs, it was thus a rational decision to incorporate the polycyclic cage structures into the novel drugs envisaged for synthesis. In the current study the focus will therefore be on cage compounds that have potential to be therapeutic in the treatment of neurodegenerative diseases, especially Parkinson's disease and Alzheimer's disease. The cage structures will include adamantylamines (e.g. amantadine and memantine) and pentacyclo-undecylamines.

2.2 Biological activity

The adamantylamines, amantadine and memantine, offer therapeutic benefit by increasing extra cellular dopamine levels and preventing excessive influx of calcium into neuronal cells. Amantadine possesses anti-parkinsonian activity since it increases extra cellular dopamine (DA) levels through DA re-uptake inhibition (Mizoguchi *et al.*, 1994) and DA release (Danysz *et al.*, 1997). Electrophysiological studies further indicated that amantadine acts through the phencyclidine (PCP) binding site located within the N-methyl-D-aspartate (NMDA) receptor/ion channel complex, blocking uptake of calcium ions into neurons (Parsons *et al.*, 1999). This antagonism is use-dependent in that the PCP site is only accessible when the ion channel pore is in an open or activated state. The channel block is accelerated by increases in open channel probability (Geldenhuys *et al.*, 2004). Memantine is used clinically in Alzheimer's disease (Kroemer *et al.*, 1998), and is a low-affinity non-competitive NMDA antagonist. Due to this classification it shows definite potential as a neuroprotective drug, by preventing excessive influx of calcium into neuronal cells (Turski *et al.*, 1991; Zuddas *et al.*, 1992; Brouillet & Beal, 1993). It exhibits rapid and strong voltage-dependent blocking kinetics. Partial trapping and release of memantine from NMDA channels, favour the occurrence of strong block only during sustained receptor stimulation (such as may occur in excitatory neurotoxic events including ischemia and brain trauma), with more limited effects expected during normal synaptic transmission (Parsons *et al.*, 1999).

Low-affinity use-dependent channel blockers (i.e. antagonists with a K_i above 200 nM) such as amantadine and memantine are generally not associated with the unacceptable side-effects, such as hallucinations (Carter, 1994), memory impairment (Trist, 2000) and neuronal vacuolisation (Bigge, 1993), and are well tolerated (Parsons *et al.*, 1999; Kroemer *et al.*, 1998). Amantadine and memantine are thus ideal lead compounds for the development of other agents with favourable indices to treat NMDA/ion channel-mediated neurodegenerative diseases (Geldenhuys *et al.*, 2004).

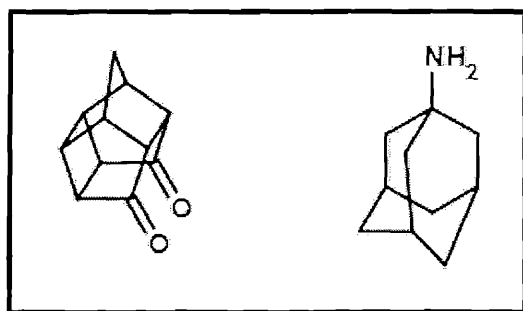


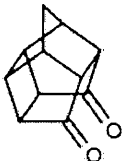
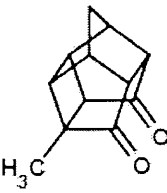
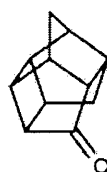
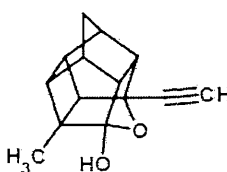
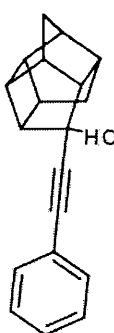
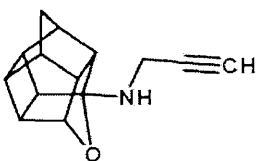
Fig. 2.2 The structures of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (left) and amantadine (right)

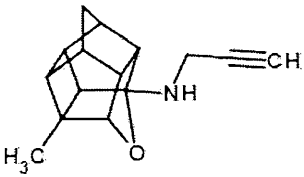
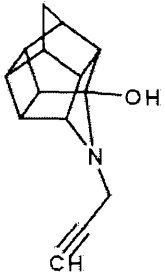
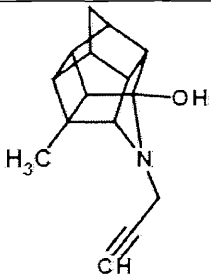
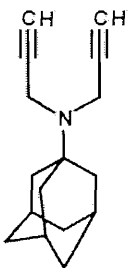
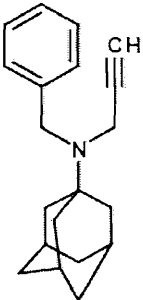
Pentacyclo-undecylamines are derived from Cookson's diketone (pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione), the so-called "bird cage" compound, that is obtained from the intramolecular [2 + 2] photocyclisation of the Diels-Alder adduct of *p*-benzoquinone and cyclopentadiene (Cookson *et al.*, 1964). The pentacyclo-undecylamines are beneficial due to their broad spectrum of therapeutic activity. They have been shown to increase cellular dopamine levels, by inhibiting DA uptake and stimulating DA release (Geldenhuys *et al.*, 2003; Geldenhuys *et al.*, 2004; Geldenhuys *et al.*, 2005). They also have very weak MAO-B inhibiting activity, and apparent neuroprotective activity. It is thus clear that these compounds can definitely contribute to the design of neuroprotective agents. The spectrum of activity within the pentacyclo-undecylamines make them ideal candidates for exploration as possible lead compounds for dual symptomatic and neuroprotective therapies against PD (Geldenhuys *et al.*, 2004) and possibly AD as well.

3 Relevant compounds to be synthesised

The compounds that were selected for this study are illustrated in Table 2.1. With the exception of **1a-c**, all the compounds were polycyclic cage compounds conjugated to propargylamine or a derivative thereof.

Table 2.1. Compounds to be synthesised

COMPOUND	STRUCTURE	NAME
1a		Pentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane-8-11-dione
1b		1-Methyl-pentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane-8-11-dione
1c		Pentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane-11-one
2		1-Methyl-8-ethynyl-11-hydroxy-8,11-oxapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane
3		8-Phenylethynyl-8-hydroxy-pentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane
4a		8-(N)-Propargylamino-8,11-oxapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane

4b		1-Methyl-8-(N)-propargylamino-8,11-oxapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane
5a		8-Hydroxy-(N)-propargyl-8,11-azapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane
5b		1-Methyl-8-hydroxy-(N)-propargyl-8,11-azapentacyclo[5.4.1.0 ^{2,6} .0 ^{3,10} .0 ^{5,9}]undecane
6		N,N-Propargyl-adamantan-1-amine
7		N-Propargyl-N-benzyl-adamantan-1-amine

3.1 Rationale of compounds to be synthesised

Compounds **1a**, **1b** and **1c** will be synthesised as precursors to test compounds **2-5**. Compounds **4a** and **5a** will be synthesised from precursor **1a**, compounds **2**, **4b** and **5b** from precursor **1b** and compound **3** from precursor **1c**. Compounds **6** and **7** will be synthesised from amantadine.

Compounds **2** and **3** will be synthesised to evaluate the activity of an acetylene group, with compound **2** synthesised to evaluate the necessity and the activity of a terminal acetylene group, and compound **3** producing insight into the activity of an acetylene group between two non-polar groups, which is not terminal.

Compounds **4a**, **4b**, **5a**, **5b**, **6** and **7** will be synthesised to evaluate the effect of different polycyclic cage structures on the activity of the propargylamine group when combined in one structure. They will also be synthesised as an approach to combine the neuroprotective activity of propargylamine with that of the polycyclic cage structures. Compounds **5a**, **5b** and **7** will also be synthesised to evaluate the activity of the tertiary propargylamine. Compound **6** will give insight into the effect of a second propargyl group on the nitrogen.

4 Concluding remarks

Developing a compound that can offer symptomatic relief as well as change the course of a disease (i.e. an anti-apoptotic drug), can be of significant importance. Since propargylamine and propargylamine derivatives are anti-apoptotic agents as well as MAO inhibitors, they could serve as possible therapeutic agents with potential. In this study a series of propargylamine derivatives will be synthesised and evaluated as anti-apoptotic agents and MAO-B inhibitors. Such multifunctional drugs may have enhanced value in the treatment of neurodegenerative disorders such as PD and AD.

CHAPTER 3

SYNTHESIS OF RELEVANT COMPOUNDS

For the synthesis of the compounds that will be tested for anti-apoptotic as well as MAO-B inhibiting activity, several experimental procedures and methods, both conventional and modern, were utilised. Each compound was synthesised to evaluate the effect of a certain functional group on the activity. Some of these groups include a terminal acetylene group, an acetylene group between two non-polar groups, a secondary propargylamine connected to a polycyclic cage and a tertiary propargylamine in an aza polycyclic structure.

1 Standard experimental procedures

1.1 Reagents and chemicals

Reagents used in the synthesis of the compounds, were obtained from Sigma-Aldrich (U.K. & U.S.A.) and Merck Chemicals (Germany). Reaction and chromatography solvents were purchased from various commercial sources.

1.2 Instrumental methods

Melting point (MP) determination: Melting points were measured with a Gallenkamp and Stuart SMP10 melting point apparatus.

Infrared (IR) absorption spectrophotometry: Infrared spectra were recorded on a Shimadzo IR prestige – 21 Fourier transform infrared spectrophotometer using KBr.

Mass spectrometry (MS): Mass spectra were recorded on an analytical VG 7070E mass spectrometer. Ionisation took place at 70 eV.

Nuclear magnetic resonance (NMR) spectrometry: ^1H and ^{13}C NMR spectra were acquired on a Bruker Avance III 600 MHz spectrometer, with the ^1H spectra recorded at a frequency of 600.170 MHz and the ^{13}C spectra at 150.913 MHz. Tetramethylsilane (TMS) was used as internal standard, with CDCl_3 as solvent. All chemical shifts are reported in parts per million (ppm), relative to the internal standard. The following abbreviations are used to indicate the multiplicities of the respective signals: s - singlet; br s - broad singlet; d - doublet; dd - doublet of doublets; t - triplet; m - multiplet; and AB-q - AB quartet. The multiplicity of the identified carbons was confirmed with DEPT-spectra.

Microwave synthesis system: Microwave synthesis was performed using a CEM Discover™ focused closed vessel microwave synthesis system.

1.3 Chromatographic methods

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

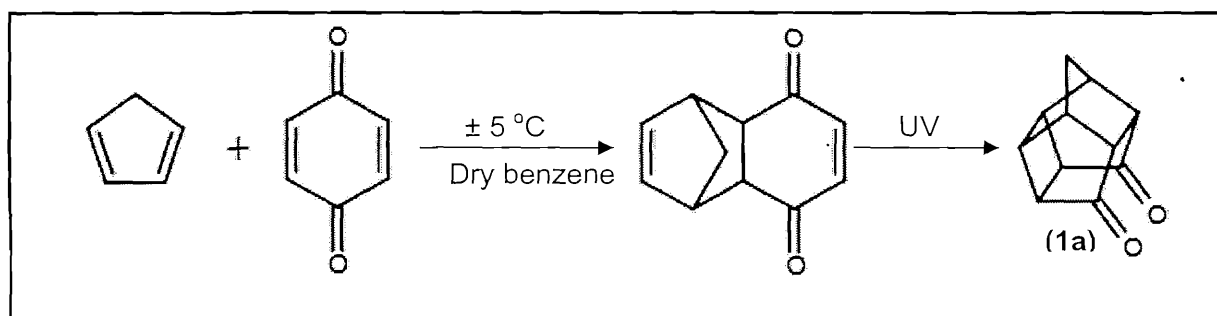
Thin layer chromatography (TLC): Thin layer chromatographic methods were used to monitor the progress of all chemical reactions. Visualisation of thin layer chromatography was achieved by making use of UV light at 254 nm and 360 nm, iodine vapours and heating the TLC plates after the application of ninhydrin 1.5%.

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

2 Synthetic procedures

2.1 Synthesis of precursor compounds

2.1.1 Synthesis of pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (1a)

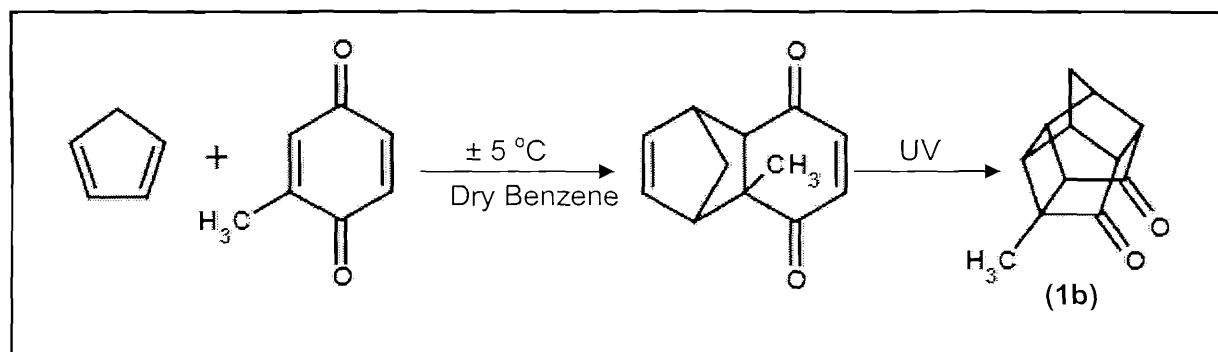


Scheme 3.1 The synthetic route of pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione

A stoichiometric volume of freshly monomerised cyclopentadiene (37.860 mL; 0.281 mol) was slowly added drop wise, in increments of 2 mL, to a solution of *p*-benzoquinone (30.400 g; 0.281 mol) in dry benzene (310 mL). The reaction mixture was maintained on an ice bath at a low temperature of $\pm 3\text{ }^{\circ}\text{C}$, to prevent the formation of the undesired Diels Alder di-adduct. The reaction mixture was protected from light and left to stir for 1 hour to reach completion, with the reaction progression being monitored by TLC. This was followed by the addition of activated charcoal (3 spatula scoops), and the mixture was stirred for a further 30–60 minutes. After filtration through Celite®, the solvent was removed under reduced

pressure and the residue, a yellow syrupy oil, was allowed to remain overnight in a dark fume hood. This allowed the full evaporation of the benzene, affording the Diels Alder adduct as yellow crystals. The Diels Alder adduct was dissolved in acetone (± 4 g/100 mL) and irradiated for ± 6 hours in a photochemical reactor (1000 W medium pressure UV lamp, Phillips HPA 1000/20). Discolouration of the solution confirmed completion of the cyclisation of the adduct. The solvent was subsequently removed under reduced pressure to afford the impure pentacyclic dione as a beige/yellow residue. This residue was purified by Soxhlett extraction in cyclohexane to produce the pure diketone as a waxy, off-white precipitate (yield: 37.890 g; 0.218 mol; 77.58%). The data from the physical characterisation of the obtained compound correlates with that of the pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (174.2 g/mol) as described by Cookson *et al.* (1958). Therefore no spectral data for this compound is presented.

2.1.2 Synthesis of 1-methyl-pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (1b)

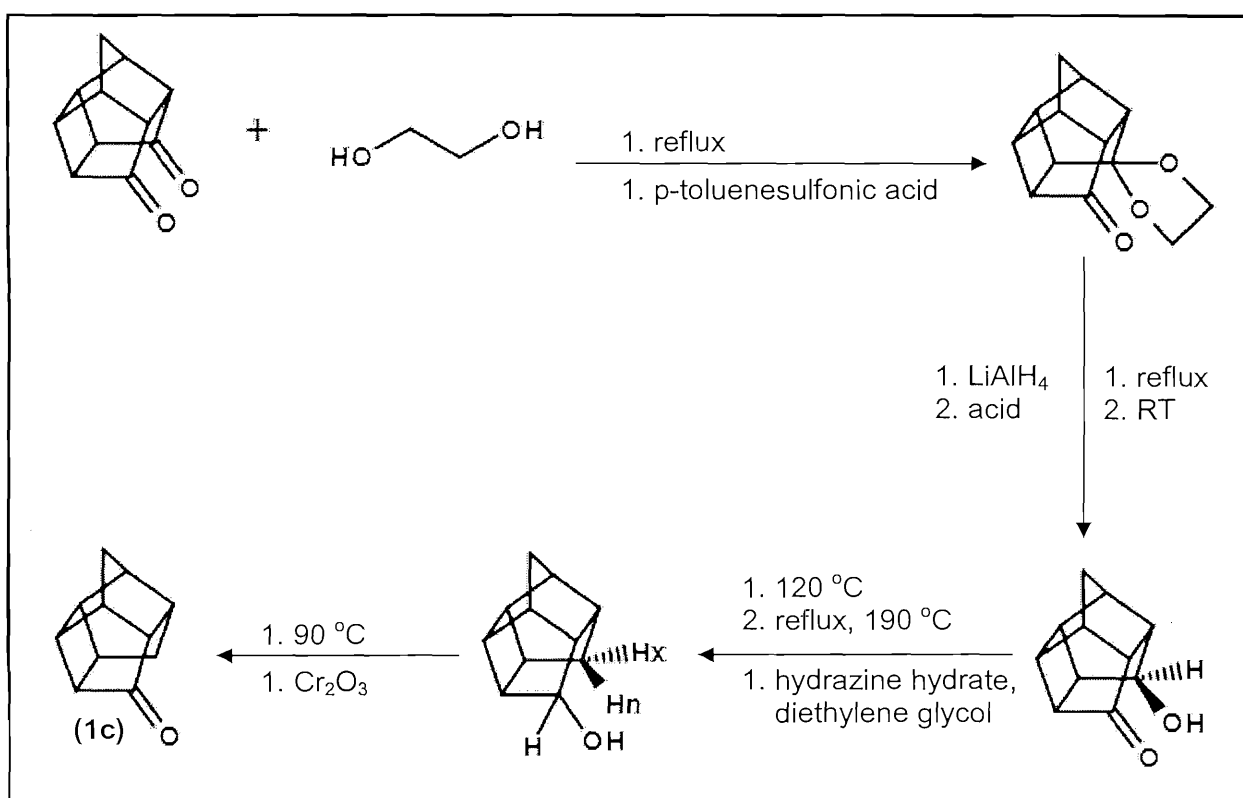


Scheme 3.2 The synthetic route of 1-methyl-pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione

A stoichiometric volume of freshly monomerised cyclopentadiene (35.610 mL; 0.265 mol) was slowly added drop wise, in increments of 2 mL, to a solution of methyl-*p*-benzoquinone (32.300 g; 0.265 mol) in dry benzene (310 mL). The reaction mixture was maintained on an ice bath at a low temperature of $\pm 3^\circ\text{C}$, to prevent the formation of the undesired Diels Alder di-adduct. The reaction mixture was protected from light and left to stir for 1 hour to reach completion, with the reaction progress being monitored by TLC. This was followed by the addition of activated charcoal (3 spatula scoops), and further stirring for 30–60 minutes. After filtration through Celite[®], the solvent was removed under reduced pressure and the formed red/brown oil was allowed to remain overnight in a dark fume hood. This allowed the full evaporation of the benzene with the Diels Alder adduct remaining as oil. The Diels Alder adduct was dissolved in acetone (± 4 g/100 mL) and irradiated for ± 6 hours in a photochemical reactor (1000 W medium pressure UV lamp; Phillips HPA 1000/20). A reduction of the colour intensity of the solution confirmed completion of the cyclisation

reaction. The solvent was subsequently removed under reduced pressure to afford the impure methyl-pentacyclic dione as an orange/brown oil. After several unsuccessful attempts of crystallisation of the product, it was purified by means of flash column chromatography on silica gel with ethyl acetate/petroleum ether/methylene chloride (1:1:1) as eluent, to afford the pure methyl-diketone as a yellow/brown oil (yield: 40.938 g; 0.217 mol; 82.23%). Data from the physical characterisation of the obtained compound correlates with that of the 1-methyl-pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (188.2 g/mol) as described by Marchand *et al.* (1984). Therefore no spectral data for this compound is presented.

2.1.3 Synthesis of pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-one (1c)



Scheme 3.3 The synthetic route of pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-one

A mixture of pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (20.000 g; 0.115 mol), ethylene glycol (7.138 g; 0.115 mol) and *p*-toluenesulfonic acid (0.263 g; 0.001 mol) was prepared in 100 mL benzene and was refluxed under Dean-Stark conditions for 3½ hours with stirring. The reaction was then cooled and slowly poured into an ice cold aqueous sodium carbonate (Na₂CO₃) solution (100 mL, 10%). This was followed by extraction with methylene chloride (DCM) (4 x 50 mL). The organic phase was dried over magnesium sulphate (MgSO₄), and removal of the solvent under reduced pressure afforded the oxo-acetal as a white precipitate (yield: 23.800 g; 94.83%). A suspension of the oxo-acetal

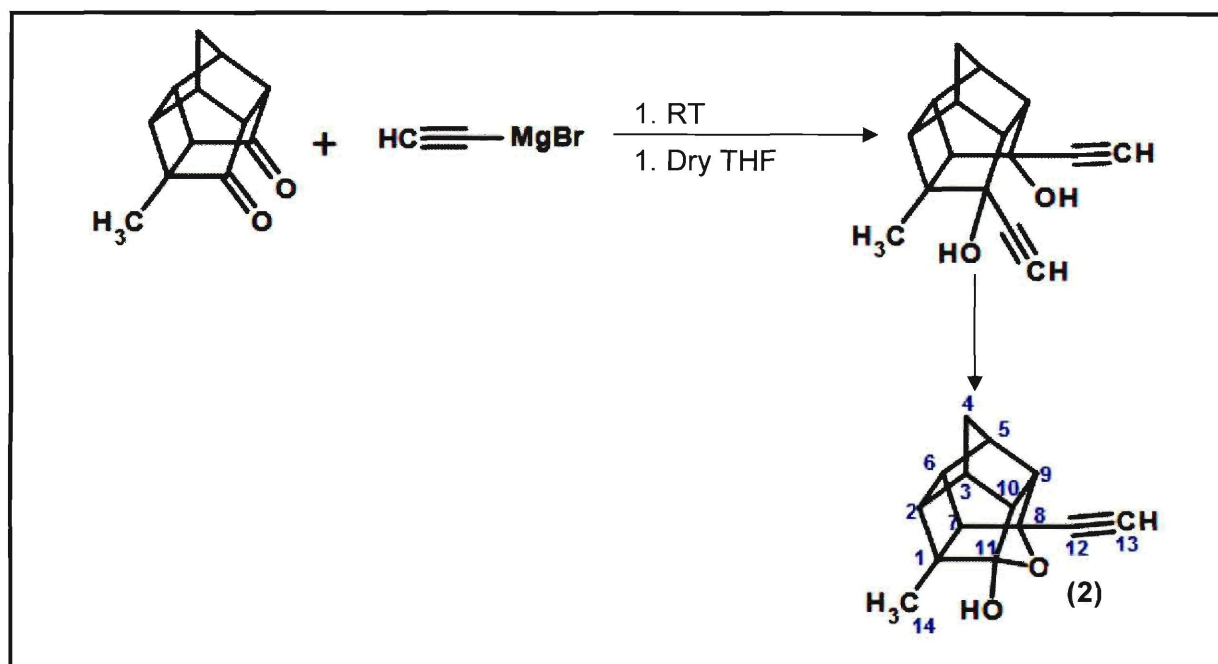
(ketal) (23.000 g; 0.105 mol) in dry ether (75 mL) was added to a stirred suspension of lithium aluminium hydride (LiAlH_4) (2.150 g; 0.057 mol) in dry ether (75 mL) over a period of 30 minutes. The reaction mixture was refluxed for 2 hours and then cooled to 0 °C. The excess LiAlH_4 was destroyed with aqueous ammonium chloride. The organic phase was concentrated under reduced pressure leaving a clear homogenous oil. The crude product was stirred in aqueous hydrochloric acid (400 mL, 6%) at room temperature (RT) for 2 hours. The reaction mixture was diluted with water (300 mL) and extracted to methylene chloride (4 x 100 mL). The organic phase was dried over MgSO_4 , and removal of the solvent gave the hydroxy-ketone (yield: 11.353 g; 61.36%). A mixture of the hydroxy-ketone (11 g; 0.062 mol) and hydrazine hydrate (22.360 mL, 98%) in diethylene glycol (180 mL) was maintained at 120 °C for 1½ hours. Potassium hydroxide (5 g) was added and the excess hydrazine and water was distilled until the temperature reached 190 °C. The reaction mixture was refluxed for 3 hours at 190 °C and then steam distilled. Extraction of the distillate with methylene chloride afforded the alcohol (yield: 3.301 g; 32.60%). Chromium trioxide (CrO_3) (3.700 g; 0.037 mol) in water (6 mL) was added to the alcohol (3 g; 0.018 mol) in aqueous acetic acid (60 mL; 94%), and the mixture was stirred at 90 °C for 4 hours. The reaction mixture was cooled, diluted with water (300 mL) and extracted with methylene chloride (3 x 40 mL). The methylene chloride extract was washed consecutively with water (2 x 100 mL), saturated aqueous sodium hydrogen carbonate (NaHCO_3) (2 x 100 mL), and water (100 mL). The organic phase was dried over MgSO_4 , and removal of the solvent under reduced pressure gave the monoketone (yield: 2.500 g; 0.016 mol; 84.38%). Data from the physical characterisation of the obtained compound correlates with that of the pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-11-one (160.2 g/mol) as described by Dekker and Oliver (1979). Therefore no spectral data for this compound is presented.

2.2 Synthesis of test compounds

2.2.1 Synthesis of 1-methyl-8-ethynyl-11-hydroxy-8,11-oxapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane (2)

A solution of methyl-pentacyclo-undecane dione (1.88 g; 10 mmol) in dry tetrahydrofuran (THF) (10 mL) was added to an excess of ethynyl magnesium bromide (1.0 M solution in THF) (2.838 mL; 22 mmol) under argon, whilst stirring. The resulting mixture was stirred at ambient temperature for 21 hours. The reaction mixture was poured over saturated aqueous ammonium chloride (NH_4Cl) solution (100 mL), and the resulting suspension was extracted with diethyl ether (3 x 25 mL). The combined organic extracts were washed sequentially with water (25 mL) and brine (25 mL). The solvent was dried (Na_2SO_4) and filtered, and the filtrate was concentrated under reduced pressure. The residue, pale yellow oil, was successfully purified by precipitation from a mixture of ethyl acetate/petroleum ether (1:3),

over a period of 24 hours at ambient temperature. This yielded the pure product as a light brown powder (yield: 0.762 g; 3.170 mmol; 15.87%).



Scheme 3.4 The synthetic route of 1-methyl-8-ethynyl-11-hydroxy-8,11-oxapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane

PHYSICAL DATA:

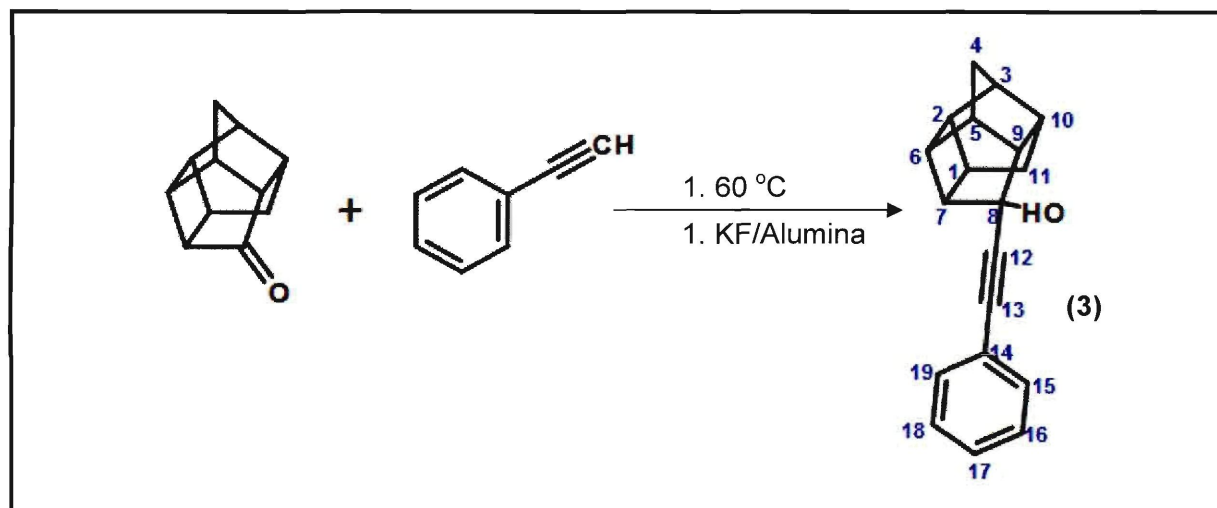
C₁₁H₁₀O₂N₁; **MW**, 214.3 g/mol; **mp.** 126 °C; **IR (KBr)** ν_{max} (Spectrum 1): 3260, 2125, 1211, 1031 cm⁻¹; **MS** (EI, 70 eV) m/z (Spectrum 9): 214 (M⁺), 186, 169, 158, 116, 91, 77, 39; **¹H NMR** (600 MHz, CDCl₃) δ_H (Spectrum 17): 3.72 (s, OH), 3.12 - 2.37 (m, 7H, H-2,3,5,6,7,9,10), 2.31 (s, 1H, H-13), 1.88:1.54 (AB-q, 2H, J = 10.4 Hz, H-4a,4b), 1.10 (s, 3H, CH₃); **¹³C NMR** (150 MHz, CDCl₃) δ_C (Spectrum 25): 116.87 (1C, C-11), 81.18 (1C, C-8), 75.30 (1C, C-12), 61.84 (1C, C-13), 57.24 (1C, C-9), 56.91 (1C, C-7), 50.75 (1C), 47.46 (1C), 45.22 (1C), 43.49 (1C, C-4), 41.56 (1C), 39.19 (1C, C-1), 15.29 (1C, C-14).

STRUCTURE ELUCIDATION:

Several characteristic groups, which include the oxa-group between carbon 8 and 11, the ethynyl group at carbon 8, the hydroxyl group at carbon 11, and the methyl group at carbon 1 of the cage as well as carbons 8, 11 and 4 were identified to confirm the structure. On the infrared spectrum the C-O bond (oxa-group) was present at 1031 cm⁻¹, the C-O bond (hydroxyl-group) at 1211 cm⁻¹, the C≡C bond (ethynyl group) at 2125 cm⁻¹, and the ≡C-H bond (ethynyl group) at 3260 cm⁻¹. On the ¹H NMR spectrum, the AB-q system of the protons on carbon 4 (1.88:1.54 ppm), the singlet of the methine proton (2.31 ppm), and the singlet integrating for the three protons of the methyl (1.10 ppm), as well as the hydrogen of

the alcohol group (3.72) was identified. On the ^{13}C NMR spectrum the unsaturated and deshielded carbons are detectable downfield from the others and include carbons 8, 11, 12 and 13. The single methyl group (C-14) is also clearly present at 15.29 ppm. The molecular ion from the MS confirmed a mass of 214.

2.2.2 Synthesis of 8-phenylethynyl-8-hydroxy-pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane (3)



Scheme 3.5 The synthetic route of 8-phenylethynyl-8-hydroxy-pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane

Neutral alumina (60–80 mesh, 30 g) in water (150 mL) was added to a stirred solution of potassium fluoride (20 g) in water (150 mL). After 30 minutes the water was evaporated in a rotary evaporator at 80 °C. When most of the water had been removed, the remaining mixture was heated to, and maintained at 140–150 °C under vacuum (5 mmHg) overnight to afford 50 g of KF/alumina reagent.

Phenylacetylene (255.350 mg; 2.500 mmol), pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-one (480,650 mg; 3 mmol), and KF/alumina (2.500 g) were mixed in a 25 mL flask at 60 °C. The progress of the reaction was monitored by TLC. After 12 hours the reaction mixture was washed with petroleum ether (PE), filtered, and the solvent was evaporated under vacuum affording a clear yellow oil. The residue was purified by means of column chromatography, by using a versa flash silica-gel column with petroleum ether/ethyl acetate (10:2) as eluent, to afford the product as a light yellow wax (yield: 480 mg; 1.830 mmol; 73.18%).

PHYSICAL DATA:

C₁₁H₁₀O₂N₁; **MW**, 262.4 g/mol; **mp.** 90 °C; **IR (KBr)** $\nu_{\max}$ (Spectrum 2): 3069, 2225, 1599, 1491, 1125, 752 cm^{-1} ; **MS** (EI, 70 eV) m/z (Spectrum 10): 262 (M^+), 196, 183, 165, 129, 115, 91, 77; **^1H NMR** (600 MHz, CDCl_3) δ_{H} (Spectrum 18): 7.36 – 7.13 (m, 5H, H-15,16,17,18,19),

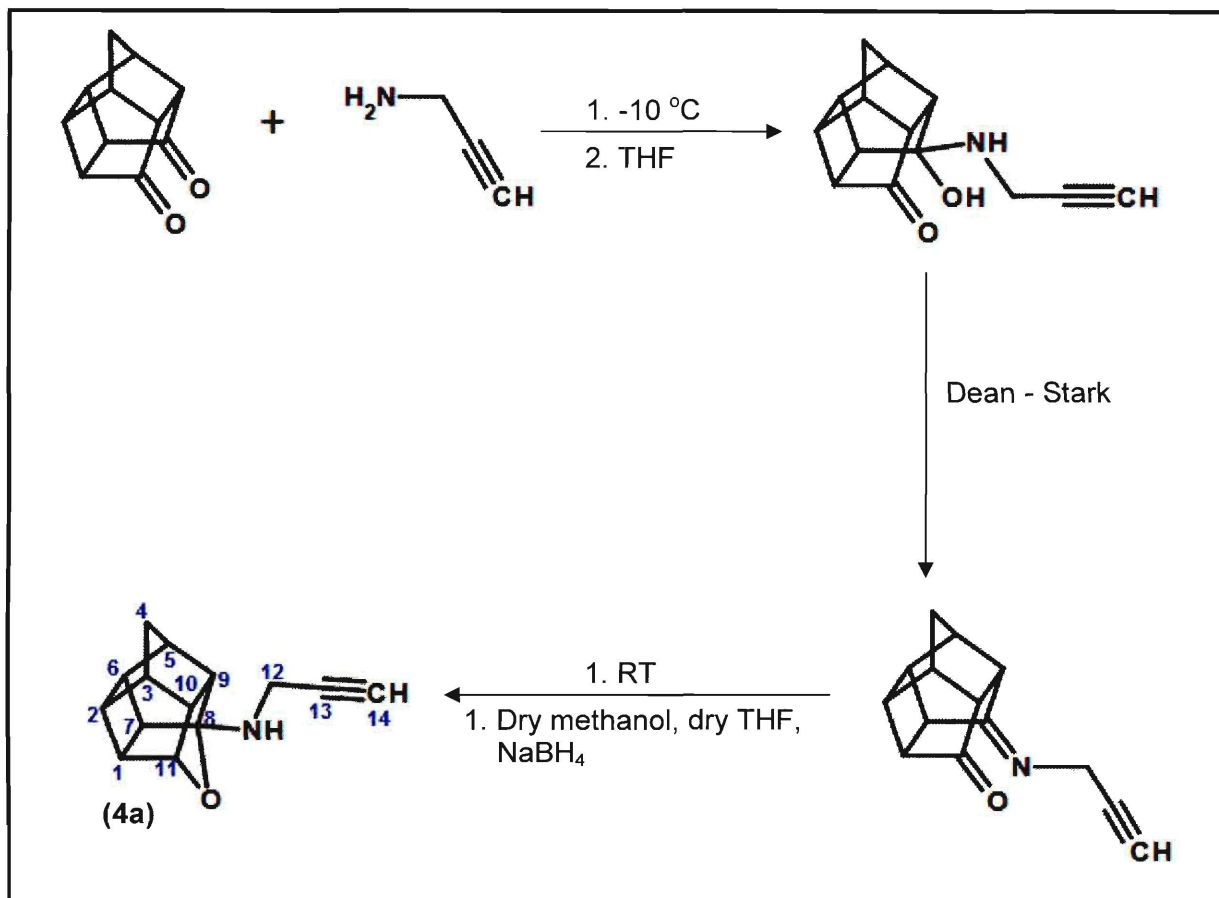
2.77 – 2.28 (m, 8H, H-1,2,3,5,6,7,9,10), 1.93:1.60 (AB-q, 2H, H-11a,11b), 1.72:1.18 (AB-q, 2H, J = 10.4 Hz, H-4a,4b); ^{13}C NMR (150 MHz, CDCl_3) δ_c (Spectrum 26): 131.51 (1C, C-14), 128.23 (2C, C-15,19), 128.10 (2C, C-16,18), 123.02 (1C, C-17), 94.15 (1C, C-8), 83.21 (1C, C-13), 75.91 (1C, C-12), 51.50 (1C), 47.27 (1C), 45.20 (1C), 44.95 (1C), 42.73 (1C, C-4), 41.50 (1C), 40.61 (1C), 36.62 (1C), 34.73 (1C), 28.98 (1C, C-11).

STRUCTURE ELUCIDATION:

Several characteristic groups including the aromatic ring, the ethynyl group between the cage and the benzene, and the hydroxyl group on carbon 8, as well as carbons 8, 11 and 4 of the cage, were identified to confirm the structure. On the infrared spectrum the C-O bond (hydroxyl-group) is present at 1125 cm^{-1} , the $\text{C}\equiv\text{C}$ bond (ethynyl group) at 2225 cm^{-1} , the C-H bonds (aromatic group) at 3069 cm^{-1} and 752 cm^{-1} , and the C=C bonds (aromatic group) at 1599 cm^{-1} and 1491 cm^{-1} . On the ^1H NMR spectrum, the AB-q system of the protons on carbon 4 (1.72:1.18 ppm) and 11 (1.93:1.60 ppm), and the aromatic hydrogens (7.36 – 7.13 ppm) were evident. On the ^{13}C NMR spectrum the aromatic and methine carbons, as well as those of the cage were identified. The molecular ion from the MS confirmed a mass of 262.

2.2.3 Synthesis of 8-(N)-propargylamino-8,11-oxapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane (4a)

Pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (5 g; 0.029 mol) was dissolved in tetrahydrofuran (THF) (50 mL) and cooled to $\pm -10\text{ }^\circ\text{C}$, while stirring on an external bath, containing an acetone/NaCl/ice mixture. Propargylamine (1.600 g; 0.029 mol) was added drop-wise, with continued stirring of the reaction mixture at lowered temperature. The carbinolamine started precipitating after approximately 15 minutes, but the reaction was allowed to stir for an additional 30 minutes to reach completion. The carbinolamine was isolated by filtration and washed with ice cold THF. Water was removed azeotropically by refluxing the material in dry benzene (60 mL), under Dean–Stark dehydrating conditions for 1 hour, or until no more water was collected in the trap. The benzene was removed under reduced pressure, which yielded the Schiff base as yellow oil. The Schiff base (imine) was then dissolved in a mixture of anhydrous methanol (MeOH) (30 mL) and anhydrous tetrahydrofuran (THF) (150 mL). Reduction was carried out by adding sodium borohydride (NaBH_4) (1.500 g; 0.040 mol) in excess, and stirring the mixture for 24 hours at room temperature. The solvents were removed under reduced pressure, the residue suspended in water (100 mL) and extracted with methylene chloride (DCM) (4 x 50 mL). The combined organic fractions were washed with water (2 x 100 mL), dried over anhydrous MgSO_4 and evaporated under reduced pressure to yield milky yellowish oil.



Scheme 3.6 The synthetic route of 8-(N)-propargylamino-8,11-oxapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane

Purification of the product mixture was accomplished using column chromatography on silica gel, with ethyl acetate/methylene chloride/petroleum ether (1:1:1) as eluent. This yielded the desired amine as a light yellow precipitate. Recrystallisation from absolute ethanol rendered the final product as a colourless microcrystalline solid (yield: 600 mg; 2.827 mmol; 9.75%).

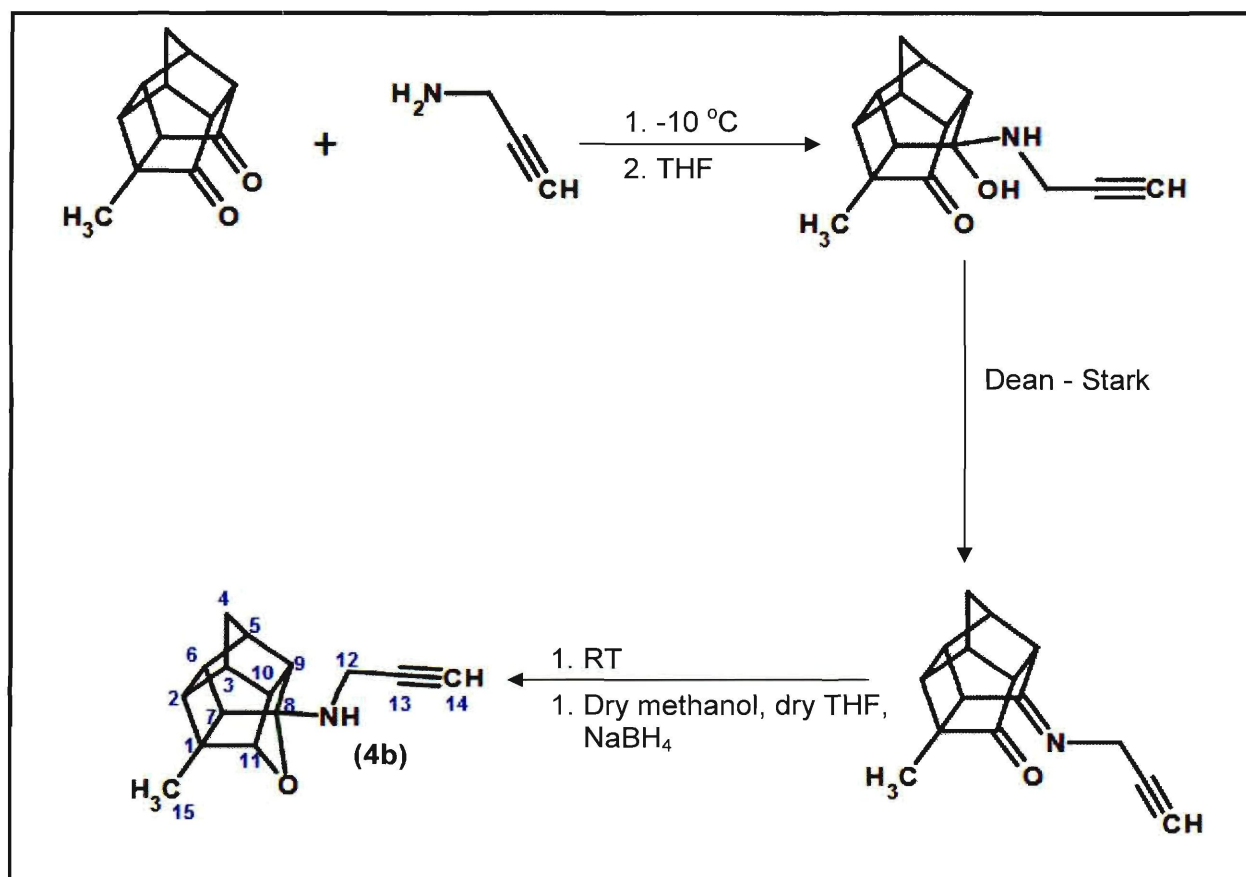
PHYSICAL DATA:

C₁₁H₁₀O₂N₁; **MW**, 212.3 g/mol; **mp.** 113 °C; **IR (KBr)** ν_{max} (Spectrum 3): 3306, 3238, 2124, 1485, 1153, 1000 cm⁻¹; **MS** (EI, 70 eV) m/z (Spectrum 11): 213 [(M⁺)+1], 212 (M⁺), 184, 134, 118, 91, 77, 39; **¹H NMR** (600 MHz, CDCl₃) δ_H (Spectrum 19): 4.63 (t, 1H, J = 5 Hz, H-11), 3.57:3.56 (dd, 2H, J = 2.5 Hz, H-12a,12b), 2.80 – 2.39 (m, 8H, H-1,2,3,5,6,7,9,10), 2.26 (br s, NH), 2.21 (s, 1H, H-14), 1.88:1.52 (AB-q, 2H, J = 10.4 Hz, H-4a,4b); **¹³C NMR** (150 MHz, CDCl₃) δ_C (Spectrum 27): 108.98 (1C, C-8), 82.81 (1C, C-11), 82.76 (1C, C-13), 70.92 (1C, C-14), 55.46 (1C, C-7/9), 54.76 (1C, C-7/9), 44.84 (1C), 44.65 (1C), 44.54 (1C), 43.3 (1C), 43.07 (1C, C-12), 42.04 (1C), 41.54 (1C), 33.07 (1C).

STRUCTURE ELUCIDATION:

In confirming the structure of **4a**, several groups, which include the oxa-group between carbon 8 and 11, the ethynyl and amine group of the propargylamine moiety, as well as carbons, 8, 11 and 4 of the cage were identified. On the infrared spectrum the C-O bond (oxa-group) is present at 1000 cm^{-1} , the $\text{C}\equiv\text{C}$ bond (propargylamine moiety) at 2124 cm^{-1} , the $\equiv\text{C-H}$ bond (propargylamine moiety) at 3238 cm^{-1} , the C-N bond (propargylamine moiety) at 1153 cm^{-1} , and the N-H bond (propargylamine moiety) at 1485 cm^{-1} and 3306 cm^{-1} . On the ^1H NMR spectrum, the AB-q system of the protons on carbon 4 (1.88:1.52 ppm), the triplet of carbon 11 (4.63 ppm) and the doublet of doublets of carbon 12 (3.57:3.56), the terminal hydrogen (2.21 ppm) of the acetylene group, as well as the nitrogen hydrogen (2.26 ppm) is evident of the structure of **4a**. The typical signals indicating C-8,11,13 and 14 were indicated on the ^{13}C NMR spectrum. The molecular ion from the MS confirmed a mass of 212.

2.2.4 Synthesis of 1-methyl-8-(N)-propargylamino-8,11-oxapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane (4b)



Scheme 3.7 The synthetic route of 1-methyl-8-(N)-propargylamino-8,11-oxapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane

1-Methyl-pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (5.460 g; 0.029 mol) was dissolved in tetrahydrofuran (THF) (50 mL) and cooled to ± -10 °C, while stirring on an external bath containing an acetone/NaCl/ice mixture. Propargylamine (1.600 g; 0.029 mol) was slowly added with continued stirring of the reaction mixture at lowered temperature. The reaction mixture was stirred for an additional 1½ hours to reach completion. The THF was removed under reduced pressure, affording the carbinolamine as red/brown oil. Water was removed azeotropically by refluxing this material in dry benzene (60 mL), under Dean–Stark dehydrating conditions for 1 hour, or until no more water was collected in the trap. The benzene was removed under reduced pressure, which yielded the Schiff base as brown oil. The Schiff base (imine) was dissolved in a mixture of anhydrous methanol (MeOH) (30 mL) and anhydrous THF (150 mL). Reduction was carried out by adding sodium borohydride (1.500 g; 0.040 mol) in excess and stirring the mixture for 24 hours at room temperature. The solvents were removed under reduced pressure, the residue suspended in water (100 mL) and extracted with methylene chloride (DCM) (4 x 50 mL). The combined organic fractions were washed with water (2 x 100 mL), dried over anhydrous MgSO₄ and evaporated under reduced pressure to yield milky dark brown oil. Purification of the product mixture was accomplished using column chromatography, with ethyl acetate/methylene chloride/petroleum ether (1:1:1) as eluent, yielding the desired amine as a dark brown oil (Yield: 1.753 g; 7.746 mmol; 26.71%).

PHYSICAL DATA:

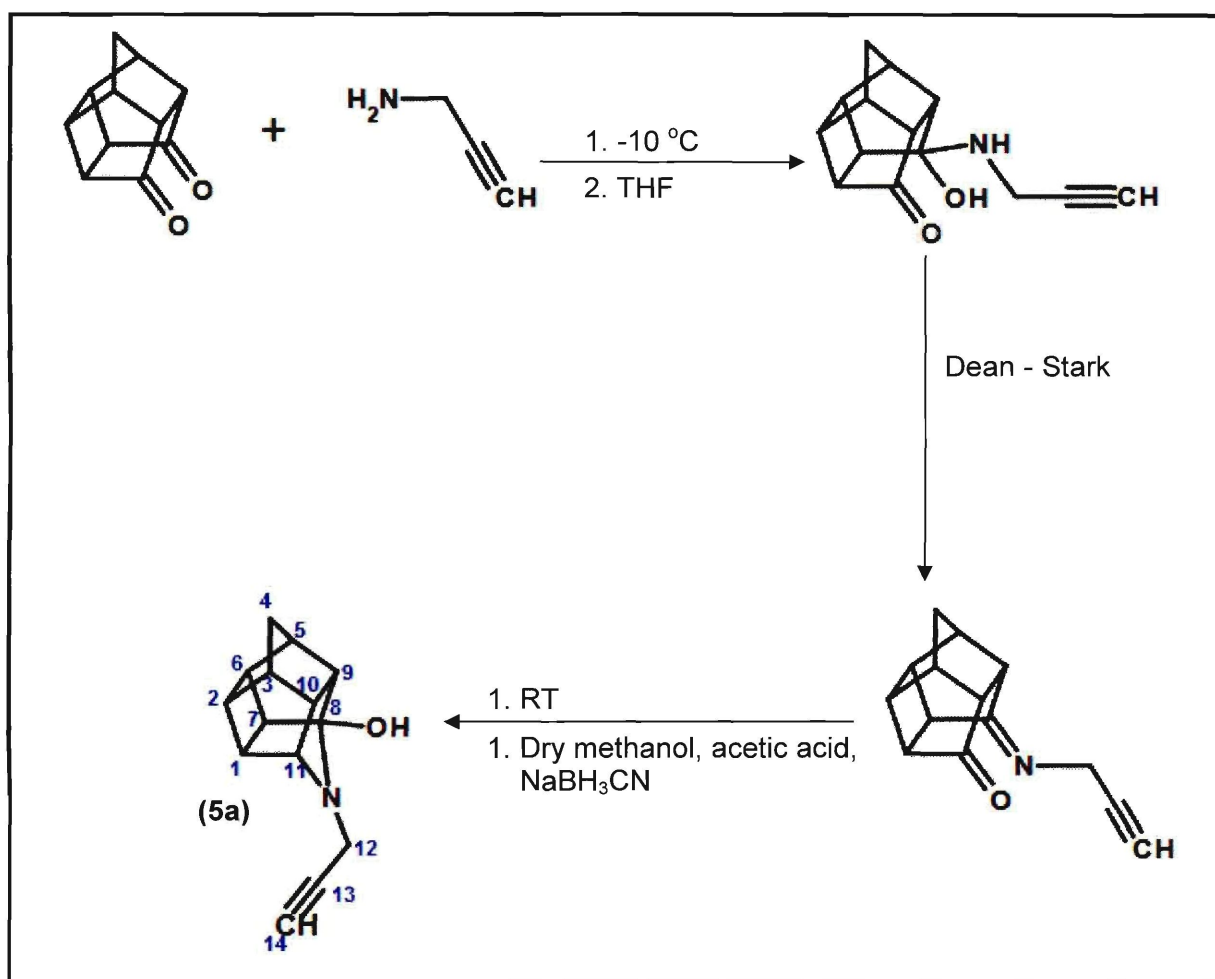
C₁₁H₁₀O₂N₁; **MW**, 226.3 g/mol; **IR (KBr)** ν_{max} (Spectrum 4): 3310, 3250, 2100, 1452, 1153, 1007cm⁻¹; **MS** (EI, 70 eV) m/z (Spectrum 12): 227 [(M⁺)+1], 226 (M⁺), 212, 198, 184, 158, 145, 131, 91, 77, 39; **¹H NMR** (600 MHz, CDCl₃) δ_H (Spectrum 20): 4.08 (d, 1H, J = 4.4 Hz, H-11), 3.57 (m, 2H, H-12a,12b), 2.73 – 2.20 (m, 7H, H-2,3,5,6,7,9,10), 2.14 (br s, NH), 2.06 (d, 1H, J = 5.28 Hz, H-14), 1.86:1.51 (AB-q, 2H, J = 10.4 Hz, H-4a,4b), 1.15 (s, 3H, CH₃); **¹³C NMR** (150 MHz, CDCl₃) δ_C (Spectrum 28): 109.73 (1C, C-8), 87.15 (1C, C-11), 82.65 (1C, C-13), 70.95 (1C, C-14), 54.97 (1C, C-9), 54.66 (1C, C-7), 50.74 (1C), 46.99 (1C), 43.58 (1C), 43.18 (1C), 38.91 (1C, C-1), 33.07 (1C, C-12), 19.85 (1C, C-15).

STRUCTURE ELUCIDATION:

In addition to the characteristic groups described under **4a**, the methyl signal was observed at 1.15 ppm on the ¹H NMR spectrum and at 19.85 ppm on the ¹³C NMR spectrum. The proton signal for C-11 showed a slight shift to 4.08 ppm on the ¹H NMR spectrum. The molecular ion from the MS confirmed a mass of 226.

2.2.5 Synthesis of 8-hydroxy-(N)-propargyl-8,11-azapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane (5a)

Pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (5 g; 0.029 mol) was dissolved in tetrahydrofuran (THF) (50 mL) and cooled to $\pm -10\text{ }^{\circ}\text{C}$, while stirring on an external bath, containing an acetone/NaCl/ice mixture. Propargylamine (1,600 g; 0.029 mol) was added slowly with continued stirring of the reaction mixture at lowered temperature. The carbinolamine started precipitating after approximately 60 minutes, but the reaction was stirred for an additional 45 minutes to reach completion. This carbinolamine was isolated by filtration.



Scheme 3.8 The synthetic route of 8-hydroxy-(N)-propargyl-8,11-azapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane

Water was removed azeotropically by refluxing this material in dry benzene (60 mL), under Dean–Stark dehydrating conditions for 1 hour, or until no more water was collected in the trap. The benzene was removed under reduced pressure and the Schiff base was used without further purification. It was dissolved in a solution of acetic acid (15 mL) and dry methanol (250 mL). To the resulting solution was added sodium cyanoborohydride

(NaBH₃CN) (2.510 g; 40 mmol) portion wise, with stirring at room temperature over a period of 5 minutes. The resulting mixture was stirred at room temperature for 2 hours. The reaction mixture was then concentrated under reduced pressure, and water (100 mL) was added to the residue. The resulting suspension was stirred and solid sodium bicarbonate was added portion wise until evolution of carbon dioxide ceased. Excess solid sodium bicarbonate (2.000 g) was added, and the aqueous suspension was extracted with methylene chloride (4 x 50 mL). The combined extracts were washed with water (2 x 100 mL), dried with anhydrous magnesium sulphate and filtered. The filtrate was concentrated under reduced pressure. A yellow solid was thereby obtained. Purification of the product mixture was accomplished using column chromatography on silica, with ethyl acetate/methylene chloride/ethanol (10:5:1) as eluent. The desired amine was obtained as an off-white powder. Recrystallisation from cyclohexane rendered the final product as an off-white microcrystalline solid (Yield: 960 mg; 4.501 mmol; 15.52%).

PHYSICAL DATA:

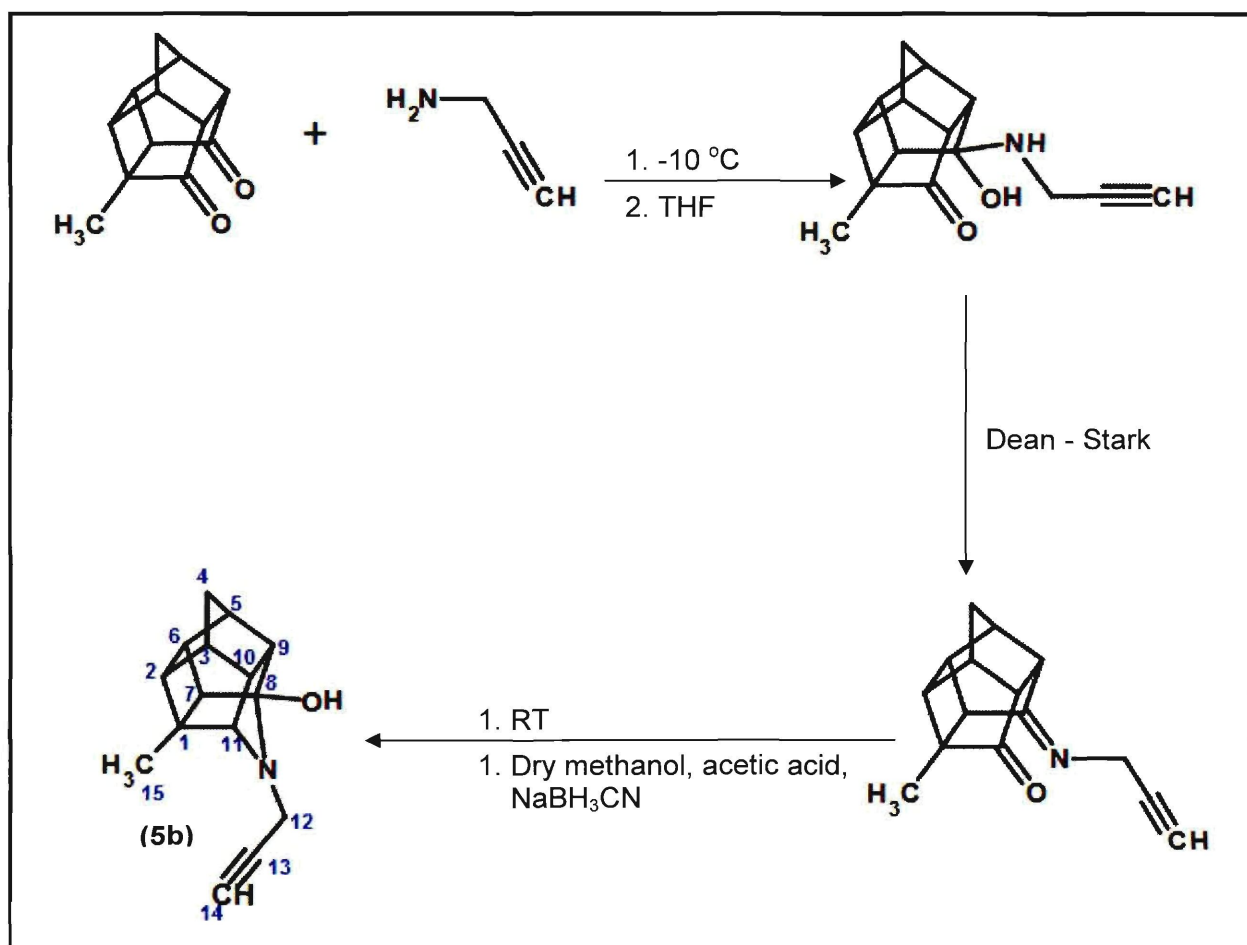
C₁₁H₁₀O₂N₁; **MW**, 213.3 g/mol; **mp.** 140 °C; **IR (KBr)** ν_{max} (Spectrum 5): 3225, 3100, 2114, 1120, 1070, cm⁻¹; **MS** (EI, 70 eV) m/z (Spectrum 13): 214 [(M⁺)+1], 213 (M⁺), 196, 174, 147, 134, 118, 91, 77, 39; **¹H NMR** (600 MHz, CDCl₃) δ_H (Spectrum 21): 3.71 (s, 1H, H-12), 3.35 (d, 1H, J = 16.5 Hz, H-11), 3.00 – 2.43 (m, 8H, H-1,2,3,5,6,7,9,10), 2.25 (s, 1H, H-14), 1.82:1.49 (AB-q, 2H, J = 10.4 Hz, H-4a,4b); **¹³C NMR** (150 MHz, CDCl₃) δ_c (Spectrum 29): 125.53 (1C, C-8), 81.23 (1C, C-13), 71.37 (1C, C-14), 65.52 (1C, C-11), 45.62 (1C, C-7/9), 43.12 (1C, C-7/9), 41.74 (1C, C-4), 41.60 (1C), 36.79 (1C), 30.33 (1C, C-12).

STRUCTURE ELUCIDATION:

Groups, which deviate from those of the oxa compounds, are the aza-group between carbon 8 and 11 and hydroxyl group on carbon 8. The C-N bond is now present at 1120 cm⁻¹ on the infrared spectrum. Instead of the N-H bond of compounds **4a** and **4b**, the aza compounds, **5a** and **5b**, have an O-H bond, which is present as a broad peak between 3600 and 2000 cm⁻¹. On the ¹H NMR spectrum, the hydrogen of carbon 11 is present as a doublet at 3.35 ppm, and the hydrogens connected to carbon 12 give a singlet at 3.71 ppm. On the ¹³C NMR spectrum carbons 8, 11, 13 and 14 all lie downfield compared to the others. The molecular ion from the MS confirmed a mass of 213.

2.2.6 Synthesis of 1-methyl-8-hydroxy-(N)-propargyl-8,11-azapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane (5b)

1-Methyl-pentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8-11-dione (10.658 g; 56.620 mmol) was dissolved in tetrahydrofuran (THF) (50 mL) and cooled to $\pm -10\text{ }^{\circ}\text{C}$, while stirring on an external bath containing an acetone/NaCl/ice mixture. Propargylamine (3.119 g; 56.620 mmol) was added drop wise with continued stirring of the reaction mixture at lowered temperature. The reaction mixture was stirred for 5½ hours to reach completion. The THF was removed under reduced pressure, affording red/brown oil, the carbinolamine. Water was removed azeotropically by refluxing this material in dry benzene (60 mL), under Dean–Stark dehydrating conditions for 1 hour, or until no more water was collected in the trap. The benzene was removed under reduced pressure, which yielded the Schiff base as dark brown oil. The Schiff base was used without further purification. It was dissolved in a solution of acetic acid (30 mL) in dry methanol (500 mL).



Scheme 3.9 The synthetic route of 1-methyl-8-hydroxy-(N)-propargyl-8,11-azapentacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}]undecane

To the resulting solution sodium cyanoborohydride (3.970 g; 63 mmol) was added portion wise with stirring at room temperature over a period of 5 minutes. The resulting mixture was

stirred at room temperature for 14 hours. The reaction mixture was then concentrated under reduced pressure, and water (150 mL) was added to the residue. The resulting suspension was stirred, and solid sodium bicarbonate was added portion wise until evolution of carbon dioxide ceased. Excess solid sodium bicarbonate (3.000 g) was added, and the aqueous suspension was extracted with methylene chloride (4 x 50 mL). The combined extracts were washed with water (2 x 100 mL), dried with anhydrous magnesium sulphate, and filtered. The filtrate was concentrated under reduced pressure, leaving deep orange oil as residue. Purification of the product mixture was accomplished using column chromatography on silica, with ethyl acetate/tetrahydrofuran (5:1) as eluent. This yielded the desired amine as light yellow oil, which precipitated when ethanol was added. Recrystallisation from ethanol (EtOH) rendered the final product as a light yellow microcrystalline solid (Yield: 555 mg; 2.442 mmol; 4.31%).

PHYSICAL DATA:

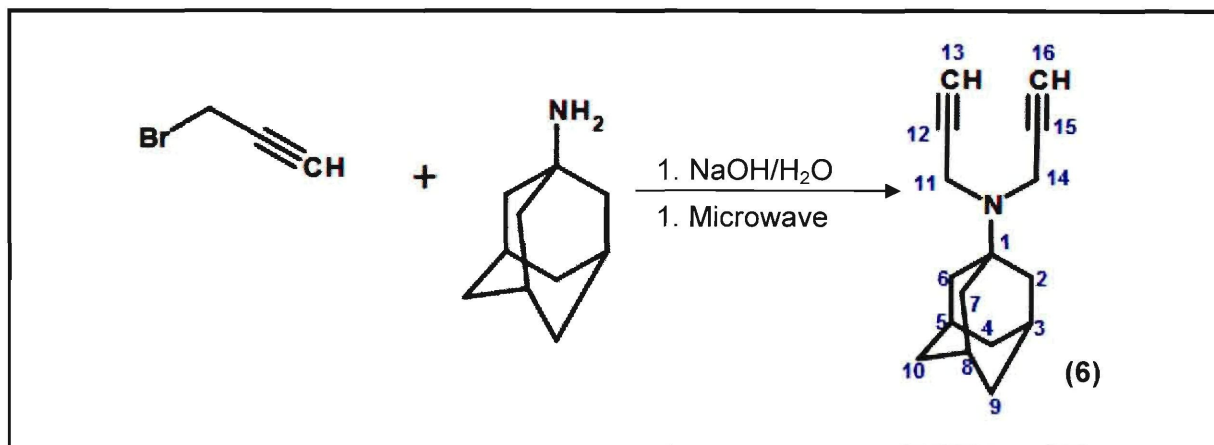
C₁₁H₁₀O₂N₁; **MW**, 227.3 g/mol; **mp.** 125 °C; **IR (KBr)** ν_{max} (Spectrum 6): 3225, 3078, 2124, 1119, 1070 cm⁻¹; **MS** (EI, 70 eV) m/z (Spectrum 14): 228 [(M⁺)+1], 227 (M⁺), 188, 134, 118, 91, 77, 39; **¹H NMR** (600 MHz, CDCl₃) δ_H (Spectrum 22): 3.46 - 3.34 (m, 1H, H-11), 3.22:3.09 (d, 2H, J = 17.3 Hz, H-12a,12b), 2.57 – 2.09 (m, 8H, H-2,3,5,6,7,9,10,14), 1.60:1.06 (AB-q, 2H, J = 10.4 Hz, H-4a,4b), 1.19 (s, 3H, CH₃); **¹³C NMR** (150 MHz, CDCl₃) δ_c (Spectrum 30): 125.53 (1C, C-8), 80.31 (1C, C-13), 72.68 (1C, C-14), 57.40 (1C, C-11), 46.62 (1C, C-9), 46.10 (1C, C-7), 45.09 (1C), 41.54 (1C, C-4), 37.63 (1C), 36.33 (1C), 34.52 (1C), 30.32 (1C, C-12), 21.98 (1C, C-15).

STRUCTURE ELUCIDATION:

Besides the characteristic groups described for **5a**, the methyl signal was observed at 1.19 ppm on the ¹H NMR spectrum and at 21.98 ppm on the ¹³C NMR spectrum. The proton signal for C-11 showed a slight shift to 3.40 ppm on the ¹H NMR spectrum.

2.2.7 Synthesis of N,N-dipropargyl-adamantan-1-amine (6)

Excess propargylbromide (0.595 mL; 6.677 mmol), amantadine (302.49 mg; 2 mmol) and aqueous sodium hydroxide (NaOH) solution (180 mg in 6 mL water; 4.5 mmol) were placed in a round-bottom glass flask equipped with a condenser and a magnetic stirrer. The flask was placed in a CEM discover focused microwave synthesis system, and subjected to microwave irradiation at 80–100 °C (power 250 Watt) for 25 minutes.



Scheme 3.10 The synthetic route of N,N-dipropargyl-adamantan-1-amine

After completion of the reaction, the product was extracted into ethyl acetate. The solvent was then removed under reduced pressure. The unreacted amantadine was removed from the residue by adding acetone to the residue and collecting the amantadine by filtration. The acetone was then removed under reduced pressure from the filtrate, affording orange crystals which were recrystallised out of ethyl acetate (EtOAc) to afford the pure product as light yellow crystals (412 mg; 1.812 mmol; 90.61%).

PHYSICAL DATA:

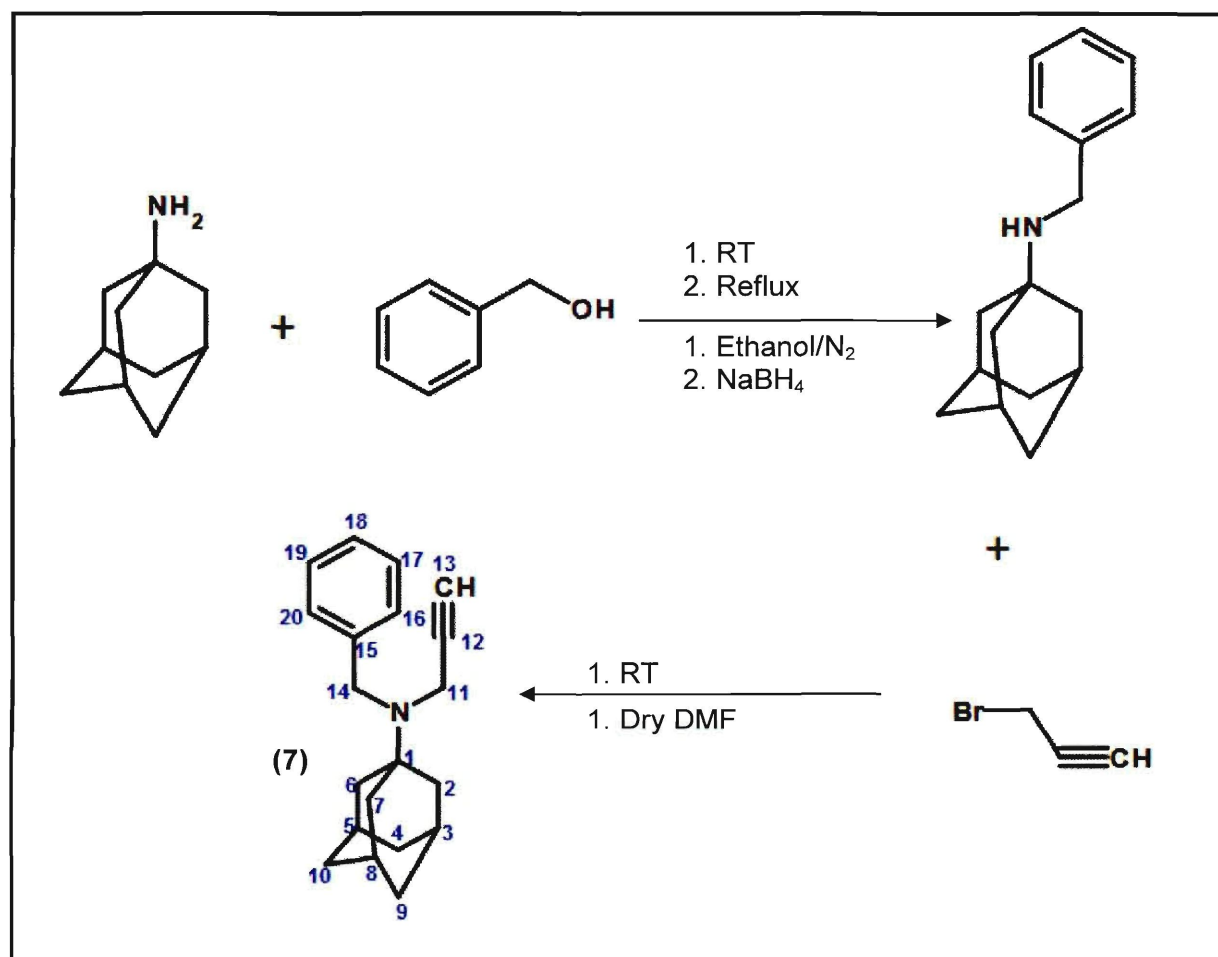
C₁₁H₁₀O₂N₁; **MW**, 227.35 g/mol; **mp.** 76 °C; **IR (KBr)** $\nu_{\max}$ (Spectrum 7): 3237, 2099, 1119 cm^{-1} ; **MS** (EI, 70 eV) m/z (Spectrum 15): 228 [(M⁺)+1], 227 (M⁺), 184, 144, 132, 91, 79, 53, 39; **¹H NMR** (600 MHz, CDCl₃) δ_{H} (Spectrum 23): 3.65 (s, 4H, H-11a,11b,14a,14b), 2.17 (s, 2H, H-13,16), 2.06 (s, 3H, H-3,5,8), 1.80 (s, 6H, H-2a,2b,6a,6b,7a,7b), 1.63 – 1.46 (m, 6H, H-4a,4b,9a,9b,10a,10b); **¹³C NMR** (150 MHz, CDCl₃) δ_{C} (Spectrum 31): 82.12 (2C, C-12,15), 72.08 (2C, C-13,16), 55.45 (1C, C-1), 39.96 (3C, C-3,5,8), 36.54 (3C, C-2,6,7), 35.03 (2C, C-11,14), 29.70 (3C, C-4,9,10).

STRUCTURE ELUCIDATION:

Characteristic groups of compound **6** to be identified, include the ethynyl and amine group of the propargylamine moiety. On the ¹H NMR spectrum the protons of the propargyl group is evident, all lying more downfield than the protons of the amantadine cage. On the ¹³C NMR spectrum the peaks of C-11, 12, 13, 14, 15 and 16 were present. The molecular ion from the MS confirmed a mass of 227.

2.2.8 Synthesis of N-propargyl-N-benzyl-adamantan-1-amine (7)

A solution of amantadine (5 g; 33.056 mmol) and benzaldehyde (3.51 g; 33.073 mmol) in ethanol (EtOH) (60 mL) was stirred for 4 days at ambient temperature under nitrogen atmosphere. Solid sodium borohydride (NaBH_4) (2.5 g; 66.085 mmol) was then added slowly in small portions over 30 minutes, and stirring of the resulting suspension was continued at room temperature for 30 minutes under nitrogen atmosphere. The reaction mixture was refluxed for 12 hours. After cooling to ambient temperature, the mixture was diluted with ethanol (EtOH) (60 mL), and the excess sodium borohydride was destroyed by adding aqueous hydrochloric acid (HCl) (10 mL, 5 M) drop wise. The reaction mixture was then made alkaline by adding aqueous sodium hydroxide (NaOH) solution, until the pH reached 12. Finally, the desired product was extracted to methylene chloride (DCM) (4 x 10 mL) and dried over anhydrous magnesium sulphate (MgSO_4). The solvent was evaporated under reduced pressure to afford the intermediate product, N-benzyl-adamantan-1-amine, as pure white crystals (yield: 4.757 g; 19.6 mmol; 59.29%). The intermediate product was used without any further purification.



Scheme 3.11 The synthetic route of N-propargyl-N-benzyl-adamantan-1-amine

A solution was made of N-benzyl-adamantan-1-amine (4.757 g; 19.6 mmol) and excess propargylbromide (2.3 ml; 25.81 mmol) in dry dimethylformamide (DMF) (30 mL), and left for 24 hours to stir at ambient temperature. After this period of time the mixture was diluted with water (50 mL), and an extraction was done in methylene chloride (DCM) (3 x 50 mL) and dried over anhydrous sodium sulphate (Na₂SO₄). The solvent was removed under reduced pressure to afford a dark orange syrupy fluid as residue. The desired product was precipitated out of the residue by the addition of chloroform (CCl₃) (30 mL), which allowed the collection of the final product by filtration as an off-white powder (yield: 1.352 g; 4.82 mmol; 24.59%).

PHYSICAL DATA:

C₁₁H₁₀O₂N₁; **MW**, 279.3 g/mol; **mp.** 81 °C; **IR (KBr)** ν_{max} (Spectrum 8): 3210, 3062, 2091, 1495, 1130, 748 cm⁻¹; **MS** (EI, 70 eV) m/z (Spectrum 16): 280 [(M⁺)+1], 279 (M⁺), 236, 222, 185, 135, 91; **¹H NMR** (600 MHz, CDCl₃) δ_H (Spectrum 24): 7.35-7.20 (m, 5H, H-16,17,18,19,20), 3.84 (s, 2H, H-14a,14b), 3.32 (s, 2H, H-11a,11b), 2.15 (s, 3H, H-3,5,8), 2.09 (s, 1H, H-13), 1.90 (s, 6H, H-2a,2b,6a,6b,7a,7b), 1.65 (s, 6H, H-4a,4b,9a,9b,10a,10b); **¹³C NMR** (150 MHz, CDCl₃) δ_C (Spectrum 32): 141.11 (1C, C-15), 128.65 (2C, C-16,20), 128.18 (2C, C-17,19), 126.66 (1C, C-18), 83.22 (1C, C-12), 72.08 (1C, C-13), 55.05 (1C, C-1), 48.98 (1C, C-14), 40.53 (3C, C-3,5,8), 36.77 (3C, C-2,6,7), 34.79 (1C, C-11), 29.86 (3C, C-4,9,10).

STRUCTURE ELUCIDATION:

Characteristic groups evident of the structure of compound **6**, include the ethynyl and amine group of the propargylamine moiety, as well as the phenyl group. The only group making the structure of **7** different from that of **6** is the phenyl group. On the infrared spectrum the aromatic C=C bonds are present at 1495 cm⁻¹, and the aromatic C-H bonds at 3062 cm⁻¹ and 748 cm⁻¹. The deshielded aromatic carbons and protons can also be identified on the ¹H and ¹³C NMR spectrum, all lying much more downfield than the other signals. The molecular ion from the MS confirmed a mass of 279.

3 Concluding remarks

In this chapter the synthesis of 8 new polycyclic cage structures are described. The structures and purity of all the compounds were confirmed with IR, MS and NMR analysis.

CHAPTER 4

BIOLOGICAL EVALUATION: APOPTOSIS & MONOAMINE OXIDASE B

The development of multifunctional compounds to treat the symptoms produced by neurodegenerative diseases and to possibly alter the underlying pathological processes that give rise to these diseases represents a challenge for medicinal chemistry. The compounds synthesised in this study were tested for anti-apoptotic and MAO-B inhibition activity. By halting the apoptotic cascade these compounds may eliminate the neuronal breakdown process and stop or slow the progression of neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD). Should these compounds also inhibit MAO-B activity, they may be effective in treating several of the symptoms produced by these diseases.

1 Apoptosis detection - DePsipher™ assay

1.1 Introduction

Apoptosis is a complex process that can be induced by many different factors, which in turn act through various cell death signalling pathways. There is an abundance of data suggesting that mitochondria play a critical role in apoptosis by releasing cytochrome c, and other proteins that are essential for the activation of pro-caspase-9 and the execution of apoptosis. Some suggest that mitochondria are the primary triggers of cell death, while others suggest that they act more as facilitators rather than essential role players in the cell death process. Either way the mitochondrion plays an important role in the execution and completion of the apoptotic process (Leist & Jaattela, 2001).

Cellular energy produced during oxidation reactions in the mitochondrial respiratory chain, is stored as a negative electrochemical gradient across the mitochondrial membrane, called delta-psi or $\Delta\Psi_m$ that enables the cell to drive the synthesis of ATP. The mitochondrial membrane of healthy cells is referred to as being polarised, and disruption of $\Delta\Psi_m$ has been shown to be one of the first intracellular changes following the onset of apoptosis. The so-called collapse of the $\Delta\Psi_m$ results in a depolarised $\Delta\Psi_m$, which causes the release of cytochrome c, making depolarisation a possible prerequisite for apoptosis to take place (LeMasters *et al.*, 1998; Facompre *et al.*, 2000).

1.2 Principle by which DePsipher™ works

DePsipher™ utilises a lipophilic cation (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyaniniodide), which can be used as a mitochondrial activity marker to evaluate the viability of a cell population, detecting early apoptosis, and evaluating the effect of drugs on the cell population. The cation aggregates upon membrane polarisation forming an orange-red fluorescent compound. If the potential is disturbed, the dye can not access the transmembrane space and remains or reverts to its green monomeric form. Thus, healthy cells present both the polymeric (orange-red) and monomeric (green) form of the cation, with the monomeric form residing in the cytoplasm and the aggregated form in the transmembrane space. It can be concluded that in comparison with healthy cells, wherein apoptosis has not been induced, the ratio of orange-red fluorescence to green fluorescence of apoptotic cells will be lower. The fluorescence can be observed and measured by flow cytometry with the red aggregates having absorption/emission maxima of 585/590 nm, and the green monomers absorption/emission maxima of 510/527 nm.

1.3 Biological material

1.3.1 Neuroblastoma cells used – SK-N-BE(2)

In the current study the SK-N-BE(2) cell line was used to evaluate the anti-apoptotic activity of the synthesised compounds.

The SK-N-BE(2) neuroblastoma cell line was established in November of 1972 from a bone marrow biopsy taken from a child with disseminated neuroblastoma, after repeated courses of chemotherapy and radiotherapy. They have a reported saturation density greater than 1×10^6 cells/cm², and the morphology of the cells varies with some cells having long processes and others being epithelioid like. The cells aggregate, form clumps and float. They grow as a mixture of floating (suspension) and adherent cells (Biedler & Splengler, 1976a, 1976b; Biedler *et al.*, 1978; Barnes *et al.*, 1981).

1.3.2 Cell cultivation

The cells were cultivated in serum-rich growing medium, consisting of F12 nutrient (F12) supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin (Pen/Strep) (10 000 units/100 mL stock) and 0.1% fungizone (FZ) (2.5 µg/mL units stock). They were incubated at 37 °C in a 5% CO₂ and 95% O₂ humidified atmosphere. The cells duplicated every 30-48 hours and when a confluency of 80% was reached, the adherent cells were detached from the flask bottom by means of trypsinisation. To ensure complete detachment of

the cells, they were incubated with trypsin for approximately 10 minutes. After trypsinisation, the cells were seeded in new flasks at a density of no less than 1/6 confluency.

1.3.3 Induction of apoptosis

In the current study trophic factor deprivation/serum starvation was used to induce apoptosis in cells. With growth factors being essential to the normal development and survival of neuronal cells, a shortage thereof would cause neurons to compete with one another for neurotrophic factors, with the unsuccessful ones dying. With no serum (neurotrophic factors) available all the cells will die in a given time. The serum deprived medium consisted of F12, Pen/Strep and FZ in the same ratio as mentioned above (see section 1.3.2).

1.3.4 Preparation of cells to be analysed by flow cytometry

1.3.4.1 Cells used to evaluate anti-apoptotic activity of compounds

Test compounds (2 – 7) and positive control

After the cells reached 80% confluency, the serum rich medium was replaced with serum deprived medium. The cells were then incubated under normal conditions (see section 1.3.2) for a period of 24 hours, which was sufficient time for apoptosis to be induced. Examining the cells under the microscope after this period of time, it was clear that their morphology had changed. None of the cells were any longer attached to the bottom of the flask, and their shape had also changed from the sprouting appearance to a spherical shape, being indicative of unhealthy cells.

After being incubated in the serum deprived medium for the indicated time, the medium was removed using centrifugation. The cells were then seeded in 24-well plates, with each well containing a concentration of 1×10^6 cells per 2 mL of serum deprived medium containing the test compounds, which were dissolved in dimethyl sulfoxide (DMSO). Final concentrations of samples to be analysed contained 1.25% (v/v) DMSO. The cells were then incubated under normal conditions for a period of 36 hours before being analysed.

The test compounds (**2,3,4a,4b,5a,5b,6,7**) were all tested in triplicate at three different concentrations (1 mM, 100 μ M and 10 μ M).

The positive control, selegiline, was tested in triplicate at two concentrations (100 μ M and 10 μ M). Included into the research was NGP 1-01 which was also tested in triplicate at only one concentration (1 mM).

1.3.4.2 Cells used for control experiments

In this study four control experiments were included.

Control experiment 1

After the cells reached 80% confluency, the serum rich medium was replaced with *serum deprived medium*. The cells were then incubated under normal conditions for a period of 24 hours. After being incubated in the *serum deprived medium*, the medium was removed using centrifugation. The cells were subsequently seeded in 24-well plates, with each well containing a concentration of 1×10^6 cells per 2 mL of *serum deprived medium* and dimethyl sulfoxide (DMSO) (1.25% (v/v)). After this the cells were incubated under normal conditions (37 °C in a 5% CO₂ and 95% O₂ humidified atmosphere) for a period of 36 hours before being analysed.

Control experiment 1 was included to evaluate the viability status of the cells, in the absence of test compound. The viability data will be compared to the data generated with the test compounds in order to determine if the test compounds attenuated the progression of the apoptotic process. With this control experiment the effect of dimethyl sulfoxide (DMSO) was also evaluated.

Control experiment 2

After the cells reached 80% confluency, the serum rich medium was replaced with *serum deprived medium*. The cells were then incubated under normal conditions for a period of 24 hours. After being incubated in the *serum deprived medium*, the medium was removed using centrifugation. The cells were subsequently seeded in 24-well plates, with each well containing a concentration of 1×10^6 cells per 2 mL of *serum deprived medium* with no DMSO content. The cells were incubated under normal conditions for a period of 36 hours before being analysed.

Control experiment 2 will be used to evaluate the effect of DMSO on the viability of the cells and on the progression of the apoptotic process.

Control experiment 3

After the cells reached 80% confluency, the serum rich medium was replaced with *serum deprived medium*. The cells were subsequently incubated under normal conditions for a period of 24 hours. After being incubated in the *serum deprived medium*, the medium was removed using centrifugation. The cells were then seeded in 24-well plates, with each well

containing a concentration of 1×10^6 cells per 2 mL of fresh *serum rich medium* with no DMSO present. After this the cells were incubated under normal conditions for a period of 36 hours before being analysed.

Since the re-introduction of serum rich medium is expected to significantly attenuate the apoptotic process, this control experiment will be used to estimate the potencies of the test compounds as anti-apoptotic agents.

Control experiment 4

After the cells reached 80% confluency, the serum rich medium was replaced with *fresh serum rich medium*. The cells were then incubated under normal conditions for a period of 24 hours. After being incubated in the *serum rich medium*, the medium was removed using centrifugation. The cells were subsequently seeded in 24-well plates, with each well containing a concentration of 1×10^6 cells per 2 mL of *serum rich medium* with no DMSO present. The cells were incubated under normal conditions for a period of 36 hours before being analysed.

Control experiment 4 was included to analyse and determine the viability status of the cells, when no apoptosis had been induced, and conditions had been favourable to the end. It was also included to ensure that the cells analysed were indeed healthy before inducing apoptosis.

1.4 Assay procedure

The cells of each sample were harvested consecutively as follows: After incubating the cells for 36 hours, the medium was removed from the cells using centrifugation. The cells that had adhered to the bottom of the flasks were removed by trypsination. The trypsin/cell mixture was added to the cells from which the medium had been removed. The trypsin was removed from the cells by centrifugation at $500 \times g$ for five minutes at room temperature. The cells of each sample were then resuspended in 1 mL of diluted DePsipher solution, consisting of 1 μ L DePsipher dye and 1 mL of pre-warmed F12 medium. The cells were incubated for 20 minutes at 37°C and 5% CO_2 . The samples were washed twice in PBS, and centrifuged at $500 \times g$ between each wash. The cells were subsequently resuspended in 1 mL PBS and immediately taken to be analysed on the flow cytometer. The samples were kept shielded from light until analysis, since the DePsipher™ agent is light sensitive.

1.5 Data analysis

Analysis of the samples were performed on a BD FACS Calibur[®] flow cytometer [Becton Dickinson, San Jose (USA)], equipped with a 15 mV 488 nm, air-cooled argon-ion laser. Cells were gated in a forward scatter/side scatter plot to exclude debris. Green and red fluorescence were detected in the corresponding FL-1 and FL-2 photomultipliers through 530 nm (FITC) or 585 nm (PE/PI) bandpass filters respectively. In generating the data, the flow cytometer was set to analyse 50 000 events/cells.

Figure 4.1 contains a representative pseudo-colour graph of every sample, which was generated using FlowJo[®], based on the data of the flow cytometry. On the x-axis the FL-1 (green) fluorescence intensity is plotted, and on the y-axis the FL-2 (red) fluorescence intensity is plotted. Both intensities are measured in MFI (mean fluorescence intensity). Each spot is representative of a single cell analysed with the flow cytometer. The graphs are divided into four separate quadrants, each containing a population of cells, which exhibit the same characteristics. In the upper left quadrant (Q1) lies all the cells which emit only red fluorescence, whereas the cells which emit only green fluorescence can be found in the lower right quadrant (Q4). The cells which emit both wavelengths of fluorescence can be found in the upper right quadrant (Q2), and the cells emitting neither of the two in the lower left quadrant (Q3).

The following can be concluded by taking note of the explanations of the different quadrants as well as the characteristics of cells in each of these quadrants: As healthy cells contain both the monomeric and polymeric form of the cation dye, and would thus have intensity in both the FL1 and FL2 channels used to analyse the samples, the MFI values of these cells will lie in Q2 (see control experiment 4). The cells with MFI values in Q1 have only red fluorescence, which might be due to low concentrations of the dye inside the cell, indicating that all the monomers were polymerised. All these cells can thus also be said to be healthy, together with these found in Q2. Cells wherein apoptosis have been induced only contain the monomeric form of the dye, and will not colour red at all, and will thus have MFI values in Q4. The cells lying in Q3 have not been dyed successfully or intensively enough, and have thus not coloured either red or green.

From the pseudo-colour graphs it is clear that there was a difference in the number of anti-apoptotic/healthy cells present in the different samples. Control experiment 1 had the least MFI values in Q1+Q2, and control experiment 4 the most. The MFI values of the other samples were either between the values of control experiments 1 and 4, or just above that of control experiment 4.

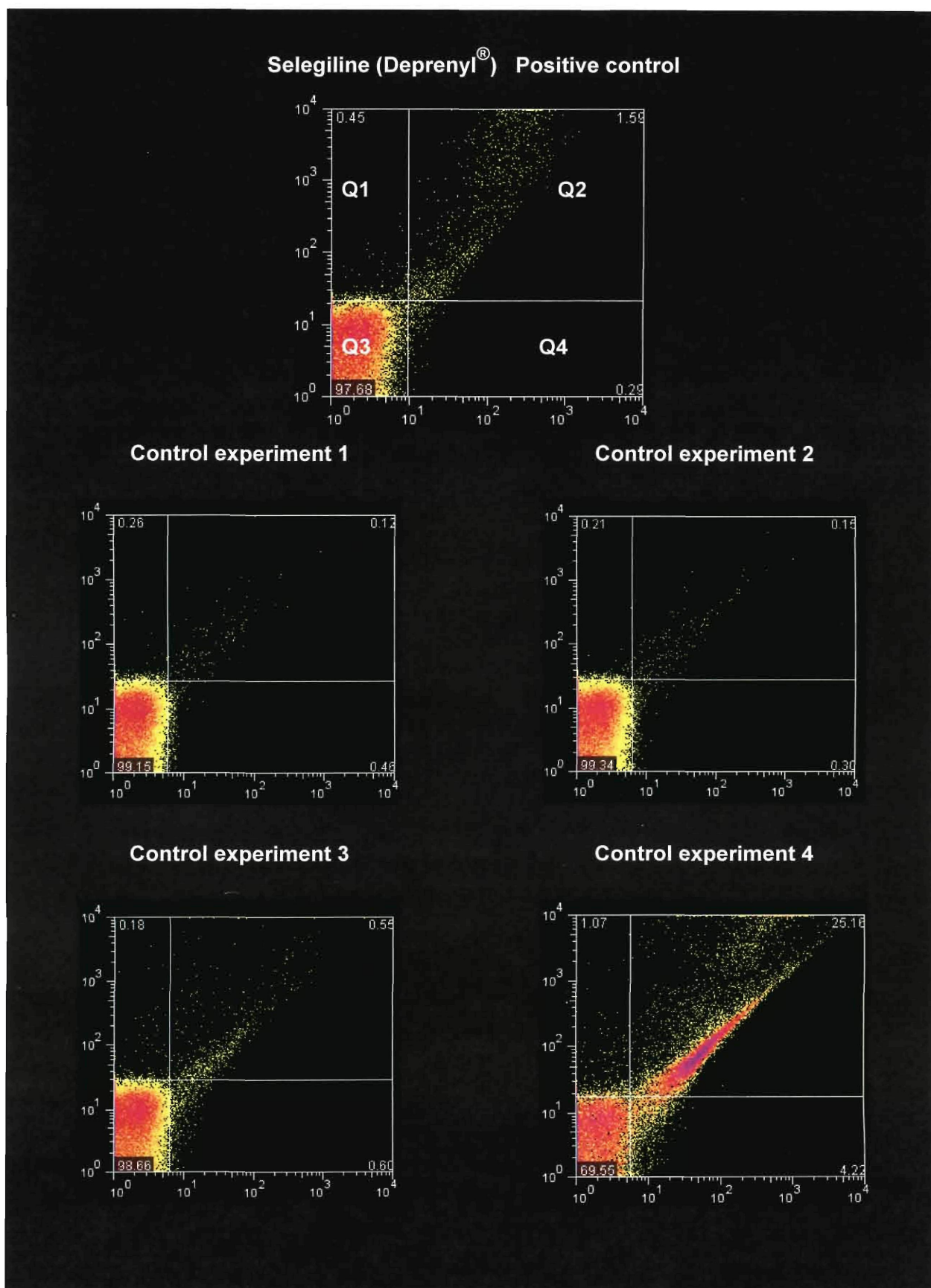


Figure 4.1: Pseudo-colour graphs of samples analysed

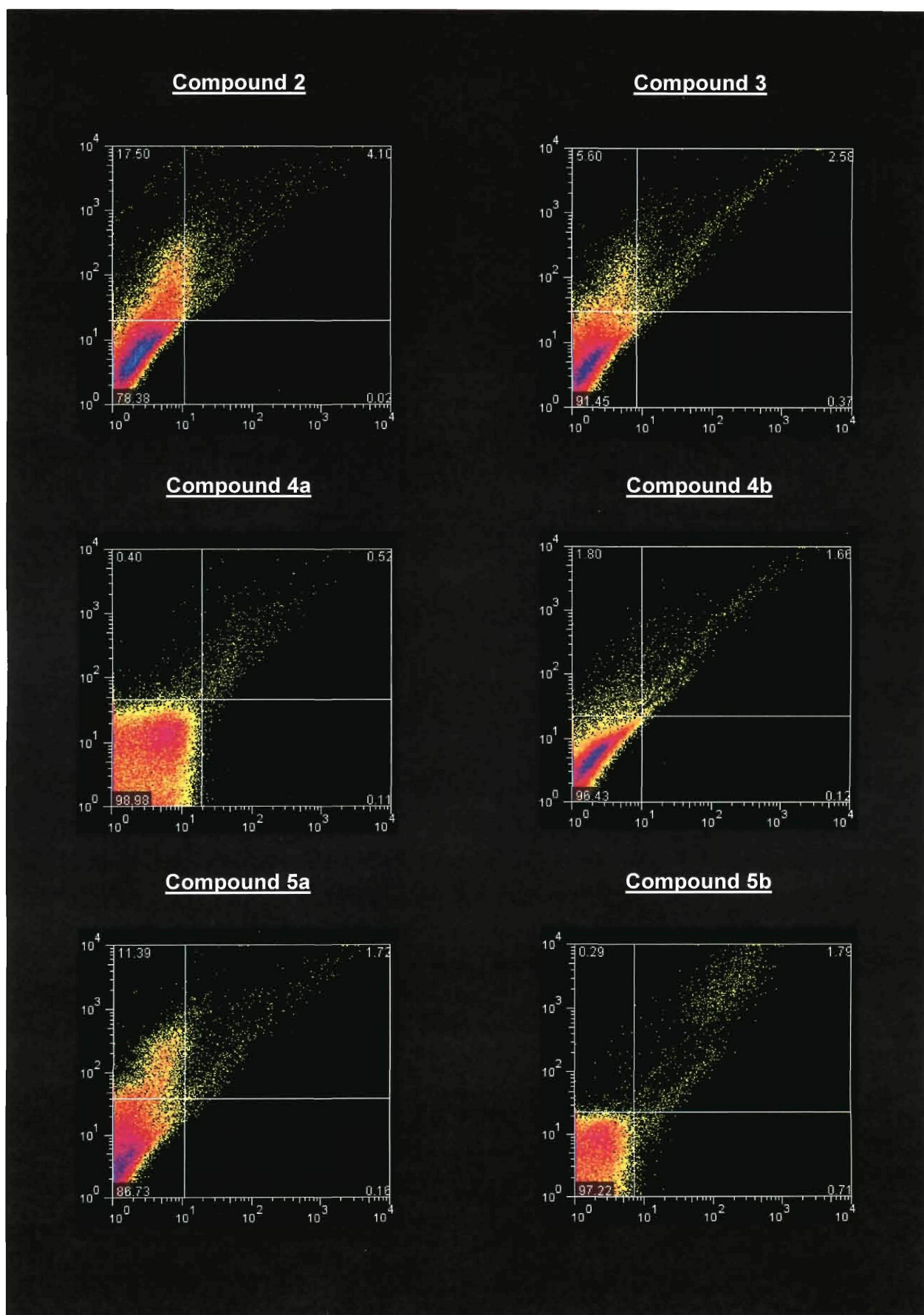


Figure 4.1: Pseudo-colour graphs of samples analysed (continued)

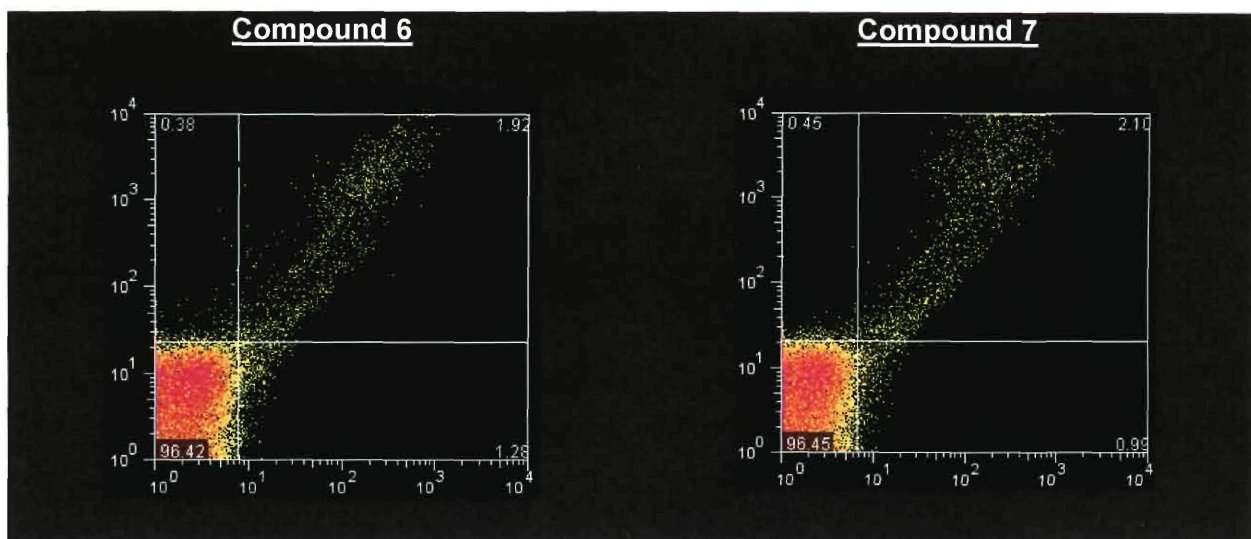


Figure 4.1: Pseudo-colour graphs of samples analysed (continued)

1.5.1 Results

The data generated by these flow cytometry experiments were used to determine if the test compounds have anti-apoptotic properties on cultured cells. The results are graphically summarised in a histogram (fig. 4.2). The percentage values used in plotting the histogram (fig. 4.2) were representative of the percentage of the coloured cells which were still viable and non-apoptotic. The values utilised to quantify cell viability were determined by the flow cytometer. Equation 4.1 was used to calculate the percentage of cells which were still viable in the samples analysed. In determining this value, the amount of cells in quadrant 1 (Q_1), quadrant 2 (Q_2) and quadrant 3 (Q_3) and the total amount of events analysed (Q_{total}) were utilised.

$$\% \text{ Viable cells} = \frac{(Q_1 + Q_2)}{(Q_{\text{total}} - Q_3)} \times 100 \quad \text{Eq. 4.1}$$

The results confirmed that a significant percentage of the cells had indeed coloured successfully with the dye. The percentage of the coloured cells that had fluorescence intensity in the FL2 channel, gave a quantitative indication of the number of healthy cells present in each sample, wherein apoptosis had not progressed or been induced.

In control experiment 1, 41.62% of the cells were apoptotic, with only 58.38% remaining healthy. This indicates that the method of apoptosis induction had indeed been successful. Comparing the values of control experiments 1 and 2, it is clear that even though the final dimethyl sulfoxide (DMSO) content in the samples was only 1.25%, the DMSO had a negative effect on the health of the cells. In control experiment 2 which did not contain any

DMSO, there was 7.19% more cells that were healthy. It can thus be said that the serum deprivation was not the only factor inducing apoptosis, but also the DMSO which served as solvent for the test compounds.

It has been shown in literature (Bar-Am *et al.*, 2005) that the anti-apoptotic effect of selegiline is dependent upon the propargylamine moiety in its structure. This anti-apoptotic activity is evident from the histogram. The positive control had 12-19% more cells that were healthy and wherein apoptosis had either not been induced or reached the level of mitochondrial depolarisation, compared to control experiment 1.

When compared to control experiment 1, compounds **2**, **3**, **4a**, **4b**, **NGP 1-01**, **5a**, **5b**, **6** and **7** all had significant anti-apoptotic activity. The anti-apoptotic activity of **2**, **3**, **4a**, **4b**, **5a**, **5b** and **6** were all extremely significant ($p < 0.001$), with that of **NGP 1-01** and compound **7** being significant ($p < 0.05$).

1.6 Discussion

In this study the contribution of several functional groups to the anti-apoptotic activity of propargylamine were evaluated. These groups included the following: a terminal acetylene group (**2**), an acetylene group between two non-polar groups (**3**), and a propargylamine connected to a cage compound (**4 – 7**). Besides the synthesised compounds an additional compound, **NGP 1-01** (Van der Schyf *et al.*, 1986), a well known calcium channel antagonist, was also evaluated for anti-apoptotic activity, as abnormally increased calcium levels acts as a trigger of apoptosis.

Even though the anti-apoptotic activity of **NGP 1-01** was significant, the activity of all the synthesised compounds, were higher. This might be due to **NGP 1-01** only inhibiting one of the triggers of apoptosis (calcium increase), whereas the synthesised compounds possibly inhibit the apoptotic cascade, directly or indirectly, at more than one point.

The activity of compounds **2** and **3** reveal that the acetylene group indeed contributes to anti-apoptotic activity, be it a terminal acetylene group (**2**) or an acetylene group between two non-polar groups (**3**). Whether this activity is solely due to the acetylene group, or the acetylene group in conjunction with a nearby electronegative atom/group, needs to be investigated further.

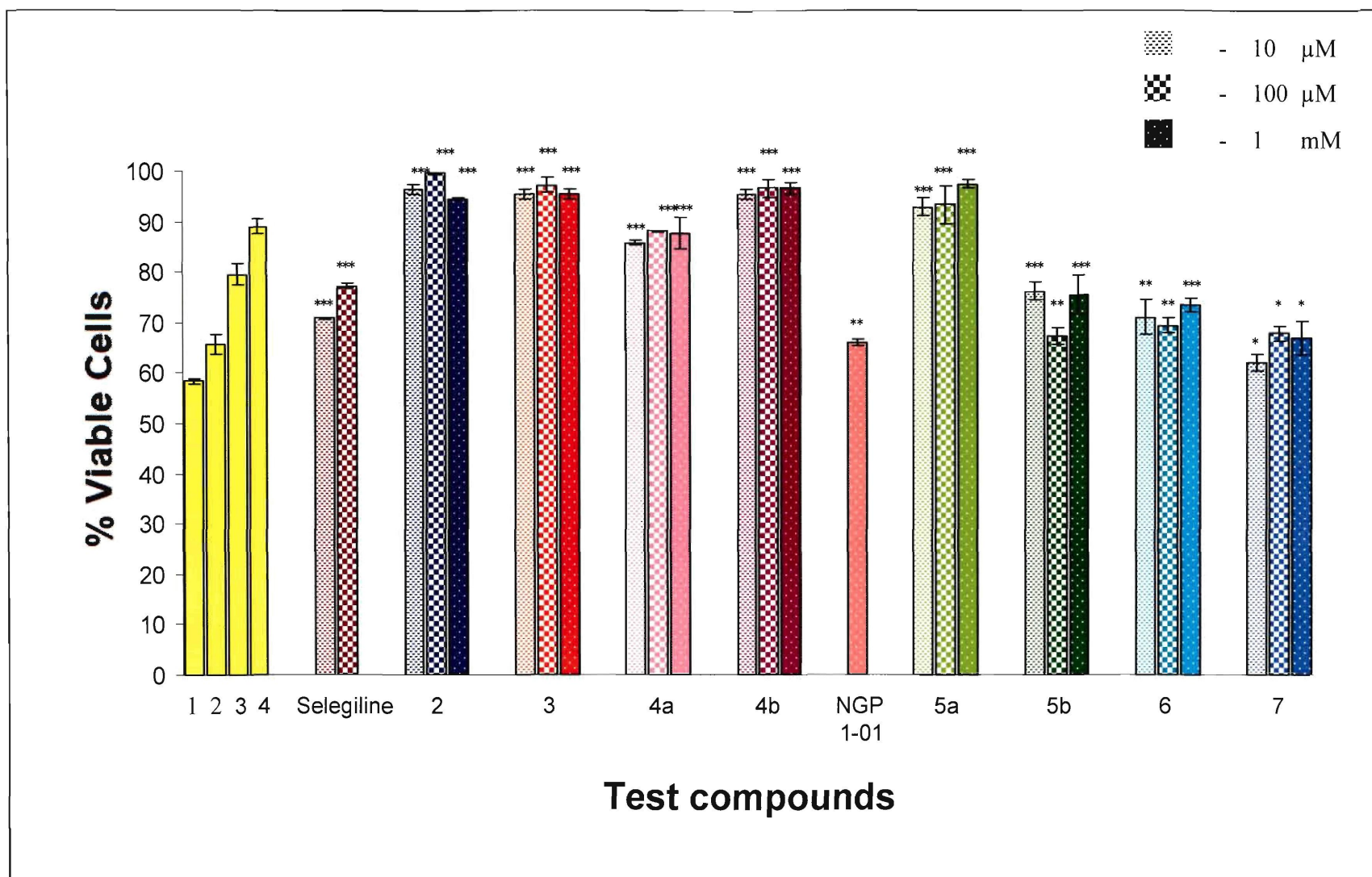


Figure 4.2: Anti-apoptotic activity of compounds (*** indicate significance with $p < 0.05$: * $p < 0.01$: ** $p < 0.001$: ***)

When comparing the anti-apoptotic activity of compounds **4a**, **4b**, **5a**, **5b**, it is clear that in this case it did not matter how the propargylamine moiety was connected to the polycyclic cage compound. Both the oxa- and aza-compounds had comparable activity. In the case of the oxa-compounds (**4a**, **4b**), the methylated cage had increased the activity. With the aza-compounds (**5a**, **5b**), this was not observed. With the activity of the oxa- and the aza-compounds being very similar, the conclusion can be made that both the secondary and tertiary propargylamine has anti-apoptotic activity.

Compounds **6** and **7** had significantly lower activities than that of compounds **2** – **5a**. With both these compounds (**6** and **7**) solubility problems were experienced at higher concentrations, which may explain the observed lower anti-apoptotic action. When looking at the structures of these two compounds, and comparing them to those of compounds **4a** - **5b**, they are different in having an additional non-polar group besides the single acetylene group. This might make the compounds unfavourable for interaction with targets in the apoptotic pathway.

Comparing the percentage healthy cells of the samples treated with the test compounds **2**, **3**, **4b** and **5a**, with that of the control experiment 4, which is representative of healthy cells in a favourable environment, it is interesting to note that these samples had more healthy cells than that of control experiment 4. This may be attributed to the compounds inhibiting even baseline apoptotic processes. The percentage of the cells which were found to be apoptotic in control experiment 4 may be, amongst others, attributed to protocol handling of the cells during change of the medium. These processes are not favourable for the cells, and may also possibly be a trigger of apoptosis.

2 Monoamine oxidase B inhibition assay

2.1 Introduction

Monoamine oxidase B (MAO-B) is an enzyme that plays a crucial role in the oxidative deamination reactions of certain monoamine neurotransmitters, which perform fundamental roles in the normal functionality and wellbeing of the human central nervous system (CNS). When its activity increases, MAO-B causes an augmentation in the levels of oxidative stress in the CNS. The hydrogen peroxide (H₂O₂) produced during amine oxidation, may interact with free iron to form highly reactive hydroxyl radicals that can damage nucleic acids, proteins, and membrane lipids leading to neuronal degeneration (Nicotra *et al.*, 2004). Inhibition of MAO-B will lead to a decrease in oxidative stress and ultimately a decrease in neuronal degeneration. Thus it can be concluded that MAO-B inhibition is beneficial as a

neuroprotective strategy, and can play an important role in the treatment and prevention of neurodegenerative disorders.

Besides its protective role in neurodegenerative diseases, such as PD and AD, MAO-B inhibition also has therapeutic potential in the treatment of the symptoms caused by the pathology of these diseases. In Parkinson's disease elevated levels of MAO-B results in increased catabolism of dopamine, which ultimately leads to reduced concentrations of dopamine compared to normal physiological levels. This dopamine depletion contributes to the symptoms of Parkinson's disease. In Alzheimer's disease MAO-B activity may also be increased. This may contribute to neuronal degeneration which ultimately leads to the loss of memory and concentration (Wells *et al.*, 2003). Incorporating the therapeutic value of MAO-B inhibitors in the design of multifunctional neuroprotective agents, would thus be a rational decision.

2.2 Preparation of biological material

The mitochondrial fraction of baboon liver tissue, as source of the MAO-B enzyme, was isolated as described previously by Salach and Wyler (Salach & Wyler, 1987), and stored at - 70 °C. Following addition of an equal volume of sodium phosphate buffer (100 mM, pH 7.4) containing glycerol (50%, w/v) to the mitochondrial isolate, the protein concentration was determined by the method of Bradford using bovine serum albumin as reference standard (Bradford, 1976). In the current study, MMTP ($K_m = 68.3 \pm 1.60 \mu\text{M}$ for baboon liver MAO-B) served as substrate for the inhibition studies.

2.3 Assay procedure

The enzymatic reactions were prepared in sodium phosphate buffer (100 mM, pH 7.4) and contained MMTP (50 μM), the mitochondrial isolate (0.15 mg protein/mL) and various concentrations of the test compounds, spanning at least three orders of magnitude (0.3 - 300 μM). The concentration of the test compounds used in this study were 0.3 μM , 1 μM , 3 μM , 10 μM , 30 μM , 100 μM and 300 μM . The final volume of the incubations was 500 μL . The stock solutions of the inhibitors were prepared in dimethyl sulfoxide (DMSO) and were added to the incubation mixtures to yield a final DMSO concentration of 4% (v/v). DMSO concentrations higher than 4% are reported to inhibit MAO-B (Gnerre *et al.*, 2000). The reactions were incubated at 37 °C for 10 minutes and then terminated by the addition of 10 μL perchloric acid (70%). The samples were centrifuged at 16,000 g for 10 minutes, and the concentrations of the MAO-B generated product, MMDP⁺ (Inoue *et al.*, 1999), were measured spectrophotometrically at 420 nm ($\epsilon = 25,000 \text{ M}^{-1}$) in the supernatant fractions.

2.4 Data analysis

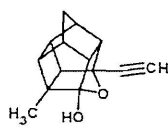
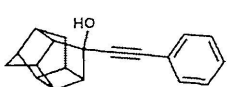
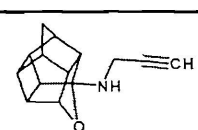
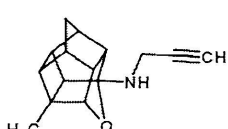
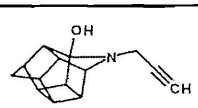
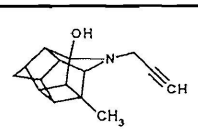
Due to the low enzyme inhibition activity of the test compounds, equation 4.2 was used to calculate the inhibition potencies of the compounds. These potencies are expressed as percentage inhibition (% inh) of the enzyme. In determining this value, the concentration of MMDP⁺ produced in the absence of a test compound (C_0) and the concentration of MMDP⁺ produced by MAO-B in the presence of a maximal concentration of the test compound (C_{300}) were utilised. All determinations were conducted in duplicate, yielding the percentage inhibition as an average of the two sets of data.

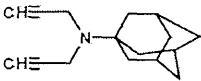
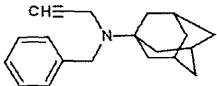
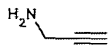
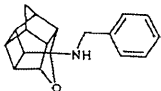
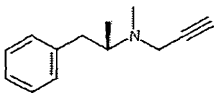
$$\% \text{ inh} = 100 - \left[\frac{C_{300} \times 100}{C_0} \right] \quad \text{Eq. 4.2}$$

2.4.1 Results

In the following table a summary of the percentage inhibition of MAO-B by the test compounds is given.

Table 4.2: Monoamine oxidase B inhibition activity of synthesised compounds

Compound	Structure	% Inhibition at 300 μ M and SEM
QRB 2		Inactive
QRB 3		73.32 $\pm$ 0.80
QRB 4a		00.00 $\pm$ 0.81
QRB 4b		11.67 $\pm$ 3.02
QRB 5a		5.83 $\pm$ 2.37
QRB 5b		10.83 $\pm$ 6.25

QRB 6		14.00 ± 0.39
QRB 7		20.13 ± 0.65
PA		19.93 ± 0.93
NGP 1-01		14.57 ± 4.46
Selegiline		93.33 ± 0.88

2.5 Discussion

The synthesised compounds were evaluated for MAO-B inhibition activity, with the aim of producing compounds with dual activity: anti-apoptotic and MAO-B inhibition activity. The only compound which had significant MAO-B inhibition activity, when compared to that of selegiline, was compound **3**. The other compounds either had no activity or exhibited little inhibition. Even though the activity of the compounds is low, valuable conclusions could still be made.

Comparing the MAO-B inhibition potencies of **4a** with **4b** and **5a** with **5b**, it can be concluded that in both cases the methyl group on the polycyclic cage, appeared to increase MAO-B inhibition activity of the compounds. This can be due to the methyl group enhancing the interaction between the compound and the non-polar regions of the enzyme. It is important to note the large standard deviations of these compounds, which emphasises need for further investigation considering the contribution of a methyl group to MAO-B inhibition. Another interesting observation is that the aza compounds (**5a** and **5b**) are slightly more favourable than the oxa-compounds (**4a** and **4b**) as MAO-B inhibitors, possibly because the hydroxyl group may act as a hydrogen bond donor within the enzyme active site. For comparison **NGP 1-01** was also evaluated as a MAO-B inhibitor. The activity of **NGP 1-01** was higher than that of compound **4a**. This indicates that the phenyl moiety of **NGP 1-01** is more effective than the propargyl moiety of compound **4a** in stabilising ligand-enzyme complexes. This trend was also observed for the other members of the synthetic series. Those containing a phenyl side chain (**3**, **7**, **NGP 1-01** and **selegiline**), were consistently better inhibitors than the other compounds in the series. This trend may be attributed to the

planar character of the phenyl ring. It has been reported that planar compounds frequently act as potent inhibitors of MAO-B (Medvedev *et al.*, 1995; Petzer *et al.*, 2009). It can be concluded that the phenyl moiety, makes a ligand more favourable for interaction with the enzyme.

Compound **3** contains both a hydroxyl, and phenyl moiety, which is most probably the reason why this compound has activity much higher than that of the other synthesised compounds, making it a possible lead compound for further investigation.

3 Concluding remarks

The compounds synthesised had exceptionally good anti-apoptotic activity, but were not potent MAO-B inhibitors. The only compound exhibiting promising MAO-B inhibiting activity was compound **3**. This compound may be considered as promising as a multifunctional neuroprotective agent, due to its significant anti-apoptotic and MAO-B inhibition activity.

CHAPTER 5

CONCLUSION

The ultimate objective of the study was to create a multifunctional neuroprotective agent with the potential to halt the neuronal breakdown process, and at the same time have the ability to treat and eliminate some of the symptoms of neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD).

Previous studies concluded that the neuronal loss found in certain areas of the brain in PD and AD, takes place by an intrinsic cell suicide program, known as apoptosis (Holbrook *et al.*, 1996). This cell death program consists of several pathways and cascades, with each one having an influence on the other. This ultimately leads to the death of the neuronal cells, and deterioration of the central nervous system.

PD is characterised by increased levels of MAO-B, which is responsible for the symptoms of the disease. Since MAO-B is a major catabolic enzyme of dopamine, inhibition of this enzyme leads to alleviation of the symptoms of PD. In AD increased levels of MAO-B has been shown to have a role in the pathology, not directly giving rise to the signs and symptoms, but contributing to the neurodegeneration. Therefore inhibition of MAO-B may also be beneficial in AD.

The aim was thus to create compounds with dual mechanisms, which would ultimately inhibit apoptosis as well as MAO-B. Such compounds may act as neuroprotective and symptomatic drugs. In designing the compounds we focused on the structures of rasagiline and selegiline, well known MAO-B inhibitors. Besides being potent MAO-B inhibitors, these drugs are also neuroprotective (Chen *et al.*, 2007). The neuroprotective ability of these agents has been shown to be dependent on the propargylamine moiety present in both structures (Bar-Am *et al.*, 2005). Based on this consideration the compounds studied, all contain the propargylamine functional group or a derivative thereof. These possible pharmacophores were linked to a polycyclic cage structure. Being very non-polar, these polycyclic structures would aid in the transport of the drug across the blood-brain barrier, as well as cell membranes into the cells where the drugs would be active. The highly non-polar polycyclic cage structures incorporated into the compounds studied were pentacyclo-undecane and adamantane.

To prepare the target compounds, propargylamine and propargylbromide or ethynyl magnesium bromide were reacted with either pentacyclo-undecane or amantadine. Both

conventional and modern methods were utilised to synthesise the proposed test compounds. Even though the reagents used in the synthetic routes were very similar, the experimental procedures utilised were very diverse. In most of the synthetic routes the percentage yield of the final products is very low, due to a high level of side-products. Due to this, considerable time was spent to purify intermediate and final products. The purification methods used included column chromatography, soxhlett extraction, recrystallisation as well as steam distillation.

In this study the following propargylamine derivatives were considered: Firstly the activity of a single terminal acetylene group (compound **2**) was evaluated, and secondly the activity of this group if placed between two non-polar groups (compound **3**). It was also evaluated what the activity of propargylamine would be, when it is linked to a polycyclic cage structure (compounds **4** - **7**) as a secondary amine (compound **4a**) and as a tertiary amine (compounds **5a**, **6** and **7**). Furthermore it was also investigated what effect a methyl substituent on the polycyclic structure (compounds **4b** and **5b**) would have on the activity of the polycyclic propargylamine.

Flowcytometric evaluation of the synthesised compounds for anti-apoptotic activity revealed surprising results. All the compounds had moderate to weak anti-apoptotic activity and compared favourably to the positive control, selegiline. It is important to consider the possibility that even though the compounds improved the survival of cell cultures and appear to have anti-apoptotic activity, these observations can also be ascribed to a mechanism wherein the test compounds improve the growth of cell cultures, and thereby increased the amount of viable cells which were present in the samples analysed. The pentacyclo-undecane derivatives had higher activity than the adamantane derivatives. This can possibly be attributed to solubility problems experienced with higher concentrations of the adamantane amines. It can also be attributed to the possibility that pentacyclo-undecanes may have an increased ability to pass cell membranes due to their higher lipophilicity. This would result in increased intracellular concentrations of the drugs. When compared to the control experiments, all the test compounds improved cell health between 9 and 41%. It can be concluded that all the compounds had anti-apoptotic activity, with the more effective concentration being in the micro molar range. The compound with the most significant activity was compound **2**. This compound was twice as potent as the positive control, and all cultures treated with **2** had only 0.5% apoptotic cells in the analysed samples. Slightly less active than compound **2** was compound **3**, with only 2.8% of the cells being apoptotic. Studying the results of these experiments, it is clear that the anti-apoptotic activity of propargylamine can most probably be attributed to the acetylene group, as compounds **2** and

3 have equivalent or even higher activity than compounds **4-7**. For activity, this acetylene group can either be terminal or intramolecular, with the terminal acetylene group having slightly higher activity. Due to solubility problems with compound **5b**, it can not accurately be determined whether the methyl group in position 1 of the pentacyclo-undecane cage had an effect on the anti-apoptotic activity of the compounds. Comparing the activity of compounds **4a** and **5a**, it can be concluded that the tertiary propargylamine had slightly higher activity than the secondary propargylamine. Even though all the synthesised compounds appeared to have moderate to weak anti-apoptotic activity, they can be used in further studies as lead compounds in the development of more potent inhibitors.

In the monoamine oxidase B assay, only one compound (**3**) had promising MAO-B inhibition activity. The assay revealed that the other synthesised compounds were not able to act as inhibitors of this enzyme. Considering the results of the MAO-B inhibition assay, the following can be concluded: The polycyclic propargylamines did not have promising MAO-B inhibiting activity, with pentacyclo-undecane and adamantane substituents decreasing the activity of the propargylamine moiety. The benzyl group was clearly a favourable substituent, increasing the activity of both the pentacyclo-undecane and adamantane derivatives. Having a methyl group in position 1 of the pentacyclo-undecane cage appeared to slightly increase the activity of the compounds (**4a** and **5b**). Inclusion of a second propargyl group does not significantly increase the activity of the polycyclic propargylamines. The promising activity of compound **3** can be ascribed to the benzyl group being linked to the cage structure by means of an acetylene linker. The acetylene group together with benzyl makes this part of the structure of compound **3** very planar, making it possible for this compound to move into the active site cavity of the enzyme, thereby inhibiting the enzyme.

In this study the aim was to create a drug acting via multiple mechanisms with MAO-B inhibiting as well as anti-apoptotic activity. Based on the results it can be concluded that only compound **3** exhibited dual actions. Compound **3** had a MAO-B inhibition percentage of 73.32% at 300 μ M, and a percentage apoptotic cells, of only 2.78% in the samples analysed. This compound represents a lead for the discovery of new treatment strategies for neurodegenerative diseases such as PD and AD.

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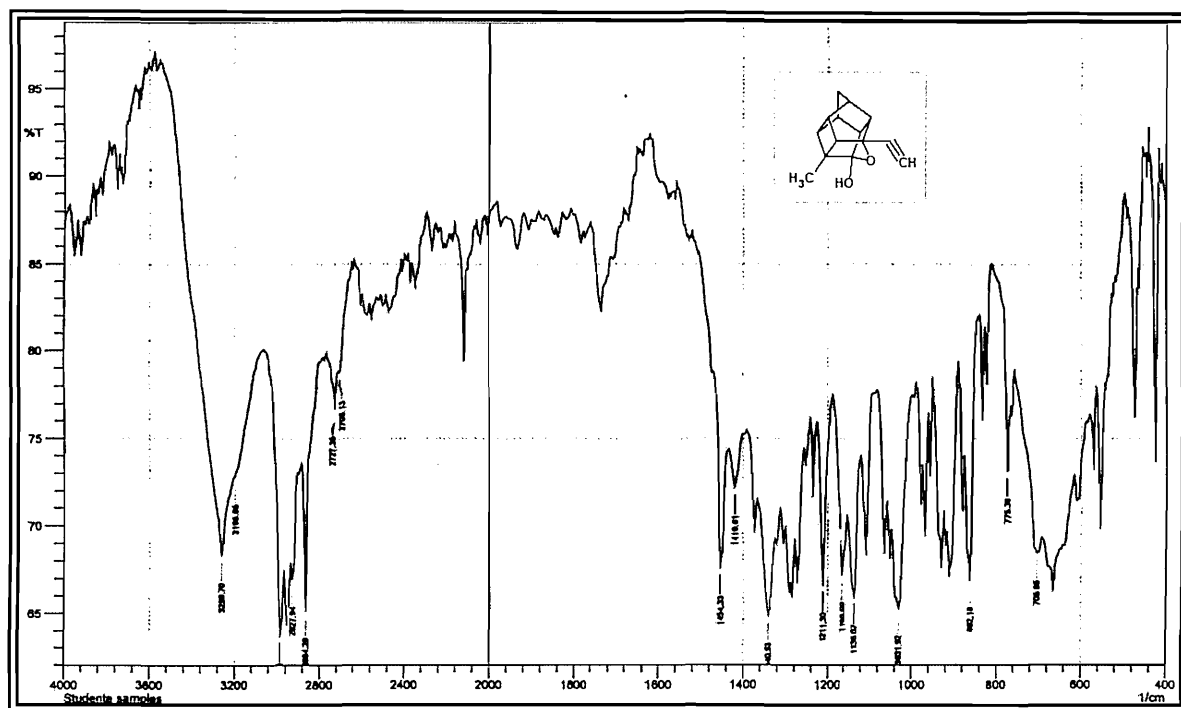
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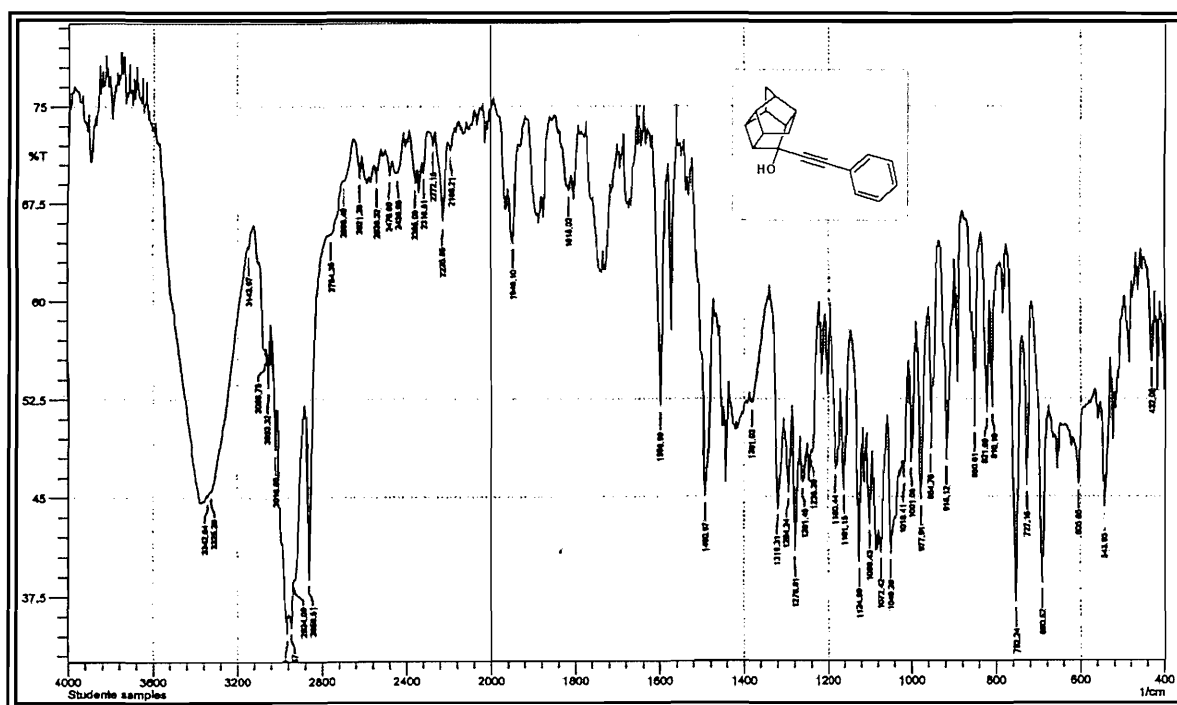
APPENDIX I

**SPECTRAL DATA:
INFRARED, MASS SPECTROMETRY,
AND
NUCLEAR MAGNETIC RESONANCE**

INFRARED



Spectrum 1



Spectrum 2

Chemical structure of 1-ethynyl-2-methylbicyclo[2.2.1]hept-5-ene-2-ol is shown in the inset:

CC12C=CC1(C2)C#CCO

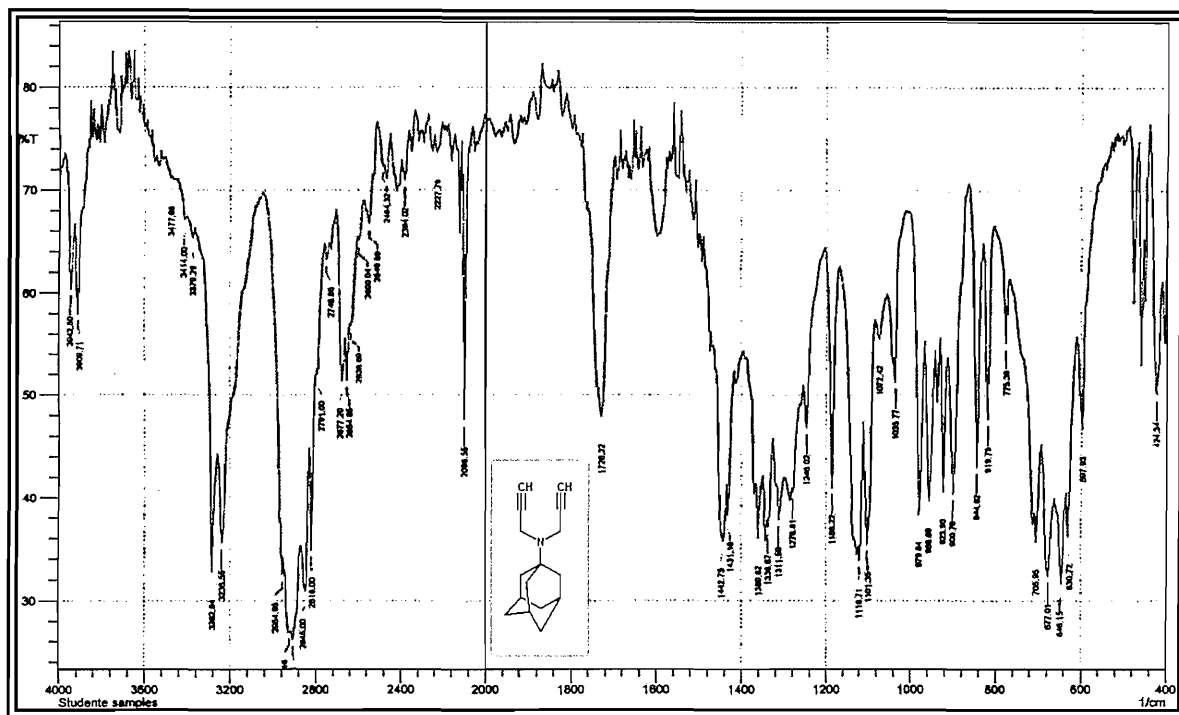
107

Chemical structure: 1-methyl-2-vinyl-7-oxabicyclo[2.2.1]heptane

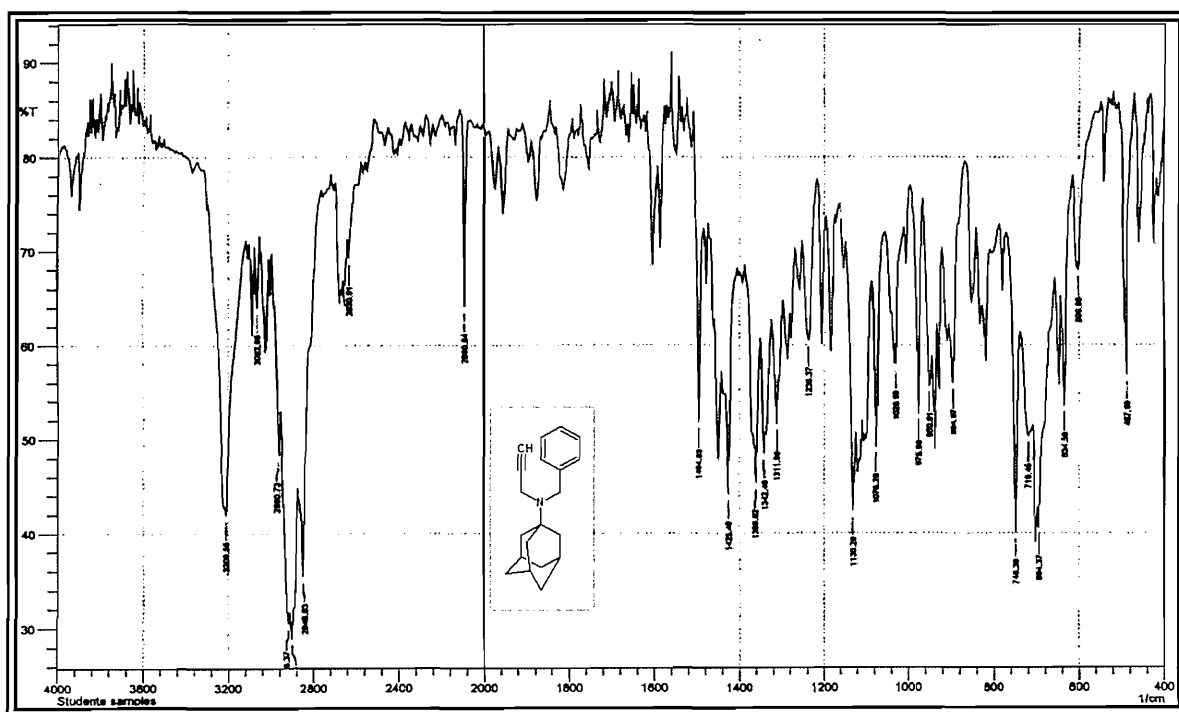
Key IR peaks (cm⁻¹):

- 3400.81, 3400.72 (O-H stretch)
- 2958.88, 2957.98, 2957.58, 2957.39, 2957.19, 2956.98, 2956.78, 2956.57, 2956.37 (C-H stretch)
- 2773.43 (C-H stretch)
- 2345.43, 2345.23 (C-H stretch)
- 2281.48, 2281.27, 2281.07, 2280.87, 2280.67, 2280.47, 2280.27, 2280.07, 2279.87, 2279.67, 2279.47, 2279.27, 2279.07, 2278.87, 2278.67, 2278.47, 2278.27, 2278.07, 2277.87, 2277.67, 2277.47, 2277.27, 2277.07, 2276.87, 2276.67, 2276.47, 2276.27, 2276.07, 2275.87, 2275.67, 2275.47, 2275.27, 2275.07, 2274.87, 2274.67, 2274.47, 2274.27, 2274.07, 2273.87, 2273.67, 2273.47, 2273.27, 2273.07, 2272.87, 2272.67, 2272.47, 2272.27, 2272.07, 2271.87, 2271.67, 2271.47, 2271.27, 2271.07, 2270.87, 2270.67, 2270.47, 2270.27, 2270.07, 2269.87, 2269.67, 2269.47, 2269.27, 2269.07, 2268.87, 2268.67, 2268.47, 2268.27, 2268.07, 2267.87, 2267.67, 2267.47, 2267.27, 2267.07, 2266.87, 2266.67, 2266.47, 2266.27, 2266.07, 2265.87, 2265.67, 2265.47, 2265.27, 2265.07, 2264.87, 2264.67, 2264.47, 2264.27, 2264.07, 2263.87, 2263.67, 2263.47, 2263.27, 2263.07, 2262.87, 2262.67, 2262.47, 2262.27, 2262.07, 2261.87, 2261.67, 2261.47, 2261.27, 2261.07, 2260.87, 2260.67, 2260.47, 2260.27, 2260.07, 2259.87, 2259.67, 2259.47, 2259.27, 2259.07, 2258.87, 2258.67, 2258.47, 2258.27, 2258.07, 2257.87, 2257.67, 2257.47, 2257.27, 2257.07, 2256.87, 2256.67, 2256.47, 2256.27, 2256.07, 2255.87, 2255.67, 2255.47, 2255.27, 2255.07, 2254.87, 2254.67, 2254.47, 2254.27, 2254.07, 2253.87, 2253.67, 2253.47, 2253.27, 2253.07, 2252.87, 2252.67, 2252.47, 2252.27, 2252.07, 2251.87, 2251.67, 2251.47, 2251.27, 2251.07, 2250.87, 2250.67, 2250.47, 2250.27, 2250.07, 2249.87, 2249.67, 2249.47, 2249.27, 2249.07, 2248.87, 2248.67, 2248.47, 2248.27, 2248.07, 2247.87, 2247.67, 2247.47, 2247.27, 2247.07, 2246.87, 2246.67, 2246.47, 2246.27, 2246.07, 2245.87, 2245.67, 2245.47, 2245.27, 2245.07, 2244.87, 2244.67, 2244.47, 2244.27, 2244.07, 2243.87, 2243.67, 2243.47, 2243.27, 2243.07, 2242.87, 2242.67, 2242.47, 2242.27, 2242.07, 2241.87, 2241.67, 2241.47, 2241.27, 2241.07, 2240.87, 2240.67, 2240.47, 2240.27, 2240.07, 2239.87, 2239.67, 2239.47, 2239.27, 2239.07, 2238.87, 2238.67, 2238.47, 2238.27, 2238.07, 2237.87, 2237.67, 2237.47, 2237.27, 2237.07, 2236.87, 2236.67, 2236.47, 2236.27, 2236.07, 2235.87, 2235.67, 2235.47, 2235.27, 2235.07, 2234.87, 2234.67, 2234.47, 2234.27, 2234.07, 2233.87, 2233.67, 2233.47, 2233.27, 2233.07, 2232.87, 2232.67, 2232.47, 2232.27, 2232.07, 2231.87, 2231.67, 2231.47, 2231.27, 2231.07, 2230.87, 2230.67, 2230.47, 2230.27, 2230.07, 2229.87, 2229.67, 2229.47, 2229.27, 2229.07, 2228.87, 2228.67, 2228.47, 2228.27, 2228.07, 2227.87, 2227.67, 2227.47, 2227.27, 2227.07, 2226.87, 2226.67, 2226.47, 2226.27, 2226.07, 2225.87, 2225.67, 2225.47, 2225.27, 2225.07, 2224.87, 2224.67, 2224.47, 2224.27, 2224.07, 2223.87, 2223.67, 2223.47, 2223.27, 2223.07, 2222.87, 2222.67, 2222.47, 2222.27, 2222.07, 2221.87, 2221.67, 2221.47, 2221.27, 2221.07, 2220.87, 2220.67, 2220.47, 2220.27, 2220.07, 2219.87, 2219.67, 2219.47, 2219.27, 2219.07, 2218.87, 2218.67, 2218.47, 2218.27, 2218.07, 2217.87, 2217.67, 2217.47, 2217.27, 2217.07, 2216.87, 2216.67, 2216.47, 2216.27, 2216.07, 2215.87, 2215.67, 2215.47, 2215.27, 2215.07, 2214.87, 2214.67, 2214.47, 2214.27, 2214.07, 2213.87, 2213.67, 2213.47, 2213.27, 2213.07, 2212.87, 2212.67, 2212.47, 2212.27, 2212.07, 2211.87, 2211.67, 2211.47, 2211.27, 2211.07, 2210.87, 2210.67, 2210.47, 2210.27, 2210.07, 2209.87, 2209.67, 2209.47, 2209.27, 2209.07, 2208.87, 2208.67, 2208.47, 2208.27, 2208.07, 2207.87, 2207.67, 2207.47, 2207.27, 2207.07, 2206.87, 2206.67, 2206.47, 2206.27, 2206.07, 2205.87, 2205.67, 2205.47, 2205.27, 2205.07, 2204.87, 2204.67, 2204.47, 2204.27, 2204.07, 2203.87, 2203.67, 2203.47, 2203.27, 2203.07, 2202.87, 2202.67, 2202.47, 2202.27, 2202.07, 2201.87, 2201.67, 2201.47, 2201.27, 2201.07, 2200.87, 2200.67, 2200.47, 2200.27, 2200.07, 2199.87, 2199.67, 2199.47, 2199.27,

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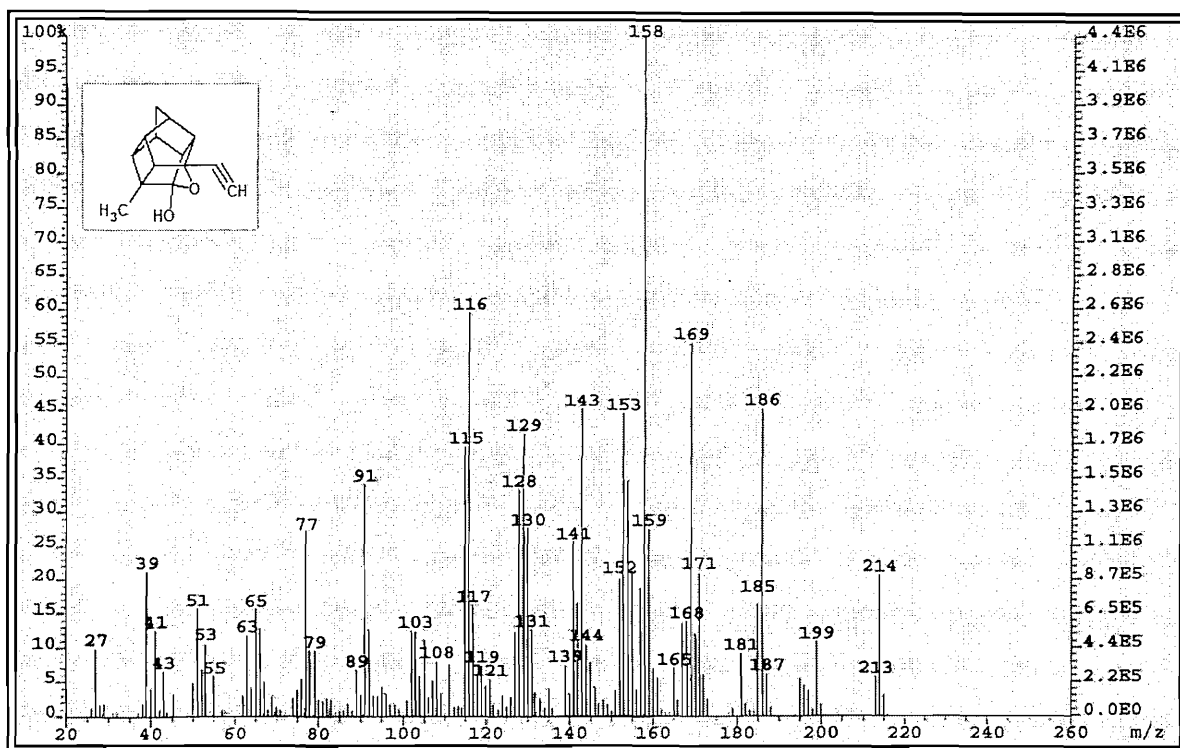


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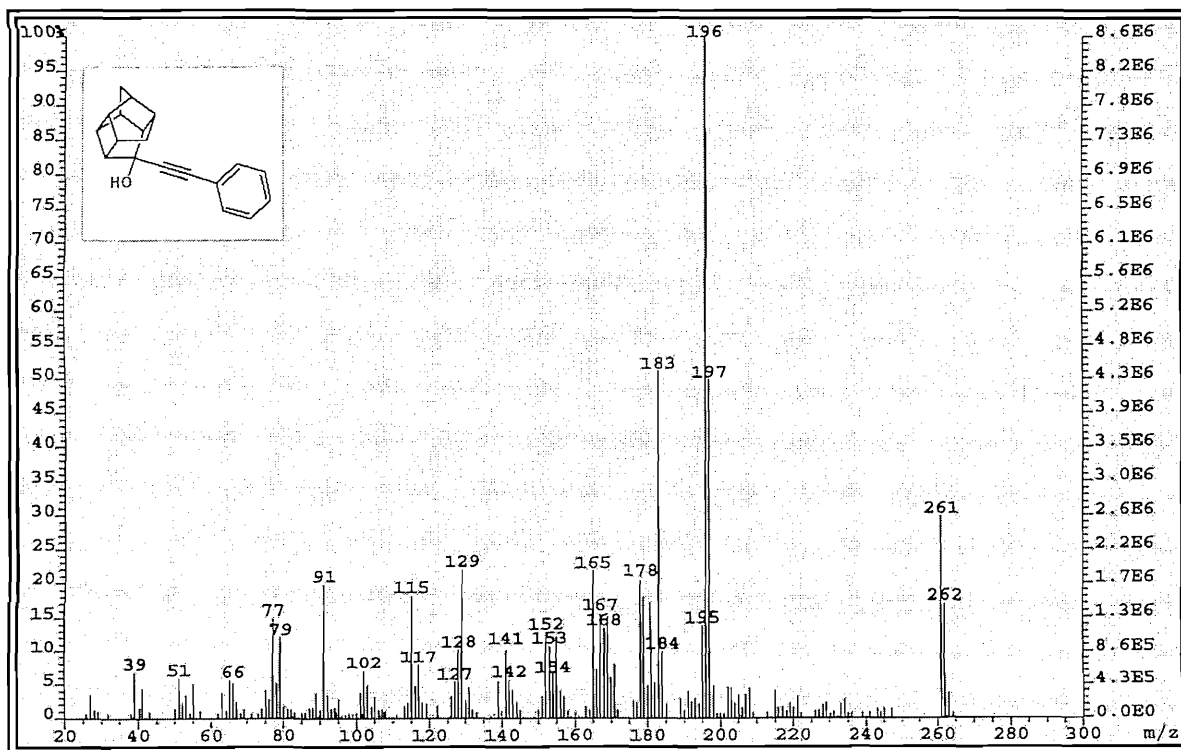


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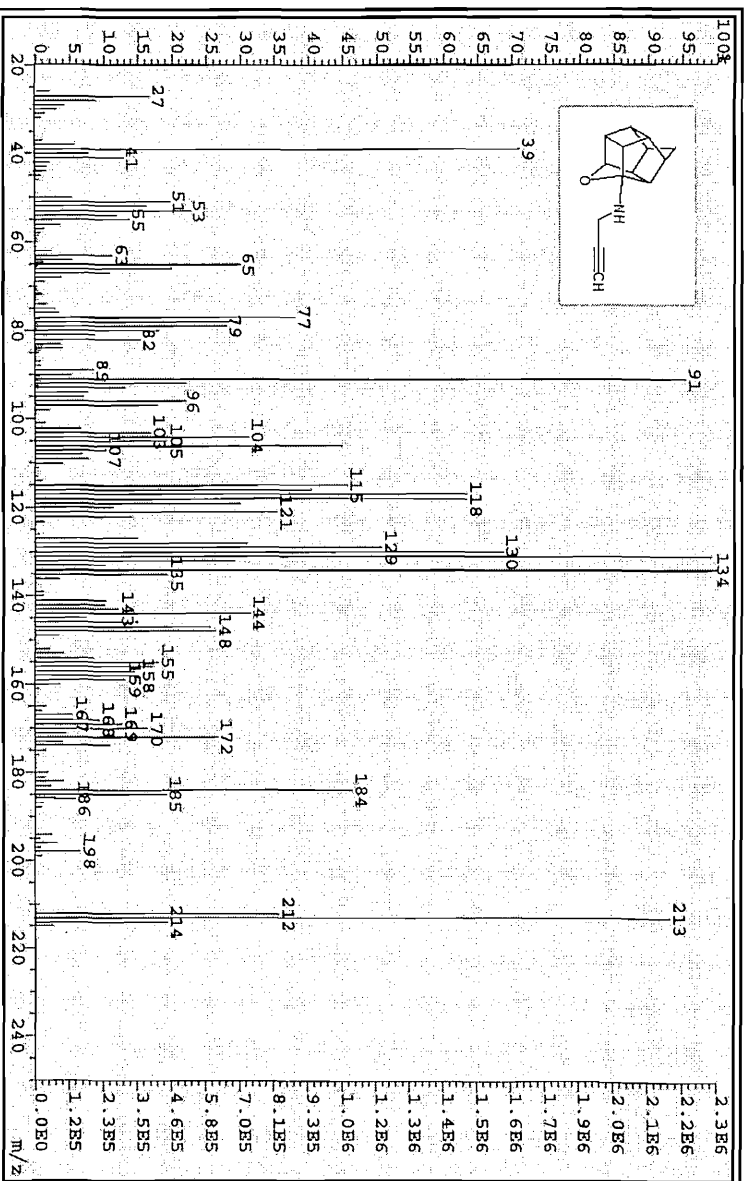
MASS SPECTROMETRY



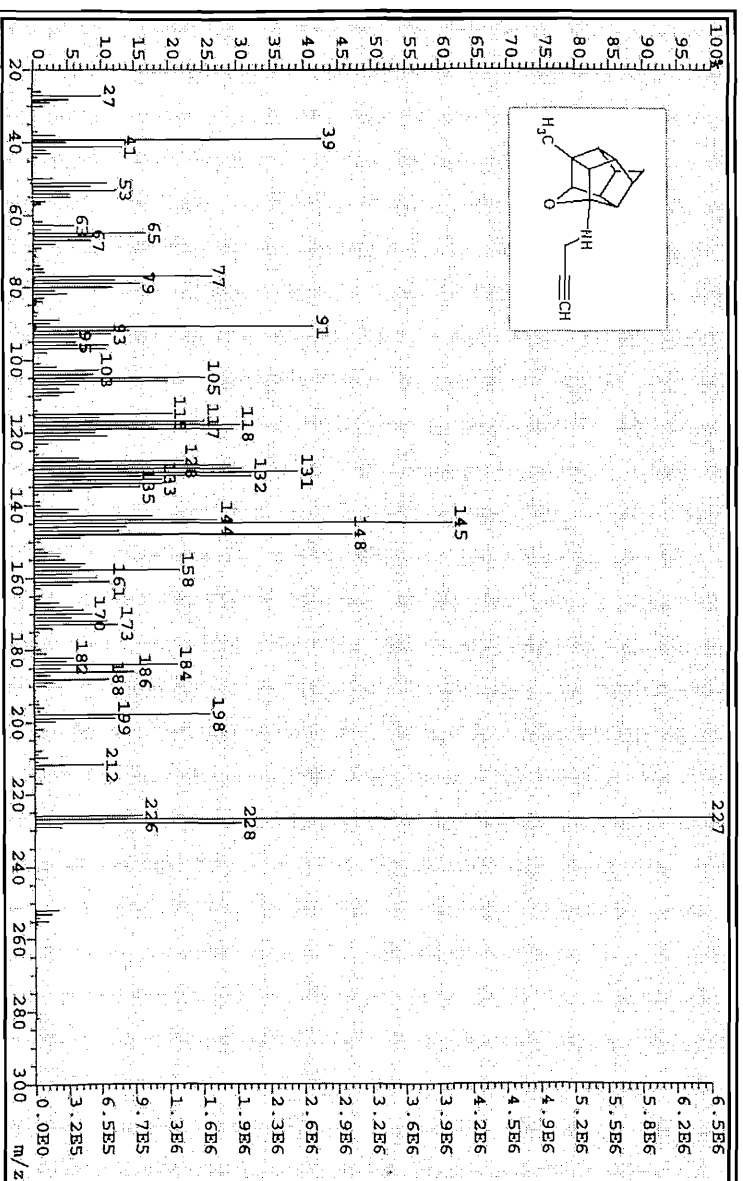
Spectrum 9



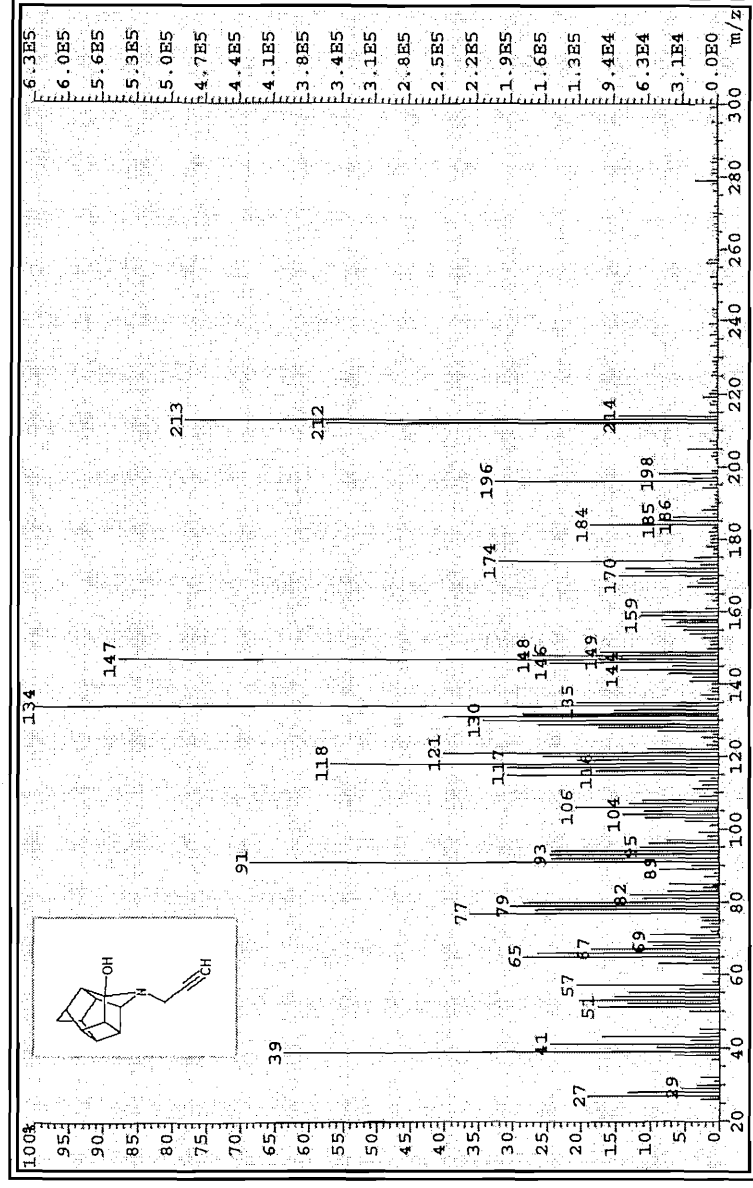
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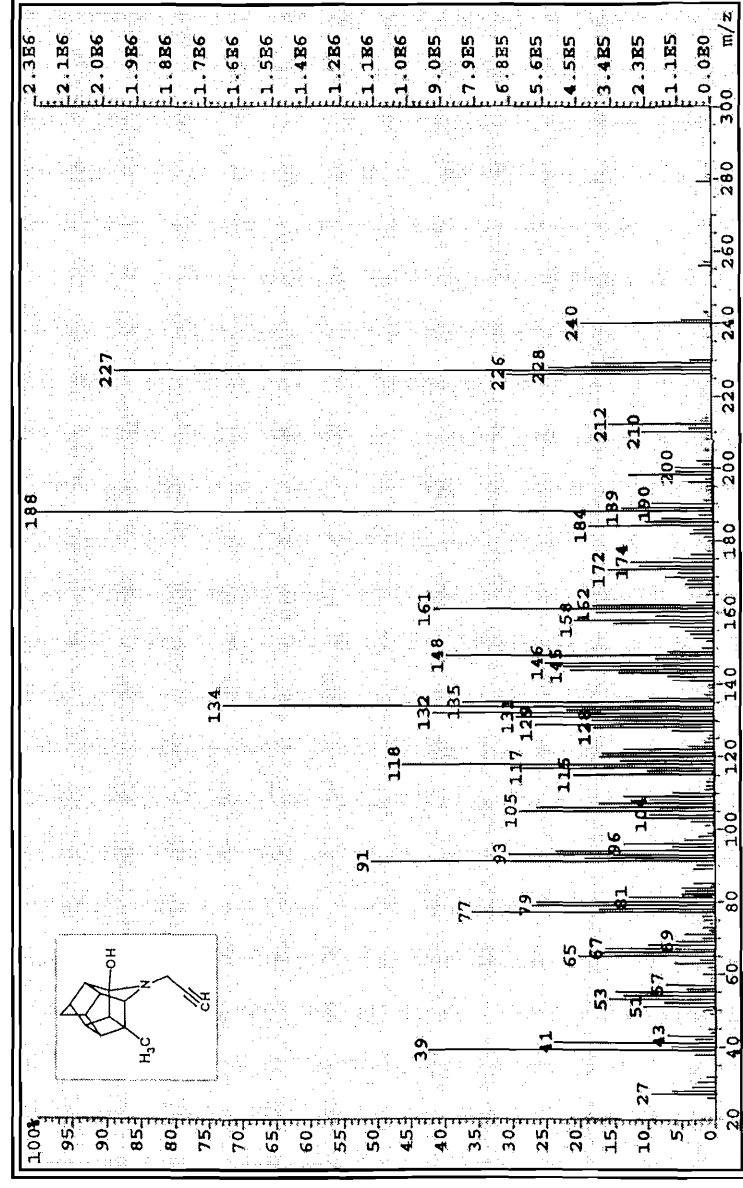
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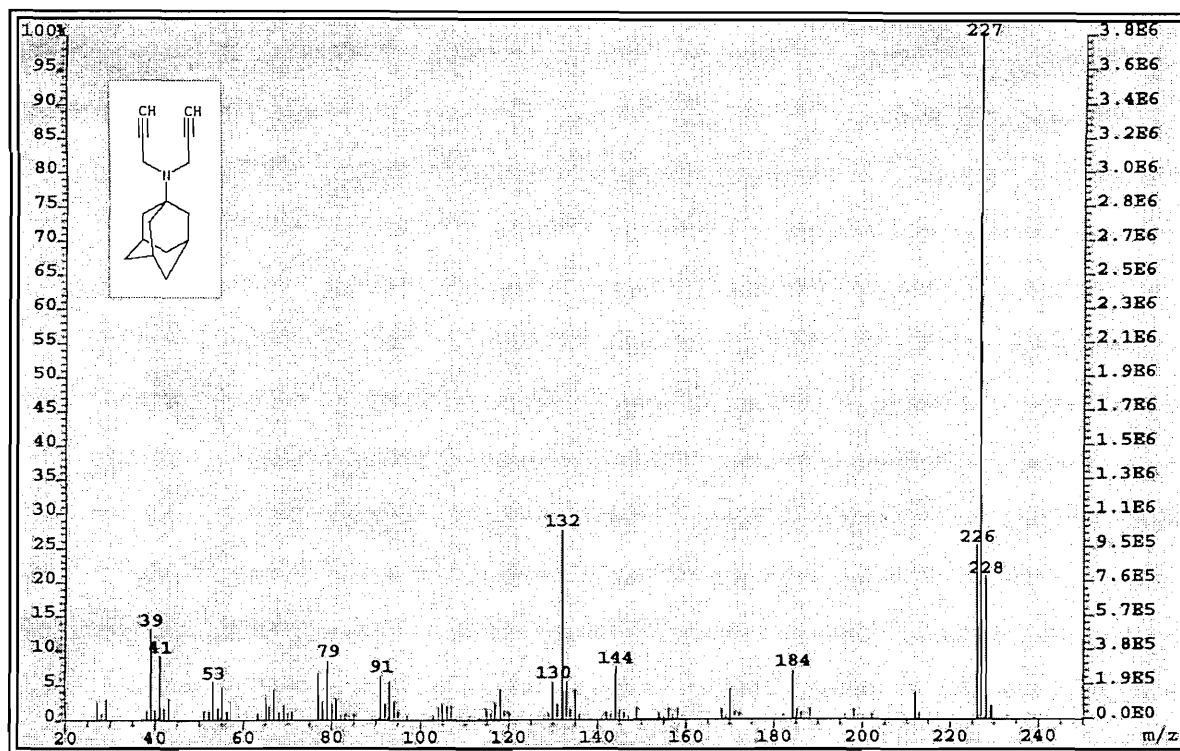
Spectrum 12



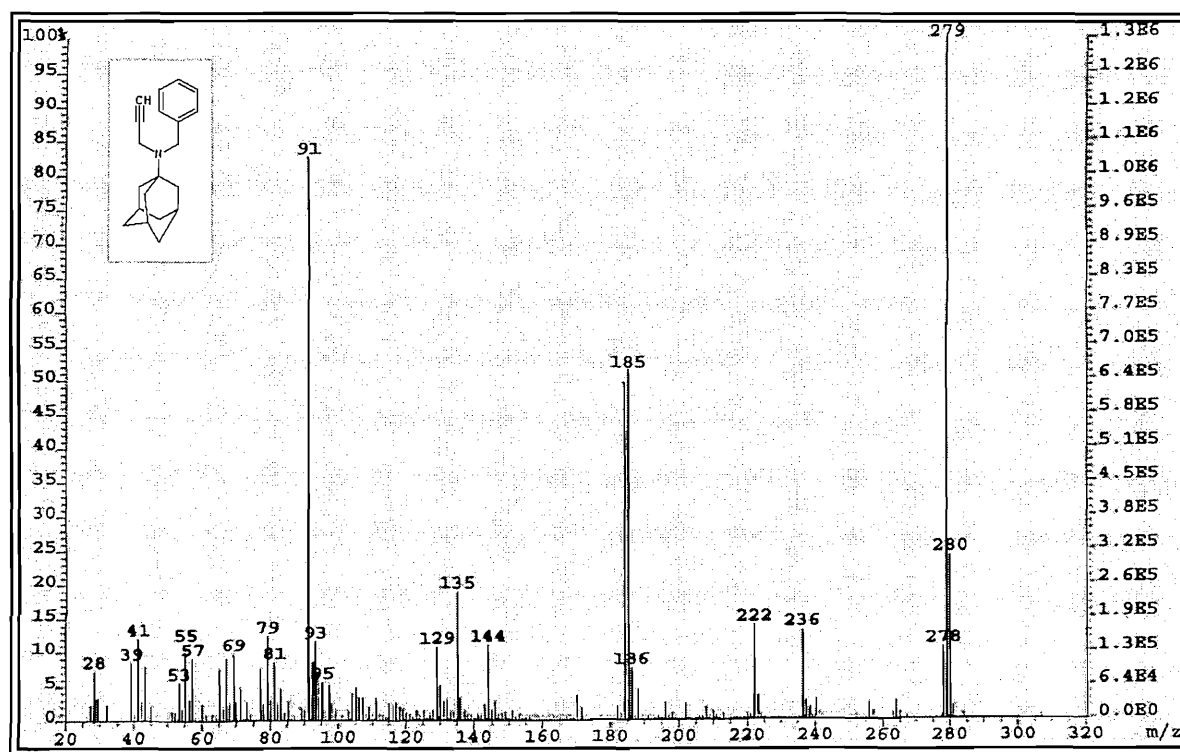
Spectrum 13



Spectrum 14

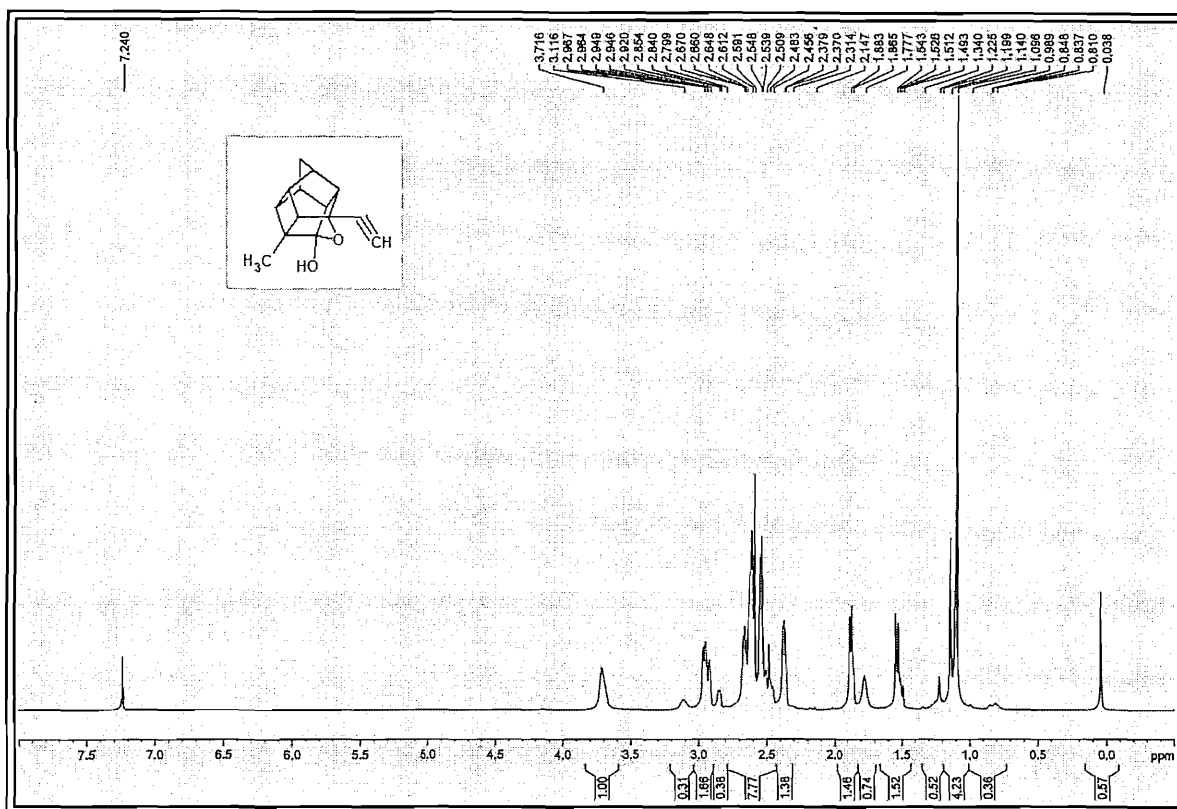


Spectrum 15

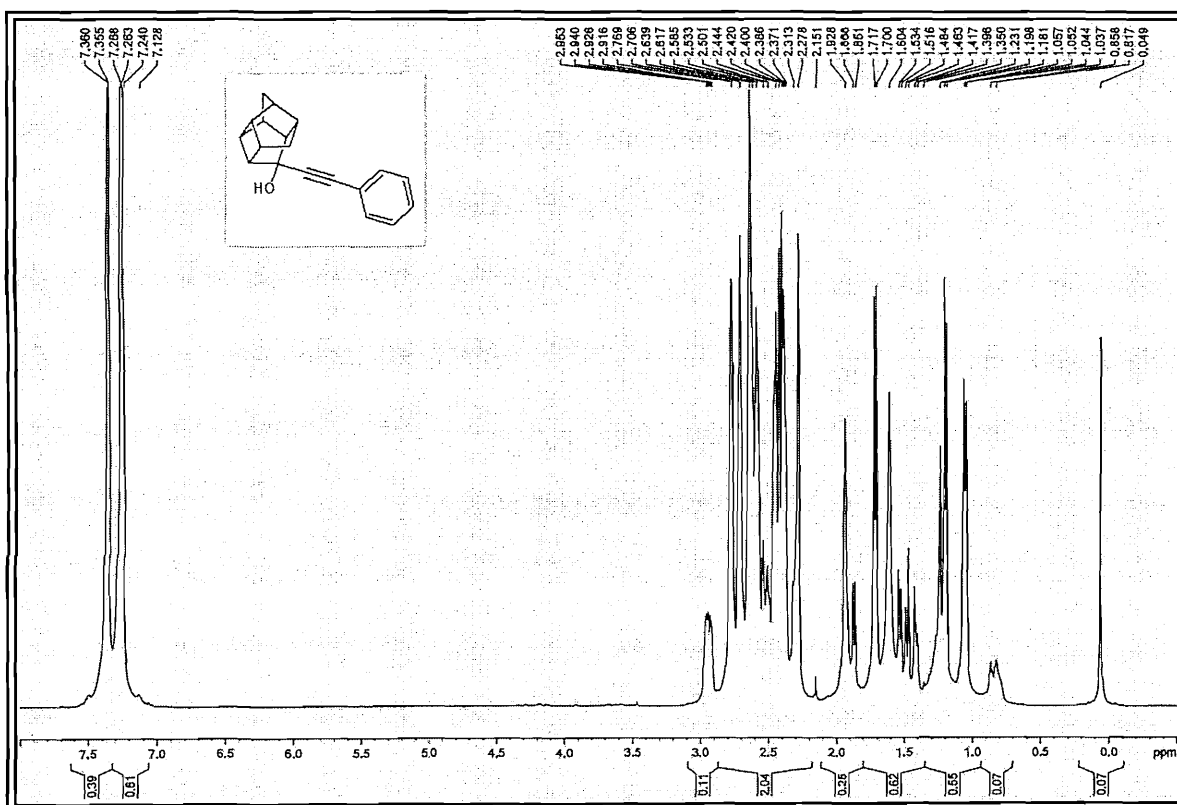


Spectrum 16

^1H
NUCLEAR MAGNETIC RESONANCE



Spectrum 17



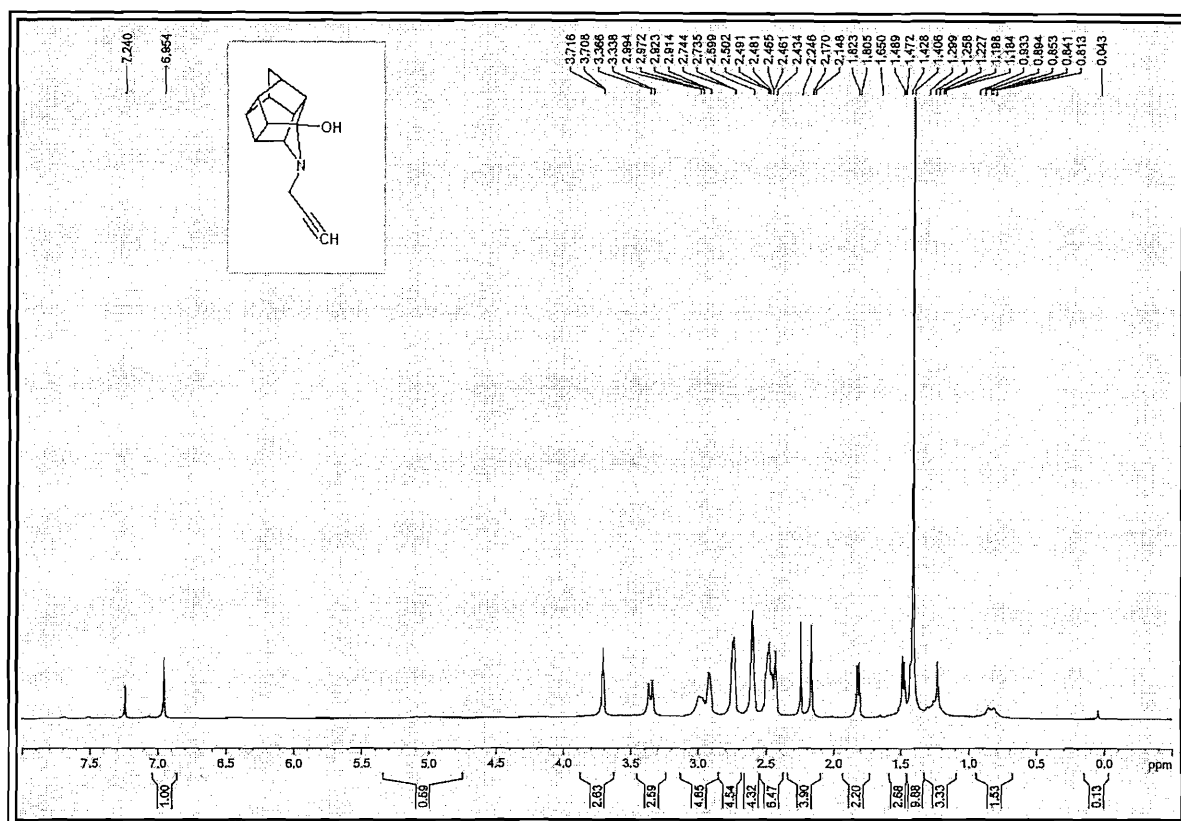
Spectrum 18

Chemical structure: CC12C(=O)C1C#CC2

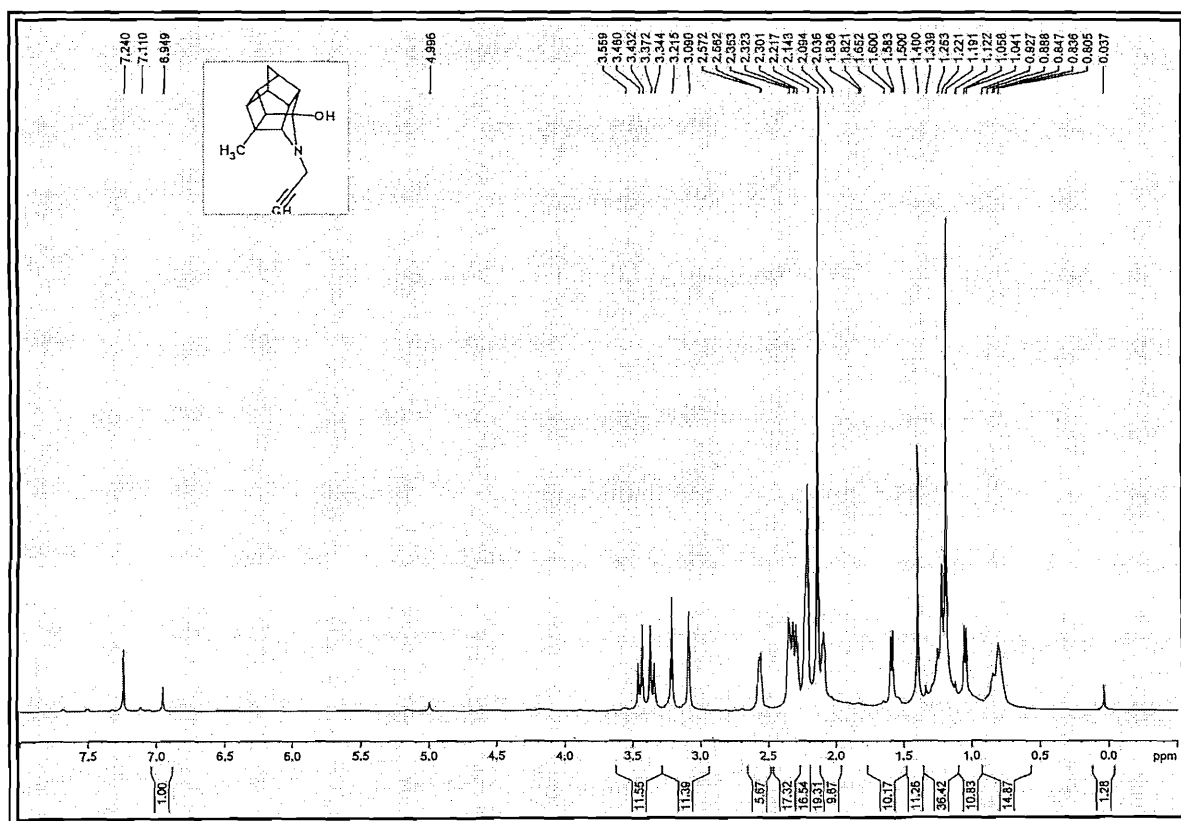
¹H NMR spectrum (CDCl₃) showing peaks from 0.0 to 7.5 ppm. Integration values are provided below the baseline.

Chemical Shift (ppm)	Integration
7.240	1.00
4.5	0.74
4.0	4.01
3.5	10.02
2.5	53.30
2.0	9.97
1.5	6.63
1.0	18.84
0.5	5.26
0.0	0.46

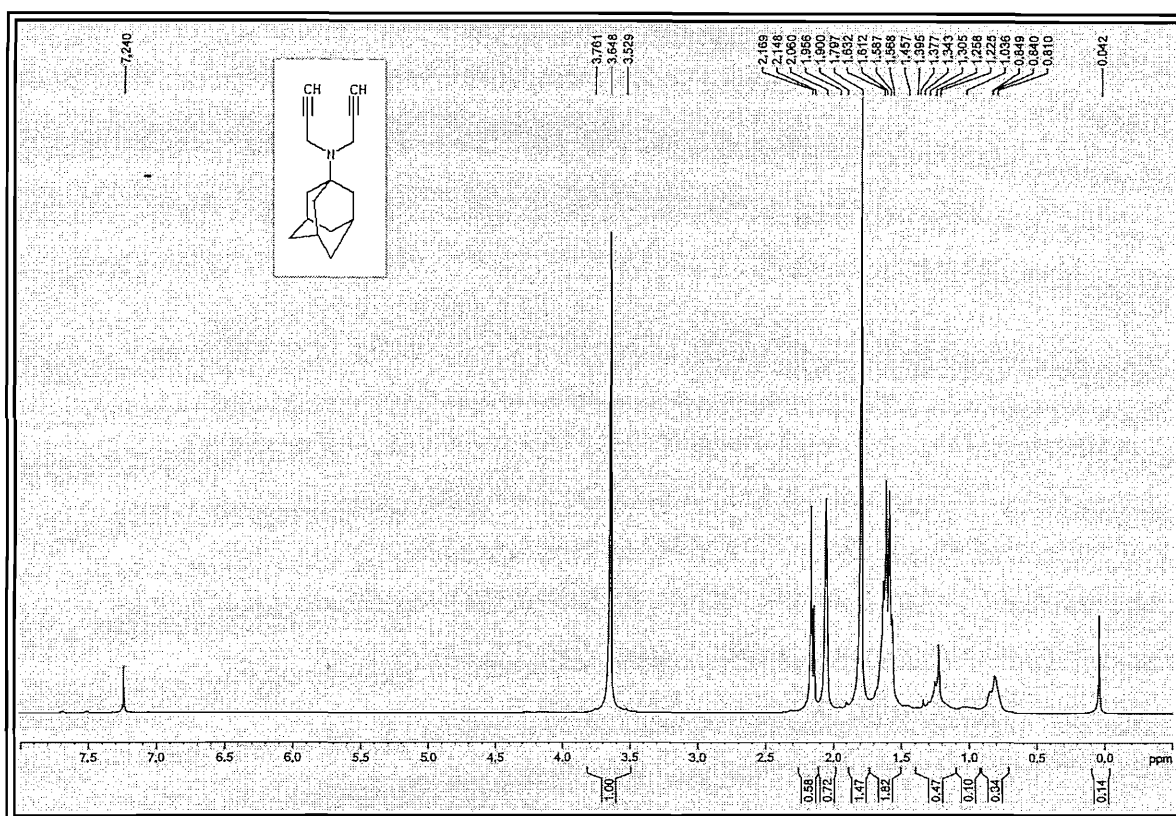
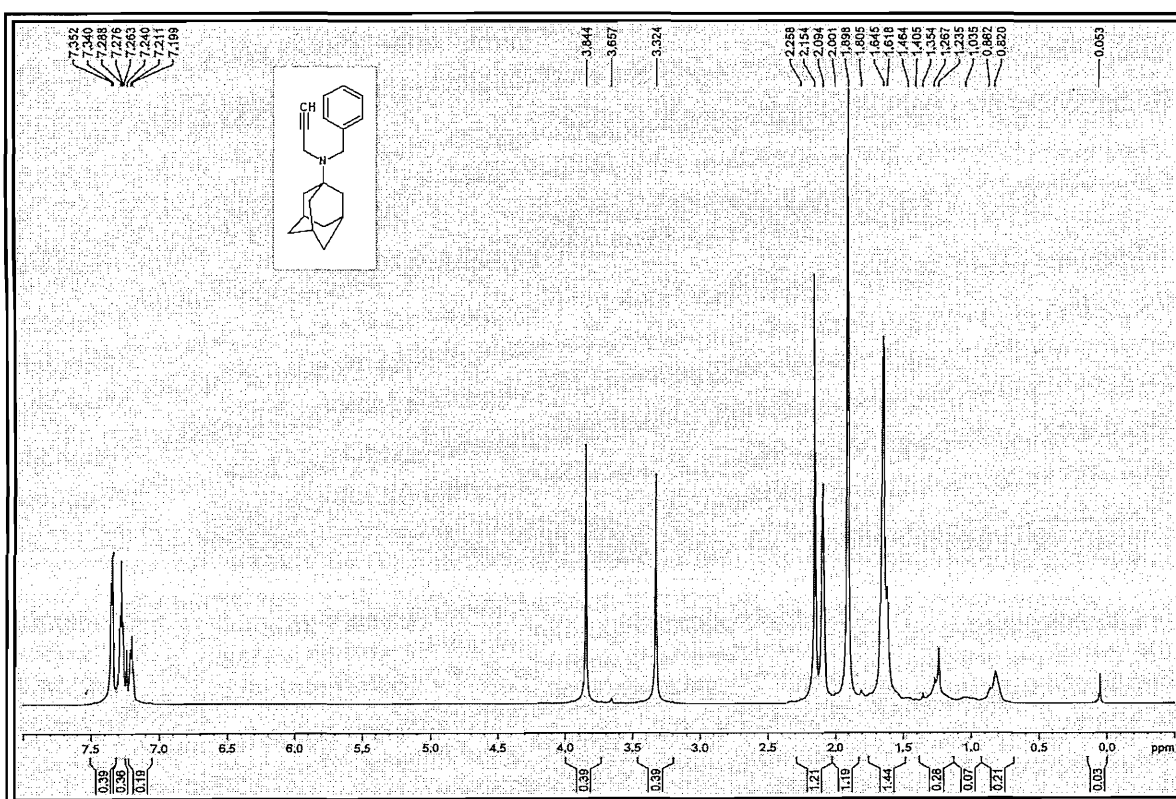
117



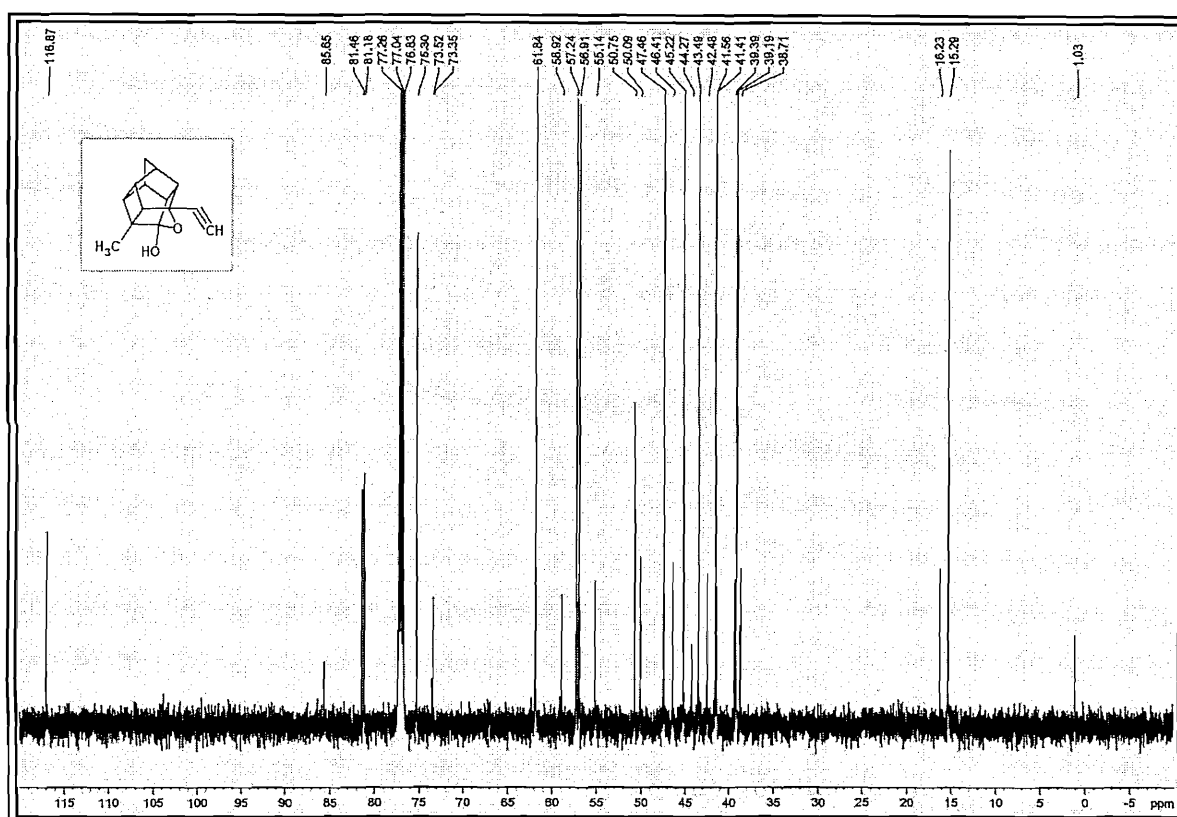
Spectrum 21



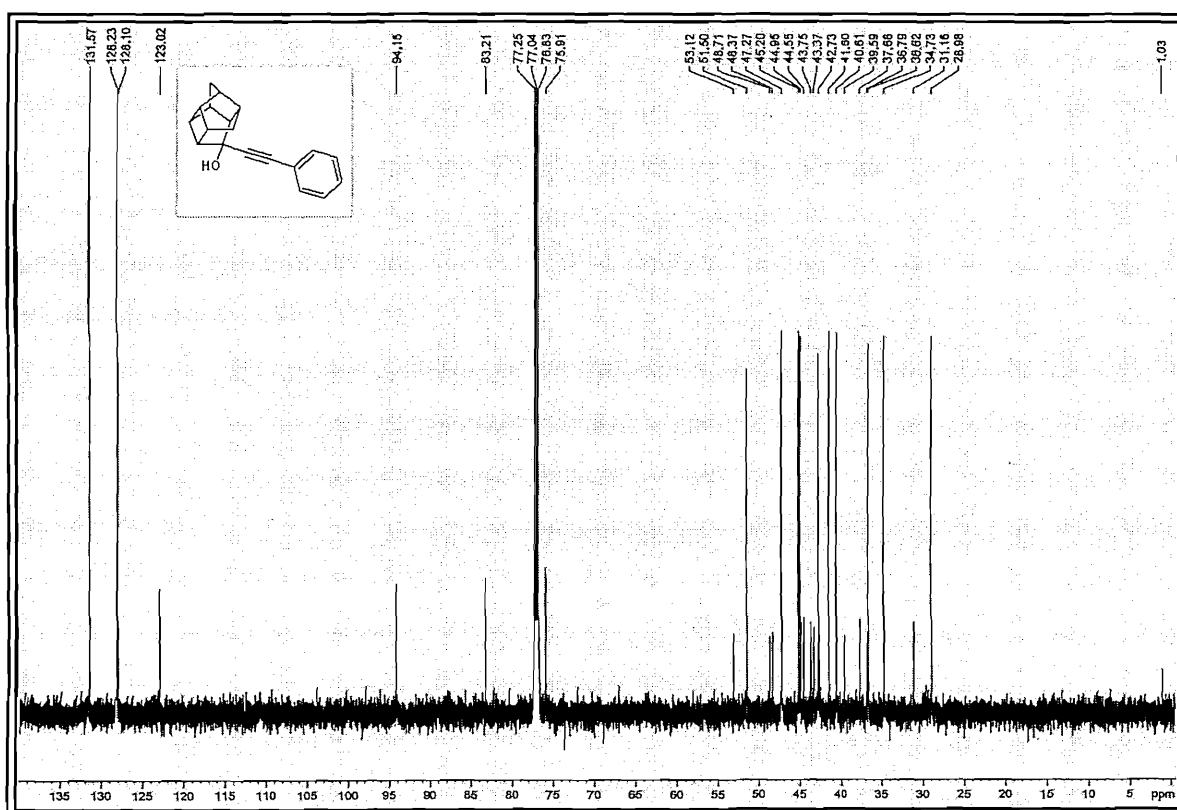
Spectrum 22

**Spectrum 23****Spectrum 24**

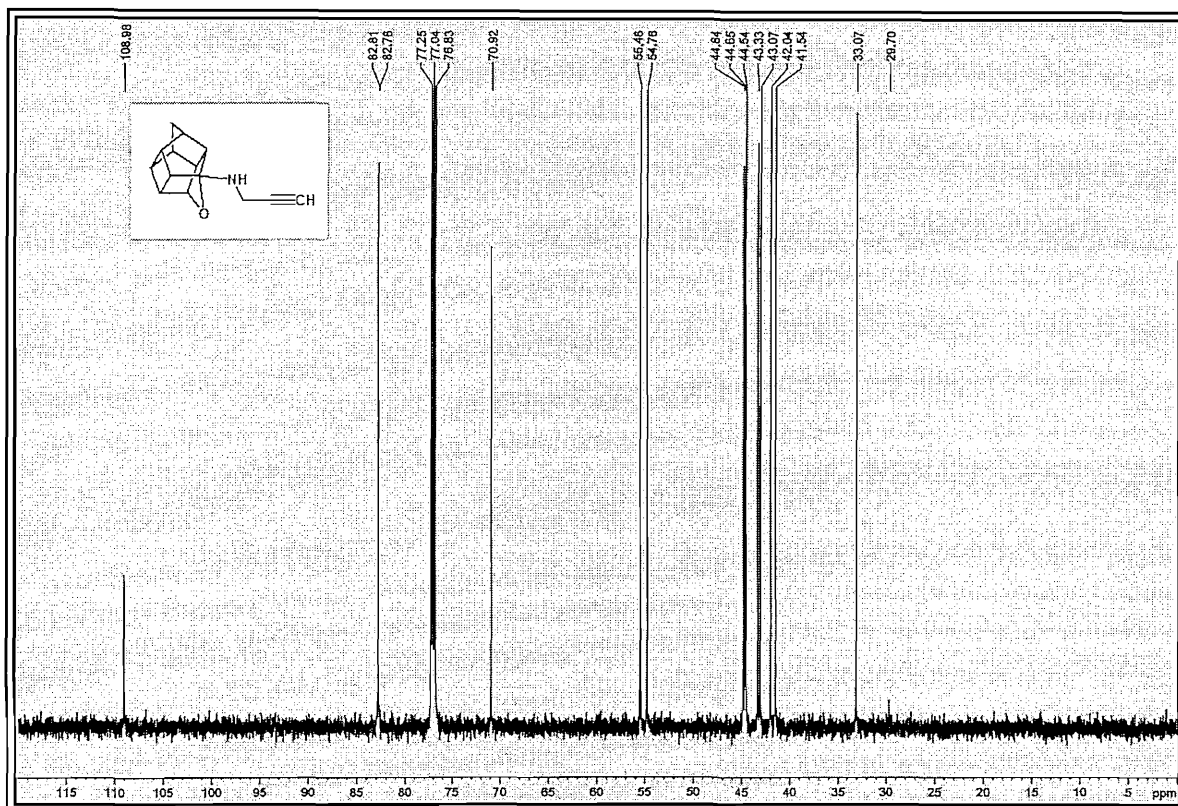
^{13}C **NUCLEAR MAGNETIC RESONANCE**



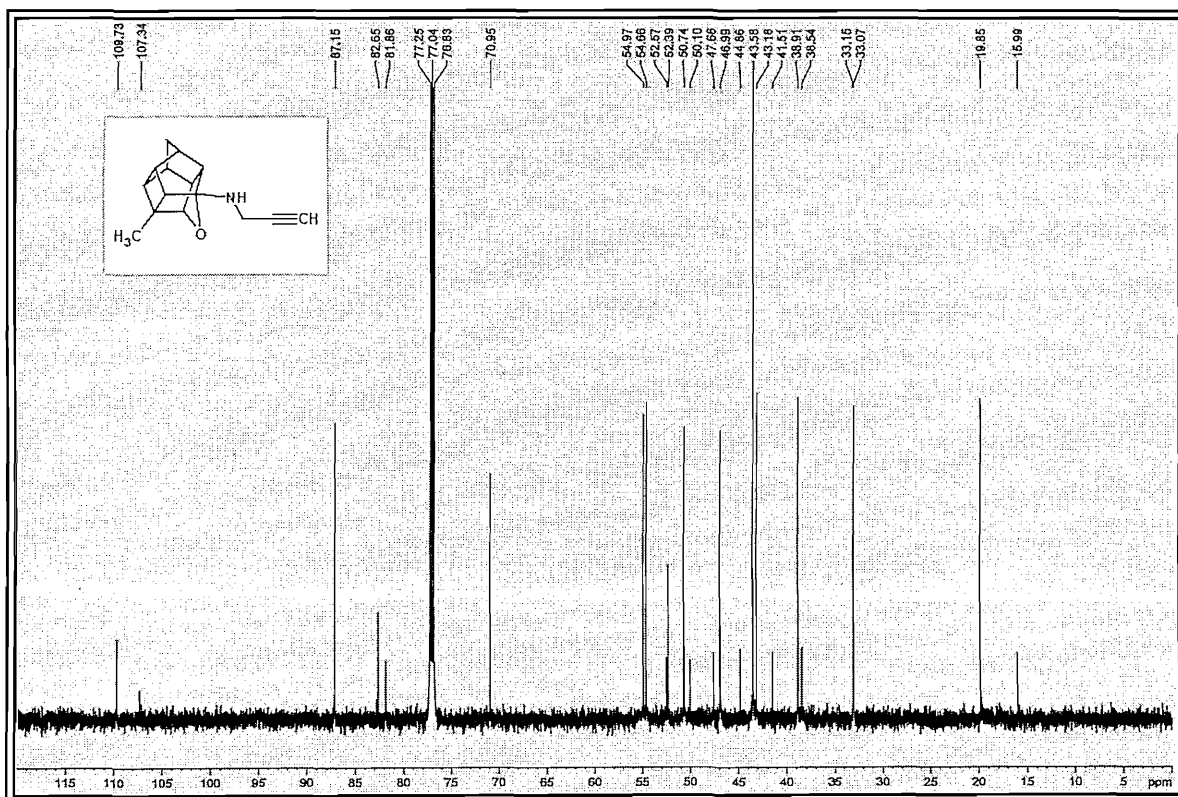
Spectrum 25



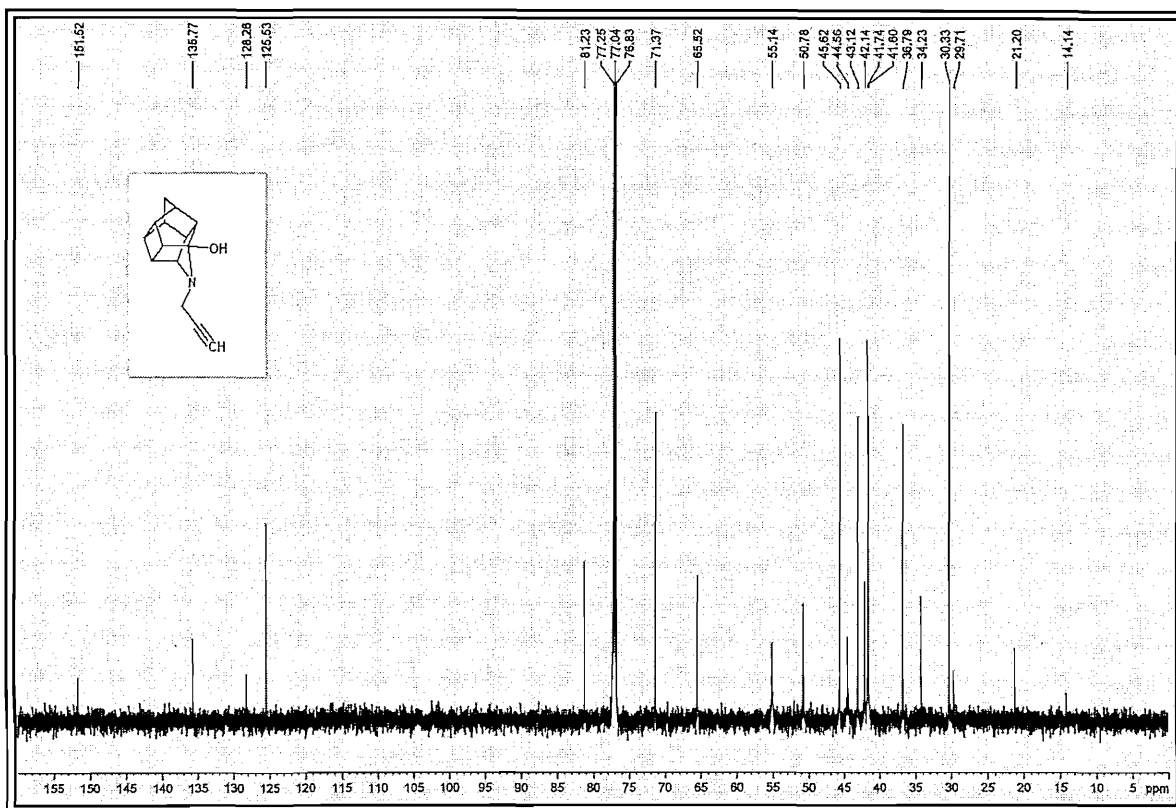
Spectrum 26



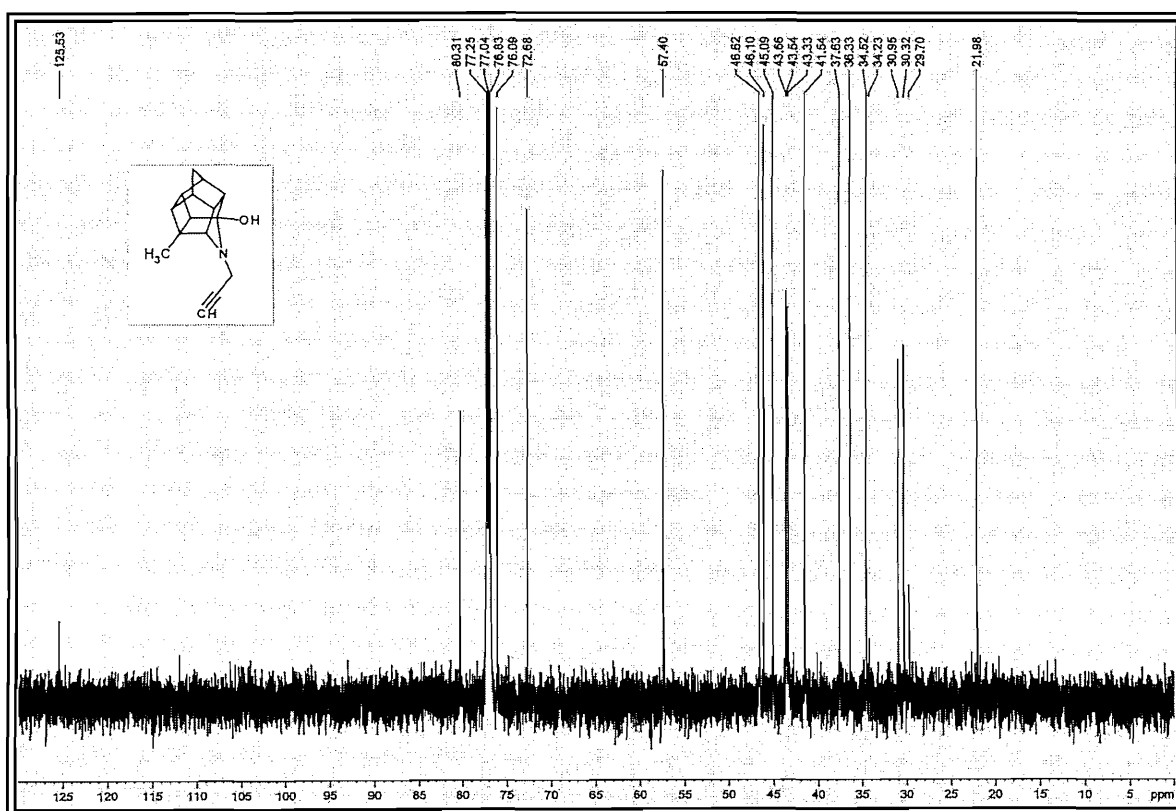
Spectrum 27



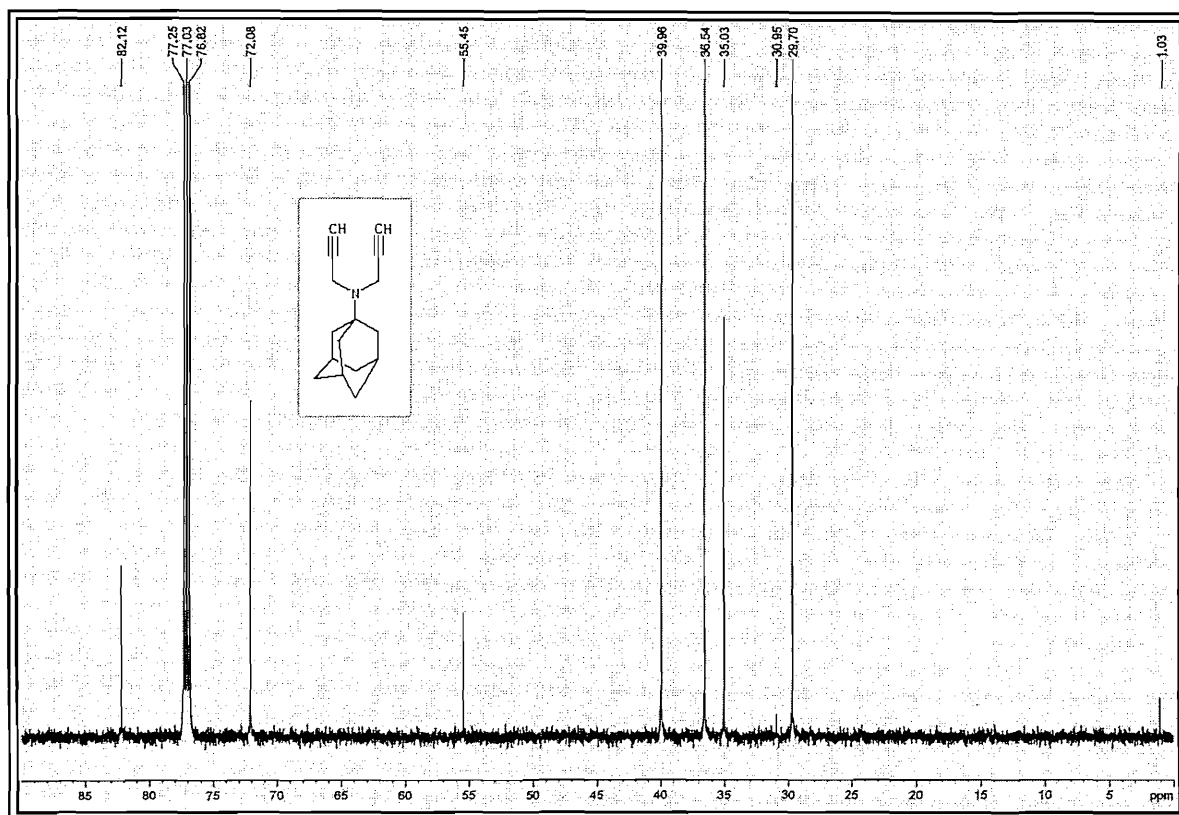
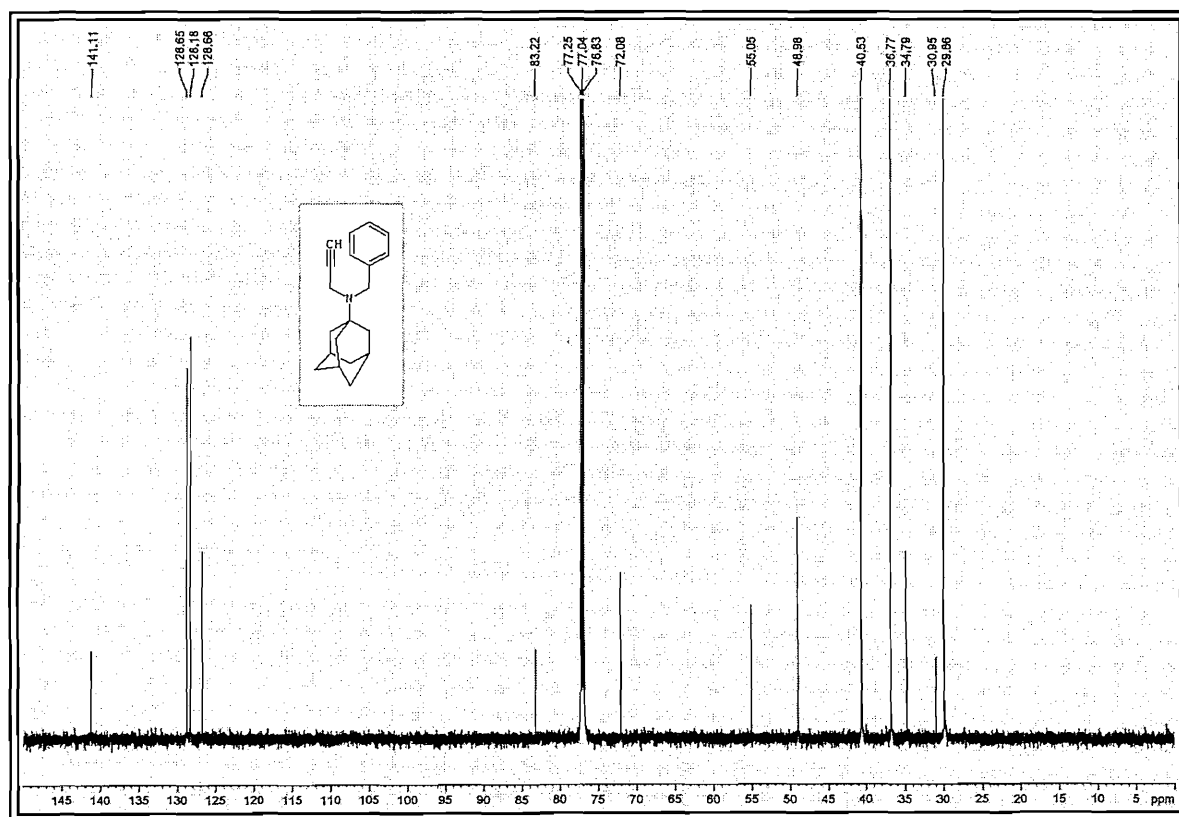
Spectrum 28



Spectrum 29



Spectrum 30

**Spectrum 31****Spectrum 32**

ACKNOWLEDGEMENTS

I dedicate this dissertation to Almighty God and to my parents, Lennon Paul Barber and Marilyn Rose Barber. They have made it possible for me to have had this wonderful opportunity!!

- ✚ May God's name be glorified by the work I have done. All glory and honour is His!!!
- ✚ I just want to thank my parents for always being there for me when I needed them most!! I want to thank them for their unconditional love, support and comfort!!
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