UNIVERSITY OF THE WESTERN CAPE

Design & Synthesis of Polycyclic Amine derivatives for Sigma Receptor Activity

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KEYWORDS

Polycyclic amine

Pentacycloundecane

Amantadine

Heterocyclic amines

Sigma receptor

Neurodegeneration

Drug addiction

Blood brain permeability

Drug Design

Ligand based

Structure activity relationship

ABSTRACT

New therapeutic strategies are needed for a diverse array of poorly understood neurological impairments. These include neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, and the psychiatric disorders such as depression, anxiety and drug dependence. Popular neuropharmacotherapies have focused on dopamine (DA), serotonin (5HT), γ-aminobutric acid (GABA) and glutamate systems (Jupp & Lawrence, 2010). However recent research points to the sigma receptor (σR) as a possible neuromodulatory system. Due to its multi-receptor action, the σR can trigger several significant biological pathways. This indicates its ideal potential as a drug target to effectively minimise drug dosage and potential side effects.

Currently there are a limited number of σR ligands available and few possess the selectivity to significantly show σR's role in neurological processes. Polycyclic amines have shown notable sigma activity and provide an advantageous scaffold for drug design that can improve pharmacodynamic and pharmacokinetic properties (Banister *et al*., 2010; Geldenhuys *et al*., 2005). Aryl-heterocycle amine groups were also shown to improve σR activity (Piergentili *et al*., 2009).

A series of pentacycloundecane compounds were synthesised which aimed at evaluating the inclusion of a amine containing aryl group in the design compared to previous pentacycloundecane structures containing only two lipophilic regions. These synthesises followed mostly bimolecular nucleophilic substitution and nucleophilic addition mechanisms. From this study we were able to gain a better understanding into synthesises involving pentacycloundecanes and devised new general methods for expanding consecutive series of these promising structures. Their affinity for the σ_1R was evaluated using the radioligand [^{3}H] (+)-pentazocine on Sprague-Dawley rat liver membranes. The difference between the oxa-and aza derivatives and piperidine versus piperazine moieties were compared.

The compound *N*-[2-(4-benzylpiperazin-1-yl) ethyl]-4-azahexacyclo [5.4.1.0^{2,6}.0^{3,1}0.0^{5,9}.0^{8,11}] dodecan-3-ol had the best affinity and suggests that the aza compounds are more favourable for σ_1R binding than their oxa counterparts. The addition of an amine containing aryl group remained inconclusive as these compounds fell within a similar range of affinities compared to other structures with two lipophilic binding regions. The synthesised compounds do not possess affinity for the σ_1R to the extent that most commercially available σ_1R ligands do.

Selectivity for the different σRs and affinity for other receptors need to be further explored to fully evaluate the potential neuroprotective effects of these structures. Additional biological activity assays are also necessary to determine their pharmacological properties and blood brain permeability.

DECLARATION

I declare that *Design & Synthesis of Polycyclic Amine derivatives for Sigma Receptor Activity* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Natasha Strydom February 2013

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I. INTRODUCTION & AIMS OF THESIS

1. Introduction

Polycyclic amines have shown to be valuable lead compounds in the development of central nervous system (CNS) acting drugs (Geldenhuys *et al*., 2005). These polycyclic compounds possess their own neuromodulatory activity on important receptor classes which have been implicated in CNS disease states such as Alzheimer's disease, Parkinson's disease, Huntington's disease, schizophrenia, stroke and disease states as intricate as drug addiction (Nguyen *et al*., 1996; Oliver *et al*., 1991; van der Schyf *et al*., 1986; Geldenhuys *et al*., 2005). They act as NMDA receptor antagonists (Geldenhuys *et al*., 2003), are able to spontaneously increase dopamine release (Geldenhuys *et al*., 2009) and block *L*-type voltage gated calcium channels (van der Schyf *et al*., 1986). They also have the ability to greatly improve the lipophilicity of their conjugates, which is helpful in increasing blood brain barrier permeability leading to increased concentration of CNS acting drugs in the brain, decreasing dosage and ultimately minimising peripheral side effects (Brookes *et al*., 1992; Zah *et al*., 2003).

Several published pentacycloundecane series have shown that both pentacycloundecane containing compounds and the amantadine structure may present with sigma receptor activity (Kassiou *et al*., 1996; Marrazzo *et al*., 2001; Kornhuber *et al*., 1993). The sigma receptor itself has been suggested as a drug target for CNS conditions such as drug addiction (Maurice *et al*., 2002), cognition (van Waarde *et al*., 2011), pain (Cendán *et al*., 2005) and depression (Urani *et al*, 2001). It also shows promise as a future cancer target (Crawford & Bowen, 2002; Wei *et al*., 2006).

The sigma receptor, which was originally thought to be an opioid receptor, is divided into two subtypes; the sigma 1 specific receptor (σ_1R) and the sigma 2 specific receptor (σ_2R), which are now classified as distinct receptors (Martin *et al*., 1976; Quiron *et al*., 1987) and do not share homology with any other known mammalian enzyme or receptor (Hellewell & Bowen, 1990; Quirion *et al*., 1992; Hanner *et al*., 1996). The σ_1R has been cloned and is implicated in intracellular signalling, synaptic transmission and is able to mediate effects on calcium conductance, NMDA activity, potassium channel activity, protein kinases and modulation of inositol phosphatases (Aydar *et al*., 2002; Hayashi & Su, 2007). The σ_2R has been implicated in calcium channel modulation, apoptosis and motor activity but with a lack of truly specific σ_2R ligands available its exact mechanism is unknown. Both receptors are located on the endoplasmic reticulum and it is likely that the σ_1R mediates its response *via* translocation from the endoplasmic reticulum to other cellular compartments. The σ_2R is richly expressed in lipid rafts and it is these lipid rafts that are probably responsible for σ_2R activity on cellular membranes (Hanner *et al*., 1996; Su *et al*., 2009). However, the exact mechanism behind sigma receptor action remains poorly understood

and there is a limited amount of sigma ligands with desirable specificity available (Jupp *et al.*, 2010). There is thus a need for novel sigma receptor ligands showing enhanced affinity and selectivity to better establish the role of the sigma receptor and to validate its potential as a drug target for new therapeutic agents involving brain and behaviour disorders.

2. Aims of thesis

The aims of the current study are as follows:

1. **Investigate the pharmacophore of the sigma receptor using extensive literature on current sigma receptor ligands to examine trends in sigma receptor binding.**

Glennon, Ablordeppey, Younes, Cobos and Zampieri have done extensive studies to investigate the pharmacophore of the sigma receptor (Ablordeppey *et al*., 1998, 2000, 2002; Glennon *et al*., 1994, Glennon, 2005; Younes *et al*., 2000; Cobos *et al*., 2008; Zampieri *et al*., 2009). Their efforts have provided a basic template for sigma receptor binding and the diagram shown in figure 1 illustrates the requirements for σ_1R receptor specific binding. This template suggests that binding is dependent on compounds presenting two lipophilic moieties joined by a basic amine that can be contained as a secondary or tertiary amine and can be present in a cyclic structure. An interesting challenge present in studies undertaken to synthesise new sigma receptor ligands is that the sigma receptor pharmacophore is relatively unspecific to various compounds and a substantial number of compounds with diverse applications bind to the sigma receptor proving its structural requirements are accessible. This is a unique limitation in drug design when considering ligand specificity in designing compounds that will strictly bind to the sigma receptor alone (*Chapter II*).

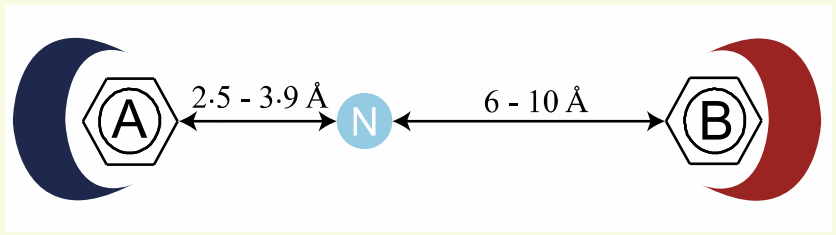
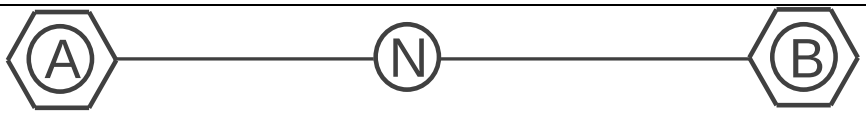
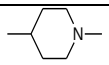
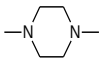


Figure 1. Illustration of optimum binding for σR affinity (Adapted and redrawn from phramacaphores proposed by Glennon *et al*., 1994, 2005)

The compounds investigated by Ablordeppey, Cobos, and Zampieri show that the addition of an aryl piperazine benzyl structure is advantageous to sigma receptor binding. It was therefore decided that the limited amount of polycyclic amines evaluated for sigma receptor activity provided the opportunity to develop a series that would bind to the basic pharmacophore while expanding into the

use of a similar aryl amine benzyl group. Table 1a shows two such compounds and their respective affinities. (Please see extended Table 1b in *Chapter II* for full list of compounds evaluated).

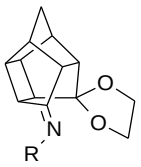
Table 1a: Compounds showing sigma receptor activity

2° binding site	2° linking chain	Basic Amine	1° linking chain	1° binding site	Binding Affinity		
					Ki (nM)		
					σR	σ_1R	σ_2R
	-CH_2-		-$CH_2(CH_2)_3CH_2$-			0.4	-
	-CH_2-		-$CH_2(CH_2)_3CH_2$-			0.6	2.8

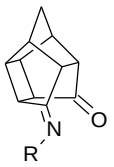
2. **Design novel pentacycloundecane derivatives based on the sigma pharmacophore that could show improved activity or provide better insight into receptor binding and point to future potential specific binding areas valuable to the current sigma receptor pharmacophore.**

We proposed the following compounds as potential sigma receptor binding substrates:

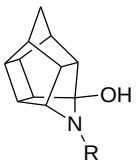
Table 2: Idealised series of polycyclic structures with sigma receptor binding potential.



Series A



Series B



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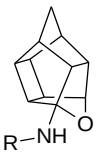
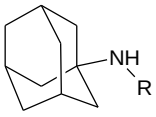
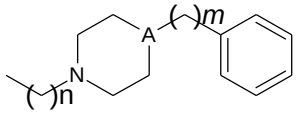


Series D



Series E

R =



	n	*m*	A
1*	1	1, 2, 3	N/C
2*	2	1, 2, 3	N/C
3*	3	1,2,3	N/C

The basic skeleton of the proposed structures stay within the guidelines initially proposed by Glennon *et al*., (1994, 2005) while including a benzyl piperazine or benzyl piperidine group that should provide a possible shift from the amine contained in the cage structure. This inclusion should help expand on our current knowledge of the sigma receptor pharmacophore (*Chapter III*).

3. Develop routes of synthesises and synthesise the selected series of compounds.

Investigation into the synthesis of pentacycloundecane, benzyl piperazine and benzyl piperidine offers the opportunity for novel synthetic pathways that can expand not only on the potential series for sigma receptor binding, but other targeted neurodegeneration receptors such as; the NMDA receptors, dopamine receptors and GABA channels. Popular synthesis routes involving amine containing aryl groups involve nucleophilic addition and synthetic routes of pentacycloundecane derivatives often utilise nucleophilic addition. This study provides the option of expanding on synthetic strategies for polycyclic and amine containing structure conjugation (*Chapter III*).

4. Evaluate the synthesised novel ligands for sigma receptor affinity.

Standardised sigma receptor binding is evaluated by radioligand markers. Dual sigma receptor binding is evaluated by [^{3}H] DTG, a dual σ_1R and σ_2R ligand with similar affinity for both receptors. Compound affinity for σ_1R binding is evaluated with σ_1R specific ligand [^{3}H] (+)-pentazocine. Many research groups are actively pursuing specific σ_2R radioligands, but none are commercially available at present. Instead σ_1R antagonist, dextrallorphan is used to block σ_1Rs and then [^{3}H] DTG is used to evaluate the number of unoccupied σ_2R receptors. For this study σ_1R radioligand binding with [^{3}H] (+)-pentazocine should be sufficient to evaluate the proposed structures' prospects as future sigma receptor ligands and validate the inclusion of pentacycloundecane moieties for improving sigma receptor binding (*Chapter IV*).

3. Conclusion

Several groups are pursuing the synthesis of more potent and specific sigma receptor ligands from previous "hit" compounds. We however, realise the lack of significant sigma ligands as the foundation to build on diversified compounds to possibly obtain new hit molecules that can be optimised at a later stage. The results of this study will help expand literature of the sigma receptor ligands and improve our ideas of how to approach this enigmatic receptor.

II. LITERATURE REVIEW

1. Sigma receptor

The sigma receptor (σR) and confusion surrounding its function and properties have come to be known as "The Sigma Enigma." Although the exact mechanism and biological pathways that the sigma receptor may be involved in are currently still unknown, the receptor has been suggested as a drug target for several diseases involved in brain and behaviour disorders and peripheral disease states such as cancer and decreasing HIV replication in drug abusers (Chavkin, 1990; Jupp & Lawrence, 2010).

1.1 History

The σR was originally discovered in 1976, but mistakenly classified. *N*-allylnormetazocine (SKF-10,047) binding to the receptor was thought to be antagonized by the universal opioid antagonist naloxone. It was therefore mislabelled as an opioid receptor. Martin *et al*. (1976) proposed that this sigma/opioid receptor provided the psychomimetic effects seen with the benzmorphans, *N*-allylnormetazocine and its analogues. Su (1982) demonstrated that the sigma/opioid receptor was able to identify a binding site for the receptor ligand SKF-10,047, however naloxone exhibits no affinity for this receptor. In reality this sigma/opioid receptor of Su (1982) was not the sigma/opioid receptor proposed by Martin *et al*. (1976) as the latter is in fact sensitive to naloxone. Later the mistaken sigma/opioid receptor of Su (1982) was renamed by Martin to sigma receptor to differentiate it from the opioid class (Su *et al*., 1988). Unfortunately the σR was still misinterpreted, this time confounded with the phencyclidine (PCP) receptor which acts on the *N*-methyl-d-aspartic acid (NMDA) calcium regulated channel due to sigma ligands used at the time possessing high PCP binding affinities (Parsons *et al*., 1999). This was later rectified with more specific σR ligands and σRs were reclassified as unique entities unlike any other neurotransmitter or hormone binding receptor (Quirion *et al*., 1987).

Two subclasses of σR's have been classified, namely the sigma 1 receptor (σ1R) and the sigma 2 receptor (σ2R), based on pharmacological profile, function and molecular size (Hellewell & Bowen, 1990; Quirion *et al*., 1992). Both receptor subtypes show high to moderate affinity for most neuroleptics with especially haloperidol (see Table 3) showing high affinity for both subtypes. The (+)-benzmorphans such as (+)-pentazocine and (+)-SKF 10,047 show greater affinity for the σ_1R subclass while (-)-benzmorphans generally do not exhibit selectivity between the two subtypes. Photo affinity labelling showed a molecular weight of 25-30 kDa for the σ_1R and 18-21 kDa for σ_2R (Hellewell & Bowen 1990; Hellewell *et al*., 1994).

The σ_1R has been cloned in humans, mice, rats and guinea pigs and is a novel protein with 90% species homology. It is unrelated to any other known receptor and has a homology with the D8,7-isomerase enzyme of fungal sterol biosynthesis (Hanner *et al*., 1996). This and the fact that its suggested natural ligands are neurosteroids, specifically progesterone, pregnelone sulphate and dehydroepiandrostrerone (DHEAS) were compelling to point to a role in the neurosteroid synthesis pathway (Moebius *et al*., 1997). This has later been discredited due to no homology with mammalian sterol synthesis enzymes or any other mammalian enzyme or receptor (Maurice *et al*., 2002).

Both receptors are found in central and peripheral tissues. They are expressed highly in the liver and moderately in the intestines, kidney, white pulp of the spleen, adrenal gland, brain, placenta, the lung and reproductive organs (Seth *et al*., 1998; Seth *et al*. 2001; Zamanillo *et al*., 2000; Wolfe & De Souza, 1993; Vilner *et al*., 1995). Both σRs have been found in very high density in tumour cells obtained from various tissues. These include neuroblastomas, glioma, melanoma, and carcinoma cell lines of breast, prostate and lung tissue (Vilner *et al*., 1995). In the brain σR distribution is wide and discrete (Vilner *et al*., 1995; Seth *et al*., 2001; Zamanillo *et al*., 2000). Its highest levels are found in hippocampal and limbic areas (Walker *et al*., 1990; Debonnel & De Montigny, 1996).

1.2 Function

The σR is found on the endoplasmic reticulum (ER), yet its action is elicited through cellular membrane ion channel responses. G-protein coupling and cytosolic factors have been rejected as the mechanism by which the sigma receptor is able to provide a considerable response away from its location. Recent research speculates that σR agonists in high concentrations cause translocation of the receptor to sub-plasma membrane proteins (Su *et al*., 2009; see figure 2 part A). What is particularly compelling is the σR's modulation ability, which helps explain why σRs are usually devoid of any effect under control conditions but have considerable effects when normal homeostasis is disturbed (Van Waarde *et al*., 2011).

The σ_1R acts *via* protein-protein interactions and modulates the activity of ion channels, G-coupled receptors and signalling molecules such as inositol phosphates, protein kinases and calcium (Aydar *et al*., 2002; Hayashi & Su, 2007). As mentioned, this is thought to be mediated *via* chaperone-like characteristics with the σR translocating to different cellular compartments (Hayashi & Su, 2007). It is also associated with a number of proteins such as ankyrin B, heat shock conjugate protein 70 (hsp70), and glucose-regulated protein (GRP78/BiP) (Hayashi & Su 2001). This suggests that σ1R's function more like growth factor receptors or receptor tyrosine kinases than classic neurotransmitter receptors (Matsumoto *et al*., 2003). The σ_2R is enriched in lipid rafts and seems to be involved in calcium signalling *via* sphingolipid products and cell cycle function (Crawford *et al*., 2002).

Dual ligands with σ_1R/σ_2R activity and σ_1R specific ligands are available but currently commercially available σ_2R ligands show cross reactivity on other receptor systems. Our knowledge surrounding the σR is therefore mostly σ_1R specific with little information on the σ_2R (Bowen, 2000; Maurice *et al*, 2002; Maurice & Su 2009; Matsumoto *et al*., 2003, 2008).

ION CHANNEL ACTIVITY

Intracellularly, σRs potentiate calcium release from the ER *via* inositol triphosphate (IP_3) action and specifically type 3 IP_3 receptors that regulate calcium signalling from the ER to mitochondria (figure 2 part B). They do not seem to have this effect on type 1 IP_3 receptors so they should not affect regular ER networks (Hayashi & Su 2007). The σR ligands inhibit all calcium channels on the plasma membrane including N-, L-, P/Q- and R-types. In previous studies, σ_1R agonists potentiated NMDA induced calcium release and this action was blocked by σ_1R antagonists. Ischemic induced $[Ca^{2+}]_i$ release was blocked by σ_1R agonists and this was reversed by σ_1R antagonists (Katnik *et al*., 2006). The σ_1R agonists further blocked acid sensing ion channels that are activated by H^+ during ischemia and resulted in increased $[Ca^{2+}]$ release. The σ_2R is not implicated in this process (Herrera *et al*, 2008).

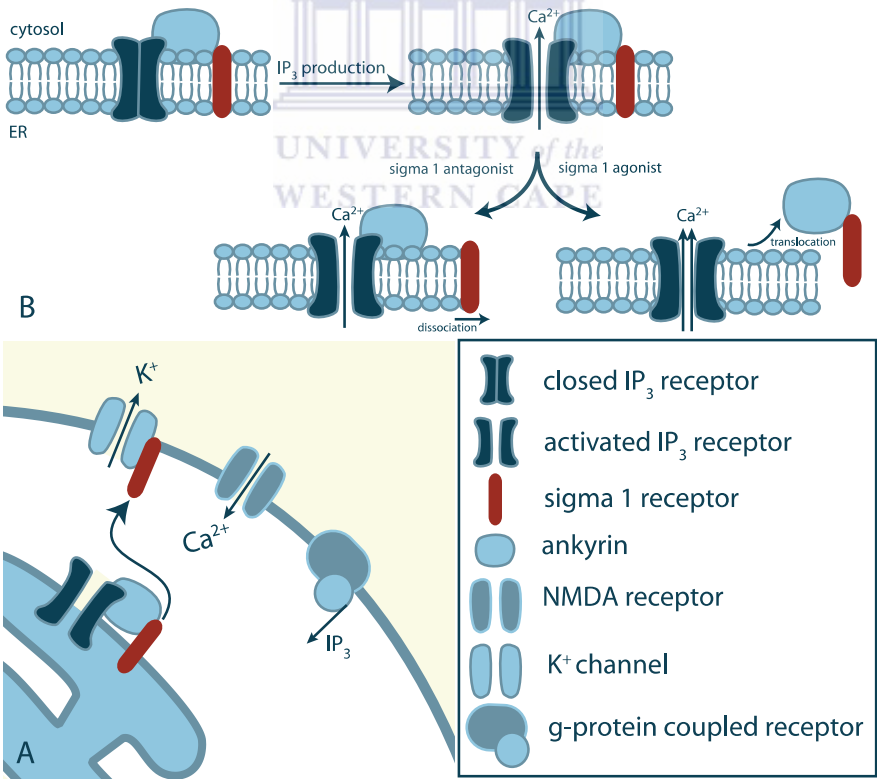


Figure 2. Sigma 1 receptor's function in cell signalling. Part A: The σ_1R is able to move from the endoplasmic reticulum (ER) to ion channels in the cellular membrane. Part B: The σ_1R is associated with ankyrin and the IP_3 receptor. The σ_1R agonists cause translocation of ankyrin and the σ_1R that causes an

increase of calcium out of the ER. The σ_1R antagonists cause dissociation of the σ_1R from ankyrin and even though it does not decrease calcium from the ER, it does impede the increase of calcium by σR agonist from the ER. Adapted and redrawn from van Waarde *et al*., 2011.

The σ_1R agonists inhibited K^+ channels without G-coupled protein participation or cytosolic second messengers (Soriani *et al*., 1999; Wilke *et al*., 1999). It was shown that σ_1R agonists inhibit persistent Na^+ currents probably *via* σ1R-G_i-protein-protein kinase signalling pathways in cortical neurons (Cheng *et al*., 2008).

Previous research has also shown that the σ_1R receptor is able to inhibit volume-regulated Cl^- channels, required for the regulation of electrical activity, cell volume, intracellular pH, immunological responses, cell proliferation and differentiation; and that σ_1R agonists further activate the channel inhibiting activity of the σ_1R (Renaudo *et al*., 2007).

In addition, the σR plays a significant role in NMDA signalling. Initially, it was thought that NMDA-induced neuronal firing in CA3 hippocampal neurons was potentiated by σ_1R agonists and was then blocked by σ_1R antagonists (Monnet *et al*., 1990). Later research pointed to σ_1R's and their associated ligands regulating NMDA receptors and long term potentiation (LTP), especially important in learning and memory by blocking the small conductance calcium activated channel (SK channel) (Chen *et al*., 2006). However more recent research has shown that the slow developing LTP was independent of the NMDA receptor but was dependent on L-type voltage-gated calcium channels and the σ_1R. More research is however necessary to understand the σR-Ca^{2+} response on the NMDA receptor (Sabeti *et al*., 2007).

NEUROTRANSMITTER MODULATION

The σR ligands show modulation of synthesis and release of monaminergic neurotransmitters especially dopamine (DA), serotonin (5HT) and to a lesser extent norepinephrine (NE) (Booth & Baldessarini, 1991; Patrick *et al*., 1993; Massamiri & Duckles, 1991), acetylcholine (Matsuno *et al*., 1995), NMDA-type glutamate receptor electrophysiology (Monnet *et al*., 1990), NMDA-stimulated neurotransmitter release (Gonzalez-Alvear & Werling, 1995), muscarinic receptor-stimulated phosphoinositide turnover and GABA (Mtchedlishvili & Kapur, 2003). Especially important in drug addiction is the σ1R effect on the DA mesocorticolimbic pathway. The nigrostriatal DA pathway is also affected which has application to neurodegeneration as shown in figure 3.

Unfortunately marked inconsistencies in research due to the different selectivity of drugs used, different administration procedures and a lack of high potency receptor specific σ_2R ligands have led to variable results.

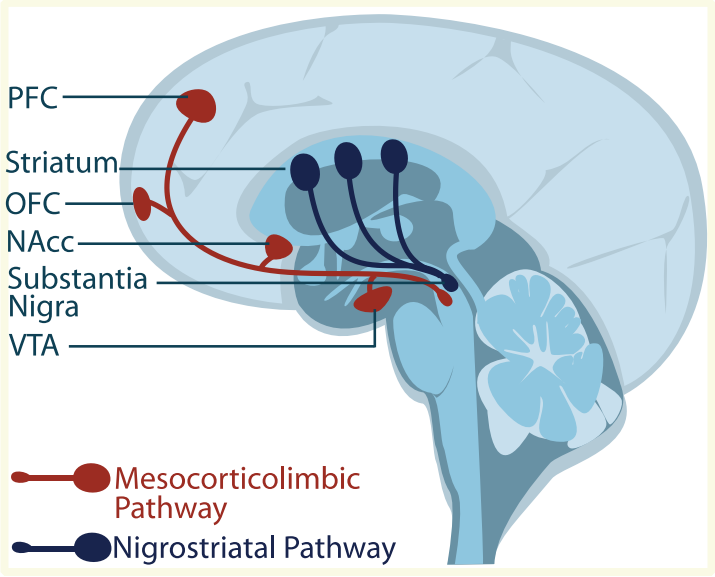


Figure 3. Dopaminergic pathways in the brain. The mesocorticolimbic pathway shown in red projects from the ventral tegmental area (VTA) to the prefrontal cortex (PFC) and passes through the amygdala (Amyg), nucleus accumbens (Nacc) and orbitofrontal cortex (OFC). The nigrostriatal pathway shown in dark blue projects from the substantia nigra to the striatum.

Research showed that σ1R's do have a significant effect on DA electric activity probably *via* opposing actions on nigrostriatal and mesolimbic DA pathways, both important pathways involved in drug addiction and neurodegeneration. Systemic administration of a σ_1R agonist reduced DA in the striatum (Kanzaki *et al*., 1992) while *in vivo* microdialysis showed that σ_1R agonists caused an increase of DA release (Patrick *et al*., 1993; Guldelsky 1995). The firing rate of DA neurons were increased by some σ_1R agonists ((+)-pentazocine and (+)-SKF-10,047) and decreased by other agonists (DTG or (+)-3-PPP), putatively acting as inverse agonists (Clark *et al* 1985, French & Cici, 1990; Steinfels & Tam, 1989). Many σR agonists had no effect on the number of spontaneous actions of DA neurons in nigrostriatal or mesolimbic pathways, but SA4503 decreased the number of nigrostriatal active neurons and increased the number of mesolimbic active neurons (Minabe *et al*., 1999). More research is necessary to understand σR activity on dopaminergic systems but compelling evidence points to the theory that these DA changes could be directly or indirectly NMDA regulated. Research showed a slight increase in NMDA-induced neuronal activation of dopaminergic neurons in the nigrostriatal and mesolimbic regions after σ_1R agonist administration. Administration of igmesine, (+)-pentazocine or 1,3-di-o-tolylguanadine (DTG) (σR agonists) produced a significant increase of NMDA-induced neuronal activation in the Nacc. These drugs also increased the suppressant effect of DA on NMDA and kainite (KA)-induced activation of accumbens neurons (Gronier & Debonnel, 1999). See Table 3, for σR ligands and their properties.

ROLE IN CELL SURVIVAL AND CELL DEATH

Both σ_1 and σ_2R's are involved in cell survival but seem to have opposing effects. To date the consensus is that σ_1R agonist promote cell survival (Yang *et al*., 2007; Tchedre & Yorio, 2008), σ_1R antagonist lead to cell death (Yang *et al*. 2007) and that σ_2R agonists promote apoptosis (Wei *et al*., 2006). The exact mechanisms behind these are still under investigation.

1.3 Pharmacological application

The σR has been implicated as a useful target in a diverse range of physiological disease such as the following:

COGNITION

Systemic administration of σ_1R agonists have anti-amnesic efficacy in several animal models of cognitive impairment (both pharmacological and pathological). These include;

a.) Cholinergic deficits (either induced by muscarinic antagonists or by lesions of the forebrain or the nucleus basalis resulting in a selective loss of cholinergic neurons),
b.) Pathology induced by direct administration of β-amyloid peptide to rodent CNS, an animal model of Alzheimer's disease,
c.) Age-induced losses of memory function, both in normal mice and senescent-accelerated mice (SAM),
d.) Neurodegeneration caused by exposure of animals to neurotoxic models such as carbon monoxide gas (results in neuronal death of CA1 area of the hippocampus), or to trimethyltin (damage to selective neural populations of the limbic system),
e.) Prenatal stress (restraint, or exposure to cocaine) and,
f.) Glutamergic, serotonergic or calcium channel deficits induced by various drugs (van Waarde *et al*., 2011).

The selective 5HT reuptake inhibitor (SSRI) fluvoxamine which possesses σ_1R activity in contrast to the SSRI paroxetine which has no σ_1R activity has been able to improve cognitive impairments in animal models of schizophrenia (Hashimoto *et al*., 2007). Fluvoxamine, but not paroxetine, was also shown to improve conditions such as lack of concentration, poor memory, slowness of mind, and poor executive function in a patient with schizophrenia (Iyo *et al*., 2008). Fluvoxamine binds to the σ_1R 50 times better than paroxetine does (Narita *et al*., 1996) and high occupancy of the σ_1R is observed after a single dose of 200 mg fluvoxamine (Ishikawa *et al*., 2007). This suggests that σ_1R agonists may be candidates for treating cognitive impairment in schizophrenia (van Waarde *et al*., 2011).

Donepezil (acetylcholine (Ach) esterase inhibitor) also acts as a σ_1R agonist. Normal therapeutic doses of donepezil result in considerable σ_1R occupancy in the human brain (Ishikawa *et al*., 2007). The σ_1R agonists could be valuable in the treatment of Alzheimer's disease as it has been proven that they are capable of alleviating cognitive deficits in animal models of cognitive impairments and provide neuroprotection, in *in vitro* cortical neurons (Marrazzo *et al*., 2005) and *in vivo* rodent studies (Meunier *et al*., 2006; Villard *et al*., 2009) and against amyloid activity (Maurice, 2002). The σ_1R agonists further powerfully suppress microglial activation (Hall *et al*., 2009) and may therefore attenuate the inflammatory component in neurodegenerative diseases. Another important application to consider for σRs is conditions such as retinal neural damage which follow similar pathways to cognitive impairment and neurodegeneration (Ola *et al*., 2001).

PAIN

Antagonists of the σR are able to attenuate formalin induced pain, indicating that they are able to decrease neuropathic pain (Cendán *et al*., 2005). The σR was found to participate in pain mediated by the mu, kappa and delta receptors. It was also shown that σR agonists' attenuate opioid analgesia and that haloperidol a σR antagonist aids in the analgesic effects of opioids. Down regulation of σRs also potentiate opioid analgesia. This shows the existence of a sigma anti-opioid system in the brain (Mei & Pasternak, 2002). The exact mechanism behind this modulation of opioid induced pain and neuropathic pain still remains unclear.

DEPRESSION

The σR also plays a role in depression and research showed that:

a.) Antagonist potentiate NMDA or cholinergic activity in a variety of amnesia models;
b.) Some antidepressants possess σR activity (Table 3). This suggests that the σR might have a role in the action of these drugs (Narita *et al*., 1996);
c.) Antidepressants such as the SSRI sertraline and the monoamine oxidase inhibitor (MAOI) clorgyline selectively potentiated the effect of NMDA in a haloperidol-sensitive manner on pyramidal neurons in the CA3 region of the rat dorsal hippocampus, an area largely implicated in depression (Bergeron *et al*., 1993).

The σR agonists have been demonstrated to be effective in depression animal models such as the forced swimming, tail suspension and conditioning fear stress test. These effects were also blocked by σ_1R antagonists (Urani *et al*, 2001; Skuza & Rogos, 2002, 2003).

HIV AND IMMUNITY

Cocaine causes enhanced human immunodeficiency virus (HIV) replication and also binds to the σ_1R. This is of great importance in understanding the mechanism of HIV as clinical studies have shown that HIV positive drug abusers experience a more rapidly progressive illness, higher viral loads, increased immune suppression and increased cognitive impairment. Opiates, cocaine and methamphetamine increase HIV replication and enhance/synergise with HIV proteins to cause glial cell activation, neurotoxicity and blood brain barrier breakdown. These findings have been confirmed *in vivo* using rat models, however clinical trials using HIV positive drug users have not been examined as such trials pose extreme limitations (Nath, 2010).

The σ_1R's role in HIV was examined in several studies. Research showed that the increase in HIV replication caused by cocaine was indeed blocked by σ_1R antagonists (Gekker *et al*., 2006). It is not clear what the σR's exact involvement in HIV replication is but these results point to a potential new role for σR ligands.

CANCER

The σR provides an interesting possibility for cancer research. They are richly expressed in some cancer cells and they could be useful in combination with radio imaging and chemotherapeutic drugs to selectively target cancer cells. Their role in cell growth and cell death could also be utilised.

The σ_2R is currently the main focus in cancer research and has been found to mediate a novel caspase-independent apoptotic pathway involving ceramide, a proapoptotic molecule, in several breast tumour cell lines (Crawford & Bowen, 2002). Haloperidol in high doses was also found to cause cell death in cancer cells *via* the σ_2R pathway (Wei *et al*., 2006). The exact mechanistic difference between σ_1R and σ_2R in cancer cells and novel selective σ_2R ligands still need further research to yield conclusive results.

PSYCHOSTIMULANT ADDICTION

Recent research has demonstrated that the σR plays an important role in the plasticity underlying reinforcing and addictive processes (Maurice *et al*., 2002). Several of the drug addiction studies focus on cocaine and methamphetamine due to their specific binding to σRs. Studies show σR antagonism to inhibit addiction behaviour by downstream signalling of dopaminergic neurotransmission in the conditioned place preference model, a model used to predict all types of addiction (Romieu *et al*., 2002). This theoretically makes it applicable to any addictive drug even if the drug itself does not have affinity for the σR.

Studies show that σR antagonists are able to attenuate cocaine induced convulsions, lethality, locomotor activity and conditioned place preference (Matsumoto *et al*., 2001, Romieu 2000, 2002). With regards to methamphetamine, studies show that σRs are involved in the stimulant action of methamphetamine. The σR antagonists block the development and expression of methamphetamine-induced sensitisation (Takahashi *et al*., 2000; Ujike *et al*., 1992) and attenuate acute locomotor stimulatory activity, possibly *via* σ_1R and σ_2R action (Nguyen *et al*., 2005). Administration of methamphetamine produces an increase of σ_1R expression in the midbrain of rats (Stefanski *et al*., 2004). What is particularly promising for the σR-methamphetamine relationship is the possible block of neurotoxic effects caused by methamphetamine. σ_2Rs are implicated in cytotoxic effects and cell death processes and the neurotoxic effects of methamphetamine have been attenuated by σR antagonists (Matsumoto *et al*., 2008). Without specific σ_2R antagonists the exact mechanism surrounding this attenuation however remains unclear.

The effects of σR's on psychostimulant actions are thought to be *via* the following processes (Matsumoto *et al*., 2003, 2008). See figure 4 for schematic representation:

a. Direct interference at the receptors, which are localized in key organ systems involved in cocaine and methamphetamine action.

The σRs are located in brain areas associated with drug addiction such as the nucleus accumbens (Nacc) and also show importance in the mesocorticolimbic pathway. The σ_1R but not the σ_2R is expressed in the heart and this is of special importance to cocaine overdose, which causes extreme effects on the cardiac system.

b. Modulation of downstream neurotransmitter systems that are involved in the action of cocaine and methamphetamine

The σR has a modulatory effect on DA, 5HT, NE, NMDA and GABA, all of which have been shown to be involved in drug addiction. These could account for promising studies regarding attenuation of psychostimulant behaviours, but the exact mechanism of these processes still remain unclear.

c. Alteration in gene expressions that are associated with long term consequences of cocaine.

Studies show that σR antagonists prevent cocaine induced changes in gene expression. These and the σR's involvement in growth factors show that σR's could be responsible for the long term effects of psychostimulants.

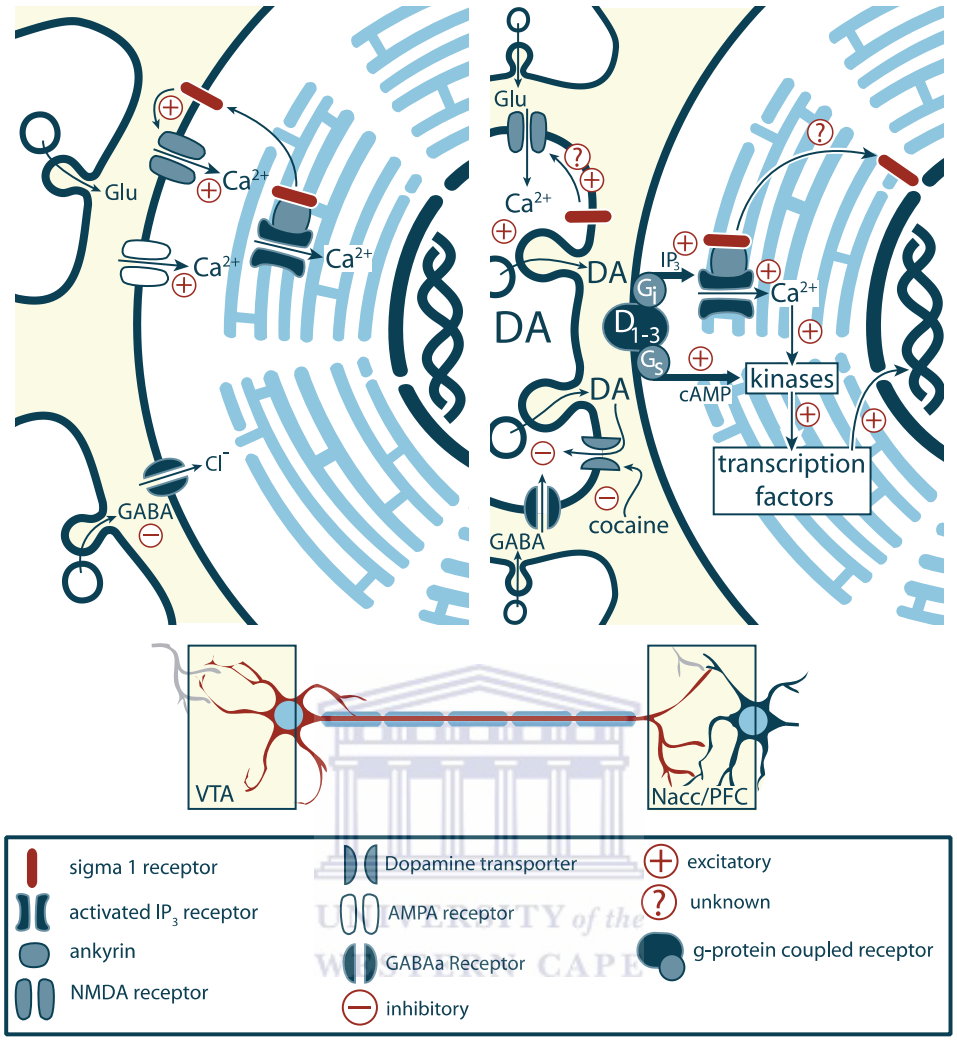


Figure 4. Schematic representation of the mechanism of action of cocaine on the dopaminergic neuron of the mesolimbic pathway and the possible involvement of the σ_1R. Glutamergic and GABAergic pathways modulate the mesocorticolimbic pathway at the ventral tegmental area (VTA) and also influence dopamine (DA) release in the nucleus accumbens (Nacc), prefrontal cortex (PFC) and amygdala (Amyg). NMDA and AMPA receptors are responsible for the mediation of cocaine-induced long term potentiation (LTP) and long-term depression (LTD). Cocaine blocks the dopamine transporter (DAT) and therefore increases DA which effect post synaptic DA receptor ($D_{1\text{-}3}$ mainly). These act *via* g-coupled proteins to increase cyclic adenosine monophosphate (cAMP) or IP_3. IP_3 causes mobilisation of intracellular ER calcium pools. cAMP and this increase of cytosolic calcium cause the activation of transcription factors responsible for short and long term effects of cocaine. The sigma 1 receptor (σ_1R) is present in the VTA, Nacc, PFC and Amyg. Cocaine can increase activation of the σ_1R either *via* direct or indirect actions increasing calcium influx, resulting in increased DA transmission. After activation the σ_1R translocates and has modulatory effect on NMDA causing NMDA stimulated DA release. This process could also occur within the presynaptic terminals in the Nacc. Within the post-synaptic neuron σ_1R could also have a modulatory role on

calcium release, NMDA activity and might have a role to play in gene expression. (Adapted and redrawn from Maurice *et al*., 2002).

NEURODEGENERATION

Neurodegeneration is the term used for any loss of progressive structure or function of neurons, including death of neurons. Diseases such as Parkinson's, Alzheimer's and Huntington's occur as a result of neurodegeneration. It's been discovered that these diseases have similarities on a sub-cellular level, which is promising for drug design research as the correct drug could successfully treat many neurodegenerative diseases. An important target that had emerged involves programmed cell death which includes the apoptosis process (Stoka *et al*., 2006).

Apoptosis is a normal function of homeostasis and is important in the management of cell growth and cell removal and the health of individual neurons is dependent on apoptotic pathways to promote the survival and growth of nerve cells (Barde, 1989; Mattson & Lindvall, 1997). However in certain disease states hyper apoptosis occurs and an abnormal amount of neurons are triggered to die. It is also triggered within specific pathways leading to detrimental effects such as loss of fine motor function controlled by the nigrostriatal pathway in Parkinson's disease.

As speculated by Bowen (2000) and confirmed by Wei (2006), σ_2R's are part of an apoptotic pathway which could play a role in regulation of cell proliferation or cell development. The σ_2R antagonists may be useful agents to lessen tardive dyskinesia which can result from chronic treatment of psychoses with typical antipsychotic drugs such as haloperidol and σ_2R agonists may be useful as anti-neoplastic agents because they induced apoptosis in breast tumour cell lines which were resistant to the common DNA-damaging anti-neoplastics (Bowen *et al*., 2000; Wei *et al*., 2006).

Research has shown that activation of the transglutaminase (TG-2) apoptotic pathway is related to σ_2R agonists (Prezzavento *et al*. 2007). An increase in calcium ion influx activates several calcium-dependant proteins of which TG-2 is one. This isoform of a family of transglutaminases catalyses the formation of ε-(γ-glutamyl)lysine cross-links between polypeptide chains which results in polymerisation, the cross-linking of dissimilar proteins and the incorporation of diamines and polyamines into proteins (Lesort *et al.*, 2000). It is also part of cell processes such as cell differentiation, signal transduction, cell survival and wound healing. Furthermore, TG-2 has a modulatory effect on apoptosis and cell response stressors, depending on the type of stimuli provoking an increase in transamidation activity (Tucholski & Johnson, 2002). The TG-2 protein is also expressed in the brain and is part of a variety of processes of the central and peripheral nervous systems (Lesort *et al.*, 2000). There are several lines of evidence suggesting that TG-2 activity may contribute to neurodegenerative diseases such Huntington's, Alzheimer's and

Parkinson's disease (Gentile & Cooper, 2004). However, there is also evidence that under certain circumstances TG-2 can show protective properties against apoptosis. Results further suggest that selective sigma ligands modulate intracellular calcium levels and eventually the up-regulation of TG-2 that is typical of several neurodegenerative diseases (Prezzavento *et al*., 2007).

1.4 Ligand structure activity relationship

Glennon (1994) initially presented an illustration for optimal σR binding in 1994 (figure 5). With the cloning of the σ_1R and an ever increasing number of σR ligands being the focus of many research groups, Glennon (2005) revisited past and present research and updated their initial illustration for σ_1R specific affinity (figure 6). Some of the structures utilised for these studies are included in Table 1b.

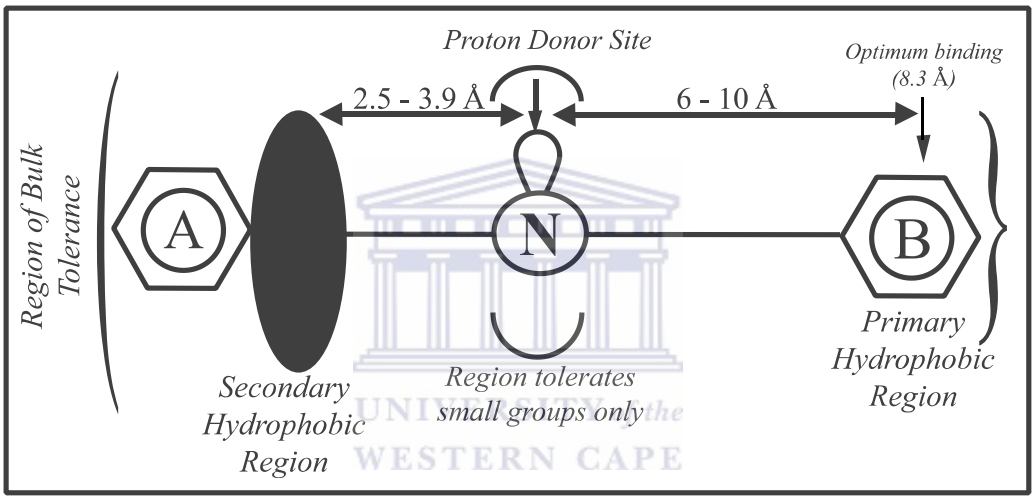


Figure 5. Optimum dual σ_1R/ σ_2R affinity (Glennon *et al.,* 1994)

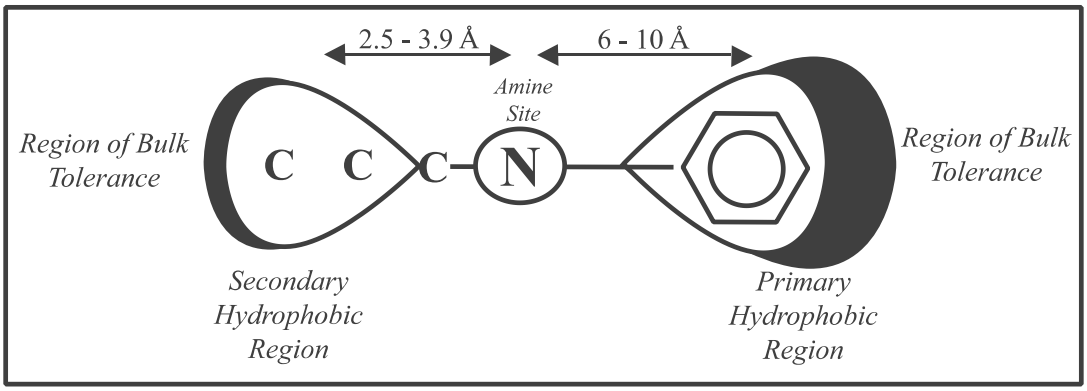
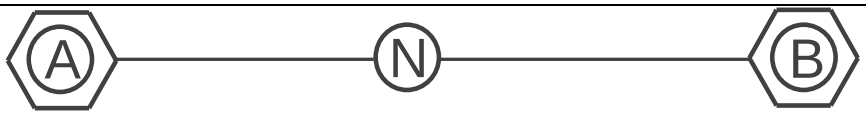
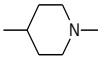
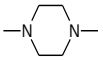
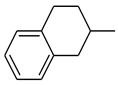
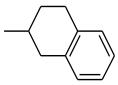
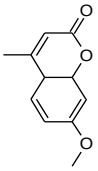
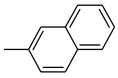
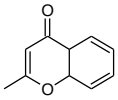
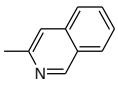
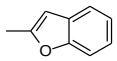


Figure 6. Optimum binding for σ_1R specific affinity (Glennon, 2005)

Table 1b serves to illustrate chemical compounds previously investigated and provide a clear idea of the Glennon *et al.*, (1994, 2005) pharmacophore profile consisting of a basic amine, two hydrophobic binding regions and the effect of chain length and halogen substitution on the primary binding region.

Table 1b: Summary of compounds studied for sigma recetor binding

##	2° binding site	2° linking chain	Basic Amine	1° linking chain	1° binding site	Binding Affinity		
						K*i* (nM)		
						σR	σ_1R	σ_2R
Basic Amine; (compounds 1- 12) (Ablordeppey *et al*., 2000; 2002)								
1*		-CH_2-		-$CH_2(CH_2)_3CH_2$-			0.4	-
2		-CH_2-		-$CH_2(CH_2)_3CH_2$-			0.6	2.8
3				-$CH_2(CH_2)_3CH_2$-			0.48	50
4				-$CH_2(CH_2)_3CH_2$-			1.0	-
5				-$CH_2(CH_2)_3CH_2$-			0.76	70
6				-$CH_2(CH_2)_3CH_2$-			6.0	89
7				-$CH_2(CH_2)_3CH_2$-			0.25	-
8				-$CH_2(CH_2)_3CH_2$-			418	7920
9				-$CH_2(CH_2)_3CH_2$-			14.0	965
10				-$CH_2(CH_2)_3CH_2$-			0.25	5
11		-CH_2-		-$CH_2(CH_2)_3CH_2$-			0.17	34
12		-CH_2-		-$CH_2(CH_2)_3CH_2$-			0.19	13
Primary and Secondary binding sites; (13, 14) (Ablordeppey *et al*., 2002); (15 -22) (Younes *et al*., 2000)								
13		-CH_2-		-$CH_2(CH_2)_3CH_2$-			0.19	13

14				$-CH_2(CH_2)_3CH_2-$			0.60	170
15		$-CH_2-$		$-CH_2-$		3		
16		$-CH_2-$		$-CH_2-$		1		
17		$-CH_2-$		$-CH_2-$		3		
18		$-CH_2-$		$-CH_2-$		3		
19		$-CH_2-$		$-CH_2-$		1		
20		$-CH_2-$		$-CH_2-$		0.4		
21		$-CH_2-$		$-CH_2(CH_2)_3CH_2-$		0.58		
22		$-CH_2-$		$-CH_2(CH_2)_3CH_2-$		0.8		

Chain Length, Secondary Chain Length; (23-26) (Ablordeppey *et al*., 2002)

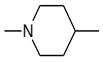
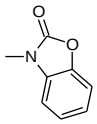
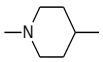
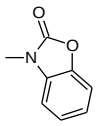
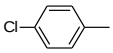
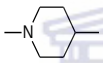
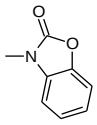
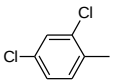
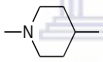
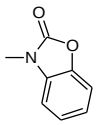
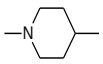
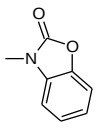
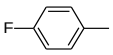
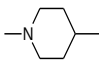
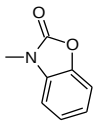
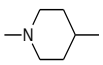
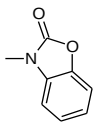
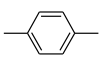
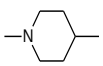
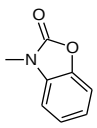
23		$-CH_2-$		$-CH_2(CH_2)_3CH_2-$			0.17	34
24		$-CH_2CH_2-$		$-CH_2(CH_2)_3CH_2-$			0.17	15
25		$-CH_2CH_2CH_2-$		$-CH_2(CH_2)_3CH_2-$			0.28	9.8
26		$-CH_2(CH_2)_2CH_2-$		$-CH_2(CH_2)_3CH_2-$			0.48	68

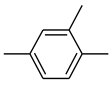
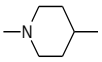
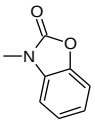
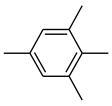
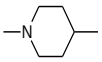
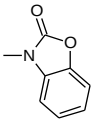
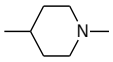
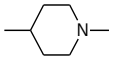
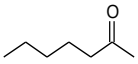
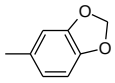
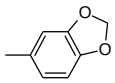
Chain Length, Primary Chain Length; (27-30) (Ablordeppey *et al*., 1998)

27		$-CH_2CH_2-$		$-CH_2CH_2CH_2-$		11.0		

28		$-CH_2CH_2-$		$-CH_2(CH_2)_2CH_2-$		2.6		
29		$-CH_2CH_2-$		$-CH_2(CH_2)_3CH_2-$		0.17	0.17	15
30		$-CH_2CH_2-$		$-CH_2(CH_2)_5CH_2-$		1.5		

Substitution of binding sites; (31-40) (Zampieri *et al*., 2009); (41-44) (Glennon, 2005)

31		$-CH_2-$		$-CH_2-$		3.6	246
32		$-CH_2-$		$-CH_2-$		61	299
33		$-CH_2-$		$-CH_2-$		0.1	427
34		$-CH_2-$		$-CH_2-$		258	382
35		$-CH_2-$		$-CH_2-$		23	213
36		$-CH_2-$		$-CH_2-$		3.9	170
37		$-CH_2-$		$-CH_2-$		33	338
38		$-CH_2-$		$-CH_2-$		2.9	116

39		-CH_2-		-CH_2-		30	187
40		-CH_2-		-CH_2-		6230	2881
41						0.15	
42						0.12	
43						0.86	554
44						0.32	63

BASIC AMINE

From figure 5 and 6 it can be seen that a basic amine is needed for optimum activity on non-specific σR and σ_1R specific ligands. This amine can be secondary or tertiary and can also be present in a cyclic structure such as pyridine, piperazine, pyrrolidine etc., as seen in compounds **1-12** (Ablordeppey *et al*., 2000, 2002; Glennon, 2005).

PRIMARY AND SECONDARY BINDING REGION

There are two binding regions on either side of the basic amine with varying optimum distances between them and the amine site. The primary binding site is further away from the amine than the secondary binding site and research shows that a vast array of lipophilic structure binding is possible in either region (**13**-**22**). A phenyl group at position B is not absolutely necessary for affinity but can improve affinity (**1** and **3**) (Ablordeppey *et al*., 2002; Younes *et al*., 2000).

CHAIN LENGTH

The distance between the amine and the binding regions are 6-10 Å and 2.5-3.9 Å to the primary and secondary binding region, respectively (for non-specific σR ligands and σ_1R ligands). The exact chain length is dependent on each unique structure due to the conformational changes that the structure can undergo. Generally an aliphatic chain of 2 carbons between the amine and secondary binding site (**23**-**26**)

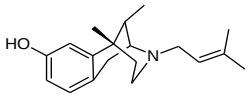
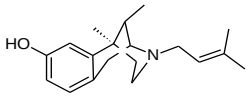
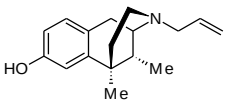
and an aliphatic chain in the range of 3-5 carbons between the amine and primary binding site seem to show optimum binding, (**27**-**30**) (Ablordeppey *et al*., 1998, 2000; Glennon, 2005).

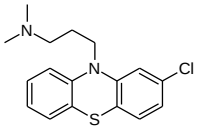
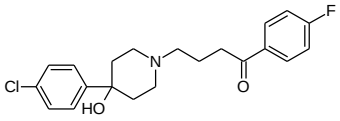
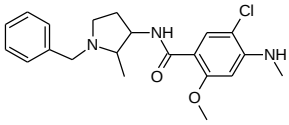
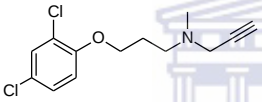
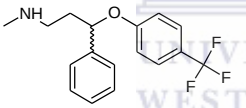
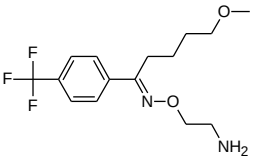
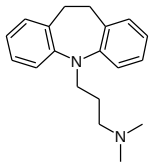
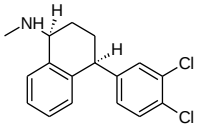
SUBSTITUTION OF BINDING SITES

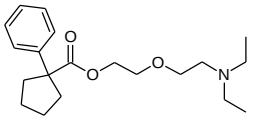
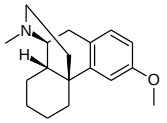
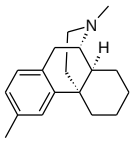
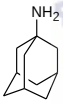
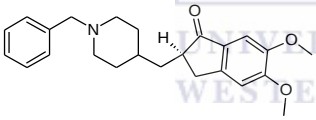
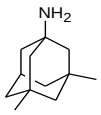
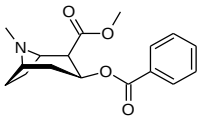
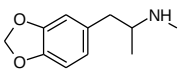
Halogenation of the B phenyl group generally increases activity (probably due to increased lipophilicity) but data on optimum halogen and position thereof differ between structures (**31**-**40**). This again could be due to conformational changes that the structure can undergo, however if a clear structure is decided on, halogen substitution can remarkably increase affinity and a definitive trend can be seen based on individual halogen substitutes and position on the phenyl group. An unsaturated alkyl chain seems to be tolerated for σ_1R activity but decreases for σ_2R activity is observed (**43** and **44**) (Ablordeppey *et al*., 2000). Carbonyl substitution here seems to make no contribution to σ_1R binding (**41** and **42**) (Glennon, 2005).

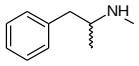
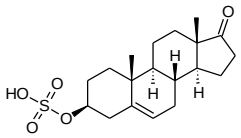
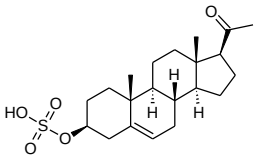
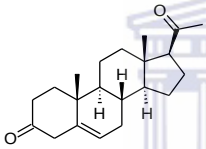
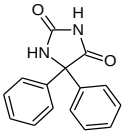
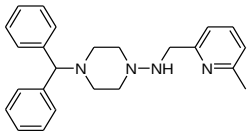
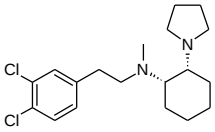
Table 3 lists substances that elicit σR activity. Some of these are commercially available for specific disorders that may or may not depend on the structure's inherent σR activity. Note where appropriate the structural adherence of the compounds with regard to a basic amine and the various lipophilic groups that could potentially add to σR affinity.

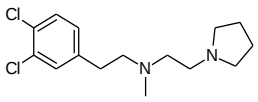
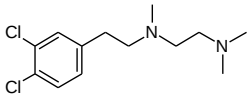
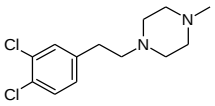
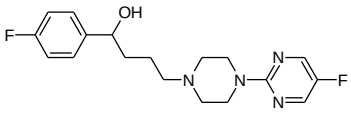
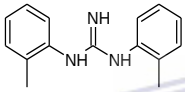
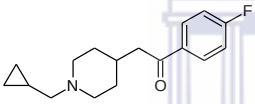
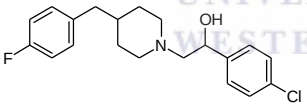
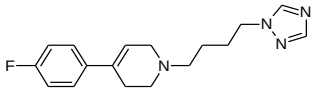
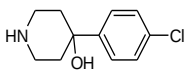
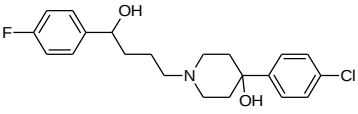
Table 3: Pharmacology of sigma receptor ligands (Reviewed by Cobos *et al*., 2008)

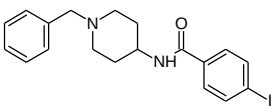
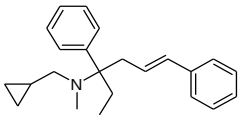
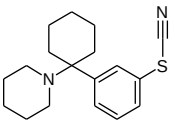
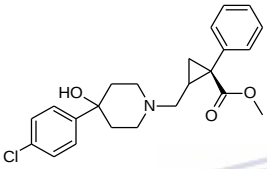
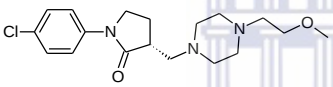
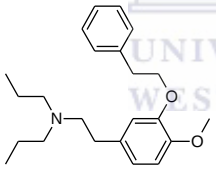
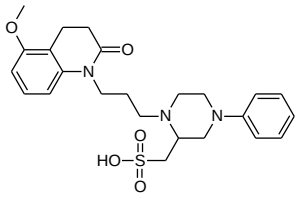
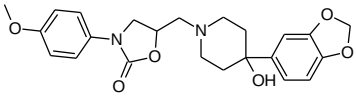
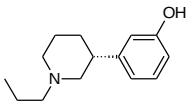
Compound	Structure	σ_1/σ_2	Affinity	σR Function	Other activities
Benzmorphans					
(+)-pentazocine		σ_1	+++	Agonist	-
(-)-pentazocine		σ_1/σ_2	++	Agonist	κ_1 agonist, μ_1, μ_2, ligand, low affinity δ, and κ_3 opioid ligand
(+)-SKF-10,047		σ_1	+++	Agonist	NMDA receptor ligand

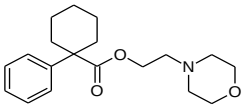
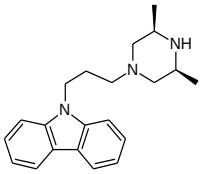
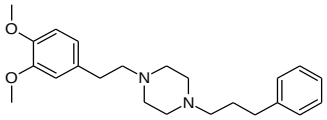
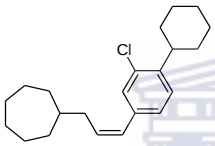
Antipsychotics					
Chlorpromazine		σ_1/σ_2	++	?	Dopamine D_2 antagonist
Haloperidol		σ_1/σ_2	+++	Agonist	Dopamine D_2 and D_3 antagonist, σ_2 agonist
Nemonapride		σ_1/σ_2	+++	?	Dopamine D_2 antagonist
Antidepressants					
Clorgyline		σ_1	+++	Agonist?	Irreversible monoamine oxidase (MAO) A inhibitor
Fluoxetine		σ_1	+	Agonist	Selective 5HT reuptake inhibitor (SSRI)
Fluvoxamine		σ_1	+++	Agonist	SSRI
Imipramine		σ_1	++	Agonist	Monoamine reuptake inhibitor
Sertraline		σ_1	++	Agonist	SSRI

Antitussives					
Carbetapentane		σ_1/σ_2	+++	Agonist	Muscarinic antagonist
Dextromethorphan		σ_1	++	Agonist	NMDA receptor allosteric antagonist
Dimemorphan		σ_1/σ_2	++	Agonist	?
Parkinson's and/or Alzheimer's disease					
Amantadine		?	+	Agonist?	NMDA antagonist, antiviral properties
Donepezil		σ_1/σ_2?	+++	Agonist	Cholinesterase inhibitor
Memantine		?	+	Agonist?	NMDA antagonist, antiviral properties
Drugs of abuse					
Cocaine		σ_1/σ_2	+	Agonist	Monoamine transporters inhibitor, among other actions
MDMA		σ_1/σ_2	+	?	Preferential SERT inhibitor, among other actions

Methamphetamine		σ_1/σ_2	+	?	Preferential DA transporter inhibitor, among other actions
Putative endogenous ligands (neurosteroids)					
DHEAS		σ_1	+	Agonist	$GABA_A$ negative modulator
Pregnelone sulphate		σ_1	+	Agonist	NMDA positive/$GABA_A$ negative modulator
Progesterone		σ_1	+	Antagonist	NMDA negative/$GABA_A$ positive modulator
Anticonvulsants					
Phenytoin		σ_1	not applicable	Allosteric Modulator	Delayed rectifier K^+ channel blocker, T-type calcium current inhibitor, Na^+ current inhibitor
Ropizine		σ_1	not applicable	Allosteric Modulator	?
Other σR drugs					
BD 737		σ_1/σ_2	+++	Agonist	-

BD 1008		σ_1/σ_2	+++	Antagonist	σ_2 agonist?
BD 1047		σ_1	+++	Antagonist	β adrenoceptor ligand
BD 1063		σ_1	+++	Antagonist	-
BMY 14802		σ_1/σ_2	++	Antagonist	$5HT_{1A}$ agonist
DTG		σ_1/σ_2	+++	?	σ_2 agonist
Dup 734		σ_1	+++	Antagonist	$5HT_2$ antagonist
Eliprodil		σ_1/σ_2	++	?	NMDA antagonist, σ_1 adrenoceptor ligand
E-5842		σ_1	+++	Antagonist	Low to moderate affinity for DA, 5HT and glutamate receptors
Haloperidol Metabolite I		σ_1	++	Antagonist	-
Haloperidol Metabolite II		σ_1/σ_2	+++	Irreversible Antagonist	Dopamine D_2 and D_3 ligand

4-IBP		σ_1/σ_2	+++	Agonist	Dopamine D_2 ligand
JO-1784 (Igmesine)		σ_1	+++	Agonist	-
Metaphit		σ_1/σ_2	++	Irreversible antagonist	Acylator of PCP and σ_2 binding sites
(+)-MR 200		σ_1/σ_2	+++	Antagonist	-
MS-377		σ_1	+++	Antagonist	-
NE-100		σ_1	+++	Antagonist	-
OPC-14523		σ_1/σ_2	+++	Agonist	Agonist of pre- and post-synaptic $5HT_{1A}$ receptors, serotonin transporter (SERT) inhibitor
Panamesine		σ_1/σ_2	+++?	Antagonist	Metabolite is a dopaminergic antagonist
(+)-3-PPP		σ_1/σ_2	++	Agonist	σ_2 agonist, NMDA receptor ligand, dopaminergic

					agonist
PRE 084		σ_1	+++	Antagonist	-
Rimcazole		σ_1/σ_2	+	Agonist	DA transporter inhibitor
SA4503		σ_1	+++	Agonist	-
SR 31742A		?	+++	?	High affinity for C8-C7 sterol isomerase

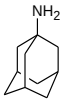
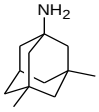
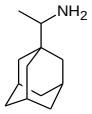
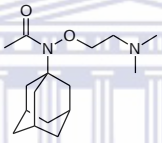
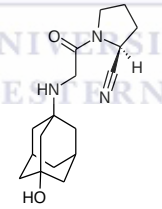
2. Polycyclic amines

The Cookson diketone has shown to be a valuable compound for the design of novel therapeutic agents. It has the ability to act as a scaffold that can improve pharmacokinetic properties and possesses an unique array of diverse receptor site interaction, ranging from neurological application in the management of Alzheimer's disease and Parkinson's disease to peripheral conditions including antiviral and potential anti-inflammatory application (Kassiou *et al*., 1996; Nguyen *et al*., 1996, 2005; Banister *et al*., 2010; Liu *et al*., 2001, 2005, 2007; Schwab *et al*., 1972; Oliver *et al*., 1991; Van der Schyf *et al*., 1986).

2.1 Background

Initial polycyclic amine cage research was prompted by the adamantine derivatives; see Table 4 for analogues in use. This led to further investigation into structures such as pentacycloundecane analogues derived from Cookson's diketone (Table 5). Adamantine compounds have shown promise in many fields including antiviral application, their initial clinical use; and treatment of Parkinson's disease. They're coveted for their modulation of diverse receptor classes and their advantageous ability to improve the pharmacokinetic properties of their analogues. They therefore have potential as novel ligands and in numerous prodrug formulations. Pentacycloundecane analogues have shown the same ability to transfer these desirable properties (Brookes *et al.*, 1992; Zah *et al*., 2003).

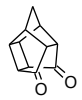
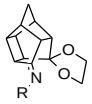
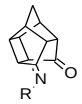
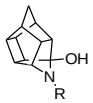
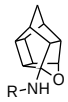
Table 4*:* Clinically available adamantane derivatives

	Compound	Structure	Indication
45	Amantadine		Viral infections including Influenza virus A, Parkinson's disease
46	Memantine		Alzheimer's disease
47	Rimantadine		Viral infection including Influenza A, Parkinson's disease
48	Tromantadine		Viral infections including herpes simplex virus
49	Vildagliptin		Anti-hyperglycaemic in maintaining diabetes

Amantadine, memantine and some pentacycloundecane analogues show affinity for the σR. They also directly modulate key receptors involved in other neurological conditions such as neurodegeneration, cognitive impairments and drug addiction. Their action is thought to stem from their NMDA antagonism, but their σR interaction should not be ruled out. Pentacycloundecane derivatives show significant σR affinity and serve as compelling prospect for elucidating the σ_2R (Kornhuber *et al.*, 1993).

Table 5: Pentacycloundecane derivatives

Compound	Structure

50	Cookson's diketone	
51	Ketal derivative	
52	Imine derivative	
53	Azapentacycloundecane	
54	Oxapentacycloundecane	
55	Trishomocubane	

2.2. Bioactivity of pentacycloundecane derivatives

Neurological disorders pose the challenge of correcting small imbalances within the brain while simultaneously not disrupting other neurological processes. Multifunctional drugs could be advantageous by working on the principle of an additive effect and administration at low doses could coordinate action on multiple implicated sites while not influencing neurochemistry to the detriment of the patient. In addition to their affinity for the σR, pentacycloundecanes have activity on multiple sites involved in neurological disorders.

DA ACTIVITY

Geldenhuys *et al.* (2009) set out to evaluate the pentacycloundecane analogues effect on DA after it was postulated that compounds such as NGP 1-01 possess neuroprotective capabilities. It was demonstrated that the phenyl ethylamine derivative inhibits DA uptake in striatal tissue (figure 7), an advantageous property with regards to treating Parkinson's disease that stems from a deficit of DA neurons in the nigrostriatal pathway. This mechanism is opposite to brain addiction pathology. Geldenhuys and co-workers then further investigated this compound's action on the DAT transport and it was found that it was able to:

a.) Significantly increase spontaneous DA;
b.) Significantly decrease methamphetamine-stimulated DA;
c.) Significantly increase DA when co-infused with 30 mM KCl;
d.) Lost the stimulatory effect of KCl-evoked DA release when calcium free buffer was used and exhibited moderate voltage-gated calcium channel blocking activity with an IC50 of 22 μM

These results show that the extracellular efflux of DA by the phenyl ethylamine derivative is not *via* the DAT and is primarily through interactions preventing DA uptake *via* calcium-dependent mechanisms. Its effect on reducing methamphetamine-evoked DA suggests that it either binds to the methamphetamine binding site thereby blocking methamphetamines action, or it allosterically interacts with the DAT inducing significant conformational changes to the DAT and decreasing methamphetamine DAT affinity. These findings suggest that this compound can act as possible lead for therapeutics designed to treat drug addiction and can also be utilized to prevent neurotoxicity associated with drug toxins that elicit their action through the DAT (Geldenhuys *et al*., 2009).

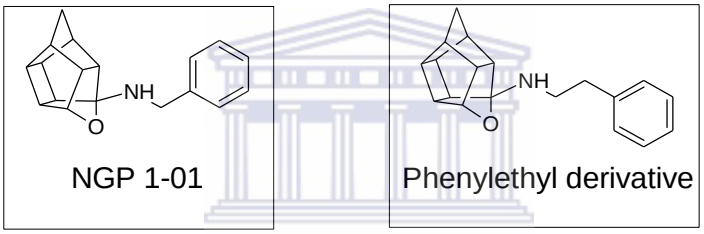


Figure 7. Compounds used in the neuroprotective study from Geldenhuys *et al.,* (2009).

NMDA RECEPTOR/ION CHANNEL

Considering memantine's NMDA antagonism, Geldenhuys *et al*. (2005) investigated pentacycloundecane's function on the NMDA calcium channel. The study included a small series of pentacycloundecane derivatives and it was found that pentacycloundecane derivatives do block calcium influx into murine synaptoneurosomes by non-competitive NMDA receptor channel antagonism. NGP1-01 showed the best potency with an IC_{50} of 2.98 μM comparable to the reference compound memantine that had an IC_{50} of 3.05 μM in the assay employed (Geldenhuys *et al.,* 2003). Memantine and adamantine interact with the PCP/TCP/MK-801/ketamine binding site inside the NMDA channel pore (Bresink *et al*., 1995). It was however demonstrated that structurally similar pentacycloundecanes (including NGP1-01) do not interact with this binding site and therefore bind to a unique and novel site on the NMDA receptor channel (Geldenhuys *et al.,* 2003). The NMDA receptor's significant involvement in mesocorticolimbic dopaminergic pathways and pentacycloundecane derivatives possessing memantine's ability of NMDA antagonism could prove valuable in treating neurodegeneration and drug addiction.

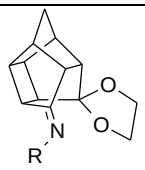
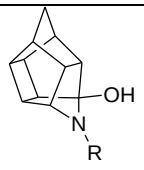
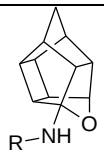
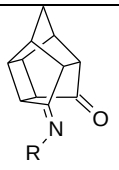
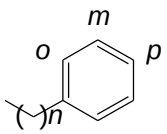
CALCIUM CHANNEL ANTAGONISM

NGP1-01 was first characterised and patented as a calcium channel antagonist in the late 1980's (Van der Schyf *et al.*, 1986). It was extensively researched as a potential cardiovascular drug that could be utilised in supraventricular tachycardias and cardiac arrhythmias as it was later discovered that they also inhibit K^+ channels. Further research showed that NGP1-01 was a lipophilic *L*-type channel blocker (van der Schyf *et al.*, 1986). This is particularly important in the process of apoptosis (programmed cell death) and their action on calcium channels, DA and NMDA receptors make them ideal candidates for neuroprotective application. In drug addiction, calcium channel antagonism could prove useful considering NMDA and σR involvement in brain addiction processes and their immediate link to the calcium system.

SIGMA RECEPTOR BINDING

Amantadine interacts with the σR binding site with a K*i* of 20.25 μM (Kornhuber *et al.*, 1993) and pentacycloundecane analogues were evaluated for σR potential based on their similar structure to adamantine analogues. Table 6 shows results from various studies that examined pentacycloundecane activity on the σR. Compounds **55-80**, and **85-88** were also examined for dopaminergic, muscarinic, serotonergic, and PCP binding. These compounds showed high affinity for the σR but no cross reactivity to other sites tested (Kassiou *et al.*, 1996; Marrazzo *et al.*, 2001). Compounds **81-84** were also tested for DAT and adrenergic activity (Banister *et al.*, 2010). Compounds **81**, **83** and **84** showed no cross reactivity to these sites but compound **39** showed significant affinity for the adrenergic receptor and moderate activity on the DAT (Banister *et al.*, 2010).

Table 6*:* Pentacycloundecanes possessing sigma reactivity.

Compound					
 **Series 1**	 **Series 2**	 **Series 3**	 **Series 4**		
R = 					
				Affinity K_i (nM)	
n	***o***	***m***	***p***	σ1R	σ2R

Series 1 (Ketal derivatives) (55-59)						
55	1	H	H	H	67	864
56	1	H	Br	H	17	208
57	1	H	I	H	124	285
58	1	I	H	H	72	246
59	2	H	H	H	15	608
Series 2 (Aza derivatives) (60-82)						
60	1	H	H	H	103	51
61	1	Br	H	H	86	176
62	1	H	Br	H	208	40
63	1	I	H	H	81	246
64	1	H	I	H	169	54
65	1	F	H	H	107	250
66	1	H	F	H	152	20
67	1	H	H	F	182	230
68	1	F	F	H	198	239
69	1	OCH_3	H	H	103	136
70	1	H	CF_3	H	270	135
71	1	H	NO_2	H	1100	242
72	1	H	CH_3	H	97	108
73	1	H	Cl	H	186	30
74	2	H	H	H	20	307
75	2	Br	H	H	10	166
76	2	H	Cl	H	21	153
77	2	Cl	Cl	H	10	233
78	2	H	F	H	10	370
79	3	H	H	H	21	238

80	4	H	H	H	9	171
81	1	H	F	H	153	31
82	2	H	F	H	12	48
Series 3 (Oxo derivatives)						
83	1	H	F	H	2280	1642
84	2	H	F	H	149	363
Series 4 (Imine derivatives)						
85	0	H	H	H	>10000	>10000
86	0	F	H	H	>10000	>10000
87	0	H	F	H	>10000	>10000
88	0	H	H	H	>10000	>10000

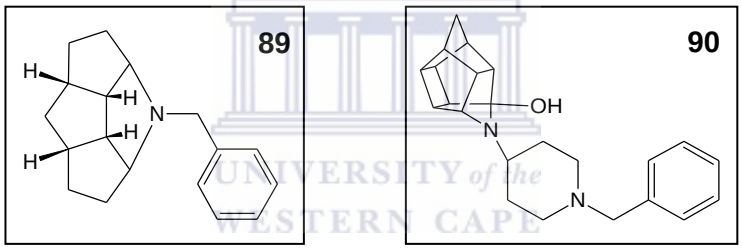


Figure 8. Additional compounds **89** and **90**

Sigma receptor affinity was consistently improved by a ketal group present and showed preferential selectivity for the σ_1R. Compounds **56** and **59** showed significant σ_1R selectivity with K*i* of 17 and 15 respectively. The azapentacycloundecanes generally had better affinity than the oxapentacycloundecanes. Chain lengthening improved σR binding at both receptor subtypes and best activity was observed for compound **80** that had the longest chain length of 4 carbon atoms. Compounds **60**, **62**, **64**, **66**, **72**, **73** showed the highest σ_2R binding. From these results it appears that meta substitution is favourable for σ_2R binding. Highest to lowest affinity by nature of substitution appears to be F>Cl>Br>I>H>CH_3. Fluoro substitution on ortho and para positions did not show σ_2R specificity. CF_3 (**70**) and NO_3 (**71**) on the meta position retained σ_2R selectivity even though affinity for the receptor diminished. The σ_2R selectivity was lost when the alkyl chain was increased with a meta fluorine present. This could lead to the conclusion that meta substitution is necessary for σ_2R selectivity, but substitution on the benzyl group should be considered inconclusive. Different chain lengths change the entire orientation of the benzyl group (see figure 9, *Chapter III*). This does however promote the theory that if an optimal chain length is established,

halogen substitution could be useful for obtaining σ_2R selectivity. The imino-ketal groups showed no affinity for the σR (Liu *et al*., 2001).

Compounds **66**, **77**, **78** and **80** were further examined for their role in amphetamine stimulated DA release and these compounds were also tested for DAT and SERT binding. Compound **89** was the only compound that had affinity for the DAT and it also showed low σR affinity with (K*i* = 3000 nM for σ_1R and K*i* > 10 000 nM for σ_2R). Compounds **66**, **77**, **78** and **80** exhibited enhanced DA release when compared to amphetamine release in a range of 30 – 45%. The most potent DA releaser was compound **66** with an IC_{50} < 100 nM. In the presence of σ_2R selective antagonist their action on DA release was diminished suggesting that they are σ_2R agonists. Compound **89** diminished the action of **78**, suggesting this compound could be a σ_2R agonist (Liu *et al*., 2001).

The study of Liu *et al.,* (2005) study also included compound **90** that showed K_i of 9.0 nM for σ_1R and 223 for σ2R. This shows interesting variation in possibly shifting the basic amine binding from the pentacycloundecane to a basic nitrogen contained in a piperidine ring. It showed good binding characteristics, but due to its low lipophilicity log $P_{7.4}$ =2.00, it is predicted by the authors that it would be unable to cross the blood brain barrier.

BLOOD-BRAIN BARRIER PERMEABILITY

Polycyclic amines are valuable in enhancing the pharmacokinetic and pharmacodynamic properties of drugs by increasing lipophilicity. This not only elevates their distribution to lipophilic areas (important with regards to crossing the blood brain barrier) but also promotes lipophilic receptor binding. Polycyclic amines can also improve patient compliance by providing metabolic stability that prolongs pharmacological action and decreases dosage frequency (Brookes *et al.*, 1992).

3. Discussion

The σR is widely implicated in an array of neurological disorders. Its specific functions and mechanisms are still inconclusive due to the small number of σR ligands available and the lack of specificity of these ligands. The σR shows potential application in neuromodulatory mechanisms and from the literature it is evident that pentacycloundecane derivatives could serve as potential σR ligands. They can assist in neuropharmacological processes not only by influencing the σR but also through their advantageous attributes on other receptor classes including NMDA receptor inhibition and, calcium channel and DAT modulation. The pentacycloundecanes' additional potential to enhance pharmacokinetic characteristics makes these structures valuable scaffolds for σR ligand design and could add value to the limited σR ligand library.

III. CHEMISTRY & SYNTHESIS

Designing and synthesising pharmacological active ligands require several considerations such as existing knowledge on the receptor's structure activity to provide a receptor binding model, and evaluation of viable synthesis routes. Factors to take into account when designing suitable synthetic pathways include time, cost of starting materials, reproducibility, purification, yield and simplicity. Various synthetic routes should be evaluated to produce the most efficient general reaction that provides a large selection of potential compounds. Three key features are critical to evaluating the final reaction:

1. The success of the reaction
2. The purification of the reaction
3. The yield of the reaction

If a reaction fails on one of these factors, adaptability is necessary to examine new potential routes.

1. Compound design and validation

1.1 Proposed structures and validation

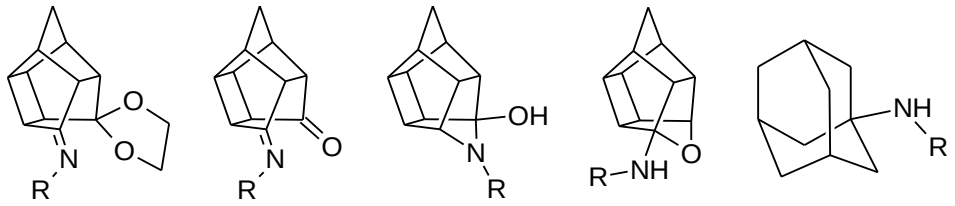
Glennon *et al.,* (1994) devised a general template for σR activity (see figure 5, *Chapter I*) that was adhered to in the design of the proposed structures regarding chain length and rotation. In addition to using polycyclic amines the objectives were also to investigate other polycyclic structures, different chain lengths and the incorporation of multiple amine binding sites. From the literature it was decided to investigate benzyl piperazine moieties that have shown to be valuable pharmacological ligands as discussed in Chapter 1 section 2.1.4. (Ablordeppey *et al*., 1998, 2000, 2002; Glennon *et al*., 1994, 2005; Cobos *et al*., 2008; Zampieri *et al*., 2009).

Originally it was envisioned that these compounds could assist in pharmacophore identification of sigma receptor 1 and 2 specific binding. But by incorporating benzyl piperazine moieties a hierarchy of objectives was necessary. This design would therefore serve as preliminary study into the potential of shifting the basic amine involved in σ_1R binding from the polycyclic amine structure to the basic amines contained in the piperazine ring. For this reason no halogen substitution on the benzyl ring was incorporated as it would detract from the goal of the initial design evaluation. We however postulate that if a more sophisticated design for the polycyclic-amine-piperazine-phenyl model can be proposed after evaluation of these pilot compounds, halogen substitution would afford specificity between receptor classes.

1. 2 Originally proposed chemical structures

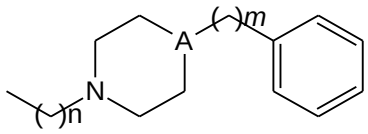
Table 7: Pentacycloundecane and amine containing aryl structures for sigma receptor binding.

Compound



Series A **Series B** **Series C** **Series D** **Series E**

R =



	n	***m***	**A**
1*	1	1, 2, 3	N
2*	2	1, 2, 3	N
3*	1	1	C
4*	2	1	C

The compound chain lengths are all within the range of the pharmacophore originally proposed by Glennon *et al*. 1996. By using the piperazine ring that contains two basic amines, the molecule can flip or shift and bind to the σR in its favoured position shown in figure 9 using aza-pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-2-(N-[2-(4-benzylpiperazin-1-yl)ethyl]) as an example. A pyridine containing analogue has been included to help predict which nitrogen the amine site binds to by comparing the affinity of the piperazine moiety compared to the pyridine moiety; and assuming that if the pyridine compounds shows improved affinity over its piperazine counterpart, the position of the pyridine's nitrogen shows preferential binding to the σR. These alternative interactions can ultimately be detrimental to affinity values by detracting from maximum binding, but provide the opportunity to further investigate σR binding which is the aim of this study.

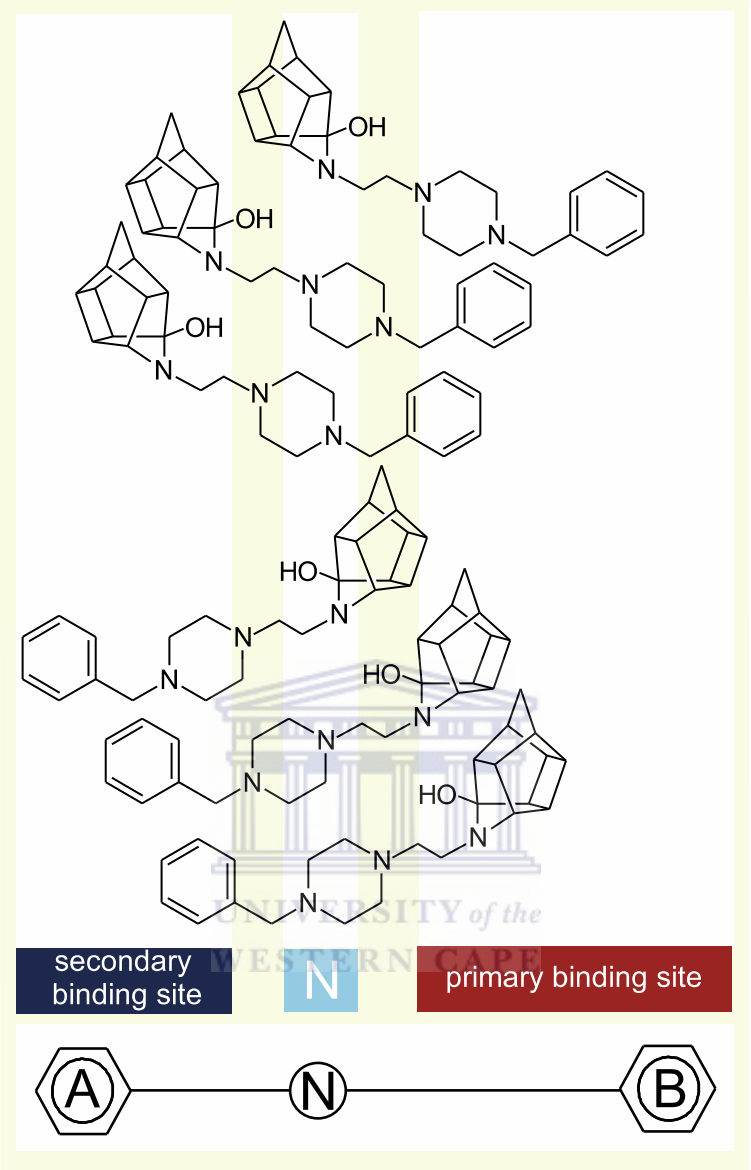


Figure 9. Alternative alignment possibilities

2. Chemical synthesis

2.1 Synthesis of pentacycloundecane analogues with potential sigma receptor affinity.

SYNTHESIS OF COOKSON'S DIKETONE

The synthesis of Cookson's diketone is well documented and was effectively standardised in 1958 (Cookson *et al*., 1958). Please see figure 10 for the complete reaction.

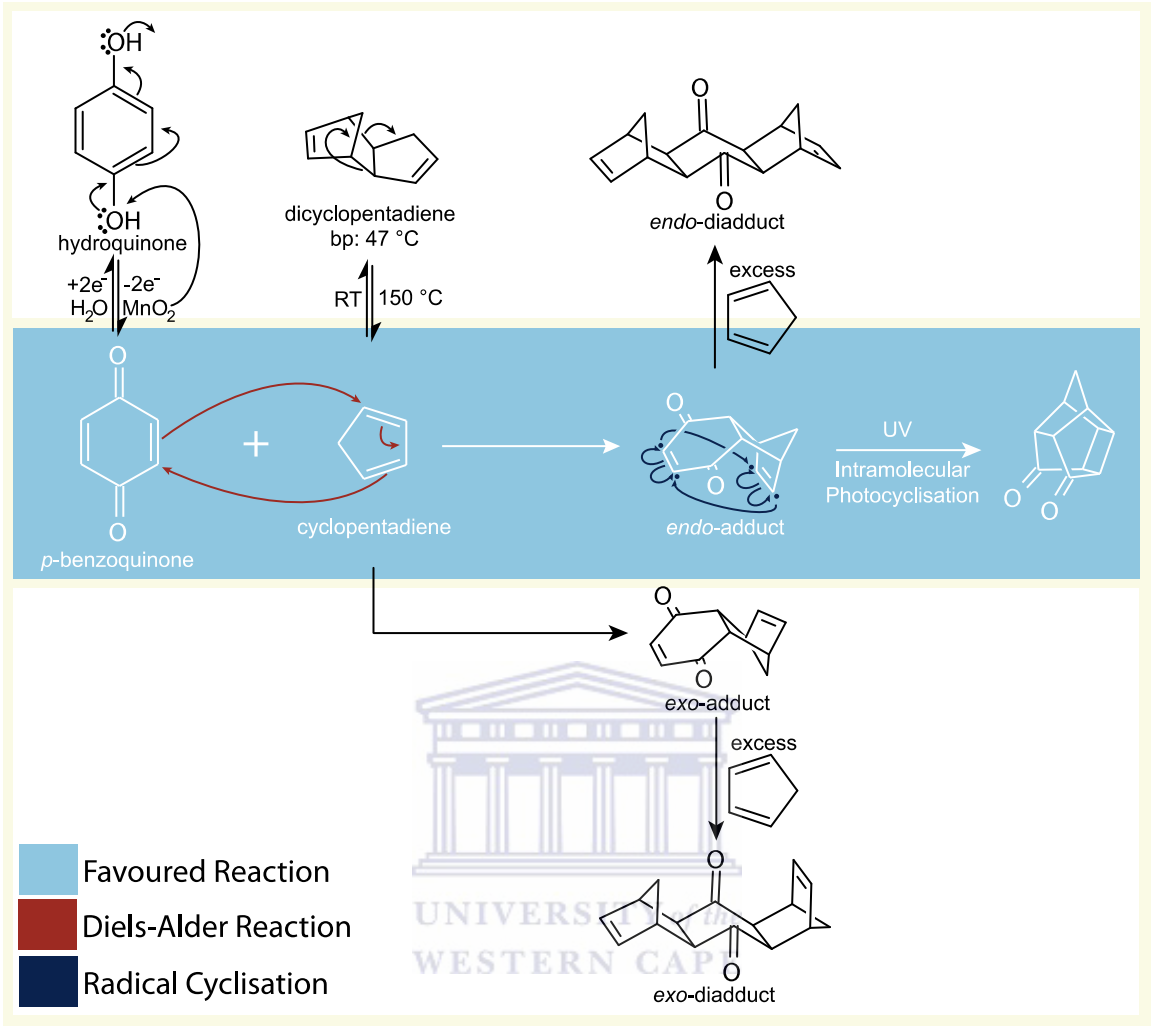


Figure 10. Synthesis of Cookson's diketone (Cookson *et al*., 1958).

Cookson's diketone is synthesised from para-benzoquinone (*p*-benzoquinone) and cyclopentadiene in an established Diels-Alder reaction. The adduct of these two compounds undergo photo cyclisation to produce Cookson's diketone. The reagent *p*-benzoquinone is commercially available but can rearrange in humid conditions to produce hydroquinone. Prior to use, it is advisable to oxidise *p*-benzoquinone to obtain a pure yield and increase its reactivity. Cyclopentadiene exists as its dimer dicyclopentadiene at room temperature and when heated to 150 °C divides into its monomer. Dicyclopentadiene and cyclopentadiene's boiling points are 170 °C and 47 °C respectively. Fractional distillation is used to separate the monomer from its dimer. A Vigreux condenser is used to slow the rate at which the hot vapours rise, giving a better separation between the different components in the distillate. Cyclopentadiene is collected and immediately used to avoid reversion of the separation. The addition of cyclopentadiene to *p*-benzoquinone is performed drop wise and on ice to promote the formation of the endo-adduct over the exo-adduct and further prevents cyclopentadiene reverting to its dimer and forming

the di-adduct. The reaction is traditionally performed in benzene but was also attempted in a 5:1 mixture of hexane-ethyl acetate (Hex, EtOAc) solvent system (Ito *et al*., 2007). The endo-adduct is insoluble in the latter solvent system and precipitates once synthesised preventing further formation of the di-adduct. It is also considered a safer solvent system than benzene but the solubility of p-benzoquinone is extremely low so a much larger volume of this solvent system is needed for the same reaction.

The Diel's Alder reaction is closely monitored by thin layer chromatography (TLC) and when complete the endo-adduct can be purified by crystallisation or immediately used in the next step where it is further reacted in a photochemical reactor. UV light generates radicals on the double bonds present and the endo-adduct undergoes intramolecular cyclisation. This process is physically impossible for the exo-adduct as the reactive pi bonds are not orientated in an adjacent position compared to the endo-adduct as shown in figure 11.

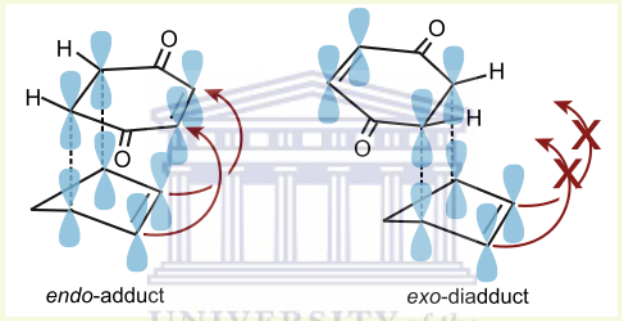


Figure 11: Spatial arrangement of p-orbitals in endo and exo-adduct

Radical cyclisation is usually dependant on the rate of reaction to favour intramolecular reactions over intermolecular bonding. This is not of concern for this particular reaction as the pi-bonds of the respective double bonds are perfectly situated in the molecule to promote cyclisation and immediately associate with the opposite double bond once the radicals are generated. A COSY NMR of the endo-adduct illustrates nuclear Overhauzer effect (NOE) between the relevant electrons, seen in figure 12. The NOE is seen in two dimensional NMR spectroscopy and is useful for 3D visualisation. The NOE differs from the application of spin-spin coupling in that the NOE occurs through space, not through chemical bonds. The NOE seen in this cosy shows a strong cross relaxation between the relevant atoms reflecting their close proximity to each other.

The final product is easily purified by Soxhlet extraction using cyclohexane as solvent. Cookson's diketone is more soluble in cyclohexane than the impurities present. In a saturated solution the Cookson's diketone will preferentially dissolve first and is syphoned away from its impurities and collected. The extraction is dependent on time with the most pure fractions collecting first and then followed by the impurities as its concentration in cyclohexane decreases. It is therefore advisable to collect the fractions at

set time intervals of approximately two hours to prevent contamination of the pure product with its impurities.

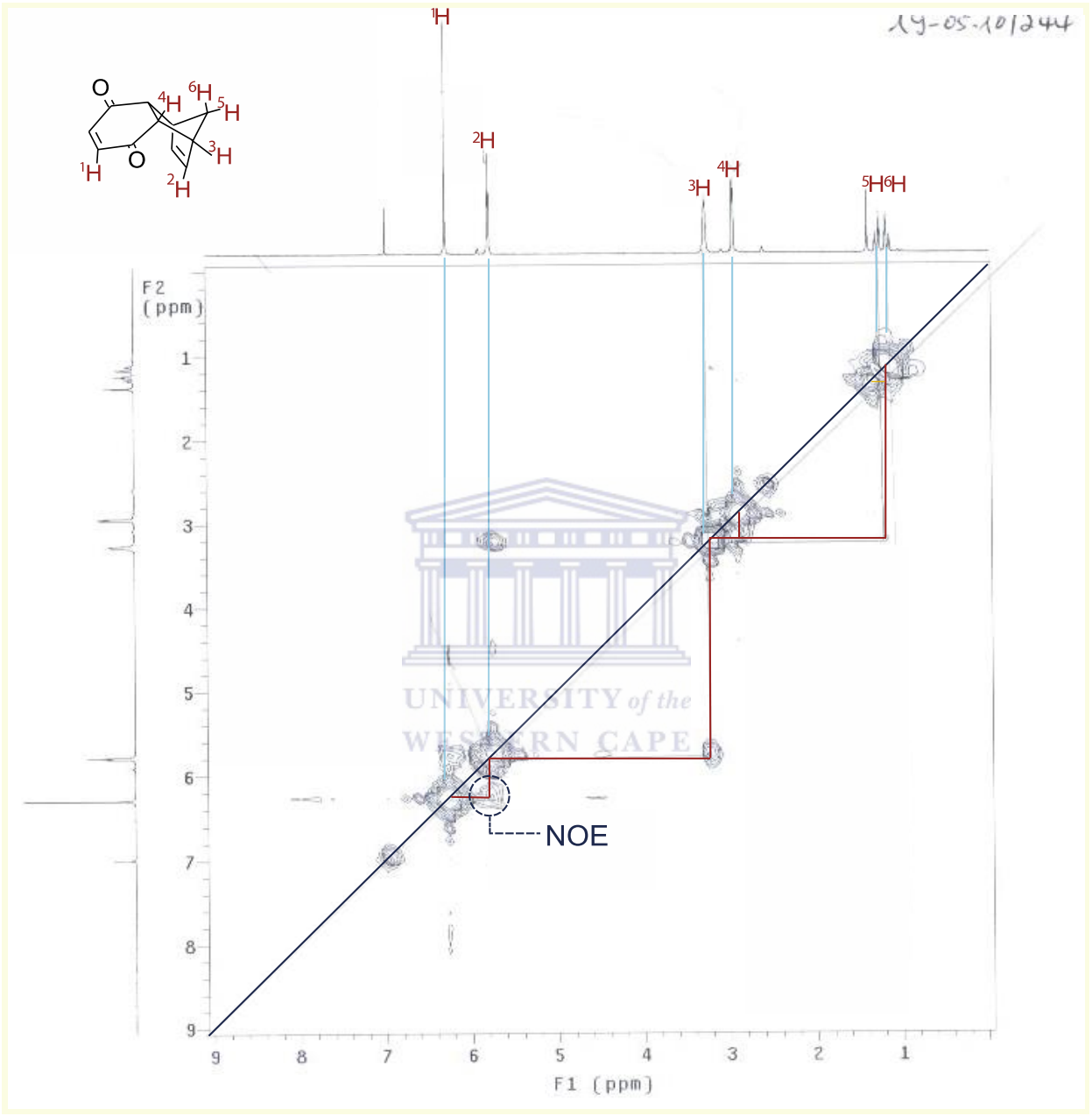


Figure 12: COSY NMR of adduct before cyclisation

PENTACYCLOUNDEACANE CONJUGATION

A popular method of synthesising Cookson's Diketone amine derivatives is by nucleophilic addition. For the resulting link to be an imine bond, a reactive amine functional group must be included in the intermediate design. To obtain the oxa and aza cage analogues a reduction step is necessary. Many

functional groups are sensitive to reductions and can be altered during the reduction step. The intermediate reacted with pentacycloundecane must therefore be as stable as possible. As illustrated in figure 13.

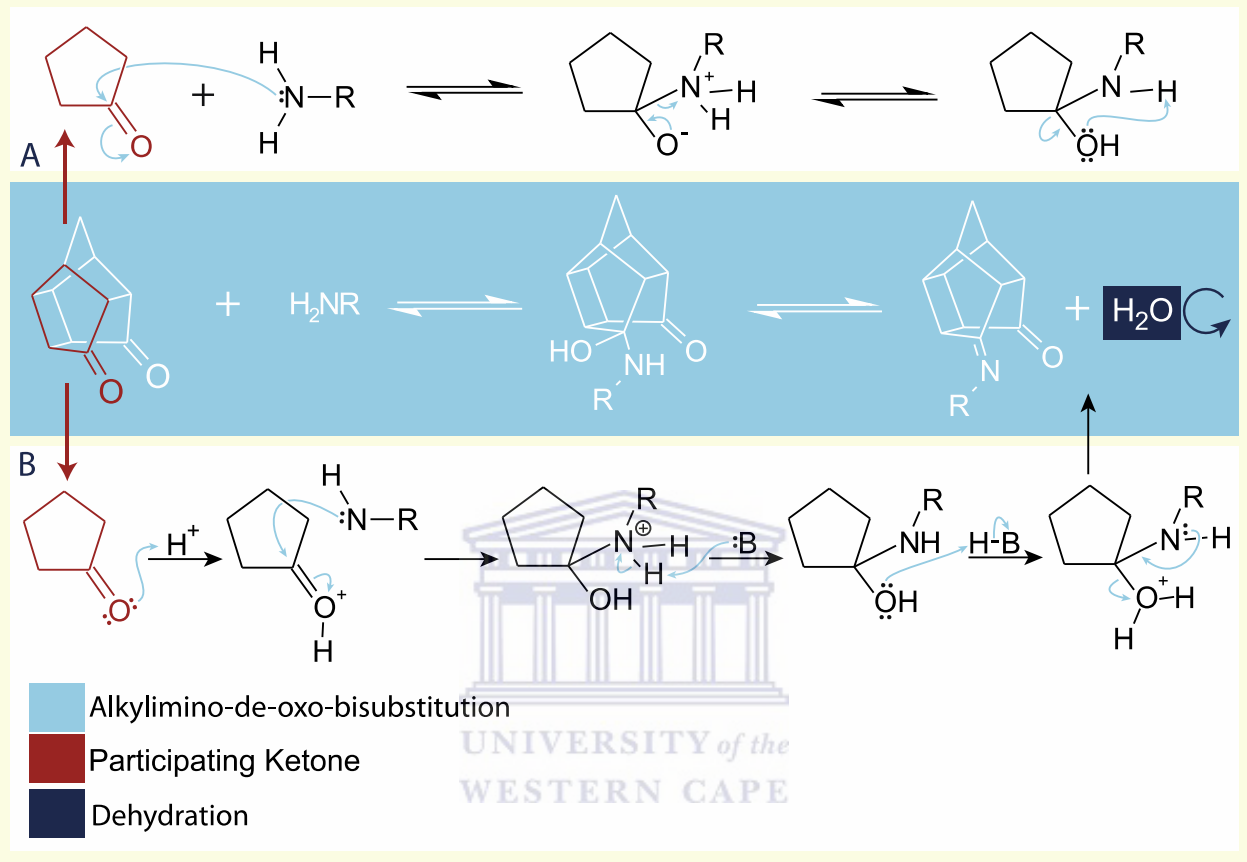


Figure 13: Primary amines react through an unstable hemiaminal intermediate which then splits of water

The reaction proceeds as the nucleophilic amine attacks the carbonyl giving a hemiaminal -C(OH)(NHR)- intermediate, followed by elimination of water to yield the imine also known as the Schiff base. The carbonyl group is open to attack by strong nucleophiles. Depending on the nucleophilicity of the intermediate introduced the reaction could require acid catalysis. Acidic conditions shown in figure 13 part B cause the carbonyl to activate and form a carbocation that is prepared to share electrons with the attacking nucleophile. Amines in addition to their nucleophilicity are also basic and will form an ammonium ion if the conditions are too acidic. For imine synthesis the equilibrium in the reaction usually favours the carbonyl compound so dehydration is necessary to push the reaction to the desired compound.

OXA, AZA AND KETAL PENTACYCLOUNDECANE DERIVATIVES SYNTHESIS

Evaluation of the effect of different pentacycloundecane moieties on σR binding is an objective of this study. The reaction mechanisms of the various pentacycloundecane structures are shown in figure 14.The oxa and aza pentacycloundecane structures are the most stable of the suggested structures.

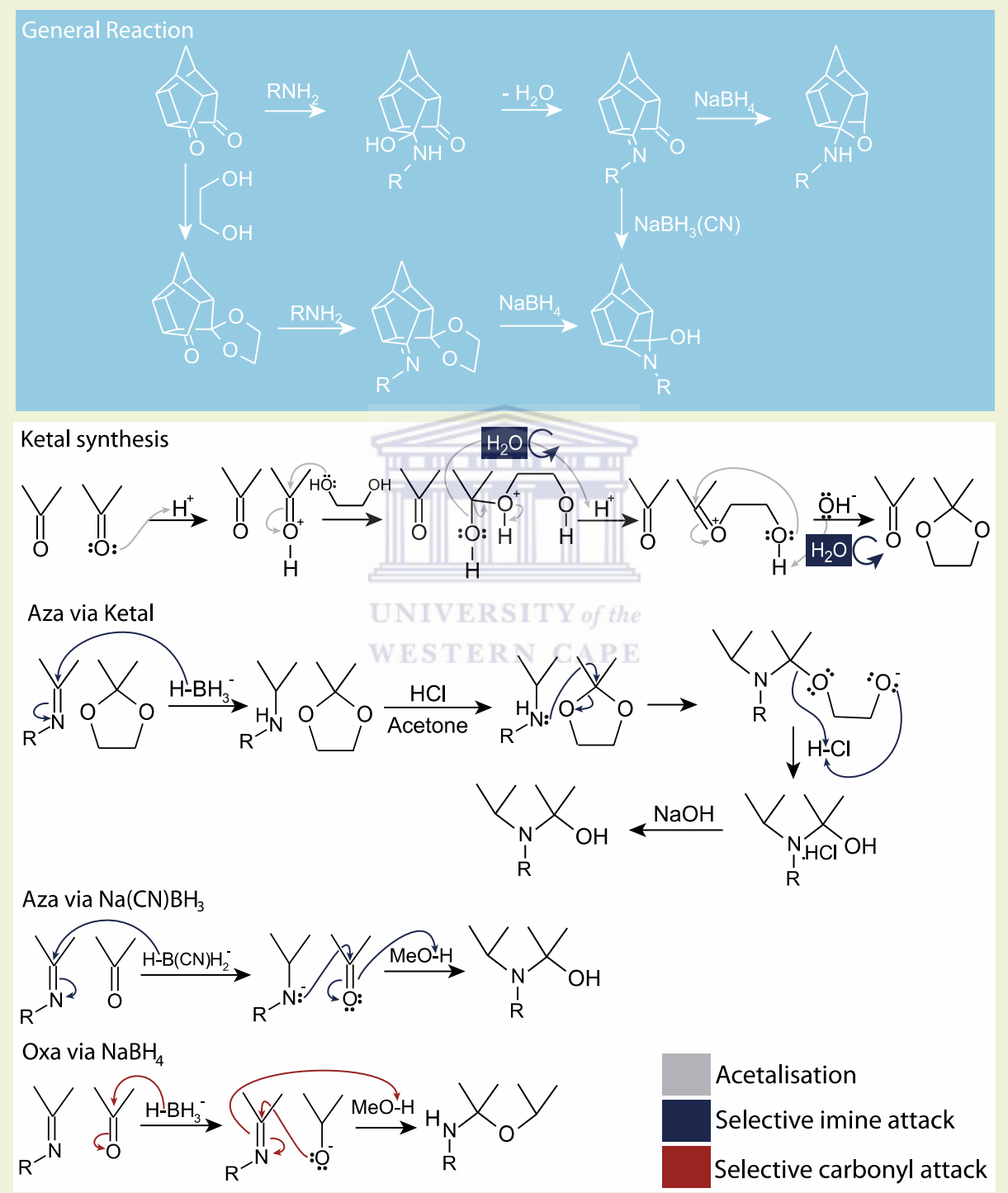


Figure 14: General reaction and mechanisms of different pentacycloundecane derivatives

To synthesise the protective ketal pentacycloundecane derivative acetalisation is necessary. The steps in nucleophilic acyl reactions are reversible and as a result the synthesis exists in equilibrium. It follows the same mechanism as imine formation by nucleophilic addition and in this case the attacking nucleophile is a hydroxyl group rather than an amine. The equilibrium favours the product containing the best nucleophile so a suitable leaving group is necessary. The reversible reaction is driven to completion by removal of water by azeotropic distillation or alternatively molecular sieves that can be placed in a Soxhlet extractor. The reaction is performed in toluene using a Dean Stark apparatus to remove water and the final product is then easily purified by crystallisation out of either methanol or diethyl ether. The ketal pentacycloundecane compound is further reacted with the selected intermediate and follows the same mechanism shown in figure 13. More extreme reaction conditions are necessary as shown by Bannister and colleagues who used a sealed vessel and ethanol as solvent to increase the pressure and boiling point of the reaction. This suggests that the ketal group does have an impact on its adjacent carbonyl making nucleophilic attack more difficult (Banister *et al.*, 2010). The ketal group could possibly affect the carbocation by inductive effects or steric hindrance.

To obtain the oxa and aza pentacycloundecane structures reduction is necessary. Sodium borohydride is a relatively strong reducing agent while sodium cyanoborohydride is comparably weak. Sodium borohydride shows selectivity towards reducing the carbonyl group and sodium cyanoborohydride is known for preferential reduction of imine groups over carbonyls. The reactive nitrogen and oxygen atoms are then capable of attacking their adjacent carbonyls forming the respective aza or oxa bridge. Alternatively the carbonyl can be protected by a ketal group, reacted with the intermediate amine and reduced by sodium borohydride which will in this case only be able to reduce the imine.

2.2 Synthesis of benzyl piperazine derivatives

An important feature of the proposed compounds is the number of amines present (see figure 5 and 9; and Table 7). These functional groups are easy to target when designing a synthesis due to their nucleophilicity, but due to multiple amines an attentive process is needed to target specific amines and prevent polymerisation. In deciding on a final approach, we considered the most convenient from the point of view of easily accessible starting materials and synthetic pathways.

NITRILE PROTECTIVE GROUP

Benzyl piperazine analogues are very popular pharmacological functional groups. They are commercially available and initial synthesis designs attempted to add a linker to benzylpiperazine analogues, see figure 15. To prevent polymerisation the terminal amine was protected as a nitrile group. This group was then to be reduced by $LiAlH_4$ to a primary amine able to react with pentacycloundecane. Unfortunately these

attempts were unsuccessful in the current study, possibly due to polymerisation to form unknown impurities or the quality of the $LiAlH_4$ used which didn't allow the reaction to proceed to completion. See Table 8 for variants of benzylpiperazine and linkers attempted.

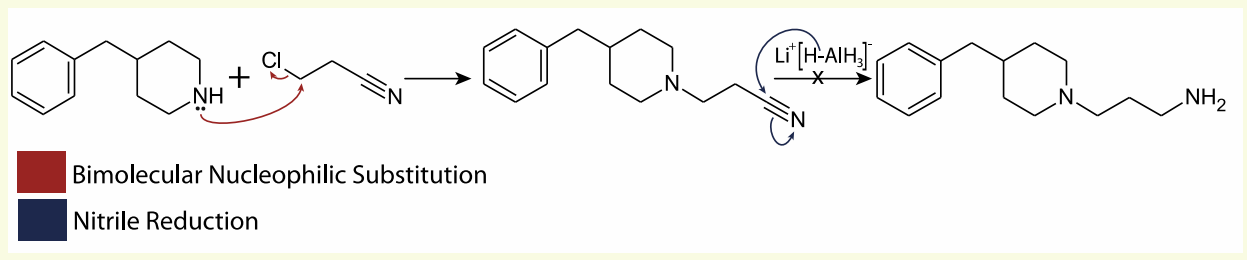


Figure 15: Failed nitrile protective group reaction

BIMOLECULAR NUCLEOPHILIC SUBSTITUTION KINETICS

Further methods to obtain the intermediates were investigated as indicated in Table 8. The most successful synthesis was achieved by taking advantage of the selectivity between primary and secondary amines in bimolecular nucleophilic substitution (Sn2) reactions. Using simple kinetic adjustments the reaction was optimised and proved more efficient in terms of yield, simplicity and cost than the initial investigated reaction (figure 16).

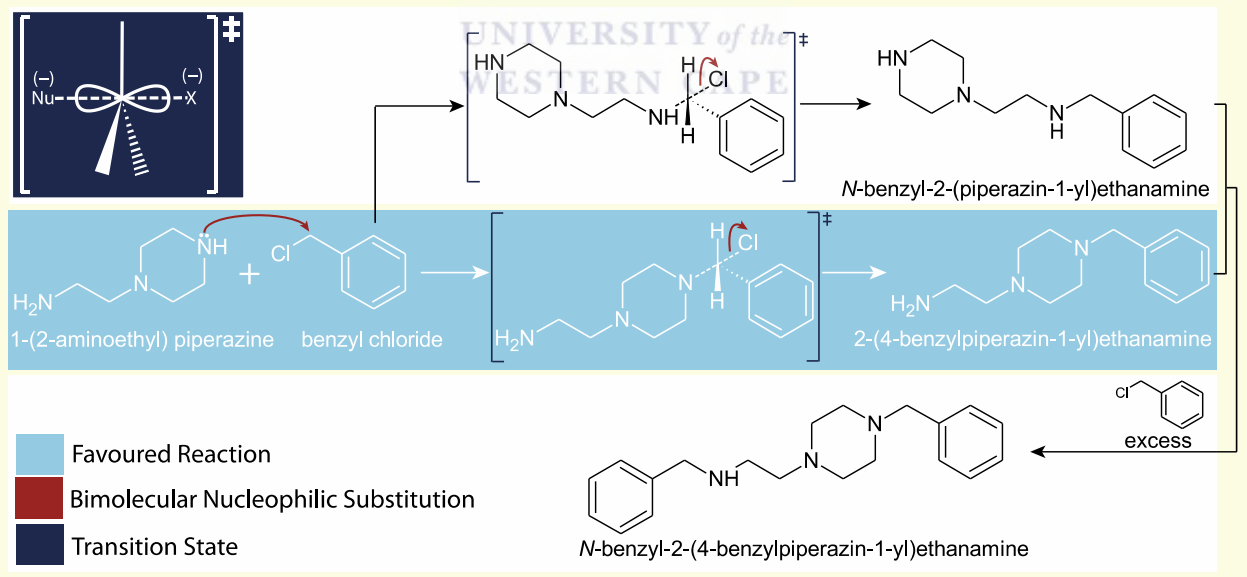


Figure 16: Sn2 reaction of amino ethylpiperazine and benzyl chloride

The Sn2 reaction is a nucleophilic substitution reaction where a nucleophile attacks an electrophilic centre, binds to it and expels another group known as the leaving group. In this reaction benzyl chloride was used, but benzyl bromide would in theory proceed faster as bromine is a better leaving group than

chlorine. The secondary amine contained in the piperazine ring attacks the electrophilic carbon centre bounded to chlorine at a rate higher than the primary amine on the ethyl carbon chain. The reasons for this are as follows;

1. A secondary amine is more basic than a primary amine due to increased alkyl inductive effects giving the secondary amine a more negative charge,
2. A secondary amine is more nucleophilic than a primary amine due to increased electron donors promoting attack and sharing of electrons,
3. The resulting product is more stable due to the rate of transient state to final product transformation occurring faster due to the secondary amines stronger push influence on the leaving group.

The Sn2 reaction follows second order kinetics making the concentration of both reagents rate limiting factors. To optimise preferential binding the benzyl chloride was added drop wise over 24 hours and 1-(2-aminoethyl)piperazine was used at a molar excess of 4:1. This slowed the rate of reaction considerably, allowing the reaction to proceed to the formation of a more stable product namely 2-(4-benzylpiperazin-1-yl)ethanamine over *N*-benzyl-2-(piperazin-1-yl)ethanamine. The molar ratio of 1-(2-aminoethyl)piperazine also prevented benzyl chloride to react with the primary amine of the formed product, as 1-(2-aminoethyl)piperazine's higher concentration lead to increase collision rate with the latter. Attempts to cool the reaction were also made but the reagents did not react at cold temperatures, making drop wise addition obsolete and the reaction was therefore carried out at room temperature.

Due to the rate of the reaction depending on the concentration of benzyl chloride, the reaction rate would decrease exponentially as the benzyl chloride present decreased after reacting. This meant that the formation of product would be highest in the first two days and then became insignificant after this time. After allowing the reaction to proceed for two weeks, there was still unreacted benzyl chloride present. The slightly acidic benzyl chloride could easily be removed from the reaction mixture by an acid/base extraction. The reaction was therefor only allowed to run for 72 hrs. as a longer time became inefficient with regard to the amount of product obtained. The final reaction mixture, in order of highest concentration, contained unreacted amino-ethyl piperazine, the desired product 2-(4-benzylpiperazin-1-yl)ethanamine, *N*-benzyl-2-(piperazin-1-yl)ethanamine, *N*-benzyl-2-(4-benzylpiperazin-1-yl)ethanamine and unreacted benzyl chloride. The reagent 1-(2-aminoethyl) piperazine is water soluble and was removed by a water/chloroform extraction. A base extraction followed to remove unreacted benzyl chloride. The *N*-benzyl-2-(4-benzylpiperazin-1-yl)ethanamine and *N*-benzyl-2-(piperazin-1-yl)ethanamine impurities were present in extremely low concentration.

The reaction mixture was then further purified by column chromatography which proved difficult due to the basicity of the desired product and impurities. A stationary phase of basic alumina or triethyl amine treated silica managed to purify the reaction mixture but poor resolution and significantly large volumes of solvents made it an inefficient purification method. Better results were obtained with flash chromatography using pure ethanol as mobile phase. The *N*-benzyl-2-(4-benzylpiperazin-1-yl)ethanamine impurity was stable and only possessed tertiary amines unlikely to react in further steps with pentacycloundecane. The *N*-benzyl-2-(piperazin-1-yl)ethanamine impurity possessed significant steric hindrance on its reactive secondary amine which would have to attack the carbonyl present on pentacycloundecane from behind. Its concentration was also very low. Comparing the NMR's of the completely purified 2-(4-benzylpiperazin-1-yl)ethanamine to the reaction mixture before column purification suggested that for its role as an intermediates subsequent column purification would be unnecessary due to the amount of product lost during the procedure. It was noticed that 2-(4-benzylpiperazin-1-yl)ethanamine would discolour to yellow if not protected from air and TLC of the product showed a new impurity of lower polarity forming. This was observed in completely purified and unpurified samples. The final product was stored at -5 °C, in the dark and under N_2 and used in subsequent reactions immediately after purification.

2.3 Conjugation of benzyl piperazine intermediate with polycyclic amines

DIRECT CONJUGATION

The next step was to react the intermediate 2-(4-benzylpiperazin-1-yl)ethanamine with the cage structure but traditional reaction conditions as depicted in figure 3 were unsuccessful. Alternative reaction conditions were attempted including pH adjustments, solvent changes, temperature changes and finally microwave irradiation. The tertiary amine in the piperazine ring is electron withdrawing and could limit the nucleophilicity and electron donating capabilities of the primary amine. Possibly steric hindrance could also be a factor making attack from behind the carbonyl group difficult, but compounds such as benzyl amine are able to react making this unlikely. Reaction using both unpurified and purified 2-(4-benzylpiperazin-1-yl)ethanamine products were attempted, so it can be assumed that the failure of the reaction was not due to interactions from impurities.

METHANESULFONYL CONJUGATION

Assembly from the pentacycloundecane structure was also attempted; see Table 8 for all attempted pentacycloundecane reactions. The reaction that proved successful involved adding a 2-aminoethyl linker to pentacycloundecane and conjugating this to a better leaving group namely methanesulfonyl chloride (MeSCl) to react with the benzylpiperazines, see figure 17. Alcohols are not good leaving groups and are

themselves nucleophilic. They can however be reacted with chlorides, bromides, and tosylate or mesylate groups which are excellent leaving groups in nucleophilic substitution reactions. The addition of the mesylate causes resonance delocalization of the developing negative charge on the original hydroxyl oxygen.

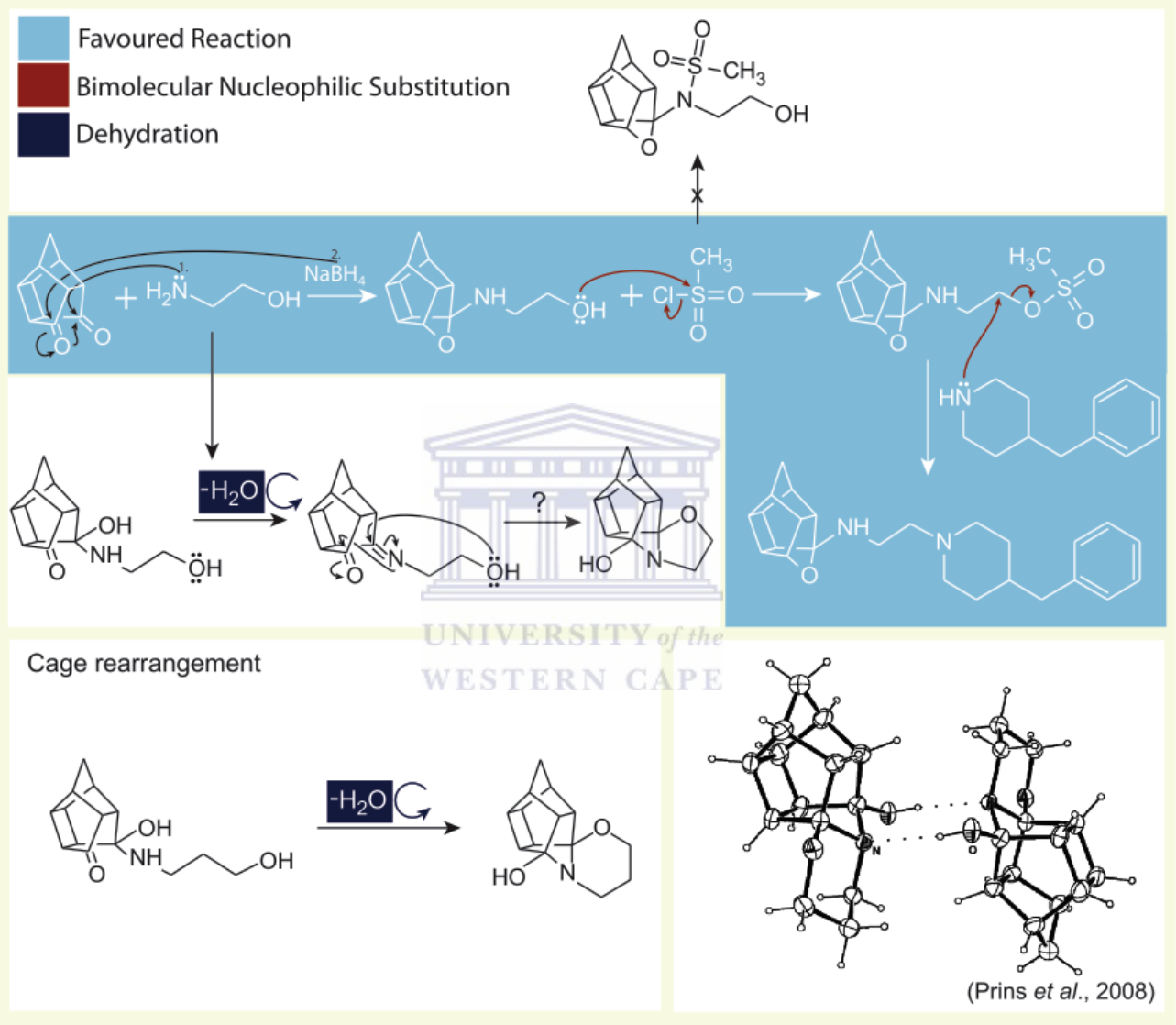


Figure 17: Protective methanesulfonyl conjugation

The reaction for conjugation with the mesylate was performed drop wise under cold condition and the solvent system that proved most successful was a triethyl amine; dichloromethane (Et_3N:DCM) combination. Tosylate and mesylate compounds selectively bind to amine groups rather than hydroxyl groups and there was concern that the mesylate would react with the secondary amine rather than hydroxyl group, but NMR confirmed that the expected final product was obtained. It can be assumed that steric hindrance from the pentacycloundecane structure had an influence on the hydroxyl selectivity. 4-

Toluenesulfonyl chloride (TSCl) was originally used but did not react, again possibly due to steric hindrance. Ideally a Dean-Stark reaction would then be performed and further reduction would yield the imine, aza and oxa cages, but when Prins and colleagues (2008) performed the step of dehydration on pentacycloundecane reacted with amino propanol, the linker reacted with the opposite hydroxyl group. This was confirmed by crystallography shown in the figure 17. To avoid a similar reaction the hydroxyl linker was immediately reduced to obtain the oxapentacycloundecane, removing the opportunity to obtain the imine and aza derivatives *via* this reaction scheme. The benzyl piperidine is then capable of reacting by Sn2 reaction (complete mechanism as described in figure 15) to displace the methanesulfonyl group and obtain the final structure.

AZA PENTACYCLOUNDECANE PIPERAZINE PHENYL SYNTHESIS

To obtain the aza derivative the pentacycloundecane was protected by a ketal group discussed in Section 3.1 of this chapter. Joining the intermediate with ketal pentacycloundecane was successful under microwave conditions. The reaction proceeded for 3 hours at a 150 W setting and maximum temperature of 100°C. (pressure 120 psi). The imine was then reduced by $NaBH_4$ in ethanol (EtOH) to obtain the endo-amine and the ketal was hydrolysed by aqueous HCl in acetone as explained in Section 3.1 to obtain the aza pentacycloundecane compound. Originally the ketal structure was to be included but due to a limited amount of reagents and time constraints, the total yield of the ketal derivative was reduced (Banister *et al.*, 2010).

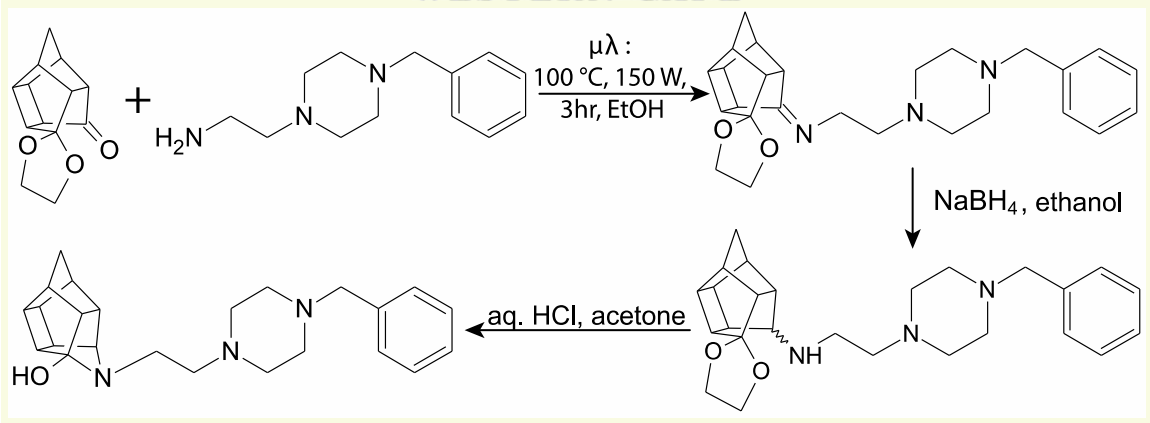


Figure 18: Ketal pentacyclundecane and intermediate reaction

AMANTADINE CONJUGATION

The research protocol postulated synthesis of a complete benzyl piperazine intermediate which was then to be reacted with different polycyclic amines. Bromoadamantane is commercially available and it was proposed that this compound could react with intermediates containing a primary amine *via* the Sn2

mechanism. Unfortunately steric hindrance on the tertiary carbon greatly limits attack from the nucleophile. Extreme conditions were necessary to allow the primary amine to position itself behind the bromide leaving group. Unfortunately the intermediate turned out to be unstable at these high temperatures and pressures and degraded before nucleophilic attack could be facilitated. The adamantine series was therefore excluded from this study.

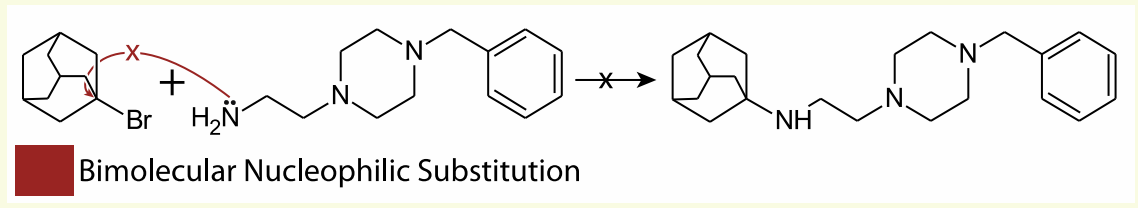


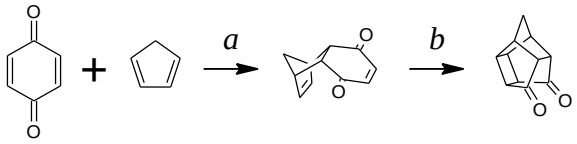
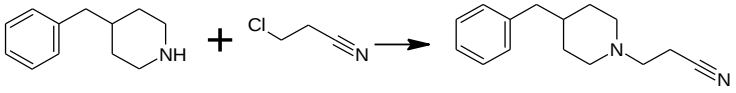
Figure 19: Failed adamantane conjugation.

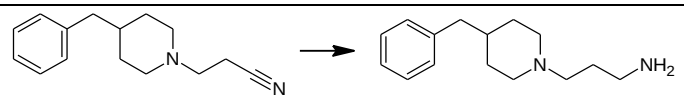
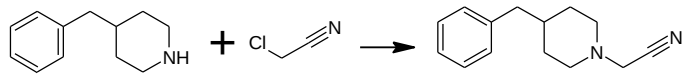
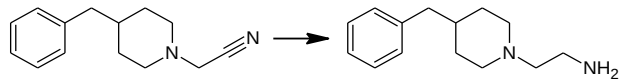
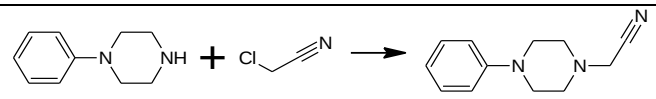
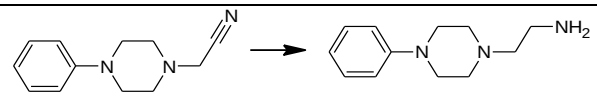
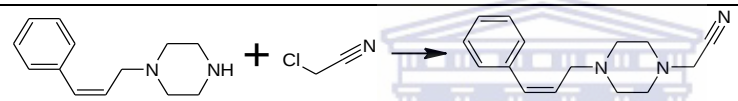
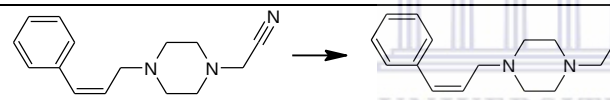
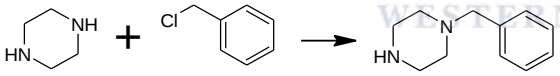
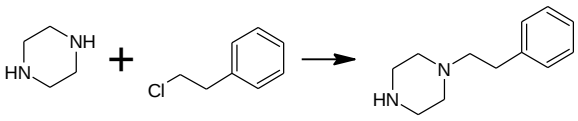
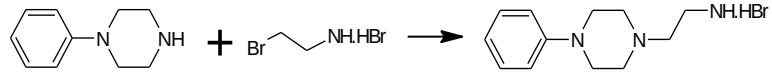
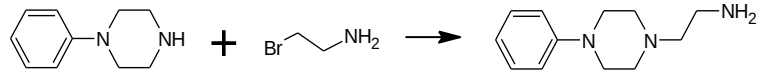
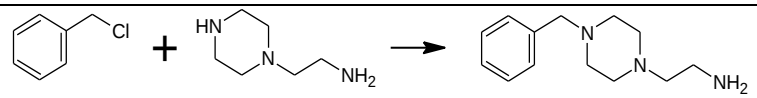
3. Experimental

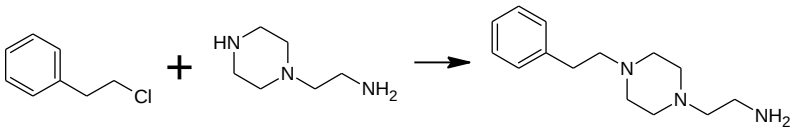
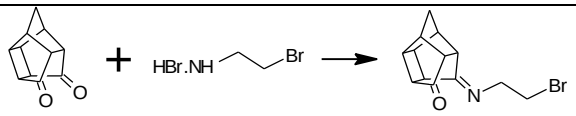
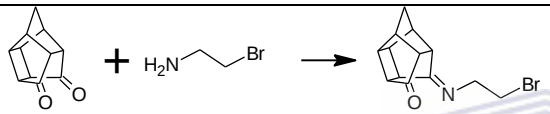
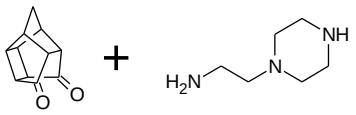
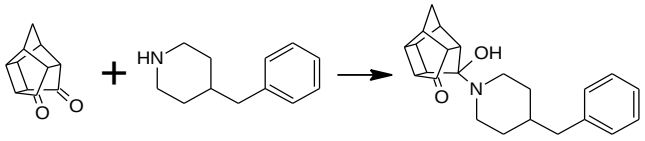
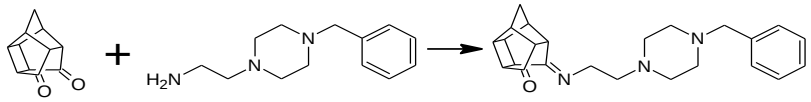
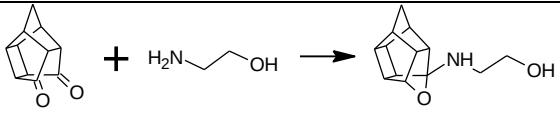
3.1. Summary of reactions attempted.

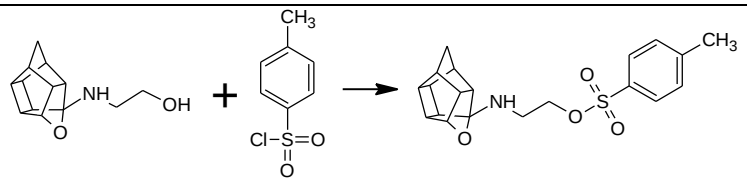
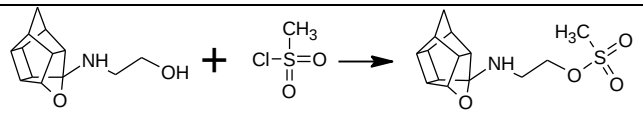
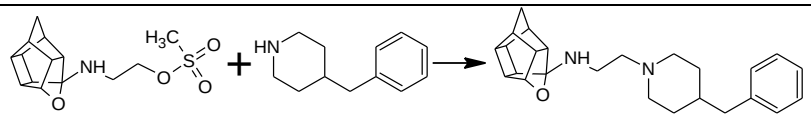
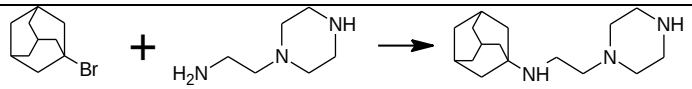
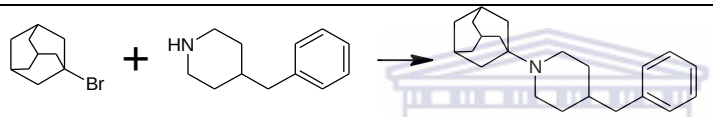
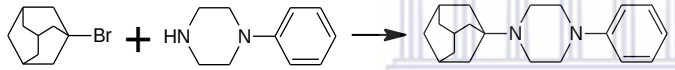
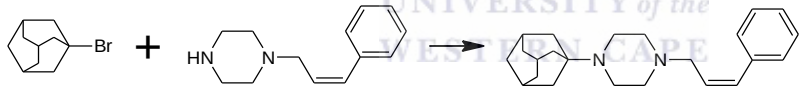
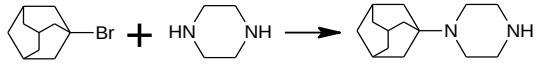
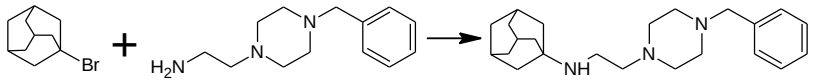
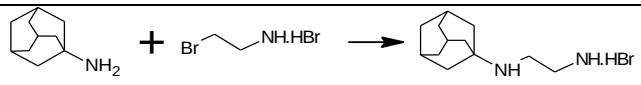
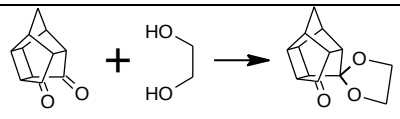
The following chemical reactions were attempted to obtain the suggested compounds.

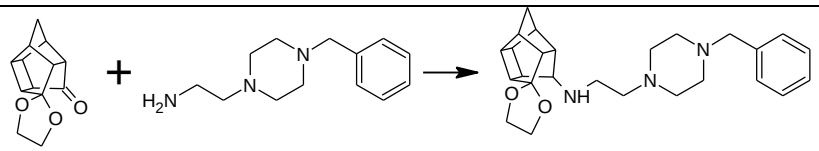
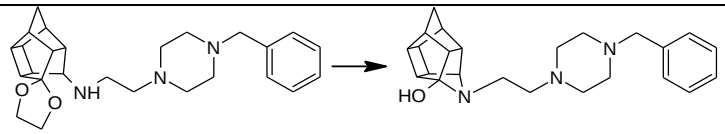
Table 8: Attempted reactions

#	Reaction Scheme	Conditions Attempted	successfully	Purification	% Yield
Cookson Cage Synthesis					
1.		*a.1*. Benzene, 4 °C, 5 h	✓	✓	86.0%[1]
		a.2. Hex:EtOAc (5:1), 4 °C, 5 h	✓	✓	96.7%[1]
		b.1.UV irradiation	✓	✓	53.3%
		b.2.UV irradiation	✓	✓	70.1%
Benzyl Piperazine Synthesis					
2.1.a.		Toluene, K_2CO_3, RT, 5 h	✓	✓	78.2%

2.1.b.		$THF, LiAlH_4$	?	×	?
		$Et_2O, LiAlH_4$	?	×	?
		$THF, NaBH_4$	?	×	?
2.2.a.		Toluene, K_2CO_3, RT, 5 h	✓	✓	63.7%
2.2.b.		$THF, LiAlH_4$	?	×	?
2.3.a.		Toluene, K_2CO_3, RT, 5 h	✓	✓	53.0%
2.3.b.		$THF, LiAlH_4$	?	×	?
2.4.a.		Toluene, K_2CO_3, RT, 8 h	✓	✓	57.2%
2.4.b.		$THF, LiAlH_4$	?	×	?
3.1.		Acetonitrile, 4 °C→reflux,7 h	✓	✓	64.4%[1]
3.2.		Acetonitrile, 4 °C→reflux, 24 h	✓	×	?
4.		ACN, K_2CO_3, reflux, 72 h	×	×	×
		EtOH, K_2CO_3, reflux, 120 h	×	×	×
5.		ACN, K_2CO_3, 4 °C, 2 h	?	×	?
6.1.		ACN, K_2CO_3, RT, 72 h	✓	✓	92%[1] 44.1%

6.2.		ACN, K_2CO_3, reflux, 120 h	✓	×	42.3%[1]
Polycyclic amine conjugation synthesis					
7.		THF, reflux, 72 h	×	×	×
		MeOH, reflux, 120 h	×	×	×
		ACN, K2CO3, reflux, 72 h	×	×	×
		µλ, 150 W, 30 m, 180 °C	×	×	×
8.		THF, 0 °C, 4 h	?	×	?
9.		THF, 4 °C→reflux, 48 h	×	×	×
		MeOH, 4°C→reflux, 48 h	×	×	×
		µλ, 250 W, 250 °C, 30 m, 210 psi[2]	×	×	×
10.		THF, 4 °C→reflux, 24 h[2]	×	×	×
		MeOH, 4°C→reflux, 72 h[2] µλ, 250 W, 250 °C, 30 m, 210 psi[2]	×	×	×
11.		THF, 4 °C→reflux, 120 h[2]	×	×	×
		MOH,4°C→reflux, 120 h[2]	×	×	×
		µλ, 250 W, 250 °C, 30 m, 210 psi[2]	×	×	×
12.		THF, $NaBH_4$, 4 °C→RT, 24 h	✓	✓	32.2%

13.a.		DCM, Et_3N, 0 °C→RT, 36 h DCM, Et_3N, 0 °C→reflux, 36 h	× ×	× ×	× ×
13.b.		Et_2O:DCM:Et_3N (5:1:4), 0 °C→RT, 24 h	✓	✓	20.0%[1]
13.c.		µλ, 100 W, 120 °C, 10 min, 90 psi	✓	✓	9.3%
14.1.		µλ, 250 W, 250 °C, 30 min, 210 psi[2]	×	×	×
14.2.		µλ, 250 W, 250 °C, 30 min, 210 psi[2]	×	×	×
14.3.		µλ, 250 W, 250 °C, 30 min, 210 psi[2]	×	×	×
14.4.		µλ, 250 W, 250 °C, 60 min, 210 psi[2]	×	×	×
14.5.		µλ, 250 W, 250 °C, 45 min, 210 psi[2]	×	×	×
14.6		EtOH, DMF etc. µλ, 250 W, 250 °C, 15 min, 210 psi[2]	×	×	×
14.7		µλ, 250 W, 250 °C, 30 min, 210 psi[2]	×	×	×
15.a		Toluene, TsOH, reflux, 5 h	✓	✓	40.1%

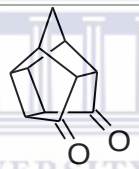
15.b		μλ, 150 W, 100 °C, 3 h, 100 psi	✓	✓	?[1]
15.c		EtOH, $NaBH_4$, RT, 18 h aq 4M HCl, acetone	✓	✓	41.0%

[1]Yield inaccurate. Product not purified and still contained impurities and or solvent.

[2] Several microwave conditions were attempted and reacted at 15 minute intervals for a total of no more than 90 min if no difference was observed by TLC and the reaction was terminated early if it was clear that one of the reactants were degraded. These conditions alternated power output between 50, 100 and 150 watts, temperatures between 70 °C and 250 °C, different solvent systems including solid state reactions and finally pH adjustments with glacial acetic acid or triethylamine.

3.2. Materials and methods

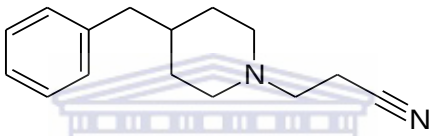
PENTACYCLO[5.4.0.0^{2,6}.0$^{3.1}$0.0^{5,9}]UNDECANE-8-11-DIONE



p-Benzoquinone (10 g, 92.51 mmol) was dissolved in dried benzene (100 ml) and oxidised with MnO_2 (50 mg) by refluxing the mixture for 60 minutes. The reaction mixture was protected from light by means of aluminium foil. Activated charcoal was added to remove impurities and the mixture was allowed to reflux for a further 5 minutes. The mixture was vacuum-filtered through Celite® to produce a clear yellow solution. This solution was cooled down to 5 °C by placing it on an external ice bath before slowly adding the freshly monomerised cyclopentadiene (12.23 g, 92.51 mmol). The reaction was monitored by means of TLC and addition of cyclopentadiene was discontinued when the di-adduct spot started to form on the TLC. The solution was protected from light and stirred for 1 hour at room temperature to ensure the reaction proceeded to completion. Activated charcoal was added and the mixture stirred at room temperature for a further 30 minutes. Removal of the activated charcoal, followed by *in vacuo* evaporation of benzene resulted in the formation of intensely coloured amber oil. Excess solvent was allowed to fully evaporate in a dark cupboard to afford the yellow Diels-Alder adduct crystals. (This entire process can alternatively be done in a hexane: ethyl acetate solution (5:1) to avoid toxic benzene as solvent.) The crystals were dissolved in ethyl acetate (4 g per 100 ml) and irradiated with UV light for 72 hours, using a photochemical reactor. Normally decolouration of the solution indicated that cyclisation of

the adduct was complete, but after 72 hours it was decided that TLC monitoring was suitable as decolourisation was not observed. Evaporation of the solution afforded a light yellow residue, which was purified by Soxhlet extraction in cyclohexane to produce the cage compound as fine light beige crystals (8.589 g, 53.3%). A solvent system of EtOAc and hexane was also evaluated. In this reaction 8 g of p-benzoquinone (74.01 mmol) was dissolved in approximately 450 ml of the solvent system. The reaction steps were followed exactly as described above. In this instance the addition of cyclopentadiene was discontinued when p-benzoquinone was no longer visible on the TLC. The reaction afforded an improved yield of Cookson's diketone but used approximately 5 times more solvent than benzene. The final crystals produced were lighter but TLC of the two compounds did not show any differences and infra-red spectra (IR) and melting points were comparable (9.037 g, 70.1%). (Cookson *et al*., 1958; Ito *et al*., 2007).

3-(4-BENZYLPIPERIDIN-1-YL)PROPANENITRILE

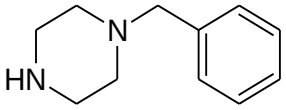


A stirred suspension of 4-benzylpiperidine (5.000 g, 28.52 mmol) 3-chloropropanenitrile (3.830 g, 42.79 mmol, 1.5 equiv.) and potassium carbonate (3.230 g, 42.79 mmol, 1.5 equiv.) in toluene (125 ml, 1 g per 25 ml) was refluxed for 5 h. Potassium carbonate was added to neutralise HCl produced by the reaction. After cooling, the mixture was poured into 50 ml of water and extracted with 3 x 25 ml dichloromethane to remove unreacted potassium carbonate and potassium salts produced by the reaction. The collected organic phases were washed with 75 ml of water and were then dried overnight with anhydrous $MgSO_4$. The $MgSO_4$ was removed by vacuum filtration and the solvent evaporated *in vacuo* to produce viscous yellow oil. The crude residue was further purified by column chromatography using a 70% ethyl acetate: chloroform mobile phase to yield the intermediate nitrile compound as a bright yellow oil (5.094 g, 78.2%).

Physical data: ^{1}H NMR (200 MHz, $CDCl_3$): δ_H: 7.39 – 7. 15 (m, 5H, H- 12, 13, 14, 15, 16, 17), 2.96 – 2.83 (d, 2H, H- 11), 2.78 – 2.65 (t, 2H, H- 7), 2.59 – 2.44 (t, 4H, H- 2, 6), 2.12- 2.96 (t, 1H, H- 4), 1.87 – 1.60 (d, 2H, H- 8), 1.43 – 1.23 (m, 4H, H- 3, 5)

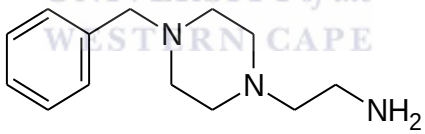
^{13}C NMR (50 MHz, $CDCl_3$): δ_c: 138.789, 128.916, 128.711, 126.663, 116.578, 53.610, 52.677, 41.962, 36.381, 28.980, 13.776

1-BENZYLPIPERAZINE



Benzyl chloride (2.351 g, 18.57mmol) was added drop wise to a stirred suspension of piperazine (8.000 g, 92.87 mmol, 5.00 equiv.) and potassium carbonate (2.804 g, 2.00 equiv.) in acetonitrile on ice over the course of 2 hours. The reaction was allowed to react for an additional 5 hours under reflux conditions. The acetonitrile was removed by *in vacuo* evaporation and 50 ml of dichloromethane was added. The unreacted piperazine, potassium carbonate and the produced potassium salts were removed by washing the organic layer with 5 x 50 ml of water. The organic layer was dried with $MgSO_4$ overnight, which was then filter off. The final mixture was evaporated *in vacuo* and yielded a yellow oil which contained one impurity visible under UV on TLC using ethyl acetate as mobile phase. The impurity was assumed to be 1,4-dibenzylpiperazine from its concentration and position on TLC. The yellow oil's infrared spectra compared to the National Institute of Standards and Technology database of the 1-benzyl piperazine compound confirmed that it was the desired 1-benzylpiperazine compound and that it was pure enough to be used in subsequent reactions (2.108 g, 64.4%). (Stein, 2013).

2-(4-BENZYLPIPERAZIN-1-YL)ETHANAMINE



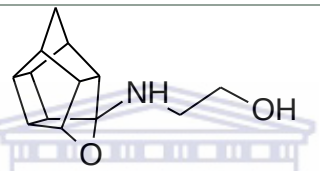
Benzyl chloride (2.000g, 15.47 mmol) in 10 ml acetonitrile was added drop wise to a suspension of neat 2-(piperazin-1-yl)ethanamine (10.030 g, 77.39 mmol, 5.00 equiv.) and potassium carbonate (1.752 g, 23.21 mmol, 1.5 equiv.) over the course of 6 hours at room temperature. The reaction was allowed to proceed for a further 72 hours. The mixture was concentrated *in vacuo* to remove acetonitrile. The suspension was added to 50 ml of chloroform and the unreacted amino ethylpiperazine, potassium carbonate; and potassium salts were removed by 2 x 25 ml water extractions. The organic phase was then washed with 2 x 50 ml 0.01 M sodium hydroxide solution to remove unreacted benzyl chloride from the mixture. The organic solution was dried over $MgSO_4$ overnight, which was then filtered off and the final solution was evaporated *in vacuo* to afford a clear oil with low viscosity. At this stage there were three compounds visible on TLC using a 95% MeOH: NH_4OH mobile phase. The two impurities present had negligible concentrations relative to the desired compound. This final mixture weighed 3.372g and estimating the impurities present were estimated at no more than 5% the theoretical yield was

approximately 92%. The compound was further purified by column chromatography using a versa flash system and absolute ethanol as mobile phase. The elution of the compounds was extremely slow and inefficient and much of the product was lost during this process (1.528 g, 44.1%). Alternatively the compound could be crystallised out of THF, but the crystallisation was inconsistent and showed very poor yield (0.277 g, 8.0%).

Physical data: ^{1}H NMR (200 MHz, $CDCl_3$): δ_H: 7.36 – 7.22 (m, 5H, H- 12, 13, 14, 15,16), 3.78 – 3.65 (s, 2H, H- 10) 2.83 – 2.73 (t, 2H, H- 7), 2.59 – 2.36 (s, 4H, H- 3, 5), 1.92 – 1.80 (t, 2H, H- 8), 1.30 – 1.18 (s, 4H, H- 6, 2).

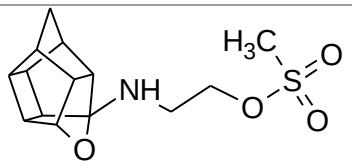
IR (υ_{max}): 2937.52, 2807.48, 1454.64, 1138.59, 1008.94

8- AMINOETHANOL-8,11- OXAPENTACYCLO[5.4.0.0 ^{2,6} .0 $^{3.1}$ 0.0 ^{5,9}]UNDECANE



2-Aminoethanol (1.774 g, 28.70 mmol) in 6 ml of THF was added drop wise to a mixture of pentacyclo[5.4.0.0^{2,6}.0$^{3.1}$0.0^{5,9}]undecane-8-11-dione (5.000 g, 28.70 mmol. 1.00 equiv.) dissolved in 30 ml of THF over the course of 30 min on an external ice bath. The carbinolamine precipitated after 10 minutes but the reaction was allowed to react for 4 hours at room temperature until completed as monitored by TLC. Sodium borohydride (1.465 g, 38.75 mmol, 1.35 equiv.) was added to the reaction mixture and it was stirred for 8 hours to reduce the product to the oxa bridged pentacycloundecane compound. The THF solvent was removed in vacuo and the product was dissolved in 25 ml dichloromethane and washed with 3 x 10 ml brine water to remove the unreacted sodium borohydride and reacted sodium salts. The product was then purified using 50% ethyl acetate; hexane as mobile phase to yield a beige coloured solid (2.033 g, 32.3%). The compound was confirmed by comparison to previous NMR confirmed reference compound by TLC and IR (Lemmer *et al.*, 2012).

8- AMINOMETHANE SULFONYLETHANE-8,11-OXAPENTACYCLO[5.4.0.0 ^{2,6} .0 $^{3.1}$ 0.0 ^{5,9}]UNDECANE

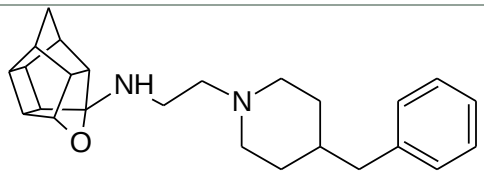


Methanesulfonyl chloride (261 mg, 2.28 mmol) dissolved in 10 ml of a 50:40:10 diethyl ether: dichloromethane: triethylamine solvent system was added drop wise to a stirred solution of ethanol-amino-4-oxapentacyclo[5.4.1.$0^{2,6}$.$0^{3,1}$0.$0^{5,9}$.$0^{8,11}$]dodecane (500 mg, 2.28 mmol, 1.00 equiv.) in 20 ml of the same solvent system placed under nitrogen gas on an external ice bath consisting of ice, acetone and sodium chloride which cooled the reaction mixture to approximately -8 °C. At the moment of addition extreme fizzing took place and the pressure increased in the reaction vessel as a large amount of gas was expelled and the reaction vessel's temperature increased despite the external ice bath. The reaction was allowed to stir overnight at room temperature. The mixture was evaporated *in vacuo* at relatively high temperature to remove the solvent system containing triethylamine. The mixture was dissolved in 10 ml of dichloromethane and washed with 2 x 10 ml of brine to remove unreacted methanesulfonyl chloride. The organic fraction was dried over $MgSO_4$ for 6 hours, filtered and evaporated *in vacuo* to produce a dark yellow oil. From TLC the product was confirmed to be pure enough for subsequent reactions and IR showed a methanesulfonate functional group present in the compound (193 mg, approximately 20%).

Physical data: ^{1}H NMR (200 MHz, $CDCl_3$): δ_H: 4.68 – 4.53 (m, 2H, H-15), 3.61 – 3.53 (t, 2H, H- 14), 3.17 – 2.94 (t, 2H, H- 20), 2.90 – 2.32 (m, 9H, H- 1, 2, 3, 5, 6, 7, 8, 9, 10, 11), 1.95 – 1.46 (AB-q, 2H, H-4a, 4b).

IR (υ_{max}): 3390.54, 2964.25, 2863.24, 1172.50, 1042.43 cm^{-1}

8- AMINO- *N*-2-(4-BENZYLPIPERIDIN-1-YL)- -8,11- OXAPENTACYCLO[5.4.0.0 ^{2,6} .0 $^{3.1}$ 0.0 ^{5,9}]UNDECANE



Methanesulfonylethane-amino-4-oxapentacyclo[5.4.1.$0^{2,6}$.$0^{3,1}$0.$0^{5,9}$.$0^{8,11}$]dodecane (193 mg, 0.65 mmol) was reacted with 4-benzylpiperidine (114 mg, 0.65 mmol, 1.00 equiv.). The reactants were dissolved in approximately 7 ml of acetonitrile and a spatula point of potassium carbonate was added. It was then reacted in a microwave reactor for 10 min at a maximum temperature of 120 °C, a pressure which fluctuated around 90 psi and power output between 50 and 100 W. The reaction mixture was then dissolved in 10 ml of dichloromethane and washed with 2 x 5 ml of water to remove the potassium salts. Further purification by column chromatography was done starting with pure hexane as mobile phase and gradually incorporating ethyl acetate into the mobile phase at increments of 10%. The product eluded at 50% ethyl acetate:hexane, but still contained impurities. The eluded compound was dissolved in 10 ml of

DCM and extracted with 3 x 10 ml 0.01M HCl solution. The acidic phases were collected and made basic with a 0.1M NaOH solution. The compound was then extracted with 3 x 30 ml of DCM. The organic fractions were collected, dried with $MgSO_4$ overnight, filtered and dried *in vacuo* to yield viscous yellow-orange oil (23 mg, 9.3%)

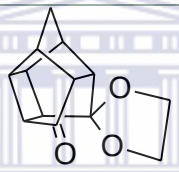
Physical data: ^{1}H NMR (200 MHz, $CDCl_3$): δ_H: 7.38 – 7.19 (m, 5H, H- 23, 24, 25, 26, 27), 4.64 – 4.59 (t, 2H, H- 11), 3.17 – 3.13 (d, 2H, H- 22), 2.79 – 2.40 (m, 21H, H- 1, 2, 3, 5, 6, 7, 8, 9, 10, 14, 15, 18, 19, 20, 21, 22), 1.90 – 1.48 (AB-q, 2H, H- 4a, 4b).

^{13}C NMR (50 MHz, $CDCl_3$): δ_c: 136.79, 128.50, 127.46, 126.28, 126.20, 82.64, 60.95, 55.05, 53.16, 51.96, 47.86, 47.59, 44.74, 44.53, 43.64, 43.40, 41.99, 41.71, 41.57, 41.29, 29.64

IR (υ_{max}): 2961.45, 2861.88, 1739.92, 1341.79, 1008.16 cm^{-1}

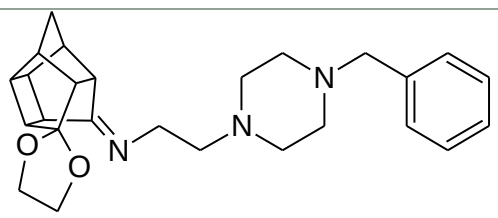
MS (ESI, 15 eV): 378.2174 (M^+ +2), 362.2292 (100%, $[M-16]^+$)

PENTACYCLO[5.4.0.0^{2,6}.0^{3,1}0.0^{5,9}]UNDECANE-8,11-DIONE ETHYLENE ACETAL



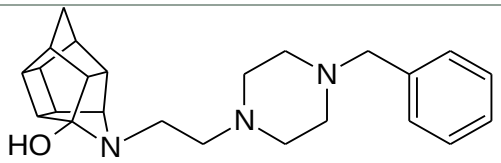
A mixture of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]undecane-8,11-dione (5.000 g, 28.66 mmol), ethylene glycol (1.60 ml, 28.66 mmol, 1.0 equiv.) and acidic catalyst *p*-toluenesulfonic acid monohydrate (54 mg, 0.28 mmol, 0.01 equiv.) in toluene (100 mL) was refluxed under Dean-Stark conditions for 5 h. The reaction mixture was neutralised with 25 ml of a saturated aqueous $NaHCO_3$ solution. The layers were separated and the aqueous layer was extracted with 3 x 25 ml dichloromethane. The combined organic layers were dried overnight with $MgSO_4$, filtered and concentrated *in vacuo*. The crude material was recrystallised from methanol as a white precipitate (2.332 g, 40.1%). The compound was compared to a previously NMR confirmed reference compound (Banister *et al.*, 2010) by TLC and IR and discovered to be correct and pure enough for subsequent reactions.

N-[2-(4-BENZYLPIPERAZIN-1-YL)ETHYL]-4-PENTACYCLO[5.4.0.0^{2,6}.0^{3,1}0.0^{5,9}]UNDECANE-11-ETHYLENE ACETAL



Pentacyclo[5.4.0.0^{2,6}.0^{3,1}0.0^{5,9}.0^{8,11}]undecane-8,11-dione ethylene acetal (200 mg, 0.917 mmol) and 1-benzylpiperazine (200 mg, 0.913 mmol, 1.00 equiv.) was reacted under microwave conditions. The reactants were dissolved in 7 ml of ethanol at a maximum temperature of 100 °C, power setting of 150 W and pressure of 100 psi for three hours. The reaction mixture was allowed to cool and directly used in the next step.

N-[2-(4-BENZYLPIPERAZIN-1-YL)ETHYL]-4-AZAHEXACYCLO [5.4.1.0^{2,6}.0^{3,1}0.0^{5,9}.0^{8,11}]DODECAN-3-OL



The cooled solution of *N*-[2-(4-benzylpiperazin-1-yl)ethyl]-4-Pentacyclo[5.4.0.0^{2,6}.0^{3,1}0.0^{5,9}]undecane-11-ethylene acetal and added $NaBH_4$ (159 mg, 4.20 mmol, 1.4 equiv.), was stirred at r.t. for 8 h. EtOH was evaporated under reduced pressure water (10 ml) was added and the mixture was extracted with DCM (3 × 10 ml). The combined organic extracts were washed with brine (10 mL), dried (Na_2SO_4), and concentrated *in vacuo*. To this crude material, acetone (25 ml) and 4 M aq. HCl (15 ml) were added. After stirring at r.t. for 12 h, the mixture was diluted with H_2O (200 ml), basified to pH 14 with 1 M aq. NaOH, and extracted with DCM (3 × 15 ml). The combined organic extracts were dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was purified by recrystallization from ethanol to yield the desired compound, as white crystals (157.86 mg, 41.0%).

Physical data: ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 7.26 – 7.16 (m, 5H, H- 23, 24, 25, 26, 27), 3.43 (s, 2H, H-21), 3.56 – 3.30 (t, 2H, H- 13), 2.96 – 2.30 (m, 19H, H- 1, 2, 3, 4, 5, 6, 7 ,8, 9, 10, 11, 14, 16, 17, 19, 20), 1.791 – 1.412 (AB-q, 2H, H- 4a, 4b).

^{13}C NMR (50 MHz, $CDCl_3$) δ_c: 137.98, 129.12, 128.21, 127.05, 70.79, 62.75, 58.66, 56.90, 52.75, 53.42, 51.53, 46.62, 45.68, 45.40, 43.32, 43.25, 42.15, 41.88, 41.69

IR (υ_{max}): 3242.86, 2949.29, 2812.87, 1319.78, 1284.36 cm^{-1}

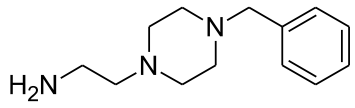
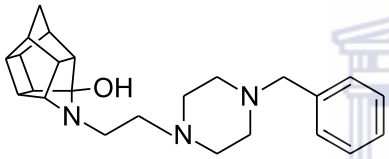
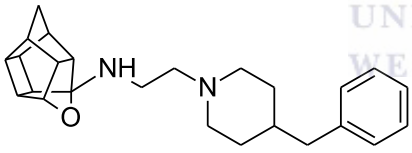
MS (ESI, 15 eV): 378.2546 (100%,[M+1]$^+$)

4. Final compounds and discussion

4.1 Final compounds

Three final compounds were obtained as shown in Table 9. All three adhered to Glennon's proposed pharmacophore and two of the compounds incorporate pentacycloundecane. The included intermediate, by comparison, will help confirm whether pentacycloundecane structure improves σR activity and should be further investigated.

Table 9: Synthesis Structures

Final Compounds	
	Compound 1: Molecular Formula = $C_{13}H_{21}N_3$ Formula Weight = 219.32594 Composition = C(71.19%) H(9.65%) N(19.16%) Nominal Mass = 219 Da
	Compound 2: Molecular Formula = $C_{24}H_{31}N_3O$ Formula Weight = 377.52244 Composition = C(76.35%) H(8.28%) N(11.13%) O(4.24%) Nominal Mass = 377 Da
	Compound 3: Molecular Formula = $C_{25}H_{32}N_2O$ Formula Weight = 376.53438 Composition = C(79.75%) H(8.57%) N(7.44%) O(4.25%) Nominal Mass = 376 Da

4.2 Discussion

It was originally planned to evaluate structures that would investigate the effect of chain length, multiple amine binding sites and different polycyclic amine structures effect on σR binding. The final synthesised compounds included an oxa and aza derivative, a piperazine and piperidine derivative to be compared but unfortunately we were unable to obtain compounds that would evaluate chain length, ketal, imine and amantadine polycyclic structures.

Reactions involving a two carbon chain between the benzyl and piperazine group (reactions 3.2 and 6.2 in Table 8) required higher temperatures than the one carbon chain reactions which would push the reaction to the formation of the di-ethylphenyl product. The reaction mixture also contained more unknown impurities at the higher temperature and could therefore not be used directly in subsequent reaction. The piperazine compounds were notoriously difficult to purify, but highly polar mobile phases and either

triethylamine treated silica or basic alumina as stationary phase were able to resolve some of the compounds, but they were never fully eluded and much of the final compound would be lost. It was for this reason that we decided to focus on the one carbon chain length moieties.

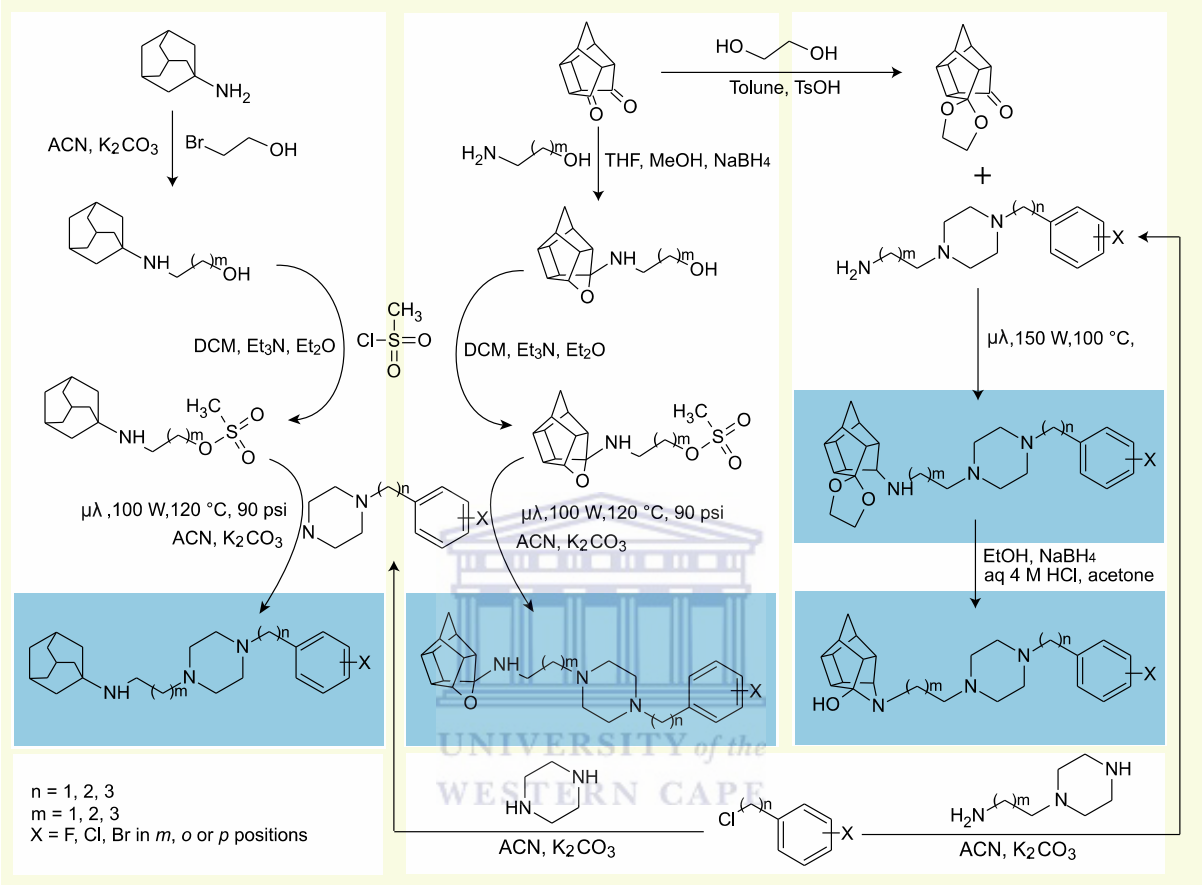


Figure 20: General reaction scheme to obtain future pentacycloundecane piperazine phenyl moieties.

These preliminary syntheses did however lead to a general reaction shown in figure 20 that can be utilised to not only obtain various chain lengths but also easily incorporate halogen substitution on the benzyl ring. This general reaction can also be used to obtain the ketal structure and points to a promising reaction to obtain the adamantane analogues. If an efficient method to obtain a hydroxyl linker with the adamantane structure is obtained the use of the methanesulfonyl protective group can be incorporated. The imine pentacycloundecane derivative remains elusive, but possibly further investigation into microwave conditions similar to the ketal reaction could be promising as the latter used a higher power output at lower temperatures for a longer duration compared to previously used high output, high temperatures for short duration.

1. Introduction

To evaluate the compounds' affinity for the sigma receptor (σR), competition radioligand binding assays are required. A radioligand is a radioactive labelled compound with known affinity to a receptor, enzyme or transporter of interest. The radioligand is first administered to a tissue sample to saturate available binding sites. The tissue sample is washed of residual radioligand that remains unbound and the compound being tested is then administered. The amount of radioligand displaced by the compound can be measured by gamma counting. A compound with higher affinity will be capable of displacing a larger proportion of radioligand. This number is used together with a nonspecific binding control that is capable of displacing a large excess of radioligand to plot the specific binding as a percentage of total binding (%B/100-%B, where %B is the percentage of bound radioligand in the absence of a competitor), against the log concentration of the competing ligand (log [L]). Non-linear regression of this graph can then be used to determine the half maximal inhibitory concentration (IC_{50}) value, which is then used to calculate the compounds' dissociation constant (K*i*) values using the Cheng–Prusoff equation ([L] = concentration of the competing ligand, KD = dissociation constant of the radioligand for the tested receptor).

$$Ki = \frac{IC_{50}}{1 + ([L] / KD)}$$

Radioligand assays used to determine σR binding are standardised and can evaluate dual σR affinity, sigma 1 specific receptor (σ_1R) affinity and sigma 2 specific receptor (σ_2R) affinities. The source of σRs tested include human cell cultures such as neuroblastomas, but animal sources such as rat liver or brain tissue is overwhelmingly more common. Previous reports show that rat brain and rat liver homogenates yield similar binding affinities for σ_1R, while rat liver is has been established as the preferred tissue for σ_2R binding assays. For dual σR, the radioligand [^{3}H] DTG is used. It possesses similar affinities for both the σ_1R and σ_2R, which makes it ideal for dual σR binding evaluation (Weber *et al*., 1986). The σ_1R agonist and radioligand [^{3}H] (+)-pentazocine is used for σ_1R specific assays (de Costa *et al*., 1989). Currently σ_2R ligands with the necessary specificity to be used as radioligands remain elusive. Instead the σ_1R agonist dextrallorphan is incubated with cell membranes before further incubation with the dual σR radioligand [^{3}H] DTG. Dextrallorphan is able to occupy the available σ_1Rs and the then administered [^{3}H] DTG binds to unoccupied σ_2R, which can be used to assess σ_2R specific receptor binding (Hellewell *et al*., 1994). To ascertain the feasibility of the synthesised structures, only σ_1R specific radioligand binding was necessary to validate pentacycloundecane structures for future development of σR ligands.

2. Sigma-1 specific receptor binding assays

To ensure comparable results, previously standardised procedures were used of which the most popular in recent publications is the method proposed by Matsumoto *et al*., (1995). In the following experiments rat liver membranes were used as the source of σRs, [^{3}H] (+)-pentazocine was used as radioligand and all stock solutions and procedures including data processing were strictly adhered to as described by Matsumoto *et al*., (1995). The final results were expressed as IC_{50} and K*i* values and further compared to known σR ligands.

2.1 Materials

ANIMALS

Male Sprague-Dawley rat livers (250-300 g) from the Comparative Medicine Unit at Northeast Ohio Medical University (NEOMED) were used in the study. The Institutional Animal Care and Use Committee (IACUC) approved the assay protocols.

RAT LIVER MEMBRANES

Membranes were prepared from the livers of male Sprague-Dawley rats (250-300 g) according to the methods described by Matsumoto *et al*., (1995). Animals were sacrificed by decapitation, and the livers were removed and minced before homogenisation. The liver tissue was homogenised in 10 volumes of ice-cold 0.32 M sucrose. The crude homogenate was centrifuged at 1000 g for 10 min at 4 °C. The supernatant was further centrifuged at 20,000 x g for 15 min at 4 °C. The pellet was resuspended in 3 volumes of ice cold 50 mM Tris-HCI / 0.32 M sucrose (pH 7.8) by vortexing and left to incubate for 30 min at 25 °C. The suspension was recentrifuged at 20,000 x g for 15 min at 4 °C and the pellet was resuspended in a final volume of 1.53 ml/g ice-cold 50 mM Tris- HCl / 0.32 M sucrose (pH 7.8).

2.2 Methods

BINDING ASSAY

Binding assays utilised optimized buffer and incubation conditions that were consistent with those reported in the literature. The compounds were prepared as 10 mM stock solutions in 100 % DMSO and diluted with Tris- HCI buffer on the day of the experiment. The final DMSO concentration in the incubation tubes was maintained at 0.1 %.

The processed rat liver membranes (20 µg of membrane protein/tube) were first incubated with 8 nM [^{3}H] (+)-pentazocine for 60 min at 37 °C. One of the four concentrations (40, 4, 0.4 and 0.004 µM)) of the

desired compound being tested in a total volume of 0.25 ml of 50 mM Tris-HCl, pH 8 was added to saturated [^{3}H] (+)-pentazocine membrane proteins and incubated for a further 60 min at 37 °C. Non-specific binding was determined separately in the presence of 10 mM unlabelled TC-1 (Tocris, USA). Assays were terminated by dilution with 2 ml ice-cold 10 mM Tris-HCl, pH 8.0, and vacuum filtered through glass fibre filters (Whatman GF/C) that were soaked in 0.3% polyethylemeimine for at least 30 min prior to use to reduce non-specific binding. The filters were rapidly washed with 2 ml ice-cold 10 mM Tris-HCL, pH 8.0, repeating the washing process three times. Following washing, the filters were transferred to scintillation vials and 3 ml scintillation cocktail was added to each sample. Filters were allowed to soak in cocktail for a minimum of 15 minutes prior to counting. The vials now containing the displaced [^{3}H] (+)-pentazocine were measured by Beckman scintillation counter. All assays, including control, non-specific binding and compounds at their various concentrations were repeated in triplicate.

DATA ANALYSIS

The competition binding data were analysed with Prism 5 (GraphPad). A plot of specific binding as a percentage of total binding against the log concentration of the competing compound was analysed using a one-site nonlinear sigmoidal regression model to determine the concentration of ligand that inhibits 50% of the specific binding of the radioligand (IC_{50} value). K*i* values were calculated from the IC_{50} using the Cheng–Prusoff equation.

3. Results and discussion

Final calculations were made from a graph of fraction [^{3}H] (+)-pentazocine bound against log drug concentration (figure 21).

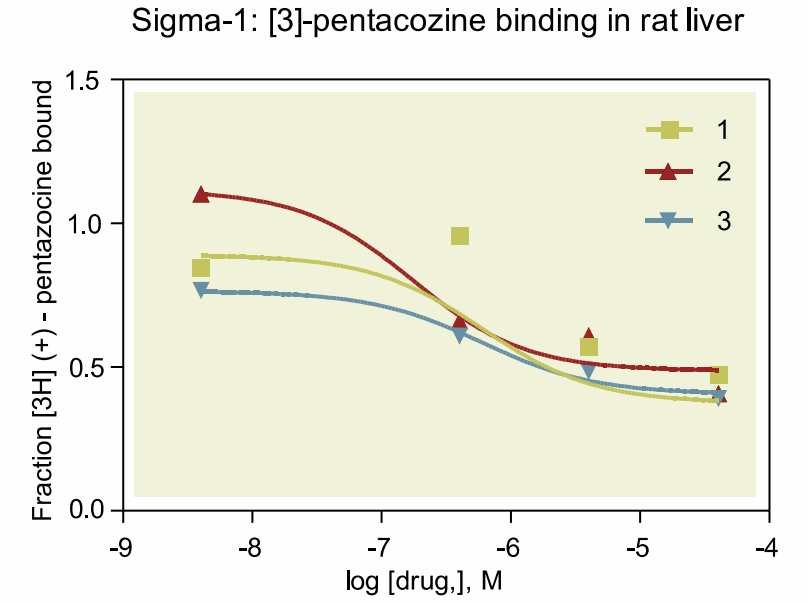
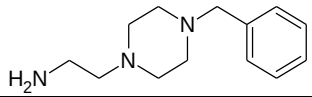
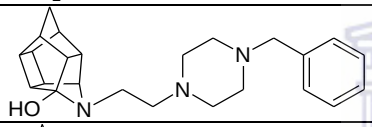
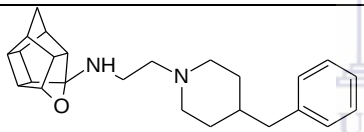


Figure 21: Fraction radioligand bound versus log concentration of final compounds (1,2 & 3) screened. Four concentrations were tested for each test compound. The graph shows how increasing concentration of the synthesised compounds decreased radioligand binding i.e. increased their occupation of the σ_1R receptor. The graph was used to calculate the respective compounds IC_{50} values which indicated the compounds' effectiveness in binding to the σ_1R. In this case the term IC_{50} is used with discretion as IC_{50} values are used to determine the efficacy of inhibitory properties of the tested compound. No pharmacological tests to obtain activity were done and therefor it is unknown whether the compounds inhibit or potentiate the σ_1R. In this case it is there only to show the compounds' potential potency on the σ_1R.

Table 10: Affinity values of final compounds

Final Compounds	K*i* (nM)	IC_{50} (µM)
1 	1132.26	2.34
2 	67.26	0.139
3 	132.10	0.273

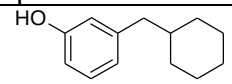
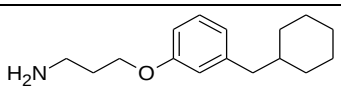
Pentacycloundecane containing compounds **2** and **3** had K*i* results of 67.26 nM and 132.10 nM respectively, and pentacycloundecane absent compound **1** had a K*i* constant of only 1132.26 nM. The pentacycloundecane addition increased ligand binding more than 10 fold for both the aza and oxa structures compared to compound **1**. This directly shows that the polycyclic cage structure has a favourable effect on σ_1R binding. This helps confirm that pentacycloundecane as a secondary lipophilic binding area is more favourable for binding than the nucleophilic primary amine present in the intermediate.

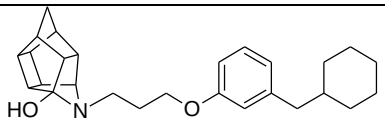
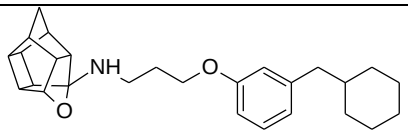
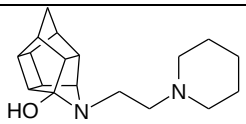
It could also be suggested that the aza compound **2** had a two fold increase in affinity compared to the oxa compound **3**, but the aza compound had a piperazine heterocycle rather than a piperidine structure as seen in the oxa compound. In *Chapter II*, Section 1.1 it was discussed that a decrease in affinity would be expected when comparing piperazine and piperidine structures, as the additional amine reduces optimum alignment. It could therefore be inferred that the increase in affinity had to be due to an overwhelming effect of the aza bridge, but these remarks are speculative and can only be confirmed with a new series of compounds that are exact in structure and only differ by an oxa or aza bridge.

The results of the new compounds compared favourably to previous studies of pentacycloundecane moieties evaluated for σR affinity that averaged within the range of 5 – 250 nM, see Table 6, *Chapter I*. In previous studies, the pentacycloundecane structures contain only the addition of one cyclic structure, usually in the form a benzyl ring that may or may not contain halogen substitution. Unfortunately our theory of increasing affinity by shifting the basic amine to a third cyclic structure remains unproven from the results obtained. The most comparable structure compound **59** that contains an unsubstituted benzyl group separated by a two carbon chain length had a K_i value of 15 nM, Table 6, *Chapter I*. This does not prove whether the addition of a third cyclic structure is advantageous, but it also doesn't discredit the hypothesis as a majority of pentacycloundecane compounds in Table 6 show an Ki value similar to compounds **2** and **3**.

A previous series of compounds synthesised by our research group which followed a similar three linear cyclic system was tested for σ_1R binding alongside the compounds from this study. The results from these compounds are included for comparison and are presented in Table 11. Compounds **2** and **3** showed improved affinity compared to compounds **4 – 8** and suggest that the pentacycloundecane followed by an amine containing aryl ring benzyl sequence is preferable for σ_1R affinity and supports the pharmacophore suggested by Glennon *et al.*, (1994, 2005). It is also important to notice that intermediate compound **5**, despite the lack of a second lipophilic binding area, was still able to have significant σ_1R binding. The aza compound **5** compared to oxa compounds **6** similar to compounds **1** and **2** showed increased σR binding. The difference was a significant increase in affinity from 11854.84 nM to 215.32 nM which strongly suggests that the aza compound is preferable for σ_1R binding which is similar to the results of compounds **1** and **2** from this present study. Intermediate compound **4** has an unexpectedly high affinity and it does not possess the necessary prescribed features of the pharmacophore including basic amine and two adjacent lipophilic regions as proposed by Glennon *et al.*, (1994, 2005) and remains an anomaly within these results. Finally compound **8** which consists of the aza pentacycloundecane and a piperidine ring separated by a two carbon linker when compared to compound **2** indicates the addition of a benzyl group opposite the piperidine amine is favourable for σ_1R affinity.

Table 11: Affinity values of previous series

Compounds		K*i* (nM)	IC_{50} (μM)
4		349.84	0.723
5		250.65	0.518

6		215.32	0.445
7		11854.84	24.5
8		469.84	0.971

4. Conclusion

The compounds synthesised in this study still require significant improvement of their affinities when compared to commercially available σ_1R ligands that have affinities ranging between 0.1 and 1.0 nM. The compounds presented in Table 1b, *Chapter I*, show that the pentacycloundecane structure unfortunately falls short to other lipophilic structures such as the benzyl groups. There were no structures that had the exact chain lengths of our final compounds so final conclusions regarding pentacycloundecanes ability to increase or decrease σ_1R binding when compared to their benzyl counterparts can't accurately be made. They do however compete strongly with other pentacycloundecane moieties tested for σR activity and considering the increased effect of halogen substitution and optimised chain length on compound affinity, these pilot compounds still have appreciable potential for further studies. The results encourage further testing of the final compounds to obtain a clearer σR binding profile.

V. SUMMARY & CONCLUSIONS

1. Introduction

Currently there are a limited number of sigma receptor (σR) ligands available and few possess the selectivity to accurately show the σR's role in biological processes. These receptors have been implicated to have a neuromodulatory effect on the dopamine, serotonin, norepinephrine and acetylcholine pathways while also having an effect on GABA and glutamate neurotransmitters. They are promising as potential therapeutic targets for the management of drug addiction, neurodegeneration and cancer therapies (Jupp & Lawrence, 2010). Polycyclic amines have shown notable σR activity and provide an advantageous scaffold for drug design that can improve pharmacodynamic and pharmacokinetic properties (Kassiou *et al*., 1996; Marrazzo *et al*., 2001). This study focused on new potential pentacycloundecane structures for σR activity with the aim to expand knowledge of the sigma receptor binding site and its interaction with pentacycloundecane derivatives and conjugates. The pentacycloundecane structure is itself a promising structure for disease states including neurodegeneration and psychiatric conditions and has proven to greatly improve blood brain permeability (Brookes *et al*., 1992; Zah *et al*., 2003). The objectives of the study were thus to investigate, design, synthesise and evaluate novel chemical compounds for sigma receptor activity using polycyclic amine structures as scaffold.

2. Methodology

Investigation into the pharmacophore of the σR provided a basic template for σR activity. It was decided that the limited amount of polycyclic amines synthesised and evaluated for sigma receptor affinity provided the opportunity to develop a series that would adhere to the basic pharmacophore while expanding into the use of an aryl piperazine group. Benzyl piperazine and benzyl piperidine moieties were synthesised with the aim of conjugating these structures with pentacycloundecane derivatives. These synthesised structures were then analysed for σ_1R specific affinity by using a standardised radioligand binding assay.

3. Chemistry

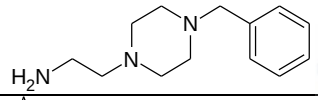
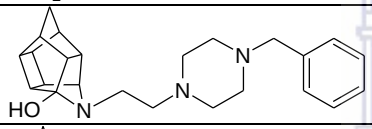
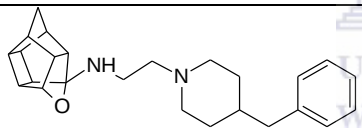
3.1 Original proposed chemical structures

The originally proposed structures were designed to investigate different polycyclic amine structures, aryl amine structures and alternative chain lengths which could further analyse the role of polycyclic amines as prospective σR ligands and to expand the σR pharmacophore.

3.2 Final compounds and general reaction

Conjugation of the polycyclic amines with benzyl piperazine moieties proved difficult, possibly due to steric hindrance and the multiple amines' effect on the process of nucleophilic addition. The basicity of the compounds impeded purification and limited the amount of final compounds synthesised. The obtained compounds are shown in Table 12. Two of the structures contained an aza and oxa derivative and piperazine and piperidine respectively. It was originally envisioned to compare the imine, ketal, aza and oxa structures, but due to limited chemical reagents and synthesised compounds, only the aza and oxa intermediates were obtained. The extensive synthetic routes developed did however produce a general reaction scheme that could be easily employed in future studies using cage derived heterocycle-benzylamine structures.

Table 12: Final compounds and their respective yields

Final Compounds	Yield
1 	44.1%
2 	41.0%
3 	9.3%

The newly designed reaction scheme should be able to efficiently produce the original proposed structures and provides the opportunity to investigate halogen substitution and various chain lengths. The reaction utilises microwave radiation in vital steps, which we believe greatly improve reaction conditions and final yield. The majority of steps should also be easy to purify by acid/base extractions which will also contribute to improved time, yield and productivity. See figure 20 for the general reaction scheme and Table 13 for a proposed series to contribute to further development of polycyclic amines for σR activity and selectivity.

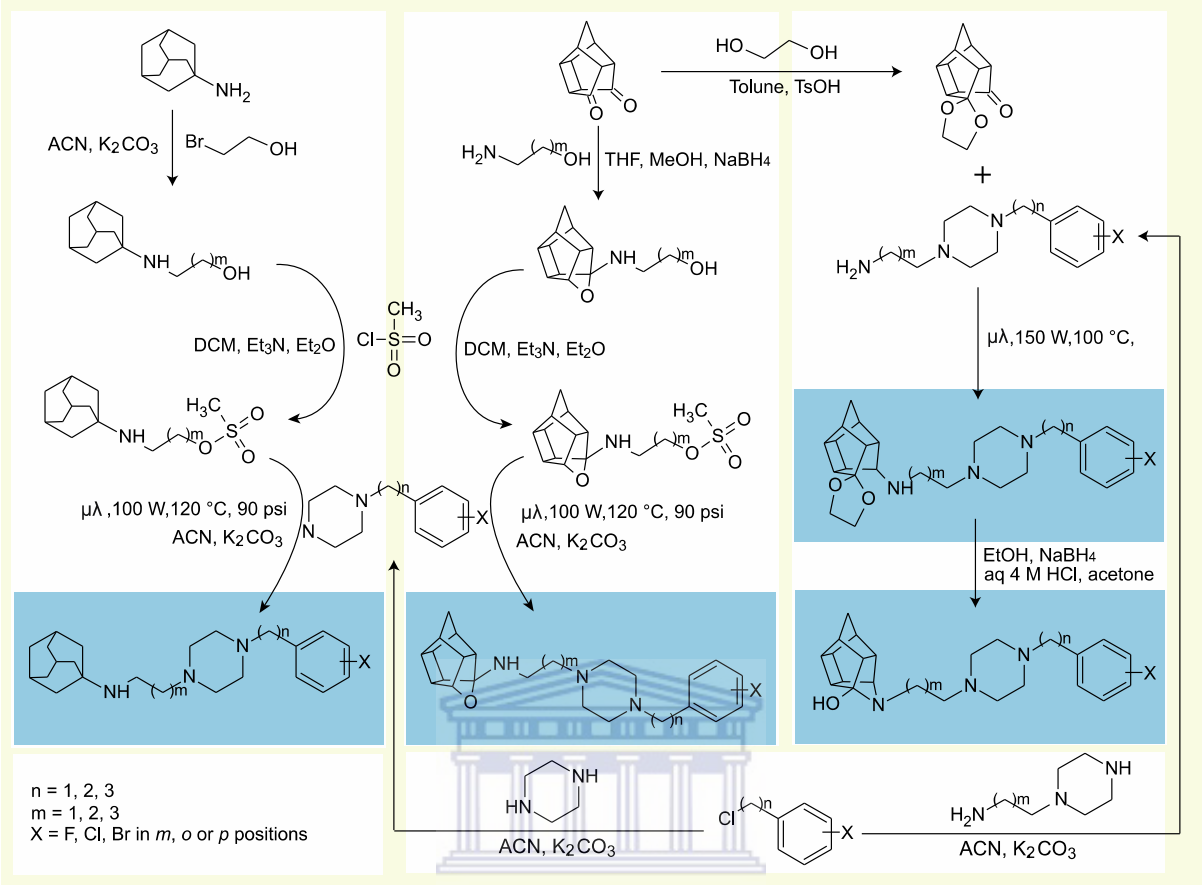
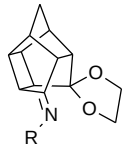
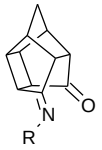
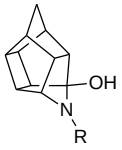
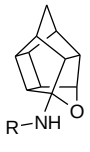
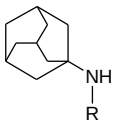
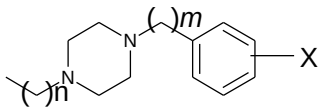


Figure 20: General reaction scheme to obtain future pentacycloundecane piperazine phenyl moieties.

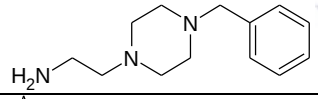
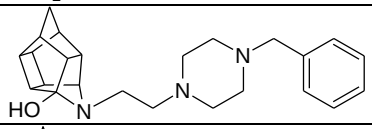
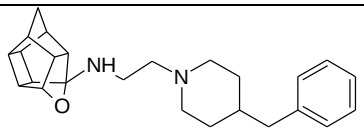
Table 13: Future compounds to be synthesised

Prospective compounds		
 **Series A**  **Series B**  **Series C**  **Series D**  **Series E** R =  X = F, Cl, Br (o, m, p)		
	n	***m***
1*	1	1, 2, 3
2*	2	1, 2, 3
3*	3	1, 2, 3

4. Biological evaluation & results

The test compounds' affinity for the σ_1R was determined by a radioligand binding study on Sprague-Dawley rat liver membranes using [^{3}H] (+)-pentazocine as marker. The compounds' different structural properties were compared. The synthesised structures' affinity fell between the ranges of 50 nM and 150 nM which validate the use of pentacycloundecanes to optimise σR binding. However, considering the differences between the oxa and aza structure, it was difficult to draw concrete conclusions on their structure activity relationships and a larger series would have to be developed to fully understand the polycyclic amines' effect on σ_1R binding. When comparing the test compounds designed in this study to previous σ_1R ligands, it confirmed that the aza structures in general have a higher affinity for the σ_1R than the oxa structures. The affinities were comparable when compared to previously published pentacycloundecane structures tested for σ_1R affinity (Kassiou *et al*., 1996; Marrazzo *et al*., 2001, Liu *et al*., 2001, 2005; Bannister *et al*., 2010). The addition of an aryl amine to improve affinity remains inconclusive and it would require a series of various chain lengths and a benzyl group with alternative halogen substitutions to prove our theory. Further comparison of the structures to commercially available σ_1R ligands shows that the affinity of the pentacycloundecane structures would have to undergo additional optimisation before being able to compete against available σR ligands.

Table 10: Affinity values of final compounds

Final Compounds		K*i* (nM)	IC_{50} (μM)
1		1132.26	2.34
2		67.26	0.139
3		132.10	0.273

5. Final remarks & future outlook

An important consideration when comparing the pentacycloundecane's affinity to other available σR ligands is their favourable effect on blood brain barrier permeability and other receptor and channel activities that were not evaluated in this present study. Pentacycloundecane compounds could show better specificity compared to their benzyl counterparts or interact favourably with other receptor targets implicated in different CNS disease states. The σR ligands are being supported as potential neuromodulatory agents and it is known that pentacycloundecane structures have increased central

concentrations compared to their polycyclic amine absent counterparts. Currently the focus of new σR ligand studies is aimed at obtaining σ_2R specific ligands. This was not one of our aims as we focused on the justification of using pentacycloundecane structures for σR ligand development. Further biological screening for dual σR activity and σ_2R specific activity should be conducted as these compounds could prove useful for σ_2R specificity. If these results are promising, further biological assays to evaluate not only their affinity for different receptor classes, but also their pharmacological activity on other relevant biological targets should be explored. The test compounds' calcium channel activity could assist in evaluating the test compounds' functionality regarding potential neuroprotective properties and determine agonist or antagonist action. Using fluorescent imaging and the fluorescent calcium channel indicator FURA2/AM in a comparative series of experiments that utilise NMDA antagonists, voltage gated calcium channel antagonist and sigma receptor agonist and antagonist, a pharmacological profile of the compounds can be established, see (Annexure 2). This protocol was devised at the start of this study, but could not be used due to time constraints. It should also prove valuable in adding to our understanding of the biological processes involved in activating the σR and its effect in cellular calcium. Towards the end of this study an article that described the crystallography of the σ_1R was published (Laurini *et al*., 2011). This is a very exciting prospect for σR ligand design as a receptor based approach using computerized molecular modelling software can now be followed to obtain σR binding in addition to the ligand based approach used in this study.

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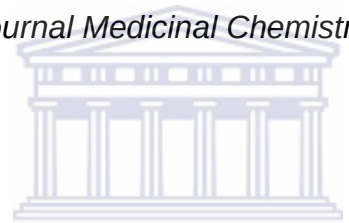
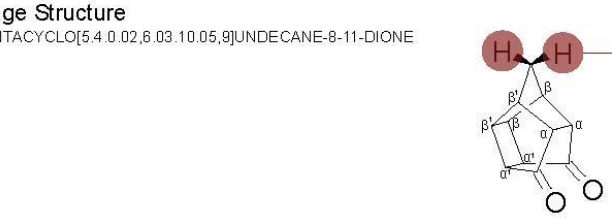
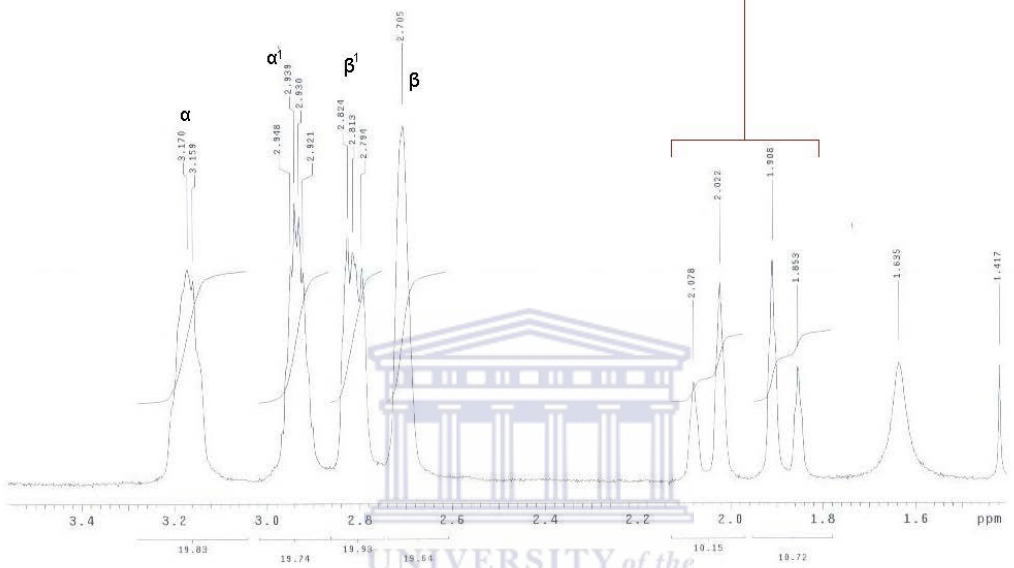


Cage Structure

PENTACYCLO[5.4.0.02,6.03,10.05,9]UNDECANE-8-11-DIONE





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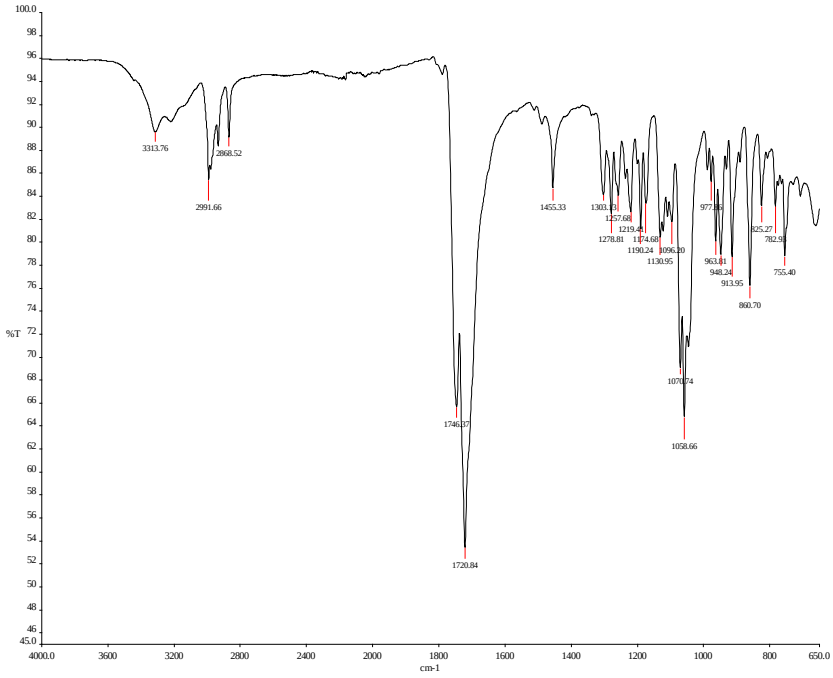
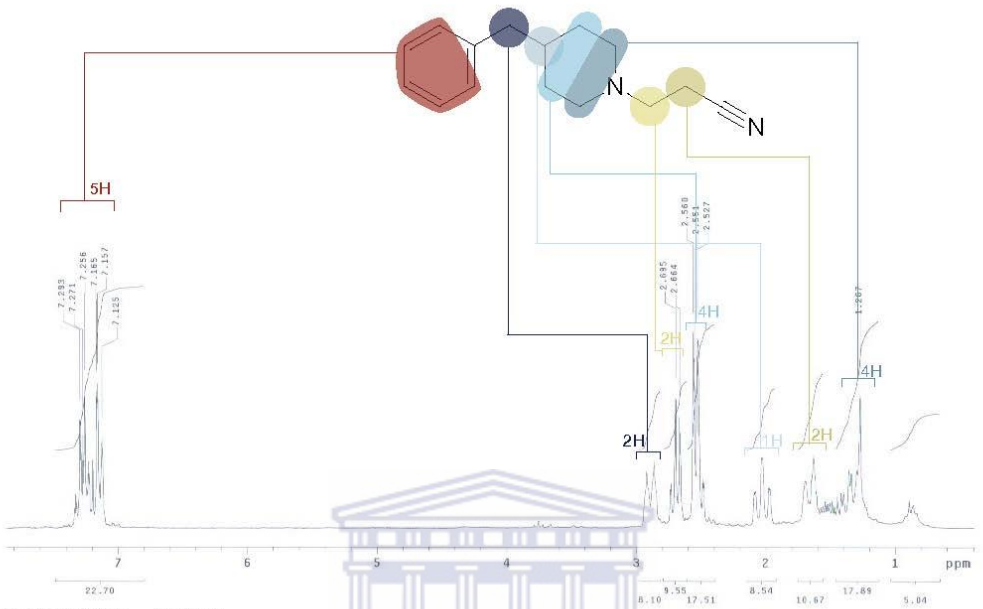


Nitrile Intermediate
(2-(4-BENZYLPIPERAZIN-1-YL)ETHANAMINE



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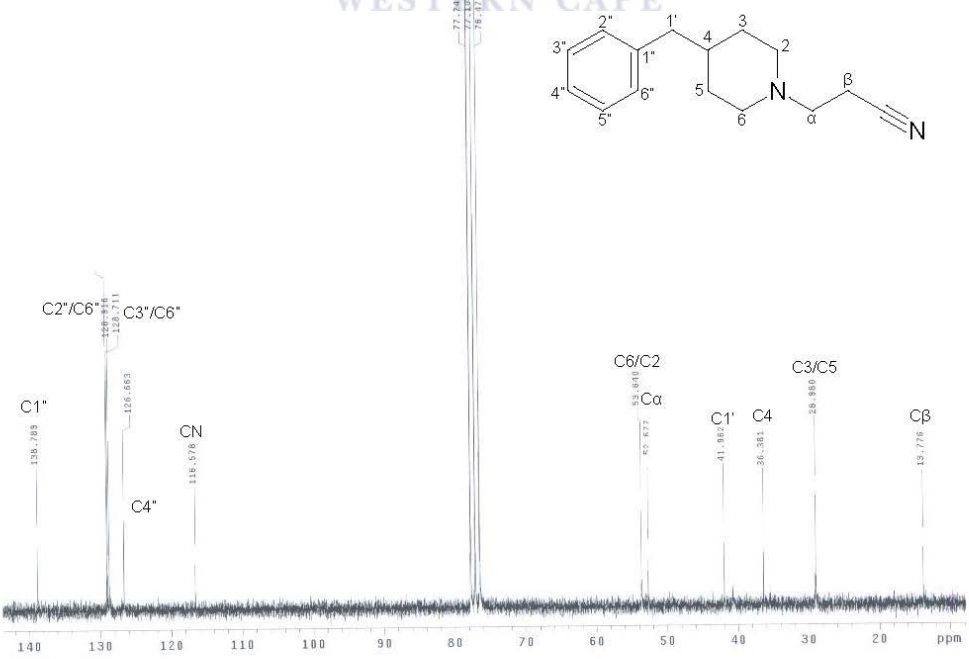

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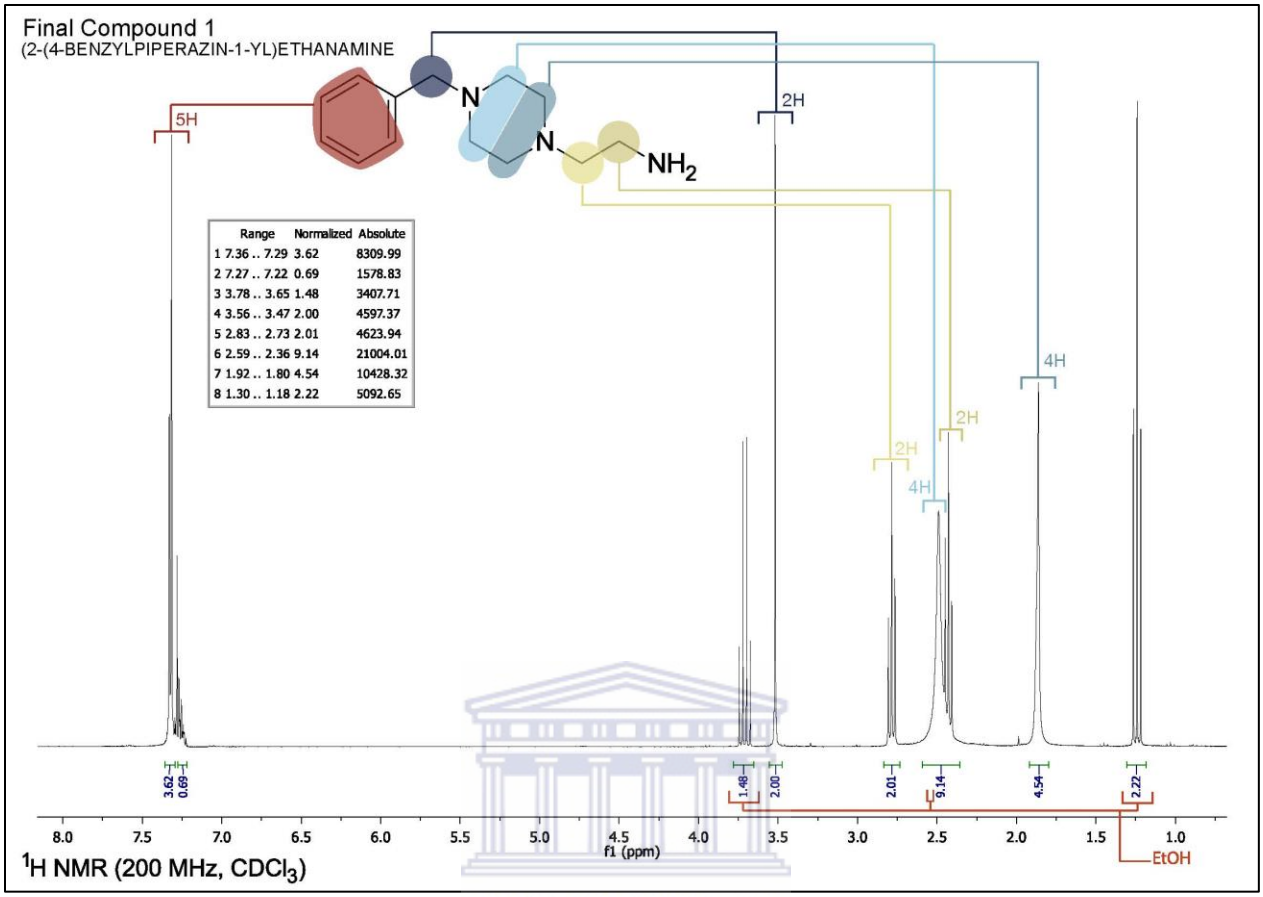

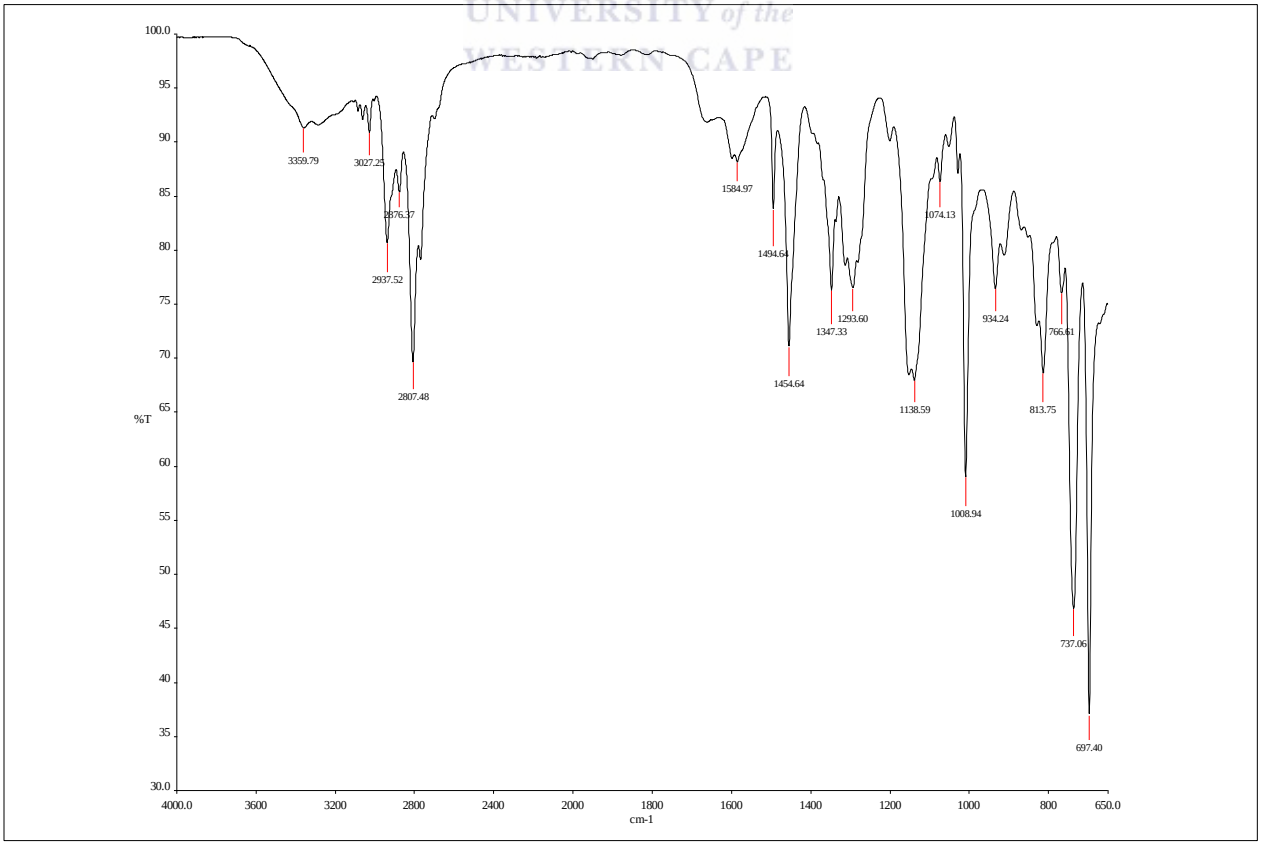

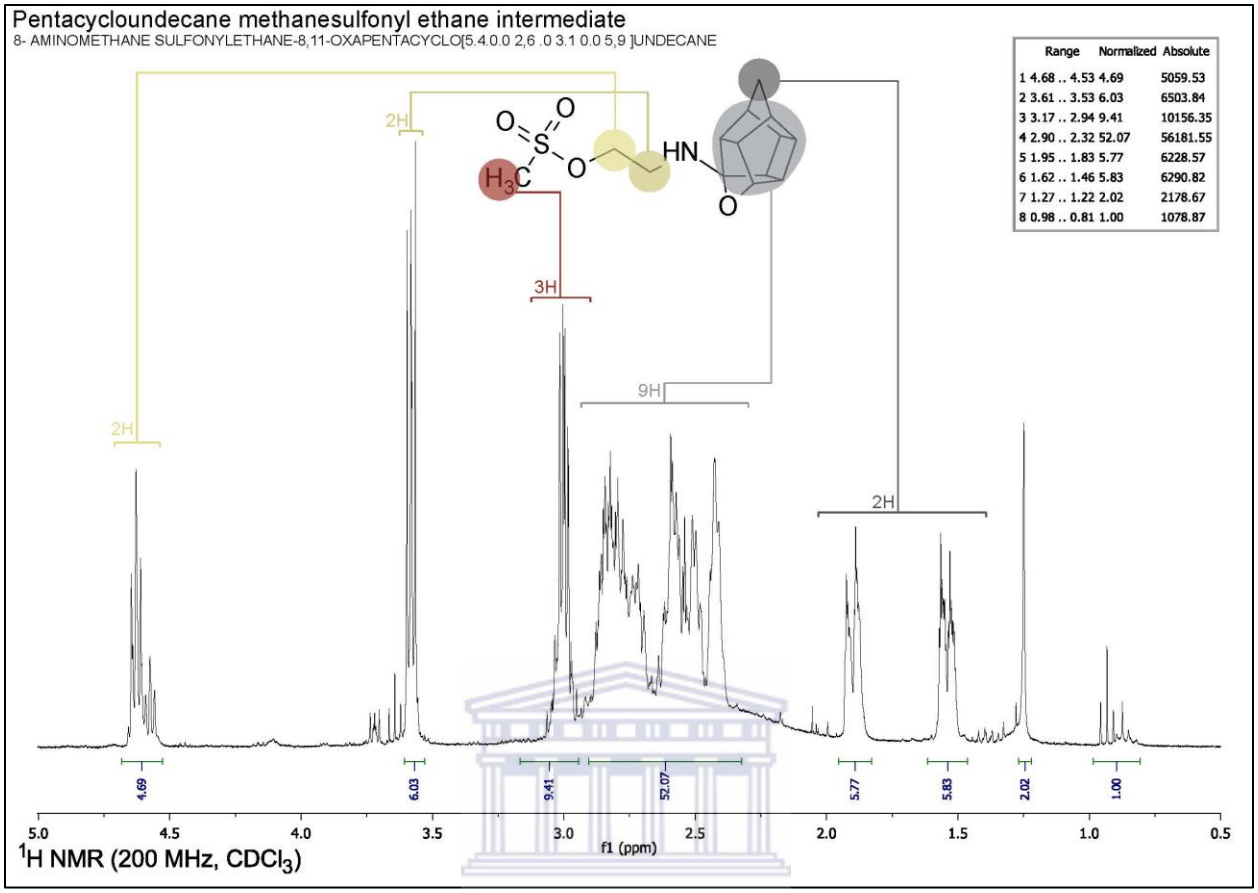

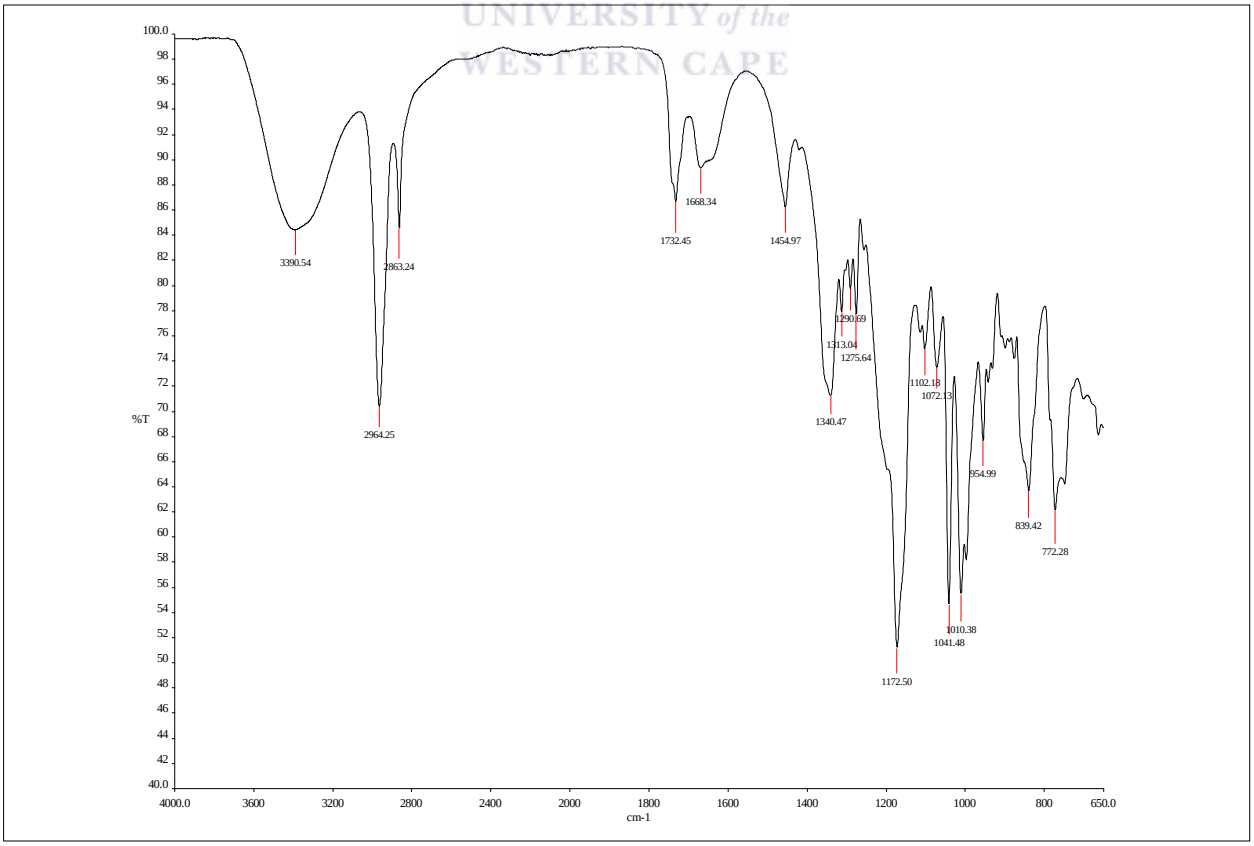

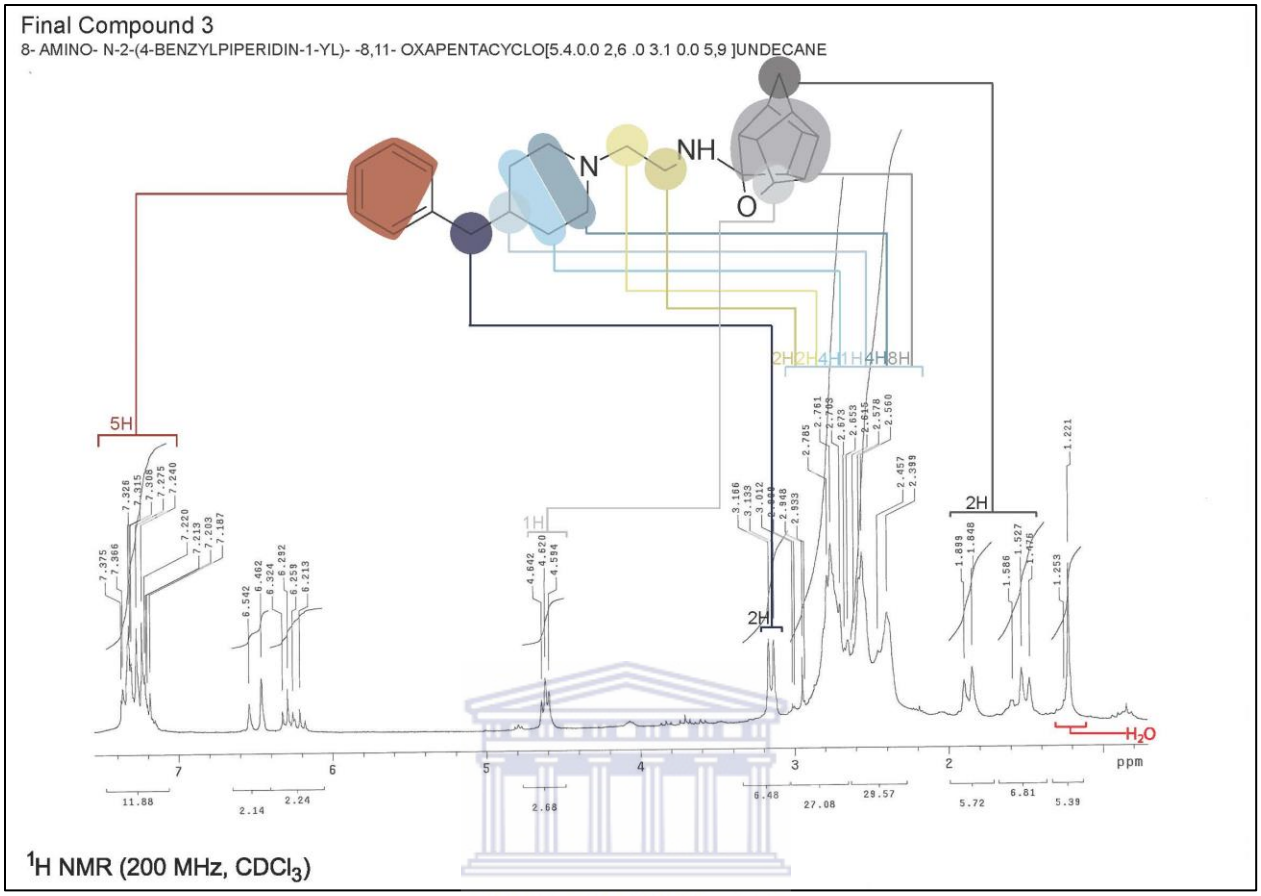

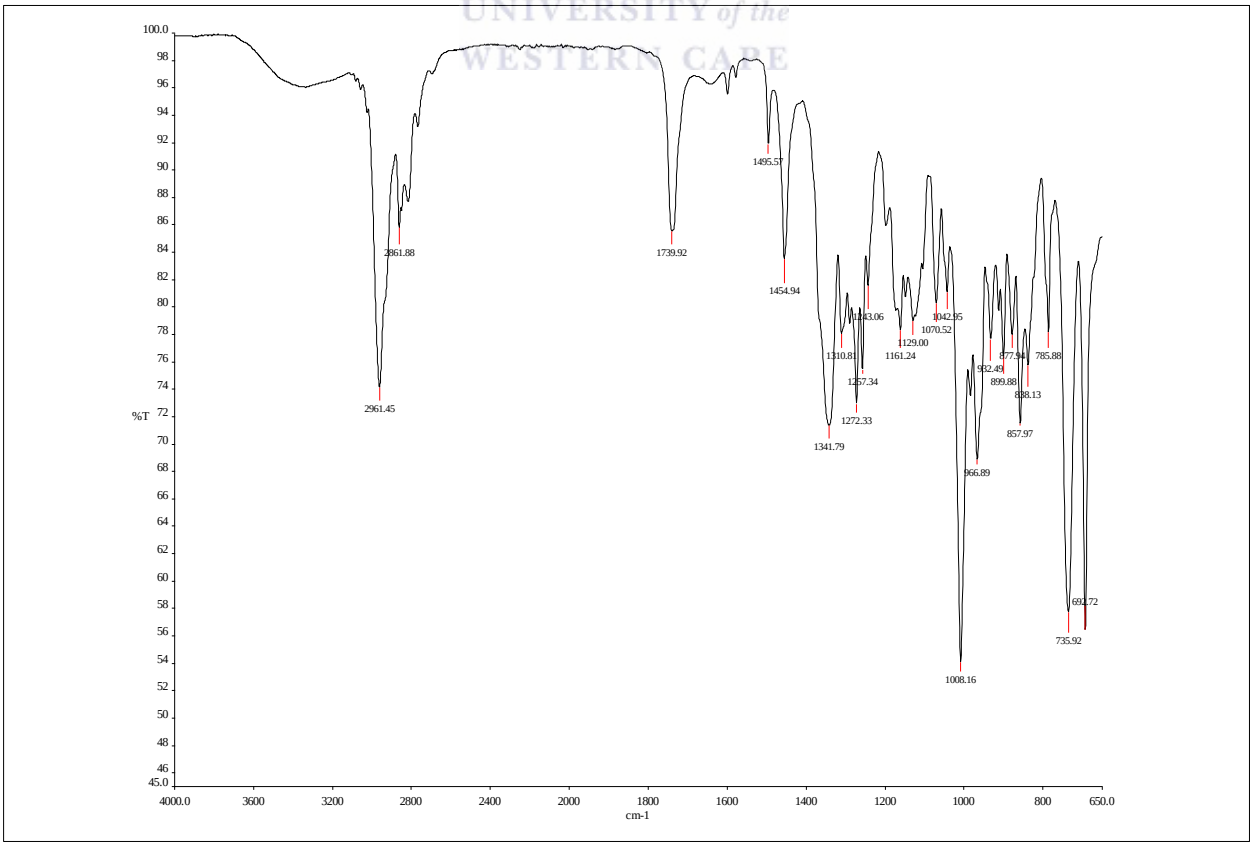

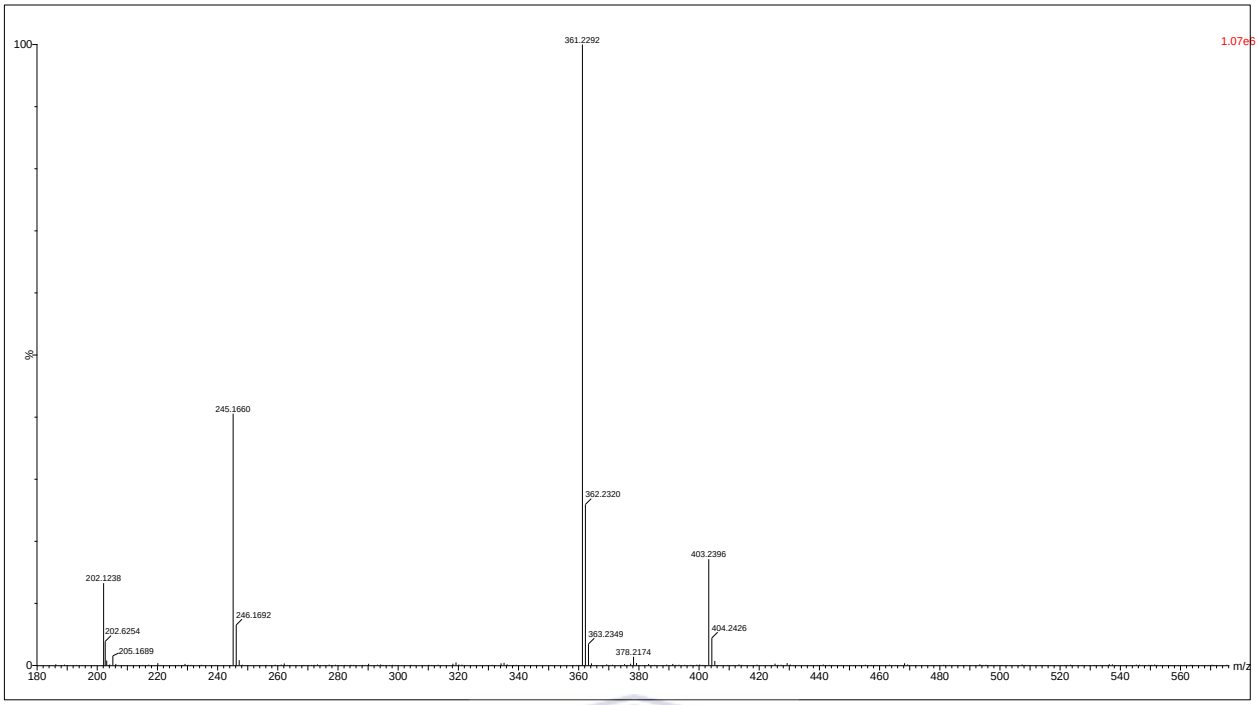

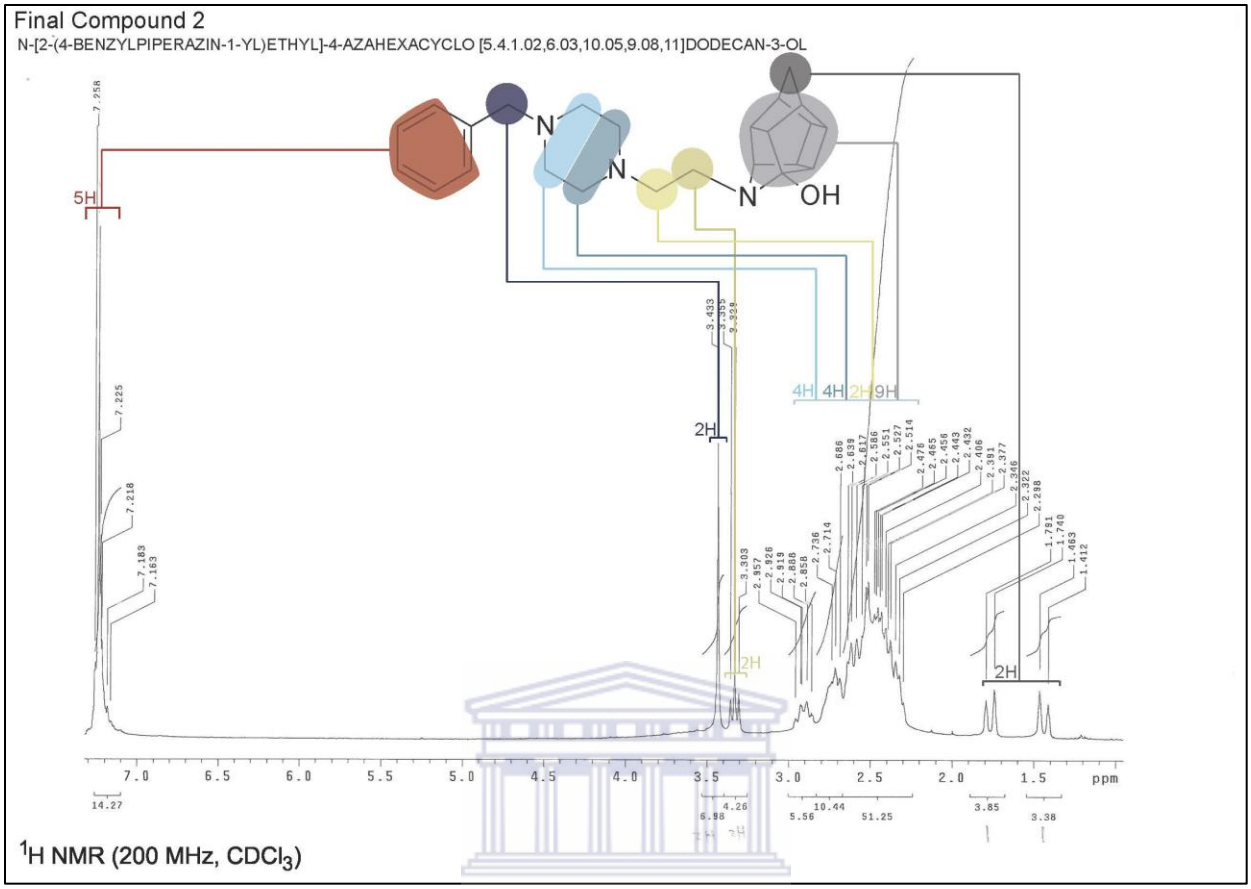

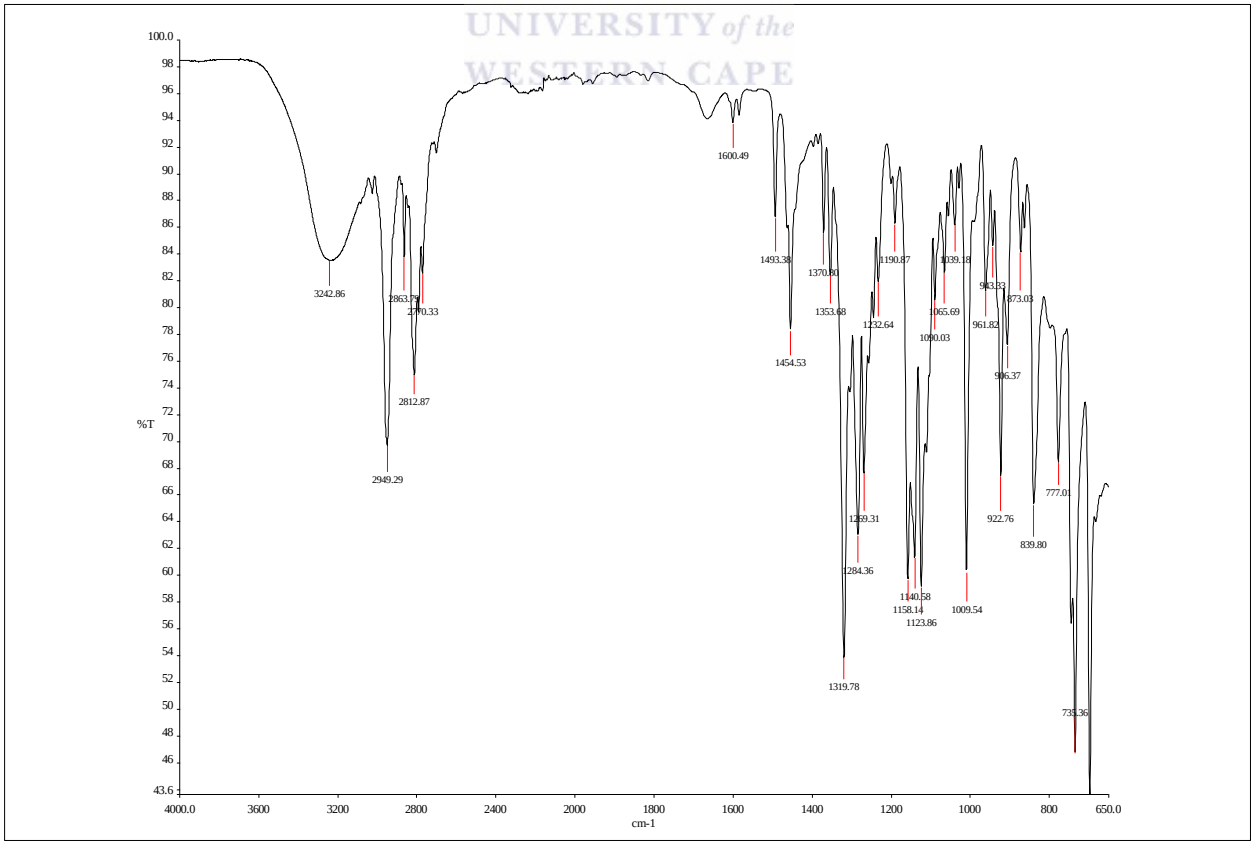

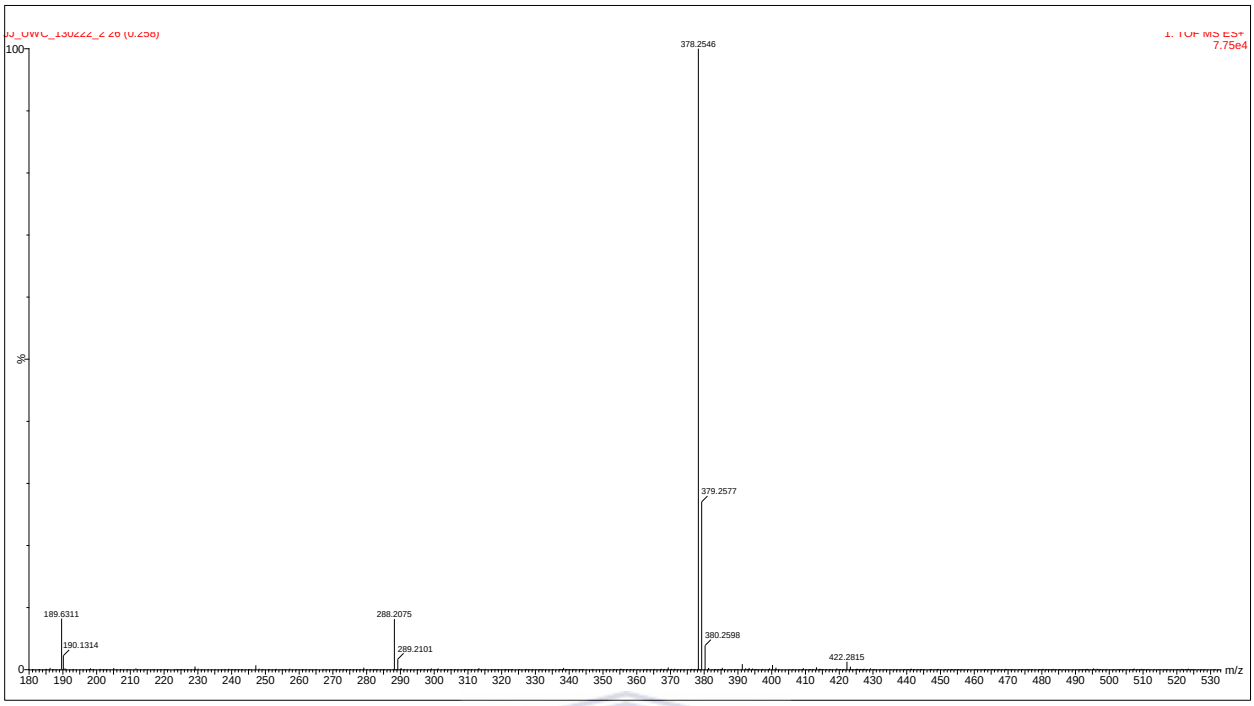

ANNEXURE B – FLUORESCENT IMAGING PROTOCOL

Calcium measurement with FURA-2/AM using SK-N-SH neuroblastoma cells

The objectives of the following assay are to evaluate novel test compounds effect on voltage gated calcium channels and *N*-Methyl-D-aspartic acid receptor channels. The test compounds were designed to show sigma receptor activity and calcium flux effects will be valuable to determine if the compounds are sigma agonists or antagonists.

Two subclasses of sigma receptors have been classified; sigma 1 and sigma 2. The sigma 1 receptor agonists increase intracellular calcium by increasing the efflux of endoplasmic reticulum calcium, specifically via IP_3 that regulate calcium signalling from the endoplasmic reticulum to mitochondria. This calcium release has been shown to be from thapsigargin-sensitive intracellular calcium stores and is mediated by the sigma 2 receptor. Sigma 1 receptor agonists increase *N*-Methyl-D-aspartic acid (NMDA) receptor channel mediated calcium influx (Hayashi & Su 2007). This increase is inhibited by sigma 1 receptor antagonist. Sigma receptor ligands also inhibit voltage gated calcium channels (VGCC), which seems to be mediated by the sigma 2 receptor (Monnet *et al*., 1990). Sigma 1 and sigma 2 receptor agonist cause a substantial rise in transient $[Ca^{2+}]_i$ (intracellular calcium concentrations) in neuroblastoma SK-N-SH cells. Prolonged exposure of cells to sigma receptor ligands was shown to result in latent and sustained rise in $[Ca^{2+}]_i$ with a pharmacological profile identical to the transient rise (Vilner & Bohen, 2000).

The test compounds are also structurally similar to the calcium channel inhibitor NGP1 – 01. Both contain pentacycloundecane and a benzyl group. NGP1 – 01 was first characterized and patented as a calcium channel antagonist in 1986 (Van der Schyf *et al*., 1986). Further research has shown that NGP1 – 01 is a lipophilic L-type channel blocker (Geldenhuys *et al.*, 2005).

Fura-2-acetoxymethyl ester, often abbreviated Fura-2AM, is a membrane-permeable derivative of the ratiometric calcium indicator Fura-2. It is extensively used in biochemistry to measure cellular calcium concentrations by fluorescence. When added to cells, Fura-2AM crosses cell membranes and once inside the cell, the acetoxymethyl groups are removed by cellular esterases. Removal of the acetoxymethyl esters gives Fura-2, the pentacarboxylate calcium indicator. Fura-2 when bound to calcium undergoes a shift in absorption from 335 and 363 nm to 340 and 380 nm. Measurement of Ca^{2+}-induced fluorescence at both 340 nm and 380 nm allows for calculation of calcium concentrations based 340/380 ratios. The use of the ratio automatically cancels out certain variables such as local differences in Fura-2 concentration or cell thickness that would otherwise lead to artefacts when attempting to image calcium concentrations in cells (Grynkiewicz *et al.*, 1985).

Human SK-N-SH neuroblastoma cells express sigma 1 and sigma 2 receptors with similar pharmacological profiles to those of rodent–derived tissue, although sigma 2 receptors exhibit some affinity differences that might suggest heterogeneity or species difference (Vilner & Bowen *et al.,* 2000). It is well established that SK-N-SH cell cultures consist of two different cell types, neuron-like and epithelium-like cells. The two cell types are easily distinguishable using phase contrast microscopy on the basis of size and shape (Ross *et al*., 1983).

Novel compounds will be evaluated for calcium channel modulation through VGCC and/or NMDAR calcium channels. The compounds' effect on intracellular calcium in the presence of sigma agonists and antagonist will also be evaluated.

Adapted methods described by Vilner & Bowen, 2000 and Larsson *et al.,* 2002 will be performed.

Materials

CELL CULTURE

SK-N-SH: Neuroblastomas from neural tissue

Fura-2/AM: 2.5 µg/mL in DBPS

Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% foetal bovine serum

1% Penicillin/Streptomycin: (Pen/Strep) 10 000 units in 100 mL stock

0.1% Fungizone: 2.5 µg/mL units stock

BUFFER SOLUTIONS

Dulbecco's phosphate buffered saline (DPBS; 136.9 mM NaCl, 2.68 mM KCl, 0.49 mM $MgCl_2$, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.904 mM $CaCl_2$, 5.55 sulphate, pH 7.2)

Cell Dissociation Solution Non-enzymatic 1x: Prepared in phosphate buffered saline without calcium and magnesium.

DEPOLARISATION SOLUTIONS

KCl depolarising solution in (mM): 55 mM KCl in normal DPBS

NMDA and glycine: 0.1 M stock solution. When needed dissolve 1 µL of stock solution in 1 mL DPBS solution to produce 100 µM.

STOCK SOLUTIONS

Cadmium Chloride (VGCC blocker): 100 µM, 50 µM, 10µM, 1 µM and 0.1 µM stock solutions in DMSO

MK – 801 (NMDAR channel antagonist): 100 µM, 50 µM, 10µM, 1 µM and 0.1 µM stock solutions in DMSO

BD 1047 (Selective sigma 1 antagonist): 100 µM, 50 µM, 10µM, 1 µM and 0.1 µM stock solutions in DMSO

PRE – 084 (Selective sigma 1 agonist): 100 µM, 50 µM, 10µM, 1 µM and 0.1 µM stock solutions in DMSO

PB 28 (Selective sigma 2 agonist): 100 µM, 30 µM, 50 µM, 10µM, 1 µM and 0.1 µM stock solutions in DMSO

Test Compounds: 100 µM, 50 µM, 10µM, 1 µM and 0.1 µM stock solutions in DMSO

Methods

THE SK-N-SH CELL CULTURE

The SK-N-SH line is cultivated in Dulbecco's Modified Eagle's Medium (DMEM) enriched 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). The cells are incubated at 37 °C in a 5% CO_2 and 95% O_2 humidified atmosphere. Once it forms a confluent monolayer, it is washed in Dulbecco's phosphate buffer solution (DPBS) and dispersed with cell dissociation solution. Cells are harvested by centrifuging and resuspending in DMEM/10% FBS at a density of 50 000 to 100 000 cells/ml.

The SK-N-SH cells are developed to be 90% enriched in the neuron-like cell type. To achieve this, mixed cells are cultured to 60 – 70% confluence. After decanting the medium, the culture is washed twice with DBPS. Non-enzymatic cell dissociation solution is added to the culture for 3 – 5 min. Because neuron-like cells lie on the epithelial-like cells, they detach more easily and earlier. The detached cells are collected and centrifuged (2000 rpm, 5 – 7 min), and the cell pellet is resuspended and replated in fresh medium. The cells are again allowed to grow to 60 – 70% confluence, and the procedure is repeated. Cells are frozen in 90% medium/10% DMSO. After reculturing, the cells can be used for experiments. Cultures prepared in this way should consist of 90 – 95% neuron-like cells (Vilner & Bowen, 2000).

MEASUREMENT OF INTRACELLULAR FREE CALCIUM CONCENTRATION

Calcium indicator Fura - 2 is used to measure changes in $[Ca^{2+}]_i$ at 24 °C on cells grown to 70 – 80% confluence (Grynkiewicz *et al*. 1985). Cells are incubated with 2.5 µM Fura – 2/AM in DPBS for 30 – 40 min, and then washed twice in DBPS and kept for an additional 10 min to ensure complete hydrolysis of Fura - 2/AM to the ratiometric indicator Fura - 2. DPBS is renewed for a final time and $[Ca^{2+}]_i$ imaging or spectrometry can be performed. Background or auto-fluorescence is measured by cells not labelled with Fura – 2 but with 0.1% DMSO present to replicate test conditions (Larson *et al*., 2002). The baseline reading of this measurement is auto-fluorescence and is repeated before each assay. Compounds that are to be incubated with the cell line are co-incubated with the Fura - 2 at this stage.

INCUBATING SK-N-SH WITH TEST COMPOUNDS

Prepare 0.1 M, 0.05 M, 0.01 M, 0.001, 0.0001 M stock solutions of relevant test compounds in solvent DMSO. When needed dissolve 1 µL of stock solution in 1 mL DPBS solution to produce 100 µM, 50 µM, 10µM, 1 µM and 0.1 µM solutions of test compounds respectively in a 0.1% DMSO final concentration.

RECORDING PARAMETERS

Fura – 2/AM

Excitation	340/380 nm
Emission	510 nm
Temperature	24 °C

ASSAYS TO BE PERFORMED

Voltage gated calcium channel (VGCC) inhibition assays and NMDAR calcium channel inhibition assays are well established (Joubert *et al*., 2011).

Standard radioligand binding studies can show affinity for the sigma receptor but cannot show the test compounds' activity or potency on a specified system. Assays to evaluate sigma 1 and 2 specific agonist alone and in combination with sigma antagonist will be used to evaluate the calcium altering properties of the sigma receptor in SK-N-SH. This will provide a pharmacological profile for sigma agonist or antagonist, which can then be compared to the properties of the test compounds. This will help determine if the test compounds are more sigma agonist or antagonist like i.e. point to their activity and possibly show whether they are sigma 1 or 2 receptor specific.

Various concentrations of test compounds and reference compounds will be tested. The IC_{50} values of the test compound can therefore be evaluated i.e. pointing to their potency when compared to known

reference standards. Affinity could be calculated by applying the Cheng-Prusoff equation to IC_{50} values to obtain apparent K_i values but preferably these results should be compared to a more accurate radioligand binding studies, considering the test compounds could bind to multiple sites.

It would be ideal to measure the same transient and latent increase in intracellular calcium observed by Vilner & Bohen, 2000. A time dependant recording of transient calcium increase will require the relevant compounds to be administered at approximately 10 seconds into a continuous recording lasting 5 – 10 min. After 10 min a $[Ca^{2+}]_i$ measurement every 10 min for up to 70 min can be determined. Alternatively a non-time dependant study can be done. This will involve incubating the test compounds with the respective compounds being evaluated for 30 – 40 min, which will provide a representation of the latent $[Ca^{2+}]_i$ increase.

Accurate comparison is difficult as most commercially available sigma receptor ligands show activity on multiple sites and as there are significant differences in binding affinity between these ligands. Sigma 1 and 2 agonists PRE-084 and PB 28 will be used as reference standards. Sigma receptor antagonism will be evaluated with BD 1047, which is a sigma 1 specific antagonist. At high concentrations BD 1047 is also able to antagonise the sigma 2 receptor sites, but to a lesser extent than sigma 1 receptor antagonism.

PRE – 084 hydrochloride is a high affinity, selective sigma 1 agonist. Its K_i values are 2.2 and 13091 nM for sigma 1 and sigma 2 receptors respectively. It is selective over PCP receptors (IC_{50} > 100000 nM) and several other receptor systems (Su *et al.,* 1991).

BD 1047 dihydrobromide is a selective, putative sigma receptor antagonist. BD-1047 has >50-fold selectivity at sigma 1 over sigma 2 and also >100-fold selectivity over opiate, phencyclidine, muscarinic, dopamine, α_1- & α_2-adrenoceptor, 5-HT_1, and 5-HT_2 (Matsumoto *et al.,* 1995).

PB 28 dihydrochloride is a high affinity sigma 2 receptor agonist with K_i values of 0.8 and 15.2 nM for sigma 2 and sigma 1 receptors respectively and displays minimal affinity at other receptors (Berardi *et al.,* 1996).

The assays will be divided into **Reference**, **Control**, **Comparison** and **Experimental**.

The **reference** assays will evaluate reference compounds at various concentrations to obtain their IC_{50} values in this test system to be compared to the test compounds.

The **control** assays will evaluate the cell cultures' normal functioning in resting and depolarised state.

The **comparison** assays will be used to evaluate similarities and differences between known reference compounds and test compounds to help classify the test compounds.

The **experimental** assays will specifically look at the test compounds' activity in the presence of various agonists and antagonists to determine its cellular properties.

Table 14: Assays to be performed for calcium fluorescent protocol

	Compound	Function	Time added	Assay Description
1	**Fura – 2 absent** 0.1% DMSO	Solvent	Incubated for 30 – 40 min	**Control**: Auto-fluorescence and DMSO effect on fluorescence **(Base line reading **)**
2	Test Compound*	Unknown	10 s into recording	**Experiment**: Inherent calcium activity in the absence of other compounds
	VGCC specific assay			
3	MK – 801 [100 µM] KCl depolarising reagent	NMDAR calcium channel antagonist Depolarise cells and activate calcium influx	Incubated for 30 – 40 min Incubated for 10 s into recording	**Control**: Measure maximum calcium influx via VGCC. Fluorescence measured is taken to be 100%. MK – 801 ensures selective evaluation of VGCC's by antagonising NMDAR
4	Cadmium Chloride* MK – 801 [100 µM] KCl depolarising reagent	VGCC blocker NMDAR calcium channel antagonist Depolarise cells and activate calcium influx	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording	**Reference**: VGCC inhibition
5	Test Compound* MK – 801 [100 µM] KCl depolarising reagent	Unknown NMDAR calcium channel antagonist Depolarise cells and activate calcium influx	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording	**Experiment**: VGCC inhibitory effect, expressed as percentage compared to 100% KCl depolarisation of control.
	NMDAR specific assay			
6	Cadmium Chloride [100 µM] NMDA/Glycine	VGCC blocker Activate NMDAR calcium influx	Incubated for 30 – 40 min 10 s into recording	**Control**: Measure maximum calcium influx via NMDAR. Fluorescence measured is taken to be 100%. Cadmium Chloride ensures selective evaluation of NMDAR by blocking

				all VGCC's.
7	MK – 801* Cadmium Chloride [100 µM] NMDA/Glycine	NMDAR calcium channel antagonist VGCC blocker Activate NMDAR calcium influx	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording	**Reference**: NMDAR calcium influx inhibition
8	Test Compound* Cadmium Chloride [100 µM] NMDA/Glycine	Unknown VGCC blocker Activate NMDAR calcium influx	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording	**Experiment**: NMDAR calcium influx inhibitory effect, expressed as percentage compared to 100% NMDA and glycine depolarisation of control.
	Selective sigma 1 receptor agonist assay			
9	PRE – 084*	Selective sigma 1 agonist	10 s into recording	**Reference**: Sigma 1 receptor effect on intracellular calcium
10	Test Compound* PRE – 084 [10 µM]	Unknown Selective sigma 1 agonist	Incubated for 30 – 40 min 10 s into recording	**Experiment**: Evaluate if test compound reverses effect of PRE-084 in a similar way to sigma receptor antagonist BD 1047.
11	Cadmium Chloride [100 µM] PRE – 084 [10 µM]	VGCC blocker Selective sigma 1 agonist	Incubated for 30 – 40 min 10 s into recording	**Comparison**: Selective sigma 1 receptor effect on intracellular calcium if VGCC effect is cancelled
12	Cadmium Chloride [100 µM] Test Compound* PRE – 084 [10 µM]	VGCC blocker Unknown Selective sigma 1 agonist	Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Experiment**: Evaluate if test compound reverses effect of PRE-084 in a similar way to sigma receptor antagonist BD 1047.
13	MK – 801 [100 µM] PRE – 084 [10 µM]	NMDAR calcium channel antagonist Selective sigma 1 agonist	Incubated for 30 – 40 min 10 s into recording	**Comparison**: Selective sigma 1 receptor effect on intracellular calcium if NMDAR effect is cancelled

14	MK – 801 [100 µM] Test Compound* PRE – 084 [10 µM]	NMDAR calcium channel antagonist Unknown Selective sigma 1 agonist	Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Experiment**: Evaluate if test compound reverses effect of PRE-084 in a similar way to sigma receptor antagonist BD 1047.
15	Cadmium Chloride [100 µM] MK 801 [100 µM] PRE – 084 [10 µM]	VGCC blocker NMDAR calcium channel antagonist Selective sigma 1 agonist	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording	**Comparison**: Selective sigma 1 receptor effect on intracellular calcium if VGCC and NMDAR effect is cancelled
16	Cadmium Chloride [100 µM] MK 801 [100 µM] Test Compound* PRE – 084 [10 µM]	VGCC blocker NMDAR calcium channel antagonist Unknown Selective sigma 1 antagonist	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Experiment**: Evaluate if test compound reverses effect of PRE-084 in a similar way to sigma receptor antagonist BD 1047.
	Selective sigma 2 receptor agonist assay			
17	PB 28*	Selective sigma 2 agonist	10 s into recording	**Reference**: Sigma 2 receptor effect on intracellular calcium
18	Test Compound* PB 28 [30 µM]	Unknown Selective sigma 2 agonist	Incubated for 30 – 40 min 10 s into recording	**Experiment**: Evaluate if test compound reverses effect of PB 28 in a similar way to sigma receptor antagonist BD 1047.
19	Cadmium Chloride [100 µM] PB 28 [30 µM]	VGCC blocker Selective sigma 2 agonist	Incubated for 30 – 40 min 10 s into recording	**Comparison**: Selective sigma 2 receptor effect on intracellular calcium if VGCC effect is cancelled
20	Cadmium Chloride [100 µM] Test Compound*	VGCC blocker Unknown	Incubated for 30 – 40 min 10 s into recording	**Experiment**: Evaluate if test compound reverses effect of PB 28 in a similar way to sigma receptor antagonist BD 1047.

	PB 28 [30 µM]	Selective sigma 2 agonist	10 s into recording	
21	MK – 801 [100 µM] PB 28 [30 µM]	NMDAR calcium channel antagonist Selective sigma 2 agonist	Incubated for 30 – 40 min 10 s into recording	**Comparison**: Selective sigma 2 receptor effect on intracellular calcium if NMDAR effect is cancelled
22	MK – 801 [100 µM] Test Compound* PB 28 [30 µM]	NMDAR calcium channel antagonist Unknown Selective sigma 2 agonist	Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Experiment**: Evaluate if test compound reverses effect of PB 28 in a similar way to sigma receptor antagonist BD 1047.
23	Cadmium Chloride [100 µM] MK 801 [100 µM] PB 28 [30 µM]	VGCC blocker NMDAR calcium channel antagonist Selective sigma 2 agonist	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording	**Comparison**: Selective sigma 2 receptor effect on intracellular calcium if VGCC and NMDAR effect is cancelled
24	Cadmium Chloride [100 µM] MK 801 [100 µM] Test Compound* PB 28 [30 µM]	VGCC blocker NMDAR calcium channel antagonist Unknown Selective sigma 2 agonist	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Experiment**: Evaluate if test compound reverses effect of PB 28 in a similar way to sigma receptor antagonist BD 1047.
	Sigma antagonist receptor assay			
25	BD 1047*	Selective sigma 1 antagonist	10 s into recording	**Reference**: Sigma 1 receptor antagonist effect on intracellular calcium
26	BD 1047 [100 µM] Test Compound*	Selective sigma 1 antagonist Unknown	Incubated for 30 – 40 min 10 s into recording	**Experiment**: Evaluate if BD 1047 reverses effect of test compound similar to PRE – 084.
27	Cadmium Chloride [100 µM]	VGCC blocker	Incubated for 30 – 40 min	**Comparison**: Selective sigma 1 receptor antagonist effect on intracellular calcium if

	BD 1047 [100 µM]	Selective sigma 1 antagonist	10 s into recording	VGCC effect is cancelled
28	Cadmium Chloride [100 µM] BD 1047 [100 µM] Test Compound*	VGCC blocker Selective sigma 1 antagonist Unknown	Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Experiment**: Evaluate if BD 1047 reverses effect of test compound similar to PRE – 084.
29	MK – 801 [100 µM] BD 1047 [100 µM]	NMDAR calcium channel antagonist Selective sigma 1 antagonist	Incubated for 30 – 40 min 10 s into recording	**Comparison**: Selective sigma 1 receptor antagonist effect on intracellular calcium if NMDAR effect is cancelled
30	MK – 801 [100 µM] BD 1047 [100 µM] Test Compound*	NMDAR calcium channel antagonist Selective sigma 1 antagonist Unknown	Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Experiment**: Evaluate if BD 1047 reverses effect of test compound similar to PRE – 084.
31	Cadmium Chloride [100 µM] MK 801 [100 µM] BD 1047 [100 µM]	VGCC blocker NMDAR calcium channel antagonist Selective sigma 1 antagonist	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording	**Comparison**: Selective sigma 1 receptor antagonist effect on intracellular calcium if VGCC and NMDAR effect is cancelled
32	Cadmium Chloride [100 µM] MK 801 [100 µM] BD 1047 [100 µM] Test Compound*	VGCC blocker NMDAR calcium channel antagonist Selective sigma 1 antagonist Unknown	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Experiment**: Evaluate if BD 1047 reverses effect of test compound similar to PRE – 084.
	Sigma receptor antagonist and sigma 1 selective agonist assay (Optional)			
33	BD 1047 [100 µM]	Selective sigma 1 antagonist	10 s into recording	**Comparison**: Antagonist reversal of sigma 1 receptor effect on intracellular calcium.

	PRE – 084 [10 µM]	Selective sigma 1 agonist	10 s into recording	
34	Cadmium Chloride [100 µM] BD 1047 [100 µM] PRE – 084 [10 µM]	VGCC blocker Selective sigma 1 antagonist Selective sigma 1 agonist	Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Comparison**: Antagonist reversal of selective sigma 1 receptor effect on intracellular calcium if VGCC effect is cancelled
35	MK – 801 [100 µM] BD 1047 [100 µM] PRE – 084 [10 µM]	NMDAR calcium channel antagonist Selective sigma 1 antagonist Selective sigma 1 agonist	Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Comparison**: Selective sigma 1 receptor effect on intracellular calcium if NMDAR effect is cancelled
36	Cadmium Chloride [100 µM] MK 801 [100 µM] BD 1047 [100 µM] PRE – 084 [10 µM]	VGCC blocker NMDAR calcium channel antagonist Selective sigma 1 antagonist Selective sigma 1 agonist	Incubated for 30 – 40 min Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Comparison**: Antagonist reversal of selective sigma 1 receptor effect on intracellular calcium if VGCC contribution and NMDAR effect is cancelled
	Sigma receptor antagonist and sigma 2 selective agonist assay (Optional)			
37	BD 1047 [100 µM] PB 28 [30 µM]	Selective sigma 1 antagonist Selective sigma 2 agonist	Incubated for 30 – 40 min 10 s into recording	**Comparison**: Antagonist reversal of sigma 2 receptor effect on intracellular calcium.
38	Cadmium Chloride [100 µM] BD 1047 [100 µM] PB 28 [30 µM]	VGCC blocker Selective sigma 1 antagonist Selective sigma 2 agonist	Incubated for 30 – 40 min 10 s into recording 10 s into recording	**Comparison**: Antagonist reversal of selective sigma 2 receptor effect on intracellular calcium if VGCC effect is cancelled
39	MK – 801	NMDAR calcium	Incubated for	**Comparison**: Selective sigma 2 receptor

	[100 µM]	channel antagonist	30 – 40 min	effect on intracellular calcium if NMDAR
	BD 1047	Selective sigma 1	10 s into	effect is cancelled
	[100 µM]	antagonist	recording	
	PB 28	Selective sigma 2	10 s into	
	[30 µM]	agonist	recording	
40	Cadmium Chloride	VGCC blocker	Incubated for	**Comparison**: Antagonist reversal of selective
	[100 µM]		30 – 40 min	sigma 2 receptor effect on intracellular
	MK 801	NMDAR calcium	Incubated for	calcium if VGCC contribution and NMDAR
	[100 µM]	channel antagonist	30 – 40 min	effect is cancelled
	BD 1047	Selective sigma 1	10 s into	
	[100 µM]	antagonist	recording	
	PB 28	Selective sigma 2	10 s into	
	[30 µM]	agonist	recording	

* Done at various concentrations [100 µM] [50 µM] [10µM] [0.1 µM] [0.01 µM] to determine dose response curves, in a log-scale, using Prism 4.0.

** A baseline reading is performed before each replicate procedure of compound to be screened.

Treatments are repeated three times on different cell preparations with three determinations in each replicate.