Chapter 9

Additional Research Outputs

The following is a depiction of research outputs directly and indirectly pertaining to the previous chapters that serves to further illustrate the potentail of fluorescent polycyclic compounds and other compounds in the study of neurodegeneration. Part one of this chapter presents a series of posters while part two consists of additional papers accepted for publication not included in this thesis.

The poster presentations represent additional research into the design and development of fluorescent polycyclic ligands and therapeutic agents for neurodegenerative disorders.

Part 1 - List of posters and presentation details:

- Poster 1: Joubert, J., Van Dyk, S., Malan, S. F. *Synthesis and evaluation of fluorescent polycyclic nitric oxide synthase (NOS) enzyme ligands* (2008), 29th APSSA conference, Hunters Rest, Rustenburg, North-West (Poster session, September 2008).
- Poster 2: Joubert, J., Van Dyk, S., Malan, S. F. *Novel fluorescent polycyclic ligands for mechanistic insights into neurodegeneration and neuroprotection* (2009), iThemba Pharmaceuticals Launch Symposium, Hilton Hotel, Santon, Gauteng (Poster session, May 2011).

Poster 1 describes the synthesis and evaluation of fluorescent ligands that may be used in the study of the NOS enzyme (expansion of Article 1, Chapter 5). Additional synthetic routes summarised in this poster, include the design and synthesis of additional pentacycloundecane fluorescent compounds (Table 3, Poster 1) as well as a pilot molecular modelling study into the NOS enzyme isoforms (Figures 7 and 8, Poster 1) that revealed interesting results, indicating that the novel compounds, especially the longer linkage compounds, may have the ability to inhibit nNOS selectively over iNOS and eNOS. This assumption was made based on the dock scores of the test compounds compared to the respective isoforms and their binding interactions with the NOS protein structure.

Poster 2 elaborated on the research done in articles 2 and 3 (Chapters 6 and 7). The poster includes additional synthetic routes of the adamantane and pentacycloundecane fluorescent

compounds (Figures 6, 7 and 8, Poster 2) and the design and development of the NMDA receptor and VGCC assays. Further development of the pentacycloundecane fluorescent derivatives in particular are currently underway. Both the improvement of synthetic procedures and biological evaluations are explored to establish the potential of these novel compounds as fluorescent ligands, in order to gain mechanistic insights into neurodegenerative disorders and aid in the design of novel neuroprotective therapeutic agents.

Part two of this chapter includes two further reseach papers that were submitted an accepted for publication during the course of this PhD study period.

Part 2 - List of additional articles accepted for publication (Both these papers will be made available upon request):

- Article A: Hendrik, J.R. Lemmer, Jacques Joubert, Sandra van Dyk, Francois, H. van der Westhuizen, Sarel, F. Malan. *S-Nitrosylation and attenuation of excessive calcium flux by pentacycloundecane derivatives*. Article in Press, Medicinal Chemistry, Bentham Publishers (June 2011).
- Article B: Jane Greeff, Jacques Joubert, Sarel F. Malan, Sandra van Dyk. *Antioxidant properties of 4-quinolones and structurally related flavones*. Bioorganic and Medicinal Chemistry 2012, 20(12), 809-818.

Article A describes the synthesis of novel nitro- and nitrate-pentacycloundecane polycyclic compounds evaluated for VGCC blocking activity and the ability to *S*-nitrosylate the NMDA receptor resulting in enhanced NMDA receptor antagonistic activity. The direct involvement in this study was the design and conduction of VGCC and NMDA receptor assays and structure elucidation of the synthesised compounds. The article was accepted for publication in Medicinal Chemistry (Bentham Publishers).

Article B was accepted for publication in Bioorganic and Medicinal Chemistry (Elservier). This study investigated the antioxidant activity of a series of quinolones and structurally related flavones as potential therapeutic agents for neurodegenerative disorders. The major contribution made to this paper was *in silico* molecular modeling to determine the bioavailability, blood-brain barrier permeability, physical-chemical properties and toxicity profiles of the series of neuroprotective antioxidants.

Synthesis and Evaluation of Fluorescent Polycyclic Nitric Oxide Synthase (NOS) Enzyme Ligands

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Introduction

Background

The medicinal potential of polycyclic compounds was realised with the discovery that amantadine ( ) exhibits antiviral activity. Subsequent to this discovery, it was found that amantadine could be benificial to patients with Parkinson's disease. It expresses its anti–Parkinsonian activity by increasing extracellular dopamine (DA) levels *via* DA re-uptake inhibition[1] or DA release and NMDA receptor antagonism.[2] Interest in the pharmacology of polycyclic cage amines was further stimulated when the dimethyl derivative of amantadine, memantine ( ), was found to be a clinically well tolerated NMDA receptor antagonist.[3] Structural similarity exists between the polycyclic cage structure of adamantane amines and that of the pentacycloundecylamines.[4] Pentacycloundecylamine derivatives ( ) are derived from pentacycloundecane-8,11-dione ( ), obtained from the intramolecular photocyclisation of the Diels Alder adduct of *p*-benzoquinone and cyclopentadiene.[5]

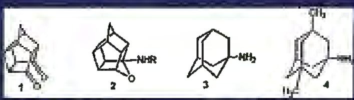


Figure 1. Structural similarities between the polycyclic compounds.[1]

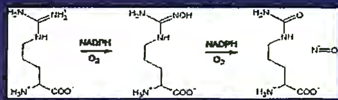


Figure 2. Reaction catalysed by NOS.[6]

Nitric oxide synthases (NOS)

NOS are multidomain proteins consisting of a heme containing catalytic oxygenase domain, a calmodulin binding linker, and a NADPH reductase domain that catalyzes the formation of NO using L-arginine, oxygen, and electrons (Figure 2). Three NOS isoforms (Table 1) have been identified sharing 50–60% sequence identity. These isoforms differ in cellular distribution, regulation and activity.[6]

Table 1. The three NOS enzyme isoforms and their roles.[7,8]

Name	Gene (s)	Category	Location	Functions
Neuronal NOS (nNOS or NOS 1)	NOS1	Constitutive, Ca^{2+} dependant	Nervous tissue	Cell communication, neurotransmission
Inducible NOS (iNOS or NOS 2)	NOS2A,B,C	Inducible, Ca^{2+} independant	Immune and Cardiovascular	Immune defense against pathogens
Endothelial NOS (eNOS or NOS 3)	NOS3	Constitutive, Ca^{2+} dependant	Endothelium	Vasodilation

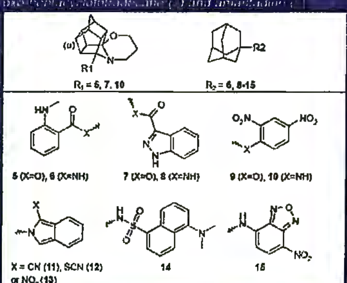
Aim and objectives

The aim of this study was to synthesise a series of novel fluorescent polycyclic derivatives for evaluation on the NOS enzyme. Polycyclic structures have been shown to increase the pharmacokinetic and pharmacodynamic properties of drugs[4] and were conjugated to fluorescent moieties to increase the blood brain barrier permeability and selectivity of the compounds. The NOS system where fluorescent techniques and fluorescent inhibitors can be used for detecting the biophysical properties of enzyme-ligand interactions during binding was targeted.

Synthesis

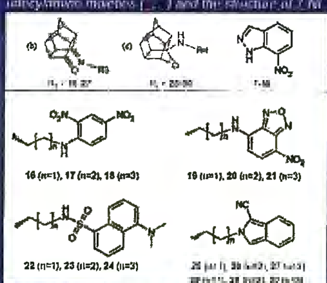
Fluorescent compounds for this study were selected on the basis of their spectroscopic properties and structural similarities to 7-Nitroindazole (7-NI, Table 3) to exhibit NOS inhibition. It is hypothesised that the novel fluorescent compounds may have the ability to selectively inhibit nNOS, as the compounds have structural similarities to 7-NI, which is a selective nNOS inhibitor.[9] The fluorescent compounds were synthesised and conjugated to 3-hydroxy-4-aza-8-oxoheptacyclotetradecane ( ), amantadine ( ) (Table 2) and pentacycloundecylamine moieties (,) (Table 3).

Table 2. Fluorescent derivatives of 3-hydroxy-4-aza-8-oxoheptacyclotetradecane ( ) and amantadine ( )



*The anthranilic and indazole structures were obtained through the intermediate complexes with CDI and DCC and yielded the fluorescent esters ( ) and amides ( ). The dinitrobenzene complex was obtained through amination ( ) and etherification ( ). The isoindoles were synthesised via reaction of o-phthalaldehyde [illegible] of the appropriate sodium salt to [illegible] (, ,). Compounds [illegible] and [illegible]

Table 3. Fluorescent derivatives of the pentacycloundecylamine moieties (,) and the structure of 7-NI



*The pentacycloundecylamine derivatives were conjugated to the selected fluorophores with appropriate diamino linkers differing in chain length. These linkers will give sufficient structural flexibility and increased water solubility – leading to a postulated increase in activity. The fluorophores were synthesised by amination and were conjugated to the cage moieties by a dehydration reaction to form the imines ( ). Compounds [illegible] were obtained by reductive amination with $NaBH_4$.

The compounds were obtained as oils or amorphous solids from chromatography or were crystallised from organic solvents and the structures were confirmed using ^1H, ^{13}C and COSY NMR and MS. A Cary Eclipse® fluorescence spectrophotometer was used for fluorescence measurements at a concentration of 10^{-5} M in absolute ethanol at 25 C.

Biological evaluation

The oxyhaemoglobin (oxyHb) assay[10] was employed to determine the activity of the novel compounds at an enzymatic level of NOS. The method is based on the reaction of oxyhaemoglobin (oxyHb) with NO to form methaemoglobin (metHb) and nitrate (equation 1), a reaction which also accounts largely for the inhibitory effect of haemoglobin on the biological effects of endogenously formed or exogenously applied NO.[11]

$$HbO^{2} + NO \rightarrow metHb + NO^{3-} \quad (1)$$

In order to determine the amount of NO formed, the change in absorbance difference between 401 and 421 nm was measured during the initial linear phase of the reaction (Figure 3). If the change in absorbance at 401 nm is plotted against time and the change in absorbance over time at 421 nm is subtracted, the slope of this resulting curve is an indication of the increase in molar amount of metHb and is equivalent to the molar amount of NO generated (Figure 4). Compounds (-) were tested for NOS activity.

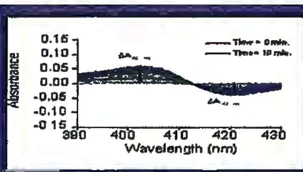


Figure 3. A spectrophotometric recording of compound [illegible]. Continuous scans between 390 nm and 430 nm were performed as the oxyHb was converted to metHb.

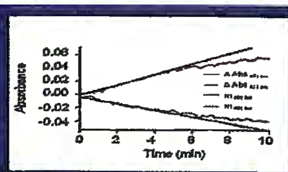


Figure 4. Change in absorbance at 401 and 421 nm vs time was calculated and the difference of the respective slope values [(mΔA(401 nm))-(mΔA(421 nm))] gave an indication of enzyme activity.

The inhibition curves of the compounds were superimposed on a single graph and the IC_{50} values were calculated. Compounds , , , and showed promise as NOS inhibitors. when compared to 7-NI (Figure 5), it is clear that none of the structures inhibit NOS as potently as 7-NI (IC_{50} = 0.111 μM). All the compounds however showed more potent inhibitory activity than aminoguanidine (IC_{50} = 19.41 μM) (Figure 5), which is reported in literature to be a selective iNOS inhibitor.[12] Compounds , and showed low or no inhibition (Figure 6). Compound proved to be the best inhibitor of the novel fluorescent compounds with a potent IC_{50} value of 0.291 μM. This 1-cyanoisoindole adamantane compound ( ) showed a ten fold increase in activity when compared to the 1-thiocyanoisoindole adamantane ( ) and 1-nitroisoindole adamantane ( ). All compounds showed an acceptable difference of excitation and emission wavelengths and Stoke shifts varied from 29 to 160 nm. Compound , the second most potent tested compound showed a Stoke shift of 80 nm and compound had the highest stoke shift of 160 nm.

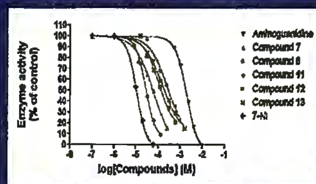


Figure 5. Superimposed inhibition curves of compounds with increased NOS inhibition activities.

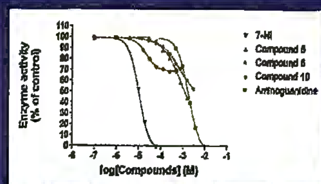


Figure 6. Superimposed inhibition curves of test compounds with low NOS inhibitory activity.

Molecular Modelling

The high level of amino acid conservation and structural similarity in the immediate vicinity of the substrate binding sites of the oxygenase domains of the NOS isoforms make the interpretation of the structural basis of inhibitor isoform specificity a challenge. Molecular modelling studies were carried out with published nNOS (pdb: 1QWC), iNOS (pdb: 1OWS) and eNOS (pdb: 2HX2)[13] crystal structures and the proposed inhibitors were docked by using the Ligandfit module of Discovery Studios 1.7® software, to identify the size and functional groups for optimum binding and selectivity to the catalytic sites of the NOS isoforms.

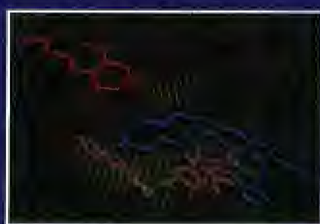


Figure 7. Ligandfit presentation of compound [illegible] ( ) docked into the active site cavity (yellowish). Biopterin is shown in [illegible] and heme in [illegible] for the nNOS protein structure.

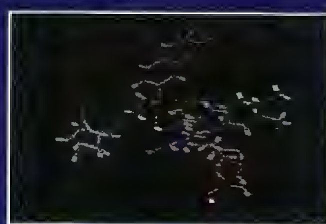


Figure 8. H-bond interactions (yellow dotted) between the synthesized compound [illegible] (top left) and the FMN co-factor for the nNOS protein structure.

The crystal structures suggest a rationale for isoform specificity that is rooted in isoform-specific residues outside the immediate active site but that nevertheless contribute to its shape. The active site is not rigid but displays some ligand induced conformational flexibility. The positioning of the cofactor H_4B, located at the isoform-specific interface between oxygenase monomers, seems to play an important role since it is involved in positioning the heme propionates, which interacts with the functional groups of the compounds. Prediction and rational design of inhibitors targeted against the active site may be achieved with compounds that are large enough ( ) to interact with isoform-specific residues in the substrate access channel or dimer interface and that fill the active site, thereby locking its conformation. It was found that the majority of the novel compounds fitted into the calculated binding pocket for the nNOS enzyme. Compounds showed higher dock scores than the smaller compounds (-), as predicted above. The compounds did bind to the other isoforms but with lower dock scores, this may be attributed to the structural similarities towards 7-NI (reported in literature to be a selective nNOS inhibitor).[9]

Conclusion

We have identified a series of fluorescent compounds with moderate to high affinity for the NOS enzyme, which may be utilized for further *in vitro* and *in vivo* studies using modern imaging techniques (e.g. confocal laser scanning microscopy, flow cytometry or multiphoton microscopy). These compounds thus have potential as useful pharmacological tools to investigate enzyme-ligand interactions in the quest for effective neuroprotective strategies. The potential of these novel fluorescent polycyclic structures as NOS inhibitors and the documented calcium channel modulation observed for selected cage structures,[4] indicate that these novel compounds may find application as multipotent drugs in neuroprotection. More structural and biochemical analysis including ligand binding affinity coupled with enzyme inhibition studies using the NOS isoforms is needed to assess the selectivity of the structures against the NOS isoforms.

References

1. [illegible]
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]
6. [illegible]
7. [illegible]
8. [illegible]
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SYNTHESIS, EVALUATION AND APPLICATION OF NOVEL FLUORESCENT POLYCYCLIC NMDA RECEPTOR AND CALCIUM CHANNEL LIGANDS

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Introduction

The N-methyl-D-aspartate receptor (NMDAR) has been suggested as a drug target through its involvement in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease.[1] Overstimulation of the NMDAR by an excess of the endogenous neurotransmitter glutamate during pathological conditions leads to excessive influx of calcium (Ca^{2+}) into neuronal cells resulting in cell death, a process known as excitotoxicity. Ca^{2+} entry through L-type Ca^{2+} channels also contributes to Ca^{2+} overload and mitochondrial disruption that lead to the recruitment or release of mediators responsible for the activation of an apoptotic cascade and ultimately, in cell death (Fig 1).[2] Excitotoxicity also leads to the activation of nitric oxide synthase (NOS). NeuronalNOS (nNOS) is physiologically activated by steroid hormones or neurotransmitters such as nitric oxide (NO), dopamine, glutamate and glycine that increase intracellular Ca^{2+} concentrations and leads to the formation of NO (a free redical) and cell death (Fig 1). It does so by synthesis of NO and L-citruline from the terminal nitrogen atom of L-arginine *via* the intermediate *NG* hydroxy-L-arginine (Fig 2). Overproduction of NO has been implicated in neurodegenerative diseases, convulsions and pain.[3]

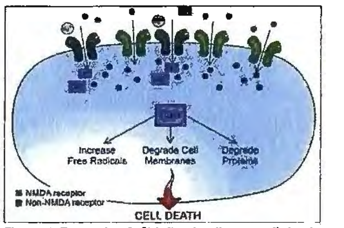

***Figure 1*: Excessive Ca^{2+} influx leading to cell death**

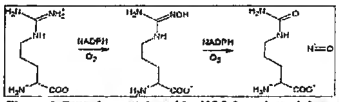

***Figure 2*: Reaction catalysed by NOS from L-arginine**

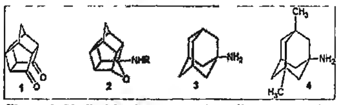

***Figure 3*: Similarities between polycyclic compounds**

Biological activity attributed to a class of polycyclic amine derivatives, the pentacycloundecylamines (**2**) (Fig 3), suggests possible neuroprotective abilities through NOS inhibition[4], modulation of voltage activated Ca^{2+} channels and interaction with NMDA receptor operated channels[5]. The pentacycloundecylamine derivatives show structural similarities to known NMDA antagonists [MK-801 (Fig 12), NGP-101 (Fig 11 and 12), amantadine (**3**) and memantine (**4**) – Fig 3, 11 and 12), L-type Ca^{2+} channel activity and NOS activity has also been described.[4,6] Although little is known about the mechanisms of CNS activity of the compounds it is postulated that these derivatives could be of therapeutic value in neurodegenerative disorders.

Aim and Objectives

The aim of this investigation is to provide fluorescent polycyclic ligands structurally related to known NMDA antagonists for use in directly quantifying ligand-receptor (NMDA) and/or ligand-enzyme interactions (nNOS). It is a further object to provide a method and reagents for use in determining neurological interactions, intracellularly and extracellularly. We aim to achieve greater insights into the neuroprotective mechanisms of these compounds, by means of fluorescent imaging.

Synthesis and Characterisation

The fluorescent moieties chosen for synthesis includes *N*-methylanthralate, Indazole, 1-Fluoro-2,4-dinitrobenzene, 1-Cyanoisoindole, Coumarin, Dansyl and NBD. These fluorescent structures were conjugated to the respective cage moieties, directly or by means of appropriate amino-linkers to provide the fluorescent polycyclic ligands with desired spectroscopic properties.

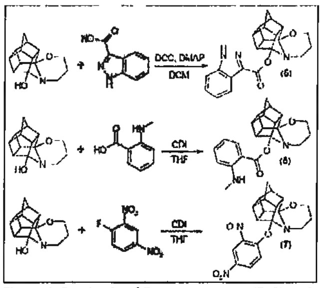

Figure 4*: Synthesis of fluorescent ester (5, 6) and ether (7) derivatives of tetradecane cage moiety

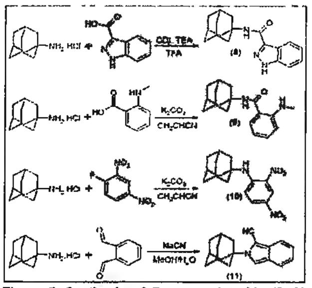

Figure 5*: Synthesis of fluorescent amide (8, 9) and amine (10, 11) amantadine derivatives

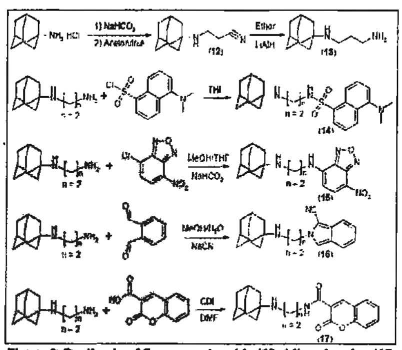

***Figure 6*: Synthesis of fluorescent amide (13, 14) and amine (15, 17, 18, 19) amantadine derivatives¥**

*N-methylantralate and indazole esters (**5**, **6**) and amides (**8**, **9**) were obtained through the intermediate complexes with CDI and DCC on reaction with the tetradecane cage (Fig. 4) and amantadine (Fig. 5), respectively. The dinitrobenzene derivative was obtained through etherfication (**7**) and amination (**10**) and the cyanoisoindole compound (**11**) was synthesised through reaction of o-phtaldialdehyde with amantadine in the presence of a NaCN.

¥3-aminopropionitrile (**12**) (Fig. 6) was synthesised by Michaels-eddition and further reduction with LiAlH produced adamantanediaminopropane (**13**). The dansyl (**14**) and NBD (**15**) compounds were obtained by amination on reaction with **13** and the cyanoisoindole (**16**) was synthesised through reaction of o-phtaldialdehyde with **13** with NaCN as catalyst. The coumarin moiety (**17**) was obtained on activation chemistry with CDI.

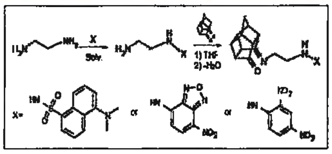

***Figure 7*: Synthesis of fluorescent pentacycloundecyl amine derivatives by amination†**

†Compounds in Fig. 7 were synthesised using an excess of the appropriate chain length diamino-linker and conjugating it with the the fluorophores by amination to produce the mono-substituted amine derivatives. These fluorophores were conjugated with pentacycloundecane-8,11-dione by reductive amination to obtain the desired cage imine moieties.

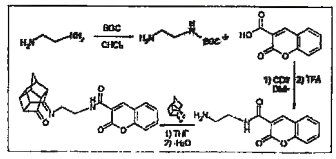

***Figure 8*: Synthesis of the fluorescent pentacyclo-undecyl coumarin derivatives‡**

‡The coumarin compounds (Fig 7) were synthesised by mono-protection of diamino-linkers with BOC and conjugation thereof with the coumarin by activation with CDI and subsequent removal of BOC by TFA. These fluorophores were conjugated with pentacyclo-undecane-8,11-dione by reductive amination to obtain the desired coumarin cage imine moieties.

The synthesised compounds were confirmed using NMR and IR. A Cary Eclipse® fluorescence spectrophotometer was used for fluorescence measurements and all the compounds showed an acceptable difference of excitation and emission wavelength and Stoke shifts varied from 29 to 215 nm.

Biological evaluation

***NOS assay*:** The oxyhaemoglobin (oxyHb) assay was employed to determine the activity of the novel compounds at an enzymatic level of NOS. The method is based on the reaction of oxyhaemoglobin (oxyHb) with NO to form methaemoglobin (metHb) and nitrate (eq. 1), the methaemoglobin concentration is then measured spectrofotometrically and is equivalent to the molar amount of NO generated.

$$HbO^2 + NO \longrightarrow metHb + NO^{3-} \quad (1)$$

The inhibition curves of the tested compounds (**5** - **11**) were superimposed on a single graph and the IC_{50} values were calculated. From the calculated IC_{50} values; compounds **7**, **13**, **17**, **18** and **19** showed promise as possible NOS inhibitors. When they are compared to **7-NI** (Fig. 9), it is clear that none of the structures inhibit NOS as potently as 7-NI (IC_{50} = 0.111 µM), a selective nNOS inhibitor.[7] All the compounds however showed more potent inhibitory activity than aminoguanidine (IC_{50} = 19.41 µM) (Fig. 9), a selective iNOS inhibitor.[8] Compounds **9**, **11**, **14** and **15** showed low or no inhibition (Fig. 8) when compared to 7-NI and aminoguanidine. Compound **17** proved to be the best inhibitor of the tested novel fluorescent compounds (IC_{50} = 0.291 µM).

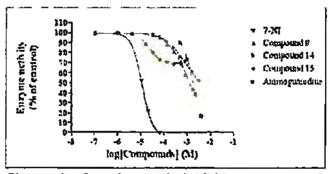

***Figure 9*: Superimposed inhibition curves of compounds with significant NOS inhibitory activity**

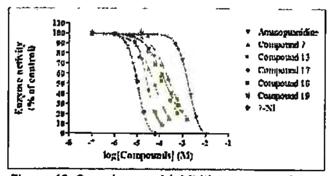

***Figure 10*: Superimposed inhibition curves of test compounds with low NOS inhibitory activity**

***Ca^{2+} and NMDA assay*:** The fluorescent ratiometric indicator, Fura-2/AM (a UV excited Ca^{2+} indicator), and a fluorescence spectrophotometer were used to evaluate the influence of calcium homeostasis *via* *L*-type Ca^{2+} channels and the NMDA receptor utilising murine synaptoneurosomes. The NMDA receptor binding studies evaluate whether the compounds are selective for the NMDA receptor or Ca^{2+} channels or has a dual Ca^{2+} blocking activity.

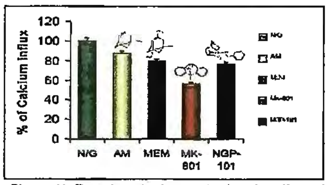

***Figure 11*: Experiments demonstrating the effect of KCl-induced depolarisation (300 mM) and Ca^{2+} influx, in the absence and presece of test compound (100 µM) and controls**

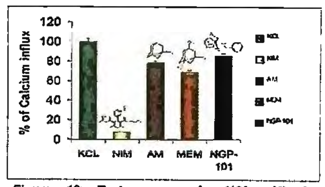

***Figure 12*: Test compounds (100 µM) for NMDA/Gly-mediated Ca^{2+} influx into synaptoneurosomes. Nimodipine was used to block *L*-type Ca^{2+} channels**

Inhibition of the Ca^{2+} flux through the *L*-type Ca^{2+} channels and NMDA channels reported in this study is consistent with findings of these structures (Fig. 11 and Fig. 12) studied by Malan *et al.*[9] The advantage of this novel assay is that the fluorescent Ca^{2+} indicator Fura-2/AM was used, over the expensive, time-consuming and hazardous radioligand binding studies used by Malan *et al.*[9] The effectiveness, accuracy and ease of this assay, makes it a great alternative over radioligand studies to test the novel compounds for their NMDA receptor and *L*-type Ca^{2+} channel activity, as well as binding affinity towards the NMDA receptor over MK-801.

Conclusion

We have synthesised a series of fluorescent polycyclic structures which may be utilised for further *in vitro* and *in vivo* studies using modern imaging techniques (e.g. confocal laser scanning microscopy, flow cytometry or multiphoton microscopy). The potential of these novel fluorescent polycyclic moieties may find application as fluorescent probes to better understand neurodegenerative and neuroprotective mechanisms. Additional assays on the NMDA receptor, voltage gated Ca^{2+} channel, NOS enzyme isoforms and blood-brain barrier permeability will furthermore elaborate on these compounds potential value. A novel displacement assay has also been developed for compound **14**.

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