

The antidepressant properties of selected methylene blue analogues

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Abstract

Keywords: Methylene blue; Azure B; Structural analogues; Antidepressant; Monoamine oxidase; Reversible inhibition.

The shortcomings of current antidepressant agents prompts the design of novel multimodal antidepressants and the identification of new antidepressant targets, especially those located at sub-cellular level. Such antidepressants should possess improved response rates as well as safety profiles. Methylene blue (MB) is reported to possess diverse pharmacological actions and is attracting increasing attention for the treatment of a variety of disorders including Alzheimer's disease, bipolar disorder, anxiety and depression. MB acts on both monoamine oxidase (MAO) and the nitric oxide (NO)-cGMP pathway, and possesses antidepressant activity in rodents. The principal goal of this study was to design a close structural analogue of MB and to evaluate the effects of these structural changes on MAO inhibition, a well-known antidepressant target. Furthermore, MAO inhibition is also responsible for cardiovascular toxicity in clinically used MAOI inhibitors. For this purpose we investigated the antidepressant properties of the synthetic MB analogue (ethyl-thioninium-chloride; ETC) as well as azure B, the major metabolite of MB, in the forced swim test (FST). ETC was synthesized with a high degree of purity from diethyl-p-phenylenediamine with 6% yield. ETC was firstly evaluated as a potential inhibitor of recombinant human MAO-A and MAO-B. Azure B and ETC were evaluated over a dosage range of 4-30 mg/kg for antidepressant-like activity in the acute FST in rats, and the results were compared to those obtained with saline, imipramine (15 mg/kg) and MB (15 mg/kg) treated rats. Locomotor activity was evaluated to ensure that changes in swim motivation are based on antidepressant response and not due to an indirect effect of the drug on locomotor activity. The results document that ETC inhibits MAO-A and MAO-B with IC_{50} values of 0.51 μ M and 0.592 μ M, respectively. Furthermore, ETC inhibits MAO-A and MAO-B reversibly, while the mode of inhibition is most likely competitive. In the acute FST, azure B and ETC were more effective than imipramine and MB in reversing immobility, without inducing locomotor effects. Azure B and ETC increased swimming behaviour during acute treatment, which is indicative of enhanced serotonergic neurotransmission. Azure B and ETC did not affect noradrenergic-mediated climbing behaviour. These results suggest that azure B may be a contributor to the antidepressant effect of MB, and acts via increasing serotonergic transmission. Secondly, small structural changes made to MB do not abolish its antidepressant effect even though ETC is a less potent MAO-A inhibitor than MB.

Uittreksel

Sleutelwoorde: Metileenblou; Azure B; Struktuuranaloo; Antidepressant; Monoamineoksidas; Selektiewe inhibisie.

As gevolg van die tekortkominge van antidepressante wat tans gebruik word, word nuwe geneesmiddels nodig wat verkieslik veelvoudige werkingsmeganismes besit. Hierdie nuwe groep antidepressante moet 'n vinniger aanvang van werking toon met 'n beter nuwe-effekprofiel. Metileenblou (MB) is 'n voorbeeld van 'n geneesmiddel wat verskeie werkingsmeganismes besit, en wat moontlik gebruik kan word vir die behandeling van verskeie siektetoestande soos Alzheimer se siekte, bipolêre gemoedsversteuring angstoestande en depressie. MB is 'n bekende inhibeerder van monoamienoksidas (MAO) en stikstofoksidasintetase (NOS), en besit betekenisvolle antidepressiewe aktiwiteit in knaagdiere. Die hoofdoel van hierdie studie was om 'n struktuuranaloo van MB te ontwerp en die effek van die strukturele veranderinge op MAO-inhibisie te evalueer. Die gesintetiseerde MB-analoo (etieltioniniumchloried, ETC) en azure B, 'n metaboliet van MB, is ook met behulp van die geforseerde swemtoets (GST) geëvalueer as potensiële antidepressante. ETC is vanaf diëtiel-p-fenileendiamien gesintetiseer met hoë suiwerheid en 'n opbrengs van 6%. Die potensie waarmee ETC rekombinante, menslike MAO-A en MAO-B inhibeer, is geëvalueer. ETC en azure B se potensiële antidepressiewe aktiwiteite is by dosisse van 4-30 mg/kg in die akute GST geëvalueer, en die resultate is vergelyk met dié verkry na saline-, imipramien- (15 mg/kg) en MB- (15 mg/kg) behandeling. Lokomotoriese aktiwiteit is geëvalueer om te verseker dat die veranderinge in swemmotivering die gevolg is van die antidepressiewe werking van die middels, en nie as gevolg van 'n indirekte effek van die toetsmiddel op lokomotoriese aktiwiteit is nie. Die resultate toon dat ETC MAO-A en MAO-B inhibeer met IC_{50} -waardes van 0.51 μM en 0,592 μM , onderskeidelik. Die resultate toon verder dat ETC 'n omkeerbare, kompeterende inhibeerder vir beide MAO-A en MAO-B is. ETC en azure B was meer effektief, vergeleke met imipramien en MB, om immobiliteit in die akute GST te verlaag, sonder om die lokomotoriese gedrag te beïnvloed. Azure B en ETC het swemgedrag verhoog tydens die akute GST, wat op 'n serotonergiese werkingsmeganisme dui. Die resultate dui daarop dat azure B en ETC waarskynlik nie katesjolaminergiese werkingsmeganismes besit nie. As metaboliet van MB, kan azure B dus bydra tot die antidepressiewe aktiwiteit van MB. Laastens kan die gevolgtrekking gemaak word dat klein veranderinge aan die struktuur van MB nie die antidepressiewe aktiwiteit van MB ophef nie, selfs al word die potensie van MAO-A-inhibisie verlaag.

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LIST OF ABBREVIATIONS AND ACRONYMS

A

ACC	Anterior cingulated cortex
ACTH	Adrenocorticotropin hormone
ANOVA	Analysis of variance

B

BDNF	Brain-derived neurotrophic factor
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C

cGMP	Cyclic guanosine monophosphate
CIEA	2-Chloroethylamine
CIAA	Chloroacetaldehyde
CREB	Cyclic adenosine monophosphate response element binding protein
CRF	Corticotrophin releasing factor
CRH	Corticotrophin releasing hormone

D

DLPFC	Dorsolateral prefrontal cortex
DMEM	Dulbecco's modified eagle medium
DSM-IV-TR	Diagnostic and statistical Manual, 4 th edition, text revision

E

ETC	Ethyl-thioninium-chloride
-----	---------------------------

F

FAD	Flavin adenine dinucleotide
FDA	Food and Drug Administration
FST	Forced swim test

G

GABA	γ -Aminobutyric acid
------	-----------------------------

H

5-HIAA	5-Hydroxyindole acetic acid
5-HT	Serotonin
HPA-axis	Hypothalamic-pituitary-adrenal-axis
HRMS	High resolution mass spectra

I

IC ₅₀	Inhibitor concentration at 50% inhibition
IMI	Imipramine
i.p.	Intraperitoneally

L

LeucoMB	Leucomethylene blue
LOPFC	Lateral orbital prefrontal cortex

M

MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MB	Methylene blue
MDD	Major depressive disorder
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NA	Noradrenaline
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-Methyl-D-aspartate
NMR	Nuclear magnetic resonance
NO-cGMP	Nitric oxide cyclic guanosine monophosphate
NOS	Nitric oxide synthases
NOS-NO-cGMP	Nitric oxide synthases- nitric oxide-cyclic guanosine monophosphate

P

PBS Phosphate-buffered saline

R

Rf Retardation factor

S

SD Standard deviation

S.E.M Standard error of mean

sGC Soluble guanylate cyclase

SNRIs Serotonin-noradrenaline reuptake inhibitors

SSRE Serotonin reuptake enhancer

SSRIs Selective serotonin reuptake inhibitors

ST Serotonin toxicity

T

TCA Tricyclic antidepressant

TLC Thin layer chromatography

V

VMPFC Ventromedial prefrontal cortex

W

WHO World Health Organization

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Chapter 1

Introduction

1.1. Problem statement

Mood disorders, such as major depression, are among the most prevalent forms of mental illness. Major depressive disorder (MDD) is a recurring psychiatric disease which affects up to 17% of the American population (Kessler *et al.*, 2005). Depression is a debilitating disease, affects family, social- and working relationships, sleep patterns, eating habits and sense of pleasure. Despite a variety of antidepressants available, the efficacy of current treatment regimens remain inadequate and a significant proportion of patients do not respond to first line therapy, or do not reach complete remission (Sartorius *et al.*, 2007). The current antidepressant agents available either inhibits the uptake of serotonin (5-HT) and/or noradrenalin (NA), or block monoamine oxidase enzymes which lead to increased synaptic levels of these monoamines. Antidepressant efficiency depends on enhanced intrasynaptic levels of monoamines, which are increased within hours after administration (Harvey, 1997; Popoli *et al.*, 2002; Harvey *et al.*, 2003). Despite this rather rapid intrasynaptic change in neurotransmitter levels, patients experience antidepressant-like effects only after 4-6 weeks from initiating treatment. This slow onset of action as well as the side effects associated with current antidepressants is responsible for poor compliance among patients. Although these agents invariably target one of more monoaminergic mechanism of action, new evidence suggests that depression is not a single transmitter illness and that a number of possible pathological processes are involved (Manji *et al.*, 2001; D'Sa & Duman, 2002; Nestler *et al.*, 2002; Duman & Monteggia, 2006; Harvey, 2008). These shortcomings prompts the design of novel multimodal antidepressants and the identification of new antidepressant targets, especially those located at sub-cellular level, with improved response rate as well as safety profiles. Dysfunction of the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) cascade is strongly linked to the neurobiology of depression (Harvey, 1996, Dhir & Kulkarni, 2011). Pre-clinical studies have shown that nitric oxide synthase (NOS) inhibitors exhibit antidepressant effects (Harkin *et al.*, 1999) while typical antidepressants also suppress hippocampal NOS activity *in vivo* (Wegener *et al.*, 2003). It has also been observed that depressed patients have elevated levels of NOS activity (Suzuki *et al.*,

2001). Drugs aimed at suppressing the NO-cGMP pathway may therefore prove to be useful in treating depression. One such drug candidate is the tricyclic compound, methylene blue (MB). MB possesses multiple pharmacological actions and as a result is used in several medical conditions. MB is a potent monoamine oxidase type A (MAO-A) inhibitor and has shown promising antidepressant and anxiolytic qualities in bipolar disorder (Naylor *et al.*, 1987). MB is also recognized as a non-selective inhibitor of NOS and guanylate cyclase (Luo *et al.*, 1995), and also is effective in correcting mitochondrial electron transfer reactions (Atamna & Kumar, 2010) thus advocating its use in clinical illnesses characterised by redox dysfunction and oxidative stress such as ifosfamide encephalopathy (Küpfer *et al.*, 1994) and methemoglobinemia (Wright *et al.*, 1999). Because the NO-cGMP system is intimately involved in cardiovascular homeostasis (Naseem, 2005) and various brain functions (Rivier, 2001; Guix *et al.*, 2005), typical agents that selectively inhibit the NO-cGMP system have great potential for adverse cardiovascular and central nervous system effects. MB, however, seems to be devoid of any significant side-effects. It is now known that mitochondrial dysfunction (Fattal *et al.*, 2006) and oxidative stress (Harvey, 2008) plays a role in the pathology of depression. MB has the ability to enhance mitochondrial function through the cycling between the reduced leucomethylene blue (leucoMB) and the oxidised MB states (Atamna & Kumar, 2010) (see Figure 2.1). It has been suggested that MB may have a dual target approach to its antidepressant response, through MAO and NOS inhibition (Harvey *et al.*, 2010). All of the above mentioned mechanisms, make MB a potential novel lead compound for the design and synthesis of new antidepressant agents.

MB is metabolised to yield N-demethylated products of which azure B, the monodemethyl species, is the major metabolite (Warth *et al.*, 2009). Azure B is an even more potent reversible MAO-A inhibitor than MB and may contribute to MB's pharmacological profile (Petzer *et al.*, 2012). Whether azure B has antidepressant actions that may contribute the clinical pharmacology of MB has not yet been characterised and is thus urgently required.

As noted earlier, MB is considered to have a good safety profile in humans with few adverse effects. However, due to its ability to inhibit MAO-A, MB may precipitate serotonin toxicity (ST) if administered with serotonergic agents (Ramsay *et al.*, 2007; Stanford *et al.*, 2009). ST is one of very few drug–drug interactions that involve therapeutic doses of commonly used drugs and which may be fatal. Another major disadvantage of MAO-A inhibitors and possibly MB is the concern for development of hypertensive crisis due to the interaction of inhibitors of MAO-A with tyramine and other monoamine-releasing compounds (Brunton *et al.*, 2010). Despite these

concerns, there is a growing interest in the clinical benefit and utility of MAO-inhibitors in the treatment of depression (Gillman, 2011). In this study we propose to design and synthesise a derivative of MB and to characterise its interactions with human MAO-A and MAO-B. The approach will be to make relatively small structural changes to MB in order to minimise the MAO-A inhibition properties of MB but at the same time retain the beneficial pharmacological effects of MB. The MAO-A inhibition properties of MB may be altered by enlarging the N-alkyl substituents, which may restrict entrance into the active site cavity of MAO-A and thus hinder MAO-A inhibition. In this way the redox chemistry and phenothiazinium structure of MB are retained in the analogue. These two structural characteristics are thought to play an important role in the biological activities of MB (Duvenhage, 2010). There would thus be great value in developing MB analogues with lower MAO-A inhibition potencies for the treatment of depression. Moreover, due to the increasing use of MB in the long term, this may lead to the development of a new class of antidepressant drugs with an overall improved safety profile.

1.2. Study aims

The principal aims of this study are:

- To design and synthesise a close structural analogue of MB;
- To evaluate the effects of structural changes to MB on MAO activity;
- To determine the physicochemical properties of the MB analogue;
- To confirm that MB does indeed induce a robust antidepressant-like response in the acute forced swim test (FST), according to previously published guidelines (Harvey *et al.*, 2010),
- To perform a dose-response analysis on the MB analogue and azure B in the acute forced swim test (FST) to determine if either possess antidepressant-like activity, whether these responses are dose dependent, and how they compare to MB as well as to a reference antidepressant, imipramine (IMI).

1.3. Study layout

One analogue of MB will be designed and synthesised in this study, viz. ethyl-thioninium-chloride (ETC). ETC will then be evaluated for its inhibitory effects on monoamine oxidase (MAO) A and B by employing an *in vitro* spectrofluorometric assay and the appropriate recombinant human MAO enzyme. Selected physicochemical properties of ETC will be measured.

Azure B and ETC will be evaluated in the acute FST using a dose-response analysis. To achieve this, male Sprague Dawley rats will be treated with four different doses of each compound. The rats will subsequently be evaluated in a Digiscan[®] animal activity monitor to determine general locomotor activity where after the acute FST will be performed. The results obtained with azure B and ETC will be compared to results obtained after treatment of rats with saline, IMI and MB. Thereafter, diverse aspects of all the above compounds will be studied with respect to their effects on immobility response in the FST as well as on various swimming behaviours in order to verify actions on serotonergic and catecholaminergic pathways (Cryan *et al.*, 2002).

1.4. Hypothesis

This study postulates that ETC, the synthetic analogue of MB will possess reduced MAO-A inhibitory potency compared to MB while retaining antidepressant-like response in the acute FST comparable to IMI. It is further postulated that azure B also will possess antidepressant-like activity in the FST. For both compounds, the responses in the FST are expected to be dose dependent.

1.5. Ethical approval

All animal procedures were approved by the Ethics Committee of the North-West University (approval number: *NWU-00024-13-S1*), and are in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

Chapter 2

Literature study

2.1. Methylene blue

2.1.1. General background

In 1876, Heinrich Caro synthesised MB for the first time to use as a cotton dye. MB was also the first synthetic drug used in medicine and was used for the treatment of malaria (Guttmann & Ehrlich, 1891). Through the years MB became famous for its staining qualities, especially for the identification of *Mycobacterium tuberculosis* by Robert Koch, for the observation of malaria parasites (Fleischer, 2004; Barcia, 2007) and the visualisation of the structural organisation of nerve tissues (Ehrlich, 1886; Cajal, 1896; Garcia-Lopez *et al.*, 2007). During the late 1800's, MB was already used for its medicinal purposes (Bodoni, 1899). More recently animal studies have found that MB has antidepressant and anxiolytic effects (Eroglu & Caglayan, 1997). These pharmacological properties were later linked to its activity as monoamine oxidase inhibitor (MAOI) (Aeschlimann *et al.*, 1996; Ramsay *et al.*, 2007) as well as an inhibitor of the nitric oxide cyclic guanosine monophosphate (NO-cGMP) cascade (Eroglu & Caglayan, 1997; Volke *et al.*, 1999). In fact, these latter actions are significant when considering the important role of NO in the neurobiology of depression (Harvey *et al.*, 1990; 1996; Eroglu & Caglayan, 1997; Dhir & Kulkarni, 2011). The principle aim of this study is to design and synthesise a MB analogue that is structurally similar to MB, and to evaluate the effect of small structural changes on MAO inhibition.

2.1.2. Physical chemistry

At room temperature MB is a green solid, and when dissolved in water it yields a blue solution. The stable oxidised form of MB gives the compound its deep blue colour with an absorbance maximum at a wavelength of 609-668 nm (Ramsay *et al.*, 2007). LeucoMB is colourless and represents the unstable reduced form of MB with no absorption in the visible spectrum (Oz *et al.*, 2009). In equilibrium, MB and leucoMB exist as a redox couple and together they form a reversible oxidation-reduction system or electron donor-acceptor couple (Oz *et al.*, 2009), as seen in Figure 2.1. The conversion of MB to leucoMB is caused by reducing agents such as nicotinamide adenine dinucleotide phosphate (NADPH) (Schirmer *et al.*, 2011). The oxidation of

leucoMB to MB may be catalysed by O₂ (Schirmer *et al.*, 2011). Each reaction cycle can lead to the production of reactive oxygen species, such as H₂O₂ (Buchholtz *et al.*, 2008; Oz *et al.*, 2009).

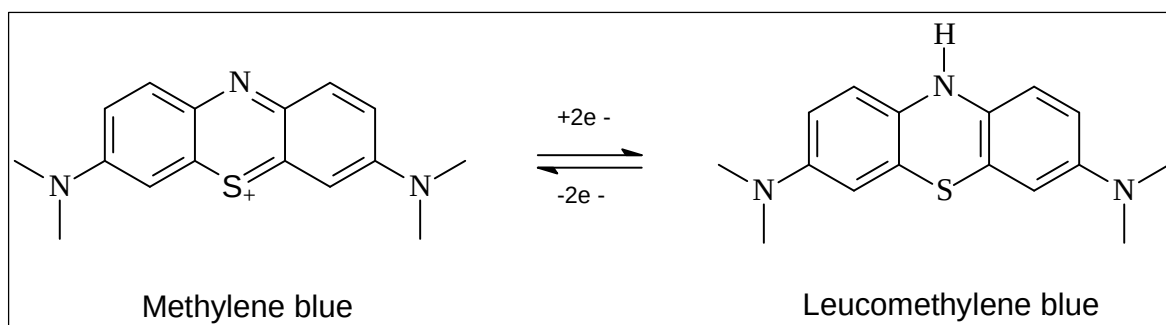


Figure 2.1: The electron donor-acceptor couple, methylene blue and its reduced state, leucoMB

MB is a cationic, tricyclic phenothiazine compound (Wainwright & Amaral, 2005) and is hydrophilic by nature (DiSanto & Wagner, 1972; Wagner *et al.*, 1998). The pKa of MB is approximately 0 to -1, it is completely ionised at physiological pH values (DiSanto and Wagner, 1972) and has a partition coefficient of -0.96 (DiSanto and Wagner, 1972). This should make it impossible for MB to cross the blood-brain barrier. However, according to Peter *et al.* (2000), MB does cross the blood-brain barrier, and there are different ways in which MB may do so. Firstly, isobolic potential curves encompassing the MB molecule (Figure 2.2.) indicate that charges on the nitrogen and sulfur atoms are not localised and are almost equally distributed on the surface of the molecule (Oz *et al.*, 2009). This may facilitate passage through the blood-brain barrier (Wagner *et al.*, 1998). Entering the brain can also be facilitated by the leucoMB form, which is uncharged and 20 times more lipophilic than MB (Harris & Peters, 1953).

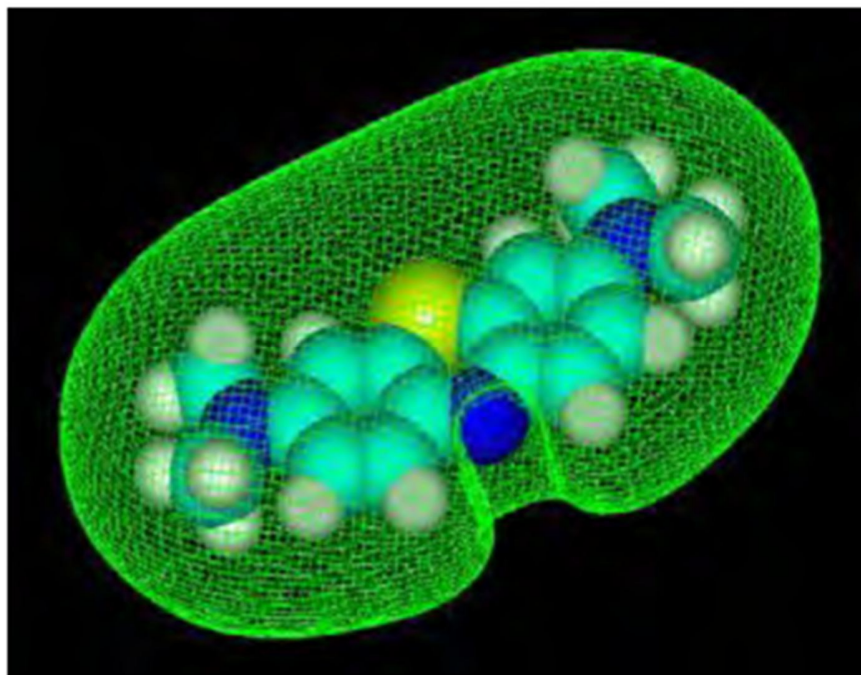


Figure 2.2: The isopotential surface surrounding the methylene blue structure in 3D space, as described by Oz *et al.* (2009).

2.1.3. Targets in the human body

2.1.3.1. Monoamine oxidase

MAO is a flavin-containing membrane bound enzyme, and is found on the outer membrane of mitochondria (Edmondson *et al.*, 2007) and is responsible for metabolising catecholamines (Baldessarini, 2001). In the 1960's it was found that MAO was not a single enzyme, but consists of two isoforms: MAO type A and MAO type B. MAO-A is responsible for metabolising serotonin and noradrenaline (Murphy *et al.*, 1987) while MAO-B metabolises dopamine (Glover *et al.*, 1977). MB is a potent inhibitor of MAO-A with an IC_{50} value of $0.07 \mu M$ for the inhibition of recombinant human MAO-A *in vitro* (Aeschlimann *et al.*, 1996; Ramsay *et al.*, 2007, Harvey *et al.*, 2010). To a lesser extent, MB inhibits human MAO-B with an IC_{50} value of $4.37 \mu M$ (Ramsay *et al.*, 2007; Harvey *et al.*, 2010). It is thought that the inhibition of MAO-A by MB is at least in part, responsible for its antidepressant effect (Harvey *et al.*, 2010). MB is metabolised in the human body to yield two metabolites: azure B as the major metabolite and azure A as the secondary metabolite (Warth *et al.*, 2009). Their structures are shown in Figure 2.3. In recent studies it was found that the major metabolite, azure B, is a reversible inhibitor of MAO-A and MAO-B. Azure B is, however, 300-fold more selective for MAO-A (IC_{50} value of 11 nM) than

MAO-B, for which it has an IC_{50} value of 968 nM (Petzer *et al.*, 2012). Petzer & co-workers (2012) also found that azure B is a superior MAO-A inhibitor compared to MB. This interaction of azure B and MB with MAO-A may be responsible for serotonin toxicity (discussed below) that may occur when MB and selective serotonin reuptake inhibitors (SSRIs) are co-administered (Ramsay *et al.*, 2007). This emphasises the need to characterise the pharmacological properties of azure B in order to determine whether azure B may contribute to MB's therapeutic and adverse effects *in vivo*.

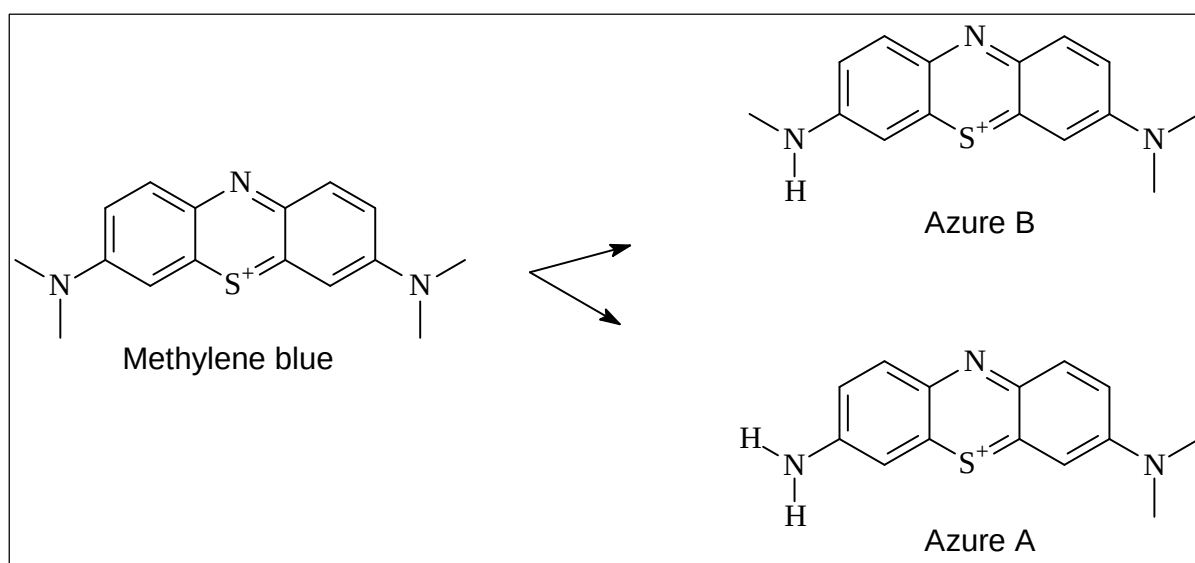


Figure 2.3: The structures of methylene blue and its metabolites azure B and azure A.

2.1.3.2. NO-cGMP cascade

It is recognised that MB is a non-selective nitric oxide synthases (NOS) and guanylate cyclase inhibitor (Luo *et al.*, 1995; Moore & Handy, 1997; Volke *et al.*, 1999). MB is known to target the heme group of iron-containing enzymes (Kelner *et al.*, 1988). That both NOS and soluble guanylate cyclase (sGC) contain stoichiometrical amounts of iron (Gerzer *et al.*, 1981; Mayer *et al.*, 1993), allows MB to inhibit both these enzymes. Mayer *et al.* (1993) found that MB has a direct inhibitory effect on NOS and is a more potent inhibitor of NOS than sGC. Thus MB inhibits the NOS-NO-cGMP pathway (Eroglu & Caglayan, 1997) which plays an important role in depressive disorders (Harvey *et al.*, 1990; 1994; Dhir and Kulkarni, 2011). Many compounds have been synthesised in the attempt to regulate the NO-cGMP pathway. These compounds include L-nitromethyl arginine, a non-specific inhibitor of NOS isoforms, N-nitro-arginine and 7-nitroindazole, selective neuronal NOS inhibitors, and aminogaunidine, a selective inducible NOS inhibitor (Oosthuizen *et al.*, 2005). There are major concerns for the use of NOS inhibitors

because they are prone to cause lethal cardiovascular toxicity and, in general, lack selectivity (Hobbs *et al.*, 1999; Ignarro *et al.*, 1999). MB, however, seems devoid of the any significant side effects associated with NOS inhibition (Narsapur & Naylor, 1983). Currently there are only a few agents that are clinically used (Ghofrani *et al.*, 2006) and which target the NO-cGMP pathway. These include the NO releasers (amyl nitrate and nitroglycerine) and agents that increase cGMP levels by inhibiting cGMP-phosphodiesterase (tadalafil and sildenafil) (Corbin, 2000). Interestingly, sildenafil and tadalafil have demonstrated antidepressant-like effects in animals as well (Brink *et al.*, 2008; Liebenberg *et al.*, 2010), further confirming that modulation of this pathway is a novel approach to treating depressive disorders (Brink *et al.*, 2008).

2.1.3.3. Cholinesterase

Acetylcholine is a neurotransmitter at cholinergic synapses and acetylcholinesterase is the enzyme responsible for inactivating acetylcholine (Oz *et al.*, 2009). Inhibition of cholinesterase increases synaptic acetylcholine levels thereby enhancing cholinergic neurotransmission that may be useful in the treatment of Alzheimer's disease (Nordberg, 2006; Holzgrabe *et al.*, 2007). MB may also modulate the cholinergic system and high concentrations MB have been associated with cholinergic activation, prompting further investigation (Pfaffendorf, 1997). Pfaffendorf *et al.* (1997) found that high concentrations of MB completely inhibit cholinesterase activity in human serum as well as purified human pseudocholinesterase. Comparatively MB inhibits bovine acetylcholinesterase to a much lesser degree (Pfaffendorf *et al.*, 1997). It was also found that the inhibitory action on cholinesterase by MB was concentration-dependent (Pfaffendorf *et al.*, 1997). The cholinergic system plays a role in the regulation of learning and memory, suggesting that MB can be useful in the treatment of Alzheimer's disease (Oz *et al.*, 2009).

2.1.4. Medical indications

Current medical indications for MB are treatment against methemoglobinemia, prevention against urinary tract infection, intraoperative visualisation of organic tissues and the prevention and treatment of ifosfamid-induced encephalopathy (Küpfer *et al.*, 1994). Currently there are 22 registered clinical trials that involve MB (<http://clinicaltrials.gov>). Pre-clinically, MB has shown activity as an antidepressant, anxiolytic (Eroglu & Caglayan, 1997), anti-psychotic (Klamer *et al.*, 2004) and as a drug for the treatment of Alzheimer's disease (Wischick *et al.*, 1996).

2.1.4.1. Methemoglobinemia

Methemoglobinemia is a disease state characterised by inadequate tissue oxygenation caused by excessive levels of blood methemoglobin. When the iron of the haemoglobin molecule is oxidized from the ferrous (Fe^{2+}) to the ferric state (Fe^{3+}) the erythrocytes are incapable of transporting oxygen through the body. Methemoglobin is the form of haemoglobin where the iron in the heme group is in the ferric state and cannot release bound oxygen, thus resulting in hypoxia and cyanosis (Wright *et al.*, 1999). MB functions by reducing methemoglobin from the ferric iron back to the ferrous state. This restores the ability of the erythrocytes to transport oxygen through the body, (Wright *et al.*, 1999) and is an effective treatment of methemoglobinemia.

2.1.4.2. Encephalopathy

MB can be used prophylactically or as treatment against ifosfamid-induced encephalopathy. Ifosfamide is an alkylating agent used in the treatment of various types of cancers (Alici-Evicimen & Breitbrat, 2007). However, the side-effects of ifosfamide are quite severe. It is known to result in neurological toxicity such as encephalopathy which may be caused by the metabolites of ifosfamide (Küpfer *et al.*, 1994; Küpfer *et al.*, 1996; Aeschlimann *et al.*, 1996). Ifosfamide-encephalopathy presents with the following symptoms: cerebellar ataxia, confusion, visual hallucinations, seizures and extrapyramidal signs (Stanford *et al.*, 2009). As a treatment, MB acts as an electron acceptor and oxidizes flavoproteins, thereby relieving the flavoprotein deficiency caused by ifosfamide and its metabolites (Küpfer *et al.*, 1994). As a prophylactic treatment MB should be administered one day before starting treatment with ifosfamide (Pelgrims *et al.*, 2000). The mechanism of action of MB as a prophylaxis is due to its ability to oxidize excessive nicotinamide adenine dinucleotide (NADH) formed during ifosfamide metabolism (Küpfer *et al.*, 1996). The re-oxidation of NADH will allow hepatic glucose production to return to normal and correct intracellular redox balance (Küpfer *et al.*, 1996). MB also inhibits the systemic and mitochondrial activation of 2-chloroethylamine (CIEA) and reverses chloroacetaldehyde (CIAA) toxicity and as such, inhibits multiple extra hepatic amine oxidases, which makes MB effective as a prophylactic treatment against ifosfamid-induced encephalopathy (Küpfer *et al.*, 1996; Aeschlimann *et al.*, 1996).

2.1.4.3. Psychotic disorders

Schizophrenia is caused by an imbalance of neurotransmitters in the central nervous system (Carlsson *et al.*, 2001), specifically that of dopamine, including deficits in frontal cortex and excessive levels in the ventral striatum (Harvey *et al.*, 1999). However, dopamine is very likely the result of a cascade of events, including altered mitochondrial function, altered glutamate activity, oxidative stress and immune-inflammatory reactions (Moller *et al.*, 2011; 2013). Klammer & colleagues (2004) found that MB has anti-psychotic-like effects in an animal model, typified as hyperactivity, increased stereotypic behaviours and episodic explosive jumping or popping (Deutsch *et al.*, 1993). MB significantly reduces these popping behaviours (Deutsch *et al.*, 1997) suggesting that NOS inhibitors or inhibitors of NO function may have anti-psychotic activity. NO and the NO-cGMP cascade are involved in various neuropathological disorders including schizophrenia (Das *et al.*, 1995; Karson *et al.*, 1996; Karatinos *et al.*, 1995; Bernstein *et al.*, 2011). NO may be involved in regulating glutamate, serotonin and dopamine mediated neurotransmission (Kano *et al.*, 1998; Wegener *et al.*, 2000; Smith & Whitton, 2000; 2001), thus supporting the role of NOS inhibitors, such as MB, and NO-cGMP pathway modifiers in the treatment of psychotic disorders. Indeed, this is supported by evidence that currently used antipsychotics modulate brain NOS activity (Nel and Harvey, 2003), while investigators have proposed that actions on altered redox systems such as the NO-system play a role in recurrent schizophrenia (Emsley *et al.*, 2013).

2.1.4.4. Mood disorders

Eroglu & Caglayan (1997) reported that in the pre-clinical setting, MB has antidepressant and anxiolytic effects. The antidepressant effect of MB can be a result of multiple mechanisms. As mentioned before MB is a potent MAO-A inhibitor (Aeschlimann *et al.*, 1996) and increases the synaptic levels of monoamines such as serotonin and noradrenaline (Harvey *et al.*, 2010). It was also found that MB increases the extracellular levels of serotonin in the hippocampus (Wegener *et al.*, 2000). Volke & colleagues (1997) found that MB increases the efflux of serotonin and dopamine. The above mentioned mechanisms for MB's antidepressant effects all support the monoamine hypothesis of depression (discussed later), where unbalanced and decreased levels of monoamines in the central nervous system may cause depression (Schildkraut, 1965; Hyman & Nestler, 1993; Randrup & Braestrup, 1997). MB is also an inhibitor of NOS and sGC and thus modulate the NO-cGMP pathway (Luo *et al.*, 1995; Moore & Handy, 1997; Volke *et al.*, 1999), which are involved in depressive disorders (Harvey *et al.*, 1990; 1994;

Eroglu & Caglayan, 1997; Suzuki *et al.*, 2001; Dhir & Kulkarni, 2011). One of the aims of this study is to investigate the antidepressant effect of a MB analogue with lower MAO-A inhibition potency compared to MB.

2.1.4.5. Cognitive disorders

For more than a century it has been known that MB has a beneficial effect in the treatment of cognitive disorders (Bodoni, 1899). Recently the focus has been on Alzheimer's disease and the fact that MB may potentially slow down cognitive decline in the illness. As with many other cognitive diseases, Alzheimer's disease may be caused by multiple components (Barten & Albright, 2008). MB influences mitochondrial function, the formation of amyloid plaques and neurofibrillary tangles, which all play a significant role in the pathology of Alzheimer's disease (Oz *et al.*, 2009). MB targets impaired mitochondrial respiration, thus improving neural energy production and memory consolidation (Oz *et al.*, 2009). MB also improves mitochondrial respiration by shuttling electrons to oxygen in the electron transport chain (Visarius *et al.*, 1997). Impaired mitochondrial respiration is associated with learning disabilities and memory deficits in various disorders such as Alzheimer's disease (Bowling *et al.*, 1995), but also depression and schizophrenia (Prabakaran *et al.*, 2004; Fattal *et al.*, 2006). The mitochondrial enzyme, cytochrome c oxidase, catalyzes the utilisation of oxygen for the electron transport chain (Kish *et al.*, 1992). It has been found that there is a decrease in cytochrome c oxidase activity in Alzheimer's disease patients (Kish *et al.*, 1992). MB has the potential to increase oxygen consumption and to compensate for decreased cytochrome c oxidase activity (Martinez *et al.*, 1978). All of the above mentioned factors make MB a promising drug for the treatment of Alzheimer's disease (Oz *et al.*, 2009).

2.1.5. Adverse effects and contra-indications

Side effects of MB include the bluish colouration of the sclera and urine, and staining of clothing (Oz *et al.*, 2009). The latter side effect is only an esthetic problem but remains one of the reasons for poor compliance among patients. Nausea and vomiting shortly after administration was shown to be a side effect in a study of 19 manic-depressive patients who received MB in a dose of 300 mg/day (Narsapur & Naylor, 1983). This was overcome by taking the drug after a meal. Another side effect is that MB may irritate the bladder and urethra, which causes dysuria and polyuria (Narsapur & Naylor 1983).

MB easily crosses the blood-milk barrier (Ziv & Heavner, 1984) and should not be used by lactating mothers. Hemolytic anemia, respiratory distress and phototoxicity are a risk in neonates exposed to MB. The use of MB during pregnancy and intra-amniotic procedures is contra-indicated due to its association with fetal intestinal atresia and other teratogenic effects (Cragan, 1999).

When using MB one must follow a strict diet, because it is known to cause the cheese reaction. The cheese reaction occurs when patients treated with an MAO-A inhibitor also consume tyramine containing food products, as shown in Table 2.1 (Wells, 2009). Under normal circumstances tyramine, a dietary amine, is metabolised by MAO in the gastrointestinal tract. In the presence of MB, which is a potent reversible MAOI, tyramine is not metabolised (Finberg *et al.*, 1981; Finberg & Tenne, 1982). The excess tyramine has access to the circulation and causes a significant release of noradrenaline. This results in a severe hypertensive crisis which can be fatal (Finberg *et al.*, 1981; Finberg & Tenne, 1982). A hypertensive crisis presents with occipital headache, stiff neck, nausea, vomiting, sweating and sharply elevated blood pressure (Wells, 2009).

Table 2.1: Dietary restrictions for patients taking MAO inhibitors

Aged cheese
Sour cream
Yogurt
Beer
Red wine (especially Chianti and sherry)
Sardines
Sauerkraut
Yeast extract and other yeast products
Raisins
Canned, aged, or processed food

A serious and fatal contra-indication of MB is the co-administration with SSRIs, which may lead to ST (Ramsay *et al.*, 2007). ST is an iatrogenic syndrome caused mostly and in the severest form when SSRIs and MAO-A inhibitors are co-administered (Gillman, 2006). It manifests with raised intrasynaptic serotonin levels and is mainly caused by reuptake inhibition of serotonin, MAO inhibition or presynaptic serotonin release (Gillman, 2006). The typical clinical features of

serotonin toxicity are displayed in Table 2.2 (Gillman & Whyte, 2004; Gillman, 2006). This adverse effect of MB highlights the need to design and develop MB analogues devoid of MAO-A inhibition.

Table 2.2.: Clinical features of serotonin toxicity

Neuromuscular hyperactivity	Tremor Clonus Myoclonus Hyperreflexia Pyramidal rigidity (advanced stage)
Autonomic hyperactivity	Diaphoresis Fever Tachycardia Tachypnea Mydriasis
Altered mental status	Agitation Excitement Confusion (advanced stage)

2.2. Depression

2.2.1. General background

Major depressive disorder can be defined as a clinical manifestation that is characterised by one or more major depressive episodes without a history of manic, mixed or hypomanic episodes (Wells, 2009). Around 400 B.C., Hippocrates used the word melancholia, which means black bile in Greek, to describe depression (Akiskal, 2000). This shows that depression has been around for several millennia yet its exact cause and how best to treat the illness remains elusive. Mood disorders, like major depression are among the most prevalent forms of mental illness. According to the World Health Organization, depression will be the second most common burden disease globally by 2020 (Murray & Lopez, 1996). One in every five South Africans is affected by mental illness (<http://www.sadag.org>) and one in every six individuals will develop clinical depression in the United States (Kessler *et al.*, 2005). This illness is burdensome and shows a recurrent nature. In fact, only a few patients go into complete

remission while they are also more prone to suffer relapses (Keller *et al.*, 2002). It was found that 23 people commit suicide every day and another 230 attempt suicide (<http://www.sadag.org>). These facts emphasise the importance of research into depression, particularly with regard to the design of novel multimodal antidepressants, with improved response rate and safety profiles.

It was also found that more women than men are prone to develop depression (Blazer, 2000). Stressful events in a person's life can add to the susceptibility to developing depressive episodes (Kraepelin, 1921). There is a 40-50% genetic risk factor for inheriting depression (Sanders *et al.*, 1999; Fava & Kendler, 2000). Other factors such as illness (Cushing's disease), side effects of drugs (such as isotretinoin) or viral infections (Borna virus) can also cause depression (Akiskal, 2000; Fava & Kendler, 2000). Kendler and colleagues (2001) linked the increased susceptibility to depression with subsequent episodes to the number of prior depressive episodes, stress related events and a high genetic risk.

Depression can consume a person's whole life, it affects family, social- and working relationships, sleep patterns, eating habits and sense of pleasure. Based on the DSM-IV-TR (Diagnostic and Statistical Manual, 4th edition, text revision, 2000) criteria for major depressive disorder (given in Table 2.3), depression can be characterised by a combination of symptoms (American Psychiatric Association, 2000). The duration of the illness is case specific and depends on the individual's particular illness. Major depression can be diagnosed when there are five or more symptoms present for a period of two weeks or longer and one of the symptoms must be either depressed mood or loss of interest or pleasure (American Psychiatric Association, 2000).

Table 2.3: The diagnostic criteria for major depressive disorder

Diagnostic and Statistical Manual of Mental Disorders: Criteria for Major Depressive Disorder Five or more of the following symptoms have been present for a period of 2 weeks and one of the symptoms is either depressed mood or anhedonia. • Depressed mood • Loss of interest or pleasure • Weight loss or weight gain • Insomnia or hypersomnia • Psychomotor agitation or retardation • Fatigue • Feelings of worthlessness or guilt • Unable to think or concentrate, or indecisiveness • Recurrent suicidal thoughts This does not include symptoms that are caused by a medical condition (hypothyroidism) or drugs (isotretinoin). The symptoms are not caused by the loss of a loved one and the symptoms persist for 2 months or longer
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According to Wells (2009) the desired outcome of treating depressive disorders, especially an acute depressive episode, is to reduce or eliminate the symptoms of depression, minimize adverse effects, to guarantee complete co-operation with the therapeutic regimen, facilitate a return to a premorbid state of mind, to prevent further episodes of depression and to reach complete remission. There is a wide range of antidepressants available but there are many problems with the current regimens. Foremost among these is the poor compliance rate because of a slow onset of action and significant side effects patients experience early in treatment (Harvey *et al.*, 2003). These include anxiety, irritability, nausea and headaches. Another problem is that approximately 30% of depressed patients do not fully respond to drug therapy and the remaining 70% never reach complete remission (Fava & Davidson, 1996; Holtzheimer & Nemerhoff, 2006). Patients experience antidepressant-like effects only after 4 to 6 weeks from starting the treatment, this despite an increase in intrasynaptic serotonin and/or noradrenaline levels within hours after administration (Harvey, 1997; Popoli *et al.*, 2002). This shows that the antidepressant effect is not a result of increased monoamines levels but rather due to subcellular changes that mediate long-term neuroplastic changes to eventually lead to

altered expression of receptors (Mongeau *et al.*, 1997). The role of the 5-HT_{1A} autoreceptors is especially important. When these receptors are stimulated, they inhibit firing of serotonin neurons, which reduces the release of serotonin (Harvey, 1997). When these receptors are exposed to higher levels of serotonin for longer periods, gradual down-regulation occurs and the result is disinhibition of serotonin release leading to increased serotonergic activity (Mongeau *et al.*, 1997).

2.2.2. The neuroanatomy of depression

Depression is a cause of neurobiological changes in the brain especially the prefrontal cortex, hippocampus and amygdala (Maletic *et al.*, 2007), shown in Figure 2.4. Abnormalities in these brain regions were found in depressed patients but not in healthy individuals (Drevets, 1998; Davidson, 2003). The prefrontal cortex, amygdala and hippocampus functions as a circuit and has a significant purpose in mood regulation, learning and contextual memory processes (Maletic *et al.*, 2007).

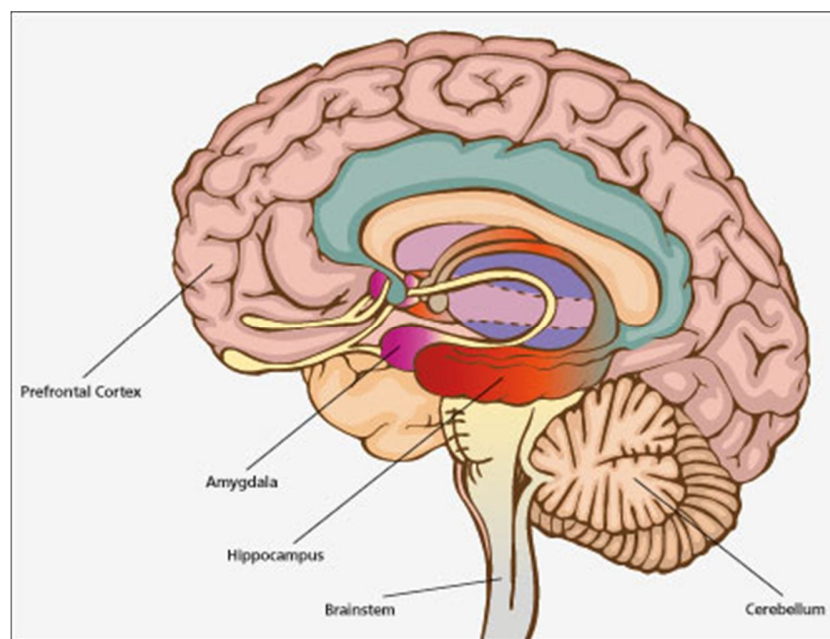


Figure 2.4: Schematic representation of the prefrontal cortex, amygdala and hippocampus (<http://www.vibrantmind.org>).

The prefrontal cortex can be divided into various subsections; the ventromedial prefrontal cortex (VMPFC), the lateral orbital prefrontal cortex (LOPFC), dorsolateral prefrontal cortex (DLPFC) and the anterior cingulate cortex (ACC) (Maletic *et al.*, 2007). Each area in the prefrontal cortex has its own purpose. The VMPFC mediates pain, aggression, sexual function and eating

behaviours (Swanson, 1987). The LOPFC assesses risk and regulates maladaptive and perseverative behaviours (Swanson, 1987). The DLFPC maintains executive function, sustained attention and memory processes (Swanson, 1987). The ACC is responsible for cognitive and/or executive functioning, assessing emotional and motivational information as well as monitoring outcomes of behavior and cognition (Bush *et al.*, 2000; McCormick *et al.*, 2006). Using regional blood flow studies, Drevets (1998) found that hyperactivity in the VMPFC and LOPFC and hypoactivity in the DLFPC exist in major depressive disorder patients compared with healthy controls. The abnormalities in these regions can be responsible for symptoms such as pain, anxiety, depressive mood, psychomotor retardation, apathy and problems with memory and attention, symptoms all associated with major depression (Maletic *et al.*, 2007).

The amygdala is associated with the regulation of emotional memory and mood. More specifically it is involved in motivation, controlling anxiety, the ability to experience pleasure and the degree of determination to reach goals (Nestler *et al.*, 2002). Abnormalities of the amygdala can be the cause of some of the symptoms which are experienced during depression such as anhedonia and anxiety (Nestler *et al.*, 2002). However, studies on structural alterations of the amygdala in patients with major depression have been inconsistent (Mervaala *et al.*, 2000; Frodl *et al.*, 2003; Frodl *et al.*, 2008).

The hippocampus plays a critical role in learning and memory as well as the regulation of motivation and emotion (Gray & McNaughton, 1983). The hippocampus is an important area in the limbic system and interacts with various regions of the cerebral cortex such as the prefrontal cortex, anterior thalamic nuclei, basal ganglia, amygdala and hypothalamus (Rosene & Van Hoesen, 1987). All of the above mentioned areas are part of the neuroanatomical network that regulates mood (Soares & Mann, 1997). There have been numerous studies on the significance of the function and volume of the hippocampus in depressive disorders. It has been reported that there is a reduction in hippocampal volume in patients suffering from depression when compared to healthy individuals (Frodl *et al.*, 2003; MacQeeun *et al.*, 2003). MacQeeun and colleagues (2003) found that there is a greater hippocampus volume reduction in patients who had multiple depressive episodes, especially from an early age, compared to patients that experience a first episode of depression (MacQeeun *et al.*, 2003). It seems that the morphological changes in the hippocampus are not present early in major depressive disorder, which suggests that antidepressant treatment is crucial during the first depressive episode to prevent damage to the hippocampal region (MacQeeun *et al.*, 2003). Santarelli and colleagues (2003) found that antidepressants can counteract the reduction in hippocampal volume by

increasing hippocampal neurogenesis. A crucial mechanism whereby antidepressants can reverse reduced hippocampal volume is by provoking the release of the neurotrophin, brain derived neurotrophic factor (BDNF) (Harvey *et al.*, 2003).

2.2.3. Neuropathological hypotheses of depression

Although there are several theories for the etiology of depression, the neuropathological basis of this disease still remains a mystery. Current antidepressant treatment is based on the monoamine hypothesis although there are a number of new hypotheses that have suggested alternative approaches to treating major depressive disorder. A few of the hypotheses for the etiology of depression will be discussed in this section.

2.2.3.1. The monoamine hypothesis

The monoamine hypothesis (Schildkraut, 1965) has been the leading theory for the etiology of depression since its proposal. This theory postulates that depression can be defined as an insufficiency of monoaminergic neurotransmission, especially noradrenaline, serotonin and dopamine in the brain (Baumeister *et al.*, 2003). There were two discoveries that led to the monoamine hypothesis, the first discovery was antidepressant-like effects of iproniazid and the second event was the observation that reserpine can cause depressive mood (Baumeister *et al.*, 2003).

Iproniazid was developed as an antitubercular agent (Fox, 1952). During clinical trials it was found that iproniazid has multiple therapeutic effects such as the destruction of *Mycobacterium tuberculosis*, an enhancement of appetite, a reversal of apathy and an increase in eudaimonia (Selikoff *et al.*, 1952). The antidepressant-like effects of iproniazid was later found to be a result of iproniazid's ability to inhibit MAO (Healy, 1997; Baldessarini, 2001). By inhibiting MAO, the monoamine levels in the brain increase leading to a reversal of depressive mood.

Reserpine is present in the *Rauwolfia Serpentina* root. During the 1930's it was found that this compound lowered blood pressure (Siddiqui & Siddiqui, 1932) and it was later used as an antihypertensive agent. Reserpine causes depletion of monoamine neurotransmitter stores and is associated with depression as an adverse effect (Schildkraut, 1965). This also supported Schildkraut's monoamine hypothesis for depression, that insufficient monoamines levels can cause depression (Schildkraut, 1965).

The monoamine hypothesis was further supported by the pharmacological mechanisms and antidepressant efficacy of the MAOIs and the tricyclic antidepressants (TCA), which immediately elevates synaptic levels of biogenic amines (Ordway *et al.*, 1998). Laboratory evidence suggested that tertiary amine containing TCAs including IMI, has a multifunctional working mechanism by inhibiting the re-uptake of both noradrenaline and serotonin, while secondary amine TCAs were more selective for NA (Carlsson *et al.*, 1969a; Carlsson *et al.*, 1969b; Ross & Renyi, 1969). It was subsequently suggested that the increased levels of serotonin may be responsible for the mood-elevating effects of the TCAs (Carlsson *et al.*, 1969b; Lapin & Oxenkrug, 1969). This was supported by medical evidence which showed the significance of serotonin in depression. The evidence included low concentrations of serotonin or its metabolite 5-hydroxyindoleacetic (5-HIAA) in the cerebrospinal fluid of depressed patients (Aschroft *et al.*, 1966; Dencker *et al.*, 1966) and the hindbrains of depressed suicide victims (Shaw *et al.*, 1967; Lloyd *et al.*, 1974; Bourne *et al.*, 1968; Korpi *et al.*, 1986). These observations prompted the development of SSRIs. Figure 2.5. show the relationship between noradrenaline, serotonin and dopamine in depression (Lanni *et al.*, 2009).

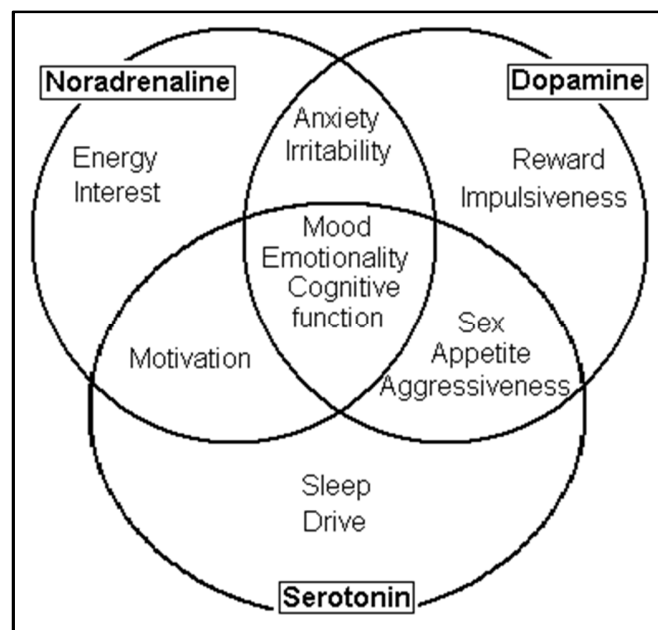


Figure 2.5: The role of noradrenaline, serotonin and dopamine in depression (Lanni *et al.*, 2009).

However, the monoamine hypothesis failed to completely explain the pathophysiology of depression and the working mechanisms of the antidepressants (Hindmarch, 2002). That antidepressants have a slow onset of action despite synaptic monoamine levels being elevated within hours after administration suggests that the working mechanism is possibly due to

secondary adaptive changes in the brain (Harvey, 1997; Popoli *et al.*, 2002). The monoamine hypothesis also fails to explain why some drugs such as cocaine and amphetamines are not effective antidepressants although they both increase monoaminergic activity (Baldessarini, 1989). Current antidepressant drugs are based on the monoamine hypothesis yet only 60-70% of patients respond to treatment (Sonnenberg *et al.*, 2008), which suggests that the mechanism of action to treat depression is far more complex than the monoamine hypothesis mechanisms might suggest (Nestler *et al.*, 2002). Finally, it does not explain why some antidepressants are effective as treatment for other diseases as well such as panic disorder, bulimia and obsessive-compulsive disorder (Sheehan *et al.*, 1993).

Although the monoamine hypothesis has significant limitations, it remains a breakthrough in understanding depression and has played a remarkable role in the development of antidepressive agents (Lanni *et al.*, 2009).

2.2.3.2. The cholinergic-adrenergic hypothesis

Janowsky and colleagues (1972) first suggested the significance of the cholinergic system in the pathophysiology of depression. They proposed that hyper-cholinergic states were responsible for depression and hypo-cholinergic states were responsible for mania (Janowsky *et al.*, 1972). It was found that cholinesterase inhibitor insecticides or organophosphate agents increased depressive-like symptoms, which resulted in the development of the cholinergic-adrenergic hypothesis (Janowsky *et al.*, 1972). Physostigmine, an acetylcholinesterase inhibitor that acts in the central nervous system evokes depressive-like behaviour, but not neostigmine an acetylcholinesterase inhibitor that only acts peripherally (Janowsky *et al.*, 1974). Recently, Furey and Drevets (2006) found that the cholinergic system is indeed hypersensitive in depressed patients thus confirming evidence that a hyper-cholinergic state is associated with depression. Acetylcholine plays an important role in regulating neuroendocrine, emotional and physiological responses to stress (Lanni *et al.*, 2009), and it is well known that depression is a stress-related disorder. Of note is that agents that enhance cGMP signaling, such as sildenafil, exert antidepressant-like effects that are amplified in the presence of a centrally acting anticholinergic agent (Brink *et al.*, 2008; Liebenberg *et al.*, 2010). Further support of the theory is that scopolamine, a muscarinic agent, has antidepressant effects in rats (Brink *et al.*, 2008; Furey & Drevets, 2006).

This theory has a few limitations such as the observation that not all anticholinergic agents are effective antidepressants. The cholinergic system only makes a contribution to the state of mind

and does not play a significant role in the regulation of mood (Picciotto *et al.*, 2008). Another limitation is that the tricyclic antidepressants' inhibition of the cholinergic system is associated with typical anticholinergic adverse effects and frequently results in discontinuation of these drugs.

2.2.3.3. The hypothalamic-pituitary-adrenal-axis hypothesis

The third hypothesis which may explain the etiology of depression is the hypothalamic-pituitary-adrenal-axis (HPA-axis) hypothesis, which suggests that chronic increased levels of corticosteroids may lead to decreased neurogenesis and thus depression (Hoschl and Hajek, 2001; Mizoguchi *et al.*, 2003; Sheline *et al.*, 1996). The HPA-axis plays an important role in the body's ability to manage stress, and acts to regulate the stress response along a sequence of events involving the hypothalamus, the anterior pituitary gland and the adrenal cortex (Hindmarch, 2002) as shown in Figure 2.6.

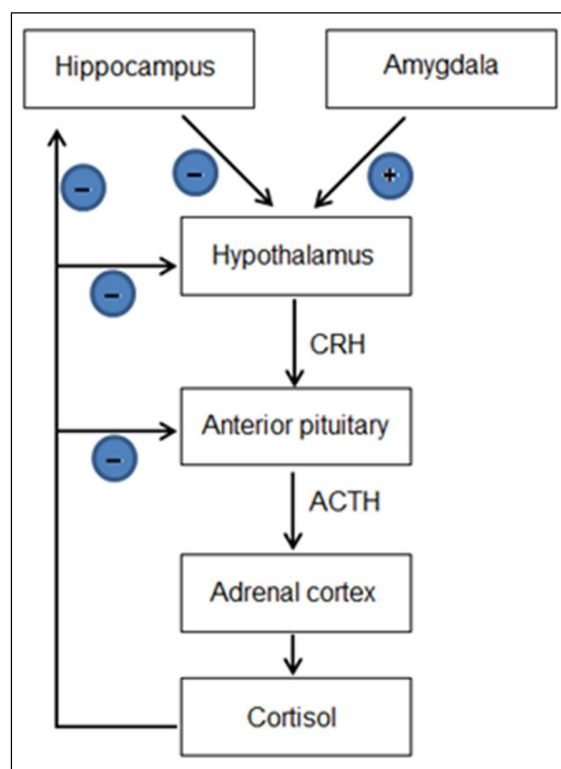


Figure 2.6: Illustration of a normal functioning HPA-axis in the human brain (Bingzheng *et al.*, 1990).

The endocrine hypothalamus is responsible for releasing corticotropin-releasing hormone (CRH) which stimulates the anterior pituitary to secrete adrenocorticotropin hormone (ACTH). ACTH acts on the cortex of the adrenal gland to stimulate secretion of the glucocorticoid hormone,

cortisol (Schimmer & Parker, 2001). Glucocorticoids in turn suppress corticotrophin-releasing factor (CRF) and ACTH synthesis and release. Impairment of this negative feedback system leads to elevated circulating cortisol levels (Pariante & Miller, 2001) which may damage hippocampal neurons (Nestler *et al.*, 2002) leading to loss of hippocampal volume (Holsboer 2000) and further dysregulation of the HPA-axis (Nestler *et al.*, 2002). Prolonged glucocorticoid secretion indirectly modulates the glutamatergic cascade (Sapolsky, 2000). Glutamate mediates the activation of subcellular calcium-dependent pathways, especially NO and cGMP (Harvey, 1996; McLeod *et al.*, 2001; Paul, 2001). With excessive glutamate release and thus increased NO production, NO reacts with superoxide to form the highly neurotoxic peroxynitrite anion (Dawson & Dawson, 1995). Cortisol-mediated damage to neurons occurs by overt release of glutamate and NO production, excessive activation of N-methyl-D-aspartate (NMDA) receptors, inhibition of glucose transport and an increase production of reactive oxygen radicals (Sapolsky, 2000) (see Figure 2.7.). Indeed, stress-induced increases in NOS activity are prevented by blocking cortisol synthesis (Harvey *et al.*, 2004). Elevated cortisol levels have been found in depressed patients (Board *et al.*, 1996) while administration of dexamethasone fails to suppress endogenous cortisol secretion (Carrol *et al.*, 1981). This dysfunction of the negative feedback system of the HPA-axis and the failure to suppress cortisol secretion is the cornerstone of the HPA-axis hyperactivity hypothesis. It has been found that cortisol levels return to normal once the depressed patient has reach remission (Hoschl & Hajek, 2001; Caetano *et al.*, 2004) and is possibly good evidence that the HPA-axis hyperactivity hypothesis has relevance.

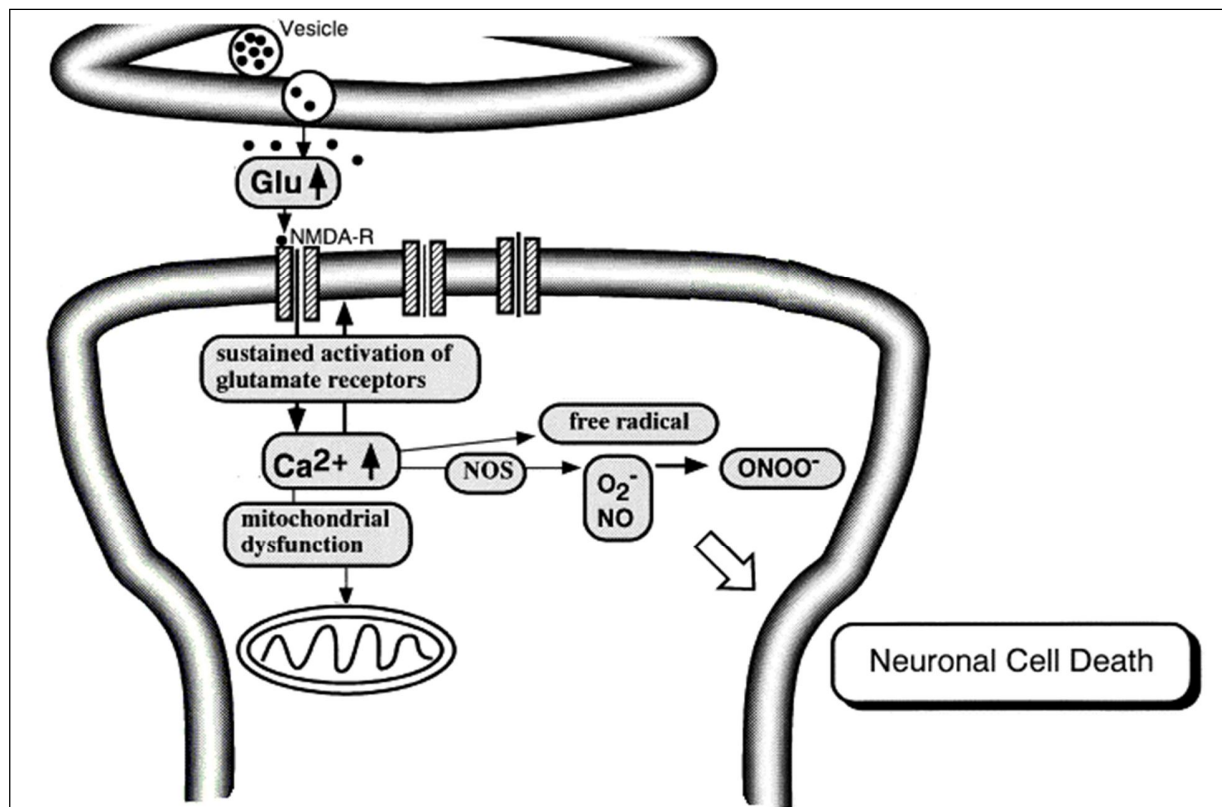


Figure 2.7: An illustration of elevation of extracellular glutamate induces various Ca^{2+} -dependent cascade reactions, increases NO production and production of reactive oxygen radicals which causes neuronal cell death (Harvey, 1996; Dawson & Dawson, 1998; Sapolsky, 2000; McLeod *et al.*, 2001; Paul, 2001). Glu: glutamate; NMDAR: NMDA-type glutamate receptor; NOS: nitric oxide synthase

2.2.3.4. The neuroplasticity hypothesis

Neuroplasticity describes the process whereby neurons in the central nervous system adapt to the changing needs of the brain. Failure of the normal function of this process may lead to various adverse neuroplastic changes, e.g. loss of synaptic interactions, increased atrophy and cell death, suppressed neural cell proliferation and changes in receptor density (Duman *et al.* 2000). This hypothesis consists of multiple theories, but which has led to a unified theory of depression where decreases in neurotrophic factors that regulate plasticity, altered monoaminergic neurotransmission and other abnormalities in neurotransmission are accounted for (Manji *et al.*, 2003; Duman & Monteggia, 2006).

Neurotrophic factors may be involved in the pathophysiology of depression (Sen *et al.*, 2008). Brain-derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate response

element binding protein (CREB) play a crucial role in the development and maintenance of the nervous system (Murer *et al.*, 2001). It has been found that BDNF modulates plasticity, inhibits cell death cascades and increases cell survival proteins responsible for neuronal proliferation and maintenance (Yulung *et al.*, 2009). BDNF plays a significant role in synaptic transmission and is important in the regulation of memory, cognition, mood and antidepressant response (Yulung *et al.*, 2009). The significance of chronic stress is well known in depression and it has been found that stress reduces neurogenesis and neurotrophic factors in the brain (Duman, 2004; Sen *et al.*, 2008). Duman & Monteggia (2006) found that chronic stress leads to reduced BDNF levels in the hippocampus. It has been observed that patients with long-term severe depression have atrophy of the hippocampus (Sheline, 2000; Steffens *et al.*, 2000; MacQueen *et al.*, 2003). Consistently high levels of glucocorticoids (Barbany & Persson, 1992; Schaaf *et al.*, 1998) and pro-inflammatory cytokines (Barrientos *et al.*, 2003) cause down-regulation of BDNF expression very likely as a consequence of hypercortisolemia (Gibbins & McHugh, 1962; Varghese & Sherwood Brown, 2001). The normal expression of BDNF can be restored through chronic antidepressant treatment (Duman & Monteggia, 2006; Warner-Schmidt & Duman, 2007).

Glutamate and γ -aminobutyric acid (GABA) play an important role in neural function and activation in the central nervous system (Harvey *et al.*, 2003; Harvey, 2008). As mentioned, glutamate release is promoted by glucocorticoids (Sapolsky, 2000), while the released glutamate regulates the release of neurotransmitters such as dopamine, serotonin, acetylcholine and noradrenaline (Flott & Seifert, 1991; Prast & Phillippu, 2001), which suggests that glutamate may play a profound role in the regulation of mood and thus depression. Glutamate NMDA receptor antagonists such as ketamine have been shown to induce a rapid antidepressant response in treatment refractory patients (Machado-Vieira *et al.*, 2009). Indeed, clinical studies have indicated altered glutamate levels in depression (Altamura *et al.*, 1995) while such patients present with platelet glutamate receptor super-sensitivity (Berk *et al.*, 2001).

Abnormalities in GABA concentrations are also associated with depressive disorders. It was found that depressed patients have reduced GABA levels (Gernet & Hare, 1981; Petty *et al.*, 1992), with decreased GABA levels a result of decreased synthesis or an overactive metabolism of GABA (Sanacora & Saricicek, 2007). Glutamate is involved in hippocampal neurogenesis (Balu & Lucki, 2009) by increasing hippocampal cell proliferation (Gould *et al.*, 1994; Cameron *et al.*, 1995). It has been suggested that excessive glutamatergic activity may be responsible for the progressive, deleterious neurocognitive effects associated with chronic

stress and major depressive disorders because of the high concentration of NMDA receptors in the hippocampus and amygdala (Magarinos & McEwen, 1995; Maes *et al.*, 1998). This has prompted the theory that depression may be brought on by excessive activation of the glutamate NMDA receptor cascade or reduced GABA (Harvey *et al.*, 2003; Harvey, 2008). In fact chronic antidepressant treatment reduces glutamate serum levels (Maes *et al.*, 1998) while GABA abnormalities can be corrected by antidepressant therapy such as SSRIs (Sanacora *et al.*, 2002).

It was found that NO regulates the release of neurotransmitters such as noradrenaline, serotonin, dopamine and acetylcholine in the central nervous system (Prast & Phillippu, 2001; Heiberg *et al.*, 2002). Evidence suggests that in depression there are abnormalities in the concentrations of nitric oxide or NOS in the central nervous system. Suzuki and colleagues (2001) found elevated levels of nitric oxide metabolites in depressed patients, although decreased levels of NOS have also been reported (Xing *et al.*, 2002). However, a paradoxical effect of NO in depression has been proposed, especially based on data from animal models. To illustrate this, NOS inhibitors (that reduce NO-cGMP signalling) are antidepressant (Harkin *et al.*, 1999), yet cGMP-PDE inhibitors (that increase NO-cGMP signalling) are also antidepressant (Brink *et al.*, 2008; Liebenberg *et al.*, 2010). It was also found that patients suffering from depression have a reduction in NOS-containing neurons in the hypothalamus (Bernstein *et al.*, 1998). Not only does glutamate increase NO release (Harvey, 1996; McLeod *et al.*, 2001; Paul, 2001), but the actions of glutamate are potentiated by NO through the inhibition of synaptic reuptake of released glutamate into the neuron (Pogun *et al.*, 1994) (see Figure 2.8). These modulatory actions of NO highlight its potential role in the regulation of mood, anxiety, motor activity as well as depression (Karatinos *et al.*, 1995).

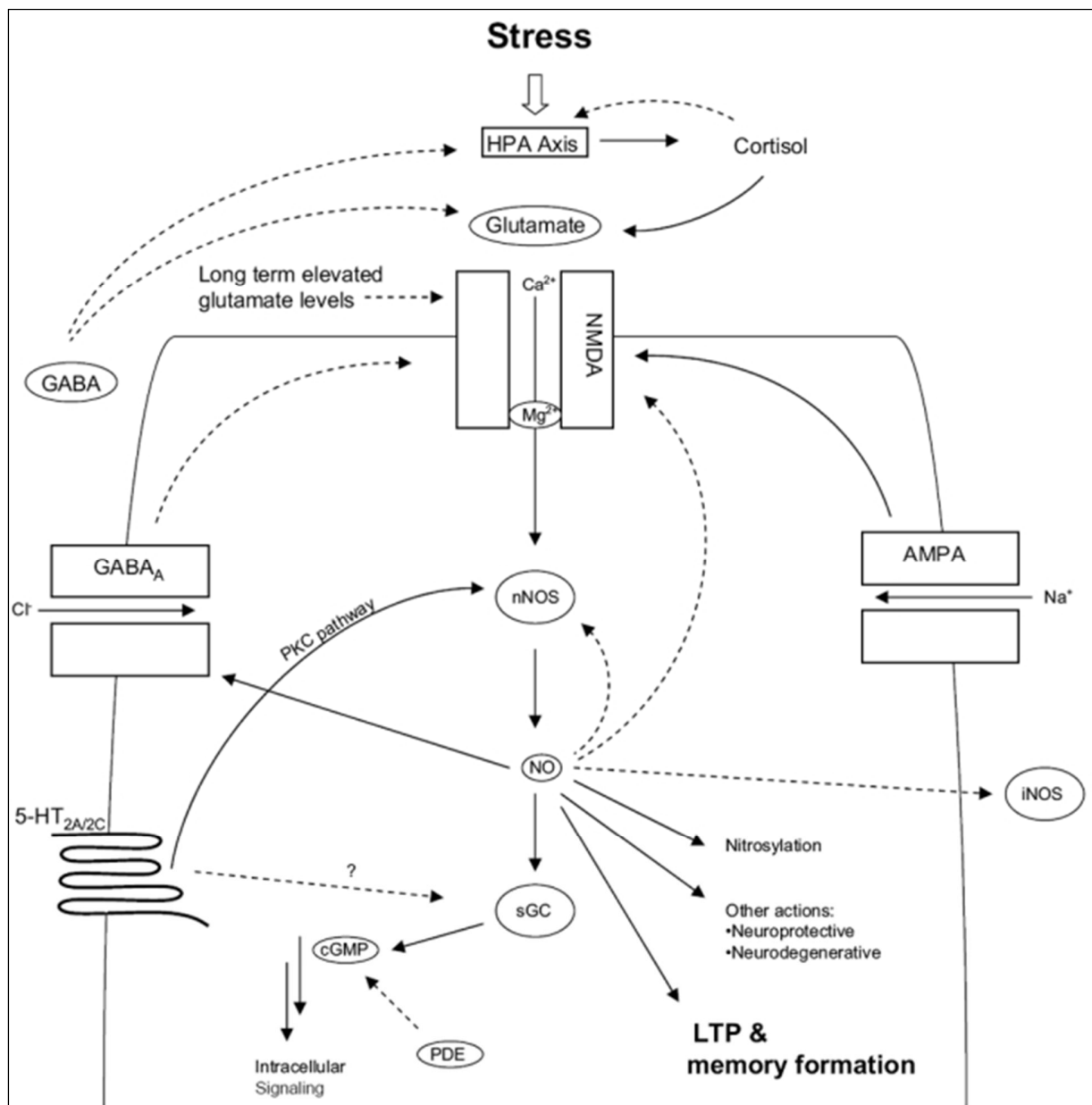


Figure 2.8: Activation of nNOS in the central nervous system as depicted by Oosthuizen *et al.*, (2005). NO is released via the action of adrenal glucocorticoids and the sequential release of glutamate in the hippocampus, activation of NMDA receptors, and the influx of Ca^{2+} leading to the activation of nNOS receptors leading to the Ca^{2+} -dependent activation of NOS and NO release. NO then acts as a modulator of further glutamate and GABA release, as well as promotes cellular plasticity, via the second messenger cGMP.

Preclinical studies show that NMDA antagonists (Skolnick, 1999; Rogoz *et al.*, 2008), NOS-inhibitors (Harkin *et al.*, 1999), guanylyl cyclase-cGMP inhibitors (Heiberg *et al.*, 2002) and

infusion of BDNF into the brain (Shirayama *et al.*, 2002) all lead to antidepressant-like effects. On the other hand, there is evidence that all classes of antidepressants, including electroconvulsive therapy, suppress NMDA receptor activity (Skolnick, 1999; Stewart & Reid, 2002), possibly by inducing adaptations of glutamate-dependent calcium-calmodulin-NOS-guanylyl cyclase signaling pathways (Skolnick, 1999; Paul, 2001). This statement is supported by the fact that major depressive disorder is associated with abnormalities in BDNF levels, glutamate signalling and subcellular NOS signalling. The glutamate-NO cascade therefore offers many possible explanations for the neuropathology of depression, which makes it an attractive target for novel antidepressant development.

2.2.3.5. The oxidative stress hypothesis

Morphologic and functional abnormalities associated with mitochondrial dysfunction and oxidative stress may be associated with depression (Harvey, 2008; Ng *et al.*, 2008). Alterations in multiple biomarkers of oxidative stress, including enzymes involved in the production or clearance of free radical species, have been observed in depression (Selek *et al.*, 2008; Kunz *et al.*, 2008; Wang *et al.*, 2009). Oxidative stress occurs when the redox balance favours the overbalance of free radicals, due to either their overproduction or deficiencies in antioxidant defence (Sies, 1997). Neuronal damage can directly be linked to excessive levels of reactive oxygen species (ROS), reactive nitrogen species (RNS) as well as some non-radicals that readily convert into radicals (Halliwell, 2006; Pacher *et al.*, 2007). A fine balance in the redox state of the brain is essential. The high metabolic activity of the brain is a persistent source of oxidative species, as utilisation of O₂ by energy generating mitochondria constantly generates oxygen free radicals (Raichle & Gusnard, 2002; Herculano-Houzel, 2011). Most free radicals in the brain are produced as a byproduct of energy production by the mitochondria, and dysfunction of mitochondrial function can significantly elevate oxidative stress (Berk *et al.*, 2013). Increased oxidative stress results in a cascade of neurodegenerative events, resulting in cell death (Beckman & Ames 1998; Di Mascio *et al.*, 1991; Zhao *et al.*, 2005). The hippocampus is particularly susceptible to oxidative stress, resulting in deficits in cognition (Avila-Costa *et al.*, 1999; Thiels & Klann 2002). As mentioned before, there is a reduction in hippocampal volume in patients suffering from depression when compared to healthy individuals (Frodl *et al.*, 2003; MacQueen *et al.*, 2003), which support the oxidative stress hypothesis.

MB has the ability to enhance mitochondrial function through the cycling between the reduced leucoMB and the oxidised MB states (Atamna & Kumar, 2010). NADH-dependent

dehydrogenases can reduce MB to leucoMB or O_2 to superoxide radical (Grivennikova & Vinogradov, 2006). MB and leucoMB serve as electron carriers within the mitochondrial electron transfer chain and inhibit the production of superoxide radicals by competing with O_2 at the site of free radical production at NADH-dehydrogenase (Grivennikova & Vinogradov, 2006).

2.2.4. Treatment of depression

Antidepressant drugs play a crucial role in the management of depression and long term treatment with antidepressants has proven to be more successful than short term treatment (Thase, 1992; Montgomery, 1994; Hirschfeld, 1994). Only 70% of patients with depression respond to current antidepressant treatment and not all of the patients that respond achieve complete remission (Fava & Davidson, 1996; Holtzheimer & Nemerhoff, 2006). A basic treatment regimen for major depressive episodes is shown in figure 2.9. There is still a great need for new antidepressant agents with faster onset of actions and higher remission rates. However, in this section the currently available antidepressants will be reviewed.

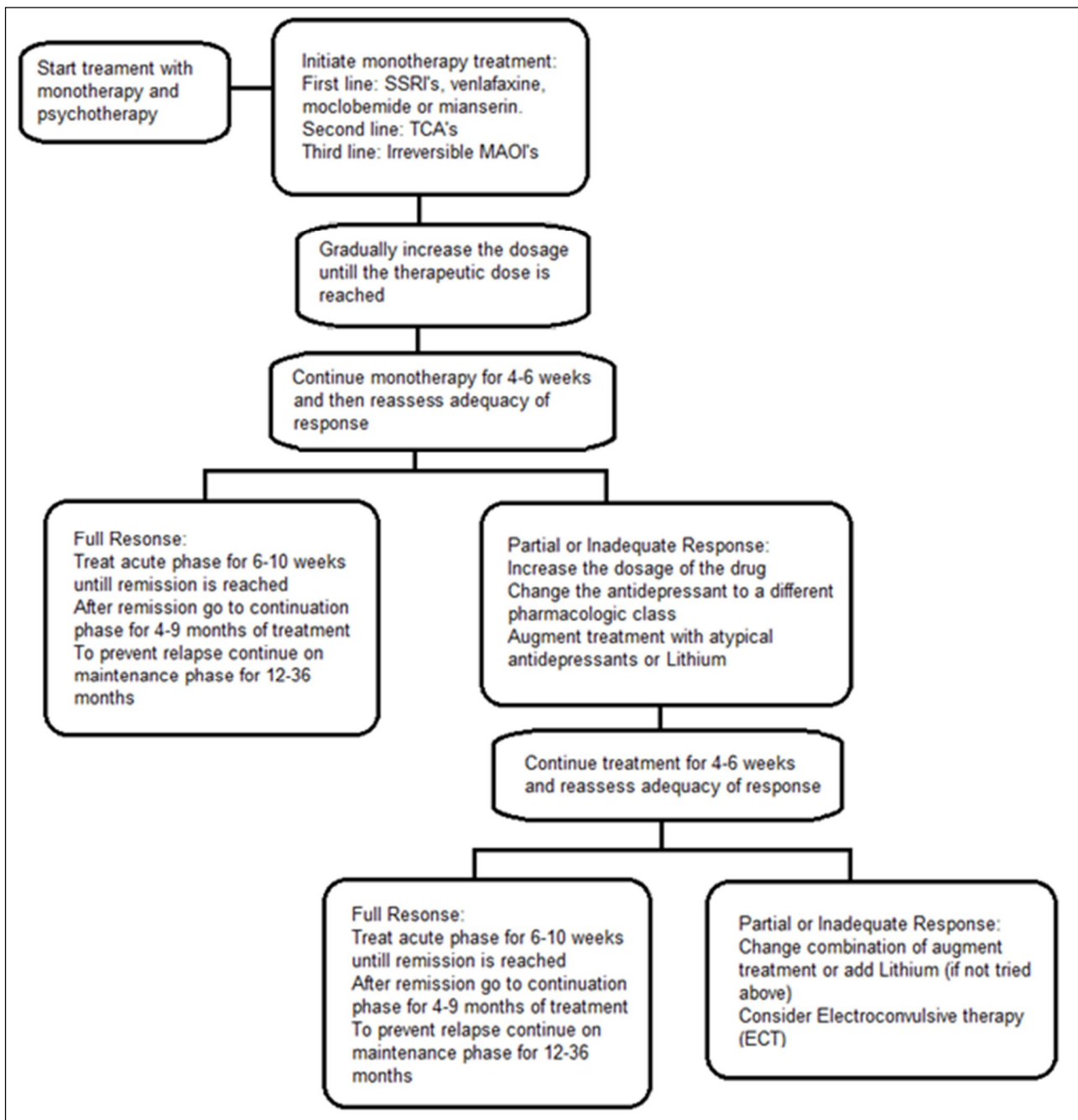


Figure 2.9: The treatment regimen for major depressive disorder (American Psychiatric Association; Priest *et al.*, 1992; Wells, 2009).

2.2.4.1. Monoamine oxidase inhibitors

Non-selective monoamine oxidase inhibitors (MAOI) were the first drugs used to treat depression. Iproniazid (figure 2.10.) was developed for the treatment of tuberculosis in 1951, but following the discovery of its antidepressant-like effect it was successfully used for the treatment

of depression in the 1950's. Iproniazid's mood-elevating qualities were later recognised to be the result of inhibition of monoamine oxidase (MAO) (Healy, 1997; Baldessarini, 2001).

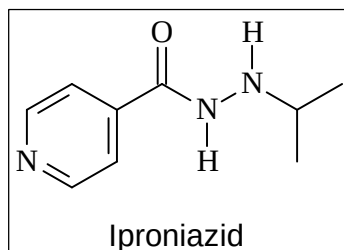


Figure 2.10: The chemical structure of iproniazid

MAO is a flavin-containing membrane bound enzyme found on the outer membrane of mitochondria (Edmondson *et al.*, 2007) of nerve terminals (Cesura & Pletscher, 1992; Baldessarini, 2001) and is responsible for metabolising catecholamines (Baldessarini, 2001). In the 1960's it was found that MAO is not a single enzyme, but consists of two isoforms: MAO type A and MAO type B. MAO-A is responsible for metabolising serotonin and noradrenaline and MAO-A inhibitors are used for the treatment of depression (Murphy *et al.*, 1987). MAO-B metabolises dopamine and plays an important role in the therapy of Parkinson's disease. As antidepressants the MAOIs increase the concentration of amines in the brain and support the monoamine hypothesis (Schildkraut, 1965). The MAOIs have a poor safety profile which is mostly due to the cheese reaction. As mentioned, some food products such as cheese and red wine contain tyramine, a dietary amine, which is metabolised by MAO-A in the gut. MAOI blocks the metabolism of tyramine and thus tyramine has access to the circulation. Tyramine causes the release of noradrenaline and this results in a severe hypertensive crisis which can be fatal (Finberg *et al.*, 1981; Finberg & Tenne, 1982).

Selective reversible MAO-A inhibitors, such as moclobemide (Figure 2.11), have been developed to inhibit MAO and elevate serotonin and noradrenaline levels without provoking the cheese reaction (Haefely *et al.*, 1992). Reversible inhibitors allow competition with dietary amines, which enables dietary amines such as tyramine to displace the reversible inhibitor from MAO-A and to be metabolised normally. Reversible inhibitors are thus less likely to provoke the cheese reaction (Youdim & Bakle, 2006).

The use of MAOIs, especially the irreversible inhibitors, has declined as a result of their poor safety profile. Their most notable adverse effects are hypertensive crisis when ingested with tyramine containing foods, sexual dysfunction, postural hypotension and the ST (discussed in

section 2.1.5.) when administered with either TCAs or SSRIs (Wells, 2009). Irreversible MAOIs, such as tranylcypromine and phenelzine, shown in Figure 2.11, are only used for “atypical” depression (Holtzheimer & Nemerhoff, 2006), although the reversible MAOI, moclobemide, can be used as a first line antidepressant (Priest *et al.*, 1992.).

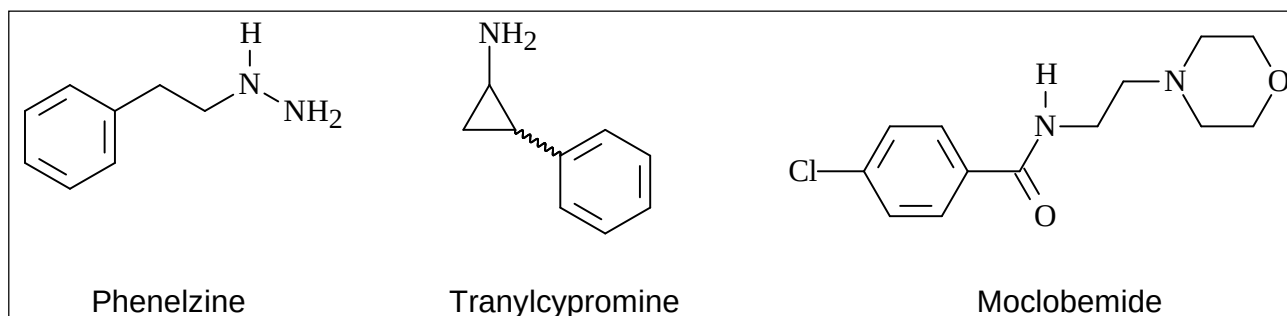


Figure 2.11: The chemical structures of phenelzine, tranylcypromine and moclobemide

2.2.4.2. Tricyclic antidepressants

During the process of developing new antihistamines, sedatives or analgesics, IMI (Figure 2.12.) was serendipitously discovered (Baldessarini, 2001). IMI was an ineffective antipsychotic, but had remarkable antidepressant qualities (Baldessarini, 2001) and became the archetypal TCA.

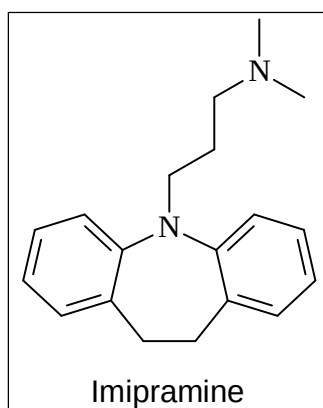


Figure 2.12: The chemical structure of imipramine

The working mechanism of TCAs is to inhibit the reuptake of noradrenaline and/or serotonin (Barker & Barkley, 1995). The TCAs can be divided into 2 groups; a group with secondary amine side chains with desipramine and nortriptyline as examples, and a group with a tertiary amine side chains with amitriptyline, clomipramine and IMI as examples (Wells, 2009). The secondary amine containing TCAs inhibit the neural transport of noradrenaline while the tertiary amine containing TCAs inhibit the reuptake of noradrenaline and serotonin (O'Donnell &

Shelton, 2011). However, tertiary amine derived TCAs are metabolised in the body to an active secondary amine (Rudorfer & Potter, 1997). The structures of these metabolites are shown in Figure 2.13. The TCAs are not selective and are also antagonists for adrenergic-, cholinergic- and histaminergic receptors (Wells *et al*, 2009), which may result in many adverse effects (Table 2.4).

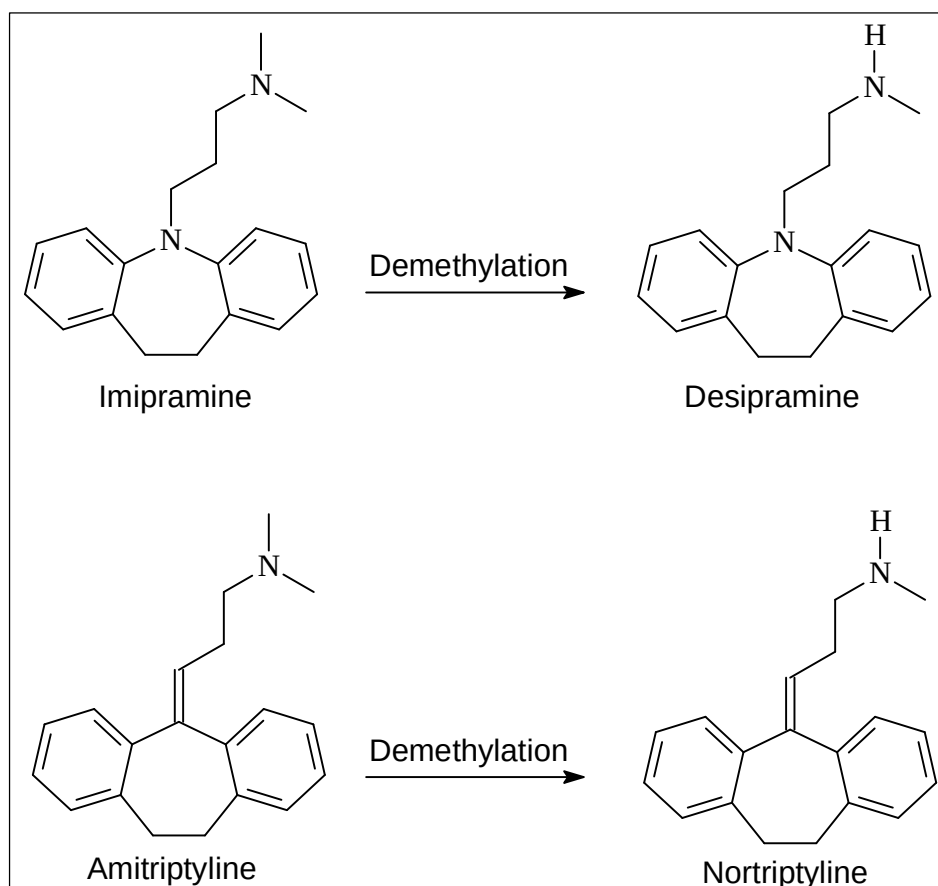


Figure 2.13: The demethylation of IMI to desipramine and amitriptyline to nortriptyline

TCAs can be used for all depressive subtypes, but their use is limited by their poor safety profile and the availability of equally effective, safer antidepressants. Another drawback with TCAs is their cardiac toxicity with overdose. MAOIs should also not be used in combination with TCAs in order to prevent the risk of toxicity. Other side effects that are associated with TCAs are summarised in Table 2.4. (Holzheimer & Nemerhoff, 2006; Wells, 2009).

Table 2.4: Side effects of tricyclic antidepressants mediated by non-specific receptor interactions, as indicated

Receptor	Side effects
Cholinergic receptor	• Dry mouth • Blurred vision • Constipation • Urinary retention • Tachycardia • Memory impairment • Delirium
α_1 -Adrenergic receptor	• Orthostatic hypotension • Cardiac conduction delay • Heart block
Histaminergic receptor	• Sedation • Weight gain
Other	• Sexual dysfunction

2.2.4.3. Selective serotonin reuptake inhibitors

The original theory was that insufficient noradrenaline and/or dopamine were responsible for depressive disorders. When it was found that the tertiary amine TCAs also block serotonin reuptake it raised the question as to the role of serotonin in depression (Nestler *et al.*, 2002). The SSRIs were developed in answer to this question (Nestler *et al.*, 2002). Their working mechanism of action is to selectively block the presynaptic reuptake of serotonin (Holtzheimer & Nemerhof, 2006). In 1972 Wong and colleagues (1974) from Eli Lilly and Company found that fluoxetine, a derivate of N-methyl-phenoxyphenylpropylamine (Figure 2.14.), is a potent selective serotonin reuptake inhibitor. This publication led to the clinical development of fluoxetine (Prozac™) for the treatment of depression and soon led to the synthesis of many other SSRIs (Wong *et al.*, 1995). Their chemical structures are shown in Figure 2.15.

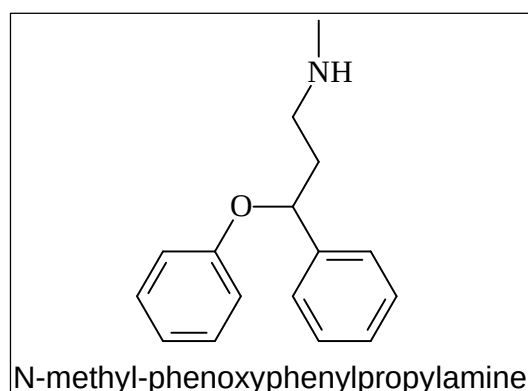


Figure 2.14: The chemical structure of N-methyl-phenoxyphenylpropylamine

Fluoxetine was approved by the Food and Drug Administration (FDA) of the United States in 1987 as an antidepressant (Wong *et al.*, 1995). Subsequently released SSRIs included zimeldine (later withdrawn for reasons of toxicity), fluvoxamine, paroxetine, sertraline, citalopram and escitalopram. The SSRIs quickly replaced the older antidepressants and became the first line treatment for depression (Tollenfson & Rosenbaum, 1998). The SSRIs have a safer overdose profile and are more tolerable. Although they had fewer side effects, specific adverse effects were quickly noted, including nausea, vomiting, diarrhea, headache, insomnia, fatigue and sexual dysfunction (Wells, 2009).

Contra-indications of the SSRIs include the co-administration with drugs that elevate serotonin levels such as MAOIs, TCAs, amphetamine, MB and even some analgesics such as meperidine and tramadol (Gillman, 2005). Such a combination leads to raised intrasynaptic serotonin levels and has the risk of causing ST that may be fatal (Gillman, 2006). ST is mostly associated with SSRI-MAOI combinations (Gillman, 2006), the most typical clinical features being tremor, clonus, myoclonus, hyperreflexia, diaphoresis, tachycardia, tachypnea and agitation (Gillman & Whyte, 2004; Gillman, 2006). During the advanced stage of ST pyramidal rigidity and confusion are also present (Gillman & Whyte, 2004; Gillman, 2006). For an accurate diagnosis of ST only a few symptoms need to be present such as clonus, agitation, diaphoresis, tremor and hyperreflexia (Whyte, 2004).

The indications for the use of SSRIs, especially fluoxetine, are major depression, obsessive-compulsive disorder, bulimia nervosa and panic disorder (Whong *et al.*, 1995).

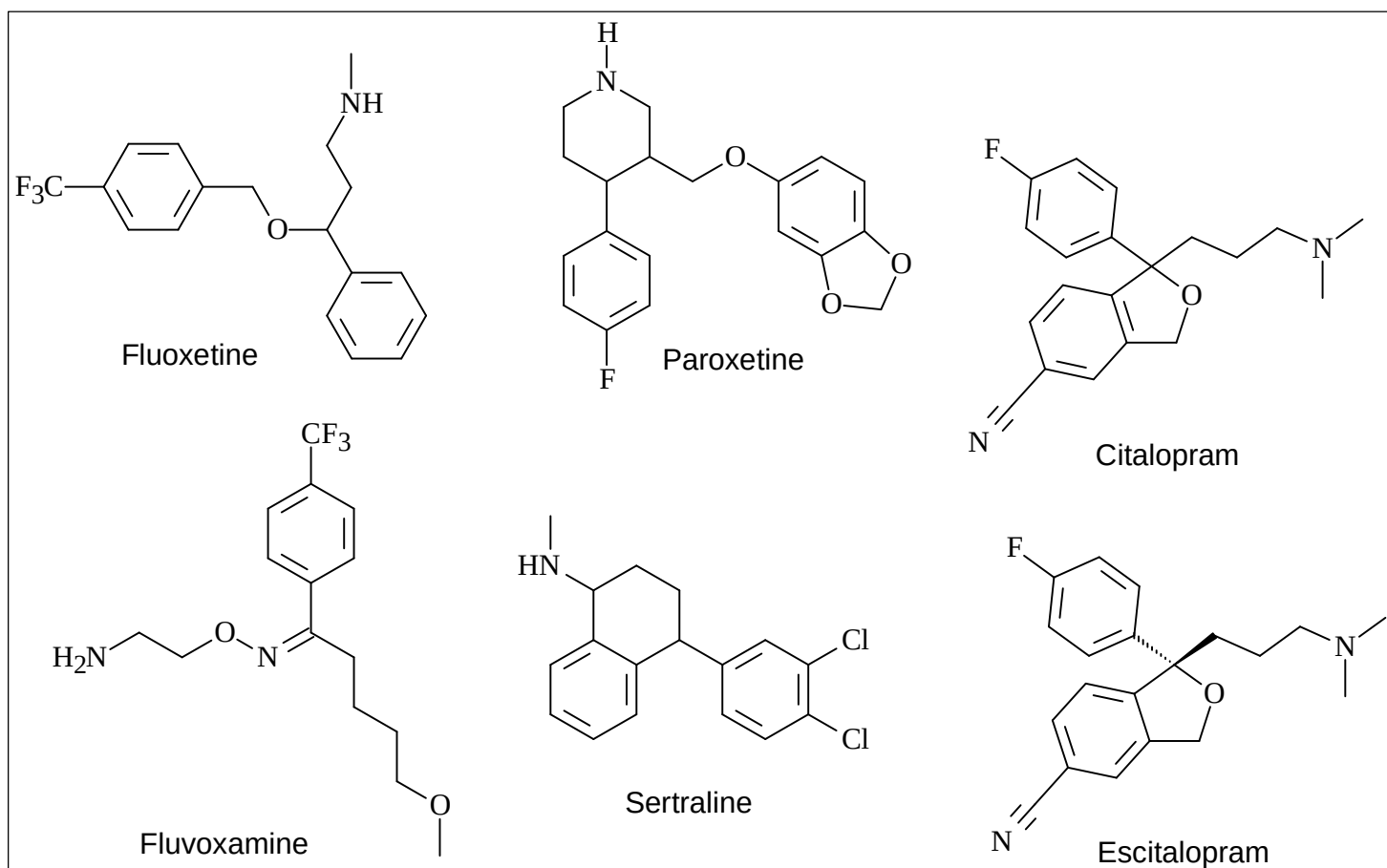


Figure 2.15: The chemical structures of fluoxetine, paroxetine, citalopram, escitalopram, sertraline and fluvoxamine.

2.2.4.4. Serotonin-noradrenaline reuptake inhibitors

The serotonin noradrenaline reuptake inhibitors (SNRIs) were developed to mimic the working mechanism of the tertiary amine TCAs but without antagonism of other receptors (O'Donnell & Shelton, 2011). Their mode of action includes the inhibition of both the neural serotonin and noradrenaline transporter (O'Donnell & Shelton, 2011) resulting in an increase of both serotonin and noradrenaline, thus yielding an antidepressant affect. Venlafaxine and duloxetine are members of the SNRIs and their chemical structures are shown in Figure 2.16.

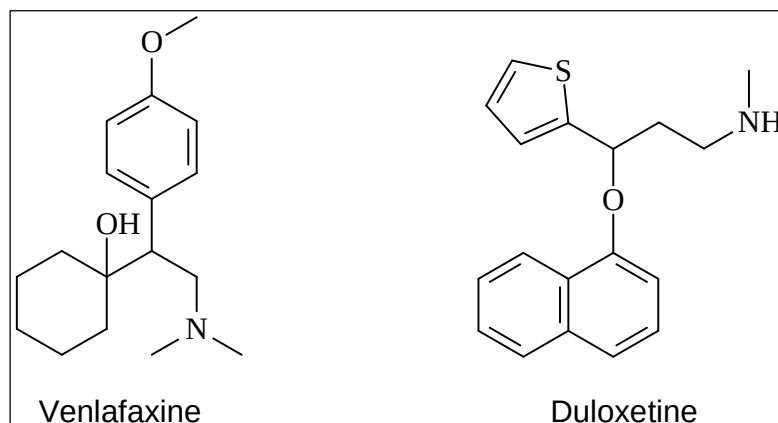


Figure 2.16: The chemical structures of venlafaxine and duloxetine

The advantages of these drugs are their shorter wash-out period, seven days for venlafaxine and five days for duloxetine, before the initiation of treatment with a MAOI (O'Donnell & Shelton, 2011). Millan (2004) stated that venlafaxine may have a faster onset of action and be more efficacious than the SSRIs, although its faster action and improved efficacy have been advocated at higher than nominal dosages (Mbaya, 2002). The SSRIs and SNRIs share a similar side effect profile such as sexual dysfunction, nausea, vomiting, insomnia and fatigue, although venlafaxine is also associated with increased diastolic pressure while duloxetine may cause increased sweating (Wells, 2009). Currently venlafaxine can be used as a first line drug for the treatment of major depression, anxiety and pain (O'Donnell & Shelton, 2011).

2.2.4.5. Atypical antidepressants

The atypical antidepressants are a group of drugs with chemical structures that differ from the well-known classes of antidepressants (shown in Figure 2.17.), while often having a different mechanism of action. These drugs are nevertheless efficacious in treating depression (Fava & Kendler, 2000), and includes bupropion, mirtazapine, nefazodone, trazodone and agomelatine.

Bupropion is a monocyclic phenylbutylamine of the aminoketone group and is structurally different from any other antidepressant (Ruiz *et al.*, 2011). Bupropion selectively inhibits the reuptake of dopamine (Ascher *et al.*, 1995). An advantage of bupropion over other antidepressants is that bupropion does not cause weight gain, since it has no affinity for histaminergic or serotonergic receptors (Masand & Gupta, 2002). It also does not have an effect on serotonin receptors especially 5-HT_{2A/C}, which antagonises dopaminergic function in the cortex and striatum and thus does not cause sexual dysfunction (Harvey, 1996; Baldessarini, 2001; Harvey *et al.*, 2003). The most common adverse effects of bupropion are nausea,

vomiting, tremor, insomnia, dry mouth and skin reactions (Wells, 2009). Bupropion is known to cause seizures, although this is dose dependent and can be triggered by previous head trauma or a tumor in the central nervous system (Wells, 2009). The contra-indications for bupropion are bulimia nervosa and anorexia nervosa (Wells, 2009). According to O'Donnell & Shelton (2011) bupropion's indications are the treatment of major depressive disorder, prophylactic treatment of seasonal depression and as a smoking cessation treatment. Bupropion and SSRIs can be used in combination to overcome some of the side effects caused by the SSRIs such as sexual dysfunction (Jacobsen, 1996).

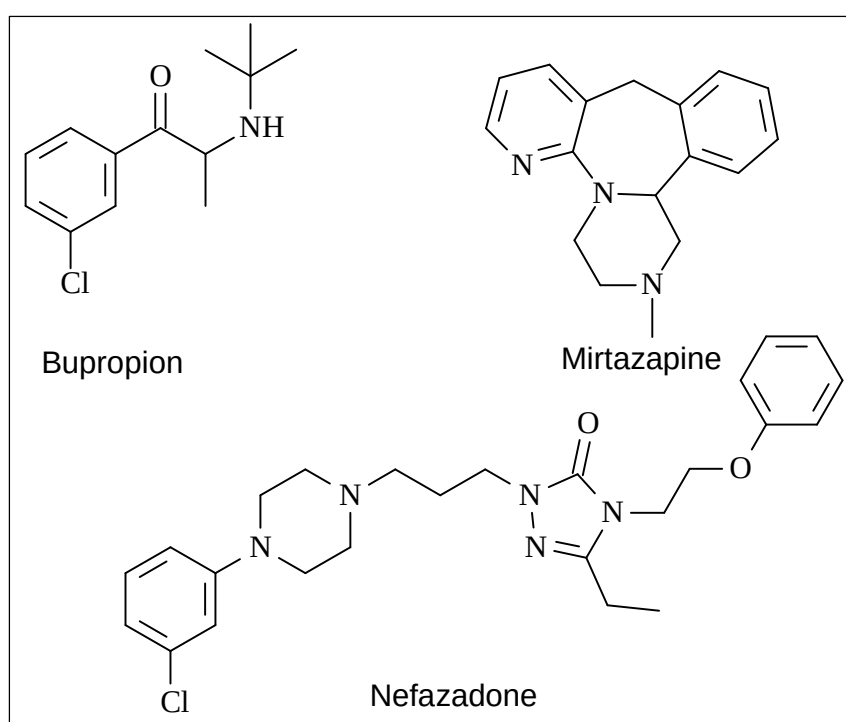


Figure 2.17: The chemical structures of bupropion, mirtazapine and nefazadone

Mirtazapine is a tetracyclic compound that is chemically classified as a piperazino-azepine (De Boer *et al.*, 1988; Mullin *et al.*, 1996). The working mechanism is associated with the ability to block presynaptic α_2 -autoreceptors and heteroreceptors, which regulates noradrenaline and serotonin release (De Boer *et al.*, 1988). The inhibition of these receptors interrupts the negative feedback mechanism responsible for decreasing noradrenaline release. Mirtazapine thus enhances noradrenaline release (Fawcett & Barkin, 1998). Noradrenaline then acts on α_1 -receptors, which enhances serotonergic firing (De Boer, 1996). As mentioned mirtazapine also blocks α_2 -heteroreceptors and subsequently prevents noradrenaline's inhibitory effect on serotonin release (Fawcett & Barkin, 1998). Mirtazapine has less serotonergic side effects than

SSRIs because it antagonises 5-HT₂ and 5-HT₃ receptors (De Boer, 1996). By blocking the 5-HT₃ type receptor, mirtazapine acts as an antiemetic and thus does not cause nausea (Barkin 1997). It is much safer than the TCAs and SSRIs and does not present with headache, anxiety, agitation or sexual dysfunction, and thus presents with a favorable side effect profile (Fawcett & Barkin, 1998). However, mirtazapine has affinity for H₁-receptors and is known to cause drowsiness (O'Donnell & Shelton, 2011) as well; as increased appetite and weight gain. In rare cases, agranulocytosis may be an adverse effect (O'Donnell & Shelton, 2011). Mirtazapine is effective in treating moderate-to-severe major depression and can be used as a first line treatment or in combination with SSRIs (O'Donnell & Shelton, 2011).

Nefazodone and trazodone are chemically classified as phenylpiperazines (Davis *et al.*, 1997). Nefazodone's pharmacological effect is a result of the inhibition of the postsynaptic 5-HT_{2A} receptors (Eison *et al.*, 1990) and inhibition of serotonin reuptake (Eison *et al.*, 1990; Owens & Nemerhoff, 1994). Trazodone may cause priapism in rare cases (O'Donnell & Shelton, 2011), while it may also cause panic and severe anxiety (due to its metabolite, *meta*-Chlorophenylpiperazine) (Kennett *et al.*, 1989). Nefazodone is associated with a lower incidence of anticholinergic and cardiovascular effects as well as agitation, anxiety, nausea and sexual dysfunction (Davis *et al.*, 1997). Adverse effects of nefazodone are dizziness, dry mouth, constipation and confusion (Davis *et al.*, 1997). In some countries nefazodone have been withdrawn from the market because it may cause liver failure in rare cases (O'Donnell & Shelton, 2011). It was found that nefazodone is just as effective in treating depression as the SSRIs (Davis *et al.*, 1997), but its use is limited due to hepatotoxicity. Trazodone is often used for its sedative properties.

2.2.4.6. Agomelatine

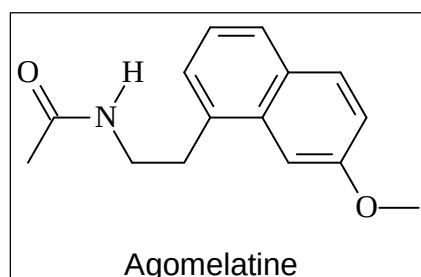


Figure 2.18: The chemical structures of agomelatine

Agomelatine (Figure 2.18.) is the first clinically available antidepressant without any monoaminergic mechanism of action, which targets the perturbed biological rhythms linked to

depressed states (De Bodinat *et al.*, 2010). Agomelatine is a potent agonist of the melatonergic MT₁ /MT₂ receptors, as well as an antagonist of 5-HT_{2C} receptors (Racagni *et al.*, 2011). Both the melatonergic and 5-HT_{2C} effects are important for agomelatine's antidepressant effect (Racagni *et al.*, 2011) by resynchronisation of circadian rhythms, increasing extracellular dopamine and NA in the frontal cortex and induction of neurogenesis (De Bodinat *et al.*, 2010). Agomelatine is as efficacious as other well-established antidepressants and improves mood, anxiety, daytime alertness and quality of sleep from initiation of therapy to remission (Demyttenaere, 2011). The safety and tolerability of agomelatine has been shown to be good in individual and pooled trials (Kennedy & Rizvi, 2010). Agomelatine does not cause the most common side-effects associated with antidepressants used, such as, sexual dysfunction and weight gain (Kennedy *et al.*, 2008; Montejo *et al.*, 2010; Kennedy & Rizvi, 2010).

Agomelatine is contraindicated in patients with hepatic impairment and to prevent liver damage, it is advised that liver function tests should be performed in all patients (Kennedy and Rizvi, 2010). The discontinuation rate of agomelatine were found to be lower compared to venlafaxine and sertraline

2.3. Monoamine oxidase

2.3.1. General background

MAO was discovered in 1928 when Mary Hare-Bernheim described an enzyme involved in the metabolism of tyramine, which she called tyramine oxidase (Youdim *et al.*, 1988). A few years later Zeller named it mitochondrial monoamine oxidase after Blascko found that tyramine oxidase, noradrenaline oxidase and aliphatic oxidase was one and the same enzyme (Youdim *et al.*, 1988).

During the late 1960's it was discovered that MAO consisted of two isoforms (Johnston, 1968). The two isoforms have different affinities for different substrates and inhibitors. MAO type A is blocked by clorgyline and metabolises noradrenaline and serotonin, and MAO type B is not inhibited by clorgyline and favours benzylamine as a substrate (Johnston, 1968).

2.3.2. The three dimensional structure of MAO

MAO is located on the outer mitochondrial membrane, and is a flavo-protein with flavin adenine dinucleotide (FAD) as a co-factor (Youdim *et al.*, 2006). The flavin binds to MAO-A and MAO-B covalently via a thioether linkage between a cysteinyl residue and the 8 α -methylene of the

isoalloxazine ring (Kearney *et al.*, 1971). The chemical structure of FAD is shown in figure 2.19. In MAO-B, Cys397 is the residue involved, and in MAO-A it is Cys406 that forms the linkage with the flavin (Bach *et al.*, 1988).

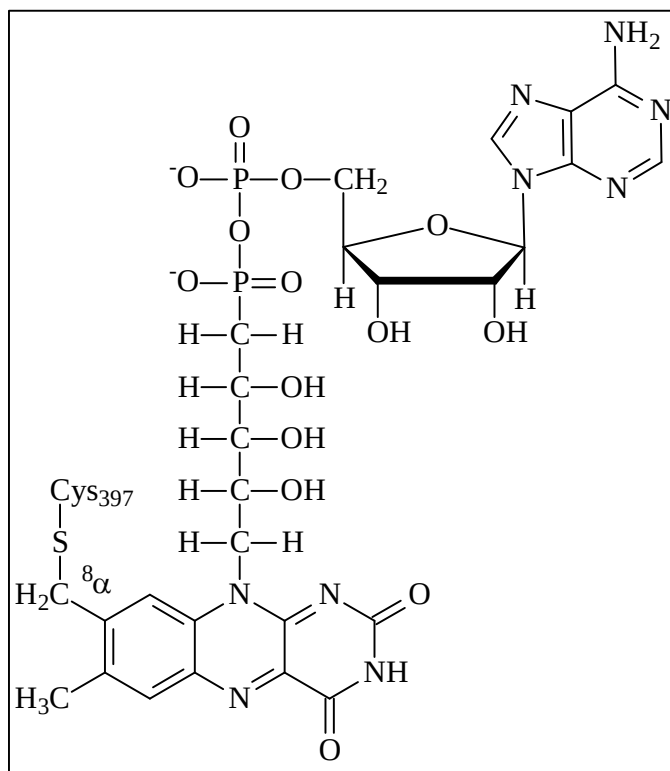


Figure 2.19: The chemical structure of FAD

Human MAO-A crystallises as a monomer (DeColibus *et al.*, 2005). As shown in Figure 2.20., MAO-A has one substrate binding cavity, with a cavity size of $550 \text{ \AA}^3$ and a protein loop at the entrance (Edmondson *et al.*, 2007). The substrate binding cavity of MAO-A has a “round” shape and is mostly hydrophobic (Edmondson *et al.*, 2007). In contrast MAO-B crystallises as a dimer (Edmondson *et al.*, 2007), as shown in Figure 2.21. MAO-B has two active site cavities, an “entrance cavity” which is hydrophobic and has a capacity of $290 \text{ \AA}^3$, and a “substrate cavity” which is also mostly hydrophobic and has a volume of $390 \text{ \AA}^3$ (Edmondson *et al.*, 2007). There is also a protein loop at the entrance of the entrance cavity and an Ile-199 side chain that acts as a “gate” between the two cavities of MAO-B (Edmondson *et al.*, 2007). When the Ile-199 side chain is in the “open” position, the active site of MAO-B exists as two separate cavities (Edmondson *et al.*, 2007). When the Ile-199 side chain is in the “closed” position, the active site of MAO-B exists as a single elongated cavity. A comparison of the active sites of human MAO-A and MAO-B is shown in Figure 2.22.

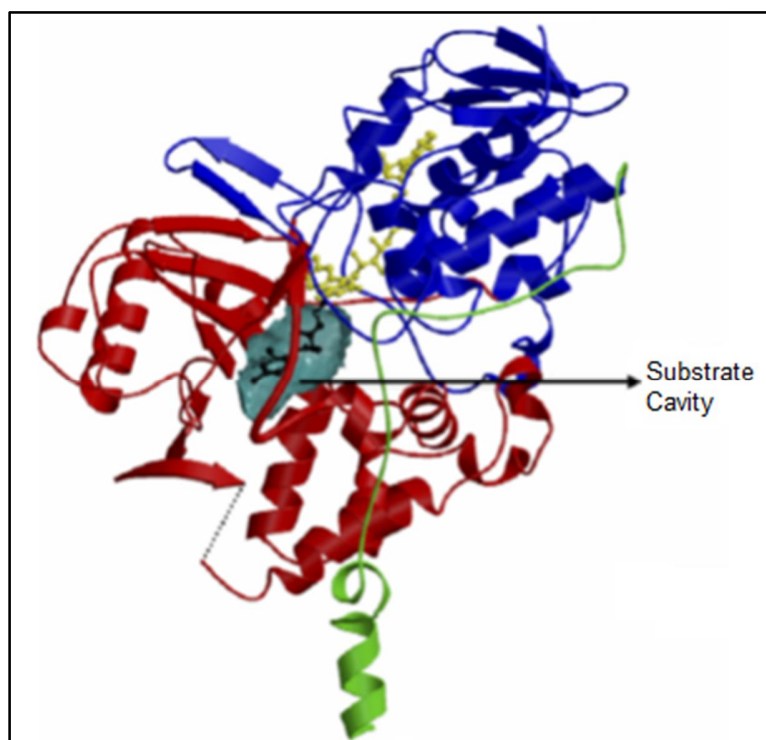


Figure 2.20: A ribbon diagram of the human MAO-A structure (Edmondson *et al.*, 2007).

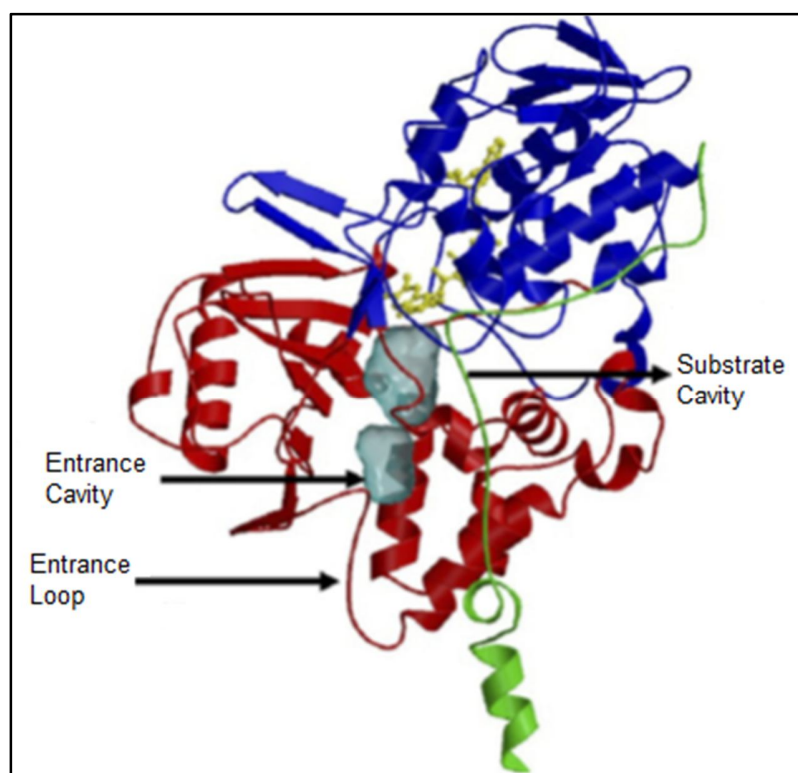


Figure 2.21: A ribbon diagram of the monomeric unit of the human MAO-B structure (Edmondson *et al.*, 2007).

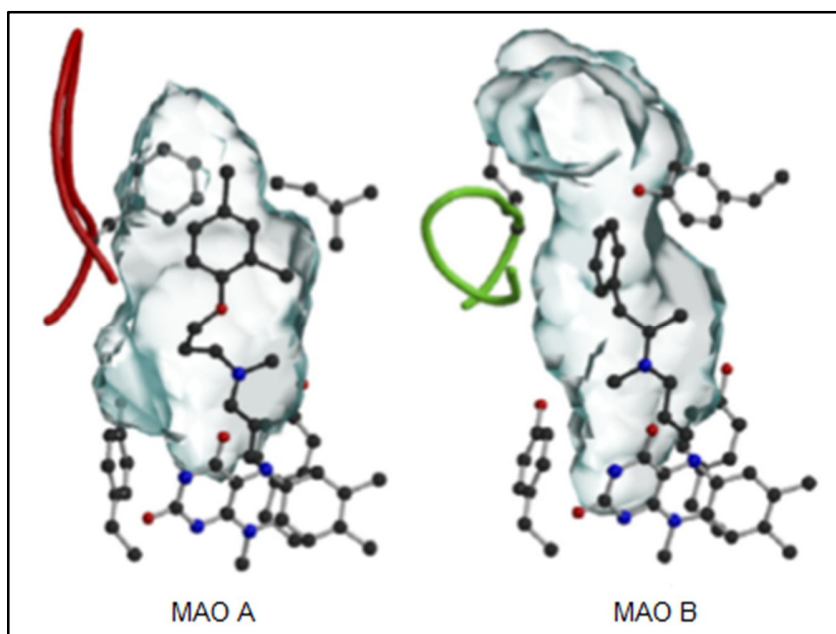


Figure 2.22: A comparison of the active site cavities of human MAO-A (left) and human MAO-B (right). Clorgyline is in the active site of MAO-A and deprenyl is in the active site of MAO-B. The active site “shaping loop” structure is in red for MAO-A and green for MAO-B (Edmondson *et al.*, 2007).

2.3.3. Inhibitors of MAO

MAO inhibitors have made a significant contribution to the treatment of both psychiatric and neurological disorders. The MAOIs can be classified into three groups, which are showed in Table 2.5 (Riederer *et al.*, 2004).

Table 2.5: The available MAOIs classified into three groups

Older, irreversible, nonselective agents	Reversible, selective MAO-A inhibitors	Irreversible, selective MAO-B agents	Irreversible, selective MAO-A agents
Phenelzine Tranylcypromine Iproniazid Isocarboxazid Nialamide Safrazine	Moclobemide Toloxatone	Selegiline Rasagiline	Pargyline Clorgyline

The older, irreversible, nonselective agent, iproniazid was the first antidepressant to be developed and made a significant contribution to the development of the monoamine hypothesis of depression, on which current antidepressant treatment is based. The main indication for this group of MAOIs is depression, more specifically atypical depression and therapy resistant depression (Paykel & White, 1989; Vink *et al.*, 1994). However, safety issues with the irreversible, nonselective MAOIs limits their use (Ballenger *et al.*, 1998). As discussed above, the “cheese reaction” is responsible for non-compliance among patients. Other limitations are the interactions between MAOIs and sympathomimetic agents such as ephedrine and pseudoephedrine, as well as SSRIs and other serotonin drugs (Dingemans, 1993). The general side-effects of the irreversible, non-selective MAOIs are orthostatic hypotension, sleep disturbances and agitation (Remick *et al.*, 1989). Great care should be taken when patients with hepatic impairment use MAOIs, in which case the dosage should be adjusted to a third of the nominal dose (Atkinson & Ditman, 1965; Fitton *et al.*, 1992).

The reversible, selective MAO-A inhibitors such as moclobemide are used in the treatment of severe depression (Angst *et al.*, 1995) and social phobias (Riederer *et al.*, 2004). Dietary restrictions are unnecessary because it does not provoke the “cheese reaction” (Riederer *et al.*, 2004). However, great caution should be exercised when moclobemide and SSRIs are co-administered due to the risk of ST (Dingemans, 1993). Adverse effects associated with moclobemide are headaches, insomnia, agitation, dizziness and nausea (Laux *et al.*, 1995). Moclobemide does not have effects on the cardiovascular or cholinergic system (Moll & Hentzel, 1990; Fulton & Benfield, 1996) and has a superior tolerability and safety profile compared to the TCAs and the nonselective MAOIs (Riederer *et al.*, 2004). The dose of moclobemide in patients with hepatic impairment should be reduced to one-third when moclobemide is used (Atkinson & Ditman, 1965; Fitton *et al.*, 1992).

Selective MAO-B inhibitors such as selegiline are used in the treatment of Parkinson's disease (Knoll & Magyar, 1972). Birkmayer and colleagues (1975) found that the co-administration of L-dopa and selegiline improves the treatment outcome in Parkinson's disease. Selegiline as monotherapy is used to control motor symptoms, and in combination with L-dopa it is used to treat major complications in motor performance associated with L-dopa treatment, and to prevent the progression of Parkinson's disease (Riederer *et al.*, 2004). MAO-B inhibitors such as selegiline may be neuroprotective by altering oxidative stress which plays a role in the pathogenesis of Parkinson's disease (Gerlach *et al.*, 1992; Olanow, 1996). Side effects associated with selegiline are headache, nausea, dry mouth and constipation (Bhattacharya *et*

al., 2003). Dopaminergic side effects such as dyskinesia and hallucinations are associated with the combination of MAOIs and L-dopa and can be avoided by lowering the L-dopa dosage (Riederer *et al.*, 2004).

2.4. Summary

In this chapter, the pharmacology of the charged molecule, MB, was discussed including its targets in the human body and most common adverse-effects. MB has a multi-mode of action by targeting the nitricoxide and monoaminergic systems and has shown promising antidepressant and anxiolytic qualities in clinical trials (Naylor *et al.*, 1986). The hypotheses for the etiology of depression as well as the current antidepressant agents available were also discussed in this chapter. The shortcomings of current treatment regimens enhance the need for the development of new antidepressants with a faster onset of action and improved safety profiles. There is a growing interest in the clinical benefit and utility of MAO-inhibitors in the treatment of depression (Gillman, 2011), which makes MB an ideal lead compound for the design and synthesis of new antidepressant agents.

Chapter 3

Synthesis

3.1. Introduction

As discussed in the introduction, MB acts at multiple cellular and molecular targets and as a result possesses diverse medical applications. Among these is a high potency reversible inhibition of MAO-A that may, at least in part, underlie its adverse effects but also its antidepressant-like actions. MB has been described as having a dual antidepressant action, by inhibiting MAO and NOS (Harvey *et al.*, 2010), both mechanisms having antidepressant effects independently. Since MAO-A inhibition is associated with several side effects, it may be advantageous to “design-out” the MAO-A inhibition properties of MB in order to improve its safety profile, yet retain its antidepressant effects via actions on NO synthase. In this study we propose to design and synthesise a derivative of MB and to characterise its interactions with human MAO-A. The principal goal of this study will be to design a close structural analogue of MB that inhibits MAO-A to a lesser degree compared to MB, but still retains an antidepressant activity. Such a compound may represent an ideal pharmacological tool to probe the antidepressant mechanism of MB, and may serve as lead compound for the design of MB-derived drugs with an improved safety profile.

The aim of this study is therefore to eliminate or reduce the MAO-A inhibitory component of MB in the hope of retaining its other beneficial pharmacological properties, such as its NOS inhibitory actions as well as its effects on redox chemistry. For this purpose a close structural analogue of MB will be synthesised and characterised as a potential inhibitor of human MAO-A and MAO-B. The approach that will be followed will be to make a relatively small structural change to MB in order to minimise or remove completely the MAO-A inhibition properties of MB but at the same time to retain its antidepressant efficacy, as will be assessed using the rat forced swim test (FST). It is hoped that the resulting compound will thus possess antidepressant activity which is not dependent on the inhibition of MAO-A, thus heralding a new class of antidepressant compounds. It has been shown that relatively small structural modifications to a lead compound frequently have a large effect on MAO inhibition potency (Strydom *et al.*, 2010). The focus will be to modify only the N-alkyl groups as shown in Figure 3.1. In this way the redox chemistry and phenothiazinium structure of MB is retained in the analogue. These two structural

characteristics are thought to play important roles in the biological activities of MB (Duvenhage, 2010). In this study the tetramethyl substitution of the amine nitrogens of MB will be replaced by tetraethyl substitution. This will increase the size of the structure, which may lead to reduced MAO-A inhibition. The active site cavity of MAO-A is reported to be relatively small, and in general accommodates smaller inhibitors better than larger inhibitors (Milczek *et al.*, 2011). In addition, larger compounds may not bind well to MAO-A because, in the MAO-A active site, the residue corresponding to Ile199 in MAO-B is Phe208. The increased size of the Phe side chain compared to the side chain of Ile prevents it from rotating from the MAO-A active site cavity into an alternative conformation as does Ile199 in MAO-B. The binding of larger inhibitors may thus be hindered by Phe208 in the MAO-A active site (Milczek *et al.*, 2011). It is thus envisioned that a larger MB analogue will display weaker interaction with MAO-A than MB.

The synthetic MB analogue, ethylthioninium chloride (ETC), which will be prepared and evaluated as a MAO-A and MAO-B inhibitor in this study is illustrated in Figure 3.1. As shown in the figure, ETC differs from MB only in substitution on the amine nitrogens.

Considering the structural features of ETC, the following structure-activity relationships may be determined:

- The effect that the addition of steric bulk to the amine nitrogens will have on the MAO-A and MAO-B inhibition potencies.
- The effect the addition of adding steric bulk to the amine nitrogens will have on the antidepressant-like effect in the FST.

The principal goal of this study is thus to synthesise ETC as a close structural analogue of MB, and to evaluate the effects of the structural differences between MB and ETC on MAO-A and MAO-B inhibition and its antidepressant-like effect in the FST.

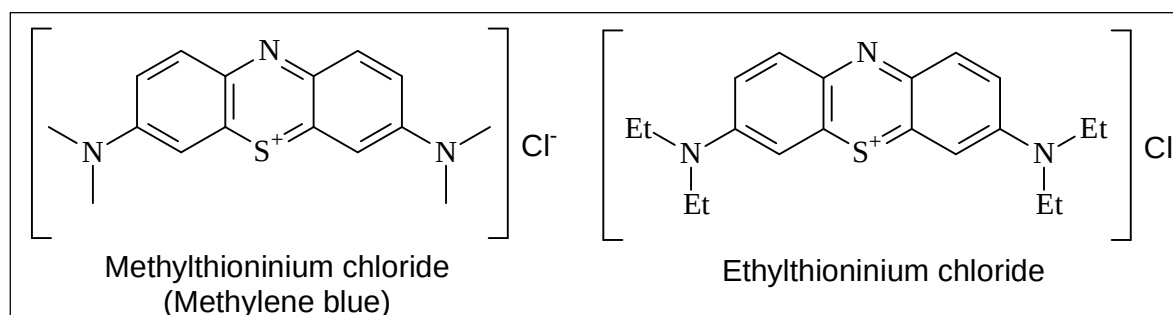


Figure 3.1: The structures of MB and ETC.

3.2. General approaches to the synthesis of MB analogues

A number of routes for the synthesis of MB derivatives have been reported in the literature (Wischik *et al.*, 2006). During a pilot study, two synthetic routes were evaluated in order to find a suitable route for the synthesis of ETC. In the first synthetic route (Figure 3.2.) a nitrosodiethylaniline is prepared from diethylaniline by treatment with NaNO_2 in the presence of HCl . The nitroso compound is reduced to yield p-aminodiethylaniline, which is reacted with $\text{Na}_2\text{S}_2\text{O}_3$ in the presence of $\text{Na}_2\text{Cr}_2\text{O}_7$ to yield the thiosulfonic acid intermediate. After reaction with N,N-diethylaniline and subsequent treatment with CuSO_4 in the presence of $\text{Na}_2\text{S}_2\text{O}_3$, the target compound, ETC, may be obtained (Wischik *et al.*, 2006). This synthetic route did not yield a pure product and the mixture could not be purified by chromatography.

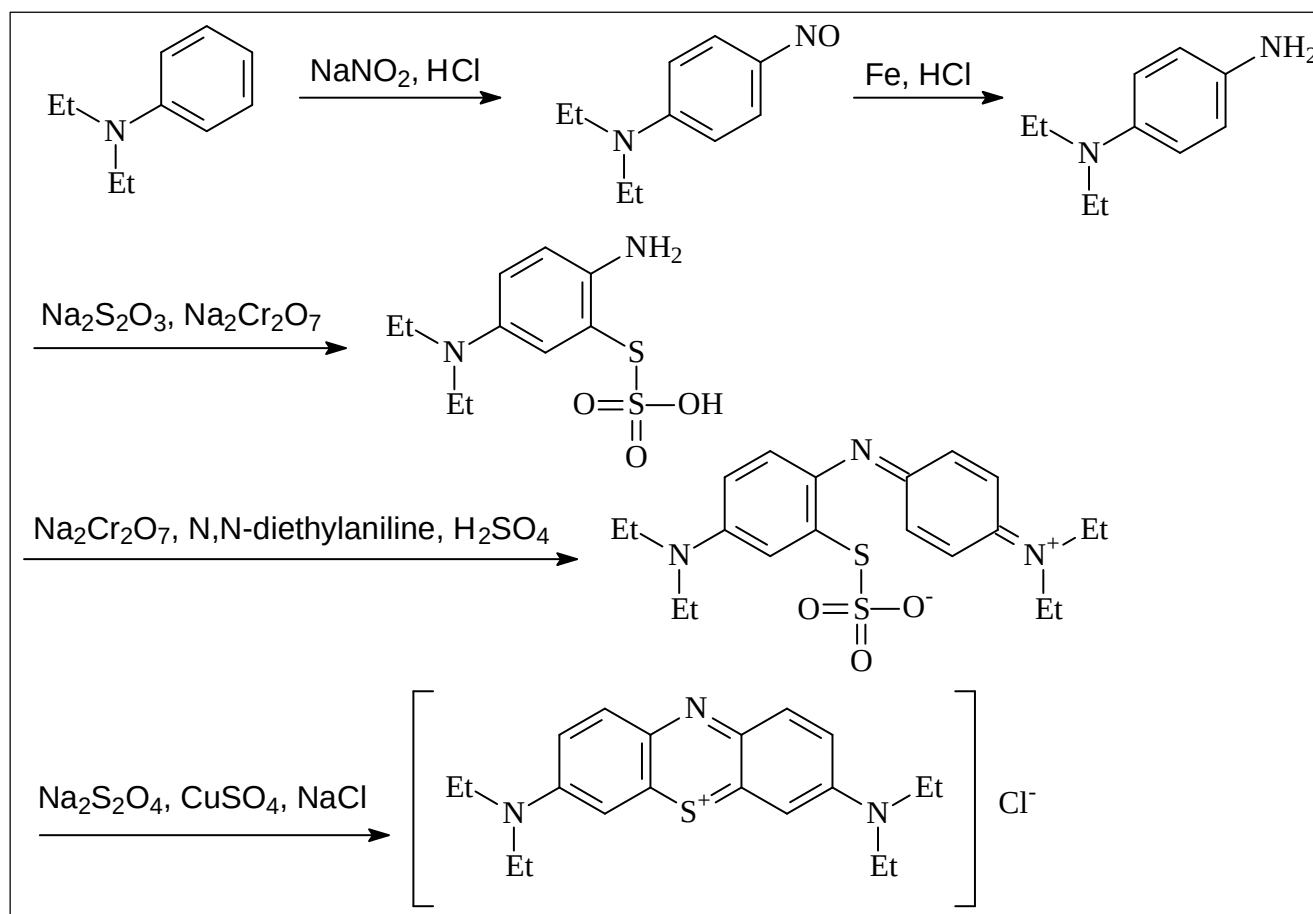


Figure 3.2: First synthetic route for the synthesis of ETC.

The second synthetic route (Figure 3.3.) was used in this study to synthesise ETC. This particular route was selected because it produced the purest product although a relatively low

percentage yield was obtained. To synthesise ETC, the HCl salt of N,N-diethyl-*p*-phenylenediamine was treated with sodium sulphide and iron(III)chloride to yield ETC.

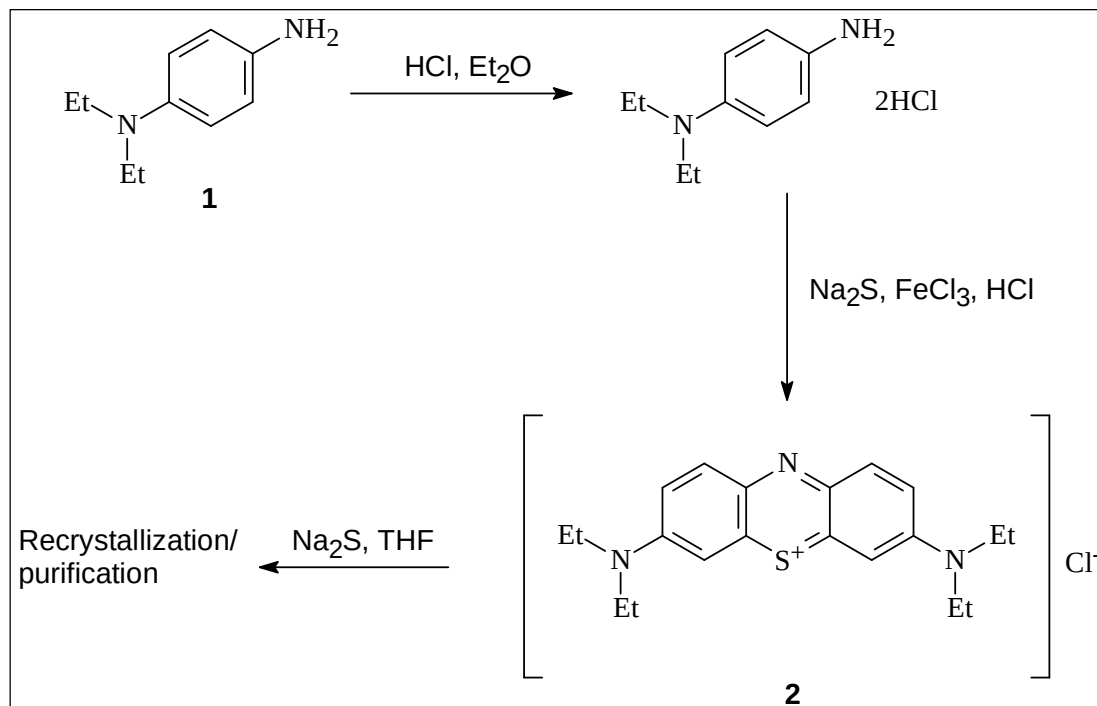


Figure 3.3: Second synthetic route for the synthesis of ETC

3.3. Materials and instrumentation

3.3.1. Materials:

All starting reagents and solvents were obtained from Sigma-Aldrich and were used without further purification.

3.3.2. Thin layer chromatography (TLC):

TLC was used to determine whether the reactions proceeded to completeness. Silica gel 60 (Merck) TLC sheets were employed with a mobile phase consisting of 85% methanol, 15% water and 3% ammonium acetate. The developed TLC sheets were visually observed as ETC forms a blue spot with a R_f value of 0.53.

3.3.3. Mass spectra:

High resolution mass spectra (HRMS) were obtained on a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionization (APCI) mode.

3.3.4. Nuclear magnetic resonance (NMR):

Proton (^1H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance III 600 spectrometer. ^1H NMR spectra were recorded at a frequency of 600 MHz and ^{13}C NMR spectra were recorded at frequency of 150 MHz. All NMR measurements were conducted in $\text{DMSO-}d_6$ and the chemical shifts are reported in parts per million (δ).

3.4. Synthesis of the methylene blue analogue, ETC:

The following is an account of the method used for the synthesis of the methylene blue analogue, ETC. The synthetic route is shown in figure 3.3.

N,N-Diethyl-*p*-phenylenediamine (73.2 mmol) was dissolved in 60 ml diethyl ether. A volume of 12 ml HCl (32%) was subsequently added and the mixture was stirred for 15 min at room temperature. The resulting solution was concentrated by rotary evaporation to give N,N-diethyl-*p*-phenylenediamine dihydrochloride as a thick red material.

N,N-Diethyl-*p*-phenylenediamine dihydrochloride (73.2 mmol) was dissolved in 360 ml H_2O . The pH of the solution was adjusted to 1.6 using 10% aqueous HCl. The reaction turned pink in colour. Sodium sulphide (73.2 mmol) was added to the reaction and the reaction turned to a light yellow solution with a green precipitate. An aqueous solution of iron(III) chloride (109.5 mmol) in 120 ml H_2O was prepared and added to the reaction. The reaction immediately turned blue. The reaction was subsequently aerated for 1 h and another portion of iron(III)chloride solution (109.5 mmol in 120 ml H_2O) was added to the reaction. The reaction turned dark blue and was cooled to 5 °C, and was subsequently filtered. The residue was washed with H_2O and the filtrate fractions were pooled. Sodium chloride (2.07 mol) was added to the filtrate and the resulting mixture was stirred for 15 min before being cooled on ice for 30 min. The mixture turned purple and a precipitate formed. The mixture was filtered and the solid residue collected. The solid residue was air-dried overnight and was dissolved in a mixture of 300 ml CH_2Cl_2 and 30 ml CH_3OH . The solution was stirred for 1 h and was subsequently filtered. The filtrate was concentrated with under reduced pressure to give the product,

ethylthioninium chloride (ETC) as a green solid (Wischik *et al.*, 2006). TLC was performed and indicated that the reaction has proceeded to completion.

The following method was used to purify and recrystallise ETC. ETC (4.17 mmol) was dissolved in 72 ml H₂O and heated to 60 °C. The dark blue mixture was allowed to cool to room temperature. The mixture was treated with a solution of sodium sulphide (0.32 mmol) dissolved in 6 ml H₂O, and stirring was continued for 15 min. The mixture was filtered and the filtrate was treated with 20 ml HCl (32%) and 60 ml tetrahydrofuran (THF). The solution was allowed to recrystallise at room temperature for approximately 3 weeks. Small dark green crystals of ETC were collected by filtration. Yields of 5.7-6.2% were typically obtained (Wischik *et al.*, 2006).

3.5. Interpretation of the TLC sheet

TLC was used to determine whether the reactions proceeded to completeness. Silica gel 60 TLC sheets were employed with a mobile phase consisting of 85% methanol, 15% water and 3% ammonium acetate. The developed TLC sheets were visually observed as ETC forms a blue spot (Figure 3.4.) with a *R_f* value of 0.53. The equation used to calculate the *R_f* value is given below (Equation 3.1).

$$R_f = \frac{\text{migration distance of substance}}{\text{migration distance of solvent}}$$

Equation 3.1.: The equation for the calculation of *R_f* values.

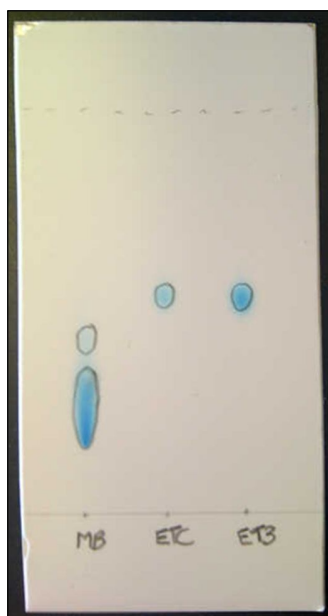
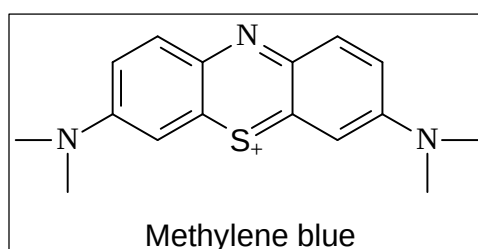


Figure 3.4: A developed Silica gel 60 TLC sheet with MB (left) and ETC (middle and right).

3.6. Results of the NMR spectra

The NMR spectra of MB and ETC are given in Appendix A. The structure of MB is given below and is correlated with the ^1H NMR and ^{13}C NMR spectra. The NMR data of MB is provided here for comparison with those obtained for ETC.



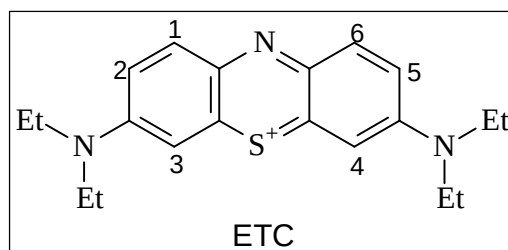
MB was purchased from Sigma-Aldrich (St. Louis, USA).

Methylene blue: ^1H NMR (Bruker Avance III 600, DMSO- d_6) δ 2.49 (12H); 7.34 (4H), 7.72 (2H), ^{13}C NMR (Bruker Avance III 600, DMSO- d_6) 41.1, 106.7, 118.8, 133.3, 134.8, 137.6, 153.6.

Table 3.1: Correlation of the NMR spectra with the structure of methylene blue

^1H NMR:	^{13}C NMR:
• The methyl protons correspond to the signal at 2.49 ppm (the signal integrates for 12 protons). • The aromatic protons correspond to the two signals at 7.34 and 7.72 ppm (the signals integrate for 4 protons and 2 protons, respectively).	• The methyl carbons are represented by the signal at 41.1 ppm. • The aromatic carbons are represented by the signals at 106.7, 118.8, 133.3, 134.8, 137.6 and 153.6 ppm.

The structure of synthesised ETC is given below and correlated with the ^1H NMR and ^{13}C NMR data. As shown below, the appropriate ^1H NMR and ^{13}C NMR signals were observed for the compound. Based on the NMR data, it may thus be concluded that the structure of the target MB derivative, ETC is as proposed in this dissertation.



Ethylthioninium chloride was prepared from N,N-diethyl-*p*-phenylenediamine in a yield of 6%; ^1H NMR (Bruker Avance III 600, D_2O) δ 1.16 (12H); 3.39 (8H), 6.71 (2H), 6.91 (2H), 7.08 (2H), ^{13}C NMR (Bruker Avance III 600, $\text{DMSO}-d_6$) 12.5, 45.3, 105.7, 117.9, 132.5, 133.2, 137.0, 150.6.

Table 3.2: Correlation of the NMR spectra with the structure of ETC

^1H NMR:	^{13}C NMR:
- The methyl protons correspond to the signal at 1.16 ppm (the signal integrates for 12 protons). - The methylene protons correspond with the signal at 3.39 ppm (the signal integrates for 8 protons). - The aromatic protons correspond to the signals at 6.71, 6.91 and 7.08 ppm (the signals collectively integrate for 6 protons).	- The methyl carbons are represented by the signal at 12.5 ppm. - The methylene carbons are represented by the signal at 45.3 ppm. This assignment is made based on the observation that the methyl carbons, attached to N, of MB are found at 41.1 ppm. - The aromatic carbons are represented by the signals at 105.7, 117.9, 132.5, 133.2, 137.0 and 150.6 ppm.

3.7. Interpretation of the mass spectra

ETC was characterised by mass spectrometry. The calculated and experimentally determined masses are given in Table 3.3. The small differences between these masses indicate that the structure of the compound correspond to that given in Figure 3.1.

Tabel 3.3: The calculated and experimentally determined high resolution masses of the synthesised compound

	Mass spectrometry			
	Calculated	Found	Formula	ppm
ETC	340.1847	340.1836	C ₂₀ H ₂₆ N ₃ S	1.6

ppm = [(found - calcd.)/calcd]. x 1000000.

In general a ppm difference smaller than 5 is considered to be good agreement.

3.8. Conclusion

ETC, a structural analogue of MB, was successfully synthesised from N,N-diethyl-*p*-phenylenediamine. Although ETC was obtained in low yield, the compound exhibited a high degree of purity as judged by TLC. The structure of ETC was characterised by MS and NMR analyses. The ¹H NMR and ¹³C NMR spectra correspond to the proposed structure ETC and the experimentally determined mass corresponds with the calculated mass. Thus, it can be concluded that the proposed structure of ETC corresponds with the synthesised compound.

Chapter 4

Enzymology

4.1. Introduction

In this chapter the MB analogue, ETC, which was synthesised (as seen in Chapter 3), will be investigated as an inhibitor of human MAO-A and MAO-B. The main goal is to evaluate what the effect of small structural changes to the structure of MB will be on MAO-A and MAO-B inhibition. Selected physicochemical properties of ETC, MB and azure B will also be measured and compared to one another. The biological and physicochemical properties may be used in future studies to guide structure modification of MB in order to improve its properties, antidepressant activity and safety profile. The following biological and physicochemical properties will be measured:

- In this study, the IC_{50} values for the inhibition of MAO-A and MAO-B will be determined for ETC. This will be done to determine the potency by which ETC inhibits MAO-A and MAO-B.
- In order to determine if ETC interacts reversibly or irreversibly with MAO-A and MAO-B, the recovery of enzyme activity after dilution of enzyme-inhibitor complexes will be examined.
- To evaluate the mode of inhibition of MAO-A and MAO-B by ETC, sets of Lineweaver-Burk plots for the inhibition of MAO-A and MAO-B will be constructed.
- The LogD values of ETC and MB will be measured to evaluate the effect of structural changes on the lipophilicity of MB.
- Determination of the solubilities of ETC, MB and azure B will be carried out and compared to one another.
- The pKa value of ETC will be determined and will be compared to the pKa value of MB.
- The effects of ETC, MB and azure B on cell viability of cultured cells will be measured.

4.2. Chemicals and instrumentation

- For fluorescence spectrophotometry, a Varian Cary Eclipse fluorescence spectrophotometer was employed. Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg/ml) and kynuramine.2HBr were obtained from Sigma-

Aldrich. The Graphpad Prism 5 software package was used to construct sigmoidal dose-response curves and to determine the IC₅₀ values.

- n-Octanol (analytical reagent) was obtained from Sigma-Aldrich.
- For UV-Vis spectrophotometry, a Shimadzu MultiSpec-1501 UV-Vis photodiode array spectrophotometer was used.
- MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich.
- Cell culture media (Dulbecco's Modified Eagle Medium; DMEM), fetal bovine serum, penicillin (10 000 units/ml)/streptomycin (10 mg/ml), fungizone (250 µg/ml) and trypsin/EDTA (0.25%/0.02%) were from Gibco.
- A Multiscan RC UV/Vis platereader (from Labsystems) were used to measure the absorbances in 96-well microplates.
- 24-Well and 96-well plates were from Corning.
- Sterile syringe filters (0.22 µm) were obtained from Pall Corporation Life Sciences.

4.3. The determination of IC₅₀ values for the inhibition of the MAOs by ETC

A fluorometric assay was used to determine the IC₅₀ value (concentration of the inhibitor that produces 50% inhibition) of ETC for the inhibition of the MAO enzymes. The substrate used for the assay was kynuramine, which is nonfluorescent until undergoing oxidative deamination by MAO-A and MAO-B. This yields the fluorescent metabolite, 4-hydroxyquinoline. Addition of an inhibitor is expected to reduce the formation of 4-hydroxyquinoline and thus the fluorescence. The concentrations of 4-hydroxyquinoline were measured fluorometrically at an excitation wavelength of 310 nm and an emission wavelength of 400 nm.

4.3.1. Method

Incubations: Recombinant human MAO-A (5 mg/ml) and MAO-B (5 mg/ml) were pre-aliquoted and stored at -70 °C. The incubations were prepared in 500 µl potassium phosphate buffer and contained MAO-A (0.0075 mg/ml) or MAO-B (0.0075 mg/ml), various concentrations of the test inhibitor (0-100 µM) and 4% DMSO as co-solvent. Kynuramine was also added to each incubation, at concentrations of 30 µM and 45 µM for MAO-B and MAO-A, respectively. The reactions were incubated for 20 min at 37 °C and 400 µl NaOH (2 N) was added to terminate the reactions. Finally 1000 µl distilled water was added to each reaction.

Fluorometric measurement: Concentration measurements of 4-hydroxyquinoline in each incubation were carried out spectrofluorometrically. This was done by measuring the fluorescence of the supernatant at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. For this purpose, the PMT voltage of the fluorescence spectrophotometer was set to medium and the excitation and emission slit widths were set to 5 mm.

Calibration curve: Quantitative estimations were made by means of a linear calibration curve ranging from 0.047–1.560 μM of 4-hydroxyquinoline dissolved in 500 μl potassium phosphate buffer. 400 μl NaOH (2 N) and 1000 μl water were added to each calibration standard. To confirm that the fluorescence of 4-hydroquinoline is not quenched by the test inhibitors, control samples were included.

Data analysis: The IC_{50} values were determined by plotting the initial rate of oxidation versus the logarithm of the inhibitor concentration to obtain a sigmoidal dose response curve. This kinetic data were fitted to the one site competition model incorporated into the Prism (Graphpad) software package. For each sigmoidal curve, 6 different inhibitor concentrations spanning at least 3 orders of magnitude were used. The IC_{50} values were determined in triplicate and expressed as mean $\pm$ standard deviation (SD). See Figure 4.1. for a diagrammatic overview of the IC_{50} value determination method.

Determination of the IC ₅₀ values	Preparation of KH₂PO₄ buffer
	- 100 mM, pH 7.4, made isotonic with KCl 20.2 mM
	Preparation of incubations
	- Prepare incubations in 500 µl KH ₂ PO ₄ buffer
	- Include 6 different concentrations of 0-100 µM of test inhibitor
	- Add 4% DMSO as cosolvent
	Enzyme and substrate concentrations
	- Add 45 µM kynuramine for MAO-A and 30 µM kynuramine for MAO-B as substrate
	- Add MAO-A or MAO-B [0.0075 mg/ml]
	Incubate enzymatic reactions
	- 20 min incubation at 37 °C
	- Termination of reactions by addition of 400 µl NaOH (2 N)
	- Add 1000 µl distilled water to each incubation
	Fluorescent measurement
	- Measure 4-hydroxyquinoline concentrations spectrofluorometrically
	- Use excitation and emission wavelengths of 310 nm and 400 nm, respectively
	Quantitative estimations
	- A calibration curve was prepared by measuring the fluorescence of increasing concentrations of 4-hydroxyquinoline
	Calculate IC₅₀ values
	- Plot initial rate of kynuramine oxidation versus the logarithm of inhibitor concentration
	- Determine IC ₅₀ values in triplicate and express as mean ± SD

Figure 4.1: Diagrammatic representation of the method for determining IC₅₀ values for the inhibition of MAO-A and MAO-B

4.3.2. Results

The IC₅₀ values obtained for the inhibition of MAO-A and MAO-B by ETC are listed in Table 4.1. Also shown are the IC₅₀ values for the inhibition of the MAOs by MB and azure B which were

obtained in earlier studies in our laboratory (Harvey *et al.*, 2010, Petzer *et al.*, 2012). The IC₅₀ values are given as the mean $\pm$ SD and the unit of measurement is μM .

Table 4.1: The IC₅₀ values for the inhibition of human MAO-A and MAO-B by ETC, MB and azure B

	IC ₅₀ values (μM) ^a	
	MAO-A	MAO-B
ETC	0.51 $\pm$ 0.045	0.59 $\pm$ 0.12
MB	0.07 $\pm$ 0.018	4.37 $\pm$ 0.14
Azure B	0.01 $\pm$ 0.001	0.97 $\pm$ 0.12

^a All values are expressed as the mean $\pm$ SD of triplicate determinations

The sigmoidal dose-response curve employed for the determination of the IC₅₀ value for the inhibition of MAO-A and MAO-B by ETC are shown in Figures 4.2 and 4.3, respectively. The rate of the MAO catalysed oxidation of kynuramine is plotted against the logarithm of the inhibitor concentration. The IC₅₀ values were determined by calculating the inhibitor concentration that produces a 50% reduction of the enzyme catalytic rate. These determinations were carried out in triplicate and the values are expressed as the mean $\pm$ SD.

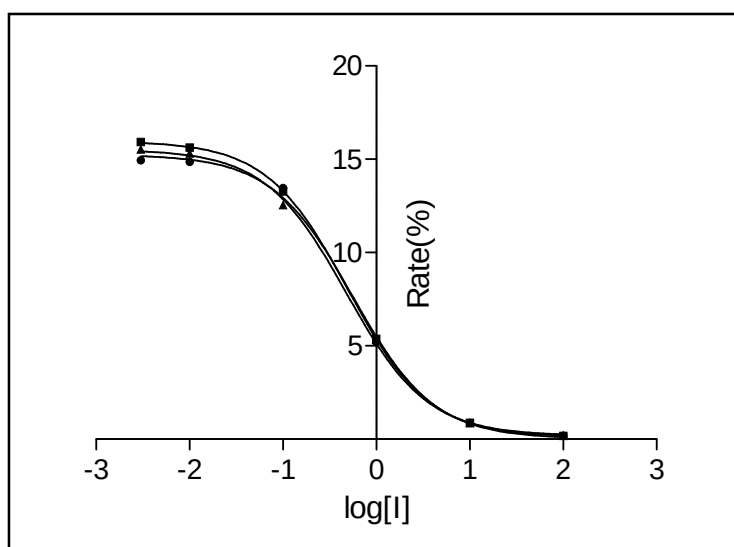


Figure 4.2: A sigmoidal dose-response curve employed to determine the IC₅₀ value of ETC for the inhibition of MAO-A. The determinations were carried out in triplicate and the values are expressed as the mean $\pm$ SD.

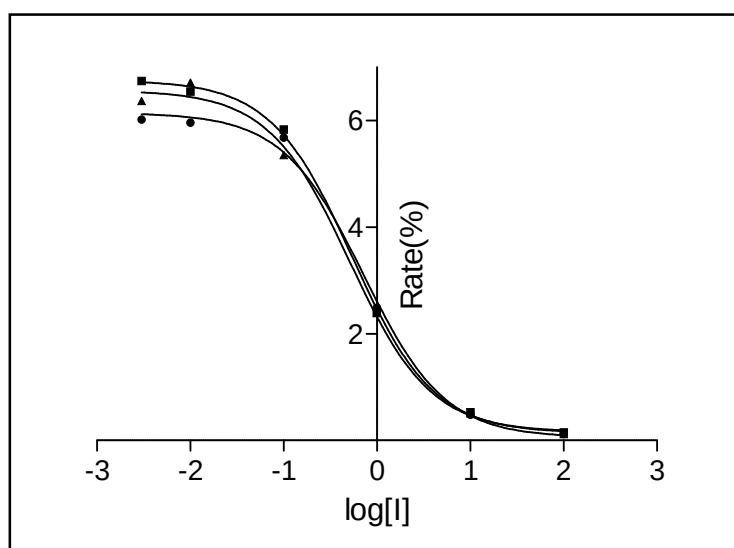


Figure 4.3: A sigmoidal dose-response curve employed to determine the IC_{50} value of ETC for the inhibition of MAO-B. The determinations were carried out in triplicate and the values are expressed as the mean $\pm$ SD

From the results, the following observations and conclusions may be made:

The IC_{50} value for the inhibition of MAO-A by ETC is $0.51 \mu\text{M}$. When compared to MB, which inhibits MAO-A with an IC_{50} value of $0.07 \mu\text{M}$, ETC is 7-fold less potent than MB. When compared to azure B, which inhibits MAO-A with an IC_{50} value of $0.011 \mu\text{M}$, ETC is 46 times less potent. This result correlates the reported structure of the MAO-A active site (Son *et al.*, 2008). The MAO-A active site is reported to consist of a single cavity, which accommodates smaller inhibitors, such as harmine, better than larger inhibitors with extended structures. The reason for this is that residue Phe-208 in the MAO-A active site obstructs the binding of larger inhibitors to MAO-A. In MAO-B, the residue corresponding to Phe-208 in MAO-A is Ile-199. The side chain of Ile is smaller than that of Phe, and can rotate from the active site cavity to create more space for the binding of larger inhibitors. Since larger structures are not well accommodated in the MAO-A active site, increasing the size of the structure of MB to yield ETC should result in lower inhibition potency. In contrast, reducing the size of MB to yield azure B should result in higher MAO-A inhibition potency.

The IC_{50} value for the inhibition of MAO-B by ETC is $0.592 \mu\text{M}$, which is 7-fold more potent when compared to MB (IC_{50} value = $4.37 \mu\text{M}$). Compared to azure B, which has an IC_{50} value of $0.97 \mu\text{M}$, ETC is 1.6 times more potent. This result also correlates to literature. As discussed above, the MAO-B active site may accommodate larger inhibitors since the Ile-199 side chain can rotate from the active site thus creating a larger cavity. Being larger than MB and azure B,

ETC should undergo more productive Van der Waals interactions with the MAO-B active site, especially since the MAO-B active site is reported to be largely hydrophobic.

It is interesting to note that ETC may be considered as a non-selective MAO inhibitor since it inhibits both MAO-A and MAO-B with similar potencies. Non-selective MAO inhibitors are particularly desirable for antiparkinsonian therapy since dopamine is metabolised by both MAO isoforms in the brain. Inhibition of both MAO-A and MAO-B may thus lead to enhanced protection against dopamine depletion than a selective MAO-B inhibitor. Further studies on the potential of ETC in Parkinson's Disease are recommended.

4.4. Reversibility of inhibition

The recovery of the enzymatic activity after dilution of enzyme-inhibitor complexes was evaluated for the inhibition of MAO-A and MAO-B by ETC. When 90% or more enzyme activity is recovered after dilution to a concentration equal to $0.1 \times IC_{50}$ value of the inhibitor, the inhibitor is considered to be a reversible inhibitor. MAO inhibitors with a reversible mode of action may possess certain advantages over irreversible MAO inhibitors, such as an improved safety profile.

4.4.1. Method

Preincubation: For this study, IC_{50} values of 0.51 μ M and 0.59 μ M for the inhibition of MAO-A and MAO-B, respectively, by ETC were employed. ETC was preincubated at concentrations equal to $10 \times IC_{50}$ and $100 \times IC_{50}$ with recombinant human MAO-A or MAO-B (0.75 mg/ml) for 30 min at 37 °C. These preincubations contained DMSO (4%) as co-solvent.

Diluting: The reactions were subsequently diluted 100-fold with the addition of a solution of kynuramine (in K_2HPO_4/KH_2PO_4 , pH 7.4, 100 mM) to yield final concentrations of the test inhibitor equal to $1.0 \times IC_{50}$ and $0.1 \times IC_{50}$. After dilution, the final concentration of kynuramine was 45 μ M (MAO-A) or 30 μ M (MAO-B) and the final concentration of MAO-A or MAO-B were 0.0075 mg/ml. The volumes of these diluted reactions were 500 μ l each.

Residual enzyme activity: The reactions were incubated for a further 20 min at 37 °C, terminated and the residual rates of 4-hydroxyquinoline formation were measured. This was accomplished by adding 400 μ l NaOH and 1000 μ l of distilled water to each reaction. Concentration measurements of the 4-hydroxyquinoline in each incubation were carried out spectrofluorometrically. This was done by measuring the fluorescence of the supernatant at an

excitation wavelength of 310 nm and an emission wavelength of 400 nm. For this purpose, the PMT voltage of the fluorescence spectrophotometer was set to medium and the excitation and emission slit widths were set to 5 mm.

Calibration curve: Quantitative estimations were made by means of a linear calibration curve ranging from 0.047–1.56 μM of 4-hydroxyquinoline dissolved in 500 μl potassium phosphate buffer. 400 μl NaOH (2 N) and 1000 μl water were added to each calibration standard.

Control reactions: For comparison, the irreversible inhibitors, pargyline ($\text{IC}_{50} = 12.97 \mu\text{M}$) for MAO-A and R-deprenyl ($\text{IC}_{50} = 0.079 \mu\text{M}$) for MAO-B, were used. The irreversible inhibitors were similarly preincubated at a concentration of $10 \times \text{IC}_{50}$ with MAO-A or MAO-B, diluted to $0.1 \times \text{IC}_{50}$ and the residual MAO-A or MAO-B catalytic activity was measured as above. Similar reactions, which served as negative controls, were also conducted in the absence of inhibitor (Petzer *et al.*, 2012). See Figure 4.4. for a diagrammatic overview of the measurement of recovery of enzyme activity after dilution.

Recovery of enzyme activity after dilution	Preparation of KH_2PO_4 buffer - 100 mM, pH 7.4, made isotonic with KCl 20.2 mM Preparation of incubations - Prepare incubations in KH_2PO_4 buffer - Include the test inhibitor at concentrations equal to $10 \times \text{IC}_{50}$ and $100 \times \text{IC}_{50}$ - Add 4% DMSO as cosolvent - Add MAO-A or MAO-B [0.0075 mg/ml] - Pre-incubate at 37°C for 30 min - Dilute the reactions to concentrations equal to $0.1 \times \text{IC}_{50}$ and $1.0 \times \text{IC}_{50}$ Incubate enzymatic reactions - 20 min incubation at 37°C - Termination of reactions by addition of 400 μl NaOH (2 N) - Add 1000 μl distilled water to each incubation - Add 45 μM kynuramine for MAO-A and 30 μM kynuramine for MAO-B as substrate Fluorescent measurement - Measure 4-hydroxyquinoline concentrations spectrofluorometrically - Use excitation and emission wavelengths of 310 nm and 400 nm, respectively Quantitative estimations - A calibration curve was prepared by measuring the fluorescence of increasing concentrations of 4-hydroxyquinoline Dilution studies - Plot the enzyme-activities recorded in the absence and presence of the test inhibitors as well as in the presence of pargyline or deprenyl on a histogram
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Figure 4.4: Diagrammatic overview of the measurement of recovery of enzyme activity after dilution of enzyme-inhibitor complexes

4.4.2. Results

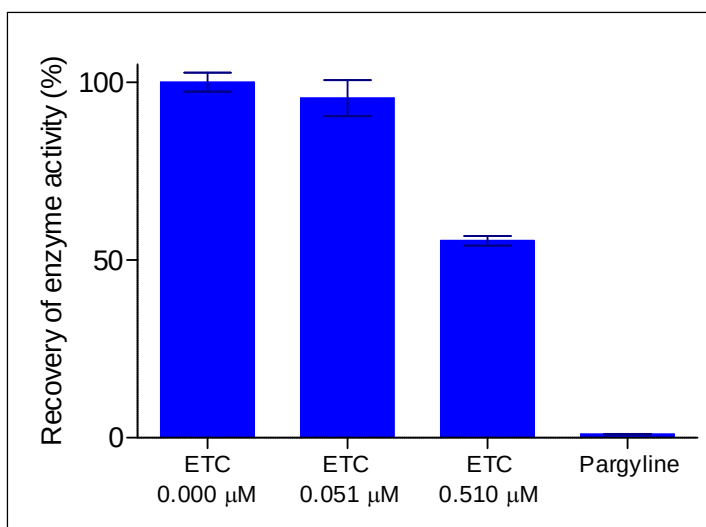


Figure 4.5: The reversibility of inhibition of MAO-A by ETC. MAO-A was preincubated with ETC at $10 \times \text{IC}_{50}$ and $100 \times \text{IC}_{50}$ for 30 min and then diluted to $0.1 \times \text{IC}_{50}$ and $1.0 \times \text{IC}_{50}$, respectively. For comparison, the irreversible MAO-A inhibitor, pargyline, at $10 \times \text{IC}_{50}$, was similarly incubated with MAO-A and diluted to $0.1 \times \text{IC}_{50}$. The residual activities of MAO-A were subsequently measured.

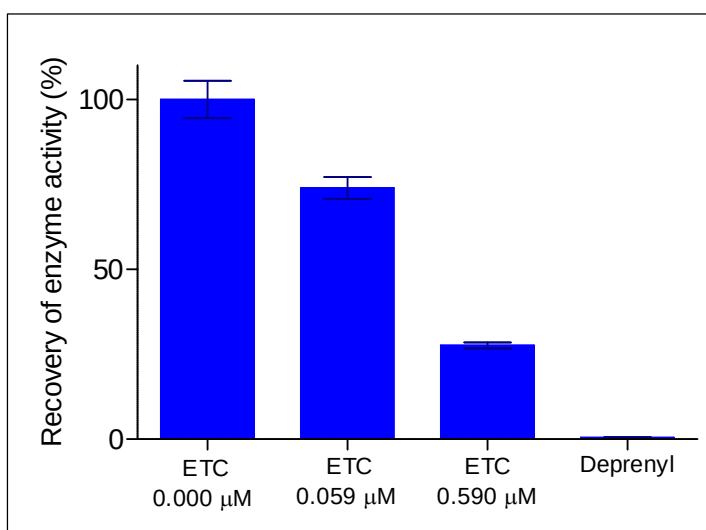


Figure 4.6: The reversibility of inhibition of MAO-B by ETC. MAO-B was preincubated with ETC at $10 \times \text{IC}_{50}$ and $100 \times \text{IC}_{50}$ for 30 min and then diluted to $0.1 \times \text{IC}_{50}$ and $1.0 \times \text{IC}_{50}$, respectively. For comparison, the irreversible MAO-B inhibitor, deprenyl, at $10 \times \text{IC}_{50}$, was similarly incubated with MAO-B and diluted to $0.1 \times \text{IC}_{50}$. The residual activities of MAO-B were subsequently measured.

Since MB is reported to be a reversible inhibitor of MAO-A and MAO-B (Aeschlimann *et al.*, 1996; Ramsay *et al.*, 2007; Harvey *et al.*, 2010), it was necessary to evaluate the reversibility of ETC inhibition towards MAO-A and MAO-B. The results of these reversibility studies are given

in Figures 4.5. and 4.6. The recovery of the enzymatic activity after dilution of the enzyme-inhibitor complex was evaluated. When 90% or more enzyme activity is recovered after dilution to a concentration equal to $0.1 \times IC_{50}$ of the inhibitor, the mode of inhibition is reversible. After dilution to a concentration equal to 0.1-fold ETC's IC_{50} value, MAO-A activity is recovered to a level of 95.5% of the control value. This behaviour is consistent with a reversible interaction of ETC with MAO-A. As shown in the figure, with the irreversible inhibitor, pargyline, MAO-A activity is not recovered after dilution of the inhibitor to a concentration of $0.1 \times IC_{50}$. After dilution of pargyline to $0.1 \times IC_{50}$, the enzyme residual activity is only 1% of the control, conducted in absence of inhibitor. Also shown is that after dilution of ETC to a concentration equal to $1.0 \times IC_{50}$, MAO-A activity is recovered to a level of 55.4% of the control value. This behaviour is consistent with a reversible interaction of ETC with MAO-A since it is expected that after dilution of the enzyme-inhibitor mixtures to inhibitor concentrations of $1.0 \times IC_{50}$, enzyme activity would recover to a value of approximately 50%.

After dilution to a concentration equal to 0.1-fold ETC's IC_{50} value, MAO-B activity is recovered to a level of 73.9% of the control value. This behaviour also is indicative of a reversible interaction of ETC with MAO-B. As shown, with the irreversible inhibitor, deprenyl, MAO-B activity is not recovered after dilution of the inhibitor to a concentration of $0.1 \times IC_{50}$. After dilution to of deprenyl to $0.1 \times IC_{50}$, the enzyme residual activity is only 0.48% of the control, conducted in absence of inhibitor. Also shown is that after dilution of ETC to a concentration equal to $1.0 \times IC_{50}$, MAO-B activity is recovered to a level of 27.6% of the control value. This behaviour is consistent with a reversible interaction of ETC with MAO-B since it is expected that after dilution of the enzyme-inhibitor mixtures to inhibitor concentrations of $1.0 \times IC_{50}$, enzyme activity would recover to a value of approximately 50%.

The implications of a reversible mode of inhibition of MAO-A should be further considered. A reversible inhibition of MAO-A is a desirable since reversible MAO-A inhibitors, in general, do not precipitate the “cheese-reaction” (Anderson *et al.*, 1993). In contrast, irreversible inhibition of MAO-A is associated with the “cheese-reaction”. While unlikely to cause the “cheese-reaction”, ETC may still, however” precipitate ST since MB, also a reversible MAO-A inhibitor has been reported to exert a powerful serotonergic action in animals (Harvey *et al.*, 2010) and hence to precipitate ST in humans (Ramsay *et al.*, 2007). ETC, however, is a weaker MAO-A inhibitor than MB, and thus in theory less likely to lead to ST.

4.5. Lineweaver-Burk plots

To evaluate the mode of inhibition by ETC, sets of Lineweaver-Burk plots for the inhibition of MAO-A and MAO-B were constructed. The initial rates of the MAO-catalysed oxidation of kynuramine at four different substrate concentrations in the absence and presence of three different concentrations of ETC were measured.

4.5.1. Method

Incubations: The mode of MAO-A and MAO-B inhibition was examined by constructing a set of four Lineweaver-Burk plots. The first plot was constructed in the absence of inhibitor while the remaining three plots were constructed in the presence of different concentrations of ETC. These inhibitor concentrations were $\frac{1}{4} \times IC_{50}$, $\frac{1}{2} \times IC_{50}$ and $1 \times IC_{50}$. Recombinant human MAO-A and MAO-B (5 mg/ml), obtained from Sigma-Aldrich, were pre-aliquoted and stored at -70 °C. The incubations were conducted in 500 μ l potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl), and contained the substrate, kynuramine, MAO-A or MAO-B (0.015 mg/ml) and three selected concentrations of the test inhibitor (MAO-A: 0.1275 μ M, 0.2550 μ M and 0.5100 μ M and MAO-B: 0.1479 μ M, 0.2958 μ M and 0.5917 μ M). A Lineweaver-Burk plot was also constructed in the absence of inhibitor. Kynuramine was included in the incubations at four different concentrations (15–90 μ M). These reactions were subsequently incubated for 20 min at 37 °C and terminated by the addition of 400 μ l NaOH and 1000 μ l distilled water. The initial rates by which MAO catalyses the oxidation of kynuramine were subsequently determined spectrofluorometrically.

Calibration curve: The concentration of 4-hydroxyquinoline in each incubation was determined by measuring the fluorescence of the supernatant at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. The PMT voltage of the spectrofluorometer was set to medium with an excitation and emission slit width of 5 mm each. A calibration curve was constructed with known amounts (0.047–1.56 μ M) of 4-hydroxyquinoline dissolved in 500 μ l potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl 20.2 mM). To each calibration standard, volumes of 400 μ l NaOH (2 N) and 1000 μ l water were added.

Data analysis: Lineweaver-Burk plots were constructed from these data sets and linear regression analysis was performed using the GraphPad Prism 5 software package. A Diagrammatic overview of the method used for construction of Lineweaver Burk plots is shown in Figure 4.7.

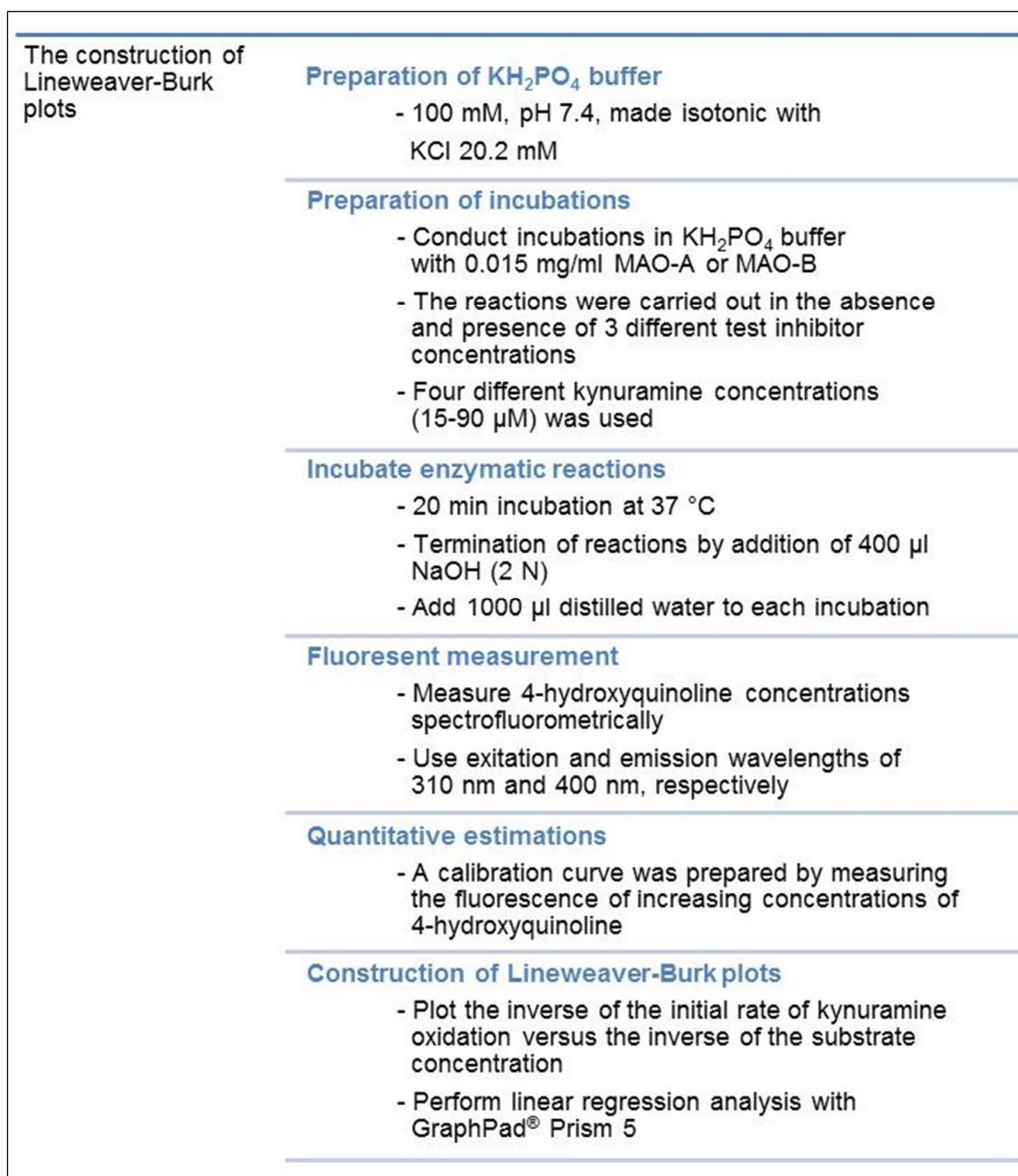


Figure 4.7: Diagrammatic presentation of the method used for the construction of Lineweaver-Burk plots

4.5.2. Results

To examine the mode of inhibition for MAO-A and MAO-B by ETC, sets of Lineweaver-Burk plots for the inhibition of MAO-A and MAO-B were constructed. The inverse of the measured

rates were plotted versus the inverse of the substrate concentrations used. The plots were constructed in the absence of inhibitor and presence of 3 different inhibitor concentrations. Competitive inhibition is evident from the intersecting set of Lineweaver-Burk plots. The set of Lineweaver-Burk plots for MAO-A inhibition is given in Figure. 4.8. and the set of Lineweaver-Burk plots for MAO-B inhibition is given in Figure 4.9. The observation that the lines are linear and intersect towards the y-axis suggests that ETC is a competitive inhibitor of both human MAO-A and MAO-B. Since competitive inhibitors bind reversibly to enzymes, this is further evidence that ETC is a reversible MAO-A and MAO-B inhibitor.

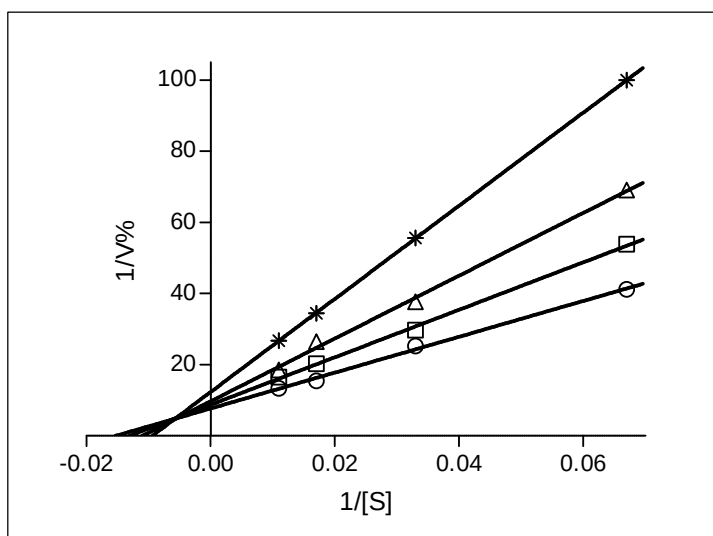


Figure 4.8: Lineweaver-Burk plots for the inhibition of human MAO-A by ETC. The plots were constructed in the absence (open circles) and presence of various concentrations of ETC

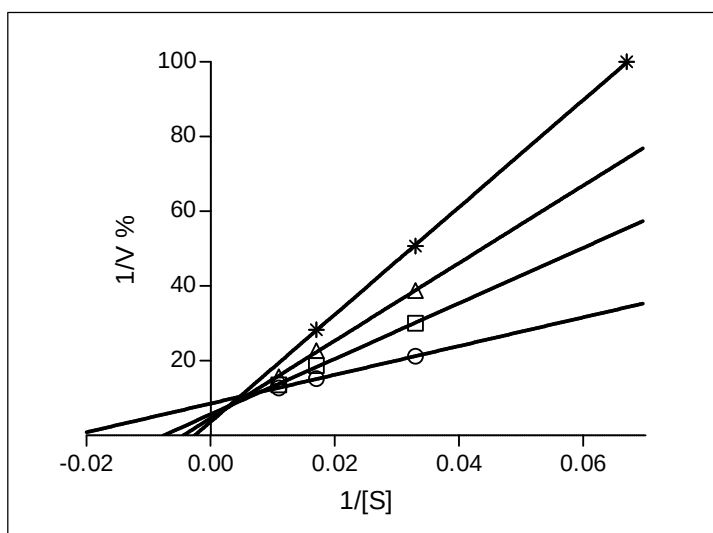


Figure 4.9: Lineweaver-Burk plots for the inhibition of human MAO-B by ETC. The plots were constructed in the absence (open circles) and presence of various concentrations of ETC

4.6. Lipophilicity (LogD)

LogD is the logarithm of the partition coefficient of a compound at a specific pH value and is used to express the lipophilicity of a compound. A partition coefficient is the ratio of concentrations of a compound in the two phases of a mixture of two liquids at equilibrium. The octanol/buffer partition coefficient (D) of ETC was measured. Buffer (pH 7.0, 7.4 and 7.8) and deionised water (MilliQ) were selected for the hydrophilic phase and octanol for the hydrophobic phase. Hence the partition coefficient is a measure of how hydrophilic or hydrophobic the compound is.

4.6.1. Method

n-Octanol and the appropriate buffer or water were mutually saturated in a 30 ml screw-cap tube. For this study potassium phosphate buffer at pH 7.0, 7.4 and 7.8 and deionised water (MilliQ) served as the aqueous buffer phases. In a 10 ml glass vessel, 2 ml of n-octanol was placed followed by 1.6 ml of buffer and 400 µl ETC (10 mM). The vessels were shaken by hand for 5 min and centrifuged at 4000 g for 10 min. The n-octanol phase was diluted 60-fold into neat n-octanol and the absorbance of the resulting solution was recorded at a wavelength of maximal absorbance of 662 nm. The buffer/water phase was diluted 60-fold into neat buffer/water and the absorbance of the resulting solution was recorded at a wavelength of maximal absorbance of 671 nm. A diagrammatic overview of the shake-flask method for LogD determination is given in Figure 4.10.

The concentrations of ETC in the n-octanol and buffer/water phases were determined by employing the molar extinction coefficients recorded in n-octanol, in each of the three buffers (pH 7.0, 7.4 and 7.8) and in deionised water (MilliQ). The molar extinction coefficients of ETC are given in Table 4.2. The LogD value at a given pH is equal to the logarithm of the octanol/buffer partition coefficients (D). The equation used to calculate the Log D values is given below (Equation 4.1). The partition coefficient is the ratio of the concentration of ETC in the n-octanol phase to the buffer/water phase. LogD values are given as mean ± SD of triplicate determinations.

$$\text{LogD} = \log \left(\frac{[\text{analyte}]_{\text{octanol}}}{[\text{analyte}]_{\text{buffer}}} \right)$$

Equation 4.1.: The equation for the calculation of LogD values.

Using the same protocol as above the logD values at pH 7.0, 7.4 and 7.8 and in deionised water (MilliQ) for MB were also determined. The molar extinction coefficient of MB in n-octanol in the three buffers (pH 7.0, 7.4 and 7.8) are given in Table 4.2.

Table 4.2: The molar extinction coefficients for ETC and MB

		Extinction coefficient (M^{-1})			
		Water	pH 7.0	pH 7.4	pH 7.8
	n-Octanol	50735 $\pm$ 4443	50202 $\pm$ 1315	50232 $\pm$ 1272	49362 $\pm$ 2502
ETC	Buffer	44023 $\pm$ 2048	49257 $\pm$ 6692	49227 $\pm$ 2577	43153 $\pm$ 902
MB	Buffer	74741 $\pm$ 6453	72521 $\pm$ 5690	75836 $\pm$ 4056	78355 $\pm$ 7534

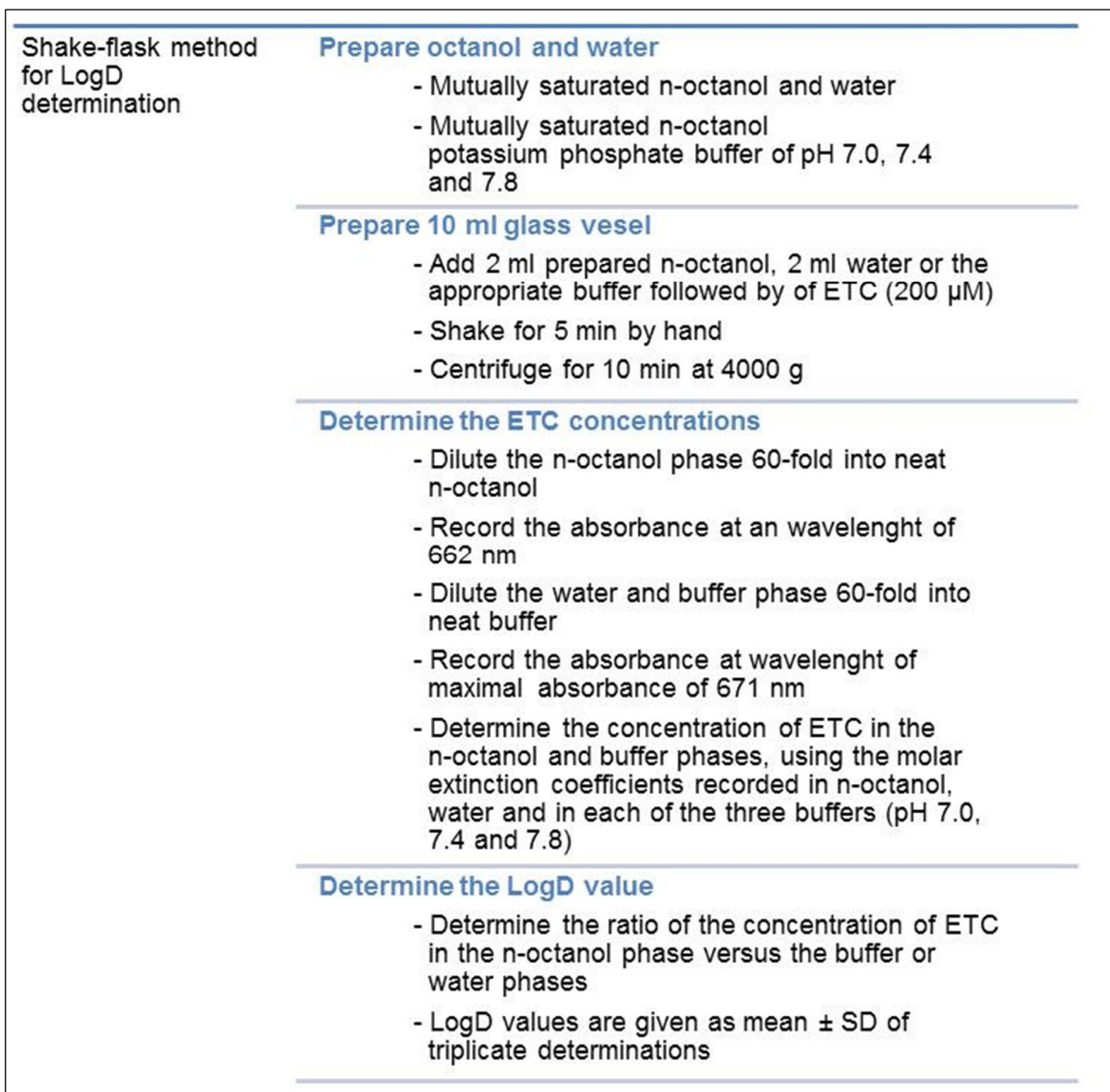


Figure 4.10: Diagrammatic overview of the shake-flask method for LogD determination

4.6.2. Results

As shown in Table 4.3, ETC and MB may be viewed as hydrophilic since they display LogD values which are smaller than 0. The LogD values of ETC ranges from -0.13 to -0.21. The LogD values at different pH values and in water are quite similar because it is expected that ETC would be completely ionized at physiological pH values. ETC and MB are hydrophilic because they are charged molecules. ETC is, however, slightly less hydrophilic than MB. This finding is in agreement with the structural differences between MB and ETC. ETC is a larger structure than MB and contains tetraethyl substitution on the amine nitrogens. MB contains tetramethyl

substitution on the amine nitrogens and is thus expected to be less lipophilic than ETC. In general the addition of methyl groups to a structure is expected to enhance lipophilicity. Since the ideal range for good oral bioavailability is LogD values between 0-3 (Kerns & Di, 2008), the high degree of hydrophilicity of ETC reduces the permeation of this compound through biological membranes. As mentioned before, although MB is hydrophilic, it still crosses the blood-brain barrier (Peter *et al.*, 2000). Entering the brain can be facilitated by the leucoMB form, which is uncharged and 20 times more lipophilic than MB (Harris & Peters, 1953). It is expected that ETC may also be reduced to its leuco-form, which will be more lipophilic and thus allow penetration through the blood-brain barrier.

Table 4.3: The LogD values of the ETC and MB in buffer at different pH values and in water

	Water	pH 7.0	pH 7.4	pH 7.8
ETC	-0.21 ± 0.006	-0.20 ± 0.015	-0.13 ± 0.067	-0.20 ± 0.006
MB	-2.02 ± 0.200	-1.53 ± 0.584	-1.82 ± 0.022	-1.73 ± 0.035

Values are given as mean ± standard deviation of triplicate determinations

4.7. Solubility

Solubility plays an important role in drug absorption from the gastrointestinal tract, for accurate results in biological *in vitro* assays and dosage form selection for *in vivo* administration (Kerns & Di, 2008). To measure the solubility of ETC, MB and azure B an equilibrium shake-flask thermodynamic solubility method were used. The following solubility classification ranges are suggested:

- <10 µg/ml Low solubility
- 10-60 µg/ml Moderate solubility
- >60 µg/ml High solubility

4.7.1. Method

To determine the solubility of the ETC, MB and azure B, 2 mg of the compounds were placed into a polypropylene tube and 500 µl water or potassium phosphate buffer (pH 7.4) was added. The tubes were agitated for 24 h in a shaking water bath (85 rpm) at 25 °C, and subsequently centrifuged at 16000 g for 10 min. The samples were filtered through a 0.22 µm syringe filter and diluted 500-fold into water or potassium phosphate buffer (100 mM, pH 7.4). The absorbances of the resulting solutions for ETC, MB and azure B were recorded at wavelengths

of maximal absorbance of 671 nm, 665 nm and 646 nm, respectively. A diagrammatic overview of the method to determine solubility is given in Figure 4.11.

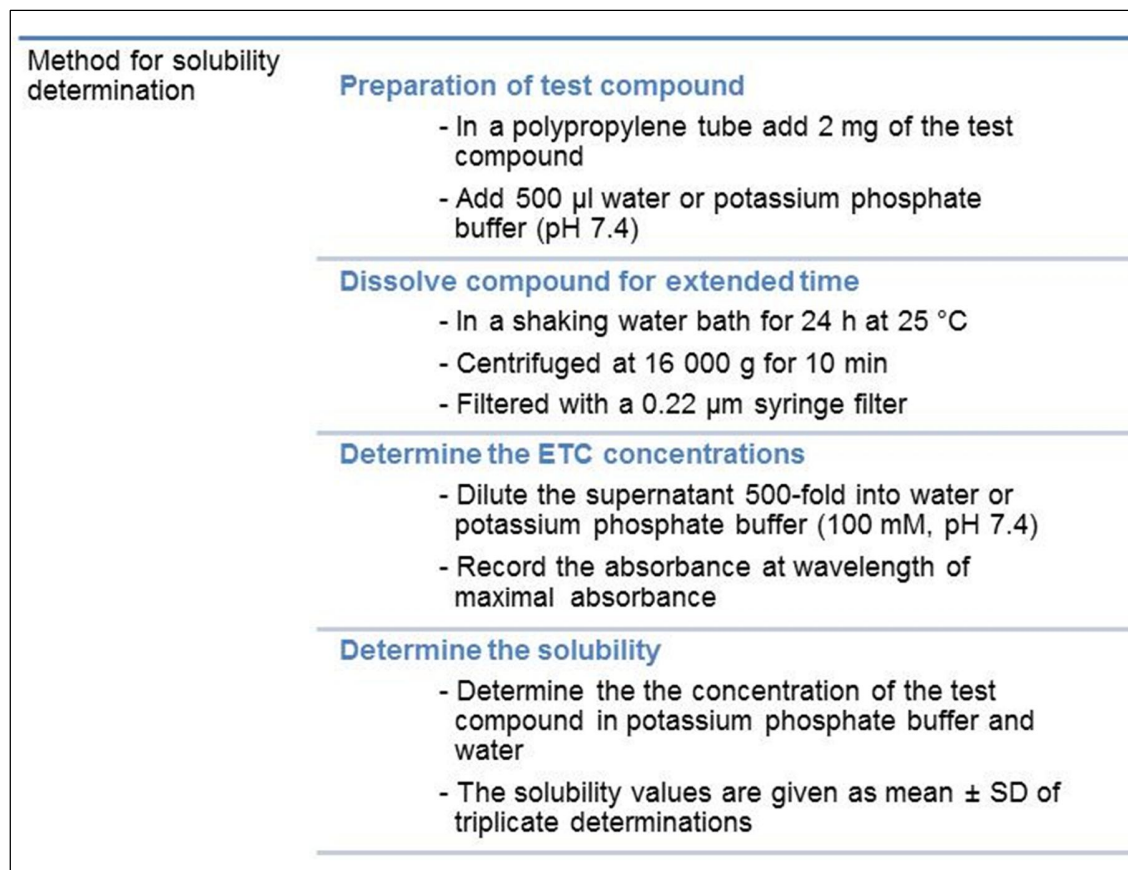


Figure 4.11: Diagrammatic overview of the method for solubility determination

The concentration of ETC, MB and azure B were determined by employing the molar extinction coefficients measured in water and potassium phosphate buffer (100 mM, pH 7.4), and are given in Table 4.2. The solubility values are given as mean ± SD of triplicate determinations.

4.7.2. Results

The concentrations of ETC, MB and azure B in the water and buffer phases are shown in table 4.4. The results show that ETC, MB and azure B are highly soluble in water and buffer since the concentrations recorded in water and buffer are well above 60 µg/ml. It should be noted that the increase in structure size of ETC compared to MB and azure B, is most likely responsible for the decreased aqueous solubility. In addition ETC is expected to be less soluble in aqueous media than MB and azure B since ETC has been found to be more lipophilic than MB and azure B. As

mentioned above, the addition of additional methyl groups to a structure ETC is expected to enhance lipophilicity, and as a result lower aqueous solubility. In spite of this ETC may still be viewed as a compound with a high degree of aqueous solubility. The high degree of aqueous solubility will result in an improvement in the oral bioavailability of ETC.

Table 4.4.: The concentrations of ETC, MB and azure B in water and aqueous buffer at pH 7.4

	Concentration ($\mu\text{g/ml}$)	
	Water	Buffer pH 7.4
ETC	3295 $\pm$ 72	3328 $\pm$ 187
MB	4465 $\pm$ 363	4523 $\pm$ 188
Azure B	5189 $\pm$ 786	4912 $\pm$ 286

Values are given as mean $\pm$ SD of triplicate determinations

4.8. Ionization constant (pKa)

The pKa value of ETC should give an indication of the degree of ionization of ETC at the specific pH values in the gastrointestinal tract and plasma. As previously mentioned, MB is completely ionized at physiological pH values with a pKa value between 0 and -1 (DiSanto and Wagner, 1972). In this section the pKa value of ETC will be determined by employing a spectrophotometric protocol. The pKa of ETC will subsequently be compared to the pKa value of MB. The spectrophotometric protocol is a suitable method for compounds with particularly low pKa values. The method depends on the direct determination of the ratio of molecular species (neutral molecule) to ionized species in a series of non-absorbing buffer solutions with known pH values (Albert & Serjeant, 1984). The acidity function (H_0) devised by Hammett & Deyrup (1932) take the place of the buffer solutions mentioned above for determination of pKa values in highly acidic regions. The H_0 values are negative logarithms and form an extension of the pH scale. The H_0 values of the solutions in table 4.5. were obtained from Albert & Serjeant (1984).

Table 4.5: Solutions of known acidity function (H_0) at 25 °C

H_2SO_4 (wt%)	Normality	H_0
0.0049	0.001	3.03
0.0470	0.010	2.08
0.6120	0.130	0.09
8.3000	1.800	-0.23
10.7500	2.360	-0.42
11.8000	3.080	-0.64
17.8000	4.080	-0.90
22.2000	5.220	-1.21
25.1000	6.040	-1.41
28.7000	7.100	-1.69
33.6000	8.580	-2.03
35.6000	9.220	-2.22
38.6000	10.180	-2.45
42.0000		-2.69
47.0000		-3.13
52.0000		-3.60
60.0000		-4.51

4.8.1. Method

To determine the ionisation constant of the ETC, a 1 mM solution in water was prepared. ETC was subsequently diluted to 200 μ M into H_2SO_4 solutions of known acidity function (H_0). The absorbance spectra of ETC is subsequently recorded. The spectrum of the ionised species (A_I) was first recorded in 100% H_2SO_4 , where after the spectrum of the molecular species (A_M) was recorded in water. From the spectra of A_I and A_M , the analytical wavelength can be found at which the two species differ most in absorbance from one another. This analytical wavelength was selected to be 670 nm for ETC. Appropriate H_2SO_4 solutions were subsequently prepared for the concentrations needed to meet the H_0 values shown in Table 4.6. The absorbance spectra of ETC is recorded in these acidic solutions and the absorbance values (A) recorded at a wavelength of 670 nm. The equation used to calculate the pKa value is given below (Equation 4.2).

$$pKa = pH + \log\left(\frac{A - A_M}{A_I - A}\right)$$

Equation 4.2.: The equation for the calculation of pKa values.

4.8.2. Results

Table 4.6: The determination of the ionization constant (pKa value) of ETC

H ₂ SO ₄ (wt%)	pH	A	pK _a
0.0049	3.03	0.617	
0.0470	2.08	0.622	
0.6120	1.06	0.560	0.122
5.1000	0.09	0.294	0.257
8.3000	-0.23	0.228	0.174
10.7500	-0.42	0.204	0.084
11.8000	-0.64	0.196	-0.100
17.8000	-0.90	0.183	-0.297
22.2000	-1.21	0.172	-0.549
25.1000	-1.41	0.167	-0.722
28.7000	-1.69	0.163	-0.978
33.6000	-2.03	0.161	-1.306
35.6000	-2.22	0.123	-1.208
38.6000	-2.45	0.144	-1.615
42.0000	-2.69	0.121	-1.658
47.0000	-3.13	0.101	-1.833
52.0000	-3.60	0.073	
60.0000	-4.51	0.070	
Where A _I is 0.075 and A _M is 0.616			

As shown in Table 4.6., the experimentally determined ionization constant of ETC ranges from 0.122 to -1.833. Similar to MB, the low pKa value of ETC is expected since the molecule already possesses a positive charge. It should be mentioned that, as for the reported value for MB, the pKa value range determined here for ETC has a relatively large error because of “medium shifts” as water becomes decreasingly available. In addition, the glass pH electrode may become less accurate at very low pH values. The pKa value given here, however, demonstrates that the acid-base properties of MB and ETC are similar.

4.9. Cell viability

The safety profiles of drugs are a great concern for pharmaceutical companies and toxicity remains a significant cause for the cessation during drug development. The increase in the interest in MB as a therapy for a variety of diseases emphasises the need to determine its cytotoxicity in human cells. To estimate the toxicity of MB, ETC and azure B, their effects on cell

viability of cultured cells were examined. For this purpose, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was used (Mosmann, 1983). The MTT assay is a standard cell viability assay, measuring mitochondrial activity in live (metabolically viable) cells. Metabolically active mitochondria in viable cells have the ability to reduce MTT to coloured formazan crystals that can be measured spectrophotometrically. The presence of a toxic agent will reduce cell viability and thus reduce the reduction of MTT to the formazan product. For this study HeLa cells were selected. This selection was based on the high growth rate of HeLa cells and on the observation that these cells are frequently used to measure cell viability in the presence of toxic agents (Rahbari *et al.*, 2009).

4.9.1. Method

For the cell viability assay, three types of experiments with triplicate observations of each condition are recommended. Two types of controls are needed, namely the negative control (100% cell viability with no drug treatment) and positive control (100% cell death, induced by 0.03% formic acid). The third type of experiment is the test experiments where the viability of cells treated with the test drug is measured. HeLa cells were maintained in 250 cm² flasks in 30 ml DMEM containing 10% fetal bovine serum, 1% penicillin (10 000 units/ml)/streptomycin (10 mg/ml) and 0.1% fungizone (250 µg/ml). The cells were incubated at 37 °C in an atmosphere of 10% CO₂. Once confluent, the cells were seeded at 500 000 cells/well in 24-well plates and incubated for 24 h. A volume of 3 ml trypsin/EDTA (0.25%/0.02%) was used to facilitate cell detachment (from the 250 cm² flasks) and the counting of cells was done with a haemocytometer. The wells were subsequently rinsed with 0.5 ml DMEM containing no fetal bovine serum. A volume of 0.99 ml DMEM (containing no fetal bovine serum) was subsequently added to each well followed by 10 µl of the test drug. Stock solutions of the test drugs were prepared in deionised water and sterilised by filtration through a 0.22 µm syringe filter (Pallman). In each 24-well plate, wells were reserved as positive controls (100% cell death via lyses with 0.03% formic acid) and negative controls (0% cell death as a result of no treatment). The well-plates were incubated for a further 24 h after which the culture medium was aspirated from the cells. The wells were washed twice with 0.5 ml/well phosphate-buffered saline (PBS) and 200 µl of 0.5% MTT reagent in PBS was added to each well. The well-plates were incubated at 37 °C for 2 h in the dark, the MTT-reagent was aspirated from the wells and 250 µl isopropanol was added to each well. The well-plates were then incubated at room temperature for 5 minutes to dissolve the blue formazan crystals, where after 100 µl of the isopropanol phase was transferred to a 96-well plate. The absorbance of each isopropanol phase was measured

spectrophotometrically at 560 nm. The effects on cell viability of MB, ETC and azure B were tested in triplicate. See Figure 4.12 for a diagrammatic overview of the method for cell viability determination. The mitochondrial activity was determined and expressed as a percentage of the negative control (100% cell viability) by means of the following equation (where Abs = absorbance as read by the spectrophotometer):

$$\% \text{ Viable cells} = 100 \times \frac{Abs_{(sample)} - Abs_{(positive\ control)}}{Abs_{(negative\ control)} - Abs_{(positive\ control)}}$$

Equation 4.3: The equation for the calculation of % cell viability.
Where $Abs_{(sample)}$ = absorbance from spectrophotometer of the sample, $Abs_{(positive\ control)}$ = absorbance of the cells treated with 0.03% formic acid (100% cell death), $Abs_{(negative\ control)}$ = absorbance of cells without treatment.

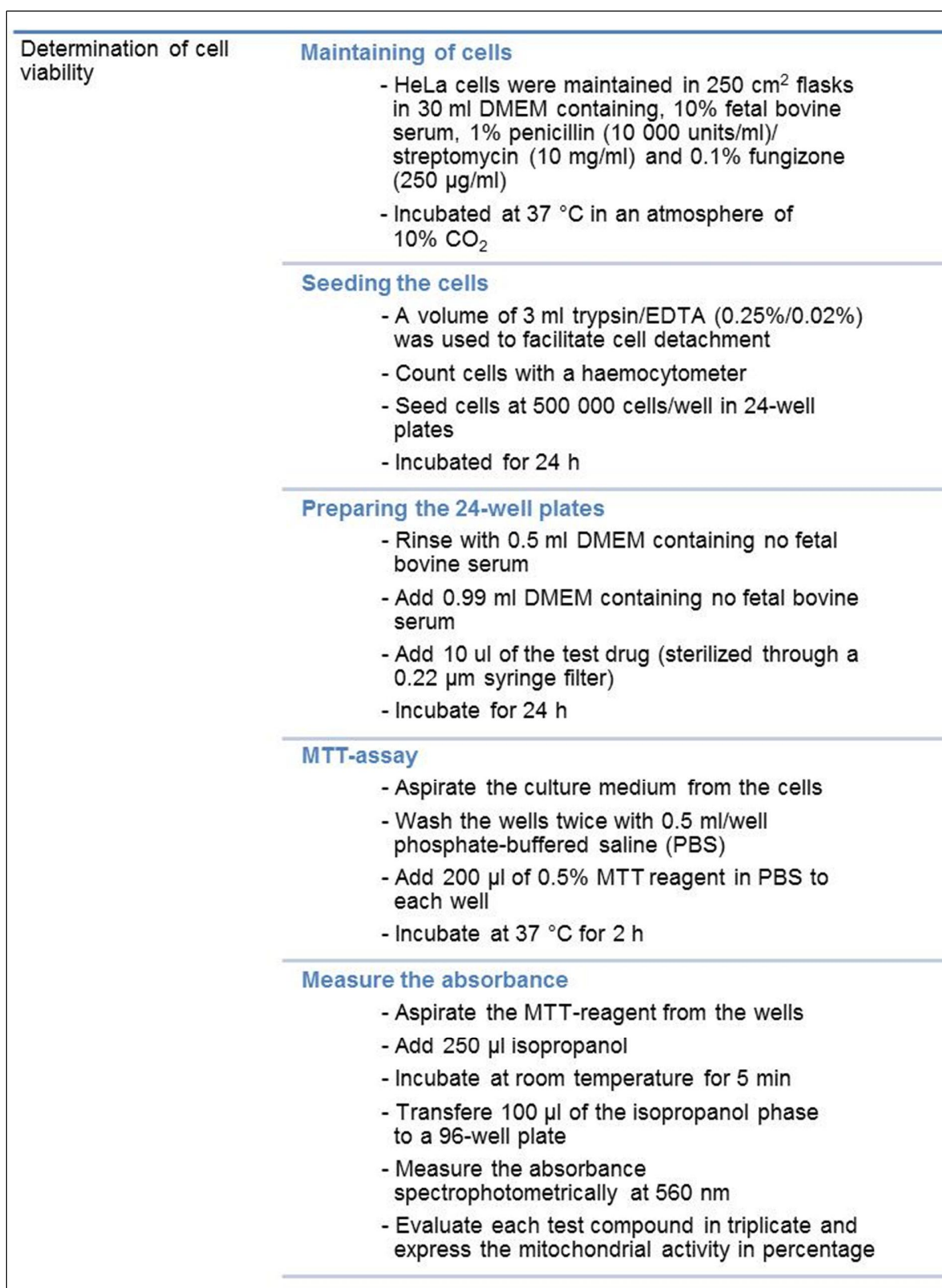


Figure 4.12: A diagrammatic overview of the method for cell viability determination

4.9.2. Results

The percentage viable cells after treatment with the MB, ETC and azure B were calculated by using Equation 4.3 above. The results are shown in Table 4.7. MB was relative non-toxic at 1 μ M with 85.9% viable cells compared to untreated controls. Azure B and ETC were, however, more toxic at all the concentrations with 44.1% and 38.3% viable cells remaining at 1 μ M concentration, respectively. These results indicate that ETC and azure B are significantly more toxic for cultured cells than MB at all the concentrations tested. A possible explanation for the enhanced toxicity of ETC compared to MB is that ETC more readily permeates into the cultured cells, and thus attains higher concentrations in the cytosol than MB. Since ETC is more lipophilic than MB, ETC is expected to display a higher degree of passive diffusion permeability than MB. Reasons for the enhanced cytotoxicity of azure B compared to MB are not apparent. Being the primary metabolite of MB, it is expected to be less toxic but further investigation is required to address this issue.

Interestingly, at 10 μ M and 100 μ M, all three compounds, MB, azure B and ETC, are toxic to cultured cells with only 4-26% viable cells remaining. The mechanism by which these compounds are toxic to the HeLa cells is unknown, and should be investigated further. This is especially important in the light that MB is considered to possess a good safety profile.

Table 4.7: The percentage viable cells remaining after treatment with ETC, MB and azure B

	% Viable cells			
	Control	1 μ M	10 μ M	100 μ M
ETC	100	38.32	4.09	4.09
MB	100	85.90	21.61	9.22
Azure B	100	44.14	26.48	15.81

4.10. Conclusion

The present study shows that ETC (IC_{50} = 0.51 μ M) is a less potent MAO-A inhibitor than MB (IC_{50} = 0.07 μ M) and azure B (IC_{50} = 0.01 μ M). ETC (IC_{50} = 0.59 μ M) was, however, found to be a more potent MAO-B inhibitor compared to MB (IC_{50} = 4.37 μ M) and azure B (IC_{50} = 0.97 μ M). Results also show that ETC is a reversible and competitive inhibitor for both MAO-A and MAO-B. Studies on its physicochemical properties show that ETC is highly hydrophilic and

highly water soluble and has a pKa value between -1.883 and 0.122. Finally, MB, ETC and azure B is all relatively toxic to cultured cells.

Chapter 5

Animal behavioural studies

5.1. Introduction

This chapter presents the experiments carried out with experimental animals, including animals, materials, drugs used and their dosages, behavioural studies and results. The treatment of the animals and behavioural testing were conducted at the Vivarium at the Potchefstroom campus of the North-West University in accordance with the ethics application, *NWU-00024-13-S1*, as approved by the Research Ethics Committee of the North-West University.

This will be an acute treatment study with the aim to confirm the antidepressant action of MB in the FST and to determine if ETC and azure B also possess antidepressant-like activity. In addition it will be determined whether the antidepressant-effect of ETC and azure B are dose dependent. The results obtained will be compared to results obtained with the reference antidepressant, IMI. IMI was also chosen because, like the compounds being tested, it is also presents with a similar tricyclic structure. To achieve the goals of this chapter, male Sprague Dawley rats will be treated with four different doses of each compound. The rats will subsequently be evaluated in a Digiscan[®] animal activity monitor to determine general locomotor activity where after the acute FST will be performed. The results obtained with ETC and azure B will be compared to results obtained after treatment of rats with saline, IMI and MB.

5.2. Animals and materials used

5.2.1. Animals

Male Sprague-Dawley rats weighing between 230-270 g were used for the acute FST. In all cases, rats were provided by the Vivarium of the North-West University. Ethical approval for the study was granted by the Ethical Committee of the North-West University (no: *NWU-00024-13-S1*). Suffering and discomfort to animals were minimised and the number of animals per group was the minimum needed for meaningful statistical evaluation of the data. The rats were housed in cages (4 rats per cage) with a width of 28 cm, a length of 44.5 cm and a height of 12.5 cm. The conditions in the animal centre were controlled at 21 ± 0.5 °C and $50\pm5\%$ humidity. Full spectrum cold white light, with a light intensity of 350-400 lux was provided over a 12 hour light-12 hour dark cycle (06:00-18:00 light). A positive air pressure was maintained in the laboratory

with air filtration 99.7% effective for a particle size of 2 μm and 99.9% for a particle size of 5 μm . All animals had free access to food and water and were acclimatised to the laboratory conditions before the experiments. For the acute FST, animals were randomised into 11 groups comprising 8 animals each, and destined to receive acute treatment with saline, IMI, MB, azure B and ETC.

5.2.2. Drug treatment

IMI, MB and azure B were purchased from Sigma-Aldrich (St. Louis, USA) and ETC was synthesised as detailed in Chapter 3. All animals were weighed each morning, and their respective dosages were calculated accordingly per kg body weight. Drug solutions were prepared fresh on the day of treatment. In the acute FST treatment protocol, MB, IMI, azure B and ETC were dissolved in a 0.9% saline solution and administered intraperitoneally (i.p.) at a maximum volume of administration of 0.2 ml.

IMI was used as a positive control mainly because of its known positive response in the FST. Normal saline was used as a solvent and as control. The doses of the compounds were determined according to previous studies (Naylor *et al.*, 1986; Harvey *et al.*, 2010). Some of these compounds have not been widely researched and data on their bioavailability and metabolism are not available. In order to improve safety and tolerability in the animals, four different dosages of each compound were selected and administered.

- IMI was administered i.p. at a dose of 15 mg/kg in the FST, (Harvey *et al.*, 2002; 2006; Kulkarni & Dhir, 2007; Brink *et al.*, 2008; Harvey *et al.*, 2010) and was used as a reference antidepressant (positive control).
- The i.p. dose of 15 mg/kg of MB was used, and in accordance to the work of Harvey and colleagues (2010).
- The dosages used for the administration of azure B (i.p.) were 4.0, 7.5, 15.0 and 30.0 mg/kg. This dosage range (7.5, 15.0 and 30.0 mg/kg) was selected based on previous research in our laboratories using various MB analogues (Harvey *et al.*, 2010), while the 4 mg/kg dose was specifically selected since it corresponds to the clinically used dose of 300 mg/day for MB (corresponding to 4.3 mg/kg for a 70 kg person). This dose has been administered to humans for one year without significant side effects (Naylor *et al.*, 1986; Riha *et al.*, 2005).
- ETC was administered in the same dosage range as azure B, viz. 4.0, 7.5, 15.0 and 30.0 mg/kg (i.p.).

5.2.3. Instruments

The following instruments were used during this study:

- Perspex[®] cylinders: diameter 180 mm, height 600 mm (Porsolt FST apparatus)
- Digiscan[®] Animal Activity Monitor (AccuScan[®] Instruments, Columbus, OH, USA)
- Video cameras were mounted directly opposite the FST apparatus to record swimming behaviour in the FST

5.3. The rat forced swim test

5.3.1. Introduction

The FST is a standard, validated test to assess depressive-like behaviour in rodents (Porsolt *et al.*, 1978, Porsolt, 2000). This well-described test is robust and shows good inter-laboratory repeatability, and has been well studied in our laboratory (Brink *et al.*, 2008; Mokoena *et al.*, 2010; Liebenberg *et al.*, 2010; Harvey *et al.*, 2010). This antidepressant screening model is based on the observation that when rats are forced to swim in a restricted space from which there is no possibility of an escape, they eventually cease to struggle, surrendering themselves to helplessness (Kulkarni & Dhir, 2007). This state is similar to some of the characteristics symptoms of depression, especially despair, lack of goal driven behaviour and learned helplessness (Porsolt *et al.*, 1978; American Psychiatric Association, 1994). This condition is then used to evaluate whether a putative antidepressant drug is able to reverse this state by promoting struggling and swimming or escape driven behaviour. Antidepressants that primarily potentiate serotonin mediated neurotransmission increase swimming behaviour while those with primary actions on catecholaminergic neurotransmission increase climbing behaviour (Cryan *et al.*, 2002).

5.3.2. Method

On day one of the challenge, the rats were allowed to habituate to their surroundings for a period of 30 min. Thereafter each animal was subjected to 15 minutes of pre-swimming in transparent Perspex[®] cylinders (diameter 180 mm, height 600 mm) containing clean water (25 °C) at a depth of 30 cm. After the pre-swim, the animals were dried and returned to their home cages. Immediately thereafter, each rat received their first i.p. injection of the test drug. This time point was 24 h prior to the final swim test. The animals received subsequent treatments at the same time each day (between 13:00 and 14:00 am) for the 24 h treatment

period. On day 2, 6 h and again 1 h prior to the final 5 min test swim, the rats received their second and third i.p. injections of the test drug. After the last injection the rats were assessed in the Digiscan[®] animal activity monitor to evaluate general locomotor activity. The rats were then left in the room to habituate for a further 30 min before the final 5 min test swim in the FST commenced. For the final five min swim test, rats were placed in the same cylinders (containing clean water at 25 °C and a depth of 30 cm) as before and were allowed to swim for 5 min. A diagrammatical presentation of the method is given in Figure 5.1. During this period, all swimming behaviours were digitally recorded for later evaluation by 3 blinded investigators, 2 of whom were not involved in the study. In the present study, the performance of each rat in the FST was video-taped using a digital camera and scored individually. The most prominent of the behavioural components were identified and measured in 5 sec intervals and expressed in terms of the amount of time (in seconds) spent performing this behaviour over a total period of 5 min. Subsequently, the combined time of the three components observed added up to 300 seconds. Three specific behavioural components were distinguished and measured (shown in Figure 5.2.):

- Immobility is defined when no additional activity is observed other than that required to keep the animal's head above the water.
- Climbing behaviour (also known as struggling) is defined as upward directed movements of the forepaws along the side of the cylinder.
- Swimming behaviour is defined as swimming movements (usually horizontal) throughout the cylinder.

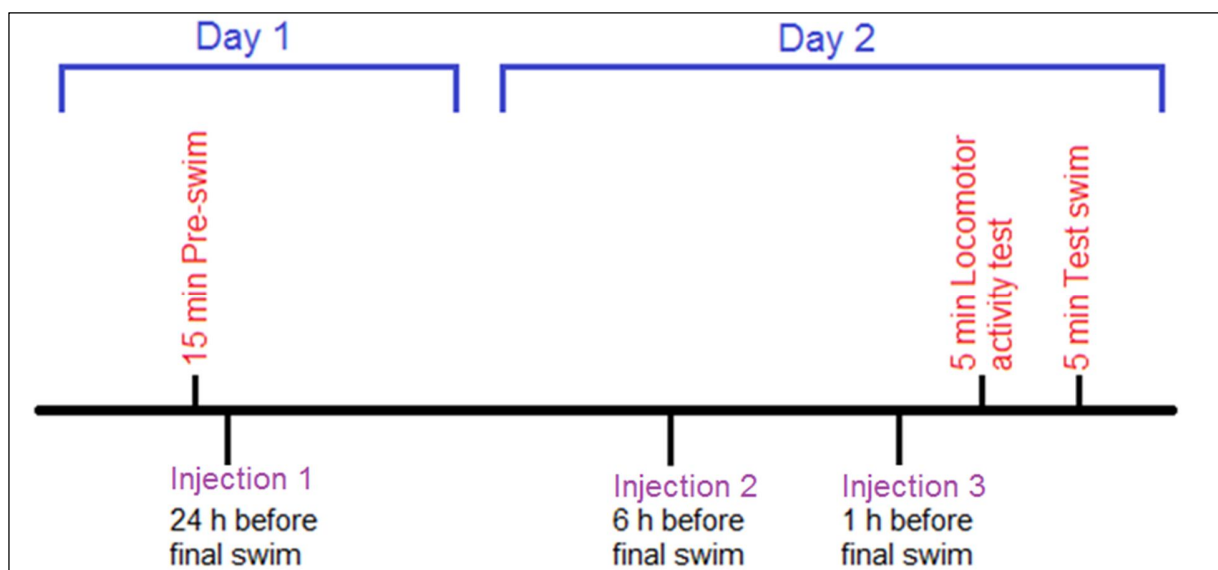


Figure 5.1: The diagrammatical presentation of the experimental layout for the FST

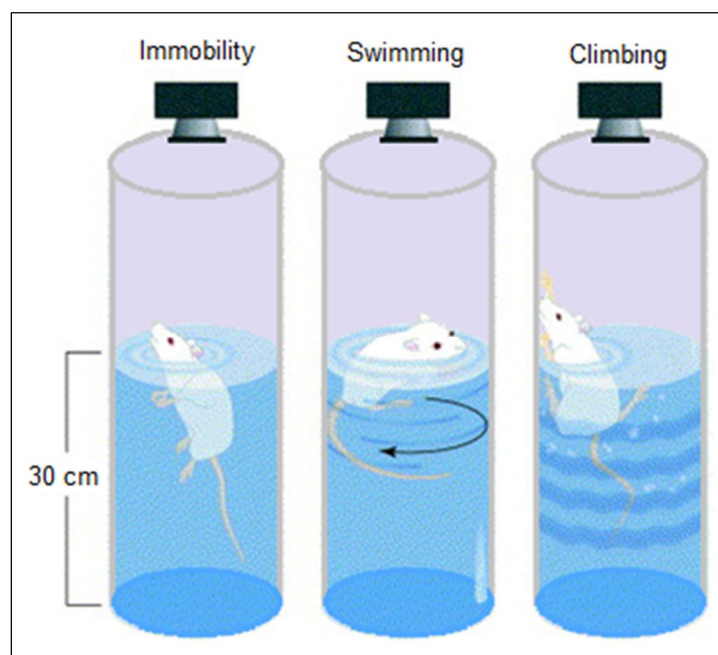


Figure 5.2: The behavioural parameters measured in the modified FST (Cryan *et al.*, 2002).

5.3.3. Results

The aim of this study was to confirm the antidepressant efficacy of MB in the FST, as previously reported (Eroglu & Caglayan, 1997, Harvey *et al.*, 2010), and thereafter to determine whether ETC and azure B present with similar antidepressant-like activity. Secondly, we determined the dosage at which ETC and azure B are effective as antidepressants using a dose-response analysis. Finally, to determine if the nature of the swimming activity observed in the FST relates to catecholaminergic or serotonergic driven mechanisms, swimming and climbing behaviour was further analysed by a process of behavioural sampling, as described by Cryan and colleagues (2002), whereby duration of immobility, climbing and swimming behaviours were specifically measured. In all instances IMI (15 mg/kg) was used as the positive control. Data were analysed by means of one-way analysis of variance (ANOVA) across all groups, and were subsequently subjected to the Tukey's post-test. GraphPad Prism® was used for all statistical analyses and graphical representations. Data are presented in table 5.1. as averages $\pm$ S.E.M. (standard error of mean) with a value of $p < 0.05$ taken as statistically significant.

One way analysis of variance of the data revealed a significant effect of treatment on behaviour as illustrated in figure 5.3. Post hoc analysis (see Table 5.1. and Figure 5.3.) revealed a significant decrease in immobility time for IMI (15 mg/kg; positive control) and MB (15 mg/kg) versus saline treatment. ETC demonstrated a decrease in immobility, with a significant

response compared to saline at all the doses examined: 4 mg/kg (from 221 ± 13.95 sec to $35.00 \pm 6.81^*$ sec, $*p < 0.05$), 7.5 mg/kg (from 221 ± 13.95 sec to $46.25 \pm 23.96^*$ sec, $*p < 0.05$), 15 mg/kg (from 221 ± 13.95 sec to $31.88 \pm 8.86^*$ sec, $*p < 0.05$), and at 30 mg/kg (from 221 ± 13.95 sec to $15.71 \pm 7.67^*$ sec, $*p < 0.05$) (Table 5.1. and Figure 5.3.). Compared to IMI, ETC demonstrated a significantly greater decrease in immobility at all doses examined: 4 mg/kg (from 126.30 ± 24.42 sec to $35.00 \pm 6.81^*$ sec, $*p < 0.05$), 7.5 mg/kg (from 126.30 ± 24.42 sec to $46.25 \pm 23.96^*$ sec, $*p < 0.05$), 15 mg/kg (from 126.30 ± 24.42 sec to $31.88 \pm 8.86^*$ sec, $*p < 0.05$), and at 30 mg/kg (from 126.30 ± 24.42 sec to $15.71 \pm 7.67^*$ sec, $*p < 0.05$) (Table 5.1. and Figure 5.3.). The difference in immobility between IMI and MB treated rats, however, was not significant, suggesting therapeutic equivalence (Table 5.1. and Figure 5.3.).

Table 5.1: The effect of ETC and azure B on immobility of rats in the FST, where * denotes $p < 0.05$ vs saline-treated animals and † denotes $p < 0.05$ vs IMI treated animals (n=8)

Treatment group	Mean $\pm$ SEM, immobility time (sec)
Saline	221.90 ± 13.95
IMI	$126.30 \pm 24.42^*$
MB	$70.63 \pm 35.78^*$
ETC (4.0 mg/kg)	$35.00 \pm 6.81^{* \dagger}$
ETC (7.5 mg/kg)	$46.25 \pm 23.96^{* \dagger}$
ETC (15.0 mg/kg)	$31.88 \pm 8.86^{* \dagger}$
ETC (30.0 mg/kg)	$15.71 \pm 7.67^{* \dagger}$
Azure B (4.0 mg/kg)	$30.00 \pm 8.81^{* \dagger}$
Azure B (7.5 mg/kg)	$21.25 \pm 2.26^{* \dagger}$
Azure B (15.0 mg/kg)	$15.63 \pm 6.51^{* \dagger}$
Azure B (30.0 mg/kg)	$36.25 \pm 13.98^{* \dagger}$

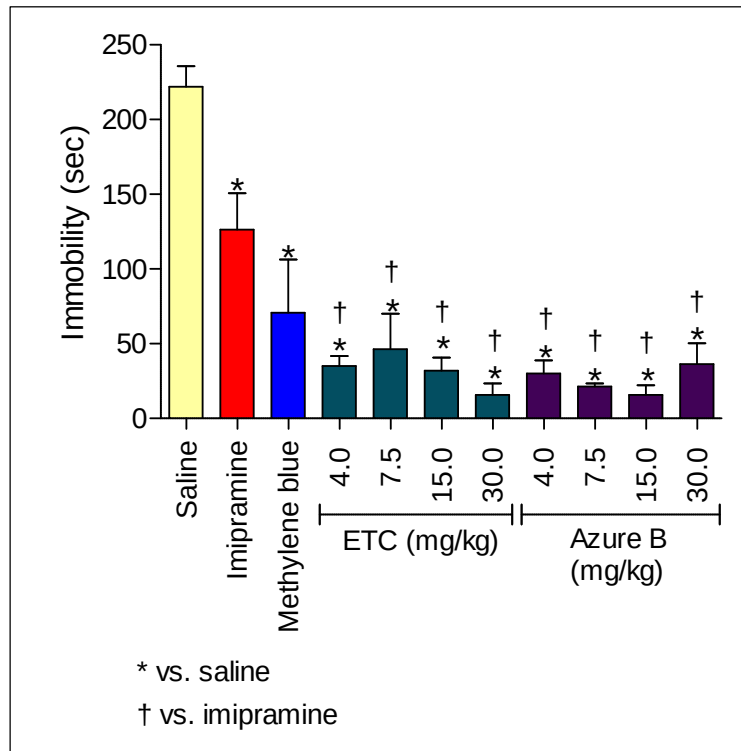


Figure 5.3: Effect of various doses of ETC and azure B, as indicated, on the duration of immobility in the FST, compared to saline (* $p < 0.05$) and IMI ($^{\dagger} p < 0.05$) treated animals ($n = 8/\text{group}$).

The effect of azure B on immobility of rats in the FST (Table 5.1.) is expressed as the mean $\pm$ S.E.M. with a value of $p < 0.05$ taken as statistically significant. One way ANOVA of the data revealed a significant effect of treatment with azure B, as illustrated in Figure 5.4. Post-hoc analysis indicated that, compared to saline, azure B demonstrated a decrease in immobility, with a significant response (compared to saline treated animals) at all the doses examined: 4.0 mg/kg (from 221 ± 13.95 sec to $30.00 \pm 8.81^*$ sec, $*p < 0.05$), 7.5 mg/kg (from 221 ± 13.95 sec to $21.25 \pm 2.26^*$ sec, $*p < 0.05$), 15.0 mg/kg (from 221 ± 13.95 sec to $15.63 \pm 6.51^*$ sec, $*p < 0.05$), and at 30.0 mg/kg (from 221 ± 13.95 sec to $36.25 \pm 13.98^*$ sec, $*p < 0.05$) (Table 5.2. and Figure 5.4.). Azure B demonstrated a decrease in immobility, with a significant response (compared to IMI treated animals) at all the doses examined: 4.0 mg/kg (from 126.30 ± 24.42 sec to $30.00 \pm 8.81^*$ sec, $*p < 0.05$), 7.5 mg/kg (from 126.30 ± 24.42 sec to $21.25 \pm 2.26^*$ sec, $*p < 0.05$), 15.0 mg/kg (from 126.30 ± 24.42 sec to $15.63 \pm 6.51^*$ sec, $*p < 0.05$), and at 30.0 mg/kg (from 126.30 ± 24.42 sec to $36.25 \pm 13.98^*$ sec, $*p < 0.05$) (Table 5.1. and Figure 5.3.). The difference in immobility between IMI and MB treated animals, however, was not significant, suggesting therapeutic equivalence.

When considering the effects of ETC and azure B treatment on swimming and climbing behaviour in the FST, data were analysed by means of one-way (ANOVA) across all groups, and were subsequently subjected to the Dunnett's post-test. GraphPad Prism® was used for all statistical analyses and graphical representations. Data are presented in table 5.2. and table 5.3. as mean $\pm$ S.E.M. with a value of $p < 0.05$ taken as statistically significant.

One way ANOVA revealed a significant effect of treatment on swimming behaviour, while post hoc analysis with Dunnetts (Table 5.2) indicated a significant increase (compared to saline treated animals) in the swimming behaviour at all the doses for ETC and azure B. It was also noted that there was significant differences in the swimming behaviour after treatment with either IMI or MB compared to saline. The effect of various doses of ETC and azure B on swimming behaviour in the FST, compared to saline (* $p < 0.05$) treated animals is shown in Figure 5.4.

One way ANOVA revealed a significant effect of treatment on climbing behaviour, while post hoc analysis with Dunnetts (table 5.3) revealed no significant differences in climbing behaviour after treatment with IMI, MB, ETC or azure B at the doses tested, compared to saline treated animals. The effect of various doses of ETC and azure B on the climbing behaviour in the FST, compared to saline treated animals is shown in Figure 5.5.

Table 5.2: The effect of ETC and azure B on the swimming behaviour of rats in the FST, where * denotes $p < 0.05$ vs saline-treated (n=8).

Treatment group	Mean $\pm$ SEM, swimming time (sec)
Saline	30.0 $\pm$ 9.73
IMI	126.9 $\pm$ 24.91*
MB	201.9 $\pm$ 33.06*
ETC (4.0 mg/kg)	243.8 $\pm$ 8.70*
ETC (7.5 mg/kg)	209.4 $\pm$ 27.31*
ETC (15.0 mg/kg)	233.1 $\pm$ 10.98*
ETC (30.0 mg/kg)	257.1 $\pm$ 11.07*
Azure B (4.0 mg/kg)	233.1 $\pm$ 8.18*
Azure B (7.5 mg/kg)	258.8 $\pm$ 7.55*
Azure B (15.0 mg/kg)	268.8 $\pm$ 6.39*
Azure B (30.0 mg/kg)	224.4 $\pm$ 12.69*

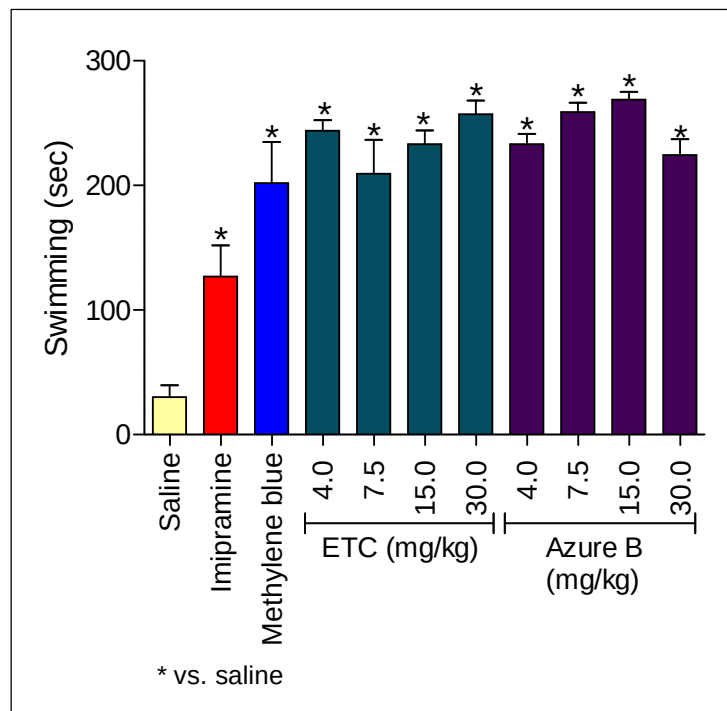


Figure 5.4: Effect of various doses of ETC and azure B, as indicated, on swimming behaviour in the FST, compared to saline treated animals (*p < 0.05) (n = 8/group)

Table 5.3: The effect of ETC and azure B on the climbing behaviour of rats in the FST, where *p < 0.05 vs saline-treated animals (n=8).

Treatment group	Mean $\pm$ SEM, climbing time (sec)
Saline	48.13 $\pm$ 13.76
IMI	47.50 $\pm$ 12.85
MB	26.88 $\pm$ 7.45
ETC (4.0 mg/kg)	21.25 $\pm$ 3.50
ETC (7.5 mg/kg)	43.75 $\pm$ 22.71
ETC (15.0 mg/kg)	35.00 $\pm$ 6.94
ETC (30.0 mg/kg)	27.86 $\pm$ 9.05
Azure B (4.0 mg/kg)	36.25 $\pm$ 10.85
Azure B (7.5 mg/kg)	19.38 $\pm$ 7.04
Azure B (15.0 mg/kg)	15.63 $\pm$ 5.86
Azure B (30.0 mg/kg)	40.00 $\pm$ 12.54

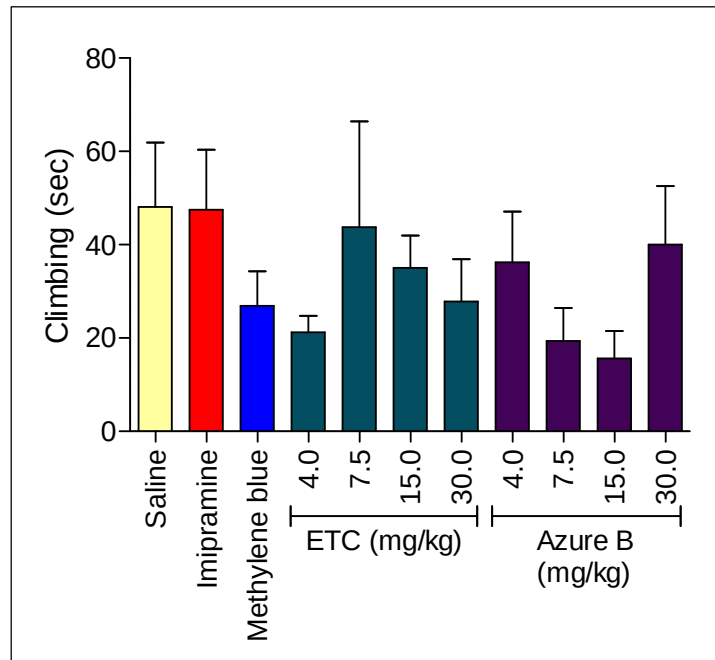


Figure 5.5: Effect of various doses of ETC and azure B, as indicated, on climbing behaviour in the FST, compared to saline treated animals (n = 8/group)

5.4. General locomotor activity

5.4.1. Introduction

Locomotor activity was routinely evaluated to ensure that changes in swim motivation were based only on antidepressant-like response and not due to an indirect effect of the drug on locomotor activity. The Digiscan[®] animal activity monitor (AccuScan[®] Instruments, Columbus, OH, USA) (Figure 5.6.) was employed to measure locomotor activity of the animals. The Digiscan[®] animal activity monitor provides automated and continual computerised monitoring of animal movement. It is more sensitive than visual observation and without the risks of investigator bias (Sanberg *et al.*, 1983; 1987). The Digiscan[®] animal activity monitor boxes have a series of cross-sectional horizontal infrared light beams at ground level plus additional beams 10 cm above ground level. These infrared beams enable the computerised collection of all locomotor activity, to provide a dynamic picture of all the aspects of the animal's horizontal and vertical movement (locomotor activity) throughout the observation period. By breaking any one of the beams, the action is interpreted as an activity score, whereas the breaking of two or more consecutive beams is recorded as a movement score (Korff *et al.*, 2008). In the current study horizontal activity (number of beam breaks) and the total distance covered (measured in centimetres) during the five minute trial were recorded.

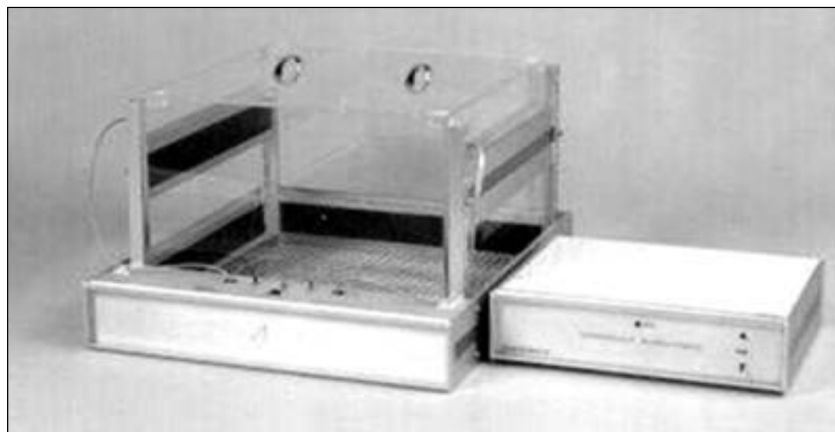


Figure 5.6: The Digiscan[®] animal activity monitor, as implemented in the current study.

5.4.2. Method

After the last injection on day two, the animals were habituated in a room for 20 min, and their general locomotor activity was recorded. Each rat was placed in the centre of a separate Digiscan[®] box and left to explore the arena for 5 min during which time horizontal movement and the total distance covered was measured.

5.4.3. Results

Post hoc analysis with Dunnetts (Table 5.4. and 5.5.) revealed no significant difference in the locomotor activity (horizontal activity or total distance) in MB, ETC and azure B treated animals compared to saline treated animals. However, IMI decreased horizontal activity significantly compared to saline treated animals as shown in Figure 5.7. The effect of various doses of ETC and azure B on the locomotor activity, compared to saline treated animals is shown in Figure 5.7. and 5.8.

Table 5.4: The effect of ETC and azure B on horizontal activity of rats in the locomotor activity test, where *p < 0.05 vs saline-treated animals (n=8)

Treatment group	Mean $\pm$ SEM, horizontal activity(sec)
Saline	2245 $\pm$ 120.5
IMI	1374 $\pm$ 236.2*
MB	2580 $\pm$ 210.6
ETC (4.0 mg/kg)	2757 $\pm$ 187.8
ETC (7.5 mg/kg)	2542 $\pm$ 252.7
ETC (15.0 mg/kg)	2192 $\pm$ 78.6
ETC (30.0 mg/kg)	2295 $\pm$ 317.8
Azure B (4.0 mg/kg)	2111 $\pm$ 198.8
Azure B (7.5 mg/kg)	1701 $\pm$ 164.7
Azure B (15.0 mg/kg)	1653 $\pm$ 192.1
Azure B (30.0 mg/kg)	1559 $\pm$ 176.0

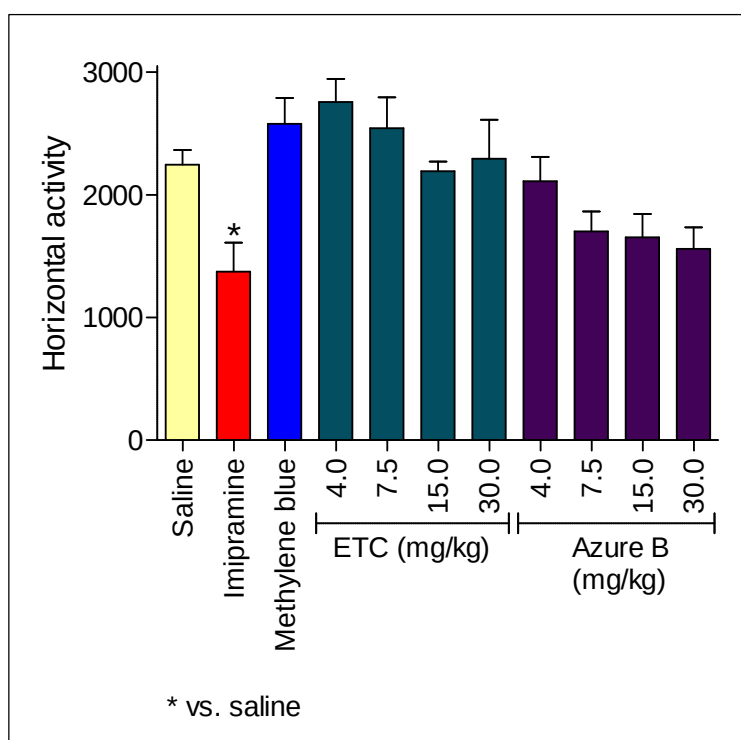


Figure 5.7: Effect of various doses of ETC and azure B, on horizontal activity, compared to saline treated animals (*p < 0.05) (n = 8/group).

Table 5.5: The effect of ETC and azure B on total distance covered of rats in the locomotor activity test (n=8)

Treatment group	Mean $\pm$ SEM, total distance (cm)
Saline	1164 $\pm$ 77.0
IMI	640 $\pm$ 126.1
MB	1681 $\pm$ 282.1
ETC (4.0 mg/kg)	1809 $\pm$ 221.4
ETC (7.5 mg/kg)	1651 $\pm$ 301.4
ETC (15.0 mg/kg)	1174 $\pm$ 80.7
ETC (30.0 mg/kg)	1716 $\pm$ 402.2
Azure B (4.0 mg/kg)	1441 $\pm$ 235.1
Azure B (7.5 mg/kg)	782 $\pm$ 90.8
Azure B (15.0 mg/kg)	1853 $\pm$ 170.4
Azure B (30.0 mg/kg)	778 $\pm$ 122.6

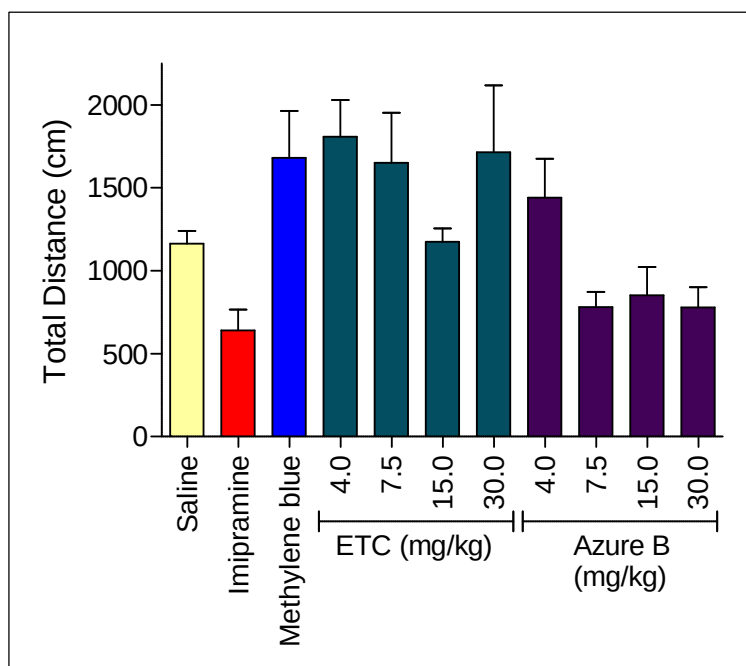


Figure 5.8: Effect of various doses of ETC and azure B, on total distance measured, compared to saline treated animals (n = 8/group)

5.4. Conclusion

ETC and azure B were evaluated over a dosage range of 4-30 mg/kg in the acute FST and compared to saline, IMI (15 mg/kg) and MB (15 mg/kg) treatment. In the acute FST, IMI was found to be an effective antidepressant, although ETC (at all doses tested) and azure B were more effective than IMI in reversing immobility. IMI increased swimming but not climbing behaviour in the FST, indicative of enhancing serotonergic activity. ETC and azure B (at all doses tested) similarly increased swimming behaviour, and was statistically superior to that of IMI. At all the doses tested, ETC and azure B increased swimming behaviour, and was numerically but not statistically superior to that of MB. This significant decrease in depressive-like behaviour as observed in the FST are supported by the data from the locomotor activity, since the results show that azure B and ETC do not significantly enhance locomotor activity compared to saline treated animals.

Chapter 6

Conclusion

6.1. Introduction

Mood disorders, such as major depression are among the most prevalent forms of mental illness. According to the WHO, depression will be the second leading burden of disease globally by 2020 (Murray & Lopez, 1996). Despite the wide variety of antidepressants available, more effective drugs with a quick onset of action and minimal side effects are needed. MB acts at multiple cellular and molecular targets and as a result possesses diverse medical applications. MB possesses promising antidepressant (Harvey *et al.*, 2010) and anxiolytic (Eroğlu and Caglayan, 1997) activity in pre-clinical models and has shown promise in clinical trials for bipolar disorder (see <http://clinicaltrials.gov/show/NCT00214877>) and depressive illness (Naylor *et al.*, 1987). MB is a potent reversible inhibitor of MAO-A as well as inhibitor of NOS, which together may underlie its adverse effects but also its antidepressant-like actions. MB is metabolised to yield N-demethylated products of which azure B, the monodemethyl species, is the major metabolite. Similar to MB, azure B also displays a variety of biological activities and may therefore contribute to the pharmacological profile of MB.

In this study a close structural analogue of MB (ETC), that inhibits MAO-A to a lesser degree compared to MB, was designed and synthesised. These structural modifications were made in order to improve the safety profile of MB, since the MAO-A inhibitory component of MB is associated with several side effects. Although NOS inhibition is associated with a risk for cardiovascular and other CNS adverse effects, MB seems to be at less risk of inducing these problems. The approach that was followed was to make a relatively small structural change to MB in order to minimise or remove the MAO-A inhibition properties of MB and to establish whether its antidepressant activity could be retained. The tetramethyl substitution pattern of the amine nitrogens of MB was thus replaced by tetraethyl substitution. This modification increases the size of the structure, which may lead to reduced MAO-A inhibition. The active site cavity of MAO-A is reported to be relatively small, and in general accommodates smaller inhibitors better than larger inhibitors (Milczek *et al.*, 2011). In addition, larger compounds may not bind well to MAO-A because, in the MAO-A active site, the residue corresponding to Ile199 in MAO-B is Phe208. The increased size of the Phe side chain compared to the side chain of Ile prevents it from rotating from the MAO-A active site cavity into an alternative conformation as does Ile199

in MAO-B. The binding of larger inhibitors may thus be hindered by Phe208 in the MAO-A active site (Milczek *et al.*, 2011). It was thus envisioned that since ETC is larger than MB, it may display weaker interaction with MAO-A than MB.

6.2. Specific findings and conclusions

The following is an overview of experiments conducted in this study, with an accompanying discussion at each point:

The synthesis of the MB analogue: ETC was synthesised from the HCl salt of N,N-diethyl-*p*-phenylenediamine. N,N-Diethyl-*p*-phenylenediamine was treated with sodium sulphide and iron(III)chloride to yield ETC according to the published method (Wischik *et al.*, 2006). Although ETC was obtained in low yield (6%), the compound exhibited a high degree of purity.

ETC was subsequently evaluated as an inhibitor of human MAO-A and MAO-B. The MAO activity measurements were based on measuring the rate by which kynuramine undergoes oxidative deamination to yield a fluorescent product, 4-hydroxyquinoline. The results show that ETC, with an IC_{50} value of 0.51 μ M, is a weaker MAO-A inhibitor than MB (IC_{50} = 0.07 μ M) and azure B (IC_{50} = 0.01 μ M). This suggests that ETC would have a lower probability of causing adverse effects associated with MAO-A inhibition such as the cheese reaction and ST. This outcome was set as one of the goals of this study, since ETC should show weaker MAO-A inhibitory potency than MB. It was also found that ETC (IC_{50} = 0.59 μ M) is a more potent inhibitor of MAO-B than MB (IC_{50} = 4.37 μ M) and azure B (IC_{50} = 0.97 μ M). Being larger than MB and azure B, ETC should undergo more productive Van der Waals interactions with the MAO-B active site, especially since the MAO-B active site is reported to be largely hydrophobic. It is interesting to note that ETC may be considered as a non-selective MAO inhibitor since it inhibits both MAO-A and MAO-B with similar potencies. Non-selective MAO inhibitors are particularly desirable for antiparkinsonian therapy since dopamine is metabolised by both MAO isoforms in the brain. Inhibition of both MAO-A and MAO-B may thus lead to enhanced protection against dopamine depletion than a selective MAO-B inhibitor.

The recovery of the enzymatic activity after dilution of enzyme-inhibitor complexes was evaluated for the inhibition of MAO-A and MAO-B by ETC. The results indicate that ETC binds reversibly to the MAO-A and MAO-B enzymes. This finding suggests that ETC may be a relatively safe MAO-A inhibitor in the clinical setting since reversible MAO inhibitors are less likely to cause the cheese reaction than irreversible MAO inhibitors. Sets of Lineweaver-Burk

plots were also constructed for the inhibition of MAO-A and MAO-B by ETC. The initial rates of the MAO-catalysed oxidation of kynuramine at four different substrate concentrations in the absence and presence of three different concentrations of ETC were measured. The Lineweaver-Burk plots were found to be linear and intersected towards the y-axis, this suggests that ETC is a competitive inhibitor of both human MAO-A and MAO-B. Since competitive inhibitors bind reversibly to enzymes, this is further evidence that ETC is a reversible MAO-A and MAO-B inhibitor.

The lipophilicity (LogD) of ETC and MB were determined. The partition coefficient is a measure of how hydrophilic or hydrophobic a compound is. ETC and MB were found to be hydrophilic since they display LogD values smaller than 0. The LogD values of ETC ranges from -0.13 to -0.21. The LogD values at different pH values and in water are quite similar because it is expected that ETC would be completely ionised at physiological pH values. The hydrophilic nature of ETC and MB are to be expected since these molecules are charged. ETC is, however, slightly less hydrophilic than MB. This finding is in agreement with the structural differences between MB and ETC. Compared to the tetramethyl substitution of MB, tetraethyl substitution, as found with ETC, should enhance lipophilicity. As previously mentioned, although MB is hydrophilic, it still crosses the blood-brain barrier (Peter *et al.*, 2000). Permeation can be facilitated by the leucoMB form, which is uncharged and 20 times more lipophilic than MB (Harris and Peters, 1953). It is expected that ETC may also be reduced to its leuco-form, which will be more lipophilic and thus allow penetration through the blood-brain barrier.

To measure the solubility of ETC, MB and azure B, an equilibrium shake-flask thermodynamic solubility method was used. The results show that ETC, MB and azure B are highly soluble in water and buffer since the concentrations recorded in water and buffer are well above 60 µg/ml. The results showed that ETC has a lower aqueous solubility compared to MB and azure B. ETC is expected to be less soluble in aqueous media than MB and azure B since ETC has been found to be more lipophilic than MB and azure B. The high degree of aqueous solubility of ETC will facilitate oral bioavailability.

The ionisation constant (pKa) of ETC was determined. The pKa value of ETC should give an indication of the degree of ionisation of ETC at the specific pH values in the gastrointestinal tract and plasma. The experimentally determined ionisation constant of ETC ranges from 0.122 to -1.833. Similar to MB (pKa = -1 to 0), the low pKa value of ETC is expected since the molecules

already possess a positive charge. The pKa value of ETC demonstrates that the acid-base properties of MB and ETC are similar.

The toxicity of ETC, MB and azure B towards cultured cells were determined. For this purpose, the MTT cell viability assay was used (Mosmann, 1983). The MTT assay is a standard cell viability assay, measuring mitochondrial activity in live cells. It was found that MB was relative non-toxic at 1 μ M with 85.9% viable cells. Azure B and ETC was however, more toxic at 1 μ M with 44.1% and 38.3% viable cells remaining, respectively, compared to untreated controls. ETC is more lipophilic than MB, which may explain the enhanced toxicity. ETC may permeate more readily into the cultured cells, and thus may attain higher concentrations in the cytosol than MB. Higher intracellular concentrations are expected to lead to a higher degree of toxicity. The exact molecular mechanisms of cytotoxicity of ETC and azure B are not apparent and further investigation is required to address this issue.

Azure B and ETC were evaluated in the acute FST to determine if ETC and azure B possess antidepressant-like activity similar to MB. ETC and azure B were evaluated over a dosage range of 4-30 mg/kg in the acute FST and compared to saline, IMI (15 mg/kg) and MB (15 mg/kg) treated rats. In the acute FST, ETC and azure B were more effective than IMI in reversing immobility, without confounding effects on locomotor activity, suggesting that the animals have an increased drive to escape the water. The significant decrease in depressive-like behaviour as observed in the FST is supported by the data from the locomotor activity, since the results show that azure B and ETC do not significantly enhance locomotor activity compared to saline treated animals. ETC and azure B increased swimming behaviour but not climbing, which is indicative of a selective action to increase serotonergic neurotransmission. This is in line with earlier work in animals (Harvey *et al.*, 2010) as well as the ability of MB to cause ST when combined with serotonergic agents (Ramsay *et al.*, 2007; Stanford *et al.*, 2010).

The hypothesis of this study, as mentioned in Chapter 1, was that ETC will possess reduced MAO-A inhibitory potency compared to MB while retaining an antidepressant-like response in the acute FST. It was further postulated that azure B would also possess antidepressant-like activity in the FST. It may be concluded that the hypothesis of this study has been confirmed. ETC is indeed a weaker MAO-A inhibitor than MB, while possessing equipotent antidepressant-like activity with a known antidepressant in the acute FST. Azure B also possesses antidepressant-like properties in the FST, which suggests that azure B may be a contributor to the antidepressant-effect of MB.

6.3. Future recommendations and future studies

In the current study we provide evidence that ETC and azure B are novel antidepressants in the acute rat FST, and that charged tricyclic structures similar to MB may be novel lead antidepressant compounds. This evidence suggests that azure B may be a contributor to MB's antidepressant effect. This finding warrants further investigation using different animal models of depression, as well as other preclinical studies to clarify the complete pharmacological and toxicological profile of ETC and azure B.

Based on the outcome of the current study, several strategies for future studies are proposed:

- Chemical synthesis of novel compounds based on the MB structure, with increased structure size, which may lead to reduced MAO-A inhibition.
- Perform subsequent pharmacological and behavioural studies on these novel synthetic MB derivatives.
- Studying the effects of ETC and azure B in animal models that simulate more closely the chronic disease, such as the genetic model of depression, the Flinders Sensitive Line (FSL) rats (Overstreet, 1993).
- Studying monoamine, GABA and glutamate release following acute challenge with the above compounds or after chronic treatment, using *in vivo* microdialysis.
- Analysis of the effects of MB, ETC, azure B and other selected MB analogues on brain NOS.

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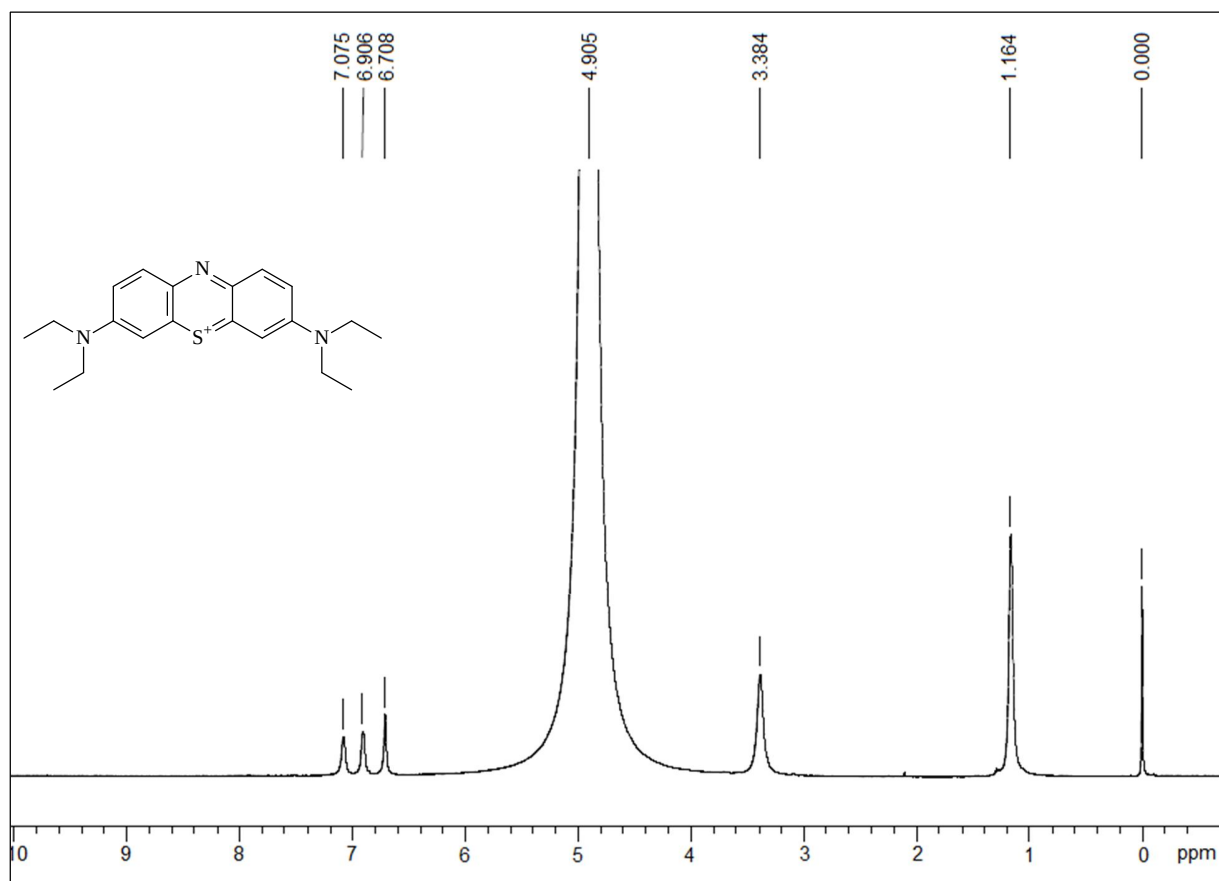
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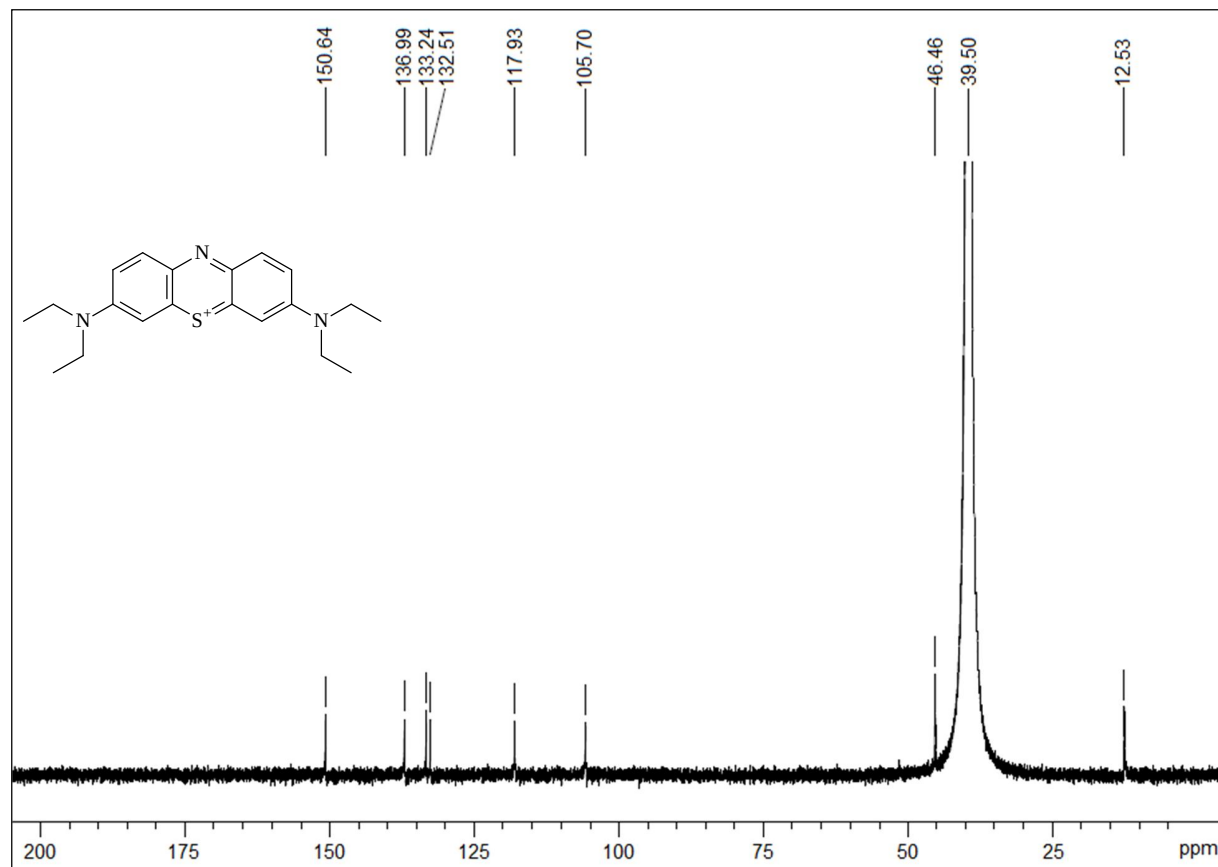
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Appendix A

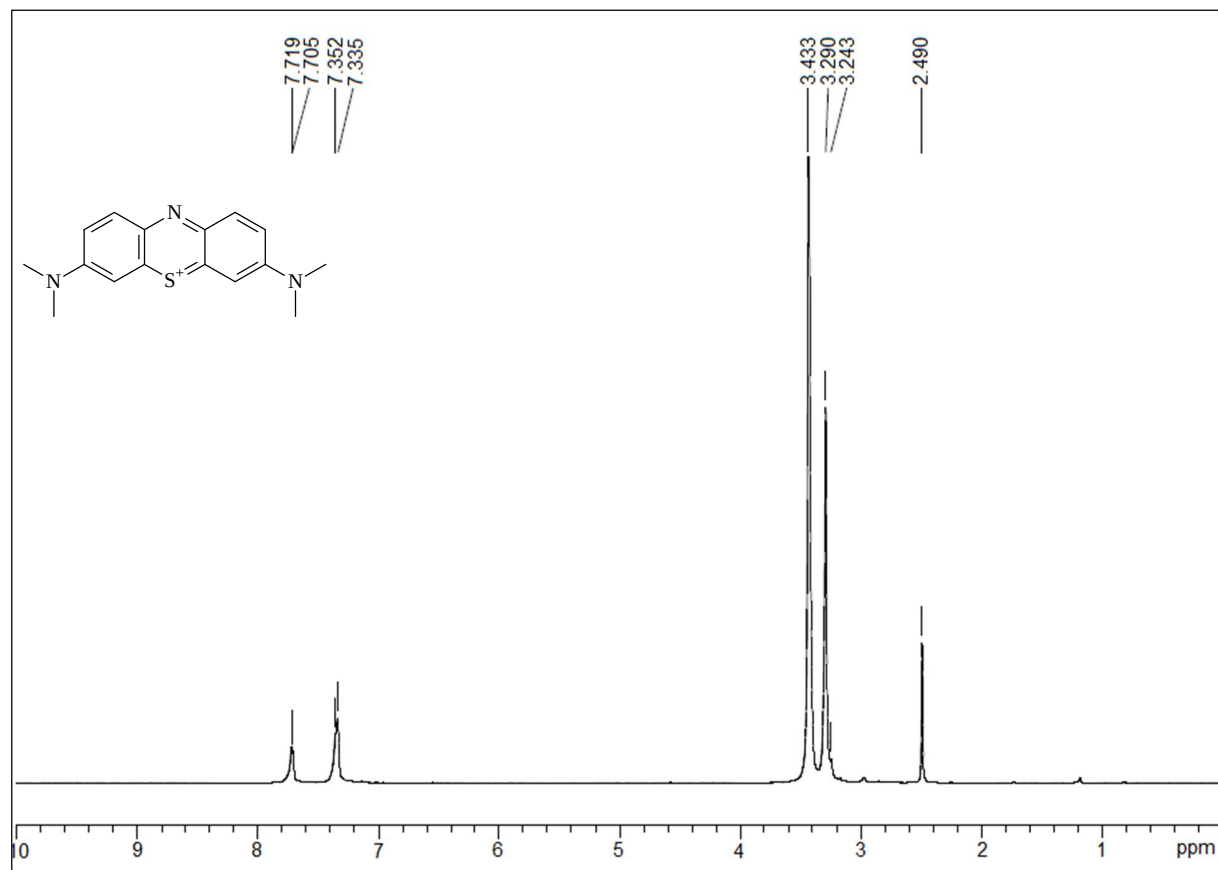
The ^1H NMR spectra of ETC



The ^{13}C spectra of ETC



The ^1H spectra of MB



The ^{13}C spectra of MB

