



**An investigation of plant derived compounds as
inhibitors of monoamine oxidase
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Preface:

This dissertation is submitted in article format and contains an original research article. All the results obtained during this study is presented in the article. The article was submitted for publication to the academic journal Chemistry & Biodiversity. The author guidelines can be found in annexure B. The research described in this article was conducted by Ms D. Prinsloo at the North-West University, Potchefstroom campus.

The Letter of agreement from the co-authors of the research article is included in annexure A.

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*“Now to Him who is able to do immeasurably more
than all we ask or imagine, according to His Power
that is at work within us,
to Him be glory in the church and in
Christ Jesus throughout all generations,
forever and ever! Amen.”*

- Ephesians 3:20

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Abstract:

Computational chemistry, among other things, elucidates possible drug-molecular target interactions when an algorithm is run on a molecular modelling program. Although it is just a projection of reality, it enables researchers to eliminate compounds that have no specific interaction with the chosen molecular target before expensive chemical and biological screenings are done. The combination of computational chemistry and biological chemistry redefines drug design as a more secure method of identifying target specific compounds. Novel drug design often relies on compounds isolated from natural products to produce viable leads for the treatment of various diseases and ailments. Traditional healers all over the world have used indigenous vegetation as medicine to the point that researchers have started to investigate the reported medicinal value of specific fauna or flora. Testing the major constituents of natural products may provide new drug leads and sources.

Parkinson's disease is described as a progressive neurodegenerative disease with an unknown aetiology. The depletion of the neurotransmitter, dopamine, in the striatum is responsible for the motor symptoms of Parkinson's disease and constitutes the focus of current and novel treatment regimes. The disease onset is usually met with an occasional tremor in the hands or fingers and gradually worsens over time to complete motor dysfunctionality and mental incapacity. The unknown aetiology limits researchers to symptomatic treatments that will either mimic the effects of dopamine at dopaminergic receptors or enhance the levels of dopamine in the affected regions of the brain.

One of the current treatment strategies include the use of monoamine oxidase (MAO) inhibitors. MAO is an outer-mitochondrial bound enzyme responsible for the regulation and deamination of neurotransmitters throughout the body. By inhibiting this enzyme in the central nervous system the metabolism of dopamine is reduced, and thus dopaminergic neurotransmission is enhanced. The two known isoforms of the MAO enzyme, MAO-A and MAO-B, both metabolise dopamine in the brain. The difference in substrate specificity of the two MAO isoforms provides a rationale for targeting them for different therapeutic applications. MAO-A, for example, is responsible for the

degradation of serotonin, tyramine and norepinephrine. Inhibition of MAO-A is indicated for diseases such as depression and anxiety. MAO-B inhibition, on the other hand, results in a significant increase in dopamine levels in the brain, and MAO-B specific inhibitors are thus used in the treatment of Parkinson's disease.

The aim of this study was to select the major constituents or metabolites of natural products and to evaluate them as potential MAO inhibitors. The chemical structures of these compounds can then be used in future studies as possible lead compounds for the design of MAO inhibitors.

The results shows that DL-kavain, from the roots of the kava plant, is a good potency *in vitro* inhibitor of human MAO-B with an IC_{50} of 5.43 μ M. DL-Kavain is a weaker MAO-A inhibitor with an IC_{50} of 19.0 μ M. Under the same experimental conditions, the known MAO inhibitor, curcumin, displays IC_{50} values of 5.02 μ M and 2.56 μ M for the inhibition of MAO-A and MAO-B, respectively. It was further established that DL-kavain interacts reversibly and competitively with MAO-A and MAO-B with enzyme-inhibitor dissociation constants (K_i) of 7.72 and 5.10 μ M, respectively. Curcumin in turn, displays a K_i value of 3.08 μ M for the inhibition of MAO-A. Other natural products that exhibited MAO inhibition were tanshinone I, myrtenol and ellagic acid.

Molecular docking studies was used to investigate possible binding modes and interactions of DL-kavain and curcumin with the MAO enzymes. It may be concluded that some of the central effects (e.g. anxiolytic) of kava may be mediated by MAO inhibition. Furthermore, natural products with MAO inhibition properties may serve as leads for future studies that aims to discover high potency MAO inhibitors.

Keywords: Monoamine oxidase, Natural products, Neurodegenerative diseases, Parkinson's disease, *Piper methysticum*, Molecular modelling, Kavain, Curcumin.

Uittreksel:

Molekulêre modelleringsprogramme poog om interaksies wat moontlik tussen geneesmiddels en bindingsetels bestaan, met behulp van wetenskaplike-gebaseerde algoritmes te identifiseer. Alhoewel hierdie simulasies net 'n projeksie van die werklikheid is, word dit gewoonlik op groot hoeveelhede molekules uitgevoer voordat duur chemiese en biologiese studies gedoen word. Sodoende word die proses van geneesmiddelontdekking meer prakties benader deur onnodige verbindings uit te skakel wat geen spesifieke interaksies met die gekose molekulêre teikens toon nie. Die kombinasie van modellering en biochemie identifiseer veiliger en meer teikenspesifieke geneesmiddels, en maak dikwels van verbindings wat uit natuurprodukte geïsoleer is gebruik, om sodoende lewensvatbare leidrade vir verskeie siektetoestande te ontwikkel. Tradisionele genesers gebruik al vir dekades verskeie inheemse plante vir medisinale doeleindes en navorsers ondersoek gereeld sekere fauna en flora as bronne vir nuwe medikasie. Deur die aktiewe verbindings in plante en ander natuurbronne te toets, kan nuwe leidraadverbindings geïdentifiseer word.

Parkinson se siekte word as 'n progressiewe neurodegeneratiewe siekte van onbekende etiologie beskryf. Die verlaagde konsentrasies dopamien in die striatum is verantwoordelik vir die motoriese simptome wat met Parkinsonisme geassosieer word, en dit vorm die fokus van huidige en nuwe behandelingstrategieë. Die siekte begin gewoonlik met 'n sporadiese bewing in die hande of vingers, en vererger geleidelik tot volledige motoriese disfunksionaliteit en verstandelike onvermoë. Die onbekende etiologie beperk navorsers tot simptomatiese behandeling wat gewoonweg die effekte van dopamien naboots of die vlakke van dopamien in die brein verhoog.

Een van die behandelingstrategieë is die gebruik van monoamienoksidase- (MAO-) inhibeerders. MAO is 'n mitochondrion-gebonde ensiem wat vir die regulering en deaminering van monoamiensubstrate oral in die liggaam verantwoordelik is. Deur hierdie ensiem in die sentrale senuweestelsel te inhibeer, word die metabolisme van dopamien verlaag, en sodoende word dopaminergiese neurotransmissie verbeter. Beide die twee isovorme van die MAO-ensiem, MAO-A en MAO-B, metaboliseer dopamien in die brein. Die verskil in die substraat-spesifisiteit van die twee MAO-

isovorme veroorsaak dat hulle vir verskillende siektetoestande geteiken kan word. MAO-A metaboliseer serotonien, tiramien en noradrenalin en MAO-A-inhibeerders word dus vir toestande soos depressie en angs gebruik. MAO-B inhibisie, aan die ander kant, lei tot 'n beduidende toename in dopamienvlakke in die brein en MAO-B-inhibeerders word dus vir die behandeling van Parkinson se siekte gebruik.

Die doel van hierdie studie was om die hoofkomponente of metaboliete van natuurprodukte te selekteer en hulle as potensiële MAO-inhibeerders te evalueer. Die chemiese strukture van aktiewe verbindings kan in toekomstige studies as moontlike leidraadverbindings vir die ontwerp van MAO-inhibeerders gebruik word.

Die resultate het getoon dat DL-kavain, vanaf die kava-plant, 'n goeie *in vitro* inhibeerder van menslike MAO-B is, met 'n IC_{50} -waarde van 5.43 μ M. DL-kavain is 'n swakker MAO-A-inhibeerder met 'n IC_{50} -waarde van 19.0 μ M. Onder dieselfde eksperimentele kondisies het die bekende MAO-inhibeerder, curcumin, IC_{50} -waardes van 5.02 μ M en 2.56 μ M vir die inhibisie van onderskeidelik MAO-A en MAO-B getoon. Daar is verder vasgestel dat DL-kavain as 'n omkeerbare en kompeterende MAO-inhibeerder optree met ensiem-inhibeerder-dissosiasiekonstantes (K_i) van 7.72 en 5.10 μ M vir onderskeidelik MAO-A en MAO-B. Curcumin toon weer 'n K_i -waarde van 3.08 μ M vir die inhibisie van MAO-A. Ander natuurprodukte wat MAO-inhibisie getoon het was tansjinoon I, mirtenol en ellagiensuur.

Molekulêre modelleringstudies is gebruik om moontlike bindingoriëntasies en -interaksies van DL-kavain en curcumin met die MAO-ensieme te ondersoek. Hierdie studie maak die gevolgtrekking dat sommige van die sentrale effekte (bv. ansiolitiese effekte) van kava, deur die sentrale inhibisie van MAO veroorsaak word. Verder kan ander natuurprodukte wat MAO-inhibisie in hierdie studie getoon het, as leidraadverbindings dien vir toekomstige studies wat daarop gemik is om potente MAO-inhibeerders te ontdek.

Sleutelwoorde: Monoamienoksidase, Natuurprodukte, Neurodegeneratiewe siektes, Parkinson se siekte, *Piper methysticum*, Molekulêre modellering, Kavain, Curcumin.

LIST OF ABBREVIATIONS	
3D	Three dimensional
4-HQ	4-Hydroxyquinoline
[]	Concentration of
A	
AChE	Acetylcholinesterase
AD	Alzheimer's disease
Ala	Alanine
Arg	Arginine
Asn	Asparagine
B	
BBB	Blood–brain barrier
C	
clogP	Calculated partition coefficient
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
Cys	Cysteine
D	
DA	Dopamine
DOPA	Levodopa
DOPAC	3,4-Dihydroxyphenyl acetic acid
DMSO	Dimethyl sulfoxide
E	
E	Enzyme
F	
FAD	Flavin adenine dinucleotide
FDA	Food and Drug Administration
G	
GABA	Gamma-aminobutyric acid
Gln	Glutamine
Gly	Glycine

GP	Globus pallidus
Gpe	Globus pallidus externa
Gpi	Globus pallidus interna
GSH	Glutathione
H	
HTS	High-throughput screening
I	
I	Inhibitor
IC ₅₀	Inhibitor concentration that produces 50% inhibition of an enzyme
Ile	Isoleucine
I _{max}	Maximal rate of inactivation
K	
K _d	Dissociation constant
K _i	Inhibition constant
K _m	Michaelis constant
L	
LD ₅₀	Half lethal dose
Leu	Leucine
Lys	Lysine
M	
MAO	Monoamine oxidase
mg/kg	Milligram/kilogram
μM	Micromolar
MPP ⁺	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
N	
n	Number of selected hits
N	Number of total hits
NA	Norepinephrine
ND	Not determined
nM	Nanomolar
P	

PD	Parkinson's disease
PEA	Phenylethylamine
pIC ₅₀	−log IC ₅₀
R	
rMAO	Rat monoamine oxidase
RMSD	Root-mean-square deviation
ROS	Reactive oxygen species
ROC curve	Receiver operating characteristics curve
S	
S	Substrate
SD	Standard deviation
Se	Sensitivity
Ser	Serine
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticularis
Sp	Specificity
T	
ThC	Tetrahydrocurcumin
Tyr	Tyrosine
Trp	Tryptophan
V	
v _i	Initial velocity
V	Reaction velocity
V _{max}	Maximum reaction rate (Maximal velocity)

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CHAPTER 1

Title

An investigation of plant derived compounds as inhibitors of monoamine oxidase.

1. Introduction

1.1. Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder characterised by various movement abnormalities such as tremors, slow movement and muscle rigidity that gradually worsens as the disease progresses. The main neurochemical hallmark of PD is a deficiency of dopamine in the brain, which is responsible for most of the motor symptoms of PD. The drastic decline in neuronal dopamine is mainly found in the substantia nigra pars compacta in the basal ganglia, but other regions in the brain are also affected in the late stages of the disease. The cause of PD remains elusive but oxidative stress, mitochondrial dysfunction and protein aggregation appears to be central factors in the pathogenesis. The symptoms of PD only surface after more than 60% of the nigrostriatal dopaminergic neurons have died. There is no cure for PD and treatment regimens focus on symptomatic treatment. Levodopa, dopamine agonists, catechol-O-methyltransferase (COMT) inhibitors and monoamine oxidase (MAO) inhibitors are drugs that either enhance dopamine levels in the central nervous system or mimics the function of dopamine at dopaminergic receptors. The rationale behind the treatment options is that dopamine receptor stimulation reduces motor impairment. Dopamine is a water-soluble molecule and cannot be used in PD therapy as it is not able to cross the blood-brain barrier. Levodopa is thus the drug of choice in most PD cases since it gains access to the brain via amino acid transporter systems and is converted to dopamine after it has crossed the blood-brain barrier ([Dauer & Przedborski, 2003](#); [Fox *et al.*, 2018](#)).

1.2. Monoamine oxidase

MAO is a mitochondrial flavoenzyme found in most tissues and is attached to the outer membranes of mitochondria. MAO plays a vital role in the oxidative deamination of biological amines, exogenous amines and amine neurotransmitters (e.g. serotonin, dopamine and epinephrine) in the central nervous system as well as the peripheral tissues. Consequently, the MAOs are significant targets for the development of treatments for a variety of disease states. These include depression,

PD and Alzheimer's disease, disorders of the central nervous system that are linked to dysfunctional neurotransmitter function. Studies have linked certain personality traits such as impulsiveness, sensation-seeking behaviour or heightened reactivity toward provocative situations with increased activity of platelet MAO ([Binda et al., 2001](#); [McAllister & Nichols, 2018](#))

MAO consists of two isoforms, MAO-A and MAO-B. These isoforms are encoded by distinct genes that are approximately 70% identical. Based on differences of the amino acids in their active site cavities, the MAO isoforms exhibit different substrate and inhibitor specificities. For example, MAO-A selectively metabolises serotonin while MAO-B selectively metabolises benzylamine and phenylethylamine. Amine neurotransmitters such as dopamine, epinephrine, norepinephrine and tyramine are equally well metabolised by both MAO-A and MAO-B, resulting in an overlap of the MAO substrate specificities ([Edmondson & Binda, 2018](#); [Krishnan, 2017](#)).

Interestingly, the level of expressed enzyme varies with tissue type and the concentration of the MAO-B enzyme is tripled in neuronal tissue of the elderly. Increased levels of MAO, especially MAO-B, are associated with an increase of all the products formed during the catalytic reaction of MAO, including hydrogen peroxide. During a MAO-catalysed reaction, oxygen (O_2) acts as the electron acceptor and is thus converted to hydrogen peroxide (H_2O_2) by MAO. The oxidation of the amine substrates leads to the reduction of flavin adenine dinucleotide (FAD). The product of amine oxidation is an imine, which is further hydrolysed, in a non-enzymatic process, to yield ammonia and an aldehyde. When the aldehyde undergoes further oxidation by aldehyde dehydrogenase, a carboxylic acid is formed. It is important to note that the reaction of iron and other transition metals (cobalt, nickel, iron) with hydrogen peroxide may yield hydroxyl radicals, which are highly reactive and thought to contribute to neurodegeneration in PD ([Edmondson, 2014](#); [Edmondson et al., 2009](#); [Fišar, 2016](#); [Nagy and Kalász, 2017](#)).

MAO inhibitors may be classified as either reversible or irreversible inhibitors. Selegiline, tranylcypromine, rasagiline and safinamide are examples of inhibitors that have been approved for clinical use. Reversible inhibitors usually act as competitive inhibitors and have the advantage that inhibition is terminated with withdrawal of the

drug. Irreversible inhibitors, also known as k_{cat} or suicide inhibitors, initially bind in a reversible, competitive manner but the enzyme oxidises them to reactive intermediates that covalently binds to the active site of the enzyme. Enzyme activity thus only recovers by *de novo* synthesis of the MAO enzyme and inhibition may persist for several weeks after drug withdrawal and washout (Edmondson *et al.*, 2009; Krishnan, 2017).

One of the most serious adverse effects of MAO inhibition is tyramine-induced hypertension, also known as the cheese reaction. MAO-A inhibition in peripheral tissues block the metabolism of dietary tyramine, an amine found in food such as cheese and wine. This results in an elevation of tyramine concentrations in the systemic circulation, and since tyramine releases norepinephrine from peripheral neurons, tyramine significantly elevates blood pressure. This causes a series of hypertensive effects which can be fatal to certain individuals, especially those with cardiovascular problems. It is important to note that tyramine-induced hypertension is only associated with irreversible MAO-A inhibition, and MAO-B inhibitors, reversible and irreversible, are generally not associated with this adverse effect (Fox *et al.*, 2018; Katzung, Masters and Trevor, 2012; Krishnan, 2017)

1.3. Natural products in Parkinson's disease

In South Africa, more than 1000 plant species are used by a large part of the population as herbal medication. As modern health care in South Africa is not always regarded in a positive light by certain cultures, certain disorders, such as mental health problems, are considered to be one the diseases that should solely be treated with natural products administered by traditional healers (Enogieru *et al.*, 2018). These African traditional healers, known as sangomas, diagnose and devise treatment regimens for mental disorders such as hysteria, mental disturbances and anxiety using a wide variety of indigenous plants. The roots, leaves, bulb or corm of the plants are either eaten in whole, burned as incense or prepared as extracts in water or alcohol, to only name a few methods in which traditional medications are administered and formulated. The FDA approved more than two dozen natural products in the past 10 years. Out of the twenty-six approved products, seven products were indicated for neurodegenerative disorders and three of the seven

products focus on PD related symptomatic treatment. Focus is placed on plant-derived compounds that offer neuroprotective effects. With these medications, neurons may be provided with protection against mechanisms such as oxidative stress and excitotoxicity that cause cell damage and lead to irreparable cell loss as seen in PD (Khatoon *et al.*, 2018; Van Wyk, 2017).

1.4. Natural products as MAO inhibitors

Isolated small molecules derived from plants and other natural products acts as a rich source for novel drug leads. Flavonoids, xanthonenes, tannins, alkaloids, cannabinoids and curcumin are typical examples of compounds derived from natural sources with MAO inhibition activity (Dey *et al.*, 2017). Flavonoids (Fig. 1) reduce free radical formation by chelating metal ions and is also known for their antioxidant activity that interferes in processes that cause oxidative stress. Curcumin possesses good anti-depressant properties, which is due to the inhibition of MAO activity. Curcumin remains therapeutically inadequate since it demonstrates rapid metabolism and poor bioavailability. Alkaloids are composed of carbon, hydrogen nitrogen and oxygen, the majority of them are have excellent physiological properties but display toxic profiles . Morphine, atropine, reserpine and physostigmine are examples of these alkaloids and are essential medicinal drugs. Alkaloids isolated from the venom of scorpions and spiders have been tested positive as MAO inhibitors and various studies have included nicotine, caffeine and quinines as additional MAO inhibitors and potential neuroprotective agents (Carradori *et al.*, 2014). Cannabinoids are classified into three classes: endogenous cannabinoids, synthetic cannabinoids and herbal cannabinoids. There is a standing hypothesis that cannabinoids modulate monoaminergic neurotransmission by inhibiting MAO directly (Fišar, 2010). The pharmaceutical potential of MAO inhibitors found in plants all over the world has been recognised and the search for novel active MAO inhibitors has increased to a great extent (Butterweck *et al.*, 2002). Investigation of other herbal remedies specifically used for mental disorders such as *Acorus gramineus* Sol, ex Aiton (Tao *et al.*, 2005) *Rhazya stricta* Decne, (Al-Dabbagh *et al.*, 2018) *Uncaria rhynchophylla* (Miq.) Havil. (Lin *et al.*, 2003), *Gentiana lutea* L. (Haraguchi *et al.*, 2004), *Ruta graveolens* L, *Schotia brachypetala* Sond. and *Mentha aquatic* L., has shown that these remedies exhibit MAO inhibitory activity (Stafford *et al.*, 2007).

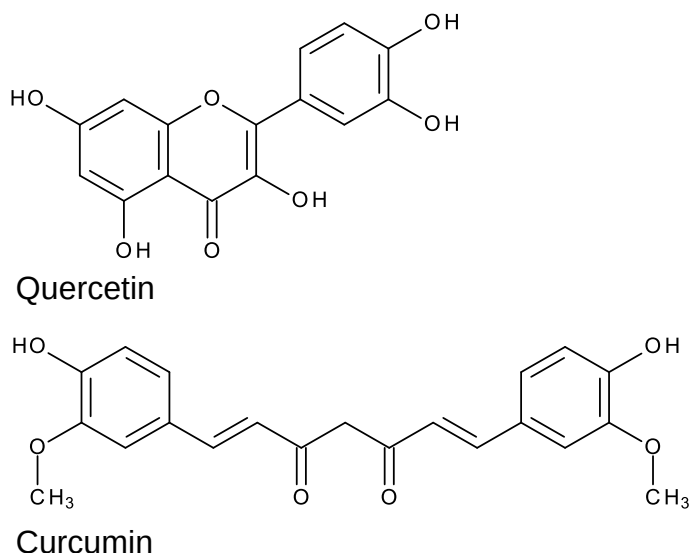


Figure 1.1 The structure of the flavonoid quercetin, and curcumin. (Engelbrecht *et al.*, 2018) Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

1.5. Molecular docking in drug discovery

In drug design it is vital to identify the molecular target, that is, the receptor site or enzyme that may be modified to manipulate the outcome of a certain disease. Molecular modelling is a computational method utilised in drug design to study the three-dimensional structure of molecules and their effect on the molecular target. Based on the importance of MAO inhibitors in disorders such as depression and PD, the present study aims to discover novel leads for the design and development of MAO inhibitors. The approach that will be followed is to select known compounds from natural origin and evaluate them as *in vitro* inhibitors of human MAO-A and MAO-B. The selection of the compounds will be based on (1) chemical similarity to known MAO inhibitors, (particularly with respect to planarity and the presence of hydrogen bond acceptors and donors) (2) novelty with respect to classes that have not yet been evaluated as MAO inhibitors, (3) commercial availability and (4) cost. The mentioned structural features are frequently found in the structures of MAO inhibitors. To aid in compound selection, similarity of the library of natural compounds to known MAO inhibitors may also be assessed by computer-assisted chemical “fingerprinting”, a subfunction of virtual screening. Ligands assessed in fingerprinting are presented in their most basic forms for comparison of active molecules against unknown or *de novo* molecules, which aims to identify the

molecular presence and absence of specific substructures in the two-dimensional and three-dimensional field planes.

1.6. Hypothesis of this study

MAO inhibitors increase the levels of monoamine neurotransmitters in the central nervous system by preventing their MAO-catalysed metabolism. In addition, MAO inhibitors may also reduce the formation of toxic by-products of the MAO catalytic cycle. MAO inhibitors, particularly inhibitors of the MAO-B isoform, are thus of value in neurodegenerative disorders, not only a symptomatic treatment but also as possible disease-modifying agents. The present study aims to discover novel leads for the design and development of MAO inhibitors. The approach that will be followed is to select known compounds from natural origin and evaluate them as *in vitro* inhibitors of human MAO-A and MAO-B. It is hypothesised that, among appropriately selected natural products, some novel compounds will exhibit MAO inhibition.

1.7. Aim and objectives

The objective of the study is to evaluate selected compounds derived from natural products as potential MAO inhibitors. The specific aims are as follows:

- The selection of compounds from natural products that will be evaluated as potential MAO inhibitors. A virtual library of commercially available natural products will be constructed and using computer-assisted “fingerprinting”, compounds with chemical similarity to known MAO inhibitors will be identified. Important considerations are planarity and the presence of hydrogen bond acceptors and donors.
- Biological evaluation of selected compounds will be carried out by measuring *in vitro* IC₅₀ values for the inhibition of the MAOs.
- The reversibility of inhibition by active compounds will be examined by dialysis and the construction of Lineweaver-Burk plots.
- Molecular modelling will be carried out to determine possible binding orientations of active inhibitors in the active sites of the MAOs.

CHAPTER 2

2.1 Parkinson's disease

2.1.1 General background

Parkinson's disease was first described as early as the 12th century B.C but the various symptoms were regarded as separate afflictions occurring at random to isolated individuals with no correlating environmental exposures. "Festinating gait" was described by Aelius Galen (a famous Roman physician in 130 - 210 A.D) and Leonardo da Vinci described involuntary unconscious movement in the late 1400s. It was only in the 19th century when a member of the Royal College of Surgeons, James Parkinson, studied six individuals who showed certain movement dysfunctionalities. His study formed part of the foundation for the discovery and classification of neurodegenerative diseases. In an extract from his famous assay on *paralysis agitans*, he coined the term "shaking palsy" by describing patients who had "involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace; the senses and intellects being uninjured." Jean Martin Charcot, in 1877, further made special reference that the characteristic symptoms occur in the elderly, mostly people above the age of 50. "Shaking palsy", which James described in 1817, was termed Parkinson's disease (PD). Charcot stated, contradictory to Parkinson's findings, that the intellect becomes foggy and the patient experiences severe memory loss in the last stages of the disease. Years later, , a sub-division of PD, namely early-onset Parkinsonism could be described as the same set of symptoms occurring before the age of 40 (Fahn, 2015; Sveinbjornsdottir, 2016).



Figure 2.1 Side view of the brain identifying key anatomical structures. Image obtained and modified from a public domain graphic site see link for further information. <https://publicdomainvectors.org/>

2.1.1.1 Neurochemical and neuropathological features:

Peptides and small molecules of organic origin are involved in neural activity and are called neurochemicals. Neurochemicals include neuromodulators and neurotransmitters. Serotonin, norepinephrine, dopamine and histamine are more specifically known as the monoamine neurotransmitters. Neurotransmitters convey a specific message in order for an action to be completed. Messages are received by the dendrites and conveyed to the axons via the cell body. Dendrites, cell bodies and axons make up the three-part construction of nerve cells. Once there, neurons transport information through chemical and electrical signals by exchanging neurotransmitters across the synapse where it binds to receptors to eventually have their intended action (Poewe *et al.*, 2017).

The brain is divided into three main parts: the brainstem, the cerebellum and the cerebrum (Fig 2.1). Ten of the twelve cranial nerves originate from the brainstem and most autonomic functions are performed here. The brainstem connects the cerebellum and the cerebrum to the spinal cord and is divided into the midbrain, medulla and pons. The subcortical section of the midbrain contains the basal ganglia. The neostriatum, subthalamic nucleus, globus pallidum segments and the substantia nigra are found in the basal ganglia (Tian *et al.*, 2017).

The primary input structure, the striatum, in the basal ganglia mainly expresses acetylcholine receptors and dopaminergic (D1 and D2) receptors. The internal globus pallidus (GPi) and the substantia nigra pars reticulata (SNpr) serve as output nuclei in the basal ganglia in the midbrain. They receive information from the subthalamic nucleus via the thalamus which is interconnected with afferent neurons. Glutaminergic stimulation from various areas of the cerebral cortex activate the projection neurons that contain the dopamine receptors. Two pathways represent communication in the basal ganglia, the direct pathway and the indirect pathway. Dopamine D1 receptors inhibit GPi/SNpr neurons directly from the putamen whereas dopamine D2 receptors exert inhibitory activity by connecting the putamen to the GPi/SNpr neurons indirectly via synaptic transmissions of the external globus pallidus (GPe) and the subthalamic nucleus. Stimulating the neurons in the indirect pathway results in activation of the GPi/SNpr by first inhibiting GPe and activating

(disinhibiting) the subthalamic nucleus (Fig 2.2). The brainstem along with the thalamo-cortical neurons exert their desired action on motoric activity by the opposing mechanisms of the different pathways. The net effect of a healthy brain is to obtain homeostasis by stimulating the indirect pathway to reduce the excitatory outflow of glutamate from the thalamus to the cerebral cortex. When dopaminergic neurons are depleted, as in PD, the direct pathway is less active and activity in the indirect pathway is increased causing the SNpc/GPi neurons to activate, release GABA and inhibit the excitatory activity of the thalamus to the cerebral cortex (Goodman et al., 2018; Obeso et al., 2008; Poewe et al., 2017; Tian et al., 2017).

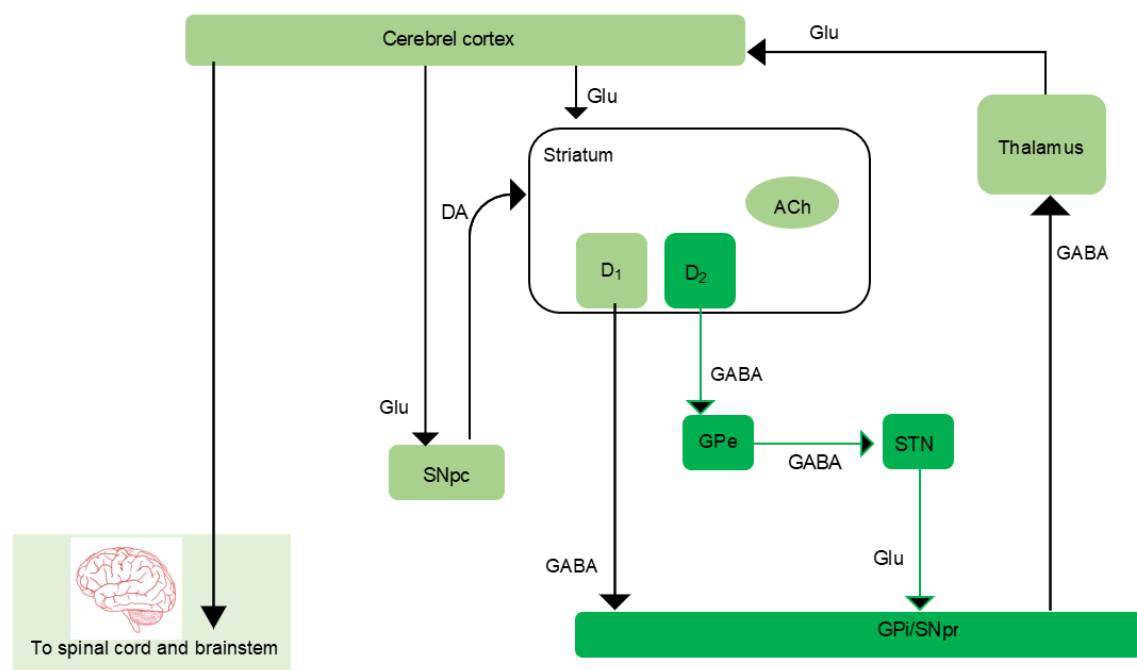
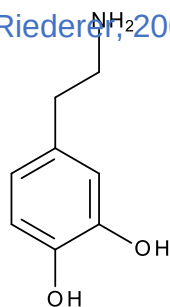


Figure 2.2 Direct and indirect pathway of dopamine transmission in the brain (Katzung, 2012). <https://publicdomainvectors.org/>

Neurotransmitters exercise their influence on cognitive processes, movement, executive function, body temperature regulation, mood, sleep, appetite, pain and arousal. The functions the neurotransmitters have in the body are directly correlated to certain diseases when the neurotransmitters are not functioning optimally. The neuropathological interest in monoamine neurotransmitters is their involvement in diseases such as Alzheimer's disease, PD and depression. In the deficit state, neurotransmitters may cause drastic alterations of the brain's topology as seen on computed tomography CT scans. Researchers are investigating innovative ways to manipulate processes in the brain using these transmitters and their receptors either

by administering more neurotransmitters or interfering in their degradation (Dickson *et al.*, 2009; Goodman *et al.*, 2018; Katzung, 2012; Poewe *et al.*, 2017).

Dopamine (Fig. 2.3) is mainly found in three functional areas: the substantia nigra, arcuate nuclei and the ventral tegmental area (VTA). In PD, dopamine is produced in inadequate quantities in the substantia nigra pars compacta (SNpc). Dopaminergic pathways are responsible for locomotor movement, pituitary gland hormone secretion and reward and motivation. The three pathways are the tubero-infundibular, mesocorticol-mesolimbic and nigro-striatal pathways. The nigrostriatal pathway connects the SNpc with the dorsal striatum. The low concentration of dopamine in the striatum of PD patients is due to loss of dopaminergic neurons. Dopamine in the dorsal striatum is found to be practically non-existing in PD (Gille & Riederer, 2005).



Dopamine

Figure 2.3 The chemical structure of the monoamine neurotransmitter, dopamine (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

In PD, neurodegeneration also occurs within other regions affecting not only the nigrostriatal pathway or the striatal terminals. The locus coeruleus, raphe, Meynert's basal nucleus, cortices of the cingulate, olfactory bulbs and even the autonomic nervous system are some of the other systems that may also be affected in PD. Inevitably these regions will also undergo degeneration in the late stages of PD (Dauer & Przedborski, 2003; Katzung, 2012; Poewe *et al.*, 2017).

2.1.1.2 Etiology:

Two main hypotheses regarding the causative nature of PD has been developed:

- Environmental toxin hypothesis:
 - Chronic toxin exposure
 - Once-off toxin exposure
- Genetic susceptibility hypothesis
 - Endogenous toxin exposure

The environmental toxin hypothesis suggests the exposure to dopaminergic neurotoxins causes PD. Whether neurotoxins must be chronically present for a set period or if exposure is a one-time occurrence remains unknown. The latter exposure option supposes that only initiation is needed for PD to develop and little can be done to prevent the deleterious cascade from happening ([Betarbet et al., 2000](#); [Poewe et al., 2017](#)).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxic compound which ultimately causes dopaminergic neurons in the SNpc to die ([Obeso et al., 2008](#)). MPTP serves as a good example of a compound inducing neurodegenerative disease. Monkeys are treated with MPTP in toxin-based models prior to being treated with possible clinically efficacious drugs. 6-Hydroxydopamine, paraquate, cyanide, 3-propionic acid (3-NP), malonic acid and rotenone are additional examples of compounds used in toxin based-models to induce dopaminergic neurodegeneration by generating reactive oxygen species (ROS). Most toxins identified to be responsible for PD are found to be present in herbicides or insecticides. ([Dauer & Przedborski, 2003](#); [Katzung, 2012](#))

Endogenous toxins on the other hand, are usually compounds converted to toxic metabolites within the body. These toxins accumulate over time and negative effects are only observed long after the damage has already been done. For example, dopamine metabolism causes the formation of ROS. If the reactive intermediates cannot be eliminated from the body, oxidative stress occurs. Tetrahydroisoquinoline

(TIQ) is a classic example of endogenous toxin. TIQ yields oxygen radicals by inhibiting complex 1¹ of the electron transport system, which leads to lowered ATP production. Less ATP production indirectly leads to more oxygen radical production. Environmental factors can also influence PD prevalence inversely. There are various plant-based diets that are known to have neuroprotective effects. Foods known as superfoods are popular in this category and chia seeds and blueberries are antioxidants used daily for this specific purpose. Neuroprotection in PD has also been associated with coffee usage and the smoking cigarettes (Blesa *et al.*, 2015; Hernán *et al.*, 2002; Katzung, 2012; Poewe *et al.*, 2017; Van Eeden *et al.*, 2003).

2.1.1.3 The pathogenesis of PD:

James Parkinson suggested the possible origin for PD in the part of the *medulla spinalis* where a canal is formed by the superior cervical vertebrae. He further explained with disease progression the affected regions will extend all the way to the *medulla oblongata*. The nigrostriatal pathway and the substantia nigra (SN) play contributory parts in the progression of PD (Fahn, 2015).

Increased age is only a contributing factor in the development of neurodegenerative diseases such as PD. Cell loss in the substantia nigra is seen in both healthy individuals as well as in individuals with neurodegenerative diseases. “Healthy” cell death is seen to originate in the dorsomedial part of the SNpc, and in PD it occurs in the ventrolateral and caudal regions. SNpc depigmentation indicates the extent of dopaminergic neuron depletion or rather, it shows the degree of decreased dopamine and homovanillic acid (HVA) concentrations. Neuromelanin is a by-product released during neuron degeneration and catecholamine catabolism and it accumulates through the auto-oxidation of catecholamines. Neuromelanin is responsible for the dark colouring seen in the nerve cells of normal and healthy SN and other catecholaminergic cell structures. Depigmentation is due to the low quantities of neuromelanin and is thus indicative of SNpc-dopaminergic neuron cell

¹Complex 1, also known as nicotinamide adenine dinucleotide (NADH) dehydrogenase, of the electron transport system is a flavoprotein containing flavin mononucleotide (FMN). When mitochondrial NADH cannot be oxidised, electrons can't be transferred through FMN to eventually synthesise ATP (energy).

death. During oxidative stress (both intracellular and extracellular), neuromelanin protects the nerve cells by binding to redox-active metal ions. Neuromelanin, however, may also cause further cellular death when it releases bound redox-active metals which produces hydrogen peroxide (H_2O_2). Neuromelanin can be utilised as a guide toward determining cellular health since PD symptoms only become evident after 60-80% of dopaminergic neurons have died and the available dopamine has been depleted. By determining neuromelanin concentrations an estimation can be made of disease progression or the phase of the disease the individual currently experience, which in turn could optimise treatment regimens ([Dauer & Przedborski, 2003](#); [Fahn, 2015](#); [Goswami et al., 2017](#); [Poewe et al., 2017](#))

Mechanisms of neurodegeneration are only theoretically understood and no foundation towards the direct molecular cause of PD can be given. Varying cellular dysfunctionalities and diverse mechanism of actions all play contributory roles in explaining the pathogenesis in PD. Some researchers explain neurodegeneration in PD by two mechanisms:

- misfolding with protein aggregation and
- mitochondrial dysfunction during oxidative stress.

Other researchers differentiate three mechanisms:

- metabolic compromise (which entails mitochondrial dysfunction)
- excitotoxicity and
- oxidative stress ([Blesa et al., 2015](#)).

These differing hypotheses regarding the mechanisms of neurodegeneration all indefinitely lead to PD. Below is a brief explanation of these mechanisms.

2.1.1.3.1 Misfolded proteins and protein aggregation:

The striatal dopaminergic nerve terminal is the actual target in the PD degenerative proses and when protected, cell death and associated symptoms could be ceased if not completely avoided altogether. The whole protein manufacturing system which begins at protein synthesis and ends with protein degradation, lose their optimal functioning when we age. This leads to the formation of misfolded proteins that

eventually accumulates and produces aggregates. Normally, misfolded proteins are eliminated by nucleoplasmin and proteasomes. In advanced age the function of these two mechanisms drastically decline. Nucleoplasmin is known as a “molecular chaperone”. This protein can be found at the cell nucleus where it binds to histones to prevent unsuitable interactions from taking place during nucleosome formation at the DNA binding site. It escorts other proteins to their designated places and ensures that designated actions are executed properly. In a nutshell, chaperones prevent aggregate formation and assist in the correct folding of newly created proteins in order to obtain and maintain protein complexes. If protein misfolding occurs, poly-ubiquitin chains are automatically attached to the misfolded protein which marks it for degradation so that it can be naturally eliminated from the body (Dauer & Przedborski, 2003; Gauss *et al.*, 2006; Reynaud, 2010; Sherman & Goldberg, 2001; Poewe *et al* 2017)

In inherited PD, misfolded proteins are caused by defective conformation commands embedded in the DNA. Selected proteins are thus already corrupted within the nucleus during protein synthesis and negatively influence other correctly folded proteins once they leave the cell nucleus towards their place of functionality. By interfering in the degradation of misfolded proteins, the content of misfolded proteins is indirectly increased in PD. By ignoring or accepting their existence, the proteins are not marked for degradation and are thus not eliminated from the body (Poewe *et al.*,2017).

Lewy bodies are protein aggregates consisting of numerous proteins and are found in patients that suffer from PD. Synucleinopathy is the term used to explain the excessive accumulation of the α -synuclein protein in neurons during neurodegenerative diseases. α -Synuclein is a major component of Lewy bodies. The typical Lewy Body can be found in specific regions of the brain, namely the autonomic ganglia, basal forebrain, brainstem, cerebral cortex and the diencephalon regions in monoaminergic and cholinergic neurons. The Lewy bodies are responsible for neurotoxicity on a microcellular level. Lewy bodies damage the healthy proteins needed in specific cells and expand in their aggregated mass via protein inclusions. This directly and indirectly causes cell death. They also prevent transport in and between cells (Dauer & Przedborski, 2003; Kalia, 2015).

2.1.1.3.2 Dysfunctional mitochondrial respiration and oxidative stress:

Mitochondria convert energy obtained from macronutrients to adenosine triphosphate (ATP). Aerobic respiration is more efficient than anaerobic metabolic processes, yielding 15-times more ATP molecules. For this metabolic reaction to occur via oxidative phosphorylation, oxygen (O_2) is required. In the final stage of cellular respiration oxidative phosphorylation chemiosmotically combines ATP synthesis and electron transport via the electron transport chain (ETC). The ETC consists of protein complexes embedded in the inner membrane of mitochondrial organelles that ensures that an electrochemical protein gradient is established by allowing electrons (donated from the reduced electron carriers NADH and $FADH_2$) to move from one protein to another whilst protons are pumped across the membrane into the intermembrane space. Free energy is released and captured by the resulting proton gradient. The protons flow back across the membrane via ATP synthase, which uses the free energy and “powers” ATP formation from ADP and inorganic phosphate (Burke *et al.*, 2007; Poewe *et al.*, 2017; Schulz *et al.*, 2016)

During the mitochondrial respiration of ATP formation, by-products such as superoxide radicals and peroxide are also produced. The radicals either attack lipids, nucleic acids and proteins, causing severe cellular damage or increase reactive oxygen species (ROS) production by targeting the electron transport chain, and damaging mitochondria beyond repair. Post-mortem SNpc dissections of PD patients show elevated ROS and substantially decreased glutathione (an anti-oxidant) when compared to a non-PD brains. Glutathione inactivates H_2O_2 via glutathione peroxidase. The direct cause of sporadic PD is uncertain but there is a definitive correlation between oxidative stress and the misfolding of abnormally oxidised proteins. Oxidative stress could be caused by a toxin that is able to inhibit the electron transport chain. MPTP has been shown to be such a toxin. This not only leads to energy failure but also to increased amounts of ROS production. Energy failure causes cytosolic dopamine concentrations to rise by disrupting their storage vesicles. The resulting increase in dopamine metabolism produces H_2O_2 and

superoxide radicals making dopaminergic neurons susceptible to cellular damage. The increase in ROS leads to more misfolded proteins. As mentioned, the loss of dopaminergic neurons in the SNpc could be caused by the aggregates produced from misfolded proteins or the toxins released from dopamine itself via oxidative stress. The current belief is that both mechanisms of neuronal cell death play contributory roles in the pathogenesis of PD. One mechanism causes the other to originate and from there have deleteriously cascading effects. Protein aggregates are found in multiple diseases and cannot be isolated to PD alone. Toxicity occurs either through cell deformation (direct cause) or through obstructed intercellular mechanisms (indirect cause), such as proteasome dysfunction ([Burke et al., 2007](#); [Poewe et al., 2017](#); [Schulz et al., 2016](#))

2.1.1.3.3 Excitotoxicity:

Excitotoxicity has been implicated in diseases such as stroke and neurodegenerative diseases. Excitotoxicity is explicitly relevant to the excitatory glutamate neurotransmitter. Overactivation of N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors damages and kills neurons by permitting a high influx of calcium ions into the cell. Damage is caused by the Ca^{2+} influx which stimulates enzymes such as endonucleases, calpain (proteases) and phospholipases. These enzymes damage crucial cell structures including DNA and the cytoskeleton via lipid peroxidation, free radicals and iron released from ferritin. Calbindin is a series of calcium-binding proteins and is seen in increased levels in dopaminergic neurons in autopsied PD brains. This provides evidence of the role of excitotoxicity in PD. Calcium channel blockers and glutamate receptor antagonists may become future treatment options ([Burke et al., 2007](#); [Poewe et al., 2017](#); [Schulz et al., 2016](#))

2.1.1.3.4 Apoptosis:

Apoptosis is programmed cell death. In short, apoptotic factors are contributory to the rapid worsening of PD in the late stages. In the late stages of PD, cellular communication becomes dysfunctional and the remaining cells are targeted for apoptosis. Apoptotic and autophagic cell death pathways are thought to be the end

result of the PD pathogenesis mechanisms and could be seen in end-stage PD. Neuroprotection via this pathway with anti-apoptotic drugs such as minocycline and TCH346 could result in neuroprotection in PD. Minocycline is a second-generation semisynthetic tetracycline that shows protective effects towards the destruction of nigrostriatal dopaminergic pathways in MPTP treated mice. TCH346 is a novel drug that resembles selegiline in that it also contains the propargylamine functional group. In animal models, TCH346 protects against dopamine neuron degeneration by preventing apoptosis. ([Burke et al., 2007](#); [Poewe et al., 2017](#); [Schulz et al., 2016](#); [Yacoubian & Standaert, 2009](#))

2.1.1.3.5 Trophic factors:

Brain-derived neurotrophic factor, glial-derived neurotrophic factor (GDNF) and nerve growth factor² are the main trophic factors found to be diminished within nigral areas of PD brains. The discovery of trophic factor involvement in PD led to growth factors being investigated as possible treatment to reverse damage to dopaminergic neurons regardless of the cell death mechanism. Neurturin and GDNF have entered clinical trial phases as potential neuroprotectants in PD ([Dauer & Przedborski, 2003](#); [Liu, 2018](#)).

2.1.1.3.6 Neuroinflammation:

T-Lymphocytes, pro-inflammatory mediators and activated microglial cells are found in the post-mortem brain in multiple neurodegenerative diseases such as PD, Huntington's disease and progressive supranuclear palsy. Neuroinflammation can thus not be the principal cause of PD development but is recognised as a contributory factor since innate and adaptive immune responses are believed to be triggered by oxidative stress, cellular death and neuronal loss. Neuromelanin, a by-product released during neuron degeneration and catecholamine catabolism, is

² Neurotrophic factors are peptides and proteins that are responsible for supporting the growth, differentiation and survival of neurons. GDNF and Neurturin are proteins of the neurotrophic factors whereas nerve growth factor is a neuropeptide of the neurotrophic factors ([Liu, 2018](#)).

engulfed by macrophages (also known as microglial cells) which stimulate the up-regulation of p38 mitogen activated protein kinase (MAPK), NF- κ B signalling pathways³ and pro-inflammatory mediators. Neuroinflammation in PD might be reduced by nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, aspirin and statins (such as simvastatin). (Hirsch *et al.*, 2012; Taniguchi & Karin, 2018; Yacoubian & Standaert, 2009)

2.1.1.4 Genetics and epidemiology:

Only 4% of PD cases occur before the age of 50 (early-onset PD). The mean onset age is between 55 and 60. Women are one and a half times less likely to develop PD. PD is the second most prevalent neurodegenerative disease affecting all ethnic groups with up to 10 million people worldwide presenting with PD. There are 50 000 to 60 000 new cases diagnosed yearly. More than 25 people are diagnosed daily. Statistical analysis done in Canada revealed that by the year 2031 the number of Canadians diagnosed with PD will be double compared to 2011. A comprehensive study done in 2003 showed that PD is most prevalent in caucasian male individuals and least prevalent in female Asians. Native Americans was one of the populations that showed the least tendency to be diagnosed with PD. The over-all annual incidence of PD development in the differing age groups has shown a steady increase (Michel *et al.*, 2016; Poewe *et al.*, 2017; Schulz *et al.*, 2016)

A fifth of PD cases are related to genetics. The main genes responsible for susceptibility to develop PD are α -synuclein, leucine rich repeat kinase 2 (LRRK-2) and glucocerebrosidase (GBA)⁴. Genes that are frequently present in parkinsonian-like diseases are parkin, PTEN-induced putative kinase 1 (PINK1), DJ-1 and ATP13A2⁵. Certain mutations found in these genes are responsible for the increased

³ MAPK is communicating proteins that sends signals from the receptor surface to the DNA in a nucleus. NF- κ B is also a protein complex but is mostly responsible for cell survival through transcription of DNA and is important for regulating the immune response to infection (Taniguchi & Karin, 2018).

⁴ α -Synuclein is a neuronal protein found abundantly in the brain. LRRK-2 is an enzyme that encodes several proteins and is involved macroautophagy. GBA is an enzyme involved in glycolipid metabolism(via hydrolyses of glucocerebroside) in cell membranes.

⁵ PINK-1 is a mitochondrial enzyme that protects cells from mitochondrial dysfunction. DJ-1 is an enzyme designed to protect cells from α -synuclein aggregation.ATP13A2 is an enzyme involved in the transportation of metal cations.

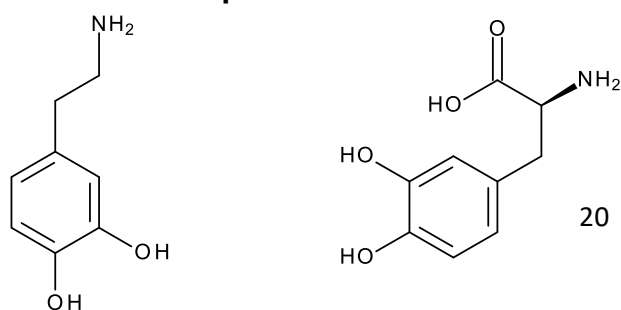
susceptibility of developing PD. The role genetics plays sheds light on inherited PD aetiology. Different types of PD occur with each mutation. In the LRRK-2 gene it is the Gly2019Ser mutation that researchers most commonly encounter in inherited PD. There are five other mutations of the LRRK-2 gene that have been documented but these do not lead to the development of PD ([Burke et al., 2007](#); [Zimprich et al., 2004](#))

The α -synuclein gene shows dominant point mutations and gene multiplications which cause the α -synuclein protein to aggregate. The resulting pathology closely resembles sporadic PD. Individuals that have a GBA loss-of-function mutation is at greater risk of developing PD. Loss-of-function mutations in parkin, PINK1, DJ-1 and ATP13A2 are responsible for recessive forms of PD. The course of the disease is less aggressive, and individuals tend to not exhibit any speech slurring, chewing difficulties or severe forms of dementia. Among these genes, parkin tends to be the most prominent gene involved in inherited PD, and its mutations show less to no brain stem neuronal loss, neurofibrillary degradation or Lewy bodies ([Burke et al., 2007](#); [Dauer & Przedborski, 2003](#); [Poewe et al., 2017](#))

2.1.2. Symptomatic treatment of PD

The symptomatic treatment of PD includes agents such as levodopa, dopamine agonists, carbidopa and benserazide, catechol-O-methyltransferase (COMT) inhibitors, MAO-B inhibitors, anticholinergic drugs, adenosine A_{2A} receptor antagonists and amantadine. Treatment regimens are focused on symptomatic treatment only since there is no cure for PD. The current hypothesis states that dopamine receptor stimulation will lessen motor complications. Drugs are broadly classified as drugs that enhances dopamine levels in the brain or drugs that mimic dopaminergic effects. Dopamine itself is not recognised as a drug-treatment option since it cannot cross the blood-brain barrier to exert an effect on dopaminergic neurons in the basal ganglia ([Fox et al., 2018](#); [Manning, 2017](#))

2.1.2.1. Levodopa



Dopamine

Levodopa

Figure 2.4 The chemical structures of dopamine and levodopa ([Engelbrecht et al, 2018](#)). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Levodopa (L-dopa) is a prodrug that is the most effective drug for the symptomatic treatment of PD, particularly for the bradykinesia and rigidity symptoms. It is metabolised to dopamine or 3-O-methyldopa via aromatic-L-amino acid decarboxylase (AADC) and COMT, respectively. To increase the amount of striatal dopamine, levodopa needs to cross the blood-brain barrier before conversion to dopamine must occur ([Fox et al., 2018](#); [Manning, 2017](#); [Poewe et al., 2017](#)).

Levodopa can, via autoxidation or oxidative metabolism after conversion to dopamine, produce cytotoxic ROS that can damage or kill dopaminergic neurons. This will worsen the degeneration of the basal ganglia in PD. Levodopa also worsens motor fluctuations and dyskinesia, which seem to occur erratically at different levodopa plasma concentrations. Inconsistent stimulation of dopamine receptors in the basal ganglia may occur for three reasons: dopamine denervation, poor levodopa absorption and distribution to the brain and inconsistent receptor stimulation because of irregular distribution of levodopa ([Fox et al., 2018](#); [Katzung, 2012](#); [Manning, 2017](#); [Poewe et al., 2017](#)).

Even with its limitations, levodopa was the first drug used to treat PD and has been used since the 1970s. Since 1980 focus shifted to continuous dopamine stimulation (CDS) and continuous dopamine delivery (CDD) to reduce response fluctuations and “on” and “off” periods. No evidence suggests an advantage towards differing dosage forms of levodopa (e.g. slow release of levodopa versus immediate release of levodopa) ([Dauer & Przedborski, 2003](#); [Katzung, 2012](#))

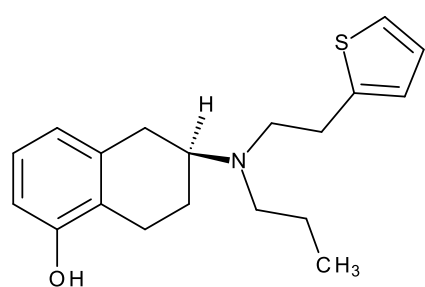
AADC inhibitors are often used in combination with levodopa and reduces the peripheral metabolism of levodopa to dopamine. In this instance 3-O-methylation by COMT becomes the dominant metabolic route of levodopa. COMT inhibitors are thus also used in combination with levodopa. These two strategies ensure that higher

concentrations of levodopa will be able to cross the blood-brain barrier ([Fabbri et al., 2018](#))

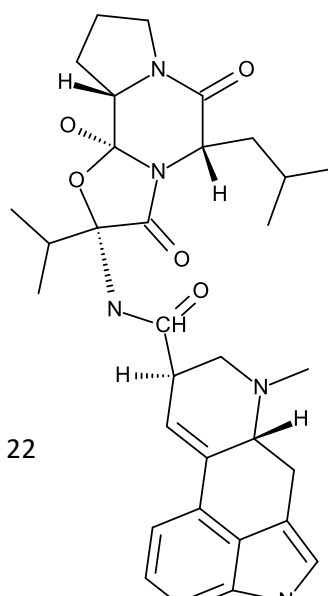
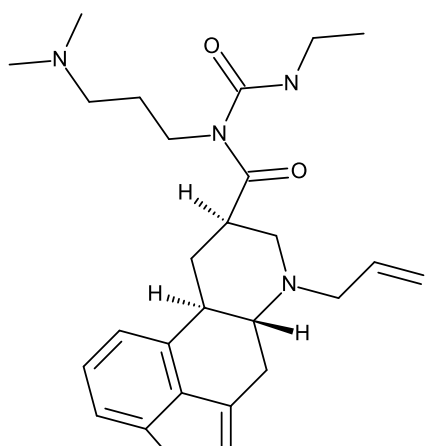
Amino acids interfere with the absorption of levodopa by interacting with the amino acid transporters at both the gastrointestinal tract and the blood-brain barrier. High protein diets should be avoided, and normal protein consumption needs to be spaced throughout the day in equal amounts. Other products to avoid while on levodopa treatment are foods and supplements containing vitamin B6 as pyridoxine reduces the effectiveness of levodopa ([Dauer & Przedborski, 2003](#); [Katzung, 2012](#))

The dextrorotatory isomer of levodopa causes haematological changes not seen in the levo-conformation. Reports on both peripheral as well as central adverse effects included gastrointestinal problems, mental effects, involuntary movements, nausea, vomiting and anorexia. The well-known mental effects are paranoia, insomnia, irrational hostility and anger, characterised by underlying irritability. Chorea, dystonia, myoclonus and hemiballism are grouped together under the term dyskinesia. These adverse effects recede once the dosage is lowered, and some completely disappear when the dose is increased at a slower rate ([Dauer & Przedborski, 2003](#); [Fox et al., 2018](#); [Katzung, 2012](#); [Manning, 2017](#); [Poewe et al., 2017](#)).

2.1.2.2. Dopamine agonists



Rotigotine



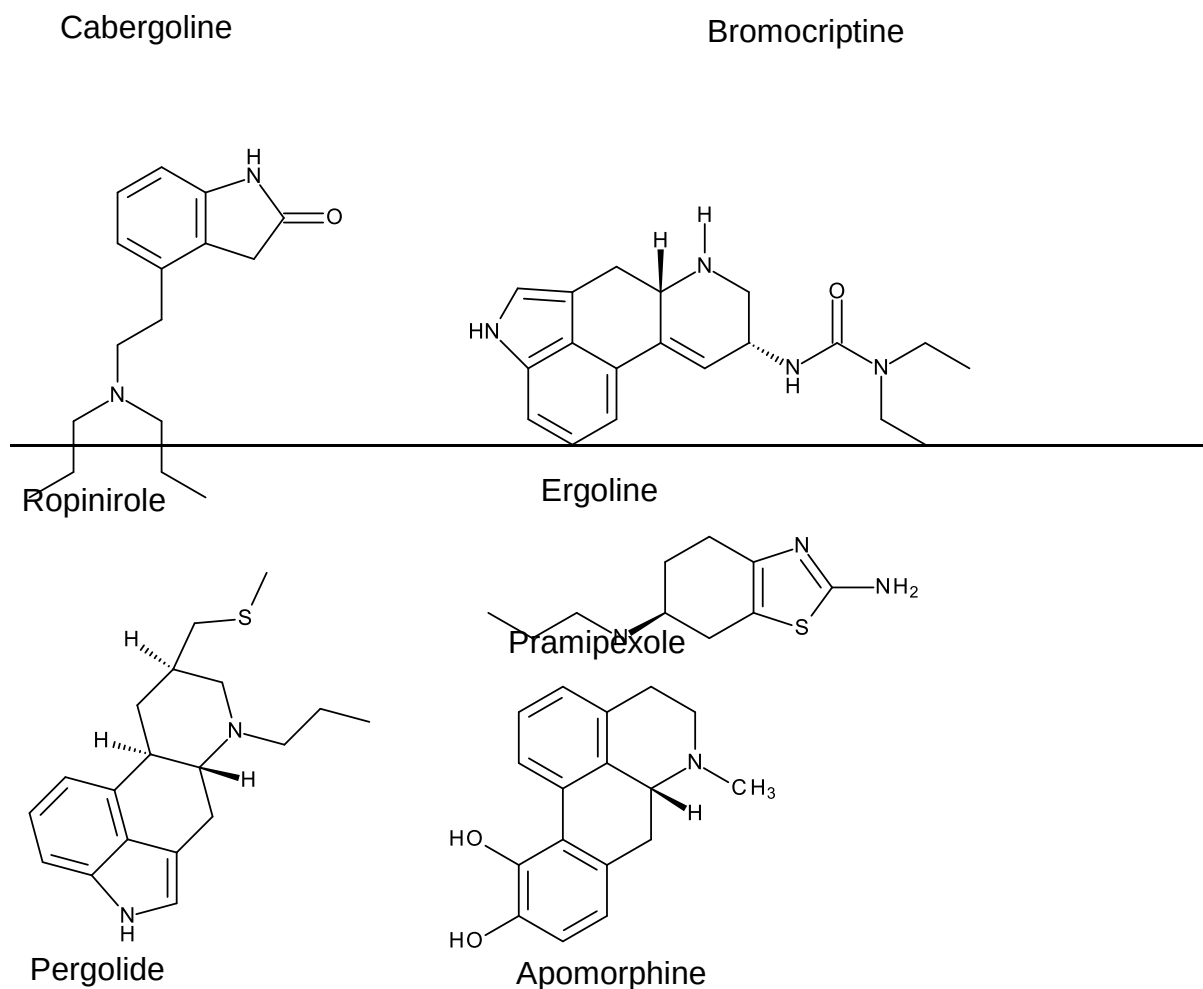


Figure 2.5 The chemical structures of dopamine agonists ([Engelbrecht et al, 2018](#)). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Dopamine agonists may replace levodopa since they are effective against all symptomatic features of PD. Dopamine agonists can also be used in combination with levodopa for a better clinical effect. Dopamine agonists used in PD include pramipexole and ropinirole (oral formulation), apomorphine (an injection), bromocriptine and pergolide (now withdrawn from the market). Pramipexole, ropinirole and bromocriptine needs to be taken 2-4 times a day. These drugs reduce symptom severity and is commonly used in early-onset PD and advanced PD.

Daytime sleepiness is a major negative effect which may cause accidents during monotonous activities. Hallucinations are commonly observed as the mental disturbance most likely to precipitate in dopaminergic treatment strategies. Patients have also complained of excessive leg swelling that increases their discomfort. The increased tendency towards compulsiveness (extreme shopping-sprees, gluttony and irresponsible gambling) are behavioural side effects that drastically influence their occupation and personal life. Apomorphine has rapid effects, but only lasts for up to an hour. This is a useful drug in emergency treatments when quick results are needed. Patients experiencing extreme “off-periods”⁶ also use this drug when other drugs are ineffective. Anti-nausea medication needs to be used before apomorphine injection as most patients experience nausea. Side effects are even more pronounced with apomorphine compared to other dopamine agonists. Beginning at the lowest dose and up-regulating every 5-7 days with all dopamine agonist drugs will eliminate most negative effects, and maximum benefit at the lowest possible dose will be achieved. Stimulation of D₂-autoreceptors in the substantia nigra by dopamine agonists can reduce oxidative stress by limiting dopamine release, which indicates a possible neuroprotective effect ([Katzung, 2012](#); [Manning, 2017](#); [Yacoubian & Standaert, 2009](#))

2.1.2.3. Carbidopa and benserazide

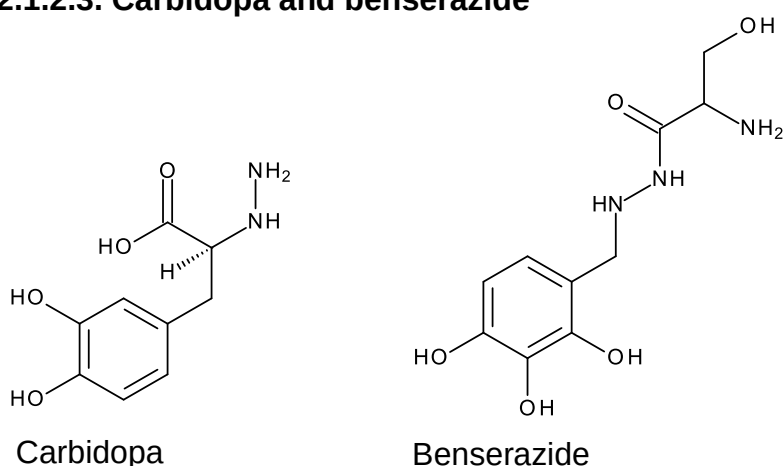


Figure 2.6 The chemical structures of carbidopa and benserazide ([Engelbrecht et al, 2018](#)). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

⁶ Periods when PD patients experience motor function fluctuations. PD symptoms seem to return even when on medication.

Carbidopa is an AADC inhibitor and prevents the metabolic breakdown of levodopa to dopamine in the peripheral tissues. Levodopa is normally absorbed in the small intestine and is quickly metabolised to dopamine and 3-O-methyldopa via AADC and COMT. By inhibiting AADC, the levels of levodopa that reaches the blood plasma increases which makes it possible that enough levodopa is constantly available to cross the blood-brain barrier. This results in regular and sufficient dopaminergic stimulation. Benserazide is another AADC inhibitor that is used in combination with levodopa to increase the amount of levodopa available for crossing the blood-brain barrier. Both these drugs are unable to cross the blood-brain barrier and are thus target specific the peripheral tissues. Benserazide and carbidopa are not used as monotherapy and must be used in combination with levodopa (Brooks *et al.*, 2005; Katzung, 2012; Rasameesoraj *et al.*, 2018).

2.1.2.4. COMT inhibitors

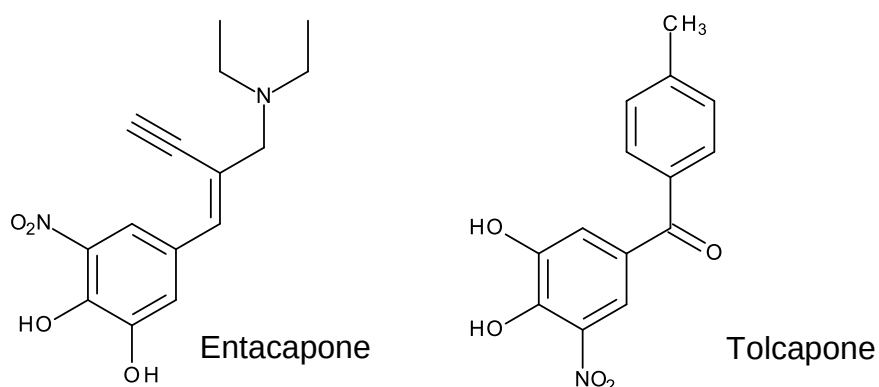


Figure 2.7 The chemical structures of entacapone and tolcapone (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

COMT is a monomeric enzyme present in most mammalian tissues and has two isoforms, membrane bound-COMT (mostly present in the brain) and soluble-COMT (mostly present in the peripheral areas such as the liver, kidneys and the gastrointestinal tract). COMT, encoded by the COMT gene, is involved in the metabolism of catecholamines, catechol estrogens and hydroxylated metabolites. A methyl group from S-adenosyl-methionine (SAM), in the presence of magnesium, is added to the phenolic group of neurotransmitters and metabolites, and this addition

renders them inactive. COMT inhibitors mostly target the soluble-enzyme isomer, thus increasing levodopa's concentration in the periphery. This makes more levodopa available to cross the blood-brain barrier and reduces the amount of levodopa that is converted to the 3-O-methylated metabolite. The action of these drugs is limited to the periphery because of the polarity they exhibit. First-generation COMT inhibitor drugs have a short half-life and lack selectivity. Nitrocatechol type inhibitors are more commonly known as the second generation COMT inhibitors which features a nitro group on the benzene ring (Fig 2.7). Of the four inhibitors that were developed in 1989, only two are commercially available, tolcapone and entacapone. COMT inhibitors do not have any effect on the symptoms of PD when used alone. Dyskinesia may develop upon initiating treatment with COMT inhibitors. COMT inhibitors are, however, better known to worsen the side effects of levodopa rather than have their own side effect profile. Orange discolouration of urine is the only characteristic adverse effect of COMT inhibitors. Tolcapone is strongly associated with hepatotoxicity. Nebicapone is another second-generation COMT inhibitor with low activity that makes it ineffective in clinical practice. Opicapone is a third-generation drug with a long half-life and potentially better clinical effect than the second generation COMT inhibitors (Dauvilliers *et al.*, 2015; Fabbri *et al.*, 2018; Katzung, 2012).

2.1.2.5. MAO-B inhibitors

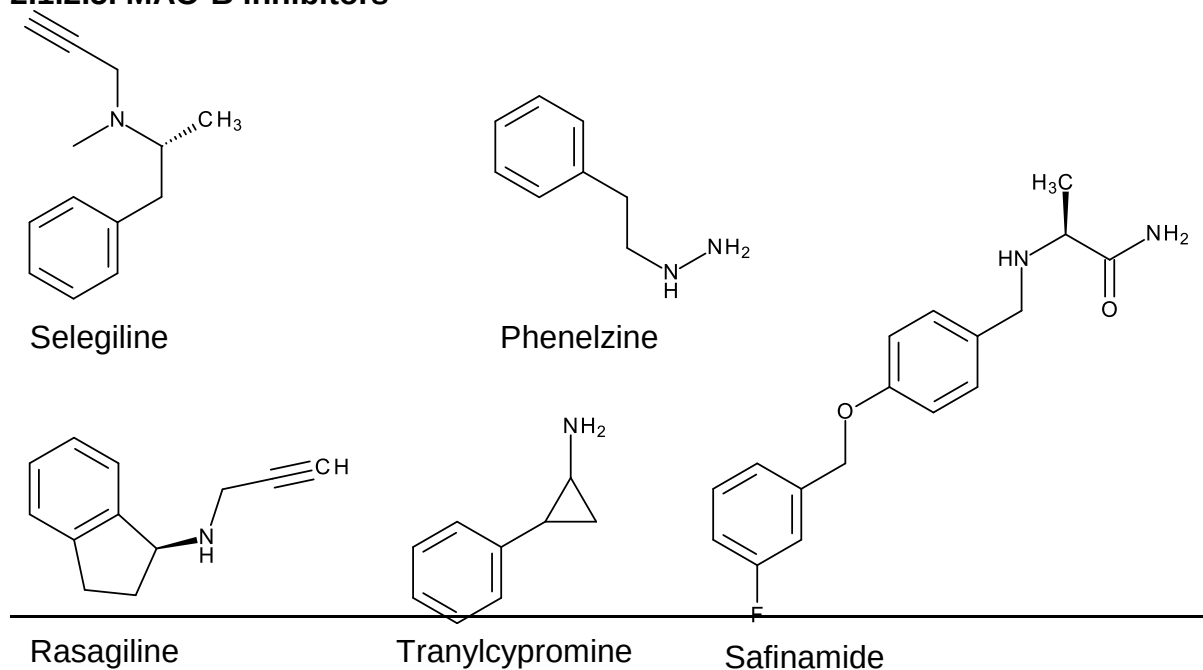


Figure 2.8 The chemical structures of MAO-B inhibitors, selegiline, rasagiline,

phenelzine, safinamide and tranylcypromine ([Engelbrecht et al, 2018](#)). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Selegiline, rasagiline and safinamide are MAO-B inhibitors used in the treatment of PD. Safinamide has a dual action in PD and affects the glutamate pathway in addition to blocking dopamine re-uptake and inhibiting MAO-B activity. The mechanism of action of selegiline and rasagiline involves the selective and irreversible inhibition of the MAO enzymes, especially the MAO-B isoform. Dopamine is normally degraded in the brain by MAO and by inhibiting their action, dopamine has a longer time-frame in which to reduce parkinsonian symptoms. Selegiline and rasagiline are both used as monotherapy but also in combination with levodopa in severe PD. ([Youdim & Weinstock, 2004](#))

Selegiline, also known as L-deprenyl, is an effective inhibitor of MAO-B in the brain. Dextro-selegiline is less active than levo-selegiline and the three main metabolites of selegiline are desmethylselegiline, levoamphetamine and levomethamphetamine. The elimination half-time of selegiline is 1.5 hours and MAO-B platelet activity is completely inhibited after four hours of initial administration. After a single dose, inhibition of MAO-B continues for at least 14 days. Selegiline compared to rasagiline is less bioavailable and therefore less potent. Desmethylselegiline supposedly also inhibits MAO-B, but to a lesser extent than selegiline. Transdermal delivery of selegiline avoids “first-pass metabolism” and reduces metabolite formation. Amphetamine metabolites derived from selegiline do not cause neurotoxicity, but caution should still be exercised since amphetamine metabolites may affect dopamine release. Selegiline is also thought to possess neuroprotective properties and thus may delay the initiation of levodopa therapy ([Katzung, 2012](#); [Manning, 2017](#); [Riederer & Müller, 2017](#); [Youdim & Bakhle, 2006](#)).

Rasagiline is a newer MAO-B inhibitor and is not metabolised to yield amphetamine metabolites. Rasagiline metabolites may possess antioxidant properties, making rasagiline a better treatment option and neuroprotectant compared to selegiline. The reversible MAO-B inhibitor, lazabemide, is a short-acting MAO-B inhibitor and, just like rasagiline, does not produce amphetamine metabolites. Lazabemide also delays

the initiation of levodopa treatment, but whether this property is dependent on symptomatic remediation or true neuroprotection, is unclear (Katzung, 2012; Manning, 2017; Riederer & Müller, 2017; Youdim & Bakhle, 2006).

2.1.2.6. Anticholinergic drugs

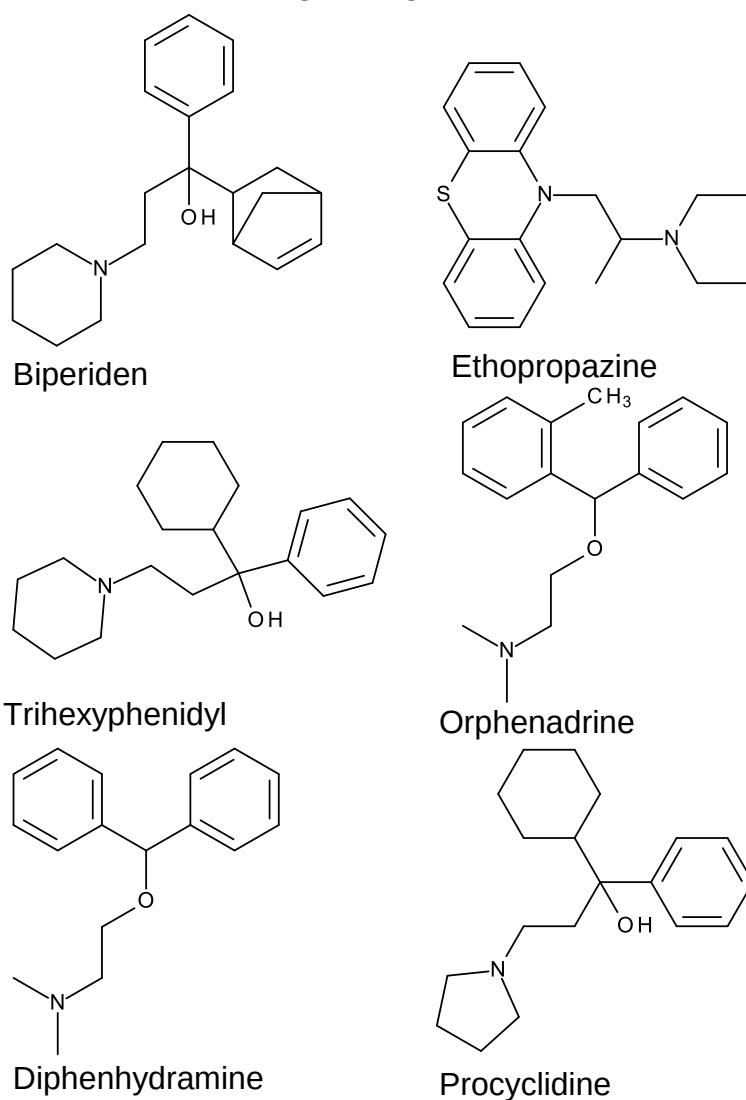
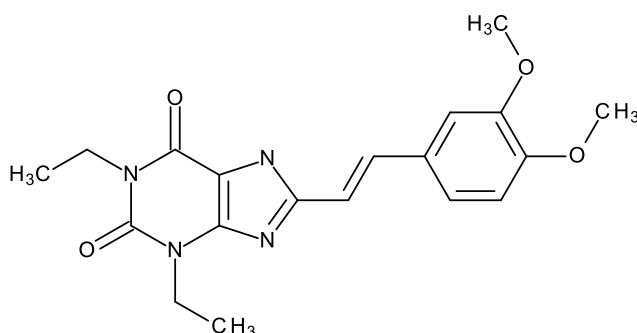


Figure 2.9 The chemical structures of anticholinergic drugs, trihexyphenidyl, ethopropazine, biperiden, procyclidine, diphenhydramine and orphenadrine (Engelbrecht *et al*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Anticholinergic drugs reduce tremor and rigidity but does not affect the movement or

postural stability in PD. Anticholinergic drugs can enhance confusion experienced by patients suffering of dementia and elderly PD patients by causing cholinergic deficiency in the cortex and hippocampus of the brain. More than 30% of PD patients suffer from intellectual impairment or subcortical dementia because of lesions in the basal ganglia. As the disease progresses anticholinergic drugs should be avoided in PD. These drugs competitively antagonise the muscarinic receptors, and by reducing the degree of neurotransmission mediated by neostriatal acetylcholine corrects the imbalance between the cholinergic and dopaminergic neurological pathways. Anticholinergic drugs used in PD are procyclidine, orphenadrine, benztropine, biperiden, diphenhydramine and trihexyphenidyl ([Katzung, 2012](#); [Manning, 2017](#)).

2.1.2.7. Adenosine A_{2A} receptor antagonists



Istradefylline

Figure 2.10 The chemical structures of the adenosine A_{2A} receptor antagonist drug, istradefylline ([Engelbrecht et al, 2018](#)). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Adenosine A_{2A}⁷ receptors are selectively expressed in the basal ganglia and with the antagonism of these receptors locomotor function improves without causing dyskinesia. When used in combination with levodopa, studies suggest that a reduced “off” time is experienced with adenosine A_{2A} receptor antagonists. Adenosine A_{2A} receptor antagonists modulate the neuronal activity of striatal GABA releasing output neurons. Caffeine is a known antioxidant with beneficial effects against oxidative stress and ROS formation. Caffeine is also an adenosine receptor antagonist and while exhibiting no significant adenosine receptor selectivity, it acts as lead for the

⁷ Adenosine A_{2A} receptor is part of the G-coupled protein receptors and it features a ligand binding pocket. It is involved in the synthesis of intracellular cAMP.

design and discovery of adenosine A_{2A} receptor antagonists. Istradefylline is an example of a caffeine-derived compound with potent adenosine A_{2A} receptor antagonistic properties, and has entered clinical trials for the treatment of PD (Katzung, 2012; LeWitt *et al.*, 2008; Manning, 2017)

2.1.2.8. Amantadine

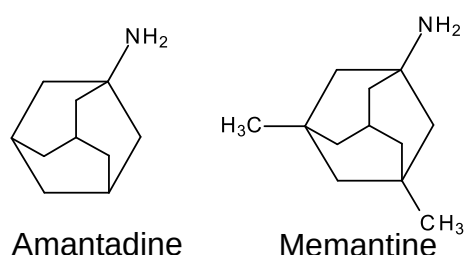


Figure 2.11 The chemical structures of amantadine and memantine (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

An antiviral drug used to treat influenza A, amantadine, is also used to treat extrapyramidal symptoms such as dyskinesia and motor fluctuations in all stages of PD. The complete mechanism of action of amantadine is not known, but amantadine does not exhibit anticholinergic activity and significantly increases dopamine release. Amantadine is also involved in dopamine synthesis and dopamine re-uptake inhibition. Normally, after a high frequency signal, glutamate will be released from its synapse and bind both α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors. AMPA receptors are more bound since positively charged magnesium ions are attracted by the negative resting membrane potential (RMP) and binds and blocks NMDA receptors. Prolonged AMPA stimulation mediates its own depolarization by allowing sodium ion influx, making the whole neuronal membrane less negative and removing the Mg^{2+} blockade at the NMDA receptor. The NMDA receptor channel then indirectly mediates multiple processes involved in memory formation and learning activities via Ca^{2+} influx. Both glutamate receptors play fundamental excitotoxicity roles when receptor overactivation occurs. Neuronal dysfunction will eventually lead to severe cellular death and total cognitive

impairment. Antiglutamatergic drugs and NMDA antagonists such as amantadine and memantine can exhibit neuroprotective effects by blocking neuronal damage caused by overstimulation of the glutamatergic receptors ([Katzung, 2012](#); [Manning, 2017](#); [Weintraub et al., 2010](#)).

2.2 Monoamine oxidase:

2.2.1 General background and tissue distribution

Mary Hare-Berheim discovered tyramine oxidase in 1928, the compound responsible for the oxidative deamination of tyramine. In the 1930s, Hugh Blaschko, realised the oxidative deamination of norepinephrine and aliphatic amines are all catalysed by the “tyramine oxidase” enzyme. This recognition redefined the function of this now single enzyme as one that could metabolise primary, secondary and tertiary amines. Histamine and all the other diamines do not follow this specific metabolising rule, which later led to the enzyme being renamed as mitochondrial MAO.

MAO is a mitochondrial flavoenzyme with FAD as cofactor. It is responsible for the oxidative deamination of biogenic amines, exogenous arylalkylamines and amine neurotransmitters such as serotonin, dopamine and epinephrine in the central nervous system (CNS) as well as the peripheral tissues. Both MAO enzymes are bound to outer mitochondrial membranes through C-terminal transmembrane polypeptide segments. Whether the MAO enzymes exist in the dimeric form (as observed in crystallographic studies) at the mitochondrial membrane as its natural oligomeric form, is yet to be determined. As will be discussed below, the MAO enzymes have three regions, the flavin binding domain, the substrate binding domain and the membrane binding domain ([Binda et al., 2001](#); [Edmondson et al., 2004](#); [Yamada & Yasuhara, 2004](#))

2.2.2 The three-dimensional structure of MAO-B:

The dimeric MAO-B enzyme consists of 520 amino acids that fold into a dense structure. Its active site consists of a 420 Å³ hydrophobic substrate cavity that is

joined to an entrance cavity of 290 Å³ (fig 2.12). The substrate and the entrance cavity are situated in the front of the flavin ring and underneath the protein surface. Between the two cavities is the Ile-199 residue that separates or fuses the entrance cavity and the substrate cavity into one enlarged cavity. An aromatic cage is formed by Tyr398 and Tyr435, and this is the recognition site for the amine group of a substrate. The peptide bond between the Tyr398 and Cys397 residues is in the cis-conformation. The amino acid, Gly110, is responsible for the elasticity of the entrance loop which is needed for substrate access. The crystal structure of the human MAO-B enzyme (fig 2.13) shows that Tyr326 and Ile199 determines the size and the shape of the MAO-B binding cavity whereas the residues Gln206, Tyr398 and Phe343 are involved in co-planar interactions with the aromatic rings on opposite sides of substrates and inhibitors. Two types of flavin adducts form with different types of irreversible MAO-B inhibitors. The first is the formation of a covalent N(5) flavin adduct (e.g. selegiline) and the other is a covalent C(4a) flavin adduct (e.g. tranylcypromine). The crystal structure of MAO-B illustrates the oxidised flavin ring exist in a bent confirmation. The flavin ring is found to be in a constant twisted non-planar conformation whether the enzyme is in its oxidised form or in adduct formations that are expected to be similar to the reduced form. Adaptability toward inhibition can be directly correlated to the elasticity of the MAO-B active site cavity since the cavity can host both small inhibitors such as tranylcypromine and cavity filling ligands such as safinamide. Inhibitors that only partially fill the MAO-B active site such as selegiline are also accommodated. Larger ligands are bulky enough to push the gating residue, Ile199, into an open conformation. This allows for binding to both the entrance and substrate cavities. Such larger inhibitors which are cavity filling ligands are highly specific for MAO-B rather than MAO-A (Binda *et al.*, 2001; De Colibus *et al.*, 2005; Iacovino *et al.*, 2018; Son *et al.*, 2008)

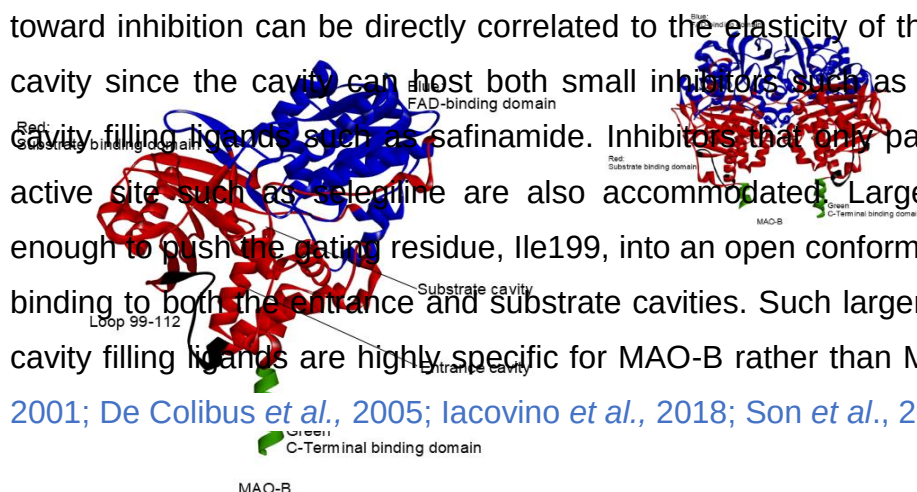


Figure 2.12 Three-dimensional structure of the MAO-B monomeric unit. The FAD-binding domain (residues 4-79, 211-285 and 391-453) is shown in blue, the substrate domain (residues 80-210, 286-390, 454-488) is shown in red, and C-terminal binding domain (residues 489-500) is shown in green. The substrate and entrance cavities are indicated in the red binding domain. Dassault Systèmes BIOVIA, BIOVIA Discovery Studio Visualizer, V16.1.0.15350, San Diego: Dassault Systèmes, 2015.

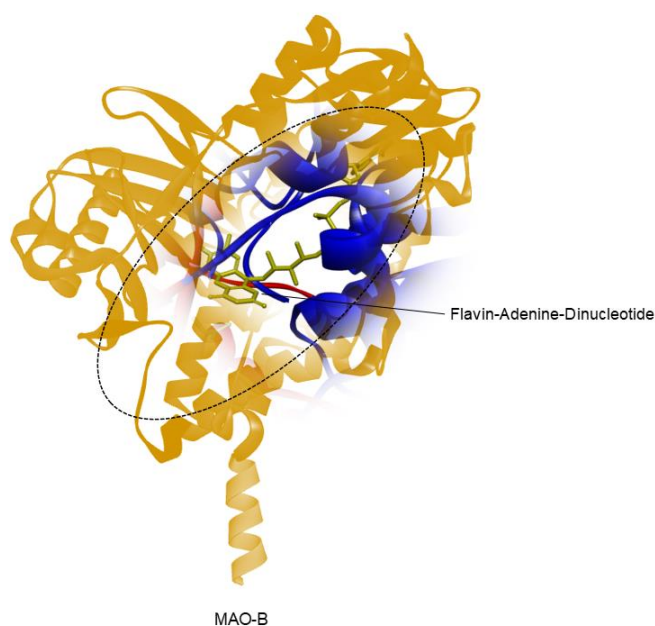


Figure 2.13 Three-dimensional structure of human MAO-B in complex with safinamide (inhibitor). Dassault Systèmes BIOVIA, BIOVIA Discovery Studio Visualizer, V16.1.0.15350, San Diego: Dassault Systèmes, 2015.

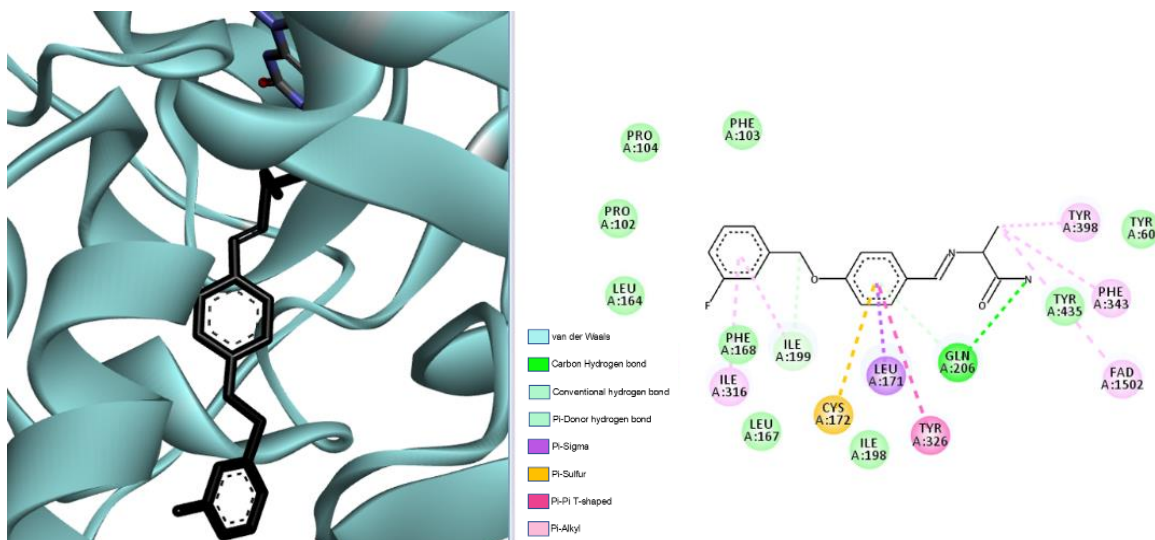


Figure 2.14 Three-dimensional structure of human MAO-B in complex with safinamide showing interactions with various residues and the FAD co-factor. Dassault Systèmes BIOVIA, BIOVIA Discovery Studio Visualizer, V16.1.0.15350, San Diego: Dassault Systèmes, 2015.

2.2.3 The three-dimensional structure of MAO-A:

MAO-A is predominantly found in the small intestine but it also metabolises dietary amines in the placenta, lungs, kidneys, liver and adrenal glands, and in the CNS. The MAO-A enzyme consists of 527 amino acids, 16 of which surround the substrate cavity. The substrate cavity has a total volume of 550 Å³ which is larger than the rat MAO-A cavity (450 Å³). The cavity is hydrophobic and is a single unit that stretches from the flavin ring to the cavity-shaping loop. The cavity is hydrophobic since it is lined by 5 aromatic and 11 aliphatic residues. The cavity-shaping loop consists of residues 210-216. The cavity of MAO-A differs considerably from the MAO-B cavity because of conformational loop alternations and amino acid replacements. Human and rat MAO-A are monomeric and dimeric enzymes, respectively. The single substrate cavity of human MAO-A is shorter and wider than the MAO-B active site. MAO-A has no gating residue that can fuse or separate cavities as with MAO-B. As seen with the crystal form of human MAO-B, an aromatic cage is formed by Tyr407 and Tyr444 making this the recognition site for the amine group of a substrate. The residues Ile335 and Phe208 restrict the size and shape of the inhibitors that could fit into the active site cavity, similar to Tyr326 and Ile199 in MAO-B. Slight movement of these residues are responsible for induced fit to accommodate inhibitors in the MAO-A active site (De Colibus *et al.*, 2005; Son *et al.*, 2008).

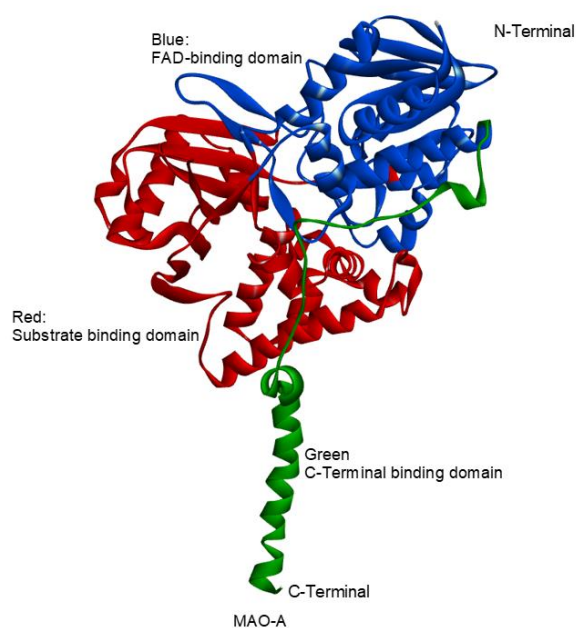


Figure 2.15 Three-dimensional structure of MAO-A. The FAD-binding domain (residues 13-88, 220-294 and 400-462) is shown in blue, the substrate domain (residues 89-219, 295-399) is shown in red, and the C-terminal binding domain (residues 463-506) is shown in green. The substrate cavity is located in the red binding domain. Dassault Systèmes BIOVIA, BIOVIA Discovery Studio Visualizer, V16.1.0.15350, San Diego: Dassault Systèmes, 2015.

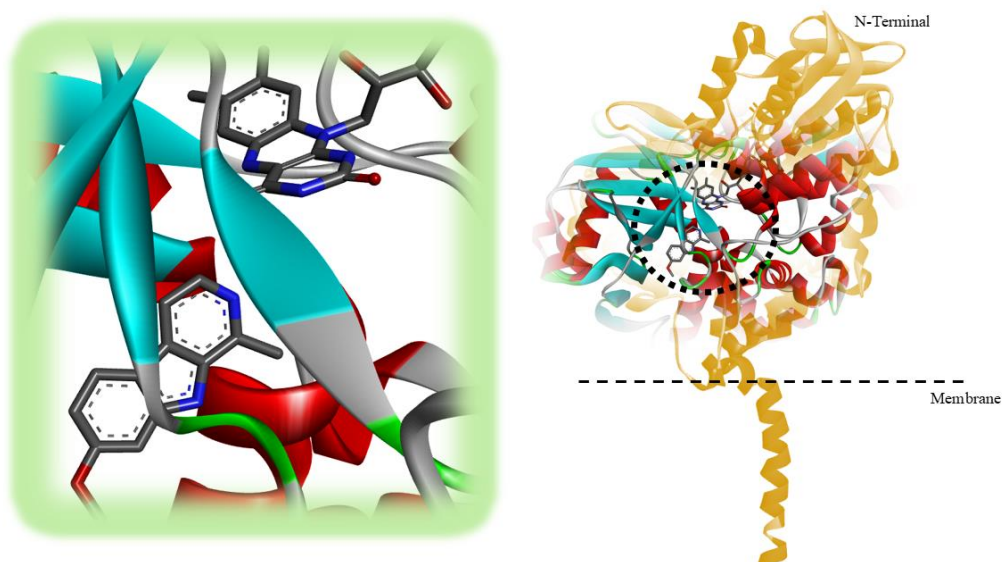


Figure 2.16 Three-dimensional structure of human MAO-A in complex with harmine (inhibitor). Dassault Systèmes BIOVIA, BIOVIA Discovery Studio Visualizer, V16.1.0.15350, San Diego: Dassault Systèmes, 2015.

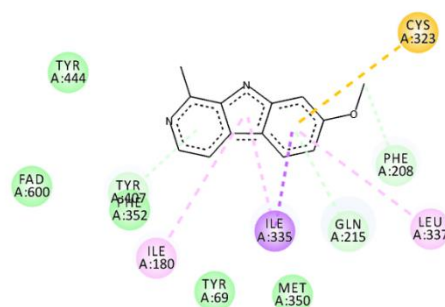
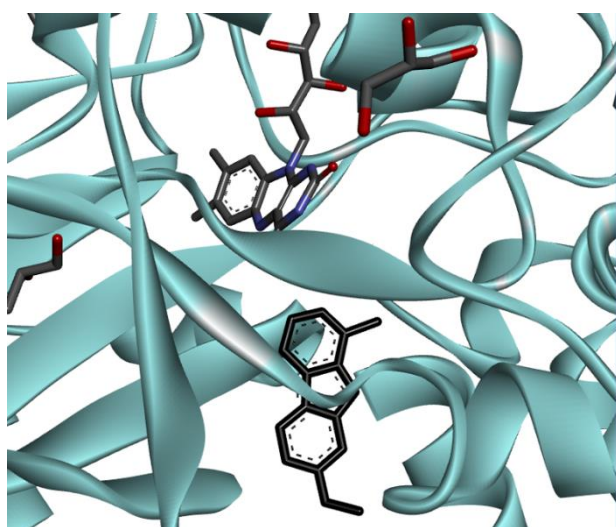


Figure 2.17 Three-dimensional structure of human MAO-A in complex with harmine showing interactions with various residues and the FAD co-factor. Dassault Systèmes BIOVIA, BIOVIA Discovery Studio Visualizer, V16.1.0.15350, San Diego: Dassault Systèmes, 2015.

In summary, active site residues most important for catalysis, and substrate and inhibitor specificity are:

1. The tyrosine aromatic sandwich pair at the flavin ring (Tyr407 and Tyr444 in MAO-A, and Tyr 398 and Tyr435 in MAO-B)
2. A lysine residue hydrogen bonded (by means of a H₂O molecule) to the N-5 position on the flavin ring (Lys305 and Lys296 in MAO-A and MAO-B, respectively).
3. The Phe208/Ile335 pairs in MAO-A and Ile199/Tyr326 pairs in MAO-B which determines the substrate-inhibitor specificities of the two enzymes. ([Binda et al., 2001](#); [Binda et al., 2007](#); [Hubálek et al., 2005](#))

2.2.3.1. Substrate specificities:

MAO-A and MAO-B are encoded by separate genes. Since the MAO enzymes exhibit differing substrate specificities and tissue distribution, they represent targets for the treatment of different disease states. MAO-A selectively deaminates serotonin and MAO-B selectively deaminates benzylamine and phenylethylamine. Some amine neurotransmitters such as dopamine, epinephrine and tyramine act as substrates for both enzymes. The partial overlapping of substrate specificity of MAO-A and MAO-B suggests that non-specific inhibitors may be more appropriate for the treatment of disease states where there is a deficiency of shared substrates such as dopamine. (Binda *et al.*, 2001; De Colibus *et al.*, 2005; Iacovino *et al.*, 2018; Son *et al.*, 2008)

Table 2.1 Substrates of MAO-A and MAO-B.

Substrate	Metabolic enzyme
Norepinephrine	MAO-A
Serotonin	MAO-A
Dopamine	MAO-A & MAO-B
Tyramine	MAO-A & MAO-B
Phenylethylamine	MAO-B
Benzylamine	MAO-B

2.2.3.2. Genes and MAO:

The genes encoding the MAO enzymes are located on the Xp11.23-11.4 chromosome. The duplication and evolution of an ancestral gene is believed to have caused the same inter-neuron (exon-intron) layout which comprises 15 exons each, but differences in their promotor regions are believed to cause variances in expression patterns and levels. The mitogen-activated protein kinase pathway in conjunction with various other enzymatic pathways such as MEK-1, 3 and 7 and protein kinase C have contributory roles in regulating MAO-B expression. Low platelet MAO-B is linked to personality dysfunctions such as substance abuse and is

commonly seen in long-term smokers ([Andres et al.,2004](#); [Naoi et al.,2017](#))

The regulation of MAO-A expression is subject to certain hormones in the reproductive tissues, capillary endothelium cells, endometrium, brain and the fibroblasts. When glucocorticoids, progesterone, testosterone and corticosterone are elevated in these regions, MAO-A levels increase, and studies show that castration (of mice) lead to triplicated MAO-A levels. Depleted or lacking expression of MAO-A causes abundant serotonin availability and may result in a compulsive aggressive phenotype. Low MAO-A can lead to impulsive violence which implies a compensatory-hypotheses in the brain where high levels serotonin exuberates aggressiveness. A hemizygous mutation on exon 8 (chain termination) of the MAO-A gene has been implicated in hypersexual behaviour, mental retardation, aggression and arson. Research done on Norrie disease where MAO is absent, has shown that MAO is not essential for survival ([Andres et al.,2004](#); [Dauer & Przedborski.,2003](#); [Naoi et al.,2017](#))

2.2.4. Biological function of MAO-A:

2.2.4.1. The cheese reaction:

One of the most serious adverse effects of MAO-A inhibition is the “cheese reaction”. MAO-A metabolises tyramine in the periphery and when MAO-A is inhibited, tyramine levels in the systemic circulation are elevated. When MAO-A inhibitors are used, tyramine levels are significantly elevated, and since tyramine is an indirectly acting sympathomimetic agent it releases norepinephrine from peripheral neurons. This causes a hypertensive effect which can be fatal to certain individuals, especially those with cardiovascular problems. Only nonselective and irreversible MAO-A inhibitors cause this hypertensive reaction. The newer selective and reversible drugs make it possible for tyramine to be metabolised normally in the periphery and thus avoid this severe adverse effect. With reversible MAO-A inhibitors, tyramine will compete and displace the reversible inhibitor from the MAO enzyme. Aged cheeses, tofu, non-pasteurised beer and dried or smoked chicken, fish, meats and curated foods are direct dietary restrictions when prescribing MAO-A inhibitors since these foods contain considerable amounts of tyramine. Safe foods are fresh meats, bananas, avocados, raspberries and all other vegetables. Certain drugs that patients

take for unrelated reasons can enhance adrenergic levels and precipitate hypertensive reactions in combination with MAO-A inhibitors. These drugs include decongestants, certain stimulants, norepinephrine containing anaesthetics and antidepressants. (Fox *et al.*, 2018; Katzung, Masters and Trevor, 2012; Krishnan, 2017; Nagatsu, 2004)

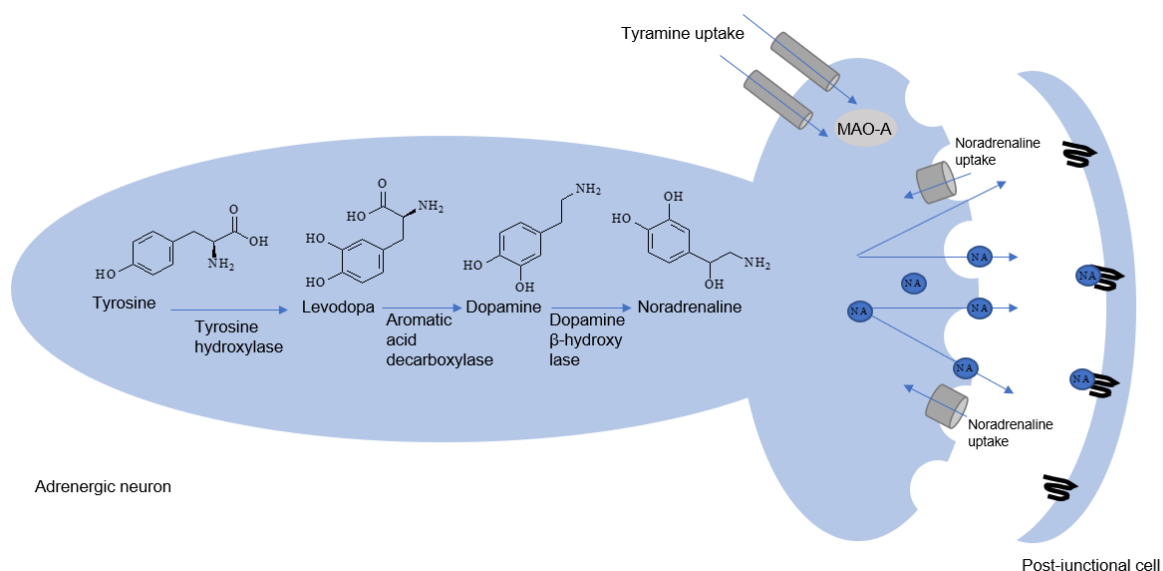


Figure 2.18 Illustration of the cheese reaction within the adrenergic neuron. When tyramine is not sufficiently metabolised by MAO-A in the gut, it reaches the systemic circulation and causes norepinephrine release at the adrenergic synapse. A possible fatal hypertensive crisis may arise. Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016. (Katzung, Masters & Trevor, 2012; Youdim & Bakhle, 2006)

2.2.4.2. MAO-A in depression

MAO is a drug target for the treatment of depression since it metabolises serotonin, norepinephrine and dopamine in the brain. Depression is a disease of major concern since it ranks under the top 5 causes of death and injury and has a yearly prevalence of more than 2%. Depressive disorders can be sub-categorised into three types:

major depression, refractory depression and atypical depression. Anxiety disorders on the other hand constitutes social phobia, panic disorder, post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder (OCD). Among these disorders, only PTSD and panic disorder are treated with MAO inhibitors, which represent the treatment of choice. Reduced monoamine levels has been implicated as one of the main contributing factors to major depression while increased densities of MAO-A in the brain may also play a role in the development of major depressive disorders. MAO-A inhibitors are considered highly effective drugs for the treatment of major depression with moclobemide, toloxatone and befloxatone representing reversible MAO-A inhibitors. Phenylezine and tranylcypromine are irreversible MAO-A inhibitors and show higher efficacy for the treatment of depression. Non-selective MAO inhibitors tend to be more effective in treating atypical depression and phobic anxieties such as bulimia, rejection impressions and hysteria ([Goodman et al., 2018](#); [Katzung, Masters & Trevor, 2012](#); [Meyer et al., 2006](#); [Youdim & Bakhle, 2006](#))

2.2.4.3. The serotonin syndrome

When combining MAO inhibitors with selective serotonin reuptake inhibitors (SSRIs) a possible life-threatening adverse effect, known as serotonin syndrome or serotonin toxicity, may develop. This syndrome is characterised by an excessive extracellular concentration of serotonin in the CNS. This occurs because the metabolism of serotonin by MAO-A is inhibited while the reuptake of serotonin into neurons is also blocked. Autonomic hyperactivity, neuromuscular hyperactivity and an altered mental state are the diverse and nonspecific symptoms of serotonin syndrome. The most dangerous aspect of this disorder is that the symptoms will present themselves abruptly and progress quickly ([Katzung, Masters & Trevor, 2012](#))

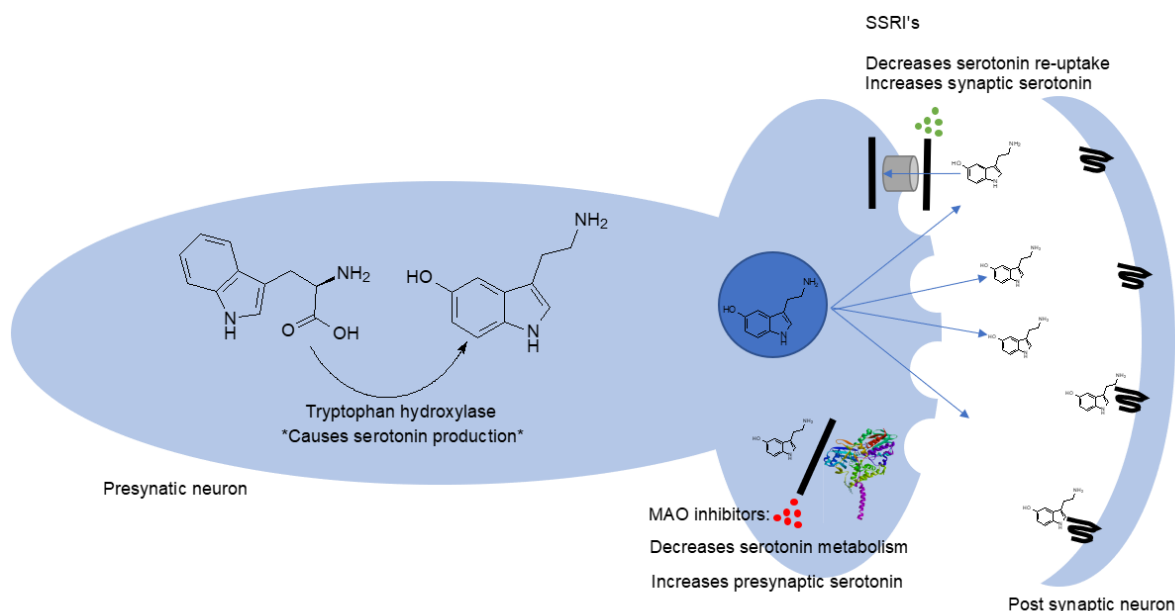


Figure 2.19 Illustration of serotonin syndrome. MAO-A inhibitors decrease serotonin metabolism and increase the amount of available (presynaptic) serotonin. SSRIs, in turn, decrease serotonin re-uptake, which further enhance synaptic serotonin levels. Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016. ([Katzung, Masters & Trevor, 2012](#); [Youdim & Bakhle, 2006](#)).

2.2.5. The role of MAO-B in PD

2.2.5.1. Metabolism of dopamine

L-Tyrosine is taken up by dopaminergic neurons and converted to levodopa via tyrosine hydroxylase. Levodopa is subsequently metabolised by AADC to yield dopamine. After the re-uptake of dopamine into the nerve terminals, it is metabolised by the MAOs which are located intracellularly, attached to the outer mitochondrial membrane. In the neuron, dopamine is also metabolised by COMT, and thus both MAO and COMT reduce dopamine levels directly. The metabolism of dopamine by MAO increases the quantities of the by-products of the MAO catalytic cycle, dopanal and H_2O_2 . These by-products are involved in protein aggregation and cell apoptosis. Dopamine can also be converted to epinephrine and norepinephrine by dopamine β -hydroxylase. D1-D5 receptors are found in both the peripheral as well as the CNS with D1 receptors being the predominant receptor in the SNpc and D2 receptors the main interaction site in the postsynaptic striatal neurons. ([Katzung, Masters & Trevor, 2012](#))

2.2.5.2. Generation of toxic by-products by MAO

During a MAO-catalysed reaction, oxygen (O_2) acts as the electron acceptor and is converted to H_2O_2 by MAO. The oxidation of the amine substrates leads to the reduction of the FAD. The imine product is further hydrolysed in a non-enzymatic

process to yield ammonia and an aldehyde. When the aldehyde undergoes further oxidation by aldehyde dehydrogenase, carboxylic acids are formed. In the presence of iron and other transition metals (cobalt, nickel, iron), H_2O_2 is converted into the hydroxyl radical, which is a highly reactive and damaging molecule. By indirectly generating the hydroxyl radical, MAO catalysis may contribute to neurodegeneration in PD. Aldehydes, in turn, may also contribute to neurodegeneration if not cleared since these compounds react with, and damage cellular proteins. (Edmondson, 2014; Edmondson *et al.*, 2009; Fišar, 2016)

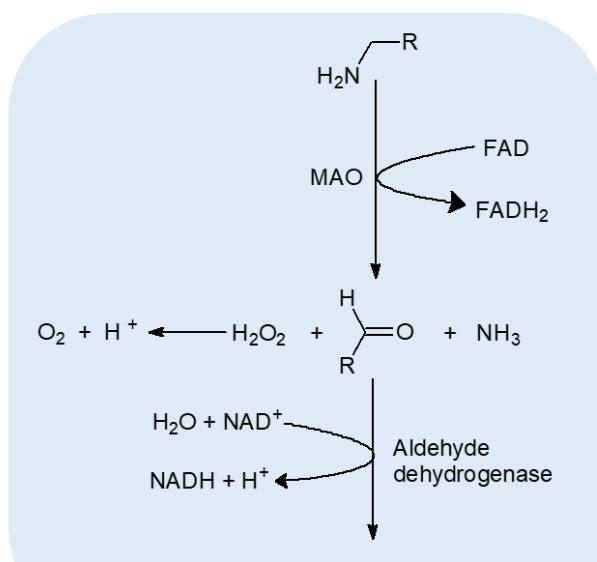
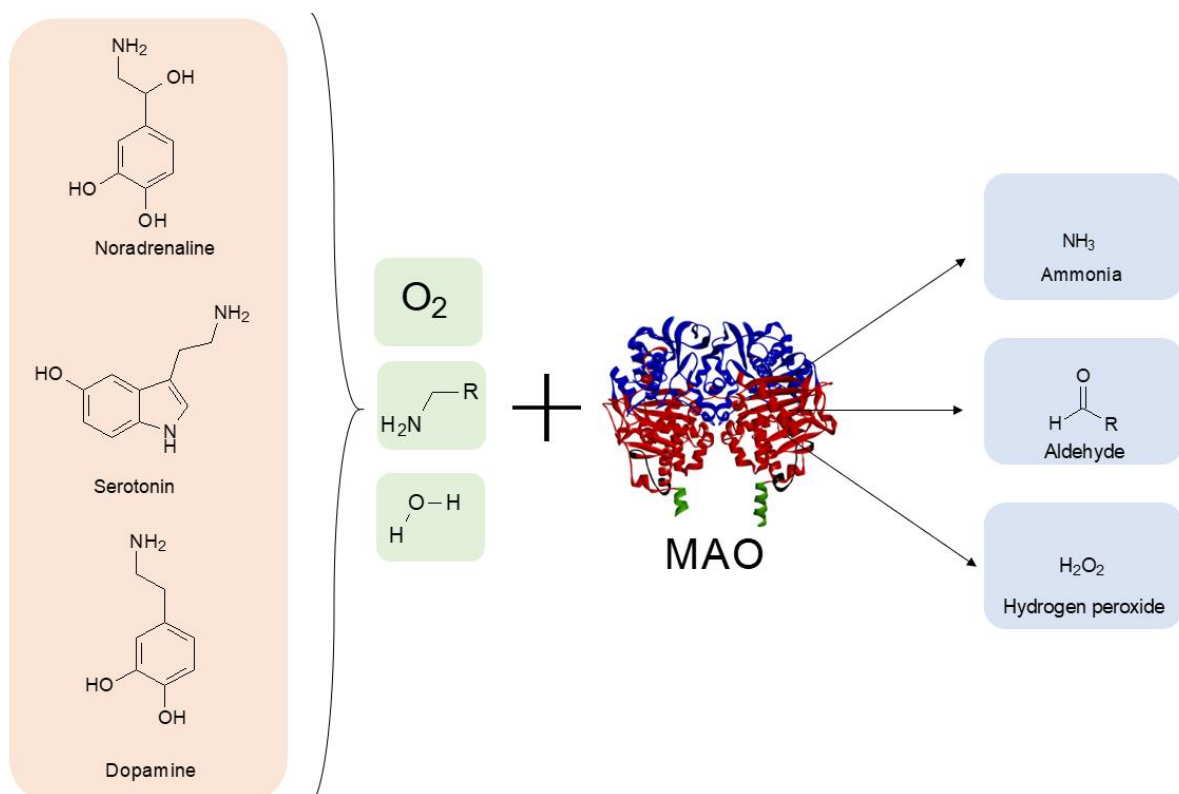


Figure 2.20 Illustration of the generation of toxic by-products by MAO. Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016. (Edmondson, 2014; Edmondson *et al.*, 2009; Fišar, 2016; Youdim & Bakhle, 2006).

2.2.5.3. MAO levels in the brain and aging

Although the level of expressed enzyme is dependent on tissue type, studies show that the levels of the MAO-B enzyme are tripled in neuronal tissue of the elderly while MAO-A levels remain constant with age. This suggests that in elderly persons, MAO-B will generate increased amounts of by-products (e.g. H_2O_2) and thus make the aged brain more susceptible to oxidative damage by the ROS. MAO-B mediated neurodegeneration may thus be more pronounced in the aged brain. (Binda *et al.*, 2001; Burke *et al.*, 2008; Fowler *et al.*, 1980; Tian *et al.*, 2017)

2.2.5.4. The role of aldehyde dehydrogenase and glutathione peroxidase

Aldehyde dehydrogenase (ALDH) converts aldehydes into carboxylic acids. Up to 19 different aldehyde dehydrogenase genes are involved in the detoxification of aldehydes generated both endogenously as well as exogenously. ALDH 1 and ALDH 2 are the two most important members of this family. ALDH thus protects against the potential toxic effects of aldehyde species such as those generated by MAO in the CNS. Glutathione peroxidase protects biological cells from oxidative damage by reducing H_2O_2 (into water) and reducing lipid hydroperoxides to their corresponding alcohols. Glutathione peroxidase is thus responsible for protection against H_2O_2 toxicity in the CNS. Glutathione peroxidase is most abundant in the glial cells and since dopaminergic cell loss in PD is associated with gliosis, glutathione peroxidase-

containing cells are increased, indicating an automatic protective action against dopaminergic damage and cell loss (Fitzmaurice *et al.*, 2013)

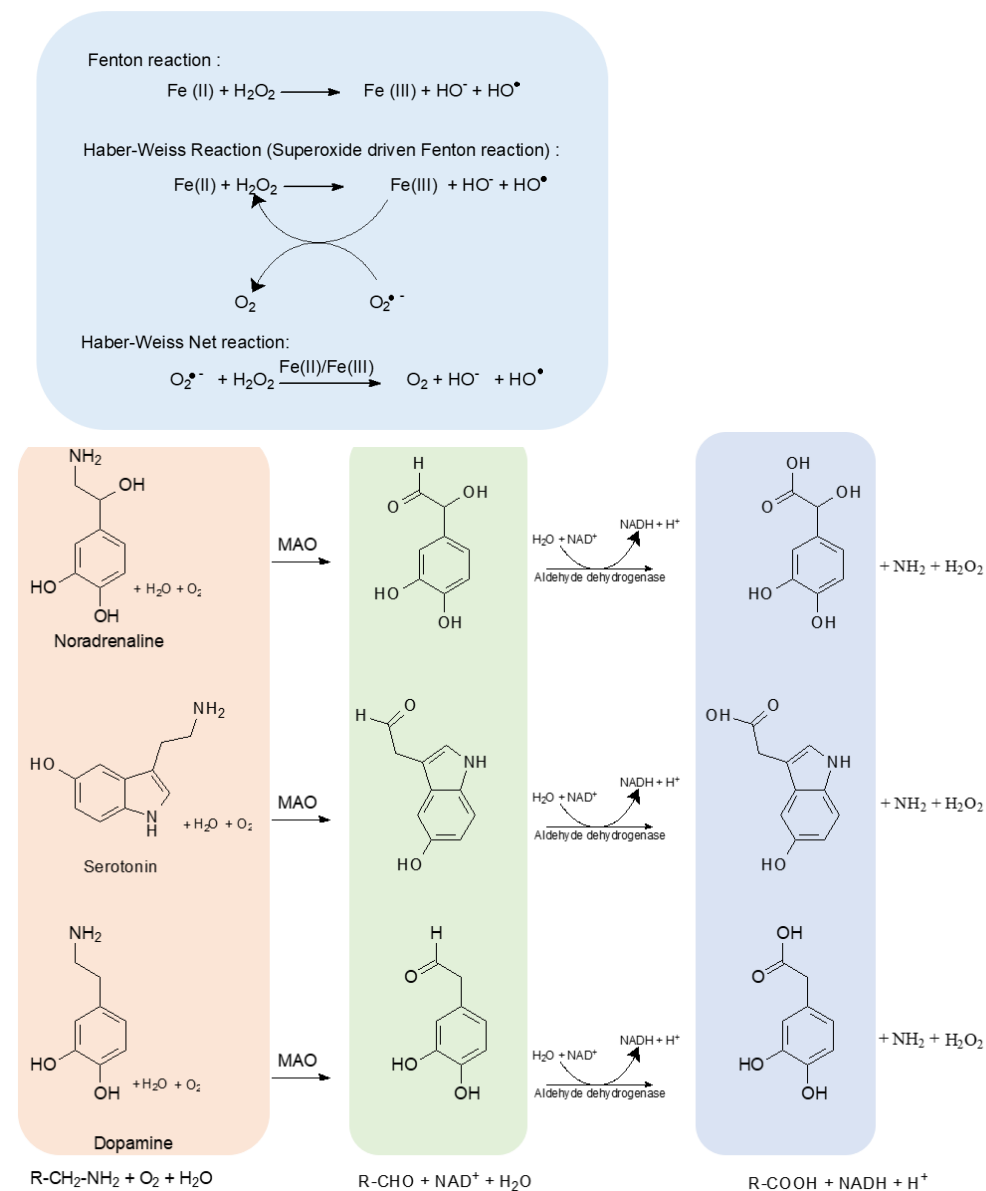


Figure 2.21 Illustration of the mechanisms of action of aldehyde dehydrogenase and the Fenton reaction. Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016 ([Fitzmaurice et al., 2013](#); [Youdim & Bakhle, 2006](#)).

2.2.6. The potential role of MAO-A in PD

Both MAO-A and MAO-B metabolise dopamine. Studies done on rodents suggest that if only one isoform is inhibited, dopamine will still be adequately metabolised within the brain by the other MAO isoform. This limits the therapeutic effect of MAO-B inhibitors in PD. The possibility of the cheese reaction, as explained earlier, has prevented MAO-A inhibitors from being used in PD. With reversible inhibitors of MAO-A, such as moclobemide and brofaromine, tyramine can “reverse” the inhibitory action of the inhibitor at the binding site by displacing the inhibitor, which significantly lowers the risk for the cheese reaction. This led to new opportunities for the potential use of MAO-A inhibitors or dual MAO-A/B inhibitors in PD. Such dual inhibitors may be more effective in enhancing dopamine levels in the CNS since the MAO-catalysed metabolism of dopamine will be much reduced compared to when only the MAO-B isoform is inhibited. Since depression is an often-encountered co-morbidity of PD, MAO-A inhibition may be of further value by representing a treatment strategy for depression ([Meyer et al., 2006](#); [Youdim & Bakhle, 2006](#))

2.2.7 Inhibitors of MAO-B

MAO inhibitors reduce the MAO-catalysed formation of H_2O_2 and increase the levels of monoamines. An increase in monoaminergic activity modulates several signalling pathways, and in PD may partially restore dopaminergic neurotransmission in the striatum. MAO inhibitors can either be reversible or irreversible with respect to its interaction with the active site of MAO. ([Yamada & Yasuhara, 2004](#))

2.2.7.1. Irreversible inhibitors of MAO-B

Irreversible MAO inhibitors are also known as k_{cat} or suicide inhibitors. They firstly bind in a reversible, competitive manner to the active site, and is subsequently

oxidised to a reactive intermediate that binds covalently to the active site of the enzyme. Amine metabolism cannot occur by the irreversibly inhibited enzyme, and only through *de novo* synthesis of the enzyme can normal physiological function again be obtained. Irreversible inhibitor drugs are thus less acceptable for medicinal purposes since enzyme activity may require several weeks to return to normal levels (Riederer and Müller, 2017)

2.2.7.1.1. Selegiline

Selegiline [also known as (R)-Deprenyl] is a selective irreversible MAO-B inhibitor that not only inhibits MAO-B activity but also prevents norepinephrine and dopamine re-uptake into the presynaptic nerve. Acting as a suicide inhibitor of MAO-B, selegiline is firstly oxidised by the enzyme and covalently binds to the FAD co-factor at the N (5) position of the isoalloxazine. Selegiline is an amphetamine derivative and is completely metabolised to methamphetamine, which exhibits sympathomimetic effects such as anorexia (via increased nausea), dry mouth, dyskinesia, orthostatic hypotension, musculoskeletal injuries and cardiac arrhythmias. Selegiline, co-administrated with levodopa, is an effective treatment option used in PD and provides relief of the symptoms associated with dopaminergic neuronal loss (Nagy and Kalász, 2017; Youdim and Weinstock, 2004).

2.2.7.1.2. Pargyline

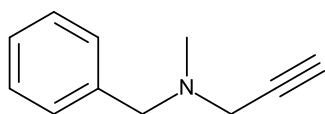


Figure 2.22 The chemical structure of Pargyline (Engelbrecht *et al*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Pargyline has antihypertensive as well as antidepressant effects and acts as an irreversible MAO inhibitor. Since pargyline also inhibits MAO-B, it may potentiate tyramine-induced hypertension (the cheese reaction as discussed previously) by inhibiting catecholamine and tyramine metabolism in the periphery. Pargyline is a suicide inhibitor and forms a covalent adduct with the FAD co-factor. Pargyline also

appears to have high affinity for I₂-imidazoline receptor sites, which is an allosteric site on the MAO enzyme (Fox *et al.*, 2018; Krishnan, 2017)

2.2.7.1.3. Rasagiline

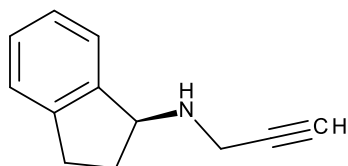


Figure 2.23 The chemical structure of Rasagiline (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Rasagiline is another example of an irreversible and selective MAO-B inhibitor and compared to selegiline generates no potentially toxic metabolites (e.g. amphetamine derivatives). Rasagiline, selegiline and pargyline are examples of propargylamine compounds which are well-known to form covalent, irreversible flavin N(5) adducts with MAO. After inhibition with these compounds, enzyme activity can only be recovered when *de novo* synthesis of the enzyme occurs. (Fox *et al.*, 2018; Krishnan, 2017; Riederer and Müller, 2017)

2.2.7.1.4. Ladostigil

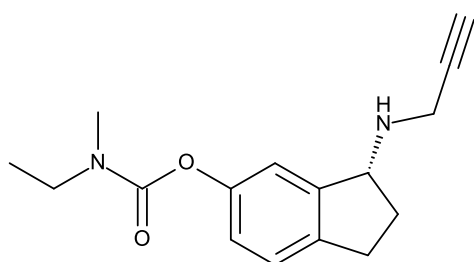


Figure 2.24 The chemical structure of Ladostigil (Youdim & Weinstock, 2004). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Ladostigil is a bi-functional compound and acts as a MAO inhibitor as well as a cholinesterase inhibitor for both butyryl and acetyl cholinesterase. Ladostigil is a nonselective irreversible MAO inhibitor and displays selective inhibition of central

MAO activity. Based on its MAO inhibition properties, ladostigil has been investigated as a potential neuroprotective agent in PD (Riederer and Müller, 2017; Youdim & Weinstock, 2004).

2.2.7.2. Reversible inhibitors of MAO-B

The reversible inhibitors are competitive with regard to their binding to the MAO active site, and often are structurally related to MAO substrates. Reversible inhibitors are more desirable than irreversible inhibitors since inhibition can be reversed by simply withdrawing the drug. This gives reversible inhibitors a better safety profile. (Riederer and Müller, 2017)

2.2.7.2.1. Lazabemide

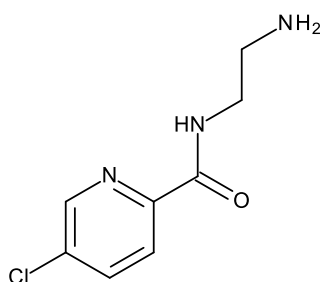


Figure 2.25 The chemical structure of Lazabemide (Youdim & Weinstock, 2004). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

The short-acting compound, lazabemide, is a highly selective reversible inhibitor of MAO-B that does not metabolise to yield active compounds. Lazabemide is a MAO-B substrate and after oxidation, the amine nitrogen is released and the resulting intermediate forms an adduct with the flavin at the N(5)-position. The crystal structure of a MAO-B inhibitor related to lazabemide, N-(2-aminoethyl)-p-chlorobenzamide, is reported and shows binding of the phenyl ring in the entrance cavity of the enzyme while the attachment occurs at the N(5)-position of the flavin. Lazabemide is, considered a reversible inhibitor since inhibition is reversed 36 hours after administration to humans. Based on MAO inhibition properties, both compounds exhibit neuroprotective effects in models of PD (Riederer and Müller, 2017; Youdim & Weinstock, 2004).

2.2.7.2.2. Isatin

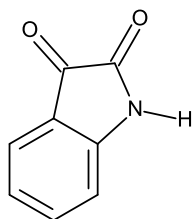
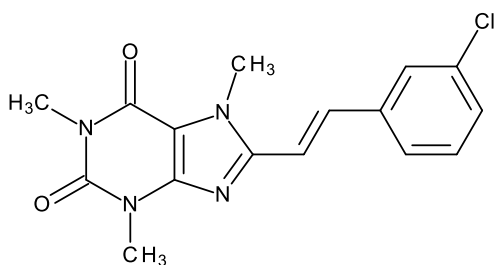


Figure 2.26 The chemical structure of Isatin (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

The endogenous indole derivative, isatin, binds to MAO-B, central benzodiazepine (BD) receptors and the atrial natriuretic peptide receptors.⁸ Other possible effects of isatin include antidiuretic, anxiolytic and supposed appetite suppressant effects. Based on crystallography studies, it has been shown that the dioxindole ring of isatin is bound in the substrate cavity of MAO-B with the oxo-groups (2-oxo and 3-oxo) of the pyrrole ring vertically projecting towards the flavin ring. The 2-oxo group and the NH are hydrogen bonded to water molecules in the active site. The Ile199 side chain which is known as the gating residue, exists in the closed position and thus separates the entrance and substrate cavities. Clinically, isatin increases the concentrations of both dopamine and acetylcholine in the striatum, which has positive effects on bradykinesia commonly seen in PD (Binda *et al.*, 2003; Riederer and Müller, 2017; Youdim & Weinstock, 2004).

2.2.7.2.3. (E)-8-(3-Chlorostyryl)caffeine



⁸ The benzodiazepine receptor site is found at γ -aminobutyric acid (GABA) – A terminal. Activation of this receptor releases the neurotransmitter, GABA, causing hypnotic, sedative and muscle relaxant properties. Atrial natriuretic peptide receptors are involved in intracellular guanyl cyclase activity where GTP is converted to cGMP to eventually induce smooth muscle relaxation.

Figure 2.27 The chemical structure of (*E*)-8-(3-chlorostyryl)caffeine (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

(*E*)-8-(3-Chlorostyryl)caffeine (CSC) is an adenosine A_{2A} receptor antagonist and is known to potently inhibit MAO-B. Since A_{2A} antagonists are also used in the treatment of PD, CSC is a dual acting compound for PD treatment. CSC also possesses neuroprotective effects in animal models, effects which may be mediated by both MAO-B inhibition and A_{2A} antagonism (Riederer and Müller, 2017; Youdim & Weinstock, 2004).

2.2.7.2.4. 1,4-Diphenyl-2-butene

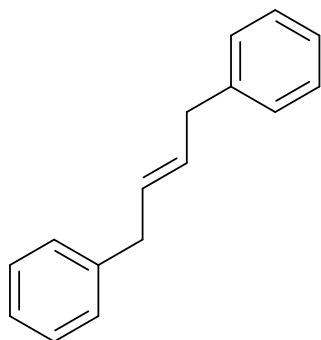


Figure 2.28 The chemical structure of 1,4-Diphenyl-2-butene (Hubálek *et al.*, 2005). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

1,4-Diphenyl-2-butene is a potent selective inhibitor of MAO-B that binds to both the entrance and substrate cavities of MAO-B. Crystallographic studies have shown that when this inhibitor is bound to MAO-B, the gating residue Ile199 is in the open conformation so that the inhibitor can span both cavities (Binda *et al.*, 2003; Hubálek *et al.*, 2005)

2.2.7.2.5. Trans, trans-farnesol

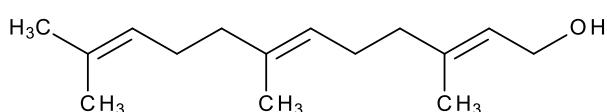


Figure 2.29 The chemical structure of Trans-trans-farnesol (Hubálek *et al.*, 2005).

Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Trans, trans-farnesol selectively inhibits human and rodent MAO-B but has no inhibitory effect on the human MAO-A enzyme. *Trans, trans*-farnesol is thus a selective, competitive and reversible MAO-B inhibitor. When this inhibitor binds to MAO-B, the gating residue Ile199 is rotated out of its normal conformation so that the two MAO-B cavities fuses into one single cavity. The alcohol group and the 1-methylene carbon are positioned close to the C(4) and the N(5) positions, respectively, of the FAD co-factor. This is the region where the amine and α -carbon of substrates are thought to bind prior to catalysis ([Hubálek et al., 2005](#))

2.2.7.2.6. Safinamide

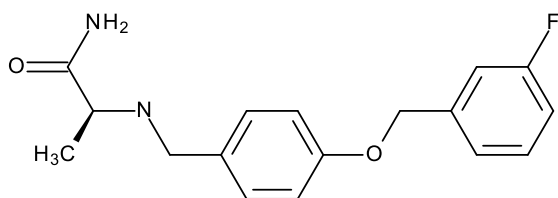


Figure 2.30 The chemical structure of Safinamide ([Engelbrecht et al, 2018](#)). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Safinamide exhibits a high degree of selectivity towards MAO-B since it spans both cavities of the MAO-B active site. The active site of MAO-A is a single cavity which does not accommodate cavity-spanning MAO-B inhibitors such as safinamide well. Safinamide possesses polar substituents that determine its orientation in the MAO-B active site. The polar amide group of safinamide binds in front of the flavin in such a manner that hydrogen bond interactions occur with protein residues as well as conserved water molecules in this region, which constitutes a hydrophilic space in the MAO-B active site. The 3-fluorobenzyloxy group interacts with the entrance cavity with the Ile199 locked in its open conformation ([Binda et al., 2007](#))

2.2.8. Inhibitors of MAO-A

MAO-A is mainly responsible for the metabolism of serotonin and norepinephrine,

and inhibition of this enzyme could lead to increased amounts of both monoamines in the brain and peripheral tissues. MAO-A inhibitors are used in the treatment of diseases such as depression where a physiological deficiency of serotonin and norepinephrine contributes to the disease pathology. MAO-A is expressed in the striatum where it also metabolises dopamine to the extent that, if MAO-B is completely inhibited, dopamine would still be adequately metabolised in normal healthy brains by MAO-A. The selective inhibition of either enzyme isoform results in an increase of dopamine release but does not affect steady state dopamine concentrations in the brain (Youdim & Bakhle, 2006; Youdim & Weinstock, 2004; Katzung, Masters & Trevor, 2012)

2.2.8.1. Clorgyline

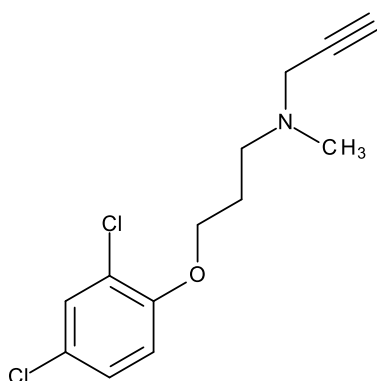


Figure 2.31 The chemical structure of Clorgyline (Engelbrecht *et al*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Clorgyline is a selective and irreversible inhibitor of MAO-A and causes an increase in serotonin and norepinephrine levels in the brain. The antidepressant effect that clorgyline exhibit is overshadowed by the risk to induce the cheese reaction, and this compound was thus not developed as a clinical antidepressant (Youdim & Bakhle, 2006; Katzung, Masters & Trevor, 2012)

2.2.8.2. Tranlycypromine and phenelzine

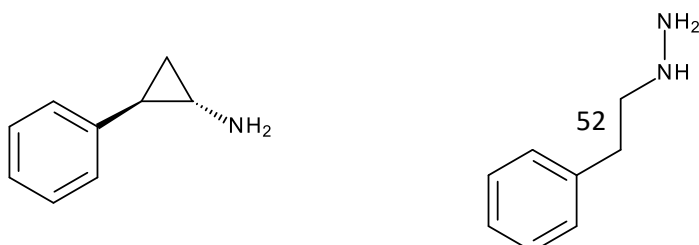


Figure 2.32 The chemical structures of tranylcypromine and phenelzine ([Engelbrecht et al, 2018](#)). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

The irreversible MAO inhibitors, tranylcypromine and phenelzine, are not selective and will bind to both MAO isoforms. These compounds also possess the risk of causing the cheese reaction. Tranylcypromine possesses a cyclopropylamine function which is oxidised by MAO to yield an intermediate that covalently binds to the C(4) position of the flavin. This interaction is responsible for the irreversibility of inhibition. Phenelzine possesses the hydrazine function that interacts with the flavin of MAO in a similar manner to form an adduct at the C(4) position. Both drugs are still used as antidepressants. Since tranylcypromine and phenelzine are small molecules, they bind only to the substrate cavity of MAO-B (leaving the entrance cavity unoccupied) with the Ile199 residue in a closed conformation ([Binda et al.,2003](#))

2.2.8.3. Befloxatone, moclobemide and brofaromine

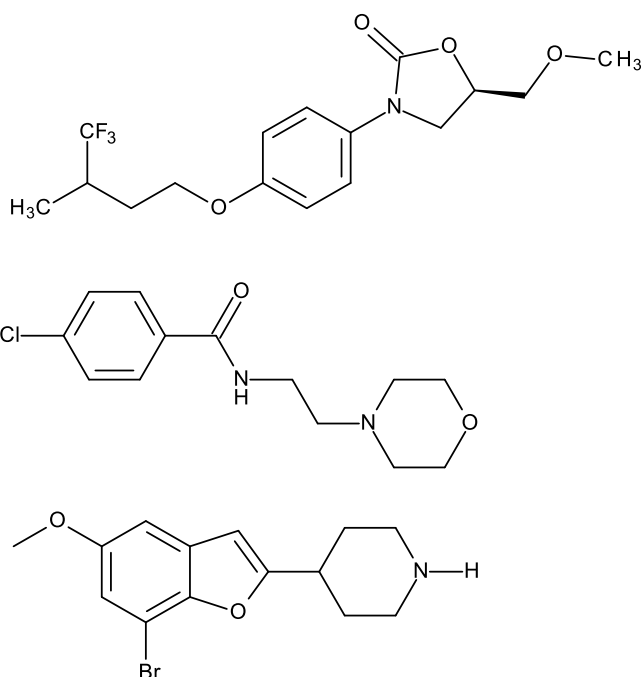


Figure 2.33 The chemical structures of befloxatone, moclobemide and brofaromine (Flockhart, 2012). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Befloxatone is an oxazolidinone compound with similarity to toloxatone and cimotoxone. Oxazolidinones were first introduced as selective and reversible MAO inhibitors without an amine function. The absence of an amine, as seen with these oxazolidinone derivatives, explains the reversible action. When present, the amine forms an irreversible adduct with MAO after being oxidised into an amine radical cation which is trapped by the enzyme giving it an irreversible binding action. Only through *de novo* synthesis can MAO activity be recovered. The phenyl ring and the oxazolidinone ring of befloxatone are co-planar which contributes to stability. The reversible inhibition of MAO-A by befloxatone is proposed to occur via a double mechanism. Intermolecular hydrogen bonds in the hydrophobic region of the cavity and the involvement of an interaction of the FAD with befloxatone explains the high affinity and potency of this compound as a MAO-A inhibitor (Fowler *et al.*, 2015).

Moclobemide exhibits adverse effects such as sleep disturbances, anxiety, restlessness, and headaches, but completely lack the cheese reaction since tyramine can compete and displace reversible MAO-A inhibitors such as moclobemide. Dopamine itself is also able to challenge and displace moclobemide from the MAO-A substrate cavity, which makes moclobemide a better complementary drug in levodopa treatment. Moclobemide is symptomatically beneficial in PD and other neurological diseases by reducing motor output fluctuations. The main indication of moclobemide, however, is for the treatment of depression since it is a MAO-A inhibitor (Fowler *et al.*, 2015).

Brofaromine, a piperidine derivative, is a reversible MAO-A inhibitor which binds tightly to the MAO-A active site. Brofaromine also inhibits serotonin re-uptake and thus leads to an increase in functional serotonin levels. This compound thus has a dual mechanism in the treatment of depression (Youdim & Bakhle, 2006; Youdim & Weinstock, 2004)

2.2.8.4. Iproniazide

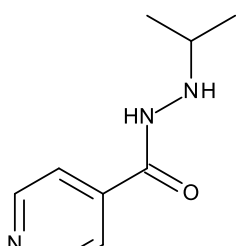


Figure 2.34 The chemical structure of iproniazide (Youdim & Bakhle, 2006). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

Iproniazid is a non-selective, irreversible MAO inhibitor, which was initially used to treat tuberculosis and depressive disorders. The hydrazine functionality of iproniazid is converted to a toxic metabolite that may precipitate hepatotoxicity and liver necrosis. High toxicity levels directly correlate to its hydrazine structure and rendered it unusable in the clinical practice (Youdim & Bakhle, 2006)

2.2.9. Natural products that inhibit MAO

More than a 1000 plant species are used as traditional herbal medication in South Africa by a large part of the country's population. African traditional healers, known as sangomas, diagnose and devise treatment regimens for mental disorders such as hysteria, mental disturbances and anxiety using a wide variety of indigenous plants. The roots, leaves, bulb or corm of the plants are either eaten whole, burned as incense or prepared as extracts in water or alcohol. The pharmaceutical potential of MAO inhibitors found in plants all over the world have in the last decade been recognised and the search for novel active compounds has increased a great deal. (Butterweck *et al.*, 2002). Investigation of some herbal remedies specifically used for mental disorders include *Acorus gramineus* Sol, ex Aiton (Tao *et al.*, 2005), *Rhazya stricta* Decne, (Al-Dabbagh *et al.*, 2018), *Uncaria rhynchophylla* (Miq.) Havil. (Lin *et al.*, 2003), *Gentiana lutea* L. (Haraguchi *et al.*, 2004), *Ruta graveolens* L, *Schotia brachypetala* Sond. and *Mentha aquatic* L. These remedies have been found to exhibit MAO inhibitory activity (Stafford *et al.*, 2007) and special attention is paid to the modulatory monoaminergic neurotransmission activity of cannabinoids (Fišar, 2010). Compounds containing certain chemical structures such as alkaloids, anthocyanins, anthraquinones, chalcones, coumarins, flavonoids and terpenoids function as natural MAO inhibitors. These chemical structures are ideal leads for

synthesising derivatives that can be more potent inhibitors with better side effect profiles (Dey *et al.*, 2017; Van Wyk *et al.*, 2018; Zhi *et al.*, 2014)

2.3 Enzyme Kinetics

2.3.1 Michaelis-Menten Kinetics

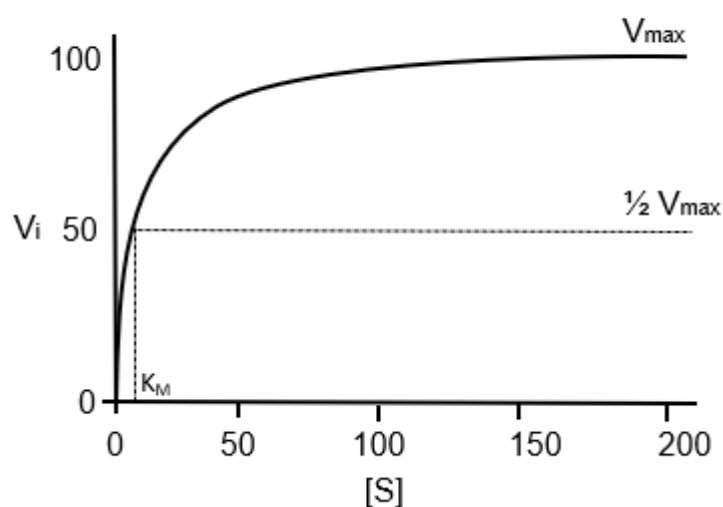
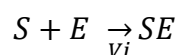


Figure 2.35 A graph showing the relationship between $V_{\max}$ and K_m (Stein, 2011)

Enzyme kinetics is the study of the rates by which enzymes catalyse chemical reactions. Binding affinities of both substrates and inhibitors, and the mechanism of actions of enzymes are included in the study of enzyme kinetics. A simple enzyme catalysed reaction may be illustrated as follows:



The letters S and E are the substrate and enzyme, respectively. The term v_i is representative of the initial velocity of the forward reaction. The relationship between the initial velocity (v_i) and the substrate concentration ($[S]$) can be represented with a mathematical equation known as the Michaelis-Menten equation (Burlingham & Widlanski, 2003)

$$v_i = \frac{V_{max} [S]}{K_m + [S]}$$

K_m or the Michaelis constant denotes an equilibrium constant during reversible reactions of enzymes and substrates. K_m also represents the substrate concentration that leads to half-maximal velocity. The other constant, V_{max} , represents maximal velocity during enzyme saturation. These two constants are directly responsible for predicting the rate with which a reaction takes place.

2.3.2. K_i determination, competitive inhibition and the Lineweaver-Burk plot

The reaction between a substrate and an enzyme follows first order kinetics, which results in low initial velocity (V_i) values since substrate concentrations are initially low. Complex formation follows a 1:1 binding order when every substrate binding site on the enzyme is occupied by a single substrate molecule. Substrate concentration increases and saturates the enzyme, velocity “separates” itself from the first order kinetics law when it reaches its maximum velocity (V_{max}) and follows zero order kinetics which is independent of $[S]$. When the substrate concentration is plotted against velocity, a hyperbolic shaped graph is obtained (Fig. 2.35). The maximum velocity can thus only be estimated by extrapolation. The Michaelis-Menten approach is modified using the $y = mx + c$ equation to construct a straight line (Burlingham & Widlanski, 2003) :

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}} \right) \left(\frac{1}{[S]} \right) + \frac{1}{V_{max}}$$

This equation, more commonly known as the Lineweaver-Burk double-reciprocal plot, places both the velocity and substrate as the denominators to allow K_m/V_{max} to

define the slope. Both values can thus be determined via straight line extrapolation (Bisswanger, 2017; Burlingham & Widlanski, 2003; Stein, 2011).

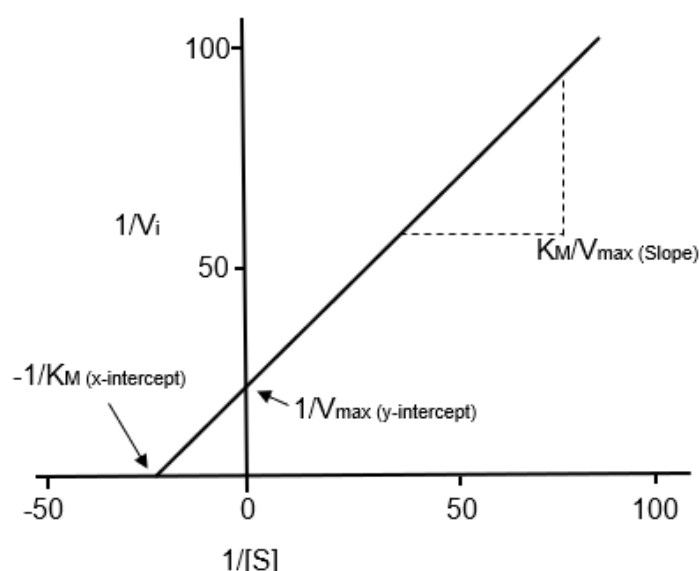


Figure 2.36 The Lineweaver-Burk plot. (Bisswanger, 2017; Burlingham & Widlanski, 2003; Stein, 2011).

Competitive inhibitors seek to replace or displace the substrate from the enzyme active site in a reversible manner. Since both the inhibitor and substrate bind to the same active site, the inhibitor slows the catalytic rate of the enzyme. The Michaelis-Menten equation may be modified as follows to describe the effect of a competitive inhibitor on enzyme catalytic rate:

$$v = \frac{V_{max}[S]}{[S] + K_m \left(1 + \frac{[I]}{K_i} \right)}$$

K_i is the inhibitor or dissociation constant that indicates the potency of an inhibitor during enzyme-inhibitor complex breakdown. Small K_i values are indicative of good potency. The presence of an inhibitor will lower the velocity rate of the total reaction and smaller dissociation constants will increase inhibitor potency since an increase in $[I]$ will increase the apparent Michaelis-constant (K_m) via the factor $\left(1 + \frac{[I]}{K_i} \right)$. With a Lineweaver Burk plot (Fig. 2.36) it can easily be illustrated that velocity will decrease at any given inhibitor concentration. Since all the available enzyme is bound at high substrate concentrations, V_{max} remains stable, unaffected by inhibitor concentration.

This results in identical y-intercept values for all competitive inhibitor concentrations. The x-intercept is defined by the K_m value and the x-intercept decreases as the inhibitor concentration increases. Using this x-intercept $x = \frac{-1}{K_m} \left(1 + \frac{[I]}{K_i}\right)$ equation, K_i can be calculated by determining the K_m values in both the presence and absence of an inhibitor (Fig.2.37) (Bisswanger, 2017; Burlingham & Widlanski, 2003; Stein, 2011)

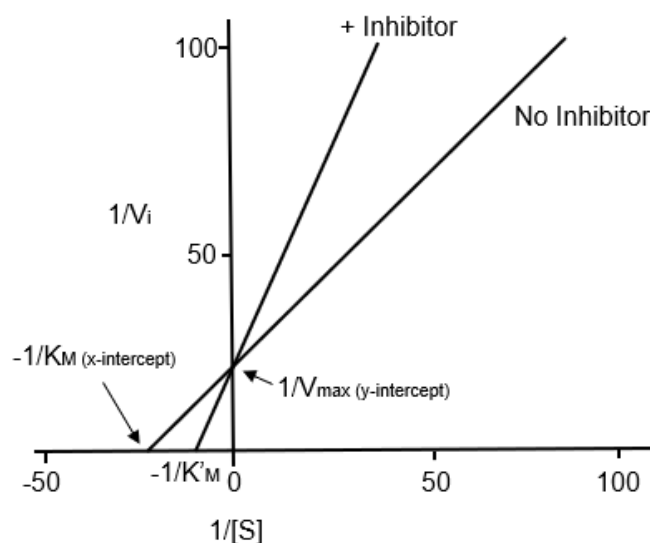


Figure 2.37 Lineweaver-Burk plots illustrating competitive inhibition (Bisswanger, 2017; Burlingham & Widlanski, 2003; Stein, 2011)

2.3.3. IC_{50} determination

IC_{50} values are the concentrations of inhibitors that represent 50% enzyme inhibition in the presence of a substrate. Small IC_{50} values are indicative of potent inhibitors

with high binding affinities for the enzyme active site. These values will be used in this study to express the potencies by which the active drugs inhibit MAO-A and MAO-B. (Burlingham & Widlanski, 2003; Stein, 2011)

$$IC_{50} = \left(1 + \frac{[S]}{K_m}\right) K$$

Figure 2.38 A graph showing the determination of the IC_{50} value (Bisswanger, 2017; Burlingham & Widlanski, 2003; Stein, 2011).

2.4 Summary

In this chapter a broad overview of PD was given with respect to general history, possible mechanisms of neurodegeneration, pathogenesis and symptomatic treatment options. PD symptoms result from the diminished quantities of dopamine in specific brain regions, including the substantia nigra and striatum. Levodopa is the most effective drug therapy for the symptomatic relief of PD. Various enzymes target levodopa for degradation in the periphery. This reduces the effects exerted by levodopa in the central nervous system due to insufficiently low concentrations of levodopa available to cross the blood-brain barrier. MAO inhibitors, specifically MAO-B inhibitors, decrease the degradation of dopamine in the central nervous system which increases the amount of available dopamine and prolongs the action of administered levodopa. Known inhibitors of both MAO isoforms are discussed in this chapter and some of their uses in cardiac and neurological diseases are listed. The three-dimensional structures of MAO-A and MAO-B are explored, and key residues are highlighted. This chapter thus serves as background for the evaluation of the MAO inhibition properties of selected natural products.

CHAPTER 3

Monoamine oxidase inhibition by kavalactones from kava (*Piper methysticum*)

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Abstract

Monoamine oxidase (MAO) is a mitochondrial-bound flavoenzyme that exists as two distinct isoforms, MAO-A and MAO-B. The MAOs are key metabolic enzymes for neurotransmitter and dietary amines in both the peripheral tissues and the central nervous system and are targets for the treatment of neuropsychiatric and neurodegenerative disorders such as depression and Parkinson's disease (PD). For the discovery of novel MAO inhibitors, natural products have often been considered as leads. Numerous natural products have been found to act as good potency MAO inhibitors among which kaempferol, curcumin and chrysin are examples. The present study examines the MAO inhibition potential of a small series of natural products and found that DL-kavain, from the roots of the kava plant, is a good potency *in vitro* inhibitor of human MAO-B with an IC_{50} of 5.43 μ M. DL-Kavain is a weaker MAO-A inhibitor with an IC_{50} of 19.0 μ M. Under the same experimental conditions, the known MAO inhibitor, curcumin, displays IC_{50} values of 5.02 μ M and 2.56 μ M for the inhibition of MAO-A and MAO-B, respectively. It was further established that DL-kavain interacts reversibly and competitively with MAO-A and MAO-B with enzyme-inhibitor dissociation constants (K_i) of 7.72 μ M and 5.10 μ M, respectively. Curcumin in turn, displays a K_i value of 3.08 μ M for the inhibition of MAO-A. Based on these findings, other kavalactones (dihydrokavain, methysticin, dihydromethysticin, yangonin and desmethoxyyangonin) were also evaluated as MAO inhibitors in this study. Yangonin proved to be the most potent MAO inhibitor with IC_{50} values of 1.29 and 0.086 μ M for MAO-A and MAO-B, respectively. Molecular docking studies suggest possible binding modes and interactions of DL-kavain and yangonin with the MAO enzymes. It may be concluded that some of the central nervous system effects (e.g. anxiolytic) of kava may be mediated by MAO inhibition.

1. Introduction

Monoamine oxidase (MAO) is a flavin adenine dinucleotide (FAD) containing enzyme which is attached to the outer mitochondrial membrane. The MAOs are key metabolic enzymes and regulates the levels of neurotransmitters and dietary amines in both the central and peripheral tissues (Youdim & Bakhle, 2006). MAO exists as two distinct isoforms, MAO-A and MAO-B, which are encoded by separate genes. Although the MAO isoforms are approximately 70% identical on the amino acid sequence level, in many instances they exhibit different substrate specificities (Shih *et al.*, 1999). In this regard, serotonin is a specific substrate for MAO-A while 2-phenylethylamine and benzylamine are specific substrates for the MAO-B isoform (Fig. 3.1) (Youdim *et al.*, 2006). Considerable overlap in substrate specificity also occur with for example dopamine, epinephrine, norepinephrine and tyramine acting as substrates for both isoforms. The MAOs often also display different inhibitor specificities. The irreversible inhibitors clorgyline and selegiline are thus specific inhibitors of MAO-A and MAO-B, respectively, and have been used to distinguish between the two isoforms in pharmacological studies (Fig. 3.2).

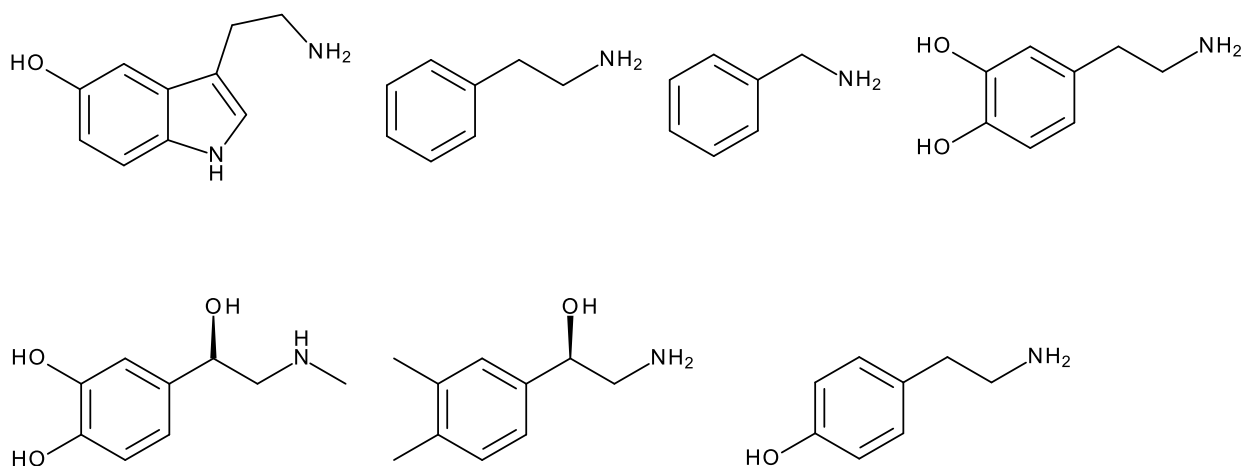


Figure 3.1. The structures of MAO substrates. Serotonin, 2-Phenylethylamine, Benzylamine, Dopamine, Epinephrine, Norepinephrine, Tyramine (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

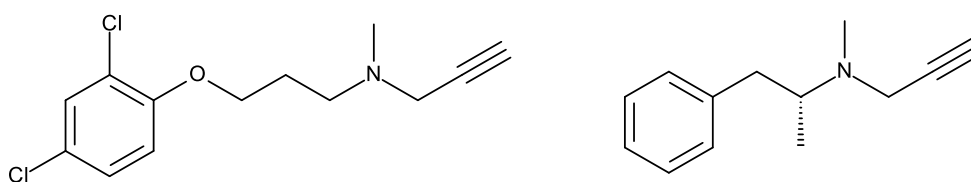


Figure 3.2. The structures of clorgyline and selegiline (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

The structural basis for differing MAO specificities for several reversible inhibitors have been defined by crystallographic studies (Binda *et al.*, 2001; Son *et al.*, 2008). The Ile-335 and Phe-208 residues in MAO-A play an important role in substrate and inhibitor specificity between MAO-A and MAO-B. The corresponding residues in MAO-B are Tyr-326 and Ile-199, respectively (Fig. 3.3). The large size of Phe-208 restricts the binding of certain MAO-B specific inhibitors (e.g. 1,4-diphenyl-2-butene) to MAO-A, while Tyr-326 in MAO-B restricts the binding of certain MAO-A specific inhibitors (e.g. harmine) to MAO-B (Son *et al.*, 2008). Furthermore, the active site of MAO-A consists of a single cavity while the MAO-B active site consists of a smaller entrance followed by a larger substrate cavity (Binda *et al.*, 2001). The gating residue separating the MAO-B cavities is Ile-199, of which the side chain may adopt differing conformations based on the inhibitor bound (Hubálek *et al.*, 2005; Binda *et al.*, 2007). When larger inhibitors such as safinamide and *trans*, *trans*-farnesol are bound to MAO-B, the side chain of Ile-199 exists in the open position allowing the two cavities to fuse. Such inhibitors are cavity-spanning inhibitors and often are MAO-B specific inhibitors. Smaller inhibitors such as isatin bind only to the substrate cavity of MAO-B, leaving the entrance cavity unoccupied. In this instance, the side chain of Ile-199 exists in the closed position. Such smaller inhibitors are thus often non-specific inhibitors of the two MAO isoforms (Hubálek *et al.*, 2005).

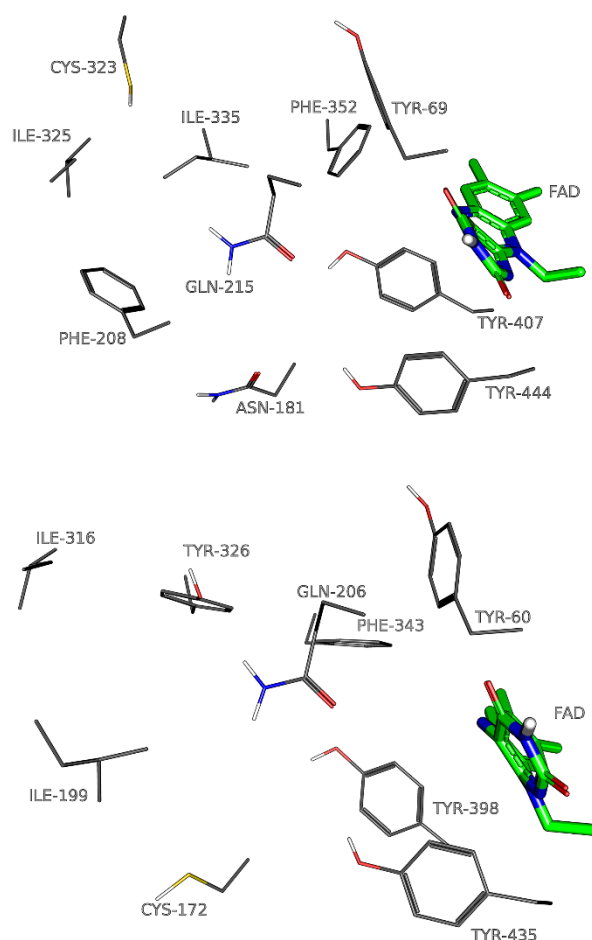


Figure 3.3. Active site models of MAO-A (top) and MAO-B (bottom) showing residues Ile-335/Phe-208 and Tyr-326/Ile-199 that determines inhibitor specificity. The positions of the FAD co-factor and selected residues are also shown.

Since the MAOs are involved in the metabolic breakdown of neurotransmitters, they are of considerable pharmacological and therapeutic interest (Youdim *et al.*, 2006). MAO-A inhibitors (e.g. tranylcypromine, phenelzine, moclobemide and toloxatone) have been used clinically to treat depressive illness and act by inhibiting the central metabolism of serotonin and noradrenaline thereby elevating their levels. MAO-B inhibitors (e.g. selegiline and rasagiline), in turn, have been used to treat Parkinson's disease and act by reducing the central metabolism of dopamine (LeWitt & Taylor, 2008). MAO-B inhibitors are thus frequently combined with L-dopa, the metabolic precursor of dopamine, in Parkinson's disease treatment (Henchcliffe *et al.*, 2005). MAO inhibitors may also reduce the formation of metabolic by-products of MAO catalysis, most notably hydrogen peroxide (Youdim & Bakhle, 2006). The MAO-

mediated formation of hydrogen peroxide and its role in generating reactive oxygen species (ROS) have been implicated in cellular- and neurodegeneration in cardiovascular disease and Parkinson's disease, and MAO-A inhibitors have thus been advocated as potential treatment of congestive heart failure while MAO-B inhibitors may act as potential neuroprotective agents in neurodegenerative disorders (Umbarkar *et al.*, 2015; Kaludercic *et al.*, 2014; Manni *et al.*, 2016; Youdim & Bakhle, 2006)., MAO-A activity was found to be increased in types of cancer, and MAO-A inhibitors have thus been investigated as potential treatment of prostate cancer (Flamand *et al.*, 2010).

Based on their pharmacological roles and clinical applications, MAO inhibitors remain of interest and the discovery of MAO inhibitors are pursued in both the industry and academia (Carradori & Silvestri, 2015; Carradori & Petzer, 2015). Natural products have often been considered as leads for the discovery of novel MAO inhibitors. Numerous natural products were found to act as good potency MAO inhibitors among which kaempferol, curcumin and chrysin are recent examples (Fig. 3.4). These compounds exhibit IC₅₀ values for the inhibition of the MAO-A of 0.525, 5.02 and 0.77 μ M, respectively (Gidaro *et al.*, 2016; Khatri & Juvekar, 2016; Xu *et al.*, 2005; Engelbrecht *et al.*, 2018). While kaempferol does not inhibit MAO-B (up to tested concentration of 100 μ M), curcumin and chrysin exhibit IC₅₀ values of 2.56 and 0.79 μ M. Harmine (IC₅₀ = 0.0029 μ M), another natural compound, represents a highly potent and specific MAO-A inhibitor, and is of particular interest since the X-ray crystal structure of harmine bound human MAO-A has been reported (Son *et al.*, 2008; Carradori *et al.*, 2016). Isatin, another natural compound, is a non-specific MAO inhibitor with IC₅₀ values of 31.8 and 12.4 μ M for the inhibition of human MAO-A and MAO-B, respectively (Manley-King *et al.*, 2011). The X-ray crystal structure of isatin complexed to human MAO-B has also been reported (Binda *et al.*, 2003). Structures of MAO-inhibitor complexes has contributed vastly to our understanding of the architecture of the active sites of the MAOs and the potential interactions that substrates and inhibitors may undergo with the MAO enzyme. This has, in turn, facilitated the discovery of numerous new MAO inhibitors. Based on these considerations, the present study evaluated a series of 14 natural products as potential inhibitors of human MAO-A and MAO-B. The aim of this study was to discover natural products that may serve as future leads for the design of MAO

inhibitors. Natural products are particularly attractive as lead compounds for the design of new MAO inhibitors for depression and Parkinson's disease since many natural compounds also possess additional pharmacological activities that may be relevant to these disorders. Since oxidative damage is implicated in the neurobiology of both depression and Parkinson's disease, the antioxidant activities displayed by many natural products may be beneficial by enhancing the potential neuroprotective actions of MAO inhibitors as discussed above (Kelsey *et al.*, 2010).

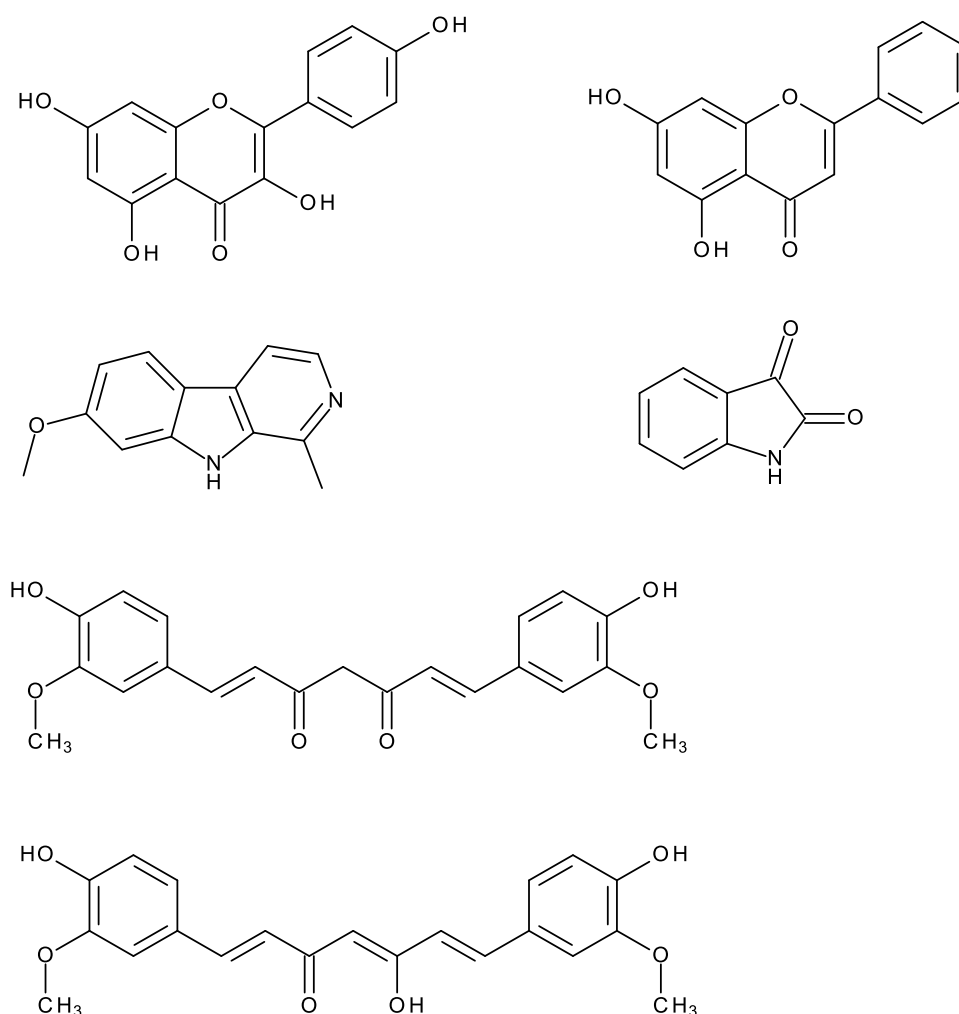


Figure 3.4. The structures of selected natural compounds with MAO inhibition activities. Kaempferol, chrysin, harmine, isatin, curcumin (keto-form), curcumin (enol-form) (Engelbrecht *et al.*, 2018). Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

As will be shown in the results section of this study, DL-kavain was found to be the most potent MAO inhibitor among the natural compounds evaluated with IC₅₀ values of 19.0 and 5.43 μ M for the inhibition of human MAO-A and MAO-B, respectively (Fig. 3.5). DL-Kavain is a major constituent of *Piper methysticum* Forst. f. (Fam. Piperaceae) a pepper plant called kava (Lebot *et al.*, 1997; Cairney *et al.*, 2002). The root of the kava plant is a cultural beverage prepared in water or coconut milk and is used for religious, social and medicinal purposes by South Pacific island inhabitants. The recreational use of kava is associated with a calming effect and kava extracts have subsequently been evaluated in clinical trials for the treatment of anxiety disorder (Sarris *et al.*, 2009; Sarris *et al.*, 2013a; Sarris *et al.*, 2013b; Savage *et al.*, 2015). In some western societies, kava is used to relieve stress-induced anxiety and insomnia. Other pharmacological effects of extracts of the kava plant include anti-inflammatory and analgesic properties (Wu *et al.*, 2002; Jamieson & Duffield, 1990). The constituents of kava exert neurological effects, in part, due to their lipophilic profile which facilitates entry into the central nervous system (Keledjian *et al.*, 1988). The major constituents are subdivided into three groups, kavalactones, chalcones and conjugated diene ketones. The kavalactones (also known as kavapyrones) include (+)-kavain, (+)-7,8-dihydrokavain, (+)-methysticin, (+)-7,8-dihydromethysticin, yangonin, desmethoxyyangonin and (+)-5,6,7,8-tetrahydroyangonin as the most abundant kavalactones (Smith *et al.*, 2004). The kavalactones undergo four degradation processes which are hydroxylation of the lactone ring and the aromatic ring at C-12, demethylation of the 4-methoxyl group, 7,8-double bond reduction and dehydration. The molecular targets of the kavalactones include histamine receptors, voltage gated channels (Na⁺ and Ca²⁺), opioid receptors, the dopamine type-2 receptor and γ -aminobutyric acid type A receptors (Dinh *et al.*, 2001; Jussofie *et al.*, 1994; Chua *et al.*, 2016; Friese & Gleitz, 1998; Schirmacher *et al.*, 1999). A literature survey reveals these kavalactones were found to act as inhibitors of human platelet MAO-B (Uebelhack *et al.*, 1998). The inhibition of MAO-A by kavalactones have, however, not been reported. This study therefore investigates the human MAO-A inhibition properties of the kavalactones that are most abundant in the kava plant, and examines the reversibility and mode of MAO inhibition by DL-kavain and yangonin. Curcumin was included in this study as reference MAO inhibitor (Khatri & Juvekar, 2016). The interaction of DL-kavain and yangonin on the molecular level will be investigated by molecular docking and dynamics simulations. The potential

MAO-A inhibition properties of kavalactones may be particularly relevant in defining the mechanism by which kava extracts exert an anxiolytic effect.

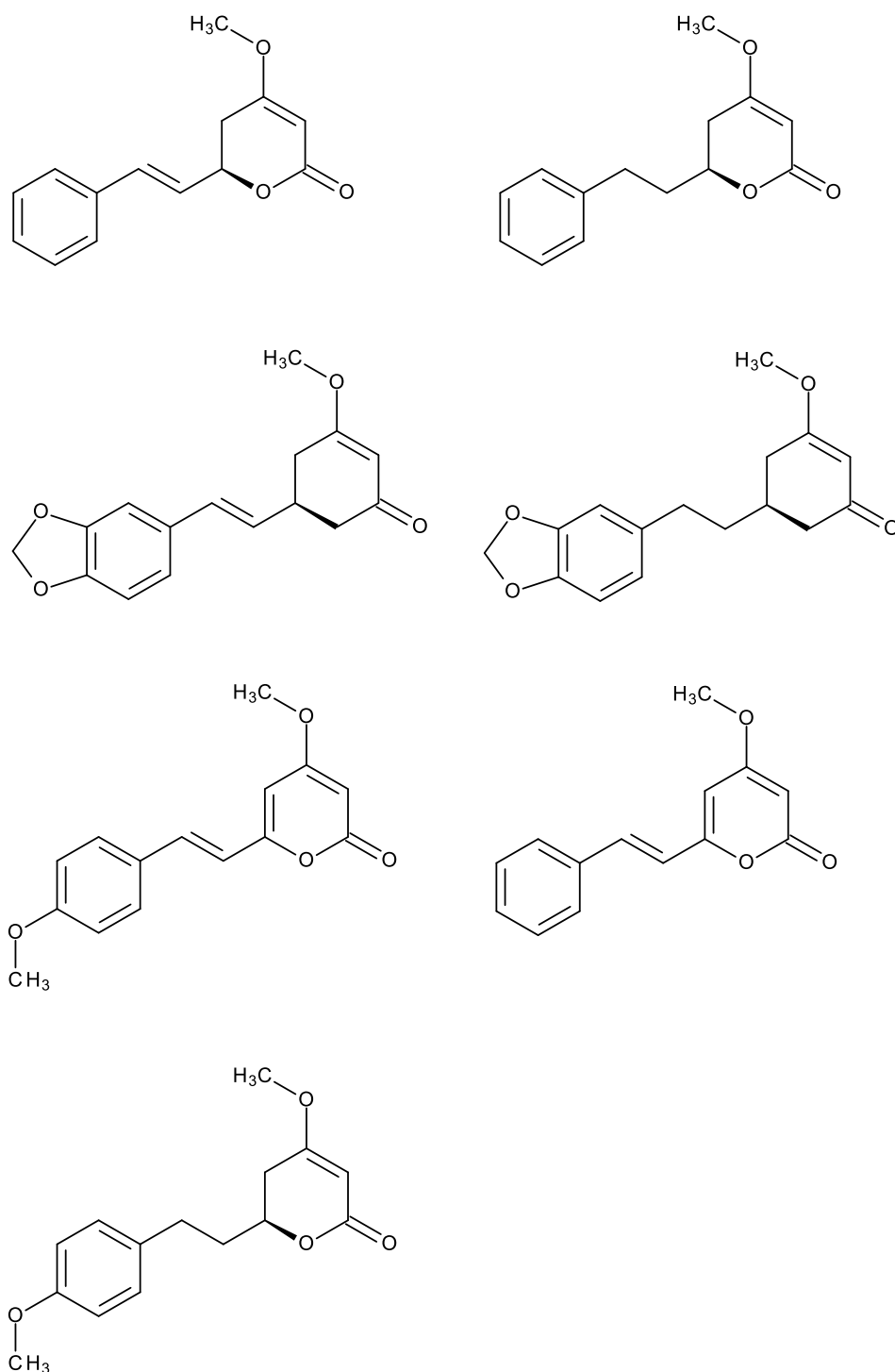


Figure 3.5. The structures of kavalactones from *Piper methysticum*. (+)-Kavain, (+)-7,8-Dihydrokavain, (+)-Methysticin, (+)-Dihydromethysticin, Yangonin, Desmethoxyyangonin, (+)-5,6,7,8-Tetrahydroyangonin. Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

2. Results and discussion

2.1. IC_{50} values

The present study evaluated a series of 14 natural products as potential inhibitors of human MAO-A and MAO-B. The aim of this study was to discover natural products that may serve as future leads for the design of MAO inhibitors. The natural products that were selected for this study are listed in [table 3.1](#). The inhibition studies were carried out with recombinant human MAO as enzyme sources and kynuramine served as the non-selective substrate ([Novaroli et al., 2005](#)). Kynuramine is oxidised by the MAO enzymes to yield the intermediate iminium which is converted to the corresponding aldehyde intermediate [3-(2-aminophenyl)-3-oxo-propionaldehyde] after hydrolysis ([Fig. 3.6](#)). Ring closure yields 4-hydroxyquinoline, a metabolite that fluoresces in basic media. By measuring the fluorescence of 4-hydroxyquinoline after the enzymatic reactions have been terminated at end-point (20 min) with sodium hydroxide (2 M NaOH), the formation of 4-hydroxyquinoline could be quantitated and the rate of MAO catalysis calculated ([Mostert et al., 2015](#)). After the measurement of the catalytic rates of MAO-A and MAO-B in the absence and presence (0.003–100 μ M) of the test inhibitors, the rate data were fitted to the one site competition model incorporated into the Prism 5 software package (GraphPad). IC_{50} values were estimated from the resulting sigmoidal curves of catalytic rate versus the logarithm of inhibitor concentration ($\log[I]$) and were expressed as the means $\pm$ standard deviation (SD) of triplicate determinations. Examples of sigmoidal curves thus obtained are given in [fig 3.7](#). For this study, the natural product and known MAO inhibitor, curcumin, served as reference inhibitor ([Khatri & Juvekar, 2016](#)).

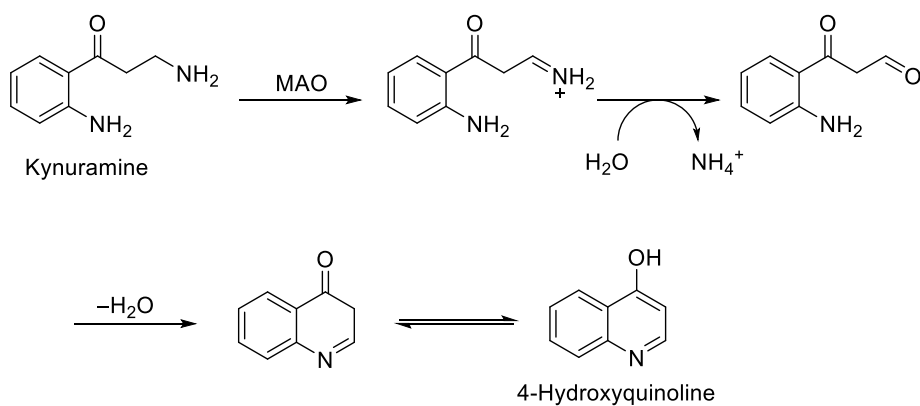


Figure 3.6. The oxidation of kynuramine to yield 4-hydroxyquinoline. Dassault Systèmes BIOVIA, BIOVIA Draw, Version 17.2.NET(64bit), San Diego: Dassault Systèmes, 2016.

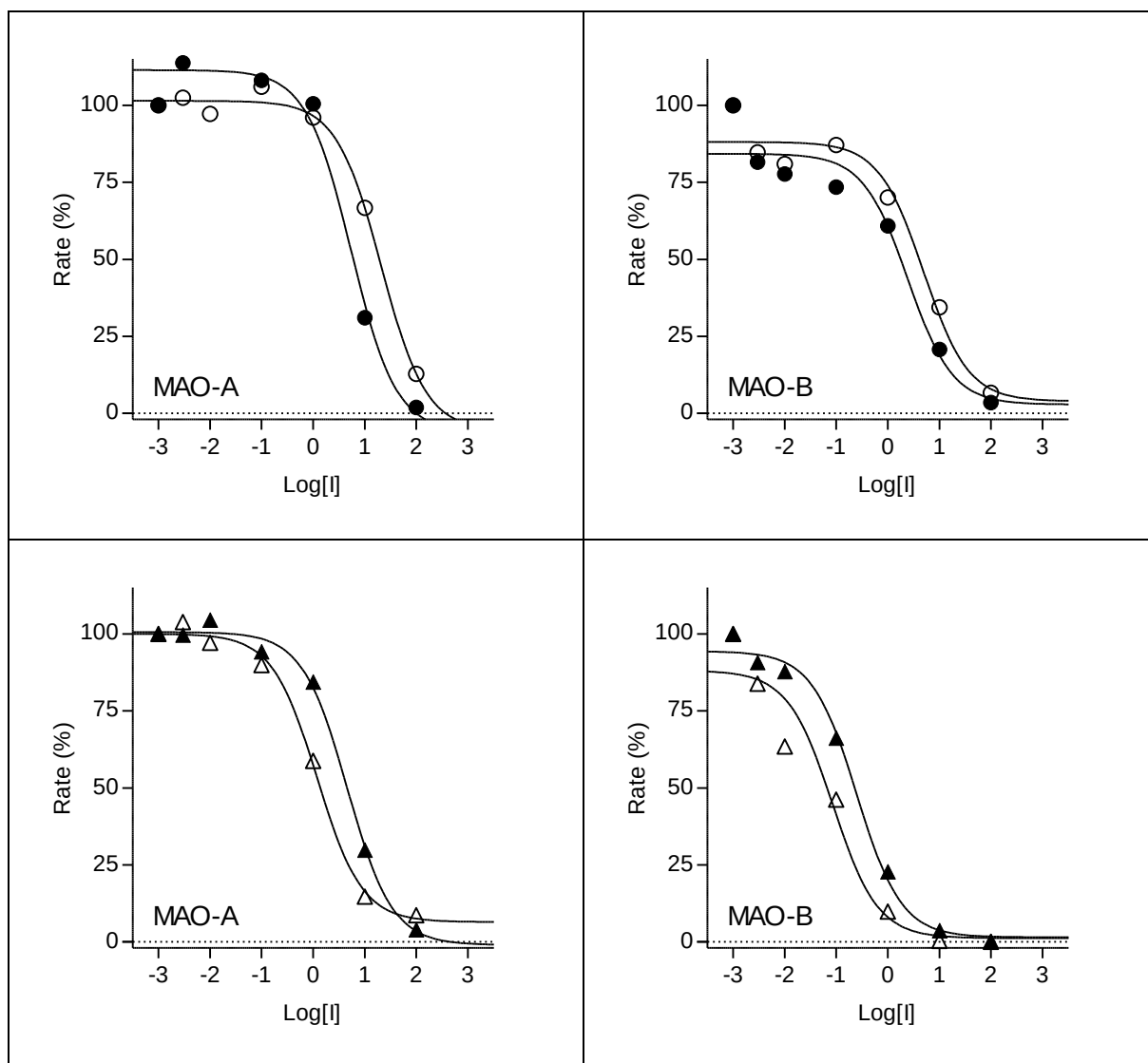


Figure 3.7. Sigmoidal plots for the inhibition of MAO-A (left) and MAO-B (right) by kavain (open circles), curcumin (filled circles), yangonin (open triangles) and desmethoxyyangonin (filled triangles). Each data point represents a mean $\pm$ SD of triplicate determinations.

The results of the MAO inhibition studies are given in [table 3.1](#) and show that tanshinone I, myrtenol, ellagic acid and kavain are active MAO inhibitors among the natural products that were tested. Among these, kavain is a noteworthy inhibitor of MAO-B with an IC_{50} value of 5.43 μ M. This inhibition potency is within the same range as that of the reference MAO inhibitor curcumin, which exhibits IC_{50} values of 5.02 and 2.56 μ M for the inhibition of MAO-A and MAO-B, respectively. A literature survey shows that ellagic acid and kavain are known to inhibit MAO-B ([Khatri & Juvekar, 2016](#); [Uebelhack et al., 1998](#)). The MAO-B inhibition properties of kavain as well as other kavalactones from the kava plant (dihydrokavain, methysticin, dihydromethysticin, yangonin and desmethoxyyangonin) were thus reported and the literature IC_{50} values are given in [table 3.2](#). The inhibition of MAO-A by kavain and kavalactones have, however, not been reported. As shown in [table 3.1](#), kavain inhibits MAO-A with an IC_{50} of 19.0 μ M. As mentioned in the introduction, the potential MAO-A inhibition properties of kavalactones may be particularly relevant in defining the mechanism by which kava extracts exert an anxiolytic effect. This study therefore set out to determine the MAO-A inhibition properties of other kavalactones that are present in kava. The results are given in [table 3.2](#) and show that some of the kavalactones are indeed inhibitors of MAO-A with yangonin ($IC_{50} = 1.29 \mu$ M) and desmethoxyyangonin ($IC_{50} = 4.44 \mu$ M) acting as the most potent inhibitors. These inhibition potencies are in the same range as that recorded for the reference MAO-A inhibitor, toloxatone. It is interesting to note that yangonin and desmethoxyyangonin are the only planar compounds among the kavalactones (e.g. possessing the aromatic 2-pyrone), which suggest that these compounds fit and interact better in the MAO-A active site. This observation may explain their good MAO-A inhibition potencies. The MAO-B inhibition potencies recorded for the kavalactones are significantly better than those reported ([Uebelhack et al., 1998](#)). Yangonin, in particular is a high potency MAO-B inhibitor ($IC_{50} = 0.086 \mu$ M). In fact, four of the six kavalactones inhibit MAO-B with IC_{50} values in the submicromolar range.

2.2. Reversibility of MAO inhibition

Reversibility of MAO inhibition, particularly of the MAO-A isoform, is an important factor of MAO inhibitors to consider. Irreversible MAO-A inhibitors are associated with the “cheese reaction”, which is a potentially fatal increase in blood pressure that may occur when tyramine-containing foods are taken with irreversible MAO-A

inhibitors (Da Prada *et al.*, 1988; Flockhart, 2012). Reversible MAO-A inhibitors and MAO-B inhibitors do not cause the cheese reaction (Youdim & Bakhle, 2006; Da Prada *et al.*, 1988). The reversibility of MAO-A and MAO-B inhibition was investigated for kavain, yangonin and curcumin, the latter serving as reference inhibitor. For this purpose, dialysis experiments were carried out.

MAO-A or MAO-B and the test inhibitors (kavain, yangonin and curcumin) were firstly pre-incubated for 15 min at 37 °C with the inhibitor concentration set to $4 \times IC_{50}$. These pre-incubations were subsequently placed in dialysis cassettes and dialysed for 24 h at 4 °C. Following dialysis, the incubation mixtures were diluted twofold with the addition of kynuramine (50 μ M) to yield an inhibitor concentration of $2 \times IC_{50}$. These reactions were incubated for 20 min and the residual MAO-A and MAO-B activities were determined by measuring the formation of 4-hydroxyquinoline by fluorescence spectrophotometry (as discussed above and in the experimental section). For comparison, the residual MAO-A and MAO-B activities in undialysed pre-incubations of the enzyme and test inhibitors were also measured. As negative and positive controls, respectively, similar dialysis experiments were carried out in the absence of test inhibitor and presence of the irreversible MAO-A and MAO-B inhibitors, pargyline and selegiline, respectively

The results of the dialysis experiments are given in **figs. 3.8 and 3.9**, and show that kavain, yangonin and curcumin are reversible inhibitors of MAO-A. After dialysis, MAO-A activity is recovered to 110%, 99% and 83% compared to the negative control value (100%) for kavain, yangonin and curcumin, respectively. As anticipated, inhibition of MAO-A persists in undialysed pre-incubations with the residual activities at 26%, 46% and 30%, respectively. For the positive control pargyline, dialysis does not restore catalytic activity with the residual activity at 1.2–2.2%. It may be concluded that kavain, yangonin and curcumin are reversible inhibitors of MAO-A.

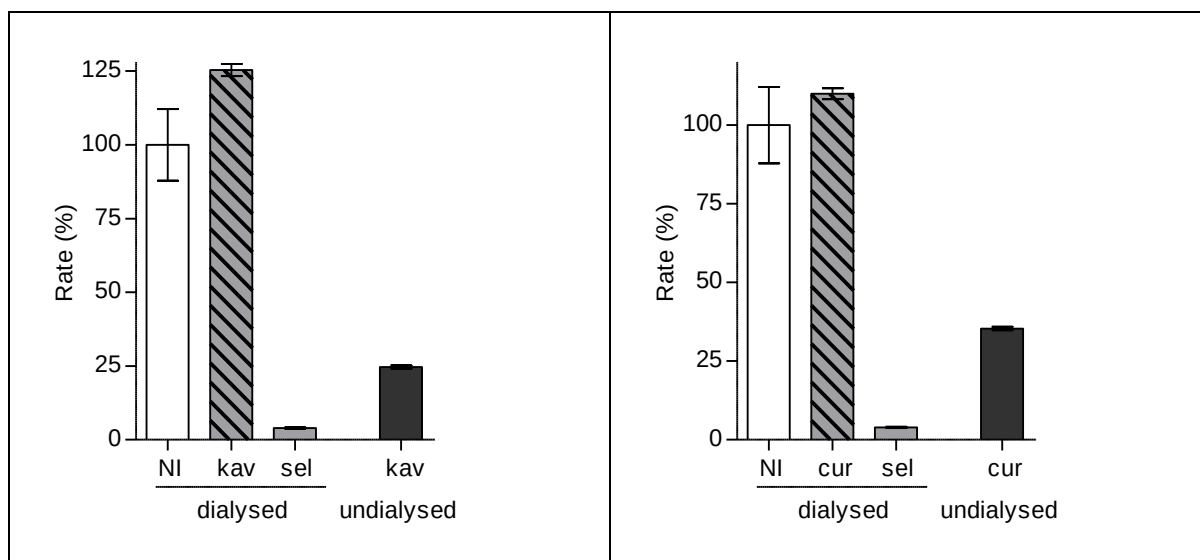


Figure 3.8. Reversibility of MAO-A and MAO-B inhibition by kavain and curcumin. The MAO enzymes and the test inhibitors (at a concentration of $4 \times IC_{50}$) were incubated for 15 min, dialysed for 24 h and the residual enzyme activities were measured (kav/cur dialysed). Similar incubation and dialysis of the enzyme in the absence (NI dialysed) and presence of the irreversible inhibitors, pargyline and selegiline (parg/sel dialysed), were also carried out. The residual activities of undialysed mixtures of the MAO enzyme and the test inhibitors were also recorded (kav/cur undialysed).

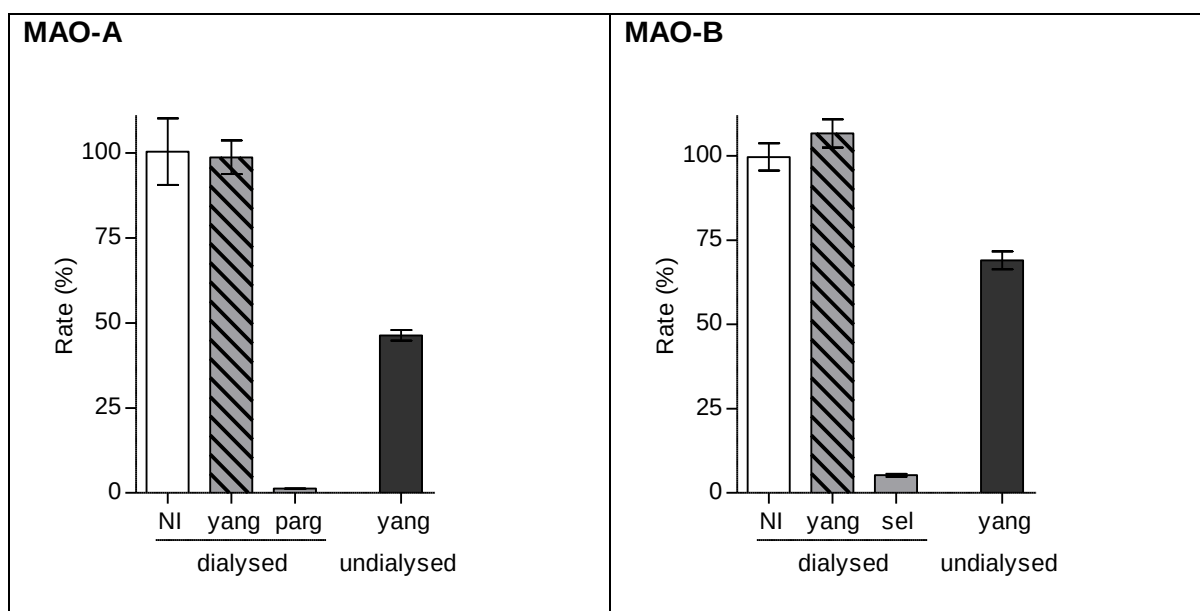


Figure 3.9. Reversibility of MAO-A and MAO-B inhibition by yangonin. The MAO enzymes and the test inhibitor (at a concentration of $4 \times IC_{50}$) were incubated for 15 min, dialysed for 24 h and the residual enzyme activities were measured (yang dialysed). Similar incubation and dialysis of the enzyme in the absence (NI dialysed) and presence of the irreversible

inhibitors, pargyline and selegiline (parg/sel dialysed), were also carried out. The residual activities of undialysed mixtures of the MAO enzyme and the test inhibitor were also recorded (yang undialysed).

Kavain, yangonin and curcumin were also found to be reversible inhibitors of MAO-B. After dialysis, MAO-B activity is restored to 125%, 106% and 110% compared to the negative control for kavain, yangonin and curcumin, respectively. In contrast, after pre-incubation of MAO-B with selegiline, dialysis does not restore catalytic activity with the residual activity at 3.9–5.2%. Inhibition of MAO-B by kavain, yangonin and curcumin also persists in undialysed pre-incubations with the residual activities at 25%, 69% and 35%, respectively. It may be concluded that kavain, yangonin and curcumin are also reversible inhibitors of MAO-B.

2.3. Mode of MAO inhibition

To determine whether kavain, yangonin and the reference inhibitor, curcumin, are competitive inhibitors of MAO-A, sets of Lineweaver-Burk plots were constructed (Figs. 3.10 and 3.12). For each plot, MAO-A catalytic rates were recorded at 8 different kynuramine concentrations (15–250 μM). Five plots were constructed with the first constructed in the absence of inhibitor, and the remaining five at five different concentrations ($\frac{1}{4} \times \text{IC}_{50}$, $\frac{1}{2} \times \text{IC}_{50}$, $\frac{3}{4} \times \text{IC}_{50}$, $1 \times \text{IC}_{50}$ and $1\frac{1}{4} \times \text{IC}_{50}$) of kavain, yangonin and curcumin. The Lineweaver-Burk plots for the inhibition of MAO-A by kavain, yangonin and curcumin were found to be linear and to intersect on the y-axis. This suggests that the test inhibitors are competitive inhibitors of MAO-A, and further supports the findings of the dialysis study which concluded that these inhibitors are reversible MAO-A inhibitors. From replots of the slopes of the Lineweaver-Burk plots versus inhibitor concentration, K_i values ($K_i = -x$ when $y = 0$) of 7.72, 1.12 and 3.08 μM are estimated for kavain, yangonin and curcumin, respectively.

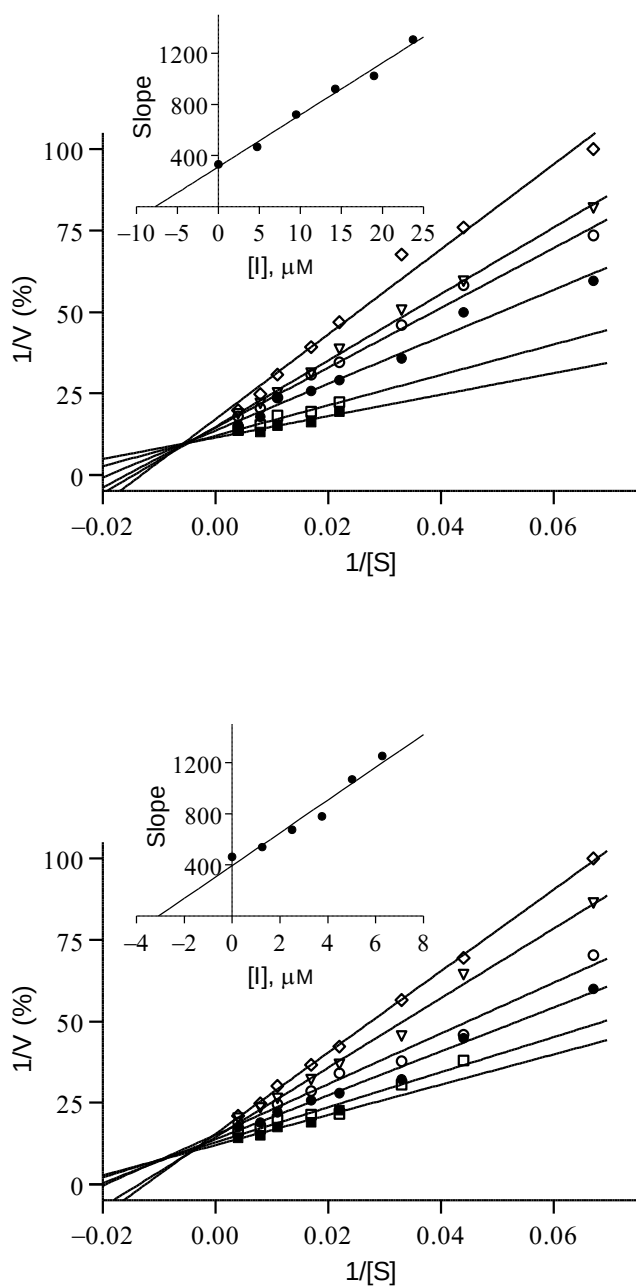


Figure 3.10. Lineweaver-Burk plots for the inhibition of MAO-A by kavain (top) and curcumin (bottom). Plots were constructed in the absence of inhibitors (filled squares) and presence of various concentrations of the test inhibitors ($1/4 \times IC_{50}$, $1/2 \times IC_{50}$, $3/4 \times IC_{50}$, $1 \times IC_{50}$ and $1.5 \times IC_{50}$). The insets are graphs of the slopes of the Lineweaver-Burk plots versus inhibitor concentration. From these replots, K_i values of 7.72 and 3.08 μM are estimated for kavain and curcumin, respectively.

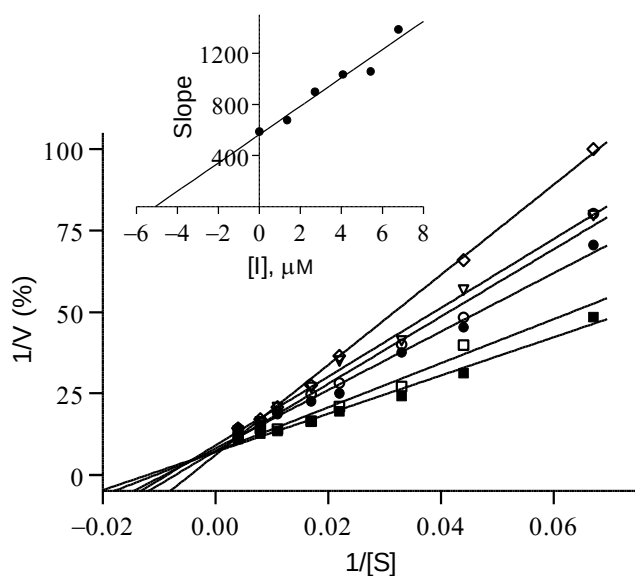
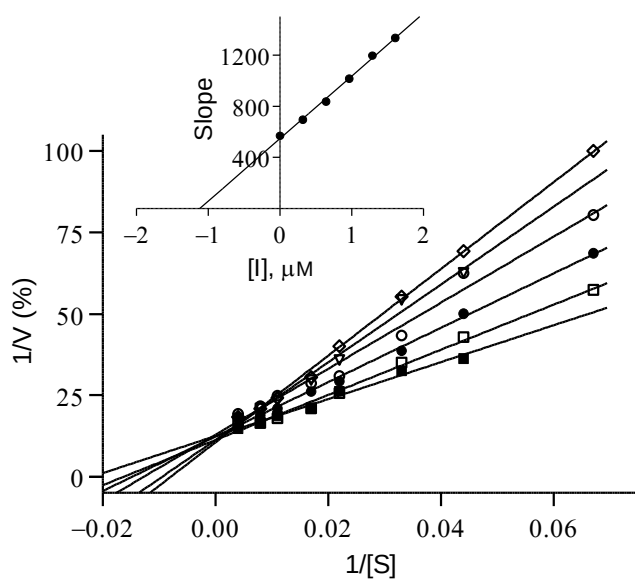


Figure 3.11. Lineweaver-Burk plots for the inhibition of MAO-B by kavain. Plots were constructed in the absence of inhibitor (filled squares) and presence of various concentrations of kavain ($\frac{1}{4} \times IC_{50}$, $\frac{1}{2} \times IC_{50}$, $\frac{3}{4} \times IC_{50}$, $1 \times IC_{50}$ and $1\frac{1}{4} \times IC_{50}$). The insert is a graph of the slopes of the Lineweaver-Burk plots versus inhibitor concentration. From this replot, a K_i value of 5.10 μM is estimated for kavain.



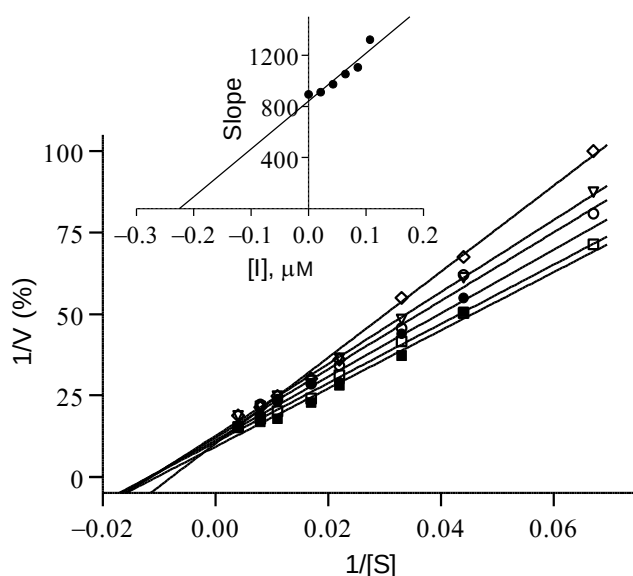


Figure 3.12. Lineweaver-Burk plots for the inhibition of MAO-A (top) and MAO-B (bottom) by yangonin. Plots were constructed in the absence of inhibitor (filled squares) and presence of various concentrations of the test inhibitor ($\frac{1}{4} \times \text{IC}_{50}$, $\frac{1}{2} \times \text{IC}_{50}$, $\frac{3}{4} \times \text{IC}_{50}$, $1 \times \text{IC}_{50}$ and $1\frac{1}{4} \times \text{IC}_{50}$). The insets are graphs of the slopes of the Lineweaver-Burk plots versus inhibitor concentration. From these replots, K_i values of 1.12 and 2.26 μM are estimated for the inhibition of MAO-A and MAO-B, respectively.

A similar set of Lineweaver-Burk plots were constructed for the inhibition of MAO-B by kavain and yangonin. These plots are given in figs. 3.11 and 3.12 and show that kavain and yangonin also are competitive inhibitors of MAO-B with a K_i values of 5.10 and 2.26 μM . This finding is in accordance to the literature report on the inhibition of human platelet MAO-B by kavain and other kavalactones.

2.4. Molecular modelling – validation of protocol

This study shows that kavain acts as an inhibitor for both MAO-A and MAO-B. To provide additional insight, the binding modes of kavain in crystal structure models of human MAO-A and MAO-B were examined using molecular docking. Curcumin, also an inhibitor of both MAO isoforms, was included in this study for comparison. Based on the knowledge of the active site structures of MAO-A and MAO-B, it may be expected that the 2-pyrone moiety of kavain would bind in the polar regions of the MAO active sites, the region in proximity to the FAD. The styryl side chain would

project towards the entrance of the active site, where stabilisation of the inhibitor would mostly be via Van der Waals interactions. This binding mode has been demonstrated for a number of ligands bound to MAO-B including safinamide, *trans,trans*-farnesol and pioglitazone (Carradori & Silvestri, 2015).

Molecular docking was carried out with the Discovery Studio 3.1 modelling software (Accelrys). The structures of human MAO-A co-crystallised with harmine (PDB entry: 2Z5X) (Son *et al.*, 2008) and human MAO-B co-crystallised with safinamide (PDB entry: 2V5Z) (Binda *et al.*, 2007) were selected and molecular docking was carried out according to a modification of a previously reported protocol with the CDOCKER algorithm application of the Discovery Studio. These models were selected based on the high resolution of the crystallographic structures. After calculation of the pKa values and protonation of the enzyme models, they were energy minimised with the protein backbone constrained. The structures of the ligands were drawn in Discovery Studio, and after docking with CDOCKER, the docking orientations were refined using *in situ* ligand minimisation. For each ligand, the highest ranked orientation was selected among the ten solutions generated.

The accuracy of the docking procedure was evaluated by redocking the co-crystallised ligands, harmine and safinamide, into the active sites of MAO-A and MAO-B, respectively. The root-mean-square deviation (RMSD) of the docked orientation from the position of the co-crystallised ligand was subsequently calculated. In addition, various other co-crystallised ligands were also docked into the binding site of MAO-B in complex with safinamide. After the crystal structures were superimposed, the RMSD from the original orientation was measured. The RMSD values are given in table 3.3. The best ranked orientations of harmine and safinamide exhibited RMSD values of 0.257 Å and 0.558 Å, respectively from the position of the co-crystallised ligand (Figs. 3.13 and 3.14). In a second scenario, the crystal water was removed from the models with the exception for HOH 710, 718 and 739 in MAO-A, and HOH 1155, 1170 and 1351 in the A-chain of MAO-B. These waters are active site waters and are conserved and non-displaceable. With crystal waters removed, harmine and safinamide exhibited RMSD values of 2.83 Å and 0.548 Å, respectively. The binding position of safinamide can be predicted when the waters are present as well as absent, the orientation of harmine is not well predicted

when the crystal waters are removed from MAO-A. For the other co-crystallised ligands which were also docked into the binding site of MAO-B in complex with safinamide, RMSD values ranged from 0.8 to 3.68 Å, with similar RMSD values recorded for the simulations carried out in the presence and absence of the water molecules. The only exception was for *trans,trans*-farnesol, which exhibited a reversed orientation (RMSD = 8.56 Å) when the waters were retained. Based on these results it may be concluded that the selected protocol is suitable for the docking of inhibitors into the active sites of MAO-A and MAO-B, and that more accurate results may be obtained by retaining the crystal waters for MAO-A, while removing the waters for simulations with MAO-B. This study, however, examines the binding modes of kavain and curcumin with both scenarios.

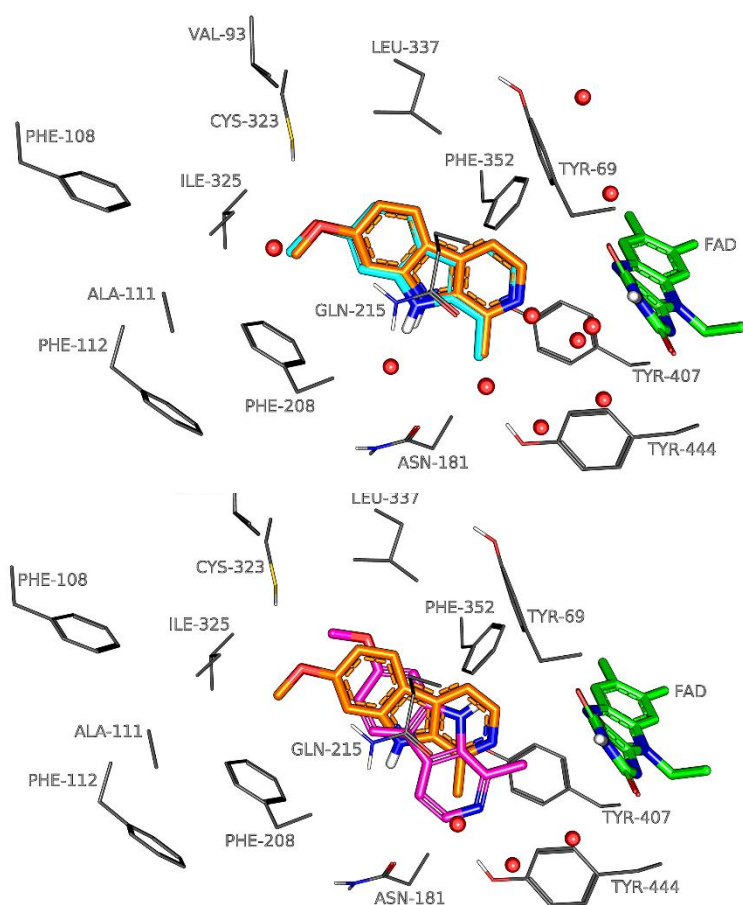


Figure 3.13. The docked binding orientation of harmine in MAO-A compared to the orientation of harmine (orange) in the X-ray crystal structure (2Z5X). Docking was carried out with all crystal waters present (top panel, structure in cyan) and with only three conserved active site waters present (bottom panel, structure in magenta).

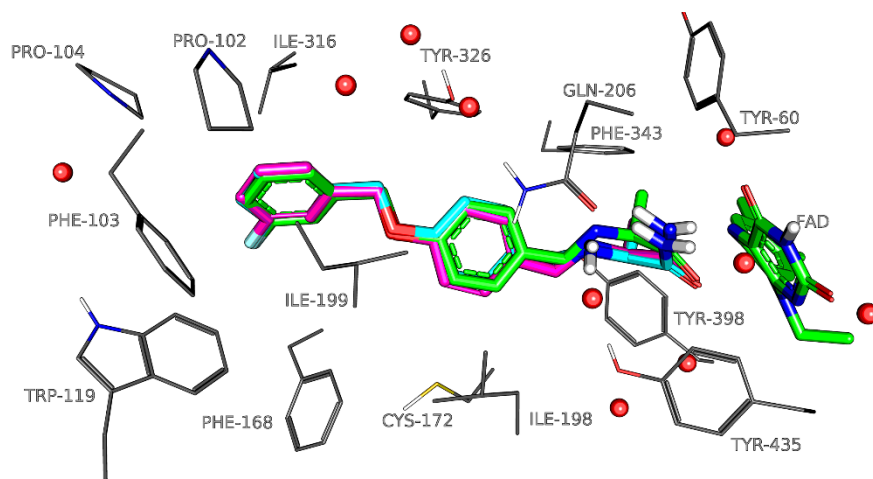


Figure 3.14. The docked binding orientation of safinamide in MAO-B compared to the orientation of safinamide (green) in the X-ray crystal structure (2V5Z). Docking was carried out with all crystal waters present (cyan) and with only three conserved active site waters present (magenta).

2.5. Molecular docking – kavain and curcumin

2.5.1. Docking with crystal waters retained

Both enantiomers of kavain were considered while both the keto and enol forms of curcumin were also included in the docking study. CDOCKER was unable to dock curcumin (both keto and enol forms) into MAO-A, even though curcumin acts as an *in vitro* MAO-A inhibitor. Considering that larger inhibitors are normally not expected to bind to MAO-A, presumably because of the restriction imposed by Phe-208, the binding mode of curcumin to MAO-A would either involve an allosteric site or the movement of active site residues that was not predicted by CDOCKER. The Lineweaver-Burk plots constructed for the interaction of curcumin with MAO-A, however, suggests that this compound is a competitive inhibitor, and thus binds to the MAO-A active site. In contrast, both enantiomers of kavain docked into MAO-A (Fig. 3.15). The binding orientations of the enantiomers are highly similar, with the

pyrone carbonyl directed towards the top of the active site cavity, where it establishes a hydrogen bond with a water molecule. As predicted, the 2-pyrone moiety is placed in the polar region of the active site, in proximity to the FAD. An analysis of the interaction energies of the inhibitor with active site residues show that the most productive interaction of both kavain enantiomers occur with Gln-215 with energies of -4.08 and -4.68 kcal/mol, respectively (Table 3.4). However, (R)-kavain has a large steric conflict with several residues among which Phe-352 is most significant, which results in a positive total interaction energy (669 kcal/mol) with MAO-A and thus weak binding. Although (S)-kavain also has a steric conflict with Phe-352 (20.2 kcal/mol), it is much lower so that this compound still exhibits a negative interaction energy (-7.99 kcal/mol) with MAO-A. Based on this analysis it may be postulated that (S)-kavain is a more potent MAO-A inhibitor compared to the (R)-enantiomer, and thus responsible for the observed inhibition.

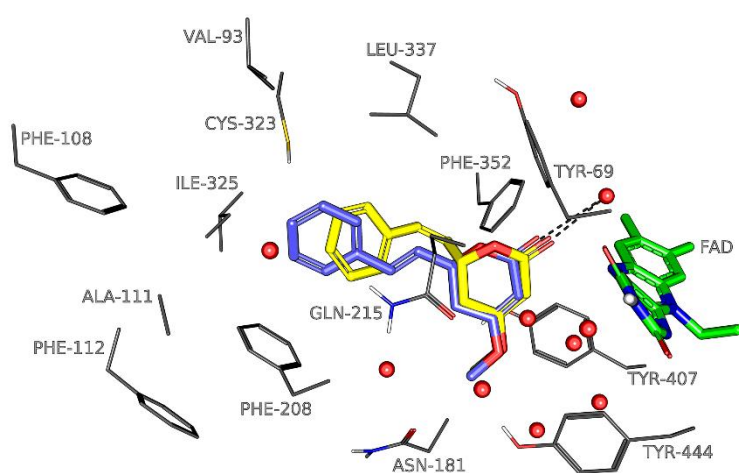


Figure 3.15. The docked binding orientations of (R)-kavain (yellow) and (S)-kavain (purple) in MAO-A. Docking was carried out with all crystal waters present.

In MAO-B, the 2-pyrone moiety of kavain is also placed in proximity to the FAD, but the two enantiomers exhibit differing orientations with respect to the binding of the 2-pyrone (Fig. 3.16). For (R)-kavain, the carbonyl is directed towards the top of the active site, while for (S)-kavain, the carbonyl is directed to the bottom of the active site cavity. The placement of the styryl side chains of the two enantiomers in the entrance cavity is virtually superimposable. For curcumin, the keto and enol forms bind with very similar orientations with both orientations forming π - π interactions with Tyr-398 (Fig. 3.17). The enol hydroxyl group establishes a close polar contact (1.6 Å) with Cys-172. Of significance is the placement of the inhibitor deep within the

entrance cavity, where stabilisation via Van der Waals interactions is possible. An analysis of the interaction energies of kavain with active site residues shows that both enantiomers has negative total interaction energies and thus are expected to bind to the MAO-B active site (Table 3.5). For both kavain enantiomers, a relatively small steric conflict occurs with Gln-206. The most productive interaction occurs with Ile-199 the gating residue in MAO-B. Based on this analysis it may be expected that both (R)- and (S)-kavain contribute to the observed inhibition of MAO-B. A similar analysis of interaction energies for curcumin shows positive total interaction energies and suggests that neither the keto- or enol-forms bind well in the MAO-B active site (Table 3.6). The enol-form in particular shows a high positive interaction energy (15585 kcal/mol) resulting from a large steric conflict with Leu-167. Leu-164, Ile-316 and an active site water also represents steric conflicts for the binding of curcumin to MAO-B. This analysis suggests that the keto-form has a higher affinity for MAO-B than the enol-form and may be the bioactive tautomer. Interestingly, curcumin forms a significant productive interaction with the FAD, presumably because it binds in very close proximity to the cofactor. The poor binding of curcumin to MAO-B is surprising considering that the experimental data shows that curcumin is a potent MAO-B inhibitor.

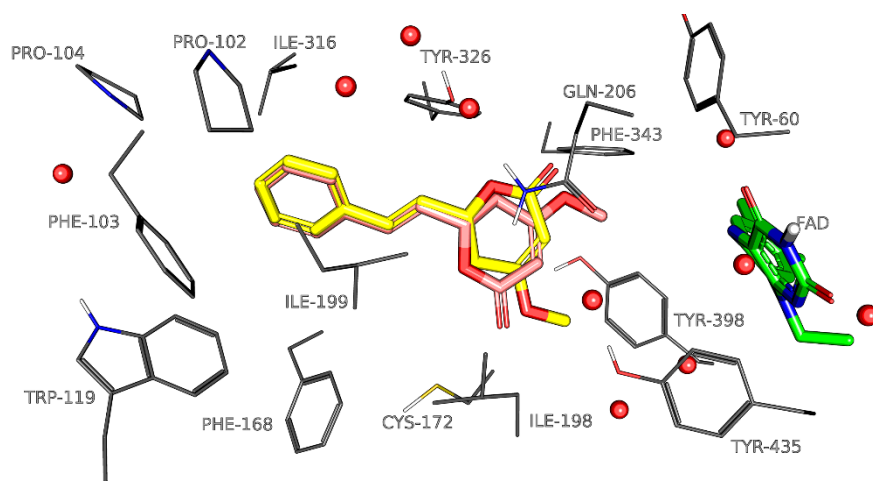


Figure 3.16. The docked binding orientations of (R)-kavain (yellow) and (S)-kavain (pink) in MAO-B. Docking was carried out with all crystal waters present.

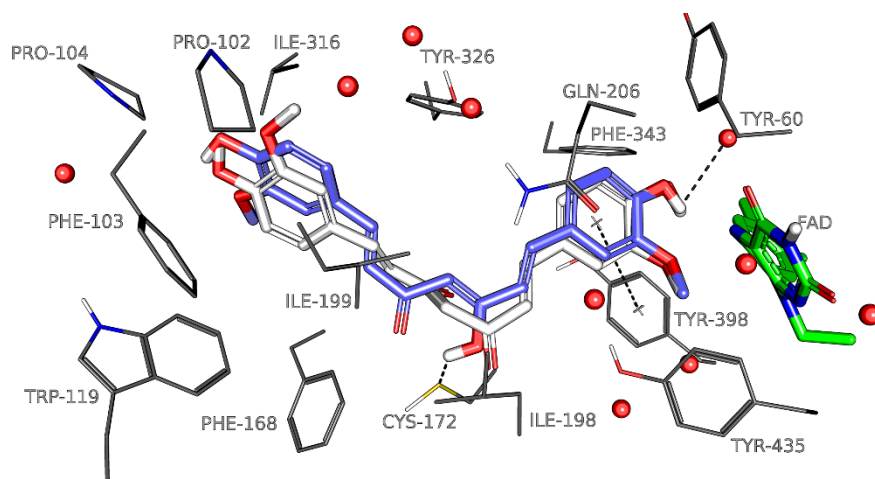


Figure 3.17. The docked binding orientations of curcumin (white) and the enol form of curcumin (purple) in MAO-B. Docking was carried out with all crystal waters present.

2.5.2. Docking with crystal waters removed

Similarly to the docking experiments conducted in the presence of all active site waters, CDOCKER was also unable to dock curcumin (both keto and enol forms) into MAO-A when only three conserved active site waters were retained., Kavain exhibited a reversed binding orientation in MAO-A compared to when the active site waters were retained. In this respect, the 2-pyrone moiety of kavain is directed towards the entrance of the active site (Fig. 3.18). The two enantiomers also bind differently with the 2-pyrone carbonyl of the (S)-kavain directed towards the bottom of the active site while the carbonyl of (R)-kavain is directed towards the top. The (S)-enantiomer also forms more polar contacts with MAO-A compared to the (R)-enantiomer and includes a hydrogen bond with Cys-323, a π - π interaction with Tyr-444 and a sigma- π interaction with Phe-208. An analysis of the interaction energies shows that the most productive interaction of both kavain enantiomers occur with Gln-215 with energies of -4.39 and -3.58 kcal/mol, respectively (Table 3.7). This was also observed when docking was carried out in the presence of all waters. The only significant steric conflict occurs between (S)-kavain and Ile-335. As mentioned, with the waters present, steric conflicts occur with Phe-352. For both kavain enantiomers, negative total interaction energies (-25.9 and -23.2 kcal/mol) with MAO-A were recorded which shows that these compounds fit better in the MAO-A active site when the waters are removed compared to when the waters are present. This analysis suggests that (R)- and (S)-kavain contribute equally to the MAO-A inhibition observed in the laboratory experiments.

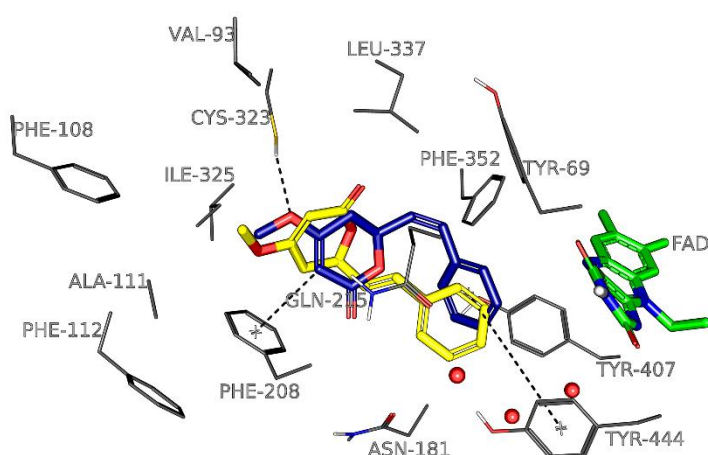


Figure 3.18. The docked binding orientations of (R)-kavain (yellow) and (S)-kavain (blue) in MAO-A. Docking was carried out with three conserved crystal waters present.

In MAO-B, the binding modes of (R)- and (S)-kavain are almost identical to that observed when the active site waters were retained (Fig. 3.19). In this respect, the carbonyl of (R)-kavain is directed towards the top of the active site, while for (S)-kavain, the carbonyl is directed to the bottom of the active site cavity. The styryl side chains of the two enantiomers are placed in the entrance cavity. An analysis of the interaction energies of kavain with active site residue are similar to that when the waters are present and shows that both enantiomers have negative total interaction energies and are expected to bind to the MAO-B active site (Table 3.8). For both kavain enantiomers, a relatively small steric conflict occurs with Gln-206. The most productive interaction occurs with Leu-171 and Ile-199. As concluded above, it may be expected that both (R)- and (S)-kavain contribute to the observed inhibition of MAO-B.

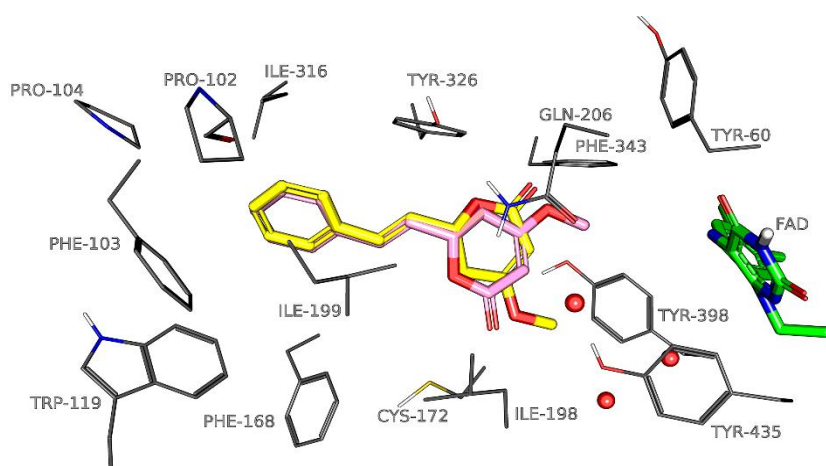


Figure 3.19. The docked binding orientations of (R)-kavain (yellow) and (S)-kavain (pink) in MAO-B. Docking was carried out with three conserved crystal waters present.

The binding of curcumin and its enol-form are also similar to those observed when the active site waters were retained (Fig. 3.20). The same polar interactions are observed, π - π interactions with Tyr-398, and a close polar contact (1.9 Å) of the enol hydroxyl with Cys-172. An additional contact (hydrogen bond) with Pro-102 is established a phenolic OH of both curcumin tautomers. An analysis of interaction energies for curcumin shows a negative total interaction energy (-4.00 kcal/mol) for the enol-form which suggests that the enol-form binds better in the MAO-B active site than the keto-form (58.7 kcal/mol) (Table 3.9). In fact, the keto-form of curcumin exhibits several steric conflicts with the most significant being with Ile-316 (50.8 kcal/mol) and Leu-328 (11.8 kcal/mol). The enol-form also has steric conflicts (with Cys-172 and Tyr-398). With the waters removed, the enol-form seems to be the bioactive tautomer since it exhibits a lower total interaction energy (-4.00 kcal/mol) than the keto-form (58.7 kcal/mol). This is in contrast to the finding of the docking study carried out in the presence of the water molecules and demonstrates that waters plays an important role in the placement of ligands in binding sites.

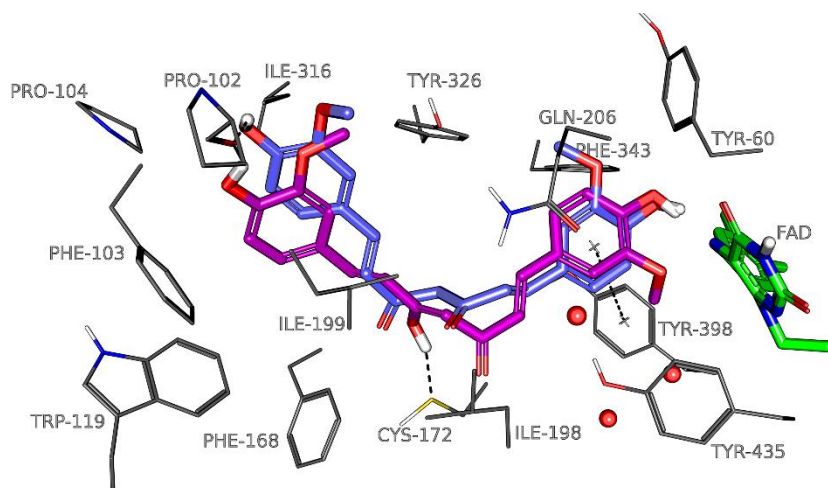


Figure 3.20. The docked binding orientations of curcumin (light blue) and the enol form of curcumin (purple) in MAO-B. Docking was carried out with three conserved crystal waters present.

2.6. Molecular dynamics simulation

The docking results predict that (R)- and (S)-kavian binds equally well in the MAO-B active site, although they exhibit differing binding modes. These docking results are

most likely representative of their true binding orientations since virtually identical results were obtained with the active site waters present and absent. For the binding of (R)- and (S)-kavain to MAO-A, less reliable results were obtained since differing results were obtained when the docking was carried out in the absence and presence of the active site waters. For this reason, the binding interactions of (R)- and (S)-kavain with MAO-B were further explored by molecular dynamics simulation. For this purpose, the highest ranked docked orientations of the inhibitors in MAO-B with three conserved crystal waters present were selected (Fig. 3.19). The structural integrity of these docking solutions were examined with short (500 ps) molecular dynamics simulations of the enzyme–inhibitor complexes. Frames of the production run were recorded at each 1 ps, to yield 500 frames.

An analysis of the trajectories of (R)- and (S)-kavain shows that their orientations and placement in the MAO-B active site are relatively stable with average RMSDs from the initial docked orientation of 0.51 ± 0.13 Å and 1.0 ± 0.24 Å, respectively. For (R)-kavain, hydrogen bonding occurs mostly between with Tyr-326 and the lactone oxygen of (R)-kavain and is present in 110 frames (of a total of 500 frames). Some hydrogen bonding between Tyr-326 and the carbonyl oxygen (4 frames) is also observed as well as hydrogen bonding between Cys-172 and the methoxy oxygen (11 frames). The methoxy also undergoes hydrogen bonding with an active site water (5 frames). Sigma– π and π – π interactions have also been recorded with Ile-199 and Tyr-326, respectively. From these results it may be concluded that (R)-kavain undergoes limited polar interactions with MAO-B, with Tyr-326 being the residue mostly involved. For (S)-kavain, hydrogen bonding mostly between with Cys-172 and the carbonyl oxygen of (S)-kavain and is present in 85 frames (of a total of 500 frames). Hydrogen bonding of the carbonyl with water molecules is also frequently observed (111 frames) while hydrogen bonding with Tyr-435 is seen in 2 frames. Sigma– π and π – π interactions with Tyr-326 is observed in 10 frames, while sigma– π interactions with Ile-316 and Ile-119 have also been recorded. As for (R)-kavain, it may be concluded that (S)-kavain undergoes limited polar interactions with MAO-B, but Cys-172 is the residue mostly involved. The moderate MAO-B inhibition potency of kavain may be explained by the relatively low degree of anchoring of the inhibitors by hydrogen bonding and π interactions. In agreement with this, hydrogen

bond interactions contribute only to 5.9% and 4.5% of the total interaction energy between (R)- and (S)-kavain, respectively, and the MAO-B active site.

The most prominent Van der Waals interactions of (R)- and (S)-kavian with MAO-B occur with Leu-171, Ile-199, Gln-206 and Tyr-326 (Table 3.10). These interactions contribute to 42.6 and 45.3% of the total interaction energy between (R)- and (S)-kavian, respectively, and the MAO-B active site. It is interesting to note that the average interaction energy recorded for Gln-206 is highly favourable (–3.34 and –3.55 kcal/mol), which contrasts with the finding of the docking study (Table 3.8). In fact, when considering the average interaction energies, no unfavourable interactions with active site residues were observed. This underscores the importance of molecular dynamics simulation, which to some degree considers the variations in interaction that may occur with movement of the ligand in the binding site. Based on this analysis it may be concluded that (R)- and (S)-kavian are stabilised to similar extents by Van der Waals interactions, and mostly interacts with the same residues in the MAO-B active site. Furthermore, no significant unfavourable interactions between the MAO-B active site and kavain exists.

Since yangonin was the most potent MAO-A and MAO-B inhibitor of this study, it was docked into the active sites of MAO-A and MAO-B under conditions where the three conserved active site waters were retained (Fig. 3.21). The binding interactions of yangonin with MAO-A and MAO-B were further explored by molecular dynamics simulation. In MAO-A, yangonin binds with the 2-pyrone moiety directed towards FAD, with the 2-pyrone carbonyl directed towards the top of the active site. Polar contacts include a hydrogen bond with Cys-323 and a π – π interaction with Tyr-407. In MAO-B, the binding mode differs, and the 2-pyrone moiety is placed the entrance cavity and a π – π interaction with Tyr-398 is formed.

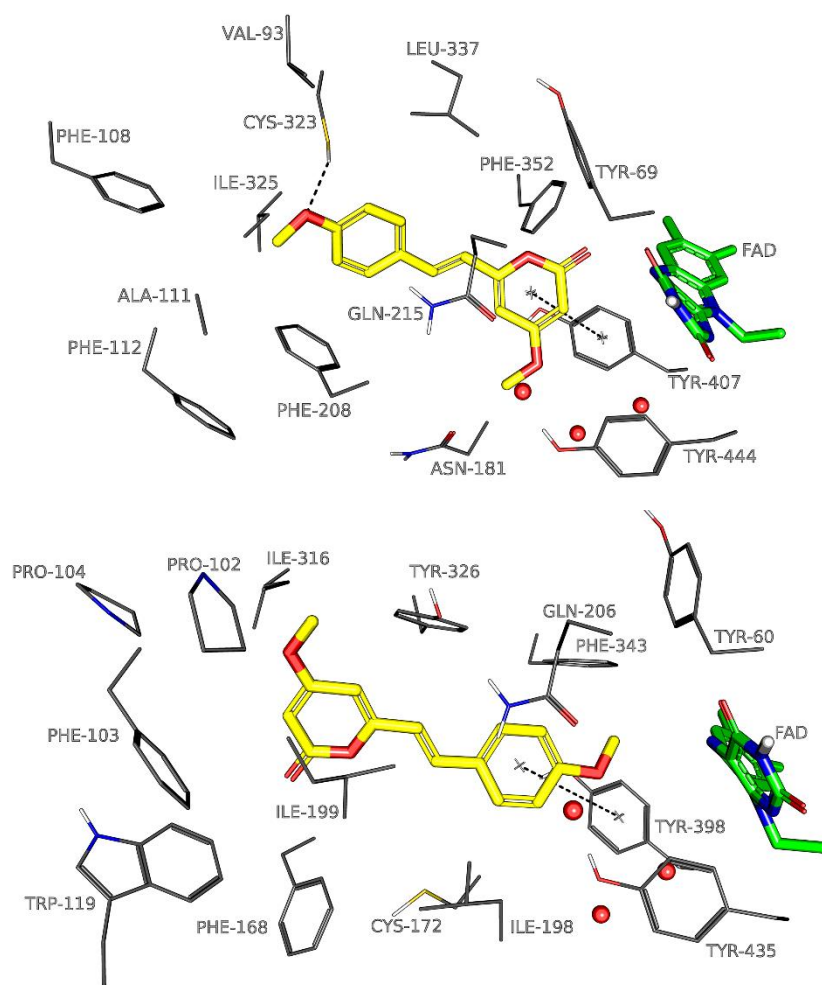


Figure 3.21. The docked binding orientations of yangonin in MAO-A and MAO-B. Docking was carried out with three conserved crystal waters present.

The results of the molecular dynamics simulation are given in [Table 3.11](#) and show that the most prominent Van der Waals interactions of yangonin with MAO-B occur with Ile-199, which contribute 13.9% of the total interaction energy. It is interesting to note that the same residues (Leu-171, Ile-199, Gln-206 and Tyr-326) are involved in the most productive interactions with both kavain and yangonin. In MAO-A, Gln-215 and Phe-208 exhibit the most significant Van der Waals interactions with yangonin. These residues contribute 18.9% of the total interaction energy. Ile-199 in MAO-B and Phe-208, the analogous residue in MAO-A, thus are important residues for inhibitor stabilisation.

The binding of yangonin is relatively stable in MAO-A and MAO-B with RMSDs from the initial docked orientation of 0.65 ± 0.16 Å and 0.98 ± 0.52 Å, respectively. In MAO-A hydrogen bonding occurs mostly with Cys-323 (123 frames) and Tyr-444 (89

frames). Hydrogen bonding with active site waters also occur (63 frames). A key interaction appears to be a π - π interaction with Tyr-407 which is present in 445 frames. π - π interactions with Tyr-444 is present in only 7 frames. Sigma- π interactions have also been recorded with Ile-335 (45 frames) and Phe-352 (22 frames). It may be concluded that the relatively good MAO-A inhibition observed with yangonin may be due to key stabilising interactions with Tyr-407 and Cys-323 at opposite ends of the active site cavity. These interactions are likely facilitated by the planar conformation of yangonin and are not possible for kavain, which is non-planar. In MAO-B, yangonin is mostly hydrogen bonded to water molecules (HOH1351, 213 frames; HOH1155, 13 frames; HOH1170, 4 frames). π - π interactions with Tyr-398 is present in 144 frames, while π - π interactions with Tyr-435 is present in 6 frames. Sigma- π have also been recorded with Ile-199 (59 frames), Leu-171 (2 frames), and interestingly with the FAD (3 frames).

3. Conclusion

This study investigated the MAO inhibition potential of several natural compounds and found that DL-kavain is a moderately potent MAO inhibitor. Although MAO-B inhibition by kavain is known, it is shown for the first time that this compound also inhibits MAO-A. Other kavalactones were also evaluated as MAO inhibitors and yangonin proved to be the most potent MAO inhibitor. In fact, yangonin may be considered a high potency MAO-B inhibitor, comparable to the reference MAO-B inhibitor, lazabemide. The MAO-A inhibition potency of yangonin may also be considered good and is in a similar range as toloxatone. The molecular modelling studies suggest that the planarity of yangonin may mediate its higher MAO inhibition potencies compared to kavain. It may be concluded that some of the central effects (e.g. anxiolytic) of kava may be mediated by MAO inhibition.

4. Experimental section

4.1. Materials and instrumentation

Fluorescence spectrophotometry was carried out using a Varian Cary Eclipse fluorescence spectrophotometer. Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg/mL) and kynuramine dihydrobromide

were obtained from Sigma-Aldrich. All molecular modelling simulations were carried out with the Discovery Studio 3.1 modelling software (Accelrys).

4.2. IC_{50} value determinations:

IC_{50} values for the inhibition of MAO were measured by using the recombinant human MAO-A and MAO-B enzymes (Mostert *et al.*, 2015). The enzyme reactions were carried out in white 96-well microtiter plates (Eppendorf) in potassium phosphate buffer (pH 7.4, 100 mM, made isotonic with KCl). The final volume of the reactions was 200 μ L and contained kynuramine (50 μ M), the test inhibitors (0.003–100 μ M) and MAO-A (0.0075 mg protein/mL) or MAO-B (0.015 mg protein/mL). Stock solutions of the test inhibitors were prepared in DMSO and added to the reactions to yield a final stock solution concentration of 4%. Reactions serving as negative controls were carried out in the absence of inhibitor. The enzyme reactions were initiated with the addition of the MAO enzymes, and were subsequently incubated for 20 min at 37 °C in a convection oven. At endpoint, the reactions were terminated with the addition of 80 μ L sodium hydroxide (2 N) and the concentration of 4-hydroxyquinoline, the product of kynuramine oxidation by MAO, was measured by fluorescence spectrophotometry (λ_{ex} = 310 nm; λ_{em} = 400 nm) (Novaroli *et al.*, 2005). For this purpose, a linear calibration curve was constructed with authentic 4-hydroxyquinoline (0.047–1.56 μ M). The rates of MAO-catalysed 4-hydroxyquinoline formation thus measured were fitted to the one site competition model of the Prism 5 software package (GraphPad). This gave sigmoidal plots of rate versus logarithm of inhibitor concentration from which the IC_{50} values were estimated. IC_{50} values were measured in triplicate and are reported as the mean $\pm$ standard deviation (SD).

4.3. Dialysis studies

Dialysis was carried out with Slide-A-Lyzer dialysis cassettes (Thermo Scientific) with a molecular weight cut-off of 10 000 and a sample volume capacity of 0.5–3 mL (Mostert *et al.*, 2015). The test inhibitor (at a concentration equal to $4 \times IC_{50}$) and MAO (0.03 mg protein/mL) were prepared to a final volume of 0.8 mL in the dialysis buffer (potassium phosphate buffer (100 mM, pH 7.4, 5% sucrose). Stock solutions of the inhibitor were prepared in DMSO and added to the buffer to yield a final concentration of 4% DMSO. These samples were preincubated for 15 min at 37 °C and were subsequently dialysed at 4 °C in 80 mL of dialysis buffer. The dialysis buffer was replaced with fresh buffer at 3 h and 7 h after the start of dialysis. As

positive controls, MAO-A and MAO-B were similarly preincubated and dialysed in the presence of the irreversible inhibitor, pargyline ($IC_{50} = 13 \mu M$) and (R)-deprenyl ($IC_{50} = 0.079 \mu M$), respectively (Strydom *et al.*, 2012; Petzer *et al.*, 2012). As negative control, dialysis of the enzyme was carried out in the absence of the inhibitor. After 24 h of dialysis, 250 μL of the dialysed samples were diluted twofold with the addition of 250 μL kynuramine (dissolved in potassium phosphate buffer, 100 mM, pH 7.4, made isotonic with KCl) to yield reactions with a final volume of 500 μL and containing kynuramine (50 μM), MAO (0.015 mg protein/mL) and test inhibitor ($2 \times IC_{50}$). The reactions (prepared in 1.5 mL microcentrifuge tubes) were incubated at 37 °C in a water bath and after 20 min were terminated with the addition of 400 μL sodium hydroxide (2 N). After addition of 1000 μL water, the concentrations of 4-hydroxyquinoline were measured by fluorescence spectrophotometry ($\lambda_{ex} = 310$ nm; $\lambda_{em} = 400$ nm) employing a 3.5 mL quartz cuvette (pathlength 10 $\times$ 10 mm). To quantify 4-hydroxyquinoline, a linear calibration curve was constructed with authentic 4-hydroxyquinoline (0.047–1.56 μM). For comparison, undialysed mixtures of MAO and the test inhibitor were maintained at 4 °C for 24 h and subsequently diluted twofold and assayed as above. All reactions were carried out in triplicate and the residual enzyme catalytic rates were expressed as mean $\pm$ SD.

4.4. Lineweaver-Burk plots and K_i value calculations

To determine the mode of MAO inhibition of selected inhibitors, sets consisting of six Lineweaver-Burk plots were constructed (Mostert *et al.*, 2015). The first plot was constructed in the absence of inhibitor while the remaining five plots were constructed in the presence of different concentrations of the test inhibitor: $\frac{1}{4} \times IC_{50}$, $\frac{1}{2} \times IC_{50}$, $\frac{3}{4} \times IC_{50}$, $1 \times IC_{50}$ and $1\frac{1}{4} \times IC_{50}$. The enzyme substrate, kynuramine, was used at concentrations ranging from 15 to 250 μM while the final enzyme concentration was 0.015 mg/mL. All incubations were carried out in 1.5 mL microcentrifuge tubes to a volume of 500 μL . The MAO catalytic activities were measured by fluorescence spectrophotometry as described for the dialysis study. K_i values were estimated from plots of the slopes of the Lineweaver-Burke plots versus inhibitor concentration, where the x-axis intercept equals $-K_i$.

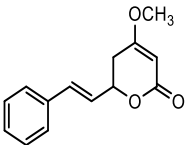
4.5. Molecular docking and dynamics simulations

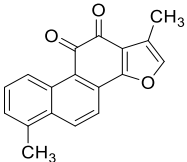
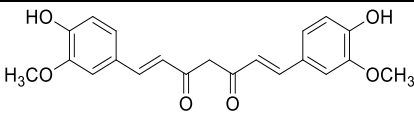
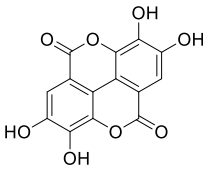
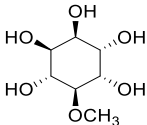
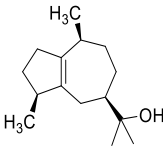
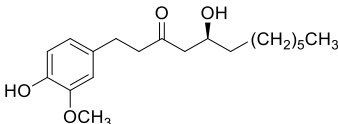
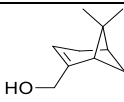
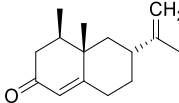
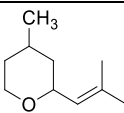
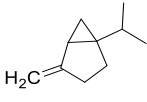
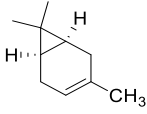
All modelling calculations were carried out with the Windows-based Discovery Studio 3.1 software package (Accelrys). Unless otherwise specified, all the applications within Discovery Studio were set to their default values. The X-ray crystal structures of human MAO-A (PDB code 2Z5X) (Son *et al.*, 2008) and human MAO-B (PDB code 2V5Z) (Binda *et al.*, 2007) were obtained from the Brookhaven Protein Data Bank. The pKa values and protonation states of the ionisable amino acids were calculated and hydrogen atoms were added at pH 7.4 to the protein models. When necessary, the valences of the FAD cofactors (oxidised state) and co-crystallised ligands were corrected, the protein models were automatically typed with the Momany and Rone Chemistry at Harvard Macromolecular Mechanics (CHARMm) forcefield and a fixed atom constraint was applied to the backbone. The models were subsequently energy minimised using the Smart Minimizer algorithm with the maximum number of steps set to 50000. The implicit generalized Born solvation model with molecular volume was used during the minimisation procedure. The co-crystallised ligands, waters and the backbone constraints were removed from the models and the binding sites were identified from an analysis of the enzyme cavities. In each model, three active site waters are considered to be conserved and were retained. These waters are HOH 710, 718 and 739 in the MAO-A active site, and HOH 1155, 1170 and 1351 in the A-chain of the MAO-B active site. For some simulations all waters were retained (see text). The structures of the test compounds were drawn in Discovery Studio, the geometries were briefly optimised using a Dreiding-like forcefield (5000 iterations) and the structures were submitted to the Prepare Ligands protocol. Atom potential types and partial charges were subsequently assigned to the structures with the Momany and Rone CHARMm forcefield. Docking of the test inhibitors into the MAO models was carried out with the CDOCKER algorithm. For this purpose, ten random ligand conformations were generated, the heating target temperature was set to 700 K and full potential mode was employed. The docking solutions were finally refined using in situ ligand minimisation with the Smart Minimizer algorithm.

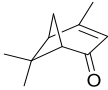
The highest ranked docking solutions were subjected to a short (500 ps) molecular dynamics simulation. Fixed atom constraints were applied to the backbone of the protein models and the residues that are further than 5 Å from the docked ligand. The models were automatically typed with the Momany and Rone CHARMm

forcefield. The standard dynamics cascade was used for this study and consisted of a steepest descent minimisation (maximum steps, 2500) followed by a conjugate gradient minimisation (maximum steps, 2500; RMS gradient, 0.01). Heating was subsequently carried out in 2000 steps (time step, 0.001 ps) from 50 K to 300 K, and equilibration of the system was allowed for 10000 steps (time step, 0.001 ps). The production runs were carried out for 500 000 steps (time step, 0.001 ps), applying the SHAKE algorithm to all bonds involving hydrogen atoms. The equations for motion were integrated with the Leapfrog Verlet algorithm. The implicit generalised Born solvation model with simple switching was used during the dynamics calculations. The conformation of the complex was saved every 1 ps, yielding 500 frames. The illustrations were generated with PyMOL and represent the docked orientations prior to the molecular dynamics simulation ([DeLano, 2002](#)).

Table 3.1. The IC₅₀ values for the inhibition of recombinant human MAO-A and MAO-B by selected natural products.

Structure	IC ₅₀ (μM) ^a		SI ^b
	MAO-A	MAO-B	
Kavain (Smith et al., 2004) 	19.0 ± 0.61	5.43 ± 1.28	3.5

Tanshinone I (Kishikawa et al., 2017)		15.7 ± 2.67	NI ^c	-
Curcumin (Engelbrecht et al., 2018)		5.02 ± 0.45	2.56 ± 0.21	2.0
Ellagic acid (Dalvi et al., 2017)		80.1 ± 1.82	35.9 ± 2.09	2.2
D-Pinitol (Rengarajan et al., 2015)		NI ^c	NI ^c	-
Eucalyptol (Imad, Israa and Hawraa, 2015)		NI ^c	NI ^c	-
Guaiol (Panidthananon et al., 2018)		NI ^c	NI ^c	-
[8]-Gingerol (Choi et al., 2017)		NI ^c	NI ^c	-
Myrtenol (Gomes et al., 2017)		30.0 ± 1.55	NI ^c	-
Nootkatone (Rengarajan et al., 2015)		NI ^c	NI ^c	-
Rose oxide (Alsters et al., 2010)		NI ^c	NI ^c	-
Sabinene (Rengarajan et al., 2015)		NI ^c	NI ^c	-
(+)-3-Carene (Rengarajan et al., 2015)		NI ^c	NI ^c	-

Verbenone (de Melo et al., 2017)		NI ^c	NI ^c	-
Toloxatone		3.92 ^d	-	-
Lazabemide		-	0.091 ^d	-

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

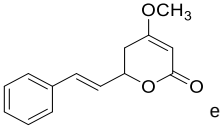
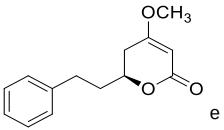
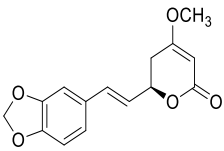
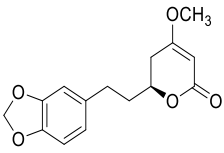
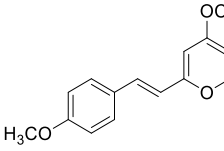
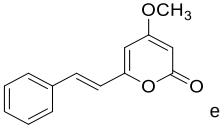
^b Selectivity index (SI) = $IC_{50}(\text{MAO-A})/IC_{50}(\text{MAO-B})$.

^c No inhibition observed at maximum tested concentration of 100 μM .

^d Values taken from reference (Petzer et al., 2013).

Table 3.2. The IC_{50} values for the inhibition of recombinant human MAO-A and MAO-B by constituents of *Piper methysticum*.

		IC_{50} (μM) ^a		SI ^b
		MAO-A	MAO-B	

DL-kavain		19.0 ± 0.61	5.43 ± 1.28 (40.5) ^c	3.5
(+)-7,8-Dihydrokavain		$>100^d$	8.37 ± 1.95	>11.9
(+)-Methysticin		8.12 ± 0.375	0.356 ± 0.054 (0.67 racemate) ^c	22.8
(+)-7,8-Dihydromethysticin		23.3 ± 1.97	0.856 ± 0.034	27.2
Yangonin		1.29 ± 0.106	0.086 ± 0.0087	15.1
Desmethoxyyangonin		4.44 ± 0.151	0.251 ± 0.0086 (0.12) ^c	17.7

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

^b Selectivity index (SI) = $IC_{50}(\text{MAO-A})/IC_{50}(\text{MAO-B})$.

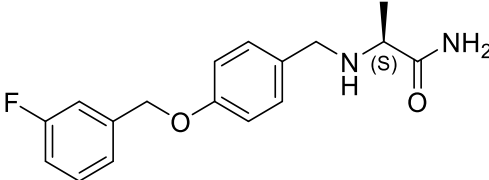
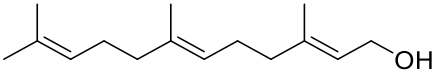
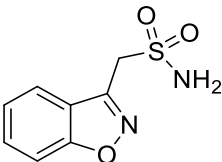
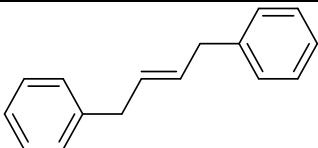
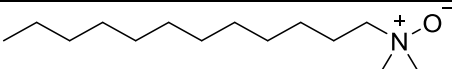
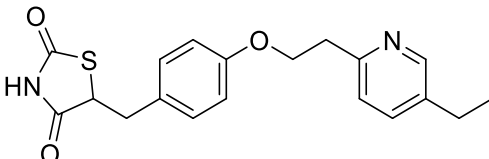
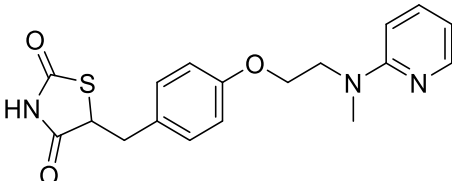
^c Values obtained from reference ([Uebelhack et al., 1998](#)).

^d No inhibition observed at maximum tested concentration of 100 μM .

^e Reference to structures : ([Smith et al., 2004](#))

Table 3.3. The RMSD of various co-crystallised ligands which were docked into the binding site of MAO-B in complex with safinamide (2V5Z). After the crystal structures were

superimposed, the RMSD from the original orientation was measured. The RMSD of harmine redocked into the crystal structure of MAO-A is also given.

Co-crystallised inhibitor		PDB entry	RMSD (Å) 3 waters	RMSD (Å) all waters	Resolution (Å)
Safinamide (Binda et al., 2007)		2V5Z	0.5475	0.558	1.6
Trans, trans-Farnesol (Hubálek et al., 2005)		2BK3	1.883	8.562	1.8
Zonisamide (Binda et al., 2011b)		3PO7	2.683	2.442	1.8
1,4-Diphenyl-2-butene (Binda et al., 2003)		1OJ9	1.208	1.225	2.3
Lauryldimethylamine-N-oxide (LDAO) (Binda et al., 2003)		1OJD	1.945	1.343	3.1
(R)-Pioglitazone (Binda et al., 2011a)		4A79	3.342	3.677	1.89
(R)-Rosiglitazone (Binda et al., 2011a)		4A7A	2.407	1.751	1.7

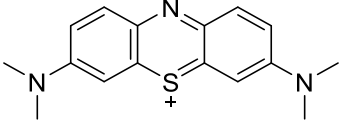
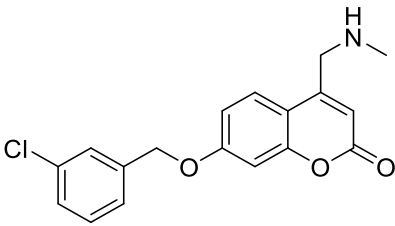
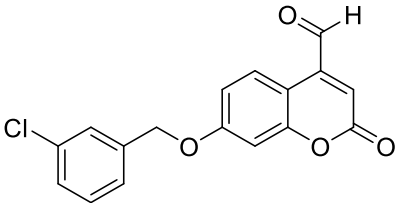
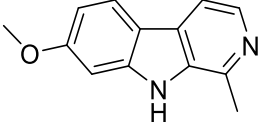
Methylene blue		3ZYX	2.792	2.802	2.2
7-(3-Chlorobenzoyloxy)-4-(methylamino)methyl-coumarin (Binda et al., 2007)		2V61	2.884	2.857	1.7
7-(3-Chlorobenzoyloxy)-4-carboxaldehyde-coumarin (Binda et al., 2007)		2V60	0.858	0.800	2.0
Harmine (Son et al., 2008)		2Z5X	2.830	0.257	

Table 3.4. The interaction energies (kcal/mol) between key MAO-A residues and kavain. Docking was carried out with all crystal waters present

	(R)-Kavain	(S)-Kavain
TYR69	-1.99	-1.37
ILE180	-3.13	-3.25
ASN181	-1.86	-2.03
PHE208	-2.53	-0.72
GLN215	-4.08	-4.68
ILE335	5.15	-2.65
LEU337	-2.16	-1.68
PHE352	669	20.2
TYR407	1.55	-2.39
HOH766	9.69	1.41
Total interaction energy	656	-7.99

Table 3.5. The interaction energies (kcal/mol) between key MAO-B residues and kavain. Docking was carried out with all crystal waters present.

	(R)-Kavain	(S)-Kavain
LEU171	-4.79	-3.01
CYS172	-2.10	-1.11
ILE198	-2.64	-2.90
ILE199	-5.12	-5.21
GLN206	3.80	0.26
TYR326	-1.85	-3.64
Total interaction energy	-26.3	-31.6

Table 3.6. The interaction energies (kcal/mol) between key MAO-B residues and curcumin. Docking was carried out with all crystal waters present.

	Keto-form	Enol-form
TYR60	-2.12	-2.26
LEU164	27.0	3520
LEU167	-1.69	11559
PHE168	-2.64	18.1
LEU171	22.93	-4.22
CYS172	4.69	26.9
ILE198	1.63	-3.31
ILE199	-2.28	0.500
GLN206	-2.71	-2.56
ILE316	144	112
TYR326	-2.61	-2.53
TYR398	-3.56	-3.51
TYR435	-2.59	-2.13
FAD1502	-4.19	-3.72
HOH1351	237	378
Total interaction energy	403	15585

Table 3.7. The interaction energies (kcal/mol) between key MAO-A residues and kavain. Docking was carried out with three conserved crystal waters present.

	(R)-Kavain	(S)-Kavain
LEU97	0.900	-0.638
ILE180	-2.06	-3.29
ASN181	-2.27	-0.793
TYR197	-0.049	0.118
PHE208	-0.758	-4.93
GLN215	-4.39	-3.58
ILE335	0.464	6.97
LEU337	-1.49	-2.05
TYR407	-1.95	-0.817
Total interaction energy	-25.9	-23.2

Table 3.8. The interaction energies (kcal/mol) between key MAO-B residues and kavain. Docking was carried out with three conserved crystal waters present.

	(R)-Kavain	(S)-Kavain
PHE168	-1.78	-1.90
LEU171	-5.24	-2.57
CYS172	-2.16	-1.18
ILE198	-2.68	-2.87
ILE199	-4.92	-5.07
GLN206	3.22	0.398
LYS296	-0.353	0.259
TYR326	-1.95	-3.65
TYR398	2.089	-1.15
HOH1155	1.56	-0.204
Total interaction energy	-25.4	-30.4

Table 3.9. The interaction energies (kcal/mol) between key MAO-B residues and curcumin. Docking was carried out with three conserved crystal waters present.

	Keto-form	Enol-form
PRO102	-2.97	-1.86
PRO104	-1.15	1.28
PHE168	3.49	-4.04
LEU171	4.97	-0.904
CYS172	-2.40	11.6
ILE198	-2.95	-3.72
ILE199	-6.33	-3.86
GLN206	-3.78	-3.00
ILE316	50.8	-2.70
TYR326	1.88	0.104
LEU328	11.8	-0.473
PHE343	15.2	-0.716
TYR398	6.72	16.4
TYR435	-1.00	-1.99
FAD1502	-2.25	-2.43
HOH1351	-0.566	2.13
Total interaction energy	58.7	-4.00

Table 3.10. The interaction energies between key MAO-B residues and kavian recorded over a period of 500 ps.

	Interaction energy (kcal/mol)	
	(R)-Kavian	(S)-Kavian
Leu-171	-4.46 ± 0.74	-4.01 ± 0.82
Ile-199	-4.02 ± 0.73	-4.60 ± 0.37
Gln-206	-3.34 ± 0.45	-3.55 ± 0.56
Tyr-326	-3.96 ± 0.95	-3.92 ± 0.51
Total interaction energy	-37.09	-35.51

All values are expressed as the mean ± SD of 500 frames.

Table 3.11. The interaction energies between key MAO-A and MAO-B residues and yangonin recorded over a period of 500 ps.

	Interaction energy (kcal/mol)
MAO-A	
Ile-180	-2.79 ± 0.25
Phe-208	-3.30 ± 0.87
Gln-215	-4.28 ± 0.58
Ile-335	-2.29 ± 0.28
Tyr-407	-2.80 ± 0.37
Total interaction energy	-40.06
MAO-B	
Leu-171	-3.85 ± 0.50
Ile-199	-5.46 ± 62
Gln-206	-2.42 ± 47
Tyr-326	-3.56 ± 0.53
Total interaction energy	-39.33

All values are expressed as the mean $\pm$ SD of 500 frames.

CHAPTER 4

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterised by tremors, slow movement and muscle rigidity. Deficiency of dopamine in the brain is responsible for most of the motor symptoms of PD. The substantia nigra pars compacta in the basal ganglia is the main region affected by the neurodegenerative process in PD. Other regions in the brain only start to degenerate in the late stages of the disease. Oxidative stress, mitochondrial dysfunction and protein aggregation appears to be central factors in the pathogenesis of PD, but the direct cause remains elusive. PD cannot be cured thus, focus is placed on increasing quality of life through symptomatic treatment regimes. Levodopa, dopamine agonists, catechol-O-methyltransferase (COMT) inhibitors and monoamine oxidase (MAO) inhibitors are drugs that either enhance dopamine levels in the central nervous system or mimic the function of dopamine at dopaminergic receptors to improve motor function (Fox *et al.*, 2018).

MAO is a flavoenzyme attached to the outer membranes of mitochondria and is responsible for the oxidative deamination of amines in the central nervous system and the peripheral tissues. The two isoforms, MAO-A and MAO-B, exhibit different tissue distributions, and substrate and inhibitor specificities. The concentration of the MAO-B enzyme is higher in neuronal tissue than MAO-A, and MAO-B is thus targeted for the treatment of neurological disorders such as PD. Since PD is a disorder linked to dysfunctional neurotransmitter function in the central nervous system, MAO inhibitors can be used to enhance neurotransmitter function (Dauer & Przedborski, 2003; Fox *et al.*, 2018).

This study examined the MAO inhibition potential of a series of natural products with the specific aim of discovering natural products that may serve as future leads for the design of MAO inhibitors. Natural products are known to have pharmacological activities relevant to the central nervous system which makes them viable leads in drug discovery projects that focus on targeting neurodegenerative diseases. Special interest is placed on their effects on enzymes such as MAO that modulate the levels of neurotransmitters. An additional benefit that some natural products have is

antioxidant activity that could provide a neuroprotective action in neurodegenerative disorders.

DL-Kavain, the major constituent of *Piper methysticum*, was evaluated as an *in vitro* inhibitor of human MAO-A and MAO-B and found to exhibit IC₅₀ values of 5.43 μ M and 19.0 μ M, respectively. This is the first report of the MAO-A inhibition properties of DL-kavain. Curcumin was examined under the same experimental conditions and served as a reference compound. Curcumin inhibits MAO with IC₅₀ values of 2.56 μ M for MAO-B and 5.02 μ M for MAO-A. Curcumin inhibits MAO-A with a K_i value of 3.08 μ M while DL-kavain inhibits MAO-A and MAO-B competitively with K_i values of 7.72 μ M and 5.10 μ M, respectively. Dialysis studies established that DL-kavain is a reversible inhibitor of MAO-A and MAO-B. Dihydrokavain, methysticin, dihydromethysticin, yangonin and desmethoxyyangonin are also constituents of the *Piper methysticum* plant and were thus evaluated as MAO inhibitors. Other natural products that were found to act as MAO inhibitors were tanshinone I and myrtenol with *in vitro* IC₅₀ values of 15.7 μ M and 30.0 μ M, respectively, for the inhibition of MAO-A and no inhibitory activity for MAO-B. Ellagic acid also showed inhibitory activity for both MAO isoforms with IC₅₀ values at 80.1 μ M for MAO-A and 35.9 μ M for MAO-B.

The possible binding modes and interactions of DL-kavain and curcumin were examined by molecular docking and molecular dynamics simulations. Although curcumin is a known competitive MAO-A inhibitor, both keto and enol enantiomers were unable to fit in the active site of MAO-A. This suggests that induced fit should be considered when curcumin binds to MAO-A. Alternatively, the binding mode of curcumin to MAO-A is not competitive and the inhibitor may bind to an allosteric site on the enzyme. Curcumin, however, fits well in the active site of MAO-B and exhibits a cavity-spanning mode of binding where the inhibitor binds to both the substrate and entrance cavities. Both enantiomers of kavain, fit in the MAO-A active site, although differing orientations are observed when the active site waters are retained and removed. This may be due to induced fit that were not considered during the docking simulations. In MAO-B, the binding modes of (R)- and (S)-kavain are almost identical irrespective of whether the active site waters are removed or retained. The carbonyl of (R)-kavain is directed towards the top of the active site, while for (S)-

kavain, the carbonyl is directed to the bottom of the active site cavity. The styryl side chains of the two enantiomers are placed in the entrance cavity.

The objective of this study was to discover novel natural products that inhibit the MAO enzymes. The most significant discovery was that DL-kavain is a moderately potent MAO-B inhibitor. Although its MAO-B inhibition properties are known, this study shows for the first time that DL-kavain also inhibits MAO-A. Curcumin is another compound that was shown to possess potent MAO inhibition activity, although this is known in literature. It may be concluded that DL-kavain may act as a lead for the development of MAO-A and MAO-B inhibitors. Such compounds may serve as future antidepressants and therapy for PD. The hypothesis of this study which states that among the selected natural products, some novel compounds will exhibit MAO inhibition, has thus been proved.

Although kavain is a well-known anxiolytic, other pharmacological activities are also noteworthy. These include analgesic and anti-inflammatory properties, as well as the possibility of GABA receptor stimulatory activity which could be beneficial towards the treatment of convulsions. Furthermore, kavain has been included in numerous studies investigating other receptor stimulatory and inhibitory activities. These include the evaluation of kavain as a selective serotonin reuptake inhibitor for the improvement of depressive disorders, an inhibitor of the calcium channels of vascular smooth muscle and a stimulator of mesolimbic dopamine neurons which relaxes skeletal muscles. Curcumin is believed to reduce the risk of certain cancers and may possess antioxidant activity. Anti-inflammatory properties, enhanced wound healing benefits and anti-angiogenic activities are some of the major benefits that are closely related with the use of curcumin. Besides the inhibition of MAO-A and MAO-B, some of the above pharmacological activities may contribute to the antidepressant and antiparkinsonian activities of MAO inhibitors. In particular, the anxiolytic and serotonin reuptake inhibition activity of kavain may be relevant in the treatment of depression while anti-inflammatory and antioxidant properties of kavain and curcumin may be relevant to inflammatory and oxidative processes associated with depression and Parkinson's disease ([Chua et al., 2016](#)).

A consideration for future studies is to determine the contribution of the respective enantiomers of kavain to MAO-A and MAO-B inhibition. From the molecular docking studies, it may be expected that both (R)- and (S)-kavain contribute to the observed inhibition of MAO-A and MAO-B. It is suggested that a method for the separation of kavain enantiomers be developed to assist in the determination of the MAO inhibition properties of the individual enantiomers.

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ANNEXURES A

Letter of permission

Ms. D. Prinsloo

October 2018

To whom it may concern:

Dear Sir / Madam

CO-AUTHORSHIP ON RESEARCH PAPERS

The undersigned, as co-authors of the research article listed below, hereby give permission to Ms. Denise Prinsloo to submit the paper as part of the degree M.Sc. in Pharmaceutical Chemistry at the North-West University, Potchefstroom Campus.

I. Monoamine oxidase inhibition by kavalactones from kava (*Piper methysticum*).

Yours sincerely,

Prof. S van Dyk

Prof. J.P. Petzer

Prof. A. Petzer

ANNEXURE B

Author guidelines



Instructions for Authors (2018)

General Policy

Chemistry & Biodiversity publishes field-specific and interdisciplinary high-quality contributions on all research fields that straddle the border between the chemical and the biological sciences, with the ultimate goal of broadening our understanding of how nature works at a molecular level.

Considered for publication will be submissions on all aspects of **biologically relevant chemistry** (see Table 1) in the form of full papers, invited reviews, short communications, and commentaries.

Table 1. Coverage of *Chemistry & Biodiversity* by Means of Target Keywords (not restrictive)

Fields	Substance Classes
Biocatalysis	Alkaloids
Biological Chemistry	Antibiotics
Biomaterials	Bioactive Compounds
Biomimetic Reactions	Bioassemblies
Chirality*	Biopolymers
Combinatorial Chemistry*	Carbohydrates
Computational Chemistry*	Flavors And Fragrances
Ecology**	Glycosides
Environmental Chemistry	Hormones
Evolution**	Lipids
Food and Nutrition	Membranes
Green Chemistry	Neurotransmitters
Immunochemistry	Nucleosides, Nucleotides, etc.
Medicinal Chemistry	Peptides and Amino Acids
Metabolic Pathways	Pheromones
Molecular Recognition	Pollutants
Molecular Toxicology	Porphyryns
Photochemistry*	Proteins
Prebiotic Chemistry	Ribozymes
Structure/Topology of Biomolecules	Steroids
Structure–Activity Relationships	Terpenes
Supramolecular Chemistry	Toxins* (natural or synthetic)
	Vitamins

* Biorelevant Aspects Only. ** Chemical Aspects Only.

Reviews deal with topics of current interest in any of the areas covered by *Chemistry & Biodiversity*. Rather than an assemblage of detailed information with a complete literature survey, a critically selected treatment of the material is desired. Unsolved problems and possible developments should also be discussed.

A strong chemical focus and proper manuscript preparation (see below) are absolute prerequisites for publication. Articles should be written to meet the interests of readers of both the chemistry and the life-science communities.

Contributions will be immediately rejected if:

1. The manuscript does not fall into any of the above areas (see Table 1).
2. Activity data are reported without comparison to a recognized positive control.
3. No statistical analysis is present where absolutely necessary (e.g., in cytotoxicity assays).
4. The manuscript is not well written and does not adhere to the format and style of *Chemistry & Biodiversity*.

The manuscript should be submitted online via the online submission service *Editorial Manager* at <http://www.editorialmanager.com/chembiodiv/default.aspx>

The corresponding author, submitting the manuscript, should follow these instructions. Manuscripts must be formatted using the *New Chemistry & Biodiversity Template* as a **single Microsoft Word file** that contains the text, **all** figures, schemes, and tables. These elements should be integrated into the text (i.e., inserted where first mentioned), to facilitate evaluation of the article by the editors and reviewers. PDF files are not acceptable. Please include a cover letter justifying why your article should appear in *Chemistry & Biodiversity*. The text should not merely repeat the abstract but should clearly describe the significance of the study for those working within/outside the field. Additional files containing *Supporting Information* associated with the manuscript should be uploaded separately.

Chemistry & Biodiversity does not publish manuscripts that have already appeared in print or electroni-

cally (including those deposited in preprint archives). The author must inform the editor of manuscripts submitted, soon to be submitted, or in press at other journals that have a bearing on the manuscript being submitted to *Chemistry & Biodiversity*. The *Ethical Guidelines for Publication* issued by the *European Association for Chemical and Molecular Sciences* are followed and applied by the editors of *Chemistry & Biodiversity*. Authors should reveal all sources of funding for the work presented in the manuscript and should declare any conflict of interest.

All manuscripts submitted to the journal are screened by an antiplagiarism software (iThenticate).

If the manuscript is a revised/extended version of a manuscript previously rejected by *Chemistry & Biodiversity*, the author must inform the editor about the previous submission in the cover letter and explain in detail which changes have been made. Alerting us on any previous versions may facilitate evaluation of the article.

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Preparation of Manuscripts

General Considerations. Manuscripts must be submitted in English. American or British spelling can be used provided that only one spelling style is consistently used. Manuscripts must be grammatically and linguistically correct,

and authors less familiar with English usage are advised to seek the help of English-speaking colleagues. Non-native English speakers authors may choose to have their manuscript professionally edited before submission or during the review process. Help and suggestions for language services can be found at <http://wileyeditingservices.com/en>. Careful preparation of the manuscript and adherence to the format and conventions of *Chemistry & Biodiversity* as outlined in these *Instructions for Authors* is required. Authors investigating the chemistry of a single species should aim to publish their results in a single manuscript rather than in a series of papers. The manuscript should not report parts of a larger study.

A representative structural formula, scheme, or figure should be provided for the Table of Contents. The maximum available space for this **graphical abstract** is 7 × 17.4 cm.

Suggestions for the **cover picture** (18.5 × 14 cm, 600 dpi) with an explanatory text (300–1000 characters) are welcome. Part of the additional costs for the publication of the cover picture must be paid by the author.

Manuscripts should be kept to minimum length, and, for clarity, each work should be subdivided into labeled sections, e.g., **Introduction, Results and Discussion, Conclusions, Experimental Section, Acknowledgement, Author Contribution Statement, References**.

Special types of print should be used as follows:

- **Boldface:** headings, designated numbers of chemical compounds.
- *Italic:* subheadings, configurational prefixes ((*R*)-, (*S*)-, *cis*-, *trans*-, etc.), Latin words or abbreviations, trade names of chemical compounds (first letter should be capitalized), names of authors if mentioned in the text.
- **SMALL CAPITAL:** symbols of molar and normal concentrations (M and N), D and L, the names of the discoverer in the nomenclature of genera, species, or varieties.
- **Boldface italic:** the italicized terms and prefixes in headings.

Title. a) The title of a manuscript, being of great importance for attracting readers' interest and for information retrieval, should clearly and accurately provide information on the content and emphasis of the work. The use of abbreviations, symbols, chemical formulae, and references in a title is strongly discouraged. First letters of nouns, adjectives, and verbs are capitalized. b) The authors' full first names, middle initials, and last names should be given, followed by the address(es) of the contributing laboratory or laboratories. The author to whom correspondence and/or inquiries should be directed is indicated with an asterisk (*). Footnotes may

be added to specify the present mailing address(es) of the author(s). The corresponding author's mailing address and **e-mail address** should also be included.

Abstract. The abstract should state briefly the purpose of the research, the principal results, and major conclusions; it should be self-explanatory and intelligible without reference to the text. For a typical contribution, an 800- to 1000-character abstract is usually adequate.

Keywords. Authors can ensure that a keyword search within Wiley Online Library (WOL) leads to a list that is as complete as possible of relevant publications in many Wiley-VCH journals (see the list at the start of the [common keyword catalogue](#)) by preferably using keywords from this catalogue. The catalogue is subdivided to facilitate the search for keywords but can also be completely searched. Some of the keywords are used in more than one area. As with all such records, a few guidelines facilitate its use, and these are briefly explained below:

1. At least two of the maximum of five keywords assigned to an article must come from this list.
2. Named reactions will be incorporated only in exceptional cases. Generally, the reaction type is selected instead. For example, 'cycloadditions' instead of 'Diels–Alder reactions' and 'rearrangement' instead of 'Claisen rearrangement'.
3. Heteroanalogues of compounds are mainly classified under the C variants, for example, (hetero)cumulenes, (hetero)dienes. A few aza and phosphorus derivatives are exceptions.
4. Compounds with inorganic components that are central to the article are listed under the element, for example, iron complexes under 'iron' and, if appropriate, the ligand type. Some group names such as 'alkali metals' exist alongside the names of important members of the group, for example, 'lithium'. In such cases the group name is used for these members only when comparative studies are described. The members that do not appear separately are also categorized under the group name.
5. A keyword in the form 'N ligand' is only chosen if a considerable portion of the article deals with the coordination of any ligand that coordinates through the atom concerned (in the example, nitrogen).
6. Spectroscopic methods are assigned as keywords only if the article is about the method itself or if the spectroscopic technique has made an important contribution to the problem under investigation.

7. 'Structure elucidation' is intended only if the crux of the paper is a structural elucidation or if a combination of several spectroscopic techniques were needed for conclusive solution of the structure.
8. An attempt has been made to avoid synonyms and to select more general concepts rather than specialized terms. Thus the term 'double-decker complexes' is excluded in favor of 'sandwich complexes'.
9. Enzymes should be assigned to one of the six main enzyme classes (hydrolases, isomerases, ligases, lyases, oxidoreductases, transferases).
10. Add the scientific name and the family of the organism(s) investigated.

Introduction. The introduction should state the purpose of the investigation. The status of the latest research of the topic to be discussed should be briefly presented with leading references.

Results and Discussion. The results and discussion may be combined or kept separate and may be further divided by subheadings. This section should not be cluttered with technical details. Abbreviations and acronyms should be used sparingly and consistently. Where they first appear in the text, they should – apart from the most common ones, such as IR, UV, and NMR – be defined; you may prefer to explain large numbers of abbreviations and acronyms in a footnote on the first page.

Conclusion(s) should present the summing up of the achieved points, demonstrate the importance of your ideas, and propel the reader to a new view of the subject.

Acknowledgements should be brief. A person can be thanked for assistance or for comments. Acknowledgements can contain grant and contribution numbers.

Author Contribution Statement. Authors are required to include a statement describing the contribution of each author to the manuscript.

Example: A.-B. C. and D. E. performed the experiments, analyzed the data, and wrote the article. F. G. contributed samples/reagents/materials/analysis tools and analyzed the data. H. I. and J. K. conceived and designed the experiments.

Experimental Section. The *Experimental Section* should only contain the most essential parts of your experimental procedures; the rest should be reported into the *Supporting Information*. Taken together, the experimental data in the main manuscript and the *Supporting Information* should be given in sufficient detail to enable others to repeat your work. In theoretical reports, technical details such as computational methods should likewise be confined to an appropriately named section.

Equipment and conditions used for the measurement of physical data should be described at the beginning of the *Experimental Section* in the *General Section*. Suppliers for major equipment, cell lines, biochemical reagents, and major disposables should be indicated. Quantities of reactants, solvents, etc. should be included in parentheses (e.g., triphenylstannyl chloride (0.964 g, 2.5 mmol) in toluene (20 ml)) rather than in the running text. Physical data should be quoted with decimal points and negative exponents (e.g., 25.8 JK⁻¹mol⁻¹). The identity of all new compounds must be fully characterized by appropriate analytical methods (e.g., NMR spectroscopy, X-ray crystal structure analysis, elemental analysis). The purity of all new compounds should be verified by elemental analysis, to an accuracy of within $\pm 0.4\%$. In special cases, for instance, when the compound is unstable or not available in sufficient quantities for complete analysis, the exact relative molecular mass obtained from a high-resolution mass spectrum (HR-MS) and a clean ¹³C-NMR spectrum (as *Supporting Information* for inspection by the referees) should be supplied. These data should be given in the *Supporting Information* in the event that they exceed the scope of the *Experimental Section*.

Detailed presentation of physical data: $R_f = 0.38$ (CHCl₃/MeOH 9:1). M.p. 20–21 °C. $[\alpha]_D^{20} = -13.5$ ($c = 0.2$, acetone). UV (MeOH): 320 (5000). IR (KBr): 1780, 1790 (C=O). ¹H-NMR (400 MHz, (D₆)THF): 2.41–2.32 (m, H-C(5)); 1.33 (q, ³J(H,H) = 8.0, CH₂); 0.92 (t, ³J(H,H) = 8.1, Me). ¹³C-NMR (100 MHz, CDCl₃): 72.5 (d, CCH); 26.8 (s, Me); 6.5 (d, ¹J(C,P) = 156.9, CHP). HR-MS: 315.1495 ([M + H]⁺, C₂₁H₁₉N₂O⁺; calc. 315.1497). Anal. calc. for C₂₁H₁₉BrNOS (296.18): C 48.66, H 3.40, N 4.73; found: C 48.41, H 3.22, N 4.82. Please give data in this order.

Please note that *Chemistry & Biodiversity* represents **multiplets** in the ¹H-NMR part as ranges as shown in this example: 4.23–4.29 (m, 2 H).

Documentation of plants and other organisms or starting materials is essential. Correct scientific nomenclature has to be used. The person **who identified** the material as well as date and place of collection has to be indicated. The manuscript must include references to voucher specimens of the plants (deposited in a major regional herbarium) or the material examined including their **registration number(s)**. It should be also stated which plant parts have been used.

Manuscripts containing **animal experiments** must include a permission statement which was obtained from the relevant national or local authorities. The institutional committees that have approved the experiments must be identified and the **accreditation number** of the laboratory or of the investigator given

where applicable. If no such rules or permissions are in place in the country where the experiments were performed, then this must also be clearly indicated.

Chemistry & Biodiversity will only consider manuscripts in which conclusions are based on adequate **statistical analysis**.

The description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and *P* values, the number *n* of independent experiments underlying each data point (not replicate measures of one sample), and the actual *P* value for each test (not merely 'significant' or '*P* < 0.05'). Discussion of statistical methodology can be reported in the *Experimental Section*, but figure and table **legends should contain a basic description of data presentation (mean $\pm$ SD or SEM), *n*, *P*, and the test applied**.

Descriptive statistics should include a clearly labelled measure of centre (such as the mean or the median), and a clearly labelled measure of variability (such as standard deviation or range). Ranges are more appropriate than standard deviations or standard errors for small data sets. Standard error or confidence interval is appropriate to compare data to a control.

We recommend that the actual individual data from each experiment should be plotted if *n* < 5, alongside an error bar. In cases where *n* is small, a justification for the use of the statistical test employed has to be provided. Presenting a single 'typical result' of *n* experiments is sometimes unavoidable, but should be accompanied by an indication of the variability of data between independent experiments. If *n* is not based on independent experiments (that is, *n* merely represents replicates of a measurement), statistics may still be useful, but a detailed description of the repeated measurement is required.

Use of the word 'significant' should always be accompanied by a *P* value; otherwise, use 'substantial', 'considerable', etc.

For more information on the appropriate use of standard deviation, standard error, and confidence intervals please refer to *Cumming et al.*

Compounds should follow accepted guidelines when represented as '**active**'. For example, the cytotoxic effect of a pure substance when tested against a cancer cell line would exhibit an **IC₅₀ value of < 10 μ M**. In each case, positive controls (reference compounds) have to be used and the **dose-activity dependence** should be shown. Authors should pay attention to the following definitions: Compounds that suppress the growth of or kill isolated tumor cell lines grown in culture should be referred to as either '**cytostatic**' or '**cy-**

tototoxic, as appropriate. Only compounds that inhibit the growth of tumors in animal-based models should be called '**antitumor**'. The term '**anticancer**' should be reserved for compounds that show specific activity in human-based clinical studies.

References. The citations should be up to date and informative, but not excessive. The list of references should be numbered **sequentially** in the order as they appear in the text including table and figure legends. The numbers should be set in brackets, thus [2], [3][14], or [5–8]. References are to be collected in numerical order at the end of the main text. Titles of journals must be abbreviated according to *Chemical Abstracts* (cf. *Chemical Abstracts Service Source Index (CASSI)*). After the authors' names, the title of the article (in simple 'quotation marks') should follow. Then, the journal title (in italics) should be followed (no comma) by the year of publication (in boldface), comma, volume number (in italics), comma, first page till last page, period.

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Examples of references to book chapters,^[6] books,^[7] patents,^[8] computer programs,^[9] Ph.D. theses,^[10] and electronic sources^[11] are also given.

For users of **Endnote**, please download the *Helvetica Chimica Acta Endnote Style-File* and put it in your Styles' directory on your computer.

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In the text, reference to author(s) of cited works should be made without giving initials, e. g., '... as shown by Kamata and Wasada^[8]'. If the reference carries the names of three or more authors it should be quoted as '*Bourgeois et al.*^[4]', if *Bourgeois* is the first author, or as '*Diederich and coworkers*^[4]', if *Diederich* is the senior author.

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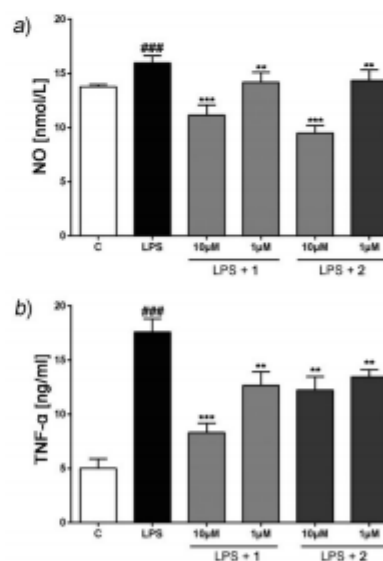


Figure 1. Effects of **1** and **2** on NO a) and TNF- α b) expression in LPS-stimulated RAW264.7 cells. Data represent the mean values of three experiments ($\pm$ SD). *** $P < 0.001$, significant as compared to the control group. ** $P < 0.01$, *** $P < 0.001$ compared to the group treated with LPS alone.

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