The synthesis and evaluation of dual inhibitors of monoamine oxidase and catechol-O-methyltransferase

I Engelbrecht

orcid.org 0000-0001-8007-1594

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Promoter: Dr A Petzer
Co-promoter: Prof JP Petzer

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Student number: 21639159

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Preface

This thesis is submitted in article format and consists of three original research articles as separate entities. The written declaration from the co-authors of the research articles is included.

All scientific research conducted for this thesis (synthesis of compounds, the biological evaluation of the synthesised, natural and library compounds, writing of the thesis as well as the articles presented) was conducted by Miss I. Engelbrecht at the North-West University, Potchefstroom campus. Guidance and assistance in the preparation of this thesis was provided by the promoter and co-promoter, Prof. Anél Petzer and Prof. Jacques Petzer. Mr. André Joubert and Dr. Johan Jordaan from the SASOL Centre for Chemistry, North-West University, recorded the NMR and MS spectra. Support with the HPLC purity analyses of the synthesised compounds was provided by Prof. Jan du Preez of the Analytical Technology Laboratory, North-West University. Support with the preparation of liver tissue for the COMT activity measurements was provided by Mrs. Sharlene Lowe of Pharmacen, North-West University. Assistance with the COMT activity measurements was provided by Miss Denise Prinsloo, fellow postgraduate student at Pharmaceutical Chemistry, North-West University.

Declaration

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Chemistry, at the School of Pharmacy, North-West University.

I, Idalet Engelbrecht, hereby declare that the thesis with the title: "**The synthesis and evaluation of dual inhibitors of monoamine oxidase and catechol-O-methyltransferase**" is my own work and has not been submitted at any other University either whole or in part.

I. Engelbrecht

Signed at Potchefstroom on the 8 day of November, 2017.

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Letter of permission

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Department of Pharmaceutical Chemistry

Tel: +27 18 299 4464

Fax: +27 18 299 4243

Email: 12264954@nwu.ac.za

8 November 2017

To whom it may concern,

Dear Sir/Madam,

CO-AUTHORSHIP ON RESEARCH PAPERS

The undersigned, as co-authors of the research papers listed below, hereby give permission to Miss Idalet Engelbrecht to submit the papers as part of the degree Doctor of Philosophy in Pharmaceutical Chemistry at the North-West University, Potchefstroom campus:

- **The synthesis and evaluation of nitrocatechol derivatives of chalcone as dual inhibitors of monoamine oxidase and catechol-O-methyltransferase**
- **The evaluation of selected natural compounds as potential dual inhibitors of catechol-O-methyltransferase and monoamine oxidase**
- **The evaluation of structurally diverse monoamine oxidase inhibitors as potential dual inhibitors of catechol-O-methyltransferase**

Yours sincerely,

Petzer

Prof. A. Petzer

Prof. J.P. Petzer

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List of abbreviations

3-OMD	3-O-Methyldopa
6-OHDA	6-Hydroxydopamine
AADC	Aromatic-L-amino acid decarboxylase
APCI	Atmospheric-pressure chemical ionisation
Asn	Asparagine
Asp	Aspartartic acid
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
COMT	Catechol-O-methyltransferase
Cys	Cysteine
DAT	Dopamine transporter
Depr dialysed	Dialysis of the enzyme in the presence of the irreversible inhibitor selegiline
DNA	Deoxyribonucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid
EDTA	Ethylenediamine tetra-acetate
FAD	Flavin adenine dinucleotide
GABA	γ-Aminobutyric acid
GBA	Glucocerebrosidase
Glu	Glutamic acid
Gly	Glycine
GSH	Glutathione
GSSH	Oxidised glutathione
His	Histidine
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectra
Ile	Isoleucine
iNOS	Inducible nitric oxide synthase
L-Dopa	Levodopa
Leu	Leucine
LID	L-Dopa-induced dyskinesia
LRRK-2	Leucine rich repeat kinase 2
MAO	Monoamine oxidase
Met	Methionine
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NI	Dialysis of the enzyme in the absence of the irreversible inhibitor selegiline
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NWU	North-West University
Phe	Phenylalanine
PINK1	PTEN-induced putative kinase 1
Pro	Proline
ROS	Reactive oxygen species
SAM	S-Adenosyl-L-methionine
SAR	Structure-activity relationship
SD	Standard deviation
Ser	Serine
SI	Selectivity index
Thr	Threonine
TMMP	1-Methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine
TNFα	Tumor necrosis factor-alpha
Trp	Tryptophan
Tyr	Tyrosine
UCHL-1	Ubiquitin C-terminal hydrolase L1
UFLC	Ultra fast liquid chromatograph

Abstract

Parkinson's disease is the second most prevalent neurodegenerative disorder after Alzheimer's disease. Parkinson's disease is a debilitating, incurable, bradykinetic disorder which seriously inhibits a patient's quality of life. The aetiology of the disease is still unknown, although it is widely accepted that Parkinson's disease may be caused by a multifactorial cascade of events. The main pathological hallmark is the degeneration of dopaminergic neurons, mainly in the nigrostriatal pathway of the brain. This degeneration subsequently leads to reduced levels of central dopamine, which give rise to the characteristic symptoms pertaining to movement in Parkinson's disease. Parkinson's disease treatment mainly focusses on the elevation of central dopamine levels by either dopamine replacement therapy which consists of either levodopa (L-dopa) and dopamine agonists, or by inhibiting the metabolism of dopamine in the central nervous system through inhibition of either monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT). L-Dopa is still considered the mainstay of Parkinson's disease treatment, but due to extensive metabolism in the periphery by aromatic-L-amino acid decarboxylase (AADC) and COMT, less than 1% of L-dopa reaches the brain unchanged. By inhibiting these metabolic routes of peripheral L-dopa degradation, L-dopa uptake into the brain can be increased, which subsequently elevates central dopamine levels.

COMT catalyses the metabolism of endogenous catecholamines (such as dopamine) and exogenous compounds with a catechol structure. Through inhibition of peripheral COMT, higher levels of L-dopa can enter the brain for conversion to dopamine. In the brain L-dopa-derived dopamine is metabolically inactivated by MAO through oxidative deamination. Thus, MAO inhibition would serve to elevate central dopamine levels by decreasing the metabolism thereof and may serve to be neuroprotective by decreasing the formation of injurious metabolic by-products. Central dopamine may also be metabolically inactivated by COMT present in the central nervous system. Thus, peripheral as well as central COMT inhibition may be beneficial in the treatment of Parkinson's disease. Selected metabolic routes of dopamine and the enzymes involved therein will serve as drug targets in this study in order to discover new inhibitors with dual inhibition of MAO and COMT.

Current treatment options available for the management of Parkinson's disease focus on symptomatic relief with only a limited number of drugs on the market. Thus, there exists a

need for new treatment strategies. This thesis will contribute in this regard by synthesising novel compounds and investigating their inhibitory potencies towards MAO and COMT.

Literature reports that chalcones act as potent reversible MAO-B inhibitors. A series of chalcones were thus synthesised in this study and their IC_{50} values for the inhibition of both isoforms of human MAO (A and B) were determined *in vitro*. The most potent MAO-B inhibitor was (2*E*)-3-(3-bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one with an IC_{50} value of 13.89 μM, while (2*E*)-3-(4-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one was the most potent inhibitor of MAO-A with an IC_{50} value of 32.37 μM. Since COMT inhibitors currently on the market (tolcapone and entacapone) contain the nitrocatechol moiety, this structural feature was also incorporated into the chalcones synthesised in this study. The chalcones were thus also investigated as potential COMT inhibitors with the aim of discovering compounds with dual inhibition activity towards MAO and COMT. Such compounds are known as multi-target-directed inhibitors and may have enhanced value in the management of Parkinson's disease. All of the synthesised 3,4-dihydroxy-5-nitrochalcones displayed potent inhibition activity towards rat liver COMT, with the most potent inhibitor, (2*E*)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(3-methoxyphenyl)prop-2-en-1-one, exhibiting an IC_{50} value of 0.07 μM. Nine compounds of the synthesised series exhibited mixed MAO and COMT inhibitory activities and can thus be classified as multi-target-directed inhibitors. Such inhibitors may be structurally modified in future studies with the aim of designing more potent multi-target-directed inhibitors.

The MAO inhibitory activities of several naturally occurring compounds have been reported. As a second objective, this thesis evaluated selected commercially available natural compounds with unique structures with the aim to discover novel compounds that may act as multi-target-directed inhibitors of MAO and COMT. The inhibitory potencies of the selected natural compounds for MAO and COMT were determined. The most potent MAO inhibitor among the natural compounds is chrysin which inhibits MAO-A with an IC_{50} value of 0.77 μM, while an IC_{50} value 0.79 μM was recorded for MAO-B. The most potent COMT inhibitor among the forty-two natural compounds examined was (+)-catechin with an IC_{50} value of 0.86 μM. Another natural compound, alizarin, also inhibits COMT with an IC_{50} value of 0.88 μM. Three of the forty-two tested compounds exhibit dual inhibition of MAO and COMT. These compounds are morin, fisetin and alizarin, and exhibits potent MAO-A inhibition, in addition to acting as COMT inhibitors. Thus, these compounds can be used as leads in the future design of multi-target-directed inhibitors of MAO and COMT.

Lastly, our research group has discovered numerous synthetic compounds from various chemical classes which act as potent inhibitors of MAO. These compounds were evaluated in the present study as potential inhibitors of COMT, again with the aim of discovering compounds with dual inhibitory activity towards MAO and COMT. Even though none of the compounds selected for this study inhibited COMT, this study demonstrates the importance of the catechol structure for COMT inhibition.

Keywords: Parkinson's disease, dopamine, L-dopa, monoamine oxidase, MAO, catechol-O-methyltransferase, COMT, inhibition, multi-target-directed, chalcone, natural compounds.

Chapter 1
Introduction

1.1. General background

Parkinson's disease is the second most common neurodegenerative disorder (Dauer & Przedborski, 2003; Fahn & Przedborski, 2000). This disease affects 1–2% of the human population over 50 years of age, and the prevalence is expected to double by 2030 (de Rijk *et al*., 1995; Dorsey *et al*., 2007; Jenner *et al*., 2009; Smeyne & Jackson-Lewis, 2005). The primary cause of Parkinson's disease is the death of dopaminergic neurons situated in the substantia nigra pars compacta of the brain (Kaiser *et al*., 2000; Young & Penney, 1993). This results in the depletion of striatal dopamine (Le & Jankovic, 2001; Lees, 2005; Olanow, 2004). Parkinson's disease symptoms only manifest when approximately 60% of the dopaminergic neurons in the substantia nigra pars compacta have degenerated and 70% of responsiveness to dopamine has disappeared (German *et al*., 1989; Ma *et al*., 2002; Uhl *et al*., 1985). Early diagnosis is often not made since the early symptoms of Parkinson's disease are similar to manifestations of normal aging (Pahwa, 2006). The clinical manifestation of the disease consists of a tetrad of symptoms namely tremor at rest, slowness of movement or bradykinesia, rigidity and postural instability or gait impairment (Braak *et al*., 2003; Foley *et al*., 2000; Lees, 2005). Apart from the distinct motor symptoms, Parkinson's disease is also characterised by non-motor symptoms such as cognitive dysfunction, fatigue, sleep disturbances, autonomic dysfunction and anosmia (Rao *et al*., 2006; Schwarzschild *et al*., 2006; Yacoubian & Standaert, 2009). These non-motor symptoms represent an area of unmet therapeutic need. Non-motor complications do not respond to dopaminergic innervation and thus prove to be treatment challenging in the management of Parkinson's disease (Braak *et al*., 2003; Chaudhuri *et al*., 2006; Yacoubian & Standaert, 2009). Currently, no drugs have been approved for neuroprotective use in Parkinson's disease, and disease progression thus remains untreated (Jenner *et al*., 2009).

The current treatment options available for Parkinson's disease focuses on symptomatic treatment rather than the prevention of neurodegeneration (Ahlskog & Muenter, 2001; Dauer & Przedborski, 2003). Considering that the key molecular events leading to neurodegeneration have not been firmly established, development of neuroprotective therapies have been limited. In 1958 Arvid Carlsson discovered dopamine in the mammalian

brain, which accelerated the pace of discovering novel treatment strategies for Parkinson's disease (Dauer & Przedborski, 2003). Since dopamine is a neurotransmitter that is involved in motor neuron stimulation, the motor nervous system will be incapable to control movement and coordination if dopamine levels are depleted (Kiss & Soares-da-Silva, 2014; Orth & Schapira, 2002). Almost all therapies currently used in the treatment of Parkinson's disease focus on restoring striatal dopamine. This can be accomplished by increasing dopamine supply through levodopa (L-dopa) administration, dopamine receptor stimulation with dopamine agonist treatment, inhibiting dopamine reuptake and inhibiting the enzymes involved in the metabolism of dopamine (Lees, 2005; LeWitt & Nyholm, 2004). The different treatment options currently available for Parkinson's disease include L-dopa, dopamine agonists, aromatic-L-amino acid decarboxylase (AADC) inhibitors, catechol-O-methyltransferase (COMT) inhibitors, anticholinergic agents, monoamine oxidase (MAO) inhibitors and amantadine (Laurencin *et al*., 2016). The treatment of Parkinson's disease usually employs a combination of antiparkinsonian drugs; therefore, polypharmacy is common with disease progression (Tuite & Riss, 2003). The discovery of L-dopa in 1967 transformed the treatment of Parkinson's disease (Barbeau *et al*., 1961; Birkmayer & Hornykiewicz, 1961; Cotzias *et al*., 1969; Sano, 1960). When L-dopa is administered in the initial stages of Parkinson's disease, most motor symptoms subside, and this significantly improves the patient's quality of life (Colosimo & De Michele, 1999; Fahn, 1974; Marsden & Parkes, 1976; Shaw *et al*., 1980). However, after long-term treatment with L-dopa and other dopamine replacement therapies, patients will develop daily motor fluctuations in mobility and involuntary movements termed "dyskinesia" or "L-dopa-induced dyskinesia" (LID) (Ahlskog & Muenter, 2001; Olanow & Jankovic, 2005). Dyskinesia and other motor complications reduce patient function, quality of life and increase treatment costs (Tse, 2006). Although L-dopa is used in conjunction with dopamine agonists in the primary treatment for motor symptoms, COMT inhibitors and MAO inhibitors are employed to control wearing-off, but do not increase on-time or improve LID (Huot *et al*., 2016; Romrell *et al*., 2003).

MAO-B inhibitors such as rasagiline and selegiline can be used as monotherapy in the initial stages of Parkinson's disease or employed as adjunctive therapy to L-dopa (Jankovic & Stacy, 2007; Shoulson, 1998; Weinreb *et al*., 2010). These compounds are proposed to delay disease progression, enhance life span and exert possible disease-modifying effects (Adeyemo *et al*., 1993; Shoulson, 1998). In this regard MAO-B inhibitors increase dopamine concentrations in the brain by reducing the MAO-B-catalysed degradation of dopamine. The potential side effects that may be experienced due to the irreversible mechanism of inhibition

of the abovementioned inhibitors (selegiline and rasagiline), is the driving force for the discovery of novel reversible and selective MAO-B inhibitors (Gaspar *et al*., 2011; Rezak, 2007; Riederer *et al*., 2004). The COMT inhibitors utilised in the treatment of Parkinson's disease include tolcapone, entacapone and nebicapone (Kiss *et al*., 2010; Kiss & Soares-da-Silva, 2014). These COMT inhibitors are clinically useful as adjunctive treatment in Parkinson's disease (Calne, 1993; Männistö & Kaakkola, 1989).

Another debilitating factor of Parkinson's disease is the development of co-morbid disorders such as depression and dementia in elderly patients (Dauer & Przedborski, 2003). Approximately half of all patients suffering from Parkinson's disease present with depression and require antidepressants daily (Paumier *et al*., 2015; Ravina *et al*., 2007). MAO inhibitors exhibit antidepressant action which may be beneficial for patients suffering from Parkinson's disease (Youdim & Bakhle, 2006). When a MAO-B inhibitor such as selegiline is combined with a COMT inhibitor, catecholamine levels may be increased significantly in the brain and alleviate depression (Tom & Cummings, 1998).

MAO and COMT are the two enzymes primarily responsible for the metabolic inactivation of catecholamines in mammalian tissue (Hirsch, 1994; Männistö & Kaakkola, 1999; Yan *et al*., 2002). Thus, dual inhibition of MAO and COMT may be a novel treatment strategy for certain neurological disorders. In Parkinson's disease, centrally acting COMT inhibitors have minimal beneficial effect alone, and thus have to be administered in combination with another inhibitor such as a MAO inhibitor (Learmonth *et al*., 2002; Lees *et al*., 2009; Miyasaki, 2006; Rascol *et al*., 2002). Furthermore, dual inhibition of MAO and COMT may exert a neuroprotective effect. Inhibition of extraneuronal and neuronal MAO or the predominantly glial located COMT, may enhance dopamine levels which increase the biosynthesis of neurotrophic factors. However, COMT inhibition may intensify catecholamine metabolism in neurons by MAO resulting in increased levels of free radicals and oxidative stress. For this reason, centrally active COMT inhibitors should only be used in conjunction with MAO inhibitors in neurodegenerative disorders (Müller *et al*., 1993). Additionally, the inhibition of MAO activity may be neuroprotective by decreasing oxidative stress (Mazzio *et al*., 1998).

Dual inhibition of MAO-B and COMT would be greatly beneficial in L-dopa therapy. COMT inhibition reduces the formation of 3-O-methyldopa (OMD) from L-dopa, which improves the

bioavailability of L-dopa (Bonifácio *et al*., 2002; Deleu *et al*., 2002; Robinson *et al*., 2012). A COMT inhibitor may further enhance the effect of L-dopa by delaying the metabolism of dopamine derived from L-dopa in the brain (Männistö & Kaakkola, 1999). In the periphery L-dopa undergoes its most extensive metabolic breakdown which can be reduced by COMT inhibition (Learmonth & Freitas, 2002). Inhibition of either MAO-A or MAO-B alone does not significantly alter the central dopamine levels, but a rise in dopamine levels can only be observed when both isoforms are inhibited. Thus, dual MAO-A and MAO-B inhibitors may be of value in future therapies (Green *et al*., 1977; Riederer & Youdim, 1986; Youdim *et al*., 2006). Although non-selective MAO inhibition may represent an attractive strategy to enhance central dopamine levels in Parkinson's disease, these non-selective MAO inhibitors should display a reversible mode of inhibition, since irreversible inhibition of MAO-A may lead to serious adverse effects such as the "cheese reaction" (Da Prada *et al*., 1988; Di Monte *et al*., 1996; Finberg *et al*., 1998; Youdim & Weinstock, 2004).

1.2. Structures known to inhibit MAO and COMT

1.2.1. Chalcones: Chalcones (1,3-diphenyl-2-propen-1-ones) has emerged as a valid scaffold in the design and development of novel potent MAO-B inhibitors (Choi *et al*., 2015; Gao *et al*., 2001; Haraguchi *et al*., 2004; Mathew *et al*., 2015; Morales-Camilo *et al*., 2015; Tanaka *et al*., 1987). Chalcones are found in nature and are the precursors of flavonoid biosynthesis as well as of coumarins (Batovska & Todorova, 2010; Helguera *et al*., 2013). Chalcones consist of open-chain flavonoids with two aromatic rings and an α,β-unsaturated carbonyl system (Dimmock *et al*., 1999; Go *et al*., 2005). The various biological activities of chalcones have been recorded (Batovska & Todorova, 2010; Dimmock *et al*., 1999; Go *et al*., 2005). Chalcones have antifungal, antimalarial, anticancer, antilipedemic, antiviral and anti-inflammatory action, and may also be neuroprotective (Batovska *et al*., 2007; Kim *et al*., 2012; Lahtchev *et al*., 2008; Nobre-Júnior *et al*., 2009; Trivedi *et al*., 2007). The MAO inhibitory activity of chalcones and related chalcone analogues such as furanochalcones has previously been reported (Chimenti *et al*., 2009a; Robinson *et al*., 2013). Literature reports that, with appropriate substitution, most chalcones are potent, reversible and selective inhibitors of MAO-B (Fioravanti *et al*., 2010; Gökhan-Kelekçi *et al*., 2009; Kneubühler *et al*., 1995; Pisani *et al*., 2009; Prins *et al*., 2010; Wouters *et al*., 1997). A potent chalcone-derived MAO-B inhibitor, (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-4-methoxyphenyl)prop-2-en-1-one, was synthesised by Chimenti and colleagues (Chimenti *et al*., 2009a). This compound possesses an IC_{50} value for the inhibition of human MAO-B of 0.0044 μM. Accordingly, the synthesis of novel chalcones has yielded a number of potent MAO-B selective inhibitors.

Robinson and co-workers synthesised a series of furanochalcones to determine the potential effect of heterocyclic substitution on MAO inhibition potency. The resulting furanochalcones proved to be potent and selective MAO-B inhibitors with the most potent compound, [2(*E*)-3-(5-chlorofuran-2-yl)-1-(3-chlorophenyl)prop-2-en-1-one], displaying an IC_{50} value of 0.174 µM for the inhibition of MAO-B (Robinson *et al*., 2013). Chalcones with hydroxy or methoxy groups in the C2 and C5 (or C6) positions of aromatic ring A have moderate inhibitory activity against MAO, while substitutions on the second aromatic ring, ring B, with an electron-withdrawing group (CF_3, F or Cl) results in higher inhibitory activity against human MAO-B than substitution with electron-donating groups (OMe) (Choi *et al*., 2015; Morales-Camilo *et al*., 2015). In order to further investigate the potential MAO inhibitory activity of chalcones, a series which is disubstituted with the hydroxy group on ring A in positions 3 and 4, and a nitro group on position 5, will be synthesised in this study. These chalcones are thus nitrocatechol derivatives. The COMT inhibitory activity of nitrocatechol compounds has previously been reported, with all the COMT inhibitors available on the market possessing this moiety (Kiss *et al*., 2010; Learmonth *et al*., 2004; Müller, 2015). Since the chalcones of the present study are nitrocatechol derivatives, the possibility exist that these compounds may possess dual MAO-B and COMT inhibitory activities. In this respect, the chalcones are known to inhibit MAO-B while nitrocatechol compounds display inhibition of COMT.

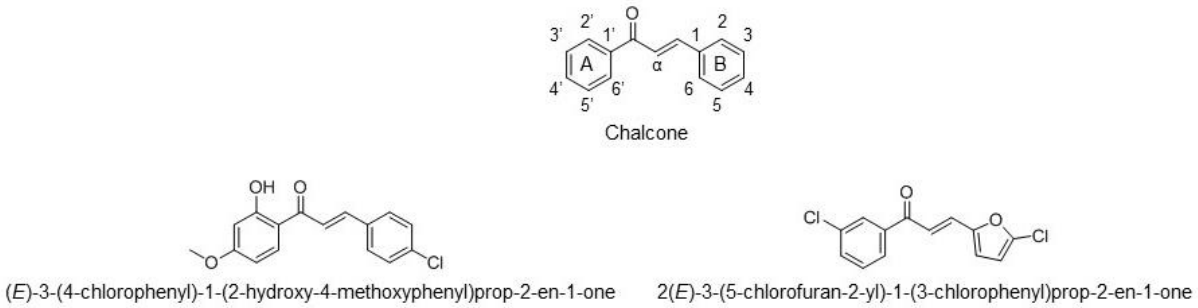

Figure 1.1. The structures of chalcones discussed in text.

1.2.2. Flavonoids, flavones and natural compounds: Flavonoids are a group of polyphenolic compounds which can only be biosynthesised by plants. These polyphenolic compounds' effect on human health is highly dependent upon their chemical, physical and structural properties (Carradori *et al*., 2014). Thus, the pharmacokinetic properties of the polyphenol, especially its lipophilicity and charge, should be considered when utilising it as a chemical entity for specific diseases. In most instances it is essential for the polyphenol to cross the blood-brain barrier to exert a neuroprotective action, and target specificity is thus an important consideration (Essa *et al*., 2012).

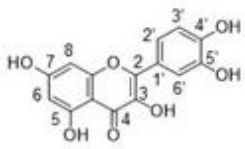

Figure 1.2. The basic structure of a flavonoid.

Flavonoids are well-known for their antioxidant action which may be attributed to the highly reactive hydroxy group in their chemical structure, which can be oxidised by electron transfer in order to stabilise free radicals. Literature suggest that flavonoids may delay or even prevent the onset of diseases in which oxidative stress is a causing factor. In addition, flavonoids also have chelating properties which may be of value in the future treatment of diseases where divalent metal ions play a pivotal role (Carradori *et al*., 2014). The biological activity of flavonoids and related structures have been widely reported (Batovska & Todorova, 2010; Dimmock *et al*., 1999; Go *et al*., 2005). Mazzio and colleagues (1998) previously described the potential application of certain flavonoids in Parkinson's disease. The study indicated that dietary compounds can dissipate peroxide production in glial cells by two distinct mechanisms, either by inhibition of MAO or by functioning as free radical scavengers (Mazzio *et al*., 1998). Literature reports that flavonoids such as quercitrin, isoquercitrin, rutin, quercetin, (+)-catechin and (-)-epicatechin, as well as flavones such as apigenin and luteolin and flavononol derivatives such as taxifolin and aromadendrin have weak to moderately potent MAO inhibitory activity. The MAO-B inhibitory activities for the abovementioned compounds range from 3.89–88.6 μM (Carradori *et al*., 2014).

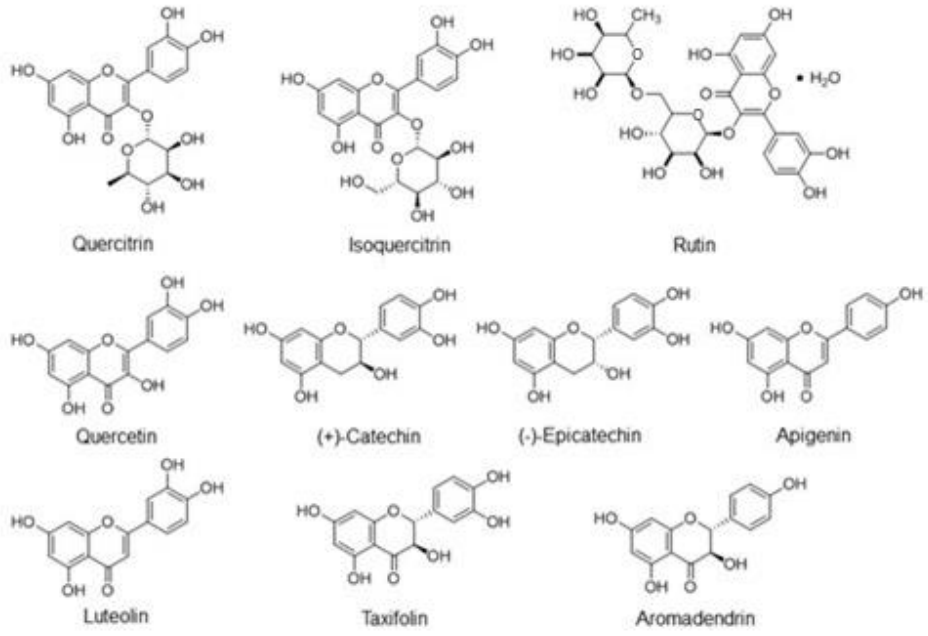

Figure 1.3. The chemical structures of compounds discussed in text.

The MAO inhibitory activity of flavonoids decreases with the presence of hydroxy groups on the B-ring of the flavone. The inhibition activity is dependent upon the presence of a phenyl or hydroxyphenyl ring on the C2 position in conjunction with a double bond in the C2 and C3 positions of the molecule (Carradori *et al*., 2014). Most flavonoids inhibit MAO-B specifically which may be attributed to its coumarin-related structure (Chimenti *et al*., 2006; Chimenti *et al*., 2009a; Chimenti *et al*., 2009b; Chimenti *et al*., 2010; Secci *et al*., 2011). Flavonoids represent ideal lead compounds for the design of dual inhibitors and provide the pharmacophoric requirements to obtain novel multi-functional agents (Carradori *et al*., 2014). The main feature required for MAO-B inhibition activity is the lipophilic character of the substituents on the phenyl ring of the molecule. In the presence of a sulfonic ester function, the MAO-A inhibitory activity increases (Gnerre *et al*., 2000). Previous studies established that tea catechins exert potent inhibitory activity towards COMT (Chen *et al*., 2005; Kang *et al*., 2013; Lu *et al*., 2003; Nagai *et al*., 2004; van Duursen *et al*., 2004).

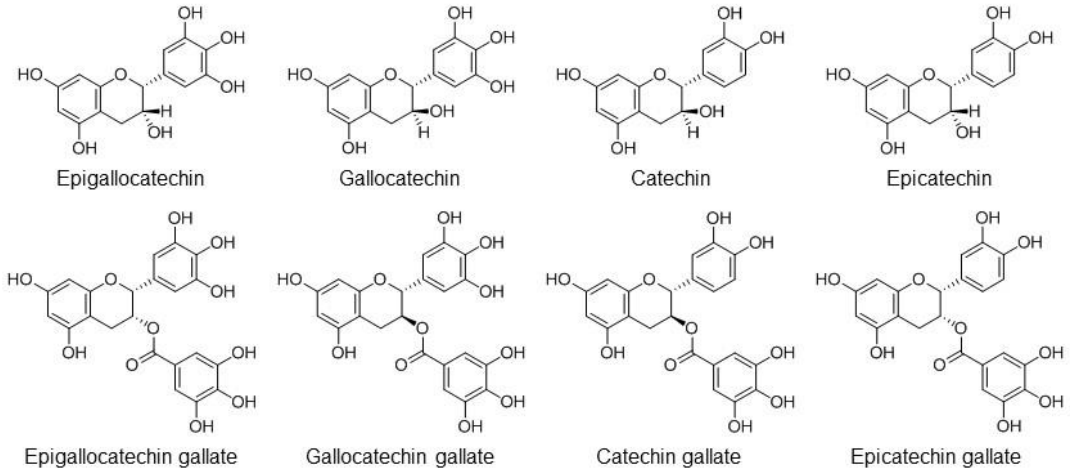

Figure 1.4. The chemical structures of some tea catechins and flavonoids known to inhibit COMT.

It is thus postulated that compounds with a similar polyphenol catechol structure, such as flavonoids, will also exhibit strong COMT inhibition activity. In fact, flavonoids have been shown to inhibit COMT (Guldberg & Marsden, 1975).

In consideration of the limited preventative tools available for delaying Parkinson's disease onset and progression, an intervention approach using antioxidant and flavonoid-rich natural products may be of future importance (Essa *et al*., 2012). The application of natural products

either directly as drugs or as a source of novel lead compounds for the design and development of chemical entities has a long-standing tradition (Chen & Decker, 2013; Newman & Cragg, 2007; Paterson & Anderson, 2005). Several data have been published on the use of natural products as therapeutics and lead compounds in neurodegenerative disorders (Chen & Decker, 2013; Joyner & Cichewicz, 2011; Williams *et al*., 2011). A benefit of utilising naturally occurring substances is their capability to interact with multiple targets in the body to obtain expedient advantageous outcomes (Kang *et al*., 2013; Schmidt *et al*., 2008). Based on this, a series of natural compounds with diverse chemical structures will be evaluated as inhibitors of MAO and COMT. While flavonoids and related structures will be included in this study, other natural products will also be considered. Considering the potential of MAO and COMT inhibitors in Parkinson's disease, natural products with dual inhibition may be particularly valuable. In addition, considering the wide range of biological activities of natural products, some dual MAO/COMT inhibitors indeed may possess additive activities (e.g. antioxidant) relevant to the treatment of Parkinson's disease.

1.2.3. Nitrocatechol compounds: Since COMT is a rather malleable enzyme, it is a difficult drug target (Ehler *et al*., 2014). It has been shown that a wide variation of flavonoids with different basic structures can inhibit COMT with the most powerful inhibitors containing the catechol structure. Flavonoids devoid of a 3-hydroxy group exhibit a less pronounced but significant inhibition while flavonoids lacking neighbouring hydroxy groups undergo no COMT catalysis but inhibit COMT in a mixed type non-competitive manner (Guldberg & Marsden, 1975; Schwabe & Flohé, 1972).

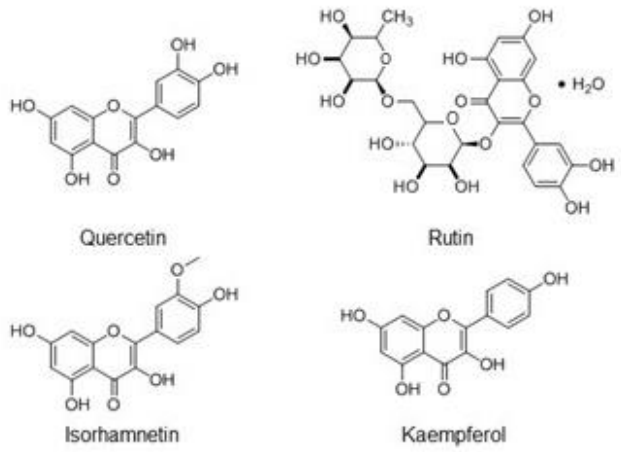

Figure 1.5. The chemical structures of flavonoids with neighboring hydroxy groups exhibiting a catechol structure (quercetin and rutin) and flavonoids devoid of a 3-hydroxy group (isorhamnetin and kaempferol).

Catechols with electronegative substituents such as NO_2, CN or F are potent inhibitors and poor substrates of COMT (Bäckström *et al*., 1989; Borgulya *et al*., 1989). Catechols with the NO_2 group in another position than the "classic" *ortho* site exhibits COMT inhibition activity that is generally lower than those catechols with the NO_2 in the "classic" position (figure 1.6a). Disadvantages of nitrocatechols are their poor intracellular availability, their weak acidic nature and low water solubility. They also undergo extensive metabolism with only a small fraction excreted intact in the urine (Männistö & Kaakkola, 1999). Compounds with a NO_2 group *ortho* to a hydroxy group (the "classic" position) are superior in potency as COMT inhibitors compared to compounds substituted at other positions (figure 1.6b) (Bäckström *et al*., 1989; Kiss & Soares-da-Silva, 2014). Nitrocatechol compounds bearing a carbonyl group, especially those conjugated with the benzene ring directly or through a carbon-carbon double bond, exhibit potent inhibitory activity (figure 1.6c). COMT inhibitors which have a multiple conjugated system throughout the molecule are highly active. Substitution at R^1, in order to increase lipophilicity, also increases the inhibition activity (figure 1.6a) (Bäckström *et al*., 1989). COMT inhibitors with a nitrocatechol structure are generally poor substrates of the enzyme although they bind well to the active site (Bonifácio *et al*., 2002; Kiss & Soares-da-Silva, 2014). The electronegative nitro group strongly stabilises the ionised catechol-COMT complex and subsequently increases the activation energy for the methylation step of COMT catalysis (Ma *et al*., 2013; Ovaska & Yliniemelä, 1998; Vidgren & Ovaska, 1997).

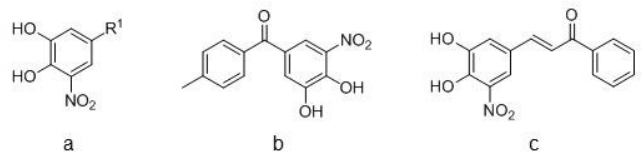

Figure 1.6. The chemical structures of "classic" nitrocatechol compounds that inhibit COMT.

Increasing chain length on nitrocatechol structures has a profound effect on the selectivity and duration of COMT inhibition. The conjugation of an unmodified carbonyl moiety to the 3,4-dihydroxy-5-nitrophenyl pharmacophore, as found in 1-(3,4-dihydroxy-5-nitrophenyl)-2-phenylethanone, is absolutely essential for high and prolonged COMT inhibitory activity. The unsubstituted methylene carbon atom α to the carbonyl group is required to achieve selective peripheral COMT inhibition (Learmonth *et al*., 2002). Compounds with oxygen in the side chains are highly potent and long-acting COMT inhibitors with selectivity towards peripheral COMT (Learmonth *et al*., 2004). The high permeation of tolcapone across the blood-brain barrier may be attributed to the lipophilic 4-methylbenzoyl side chain (Kiss & Soares-da-Silva, 2014).

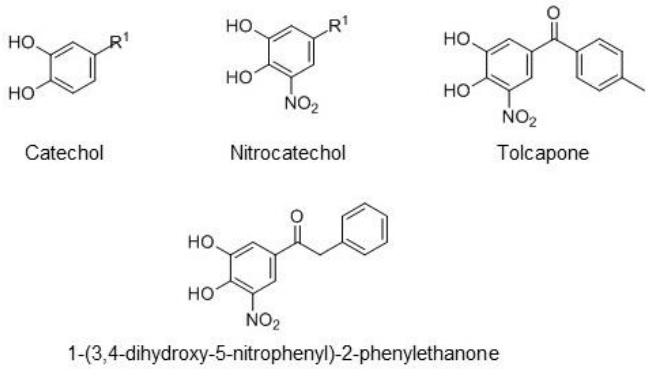

Figure 1.7. The chemical structures of nitrocatechol compounds discussed in text.

Compounds such as tropolones, which are isosteric with catechols, also inhibit COMT, but are short-acting and toxic to man (Belleau & Burba, 1963; Mavridis *et al*., 1963; Ross & Haljasmaa, 1964). 8-Hydroxyquinoline is 1.5-fold more potent as a COMT inhibitor than pyrogallol (Ross & Haljasmaa, 1964). 8-Hydroxyquinolines inhibit COMT in a non-competitive manner. One of the hydroxy groups on the catechol rings of 8-hydroxyquinolines may be substituted with a heteroatom without losing COMT inhibition activity. This substitution results in a reversible and tight-binding inhibitor (Guldberg & Marsden, 1975).

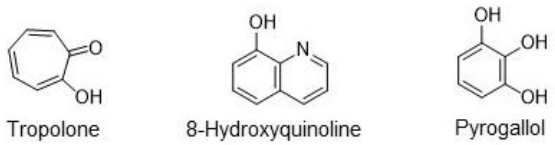

Figure 1.8. The chemical structures of tropolone, 8-hydroxyquinoline and pyrogallol.

1.2.4. Bisubstrate inhibitors: Bisubstrate inhibitors are obtained by covalently linking a substrate analogue and a cofactor analogue (Lerner *et al*., 2003). Bisubstrate inhibitors should incorporate an adenosine as well as a catechol portion in order to bind to both COMT binding pockets (Ma *et al*., 2013). In relation to bisubstrate COMT inhibitors which transverses both cavities, rigidification of the spacer by incorporating a double bond has a tremendous effect on the binding affinity of the inhibitor (Lerner *et al*., 2001; Lerner *et al*., 2003). The flexibility of the side chains of Met40 and Trp143 should be taken into account when designing bisubstrate inhibitors (Lerner *et al*., 2001).

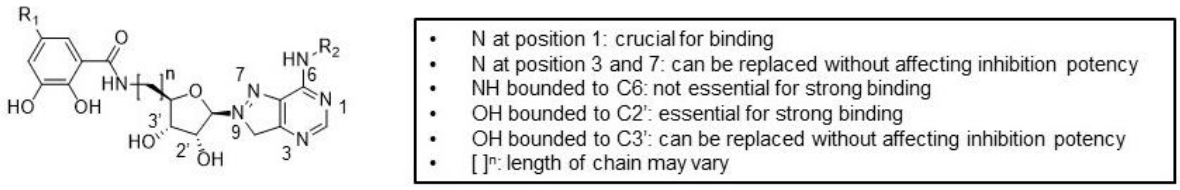

Figure 1.9. A summary of the structure-activity relationships of bisubstrate inhibitors of COMT.

1.3. Hypothesis of the study

It is postulated that a series of 3,4-dihydroxychalcone derivatives containing a 5-nitro moiety will exhibit potent MAO and COMT inhibitory activity, in accordance with literature which reports that chalcones are potent MAO-B inhibitors while nitrocatechol compounds are COMT inhibitors (Bäckström *et al*., 1989; Chimenti *et al*., 2009a; Choi *et al*., 2015; Gao *et al*., 2001; Haraguchi *et al*., 2004; Kiss & Soares-da-Silva, 2014; Mathew *et al*., 2015; Morales-Camilo *et al*., 2015; Robinson *et al*., 2013; Tanaka *et al*., 1987). It is further hypothesised that selected natural compounds exhibiting a flavonoid-like structure would display MAO inhibition activity as well as COMT inhibition activity as described in literature (Batovska & Todorova, 2010; Carradori *et al*., 2014; Chen *et al*., 2005; Chimenti *et al*., 2006; Chimenti *et al*., 2010; Secci *et al*., 2011; Essa *et al*., 2012; Guldberg & Marsden, 1975; Kang *et al*., 2013; Lu *et al*., 2003; Mazzio *et al*., 1998; Nagai *et al*., 2004; van Duursen *et al*., 2004). Flavonoids have been reported to inhibit MAO and COMT, although dual activity has not been described. Furthermore, it is postulated that natural compounds that are not closely related to flavonoids could be discovered which display dual MAO/COMT inhibition. Since COMT is a malleable enzyme (Ehler *et al*., 2014), it is postulated that several novel compounds with distinct chemical structures may be possible inhibitors of COMT. In this respect, a series of previously synthesised compounds with established MAO inhibitory activity, will be screened for potential COMT inhibititory activity.

1.4. Aims of the study

The focus of this thesis is to discover multi-target-directed drugs to improve the future treatment of Parkinson's disease. The multi-target-directed strategy is applied to two enzymes involved in the metabolism of dopamine, namely MAO and COMT. This thesis aims to design or discover novel MAO inhibitors with non-selective and reversible modes of

action with accompanied COMT inhibitory activity. In accordance to the discussion above, the objectives of the study are:

- In the present study, 3,4-dihydroxy-5-nitrochalcone analogues will be synthesised and evaluated as potential dual MAO and COMT inhibitors.
- A variety of commercially available natural compounds with divergent structures will be evaluated as potential inhibitors of MAO and COMT.
- Various compounds from a library of compounds (previously synthesised in our laboratory and evaluated as MAO inhibitors) will be evaluated as potential inhibitors of COMT.

1.5. Summary

Parkinson's disease is considered an incurable and progressive degenerative disease (Fahn *et al*., 2004; Lees *et al*., 2009). MAO and COMT are the two enzymes primarily responsible for the metabolic inactivation of catecholamines such as dopamine (Hirsch, 1994; Männistö & Kaakkola, 1999; Mazzio *et al*., 1998; Yan *et al*., 2002). Thus, these enzymes represent valid drug targets for the treatment of Parkinson's disease. When a MAO-B inhibitor such as selegiline is combined with a COMT inhibitor, catecholamine levels may be increased significantly in the brain and thus alleviate depression and motor symptoms associated with Parkinson's disease (Lees *et al*., 2009; Tom & Cummings, 1998). Inhibition of either MAO-A or MAO-B alone, does not significantly alter the central dopamine levels, but a rise in dopamine levels can only be observed when both isoforms are inhibited. Thus dual MAO-A and MAO-B inhibitors may be of value in future therapies (Green *et al*., 1977; Riederer & Youdim, 1986; Youdim *et al*., 2006; Youdim & Bakhle, 2006). The discovery of novel treatment strategies for Parkinson's disease is the main focus of many research groups. The design of novel compounds and the evaluation of known natural and synthetic compounds as potential dual inhibitors of MAO and COMT may therefore lead to drugs that may be of enhanced value in the management of Parkinson's disease.

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Chapter 2

Literature Overview

2.1. General background of Parkinson's disease

James Parkinson was first to describe Parkinson's disease, a syndrome associated with specific neuropathological lesions (Blum *et al*., 2001; Parkinson, 1817). Parkinson's disease is regarded as the second most common degenerative disorder of the aging brain (Dauer & Przedborski, 2003; Fahn & Przedborski, 2000) and affects 1–2% of the human population over 50 years of age (de Rijk *et al*., 1995; Smeyne & Jackson-Lewis, 2005). Parkinson's disease is one of the most common causes of neurological disability affecting 120–190/100000 of the human population (MacDonald *et al*., 2000; Niccolini *et al*., 2015), with an estimated 4 million patients worldwide (Papapetropoulos, 2012; Romrell *et al*., 2003). The median age of onset is 55 to 60 years with the incidence of Parkinson's disease rising steeply with age (Lees *et al*., 2009; Przedborski, 2005). The duration of the disease from diagnosis to death is approximately 15 years (Katzenschlager *et al*., 2008; Lees *et al*., 2009). Parkinson's disease patient numbers are expected to double by 2030 (Dorsey *et al*., 2007; Jenner *et al*., 2009). The clinical picture of the disease consists of a tetrad of symptoms: tremor at rest, slowness of movement or bradykinesia, rigidity and postural instability or gait impairment form the characteristic clinical manifestation of Parkinson's disease (Braak *et al*., 2003; Foley *et al*., 2000a; Lees, 2005). Akinesia is considered the most significant symptom of Parkinson's disease and is characterised by decreased locomotor activity, slowness of movement, awkwardness and freezing (Nomoto, 1996). Patients younger than 40 years frequently present with tremor of the legs, whereas patients over 70 years of age tend to have tremor of the jaw, chin, lips and tongue (Grosset & Lees, 2005; Lees *et al*., 2009).

The primary cause of the development of Parkinson's disease is the death of neuromelanin-containing dopaminergic neurons in the substantia nigra pars compacta (Kaiser *et al*., 2000; Young & Penney, 1993) and resulting in depletion of striatal dopamine (Le & Jankovic, 2001; Lees, 2005; Olanow, 2004). The nigrostriatal dopaminergic pathway consists of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta and whose projecting axons and nerve terminals are located in the striatum (Bové *et al*., 2005; Marsden, 1983; Przedborski, 2005). The striatum is considered the main input and

information processing structure in the basal ganglia. The dorsal part of the striatum contains two subtypes of striatal γ-aminobutyric acid (GABAergic) efferent neurons which give rise to two dorsal efferent systems. The dorsal efferent systems connect the dorsal striatum with the output structures of the basal ganglia, the substantia nigra pars reticulata as well as the internal segment of the globus pallidus. These various structures form the “direct” and “indirect” pathways in the brain (Gerfen, 2004; Müller & Ferré, 2007).

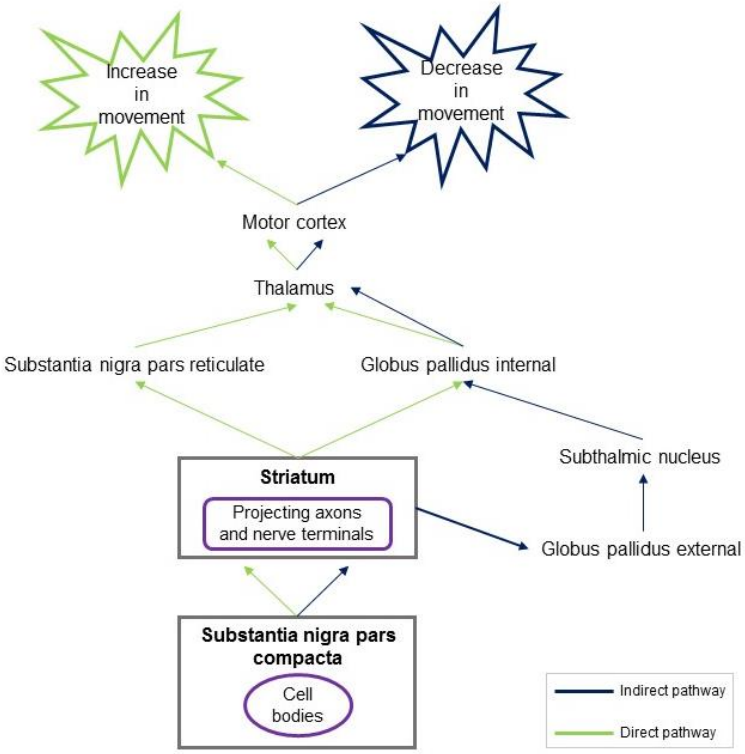

Figure 2.1. The “direct” and “indirect” pathways in the brain.

Parkinson’s disease is considered an incurable and progressive disease (Fahn *et al*., 2004; Lees *et al*., 2009). Clinical manifestation of Parkinson’s disease occurs when approximately 60% of the neurons in the substantia nigra pars compacta have died and 70% of responsiveness to dopamine has disappeared (German *et al*., 1989; Ma *et al*., 2002; Uhl *et al*., 1985). Early diagnosis is difficult since the key Parkinson’s disease symptoms also present as manifestations of normal aging (Pahwa, 2006). Noradrenergic neurons of the locus coeruleus also degenerate early in Parkinson’s disease which may contribute to the development of the syndrome (German *et al*., 1992; Mavridis *et al*., 1991; Zarow *et al*., 2003).

Parkinson's disease is also characterised by non-motor symptoms such as cognitive dysfunction, fatigue, sleep disturbances, autonomic dysfunction (Rao *et al*., 2006; Schwarzschild *et al*., 2006) and anosmia (Yacoubian & Standaert, 2009). An overlooked non-motor complication of Parkinson's disease is oral hygiene. Since patients experience motor complications, maintaining oral hygiene is extremely difficult. This results in disorders like gingivitis, dental disease, diurnal and nocturnal sialorrhea and drooling, xerostomia, orofacial pain, burning mouth syndrome and bruxism (Debowes *et al*., 2013; Zlotnik *et al*., 2015). Non-motor symptoms reflect the neuropathological involvement of non-motor areas of the brain as well as the degeneration of non-dopaminergic neurotransmitter nuclei (Ruitenberg *et al*., 2015; Tremblay *et al*. 2010). These non-motor symptoms represent an area of unmet therapeutic need. In general, non-motor complications do not respond to dopaminergic innervation and thus lead to a major challenge in the management of Parkinson's disease (Braak *et al*., 2003; Chaudhuri *et al*., 2006; Yacoubian & Standaert, 2009). With no drugs currently approved for neuroprotective use in Parkinson's disease, disease progression remains untreated (Jenner *et al*., 2009).

2.2. The treatment of Parkinson's disease

Current treatment available focuses on symptomatic treatment rather than the prevention of dopaminergic neuron degeneration (Ahlskog & Muenter, 2001; Dauer & Przedborski, 2003). A limited understanding of the key molecular events leading to neurodegeneration in the disease presents an obstacle for developing neuroprotective therapies. After Arvid Carlsson's discovery of dopamine in the mammalian brain in 1958, the pace of discovering novel treatment strategies for Parkinson's disease accelerated (Dauer & Przedborski, 2003). Dopamine is a neurotransmitter that is involved in motor neuron stimulation. If dopamine levels are depleted, the motor system nerves are unable to control movement and coordination (Kiss & Soares-da-Silva, 2014; Orth & Schapira, 2002). The marker of a dopaminergic neuron, tyrosine hydroxylase, is also a key enzyme in the production of dopamine (Bowling *et al*., 2008; Calvo *et al*., 2011; Ren *et al*., 2015). Neuronal death occurs at a chronic and slow pace in Parkinson's disease and is responsible for altered neurotransmission of various biogenic amines, of which dopamine is the most important (Müller, 2015a). Dopamine is classified as a monoamine, together with norepinephrine, epinephrine and serotonin (Dorszewska *et al*., 2013; Gainetdinov & Caron, 2003). These monoamines are involved in various physiological processes and are implicated in several clinical conditions which include hypertension (Sjoerdsma, 1961), endogenous depression (Kety, 1966; Schildkraut & Kety, 1967) as well as Parkinson's disease (Guldberg & Marsden,

1975; Hornykiewicz, 1971). Most therapies currently used in the treatment of Parkinson's disease focus on restoring striatal dopamine. Striatal dopamine restoration can be accomplished by increasing dopamine supply through L-dopa administration, dopamine receptor stimulation with dopamine agonist treatment or by inhibiting dopamine reuptake and metabolism (Lees, 2005; LeWitt & Nyholm, 2004).

2.2.1. L-Dopa

The different treatment options currently available for Parkinson's disease include L-dopa, dopamine agonists, COMT inhibitors, anticholinergic agents, MAO inhibitors and amantadine (Laurencin *et al*., 2016). Polypharmacy is common with disease progression and usually employs a combination of antiparkinsonian agents (Tuite & Riss, 2003). The introduction of L-dopa in 1967 revolutionised the treatment of Parkinson's disease (Barbeau *et al*., 1961; Birkmayer & Hornykiewicz, 1961; Cotzias *et al*., 1969; Sano, 1960) and is still considered the mainstay of Parkinson's disease treatment (Colosimo & De Michele, 1999; Huot *et al*., 2016). L-Dopa administered in the initial stages of the Parkinson's disease reduces most motor symptoms and significantly improves the patient's quality of life (Colosimo & De Michele, 1999; Fahn, 1974; Marsden & Parkes, 1976; Shaw *et al*., 1980). After long-term treatment with L-dopa or other dopamine replacement therapies, 40–50% of patients suffering from Parkinson's disease will develop daily motor fluctuations in mobility and involuntary movements termed "dyskinesia" or LID (Ahlskog & Muenter, 2001; Olanow & Jankovic, 2005). Dyskinesia and other motor complications reduce patient function, quality of life and increase treatment costs (Tse, 2006). The mechanisms underlying the development of LID are not clearly understood, which complicates the treatment thereof (Calabresi *et al*., 2010; Niccolini *et al*., 2015). One theory proposes that, as the disease progress and dopaminergic neurons degenerate, the serotonergic nerve terminals present in the brain may misinterpret synaptic dopamine levels. This misinterpretation of neurotransmitter signalling may cause LID (Carta *et al*., 2007; Niccolini *et al*., 2015). Chronic overstimulation of extremely sensitised dopamine D_1 receptors by L-dopa may also lead to LID development (Nagatsua & Sawadab, 2009). Overactive glutamatergic transmission is also believed to contribute to the development of LID. As a result, the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, amantadine, is used for reducing the occurrence of LID (Chen & Swope, 2007; Dekundy *et al*., 2007; Romrell *et al*., 2003). Due to the antagonism of NMDA receptors, amantadine may limit excitotoxic reactions resulting from excessive glutamatergic stimulation and thus may also be neuroprotective (Lees, 2005; Uitti *et al*., 1996). Amantadine has several mechanisms of action other than its action on NMDA receptors. It enhances

dopamine release and blocks dopamine reuptake, and has mild anticholinergic effects (Lees, 2002). It can be used in conjunction with L-dopa or anticholinergic therapy as well as dopamine agonists (Deleu *et al*., 2002; Lees, 2005).

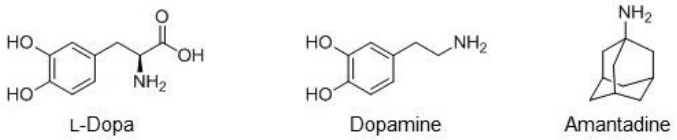

Figure 2.2. The chemical structures of L-dopa, dopamine and amantadine.

L-Dopa in conjunction with dopamine agonists form the primary treatment regime for controlling motor symptoms in Parkinson's disease (Ahlskog & Muenter, 2001; Jenner *et al*., 2009). COMT inhibitors and MAO inhibitors are currently used to control wearing-off, but do not increase on-time or improve LID (Huot *et al*., 2016; Romrell *et al*., 2003; Tse, 2006). Rasagiline, a MAO-B inhibitor, is an alternative to a COMT inhibitor such as entacapone in the treatment of the wearing-off phenomenon. Rasagiline can also be used in combination with entacapone as an approach to delay the onset of and improve the severity of motor complications (Müller, 2014). Entacapone, selegiline (a MAO-B inhibitor) and the combination of entacapone plus selegiline adjunctive to L-dopa and an AADC inhibitor improves clinical disability relative to L-dopa monotherapy (Hauser *et al*., 1998; Lyytinen *et al*., 1997; Lyytinen *et al*., 2000). The development of motor complications with L-dopa treatment, however, is inevitable. Introducing a dopamine agonist early in the treatment, as well as judicious use of L-dopa reduces the incidence of severe dyskinesia (Constantinescu *et al*., 2007; Hauser *et al*., 2007; Hely *et al*., 2005). Surgical approaches such as deep brain stimulation can also be considered to prevent the development of severe dyskinesia (Fox *et al*., 2011; Lang & Widner, 2002; Rascol *et al*., 2015) or for patients which are refractory to pharmaceutical intervention (Pahwa, 2006). Deep brain stimulation activates the subthalmic nucleus which improves the cardinal symptoms of Parkinson's disease and LID. In addition, bilateral pallidal deep brain stimulation considerably improves Parkinson's disease associated generalised dystonia (Yokochi, 2009). New L-dopa formulations such as intrajejunal L-dopa-carbidopa infusion or bilayered extended release L-dopa-carbidopa have been shown to reduce the occurrence of LID (Caceres-Redondo *et al*., 2014; Fernandez *et al*., 2015). A prodrug of L-dopa, L-dopa-α-lipoic acid, results in continuous dopaminergic stimulation which avoids the development of motor complications (D'Aurizio *et al*., 2011; Di Stefano *et al*., 2006).

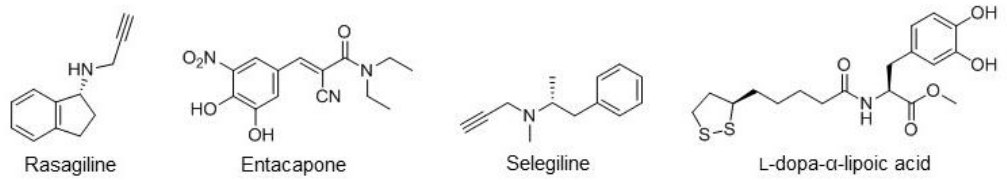

Figure 2.3. The chemical structures of rasagiline, entacapone, selegiline and L-dopa-α-lipoic acid.

2.2.2. Dopamine agonists

Dopamine agonists all act on dopamine D_2-like receptors. Antiparkinsonian activity is associated with postsynaptic D_2 receptor stimulation while presynaptic stimulation has been claimed to have neuroprotective effects (Deleu *et al*., 2002; Lees, 2005). Motor activation by dopamine and dopamine agonists are mediated by stimulating the direct pathway and depressing the indirect pathway in the brain (Agnati *et al*., 2003; Gerfen, 2004; Müller & Ferré, 2007). In contrast to L-dopa, dopamine agonists do not require carrier-mediated transport for absorption into the brain, nor do they produce potentially toxic metabolites or free radicals (Deleu *et al*., 2002; Lees, 2005). Dopamine agonists possess long elimination half-lives relative to L-dopa, which may be deemed an advantage (Lees, 2005; Pfeiffer, 2002). As monotherapy, dopamine agonists do not cause dyskinesia or severe "on-off" effects (Holloway *et al*., 2004; Lees, 2005). As adjunctive treatment to L-dopa, dopamine agonists allow for the reduction of the efficacious L-dopa dose. Treatment with dopamine agonists as initial antiparkinsonian therapy is considered valuable in patients under 50 years of age (Lees, 2005; Rascol *et al*., 2002; Romrell *et al*., 2003). Dopamine therapy in older patients should be initiated with care (Chan, 2003), since these patients experience a higher rate of intolerable adverse effects which include hallucinations, orthostatic hypotension, somnolence and oedema (Lees, 2005; MacMahon, 2003; Pahwa, 2006). Psychosis and dopamine dysregulation syndromes caused by dopamine agonist treatment can be treatment limiting (Jenner *et al*., 2009; Yamamoto & Schapira, 2008). This further stress the need for novel treatment strategies (Ruitenberg *et al*., 2015). Dopamine agonists are divided into two groups: ergoline agonists with an ergot-like structure, and nor-ergoline dopamine agonists. Bromocriptine, cabergoline, lisuride and pergolide are the most common ergoline dopamine agonists in use (Lees, 2005).

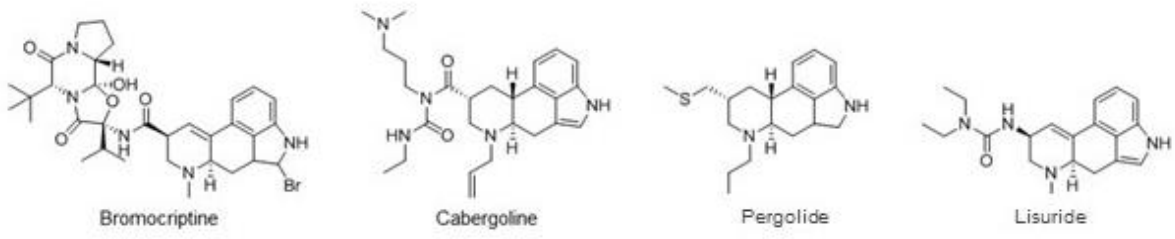

Figure 2.4. The chemical structures of ergoline dopamine agonists.

Nor-ergoline dopamine agonists include apomorphine, pramipexole, ropinirole and piribedil. In early Parkinson's disease therapy, pergolide, pramipexole and ropinirole can be used as monotherapy while cabergoline, pergolide and pramipexole are used as adjunctive therapy in patients receiving L-dopa. In patients experiencing L-dopa-induced motor fluctuations and dyskinesia, pergolide, pramipexole and ropinirole can be introduced as adjunctive therapy. *In vitro* animal studies suggest that pergolide, pramipexole and ropinirole may have neuroprotective properties (Bhatia *et al*., 2001; Le & Jankovic, 2001; LeWitt & Taylor, 2008; Rascol *et al*., 2002).

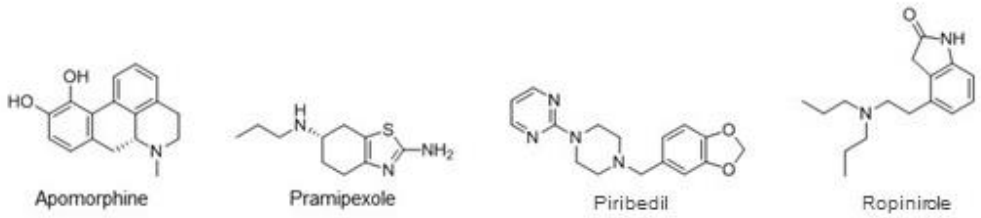

Figure 2.5. The chemical structures of nor-ergoline dopamine agonists.

2.2.3. MAO inhibitors

MAO-B inhibitors such as rasagiline and selegiline are used as monotherapy in the initial stages of Parkinson's disease or as adjunctive therapy to L-dopa (Jankovic & Stacy, 2007; Shoulson, 1998; Weinreb *et al*., 2010). These compounds are thought to delay disease progression, enhance life span and have possible disease-modifying effects (Adeyemo *et al*., 1993; Shoulson, 1998). For example, selegiline may possess protective properties by scavenging the hydroxyl radical (Wu *et al*., 1996) and by inducing the activity of the catalase enzyme (Kitani *et al*., 1994). Thus, selegiline may reduce the formation of excessive toxic radicals and subsequently protect against neurodegeneration. Selegiline also retards the necessity for L-dopa treatment in early Parkinson's disease (Knoll, 1993; Mazzio *et al*., 1998). In this regard MAO-B inhibitors increase dopamine concentrations in the brain by

reducing the MAO-B-catalysed degradation of dopamine. The potential side effects that may be experienced due to the irreversible mechanism of inhibition of the abovementioned inhibitors (selegiline and rasagiline), is the driving force for the discovery of novel reversible and selective MAO-B inhibitors (Gaspar *et al*., 2011; Rezak, 2007; Riederer *et al*., 2004).

2.2.4. COMT inhibitors

First generation COMT inhibitors are poorly selective and have poor efficacy *in vivo* (Ericson, 1971; Kiss *et al*., 2010; Zhu *et al*., 1994a). Compounds such as pyrogallol (Crout, 1961), tropolone (Belleau & Burba, 1963) and gallic acid competitively inhibit COMT, but due to their unfavourable pharmacokinetic properties and questionable efficacies against cerebral catecholamine metabolism, none of the first generation COMT inhibitors have advanced into clinical development (Guldberg & Marsden, 1975).

Figure 2.6. The chemical structures of the first generation COMT inhibitors.

Second generation COMT inhibitors include tolcapone, entacapone and nebicapone (Kiss *et al*., 2010; Kiss & Soares-da-Silva, 2014). The second generation COMT inhibitors are clinically useful as adjunctive treatment in Parkinson's disease (Calne, 1993; Männistö & Kaakkola, 1989). Entacapone is short-acting and acts only in the peripheral tissues (Keranen *et al*., 1994), while tolcapone is more potent, longer-acting and act both centrally and peripherally (Assal *et al*., 1998; Männistö & Kaakkola, 1999). Tolcapone is used with caution in the clinic due to the risk of hepatotoxicity (Assal *et al*., 1998; Kiss *et al*., 2010; Männistö & Kaakkola, 1999). Nebicapone is a longer acting inhibitor of peripheral COMT compared to entacapone and has more limited access to the central nervous system than tolcapone (Kiss *et al*., 2010). Nitecapone, another second generation COMT inhibitor, is a tight-binding inhibitor which is strictly confined to the periphery with no risk of acute toxicity. Entacapone is longer acting than nitecapone, but due to inadequate physiochemical properties it has limited therapeutic application (Männistö & Kaakkola, 1999; Parashos *et al*., 2004). Dopaminergic and gastrointestinal tract adverse effects are the most common disadvantages associated with entacapone and tolcapone. The most commonly observed dopaminergic adverse effect is the worsening of LID, while nausea, vomiting, orthostatic

hypotension, sleep disorders and hallucinations are rare (Männistö & Kaakkola, 1999). The most common non-dopaminergic adverse effect is diarrhoea (Robinson *et al*., 2012). Opicapone is the only COMT inhibitor currently in phase III clinical trials (Ferreira *et al*., 2012; Kiss & Soares-da-Silva, 2014; Lees *et al*., 2012).

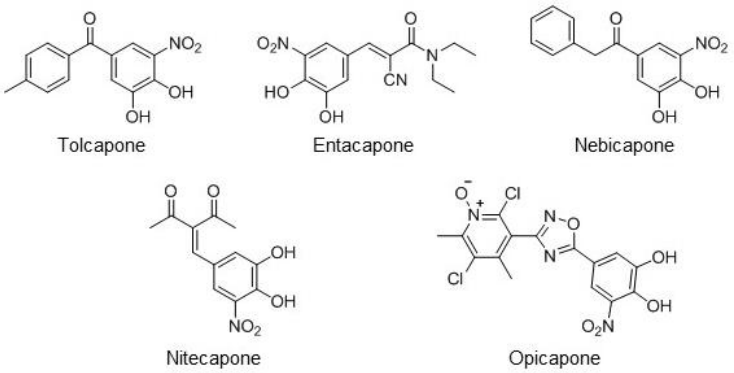

Figure 2.7. The chemical structures of second generation COMT inhibitors.

2.2.5. Adenosine A_{2A} receptor antagonists

Drugs that target non-dopaminergic systems have emerged as promising add-on therapy (Müller & Ferré, 2007). Adenosine A_{2A} receptor antagonists, which target non-dopaminergic systems, can be used to provide greater benefit in patients with advanced Parkinson's disease (Müller & Ferré, 2007; Vorovenci & Antonini, 2015). Adenosine A_{2A} receptor localisation in the basal ganglia is restricted to GABAergic neurons of the indirect pathway (Augood & Emson, 1994; Ciruela *et al*., 2006; Rosin *et al*., 2003). Overexpression of adenosine A_{2A} receptors is associated with the development of LID. Adenosine A_{2A} receptor antagonists block A_{2A} receptors and striatopallidal neurons, dampening their excessive activity (Mori & Shindou, 2003) and restoring balance between striatonigral and straitopallidal neurons, which relieves thalomo-cortical activity (Jenner *et al*., 2009).

2.2.6. Anticholinergic therapy

Drugs targeting the acetylcholine receptor with the subsequent inhibition of the effects of the neurotransmitter acetylcholine, was the leading treatment option for Parkinson's disease until the discovery of L-dopa in 1960. Anticholinergics can be classified as either antinicotinic or antimuscarinic agents depending on the receptor they target (Giugni & Rodriguez-Cruz, 2016; Standaert & Roberson, 1995). Antimuscarinic drugs that have an effect centrally, such as trihexyphenidyl, benztropine and procyclidine, are used for the treatment of Parkinson's

disease and were the first synthetic drugs used for Parkinson's disease treatment (Giugni & Rodriguez-Cruz, 2016; Schwabe & Tillmann, 1949). These drugs act by correcting the disequilibria between striatal dopamine and acetylcholine activity (Lees, 2005), considering that the dopaminergic and cholinergic systems are closely related and should be in perpetual equilibrium (Aosaki *et al*., 2010; Giugni & Rodriguez-Cruz, 2016). Anticholinergic therapy is successfully applied in the treatment of tremor in Parkinson's disease (Lees, 2005) as well as sialorrhea and urinary urgency (Romrell *et al*., 2003). The most commonly used anticholinergics are trihexyphenidyl (benzhexol), benztropine, orphenadrine and procyclidine, although adverse effects limit their use (Lees, 2005).

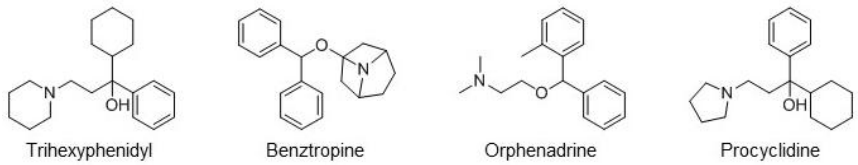

Figure 2.8. The chemical structures of anticholinergic therapy used in Parkinson's disease.

2.2.7. Antidepressant drugs

Parkinson's disease results in common co-morbid disorders such as depression (Paumier *et al*., 2015; Ravina *et al*., 2007) while dementia is significantly more frequent in elderly patients (Dauer & Przedborski, 2003). Risk factors associated with developing depression in Parkinson's disease include right-sided hemiparkinsonism, akinesia and increased severity of disability, anxiety and psychosis. Early onset Parkinsonism, atypical Parkinson's disease, the use of L-dopa and gender can also contribute to the risk for depression (Tom & Cummings, 1998). Almost half of all patients suffering from Parkinson's disease present with depression and require antidepressants daily (Paumier *et al*., 2015; Ravina *et al*., 2007). Studies have shown that psychopharmacological agents such as antidepressants modulate certain signalling pathways involved in cell survival and plasticity (Nibuya *et al*., 1995; Nibuya *et al*., 1996; Xu *et al*., 2003). Such changes at cell level may induce neuroprotection in neurodegenerative diseases such as Parkinson's disease. Parkinson's disease associated depression is effectively treated with tricyclic antidepressants such as amitriptyline (Chung *et al*., 2010; Menza *et al*., 2009). Amitriptyline also delays the need to initiate dopaminergic therapy in early Parkinson's disease thus confirming it has disease-modifying abilities (Paumier *et al*., 2012; Paumier *et al*., 2014). In the intact nigrostriatal system, amitriptyline stimulates brain-derived neurotrophic factor (BDNF) to a higher extent than glial cell-line derived neurotrophic factor (Paumier *et al*., 2015). BDNF, nerve growth factor and neurotrophin 3 promote neuron survival and function in the peripheral and central nervous

system. Dopamine and other biogenic amines induce the biosynthesis of these neurotrophic factors in glial and neuronal cells (Müller *et al*., 1993).

Certain antidepressants also possess anti-inflammatory capabilities such as fluoxetine, which may also be neuroprotective in Parkinson's disease (Chung *et al*., 2011; Sanchez-Guajardo *et al*., 2015; Zhang *et al*., 2012). MAO inhibitors exhibit antidepressant action which may be beneficial for patients suffering from Parkinson's disease (Youdim & Bakhle, 2006). For example, selegiline enhances catecholamine levels in the central nervous system which may improve depression (LeWitt & Taylor, 2008; Tom & Cummings, 1998). When a MAO-B inhibitor such as selegiline is combined with a COMT inhibitor, catecholamine levels may be increased significantly in the brain and alleviate depression (Tom & Cummings, 1998). Normally MAO inhibitors exhibit antidepressant activity through the selective inhibition of MAO-A in the central nervous system (Youdim *et al*., 2006), which elevate central dopamine levels (Youdim & Bakhle, 2006). Non-selective MAO inhibitors (phenelzine and tranylcypromine) are, however, still used clinically (Mertens *et al*., 2014; Youdim *et al*., 2006) along with MAO-A selective inhibitors such as moclobemide, befloxatone and toloxatone. These inhibitors are particularly effective in the treatment of depression in elderly patients (Gareri *et al*., 2000), as well as phobic anxiety and atypical depression (Galter *et al*., 2003). The use of MAO inhibitors with uptake inhibitors such as tricyclic antidepressants or serotonin-selective reuptake inhibitors should be avoided as this combination may provoke the "serotonin syndrome", a severe adverse reaction (Boyer & Shannon, 2005; Youdim & Bakhle, 2006). Using animal models of depression, it was established that COMT inhibition also may lead to cognition improving effects and may have antidepressant effects (Bonifácio *et al*., 2002; Männistö *et al*., 1995; Moreau *et al*., 1994).

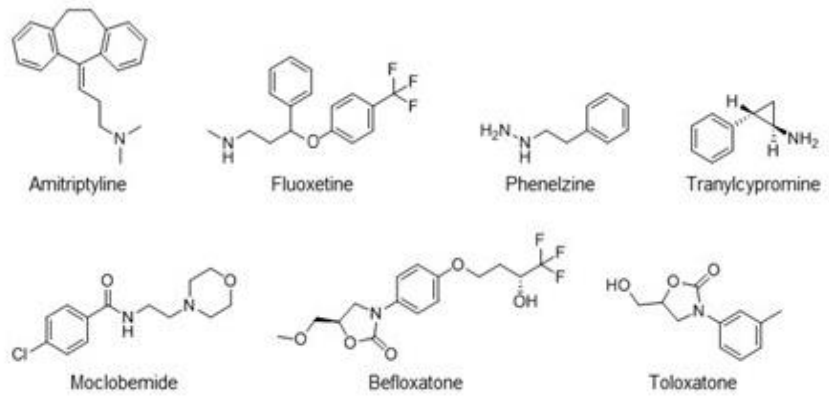

Figure 2.9. The chemical structures of the antidepressants used in Parkinson's disease.

2.2.8. Drug treatment and quality of life

The risk for developing co-morbid dementia in Parkinson's disease depends on the age of the patient and not the duration of disease (Kempster *et al*., 2007; Levy, 2007). It is estimated that dementia occurs in 30% of all Parkinson's disease cases (Aarsland *et al*., 1996; Blum *et al*., 2001). In Parkinson's disease, quality of life is a patient-recorded outcome that is an important variable which reflects the disease impact and treatment success from the patients' perspective. Quality of life can be used to indicate the beneficial effect of treatment strategies (Martinez-Martin *et al*., 2015). Some studies indicate that the quality of life of patients suffering from Parkinson's disease is increased after receiving COMT inhibitor therapy (Baas *et al*., 1997; Männisto & Kaakkola, 1999; Waters *et al*., 1997; Welsh *et al*., 1995). Patients with motor complications significantly benefit from the use of tolcapone, while nonfluctuating patients tolerate entacapone better. In the early stages of the disease, rasagiline as monotherapy improves patients' quality of life, but not in advanced stages (Gallagher & Schrag, 2008). Rasagiline enhances dopamine release, retards dopamine catabolism and antagonises several cellular processes involved in the cascade of events leading to apoptosis (LeWitt & Taylor, 2008; Mandel *et al*., 2003; Maruyama *et al*., 2002). Rotigotine improves the quality of life in the early stages of Parkinson's disease as well as in patients with motor fluctuations (Gallagher & Schrag, 2008). Another aspect of treatment to consider is patient compliance. Multivariate analyses carried out in patient groups aged 65 to 74 years, 75 to 84 years and above 85 years indicate that the first and last groups are less likely to comply with antiparkinsonian therapy. Patients with a high level of education were also more compliant than those without (Wei *et al*., 2010).

2.3. Aetiology of Parkinson's disease

2.3.1. General

The aetiology of Parkinson's disease is unknown, however, major biochemical processes such as oxidative stress and mitochondrial inhibition have been described (Blum *et al*., 2001). The major risk factor for Parkinson's disease is aging (Bower *et al*., 1999; de Rijk *et al*., 1995; Lees *et al*., 2009). Before the symptoms in Parkinson's disease occur, a subclinical phase develops where increased neuronal activity and sensitisation of dopaminergic neurons occur. This is described as the striatal compensatory phenomena (Agid *et al*., 1990; Anglade *et al*., 1995; Blum *et al*., 2001). Hence the appearance of the clinical manifestation of Parkinson's disease occurs only when 50–70% of dopaminergic neurons have been lost. The first symptoms appear after advanced degeneration, although the onset time point of this degeneration cannot be determined (Blum *et al*., 2001). Since the

early physical signs of Parkinson's disease may also be attributed to old age, a lag of 2–3 years from the first symptoms to diagnosis often occur (Lees *et al*., 2009). This leads to a difficulty in establishing the exact aetiology of the disease, which in turn prevents the possibility of developing preventative tools (Blum *et al*., 2001). Most patients suffering from Parkinson's disease experience extensive motor disability after 5–10 years of the disease, even with therapeutic intervention (Dauer & Przedborski, 2003). The aetiology of sporadic Parkinsonism is considered multifactorial (Białecka *et al*., 2004; Fahn & Cohen, 1992; Tan *et al*., 2000). Environmental and genetic factors might contribute to the aetiology and subsequently the pathogenesis of Parkinson's disease (Béné *et al*., 2009; Blum *et al*., 2001; Dauer & Przedborski, 2003). 95% of Parkinson's disease cases are considered to be sporadic with no genetic linkage (Dauer & Przedborski, 2003).

2.3.2. Environmental factors

Environmental involvement was established after the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a dopaminergic neurotoxin (Blum *et al*., 2001; Dauer & Przedborski, 2003; Langston *et al*., 1983). Chronic or limited neurotoxin exposure can result in progressive neurodegeneration, initiating a self-perpetuating cascade of deleterious events (Dauer & Przedborski, 2003). MPTP produces selective nigral neuronal death in humans as well as in experimental models of Parkinson's disease (Blum *et al*., 2001; Gerlach & Riederer, 1996). The motor symptoms caused by MPTP mimic those of Parkinson's disease (Blum *et al*., 2001; Langston *et al*., 1983). However, although MPTP and its analogues (Naoi *et al*., 1993; Niwa *et al*., 1987) as well as rotenone, a common pesticide (Betarbet *et al*., 2000), can produce dopaminergic brain lesions, none of these compounds are responsible for the majority of Parkinson's disease cases. Many exogenous and endogenous compounds may induce Parkinson's disease or a similar syndrome when a susceptible background is present (Blum *et al*., 2001).

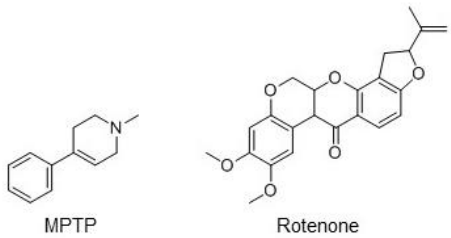

Figure 2.10. The chemical structures of MPTP and rotenone, environmental factors causing Parkinson's disease.

2.3.3. Endogenous toxins

Endogenous toxins may cause neurodegeneration in Parkinson's disease. Normal metabolism may create toxic substances when its function is distorted through environmental exposure or inherited differences in certain metabolic pathways. Dopamine may act as an endogenous toxin through its normal metabolism which generates harmful reactive oxygen species (ROS) (Cohen, 1984; Dauer & Przedborski, 2003). Dopamine metabolism produces H_2O_2, superoxide radicals and dopamine quinone, a molecule which reacts with cysteine-residues and damages proteins (Dauer & Przedborski, 2003; Graham, 1978). Therefore, dopamine may be pivotal in rendering dopaminergic neurons in the substantia nigra pars compacta particularly susceptible to oxidative attack (Dauer & Przedborski, 2003). ROS and free radical-mediated oxidative damage of cell membranes, deoxyribonucleic acid (DNA) and proteins in degenerative processes relating to aging, cancer and atherosclerosis is a cause for concern, since this can lead to the development of Parkinson's disease (Finkel & Holbrook, 2000; Finkel, 2005; Hussain *et al*., 2003; Qian *et al*., 2011; Rice-Evans & Diplock, 1993).

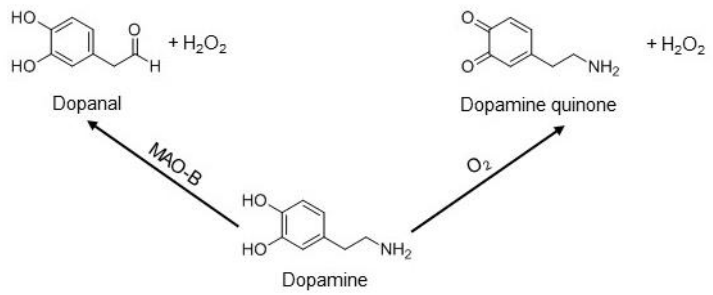

Figure 2.11. The metabolic route of dopamine and the formation of toxic by-products.

2.3.4. Genetic factors

The genetic contribution stems from 5–10% of Parkinson's disease cases that has a familial linkage (Blum *et al*., 2001; Dauer & Przedborski, 2003). This is mainly due to gene mutations that cause protein misfolding and dysfunction of the ubiquitin-proteasome pathway or chaperone system (Przedborski, 2005). Inherited Parkinson's disease express pathogenic mutations that directly result in toxic protein conformations or indirectly by interfering with the processes normally responsible for the identification and processing of misfolded proteins (Bussell & Eliezer, 2001). With aging, there is a decline in the cell's ability to induce chaperones to refold misfolded proteins as well as an impaired activity of the proteasome to degrade misfolded proteins (Sherman & Goldberg, 2001). Proteasomal dysfunction and the

subsequent accumulation of misfolded proteins provoke a deleterious cycle leading to neuronal death (Dauer & Przedborski, 2003).

No linkage to sporadic Parkinsonism has been established. Parkinson's disease cannot be categorised as an inherited disease, since genetic studies performed on monozygotic twins contradicted a familial linkage (Marttila *et al*., 1988; Tanner *et al*., 1999). Hence the majority of Parkinson's disease cases can be categorised as sporadic and without any family history (Blum *et al*., 2001). Clinically, any syndrome that pertains striatal dopamine deficiency or direct striatal damage may result in Parkinsonism (Dauer & Przedborski, 2003).

Patients vary widely in their response to L-dopa therapy (Deleu *et al*., 2002) which may be genetic in origin (Goetz *et al*., 2001; Kaiser *et al*., 2003; Wang *et al*., 2001). Seven gene mutations have been linked to L-dopa-responsive Parkinsonism (Lees *et al*., 2009) and a few cases of autosomal dominant transmission exist (Blum *et al*., 2001). In 1997 an α-synuclein gene mutation has been identified (Dauer & Przedborski, 2003) on chromosome 4q21–q23 (Blum *et al*., 2001; Polymeropoulos *et al*., 1996). This mutation consists of an Ala53Thr substitution in the encoding protein for α-synuclein (Blum *et al*., 2001; Polymeropoulos *et al*., 1997). Another mutation substitutes Ala30Pro (Blum *et al*., 2001; Kruger *et al*., 1998). This contributes to the α-synuclein associated pathogenesis of Parkinson's disease. Different experimental models involving mice (Abeliovich *et al*., 2000), flies and *Drosophila* nerve cells (Feany & Bender, 2000) determined that a mutation or dysfunction of α-synuclein results in the abnormal functioning of the nigrostriatal system and age-dependent dopaminergic neuron loss. These models also confirm the presence of intraneuronal inclusions resembling Lewy bodies (Dauer & Przedborski, 2003; Spillantini *et al*., 1998). Thus, α-synuclein and its mutated forms may contribute to nigral degeneration and subsequently the development of Parkinson's disease (Blum *et al*., 2001).

In striatal dopaminergic terminals, α-synuclein is involved in the modulation of synaptic function (Abeliovich *et al*., 2000; Dauer & Przedborski, 2003) where α-synuclein binds to lipid membranes and changes the previously unfolded N-terminus of the protein to a stable α-helical secondary structure (Davidson *et al*., 1998; Eliezer *et al*., 2001). This conformational change may be responsible for its neurotoxicity since it can easily misfold or form amyloid fibrils. α-Synuclein can also form protofibrils (non fibrillar oligomers) (Conway *et al*., 1998), which permeabilise synaptic vesicles and allows for dopamine to leak into the cytoplasm

(Lashuel *et al*., 2002; Volles *et al*., 2001). This in turn results in oxidative stress within the neurons (Dauer & Przedborski, 2003; Sanchez-Gaujardo *et al*., 2015). Three enzymes have been identified in the degradation of extracellular α-synuclein namely plasmin (Kim *et al*., 2012), metalloproteinase (Sung *et al*., 2005) and neurosin (Sanchez-Guajardo *et al*., 2015; Tatebe *et al*., 2010). These enzymes may be of relevance in future therapies (Sanchez-Guajardo *et al*., 2015). Microglial response can be activated by neurons expressing α-synuclein, which may lead to mishandling of α-synuclein. Furthermore, microglial activation promotes α-synuclein pathology by increased nitric oxide formation, resulting in cell death (Sanchez-Guajardo *et al*., 2015; Shavali *et al*., 2006). Extracellular α-synuclein induces a pro-inflammatory profile in microglia which increases TNFα, interleukin 1b, interleukin 6, cyclooxygenase 2 and inducible nitric oxide synthase (iNOS) levels, resulting in an increase in ROS and cell toxicity (Lee *et al*., 2010; Sanchez-Guajardo *et al*., 2015; Su *et al*., 2008). Mutated forms of α-synuclein may also interfere with the phagocytic ability of microglia, further impairing its action (Sanchez-Guajardo *et al*., 2015). Neurons, as well as microglia can produce α-synuclein (Austin *et al*., 2006).

Another gene related to Parkinson's disease is parkin. A relevant mutation on this gene is located on the locus 6q25.3–q27 (Blum *et al*., 2001; Kitada *et al*., 1998). Parkin gene mutations are found in patients without a family history of Parkinson's disease, and are associated with early onset parkinsonism before the age of 30 years (Dauer & Przedborski, 2003; Lees *et al*., 2009; Mizuno *et al*., 2001). Parkin is the second most common genetic cause of L-dopa-responsive parkinsonism (Lees *et al*., 2009) and is associated with nigrostriatal dopaminergic neuron loss without the presence of Lewy bodies (Dauer & Przedborski, 2003; Mizuno *et al*., 2001).

Additional rare Parkinson's disease-causing genes have recently been identified with all of these genes operating through a common molecular pathway, the ubiquitin-proteasome pathway (Dauer & Przedborski, 2003). Altered mitochondrial function is associated with mutations in PTEN-induced putative kinase 1 (PINK1) and DJ–1 gene (Canet-Avilés *et al*., 2004; LeWitt & Taylor, 2008; Przedborski, 2005). The PINK1 and parkin genes share the same mitochondrial pathway (Clark *et al*., 2006; Lees *et al*., 2009; Park *et al*., 2006), whereas DJ–1 causes missense mutations. DJ–1 incorporates a proline into the α-helical region of a protein which subsequently causes the protein to accumulate in mitochondria resulting in oxidative stress (Dauer & Przedborski, 2003).

Ubiquitin C-terminal hydrolase L1 (UCHL–1) may also be potentially linked to Parkinson's disease. UCHL–1 catalyses the hydrolysis of C-terminal ubiquitin esters and is involved in the recycling of misfolded proteins after proteasome degradation (Dauer & Przedborski, 2003; Wilkinson, 2000).

Six pathogenic mutations in leucine rich repeat kinase 2 (LRRK–2) have been identified with the Gly2019Ser mutation being the most common (Healy *et al*., 2008; Lees *et al*., 2009; Paisan-Ruiz *et al*., 2004) and is attributed to 2% of all Parkinson's disease cases (Deng *et al*., 2005; Gilks *et al*., 2005; Kachergus *et al*., 2005). Patients with this mutation have a more benign course of Parkinson's disease, with decreased risk of developing dementia (Lees *et al*., 2009; Williams *et al*., 2005). The risk for developing Parkinson's disease with the Gly2019Ser mutation is only 28% when younger than 60 years of age, but the risk rises to 74% at 79 years of age (Healy *et al*., 2008; Lees *et al*., 2009). Loss of function of glucocerebrosidase (GBA) increases the risk for Parkinson's disease 5-fold (Goker-Alpan *et al*., 2004; Lees *et al*., 2009).

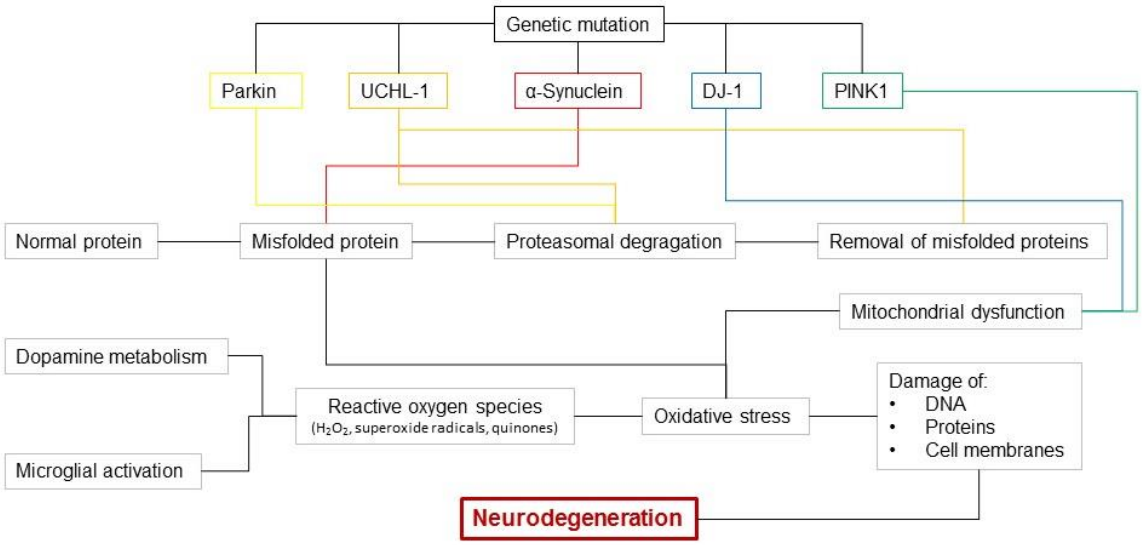

Figure 2.12. Simplified diagram explaining the genetic factors and the subsequent pathogenetic pathways leading to neurodegeneration.

2.3.5. Smoking and coffee consumption

Interestingly, smoking and coffee consumption lowers the risk for developing Parkinson's disease. Patients who has never smoked are twice as likely to develop Parkinson's disease (Allam *et al*., 2004; Hernàn *et al*., 2001), as well as men and postmenopausal women who

are not taking hormone replacement therapy with little to no daily caffeine intake (Ascherio *et al*., 2003; Ascherio *et al*., 2004; Lees *et al*., 2009). Potential contributing factors to the protective effect of smoking and coffee are the observation that MAO-B activity in the brain of smokers is 40% lower relative to non-smokers since nicotine inhibits MAO-B (Fowler *et al*., 1996; Lees *et al*., 2009; Mazzio *et al*., 1998), while caffeine acts as an adenosine A_{2A} receptor antagonist (Jankovic, 2008; Lees *et al*., 2009).

2.3.6. Additional contributing factors to the development of Parkinson's disease

It has been reported in previous studies that the use of non-aspirin non-steroidal anti-inflammatory drugs decreases the risk of developing Parkinson's disease (Rees *et al*., 2012; Sanchez-Guajardo *et al*., 2015).

Dopamine antagonist therapy (e.g. metoclopramide and haloperidol) (Young, 1999) as well as herbal remedies such as the Western Pacific sedative kava kava (Lees et al., 2009; Meseguer *et al*., 2002), or the Indian snake root *Rauwolfia serpentina* can cause reversible secondary parkinsonism (Lees *et al*., 2009; Sourkes, 1999). Cardiovascular disease such as multiple lacunar strokes may also result in secondary Parkinsonism (Young, 1999).

2.4. Mechanisms leading to neuronal death in Parkinson's disease

2.4.1. Multiple mechanisms underlie Parkinson's disease pathogenesis

The main pathological hallmark of idiopathic Parkinson's disease is the loss of dopaminergic neurons (Blum *et al*., 2001; Mazzio *et al*., 1998) with the presence of eosinophilic intraneuronal inclusions called Lewy bodies (Marsden, 1983; Tretiakoff, 1919). Lewy bodies are composed of α-synuclein, parkin (Dauer & Przedborski, 2003; Forno, 1996; Spillantini *et al*., 1998), neurofilaments (Blum *et al*., 2001; Goldman *et al*., 1983; Pappolla, 1986) and ubiquitin (Kuzuhara *et al*., 1988; Lowe *et al*., 1988; Mayer *et al*., 1989). Lewy bodies are found in the central as well as the peripheral nervous system (Vanderhaegen *et al*., 1970) and is not restricted to Parkinson's disease but can be present in other neurodegenerative diseases such as Lewy body disease, amyotrophic lateral sclerosis (Blum et al., 2001), Alzheimer's disease, dementia with Lewy body disease and in people with advanced age (Dauer & Przedborski, 2003; Gibb & Lees, 1988). The neurodegeneration in Parkinson's disease follows a distinct pattern with the more susceptible area located laterally in the ventral part of the substantia nigra pars compacta (Fearnley & Lees, 1991; Goto *et al*., 1989; Hirsch *et al*., 1988). Neurodegeneration results in severe striatal dopamine depletion

(Ehringer & Hornykiewicz, 1960) which causes the motor symptoms associated with Parkinson's disease (Blum *et al*., 2001; Lee *et al*., 1994). Neurodegeneration and the presence of Lewy bodies are not restricted to the nigrostriatal pathway, and can be found in the locus coeruleus (Greenfield & Bosanquet, 1953), nucleus basalis (Candy *et al*., 1983), hypothalamus, cerebral cortex, cranial nerve motor nuclei and central and peripheral components of the autonomic nervous system (Damier *et al*., 1999; Kaiser *et al*., 2000; Przedborski, 2005). These lesions give rise to the cognitive and psychological impairments of Parkinson's disease such as dementia (Aarsland *et al*., 1996; Blum *et al*., 2001). The pathogenesis in Parkinson's disease involves strong oxidative stress, reduced antioxidant levels, mitochondrial defects (Blum et al., 2001; Dauer & Przedborski, 2003), abnormal protein aggregation and misfolding, inflammation, excitotoxicity and loss of trophic support leading to apoptosis (Merad-Boudia *et al*., 1998; Yacoubian & Standaert, 2009). Parkinson's disease has no primary pathogenic factor with all these mechanisms likely acting synergistically through complex interactions to promote neurodegeneration (Yacoubian & Standaert, 2009).

2.4.2. Dopamine metabolism yields injurious by-products

Dopaminergic neuronal loss in Parkinson's disease has a characteristic topology that differs from the pattern seen in normal aging (Dauer & Przedborski, 2003; Fearnley & Lees, 1991). Furthermore, the degree of terminal loss in the striatum is more pronounced than the magnitude of dopaminergic neuron loss in the substantia nigra pars compacta suggesting that striatal dopaminergic neurons are the primary degenerative target (Bernheimer *et al*., 1973; Dauer & Przedborski, 2003; Müller & Ferré, 2007). Interestingly, nigral dopaminergic neurons are particularly susceptible to degeneration since basal ROS levels are high inside neurons (Blum *et al*., 2001; Hirsch *et al*., 1997; Uhl, 1998).

There exists a linear relationship between dopamine levels and those of destructive free radicals (Mazzio *et al*., 1998; Smith *et al*., 1994). Mechanisms by which dopamine may be toxic to nerve cells include oxidation and MAO-catalysed deamination, both of which lead to oxidative damage (Graham *et al*., 1978; Hastings & Zigmond, 1994; Offen *et al*., 1996). MAO oxidises dopamine to yield neurotoxic by-products such as H_2O_2, hydroxyl radicals (Mazzio *et al*., 1998; Smith *et al*., 1994), superoxide (Hastings *et al*., 1996; Sulzer & Zecca, 2000; Youdim & Lavie, 1994) and 3,4-dihydroxyphenylacetaldehyde (dopanal), which are highly toxic to catecholaminergic cells (Blum *et al*., 2001; Mattamal *et al*., 1995). The oxidation of the catechol moeity of dopamine, in turn, may lead to the formation of semiquinones

(Klegeris *et al*., 1995; Mazzio *et al*., 1998). The formation of these reactive species may contribute to oxidative stress and cell death (Chiueh *et al*., 1994; Hastings & Zigmond, 1997; Jellinger *et al*., 1995; Jellinger, 1999; Slivka & Cohen, 1985).

2.4.3. Role of iron

In the Parkinsonian substantia nigra pars compacta there is increased iron levels (Dexter *et al*., 1989; Good *et al*., 1992; Hirsch *et al*., 1991), possibly related to a decrease in cellular ferritin, an iron-binding protein (Dexter *et al*., 1990; Levay & Bodell, 1993; Riederer *et al*., 1989), and an increase in lactoferritin receptor expression (Blum *et al*., 2001; Leveugle *et al*., 1996). Dysregulation of iron metabolism, iron-induced oxidative stress and free radical formation are major pathogenic factors in the progression of Parkinson's disease. These factors trigger a cascade of deleterious events which leads to neuronal death and biochemical disturbances associated with Parkinson's disease (Götz *et al*., 1994; Keyer & Imlay, 1966; Youdim *et al*., 1990). An increase in iron levels may increase ROS formation (Blum *et al*., 2001) through the Fenton reaction and subsequently lead to lipid peroxidation, protein and nucleic alteration (Moldeus *et al*., 1983; Velez-Pardo *et al*., 1997), and ultimately neurodegeneration (Chiueh *et al*., 1994; Mazzio *et al*., 1998). Nigral neurons contain neuromelanin (Bharath *et al*., 2002) which displaces iron from cellular ferritin, further increasing free cellular iron levels which in turn reinforces the chemical reaction between iron and dopamine (Blum *et al*., 2001). Hydroxyl radicals generated by the Fenton reaction, have the ability to extract methylene hydrogens from polyunsaturated fats present in neural membrane phospholipids. This extracting ability initiates lipid peroxidation and subsequent cell death (Bharath *et al*., 2002; Double *et al*., 2000).

2.4.4. Lowered antioxidant status and glutathione levels

Glutathione (GSH) and glutathione peroxidase act as endogenous protective mechanisms in the substantia nigra (Perry & Yong, 1986; Sian *et al*., 1994; Sofic *et al*., 1992). GSH acts as a free radical reducing agent which lessens the damaging effects of oxidative stress in the central nervous system (Smeyne & Smeyne, 2013). In the vulnerable area of the substantia nigra pars compacta there is a decrease in cells synthesising GSH peroxidase (Blum *et al*., 2001; Damier *et al*., 1993). In total a 40–50% decrease in GSH levels can be observed in the Parkinson's disease patients' brain (Riederer *et al*., 1989; Sian *et al*., 1994; Smeyne & Smeyne, 2013; Sofic *et al*., 1992). This results in defective detoxification capabilities of the basal ganglia, leading to oxidative stress and degeneration. Furthermore, dopamine turnover

could increase basal production of H_2O_2 which depletes GSH (Blum *et al*., 2001). The ratio of reduced GSH and oxidised GSH (GSSG) is reduced during nigral degeneration which leads to an increase in the formation of toxic hydroxyl radicals (Sian *et al*., 1994; Sofic *et al*., 1992). GSH-dependent detoxification in neurons is impaired possibly due to increased dopamine metabolism which increases the production of basal hydrogen peroxide which, in turn, depletes GSH (Blum *et al*., 2001; Lee *et al*., 2001a; Riederer *et al*., 1989). GSH depletion also lowers catalase (Ambani *et al*., 1975) and GSH peroxidase expression in neurons (Kish *et al*., 1985). This deleterious cycle increases ROS levels and subsequently damage cellular and neuronal cell functions (Blum *et al*., 2001). Furthermore, dopamine metabolism may contribute to the reduction of GSH levels. The quinone generated through dopamine metabolism can react with the cysteine residue of GSH and covalently modify the peptide (Rabinovic & Hastings, 1998; Stokes *et al*., 1999).

2.4.5. The role of NOS

The NOS family consists of three different isoforms namely neuronal NOS, iNOS, and endothelial NOS. All of these isoforms are present in the mammalian brain, although not in equal amounts (Przedborski & Dawson, 2001; Tieu *et al*., 2003). Activated microglia could produce nitric oxide (NO) and cytokines which give rise to free radicals (McGeer *et al*., 1988a; McGeer *et al*., 1988b). iNOS is elevated in the Parkinsonian brain (Hunot *et al*., 1996) resulting in elevated NO levels and subsequent generation of the harmful peroxynitrite radical (Blum *et al*., 2001). NO acts as an atypical neural modulator (Zhang *et al*., 2006). It has different signalling functions in the nervous system such as neurotransmitter release, neuronal excitability and is involved in learning and memory processes (Boehning & Snyder, 2003; Kiss & Vizi, 2001; Prast & Philippu, 2001). Glial cell activation, stimulated by NO, could furthermore increase cytokine levels as well as produce ROS or even activate the cell death pathways directly (Boka *et al*., 1994; Hunot *et al*., 1997; Mogi *et al*., 1994). ROS could induce secondary excitotoxicity by increasing free cellular calcium levels, which in turn increases cellular NO levels (Blum *et al*., 2001). Glutamate, the primary excitatory transmitter in the mammalian brain, could increase intracellular calcium levels through excessive NMDA receptor activation (Mody & MacDonald, 1995) which promote peroxynitrite production through iNOS activation (Dawson & Dawson, 1996). Subsequently peroxynitrite reacts with other compounds, generating other toxic peroxide products which cause DNA damage and may activate caspase dependent as well as caspase independent cell death pathways (Beckman & Koppenol, 1996; Hong *et al*., 2004). Furthermore, NO can cause cell damage by protein modification through nitrosylation and nitration (Zhang *et al*., 2006).

2.4.6. Protein deposition

Protein deposition feature in several age-related neurodegenerative diseases and causes neuronal cell death. Proteins often appear to be natively unfolded with certain genes implicated in the aggregation of specific proteins (Uversky, 2003). Familial Parkinson's disease may be caused by triplication at the α-synuclein locus (Singleton *et al*., 2003). Covalent protein modification may occur which facilitates protein aggregation, especially in the presence of oxidative stress. Oxidative modification of α-synuclein may occur in the presence of the by-products of dopamine catabolism, increasing the likelihood of aggregation (Conway *et al*., 2001). Nitration of α-synuclein may also occur (Giasson *et al*., 2000). Another protein which may be associated with aggregation and subsequent pathogenesis of Parkinson's disease, is parkin. Parkin mutations is commonly associated with siblings presenting with juvenile Parkinson's disease (Forloni *et al*., 2002). The deposition of α-synuclein and parkin is discussed in more detail in section 2.3.4. Protein aggregates can directly damage neurons by deforming its structure or through interfering with intracellular trafficking in the neuron. Other proteins can also be sequestered into the neuron by protein inclusions already present in the neuronal structure leading to further cell damage. The ability of cells to manage misfolded proteins is compromised in older patients (Sherman & Goldberg, 2001). In the healthy patient, misfolded proteins are either refolded by a chaperone-inducing system or degraded by proteasomal polyubiquitination, yet these systems are impeded in older patients (Dauer & Przedborski, 2003).

2.4.7. Dysfunctional mitochondrial respiration

Mitochondria are vital organelles involved in many functions of neuronal survival and activity (Ren *et al*., 2015). Mitochondrial dysfunction on DNA level may contribute to Parkinson's disease, however, these mutations have been described only in a small percentage of Parkinson's disease cases (Morais & Strooper, 2010; Park *et al*., 2009). Molecular oxygen is consumed almost fully by mitochondrial respiration producing powerful oxidants (H_2O_2 and superoxide radicals) as by-products. Dysfunction and inhibition of mitochondrial nicotinamide adenine dinucleotide coenzyme Q reductase, or complex I, increases ROS formation (Ren *et al*., 2015; Xie *et al*., 2010) which in turn may form toxic hydroxyl radicals or react with NO to form peroxynitrite. These oxidants and by-products cause cellular damage by reacting with nucleic acids, proteins and lipids. The electron transport chain may be targeted (Cohen, 2000) which subsequently causes mitochondrial damage with additional ROS production. Accumulation of ROS increases the amount of misfolded proteins which increase the demand on the ubiquitin-proteasome system (Dauer & Przedborski, 2003).

The main mitochondrial defects observed in Parkinson's disease are located at complex I. Complex I form part of the oxidative phosphorylation system which produces cellular adenosine triphosphate (ATP). Complex I activity and immunoreactivity are reduced in the substantia nigra pars compacta of patients suffering from Parkinson's disease (Hattori *et al*., 1991; Mizuno *et al*., 1989; Schapira *et al*., 1989; Schapira *et al*., 1990). This may be due to mitochondrial DNA mutations which make certain individuals more susceptible to the development of Parkinson's disease (Blum *et al*., 2001). Whatever the nature of the mitochondrial defect, it may result in apoptosis, a physiological and regulated mode of cell death, through decreased ATP levels and impairment of proton pumping (Desagher & Martinou, 2000; Ren *et al*., 2015). Apoptosis as a pathogenic factor of Parkinson's disease remains controversial since a high rate of apoptotic cell death could also occur in the substantia nigra pars compacta of patients with a rapid dopaminergic neuron loss through normal aging or induced by an undefined toxic event (Blum *et al*., 2001; Dauer & Przedborski, 2003).

2.4.8. Monoamine oxidase may produce toxic metabolic by-products

Epidemiological studies have shown that with aging, MAO activity may be linked to neurodegenerative diseases such as Parkinson's disease (Mazzio *et a*l., 1998). MAO-B has a double role in the pathogenesis of Parkinson's disease since it is the main enzyme responsible for dopamine metabolism and it generates free radical and other neurotoxic species during its normal catalytic cycle (Novaroli *et al*., 2005). ROS generated by MAO activity and auto-oxidation of dopamine may be amplified by iron that catalyses the Fenton reaction (Fahn & Cohen, 1992; Jellinger *et al*., 1992; Jenner *et al*., 1992). Since MAO-B levels increase 4–5 fold with aging (Fowler *et al*., 1997; Fowler *et al*., 2003; Kalaria *et al*., 1988), it is expected that dopamine levels in the brain will diminish and the generation of toxic by-products will increase with age (Edmondson *et al*., 2009). Dopanal, the oxidation product of dopamine, has been implicated in the aggregation of α-synuclein (Burke *et al*., 2008) while increased H_2O_2 levels promote apoptotic signalling events, both events that may lead to neurodegeneration in Parkinson's disease (Edmondson *et al*., 2009; Mallajosyula *et al*., 2008).

2.4.9. 6-Hydroxydopamine toxicity as a model for cytotoxicity of catecholamines

The molecular pathways involved in the development of Parkinson's disease have essentially been studied using experimental animal models reproducing the human disease. These models are created by neurotoxic compounds, i.e. 6-hydroxydopamine (6-OHDA), MPTP and rotenone (Blum *et al*., 2001; Bové *et al*., 2005; Smeyne & Jackson-Lewis, 2005). These models mimic Parkinson's disease to some extent on histological and biochemical levels, thus assisting in determining the cardinal cellular processes of cell death and nigral degeneration. These models also assist in studying possible neuroprotective strategies that will halt disease progression (Blum *et al*., 2001). Nigral dopaminergic neurons contain significant levels of dopamine, H_2O_2 and free iron. A non-enzymatic reaction between these elements may lead to 6-OHDA formation (Jellinger *et al*., 1995; Linert *et al*., 1996; Slivka & Cohen, 1985).

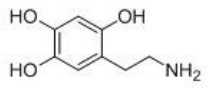

Figure 2.13. The chemical structure of 6-OHDA.

Melanin can increase free iron levels by displacing it from ferritin. Dopamine can be oxidised by various H_2O_2-dependent systems in the presence of nitrite ions to generate 6-OHDA and 6-nitrodopamine (Palumbo *et al*., 1999). Manganese can also stimulate dopamine auto-oxidation and 6-OHDA formation (Blum *et al*., 2001; Garner & Nachtman, 1989). Under physiological conditions, 6-OHDA produces oxidative stress by rapid and non-enzymatic auto-oxidation (Heikkila & Cohen, 1972; Seitz *et al*., 2000; Soto-Otero *et al*., 2000) which generates several toxic species such as quinones (Saner & Thoenen, 1971), superoxide radicals, H_2O_2 and the highly reactive hydroxyl radical (Blum *et al*., 2001; Cohen & Heikkila, 1974). Thus, the cytotoxicity of catecholamines directly correlates with their rates of auto-oxidation (Blum *et al*., 2001; Graham *et al*., 1978; Soto-Otero *et al*., 2000). Iron may contribute to 6-OHDA toxicity since an iron-chelating agent prevents the deleterious effects caused by 6-OHDA (Ben Shachar *et al*., 1991; Blum *et al*., 2001; Borisenko *et al*., 2000). ROS produced by 6-OHDA causes DNA strand break (Bruchelt *et al*., 1991), mutations (Gee *et al*., 1992), disorganisation of the cytoskeleton (Davidson *et al*., 1986) and impairment of glucose and GABA uptake (Vroegop *et al*., 1995). The basal mechanism of dopamine toxicity closely resembles that of 6-OHDA and involve extracellular as well as intracellular

ROS generation through oxidation of the catechol moiety and possible mitochondrial inhibition (Blum *et al*., 2001).

2.4.10. Potental toxicity of L-dopa

L-Dopa catabolism generates oxyradicals which may promote neuronal degeneration (Gnerre *et al*., 2000; LeWitt & Taylor, 2008; Walkinshaw & Waters, 1995). Chronic L-dopa therapy increases homocysteine plasma levels, which also may contribute to Parkinson's disease due to concomitant onset of neuropsychiatric symptoms and co-morbidities such as vascular disease (Müller, 2008). In contrast, some reports suggest that L-dopa has antioxidant effects in the striatum which suggest it has a beneficial effect against oxidative stress and consequently neurodegeneration (Camp *et al*., 2000; LeWitt & Taylor, 2008). Further studies are required to establish the relationship of L-dopa in the progression of Parkinson's disease (LeWitt & Taylor, 2008; Yacoubian & Standaert, 2009), since evidence is lacking for L-dopa toxicity in Parkinson's disease (Gnerre *et al*., 2000).

2.5. Metabolism of L-dopa and dopamine

L-Dopa metabolism by AADC occurs in the gastrointestinal tract, kidney and liver, with pyridoxine as cofactor (Halkias *et al*., 2007). Gastric emptying may be slow and erratic in Parkinson's disease which in turn influences the absorption of L-dopa from the proximal small intestine (Kurlan *et al*., 1988; Rivera-Calimlim *et al*., 1971; Sasahara *et al*., 1981). 70–80% of the L-dopa dose is excreted in the urine (Halkias *et al*., 2007). L-Dopa demonstrates significant intra- and inter-individual variations in the rate and extent of absorption (Abbruzzese, 2008; Hauser, 2009; Juncos, 1992; LeWitt, 2008). Variations in the absorption of L-dopa results primarily from food-drug interactions in the intestine where L-dopa competes with amino acids for uptake by amino acid transporters (Crevoisier *et al*., 2003; Kempster & Wahlqvist, 1994; Nomoto *et al*., 2009; Wang *et al*., 2013). L-Dopa is rapidly converted metabolically at both the peripheral and central levels (Gnerre *et al*., 2000). Apoproximately 70% of the orally administered L-dopa dose is degraded through peripherally acting metabolic enzymes (Kiss & Soares-da-Silva, 2014).

L-Dopa therapy ameliorates Parkinson's disease symptoms by compensating for the disease related loss of endogenous dopamine (Heeringa *et al*., 1997). In this respect, L-dopa is the biological precursor of dopamine and can be used as an artificial means to manipulate the

endogenous cerebral dopamine levels (Birkmayer & Hornykiewicz, 1961; Kiss & Soares-da-Silva, 2014). As Parkinson's disease progresses, the degeneration of dopamine neurons also increases, and the amount of endogenous brain dopamine will start to follow the pharmacokinetic properties of L-dopa (Heeringa *et al*., 1997; Müller & Ferré, 2007). This phenomenon results in a sudden end-of-dose hypokinesia (Heeringa *et al*., 1997). The metabolic inactivation of L-dopa hampers its therapeutic potential (Müller & Ferré, 2007). Thus, L-dopa is typically administered in conjunction with AADC inhibitors such as benserazide and carbidopa (Gnerre *et al*., 2000; Heeringa *et al*., 1997), or in combination with selective reversible COMT inhibitors such as entacapone and tolcapone (Gnerre *et al*., 2000; LeWitt, 2007). L-Dopa is also combined with selective irreversible MAO-B inhibitors such as selegiline (Drucharch & van Muiswinkel, 2000; Nagatsua & Sawadab, 2009). Benserazide is much more potent than carbidopa, and in combination with L-dopa constitutes the best available therapy for Parkinson's disease (Da Prada *et al*., 1984). Despite this, treatment with L-dopa and a decarboxylase inhibitor still results in fluctuating L-dopa plasma concentrations. This can be overcome by administrating L-dopa with both a decarboxylase inhibitor and a COMT inhibitor (Birkmayer & Hornykiewicz, 1961; Foley *et al*., 2000b; Riederer *et al*., 2007). With this treatment regime L-dopa is metabolised to a lesser extent in the periphery (Männistö *et al*., 1992; Messiha *et al*., 1972; Sharpless *et al*., 1973).

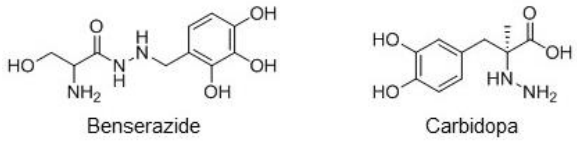

Figure 2.14. The chemical structures of typical AADC inhibitors used in the treatment of Parkinson's disease.

Part of the L-dopa spared from COMT catabolism is metabolised through alternative metabolic pathways (Da Prada *et al*., 1984; Fahn, 1974; Kuruma *et al*., 1972). When present in the brain, catechol-type neurotransmitters are removed from the synapse by three main metabolic routes. In the striatum these neurotransmitters are taken up into the neuron predominantly by the dopamine transporter (DAT), while in the prefrontal cortex inactivation is achieved enzymatically through oxidation by MAO and aldehyde dehydrogenase as well as through methylation by COMT (Ehler *et al*., 2014; Männistö & Kaakkola, 1999).

Although L-dopa can cross the blood-brain barrier by means of amino acid transporters, dopamine derived from L-dopa in the periphery is unable to penetrate the blood-brain barrier

(Lerner *et al*., 2003; Männistö *et al*., 1992; Männistö & Kaakkola, 1999). Only a fraction, 5–10%, of administered L-dopa thus reaches the brain (Bonifácio *et al*., 2002; Männistö & Kaakkola, 1999). The efficacy of L-dopa depends on the level of dopamine converted from it (Lerner *et al*., 2003). 10% of orally administered L-dopa is converted to 3-OMD by peripheral COMT (Bäckström *et al*., 1989; Deleu *et al*., 2002; Kiss & Soares-da-Silva, 2014). 3-OMD accumulates in large amounts in the plasma due to its long elimination half-life (Kuruma *et al*., 1971) and competes with L-dopa uptake across the blood-brain barrier (Gervas *et al*., 1983; Nutt & Fellman, 1984; Reches & Fahn, 1982; Wade & Katzman, 1975). This further reduces the bioavailability of L-dopa to the brain. A close relationship exists between 3-OMD accumulation and the "end-of-dose" or "wearing-off" syndrome experienced with L-dopa treatment (Learmonth *et al*., 2002; Toghi *et al*., 1991). Thus, peripheral inhibition of COMT is proposed to prolong the half-life of L-dopa and decrease the amount of 3-OMD peripherally which will increase the bioavailability and therapeutic benefit of L-dopa (Bäckström *et al*., 1989; Bonifácio *et al*., 2002; Männistö & Kaakkola, 1999). Once L-dopa crosses the blood-brain barrier it is further metabolised to dopamine in the brain (Heeringa *et al*., 1997). This conversion occurs at the surviving dopaminergic nerve terminals as well as at serotonergic and adrenergic nerve terminals containing decarboxylase enzymes (Halkias *et al*., 2007).

Dopamine released from L-dopa is metabolically inactivated in the brain by MAO through oxidative deamination (Hirsch, 1994; Mazzio *et al*., 1998). Dopamine is a substrate for both subtypes of MAO (MAO-A and MAO-B) with equal affinities (Fowler & Tipton, 1984; Green *et al*., 1977; Yang & Neff, 1974). Furthermore, central COMT also metabolises dopamine (Guldberg & Marsden, 1975; Männistö & Kaakkola, 1999). Therefore, peripheral as well as central COMT inhibition will diminish the metabolism of dopamine and increase L-dopa's therapeutic potency (Heeringa *et al*., 1997).

Dopamine generates three distinct metabolites in appreciable amounts in the central nervous system, namely the deaminated metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), the deaminated O-methylated metabolite homovanillic acid and the intermediate 3-methoxytyramine (Anden *et al*., 1963; Carlsson & Waldeck, 1964; Rosengren, 1960).

Studies have shown that MAO-A and COMT inhibition potentiates L-dopa-induced turning behaviour, while the effect of MAO-B is less profound (Heeringa *et al*., 1997). COMT inhibitors are also useful for decreasing the dosage of L-dopa required for a therapeutic

effect in Parkinson's disease (Hamaue *et al*., 2010). In combination with L-dopa, MAO-B inhibition may enhance the L-dopa-induced dopamine increase and may allow for a reduction in the dosage of L-dopa required for a therapeutic response. Since dopamine is an equally favoured substrate of MAO-A and MAO-B, MAO-A inhibition also enhances endogenous dopamine levels (Di Monte *et al*., 1996; Finberg *et al*., 1998; Marchitti *et al*., 2007). In conclusion, patients with advanced Parkinson's disease benefit significantly from the use of a COMT or MAO-B inhibitor in conjunction with L-dopa compared to L-dopa therapy alone (Lees *et al*., 2009; Miyasaki, 2006; Rascol *et al*., 2002; Talati *et al*., 2009). Peripheral COMT inhibitors may be of enhanced value in the treatment of Parkinson's disease, since L-dopa undergoes its most extensive metabolic breakdown in the periphery (Learmonth & Freitas, 2002).

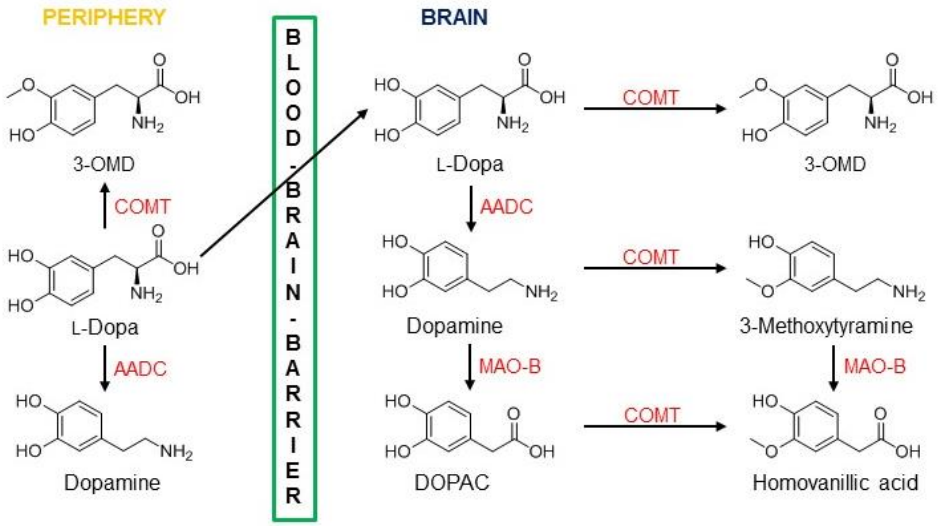

Figure 2.15. Diagram representing the metabolic routes for L-dopa and dopamine in the periphery and brain (Adapted from Solla *et al*., 2010).

2.6. A case for multi-target-directed inhibitors for Parkinson's disease

MAO and COMT are the two enzymes primarily responsible for the initial metabolic inactivation of endogenous as well as infused catecholamines in the blood and tissue of mammals (Hirsch, 1994; Männistö & Kaakkola, 1999; Mazzio *et al*., 1998; Yan *et al*., 2002). Therefore, dual inhibition of MAO and COMT may be a novel strategy in the treatment of certain neurological disorders. In Parkinson's disease, COMT inhibitors entering the brain have minimal beneficial effect alone, and thus must be administered in conjunction with another inhibitor such as a MAO inhibitor (Learmonth *et al*., 2002; Lees *et al*., 2009; Miyasaki, 2006; Rascol *et al*., 2002). Furthermore, dual MAO and COMT inhibition may exert

a neuroprotective effect. Since dopamine and other biogenic amines induce the biosynthesis of neurotrophic factors implicated in neuroprotection, inhibition of extraneuronal and neuronal MAO or the predominantly glial located COMT, may enhance dopamine levels and thus also increase the biosynthesis of these factors. In fact, the inhibition of COMT alone may slow the metabolism of biogenic amines in glial cells and thus increase the biosynthesis of neurotrophic factors. COMT inhibition, however, may intensify catecholamine metabolism in neurons by MAO resulting in increased levels of free radicals and oxidative stress. For this reason, centrally active COMT inhibitors should only be used in conjunction with MAO inhibitors in neurodegenerative disorders (Müller *et al*., 1993). Additionally, the inhibition of MAO activity may be neuroprotective by decreasing oxidative stress (Mazzio *et al*., 1998).

Dual inhibition of MAO-B and COMT would be greatly beneficial in L-dopa therapy. Peripheral and central COMT inhibition reduces the formation of 3-OMD from L-dopa, thus improving the bioavailability of L-dopa by both increasing its entry into the brain and prolonging its half-life (Bonifácio *et al*., 2002; Deleu *et al*., 2002; Robinson *et al*., 2012). A central-acting COMT inhibitor may further potentiate the effect of L-dopa by delaying the metabolism of dopamine derived from L-dopa (Männistö & Kaakkola, 1999). Since COMT inhibition improves the bioavailability of L-dopa, a concomitant increase in central dopamine levels is expected (Ellerman *et al*., 2011; Männistö *et al*., 1992; Männistö & Kaakkola, 1999). Peripheral selective COMT inhibitors may be of enhanced value in the treatment of Parkinson's disease, since L-dopa undergoes its most extensive metabolic breakdown in the periphery (Learmonth & Freitas, 2002). Inhibition of either MAO-A or MAO-B does not significantly alter the steady state of central dopamine levels. Only when both isoforms are inhibited are dopamine levels increased significantly. Thus, dual MAO-A and MAO-B inhibitors may be of value in future therapies (Green *et al*., 1977; Riederer & Youdim, 1986; Youdim *et al*., 2006; Youdim & Bakhle, 2006). Dopamine is metabolised by both isoforms of MAO in the human brain, thus non-selective MAO inhibition may represent an attractive strategy to enhance central dopamine levels in Parkinson's disease (Di Monte *et al*., 1996; Finberg *et al*., 1998). Non-selective MAO inhibitors should however possess a reversible mode of inhibition, since irreversible inhibition of MAO-A may lead to adverse effects such as the "cheese reaction" (Da Prada *et al*., 1988; Youdim & Weinstock, 2004; Youdim & Bakhle, 2006). Furthermore, reversible non-selective MAO inhibition leads to better controllability (Mertens *et al*., 2014). Interestingly, dual-target directed MAO inhibitors which addresses a second target such as adenosine A_{2A} receptor antagonism have been developed (Mertens *et al*., 2014; Stößel *et al*., 2013).

Since many patients suffering from Parkinson's disease tend to develop depression, dual MAO and COMT inhibition will be of further value. When a MAO-B inhibitor such as selegiline is combined with a COMT inhibitor, catecholamine levels may be increased significantly in the brain which may contribute to depression therapy (Tom & Cummings, 1998). COMT inhibitors may also extend the action of compounds with a catechol structure such as endogenous catecholamines. Thus, central inhibition of COMT could restore noradrenaline levels, subsequently relieving the symptoms of depression. Correction of this deficit through COMT inhibition, in conjunction with either tricyclic antidepressants or MAO inhibitors, presents a novel way to treat depression (Männistö & Kaakkola, 1999).

2.7. The biology of MAO

2.7.1. General background

Mary Hare-Bernheim described a catalytic enzyme responsible for the oxidative deamination of tyramine in 1928. Later Hugh Blaschko concluded that tyramine oxidase, noradrenaline oxidase and aliphatic amine oxidase were the same enzyme. Eventually, Zeller named the enzyme mitochondrial MAO (Nagatsu, 2004; Schnaitman *et al*., 1967; Youdim *et al*., 1988; Youdim *et al*., 2005). All mammals contain MAO-A and MAO-B in various tissues with both of these enzymes localised on the outer mitochondrial membrane (Nicotra *et al*., 2004; Setini *et al*., 2005; Strolin Benedetti *et al*., 1992; Tsang *et al*., 1986). Both MAOs contain the flavin adenine dinucleotide (FAD) cofactor covalently bound via a thio-ether linkage between the side chain of a specific cysteinyl residue and the C8α-position of the FAD (Edmondson *et al*., 2004a; Edmondson *et al*., 2007; Kearney *et al*., 1971; Youdim *et al*., 2006). In both MAO-A and MAO-B this cysteinyl residue is part of a conserved pentapeptide Ser-Gly-Gly-Cys-Tyr (Bach *et al*., 1988; Chen *et al*., 1991; Nagatsu, 2004; Shih *et al*., 1999). The two isoforms of MAO are encoded by different genes that correspond to different amino acid sequences with 70% identity between them (Binda *et al*., 2007; Wong *et al*., 2002; Zhu *et al*., 1994b). The MAO-A and MAO-B genes consist of 15 exons with each having an identical exon-intron organisation. Exon 12 in both isoforms codes for the covalent FAD-binding site and is the most conserved exon with 93.9% amino acid identity between the two isoforms. This suggests that both isoforms were derived from the duplication of a common ancestral gene (Grimsby *et al*., 1991; Nagatsu, 2004). MAO-A and MAO-B represent key metabolic pathways to control amine neurotransmitter levels in cells (Kumar *et al*., 2003; Pisani *et al*., 2013; Youdim *et al*., 1988; Youdim *et al*., 2006). The MAOs metabolise primary, secondary and tertiary monoamines (Blaschko *et al*., 1937; Carradori *et al*., 2014; Youdim *et al*., 2005)

and also catalyse the oxidation of monoamines such as serotonin, histamine, and the catecholamines dopamine, adrenaline and noradrenaline (Shih *et al*., 1999; Tipton *et al*., 2004; Zeller, 1938). Additionally, the MAOs are also responsible for oxidation of ingested amines such as phenylethylamine and tyramine (Chen & Swope; 2007; Helguera *et al*., 2013; Waldmeier, 1987; Youdim *et al*., 2006). Dopamine, adrenaline, noradrenaline, tryptamine and tyramine are essentially oxidised by both MAO isoforms in most species (Glover *et al*., 1977; Tipton *et al*., 2004; Youdim *et al*., 1988). MAO-A and MAO-B exhibit different substrate and inhibitor specificities (Chen *et al*., 2007; Elmer & Bertoni, 2008; Helguera *et al*., 2013; Hubálek *et al*., 2005). Typically, MAO-A is inhibited by low concentrations of clorgyline and catalyses the oxidation of serotonin, whereas MAO-B is inhibited by low concentrations of selegiline and catalyses the oxidation of benzylamine and 2-phenylethylamine (Cesura & Pletscher, 1992; Fariello & Lieberman, 2006; Johnston, 1968; Yamada & Yasuhara, 2004). Interestingly, the MAOs function as metabolic barriers with MAO-A in the placental tissue protecting the foetus from transfer of biogenic or bioactive amines across the placenta, while MAO-B in the microvasculature of the brain may prevent false neurotransmitters (e.g. 2-phenylethylamine) from reaching the central tissues (Abell & Kwan, 2000; Yan *et al*., 2002).

2.7.2. The "cheese reaction"

Since the discovery of the first MAO inhibitor, iproniazid, countless other MAO inhibitors have been developed with various structures (Youdim *et al*., 1988; Youdim & Bakhle, 2006; Youdim *et al*., 2006). Irreversible MAO-A inhibitors such as tranylcypromine, a non-hydrazine MAO inhibitor, induce a severe adverse reaction known as the "cheese reaction". This reaction is caused by tyramine and other indirectly acting sympathomimetic amines present in certain foods and fermented drinks (Da Prada *et al*., 1988; Youdim *et al*., 1988; Youdim & Bakhle, 2006). Under normal physiological conditions, these dietary amines would be deactivated by MAO-A present in the gut wall and liver, thus preventing their entrance into the systemic circulation. With MAO-A inhibition, this protective system is inactivated, and tyramine and other monoamines are not metabolised and enter the circulation where they induce noradrenaline release from peripherally located adrenergic neurons (Finberg *et al*., 1981; Finberg & Tenne, 1982; Youdim & Weinstock, 2004). This results in a severe hypertensive response, which may be fatal (Da Prada *et al*., 1988; Youdim & Bakhle, 2006). This adverse effect is primarily a concern of irreversible MAO-A inhibition since tyramine is metabolised by MAO-A in the gut. Thus, selective MAO-B inhibition will not elevate systemic tyramine levels or cause the "cheese reaction" (Youdim & Weinstock, 2004).

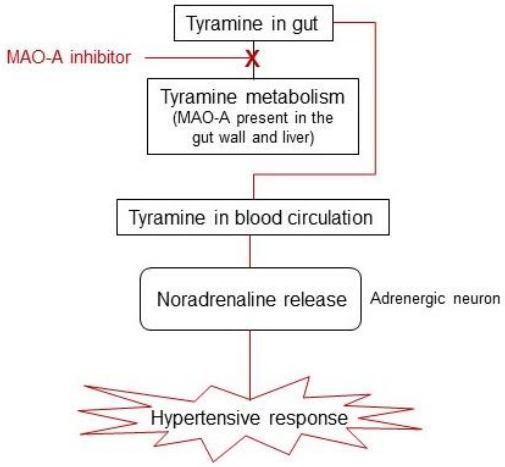

Figure 2.16. Diagram representing the "cheese reaction" (Adapted from Youdim & Bakhle, 2006 and Youdim *et al*., 2006).

2.7.3. Tissue distribution of the MAOs

For reasons that remain unclear, only one isoform of the enzyme is present in a specific cell type of a specific tissue (Inoue *et al*., 1999; Trendelenburg *et al*., 1987; Yu *et al*., 1992). MAO-A is the predominant isoform in the gastrointestinal tract (Chen *et al*., 2007; Saura *et al*., 1996b), whereas MAO-B is predominant in the brain (Collins *et al*., 1970; Fowler *et al*., 1980; Kalaria *et al*., 1988). MAO-A is predominantly present in catecholaminergic neurons, whereas MAO-B is primarily located in serotonergic neurons and astrocytes in the central nervous system (Bach *et al*., 1988; Binda *et al*., 2004a; Johnston, 1968; Murphy, 1978). MAO-B activity and expression levels, but not that of MAO-A, increase in the human brain with age (Fowler *et al*., 1997; Novaroli *et al*., 2006; Shih, 1979; Strolin Benedetti *et al*., 1992). MAO-B's activity is particularly high in the substantia nigra pars compacta of patients suffering from Parkinson's disease (Choi *et al*., 2015; Duty & Jenner, 2011; Finberg, 2014). Since MAO-B is predominantly located in glial cells, the increase may be attributed to glial cell proliferation in response to neuronal loss (Fowler *et al*., 1995; Fowler *et al*., 1998; Mellick *et al*. 1999). This may cause an increase in oxidative stress (Barnham *et al*., 2004; Mallajosyula *et al*., 2008). Ontogenetic studies established that MAO-B activity only start to increase after the 60th year of life and then increases non-linearly (Delumeau *et al*., 1994; Strolin Benedetti & Dostert, 1989). Increased MAO-B levels can also be observed in other degenerative diseases such as Alzheimer's disease (Novaroli *et al*., 2005; Saura *et al*., 1996a). MAO-A levels have been found to increase 9-fold in the hearts of aged rats (Edmondson *et al*., 2009; Maurel *et al*., 2003), thus suggesting that MAO-A could serve as a potential drug target in cardiac cellular degeneration (Edmondson *et al*., 2009; Gentili *et al*.,

2006). 80% of the total MAO activity is found in the human liver and can be attributed to MAO-B (Inoue *et al*., 1999; Saura *et al*., 1996a). MAO-B is the principle enzyme in all liver and brain preparations of various species, while human liver contains the highest level of MAO-A activity. It has been shown that different regions in the human brain can contain up to 20% of MAO-A activity (Inoue *et al*., 1999; Kalaria *et al*., 1988), although MAO-B is the dominant enzyme present in all species. The MAO activity profiles between human and rats are much more similar than that between humans and subhuman primates (Inoue *et al*., 1999). Thus, extrapolating results obtained from one species to another should be done with caution (Hubálek *et al*., 2005; Youdim *et al*., 2006).

2.7.4. The catalytic mechanism of MAO

It is proposed that both isoforms of MAO function by a proton abstraction mechanism (Edmondson *et al*., 2007). The catalytic mechanism of MAO catalysis consists of a reductive half reaction where cleavage of the amine Cα–H bond generates the protonated imine and the covalently bound 8α-S-cysteinyl FAD cofactor is reduced to the flavin hydroquinone (Edmondson *et al*., 2000; Edmondson *et al*., 2007; Miller & Edmondson, 1999). It is evident that it is the deprotonated form of the amine substrate that enters the substrate binding site for interaction with the flavin cofactor (Edmondson *et al*., 1993; Edmondson *et al*., 2000; Miller & Edmondson, 1999). To date, no evidence exists for the deprotonation process of the amine substrates and, at physiological pH values, the amine substrate would predominantly be in its protonated form in solution (Edmondson *et al*., 2007). MAO-A and MAO-B utilise oxygen as an electron acceptor which reacts with the reduced flavin hydroquinone to yield the oxidised flavin and H_2O_2, respectively. This completes the catalytic cycle. The dissociated protonated imine is released from the enzyme and undergoes a non-catalysed hydrolysis which generates ammonium and the corresponding aldehyde (Edmondson *et al*., 1993; Edmondson *et al*., 2009; Youdim & Bakhle, 2006). The MAO catalytic reaction for most substrates can be summarised as follows, $RCH_2NR_1R_2 + H_2O + O_2 \rightarrow RCHO + NHR_1R_2 + H_2O_2$. The identity of the generated aldehyde is thus dependent upon the identity of the substrate (Holt *et al*., 1997; Nagatsu, 2004; Tabor *et al*., 1954). In the catalytic reaction, 1 mole dopaldehyde and 1 mole H_2O_2 are produced for each mole of dopamine oxidised (Gesi *et al*., 2001; Götz *et al*., 1990).

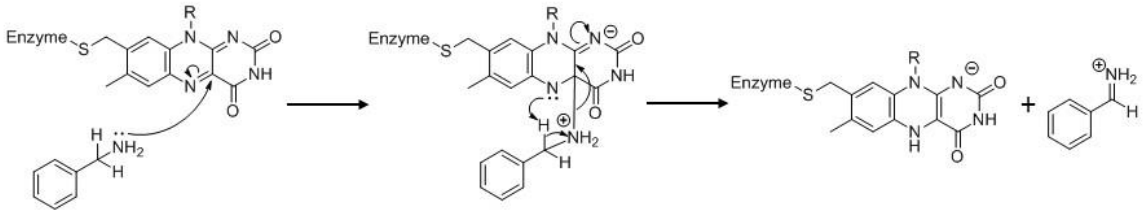

Figure 2.17. The polar nucleophilic mechanism of MAO catalysis (Adapted from Edmondson *et al*., 2004b; Erdem *et al*., 2006; Miller & Edmondson, 1999).

In either enzyme, the isoalloxazine ring of the FAD is in a "bent" conformation and deviates approximately 30° from planarity about the N(5)–N(10) axis whereas the ring geometry is in an energetically favourable flat conformation either free in solution or in most other flavoenzymes (Binda *et al*., 2003; De Colibus & Mattevi, 2006). This bent conformation of the isoalloxazine ring results in a higher election density at N(5) and lowered electron density at the C(4a) position of the flavin ring. This facilitates the nucleophilic attack of the basic substrate amine lone pair on the C(4a) position of the FAD cofactor resulting in a flavin-substrate adduct. For a flavin ring to acquire such a conformation, it must accept a proton from the N(5) position (Edmondson *et al*., 2007). In the subsequent step, α-CH bond cleavage occurs with the flavin N(5) position functioning as the base (Edmondson *et al*., 2004b; Hubálek *et al*., 2005). This mechanistic pathway is termed the polar nucleophilic mechanism.

Another potential reaction mechanism is the single electron transfer mechanism proposed by Silverman and co-workers (Edmondson *et al*., 2007; Lu *et al*., 2002). For this mechanism the α-CH protons of the amine require a very strong base for abstraction (Edmondson *et al*., 2007; Edmondson *et al*., 2009; Lu *et al*., 2002). Basic amino side chains would most likely not suffice as a strong base. Thus, to lower the pKa values of these protons, the amine first has to be oxidised by one electron to the amine cation radical which results in acidification of the α-CH group and subsequently allows H^+ abstraction. In the second reaction, a second electron is transferred to the flavin semiquinone to yield the imine product and flavin hydroquinone (Edmondson *et al*., 2009).

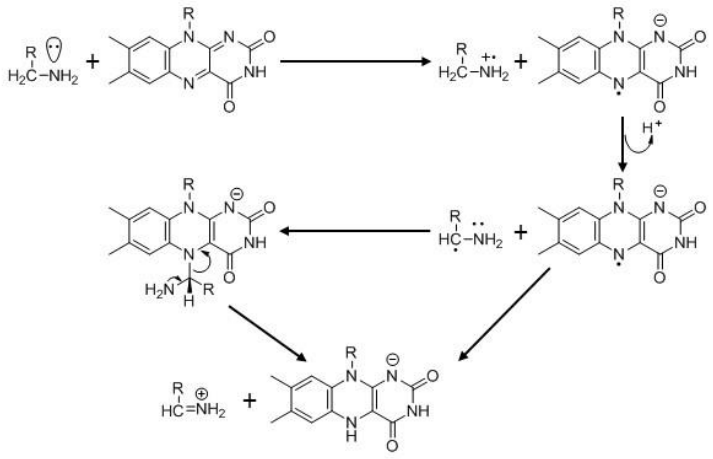

Figure 2.18. The single electron transfer mechanism of MAO catalysis (Adapted from Edmondson *et al*., 2004b; Lu *et al*., 2002; Vintem *et al*., 2005).

2.7.5. Expression systems of the MAOs

The first reported heterologous expression of human MAO activity was in 1990, using *Saccharomyces cerevisiae* as the host expression system (Newton-Vinson *et al*., 2000; Urban *et al*., 1991; Weyler *et al*., 1990). Even though the enzymes were bound to the outer mitochondrial membrane and exhibited sensitivities to classic MAO inhibitors, this system was unable to express large quantities of human MAO-B (Edmondson *et al*., 2007). MAO-B has been successfully expressed from human MAO-B-cDNA through various transfer vectors such as plasmids (Newton-Vinson *et al*., 2000) and the baculovirus expression systems (Novaroli *et al*., 2005; Rebrin *et al*., 2001).

A breakthrough in MAO expression came after successful demonstration of heterologous expression of human MAO-B initially (Newton-Vinson *et al*., 2000), and later MAO-A (Li *et al*., 2002), in large quantities using the methylotrophic yeast *Pichia pastoris* (Edmondson *et al*., 2007; Novaroli *et al*., 2005). The purified proteins using this expression system contain all the post-translational modifications as those found in natural source preparations with comparable activity levels. This expression system serves as a reliable and convenient source of the human MAOs and additionally allows the production of site-directed mutants (Edmondson *et al*., 2007; Newton-Vinson *et al*., 2000).

Since platelet MAO-B has the same amino acid sequence as human brain MAO-B (Chen *et al*., 1993), it can be utilised as a peripheral model to indirectly assess MAO-B activity in the central nervous system (Novaroli *et al*., 2005; Novaroli *et al*., 2006; Wirz-Justice, 1988). The mitochondrial microenvironment is important for enzyme activity (Newton-Vinson *et al*., 2000), catalytic properties and inhibitor specificity of recombinant as well as natural MAO-B. Human recombinant MAO-B is a reliable and efficient source for inhibitor screening and is now commercially available (Newton-Vinson *et al*., 2000; Novaroli *et al*., 2005).

Schumacher *et al*. has shown that exposure of *Caenorhabditis elegans* to selegiline in order to block dopamine and serotonin degradation, resulted in accumulation of the respective neurotransmitters. Thus, this model may be utilised to investigate drug-mediated modulation of the dopamine and serotonin system to identify compounds with neuroprotective properties (Schumacher *et al*., 2015).

2.7.6. MAO gene polymorphisms are related to the development of Parkinson's disease

Gene polymorphisms in dopamine metabolism may contribute to the development of certain neurodegenerative diseases (Białecka *et al*., 2005; Moreau *et al*., 2015). MAO-B polymorphisms in Parkinson's disease are commonly associated with older patients (Goudreau *et al*., 2002). The rs1799836 in the gene encoding MAO-B has been identified as a possible detrimental polymorphism (Moreau *et al*., 2015) which increases the risk for Parkinson's disease especially in patients possessing the AA genotype (Kiyohara *et al*., 2011). It was established that Chinese patients possessing polymorphisms of MAO-B genes experience differences in drug responses (Hao *et al*., 2015). Hao and colleagues determined that patients suffering from Parkinson's disease with developed dyskinesia have a statistically higher frequency of the MAO-B genotype (Hao *et al*., 2014). Polymorphisms in the MAO-B genotype or in the transporters responsible for monoamine transport (e.g. DAT) result in variable levels of the biogenic amines and their metabolic by-products (Bugaj *et al*., 2011; Lee *et al*., 2001a; Lin *et al*., 2002; Nakamura *et al*., 2000; Parsian *et al*., 2004). The distribution of the MAO-B allele A is slightly higher in patients suffering from Parkinson's disease (Balciuniene *et al*., 2002; Białecka *et al*., 2007; Ho *et al*., 1995; Torkaman-Boutorabi *et al*., 2012a). A polymorphism on MAO-B, A644G, influences the risk and treatment of Parkinson's disease and affects the patients' response to levodopa therapy (Costa *et al*., 1997; Singh *et al*., 2008; Torkaman-Boutorabi *et al*., 2012b; Wu *et al*., 2001).

2.7.7. MAO inhibition and neuroprotection

It is proposed that the specific inhibition of MAO-B is neuroprotective in Parkinson's disease (Chen & Swope, 2007; Lees, 2005; Stocchi & Olanow, 2003). When the catalytic by-products produced by dopamine catabolism (dopaldehyde and H_2O_2) are not rapidly inactivated by centrally located aldehyde dehydrogenase (Gesi *et al*., 2001) and GSH peroxidase (Götz *et al*., 1990), respectively, these by-products may exert neurotoxic effects (Grunblatt *et al*., 2004; Riederer *et al*., 1989; Youdim & Bakhle, 2006). Since aldehyde dehydrogenase and GSH peroxidase activity is impaired in patients suffering from Parkinson's disease, these neurotoxic by-products could accumulate in the brain (Galter *et al*., 2003; Grunblatt *et al*., 2004). Thus MAO-B inhibition may be neuroprotective by stoichiometrically decreasing dopaldehyde and H_2O_2 production in the brain (Chazot, 2001; Dingemanse *et al*., 1997; Ebadi *et al*., 2006; Rabey *et al*., 2000). This proposal was supported through studies done on pre-Parkinson's patients where selegiline and rasagiline were effective in delaying the development of Parkinson's disease, although the disease could not be prevented (Birkmayer *et al*., 1985; Heikkila *et al*., 1985; Walkinshaw & Waters, 1995).

2.7.8. Inhibitors of MAO in Parkinson's disease

Both selegiline and rasagiline are propargylamines that form covalent adducts with the N(5) of the covalently bound FAD cofactor of MAO-B (Maycock *et al*., 1976; Riederer *et al*., 1982; Youdim, 1978; Youdim *et al*., 2005), which render the enzyme inactive (Binda *et al*., 2003; Pisani *et al*., 2013). Both the abovementioned compounds are selective and irreversible MAO-B inhibitors and possess no pharmacological action on MAO-A (Kalir *et al*., 1981; Sabbagh & Youdim, 1978; Weinreb *et al*., 2010). Selegiline is the most commonly used MAO-B inhibitor in Parkinson's disease, but has some disadvantages. It has low bioavailability, undergoes extensive first-pass metabolism and is metabolised to L-*N*-desmethylselegiline, L-metamphetamine and L-amphetamine which may cause psychotoxic adverse effects (Lees, 2005; Waters *et al*., 2004). Rasagiline, although structurally related to selegiline, is not metabolised to amphetamine derivatives and is therefore devoid of amphetamine-like adverse effects (Lees, 2005). Safinamide, another specific MAO-B inhibitor, also functions effectively as a neuroprotectant (Edmondson *et al*., 2009). Furthermore, safinamide has a novel mode of action as a dopamine modulator by selectively and reversibly inhibiting MAO-B, and blocking dopamine reuptake (Mertens *et al*., 2014, Müller, 2015a; Schapira, 2011). This is further complemented by an effect on the glutamate pathway (Binda *et al*., 2007; Caccia *et al*., 2006; Onofrj *et al*., 2008). Unlike selegiline and

rasagiline, safinamide does not form a covalent adduct with the flavin cofactor and is thus a reversible inhibitor (Binda *et al*., 2007).

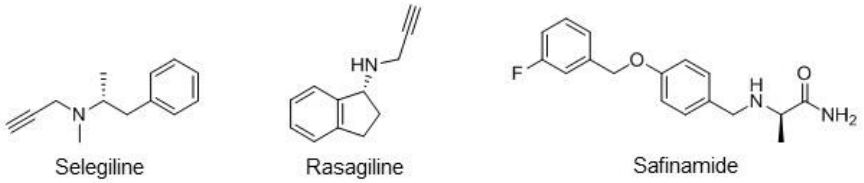

Figure 2.19. The chemical structures of selegiline, rasagiline and safinamide.

Reversible MAO-B inhibitors are advantageous over inactivators since enzyme activity can be regained relatively quickly after inhibitor withdrawal. With irreversible inhibition by inactivators, enzyme activity requires *de novo* synthesis of the MAO-B protein, a process which may require several weeks (Arnett *et al*., 1987; Corte & Tipton, 1980; Fowler *et al*., 1994; Neff & Goridis, 1972). The MAO-B activity recovers quickly in the small intestine, while in the brain it recovers more slowly (Weinreb *et al*., 2010; Youdim *et al*., 2001). In contrast, with irreversible MAO-A inhibition, the brain enzyme activity recovers significantly faster (Freedman *et al*., 2005; Youdim & Tipton, 2002).

2.7.9. The structure of MAO-B

Human MAO-B crystallises as a dimer (Edmondson *et al*., 2007; Edmondson *et al*., 2009; Youdim *et al*., 2006). When a substrate binds to the enzyme, it must first negotiate a protein loop at the entrance of one of the two cavities to reach the flavin cofactor. The first cavity, namely the “entrance cavity” is very hydrophobic in nature and has a volume of 290 Å³ (Binda *et al*., 2004a; Hubálek *et al*., 2005; Novaroli *et al*., 2006). The second cavity termed the “substrate cavity” exhibits a volume of 390 Å³ with the side chain of the isoleucine (Ile) 199 amino acid residue separating the two cavities (Binda *et al*., 2001; Binda *et al*., 2003; Veselovsky *et al*., 2004). The MAO-B active site cavity has a bipartite character that occupies a combined volume of 700 Å³ when the Ile199 residue is in its open conformation (Edmondson *et al*., 2007; Edmondson *et al*., 2009). This Ile199 can either exhibit an open or closed form depending on the substrate or bound inhibitor (Binda *et al*., 2003; Hubálek *et al*., 2005). The conformation of Ile199 is important in defining inhibitor specificity in human MAO-B (Binda *et al*., 2003; Edmondson *et al*., 2007; Hubálek *et al*., 2005). MAO-B can either host small inhibitors such as isatin and tranylcypromine or cavity-filling ligands such as safinamide (Edmondson *et al*., 2009; Hubálek *et al*., 2003). Rasagiline and selegiline exhibit

a midspan type of binding where they push Ile199 into an open conformation (De Colibus *et al*., 2005; Edmondson *et al*., 2009; Hubálek *et al*., 2004). The entrance and substrate cavities have been shown to be very hydrophobic with sites for favourable amine binding at the flavin involving two nearly parallel tyrosine (Tyr398 and 435) residues (Edmondson *et al*., 2007; Edmondson *et al*., 2009; Youdim *et al*., 2006). The amide linkage between cysteine 397 (Cys397) and Tyr398 is in a *cis*-conformation (Abell & Kwan, 2000; Nagatsu, 2004). These two Tyr-residues form an "aromatic cage" which has catalytic significance. This aromatic cage may act in polarising the amine moiety of the substrate in order to generate a more effective nucleophile in the MAO catalytic mechanism, while guiding a path for the substrate amine towards the reactive positions on the flavin ring. In the active site, Tyr326 produces a restriction for substrate binding although it is not directly involved in the separation of the two cavities (Edmondson *et al*., 2009; Novaroli *et al*., 2006). Human MAO-B is mostly hydrophobic with a small hydrophilic area in front of the *re* face of the flavin (Binda *et al*., 2003), which is occupied by highly conserved water molecules. Three water molecules present in the MAO-B structure are buried near the FAD with two involved in multiple hydrogen bond networks and the third fixed by the π-systems of the aromatic side chains of Tyr398 and Tyr435 and the central heterocyclic conjugated ring of the FAD (Binda *et al*., 2003; Binda *et al*., 2004b; Novaroli *et al*., 2006). The other water molecules form hydrogen bonds with the side chain oxygen of Gln206 and the O(4) atom of the flavin (Binda *et al*., 2003; Binda *et al*., 2004b).

The MAO-B substrate cavity has the shape of an ellipsoidal disk defined by leucine 171 (Leu171), Cys172 and Tyr398 on one side of the cavity and Ile198, Ile199 and Tyr435 on the other side (Binda *et al*., 2004a). The substrate cavity is mainly polar with various polar side chains as accessible hydrogen bonding groups in addition to the flavin nucleus and the three buried water molecules. Only the area containing three apolar residues (Tyr60, phenylalanine 343 [Phe343] and Tyr398) represents a hydrophobic environment in the substrate cavity (Novaroli *et al*., 2006). In conjunction with the side chain of Tyr188, these apolar residues form the floor and the roof of the substrate cavity, respectively (Binda *et al*., 2004a).

In contrast to the hydrophilic substrate cavity, the entrance cavity is defined by a pocket coated by Phe103, tryptophan 119 (Trp119), Leu164, Leu167, Phe168 and Ile316 giving rise to a highly hydrophobic environment (Novaroli *et al*., 2006; Youdim *et al*., 2006). Another pocket situated towards the outside of the protein is surrounded by polar residues and amino

acids with hydrogen bonding capacity. This pocket consists of glutamic acid 84 (Glu84), glycine 101 (Gly101), proline 102 (Pro102), serine 200 (Ser200), threonine 201(Thr201), Thr202 and Tyr 326 (Novaroli *et al*., 2006). It is proposed that histidine 382 (His382) and Thr158 are relevant residues in MAO-B catalysis (Cesura *et al*., 1998; Nagatsu, 2004).

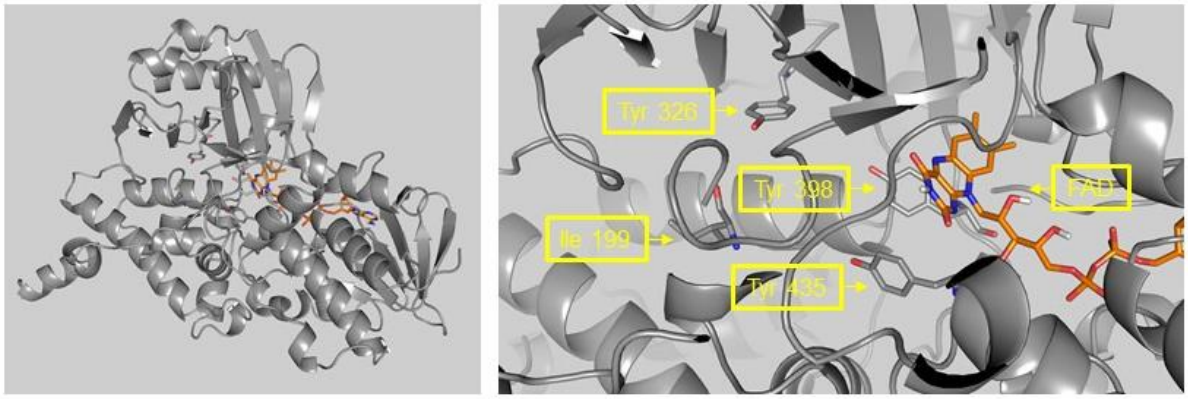

Figure 2.20. Diagram representing the MAO-B protein with key active site residues indicated.

2.7.10. The structure of MAO-A

MAO-A, in contrast to MAO-B, crystallises as a monomer (De Colibus *et al*., 2005; Son *et al*., 2008; Youdim *et al*., 2006) and contains only one substrate binding cavity. Human MAO-A exhibits a volume of 550 Å^3 with the *re* face of the covalent FAD situated as one of the faces of the substrate binding site opposite the entrance to the cavity. Similar to MAO-B, MAO-A also possesses an “aromatic cage” which is comprised of two nearly parallel Tyr-residues, 407 and 444, in front of the flavin (Edmondson *et al*., 2007). In contrast to the Ile199 residue in MAO-B, the corresponding Phe208 residue in MAO-A does not function as a gating residue (Hubálek *et al*., 2005; Pisani *et al*., 2013; Son *et al*., 2008). In MAO-A, the amide linkage between Cys406 (Abell & Kwan, 2000) and Tyr407 is in a *cis*-conformation (Edmondson *et al*., 2009; Nagatsu, 2004). Both substrate binding sites in MAO-A and MAO-B are relatively hydrophobic. MAO-A has a single cavity (Edmondson *et al*., 2007; Youdim *et al*., 2006) with a rounder shape than that in MAO-B which has a larger volume (Edmondson *et al*., 2007). In the active site of MAO-A, Ile335 occupies the position of Tyr326 in MAO-B which has a less restricting effect on substrate and inhibitor binding than observed in MAO-B (Edmondson *et al*., 2009).

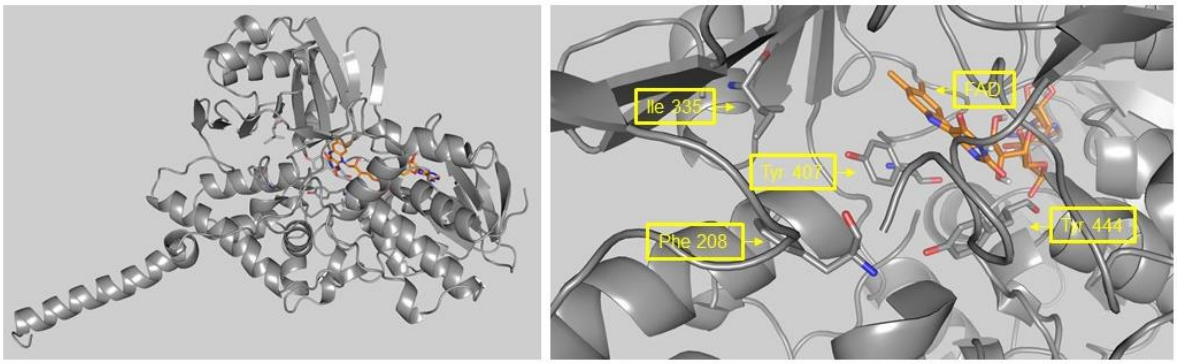

Figure 2.21. Diagram representing the MAO-A protein with key active site residues indicated.

The structural differences between MAO-A and MAO-B can be attributed to a six residue segment, residues 200–215, that constitutes a “cavity shaping loop”. This loop has a more extended conformation in MAO-A and a more compact conformation in MAO-B (Binda *et al*., 2001; Edmondson *et al*., 2007; Edmondson *et al*., 2009; Son *et al*., 2008), which gives a substrate less freedom for rotation in the MAO-B active site than in MAO-A. Both entrances to MAO-A or MAO-B are situated on the surface of the negatively charged mitochondrial outer membranes which could increase the effective substrate concentration by electrostatic attraction (Edmondson *et al*., 2007). The membrane binding motifs of the enzyme isoforms are located in a group of 35–40 C-terminal residues. In human MAO-B this transmembrane region folds into an α-helix which is inserted into the outer membrane of the mitochondrion (Binda *et al*., 2004b; Mitoma & Ito, 1992; Nagatsu, 2004). The last 20 residues of this motif are too disordered to provide definite electron density (Binda *et al*., 2001), whereas in human MAO-A the electron density is sufficiently defined to provide a complete view of the transmembrane α-helix (Edmondson et al., 2009; Son *et al*., 2008). The FAD cofactors’ position is highly conserved. In both isoenzymes certain active site residues are conserved which act as major determinants in dictating the substrate and inhibitor specificities for the two enzymes (De Colibus *et al*., 2005; Edmondson *et al*., 2007; Son *et al*., 2008). The Tyr-pair of the aromatic sandwich at the *re* face of the FAD cofactor and the Lys (Lys305 in MAO-A and Lys296 in MAO-B) residues which are bridged via a water molecule to the N(5) position of the flavin through hydrogen bonding, are highly conserved in both isoenzymes. In MAO-A, Phe208 and Ile335 is involved in determining enzyme specificity, while in MAO-B, Ile199 and Tyr326 determine specificity for substrates and inhibitors (Edmondson *et al*., 2009; Son *et al*., 2008). In spite of these differences, the active site cavities of MAO-A and MAO-B are highly conserved with only 6 of the 16 amino acid residues differing between them (Son *et al*., 2008).

2.8. COMT

2.8.1. General background and tissue distribution

Julius Axelrod first postulated that endogenous catecholamines undergo O-methylation in the late 1950's. This led to the discovery of COMT (Armstrong *et al*., 1957; Axelrod, 1957; Axelrod & Tomchick, 1958). COMT is encoded by a single gene which encodes for both isoforms, soluble COMT and membrane-bound COMT (Lundström *et al*., 1991; Männistö & Kaakkola, 1999; Salminen *et al*., 1990), using different promoters and translational regulation (Tenhunen & Ulmanen, 1993; Tenhunen *et al*., 1993; Tenhunen *et al*., 1994). The COMT gene is located on chromosome 22 band q11.2 (Grossman *et al*., 1992; Kiss & Soares-da-Silva, 2014). The only difference between the two isoforms is the inclusion of an additional 50 hydrophobic amino acid sequence in membrane-bound COMT (Chen *et al*., 2011; Lotta *et al*., 1995; Ma *et al*., 2013; Tunbridge *et al*., 2004). Early research has shown that COMT is highly localised in the soluble fraction of the cell (Axelrod & Tomchick, 1958; Guldberg & Marsden, 1975). Later investigations identified the membrane-bound form of COMT which did not appear to differ in biochemical and immunological characteristics from soluble COMT (Borchardt *et al*., 1974; Guldberg & Marsden, 1975). In the central nervous system, COMT is also generally assumed to be a cytoplasmic enzyme with nonspecific localisation (Bertocci *et al*., 1991; Lundström *et al*., 1991; Ulmanen & Lunström, 1991). The extra 50 amino acids of membrane-bound COMT is attached to the cytoplasmic side of intracellular membranes (Männistö & Kaakkola, 1999; Ulmanen & Lunström, 1991), thus oriented towards the cytoplasm (Kiss & Soares-da-Silva, 2014).

COMT is found in several invertebrate tissues, while in vertebrates the greatest activity is found in the liver (Axelrod & Tomchick, 1958; Männistö *et al*., 1992). COMT activity and localisation is significantly lower in the central nervous system than in peripheral tissues (Kiss & Soares-da-Silva, 2014). Vertebrate COMT activity appears to be mostly in the soluble COMT form, with only a minor fraction attributed to membrane-bound COMT (Ding *et al*., 1996; Karhunen *et al*., 1994; Rivett *et al*., 1983; Roth, 1992). Species differences exist in the physiochemical properties as well as level of enzyme activity. COMT activity increases 10-fold from birth to adulthood while the greatest increase occurs from 6–20 years with a decline after 60 years of age (Agathopoulous *et al*., 1971; Guldberg & Marsden, 1975; Männistö & Kaakkola, 1999).

In the human brain, 70% of the total COMT activity is attributed to membrane-bound COMT while 30% is due to soluble COMT activity. COMT activity seems to be absent in presynaptic dopaminergic neurons, while postsynaptic neurons and glial cells show some activity (Männistö & Kaakkola, 1999). The highest COMT activity in the human is found in the liver, followed by the kidneys and gastrointestinal tract (Bonifácio *et al*., 2002; Nissinen *et al*., 1988; Schultz & Nissinen, 1989). COMT activity in the liver of males appears to be 30% higher than that in females (Boudikova *et al*., 1990; Männistö & Kaakkola, 1999), while the liver contains 3–4 fold higher COMT activity than any other peripheral tissue (Kiss & Soares-da-Silva, 2014). Membrane-bound COMT is considered a more important drug target since it is able to methylate catecholamines at their physiological concentration (Roth, 1992), whereas soluble COMT is more important in non-physiological conditions such as when the substrate concentration suddenly increases (i.e. after L-dopa treatment) or when a higher methylation reaction rate is needed (Huotari *et al*., 2002; Lotta *et al*., 1995; Ma *et al*., 2013). Under normal physiological conditions, a deficiency in COMT activity does not appear to significantly affect central dopamine or noradrenaline levels, only when additional dopaminergic therapy is added (Huotari *et al*., 2002).

2.8.2. The reaction catalysed by COMT

COMT plays a vital role in the metabolism of catecholamines and the deactivation of exogenous compounds with a catechol structure (Aoyama *et al*., 2005; Jeffery & Roth, 1987; Lautala *et al*., 2001; Masjost *et al*., 2000). At physiological catecholamine concentrations, membrane-bound COMT may be more important for their metabolism since it is the predominant isoform present at regions where dopamine concentrations are <10 mM and noradrenaline concentrations are <300 mM (Männistö & Kaakkola, 1999; Roth, 1992). COMT metabolises catechol structures by methylation of only one of the O-hydroxy groups, employing S-adenosyl-L-methionine (SAM) as a methyl donor (Ehler *et al*., 2014; Vidgren *et al*., 1994; Woodard *et al*., 1980). This is classified as phase II metabolism (Ma *et al*., 2013; Männistö & Kaakkola, 1999). The catechol neurotransmitter is inactivated with SAM converted to S-adenosylhomocysteine (Coward & Ying-Hsiueh Wu, 1973; Guldberg & Marsden, 1975; Vidgren *et al*., 1994). COMT activity is dependent on the SAM concentration which should be high enough to saturate COMT, while the reaction rate is dependent on Mg^{2+} concentration (Creveling & Daly, 1971; Guldberg & Marsden, 1975). Woodard and co-workers concluded that the reaction is sequentially ordered (Lotta *et al*., 1995; Woodard *et al*., 1980) while catalysis proceeds via a simple S_N2 transfer of an electrophilic methyl group,

supplied by SAM, onto a nucleophilic O-atom of a catechol hydroxy group (Ehler *et al*., 2014; Palma *et al*., 2006; Woodard *et al*., 1980).

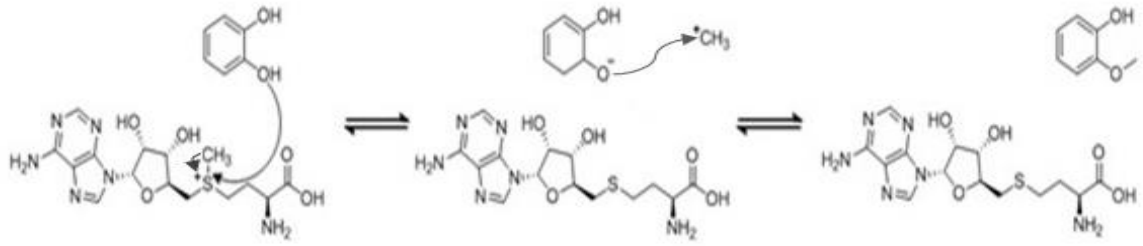

Figure 2.22. The simple S_N2 reaction mechanism of COMT showing only the SAM cofactor and catechol (Adapted from Williams, 2010).

The transfer is facilitated through in-line juxtaposition of the reactants while lowering the pKa of the catechol upon binding to the Lewis acid (the Mg^{2+} ion). The acidity of the Lewis Mg^{2+} acid must be low enough to not interact with the substrate phenolate, but within range to allow generation of a nucleophile in conjunction with Lys187 (Borgulya *et al*., 1989; Masjost *et al*., 2000; Vidgren *et al*., 1994). The Mg^{2+} ion exerts a double role in COMT catalysis. On the one hand it has a structural role by organising the active site of COMT and bringing together SAM and the catechol substrate; on the other hand, it has a functional role by facilitating the deprotonation of the catechol hydroxy group and lowering the pKa of Lys144. The side chain NH_2 of Lys144 is proposed to act as the catalytic base to abstract the proton from the hydroxy group of the catechol (Palma *et al*., 2006; Zheng & Bruice, 1997). Thus, the order of the catalytic reaction is as follows, SAM firstly binds to the enzyme, followed by the Mg^{2+} ion and the substrate (Ma *et al*., 2013; Tsao *et al*., 2011; Zheng & Bruice, 1997). Although COMT possesses broad substrate specificity, all known substrates are characterised by a catechol configuration regardless of other substituents on the aromatic nucleus (Guldberg & Marsden, 1975; Ma *et al*., 2013). Furthermore, COMT shows no stereospecificity with respect to D- and L-isomers of catecholamines (Axelrod & Tomchick, 1958; Garg *et al*., 1971; Guldberg & Marsden, 1975). Both COMT isoforms favour 3-O-methylation (*m*-O-methylation) with membrane-bound COMT being more regioselective than soluble COMT (Männistö & Kaakkola, 1999).

As mentioned, the O-methylation of catecholamines result almost exclusively in the generation of *m*-O-methylated metabolites (Axelrod, 1966; Guldberg & Marsden, 1975), thus the reaction proceeds with high regioselectivity (Kiss & Soares-da-Silva, 2014; Palma *et al*.,

2006). Certain xenobiotic catechols (Daly *et al*., 1960) and physiological substrates also produce *p*-O-methylated derivatives by the catalytic action of COMT (Creveling *et al*., 1972; Kuehl *et al*., 1964; Senoh *et al*., 1959; Winckle & Friedhoff, 1968). The reason for this anomaly between different ring substitutions is unknown. The ratio of *p*- versus *m*-O-methylated metabolites *in vitro* is dependent on the nature of the aromatic substrate and the pH of the reaction mixture. *m*-O-methylated products is predominantly generated with substrates containing a highly polar substituent (Creveling *et al*., 1970). Non-polar substituents yield an almost equal amount of *m*- and *p*-O-methylated metabolites (Guldberg & Marsden, 1975; Kiss & Soares-da-Silva, 2014). Modelling studies have confirmed that COMT favours *m*-O-methylation over *p*-O-methylation, since the binding of the *p*-hydroxy group of the catechol nucleus to the SAM methyl group forces the side chain in an unfavourable position which results in repulsive interactions with the protein residues of the enzyme (Kiss & Soares-da-Silva, 2014; Vidgren *et al*., 1994). Nitrocatechol inhibitors can also undergo O-methylation by COMT, although they act as poor substrates, yielding mono-O-methylether derivatives (Da Prada *et al*., 1994; Dingemanse *et al*., 1996; Palma *et al*., 2003; Palma *et al*., 2006).

2.8.3. The purification of the COMT enzyme

Axelrod & Tomchick were the first to purify COMT from rat liver with a 30-fold purification (Axelrod & Tomchick, 1958). By applying additional techniques (ion-exchange chromatography) it was possible to obtain a 200-fold purification (Anderson & D'Iorio, 1968). Since COMT is unstable in its purer forms, the lability of this preparation prevented further attempts at purification. Human liver COMT can be stabilised by the addition of $MgCl_2$ and ethylenediamine tetra-acetate (EDTA) in equimolar concentrations (Ball *et al*., 1971; Guldberg & Marsden, 1975). Expression of rat soluble COMT and membrane-bound COMT has previously been described by Ulmanen & Lundström (1991). Expression of human COMT in primary neurons can be achieved by cloning membrane-bound COMT cDNA (Ulmanen & Lundström, 1991) into the *Bam*HI restriction site of Semliki Forest virus expression vector pSFV1 (Liljeström & Garoff, 1991).

COMT expression in monkey COS-7 cells has also been described (Ulmanen *et al*., 1997). The construction of an expression plasmid carrying human COMT has been successfully designed by utilising Hep2 cells from porcine liver (Bertocci *et al*., 1991).

Exposure of *Caenorhabditis elegans* to tolcapone, an inhibitor of COMT, results in accumulation of dopamine and serotonin, making this model well-suited to investigate drug-mediated modulation of the dopamine and serotonin system in order to identify compounds with neuroprotective properties (Schumacher *et al*., 2015). The expression of recombinant COMT has preferentially been carried out using *Escherichia coli* due to the lack of inherent COMT activity (Lundström *et al*., 1992; Lundström *et al*., 1995). Immunoreactive and enzymatically active soluble and membrane-bound COMT have been expressed in eukaryotic expression systems using a baculovirus vector pAcYM1 (Lundström *et al*., 1995; Matsuura *et al*., 1987).

2.8.4. Inhibitors of COMT

Many catechol compounds and derivatives thereof are suitable as inhibitors and substrates of COMT (Guldberg & Marsden, 1975; Kiss & Soares-da-Silva, 2014). The parent catechol compound acts as a competitive substrate for COMT (Bacq *et al*., 1959) while acting as an inhibitor *in vivo* (Ozawa & Suzuki, 1971). The degradation products of adrenaline, adnamine and noradnamine (Abbs *et al*., 1967), are potent COMT inhibitors, while metanephrine is a weak inhibitor of COMT (Allen *et al*., 1969; Nikodwevic *et al*., 1970; Schwabe & Flohé, 1972). Catechol ketones inhibit COMT (Moffet *et al*., 1964), while the ketone analogues of adrenaline and noradrenaline, adrenalone and arterenone respectively, are *in vitro* COMT inhibitors with greater affinity for COMT than catechol (Guldberg & Marsden, 1975; Wylie *et al*., 1960). Some commonly used drugs are COMT inhibitors and include L-dopa, L-α-methyl-dopa-α-hydrazine, isoprenaline, apomorphine and desmethylpapaverine, as well as certain metabolites such as 2-hydroxyestrone and 7,8-dihydroxychlorpromazine (Guldberg & Marsden, 1975; Männistö & Kaakkola, 1999).

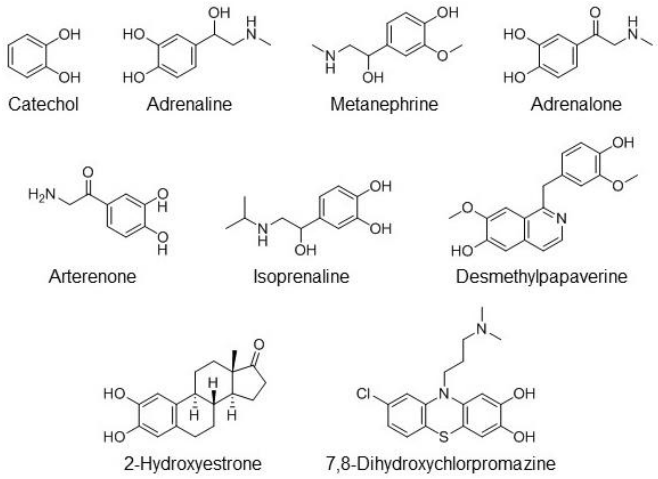

Figure 2.23. The chemical structures of various COMT inhibitors.

All the abovementioned compounds contain the catechol moiety and thus would be expected to inhibit COMT by competing with the substrate for the active sites of the enzyme. L-Dopa is a substrate for COMT (Axelrod & Tomchick, 1958) and inhibits COMT by competing for the active site with other substrates. Thus, L-dopa represents a weak COMT inhibitor (Guldberg & Marsden, 1975). L-Dopa can also indirectly inhibit COMT activity by depleting tissue concentrations of SAM (Chalmers *et al*., 1971; Wurtman *et al*., 1970) and inhibiting methionine (Met) uptake (Baldessarini & Karobath, 1972), which results in decreased synthesis of SAM and thus reduced COMT activity. In 1972 the hydrolysis product of SAM, S-adenosylhomocysteine, was identified as a potent inhibitor of COMT (Coward *et al*., 1972; Guldberg & Marsden, 1975). In the late 1980's several compounds were developed exhibiting potent COMT activity with reversible and selective inhibition of COMT. These compounds were clinically introduced in the late 1990's as second generation COMT inhibitors, namely tolcapone and entacapone (Müller, 2015b). Entacapone is the only selective peripheral COMT inhibitor which is usually administered in conjunction with L-dopa and an AADC inhibitor (Müller *et al*., 1993). Tolcapone, a centrally active COMT inhibitor, is also utilised clinically as adjunctive therapy to L-dopa for LID treatment. Opicapone can be characterised as a third generation COMT inhibitor, although it possesses a nitrocatechol structure. Opicapone is a purely peripheral COMT inhibitor with high binding affinity and slow dissociation constant, thus it has a longer inhibitory effect than observed for either tolcapone or entacapone (Müller, 2015b).

2.8.5. The therapeutic actions of COMT

COMT inhibitors are proposed to have antidepressant (Männistö *et al*., 1995; Moreau *et al*., 1994) and cognition improving effects (Khromova *et al*., 1995; Khromova *et al*., 1997), especially tolcapone. The nitrocatechol COMT inhibitors such as entacapone and nitecapone are effective antioxidants (Suzuki *et al*., 1992), NO scavengers (Marcocci *et al*., 1994) and iron chelators (Haramaki *et al*., 1995; Orama *et al*., 1997). Furthermore, nitrocatechols inhibit oxidative phosphorylation and uncouple mitochondrial energy production. Thus, these medications may protect cells from lipid peroxidation (Haramaki *et al*., 1995). These protective effects do not appear to be related to COMT inhibition (Männistö & Kaakkola, 1999).

Clinically COMT inhibitors have various applications in the treatment of central and peripheral nervous system disorders (Jatana *et al*., 2013) such as Parkinson's disease (Calne, 1993; Männistö & Kaakkola, 1989), restless leg syndrome (Sharif, 2002), mood

disorder (Fava *et al*., 1999), cognition improvement (Lachman *et al*., 1996), bipolar disorder (Berk *et al*., 2007), oedema formation state (Vieira-Coelho *et al*., 2001), gastrointestinal disturbances (Larsen *et al*., 1998) and other dopamine deficiency-related diseases such as attention deficit disorder (Talan, 2009) and attention deficit hyperactivity disorder (DiMaio *et al*., 2003; Kiss & Soares-da-Silva, 2014). COMT metabolises dopamine at cortical levels suggesting that COMT inhibition may serve as a promising adjunct therapy for schizophrenia (Egan *et al*., 2001; Meyer-Lindenberg & Weinberger, 2006; Tunbridge *et al*., 2004; Yavich *et al*., 2007).

Since the peripheral inhibition of soluble COMT by tolcapone has been identified as a possible cause for its hepatotoxicity (Chen *et al*., 2011), the identification or development of membrane-bound specific COMT inhibitors has been proposed as a possibility for future therapies. Tolcapone is the most potent membrane-bound COMT inhibitor and combines with COMT in a tight-binding manner, thus inhibition increases the longer the enzyme and drug interacts (Borges *et al*., 1997; Robinson *et al*., 2012). The hepatoxicity of tolcapone may also originate from the methylation and oxidation reaction it undergoes to yield amine and acetylamine metabolites. These metabolites are further metabolised to reactive intermediates which form covalent adducts to hepatic proteins resulting in liver tissue damage (Lin *et al*., 2012).

2.8.6. COMT gene polymorphisms are implicated in the development of Parkinson's disease

Certain gene polymorphisms have been identified for COMT (Białecka *et al*., 2005). Polymorphisms of COMT are commonly found in younger patients with Parkinson's disease and in women (Goudreau *et al*., 2002). The substitution of Met at codon 158 on the COMT gene results in a 40% decrease in COMT activity relative to the valine (Val) allele and thereby increasing cortical dopamine levels (Grant *et al*., 2013; Lotta *et al*., 1995; Scanlon *et al*., 1979). Carriers of the Val allele have been shown to exhibit less efficient prefrontal neuronal signalling and relative deficits in cognitive functioning (Bertolino *et al*., 2006; Diaz-Asper *et al*., 2008; Dumontheil *et al*., 2011). The nitrocatechol COMT inhibitor, tolcapone, has been shown to improve executive functioning as well as the efficacy of processing cortical information in individuals with the Val allele of COMT (Apud *et al*., 2007; Farrell *et al*., 2012; Giakoumaki *et al*., 2008).

Another gene implicated for dopamine metabolism is rs4680 in the COMT gene (Klebe *et al*., 2013; Moreau *et al*., 2015) which increases the risk for Parkinson's disease (Kiyohara *et al*., 2011). Hao *et al*. established that Chinese patients possessing polymorphisms of COMT genes experience differences in drug responses (Hao *et al*., 2015). Furthermore, COMT polymorphisms are more common among patients suffering from Parkinson's disease with "wearing-off" compared to patients without "wearing-off" effects (Hao *et al*., 2014; Watanabe *et al*., 2003). Polymorphisms of COMT genes may change the levels of the biogenic amines it metabolises as well as their metabolic products (Lee *et al*., 2001b; Lin *et al*., 2002; Nakamura *et al*., 2000). The G1947A polymorphism of COMT influences the response to L-dopa and subsequently the treatment efficacy of Parkinson's disease (Costa *et al*., 1997; Singh *et al*., 2008; Torkaman-Boutorabi *et al*., 2012b; Wu *et al*., 2001).

2.8.7. The structure of COMT

The structure of COMT consists of a single domain α/β-folded structure with 8 α-helices arranged around a central mixed β-sheet (Männistö & Kaakkola, 1999; Vidgren *et al*., 1994). The central β-sheet is sandwiched between helices α1–5 on one side and α6–8 on the other. In the β-sheet, strand 7 is antiparallel to the other 6 sheets (Ma *et al*., 2013). The active site of the enzyme consists of the SAM-binding domain and the actual catalytic site (Ehler *et al*., 2014; Kiss & Soares-da-Silva, 2014). Thus, the active site of COMT has a bipartite character (Ehler *et al*., 2014; Vidgren *et al*., 1994). The catalytic site consists of a few amino acids involved in substrate binding, water and Mg^{2+} involved in the catalytic reaction of O-methylation (Männistö & Kaakkola, 1999; Masjost *et al*., 2000). The Mg^{2+} binds to COMT after SAM-binding and transforms one of the catechol hydroxy groups to be more easily ionisable (Bonifácio *et al*., 2002; Männistö & Kaakkola, 1999; Zheng & Bruice, 1997). Lys144 accepts the proton from the hydroxy of the substrate and subsequently the methyl group from SAM is transferred to the catechol hydroxy group (Bonifácio *et al*., 2002; Masjost *et al*., 2000). Thus, the Lys residue acts as a catalytic base in the base-catalysed nucleophilic reaction. The Mg^{2+} ion is octahedrally situated between two aspartic acid residues (Asp141 and Asp169), one asparagine (Asn170), both catechol hydroxy groups and a water molecule (Ma *et al*., 2013; Männistö & Kaakkola, 1999; Rutherford *et al*., 2008). Thus, the Mg^{2+} ion is also responsible for the orientation of the catechol moiety in the active site. COMT, as with MAO, has "gatekeeper" residues that form hydrophobic walls and define the selectivity of COMT towards the substrate and keeps the planar catechol ring in the correct position for the methylation reaction. These residues, Trp38, Trp143 and Pro174, are also involved in the binding of substrates and subsequently inhibitors to the active site of COMT (Vidgren *et*

al., 1991; Vidgren & Ovaska, 1997; Vidgren *et al*., 1999). The interaction of the catechol aromatic ring with Trp38 is essential for high binding affinity (Kiss & Soares-da-Silva, 2014; Ma *et al*., 2013). The active sites of both soluble COMT and membrane-bound COMT are located on the outer surface of the enzyme and is a shallow groove. The SAM-binding pocket is deeper within the protein than the Mg^{2+} site (Bonifácio *et al*., 2002; Vidgren *et al*., 1994; Vidgren & Ovaska, 1997), hence the sequential ordered catalytic mechanism where SAM binds first, followed by Mg^{2+} and finally the catechol substrate (Kiss & Soares-da-Silva, 2014; Männistö & Kaakkola, 1999). The adenine moiety of SAM has favourable van der Waals interactions with Met91, His142 and Trp143, while the ribose moiety interacts with Trp143 (Bonifácio *et al*., 2002). The adenine ring of SAM is hydrogen bonded to Ser119 and Gln120, with the Met portion of the cofactor hydrogen bonded to Val42, Ser72 and Asp141 (Kiss & Soares-da-Silva, 2014; Ma *et al*., 2013).

The catalytic sites of soluble COMT and membrane-bound COMT have identical amino acid sequences with membrane-bound COMT having more favourable binding interactions with substrates even though no conformational change is evident in the basic structures of the enzymes (Kiss & Soares-da-Silva, 2014; Männistö & Kaakkola, 1999). Soluble COMT has 221 amino acids (Bonifácio *et al*., 2002; Lotta *et al*., 1995; Vidgren *et al*., 1994), whereas membrane-bound COMT has a 50-residue terminal responsible for the hydrophobic anchor region, a single-pass helical membrane anchor (Kiss & Soares-da-Silva, 2014). There is marked kinetic differences between soluble COMT and membrane-bound COMT, e.g. membrane-bound COMT exhibits a higher substrate affinity than soluble COMT (Kiss & Soares-da-Silva, 2014).

COMT is a rather slow enzyme with sizeable conformational changes during the catalytic cycle (Ehler *et al*., 2014; Vidgren *et al*., 1994). This may be due to the bi-bi mechanism where SAM binds first to COMT then the catechol substrate (Ehler *et al*., 2014; Tunnicliff & Ngo, 1983). The apo form, the semi-holo form (SAM bounded but not Mg^{2+}) and the holo form (SAM and Mg^{2+} bounded) are all obligatory intermediates within the reaction mechanism (Ehler *et al*., 2014). Conformational variations in COMT in the presence of inhibitors are limited since they mostly represent a closed conformation with both substrate binding sites occupied (Tsuji *et al*., 2009). The human apo intermediate showed that the α2/α3 loop is disordered while the β5/α9 and β6/β7 loop regions are well defined. It is proposed that this apo form is generated after the reaction products have dissociated from COMT. COMT exhibits structural plasticity which is not restricted to loops α2/α3, β5/α9 and

β6/β7 (Ehler *et al*., 2014). Entire subdomains in COMT can be swapped to create dimers (Ellerman *et al*., 2011). Although the β6/β7 hinge region is flexible, human COMT does not exhibit a high propensity for domain swapping. These domain swaps occur at the hinge regions β6/β7 and α2/α3 mostly upon ligand binding. Since these regions are involved in ligand binding, each one of them are a drug target for future inhibitors with novel binding modes and inhibition mechanisms (Ehler *et al*., 2014).

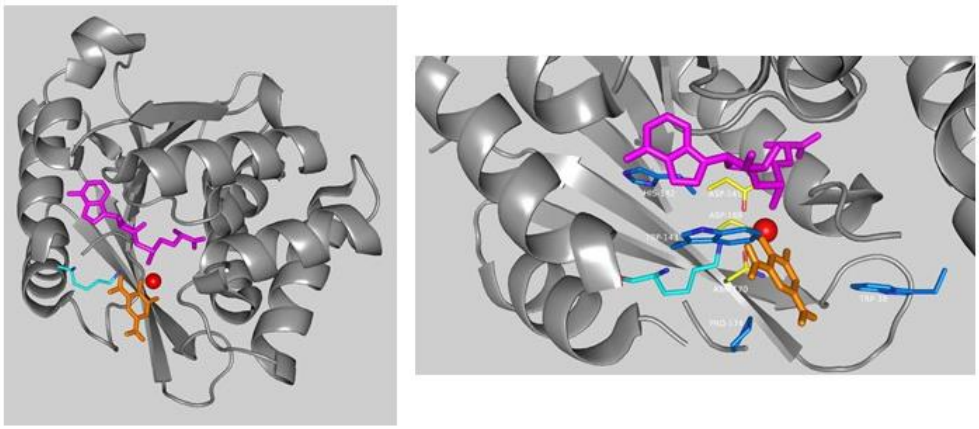

Figure 2.24. Diagram representing the structure of human COMT showing the active site architecture. SAM is shown in magenta, the inhibitor (3,5-dinitrocatechol) in orange and Lys144 in cyan (Adapted from Rutherford *et al*., 2008).

2.9. Summary

Parkinson's disease is considered as one of the most common neurodegenerative diseases of which the aetiology and pathogenesis is still largely unresolved. Parkinson's disease pathogenesis is multifactorial and as such a vast array of strategies have been postulated as potentially neuroprotective, while several symptomatic treatment options have been investigated. Since MAO and COMT play vital parts in the development and neurochemistry of Parkinson's disease, these enzymes are interesting drug targets. It has been established that inhibitors of MAO that specifically target the MAO-B isoform of the enzyme are of significant value in Parkinson's disease treatment. As such, inhibitors of MAO-B block the central metabolism of dopamine and thus enhance dopaminergic neurotransmission. Inhibitors of COMT have a pivotal role as adjunct therapy for the treatment of Parkinson's disease and reduce the metabolism of both L-dopa and dopamine. Thus, a multi-target-directed approach that target both abovementioned enzymes may present an interesting and relevant approach to future Parkinson's disease treatment.

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Chapter 3

Article 1

The synthesis and evaluation of nitrocatechol derivatives of chalcone as dual inhibitors of monoamine oxidase and catechol-O-methyltransferase

Idalet Engelbrecht,[1] Jacobus P. Petzer,[1] Anél Petzer [1,*]

1. *Pharmaceutical Chemistry, School of Pharmacy and Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa*

*Corresponding author: Anél Petzer, Tel.: +27 18 2994464

E-mail address: 12264954@nwu.ac.za

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Abstract

Parkinson's disease is regarded as the second most common neurodegenerative disorder after Alzheimer's disease. The clinical syndrome consists of a tetrad of symptoms namely tremor at rest, slowness of involuntary movements, rigidity and postural instability. Even though the aetiology of Parkinson's disease is still unknown, the neurochemical deficits and neuropathological defects are well defined. Parkinson's disease is the result of the degeneration of dopaminergic neurons located in the substantia nigra pars compacta, resulting in a reduction in brain dopamine levels. The prevention of Parkinson's disease is almost impossible since clinical signs are only observed when neural degeneration is in the advanced stages. Available therapeutics are essentially symptomatic, with no treatment

available to slow or halt the degenerative process. The discovery of levodopa (L-dopa) transformed the treatment of Parkinson's disease. The efficacy of L-dopa depends on its metabolic conversion to dopamine in the brain, however extensive peripheral metabolism of L-dopa diminishes its availability for uptake into the brain. L-Dopa is extensively decarboxylated in the gastrointestinal tract and peripheral tissues by the enzyme aromatic-L-amino acid decarboxylase (AADC), and AADC inhibitors are thus frequently combined with L-dopa therapy. When AADC is inhibited, 3-O-methylation catalysed by catechol-O-methyltransferase (COMT) becomes a dominant metabolic pathway for L-dopa, and COMT inhibitors are thus also used as adjuncts to L-dopa in Parkinson's disease. Since COMT and monoamine oxidase (MAO) metabolises dopamine in the brain, inhibitors of these enzymes may be of further value in Parkinson's disease by conserving the depleted supply of dopamine in the brain. Based on the roles of COMT and MAO in the metabolism of dopamine, the present study attempts to discover novel dual inhibitors of these enzymes. For this purpose, nitrocatechol derivatives of chalcone were synthesised and evaluated as inhibitors of COMT and MAO. The chalcone class of compounds is well known to potently inhibit MAO-B, while nitrocatechol derivatives (e.g. tolcapone and entacapone) are clinically used COMT inhibitors. The results documented that all of the derivatives are high potency *in vitro* inhibitors of rat liver COMT with IC_{50} values ranging from 0.07 to 0.29 µM. Under these experimental conditions, tolcapone and entacapone display IC_{50} values of 0.26 µM and 0.25 µM, respectively. The chalcones were, however, much less potent as inhibitors of MAO with IC_{50} >13.9 µM for the *in vitro* inhibition of human MAO-B. Although none of the compounds acts as potent MAO inhibitors, this study shows that nitrocatechol derivatives of chalcone are a promising class of COMT inhibitors.

1. Introduction

Parkinson's disease is regarded as the second most common neurodegenerative disorder after Alzheimer's disease and affects 1-2% of the human population over 50 years of age (de Rijk *et al*., 1995). The disease clinically present as tremor at rest, slowness of movement or bradykinesia, rigidity and postural instability or gait impairment (Lees, 2005). The primary cause of Parkinson's disease is the degeneration of dopaminergic neurons, specifically those of the nigrostriatal pathway which delivers dopamine to the striatum. This results in a functional deficit of dopamine in the striatum (Dauer & Przedborski, 2003). No drugs are currently approved as neuroprotective agents in Parkinson's disease and disease progression remains untreated. Parkinson's disease is also characterised by non-motor symptoms which is easily overlooked since it closely resembles normal aging. Non-motor symptoms include cognitive dysfunction, fatigue, sleep disturbances, autonomic dysfunction

and anosmia (Sprenger & Poewe, 2013). The treatment of these non-motor complications prove difficult since they do not respond to dopaminergic innervation (Chaudhuri *et al*., 2006). Thus, these non-motor symptoms represent an area of unmet therapeutic need.

The treatment of the motor symptoms of Parkinson's disease focuses on restoring striatal dopaminergic neurotransmission. This may be achieved by increasing dopamine supply through L-dopa administration, dopamine receptor stimulation with dopamine agonist therapy or by inhibiting dopamine reuptake and metabolism (Lees, 2005). As mentioned, current treatment options are purely symptomatic and do not prevent neuronal degeneration. After the introduction of levodopa (L-dopa) in 1967, this drug is still considered the gold standard of Parkinson's disease treatment (Freitas *et al*., 2016; Poewe & Antonini, 2015). L-Dopa is the metabolic precursor of dopamine and, in contrast to dopamine, permeates the blood-brain barrier by carrier-mediated transport (Di Stefano *et al*., 2011). In the brain, L-dopa is converted to dopamine, thus effectively replacing the lost dopamine in the striatum (Di Stefano *et al*., 2009). Unfortunately, L-dopa undergoes rapid metabolism at both the peripheral and central levels, which limits the therapeutic potential thereof (Freitas *et al*., 2016; Contin & Martinelli, 2010). Approximately 70% of the oral L-dopa dose is converted to dopamine in the peripheral tissues which limits the amount that remains intact for passage into the brain (Kiss & Soares-da-Silva, 2014). Since the predominant metabolic pathway for L-dopa is decarboxylation by the enzyme aromatic-L-amino acid decarboxylase (AADC), peripheral AADC inhibitors (e.g. benserazide and carbidopa) are thus frequently combined with L-dopa therapy. This improves the bioavailability of L-dopa and reduces peripheral dopaminergic side effects such as cardiac arrhythmias, hypotension, nausea and vomiting (due to the peripheral conversion of L-dopa to dopamine) (Seeberger & Hauser, 2015). When AADC is inhibited, 3-O-methylation catalysed by catechol-O-methyltransferase (COMT), however, becomes a dominant metabolic pathway for L-dopa, and due to metabolism by COMT less than 10% of the oral L-dopa dose reaches the brain (Nutt & Fellman, 1984). The peripheral inhibition of COMT is thus proposed to prolong the half-life of L-dopa and decrease the formation of the metabolic product, 3-O-methyldopa, which competes with L-dopa for uptake at the blood-brain barrier (Learmonth *et al*., 2004; Nissinen *et al*., 1992). Several clinical observations have shown that poor response to L-dopa therapy is associated with high plasma levels of 3-O-methyldopa (Tohgi *et al*., 1991). Peripheral COMT inhibition will thus increase the availability of L-dopa to the brain, its therapeutic benefit and also allow a reduction in L-dopa dose (Guldberg & Marsden, 1975; Männistö & Kaakkola, 1999).

COMT exists in two isoforms, namely soluble COMT and membrane-bound COMT which is encoded by a single gene (Lundström *et al*., 1991; Männistö & Kaakkola, 1999; Salminen *et al*., 1990). Both isoforms are identical except for the inclusion of an additional 50 hydrophobic amino acid sequence in membrane-bound COMT which is attached to the cytoplasmic side of intracellular membranes (Chen *et al*., 2011; Tunbridge *et al*., 2004; Ulmanen & Lundström, 1991). Early research indicated that COMT is localised in the soluble fraction of the cell (Axelrod & Tomchick, 1958; Guldberg & Marsden, 1975). Later investigations identified membrane-bound COMT. Membrane-bound COMT does not appear to differ in biochemical and immunological characteristics from soluble COMT (Borchardt *et al*., 1974; Guldberg & Marsden, 1975). This indicates that results may be extrapolated between the isoforms without compromising the integrity of the results. COMT activity in vertebrates is the greatest in the liver (Axelrod & Tomchick, 1958; Männistö *et al*., 1992). Vertebrate COMT activity occur mostly in the soluble form, with only a minor fraction attributed to membrane-bound fraction of COMT (Ding *et al*., 1996; Karhunen *et al*., 1994; Rivett *et al*., 1983; Roth, 1992). Species differences exist in the physiochemical properties as well as level of enzyme activity which indicates that extrapolating results between species should only be done with caution (Agathopoulous *et al*., 1971; Guldberg & Marsden, 1975; Männistö & Kaakkola, 1999). COMT activity and localisation is significantly lower in the central nervous system than in the peripheral tissues (Kiss & Soares-da-Silva, 2014). In humans, 70% of the total centrally located COMT is membrane-bound while 30% is attributed to soluble COMT activity (Männistö & Kaakkola, 1999). Membrane-bound COMT is considered to be a more important drug target since it is able to methylate catecholamines at their physiological concentration (Roth, 1992). Apart from this, soluble COMT plays a more important role in non-physiological conditions such as when the substrate concentration suddenly increases (i.e. after L-dopa treatment) or when a higher methylation reaction rate is needed (Huotari *et al*., 2002; Lotta *et al*., 1995; Ma *et al*., 2013).

It is important to note that dopamine also is a substrate for COMT and therefore it may be argued that, while peripheral inhibition of COMT is the appropriate strategy for reducing the metabolism of L-dopa, central COMT inhibition will block the metabolism of dopamine in the brain and thus exert a dopamine sparing effect (Guldberg & Marsden, 1975; Männistö & Kaakkola, 1999). This in turn will enhance dopaminergic neurotransmission and the efficacy of L-dopa therapy. As adjuvants to L-dopa, inhibitors that inhibit both central and peripheral COMT may be of enhanced value (Heeringa *et al*., 1997).

Another approach to improve the therapeutic efficacy of L-dopa is to inhibit the monoamine oxidase (MAO)-catalysed metabolism of dopamine in the brain. MAO inhibitors may enhance dopamine levels derived from L-dopa and thus not only improve the therapeutic effect but also allow for a reduction in the L-dopa dosage required for an effective therapeutic effect (Youdim *et al*., 2006; Youdim & Bakhle, 2006). All mammals contain MAO-A and MAO-B with various tissue distribution and activity (Nicotra *et al*., 2004; Setini *et al*., 2005; Strolin Benedetti *et al*., 1992; Tsang *et al*., 1986). Both isoforms of MAO contain the flavin adenine dinucleotide (FAD) cofactor (Edmondson *et al*., 2004; Edmondson *et al*., 2007; Kearney *et al*., 1971; Youdim *et al*., 2006). In contrast to COMT where both forms are encoded by the same gene, the two isoforms of MAO are encoded by different genes that correspond to different amino acid sequences with 70% identity between them (Binda *et al*., 2007; Wong *et al*., 2002; Zhu *et al*., 1994).

MAO metabolise primary, secondary and tertiary monoamines and catalyses the oxidation of serotonin, histamine, and the catecholamines dopamine, adrenaline and noradrenaline (Blaschko *et al*., 1937; Carradori *et al*., 2014; Shih *et al*., 1999; Tipton *et al*., 2004; Youdim *et al*., 2005; Zeller, 1938). In most species dopamine, adrenaline, noradrenaline, tryptamine and tyramine are oxidised by both MAO isoforms (Glover *et al*., 1977; Tipton *et al*., 2004; Youdim *et al*., 1988). Although both MAO isoforms metabolise dopamine in the brain, MAO-B inhibitors (e.g. selegiline and rasagiline) are used in Parkinson's disease. MAO-A inhibitors are avoided due to a potentially fatal hypertensive crisis that may arise when irreversible MAO-A inhibitors are combined with tyramine containing food, and concerns over elevations in blood-pressure when these drugs as coadministered with L-dopa (Flockhart, 2012). Besides a dopamine sparing effect, MAO-B inhibitors may also, by reducing the MAO-catalysed formation of hydrogen peroxide and ensuing oxidative damage in the brain, represent potential neuroprotective agents in Parkinson's disease (Youdim & Bakhle, 2006). Oxidative damage appears to be an important factor in the neurodegenerative processes associated with Parkinson's disease (Dauer & Przedborski, 2003). The inhibition of MAO-B is a particularly relevant strategy when considering that MAO-B activity and density increase in the brain with aging (Fowler *et al*., 1997). It is noteworthy that MAO-B inhibitors are also used as monotherapy in the early stages of Parkinson's disease and may delay the emergence of the motor symptoms that require the initiation of L-dopa therapy (Robakis & Fahn, 2015).

Based on the roles of COMT and MAO-B in in the metabolism of dopamine, the present study attempts to discover novel dual inhibitors of these enzymes. Compared to specific inhibition of either enzyme, dual inhibition may have enhanced value in Parkinson's disease

particularly as adjuvants to L-dopa. In this respect, both peripheral and central enzymes may be targeted, which would result in the enhanced availability of L-dopa for uptake into the brain as well as the sparing of depleted dopamine in the brain. This approach would enhance the therapeutic efficacy of L-dopa, but also allow for the effective L-dopa dosage to be reduced. A reduction of L-dopa dosage would greatly decrease the potential for L-dopa-associated adverse effects such as dyskinesia. The multi-target-directed approach as considered here has been advocated as particularly relevant for the design of therapies for neurodegenerative disorders where multiple drugs are often co-prescribed (Cavalli *et al*., 2008).

For dual inhibition of COMT and MAO-B, the present study considers nitrocatechol derivatives of chalcone (**1a–k**). The general structure of the compounds investigated here are shown in figure 1. As shown, the A-ring consists of the nitrocatechol moiety while limited substitution will be explored on ring B.

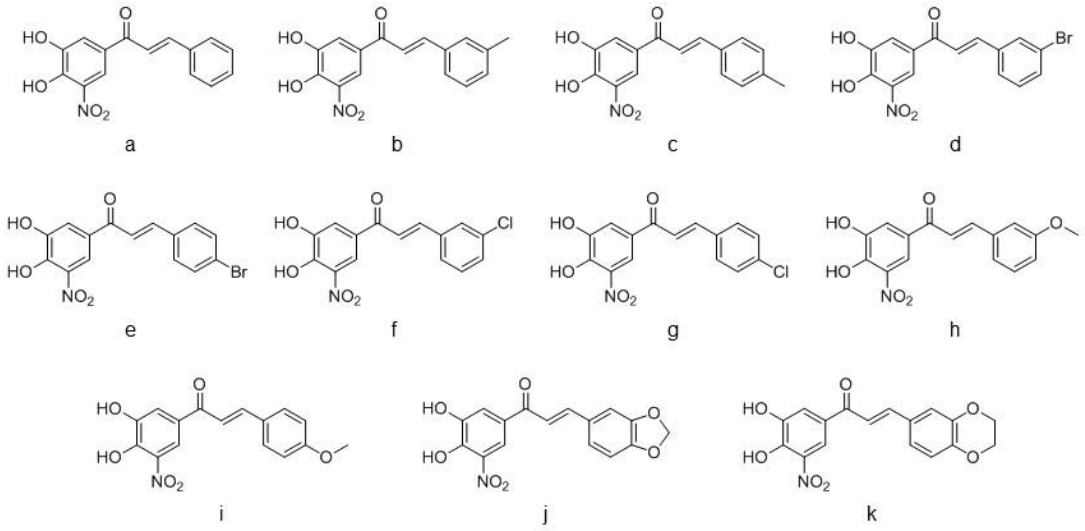


Figure 1. The structures of the nitrocatechol derivatives of chalcone (**1a–k**) that were investigated in this study.

The chalcone class of compounds is well known to potently inhibit MAO-B. For example, chalcone **2** is an example of a potent inhibitor of human MAO-B with an IC_{50} value of 0.0051 µM (figure 2) (Chimenti *et al*., 2009). This compound is a much weaker human MAO-A inhibitor with an IC_{50} value of 4.95 µM. In general, potent MAO-B inhibition is obtained with substitution with polar groups (e.g. OH) on the A-ring and halogens on ring B.

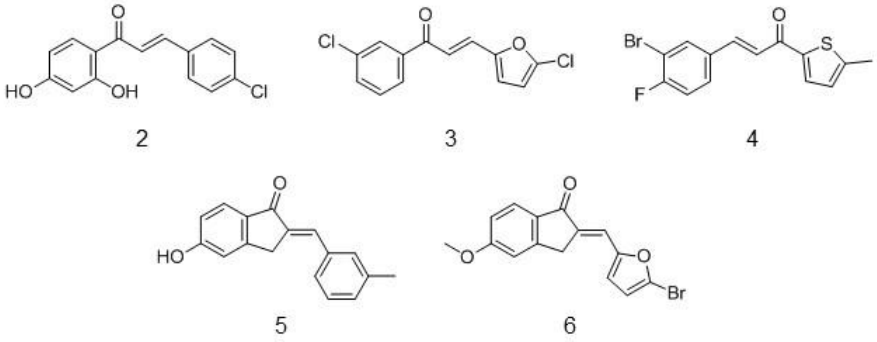


Figure 2. The structures of chalcone derivatives known to inhibit MAO.

A variety of studies have demonstrated MAO inhibition by chalcone and chalcone derivatives (Choi *et al*., 2015; Gao *et al*., 2001; Hammuda *et al*., 2016; Haraguchi *et al*., 2004; Mathew *et al*., 2016a; Mathew *et al*., 2016b; Pan *et al*., 2000; Tanaka *et al*., 1987). These include heterocyclic chalcone derivatives as well as cyclic derivatives of chalcone (Robinson *et al*., 2013; Minders *et al*., 2015; Morales-Camilo *et al*., 2015; Huang *et al*., 2012; Nel *et al*., 2016a; Nel *et al*., 2016b). For example, heterocyclic chalcone derivatives **3** and **4** inhibit MAO-B with IC_{50} values of 0.174 µM and 0.067 µM, respectively, while cyclic chalcones **5** and **6** display IC_{50} values of 0.0052 µM and 0.0044 µM, respectively (Minders *et al*., 2015; Nel *et al*., 2016a; Nel *et al*., 2016b).

Nitrocatechol compounds, in turn, are known to act as COMT inhibitors. COMT inhibitors containing the 3-nitrocatechol moiety that have been developed and introduced into the market (or proceeded to clinical trials) include tolcapone, entacapone, opicapone, nebicapone and nitecapone (figure 3) (Kiss & Soares-da-Silva, 2014). These are the so-called second generation COMT inhibitors and have been successfully used as adjuvants to L-dopa in the treatment of Parkinson's disease. The possibility that members of the chalcone class of compounds may inhibit COMT is supported by reports that the flavonoid, quercetin (**7**, K_i = 8.4 µM), is a COMT inhibitor (Gugler & Dengler, 1973). Quercetin may be viewed as a ring-closed chalcone derivative. Furthermore, chalcone **8** (IC_{50} = 5 nM) is reported to inhibit rat brain COMT (Bäckström *et al*., 1989). In contrast to the study compounds where the A-ring consists of the nitrocatechol moiety, the ring B of **8** represents the nitrocatechol moiety. Chalcones (e.g. **9**) with nitro substitution in the *ortho* position of ring B are also known COMT inhibitors (Pérez *et al*., 1993). It may also be noted that tolcapone and nebicapone, although not chalcones, structurally resemble chalcone and in particular, bear the carbonyl in the benzylic position with respect to the nitrocatechol moiety, which is similar to the compounds of the present study.

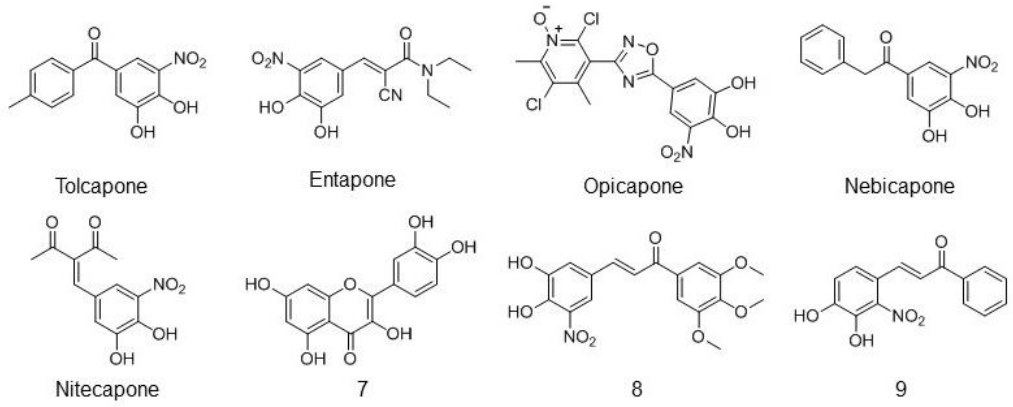


Figure 3. The structures of 3-nitrocatechol derivatives known to inhibit COMT.

2. Results

2.1. Chemistry

In the present study a series of 3-nitrocatechol derivatives of chalcone (e.g. 3,4-dihydroxy-5-nitrochalcones) were synthesised with the aim of discovering compounds that inhibit both COMT and MAO. The synthetic route consisted of three steps (figure 4). Firstly, nitration of 4-hydroxy-3-methoxyacetophenone (apocynin; **10**) was carried out with 60% nitric acid in the presence of acetic acid according to the literature description to yield the nitro derivative **11** (5-nitroapocynin). In the second step, demethylation of **11** was carried out with $AlCl_3$/pyridine to yield the 5-acetyl-3-nitrocatechol **12** (Kiss *et al*., 2010). The target chalcones **1a**–**k** was obtained via the Claisen-Schmidt condensation reaction between **12** and an appropriately substituted aldehyde in ethanol. Potassium hydroxide (60%) served as the base. HCl was used to acidify the reaction and was added during workup of the reaction (Klinke & Gibian, 1961).

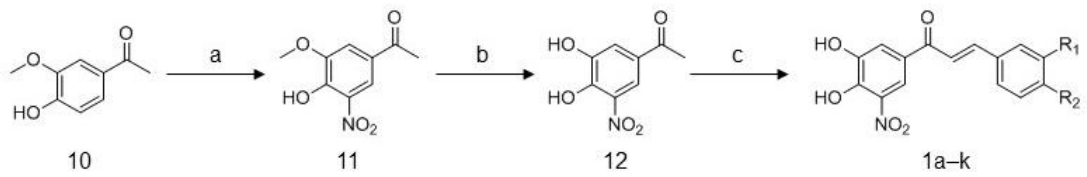


Figure 4. The synthetic route for the synthesis of nitrocatechol derivatives of chalcone (**1a**–**k**). Reagents and conditions: (a) 60% HNO_3, acetic acid; (b) $AlCl_3$, pyridine, ethyl acetate, 80 °C, HCl; (c) appropriately substituted benzaldehyde, ethanol, 60% KOH.

The target 3,4-dihydroxy-5-nitrochalcone derivatives were purified by crystallisation from an appropriate solvent. Even though the overall yields were low, the recrystallised compounds were of high purity as found by the high-performance liquid chromatography (HPLC) analyses (see experimental). In each instance, the structures of the target compounds were verified by ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR and mass spectrometry. In particular, the signal of the carbonyl carbon was observed at ~186 ppm, while the *trans* geometry of the conjugated double bond was confirmed by coupling constants of ~15.5 Hz for the vinylic protons.

2.2. MAO inhibition studies

The MAO inhibitory properties of the 3-nitrocatechol derivatives of chalcone (**1a**–**k**) were investigated with the recombinant human MAOs as enzyme sources. For the activity measurements, kynuramine was used as substrate for both MAO-A and MAO-B, and the MAO-catalysed oxidation product, 4-hydroxyquinoline, was measured by fluorescence spectrophotometry (Mostert *et al*., 2015; Novaroli *et al*., 2005). By measuring enzyme catalytic rates in the absence and presence of various concentrations of the test inhibitors, sigmoidal plots of rate versus inhibitor concentration (Log[I]) were constructed from which IC_{50} values were estimated (figure 5). IC_{50} values were measured in triplicate and are given as mean ± standard deviation (SD).

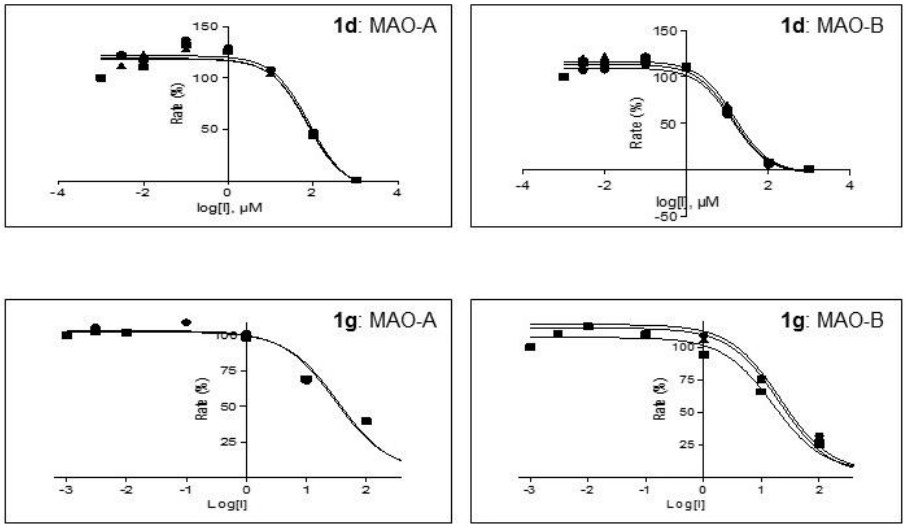
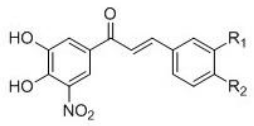


Figure 5. Sigmoidal plots for the inhibition of MAO-A and MAO-B by compound **1d** and **1g**.

The IC_{50} values thus recorded are given in Table 1. The selectivity index (SI) for each compound was also calculated and indicates the specificity for inhibition of the MAO-B isoform. It is clear from the inhibition data that 3-nitrocatechol derivatives of chalcone are not potent inhibitors of either MAO-A or MAO-B with IC_{50} values >32.4 µM and >13.9 µM, respectively. The most potent inhibition was observed for **1d** which displays an IC_{50} of 13.9 µM for MAO-B inhibition. This compound also displays the highest degree of specificity with a SI value of 5.48. Since chalcones are in general good potency inhibitors of MAO-B, particularly compounds containing polar groups on the A-ring (e.g. OH) and halogens in the B-ring (e.g. Cl), it may be concluded that nitro substitution on ring A virtually abolished MAO-B inhibition (Chimenti *et al*., 2009). This is most apparent when comparing the inhibition potencies of **1f** to chalcone **2**.

Table 1

The IC_{50} values for the inhibition of recombinant human MAO-A and MAO-B, and the IC_{50} values for the inhibition of rat liver COMT by the synthesised 3,4-dihydroxy-5-nitrochalcone derivatives (**1a**–**k**).



	R^1	R^2	IC_{50} (µM)[a]		SI[b]	IC_{50} (µM)[a]
			MAO-A	MAO-B		COMT
1a	–H	–H	59.0 ± 8.19	56.1 ± 5.14	1.05	0.17 ± 0.05
1b	$–CH_3$	–H	46.6 ± 3.25	31.8 ± 3.80	1.46	0.12 ± 0.01
1c	–H	$–CH_3$	37.6 ± 0.73	15.7 ± 1.04	2.40	0.13 ± 0.07
1d	–Br	–H	76.1 ± 4.13	13.9 ± 0.89	5.48	0.29 ± 0.01
1e	–H	–Br	No inhibition[c]	17.7 ± 7.33	-	0.09 ± 0.03
1f	–Cl	–H	48.0 ± 9.92	16.6 ± 3.19	2.89	0.17 ± 0.07
1g	–H	–Cl	32.4 ± 2.76	19.0 ± 2.77	1.71	0.08 ± 0.03
1h	$–OCH_3$	–H	67.6 ± 8.20	56.0 ± 2.26	1.21	0.07 ± 0.03
1i	–H	$–OCH_3$	41.7 ± 4.35	57.8 ± 5.10	1.39	0.11 ± 0.03
1j	3’,4’-methylenedioxy		46.6 ± 1.39	No inhibition[c]	-	0.15 ± 0.06
1k	3’,4’-ethylenedioxy		58.5 ± 2.73	38.9 ± 0.74	1.50	0.12 ± 0.02
	Tolcapone		-	-	-	0.26 ± 0.01
	Entacapone		-	-	-	0.25 ± 0.15

[a] All values are expressed as the mean ± SD of triplicate determinations.
[b] Selectivity index (SI) = IC_{50}(MAO-A)/ IC_{50}(MAO-B).
[c] No inhibition observed at maximum tested concentration of 100 µM.

In spite of their weak MAO inhibition potencies, some structure-activity relationships (SARs) may be derived. Substitution of the B-ring of the chalcone with a halogen (Br, Cl) results in more potent MAO-B inhibition (**1d–g** versus **1a**). The position of the halogen (*meta* versus *para*) does not affect MAO-B inhibition to a large degree. Interestingly, substitution with a methyl group in *para* position of the B-ring resulted in more potent MAO-B inhibition than *meta* substitution. This trend is also observed for MAO-A inhibition. For substitution with methoxy, the position (*meta* versus *para*) does not affect MAO-A and MAO-B inhibition to a large degree.

2.3. Reversibility of MAO-B inhibition

It has previously been reported that chalcone derivatives act as reversible MAO-B inhibitors (Chimenti *et al*., 2009; Huang *et al*., 2012; Minders *et al*., 2015; Morales-Camilo *et al*., 2015; Nel *et al*., 2016a; Nel *et al*., 2016b; Robinson *et al*., 2013). To verify that this property is shared by nitrocatechol chalcone derivatives, the reversibility of inhibition of a representative compound, **1d**, was examined. For this purpose, MAO-B and the selected inhibitor was pre-incubated for 15 min at 37 °C. The inhibitor concentration was set to to 4 × IC_{50}. The reactions were subsequently placed into dialysis cassettes and dialysed for 20–25 h at 4 °C. After dialysis, the samples were diluted twofold to yield an inhibitor concentration of 2 × IC_{50} by adding kynuramine and the residual MAO-B activities were measured. For this purpose, the reactions were incubated for 20 min, and 4-hydroxyquinoline generated from the MAO-B-catalysed oxidation of kynuramine was quantified by fluorescence spectrophotometry. For comparison, the residual MAO-B activities in non-dialysed enzyme-inhibitor complexes were also recorded. As negative and positive controls, respectively, similar dialysis experiments were carried out in the absence of inhibitor and presence of the irreversible inhibitor selegiline.

The results of the dialysis experiments are shown in figure 6. The results indicate that **1d** is a reversible inhibitor of MAO-B since enzyme activity is recovered by dialysis, with the activitiy at 84% compared to the negative control value (100%). In contrast, dialysis failed to restore catalytic activity when MAO-B was incubated in the presence of selegiline (residual activity of 3.5%). For comparison, inhibition of MAO-B by **1d** persists in undialysed samples with the activities at 21%. From this result, it may be concluded that nitrocatechol derivatives of chalcone act as reversible inhibitors of MAO-B.

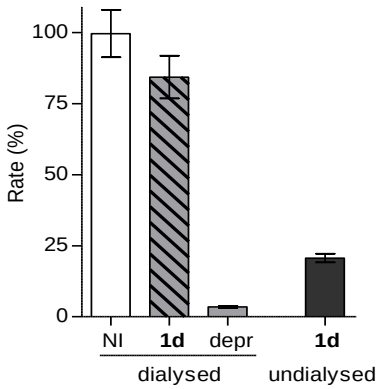


Figure 6. Reversibility of inhibition of MAO-B by **1d**. MAO-B and **1d** (at a concentration of 4 × IC_{50}) were incubated for 15 min, dialysed for 24 h and the residual enzyme activity was measured (**1d** dialysed). Similar incubation and dialysis of the enzyme in the absence (NI dialysed) and presence of the irreversible inhibitor, selegiline (Depr dialysed), were also carried out. The residual activity of undialysed mixtures of the enzyme and **1d** was also recorded (**1d** undialysed).

2.4. Mode of MAO-B inhibition

To further examine the mode of MAO-B inhibition of nitrocatechol derivatives of chalcone, sets of Lineweaver-Burk plots were constructed for a selected inhibitor, compound **1d** (figure 7). For this purpose, the MAO-B catalytic rates were recorded at 8 different kynuramine concentrations (15–250 µM) in the absence of inhibitor, and presence of five different inhibitor concentrations (¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}) of **1d**. Inspection of the Lineweaver-Burk plots suggests that compound **1d** inhibits MAO-B competitively since the plots are linear and intersect at the y-axis. These findings lend further support that **1d** acts reversibly with the active site of MAO-B. For **1d**, a K_i value for the inhibition of MAO-B of 4.6 µM is estimated.

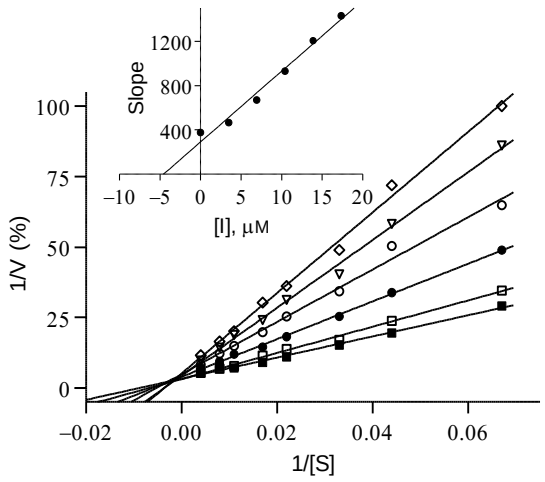


Figure 7. Lineweaver-Burk plots of MAO-B activity in the absence (filled squares) and presence of various concentrations of **1d**. For these studies the concentrations of the inhibitor were ¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}. The inset is a graph of the slopes of the Lineweaver-Burk plots versus inhibitor concentration. From the replot, a K_i value of 4.6 μM is estimated.

2.5. COMT inhibition studies

The COMT inhibition properties of the 3-nitrocatechol derivatives of chalcone (**1a–k**) were investigated using the soluble fraction obtained from rat liver homogenates (Hirano *et al*., 2005; Zhu *et al*., 2010). The measurement of COMT activity was based on measuring normetanephrine generated through the enzyme-catalysed methylation of (-)-norepinephrine. The enzyme reactions thus contained the substrate, (-)-norepinephrine, $MgCl_2$, S-adenosyl-L-methionine and the test inhibitor at various concentrations (0.01–100 μM). The reactions were initiated with the addition of the COMT enzyme and after 15 min terminated with perchloric acid. Normetanephrine generated by COMT was subsequently quantitated by HPLC with fluorescence detection (Aoyama *et al*., 2005). A chromatogram routinely obtained is provided as example (figure 8).

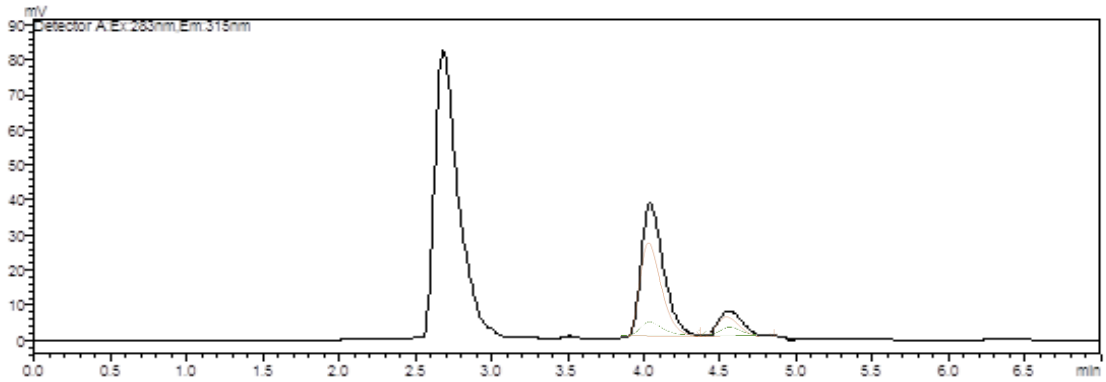


Figure 8. A chromatogram routinely obtained for the detection and quantitation of normetanephrine generated through the COMT-catalysed methylation of (-)-norepinephrine. The chromatograms indicate that the retention time for (-)-norepinephrine and normetanephrine is at 2.8 min and 4.1 min, respectively. The chromatogram in black represents an enzymatic reaction with an inhibitor concentration of 0 µM, the orange chromatogram represents an enzymatic reaction with an inhibitor concentration of 0.1 µM and the green represents an enzymatic reaction with an inhibitor concentration of 0.3 µM.

As for MAO, sigmoidal plots of enzyme catalytic rate versus inhibitor concentration (Log[I]) were constructed from which IC_{50} values were estimated (figure 9). IC_{50} values were measured in triplicate and expressed as the mean ± SD.

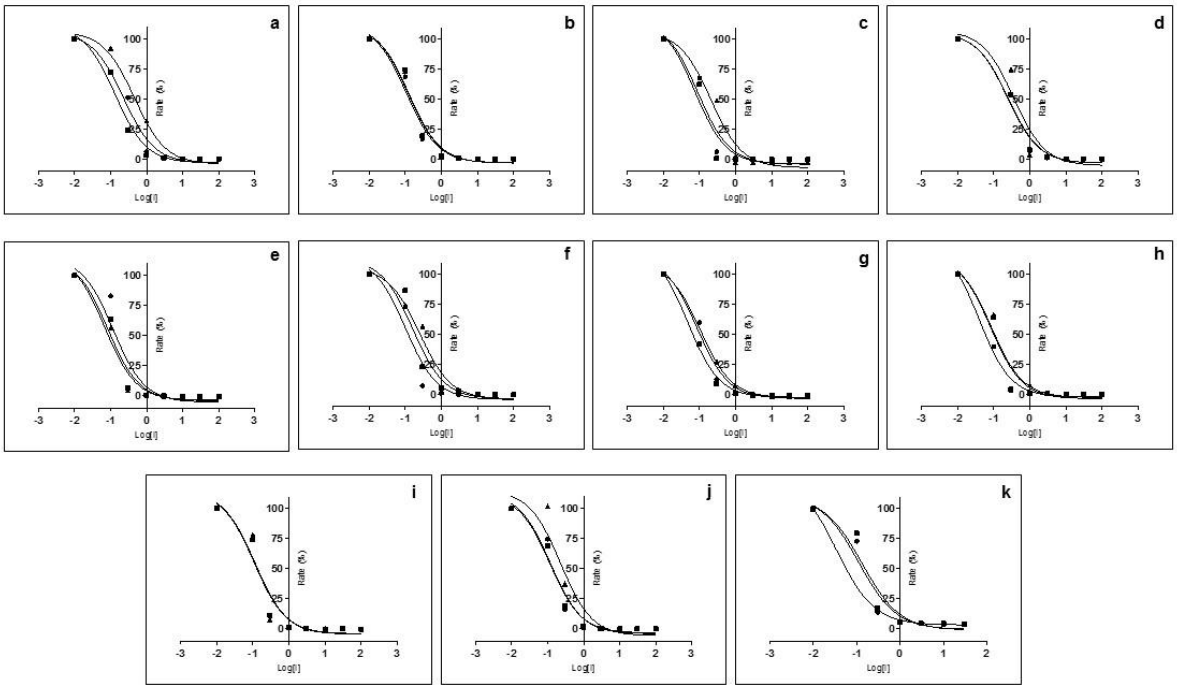


Figure 9. Sigmoidal plots for the inhibition of COMT by derivatives **1a**–**k**. Each data point represents a mean ± SD of triplicate determinations.

The IC_{50} values for the inhibition of COMT are given in Table 1. The results show that all 3-nitrocatechol derivatives of chalcone (**1a**–**k**) are highly potent inhibitors of COMT with IC_{50} values <0.29 µM. This is in accordance with expectation since compounds containing the 3-nitrocatechol moiety are well known to inhibit COMT (Kiss & Soares-da-Silva., 2014). The most potent inhibitor, compound **1h**, exhibits an IC_{50} value of 0.07 µM. Under the present experimental conditions, the reference inhibitors tolcapone and entacapone exhibit IC_{50} values of 0.26 and 0.25 µM, respectively. Compound **1h** is therefore 3.5-fold more potent than the reference COMT inhibitors. In fact, except for **1d** (IC_{50} = 0.29 µM), all synthesised compounds are more potent COMT inhibitors than the reference inhibitors. Compound **1d**, however, have the distinction of being the most potent MAO-B inhibitor of the series, and thus the most suitable dual COMT/MAO-B inhibitor discovered here. The results further show that substitution on the B-ring of the chalcone derivatives does not significantly alter COMT inhibition potency. In this regard, the COMT inhibition potency of unsubstituted chalcone **1a** (IC_{50} = 0.17 µM) is similar to those containing substituents on ring B (**1b**–**k**, IC_{50} = 0.07–0.29 µM). Substitution of the B-ring with sesamol (**1j**) and benzodioxane (**1k**) also does not affect COMT inhibition to a high degree, which further suggests that simple substitution on, or modification of ring B is unlikely to further improve COMT inhibition, and likely also would not decrease COMT inhibition. The B-ring thus represents a suitable point for substitution and modification to improve the MAO-B inhibition potencies of this class of compounds.

For COMT inhibition by the nitrocatechol derivatives of chalcone (**1a**–**k**), the following SARs may be derived. The results indicate that substitution on the *para* position of the B-ring with a halogen (Br, Cl) results in more potent inhibition compared to *meta* substitution (compare **1e** vs. **1d**; **1g** vs. **1f**). *Para* and *meta* substitution with the methyl group yields equipotent COMT inhibition (e.g. **1b** and **1c**). *Meta* methoxy substitution on the B-ring in turn yields more potent inhibition compared to *para* substitution (compare **1h** vs. **1i**). It is thus evident that 3-nitrocatechol derivatives of chalcone may serve as leads for the future design of potent COMT inhibitors.

3. Discussion and conclusion

In the present study a series of 3-nitrocatechol derivatives of chalcone (**1a**–**k**) were synthesised and evaluated as potential dual inhibitors of COMT and MAO. The results indicated that the synthesised compounds are relatively weak inhibitors of MAO-A and MAO-B with IC_{50} values >32.4 µM and >13.9 µM, respectively. For comparison, when examined

under identical experimental conditions, the reference inhibitors toloxatone and lazabemide, exhibit IC_{50} values of 3.92 µM and 0.091 µM for the inhibition of MAO-A and MAO-B, respectively (Petzer *et al*., 2013). In contrast to their MAO inhibition potencies, the chalcone derivatives are potent inhibitors of COMT with IC_{50} values <0.29 µM. Except for **1d**, all derivatives are more potent than the reference inhibitors, tolcapone and entacapone. An interesting SAR is that substitution on, or modification of the B-ring of the chalcone derivatives do not affect COMT inhibition to a large degree. Since the MAO-B inhibition potency of this class of compounds should be improved for effective dual COMT/MAO inhibition, the B-ring represents a suitable site for structural modification. Functional groups known to confer MAO inhibition (e.g. propargylamine for irreversible inhibition) may thus be substituted on ring B to enhance MAO inhibition, while retaining the potent COMT inhibition activity of 3-nitrocatechol derivatives of chalcone.

The design of dual inhibitors of COMT and MAO appears to be an attractive approach for the treatment of Parkinson's disease, particularly as adjuvants to L-dopa. Dual inhibitors that function in both the peripheral and central tissues are expected to not only reduce undesired peripheral metabolism of L-dopa, but also conserve the dopamine supply in the brain. Furthermore, the dual inhibition of MAO and COMT will be of further value in the treatment of co-morbid depression in Parkinson's disease. Since the central actions of endogenous catecholamines are prolonged by both COMT and MAO inhibition, dual inhibitors may be more effective as antidepressants. Literature reports that when a MAO-B inhibitor such as selegiline is combined with a COMT inhibitor, catecholamine levels may be increased significantly in the brain (Tom & Cummings, 1998). It should be noted that both MAO-A and MAO-B inhibitors are antidepressants (Bied *et al*., 2015). In conclusion, patients with advanced Parkinson's disease will benefit significantly from the use of a COMT or MAO-B inhibitor in conjunction with L-dopa compared to L-dopa therapy alone (Miyasaki, 2006; Talati *et al*., 2009). The administration of a COMT inhibitor alone has minimal beneficial effect and COMT inhibitors thus have to be administered in combination with another antiparkinsonian drug such as a MAO inhibitor (Learmonth *et al*., 2002; Lees *et al*., 2009; Miyasaki, 2006; Rascol *et al*., 2002). The design of dual inhibitors of COMT and MAO should thus be further pursued, and this study shows that 3-nitrocatechol derivatives of chalcone are a suitable class to reach this objective.

4. Experimental section

4.1. Chemicals and instrumentation

Unless otherwise indicated, all starting materials and solvents were obtained from Sigma-Aldrich and were used without further purification. The undeuterated solvent (DMSO-*d6*) used for the preparation of NMR samples was purchased from Merck. NMR spectra were recorded with a Bruker Avance III spectrometer at frequencies of 600 MHz for the proton (^{1}H) spectra and 150 MHz for the carbon (^{13}C) spectra. The processing and analyses of the NMR data were carried out with MestreNova. All chemical shifts (δ) are given in parts per million (ppm) and were referenced to the signal of the residual undeuterated solvent, DMSO-*d6*, at 2.5 ppm for ^{1}H NMR and 39.5 ppm for ^{13}C NMR. Spin multiplicities are given as singlet (s), doublet (d), doublet of doublets (dd), triplet (t) and multiplet (m). A Bruker micrOTOF-Q II mass spectrometer functioning in atmospheric-pressure chemical ionisation (APCI) mode was used to record high resolution mass spectra (HRMS). Melting points were determined using a Büchi B-545 melting point apparatus and are uncorrected. Thin layer chromatography was carried out on silica gel 60 F254 precoated aluminium sheets (0.25 mm, Merck) with a mixture of ethyl acetate and benzene (70:30) as mobile phase. To determine the purities of the synthesised compounds, HPLC analyses were carried out on an Agilent 1100 HPLC system equipped with a quaternary pump and an Agilent 1100 series diode array detector. HPLC grade acetonitrile (Merck) and Milli-Q water (Millipore) was used for the chromatography.

For the MAO inhibition studies, a Varian Cary Eclipse fluorescence spectrophotometer was employed. Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg protein/ml) and kynuramine dihydrobromide were obtained from Sigma-Aldrich. For the COMT inhibition studies, (-)-norephinephrine, DL-normetanephrine, S-adenosyl-L-methionine and $MgCl_2$ were purchased from Sigma-Aldrich. A Shimadzu Ultra Fast Liquid Chromatograph (UFLC) prominence HPLC system, equipped with a Shimadzu communications bus module (CBM-20A), Shimadzu degasser (DGU-20A_5), Shimadzu pump (LC-20AD), Shimadzu auto-sampler (SIL-20AC), Shimadzu column oven (CTO-20A) and Shimadzu RF-10AXL fluorescence detector, with a USP L1 Luna C18 column (250 × 4.6 mm, 5 µm) (Phenomenex, Torrance, CA) was used for chromatographic separation and detection of normetanephrine. The processing and analyses of the COMT data were carried out with LabSolutions.

4.2. Synthesis of 4-hydroxy-3-methoxy-5-nitroacetophenone (5-nitroapocynin) (11)

4-Hydroxy-3-methoxyacetophenone (apocynin; **10**) (30 mmol; 5 g) was dissolved in acetic acid (50 ml). 60% nitric acid (1.45 ml) was added dropwise to the solution. The reaction was stirred at room temperature for 30 min and poured into cold water. The resulting precipitate was collected by filtration and dried to yield the nitro derivative **11** (5-nitroapocynin) (Kiss *et al*., 2010).

4.3. Synthesis of 3,4-dihydroxy-5-nitroacetophenone (12)

4-Hydroxy-3-methoxy-5-nitroacetophenone (5-nitroapocynin; **11**) (23.7 mmol; 5 g) was dissolved in ethyl acetate (68 ml). Aluminium chloride (28.4 mmol; 3.8 g) was added in one portion at room temperature resulting in an orange-red coloured mixture. Pyridine (94.7 mmol; 7.7 ml) was added dropwise to the mixture. The reaction was refluxed for 2 h at 77 °C. After the mixture was cooled to room temperature, ice cold hydrochloric acid was added. The resulting yellow precipitate was collected by filtration and dried to yield the demethylated derivative **12** (3,4-dihydroxy-5-nitroacetophenone) (Learmonth *et al*., 2002).

4.4. Synthesis of 3-nitrocatechol derivatives of chalcone (1a–k)

The 3-nitrocatechol derivatives of chalcone were prepared by the Claisen-Schmidt condensation reaction. 3,4-Dihydroxy-5-nitroacetophenone (**12**; 2.54 mmol; 0.5 g) was dissolved in 0.9 ml ethanol. The appropriately substituted aldehyde (1.52 mmol) were added to the solution and potassium hydroxide (60%) was added as the base. The mixture was left at room temperature for approximately 24 h after which acidification was carried out with 0.5 N hydrochloric acid solution (Chimenti *et al*., 2008; Cocconcelli *et al*., 2008; Klinke & Gibian, 1961). The subsequent precipitate was collected by filtration and dried. The 3-nitrocatechol derivatives of chalcone were purified by crystallisation from acetonitrile.

1-(4-Hydroxy-3-methoxy-5-nitrophenyl)ethanone (11)

The title compound (yellow powder) was prepared from 4-hydroxy-3-methoxyacetophenone (apocynin; **10**) and 60% nitric acid in a yield of 70.1%: mp 158–164 °C, mp (literature) 129–130 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ) δ 11.10 (s, 1H), 8.27 (d, *J* = 1.3 Hz, 1H), 7.73 (s, 1H), 3.98 (s, 3H), 2.60 (s, 3H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ

194.90, 150.35, 150.05, 132.89, 128.29, 117.72, 115.18, 56.85, 25.98; APCI-HRMS *m/z*: calcd for $C_9H_{10}NO_5$ (MH^+), 212.0553, found 212.0574.

1-(3,4-Dihydroxy-5-nitrophenyl)ethanone (12)

The title compound (yellow powder) was prepared from 1-(4-hydroxy-3-methoxy-5-nitrophenyl)ethanone (**11**) in the presence of $AlCl_3$ and pyridine in a yield of 28.5%: mp 153.1–158.2 °C, mp (literature) 161–169 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 7.94 (s, 1H), 7.56 (s, 1H), 2.52 (s, 3H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 195.38, 147.64, 145.76, 137.08, 127.29, 117.14, 116.44, 26.28; APCI-HRMS *m/z*: calcd for $C_8H_8NO_5$ (MH^+), 198.0397, found 198.0410.

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-phenylprop-2-en-1-one (1a)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and benzaldehyde in a yield of 6.3%: mp 185.4–189.8 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 7.24 (s, 1H); 7.39–7.47 (m, 3H); 7.59 (d, *J* = 15.5 Hz, 1H); 7.80–7.86 (m, 3H); 8.30 (d, *J* = 1.0 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 107.91, 117.98, 121.41, 122.02, 128.53, 128.92, 129.94, 133.09, 135.25, 141.08, 152.15, 162.17, 185.29; APCI-HRMS *m/z*: calcd for $C_{15}H_{12}NO_5$ (MH^+), 286.0710, found 286.0693; Purity (HPLC): 99.23%.

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-methylphenyl)prop-2-en-1-one (1b)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and *m*-tolualdehyde in a yield of 56.9%: mp 175.8–183 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 2.34 (s, 3H); 7.25 (d, *J* = 7.5 Hz, 1H); 7.33 (t, *J* = 7.6 Hz, 1H); 7.67 (m, 2H); 7.70–7.75 (m, 2H); 7.88 (d, *J* = 15.5 Hz, 1H); 8.26 (d, *J* = 1.5 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 20.89, 116.64, 117.46, 121.05, 126.45, 127.95, 128.79, 129.22, 131.41, 134.57, 137.57, 138.17, 144.23, 145.68, 147.81, 186.27; APCI-HRMS *m/z*: calcd for $C_{16}H_{14}NO_5$ (MH^+), 300.0866, found 300.0874; Purity (HPLC): 96.7%.

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(4-methylphenyl)prop-2-en-1-one (1c)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and *p*-tolualdehyde in a yield of 22.7%: mp 196.8–198.4 °C. ^{1}H NMR (Bruker Avance III 600,

DMSO-*d6*) δ 2.34 (s, 3H); 7.26 (d, *J* = 7.9 Hz, 2H); 7.72 (d, *J* = 10.1 Hz, 2H); 7.78 (d, *J* = 8 Hz, 2H); 7.87 (d, *J* = 15.5 Hz, 1H); 8.27 (d, *J* = 1.9 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 21.16, 116.67, 117.45, 120.24, 128.04, 129.09, 129.58, 131.99, 137.55, 140.83, 144.20, 145.73, 147.84, 186.30; APCI-HRMS *m/z*: calcd for $C_{16}H_{14}NO_5$ (MH^+), 300.0866, found 300.0856; Purity (HPLC): 99.8%.

(2*E*)-3-(3-Bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1d)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and 3-bromobenzaldehyde in a yield of 9.1%: mp 173.2–175.7 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 7.40 (t, *J* = 7.8 Hz, 1H); 7.62 (d, *J* = 7.9 Hz, 1H); 7.67 (d, *J* = 15.5 Hz, 1H); 7.73 (d, *J* = 1.9 Hz, 1H); 7.86 (d, *J* = 7.8 Hz, 1H); 8.00 (d, *J* = 15.6 Hz, 1H), 8.20 (s, 1H), 8.31 (d, *J* = 1.8 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 116.90, 117.40, 122.39, 122.79, 127.74, 128.42, 130.85, 130.92, 133.07, 137.18, 137.61, 142.28, 145.84, 147.83, 186.15; APCI-HRMS *m/z*: calcd for $C_{15}H_{11}BrNO_5$ (MH^+), 363.9815, found 363.9816; Purity (HPLC): 95.9%.

(2*E*)-3-(4-Bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1e)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and 4-bromobenzaldehyde in a yield of 31.8%: mp 176.3–180.9 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 7.64 (s, 2H); 7.71 (d, *J* = 12.4 Hz, 2H); 7.86 (d, *J* = 8.3 Hz, 2H); 7.96 (d, *J* = 15.5 Hz, 1H); 8.30 (s, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 116.84, 117.40, 122.07, 124.03, 127.81, 130.91, 131.85, 133.98, 137.53, 142.65, 145.83, 147.84, 186.17; APCI-HRMS *m/z*: calcd for $C_{15}H_{11}BrNO_5$ (MH^+), 363.9815, found 363.9801; Purity (HPLC): 90.6%.

(2*E*)-3-(3-Chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1f)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and 3-chlorobenzaldehyde in a yield of 17.9%: mp 191.6–194.9 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 7.38–7.53 (m, 2H); 7.70 (m, 2H); 7.81 (d, *J* = 7.2 Hz, 1H); 8.00 (d, *J* = 15.6 Hz, 1H); 8.07 (s, 1H); 8.31 (d, *J* = 1.8 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 116.94, 117.40, 122.84, 127.75, 128.01, 128.09, 130.22, 130.71, 133.83, 136.94, 137.62, 142.36, 145.91, 147.88, 186.21; APCI-HRMS *m/z*: calcd for $C_{15}H_{11}ClNO_5$ (MH^+), 320.0320, found 320.0309; Purity (HPLC): 97.9%.

(2*E*)-3-(4-Chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1g)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and 4-chlorobenzaldehyde in a yield of 24.2%: mp 191.3–213.5 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 7.50 (d, J = 8.4 Hz, 2H); 7.71 (t, *J* = 9.2 Hz, 2H); 7.94 (t, *J* = 11.2 Hz, 3H); 8.29 (d, *J* = 1.4 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 116.83, 117.41, 122.02, 127.82, 128.92, 130.70, 133.65, 135.13, 137.53, 142.56, 145.83, 147.84, 186.16; APCI-HRMS *m/z*: calcd for $C_{15}H_{11}ClNO_5$ (MH^+), 320.0320, found 320.0320; Purity (HPLC): 92%.

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (1h)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and *m*-anisaldehyde in a yield of 28.3%: mp 182.5–185.9 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 3.81 (s, 3H); 7.02 (dd, *J* = 8.1 Hz, 2.0 Hz, 1H); 7.35 (t, *J* = 7.9 Hz, 1H); 7.42–7.48 (m, 2H); 7.71 (m, 2H); 7.91 (d, *J* = 15.6 Hz, 1H), 8.26 (d, *J* = 1.9 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 55.36, 113.78, 116.59, 116.75, 117.49, 121.63, 121.75, 127.93, 129.96, 136.08, 137.59, 144.12, 145.76, 147.84, 159.68, 186.40; APCI-HRMS *m/z*: calcd for $C_{16}H_{14}NO_6$ (MH^+), 316.0816, found 316.0804; Purity (HPLC): 89.9%.

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (1i)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and *p*-anisaldehyde in a yield of 19.1%: mp 234.5–235.9 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 3.82 (s, 3H); 7.01 (d, *J* = 8.7 Hz, 2H); 7.72 (dd, *J* = 14.7 Hz, 8.6 Hz, 2H); 7.79 (d, *J* = 15.5 Hz, 1H); 7.86 (d, *J* = 8.7 Hz, 2H); 8.26 (d, *J* = 1.9 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 55.39, 114.39, 116.48, 117.49, 118.74, 127.33, 128.20, 130.94, 137.51, 144.09, 145.54, 147.76, 161.42, 186.13; APCI-HRMS *m/z*: calcd for $C_{16}H_{14}NO_6$ (MH^+), 316.0816, found 316.0822; Purity (HPLC): 99.1%.

(2*E*)-3-(2*H*-1,3-Benzodioxol-5-yl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1j)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and piperonal in a yield of 37.7%: mp 235.1–237.4 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 6.11 (s, 2H); 6.98 (d, *J* = 8.0 Hz, 1H); 7.32 (dd, *J* = 8.1 Hz, 1.4 Hz, 1H); 7.68 (m, 2H); 7.73 (d, *J* = 2.1 Hz, 1H); 7.80 (d, *J* = 15.4 Hz, 1H); 8.29 (d, *J* = 2.0 Hz, 1H); ^{13}C

NMR (Bruker Avance III 600, DMSO-*d6*) δ 102.15, 107.48, 108.96, 117.01, 117.96, 119.66, 126.61, 128.64, 129.69, 137.99, 144.59, 146.01, 148.21, 148.58, 150.08, 186.53; APCI-HRMS *m/z*: calcd for $C_{16}H_{12}NO_7$ (MH^+), 330.0608, found 330.0606; Purity (HPLC): 97.7%.

(2*E*)-3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1k)

The title compound (yellow crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and 1,4-benzodioxan-6-carboxaldehyde in a yield of 28.9%: mp 223.9–224.5 °C. ^{1}H NMR (Bruker Avance III 600, DMSO-*d6*) δ 4.26–4.31 (m, 4H); 6.91 (d, *J* = 8.4 Hz, 1H); 7.36 (dd, *J* = 8.3 Hz, 1.3 Hz, 1H); 7.52 (s, 1H); 7.63 (d, *J* = 15.4 Hz, 1H); 7.75 (m, 2H); 8.28 (d, *J* = 1.5 Hz, 1H); ^{13}C NMR (Bruker Avance III 600, DMSO-*d6*) δ 63.96, 64.43, 116.57, 117.26, 117.41, 117.46, 119.32, 123.29, 128.16, 128.19, 137.57, 143.63, 144.00, 145.49, 145.91, 147.73, 186.06; APCI-HRMS *m/z*: calcd for $C_{17}H_{14}NO_7$ (MH^+), 344.0765, found 344.0760; Purity (HPLC): 94%.

4.5. MAO-A and MAO-B inhibition studies

Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg protein/ml) were obtained from Sigma-Aldrich, pre-aliquoted, and stored at –70 °C. All enzyme reactions were carried out in white polypropylene 96-well microtiter plates to a final volume of 200 µl. The reactions contained potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl (20.2 mM), kynuramine (50 µM) and the test inhibitors at various concentrations (0.003–100 µM). Stock solutions of the inhibitors were prepared in DMSO and added to the reactions to yield a final concentration of 4% (v/v). Control reactions carried out in the absence of inhibitor were included and contained 4% DMSO. The reactions were incubated for 30 min at 37 °C and were subsequently initiated with addition of MAO-A and MAO-B to yield a final concentration of 0.0075 mg protein/ml and 0.015 mg protein/ml, respectively. After a further 20 min of incubation at 37 °C, the reactions were terminated by the addition of 80 µl NaOH (2 N). The concentration of 4-hydroxyquinoline generated by MAO was measured by fluorescence spectrophotometry at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. To quantitate 4-hydroxyquinoline, a linear calibration curve was constructed with authentic 4-hydroxyquinoline (0.047–1.50 µM). In order to confirm that the test compounds do not fluoresce or quench the fluorescence of 4-hydroxyquinoline, control samples were included in the assay. These control samples (200 µl) contained 4-hydroxyquinoline (1.50 µM), the test compound (100 µM) and 80 µl NaOH.

By employing the calibration curve, the enzyme catalytic rates were determined and sigmoidal plots of rate versus the logarithm of the concentration of the test inhibitor was constructed. This kinetic data was fitted to a one site competition model incorporated into the Prism software package (GraphPad) and the corresponding IC_{50} values of the test compounds were estimated. The IC_{50} values were determined in triplicate and expressed as mean ± SD.

4.6. Reversibility of MAO-B inhibition

To examine the reversibility of inhibition of nitrocatechol derivatives of chalcone, compound **1d** was selected as representative compound. The reversibility of the MAO-B inhibition by **1d** was examined by dialysis. 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 were used for the dialysis. The selected inhibitor at a concentration equal to 4 × IC_{50} was pre-incubated with MAO-B (0.03 mg protein/ml) for 15 min at 37 ºC. The enzyme-inhibitor incubations were carried out in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl) containing 5% sucrose. Control incubations were conducted in the absence of inhibitor and presence of the irreversible inhibitor selegiline (IC_{50} = 0.079 µM). DMSO (4%) was added as co-solvent to all pre-incubations, and the final volume of the incubations was 0.8 ml. The enzyme-inhibitor complexes were dialysed for 20–25 h at 4 °C in 100 ml outer buffer (100 mM potassium phosphate, pH 7.4, 5% sucrose). The sucrose buffer was replaced at 3 h and 7 h after the start of dialysis. To determine the residual MAO-B activity, the reactions were diluted twofold with the addition of kynuramine. The final concentration of MAO-B was 0.015 mg protein/ml and the final kynuramine concentration was 50 µM. The reactions were incubated for 20 min and terminated with the addition of 400 µl NaOH (2 N) and 1000 µl deionised water. The concentrations of the MAO-generated 4-hydroxyquinoline were measured spectrofluorometrically at an excitation wavelength of 310 nm and an emission wavelength of 400 nm, as described above. These reactions were carried out in triplicate and the residual catalytic rates were expressed as mean ± SD. For comparison, non-dialysed enzyme-inhibitor mixtures were maintained at 4 °C for the same period and the residual MAO-B activity was measured as described above.

4.7. Mode of MAO-B inhibition

To evaluate the mode of inhibition of MAO-B by the nitrocatechol derivatives of chalcone, Lineweaver-Burk plots were constructed for a representative inhibitor, **1d**. For this purpose, the enzyme reactions were conducted in potassium phosphate buffer (100 mM, pH 7.4,

made isotonic with KCl 20.2 mM) to a final volume of 500 µl. The reactions contained kynuramine as substrate (15–250 µM), MAO-B (0.015 mg/ml), the representative inhibitor, **1d**, at various concentrations (¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}) and DMSO (4% v/v) as co-solvent. Control incubations were conducted in the absence of inhibitor. The reactions were incubated at 37 °C for 20 min and terminated by the addition of 400 µl NaOH (2 N) and 1000 µl deionised water. The MAO-B-generated 4-hydroxyquinoline was measured by fluorescence spectrophotometry as described above.

4.8. COMT inhibition studies

To investigate the COMT inhibition properties, the soluble fraction of rat liver homogenate served as enzyme source. Sprague Dawley rats were bred, supplied and housed at the Vivarium at the Potchefstroom campus of the North-West University (NWU) (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019) of the Preclinical Drug Development Platform of the NWU. Experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) at the NWU. All animals were maintained, and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa, and complied with national legislation. Ethical approval for the collection and use of animal tissue was obtained from the Research Ethics Committee, NWU. Ethics approval numbers: NWU-00438-16-S5 and NWU-00267-16-A5.

The liver tissue was prepared as reported in literature by homogenisation in 25 mM sodium phosphate buffer (pH 7.8, containing 0.5 mM dithiothreitol) followed by centrifugation (Hirano *et al*., 2005; Zhu *et al*., 2010). Protein determination was carried out by the method of Bradford (Bradford, 1970). A modification of the literature HPLC protocol was employed to measure normetanephrine generated by the action of COMT on (-)-norepinephrine (Aoyama *et al*., 2005). The enzyme reactions (110 µl) contained $MgCl_2$ (2 mM), (-)-norepinephrine (250 µM), S-adenosyl-L-methionine (200 µM) and the test inhibitor at various concentrations (0.01–100 µM). Inhibitor stock solutions were prepared in DMSO and added to the reactions to yield a final DMSO concentration of 4%. Control reactions carried out in the absence of inhibitor were included and also contained 4% DMSO. The reactions were pre-incubated for 10 min at 37 °C, after which 15 µl of the COMT enzyme was added to each incubation to yield a final volume of 125 µM and a final enzyme concentration of 30 mg protein/ml. After a further 15 min incubation at 37 °C, the enzyme reactions were terminated with the addition of 12.5 µl perchloric acid (1 M). The samples were centrifuged at 20000 × g for 30 min and the supernatants were analysed by HPLC with fluorescence detection (λ_{ex} 283 nm; λ_{em} 315

nm). The peak areas of normetanephrine were recorded and quantitated with the aid of a calibration curve (0.5–90 µM). After calculating the enzyme catalytic rates, sigmoidal plots of rate versus logarithm of inhibitor concentration were constructed. This was carried out by fitting the calculated rates to a one site competition model incorporated into the Prism software package (GraphPad). The IC_{50} values were estimated in triplicate from these plots and expressed as mean ± SD.

To ensure that normetanephrine measured in the enzymatic reactions are indeed formed from the action of COMT on (-)-norepinephrine the following controls are included as examples (figure 10): **a**. No enzyme added to the reaction, and thus no normetanephrine was formed and the peak observed represents the substrate, (-)-norepinephrine. **b**. A chromatogram of a mixture containing only $MgCl_2$ (2 mM), (-)-norepinephrine (10 µM), S-adenosyl-L-methionine (200 µM), DMSO (4%) and perchloric acid (1 M). **c**. A chromatogram of a sample of normetanephrine (10 µM) showing a longer retention time compared to (-)-norepinephrine. **d** and **e**. Chromatograms on enzyme reactions that do not contain (-)-norepinephrine (250 µM) showing that when substrate is absent from the enzymatic mixture, no normetanephrine is generated as indicated by the absence of a peak at the expected retention time for normetanephrine. **f** and **h**. Chromatograms represents samples of S-adenosyl-L-methionine (5 µM), perchloric acid and buffer, respectively, showing no interfering peaks at the retention time expected for normetanephrine.

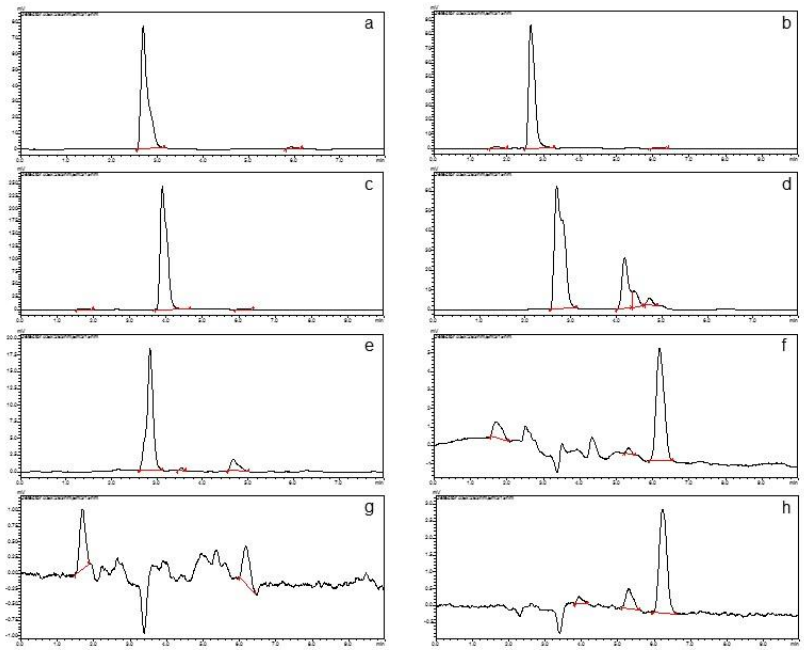


Figure 10. Chromatograms obtained as controls for the enzyme reactions where the formation of normetanephrine from the COMT-catalysed methylation of (-)-norepinephrine, is measured.

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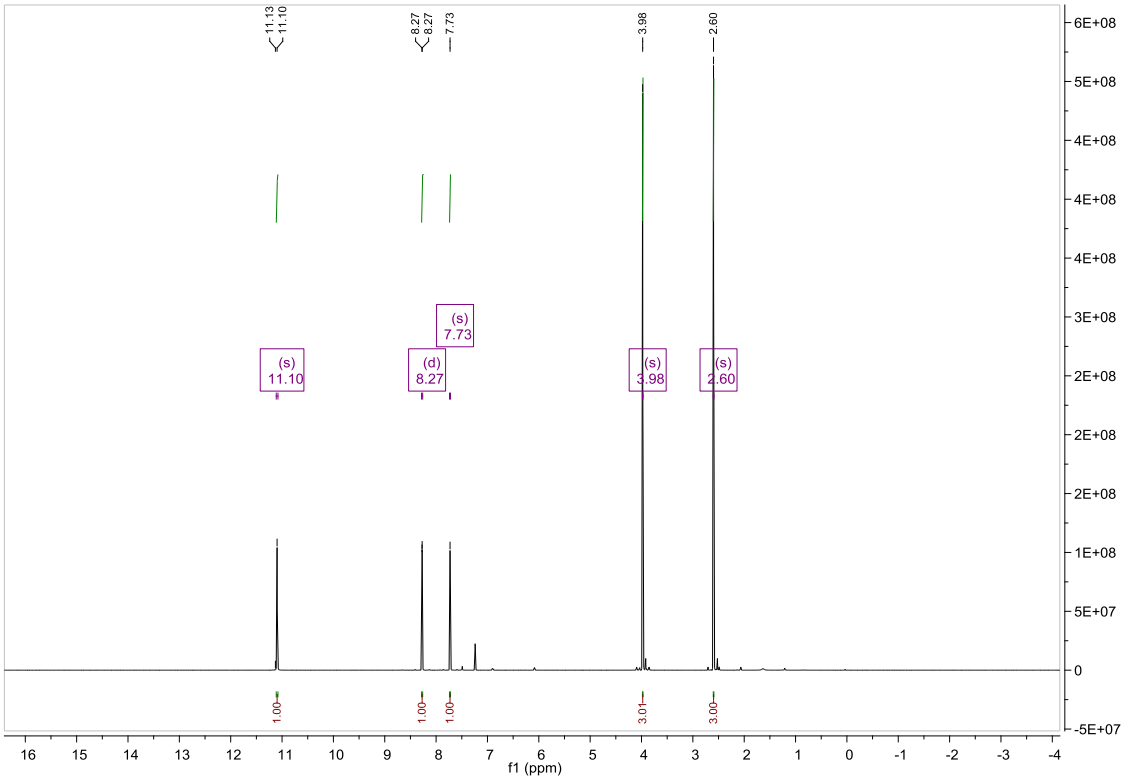
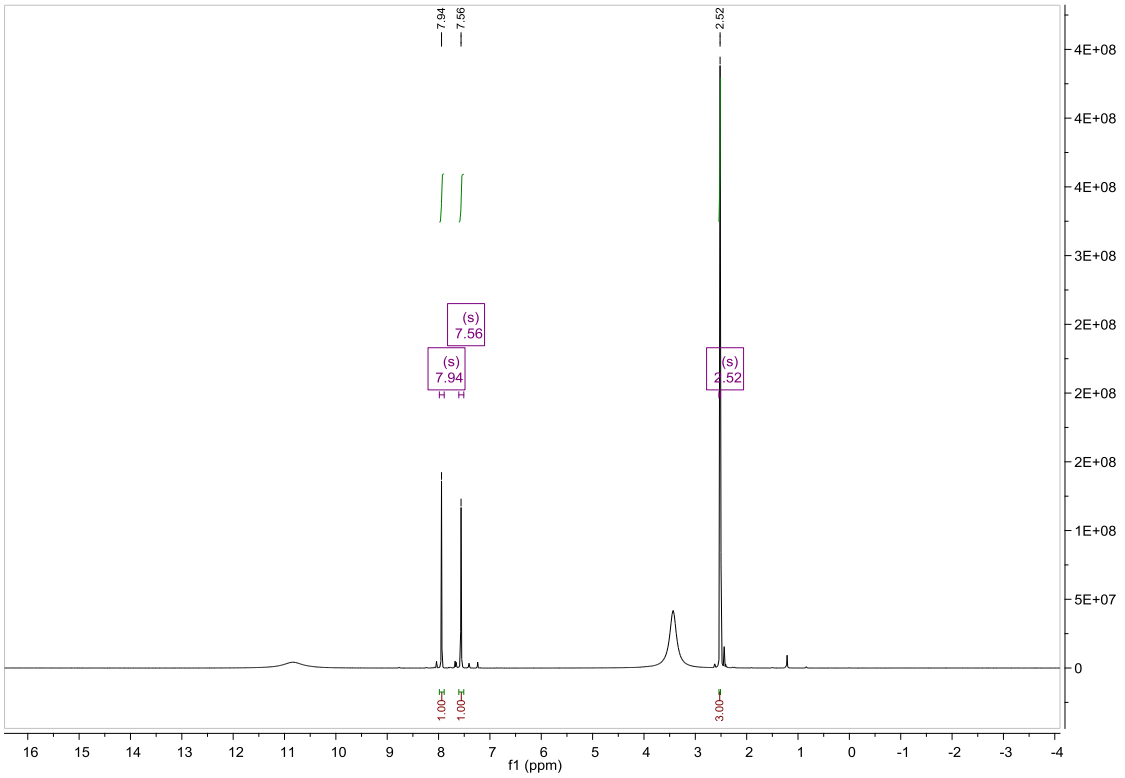
Supplementary material - ^{1}H NMR

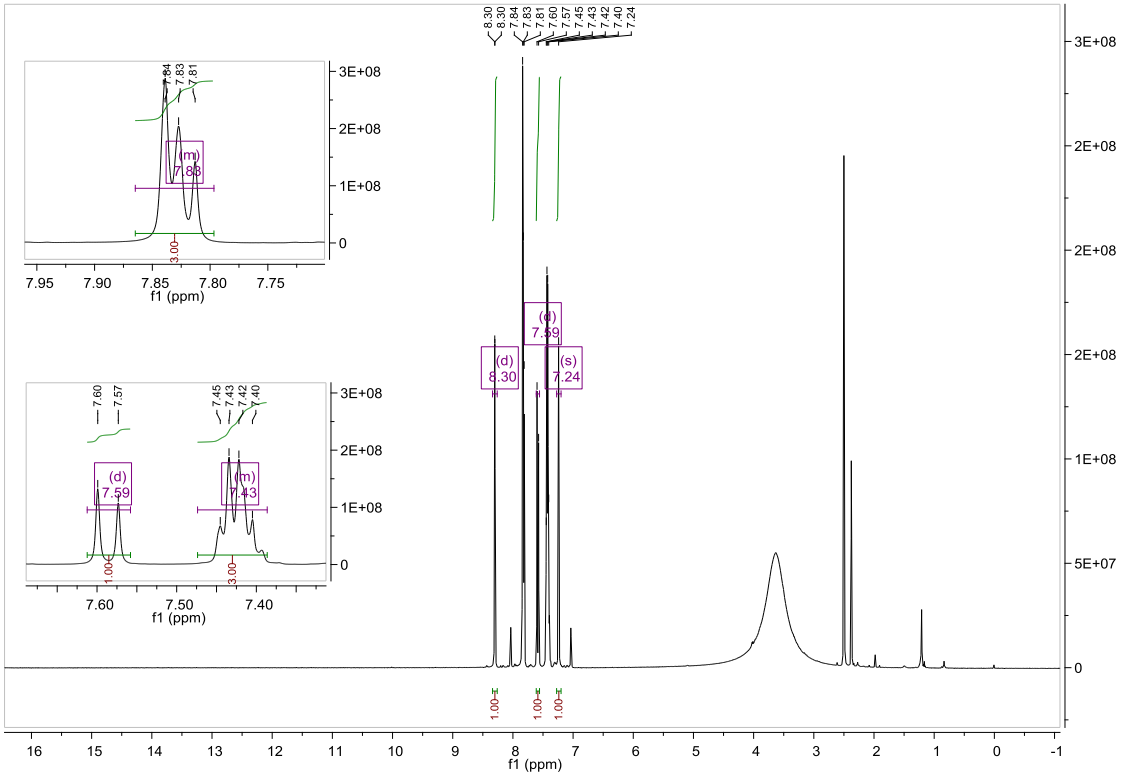
1-(4-Hydroxy-3-methoxy-5-nitrophenyl)ethanone (11)



1-(3,4-Dihydroxy-5-nitrophenyl)ethanone (12)



(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-phenylprop-2-en-1-one (1a)



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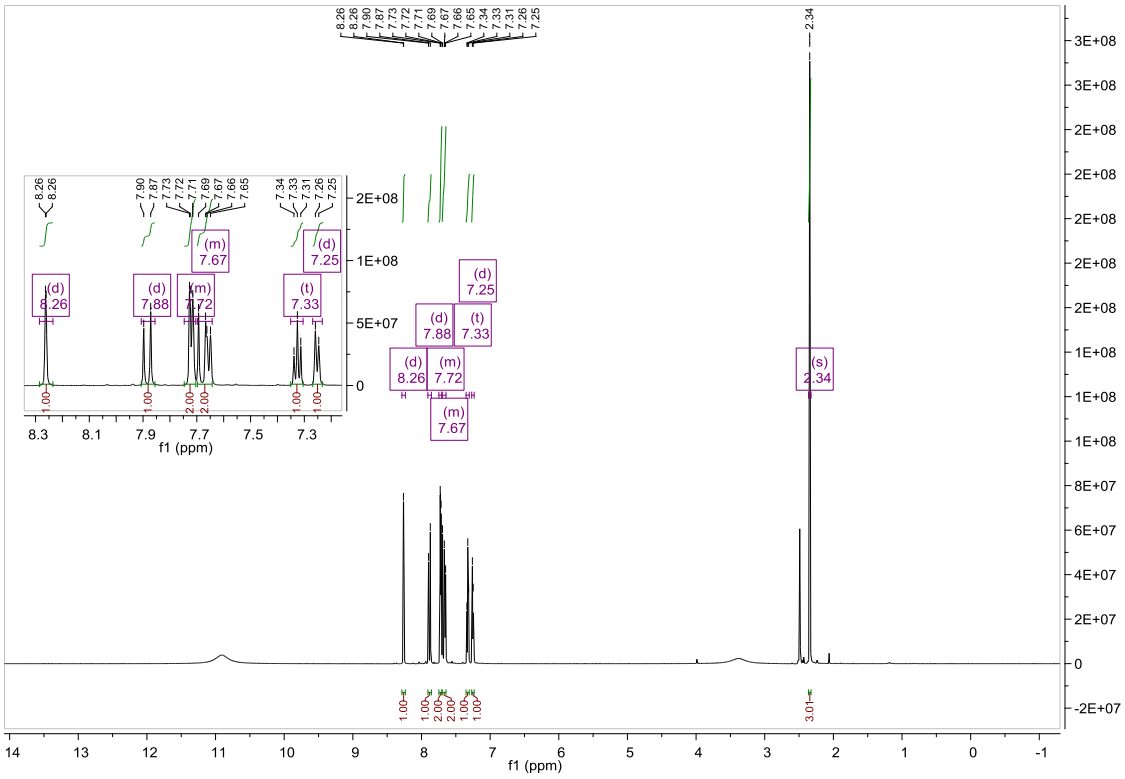

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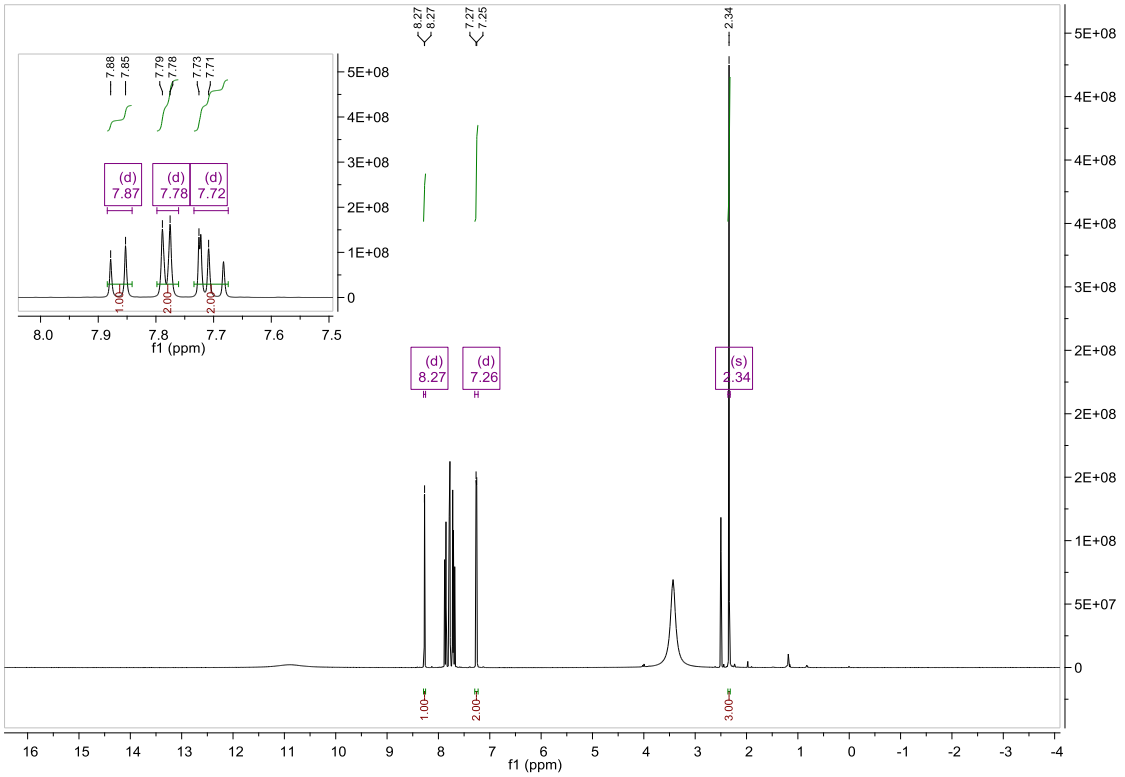

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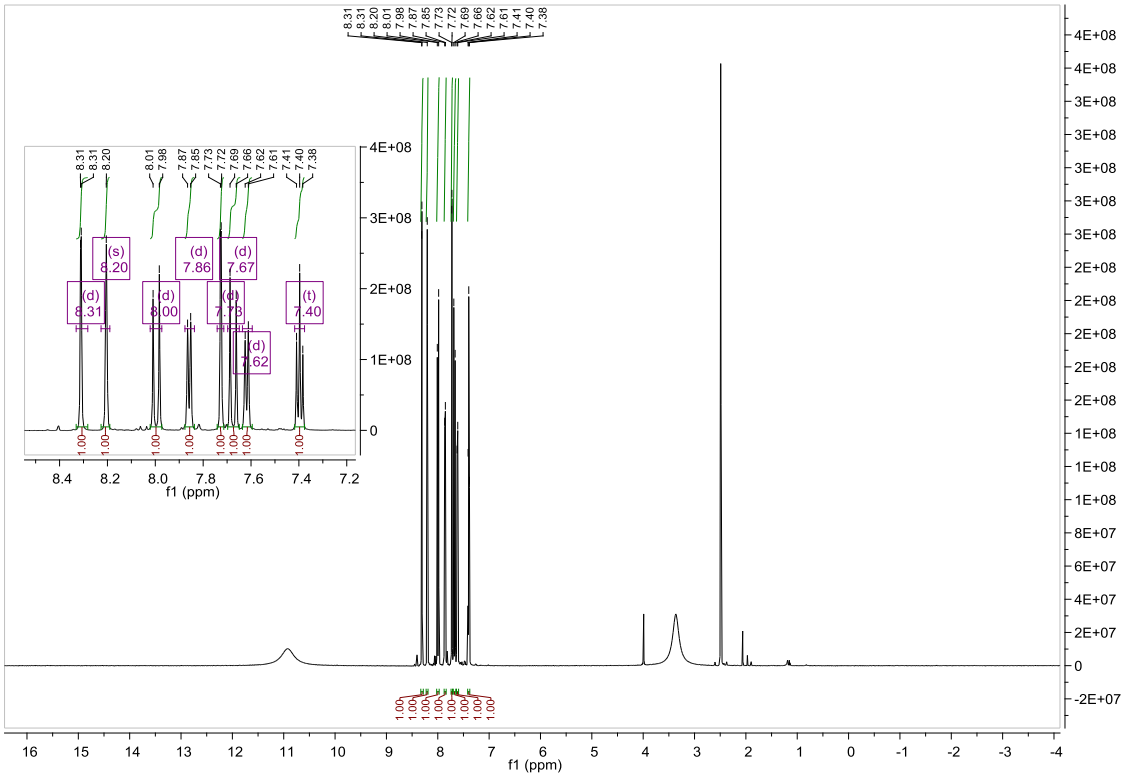

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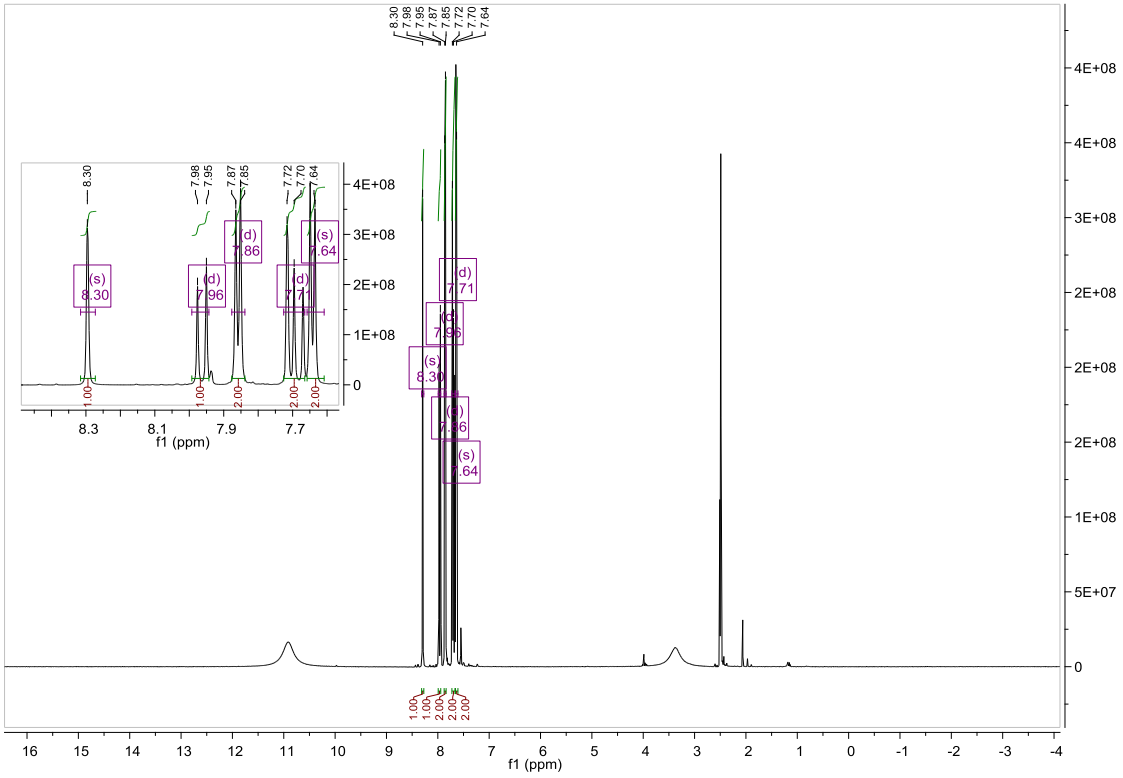

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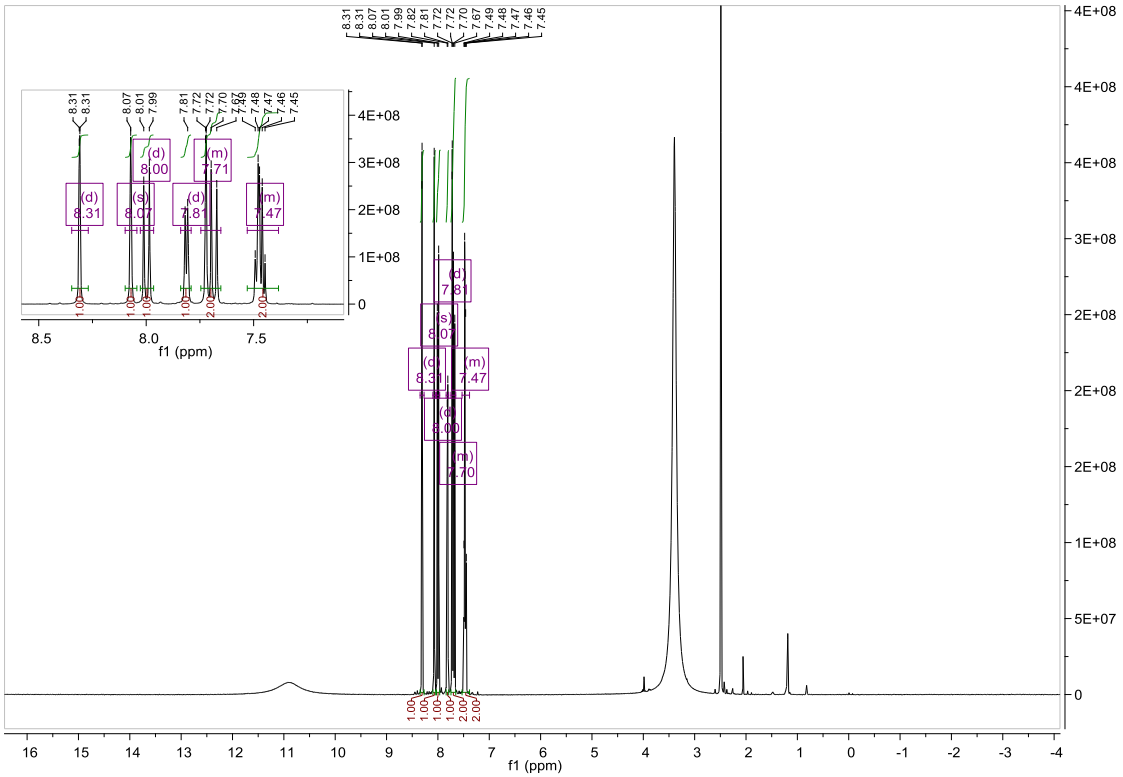

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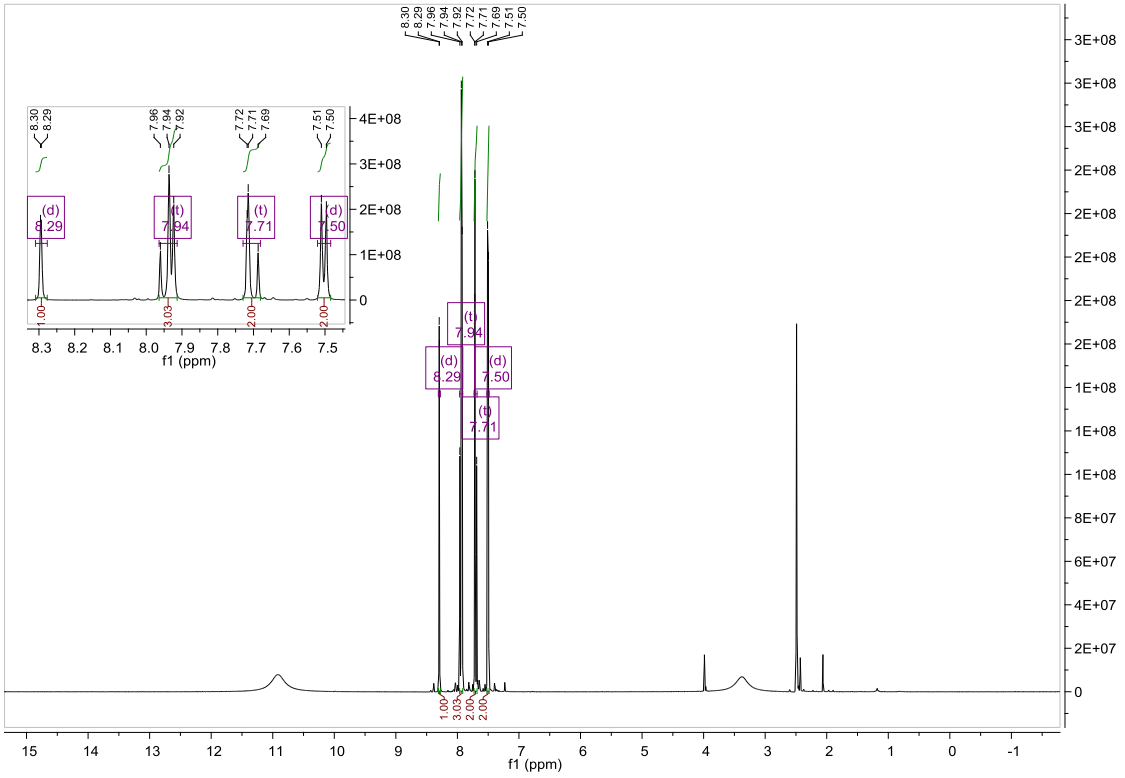

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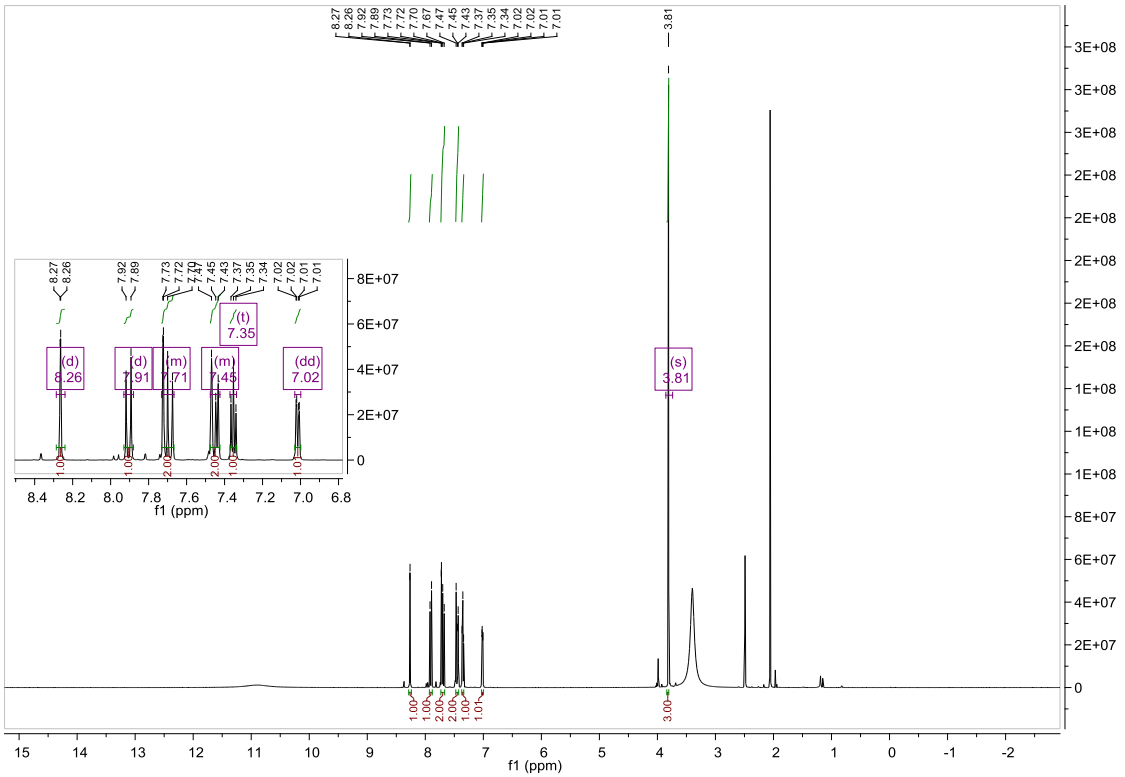

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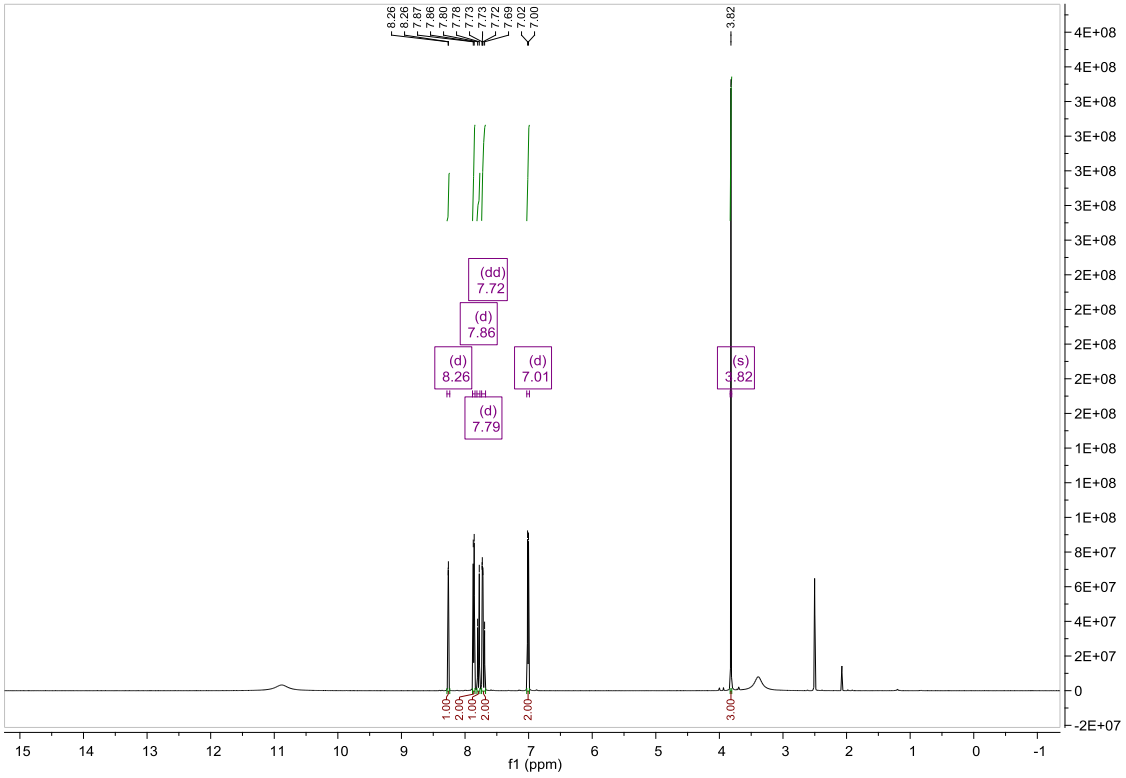

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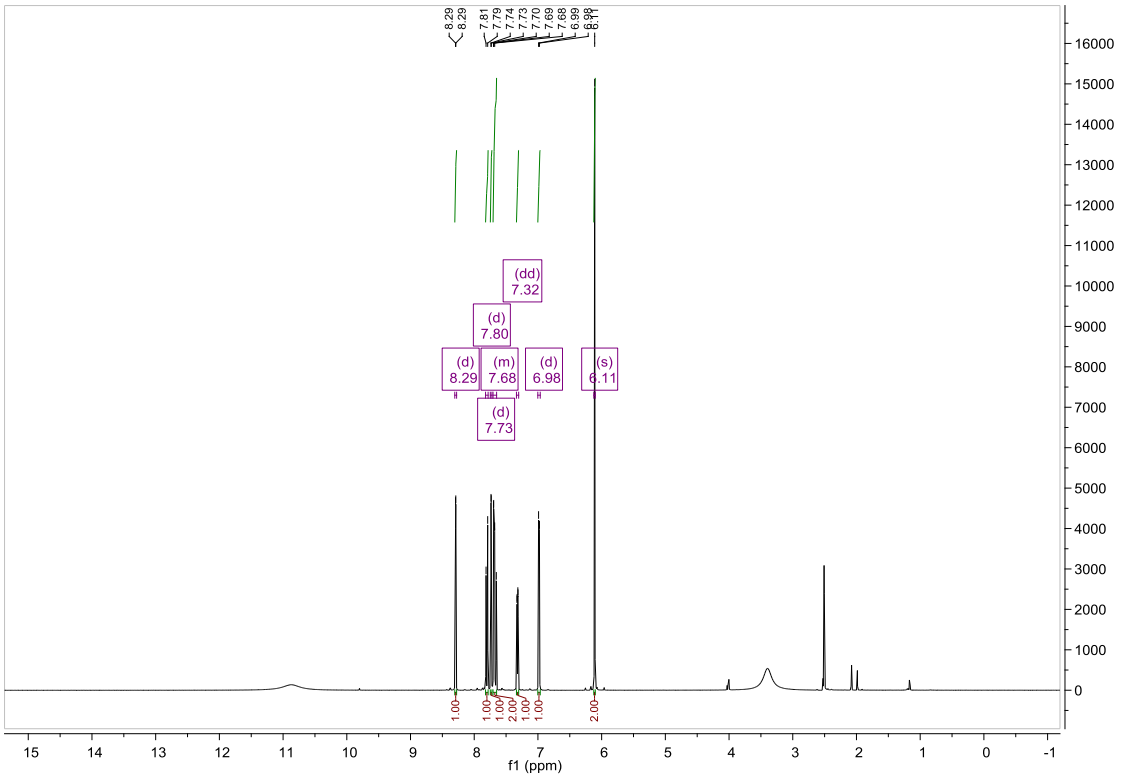

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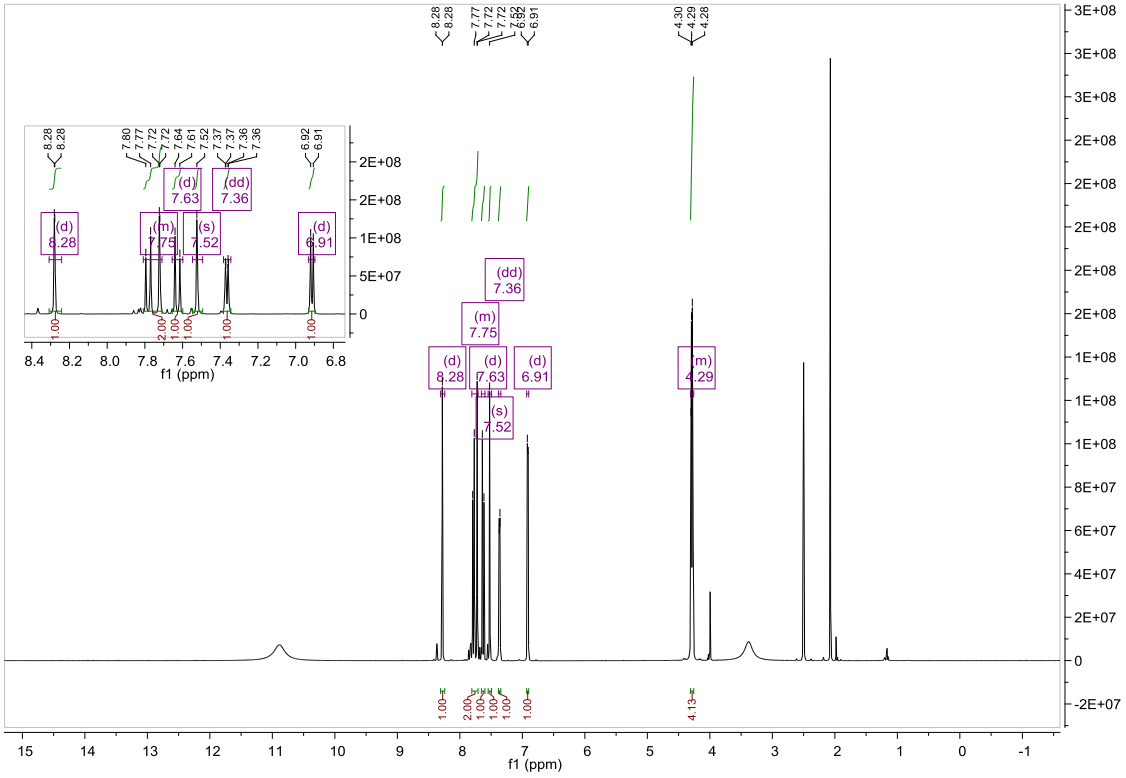
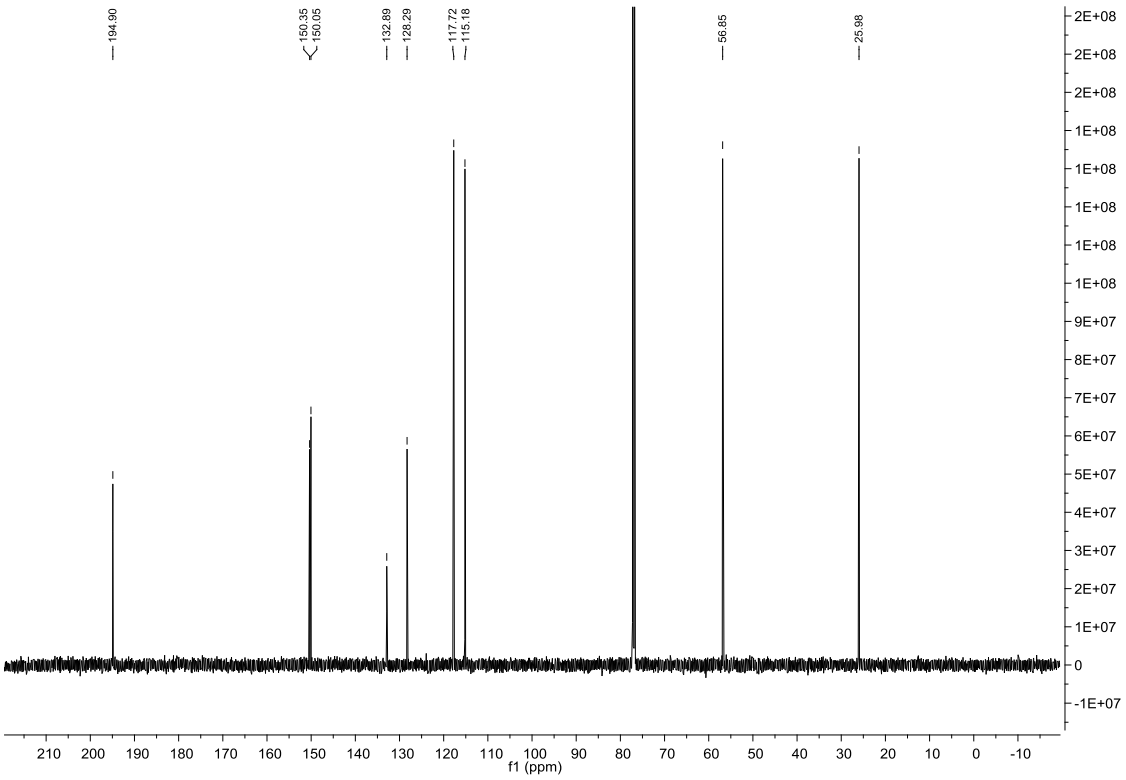
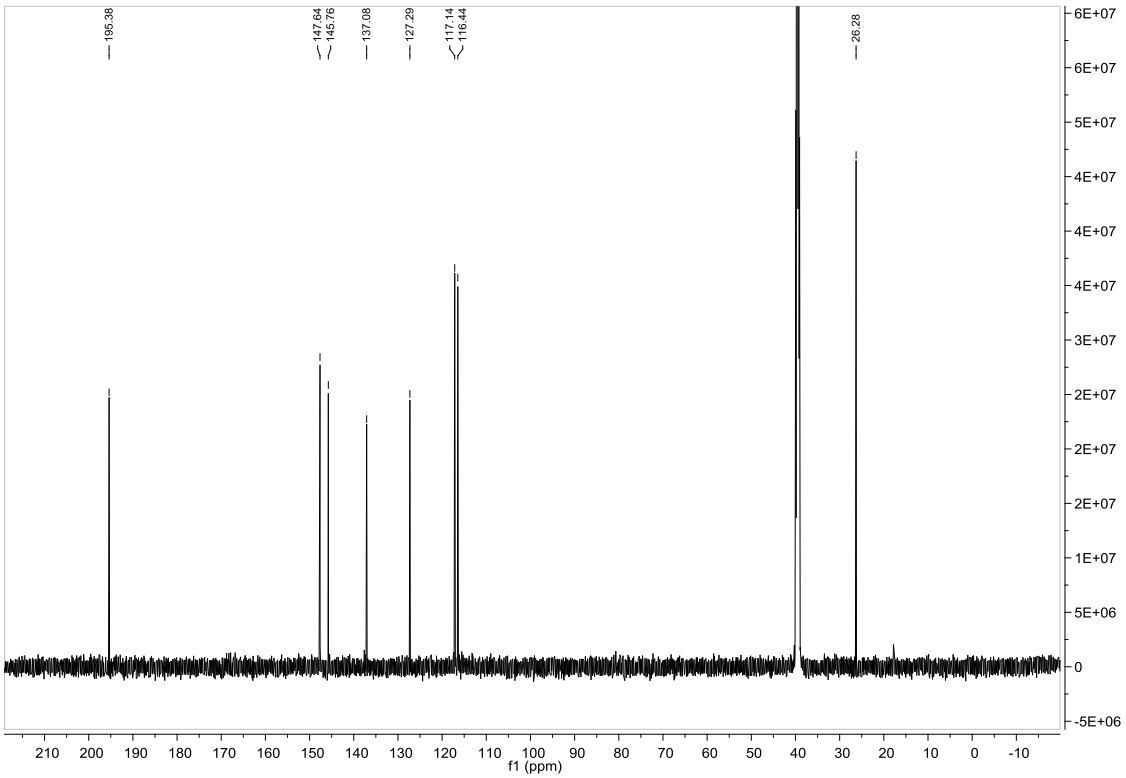


^{13}C NMR

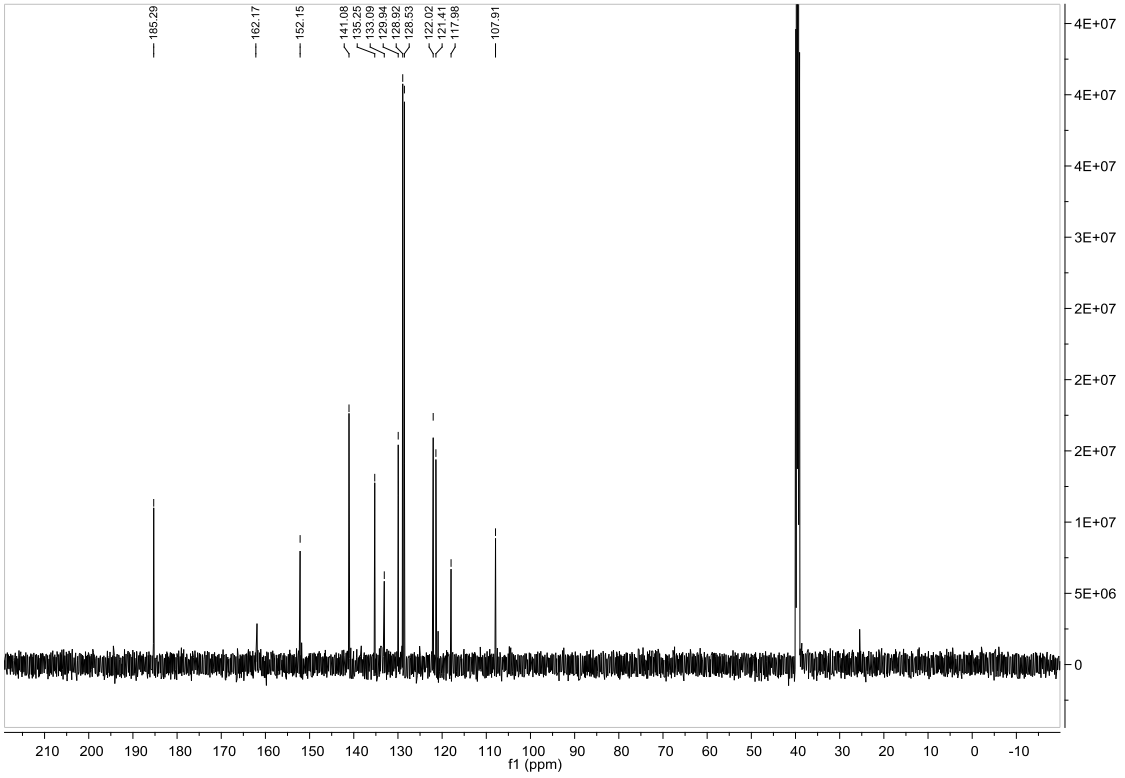
1-(4-Hydroxy-3-methoxy-5-nitrophenyl)ethanone (11)



1-(3,4-Dihydroxy-5-nitrophenyl)ethanone (12)



(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-phenylprop-2-en-1-one (1a)



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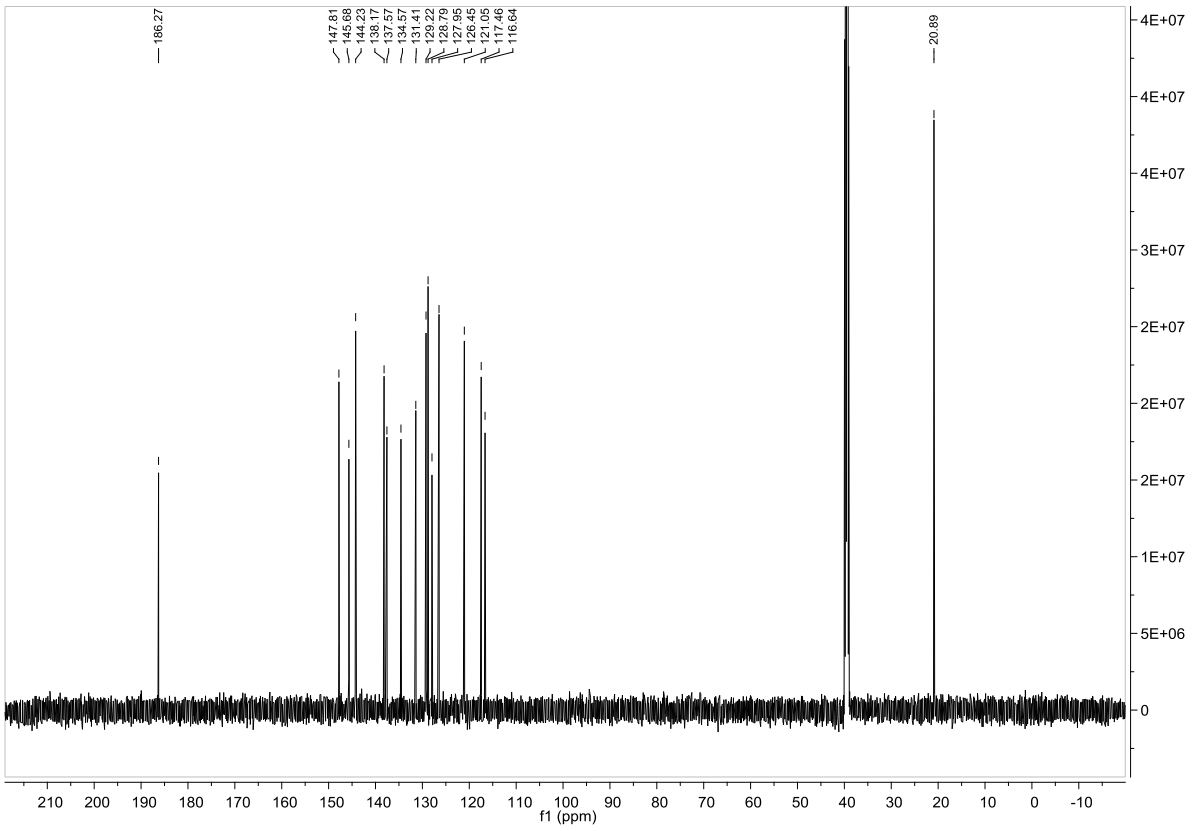

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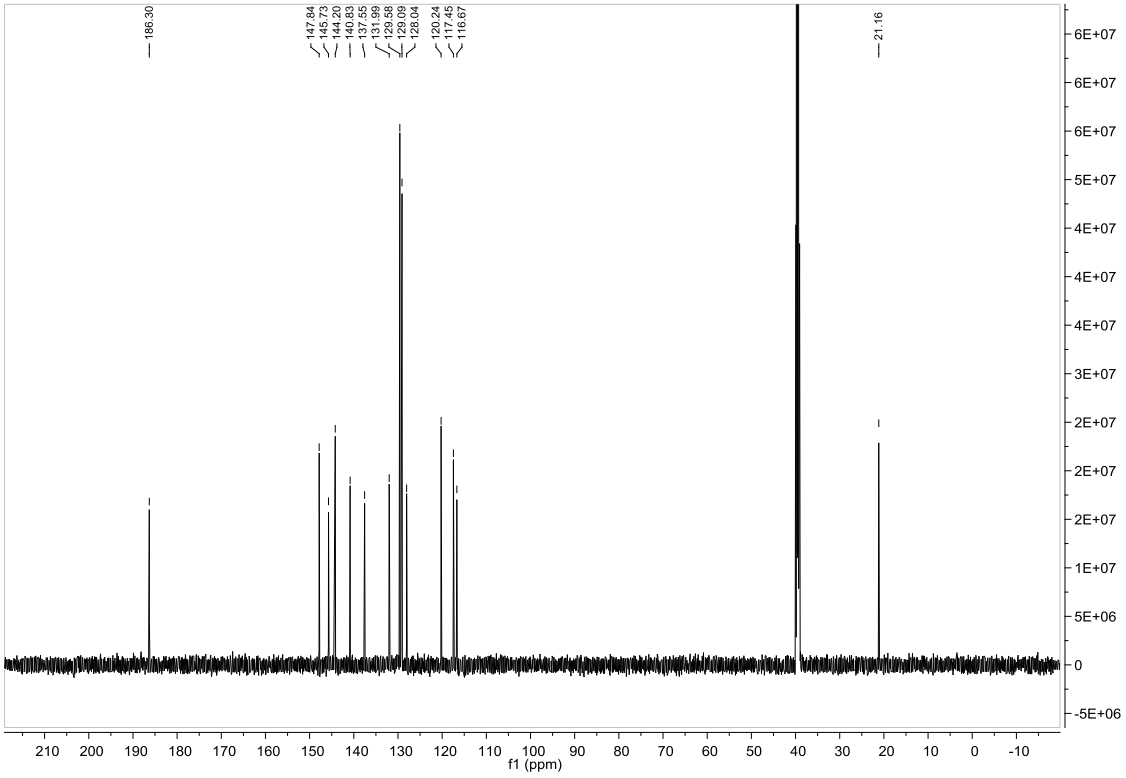
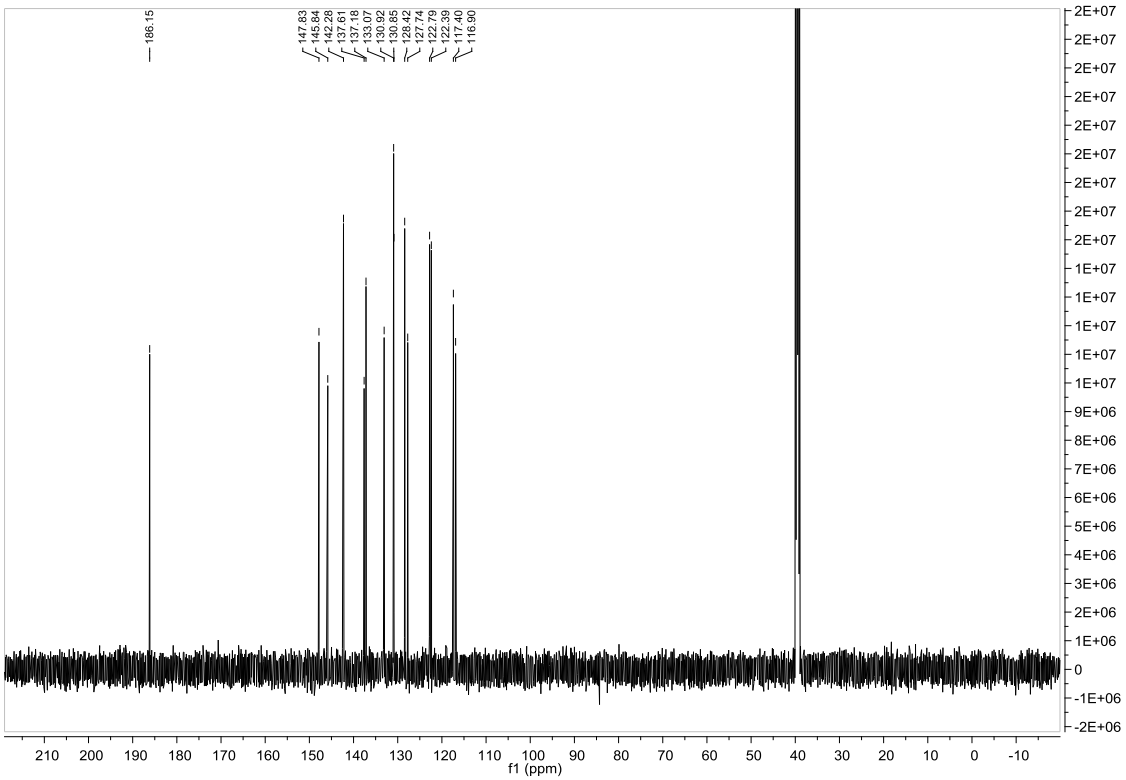
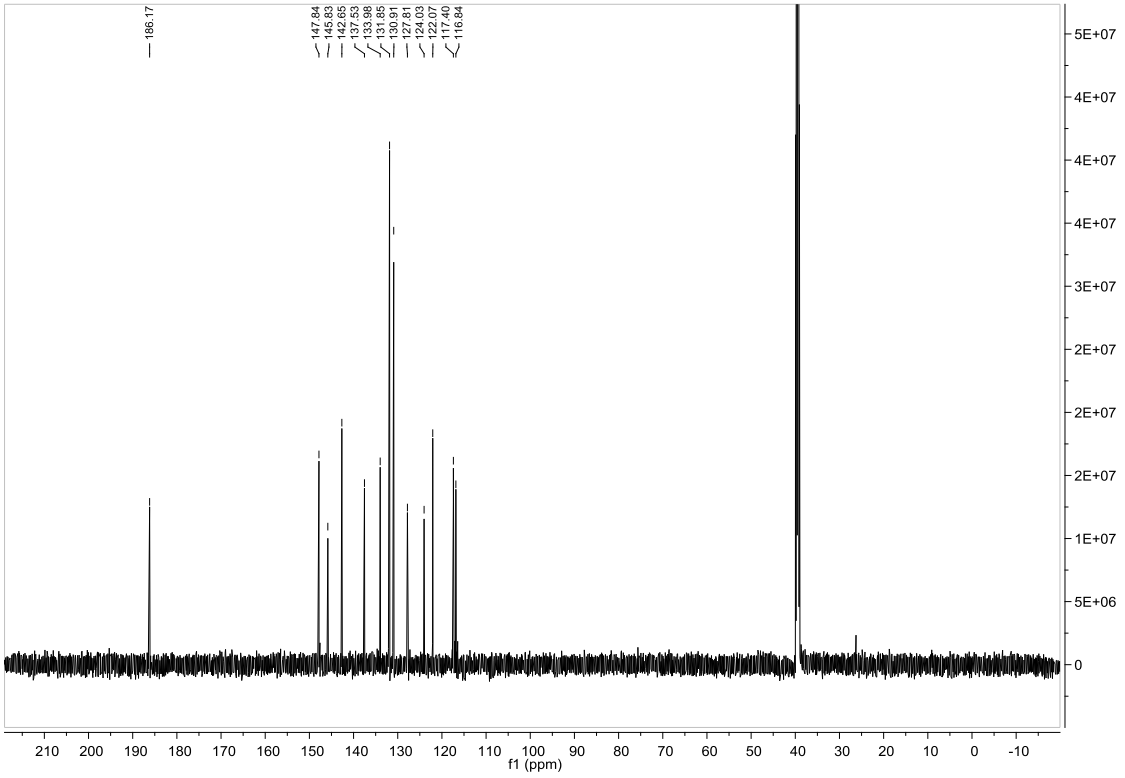


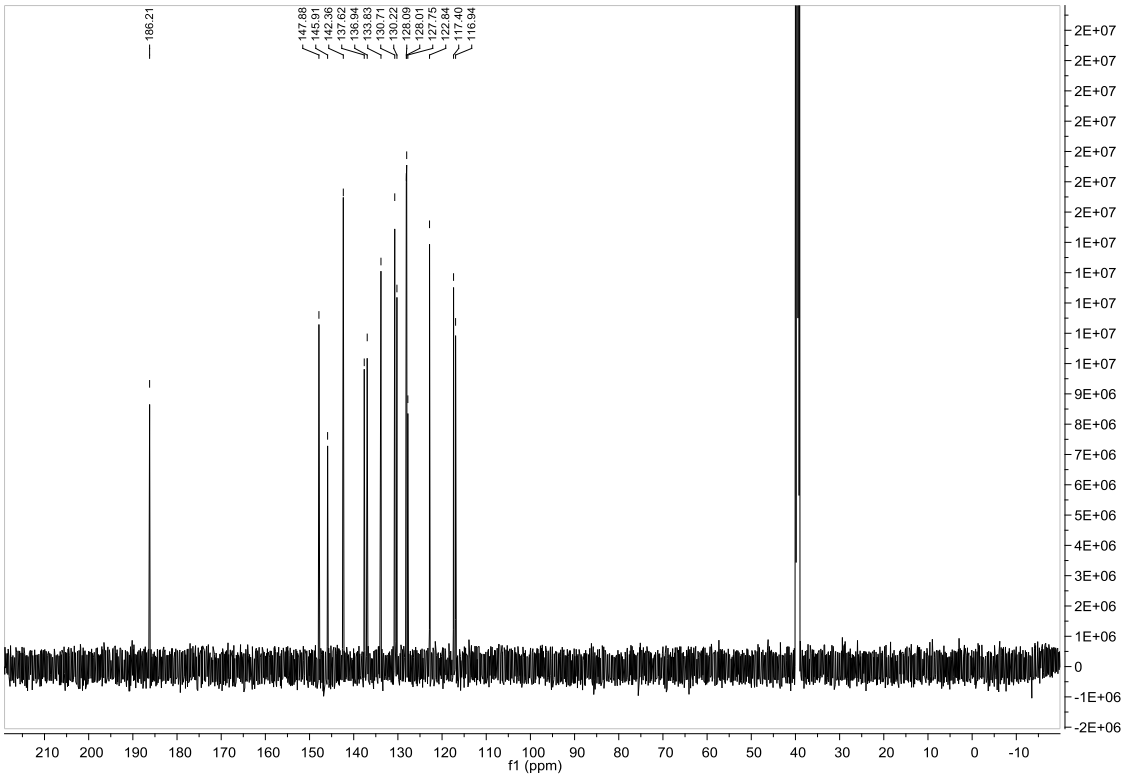
(2*E*)-3-(3-Bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1d)



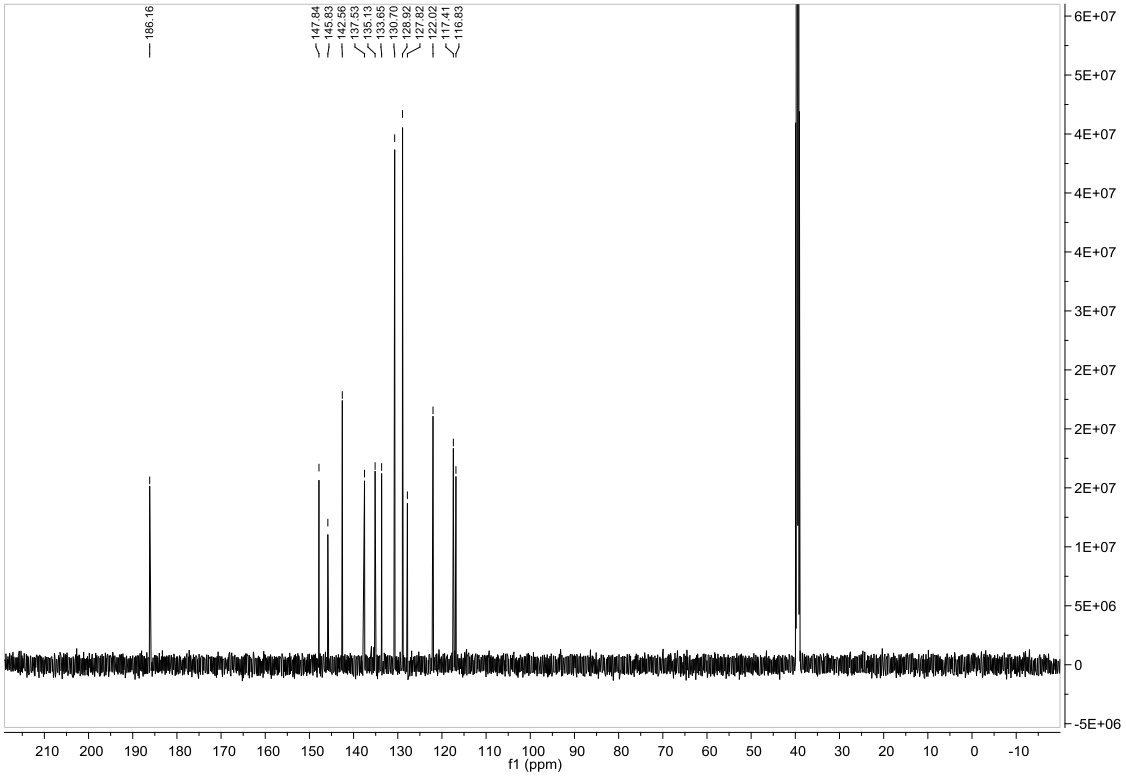
(2E)-3-(4-Bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1e)



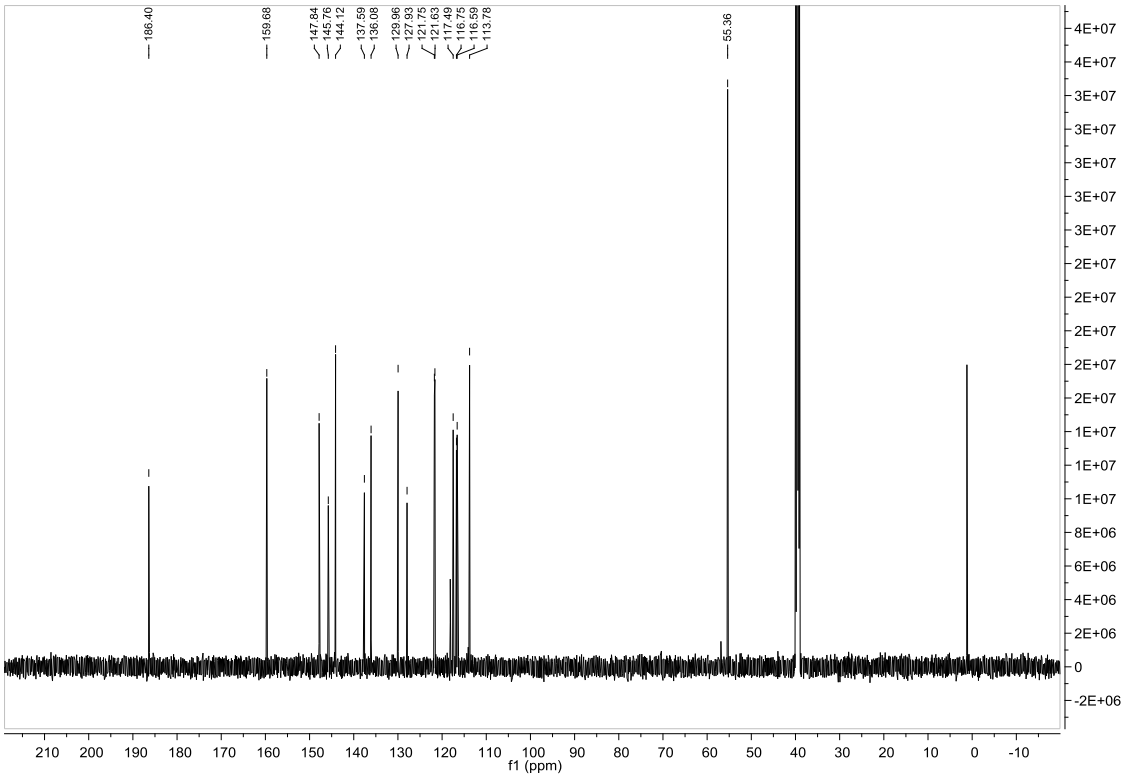
(2*E*)-3-(3-Chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1f)



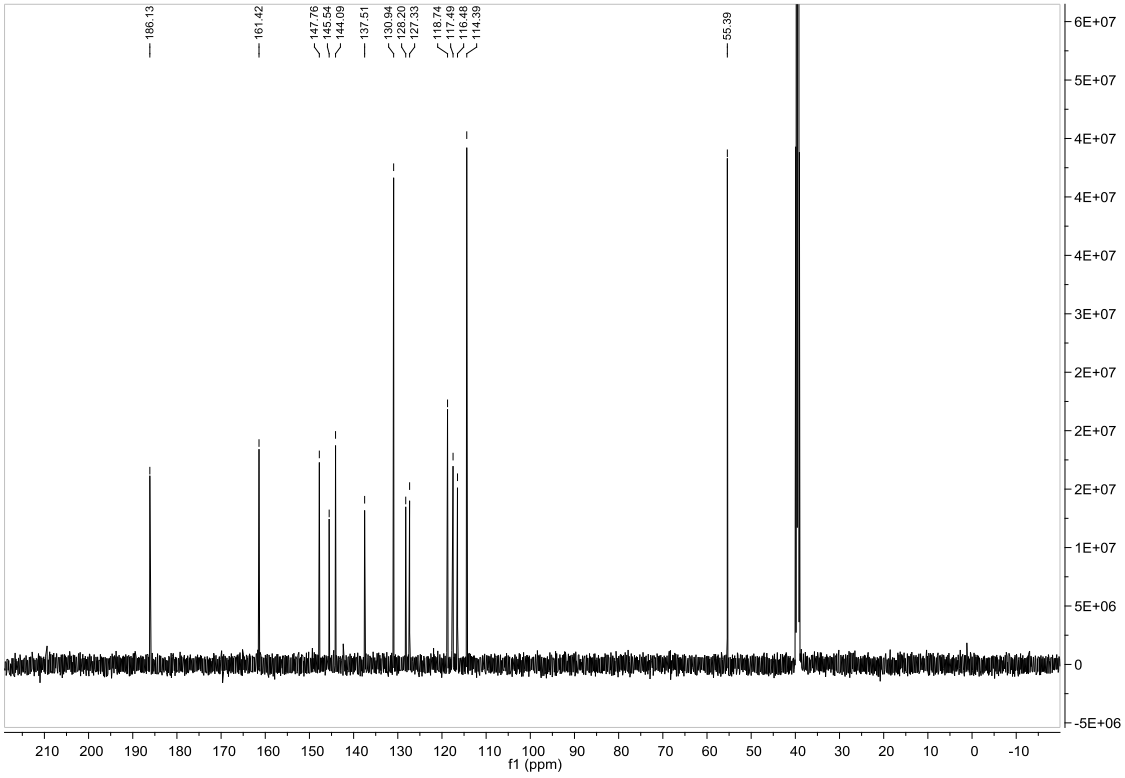
(2*E*)-3-(4-Chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1g)



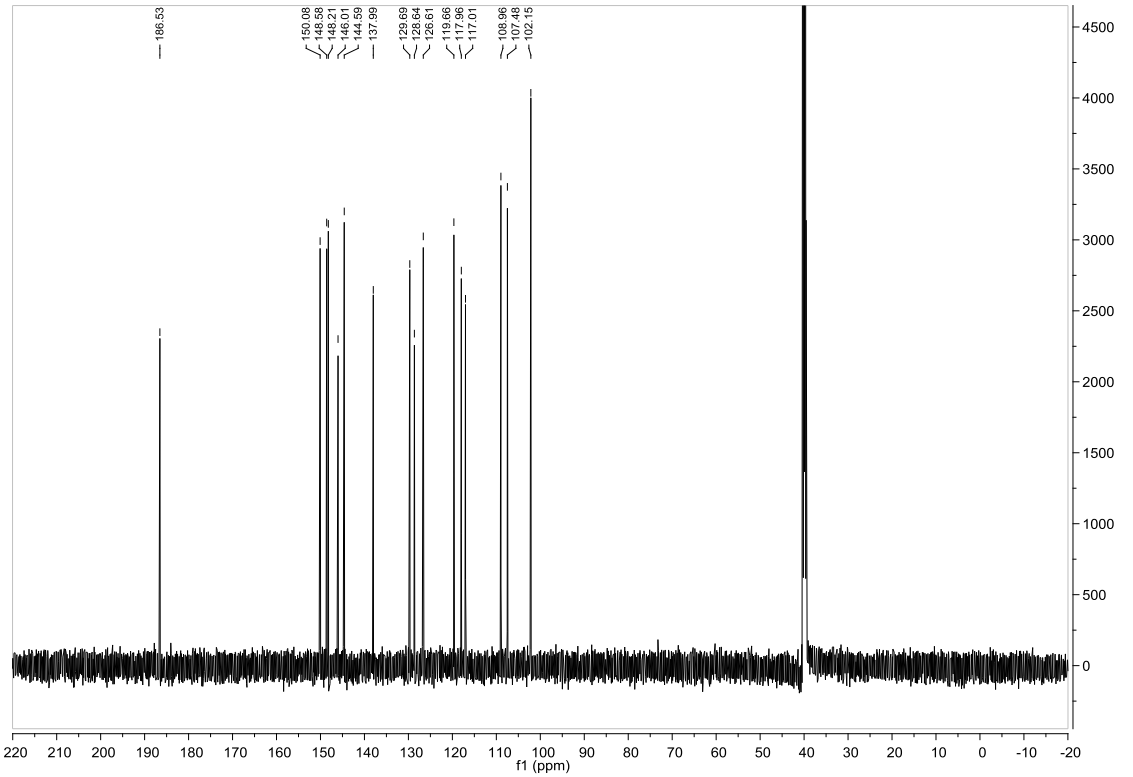
(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (1h)



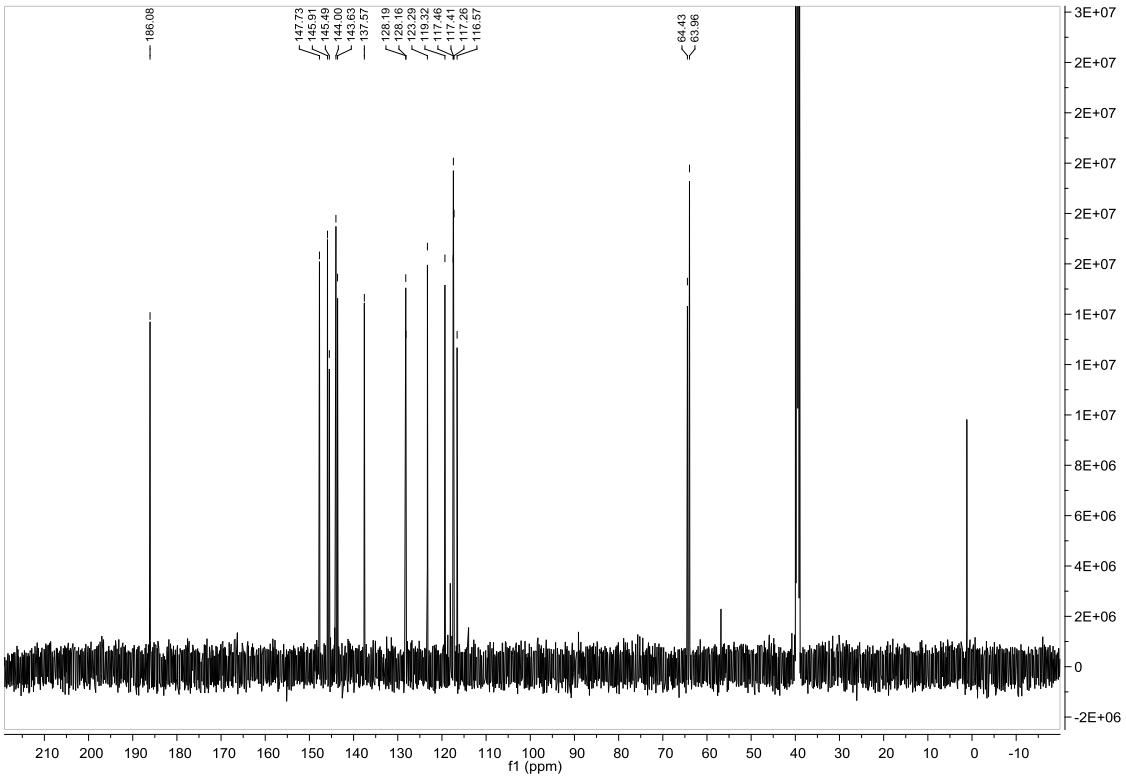
(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (1i)



(2*E*)-3-(2*H*-1,3-Benzodioxol-5-yl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1j)

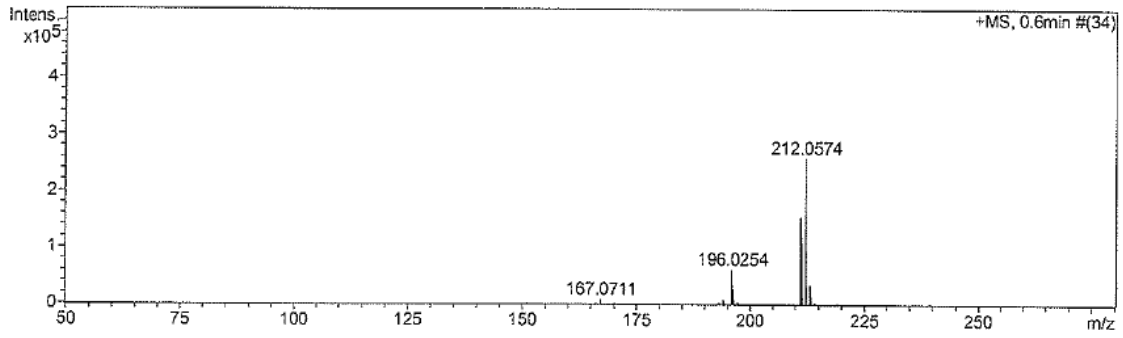


(2*E*)-3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1k)



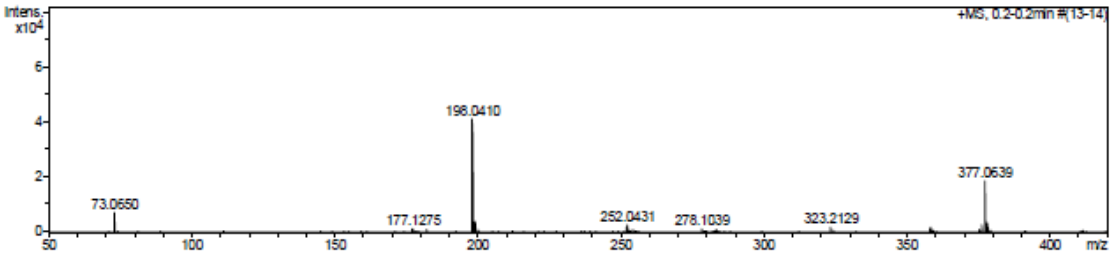
Mass spectrometry Spectra

1-(4-Hydroxy-3-methoxy-5-nitrophenyl)ethanone (11)



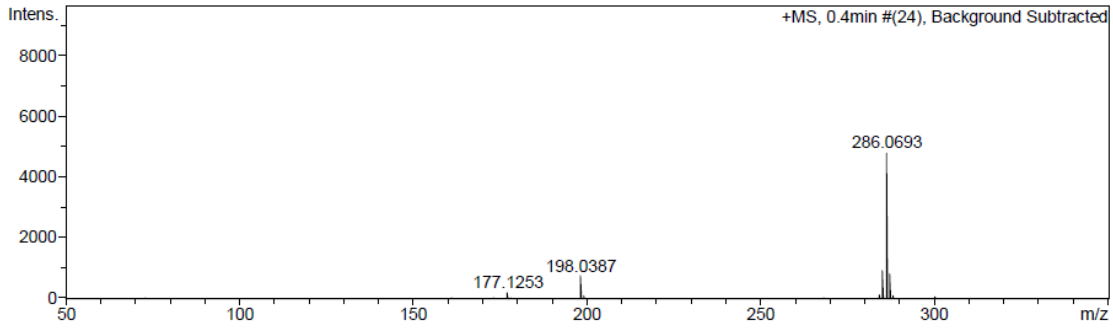
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
212.0574	1	C 9 H 10 N O 5	100.00	212.0553	-2.1	-9.7	21.3	5.5	ok	even

1-(3,4-Dihydroxy-5-nitrophenyl)ethanone (12)



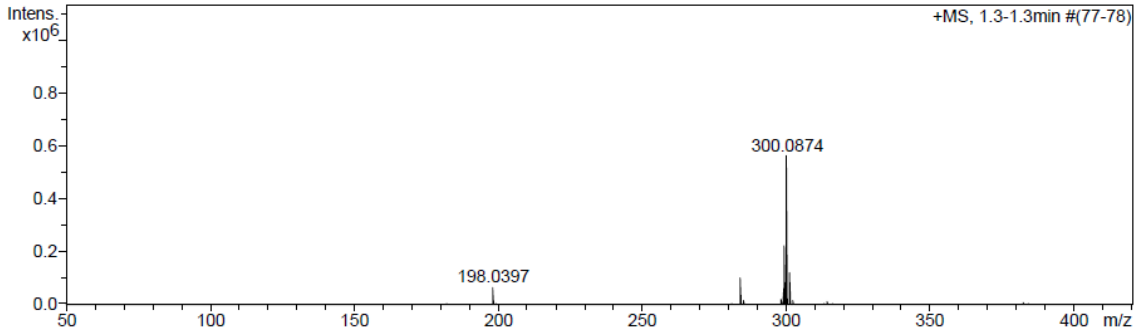
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
198.0410	1	C 8 H 8 N O 5	100.00	198.0397	-1.3	-6.6	1.7	5.5	ok	even
377.0639	1	C 16 H 13 N 2 O 9	100.00	377.0616	-2.3	-6.1	6.3	11.5	ok	even
	2	C 21 H 13 O 7	86.99	377.0656	1.7	4.6	33.4	15.5	ok	even

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-phenylprop-2-en-1-one (1a)



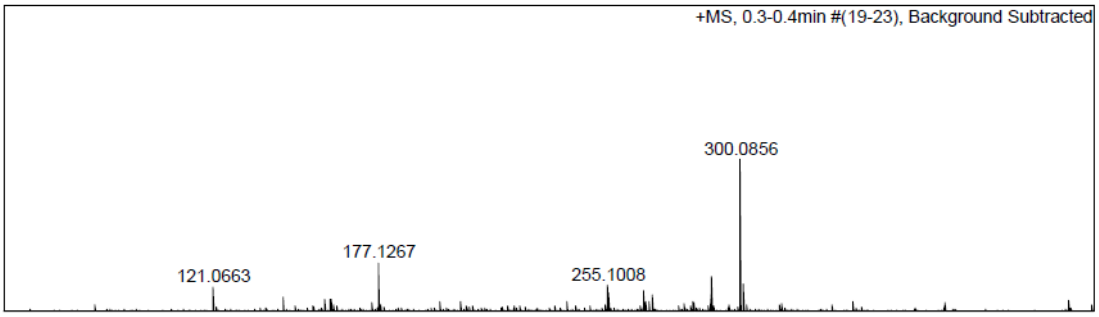
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
286.0693	1	C 15 H 12 N O 5	81.31	286.0710	1.7	5.9	6.0	10.5	ok	even
	2	C 11 H 8 N 7 O 3	100.00	286.0683	-1.0	-3.5	18.6	11.5	ok	even

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-methylphenyl)prop-2-en-1-one (1b)



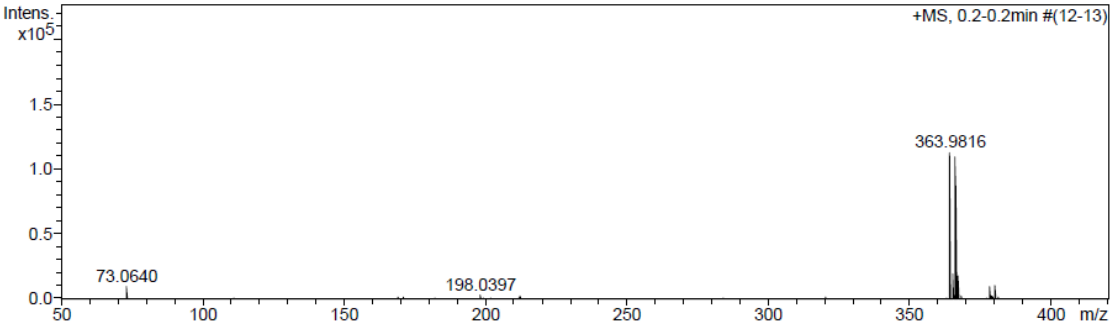
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
198.0397	1	C 8 H 8 N O 5	100.00	198.0397	0.0	0.2	6.3	5.5	ok	even
	2	C 5 H 10 O 8	13.14	198.0370	-2.6	-13.4	24.2	1.0	ok	odd
300.0874	1	C 16 H 14 N O 5	100.00	300.0866	-0.7	-2.5	25.5	10.5	ok	even

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(4-methylphenyl)prop-2-en-1-one (1c)



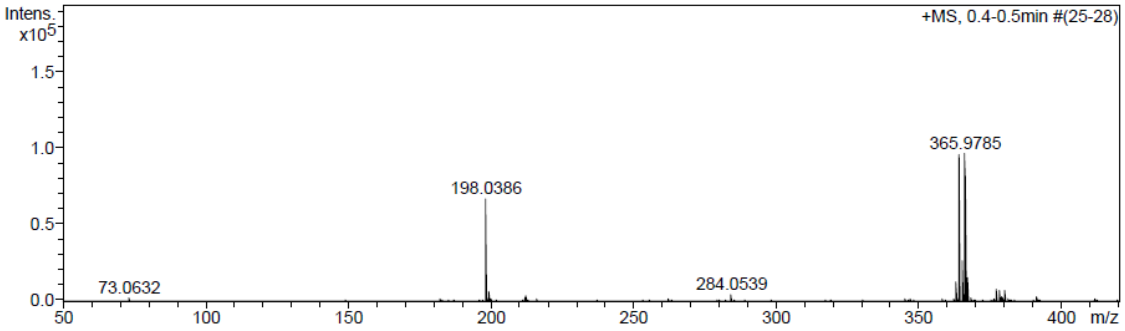
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
300.0856	1	C 16 H 14 N O 5	100.00	300.0866	1.1	3.6	15.1	10.5	ok	even

(2*E*)-3-(3-Bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1d)



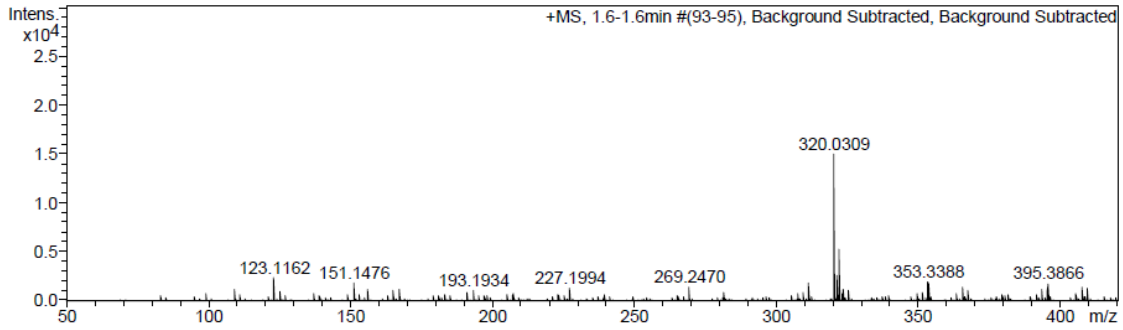
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
363.9816	1	C 15 H 11 Br N O 5	100.00	363.9815	-0.1	-0.3	10.8	10.5	ok	even

(2*E*)-3-(4-Bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1e)



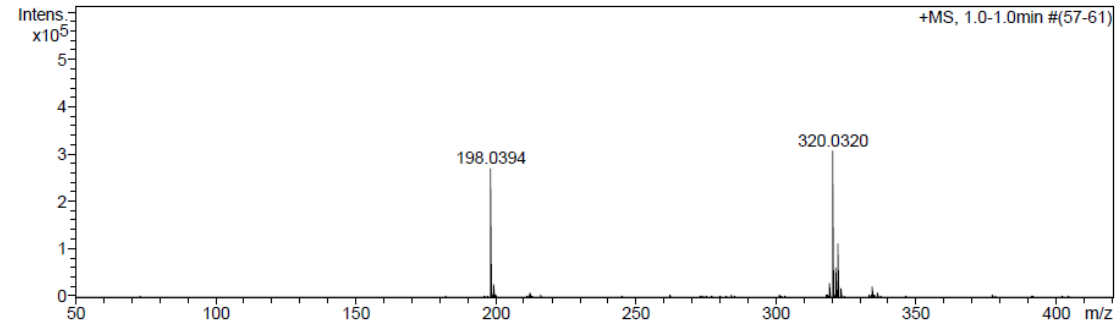
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
363.9801	1	C 15 H 11 Br N O 5	100.00	363.9815	1.4	3.8	47.5	10.5	ok	even
	2	C 12 H 13 Br O 8	60.89	363.9788	-1.3	-3.6	64.6	6.0	ok	odd
	3	C 10 H 6 N O 14	0.00	363.9783	-1.8	-5.1	564.2	8.5	ok	even

(2*E*)-3-(3-Chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1f)



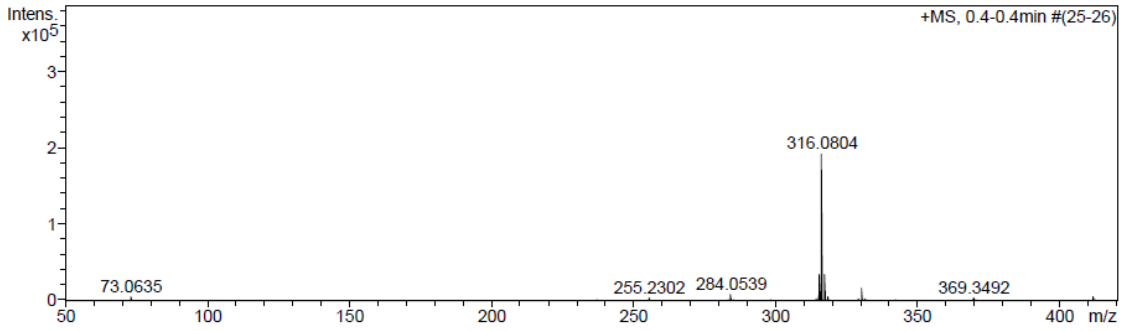
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
320.0309	1	C 15 H 11 Cl N O 5	100.00	320.0320	1.2	3.7	25.1	10.5	ok	even

(2*E*)-3-(4-Chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1g)



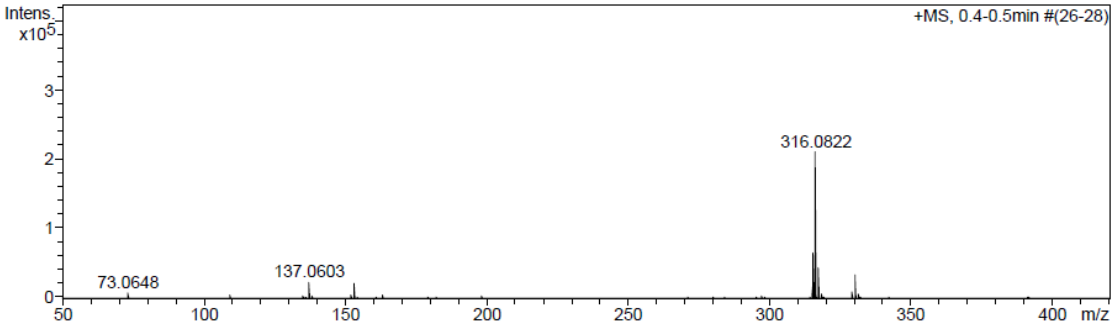
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
198.0394	1	C 8 H 8 N O 5	100.00	198.0397	0.3	1.6	5.5	5.5	ok	even
	2	C 5 H 10 O 8	18.14	198.0370	-2.4	-11.9	25.8	1.0	ok	odd
320.0320	1	C 15 H 11 Cl N O 5	100.00	320.0320	0.0	0.1	19.4	10.5	ok	even
	2	C 12 H 13 Cl O 8	13.28	320.0293	-2.6	-8.2	34.7	6.0	ok	odd

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (1h)



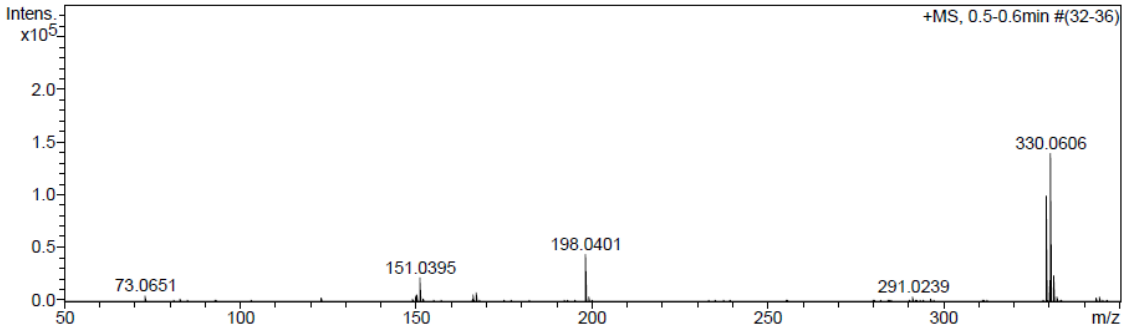
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
316.0804	1	C 16 H 14 N O 6	100.00	316.0816	1.2	3.8	3.2	10.5	ok	even

(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (1i)



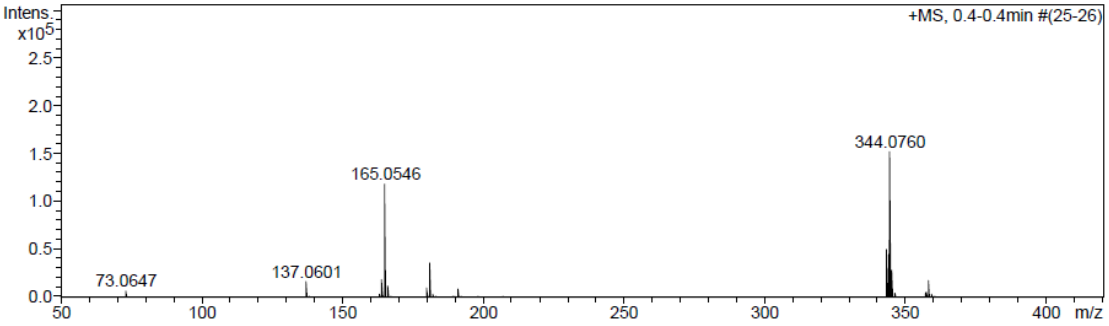
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
316.0822	1	C 16 H 14 N O 6	100.00	316.0816	-0.6	-1.9	17.5	10.5	ok	even

(2*E*)-3-(2*H*-1,3-Benzodioxol-5-yl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1j)



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e¯ Conf
330.0606	1	C 16 H 12 N O 7	100.00	330.0608	0.2	0.7	2.1	11.5	ok	even
	2	C 20 H 10 O 5	0.00	330.0523	-8.3	-25.2	25.0	16.0	ok	odd
	3	C 23 H 8 N O 2	0.19	330.0550	-5.6	-17.1	45.9	20.5	ok	even
	4	C 24 H 10 O 2	0.02	330.0675	6.9	21.0	50.1	20.0	ok	odd

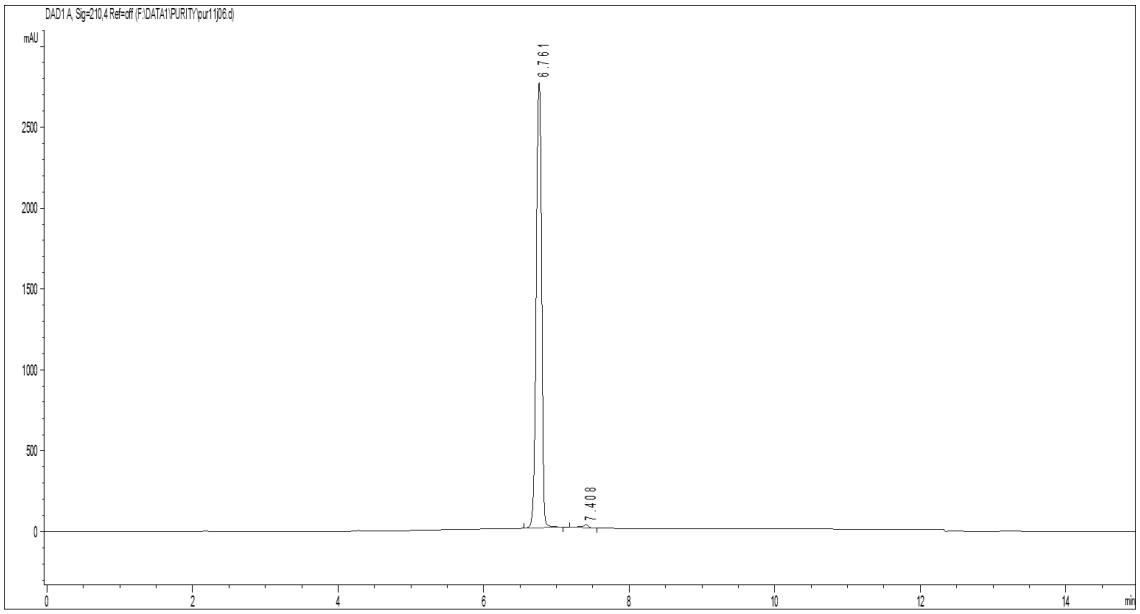
(2*E*)-3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1k)



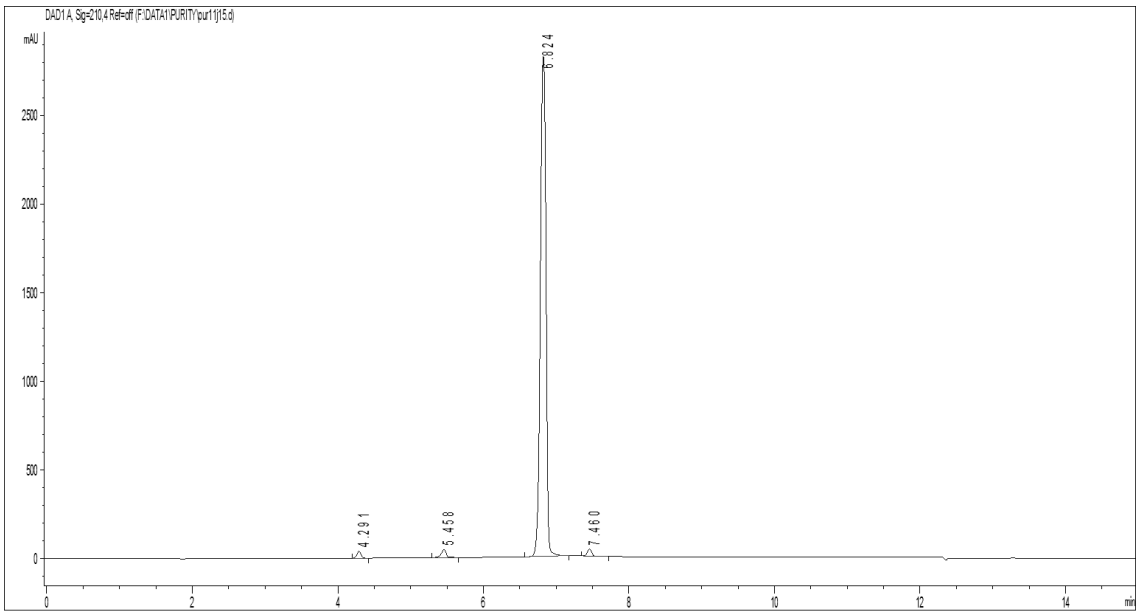
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
165.0546	1	C 9 H 9 O 3	100.00	165.0546	-0.0	-0.2	2.7	5.5	ok	even
	2	C 12 H 7 N	13.93	165.0573	2.7	16.1	18.3	10.0	ok	odd
344.0760	1	C 17 H 14 N O 7	100.00	344.0765	0.5	1.5	1.6	11.5	ok	even

HPLC Purity analysis

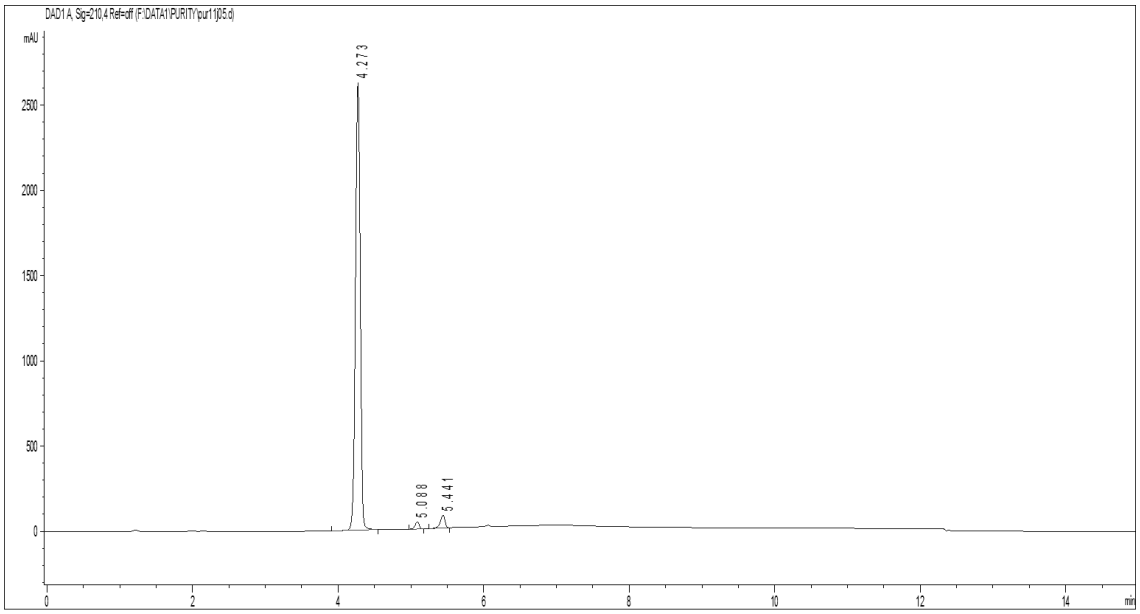
(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-phenylprop-2-en-1-one (1a)



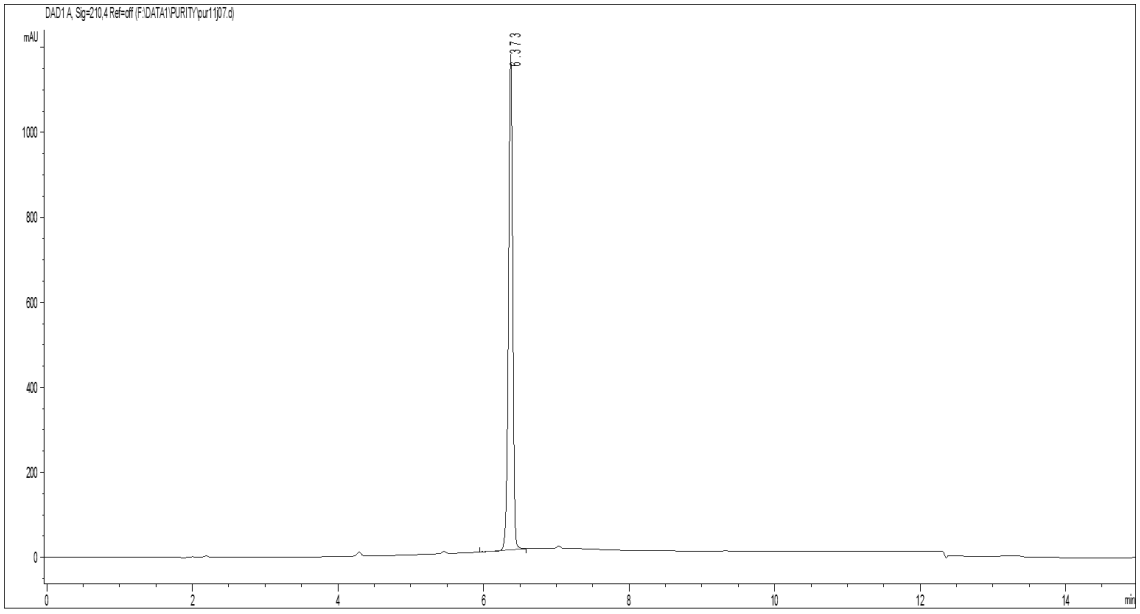
(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-methylphenyl)prop-2-en-1-one (1b)



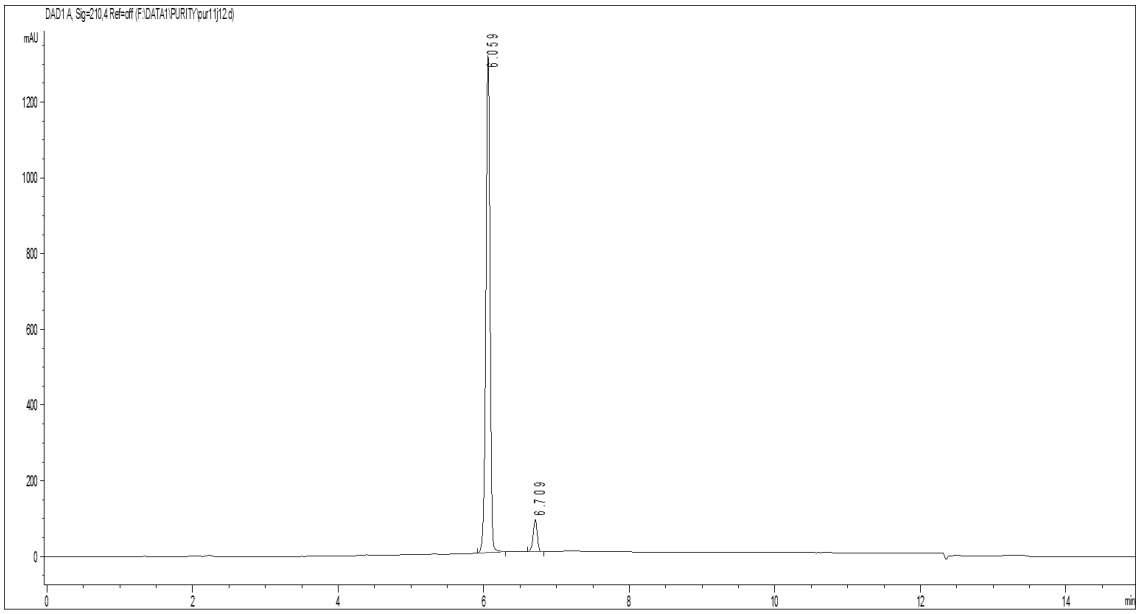
(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(4-methylphenyl)prop-2-en-1-one (1c)



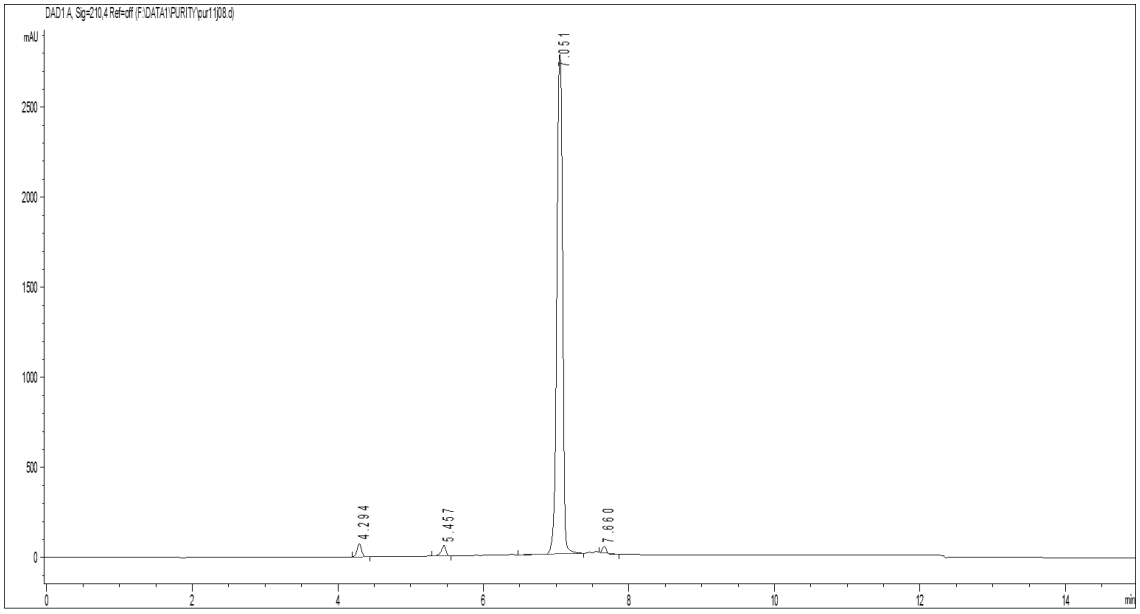
(2*E*)-3-(3-Bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1d)



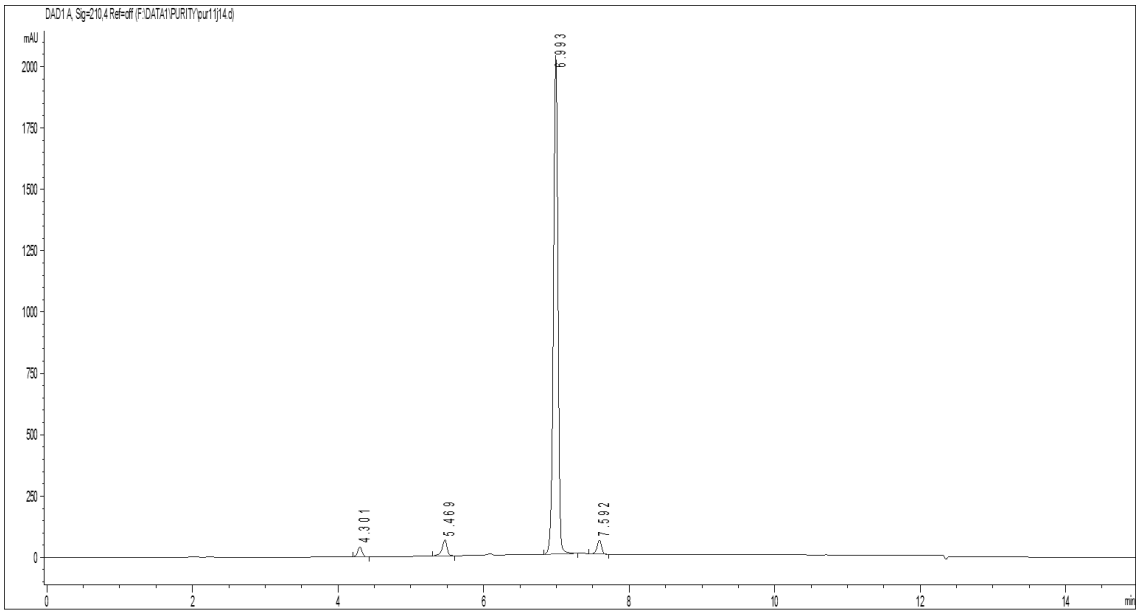
(2*E*)-3-(4-Bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1e)



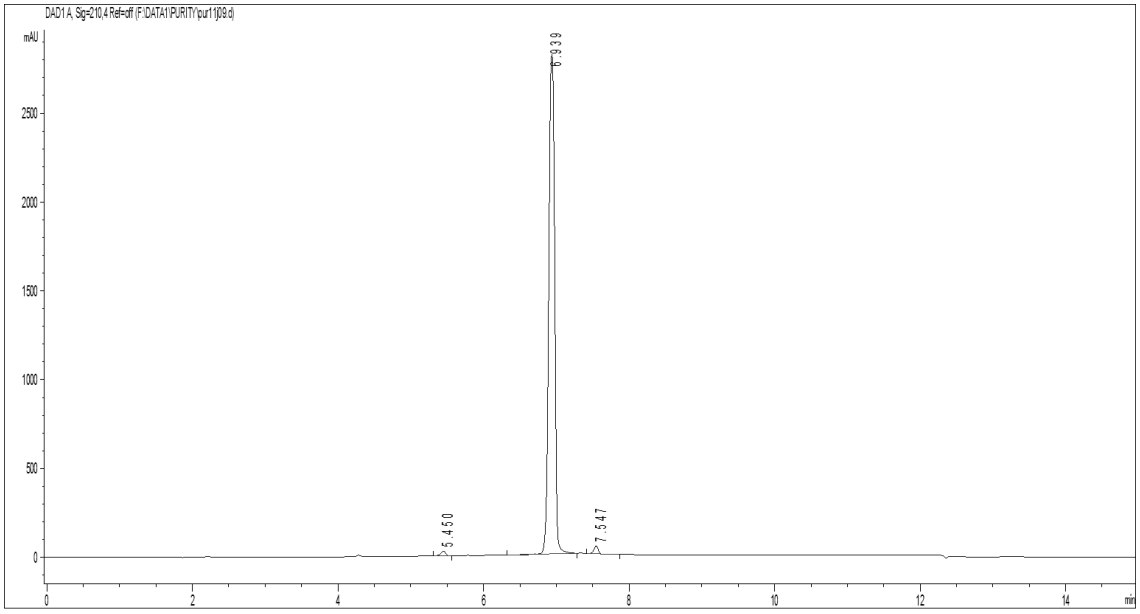
(2*E*)-3-(3-Chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1f)



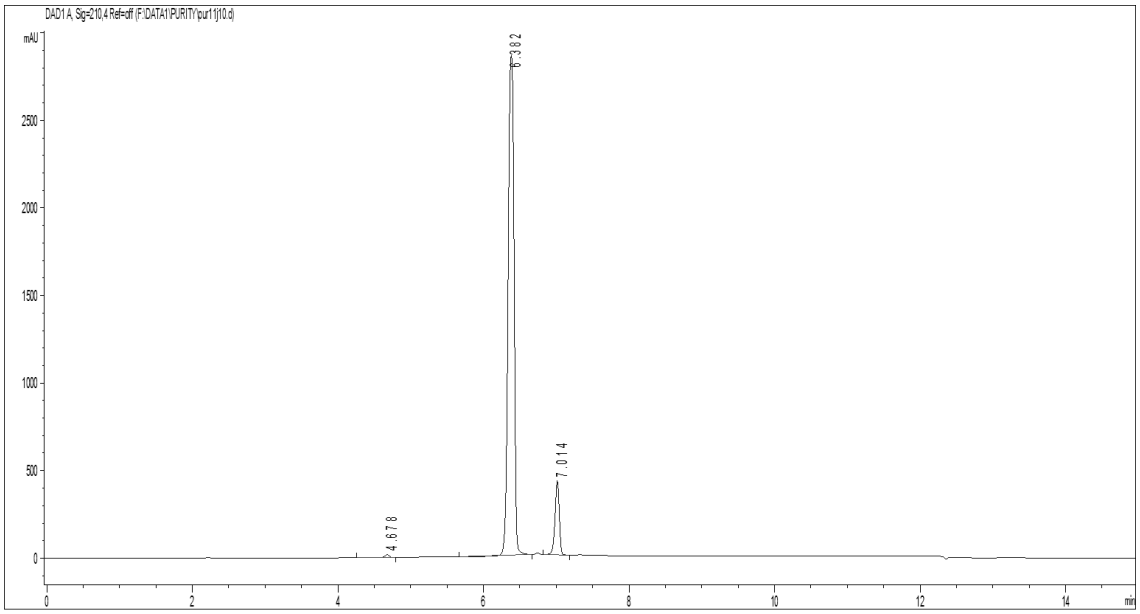
(2*E*)-3-(4-Chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1g)



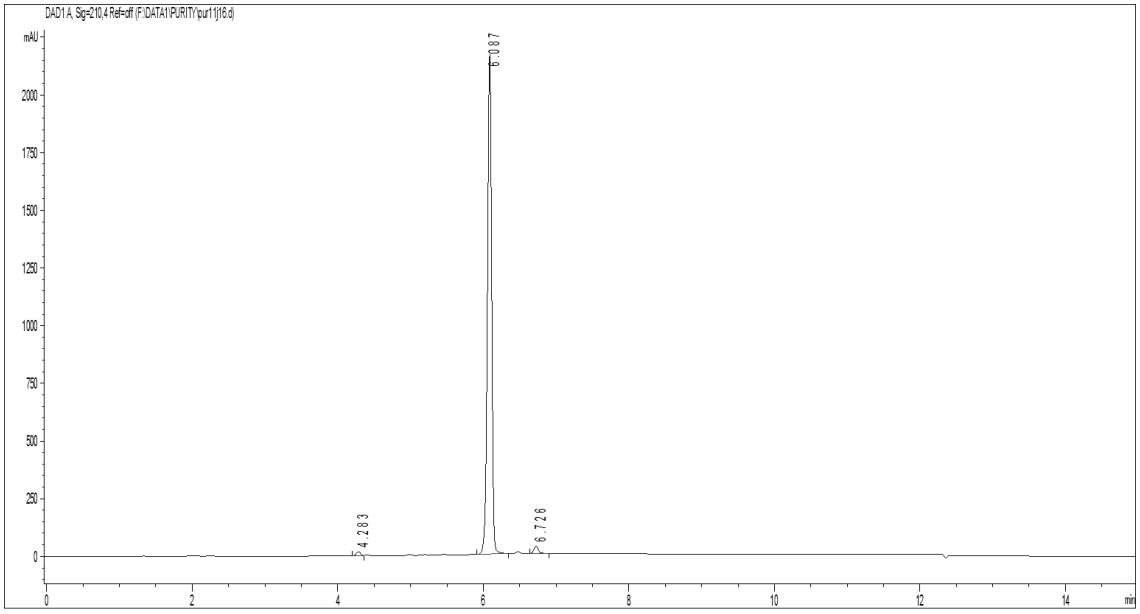
(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (1h)



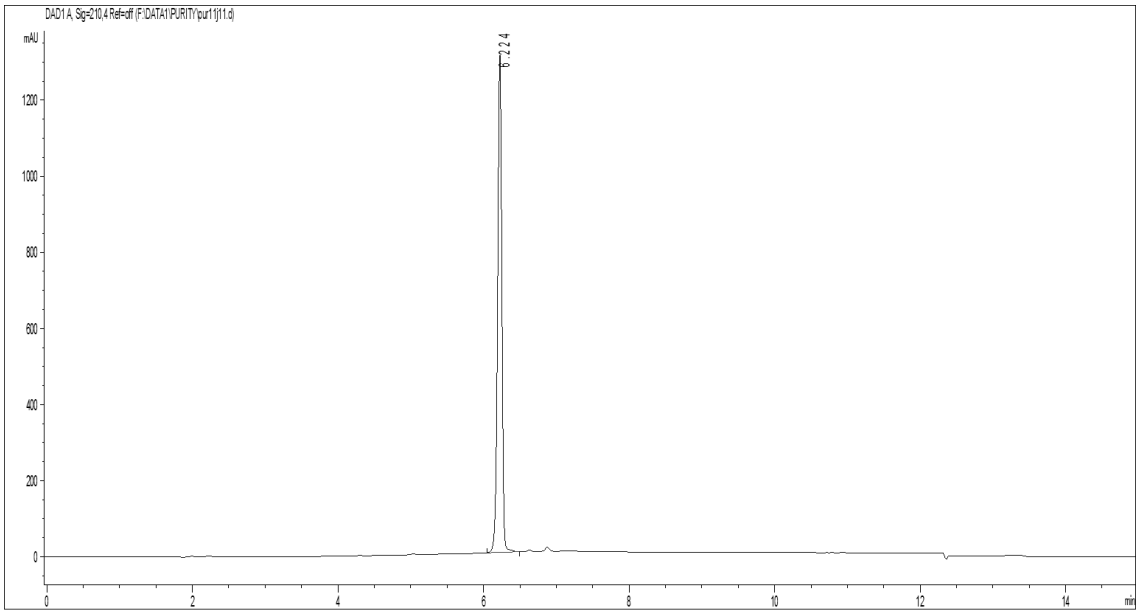
(2*E*)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (1i)



(2*E*)-3-(2*H*-1,3-Benzodioxol-5-yl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1j)



(2*E*)-3-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (1k)



Chapter 4

Article 2

The evaluation of selected natural compounds as potential dual inhibitors of catechol-O-methyltransferase and monoamine oxidase

Idalet Engelbrecht,[1] Jacobus P. Petzer,[1] Anél Petzer [1,*]

[2.] *Pharmaceutical Chemistry, School of Pharmacy and Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa*

*Corresponding author: Anél Petzer, Tel.: +27 18 2994464

E-mail address: 12264954@nwu.ac.za

Running title: MAO and COMT inhibition by natural products

Keywords: monoamine oxidase, MAO, inhibition, catechol-O-methyltransferase, COMT, multi-target-directed, Parkinson's disease, L-dopa, dopamine, natural compounds

Abstract

Parkinson's disease is characterised by the loss of nigrostriatal dopaminergic neurons and depletion of striatal dopamine. The most effective symptomatic treatment relies on the 'artificial' replacement of central dopamine with its metabolic precursor, levodopa (L-dopa). The efficacy of L-dopa is however compromised by extensive peripheral metabolism by aromatic-L-amino acid decarboxylase (AADC) and to a lesser extent catechol-O-methyltransferase (COMT). To enhance central L-dopa levels and promote its conversion to dopamine in the brain, L-dopa is often combined with inhibitors of these enzymes. Another approach to enhance the therapeutic effect of L-dopa is to inhibit monoamine oxidase (MAO)

B, a key metabolic enzyme of dopamine in the brain. MAO-B inhibitors, as monotherapy or as adjunct to L-dopa, is thus established therapy in Parkinson's disease. The present study attempts to discover compounds that exhibit dual inhibition of COMT and MAO-B among a small library of 43 structurally diverse natural compounds. Such dual acting inhibitors may be effective as adjuncts to L-dopa and offer enhanced value in the management of Parkinson's disease. Among the natural compounds morin (IC_{50} = 1.32 µM), chlorogenic acid (IC_{50} = 6.17 µM), (+)-catechin (IC_{50} = 0.86 µM), alizarin (IC_{50} = 0.88 µM), fisetin (IC_{50} = 5.78 µM) and rutin (IC_{50} = 25.3 µM) exhibited COMT inhibition. Among these active COMT inhibitors only morin (IC_{50} = 16.2 µM), alizarin (IC_{50} = 8.16 µM) and fisetin (IC_{50} = 7.33 µM) were noteworthy MAO inhibitors, with specificity for MAO-A. The most potent MAO inhibition was observed with chrysin, which inhibited both MAO-A (IC_{50} = 0.77 µM) and MAO-B (IC_{50} = 0.79 µM). Unfortunately, this compound was not an inhibitor of COMT. Although none of the natural products investigated here are dual COMT/MAO-B inhibitors, active COMT inhibitors may serve as leads for the future development of dual acting compounds.

1. Introduction

Parkinson's disease is a progressive neurodegenerative disorder associated with specific neuropathological lesions, particularly the loss of dopaminergic neurons situated in the substantia nigra pars compacta (Dauer & Przedborski, 2003). Since these neurons project to the striatum, Parkinson's disease presents with a functional loss of dopamine in this region, which leads to severe motor impairment. Clinically Parkinson's disease presents with a tetrad of symptoms namely tremor at rest, slowness of movement or bradykinesia, rigidity and postural instability or gait impairment (Fahn & Przedborski, 2000; Gaspar *et al*., 2011; Yacoubian & Standaert, 2009). Clinical manifestation of the disease occurs when approximately 60% of the dopaminergic neurons in the substantia nigra pars compacta have perished and responsiveness to dopamine has decreased to 70% (German *et al*., 1989; Smeyne & Jackson-Lewis, 2005; Uhl *et al*., 1985). Parkinson's disease is the second most common cause of neurological disability of the aging brain and affects 1–2% of the human population over 50 years of age. The average age of onset of the disease is 55 to 60 years with the incidence rising steeply with age. The duration of the disease is approximately 15 years from diagnosis to death (Katzenschlager *et al*., 2008; Lees *et al*., 2009).

The aetiology of Parkinson's disease is considered multifactorial with the involvement of oxidative stress, reduced antioxidant levels, mitochondrial dysfunction, abnormal protein aggregation and misfolding, inflammation, excitotoxicity and loss of trophic support leading to apoptosis (Blum *et al*., 2001). Interestingly, dopamine may function as an endogenous

toxin through its normal metabolism which generates harmful reactive species (Dauer & Przedborski, 2003). The metabolism of dopamine by monoamine oxidase (MAO) in the brain produces hydrogen peroxide (H_2O_2) and 3,4-dihydroxyphenylacetaldehyde as by-products. The auto-oxidation of dopamine also yields hydrogen peroxide as product (Hermida-Ameijeiras *et al*., 2004). Hydrogen peroxide generated by MAO and auto-oxidation serves as a substrate in the Fenton reaction which generates the highly reactive and injurious hydroxyl radical, while 3,4-dihydroxyphenylacetaldehyde is highly toxic to catecholaminergic cells (Mattamal *et al*., 1995). Furthermore, the oxidation of the catechol moiety of dopamine may lead to the formation of semiquinones, which promote radical formation and neuronal injury (Klegeris *et al*., 1995; Terland *et al*., 1997). Dopamine thus may play a crucial role in rendering dopaminergic neurons in the substantia nigra vulnerable to oxidative damage (Dauer & Przedborski, 2003). Since glutathione and glutathione peroxidase activity is reduced in the substantia nigra in Parkinson's disease, protection against oxidative damage by glutathione may be compromised in this brain region (Damier *et al*., 1993). Also, hydrogen peroxide produced by dopamine metabolism depletes glutathione leading to a detrimental cycle involving increased oxidative damage and lowered capacity of oxidant buffer systems (Blum *et al*., 2001).

Although no current treatment exists that halt or slow neurodegeneration in Parkinson's disease, several effective strategies are available for the symptomatic treatment of the motor symptoms. Most of the therapies currently approved aim to restore striatal dopamine levels with levodopa (L-dopa), the direct metabolic precursor of dopamine, to stimulate dopamine receptors directly with dopamine agonist treatment or to inhibit dopamine reuptake and metabolism (Lees, 2005). Since its first use in 1967, L-dopa remains the backbone of Parkinson's disease treatment regimens and particularly in the initial stages of the disease, greatly improves motor symptoms, which in turn significantly enhances the patient's quality of life (Colosimo & De Michele, 1999; Fahn, 1974; Huot *et al*., 2016; Marsden & Parkes, 1976; Shaw *et al*., 1980). Despite these advantages of L-dopa, 40–50% of patients with Parkinson's disease will develop daily motor fluctuations and involuntary movements termed dyskinesia (Ahlskog & Muenter, 2001; Olanow & Jankovic, 2005). Furthermore, L-dopa undergoes extensive peripheral decarboxylation and 3-O-methylation which reduce its therapeutic efficiency (Da Prada *et al*., 1984; Deleu *et al*., 2002). In Parkinson's disease therapy, L-dopa is thus frequently combined with inhibitors of peripheral aromatic-L-amino acid decarboxylase (AADC) and catechol-O-methyltransferase (COMT). These combinations greatly increase L-dopa's availability for uptake into the brain and allow for a reduction of the therapeutic L-dopa dose (Bonifácio *et al*., 2002; Heeringa *et al*., 1997; Robinson *et al*.,

2012). Furthermore, AADC inhibitors reduce the peripheral conversion of L-dopa to dopamine and thus dopaminergic side effects such as cardiac arrhythmias, hypotension, nausea and vomiting (Seeberger & Hauser, 2015). Although metabolism by COMT represents a lesser metabolic route compared to AADC, COMT inhibitors reduce the peripheral formation of 3-O-methyldopa which accumulates in the plasma, partially due to its long elimination half-life (Gervas *et al*., 1983; Kuruma *et al*., 1971; Reches & Fahn, 1982; Wade & Katzman, 1975). 3-O-Methyldopa is associated with a poor response to L-dopa therapy presumably because it may compete with L-dopa for uptake transport across the blood-brain barrier (Nutt & Fellman, 1984). The AADC inhibitors, benserazide and carbidopa, and the COMT inhibitors, entacapone and tolcapone, have been used in the clinic as adjuvants to L-dopa (figure 1) (Calne, 1993; Gnerre *et al*., 2000).

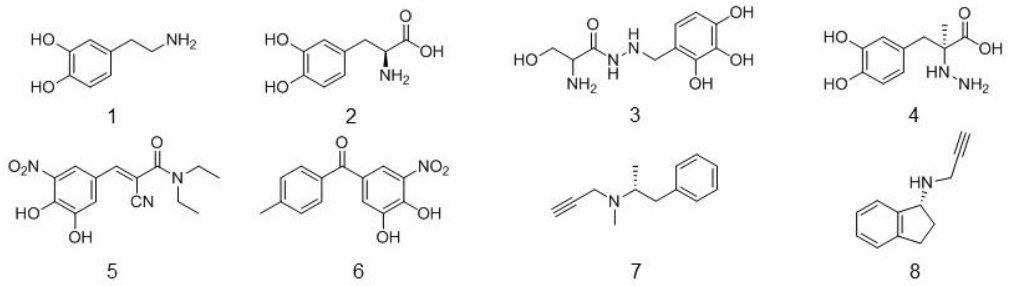


Figure 1. The structures of compounds discussed in the text: dopamine (**1**), L-dopa (**2**), benserazide (**3**), carbidopa (**4**), entacapone (**5**), tolcapone (**6**), selegiline (**7**) and rasagiline (**8**).

The COMT enzyme exists as two isoforms encoded by a single gene (Lundström *et al*., 1991; Salminen *et al*., 1990). Both soluble and membrane-bound COMT is identical except for the inclusion of an additional 50 hydrophobic amino acid sequence in membrane-bound COMT (Ma *et al*., 2013; Tunbridge *et al*., 2004; Ulmanen & Lundström, 1991). It was determined in early research that COMT is highly localised in the soluble fraction of the cell (Axelrod & Tomchick, 1958; Guldberg & Marsden, 1975). Later research identified membrane-bound COMT which does not appear to differ in biochemical and immunological characteristics from the soluble fraction of the enzyme (Borchardt *et al*., 1974; Guldberg & Marsden, 1975). Based on this, experimental findings may be extrapolated between the isoforms without compromising the integrity of the results.

In vertebrates, COMT activity is greatest in the liver which is mostly attributed to the soluble isoform with only a minor fraction attributed to the membrane-bound fraction (Ding *et al*., 1996; Karhunen *et al*., 1994; Rivett *et al*., 1983). In humans, 70% of the total COMT activity in the brain can be attributed to the membrane-bound fraction, while 30% is attributed to soluble COMT activity (Männistö & Kaakkola, 1999). Although both isoforms of COMT are identical in biochemical and immunological characteristics, differences between species exist in the physiochemical properties as well as level of enzyme activity. Thus, extrapolating results between species should only be done with caution (Agathopoulous *et al*., 1971). The localisation and degree of activity of COMT is significantly lower in the central nervous system than in the peripheral tissues (Kiss & Soares-da-Silva, 2014). The membrane-bound isoform of the enzyme is considered to be a more important drug target than the soluble isoform, since the membrane-bound fraction is able to methylate catecholamines at their physiological concentration (Roth, 1992). The soluble fraction plays a more important role in non-physiological conditions such as when the substrate concentration suddenly increases (i.e. after L-dopa treatment) or when a higher methylation reaction rate is needed (Huotari *et al*., 2002; Lotta *et al*., 1995).

Medications that inhibit the metabolic breakdown of dopamine also represent a strategy to enhance the therapeutic efficacy of L-dopa. In this respect, MAO inhibitors such as selegiline and rasagiline are used as monotherapy in the initial stages of Parkinson's disease or as adjunctive therapy to L-dopa (Jankovic & Stacy, 2007; Shoulson, 1998; Weinreb et al., 2010). MAO inhibitors elevate dopamine levels in the brain by suppressing the MAO-catalysed catabolism of dopamine, thus enhancing and possibly prolonging the dopaminergic effect of L-dopa (Knoll, 1993; Mazzio *et al*., 1998). MAO inhibitors also reduce the MAO-catalysed formation of hydrogen peroxide and 3,4-dihydroxyphenylacetaldehyde in the brain, and have thus been advocated as potential neuroprotective agents in Parkinson's disease (Chazot, 2001; Ebadi *et al*., 2006; Rabey *et al*., 2000). Partly due to serious adverse effects of nonspecific irreversible MAO inhibition, inhibitors of the MAO-B isoform are used in Parkinson's disease therapy. Nonspecific inhibitors that also inhibit the MAO-A isoform may potentiate the sympathomimetic effect of dietary tyramine leading to a potentially fatal increase in blood-pressure (Da Prada *et al*., 1988; Youdim *et al*., 1988; Youdim & Bakhle, 2006). It is important to note that dopamine is also metabolised by COMT, and in addition to preventing the breakdown of L-dopa, COMT inhibitors may, similar to MAO, exert a dopamine-sparing effect (figure 2) (Männistö & Kaakkola, 1999). COMT inhibitors such as tolcapone that act in both peripheral and central tissues may thus have enhanced therapeutic value in Parkinson's disease (Zürcher *et al*., 1990). Centrally active COMT

inhibitors should only be administered in combination with MAO inhibitors in neurodegenerative disorders since COMT inhibition may significantly enhance the level of catecholamine metabolism by MAO, consequently increasing oxidative damage by by-products of MAO catalysis (Müller *et al*., 1993).

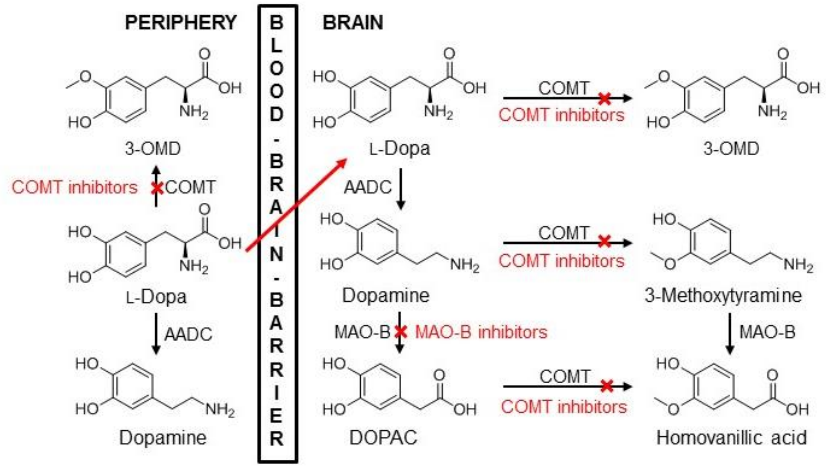


Figure 2. The metabolism of L-dopa in the periphery and central nervous system.

Based on the therapeutic roles of COMT and MAO, the present study attempts to discover compounds that exhibit dual inhibition of COMT and MAO-B among a small library of 43 structurally diverse natural compounds (Table 1). Compared to monotherapy with either inhibitor class, the combination of COMT and MAO-B inhibition may more effectively conserve depleted dopamine stores in the brain, enhance dopamine levels 'artificially' derived from exogenous L-dopa and prevent oxidative damage due to central catecholamine metabolism. Furthermore, both COMT and MAO inhibitors are known to exert antidepressant effects and, in this way, may be of future value where patients with Parkinson's disease present with depression as co-morbidity (Tom & Cummings, 1998). If fact, COMT inhibition in conjunction with either tricyclic antidepressants or MAO inhibitors, has been suggested as an innovative approach to treat depression (Männistö & Kaakkola, 1999). The multi-target-directed approach as considered here has been advocated as particularly relevant for the design of therapies for neurodegenerative disorders where multiple drugs are often co-prescribed (Cavalli *et al*., 2008).

Natural products either directly as drugs or as sources of novel lead compounds for the design and development of drugs have a long-standing tradition (Newman & Cragg, 2007; Paterson & Anderson, 2005). A large number of literature reports suggests that natural products may serve as therapeutics and lead compounds in neurodegenerative disorders (Chen & Decker, 2013). An often-observed benefit of natural compounds is their capability to

interact with multiple targets such as signalling pathways, protein folding and neuroinflammation (Essa *et al*., 2012). Natural compounds also frequently present with antioxidant activities, which is particularly relevant to oxidative stress-related disorders such as Parkinson's disease (Mazzio *et al*., 1998). Furthermore, several natural compounds have been shown to inhibit the COMT and MAO enzymes (figure 3). For example, tea catechins such as epigallocatechin, gallocatechin, catechin, epicatechin, epigallocatechin gallate, gallocatechin gallate, catechin gallate and epicatechin gallate are reported to act as potent inhibitors of COMT, and it is postulated that compounds with a similar polyphenol catechol structure will also exhibit strong COMT inhibition activity (Chen *et al*., 2005; Kang *et al*., 2013; van Duursen *et al*., 2004). Numerous MAO inhibitors from natural sources are also described in literature. For example, the naturally occurring flavonoids apigenin and diosmetin have recently been reported to act as potent reversible and competitive inhibitors of MAO-A and MAO-B (Carradori *et al*., 2016). Similarly, kaempferol, also a flavonoid, acts as a potent reversible human MAO-A inhibitor with a K_i value of 0.362 µM (Gidaro *et al*., 2016).

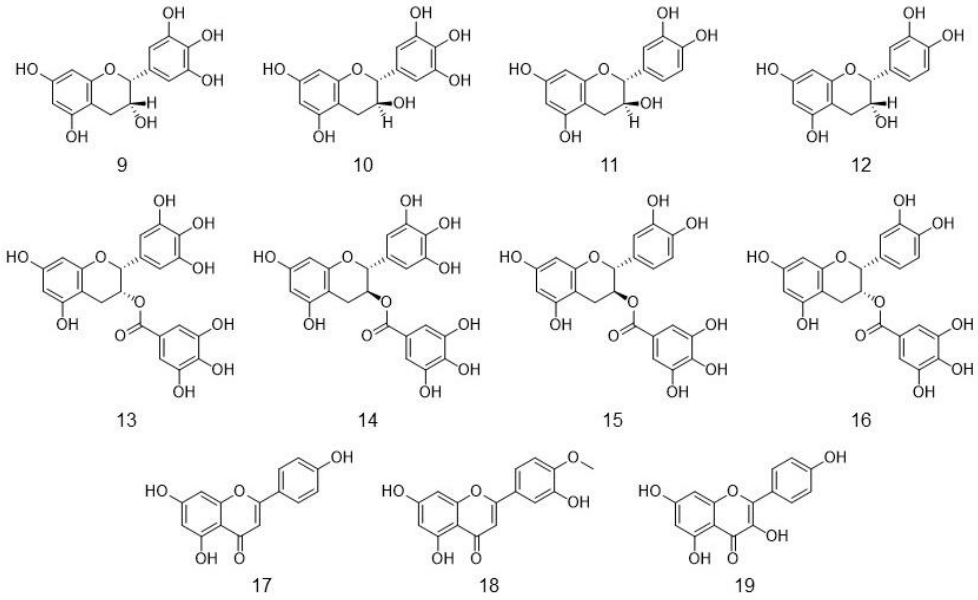


Figure 3. Natural compounds known to inhibit COMT and MAO: epigallocatechin (**9**), gallocatechin (**10**), catechin (**11**), epicatechin (**12**), epigallocatechin gallate (**13**), gallocatechin gallate (**14**), catechin gallate (**15**), epicatechin gallate (**16**), apigenin (**17**), diosmetin (**18**) and kaempferol (**19**).

In view of the limited drugs available for the symptomatic and potentially neuroprotective therapy of Parkinson's disease, this study will, for the first time, examine the possibility of discovering leads for dual COMT and MAO inhibition among naturally occurring compounds.

2. Results

2.1. MAO inhibition studies

To investigate the MAO inhibition properties of the selected natural compounds, recombinant human MAO-A and MAO-B were used as enzyme sources, and the published protocol was followed (Mostert *et al*., 2015; Novaroli *et al*., 2005). Kynuramine served as substrate for both MAO isoforms. MAO oxidises kynuramine to yield 4-hydroxyquinoline as final product. Since 4-hydroxyquinoline fluoresces in basic media, the formation of this metabolite by the action of MAO on kynuramine may be measured by fluorescence spectrophotometry. The enzyme reactions typically contained kynuramine (50 µM) and various concentrations of the test inhibitor (0.003–100 µM) in potassium phosphate buffer and were initiated with the addition of enzyme (0.0075–0.015 mg protein/ml). After 20 min incubation, the reactions were terminated with the addition of sodium hydroxide and the concentration of 4-hydroxyquinoline was measured by fluorescence spectrophotometry. Sigmoidal plots of enzyme catalytic rate versus the logarithm of inhibitor concentrations were constructed from which IC_{50} values were estimated (figure 4).

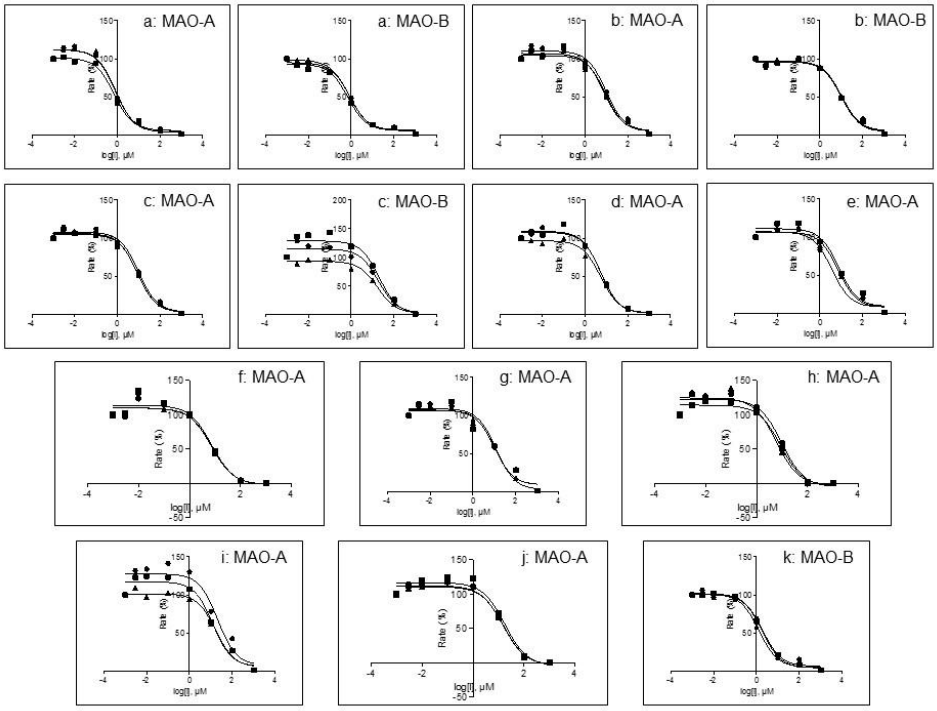
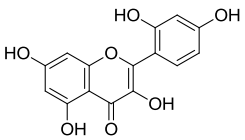
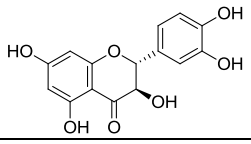
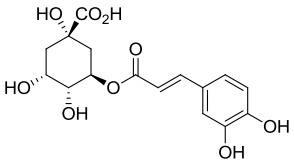
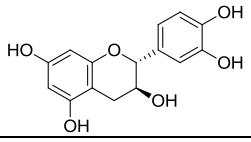
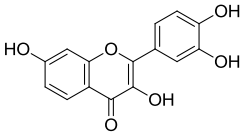
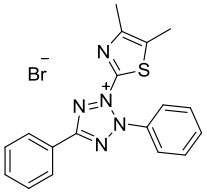


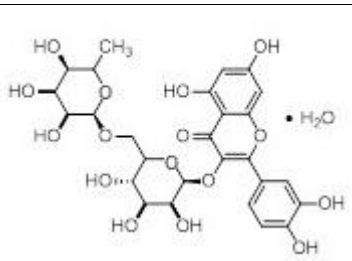
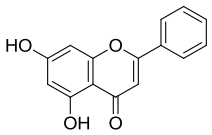
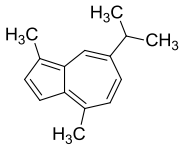
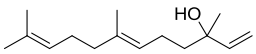
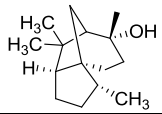
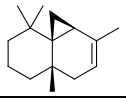
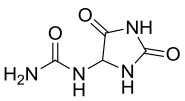
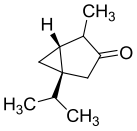
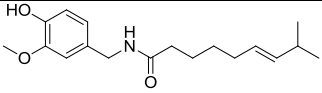
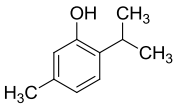
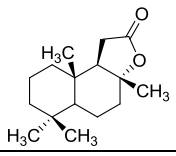
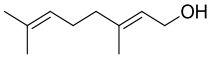
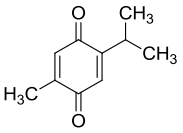
Figure 4. Sigmoidal plots for the inhibition of MAO-A and MAO-B by chrysin (a), 5-methoxypsoralen (b) and 8-methoxypsoralen (c). Sigmoidal plots for the inhibition of MAO-A by thiozolyl blue tetrazolium (d), rhein (e), fisetin (f), chrysophanol (g), alizarin (h), (+)-cedrol (i) and morin (j). Sigmoidal plot for the inhibition of MAO-B by 1,8-dihydroxy-3-methylanthraquinone (k). Each data point represents a mean ± SD of triplicate determinations.

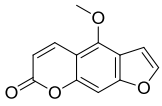
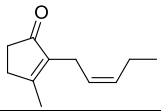
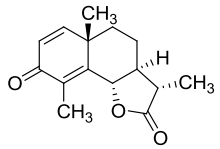
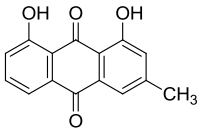
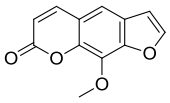
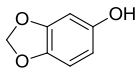
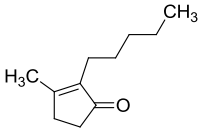
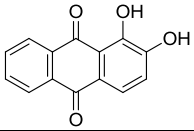
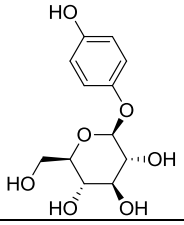
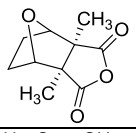
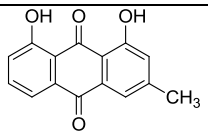
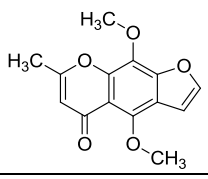
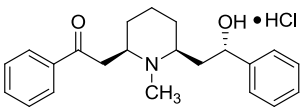
The IC_{50} values for the inhibition of the MAOs by the selected natural compounds as well as the selectivity index (SI) values are given in table 1. The SI of a given compound indicates the specificity of inhibition of the MAO-B isoform, and is the ratio of the IC_{50} value for the inhibition of MAO-A to the IC_{50} value for the inhibition of MAO-B. The results indicate that among the natural compounds, five compounds display selective inhibition of MAO-A, while six compounds exhibited specificity for MAO-B. Nine compounds inhibit both MAO-A and MAO-B although to different extents, while twenty-three compounds exhibited no inhibition towards MAO at a maximum concentration of 100 μM.

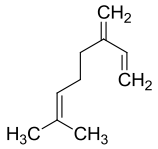
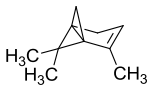
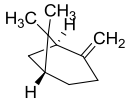
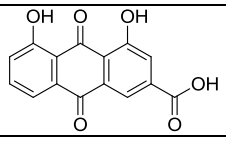
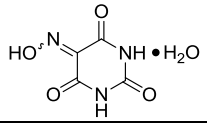
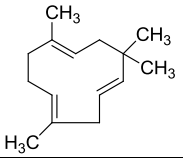
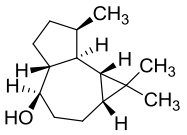
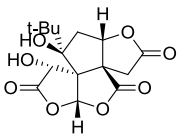
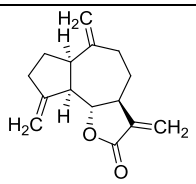
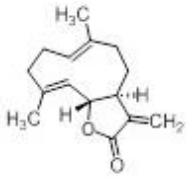
Table 1

The IC_{50} values for the inhibition of recombinant human MAO-A and MAO-B, and rat liver COMT by the selected natural compounds.

Name (% Purity)	Structure	IC_{50} (μM)[a]		SI[b]	IC_{50} (μM)[a] COMT
		MAO-A	MAO-B		
Morin (85%)		16.2 ± 1.37	59.2 ± 4.68	3.65	1.32 ± 1.55
Taxifolin (85%)		139 ± 3.13	No inhibition[c]	-	No inhibition[c]
Chlorogenic acid (95%)		158 ± 24.5	No inhibition[c]	-	6.17 ± 2.23
(+)-Catechin (99%)		200 ± 39.9	No inhibition[c]	-	0.86 ± 0.12
Fisetin (98%)		7.33 ± 0.11	74.4 ± 19.5	10.15	5.78 ± 0.46
Thiazolyl blue tetrazolium* (98%)		5.44 ± 0.13	43.5 ± 4.45	7.99	No inhibition[c]

Rutin (94%)		No inhibition[c]	No inhibition[c]	-	25.3 ± 6.25
Chrysin (97%)		0.77 ± 0.05	0.79 ± 0.09	1.03	No inhibition[c]
Guaiazulene (99%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Nerolidol (98%)		No inhibition[c]	27.6 ± 1.48	-	No inhibition[c]
(+)-Cedrol (99%)		16.7 ± 4.17	No inhibition[c]	-	No inhibition[c]
(-)-Thujopsene (97%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Allantoin (98%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
α,β-Thujone (80%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Capsaicin (95%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Thymol (98.5%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
(3aR)-Sclareolide (97%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Geraniol (98%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Thymoquinone (98%)		No inhibition[c]	47.3 ± 1.45	-	No inhibition[c]

5-Methoxypsoralen (99%)		8.76 ± 0.94	10.1 ± 0.13	1.15	No inhibition[c]
Jasmone (90%)		77.2 ± 8.41	No inhibition[c]	-	No inhibition[c]
(-)-α-Santonin (99%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
1,8-Dihydroxy-3-methylanthraquinone (98%)		No inhibition[c]	1.67 ± 0.31	-	No inhibition[c]
8-Methoxypsoralen (98%)		9.17 ± 0.79	18.6 ± 1.77	2.03	No inhibition[c]
Sesamol (98%)		No inhibition[c]	74.9 ± 8.60	-	No inhibition[c]
Dihydrojasmone (98%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Alizarin (97%)		8.16 ± 2.02	78.1 ± 5.94	9.57	0.88 ± 0.15
Arbutin (98%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Cantharidin (98%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Chrysophanol (98%)		10.8 ± 1.19	43.5 ± 15.4	4.03	No inhibition[c]
Khellin (98%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
(-)-Lobeline hydrochloride (98%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]

Myrcene (95%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
(+)-α-Pinene (99%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
(+)-β-Pinene (98.5%)		No inhibition[c]	94.5 ± 5.46	-	No inhibition[c]
Rhein (95%)		7.30 ± 0.49	97.0 ± 6.67	13.29	No inhibition[c]
Violuric acid (97%)		No inhibition[c]	90.5 ± 10.7	-	No inhibition[c]
α-Humelene (96%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
(-)-Epiglobulol (95%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Bilobalide (96%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Dehydrocostus lactone (98%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]
Costunolide (97%)		No inhibition[c]	No inhibition[c]	-	No inhibition[c]

[a] All values are expressed as the mean ± SD of triplicate determinations.

[b] Selectivity index (SI) = IC_{50}(MAO-A)/ IC_{50}(MAO-B). This value indicates specificity for the MAO-B isoform.

[c] No inhibition observed at a maximum tested concentration of 100 µM.

* Compound does not occur naturally.

The most potent inhibitor is chrysin with an IC_{50} value of 0.77 µM for MAO-A, and an IC_{50} value of 0.79 µM for MAO-B. Chrysin thus may be considered to be a nonspecific MAO

inhibitor. The most potent MAO-A inhibitor is thiazolyl blue tetrazolium with an IC_{50} value of 5.44 µM, although it is not a natural compound. Other noteworthy compounds are rhein (IC_{50} = 7.30 µM), fisetin (IC_{50} = 7.33 µM), chrysophanol (IC_{50} = 10.8 µM), alizarin (IC_{50} = 8.16 µM), 8-methoxypsoralen (IC_{50} = 9.17 µM), 5-methoxypsoralen (IC_{50} = 8.76 µM), (+)-cedrol (IC_{50} = 16.7 µM) and morin (IC_{50} = 16.2 µM), which display potent MAO-A inhibition compared to the other natural compounds of this study. The MAO-A inhibition potencies recorded here for some of the natural compounds are similar to that of the reference inhibitor, toloxatone (IC_{50} = 3.92 µM), evaluated under identical experimental conditions (Petzer *et al*., 2013). Besides chrysin, potent MAO-B inhibitors include 8-methoxypsoralen (IC_{50} = 18.6 µM), 5-methoxypsoralen (IC_{50} = 10.1 µM) and 1,8-dihydroxy-3-methylanthraquinone (IC_{50} = 1.67 µM). Although the MAO inhibition potencies of the most potent natural products are, except for chrysin, in the micromolar range, these compounds represent unique leads for the development of MAO inhibitors.

2.2. Reversibility of MAO inhibition by dialysis

The study further aimed to investigate whether the observed inhibition of MAO-A and MAO-B by selected natural products are reversible or irreversible. For this purpose, MAO-A was combined with the selected natural compounds, chrysin, morin, alizarin and fisetin, and pre-incubated for 15 min at 37 °C. The concentrations of the natural compounds were equal to 4 × IC_{50}. These mixtures were subsequently dialysed for 24 h and diluted twofold with the addition of kynuramine to yield an inhibitors concentration of 2 × IC_{50}. The enzyme reactions were incubated for 20 min, and after termination the residual enzyme catalytic rates were determined. For comparison, the residual MAO-B activities in non-dialysed enzyme-inhibitor complexes were also recorded. As negative and positive controls, respectively, similar dialysis experiments were carried out in the absence of inhibitor and presence of the irreversible inhibitors, pargyline and selegiline.

The results of the dialysis studies are given in figure 5. As shown by the graphs, both MAO-A and MAO-B activities are recovered by dialysis with the activities at 79–89% of the negative control value (100%). In contrast, inhibition of the MAOs by natural compounds persist in undialysed samples with the activities at 13–24%. Since dialysis lead to a recovery in enzyme activity, it may be concluded that the selected natural compounds are reversible MAO inhibitors. As anticipated, dialysis failed to restore enzyme activity when the MAOs were incubated in the presence of pargyline and selegiline, with residual activities at 1.9–3%.

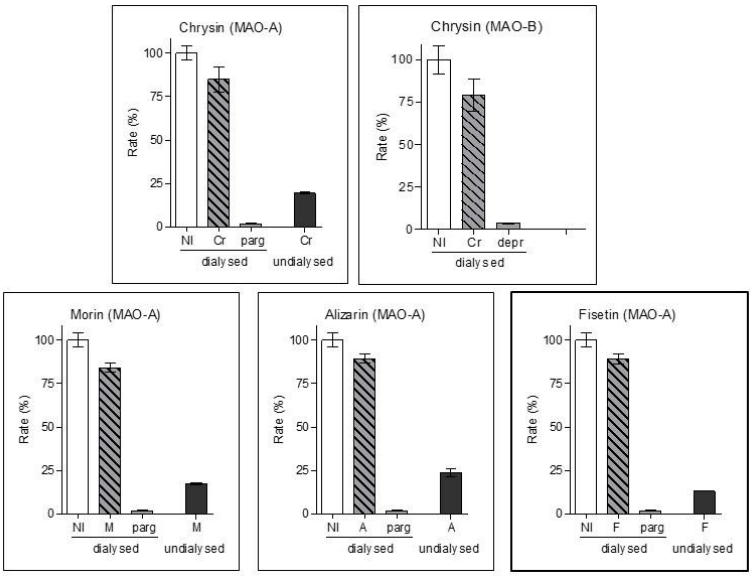


Figure 5. Reversibility of inhibition of MAO-A and MAO-B by chrysin (Cr), morin (M), alizarin (A) and fisetin (F). The MAO enzymes were pre-incubated in the presence of the natural compounds. After dialysis, the residual enzyme activities were measured. As negative and positive controls, similar dialysis of the MAOs was carried out in the absence of inhibitor and presence of irreversible MAO inhibitors (pargyline and selegiline), respectively. For comparison, the MAO activities of undialysed mixtures of the MAOs and the test inhibitors were also measured.

2.3. Mode of inhibition studies

To provide further support that chrysin is a reversible inhibitor of both MAO-A and MAO-B, and morin, alizarin and fisetin are reversible inhibitors of MAO-A, the modes of inhibition were examined. For this purpose, sets of Lineweaver-Burk plots were constructed for each selected inhibitor (figure 6). The enzyme catalytic rates were recorded at 8 different kynuramine concentrations (15–250 µM) in the absence of inhibitor, and presence of five different concentrations (¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}) of the selected natural compounds. Inspection of the Lineweaver-Burk plots suggests that chrysin inhibits both MAO-A and MAO-B, and morin, alizarin and fisetin inhibits MAO-A competitively since the plots are linear and intersect at the y-axis. These findings lend further support that these naturally occurring compounds acts reversibly with the active sites of MAO-A and MAO-B, respectively. From analyses of the Lineweaver-Burk plots, the following K_i values were estimated for the inhibition of MAO-A: chrysin (0.69 µM), morin (6.49 µM), alizarin (3.26 µM), fisetin (2.12 µM). For the inhibition of MAO-B by chrysin, a K_i of 1.53 µM is estimated.

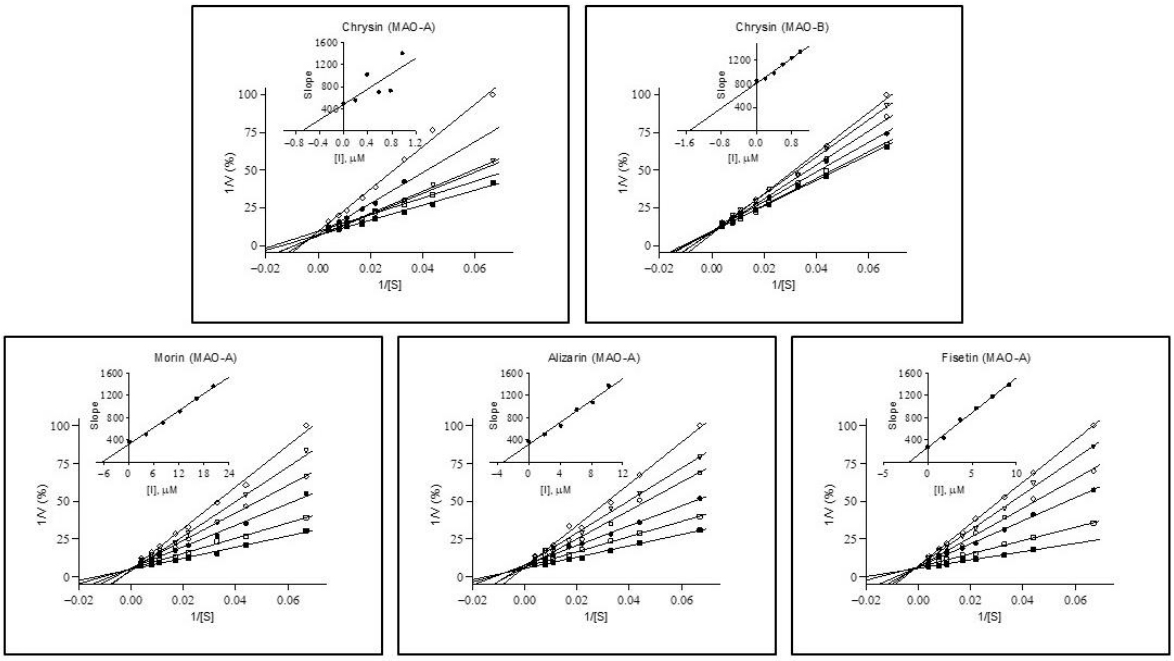


Figure 6. Lineweaver-Burk plots of the catalytic activities of the MAOs recorded in the absence (filled squares) and presence of various concentrations of chrysin, morin, alizarin and fisetin. For these studies the concentrations of the natural compounds were ¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}. The inset is a graph of the slopes of the Lineweaver-Burk plots versus inhibitor concentration.

2.4. COMT inhibition studies

To evaluate the natural compounds as potential inhibitors of COMT, the soluble fraction obtained from rat liver homogenate was used as enzyme source (Hirano *et al*., 2005; Zhu *et al*., 2010). A protocol for the measurement of COMT activity in the absence and presence of test inhibitors was developed by modification of literature procedures (Aoyama *et al*., 2005). To measure COMT activity, (-)-norepinephrine was used as substrate and normetanephrine, generated by the methylation of (-)-norepinephrine, was quantitated by high-performance liquid chromatography (HPLC) with fluorescence detection. Typical enzyme reactions contained (-)-norepinephrine (250 µM), $MgCl_2$, S-adenosyl-L-methionine and the test inhibitor at various concentrations (0.01–100 µM). The reactions were initiated with the addition of the COMT enzyme (30 mg protein/ml) and incubated for 15 min. After the reactions were terminated with the addition of perchloric acid, normetanephrine generated by COMT was measured. A chromatogram routinely obtained is provided as example (figure 7). Sigmoidal plots of enzyme catalytic rate versus the logarithm of inhibitor concentration were constructed from which IC_{50} values were estimated (figure 8).

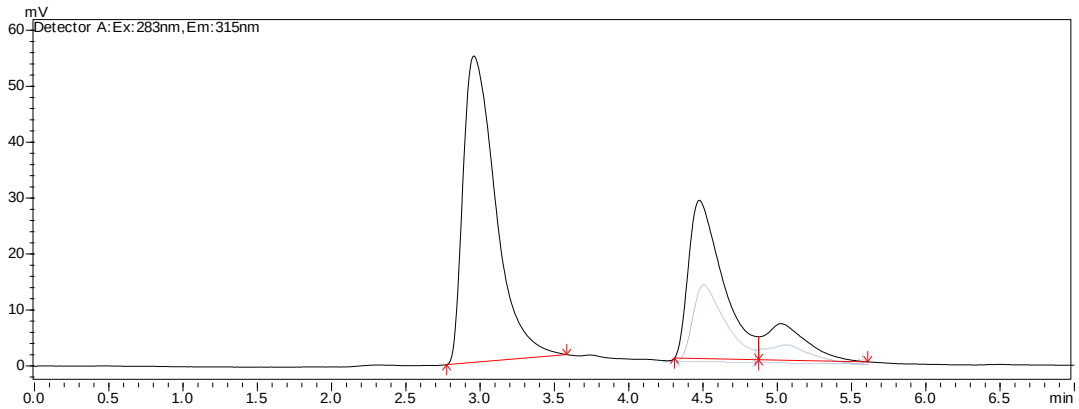


Figure 7. A chromatogram routinely obtained for the detection and quantitation of normetanephrine generated through the COMT-catalysed methylation of (-)-norepinephrine. The chromatogram in black represents an enzymatic reaction carried out in the absence of inhibitor, while the blue chromatogram represents an enzymatic reaction with an inhibitor concentration of 0.1 µM.

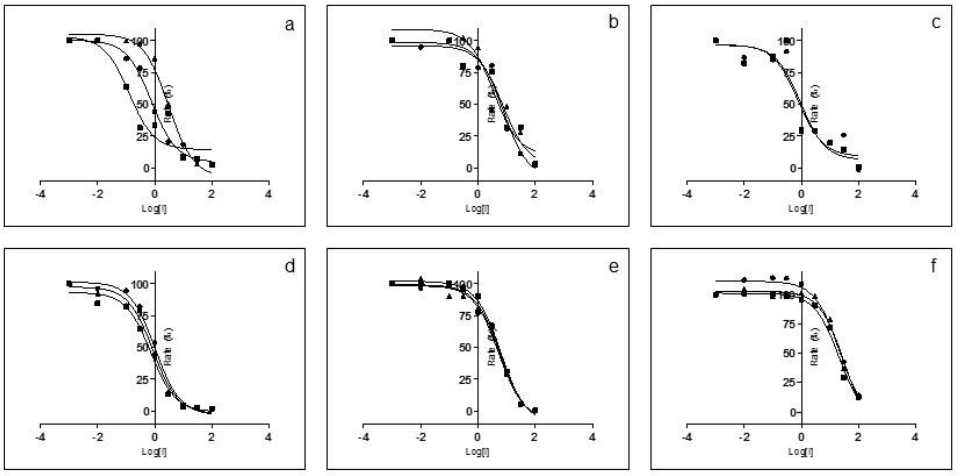


Figure 8. Sigmoidal plots for the inhibition of COMT by morin (a), chlorogenic acid (b), (+)-catechin (c), alizarin (d), fisetin (e) and rutin (f). Each data point represents a mean ± SD of triplicate determinations.

The IC_{50} values for COMT inhibition by the natural compounds are given in table 1. The results indicated that among the selected natural compounds, six are noteworthy inhibitors of COMT. These are morin (IC_{50} = 1.32 µM), chlorogenic acid (IC_{50} = 6.17 µM), (+)-catechin (IC_{50} = 0.86 µM), alizarin (IC_{50} = 0.88 µM), fisetin (IC_{50} = 5.78 µM) and rutin (IC_{50} = 25.3 µM). The most potent COMT inhibitors of the study are thus (+)-catechin and alizarin, which exhibit IC_{50} values in the submicromolar range. These compounds are, however, significantly

less potent than the reference COMT inhibitors, tolcapone and entacapone, which exhibit IC_{50} values of 0.26 µM and 0.25 µM, under these experimental conditions. From the results it is evident that the structural requirements for COMT inhibition are strict, and in the present study limited to compounds with the catechol structure, which is present in all six active inhibitors.

3. Discussion

The present study explores the possibility of discovering compounds that exhibit dual COMT and MAO-B inhibition among forty-two selected natural compounds. A number of compounds with potent MAO-B inhibition were discovered as exemplified by chrysin, a nonspecific MAO inhibitor. Unfortunately, this compound did not inhibit COMT. Among the natural compounds, potent COMT inhibitors were also found with (+)-catechin and alizarin representing the most potent inhibitors. It was evident that the active COMT inhibitors all possessed the catechol structure which showed the strict structural requirements for COMT inhibition compared to MAO inhibition. Although no noteworthy dual inhibition of COMT and MAO-B was observed for any of the natural compounds, morin, alizarin and fisetin inhibited both COMT and MAO-A. Considering that MAO-A also metabolises dopamine in the brain, such compounds may possess enhanced value in Parkinson's disease compared to compounds that inhibit exclusively the COMT enzyme (Glover *et al*., 1977; Tipton *et al*., 2004; Youdim *et al*., 1988). Furthermore, it is well known that the inhibition of MAO-A results in antidepressant effects which may alleviate depression, which is a co-morbid disorder of Parkinson's disease (Youdim *et al*., 2006).

In conclusion, patients with advanced Parkinson's disease may benefit significantly from the use of a dual COMT and MAO inhibitor. In Parkinson's disease, monotherapy with a COMT inhibitor has minimal beneficial effect and COMT inhibitors thus have to be administered in conjunction with another inhibitor such as a MAO inhibitor (Lees *et al*., 2009; Miyasaki, 2006; Rascol *et al*., 2002). It is noteworthy that the evaluation of natural compounds as potential dual inhibitors of COMT and MAO underscores the possibility of discovering or designing such multi-target-directed inhibitors. Natural compounds are an inexhaustible source of lead compounds that may be structurally modified to enhance the inhibition potencies.

4. Experimental section

4.1. Chemicals and instrumentation

Unless otherwise indicated, all starting materials and solvents were obtained from Sigma-Aldrich and were used without further purification. For the MAO inhibition studies, a Varian Cary Eclipse fluorescence spectrophotometer was employed. Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg protein/ml) and kynuramine dihydrobromide were obtained from Sigma-Aldrich. For the COMT inhibition studies, (-)-norephinephrine, S-adenosyl-L-methionine and $MgCl_2$ were purchased from Sigma-Aldrich. A Shimadzu Ultra Fast Liquid Chromatograph (UFLC) prominence HPLC system, equipped with a Shimadzu communications bus module (CBM-20A), Shimadzu degasser (DGU-$20A_5$), Shimadzu pump (LC-20AD), Shimadzu auto-sampler (SIL-20AC), Shimadzu column oven (CTO-20A) and Shimadzu RF-10AXL fluorescence detector, with a USP L1 Luna C18 column (250 × 4.6 mm, 5 µm) (Phenomenex, Torrance, CA) was used for chromatographic separation and detection of normetanephrine. The processing of the COMT data was done using LabSolutions.

4.2. Recombinant human MAO-A and MAO-B inhibition studies

IC_{50} values for the inhibition of the MAOs were measured as described previously (Mostert *et al*., 2015). Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg protein/ml) served as enzyme sources and kynuramine served as substrate. All enzyme reactions were carried out in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl) to a volume of 200 µl and contained kynuramine (50 µM) and the test inhibitors (0.003–100 µM). Stock solutions of the inhibitors were prepared in DMSO and added to the reactions to yield a final concentration of 4% (v/v). The reactions were incubated for 30 min at 37 °C and were subsequently initiated with addition of MAO-A and MAO-B to yield a final concentration of 0.0075 mg protein/ml and 0.015 mg protein/ml, respectively. After a further 20 min of incubation at 37 °C, the reactions were terminated by the addition of 80 µl NaOH (2 N) and 4-hydroxyquinoline was quantitated by fluorescence spectrophotometry (λ_{ex} = 310 nm; λ_{ex} = 400 nm). For this purpose, a calibration curve was constructed with authentic 4-hydroxyquinoline (0.047–1.50 µM). After the enzyme catalytic rates were calculated, sigmoidal plots of rate versus the logarithm of the concentration of the test inhibitor were constructed by fitting the rate data to a one site competition model incorporated into the Prism software package (GraphPad). From these plots, IC_{50} values were estimated in triplicate and expressed as mean ± 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. The selected natural compounds, at concentrations equal to 4 × IC_{50} were pre-incubated with recombinant human MAO-A or MAO-B (0.03 mg protein/ml) for 15 min at 37 ºC. For this purpose, potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl) containing 5% sucrose served as reaction medium. Control incubations were conducted in the absence of inhibitor and presence of the irreversible inhibitors pargyline [IC_{50}(MAO-A) = 13 µM] and selegiline [IC_{50}(MAO-B) = 0.079 µM]. DMSO (4%) was added as co-solvent, and the final volume of the incubations was 0.8 ml. The enzyme-inhibitor complexes were dialysed for 20–25 h at 4 °C in 80 ml buffer, with the buffer being replaced at 3 h and 7 h after the start of dialysis. To determine the residual enzyme activity, the reactions were diluted twofold with the addition of kynuramine to yield a final enzyme concentration of 0.015 mg protein/ml and a final kynuramine concentration of 50 µM. The reactions were incubated for 20 min and terminated with the addition of 400 µl NaOH (2 N) and 1000 µl deionised water. The concentrations of the MAO-generated 4-hydroxyquinoline were measured spectrofluorometrically at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. These reactions were carried out in triplicate and the residual catalytic rates were expressed as mean ± SD. For comparison, non-dialysed enzyme-inhibitor complexes were maintained at 4 °C for the same period and the residual enzyme activity was measured spectrofluorometrically as described above.

4.4. Mode of inhibition studies

To evaluate the mode of inhibition of MAO-A and MAO-B by the selected natural compounds, Lineweaver-Burk plots were constructed. The enzymatic reactions were conducted in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl 20.2 mM) to a final volume of 500 µl. The reactions contained kynuramine as substrate (15–250 µM final concentration), MAO-A or MAO-B (0.015 mg/ml final concentration), the selected compounds at various concentrations (¼ × IC_{50}, ½ × IC_{50}, ¾ × IC_{50}, 1 × IC_{50} and 1¼ × IC_{50}) and DMSO (4%) as co-solvent. Control incubations were conducted in the absence of inhibitor. The reactions were incubated at 37 °C for 20 min and terminated by the addition of 400 µl NaOH (2 N) and 1000 µl deionised water. The initial rates by which MAO catalyses the oxidation of kynuramine were determined spectrofluorometrically as described in previous sections.

4.5. COMT inhibition studies

Sprague Dawley rats were bred, supplied and housed at the Vivarium at the Potchefstroom campus of the North-West University (NWU) (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019) of the Preclinical Drug Development Platform of the NWU. Experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) at the NWU. All animals were maintained, and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa, and complied with national legislation. Ethical approval for the collection and use of animal tissue was obtained from the Research Ethics Committee, NWU. Ethics approval numbers: NWU-00438-16-S5 and NWU-00267-16-A5.

The soluble fraction of rat liver homogenate was prepared as reported in literature (Hirano *et al*., 2005; Zhu *et al*., 2010). For this purpose, homogenisation was carried out in 25 mM sodium phosphate buffer (pH 7.8, containing 0.5 mM dithiothreitol). Protein determination was carried out by the method of Bradford (Bradford, 1970). The enzyme reactions were carried out to a final volume of 125 µl and contained $MgCl_2$ (2 mM), (-)-norepinephrine (250 µM), S-adenosyl-L-methionine (200 µM) and the test inhibitor at various concentrations (0.01–100 µM). Inhibitor stock solutions were prepared in DMSO and added to the reactions to yield a final DMSO concentrations of 4%. The COMT enzyme was added to yield a final enzyme concentration of 30 mg protein/ml, and after 15 min incubation at 37 °C, the enzyme reactions were terminated with the addition of 12.5 µl perchloric acid (1 M). The samples were centrifuged, and the supernatants were analysed by HPLC with fluorescence detection (λ_{ex} 283 nm; λ_{em} 315 nm) (Aoyama *et al*., 2005). Quantification of the COMT-generated normetanephrine was carried out with a calibration curve (0.5–90 µM). After calculating the enzyme catalytic rates, sigmoidal plots of rate versus logarithm of inhibitor concentration were constructed by fitting to a one site competition model incorporated into the Prism software package (GraphPad). The IC_{50} values were estimated in triplicate from these plots and expressed as mean ± SD.

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Chapter 5

Article 3

The evaluation of structurally diverse monoamine oxidase inhibitors as potential dual inhibitors of catechol-O-methyltransferase

Idalet Engelbrecht,[1] Jacobus P. Petzer,[1] Anél Petzer [1,*]

[3.] *Pharmaceutical Chemistry, School of Pharmacy and Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa*

*Corresponding author: Anél Petzer, Tel.: +27 18 2994464, fax: +27 18 2994243
E-mail address: 12264954@nwu.ac.za

Running title: Dual MAO and COMT inhibition

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Abstract

Parkinson's disease is a neurodegenerative disorder and occurs when dopaminergic neurons located in the substantia nigra pars compacta degenerate. The development of the disease cannot be prevented since the clinical features resemble those of normal aging and the aetiology of the disease is not clearly defined. The drugs that are available for the treatment of Parkinson's disease are essentially antisymptomatic, with no convincing neuroprotective effects. Levodopa (L-dopa) is considered to be the gold standard of Parkinson's disease treatment although it has certain limitations. The efficacy of L-dopa depends on its conversion to dopamine in the brain. However, extensive metabolism by aromatic-L-amino acid decarboxylase (AADC) and catechol-O-methyltransferase (COMT) in the periphery diminishes the amount of L-dopa that reaches the brain. Furthermore, dopamine is metabolically deactivated by either oxidative deamination by monoamine oxidase (MAO) or methylation by COMT. The inhibition of MAO and COMT is therefore expected to increase central dopamine levels artificially derived from exogenous L-dopa,

while also conserving endogenous dopamine already present in the brain. In the search for novel treatment strategies for Parkinson's disease, the dual inhibition of MAO and COMT may represent a viable strategy. The present study attempts to discover dual MAO and COMT inhibitors by screening of a series of structurally diverse MAO inhibitors in our in-house library for potential inhibitory action against rat liver COMT. This approach may identify new lead compounds for the future design of dual MAO and COMT inhibitors. Although none of the library compounds exhibited inhibition activity towards COMT, this study concludes that the structural requirements for COMT inhibition is extremely strict compared to those for MAO inhibition, and the presence of the catechol moiety is almost an absolute requirement for potent COMT inhibition.

1. Introduction

Parkinson's disease was first described in 1817 and is today regarded as the second most common neurodegenerative disorder of the aging brain (Dauer & Przedborski, 2003; Fahn & Przedborski, 2000). The prevalence of Parkinson's disease is 120–190/100000 of the human population (MacDonald *et al*., 2000; Niccolini *et al*., 2015). The clinical syndrome presents as a tetrad of symptoms namely tremor at rest, slowness of movement or bradykinesia, rigidity and postural instability or gait impairment (Braak *et al*., 2003; Foley *et al*., 2000). The primary pathology of Parkinson's disease is the death of dopaminergic neurons situated in the substantia nigra pars compacta, which results in depletion of striatal dopamine (Kaiser *et al*., 2000; Le & Jankovic, 2001; Olanow, 2004; Young & Penney, 1993). The clinical manifestation of the disease only occurs when approximately 60% of the neurons in the substantia nigra pars compacta have died and 70% of responsiveness to dopamine has been lost (German et al., 1989; Ma *et al*., 2002; Uhl *et al*., 1985). The most important risk factor for Parkinson's disease is aging (Bower *et al*., 1999; de Rijk *et al*., 1995; Lees *et al*., 2009). 95% of Parkinson's disease cases are considered to be sporadic with no genetic linkage (Dauer & Przedborski, 2003). Environmental as well as genetic factors may however contribute to the aetiology and subsequent pathogenesis of Parkinson's disease (Béné *et al*., 2009).

The cause of neuronal death in Parkinson's disease is still not established, however, the major biochemical processes involved are oxidative stress and mitochondrial dysfunction (Blum *et al*., 2001). Furthermore, dopamine may act as an endogenous neurotoxin since its metabolism by monoamine oxidase (MAO) in the brain produces hydrogen peroxide (H_2O_2), an oxygen species which may act as a substrate in the Fenton reaction to produce the highly reactive and destructive hydroxyl radical. Furthermore, dopamine auto-oxidation in the brain

also produces hydrogen peroxide (and possibly superoxide radicals) as well as dopamine quinone, which reacts with cysteine-residues resulting in protein damage (figure 1) (Dauer & Przedborski, 2003; Graham, 1978). In this regard, dopamine may render dopaminergic neurons particularly susceptible to oxidative attack (Dauer & Przedborski, 2003). The formation of reactive oxygen species and the subsequent free radical-mediated oxidative damage of cell membranes, deoxyribonucleic acid (DNA) and proteins is thus central to the mechanism of neuronal damage and death in Parkinson's disease (Finkel & Holbrook, 2000; Finkel, 2005; Hussain *et al*., 2003; Qian *et al*., 2011; Rice-Evans & Diplock, 1993).

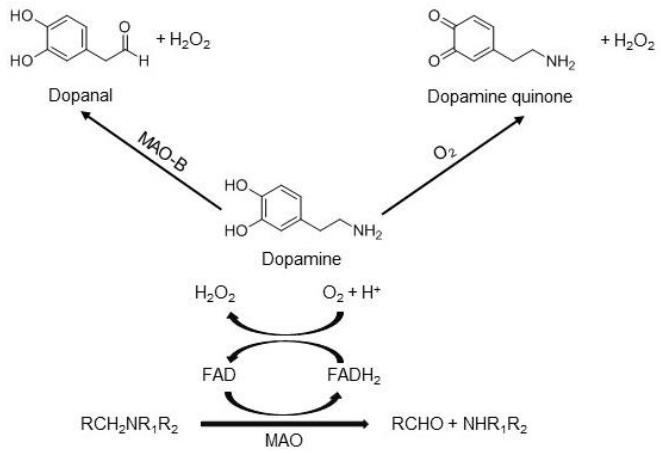


Figure 1. The metabolic route of dopamine and the formation of toxic by-products.

The treatment options currently available for Parkinson's disease focuses on symptomatic treatment rather than the prevention of dopaminergic neuron degeneration (Ahlskog & Muenter, 2001). The molecular events leading to neurodegeneration in Parkinson's disease is not clearly understood, which presents an obstacle for developing neuroprotective therapies. After the discovery of dopamine in the mammalian brain, novel treatment strategies for Parkinson's disease were rapidly introduced (Dauer & Przedborski, 2003). These therapies focus on restoring striatal dopamine levels through levodopa (L-dopa) administration, dopamine receptor stimulation by dopamine agonists or by inhibiting dopamine reuptake and metabolism (figure 2) (Lees, 2005; LeWitt & Nyholm, 2004). The most important treatment options currently available for Parkinson's disease include L-dopa, dopamine agonists, aromatic-L-amino acid decarboxylase (AADC) inhibitors, catechol-O-methyltransferase (COMT) inhibitors, anticholinergic agents, MAO inhibitors and amantadine (Laurencin *et al*., 2016).

The discovery of L-dopa in 1967 transformed the treatment of Parkinson's disease and is still considered the gold standard of Parkinson's disease treatment (Barbeau *et al*., 1961; Birkmayer & Hornykiewicz, 1961; Cotzias *et al*., 1969; Sano, 1960). In the initial stages of the disease, L-dopa markedly reduces the motor symptoms of Parkinson's disease and improves the patient's quality of life (Colosimo & De Michele, 1999; Fahn, 1974). L-Dopa therapy, however, leads to the development of daily motor fluctuations in mobility and involuntary movements termed dyskinesia (Olanow & Jankovic, 2005). Dyskinesia in particular reduce patient function, quality of life and increase treatment costs (Tse, 2006).

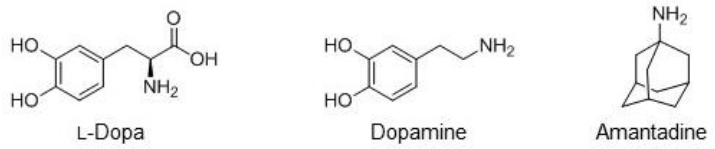


Figure 2. The chemical structures of L-dopa, dopamine and amantadine.

L-Dopa is usually administered in conjunction with dopamine agonists for controlling motor symptoms in Parkinson's disease, while COMT inhibitors and MAO inhibitors are used as adjuvants to control wearing-off symptoms (Huot *et al*., 2016; Jenner *et al*., 2009; Romrell *et al*., 2003). The development of motor complications and dyskinesia with L-dopa treatment, however, is inevitable, and only by introducing a dopamine agonist early in the treatment, as well as judicious use of L-dopa, the incidence of severe dyskinesia can be reduced (Constantinescu *et al*., 2007; Hauser *et al*., 2007; Hely *et al*., 2005). MAO-B inhibitors also represents a useful treatment option and may be used as monotherapy in the initial stages of Parkinson's disease or as adjunctive therapy to L-dopa (Jankovic & Stacy, 2007; Weinreb *et al*., 2010). Certain MAO-B inhibitors such as rasagiline and selegiline are proposed to delay disease progression, enhance life span and have possible disease-modifying effects (figure 3) (Adeyemo *et al*., 1993; Shoulson, 1998). By inhibition of the MAO-catalysed metabolism of dopamine, MAO-B inhibitors increase dopamine concentrations in the brain, which reduces the formation of injurious by-products, most notably hydrogen peroxide. The potential side effects related to the irreversible mechanism of inhibition of the abovementioned MAO-B inhibitors (selegiline and rasagiline), underscore the need for the discovery of novel reversible and selective MAO-B inhibitors (Gaspar *et al*., 2011; Rezak, 2007; Riederer *et al*., 2004).

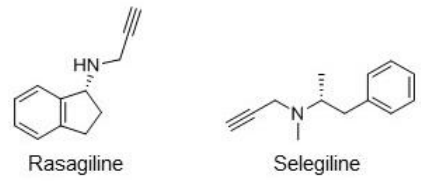


Figure 3. The chemical structures of the MAO-B inhibitors, rasagiline and selegiline.

Second generation COMT inhibitors are also used as adjunctives to L-dopa in the treatment of Parkinson's disease (figure 4) (Calne, 1993; Männistö & Kaakkola, 1989). Entacapone is a short-acting inhibitor that inhibits COMT in the periphery, while tolcapone is a more potent, longer acting COMT inhibitor which functions in both the peripheral and central tissues (Assal *et al*., 1998; Keranen *et al*., 1994; Männistö & Kaakkola, 1999). Nebicapone is longer acting compared to entacapone, while nitecapone is a tight-binding peripheral COMT inhibitor. Some adverse effects limit the clinical use of entacapone and tolcapone. The most commonly observed dopaminergic adverse effect is the worsening of L-dopa-induced dyskinesia, while the most common non-dopaminergic adverse effect is diarrhoea (Kiss *et al*., 2010; Männistö & Kaakkola, 1999; Robinson *et al*., 2012). Opicapone, another second generation COMT inhibitor, is currently undergoing phase III clinical trials (Ferreira *et al*., 2012; Kiss & Soares-da-Silva, 2014; Lees *et al*., 2012).

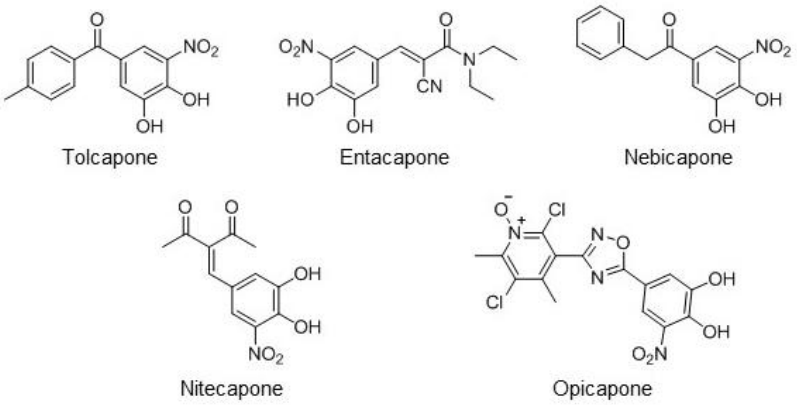


Figure 4. The chemical structures of second generation COMT inhibitors.

Quality of life is a patient-recorded outcome that is an important variable which reflects the disease impact and treatment success from the patients' perspective. An assessment of the quality of life indicates the beneficial effect of treatment strategies (Martinez-Martin *et al*., 2015). With COMT inhibitor therapy, some studies indicate that the quality of life of patients with Parkinson's disease significantly increases, and patients with motor complications

benefit more from the use of tolcapone, while nonfluctuating patients tolerate entacapone better (Baas *et al*., 1997; Waters *et al*., 1997; Welsh *et al*., 1995).

2. The rationale for multi-target-directed inhibitors of MAO and COMT

Two enzymes are primarily responsible for the initial metabolic inactivation of catecholamines in the blood and tissue of mammals, namely MAO and COMT (Hirsch, 1994; Yan *et al*., 2002). Since catecholamines such as dopamine is involved in motor function in Parkinson's disease, the inhibition of MAO and COMT, respectively or dual inhibition of these enzymes, may be a novel treatment strategy. In the treatment of Parkinson's disease, the administration of COMT inhibitors alone have minimal beneficial effect, and therefore should be administered in combination with another antiparkinsonian drug such as a MAO inhibitor (Miyasaki, 2006; Orth & Schapira, 2002; Rascol *et al*., 2002). Besides conserving the dopamine supply in the brain and boosting dopamine levels derived from exogenously administered L-dopa, the inhibition of MAO may potentially exert a neuroprotective effect. As mentioned, the inhibition of MAO may be neuroprotective by decreasing oxidative damage as a result of the formation of injurious by-products generated by normal MAO-catalysed metabolism (Mazzio *et al*., 1998). Peripheral COMT inhibitors, in turn, prolong the half-life of L-dopa and decrease the formation of 3-O-methyldopa, the product of L-dopa metabolism by COMT. 3-O-Methyldopa competes with L-dopa for uptake at the blood-brain barrier and thus reduces the penetration of L-dopa into the brain (Learmonth *et al*., 2004; Nissinen *et al*., 1992). Several clinical observations have shown that poor response to L-dopa therapy is associated with high plasma levels of 3-O-methyldopa (Tohgi *et al*., 1991). Furthermore, the inhibition of centrally located COMT may further potentiate the effect of L-dopa by diminishing the degradation of L-dopa-derived dopamine (Männistö & Kaakkola, 1999).

In L-dopa therapy, the dual inhibition of MAO-B and COMT may thus be greatly beneficial. By reducing the metabolism of both L-dopa and dopamine, the dual inhibition of MAO-B and COMT may allow for a reduction of the L-dopa dose required for relief of motor symptoms. Parkinson's disease is commonly associated with co-morbid disorders such as depression (Paumier *et al*., 2015; Ravina *et al*., 2007). In this regard, the dual inhibition of MAO and COMT will be of further value. It has previously been established that the administration of a MAO-B inhibitor (such as selegiline) in combination with a COMT inhibitor markedly increased catecholamine levels in the brain, which may lead to an antidepressant effect (Tom & Cummings, 1998). The inhibition of COMT may potentially extend the action of catechol-like compounds such as endogenous catecholamines (specifically noradrenaline).

This could result in enhanced noradrenaline levels, subsequently relieving the symptoms of depression. Furthermore, it has been concluded that the administration of COMT inhibitors in conjunction with either tricyclic antidepressants or MAO inhibitors, presents a novel way to treat depression (Männistö & Kaakkola, 1999). While MAO-A inhibitors are established therapy for depression, MAO-B inhibitors such as selegiline has also recently been shown to be effective in this regard (LeWitt & Taylor, 2008; Tom & Cummings, 1998).

3. The selection of known MAO inhibitors for evaluation as potential COMT inhibitors

In the search for multi-target-directed drugs for the treatment of Parkinson's disease, the natural methylxanthine derivative, caffeine, has emerged as a valid lead compound (Fredholm et al., 1999; Petzer *et al*., 2009). Caffeine (**1**) is a moderately potent non-selective adenosine receptor antagonist with weak inhibition activity towards MAO-A and MAO-B (Baraldi *et al*., 2003; Müller *et al*., 1997). Appropriate structural modification of caffeine, however, yielded numerous compounds with enhanced MAO-B inhibition potency as well as compounds endowed with more potent and selective adenosine-receptor antagonistic properties (Baraldi *et al*., 2003; Booysen *et al*., 2011; Chen *et al*., 2001; Mostert *et al*., 2012; Petzer *et al*., 2003; Petzer *et al*., 2009; Pretorius *et al*., 2008; Strydom *et al*., 2010; Strydom *et al*., 2011; van den Berg *et al*., 2007). Literature reports that the majority of A_{2A} antagonists are 1,3-disubstituted xanthinyl analogues substituted on C8 with a (*E*)-styryl moiety (Chen *et al*., 2001; Petzer *et al*., 2003; Vlok *et al*., 2006). Based on this, (*E*)-8-styrylcaffeine derivatives were included in the current study with the aim of discovering dual MAO and COMT inhibitors (figure 5). Utilising (*E*)-8-(3-chlorostyryl)caffeine (CSC, **2**) as a lead compound, previous studies have designed and evaluated several (*E*)-8-styrylxanthinyl analogues as MAO inhibitors (Booysen *et al*., 2011; Mostert *et al*., 2012; Petzer *et al*., 2003; Petzer *et al*., 2009; Pretorius *et al*., 2008; Strydom *et al*., 2010; Strydom *et al*., 2011; van den Berg *et al*., 2007; van der Walt *et al*., 2009). Among these, the following were included in the present study: (*E*)-7-methyl-8-styrylxanthines (**2**–**4**) (Petzer *et al*., 2003), purino[7,8-*c*]quinozoline-8,10(9*H*,11*H*)dione analogue (**5**) (van den Berg *et al*., 2007), 8-phenylcaffeine (**6**) (Pretorius *et al*., 2008), (*E,E*)-8-(4-phenylbutadien-1-yl)caffeine (**7**), 1,3-diethyl-(*E,E*)-8-(4-phenylbutadien-1-yl)caffeine (**8**) (Petzer *et al*., 2009), 8-chlorocaffeine (**9**) (Strydom, 2009), 8-benzyloxycaffeine (**10**) (Strydom *et al*., 2010), 8-[2-(benzyloxy)ethoxy]caffeine (**11**), 8-{2-(4-chlorophenoxy)ethoxy]caffeine (**12**) (Strydom *et al*., 2011), 8-(benzylsulfanyl)caffeine (**13**), 8-[(2-phenylethyl)sulfanyl]caffeine (**14**), 8-[(2-phenylethyl)amino]caffeine (**15**), 8-[methyl-(2-phenylethyl)amino]caffeine (**16**) (Booysen *et al*., 2011), 8-(benzylsulfinyl)caffeine (**17**) (Mostert *et al*. 2012) and 8-sulfonylcaffeine (**18**) (Mentz, 2013). A novel compound

structurally related to 8-chlorocaffeine, 8-chloro-1,3-diethyl-7-methylxanthine (**19**) (unpublished data) has also been included in the present study to be evaluated as a potential inhibitor of COMT.

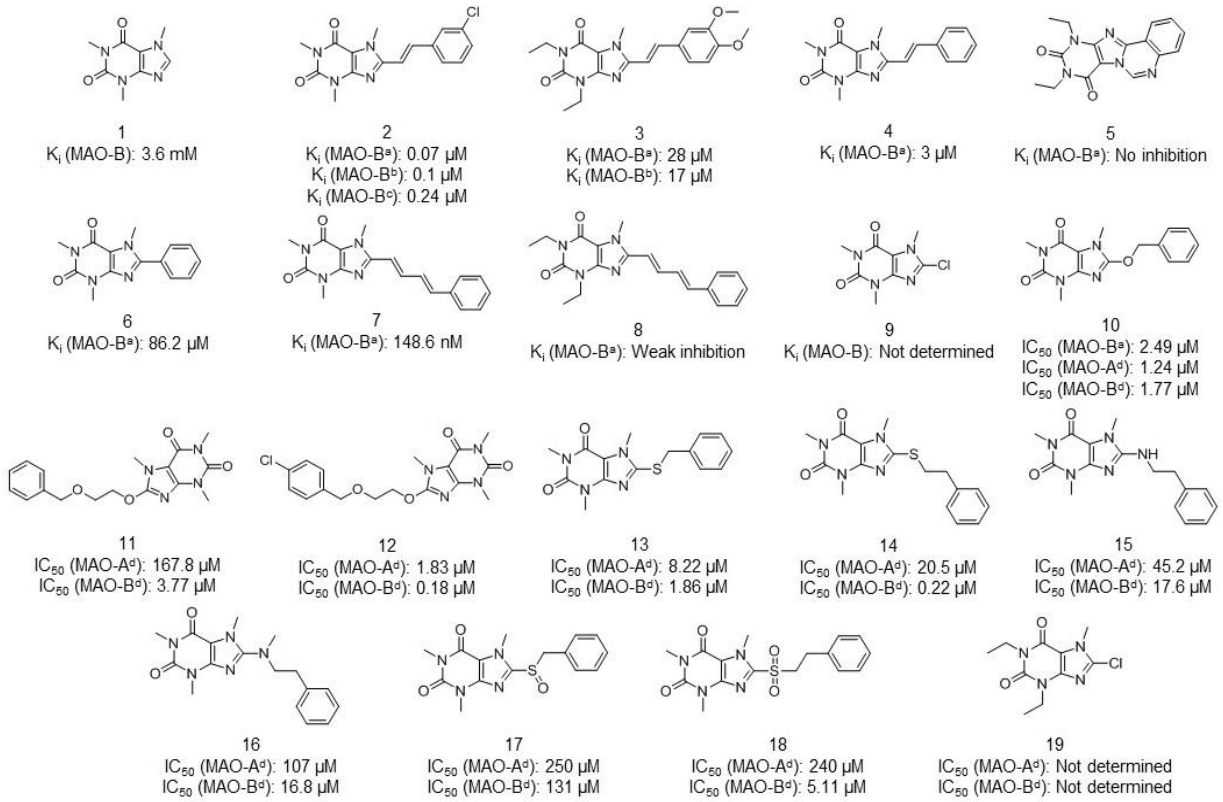


Figure 5. The chemical structures of caffeine and caffeine analogues which were selected for evaluation as potential COMT inhibitors in the present study ([a] baboon liver mitochondria; [b] mouse brain mitochondria; [c] human liver mitochondria; [d] recombinant human MAO-A and MAO-B).

Literature reports that 2-styrylbenzimidazoles act as reversible selective inhibitors of MAO-B (Petzer *et al*., 2003; van den Berg *et al*., 2007). Certain compounds in this chemical class were thus selected and evaluated as COMT inhibitors (figure 6). The compounds that were selected are 2-styryl-1*H*-benzimidazole (**20**) and 2-styryl-1-methylbenzimidazole (**21**) (Petzer *et al*., 2003). An anilide derivative (**22**), which has some structural resemblance to benzimidazole was also included (Legoabe *et al*., 2011). Another novel structure, related to the benzimidazole class, 2-[(*E*)-2-phenylethenyl]-3*H*-imidazo[4,5*c*]pyridine (**23**), has previously been synthesised in our laboratory (unpublished data) and was included in the current study.

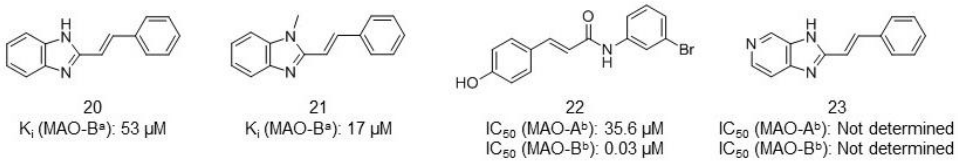


Figure 6. The chemical structures of the 2-styrylbenzimidazoles and related structures which were selected for evaluation as potential COMT inhibitors in the present study ([a] baboon liver mitochondria; [b] recombinant human MAO-A and MAO-B).

Isatin (**24**), an endogenous small molecule, is a reversible non-selective inhibitor of MAO (Hubálek *et al*., 2005). Based on this, isatin has been used as a lead compound for the design of novel reversible MAO inhibitors (Manley-King *et al*., 2011; van der Walt *et al*., 2009). In the present study (*E*)-5-styrylisatin (**25**) and (*E*)-6-styrylisatin (**26**) have been selected for evaluation as potential inhibitors of COMT (van der Walt *et al*., 2009). Additional derivatives of isatin substituted on C5 and C6 were also included (compounds **27**–**34**) (Manley-King *et al*., 2011). A novel compound, *N*-methyl-2-phenylmaleimide (**35**), which is structurally related to isatin, was also evaluated as a potential COMT inhibitor (figure 7) (Manley-King *et al*., 2009).

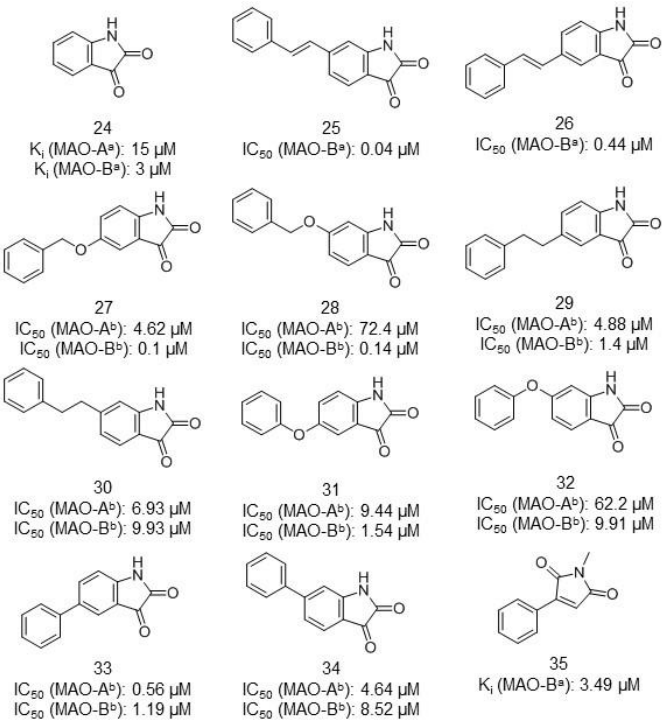


Figure 7. Isatin and its derivatives which were selected for evaluation as potential COMT inhibitors in the present study ([a] baboon liver mitochondria; [b] recombinant human MAO-A and MAO-B).

Phthalide (**36**) is structurally related to isatin and has previously been used as a lead compound for the design of potent MAO inhibitors (Strydom *et al*., 2013). Based on this, phthalide derivatives containing various substituents were included in the present study. The phthalide derivatives that were selected are: 6-benzyloxyphthalide (**37**), 6-(phenylethoxy)phthalide (**38**), 6-[4-chloro-(phenoxyethoxy)]phthalide (**39**) and 6-benzylaminophthalide (**40**) (Strydom *et al*., 2013). Sesamol (**41**) and benzodioxane (**42**) closely resemble the chemical structure of phthalide and has been previously used as lead compounds for the design of MAO inhibitors (Engelbrecht *et al*., 2015). To establish whether these compounds may be used for the design of COMT inhibitors, two sesamol derivatives (**43** and **44**) and three benzodioxane derivatives (**45**, **46** and **47**) from the compound library were evaluated as potential inhibitors of COMT (figure 8).

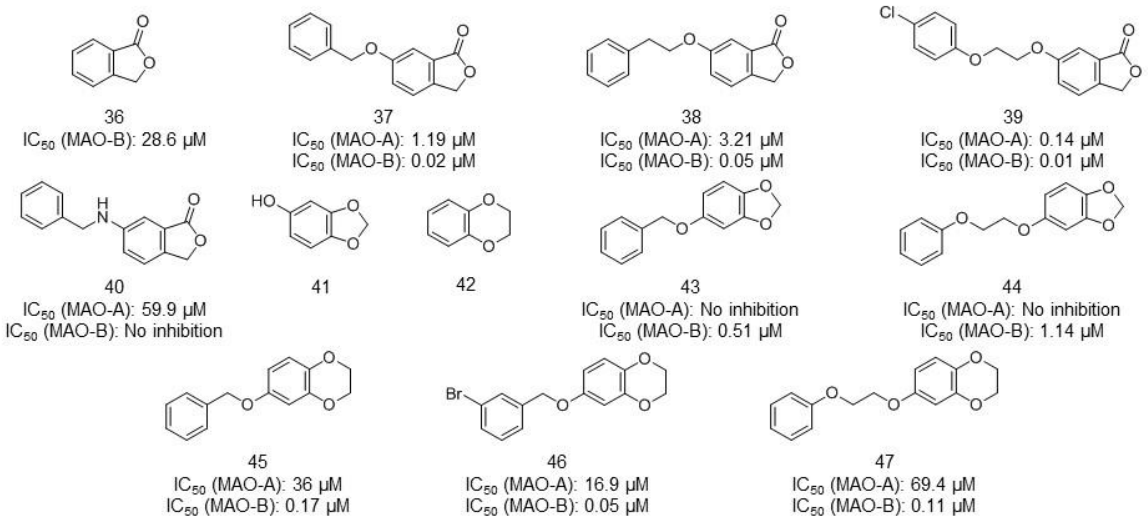


Figure 8. The chemical structures of phthalide and its derivatives which were selected for evaluation as potential COMT inhibitors in the present study.

In the search for novel structures that may serve as lead compounds in the design of MAO inhibitors, 2*H*-1,3-benzoxathiol-2-one has been identified (Mostert *et al*., 2016). Two derivatives of this chemical class, **48** and **49**, has thus been included in this study (figure 9).

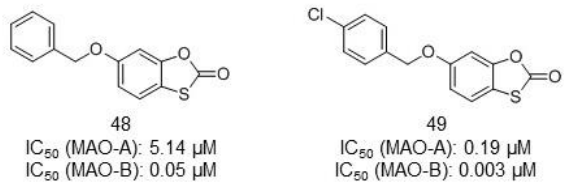


Figure 9. The 2*H*-1,3-benzoxathiol-2-one derivatives which were selected for evaluation as potential COMT inhibitors in the present study.

A variety of heterocycles containing oxygen and nitrogen have been identified as suitable inhibitors of MAO-B (Gnerre *et al*., 2000). Among these chromone (benzopyran-4-one, **50**) has emerged as a valid lead compound (Gaspar *et al*., 2011; Legoabe *et al*., 2012a). Thus, in order to establish whether chromone derivatives may act as inhibitors of COMT, three compounds previously synthesised in our laboratory have been selected for inclusion in the present study (figure 10). These compounds are: 3-bromo-2-hydroxy-2,3-dihydro-1-benzopyran-4-one (**51**) (Legoabe *et al*., 2012a), 6-(benzyloxy)-4*H*-chromen-4-one (**52**) (Legoabe *et al*., 2012b) and an 2-acetylphenol analogue, 2-acetyl-5-(4-bromobenzyloxy)phenol (**53**), which may be viewed as an open-ring analogue of chromone (Legoabe *et al*., 2012a; Legoabe *et al*., 2015a). 6-(2-Phenoxyethoxy)-3,4-dihydro-2*H*-1-benzopyran-4-one (**54**) (unpublished compound) from the compound library has also been included in the present study.

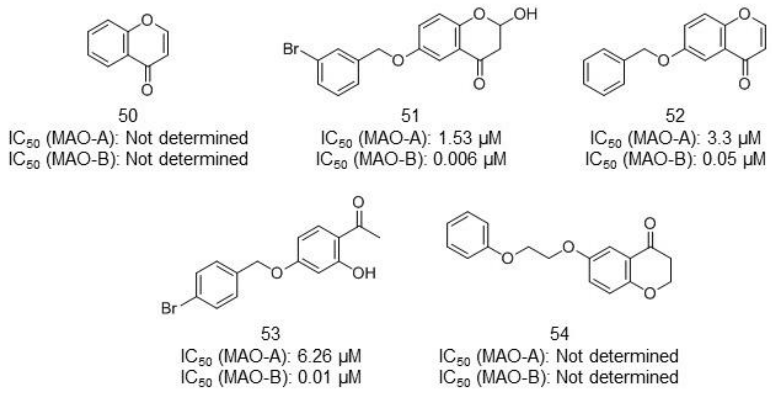


Figure 10. The chemical structures of chromone and the selected chromone analogues which were selected for evaluation as potential COMT inhibitors in the present study.

It has previously been established that 1-tetralone (**55**) is a valid scaffold for the design of MAO inhibitors (Legoabe *et al*., 2014). Accordingly, a 7-benzyloxy-1-tetralone derivative (**56**) previously synthesised in our laboratory was selected for the current study (Legoabe *et al*., 2015b). 1-Indanone (**57**) structurally resembles 1-tetralone, and subsequently an indanone derivative (**58**) from our compound library was included in the present study (figure 11) (Mostert et al., 2015).

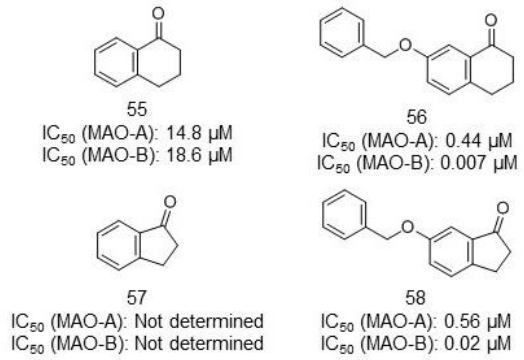


Figure 11. The chemical structures of 1-tetralone, 1-indanone and derivatives thereof which were selected for evaluation as potential COMT inhibitors in the present study.

In the search for novel lead compounds for the design of MAO inhibitors, coumarin (1-benzopyran-2-one, **59**) has emerged as a valid scaffold (Gnerre *et al*., 2000). Based on the structural similarity with coumarin, 3,4-dihydro-2(1*H*)-quinolinone (**60**) has also been shown to be a suitable lead for the design of MAO inhibitors (Meiring *et al*., 2013). In the present study, two 3,4-dihydro-2(1*H*)-quinolinone derivatives (**61** and **62**) have been evaluated as potential inhibitors of COMT (figure 12).

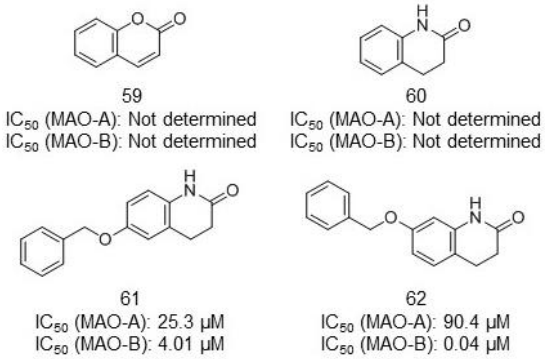
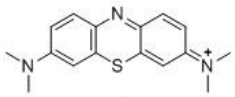


Figure 12. The structures of coumarin, 3,4-dihydro-2(1*H*)-quinolinone and the two selected 3,4-dihydro-2(1*H*)-quinolinone derivatives which were selected for evaluation as potential COMT inhibitors in the present study.

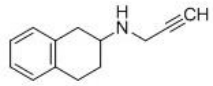
Methylene blue (**63**) has been used as drug since 1899 and has diverse medical applications (Bodini, 1899). Among these, methylene blue is used to treat malaria and certain neuropsychiatric illnesses such as depression and Alzheimer's disease (Eroglu & Caglayan, 1997; Oz *et al*., 2009). Furthermore, methylene blue acts as an inhibitor of MAO (Oxenkrug *et al*., 2007; Ramsay *et al*., 2007). In order to further investigate the application of methylene blue in the treatment of neuropsychiatric illnesses, it was included in the present study to be evaluated as an inhibitor of COMT (figure 13).



63
IC_{50} (MAO-A): 0.07 µM
IC_{50} (MAO-B): 4.37 µM

Figure 13. The chemical structure of methylene blue.

Rasagiline and selegiline are classified as mechanism-based inhibitors of MAO and contain the propargylamine functional group (Youdim *et al*., 2006). Based on the successful use of these compounds in the treatment of Parkinson's disease, *N*-propargylamine-2-aminotetralin (**64**) was previously synthesised and evaluated as a MAO inhibitor in our laboratory (Meiring, 2016). *N*-Propargylamine-2-aminotetralin was thus also included in this study (figure 14).



64
IC_{50} (MAO-A): 0.72 µM
IC_{50} (MAO-B): 14.6 µM

Figure 14. The chemical structure of *N*-propargylamine-2-aminotetralin.

4. Experimental section

4.1. Materials and methods

For the COMT inhibition studies, (-)-norephinephrine, DL-normetanephrine, S-adenosyl-L-methionine and $MgCl_2$ were purchased from Sigma-Aldrich. A Shimadzu Ultra Fast Liquid Chromatograph (UFLC) prominence high-performance liquid chromatography (HPLC) system, equipped with a Shimadzu communications bus module (CBM-20A), Shimadzu degasser (DGU-$20A_5$), Shimadzu pump (LC-20AD), Shimadzu auto-sampler (SIL-20AC), Shimadzu column oven (CTO-20A) and Shimadzu RF-10AXL fluorescence detector, with a USP L1 Luna C18 column (250 × 4.6 mm, 5 µm) (Phenomenex, Torrance, CA) was used for chromatographic separation and detection of normetanephrine. The processing of the COMT data was done using LabSolutions.

4.2. COMT inhibition studies

Sprague Dawley rats were bred, supplied and housed at the Vivarium at the Potchefstroom campus of the North-West University (NWU) (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019) of the Preclinical Drug Development Platform of the NWU. Experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) at the NWU. All animals were maintained, and procedures

performed in accordance with the code of ethics in research, training and testing of drugs in South Africa, and complied with national legislation. Ethical approval for the collection and use of animal tissue was obtained from the Research Ethics Committee, NWU. Ethics approval numbers: NWU-00438-16-S5 and NWU-00267-16-A5.

The soluble fraction of rat liver homogenate was prepared as reported in literature (Hirano *et al*., 2005; Zhu *et al*., 2010). For this purpose, homogenisation was carried out in 25 mM sodium phosphate buffer (pH 7.8, containing 0.5 mM dithiothreitol). Protein determination was carried out by the method of Bradford (Bradford, 1970). The enzyme reactions were carried out to a final volume of 125 µl and contained $MgCl_2$ (2 mM), (-)-norepinephrine (250 µM), S-adenosyl-L-methionine (200 µM) and the test inhibitor at various concentrations (0.01–100 µM). Inhibitor stock solutions were prepared in DMSO and added to the reactions to yield a final DMSO concentration of 4%. The COMT enzyme was added to yield a final enzyme concentration of 30 mg protein/ml, and after 15 min incubation at 37 °C, the enzyme reactions were terminated with the addition of 12.5 µl perchloric acid (1 M). The samples were centrifuged, and the supernatants were analysed by HPLC with fluorescence detection (λ_{ex} 283 nm; λ_{em} 315 nm) (Aoyama *et al*., 2005). Quantification of normetanephrine generated by COMT was carried out using a calibration curve (0.5–90 µM). After calculating the enzyme catalytic rates, sigmoidal plots of rate versus logarithm of inhibitor concentration were constructed by fitting to a one site competition model incorporated into the Prism software package (GraphPad). The IC_{50} values were estimated in triplicate from these plots and are expressed as mean ± standard deviation (SD).

5. Results

5.1. MAO inhibition

The compounds that were selected for the present study have previously been examined as potential MAO inhibitors, and for the most part the inhibition potencies (expressed as K_i or IC_{50}) are reported in literature. It should be noted that the enzyme source and substrate employed may differ from study to study. For compound **2**, two different enzyme sources were used for the determination of MAO inhibition potency (e.g. baboon liver mitochondria and mouse brain mitochondria). Both these assays employed 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as substrate. The MAO inhibition properties of compound **3** was examined with three different enzyme sources, namely baboon liver mitochondria, mouse brain mitochondria and human liver mitochondria. For compound **3**, MPTP was employed as substrate (Petzer *et al*., 2003). For compounds **4**–**10**, and compounds **25**, **26** and **35**,

baboon liver mitochondria served as MAO-B source, and 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP) was used as substrate (Petzer *et al*., 2003; Petzer *et al*., 2009; Pretorius *et al*., 2008; Manley-King *et al*., 2009; Strydom *et al*., 2010; van den Berg *et al*., 2007; van der Walt *et al*., 2009). Compound **10** was evaluated as a potential MAO inhibitor by using both baboon liver mitochondria and recombinant human MAO-A and MAO-B as enzyme sources, and kynuramine as mixed MAO-A/B substrate (Strydom *et al*., 2010). For the remainder of the compounds (**11**–**18**, **20**–**23**, **27**–**34**, **37**–**40**, **43**–**49**, **51**–**53**, **56**, **58**, **61**, **62**, **64**), the human recombinant MAO enzymes served as enzyme sources and kynuramine was used as substrate (Booysen *et al*., 2011; Engelbrecht *et al*., 2015; Legoabe *et al*., 2011; Legoabe *et al*., 2012a; Legoabe *et al*., 2012b; Legoabe *et al*., 2014; Legoabe *et al*., 2015a; Legoabe *et al*., 2015b; Manley-King *et al*., 2011; Meiring *et al*., 2013; Meiring, 2016; Mentz, 2013; Mostert *et al*., 2012; Mostert et al., 2015; Mostert *et al*., 2016; Strydom *et al*., 2011; Strydom *et al*., 2013).

5.2. COMT inhibition studies

The COMT inhibitory activities of the selected compounds included in this study were investigated using rat liver as enzyme source and (-)-norepinephrine as substrate. The rat liver tissue was prepared according to the literature protocol and the soluble fraction of the COMT enzyme was used for the determination of the IC_{50} values (Hirano *et al*., 2005; Zhu *et al*., 2010). The catalytic activity of COMT was determined by measuring the extent to which (-)-norepinephrine is O-methylated to yield normetanephrine, which was separated and quantitated by HPLC with fluorescence detection (Aoyama *et al*., 2005). The enzyme reactions contained (-)-norepinephrine (250 µM), $MgCl_2$, S-adenosyl-L-methionine and the test inhibitor at various concentrations (0.01–100 µM). The reactions were initiated with the addition of the COMT enzyme (30 mg protein/ml), incubated for 15 min and terminated with the addition of perchloric acid. A chromatogram routinely obtained is provided as example (figure 15). From the measurements of the concentrations of normetanephrine in the incubation mixtures, the catalytic rates of COMT was calculated. Sigmoidal plots of enzyme catalytic rate versus the logarithm of inhibitor concentration were subsequently constructed from which IC_{50} values could be estimated (figure 16).

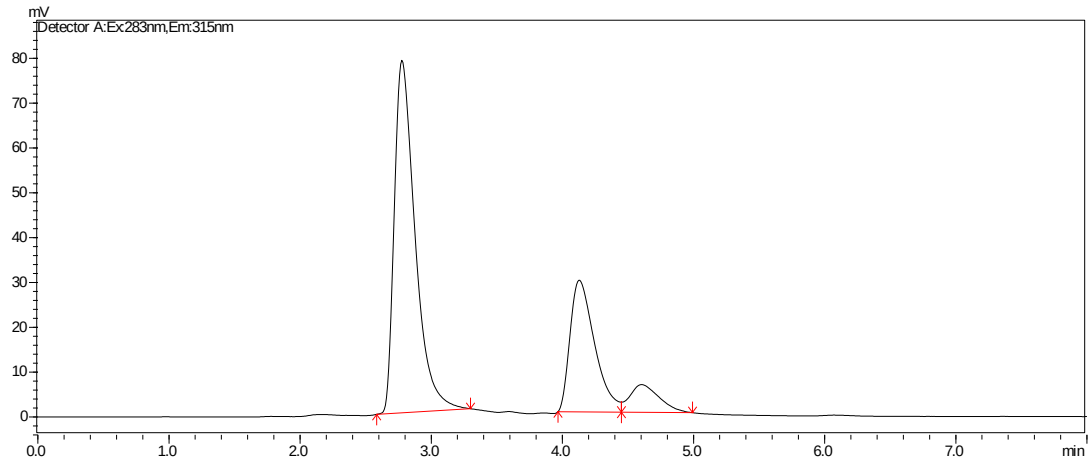


Figure 15. A chromatogram routinely obtained for the detection and quantitation of normetanephrine generated through the COMT-catalysed methylation of (-)-norepinephrine.

The results of the inhibition studies showed that none of the selected compounds act as COMT inhibitors. Shown in figure 16 are the sigmoidal plots for the inhibition of COMT by the reference inhibitor, tolcapone. As example, the plots of a selected compound (compound **20**) are also given to show lack of inhibition activity.

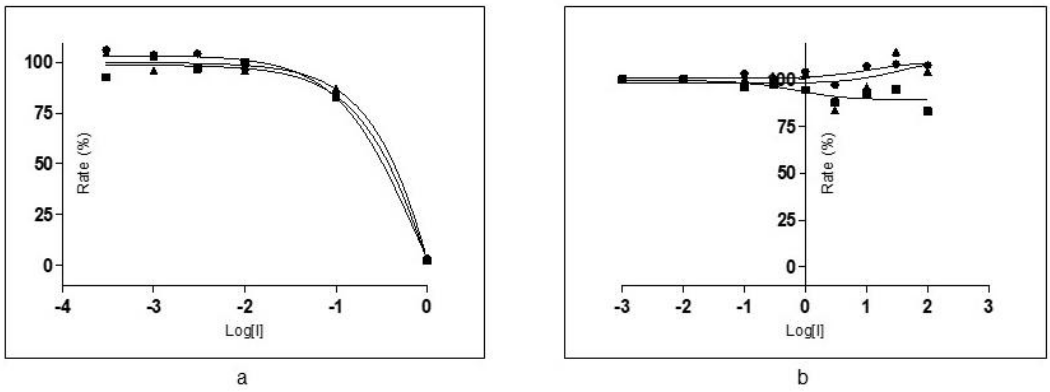


Figure 16. Sigmoidal plots for the inhibition of COMT by tolcapone (a), as well as the corresponding plots constructed for compound **20** (b), included to show no inhibition. Each data point was recorded in triplicate.

6. Discussion

The MAO inhibitory potencies of the selected compounds have previously been reported. Compounds **2**, **10**–**14**, **22**, **27**–**29**, **31**, **33**, **37**–**39**, **43**–**49**, **51**–**53**, **56**, **58**, **61** and **62** are potent inhibitors of MAO-B with IC_{50} < 4 µM, while compounds **5** and **40** exhibit no inhibition activity towards MAO-B. The remainder of the selected compounds exhibit weak to moderate MAO-B inhibition activity as previously described. Since in vertebrates the greatest COMT activity is found in the liver, the rat liver enzyme was used to determine the inhibition

potencies of the selected compounds towards COMT (Axelrod & Tomchick, 1958; Männistö *et al*., 1992). Vertebrate COMT activity appears to be mostly in the soluble form, with only a minor fraction attributed to membrane-bound fraction of the enzyme (Ding *et al*., 1996; Rivett *et al*., 1983). Differences between species exist and extrapolation of the results obtained should only be done with caution (Agathopoulous *et al*., 1971; Guldberg & Marsden, 1975). COMT plays a vital role in the metabolism of catecholamines and the deactivation of exogenous compounds with a catechol structure (Jeffery & Roth, 1987; Lautala *et al*., 2001; Masjost *et al*., 2000). In this respect, COMT specifically degrades compounds with catechol structures, and for neurotransmitters this leads to the termination of their actions. The COMT enzyme results in methylation of only one of the O-hydroxy groups of catechol substrates, employing S-adenosyl-L-methionine (SAM) as a methyl donor (Ehler *et al*., 2014; Vidgren *et al*., 1994; Woodard *et al*., 1980). Thus, many catechol compounds and derivatives thereof are suitable as competitive inhibitors and substrate inhibitors of COMT (Guldberg & Marsden, 1975; Kiss & Soares-da-Silva, 2014). For example, the parent catechol compound often acts as a substrate inhibitor, while the O-methylated products of certain substrates such as adrenaline, adnamine and noradnamine, are potent COMT inhibitors (Abbs *et al*., 1967; Ozawa & Suzuki, 1971). Metanephrine is a weak inhibitor of COMT, while the ketone analogues of adrenaline and noradrenaline, adrenalone and arterenone, respectively, are *in vitro* COMT inhibitors (Allen *et al*., 1969; Nikodwevic *et al*., 1970; Schwabe & Flohé, 1972; Wylie *et al*., 1960). Some commonly used drugs act as inhibitors of COMT and include L-dopa, L-α-methyl-dopa-α-hydrazine, isoprenaline, apomorphine and desmethylpapaverine, as well as certain metabolites such as 2-hydroxyestrone and 7,8-dihydroxychlorpromazine (Guldberg & Marsden, 1975; Männistö & Kaakkola, 1999).

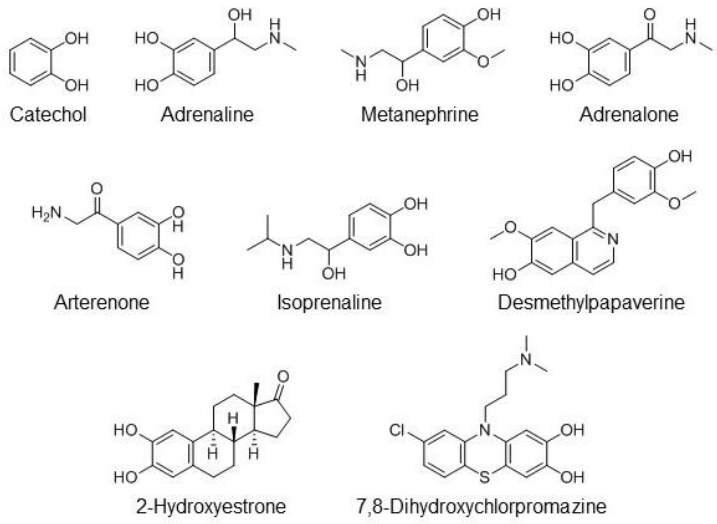


Figure 17. The chemical structures of various COMT inhibitors.

All the abovementioned compounds contain the catechol moiety and thus would be expected to inhibit COMT by competing with the substrate for binding to the active site of the enzyme. The active site structure of COMT consists of the SAM-binding domain and the actual catalytic site (Ehler *et al*., 2014; Kiss & Soares-da-Silva, 2014). The active site of COMT thus has a bipartite character (Ehler *et al*., 2014; Vidgren *et al*., 1994). Although the catalytic sites of soluble COMT and membrane-bound COMT are identical with respect to the amino acid residues that line the cavities, membrane-bound COMT has more favourable binding interactions with substrates even though no conformational change is evident in the basic structures of the enzymes (Kiss & Soares-da-Silva, 2014; Männistö & Kaakkola, 1999). This suggests that even if only the soluble fraction of the enzyme is used to determine the inhibition potencies of selected compounds, the data may be extrapolated to the membrane-bound fraction of the enzyme.

COMT is a malleable enzyme, which makes it a difficult drug target (Ehler *et al*., 2014). It is noteworthy that most COMT inhibitors contain the catechol structure. The observation that none of the selected compounds display inhibition of COMT and do not possess the catechol structure, shows that the catechol moiety is a required functional group for COMT inhibition. Even though compounds **22**, **51** and **53** possess the hydroxy group, they do not exhibit inhibition activity towards COMT. This shows that deviation from the catechol structure is not tolerated for COMT inhibition. Furthermore, isatin (**24**–**34**) and phthalide (**36**–**40**) derivatives also resembles the catechol structure with respect to the *ortho* placement of carbonyl oxygens (for isatins) and carbonyl and ether oxygens (for phthalide). The observation that these derivatives do not inhibit COMT further underscores the importance of the catechol and *ortho* placement of specifically hydroxy groups.

It has previously been reported that 4-pyridinone derivatives possess weak inhibition activity towards COMT (Robinson *et al*., 2012). The caffeine and benzimidazole derivatives **2**–**23** are related to 4-pyridinone, and it was speculated that these chemical classes may exhibit COMT inhibition. Unfortunately, no inhibition was observed. Although it was previously reported that ketone analogues of endogenous neurotransmitters are potent inhibitors of COMT, compounds **25**–**35**, **37**–**40**, **48**, **49**, **51**–**54**, **56**, **58**, **61** and **62**, which contains ketones as part of their chemical structures, do not exhibit any inhibition potency towards COMT (Guldberg & Marsden, 1975; Wylie *et al*., 1960). The reason for this behaviour may again be attributed to the presence of the catechol moiety in the endogenous neurotransmitters, while being absent from the study compounds.

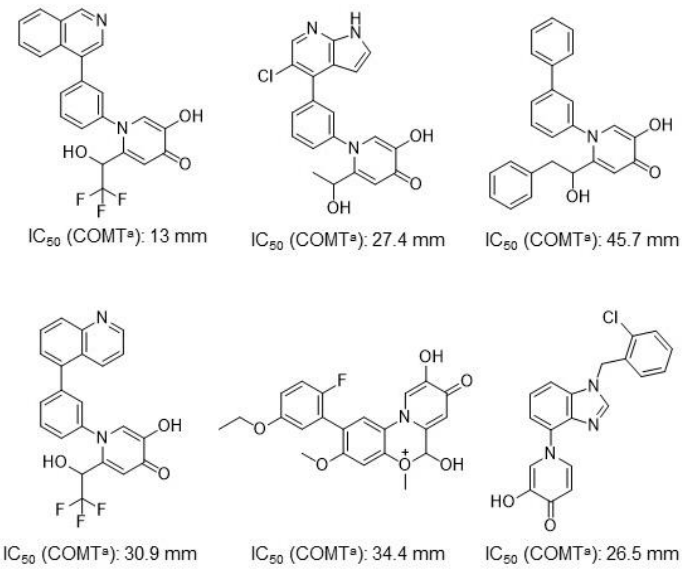


Figure 18. The structures of 4-pyridinone COMT inhibitors ([a]: rat membrane-bound COMT).

Even though none of the compounds selected for this study inhibited COMT, this study demonstrates the importance of the catechol structure for COMT inhibition. This study also shows that caffeine and the related 2-styrylbenzimidazole derivatives, isatins and the related phthalides, sesamol and benzodioxane derivatives, 2*H*-1,3-benzoxathiol-2-ones, chromone and the related 1-tetralone and 1-indanone derivatives, 3,4-dihydro-2(1*H*)-quinolinone derivatives, as well as methylene blue and *N*-propargylamine-2-aminotetralin cannot be used as valid lead compounds for the design of COMT inhibitors. In conclusion, a catechol moiety is critical for inhibition activity towards COMT.

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Chapter 6

Conclusion

Parkinson's disease is the second most prevalent neurodegenerative disorder. The aetiology of the disease cannot be ascribed to a common cause and Parkinson's disease may be induced by a multifactorial cascade of events which leads to motor disability. The primary pathological hallmark of Parkinson's disease is the degeneration of the dopaminergic neurons in the nigrostriatal pathway of the brain which leads to reduced levels of striatal dopamine. This event is responsible for the characteristic symptoms pertaining to movement in Parkinson's disease. The current treatment of Parkinson's disease focusses on restoring depleted central dopamine levels by either dopamine replacement therapy, mainly via the administration of L-dopa and dopamine agonists, or by inhibiting the metabolism of central dopamine through inhibition of either MAO or COMT. L-Dopa is still considered to be the most effective treatment for Parkinson's disease, although extensive peripheral metabolism by AADC and COMT decreases the bioavailability of L-dopa to a very large extent. Thus, by the inhibition of the enzymes responsible for L-dopa and dopamine metabolism, dopaminergic neurotransmission in the brain may be enhanced leading to alleviation of the motor symptoms in Parkinson's disease.

As mentioned above, COMT and MAO are important enzymes involved in the degradation of L-dopa and subsequently dopamine. COMT catalyses the metabolism of endogenous catecholamines, including L-dopa and dopamine, and exogenous compounds with a catechol structure. Accordingly, inhibition of peripheral COMT will result in higher levels of L-dopa available for conversion to dopamine in the brain. In the brain L-dopa-derived dopamine is enzymatically degraded by MAO through oxidative deamination. Hence the inhibition of MAO would elevate central dopamine levels. Dopamine present in the brain may also be metabolically inactivated by centrally located COMT. Thus, peripheral as well as central COMT inhibition may be beneficial in the treatment of Parkinson's disease. These metabolic routes of L-dopa and dopamine and the COMT and MAO enzymes involved served as drug targets in this study which aimed to discover potential new inhibitors with dual inhibition of MAO and COMT.

Current treatment options available for the management of Parkinson's disease focus on symptomatic relief. It may be argued that drugs that modulate more than one target relevant to Parkinson's disease may have enhanced value compared to compounds that only act at a specific target. Such compounds follow a multi-target-directed approach and, in neurodegenerative disorders where multiple drugs are often used simultaneously, may exhibit enhanced efficacy. This thesis contributes in this regard. This study synthesised novel chalcone derivatives and investigated their inhibitory potencies towards MAO and COMT. In addition, various natural and synthetic compounds with distinct chemical structures have been evaluated for potential dual inhibition of MAO and COMT. The results of these studies were presented as three journal articles.

The first article examined chalcone derivatives as potential MAO and COMT inhibitors. Based on literature reports, chalcones has emerged as useful lead compounds in the design of potent MAO inhibitors. A series of chalcones were thus synthesised and their IC_{50} values for the inhibition of both isoforms of human MAO (A and B) were determined *in vitro*. The results indicated that most compounds are non-selective MAO inhibitors although they exhibit higher affinity for MAO-B. The most potent MAO-B inhibitor was (2*E*)-3-(3-bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (compound **1d**) with an IC_{50} value of 13.9 μM, while (2*E*)-3-(4-chlorophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (compound **1g**) was the most potent inhibitor of MAO-A with an IC_{50} value of 32.4 μM. The study further established that compound **1d** is a reversible and competitive inhibitor of MAO-B. Thus, it can be concluded that nitrocatechol derivatives of chalcone act reversibly and competitively with the MAO-B enzyme.

The chalcones were also investigated as potential COMT inhibitors. Since COMT inhibitors currently on the market (tolcapone and entacapone) contain the nitrocatechol moiety, this structural feature was incorporated into the chalcones synthesised in this study. The most potent inhibitor, (2*E*)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (compound **1h**), exhibited an IC_{50} value of 0.07 μM towards rat liver COMT. In fact, all of the derivatives were found to be highly potent *in vitro* inhibitors of rat liver COMT with IC_{50} values ranging from 0.07 to 0.29 μM. Although none of the compounds acted as potent MAO inhibitors, this study shows that nitrocatechol derivatives of chalcone are a promising class of COMT inhibitors. In this respect, the MAO inhibition potencies of the nitrocatechol derivatives of chalcone should be further improved.

This study found that compound **1d** is the most promising dual inhibitor of COMT and MAO-B among the chalcone derivatives synthesised and may thus serve as a possible lead compound for the future design of multi-target-directed inhibitors of MAO and COMT. Compound **1d** inhibits MAO-B and COMT with IC_{50} values of 13.9 μM and 0.29 μM, respectively. Although compound **1d** inhibited COMT to a lesser extent than the other nitrocatechol derivatives, this compound displayed similar inhibition activity to the reference compounds, tolcapone (IC_{50} = 0.26 μM) and entacapone (IC_{50} = 0.25 μM). An important observation was that substitution on the B-ring of chalcone does not significantly alter COMT inhibition activity, which suggests that substitution on the B-ring is a potential approach to enhance the MAO-B inhibition activity of compound **1d** with the aim of designing more potent multi-target-directed inhibitors of MAO and COMT. Since it was established that compound **1d** acts reversibly and competitively with MAO-B, it may be concluded that other structurally similar nitrocatechol chalcone derivatives will also display reversible and competitive modes of MAO-B inhibition.

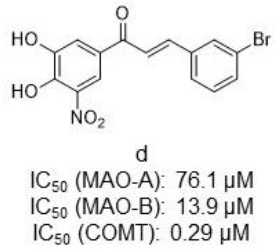
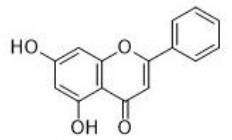


Figure 6.1. The chemical structure of (2*E*)-3-(4-bromophenyl)-1-(3,4-dihydroxy-5-nitrophenyl)prop-2-en-1-one (compound **1d**).

Compound **1d** may serve as a potential lead for the design of future inhibitors of MAO and COMT. Such compounds may be useful in the treatment of neurodegenerative disorders such as Parkinson's disease and depression where the metabolic breakdown of neurotransmitters such as dopamine by these enzymes plays crucial roles in the development and pathogenesis of these disorders. The design of multi-target-directed inhibitors of MAO and COMT is an interesting approach for the treatment of Parkinson's disease, since these inhibitors may serve as adjuvants to L-dopa. Compounds that inhibit these enzymes in both the peripheral and central tissues may reduce the undesired peripheral breakdown of L-dopa, and subsequently conserve the dopamine supply in the brain. Additionally, the dual inhibition of MAO and COMT may be valuable in the treatment of depression. Since the central inhibition of COMT and MAO prolongs the functions of

endogenous catecholamines such as dopamine and noradrenaline, dual inhibitors of MAO and COMT may be more effective as antidepressants. Catecholamine levels in the brain are significantly increased when a MAO-B inhibitor such as selegiline is coadministered with a COMT inhibitor. In conclusion, the dual inhibition of MAO and COMT by a multi-target-directed inhibitor such as compound **1d** may be valuable in the treatment of patients with advanced Parkinson's disease and depression. The design of multi-target-directed inhibitors of MAO and COMT should thus be further pursued to reach this objective.

A second goal of this study was to investigate the MAO and COMT inhibition potencies of selected natural compounds as presented in the second article. Considering literature reports of the MAO inhibitory activities of several naturally occurring compounds, this thesis evaluated a series of selected commercially available natural compounds. This series included diverse compounds with unique structures with the aim to discover multi-target-directed inhibitors of MAO and COMT. The MAO and COMT inhibitory potencies of the selected natural compounds were determined and the results showed that the most potent MAO inhibitor, chrysin, exhibited non-selective inhibition of MAO-A and MAO-B with almost equivalent IC_{50} values (0.77 µM for MAO-A and 0.79 µM for MAO-B). It was further established that chrysin is a reversible and competitive inhibitor of both MAO-A and MAO-B. Chrysin can thus be used as a lead compound in the future design of non-selective reversible and competitive MAO inhibitors for the treatment of Parkinson's disease and co-morbid depression. MAO-B inhibition is necessary for alleviating motor symptoms associated with Parkinson's disease, while MAO-A and MAO-B inhibition may alleviate depression since both these enzymes are responsible for neurotransmitter breakdown, especially dopamine.



IC_{50} (MAO-A): 0.77 µM
IC_{50} (MAO-B): 0.79 µM

Figure 6.2. The chemical structure of chrysin.

Among the forty-two natural compounds selected for this study, (+)-catechin was the most potent COMT inhibitor with an IC_{50} value of 0.86 µM. Another natural compound, alizarin (IC_{50} = 0.88 µM), inhibited COMT with a potency similar to that of (+)-catechin. Three of the forty-two compounds included in this study exhibited inhibition activity for either MAO-A or

MAO-B and COMT. Morin, fisetin and alizarin were found to be potent MAO-A inhibitors, moderate inhibitors of MAO-B and potent COMT inhibitors.

Morin exhibited inhibition activity for both MAO isoforms with selectivity towards MAO-A (IC_{50} MAO-A: 16.2 µM and MAO-B: 59.2 µM). It was determined that morin acts reversibly with MAO-A with a competitive mode of inhibition. Additionally, morin also inhibited COMT with an IC_{50} value of 1.32 µM. This indicates that morin may be used as a lead compound in the design of multi-target-directed inhibitors that target MAO-A and COMT. This combination of inhibition will serve to alleviate depression since both these enzymes are responsible for the metabolism of neurotransmitters such as dopamine and noradrenaline, which are implicated in the pathogenesis of depression. COMT inhibition by morin and derivatives thereof may be useful as adjuvants to L-dopa in the treatment of Parkinson's disease.

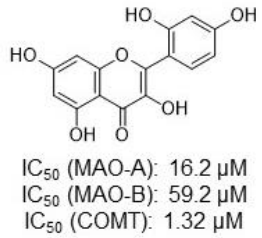
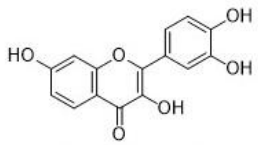


Figure 6.3. The chemical structure of morin.

Fisetin acts as a reversible and competitive inhibitor of MAO-A with an IC_{50} value of 7.33 µM. Fisetin also inhibits MAO-B (IC_{50} = 74.4 µM) but is 10-fold more selective towards MAO-A. Fisetin displays moderate inhibition of COMT with an IC_{50} value of 5.78 µM. Thus, fisetin may be used as a lead compound in the design of dual-acting inhibitors of MAO-A and COMT. As mentioned, the dual inhibition of these enzymes may serve to alleviate the symptoms of Parkinson's disease since dual inhibitors that function in both the peripheral and central tissues are expected to not only reduce undesired peripheral metabolism of L-dopa, but also conserve the dopamine supply in the brain. Similar to MAO-B, MAO-A is also relevant to Parkinson's disease since both MAO-A and MAO-B degrade the neurotransmitter, dopamine, in the brain and periphery. Furthermore, the inhibition of both MAO-A and COMT will be of potential value in the treatment of co-morbid depression in Parkinson's disease. Since dual inhibition of COMT and MAO may prolong the central actions of endogenous catecholamines to a greater degree than inhibition of either enzyme, these multi-target-directed drugs may be more effective as antidepressants. Fisetin may thus

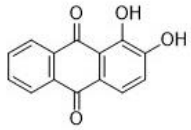
serve as a lead compound in the design of dual inhibitors of MAO-A and COMT for adjuvant therapy to L-dopa in the treatment of Parkinson's disease, or as effective antidepressants.



IC_{50} (MAO-A): 7.33 µM
IC_{50} (MAO-B): 74.4 µM
IC_{50} (COMT): 5.78 µM

Figure 6.4. The chemical structure of fisetin.

Like morin and fisetin, alizarin also displays inhibition activity towards MAO and COMT. Alizarin inhibits MAO-A with an IC_{50} value of 8.16 µM and MAO-B with an IC_{50} value of 78.1 µM, while displaying potent COMT inhibition with an IC_{50} value of 0.88 µM. Alizarin is 9-fold more selective towards MAO-A and may thus be regarded as a selective MAO-A inhibitor. Furthermore, since alizarin displays COMT inhibition activity, it may be regarded as a multi-target-directed inhibitor of MAO-A and COMT. Alizarin may be used as a lead compound in the design of selective, reversible and competitive inhibitors of MAO-A, or as a lead compound in the design of dual inhibitors of MAO-A and COMT. It may thus be concluded that similar to morin and fisetin, alizarin may be used as a lead compound for the future design of dual-acting inhibitors of MAO-A and COMT for the treatment of Parkinson's disease and depression.



IC_{50} (MAO-A): 8.16 µM
IC_{50} (MAO-B): 78.1 µM
IC_{50} (COMT): 0.88 µM

Figure 6.5. The chemical structure of alizarin.

It should be noted that morin and fisetin are flavonoids and have similar structures. These compounds and alizarin possess the dihydroxyphenyl moiety, which characterises the catechol structure. This structural feature is crucial for COMT inhibition activity as most of the natural compounds selected for this study do not inhibit COMT and do not possess catechol-like structures. In conclusion, morin, fisetin and alizarin can be used in the future design of dual-target directed inhibitors of COMT and MAO. Natural compounds are an

inexhaustible source of lead compounds which may be structurally modified to enhance the inhibition potencies of the proposed drug target. In this respect, this study shows that natural compounds are a viable source of leads for the discovery of dual inhibitors of COMT and MAO.

The third article investigated the COMT inhibitory activity of synthetic compounds from various chemical classes previously synthesised by our research group. For these compounds, the MAO inhibition activities have already been established. Most of the compounds selected for this study have previously been found to be potent and reversible inhibitors of MAO. These compounds were evaluated in the present study as potential inhibitors of COMT to discover new lead compounds with novel structures for the future design of multi-target-directed inhibitors of MAO and COMT. None of the fifty-four compounds exhibited inhibition activity towards COMT. Examination of the chemical structures of these compounds showed that none possess a catechol-like structure, which suggest that the catechol structure is crucial for the COMT inhibition of most known COMT inhibitors. This study thus showed that caffeine and related 2-styrylbenzimidazole derivatives, isatins and the related phthalides, sesamol and benzodioxane derivatives, 2*H*-1,3-benzoxathiol-2-ones, chromone and the related 1-tetralone and 1-indanone derivatives, 3,4-dihydro-2(1*H*)-quinolinone derivatives as well as methylene blue and *N*-propargylamine-2-aminotetralin are not suitable lead compounds for the design of COMT inhibitors.

In conclusion, a number of nitrocatechol derivatives of chalcone have been shown to act as multi-target-directed inhibitors with inhibitory activities towards MAO and COMT. Furthermore, morin, fisetin and alizarin act as potent COMT inhibitors with inhibitory activity towards MAO-A. This study also established that the catechol moiety is essential for a compound to possess COMT inhibition activity. The aim of this study was to examine different classes of compounds as potential dual inhibitors of MAO and COMT with the objective to discover promising lead compounds for the design of novel multi-target-directed compounds. This study successfully identified several novel compounds which can be used as lead compounds for the future design of specific inhibitors of MAO and COMT as well as dual inhibitors of these enzymes.

Future perspective

The current study concluded that nitrocatechol derivatives of chalcone can be used in the future design of multi-target-directed inhibitors of COMT and MAO. In this respect the structures should be modified to enhance MAO inhibition activity especially towards MAO-B. Furthermore, (+)-catechin can be used to design novel and potent COMT inhibitors while morin, fisetin and alizarin may be suitable for the future design of dual COMT and MAO inhibitors. It is especially important that these compounds be evaluated further to determine their physicochemical properties (logP, solubility, lipophilicity) in order to evaluate their feasibility as drugs for the treatment of Parkinson's disease. Furthermore, the antioxidant properties of the different compounds should be evaluated which will establish whether these compounds may exert neuroprotective properties by scavenging destructive free radicals. This will provide a further mechanism by which these compounds are relevant to Parkinson's disease and may be particularly applicable to natural compounds which frequently exhibit antioxidant activity.

Annexure

To whom it may concern,

Dear Sir/Madam,

SUBMISSION OF RESEARCH PAPERS

This thesis is submitted in article format consisting of three original research articles, listed below, as separate entities. The written declaration from the co-authors of the research articles are included. The specific journals in which these research articles will be included, is still undecided. Thus no author notes and instructions are included in this thesis.

The three original research articles are:

- **The synthesis and evaluation of nitrocatechol derivatives of chalcone as dual inhibitors of monoamine oxidase and catechol-O-methyltransferase**
- The evaluation of **selected natural compounds as potential dual inhibitors of catechol-O-methyltransferase and monoamine oxidase**
- The evaluation of **structurally diverse monoamine oxidase inhibitors as potential dual inhibitors of catechol-O-methyltransferase**

Yours sincerely,

Prof. A. Petzer

Prof. J.P. Petzer

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