



Design, synthesis and evaluation of novel levodopa pro-drugs for the treatment of Parkinson's disease

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LIST OF ABBREVIATIONS AND ACRONYMS

A

AADC	Aromatic L-amino acid decarboxylase
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
APCI	Atmospheric-pressure chemical ionisation
A _{2A}	Adenosine A _{2A} receptor
Å	ångström

B

Boc ₂ O	Di-t-butyl dicarbonate
BOP	Benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate

C

Caco-2	Heterogeneous human epithelial colorectal adenocarcinoma cells
Ca ²⁺	Calcium
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CO ₂	Carbon dioxide
CoQ10	Coenzyme Q ₁₀ /ubiquinone
CTZ	<i>Chemoreceptor trigger zone</i>
CYP2B6	<i>Cytochrome P450 2B6</i>
CYP2C19	<i>Cytochrome P450 2C19</i>
Cys	Cysteine

D

D1	Dopamine receptor 1
D2	Dopamine receptor 2
D3	Dopamine receptor 3

D4	Dopamine receptor 4
Da	Dalton
DA	Dopamine
DAT	Dopamine transporter
DATATOP	(R)-deprenyl and tocopherol antioxidative therapy of Parkinsonism
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DH	Dopamine β -hydroxylase
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DOPAC	Dihydroxyphenyl acetic acid

E

EDTA	Ethylenediaminetetraacetic acid
EI	Electron ionisation
ER	Extended-release

F

FAD	Flavin adenine dinucleotide
FDA	Food and drug administration

G

GABA	gamma-Aminobutyric acid
GPCR	G-protein-coupled receptors
g	Gram

H

H	Hour
H ₂ O ₂	Hydrogen peroxide
HCl	Hydrochloric acid
HeLa	Henrietta Lacks (uterine cell variety)
5-HIAA	5-Hydroxyindole acetic acid
HPLC	High-performance liquid chromatography

5-HT	Serotonin
HRMS	High resolution mass spectrometry
HVA	Homovanillic acid

I

IC ₅₀	Inhibitor concentration at 50% inhibition
Ile	Isoleucine
i.p.	Intraperitoneal

K

K _i	Inhibitor constant
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L

LAT	Large amino acid transporter
LBs	Lewy bodies
Leu	Leucine
L-Dopa	Levodopa
LogD	Partition coefficient of ionisable solutes
LogP	Partition coefficient of permeability
LNAA	Large neutral amino acid
Lys	Lysine

M

MAO	Monoamine oxidase
MHz	Megahertz
MilliQ	Millipore corporation ultrapure water
Min	Minutes
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NA	Noradrenaline
NE	Norepinephrine
NMM	N-Methylmorpholine
NMDA	N-Methyl-D-aspartate
NMR	Nuclear magnetic resonance

O

O ₂	Oxygen
3-O-MD	3-Methoxy-4-hydroxy-L-phenylalanine

P

PAMPA	Parallel artificial membrane permeability assay
PBS	Phosphate buffered saline
P _e	Permeability coefficient
pK _a	Acid dissociation constant
pH	Power of hydrogen
Phe	Phenyl
PNMT	Phenylethanolamine-N-methyltransferase
PD	Parkinson's disease
Ppm (δ)	Parts per million
PS	Parkinson se siekte
Pro	Proline
PTAPT	Peptide transporter associated prodrug therapy

Q

QSAR	Quantitative structure-activity relationship
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S

SD	Standard deviation
SNpc	Substantia nigra pars compacta

T

TBDMS	Tert-butyldimethylsilyl
TFA	Trifluoroacetic acid
TH	Tyrosine hydroxylase
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Trp	Tryptophane
Tyr	Tyrosine

U

UCH-L1	Ubiquitin C-terminal hydrolase L1
UPDRS	Unified Parkinson's disease rating scale
UPS	Ubiquitin-proteasome system
UV/Vis	Ultraviolet-visible

α

α-syn	α-synuclein
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μ

μM	Micro molar
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%	Percentage
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Abstract

Parkinson's disease is a slowly progressive neurodegenerative disorder of unknown cause that selectively affects the dopaminergic, extrapyramidal nigrostriatal pathway. Parkinson's disease is a mid- or late life disease, presenting most often at ages 55-65, affecting 1-2% of the population over the age of 65. Current therapy is essentially symptomatic, and L-dopa, the direct precursor of dopamine, is the treatment of choice in more advanced stages of the disease. The oral bioavailability of L-dopa is estimated to be about 10% and less than 1% of the administered oral dose reaches the brain unchanged. In an attempt to overcome the problems with peripheral L-dopa metabolism, delivery difficulties and insufficient conversion of L-dopa to dopamine in the brain tissue, L-dopa prodrugs are proposed in this study.

An L-dopa-lazabemide prodrug was thus proposed to overcome the problems associated with L-dopa absorption and delivery to the brain. Lazabemide, a monoamine oxidase (MAO) B inhibitor, slows depletion of dopamine stores and elevates dopamine levels produced by exogenously administered L-dopa. L-Dopa was linked at the carboxylate with the primary aminyl functional group of lazabemide via an amide, a strategy which is anticipated to protect L-dopa against peripheral decarboxylation and possibly also enhance the membrane permeability of the prodrug. Selected physicochemical and biochemical properties of the prodrug were determined. Although oral and i.p. treatment of mice with the prodrug did not result in an enhancement of striatal dopamine levels, DOPAC (Dihydroxyphenyl acetic acid) levels were significantly depressed compared to saline, L-dopa and carbidopa/L-dopa treatment.

Secondly, in another attempt to overcome L-dopa's limited bioavailability and brain penetration, the present study synthesises four carrier-linked prodrugs of L-dopa in which 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine are linked to the carboxylate of L-dopa. Key physicochemical and biochemical parameters of the prodrugs were evaluated in an attempt to assess the potential of these prodrugs as vehicles to enhance the absorption and central delivery of L-dopa.

Although the development of lazabemide has been discontinued, this compound is still used as reference MAO-B inhibitor in the *in vitro* screening of experimental MAO inhibitors. The third section of the present study aimed to characterise the *in vitro* MAO inhibition properties of lazabemide with respect to potency, isoform selectivity and reversibility. The results show that lazabemide is a selective inhibitor of human MAO-B with an IC_{50} value of 0.091 μ M. For

human MAO-A, lazabemide exhibits an IC_{50} of $>100 \mu\text{M}$. Interestingly, dialysis restores MAO-B activity only to a very small extent following inhibition by lazabemide, which shows that, *in vitro* inhibition persists and lazabemide may be viewed as an irreversible MAO-B inhibitor.

Irreversible MAO inhibitors of the well-known propargylamine class include drugs that have been used clinically such as pargyline, selegiline and rasagiline, specifically for the treatment of depression and as adjuvants to L-dopa in Parkinson's disease. Due to their importance as MAO inhibitors, the fourth part of the present study synthesises a small series of novel propargylamine compounds that incorporate the pyridyl moiety. Pyridyl-derived propargylamines have not yet been investigated as potential MAO inhibitors. This study finds that the pyridyl-derived propargylamines do not inhibit either of the human MAO isoforms.

Key words: Parkinson's disease, L-dopa, lazabemide, prodrugs, propargylamine

Uittreksel

Parkinson se siekte (PS) is 'n stadig vorderende neurodegeneratiewe siekte van onbekende oorsaak wat die dopaminergiese, ekstrapirimidale nigrostriatale baan selektief affekteer. PS kom voor in die middel tot laat fase van lewe, om en by die ouderdom van 55-65 en affekteer 1-2% van die populasie oor die ouderdom van 65. Huidige behandeling is hoofsaaklik simptomaties en L-dopa, die direkte voorganger van dopamien, is die behandeling van keuse in meer gevorderde stadiums van die siektestoestand. Die orale biobeskikbaarheid van L-dopa is beraam om 10% te wees, waarvan slegs 1% van die toegediende orale dosis die brein onveranderd bereik. Om te probeer om die probleme met die perifere L-dopa metabolisme, aflewering probleme en onvoldoende omskakeling van L-dopa na dopamien in die brein te oorkom, is L-dopa progeneesmiddels voorgestel vir hierdie studie.

'n L-dopa-lazabemied progeneesmiddel is daarom voorgestel om die probleme wat geassosieer is met L-dopa absorpsie en aflewering in die brein te oorkom. Lazabemied is 'n monoamienoksidase (MAO) B inhibeerder wat die uitputting van dopamienstore vertraag en verhoog dopamienvlakke wat geproduseer word deur ekstern toegediende L-dopa. L-Dopa was by die karboksilaat verbind met die primêre aminiële funksionele groep van lazabemied via 'n amied. Hierdie strategie verwag dat die L-dopa beskerm sal word teen perifere dekarboksilasie en moontlik ook die membraandeurloosbaarheid van die progeneesmiddel verbeter. Sekere fisieschemiese en biochemiese eienskappe van die progeneesmiddel is vasgestel. Ten spyte daarvan dat oraal en intraperitoneaal behandelde muis met die progeneesmiddel, nie die striatale dopamienvlakke verhoog het nie, was DOPAC (Dihidroksifeniel asynsuur) vlakke aansienlik laer in vergelyking met die sout, L-dopa en karbidopa/L-dopa behandelde muis.

Tweedens, in nog 'n poging om die beperkte biobeskikbaarheid en breinpenetrasie van L-dopa te oorkom, het die huidige studie 4 draergekoppelde L-dopa progeneesmiddels gesintetiseer waaraan 4-piridielmetielamien, 2-(4-piridiel)etielamien, 2-(2-piridiel)etielamien en 3-feniel-1-propielamien verbind is aan die karboksilaat van L-dopa. Sleutel fisieschemiese en biochemiese grense van die geneesmiddel is geëvalueer in 'n poging om die potensiaal van hierdie progeneesmiddels as voertuie om die absorpsie en sentrale aflewering van L-dopa, vas te stel.

Ten spyte daarvan dat die ontwikkeling van lazabemied gestaak is, word hierdie verbinding steeds gebruik as 'n verwysing MAO-B-inhibeerder in die *in vitro* keuring van eksperimentele

MAO-inhibeerders. Die derde gedeelte van die huidige studie het gemik om *in vitro* MAO-inhiberende eienskappe van lazabemied te karakteriseer met betrekking tot sterkte, isoform-selektiwiteit en omkeerbaarheid. Die resultate het aangedui dat lazabemied 'n selektiewe inhibeerder van menslike MAO-B is, met 'n IC_{50} waarde van $0.091 \mu\text{M}$. Lazabemied toon 'n IC_{50} waarde van $>100 \mu\text{M}$ vir menslike MAO-A. 'n Interessante observasie is dat met dialise, MAO-B aktiwiteit herstel tot 'n baie klein mate na inhibisie met lazabemied, wat wys dat *in vitro* inhibisie voortgaan en lazabemied kan gesien word as 'n onomkeerbare MAO-B-inhibeerder.

Onomkeerbare MAO-inhibeerders van die bekende propargielamiengroep sluit geneesmiddels in wat al klinies gebruik is soos pargilien, selegilien en rasagilien, spesifiek vir die behandeling van depressie en as 'n toevoeging tot L-dopa in PS. Omdat hulle belangrike MAO-inhibeerders is, het die vierde gedeelte van die huidige studie 'n klein reeks nuwe propargielamien verbindings gesintetiseer wat 'n piridielgedeelte bevat. Piridiel-afgeleide propargielamiene is nog nooit voorheen ondersoek as potensiele MAO-inhibeerders nie. Hierdie studie het bevind dat die piridiel-afgeleide propargielamiene nie een van die menslike MAO isoforms geinhibeer het nie.

Sleutelwoorde: Parkinson se siekte, L-dopa, lazabemied, progeneesmiddels, propargielamien.

Preface

This doctoral thesis is submitted in article format. All four articles are research articles and have been prepared for submission to academic journals. All the scientific research for this thesis was conducted by Mrs Monique Strydom. The relevant contribution of co-authors are also stated as well as permission granted by the respective journals and authors for the inclusion of these articles and figures in this thesis.

I would like to express my sincere gratitude towards the North-West University, especially towards the School of Pharmacy and Prof Jeanetta du Plessis for granting me the opportunity to pursue and complete my doctoral studies. During the period of this study, their assistance, guidance and support were of great worth, and I would like to thank all the people who aided to complete this thesis and fulfil my dreams. These people include members of the Sasol Centre for Chemistry for the numerous NMR and MS spectra, especially Dr Johan Jordaan en Mr Andre Joubert. Prof JL du Preez at the Analytical Technology Laboratory for his assistance in recording HPLC spectra and Mr Francois Viljoen from the division of Pharmacology for assistance with the monoamine determinations. Me Antoinette Fick and Mr Hylton Bunting and all the other NWU Vivarium staff for assistance with the animal studies. Thank you to the National Research Foundation for their highly appreciated financial support. This doctoral study and thesis would not have been a reality if it was not for my promoter, Prof A Petzer, and co-promoter, Prof JP Petzer, I am truly grateful for their treasured and respected assistance during the whole study period.

Lastly, I want to express my utmost thanks to my husband, Tiaan Strydom, parents Deon and Annette Hoon and all my friends and family for wiping away my tears and giving me words of encouragement when there were hardships, and celebrating with me when there were victories. This thesis can attest to 2 Cor 12:9, which states "My grace is sufficient for you, for (My) power is perfected in weakness."

"All our dreams can come true if we have the courage to pursue them." *Walt Disney*

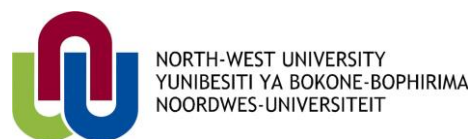
Declaration

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

I, Monique Strydom hereby declare that the dissertation with the title: **Design, synthesis and evaluation of novel levodopa pro-drugs for the treatment of Parkinson's disease** is my own work and has not been submitted at any other university either whole or in part.

M. Strydom

Letter of agreement



Privaatsak X6001, Potchefstroom
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Oktober 2017

To whom it may concern,

Dear Sir/Madam

CO-AUTHORSHIP ON RESEARCH PAPERS

The undersigned as co-authors of the research articles listed below, hereby give permission to M. Strydom to submit these articles as part of the degree PhD in Pharmaceutical Chemistry at the North-West University, Potchefstroom campus.

- ❖ The design and evaluation of an L-dopa-lazabemide prodrug for the treatment of Parkinson's disease
- ❖ An investigation of the in vitro reversibility of MAO inhibition by lazabemide
- ❖ The synthesis and property evaluation of novel L-dopa prodrugs for the treatment of Parkinson's disease
- ❖ The synthesis and evaluation of novel propargylamine MAO inhibitors incorporating the pyridyl moiety

Yours sincerely,

Prof. A. Petzer

Prof. J.P. Petzer

Mr. F. Viljoen

Chapter 1

Introduction

1.1. Background

Parkinson's disease (PD) is a progressive, neurodegenerative disorder which is caused by the loss of dopaminergic neurons from the substantia nigra pars compacta in the brain (Dauer & Przedborski, 2003). The dopaminergic neurons which degenerate in PD are specifically those of the nigrostriatal pathway which deliver dopamine to the striatum. The resulting functional deficit of dopamine (DA) in the striatum is responsible for the motor symptoms observed in PD (Dauer & Przedborski, 2003). Ever since its early clinical use in the 1960s, L-dopa has remained the most effective treatment for PD (Freitas *et al.*, 2016; Poewe & Antonini, 2015). L-Dopa (3,4-dihydroxy-L-phenylalanine) is a naturally occurring amino acid first isolated from the bean of *Vicia faba* in 1910–1911 (Hornykiewicz, 2010). In humans, dietary and clinically administered L-dopa is absorbed from the gastrointestinal tract via the amino acid transport machinery. L-Dopa also gains access to the central nervous system (CNS) via amino acid transporters at the blood-brain barrier and thus dietary amino acids may compete with L-dopa for transport in the intestine and at the blood-brain barrier (Camargo *et al.*, 2014). L-Dopa is extensively metabolised with approximately 70% of the oral dose undergoing pre-systemic decarboxylation to DA by the enzyme, aromatic L-amino acid decarboxylase (AADC), present in the stomach, lumen of the intestine, kidney and liver (Khor & Hsu, 2007; Contin & Martinelli, 2010). Another prominent metabolic pathway for L-dopa is 3-O-methylation by hepatic catechol-O-methyltransferase (COMT) to yield 3-O-methyldopa (Nutt & Fellman, 1984). L-Dopa thus has a short half-life of approximately 0.7 to 1.4 h (Contin *et al.*, 1990).

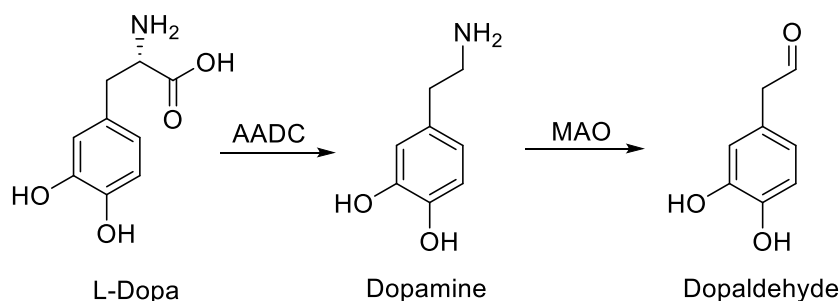


Figure 1.1: DA biosynthesis from L-dopa and the MAO-B catalysed catabolism thereof.

To overcome the poor bioavailability (~1%) of L-dopa, novel prodrugs will be designed, synthesised and evaluated. These prodrugs will be designed to improve the absorption of L-dopa from the gastrointestinal tract, protect L-dopa against peripheral metabolism and will allow L-dopa to permeate the blood-brain barrier more readily. In particular, L-dopa will be conjugated to a variety of carrier molecules including lazabemide, a selective and high affinity inhibitor of monoamine oxidase (MAO) B. Lazabemide will have the additional advantage that it may conserve the depleted supply of DA in the brain and enhance DA levels derived from L-dopa. Such L-dopa prodrugs may significantly improve the treatment of PD. Based on the interest and therapeutic potential of L-dopa prodrugs, the present study synthesises four carrier-linked prodrugs of L-dopa in which 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine are linked to the carboxylate of L-dopa.

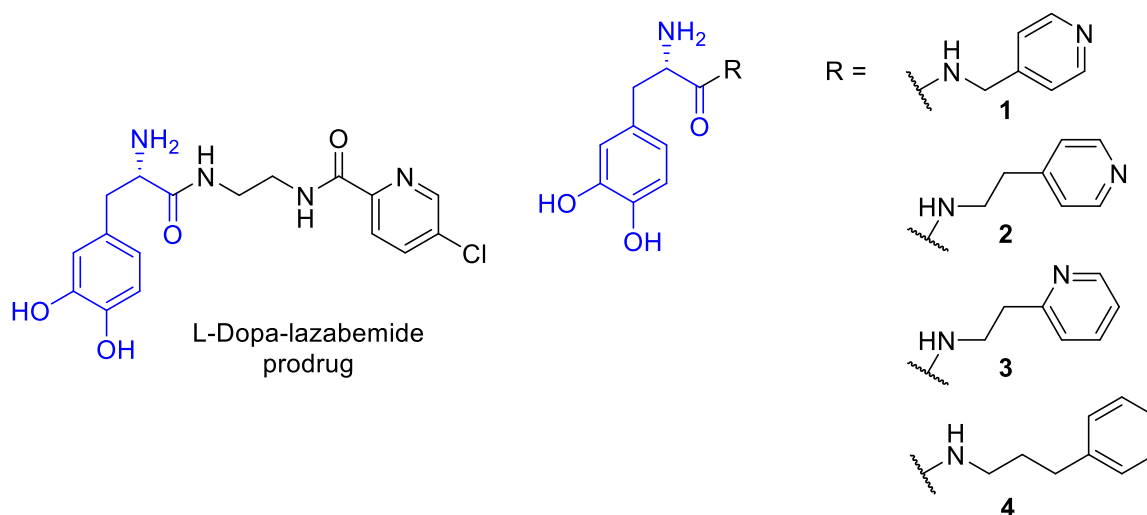


Figure 1.2: The structures of L-dopa-lazabemide prodrug and four additional L-dopa prodrugs investigated in this study.

Irreversible MAO inhibitors of the propargylamine class are well-known and include drugs that have been used clinically such as pargyline, selegiline and rasagiline, specifically for the treatment of depression and as add on therapy to L-dopa in Parkinson's disease. Based on the academic and clinical interest in propargylamines as MAO inhibitors, the present study synthesises a small series of novel propargylamine compounds that integrate the pyridyl moiety. Pyridyl-derived propargylamines have never before been investigated as potential MAO inhibitors. As part of an initial exploratory approach, N-(4-pyridylmethyl)propargylamine (**5a**), N-(2-pyridylmethyl)propargylamine (**5b**) and N-(3-pyridylmethyl)propargylamine (**5c**) were considered for this study (Fig. 1.3). Also included in this study are the N,N-dipropargyl analogues, compounds **6a–d**, isolated during the synthesis of **5**.

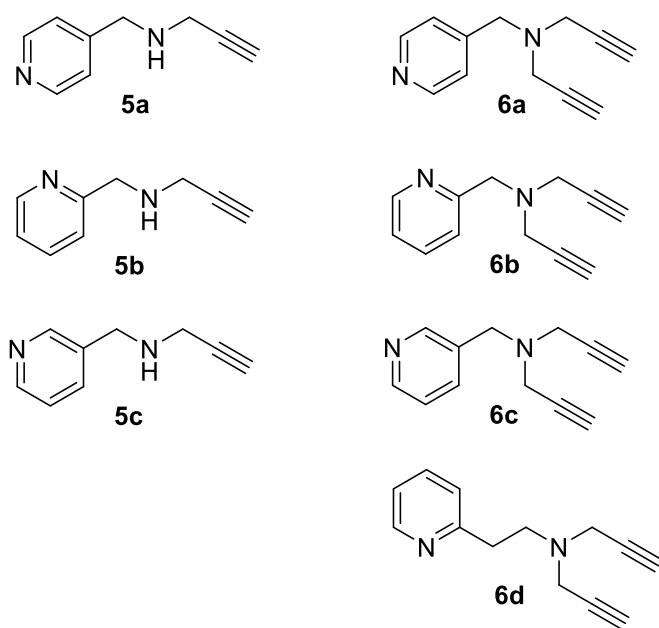


Figure 1.3: The structures of the propargylamine compounds that were synthesised and investigated in this study.

1.2. Lazabemide

Lazabemide [Ro 19-6327; N-(2-aminoethyl)-5-chloro-2-pyridinecarboxamide], a MAO-B specific inhibitor, was discovered in the 1980s (Cesura *et al.*, 1990; Cesura *et al.*, 1999). Lazabemide and related N-(2-aminoethyl)carboxamides (e.g. Ro 41-1049, Ro 16-6491) have the distinction of acting as mechanism-based inhibitors with a reversible mode of action. These inhibitors exhibit an initial competitive mode of binding, but are subsequently activated by MAO to form reversible adducts with the enzyme. The result is rapid and comprehensive MAO-B inhibition with enzyme activity only returning to baseline values 36 h after drug discontinuation (Dingemans *et al.*, 1997; Fowler *et al.*, 1993). Following inhibition with irreversible MAO-B inhibitors, the recovery period can be 40 days (Fowler *et al.*, 2005; Fowler *et al.*, 2015). Furthermore, for a pharmacological effect >90% MAO-B should be inhibited (Ramsay *et al.*, 2016; Fowler *et al.*, 2005). A dose of at least 0.4 mg/kg lazabemide given every 12 h provides >90% inhibition of brain MAO B in patients with early PD (Fowler *et al.*, 1993). Unfortunately, the development of lazabemide has been discontinued due to liver toxicity (Berlin *et al.*, 2002). The mechanism by which lazabemide inhibits MAO-B is not completely understood. The present study proposes to characterise the *in vitro* MAO inhibition properties of lazabemide with respect to potency, isoform selectivity and reversibility.

The present study also proposes a novel L-dopa-lazabemide prodrug to overcome the problems associated with L-dopa absorption and metabolism. As mentioned, lazabemide is a specific inhibitor of MAO-B, with a reversible mechanism-based mode of action (Cesura *et al.*, 1990; Cesura *et al.*, 1999; Binda *et al.*, 2003; Edmondson *et al.*, 2004). MAO-B inhibitors such as lazabemide are considered useful agents in the therapy of PD and are frequently combined with L-dopa (Youdim *et al.*, 2006). By blocking the central MAO-B-catalysed metabolism of DA, these drugs are thought to slow the depletion of dopamine stores and to elevate DA levels produced by exogenously administered L-dopa (Finberg *et al.*, 1998). In addition to enhancing the absorption and delivery of L-dopa, lazabemide released after activation of the prodrug will further bolster DA levels derived from L-dopa.

1.3. L-Dopa prodrugs

L-Dopa is extensively metabolised with approximately 70% of an oral dose undergoing pre-systemic decarboxylation to DA by the enzyme, AADC, present in the stomach, lumen of the intestine, kidney and liver (Khor & Hsu, 2007; Contin & Martinelli, 2010). Another prominent metabolic pathway for L-dopa is 3-O-methylation by hepatic COMT to yield 3-O-methyldopa (Nutt & Fellman, 1984). L-Dopa thus has a short half-life of approximately 0.7 to 1.4 h (Contin *et al.*, 1990). Despite these and other shortcomings, L-dopa is used as DA replacement therapy in PD and since its first use in the 1960s, remains the most effective treatment (Freitas *et al.*, 2016; Poewe & Antonini, 2015). To enhance bioavailability and limit peripheral metabolism, L-dopa is co-administered with inhibitors of AADC such as carbidopa or benserazide (Seeberger & Hauser, 2015). This greatly enhances the systemic bioavailability of an oral L-dopa dose. The metabolism of L-dopa may be further reduced and efficacy enhanced by administering COMT inhibitors such as entacapone (Nutt, 2000; Learmonth *et al.*, 2004; Nissinen *et al.*, 1992). Several experimental prodrugs of L-dopa have also been designed and evaluated (Di Stefano *et al.*, 2011). For example, a prodrug in which L-dopa is linked via a biodegradable carbamate to entacapone has been reported (Savolainen *et al.*, 2000; Leppänen *et al.*, 2002). In this respect, prodrugs with benserazide linked to L-dopa have also been designed (Di Stefano *et al.*, 2006).

Based on the interest and therapeutic potential of L-dopa prodrugs, the present study synthesises four carrier-linked prodrugs of L-dopa in which 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine are linked to the carboxylate of L-dopa (Fig. 1.2). The key physicochemical and biochemical parameters of the prodrugs will subsequently be evaluated to assess the potential of these prodrugs as vehicles to enhance the absorption and central delivery of L-dopa. These selected carriers will be linked

to L-dopa at the carboxylate with the primary aminyl functional group. This would protect the carboxylic acid of L-dopa against peripheral decarboxylation and possibly enhance passive diffusion permeability by elimination of the carboxylate charge. Additionally, unlike L-dopa, the prodrugs do not contain the carboxylate group, which is known to reduce membrane permeation of small organic compounds (Gleeson, 2008; Manallack *et al.*, 2013).

1.4. Propargylamine MAO inhibitors

Propargylamine compounds are well known to act as inhibitors of the MAO enzymes and have been used in the clinic to treat neuropsychiatric and neurodegenerative disorders such as major depressive disorder and PD (Youdim *et al.*, 2006). In this regard, the propargylamine compound, clorgyline, is a MAO-A specific inhibitor while selegiline and rasagiline, also propargylamines, exhibit specificity for MAO-B. Pargyline, in turn, is a non-specific propargylamine inhibitor (Youdim *et al.*, 2006). In depressive illness and PD, MAO inhibitors act by reducing the MAO-catalysed metabolism of the relevant neurotransmitters and thereby elevating neurotransmitter levels in the brain (Ramsay *et al.*, 2016). Thus, MAO-A inhibitors are used for the treatment of depression since they enhance central levels of serotonin and noradrenaline (Lum & Stahl, 2012), while MAO-B inhibitors block the metabolism of central DA and are applied in PD therapy (Youdim *et al.*, 2006; Youdim & Bakhle, 2006). In this respect, MAO-B inhibitors are often used as adjuvants to L-dopa, the direct metabolic precursor of DA, in an effort to further enhance DA levels in the brain. Currently, selegiline and rasagiline are registered for the treatment of PD while pargyline, now discontinued, has been used as an antihypertensive drug.

Based on the academic and clinical interest in propargylamines as MAO inhibitors, the present study synthesises a small series of novel propargylamine compounds that incorporate the pyridyl moiety. Pyridyl-derived propargylamines have not thus far been investigated as potential MAO inhibitors. As part of a preliminary exploratory approach, N-(4-pyridylmethyl)propargylamine (**5a**), N-(2-pyridylmethyl)propargylamine (**5b**) and N-(3-pyridylmethyl)propargylamine (**5c**) will be considered for this study (Fig. 1.3). Also included in this study are the N,N-dipropargyl analogues, compounds **6a–d**, isolated during the synthesis of **5a–c**. This study thus investigated the effect of the pyridyl moiety on the MAO inhibition properties of pyridyl-derived propargylamines.

1.5. Rationale for this study

In an effort to overcome the problems with peripheral L-dopa metabolism, delivery difficulties and insufficient conversion of L-dopa to DA in the brain tissue, DA prodrugs and L-dopa prodrugs have been previously proposed (Di Stefano *et al.*, 2008). From these studies, a promising approach that emerged was the design of a L-dopa prodrug in which L-dopa is linked to benserazide, a peripheral decarboxylase inhibitor (Di Stefano *et al.*, 2006). The L-dopa-benserazide prodrug was designed to improve the absorption of L-dopa from the gastrointestinal tract and then release L-dopa and benserazide in the peripheral tissues. This approach has the advantage over L-dopa monotherapy because benserazide inhibits the peripheral metabolism of L-dopa and thereby increases the available L-dopa for uptake into the brain. The most important disadvantage of this approach is that the prodrug does not deliver L-dopa in the brain. Subsequently L-dopa does not diffuse freely across the blood-brain barrier, and is dependent upon uptake by the L-amino acid transport system, its bioavailability to the brain remains meager. To overcome these problems, the present study proposes new L-dopa prodrugs. A potential approach is to design a L-dopa prodrug in which L-dopa is conjugated to a suitable carrier molecule. Ideally the carrier molecule confers good properties to the prodrug such as good solubility, high permeability and a LogD of 1-3. This may yield a prodrug with excellent physicochemical properties and good potential to deliver L-dopa to the brain (Hoon, 2013). The envisioned prodrugs may have the following advantages:

- (1) Enhanced lipophilicity and therefore absorption from the gastrointestinal tract.
- (2) Protection against peripheral decarboxylation in the liver since the carrier is linked to the carboxyl group of L-dopa. Peripheral inhibitors of amino acid decarboxylase (carbidopa and benserazide) may therefore not be necessary.
- (3) Based on the enhanced lipophilicity of the prodrugs, the prodrugs may, in contrast to L-dopa, diffuse freely across the blood-brain barrier.
- (4) Since L-dopa and the carrier drugs are linked via a more stable amide, more time is allowed for the prodrugs to diffuse into the brain before hydrolysis. This would limit peripheral hydrolysis of the prodrugs and maximise release of L-dopa in the brain. It is important to note that, in contrast to other amides, in general amides formed with amino acids (such as L-dopa) are labile enough to be metabolically hydrolysed *in vivo*. Amides of amino acids are, however, stable enough to allow for equilibration across the blood-brain barrier.
- (5) Since the prodrugs may enhance the efficacy and delivery of L-dopa, lower doses will be permitted and therefore an improved safety and side effect profile for L-dopa will be obtained.

The second section of this study will synthesise a small series of novel propargylamine compounds that incorporate the pyridyl moiety. The pyridine heterocycle has a low pKa, and is thus weakly basic. At physiological pH the pyridyl moiety is expected to be uncharged, which is an advantage for MAO inhibition since MAO substrates are thought to bind in the unionised form to the active site (Edmondson *et al.*, 2009). Furthermore, due to the electronegative nitrogen, pyridine is relatively electron deficient and would be expected to undergo differing intermolecular interaction compared to the phenyl found in inhibitors such as pargyline and selegiline. This study will therefore compare the MAO inhibition profiles of these very different classes of propargylamine-containing compounds (e.g. pyridinyl- versus phenyl-containing compounds).

1.6. Study objectives

1. An L-dopa-lazabemide prodrug will be designed and synthesised. Key physicochemical and biochemical properties of the prodrug including lipophilicity (log D), solubility, passive diffusion permeability, pKa, chemical and metabolic stability as well as cytotoxicity will be measured. An *in vivo* study in mice will evaluate the potential of the prodrug to alter central monoamine neurotransmitter levels with L-dopa as comparator.
2. Four carrier-linked prodrugs of L-dopa in which 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine are linked to the carboxylate of L-dopa will be synthesised. Key physicochemical and biochemical parameters of the prodrugs will be evaluated to assess the potential of these prodrugs as vehicles to enhance the absorption and central delivery of L-dopa. These parameters are lipophilicity (logD), passive diffusion permeability, pKa, chemical and metabolic stability as well as cytotoxicity.
3. In this study, the behaviour of lazabemide in *in vitro* MAO inhibition studies will be characterised. Potency, isoform selectivity and reversibility will be evaluated.
4. This study will also synthesise a small series of novel propargylamine compounds that incorporate the pyridyl moiety. This study will thus investigate the effect of the pyridyl moiety on the MAO inhibition for comparison with phenyl-containing propargylamine inhibitors such as pargyline.

Chapter 2

Literature review

2.1. Introduction

The British physician, James Parkinson published “An Essay on the Shaking Palsy” in 1817 that first described the clinical features of Parkinson’s disease (PD), the second most common neurodegenerative disorder (Parkinson, 1817). A resting tremor, disturbances of posture, and paucity or slowing of volitional movement, characterises PD. The primary cause of PD is unknown, but the neuropathology is characterised by progressive degeneration of pigmented neurons of nigrostriatal pathway which projects from the substantia nigra to the extrapyramidal motor control centre of the basal ganglia (caudate-putamen and other parts of the corpus striatum). This pathway consists of dopaminergic neurons, and degeneration thereof results in a functional loss of dopamine (DA) in the striatum. A variable loss of other pigmented monoaminergic neurons in the brainstem, in particular those producing norepinephrine, also exists (Greenamyre & Hastings, 2004).

Pharmacotherapy for PD has rationally been based on the replacement of DA lost due to selective and idiopathic degeneration of dopaminergic neurons. Since the early 1960’s, this has been accomplished by administering large oral doses of L-dihydroxyphenylalanine (L-dopa), the direct metabolic precursor of DA. Later, synthetic DA receptor agonists and agents that inhibit the metabolic breakdown of DA (or L-dopa) were also employed. The pathophysiological mechanisms underlying neuronal degeneration in PD is still unknown, with pharmacotherapy for PD remaining palliative and symptomatic. Both the effectiveness and tolerability of current treatments limit pharmacotherapy, especially late in the progression of the disease. A better understanding of the fundamental pathogenesis of PD will lead to improved symptomatic and anticipated curative pharmacotherapy (Olanow *et al.*, 2009).

2.2 Parkinson’s disease

PD typically presents in mid- or late life, most often at ages 55-65. The incidence in the population over the age of 65 is approximately 1-2%, with the incidence increasing to 3-5% at the age of 84 (Alves, *et al.*, 2008). A tetrad of symptoms characterises PD: (1) bradykinesia or slow-initiation and paucity of voluntary movements, (2) resting tremor that improves with voluntary activity, (3) postural disturbances including falls, and (4) rigidity of muscle and joint motility. In some cases, dysfunction of autonomic functions mediated by the potentially

affected central noradrenergic sympathetic nervous system, with loss of norepinephrine neurons of the locus coeruleus, may be present (Forno *et al.*, 1996). Dementia is approximately six-fold more prevalent among elderly PD patients, and other neuropsychiatric disturbances, including hallucinations and depression, can exist (Olanow *et al.*, 2009). The etiologically diverse condition, parkinsonism, includes other idiopathic degenerative disorders such as idiopathic Lewy body dementia and multiple system atrophy with dysautonomia (Shy-Drager syndrome) as well as post encephalitic parkinsonism, such as Van Economo's encephalitis lethargica, that arose with influenza epidemics of the early 20th century. Also, included in parkinsonism are the effects of neurotoxins such as certain heavy metals (manganese), pyridiniums such as 1-methyl-4-phenylpyridinium, and the marine cyanobacteria product, β -N-methylamino-L-alanine (Olanow *et al.*, 2009).

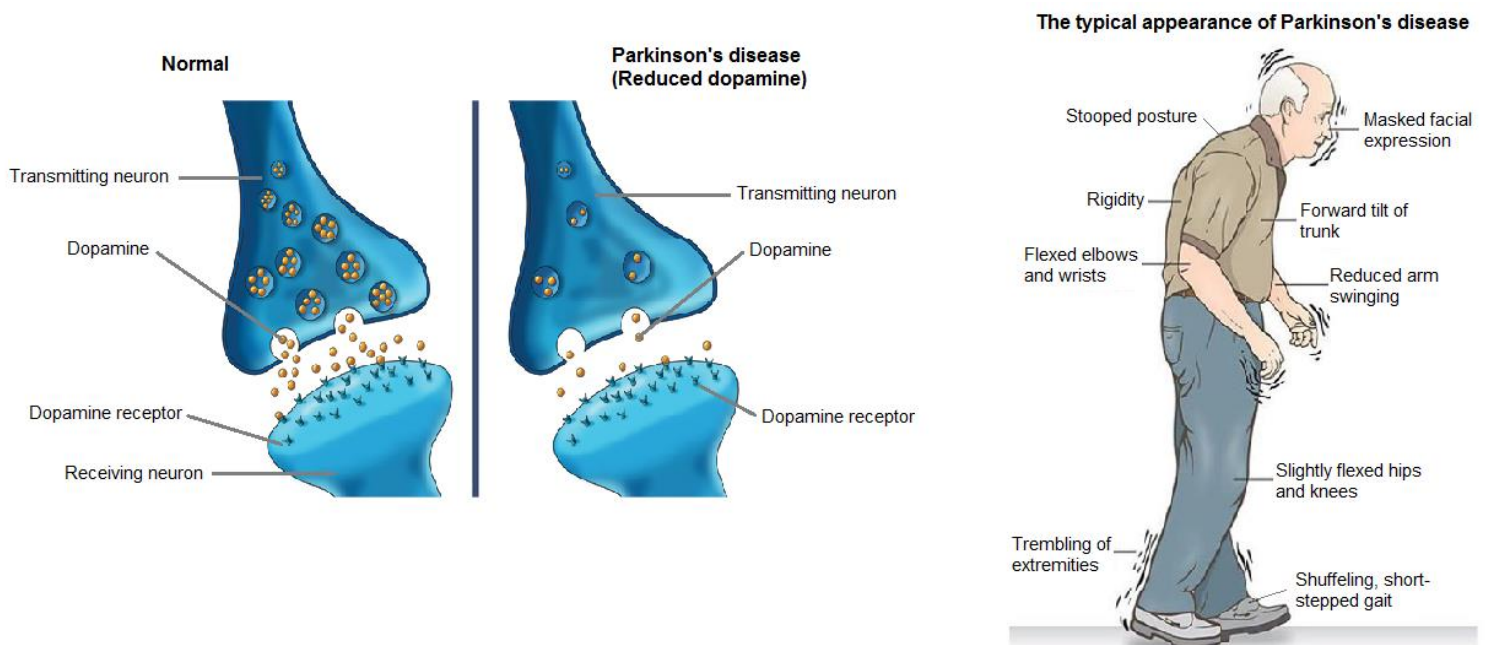


Figure 2.1: A normal vs. a PD dopaminergic neuron and the typical appearance of PD (Massing, 2016).

2.3. Background, pathology and symptoms of PD

The loss of dopaminergic neurons of the substantia nigra is the hallmark of PD, and is also associated with degeneration of many brainstem, limbic and midbrain neurons that leads to alterations in the activity of brain networks that control movement. Dysregulation of interacting inhibitory and excitatory pathways is the consequence of this degenerative process and leads to a movement disorder that is characterised by difficulty to initiate movements, muscular rigidity, balance problems, tremor, autonomic disturbances and cognitive impairment. Initial treatment that benefits most patients is the pharmacological facilitation of dopaminergic

neurotransmission, although advanced PD patients often develop unacceptable drug-related complications such as dyskinesia and motor fluctuations. Interventions that directly increase dopaminergic neurotransmission might worsen dyskinesia and other dopamine-related complications such as hallucinations, once these complications have begun (Kaplitt *et al.*, 2007). Environmental risk factors for PD have received considerable attention, although the importance of the genetics underlying susceptibility to PD is increasingly being recognised. Familial forms of PD (<10% of cases) are relatively rare, however, identification of single genes linked to the disease has yielded crucial insights into possible mechanisms of PD pathogenesis (Greenamyre & Hastings, 2004).

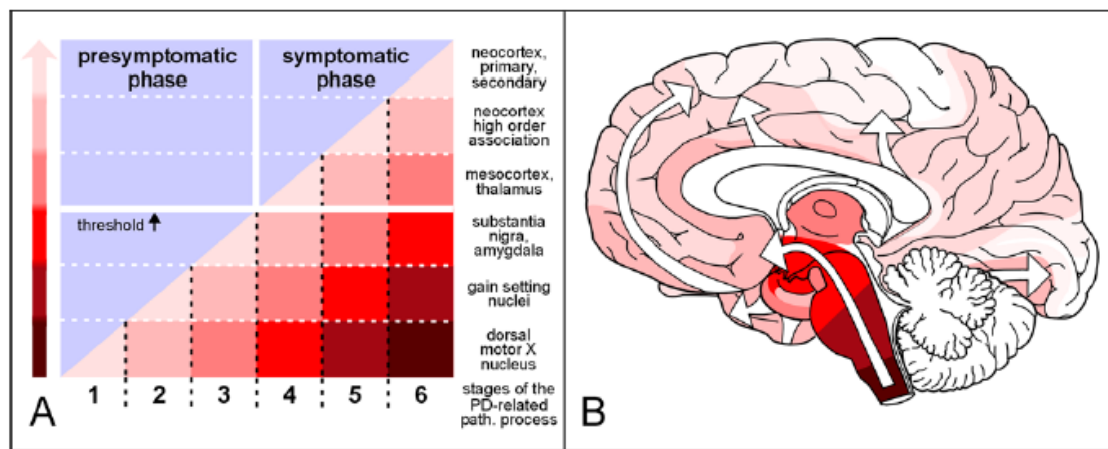


Figure 2.2: Parkinson's disease presymptomatic and symptomatic phases. (A) The appearance of Lewy bodies marks the presymptomatic phase in the brain of asymptomatic persons. Black arrows indicate the individual neuropathological threshold in the symptomatic phase. On the right, the increasing slope and intensity of the coloured areas below the diagonal indicates the growing severity of the pathology in vulnerable brain regions. The darker degrees of shading in the coloured arrow, on the left, shows the severity of the pathology. (B) This diagram shows the ascending pathology process in white arrows, where the shading intensity of the coloured areas corresponds to those in A (Braak *et al.*, 2004).

2.4. Mechanisms of pathogenesis in PD

Oxidative damage has consistently been implicated from post-mortem studies in PD pathogenesis, however, the source of the damage has not been clear. The leading reactive oxygen producing species implicated in PD are dopamine metabolism and dysfunction of mitochondria (Jenner, 2003). After a group of intravenous drug users developed acute, permanent parkinsonism from injecting a contaminant [(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)] of a synthetic opiate in 1982, it became obvious that “environmental” chemicals might be the culprits in some cases. Environmental chemicals,

such as pesticides, might be contributing factors in PD pathogenesis according to epidemiological studies. It was discovered that MPTP inhibits the first enzyme complex of the mitochondrial electron transfer chain (complex 1), which prompted several groups to uncover complex 1 mitochondrial defects in the brains and platelets of patients with PD (Langston *et al.*, 1983). Oxidative stress, disrupted mitochondrial complex 1 activity and environmental chemicals may all participate in the dopaminergic neuron death in PD. PD genetic studies have led in other directions and the first causative, but rare mutation, was found in the α -synuclein gene. Subsequently, it was shown that Lewy bodies consist mainly of α -synuclein, a phosphoprotein of uncertain function, even in the more common sporadic cases of PD where no mutation has been found (Dawson & Dawson, 2003).

Another cause of PD is the overexpression of wild-type α -synuclein. The proteins appear to be nitratively and oxidatively modified and cross-linked to form insoluble aggregates in PD patients. In this process, the formation of dopamine-quinone adducts may be important.

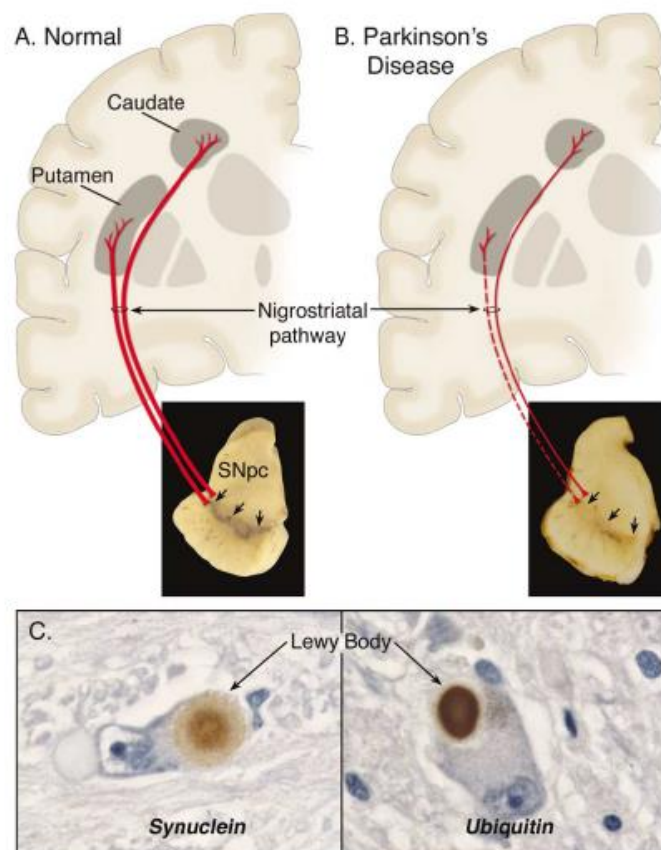


Figure 2.3: Neuropathology of Parkinson's disease (Greenamyre & Hastings, 2004).

Another familial PD mutation affects ubiquitin carboxyl-terminal hydrolase-1 (UCHL1) which is a component of the cell's ubiquitin-proteasome system (UPS) that degrades damaged proteins. A much more common causative mutation in an ubiquitin E3 ligase called parkin,

affects another component of the UPS. Finally, DJ-1, a protein that participates in the oxidative stress response, has been reported to possess pathogenic mutations in PD. Disease-causing mutations thus implicate aberrant protein handling and oxidative stress as key events in PD pathogenesis. Normal mitochondrial activity may be affected by environmental chemicals (both natural and synthetic) as well as by mitochondrial DNA polymorphisms and mutations in nuclear genes. α -Synuclein overexpression and inactivation of parkin can also cause mitochondrial dysfunction (Singleton *et al.*, 2003).

Increased production of reactive oxygen species, common by-products of many types of mitochondrial impairment, may be the source of the oxidative damage found in PD brains. Mitochondrial complex 1 inhibition, in turn, leads to increased production and aggregation of α -synuclein. Aggregation may be promoted by dopamine metabolites and, perhaps, by the formation of highly reactive dopamine-quinones in dopaminergic neurons, which can form adducts with proteins such as α -synuclein, crosslink them, and facilitate their aggregation. There seems to be multiple, diverse causes of PD, yet the pathogenesis of this disease appears to be converging on common mechanisms such as mitochondrial impairment, oxidative stress and protein mishandling, which are all tightly linked (Greenamyre & Hastings, 2004).

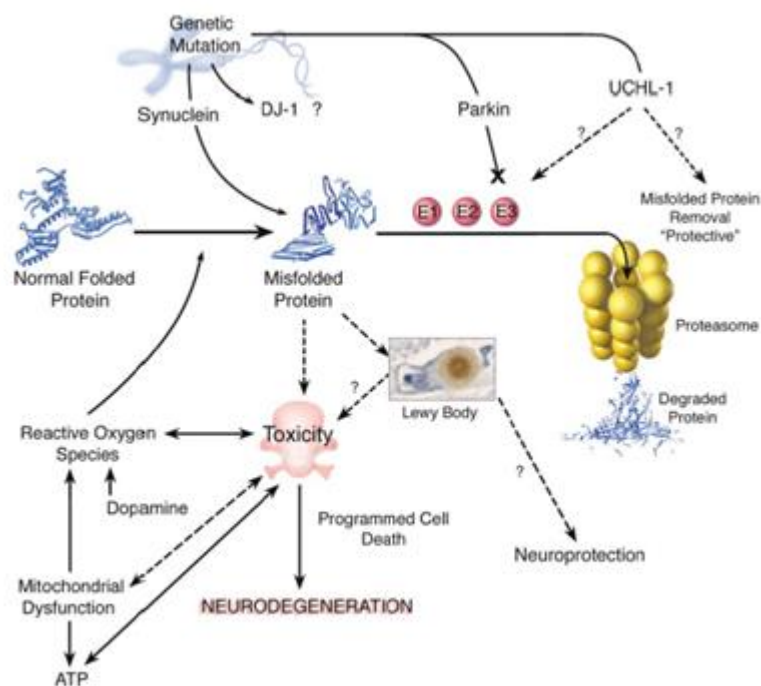


Figure 2.4: Mechanism of neurodegeneration in PD (Greenamyre & Hastings, 2004).

2.5. Treatment

2.5.1. L-Dopa therapy

More than 40 years after its introduction, L-dopa remains the most effective symptomatic pharmacotherapy for PD (Olanow *et al.*, 2009). Although long-term efficacy, adverse effects, and even potential neurotoxicity remain controversial for this amino acid precursor of DA, most PD patients derive a substantial benefit from L-dopa throughout their illness. Life expectancy increases with L-dopa usage among PD patients, especially if instituted early in the illness course (Rajput *et al.*, 1997). L-Dopa treatment is only effective when it penetrates the central nervous system (CNS) and is locally decarboxylated to DA. DA cannot cross the blood-brain diffusion barrier, because its amino moiety is protonated under physiological conditions ($pK_a = 10.6$), which makes it excessively hydrophilic (Nagatsu *et al.*, 1973). On the other hand, L-dopa, the amino acid precursor of DA, is less basic and polar at physiological pH, and can penetrate the CNS more freely, in part facilitated by transport into the brain by aromatic and neutral aliphatic amino acid transport systems (Baldessarini & Fischer, 1977).

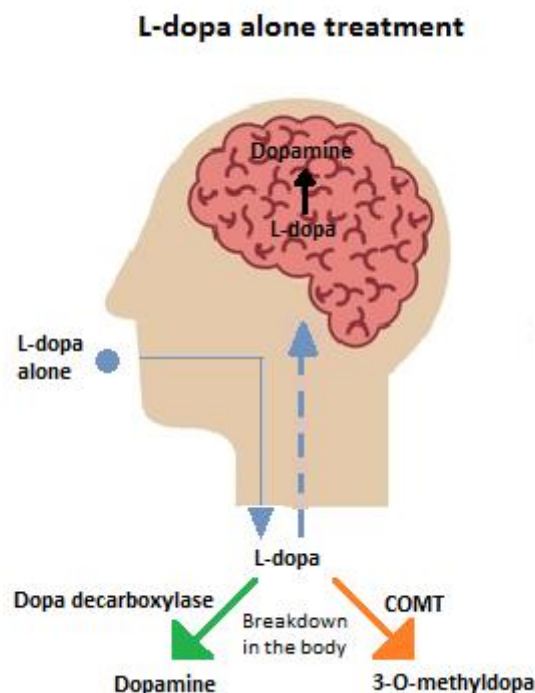


Figure 2.5: An illustration of the breakdown of L-dopa in the body (Satoh *et al.*, 2015).

2.5.2. DA agonists

2.5.2.1. Apomorphine-type DA receptor agonists.

As a useful adjunct in the therapy of PD, apomorphine (an agonist for both D1- and D2-type DA receptors) was resurrected after years of neglect following promising early observations (Cotzias *et al.*, 1967; Schwab *et al.*, 1951). Its clinical use was discouraged because of a lack

of oral bioavailability, short duration of action and potent central emetic action. Despite this, apomorphine was approved in the UK for the control of refractory motor dysfunction and fluctuations in responses to L-dopa or DA agonist treatment (“on-off” syndrome) (Colosimo *et al.*, 1994; Mouradian & Chase, 1997; Stocchi *et al.*, 2008). An acute dose of apomorphine leads to improved motility in response to L-dopa treatment (Frankel *et al.*, 1990; Hughes *et al.*, 1993). Apomorphine has a pKa of 9 and is sufficiently lipophilic to cross the blood-brain barrier (Campbell *et al.*, 1982).

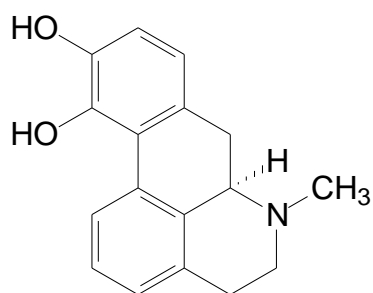


Figure 2.6: The structure of apomorphine.

2.5.2.2. Ergot-type DA receptor agonists

Bromocriptine, a partial-agonist at D₂ and D₃ DA receptors, is an ergot alkaloid-peptide (Newman-Tancredi *et al.*, 2002). Bromocriptine along with other D₂ partial-agonist ergolines act as D₂ agonists with antiparkinsonian and some mood-elevating effects (Baldessarini & Tarsy, 1980). After oral administration, bromocriptine is absorbed, where after approximately 90% of the dose undergoes extensive first-pass hepatic metabolism. The remaining 10% is hydrolysed in the liver to inactive metabolites and eliminated via the bile. Cabergoline is another ergot-type DA agonist and acts as a full D₂ receptor agonist and partial D₃ and D₄ receptor agonist (Millan *et al.*, 2002; Newman-Tancredi *et al.*, 2002). This drug has a 48-hour half-life (Olanow *et al.*, 2009), however it has a poor efficacy compared to L-dopa (Hutton *et al.*, 1993).

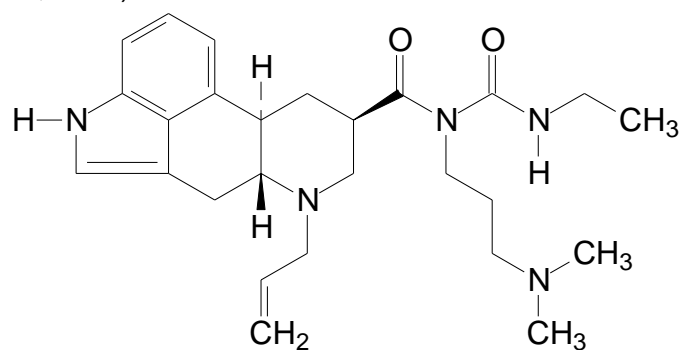


Figure 2.7: The structure of cabergoline.

2.5.2.3. Other small-molecule DA receptor agonists

The most commonly prescribed and relatively well tolerated direct DA agonists for PD is pramipexole and ropinirole (Standaert & Young, 2006). The introduction of these drugs for the advanced stages of PD was to limit fluctuations in response to L-dopa therapy as well as a “rescue” therapy when L-dopa became ineffective. Potential damage to DA neurons by L-dopa have encouraged the use of these agents as first-line treatments. A relatively prolonged dopaminergic action (long half-life) is an added advantage of these agents, as this provides more sustainable clinical benefits with less risk of fluctuation of neurological status than with L-dopa (Rascol *et al.*, 2000). Some adverse effects include, initial nausea and vomiting, postural hypotension and fatigue. Additional psychotic reactions when DA agonists are given alone or with L-dopa include hallucinations, confusion, delusions and delirium.

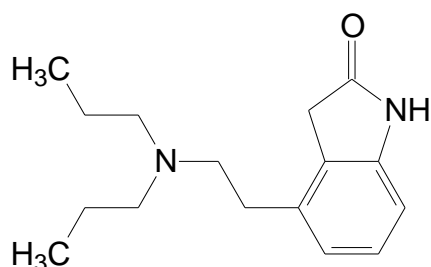


Figure 2.8: The structure of ropinirole.

2.5.3. Aromatic amino acid decarboxylase (AADC) inhibitors

The most effective treatment for PD currently are the combinations of benserazide and L-dopa and carbidopa and L-dopa. Carbidopa is tenfold less potent than benserazide as a peripheral AADC inhibitor in both animals and humans. Benserazide inhibits the decarboxylation of L-dopa only in the extracerebral tissues, thus permitting the formation of DA in the striatum and in the hypothalamus. Because benserazide is well tolerated, relatively nontoxic even when used chronically and is the most potent peripheral AADC inhibitor presently available, it appears to be the drug of choice for the development of controlled release formulations in which L-dopa is combined with a peripheral AADC inhibitor. These controlled-release systems may reduce the clinical fluctuations in patients where the “wearing-off” and “on-off” phenomena occurs (Da Prada *et al.*, 1987).

L-dopa plus AADC inhibitor (benseraside)

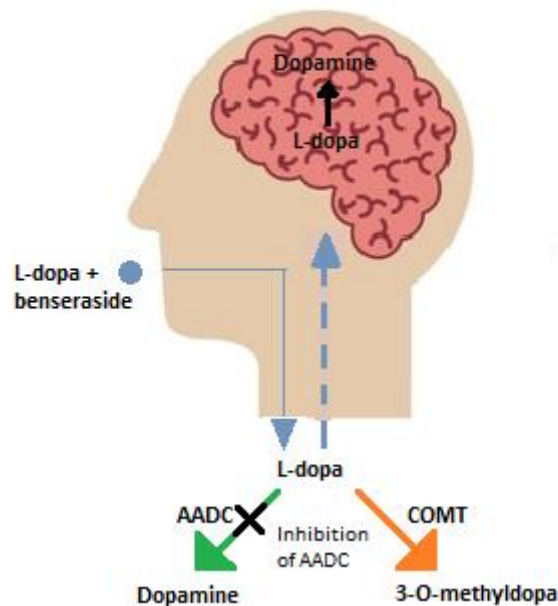


Figure 2.9: An illustration of the breakdown of L-dopa in the presence of an AADC inhibitor in the body (Sato *et al.*, 2015).

2.5.4. Catechol-O-methyltransferase (COMT) inhibitors

When L-dopa is given as monotherapy, its extensive peripheral metabolism results in very limited access of the amino acid to the CNS. L-Dopa is rapidly decarboxylated by AADC and 3-O-methylated by COMT. The COMT enzyme converts L-dopa and catecholamines to their methoxy derivatives to yield reaction products such as 3-O-methyl-dopa and 3-O-methyl-DA, as well as the 3-O-methylated, deaminated compound homovanillic acid (HVA), the major final metabolite of DA metabolism in humans (Factor *et al.*, 2001; Kuno, S., 1997; Standaert & Young, 2006; Teräväinen *et al.*, 2001). Reversible COMT inhibitors are currently used clinically in PD therapy and include tolcapone and entacapone. Entacapone has a shorter duration of action (2 hours) and acts mostly in the periphery, where tolcapone has a relatively longer duration of action (8-12 hours) and acts in both the brain and periphery. The common adverse effects of these agents are nausea, vivid dreams, confusion and hallucinations, which can be attributed to an increase in brain DA (Olanow & Watkins, 2007).

L-dopa plus COMT inhibitor (tolcapone)

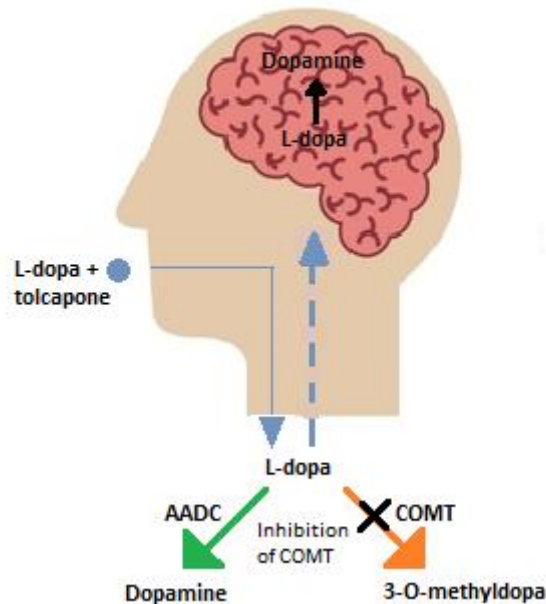


Figure 2.10: An illustration of the breakdown of L-dopa in the presence of a COMT inhibitor in the body (Sato *et al.*, 2015).

2.5.5. Monoamine oxidase (MAO) inhibitors

MAO-A and MAO-B are enzymes that catalyse the oxidation of biogenic and xenobiotic amines (Bortolato & Shih, 2011; Edmondson *et al.*, 2007). These two isoforms of MAO have different substrate preference and inhibitor selectivity. For example, the irreversible inhibitor, clorgyline, inhibits the oxidation of serotonin and norepinephrine that is catalysed by MAO-A. MAO-B on the other hand uses benzylamine and phenylethylamine as substrates, and is irreversibly inhibited by selegiline, also an irreversible MAO inhibitor. DA is the common substrate for both isoforms (Youdim & Bakhle, 2006). DA is, however, preferentially deaminated by MAO-B in the human nigrostriatal system, and MAO-B inhibitors are thus used to block the MAO-catalysed metabolism of DA and increase DA bioavailability in the PD brain. MAO-B inhibitors therefore are expected to increase DA levels and thus compensate the nigrostriatal deficit in DA and consequently provide symptomatic relief of the motor symptoms of PD (Finberg, 2014; Riederer & Laux, 2011; Robakis & Fahn, 2015; Youdim & Bakhle, 2006). Selegiline and the second-generation drug, rasagiline, are currently used for the treatment of PD and is often combined with L-dopa. Modest improvement in an early phase of PD and adjunctive enhancement of dopaminergic function in later stages are provided by MAO-B inhibitors.

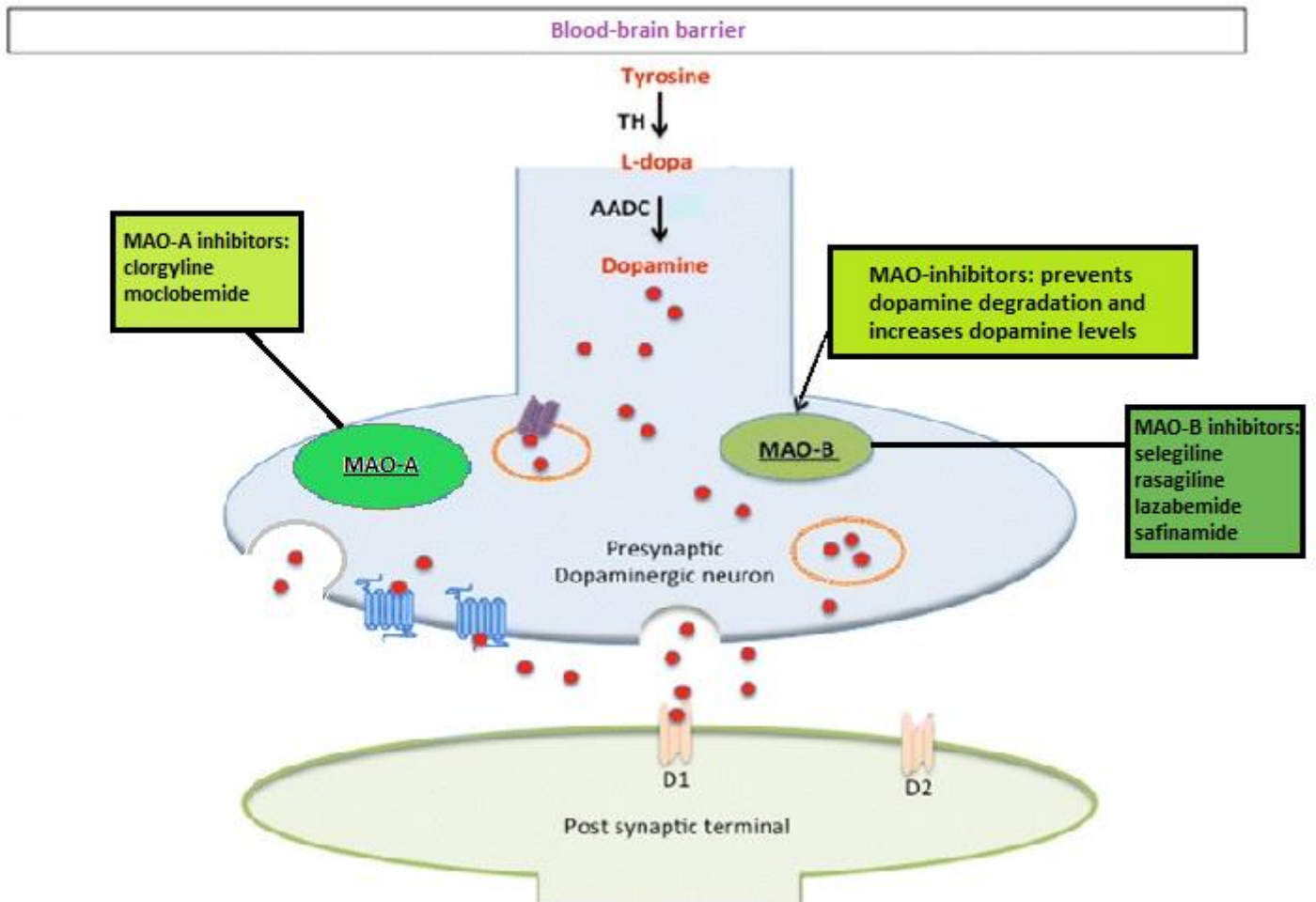


Figure 2.11: A schematic diagram of dopaminergic neurotransmission and the role of MAO-inhibitors (Ng *et al.*, 2014).

2.5.6. Other agents for PD therapy

2.5.6.1. Anticholinergic agents

An increase in cholinergic neurotransmission (e.g. the cholinesterase inhibitor physostigmine and the direct agonist carbachol) is known to aggravate parkinsonism in humans, whereas centrally active muscarinic antagonists (such as belladonna alkaloids, including atropine), have beneficial effects (Felder *et al.*, 2000; Standaert & Young, 2006). Before L-dopa was discovered, drug therapy for parkinsonism and PD depended on the limited efficacy of the natural belladonna alkaloids and newer synthetic antimuscarinic alkaloids, as well as antihistamines that also exert central antimuscarinic actions. Benztropine, biperiden, the antihistamine diphenhydramine, the phenothiazine ethopropazine, orphenadrine, procyclidine and trihexylphenidyl are synthetic central anticholinergic agents. These drugs are used to control parkinsonism and the adverse extrapyramidal neurological effects of potent D2-

receptor antagonist antipsychotic agents, for which they are quite effective (Miyawaki *et al.*, 1997; Standaert & Young, 2006). Central antimuscarinic agents, however, have limited therapeutic benefit in PD. These drugs have a range of adverse effects because of their blockade of peripheral parasympathetic function, including dry mouth, impaired visual accommodation, urinary retention, constipation and tachycardia. Delirium, marked confusion, memory impairment and psychotic symptoms are some of the CNS adverse effects. These agents are still sometimes employed in the treatment of PD in combination with L-dopa, to help control the tremor, despite their relatively unfavourable benefit to risk ratio (Felder *et al.*, 2000).

2.5.6.2. Adenosine receptor antagonists

The nucleoside signalling molecule, adenosine, acts at four G-protein-coupled receptor (GPCR) subtypes, A₁, A_{2A}, A_{2B} and A₃. A high concentration of the A_{2A} receptor can be found in the striatum, nucleus accumbens, olfactory tubercle and it has been shown that the A_{2A} receptor colocalises with D2 receptors in these brain regions (Fink *et al.*, 1992). It is expected that antagonism of A_{2A} receptors would increase GABA-mediated inhibition of the medium spiny output neurons to help compensate for the loss of D1 receptor-stimulated GABA release and D2 receptor-mediated inhibition of these neurons in PD (Richardson *et al.*, 1997).

A_{2A} receptor activation, in turn, stimulates the release of acetylcholine in the striatum (Kurokawa *et al.*, 1996). By reducing striatal cholinergic neurotransmission, A_{2A} receptor antagonists may have additional benefits in PD because muscarinic acetylcholine receptor antagonists can ameliorate some PD symptoms (Trevitt *et al.*, 2009). A potent and selective adenosine A_{2A} receptor antagonist, istradefylline, was shown to improve motor disability in primate models of PD (Kanda *et al.*, 1998). In a double-blind, placebo-controlled clinical trial, patients that are stabilised on L-dopa and other PD drug regimens exhibited significant reductions in motor fluctuations when treated with istradefylline. Istradefylline was also well tolerated (Hauser *et al.*, 2008).

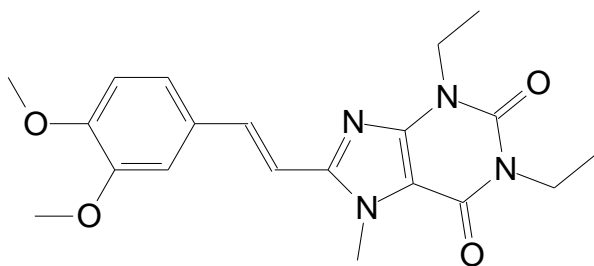


Figure 2.12: The structure of istradefylline.

2.5.6.3. Serotonin 5-HT_{1A} agonists

Neurotransmission mediated by 5-hydroxytryptamine (5-HT; serotonin) may be dysfunctional in the basal ganglia of patients with PD, and excessive serotonergic transmission may contribute to dyskinesia associated with dopaminergic treatments (Melamed *et al.*, 1996). Presynaptic expression of 5-HT_{1A} receptors on 5-HT terminals limits serotonin release via autoreceptors (Blier *et al.*, 1998). Alleviation of dopaminergic dyskinesia in PD occurs with the activation of 5-HT_{1A} receptors that decreases 5-HT release (Bibbiani *et al.*, 2001). Parkinsonism-like catalepsy induced by haloperidol can be reversed by 5-HT_{1A} receptor stimulation (Christoffersen *et al.*, 1998), while loss of nigrostriatal DA neurotransmission in PD may also be counteracted by 5-HT_{1A} receptor activation. An aminomethylchroman derivative, sarizotan, is a full-efficacy agonist relative to 5-HT and is well characterised regarding its neurobiochemical activity (Bartoszyk *et al.*, 2004). Sarizotan has appreciable affinity at human DA D₂, D₃, and D₄ receptors and is a partial D₂ agonist with no apparent activation of D₃ and D₄ receptors. In PD patients receiving L-dopa therapy, the addition of sarizotan pharmacotherapy produces significant elevation in periods of time without dyskinesia, and significant reduction in periods of time with troublesome dyskinesia (Olanow *et al.*, 2004).

2.5.6.4. Glutamate antagonists

The over-activity of thalamocortical excitatory glutamatergic input to the motor cerebral cortex may contribute to dyskinesia associated with L-dopa therapy. It is hypothesised that excessive release of glutamate due to synaptic over-activity leads to “exitotoxicity”, the result of excess neuronal Ca²⁺ influx due to the opening of N-methyl-D-aspartate (NMDA) ion channel receptors (Gardoni & Di Luca, 2006), where glutamate is a co-agonist (along with glycine). Amantadine and the adamantane derivative, memantine (dimethylated congener), are antagonists of glutamate at NMDA receptors and may provide neuroprotective effects (Merello *et al.*, 1999). Amantadine and memantine both have moderately beneficial effects in early PD, can enhance the effects of L-dopa and maybe even limit the severity of dyskinesia induced by L-dopa therapy (Paci *et al.*, 2001).

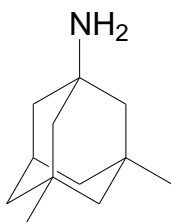


Figure 2.13: The structure of memantine.

2.6. L-Dopa

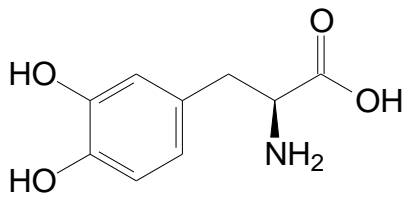


Figure 2.14: The structure of L-dopa.

Ehringer and Hornykiewicz assayed DA in the brains of dying PD patients in 1960, and found that tissue concentrations of DA in the striata of many of these patients averaged only 20% of normal levels (Ehringer & Hornykiewicz, 1960). The behavioural changes in rats treated with reserpine or other amine-depleting agents resembled the signs of PD in patients. These findings led to the administration of high oral doses of racemic dopa to patients with PD. Subsequent clinical trials confirmed the beneficial symptomatic effects of racemic dopa in PD. The physiological enantiomer, L-dopa, demonstrated greater potency and safety (Cotzias *et al.*, 1969; Barbeau, A., 1969).

In the biosynthesis of catecholamines, the trace intermediary metabolite is normally L-dopa, which is formed in a rate limiting hydroxylation step by tyrosine hydroxylase, a phosphorylation-activated cytoplasmic mono-oxygenase, from the essential amino acid L-tyrosine. Decarboxylation of L-dopa by the cytoplasmic enzyme, AADC, yields DA. After systemic administration of L-dopa, certain effects were observed and have been attributed to its peripheral and cerebral metabolites, mainly DA. L-dopa undergoes less conversion to norepinephrine (by β -hydroxylation) and epinephrine, which are formed by N-methylation of norepinephrine by phenylethanolamine-N-methyltransferase (Chalmers *et al.*, 1971; Nagatsu, 1973). L-3-O-Methyldopa (L-3-methoxytyrosine), which is formed by the COMT-catalysed methylation of L-dopa, accumulates in the CNS because of its long half-life. Decarboxylation occurs rapidly for most exogenous L-dopa to yield DA in peripheral tissues including liver, heart, lung and kidney.

L-dopa by itself has a very limited dose effectiveness, because only approximately 1% of an administered dose reaches the brain (Vogel, 1970). Only when L-dopa is administered in doses high enough (3-6 g daily) to compensate for losses caused by peripheral metabolism, can appreciable quantities enter the brain. Competitive inhibition of AADC blocks the decarboxylation of L-dopa and significantly increases therapeutic efficacy. This is most often achieved by co-administration of carbidopa (combined with levodopa in Sinemet and other products) or benserazide (combined with L-dopa in Prolopa and others, all in countries other

than the US). These polar decarboxylase inhibitors do not inhibit cerebral decarboxylase because they do not appreciably penetrate the brain, thus the proportions of L-dopa that reaches the brain for conversion to DA markedly increase. This allows for a 2.5-30-fold lower dose (0.2-1.2g/day) of L-dopa compared to L-dopa monotherapy (Standaert & Young, 2006).

Other aromatic and neutral aliphatic amino acids compete with L-dopa for absorption and uptake into the brain, and L-dopa bioavailability to the brain can be decreased substantially by a protein meal. A cofactor for AADC is pyridoxine (vitamin B6), and in high doses, pyridoxine can increase peripheral decarboxylase activity and thus decrease the therapeutic effects of L-dopa. Carbidopa or benserazide minimises this potential effect of pyridoxine by competitively blocking the peripheral decarboxylation. Rapid metabolism of DA to its principle inactive by-products occurs by the action of MAO (particularly MAO-A in mitochondria of aminergic nerve terminals) as well as by extra neuronal COMT (Matthysse & Baldessarini, 1972).

3,4-Dihydroxyphenylacetic acid (DOPAC) and HVA are the main by-products of DA metabolism. Nausea and vomiting, possibly because of gastrointestinal irritation, are common adverse effects of L-dopa therapy. These adverse effects also may result from the stimulation by DA (and perhaps L-dopa) of the chemoreceptor trigger zone (CTZ) in the postrema area of the brainstem, an emesis inducing centre that is largely unprotected by the blood-brain barrier. The reduction of required doses of L-dopa result is a lowered risk of emesis or other adverse effects associated with peripheral formation of excess DA. This is an important advantage of combining L-dopa with a peripheral decarboxylase inhibitor (Standaert & Young, 2006).

Approximately 50% of PD patients develop fluctuating motor responses after approximately 5 years of continuous treatment with L-dopa, and almost three-quarters do so within 15 years. These "on-off" fluctuations include "on" periods with abnormal involuntary movements or dyskinesia and "off" periods of immobility. The disease progression is reflected by these phenomena with more severe striatal nerve terminal degeneration and further loss of DA, as well as increased receptor sensitivity. Hypersexuality, mania, visual hallucinations and paranoid psychosis are common psychiatric disturbances, and sometimes severe adverse responses to treatment with L-dopa or direct DA agonists occur. Excessive stimulation of DA receptors in the mesolimbic or mesocortical DA systems are reflections of these behavioural disturbances (Miyawaki *et al.*, 1997).

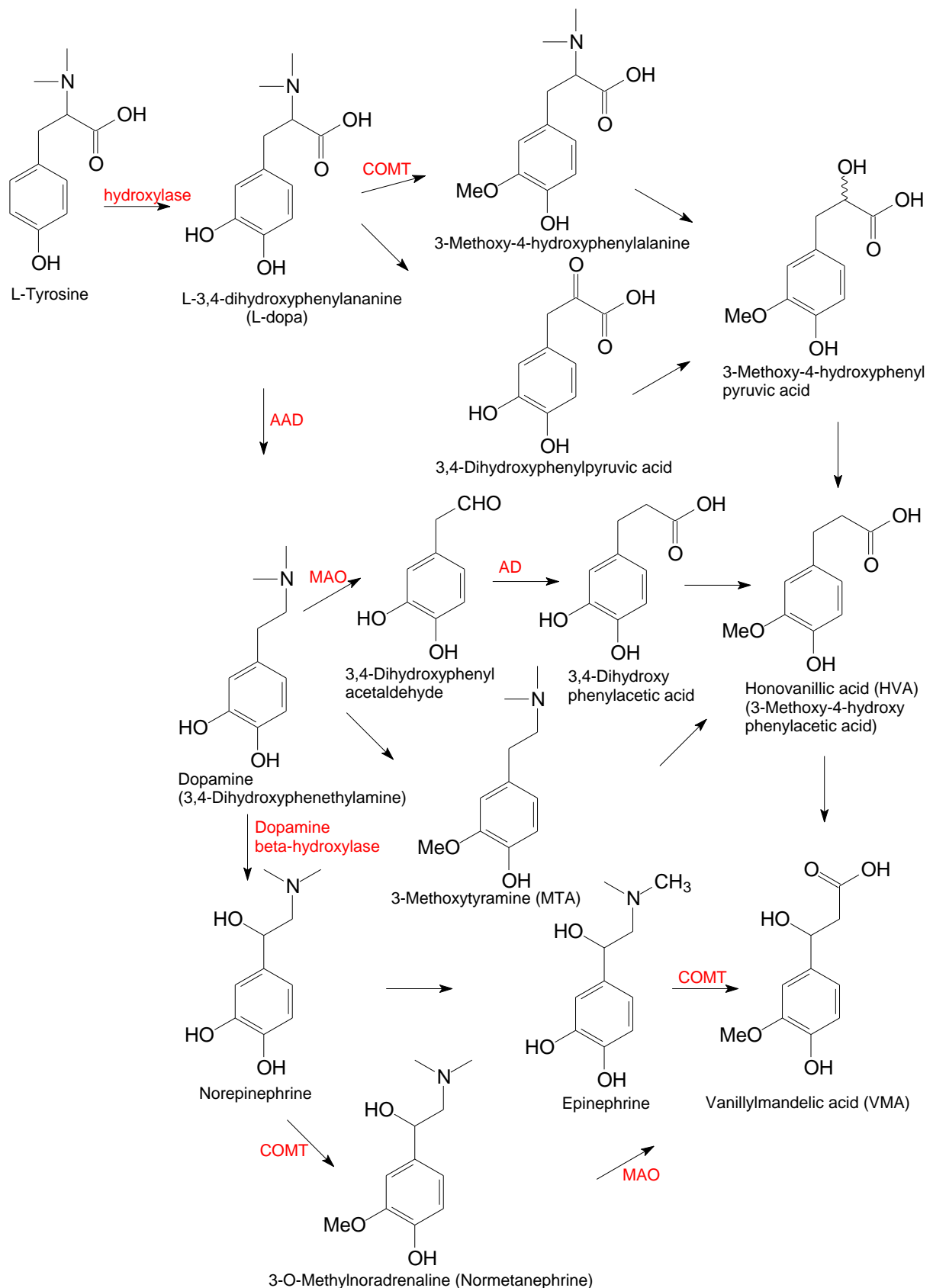


Figure 2.15: The metabolism of L-dopa and its major decarboxylated product DA. Heavy and light arrows indicate major or minor reactions. AD, aldehyde dehydrogenase; AAD, aromatic amino acid decarboxylase; COMT, catechol-O-methyltransferase; DH, DA β -hydroxylase; MAO, monoamine

oxidase; PNMT, phenylethanolamine-*N*-methyltransferase (Broadley, 2010; Lindemann & Hoener, 2005).

2.7. Monoamine Oxidase Inhibitors

2.7.1. Background

The catabolism of DA by MAO leads to the formation of neurotoxic oxidation products. Thus, MAO inhibition can potentially boost DA levels in the brain and prevent the formation of damaging MAO-derived oxidation products. MAO inhibitors can extend the duration of response to L-dopa and allow for the use of lower doses by reducing the oxidation of DA (Macleod *et al.*, 2005). Phenzelzine and tranylcypromine are long-acting, irreversible, nonselective MAO inhibitors, which are contraindicated in combination with L-dopa because of the risk of inducing hypertensive crises and delirium (Standaert & Young, 1996). The MAO isozyme present in DA nerve terminal remains uncertain, however, human brain MAO-A is found in noradrenergic neurons and MAO-B is found in serotonergic or histaminergic neurons and in glial cells (Saura *et al.*, 1996). MAO-A and MAO-B are equally active towards dopamine and tyramine. MAO-A, which is the main isoform found in non-CNS organs, deaminates tyramine in the gut. With MAO-A inhibition, tyramine is not sufficiently metabolised in the gut and, after absorption, it is taken up by the norepinephrine nerve terminals and acts as a false neurotransmitter that can lead to a hypertensive crisis. This hypertensive crisis is also known as the “cheese effect” because it was first associated with the ingestion of certain cheeses (which contain tyramine). Because MAO-B inhibitors do not affect tyramine metabolism in the gut, it may be used in the treatment of PD without the restriction of a low-tyramine diet.

The substantia nigra hosts both isoforms, with MAO-A localised to the pars compacta and MAO-B being the predominant isoform in the pars reticulata. Whether to target MAO-A or MAO-B for the treatment of PD is uncertain, and little is known about the structural requirements for highly specific reversible MAO-A inhibitors (Edmondson *et al.*, 2009), when compared to selective MAO-B inhibitors, several of which are beneficial in PD. Among the propargylamine-type selective irreversible inhibitors of MAO-B are selegiline and rasagiline. MAO-B inhibitors may prevent the formation of neurotoxic oxidation products of DA and slow neurodegeneration in PD. Additionally MAO-B inhibitors potentiate DA actions and allow for the reduction of L-dopa dose (Macleod *et al.*, 2005; Olanow, 2009). Because of their L-dopa-sparing effect, MAO-B inhibitors also have a beneficial effect on motor fluctuations (Macleod *et al.*, 2005). Selegiline undergoes extensive hepatic metabolism and is N-dealkylated via CYP2B6 and CYP2C19 to L-methamphetamine and then to L-amphetamine, which may, like D-amphetamine, lead to vasoactive activity. (Glezer & Finberg, 2003). The amphetamine metabolites from this reaction may contribute to other pharmacological properties such as DA

and norepinephrine reuptake inhibition, which may potentiate the effects of L-dopa (Castagnoli *et al.*, 1997). After careful assessment of rasagiline's ability to provide neuroprotection, it was found that, at a dose of 1 mg/day, this drug met the endpoints consistent with neuroprotection. The α -aminoamide derivative, safinamide, is a reversible selective MAO-B inhibitor that has been shown to provide benefits in early PD (Caccia *et al.*, 2006; Stocchi *et al.*, 2004). Additionally, safinamide blocks voltage-dependent sodium and calcium channels and inhibits glutamate release (Caccia *et al.*, 2006).

2.7.2. MAO-B inhibitors

For the treatment of PD, MAO-B inhibitors occupy a prominent place. The first MAO-B inhibitor to be used therapeutically was selegiline, while a second-generation drug, rasagiline, showed higher potency and selectivity. Safinamide is still under investigation for its MAO-B inhibitory properties. Safinamide also acts via other mechanisms relevant to PD and may provide more advantages than selegiline and rasagiline. MAO-B inhibitors, as a class, are considered safe and well tolerated and provide symptomatic benefit as monotherapy as well as in combination with other antiparkinsonian medications from early to late stages of the disease. The combination of L-dopa and MAO-B inhibitors may improve motor fluctuations and allow for lower doses of L-dopa. MAO-B inhibitors have also shown promise as disease-modifying agents although the clinical trial evidence is not compelling (Robakis & Fahn, 2015).

2.7.2.1. Selegiline

In 1965, Knoll and Magyar described selegiline as a selective MAO-B inhibitor that does not cause the cheese effect. It was the first MAO-B inhibitor to obtain FDA (Food and Drug Administration) approval for the treatment of PD, either as monotherapy or as an adjunct to L-dopa in moderately advanced PD. Selegiline is a selective, irreversible MAO-B inhibitor which, unlike rasagiline, is metabolised to L-methamphetamine and L-amphetamine. Selegiline may delay disease progression, as suggested by clinical studies and experimental animal models, by reducing oxidative stress-related pathways that are implicated in dopaminergic neuronal death in the substantia nigra. A prospective investigation of the effects of selegiline on the natural progression of PD, is the extensive DATATOP study. This study tested the hypothesis that the time until disability that requires therapy with L-dopa (primary end point) can be extended with long-term treatment of patients suffering from early PD with selegiline, and was the first double-blind placebo-controlled neuroprotection trial. This study found that after 12 months, 47% of those treated with placebo needed to initiate L-dopa therapy compared with 26% in the selegiline group. For those patients receiving selegiline, the need for symptomatic treatment with L-dopa was delayed by 9 months. A follow-up long-term study to the DATATOP

re-randomised 368 patients who already required L-dopa to either placebo or selegiline for 2 years. A slower progression of motor symptoms according to the Unified Parkinson Disease Rating Scale (UPDRS) as well as less freezing of gait (16%) was experienced by the selegiline group compared to the placebo group (29%), however the rate of dyskinesias was higher (34 vs. 19%). Selegiline plus L-dopa is associated with decreased L-dopa requirements over time compared to L-dopa alone. Selegiline has been associated with slower disease progression and with slower accumulation of disability in addition to lowering L-dopa requirements. One of the most common side effects of selegiline is insomnia because of the effect of the amphetamine metabolites on sleep. To summarise, for selegiline, good evidence exists for a delay in disease progression, a mild reduction in L-dopa requirements and mild symptomatic benefits in PD (Robakis & Fahn, 2015).

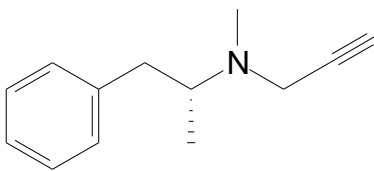


Figure 2.16: The structure of selegiline.

2.7.2.2. Rasagiline

Another selective irreversible MAO-B inhibitor is rasagiline, which was FDA approved in 2006 for PD treatment. Like selegiline, it may be used alone or in combination with other PD medications from early to late stages of the disease. Rasagiline has a significant beneficial effect on motor symptoms as adjunctive therapy compared to placebo as measured by UPDRS scores, and significantly decreases daily “off” time. Improved motor function in PD over at least 5 years has been conclusively demonstrated by rasagiline, however, any potential neuroprotective benefits have yet to be confirmed (Robakis & Fahn, 2015).

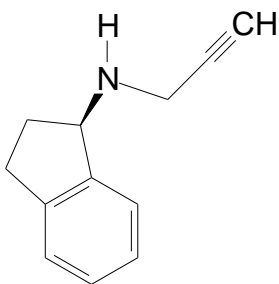


Figure 2.17: The structure of rasagiline.

2.7.2.3. Lazabemide

Lazabemide [N-(2-aminoethyl)-5-chloro-2-pyridinecarboxamide; Ro 19-6327] is a potent and reversible (complete recovery of enzyme activity within 24 hours), and extremely selective inhibitor of MAO-B. Unlike selegiline, lazabemide does not potentiate the pressor effect of tyramine. Lazabemide also demonstrates effective concentration dependent antioxidant activity, completely independent of its interaction with MAO-B. Greater antioxidant activity was noted compared to either selegiline or vitamin E under identical experimental conditions. Lazabemide interferes with the efficient propagation of free radicals by various biochemical and biophysical mechanisms, which involves the intercalation into the membrane lipid bilayer (Mason *et al.*, 2000).

Lazabemide's interaction with MAO-B appears to be like that of substrates, thus, it is oxidised by MAO-B to form tightly bound, but reversible, adducts with the active site of the enzyme. The reduction of the enzyme-inhibitor adducts with NaBH₃CN at acidic pH causes the irreversible incorporation of a fraction of the bound inhibitor into MAO-B, thereby irreversibly inactivating the enzyme, which is an interesting property of this class of inhibitor (Cesura *et al.*, 1996). Lazabemide inhibits MAO-B in the brain 100-fold more potently than selegiline, it prevents dopaminergic neurotoxicity induced by MPTP and potentiates the effect of L-dopa. A phase II dose-finding study of lazabemide as an aid to smoking cessation was discontinued due to liver toxicity observed in other studies (Berlin *et al.*, 2002). Headache was the most frequent adverse effect at higher doses, but otherwise lazabemide was well tolerated at all dose levels with no clinically relevant changes in vital signs or laboratory parameters. Rapid absorption and elimination by mixed linear and non-linear pathways occurred and steady state plasma concentrations were achieved on the third day of dosing (Dingemans *et al.*, 1997).

Reversible MAO-B inhibition by lazabemide has been shown *in vivo* by rapid recovery of platelet MAO-B activity after single doses as well as after 7 days of treatment. The absence of a significant regulatory system controlling MAO-B activity is suggested because of the absence of change in the parameters of the interaction over a period of 7 days of almost complete inhibition of the platelet enzyme (Holford *et al.*, 1994). No serious adverse experiences are associated with lazabemide treatment and a significant improvement in the activities of daily living after 4 weeks of lazabemide treatment was found (Kiebertz, 1993). By affinity labelling and site-directed mutagenesis, the structural features of the active site of human MAO-B was investigated. As mentioned, lazabemide can be linked, irreversibly, to MAO-B by reduction of the enzyme-inhibitor complex with NaBH₃CN. Early analysis of [³H]-lazabemide-labelled human MAO-B flavin spectrum, indicated that insertion of the inhibitor does not occur into the isoalloxazine ring of FAD. [³H]-Lazabemide was suggested to be

incorporated into the MAO-B peptide stretch containing the FAD-modified Cys397 (Cesura *et al.*, 1996). Recently structural evidence has shown that this class of inhibitors are indeed covalently linked to the isoalloxazine ring of FAD, and that adduct formation occurs at the N(5) position (Binda *et al.*, 2002; Edmondson *et al.*, 2007).

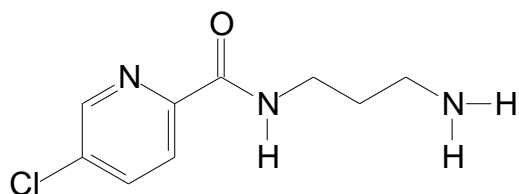


Figure 2.18: The structure of lazabemide.

2.7.2.4. Safinamide

Another MAO-B inhibitor, safinamide, was recently approved by the European Commission. This drug was approved as an add-on to L-dopa or in combination with other PD medications in mid- to late stage PD patients with motor fluctuations. The FDA is currently reviewing this drug for early and mid- to late-stage PD use at doses of 50 and 100 mg per day. Safinamide is a selective, reversible inhibitor of MAO-B with additional pharmacological properties. Safinamide inhibits glutamate release by blocking voltage-dependent sodium channels. Its anti-glutamate activity may provide a potential advantage over other MAO-B inhibitors, namely an antidyskinetic effect. However, more data is required to elucidate the potential antidyskinetic properties of safinamide in PD patients. Safinamide has also shown prevention of neuronal death in animal and tissue culture models, which suggests that it may possess neuroprotective properties (Robakis & Fahn, 2015).

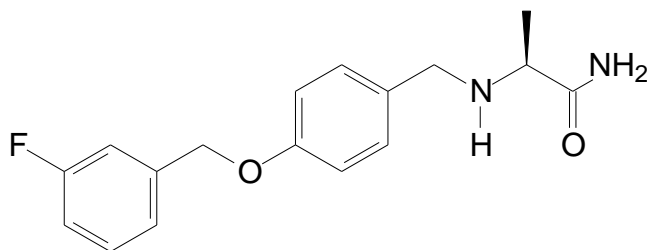


Figure 2.19: The structure of safinamide.

2.7.3. MAO-A inhibitors

Although dopamine is well metabolised by MAO-A and MAO-B, the striatum contains lower levels of MAO-A and as a result little attention has been paid to MAO-A inhibition as a means of controlling dopamine levels in the brain (Green *et al.*, 1977). Another factor that discouraged the use of MAO-A inhibitors is that MAO-A inhibition induces the cheese reaction. Results indicated that when one MAO isoform is fully inhibited, the other isoform would metabolise dopamine adequately, thus, with selective MAO-A or MAO-B inhibition the level of dopamine will not change drastically in the human striatum (Riederer & Youdim, 1986).

2.7.3.1. Moclobemide

Moclobemide is a reversible MAO-A inhibitor, which does not provoke the cheese reaction. The reversibility allows competition with ingested tyramine (or other dietary amines) and the amine substrate can displace the inhibitor from the enzyme and be metabolised in the normal way in the gut and liver. Microdialysis studies in rodents showed a clear increase of dopamine release after moclobemide or rasagiline treatment, however, selective inhibition of MAO-A or MAO-B does not affect the steady state level of dopamine in the brain. As an addition to therapy with L-dopa, moclobemide has a mild symptomatic benefit, mostly a reduction of motor functions. Moclobemide was first assessed as an antidepressant and found to be effective by improving vigilance, psychomotor speed and long-term memory as well as choice reaction time in elderly patients. This drug is considered to be a mild antidepressant, better tolerated by older patients, but appears to be safe and devoid of major side effects. A significant proportion (40-60%) of PD patients exhibits signs of depression and this antidepressant action of moclobemide is a useful and beneficial effect (Youdim & Bakhle, 2006).

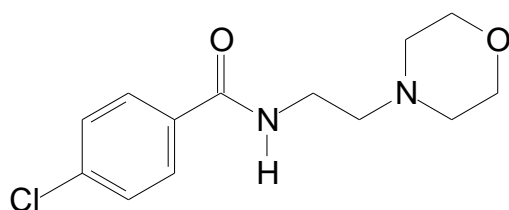


Figure 2.20: The structure of moclobemide.

2.7.3.2. Brofaromine

Brofaromine [4-(7-bromo-5-methoxy-2-benzofuranyl)piperidine] is a reversible MAO-A inhibitor. The inhibition occurs reversibly, making brofaromine pharmacologically and structurally distinct from most of the currently available MAO inhibitors such as phenelzine and

tranylcypromine. Additionally, it has significantly fewer problems than other MAO inhibitors in terms of hepatic toxicity and interaction with tyramine. Brofaromine is well tolerated and appears to be comparatively free from unwanted physical side-effects according to clinical studies (Kerr *et al.*, 1993).

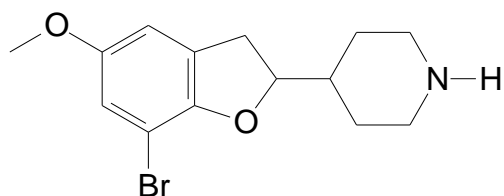


Figure 2.21: The structure of brofaromine.

2.7.3.3. Befloxatone

An oxazolidinone derivative, befloxatone, is another potent reversible MAO-A inhibitor and is active in both animals and humans. Befloxatone induces a dose-dependent increase in endogenous levels of noradrenaline, 5HT and DA, with a decrease in the corresponding catabolites (HVA, 5-hydroxyindoleacetic acid (5HIAA), DOPAC), in the rat brain. This drug increases extracellular levels of DA in the striatum and of noradrenaline in the frontal cortex. Befloxatone's pharmacological profile shows evidence of potential antidepressant activity in behavioural mice and rat tests, without other central (sedation, stimulation or anticholinergic) or peripheral (cardiovascular, gastrointestinal or renal) effects. The clinical and laboratory safety data for this drug is thus satisfactory (Patat *et al.*, 1995).

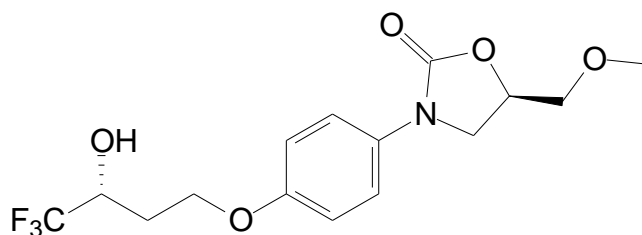


Figure 2.22: The structure of befloxatone.

2.7.4. The structures of MAO-A and MAO-B

The MAO-A and MAO-B enzymes are attached to the outer mitochondrial membrane. X-Ray crystal data suggest that human MAO-B possibly exists as a dimer (Upadhyay *et al.*, 2008). Substrates and inhibitors bind to the active site cavity of MAO in proximity to the flavin, which

is present at the end of the active site cavity. Both isoforms are anchored via a C-terminal sequence of 35-40 amino acids to the outer mitochondrial membrane. The active site of MAO-A consists of a single cavity whereas the MAO-B active site has a bipartite structure, consisting of an entrance cavity and inner substrate cavity. In MAO-B, the residue Ile199 acts as a gate that may separate the entrance and substrate cavities. Depending on the conformation of the side chain of Ile199, the entrance and substrate cavities of MAO-B are either fused to form a single large chamber or separated into two distinct chambers. The entrance cavity of MAO-B is relatively small and permits access to smaller hydrophobic molecules, whereas MAO-A has a wider cavity which enables binding of larger substrates and inhibitors (Finberg, 2014).

Although human MAO-A and MAO-B differ in their substrate specificities, they share high sequence similarity of approximately 70%. Serotonin, among the bulkier endogenous amine neurotransmitters, is degraded by MAO-A, whereas MAO-B mainly acts on small exogenous amines. Leu171 (MAO-B)/Ile180 (MAO-A), Cys172/Asn181, Ile199/Phe208 and Tyr326/Ile335 are residues in MAO-B and MAO-A that form the lining of the substrate cavity and are responsible for the main differences between the human MAO-A and MAO-B active sites. The entrance cavity and the substrate cavity of MAO-B are separated by three of these residues (Leu171, Ile199 and Tyr326). The steric accommodation of the substrate can be affected by side chain changes in these positions, which may lead to changes in the separation of the substrate cavity from the entrance cavity. In this respect, the alteration of the side chains of these residues may lead to the fusing of the two cavities to form a single larger cavity that would accommodate larger substrates and inhibitors. Alterations in the substrate and inhibitor specificities to resemble those of MAO-A are accomplished by mutations of the cavity-separating residues in MAO-B (Binda *et al.*, 2002).

2.7.4.1. The structure of MAO-B

MAO-B consists of 520 amino acids that fold into a compact structure and exhibits a topology which was initially found in p-hydroxybenzoate hydroxylase and then observed in several flavoproteins. The closest structural matches of MAO-B are L-amino acid oxidase and polyamine oxidase with root mean square deviations of 2.7 Å and 3.5 Å, respectively. The enzyme is dimeric, as shown by the crystal structure, however it is unlikely to be a crystal packing artefact because the dimer is present in the orthorhombic and triclinic crystal forms employed in the structure determination. In addition, the monomer-monomer interactions are extensive and based on these findings the dimer form is thus suggested to be the quaternary assembly present *in vivo* (Binda *et al.*, 2002).

The need for digestion of phospholipids for its efficient detergent extraction is evidence that MAO-B is tightly bound to the outer mitochondrial membrane. The C-terminal amino acids 461-520 is the protein region responsible for membrane attachment (Mitoma & Ito, 1992). It is predicted that residues 489-511 form a transmembrane helix, 27 amino acids long, which has similar values to transmembrane helices in membrane proteins of known three-dimensional structures. C-Terminal truncation mutagenesis experiments show that deletion of the C-terminal residues does not completely abolish the ability of the enzyme to bind to the membrane, therefore MAO-B is expected to contain additional membrane interaction sites (Rebrin *et al.*, 2001). Evidence suggest that membrane attachment involves additional hydrophobic patches of the protein surface in addition to the C-terminal helix (Binda *et al.*, 2002). It was observed that the helix of each monomer protrudes from the basal face of the dimer, with each helical axis approximately parallel to the molecular two-fold axis. This further suggests that the C-terminal helices are inserted in the lipid bilayer and the MAO-B dimer binds to the membrane with its two-fold axis perpendicular to the membrane plane. The exact depth of protein insertion into the bilayer is not defined in the current structural data.

Pargyline covalently binds to the N5 atom on the reverse side of the flavin in a solvent inaccessible environment. A flat cavity with a volume of 420 Å³ forms the substrate binding site. Substrate specificity and quantitative structure-activity relationship (QSAR) studies predicted that this cavity is lined by several aromatic and aliphatic amino acids that provide the highly hydrophobic environment (Walker & Edmondson, 1994).

A separate, smaller hydrophobic cavity (volume of 290 Å³) is adjacent to the substrate cavity and is lined by residues Phe103, Pro104, Trp119, Leu164, Leu167, Phe168, Leu171, Ile199, Ile316 and Tyr326. This second cavity is located between the protein surface and the active site, and is shielded from solvent by loop 99-112. Loop 99-112 thus functions as a “gating switch” to the entrance cavity. As mentioned, the residues that separate the two cavities are Tyr326, Ile199, Leu171 and Phe168. A mechanism for admission of the substrate into the active site initially involves the movement of loop 99-112 to allow direct access to the smaller cavity (also known as the “entrance cavity”). A transient movement of the four residues separating the substrate cavity from the entrance cavity must occur to allow diffusion of the substrate into the active site, after the substrate reaches the entrance cavity. The substrate must migrate a distance of approximately 20 Å from the surface of the entrance cavity to the flavin ring (Binda *et al.*, 2002).

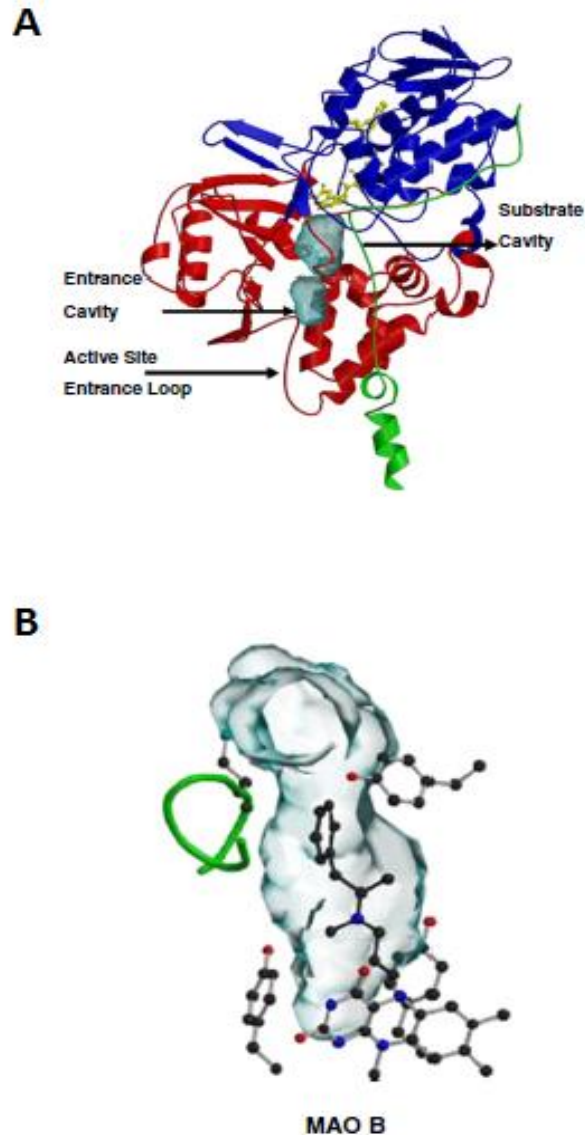


Figure 2.23: The molecular structure of MAO-B and the amine binding site. (A) A ribbon diagram of human MAO-B. The yellow ball and stick model is the covalent flavin moiety. The blue indicates the flavin binding domain, the red shows the substrate domain and the green is the mitochondrial membrane binding domain. (B) The active site cavities of human MAO-B with selegiline in the active site. The inhibitor forms a covalent N(5) flavocyanine adduct with the flavin coenzyme. The green indicates the active site “shaping loop” structure (Edmondson *et al.*, 2007).

2.7.4.2. The structure of MAO-A

New insights into the differences that exist between human MAO-A and MAO-B has been provided after elucidation of the structure of human MAO-A to a resolution of 3 Å (DeColibus *et al.*, 2005). Human MAO-A crystallises as a monomer, while human MAO-B crystallises as a dimer. A human-selective mutation was observed on comparison of the human MAO-A

sequence with those of other primates and mammals (Andres *et al.*, 2004). The study found that a conserved glutamate residue in all sources of MAO-A and MAO-B is specifically mutated to a lysyl residue in human MAO-A, and it was proposed that this human-selective mutation results in the disruption of the dimer structure to that of a monomer.

The human MAO-A active site differs from human MAO-B in that it has a monopartite cavity with a total volume of 550 Å³. The substrate binding site for human MAO-A is slightly bigger than that of human MAO-B. The largest differences between the active sites of MAO-A and MAO-B occurs in the conformation of residue loop 201-216 in human MAO-A, which is referred to as the “cavity-shaping loop”. The different substrate and inhibitor specificities of the two enzymes may be attributed to the conformation of this loop, which has a significant effect on the shape and volume of the substrate binding cavity (Edmondson *et al.*, 2007).

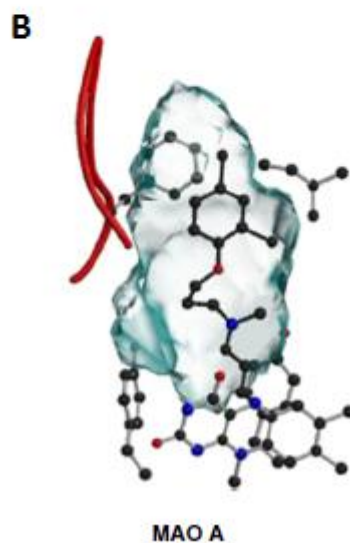
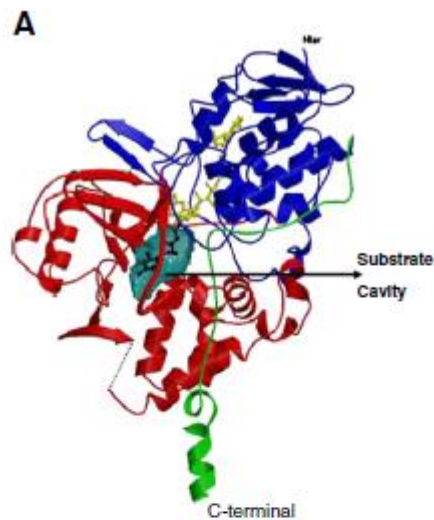


Figure 2.24: The molecular structure of MAO-A and the amine binding site. (A) A ribbon diagram of human MAO-A. The yellow ball and stick model indicates the covalent flavin moiety. The blue area shows the flavin binding domain, the red indicates the substrate domain and the green area shows the mitochondrial membrane binding domain. (B) The active site cavities of human MAO-A with clorgyline in the active site, where this inhibitor forms a covalent N(5) flavocyanine adduct with the flavin coenzyme. The red structure is the active site “shaping loop” structure for MAO-A (Edmondson *et al.*, 2007).

2.8. Prodrugs

2.8.1. Background of prodrugs

Prodrugs can be defined as modifications of active drugs designed to enhance the pharmacokinetic, pharmacodynamic or pharmaceutical properties of molecules. The drug's characteristics such as solubility, permeability, chemical or metabolic stability are improved by these chemical modifications, thus typically enhancing its absorption and bioavailability. *In vivo* enzymatic and/or chemical transformations should ideally convert a pharmacologically inactive prodrug to the active parent drug. The prodrug releases the active drug and a promoiety, which is preferably physiologically inert and readily eliminated, through bioconversion (Nofsinger *et al.*, 2014).

Drugs that are administered systemically can reach the brain by crossing one of two physiological barriers resistant to free diffusion for most molecules from blood to CNS: the epithelial blood-cerebrospinal fluid barrier or the endothelial blood-brain barrier. These barriers are both enzymatic as well as transport barriers making the brain the least accessible organ for delivery of active pharmacological compounds. A resourceful chemical/biochemical approach to overcome limitations in the effectiveness of a parent drug, including solubility, is the design of new prodrugs. These drugs are defined as therapeutically inactive agents that can be predictably transformed into their active metabolites. The prodrug, with no intrinsic activity, undergoes enzymatic and/or chemical/spontaneous processes in a predictable way to transform into active agents *in vivo*. A covalent link between the drug and the strategically selected chemical transport moiety or carrier molecule is usually employed in the design of “carrier-linked” prodrugs. Ideally the inactive prodrug should release the active agent either non-enzymatically at the site of action or by means of target specified enzymes that are more abundant at the target site than anywhere else in the body. The prodrug must have easy access to the target tissue. An increase of the parent drug lipophilicity is the most common strategy for designing a prodrug with enhanced blood-brain barrier permeation. However, failure will incur if lipophilicity is increased without a concomitant increase in rate and selectivity

of prodrug bioconversion in the brain. In 1997, Lipinski's "rule of five" was introduced and is widely used to estimate the bioavailability of drugs, and thus also prodrugs. There are four factors to consider: hydrogen bond donors, hydrogen bond acceptors, molecular weight and LogP (Pavan *et al.*, 2008).

- 1) To determine free diffusion of molecules across the blood-brain barrier, the molecular weight must be taken into account. The molecular weight should preferably be lower than 400-500 Da for CNS-directed drugs.
- 2) For a drug to permeate the blood-brain barrier, it should have a desirable LogD of 0-3 at physiological pH values.
- 3) Another important factor that determines the availability of a drug is the plasma pharmacokinetics and the plasma area under the concentration curve (AUC). The concentration of the drug in the brain is directly relative to both the plasma AUC and the blood-brain barrier permeability coefficient (P_e).

Because a prodrug needs conversion into the parent drug to be active, it is extremely important to consider the role played by the biological or chemical processes in the brain tissue and in the barriers for a successful approach. A variety of oxidative enzymes, including xanthine oxidase, monoamine oxidase and cytochrome-P450 enzymes, in addition to esterase and adenosine deaminase, are of interest for their role in the enzymatic activity of the blood-brain barrier. These enzymes are used as biotransformation systems for the activation of prodrugs that are able to cross the blood-brain barrier (Pavan *et al.*, 2008).

To access the CNS, nutrients, vitamins or hormones are transported via carrier mediated transport across the blood-brain barrier. In the blood-brain barrier, large neutral amino acids (phenylalanine, tyrosine or leucine) are transported by the large amino acid transporter (LAT) system, which acts as sodium-ion-independent transporters. Two subtypes of LAT exist, LAT1 and LAT2, with the principal transporter at the blood-brain barrier being LAT1. L-Dopa is a classic example of a modification of the drug structure for brain targeting of DA, which does not cross the blood-brain barrier. L-Dopa thus represents a prodrug of DA and accesses the brain via LAT1. L-Dopa undergoes subsequent decarboxylation by AADC to deliver DA to the brain (Pavan *et al.*, 2008).

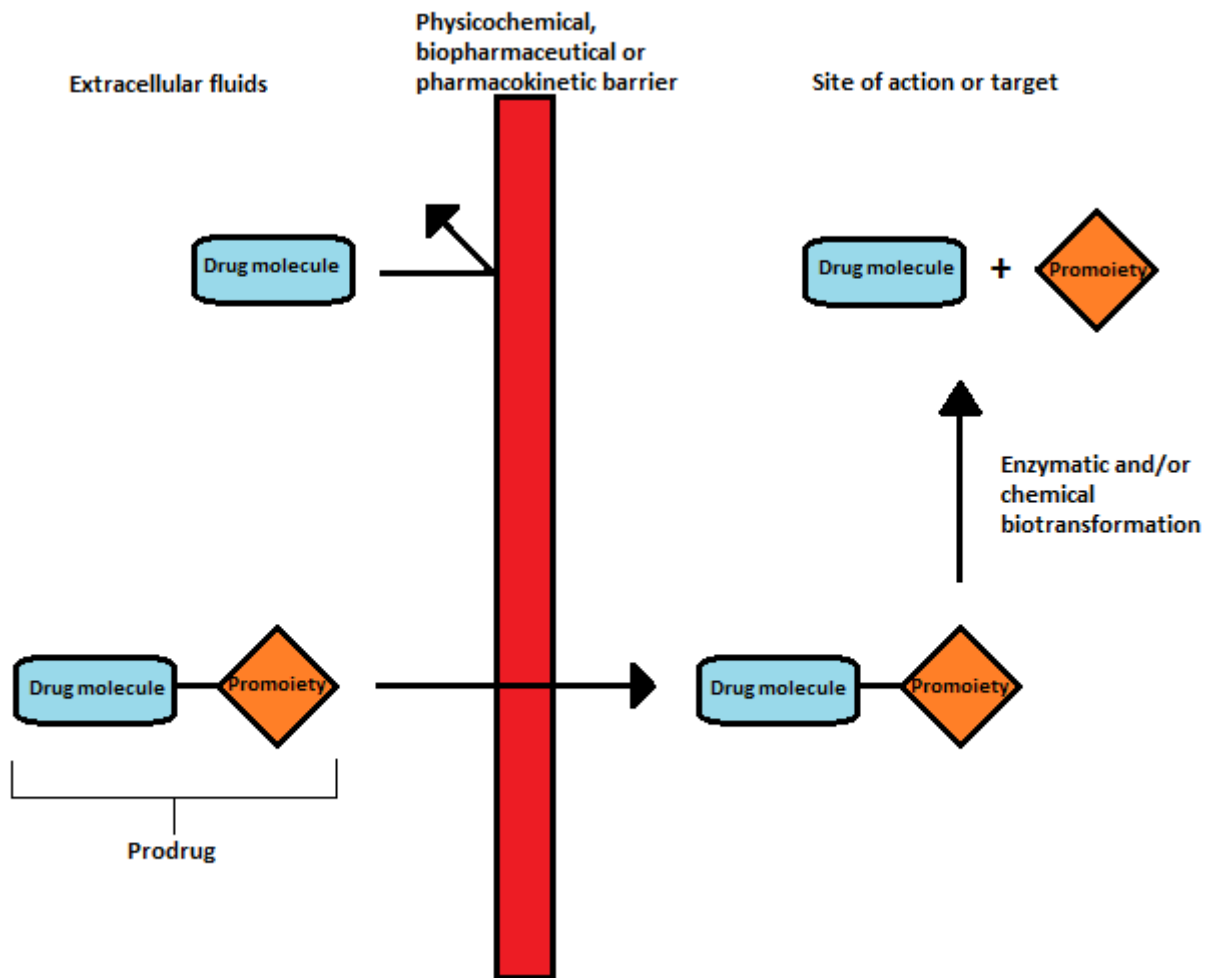


Figure 2.25: A schematic diagram of the rationale for prodrug design.

2.8.2. L-Dopa and DA prodrugs

Targeted prodrug design represents a novel strategy for efficient and directed drug delivery. Targeting prodrugs to a specific enzyme and/or a membrane transporter has potential as a selective drug delivery system in cancer chemotherapy or as an efficient oral drug delivery system. The simultaneous use of gene delivery to express the requisite enzymes or transporters enhances site-selective targeting with prodrugs. To overcome various undesirable drug properties, prodrugs can be designed to target specific enzymes or carriers by taking into account the enzyme-substrate specificity or carrier-substrate specificity. To execute this type of “targeted-prodrug” design, considerable knowledge of the enzymes or carrier systems, including their molecular and functional characteristics are required (Han & Amidon, 2000).

Enzymes can be recognised as presystemic metabolic sites or prodrug-drug *in vivo* reconversion sites, in prodrug design. Irreversible chemical alteration rather than by a prodrug approach is more successful to reduce presystemic metabolism. Oral drug absorption as well as site-specific drug delivery can be improved by the enzyme-targeted prodrug approach. Gastrointestinal enzymes may be the main targets for prodrug design for improving oral drug absorption (Amidon *et al.*, 1980). Prodrug use have been actively used to achieve direct and precise effects at the “site of action”, with minimal side effects. For site specific delivery of drugs by using the prodrug approach, at least 3 factors should be enhanced (Stella & Himmelstein, 1982; Friend & Chang, 1984).

1. Voluntary transport of the prodrug to the site of action and rapid uptake to the site that is essentially perfusion rate limited.
2. The prodrug must be selectively cleaved to the active drug, once at the site, relative to its conversion at other sites.
3. The active drug must be slightly retained by the tissue once selectively generated at the site of action.

To obtain site-specific drug delivery from tissue-specific activation of a prodrug, metabolism by an enzyme that is either unique for the tissue or present at a higher concentration compared to the other tissues, should occur.

Transporters designed for facilitating membrane transport of polar nutrients such as amino acids and peptides are also used for a targeted prodrug approach. The evidence suggests the participation of carrier-mediated membrane transport mechanisms have a positive outcome, where several hydrophilic compounds seem to be absorbed efficiently via specific transporters (Mizuma *et al.*, 1992), therefore being beneficial when prodrugs are polar or charged.

The Peptide Transporter Associated Prodrug Therapy (PTAPT) is a prodrug strategy that targets peptide transporters (Amidon *et al.*, 1980). Conversion of a polar drug with low membrane permeability through passive diffusion, into a prodrug that is absorbed via the peptide transporter into the mucosal cell is typical of this approach. This prodrug may still be very polar and possess sufficient solubility in the gastrointestinal lumen, but may also be well absorbed across the intestinal epithelium via peptide transporters. The prodrug may be hydrolysed by enzymes in the mucosal cells, blood or liver after membrane transport. The membrane permeability and systemic availability of polar α -methyl dopa through peptidyl derivatives has been effectively improved by this prodrug strategy (Swaan & Tukker, 1995; Hu & Borchardt, 1990).

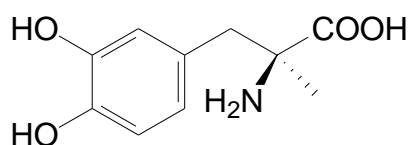


Figure 2.26: The structure of L- α -methyldopa.

L- α -Methyldopa has a low membrane permeability and is insufficiently absorbed via Na⁺-coupled neutral amino acid transporters (Bai, 1995). Absorption has been meaningfully enhanced by peptidyl prodrugs such as Phe- α -methyldopa, α -methyldopa-Phe and α -methyldopa-Pro in rats by means of peptide transporters (Swaan & Tukker, 1995; Hu & Borchardt, 1990). Similarly, peptide prodrugs of L-dopa were designed to be absorbed via peptide transporters and converted to L-dopa by peptidases, to minimise the extensive metabolism of L-dopa in the gut wall (Ganapathy *et al.*, 1998).

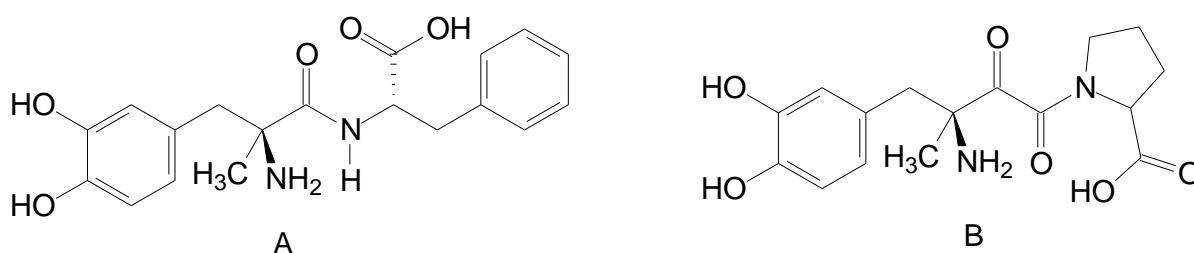


Figure 2.27: The structures of A) α -methyldopa-Phe and B) α -methyldopa-Pro (Swaan & Tukker, 1995; Hu & Borchardt, 1990).

L-Dopa is the immediate precursor of DA, which is still considered the primary drug of choice for the treatment of PD. L-Dopa therapy, however, results in several acute problems. To increase the bioavailability after oral administration, multi-protected L-dopa prodrugs were designed, which can release the drug by spontaneous chemical or enzyme catalysed hydrolysis. These compounds were synthesised and evaluated for their LogP, water solubility, chemical stability and enzymatic stability. The incorporation of L-dopa into imidazoline-4-one rings yields prodrugs that are sufficiently stable to potentially cross unchanged through the acidic environment of the stomach, and be absorbed from the intestine. L-Dopa's release in human plasma after enzymatic hydrolysis is also a possibility. These prodrugs may also have the ability to increase basal levels of striatal DA, and influence brain neurochemistry associated with dopaminergic activity, following oral administration (Giorgionia, *et al.*, 2010).

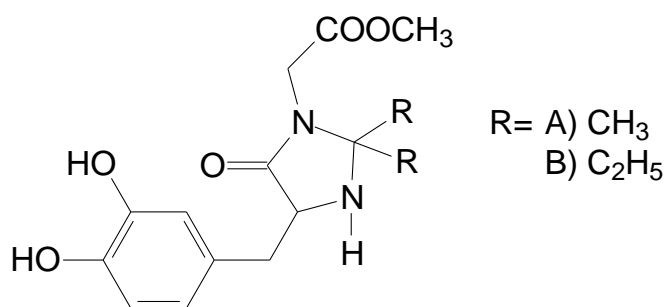


Figure 2.28: The structure of an imidazoline-4-one ring as potential L-dopa prodrugs (Giorgionia, *et al.*, 2010).

Other studies reported a series of di- and tripeptides containing L-dopa and examined them for their antiparkinsonian activity in mice. The structures shown in figure 2.29 were more effective than L-dopa in reversing reserpine-induced catatonia.

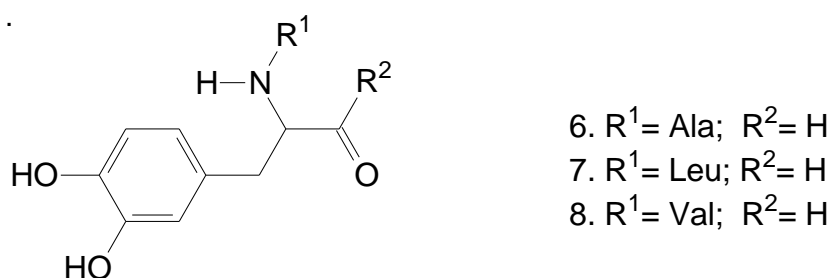


Figure 2.29: Chemical structures of di- and tripeptide prodrugs of L-dopa (Di Stefano *et al.*, 2009).

These prodrugs are relatively non-toxic and result in a low degree of stereotypical behaviour compared to L-dopa. The L-dopa esters also seem to effectively prevent the metabolism of L-dopa prior to or during the absorption process, which results in better bioavailability of the drug. Thus, improved bioavailability, decreased side effects and potentially enhanced CNS delivery are possible therapeutic advantages of these L-dopa prodrugs (Di Stefano *et al.*, 2009).

DA is extensively metabolised by the liver following oral administration, and at physiological pH, DA is completely ionised due to the amine moiety. This means that DA displays poor blood-brain barrier and other cell membrane permeation, and therefore cannot be used as PD treatment. To overcome these problems and to be used in PD, hypertension and renal failure therapy, a series of lipophilic 3,4-O-diester, **1-5** (figure 2.30), were proposed. Administration of the catechol protected DA prodrugs to experimental animals resulted in brain-specific, high and sustained prodrug concentrations. The systemic concentrations of these prodrugs

decreased rapidly with a $t_{1/2}$ of less than 30 min, and significant sustained dopaminergic activity in the brain was observed (Di Stefano *et al.*, 2009).

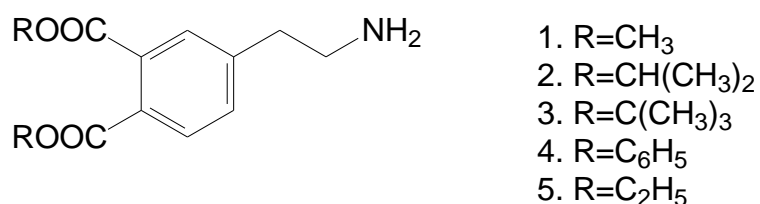


Figure 2.30: The structures of selected dopamine prodrugs (Di Stefano *et al.*, 2009)

2.9. Conclusion

PD is the second most common chronic, progressive neurodegenerative disease, and is caused by the degeneration of the nigrostriatal neurons of the brain. L-Dopa, the direct metabolic precursor of DA, is the most effective agent in the treatment of PD. However, the oral bioavailability of L-dopa is about 10% and less than 1% of the administered oral dose reaches the brain unchanged. To overcome the problems with peripheral L-dopa metabolism, delivery difficulties and insufficient L-dopa to DA conversion in the brain tissue as well as the adverse effects associated with continued use, DA prodrugs and L-dopa prodrugs may potentially be used. Thus, the therapeutic benefits of L-dopa can be enhanced by using a prodrug approach. A prodrug may allow for a lower initial dose of the prodrug (compared to L-dopa), thus delaying the need for higher doses of L-dopa and the occurrence of its side effects. According to literature reports, several prodrugs of DA, L-dopa and DA agonists possess superior therapeutic properties in PD models compared to L-dopa.

References for chapter 1 and 2:

- Alves, G., Forsaa, E.B., Pedersen, K.F., Dreetz Gjerstad, M. & Larsen, J.P. 2008. Epidemiology of Parkinson's disease. *Journal of neurology*, 5:18-32.
- Amidon, G.L., Leesman, G.D. & Elliott, R.L. 1980. Improving intestinal absorption of water-insoluble compounds: a membrane metabolism strategy. *Journal of pharmaceutical science*, 69:1363-1368.
- Andres, A.M., Soldevila, M., Navarro, K.K.K., Oliva, B. & Bertranpetit, J. 2004. Positive selection in MAO A gene is human exclusive: determination of the putative amino acid change selected in the human lineage. *Human genetics*, 115: 377-386.
- Bai, J.P.F. 1995. PGlu-L-dopa-pro: a tripeptide prodrug targeting the intestinal peptide transporter for absorption and tissue enzymes for conversion. *Pharmaceutical research*, 12:1101-1104.
- Baldessarini, R.J. & Fischer, J.E. 1977. Substitute and alternative neurotransmitters in neuropsychiatric illness. *Archives of general psychiatry*, 34:958-964.
- Baldessarini, R.J. & Tarsy, D. 1980. Dopamine and the pathophysiology of dyskinesias induced by antipsychotic drugs. *Annual review of neuroscience*, 3:23-41.
- Barbeau, A. 1969. L-Dopa therapy in Parkinson's disease: a critical review of nine years' experience. *Canadian medical association journal*, 101:59-68.
- Bartoszyk, G.D., van Amsterdam, C., Greiner, H.E., Rautenberg, W., Russ, H. & Seyfried, C.A. 2004. Sarizotan, a serotonin 5-HT_{1A} receptor agonist and dopamine receptor ligand: 1. Neurochemical profile. *Journal of neural transmission*, 111:113-126.
- Berlin, I., Aubin, H., Pendarriosse, A. & Rames, A. 2002. Lazabemide, a selective, reversible monoamine oxidase B inhibitor, as an aid to smoking cessation. *Addiction*, 97:1347-1354.
- Bibbiani, F., Oh, J.D. & Chase, T.N. 2001. Serotonin 5-HT_{1A} agonist improves motor complications in rodent and primate parkinsonian models. *Neurology*, 57:1829-1834.
- Binda, C., Li, M., Hubalek, F., Restelli, N., Edmondson, D.E. & Mattevi, A. 2003. Insights into the mode of inhibition of human mitochondrial monoamine oxidase B from high-resolution crystal structures. *Proceedings of the national academy of science of the United States of America*, 100(17):9750-9755.
- Binda, C., Newton-Vinson, P., Hubálek, F., Edmondson, D.E. & Mattevi, A. 2002. Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders. *Nature structural biology*, 9:22-28.

- Blier, P., Piñeyro, G., el Mansari, M., Bergeron, R. & de Montigny, C. 1998. Role of somatodendritic 5-HT autoreceptors in modulating 5-HT neurotransmission. *Annals of the New York academy of sciences*, 861:204-216.
- Bortolato, M. & Shih, J.C. 2011. Behavioral outcomes of monoamine oxidase deficiency: preclinical and clinical evidence. *International review of neurobiology*, 100:13-42.
- Braak, H., Ehebremdhin, E., Rüb, U., Bratzke, H. & Del Tedici, K. 2004. Stages in the development of parkinson's disease-related pathology. *Cell and tissue research*, 318:121-134.
- Broadley, K.J. 2010. The vascular effects of trace amines and amphetamines. *Pharmacology & therapeutics*, 125(3):363–375.
- Caccia, C., Maj, R., Calabresi, M., Maestroni, S., Faravelli, L., Curatolo, L., Salvati, P. & Fariello, R.G. 2006. Safinamide: from molecular targets to a new anti-Parkinson drug. *Neurology*, 67:18-23.
- Camargo, S.M., Vuille-dit-Bille, R.N., Mariotta, L., Ramadan, T., Huggel, K., Singer, D., Götze, O. & Verrey, F. 2014. The molecular mechanism of intestinal levodopa absorption and its possible implications for the treatment of Parkinson's disease. *Journal of pharmacology and experimental therapeutics*, 351(1):114-123.
- Campbell, A., Baldessarini, R.J., Ram, V.J. & Neumeyer, J.L. 1982. Behavioral effects of (-)-10,11-methylenedioxy-N-n-propylnoraporphine, an orally effective long-acting agent active at central dopamine receptors, and analogous aporphines. *Neuropharmacology*, 21:953-961.
- Castagnoli, N Jr., Rimoldi, J.M. & Bloomquist, J. 1997. Potential metabolic bioactivation pathways involving cyclic tertiary amines and azaarenes. *Chemical research in toxicology*, 10:924-940.
- Cesura, A.M., Borroni, E., Gottowik, J., Kuhn, C., Malherbe, P., Martin, J. & Richards, J.G. 1999. Lazabemide for the treatment of Alzheimer's disease: rationale and therapeutic perspectives. *Advances in neurology*, 80:521-528.
- Cesura, A.M., Gottowik, J., Lahm, H., Lang, G., Imhof, R., Malherbe, P., Rothlisberger, U. & Da Prada, M. 1996. Investigation on the structure of the active site of monoamine oxidase-B by affinity labelling with the selective inhibitor lazabemide and by site-directed mutagenesis. *European journal of biochemistry*, 236:996-1002.

- Cesura, A.M., Muggli-Maniglio, D., Lang, G., Imhof, R. & Da Prada, M. 1990. Monoamine oxidase inhibition by moclobemide and 2-amino-ethyl carboxamide derivatives: mode of action and kinetic characteristics. *Journal of neural transmission*, 32:165-170.
- Chalmers, J.P., Baldessarini, R.J. & Wurtman, R.J. 1971. Effects of L-dopa on norepinephrine metabolism in the brain. *Proceedings of the national academy of sciences of the United States of America*, 68:662-666.
- Christoffersen, C.L. & Meltzer, L.T. 1998. Reversal of haloperidol-induced extrapyramidal side effects in cebus monkeys by 8-hydroxy-2-(di-n-propylamino)tetralin and its enantiomers. *Neuropsychopharmacology*, 18:399-402.
- Colosimo, C., Merello, M. & Albanese, A. 1994. Clinical usefulness of apomorphine in movement disorders. *Clinical neuropharmacology*, 17:243-259.
- Contin, M. & Martinelli, P. 2010. Pharmacokinetics of levodopa. *Journal of neurology*, 257(2):253-261.
- Contin, M., Riva, R., Martinelli, P., Procaccianti, G., Cortelli, P., Avoni, P. & Baruzzi, A. 1990. Response to a standard oral levodopa test in parkinsonian patients with and without motor fluctuations. *Clinical neuropharmacology*, 13(1):19-28.
- Cotzias, G.C., Papavasiliou, P.S. & Gellene, R. 1969. Modification of Parkinsonism—chronic treatment with L-dopa. *New England journal of medicine*, 280:337-45.
- Cotzias, G.C., Van Woert, M.H. & Schiffer, L.M. 1967. Aromatic amino acids and modification of parkinsonism. *New England journal of medicine*, 276:374-379.
- Da Prada, M., Kettler, R., Zurcher, G., Schaffner, R. & Haefely, W.E. 1987. Inhibition of decarboxylase and levels of dopa and 3-o-methyldopa: A comparative study of benserazide versus carbidopa in rodents and of Madopar standard versus Madopar HBS in volunteers. *European neurology*, 27:9-20.
- Dauer, W. & Przedborski, S. 2003. Parkinson's Disease: mechanisms and models. *Neuron*, 39:889-909.
- Dawson, T.M. & Dawson, V.L. 2003. Molecular pathways of neurodegeneration in Parkinson's disease. *Science*, 302:819-822.
- DeColibus L., Li, M., Binda, C., Edmondson, D.E. & Mattevi, A. 2005. Three-dimensional structure of human monoamine oxidase A (MAO A): relation to the structures of rat MAO A and human MAO B. *Proceedings of the national academy of science of the United States of America*, 102:12684-12689.

- Di Stefano, A., Sozio, P. & Cerasa, L.S. 2008. Antiparkinson prodrugs. *Molecules*, 13(1):46-68.
- Di Stefano, A., Sozio, P., Cerasa, L.S. & Iannitelli, A. 2011. L-Dopa prodrugs: an overview of trends for improving Parkinson's disease treatment. *Current pharmaceutical design*, 17(32):3482-3493.
- Di Stefano, A., Sozio, P., Iannitelli, A. & Cerasa, L.S. 2009. New drug delivery strategies for improved Parkinson's disease therapy. *Expert opinion on drug delivery*, 6:389-404.
- Di Stefano, A., Sozio, P., Iannitelli, A., Cocco, A., Orlando, G. & Ricciutelli, M. 2006. Synthesis and preliminary evaluation of Ldopa/benserazide conjugates as dual acting codrugs. *Letters in drug design and discovery*, 3(10):747-752.
- Dingemans, J., Wood, N., Jorga, K. & Kettlefi, R. 1997. Pharmacokinetics and pharmacodynamics of single and multiple doses of the MAO-B inhibitor lazabemide in healthy subjects. *British journal of clinical pharmacology*, 43:41-47.
- Edmondson, D. E., Binda, C., & Mattevi, A. 2007. Structural insights into the mechanism of amine oxidation by monoamine oxidases A and B. *Archives of biochemistry and biophysics*, 464:269-276.
- Edmondson, D.E., Binda, C., Wang, J., Upadhyay, A.K. & Mattevi, A. 2009. Molecular and mechanistic properties of the membrane-bound mitochondrial monoamine oxidases. *Biochemistry*, 48:4220-4230.
- Edmondson, D.E., DeColibus, L., Binda, C., Li, M. & Mattevi, A. 2007. New insights into the structures and functions of human monoamine oxidase A and B. *Journal of neural transmission*, 114:703-705.
- Edmondson, D.E., Mattevi, A., Binda, C., Li, M. & Hubálek, F. 2004. Structure and mechanism of monoamine oxidase. *Current medicinal chemistry*, 11(15):1983-93.
- Ehringer, H. & Hornykiewicz, O. 1960. Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system. *Weiner klinische wochenschrift*, 38:1236-1239.
- Factor, S.A., Molho, E.S., Feustel, P.J., Brown, D.L. & Evans, S.M. 2001. Long-term comparative experience with tolcapone and entacapone in advanced Parkinson's disease. *Clinical neuropharmacology*, 24:295-299.

Felder, C.C., Bymaster, F.P., Ward, J. & DeLapp, N. 2000. Therapeutic opportunities for muscarinic receptors in the central nervous system. *Journal of medicinal chemistry*, 43:4333-4353.

Finberg, J.P., Wang, J., Bankiewicz, K., Harvey-White, J., Kopin, I.J. & Goldstein, D.S. 1998. Increased striatal dopamine production from L-DOPA following selective inhibition of monoamine oxidase B by R(+)-N-propargyl-1-aminoindan (rasagiline) in the monkey. *Journal of neural transmission*, 52:279-285.

Finberg, J.P.M. 2014. Update on the pharmacology of selective inhibitors of MAO-A and MAO-B: focus on modulation of CNS monoamine neurotransmitter release. *Pharmacology & therapeutics*, 143:133-152.

Fink, J.S., Weaver, D.R., Rivkees, S.A., Peterfreund, R.A., Pollack, A.E., Adler, E.M. & Reppert, S.M. 1992. Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Brain research. Molecular brain research*, 14:186-195.

Forno, L.S. 1996. Neuropathology of Parkinson's disease. *Journal of neuropathology: Experiments in neurology*, 55:259-272.

Fowler, J.S., Logan, J., Shumay, E., Alia-Klein, N., Wang, G.J. & Volkow, N.D. 2015. Monoamine oxidase: radiotracer chemistry and human studies. *Journal of labelled compounds and radiopharmaceuticals*, 58(3):51-64.

Fowler, J.S., Logan, J., Volkow, N.D. & Wang, G.J. 2005. Translational neuroimaging: positron emission tomography studies of monoamine oxidase. *Molecular imaging and Biology*, 7(6):377-387.

Fowler, J.S., Volkow, N.D., Logan, J., Schlyer, D.J., MacGregor, R.R., Wang, G.J., Wolf, A.P., Pappas, N., Alexoff, D. & Shea, C. 1993. Monoamine oxidase B (MAO B) inhibitor therapy in Parkinson's disease: the degree and reversibility of human brain MAO B inhibition by Ro 19 6327. *Neurology*, 43(10):1984-1992.

Frankel, J.P., Lees, A.J., Kempster, P.A. & Stern, G.M. 1990. Subcutaneous apomorphine in the treatment of Parkinson's disease. *Journal of neurology neurosurgery psychiatry*, 53:96-101.

Freitas, M.E., Ruiz-Lopez, M. & Fox, S.H. 2016. Novel levodopa formulations for parkinson's disease. *CNS Drugs*, 30(11):1079-1095.

- Friend, D.R. & Chang, G.W. 1984. A colon-specific drug-delivery system based on drug glycosides and the glycosidases of colonic bacteria. *Journal of medicinal chemistry*, 27:261-266.
- Ganapathy, M.E., Huang, W., Wang, H., Ganapathy, V. & Leibach, F.H. 1998. Valacyclovir: a substrate for the intestinal and renal peptide transporters PEPT1 and PEPT2. *Biochemical and biophysical research communications*, 246:470-475.
- Gardoni, F. & Di Luca, M. 2006. New targets for pharmacological intervention in the glutamatergic synapse. *European journal of pharmacology*, 545:2-10.
- Giorgionia, G., Claudia, F., Ruggiera, S., Ricciutellia, M., Palmiera, G.F., Di Stefano, A., Sozio, P., Cerasa, L.S., Chiavaroli, A., Ferrante, C., Orlando, G. & Glennonc, R.A. 2010. Design, synthesis, and preliminary pharmacological evaluation of new imidazolinones as L-DOPA prodrugs. *Bioorganic & medicinal chemistry*, 18:1834-1843.
- Gleeson, M.P. 2008. Generation of a set of simple, interpretable ADMET rules of thumb. *Journal of medicinal chemistry*, 51(4):817-834.
- Glezer, S. & Finberg, J.P. 2003. Pharmacological comparison between the actions of methamphetamine and 1-aminoindan stereoisomers on sympathetic nervous function in rat vas deferens. *European journal of pharmacology*, 472:173-177.
- Green, A.R., Mitchell, B.D., Tordoff, A.F. & Youdim, M.B. 1977. Evidence for dopamine deamination by both type A and type B monoamine oxidase in rat brain in vivo and for the degree of inhibition of enzyme necessary for increased functional activity of dopamine and 5 hydroxytryptamine. *British journal of pharmacology*, 60:343-349.
- Greenamyre, J.T. & Hastings, T.G. 2004. Parkinson's-divergent causes, convergent mechanisms. *Science*, 304:1120-1122.
- Han, H. & Amidon, G.L. 2000. Targeted prodrug design to optimize drug delivery. *AAPS PharmSciTech*, 2:1-11.
- Hauser, R.A., Shulman, L.M., Trugman, J.M., Roberts, J.W., Mori, A. & Ballerini, R. 2008. Study of istradefylline in patients with Parkinson's disease on levodopa with motor fluctuations. *Movement disorders*, 23:2177-85.
- Holford, N.H.G., Guentert, T.W., Dingemans, J. & Kettler, R. 1994. Pharmacodynamics of lazabemide, a reversible and selective inhibitor of monoamine oxidase B. *British journal of clinical pharmacology*, 37:553-557.

- Hoon, M. 2013. Design of novel levodopa prodrugs for the treatment of Parkinson's disease. Dissertation submitted to North-West University – Potchefstroom Campus. November 2013.
- Hornykiewicz, O. 2010. A brief history of levodopa. *Journal of neurology*, 257(2):249-252.
- Hu, M. & Borchardt, R.T. 1990. Mechanism of L-a-methyldopa transport through a monolayer of polarized human intestinal epithelial cells (Caco-2). *Pharmaceutical research*, 7:1313-1319
- Hughes, A.J., Bishop, S., Kleedorfer, B., Turjanski, N., Fernandez, W., Lees, A.J. & Stern, G.M. 1993. Subcutaneous apomorphine in Parkinson's disease: response to chronic administration for up to five years. *Movement disorders*, 8:165-170.
- Hutton, J.T., Morris, J.L. & Brewer, M.A. 1993. Controlled study of the antiparkinsonian activity and tolerability of cabergoline. *Neurology*, 43:613-616.
- Jenner, P. 2003. Oxidative stress in Parkinson's disease. *Annals of neurology*, 53:526-536.
- Kanda, T., Jackson, M.J., Smith, L.A., Pearce, R.K., Nakamura, J., Kase, H., Kuwana, Y. & Jenner, P. 1998. Adenosine A_{2A} antagonist: a novel antiparkinson's agent that does not provoke dyskinesia in parkinsonian monkeys. *Annals of neurology*, 43:507-513.
- Kaplitt, M.G., Feigin, A., Tang, C., Fitzsimons, H.L., Mattis, P., Lawlor, P.A., Bland, R.J., Young, D., Strybing, K., Eidelberg, D. & During, M.J. 2007. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *Lancet*, 369:2097-2105.
- Kerr, J.S., Fairweather, D.B. & Hindmarch, I. 1993. The effects of brofaromine alone and in conjunction with alcohol on cognitive function, psychomotor performance, mood and sleep in healthy volunteers. *Human psychopharmacology*, 8:107-116.
- Khor, S.P. & Hsu, A. 2007. The pharmacokinetics and pharmacodynamics of levodopa in the treatment of Parkinson's disease. *Current clinical pharmacology*, 2(3):234-243.
- Kieburtz, K. 1993. A controlled trial of lazabemide (RO19-6327) in untreated Parkinson's disease. *Annals of neurology*, 33:350-356.
- Kuno, S. 1997. Differential therapeutic effects of dopamine D1 and D2 agonists in MPTP induced parkinsonian monkeys: clinical implications. *European neurology*, 38:18-22.
- Kurokawa, M., Koga, K., Kase, H., Nakamura, J. & Kuwana, Y. 1996. Adenosine A_{2a} receptor mediated modulation of striatal acetylcholine release in vivo. *Journal of neurochemistry*, 66:1882-1888.

Langston, J.W., Ballard, P., Tetrud, J.W. & Irwin, I. 1983. Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*, 219:979-980.

Learmonth, D.A., Palma, P.N., Vieira-Coelho, M.A. & Soares-da-Silva, P. 2004. Synthesis, biological evaluation, and molecular modeling studies of a novel, peripherally selective inhibitor of catechol-O-methyltransferase. *Journal of medicinal chemistry*, 47(25):6207-6217.

Leppänen, J., Huuskonen, J., Nevalainen, T., Gynther, J., Taipale, H. & Järvinen, T. 2002. Design and synthesis of a novel L-dopa-entacapone codrug. *Journal of medicinal chemistry*, 45(6):1379-1382.

Lindemann, L. & Hoener, M.C. 2005. A renaissance in trace amines inspired by a novel GPCR family. *Trends in pharmacological sciences*, 26:274-281.

Lum, C.T. & Stahl, S.M. 2012. Opportunities for reversible inhibitors of monoamine oxidase-A (RIMAs) in the treatment of depression. *CNS Spectrums*, 17(3):107-120.

Macleod, A.D., Counsell, C.E., Ives, N. & Stowe, R. 2005. Monoamine oxidase B inhibitors for early Parkinson's disease. *Cochrane database of systemic reviews*, 3:CD004898.

Manallack, D.T., Prankerd, R.J., Yuriev, E., Oprea, T.I. & Chalmers, D.K. 2013. The significance of acid/base properties in drug discovery. *Chemical society reviews*, 42(2):485-496.

Mason, R.P., Olmstead Jr, E.G. & Jacob, R.F. 2000. Antioxidant activity of the monoamine oxidase B inhibitor lazabemide. *Biochemical pharmacology*, 60:709-716.

Massing, T. 2016. Pesticides and Parkinson's disease. *The clinical advisor*, 492896:1-2.

Matthysse, S. & Baldessarini, R.J. 1972. S-Adenosylmethionine and catechol-O-methyltransferase in schizophrenia. *American journal of psychiatry*, 128:1310-1312.

Melamed, E., Zoldan, J., Friedberg, G., Ziv, I. & Weizmann, A. 1996. Involvement of serotonin in clinical features of Parkinson's disease and complications of L-dopa therapy. *Advances in neurology*, 69:545-550.

Merello, M., Nouzeilles, M.I., Cammarota, A. & Leiguarda, R. 1999. Effect of memantine (NMDA antagonist) on Parkinson's disease: a double-blind crossover randomized study. *Clinical neuropharmacology*, 22:273-276.

Millan, M.J., Maiorini, L., Cussac, D., Audinot, V., Boutin, J. & Newman-Tancredi, A. 2002. Differential actions of antiparkinson agents at multiple classes of monoaminergic receptor. I.

- A multivariate analysis of the binding profiles of 14 drugs at 21 native and cloned human receptor subtypes. *Journal of pharmacology and experimental therapeutics*, 303:791-804.
- Mitoma, J. & Ito, A. 1992. Mitochondrial targeting signal of rat liver monoamine oxidase B is located at its carboxy terminus. *Journal of biochemistry*, 111:20-24.
- Miyawaki, E., Lyons, K. & Pahwa, R. 1997. Motor complications of chronic levodopa therapy in Parkinson's disease. *Clinical neuropharmacology*, 20:523-530.
- Mizuma, T., Ohta, K., Hayashi, M. & Awazu, S. 1992. Intestinal active absorption of sugar-conjugated compounds by glucose transport system: implication of improvement of poorly absorbable drugs. *Biochemical pharmacology*, 43:2037-2039.
- Mouradian, M.M. & Chase, T.N. 1997. Gene therapy for Parkinson's disease: An approach to the prevention or palliation of levodopa-associated motor complications. *Experimental neurology*, 144:51-57.
- Nagatsu, T. 1973. Biochemistry of Catecholamines: The Biochemical Method. 289-299. University Park Press, Baltimore.
- Newman-Tancredi, A., Cussac, D., Audinot, V., Nicolas, J.P., De Ceuninck, F., Boutin, J.A. & Millan, M.J. 2002. Differential actions of antiparkinson agents at multiple classes of monoaminergic receptor. II. Agonist and antagonist properties at subtypes of dopamine D (2)-like receptor and alpha (1)/alpha (2)-adrenoceptor. *Journal of pharmacology experimental therapeutics*, 303:805-814.
- Ng, J., Heales, S.J.R. & Kurian, M.A. 2014. Clinical features and pharmacotherapy of childhood monoamine neurotransmitter disorders. *Pediatric drugs*, 16:275-291.
- Nissinen, E., Lindén, I.B., Schultz, E. & Pohto, P. 1992. Biochemical and pharmacological properties of a peripherally acting catechol-O-methyltransferase inhibitor entacapone. *Naunyn-Schmiedeberg's archives of pharmacology*, 346(3):262-266.
- Nofsinger, R., Clas, S., Sanchez, R.I., Walji, A., Manser, K., Nissley, B., Balsells, J., Nair, A., Dang, Q., Bennett, D.J., Hafey, M., Wang, J., Higgins, J., Templeton, A., Coleman, P., Grobler, J., Smith, R. & Wu, Y. 2014. Design of prodrugs to enhance colonic absorption by increasing lipophilicity and blocking ionization. *Pharmaceuticals*, 7:207-219.
- Nutt, J.G. 2000. Effect of COMT inhibition on the pharmacokinetics and pharmacodynamics of levodopa in parkinsonian patients. *Neurology*, 55(4):33-37.
- Nutt, J.G. & Fellman, J.H. 1984. Pharmacokinetics of levodopa. *Clinical neuropharmacology*, 7(1):35-49.

- Olanow, C.W. 2009. Can we achieve neuroprotection with currently available anti-parkinsonian interventions? *Neurology*, 72:59-64.
- Olanow, C.W. & Watkins, P.B. 2007. Tolcapone: an efficacy and safety review. *Clinical neuropharmacology*, 3:287-94.
- Olanow, C.W., Damier, P., Goetz, C.G., Mueller, T., Nutt, J. & Rascol, O. 2004. Multicenter, open-label trial of sarizotan in Parkinson's disease patients with levodopa induced dyskinesias (SPLENDID study). *Clinical neuropharmacology*, 27:58-62.
- Paci, C., Thomas, A. & Onofrj, M. 2001. Amantadine for dyskinesia in patients affected by severe Parkinson's disease. *Neurological sciences*, 22:75-76.
- Parkinson J. 1817. An essay on the shaking palsy. Whittingham and Rowland for Sherwood, Neely and Jones, London. Parkinson's disease. *Journal of neurology*, 255:18-32.
- Patat, A., Gandon, J.M., Durrieu, G., Le Coz, F., Curet, O., Cimatosti, I. & Allain, H. 1995. Effects of single and multiple doses of a new reversible MAO-A inhibitor, befloxatone, on psychomotor performance and memory in healthy subjects. *Human psychopharmacology*, 10:111-125.
- Pavan, B., Dalpiaz, A., Ciliberti, N., Biondi, C., Manfredini, S. & Vertuani, S. 2008. Progress in drug delivery to the central nervous system by the prodrug approach. *Molecules*, 13:1035-1065.
- Poewe, W. & Antonini, A. 2015. Novel formulations and modes of delivery of levodopa. *Movement disorder*, 30(1):114-120.
- Rajput, A.H., Uitti, R.J. & Offord, K.O. 1997. Timely levodopa administration prolongs survival in Parkinson's disease. *Parkinsonism and related disorders*, 3:159-165.
- Ramsay, R.R. 2016. Molecular aspects of monoamine oxidase B. *Progress in neuro-psychopharmacol & biological Psychiatry*, 69:81-89.
- Rascol, O., Brooks, D.J., Korczyn, A.D., De Deyn, P.P., Clarke, C.E. & Lang, A.E. 2000. A five-year study of the incidence of dyskinesia in patients with early Parkinson's disease who were treated with ropinirole or levodopa. 056 Study Group. *New England journal of medicine*, 342:1484-1491.
- Rebrin, I., Geha, R.M., Chen, K. & Shih, J.C. 2001. Effects of carboxyl-terminal truncations on the activity and solubility of human monoamine oxidase B. *The journal of biological chemistry*, 276:29499-29506.

- Richardson, P.J., Kase, H. & Jenner, P.G. 1997. Adenosine A_{2A} receptor antagonists as new agents for the treatment of Parkinson's disease. *Trends in pharmacological sciences*, 18:338-344.
- Riederer, P. & Laux, G. 2011. MAO inhibitors in Parkinson's disease. *Experimental neurobiology*, 20:1-17.
- Riederer, P. & Youdim, M.B. 1986. Monoamine oxidase activity and monoamine metabolism in brains of parkinsonian patients treated with l-selegiline. *Journal of neurochemistry*, 46:1359-1365.
- Robakis, D.M. & Fahn, S. 2015. Defining the role of the monoamine oxidase-B inhibitors for Parkinson's disease. *CNS drugs*, 29:433-441.
- Satoh, M., Kuzuu, A., Doi, H., Masaka, T. & Sakakibara, R. 2015. Monitoring of blood L-dopa and L-dopa metabolite concentrations and adverse events in patients with advanced Parkinson's disease receiving L-dopa and amantadine combination therapy: A clinical study. *Research and reviews: journal of pharmacy and pharmaceutical sciences*, 4:88-96.
- Saura, J., Bleuel, Z., Ulrich, J., Mendelowitsch, A., Chen, K., Shih, J.C., Malherbe, P., Da Prada, M. & Richards, J.G. 1996. Molecular neuroanatomy of human monoamine oxidases A and B revealed by quantitative enzyme radioautography and in situ hybridization histochemistry. *Neuroscience*, 70:755-774.
- Savolainen, J., Leppanen, J., Forsberg, M., Taipale, H., Nevalainen, T., Huuskonen, J., Gynther, J., Mannisto, P.T. & Jarvinen, T. 2000. Synthesis and in vitro/in vivo evaluation of novel oral N-alkyl- and N,N-dialkyl-carbamate esters of entacapone. *Life science* 67(2):205-216.
- Schwab, R.S., Amador, L.V. & Lettvin, J.Y. 1951. Apomorphine in Parkinson's disease. *Transactions of the American neurology association*, 56:251-253.
- Seeberger, L.C. & Hauser, R.A. 2015. Carbidopa levodopa enteral suspension. *Expert opinion pharmacotherapy*, 16(18):2807-2817.
- Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muenter, M., Baptista, M., Miller, D., Blancato, J., Hardy, J. & Gwinn-Hardy, K. 2003. Alpha-Synuclein locus triplication causes Parkinson's disease. *Science*, 302:841.

Standaert, D.G. & Young, A.B. 2006. Treatment of central nervous system degenerative disorders. pp 527-546 in Brunton, Laurence L., John S. Lazo, and Keith L. Parker, eds. Goodman and Gilman's The Pharmacological Basis of Therapeutics. 2006. McGraw-Hill, New York.

Stella, V.J. & Himmelstein, K.J. 1982. Critique of prodrugs and site-specific delivery. In: Bundgaard H, ed. Optimization of Drug Delivery. Alfred Benzon Symposium 17. Copenhagen, Munksgaard; 134-155.

Stocchi, F. 2008. Use of apomorphine in Parkinson's disease. *Neurology science*, 29:383-386.

Stocchi, F., Arnold, G., Onofrij, M., Kwiecinski, H., Szczudlik, A., Thomas, A., Bonuccelli, U., Van Dijk, A., Cattaneo, C., Sala, P. & Fariello, R.G. 2004. Improvement of motor function in early Parkinson disease by safinamide. *Neurology*, 63:746-748.

Swaan, P.W. & Tukker, J.J. 1995. Carrier-mediated transport mechanism of foscarnet (trisodium phosphonoformate hexahydrate) in rat intestinal tissue. *Journal of pharmacology and experimental therapy*, 272:242-247.

Teräväinen, H., Rinne, U. & Gordin, A. 2001. Catechol-O-methyltransferase inhibitors in Parkinson's disease. *Advanced neurology*, 86:311-325.

Trevis, J., Kawa, K., Jalali, A. & Larsen, C. 2009. Differential effects of adenosine antagonists in two models of parkinsonian tremor. *Pharmacology biochemistry and behaviour*, 94:24-29.

Upadhyay, A.K., Borbat, P.P., Wang, J., Freed, J.H. & Edmondson, D.E. 2008. Determination of the oligomeric states of human and rat monoamine oxidases in the outer mitochondrial membrane and octyl beta-D-glucopyranoside micelles using pulsed dipolar electron spin resonance spectroscopy. *Biochemistry*, 47:1554-1566.

Vogel, W.H. 1970. Determination and physiological disposition of p-methoxyphenylethylamine in the rat. *Biochemical pharmacology*, 19:2663-2665.

Walker, M.C. & Edmondson, D.E. 1994. Demonstration of isoleucine 199 as a structural determinant for the selective inhibition of human monoamine oxidase B by specific reversible inhibitors. *Biochemistry*, 33:7088-7098.

Youdim, M.B., Edmondson, D. & Tipton, K.F. 2006. The therapeutic potential of monoamine oxidase inhibitors. *Nature reviews neuroscience*, 7(4):295-309.

Youdim, M.B.H. & Bakhle, Y.S. 2006. Monoamine oxidase: isoforms and inhibitors in Parkinson's disease and depressive illness. *British journal of pharmacology*, 147: S287-S296.

Chapter 3

Article 1

The design and evaluation of an L-dopa-lazabemide prodrug for the treatment of Parkinson's disease

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Running title: L-Dopa-lazabemide prodrug for PD

Abstract

L-Dopa, the metabolic precursor of dopamine (DA), is the treatment of choice for the symptomatic relief of the advanced stages of Parkinson's Disease (PD). The oral bioavailability of L-dopa, however, is only about 10% and less than 1% of the oral dose reaches the brain unchanged. L-Dopa's physicochemical properties are responsible for its poor bioavailability, short half-life and the wide range of inter- and intra-patient variations of plasma levels. An L-dopa-lazabemide prodrug is proposed to overcome the problems associated with L-dopa absorption. Lazabemide is a monoamine oxidase (MAO) B inhibitor, a class of compounds which slows the depletion of DA stores in PD and elevates DA levels produced by exogenously administered L-dopa. L-Dopa was linked at the carboxylate with the primary aminyl functional group of lazabemide via an amide, a strategy which is anticipated to protect L-dopa against peripheral decarboxylation and possibly also enhance the membrane permeability of the prodrug. Selected physicochemical and biochemical properties of the prodrug were determined and included lipophilicity (logD), solubility, passive diffusion permeability, pKa, chemical and metabolic stability as well as cytotoxicity. Although oral and i.p. treatment of mice with the prodrug did not result in enhanced striatal DA levels, DOPAC levels were significantly depressed compared to saline, L-dopa and carbidopa/L-dopa treatment. Based on these data, further preclinical evaluation of the L-dopa-lazabemide prodrug should be undertaken with the aim of discovering prodrugs that may be advanced to the clinical stages of development.

Keywords: lazabemide, L-dopa, prodrug, monoamine oxidase, MAO, inhibition, physicochemical properties

3.1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disorder which is caused by the loss of dopaminergic neurons from the substantia nigra pars compacta in the brain (Dauer & Przedborski, 2003). The dopaminergic neurons which degenerate in PD are specifically those of the nigrostriatal pathway and delivers dopamine (DA) to the striatum. The resulting functional deficit of DA in the striatum is responsible for the motor symptoms observed in PD (Dauer & Przedborski, 2003). Ever since its early clinical use in the 1960s, L-dopa has remained the most effective treatment for PD (Fig. 3.1) (Freitas *et al.*, 2016; Poewe & Antonini, 2015). L-Dopa is the direct metabolic precursor of DA and, in contrast to DA, permeates the blood-brain barrier by carrier-mediated transport (Di Stefano *et al.*, 2011). Once in the brain, L-dopa can be converted to DA, thus effectively replacing the lost DA in the striatum (Di Stefano *et al.*, 2008). Although L-dopa is absorbed from the gastrointestinal tract via the large neutral amino acid (LNAA) transport system, it is extensively decarboxylated in the gastrointestinal tract by the enzyme L-amino acid decarboxylase (AADC) and only approximately 30% of the L-dopa dose reaches the systemic circulation unchanged (Freitas *et al.*, 2016; Contin & Martinelli, 2010). L-Dopa is further decarboxylated in the peripheral tissues, which further reduces the bioavailability to the brain. It is estimated that less than 1% of the administered oral dose of L-dopa reaches the brain unchanged (Di Stefano *et al.*, 2011). To improve the bioavailability of L-dopa and to reduce peripheral dopaminergic side effects such as cardiac arrhythmias, hypotension, nausea and vomiting (due to the peripheral conversion of L-dopa to DA), L-dopa is combined with an AADC inhibitor such as carbidopa or benserazide (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 the resulting metabolite, 3-O-methyldopa accumulates in the peripheral and central tissues (Nutt & Fellman, 1984). 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). This is most likely because 3-O-methyldopa competes with L-dopa for transport across the blood-brain barrier. COMT inhibitors (e.g. entacapone), are thus also used as adjuncts to L-dopa in PD (Learmonth *et al.*, 2004; Nissinen *et al.*, 1992).

Although oral L-dopa is a highly effective treatment for PD, long-term use is associated with response fluctuations and involuntary movements, termed L-dopa-induced dyskinesias. These are, in part, due to irregular absorption (due to erratic gastric emptying), peripheral metabolism, a short half-life of approximately 90 min and limited transport across the blood-brain barrier (Shoulson *et al.*, 1975; Nutt, 1987).

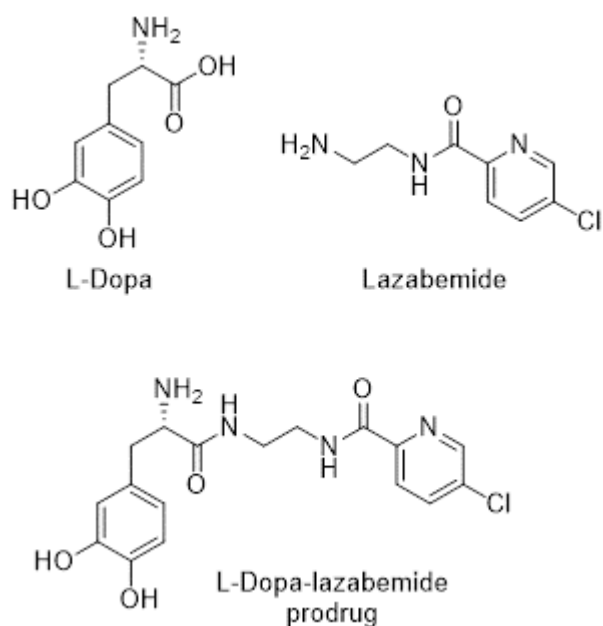


Fig. 3.1: The structures of L-dopa, lazabemide and the L-dopa-lazabemide prodrug.

In this respect, competition with dietary amino acids at transporters in the gastrointestinal tract and brain microvasculature may significantly affect L-dopa absorption and delivery to the brain (Leenders *et al.*, 1986; Nutt *et al.*, 1984). Efforts have thus been made to develop new oral L-dopa formulations which may address some of the absorption, metabolism and delivery issues of L-dopa (Freitas *et al.*, 2016; Poewe & Antonini, 2015). Several of these have entered clinical trials with IPX066, a L-dopa–carbidopa oral formulation combining immediate-release (IR) and extended-release (ER) LD/CD, having recently been approved (Freitas *et al.*, 2016). Another approach to overcome the problems with peripheral L-dopa metabolism and delivery difficulties, is to alter the physicochemical properties of L-dopa by designing prodrugs (Di Stefano *et al.*, 2011). For example, XP21279 is a prodrug that is absorbed from the small and large intestine by high-capacity nutritional transporters (Poewe & Antonini, 2015). After absorption, this compound is rapidly metabolised to L-dopa with a relative bioavailability of approximately 90% compared to L-dopa–carbidopa combination therapy (Lewitt *et al.*, 2012; Lewitt *et al.*, 2014).

The present study proposes a novel L-dopa-lazabemide prodrug to overcome the problems associated with L-dopa absorption and metabolism. Lazabemide is a specific inhibitor of monoamine oxidase (MAO) B, with a reversible mechanism-based mode of action (Fig. 3.1) (Cesura *et al.*, 1990; Cesura *et al.*, 1999; Binda *et al.*, 2003; Edmondson *et al.*, 2004). Lazabemide causes rapid and complete MAO-B inhibition with enzyme activity returning to baseline values by 36 h after drug discontinuation (Dingemans *et al.*, 1997; Fowler *et al.*, 1993). A dose of at least 0.4 mg/kg lazabemide given every 12 h provides >90% inhibition of

brain MAO-B in patients with early PD (Fowler *et al.*, 1993). MAO-B inhibitors are considered useful agents in the therapy of PD and are frequently combined with L-dopa (Youdim *et al.*, 2006). By blocking the central MAO-B-catalysed metabolism of DA, these drugs are thought to slow the depletion of DA stores and to elevate DA levels produced by exogenously administered L-dopa (Finberg *et al.*, 1998). In addition, MAO-B inhibitors may also protect against neurodegeneration in PD, presumably by reducing the formation of injurious by-products of the MAO-B catalytic cycle (Youdim & Bakhle, 2006). These by-products, hydrogen peroxide and aldehyde species, may lead to neuronal death if not efficiently cleared from the central nervous system (CNS). Considering that MAO-B activity increases with age, the inhibition of this enzyme seems particularly relevant in PD (Fowler *et al.*, 1997). MAO-B inhibitors that are currently registered for the treatment of PD are selegiline and rasagiline, irreversible mechanism-based inhibitors, and safinamide, a reversibly acting drug (Fig. 3.2) (Muller, 2016; Robakis & Fahn, 2015).

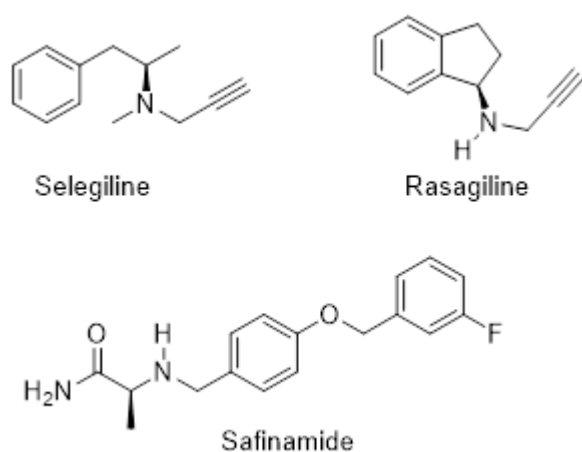


Fig. 3.2: The structures of selegiline, rasagiline and safinamide.

For the L-dopa-lazabemide prodrug, we have selected to link L-dopa at the carboxylate with the primary aminyl functional group of lazabemide via an amide (Fig. 3.1). This would protect the carboxylic acid of L-dopa against peripheral decarboxylation and, since lazabemide is amphiphilic and associates with the phospholipid bilayer of membranes (Mason *et al.*, 2000), the prodrug may exhibit enhanced membrane permeability compared to L-dopa. With the presence of the ionisable aminyl functional group and the lipophilic cloropyridinyl moiety, it is expected that the amphiphilic nature of lazabemide will be retained in the prodrug. Although amides, in general, do not hydrolyse readily, amides of amino acids (e.g. L-dopa) are known to hydrolyse *in vivo* and the proposed prodrug is expected to be activated after administration (Silverman, 2004). The increased stability of the amide link compared to the more traditional ester between a drug and its carrier could possibly allow the prodrug more time to diffuse into the brain prior to this hydrolysis event. Furthermore, unlike L-dopa, the prodrug does not

contain the acidic carboxylate group. Acidic moieties are known to reduce membrane permeation of small organic compounds (Kerns & Di, 2008). With an enhanced lipophilicity, the prodrug may be absorbed from the gastrointestinal tract by passive diffusion, thus leading to improved bioavailability compared to L-dopa (Kerns & Di, 2008). It is therefore anticipated that the prodrug will facilitate improved absorption and delivery of L-dopa to the brain and reduce peripheral decarboxylation. In addition, after activation of the prodrug, the released lazabemide may inhibit central MAO-B and thus enhance the levels of DA derived from L-dopa. Although the development of lazabemide has been discontinued due to liver toxicity, the L-dopa-lazabemide prodrug could serve as proof-of-concept for the feasibility of a prodrug that combines L-dopa with a MAO-B inhibitor (Berlin *et al.*, 2002).

3.2. Materials and methods

3.2.1. The synthesis of lazabemide

Lazabemide, as the free base, was synthesised from 5-chloro-2-cyanopyridine according to the published method (Soriato *et al.*, 2008).

3.2.2. The synthesis of the L-dopa-lazabemide prodrug

3.2.2.1. Chemicals and instrumentation

High resolution mass spectra (HRMS) were recorded with a DFS high resolution magnetic sector mass spectrometer (Thermo Electron Corporation) in electron ionization (EI) mode. Proton (^1H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance III 600 spectrometer at a frequency of 600 MHz for ^1H NMR spectra and 150 MHz for ^{13}C NMR spectra. All chemical shifts are reported in parts per million (δ) and were referenced to the residual solvent signal (DMSO- d_6 : 2.50 and 39.52 ppm for ^1H and ^{13}C , respectively). Spin multiplicities are given as s (singlet), d (doublet), m (multiplet) or broad singlet (brs). All chemicals and reagents were obtained from Sigma-Aldrich, and were used without further purification.

3.2.2.2. The synthesis of L-dopa(TBDMS) $_2$

L-Dopa (8 mmol) was added to a solution of tert-butyldimethylsilyl chloride (TBDMS-Cl, 23.2 mmol) in acetonitrile (18 ml). The suspension was stirred and cooled to 0 °C, and 3.6 ml of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 24 mmol) was added. The reaction mixture was then stirred for 18 h at room temperature and was subsequently filtered. The precipitate was collected and dried at 50 °C to yield L-dopa(TBDMS) $_2$ as a white powder in a yield of 37% (Nakonieczna *et al.*, 1994).

3.2.2.3. The synthesis of Boc-L-dopa(TBDMS)₂

Di-tert-butyl dicarbonate (Boc₂O, 8.8 mmol) was dissolved in 20 ml THF and added to a suspension L-dopa(TBDMS)₂ (7.4 mmol) in 20 ml of water containing 8 mmol of NaHCO₃. The reaction mixture was stirred for 24 h at room temperature and the THF was subsequently evaporated under reduced pressure. Water (10 ml) was added to the residue and the solution was extracted to diethyl ether (30 ml). The aqueous layer was acidified with citric acid (20%) to pH 5-6 and extracted three times with ethyl acetate (30 ml). Drying of the combined extracts over MgSO₄ and removal of the solvent under reduced pressure yielded Boc-L-dopa(TBDMS)₂ in a yield of 67% as an amorphous solid (Nakonieczna *et al.*, 1994).

3.2.2.4. The conjugation of protected L-dopa with lazabemide

Protected L-dopa [Boc-L-dopa(TBDMS)₂] (2 mmol), lazabemide (2 mmol) and 2 mmol of the BOP reagent [(1*H*-benzotriazole-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate] was added to 15 ml dichloromethane. N-Methylmorpholine (NMM, 4 mmol) was added to the reaction mixture, and the reaction was stirred for 24 h at room temperature. The dichloromethane was evaporated under reduced pressure and 5 ml of water added to the residue. The residue was subsequently extracted to ethyl acetate (35 ml). The organic phase was washed successively with 2% citric acid, water, a 3% aqueous NaHCO₃ solution and water, and was finally dried over MgSO₄. After concentration of the solution under reduced pressure the crude substance was incubated overnight upon which it solidified. The product, the protected L-dopa-lazabemide prodrug [Boc-L-dopa(TBDMS)₂-lazabemide] was obtained in a yield of 80% (Nakonieczna *et al.*, 1994).

3.2.2.5. Removal of the TBDMS and Boc protective groups from Boc-L-dopa(TBDMS)₂-lazabemide

The protected L-dopa-lazabemide prodrug [Boc-L-dopa(TBDMS)₂-lazabemide] (150 mg) was dissolved in 1.5 ml HCl solution (4 M in dioxane) and stirred for 48 h at 55 °C. The deprotecting agent was evaporated under reduced pressure and the residue was washed three times with diethyl ether (30 ml). In each instance, the diethyl ether was removed under reduced pressure. A white crystalline product, as the hydrochloric acid salt, was obtained in good yield (61 mg; 32.2%) (Nakonieczna *et al.*, 1994). ¹H NMR (Bruker Avance III 600, DMSO-d₆) δ 2.75–2.78 (m, 1H), 2.86–2.89 (m, 1H), 3.20 (m, 1H), 3.30–3.36 (m, 3H), 3.75 (m, 1H), 6.45 (d, 1H, J = 7.9 Hz), 6.64 (m, 2H), 8.03 (d, 1H, J = 8.3 Hz), 8.07 (brs, 3H), 8.11 (d, 1H, J = 8.3 Hz), 8.63 (s, 1H), 8.69 (s, 1H), 8.86 (m, 1H), 8.91 (brs, 3H). ¹³C NMR (Bruker Avance III 600, DMSO-d₆) δ 36.6, 38.4, 38.6, 54.1, 115.7, 116.9, 120.2, 123.5, 125.8, 134.0, 137.6, 144.5, 145.2, 147.1, 148.4, 163.3, 168.6. EI-HRMS m/z calcd for C₁₇H₁₉O₄N₄Cl (M⁺), 378.1095, found 378.1089.

3.3. Determination of physicochemical and biological properties

3.3.1. Materials and instrumentation

UV-Vis spectrophotometry was carried out with a Shimadzu MultiSpec-1501 UV-Vis photodiode array spectrophotometer. A Multiscan RC UV/Vis plate reader (Labsystems) was used to measure the absorbances in 96-well microplates. For potentiometric titration, a Hanna HI1230B general purpose electrode was employed and standardised KOH (0.1 N) was obtained from Merck. Precoated parallel artificial membrane permeability assay (PAMPA) plate systems were obtained from BD Biosciences while Phree phospholipid removal tubes were from Phenomonex. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich. Cell culture media (Dulbecco's Modified Eagle Medium; DMEM), fetal bovine serum, penicillin (10 000 units/ml)/streptomycin (10 mg/ml), fungizone (250 µg/ml) and trypsin/EDTA (0.25%/0.02%) were from Gibco. 24-Well and 96-well plates were from Corning while sterile syringe filters (0.22 µm) were obtained from Pall Corporation Life Sciences.

3.3.2. Ethics consideration

The treatment of the animals was conducted at the Vivarium at the Potchefstroom campus of the North-West University (NWU). Animals were bred, supplied and housed at the Vivarium (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 human blood was obtained from the Research Ethics Committee, NWU (ethics approval numbers: NWU-00325-15-A5, NWU-00326-15-A5; NWU-00056-11-S5).

3.3.3. Shake-flask method for logD determination

n-Octanol (analytical reagent from Sigma-Aldrich) and the appropriate buffer were mutually saturated in a separatory funnel. Potassium phosphate buffer at pH 6.4, 7.4 and 7.8 served as the aqueous buffer phases. In a 10 ml glass vessel, 4 ml of n-octanol was placed followed by 4 ml of buffer containing 2 mM of the L-dopa-lazabemide prodrug. The vessels were shaken by hand for 5 min and centrifuged at 4,000 g for 10 min. The n-octanol phase was diluted 30-fold into neat n-octanol and the absorbance of the resulting solution was recorded at an absorbance maximum of 276 nm. The buffer phase also was diluted 30-fold into neat buffer

and the absorbance of the resulting solution was recorded at an absorbance maximum of 276 nm. The concentrations of the L-dopa-lazabemide prodrug in the n-octanol and buffer phases were determined by employing the molar extinction coefficients recorded in n-octanol and in each of the three buffers (pH 6.4, 7.4 and 7.8) employed. The molar extinction coefficient of the prodrug in n-octanol was found to be 12493 M^{-1} . The molar extinction coefficients of the prodrug in the three buffers were similar and were found to be 13583 M^{-1} . The logD value at a given pH is equal to the logarithm of the octanol/buffer partition coefficients (D). The partition coefficient is the ratio of the concentration of the L-dopa-lazabemide prodrug in the n-octanol phase to the buffer phase. LogD values are given as mean \pm standard deviation of triplicate determinations. Using the same protocol as above the logD values at pH 6.4, 7.4 and 7.8 for lazabemide were also determined. The molar extinction coefficient of lazabemide in n-octanol was found to be 9666 M^{-1} . The molar extinction coefficients of lazabemide in the three buffers were similar and were found to be 11159 M^{-1} .

3.3.4. Determination of solubility

To determine the solubility of the L-dopa-lazabemide prodrug, 18 mg of the drug was placed into a polypropylene tube and 3 ml water or potassium phosphate buffer (pH 7.4) was added. This yielded a theoretical concentration of 6.0 mg/ml. The tubes were agitated for 24 h in a water bath at $37 \text{ }^{\circ}\text{C}$ and were subsequently centrifuged at 16,000 g for 10 min. The samples were filtered through a $0.22 \text{ }\mu\text{m}$ syringe filter and diluted 150-fold into water or potassium phosphate buffer (100 mM, pH 7.4). The absorbances of the resulting solutions were recorded at a wavelength of maximal absorbance of 276 nm. The concentration of the L-dopa-lazabemide prodrug was determined by employing the molar extinction coefficient measured in water (13956 M^{-1}) and that cited above (13583 M^{-1}) for potassium phosphate buffer (100 mM, pH 7.4). The solubility values are given as mean \pm standard deviation of triplicate determinations.

3.3.5. Determination of ionisation constant, pKa

A 10 mM solution of the L-dopa-lazabemide prodrug in 10 ml water was prepared and potentiometrically titrated with a standardised solution of KOH (0.1 N). The prodrug was titrated in 0.1 ml increments and the pH was recorded after each addition. A total of 1 ml KOH was added to fully neutralise the primary amine group of the prodrug. The results were subsequently tabulated according to the method described in literature (Albert, 1984), and the pKa value was calculated.

3.3.6. Determination of toxicity towards cultured cells

HeLa cells were maintained in 250 cm² flasks in 30 ml Dulbecco's Modified Eagle Medium (DMEM) containing, 10% fetal bovine serum, 1% penicillin (10 000 units/ml)/streptomycin (10 mg/ml) and 0.1% fungizone (250 µg/ml). The cells were incubated at 37 °C in an atmosphere of 10% CO₂. Once confluent, the cells were seeded at 500 000 cells/well in 24-well plates and incubated for 24 h. A volume of 3 ml trypsin/EDTA (0.25%/0.02%) was used to facilitate cell detachment and the counting of cells was done with a haemocytometer. The wells were subsequently rinsed with 0.5 ml DMEM containing no fetal bovine serum. A volume of 0.99 ml DMEM (containing no fetal bovine serum) was subsequently added to each well followed by 10 µl of the test drug. Stock solutions of the test drugs were prepared in deionised water and sterilised via a 0.22 µm syringe filter. In each 24-well plate, wells were reserved as positive controls (100% cell death via lyses with 0.3% formic acid) and negative controls (0% cell death as a result of no treatment). The well-plates were incubated for a further 24 h after which the culture medium was aspirated from the cells. The wells were washed twice with 0.5 ml/well phosphate-buffered saline (PBS) and 200 µl of 0.5% MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in PBS was added to each well. The well-plates were incubated at 37 °C for 2 h in the dark, the MTT-reagent was aspirated from the wells and 250 µl isopropanol was added to each well. The well-plates were then incubated at room temperature for 5 min to dissolve the purple formazan crystals, where after 100 µl of the isopropanol phase was transferred to a 96-well plate. The absorbance of each isopropanol phase was measured spectrophotometrically at 560 nm. The effect on cell viability of each drug (L-dopa and the L-dopa-lazabemide prodrug) was tested in triplicate (Brink *et al.*, 2008).

3.3.7. High performance liquid chromatography (HPLC)

To measure the concentrations of the L-dopa-lazabemide prodrug, lazabemide and L-dopa in buffer and biological matrices, a HPLC method was developed. The HPLC system consisted of an Agilent 1200 series HPLC with a binary gradient pump, auto sampler and vacuum degasser coupled to an Agilent 1200 series variable wavelength detector. The analyses were carried out with a Venusil XBP C18 column (4.60 × 150 mm, 5 µm). For the analyses of the L-dopa-lazabemide prodrug (retention time 5.2 min) and lazabemide (retention time 3.3 min), the mobile phase consisted of 60% sodium acetate buffer (50 mM, pH 4.7) and 40% methanol at a flow rate of 1 ml/min. The effluent was monitored at 276 nm. For the analysis of L-dopa (retention time 3.0 min), the mobile phase consisted of 95% sodium acetate buffer (50 mM, pH 4.7) and 5% methanol at a flow rate of 1 ml/min. The effluent was monitored at 280 nm. A volume of 20 µl of the samples was injected into the HPLC system.

3.3.8. Determination of passive diffusion permeability

The pre-coated parallel artificial membrane permeability assay (PAMPA) plate system was obtained from BD Biosciences. Prior to use, the pre-coated PAMPA plate system was warmed to room temperature for at least 30 min. Once warmed to room temperature, the plate system was used within 24 h. A volume of 300 μ l of the test compound solution in potassium phosphate buffer (100 mM, pH 6.4; 7.4 and 7.8) or in sodium acetate buffer (50 mM, pH 3.7; 4.7 and 5.7) was added per well in the receiver/donor plate (bottom). The final concentration of the test compounds was 200 μ M in each well. A volume of 200 μ l of potassium phosphate buffer (100 mM, pH 6.4; 7.4 and 7.8) or in sodium acetate buffer (50 mM, pH 3.7; 4.7 and 5.7) was then added per well to the filter/acceptor plate (top). The filter plate was placed on the receiver plate by slowly lowering the pre-coated PAMPA plate until it was seated on the receiver plate. The assembly was incubated at room temperature for 5 h. The pre-coated PAMPA plate and the receiver plate were separated. The compound concentrations were determined using the HPLC system described above. The permeability values are given as mean \pm standard deviation of triplicate determinations (Wohnsland & Faller, 2001).

3.3.9. Determination of chemical stability

To determine the chemical stability of the L-dopa-lazabemide prodrug, a 10 mM stock solution of the prodrug in aqueous buffer was prepared. Potassium phosphate buffer (100 mM, pH 6.4; 7.4 and 7.8) or sodium acetate buffer (50 mM, pH 3.7; 4.7 and 5.7) served as the aqueous buffer phases. The stock solution was immediately diluted to 50 μ M and the resulting solution was analysed by the HPLC method described above. The first injection served as reference point, after which the same solution was again injected at 1 h, 3 h, 6 h, 9 h and 11 h. During this time, the test solution remained at room temperature in the auto-sampler tray. The stabilities of the prodrug in the selected buffer phases are reported as the percentage mean \pm standard deviation of the first injection, which served as reference point.

3.3.10. Determination of plasma and tissue stability

Plasma was collected in 0.5 ml MiniCollect® Heparin plasma tubes and centrifuged at 5500 rpm. Liver and brain tissue were collected, placed on ice and rinsed with 0.25 M sucrose dissolved in water. Sucrose solution was added to the tissue at a ratio of 1:3 (w/v), the tissue was cut into small pieces using scissors and homogenised by 2 passes (60 s each) using a Potter-Elvehjem homogeniser (1200 rev/min) (Barrow & Holt, 1971). Care was taken to cool the homogeniser in ice during this procedure. The plasma was stored at -80 °C until the day of the experiment while the tissue homogenate was used immediately. To determine the plasma and tissue stabilities of the L-dopa-lazabemide prodrug, a 10 mM stock solution of the

prodrug in MilliQ water was prepared. The stock solution was immediately diluted to approximately 200 μ M into plasma or tissue homogenate (preheated to 37 °C) and the resulting mixtures was analysed by the HPLC method described above. For this purpose, 100 μ l of the spiked plasma or tissue homogenate was added to 300 μ l acetonitrile (containing formic acid 1%) in a Phree tube. The tube was vortexed for 2 min and filtered under vacuum (9–10 mmHg). The collected solvent was evaporated under a stream of air, and the residue was redissolved in 100 μ l methanol. A volume of 300 μ l water was added and 20 μ l of the resulting solution was analysed by the HPLC system. The first injection occurred at 5 min after preparation of the spiked plasma sample, after which aliquots of the same plasma sample or tissue homogenate was again subjected to the Phree work-up and injected at various time points. During this time, the plasma solution and tissue homogenate remained at 37 °C in a waterbath. The stabilities of the prodrug are reported as the mean \pm standard deviation of the remaining concentration of the prodrug in the plasma sample or tissue homogenate. The concentration of lazabemide was also determined.

3.4. Animal studies

Male C57BL/6 mice were weighed and allocated into 4 groups of 3 mice each. The 4 groups were treated by oral gavage with saline, L-dopa, L-dopa and carbidopa, and the L-dopa-lazabemide prodrug. L-Dopa and the L-dopa-lazabemide prodrug were given at a dose of 63.5 μ mol/kg and carbidopa was given at 10 mg/kg. A second 4 groups (n = 3) were treated in the same manner by intraperitoneal injection. All solutions were prepared in sterile saline and administered at volumes of 0.1 ml for oral gavage and 0.2 ml for intraperitoneal injection. The animals were euthanized 60 min after treatment by cervical dislocation, the striata were dissected and stored at –80 °C until the day of the experiment. The concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), noradrenaline, serotonin and 5-hydroxyindoleacetic acid (5-HIAA) were determined according to the published protocol, and are reported as mean \pm standard deviation of each group (n = 3 mice/group) (Harvey *et al.*, 2006). Prism version 5 (GraphPad Software, San Diego, California, USA) was used for the statistical analyses and for the preparation of graphical presentations. Data were analysed by one-way analysis of variance (ANOVA) across all groups, and were subsequently subjected to the Dunnett's post-test to determine statistical differences between the means. A p-value of <0.05 indicates statistical significant differences.

3.5. Results

3.5.1. Synthesis of the L-dopa-lazabemide prodrug

The synthesis of lazabemide has been reported (Soriato *et al.*, 2008). Lazabemide was synthesised by reacting 5-chloro-cyanopyridine (**1**) with p-toluenesulfonic acid and

ethylenediamine (**2**) to yield the imidazoline derivative **3**. This reaction takes place at 80 °C in an atmosphere of argon. The product, 2-(5-chloropyridine-2-yl)-1*H*-imidazoline (**3**) was obtained in good yields of 88%. This reaction is illustrated in Fig. 3.3. In the second step, **3** is hydrolysed with aqueous NaOH to yield lazabemide as the free base.

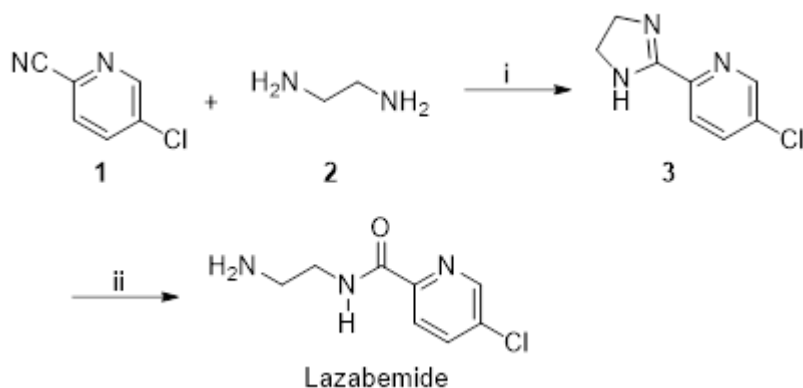


Fig. 3.3: The synthetic route to lazabemide. Key: (i) p-toluenesulfonic acid, 80 °C, argon; (ii) NaOH, reflux, 2 h.

Lazabemide was subsequently conjugated to the carboxylic acid functional group of L-dopa via an amide (Fig. 3.4). A literature review showed that similar conjugation reactions have previously been carried out (Nakonieczna *et al.*, 1994) and the carboxylic acid of L-dopa was, for example, conjugated to leucine, phenylalanine and valine. L-Dopa was firstly protected at the catechol OH groups using TBDMS-Cl in acetonitrile. Both phenolic positions on free L-dopa are selectively silylated by the reaction with TBDMS-Cl in the presence of DBU. This step yields L-dopa protected on both phenolic positions, denoted as L-dopa(TBDMS)₂ (**4**). The aminyl NH₂ group of L-dopa was subsequently protected using Boc₂O in THF. This introduces the tert-butoxycarbonyl (t-Boc) group on the primary amine of L-dopa(TBDMS)₂ yielding the Boc-L-dopa(TBDMS)₂ (**5**).

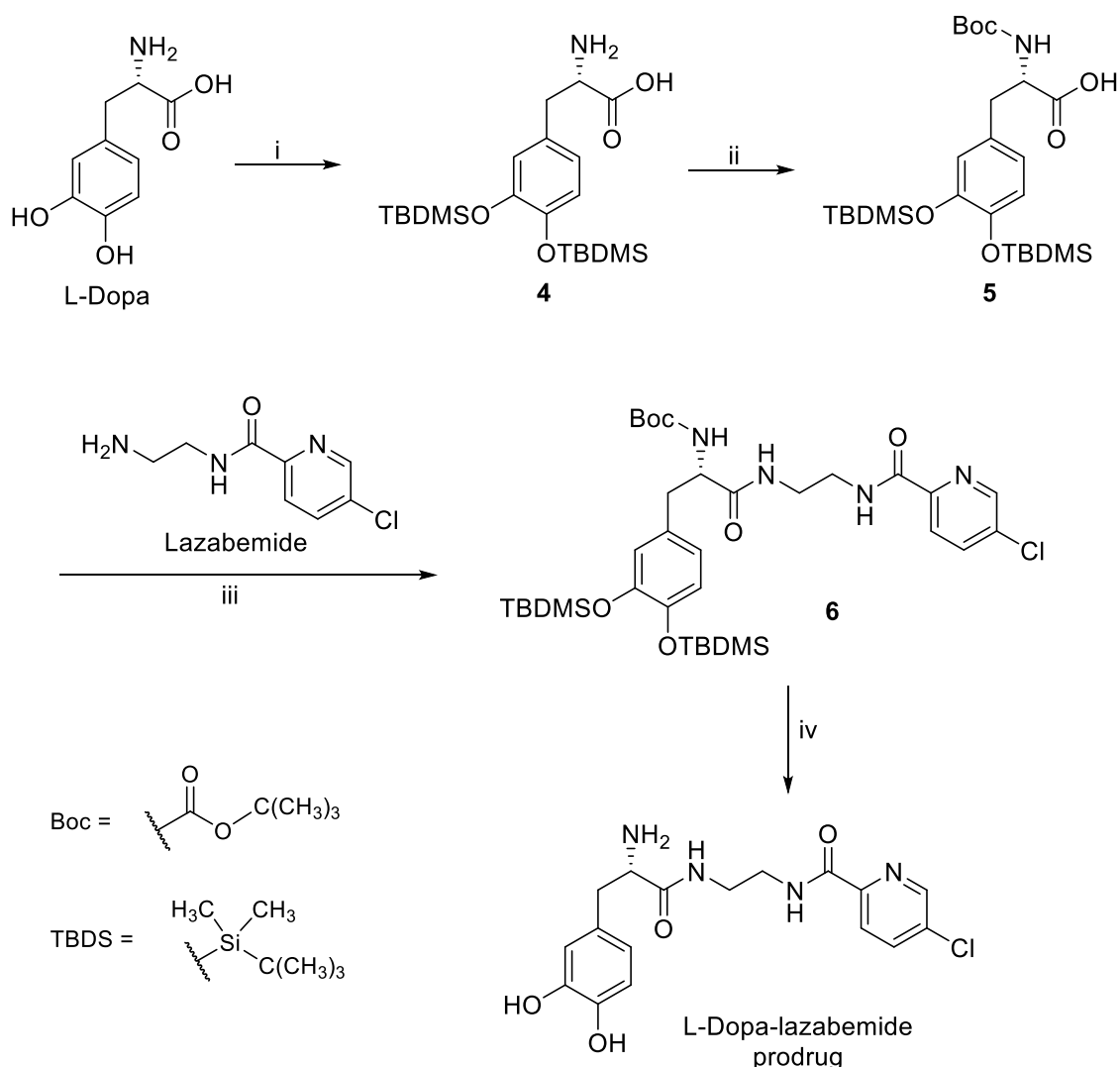


Fig. 3.4: The protection of L-dopa and the synthesis of the L-dopa-lazabemide prodrug. Key: (i) rt, 18 h, DBU, TBDMS-Cl; (ii) THF/H₂O, NaHCO₃, di-tert-butyl dicarbonate (Boc₂O); (iii) BOP, NMM, CH₂Cl₂. 24 h; (iv) 4 M HCl (dioxane); 48 h, 55 °C.

In the next step lazabemide was conjugated to the protected L-dopa, Boc-L-dopa(TBDMS)₂ (**5**), in the presence of BOP with dichloromethane as solvent. NMM served as base in this reaction. This reaction yielded the protected L-dopa-lazabemide prodrug (**6**). The removal of the TBDMS and Boc protective groups from the protected L-dopa-lazabemide prodrug (**6**) was the last step of the reaction sequence. The protected L-dopa-lazabemide prodrug (**6**) was treated with a variety of deprotection agents of which complete deprotection was achieved with 95% TFA after 24 h. Another useful reagent was HCl (4 M in dioxane). After treatment of **6** with the HCl reagent for 48 h at 55 °C, deprotection to a level of 95% was achieved. It may thus be concluded that both TFA and HCl are appropriate for the deprotection of protected L-dopa dipeptides. The deprotection reaction yielded the final product, the L-dopa-lazabemide prodrug.

The structure of the L-dopa-lazabemide prodrug was characterised by ^1H NMR and ^{13}C NMR data, as well as by mass spectrometry. In both the ^1H NMR and ^{13}C NMR spectra, the appropriate signals were observed for the proposed prodrug. In the ^{13}C NMR spectrum, the number of signals and their chemical shifts are in correspondence to what is expected for the proposed structure of the L-dopa-lazabemide prodrug. In the ^1H NMR spectrum, the number of signals, their integration values, multiplicities and their chemical shifts are in correspondence to what is expected for the proposed structure of the L-dopa-lazabemide prodrug. In this respect, the CH_2 protons of the L-dopa moiety (C7) corresponds to the signals at 2.75–2.78 and 2.86–2.89 ppm (the signals integrate for 1 proton each), while the CH α -carbon (C8), corresponds to part of the multiplet at 3.20–3.36 ppm (the signal integrates for 3 protons) (Fig. 3.5). The aromatic L-dopa protons (on C1, C4 and C6) correspond to the doublet at 6.45 ppm (1H) and the multiplet at 6.64 ppm (2H). Based on literature, the C6 CH corresponds to the signal at 6.45 ppm, while the protons at C1 and C4 correspond to the signal at 6.64 ppm (Nakonieczna *et al.*, 1994). For the lazabemide moiety, two of the protons of the $\text{CH}_2\text{-CH}_2$ (C12/13) correspond to the multiplets at 3.20 and 3.75 ppm (the signals integrate for 1 proton each). The remaining 2 protons are most likely part of the multiplet at 3.20–3.36 ppm. The complex nature of these signals may be attributed to the chirality of the α -carbon, C8. The aromatic protons at C20 and C21 of the pyridyl ring correspond to the two doublets at 8.03 ppm (1H) and 8.11 ppm (1H). It is not clear why *meta*-coupling with C18 is not observed in this spectrum. The aromatic proton on C18 of the pyridyl ring corresponds to the signal at 8.69 ppm (1H). As mentioned above, in this spectrum *meta*-coupling with C20 is not observed. In the ^1H NMR spectrum of the trifluoroacetic acid salt of the prodrug, *meta*-coupling was, however, observed between the signal at 8.11 ppm and the signal at 8.69 ppm (data not shown). The hydrochloric acid of the prodrug contains 7 exchangeable protons (NH, NH_3^+ and OH groups). These signals are most likely represented by the broad signals observed in the spectrum at 8.07 ppm (3H), 8.63 ppm (1H) and 8.91 ppm (3H).

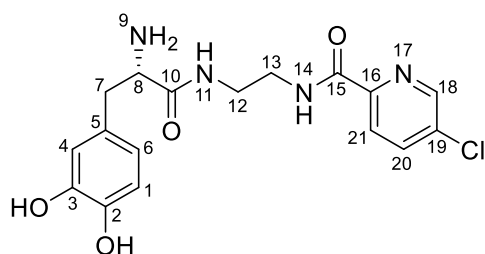


Figure. 3.5: Atom numbering scheme for the L-dopa-lazabemide prodrug.

In the ^{13}C NMR, the carbonyl carbons (C10 and C15) are represented by the signals at 163.3 and 168.6 ppm. The methylene and methene carbons (C7, C8, C12 and C13) correspond to

the signals at 36.6, 38.4, 38.6 and 54.1 ppm. Based on the DEPT 135 spectrum, the methylene CH₂ groups at C7, C12 and C13 correspond to the signals at 36.6, 38.4 and 38.6 ppm, while the methene CH group (C8) is represented by the signal at 54.1 ppm. Aromatic CH carbons (6 carbons) are represented by signals at 115.7, 116.9, 120.2, 123.5, 137.6, 147.1 ppm. Aromatic C carbons (5 carbons) are represented by signals at 125.8, 134.0, 144.5, 145.2 and 148.4 ppm.

Mass spectrometry reveals an experimental mass (m/z) of 378.1089 Da, which best corresponds to an empirical formula of C₁₇H₁₉O₄N₄Cl (378.1095 Da). The differences (1.59 ppm) between the calculated and experimentally determined masses are indicative that the structure of the analysed compound corresponds to that of the L-dopa-lazabemide prodrug. Also of interest is the satellite signal at 380.10771, which corresponds to the L-dopa-lazabemide prodrug containing the ³⁷Cl isotope.

3.5.2. Physicochemical properties

3.5.2.1. LogD

As shown in table 3.1, the L-dopa-lazabemide prodrug may be viewed as hydrophilic at pH 6.4 since it displays logD <0. As a general guide, LogP values ranging from 0–3 are optimal for passive diffusion permeability and such compounds are expected to display good oral bioavailability and blood-brain barrier permeation (Kerns & Di, 2008). At pH 7.4 and 7.8, the logD values are 0.199 and 0.319, respectively. This suggests that the L-dopa-lazabemide prodrug may permeate biological membranes by passive diffusion at these pH values. It is interesting to note that lazabemide also is a hydrophilic compound at the pH values evaluated. In fact, at pH 7.4 and 7.8, lazabemide is more hydrophilic than the L-dopa-lazabemide prodrug. In spite of this, lazabemide is an orally active MAO-B inhibitor which acts in the CNS.

Table 3.1: The logD values of the L-dopa-lazabemide prodrug and lazabemide at different pH values.

pH value	LogD - prodrug	LogD - lazabemide
6.4	-4.58 ± 0.002	-1.18 ± 0.032
7.4	0.199 ± 0.002	-0.68 ± 0.019
7.8	0.319 ± 0.003	-0.63 ± 0.009

Values are given as mean ± standard deviation of triplicate determinations

3.5.2.2. Solubility

The L-dopa-lazabemide prodrug was dissolved to a theoretical concentration of 6 mg/ml in water and buffer, and the concentration of material that dissolved was subsequently measured. These concentrations of the L-dopa-lazabemide prodrug in the water and buffer phases are shown in table 3.2. The results show that the L-dopa-lazabemide prodrug is highly soluble in water and buffer since the concentrations recorded in water and buffer are, within experimental error, equal to 6 mg/ml. It may thus be concluded that the minimum solubility of the prodrug is 6 mg/ml, which may be viewed as good aqueous solubility.

Table 3.2: The solubility of the L-dopa-lazabemide prodrug in water and aqueous buffer at pH 7.4.

Medium	Amount dissolved ($\mu\text{g/ml}$)	Amount measured ($\mu\text{g/ml}$)
Water	6000	6277 ± 747
pH 7.4	6000	6142 ± 730

Values are given as mean \pm standard deviation of triplicate determinations

3.5.2.3. Ionisation constant

The pKa of the L-dopa-lazabemide prodrug was found to be 7.25 ± 0.16 . At physiological pH 7.4, approximately 59% of the prodrug will exist as the neutral uncharged species. Considering that the neutral species should display better membrane permeability than the ionised aminium, a relatively large fraction of the prodrug is available for passive diffusion. This result suggests that ionisation of the prodrug at pH 7.4 should not be a significant barrier for permeation through the blood-brain barrier. At the pH values found in the gastrointestinal tract (for example pH 5.5), however, only 1–2% of the prodrug will exist as the neutral uncharged species. This result suggests that ionisation of the prodrug in the gastrointestinal tract may represent a barrier for absorption into the systemic circulation.

3.5.2.4. Cell viability

The toxicity of the L-dopa-lazabemide prodrug towards cultured cells was examined and compared to that of L-dopa. For this purpose, the MTT cell viability assay was used (Mosmann, 1983). The MTT assay is a standard cell viability assay, measuring mitochondrial activity in live (metabolically viable) cells. Metabolically active mitochondria in viable cells have the ability to reduce MTT to coloured formazan crystals that can be quantitated spectrophotometrically. The presence of a toxic agent will reduce cell viability and thus limit the reduction of MTT to

the formazan product. For this study HeLa cells were selected. This selection was based on the high growth rate of HeLa cells and on the observation that these cells are frequently used to measure cell viability in the presence of toxic agents (Brink *et al.*, 2008).

The percentage viable cells after treatment with the L-dopa-lazabemide prodrug and L-dopa are shown in table 3.3. It is evident from the results that at concentrations of 1 μM , neither the L-dopa-lazabemide prodrug nor L-dopa are toxic for the cultured cells. At concentrations of 10 μM , the L-dopa-lazabemide prodrug displays toxicity for the cultured cells (viability of 52.1%) while L-dopa is non-toxic. At concentrations of 100 μM , both the L-dopa-lazabemide prodrug and L-dopa display toxicity for the cultured cells with viabilities of 35% and 79%, respectively. These results indicate that the L-dopa-lazabemide prodrug is significantly more toxic for cultured cells at 10 μM and 100 μM than L-dopa. A possible explanation for the higher toxicity observed for the L-dopa-lazabemide prodrug is that the prodrug may permeate the cell membrane of the cultured cell more readily than L-dopa, and thus reach higher concentrations within the cytosol. This greater degree of intracellular exposure may lead to higher toxicity. More investigation to clarify this observation is, however, required.

Table 3.3: The percentage viable cells remaining after treatment with the L-dopa-lazabemide prodrug and L-dopa.

	Concentration of the test drug		
	1 μM	10 μM	100 μM
L-Dopa	102 \pm 4.19	105 \pm 5.24	79.3 \pm 13.0
L-Dopa-lazabemide prodrug	112 \pm 12.0	52.1 \pm 14.0	34.9 \pm 7.94

Values are given as mean (percentage) \pm standard deviation of triplicate determinations

3.5.2.5. Passive diffusion permeability

The permeabilities of the L-dopa-lazabemide prodrug, lazabemide and L-dopa were evaluated via PAMPA and are given in table 3.4. From the data it is evident that L-dopa displays poor permeability at all pH values with LogP_e values smaller than -7.99 . Higher LogP_e values are indicative of increased permeability. For example, propranolol, a drug considered to have good permeability is reported to have a LogP_e value of -4.4 at pH 6.8 (Wohnsland & Faller, 2001). In contrast to L-dopa, lazabemide displays better permeability with LogP_e values of up to 5.67 at pH 7.8. It is interesting to note that the permeability of lazabemide increases with increasing pH. This is consistent with a lower degree of ionisation of this compound as pH increases. The L-dopa-lazabemide prodrug also displayed better permeability than L-dopa with the best permeability at pH 7.8 ($\text{LogP}_e = -7.33$). As with lazabemide, the permeability of the prodrug

increases with increasing pH, which is consistent with a lower degree of ionisation at higher pH values. Compared to propranolol and lazabemide, the L-dopa-lazabemide prodrug, however, displays poor permeability.

Table 3.4: The permeability (P_e) of L-dopa-lazabemide prodrug, lazabemide and L-dopa at selected pH values.

	Permeability (cm/s) expressed as $\text{Log}P_e$					
	pH 3.7	pH 4.7	pH 5.7	pH 6.4	pH 7.4	pH 7.8
L-Dopa	-7.99 ±	-8.05 ±	-8.22 ±	-8.62 ±	-8.17 ±	-8.56 ±
	0.28	0.19	0.14	0.87	0.56	0.07
Prodrug	-8.02 ±	-7.81 ±	-7.71 ±	-7.71 ±	-7.53 ±	-7.33 ±
	0.39	0.18	0.14	0.06	0.04	0.30
Lazabemide	-7.37 ±	-7.06 ±	-6.83 ±	-6.45 ±	-5.79 ±	-5.67 ±
	0.24	0.05	0.18	0.28	0.03	0.06

Values are given as mean ± standard deviation of triplicate determinations

3.5.2.6. Chemical stability

The chemical stability of the L-dopa-lazabemide prodrug was determined in aqueous buffer. These measurements were done in order to determine if the prodrug may undergo non-enzymatic hydrolysis. Hydrolysis of the L-dopa-lazabemide prodrug will yield L-dopa and lazabemide. Such an event may be viewed as undesirable since premature hydrolysis prior to absorption may reduce the amount of the prodrug available for absorption into the systemic circulation. The prodrug should therefore be stable in aqueous solution. In addition, the prodrug should be relatively stable in plasma in order to provide enough exposure time of the prodrug to the blood-brain barrier. If the prodrug is activated too quickly in the plasma, a relatively small amount will gain access to the CNS. In general, non-enzymatic hydrolysis of the prodrug is undesirable, while enzymatic hydrolysis is the preferred method of activation.

The results of the chemical hydrolysis study are given in fig. 3.6 and show that the L-dopa-lazabemide prodrug is stable at pH 3.7–7.4, with no significant decrease of the peak area and concentration of the prodrug (up to 11 h) after the preparation of the solution. This indicates that the prodrug does not significantly hydrolyse at these pH values, and does not undergo other chemical transformations such as oxidation at these pH values. At a pH of 7.8, the concentration of the L-dopa-lazabemide prodrug, however, does decrease in a time-dependent manner. At 11 h after the preparation of the solution, the prodrug concentration has decreased to 90.3%. The decrease of the prodrug concentration was not accompanied

by the appearance of the peak of lazabemide, which suggests that at higher pH values, the prodrug undergoes different chemical reactions than hydrolysis of the amide functionality.

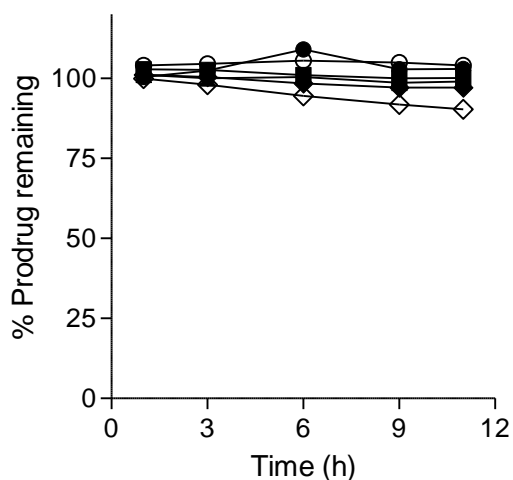


Figure. 3.6: The chemical stability of the L-dopa-lazabemide prodrug at different pH values. Key: pH 3.7 (filled circles); pH 4.7 (open circles); pH 5.7 (triangles); pH 6.4 (squares); pH 7.4 (filled diamonds); pH 7.8 (open diamonds). Values are given as mean (percentage) \pm standard deviation of triplicate determinations.

3.5.2.7. Plasma and tissue stability

Although the L-dopa-lazabemide prodrug should be stable in aqueous solution, the prodrug should undergo hydrolysis in plasma or tissue. It is preferable that the prodrug does not hydrolyse very quickly (within minutes) in plasma, but be relatively stable in order to provide enough exposure time of the prodrug to the blood-brain barrier. As mentioned above, if the prodrug is activated too quickly, a relatively small amount will gain access to the CNS. Slow enzymatic hydrolysis is thus the preferred method of activation of the L-dopa-lazabemide prodrug.

To evaluate plasma stability, three experiments were conducted. Two plasma stability experiments were carried out with human plasma (from different donors) and one study was carried out with rat plasma. The results of the plasma stability study shows that the L-dopa-lazabemide prodrug is stable in human plasma, with no significant decrease of the peak area and concentration of the prodrug at up to 8 h incubation in plasma (Fig. 3.7). The appearance and increase of the peak of lazabemide, however, shows that small amounts of the prodrug are indeed hydrolysed. The increase of the concentration of lazabemide is time-dependent. At 17 h after the preparation of the spiked plasma samples, the prodrug concentration has decreased to 64–68 μM in human plasma and the concentration of lazabemide has increased to 13–15 μM . The relatively small concentration of lazabemide at 17 h, indicates that

lazabemide may undergo metabolism in the plasma or the prodrug also undergoes different chemical reactions than hydrolysis of the amide functionality, thus not yielding lazabemide. Further investigation is necessary to clarify this point.

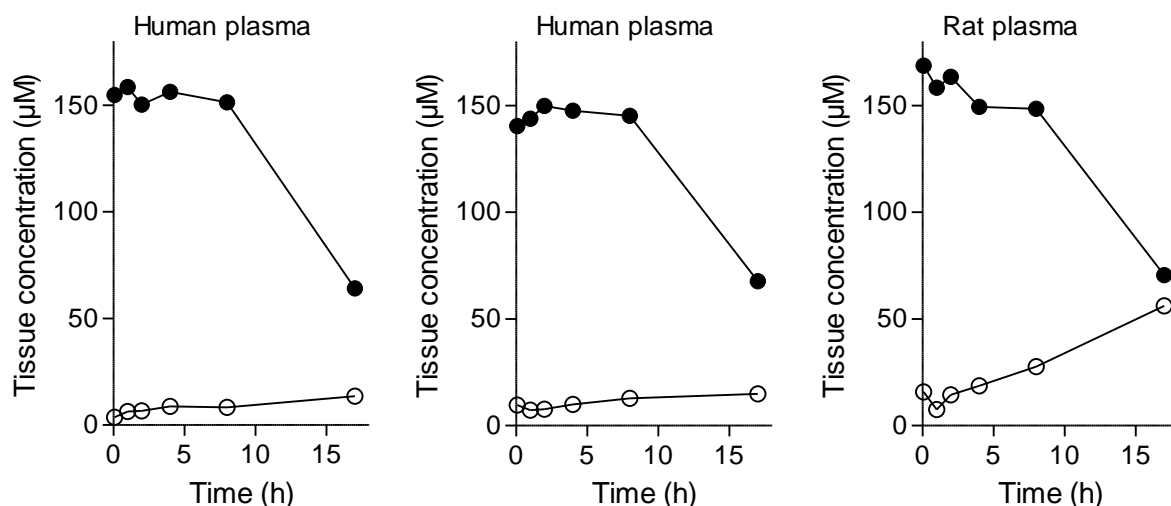


Figure. 3.7: The metabolic stability of the L-dopa-lazabemide prodrug in human and rat plasma. Key: lazabemide (open circles); prodrug (filled circles). Values are given as mean (μM) \pm standard deviation of triplicate determinations.

Equivalent results were obtained with rat plasma. The results document that the L-dopa-lazabemide prodrug is relatively stable in rat plasma, with a slow decrease of the concentration of the prodrug up to 8 h of incubation. After 17 h of incubation of the prodrug in rat plasma, the prodrug concentration has decreased to 70.5 μM . The degree of reduction in concentration of the prodrug is similar to that obtained with the human plasma. As with the human plasma, the decrease of prodrug concentration is accompanied with the appearance of the peak of lazabemide. Although more lazabemide is detected in rat plasma (56 μM) after 17 h than in human plasma, the concentration of lazabemide is still smaller than what would be expected if the prodrug has undergone only hydrolysis. The potential metabolism of lazabemide and/or the prodrug in rat plasma may explain this discrepancy.

To evaluate tissue stability, experiments with both rat brain and liver homogenates were carried out. As in plasma, the concentration of the prodrug declines in a time-dependent manner in liver tissue with 37% remaining after 4 h incubation (Fig. 3.8). During this time, the concentrations of lazabemide and L-dopa increase in a time-dependent manner. In brain tissue homogenate, an increase in the concentrations of lazabemide and L-dopa were also observed with increased incubation time of the prodrug with the tissue. After 4 h incubation the concentrations of lazabemide and L-dopa have increased 5-fold and 8-fold, respectively

compared to the concentrations at 5 min. These data indicate that, in both plasma and tissue, the prodrug may indeed undergo activation to yield lazabemide and L-dopa.

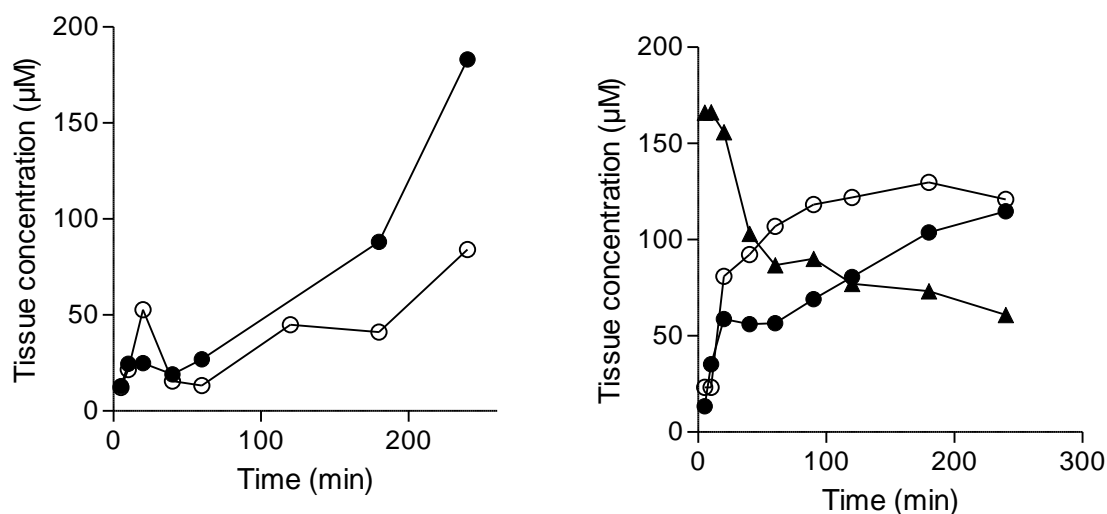


Figure. 3.9: The metabolic stability of the L-dopa-lazabemide prodrug in rat brain (left) and liver (right) homogenates. Key: lazabemide (open circles); L-dopa (filled circles); prodrug (triangles). Values are given as mean (μM) \pm standard deviation of triplicate determinations.

3.5.3. *In vivo* effect of the prodrug on brain monoamines

C57BL/6 mice were allocated into 4 groups ($n = 3$ mice/group) and each group was treated by oral gavage with either saline, L-dopa, L-dopa and carbidopa, or the L-dopa-lazabemide prodrug. L-dopa and the L-dopa-lazabemide prodrug were given at a dose of $63.5 \mu\text{mol/kg}$ and carbidopa was given at 10 mg/kg , a dosage regimen similar to that reported in literature (Zhou *et al.*, 2010). Another 4 groups ($n = 3$ mice/group) were treated in the same manner by intraperitoneal injection. The animals were sacrificed 60 min after treatment, the striata were dissected and the concentrations of DA, DOPAC, HVA, noradrenaline, serotonin and 5-HIAA were measured (Harvey *et al.*, 2006). The time point of 60 min was selected based on literature reports that after oral administration of L-dopa prodrugs to rats, the plasma half-life of L-dopa is 51–97 min (Zhou *et al.*, 2010). In mice, L-dopa levels reached peak plasma levels 30–60 min after oral administration of L-dopa and carbidopa (Käenmäki *et al.*, 2009). The results given in Fig. 3.9 show that, while treatment with the prodrug does not enhance striatal DA levels, a significant reduction (compared to saline) in DOPAC is observed following intraperitoneal treatment. In this respect, one-way ANOVA [$F(3,7) = 16.10$; $p = 0.0016$] of the DOPAC concentration data revealed a significant effect of treatment and post-hoc (Dunnett's test) analysis indicated a significant decrease ($p = 0.016$) in DOPAC levels after intraperitoneal treatment with the prodrug versus saline treated controls. Interestingly, intraperitoneal L-dopa

treatment significantly enhanced ($p = 0.049$) DOPAC levels. None of the other monoamines measured were significantly altered by treatment.

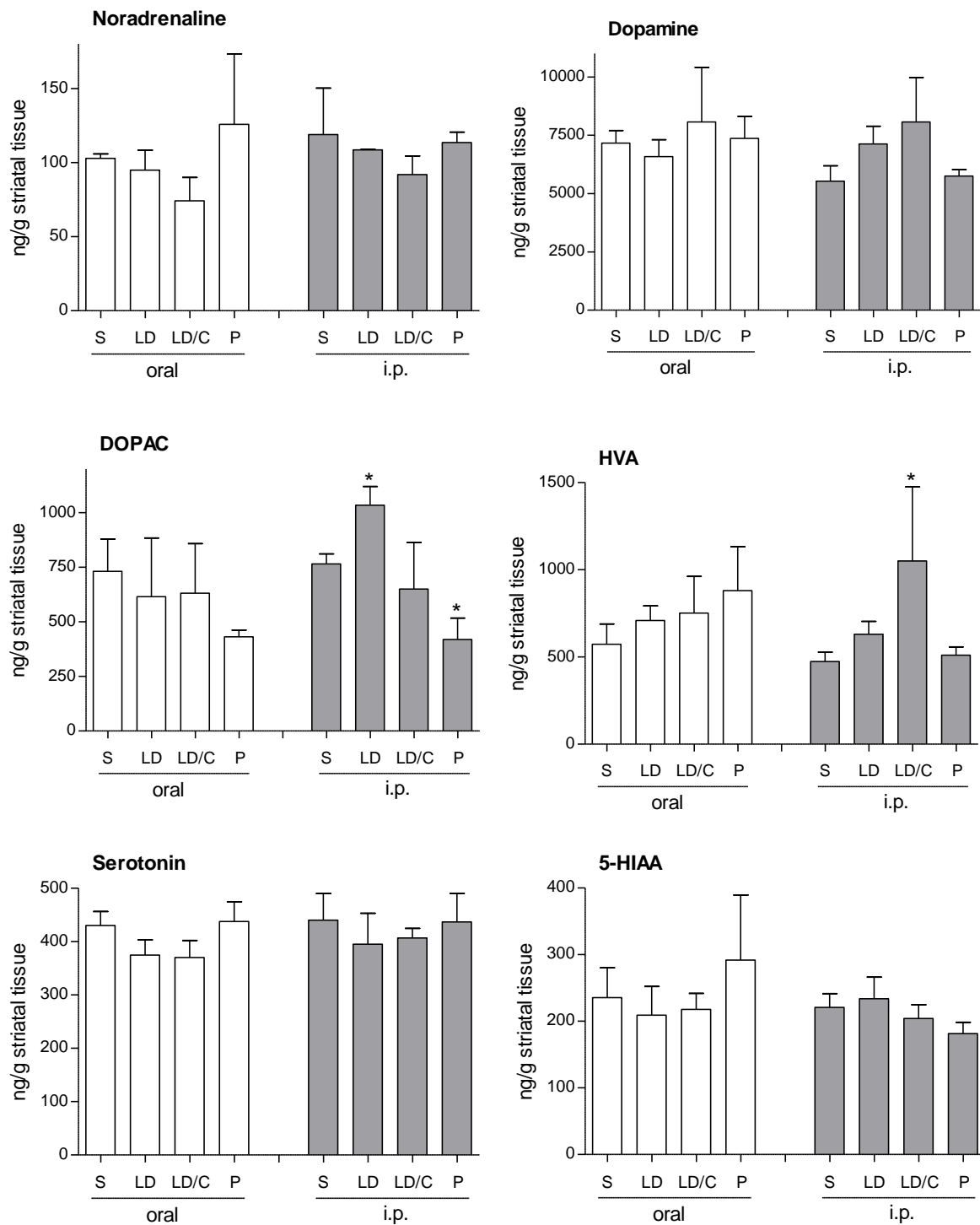


Figure. 3.9: The concentrations of selected monoamines and metabolites in the striatum of mice following oral and intraperitoneal (i.p.) treatment with saline (S), L-dopa (LD), L-dopa and carbidopa (LD/C), or the L-dopa-lazabemide prodrug (P). Values are given as mean \pm standard deviation with statistical comparisons to the saline treated animals indicated (* $p < 0.05$; $n = 3$ /group).

3.6. Discussion and conclusion

In the present study, an L-dopa-lazabemide prodrug is proposed to enhance the delivery of L-dopa to the brain after oral administration. As shown in the introduction, the prodrug is designed to possess enhanced permeability and metabolic stability compared to L-dopa. In addition, lazabemide, liberated by the activation of the prodrug may protect against MAO-B catalysed depletion of central DA, and possibly also reduce potentially neurotoxic species produced by the MAO catalytic cycle. An analysis of the properties of the prodrug shows that the prodrug possesses appropriate lipophilicity (logD) and solubility profiles for oral absorption, although passive diffusion permeability, as evaluated by PAMPA, is comparatively low. In spite of this, the prodrug displays higher toxicity to cultured cells than L-dopa, possibly indicating higher intracellular exposure of the prodrug as a result of better permeability. Further experiments show that the prodrug is stable towards hydrolysis in aqueous buffer and undergoes slow activation in plasma and tissue (liver and brain). Although oral and i.p. treatment of mice with the prodrug did not result in enhanced striatal DA levels, DOPAC levels were significantly depressed compared to saline, L-dopa and carbidopa/L-dopa treatment. This suggests that the prodrug may reduce DA metabolism, most likely as a result of MAO-B inhibition by lazabemide. After i.p treatment with L-dopa, DOPAC levels are significantly increased, likely due to increased central DA in response to L-dopa. This behaviour is not observed with the prodrug as lazabemide is expected to block the metabolic route leading from DA to DOPAC. Based on these data, further preclinical evaluation of the L-dopa-lazabemide prodrug should be undertaken with the aim of discovering prodrugs that may be advanced to the clinical stages of development.

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Conflict of interest

The authors declare no conflicts of interest in this work.

References

1. Albert, A. & Serjeant, E.P. 1984. The determination of ionization constants: a laboratory manual. 3rd ed. London: Chapman and Hall. pp. 176-191.
2. Barrow, P.C. & Holt, S.J. 1971. Differences in distribution of esterase between cell fractions of rat liver homogenates prepared in various media. Relevance to the lysosomal location of the enzyme in the intact cell. *Biochemistry journal*, 125(2):545-555.
3. Berlin, I., Aubin, H.J., Pedarriosse, A.M., Rames, A., Lancrenon, S. & Lagrue, G. 2002. Lazabemide in smoking cessation study investigators. Lazabemide, a selective, reversible monoamine oxidase B inhibitor, as an aid to smoking cessation. *Addiction*, 97(10):1347-1354.
4. Binda, C., Li, M., Hubalek, F., Restelli, N., Edmondson, D.E. & Mattevi, A. 2003. Insights into the mode of inhibition of human mitochondrial monoamine oxidase B from high-resolution crystal structures. *Proceedings of the national academy of science of the United States of America*, 100(17):9750-9755.
5. Brink, C.B., Pretorius, A., van Niekerk, B.P., Oliver, D.W. & Venter, D.P. 2008. Studies on cellular resilience and adaptation following acute and repetitive exposure to ozone in cultured human epithelial (HeLa) cells. *Redox Report*, 13(2):87-100.
6. Cesura, A.M., Borroni, E., Gottowik, J., Kuhn, C., Malherbe, P., Martin, J. & Richards, J.G. 1999. Lazabemide for the treatment of Alzheimer's disease: rationale and therapeutic perspectives. *Advances in neurology*, 80:521-528.
7. Cesura, A.M., Muggli-Maniglio, D., Lang, G., Imhof, R. & Da Prada, M. 1990. Monoamine oxidase inhibition by moclobemide and 2-amino-ethyl carboxamide derivatives: mode of action and kinetic characteristics. *Journal of neural transmission*, 32:165-170.
8. Contin, M. & Martinelli, P. 2010. Pharmacokinetics of levodopa. *Journal of neurology*, 257(2):253-261.
9. Dauer, W. & Przedborski, S. 2003. PD: mechanisms and models. *Neuron*, 39:889-909.
10. Di Stefano, A., Sozio, P., Cerasa, L.S. & Iannitelli, A. 2011. L-Dopa prodrugs: an overview of trends for improving PD treatment. *Current pharmaceutical design*, 17(32):3482-3493.
11. Di Stefano, A., Sozio, P. & Cerasa, L.S. 2008. Antiparkinson prodrugs. *Molecules*, 13(1):46-68.
12. Dingemans, J., Wood, N., Jorga, K. & Kettler, R. 1997. Pharmacokinetics and pharmacodynamics of single and multiple doses of the MAO-B inhibitor lazabemide in healthy subjects. *British journal of clinical pharmacology*, 43(1):41-47.

13. Edmondson, D.E., Mattevi, A., Binda, C., Li, M. & Hubálek, F. 2004. Structure and mechanism of monoamine oxidase. *Current medicinal chemistry*, 11(15):1983-93.
14. Finberg, J.P., Wang, J., Bankiewicz, K., Harvey-White, J., Kopin, I.J. & Goldstein, D.S. 1998. Increased striatal DA production from L-DOPA following selective inhibition of monoamine oxidase B by R(+)-N-propargyl-1-aminoindan (rasagiline) in the monkey. *Journal of neural transmission*, 52:279-285.
15. Fowler, J.S., Volkow, N.D., Logan, J., Schlyer, D.J., MacGregor, R.R., Wang, G.J., Wolf, A.P., Pappas, N., Alexoff, D. & Shea, C. 1993. Monoamine oxidase B (MAO B) inhibitor therapy in PD: the degree and reversibility of human brain MAO B inhibition by Ro 19 6327. *Neurology*, 43(10):1984-1992.
16. Fowler, J.S., Volkow, N.D., Wang, G.J., Logan, J., Pappas, N., Shea, C. & MacGregor, R. 1997. Age-related increases in brain monoamine oxidase B in living healthy human subjects. *Neurobiological Aging*, 18(4):431-435.
17. Freitas, M.E., Ruiz-Lopez, M. & Fox, S.H. 2016. Novel levodopa formulations for PD. *CNS Drugs*, 30(11):1079-1095.
18. Harvey, B.H., Brand, L., Jeeva, Z. & Stein, D.J. 2006. Cortical/hippocampal monoamines, HPA-axis changes and aversive behavior following stress and restress in an animal model of post-traumatic stress disorder. *Physiology & behaviour*, 87(5):881-890.
19. Käenmäki, M., Tammimäki, A., Garcia-Horsman, J.A., Myöhänen, T., Schendzielorz, N., Karayiorgou, M., Gogos, J.A. & Männistö, P.T. 2009. Importance of membrane-bound catechol-O-methyltransferase in L-DOPA metabolism: a pharmacokinetic study in two types of Comt gene modified mice. *British journal of pharmacology*, 158(8):1884-1894.
20. Kerns, E. H. & Di, L. 2008. Drug-like properties: concepts, structure design and methods: from ADME to toxicity optimization. Amsterdam: Academic Press.
21. Learmonth, D.A., Palma, P.N., Vieira-Coelho, M.A. & Soares-da-Silva, P. 2004. Synthesis, biological evaluation, and molecular modeling studies of a novel, peripherally selective inhibitor of catechol-O-methyltransferase. *Journal of medicinal chemistry*, 47(25):6207-6217.
22. Leenders, K.L., Poewe, W.H., Palmer, A.J., Brenton, D.P. & Frackowiak, R.S. 1986. Inhibition of L-[¹⁸F]fluorodopa uptake into human brain by amino acids demonstrated by positron emission tomography. *Annals of Neurology*, 20(2):258-262.
23. LeWitt, P.A., Ellenbogen, A., Chen, D., Lal, R., McGuire, K., Zomorodi, K., Luo, W. & Huff, F.J. 2012. Actively transported levodopa prodrug XP21279: a study in patients with parkinson disease who experience motor fluctuations. *Clinical neuropharmacology*, 35(3):103-110.

24. LeWitt, P.A., Huff, F.J., Hauser, R.A., Chen, D., Lissin, D., Zomorodi, K. & Cundy, K.C. 2014. Double-blind study of the actively transported levodopa prodrug XP21279 in PD. *Movement disorder*, 29(1):75-82.
25. Mason, R.P., Olmstead, E.G. & Jacob, R.F. 2000. Antioxidant activity of the monoamine oxidase B inhibitor lazabemide. *Biochemistry & pharmacology*, 60(5):709-716.
26. Mosmann, T. 1983. Rapid colourimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, 65(1-2):55-63.
27. Müller, T. 2016. Emerging approaches in PD - adjunctive role of safinamide. *Therapeutics and clinical risk management*, 12:1151-1160.
28. Nakonieczna, L., Przychodzeń, W. & Chimiak, A. 1994. A New convenient route for the synthesis of DOPA peptides. *Liebigs Annalen der chemie*, 1994:1055-1058.
29. Nissinen, E., Lindén, I.B., Schultz, E. & Pohto, P. 1992. Biochemical and pharmacological properties of a peripherally acting catechol-O-methyltransferase inhibitor entacapone. *Naunyn-Schmiedeberg's archives of pharmacology*, 346(3):262-266.
30. Nutt, J.G. & Fellman, J.H. 1984. Pharmacokinetics of levodopa. *Clinical neuropharmacology*, 7(1):35-49.
31. Nutt, J.G., Woodward, W.R., Hammerstad, J.P., Carter, J.H. & Anderson, J.L. 1984. The "on-off" phenomenon in PD. Relation to levodopa absorption and transport. *New England journal of medicine*, 310(8):483-488.
32. Nutt, J.G. 1987. On-off phenomenon: relation to levodopa pharmacokinetics and pharmacodynamics. *Annals of neurology*, 22(4):535-540.
33. Poewe, W. & Antonini, A. 2015. Novel formulations and modes of delivery of levodopa. *Movement disorders*, 30(1):114-120.
34. Robakis, D. & Fahn, S. 2015. Defining the role of the monoamine oxidase-B inhibitors for PD. *CNS Drugs*, 29(6):433-441.
35. Seeberger, L.C. & Hauser, R.A. 2015. Carbidopa levodopa enteral suspension. Expert opinion on pharmacotherapy, 16(18):2807-2817.
36. Shoulson, I., Glaubiger, G.A. & Chase, T.N. 1975. On-off response. Clinical and biochemical correlations during oral and intravenous levodopa administration in parkinsonian patients. *Neurology*, 25(12):1144-1148.
37. Silverman, R.B. 2004. The organic chemistry of drug design and drug action. 2nd Edition. Elsevier Academic Press, p. 503.
38. Soriato, G., Focati, M.P., Brescello, R., Cotarca, L. & Giovanetti, R. 2008. Pharmaceutical preparations of crystalline lazabemide. Patent WO/2008/010794.

39. Tohgi, H., Abe, T., Kikuchi, T., Takahashi, S. & Nozaki, Y. 1991. The significance of 3-O-methyldopa concentrations in the cerebrospinal fluid in the pathogenesis of wearing-off phenomenon in PD. *Neuroscience letters*, 132(1):19-22.
40. Wohnsland, F. & Faller, B. 2001. High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. *Journal of medicinal chemistry*, 44(6):923-930.
41. Youdim, M.B. & Bakhle, Y.S. 2006. Monoamine oxidase: isoforms and inhibitors in PD and depressive illness. *British journal of pharmacology*, 147(1):287-296.
42. Youdim, M.B., Edmondson, D. & Tipton, K.F. 2006. The therapeutic potential of monoamine oxidase inhibitors. *Nature reviews neuroscience*, 7(4):295-309.
43. Zhou, T., Hider, R.C., Jenner, P., Campbell, B., Hobbs, C.J., Rose, S., Jairaj, M., Tayarani-Binazir, K.A. & Syme, A. 2010. Design, synthesis and biological evaluation of L-dopa amide derivatives as potential prodrugs for the treatment of PD. *European journal of medicinal chemistry*, 45(9):4035-4042.

Supplementary material

Figure S3.1. High resolution mass spectrum of the L-dopa-lazabemide prodrug.

AP1_HR2-c1 #90 RT: 1.19 AV: 1 NL: 7.69E5
T: + c EI Full ms [367.50-385.50]

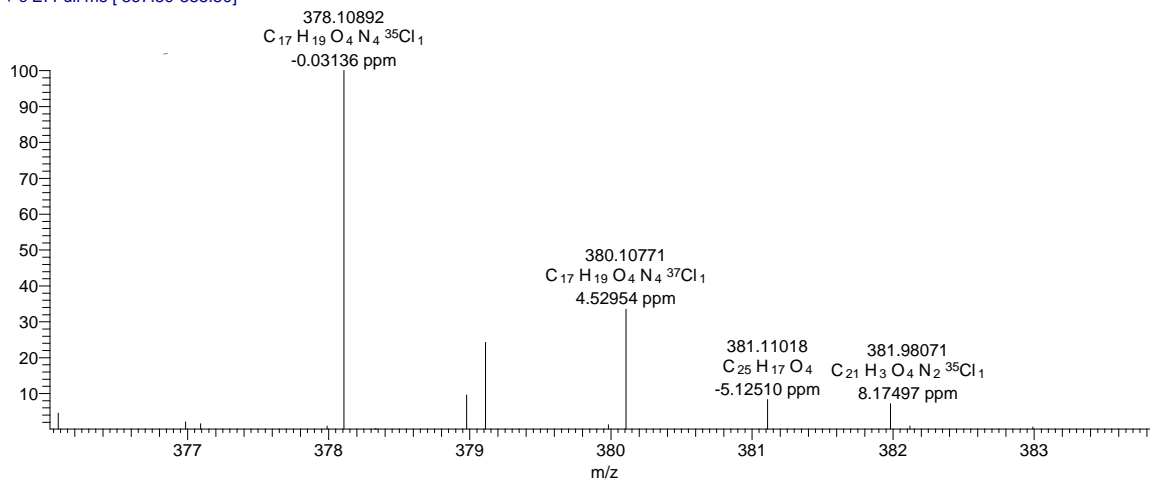
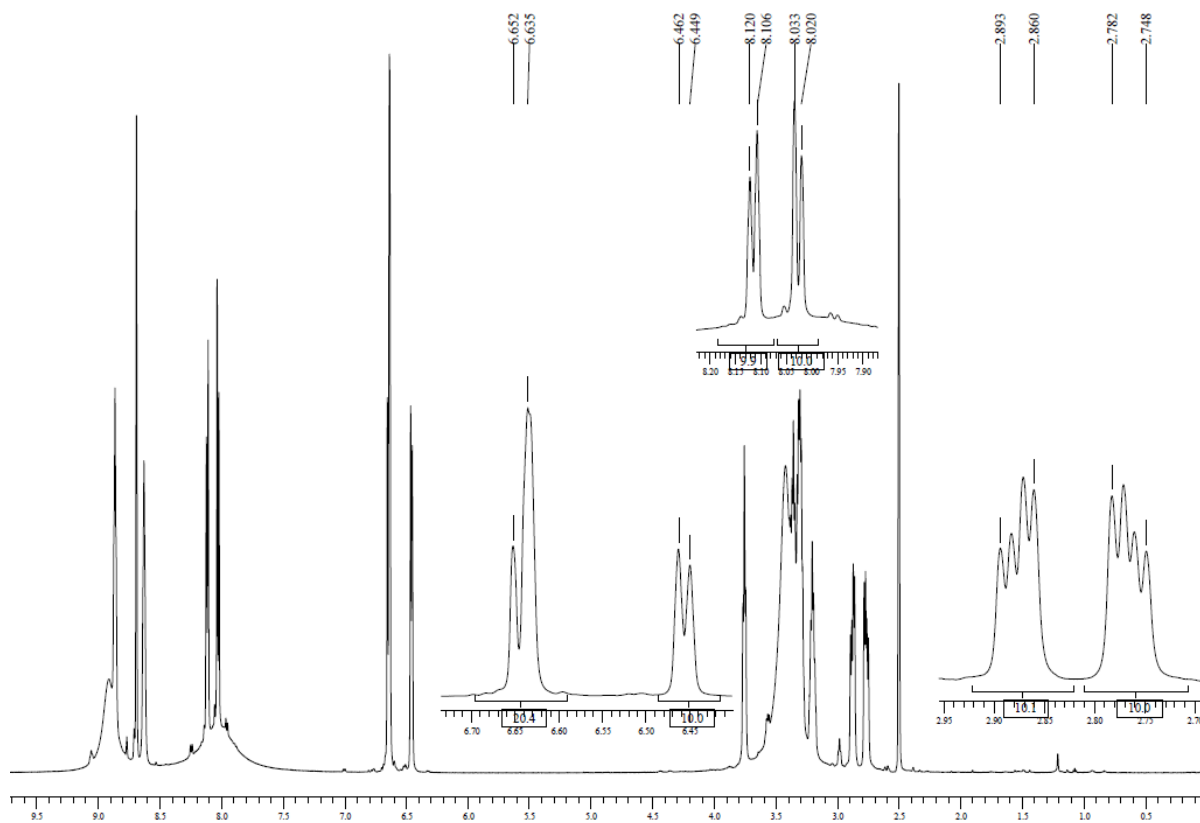
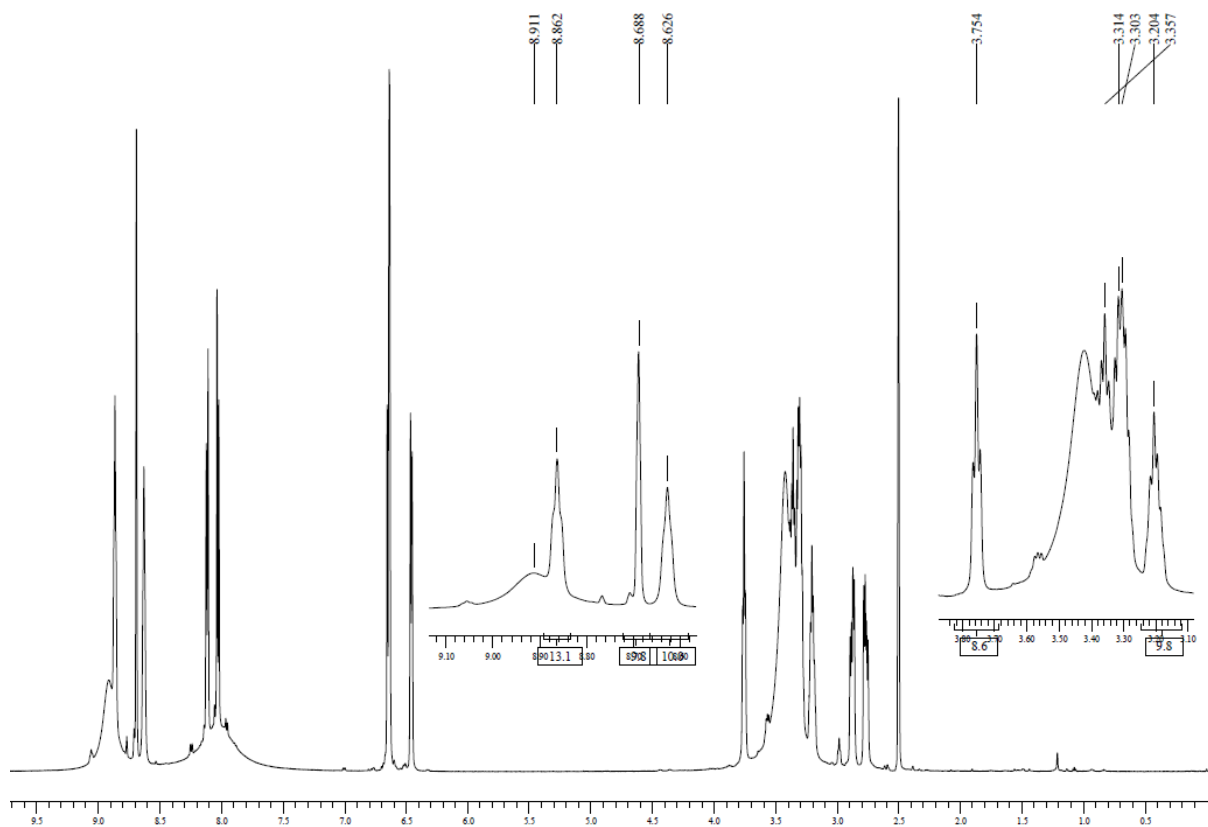


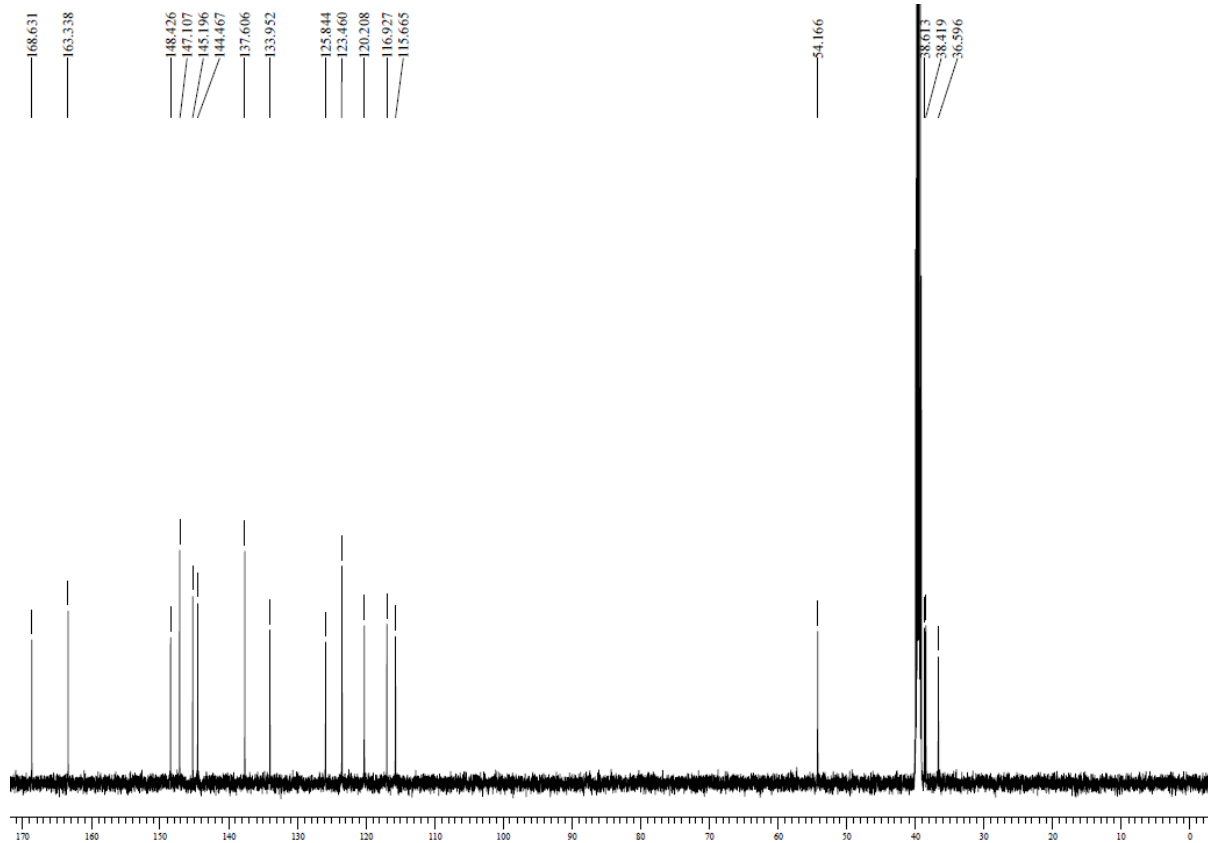
Figure S3.2. NMR spectra of the L-dopa-lazabemide prodrug

1H NMR

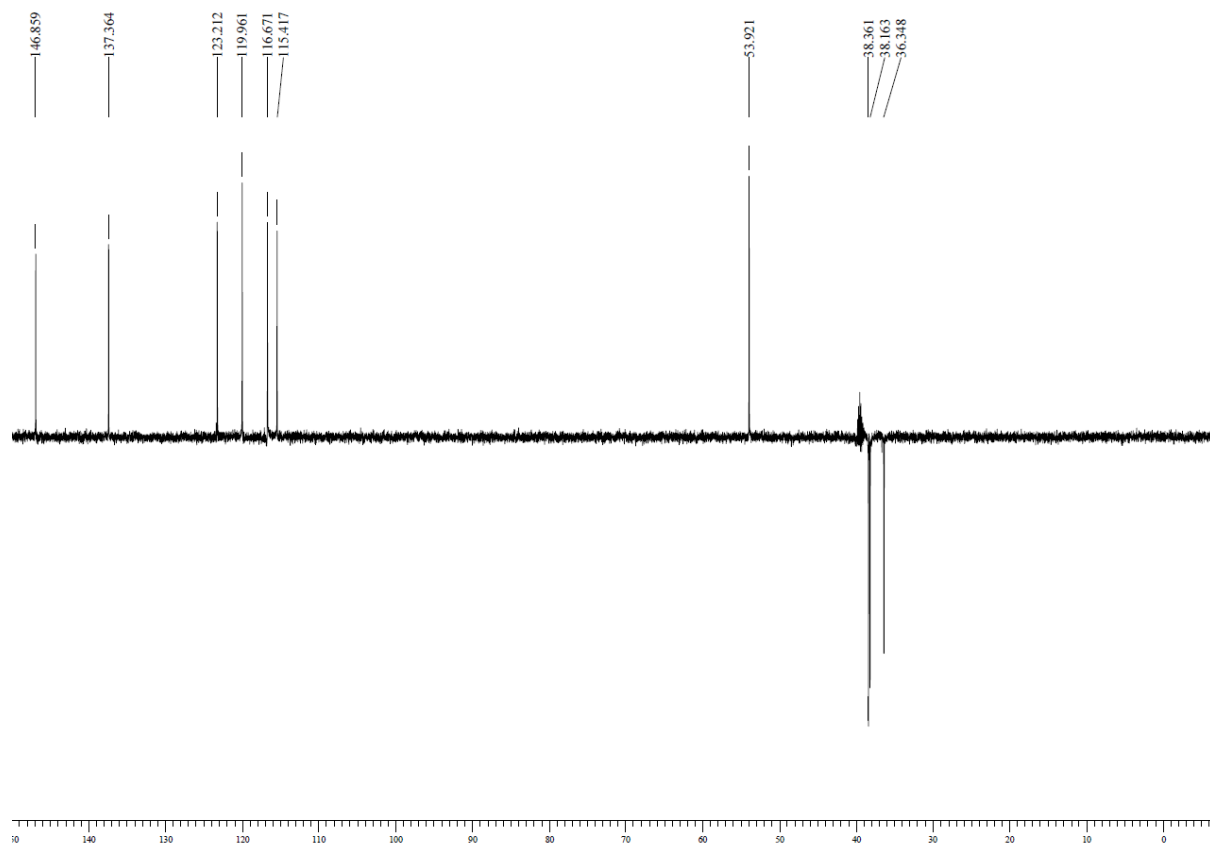




¹³C NMR



¹³C DEPT135



Chapter 4

Article 2

An investigation of the *in vitro* reversibility of MAO inhibition by lazabemide

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Abstract

Lazabemide [Ro 19-6327; N-(2-aminoethyl)-5-chloro-2-pyridinecarboxamide] is a selective and high affinity inhibitor of monoamine oxidase (MAO) B. Lazabemide and related N-(2-aminoethyl)carboxamides (e.g. Ro 41-1049, Ro 16-6491) exhibit an initial competitive mode of binding, but are subsequently activated to form reversible adducts with the enzyme. Structural evidence suggests that adduct formation occurs at the N(5) position of the flavin. Lazabemide may thus be classified as a reversible mechanism-based inhibitor of MAO-B. The reversibility of MAO-B inhibition by lazabemide is corroborated by clinical findings of complete but reversible inhibition of platelet MAO-B with a dose-dependent duration. Although the development of lazabemide has been discontinued, this compound is still used as reference MAO-B inhibitor in the *in vitro* screening of experimental MAO inhibitors. For comparison with experimental inhibitors, the behaviour of lazabemide in *in vitro* MAO inhibition studies should thus be defined. The present study aimed to characterise the *in vitro* MAO inhibition properties of lazabemide with respect to potency, isoform selectivity and reversibility. The results show that lazabemide is a selective inhibitor of human MAO-B with an IC₅₀ value of 0.091 μM. For human MAO-A, lazabemide exhibits an IC₅₀ of >100 μM. Interestingly, dialysis restores MAO-B activity only to a very small extent following inhibition by lazabemide, which shows that *in vitro* inhibition persists and lazabemide may be viewed as an irreversible MAO-B inhibitor. These results should be taken into consideration when using lazabemide as reference inhibitor in *in vitro* screening studies.

4.1. Introduction

The monoamine oxidase (MAO) enzymes exist as two isoforms, MAO-A and MAO-B, and are key enzymes for the metabolism of monoamine neurotransmitters in the peripheral and central tissues. Serotonin is a specific substrate of MAO-A while the false neurotransmitter, β -phenylethylamine, is a MAO-B specific substrate. The catecholamines, dopamine (DA), noradrenaline and adrenaline, as well as the dietary amines, tryptamine and tyramine, are substrates for both MAO isoforms (Youdim *et al.*, 2006). Inhibitors of MAO have been used as antidepressant drugs for over 50 years and act by raising neurotransmitter levels in the brain (Ramsay *et al.*, 2016). In this respect, MAO-A specific inhibitors are used for major depressive disorders and therapy-resistant depression (Lum & Stahl, 2012). Specific inhibitors of MAO-B are used in Parkinson's disease (PD) and act by preventing the MAO-B-catalysed depletion of central DA (Youdim *et al.*, 2006; Youdin & Bakhle, 2006). In PD, MAO-B inhibitors are frequently combined with L-dopa in an effort to bolster the enhancement of DA levels in the brain. Currently two MAO-B inhibitors are registered for the treatment of PD, selegiline and rasagiline (Fig. 4.1). These are irreversible mechanism-based inhibitors of the propargylamine class. A reversible inhibitor, safinamide, was recently approved in Europe for the treatment of PD (Müller, 2016).

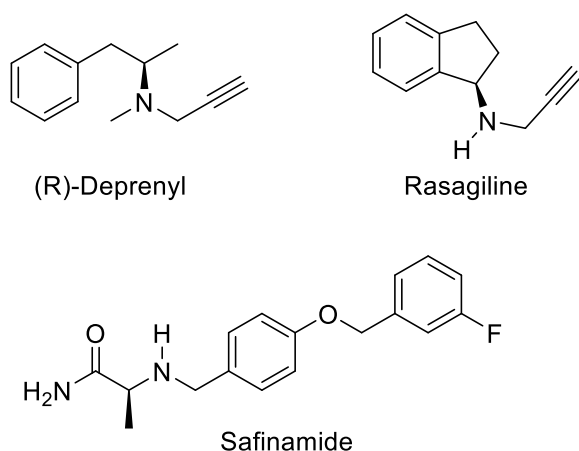


Figure 4.1: The structures of (R)-deprenyl (selegiline), rasagiline and safinamide.

MAO inhibitors may also find future applications in other disease states. For example, MAO-A levels are elevated in certain types of cancer tissue such as prostate cancer, and MAO-A inhibition may, in synergism with surviving suppressants, inhibit cancer cell growth, migration and invasion (Xu *et al.*, 2015; Wu *et al.*, 2014). MAO-B inhibitors, in turn, are under investigation for the treatment of Alzheimer's disease, possibly acting by reducing hydrogen peroxide and aldehyde intermediates formed by the MAO catalytic cycle (Sturm *et al.*, 2016). These are potentially injurious to neuronal cells and may contribute to disease pathogenesis.

By similarly decreasing the MAO-B-catalysed generation of these injurious species in the Parkinsonian brain, MAO-B inhibitors have been proposed to be neuroprotective in PD (Youdim & Bakhle, 2006; Edmondson, 2014). For neurodegenerative disorders such as Alzheimer's disease and PD, the MAO-B isoform seems to be the more relevant target for neuroprotection since MAO-B activity in the brain increases with age while MAO-A activity remains largely unchanged (Fowler *et al.*, 1997). Conversely, hydrogen peroxide formed by MAO-A in the heart has been linked to age-related cardiac cellular degeneration in rats (Maurel *et al.*, 2003), thus providing a possible role for MAO-A inhibitors in the therapy for certain cardiomyopathies. Interestingly, MAO-B inhibitors have also been advocated as an aid to smoking cessation (Berlin *et al.*, 2002).

Due to the role of MAO in neurotransmitter metabolism, and the potential applications of MAO inhibitors in various disease states, the discovery of novel compounds that potently inhibit the MAOs has been pursued for many decades (Ramsay, 2013). In this effort, compounds that exhibit potentially useful mechanisms of inhibitory action are of particular interest. One such compound is lazabemide [Ro 19-6327; N-(2-aminoethyl)-5-chloro-2-pyridinecarboxamide], a MAO-B specific inhibitor discovered in the 1980s (Fig.4.2) (Cesura *et al.*, 1990; Cesura *et al.*, 1999). Lazabemide and related N-(2-aminoethyl)carboxamides (e.g. Ro 41-1049, Ro 16-6491) have the distinction of acting as mechanism-based inhibitors with a reversible mode of action. These inhibitors exhibit an initial competitive mode of binding, but are subsequently activated by MAO to form reversible adducts with the enzyme. The result is rapid and complete MAO-B inhibition with enzyme activity returning to baseline values by 36 h after drug discontinuation (Dingemans *et al.*, 1997; Fowler *et al.*, 1993). Following inhibition with irreversible MAO-B inhibitors, the recovery period can be 40 days (Fowler *et al.*, 2005; Fowler *et al.*, 2015). Furthermore, for a pharmacological effect >90% of MAO-B should be inhibited (Ramsay *et al.*, 2016; Fowler *et al.*, 2005). A dose of at least 0.4 mg/kg lazabemide given every 12 h provides >90% inhibition of brain MAO B in patients with early PD (Fowler *et al.*, 1993). Unfortunately the development of lazabemide has been discontinued due to liver toxicity (Berlin *et al.*, 2002).

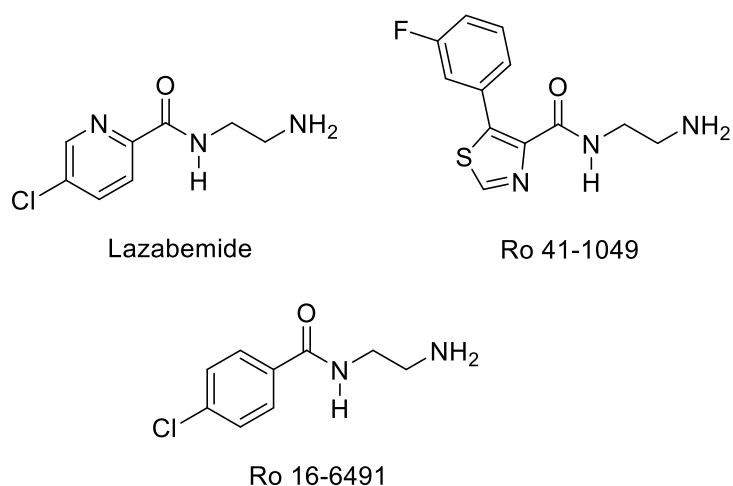


Figure 4.2: The structures of lazabemide, Ro 41-1049 and Ro 16-6491.

The mechanism by which lazabemide inhibits MAO-B is not completely understood. Structural evidence with N-(2-aminoethyl)-p-chlorobenzamide (Ro 16-6491) shows that an adduct forms at the N(5) position of the flavin, which is support for a mechanism-based mode of inhibition (Fig. 4.3) (Binda *et al.*, 2003; Edmondson *et al.*, 2004). The N(5) position also is the site of covalent attachment of virtually all irreversible MAO inhibitors (including propargylamines). The only exceptions are cyclopropylamine inhibitors which form a flavin C(4a) adduct (Edmondson *et al.*, 2009). Evidence for the reversibility of MAO-B inhibition by lazabemide is provided by the relatively short enzyme recovery period after drug discontinuation in clinical studies (Dingemans *et al.*, 1997; Fowler *et al.*, 1993). Furthermore, after denaturation of the enzyme, no covalent adducts with the flavin are found. A covalent adduct is only detected in peptide fragments after borohydride reduction (Cesura *et al.*, 1989). With MAO-B in brain and platelet membranes, the binding of radiolabeled Ro 16-6491 is fully reversible. However, irreversible attachment to the membranes occurs by treatment with borohydride (Cesura *et al.*, 1988).

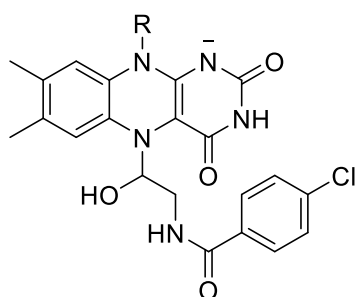


Figure 4.3: The adduct that forms with the inhibition of human MAO-B by N-(2-aminoethyl)-p-chlorobenzamide (Ro 16-6491) (Edmondson *et al.*, 2004).

Although the development of lazabemide has been discontinued, this [and other N-(2-aminoethyl)carboxamides] are still being used as reference MAO-B inhibitors in the *in vitro* screening of experimental MAO inhibitors (Petzer *et al.*, 2013). For comparison with experimental inhibitors, the behaviour of lazabemide in *in vitro* MAO inhibition studies should thus be defined, particularly with respect to reversibility of inhibition on the time scale (10–60 min) of a typical *in vitro* experiment. An important consideration when comparing *in vitro* IC₅₀ values of experimental and reference inhibitors is reversibility of inhibition. The IC₅₀ value recorded for an irreversible inhibitor will vary with different exposure times of the enzyme to the inhibitor prior to the addition of the substrate. Typically the longer the enzyme and inhibitor are preincubated, the higher the degree of inhibition in as much that enzyme activity may be suppressed to a level much lower than expected from a literature IC₅₀ value. With this in mind, the present study aimed to characterise the *in vitro* MAO inhibition properties of lazabemide with respect to potency, isoform selectivity and reversibility.

4.2. Experimental section

4.2.1. The synthesis of lazabemide

2-(5-Chloropyridine-2-yl)-1H-imidazoline: In a 100 mL round bottom flask, 5-chloro-cyanopyridine (28.9 mmol) was placed, and the atmosphere was replaced with argon. Toluene (35 mL) and ethylenediamine (54.2 mmol) was added to the reaction. This was followed by the addition of a catalytic amount of p-toluenesulfonic acid (2.58 mmol). The reaction mixture was heated under reflux to 80 °C for a period of 4.5 h. The reaction was subsequently cooled to room temperature and acetone (32 mL) was added over a period of 30 min. A light yellow precipitate formed and the reaction was incubated for a further 1 h at 0 °C. The precipitate was then filtered under vacuum and washed with acetone/toluene mixture (24 mL/342 mL) and cooled to 0 °C. *2-(5-Chloropyridine-2-yl)-1H-imidazoline* was dried under vacuum (Soriato *et al.*, 2008).

Hydrolysis to yield lazabemide: *2-(5-Chloropyridine-2-yl)-1H-imidazoline* (17.1 mmol) was added to a solution of NaOH (0.68 mmol) in 24 mL water. The reaction mixture was heated at reflux for 2 h and then cooled to room temperature. A solution of saturated NaCl (50 mL) was added to the reaction, and the product was extracted to dichloromethane (3 × 30 mL). The dichloromethane phase was dried over anhydrous sodium sulfate and was subsequently removed under reduced pressure. The residue, a waxy solid, was allowed to solidify overnight. Lazabemide as the free base appeared as a light yellow powder. ¹H NMR (600 MHz, DMSO-d₆) δ 8.97 – 8.78 (m, 1H), 8.71 – 8.63 (m, 1H), 8.11 (dd, J = 8.4, 2.6 Hz, 1H), 8.03 (d, J = 8.3 Hz, 1H), 3.37 – 3.26 (m, 2.44H), 3.12 (s, 0.67H), 2.71 (t, J = 6.5 Hz, 1.46H). ¹³C NMR (151 MHz, DMSO-d₆) δ 163.06, 148.60, 147.01, 137.53, 133.80, 123.35, 41.79, 40.83.

4.2.2. Measurement of IC₅₀ values

For these studies, the recombinant human MAO-A and MAO-B enzymes were obtained from Sigma-Aldrich (St. Louis, MO, USA). The IC₅₀ values were measured according to a recently reported protocol (Meiring *et al.*, 2017). In short, the enzyme reactions (200 µL) contained kynuramine (50 µM), the test inhibitors (0.003–100 µM) and MAO-A (0.0075 mg protein/mL) or MAO-B (0.015 mg protein/mL). Stock solutions of the test inhibitors were prepared in DMSO and added to the reactions to yield a final DMSO concentration of 4%. The reactions were initiated with the addition of the MAO enzymes and were subsequently incubated for 20 min at 37 °C. After termination at endpoint (with 80 µL NaOH, 2 N), the concentration of 4-hydroxyquinoline, the product of kynuramine oxidation by MAO, was measured by fluorescence spectrophotometry. The inhibition data were fitted to the one site competition model of the Prism 5 software package (GraphPad, San Diego, CA, USA) and the IC₅₀ values, measured in triplicate, are reported as the mean ± standard deviation (SD).

4.2.3. Dialysis

Dialysis studies were carried out with Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Waltham, MA, USA) with a molecular weight cut-off of 10 000 according to the literature protocol (Meiring *et al.*, 2017). In short, the test inhibitors (at a concentration equal to 4 × IC₅₀) and the MAO enzymes (0.03 mg protein/mL) were preincubated for 15 min and were subsequently dialysed at 4 °C for 24 h. Potassium phosphate buffer (100 mM, pH 7.4, 5% sucrose) served as dialysis buffer, and was replaced with fresh buffer at 3 h and 7 h after the start of dialysis. Stock solutions of the test inhibitors were prepared in DMSO and added to the buffer to yield 4% DMSO. As positive controls, MAO-A and MAO-B were similarly preincubated and dialysed in the presence of the irreversible inhibitors, pargyline (IC₅₀ = 13 µM) and selegiline (IC₅₀ = 0.079 µM), respectively (Strydom *et al.*, 2012; Petzer *et al.*, 2012). As negative control, dialysis of the enzymes was carried out in the absence of the test inhibitors. After dialysis, the samples were diluted twofold with the addition of kynuramine and the reactions were incubated for 20 min at 37 °C. After termination at endpoint, the concentration of 4-hydroxyquinoline was measured by fluorescence spectrophotometry as described above. For comparison, undialysed mixtures of MAO enzyme and the test inhibitors were maintained at 4 °C for 24 h and subsequently diluted and assayed as above. All reactions were carried out in triplicate and the residual enzyme catalytic rates were expressed as mean ± SD.

4.3. Results

4.3.1. The synthesis of lazabemide

A literature survey shows that a procedure for the synthesis of lazabemide has been reported. Lazabemide was synthesised from 5-chloro-2-cyanopyridine according to the published method (Soriato *et al.*, 2008). In the first step, 5-chloro-cyanopyridine was reacted with p-toluenesulfonic acid and ethylenediamine to yield the imidazoline intermediate (Fig. 4.4). This reaction takes place at 80 °C in an atmosphere of argon. The product, 2-(5-chloropyridine-2-yl)-1*H*-imidazoline, was obtained in good yields of 88%. In the second step, 2-(5-chloropyridine-2-yl)-1*H*-imidazoline is hydrolysed with aqueous NaOH to yield lazabemide as the free base.

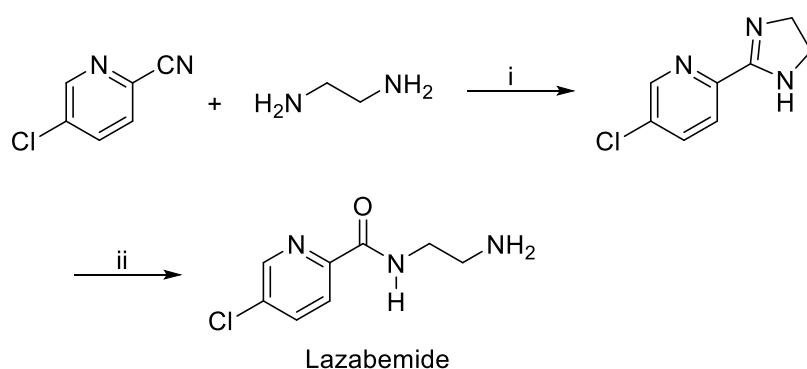


Figure 4.4: The synthesis of lazabemide. Key: (i) p-toluenesulfonic acid, 80 °C, argon; (ii) NaOH, reflux, 2 h.

4.3.2. IC₅₀ values for the inhibition of human MAO

For the MAO inhibition studies, the recombinant human enzymes were used, with kynuramine serving as substrate for both MAO isoforms. Kynuramine is metabolised by the MAOs to ultimately yield 4-hydroxyquinoline, a compound which may be measured by fluorescence spectrophotometry. By measuring the MAO-catalysed formation of 4-hydroxyquinoline in the presence of different inhibitor concentrations (0.003–100 μM), sigmoidal plots of enzyme catalytic rate versus logarithm of inhibitor concentration may be constructed from which IC₅₀ values are estimated. As shown in Fig. 4.5, in *in vitro* experiments, lazabemide is a highly selective inhibitor of human MAO-B with an IC₅₀ value of 0.091 ± 0.015 μM (Petzer *et al.*, 2013). Lazabemide is a much less potent inhibitor of human MAO-A with an IC₅₀ value of >100 μM. For these experiments the reactions were initiated with the addition of the MAO enzymes with no prior exposure of the enzyme to lazabemide. Since lazabemide is reported to inhibit MAO-B irreversibly, it may be expected that inhibition is time-dependent and the IC₅₀ value will be much decreased with prior exposure of the enzyme to lazabemide.

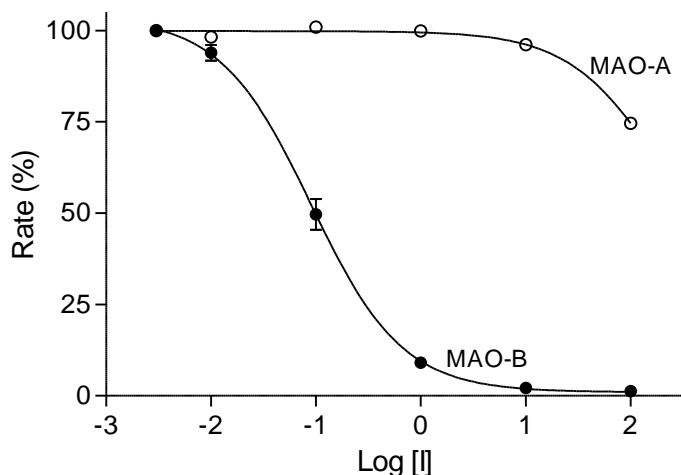


Figure 4.5: Sigmoidal curves for the inhibition of human MAO-A and MAO-B by lazabemide. Data points are shown as mean \pm SD.

4.3.3. Reversibility of MAO-B inhibition by dialysis

As mentioned, although lazabemide is a mechanism-based inhibitor of MAO-B, the adduct formed with the enzyme's flavin cofactor is reversible. To better interpret the *in vitro* IC_{50} value recorded for MAO-B, the reversibility of inhibition in the *in vitro* setting was further examined. For this purpose dialysis was used. Lazabemide (at a concentration of $4 \times IC_{50}$) was combined with MAO-B, preincubated for 15 min and subsequently dialysed for 24 h. The dialysed mixtures were diluted twofold with the addition of kynuramine and the residual enzyme catalytic rates, the rates of formation of 4-hydroxyquinoline, were measured. As negative control, dialysis experiments were carried out in the absence of inhibitor while as positive control, dialysis of MAO-B was carried out in the presence of the irreversible inhibitor, (R)-selegiline. As third control, the residual MAO-B activity of undialysed mixtures of MAO-B and lazabemide was recorded.

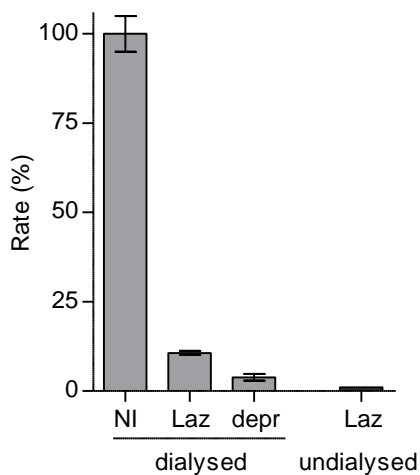


Figure 4.6: The reversibility of the inhibition of human MAO-B by lazabemide. MAO-B and lazabemide (at $4 \times IC_{50}$) were preincubated for 15 min, dialysed for 24 h and the residual enzyme activity was measured (Laz–dialysed). MAO-B was similarly preincubated in the absence (NI–dialysed) and presence of the irreversible inhibitor, (R)-deprenyl (depr–dialysed), and dialysed. For comparison, the residual MAO-B activity of undialysed mixtures of MAO-B with lazabemide is also shown (Laz–undialysed).

As shown in Fig. 4.6, after inhibition of MAO-B by lazabemide, dialysis does not restore enzyme activity, with the residual rate at only 10.6% of the negative control (100%). For reversible inhibition, dialysis is expected to restore enzyme activity to 100% of the negative control. Following inhibition with selegiline, enzyme activity is also not restored by dialysis with the residual activity at 3.9%. Interestingly, in undialysed mixtures of MAO-B and lazabemide, the residual enzyme activity was only 1.1% of the negative control value. Since the inhibitor concentration in the undialysed reactions is $2 \times IC_{50}$, the degree of inhibition for reversible interaction should be 25%. The degree of inhibition observed in this study is much higher than the 25%, which indicates time-dependent, and therefore irreversible, inhibition of MAO-B by lazabemide. These results thus show that, *in vitro*, the inhibition of MAO-B by lazabemide is essentially irreversible, for at least a period of 24 h.

4.4. Discussion and conclusion

As mentioned, lazabemide and related N-(2-aminoethyl)carboxamides are mechanism-based inhibitors of MAO-B. While inhibition is irreversible *in vitro*, *in vivo*, enzyme activity returns to baseline values by 36 h after drug discontinuation (Dingemans *et al.*, 1997; Fowler *et al.*, 1993). Since enzyme recovery may require as much as 40 days following inhibition with irreversible inhibitors, this demonstrates that lazabemide inhibition *in vivo* is essentially reversible (Fowler *et al.*, 2005; Fowler *et al.*, 2015). Due to its unique mechanism of action, lazabemide results in rapid and complete MAO-B inhibition, which highlights its potential efficacy in the *in vivo* setting. A further point of interest is that lazabemide is a highly specific inhibitor of MAO-B over the MAO-A isoform. Considering its reversibility of *in vivo* inhibition, potential high efficacy and complete MAO-B inhibition, and high specificity, lazabemide may be considered an ideal MAO-B inhibitor for the treatment of neurodegenerative disorders such as Alzheimer's disease and PD. This compound will be expected to possess a very low risk of causing adverse effects such as the potentiation of tyramine-induced hypertension, which is associated with MAO-A inhibition, particularly irreversible MAO-A inhibition (Da Prada *et al.*, 1988; Flockhart, 2012). Although the development of lazabemide has been discontinued, related compounds in this class may still be considered for future development as MAO-B specific inhibitors. Furthermore, lazabemide may still be useful for experimental work and a

clear understanding of its mechanism of action is therefore required. By characterising the *in vitro* interaction of lazabemide with human MAO-B, the present study contributes to knowledge in this respect.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interest in this work.

References

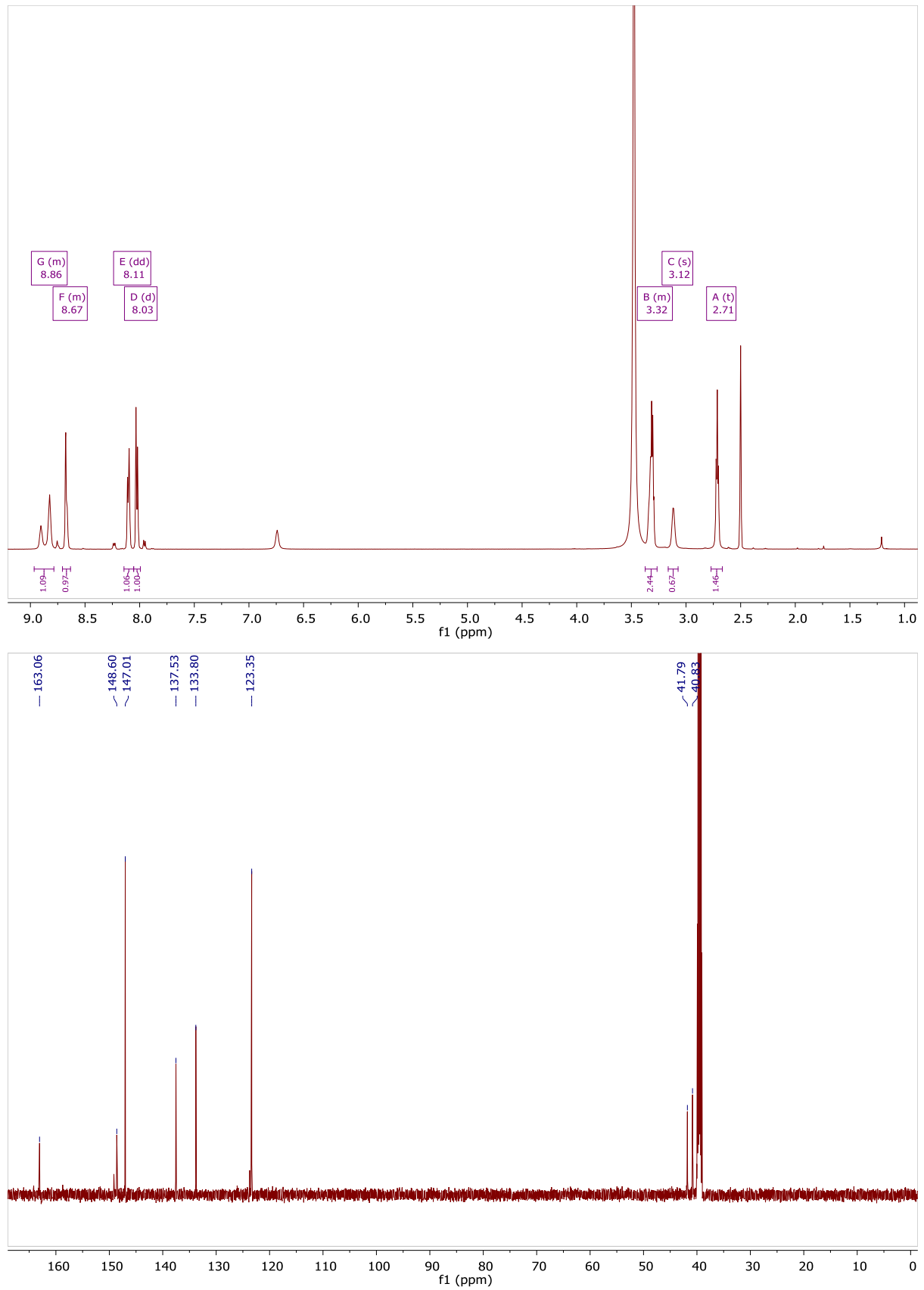
1. Berlin, I., Aubin, H.J., Pedarriosse, A.M., Rames, A., Lancrenon, S. & Lagrue, G. 2002. Lazabemide in Smoking Cessation Study Investigators. Lazabemide, a selective, reversible monoamine oxidase B inhibitor, as an aid to smoking cessation. *Addiction*, 97(10):1347-1354.
2. Binda, C., Li, M., Hubalek, F., Restelli, N., Edmondson, D.E. & Mattevi, A. 2003. Insights into the mode of inhibition of human mitochondrial monoamine oxidase B from high-resolution crystal structures. *Proceedings of the national academy of science of the United States of America*, 100(17):9750-9755.
3. Cesura, A.M., Borroni, E., Gottowik, J., Kuhn, C., Malherbe, P., Martin, J. & Richards, J.G. 1999. Lazabemide for the treatment of Alzheimer's disease: rationale and therapeutic perspectives. *Advances in neurology*, 80:521-528.
4. Cesura, A.M., Galva, M.D., Imhof, R., Kyburz, E., Picotti, G.B. & Da Prada, M. 1989. [³H]Ro 19-6327: a reversible ligand and affinity labelling probe for monoamine oxidase-B. *European journal of pharmacology*, 162(3):457-465.
5. Cesura, A.M., Imhof, R., Takacs, B., Galva, M.D., Picotti, G.B. & Da Prada, M. 1988. [³H]Ro 16-6491, a selective probe for affinity labelling of monoamine oxidase type B in human brain and platelet membranes. *Journal of neurochemistry*, 50(4):1037-1043.
6. Cesura, A.M., Muggli-Maniglio, D., Lang, G., Imhof, R. & Da Prada, M. 1990. Monoamine oxidase inhibition by moclobemide and 2-amino-ethyl carboxamide derivatives: mode of action and kinetic characteristics. *Journal of neural transmission*, 32:165-170.

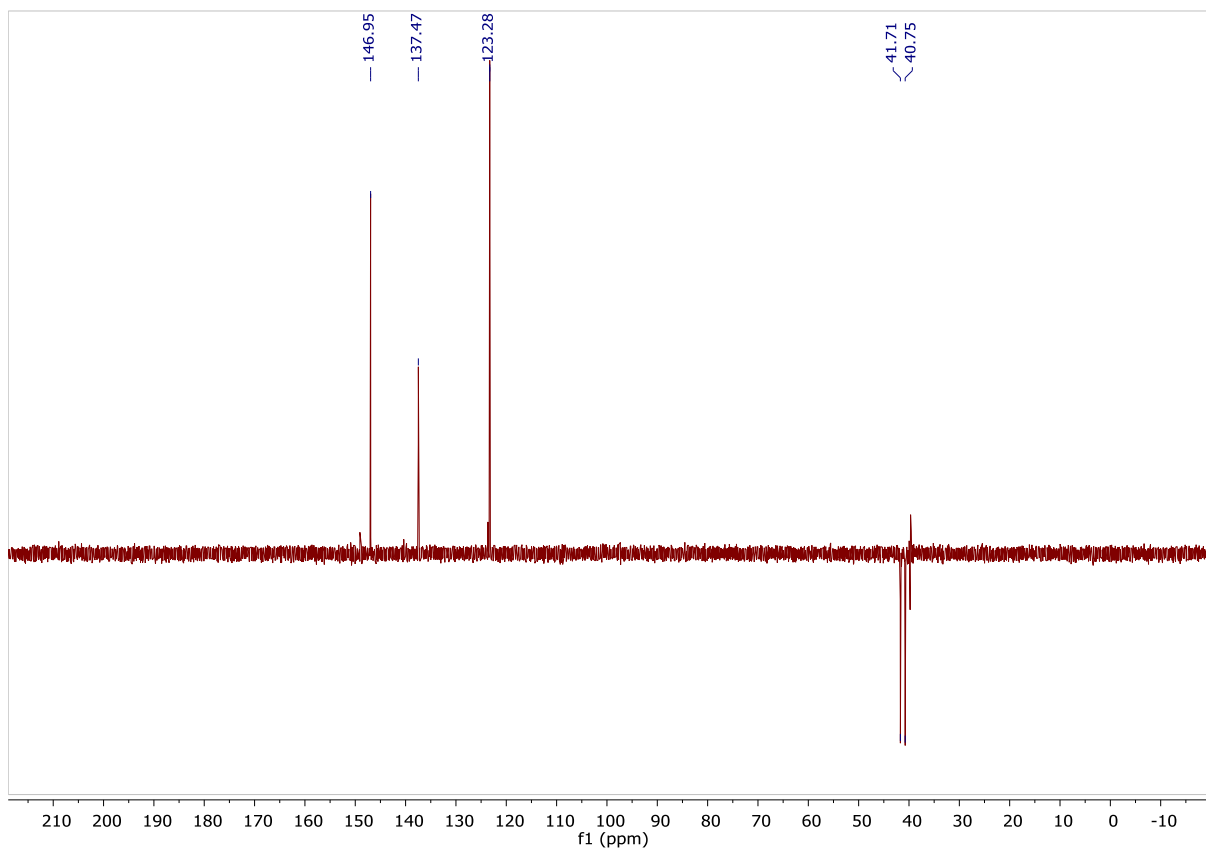
7. Da Prada, M., Zürcher, G., Wüthrich, I. & Haefely, W.E. 1988. On tyramine, food, beverages and the reversible MAO inhibitor moclobemide. *Journal of neural transmission*, 26:31-56.
8. Dingemans, J., Wood, N., Jorga, K. & Kettler, R. 1997. Pharmacokinetics and pharmacodynamics of single and multiple doses of the MAO-B inhibitor lazabemide in healthy subjects. *British journal of clinical pharmacology*, 43(1):41-47.
9. Edmondson, D.E., Binda, C., Wang, J., Upadhyay, A.K. & Mattevi, A. 2009. Molecular and mechanistic properties of the membrane-bound mitochondrial monoamine oxidases. *Biochemistry*, 48(20):4220-4230.
10. Edmondson, D.E., Mattevi, A., Binda, C., Li, M. & Hubálek, F. 2004. Structure and mechanism of monoamine oxidase. *Current medicinal chemistry*, 11(15):1983-93.
11. Edmondson, D.E. 2014. Hydrogen peroxide produced by mitochondrial monoamine oxidase catalysis: biological implications. *Current pharmaceutical design*, 20(2):155-160.
12. Flockhart, D.A. 2012. Dietary restrictions and drug interactions with monoamine oxidase inhibitors: an update. *Journal of clinical Psychiatry*, 73(1):17-24.
13. Fowler, J.S., Logan, J., Shumay, E., Alia-Klein, N., Wang, G.J. & Volkow, N.D. 2015. Monoamine oxidase: radiotracer chemistry and human studies. *Journal of labelled compounds and radiopharmaceuticals*, 58(3):51-64.
14. Fowler, J.S., Logan, J., Volkow, N.D. & Wang, G.J. 2005. Translational neuroimaging: positron emission tomography studies of monoamine oxidase. *Molecular imaging and Biology*, 7(6):377-387.
15. Fowler, J.S., Volkow, N.D., Logan, J., Schlyer, D.J., MacGregor, R.R., Wang, G.J., Wolf, A.P., Pappas, N., Alexoff, D. & Shea, C. 1993. Monoamine oxidase B (MAO B) inhibitor therapy in PD: the degree and reversibility of human brain MAO B inhibition by Ro 19 6327. *Neurology*, 43(10):1984-1992.
16. Fowler, J.S., Volkow, N.D., Wang, G.J., Logan, J., Pappas, N., Shea, C. & MacGregor, R. 1997. Age-related increases in brain monoamine oxidase B in living healthy human subjects. *Neurobiological Aging*, 18(4):431-435.
17. Lum, C.T. & Stahl, S.M. 2012. Opportunities for reversible inhibitors of monoamine oxidase-A (RIMAs) in the treatment of depression. *CNS Spectrums*, 17(3):107-120.
18. Maurel, A., Hernandez, C., Kunduzova, O., Bompard, G., Cambon, C., Parini, A. & Francés, B. 2003. Age-dependent increase in hydrogen peroxide production by cardiac monoamine oxidase A in rats. *The American journal of physiology - Heart circulatory physiology*, 284(4):1460-1467.

19. Meiring, L., Petzer, J.P. & Petzer, A. 2017. C6- and C7-Substituted 3,4-dihydro-2(1H)-quinolinones as inhibitors of monoamine oxidase. *Drug Research (Stuttg)*, 67(3):170-178.
20. Müller, T. 2016. Emerging approaches in PD - adjunctive role of safinamide. *Therapeutics and clinical risk management*, 12:1151-1160.
21. Petzer, A., Harvey, B.H., Wegener, G. & Petzer, J.P. 2012. Azure B, a metabolite of methylene blue, is a high-potency, reversible inhibitor of monoamine oxidase. *Toxicology and applied pharmacology*, 258(3):403-409.
22. Petzer, A., Pienaar, A. & Petzer, J.P. 2013. The inhibition of monoamine oxidase by esomeprazole. *Drug Research (Stuttg)*, 63(9):462-467.
23. Ramsay, R.R. 2013. Inhibitor design for monoamine oxidases. *Current pharmaceutical design*, 19(14):2529-2539.
24. Ramsay, R.R. 2016. Molecular aspects of monoamine oxidase B. *Progress in neuro-psychopharmacology and biological Psychiatry*, 69:81-89.
25. Soriato, G., Focati, M.P., Brescello, R., Cotarca, L. & Giovanetti, R. 2008. Pharmaceutical preparations of crystalline lazabemide. Patent WO/2008/010794.
26. Strydom, B., Bergh, J.J. & Petzer, J.P. 2012. The inhibition of monoamine oxidase by 8-(2-phenoxyethoxy)caffeine analogues. *Arzneimittelforschung*, 62(11):513-518.
27. Sturm, S., Forsberg, A., Nave, S., Stenkrona, P., Seneca, N., Varrone, A., Comley, R.A., Fazio, P., Jamois, C., Nakao, R., Ejduk, Z., Al-Tawil, N., Akenine, U., Halldin, C., Andreasen, N. & Ricci, B. 2016. Positron emission tomography measurement of brain MAO-B inhibition in patients with Alzheimer's disease and elderly controls after oral administration of sebragiline. *European journal of nuclear medicine and molecular imaging*, [Epub ahead of print].
28. Wu, J.B., Shao, C., Li, X., Li, Q., Hu, P., Shi, C., Li, Y., Chen, Y.T., Yin, F., Liao, C.P., Stiles, B.L., Zhau, H.E., Shih, J.C. & Chung, L.W. 2014. Monoamine oxidase A mediates prostate tumorigenesis and cancer metastasis. *Journal of clinical investigation*, 124(7):2891-2908.
29. Xu, S., Adisetiyo, H., Tamura, S., Grande, F., Garofalo, A., Roy-Burman, P. & Neamati, N. 2015. Dual inhibition of survivin and MAOA synergistically impairs growth of PTEN-negative prostate cancer. *British journal of Cancer*, 113(2):242-251.
30. Youdim, M.B. & Bakhle, Y.S. 2006. Monoamine oxidase: isoforms and inhibitors in PD and depressive illness. *British journal of pharmacology*, 147(1):287-296.
31. Youdim, M.B., Edmondson, D. & Tipton, K.F. 2006. The therapeutic potential of monoamine oxidase inhibitors. *Nature reviews neuroscience*, 7(4):295-309.

Supplementary material

Figure S4.1. ^1H NMR, ^{13}C NMR and DEPT-135 spectra for lazabemide





Chapter 5

Article 3

The synthesis and property evaluation of novel L-dopa prodrugs for the treatment of Parkinson's disease

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Running title: Properties of L-dopa prodrugs

Abstract

Fifty years after its introduction to the clinic, L-dopa remains the “gold standard” in the drug treatment of Parkinson’s disease (PD). L-Dopa is the metabolic precursor of dopamine (DA) and is activated by decarboxylation by the enzyme, aromatic L-amino acid decarboxylase (AADC), to yield DA. In the central nervous system, this reaction sequence replenishes striatal DA and provides symptomatic relief for the motor deficits of the disorder. Unfortunately, L-dopa is also decarboxylated in the peripheral tissue which limits its availability for entry into the brain and increases peripheral dopaminergic side effects. Other factors that limit L-dopa’s bioavailability are irregular absorption from the gastrointestinal tract, a short half-life and 3-O-methylation catalysed by catechol-O-methyltransferase (COMT). In an attempt to rectify this, L-dopa is administered with AADC and COMT inhibitors. Another approach to improve L-dopa bioavailability is more effective formulations for oral absorption, alternative routes of administration and prodrugs that may protect L-dopa from peripheral conversion. Based on these observations, the present study synthesises four carrier-linked prodrugs of L-dopa in which 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine are linked to the carboxylate of L-dopa. Key physicochemical and biochemical parameters of the prodrugs were evaluated in an attempt to assess the potential of these prodrugs as vehicles to enhance the absorption and central delivery of L-dopa.

Keywords: L-dopa, levodopa, prodrug, physicochemical properties, decarboxylation, lipophilicity, solubility, permeability, stability

5.1. Introduction

L-Dopa (3,4-dihydroxy-L-phenylalanine) is a naturally occurring amino acid first isolated from the bean of *Vicia faba* in 1910–1911 (Fig. 5.1) (Hornykiewicz, 2010). In the human, dietary and clinically administered L-dopa is absorbed from the gastrointestinal tract via the amino acid transport machinery. L-Dopa also gains access to the central nervous system via amino acid transporters at the blood-brain barrier and thus dietary amino acids are avoided to decrease competition for transport in intestine and at the blood-brain barrier (Camargo *et al.*, 2014). L-Dopa is extensively metabolised with approximately 70% of an oral dose undergoing pre-systemic decarboxylation to DA by the enzyme, aromatic L-amino acid decarboxylase (AADC), present in the stomach, lumen of the intestine, kidney and liver (Khor & Hsu, 2007; Contin & Martinelli, 2010). Another prominent metabolic pathway for L-dopa is 3-O-methylation by hepatic catechol-O-methyltransferase (COMT) to yield 3-O-methyldopa (Nutt & Fellman, 1984). L-Dopa thus has a short half-life of approximately 0.7 to 1.4 h (Contin *et al.*, 1990). In spite of these and other shortcomings, L-dopa is used as DA replacement therapy in PD and since its first use in the 1960s, remains the most effective treatment (Freitas *et al.*, 2016; Poewe & Antonini, 2015). To enhance bioavailability and limit peripheral metabolism, L-dopa is co-administered with inhibitors of AADC such as carbidopa or benserazide (Seeberger & Hauser, 2015). This greatly enhances the systemic bioavailability of an oral L-dopa dose. The metabolism of L-dopa may be further reduced and efficacy enhanced by administering COMT inhibitors such as entacapone (Nutt, 2000; Learmonth *et al.*, 2004; Nissinen *et al.*, 1992). Of great clinical significance is the observation that DA itself cannot be used in the treatment of PD because of its inability to penetrate the blood-brain barrier. DA generated in the periphery from L-dopa, thus does not have access to the brain, and enhances peripheral dopaminergic side effects (Hornykiewicz, 2010). L-Dopa thus is a prodrug which enters the brain and is decarboxylated to yield DA.

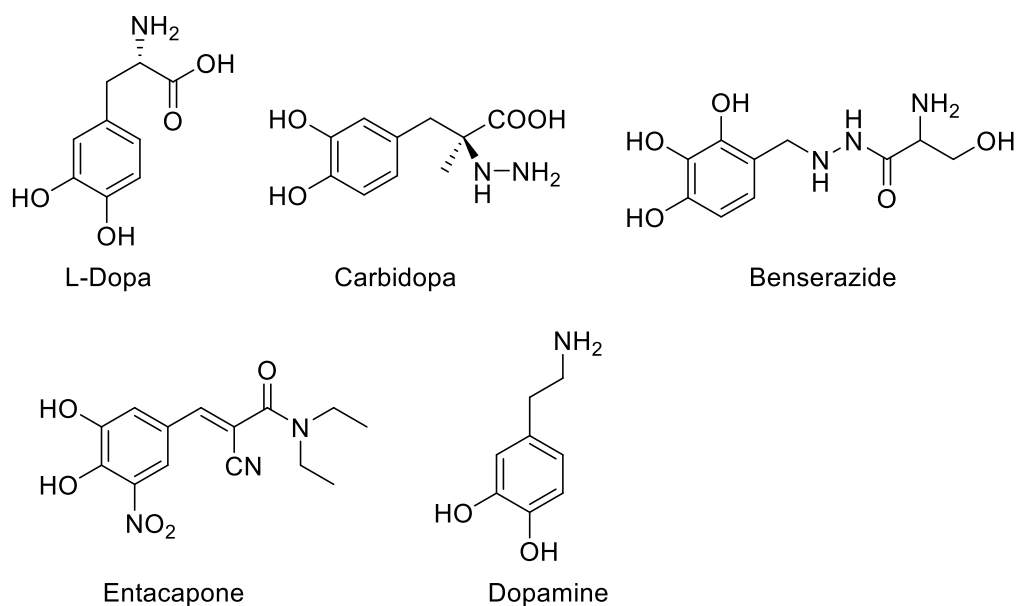


Figure 5.1: The structures of L-dopa and other compounds discussed in the text.

Recently, much effort has been devoted to improving the pharmacokinetic profile of L-dopa by novel formulations to improve absorption, exploring nonoral routes of administration and reducing peripheral metabolism (Freitas *et al.*, 2016; Poewe & Antonini, 2015). For example, IPX066 is a novel extended-release oral formulation of L-dopa/carbidopa that combines immediate-release with extended-release (Freitas *et al.*, 2016; Hauser *et al.*, 2013). This formulation has recently been approved in the USA and the EU. XP21279 is an orally active prodrug of L-dopa that is absorbed from the small and large intestine by high-capacity nutrient transporters (Lewitt *et al.*, 2012). Unfortunately the development of this prodrug has been discontinued. ODM-101, an oral formulation of L-dopa/carbidopa/entacapone, delivers a higher dose of carbidopa. AP09004 is an extended release 'accordion pill' formulation of L-dopa/carbidopa with gastroretentive properties (Freitas *et al.*, 2016). DM-1992 is a bilayer formulation and consists of immediate-release and extended-release layers of L-dopa/carbidopa (Verhagen Metman *et al.*, 2015). An intestinal gel, which is infused directly into the proximal jejunum, contains a suspension of L-dopa/carbidopa in carboxymethyl-cellulose and represents an approved therapy (Olanow *et al.*, 2014). ND0612 is a liquid formulation of L-dopa/carbidopa for subcutaneous administration by a patch-pump device, while CVT-301 is a L-dopa inhalation powder with rapid onset of action (Freitas *et al.*, 2016; LeWitt *et al.*, 2016).

A number of experimental prodrugs of L-dopa have also been designed and evaluated (Di Stefano *et al.*, 2011). For example, a prodrug (**1**) in which L-dopa is linked via a biodegradable carbamate to entacapone has been reported (Fig. 5.2) (Savolainen *et al.*, 2000; Leppänen *et*

et al., 2002). In this respect, prodrugs (**2–3**) with benserazide linked to L-dopa have also been designed (Di Stefano *et al.*, 2006). Peptidyl prodrugs such as the tripeptide mimetic prodrug, in which D-p-hydroxyphenylglycine and L-proline is linked to L-dopa (**4**), was designed as a delivery system for improved L-dopa oral absorption (Wang *et al.*, 1995). L-Dopa has also been linked via an amide bond with glutathione (**5**), in an attempt to reduce oxidative stress, a process that has been linked to neurodegeneration in PD (Pinnen *et al.*, 2007). Glycosyl prodrugs such as **6**, in turn, have been designed for active transport across the blood-brain barrier (Di Stefano *et al.*, 2008). Cyclic prodrugs of L-dopa such as **7** may possess enhanced absorption and metabolic resistance toward AADC (Cingolani *et al.*, 2000). The general approach to designing L-dopa prodrugs is attaching appropriate carriers at the aminium, carboxylate or phenolic hydroxyl groups. These points of attachment should be biodegradable to allow for the release of L-dopa and the carrier. In this respect, carriers are most frequently linked to L-dopa via the ester, amide (peptide) and carbamate functions (Di Stefano *et al.*, 2011).

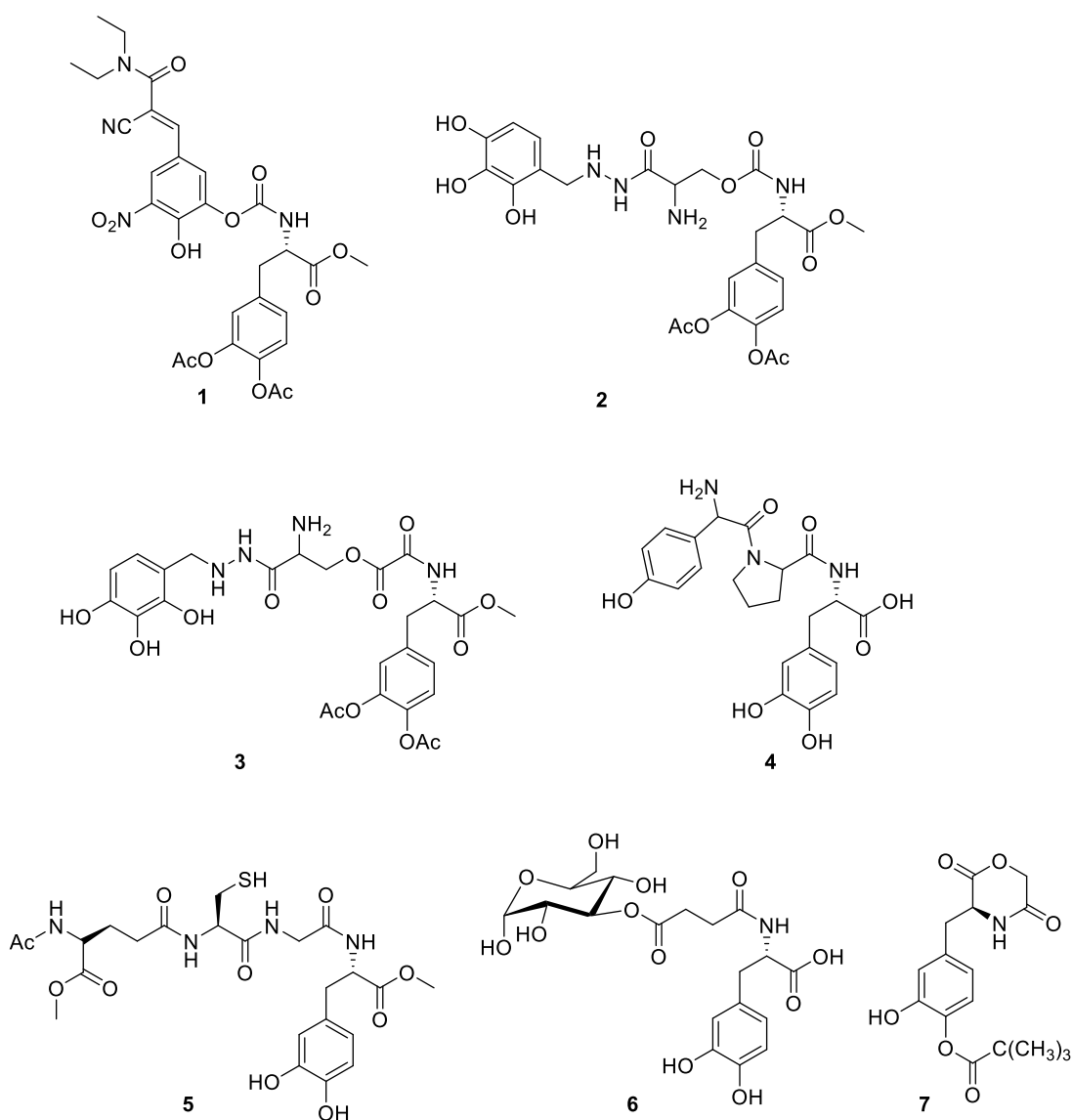


Figure 5.2: The structures of selected experimental prodrugs of L-dopa (Di Stefano *et al.*, 2011).

Based on the interest and therapeutic potential of L-dopa prodrugs, the present study synthesises four carrier-linked prodrugs (**8–11**) of L-dopa in which 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine are linked to the carboxylate of L-dopa (Fig. 5.3). The key physicochemical and biochemical parameters of the prodrugs were subsequently evaluated in an attempt to assess the potential of these prodrugs as vehicles to enhance the absorption and central delivery of L-dopa. These selected carriers were linked to L-dopa at the carboxylate with the primary aminyl functional group. This would protect the carboxylic acid of L-dopa against peripheral decarboxylation and possibly enhance passive diffusion permeability by elimination of the carboxylate charge. Furthermore, unlike L-dopa, the prodrugs do not contain the carboxylate group, which is known to reduce membrane permeation of small organic compounds (Gleeson, 2008; Manallack *et al.*, 2013). A further consideration is that the relative stability of the amide link may allow the prodrug more time to diffuse into the brain prior to activation, thus effectively delivering L-dopa in the brain. Since the prodrugs are expected to be more lipophilic than L-dopa, absorption from the gastrointestinal tract by passive diffusion and enhanced penetration of the blood-brain barrier are probable. The selection of the pyridine-containing carriers in this study was based on the high Caco-2 permeability of pyridine (Chen *et al.*, 2006). Although chemical substitution of pyridine reduces permeability, the overall effect of the addition of the pyridine moiety to the prodrug would be an enhancement of permeability. The 3-phenyl-1-propylamine carrier was included as a comparator for the prodrugs incorporating the pyridine function.

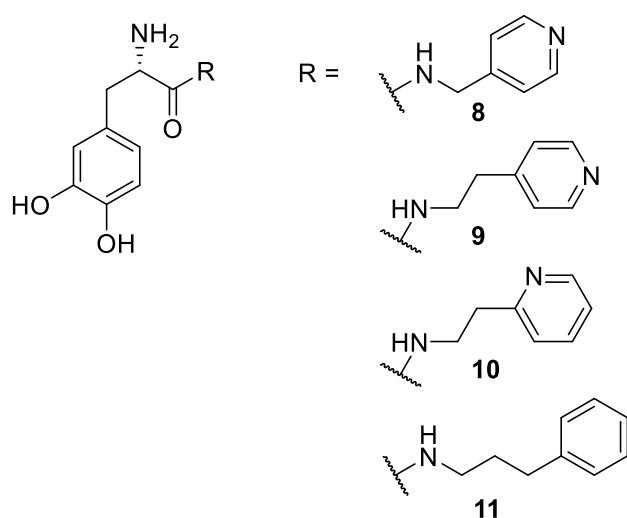


Figure 5.3: The structures of the L-dopa prodrugs (**8–11**) examined in this study.

5.2. Materials and methods

5.2.1. The synthesis of the L-dopa prodrugs

5.2.1.1. Chemicals and instrumentation

High resolution mass spectra (HRMS) were recorded with a DFS high resolution magnetic sector mass spectrometer (Thermo Electron Corporation) in electron ionisation (EI) mode. Proton (^1H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance III 600 spectrometer at a frequency of 600 MHz for ^1H NMR spectra and 150 MHz for ^{13}C NMR spectra. All chemical shifts are reported in parts per million (δ) and were referenced to the residual solvent signal (DMSO- d_6 : 2.50 and 39.52 ppm for ^1H and ^{13}C , respectively). Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), dq (doublet of quartets), p (pentet), hept (heptet), dhept (doublet of heptets), m (multiplet). All chemicals and reagents were obtained from Sigma-Aldrich, and were used without further purification.

5.2.2. The synthesis of L-dopa prodrugs 8–11

The L-dopa prodrugs were prepared according to the procedure detailed in the literature (Nakonieczna *et al.*, 1994; Strydom *et al.*, 2017). L-Dopa was firstly protected at the catechol OH groups using tert-butyldimethylsilyl chloride (TBDMS-Cl) in acetonitrile. This reaction was carried out in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and yielded L-dopa(TBDMS) $_2$ (**12**) in a yield of 37%. In the second step, the aminium group of L-dopa was protected using di-tert-butyl dicarbonate (Boc $_2$ O) in a mixture of THF/water/NaHCO $_3$. This reaction introduces the tert-butoxycarbonyl (t-Boc) group on the primary amine of L-dopa(TBDMS) $_2$, giving the Boc-L-dopa(TBDMS) $_2$ (**13**) in a yield of 67%. The carrier molecules, 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine, were subsequently reacted with the protected L-dopa (**13**) in the presence of BOP [(1*H*-benzotriazole-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate] with dichloromethane as solvent and N-methylmorpholine (NMM) serving as base. This reaction was carried out for 24 h at room temperature and yielded the protected L-dopa prodrugs (**14**–**17**). The removal of the TBDMS and Boc protective groups from the protected L-dopa prodrugs was facilitated by HCl (4 N in dioxane). This reaction was carried out for 48 h at 55 °C to yield the L-dopa prodrugs (**8**–**11**) as the hydrochloric acid salts in yields of 35.3-69.26% (final step).

5.2.2.1. *L-Dopa-4-pyridylmethylamine hydrochloride* (**8**): ^1H NMR (600 MHz, DMSO- d_6) δ 9.45 (t, J = 6.0 Hz, 1H), 8.75 (d, J = 5.9 Hz, 2H), 8.60 – 8.42 (m, 5H), 7.63 (d, J = 5.8 Hz, 2H), 6.70 (d, J = 8.0 Hz, 1H), 6.64 (d, J = 2.1 Hz, 1H), 6.50 (dd, J = 8.1, 2.1 Hz, 1H), 4.60 (dd, J = 17.7, 6.2 Hz, 1H), 4.47 (dd, J = 17.7, 5.6 Hz, 1H), 4.13 – 3.95 (m, 1H), 2.95 (dd, J = 13.4, 6.3 Hz, 1H), 2.86 (dd, J = 13.5, 8.4 Hz, 1H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 168.83, 158.52, 145.32,

144.57, 141.53, 125.61, 124.64, 120.32, 117.11, 115.93, 53.91, 41.57, 36.38. C₁₅H₁₇N₃O₃, 286.31, found 69.26% yield.

5.2.2.2. *L-Dopa-2-(4-pyridyl)ethylamine hydrochloride (9)*: ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.96 (d, *J* = 4.8 Hz, 2H), 8.53 (s, 3H), 7.27 (t, *J* = 7.6 Hz, 2H), 7.20 – 7.11 (m, 3H), 6.67 (d, *J* = 8.0 Hz, 1H), 6.62 (d, *J* = 2.1 Hz, 1H), 6.46 (dd, *J* = 8.0, 2.1 Hz, 1H), 4.11 (dd, *J* = 7.5, 5.9 Hz, 1H), 3.01 (dd, *J* = 14.0, 5.8 Hz, 1H), 2.88 (dd, *J* = 14.0, 7.6 Hz, 1H), 2.55 (d, *J* = 6.7 Hz, 2H), 1.81 (p, *J* = 6.8 Hz, 2H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 169.19, 145.34, 144.69, 141.05, 128.37, 125.93, 120.16, 116.74, 115.72, 53.53, 35.54, 31.15, 29.50. C₁₆H₁₈N₃O₃, 300.34, found 59.1% yield.

5.2.2.3. *L-Dopa-2-(2-pyridyl)ethylamine hydrochloride (10)*: ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.84 (t, *J* = 5.8 Hz, 1H), 8.73 (d, *J* = 5.6 Hz, 1H), 8.40 (t, *J* = 7.6 Hz, 1H), 8.33 (s, 3H), 7.85 (t, *J* = 6.7 Hz, 1H), 7.81 (d, *J* = 8.0 Hz, 1H), 6.64 (d, *J* = 8.0 Hz, 1H), 6.59 (d, *J* = 2.0 Hz, 1H), 6.41 (dd, *J* = 8.1, 2.0 Hz, 1H), 3.85 – 3.71 (m, 1H), 3.52 (dq, *J* = 12.2, 6.0 Hz, 1H), 3.46 (dq, *J* = 13.2, 6.3 Hz, 1H), 3.14 (hept, *J* = 7.9, 7.1 Hz, 2H), 2.81 (dd, *J* = 13.7, 7.2 Hz, 1H), 2.75 (dd, *J* = 13.7, 6.9 Hz, 1H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 168.31, 154.46, 145.38, 145.17, 144.48, 141.78, 127.42, 125.62, 124.94, 120.27, 116.99, 115.82, 54.00, 38.08, 36.33, 32.96. C₁₈H₂₁N₂O₃, 313.38, found 36.2% yield.

5.2.2.4. *L-Dopa-3-phenyl-1-propylamine hydrochloride (11)*: ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.91 (s, 2H), 8.54 (t, *J* = 5.6 Hz, 1H), 7.26 (t, *J* = 7.6 Hz, 2H), 7.20 – 7.12 (m, 3H), 6.70 – 6.63 (m, 2H), 6.49 (dd, *J* = 8.0, 2.1 Hz, 1H), 3.83 (t, *J* = 7.1 Hz, 1H), 3.35 (dq, *J* = 12.8, 6.7 Hz, 1H), 3.10 - 2.97 (m, 2H) 2.85 (qd, *J* = 13.7, 7.0 Hz, 2H), 1.64 (dhept, *J* = 13.6, 7.0 Hz, 2H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 168.10, 145.18, 144.46, 141.62, 128.40, 128.32, 125.81, 125.77, 120.24, 117.00, 115.65, 54.05, 38.22, 36.70, 32.35, 30.50. C₁₇H₁₉N₂O₃, 299.35, found 35.3% yield.

5.3. Determination of physicochemical and biochemical properties

5.3.1. Materials and instrumentation

UV-Vis spectrophotometry was carried out with a Shimadzu MultiSpec-1501 UV-Vis photodiode array spectrophotometer and a Multiscan RC UV/Vis platereader (Labsystems). Potentiometric titration was carried out with a Hanna HI1230B general purpose electrode and standardised KOH (0.1 N) (Merck). Precoated parallel artificial membrane permeability assay (PAMPA) plate systems were obtained from BD Biosciences while Phree phospholipid removal tubes were from Phenomonex. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and phosphate-buffered saline (PBS) were obtained from

Sigma-Aldrich. Cell culture media and reagents were from Gibco. 24-Well and 96-well plates were from Corning while sterile syringe filters (0.22 μm) were obtained from Pall Corporation Life Sciences.

5.3.2. Shake-flask method for logD determination

LogD values were measured using a scaled-down version of the shake-flask method as recently reported (Strydom *et al.*, 2017). n-Octanol (analytical reagent from Sigma-Aldrich) served as the lipophilic phase while potassium phosphate buffer (100 mM, pH 7.4 and 7.8) or sodium acetate buffer (50 mM, pH 4.7 and 5.7) served as the aqueous phases. A mixture of n-octanol (4 ml), buffer (4 ml) and L-dopa prodrug (2 mM) was shaken by hand for 5 min and centrifuged to separate the phases. After appropriate dilution of the aqueous phase (30-fold), the concentrations of the L-dopa prodrugs were measured by spectrophotometry. The octanol phase was analysed without further dilution. The wavelengths of maximal absorption and molar extinction coefficients of the prodrugs in n-octanol were as follows: **8** (206 nm, 2679 M^{-1}); **9** (206 nm, 2869 M^{-1}); **10** (206 nm, 4677 M^{-1}); **11** (206 nm, 2049 M^{-1}). The wavelengths of maximal absorption and molar extinction coefficients of the prodrugs in aqueous buffer were as follows: **8** (206 nm, 2158 M^{-1}); **9** (206 nm, 2372 M^{-1}); **10** (206 nm, 5401 M^{-1}); **11** (206 nm, 2323 M^{-1}).

5.3.3. Determination of ionisation constant, pKa

The pKa values of the L-dopa prodrugs were measured and calculated according to the method described in literature (Albert, 1984). For this purpose, 10 mM solutions of the L-dopa prodrugs in 10 ml water were prepared and potentiometrically titrated with a standardised solution of KOH (0.1 N). Titration was carried out in 0.1 ml increments and a total of 1 ml KOH was added to fully neutralise the primary aminium of the prodrug **11**, while 2 ml KOH was required to fully neutralise the pyridyl and aminium groups of prodrugs **8–10** (Strydom *et al.*, 2017).

5.3.4. Determination of toxicity towards cultured cells

The toxicity of selected L-dopa prodrugs towards cultured cells was determined with HeLa cells as previously described (Strydom *et al.*, 2017). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum, and on the day before the experiment, were seeded at 500 000 cells/well in 24-well plates. After 24 h incubation, the cells were treated with the test prodrug, dissolved in DMEM (containing no fetal bovine serum). In each 24-well plate, wells were reserved as positive controls (100% cell death via lyses with 0.3% formic acid) and negative controls (0% cell death as a result of no treatment). Treatment lasted for 24 h and after removal of the growth medium and rinsing of the wells with phosphate-

buffered saline (PBS), the 0.5% MTT reagent (200 μ l) was added. The well-plates were incubated at 37 °C for 2 h, the MTT-reagent was aspirated from the wells and isopropanol (250 μ l) was added to each well to dissolve the purple formazan crystals. The absorbance of formazan in each well was measured spectrophotometrically at 560 nm (Brink *et al.*, 2008).

5.3.5. High performance liquid chromatography (HPLC)

Concentration measurements of the L-dopa prodrugs in buffer and biological matrices by HPLC were carried out by the protocol previously described (Strydom *et al.*, 2017). A 1200 series HPLC system equipped with a binary gradient pump, auto sampler and variable wavelength detector was used for the chromatography. A Venusil XBP C18 column (4.60 \times 150 mm, 5 μ m) was used for separation and the mobile phase consisted of 60% sodium acetate buffer (50 mM, pH 4.7) and 40% methanol at a flow rate of 1 ml/min. The injection volume was 20 μ l, and the retention times and wavelengths of detection of the L-dopa prodrugs were as follows: **8** (1.9 min; 256 nm); **9** (2.1 min; 257 nm); **10** (2.6 min; 262 nm); **11** (6.3 min; 257 nm).

5.3.6. Determination of passive diffusion permeability

The degree of passive diffusion permeability of the L-dopa prodrugs was estimated by the PAMPA as previously described (Strydom *et al.*, 2017). The assay buffers for these experiments were potassium phosphate buffer (100 mM, pH 6.4; 7.4 and 7.8) or sodium acetate buffer (50 mM, pH 3.7; 4.7 and 5.7), and the test prodrugs were evaluated at a concentration of 200 μ M. The PAMPA was carried out at room temperature for 5 h and prodrug concentrations were determined using the HPLC system described above (Wohnsland & Faller, 2001).

5.3.7. Determination of chemical stability

The chemical stabilities of the L-dopa prodrugs were estimated by the previously described protocol (Strydom *et al.*, 2017). Solutions of the L-dopa prodrugs (10–150 μ M) were prepared in potassium phosphate buffer (100 mM, pH 6.4; 7.4 and 7.8) or sodium acetate buffer (50 mM, pH 3.7; 4.7 and 5.7) and immediately analysed by the HPLC method described above. The first injection served as reference point, after which the same solution was again injected at 1 h, 3 h, 6 h, 9 h and 11 h.

5.3.8. Determination of plasma and tissue stability

The plasma and tissue stabilities of the L-dopa prodrugs were estimated by the previously described protocol (Strydom *et al.*, 2017). The L-dopa prodrugs (approximately 50–100 μ M) were added to plasma or tissue homogenate (preheated to 37 °C) and the resulting mixtures

was analysed by the HPLC method described above. For this purpose, 100 µl of the spiked plasma or tissue homogenate was added to 300 µl acetonitrile (containing formic acid 1%) in a Phree tube. The tube was vortexed, filtered under vacuum and the effluent was collected and evaporated under a stream of air. The residue was redissolved in methanol (100 µl) and water (300 µl), and analysed by HPLC. The first injection occurred at 5 min after preparation of the spiked plasma sample, after which aliquots of the same plasma sample or tissue homogenate was again subjected to the Phree work-up and injected at various time points while the plasma solution and tissue homogenate were maintained at 37 °C.

5.3.9. Ethics consideration

Animals were bred, supplied and housed at the Vivarium (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019) of the Preclinical Drug Development Platform of the Potchefstroom campus of the North-West University (NWU). Experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) at 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 (ethics approval numbers: NWU-00326-15-A5; NWU-00056-11-S5).

5.4. Results

5.4.1. Synthesis of the L-dopa prodrugs

The synthesis of the L-dopa prodrugs (**8–11**) was carried out according to the literature protocol (Nakonieczna *et al.*, 1994; Strydom *et al.*, 2017). Employing this protocol, literature reports the synthesis of an L-dopa-lazabemide prodrug and L-dopa dipeptides with the amino acids, leucine, phenylalanine and valine conjugated to the carboxylate of L-dopa (Fig. 5.4) (Nakonieczna *et al.*, 1994; Strydom *et al.*, 2017). In the first step, L-dopa is protected at the catechol OH groups using TBDMS-Cl in the presence of DBU to yield L-dopa(TBDMS)₂ (**12**) (Fig. 5.5). In the subsequent step the aminium group of **12** is protected using Boc₂O to yield Boc-L-dopa(TBDMS)₂ (**13**). The carrier molecules, 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine, were subsequently conjugated to the protected L-dopa (**13**) in the presence of BOP and NMM as base to give the protected L-dopa prodrugs (**14–17**). The removal of the TBDMS and Boc protective groups from the protected L-dopa prodrugs was facilitated by HCl (4 N in dioxane) to yield the final L-dopa prodrugs, **8–11** in yields of 35.3-69.26% (final step). The structures of **8–11** were confirmed by NMR and MS analysis as cited in the experimental section.

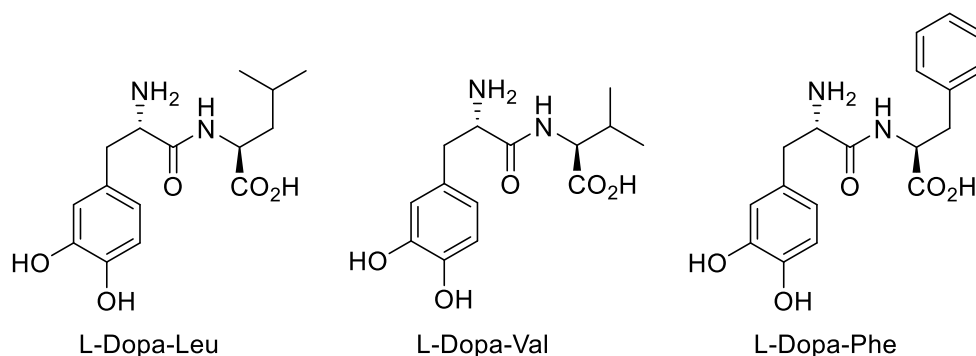


Figure 5.4: L-Dopa dipeptides with the amino acids, leucine, valine and phenylalanine (Nakonieczna *et al.*, 1994).

In both the ^1H NMR and ^{13}C NMR spectra, the appropriate signals were observed for the proposed prodrugs. In the ^{13}C NMR spectrum, the amount of signals and their chemical shifts are in correspondence to what is expected for the structures of the prodrugs. In particular, in the ^{13}C NMR, the carbonyl carbon (C10) is represented by a signal at 168–169 ppm. Employing DEPT 135°, the methylene (CH_2), methene (CH) and quaternary carbons (C) could be assigned. These assignments are tabulated in the supplementary material. In the ^1H NMR spectrum, the amount of signals, their integration values, multiplicities and their chemical shifts are in correspondence to what is expected. These include, the CH_2 protons (C7) of the L-dopa moiety at 2.75–2.88 and 2.81–3.01 ppm (1H for each signal), and the signal of the CH α -carbon (C8) at 3.80–4.11 ppm (1H). The aromatic L-dopa protons (C1, C4 and C6) correspond to the doublet of doublets at 6.41–6.50 ppm (C6, 1H) and doublets at 6.64–6.70 ppm (C1, 1H) and 6.59–6.66 ppm (C4, 1H) (Nakonieczna *et al.*, 1994). For the carrier moieties, the signals of the CH_2 (**8**), $\text{CH}_2\text{-CH}_2$ (**9**, **10**) and $(\text{CH}_2)_3$ (**11**) correspond to the signals at 1.64–4.60 ppm. The aromatic protons of the pyridyl and phenyl rings of the carrier moieties, are represented by signals at 7.11–8.75 ppm. Lastly, the exchangeable protons (NH, NH_3^+ and OH groups) are represented by, for the most part, broad signals as cited in the supplementary material. Mass spectrometry shows that the calculated masses of the prodrugs correspond well to the experimentally determined masses.

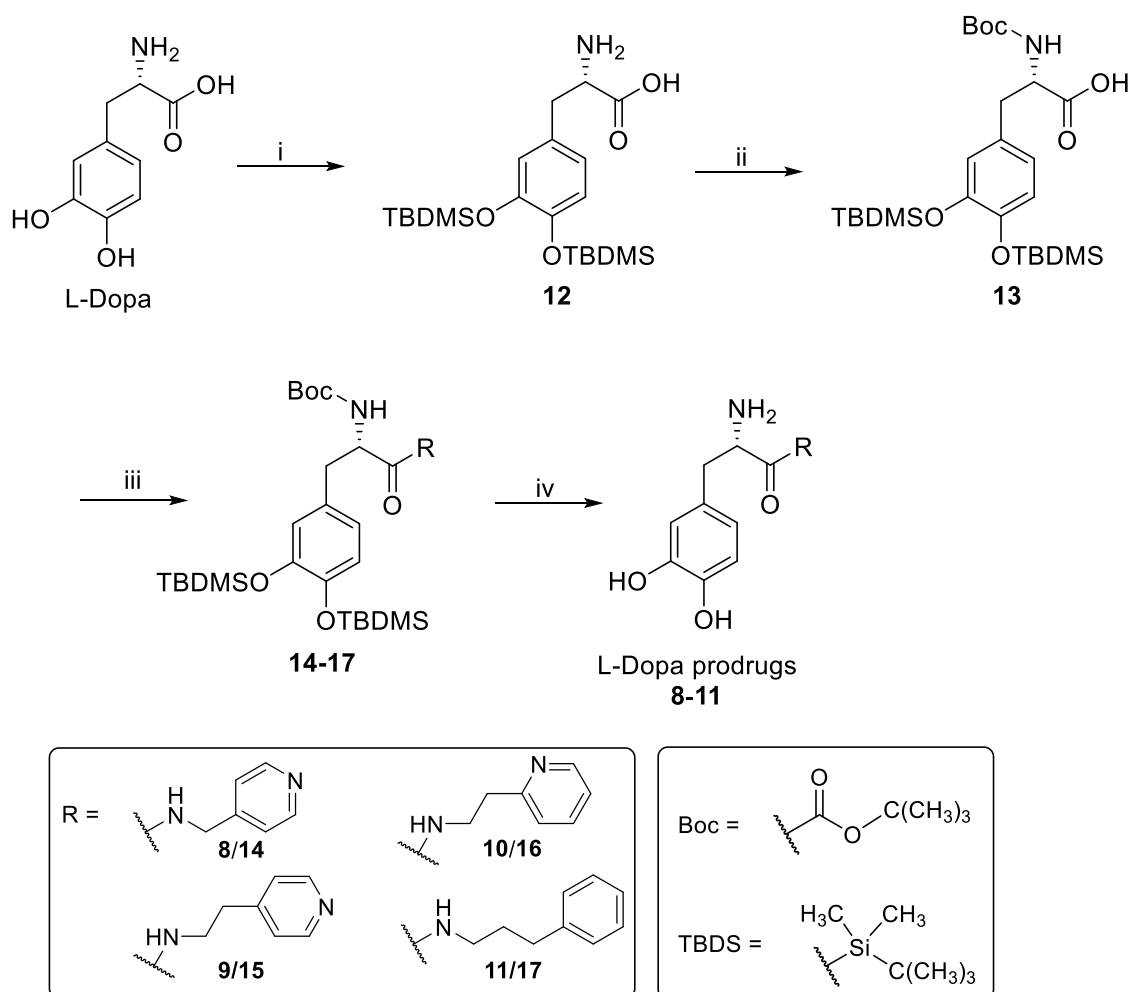


Figure 5.5: The protection of L-dopa and the synthesis of the L-dopa prodrugs, **8–11**. Key: (i) rt, 18 h, DBU, TBDMS-Cl; (ii) THF/H₂O, NaHCO₃, di-tert-butyl dicarbonate (Boc₂O); (iii) amine, BOP, NMM, CH₂Cl₂, 24 h; (iv) 4 N HCl (dioxane); 48 h, 55 °C.

5.4.2. Physicochemical properties

5.4.2.1. LogD

The logD values of the L-dopa prodrugs are tabulated in table 5.1. It is evident that the prodrugs are hydrophilic and all compounds exhibit logD < 0 at the pH values evaluated. As expected, the pyridyl containing prodrugs (**8–10**) are more hydrophilic compared to the phenyl derivative **11**. The highly hydrophilic nature of these prodrugs may limit passive diffusion permeability and thus absorption from the gastrointestinal tract and penetration of the blood-brain barrier (Kerns & Di, 2008). In general, higher bioavailability and brain exposure will be obtained for more lipophilic compounds, with the optimal logP range of 0–3 (Kerns & Di, 2008).

Table 5.1: The logD values of the L-dopa prodrugs (**8–11**) at different pH values.

pH value	8	9	10	11
4.7	-2.50 ± 0.026	-1.54 ± 0.066	-2.87 ± 0.307	-1.08 ± 0.045
5.7	-1.74 ± 0.510	-1.43 ± 0.038	-2.48 ± 0.149	-0.60 ± 0.046
7.4	-1.52 ± 0.050	-0.94 ± 0.044	-1.09 ± 0.017	-0.60 ± 0.390
7.8	-1.47 ± 0.030	-0.83 ± 0.101	-1.11 ± 0.043	-0.32 ± 0.223

Values are given as mean \pm standard deviation of triplicate determinations

5.4.2.2. Ionisation constant

The pKa values of the L-dopa prodrugs are given in table 5.2. The values show that the pyridyl nitrogen of **8–10** is weakly basic with pKa values of 5.06–5.90. At physiological pH 7.4, the pyridyl moieties are thus not expected to be charged to a large degree (<1% protonated). The amine groups of the prodrugs **8–11**, in contrast, display high pKa values of 7.45–10.1, and at physiological pH 7.4, will be protonated (53–99.8%). The charged nature of these prodrugs explains their high degree of hydrophilicity and is expected to further limit absorption and blood-brain barrier permeation by passive diffusion. For prodrug **11**, the least basic compound of the series, the degree of ionisation will be approximately 53%, with 47% existing as the neutral uncharged species. Since the neutral species should display better membrane permeability than the ionised species, a relatively large fraction of this prodrug is available for passive diffusion. This result suggests that ionisation of prodrug **11** at pH 7.4 should represent a weaker barrier for permeation through the blood-brain barrier compared to prodrugs **8–10**. At the pH values found in the gastrointestinal tract (for example pH 4–5), however, all four prodrugs will exist as the charged species. For the prodrugs **8–10** the pyridyl nitrogen will also be charged to some degree in the gastrointestinal tract (92–98% at pH 4), which will further limit absorption into the systemic circulation.

Table 5.2: The ionisation constants (pKa) of the L-dopa prodrugs (**8–11**)

	pKa (pyridyl)	pKa (amine)
8	5.32 ± 0.406	8.95 ± 0.26
9	5.90 ± 0.379	10.1 ± 0.356
10	5.06 ± 0.339	8.51 ± 0.207
11	–	7.45 ± 0.214

Values are given as mean \pm standard deviation of triplicate determinations

5.4.2.3. Cell viability

The toxicity of selected L-dopa prodrugs, compounds **10** and **11**, towards cultured HeLa cells were determined. For this purpose the MTT cell viability assay was used (Mosmann, 1983; Brink *et al.*, 2008). The cells were exposed to the compounds at 1 μ M, 10 μ M and 100 μ M for a period of 24 h and the viable cells remaining were estimated via the MTT assay. The results are given in table 5.3 as the percentage viable cells after 24 h treatment. For comparison the viability after treatment with L-dopa is also given.

At a concentration of 1 μ M, neither L-dopa nor prodrug **11** exhibit toxicity for the cultured cells. At this concentration, prodrug **10** does, however, reduce viability to 72.5%. At a concentration of 10 μ M, both prodrugs **10** and **11** exhibit some degree of toxicity with the viabilities at 73.1% and 71.4%, respectively, while L-dopa remains non-toxic. At 100 μ M, both prodrugs and L-dopa are toxic to the cultured cells. These results indicate that both L-dopa prodrugs are significantly more toxic for cultured cells at 10 μ M than L-dopa. A possible explanation for the higher toxicity of the L-dopa prodrugs for cultured cells compared to L-dopa is that the prodrugs may possess higher membrane permeabilities than L-dopa, and thus reach higher intracellular concentrations. This greater degree of intracellular exposure may lead to higher toxicity.

Table 5.3: The percentage viable cells remaining after treatment with L-dopa and the L-dopa prodrugs (**10** and **11**).

	Concentration of the test drug		
	1 μ M	10 μ M	100 μ M
L-Dopa	102 \pm 4.19	105 \pm 5.24	79.3 \pm 13.0
10	72.5 \pm 3.29	73.1 \pm 2.91	68.7 \pm 11.1
11	100.1 \pm 14.4	71.4 \pm 1.25	64.1 \pm 7.98

Values are given as mean (percentage) \pm standard deviation of triplicate determinations

5.4.2.4. Passive diffusion permeability

The permeabilities of the L-dopa prodrugs as well as the carrier molecules were evaluated by the PAMPA. The results are given in table 5.4 and shows that L-dopa displays poor permeability at all pH values with LogP_e values smaller than -7.99. Higher LogP_e values (less negative) are indicative of increased permeability. At all pH values evaluated, the four prodrugs, **8–11**, exhibit improved permeability compared to L-dopa. For example, at pH 7.4 the LogP_e value of prodrug **11** is -5.85, which shows that **11** is more than 100-fold more permeable at this pH than L-dopa. Among the prodrugs, **11** possesses the best permeability profile. Interestingly, the permeabilities of the prodrugs do not vary much with pH, which

suggests that the degree of ionisation does not affect passive diffusion in the PAMPA to a significant degree.

The permeabilities of three carrier molecules, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine were also evaluated in the PAMPA. 2-(2-Pyridyl)ethylamine was found to be the most permeable among the carriers with a LogP_e value at pH 7.4 of -4.61 . This is in similar range to compounds such as propranolol, which is considered to have good permeability ($\text{LogP}_e = 4.4$ at pH 6.8) (Wohnsland & Faller, 2001). This carrier is also more permeable than its corresponding prodrug, compound **10**. Interestingly, 2-(4-pyridyl)ethylamine and 3-phenyl-1-propylamine possess similar permeabilities compared to their corresponding prodrugs, compounds **9** and **11**.

Table 5.4: The permeability (P_e) of L-dopa, the L-dopa prodrugs (**8–11**) and the carrier molecules at selected pH values.

	Permeability (cm/s) expressed as LogP_e					
	pH 3.7	pH 4.7	pH 5.7	pH 6.4	pH 7.4	pH 7.8
L-Dopa	-7.99 ± 0.28	-8.05 ± 0.19	-8.22 ± 0.14	-8.62 ± 0.87	-8.17 ± 0.56	-8.56 ± 0.07
8	-6.26 ± 0.17	-6.42 ± 0.05	-6.48 ± 0.004	-6.41 ± 0.02	-6.26 ± 0.003	-6.38 ± 0.01
9	-6.39 ± 0.32	-7.00 ± 0.02	ND	ND	-7.24 ± 0.01	-7.15 ± 0.01
10	ND	-7.31 ± 0.01	-7.42 ± 0.01	-7.48 ± 0.01	-7.39 ± 0.01	-7.41 ± 0.02
11	-5.90 ± 0.32	-6.08 ± 0.41	-5.92 ± 0.32	ND	-5.85 ± 0.26	-5.89 ± 0.28
4-PyrEA	-6.78 ± 0.19	-7.01 ± 0.05	-6.89 ± 0.04	-7.00 ± 0.01	-6.77 ± 0.06	-6.67 ± 0.05
2-PyrEA	-4.40 ± 0.11	-4.41 ± 0.06	-4.59 ± 0.25	-4.56 ± 0.10	-4.61 ± 0.05	-4.65 ± 0.04
PhePA	ND	-6.01 ± 0.02	-5.67 ± 0.01	-5.96 ± 0.01	-5.52 ± 0.01	-5.36 ± 0.03

Values are given as mean \pm standard deviation of triplicate determinations

ND, not determined

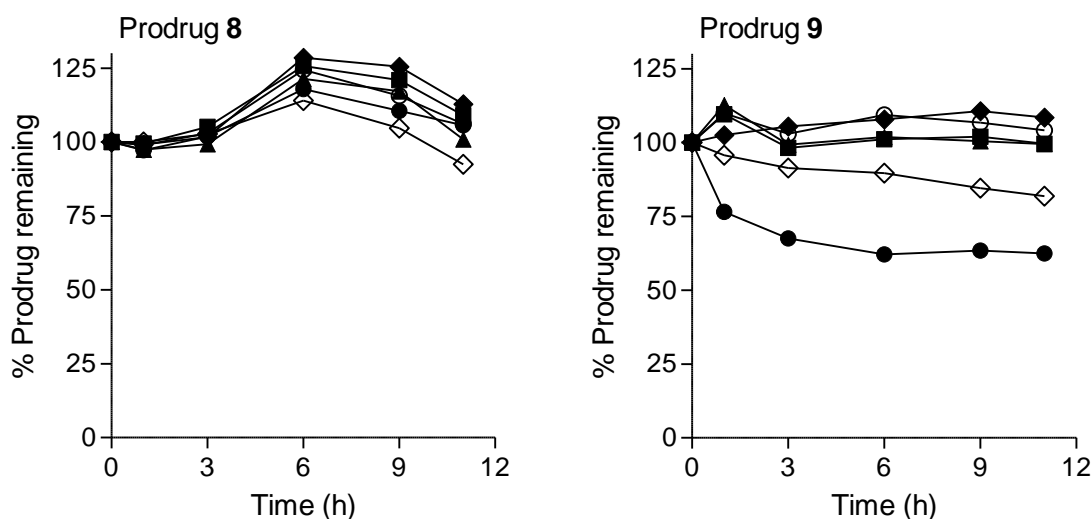
4-PyrEA, 2-(4-pyridyl)ethylamine; 2-PyrEA, 2-(2-pyridyl)ethylamine; PhePA, 3-phenyl-1-propylamine

5.4.2.5. Chemical stability

The chemical stability of the L-dopa prodrugs was determined in aqueous buffer at pH 3.7–7.8. These measurements were carried out to determine if the prodrug may undergo non-enzymatic chemical transformations such as hydrolysis or oxidation. Chemical instability of the prodrugs is undesirable since this may indicate that the prodrugs will not be orally

bioavailable and may undergo chemical breakdown in the gastrointestinal tract. Alternatively, chemical hydrolysis may activate the prodrugs prior to absorption, thus limiting their entry into the systemic circulation. Rapid chemical hydrolysis in the systemic circulation is also undesirable since this would reduce exposure time of the prodrug to the blood-brain barrier, and thus limit penetration into the brain.

The results of the chemical hydrolysis study are given in fig. 5.6. The results show that prodrug **8** is stable at all pH values with no decrease of the peak area and concentration of the prodrug (up to 11 h). Prodrug **9**, on the other hand, shows a time-dependent decrease in concentration at pH 3.7 and 7.8. At 11 h after the preparation of the solution, the prodrug concentration has decreased to 62.4% at pH 3.7 and 81.1% at pH 7.8. At the other pH values no significant decrease in concentration was observed. For prodrug **10**, a time-dependent decrease in concentration at pH 7.8 was observed and 11 h after the preparation of the solution, the prodrug concentration has decreased to 84.1%. Similarly, prodrug **11** also undergoes a time-dependent decrease in concentration at pH 7.8 with the prodrug concentration at 47.1%, 11 h after the preparation of the solution. At the other pH values evaluated, **10** and **11** did not exhibit a decrease in concentration.



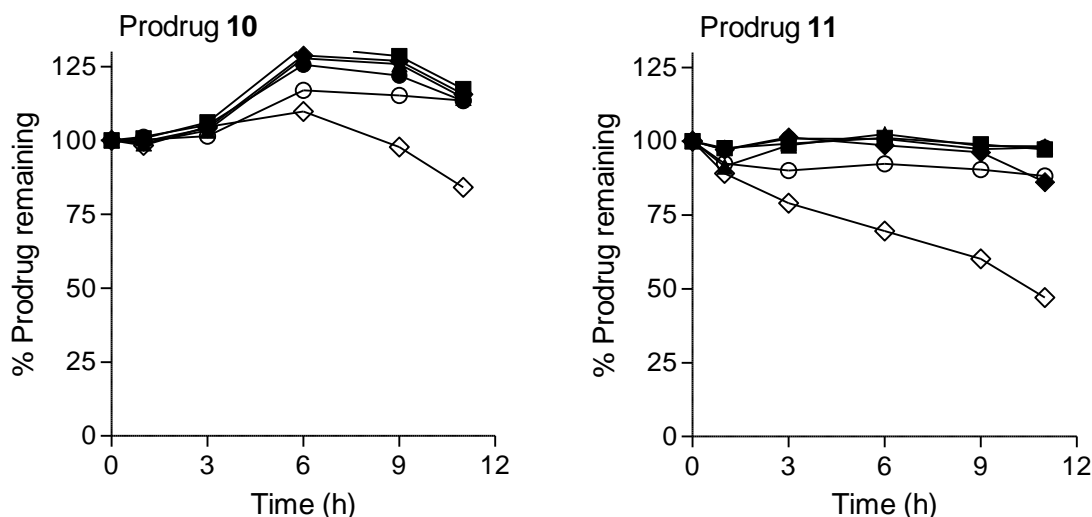


Figure 5.6: The chemical stability of the L-dopa prodrugs (**8–11**) at different pH values. Key: pH 3.7 (filled circles); pH 4.7 (open circles); pH 5.7 (triangles); pH 6.4 (squares); pH 7.4 (filled diamonds); pH 7.8 (open diamonds). Values are given as mean (percentage) \pm standard deviation of triplicate determinations.

5.4.2.6. Plasma and tissue stability

While prodrugs of L-dopa should be stable in aqueous solution, the prodrugs should undergo activation in plasma or tissue. To establish whether the prodrugs are activated in plasma and tissue, this study examined the stability of L-dopa prodrugs **8–11** in rat plasma, and rat brain and liver homogenates.

To evaluate plasma stability, the L-dopa-lazabemide prodrugs were added to freshly collected rat plasma and incubated at 37 °C. At various time points after addition, samples were taken and the residual concentrations of the prodrugs were measured. The results show that prodrug **8** is stable in rat plasma with no significant decrease in concentration after 17 h of incubation (Fig. 5.7). In contrast, **9–11** display a time-dependent decrease in concentration. This is particularly well illustrated by prodrug **10**, with a gradual decrease in concentration from 21.2 μ M to 4.9 μ M at 17 h. Similarly, for prodrug **11**, the concentration in the plasma decreases from 24.5 μ M to 5.7 μ M at 17 h. Although this study has not measured the formation of the hydrolysis products, L-dopa and the carrier molecules, it may be speculated that the observed decrease in concentrations in the plasma is due to hydrolysis.

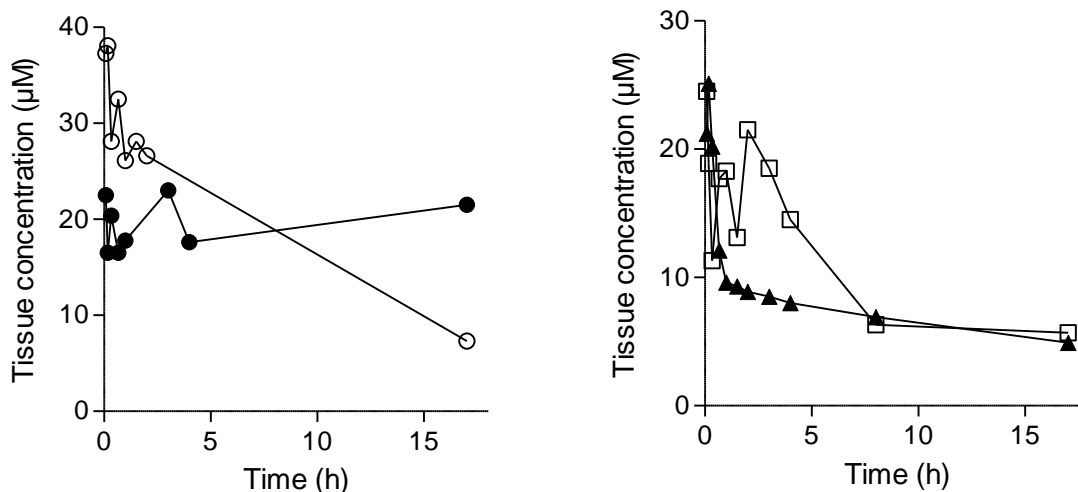


Figure 5.7: The metabolic stability of the L-dopa prodrugs in rat plasma. Key: **8** (filled circles); **9** (open circles); **10** (triangles); **11** (squares). Values are given as mean (μM) \pm standard deviation of triplicate determinations.

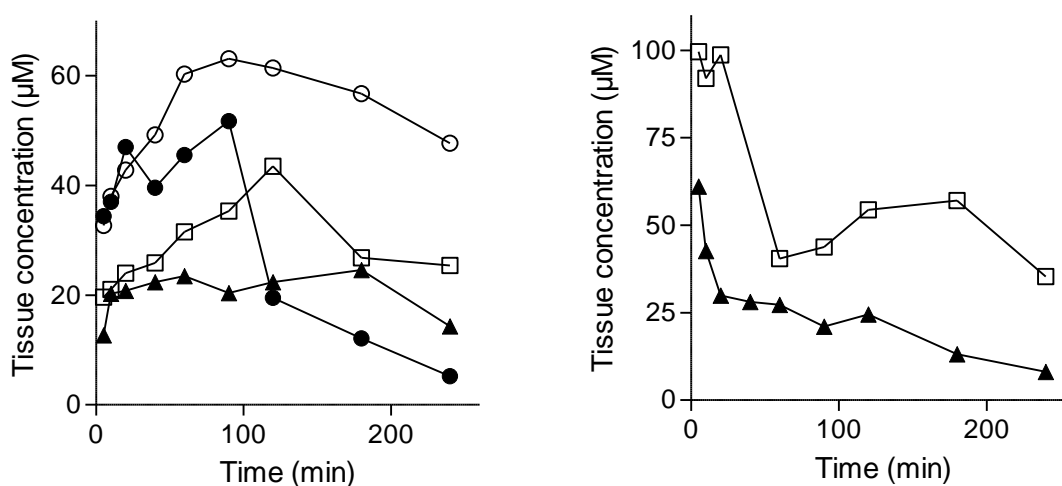


Figure 5.8: The metabolic stability of the L-dopa prodrug in rat brain (left) and liver (right) homogenates. Key: **8** (filled circles); **9** (open circles); **10** (triangles); **11** (squares). Values are given as mean (μM) \pm standard deviation of triplicate determinations.

To evaluate tissue stability of the L-dopa-lazabemide prodrugs, they were added to freshly homogenised rat brain and liver tissue and incubated at 37 °C. At various time points after addition, samples were taken and the residual concentrations of the prodrugs were measured. The results show that much variability in the concentration data exist, probably due to nonspecific binding of the prodrugs to the tissue components (Fig. 5.8). Due to the high variability of the concentration data for prodrugs **8** and **9**, the results obtained with liver

homogenate are not presented. In spite of these difficulties, a clear time-dependent decrease in concentration of prodrugs **10** and **11** is observed in liver tissue homogenate. For prodrug **10**, the concentration in the liver homogenate decreases from 61.1 μM to 8.1 μM at 240 min, while for **11**, the concentration in the liver homogenate decreases from 99.6 μM to 35.3 μM at 240 min. In the brain homogenate, only prodrug **8** displays a clear decrease in concentration with time. For prodrug **8** the concentration in the brain homogenate decreases from 34.4 μM to 5.2 μM at 240 min. As in plasma, the formation of the hydrolysis products, L-dopa and the carrier molecules, were not measured, but it may be speculated that the observed decrease in concentrations in the tissue homogenates is due to hydrolysis.

5.5. Discussion and conclusion

The present study investigated key physicochemical and biochemical parameters of four carrier-linked prodrugs of L-dopa in which 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine were linked to the carboxylate of L-dopa. The objective with this approach was to discover prodrugs with improved oral absorption, metabolic stability and brain delivery compared to L-dopa. The results document that all the prodrugs are highly hydrophilic, which may limit membrane permeability and thus absorption from the gastrointestinal tract and penetration of the blood-brain barrier. The hydrophilicity of the prodrugs may, at least in part, be attributed to protonation of the amine groups and to a lesser extent protonation of the pyridyl moieties (at lower pH values). In spite of this, the four prodrugs exhibit improved permeability compared to L-dopa, with especially prodrug **11** being more than 100-fold more permeable than L-dopa at certain pH values. Notably, this prodrug does not contain the pyridyl moiety and thus has one less ionisation centre compared to the other prodrugs evaluated and is also the least hydrophilic. The observation that the prodrugs are more permeable compared to L-dopa is supported by the finding that two prodrugs evaluated are more toxic than L-dopa to cultured cells, possibly due to higher membrane permeabilities which would result in higher intracellular concentrations. Chemical stability experiments show that none of the prodrugs are labile at several pH values, and that the amide link between L-dopa and the carrier molecules is relatively stable to chemical hydrolysis. In contrast, in plasma three of the prodrugs (**9–11**) undergo a time-dependent decrease in concentration, probably due to enzymatic hydrolysis and activation of the prodrugs. Prodrug **8**, however, appears to be stable in plasma. In liver tissue, the prodrugs (as shown with **10** and **11**) also display a time-dependent decrease in concentration, while notably in the brain tissue, only **8** undergoes a time-dependent decrease in concentration.

In conclusion, these data suggest that the prodrug approach may indeed enhance the permeability of L-dopa, yielding compounds that are stable towards chemical hydrolysis. As

exemplified by **8**, certain prodrugs may also be stable towards plasma hydrolysis while readily undergoing activation in brain tissue. With such a property profile, compound **8** would in theory undergo limited peripheral hydrolysis thus allowing more time for the prodrug to penetrate into the brain tissue. Once in the brain, the prodrug may undergo the necessary activation to deliver L-dopa in increased concentrations and possibly for a sustained period of time.

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Conflict of interest

The authors declare no conflicts of interest in this work.

References

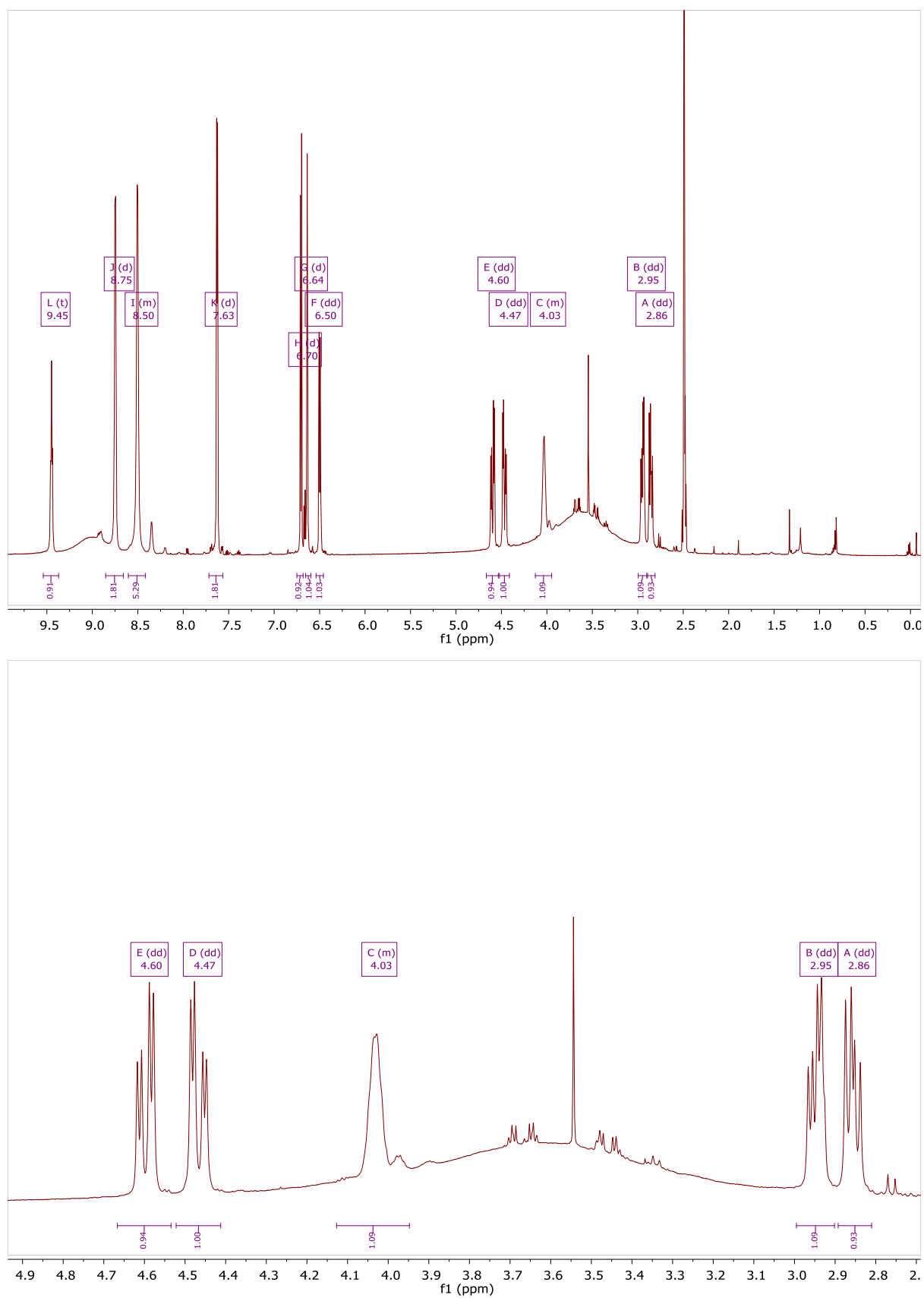
1. Albert, A. & Serjeant, E.P. 1984. The determination of ionization constants: a laboratory manual. 3rd ed. London: Chapman and Hall. pp. 176-191.
2. Brink, C.B., Pretorius, A., van Niekerk, B.P., Oliver, D.W. & Venter, D.P. 2008. Studies on cellular resilience and adaptation following acute and repetitive exposure to ozone in cultured human epithelial (HeLa) cells. *Redox Report*, 13(2):87-100.
3. Camargo, S.M., Vuille-dit-Bille, R.N., Mariotta, L., Ramadan, T., Huggel, K., Singer, D., Götze, O. & Verrey, F. 2014. The molecular mechanism of intestinal levodopa absorption and its possible implications for the treatment of PD. *Journal of pharmacology and experimental therapeutics*, 351(1):114-123.
4. Chen, I.J., Taneja, R., Yin, D., Seo, P.R., Young, D., MacKerell, A.D. & Polli, J.E. 2006. Chemical substituent effect on pyridine permeability and mechanistic insight from computational molecular descriptors. *Molecular pharmaceutics*, 3(6):745-755.
5. Cingolani, G.M., Di Stefano, A., Mosciatti, B., Napolitani, F., Giorgioni, G., Ricciutelli, M. & Claudi, F. 2000. Synthesis of L-(+)-3-(3-hydroxy-4-pivaloyloxybenzyl)-2,5-diketomorpholine as potential prodrug of L-dopa. *Bioorganic & medicinal chemistry letters*, 10(12):1385-1388.
6. Contin, M. & Martinelli, P. 2010. Pharmacokinetics of levodopa. *Journal of neurology*, 257(2):253-261.
7. Contin, M., Riva, R., Martinelli, P., Procaccianti, G., Cortelli, P., Avoni, P. & Baruzzi, A. 1990. Response to a standard oral levodopa test in parkinsonian patients with and without motor fluctuations. *Clinical neuropharmacology*, 13(1):19-28.
8. Di Stefano, A., Sozio, P. & Cerasa, L.S. 2008. Antiparkinson prodrugs. *Molecules*, 13(1):46-68.
9. Di Stefano, A., Sozio, P., Cerasa, L.S. & Iannitelli, A. 2011. L-Dopa prodrugs: an overview of trends for improving PD treatment. *Current pharmaceutical design*, 17(32):3482-3493.
10. Di Stefano, A., Sozio, P., Iannitelli, A., Cocco, A., Orlando, G. & Ricciutelli, M. 2006. Synthesis and preliminary evaluation of Ldopa/benserazide conjugates as dual acting codrugs. *Letters in drug design and discovery*, 3(10):747-752.
11. Freitas, M.E., Ruiz-Lopez, M. & Fox, S.H. 2016. Novel levodopa formulations for PD. *CNS Drugs*, 30(11):1079-1095.
12. Gleeson, M.P. 2008. Generation of a set of simple, interpretable ADMET rules of thumb. *Journal of medicinal chemistry*, 51(4):817-834.
13. Hauser, R.A., Hsu, A., Kell, S., Espay, A.J., Sethi, K., Stacy, M., Ondo, W., O'Connell, M. & Gupta, S. 2013. IPX066 advance-PD investigators. Extended-release carbidopa-levodopa (IPX066) compared with immediate-release carbidopa-

- levodopa in patients with PD and motor fluctuations: a phase 3 randomised, double-blind trial. *Lancet neurology*, 12(4):346-356.
14. Hornykiewicz, O. 2010. A brief history of levodopa. *Journal of neurology*, 257(2):249-252.
 15. Kerns, E. H. & Di, L. 2008. Drug-like properties: concepts, structure design and methods: from ADME to toxicity optimization. Amsterdam: Academic Press.
 16. Khor, S.P. & Hsu, A. 2007. The pharmacokinetics and pharmacodynamics of levodopa in the treatment of PD. *Current clinical pharmacology*, 2(3):234-243.
 17. Learmonth, D.A., Palma, P.N., Vieira-Coelho, M.A. & Soares-da-Silva, P. 2004. Synthesis, biological evaluation, and molecular modeling studies of a novel, peripherally selective inhibitor of catechol-O-methyltransferase. *Journal of medicinal chemistry*, 47(25):6207-6217.
 18. Leppänen, J., Huuskonen, J., Nevalainen, T., Gynther, J., Taipale, H. & Järvinen, T. 2002. Design and synthesis of a novel L-dopa-entacapone codrug. *Journal of medicinal chemistry*, 45(6):1379-1382.
 19. LeWitt, P.A., Ellenbogen, A., Chen, D., Lal, R., McGuire, K., Zomorodi, K., Luo, W. & Huff, F.J. 2012. Actively transported levodopa prodrug XP21279: a study in patients with parkinson disease who experience motor fluctuations. *Clinical neuropharmacology*, 35(3):103-110.
 20. LeWitt, P.A., Hauser, R.A., Grosset, D.G., Stocchi, F., Saint-Hilaire, M.H., Ellenbogen, A., Leinonen, M., Hampson, N.B., DeFeo-Fraulini, T., Freed, M.I. & Kieburtz, K.D. 2016. A randomized trial of inhaled levodopa (CVT-301) for motor fluctuations in PD. *Movement disorders*, 31(9):1356-1365.
 21. Manallack, D.T., Prankerd, R.J., Yuriev, E., Oprea, T.I. & Chalmers, D.K. 2013. The significance of acid/base properties in drug discovery. *Chemical society reviews*, 42(2):485–496.
 22. Mosmann, T. 1983. Rapid colourimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, 65(1-2):55-63.
 23. Nakonieczna, L., Przychodzeń, W. & Chimiak, A. 1994. A New convenient route for the synthesis of DOPA peptides. *Liebigs Annalen der chemie*, 1994:1055-1058.
 24. Nissinen, E., Lindén, I.B., Schultz, E. & Pohto, P. 1992. Biochemical and pharmacological properties of a peripherally acting catechol-O-methyltransferase inhibitor entacapone. *Naunyn-Schmiedeberg's archives of pharmacology*, 346(3):262-266.
 25. Nutt, J.G. 2000. Effect of COMT inhibition on the pharmacokinetics and pharmacodynamics of levodopa in parkinsonian patients. *Neurology*, 55(4):33-37.

26. Nutt, J.G. & Fellman, J.H. 1984. Pharmacokinetics of levodopa. *Clinical neuropharmacology*, 7(1):35-49.
27. Olanow, C.W., Kieburtz, K., Odin, P., Espay, A.J., Standaert, D.G., Fernandez, H.H., Vanaganas, A., Othman, A.A., Widnell, K.L., Robieson, W.Z., Pritchett, Y., Chatamra, K., Benesh, J., Lenz, R.A. & Antonini, A. 2014. LCIG Horizon Study Group. Continuous intrajejunal infusion of levodopa-carbidopa intestinal gel for patients with advanced PD: a randomised, controlled, double-blind, double-dummy study. *Lancet Neurology*, 13(2):141-149.
28. Pinnen, F., Cacciatore, I., Cornacchia, C., Sozio, P., Iannitelli, A., Costa, M., Pecci, L., Nasuti, C., Cantalamessa, F. & Di Stefano, A. 2007. Synthesis and study of L-dopa-glutathione codrugs as new anti-parkinson agents with free radical scavenging properties. *Journal of medicinal chemistry*, 50(10):2506-2515.
29. Poewe, W. & Antonini, A. 2015. Novel formulations and modes of delivery of levodopa. *Movement disorder*, 30(1):114-120.
30. Savolainen, J., Leppanen, J., Forsberg, M., Taipale, H., Nevalainen, T., Huuskonen, J., Gynther, J., Mannisto, P.T. & Jarvinen, T. 2000. Synthesis and in vitro/in vivo evaluation of novel oral N-alkyl- and N,N-dialkyl-carbamate esters of entacapone. *Life science* 67(2):205-216.
31. Seeberger, L.C. & Hauser, R.A. 2015. Carbidopa levodopa enteral suspension. *Expert opinion pharmacotherapy*, 16(18):2807-2817.
32. Strydom, M., Petzer, J.P. & Petzer, A. 2017. An investigation of the in vitro reversibility of MAO inhibition by lazabemide. In preparation.
33. Verhagen Metman, L., Stover, N., Chen, C., Cowles, V.E. & Sweeney, M. 2015. Gastroretentive carbidopa/levodopa, DM-1992, for the treatment of advanced PD. *Movement disorders*, 30(9):1222-1228.
34. Wang, H., Lee, J., Tsai, M., Lu, H. & Hsu, W. 1995. Synthesis and pharmacological activities of a novel tripeptide mimetic DA prodrug. *Bioorganic & medicinal chemistry letters*, 5(19):2195-2198.
35. Wohnsland, F. & Faller, B. 2001. High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. *Journal of medicinal chemistry*, 44(6):923-930.

Supplementary material

Figure S5.1. ¹H NMR spectra of L-dopa prodrug **8**



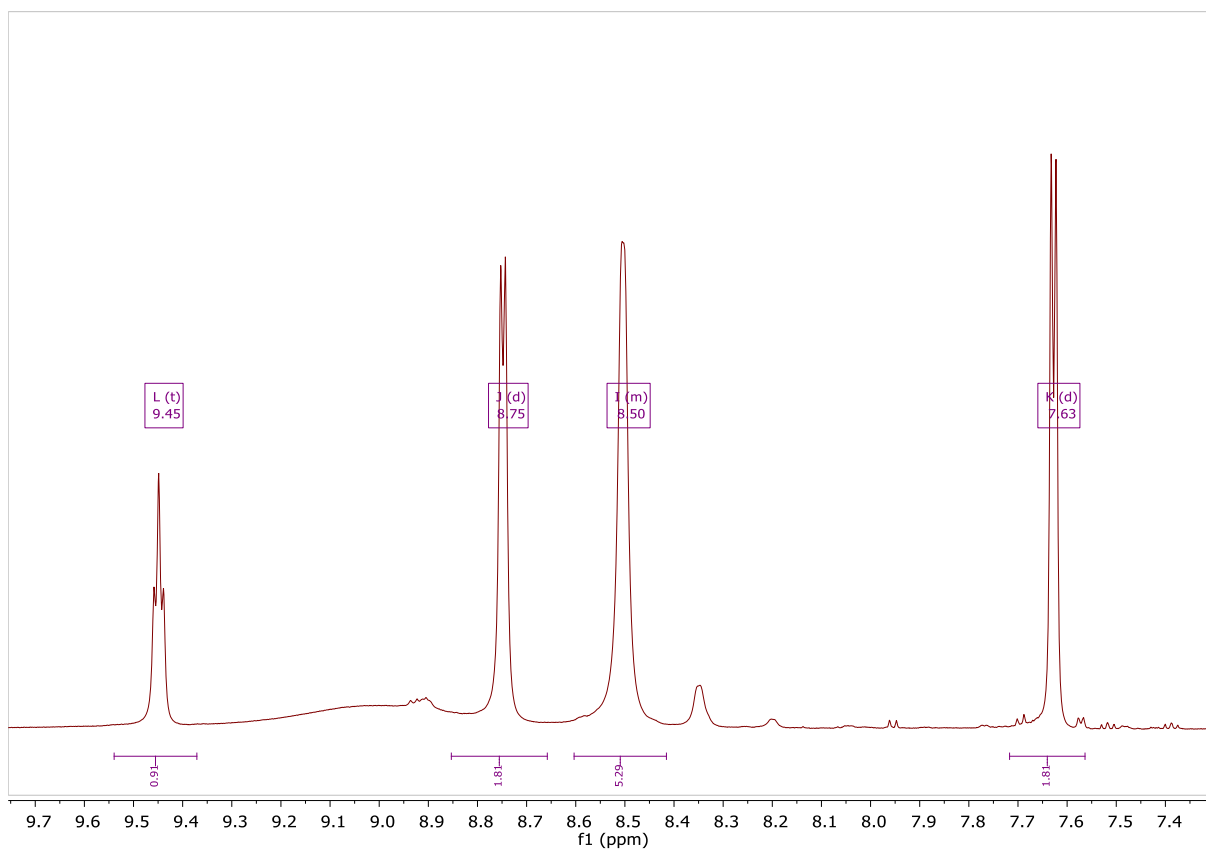
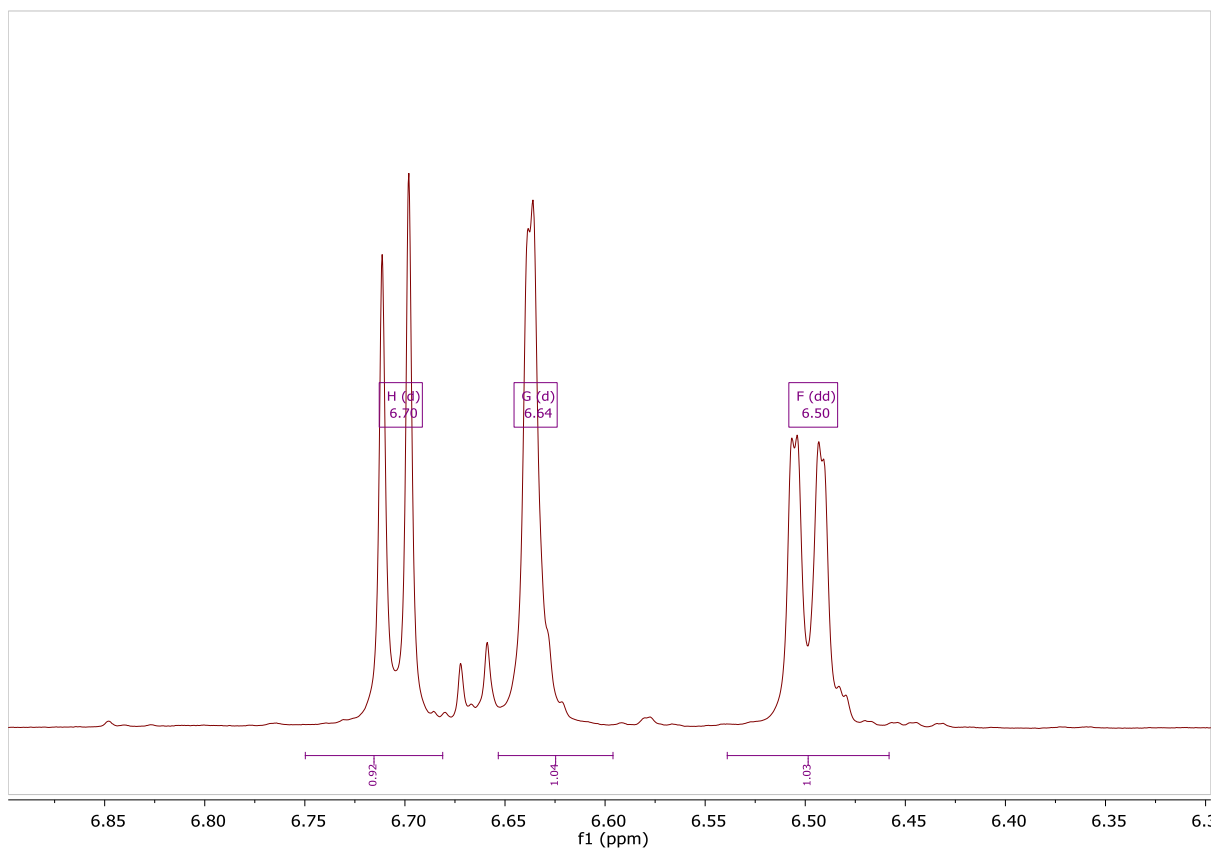


Figure S5.2. ^{13}C NMR and DEPT-135 spectra of L-dopa prodrug **8**

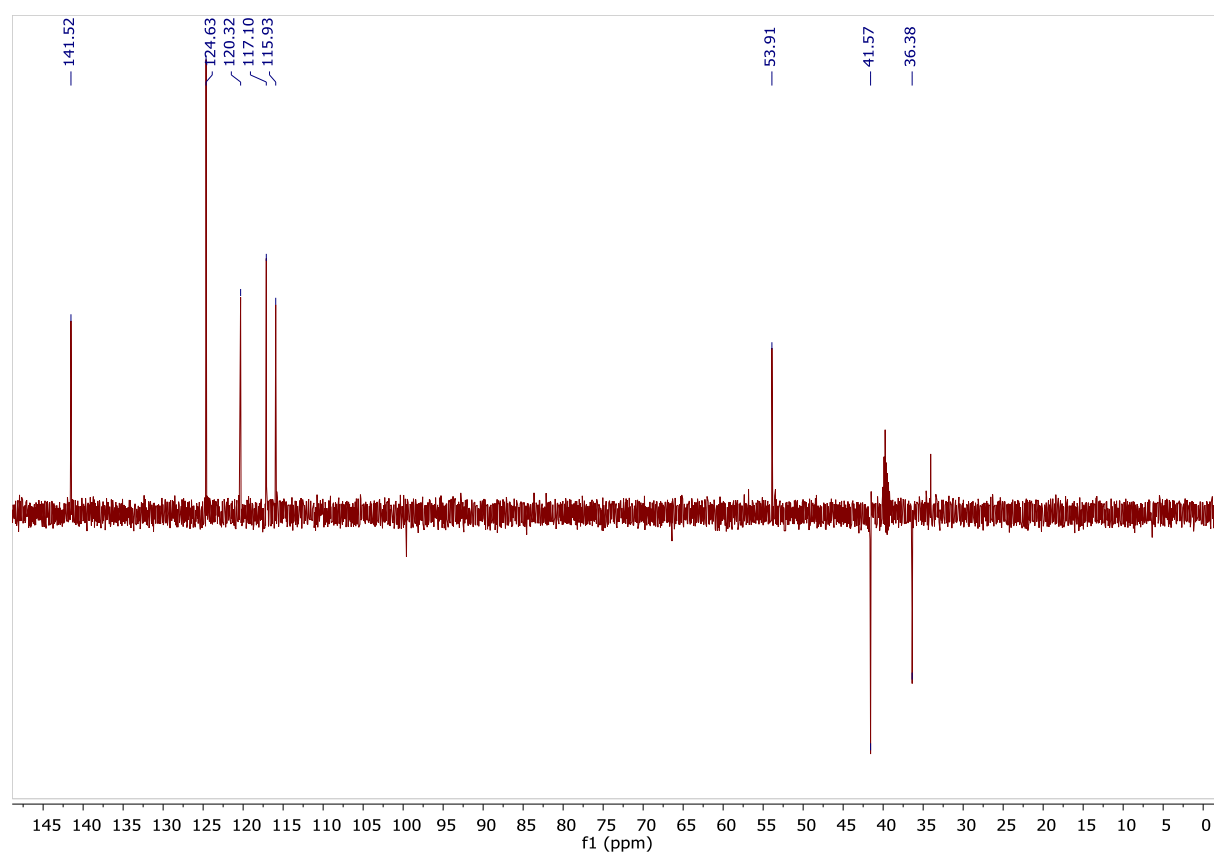
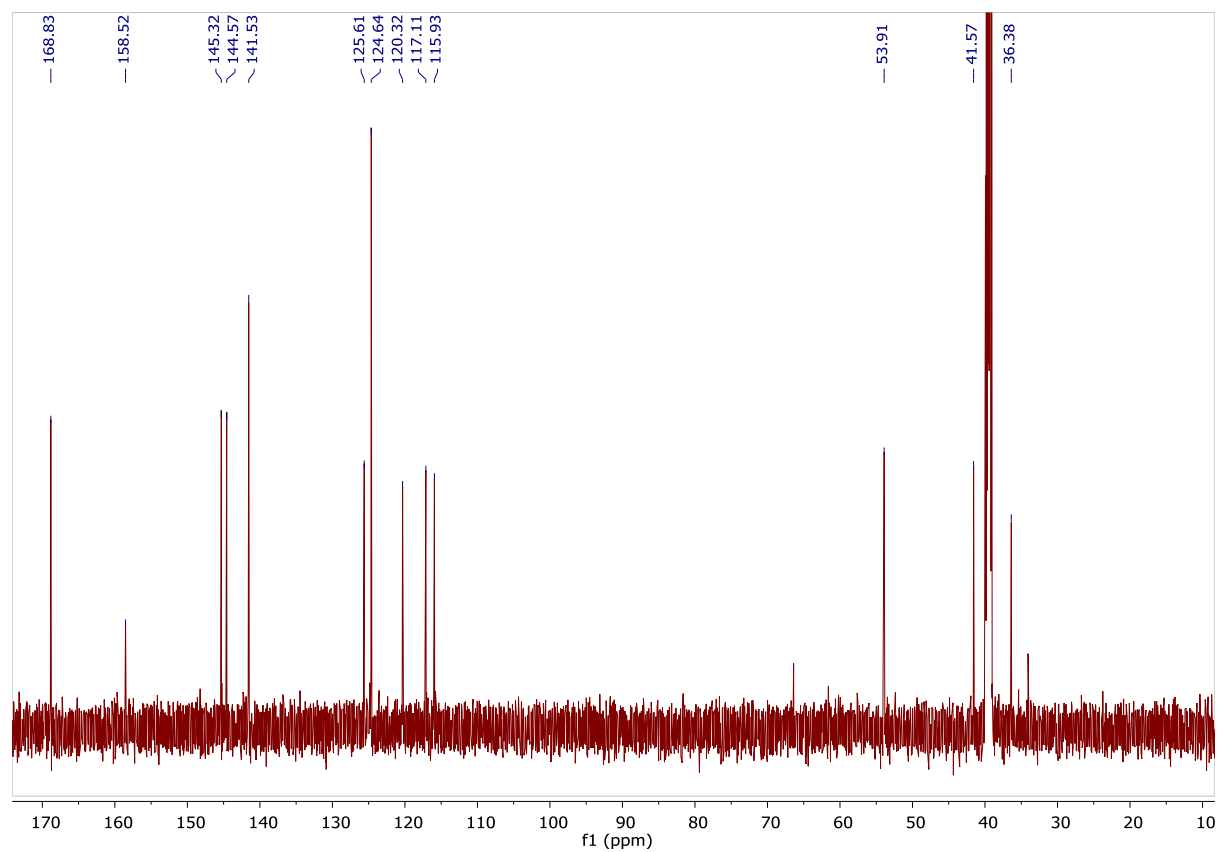
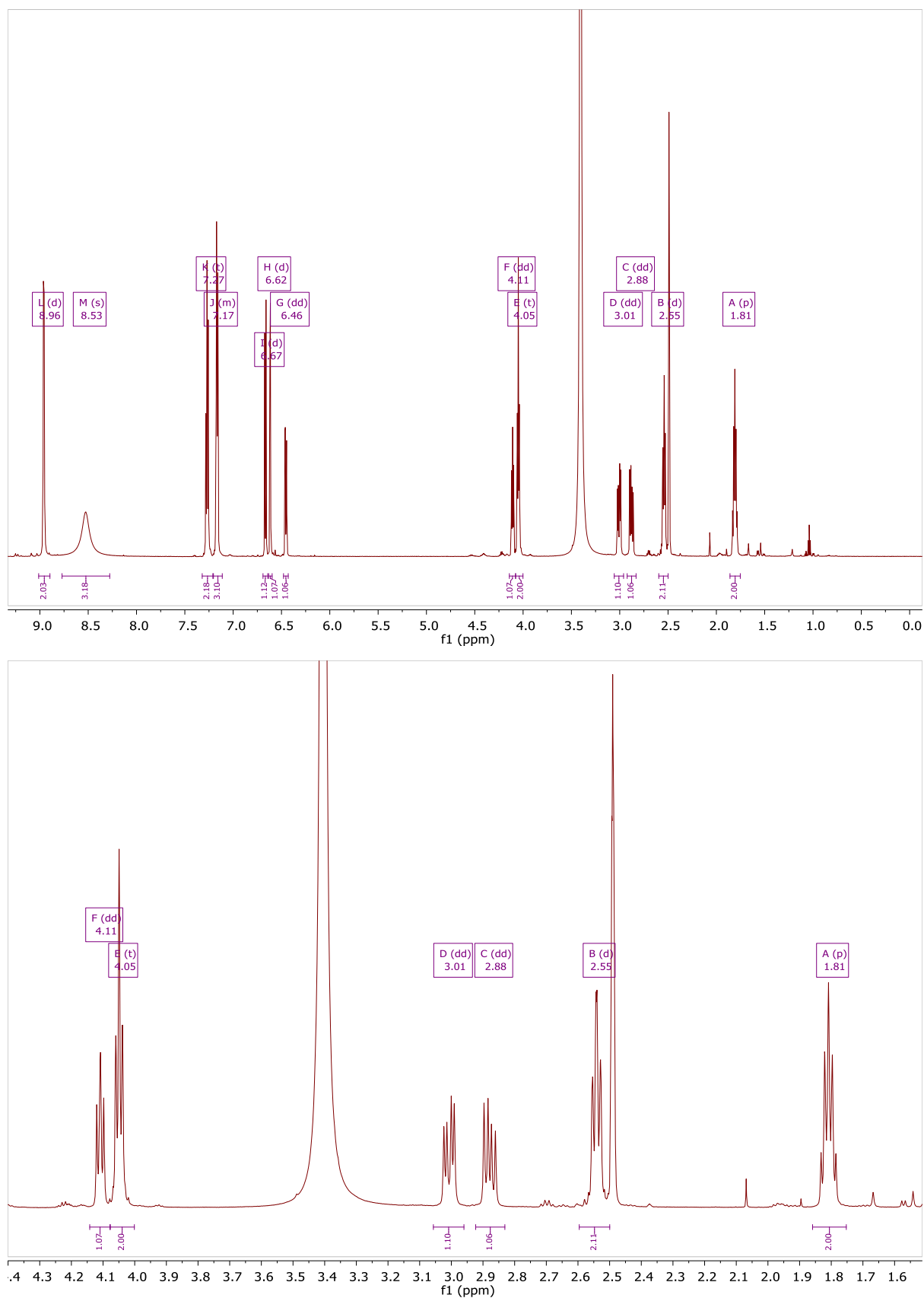


Figure S5.3. ¹H NMR spectra of L-dopa prodrug **9**



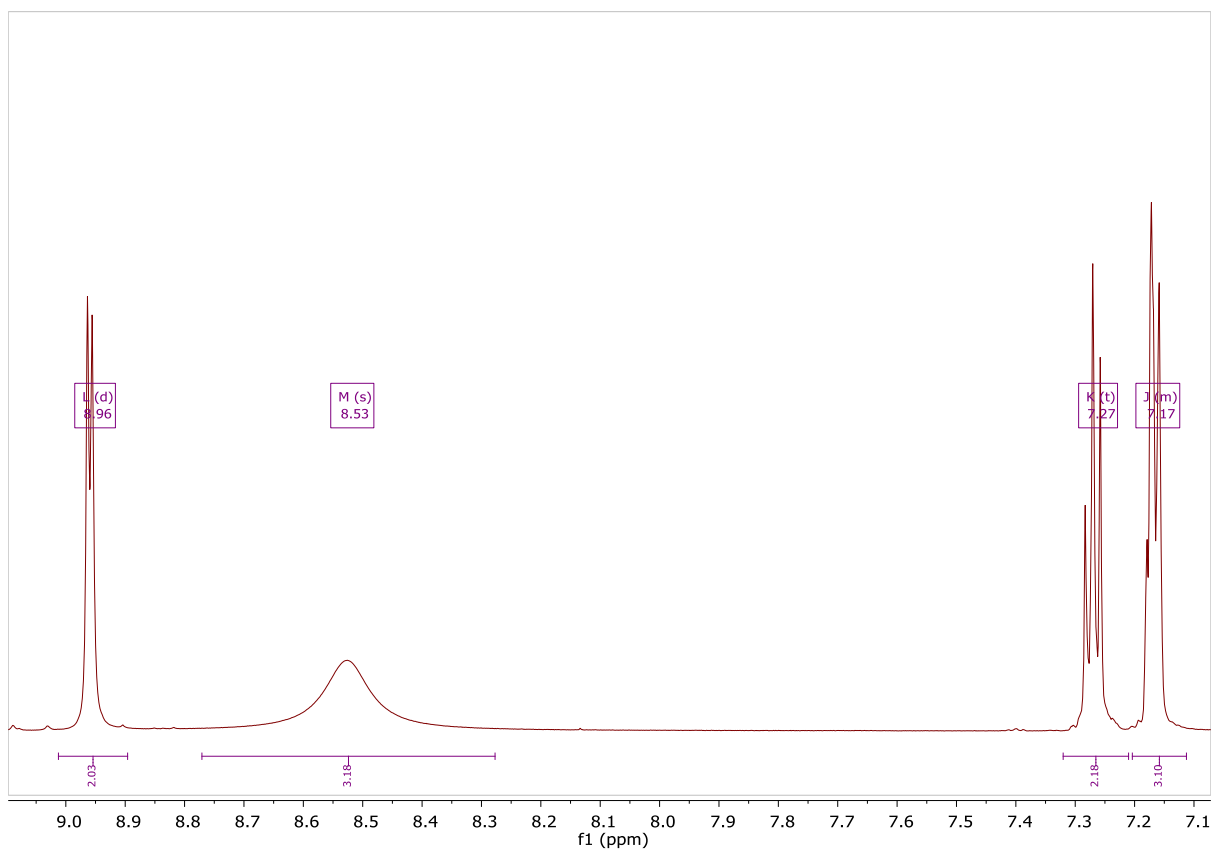
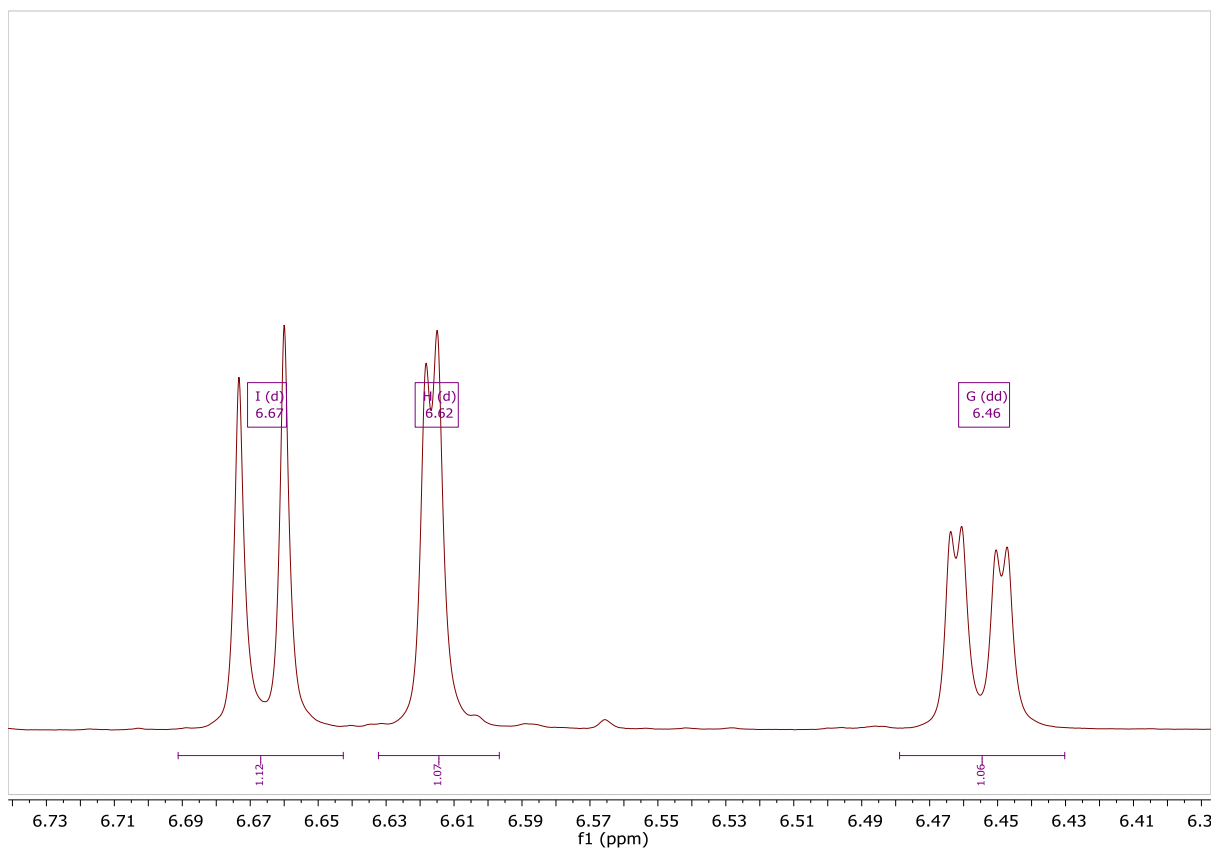


Figure S5.4. ^{13}C NMR and DEPT-135 spectra of L-dopa prodrug **9**

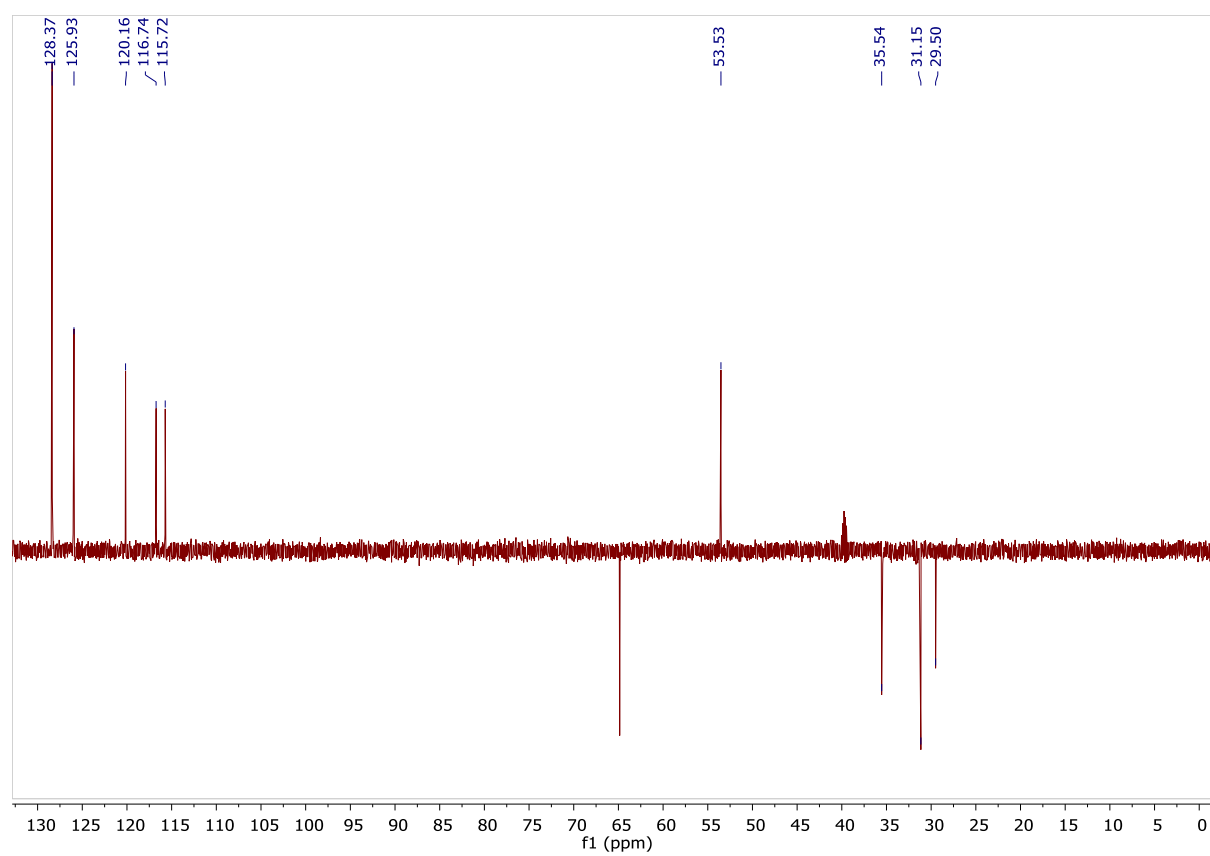
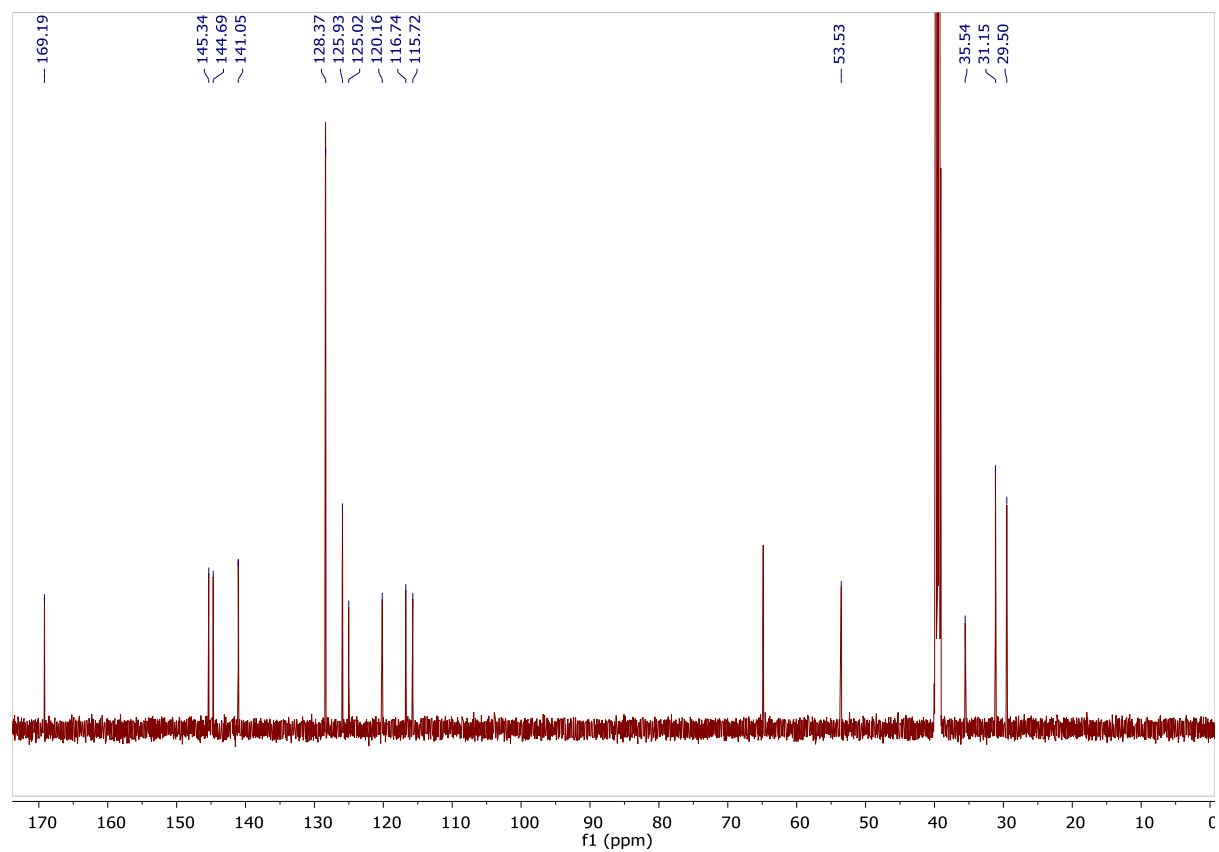
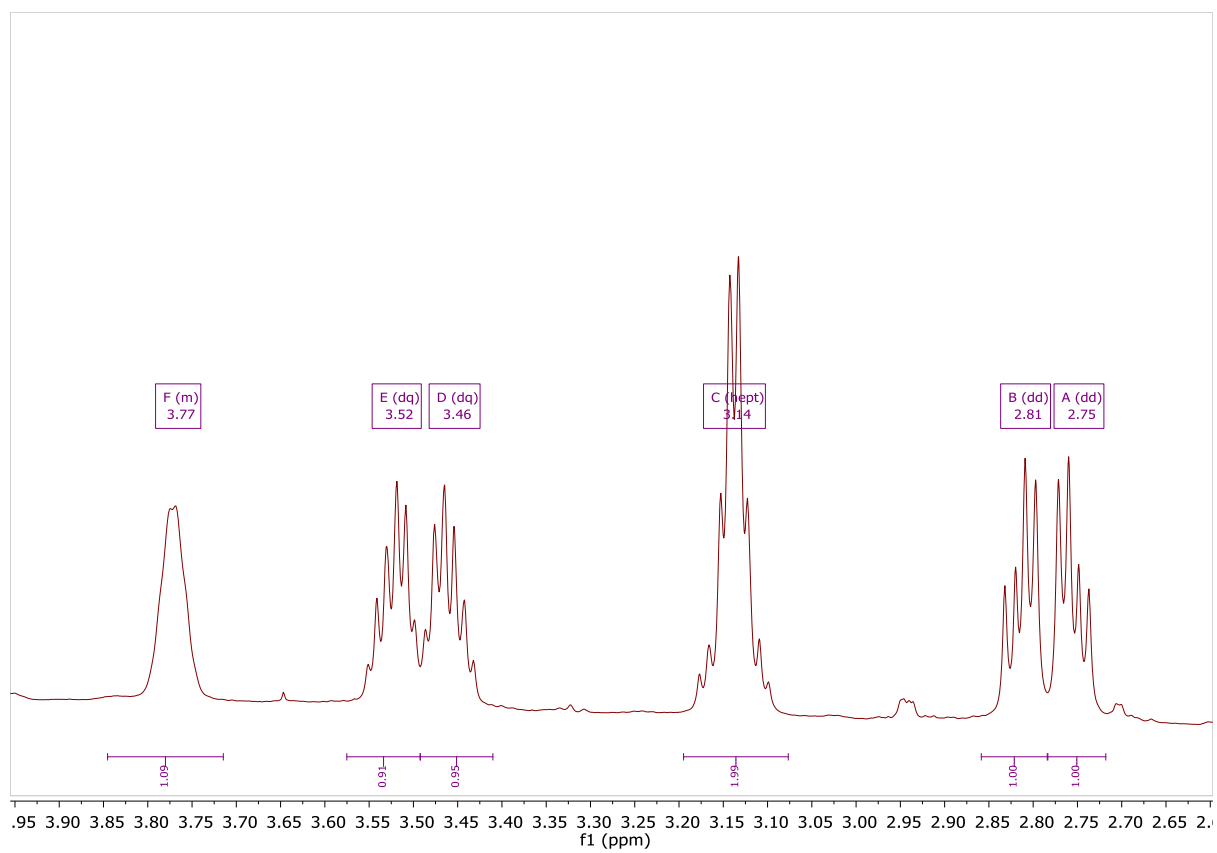
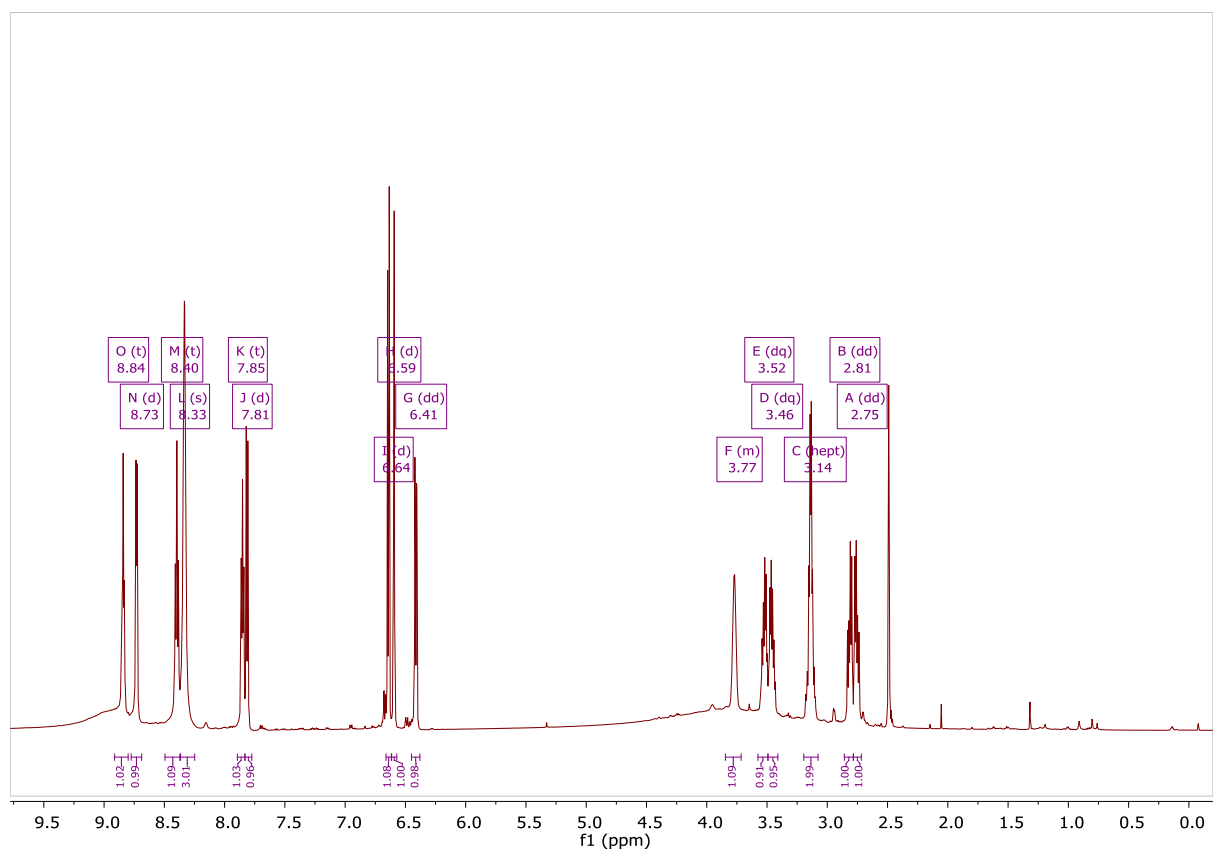


Figure S5.5. ¹H NMR spectra of L-dopa prodrug **10**



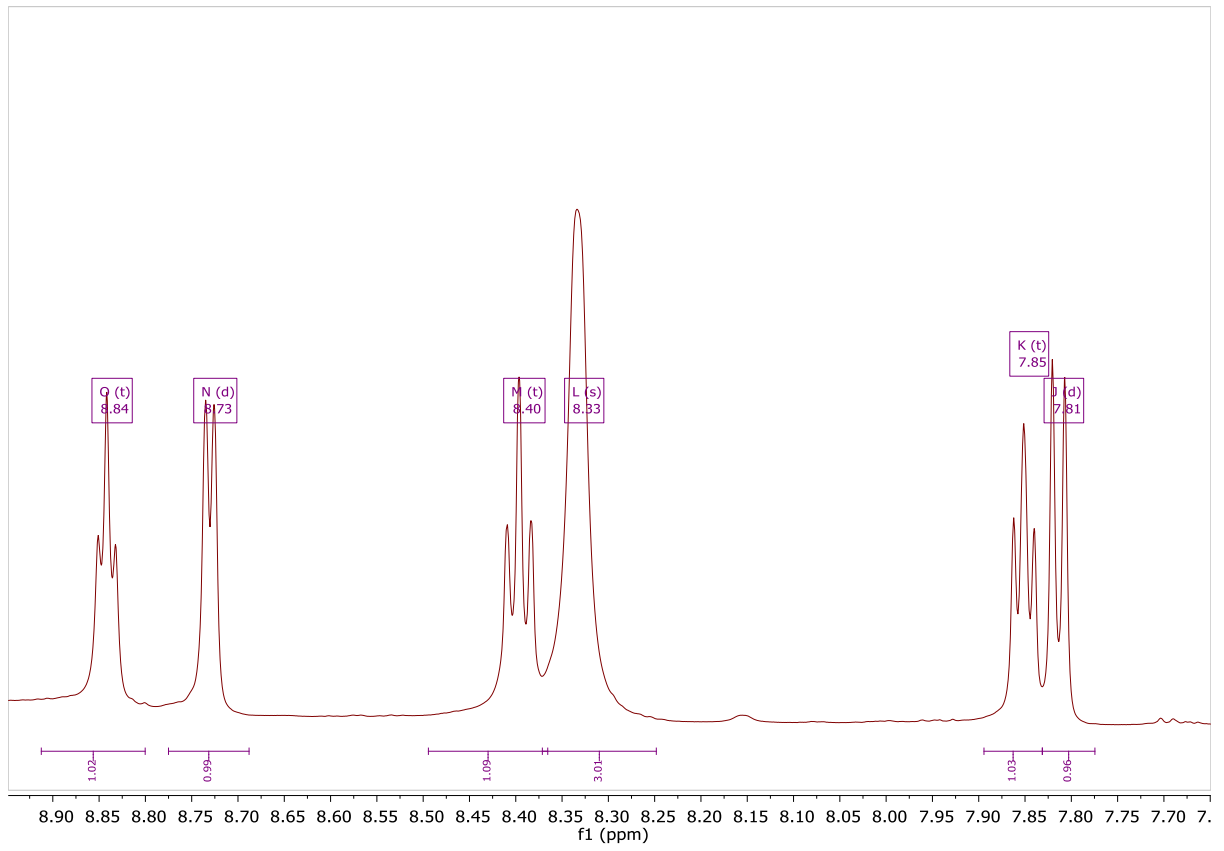
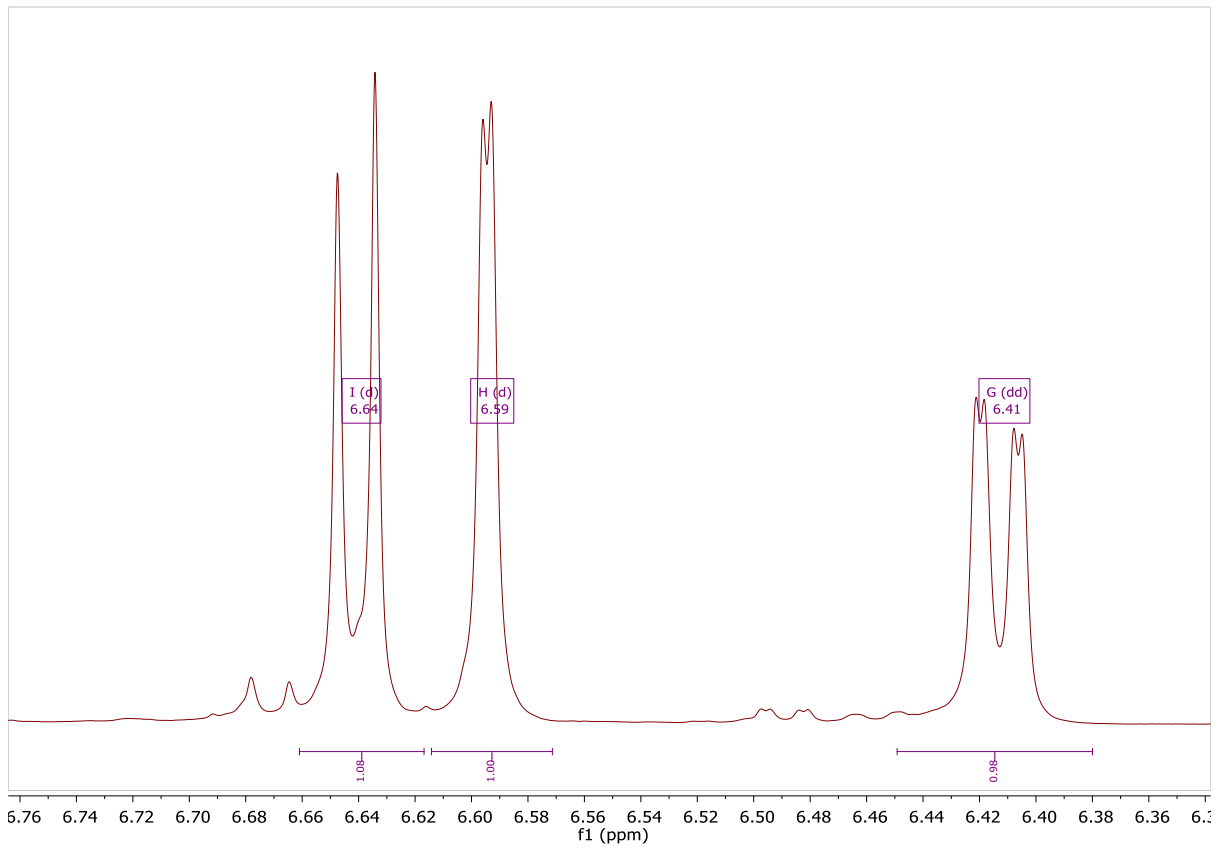


Figure S5.6. ^{13}C NMR and DEPT-135 spectra of L-dopa prodrug **10**

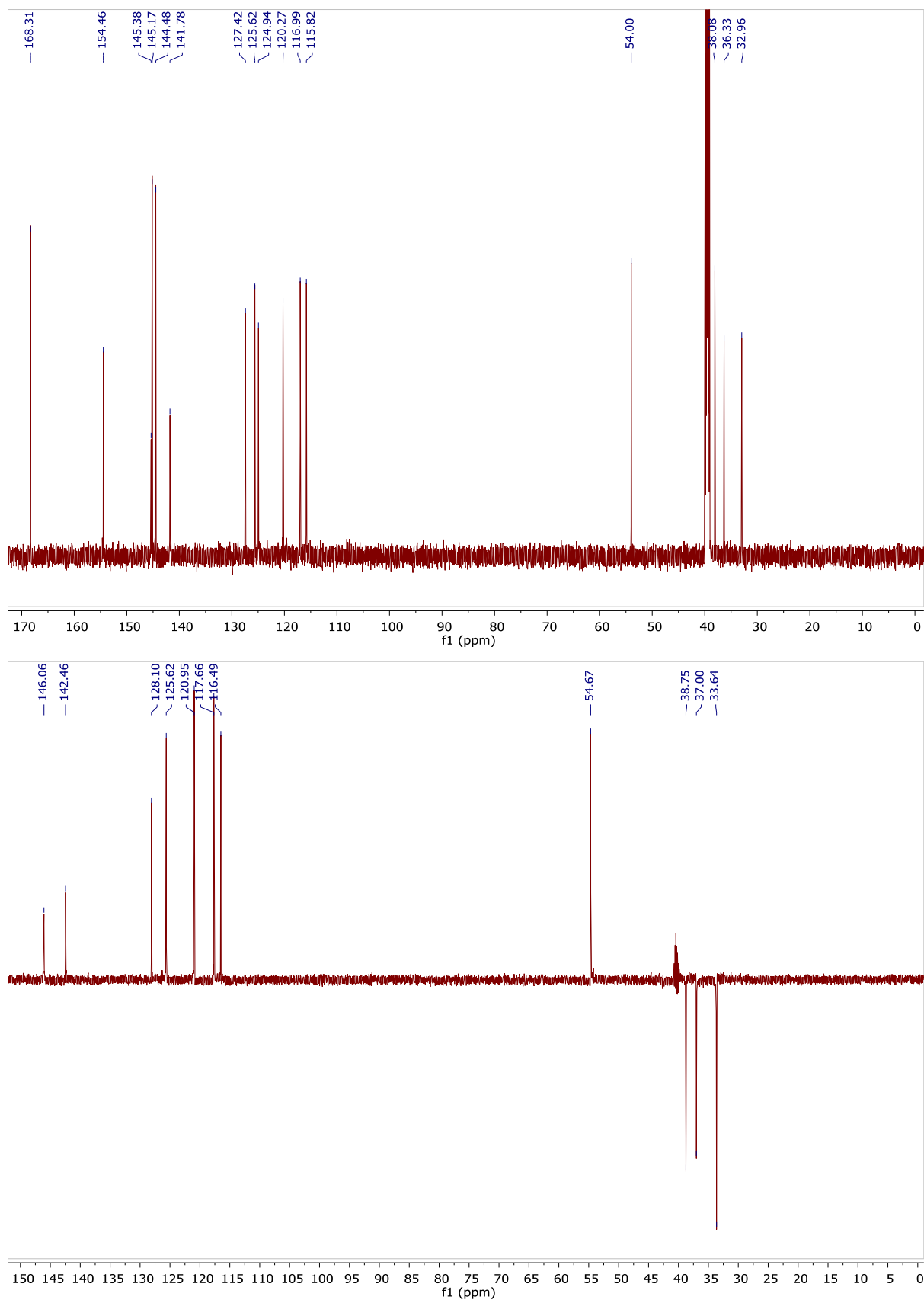
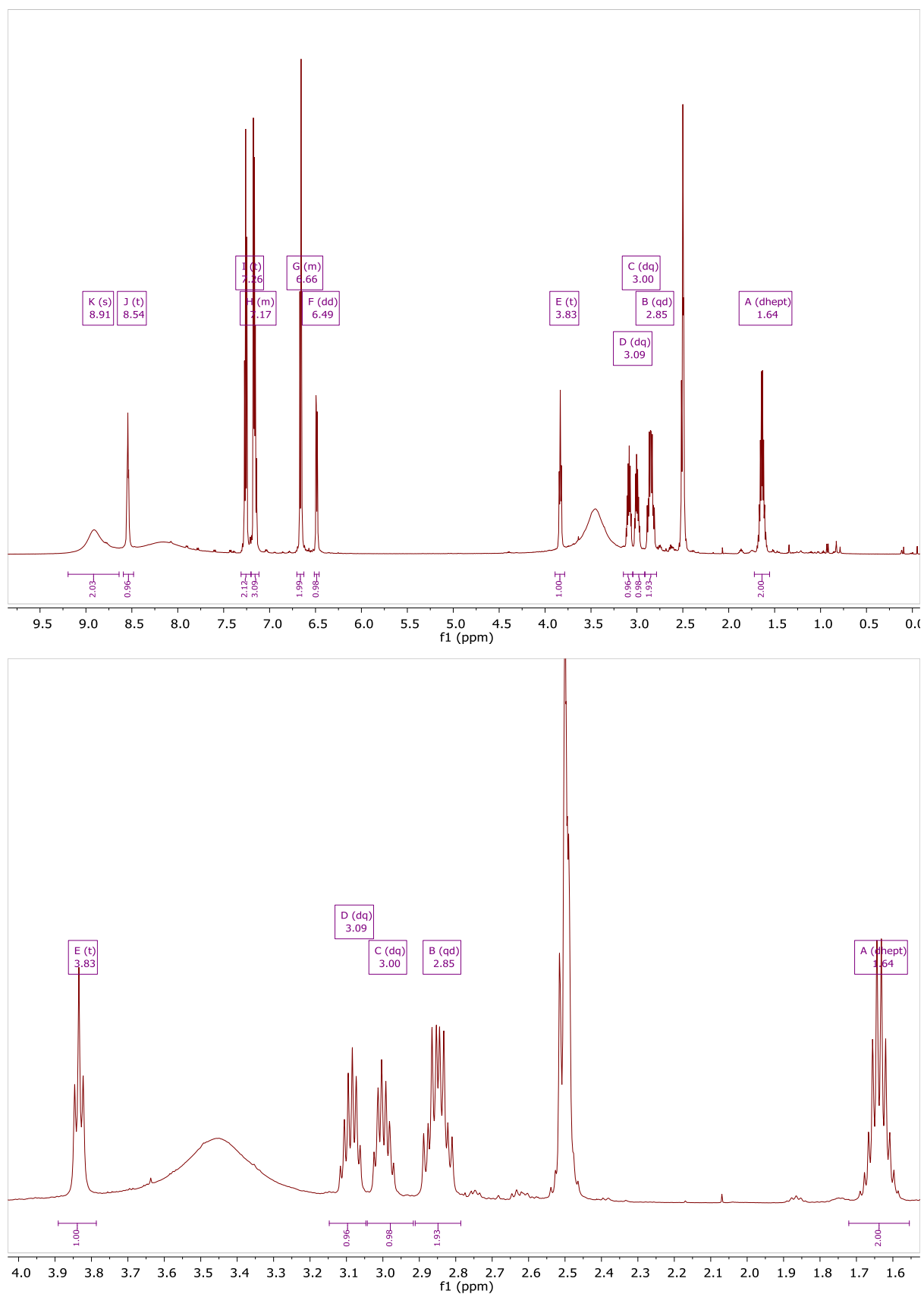


Figure S5.7. ¹H NMR spectra of L-dopa prodrug 11



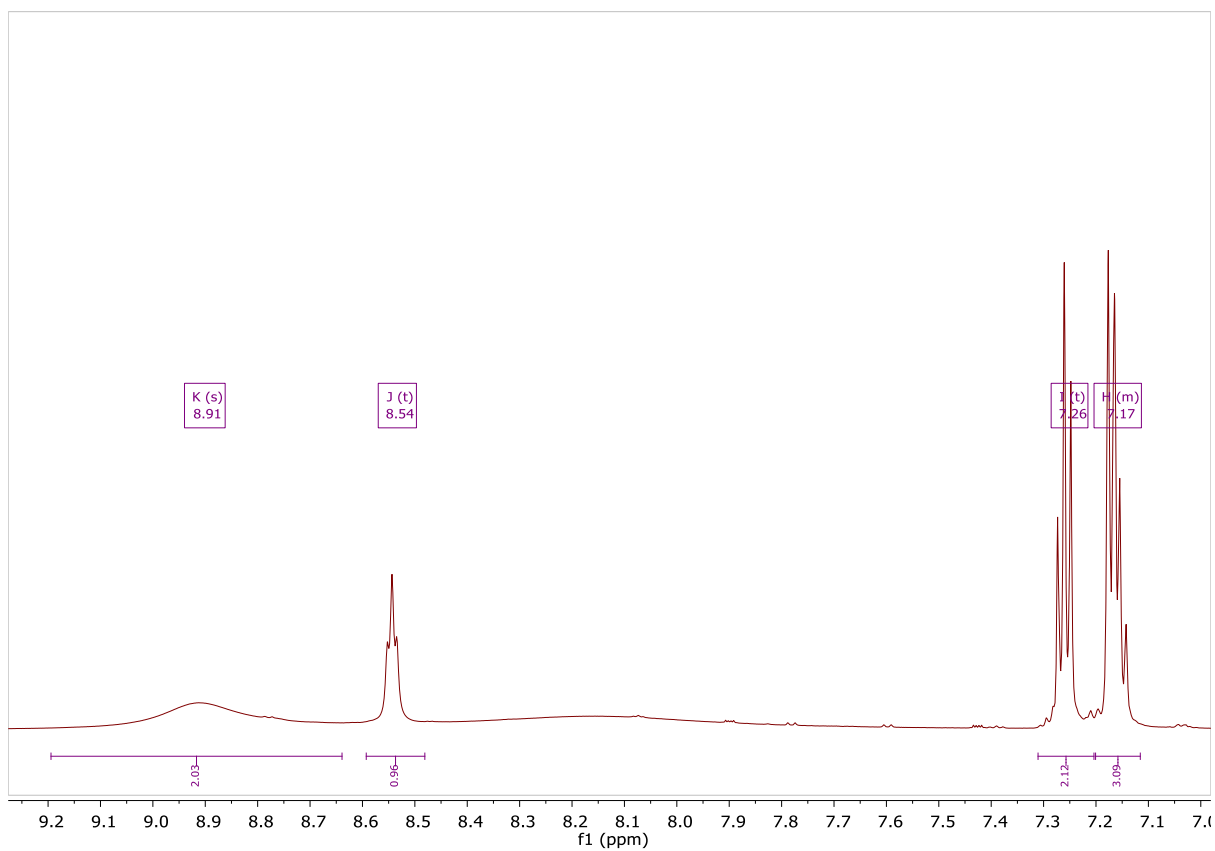
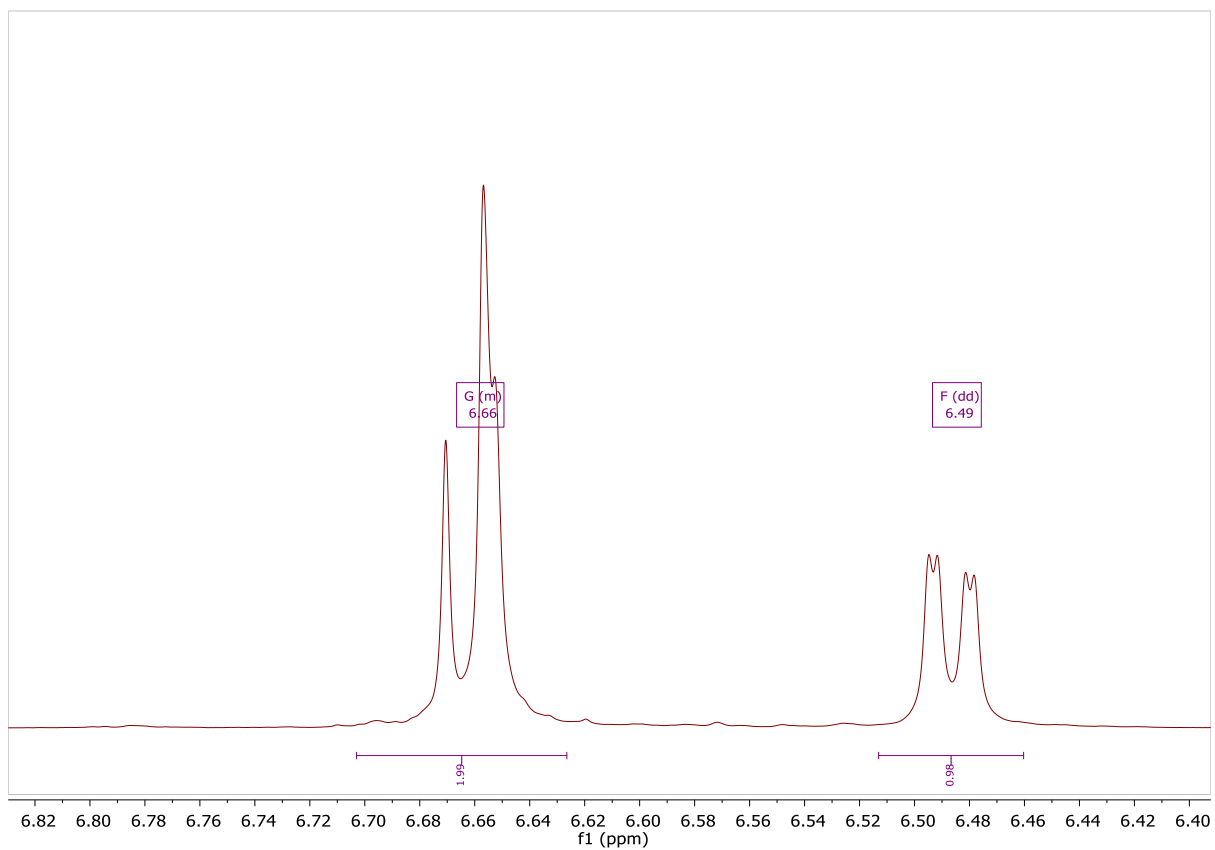


Figure S5.8. ^{13}C NMR and DEPT-135 spectra of L-dopa prodrug **11**

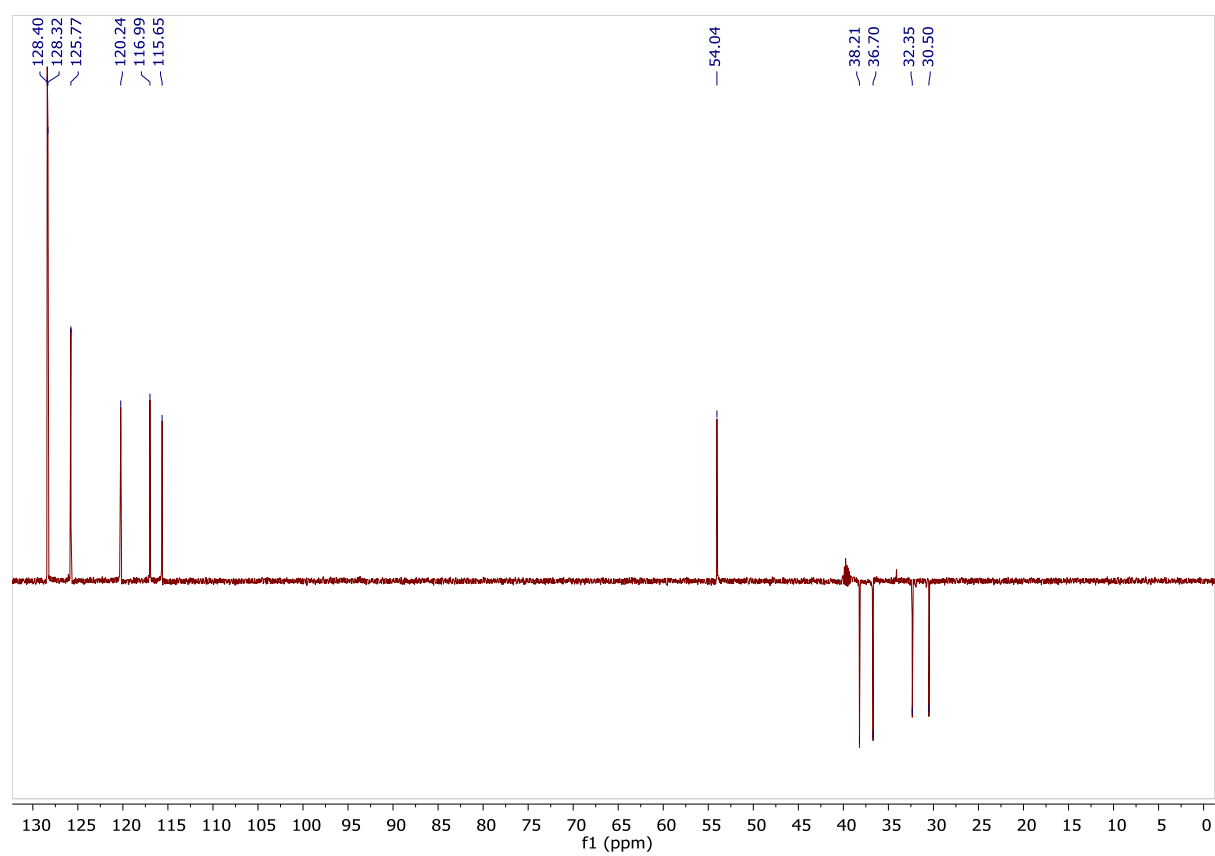
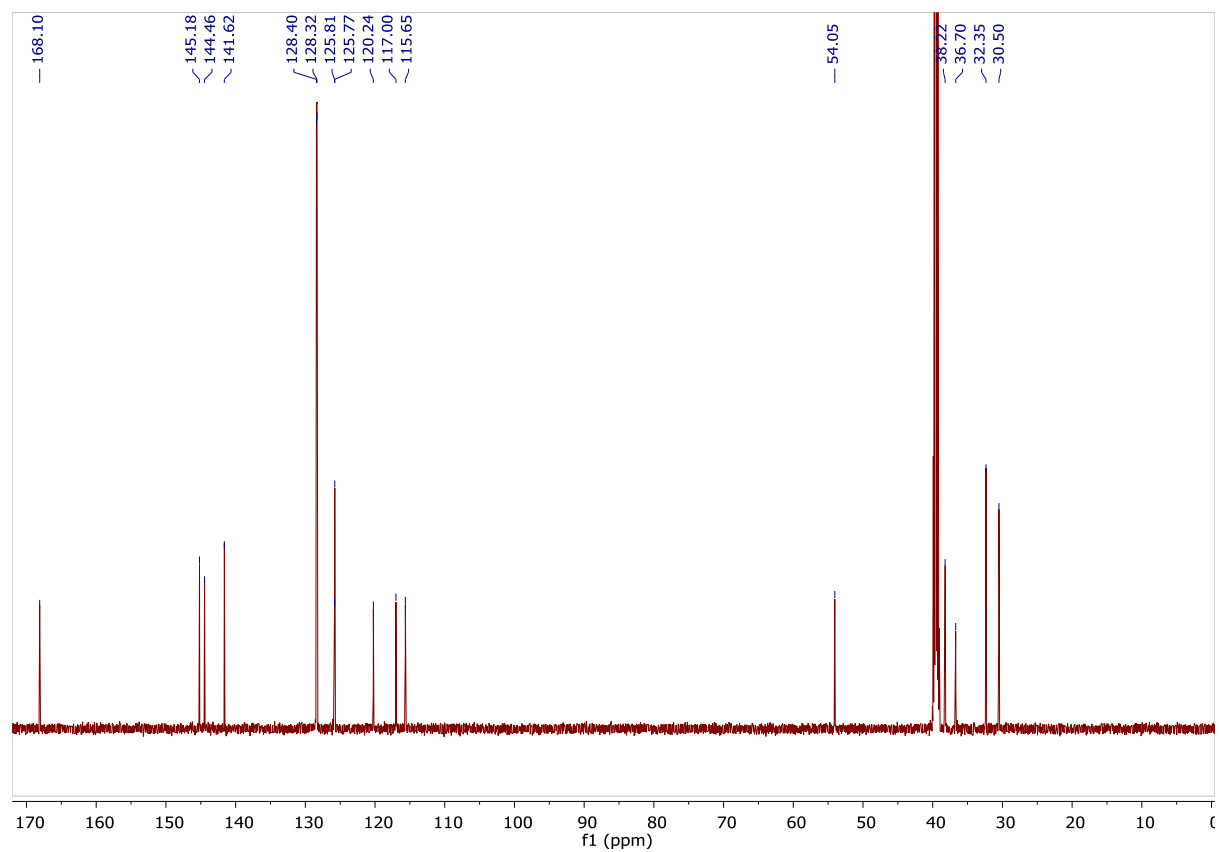
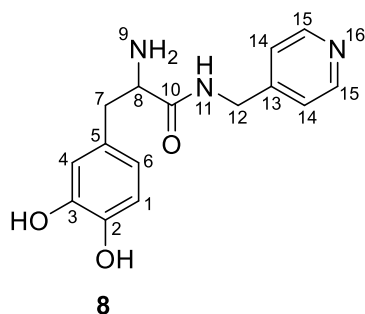


Table S5.1: Correlation of the NMR spectra with the structure of the L-Dopa prodrug **8**.



¹H NMR

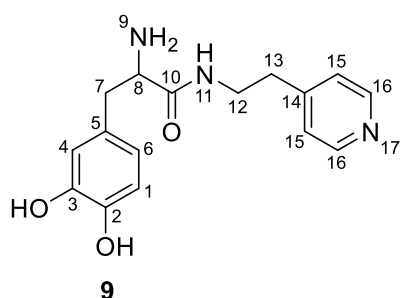
- The CH₂ group at C7 corresponds to the signals at 2.86 and 2.95 ppm (the signals integrate for 1 proton each). The assignment is based on the literature, which reports that the side chain CH₂ of L-Dopa appears as two signals at these chemical shifts (Nakonieczna *et al.*, 1994).
- The CH group at C8, the α-carbon position of L-Dopa, corresponds to the multiplet at 4.03 ppm (the signal integrate for 1 proton).
- The protons of the CH₂ group at C12 correspond to the signals at 4.47 and 4.60 ppm (the signals integrate for 1 proton each).
- The aromatic protons on the phenyl ring (C1, C4 and C6) correspond to the doublets at 6.64 ppm (C4, 1H) and 6.70 ppm (C1, 1H), and the doublet of doublets at 6.50 ppm (1H). The assignment is based on the literature, which reports that aromatic protons of L-Dopa appear as distinct signals at these chemical shifts (Nakonieczna *et al.*, 1994).
- The aromatic protons of C14 and C15 on the pyridyl ring correspond to the two doublets at 7.63 ppm (2H) and 8.75 ppm (1H). As shown by the coupling constants, the protons represented by these signals couple with each other.
- The hydrochloric acid of the prodrug contains 6 exchangeable protons (NH, NH₃⁺ and OH groups). These signals are most likely represented by the broad signals observed in the spectrum at 9.45 ppm (1H), and the broad signal at approximately 9 ppm. Exchangeable protons may also be obscured by the broad water signal at approximately 3.5 ppm.

¹³C NMR

- The carbonyl carbon at C10 is represented by the signal at 168.83 ppm.
- The methylene and methene carbons C7, C8, and C12 correspond to the signals at 53.91, 41.57 and 36.38 ppm.
- Aromatic carbons (9 carbons) on the catechol and pyridyl rings are represented by signals at 158.52, 145.32, 144.57, 141.53, 125.61, 124.64, 120.32, 117.11 and 115.93 ppm.

¹³C DEPT 135 °

- Methylene CH₂ groups at C7 and C12 correspond to the signals at 41.57 and 36.38 ppm.
- The methene CH group at C8 is represented by the signal at 53.91 ppm
- Aromatic CH carbons (5 carbons) are represented by signals at 141.53, 124.64, 120.32, 117.11 and 115.93 ppm.
- Aromatic C carbons (4 carbons) are represented by signals at 158.52, 145.32, 144.57 and 125.61 ppm.

Table S5.2: Correlation of the NMR spectra with the structure of the L-Dopa prodrug **9**.**¹H NMR**

- The CH₂ group at C7 corresponds to the signals at 2.88 and 3.01 ppm (the signals integrate for 1 proton each). The assignment is based on the literature, which reports that the side chain CH₂ of L-Dopa appears as two signals at these chemical shifts (Nakonieczna *et al.*, 1994).
- The CH group at C8, the α-carbon position of L-Dopa, corresponds to the signal at 4.11 ppm (the signal integrate for 1 proton).
- The protons of the CH₂CH₂ group at C12 and C13 correspond to the signals at 1.81 (2H) and 2.55 ppm (2H).
- The aromatic protons on the phenyl ring (C1, C4 and C6) correspond to the doublets at 6.62 ppm (C4, 1H) and 6.67 ppm (C1, 1H), and the doublet of doublets at 6.46 ppm (C6, 1H). The assignment is based on the literature, which reports that aromatic protons of L-Dopa appear as distinct signals at these chemical shifts (Nakonieczna *et al.*, 1994).
- The aromatic protons of C15 and C16 on the pyridyl ring correspond to the two signals at 7.27 ppm (2H) and 7.20 – 7.11 ppm (3H). For the latter signal (3H) two protons are aromatic while the other most likely represents an exchangeable proton (e.g. OH, NH).
- The hydrochloric acid of the prodrug contains 6 exchangeable protons (NH, NH₃⁺ and OH groups). These signals are most likely represented by the broad signals observed in

the spectrum at 8.53 ppm (3H), and the signal at 8.96 ppm (2H). As mentioned above one exchangeable proton may also be part of the multiplet at 7.20 – 7.11 ppm.

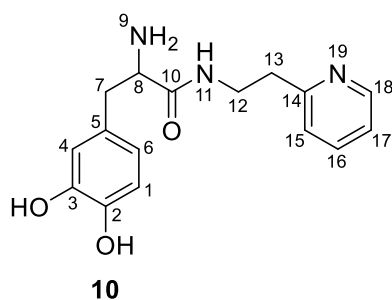
¹³C NMR

- The carbonyl carbon at C10 is represented by the signal at 169.19 ppm.
- The methylene and methene carbons C7, C8, C12 and C13 correspond to the signals at 53.53, 35.54, 31.15, 29.50 ppm.
- Aromatic carbons (9 carbons) on the catechol and pyridyl rings are represented by signals at 145.34, 144.69, 141.05, 128.37, 125.93, 125.02, 120.16, 116.74 and 115.72 ppm.

¹³C DEPT 135 °

- Methylene CH₂ groups at C7, C12 and C13 correspond to the signals at 35.54, 31.15 and 29.50 ppm.
- The methene CH group at C8 is represented by the signal at 53.53 ppm
- Aromatic CH carbons (5 carbons) are represented by signals at 128.37, 125.93, 120.16, 116.74 and 115.72 ppm.
- Aromatic C carbons (4 carbons) are represented by signals at 145.34, 144.69, 141.05 and 125.02 ppm.

Table S5.3: Correlation of the NMR spectra with the structure of the L-Dopa prodrug **10**.



¹H NMR

- The CH₂ group at C7 corresponds to the signals at 2.75 and 2.81 ppm (the signals integrate for 1 proton each). The assignment is based on the literature, which reports that the side chain CH₂ of L-Dopa appears as two signals at these chemical shifts (Nakonieczna *et al.*, 1994).
- The CH group at C8, the α-carbon position of L-Dopa, corresponds to the multiplet at 3.80 ppm (the signal integrate for 1 proton).
- The protons of the CH₂CH₂ group at C12 and C13 correspond to the multiplets at 3.14 (2H), 3.46 (1H) and 3.52 ppm (1H).

- The aromatic protons on the phenyl ring (C1, C4 and C6) correspond to the doublets at 6.59 ppm (C4, 1H) and 6.64 ppm (C1, 1H), and the doublet of doublets at 6.41 ppm (1H). The assignment is based on the literature, which reports that aromatic protons of L-Dopa appear as distinct signals at these chemical shifts (Nakonieczna *et al.*, 1994).
- The aromatic protons of C15–C18 on the pyridyl ring correspond to the signals at 7.81 ppm (1H), 7.85 (1H), 8.40 (1H) and 8.73 (1H).
- The hydrochloric acid of the prodrug contains 6 exchangeable protons (NH, NH₃⁺ and OH groups). These signals are most likely represented by the broad signals observed in the spectrum at 8.84 ppm (1H), and the broad signal at 8.33 ppm (3H). Exchangeable protons may also be obscured by the broad water signal at approximately 3.75 ppm, and the broad signal at approximately 9 ppm.

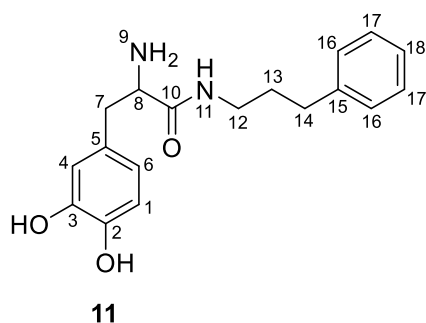
¹³C NMR

- The carbonyl carbon at C10 is represented by the signal at 168.31 ppm
- The methylene and methene carbons C7, C8, C12 and C13 correspond to the signals at 54.00, 38.08, 36.33 and 32.96 ppm
- Aromatic carbons (11 carbons) on the catechol and pyridyl rings are represented by signals at 154.46, 145.38, 145.17, 144.48, 141.78, 127.42, 125.62, 124.94, 120.27, 116.99 and 115.82 ppm.

¹³C DEPT 135 °

- Methylene CH₂ groups at C7, C12 and C13 correspond to the signals at 38.08, 36.33 and 32.96 ppm.
- The methene CH group at C8 is represented by the signal at 54.00 ppm
- Aromatic CH carbons (7 carbons) are represented by signals at 145.38, 141.78, 127.42, 124.94, 120.27, 116.99 and 115.82 ppm.
- Aromatic C carbons (4 carbons) are represented by signals at 154.46, 145.17, 144.48 and 125.62 ppm.

Table S5.4: Correlation of the NMR spectra with the structure of the L-Dopa prodrug **11**.



¹H NMR

- The CH₂ group at C7 corresponds to the signal at 2.85 ppm (the signal integrates for 2 protons). The assignment is based on the literature, which reports that the side chain CH₂ of L-Dopa appears as two signals at these chemical shifts (Nakonieczna *et al.*, 1994).
- The CH group at C8, the α-carbon position of L-Dopa, corresponds to the signal at 3.83 ppm (the signal integrate for 1 proton).
- The protons of the (CH₂)₃ group at C12–C14 correspond to the signals at 1.64 (2H), 2.85 (2H), 2.97 – 3.10 (2H) and 3.35 (1H).
- The aromatic protons on the phenyl ring (C1, C4 and C6) correspond to the two doublets, noted as a multiplet at 6.66 ppm (C4 and C1, 2H), and the doublet of doublets at 6.49 ppm (1H). The assignment is based on the literature, which reports that aromatic protons of L-Dopa appear as distinct signals at these chemical shifts (Nakonieczna *et al.*, 1994).
- The aromatic protons of C15–C18 on the phenyl ring correspond to the signals at 7.17 ppm (3H) and 7.26 (2H).
- The hydrochloric acid of the prodrug contains 6 exchangeable protons (NH, NH₃⁺ and OH groups). These signals are most likely represented by the broad signals observed in the spectrum at 8.54 ppm (1H), and the broad signal at 8.91 ppm (2H). Exchangeable protons may also be obscured by the broad water signal at approximately 3.5 ppm, and the broad signal at approximately 8.25 ppm.

¹³C NMR

- The carbonyl carbon at C10 is represented by the signal at 168.10 ppm
- The methylene and methene carbons C7, C8, C12–C14 correspond to the signals at 54.05, 38.22, 36.70, 32.35 and 30.50 ppm.
- Aromatic carbons (10 carbons) on the catechol and phenyl rings are represented by signals at 145.18, 144.46, 141.62, 128.40, 128.32, 125.81, 125.77, 120.24, 117.00 and 115.65 ppm.

¹³C DEPT 135 °

- Methylene CH₂ groups at C7 and C12–C14 correspond to the signals at 38.22, 36.70, 32.35 and 30.50 ppm.
- The methene CH group at C8 is represented by the signal at 54.05 ppm
- Aromatic CH carbons (6 carbons) are represented by signals at 128.40, 128.32, 125.77, 120.24, 117.00 and 115.65 ppm.
- Aromatic C carbons (4 carbons) are represented by signals at 145.18, 144.46, 141.62 and 125.81, ppm.

Chapter 6

Article 4

The synthesis and evaluation of novel propargylamine MAO inhibitors incorporating the pyridyl moiety

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Abstract

Irreversible monoamine oxidase (MAO) inhibitors of the propargylamine class are well-known and include drugs that have been used clinically such as pargyline, selegiline and rasagiline, specifically for the treatment of depression and as adjuvants to L-dopa in Parkinson's disease (PD). These inhibitors are classified as mechanism-based inhibitors and are activated by MAO to form irreversible adducts with the enzyme. Structural evidence shows that the N(5) position of the flavin cofactor is the site of covalent attachment of propargylamine inhibitors. Due to their importance as MAO inhibitors, the present study synthesises a small series of novel propargylamine compounds that incorporate the pyridyl moiety. Pyridyl-derived propargylamines have not yet been investigated as potential MAO inhibitors. This study finds that the pyridyl-derived propargylamines do not inhibit either of the human MAO isoforms.

6.1. Introduction

Propargylamine compounds are well known to act as inhibitors of the monoamine oxidase (MAO) enzymes and have been used in the clinic to treat neuropsychiatric and neurodegenerative disorders such as major depressive disorder and Parkinson's disease (PD) (Youdim *et al.*, 2006). The MAO enzymes exist as two isoforms, MAO-A and MAO-B, which are products of distinct genes (Shih *et al.*, 1999). Although the amino acid sequences and three-dimensional structures of MAO-A and MAO-B exhibit a high degree of similarity, the enzymes display differing inhibitor specificities (Youdim & Bakhle, 2006). In this regard, the propargylamine compound, clorgyline, is a MAO-A specific inhibitor while selegiline and rasagiline, also propargylamines, exhibit specificity for MAO-B (Fig. 6.1). Pargyline, in turn, is considered to be a non-specific propargylamine inhibitor (Youdim *et al.*, 2006). In depressive illness and PD, MAO inhibitors act by reducing the MAO-catalysed metabolism of the relevant neurotransmitters and thereby elevating neurotransmitter levels in the brain (Ramsay, 2016). Thus MAO-A inhibitors are used for the treatment of depression since they enhance central levels of serotonin and noradrenaline (Lum & Stahl, 2012), while MAO-B inhibitors block the metabolism of central DA and are applied in PD therapy (Youdim *et al.*, 2006; Youdim & Bakhle, 2006). In this respect, MAO-B inhibitors are often used as adjuvants to L-dopa, the direct metabolic precursor of DA, in an effort to further enhance DA levels in the brain. Currently, selegiline and rasagiline are registered for the treatment of PD while pargyline, now discontinued, has been used as an antihypertensive drug.

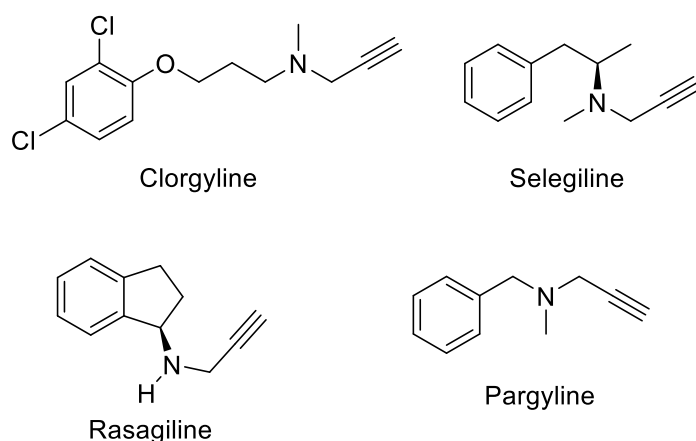


Figure 6.1: The structures of known propargylamine inhibitors.

Propargylamine inhibitors are irreversible mechanism-based inhibitors of the MAOs. These inhibitors exhibit an initial competitive mode of binding, but are subsequently activated by MAO to yield a reactive intermediate, that forms a covalent *N*(5) flavocyanine adduct with the FAD of either MAO-A or MAO-B (Fig. 6.2) (Edmondson *et al.*, 2004). The reactive intermediate may possibly be the corresponding alkyneimine, a two electron oxidation product, and nucleophilic

addition of *N*(5) of the reduced FAD across the triple bond in a Michael addition reaction would yield the observed adduct with the enzyme (Fig. 6.3) (Sigman, 1992). Alternatively, abstraction of the α -proton from the propargylamine inhibitor would yield a carbanion intermediate which could react with *N*(5) of the oxidised FAD. Protonation would then produce the flavocyanine adduct. Another possibility suggests that the propargylamine is oxidised by one electron to yield a carbon-centred radical intermediate and flavin semiquinone. The subsequent formation of a radical pair complex would result in the covalent adduct (Sigman, 1992). Structural evidence for the covalent modification of the FAD is provided by X-ray crystal structures of MAO-B, inactivated by propargylamines such as rasagiline and pargyline (Fig. 6.4) (Binda *et al.*, 2004; Binda *et al.*, 2002).

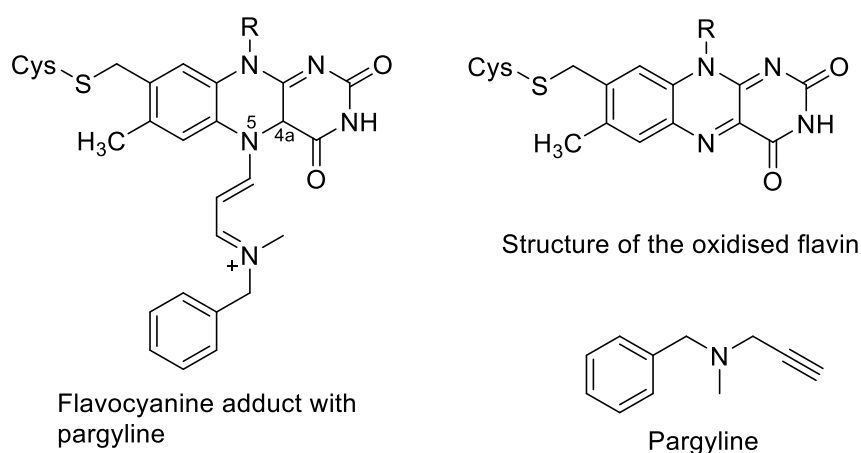
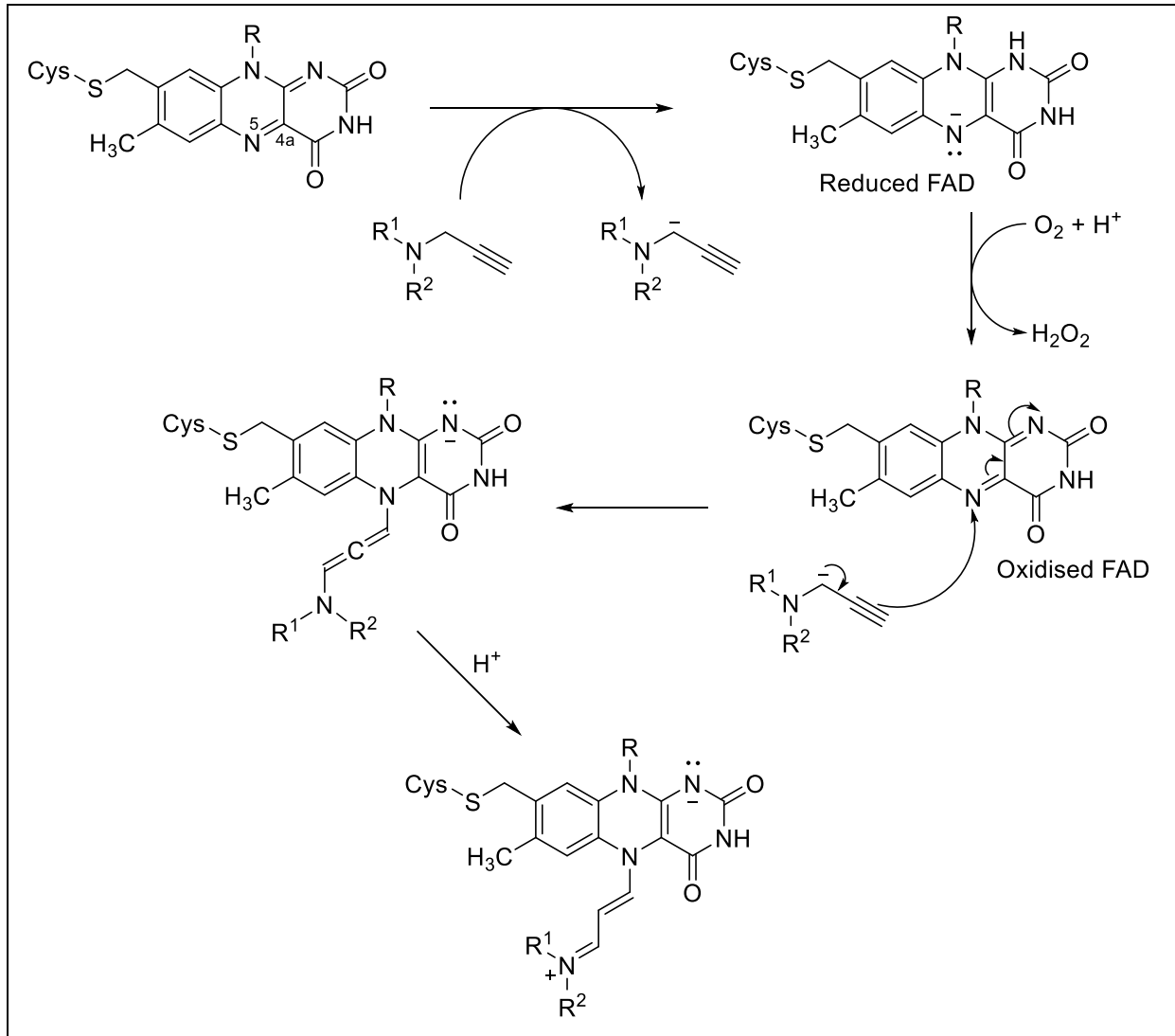
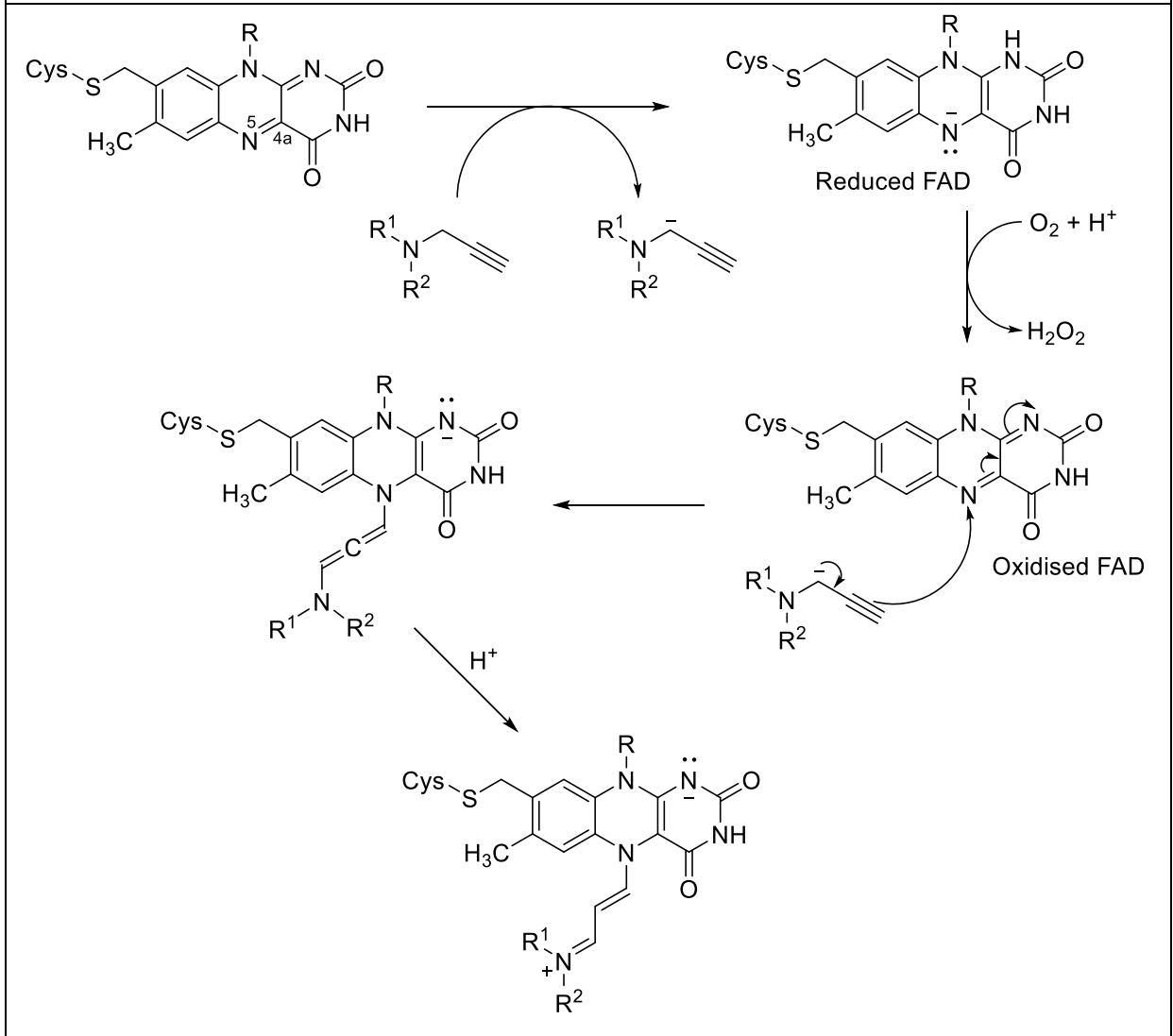
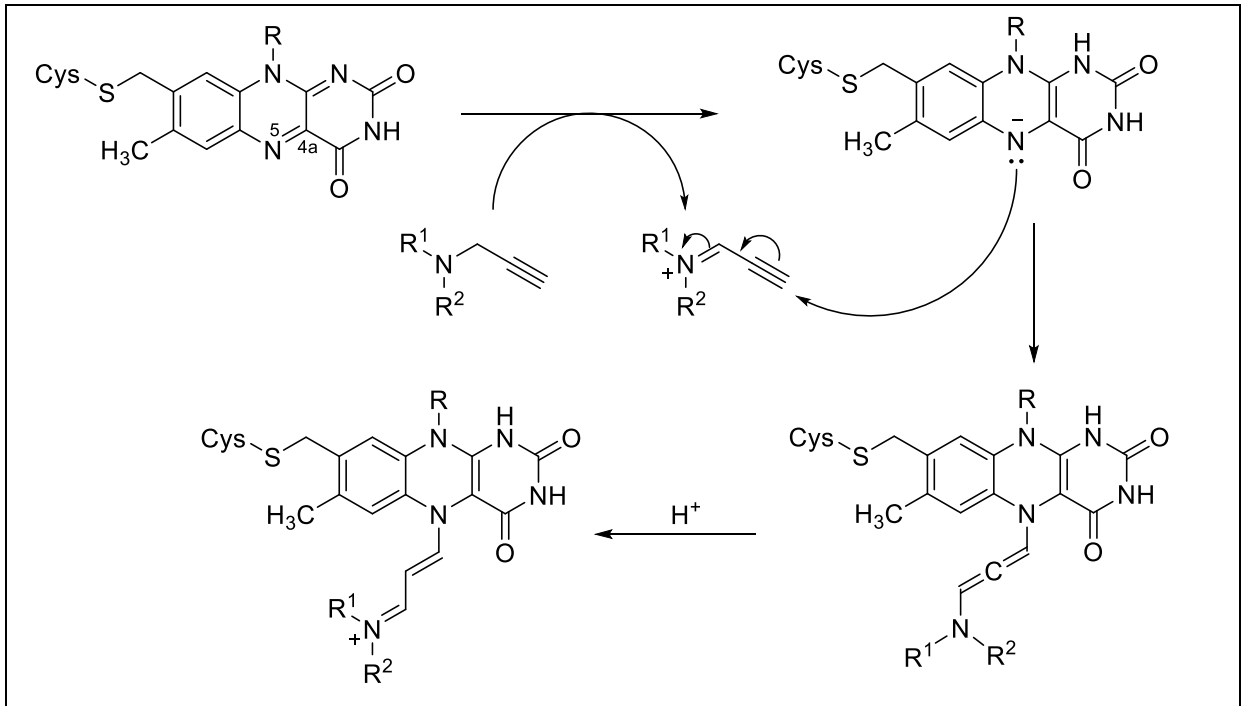


Figure 6.2: The possible structure of the covalent *N*(5) flavocyanine adduct following inactivation of MAO-B by pargyline. The structures of the oxidised flavin and pargyline are also shown (Edmondson *et al.*, 2004).

While MAO-B specific propargylamine inhibitors possess excellent safety profiles and are relatively free from adverse effects, even with chronic use, propargylamine MAO-A inhibitors and specifically irreversible inhibition of MAO-A, are associated with the potentially fatal elevation of blood-pressure when combined with tyramine containing food (Da Prada *et al.*, 1988; Flockhart, 2012; Finberg & Gillman, 2011). This event is termed the “cheese reaction” and occurs when MAO-A in intestinal (and peripheral) tissues is irreversibly inhibited, thus allowing dietary tyramine to reach high concentrations in the systemic circulation. Tyramine is a sympathomimetic amine, acting by releasing noradrenaline (and other catecholamines) from peripheral neuronal storage vesicles. This may lead to a pressor response and potential hypertensive crisis (Youdim & Bakhle, 2006). Reversible MAO-A inhibitors do not cause tyramine-induced hypertension, presumably because at higher concentrations (due to inhibition of its MAO-A-catalysed metabolism), tyramine competes with and displaces the inhibitor to allow for metabolism thereof (Bonnet, 2003; Provost *et al.*, 1992). Although

tyramine also is a substrate for MAO-B, inhibitors of this isoform do not cause the cheese reaction since tyramine is metabolised by MAO-A in the intestine (Finberg & Gillman, 2011).





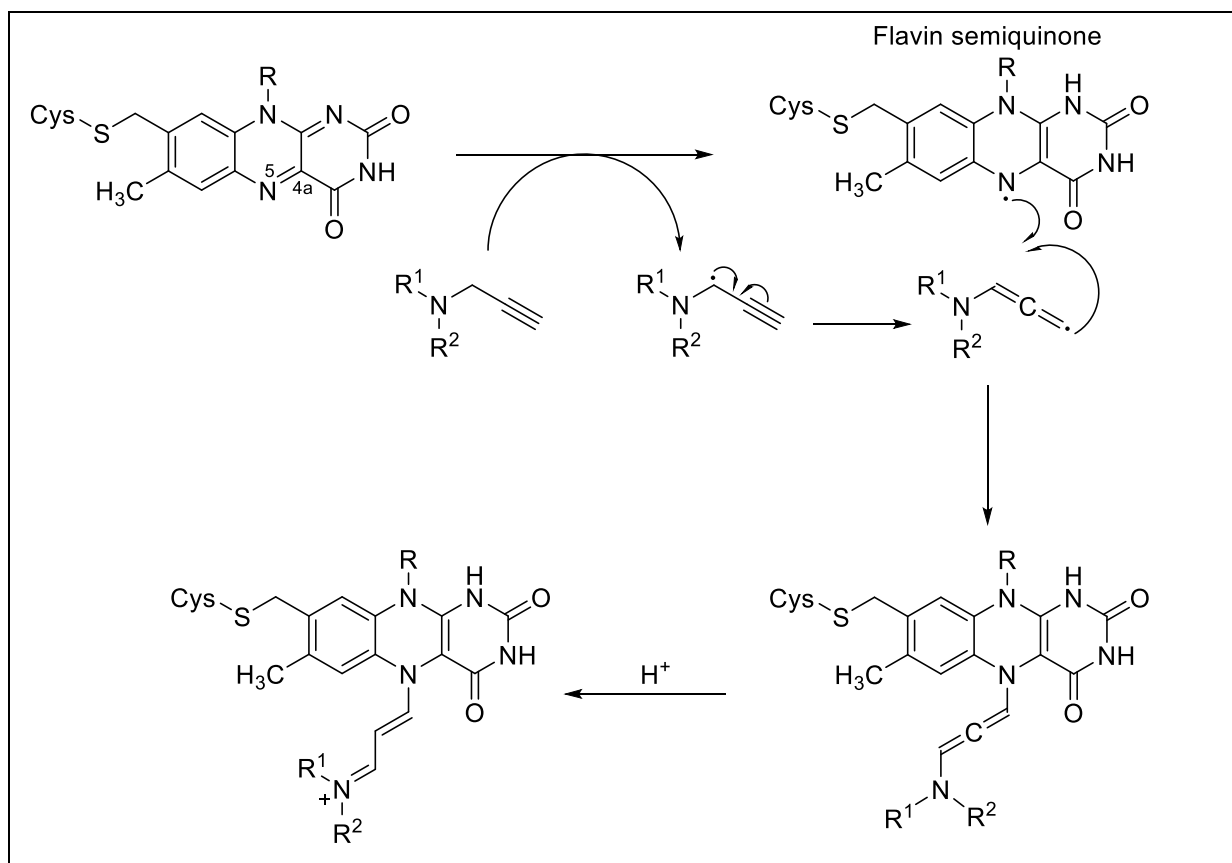


Figure 6.3: Potential mechanisms for the reaction of propargylamine inhibitors with the FAD of MAO to form covalent *N*(5) flavocyanine adducts. The mechanisms shown are the nucleophilic (top), carbanion (middle) and radical (bottom) mechanisms.

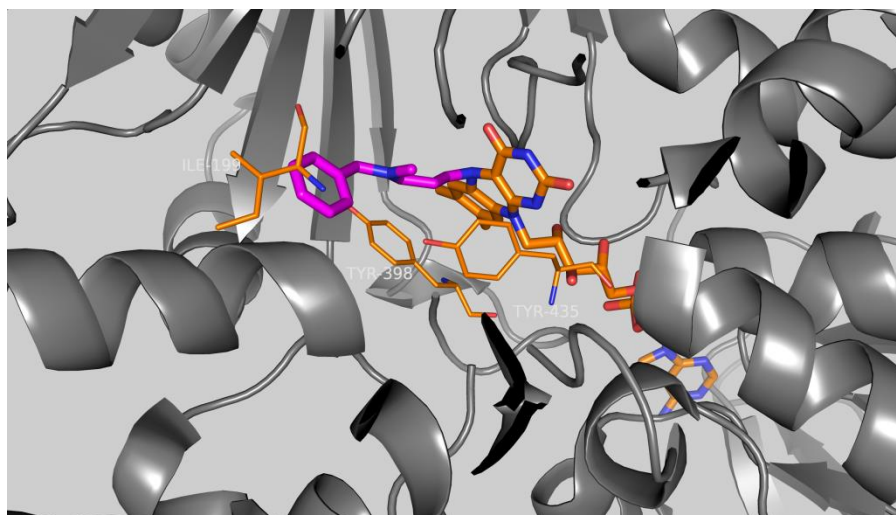


Figure 6.4: The X-ray crystal structure (1GOS) of MAO-B inactivated by pargyline (magenta) (Binda *et al.*, 2002).

Based on the academic and clinical interest in propargylamines as MAO inhibitors, the present study synthesises a small series of novel propargylamine compounds that incorporate the pyridyl moiety. Pyridyl-derived propargylamines have not yet been investigated as potential MAO inhibitors. As part of an initial exploratory approach, N-(4-pyridylmethyl)propargylamine (**1a**), N-(2-pyridylmethyl)propargylamine (**1b**) and N-(3-pyridylmethyl)propargylamine (**1c**) were considered for this study (Fig. 6.5). Also included in this study are the N,N-dipropargyl analogues, compounds **2a–d**, isolated during the synthesis of **1a–c**. The pyridine heterocycle has a low pKa, and is thus weakly basic. At physiological pH the pyridyl moiety is expected to be uncharged, which is an advantage for MAO inhibition since MAO substrates are thought to bind in the unionised form to the active site (Edmondson *et al.*, 2009). Deprotonation of amine substrates may be facilitated by the negatively charged phospholipid bilayer of the mitochondrial outer membrane, situated in proximity to the entrance of the active site. Due to the electronegative nitrogen, pyridine is relatively electron deficient and would be expected to undergo differing intermolecular interaction compared to the phenyl found in inhibitors such as pargyline and selegiline. This study thus investigated the effect of the pyridyl moiety on the MAO inhibition properties of pyridyl-derived propargylamines.

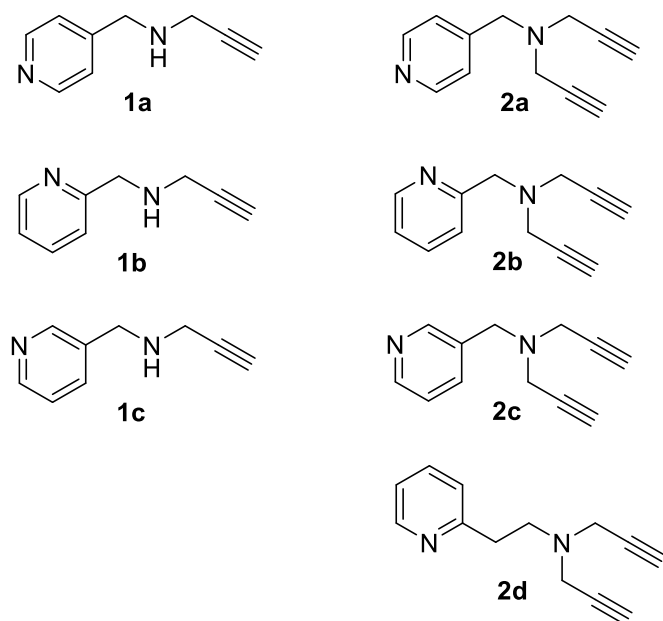


Figure 6.5: The structures of the propargylamine compounds that were synthesised and investigated in the current study.

6.2. Experimental section

6.2.1. Materials and methods

Unless otherwise noted, all starting materials and reagents were obtained from Sigma-Aldrich and were used without further purification. Proton (¹H) and carbon (¹³C) NMR spectra were

recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 MHz and 150 MHz, respectively. CDCl₃ served as NMR solvent and chemical shifts are reported in parts per million (δ), and were referenced to the residual solvent signal. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplets), ddd (doublet of doublet of doublets), t (triplet), td (triplet of doublets), or m (multiplet). High resolution mass spectra (HRMS) were recorded on a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionisation (APCI) mode. Thin layer chromatography (TLC) was performed using silica gel 60 (Merck) with UV₂₅₄ fluorescent indicator. The mobile phase consisted of ethyl acetate (100%) and the developed sheets were visualised under UV light at 254 nm. A Varian Cary Eclipse fluorescence spectrophotometer was used to carry out fluorescence spectrophotometry. Microsomes from insect cells containing recombinant human MAO-A and MAO-B (5 mg/mL) as well as kynuramine dihydrobromide were obtained from Sigma-Aldrich.

6.2.2. The synthesis of propargylamine compounds, **1a–c** and **2a–d**

The appropriate amines [4-pyridylmethylamine, 2-pyridylmethylamine, 3-pyridylmethylamine and 2-(2-pyridyl)ethylamine] (13.8 mmol) were dissolved in 15 mL tetrahydrofuran, and triethylamine (3 mL) and propargyl bromide (0.015 mmol) were subsequently added. In an atmosphere of argon, the reaction was stirred at 50 °C for 4 h. After the reaction was filtered, the filtrate was evaporated under reduced pressure. The residue was subjected to silica gel column chromatography (40 mm \times 75 mm) using a VersaFlash flash chromatography purification system (Supelco) with ethyl acetate as mobile phase. Early fractions contained the N,N-dipropargyl analogues, **2a–d** (R_f 0.38–0.52), while later fractions contained propargylamines **1a–c** (R_f 0.1) (Biedrzycki *et al.*, 1990). All compounds presented as oils at room temperature.

6.2.2.1. *N*-(4-Pyridylmethyl)propargylamine (**1a**): ¹H NMR (600 MHz, CDCl₃) δ 8.51 (d, J = 5.9 Hz, 2H), 7.25 (d, J = 5.9 Hz, 2H), 3.87 (s, 2H), 3.40 (d, J = 2.4 Hz, 2H), 2.24 (t, J = 2.4 Hz, 1H). ¹³C NMR (151 MHz, CDCl₃) δ 149.78, 148.43, 123.11, 81.45, 71.95, 50.83, 37.38. EI-HRMS m/z calcd for C₉H₁₁N₂ (MH⁺), 147.0917, found 147.0925.

6.2.2.2. *N*-(2-Pyridylmethyl)propargylamine (**1b**): ¹H NMR (600 MHz, CDCl₃) δ 8.52 (ddd, J = 5.0, 1.8, 0.9 Hz, 1H), 7.61 (td, J = 7.6, 1.8 Hz, 1H), 7.29 (d, J = 7.7 Hz, 1H), 7.13 (ddd, J = 7.6, 4.9, 1.2 Hz, 1H), 3.96 (s, 2H), 3.45 (d, J = 2.5 Hz, 2H), 2.21 (t, J = 2.4 Hz, 1H). ¹³C NMR (151 MHz, CDCl₃) δ 158.96, 149.30, 136.44, 122.37, 122.02, 81.76, 71.63, 53.58, 37.70. EI-HRMS m/z calcd for C₉H₁₁N₂ (MH⁺), 147.0917, found 147.0927.

6.2.2.3. *N*-(3-Pyridylmethyl)propargylamine (**1c**): ^1H NMR (600 MHz, CDCl_3) δ 8.55 (d, $J = 2.2$ Hz, 1H), 8.47 (dd, $J = 4.8, 1.7$ Hz, 1H), 7.66 (dt, $J = 7.9, 1.9$ Hz, 1H), 7.26 – 7.19 (m, 1H), 3.86 (s, 2H), 3.39 (d, $J = 2.4$ Hz, 2H), 2.25 (t, $J = 2.4$ Hz, 1H). ^{13}C NMR (151 MHz, CDCl_3) δ 149.85, 148.63, 136.04, 134.66, 123.35, 81.53, 71.92, 49.40, 37.25. EI-HRMS m/z calcd for $\text{C}_9\text{H}_{11}\text{N}_2$ (MH^+), 147.0917, found 147.0918.

6.2.2.4. *Bis*(propargyl)(pyridin-4-ylmethyl)amine (**2a**): ^1H NMR (600 MHz, CDCl_3) δ 8.53 (d, $J = 6.0$ Hz, 2H), 7.28 (d, $J = 5.9$ Hz, 2H), 3.68 (s, 2H), 3.39 (d, $J = 2.4$ Hz, 4H), 2.26 (t, $J = 2.4$ Hz, 2H). ^{13}C NMR (151 MHz, CDCl_3) δ 149.89, 146.98, 123.85, 78.27, 73.55, 55.81, 42.10. EI-HRMS m/z calcd for $\text{C}_{12}\text{H}_{13}\text{N}_2$ (MH^+), 185.1073, found 185.1068.

6.2.2.5. *Bis*(propargyl)(pyridin-2-ylmethyl)amine (**2b**): ^1H NMR (600 MHz, CDCl_3) δ 8.53 (ddd, $J = 4.9, 1.8, 0.9$ Hz, 1H), 7.63 (td, $J = 7.7, 1.8$ Hz, 1H), 7.42 (d, $J = 7.8$ Hz, 1H), 7.15 (ddd, $J = 7.5, 4.9, 1.2$ Hz, 1H), 3.83 (s, 2H), 3.46 (d, $J = 2.4$ Hz, 4H), 2.24 (t, $J = 2.4$ Hz, 2H). ^{13}C NMR (151 MHz, CDCl_3) δ 158.07, 149.34, 136.54, 123.21, 122.24, 78.63, 73.27, 58.86, 42.20. EI-HRMS m/z calcd for $\text{C}_{12}\text{H}_{13}\text{N}_2$ (MH^+), 185.1073, found 185.1052.

6.2.2.6. *Bis*(propargyl)(pyridin-3-ylmethyl)amine (**2c**): ^1H NMR (600 MHz, CDCl_3) δ 8.55 (d, $J = 2.2$ Hz, 1H), 8.50 (dd, $J = 4.8, 1.7$ Hz, 1H), 7.69 (dt, $J = 7.9, 2.0$ Hz, 1H), 7.24 (td, $J = 5.7, 2.9$ Hz, 1H), 3.68 (s, 2H), 3.38 (d, $J = 2.4$ Hz, 4H), 2.26 (t, $J = 2.4$ Hz, 2H). ^{13}C NMR (151 MHz, CDCl_3) δ 150.46, 148.96, 136.79, 133.17, 123.41, 78.32, 73.55, 54.17, 41.91. EI-HRMS m/z calcd for $\text{C}_{12}\text{H}_{13}\text{N}_2$ (MH^+), 185.1073, found 185.1063.

6.2.2.7. *Bis*(propargyl)[2-(pyridin-2-yl)ethyl]amine (**2d**): ^1H NMR (600 MHz, CDCl_3) δ 8.49 (ddd, $J = 5.1, 1.9, 0.9$ Hz, 1H), 7.57 (td, $J = 7.7, 1.9$ Hz, 1H), 7.17 (d, $J = 7.8$ Hz, 1H), 7.09 (ddd, $J = 7.5, 4.9, 1.1$ Hz, 1H), 3.48 (d, $J = 2.4$ Hz, 4H), 2.99 – 2.90 (m, 4H), 2.20 (t, $J = 2.4$ Hz, 2H). ^{13}C NMR (151 MHz, CDCl_3) δ 159.70, 149.21, 136.34, 123.12, 121.27, 78.64, 73.07, 52.81, 42.18, 36.40.

6.2.3. Measurement of IC_{50} values

The IC_{50} values for the inhibition of recombinant human MAO-A and MAO-B were measured as described in literature (Mostert *et al.*, 2015). The enzyme reactions were carried out in white 96-well microtiter plates (Eppendorf). Potassium phosphate buffer (pH 7.4, 100 mM, made isotonic with KCl) served as the reaction medium and the final volume of the reactions was 200 μL . The enzyme reactions contained the non-specific MAO substrate, kynuramine (50 μM), and the test inhibitors at concentrations of 0.003–100 μM . Stock solutions of the test inhibitors were prepared in DMSO and added to the reactions to yield a final concentration of

4% DMSO. Control reactions, containing 4% DMSO, were carried out in the absence of inhibitor. The enzyme reactions were initiated with the addition of MAO-A (0.0075 mg protein/mL) or MAO-B (0.015 mg protein/mL), incubated for 20 min at 37 °C in a convection oven and subsequently terminated with the addition of 80 μ L sodium hydroxide (2 N). At this endpoint, the concentration of 4-hydroxyquinoline, the oxidation product of kynuramine, was measured by fluorescence spectrophotometry ($\lambda_{\text{ex}} = 310$; $\lambda_{\text{em}} = 400$ nm) (Novaroli *et al.*, 2005). For this purpose, a linear calibration curve containing authentic 4-hydroxyquinoline (0.047–1.56 μ M) was constructed. From the concentration data, the MAO catalytic rates were calculated, which were fitted to the one site competition model of the Prism 5 software package (GraphPad). The IC₅₀ values were determined in triplicate and are expressed as mean \pm standard deviation (SD).

6.2.4. Molecular modelling

Molecular modelling was carried out with the Windows-based Discovery Studio 3.1 software package (Accelrys), with all applications within Discovery Studio set to their default values, unless otherwise specified. The X-ray crystal structures of human MAO-A (PDB code 2Z5X) (Son *et al.*, 2008) and human MAO-B (PDB code 1S2Q) (Binda *et al.*, 2004) were obtained from the Brookhaven Protein Data Bank. After calculating the pKa values and protonation states (at pH 7.4) of the ionisable amino acids, hydrogen atoms were added to the protein models. The correctness of the valences of the FAD cofactors (oxidized state) and co-crystallised ligands were verified, the protein models were automatically typed with the Momany and Rone CHARMM forcefield and a fixed atom constraint was applied to the protein backbone. The models were subsequently energy minimised using the Smart Minimiser protocol. For this purpose the maximum amount of steps was set to 50000 and the implicit generalised Born solvation model with molecular volume was employed. Following minimisation, the co-crystallised ligands and backbone constraints were removed from the models. With the exception of active site water molecules which are considered to be conserved, the waters were also removed from the models. Those retained are HOH 710, 718 and 739 in MAO-A, and HOH 614, 617, 619, 644, 769 and 811 in the A-chain of MAO-B. The structures of the ligands to be docked were drawn in Discovery Studio, their geometries were optimised using a Dreiding-like forcefield (5000 iterations) and the structures were submitted to the Prepare Ligands protocol. For the ligands, atom potential types and partial charges were calculated with the Momany and Rone CHARMM forcefield. After identifying the binding sites of the MAO enzymes from an analysis of the enzyme cavities, docking was carried out with CDOCKER, allowing for ten random ligand conformations, setting the heating target temperature to 700 K and employing full potential mode. The docking solutions were refined

using in situ ligand minimisation with the Smart Minimiser protocol and the illustrations were generated with PyMOL (DeLano, 2002).

6.3. Results

6.3.1. The synthesis of the propargylamine compounds

The propargylamine compounds (**1a–c** and **2a–d**) were synthesised by reaction of the appropriate amines [4-pyridylmethylamine, 2-pyridylmethylamine, 3-pyridylmethylamine and 2-(2-pyridyl)ethylamine] with propargyl bromide in tetrahydrofuran (Fig. 6.6). Triethylamine was added as base and the reactions were conducted in an inert atmosphere (Biedrzycki *et al.*, 1990). The reaction products were separated by silica gel column chromatography with the early fractions yielding the N,N-dipropargyl analogues **2a–d**, and the later fractions contained propargylamines **1a–c**. The yields of the target propargylamines ranged from 21.7 – 36.5%. The structures and purities of the compounds were confirmed by NMR and MS. For N,N-dipropargyl analogues **2a–d**, the ¹H NMR signals (singlets) of the propargyl CH₂ and terminal acetylenic CH protons integrated for 4H and 2H, respectively, while for **1a–c** an integration of 2H and 1H, respectively, was observed for these proton signals (singlets). On the ¹³C NMR spectra of **2a–d**, the acetylenic carbons presented at 73 and 78 ppm while for **1a–c** the signals of these carbons were observed at 71 and 81 ppm. The NMR spectra thus corresponded well with the proposed structures with respect to chemical shifts, multiplicity and integration values. The experimental high-resolution masses of the propargylamines also corresponded well with the calculated values.

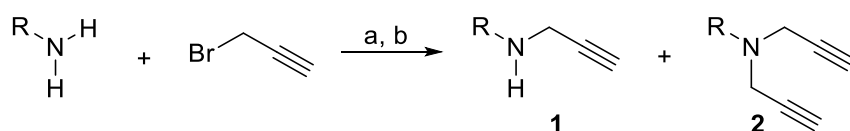


Figure 6.6: Synthetic route to the propargylamine compounds **1a–c** and **2a–d**. Key: (a) THF, triethylamine, 50 °C, 4 h, Ar; (b) silica gel column chromatography.

6.3.2. IC₅₀ values for the inhibition of human MAO

The propargylamine compounds were evaluated as potential inhibitors of human MAO. For this purpose, the recombinant human enzymes were used and kynuramine served as substrate. Kynuramine is a substrate for both MAO-A and MAO-B, and is metabolised to ultimately yield 4-hydroxyquinoline, a compound which may be measured by fluorescence spectrophotometry after alkalisation of the reaction mixtures. Employing this approach, the MAO-catalysed formation of 4-hydroxyquinoline in the presence of different inhibitor concentrations (0.003–100 μM) was measured, and sigmoidal plots of enzyme catalytic rate versus logarithm of inhibitor concentration were constructed. After fitting these data to the one

site competition model of the Prism 5 software package (GraphPad), IC_{50} values were estimated. For comparison, the irreversible MAO inhibitors, pargyline and selegiline, were included in this study as positive controls. The sigmoidal plots obtained in this study are provided in Fig. 6.7, and IC_{50} values are given in Table 6.1. The results show that, under the present experimental conditions, pargyline and selegiline are active MAO inhibitors. Pargyline displays IC_{50} values of 15.6 and 0.782 μM for the inhibition of MAO-A and MAO-B respectively while selegiline displays IC_{50} values of 29.7 and 0.095 μM . These values correspond well with those previously reported by us, with pargyline exhibiting an $IC_{50}(\text{MAO-A})$ of 13 μM and selegiline an $IC_{50}(\text{MAO-B})$ of 0.079 μM (Strydom *et al.*, 2012; Petzer *et al.*, 2012). The data also show that none of the propargylamine compounds (**1a–c** and **2a–d**) acted as MAO inhibitors and no inhibition was observed at a maximal tested concentration of 100 μM .

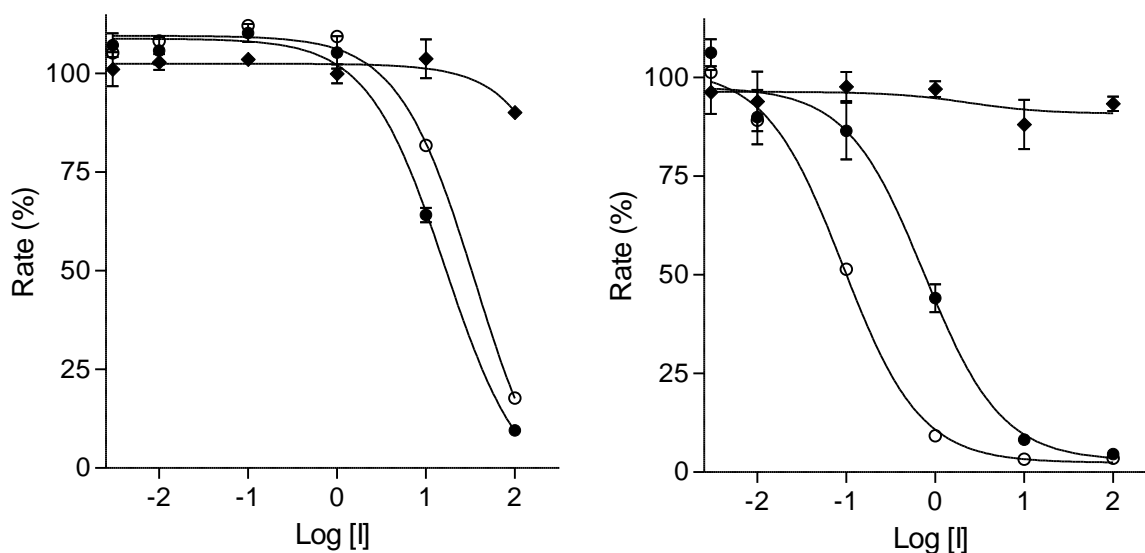
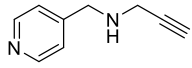
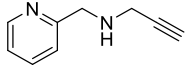
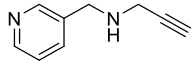
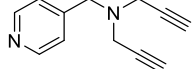
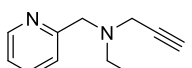
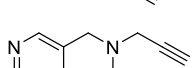

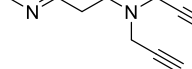
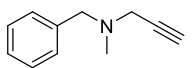


Figure 6.7: Sigmoidal curves for the inhibition of human MAO-A (left) and MAO-B (right) by pargyline (filled circles), selegiline (open circles) and **1a** (diamonds). Data points as shown as mean \pm SD.

Table 6.1: The human MAO inhibition potencies of **1a–c**, **2a–d** and reference inhibitors pargyline and selegiline.

		IC ₅₀ (μM) ^a		
		MAO-A	MAO-B	SI ^b
1a		NI ^c	NI	–
1b		NI	NI	–
1c		NI	NI	–
2a		NI	NI	–
2b		NI	NI	–
2c		NI	NI	–
2d		NI	NI	–
Pargyline		15.6 ± 0.424	0.782 ± 0.035	19.9
Selegiline		29.7 ± 1.44	0.095 ± 0.013	313

^a All values are expressed as the mean ± standard deviation (SD) of triplicate determinations.

^b The selectivity index is the selectivity for the MAO-B isoform and is given as the ratio of IC₅₀(MAO-A)/IC₅₀(MAO-B).

^c NI: no inhibition observed at a maximal concentration of 100 μM.

6.3.3. Molecular modelling

To gain insight into the differing MAO inhibition potencies of pargyline and the propargylamine compounds of this study, their reversible interactions with the MAOs were investigated on the molecular level. For the purpose of this study, **1a** and pargyline were docked into the active sites of human MAO-A and MAO-B using the CDOCKER docking algorithm of Discovery Studio 3.1 (Accelrys). The literature protocol was followed (Mostert *et al.*, 2015) using the reported crystal structure of human MAO-A (PDB code: 2Z5X) and human MAO-B (PDB code: 1S2Q) as enzyme models (Son *et al.*, 2008, Binda *et al.*, 2004). As shown in Fig. 6.8, both **1a** and pargyline adopt at least two binding orientations in the MAO-A active site, with the

propargylamine moieties directed either towards the FAD or towards the entrance of the active site. When the propargylamine moieties are directed towards the FAD, the amine is placed between residues Tyr-407 and Tyr-444, at the appropriate position for oxidation by the FAD. In this instance, the inhibitors will act as substrates and the resulting products would be able to covalently attach to the FAD. The most prominent stabilising interaction is hydrogen bonding between **1a** and Gln-215. When the propargylamine moieties are directed towards the entrance of the MAO-A active site, amine oxidation and irreversible enzyme inactivation are not possible. For these orientations, the most prominent interactions are π - π interactions of both inhibitors with Tyr-444. As shown in Fig. 6.9, the same situation exists for the interactions of **1a** and pargyline with MAO-B, and the inhibitors may adopt at least two possible orientations. When the propargylamine moieties are directed towards the FAD, the amine may be oxidised to yield a reactive intermediate that may covalently attach to the FAD. For this orientation, **1a** is hydrogen bonded to Gln-206 and an active site water. When the propargylamine moieties are directed towards the entrance of the active site, amine oxidation and irreversible enzyme inactivation are not possible. Stabilising interactions noted here are hydrogen bonding of **1a** with Gln-206 and a water molecule, and a π - π interaction of pargyline with Tyr-398. Although the modelling study does not provide an explanation for the absence of MAO inhibition by **1a**, it may be argued that the orientation where the propargylamine moiety of **1a** is directed away from the FAD is favoured compared to the placement of the propargyl in proximity to the FAD. This could possibly be attributed to stabilisation of the pyridyl nitrogen between the tyrosyl residues that constitutes the “aromatic sandwich”. Indeed, this is the site where the amine moieties of MAO substrates are predicted to bind (Binda *et al.*, 2002). With the pyridyl moiety located in the amine binding region, substrate oxidation and enzyme inactivation cannot occur. For pargyline, stabilisation of the phenyl ring by the tyrosyl residues of the “aromatic sandwich” may be less pronounced compared to the pyridyl containing compounds, and the orientation with the propargylamine moiety located in the amine binding region may thus be more probable. This would result in oxidation of pargyline and inactivation of the enzyme.

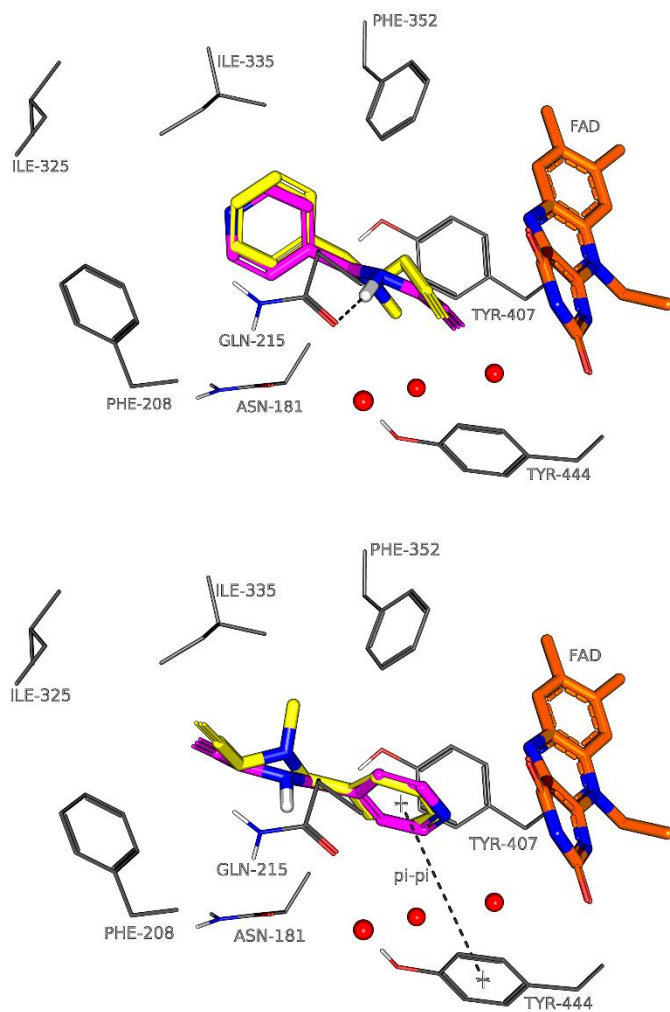
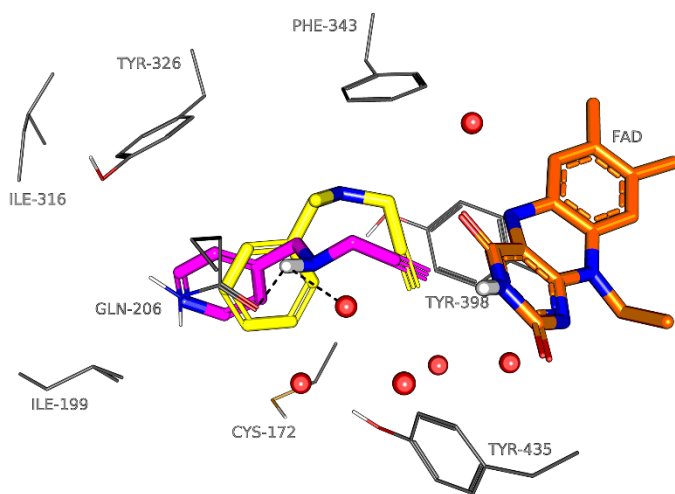


Figure 6.8: The proposed reversible interactions between pargyline (yellow) and **1a** (magenta), and MAO-A. The top figure shows the orientations of the inhibitors with the propargylamine moieties directed towards the FAD, while in the bottom figure these moieties are directed towards the entrance of the active site.



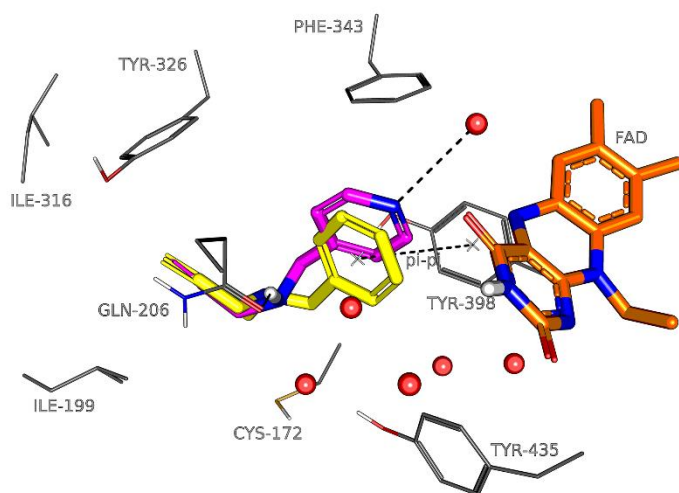


Figure 6.9: The proposed reversible interactions between pargyline (yellow) and **1a** (magenta), and MAO-B. The top figure shows the orientations of the inhibitors with the propargylamine moieties directed towards the FAD, while in the bottom figure these moieties are directed towards the entrance of the active site.

6.4. Conclusion

The present study evaluates a series of pyridyl-derived propargylamines as potential MAO inhibitors. Interestingly, the pyridyl-derived propargylamines do not inhibit either of the human MAO isoforms, while pargyline, a close structural analogue, is a nonspecific MAO inhibitor. Molecular docking suggests that the absence of MAO inhibition by the pyridyl inhibitors may be due to differing binding orientations in the MAO active sites compared to pargyline. In this respect, the pyridyl-derived inhibitors may favour binding with the propargylamine moiety distal from the FAD, which would not lead to oxidation of the inhibitor and inactivation of the enzyme. This orientation may be facilitated by stabilisation of the electron deficient pyridyl nitrogen within the amine binding region of the MAO enzymes. In contrast, the phenyl ring of pargyline may be stabilised to a lesser extent in the amine binding region, which may allow for the reversed orientations where the propargylamine moiety is placed within the amine binding region. This would lead to oxidation of pargyline and inactivation of the enzyme.

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Conflict of interest

The authors declare no conflicts of interest in this work.

References

1. Biedrzycki, M., de Bie, D.A. & van der Plas, H.C. 1990. Dihydroisobenzofurans and isoindolines by intramolecular inverse Diels-Alder reactions of pyridines. *Tetrahedron*, 46(2):607-614.
2. Binda, C., Hubálek, F., Li, M., Herzig, Y., Sterling, J., Edmondson, D.E. & Mattevi, A. 2004. Crystal structures of monoamine oxidase B in complex with four inhibitors of the N-propargylaminoindan class. *Journal of medicinal chemistry*, 47(7):1767-1774.
3. Binda, C., Newton-Vinson, P., Hubálek, F., Edmondson, D.E. & Mattevi, A. 2002. Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders. *Nature structural & molecular biology*, 9(1):22-26.
4. Bonnet, U. 2003. Moclobemide: therapeutic use and clinical studies. *CNS Drug*, 9(1):97-140.
5. Da Prada, M., Zürcher, G., Wüthrich, I. & Haefely, W.E. 1988. On tyramine, food, beverages and the reversible MAO inhibitor moclobemide. *Journal of neural transmission*, 26:31-56.
6. DeLano, W.L. 2002. The PyMOL molecular graphics system. DeLano Scientific, San Carlos, USA.
7. Edmondson, D.E., Binda, C., Wang, J., Upadhyay, A.K. & Mattevi, A. 2009. Molecular and mechanistic properties of the membrane-bound mitochondrial monoamine oxidases. *Biochemistry*, 48(20):4220-4230.
8. Edmondson, D.E., Mattevi, A., Binda, C., Li, M. & Hubálek, F. 2004. Structure and mechanism of monoamine oxidase. *Current medicinal chemistry*, 11(15):1983-1993.
9. Finberg, J.P. & Gillman, K. 2011. Selective inhibitors of monoamine oxidase type B and the "cheese effect". *International review of neurobiology*, 100:169-190.
10. Flockhart, D.A. 2012. Dietary restrictions and drug interactions with monoamine oxidase inhibitors: an update. *Journal of clinical Psychiatry*, 73(1):17-24.
11. Lum, C.T. & Stahl, S.M. 2012. Opportunities for reversible inhibitors of monoamine oxidase-A (RIMAs) in the treatment of depression. *CNS Spectrums*, 17(3):107-120.
12. Mostert, S., Petzer, A. & Petzer, J.P. 2015. Indanones as high-potency reversible inhibitors of monoamine oxidase. *ChemMedChem*, 10(5):862-873.
13. Novaroli, L., Reist, M., Favre, E., Carotti, A., Catto, M. & Carrupt, P.A. 2005. Human recombinant monoamine oxidase B as reliable and efficient enzyme source for inhibitor screening. *Bioorganic & medicinal chemistry*, 13(22):6212-6217.

14. Petzer, A., Harvey, B.H., Wegener, G. & Petzer, J.P. 2012. Azure B, a metabolite of methylene blue, is a high-potency, reversible inhibitor of monoamine oxidase. *Toxicology & applied pharmacology*, 258(3):403-409.
15. Provost, J.C., Funck-Brentano, C., Rovei, V., D'Estaque, J., Ego, D. & Jaillon, P. 1992. Pharmacokinetic and pharmacodynamic interaction between toloxatone, a new reversible monoamine oxidase-A inhibitor, and oral tyramine in healthy subjects. *Clinical pharmacology & therapeutics*, 52(4):384-393.
16. Ramsay, R.R. 2016. Molecular aspects of monoamine oxidase B. *Progress in neuro-psychopharmacol & biological Psychiatry*, 69:81-89.
17. Shih, J.C., Chen, K. & Ridd, M.J. 1999. Monoamine oxidase: from genes to behavior. *Annual review of neuroscience*, 22:197-217.
18. Sigman, D.S. 1992. The Enzymes, Volume 20: Mechanisms of Catalysis. 3rd ed. San Diego: Academic press. Inc. pp. 338-357
19. Son, S.Y., Ma, J., Kondou, Y., Yoshimura, M., Yamashita, E. & Tsukihara, T. 2008. Structure of human monoamine oxidase A at 2.2-A resolution: the control of opening the entry for substrates/inhibitors. *Proceedings of the national academy of science of the United States of America*, 105(15):5739-5744.
20. Strydom, B., Bergh, J.J. & Petzer, J.P. 2012. The inhibition of monoamine oxidase by 8-(2-phenoxyethoxy)caffeine analogues. *Arzneimittelforschung*, 62(11):513-518.
21. Youdim, M.B. & Bakhle, Y.S. 2006. Monoamine oxidase: isoforms and inhibitors in PD and depressive illness. *British journal of pharmacology*, 147(1):287-296.
22. Youdim, M.B., Edmondson, D. & Tipton, K.F. 2006. The therapeutic potential of monoamine oxidase inhibitors. *Nature reviews neuroscience*, 7(4):295-309.

Supplementary material

Figure S6.1. ¹H NMR and ¹³C NMR spectra 1a

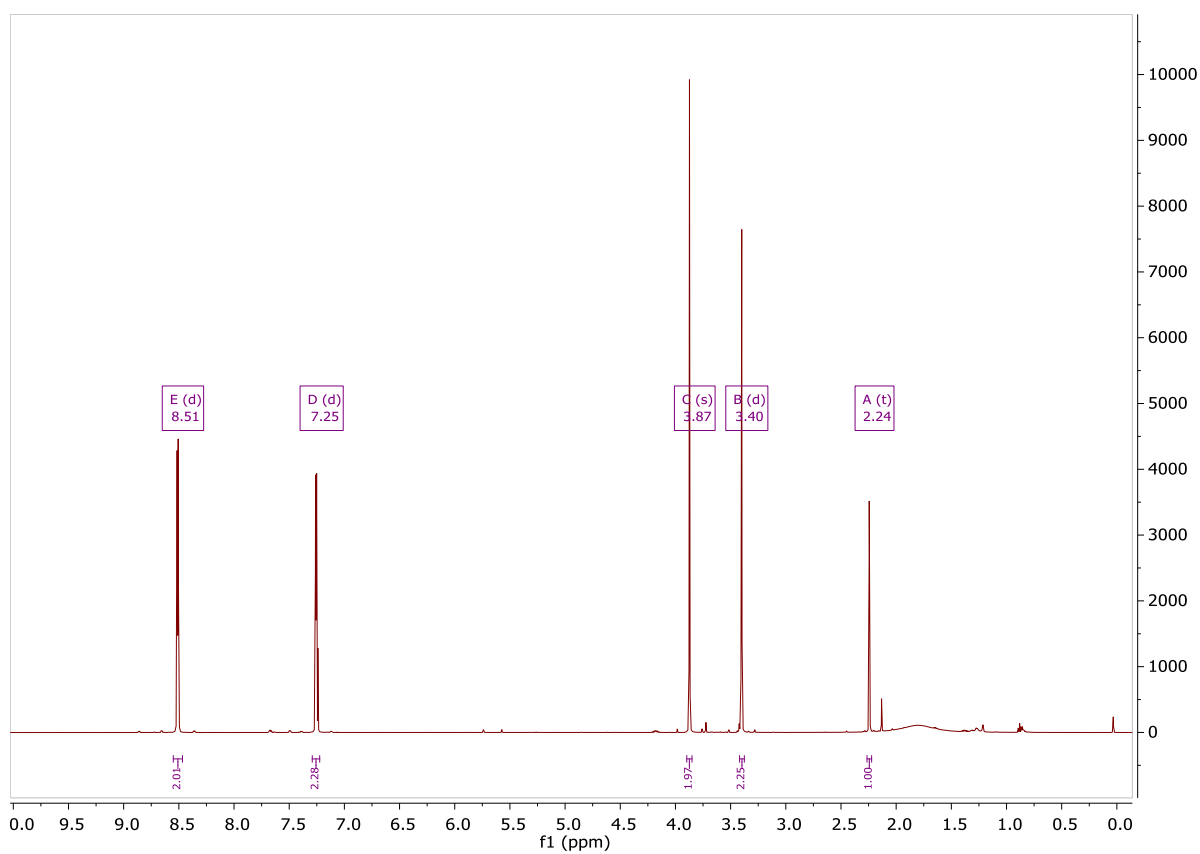
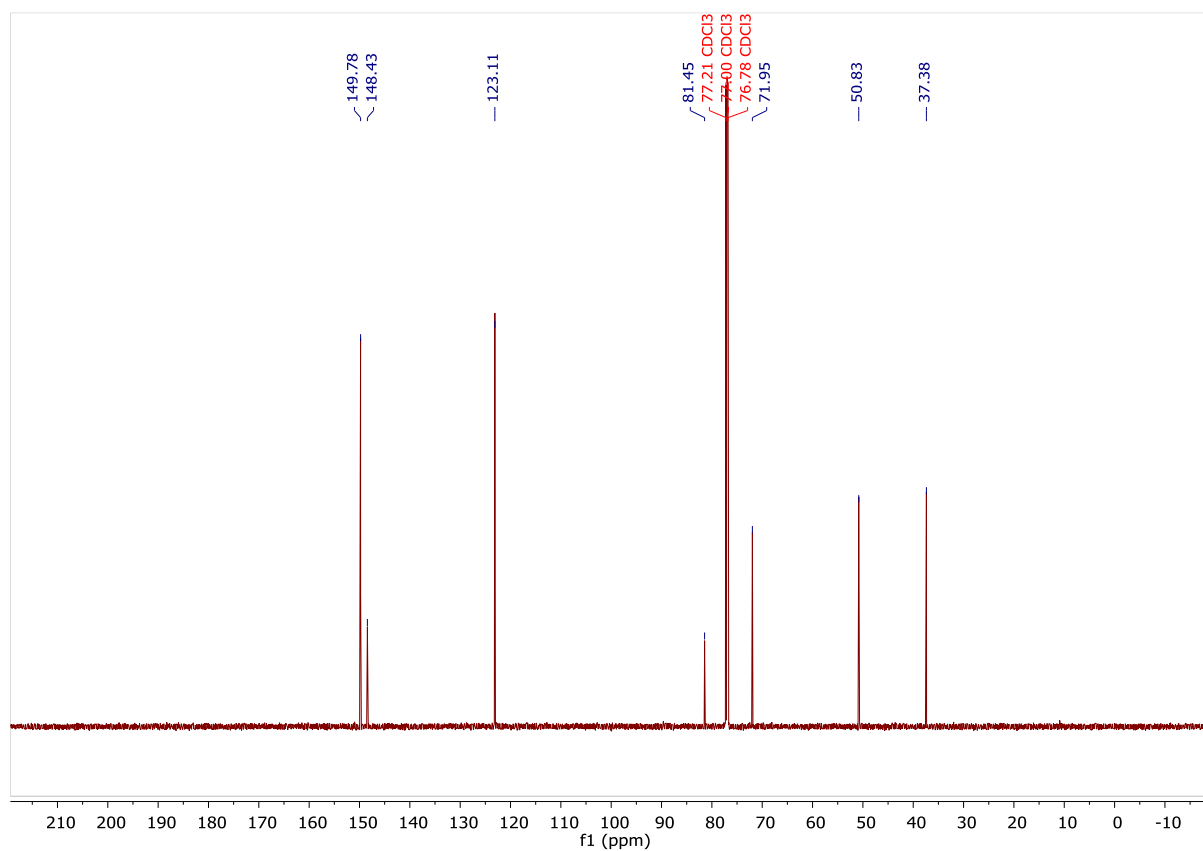


Figure S6.2. ^1H NMR and ^{13}C NMR spectra **1b**

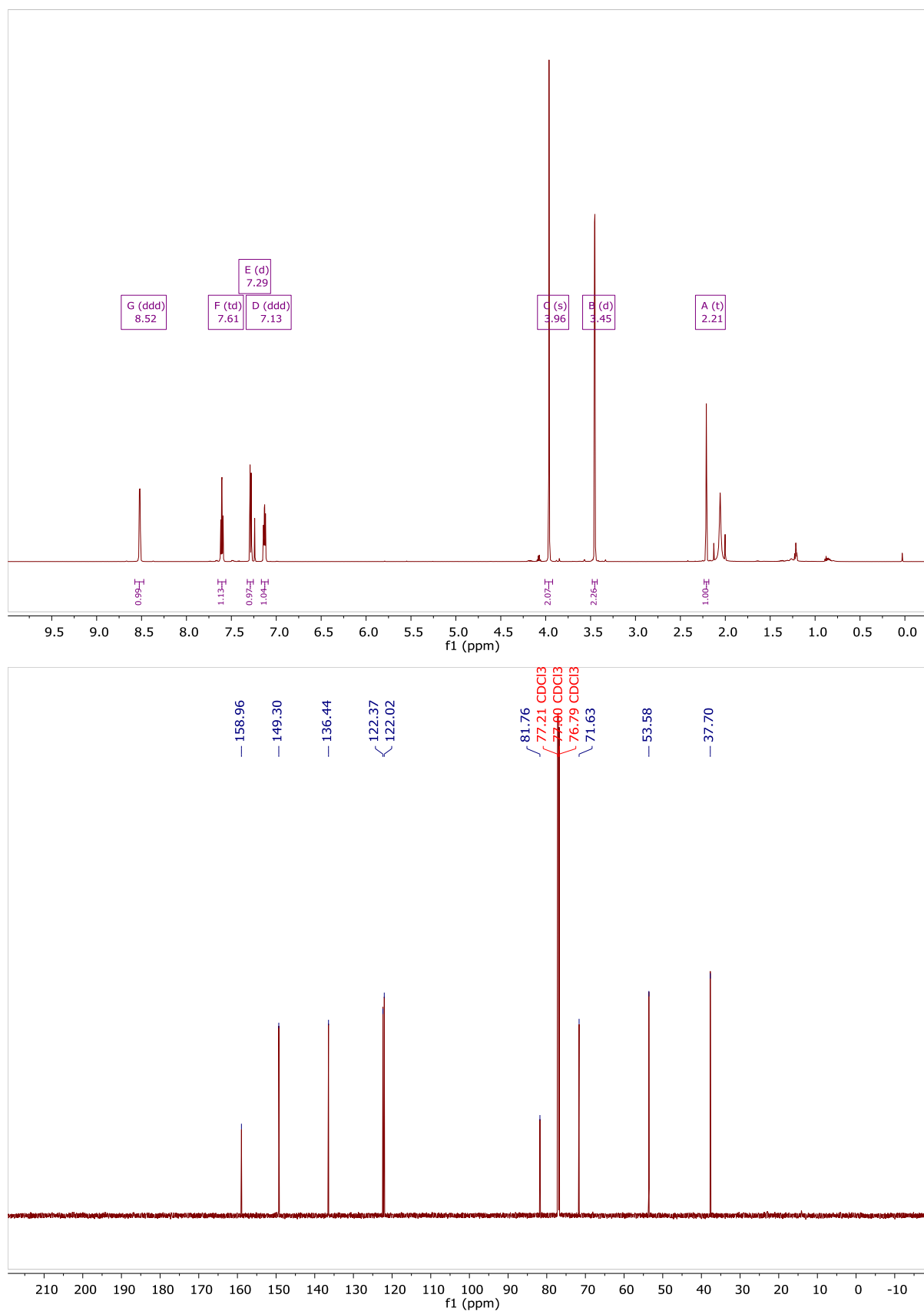


Figure S6.3. ¹H NMR and ¹³C NMR spectra 1c

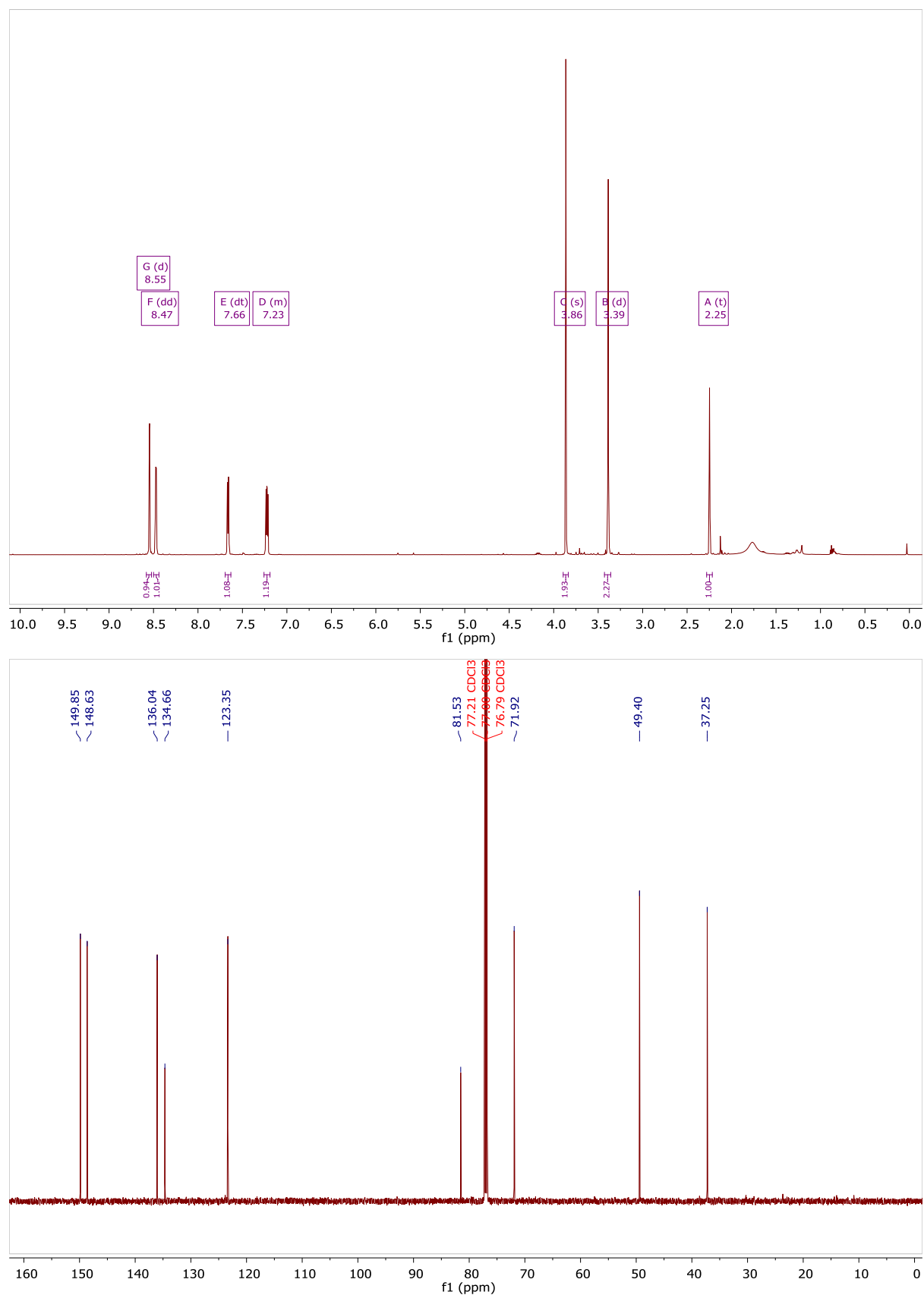


Figure S6.4. ^1H NMR and ^{13}C NMR spectra **2a**

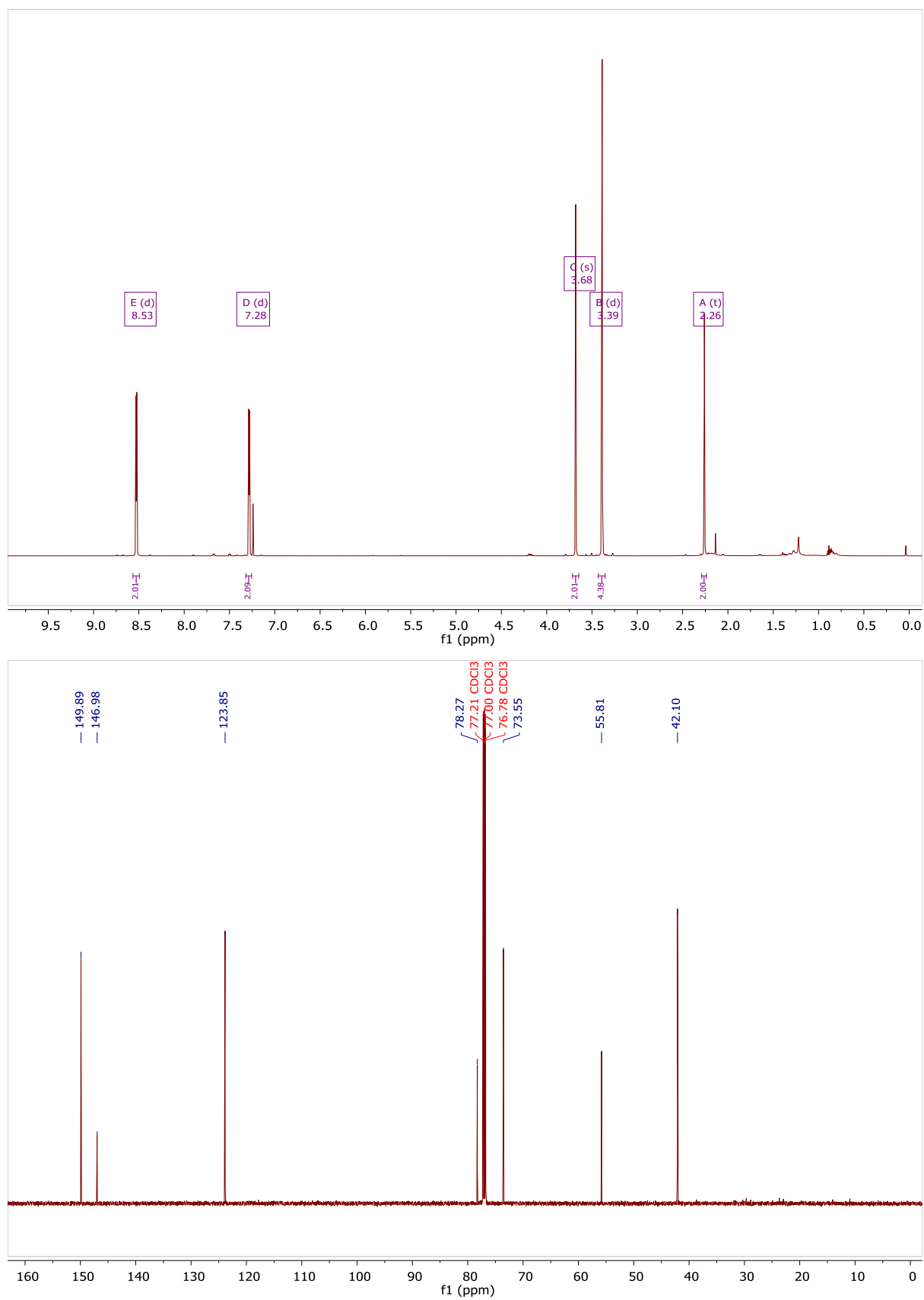


Figure S6.5. ¹H NMR and ¹³C NMR spectra 2b

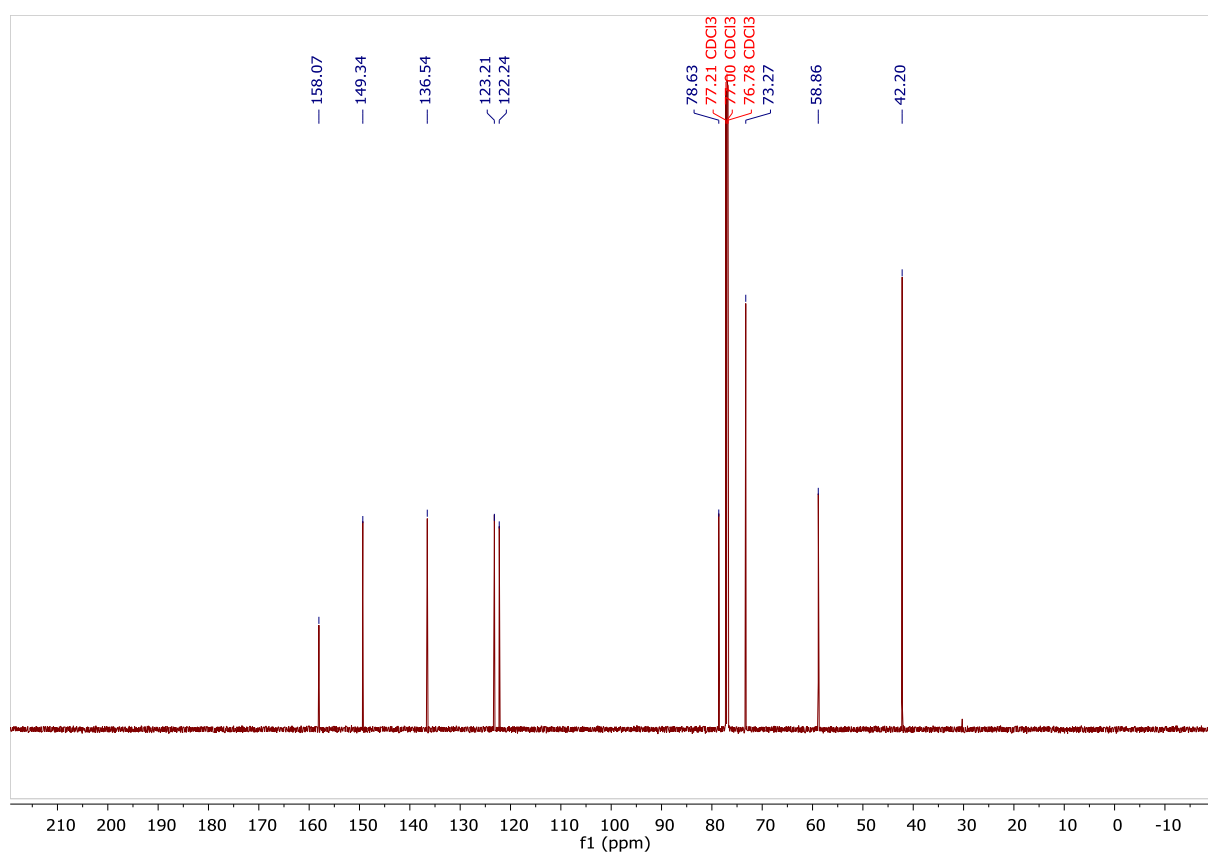
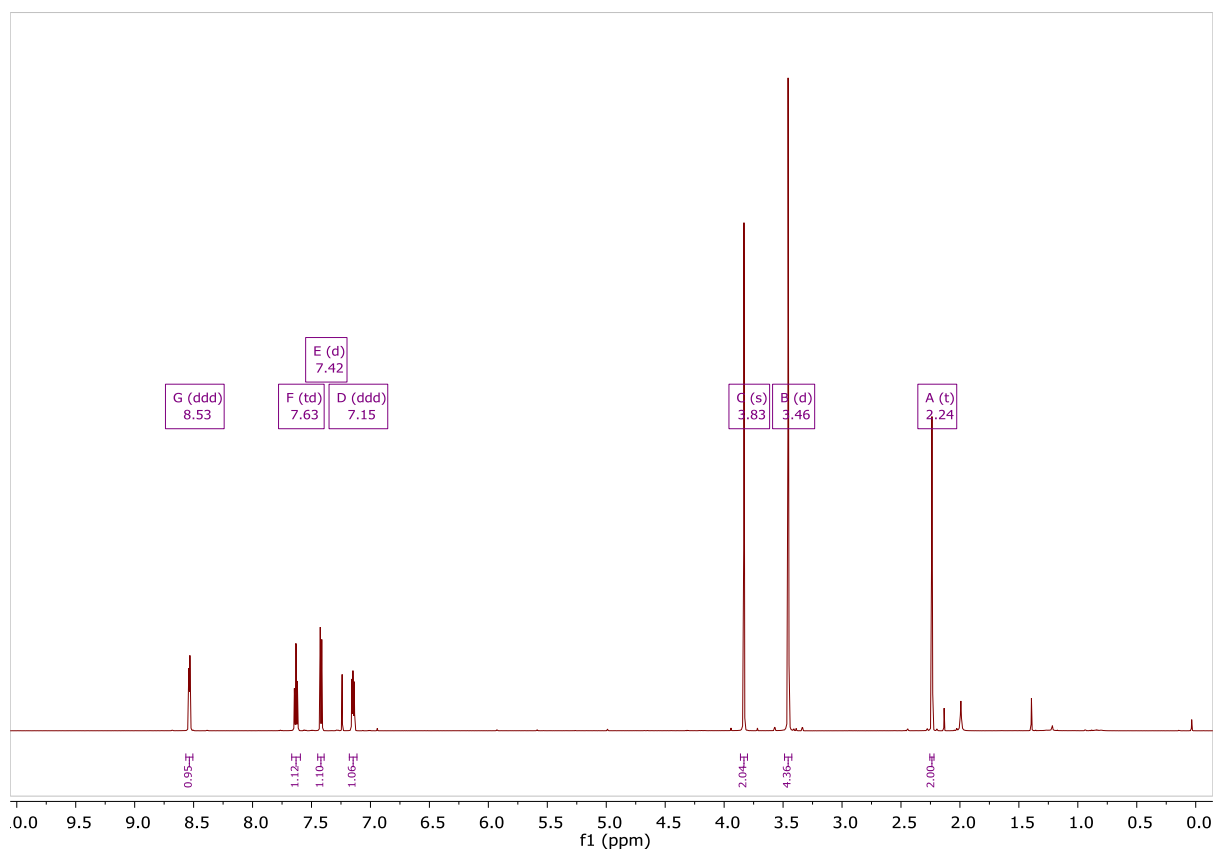


Figure S6.6. ¹H NMR and ¹³C NMR spectra 2c

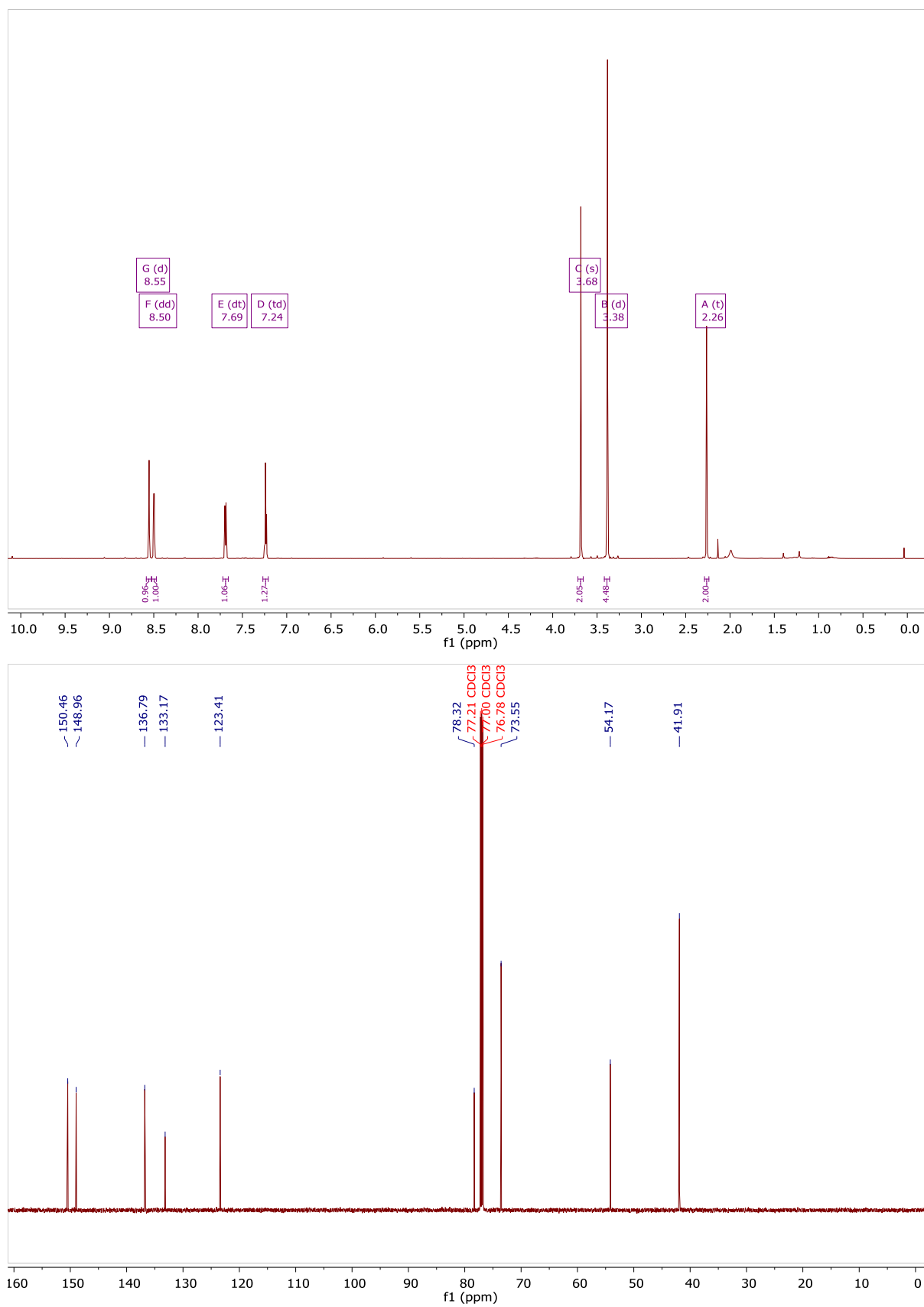
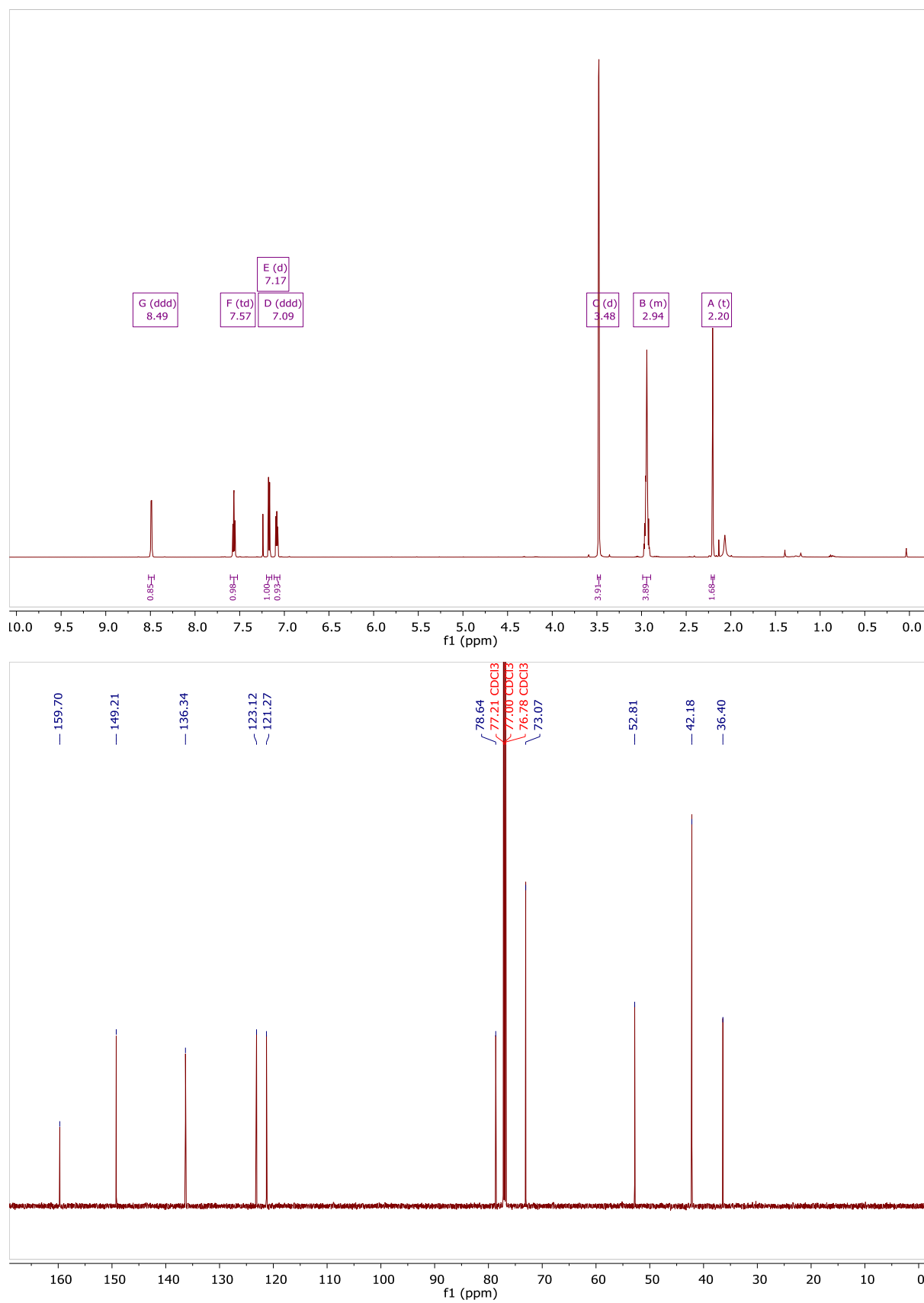


Figure S6.7. ¹H NMR and ¹³C NMR spectra 2d



Chapter 7

Conclusion

7.1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder, that progresses with time and affects approximately 1% of the over 60 population. The dopaminergic neurons of the nigrostriatal pathway degenerates specifically in PD. A functional deficit of dopamine (DA) in the striatum occurs since this neuronal pathway delivers DA to the striatum. PD symptoms such as, bradykinesia, muscle rigidity and resting tremors are mainly treated by L-dopa. As the disease progresses, sensitivity towards L-dopa decreases. The oral bioavailability of L-dopa is estimated to be approximately 10% with less than 1% of this administered oral dose reaching the brain unchanged. In this study, we attempted to overcome the problems with peripheral L-dopa metabolism, delivery difficulties and insufficient conversion of L-dopa to DA by designing novel carrier mediated L-dopa prodrugs.

In the present study, we have designed a L-dopa-lazabemide prodrug as well as four novel carrier-linked prodrugs by conjugating L-dopa at the carboxylate functionality with the primary aminyl functional group of the carrier molecule via an amide. The principal rationale for the design of the prodrugs was to protect the carboxylic acid of L-dopa against peripheral decarboxylation. It was also envisioned that the prodrugs may possess enhanced membrane permeability via passive diffusion compared to L-dopa. The fact that the carrier molecules and L-dopa are linked via an amide was an advantage because of the increased stability of the amide link compared to the more traditional ester between a drug and its carrier. This should, in theory, allow more time for the prodrugs to diffuse into the brain prior to the hydrolysis event and release of L-dopa. We also investigated the monoamine oxidase (MAO) inhibition properties of lazabemide, as the mechanism of action for this drug is not well reported. By characterising the *in vitro* interaction of lazabemide with human MAO-B, the present study may contribute to knowledge of this compound's mechanism of action. Lastly, we designed a series of pyridyl-derived propargylamine compounds as potential MAO inhibitors. Pyridyl-derived propargylamines have not yet been investigated as potential MAO inhibitors.

7.2. Specific findings and conclusions

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

- The design of an L-dopa-lazabemide prodrug – An L-dopa-lazabemide prodrug was designed in which L-dopa was linked at the carboxylate with the primary aminyl functional group of lazabemide. Analyses of the properties of the prodrug show that the prodrug possesses appropriate lipophilicity (logD) and solubility profiles for oral absorption, although passive diffusion permeability, as evaluated by PAMPA, is comparatively low. Regardless of this, the prodrug displays higher toxicity to cultured cells than L-dopa, possibly indicating higher intracellular exposure of the prodrug as a result of better permeability. Further experiments show that the prodrug is stable towards hydrolysis in aqueous buffer and undergoes slow activation in plasma and tissue (liver and brain). Although oral and i.p. treatment of mice with the prodrug did not result in enhanced striatal dopamine levels, DOPAC levels were significantly depressed compared to saline, L-dopa and carbidopa/L-dopa treatment. This suggests that the prodrug may reduce dopamine metabolism, most likely as a result of MAO-B inhibition by lazabemide. After i.p treatment with L-dopa, DOPAC levels are significantly increased, likely due to increased central dopamine in response to L-dopa. This behaviour is not observed with the prodrug as lazabemide is expected to block the metabolic route leading from dopamine to DOPAC.
- The characterisation of the MAO inhibition properties of lazabemide – In this section of the study, the *in vitro* MAO inhibition properties of lazabemide with respect to potency, isoform selectivity and reversibility were investigated. Due to its unique mechanism of action, lazabemide results in rapid and complete MAO-B inhibition, which highlight its potential efficacy in the *in vivo* setting. A further point of interest is that lazabemide is a highly specific inhibitor of MAO-B over the MAO-A isoform. Considering its reversibility of *in vivo* inhibition, potential high efficacy and complete MAO-B inhibition, and high specificity, lazabemide may be considered an ideal MAO-B inhibitor for the treatment of neurodegenerative disorders such as Alzheimer's disease and PD. Although the development of lazabemide has been discontinued, related compounds in this class may still be considered for future development as MAO-B specific inhibitors. Furthermore, lazabemide may still be useful for experimental work and a clear understanding of its mechanism of action is therefore required. This study found that lazabemide is a selective inhibitor of human MAO-B with an IC₅₀ value of 0.091 µM. For human MAO-A, lazabemide exhibits an IC₅₀ of

>100 μM . Interestingly, *in vitro* MAO-B inhibition persists and lazabemide may be viewed as an irreversible MAO-B inhibitor.

- The design of four novel carrier-linked L-dopa prodrugs – To improve the oral bioavailability, metabolic profile and brain delivery of L-dopa, this study designed four carrier-linked prodrugs of L-dopa in which 4-pyridylmethylamine, 2-(4-pyridyl)ethylamine, 2-(2-pyridyl)ethylamine and 3-phenyl-1-propylamine were linked to the carboxylate of L-dopa. The results document that all prodrugs are highly hydrophilic, which may limit membrane permeability and thus absorption from the gastrointestinal tract and penetration of the blood-brain barrier. The hydrophilicity of the prodrugs may, at least in part, be attributed to protonation of the amine groups and to a lesser extent protonation of the pyridyl moieties (at lower pH values). In spite of this, the four prodrugs exhibit improved permeability compared to L-dopa, with prodrug **11** being in excess of 100-fold more permeable than L-dopa at certain pH values. Notably, this prodrug does not contain the pyridyl moiety and thus has one less ionisation centre compared to the other prodrugs evaluated and is also the least hydrophilic. The observation that the prodrugs are more permeable compared to L-dopa is supported by the finding that two prodrugs evaluated are more toxic than L-dopa to cultured cells, possibly due to higher membrane permeabilities which would result in higher intracellular concentrations. Chemical stability experiments show that none of the prodrugs are labile at several pH values, and that the amide link between L-dopa and the carrier molecules is relatively stable to chemical hydrolysis. In contrast, in plasma, three of the prodrugs (**9–11**) undergo a time-dependent decrease in concentration, probably due to enzymatic hydrolysis and activation of the prodrugs. Prodrug **8**, however, appears to be stable in plasma. In liver tissue, the prodrugs (as shown with **10** and **11**) also display a time-dependent decrease in concentration, while notably in the brain tissue, only **8** undergoes a time-dependent decrease in concentration. In conclusion, these data suggest that the prodrug approach may indeed enhance the permeability of L-dopa, yielding compounds that are stable towards chemical hydrolysis. As exemplified by **8**, certain prodrugs may also be stable towards plasma hydrolysis while readily undergoing activation in brain tissue. With such a property profile, compound **8** would in theory undergo limited peripheral hydrolysis thus allowing more time for the prodrug to penetrate into the brain tissue. Once in the brain, the prodrug may undergo the necessary activation to deliver L-dopa in increased concentrations and possibly for a sustained period of time.
- The synthesis of propargylamine MAO inhibitors – The present study synthesises a small series of novel propargylamine compounds that incorporate the pyridyl moiety. Pyridyl-derived propargylamines have not yet been investigated as potential MAO

inhibitors. Interestingly, the pyridyl-derived propargylamines do not inhibit either of the human MAO isoforms, while pargyline, a close structural analogue, is a nonspecific MAO inhibitor. Molecular docking suggests that the absence of MAO inhibition by the pyridyl inhibitors may be due to differing binding orientations in the MAO active sites compared to pargyline. In this respect, the pyridyl-derived inhibitors may favour binding with the propargylamine moiety distal from the FAD, which would not lead to oxidation of the inhibitor and inactivation of the enzyme. This orientation may be facilitated by stabilisation of the electron deficient pyridyl nitrogen within the amine binding region of the MAO enzymes.

7.3. Future recommendations

1. Based on the results obtained with the L-dopa-lazabemide prodrug, further preclinical evaluation of the prodrug should be undertaken with the aim of discovering prodrugs that may be advanced to the clinical stages of development.
2. Based on the potency, isoform selectivity and *in vivo* reversibility of MAO-B inhibition, lazabemide analogues should be reconsidered as potential MAO-B inhibitors for the treatment of PD.
3. Further investigation of the carrier-linked L-dopa prodrugs, especially **8** should be undertaken. In this respect, the ability of the prodrug to deliver L-dopa to the brain should be evaluated *in vivo*.

Addendum 1

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Kind regards

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7/12/2017

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To: Monique Hoon <moniquehoon74@gmail.com>

Fri, Jul 7, 2017 at 10:06 AM

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