

**Synthesis and evaluation of nitrocatechol
derivatives of chalcone as inhibitors of
monoamine oxidase and
catechol-O-methyltransferase**

R Hitge

 **orcid.org/ 0000-0002-3335-7095**

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Supervisor: Prof. A. Petzer

Co-supervisor: Prof. J.P. Petzer

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Student number: 24226165

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DECLARATION

This dissertation is submitted in fulfilment of the requirements for the degree *Master of Science in Pharmaceutical Chemistry*, at the North-West University, Potchefstroom campus.

I the undersigned, Rialette Hitge, hereby declare that the dissertation with the title: “**Synthesis and evaluation of nitrocatechol derivatives of chalcone as inhibitors of monoamine oxidase and catechol-O-methyltransferase**” is my own work and has not been submitted at any other University either whole or in part.



Rialette Hitge

Signed at Potchefstroom on the 27th day of May 2019.

LETTER OF PERMISSION



School of Pharmacy
Department of Pharmaceutical Chemistry
Tel: 018-299 2260
Tel: 018-299 4464
Email: 12264954@nwu.ac.za
Email: jacques.petzer@nwu.ac.za

North-West University, Potchefstroom
South Africa, 2520
Tel: 018 299-1111/2222
Fax: 018 299-4910
Web: <http://www.nwu.ac.za>

27 May 2019

To whom it may concern

Dear Sir/Madam,

CO-AUTHORSHIP ON RESEARCH ARTICLE

The undersigned are co-authors of the research article listed below, and hereby give permission to Miss R. Hitge to submit this article as part of the degree *Magister Scientiae in Pharmaceutical Chemistry* at the North-West University (NWU) Potchefstroom campus:

- **Synthesis and evaluation of nitrocatechol derivatives of chalcone as inhibitors of monamine oxidase and catechol-O-methyltransferase**

Yours sincerely,

Prof. A. Petzer

Prof. J.P. Petzer

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“So do not fear, for I am with you; do not be dismayed, for I am your God. I will strengthen you and help you; I will uphold you with my righteous right hand.”

Isaiah 41:10

ABSTRACT

Parkinson's disease is a progressive neurological movement disorder that worsens with age. Parkinson's disease is still the most frequent neurodegenerative disorder after Alzheimer's disease. There is no known cause of Parkinson's disease, but in some cases there may be non-genetic or genetic risk factors. The non-genetic risk factors include environmental factors and exposure to organic solvents, carbon monoxide, carbon disulphide and pesticides. An example of a compound that induces a Parkinsonian syndrome in humans and animals is the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which selectively damages the dopaminergic cells in the substantia nigra. Genetic mutations in genes such as DJ-1, PINK1 and LRRK-2 can cause familial Parkinson's disease.

The main pathological features of Parkinson's disease are the degeneration and the loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of 'Lewy-bodies' in the brain. The SNpc forms part of the basal ganglia which coordinates muscle movement through the direct and indirect pathways, and connects with the motor cortex. The substantia nigra can be divided into two regions. The first region is the pars reticulata, which receives signals from the striatum, and send signals to the thalamus via the neurotransmitter, GABA (gamma-aminobutyric acid). The motor cortex receives these signals from the thalamus that initiates voluntary muscle movement. The second region is the pars compacta, which is the area that is mostly affected in Parkinson's disease. The pars compacta send signals to the striatum via the neurotransmitter, dopamine, forming the nigrostriatal pathway that stimulates the cerebral cortex and initiates movement. When the SNpc neurons die, muscle movement cannot be initiated via the direct pathway, and a decrease in movement in the indirect pathway cannot be reduced, thus resulting in slow muscle movement.

The two isoenzymes of monoamine oxidase (MAO) are MAO-A and MAO-B. They are both flavoenzymes which are responsible for the catalysis of the oxidative deamination of biogenic amines and amine neurotransmitters such as dopamine, serotonin and noradrenaline. The MAO enzyme metabolises dopamine after it has been produced in the brain and thus reduces binding of dopamine to the dopamine receptor. MAO inhibitors will bind to MAO and reduce the central metabolism of dopamine. With more dopamine available to bind to dopamine receptors in the brain, dopaminergic neurotransmission is enhanced. MAO inhibitors thus reduce dopamine depletion in the striatum of the brain. MAO-B activity in the brain increases with age and the activity is furthermore higher in the brain tissue of Parkinson's disease patients, which further depletes central dopamine.

The main clinical features of Parkinson's disease include resting tremor, rigidity, postural instability and bradykinesia. The treatment of these motor symptoms are mainly based on the re-establishment of striatal dopaminergic neurotransmission, which may be achieved by increasing the dopamine supply through levodopa administration. Levodopa is still considered the most effective treatment for Parkinson's disease. Levodopa can be administered orally and enters the systemic circulation. In the periphery, levodopa will be metabolised to dopamine by the enzyme aromatic L-amino acid decarboxylase (AADC), while catechol-O-methyltransferase (COMT) will metabolise dopamine to yield to 3-O-methyldopa. These metabolic reactions further reduce the amount of levodopa available to cross the blood-brain barrier. AADC inhibitors (carbidopa and benserazide), and COMT inhibitors (tolcapone and entacapone) will block the peripheral action of AADC and COMT, thereby reducing the conversion of levodopa to dopamine. A larger fraction of levodopa is thus available to cross the blood-brain barrier. In the central nervous system, levodopa is taken up by the nigrostriatal dopaminergic neurons, and metabolised by AADC to dopamine. MAO-B inhibitors (e.g. selegiline) and COMT inhibitors (e.g. tolcapone) prevent dopamine metabolism in the brain. The overall effect of these inhibitors is to increase the amount of dopamine available to bind to dopamine receptors in the corpus striatum, thus increasing motor activity.

The current treatments that are available for Parkinson's disease focus mostly on the management of symptoms, while there are only a few drugs available on the market for the treatment of Parkinson's disease. New treatment strategies need to be developed, and this dissertation will attempt to contribute by synthesising novel compounds that may inhibit both MAO and COMT.

In the current study we synthesised three novel nitrocatechol derivatives of chalcone as well as their corresponding pyrazoline derivatives, and investigated their MAO and COMT inhibition potencies. The inhibition potencies were expressed as IC_{50} values, and the results indicated that both the chalcone and pyrazoline derivatives are high potency inhibitors of rat liver COMT. The pyrazoline derivatives (IC_{50} = 0.048-0.079 μ M) are more potent COMT inhibitors than the chalcones (IC_{50} = 0.175-0.240 μ M). The most potent COMT inhibitor among the pyrazoline derivatives is 4-[1-acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1*H*-pyrazol-5-yl]benzonitrile, which possesses an IC_{50} value of 0.048 μ M. Furthermore, the six newly synthesised compounds are more potent COMT inhibitors compared to the reference COMT inhibitors, tolcapone (IC_{50} = 0.26 μ M) and entacapone (IC_{50} = 0.25 μ M).

The chalcone and pyrazoline derivatives were also evaluated as potential inhibitors of MAO-A and MAO-B with the aim of discovering compounds with dual inhibitory activity towards MAO and COMT. Unfortunately, the chalcone and pyrazoline derivatives that were investigated in this study were weak inhibitors for both MAO-A and MAO-B. Even though the compounds showed weak

inhibition for MAO, the pyrazoline derivatives should be further studied for their potent COMT inhibition activities, as they may represent potentially clinically valuable inhibitors of COMT.

KEYWORDS: Parkinson's disease, levodopa, dopamine, MPTP, AADC, monoamine oxidase, MAO, catechol-O-methyltransferase, COMT, inhibition, pyrazoline, chalcone, tolcapone, entacapone

UITTREKSEL

Parkinson se siekte is 'n progressiewe neurologiese bewegingsversteuring wat met ouderdom vererger. Parkinson se siekte is steeds die mees algemene neurodegeneratiewe siekte na Alzheimer se siekte. Daar is geen bekende oorsaak van Parkinson se siekte nie, maar in sommige gevalle mag daar nie-genetiese of genetiese risikofaktore wees. Die nie-genetiese risikofaktore sluit omgewingsfaktore in, asook blootstelling aan organiese oplosmiddels, koolstofmonoksied, koolstofdissulfied en onkruidodors. Die neurotoksien, 1-metiel-4-feniel-1,2,3,6-tetrahidropiridien (MPTP), is 'n voorbeeld van 'n verbinding wat Parkinsonisme by mense en diere veroorsaak en die dopaminergiese selle in die “substantia nigra” selektief beskadig. Genetiese mutasies in gene soos DJ-1, PINK1 en LRRK-2 kan familiële Parkinson se siekte veroorsaak.

Die belangrikste patologiese kenmerke van Parkinson se siekte is die degenerasie en verlies van die dopaminergiese neurone in die “substantia nigra pars compacta” (SNpc) en die teenwoordigheid van “Lewy-liggame” in die brein. Die SNpc is deel van die basale ganglia wat spierbeweging deur die direkte en indirekte weë koördineer, en verbind met die motoriese korteks. Die “substantia nigra” kan in twee dele verdeel word. Die eerste deel is die “pars reticulata”, wat seine ontvang vanaf die striatum, en seine na die talamus stuur via die neurotransmitter, GABA (gamma-aminobottersuur). Die motoriese korteks ontvang hierdie seine vanaf die talamus wat vrywillige spierbeweging inisieer. Die tweede gedeelte is die “pars compacta”, die area wat die meeste geaffekteer is in Parkinson se siekte. Die “pars compacta” stuur seine na die striatum via die neurotransmitter, dopamien, wat die nigrostriatale baan vorm en die serebrale korteks stimuleer om beweging te inisieer. Wanneer die SNpc-neurone sterf, kan spierbeweging nie geïnisieer word deur die direkte weg nie, en 'n afname in beweging in die indirekte weg kan nie verminder word nie. Dit lei tot stadige spierbeweging.

Die twee isoënsieme van monoamienoksidase (MAO) is MAO-A en MAO-B. Hulle is albei flavoënsieme wat verantwoordelik is vir die oksidatiewe deaminering van biologiese amiene en amien-neurotransmitters soos dopamien, serotonien en noradrenalin. Die MAO-ensieme metaboliseer dopamien nadat dit in die brein geproduseer is, en verminder so die binding van dopamien aan die dopamienreseptor. MAO-inhibeerders bind aan MAO en verminder die sentrale metabolisme van dopamien. Met meer dopamien beskikbaar om aan dopamienreseptore in die brein te bind, word dopaminergiese neurotransmissie verbeter. MAO-inhibeerders verhoog dus dopamienkonsentrasies in die striatum van die brein. MAO-B-aktiwiteit in die brein styg met ouderdom en die aktiwiteit is verder hoër in die breinweefsel van pasiënte met Parkinson se siekte, wat sentrale dopamienkonsentrasies verder verlaag.

Die belangrikste kliniese kenmerke van Parkinson se siekte sluit rustende bewing, rigiditeit, posturale onstabiliteit en bradikinese in. Die behandeling van hierdie motoriese simptome berus hoofsaaklik op die herstelling van striatale dopaminergiese neurotransmissie, wat bereik kan word deur die verhoging van dopamienkonsentrasies deur middel van behandeling met levodopa. Levodopa word steeds as die doeltreffendste behandeling vir Parkinson se siekte beskou. Levodopa kan oraal toegedien word en die sistemiese sirkulasie bereik. In die periferie sal levodopa gemetaboliseer word deur die ensiem, aromatiese L-aminosuurdekarboksilase (AADC) om dopamien te lewer, terwyl katesjol-O-metieltransferase (KOMT) dopamien metaboliseer om 3-O-metieldopa te lewer. Hierdie metaboliese reaksies verminder dus die hoeveelheid levodopa wat beskikbaar is om die bloedsreinskanse te kruis. AADC-inhibeerders (karbidopa en benserazied) en KOMT-inhibeerders (tolkapoon en entakapoon) sal die perifere werking van AADC en KOMT blokkeer, en sodoende die omskakeling van levodopa na dopamien verminder. 'n Groter fraksie van levodopa is dus beskikbaar om die bloedsreinskanse te kruis. In die sentrale senuweestelsel word levodopa deur die nigrostriatale dopaminergiese neurone opgeneem, en word deur AADC na dopamien gemetaboliseer. MAO-B-inhibeerders (bv. selegilien) en KOMT-inhibeerders (bv. tolkapoon) voorkom dopamien metabolisme in die brein. Die effek van hierdie inhibeerders is om die hoeveelheid dopamien wat beskikbaar is vir binding aan die dopamienreseptore in die "corpus striatum", te verhoog en sodoende motoriese aktiwiteit te verhoog.

Die huidige behandeling wat vir Parkinson se siekte beskikbaar is fokus hoofsaaklik op die behandeling van simptome, en daar is slegs 'n paar geneesmiddels wat beskikbaar is op die mark vir die behandeling van Parkinson se siekte. Nuwe behandelingstrategieë moet ontwikkel word, en hierdie verhandeling sal poog om by te dra deur nuwe verbindings te sintetiseer wat beide MAO en KOMT kan inhibeer.

Hierdie studie sintetiseer drie nuwe nitrokatesjolderivate van chalkone sowel as hul ooreenstemmende pirasolienderivate, en ondersoek hulle MAO- en KOMT-inhibisie eienskappe. Die potensie van inhibisie is uitgedruk as IC_{50} -waardes, en die resultate het aangedui dat beide die chalkoon- en pirasolienderivate potente inhibeerders van rotlewer-KOMT is. Die pirasolienderivate ($IC_{50} = 0.048\text{--}0.079\ \mu\text{M}$) is meer potente KOMT-inhibeerders as die chalkone ($IC_{50} = 0.175\text{--}0.240\ \mu\text{M}$). Die beste KOMT-inhibeerder is 4-[1-asetiel-3-(3,4-dihidroksie-5-nitrofeniel)-4,5-dihidro-1*H*-pirasol-5-yl]bensonitriël, wat 'n IC_{50} -waarde van $0.048\ \mu\text{M}$ het. Verder is die ses nuut gesintetiseerde verbindings meer potente KOMT-inhibeerders as die bekende KOMT-inhibeerders, tolkapoon ($IC_{50} = 0.26\ \mu\text{M}$) en entakapoon ($IC_{50} = 0.25\ \mu\text{M}$).

Die chalkoon- en pirasolienderivate is ook as potensiële inhibeerders van MAO-A en MAO-B geëvalueer met die doel om verbindings wat beide MAO en KOMT inhibeer, te ontdek. Ongelukkig is die chalkoon- en pirasolienderivate wat in hierdie studie ondersoek is, swak inhibeerders van

beide MAO-A en MAO-B. Alhoewel die verbindings swak inhibisie vir MAO toon, moet die pirasolienderivate verder bestudeer word vir hul potente KOMT-inhibisie aktiwiteite, aangesien dit moontlik in die toekoms waardevolle inhibeerders van KOMT kan lewer.

SLEUTELWOORDE: Parkinson se siekte, levodopa, dopamien, MPTP, AADC, monoamienoksidase, MAO, katesjol-O-metielltransferase, KOMT, inhibisie, pirasolien, chalkoon, tolkapoon, entakapoon

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LIST OF ABBREVIATIONS

ABBREVIATION:	DESCRIPTION:
°C	Degree Celsius
¹³ C-NMR	Carbon - Nuclear magnetic resonance
¹ H-NMR	Proton - Nuclear magnetic resonance
3-MT	3-Methoxytyramine
Å	Angstrom
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength
μ L	Microliter
μ M	Micromolar
A	
AADC	Aromatic L-amino acid decarboxylase
AdoHcy	S-adenosyl-L-homocysteine
AdoMet	S-adenosyl-L-methionine
Ala	Alanine
AlCl ₃	Aluminium chloride
APCI	Atmospheric-pressure chemical ionisation
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
B	
Bcl-2	B-cell lymphoma 2
C	
Ca ²⁺	Calcium ion
CNS	Central nervous system
CO ₂	Carbon dioxide
COMT	Catechol-O-methyltransferase
CYP2C19	Cytochrome P450 2C19
CYP2D6	Cytochrome P450 2D6
Cys	Cysteine
D	
d	Doublet
DA	Dopamine
DAT	Dopamine transporter
dd	Doublet of doublets
DJ-1	Protein Deglycase
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNC	Dinitrocatechol
DOPAC	3,4-Dihydroxyphenylacetic acid
E	
ED ₅₀	Effective dose that produces 50% of the maximal effect
F	
FAD	Flavin adenine dinucleotide
Fe ²⁺	Iron(II) ion
G	
g	Grams
GABA	Gamma-aminobutyric acid
Gln	Glutamine
Glu	Glutamate
H	
h	Hours
H ₂ O ₂	Hydrogen peroxide
hMAO	Human monoamine oxidase
HNO ₃	Nitric acid
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectra
HVA	Homovanillic acid
Hz	Hertz
I	
IC ₅₀	The half maximal inhibitory concentration
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
Ile	Isoleucine
iNOS	Inducible nitric oxide synthase
J	
J	Coupling constant
K	
K	Kelvin unit of temperature
K ⁺	Potassium ion
KCl	Potassium chloride
Kg	Kilogram
K _i	The dissociation equilibrium constant of the enzyme-inhibitor complex
K _m	Michaelis constant
KOH	Potassium hydroxide
L	
L/Kg	Litre per kilogram
Leu	Leucine

Log P	Partition coefficient
Log[I]	The logarithm of inhibitor concentration
LRRK-2	Leucine-rich-repeat kinase 2
Lys	Lysine
M	
M	Molar
m	Multiplet
m/z	Mass to charge ratio
MAO	Monoamine oxidase
MB-COMT	Membrane bound catechol-O-methyltransferase
Met	Methionine
mg	Milligram
mg/kg	Milligram per kilogram
Mg ²⁺	Magnesium
MHz	Megahertz
min	Minutes
ml	Millilitre
ml/min	Millilitre per minute
mm	Millimetre
mM	Millimolar
mmol	Millimole
mp	Melting point
MPDP ⁺	1-methyl-4-phenyl-2,3-dihydropyridium
MPP ⁺	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
N	
NA	Noradrenaline
Na ⁺	Sodium ion
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
nM	Nanomolar
NMDA	<i>N</i> -Methyl-d-aspartate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
O	
O ₂ ⁻ / O ₂	Superoxide ion / dioxygen
OH / ·OH / ·OH	Hydroxy group / hydroxide ion / hydroxyl radical
OONO ⁻	Peroxynitrite
P	
PDB	Protein data bank

pH	Indicates acidity
Phe	Phenylalanine
PINK1	PTEN Induced Kinase 1
pK _a	Acid dissociation constant
ppm (δ)	Parts per million
Pro	Proline
Q	
q	Quartet
R	
RMSD	Root mean square deviation
ROS	Reactive oxygen species
S	
s	Singlet
S-COMT	Soluble catechol-O-methyltransferase
SD	Standard deviation
Ser	Serine
SNpc	Substantia nigra pars compacta
T	
t	Triplet
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor alpha
Trp	Tryptophan
Tyr	Tyramine
U	
UV	Ultraviolet
V	
Val	Valine
V _d	Volume of distribution
VMAT	Vesicular monoamine transporters
V _{max}	Maximal velocity/ capacity
X	
Xp11.23	X Chromosome gene

CHAPTER 1

INTRODUCTION

1.1. BACKGROUND

Parkinson's disease is a chronic degenerative neurological disorder that mostly afflicts the aged population ([Kiss & Soares-da-Silva, 2014](#)), and is regarded as the second most common neurodegenerative disorder after Alzheimer's disease. More than a century after Parkinson's disease was first described, it was discovered that the central pathological feature of Parkinson's disease is the loss of the neurons of the *substantia nigra pars compacta* (SNpc). Later Arvid Carlsson discovered dopamine in the mammalian brain ([Dauer & Przedborski, 2003](#)), which led to the finding that the nigrostriatal dopaminergic pathway is formed by the SNpc neurons. This line of research culminated with two key discoveries. First, striatal dopamine deficiency is responsible for the major symptoms of Parkinson's disease and emerges because of the loss of SNpc neurons. Second, replenishment of striatal dopamine through the oral administration of the dopamine precursor, levodopa, alleviates most of these symptoms ([Dauer & Przedborski, 2003](#)).

Parkinson's disease is unique among the other neurodegenerative disorders because of the almost palpable anticipation of an imminent cure. So much is known about its pathophysiology that Parkinson's disease optimists argue that a definitive treatment will arrive ([LeWitt & Taylor, 2008](#)). Since the mid-1980s, treatments with potential neuroprotective capability for Parkinson's disease have been investigated in randomised, controlled, clinical trials ([LeWitt & Taylor, 2008](#)).

The treatment of the motor symptoms of Parkinson's disease - tremor, rigidity, bradykinesia, postural instability - focuses on restoring striatal dopaminergic neurotransmission. This may be achieved by increasing dopamine supply through levodopa administration, dopamine receptor stimulation with dopamine agonist therapy or by inhibiting dopamine reuptake and metabolism.

In 1967, the first oral dosing regimen of levodopa was introduced, and since then levodopa has remained the gold standard treatment for Parkinson's disease ([Freitas et al., 2016](#)). Levodopa is the biological precursor of dopamine and can be used as an "artificial" means to manipulate the cerebral levels of this neurotransmitter. Despite the beneficial effects of levodopa, one of its major disadvantages is a short *in vivo* half-life ([Kiss & Soares-da-Silva, 2014](#)). As mentioned levodopa is the metabolic precursor of dopamine and, in contrast to dopamine, permeates the blood-brain barrier by carrier-mediated transport. In the brain, levodopa is converted to dopamine, thus effectively replacing the lost dopamine in the striatum.

Absorption of levodopa occurs from the duodenum and proximal jejunum via the large neutral amino acid transport system. In the gastrointestinal tract, levodopa is rapidly decarboxylated by the enzyme aromatic L-amino acid decarboxylase (AADC) to yield dopamine, and only approximately 30% of a levodopa dose reaches the systemic circulation. The amount of oral levodopa that reaches the systemic circulation may be increased threefold by the combination of levodopa with an AADC inhibitor, either carbidopa or benserazide. This improves the bioavailability of levodopa to the brain and reduces the peripheral dopaminergic side effects (nausea, vomiting, headache, irregular heartbeat and anxiety), due to excessive dopamine formation in the peripheral tissues ([Freitas et al., 2016](#)).

The use of catechol-O-methyltransferase (COMT) inhibitors as adjuncts to levodopa therapy is based on their ability to reduce the O-methylation of levodopa to 3-O-methyldopa. When AADC is inhibited, 3-O-methylation catalysed by COMT becomes a dominant metabolic pathway for levodopa, and due to metabolism by COMT less than 10% of the oral levodopa dose reaches the brain. Several clinical observations have shown that poor response to levodopa therapy is associated with high plasma levels of 3-O-methyldopa. The duration of levodopa-induced clinical improvement is brief as a result of the short *in vivo* half-life of levodopa, which contrasts with the long half-life of 3-O-methyldopa. Additionally, 3-O-methyldopa competes with levodopa for transport across the blood-brain barrier, which further reduces the amount of an orally administered dose of levodopa that reaches the site of action, the brain ([Learmonth et al., 2012](#)).

Since COMT inhibitors block the unwanted metabolism of levodopa in peripheral tissue, they prolong the pharmacological effect of levodopa and allow for a reduced therapeutic dose of levodopa ([Kiss & Soares-da-Silva, 2014](#)). Thus COMT inhibition increases the bioavailability of levodopa, and the duration of the antiparkinsonian action is prolonged with single doses of levodopa ([Nutt, 1998](#)).

COMT exists in two isoforms, namely soluble COMT and membrane-bound COMT which are encoded by a single gene ([Männistö & Kaakkola, 1999](#); [Lundström et al., 1991](#); [Salminen et al., 1990](#)). These COMT isoforms are identical except for the inclusion of an additional 50 hydrophobic amino acid sequence in membrane-bound COMT, which is responsible for attachment to the cytoplasmic side of intracellular membranes ([Chen et al., 2011](#); [Ulmanen & Lundström, 1991](#)). Membrane-bound and soluble COMT do not differ significantly in substrate specificity but they may exhibit marked differences in their kinetic behaviour ([Kiss & Soares-da-Silva, 2014](#)). The activity and localisation of COMT is significantly lower in the central nervous system than in the peripheral tissues ([Kiss & Soares-da-Silva, 2014](#)). In humans, 70% of the total centrally located COMT is membrane bound while 30% is attributed to soluble COMT activity ([Männistö & Kaakkola, 1999](#)). It is important to note that dopamine also is a substrate for COMT and therefore it may be argued that, while peripheral inhibition of COMT is the appropriate

strategy for reducing the metabolism of levodopa, central COMT inhibition will block the metabolism of dopamine in the brain and thus exert a dopamine sparing effect (Guldborg & Marsden, 1975; Männistö & Kaakkola, 1999; Silva *et al.*, 2016). Dopaminergic neurotransmission and the efficacy of levodopa therapy will improve as a result. As adjuvants to levodopa, inhibitors that inhibit both central and peripheral COMT may be of enhanced value.

COMT inhibitors containing the 3-nitrocatechol moiety that have been developed and introduced into the market (or proceeded to clinical trials) include tolcapone (IC_{50} of 0.26 μ M, *determined in our laboratory*), entacapone (IC_{50} of 0.25 μ M, *determined in our laboratory*), opicapone, nebicapone and nitecapone. These are the so-called second generation COMT inhibitors and have been successfully used as adjuvants to levodopa in the treatment of Parkinson's disease. Structures indicated below (Fig. 1.1) are of the clinically used COMT inhibitors (tolcapone and entacapone).

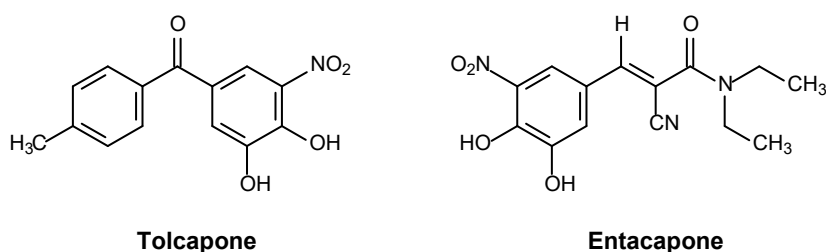


Figure 1.1: Structures of tolcapone and entacapone.

Although sharing essentially the same pharmacophore, tolcapone differs from entacapone in that it easily enters the central nervous systems and is able to inhibit central COMT as well as peripheral COMT. It could be speculated that central inhibition may be less important if the more significant action of inhibiting COMT is to prevent breakdown of levodopa in the periphery. Indeed, the use of COMT inhibitors, which do not penetrate into the brain at clinically relevant doses, may avoid potential undesired central nervous system side-effects of these agents (Learmonth *et al.*, 2012).

Another approach to improve the therapeutic efficacy of levodopa is to inhibit the monoamine oxidase (MAO)-catalysed metabolism of dopamine in the brain. Dopamine levels derived from levodopa may be enhanced by MAO inhibitors, which not only improves the therapeutic effect but also allow for a reduction in the levodopa dosage required for an effective response. MAO plays a major role in the *in vivo* inactivation of biogenic and diet-derived amines in both the central nervous system and in peripheral neurons and tissues (Foley *et al.*, 2000). The catecholamine neurotransmitters (dopamine, adrenaline and noradrenaline), serotonin and β -phenylethylamine are the most important substrates for the enzyme in the central nervous system (Foley *et al.*, 2000). Two isoenzymes of MAO are present in most mammalian tissues, MAO-A and MAO-B.

MAO isoforms are encoded by different genes and their proteins have 70% identity between them (Youdim *et al.*, 2006). They are distinguished on the basis of their substrate preferences and sensitivity to inhibition by isoform specific inhibitors (Foley *et al.*, 2000). The distribution of MAO-A and MAO-B in the mammalian brain differs, with for instance, greater MAO-B activity in basal ganglia (Youdim & Bakhle, 2006). Both isoforms of MAO contain the flavin adenine dinucleotide (FAD) cofactor (Edmondson *et al.*, 2007). Low concentrations of clorgyline inhibits MAO-A selectively and irreversibly. In the human central nervous system, MAO-A is responsible for the deamination of serotonin and noradrenaline, and catalyses the oxidation of tyramine in the intestine (Foley *et al.*, 2000). MAO-B is relatively insensitive to clorgyline, and metabolises dopamine and β -phenylethylamine in the brain (Foley *et al.*, 2000). Although MAO-A inhibitors are used in the treatment of depression, their clinical use is limited by a potentially fatal hypertensive crisis that may arise when irreversible MAO-A inhibitors are combined with tyramine-containing food. MAO-B inhibitors are used in the treatment of neurodegenerative disorders, including Parkinson's disease (Foley *et al.*, 2000).

Two classes of MAO-B inhibitors can be distinguished, reversible and irreversible inhibitors. *Reversible, competitive inhibitors* (e.g. safinamide) are structurally related to MAO substrates, and binds reversibly to the active site of the enzyme (Foley *et al.*, 2000). *Irreversible inhibitors* (e.g. selegiline, rasagiline) initially bind to MAO in a reversible, competitive manner. The inhibitor is subsequently oxidised by the enzyme to yield the active inhibitor that binds covalently to the enzyme active site via the FAD cofactor, thus rendering it permanently unavailable for amine metabolism. The inhibition is more persistent than what is achieved by reversible inhibitors (Foley *et al.*, 2000). Besides a dopamine sparing effect, MAO-B inhibitors may also, by reducing the MAO-catalysed formation of hydrogen peroxide and resulting oxidative damage in the brain, represent potential neuroprotective agents in Parkinson's disease. Oxidative damage appears to be an important factor in the neurodegenerative processes associated with Parkinson's disease. The inhibition of MAO-B is a particularly relevant strategy when considering that MAO-B activity and density increase in the brain with ageing.

The present study considers nitrocatechol derivatives of chalcones for dual inhibition of COMT and MAO-B, and will attempt to discover novel dual inhibitors of these enzymes. The chalcone class of compounds is well known to potently inhibit MAO-B. Nitrocatechol compounds, in turn, are known to act as COMT inhibitors. Compared to specific inhibition of either enzyme, dual inhibition may have enhanced value in Parkinson's disease, particularly as adjuvants to levodopa. In this respect, both peripheral and central enzymes may be targeted, which would result in the enhanced availability of levodopa for uptake into the brain as well as the sparing of depleted dopamine in the brain. This approach may enhance the therapeutic efficacy of levodopa, but also may allow for the effective levodopa dosage to be reduced. A reduction of levodopa dosage would greatly decrease the potential for levodopa-associated adverse effects such as dyskinesia.

1.2. HYPOTHESIS OF THIS STUDY

Levodopa continues to be the gold standard drug for the symptomatic treatment of Parkinson's disease, and because of this much interest in the development of inhibitors of the COMT enzyme exists. This is based on the hypothesis that inhibition of this enzyme may provide clinical improvements in Parkinson's disease patients undergoing treatment with levodopa and a peripheral AADC inhibitor ([Learmonth et al., 2012](#)).

Due to extensive peripheral metabolism by AADC and COMT, only a small fraction of the levodopa dose reaches the brain ([Kaakkola, 2000](#)). Central metabolism of dopamine by MAO further decreases the efficacy of levodopa ([Lees, 2005](#)). Thus, the dual inhibition of MAO and COMT may not only conserve endogenous dopamine levels, but may also protect levodopa against undesirable metabolism, improving its availability to the brain. This study thus hypothesises that nitrocatechol derivatives of chalcones may be designed that exhibit dual inhibition of COMT and MAO-B. The structures of the chalcones that will be investigated in this study are shown in [figure 1.2](#).

1.3. DUAL INHIBITORS THAT WILL BE INVESTIGATED IN THIS STUDY

Chalcones (*trans*-1,3-diphenyl-2-propen-1-ones) are the biogenetic precursors of all known flavonoids and are abundant in edible plants. Chemically, they consist of open-chain flavonoids in which the two aromatic rings are joined by a three-carbon α,β -unsaturated carbonyl system ([Chimenti et al., 2008](#)). They present with a broad spectrum of biological activities, such as anticancer, anti-inflammatory, antimalarial, antifungal, antilipidemic and antiviral activities ([Chimenti et al., 2008](#)). As mentioned above, this study will design nitrocatechol derivatives of chalcones that potentially exhibit dual inhibition of COMT and MAO-B.

The structures of the nitrocatechol derivatives of chalcone that will be investigated in this study are shown in [figure 1.2](#). As shown, the A-ring consists of the nitrocatechol moiety while limited substitution will be explored on B-ring. For this study, polar functional groups will be substituted on ring B since these are expected to increase inhibition of MAO-B. In particular, the benzo- and phthalonitriles are well-known to effectively inhibit MAO-B, therefore the nitrile functional group will be considered as a polar substituent ([Manley-King et al., 2011](#)). Hydroxy substitution is also known to improve the inhibition of MAO-B. This effect of polar substitution on MAO-B inhibition is due to the interaction of the polar groups with the polar region of the MAO-B active site, the region in proximity to the FAD. The positioning of the polar substituents is therefore an important consideration. Three chalcone derivatives with polar functional groups on ring B, compounds **1–3**, will thus be synthesised. These chalcones will be further converted to the pyrazoline derivatives, compounds **4–6**, since pyrazolines are also well-known to inhibit the MAO enzymes ([Chimenti et al., 2010](#)).

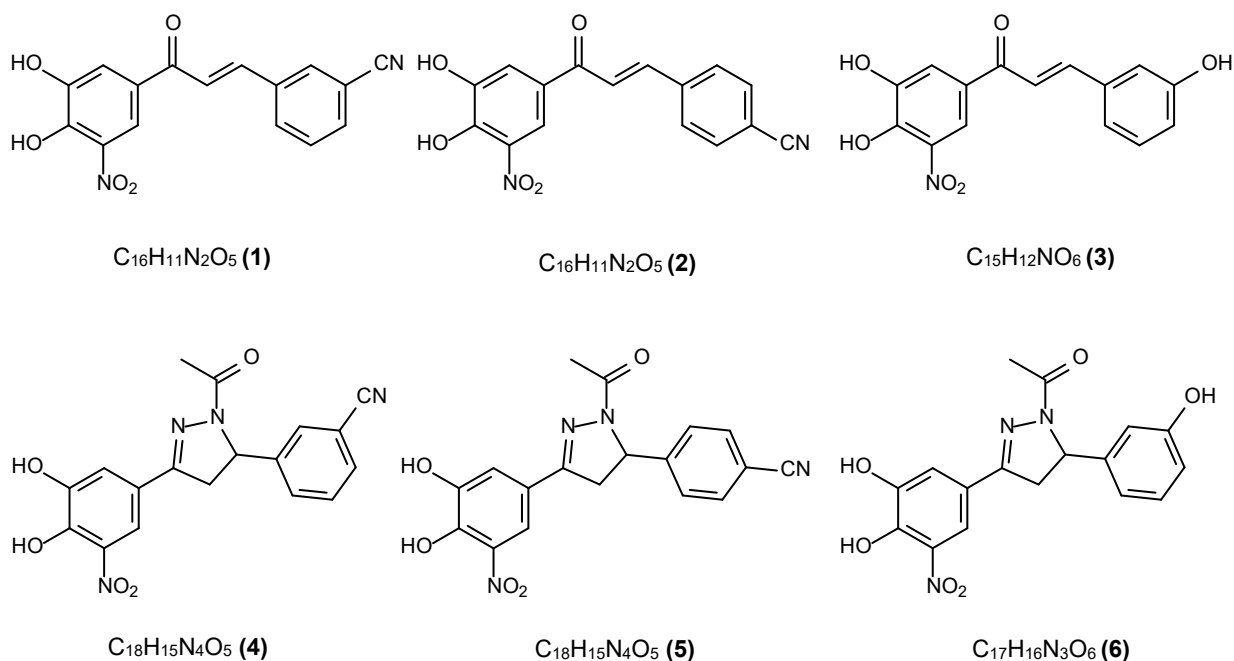


Figure 1.2: The structures of the nitrocatechol derivatives of chalcones (1–3) and pyrazoline derivatives (4–6) that will be investigated in this study.

1.4. AIMS AND OBJECTIVES

The aim of the study is:

- To discover novel dual inhibitors of MAO-B and COMT.

The objectives of this study are:

- To synthesise chalcone analogues that incorporate the nitrocatechol moiety, and to convert the chalcones to the corresponding pyrazoline compounds.
- To evaluate the nitrocatechol derivatives of chalcone and the pyrazolines as inhibitors of human MAO and rat liver COMT by measuring IC_{50} values.
- To determine possible binding orientations of selected compounds in the MAO and COMT active sites by molecular modelling studies.
- To propose potential lead compounds for the future design of dual MAO/COMT inhibitors.

CHAPTER 2

LITERATURE STUDY

2.1. PARKINSON'S DISEASE

2.1.1. General background

Parkinson's disease is a chronic degenerative neurological disorder predominantly afflicting the aged population ([Kiss & Soares-da-Silva, 2014](#)) and is regarded as the second most common neurodegenerative disorder after Alzheimer's disease. Parkinson's disease is caused by a reduction in the striatal levels of dopamine associated with the gradual degeneration or death of nigral cells in the brain. Thus, the dopaminergic neurons are gradually destroyed in a specific region of the central nervous system and this reduction of dopamine levels in the brain becomes symptomatic over a certain threshold ([Kiss & Soares-da-Silva, 2014](#)).

The cause of Parkinson's disease is still not known, but various hypotheses exist, which include genetic defects or gene mutations, impaired detoxification capacity, exposure to acute and chronic endogenous and exogenous toxins such as pesticides, deficiencies of mitochondrial function, infection by prion-like proteins, protein misfolding, inflammation and decreased neurotransmitter capacity ([Müller, 2015](#); [Blandini, 2013](#); [Halliwell, 2001](#); [Naoi et al., 2009](#)).

It is expected that Parkinson's disease will impose an increasing social and economic burden on societies as populations' age ([De Lau & Breteler, 2006](#)). The discovery of several causative monogenetic mutations has led to increased interest in Parkinson's disease and this interest has grown significantly in recent years ([De Lau & Breteler, 2006](#)).

Parkinson's disease is present in mid- or late life, most often at the age between 55 and 65. The incidence increases markedly with age from 20/100,000 overall to 120/100,000 at age 70, and affects approximately 1-2% of the population older than 65. In the population older than 84, the incidence increases by 3-5% per year ([Dauer & Przedborski, 2003](#); [Booth et al., 2003](#)). The overall prevalence of Parkinson's disease is 300/100,000 that rises from 41 people in the age range of 40-49 years to 1903 people older than 80 years of age ([Magrinelli et al., 2016](#); [De Lau & Breteler, 2006](#); [Pringsheim et al., 2014](#)). The mean duration of the disease from diagnosis to death is 15 years ([Lees et al., 2009](#)).

According to World Health, Parkinson's disease has a death rate of 2.26 per 100,000 in South Africa and is ranked 74th in the world, whereas Parkinson's disease in Finland is ranked the highest (1st in the world) with a death rate of 4.66 per 100,000. The United States of America is

ranked 4th in the world with a death rate of 4.51 per 100,000 and Egypt is ranked the lowest, 172 in the world with a death rate of 0.12 per 100,000 ([World health rankings, 27 Feb. 2018](#)).

It is expected that by the year 2030, about 8.7 million individuals will suffer from Parkinson's disease ([Sampaio et al., 2018](#); [Dorsey et al., 2007](#)).

2.1.2. Clinical features

James Parkinson published "An Essay on the Shaking Palsy" in 1817, and described the clinical features of the neurodegenerative disorder ([Booth et al., 2003](#)). The clinical features are commonly presented with impairment of dexterity or, less commonly, with a slight dragging of one foot ([Lees et al., 2009](#)). The onset is gradual, insidious and asymmetric, worsening with age and disease severity, and the earliest symptoms might be unnoticed or misinterpreted for a long time ([Lees et al., 2009](#); [Carranza et al., 2013](#)).

There are a number of clinical features of Parkinson's disease that can be recognised and will be discussed. The signs and symptoms are as follow: **Resting tremor** that improves with voluntary activity (it is often the first observed symptom and is distinguished from other forms of tremor by being unilateral. It has been reported that approximately 69% of patients with Parkinson's disease have rest tremor at disease onset, with 75% of patients having tremor during the course of their disease), **rigidity** of muscle and joint motility (also known as "cog-wheeling" and is characterised by uniform, increased resistance throughout movement and is evident in both agonist and antagonist muscles recruited for the movement), **postural instability** including falls (patients begin to lose postural reflexes and experiences persistent instability when standing and are typically present after the onset of other clinical features of Parkinson's disease), **bradykinesia** or slow initiation and paucity of voluntary movements (bradykinesia may significantly impair the quality of life because it takes much longer to perform everyday tasks), **hypokinesia** (reduction in movement amplitude), **akinesia** (absence of normal unconscious movements, such as arm swing in walking), **hypomimia** (including paucity of normal facial expression), **hypophonia** (decreased voice volume), **drooling** (failure to swallow without thinking about it), decreased size (**micrographia**) and speed of handwriting, and decreased stride length during walking ([Dauer & Prezedborski. 2003](#)). **Freezing**, the inability to begin a voluntary movement such as walking, is a common symptom of Parkinsonism. Responses to questions are delayed, and cognitive processes are slowed (**bradyphrenia**) ([Dauer & Prezedborski. 2003](#)). These signs may differ among individuals depending on their early intensity, combinations and progression. Depression is common, and dementia is about six times more frequent in Parkinson's disease, especially in elderly patients ([Carranza et al., 2013](#); [Booth et al., 2003](#); [Dauer & Prezedborski. 2003](#)).

2.1.3. Pathophysiology

The main anatomical feature of Parkinson's disease is the decrease in number of neuromelanin-containing neurons (dopamine neurons) located in the midbrain SNpc. These dopaminergic neurons project to the striatum as well as a number of other subcortical regions via the nigrostriatal pathway (Smeyne & Jackson-Lewis, 2004; Young & Penny, 1993).

In Parkinson's disease, these neurons degenerate and the loss of the nigrostriatal dopaminergic neurons and the presence of intraneuronal proteinaceous cytoplasmic inclusions termed "Lewy-bodies" are the pathological features of Parkinson's disease (Dauer & Przedborski, 2003). It has been found that the death of nigrostriatal neurons coincides with the appearance of Lewy-bodies.

The cell bodies of nigrostriatal neurons are located in the SNpc, and they project primarily to the putamen. The loss of these neurons (containing neuromelanin) produces the classic gross neuropathological findings of SNpc depigmentation (Dauer & Przedborski, 2003; Marsden, 1983). The pattern of SNpc cell loss appears to parallel the level of expression of the dopamine transporter mRNA (Uhl *et al.*, 1994), and is consistent with the finding that depletion of dopamine is most prominent in the dorsolateral putamen (Bernheimer *et al.*, 1973). Parkinson's disease symptoms first manifest when approximately 60% of the SNpc neurons have already degenerated (Smeyne & Jackson-Lewis, 2004; German *et al.*, 1989) and 70% of dopamine responsiveness disappears (Smeyne & Jackson-Lewis, 2004; Ma *et al.*, 2002).

The basal ganglia of the brain consist of five interconnected subcortical nuclei that span the telencephalon (forebrain), diencephalon and mesencephalon (mid-brain). These nuclei include the corpus neostriatum (caudate and putamen), globus pallidus, thalamus, subthalamic nucleus and midbrain substantia nigra (pars compacta and pars reticulata) (Booth *et al.*, 2003). The basal ganglia coordinates movements and adjusts the activity of the thalamus via the direct- and indirect pathway communication. The thalamus sends signals to the motor cortex which leads to the initiation of voluntary muscle activity.

The aim of the direct pathway is to increase the activity of the thalamus, causing muscle movements to increase. The aim of the indirect pathway is to reduce the activity of the thalamus, causing muscle movements to decrease. In Parkinson's disease, the substantia nigra cannot initiate more movement in the direct pathway and cannot prevent an excessive reduction in movement in the indirect pathway, thus causing the slow movements.

2.1.4. Etiology

Although the primary etiology of Parkinson's disease is unknown, its neuropathology is marked by progressive degeneration of pigmented neurons of the midbrain and brainstem, mainly those

that produce dopamine (DA) as a neurotransmitter in the midbrain substantia nigra and project to the forebrain extrapyramidal motor control centre of the basal ganglia (Booth *et al.*, 2003). There is also a notable loss of other pigmented monoaminergic neurons in the brainstem, particularly those producing norepinephrine (Booth *et al.*, 2003).

The view of etiology factors in Parkinson's disease has changed remarkably from one of a purely sporadic basis to the view that both environmental and genetic factors contribute to the onset of Parkinson's disease (Schapira & Jenner, 2011). Genetic predisposition must be seen as a major contributor to the underlying cause (Schapira & Jenner, 2011; Schapira & Tolosa, 2010; Schapira, 2009; Schapira, 2006). However, the one factor that strongly relates to the onset of Parkinson's disease is age or the aging process, but little research has been conducted to understand how it is involved (Schapira & Jenner, 2011; Obeso *et al.*, 2010). The usual explanation lies in an increased vulnerability of dopaminergic neurons to toxic insult because of increasing failure of normal cellular physiological and biochemical processes that occur with ageing (Schapira & Jenner, 2011).

There have been at least three important events related to the etiology and pathogenesis of Parkinson's disease. First, the examination of post-mortem brain material has uncovered specific components of the cell death cascade, identifying key processes that have subsequently been replicated in experimental models of Parkinson's disease and linked to the events identified in familial forms of Parkinson's disease (Schapira & Jenner, 2011; Jenner *et al.*, 1992). Second, is the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that could destroy dopaminergic neurons selectively (Schapira & Jenner, 2011; Langston *et al.*, 1984). Finally is the discovery of mutations in α -synuclein in familial Parkinson's disease, which introduced the genetic era from which the molecular events occurring as a cause and consequence of cell death have emerged (Schapira & Jenner, 2011; Polymeropoulos *et al.*, 1997).

Despite insights derived from genetic research, the exact pathogenic mechanisms underlying the selective dopaminergic cell loss in Parkinson's disease are still not understood (De Lau & Breteler, 2006). Mitochondrial dysfunction, oxidative stress, and protein mishandling have a central role in the pathogenesis of Parkinson's disease (De Lau & Breteler, 2006). To obtain a better understanding of the pathogenesis of the disease and to develop effective therapeutic strategies, deeper insight in non-genetic causes of Parkinson's disease is needed (De Lau & Breteler, 2006).

2.1.4.1. Non-genetic risk factors

Environmental factors:

Environmental influences on the occurrence of Parkinson's disease is known to differ from general to the specific factors. The general factors are industrialisation, rural environment, plant-derived

toxins, bacterial and viral infection. The specific factors occur with exposure to organic solvents, carbon monoxide and carbon disulphide (Schapira & Jenner, 2011; Corrigan *et al.*, 1998). The exposure to pesticides has gained more interest and may be linked to an increased risk of developing Parkinson's disease (Schapira & Jenner, 2011; Richardson *et al.*, 2009).

The environmental hypothesis also postulates that Parkinson's disease-related neurodegeneration results from exposure to a dopaminergic neurotoxin (Dauer & Przedborski, 2003). Theoretically, the progressive neurodegeneration of Parkinson's disease could be produced by chronic neurotoxin exposure or by limited exposure initiating a self-perpetuating cascade of adverse events (Dauer & Przedborski, 2003).

Occupational exposures: pesticides, herbicides, and heavy metals

In 1983, several people showed typical signs of Parkinson's disease after intravenous injection of drugs contaminated with MPTP. Acute and irreversible Parkinsonism were developed (De Lau & Breteler, 2006). It was later discovered that the exposure to the neurotoxic effects of MPTP led to the development of Parkinsonism. The remarkable resemblance of Parkinsonian symptoms after the intoxication of MPTP and the symptoms observed in sporadic Parkinson's disease led to the investigation of the neurotoxin's effects in various animal species (De Lau & Breteler, 2006). The discovery that MPTP damages dopaminergic cells selectively in the substantia nigra led to the hypothesis that exposure to environmental toxins may be related to the risk of developing Parkinson's disease (De Lau & Breteler, 2006).

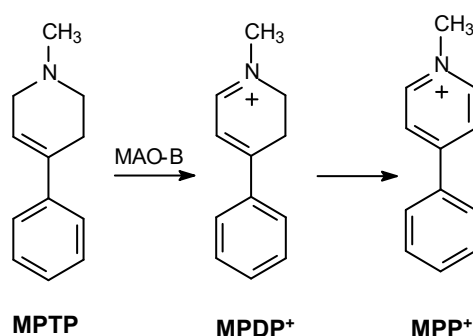


Figure 2.1: The metabolism of MPTP to yield MPDP⁺ and MPP⁺.

MPTP crosses the blood-brain barrier and permeates the glial cells where it is metabolised by the enzyme, MAO-B, to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺), which disproportionates to generate, 1-methyl-4-phenylpyridinium (MPP⁺) (Smeys & Jackson-Lewis, 2005). Endothelial cells in the microvasculature that make up the blood-brain barrier contains MAO, and also converts MPTP from its protoxin form into its neurotoxic MPP⁺ form (Fig. 2.2), thus explaining the protective effect of MAO-B inhibitors against MPTP neurotoxicity.

MPP⁺ stimulates the up-regulation of TNF- α , interleukin-1 β (IL-1 β) and IL-6, and these, in-turn, up-regulate inducible nitric oxide synthase (iNOS). Large amounts of the uncharged and lipophilic molecule, nitric oxide (NO), are produced by iNOS, which can freely pass through membranes (Smeyne & Jackson-Lewis, 2005).

The polar compound, MPP⁺, cannot exit freely from glial cells, and once MPP⁺ is released into the extracellular space, MPP⁺ is taken up into dopaminergic cells by the dopamine transporter (DAT) (Smeyne & Jackson-Lewis, 2005). Since midbrain neurons contain the highest concentration of dopamine transporters per cell, the DAT may be a control point in determining how susceptible midbrain neurons are to exogenous agents (Smeyne & Jackson-Lewis, 2005).

The free cytosolic MPP⁺ enters the mitochondria by diffusion through the mitochondrial inner membrane, inhibiting the activity of this organelle. The intracytoplasmic accumulation of MPP⁺ also depends on two intracellular trapping systems. Firstly, neuromelanin forms a complex with MPP⁺ and delays its cytoplasmic release. Secondly, the vesicular monoamine transporters (VMAT) confine the neurotoxin to synaptic vesicles (Blum *et al.*, 2001).

MPP⁺ inhibits cellular respiration in the mitochondria through the blockade of the electron transport enzyme NADH (nicotinamide adenine dinucleotide):ubiquinone oxidoreductase (complex-I) (Smeyne & Jackson-Lewis, 2005). Mitochondrial and complex-I inhibition lead to a decrease in cellular ATP levels, loss of mitochondrial membrane potential, alterations of calcium homeostasis, radical formation and subsequent cell death. Although complex-I inhibition by MPP⁺ reduces energy production within dopaminergic neurons, it is likely that this is not the immediate cause of the SNpc neuronal death (Smeyne & Jackson-Lewis, 2005). The inhibition of mitochondrial complex-I activity forms an excessive amount of superoxide radicals within the neuronal cytosol. NO produced and released by glial cells, can enter the cytosol of the neuron via membrane diffusion. The superoxide radical and NO, which are not particularly damaging by themselves, can interact to form peroxynitrite (OONO⁻), one of the most destructive oxidising molecules (Smeyne & Jackson-Lewis, 2005). Furthermore, the activation of calcium-dependent nitric oxide synthase increases NO formation, causing an increase in peroxynitrite and cell death (Blum *et al.*, 2001).

The involvement of reactive oxygen species (ROS) thus mediates MPTP-induced neurotoxicity. ROS formation may also occur by indirect excitotoxicity resulting from neuronal impairment of energy metabolism and the subsequent increase in cytoplasmic calcium (Blum *et al.*, 2001). Calcium channel blockers and *N*-methyl-D-aspartate (NMDA) receptor antagonists efficiently protect the SNpc against MPTP.

Iron may be of great importance in MPTP toxicity and triggers a Fenton reaction in dopaminergic cells. MPTP increases free iron level in SNpc, while desferrioxamine, an iron chelator, blocks the

neurotoxic effect of MPTP. MPTP also increases transferrin receptors and lactoferrin transporter expressions. MPP⁺, which is a substrate for xanthine oxidase, may lead to the formation of the MPP[•] radical (Blum *et al.*, 2001).

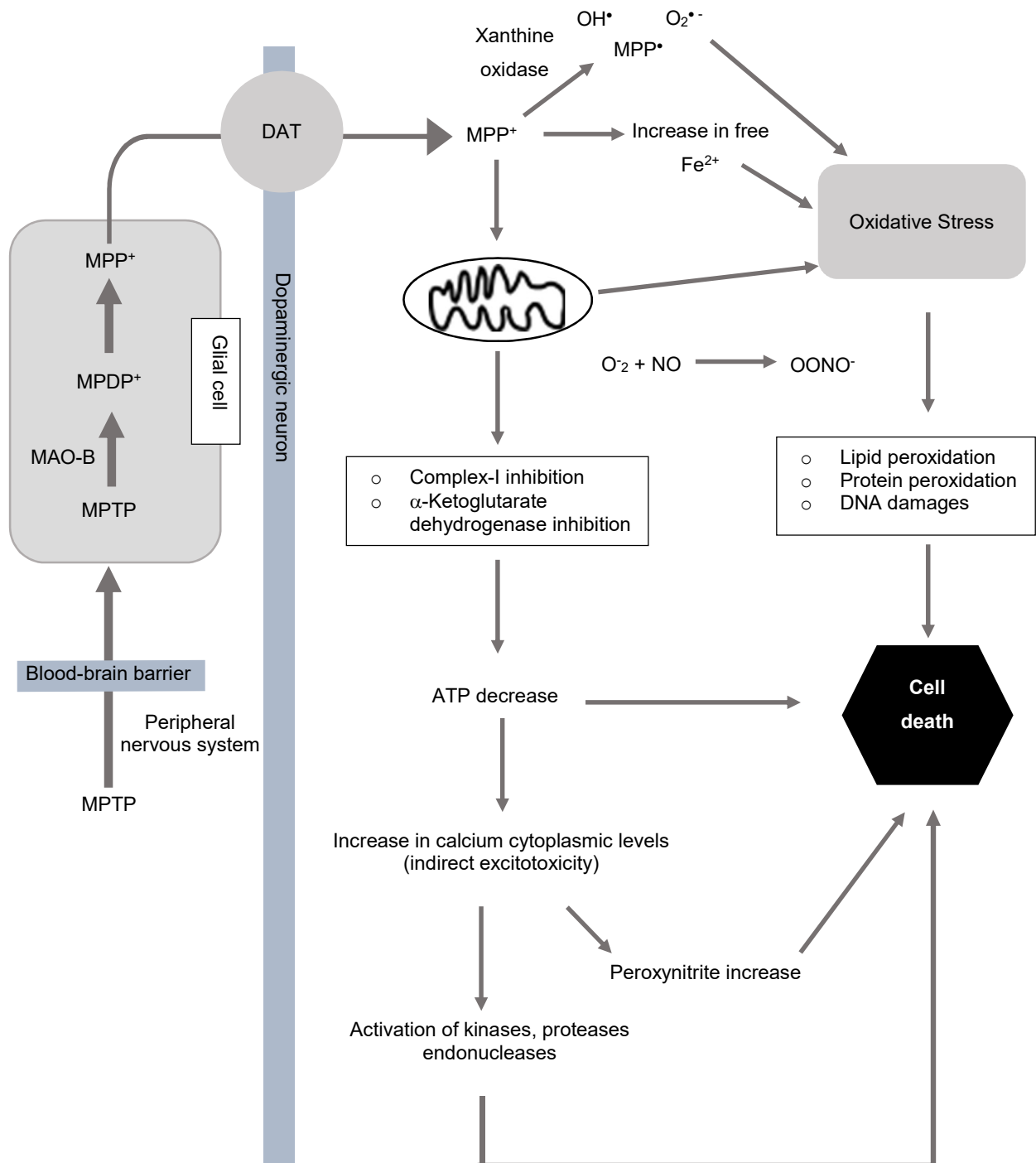


Figure 2.2: Schematic representation of the mechanism of action of MPTP toxicity.

Similar to MPP⁺, rotenone (Fig. 2.3) is also a mitochondrial poison that is present in the environment. Rotenone has been used as a pesticide for several decades to control unwanted

fish populations in lakes, in nurseries and in organic farming (Dauer & Prezedborski, 2003). Paraquat (Fig. 2.3) is structurally similar to MPP⁺ and has been used as a herbicide (Dauer & Prezedborski, 2003). MPP⁺ as well as paraquat and rotenone are selective complex-I inhibitors and induce dopamine depletion and dopaminergic neuron death in animal studies (De Lau & Breteler, 2006).

Welding and exposure to heavy metals such as iron, manganese, copper, lead, amalgam, aluminium or zinc have also been hypothesised to increase the risk of Parkinson's disease through accumulation of metals in the substantia nigra and increased oxidative stress (De Lau & Breteler, 2006).

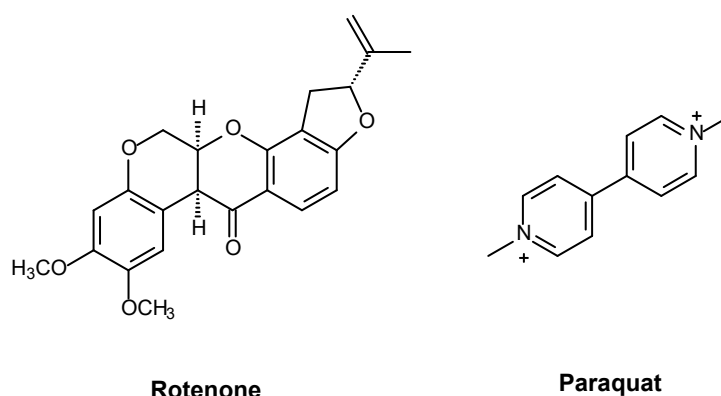


Figure 2.3: Structures of rotenone and paraquat.

Tobacco and coffee:

Many epidemiological studies have shown that there is a significantly decreased risk in the development of Parkinson's disease among cigarette smokers and coffee drinkers (De Lau & Breteler, 2006).

2.1.4.2. Genetic risk factors

Given that several neurodegenerative disorders are genetically determined, researchers have investigated possible genetic influences in Parkinson's disease. Epidemiological studies have found that apart from age, a family history of Parkinson's disease is the strongest predictor of increased risk for developing this disorder, however, shared environmental exposures in families must also be considered (Booth *et al.*, 2003).

It has been estimated that having a parent with Parkinson's disease increases the lifetime risk of developing Parkinson's disease from 2% to 6%. Most patients who is diagnosed with Parkinson's disease have no genetic cause. It is currently believed that only 5% of all Parkinson's disease cases have a genetic cause (Tugwell, 2008). Genetic studies have shown that there are several mutations in four different genes that are unequivocally associated with development of familial

Parkinson's disease (Booth *et al.*, 2003). The four genes are, DJ-1, PINK1, parkin and leucine-rich-repeat kinase 2 (LRRK-2).

One familial form of Parkinson's disease is characterised by mutations in the α -synuclein gene. The α -synuclein protein is a highly conserved 140-amino-acid polypeptide that is mainly expressed in the cerebral nerve terminals (Booth *et al.*, 2003). Aggregation of α -synuclein molecules leads to pathological inclusions that characterise many neurodegenerative disorders, including Parkinson's disease, and α -synuclein appears to play a role in regulating dopamine homeostasis, including modulation of dopamine synthesis, release and reuptake at nerve terminals (Booth *et al.*, 2003).

2.1.4.3. Oxidative stress and mitochondrial dysfunction

Oxidative stress contributes to the cascade leading to dopamine cell degeneration in Parkinson's disease, however, oxidative stress is intimately linked to other components of the degenerative process, such as mitochondrial dysfunction, excitotoxicity, nitric oxide toxicity and inflammation (Jenner, 2003). Oxidative damage to lipids, proteins and DNA occurs in Parkinson's disease and toxic products of oxidative damage, such as 4-hydroxynonenal can react with proteins to impair cell viability (Jenner, 2003).

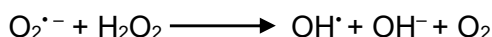
The generation of free radicals causes the impairment of proteasomal function (Jenner, 2003). Free radicals are constantly produced in eukaryotic cells and to maintain the redox homeostasis, the free radicals must be balanced by antioxidant defence (Yan *et al.*, 2013). Oxidative stress is caused by the imbalance between harmful ROS and antioxidant defences, which results in oxidative damage (Yan *et al.*, 2013). Once redox balance is lost, oxidative stress causes serious damage that leads to neuronal loss in the brain as observed in neurodegenerative diseases (Yan *et al.*, 2013). ROS, can cause nucleic acid breakage, enzyme inactivation, polysaccharide depolymerisation, lipid peroxidation, but in general ROS damage all biomolecules, and if in overabundance it will ultimately lead to cell death (Yan *et al.*, 2013).

In patients with Alzheimer's disease and Parkinson's disease, levels of glutathione and vitamin E increases in the brain as a compensatory mechanism to deal with oxidative stress (Ebadi *et al.*, 1996; Adams *et al.*, 1991). Free radicals are generated from the reduction of molecular oxygen, degradation of reactive oxygen species, from atmospheric pollutants, and from non-oxygen-containing compounds such as carbon tetrachloride or chloroform (Ebadi *et al.*, 1996; Bonorden & Pariza, 1994; Jesberger & Richardson, 1991). Exposure to an excess free radicals will ultimately lead to neuronal death (Ebadi *et al.*, 1996; Bonorden & Pariza, 1994; Jesberger & Richardson, 1991).

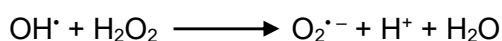
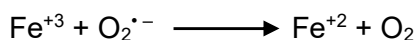
Mitochondrial dysfunction leads to oxidative stress and mitochondrial defects leads to an increase in production of ROS that consumes antioxidants such as glutathione. This cycle leads to damage of DNA, protein and lipids. Complex-I dysfunction can cause oxidative stress which in turn may lead to a disturbance in cellular Ca^{2+} homeostasis (Ebadi *et al.*, 1996; Van Der Vliet & Bast, 1992). Additionally, reduced complex-I function may cause a decrease in ATP production that could decrease the activity of ATPases, such as the Na^+/K^+ ATPase, thus resulting in neuronal depolarisation. Under these conditions, neurons are extremely vulnerable to excitotoxicity (Sherer *et al.*, 2002).

Hydroxyl radicals are the most damaging of all the free radicals, and although they exist only for a fraction of a second they are able to destroy vital enzymes (Ebadi *et al.*, 1996). Hydroxyl radicals can cause the cross-linking of DNA, the release of proteolytic enzymes, the destruction of polysaccharides and lipid peroxidation which alters membrane permeability and associated functions (Ebadi *et al.*, 1996; Warren *et al.*, 1987). There are at least nine separate reactions which may generate free radicals. The two most important implicated in Parkinson's disease are:

Haber-Weiss reaction:



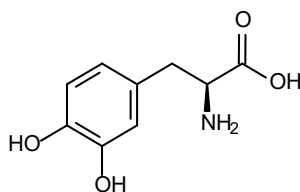
Fenton reaction:



2.1.5. Symptomatic treatment

Parkinson's disease is still an incurable progressive disease, but treatment substantially improves quality of life and functional capacity (Lees *et al.*, 2009).

2.1.5.1. Levodopa



Levodopa: $\text{C}_9\text{H}_{11}\text{NO}_4$

Figure 2.4: The structure of levodopa.

Levodopa, a dopaminergic agent, is generally reserved for the management of established symptomatic idiopathic or post-encephalitic Parkinsonism. To allow for lower dosage of levodopa, it is not used as monotherapy but is combined with an AADC inhibitor (carbidopa or benserazide) (Freitas *et al.*, 2016; Oscar & Gershanik, 2015). This approach minimises the incidence of adverse effects associated with the high doses needed when levodopa is used alone (Freitas *et al.*, 2016). A combination of levodopa/carbidopa with a COMT inhibitor (entacapone) is available and is indicated for Parkinson's disease with motor fluctuations (Oscar & Gershanik, 2015).

More than 40 years after the introduction of levodopa, it remains the most effective symptomatic pharmacotherapy for Parkinson's disease (Olanow, 2009; Booth *et al.*, 2003). Most patients that are diagnosed with Parkinson's disease gain a substantial benefit from levodopa throughout their illness, despite controversy regarding long-term efficacy, adverse effects and even potential neurotoxicity of this amino acid precursor of dopamine. Levodopa increases life expectancy among Parkinson's disease patients, particularly if introduced early in the illness (Rajput *et al.*, 1997; Booth *et al.*, 2003). Treatment with levodopa requires penetration into the central nervous system and local decarboxylation to dopamine to be effective (Booth *et al.*, 2003). Levodopa is able to penetrate the central nervous system because it is less basic ($pK_a = 8.72$ [NH_2]) and polar at physiological pH than dopamine, and is transported into the brain with other aromatic and neutral aliphatic amino acids (Booth *et al.*, 2003). In the brain, levodopa is converted by the enzyme AADC to dopamine in dopaminergic and serotonergic neurons (Müller, 2015; Riederer *et al.*, 2007).

Therapy with levodopa results in common adverse effects (nausea and vomiting), that may emerge as a result of gastrointestinal irritation, and the stimulation by of peripheral dopamine receptors by dopamine (Booth *et al.*, 2003). It has been found that after 5 years of continuous treatment with levodopa, at least half of Parkinson's disease patients develop fluctuating motor responses, and nearly three quarters of the patients develop these responses after 15 years (Booth *et al.*, 2003). These fluctuations include "off" periods of immobility, and "on" periods with abnormal involuntary movements or dyskinesia (Booth *et al.*, 2003). These phenomena may reflect progression of the disease with more severe striatal nerve terminal degeneration and further loss of dopamine, along with increased sensitivity of its receptors (Booth *et al.*, 2003).

The advent of selective peripheral inhibitors of AADC (carbidopa and benserazide) led to the development of combination formulations with levodopa that have remained the mainstay of therapy for patients with Parkinson's disease (Davis, 1998). The treatments that are currently available are Madopar® (levodopa/benserazide), Sinemet® and Carbilev® (levodopa/carbidopa), and Stalevo® (levodopa/carbidopa/ entacapone).

Levodopa may be viewed as a prodrug to increase dopamine levels for the treatment of Parkinson's disease (Elroby *et al.*, 2012). As mentioned above, although dopamine cannot cross

the blood-brain barrier, levodopa is able to cross the blood-brain barrier, and once levodopa has entered the central nervous system, it is metabolised to dopamine by AADC (Fig. 2.5) (Elroby *et al.*, 2012). Despite the effectiveness of the current levodopa/AADC inhibitor formulations, most of the orally administered levodopa is converted by COMT into the inactive metabolite 3-O-methyldopa before crossing the blood-brain barrier (Fig. 2.5) (Davis, 1998). The accumulated 3-O-methyldopa competes with levodopa for the saturable neutral amino acid active transport system in the gut wall and blood-brain barrier, and this competition may contribute to the motor fluctuations that occur in some patients with advanced Parkinson's disease (Davis, 1998). Levodopa is metabolised by four major pathways: decarboxylation, O-methylation, transamination and oxidation. The principal path is decarboxylation, whereby dopamine is formed by AADC (Fig. 2.5) (Elroby *et al.*, 2012).

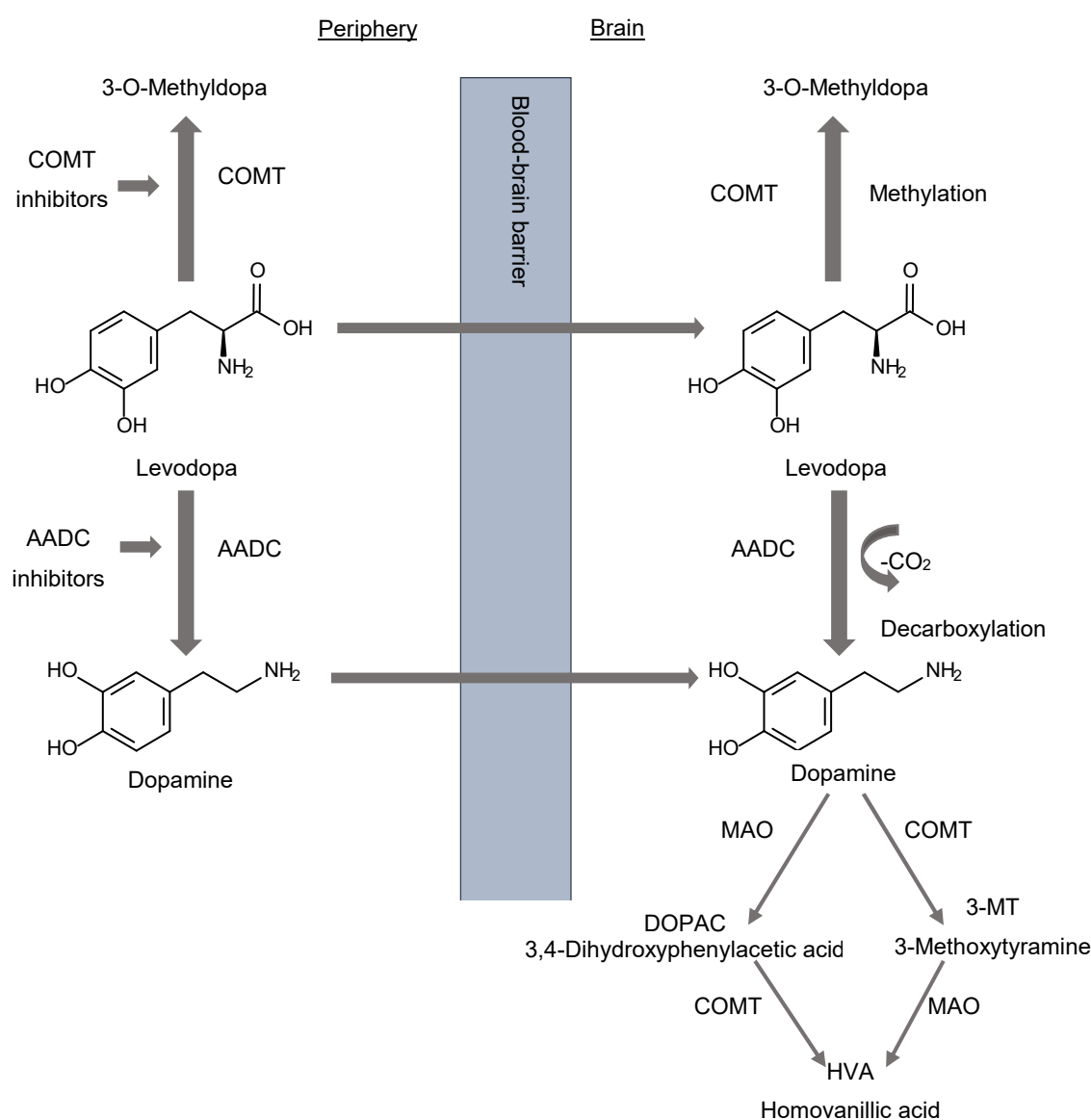
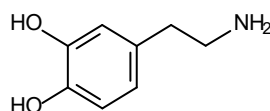


Figure 2.5: Metabolism of levodopa.

2.1.5.2. Dopamine agonists



Dopamine: C₈H₁₁NO₂

Figure 2.6: The structure of dopamine.

Dopamine (Fig. 2.6) plays a dual role in the body, occurring in both neurons and non-neuronal cells. Dopamine is a neurotransmitter in brain dopaminergic neurons, and its central role in the regulation of motor function and behaviour is well established. In the peripheral nervous system, dopamine is the first catecholamine in the biosynthesis of neurotransmitters such as norepinephrine and epinephrine (Kiss & Soares-da-Silva, 2014). The role of dopamine in the symptomatic nervous system is not limited only to providing a means for the synthesis of the neurotransmitter noradrenaline. Dopamine can also act as a co-transmitter in some circumstances (Kiss & Soares-da-Silva, 2014).

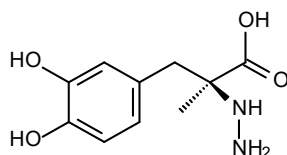
The dopamine agonists all act on dopamine D₂-like receptors, with postsynaptic D₂ receptor stimulation being linked to antiparkinsonian activity while presynaptic D₂ stimulation has been claimed to have 'neuroprotective' effects (Lees, 2005). Dopamine agonists stimulate dopamine receptors directly and do not require carrier-mediated transport for absorption into the brain, and they do not produce toxic metabolites and free radicals (Lees, 2005; Deleu *et al.*, 2002).

Dopamine does not cross the blood-brain diffusion barrier because its amino moiety is protonated under physiological conditions ($pK_a = 10.6$ [NH₂]), making it excessively hydrophilic (Booth *et al.*, 2003). The replacement of dopamine deficiency in the striatum can be accomplished by one or more of the following means: augmentation of the synthesis of brain dopamine, stimulation of dopamine release from presynaptic sites, direct stimulation of dopamine receptors, decreasing re-uptake of dopamine at presynaptic sites or decreasing the metabolism of dopamine or its precursor levodopa (Booth *et al.*, 2003). Current treatment aims to provide a more sustained, continuous stimulation of dopamine receptors (Lees, 2005). The longer elimination half-life of most dopamine agonists relative to levodopa is a potential advantage and hardly any patients develop dyskinesia or severe 'on-off' effects when receiving dopamine agonist monotherapy (Lees, 2005).

Dopamine agonists include the ergot derivative bromocriptine as well as new-generation non-ergot dopamine agonists such as ropinirole and pramipexole. These drugs stimulate dopamine receptors directly (Lees, 2005). They are often reserved for second-line treatment, but currently they are being used as first-line treatment, especially in younger patients (Lees, 2005).

The combination of levodopa and a dopamine agonist will allow for a reduction of the levodopa dose and reduce motor fluctuations. The treatments that is currently available are bromocriptine (Parlodel[®], Aspen Bromocriptine[®]), pramipexole (Pexola[®], Pexola[®] ER, Pramiola[®]) and ropinirole (Requip[®], Requip XL[®]).

2.1.5.3. Carbidopa



Carbidopa: C₁₀H₁₄N₂O₄

Figure 2.7: The structure of carbidopa.

Carbidopa (Fig. 2.7) is a drug that blocks the conversion of levodopa to dopamine outside the central nervous system and thus inhibits unwanted side effects of dopamine on organs located outside of the central nervous system during the management of Parkinson's disease (Zhu *et al.*, 2017). Carbidopa does not cross the blood-brain barrier at normal dosages. If the ratio of carbidopa to levodopa in a particular formulation is increased from 10:1 to 5:1, the tolerance to the adverse effects of levodopa generally improves (Deleu *et al.*, 2002). The amount of oral levodopa that reaches the systemic circulation may be increased threefold by the combination of levodopa with an AADC inhibitor, either carbidopa or benserazide. This increases the bioavailability of levodopa to the brain and reduces the peripheral dopaminergic side effects (nausea, vomiting, headache, irregular heartbeat and anxiety) due to excessive dopamine formation in the peripheral tissues (Freitas *et al.*, 2016). It has been found that carbidopa enhances the accumulation of 3-O-methyldopa and dopamine in skeletal muscles, but decreases that of 3,4-dihydroxyphenylacetic acid (Deleu *et al.*, 2002).

2.2. MONOAMINE OXIDASE

MAO inhibitors were among the first antidepressants to be discovered and have long been used as such (Youdim & Bakhle, 2006). MAO-A and MAO-B are outer mitochondrial membrane-bound flavoenzymes that catalyse the oxidative deamination of biogenic and dietary, xenobiotic amines to their corresponding aldehydes, with the production of hydrogen peroxide (H₂O₂) and ammonia in the process. The two isoforms are highly expressed in the human brain and have been differentiated based on their cellular/tissue localisation, substrate preference, inhibitor sensitivity and physiological roles (Li *et al.*, 2014). MAO in peripheral tissues protects the body by oxidising amines in the blood or by preventing their entry into the circulation (Youdim & Bakhle, 2006).

Together, these two enzymes assist in maintaining the homoeostasis of neurotransmitters in the brain, thereby ensuring appropriate neurological outcomes (Li *et al.*, 2014).

MAO-A and MAO-B are coded by different genes located on the X chromosome (Xp11.23). Even though they are protein products of different genes, they share a sequence identity of 70% and both contain a covalent FAD as the prosthetic group. (Inaba-Hasegawa *et al.*, 2017). The FAD is covalently bound to a cysteine residue by an 8 α -(S-cysteinyl)-riboflavin linkage (Son *et al.*, 2008). MAO plays an important role in psychiatric and neurological disorders, including depression and Parkinson's disease, and because of this they represent interesting targets for developing novel drugs to treat such illnesses (Son *et al.*, 2008). The overactivity of MAO-A and MAO-B may cause excessive production of neurotoxic by-products (H₂O₂), thus promoting neuronal dysfunction that give rise to both psychiatric disorders and neurodegenerative diseases (Li *et al.*, 2014).

The development of selective and reversible MAO inhibitors is important not only from the standpoint of treating symptoms (by increasing the biological half-life of monoamine neurotransmitters), but also with regard to the neuroprotective effects (the prevention or delay of neurodegeneration itself) (Son *et al.*, 2008). Given the role of MAO in the catabolism of dopamine, which include the production of neurotoxic oxidation products as well as the reduction of brain dopamine, MAO is an important target for the development of drugs for the treatment of Parkinson's disease. Furthermore, by reducing the oxidation of dopamine, MAO inhibition can extend the duration of response to levodopa, and allow use of lower doses (Booth *et al.*, 2003).

As mentioned, Parkinson's disease is more common in older people. It is thus noteworthy that the expression of MAO-B is increased in the brain with ageing, and MAO-B activity is significantly enhanced in the brain of Parkinson's disease patients (Li *et al.*, 2014). This further underscores the importance of MAO as a drug target in Parkinson's disease. A list of MAO-inhibitors that is currently being used for the treatment of Parkinson's disease and depression are given in the table below (table 2.1).

Table 2.1: MAO inhibitors used for the treatment of depression and Parkinson's disease, and those under development for treatment (Youdim & Bakhle, 2006).

	<i>Inhibitor selectivity</i>	<i>Mode of action</i>
<i>Antidepressant</i>		
Iproniazid	A + B	Irreversible
Phenelzine	A + B	Irreversible
Isocarboxazid	A + B	Irreversible
Tranylcypromine	A + B	Irreversible
Nialamide	A + B	Irreversible
Clorgyline	A	Irreversible
Moclobemide	A	Reversible
Brofaromine	A	Reversible
<i>Under development</i>		
Ladostigil	A + B (brain selective)	Irreversible
M30	A + B (brain selective)	Irreversible
Befloxatone	A	Reversible
<i>Anti-Parkinson's</i>		
Selegiline	B	Irreversible
Rasagiline	B	Irreversible
Lazabemide	B	Reversible
<i>Under development</i>		
M30	A + B	Irreversible
Ladostigil	A + B	Irreversible
<i>Anti-Diabetic</i>		
Pioglitazone	B	Highly selective

2.2.1. MAO-A

2.2.1.1. The biological function of MAO-A

MAO-A has unique substrate and inhibitor specificities. MAO-A is mainly localised in catecholaminergic neurons and preferentially metabolises serotonin, and is selectively inhibited by clorgyline (Li *et al.*, 2014; Son *et al.*, 2008). MAO-A also catalyses the oxidative deamination of dopamine, norepinephrine and tyramine. During this process, amine substrates are converted to their corresponding aldehydes and hydrogen peroxide (Singer & Ramsay, 1995; Wouters, 1998; Scrutton, 2004; Wang *et al.*, 2011; Biswas *et al.*, 2018). As mentioned, MAO-A is a mitochondrial membrane-bound enzyme which is present in all the mammalian cell types except erythrocytes (Shih *et al.*, 1999; Biswas *et al.*, 2018).

2.2.1.2. Three-dimensional structure of MAO-A

Human MAO-A crystallises as a monomer and the monomeric form of human MAO-A is in contrast to the structural properties of rat MAO-A, which crystallises as a dimer. Human MAO-A and rat MAO-A is in contrast with human MAO-B, since they only have single substrate binding cavities with protein loops at the entrances of either cavities (Edmondson *et al.*, 2007).

The cavity size of human MAO-A is 550 Å³, whereas the rat enzyme has a smaller active site cavity of 450 Å³ (Edmondson *et al.*, 2007). MAO-A has a single cavity that exhibits a “rounder” shape and is larger in volume than the substrate cavity of MAO-B. The substrate binding site in MAO-A is relatively hydrophobic as found for MAO-B (Fig. 2.8) (Edmondson *et al.*, 2007).

The structural basis for differences between the two cavities can be partially attributed to conformational differences of a six residue segment (residues 200-215) that constitutes what is termed a “cavity shaping loop” in MAO-A and MAO-B (Edmondson *et al.*, 2007). This loop is in a more extended conformation in MAO-A while being in a more compact conformation in MAO-B.

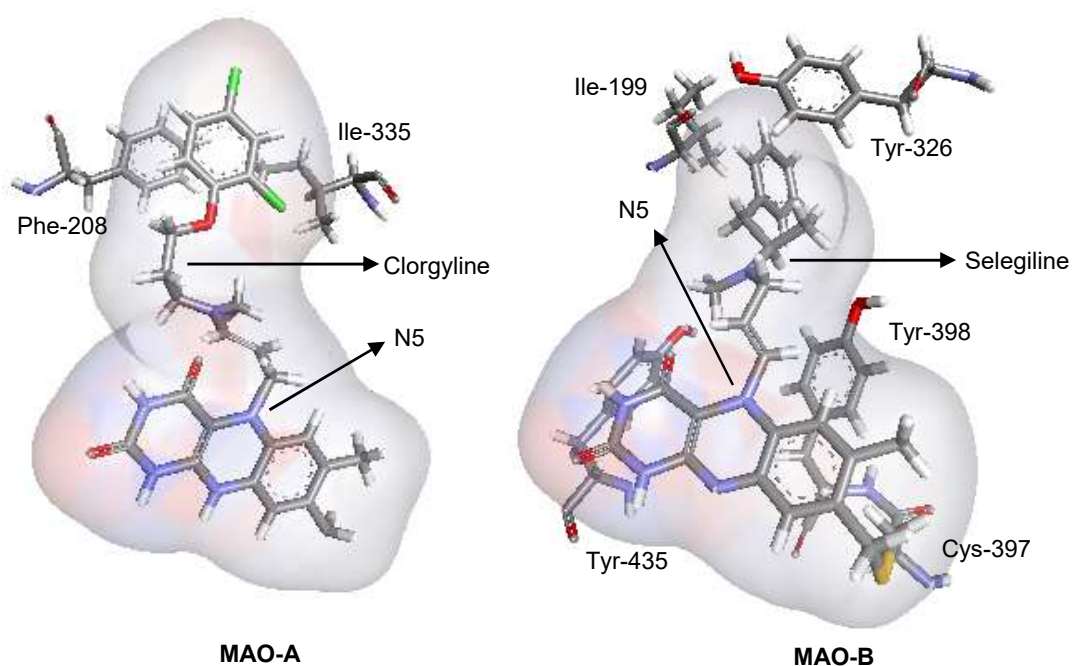


Figure 2.8: Comparison of human MAO-A and human MAO-B active site cavities (De Colibus *et al.*, 2005).

In these figures, clorgyline is in the active site of MAO-A and selegiline is in the active site of MAO-B. Both of these inhibitors form covalent N(5) flavocyanyne adducts with the respective flavin coenzymes (Edmondson *et al.*, 2007). The surface of the active site cavities is represented by the background space-filling structure (Fig. 2.8).

The overall structure of human MAO-A is shown in [figure 2.9](#). This is the X-ray structure of human MAO-A complexed with a reversible MAO-A specific inhibitor, harmine, at a resolution of 2.2 Å. Harmine, a reversible inhibitor, is located in the active site cavity of the enzyme. It interacts with Tyr-69, Asn-181, Phe-208, Val-210, Gln-215, Cys-323, Ile-325, Ile-335, Leu-337, Phe-352, Tyr-407, Tyr-444 and the FAD ([Son et al., 2008](#)). Seven water molecules occupy the space between the inhibitor and these residues. The inhibitor and the FAD are bridged through two water molecules by hydrogens bonds ([Son et al., 2008](#)).

The structure can be divided into two domains, the extra-membrane domain (shown in red and aqua, top part) and the membrane binding domain (shown in red, bottom part). The extra-membrane domain is further divided into two regions, the FAD binding region and the substrate/inhibitor binding region. The FAD and harmine are shown as ball and stick models.

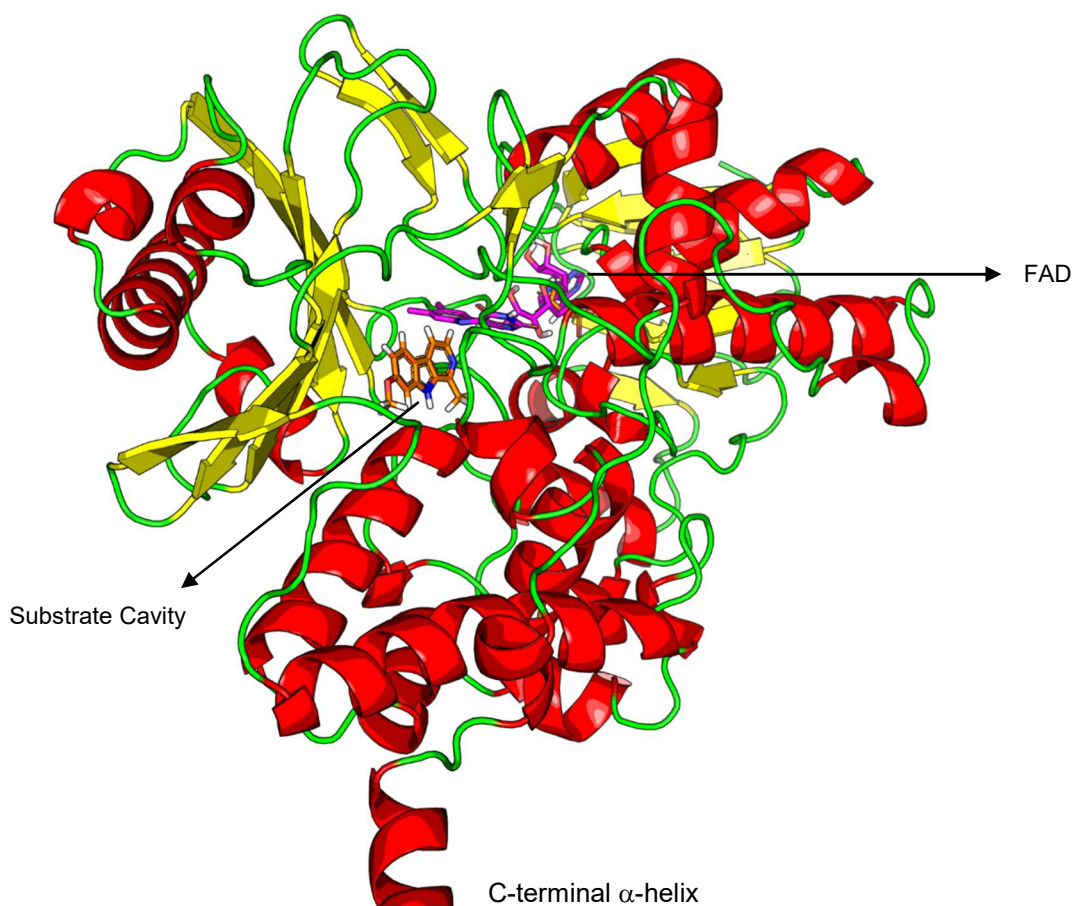
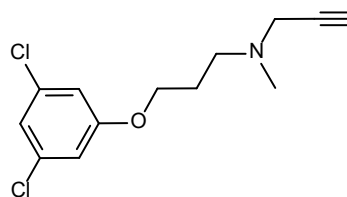


Figure 2.9: Ribbon structure of human MAO-A with harmine.

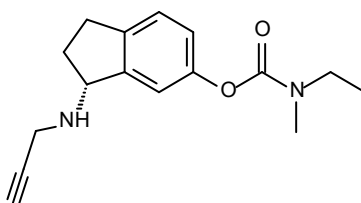
2.2.1.3. Inhibitors of MAO-A



Clorgyline: C₁₃H₁₅Cl₂NO

Figure 2.10: Structure of clorgyline.

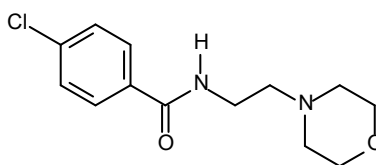
Clorgyline: MAO-A is known to be inhibited by clorgyline and metabolises noradrenaline and serotonin. Since the irreversible selective MAO-A inhibitor, clorgyline, increases brain levels of noradrenaline and serotonin, it has presented with antidepressant activity in a series of clinical trials. The consistently observed cheese reaction forced the abandonment of the development of this inhibitor as an antidepressant (Youdim & Bakhle, 2006).



Ladostigil: C₁₆H₂₀N₂O₂

Figure 2.11: Structure of ladostigil.

Ladostigil: Ladostigil is structurally related to the MAO-B inhibitor, rasagiline, and is a selective irreversible MAO-A and MAO-B inhibitor. Studies have shown that after 1-8 weeks of chronic treatment with ladostigil, both isoforms of MAO in the brain are inhibited with very little inhibition of the enzyme in gut or liver. This unexpected, tissue-selective action allowed irreversible inhibition of all MAO activity in brain, with a reduced risk for the cheese reaction, reflecting the lack of inhibition of the enzyme in gut and liver (Mandel *et al.*, 2005; Sagi *et al.*, 2005; Youdim & Bakhle, 2006). As a non-selective MAO inhibitor, ladostigil increases the levels of all three monoamines, noradrenaline, serotonin and dopamine in the hippocampus and striatum of rats and mice, and it showed antidepressant activity in animal models (Youdim & Bakhle, 2006).



Moclobemide: C₁₃H₁₇ClN₂O₂

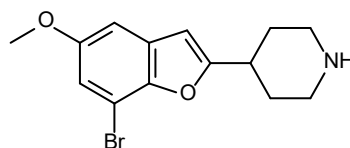
Figure 2.12: Structure of moclobemide.

Moclobemide: Moclobemide is a substrate of CYP2C19 (cytochrome P450 2C19) and is a potent reversible and selective MAO-A inhibitor (Bonnet, 2003). Moclobemide, a benzamide derivative, was the first of its kind to become clinically available. Moclobemide has been extensively evaluated in the treatment of a wide spectrum of depressive disorders and less extensively studied in anxiety disorders (Bonnet, 2003). Moclobemide is an effective antidepressant that does not cause impairment of cognitive function in elderly patients and might be beneficial to motor deficits in Parkinson's disease (Gimenez-Roldan *et al.*, 1997).

Unlike irreversible MAO-inhibitors, moclobemide has an insignificant propensity to induce hypertensive crisis after ingestion of tyramine-rich food (Bonnet, 2003). Therefore, there is no need to avoid dietary tyramine or over-the-counter decongestants with moclobemide as with older MAO inhibitors. However, with moclobemide doses above 900 mg/day the risk of interaction with ingested tyramine may become clinically relevant (Bonnet, 2003). At therapeutic doses, 300-450 mg/day, moclobemide lacks significant negative effects on psychomotor performance, cognitive function or cardiovascular system (Bonnet, 2003). Due to the relative freedom from these side effects, moclobemide is particularly attractive in the treatment of elderly patients (Bonnet, 2003).

The *in vitro* binding of moclobemide to MAO-A is weak but >167-fold more selective than for the MAO-B isozyme (Tajuma *et al.*, 1996). Moclobemide has an IC₅₀ value of 6 µM towards MAO-A and has an IC₅₀ value of >1000 µM towards MAO-B. These values indicate that there is a very selective but relatively weak inhibition of MAO-A (Kettler *et al.*, 1990). Moclobemide is almost completely absorbed after a single oral dose administration, however, oral bioavailability ranges from 44% - 69% because of substantial first-pass metabolism. The bioavailability of moclobemide increases to >80% after multiple doses, possibly due to saturation of first-pass metabolism (Tajuma *et al.*, 1996). Moclobemide is approximately 50% bound to plasma proteins and the volume of distribution ranges from 76-34 L/Kg (Tajuma *et al.*, 1996). Moclobemide is rapidly and extensively metabolised to at least 19 different metabolites, two of which have moderate MAO-A inhibitory activity. The elimination half-life of moclobemide is approximately 1-2 hours and the drug is primarily excreted renally as metabolites. Age and renal function are reported to have no

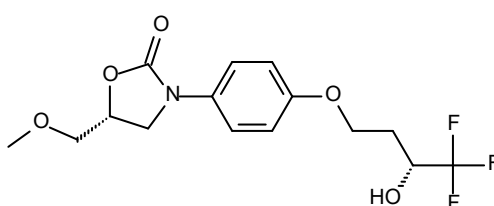
significant effect on the pharmacokinetics of moclobemide, however, elimination is impaired in patients with hepatic dysfunction (Tajuma *et al.*, 1996).



Brofaromine: C₁₄H₁₆BrNO₂

Figure 2.13: Structure of brofaromine.

Brofaromine: Brofaromine is a tight-binding, reversible inhibitor of MAO-A, and has a longer duration of action and considerably more potent activity *in vitro* than moclobemide. Encouraging results have been obtained using brofaromine as a treatment for major depression, panic disorder and social phobia (Baker *et al.*, 1999). Brofaromine is more highly protein bound than moclobemide and it has serotonin reuptake-inhibiting properties in addition to its MAO-inhibiting properties (Baker *et al.*, 1999). The major metabolic route for brofaromine is O-demethylation to yield O-desmethylbrofaromine via the action of CYP2D6, then subsequent conjugation and excretion. O-Desmethylbrofaromine is an active metabolite that is sixfold more potent than brofaromine at inhibiting serotonin uptake, but 100-fold less potent at inhibiting MAO-A in rat brain. O-Desmethylbrofaromine is extensively conjugated and the resultant derivative accounts for about 40% of the administered dose of brofaromine, with another 40% of the dose attributed to unchanged brofaromine and less than 3% to other unconjugated metabolites. The total renal excretion accounts for 76% of an oral dose of brofaromine (Baker *et al.*, 1999).



Befloxatone: C₁₅H₁₈F₃NO₅

Figure 2.14: Structure of befloxatone.

Befloxatone: Befloxatone is an oxazolidinone derivative belonging to a new generation of reversible and selective MAO-A inhibitors (Dolle *et al.*, 2003). *In vitro* and *ex vivo* studies have demonstrated that befloxatone is a potent, reversible and competitive MAO-A inhibitor with potential antidepressant properties (Dolle *et al.*, 2003). Befloxatone inhibits MAO-A selectively and competitively in human and rat brain, heart, liver and duodenum homogenates with K_i values

ranging from 1.9 nM–3.6 nM for MAO-A and 270 nM–900 nM for MAO-B (Dolle *et al.*, 2003). *In vitro*, befloxatone was more potent at inhibiting MAO-A activity than reference compounds such as moclobemide, brofaromine or harmaline. *In vivo*, befloxatone increases brain levels of norepinephrine, dopamine and serotonin and decreases the levels of the corresponding deaminated metabolites. Befloxatone is much more potent, 10-500-fold, than other clinically used reversible MAO-A inhibitors (Dolle *et al.*, 2003).

Cheese reaction: The cheese reaction (Fig. 2.15) is induced by tyramine and other indirectly acting sympathomimetic amines present in food, most commonly in certain cheeses, fermented beer and wine (Youdim & Bakhle, 2006). Normally, MAO metabolises these dietary amines in the gut wall and in the liver, and they thus suffer extensive 'first pass' inactivation and are prevented from entering the systemic circulation. At the peripheral adrenergic neurons, tyramine displaces noradrenaline from storage vesicles in the presynaptic terminals. This initiates the release of noradrenaline into the synapse in large quantities, which causes severe hypertensive crisis which is characterised by high blood-pressure, headaches, increased heart rate, diaphoresis and palpitations.

Irreversible inhibition of MAO-A, the predominant isoform in the gut, allows greatly increased amounts of tyramine to enter the systemic circulation, and from there tyramine has access to the adrenergic neurons, consequently increasing noradrenaline release and causing the cheese reaction (Youdim & Bakhle, 2006). In contrast, reversible inhibitors of MAO-A are displaced from the enzyme by tyramine which is then metabolised normally by the enzyme. With reversible MAO-A inhibitors, circulating tyramine never attains the high levels which characterise irreversible inhibition of MAO (Youdim & Bakhle, 2006).

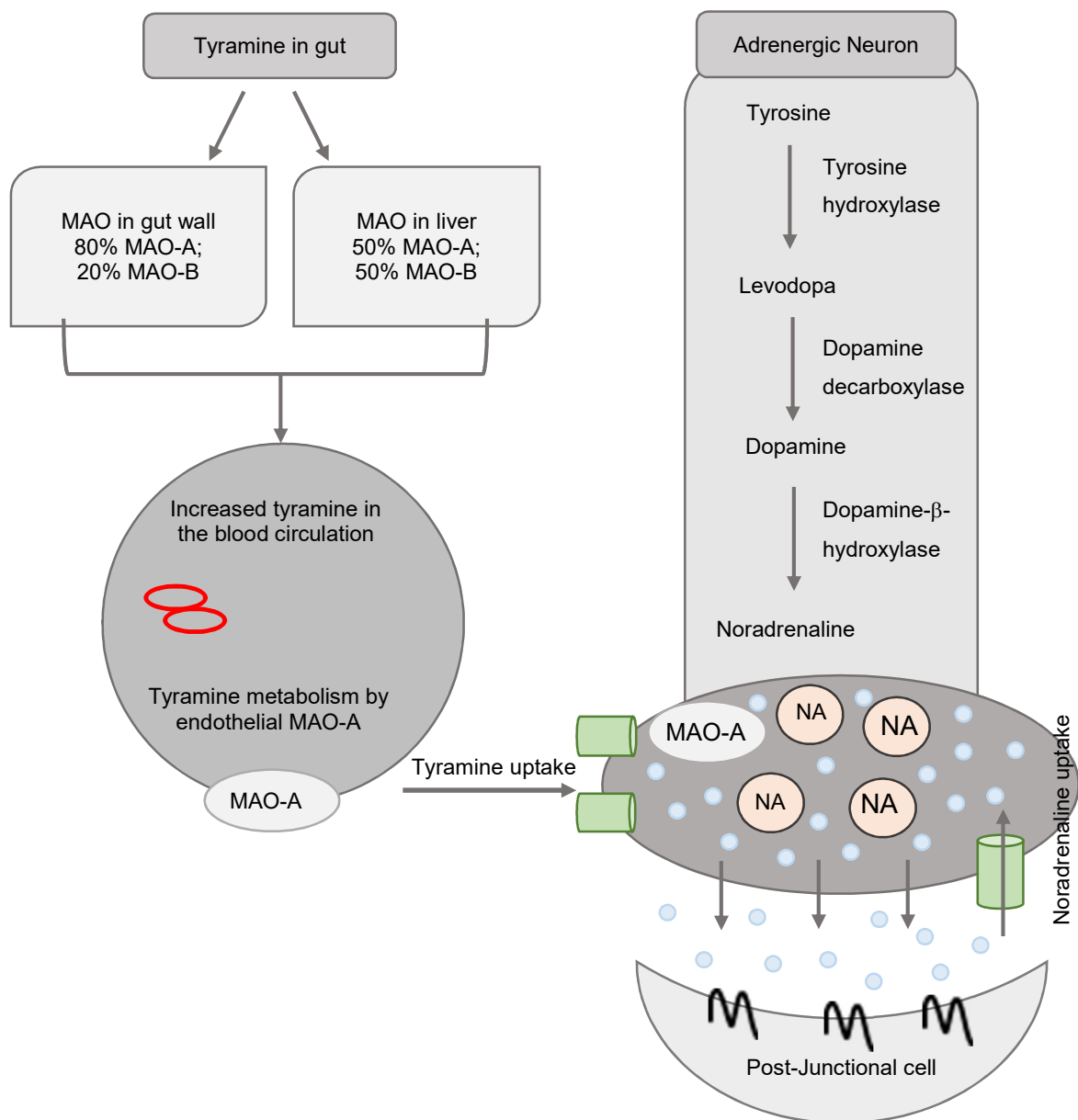


Figure 2.15: The “cheese reaction”.

2.2.2. MAO-B

2.2.2.1. The biological function of MAO-B

MAO-B is primarily found in astrocytes, serotonergic neurons, histaminergic neurons and glial cells, which suggests that MAO-B has a different role in brain function compared to MAO-A (Li *et al.*, 2014; Inaba-Hasegawa *et al.*, 2017). MAO-B acts on dopamine and β -phenylethylamine and is selectively inhibited by paraglyline, rasagiline and selegiline (Li *et al.*, 2014).

2.2.2.2. Three-dimensional structure of MAO-B

Human MAO-B crystallises as a dimer, with each monomer consisting of a globular domain anchored to the membrane through a C-terminal α -helix (Edmondson *et al.*, 2007). The active site of MAO-B consists of two cavities, the substrate cavity in front of the flavin and the entrance cavity located underneath the protein surface and closed by a loop (Edmondson *et al.*, 2007). A substrate molecule must first negotiate the protein loop at the entrance to one of two cavities before reaching the flavin coenzyme (Edmondson *et al.*, 2007). The first cavity has been termed the “entrance cavity”, and is very hydrophobic in nature and exhibits a volume of 290 Å³ (Edmondson *et al.*, 2007). The “substrate cavity” is similarly hydrophobic with a volume of 390 Å³. Separating the “entrance cavity” from the “substrate cavity” is an Ile-199 side chain which serves as a “gate” between the two cavities. Depending on the substrate or bound inhibitor, it can exist in either an open or a closed form which has been shown to be important in defining the inhibitor specificity of human MAO-B (Edmondson *et al.*, 2007). The FAD is at the end of the substrate cavity, and is covalently bound via an 8 α -thioether linkage to Cys-397. The bipartite elongated cavity occupies a combined volume close to 700 Å³ when the side chain of Ile-199 is in the “open” conformation (Fig. 2.8) (Edmondson *et al.*, 2007).

The re-face of the covalent FAD is approached by the amine functional group of the substrate molecule through an “aromatic cage”. The aromatic cage consists of two tyrosine residues which are perpendicular to the plane of the flavin ring. The “aromatic cage” is formed by Tyr-398 and Tyr-435 (Binda *et al.*, 2003; Edmondson *et al.*, 2007).

The overall structure of human MAO-B is shown in figure 2.16. This is the X-ray structure of human MAO-B complexed with salfinamide at a resolution of 1.6 Å.

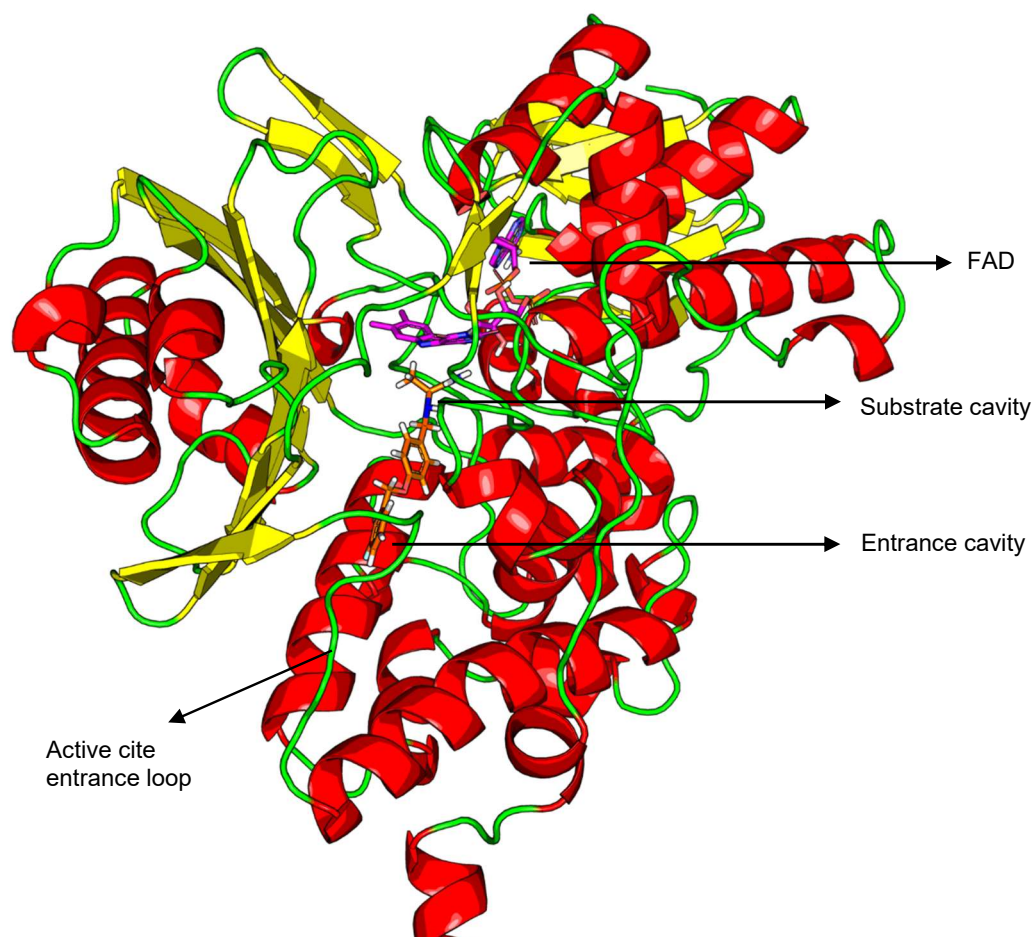


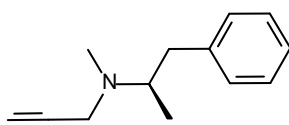
Figure 2.16: Ribbon structure of human MAO-B with salfinamide bound to the active site.

2.2.2.3. Inhibitors of MAO-B

Reversible and irreversible inhibitors: MAO-B inhibitors are used in the therapy of Alzheimer's disease and Parkinson's disease. Selective inhibitors of human MAO-B have gained interest in recent years due to the discovery of an age-related increase in human MAO-B expression after the 60th year of life, especially in glial cells (Chimenti *et al.*, 2009). Therefore, a selective human MAO-B inhibitor may contribute to neuroprotection and prevent neuronal degeneration in age-related neurodegenerative disorders (Chimenti *et al.*, 2009).

Inhibitors of MAO-B stabilise dopamine concentrations in the synaptic cleft and prolong the effects of dopamine (Szökö *et al.*, 2018; Riederer & Müller, 2018). This mode of action provides a limited effect on motor symptoms. Two irreversibly acting MAO-B inhibitors are currently available for the treatment of Parkinson's disease, selegiline and rasagiline. The reversible MAO-B inhibitor safinamide is also an inhibitor of NMDA release and acts predominantly by blocking voltage-gated sodium ion channels (Müller & Foley, 2017; Riederer & Müller, 2018). MAO-B inhibitors have a better safety profile than dopamine agonists and levodopa, and are better tolerated by patients (Müller & Foley 2017; Riederer & Müller, 2018).

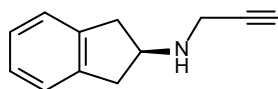
Irreversible MAO-B inhibitors may also protect neurons from cell death and may suppress mitochondrial apoptotic signalling directly by inducing anti-apoptotic Bcl-2, pro-survival neurotrophic factors and antioxidant enzymes ([Inaba-Hasegawa et al., 2017](#)).



Selegiline: C₁₃H₁₇N

Figure 2.17: Structure of selegiline.

Selegiline: Selegiline ([Fig. 2.17](#)) is a propargylamine derivative and is a selective irreversible MAO-B inhibitor which was originally evaluated as an antidepressant. At high doses selegiline loses its selectivity for MAO-B and will also inhibit MAO-A ([Youdim & Bakhle, 2006](#)). At low doses selegiline inhibits the oxidative deamination of dopamine, phenylethylamine and benzylamine, but not that of noradrenaline or serotonin, which are MAO-A substrates. At higher doses, selectivity for MAO-B is lost and MAO-A is also inhibited by selegiline ([Youdim & Bakhle, 2006](#)). Selegiline as an adjuvant to levodopa is a well-established therapy in Parkinson's disease and patients who receives the combination have a better survival rate than those treated with levodopa alone ([Youdim & Bakhle, 2006](#)).



Rasagiline: C₁₂H₁₃N

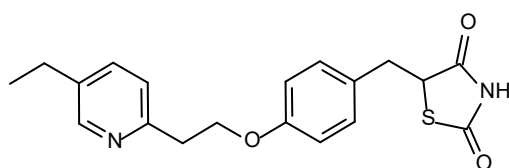
Figure 2.18: Structure of rasagiline.

Rasagiline: Rasagiline ([Fig. 2.18](#)) is a member of the propargylamine family, and is an oral, highly potent, second-generation selective irreversible MAO-B inhibitor, approved in the United States and European Union for the treatment of Parkinson's disease ([McCormack, 2014](#)). Rasagiline can be used as monotherapy in early stages of the disease or as an adjunct to other antiparkinsonian medications in early or more advanced stages of the disease ([McCormack, 2014](#)). The propargyl moiety of rasagiline inhibits MAO-B by binding covalently and irreversibly to the FAD of the enzyme, thus recovery requires *de novo* synthesis of new MAO enzyme ([Gerlach et al., 2012](#); [McCormack, 2014](#)).

Rasagiline inhibits both MAO-A and MAO-B at a high dose of 10 mg/kg, but at a therapeutic dose of 0.5-1 mg/kg that is being used in Parkinson's disease, rasagiline selectively inhibits MAO-B and does not potentiate the pressor response to oral tyramine that results from the inhibition of

MAO-A (Weinreb *et al.*, 2011; Gerlach *et al.*, 2012; McCormack, 2014). Rasagiline inhibits MAO-B with a potency 5- to 10-fold higher than selegiline, the first MAO-B inhibitor to be marketed. Unlike selegiline, rasagiline is not metabolised to yield amphetamine metabolites and therefore does not display the sympathomimetic and neurological effects seen with selegiline (Hoy & Keating, 2012; Gerlach *et al.*, 2012; McCormack, 2014).

Rasagiline demonstrated neuroprotective effects in a variety of *in vitro* and *in vivo* models of neurodegenerative diseases. This neuroprotective effects appear to be dependent on the propargyl moiety and independent of MAO-B inhibition (Hoy & Keating, 2012; Weinreb *et al.*, 2011; McCormack, 2014). The principal metabolite of rasagiline, (R)-aminoindan, may also contribute to the neuroprotective effects of rasagiline (Hoy & Keating, 2012; Weinreb *et al.*, 2011; McCormack, 2014). The exact mechanism of the neuroprotective effects is not fully understood, but rasagiline reduces oxidative stress, stabilises mitochondrial membranes and prevents apoptosis (Weinreb *et al.*, 2011; Naoi *et al.*, 2013; McCormack, 2014). Rasagiline appears to intervene in the death signalling pathway in mitochondria and induces anti-apoptotic Bcl-2 and neurotrophic factors (McCormack, 2014). It is also believed that rasagiline has neurorestorative activity and has been shown, in animal studies, to increase the proportion of tyrosine hydroxylase-immunopositive neurons (Gerlach *et al.*, 2012; McCormack, 2014).



Pioglitazone: C₁₉H₂₀N₂O₃S

Figure 2.19: Structure of pioglitazone.

Pioglitazone: Pioglitazone (Fig. 2.19) is an effective neuroprotectant in the MPTP-mouse model of Parkinson's disease (Quinn *et al.*, 2008; Binda *et al.*, 2011). Pioglitazone is a highly selective MAO-B inhibitor, highlighting potential additional pharmacological actions of this widely used drug. It has been found that (*R,S*)-pioglitazone competitively inhibits MAO-B with a submicromolar K_i value for the human enzyme (Binda *et al.*, 2011). The drug does not exhibit any inhibitory activity at ~100 μ M against either human MAO-A or rat MAO-A. Pioglitazone thus exhibits high specificity and submicromolar affinity to human MAO-B (Binda *et al.*, 2011).

The high-resolution crystal structure of human MAO-B in complex with pioglitazone show no evidence for any covalent attachment of the inhibitor thiazolidinedione ring to the flavin or to the active site residues (Binda *et al.*, 2011). The benzyl and pyridyl rings of pioglitazone extend through the substrate cavity into the entrance cavity of the bipartite active site of MAO-B, similar to safinamide. The specificity of pioglitazone binding to MAO-B and not to MAO-A, is because of

the bipartite structure of the MAO-B active site and its hydrophobic environment (Binda *et al.*, 2011).

Pioglitazone has two key features as a MAO-B inhibitor: first, its high selectivity and, secondly, it has a noncovalent binding mode (Binda *et al.*, 2011). Pioglitazone is a widely and routinely used drug that is known to reach plasma concentrations in monkeys of approximately 4.9 μM , which is ~ 10 -fold higher than the K_i value determined for MAO-B (Binda *et al.*, 2011). Pioglitazone crosses the blood-brain barrier to reach therapeutic concentrations of $\sim 0.14 \mu\text{M}$, which are in a concentration range comparable to the K_i value, 0.5 μM . These data provide a molecular basis for “repurposing” pioglitazone as a neuroprotectant in the treatment of patients in the early stages of Parkinson’s disease (Binda *et al.*, 2011).

2.3. CATECHOL-O-METHYLTRANSFERASE (COMT)

2.3.1. General background and tissue distribution

The enzyme that catalyses O-methylation of catecholamines and other catechols were first discovered in the late 1950s by Axelrod and Tomchick (Männistö & Kaakkola, 1999). They characterised an enzyme which they called catechol-O-methyltransferase (COMT) (Meco & Alessandri, 2000).

COMT is an intracellular enzyme that is widely distributed in the body and it catalyses the transfer of the methyl group from S-adenosyl-L-methionine to one of the hydroxyl groups of a catechol substrate (Axelrod, 1957; Kaakkola, 2000). The physiological substrates include levodopa, dopamine, noradrenaline, adrenaline, their hydroxylated metabolites and catecholestrogens (Guldborg & Marsden, 1975; Kaakkola, 2000).

In mammals, COMT is widely distributed throughout the organs of the body and is an intracellular enzyme. The COMT protein in vertebrates appears mostly in a soluble form (S-COMT), and is predominant in the periphery. Only a minor fraction is in the membrane-bound COMT (MB-COMT) from that predominates in the brain (Männistö & Kaakkola, 1999). Both COMT forms are coded by the same gene using two separate promoters (Borchardt *et al.*, 1974; Tenhunen *et al.*, 1993; Kiss & Soares-da-Silva, 2014). The gene encoding the human enzyme is located in chromosome 22, band q11.2 (Kiss & Soares-da-Silva, 2014). Human S-COMT consists of 221 amino acids, whereas human MB-COMT has a 50-residue-long amino-terminal extension that contains the hydrophobic anchor region (Kiss & Soares-da-Silva, 2014). Laboratory evidence indicates that S-COMT is the enzyme involved in inactivating endogenous or xenobiotic catechol in the brain and other organs (Männistö *et al.*, 1992; Roth, 1992; Lotta *et al.*, 1995; Meco & Alessandri, 2000).

The distribution and cellular localisation of COMT have been evaluated in several animal species as well as in humans (Myöhänen *et al.*, 2010; Kiss & Soares-da-Silva, 2014). COMT is considered

an ubiquitous enzyme because it can be found in almost all peripheral tissues as well as in the central nervous system (Kiss & Soares-da-Silva, 2014). In peripheral tissues, the COMT enzyme is abundantly expressed in the liver and kidney, but is also present in the lung, stomach, spleen, intestines, adrenals, heart, various glands, adipose tissue, uterus, gonads, muscles and red blood cells (Ellingson *et al.*, 1999; Guldberg & Marsden, 1975; Kiss & Soares-da-Silva, 2014). The COMT enzymatic activity in the liver is 3-4-fold higher than that in other peripheral tissues and the expression of the COMT protein and enzymatic activity in the brain is significantly lower than those in peripheral tissues (Kiss & Soares-da-Silva, 2014).

The cellular localisation of S-COMT is different from that of MB-COMT. S-COMT is mainly expressed in the cytoplasm, whereas MB-COMT is located on intracellular membranes such as the rough endoplasmic reticulum, oriented toward the cytoplasm (Kiss & Soares-da-Silva, 2014). S-COMT and MB-COMT are both active toward O-methylation reactions, and no difference in substrate specificity has been reported (Kiss & Soares-da-Silva, 2014). The primary amino acid sequences of the catalytic sites of S-COMT and MB-COMT are identical, but there is a marked difference in their kinetic behaviour. MB-COMT shows a higher substrate affinity (K_m) and a lower capacity (V_{max}), while S-COMT shows a lower substrate affinity (K_m) and a much higher capacity (V_{max}) (Kiss & Soares-da-Silva, 2014). These kinetic differences determine the roles of the two isoenzymes. MB-COMT is able to methylate catecholamines at their physiological concentrations and thus has been considered to be the physiologically more important form of the enzymes (Roth, 1992; Kiss & Soares-da-Silva, 2014). S-COMT is important in non-physiological conditions, such as when the substrate concentration suddenly increases (during levodopa treatment) or when a higher O-methylation reaction rate is needed (Huotari *et al.*, 2002; Kiss & Soares-da-Silva, 2014).

The general and main physiological function of COMT is the elimination of biologically active and potentially toxic catechols and the metabolic inactivation of endogenous catechol neurotransmitters and xenobiotic substances (Kiss & Soares-da-Silva, 2014).

2.3.2. Mechanism of action

COMT is a magnesium dependent intracellular enzyme that catalyses the transfer of a methyl group from the common methyl donor, S-adenosyl-L-methionine (AdoMet), to substrates incorporating a dihydroxybenzene (catechol) motif. This results in the formation of mono-O-methylated products and S-adenosylhomocysteine (Fig. 2.20) (Kiss & Soares-da-Silva, 2014).

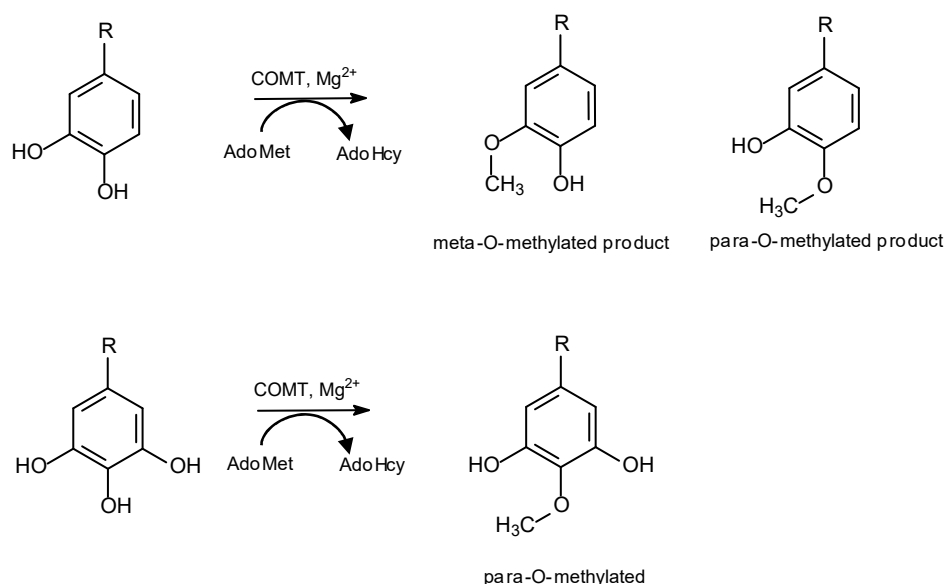


Figure 2.20: COMT catalysed methylation of catechol and pyrogallol substrates.

2.3.3. The three-dimensional structure of COMT

The methylation of catechol substrates by the COMT enzyme requires the presence of magnesium (II) ions and the cofactor S-adenosyl-L-methionine. The overall structure of rat S-COMT in complex with a nitrocatechol (3,5-dinitrocatechol; DNC) is shown in [figure 2.21](#), with the enlarged view of the catalytic site. COMT has a single domain and a mixed α/β -protein structure in which the seven-stranded central β -sheet core is surrounded by eight α -helices ([Fig. 2.21A](#)). The active site of COMT consists of the S-adenosyl-L-methionine binding domain and the catalytic region with the magnesium (II) ion. The cofactor S-adenosyl-L-methionine is located in a deeper cleft, whereas the substrate binding site occupies a shallow groove on the outer surface of the protein. It has been found that S-adenosyl-L-methionine is the first ligand to bind to the enzyme, followed by the magnesium (II) ion and finally by the substrate.

The molecular interactions with the protein are shown in [figure 2.21B](#). The adenine ring of S-adenosyl-L-methionine is hydrogen bonded to Ser-119 and Gln-120 and makes further van der Waals interactions with residues Ile-91, Ala-118 and Trp-143. The methionine fragment of S-adenosyl-L-methionine forms hydrogen bonds with Val-42, Ser-72 and Asp-141. The magnesium (II) ion is located in the centre of the catalytic site and has no interaction with the cofactor S-adenosyl-L-methionine. It is octahedrally coordinated to the oxygen atoms of the side chains of aspartic acid residues Asp-141 and Asp-169, and an asparagine residue, Asn-170, while another coordination site is occupied by a molecule of water. The fifth and sixth coordinating orbits of the magnesium (II) ion are chelated to each of the two hydroxyl groups belonging to the catechol substrate ([Kiss & Soares-da-Silva, 2014](#)).

In addition to the excellent coordination to the protein and the catechol substrate, the magnesium (II) ion lowers the substrate pKa, thereby facilitating the deprotonation of the more acidic hydroxyl group of the catechol nucleus by Lys-144. The resultant phenolate anion can be immediately methylated by S-adenosyl-L-methionine. Further important hydrogen bonding interactions have been identified between the hydroxyls of the catechol ring and the side chains of Glu-199 and Lys-144. Additional productive interactions are provided by the so-called hydrophobic “gatekeeper” residues (Trp-38, Trp-143, Pro-174 and Leu-198), which maintain the substrate correctly positioned for methylation by making favourable interactions with the catechol ring. The interaction of the catechol ring with Trp-38 was found to be absolutely essential for high binding affinity. Replacement of Trp-38 with arginine in pig COMT drastically reduces the binding affinity of the catechol substrate. From these observations, several families of inhibitors have been designed and synthesised. Overall, the analysis of the crystal structures clearly suggest that COMT can interact well with various types of inhibitors that incorporates the catechol pharmacophore. Because the catechol ring binds to the enzyme in a groove at the surface of the protein, the side chain extends out of the catalytic site cavity toward the solvent region, and it can be a target for optimization of the pharmacokinetic profile of the inhibitor ([Kiss & Soares-da-Silva, 2014](#)).

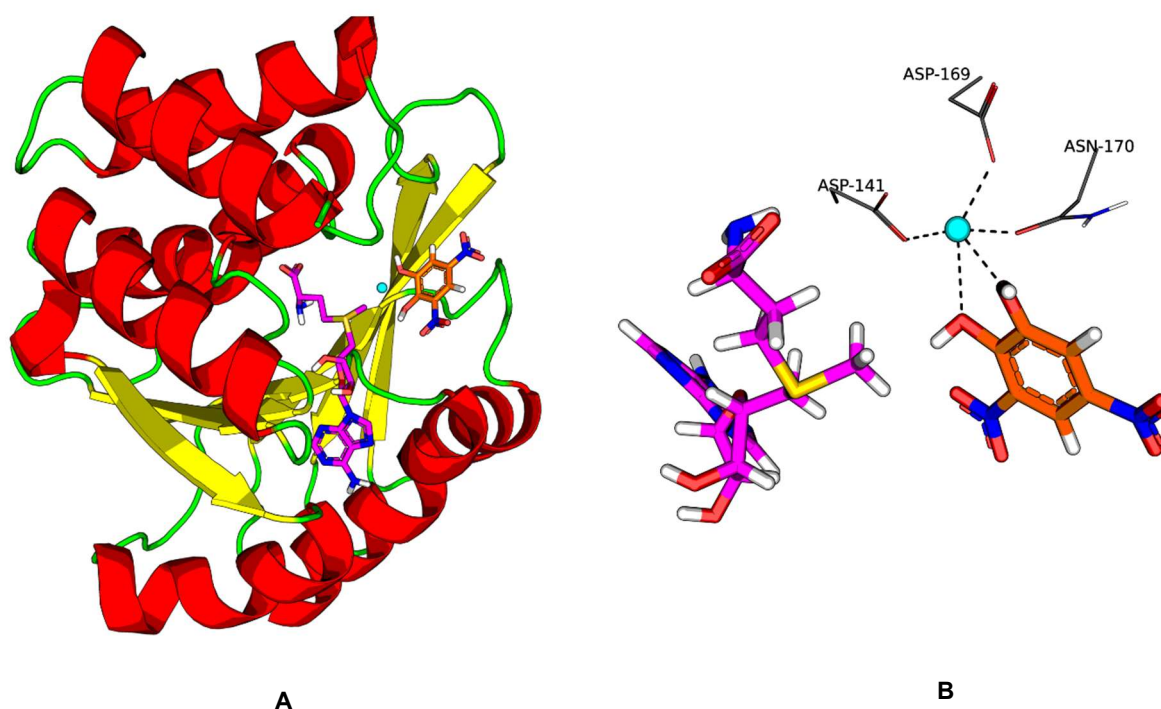


Figure 2.21: (A) Ribbon structure of COMT in complex with S-adenosyl-L-methionine (AdoMet), a Mg²⁺ ion and the ligand DNC; (B) enlarged view of the catalytic site.

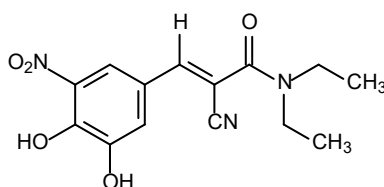
2.3.4. Inhibitors of COMT

Reversible and irreversible inhibitors: The first COMT inhibitors were described in 1960s. Several of these compounds (the gallates, U-0521 and tropolone) have been used as *in vitro* tools to investigate the pharmacology of COMT (Kaakkola, 2000). Since they are unselective, non-potent and quite toxic, they were not suitable for clinical purposes (Kaakkola, 2000). The interest in COMT inhibitors was revived in 1980s when new, potent and selective inhibitors were developed (Kaakkola, 2000). Nearly all of them have a nitrocatechol structure, two of them, namely entacapone and tolcapone have been developed for the treatment of Parkinson's disease and are now available for clinical use in many countries (Kaakkola, 2000). From a pharmacological point of view, tolcapone appears to be more successful than entacapone, with higher COMT inhibition activity, central action and a longer duration of action after oral administration to rats and humans (Müller, 2015). COMT inhibitors are used in patients with Parkinson's disease to reduce the complications associated with long-term levodopa therapy, and by inhibiting catecholamine catabolism they extend the pharmacological effects of levodopa and improve patient disability (Meco & Alessandri, 2000).

COMT inhibitors are commonly used as adjuncts to levodopa in patients with Parkinson's disease for the amelioration of 'wearing-off' symptoms (Müller, 2015). COMT is a major catabolic regulator of synaptic catecholamine neurotransmitters and, as mentioned, catalyses the transfer of a methyl group to a catecholamine substrate. Some of COMT's substrates include dopamine, norepinephrine and epinephrine (Müller, 2015).

The inhibition of COMT improves the bioavailability of levodopa and reduces motor fluctuations. It has been found that the inhibition of COMT also increases the area under the plasma-concentration time curve of levodopa without affecting the maximum concentration or the time to reach maximum concentration (Davis, 1998; Davis *et al.*, 1995; Ruottinen & Rinne, 1996). The COMT inhibitors, entacapone and tolcapone, dose-dependently inhibit the formation of the major metabolite of levodopa, 3-O-methyldopa, and improves the bioavailability and reduces the clearance of levodopa without significantly affecting its absorption. They are useful adjuncts to levodopa in patients with end-of-dose fluctuations (Deleu *et al.*, 2002). In patients treated with levodopa, the "wearing-off" symptoms can be reduced by these two COMT inhibitors.

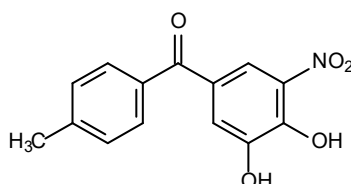
It is believed that COMT inhibition may have an important effect on cognitive functions, particularly on specific cognitive tasks connected with attention, such as learning, and short term memory and working memory (Meco & Alessandri, 2000).



Entacapone: $C_{14}H_{15}N_3O_5$

Figure 2.22: Structure of entacapone.

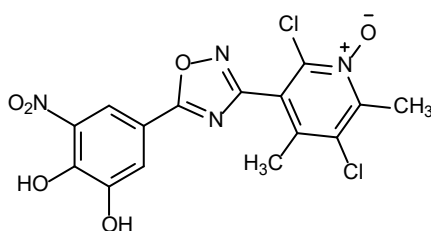
Entacapone (second generation): Entacapone (Fig. 2.22) is an effective, selective, reversible and peripheral acting COMT inhibitor. The pharmacokinetics and metabolism of entacapone have been investigated both in healthy controls and in Parkinson's disease patients. Entacapone is a less potent inhibitor of COMT than tolcapone, and the inhibition achieved is of a shorter duration (Meco & Alessandri, 2000).



Tolcapone: $C_{14}H_{11}NO_5$

Figure 2.23: Structure of tolcapone.

Tolcapone (second generation): Tolcapone (Fig. 2.23) is also an effective, reversible and selective COMT inhibitor, and in contrast to entacapone is both a peripherally and centrally acting COMT inhibitor. Furthermore, it has been found that tolcapone inhibits peripheral COMT activity more potently and longer than entacapone (Meco & Alessandri, 2000).



Opicapone: $C_{15}H_{10}Cl_2N_4O_6$

Figure 2.24: Structure of opicapone.

Opicapone (third generation): Opicapone (Fig. 2.24) is a long-acting, purely peripheral nitrocatechol COMT inhibitor. The compound possesses a high binding affinity to the enzyme with a slow dissociation constant. Opicapone produces a more potent and more prolonged inhibitory

effect of erythrocyte S-COMT than that reported for tolcapone and entacapone (Müller, 2015). Opicapone, entacapone and tolcapone may be compared with each other as shown in the table below (table 2.2).

Table 2.2: Characteristics of available and experimental COMT inhibitors (Müller, 2015).

Property:	Opicapone:	Entacapone:	Tolcapone:
Route	Oral	Oral	Oral
Frequency of administration per day	1	≤10	3
<i>In vivo</i> maximal inhibition after (hours)	3	0.5	1
COMT inhibition	Periphery	Periphery	Periphery and brain
3 h post-dose ED ₅₀ (mg/kg)	1.05 ± 0.04	1.77 ± 0.1	7.8 ± 0.7

2.4. MOLECULAR MODELLING IN DRUG DESIGN

Molecular structure-based drug design is an art and a science, it is a synthesis of scientific knowledge, experience, intuition, and aesthetics. Resources such as expanded databases, especially from crystallography and NMR (nuclear magnetic resonance), computer graphics displays, and linkage by facile web tools, has put precise molecular structures before our eyes and at our fingertips. The structural databases, which have been growing exponentially for three decades, now offer a number of interesting targets for structure-based drug design projects (Meyer *et al.*, 2000).

The development of new drugs is indeed one of the most challenging tasks of today's science. Driven by the combined efforts of the pharmaceutical industry, biotech companies, regulatory authorities, academic researchers, and other private and public sectors, the development of new drugs is a very complex and demanding interdisciplinary process (Alonso *et al.*, 2006). The discovery and manufacture of new and more effective drugs have produced a general improvement in health and has contributed to the advance of science itself, leading to the development of complex and more accurate tools and techniques for the discovery and improvement of new active compounds, and the understanding of their targets (Alonso *et al.*, 2006).

There is no unique solution to a drug design problem. The appropriate experimental techniques or computational methods to use will depend on the characteristics of the system itself and the information available (Alonso *et al.*, 2006). With a drug-design process, a variety of computational approaches can be applied at different stages. The focus on reducing the number of possible ligands, occurs at an early stage, while the emphasis on decreasing experimental cost and reducing time is occurring at the end, during the lead-optimising stage (Alonso *et al.*, 2006).

Rational drug design:

A rational approach is needed to increase the chances of finding new drugs, and to exploit the opportunities of potential new drug targets. The drug discovery process follows a well-established procedure, when the structure of the target protein is known (Alonso *et al.*, 2006). With the docking protocol, virtual screening methods are applied at an early stage to reduce the size of the large compound libraries. This procedure, which is known as enrichment, reduces the size of the library to a group of molecules that is more likely to bind the target receptor (Alonso *et al.*, 2006).

Similar compounds can be further grouped together and arranged in smaller assemblies to assist the screening process. The use of several small libraries is not only a more cost-effective approach, but can usually provide a broader chemical diversity than a single large library (Alonso *et al.*, 2006). Molecules are docked to the target receptor once an optimum library has been produced, which reduces the number of candidates further. The relative stability of the docked complexes is evaluated by the initial screening that makes use of fast ranking functions, however, it is not very accurate (Alonso *et al.*, 2006). The selected candidates, usually a few hundred, are subject to further docking experiments using more sophisticated scoring functions (Alonso *et al.*, 2006).

Docking:

Docking techniques have been designed to discover the correct conformation of a ligand and its receptor, and have been used for decades. The process of binding a small molecule to its protein target is not simple, several entropic and enthalpic factors influence the interactions between them (Alonso *et al.*, 2006). The quantitative description of the process is being complicated by the mobility of both ligand and receptor, the effect of the protein environment on the charge distribution over the ligand, and their interactions with the surrounding water molecules (Alonso *et al.*, 2006). A comprehensive set of conformations of the receptor complex needs to be generated, and ranked according to their stability (Alonso *et al.*, 2006).

Over the last two decades, more than 60 different docking tools and programs have been developed for both academic and commercial use, such as **AutoDock** (automated docking of ligand to macromolecule by Lamarckian genetic algorithm and empirical free energy scoring

function), **AutoDock Vina** (molecular docking and virtual screening), **DOCK** (to shape-based algorithms), **FlexX** (incremental build based docking program), **FRED** (systematic examination of all possible poses within the protein active site), **GOLD** (genetic algorithm based, flexible ligand, partial flexibility for protein), **Glide** (systematic search techniques based docking program), **LeDock** (flexible small molecule docking software, which performs an exhaustive search of positions, orientations and conformation of a ligand in the active site of a protein), **MOE-Dock** (provides a collection of applications for visualising and understanding detail of receptor active site and receptor-ligand interactions) and **Surflex** (based on an idealised active site ligand), and many more ([Alonso et al., 2006](#); [Pagadala et al., 2017](#)).

Among these programs, AutoDock Vina, GOLD, and MOE-Dock are considered to be particularly accurate. GOLD and LeDock are able to identify the correct ligand binding poses. Glide (XP) and GOLD predict the poses consistently with a 90.0% accuracy ([Pagadala et al., 2017](#)). For a docking process to be successful, it is necessary that both the right conformation of the ligand-receptor complex is predicted, and that the ranking of final structures is correct. The procedure needs to be able to differentiate among similar conformations of the same system, as well as to predict the relative stability of different complexes ([Alonso et al., 2006](#)).

Molecular dynamic stimulation:

Molecular dynamic simulations are one of the most resourceful and widely applied computational techniques for the study of biological macromolecules, therefore, they are very valuable for understanding the dynamic behaviour of proteins at different timescales, from fast internal motions to slow conformational changes or even protein folding processes ([Alonso et al., 2006](#)).

Time-averaged properties of the biomolecular system, such as density, conductivity, and dipolar moment, as well as different thermodynamic parameters, including interactions energies and entropies, are being obtained by the study of the effect of explicit solvent molecules on protein structure and stability ([Alonso et al., 2006](#)).

Molecular dynamics are useful not only for rationalising experimentally measured properties at the molecular level, but it is well known that most structures determined by X-ray or NMR-methods have been refined using the molecular dynamic stimulation method. Therefore, the interplay between computational and experimental techniques in the area of molecular dynamic simulations is longstanding, with the theoretical methods assisting in understanding and analysing experimental data ([Alonso et al., 2006](#)).

Protein flexibility:

The old idea of the “key and lock” interaction of a ligand and its protein receptor is not an accurate description of most biological complexes (Alonso *et al.*, 2006). The ligand-protein interactions more resemble a “hand and glove” association, where both parts are flexible and adjustable to complement each other. This is known as induced fit. They can modify their shape and mould their complementarity to increase favourable contacts and reduce adverse interactions, therefore, increasing the total binding free energy (Alonso *et al.*, 2006). It has been found that active-site regions of enzymes present areas of both low and high conformational stability. Mobile loops that close over the ligand upon binding are included within the flexible parts, while catalytic residues, for example, are usually structurally stable. This dual character of the active-site environment appears important for optimum binding (Alonso *et al.*, 2006).

Receptor conformation:

The three-dimensional structure of both ligand and protein are necessary for the application of docking techniques. While the different conformational structures of small molecules may be relatively easy to predict, the lowest energy conformation obtained may not correspond to that of the bound ligand. The structures of proteins present a bigger challenge. Although experimental techniques involving X-ray and NMR-analysis are now routine, inherent difficulties in the preparation of samples and data collection and interpretation mean we are still far from a complete automated and high-throughout process (Alonso *et al.*, 2006). Docking studies cannot be performed directly when the protein targets for drug design do not have an experimentally determined structure.

Computational techniques can be used, in some cases, to predict the three-dimensional structure of a protein when the structure of a closely related protein homolog is known (Alonso *et al.*, 2006). Homology modelling or sequence threading techniques may be used to generate models of protein structures, which can be used as docking targets, although it is not as good as experimentally determined structures. It has been reported that the use of multiple homology models constructed from different crystal structures could provide a better representation of the protein receptor and improve the docking (Alonso *et al.*, 2006).

In summary, it is of great importance to carefully prepare the structure of the protein target before the docking process. While structures of ligand-bound protein may provide the highest enrichments, the final results might be prejudiced towards particular types of ligands (Alonso *et al.*, 2006). Structure-based drug design is a powerful technique for the rapid identification of small molecules against the three-dimensional structure of the macromolecular targets available by either X-ray or NMR. Structural information of individual proteins and their interactions became very important for further drug therapy because of the abundant information regarding the

sequences and structures of the proteins. This dissertation will include a molecular modelling section using the crystal structures of human MAO-A and human MAO-B, as well as the COMT enzyme. Molecular docking and the analysis of the binding interactions and orientations will be carried out with the Discovery Studio suite of modelling softwares.

2.5. SUMMARY

In this chapter, the age-related neurodegenerative disorder, Parkinson's disease, is discussed and although Parkinson's disease is an incurable progressive disease, symptomatic treatment strategies can improve the quality of life. Treatment with MAO-B inhibitors is one strategy that can be followed, particularly since these compounds may contribute to neuroprotection and prevent neuronal degeneration in Parkinson's disease. MAO-B inhibitors have the ability to protect against dopamine metabolism thus extending the effects of dopamine in the brain. Another class of drugs that are used for the treatment of Parkinson's disease is COMT inhibitors. These compounds mainly protect against levodopa metabolism in the periphery, and thus reduces some of the complications associated with long-term therapy with levodopa. Three-dimensional structures of MAO-A, MAO-B, and COMT may play an important role in the design of inhibitors for these enzymes. In this dissertation, docking of ligands into models of these enzymes will be carried out, mainly to predict potential binding orientations and to analyse interactions of the ligands with the enzyme's active sites.

CHAPTER 3

Synthesis and evaluation of nitrocatechol derivatives of chalcone as inhibitors of monoamine oxidase and catechol-O-methyltransferase

3.1. ABSTRACT

Literature reports that several chalcones inhibit the monoamine oxidase (MAO) enzymes, mostly with specificity for the MAO-B isoform. Recently, nitrocatechol derivatives have been proposed to potentially represent dual-target-directed compounds that may inhibit both MAO-B and catechol-O-methyltransferase (COMT). Both these enzymes are key dopamine metabolising enzymes in the brain, and inhibitors are thus relevant to the treatment of Parkinson's disease. In addition, COMT metabolises levodopa, the metabolic precursor of dopamine, in the periphery and COMT inhibitors have been used to enhance the bioavailability of levodopa to the brain. Dual-target-directed compounds that inhibit both MAO-B and COMT may have enhanced value for the treatment of Parkinson's disease by more effectively conserving endogenous dopamine and by increasing levodopa's availability to the brain. The present study expands on the discovery of dual MAO-B/COMT inhibitors by synthesising three novel nitrocatechol derivatives of chalcones and converting them to the corresponding pyrazoline derivatives. The six newly synthesised compounds were evaluated as inhibitors of human MAO and COMT, and the inhibition potencies were expressed as IC_{50} values. A pyrazoline derivative, compound **5**, 4-[1-acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile, was the most potent COMT inhibitor with an IC_{50} value of 0.048 μ M. This is more potent than the reference COMT inhibitors, tolcapone and entacapone, which have IC_{50} values of 0.26 μ M and 0.25 μ M, respectively. The results indicated that the pyrazoline derivatives (IC_{50} = 0.048–0.079 μ M) are more potent COMT inhibitors than the chalcones (IC_{50} = 0.175–0.240 μ M). Unfortunately, the chalcone and pyrazoline derivatives were weak MAO inhibitors with IC_{50} values >43.9 μ M. This study concludes that the nitrocatechol derivatives investigated here are promising COMT inhibitors, while not being suitable as MAO inhibitors. Using molecular docking, potential binding modes and interactions of the inhibitors with MAO and COMT are proposed.

KEYWORDS: MAO-A, MAO-B, chalcone, inhibitors, nitrocatechol derivatives, pyrazoline derivatives, Parkinson's disease.

3.2. INTRODUCTION

Monoamine oxidase (MAO) inhibitors have been used clinically since the 1950s and were among the first antidepressants to be discovered. MAO inhibitors, however, fell out of favour because of the “cheese reaction”, which is a potentially fatal interaction of these drugs and tyramine containing food (Foley *et al.*, 2000). MAO metabolises biogenic amines in the neuronal and glial cell of the human brain, and thus plays an important role in the inactivation of amine compounds (Foley *et al.*, 2000). The inhibition of MAO leads to increased levels of biogenic amines in the synaptic cleft, which enhances neurotransmission (Müller *et al.*, 2017).

Two isoenzymes of MAO have been identified, MAO-A and MAO-B. The MAO enzymes are present in most mammalian tissues but differ in anatomical localisation, preferred substrates and inhibitor specificity (Li *et al.*, 2014). It has been found that the MAO-A and MAO-B proteins have 70% sequence identity (Youdim *et al.*, 2006), and both isoenzymes contain a covalent flavin adenine dinucleotide (FAD) as the prosthetic group (Inaba-Hasegawa *et al.*, 2017). Since the MAOs metabolise neurotransmitter amines, they play important roles in neuropsychiatric and neurodegenerative disorders. Furthermore, the overactivity of these enzymes may lead to the excessive production of neurotoxic by-products such as hydrogen peroxide, which may promote neuronal dysfunction (Li *et al.*, 2014).

In the brain, MAO-A is mainly localised in catecholaminergic neurons, while in the peripheral tissues it is the predominant isoform in the placenta and gut (Müller *et al.*, 2017). MAO-A degrades serotonin and noradrenaline in the central nervous system (CNS), while it metabolises tyramine in the intestine. In this respect, MAO-A acts as a metabolic barrier for the access of tyramine to the systemic circulation. MAO-A is specifically and irreversibly inhibited by low concentrations of clorgyline (Fig. 3.1) (Foley *et al.*, 2000). The inhibition of MAO-A in the brain is useful for the treatment of psychiatric illnesses such as depression. As mentioned, the peripheral inhibition of MAO-A may induce an acute syndrome called the “cheese reaction” (Foley *et al.*, 2000). This reaction is induced by tyramine which is present in certain cheeses, fermented beer and wine. When tyramine metabolism in the intestinal tissues by MAO-A is inhibited, tyramine gains access to the systemic circulation. At the peripheral adrenergic neurons, tyramine displaces noradrenaline from the storage vesicles in the pre-synaptic terminal. This initiates the release of noradrenaline into the synapse in large quantities, which may lead to a severe hypertensive crisis. Due to the role of MAO-A in the breakdown of serotonin, MAO-A inhibitors may also promote a centrally mediated, possibly life-threatening, serotonin syndrome when administered with selective serotonin reuptake inhibitors or other serotonin-enhancing drugs. Serotonin syndrome is characterised by hallucinations, loss of coordination, rapid heartbeat, sudden changes in blood pressure, increased body temperature, overactive reflexes, nausea, vomiting and diarrhoea (Fernandez & Chen, 2007).

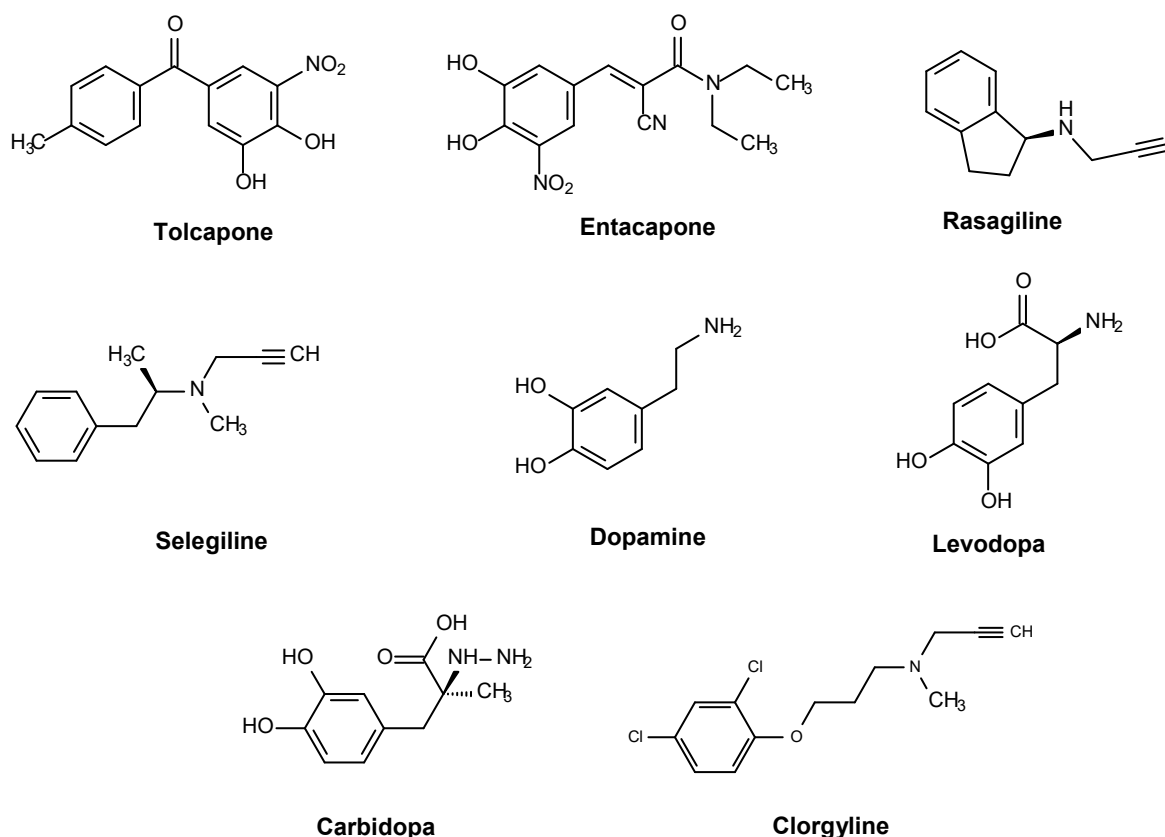


Figure 3.1: Structures of compounds referred to in the text.

MAO-B is predominantly found in the human brain, liver and platelets, and is relatively insensitive to inhibition by clorgyline. In the brain, MAO-B is a major metabolic enzyme for dopamine and β -phenylethylamine (Foley *et al.*, 2000). MAO-B finds its most significant application in the treatment of neurodegenerative disorders. There are two classes of MAO inhibitors that can be distinguished, reversible competitive inhibitors that bind reversibly to the active site of the enzyme, and irreversible inhibitors that bind MAO in a reversible competitive manner. Irreversible MAO-B inhibition is a well-accepted and proven therapeutic approach in the treatment of patients with Parkinson's disease. MAO-B inhibitors provide a modest improvement on motor symptoms, and there are currently two irreversibly acting MAO-B inhibitors clinically available, selegiline and rasagiline. These inhibitors may lose their specificity for MAO-B at high doses and may also inhibit MAO-A. At low doses, however, selegiline and rasagiline inhibit the oxidative deamination of dopamine, phenylethylamine and benzylamine (Weinreb *et al.*, 2011; Gerlach *et al.*, 2012; McCormack, 2014). Selegiline as an adjuvant to levodopa is a well-established therapy in Parkinson's disease and patients who receive the combination have a better survival rate than those treated with levodopa alone (Youdim & Bakhle, 2006).

Selective inhibitors of human MAO-B has gained interest in recent years due to the discovery of an age-related increase in human MAO-B expression after the 60th year of life, especially in glial cells (Fowler *et al.*, 1997). The MAO catalytic cycle yields hydrogen peroxide and aldehyde

species as by-products. Since these by-products may lead to cell damage under certain conditions, the increased MAO-B activity in the ageing brain may contribute to neurodegeneration and promote the development of Parkinson's disease. Therefore, a selective human MAO-B inhibitor may provide neuroprotection and prevent neuronal degeneration in age-related neurodegenerative disorders (Chimenti *et al.*, 2009). Furthermore, propargylamine compounds such as selegiline and rasagiline may protect neurons against damage by mechanisms that are unrelated to the inhibition of MAO-B. These mechanisms include the stabilisation of mitochondrial membranes and the prevention of apoptosis (Weinreb *et al.*, 2011; Naoi *et al.*, 2013; McCormack, 2014).

The main rationale for the use of specific MAO-B inhibitors in Parkinson's disease is the inhibition of dopamine metabolism and enhancement of striatal dopaminergic activity without significantly affecting MAO-A activity. This suggests that MAO-B inhibitors are a useful option for the treatment of motor symptoms in Parkinson's disease (Fernandez & Chen, 2007). A more theoretical rationale for the use of MAO-B inhibitors in patients with Parkinson's disease originates from the possibility that MAO-B may activate pro-neurotoxins present in the diet or environment. In this respect central MAO-B metabolises and activates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to firstly yield 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺), which disproportionates to generate 1-methyl-4-phenylpyridinium (MPP⁺), a potent parkinsonism-inducing neurotoxin (Fig. 3.2) (Smeyne & Jackson-Lewis, 2005). The discovery that MAO-B inhibition attenuates MPP⁺ neurotoxicity generated the theory that MAO-B inhibitors may not only protect against environmental parkinsonism-inducing neurotoxins, but may also modify the underlying processes of Parkinson's disease (Fernandez & Chen, 2007).

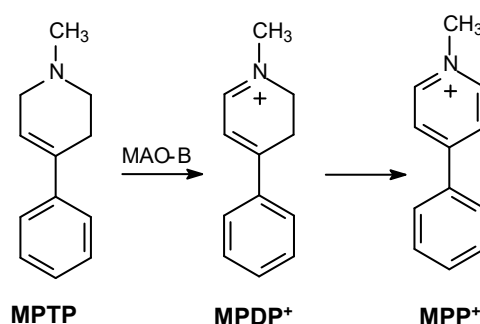


Figure 3.2: The metabolism of MPTP to yield MPDP⁺ and MPP⁺.

Patients with Parkinson's disease experience a decrease in the control of muscle movement as the most characteristic symptom. This is due to the degeneration of the nigrostriatal neurons with subsequent depletion of dopamine in the striatum. The reduced levels of dopamine are responsible for the motor symptoms in Parkinson's disease. As mentioned above, MAO-B inhibitors may provide some improvement of these symptoms by reducing the central

MAO-B-catalysed metabolism of dopamine. The most effective treatment of Parkinson's disease is, however, levodopa, the metabolic precursor of dopamine ([Fig. 3.3](#)). Unlike dopamine, levodopa gains access to the central nervous system via amino acid transporters, and is decarboxylated in the brain to yield dopamine. This restores striatal dopamine and greatly improves the motor symptoms in Parkinson's disease. The enzyme, aromatic L-amino acid decarboxylase (AADC), decarboxylates levodopa in the gastrointestinal tract and liver to yield dopamine, and subsequently only approximately 30% of levodopa reaches the systemic circulation. The combination of levodopa with an AADC inhibitor such as carbidopa or benserazide, increases the fraction of levodopa that reaches the systemic circulation, thus improving the bioavailability of levodopa to the brain ([Freitas *et al.*, 2016](#)). Levodopa is also metabolised by catechol-O-methyltransferase (COMT) in the central and peripheral tissues. In the periphery, COMT inhibitors such as tolcapone and entacapone reduce the O-methylation of levodopa to 3-O-methyldopa, and thus further enhances the fraction of levodopa that is available for uptake into the brain. Furthermore, the central inhibition of COMT may also increase the fraction of levodopa that is converted to dopamine with a subsequent improvement of therapeutic efficacy. COMT inhibitors are usually administered in conjunction with a combination of carbidopa/levodopa. The inhibition of levodopa metabolism by AADC and COMT inhibitors allows for a reduction in the levodopa dose, which greatly reduces the potential of levodopa-associated adverse effects. Levodopa continues to be the gold standard drug for the symptomatic treatment of Parkinson's disease, and because of this much interest in the development of inhibitors of the COMT enzyme exists. This is based on the hypothesis that inhibition of this enzyme may provide clinical improvements in Parkinson's disease patients undergoing treatment with levodopa and a peripheral AADC inhibitor ([Learmonth *et al.*, 2012](#)).

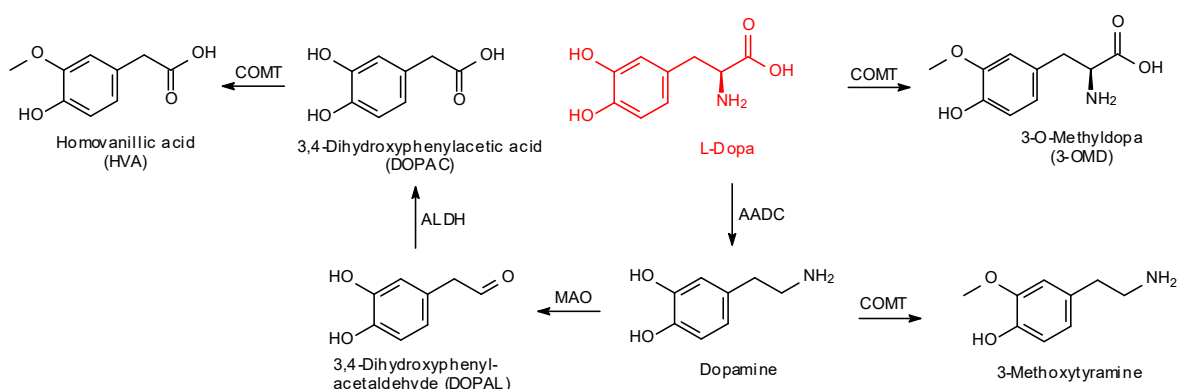


Figure 3.3: The metabolism of levodopa (L-dopa).

Due to extensive peripheral metabolism by AADC and COMT, less than 1% of the levodopa dose reaches the brain ([Kaakkola, 2000](#)). Central metabolism of dopamine by MAO further decreases the efficacy of levodopa ([Lees, 2005](#)). Thus, the dual inhibition of MAO and COMT may not only conserve endogenous dopamine levels but may also protect levodopa against undesirable

metabolism, improving its availability to the brain. This study thus hypothesises that nitrocatechol derivatives of chalcones may be designed that exhibit dual inhibition of COMT and MAO-B. The structures of the chalcones that will be investigated in this study are shown in [table 3.1](#). These compounds were selected since several chalcones are known to inhibit the MAOs, mostly with specificity for the MAO-B isoform. For example, ([Fig. 3.4](#)), chalcone **A** is a naturally occurring chalcone that has been shown to inhibit MAO using rat, bovine and hamster MAO, while chalcone **B**, a synthetic derivative was found to be a human MAO inhibitor ([Robinson *et al.*, 2013](#)). Generally, chalcones show a higher degree of inhibition for MAO-B than of MAO-A. Chalcone **B** showed high inhibitory activity with an IC_{50} value 0.0044 μ M for MAO-B, and no inhibition of MAO-A was observed at concentrations of 50 μ M ([Chimenti *et al.*, 2009](#); [Robinson *et al.*, 2013](#)). Chalcone **A** is a more potent inhibitor of MAO-A than MAO-B, with IC_{50} values of 13.9 μ M and 47.2 μ M, respectively ([Xuan *et al.*, 2000](#); [Robinson *et al.*, 2013](#)). Chalcone **C** is also a potent and selective MAO-B inhibitor and exhibits an IC_{50} value of 0.0051 μ M for MAO-B, and 4.95 μ M for MAO-A ([Chimenti *et al.*, 2009](#)).

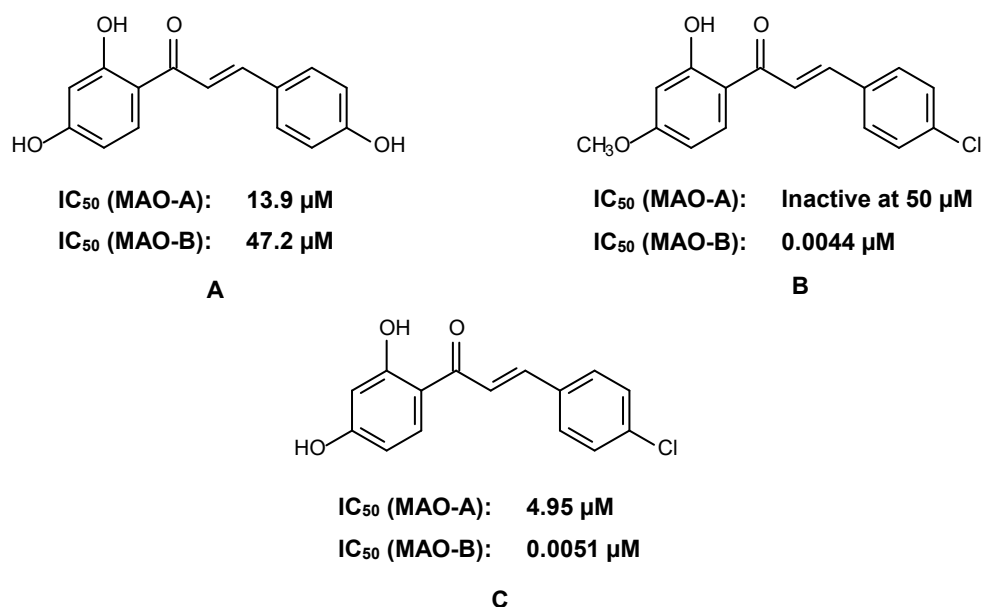


Figure 3.4: The structures of chalcone derivatives known to inhibit MAO.

The nitrocatechol moiety, in turn, is present in the clinically used COMT inhibitors, tolcapone and entacapone, and is considered privileged for the inhibition of COMT. To support the proposal that nitrocatechol derivatives of chalcones may act as dual inhibitors of MAO and COMT, a recent study showed that these compounds are high potency inhibitors of rat liver COMT, although only moderate MAO-B inhibition was observed. In that study it was shown that chalcone **D** ([Fig. 3.5](#)) is the most potent inhibitor of COMT among the compounds evaluated, with an IC_{50} value of 0.07 μ M. Tolcapone and entacapone exhibit IC_{50} values of 0.26 μ M and 0.25 μ M, respectively. Chalcone **D** is therefore 3.5-fold more potent than tolcapone and entacapone ([Engelbrecht *et al.*, 2018](#)). Chalcone **E** and **F** were also identified as potent COMT inhibitors and showed an IC_{50}

values of 0.09 μM and 0.08 μM , respectively, while only moderate MAO-B inhibition was observed. The results indicate that the substitution on ring-B of the chalcone derivatives does not significantly alter COMT inhibition potency. Chalcone **G**, showed an IC_{50} value of 0.29 μM , and is of interest since it is the most potent MAO-B inhibitor of the reported study with an IC_{50} value of 13.9 μM . Chalcone **G** is thus the most suitable dual inhibitor of COMT and MAO-B, as indicated in this recent study (Engelbrecht *et al.*, 2018).

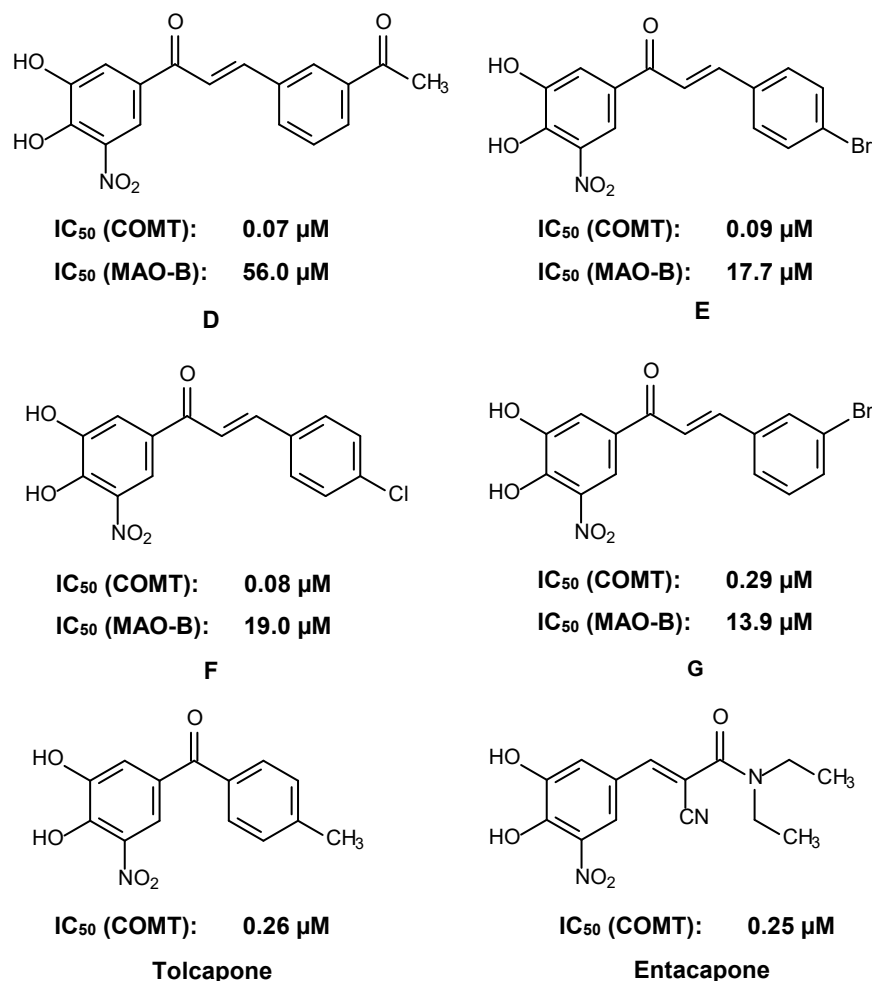


Figure 3.5: The structures of nitrocatechol derivatives of chalcone that are known to inhibit COMT (Engelbrecht *et al.*, 2018).

In this study, additional nitrocatechol derivatives of chalcone will be evaluated as inhibitors of human MAO and COMT and the inhibition potencies will be expressed as IC_{50} values (Table 3.3). Possible binding orientations of selected compounds in the MAO and COMT active sites will be determined by molecular modelling studies, and potential lead compounds for the future design of dual MAO/COMT inhibitors will be proposed. This study will also convert some of the chalcone compounds to the corresponding pyrazoline derivatives. Several literature reports have shown that pyrazolines are good potency MAO-B inhibitors. For example, pyrazoline derivatives **H**, **I** and

J (Fig. 3.6) have been reported to be inhibitors of human MAO-B with IC_{50} values ranging between 2.75 and 16.33 μM (Chimenti *et al.*, 2010).

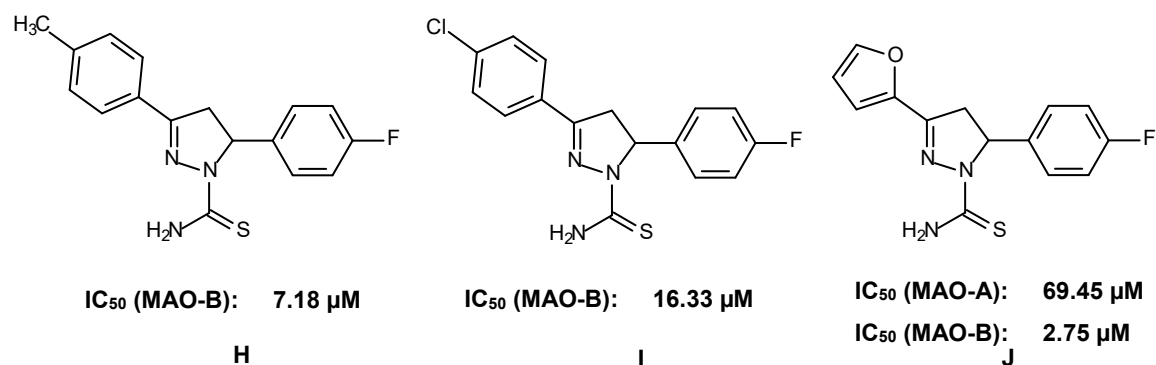


Figure 3.6: The structures of pyrazoline compounds that are known to inhibit MAO-B.

3.3. DESIGN OF THE STUDY

The structures of the nitrocatechol derivatives of chalcone and the pyrazoline derivatives which were investigated in this study are shown in table 3.1. As shown, the A-ring consists of the nitrocatechol moiety while limited substitution was explored on ring B. For this study, polar functional groups were substituted on ring B since these are expected to increase inhibition of MAO-B. In particular, the nitrile functional group was considered as polar substituent since benzo- and phthalonitriles are well-known to potently inhibit MAO-B (Manley-King *et al.*, 2011). Hydroxy substitution also is known to enhance MAO-B inhibition. This effect of polar substitution on MAO-B inhibition is due to the interaction of the polar groups with the polar region of the MAO-B active site, the region in proximity to the FAD. The positioning of the polar substituents is therefore an important consideration. As mentioned, the pyrazoline derivatives have been included in this study since this class of compounds have been reported to be good potency MAO-B inhibitors (Chimenti *et al.*, 2010).

3.4. SYNTHETIC APPROACH

A series of 3 novel nitrocatechol derivatives of chalcone (**1–3**) and 3 pyrazoline derivatives (**4–6**) were synthesised and characterised by NMR and MS, while the purity was assessed by HPLC. The series was synthesised according to the reaction schemes given in figure 3.7. The synthetic route consisted of three steps. Firstly, nitration of 4-hydroxy-3-methoxyacetophenone (apocynin; **7**) was carried out with 60% nitric acid in the presence of acetic acid according to the literature description to yield the nitro derivative **8** (5-nitroapocynin). In the second step, demethylation of **8** was carried out with $AlCl_3$ /pyridine to yield the 5-acetyl-3-nitrocatechol **9** (Kiss *et al.*, 2010). The target chalcones **1–3** were obtained via the Claisen-Schmidt condensation reaction between **9** and an appropriately substituted aldehyde in ethanol. Potassium hydroxide (60%) served as the base (Klinke & Gibian, 1961). The nitrocatechol derivatives of chalcone were

purified by crystallisation from an appropriate solvent. The yields for these reactions ranged from 27-28%. The pyrazoline derivatives **4–6** were synthesised by reacting the chalcones **1–3** with hydrazine hydrate in the presence of acetic acid. The yields for these reactions ranged from 70-86%.

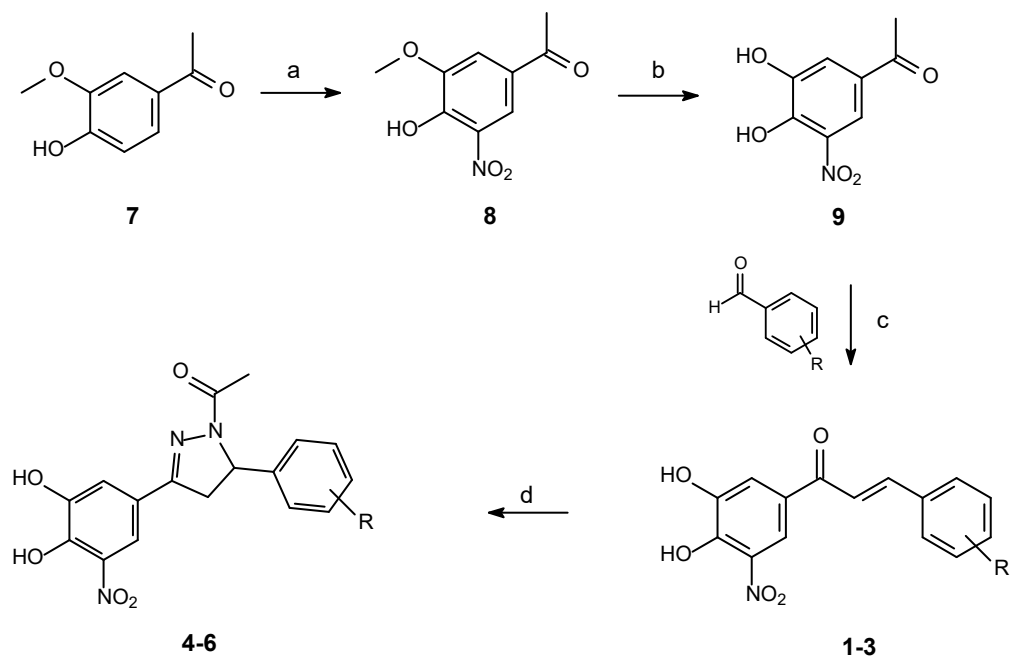
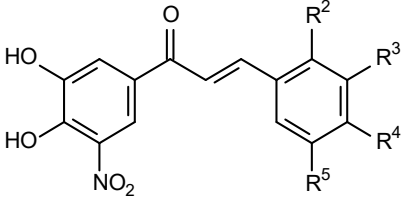
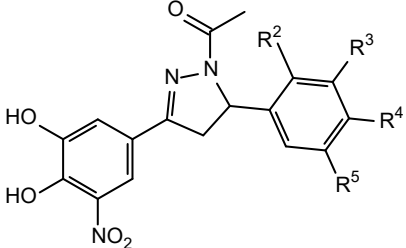


Figure 3.7: The synthetic route for the synthesis of nitrocatechol derivatives of chalcone and the pyrazoline derivatives. Reagents and conditions: (a) 60% HNO_3 , acetic acid, rt; (b) AlCl_3 , pyridine, ethyl acetate, 77 °C; (c) ethanol, 60% KOH, rt; (d) acetic acid, hydrazine hydrate, 120 °C.

Table 3.1: The structures of the nitrocatechol derivatives of chalcone and pyrazoline derivatives that were considered for this study. Substitution took place in the indicated positions on the B-ring.

									
Compound	R ²	R ³	R ⁴	R ⁵	Compound	R ²	R ³	R ⁴	R ⁵
The synthesis of the following chalcones and pyrazoline compounds were successful									
1		-CN			4		-CN		
2			-CN		5			-CN	
3		-OH			6		-OH		
The synthesis of the following chalcones were attempted, but were not successful									
i	-CN								
ii			-OH						
iii		-OH	-OH						
iv		-OH		-OH					
v		-OH	-OH	-OH					

3.5. EXPERIMENTAL SECTION

3.5.1. Materials and instrumentation

Materials: Starting materials (reagents and solvents) that were used in this study were obtained from Sigma-Aldrich and were used without further purification. All those starting materials that were prepared were purified by crystallisation.

Thin layer chromatography (TLC): TLC was carried out to determine if the reactions proceeded to completion. Silica gel sheets containing UV254 fluorescent indicator were employed with a mobile phase consisting of 100% ethyl acetate (compound **8**) or a mixture of 70% ethyl acetate and 30% benzene (compounds **1**, **2**, **3**, **4**, **5**, **6** and **9**). The developed TLC sheets were observed under an UV-lamp at a wavelength of 254 nm.

Melting points (mp): A Büchi B-545 melting point apparatus was used to measure the melting points of all of the synthesised compounds.

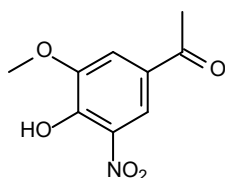
Mass spectra (MS): High resolution mass spectra (HRMS) were obtained with a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionisation (APCI) mode.

Nuclear magnetic resonance (NMR): A Bruker Avance III 600 spectrometer was used to record proton (^1H) and carbon (^{13}C) NMR spectra at frequencies of 600 MHz and 150 MHz, respectively. NMR measurements were conducted in DMSO-*d*₆ and the chemical shifts are reported in parts per million (δ). Chemical shifts were referenced to the residual solvent signal (DMSO-*d*₆) at 7.26 and 77.0 ppm for ^1H and ^{13}C , respectively. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet) or m (multiplet). The coupling constants (J) are given in Hz.

High performance liquid chromatography (HPLC): HPLC analyses were conducted with an Agilent 1100 HPLC system equipped with a quaternary pump and an Agilent 1100 series diode array detector. The HPLC analyses were used to determine the purities of the synthesised compounds. HPLC grade acetonitrile (Merck) and Milli-Q water (Millipore) was used for the chromatography. A Venusil XBP C18 column (4.60 x 150 mm, 5 μm) was used for the separation and the mobile phase consisted at the start of each run of 30% acetonitrile and 70% Milli-Q water. The flow rate was set to 1 ml/min. At the start of each run a solvent gradient program was initiated. The composition of the acetonitrile in the mobile phase was increased linearly to 80% over a period of 5 min. Each HPLC run lasted 15 min and a time period of 5 min was allowed for equilibration between runs. A volume of 20 μl of solutions of the test compounds (1 mM) was injected to the HPLC system, and the eluent was monitored at a wavelength of 254 nm. The test compounds were dissolved in acetonitrile.

3.5.2. General synthetic procedures

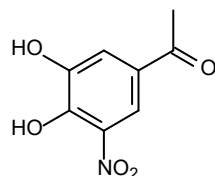
3.5.2.1. Synthesis of 4-hydroxy-3-methoxy-5-nitroacetophenone (**8**)



4-Hydroxy-3-methoxyacetophenone (**7**, 30 mmol) was weighed and dissolved in 50 ml glacial acetic acid. A solution formed which appeared colourless to very light yellow. A volume of 1.45 ml nitric acid (70%) was added dropwise to the solution. The reaction turned yellow with the first few drops and then turned into a dark brown colour and also became milky. The reaction underwent phase separation with a dark brown top layer and a brown-yellow milky bottom layer. The reaction was stirred at room temperature for 60 min after which the reaction appeared brown with a yellow

precipitate. After 60 min of stirring, the reaction was poured into ice-cold water (200 ml) and stirred for another 10 min. The resulting precipitate (brown-yellow) was collected by filtration and was air-dried overnight. Yield: 55% (yellow powder), mp: 156–158 °C; mp (literature): 158–164 °C ([Engelbrecht et al., 2018](#)); mp (literature): 159–161 °C ([Lu et al., 2011](#)).

3.5.2.2. Synthesis of 3,4-dihydroxy-5-nitroacetophenone (9)



4-Hydroxy-3-methoxy-5-nitroacetophenone (**8**, 10 mmol) was weighed and suspended in ethyl acetate (21 ml) while being cooled in an ice bath (0 °C). The reaction appeared brown-yellow. AlCl_3 (11 mmol) was weighed inside a fume cabinet and added to the reaction. The reaction turned dark brown to red. The mixture was placed in an ice bath and stirred for 10 min. The atmosphere of the reaction was replaced by argon, and pyridine (3.2 ml; 40 mmol) was added dropwise to the reaction. The reaction turned to a red-brown-orange colour. The reaction was heated at reflux for 2 h at 80 °C, and subsequently cooled to room temperature. A brown sticky precipitate formed with the reaction solvent appearing light yellow. HCl (37%) was diluted into 200 ml distilled H_2O to a concentration of 2.99 M, and cooled on an ice bath. A small volume (50 ml) of the diluted HCl was added to the reaction and the reaction was stirred for 5 min with a glass stirring rod to disperse the precipitate. The reaction was subsequently poured into the diluted HCl and stirred for a further 30 min. The resulting precipitate (brown-yellow) was collected by filtration, rinsed with distilled H_2O , and air-dried overnight. Yield: 63%. mp: 156.0–160.8 °C; mp (literature): 153.1–158.2 °C ([Engelbrecht et al., 2018](#)); mp (literature): 161–169 °C ([Bäckström et al., 1989](#)).

3.5.2.3. Synthesis of chalcone compounds (1, 2 and 3)

KOH (12 g) was dissolved in 20 ml distilled H_2O to yield a 60% KOH solution. 3,4-Dihydroxy-5-nitroacetophenone (0.591 g; 3 mmol) was weighed and placed in a round bottom flask. Ethanol (4.19 ml) was added, and while stirring, the reaction was heated with a heat gun until a complete solution was obtained. The reaction appeared dark brown. The appropriately substituted benzaldehyde (3 mmol) was added to the reaction, and while stirring, the reaction was again heated with a heat gun. A solution with a dark brown colour was obtained. A volume of 1.5 ml KOH (60%) was added to the reaction, and the reaction turned dark brown to red. The reaction was stirred for 24 h at room temperature after which a thick precipitate formed. HCl (37%) was diluted into 60 ml distilled H_2O to yield a 2.99 M solution, and cooled in an ice bath. The ice cold HCl was added to the reaction, and the yellow-mustard coloured precipitate was dispersed with a glass rod. The reaction mixture was stirred for 15 min at room temperature, and a further 15 min

while being placed on an ice bath. The resulting precipitate was collected by filtration, rinsed with distilled H₂O, and air-dried overnight. The crude product was finally purified by recrystallisation.

Recrystallisation – compound 1:

The crude product obtained from the reaction was placed in a 75 ml Erlenmeyer flask and dissolved in a mixture of acetonitrile (10 ml), ethyl acetate (40 ml) and methanol (10 ml) while being heated. The solution was filtered by warm filtration, and the clear solution was allowed to recrystallise at room temperature for 24 h. The resulting crystals were collected by filtration and were left to air-dry overnight. Yield: 28% (fine yellow round balls of crystals). mp: 244.1–244.9 °C.

Recrystallization – compound 2:

The crude product obtained from the reaction was placed in a 75 ml Erlenmeyer flask and dissolved in a mixture of acetonitrile (30 ml), and methanol (55 ml) while being heated. The solution was filtered by warm filtration, and the clear solution was allowed to recrystallise at room temperature for 24 h. The resulting crystals were collected by filtration and were left to air-dry overnight. Yield: 28%. mp: 240.1–242 °C.

Recrystallization – compound 3:

The crude product obtained from the reaction was placed in a 100 ml Erlenmeyer flask and dissolved in a mixture of acetonitrile (5 ml), ethyl acetate (5 ml) and methanol (110 ml) while being heated. The solution was filtered by warm filtration, and the clear solution was allowed to recrystallise at room temperature for 24 h. The resulting crystals were collected by filtration and were left to air-dry overnight. Yield: 27% (yellow paper like crystals). mp: 252.3–253.4 °C.

3.5.2.4. Synthesis of pyrazoline compounds (4, 5 and 6)

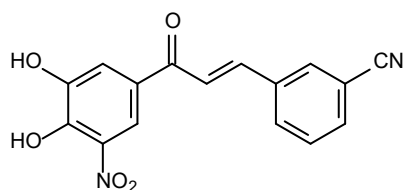
The chalcones **1–3** (0.58 mmol), were placed in a round bottom flask, and glacial acetic acid (3.48 ml) was added. While the reaction was stirred, hydrazine monohydrate (0.07 ml) was added dropwise. The reaction was heated under reflux (120 °C) for 24 h and was subsequently cooled to room temperature. Distilled H₂O (50 ml) was cooled in an ice bath, and the reaction was poured into the ice-cold water. A yellow precipitate formed and the mixture was stirred for a further 5 min. The precipitate was collected by filtration, rinsed with distilled H₂O, and allowed to air-dry overnight.

3.5.3. Physical characterisation

¹H-NMR, ¹³C-NMR and mass spectroscopy were used to verify the structures of the chalcone and pyrazoline compounds. The ¹H-NMR, ¹³C-NMR spectra as well as the mass spectra and HPLC traces are given in the appendixes. The following notations for the physical characterisation of

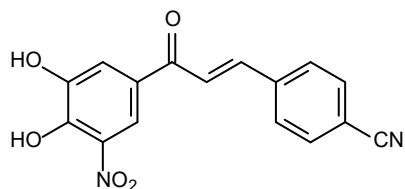
the chalcone and pyrazoline compounds are provided, while a detailed correlation of these data with the structures will be given in the results section.

3-[(1E)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (1)



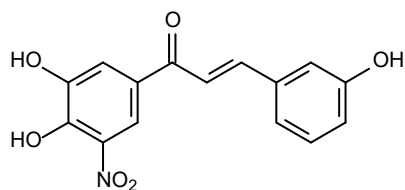
The title compound (yellow balls of crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and 3-cyanobenzaldehyde (3-formylbenzonitrile) with an average yield of 28%: mp 244.1–244.9 °C. $^1\text{H-NMR}$ (Bruker Avance III 600, $\text{DMSO-}d_6$) δ 7.64 (t, J = 7.8 Hz, 1H), 7.72 (dd, J = 8.8, 6.8 Hz, 2H), 7.88 (dd, J = 7.8, 1.4 Hz, 1H), 8.07 (d, J = 15.5 Hz, 1H), 8.15–8.23 (m, 1H), 8.33 (t, J = 1.5 Hz, 1H), 8.48 (d, J = 2.0 Hz, 1H), 10.90 (s, 2H); $^{13}\text{C-NMR}$ (Bruker Avance III 600, $\text{DMSO-}d_6$) δ 112.56, 117.55, 117.57, 117.61, 118.98, 123.94, 127.84, 130.51, 132.36, 133.93, 134.25, 136.42, 137.86, 141.92, 146.88, 148.46, 186.47. APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{11}\text{N}_2\text{O}_5$ (MH^+), 311.0662, found 311.0654; Purity (HPLC): 99.08%

4-[(1E)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (2)



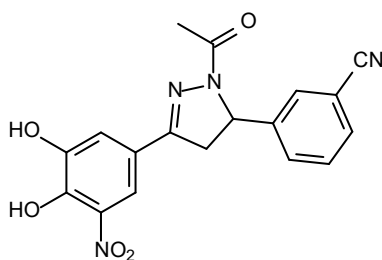
The title compound (yellow powder crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and 4-formylbenzonitrile with an average yield of 28%: mp 240.1–242 °C. $^1\text{H-NMR}$ (Bruker Avance III 600, $\text{DMSO-}d_6$) δ 7.69–7.79 (m, 2H), 7.91 (d, J = 7.6 Hz, 2H), 8.03–8.15 (m, 3H), 8.32 (s, 1H), 10.87 (s, 2H); $^{13}\text{C-NMR}$ (Bruker Avance III 600, $\text{DMSO-}d_6$) δ 112.72, 117.52, 117.68, 119.07, 125.03, 127.88, 129.93, 133.09, 137.88, 139.65, 142.10, 146.70, 148.39, 186.54. APCI-HRMS m/z : calculated for $\text{C}_{16}\text{H}_{11}\text{N}_2\text{O}_5$ (MH^+), 311.0662, found 311.0656; Purity (HPLC): 100%

(2E)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one (3)



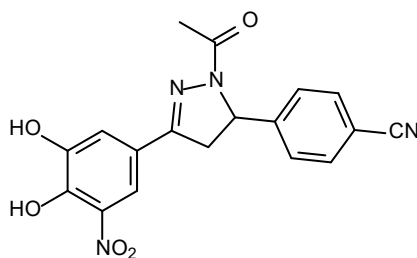
The title compound (yellow paper like crystals) was prepared from 3,4-dihydroxy-5-nitroacetophenone and 3-hydroxybenzaldehyde with an average yield of 27%: mp 252.3–253.4°C. ¹H-NMR (Bruker Avance III 600, DMSO-*d*₆) δ 6.87 (d, *J* = 8.0 Hz, 1H), 7.19–7.28 (m, 2H), 7.31 (d, *J* = 7.7 Hz, 1H), 7.63 (d, *J* = 15.5 Hz, 1H), 7.72 (d, *J* = 2.3 Hz, 1H), 7.81 (dd, *J* = 15.6, 1.8 Hz, 1H), 8.26 (d, *J* = 2.2 Hz, 1H), 9.63 (s, 1H), 10.86 (s, 2H); ¹³C-NMR (Bruker Avance III 600, DMSO-*d*₆) δ 115.72, 117.22, 117.85, 118.36, 120.56, 121.68, 128.33, 130.36, 136.44, 137.93, 144.73, 146.40, 148.36, 158.22, 186.81. APCI-HRMS *m/z*: calculated for C₁₅H₁₂NO₆ (MH⁺), 302.0659, found 302.0652; Purity (HPLC): 100%

3-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile (4)



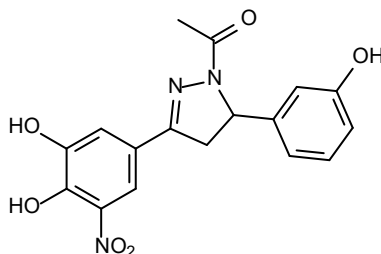
The title compound (yellow crystals) was prepared from 3-[(1E)-3-(3,4-dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (**1**) with an average yield of 86%: mp 140.6–144.1 °C. ¹H-NMR (Bruker Avance III 600, DMSO-*d*₆) δ 2.30 (s, 3H), 3.13–3.26 (m, 1H), 3.77–3.92 (m, 1H), 5.58 (dd, *J* = 12.3, 5.0 Hz, 1H), 7.54 (d, *J* = 3.1 Hz, 2H), 7.58 (d, *J* = 9.9 Hz, 2H), 7.68 (s, 1H), 7.71–7.77 (m, 1H), 10.65 (s, 2H); ¹³C-NMR (Bruker Avance III 600, DMSO-*d*₆) δ 22.13, 41.87, 59.46, 111.94, 114.55, 116.22, 119.10, 121.82, 130.03, 130.30, 131.19, 131.53, 137.65, 144.01, 144.33, 148.34, 153.38, 168.03. APCI-HRMS *m/z*: calculated for C₁₈H₁₅N₄O₅ (MH⁺), 367.1037, found 367.1041; Purity (HPLC): 89.74%

4-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile (5)



The title compound (yellow crystals) was prepared from 4-[(1E)-3-(3,4-dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (**2**) with an average yield of 81%: mp 219.9–220.8 °C. ¹H-NMR (Bruker Avance III 600, DMSO-*d*₆) δ 2.30 (s, 3H), 3.15 (dd, *J* = 18.1, 5.0 Hz, 1H), 3.84 (dd, *J* = 18.1, 12.0 Hz, 1H), 5.60 (dd, *J* = 12.1, 4.8 Hz, 1H), 7.39 (d, *J* = 7.8 Hz, 2H), 7.52–7.62 (m, 2H), 7.80 (d, *J* = 7.8 Hz, 2H), 10.69 (s, 2H); ¹³C-NMR (Bruker Avance III 600, DMSO-*d*₆) δ 22.07, 41.90, 59.81, 110.47, 114.53, 116.13, 119.13, 121.72, 127.16, 133.09, 137.67, 144.39, 147.93, 148.38, 153.35, 167.94. APCI-HRMS *m/z*: calculated for C₁₈H₁₅N₄O₅ (MH⁺), 367.1037, found 367.1037; Purity (HPLC): 91.88%

1-[3-(3,4-Dihydroxy-5-nitrophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethenone (6)



The title compound (yellow crystals) was prepared from (2E)-1-(3,4-dihydroxy-5-nitrophenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one (**3**) with an average yield of 70%: mp 143.1–144.4 °C. ¹H-NMR (Bruker Avance III 600, DMSO-*d*₆) δ 2.29 (s, 3H), 3.07 (ddd, *J* = 18.0, 4.4, 1.9 Hz, 1H), 3.71–3.82 (m, 1H), 5.38–5.49 (m, 1H), 6.54 (s, 1H), 6.61 (dd, *J* = 14.1, 7.8 Hz, 2H), 7.11 (d, *J* = 7.8 Hz, 1H), 7.57 (d, *J* = 14.7 Hz, 2H), 9.39 (s, 1H), 10.61 (s, 2H); ¹³C-NMR (Bruker Avance III 600, DMSO-*d*₆) δ 22.34, 42.45, 60.10, 112.62, 114.56, 114.79, 116.24, 116.60, 122.15, 130.29, 137.93, 144.36, 144.45, 148.57, 153.51, 158.18, 167.78. APCI-HRMS *m/z*: calculated for C₁₇H₁₆N₃O₆ (MH⁺), 358.1034, found 358.1051; Purity (HPLC): 93.46%

3.5.4. Biological evaluation

3.5.4.1. MAO inhibition studies

IC₅₀ values for the inhibition of MAO were measured by using the recombinant human MAO-A and MAO-B enzymes (Mostert *et al.*, 2015). The enzyme reactions were carried out in white 96-well microliter plates (Eppendorf) in potassium phosphate buffer (pH 7.4, 100 mM, made isotonic with KCl). The final volume of the reactions was 200 µl and 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 concentration of 4%. Reactions serving as negative controls were carried out in the absence of inhibitor. The enzyme reactions were initiated with the addition of the MAO enzymes, and were subsequently incubated for 20 min at 37 °C in a convection oven. At endpoint, the reactions were terminated with the addition of 80 µl sodium hydroxide (2 N) and the concentration of 4-hydroxyquinoline, the product of kynuramine oxidation by MAO, was measured by fluorescence spectrophotometry ($\lambda_{\text{ex}} = 310 \text{ nm}$; $\lambda_{\text{em}} = 400 \text{ nm}$) (Novaroli *et al.*, 2005). For this purpose, a linear calibration curve was constructed with authentic 4-hydroxyquinoline (0.047–1.56 µM). The rates of MAO-catalysed 4-hydroxyquinoline formation thus measured were fitted to the one site competition model of the Prism 5 software package (GraphPad). This gave sigmoidal plots of rate versus logarithm of inhibitor concentration from which the IC₅₀ values were estimated. IC₅₀ values were measured in triplicate and are reported as the mean $\pm$ standard deviation (SD).

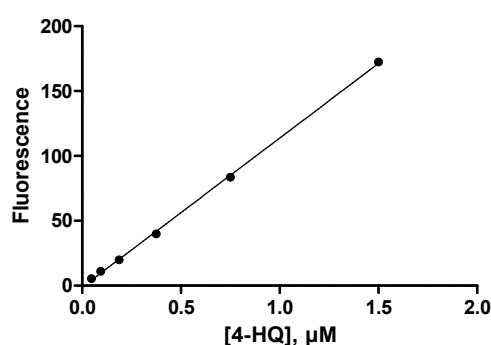


Figure 3.8: Example of a linear calibration curve used to quantitate 4-hydroxyquinoline.

3.5.4.2. COMT inhibition studies

To determine whether the synthesised compounds are inhibitors of COMT, the method described in literature (Borchardt, 1974) was used. This protocol uses esculetin (6,7-dihydroxycoumarin) as substrate for COMT. After the test inhibitor is incubated with esculetin and COMT, fluorescence spectrophotometry was used to quantify the enzymatic product, scopoletin. Sigmoidal dose-response curves of COMT activity versus the logarithm of inhibitor concentration (Log[I])

were constructed using the Prism 5.0 software package (GraphPad), and the IC₅₀ values were determined in triplicate and expressed as mean ± standard deviation (SD). As enzyme source, the soluble fraction obtained from homogenates of rat liver tissue was used. The rat liver tissue was obtained under category 0 ethical approval (AnimCare). Sprague Dawley rats were bred, supplied and housed at the Vivarium of the Preclinical Drug Development Platform at the Potchefstroom campus of the North-West University (NWU) (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019). Experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) at the NWU. All animals were maintained and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa, and complied with national legislation. Ethical approval for the collection and use of animal tissue was obtained from the Research Ethics Committee, NWU. Ethics approval number: **NWU-00561-19-S5**.

The liver tissue was prepared as reported in literature ([Hirano *et al.*, 2005](#); [Zhu *et al.*, 2010](#)). Frozen liver tissue was washed with ice cold saline, cut into smaller pieces and homogenised in three volumes sodium phosphate buffer (25 mM, pH 7.8, containing 0.5 mM dithiothreitol) for 2 min with a polytron homogeniser. The homogenate was centrifuged at 100,000 × g for 30 min at 4 °C, and the supernatant was stored as soluble COMT at –86 °C. Protein determination was carried out by the method of Bradford ([Bradford, 1970](#)).

The enzyme reactions were prepared in potassium phosphate buffer (100 mM, pH 7.4) and contained esculetin, S-adenosyl-L-methionine as co-factor, magnesium chloride, L-cysteine, the test inhibitor (0.003–80 µM) and COMT. All reactions were carried out in 96-well microtiter plates (black) to a volume of 200 µl, and stock solutions of the test inhibitors were prepared in DMSO and added to the reactions to yield a final DMSO concentration of 4%. After addition and mixing of the reagents, the 96-well plate was placed in the oven to pre-incubate at 37 °C for 15 min. The reactions were initiated with the addition of the enzyme and the fluorescence intensities were recorded continuously (λ_{ex} 355, λ_{em} 460 nm) for 500 s (8.3 min) using a SpectraMax® iD3 instrument (Molecular Devices). From plots of fluorescence intensity versus time, the slopes were recorded and used to construct sigmoidal dose-response curves of slope versus Log[I]. For this purpose, the Prism 5.0 software package (GraphPad) was used. IC₅₀ values were determined in triplicate from the sigmoidal plots, and expressed as mean ± SD.

3.5.4.3. Molecular docking

Molecular modelling was carried out with the Discovery Studio 3.1 software package (Accelrys). The structures of human MAO-A (PDB code 2Z5X) ([Son *et al.*, 2008](#)), human MAO-B (PDB code 2V5Z) ([Binda *et al.*, 2007](#)) and COMT (PDB code: 1VID) ([Vidgren *et al.*, 1994](#)) were obtained from the Brookhaven Protein Data Bank. The pKa values and protonation states of the ionisable amino acids were calculated and hydrogen atoms were added to the protein models at pH 7.4. The FAD

cofactors (oxidised state for MAO) and co-crystallised ligands valences were corrected, the protein models were automatically typed with the Momany and Rone CHARMM forcefield and a fixed atom constraint was applied to the backbone. The Smart Minimiser algorithm was used to energy minimise the models, with the maximum amount of steps set to 50000 and employing the implicit generalised Born solvation model with molecular volume. The co-crystallised ligands, waters and the backbone constraints were removed from the models and an analysis of the enzyme cavities identified the binding sites. In each MAO model, three active site waters are considered to be conserved and were retained. These waters are HOH-710, 718 and 739 in the MAO-A active site, and HOH-1155, 1170 and 1351 in the A-chain of the MAO-B active site. All the crystal waters were removed for COMT. The structures of the test compounds were drawn in Discovery Studio, the geometries were briefly optimised using a Dreiding-like forcefield (5000 iterations) and the structures were submitted to the Prepare Ligands protocol. The Momany and Rone CHARMM forcefield subsequently assigned atom potential types and partial charges to the structures. The CDOCKER algorithm was used to carry out the docking of the test inhibitors into the MAO models. For this purpose, ten random ligand conformations were generated, the heating target temperature was set to 700 K and the full potential mode was employed. The docking solutions were finally refined using *in situ* ligand minimisation with the Smart Minimizer algorithm.

3.6. RESULTS

3.6.1. Chemistry

In this present study three new nitrocatechol derivatives of chalcone (**1–3**), and three new pyrazoline derivatives (**4–6**) were synthesised with the aim of discovering compounds that inhibit both MAO and COMT. The synthetic route consisted of 4 steps (Fig. 3.7). In the first step, nitration of 4-hydroxy-3-methoxyacetophenone (**7**) was carried out with 60% nitric acid (HNO₃) in the presence of acetic acid according to the literature description to yield the nitro derivative **8**. In the second step, demethylation was carried out with aluminium chloride (AlCl₃), with the presence of pyridine and in an atmosphere of argon to yield the 3,4-dihydroxy-5-nitroacetophenone (**9**). In the third step, the target chalcones **1–3** were obtained via the Claisen-Schmidt condensation reaction between 3,4-dihydroxy-5-nitroacetophenone and an appropriately substituted benzaldehyde in ethanol. Potassium hydroxide (60%) served as the base. Three new nitrocatechol derivatives of chalcone were obtained, and were used in the final step. In the fourth and final step, the three new pyrazoline derivatives (**4–6**) were obtained by reaction of the chalcones **1–3** with hydrazine hydrate in acetic acid.

The chalcone compounds were purified by crystallisation from an appropriate solvent. The overall yields for the chalcones **1**, **2** and **3** were low (28%; 28%; 27%), while the yields for the final step in which the pyrazoline compounds (**4**, **5** and **6**) were synthesised were good (86%; 81%; 70%). In each instance, the structures of the target compounds were verified by ¹H-NMR, ¹³C-NMR and

mass spectrometry (MS). As shown in [table 3.2](#), in all instances, the calculated high resolution masses corresponded within 5 ppm with the experimental determined values. In general, a ppm difference smaller than 5 is considered to indicate good agreement between the calculated and experimental values.

The ppm was determined as follows:

$$ppm = \frac{[Found - Calculated]}{[Calculated]} \times 10^6$$

Equation 3.1: Equation to determine ppm (parts per million).

Table 3.2: The calculated and experimentally determined high resolution masses of the chalcone and pyrazoline compounds.

	Calculated	Found	Formula	ppm
1	311.0662 g/mol	311.0654 g/mol	C ₁₆ H ₁₁ N ₂ O ₅	-2.57
2	311.0662 g/mol	311.0656 g/mol	C ₁₆ H ₁₁ N ₂ O ₅	-1.93
3	302.0659 g/mol	302.0652 g/mol	C ₁₅ H ₁₂ NO ₆	-2.32
4	367.1037 g/mol	367.1041 g/mol	C ₁₈ H ₁₅ N ₄ O ₅	1.09
6	367.1037 g/mol	367.1037 g/mol	C ₁₈ H ₁₅ N ₄ O ₅	0
5	358.1034 g/mol	358.1051 g/mol	C ₁₇ H ₁₆ N ₃ O ₆	4.75

To show that the physical data corresponds to the proposed structures, the ¹H-NMR and ¹³C-NMR data for chalcone **1** and pyrazoline **4** will be discussed. In the ¹H-NMR spectrum of chalcone **1**; the signal at 10.90 ppm (s, 2H) corresponds with the catechol OH protons (these protons are deshielded). One of the vinylic protons correspond to the signal at 8.07 (d, 1H) with a coupling constant of 15.5 Hz, indicating that the geometry of the double bond is *trans*. The signals at 8.48 ppm (1H), 8.33 ppm (1H), 8.15–8.23 (1H), 7.88 ppm (1H), 7.72 ppm (2H) and 7.64 (1H), correspond to the 6 aromatic protons and one of the vinillic protons. In the ¹³C-NMR spectrum, 15 signals in the aromatic region (112–149 ppm) are observed which correspond to the 12 phenyl carbons, 2 vinillic carbons and the carbon of the nitrile functional group. The carbonyl carbon is represented by the signal at 186.47 ppm. The DEPT135 and DEPT90 spectra shows the presence of the eight CH carbons of the two phenyl rings and the vinillic group.

In the ¹H-NMR spectrum of pyrazoline **4**; the signal at 10.65 ppm (s, 2H) corresponds to the catechol OH protons. The methyl ketone corresponds with the signal at 2.30 ppm (s, 3H). The CH₂ and CH protons in the pyrazoline ring correspond to the signals at 5.58 (dd, 1H), 3.77–3.92 (m, 1H) and 3.13–3.26 (m, 1H). The complexity of these signals are due to the chiral nature of

C5. The six aromatic protons are represented by signals at 7.71–7.77 (1H), 7.68 (1H), 7.58 (2H), 7.54 (2H). On the ^{13}C -NMR spectrum, the 12 aromatic carbons, C3 of the pyrazoline ring and the nitrile carbon are represented by the signals at 153.38, 148.34, 144.33, 144.01, 137.65, 131.53, 131.19, 130.30, 130.03, 121.82, 119.10, 116.22, 114.55 and 111.94 ppm. The carbonyl carbon is represented by the signal at 168.03 ppm. The methyl of the acetyl group as well as the CH and CH₂ carbons of the pyrazoline are represented by the signals at 22.13 ppm (CH₃), 41.87 ppm (CH₂) and 59.46 ppm (CH). Analysis of the DEPT135 and DEPT90 spectra shows the presence of one CH₂ at 41.91 ppm (the CH₂ of the pyrazoline), six CH signals for the CH groups of the two phenyl rings, the CH of the pyrazoline (59.48 ppm) and the CH₃ of the acetyl group (22.16 ppm).

3.6.2. MAO inhibition

Table 3.3: The IC₅₀ values for the inhibition of human MAO-A and MAO-B, and the IC₅₀ values for the inhibition of rat liver COMT by the chalcone and pyrazoline compounds.

Compound	IC ₅₀ (μM) ± SD MAO-A	IC ₅₀ (μM) ± SD MAO-B	IC ₅₀ (μM) ± SD COMT
1	58.9 ± 2.45	60. 6 ± 5.17	0.240 ± 0.071
2	45.3 ± 2.52	56. 6 ± 7.36	0.175 ± 0.056
3	43.9 ± 4.69	55.8 ± 5.00	0.141 ± 0.037
4	70.6 ± 7.47	55.4 ± 3.21	0.079 ± 0.0089
5	76. 6 ± 7.43	83.2 ± 14.5	0.048 ± 0.028
6	87.0 ± 2.98	85.2 ± 7.48	0.075 ± 0.031
Toloxatone⁽¹⁾	3.92	-	-
Lazabemide⁽¹⁾	-	0.091	-
Tolcapone⁽²⁾	-	-	0.26
Entacapone⁽²⁾	-	-	0.25

(1) (Petzer *et al.*, 2013); (2) (Engelbrecht *et al.*, 2018)

The MAO inhibitory properties of the nitrocatechol derivatives of chalcone (**1–3**), and the pyrazoline derivatives (**4–6**) were investigated with the recombinant human MAOs as enzyme sources. The measurements of MAO activity were conducted by using kynuramine as substrate. Kynuramine is a non-selective substrate for both MAO-A and MAO-B. Kynuramine is oxidized to yield 4-hydroxyquinoline (Fig. 3.9), a metabolite that fluoresces in alkaline media (Mpitimpiti *et al.*, 2019). Kynuramine is firstly oxidised by the MAO enzymes to yield the intermediate iminium which is converted to the corresponding aldehyde intermediate [3-(2-aminophenyl)-3-oxo-propionaldehyde] after hydrolysis. As the oxidation of kynuramine is inhibited, the concentration

of 4-hydroxyquinoline is reduced in the presence of a MAO inhibitor. This decrease in concentration of 4-hydroxyquinoline can be measured fluorometrically since 4-hydroxyquinoline is fluorescent. Fluorescence will thus decrease with increasing concentration of an MAO inhibitor. The test inhibitors and kynuramine do not fluoresce at the specified assay conditions, thus fluorescence of the generated 4-hydroxyquinoline can be measured without interference from these chemical species. The enzyme reactions were initiated with the addition of the MAO enzymes and were subsequently incubated for 20 min at 37 °C in a convection oven (Mpitimpiti *et al.*, 2019). The reactions were terminated with the addition of 80 μ L sodium hydroxide (NaOH) (2 N) and the concentration of 4-hydroxyquinoline, the product of kynuramine oxidation by MAO, was quantitated by fluorescence spectrophotometry (λ_{ex} = 310 nm; λ_{em} = 400 nm) (Mpitimpiti *et al.*, 2019; Engelbrecht *et al.*, 2018). After the measurement of the catalytic rates of MAO-A and MAO-B in the absence and presence (0.003–100 μ M) of the test inhibitors, the rate data were fitted to the one-site competition model of the Prism 5 software package (GraphPad). IC₅₀ values were estimated from the resulting sigmoidal curves of catalytic rate versus the logarithm of inhibitor concentration (log[I]). The IC₅₀ values were measured in triplicate and were reported as the means $\pm$ standard deviation (SD).

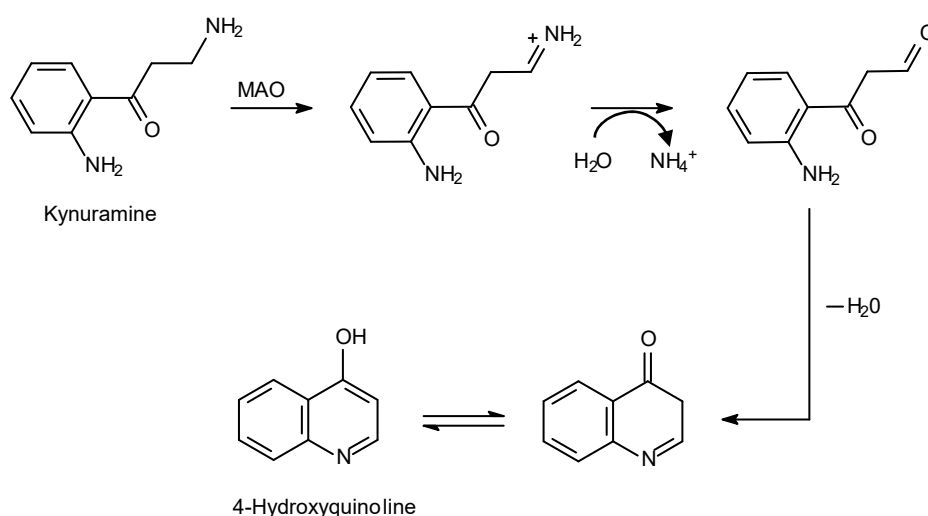


Figure 3.9: The oxidation of kynuramine to yield 4-hydroxyquinoline.

The IC₅₀ values that were recorded for MAO inhibition are given in table 3.3. It is clear from the inhibition data that nitrocatechol derivatives of chalcones (1–3) and pyrazoline derivatives (4–6) are not potent inhibitors of either MAO-A or MAO-B, with IC₅₀ values >43.9 μ M and >55.4 μ M, respectively. These values are significantly higher than the reference inhibitors, tolaxatone and lazabemide (Petzer *et al.*, 2013). These reference inhibitors inhibit MAO-A and MAO-B with IC₅₀ values of 3.92 μ M and 0.091 μ M, respectively.

It is well-known that benzo- and phthalonitriles inhibits MAO-B potently, and hydroxy substitution is also known to enhance MAO-B inhibition of chalcones. Since chalcones are in general good

potency inhibitors of MAO-B, particularly compounds containing polar groups on the A-ring (e.g. OH) and nitrile functional group (e.g. CN) on the B-ring, it may be concluded that nitro substitution on the A-ring greatly diminishes MAO-B inhibition ([Chimenti *et al.*, 2009](#)). A possible molecular basis for the low inhibition potency of these compounds will be investigated by molecular modelling below.

3.6.3. COMT inhibition

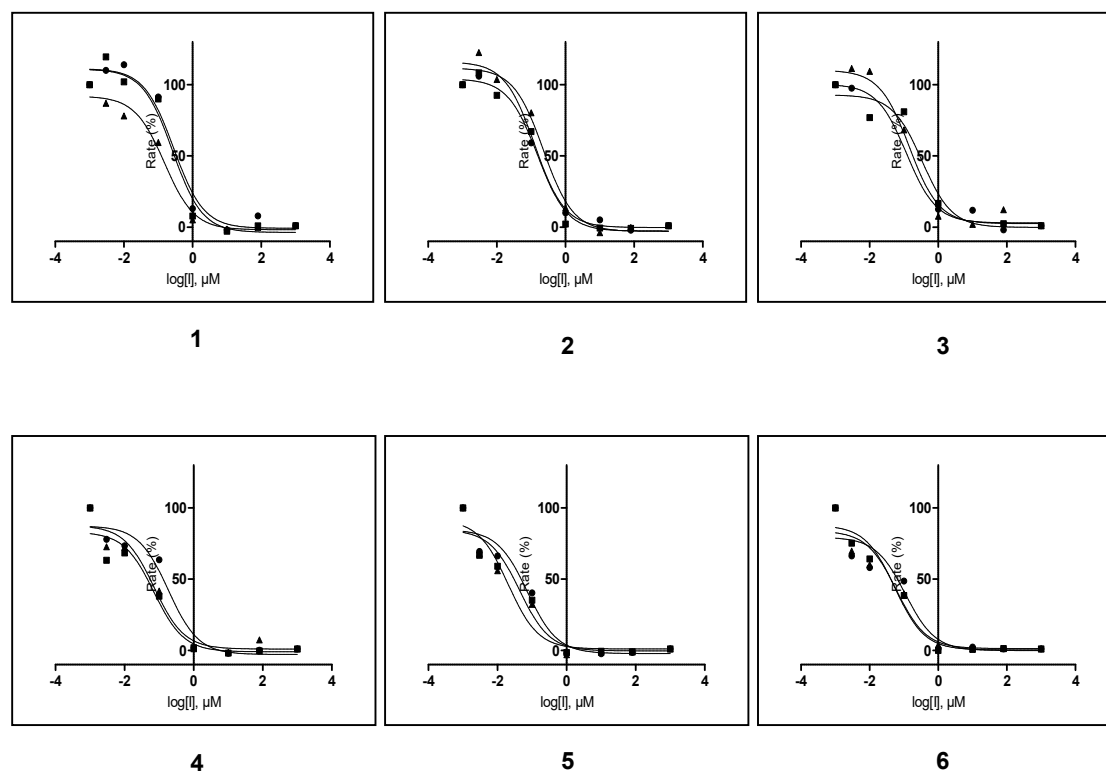


Figure 3.10: Sigmoidal plots for the inhibition of COMT by the chalcone and pyrazoline compounds. These experiments were carried out in triplicate.

The COMT inhibition properties of the nitrocatechol derivatives of chalcone (**1–3**) and the pyrazoline derivatives (**4–6**) were investigated using the soluble fraction obtained from homogenates of rat liver tissue. The enzyme reactions contained esculetin as the substrate, S-adenosyl-L-methionine as co-factor, magnesium chloride, L-cysteine and COMT. Potassium phosphate as buffer served as reaction solvent and the test inhibitors were added at concentrations ranging from 0.003–80 μM. Control reactions that were carried out in the absence of inhibitor were also included. After incubation of the reaction components at 37 °C for 15 min, the reactions were initiated with the addition of the COMT enzyme. The fluorescence intensities of the reactions were monitored continuously (λ_{ex} 355, λ_{em} 460 nm) for 500 s (8.3 min), and the slopes of plots of fluorescence intensity versus time were recorded. Sigmoidal dose-response

curves of slope versus Log[I] were constructed from which the IC₅₀ values were determined in triplicate. The IC₅₀ values are given in [table 3.3](#), while the sigmoidal plots are presented in [figure 3.10](#).

The IC₅₀ values that were recorded are given in [table 3.3](#). The results show that nitrocatechol derivatives of chalcones (**1–3**) and pyrazoline derivatives (**4–6**) are good potency inhibitors of COMT with IC₅₀ values <0.24 µM. The pyrazoline derivatives (**4–6**) are more potent inhibitors than the chalcones, with **5** being the most potent inhibitor with an IC₅₀ value of 0.048 µM. COMT inhibitors containing the 3-nitrocatechol moiety that have been developed and introduced into the market include tolcapone (IC₅₀ of 0.26 µM) and entacapone (IC₅₀ of 0.25 µM). These IC₅₀ values were determined in our laboratory. All synthesised compounds are thus more potent COMT inhibitors than tolcapone and entacapone.

3-Nitrocatechol derivatives of chalcone and the corresponding pyrazoline derivatives may serve as leads for the future design of potent COMT inhibitors. This study is the first report of COMT inhibition by pyrazoline compounds and thus proposes the further development of these nitrocatechol derivatives as potentially clinically useful COMT inhibitors. It should be kept in mind that the pyrazoline derivatives **4–6** are chiral and represent the racemates of two enantiomers. It is not clear if both enantiomers contribute equally to COMT inhibition, but future studies should separate the enantiomers of a representative inhibitor (e.g. **5**) to determine the stereochemistry of the eutomer and distomer. Should the enantiomers exhibit different potencies for COMT inhibition, the eutomer will exhibit even more potent COMT inhibition than reported here.

As mentioned in the introduction, nitrocatechol derivatives of chalcone have previously been investigated as COMT inhibitors ([Engelbrecht *et al.*, 2018](#)). The most potent COMT inhibitors of this study are shown in [figure 3.5](#). In accordance with the findings of this study, the pyrazoline compounds are significantly more potent COMT inhibitors than the chalcones investigated previously. The most potent pyrazoline compound (IC₅₀ = 0.048 µM) is approximately 1.46-fold more potent than the most potent chalcone (IC₅₀ = 0.07 µM) reported by [Engelbrecht *et al.* \(2018\)](#).

3.6.4. Physicochemical properties

Key physicochemical properties of the nitrocatechol derivatives of chalcone and pyrazoline derivatives were calculated with MarvinSketch and are presented in [table 3.4](#). These properties are the log P, polar surface area and molecular weight. Considering ‘Lipinski’s rule of 5’, for reasonable absorption or permeation of the compounds with oral administration, the hydrogen bond donors has to be less than 5 [the sum of hydroxy (OH) and amine (NH) functional groups], the hydrogen bond acceptors has to be less than 10 [the sum of nitrogen (N) and oxygen (O) atoms], the molecular weight of each compound should be less than 500 and the calculated log P should be less than 5 ([Lipinski *et al.*, 1997](#)).

According to the results in [table 3.4](#), all the compounds comply with the rules, and are thus expected to be bioavailable via the oral route. The compounds have positive log P values which is indeed smaller than 5. The positive value is an indication that the compounds are relatively lipophilic, which suggests that the compounds will be able to permeate across the blood-brain barrier via passive diffusion. For passive diffusion permeability across biological membranes, a log P value of 0–3 is the optimal range ([Kerns & Di, 2008](#)). The relatively large log P values further suggests that the compounds will be able to distribute to the tissues and will therefore exhibit larger volume of distribution (V_d) values. Larger log P values and subsequently good permeability also suggest the ability to undergo hepatic metabolism due to access to hepatocytes. In contrast more lipophilic compounds are expected to undergo lower clearance by the kidney due to the ability to be reabsorbed by passive diffusion from the tubule of the nephron.

Table 3.4: Physiochemical properties of the nitrocatechol derivatives of chalcone (**1–3**) and the pyrazoline derivatives (**4–6**).

Compound:	log P	Polar surface area	Molecular weight
1	3.08	127.14	310.27 g/mol
3	2.92	123.58	301.26 g/mol
2	3.08	127.14	310.27 g/mol
4	2.09	142.74	366.33 g/mol
5	1.93	139.18	357.32 g/mol
6	2.09	142.74	366.33 g/mol

3.6.5. Molecular modelling

Possible binding orientations and interactions of the nitrocatechol derivatives of chalcone and the corresponding pyrazoline derivatives in MAO-A, MAO-B and COMT were investigated by molecular docking experiments. Chalcones **1** and **2**, and pyrazoline **4** and **5** were selected for these studies. Both enantiomers of the pyrazoline derivatives were considered. Molecular docking was carried out with the Discovery Studio 3.1 modelling software (Accelrys). The X-ray crystal structures of human MAO-A co-crystallised with harmine (PDB entry: 2Z5X) ([Son et al., 2008](#)), human MAO-B co-crystallised with safinamide (PDB entry: 2V5Z) ([Binda et al., 2007](#)), and COMT cocrystallised with 3,5-dinitrocatechol (PDB entry: 1VID) ([Vidgren et al., 1994](#)) were selected and molecular docking was carried out according to the previously reported protocol with the CDOCKER application of the Discovery Studio ([Mostert et al., 2015](#)). The protein models were prepared by first calculating of the pKa values and protonation states of the amino acid residues,

which was followed by an energy minimisation with the protein backbone constrained. The structures of the ligands were drawn in Discovery Studio, and after docking with CDOCKER, the docked orientations were refined using *in situ* ligand minimisation. For each ligand, the highest ranked orientation was selected among the ten solutions generated.

Docking accuracy: The accuracy of the docking procedure was evaluated by redocking the co-crystallized ligands, harmine, safinamide and 3,5-dinitrocatechol into the active sites of MAO-A, MAO-B and COMT, respectively. The root mean square deviation (RMSD) of the docked orientation from the position of the co-crystallised ligand was subsequently calculated. The best ranked orientations of harmine, safinamide and 3,5-dinitrocatechol exhibited RMSD values of 2.83 Å, 0.440 Å and 0.684 Å, respectively, from the position of the co-crystallized ligand (Fig. 3.11). RMSD values <1.5 Å are considered to be successful, and this protocol was thus deemed appropriate for docking experiments with MAO-B and COMT (Hevener *et al.*, 2009).

COMT: As shown in figure 3.11, key interactions of 3,5-dinitrocatechol in the COMT active site are coordination of the catechol oxygens with the magnesium ion and hydrogen bonding with Glu-199. Lys-144 forms an attractive electrostatic interaction with the 3-nitro group, and possibly a hydrogen bond with a catechol oxygen. Pro-174 and Met-40 form pi-alkyl interactions with the catechol ring. As expected, the chalcone **1** adopts a similar binding orientation to 3,5-dinitrocatechol, and forms the same interaction network (Fig. 3.12). The B-ring of the chalcone projects out of the active site and undergoes very limited interactions, mainly a pi-alkyl interaction with Pro-174. Interestingly, for some of the chalcones an alternative binding orientation is predicted for binding to COMT. This is shown with chalcone **2** in figure 3.13. For chalcone **2**, only one of the catechol OH groups coordinates with the magnesium ion, while hydrogen bonding occurs with Glu-199. The 2-OH group undergoes hydrogen binding with both Glu-199 and Asn-170. This binding orientation is, however, considered unlikely since the X-ray crystal structure of tolcapone shows that it exhibits a similar binding mode to chalcone **1**. The pyrazoline compounds are also predicted to bind in an alternative orientation as shown in figures 3.14 and 3.15 with the S- and R-enantiomers of pyrazoline **4**. In this instance, extensive interactions between COMT and the nitro group occurs. In this respect, the nitro forms attractive electrostatic interactions with the magnesium, Lys-144, Asp-141, Asp-169 as well as the S-adenosyl-L-methionine cofactor. The bonding network formed by the nitro group most likely determines the predicted orientation for the pyrazolines. Interestingly, the phenyl of the S-enantiomer undergoes pi-pi-stacking with Trp-143, which suggests that for this compound the side chain may significantly contribute to inhibitor stabilization. As explained above, catechol compounds are expected to bind with the OH groups coordinated to the magnesium ion. In an attempt to obtain such binding modes for the S- and R-enantiomers of pyrazoline **4**, docking was carried out with AutoDock Vina (Trott & Olson, 2010). This docking function successfully predicted binding orientations for the S- and R-enantiomers of compound **4** where the catechol OH groups are coordinated to the magnesium.

Both enantiomers also form extensive hydrogen bonding, with residues Asp-141, Asn-170, Lys-144 and Glu-199 involved (Fig. 3.24). The two enantiomers bind similar with respect to the catechol and pyrazoline rings. As expected, the phenyl rings, however, protrudes into opposite directions towards the exterior of the active site cleft.

MAO-A: The predicted binding orientation of the nitrocatechol derivative of chalcone, **1**, to MAO-A is shown in figure 3.16. Chalcone **1** binds with the benzonitrile directed towards the entrance of the MAO-active site and the nitrocatechol moiety binding in proximity to the FAD. Chalcone **1** forms an extensive network of hydrogen bonding and pi-interactions with MAO-A. The nitro group is hydrogen bonded to Tyr-444 and a water molecule (HOH-710), the 1-OH is hydrogen bonded to the FAD, and the nitrile forms a hydrogen bond with Val-210. A pi-pi interaction occurs between Tyr-407 and the catechol ring while a pi-sulphur interaction occurs between Cys-323 and the benzonitrile ring. The other chalcones exhibit similar binding orientations to chalcone **1**, with the most notable difference being the interactions with Phe-208. For certain chalcones (e.g. **1**) a steric conflict occurs between Phe-208 and the benzonitrile while for others (e.g. **2**) pi-pi stacking is recorded for this interaction. Chalcone **2** also undergoes hydrogen bonding with Gln-215 (with the nitro group) and HOH-718, thus forming a slightly different network than chalcone **1** (Fig. 3.17). The enantiomers of the pyrazoline compound, **5**, exhibit reversed binding orientations to MAO-A (Figs. 3.18 and 3.19). The nitrocatechol moiety of the S-enantiomer binds in proximity to the FAD, while the R-enantiomer binds with the benzonitrile in proximity to the FAD. The nitro group of the S-enantiomer undergoes hydrogen bonding with Tyr-444, HOH-710, HOH-739, and an electrostatic interaction with Tyr-407. The catechol OH groups in turn are hydrogen bonded to HOH-710 and the FAD. A pi-pi stacking interaction occurs between the catechol ring and Tyr-407. For the R-enantiomer, hydrogen bonding only occurs between the catechol OH groups and Ala-111 and Val-210. A pi-pi interaction between the benzonitrile and Phe-352, and a pi-sulphur interaction between the catechol ring and Cys-323 are also recorded. For most pyrazolines, no solutions were predicted for binding to MAO-A, which demonstrates the limited space in the active site available for inhibitor binding.

MAO-B: The predicted binding orientation of the nitrocatechol derivative of chalcone, **1**, to MAO-B is shown in figure 3.20. Chalcone **1** binds with the nitrocatechol moiety placed in the entrance of the MAO-B active site and the benzonitrile moiety binding in proximity to the FAD. This is reversed compared to the orientation observed in MAO-A. In MAO-B, the catechol OH groups of chalcone **1** forms hydrogen bonds with Pro-102 and Ile-199 in the substrate cavity, while the benzonitrile ring forms a pi-pi interaction with Tyr-398, and a pi-sulphur bond with Cys-172 in the substrate cavity. Chalcone **2** exhibits a similar binding orientation to chalcone **1**, with additional hydrogen bonding between the nitro group and Pro-102 and Tyr-326 being formed in the entrance cavity

(Fig. 3.21). In the substrate cavity, the nitrile undergoes hydrogen bonding with HOH-1351. The enantiomers of pyrazoline compound **5** also binds with the catechol located in the entrance cavity and the benzonitrile in the substrate cavity (Figs. 3.22 and 3.23). For the R-enantiomer hydrogen bonding occurs between the catechol OH groups and Pro-102, and between the acetyl carbonyl oxygen and Cys-172. For the S-enantiomer, a catechol OH also forms hydrogen bonding with Pro-102, while hydrogen bonding between the nitro and Tyr-326 is also recorded. In the substrate cavity, the nitrile forms hydrogen bonding with HOH-1351. It is noteworthy that for chalcone **2** and the S-enantiomer of pyrazoline **5**, the nitro forms unfavourable electrostatic interactions with Glu-84. Furthermore, the orientations predicted for the chalcones and pyrazolines are reversed to those expected for these compounds. For chalcones substituted with polar functional groups, the ring containing the polar groups binds in the substrate cavity in proximity to the FAD where hydrogen bonding occurs (Chimenti *et al.*, 2009). For the compounds of the current study, the A-ring containing the polar OH groups is placed in the entrance cavity which is a lipophilic environment. This may explain, at least in part, the low MAO-B inhibition potencies of the nitrocatechol derivatives of chalcones and pyrazolines.

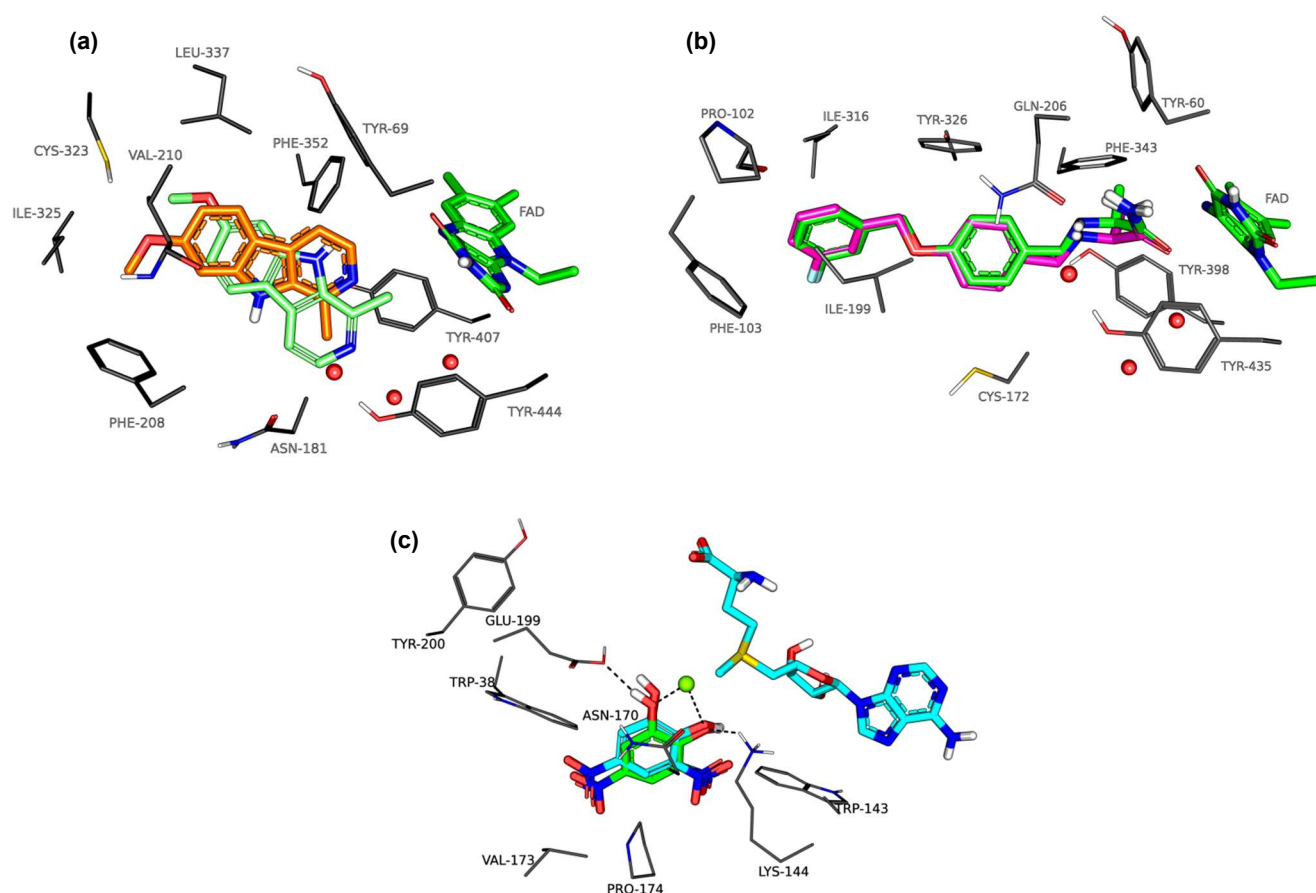


Figure 3.11: Three-dimensional representation of the predicted binding of (a) harmine, (b) safinamide and (c) 3,5-dinitro catechol to MAO-A, MAO-B and COMT, respectively.

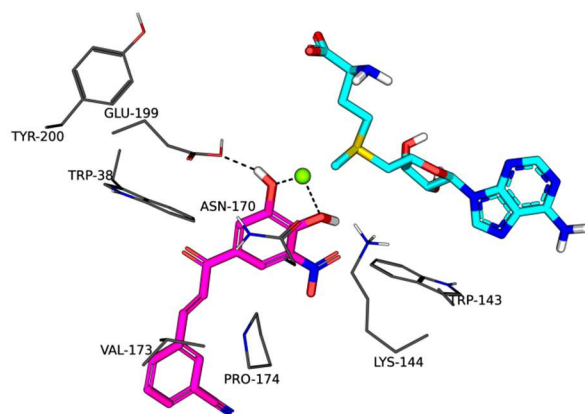


Figure 3.12: Three-dimensional representation of the predicted binding of chalcone **1** to COMT.

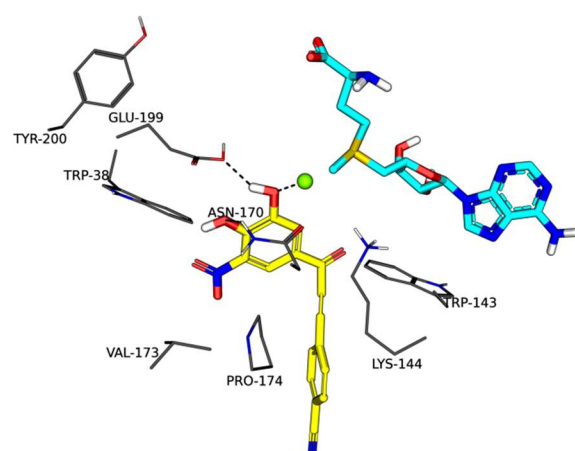


Figure 3.13: Three-dimensional representation of the predicted binding of chalcone **2** to COMT.

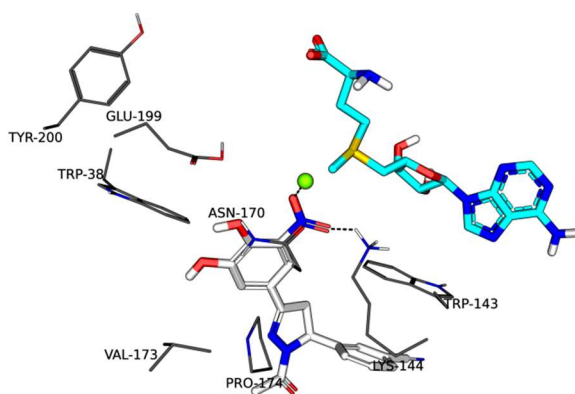


Figure 3.14: Three-dimensional representation of the predicted binding of the S-enantiomer of pyrazoline **4** to COMT.

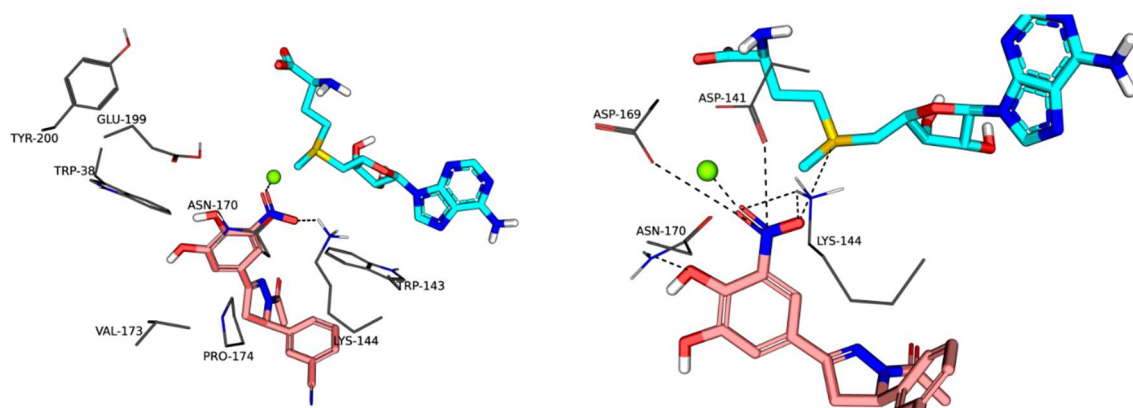


Figure 3.15: Three-dimensional representation of the predicted binding of the R-enantiomer of pyrazoline **4** to COMT (left). A close-up view of the interactions formed by the nitro group is also given (right).

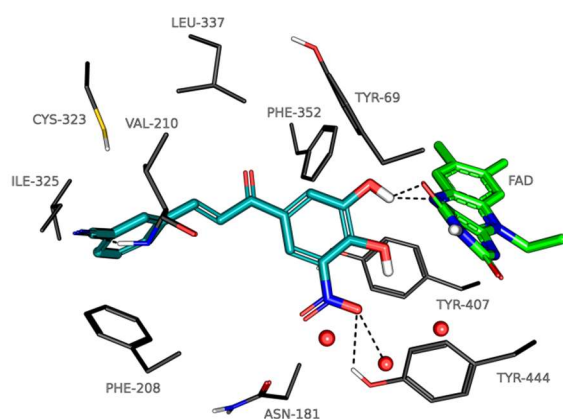


Figure 3.16: Three-dimensional representation of the predicted binding of chalcone **1** to MAO-A.

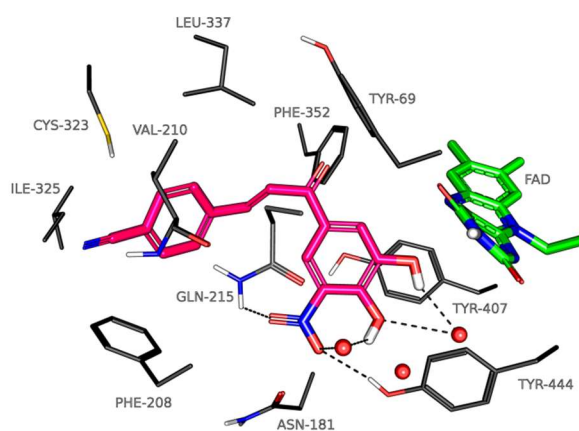


Figure 3.17: Three-dimensional representation of the predicted binding of chalcone **2** to MAO-A.

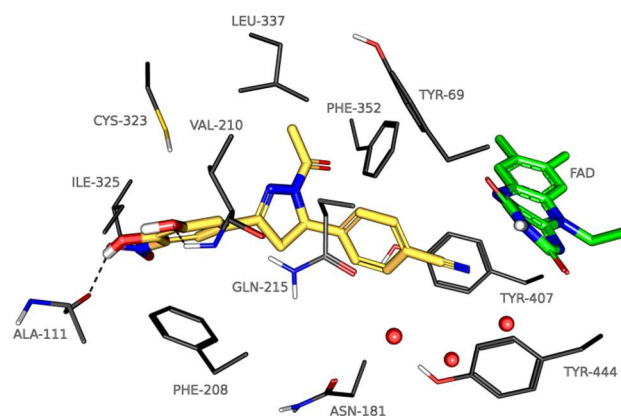


Figure 3.18: Three-dimensional representation of the predicted binding of S-enantiomer of pyrazoline **5** to MAO-A.

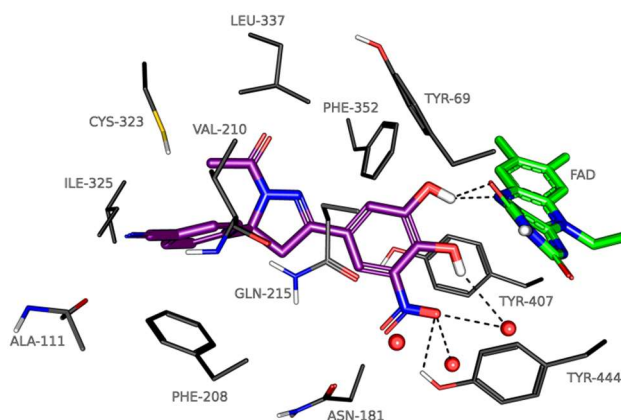


Figure 3.19: Three-dimensional representation of the predicted binding of R-enantiomer of pyrazoline **5** to MAO-A.

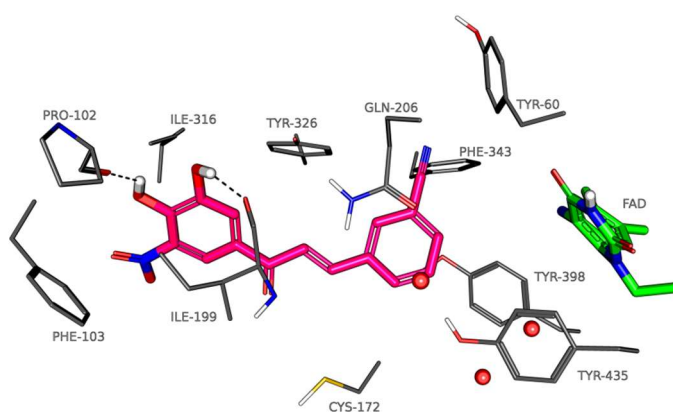


Figure 3.20: Three-dimensional representation of the predicted binding of chalcone **1** to MAO-B.

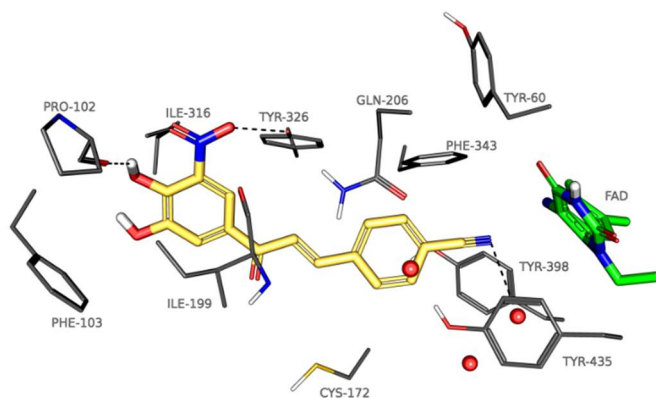


Figure 3.21: Three-dimensional representation of the predicted binding of chalcone **2** to MAO-B.

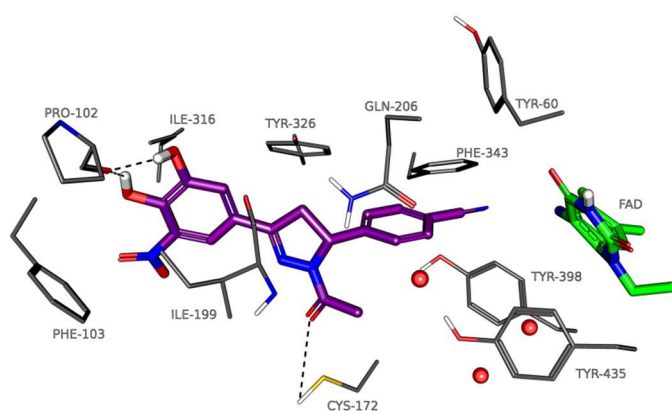


Figure 3.22: Three-dimensional representation of the predicted binding of S-enantiomer of pyrazoline **5** to MAO-B.

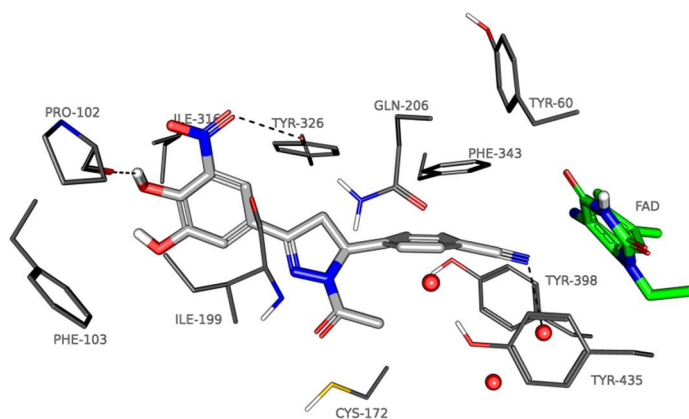


Figure 3.23: Three-dimensional representation of the predicted binding of R-enantiomer of pyrazoline **5** to MAO-B.

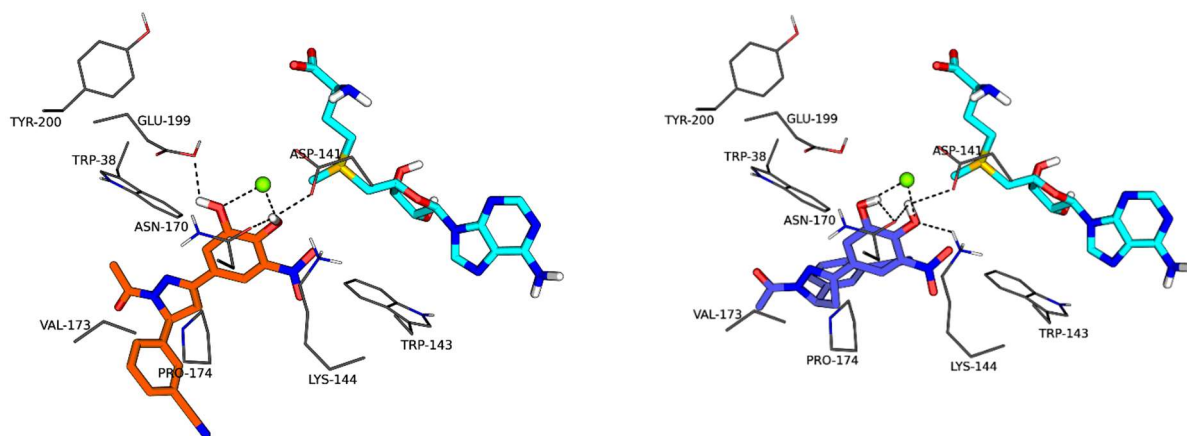


Figure 3.24: Three-dimensional representation of the predicted binding of the S-enantiomer (left) and R-enantiomer (right) of pyrazoline **4** to COMT.

3.7. DISCUSSION AND CONCLUSION

In this present study a series of three new nitrocatechol derivatives of chalcone (**1–3**) and three new pyrazoline derivatives (**4–6**) were synthesised with the aim of discovering compounds that inhibits both MAO-B, and COMT. The results show that the synthesised compounds are weak inhibitors of MAO-A and MAO-B, with IC_{50} values $>43.9 \mu\text{M}$ and $>55.4 \mu\text{M}$, respectively. In contrast to their MAO inhibition potencies, the chalcone derivatives and pyrazoline derivatives are potent inhibitors of COMT with IC_{50} values $<0.24 \mu\text{M}$. The pyrazoline derivatives are significantly more potent inhibitors than the chalcones. Chalcones contain the α,β -unsaturated carbonyl system which may be considered as a Michael acceptor. Such compounds may act as electrophiles and are thus toxic alerts from a medicinal chemistry point of view. In this regard, the pyrazolines are more desirable for the future development of COMT inhibitors, and they are expected to be less reactive than the corresponding chalcone compounds.

Pyrazoline, a five-membered, two-nitrogen-containing heterocycle ring, is widely found as the basic structure in a large variety of compounds that hold important pharmaceutical activities (Secci *et al.*, 2011; Fustero *et al.*, 2009). Pyrazoline derivatives show a variety of pharmacological properties as a result of their structural multiplicity. Pyrazoline derivatives can be considered as a cyclic hydrazine moiety and because of four positions where substituents may be attached, they offer a high degree of diversity that has proven to be useful in the search for new therapeutic agents (Secci *et al.*, 2011). Classical methods for the synthesis of substituted pyrazolines involve approaches based either on the condensation of hydrazines with 1,3-dicarbonyl compounds and their 1,3-dielectrophile equivalents, or on intermolecular [3+2] cycloadditions of 1,3-dipoles to alkynes (Secci *et al.*, 2011; Fustero *et al.*, 2009). Over the past few years, however, more efficient and broadly applicable methodologies have been developed with the aim of increasing the

regioselectivity in the preparation of substituted pyrazolines (Secci *et al.*, 2011; Fustero *et al.*, 2009). The method that was used to create the new pyrazoline derivatives (4–6) in this present study, was based on the addition of hydrazine hydrate to an α,β -unsaturated carbonyl system in acetic acid. As reported in table 3.3, the new pyrazoline derivative (4–6) showed potent inhibitory activity specifically against COMT.

The design of pyrazoline derivatives for the inhibition of COMT should thus be further pursued as this present study shows that they are a suitable class to reach this objective. Further development of the pyrazoline derivatives can result in potentially clinically useful inhibitors of COMT. As mentioned, this is the first report of COMT inhibition by the pyrazoline class of compounds.

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CHAPTER 4

CONCLUSION

In this study, chalcone analogues that incorporate the nitrocatechol moiety were synthesised, and subsequently converted to the corresponding pyrazoline derivatives. A series of three new nitrocatechol derivatives of chalcones and three new pyrazoline derivatives were successfully synthesised and evaluated as inhibitors of MAO-A, MAO-B and COMT. MAO is responsible for the oxidative deamination of endogenous monoamine neurotransmitters such as dopamine, serotonin, noradrenaline, adrenaline and trace amines ([Secci *et al.*, 2011](#)). MAO-A inhibitors are established compounds in the treatment of depression, whereas MAO-B inhibitors find application in the therapy of Parkinson's disease and other neurological disorders. The general function of COMT is the elimination of biologically active or toxic catechols and some hydroxylated metabolites. When COMT activity is inhibited, there is an improvement in the bioavailability and a decrease in the elimination of levodopa, and COMT inhibitors are thus used to enhance dopamine neurotransmission in Parkinson's disease ([Kaakkola, 2000](#)).

The hypothesis of this study states that it is possible to design nitrocatechol derivatives of chalcone that exhibit dual inhibition of COMT and MAO-B. The nitrocatechol derivatives of chalcone and the pyrazoline derivatives were evaluated as inhibitors of human MAO and rat liver COMT, and the inhibition potencies were expressed as the IC_{50} values. The IC_{50} values indicated that the synthesised compounds were weak inhibitors of MAO-A and MAO-B. The IC_{50} values of nitrocatechol derivatives of chalcone ranged from 43.9–58.9 μ M for MAO-A, and 55.8–60.6 μ M for MAO-B. The pyrazoline derivatives exhibited IC_{50} values that ranged from 70.6–87 μ M for MAO-A, and 55.4–85.2 μ M for MAO-B. The reference inhibitors, tolloxatone and lazabemide, inhibit MAO-A and MAO-B with IC_{50} values of 3.92 μ M and 0.091 μ M, respectively. This underscores the low MAO inhibition potencies of the chalcone and the pyrazoline derivatives.

The chalcone and the pyrazoline derivatives were high potency inhibitors of COMT. The nitrocatechol derivatives of chalcones exhibited IC_{50} values that ranged from 0.141–0.240 μ M, and the pyrazoline derivatives exhibited IC_{50} values that ranged from 0.048–0.079 μ M. This indicated that the pyrazoline derivatives are the more potent inhibitors for COMT compared to the chalcones. Both the chalcones and pyrazolines are more potent COMT inhibitors than the reference COMT inhibitors, tolcapone (IC_{50} = 0.26 μ M) and entacapone (IC_{50} = 0.25 μ M).

Key physiochemical properties of the chalcone and the pyrazoline derivatives were calculated, and found to comply with the 'Lipinski's rule of 5'. It is expected that the compounds will be bioavailable via the oral route, particularly since the log P values are <5. The ideal Log P range

for bioavailability is between 1 and 3. The nitrocatechol derivatives of chalcones and the pyrazoline derivatives must be able to interact with two different environments (lipophilic and aqueous) in order to be bioavailable and reach the site of action. The log P values that were calculated were all positive values (1.93–3.08) and indicated that the compounds are relatively lipophilic and will be able to permeate across the blood-brain barrier via passive diffusion.

While the synthesis of 3 nitrocatechol derivatives of chalcones and 3 pyrazoline derivatives were successfully achieved, the hypothesis was not fully achieved. In this respect, the synthesised compounds were excellent COMT inhibitors, while being weak MAO inhibitors. The study found that the most potent COMT inhibitor, a pyrazoline derivative, exhibits an IC_{50} value of 0.048 μ M. The pyrazoline derivatives may serve as lead compounds for the future design of inhibitors of COMT.

Pyrazoline refers to a class of heterocyclic ring compounds and is characterised by a 5-membered ring structure composed of three carbon atoms and two nitrogen atoms in adjacent positions (Alam *et al.*, 2015). Pyrazoline compounds display a diverse range of pharmacological activities such as analgesic, antipyretic, anticancer, antiviral, anti-inflammatory, antioxidant, antimicrobial, anti-diabetic, anticonvulsant and arrhythmic activities (Alam *et al.*, 2015). In this study, the pyrazoline derivatives are more potent COMT inhibitors compared to the chalcones. Furthermore, chalcones contain the α,β -unsaturated carbonyl system which may be considered to be a Michael acceptor. Such compounds may act as electrophiles and are thus toxic alerts from a medicinal chemistry point of view. In this regard, the pyrazolines are more desirable for the future development of COMT inhibitors, and they are expected to be less reactive than the corresponding chalcone compounds.

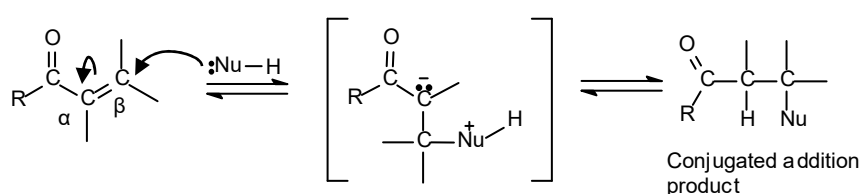


Figure 4.1: The Michael reaction (McMurry, 2012).

In figure 4.1, the α -carbon is bonded to the β -carbon via a double bond, and conjugated with the carbonyl group (McMurry, 2012). The electronegative oxygen atom of the α,β -unsaturated carbonyl compound withdraws electrons from the β -carbon, thereby making it electron-poor and more electrophilic. The net effect is the addition of the nucleophile to the $\text{C}=\text{C}$ bond, with the carbonyl group itself unchanged. The $\text{C}=\text{C}$ bond would not be activated for addition, and no reaction would occur, without the carbonyl group, which thus is important to the success of the reaction (McMurry, 2012).

Pyrazoline derivatives show a variety of pharmacological properties as a result of their structural multiplicity. Pyrazoline derivatives can be considered as a cyclic hydrazine moiety and because of four positions where substituents may be attached, they offer a high degree of diversity that has proven to be useful in the search for new therapeutic agents (Secci *et al.*, 2011). Classical methods for the synthesis of substituted pyrazolines involve approaches based either on the condensation of hydrazines with 1,3-dicarbonyl compounds and their 1,3-dielectrophile equivalents, or on intermolecular [3+2] cycloadditions of 1,3-dipoles to alkynes (Secci *et al.*, 2011; Fustero *et al.*, 2009). Over the past few years, however, more efficient and broadly applicable methodologies have been developed with the aim of increasing the regioselectivity in the preparation of substituted pyrazolines (Secci *et al.*, 2011; Fustero *et al.*, 2009).

The aim of this study was to discover novel dual inhibitors of MAO and COMT. The IC₅₀ values indicated that the compounds are weak MAO inhibitors, while exhibiting high potency COMT inhibition. This study finds that a novel approach should be followed in the design of dual MAO and COMT inhibitors, and that nitrocatechol derivatives, while being potent COMT inhibitors, are not appropriate for MAO inhibition. Since the nitrocatechol containing pyrazoline derivatives are potent COMT inhibitors, this class should be further explored as potentially clinically useful COMT inhibitors.

Future perspectives

Drug design is typically initiated with approaches to increase the pharmacodynamic properties of molecule by increasing binding to a receptor or enzyme. A compound may be found that has desired *in vitro* properties, but as unfavourable *in vivo* properties. It may be possible to alter structures of compounds to improve the pharmacokinetic properties and, thereby, transform the compound into a promising drug candidate (Silverman, 2004).

The structures of the nitrocatechol derivatives of chalcones and pyrazoline derivatives of this study should be modified in order to enhance dual inhibition of MAO-B and COMT. According to a study done by Robinson *et al.* (2013), combination with a furan or methylfuran moiety, as the A-ring of a chalcone, and a phenyl moiety, as the B-ring, substituted with electron withdrawing groups (fluorine, bromine, chlorine and trifluoromethyl groups) results in better inhibitory activity towards MAO-B. Results obtained by a study done by Minders *et al.* (2015), indicated that heterocyclic substitution of the chalcone scaffold with a methylthiophene ring is a possible design strategy, because the electron donating methyl substitution in the thiophene ring resulted in improved MAO-B inhibition. As shown in figure 4.2, these moieties may be combined with the nitrocatechol moiety in future studies in an attempt to design dual MAO/COMT inhibitors.

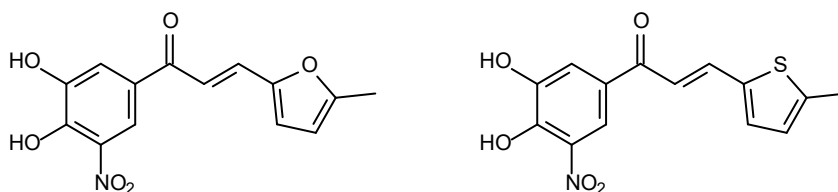


Figure 4.2: Possible structures of chalcones that contains the methylfuran and methylthiophene moiety.

Substitution of the B-ring of the nitrocatechol derivatives of chalcone with the quinoxaline structure should also be considered. Quinoxaline derivatives has showed a broad spectrum of biological and pharmaceutical applications, for example anti-inflammatory, anticancer, anticonvulsant and antibacterial characteristics. 3-Benzyl-2-substituted quinoxaline derivatives were designed and synthesised by [Hassan *et al.* \(2006\)](#), and the derivates showed good inhibitory activity towards MAO-A. The heterocyclic quinoxaline containing nitrogen can be easily synthesised and modified into different derivatives which could have potential biological, pharmacological and medicinal applications. Quinoxaline compounds are biological active and exhibit excellent and remarkable clinical and therapeutic properties, and they have diverse applications in the fields of biological, pharmacological and organic chemistry ([Irfan *et al.*, 2017](#)).

A possible structure of a chalcone that contains the quinoxaline moiety is shown in [figure 4.3](#). The A-ring constitutes the nitrocatechol moiety, while the B-ring is replaced by the quinoxaline. R¹ to R⁴ could be substituted with CN and OH as these groups were appropriate for COMT inhibition in the current study.

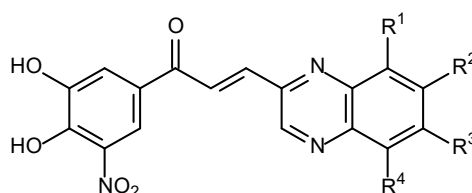


Figure 4.3: Possible structure a chalcone that contains the quinoxaline moiety.

Pyrazoline derivatives also have a broad spectrum of pharmacological activities, which is an indication that pyrazoline compounds are of interest in the design of drugs for a variety of targets. Structure modification of the pyrazoline derivatives of the current study could take place on both the A- and B-ring, as well as on the pyrazoline ring itself.

A possible structure of a pyrazoline compound that may inhibit both COMT and MAO-B is shown in [figure 4.4](#). The nitrocatechol moiety, ring-A, is substituted with an isoprenoid chain on C3 because the known properties of natural prenylated compounds are believed to reside in their enhanced interaction with biological membranes and could possibly increase affinity for target

proteins (Secci *et al.*, 2011). Substitution on the N1 of the pyrazoline derivative with a thiocarbamoyl group may further improve MAO-B inhibition (Secci *et al.*, 2011). According to Secci *et al.* (2011), the C=S group is more polarizable than the C=O group, and the presence of an unsubstituted thiocarbamoyl group on the N1 position of the pyrazoline nucleus appears to be important for the inhibitory activity against both MAO isoforms. Comparison between the N-acetyl and N-thiocarbamoyl showed a discrete inhibitory activity toward the human MAO-B isoform (Secci *et al.*, 2011). R¹ to R⁵ could be substituted with CN and OH, as they have been appropriate for COMT inhibition in the current study.

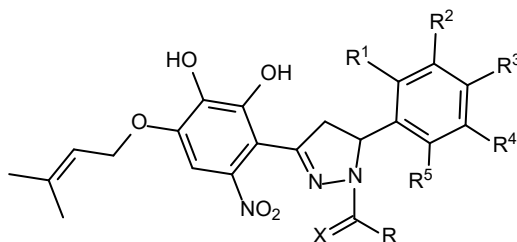


Figure 4.4: Possible structure of a pyrazoline compound that may be investigated as a dual inhibitor of COMT and MAO (X =O/S).

The current study concluded that pyrazoline derivatives can be used in the future design of inhibitors of COMT. In this respect, the structures should be modified to enhance MAO inhibition activity, especially towards MAO-B. For the future design of dual COMT and MAO inhibitors, chalcone-quinoxaline derivatives and pyrazoline compounds containing thiocarbamoyl and isoprenoid chain substitutions should be considered. Chalcones with the furan or methylfuran moiety as the B-ring may be used as an approach to enhance MAO-B inhibition, while the methylthiophene ring should also be a consideration.

If these compounds are considered for dual MAO/COMT inhibition, it is important that their physicochemical and antioxidant properties should be determined in order to evaluate their viability as drugs for the treatment of Parkinson's disease.

As a final note to this dissertation, it should be mentioned that the synthesis of several nitroatechol derivatives of chalcone containing polar groups on the B-ring were attempted, but unsuccessful. The structures of these are shown in figure 4.5. It is speculated that the basic conditions used for the chalcone synthesis caused oxidation of the B-ring of the chalcones or of the aldehyde reagents used in the synthesis. Unfortunately, these interesting chalcones and their corresponding pyrazoline derivatives could not be included and evaluated in this study.

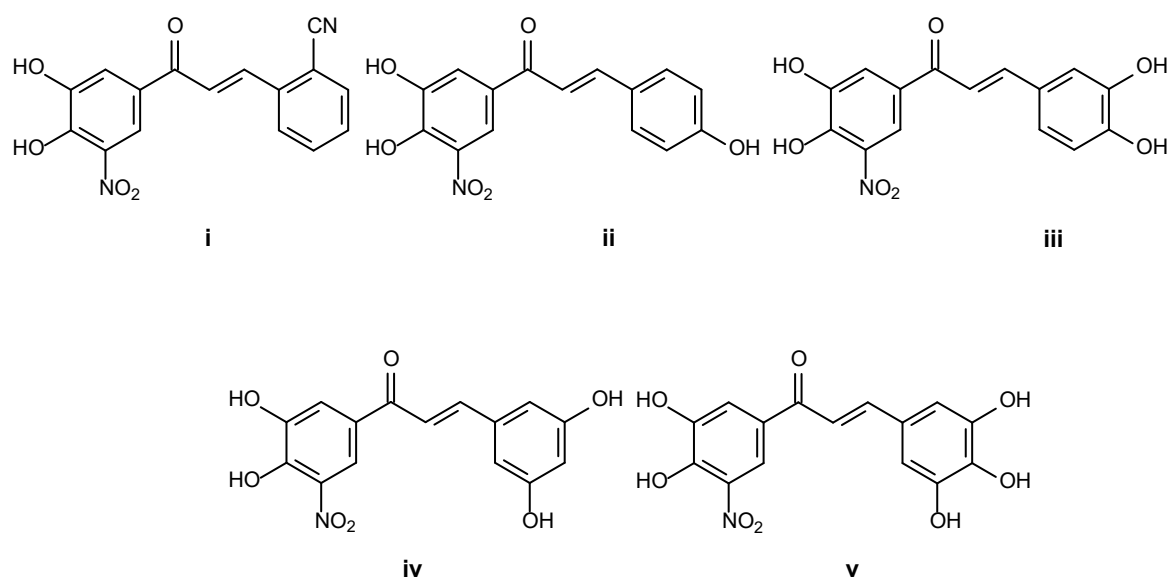


Figure 4.5: The structures of nitroatechol derivatives of chalcone of which the syntheses were attempted in this dissertation, but without success.

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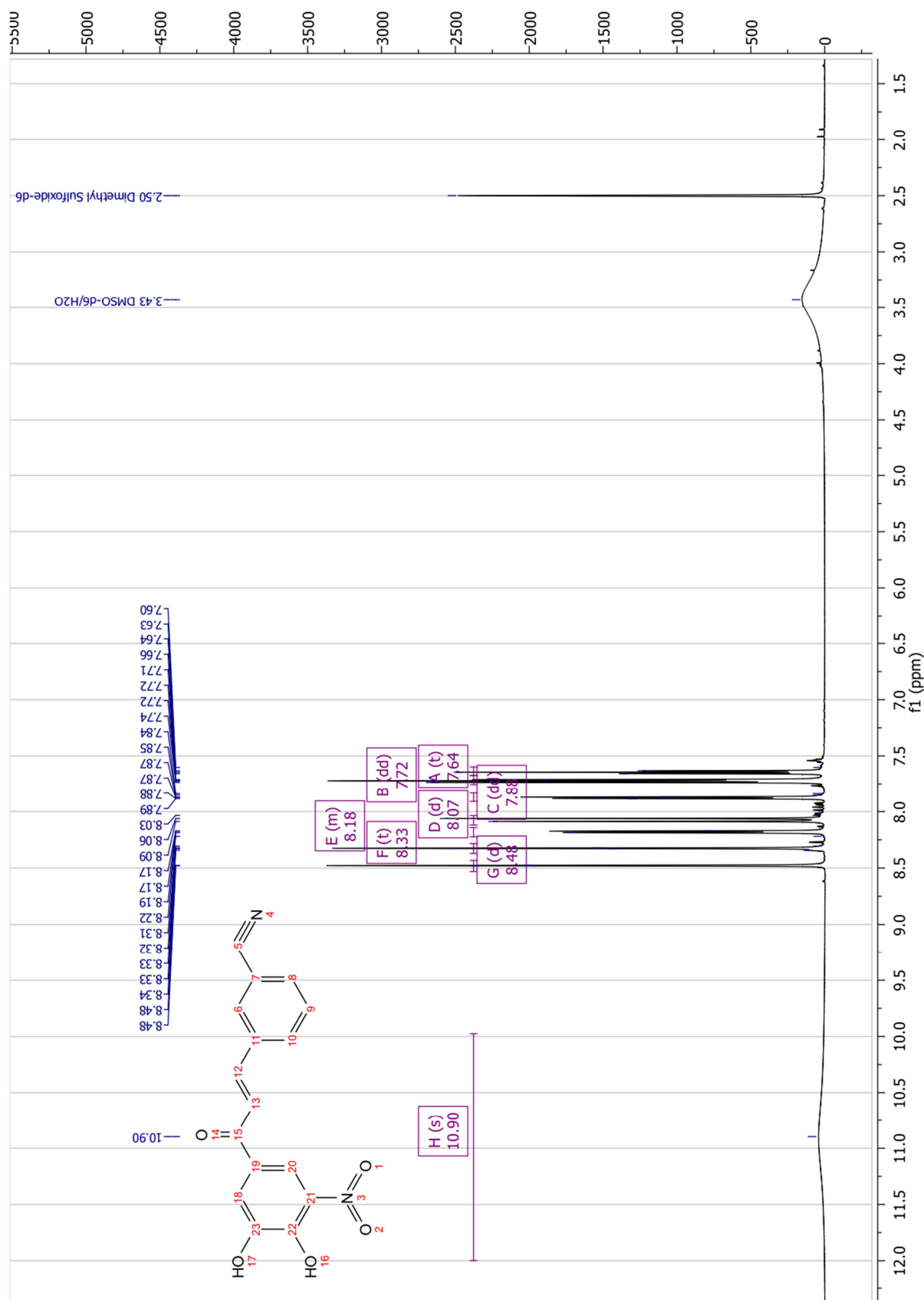
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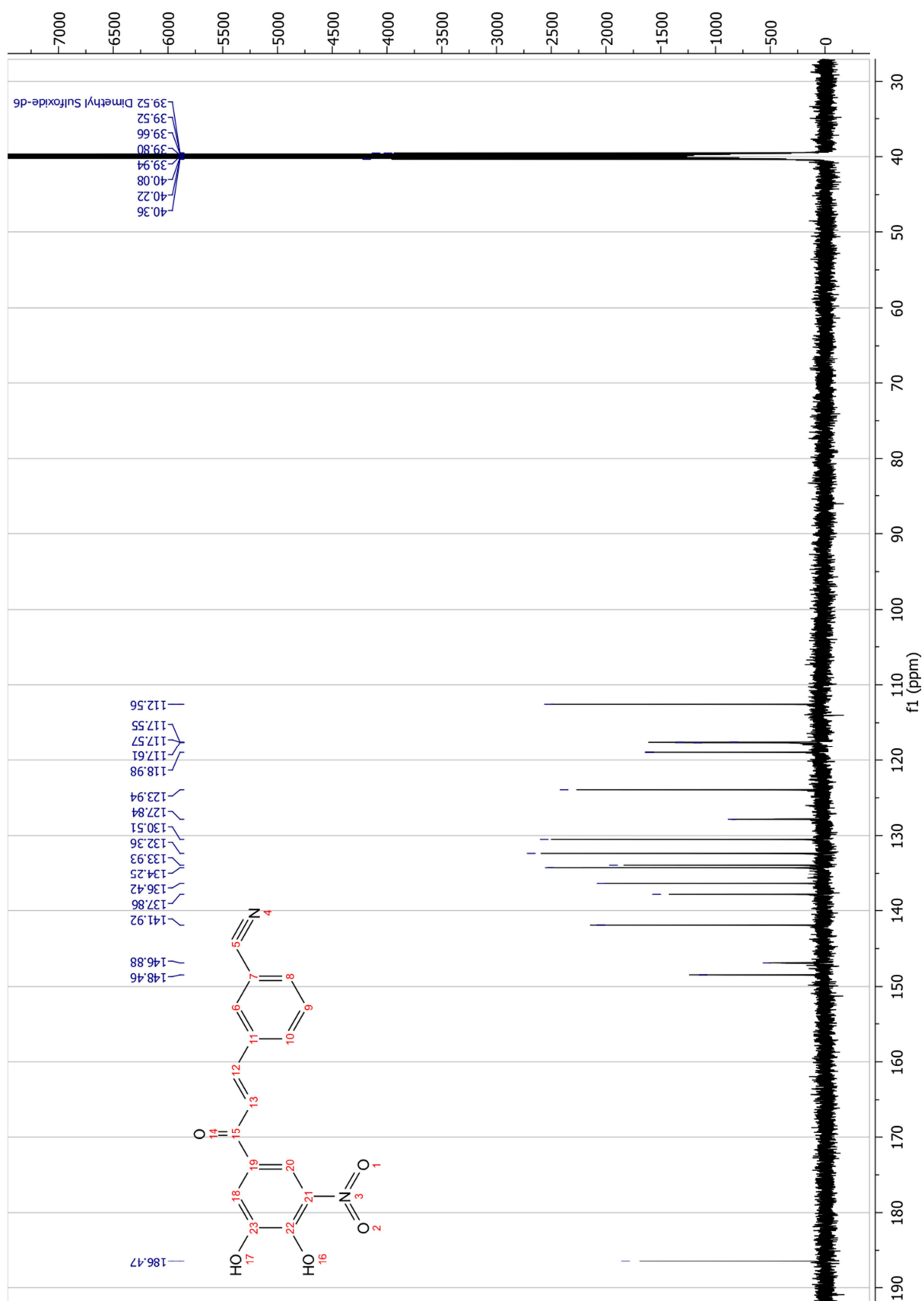
APPENDIX A

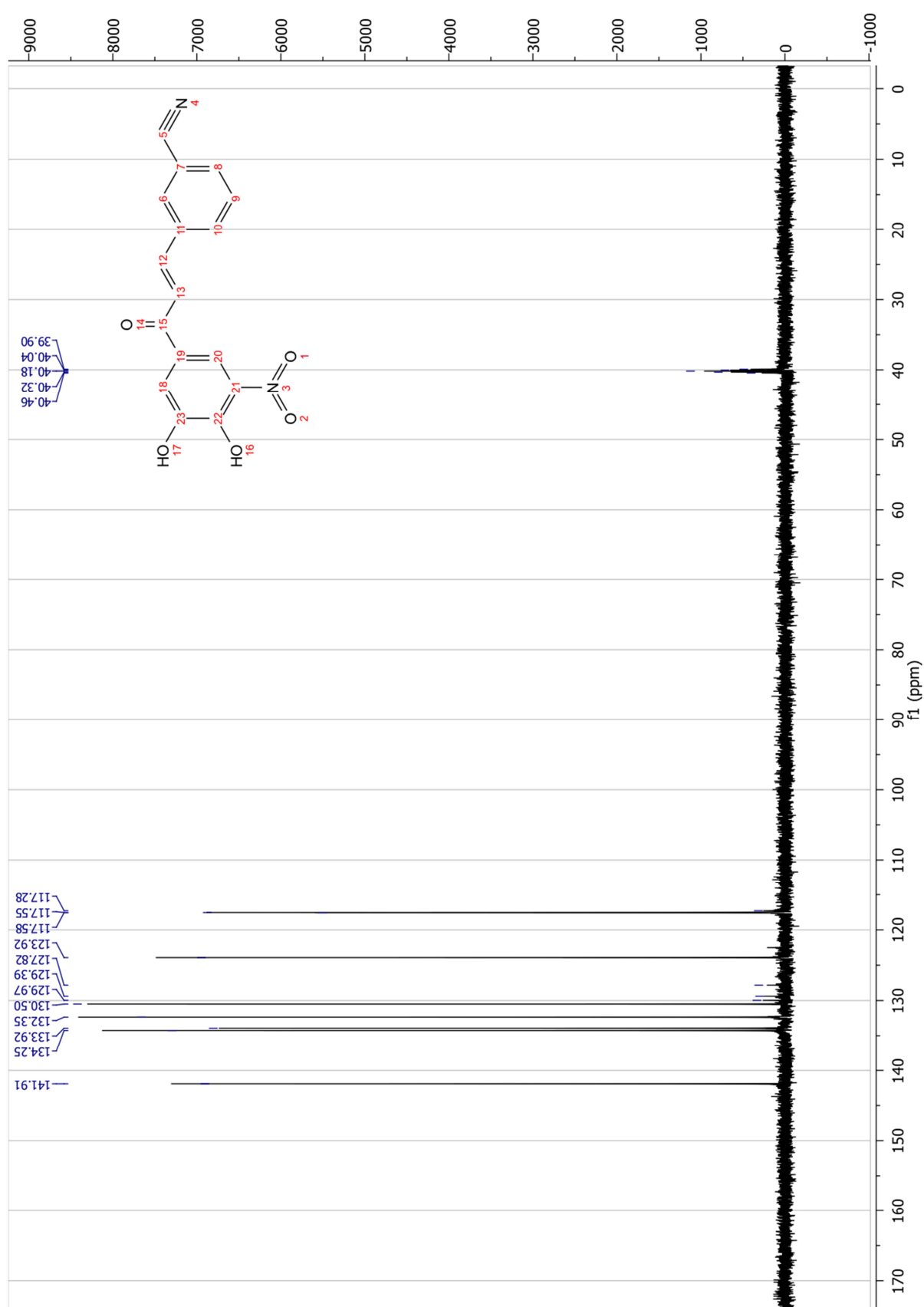
¹H-NMR & ¹³C-NMR SPECTRA

STRUCTURE NO:	STRUCTURE NAME:	PAGE NO:
1	3-[(1 <i>E</i>)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile	101
2	4-[(1 <i>E</i>)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile	105
3	(2 <i>E</i>)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one	109
4	3-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1 <i>H</i> -pyrazol-5-yl]benzonitrile	113
5	4-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1 <i>H</i> -pyrazol-5-yl]benzonitrile	117
6	1-[3-(3,4-Dihydroxy-5-nitrophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1 <i>H</i> -pyrazol-1-yl]ethenone	121

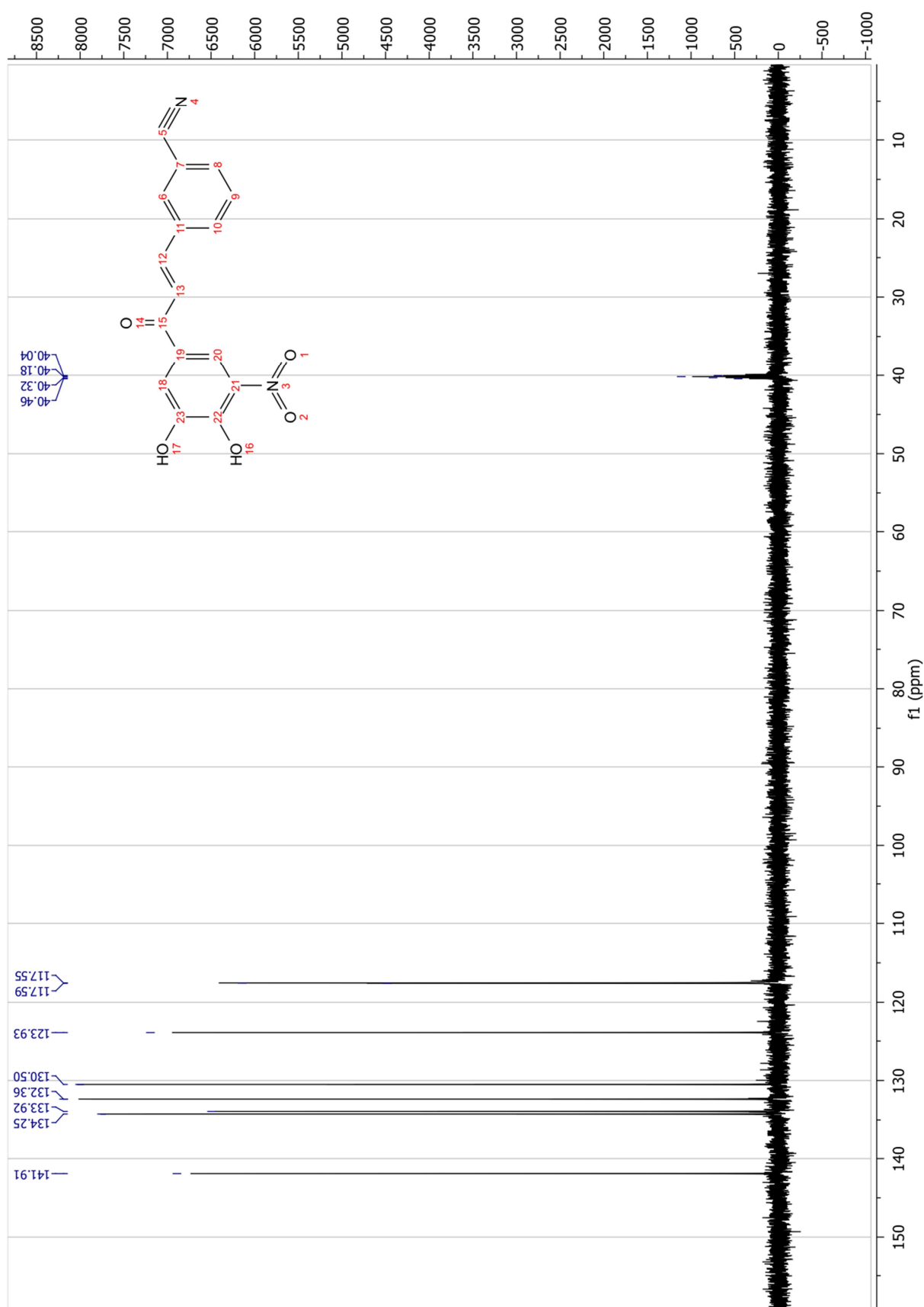
3-[(1*E*)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (1)



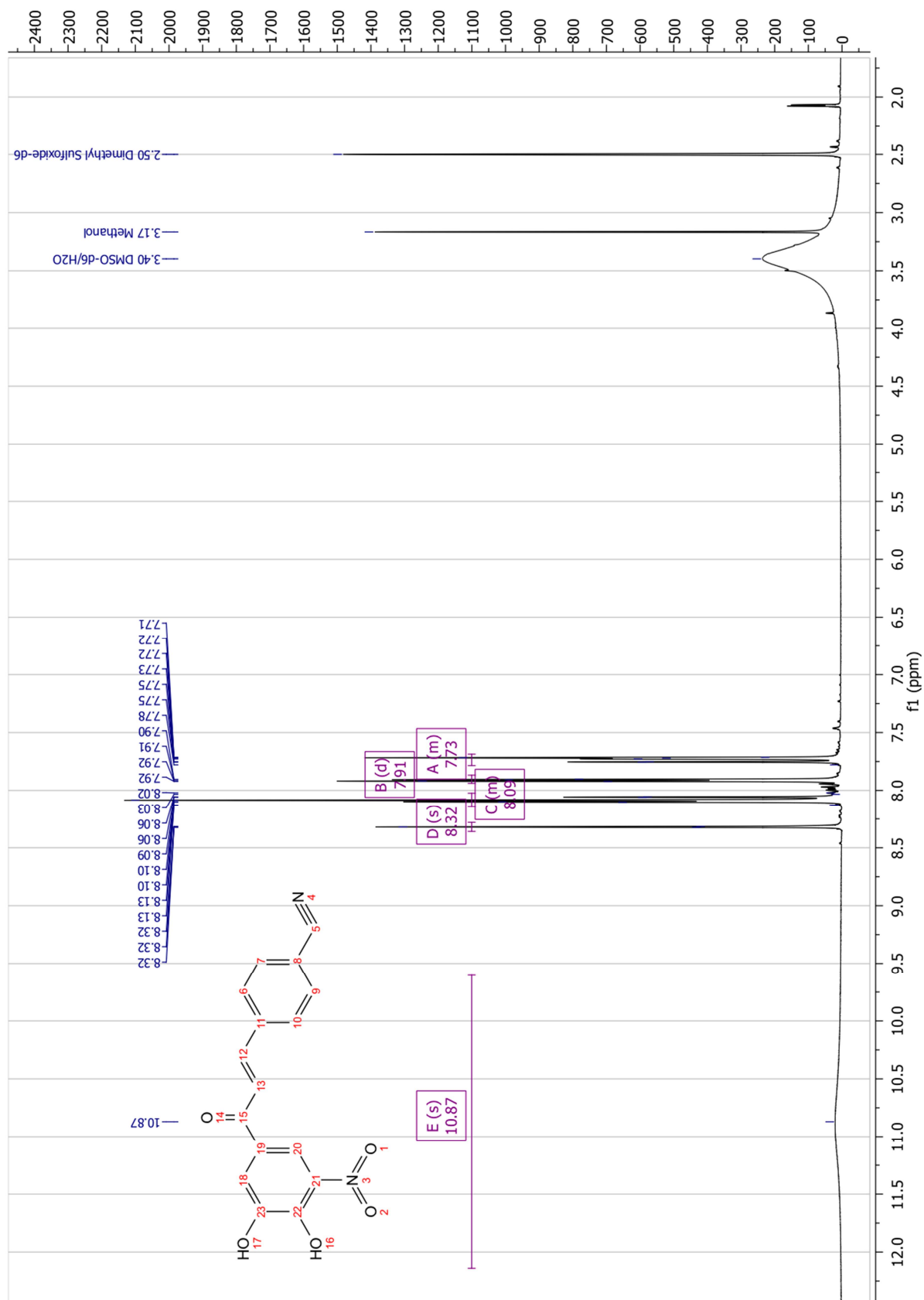


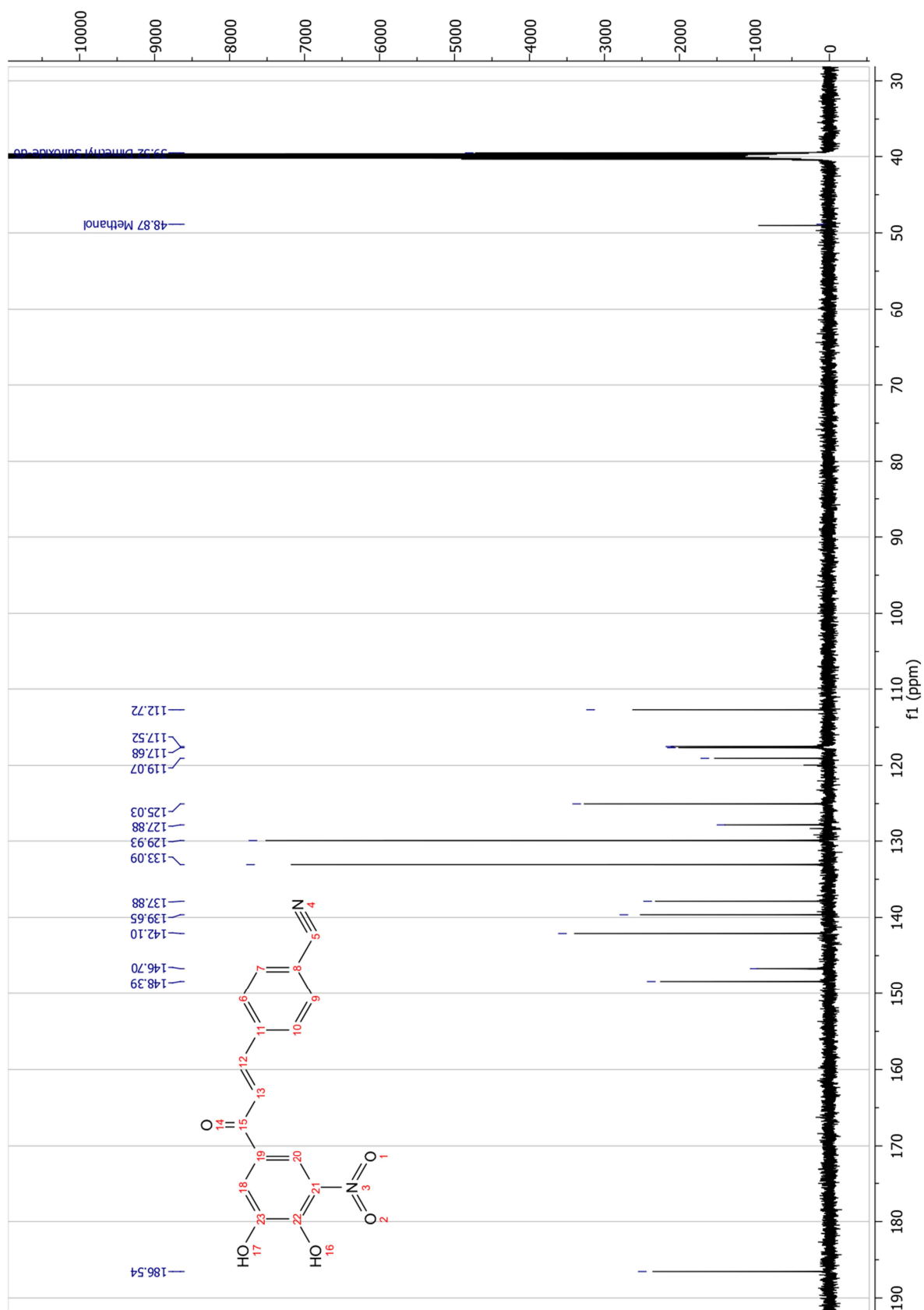


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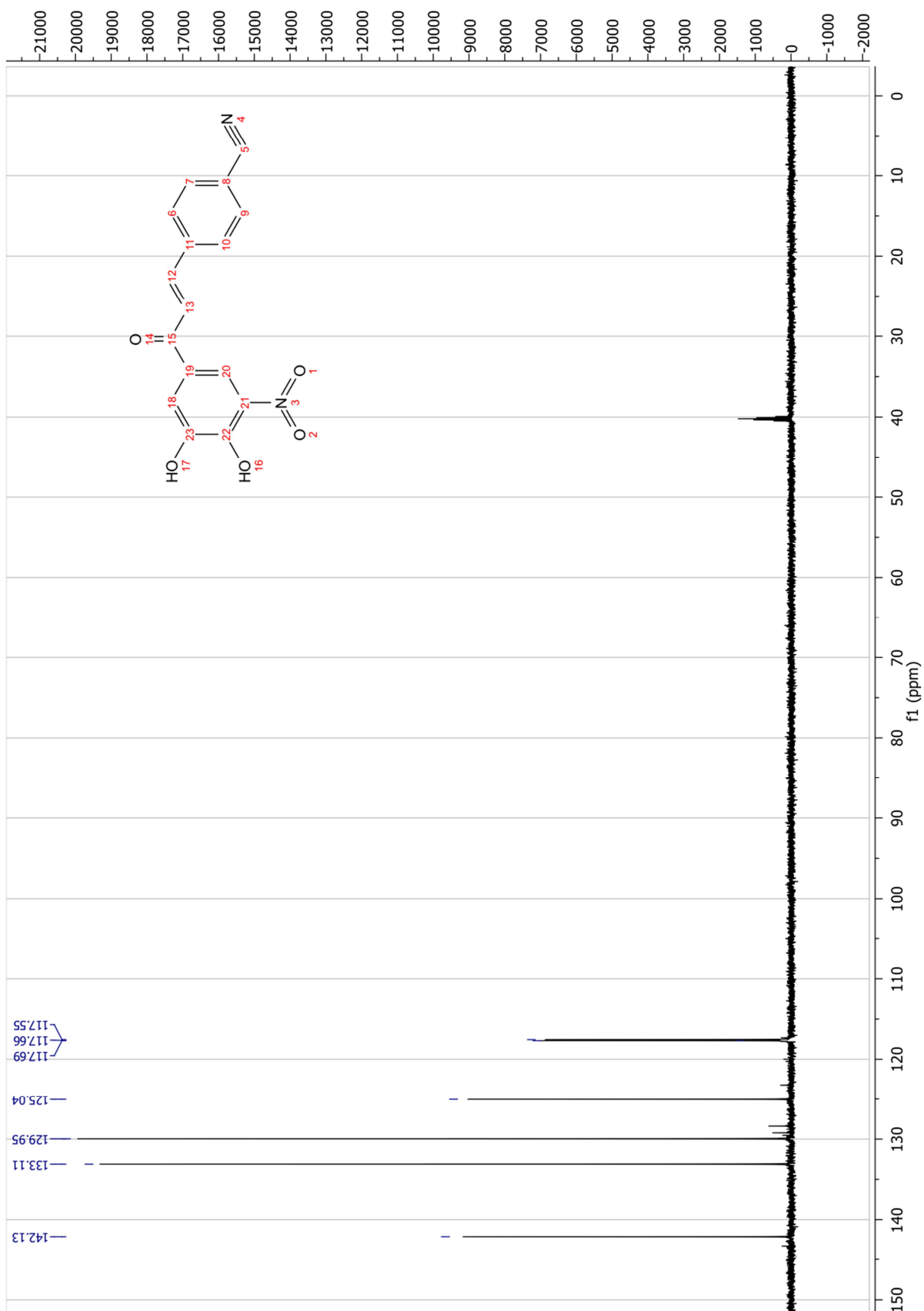


4-[(1*E*)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (2)

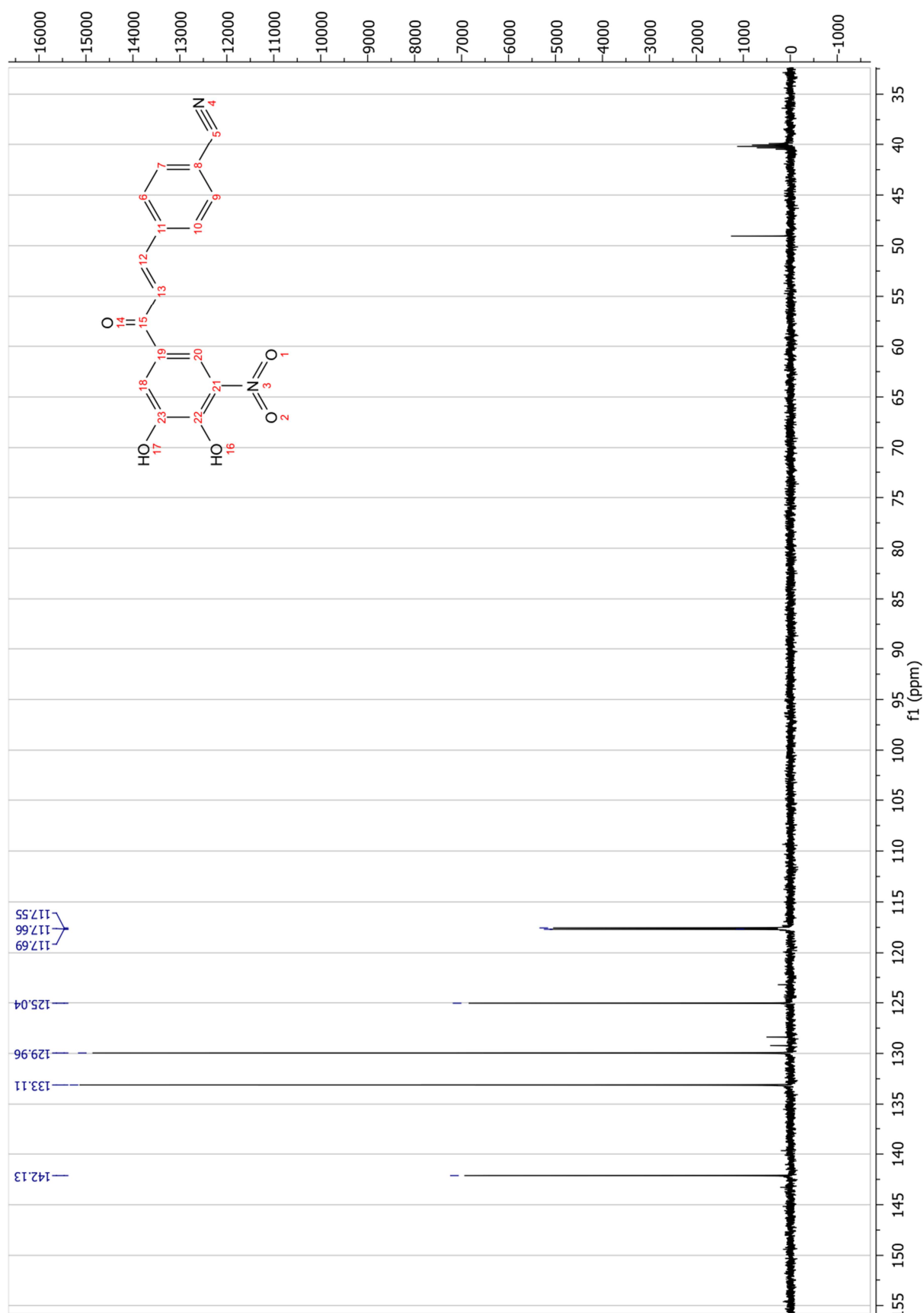




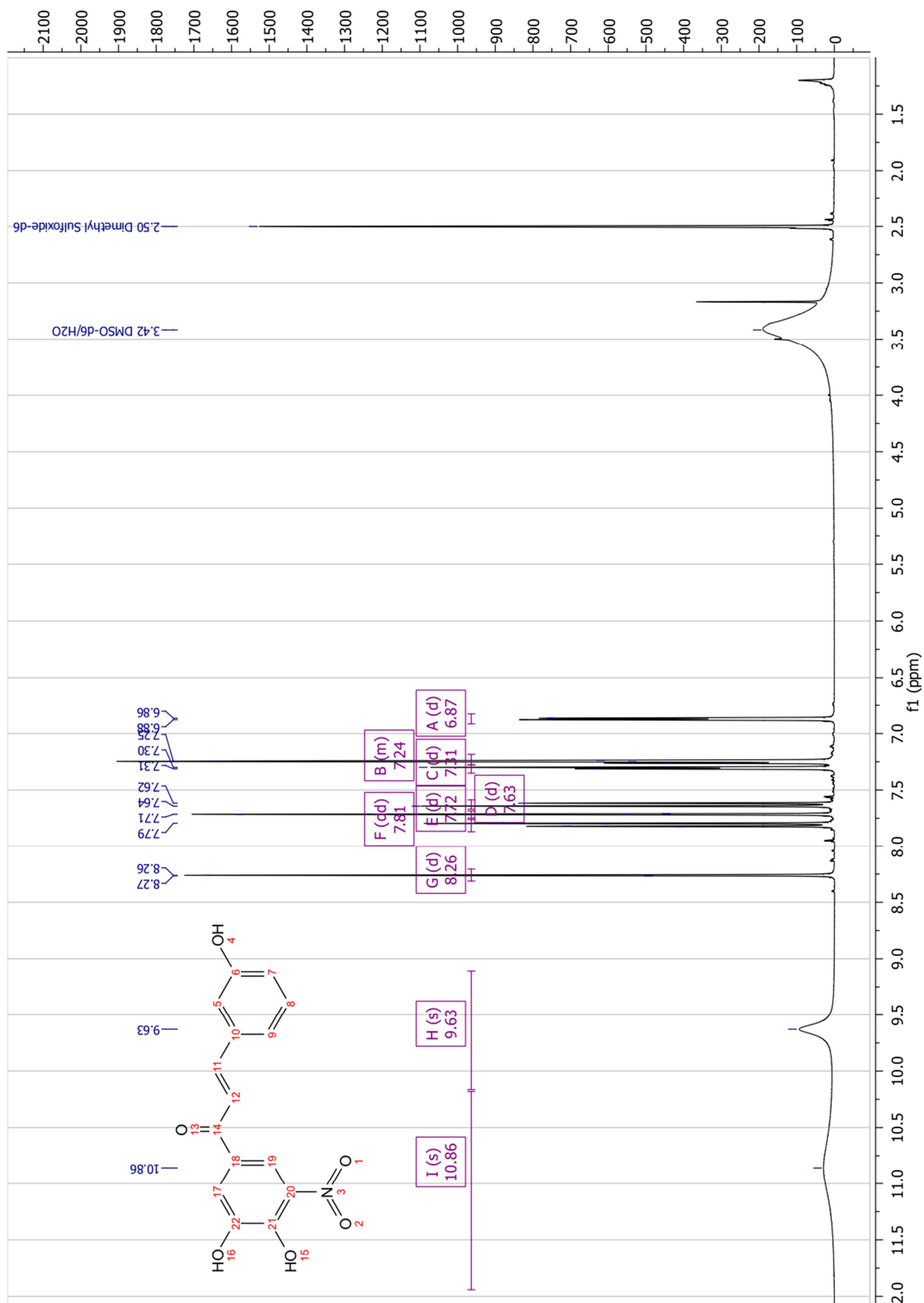
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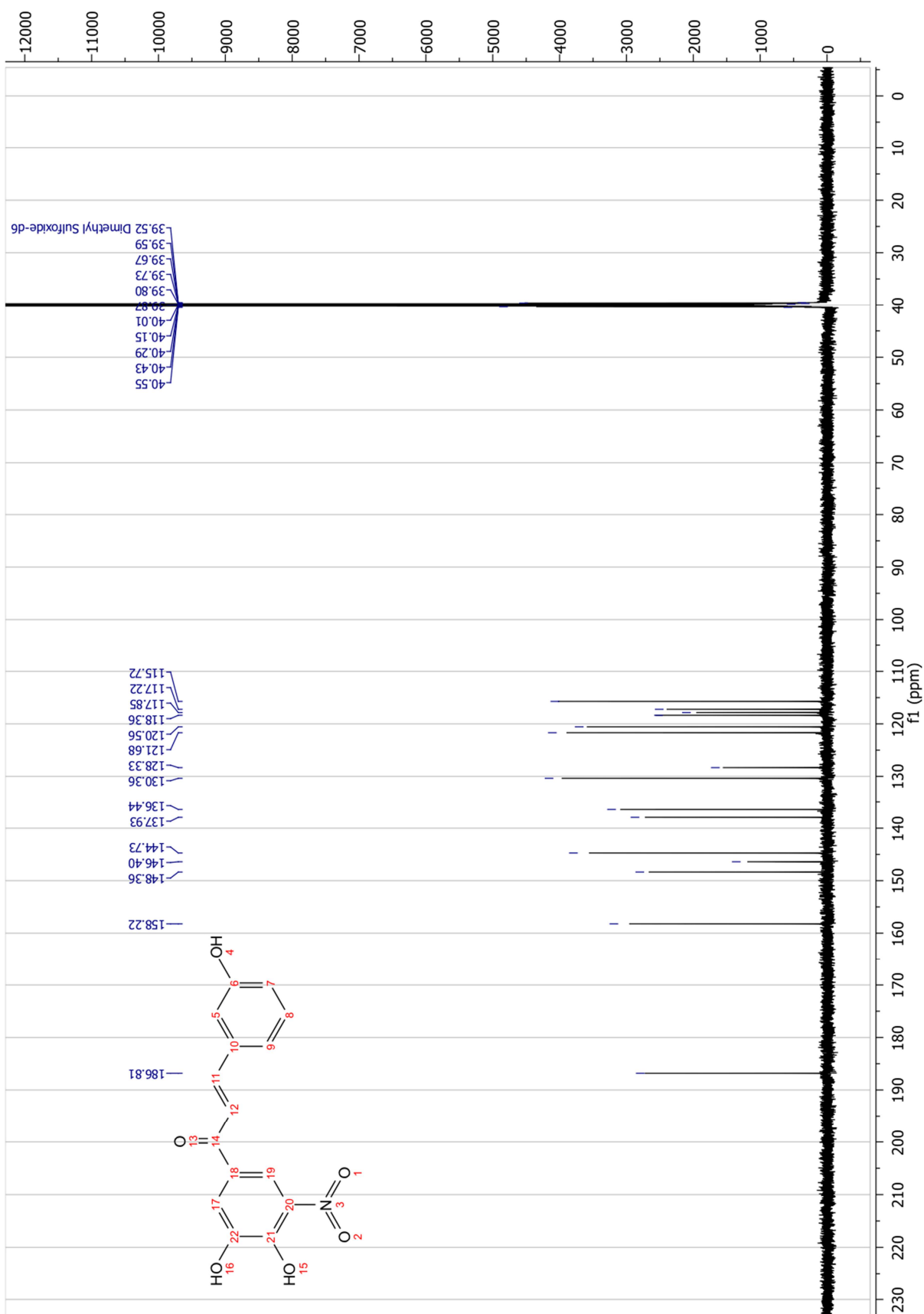


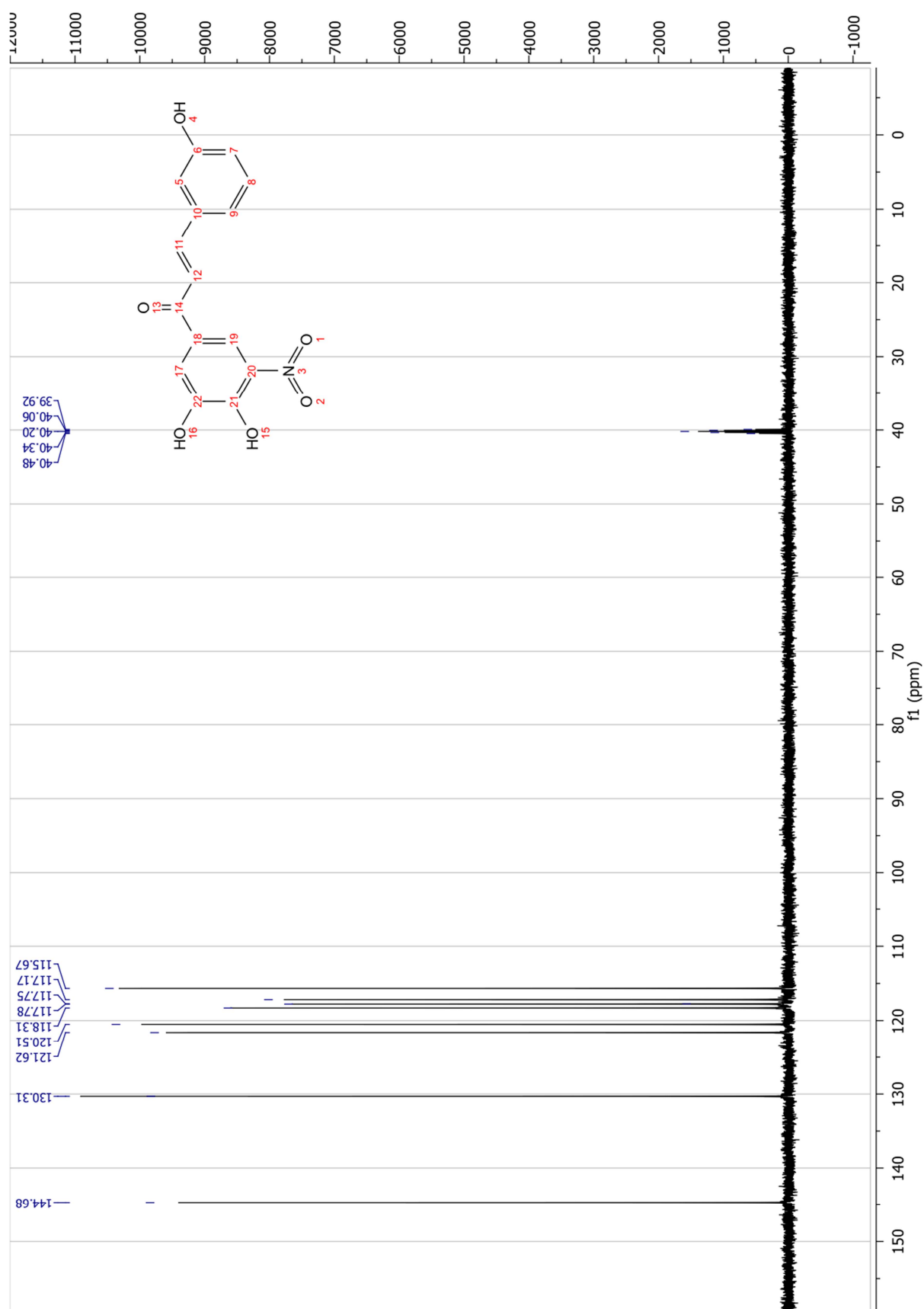
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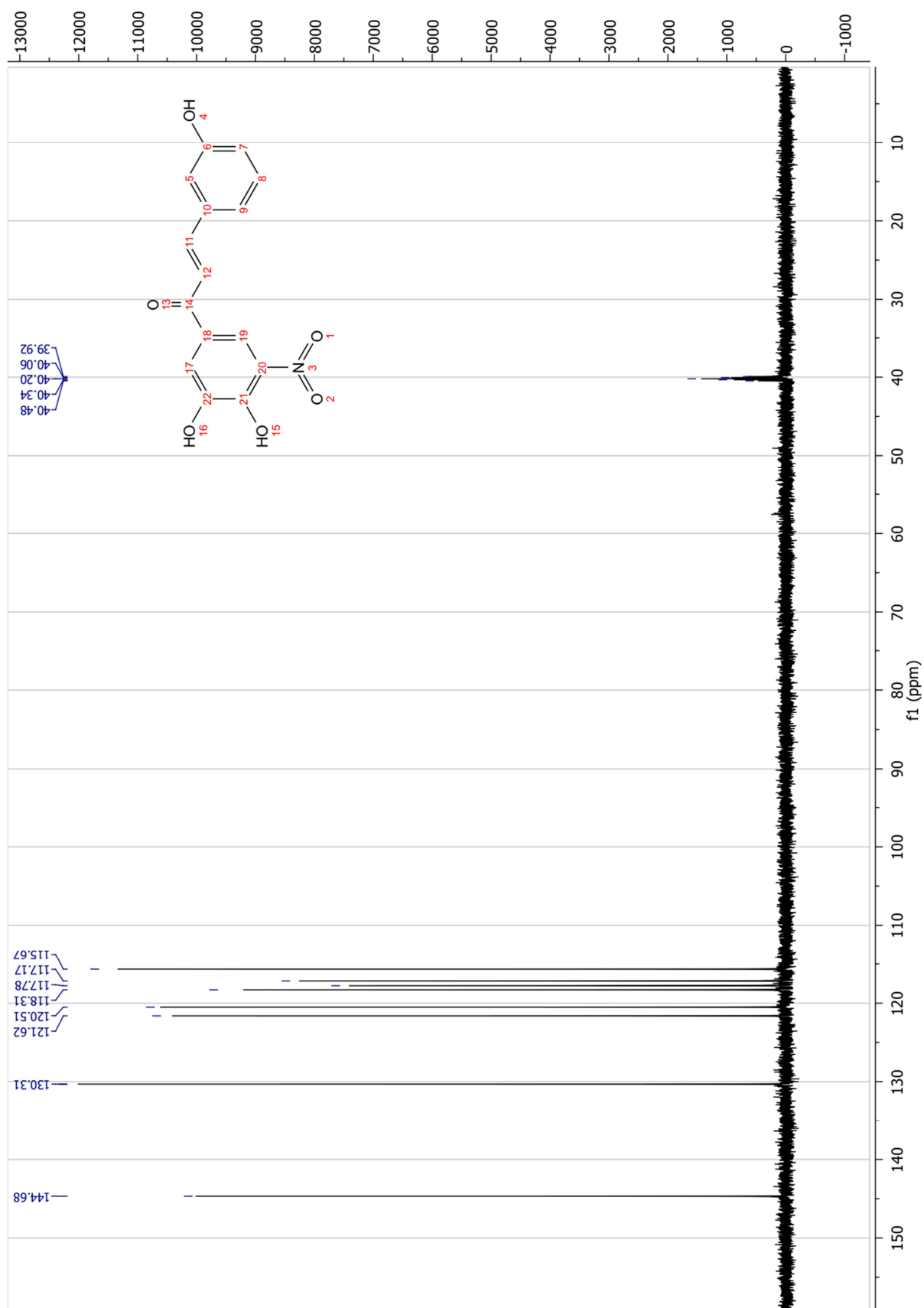


(2E)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one (3)

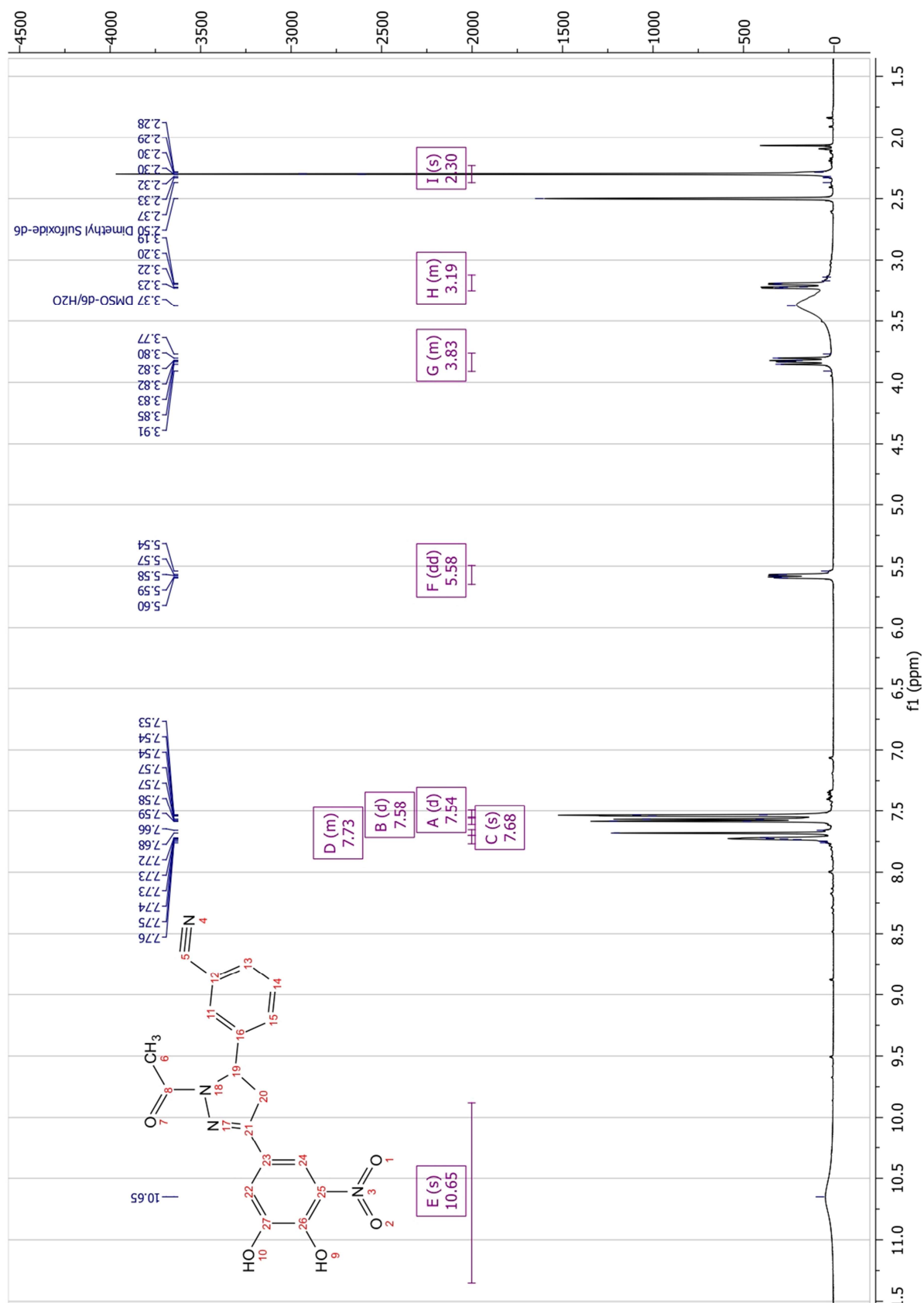


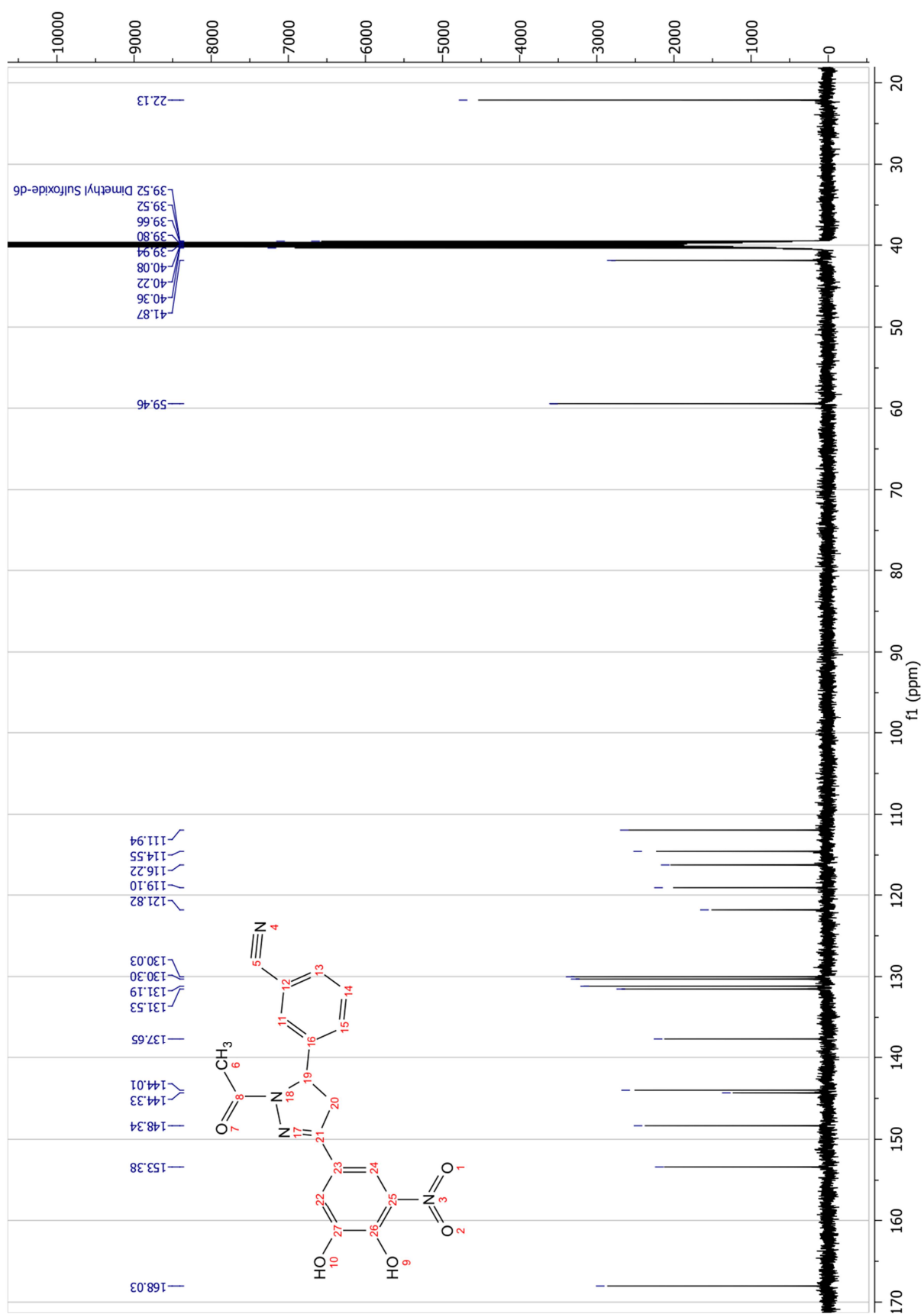


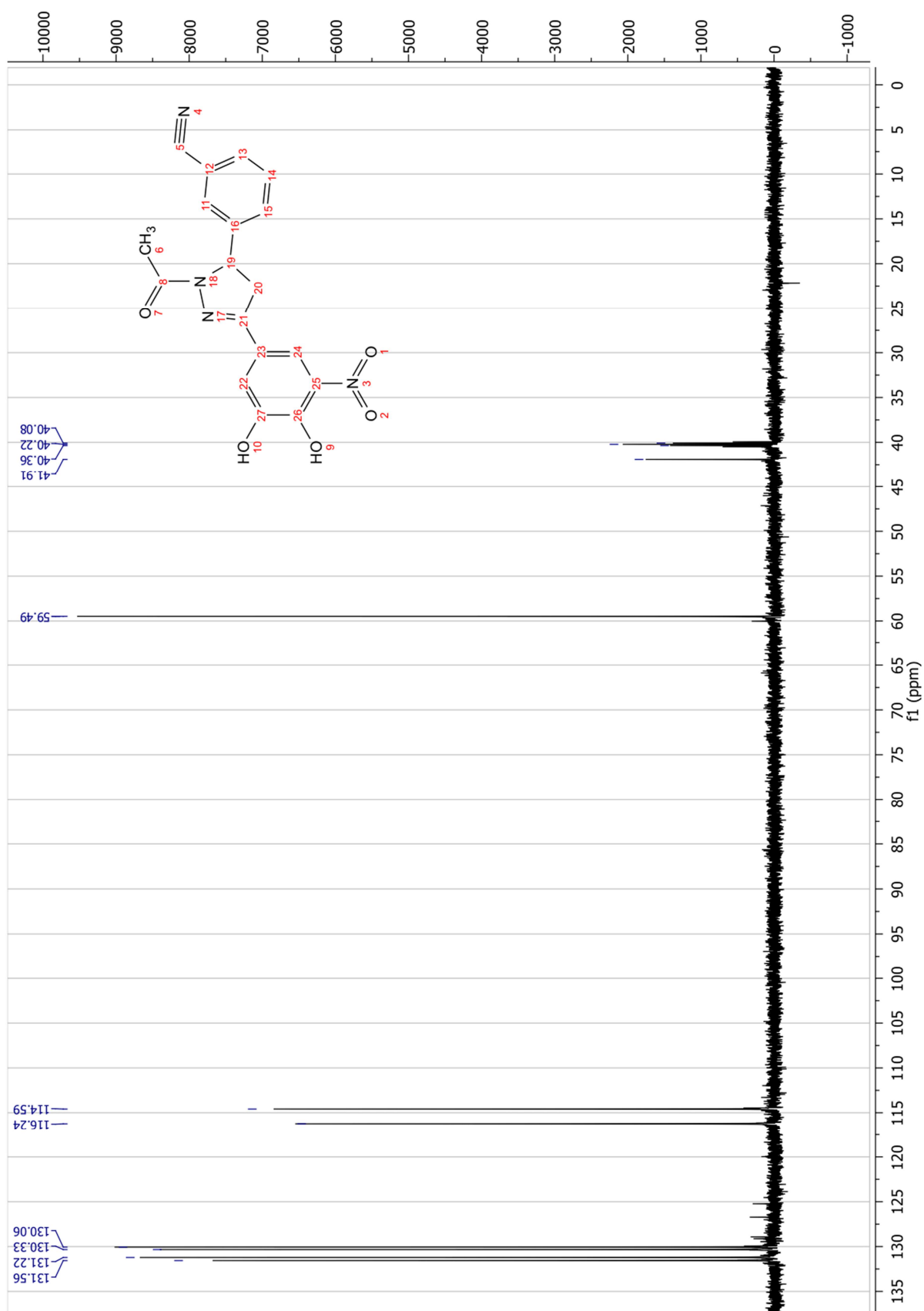


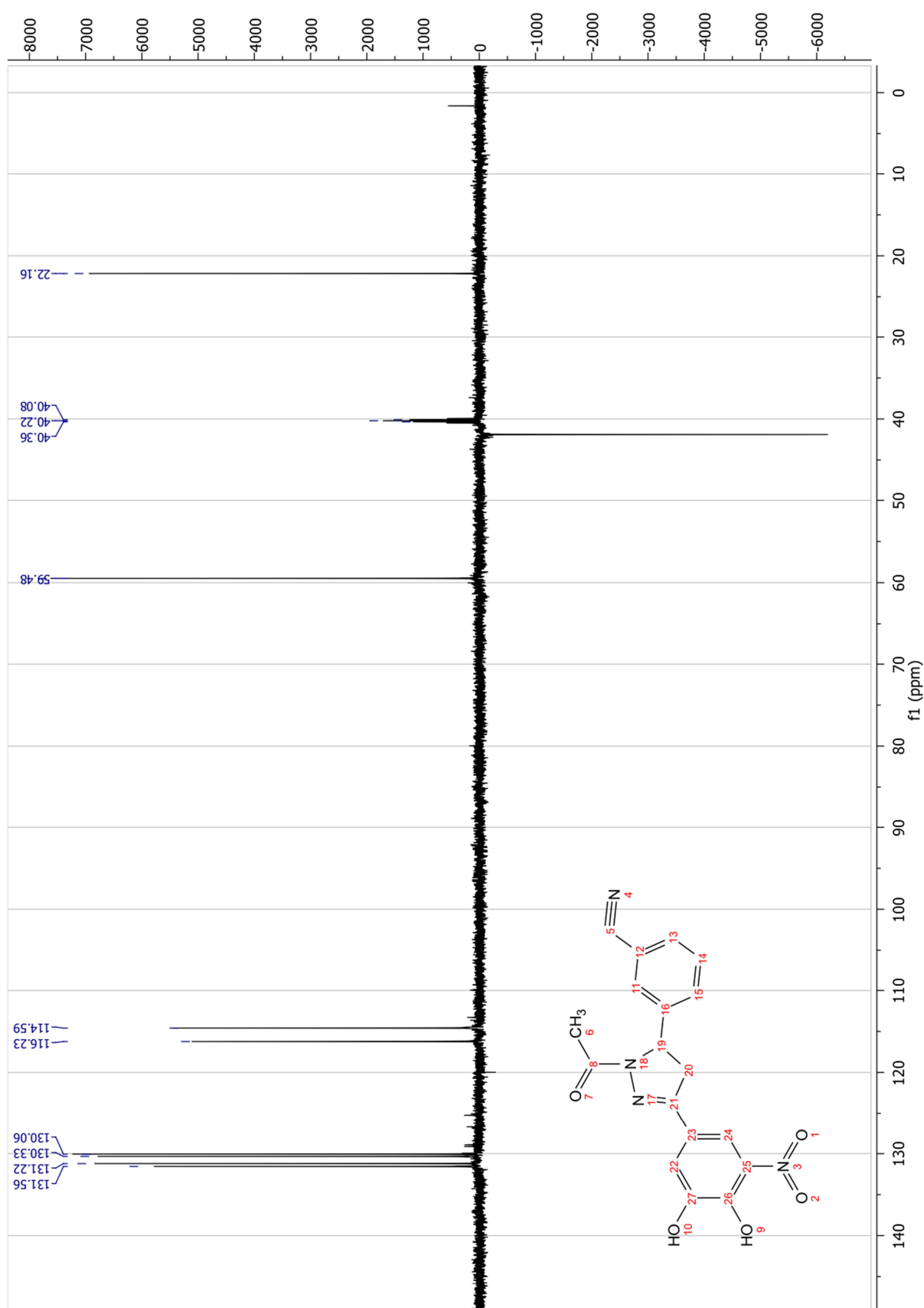


3-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile (4)

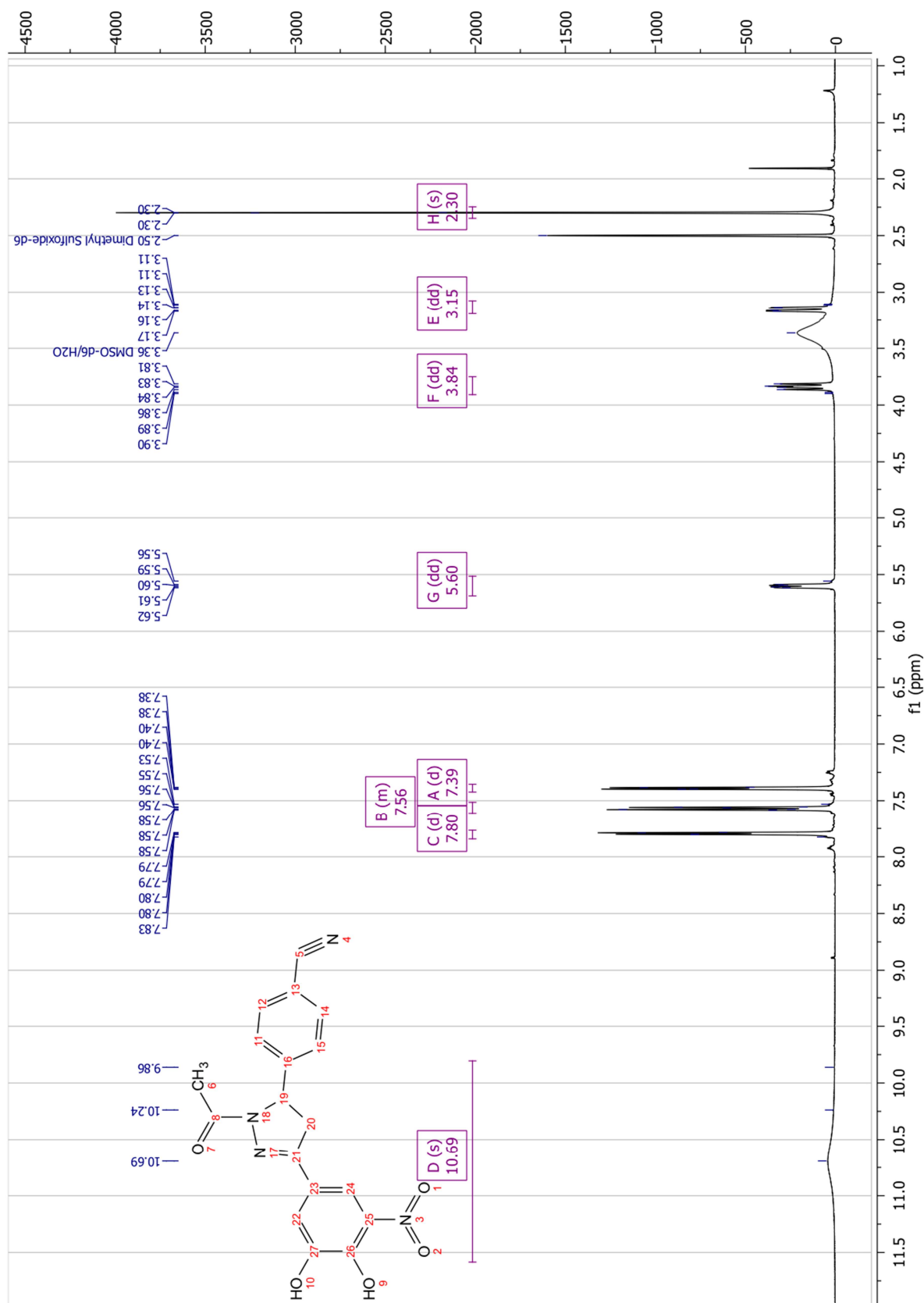


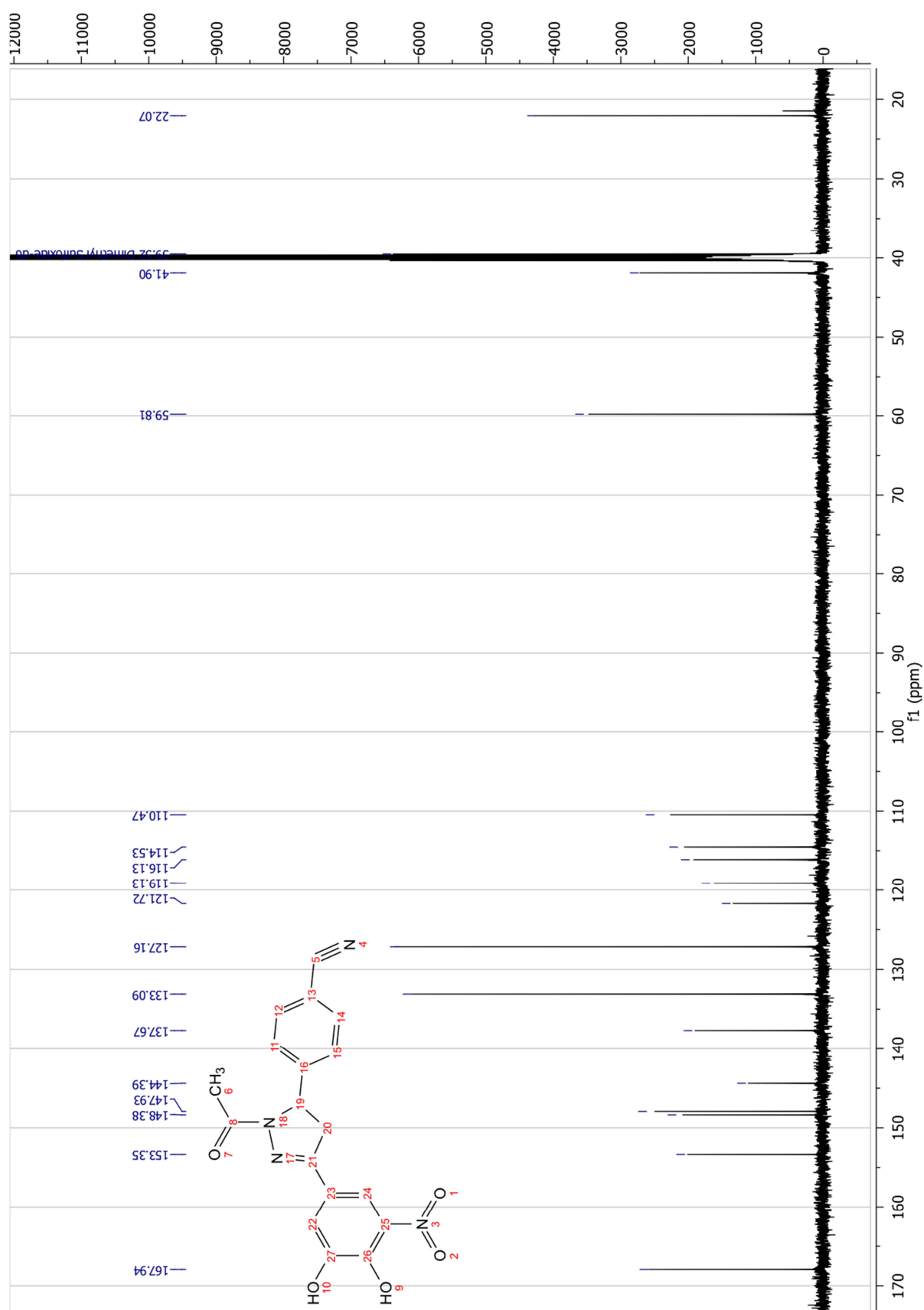


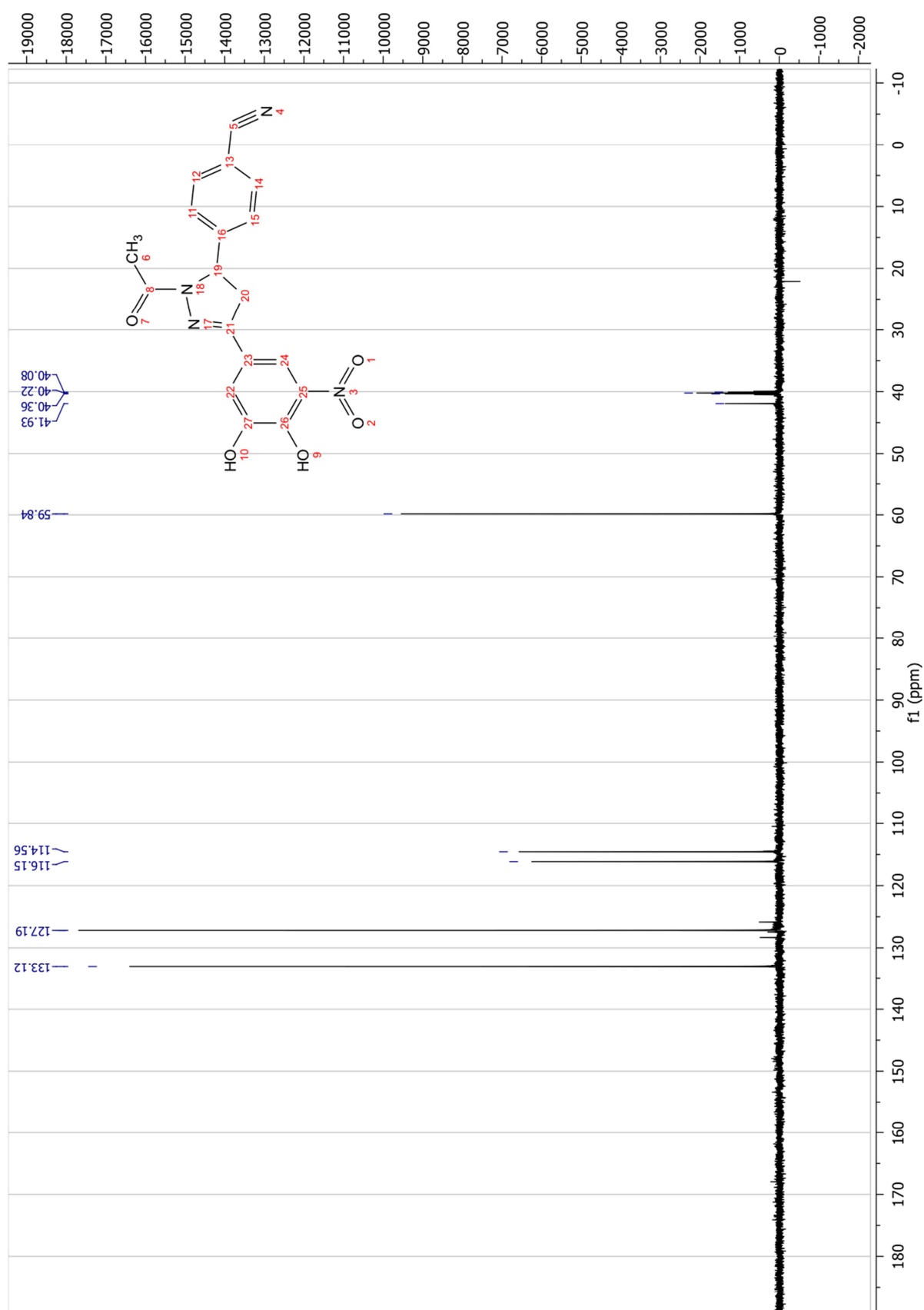


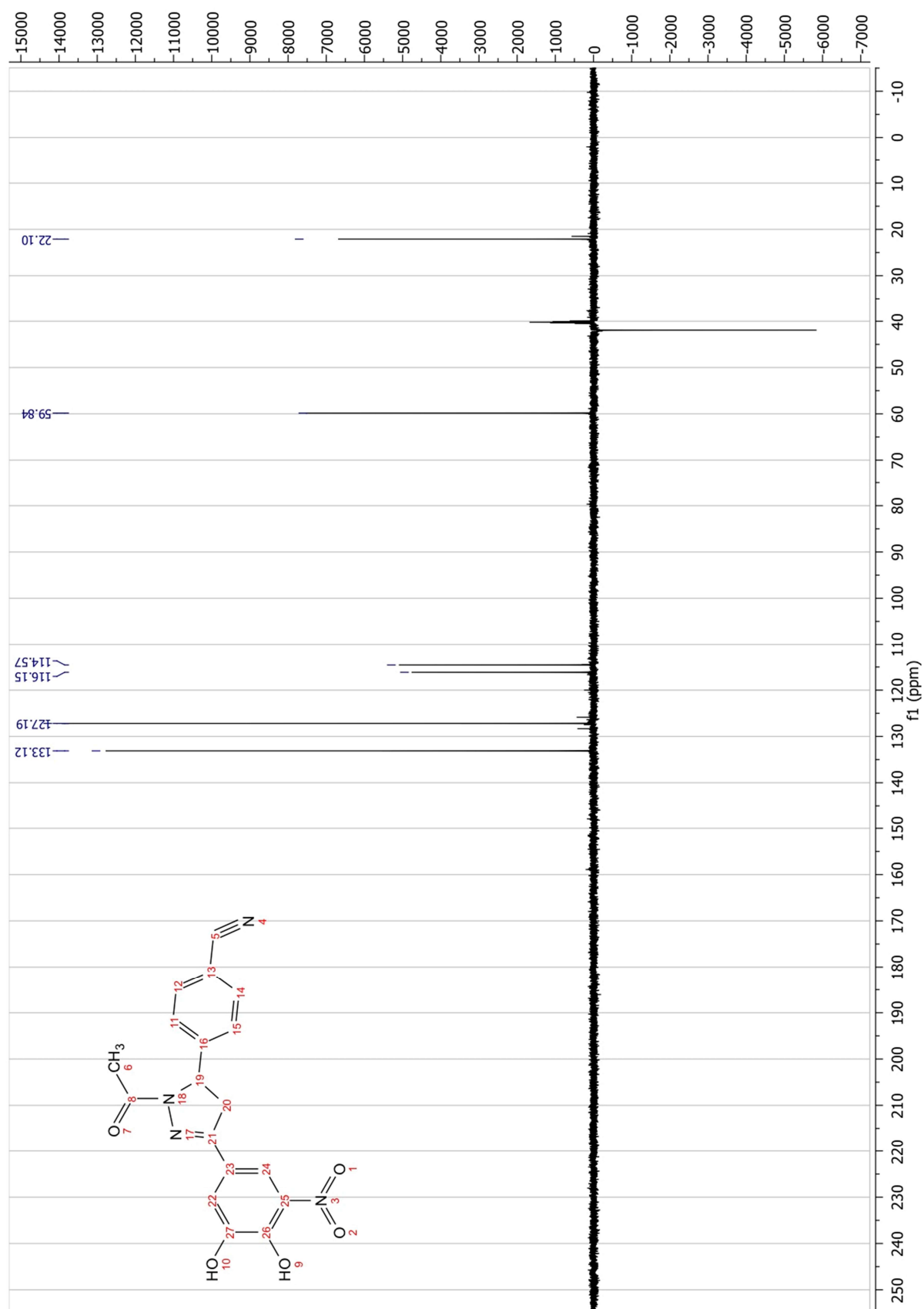


4-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile (5)

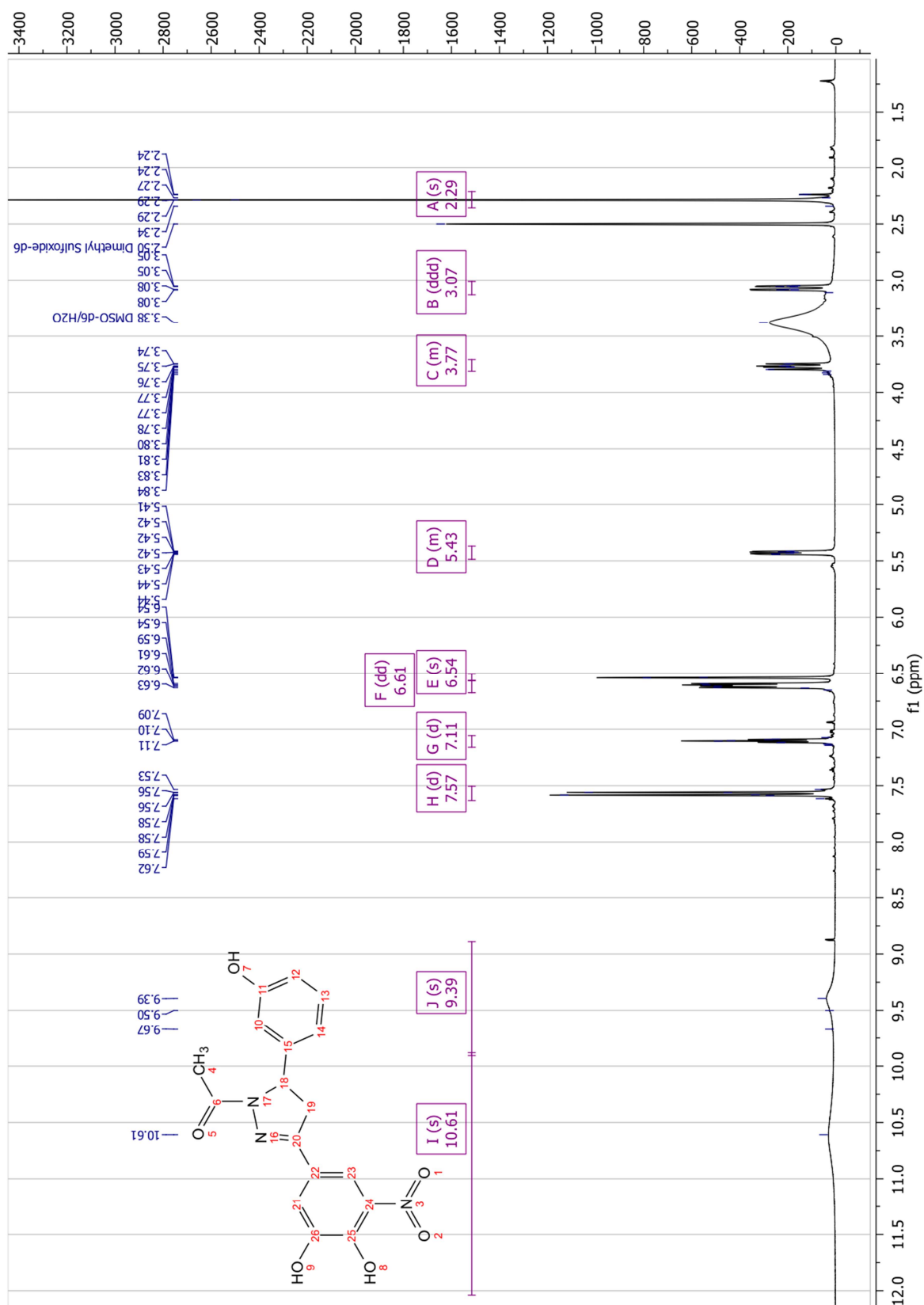


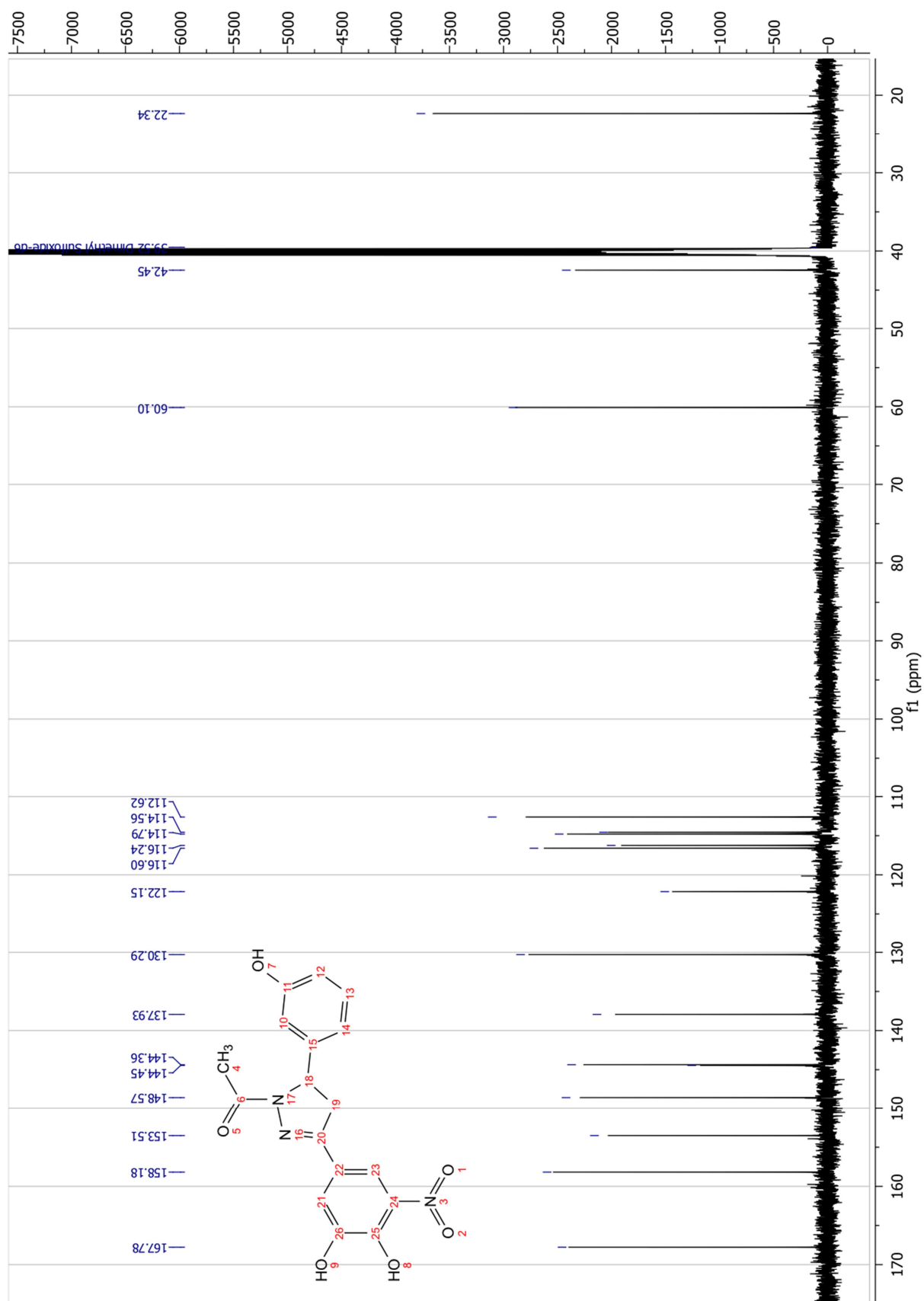


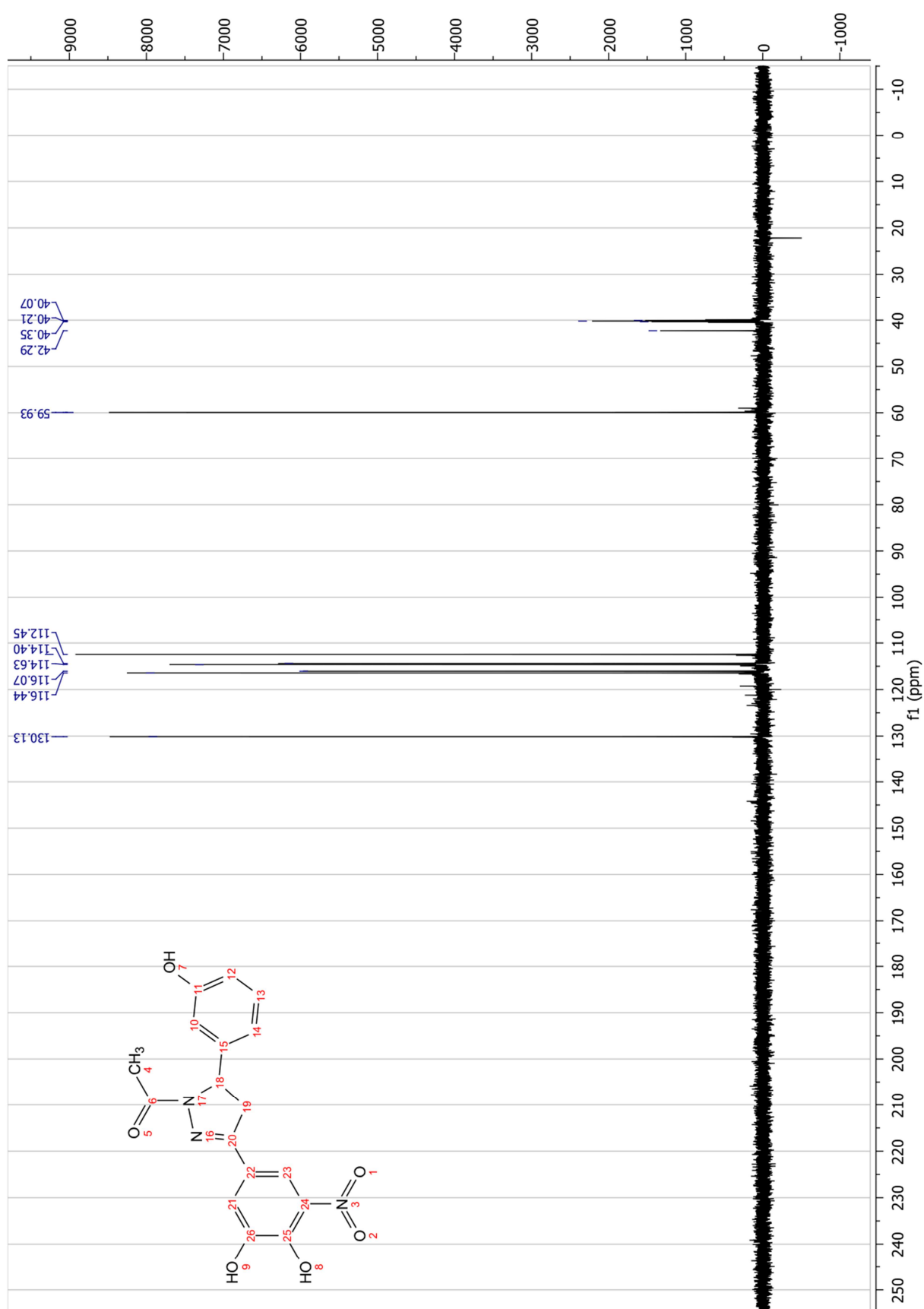


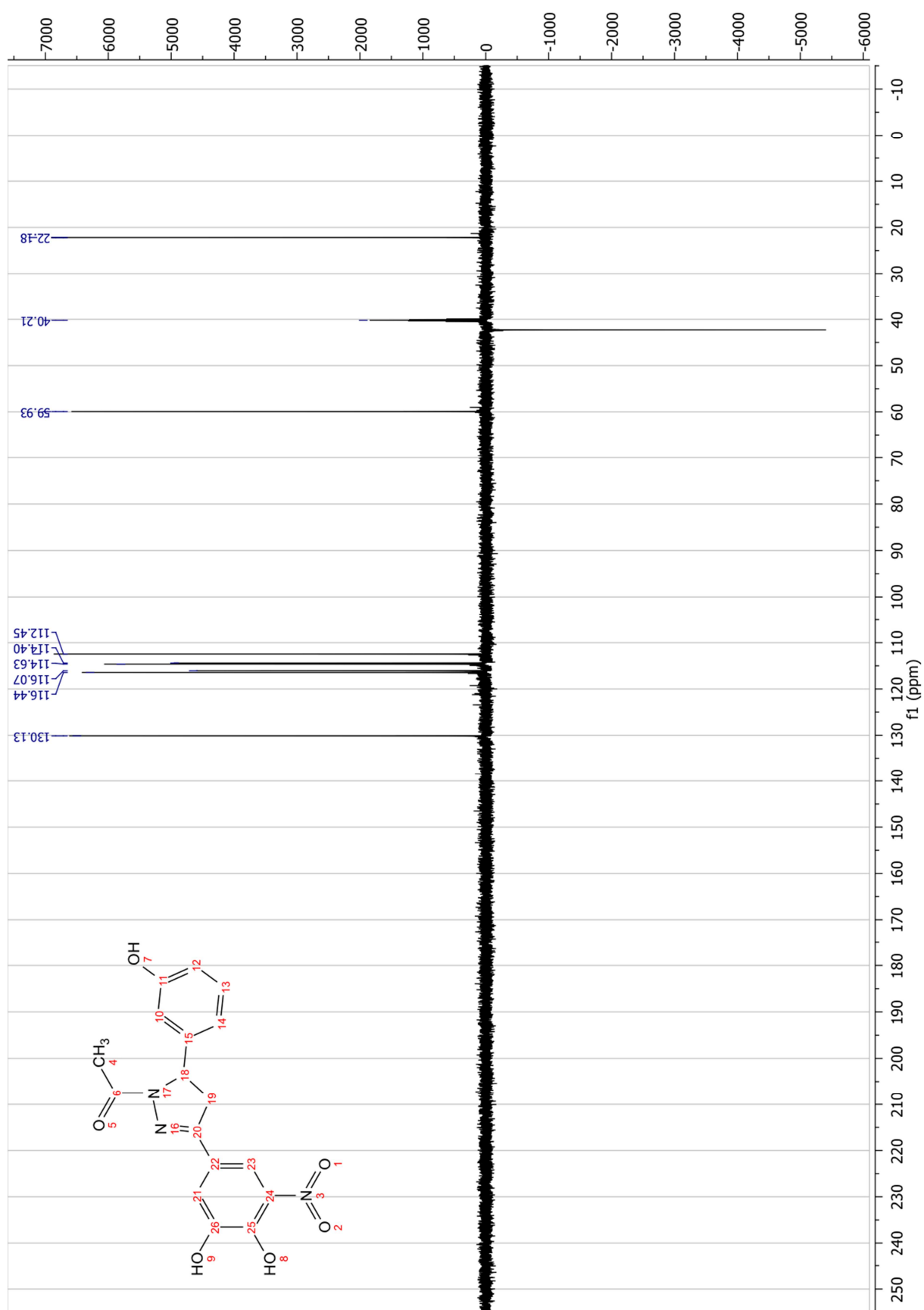


1-[3-(3,4-Dihydroxy-5-nitrophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl]ethenone (6)





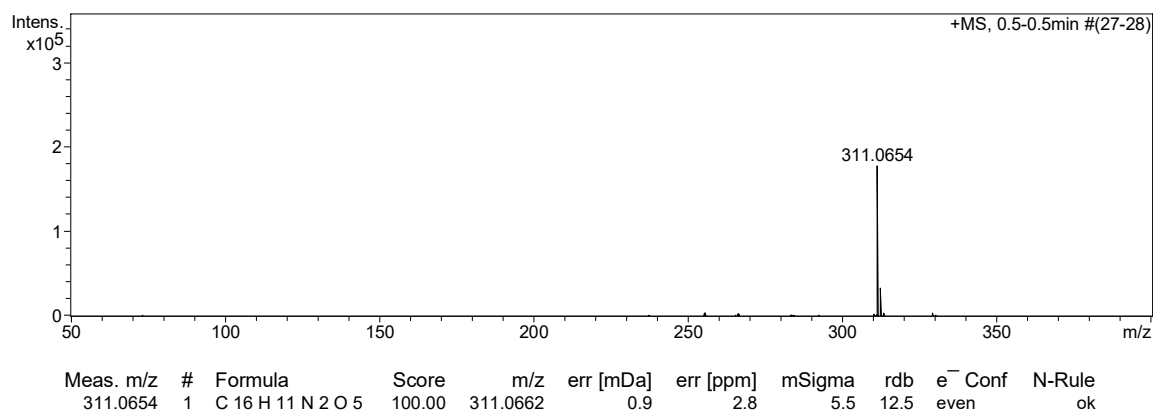




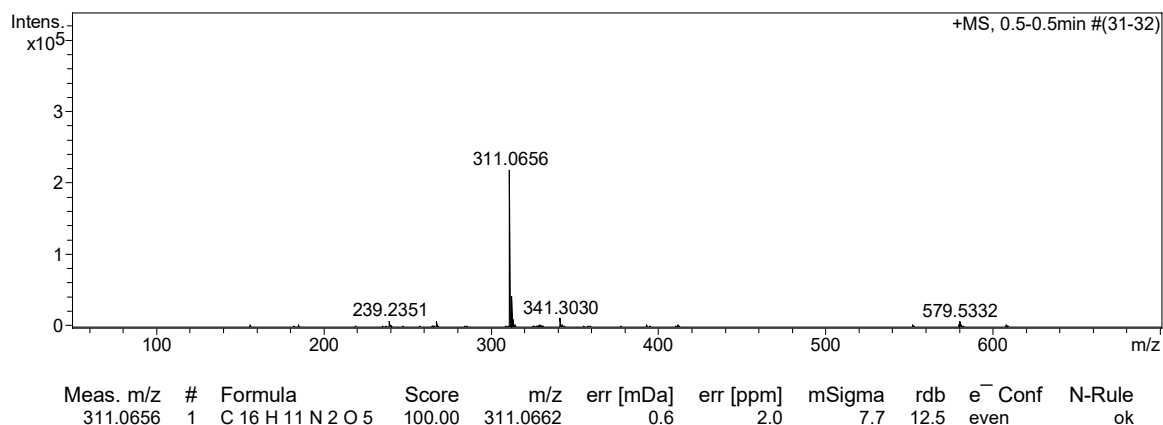
APPENDIX B

MASS SPECTRAL DATA

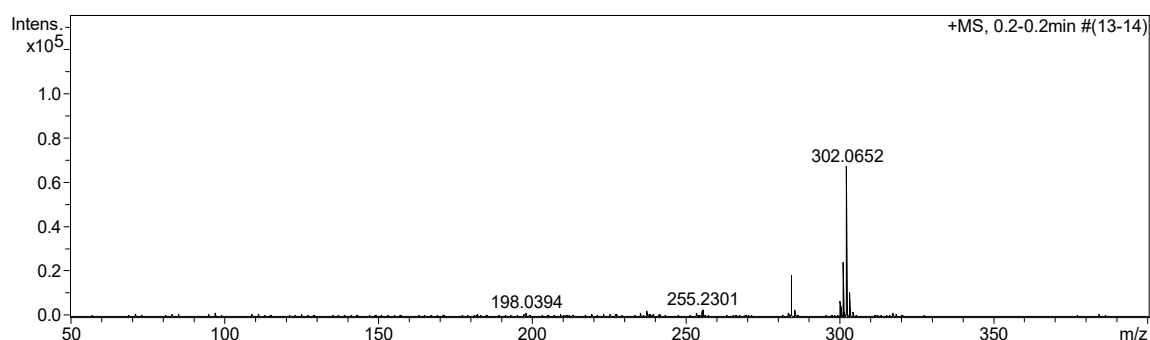
3-[(1E)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (1)



4-[(1E)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (2)

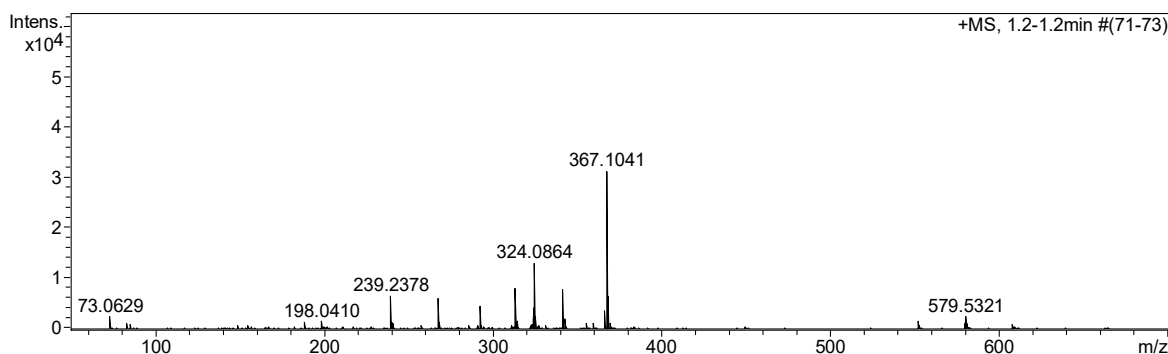


(2E)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one (3)



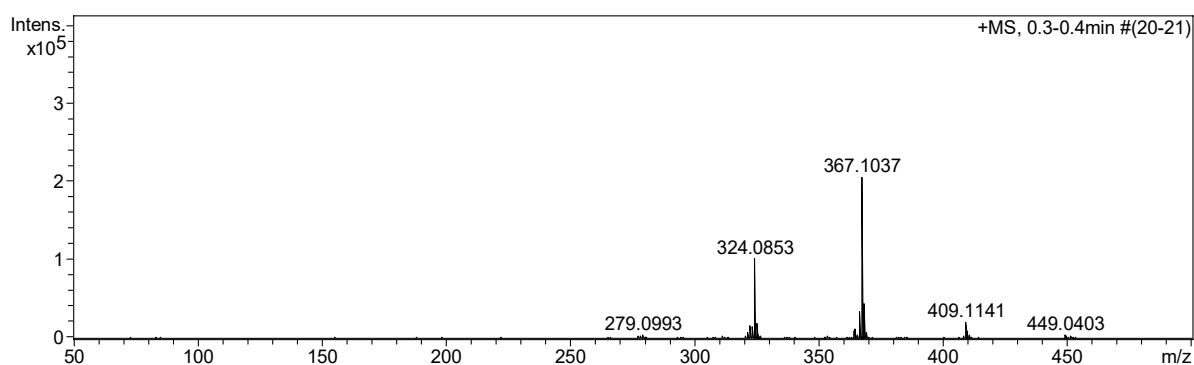
Meas. m/z	#	Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdB	e ⁻ Conf	N-Rule
284.0548	1	C 15 H 10 N O 5	100.00	284.0553	2.1	2.2	1.9	11.5	even	ok
	2	C 19 H 8 O 3	0.01	284.0468	-28.0	-27.8	21.0	16.0	odd	ok
	3	C 22 H 6 N	0.45	284.0495	-18.6	-18.4	41.9	20.5	even	ok
	4	C 23 H 8	0.01	284.0621	25.7	25.8	46.1	20.0	odd	ok
302.0652	1	C 15 H 12 N O 6	100.00	302.0659	2.2	2.7	3.0	10.5	even	ok
	2	C 19 H 10 O 4	0.01	302.0574	-26.1	-25.4	25.8	15.0	odd	ok
	3	C 22 H 8 N O	0.47	302.0600	-17.2	-16.5	46.7	19.5	even	ok
	4	C 23 H 10 O	0.01	302.0726	24.4	25.1	50.8	19.0	odd	ok

3-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile (4)



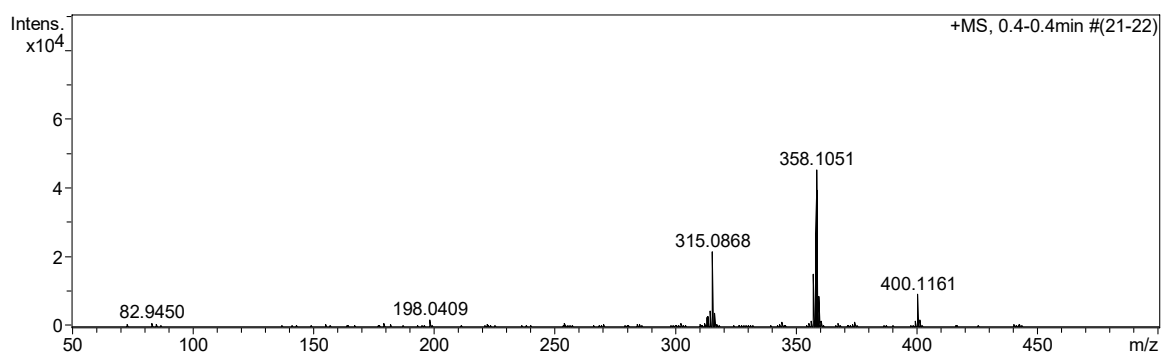
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdB	e ⁻ Conf	N-Rule
239.2378	1	C 16 H 31 O	100.00	239.2369	-0.8	-3.5	14.2	1.5	even	ok
	2	C 14 H 29 N 3	34.36	239.2356	-2.2	-9.1	22.4	2.0	odd	ok
267.2683	1	C 18 H 35 O	100.00	267.2682	-0.1	-0.4	8.1	1.5	even	ok
	2	C 16 H 33 N 3	43.52	267.2669	-1.4	-5.4	14.3	2.0	odd	ok
292.0281	1	C 19 H 4 N 2 O 2	100.00	292.0267	-1.3	-4.6	47.2	19.0	odd	ok
	2	C 24 H 4	17.64	292.0308	2.7	9.2	68.9	23.0	odd	ok
313.2739	1	C 17 H 35 N 3 O 2	49.34	313.2724	-1.5	-4.9	2.5	2.0	odd	ok
	2	C 19 H 37 O 3	100.00	313.2737	-0.2	-0.6	4.3	1.5	even	ok
	3	C 22 H 35 N	15.51	313.2764	2.5	7.9	25.0	6.0	odd	ok
324.0864	1	C 18 H 14 N O 5	100.00	324.0866	0.2	0.6	2.1	12.5	even	ok
	2	C 16 H 12 N 4 O 4	59.89	324.0853	-1.1	-3.5	4.5	13.0	odd	ok
	3	C 21 H 12 N 2 O 2	10.83	324.0893	2.9	8.9	22.9	17.0	odd	ok
341.3057	1	C 24 H 39 N	49.60	341.3077	2.0	5.9	6.8	6.0	odd	ok
	2	C 21 H 41 O 3	100.00	341.3050	-0.7	-2.0	15.0	1.5	even	ok
	3	C 19 H 39 N 3 O 2	37.06	341.3037	-2.0	-5.9	21.7	2.0	odd	ok
367.1041	1	C 18 H 15 N 4 O 5	100.00	367.1037	-0.4	-1.0	3.1	13.5	even	ok
	2	C 23 H 15 N 2 O 3	4.63	367.1077	3.6	9.9	30.3	17.5	even	ok

4-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile (5)



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e ⁻	Conf	N-Rule
324.0853	1	C ₁₆ H ₁₂ N ₄ O ₄	100.00	324.0853	-0.0	-0.1	4.4	13.0	odd		ok
	2	C ₁₈ H ₁₄ N ₄ O ₅	46.14	324.0866	1.3	4.1	10.8	12.5	even		ok
367.1037	1	C ₁₈ H ₁₅ N ₄ O ₅	100.00	367.1037	-0.0	-0.0	1.7	13.5	even		ok

1-[3-(3,4-Dihydroxy-5-nitrophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethenone (6)

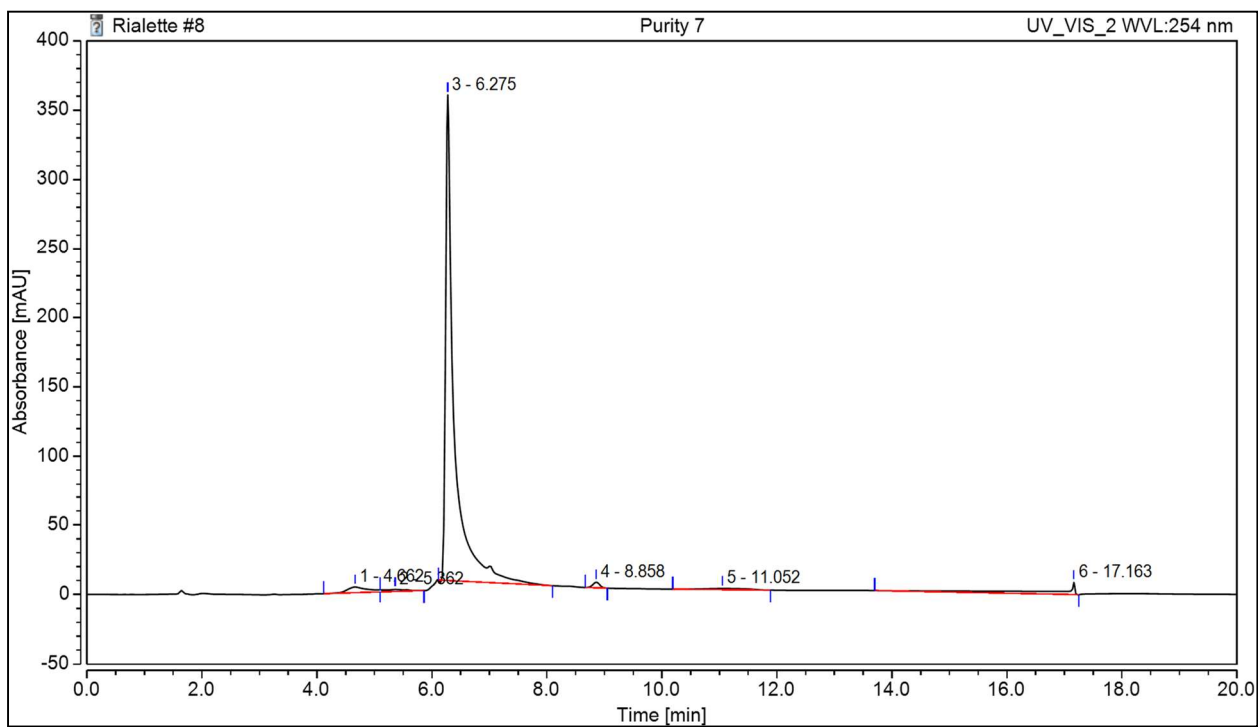


Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e ⁻	Conf	N-Rule
315.0868	1	C ₁₅ H ₁₃ N ₃ O ₅	45.28	315.0850	-1.8	-5.7	3.7	11.0	odd		ok
	2	C ₁₇ H ₁₅ O ₆	100.00	315.0863	-0.4	-1.4	3.9	10.5	even		ok
	3	C ₁₈ H ₁₁ N ₄ O ₂	60.00	315.0877	0.9	2.8	18.8	15.5	even		ok
358.1051	1	C ₁₇ H ₁₆ N ₃ O ₆	100.00	358.1034	-1.7	-4.9	0.9	11.5	even		ok
400.1161	1	C ₁₇ H ₁₆ N ₆ O ₆	13.67	400.1126	-3.5	-8.8	4.5	13.0	odd		ok
	2	C ₁₉ H ₁₈ N ₃ O ₇	44.43	400.1139	-2.2	-5.4	8.8	12.5	even		ok
	3	C ₂₁ H ₂₀ O ₈	100.00	400.1153	-0.8	-2.0	13.0	12.0	odd		ok
	4	C ₂₀ H ₁₄ N ₇ O ₃	79.81	400.1153	-0.8	-2.1	23.7	17.5	even		ok
	5	C ₂₂ H ₁₆ N ₄ O ₄	81.08	400.1166	0.5	1.3	29.6	17.0	odd		ok
	6	C ₁₂ H ₂₂ N ₃ O ₁₂	6.03	400.1198	3.7	9.3	32.4	3.5	even		ok
	7	C ₂₄ H ₁₈ N ₅ O	30.69	400.1179	1.9	4.7	35.8	16.5	even		ok
	8	C ₂₃ H ₁₂ N ₈	24.36	400.1179	1.9	4.6	44.4	22.0	odd		ok
	9	C ₂₅ H ₁₄ N ₅ O	6.31	400.1193	3.2	8.0	50.5	21.5	even		ok

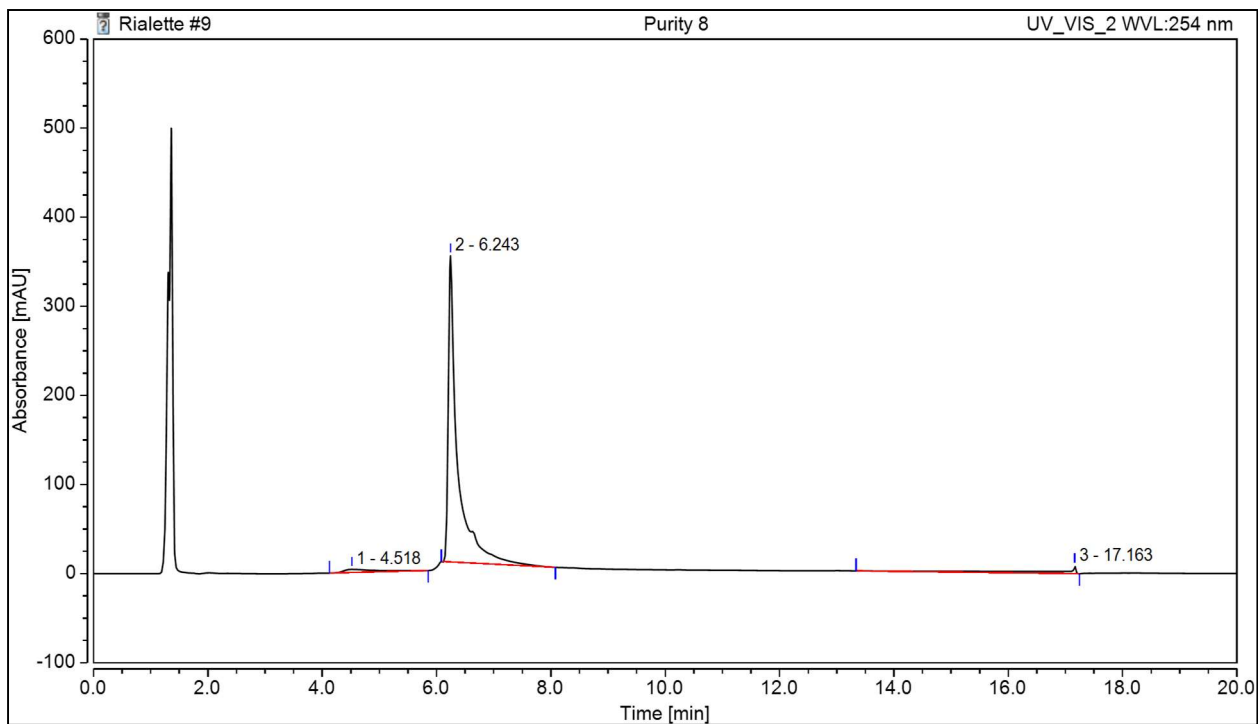
APPENDIX C

HPLC SPECTRAL DATA

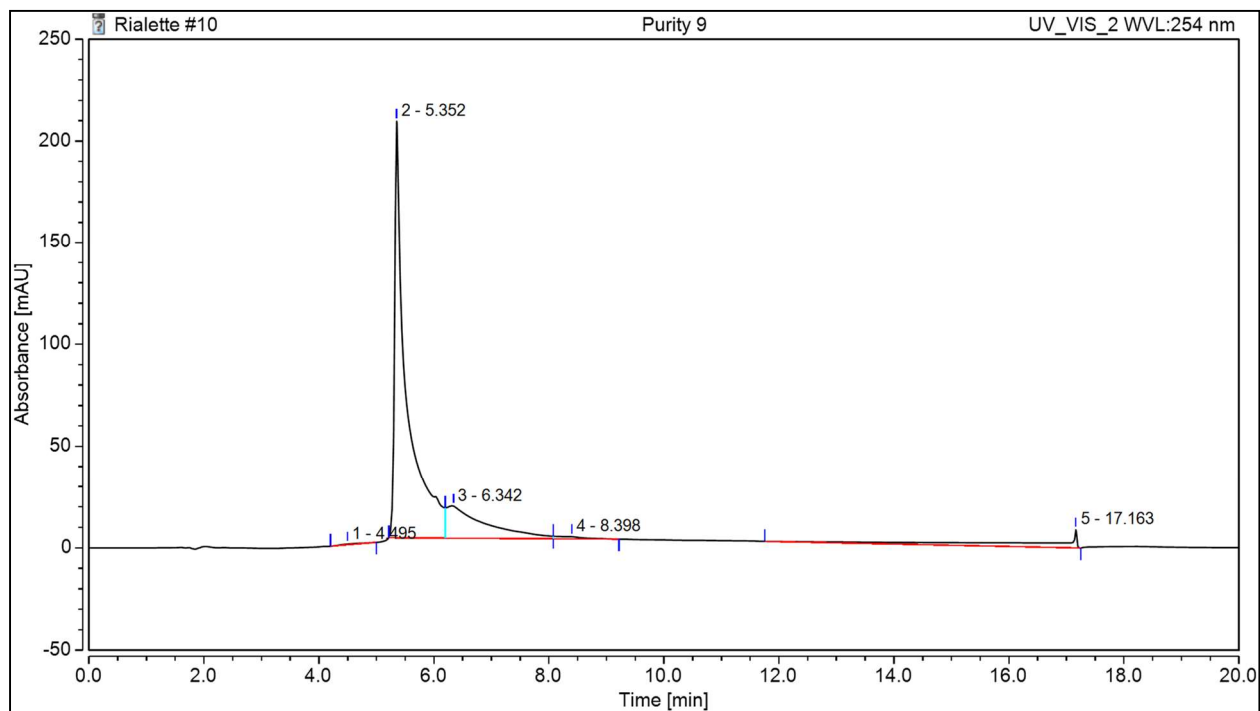
3-[(1*E*)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (1)



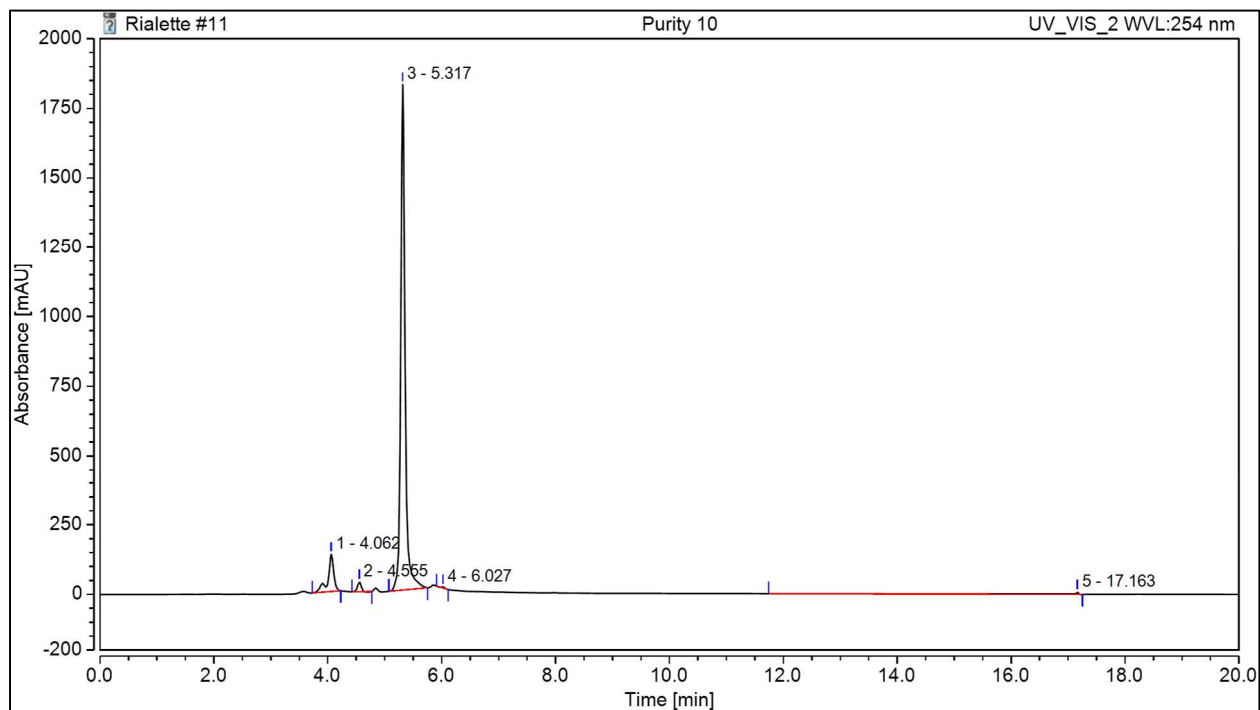
4-[(1*E*)-3-(3,4-Dihydroxy-5-nitrophenyl)-3-oxoprop-1-en-1-yl]benzonitrile (2)



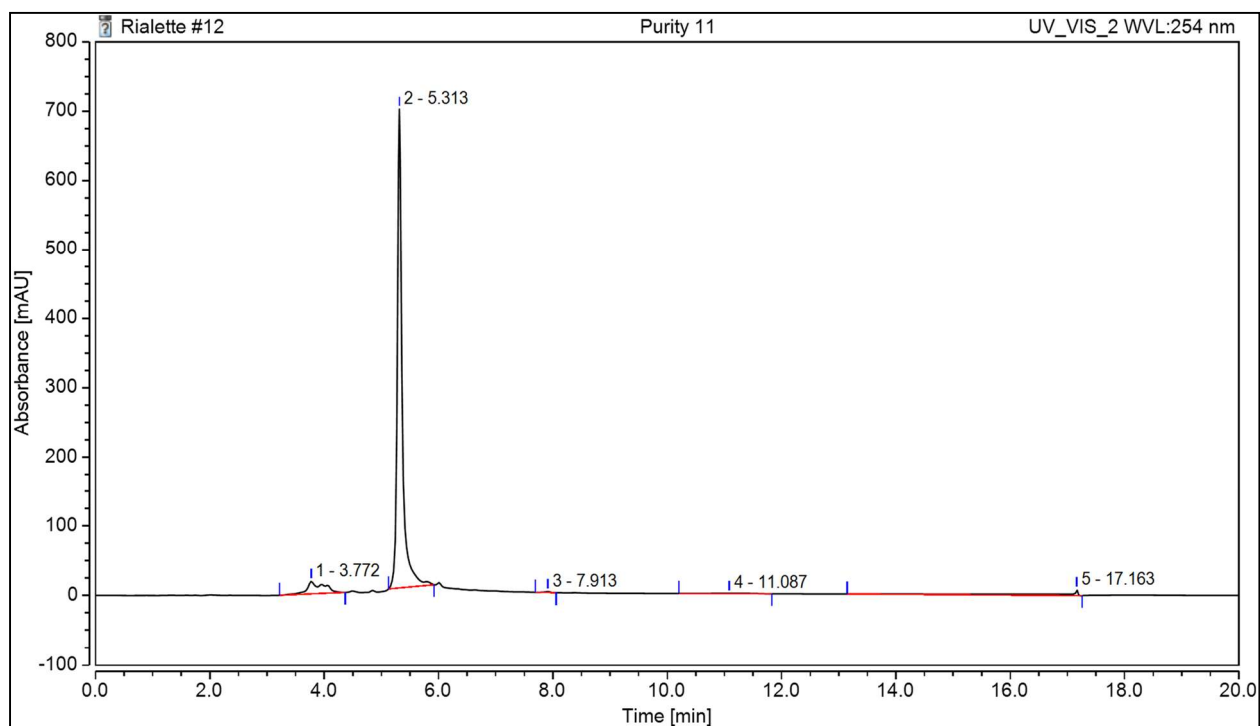
(2E)-1-(3,4-Dihydroxy-5-nitrophenyl)-3-(3-hydroxyphenyl)prop-2-en-1-one (3)



3-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile (4)



4-[1-Acetyl-3-(3,4-dihydroxy-5-nitrophenyl)-4,5-dihydro-1H-pyrazol-5-yl]benzonitrile (5)



1-[3-(3,4-Dihydroxy-5-nitrophenyl)-5-(3-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]ethenone (6)

