THE MONOAMINE OXIDASE B SUBSTRATE PROPERTIES OF SELECTED PYRROLINYL ANALOGUES

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Thesis submitted in fulfillment of the requirements for the degree Philosophiae Doctor in Pharmaceutical Chemistry, at the North-West University, Potchefstroom Campus, South Africa

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September 2008
Potchefstroom

This thesis is dedicated to the great Lord, Who is my Savior, and leads me through life, holding my hand every moment of every day.

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DECLARATION

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

I, Anél Pretorius, hereby declare that the dissertration with the title: THE MONOAMINE OXIDASE B SUBSTRATE PROPERTIES OF SELECTED PYRROLINYL ANALOGUES is my own work and has not been submitted at any other University either in whole or in part.

Signed at Potchefstroom on the 3rd November, 2008.

Anél Pretorius

November, 2008

LIST OF ABBREVIATIONS AND ACRONYMS

A	
AD	Alzheimer's disease
ATP	Adenosine triphosphate
B	
BBB	Blood-brain barrier
BPAO	Bovine plasma amine oxidase
C	
CNS	Central nervous system
D	
DA	Dopamine
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
DOPAC	Dihydroxyphenylacetic acid
E	
ECD	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid
F	
FAD	Flavine adenine dinucleotide
G	
GABA	Gamma-aminobutyric acid
GPO	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide

H

H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography

I

Ile	Isoleucine

K

K_m	The Michaelis constant

L

LBs	Lewy Bodies
L-DOPA	L-dihydroxyphenylalanine
Leu	Leucine

M

MAO	Monoamine oxidase
MOM	Mitochondrial outer membrane
$MPDP^+$	1-Methyl-4-phenyl-2,3-dihydropyridinium
MPP^+	1-Methyl-4-phenyl-pyridinium
MPPP	1-Methyl-4-phenyl-propionoxypiperidine
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

N

NAD	Nicotinamide adenine dinucleotide
NAD^+	Oxidised form of nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate

P

PD	Parkinson's disease

Q

Q	Ubiquinone
QSAR	Quantitative structure-activity relationship

R

ROS	Reactive oxygen species

S

SEM	Standard error of the mean
6-OHDA	6-Hydroxydopamine
SNpc	Substantia nigra pars compacta
[S]	Substrate

T

TPQ	2,4,5-Trihydroxyphenylalanine quinone
Tyr	Tyrosine

U

UCH-L1	Ubiquitin C-terminal hydrolase L1

V

v_i	Initial velocity
VMAT	Vesicular monoamine transporter
VMAT2	Vesicular monoamine transporter-2
V_{max}	Maximum velocity
VTA	Ventral tegmental area

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ABSTRACT

The parkinsonian inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is a cyclic tertiary allylamine that is oxidized by monoamine oxidase B (MAO-B) at the ring α-carbon to yield the 1-methyl-4-phenyl-2,3-dihydropyridinium species ($MPDP^+$). 1-Methyl-3-phenyl-3-pyrroline, another cyclic tertiary allylamine and 5-membered ring MPTP analogue, is also oxidized by MAO-B to yield the 2-electron oxidation product, 1-methyl-3-phenylpyrrole. By using the corresponding pyrroline-2,2,4,5,5-d_5 analogues as the deuterated substrates, the kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the steady-state oxidation by baboon liver MAO-B was determined for 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-fluorophenyl)-3-pyrroline. The isotope effects for the two substrates were 4.29 and 3.98 on V_{max}, and 5.71 and 3.37 on V_{max}/K_m, respectively. The values reported for the oxidation of MPTP by bovine liver MAO-B with MPTP-6,6-d_2 as deuterated substrate are 3.55 for $^D(V_{max})$ and 8.01 for $^D(V_{max}/K_m)$. We conclude that the mechanism of the MAO-B-catalyzed oxidation of pyrrolinyl substrates is similar to that of the tetrahydropyridinyl substrates and that a carbon-hydrogen bond cleavage step is, at least partially, rate-determining.

The saturated piperidinyl analogue of MPTP is not a MAO-B substrate; therefore it appears that the α-carbon 2-electron oxidation of cyclic tertiary aminyl substrates by MAO-B is dependent on the presence of the allylic π-bond. The 3,4-cyclopropyl analogue of MPTP, 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane, is the only known saturated, cyclic tertiary amine substrate of MAO-B. As part of our ongoing investigation into the MAO-B substrate properties of 5-membered azacyclic systems, we have examined the interactions of the corresponding saturated pyrrolidinyl (1-methyl-3-phenylpyrrolidine) and 3,4-cyclopropyl (3-methyl-1-phenyl-3-azabicyclo[3.1.0]-hexane) analogues of 1-methyl-3-phenyl-3-pyrroline with MAO-B. We have also examined the possibility that these saturated substrates may act as neurotoxins in the C57BL/6 mouse. The results document that both the pyrrolidinyl [K_m = 234 μM; V_{max} = 8.37 nmol/(min-mg mitochondrial protein)] and the 3,4-cyclopropyl analogues [K_m = 148 μM; V_{max} = 16.9 nmol/(min-mg mitochondrial protein)] are efficiently oxidized by baboon liver MAO-B. Since striatal dopamine

depletion was not observed while analogous treatment with MPTP resulted in 64-73% depletion, it can be concluded that, unlike MPTP, the cyclic tertiary amines examined here are not nigrostriatal toxins.

OPSOMMING

Die sikliese tersiêre allielamien, 1-metiel-4-feniel-1,2,3,6-tetrahidropiridien (MPTP), induseer parkinsonisme in beide mense en proefdiere. Monoamienoksidase B (MAO-B) oksideer die ring α-koolstof van MPTP om 1-metiel-4-feniel-2,3-dihidropiridinium ($MPDP^+$) te vorm. 'n Ander sikliese tersiêre allielamien, die 5-lid ring analoog van MPTP, 1-metiel-3-feniel-3-pirrolien word ook deur MAO-B geoksideer om die 2-elektron-oksidasieproduk, 1-metiel-3-fenielpirrool te vorm. Ons het die kinetiese deuterium-isotoopeffekte op V_{maks} en V_{maks}/K_m vir die gelykvlak oksidasietoestand van 1-metiel-3-feniel-3-pirrolien en 1-metiel-3-(4-fluorofeniel)-3-pirrolien met bobbejaanlewer MAO-B bepaal deur van die ooreenstemmende pirrolien-2,2,4,5,5-d_5 analoë gebruik te maak. Die isotoopeffekte vir die twee substrate was 4.29 en 3.98 vir V_{maks} en 5.71 en 3.37 vir V_{maks}/K_m, onderskeidelik. Die literatuurwaardes vir die oksidasie van MPTP bepaal met beeslewer MAO-B, met MPTP-6,6-d_2 as gedeutereerde substraat, is 3.55 vir $^D(V_{maks})$ en 8.01 for $^D(V_{maks}/K_m)$. Ons maak die gevolgtrekking dat die meganisme van die MAO-B gekataliseerde oksidasie van die pirroliniel-substrate soortgelyk is aan dié van die tetrahidropiridiniel-substrate en dat die breking van die koolstof-waterstofbinding, 'n belangrike faktor in die snelheidsbepaling van die reaksie is.

Die bevinding, dat die versadigde piperidinielanaloog van MPTP nie 'n MAO-B substraat is nie, dui daarop dat die α-koolstof, 2-elektronoksidasie van sikliese tersiêre amiensubstrate deur MAO-B afhanklik is van die teenwoordigheid van die alliliese π-binding. Die 3,4-siklopropielanaloog van MPTP, 3-metiel-6-feniel-3-asabisiklo[4.1.0]heptaan, is die enigste bekende versadigde, sikliese tersiêre allielamien wat 'n MAO-B-substraat is. As deel van ons belangstelling in die MAO-B-substraateienskappe van 5-lid asasikliese verbindings, het ons die interaksies van die ooreenstemmende versadigde pirrolidiniel- (1-metiel-3-fenielpirrolidien-) en 3,4-siklopropiel- (3-metiel-1-feniel-3-asabisiklo[3.1.0]heksaan-) analoë van 1-metiel-3-feniel-3-pirrolien met MAO-B ondersoek. Ons het ook die moontlikheid ondersoek dat hierdie versadigde substrate neurotoksies kan wees vir die C57BL/6 muis. Die resultate toon dat beide die pirrolidiniel- [K_m = 234 μM; V_{maks} = 8.37 nmol/(min-mg

mitochondriale proteien)] en die 3,4-siklopropiel-analoë [K_m = 148 µM; V_{maks} = 16.9 nmol/(min-mg mitochondriale proteien)] doeltreffend deur bobbejaanlewer MAO-B geoksideer word. Hierdie substrate het ook nie striatale dopamienuitputting in die C57BL/6 muis veroorsaak nie, terwyl behandeling met MPTP tot 64-73% vermindering gelei het. Hieruit kan afgelei word dat, in teenstelling met MPTP, die sikliese tersiêre amiene wat hier ondersoek is, nie neurotoksies is nie.

ACKNOWLEDGEMENTS

Throughout the completion of this study, much required help was obtained during the research and experimental analyses. I would like to take the opportunity to thank these remarkable people and institutions:

- Dr. J.P. Petzer for his assistance, guidance and demonstrating enormous patience.
- Prof. J.J. Bergh for his valuable advice and inputs.
- Dr. G. Terre'Blanche for her encouragement and willingness to help.
- Prof. J. du Preez and the staff of the Analytical Technology Laboratory, North-West University for their support and assistance.
- Prof. N. Castagnoli, Jr. who never hesitated to help us with valuable advice and inputs.
- To C. Bester and A. Fick at the Animal Research Centre, North-West University for sacrificing their time to assist me in my experimentation.
- To Judey Pretorius for her encouragement, support, and assistance whenever needed, right from day one.
- André Joubert, Johan Jordaan and Louis Fourie of the SASOL Centre for Chemistry, North-West University for the NMR and MS spectra.
- The National Research Foundation and the Medical Research Council, South Africa for the financial assistance.
- My parents, Phillip and Marie Pretorius, and brothers, Stefan and Nico Pretorius, for their love, patience, advice, encouragement, and financial support, not only the past few years, but throughout my life. Thank you for being there when I needed you.

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Dear Sir/Madam

CO-AUTHORSHIP ON RESEARCH PAPERS

The undersigned, as co-authors of the research articles listed below, hereby give permission to Miss Anél Pretorius to submit the papers as part of the degree Ph.D in Pharmaceutical Chemistry at the North-West University, Potchefstroom Campus.

1. Deuterium isotope effects for the oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues by monoamine oxidase B
2. Interactions of 1-methyl-3-phenylpyrrolidine and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane with monoamine oxidase B

Yours sincerely,

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Chapter 1
INTRODUCTION

Amine oxidations are important in a number of basic biological processes ranging from lysyl oxidation in the cross-linking of collagen to the degradative metabolism of polyamines and neurotransmitters. Biogenic amine oxidations are catalyzed by either the quinoprotein class of enzymes (usually primary amines) or by the flavin-containing amine oxidases (primary, secondary or tertiary amines), to the corresponding imines, with molecular oxygen as electron acceptor and hydrogen peroxide as reaction product formed (Binda *et al.*, 2002b). Via the oxidative cleavage of the α-CH bond of the substrate, amines are oxidized by flavin amine oxidases (flavoenzymes) to form an imine product and reduced flavin cofactor. The imine product is then hydrolyzed (non-enzymatically) to the corresponding aldehyde and ammonia (or imine for secondary and tertiary amine substrates) and the reduced flavin coenzyme reacts with oxygen to form hydrogen peroxide and the oxidized form of the flavin to complete the catalytic cycle (figure 1) (Binda *et al.*, 2002b).

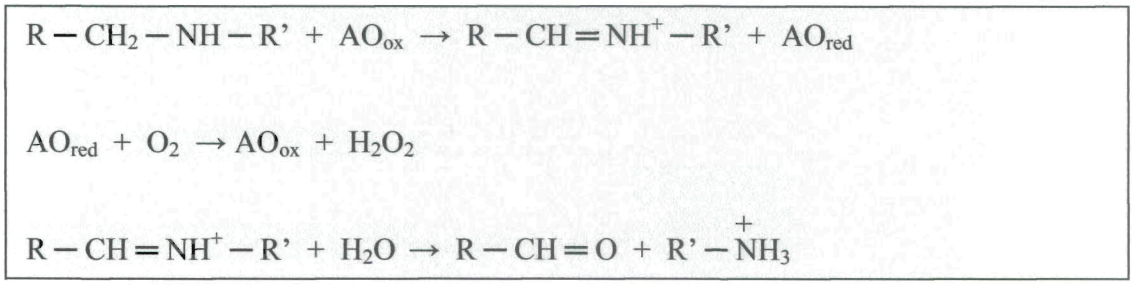

Figure 1. *A general amine oxidation reaction catalyzed by MAO (Binda et al., 2002b).*

Monoamine oxidases (MAOs) are present in two isoforms, MAO-A and MAO-B (FAD-dependent enzymes), and are responsible for the metabolism of neurotransmitters such as dopamine, serotonin, adrenaline, and noradrenaline and for the inactivation of exogenous arylalkyl amines (oxidize the primary amino groups of arylalkyl amines)

(Binda *et al.*, 2002b; Binda *et al.*, 2007). MAO-A and MAO-B share over 70% sequence identity but exhibit different substrate and inhibitor specificities (Binda *et al.*, 2002b). Serotonin is preferentially metabolized by MAO-A and dopamine by MAO-B, whereas selegiline [(R)-deprenyl] and clorgyline selectively inhibit MAO-B and MAO-A, respectively (Binda *et al.*, 2007). MAOs are bound to the mitochondrial outer membrane (MOM) and have an FAD molecule covalently bound to the protein via an 8α-thioether linkage to a cysteinyl residue. They are expressed in both a tissue-dependent and age-dependent manner (Binda *et al.*, 2002b). Due to their central role in neurotransmitter metabolism, these enzymes represent attractive drug targets in the pharmacological therapy of neurodegenerative diseases and depression (Binda *et al.*, 2002b; Binda *et al.*, 2007).

MAO-B is the principal catalyst responsible for the metabolic activation (figure 2) of the proneurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (**1**), which is oxidized at C-6 of the tetrahydropyridinyl ring (Chiba *et al.*, 1984; Heikkila *et al.*, 1984; Weisman *et al.*, 1985), to yield the α-carbon 2-electron oxidation product and the conjugated eniminium metabolite, 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) (**2**). This is followed by a second 2-electron oxidation, to form the ultimate neurotoxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (**3**) (Chiba *et al.*, 1984; Weisman *et al.*, 1985; Ramsay *et al.*, 1991; Markey *et al.*, 1984), a process that appears to take place mainly in MAO-B rich glial cells (Takada *et al.*, 1990). MPP^+ is thought to selectively damage nigrostriatal dopaminergic neurons and induces a parkinsonian syndrome in humans (Davis *et al.*, 1979; Langston *et al.*, 1983) and other susceptible mammals (Heikkila *et al.*, 1984; Nicklas *et al.*, 1985; Smeyne & Jackson-Lewis, 2005), by accumulating in nigrostriatal nerve terminals via the plasma membrane dopamine transporter (DAT). In the nerve terminals, MPP^+ localizes within the inner mitochondrial membrane and inhibits complex I of the mitochondrial respiratory chain, causing ATP depletion and oxidative stress (Singer *et al.*, 1988). This accumulation process is driven by the transmembrane electrochemical gradient and relies upon the presence of the permanent iminium charge of MPP^+ (Sayre *et al.*, 1990).

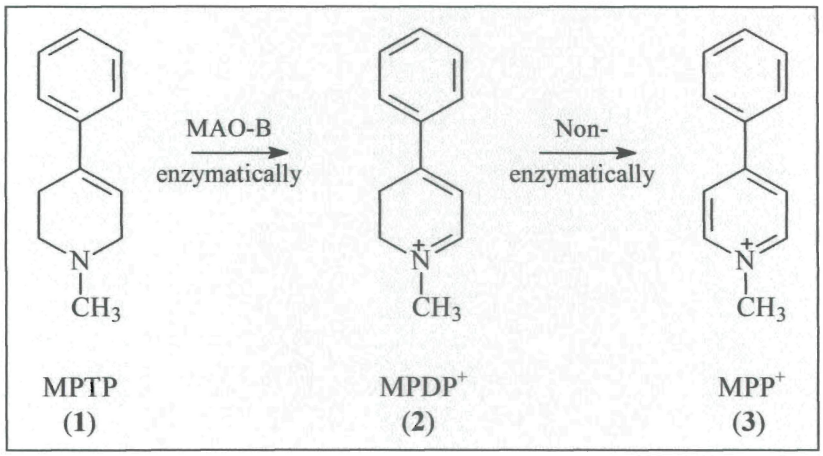

Figure 2. *The MAO-B catalyzed oxidation of MPTP (**1**).*

MPTP is the first reported cyclic tertiary allylamine that acts as a good MAO-B substrate (Chiba *et al.*, 1984). Several other tertiary aminyl substrates of MAO-B have since been described (Kalgutkar & Castagnoli, 1992; Rimoldi *et al.*, 2005). Another member of the cyclic tertiary allylamine class of MAO-B substrates is 1-methyl-3-phenyl-3-pyrroline (**8a**). This 5-membered ring analogue of MPTP has good and selective MAO-B substrate properties comparable to those of MPTP (Wang *et al.*, 1998; Ogunrombi *et al.*, 2007). The ring α-carbon oxidation of 1-methyl-3-phenyl-3-pyrroline (figure 3) results in the neutral 1-methyl-3-phenylpyrrole (**9**), an overall 2-electron process that most likely arises via the short-lived conjugate acid (**10**) of the pyrrolyl product (Ogunrombi *et al.*, 2007).

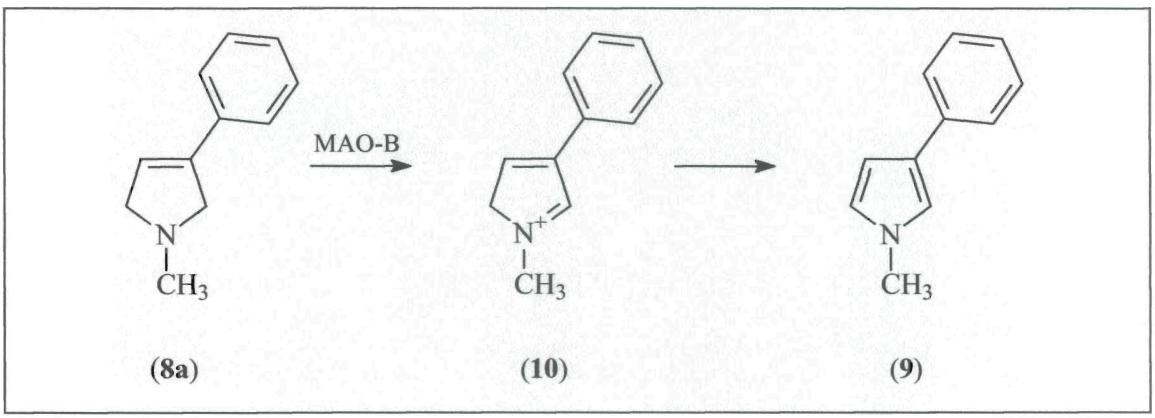

Figure 3. *The MAO-B catalyzed oxidation of 1-methyl-3-phenyl-3-pyrroline (**8a**).*

The substrate properties of the cyclic tertiary aminyl class of MAO-B substrates are thought to be dependent on the presence of the allylic π-bond. The 3,4-cyclopropyl analogue of MPTP (figure 4), 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (**6**), is the only known saturated, cyclic tertiary allylamine that acts as a good MAO-B substrate. This substrate is oxidized by MAO-B to yield the corresponding iminium metabolite, while the corresponding saturated piperidinyl analogue (**7**) of MPTP (figure 4) is devoid of MAO-B substrate properties (Heikkila *et al.*, 1985; Langston *et al.*, 1984; Rimoldi *et al.*, 2005).

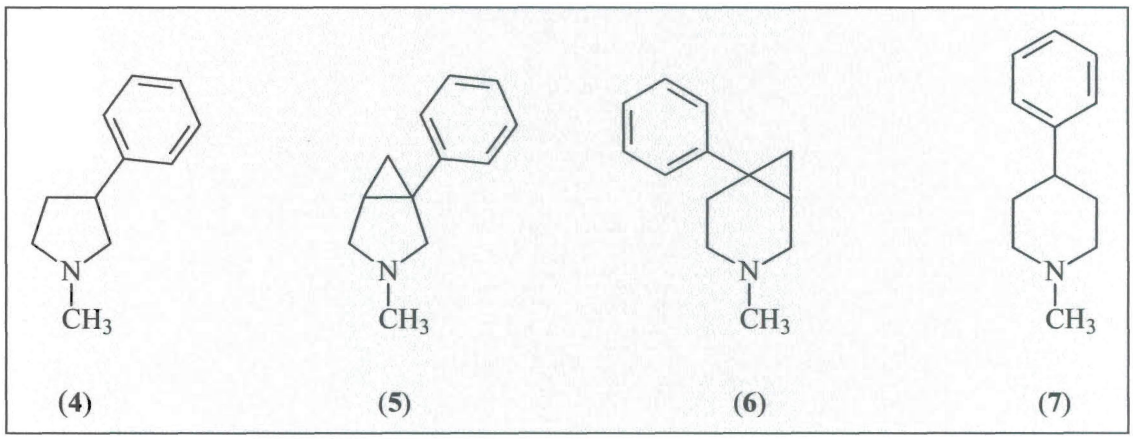

Figure 4. *The structures of the compounds: 1-methyl-3-phenylpyrrolidine (**4**), 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**5**), 3-methyl-6-phenyl-3-azabicyclo-[4.1.0]heptane (**6**) and the saturated piperidinyl analogue (**7**) of MPTP.*

It was established that only permanently charged MPP^+ analogues exhibit dopaminergic neurotoxic properties as determined by intrastriatal microdialysis studies (Rollema *et al.*, 1990; Ogunrombi *et al.*, 2007). Interestingly, 4-phenylpyridine, the neutral *N*-desmethylated form of MPP^+, is a much more potent inhibitor of complex I of the isolated respiratory chain than is MPP^+, but is not a physiological inhibitor of mitochondrial respiration, since it is not concentrated inside the mitochondria. Chronic exposure of mice to 4-phenylpyridine and 4-phenyl-1,2,3,6-tetrahydropyridine was found not to cause any reduction in striatal dopamine (Perry *et al.*, 1987). Based on these observations, 1-methyl-3-phenyl-3-pyrrolines and their MAO-B catalyzed pyrrolyl oxidation products should not be neurotoxic (Ogunrombi *et al.*, 2007). Experimental

animals treated with MPTP have become useful models for studying neurodegenerative processes. The depletion of striatal dopamine in C57BL/6 mice, measured 7 days after systemic injection (multiple or single doses) of MPTP, is indicative of the permanent loss of nigrostriatal dopaminergic cell bodies in the substantia nigra (Ogunrombi *et al.*, 2007).

The neurotoxic action of MPTP therefore depends upon its metabolic conversion by MAO-B to yield the charged pyridinium species, MPP^+ (Chiba *et al.*, 1984). The possibility exists that the saturated substrates 1-methyl-3-phenylpyrrolidine (**4**) and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**5**) may act as neurotoxins in the C57BL/6 mouse (figure 4) (Smeyne & Jackson-Lewis, 2005). The MAO-B catalyzed 2-electron oxidations of these substrates may yield charged intermediates and end products. In this study we will examine the possibility that 1-methyl-3-phenylpyrrolidine and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane could, similar to MPTP, be neurotoxic (Ogunrombi *et al.*, 2007).

Aims and objectives

1. In the present study we determined the steady-state kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the oxidation of 1-methyl-3-phenyl-3-pyrroline (**8a**) and 1-methyl-3-(4-fluorophenyl)-3-pyrroline (**8b**) by baboon liver mitochondrial MAO-B. For this purpose we employed the corresponding 1-methyl-3-phenyl-3-pyrrolinyl-2,2,4,5,5-d_5 (**8a-d_5**) and 1-methyl-3-(4-fluorophenyl)-3-pyrrolinyl-2,2,4,5,5-d_5 (**8b-d_5**) analogues as deuterated substrates (figure 5). The apparent isotope effects for the two substrates were compared to the isotope effects previously reported for the oxidation of MPTP by bovine liver mitochondrial MAO-B using MPTP-6,6-d_2 as deuterated substrate (Ottoboni *et al.*, 1989). The purpose of this section of the study was to determine whether a carbon-hydrogen bond cleavage step is rate-determining for the oxidation of pyrrolinyl substrates by MAO-B.

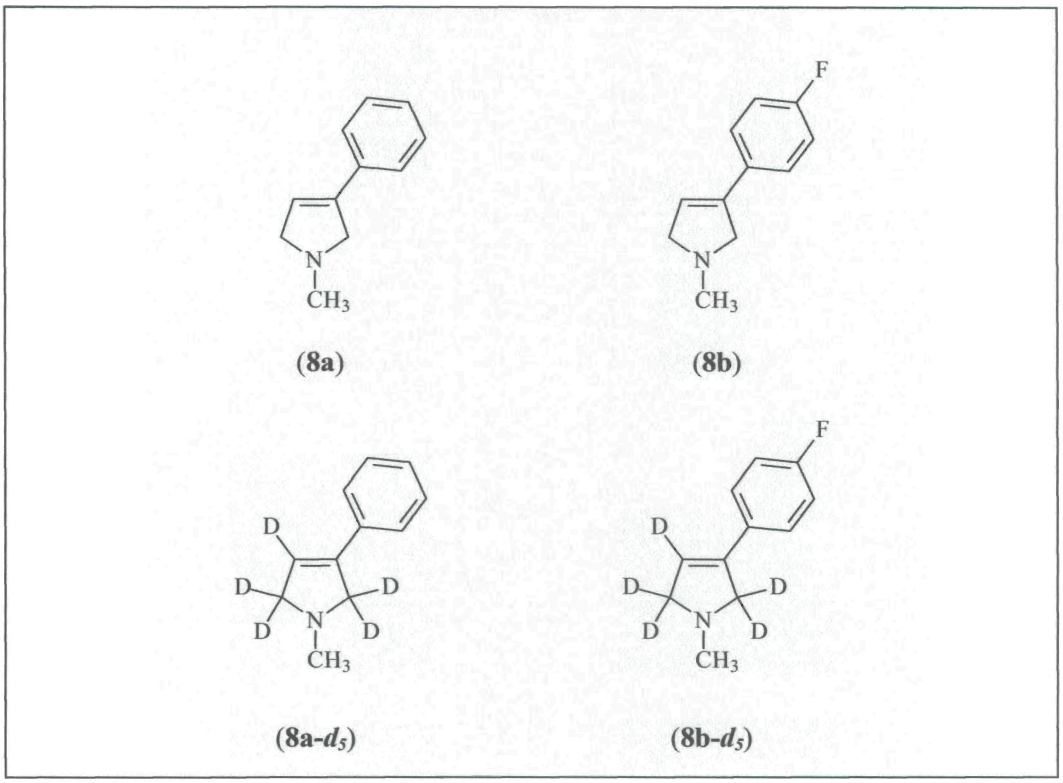

Figure 5. *The structures of 1-methyl-3-phenyl-3-pyrroline (**8a**) and 1-methyl-3-(4-fluorophenyl)-3-pyrroline (**8b**) and their deuterated analogues, 1-methyl-3-phenyl-3-pyrrolinyl-2,2,4,5,5-d_5 (**8a-d_5**) and 1-methyl-3-(4-fluorophenyl)-3-pyrrolinyl-2,2,4,5,5-d_5 (**8b-d_5**).*

2. As part of our ongoing investigation into the MAO-B substrate properties of 5-membered azacyclic systems, we examined the interactions of the corresponding saturated pyrrolidinyl (1-methyl-3-phenylpyrrolidine) (**4**) and 3,4-cyclopropyl (3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane) (**5**) analogues of 1-methyl-3-phenyl-3-pyrroline with MAO-B. In view of the π-bond characteristics of the cyclopropyl group it was expected that 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane would, analogous to the 3,4-cyclopropyl analogue of MPTP (3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane) (**6**), act as a good MAO-B substrate. On the other hand, the pyrrolidinyl (1-methyl-3-phenylpyrrolidine), analogous to the saturated piperidinyl analogue (**7**) of MPTP, was expected to be devoid of MAO-B substrate properties.

3. We examined the possibility that these saturated substrates (**4** and **5**) may act as neurotoxins in the C57BL/6 mouse (Smeyne & Jackson-Lewis, 2005). The neurotoxic action of MPTP is dependent upon its metabolic conversion by MAO-B to yield the charged pyridinium species, MPP^+ (Chiba *et al.*, 1984). Since the MAO-B catalyzed 2-electron oxidations of substrates 1-methyl-3-phenylpyrrolidine (**4**) and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**5**) may yield charged intermediates and end products, the possibility exists that these substrates could, similar to MPTP, be neurotoxic (Ogunrombi *et al.*, 2007).

Chapter 2
LITERATURE REVIEW

2.1. Monoamine oxidase (MAO)

2.1.1. Background

In 1928 Mary Hare-Bernheim described an enzyme catalyzing the oxidative deamination of tyramine, which she called tyramine oxidase. A few years later, Hugh Blaschko established that tyramine oxidase, noradrenaline oxidase and aliphatic amine oxidase was the same enzyme, capable of metabolizing primary, secondary and tertiary amines but not diamines such as histamine. Zeller eventually named this enzyme, mitochondrial monoamine oxidase (Youdim *et al.*, 2006; Youdim & Bakhle, 2006). The next significant development occurred by chance, through observation of patients undergoing treatment for tuberculosis. The antituberculosis drug, isoniazid, was found to be a potent inhibitor of monoamine oxidase (MAO). A related compound, iproniazid, became the first MAO inhibitor, thus the first antidepressant and one of the first psychotropic drugs used therapeutically. In the late 1950s and 1960s iproniazid and other MAO inhibitors demonstrated significant antidepressant action but their clinical value was seriously compromised by side effects. Iproniazid caused liver toxicity, associated with its hydrazine structure, while tranylcypromine, a non-hydrazine, MAO inhibitor, induced the 'cheese reaction' (Youdim *et al.*, 1988; Youdim & Bakhle, 2006).

Tyramine and indirect acting sympathomimetic amines present in food (certain cheeses) and fermented drink (beer and wine) induces the cheese reaction. Under normal circumstances these dietary amines are metabolized by MAO in the gut wall and liver, and thus are prevented to enter the systemic circulation. MAO-inhibitors inactivate this protective system and tyramine and other monoamines present in food enters the systemic circulation where they have access to, and induce significant release of noradrenaline

from peripheral adrenergic neurons. This leads to a severe hypertensive response which can be fatal (Finberg & Tenne, 1982; Youdim & Bakhle, 2006).

Monoamine oxidase (MAO) is of crucial interest because it is responsible for the metabolism of catecholamine neurotransmitters, noradrenaline, adrenaline, dopamine and 5-hydroxytryptamine (Youdim & Bakhle, 2006). MAO also inactivates exogenous arylalkyl amines (Binda *et al.*, 2007). MAO is not a single enzyme but has two characterized isoforms, MAO-A and MAO-B, which have different pH optima, sensitivity to heat activation, and exhibit different substrate and inhibitor specificities (Youdim *et al.*, 2006; Youdim & Bakhle, 2006). MAO-A is selectively inhibited by clorgyline and metabolize noradrenaline and 5-HT, whereas MAO-B is inhibited by (R)-deprenyl and metabolize benzylamine (Johnston, 1968; Youdim & Bakhle, 2006; Binda *et al.*, 2007). Tyramine and dopamine are metabolized equally well by both MAO-A and MAO-B (Youdim *et al.*, 2006). Selective inhibitors of MAO-B have a therapeutic role in the treatment of PD, whereas reversible inhibitors of MAO-A offer antidepressant activity (Youdim & Bakhle, 2006). These enzymes represent attractive drug targets in the pharmacological therapy of neurodegenerative diseases and depression, due to their central role in neurotransmitter metabolism (Binda *et al.*, 2007).

MAO acts on primary amines and some secondary and tertiary amines and catalyzes the following reaction: $RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$ (figure 6).

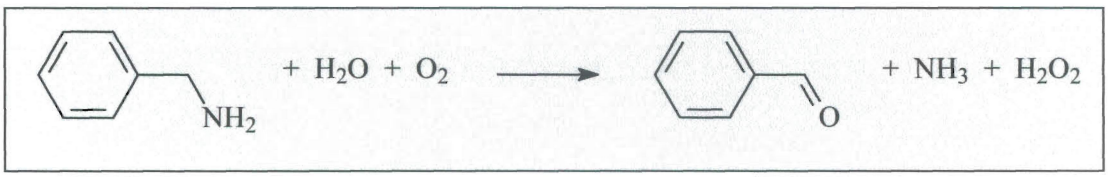

Figure 6. *The oxidation of benzylamine by MAO-B.*

MAO substrates include the slow-acting monoamine neurotransmitters (dopamine, noradrenaline, adrenaline, and serotonin) that function with rapidly-acting neurotransmitters [glutamic acid and gamma-aminobutyric acid (GABA)] to regulate movement, emotion, reward, cognition, memory and learning (Nagatsu, 2004). When

MAO-A is not functioning, MAO-B may oxidize substrates that are usually oxidized by MAO-A (Riederer & Youdim, 1990; Nagatsu, 2004). Amino acids 152-366 of MAO-B contain a domain that confers substrate and inhibitor selectivity (Chen & Shih, 1998; Nagatsu, 2004).

2.1.2. Structure of MAO-B

MAO is a flavo-protein and is bound to the mitochondrial outer membrane (MOM). MAO has an FAD cofactor covalently bound to the protein via an 8α-thioether linkage to a cysteinyl residue (figure 7) (Binda *et al.*, 2007). The FAD is the site where irreversible inhibitors of MAO (such as rasagiline and pargyline) are covalently linked (Youdim *et al.*, 2006). MAO-B appears to be a dimeric enzyme that is tightly bound to the MOM through a C-terminal transmembrane helix (a polypeptide segment) and has apolar groups located at various positions in the sequence (Binda *et al.*, 2002a). It consists of 520 amino acids (Fraaije & Mattevi, 2000; Binda *et al.*, 2002a).

The active site of MAO-B consists of a 420 $Å^3$-hydrophobic substrate cavity interconnected to an entrance cavity of 290 $Å^3$. The recognition site for the substrate amino group is an aromatic cage formed by Tyr 398 and Tyr 435 (Binda *et al.*, 2002a). The entrance cavity connects the surface of the protein to the substrate cavity in MAO-B. The substrate and entrance cavities are separated by four residues (Leu 171, Ile 199, Phe 168 and Tyr 326). Rotation of the side chains of these amino acids may alter the degree by which the substrate and entrance cavaties are separated from each other (the two cavities may fuse to form a single larger cavity that would accommodate larger substrates and inhibitors) (Wu *et al.*, 1993; Geha *et al.*, 2001; Binda *et al.*, 2002a).

Even though MAO-A and MAO-B are separate gene products, they exhibit over 70% sequence identity (Binda *et al.*, 2007), and are expressed in both a tissue-dependent and age-dependent manner (Binda *et al.*, 2002b). The level of enzyme expressed is dependent on tissue type and is elevated three-fold in aged human neuronal tissue (Fowler *et al.*, 1980; Binda *et al.*, 2002a). Elevated MAO-B levels induce apoptosis in neuronal and

kidney cells, and increased MAO-B was demonstrated in plaque-associated astrocytes obtained from the brains of Alzheimer's patients (Boulton *et al.*, 1998; Vindis *et al.*, 2000; Saura *et al.*, 1994).

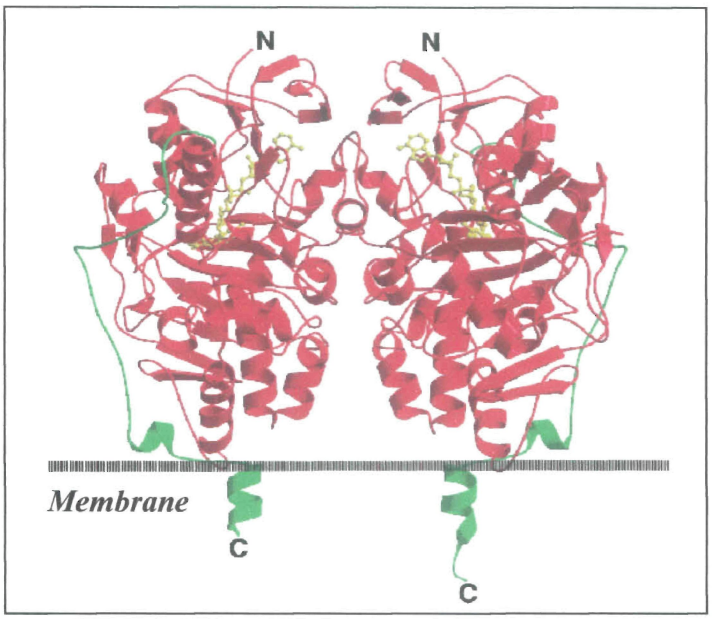

Figure 7. *The structure of human MAO-B. N represent the N-terminal and C the C-terminal. Residues 4-460 are in red and the C-terminal tail in green where the dimer is anchored to the outer mitochondrial membrane. FAD is coloured yellow (Binda et al., 2002a).*

2.1.3. Possible mechanisms of for amine oxidations

Numerous mechanisms have been proposed to describe the chemical events involved in flavin-dependent amine oxidations. One of these is the single electron transfer mechanism (figure 8) (also termed the aminium cation radical or single electron transfer mechanism). This mechanism was proposed based on the interaction of *N*-cyclopropyl-*N*-benzylamine substrate analogues with MAO-B (Binda *et al.*, 2002b). In the initial reversible step in catalysis, the flavin serves as a one-electron oxidant of the amine to form the aminium cation radical with a sufficiently acidic α-proton. This would make it possible for a basic amino acid residue in the active site to abstract the proton with subsequent radical recombination occurring to form the imine product and reduced flavin

as products (Binda *et al.*, 2002b). Little direct evidence is available to support this mechanism. Structural data of MAO-B show that none of the amino acid residues at the catalytic site would function as an active site base for a H^+ abstraction.

Mechanism-based inactivation studies of MAO-B by cyclopropylbenzylamine analogues discovered that by modification of Cys 365, which was suggested to be the active site base in MAO-B, the enzyme was inhibited. Conversely, the structural data of MAO-B show this residue (Cys 365) to be at the surface of the molecule some 20 Å away from the active site (Binda *et al.*, 2002b).

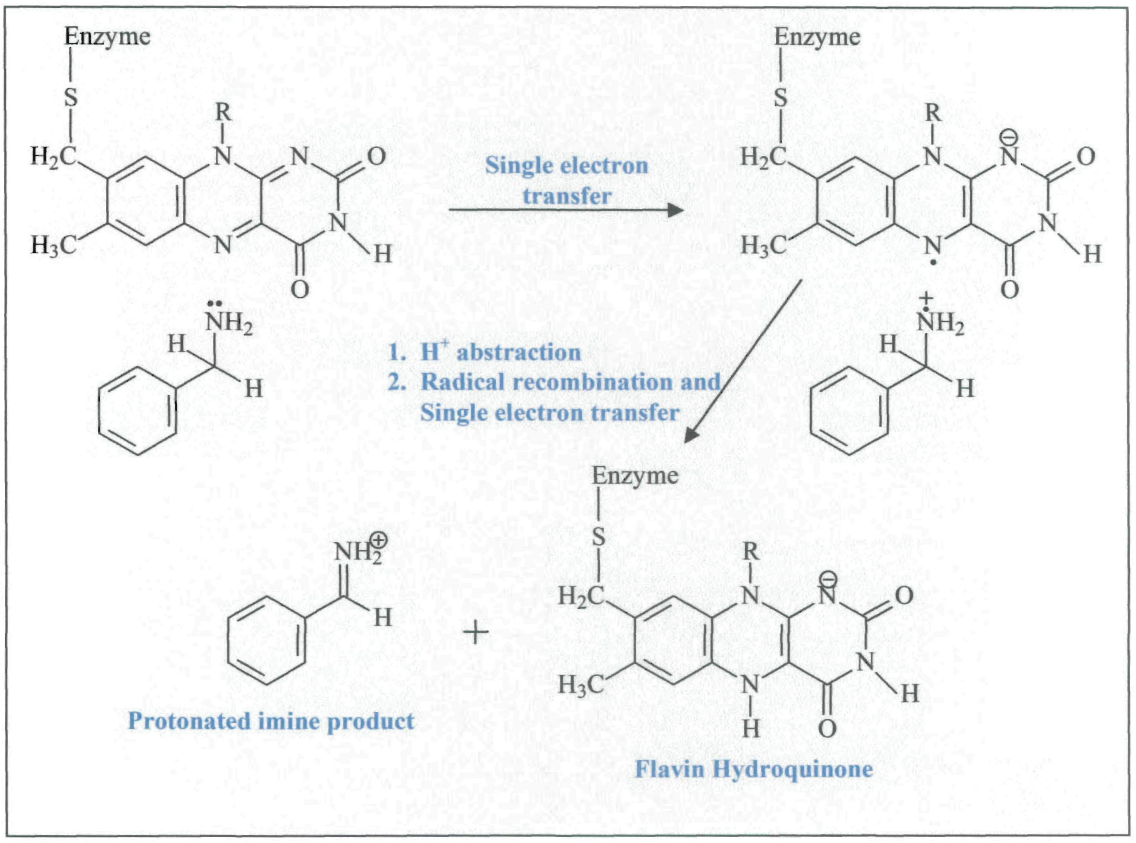

Figure 8. *The single electron transfer mechanism proposed for MAO catalysis (Binda et al., 2002b).*

Recent quantitative structure-activity relationship (QSAR) data on MAO-A with a series of *para*-substituted benzylamine analogues provide definitive evidence that α-CH bond

cleavage does occur by a proton abstraction mechanism (figure 9). The amine functionality adds to the 4a-position of the flavin in a nucleophilic manner, which activates the N5-position to function as a strong active site base. The pK_a of reduced flavins approximates that of benzyl protons, which imply that the reaction is concerted (Binda *et al.*, 2002b).

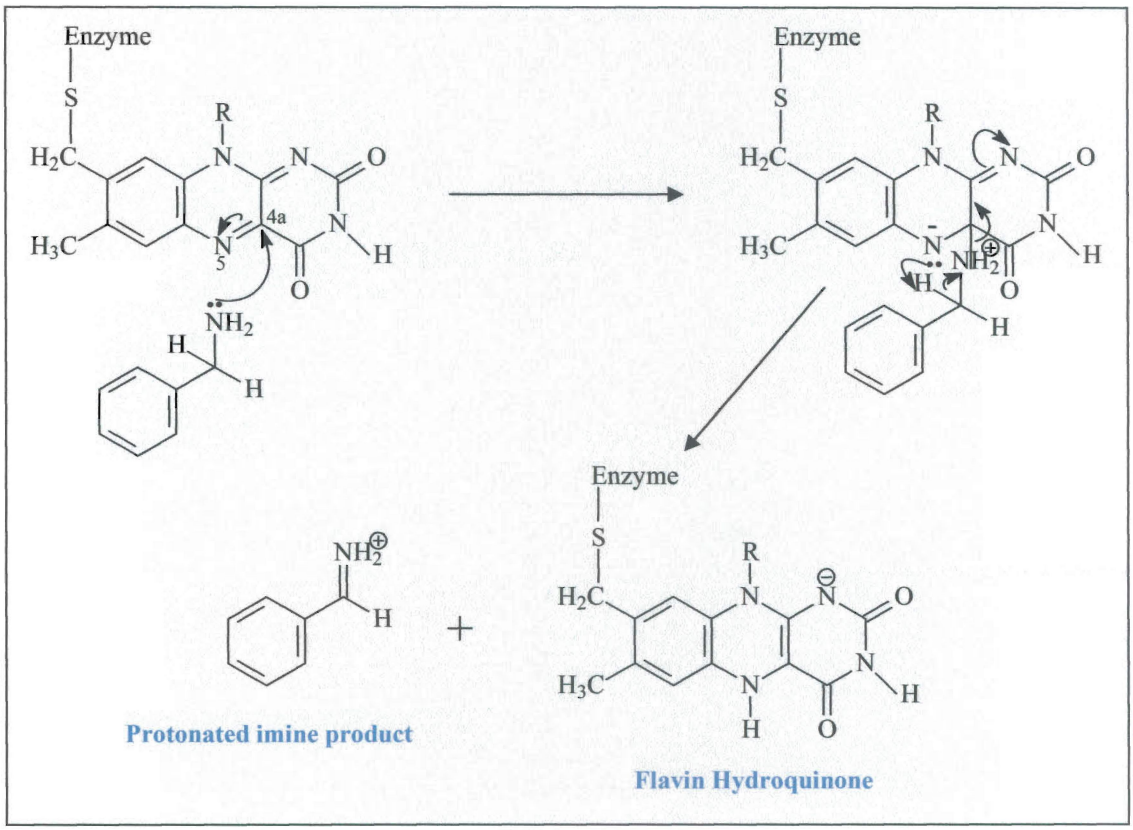

Figure 9. *Representation of the concerted covalent catalysis mechanism proposed for MAO catalysis (Binda et al., 2002b).*

2.1.4. MAO, iron and neurodegenerative diseases

Iron levels in animals and humans are known to influence the activity of MAO (Youdim *et al.*, 1975). Iron metabolism and function in the brain has a crucial role in many metabolic processes in the CNS. In many neurodegenerative diseases the sites of

neuronal death in the brain, and several mutated genes, are linked to the sites at which iron accumulates (Zecca *et al.*, 2004; Mandel *et al.*, 2005).

An increase in oxidative stress is the link between MAO, iron and neuronal damage. Hydrogen peroxide is a normal product of MAO, which is inactivated in the brain by glutathione peroxidase (GPO) which uses glutathione (GSH) as electron donor. When GSH levels are decreased, as in PD, hydrogen peroxide could accumulate and react in the Fenton reaction with iron (figure 10). Ferrous ion (Fe^{2+}) generates a highly active free hydroxyl radical from hydrogen peroxide, which depletes cellular anti-oxidants and reacts and damages lipids, proteins and DNA. Brain iron and MAO increase as age increases, thus increasing both components of the Fenton reaction and the potential of hydroxyl radical generation. Increased MAO activity is also seen in PD and AD patients (Mandel *et al.*, 2005). Inhibition of MAO decreases hydrogen peroxide production and reduces the potential for hydroxyl radical formation with the consequent oxidative stress (Youdim & Bakhle, 2006).

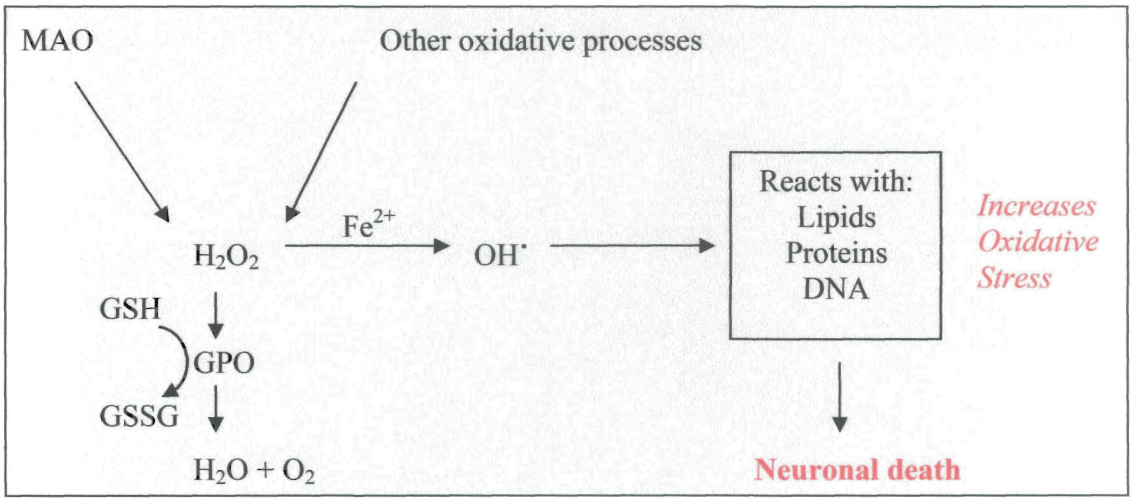

Figure 10. *The Fenton reaction (Youdim & Bakhle, 2006).*

2.1.4.1. Parkinson's disease

2.1.4.1.1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease and the clinical features was first described by James Parkinson in 1817 in his monograph "Essay of the Shaking Palsy". PD results from the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which in turn leads to striatal dopamine (DA) deficiency. The latter is the cause for the major symptoms of PD (Dauer & Przedborski, 2003). Replenishment of striatal DA by a precursor of DA, levodopa (L-3,4-dihydroxyphenylalanine), alleviates most of the symptoms but was later found to cause involuntary movements or dyskinesias after long term use. These dyskinesias are difficult to control and impair the quality of life dramatically (Dauer & Przedborski, 2003). Modern research is directed toward the prevention of dopaminergic neuron degeneration. Nevertheless, all contemporary treatments are symptomatic and do not prevent the progress of the neurodegenerative processess (Dauer & Przedborski, 2003). In the pathogenesis of PD there are three types of cellular dysfunctions that are important: oxidative stress, abnormal protein aggregation and mitochondrial respiration defect (Dauer & Przedborski, 2003).

2.1.4.1.2. Clinical characteristics of PD

PD is a progressive disease with an average age onset of 55 and increases noticeably with age. In about 95% of the cases PD onset are sporadic, with no apparent genetic linkage. In the remaining 5% the disease is inherited. Any direct striatal damage or deficient striatal DA may lead to "parkinsonism". PD accounts for approximately 80% of parkinsonism. PD symptoms are tremor at rest, rigidity, bradykinesia, hypokinesia, akinesia, postural instability, freezing and abnormalities of affect and cognition also occur (Dauer & Przedborski, 2003).

2.1.4.1.3. Neurochemical and neuropathological features of PD

Neuropathological characteristics of PD include:

- The loss of nigrostriatal dopaminergic neuron cell bodies in the SNpc which primarily project to the putamen produces SNpc depigmentation (Marsden, 1983; Dauer & Przedborski, 2003). This is associated with the loss of DA transporter (DAT) mRNA expression (Uhl *et al.*,1994; Bernheimer *et al.*, 1973; Dauer & Przedborski, 2003).
- The presence of intraneuronal proteinacious cytoplasmic inclusions termed 'Lewy Bodies' (LB) (Marsden, 1983; Dauer & Przedborski, 2003). LBs are approximately 15 μm in diameter, are spherical eosinophilic cytoplasmic protein aggregates and consists of numerous proteins, including α-synuclein, parkin, ubiquitin, and neurofilaments. They have an organized structure and contain a dense granulovesicular hyaline core surrounded by a ring of 8-10 nm fibrils (Spillantini *et al.*, 1998; Pappolla, 1986; Dauer & Przedborski, 2003). They are present in all affected brain regions (Spillantini *et al.*, 1998; Dauer & Przedborski, 2003).
- Ventrolateral and caudal portion cell loss of the SNpc (Fearnley & Lees, 1991; Dauer & Przedborski, 2003). Striatal terminal loss appears to be more pronounced than the dopaminergic neuron loss of the SNpc, suggesting that the striatal dopaminergic nerve terminals are the primary target of the degenerative process and that neuronal death in PD may result from a "dying back" process (Bernheimer *et al.*, 1973; Dauer & Przedborski, 2003).
- The neurodegeneration of the noradrenergic (locus coeruleus), serotonergic (raphe), and cholinergic (nucleus basalis of Meynert, dorsal motor nucleus of vagus) systems as well as in the cerebral cortex, olfactory bulb, and autonomic nervous system. The degeneration of hippocampal structures and cholinergic cortical inputs contribute to the high rate of dementia that accompanies PD (Dauer & Przedborski, 2003).

PD diagnosis is made on clinical grounds, but definite identification of both LB and SNpc dopaminergic neuron loss is essential (Dauer & Przedborski, 2003).

2.1.4.1.4. Etiology and pathogenesis of PD

The cause of neuronal cell death in PD still remains unknown (McNaught *et al.*, 1998). The role of environmental toxins, genetic factors and endogenous toxins in the etiology of PD is unknown. The environmental hypothesis was dominant for much of the 20th century and it argues that PD-related neurodegeneration results from either chronic exposure to a dopaminergic neurotoxin or limited exposure initiating a self-perpetuating cascade of deleterious events. The discovery of PD genes renewed interest in hereditary factors. All of the PD genes identified [α-synuclein, parkin, and ubiquitin C-terminal hydrolase L1 (UCH-L1)] appear to participate in the ubiquitin-proteasome pathway. The mutations in gene DJ-1, which causes PD, also have a potential link to the ubiquitin-proteasome pathway (Takahashi *et al.*, 2001; Dauer & Przedborski, 2003). The endogenous toxin hypothesis claims that alteration of normal metabolism might generate toxic substances due to environmental exposures or inherited differences in metabolic pathways. The normal DA metabolism generates harmful reactive oxygen species (ROS), therefore it could be seen as one source of endogenous toxins (Cohen, 1984; Dauer & Przedborski, 2003).

Modern research suggests a genetic predisposition to the actions of an environmental or endogenous toxic substance, leading to cell death by a degenerative process involving oxidative stress and mitochondrial dysfunction (McNaught *et al.*, 1998). No toxic substance related to neurodegeneration in PD has been identified, although evidence exists that the incidence of the disease parallels industrialization, the use of agrochemicals, rural residence, and the drinking of well water (McNaught *et al.*, 1998). Numerous chemical agents can induce an irreversible (e.g. manganese, carbon monoxide, and carbon dioxide) or reversible (e.g. phenothiazines and reserpine) parkinsonian-like syndrome in humans, but the clinical and pathological characteristics of these disorders are different from those of idiopathic PD (McNaught *et al.*, 1998). However, the

discovery that MPTP induces a parkinsonian syndrome in humans and experimental animals by selective destruction of the nigrostriatal pathway supports the idea that a neutoroxin may cause PD (McNaught *et al.*, 1998).

There are two major hypotheses regarding the pathogenesis of PD (Dauer & Przedborski, 2003):

1. Misfolding and aggregation of proteins are instrumental in the death of SNpc dopaminergic neurons.
2. Mitochondrial dysfunction and the consequent oxidative stress, including toxic oxidized DA species, leads to neuronal death.

2.1.5. (R)-Deprenyl, MAO-B and neuroprotection

Neuroprotection is a concept that reflects the possibility of slowing, halting or even reversing the neurodegeneration process in PD and Alzheimer's disease. Oxidative stress is a possible factor that can cause the initiation of the neurodegenerative process and the selective inhibition by MAO is therefore important to lower the oxidative stress (Youdim & Bakhle, 2006).

(R)-Deprenyl (selegiline) (figure 11), derived from propargylamine, inhibits the oxidative deamination of dopamine, phenylethylamine and benzylamine *in vivo* at low doses but not noradrenaline or 5-HT. At high doses selectivity is lost. (R)-deprenyl inhibits MAO-B at low doses and both MAO-A and MAO-B at high doses. It is thus a selective inhibitor of MAO-B, and devoid of the cheese reaction (Youdim & Bakhle, 2006).

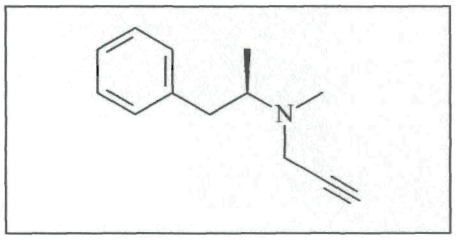

Figure 11. *Structure of (R)-deprenyl (selegiline).*

Patients receiving both (R)-deprenyl and L-DOPA treatment have a better survival rate than those treated only with L-DOPA. This suggests that (R)-deprenyl reduces the rate of dopaminergic neuron degeneration, an effect referred to as 'neuroprotection' (Birkmayer *et al.*, 1985; Youdim & Bakhle, 2006). MPTP is a neurotoxin which is selectively neurotoxic to dopaminergic neurons (in the substantia nigra of animals and humans) and induces PD-like neurodegeneration. MAO-B bioactivates MPTP to yield the active neurotoxin, MPP^+ (Melamed & Youdim, 1985). Nigrostriatal neurons in mice are protected against MPTP-induced neurotoxicity by the pretreatment with non-selective MAO inhibitors and (R)-deprenyl (selective inhibitor) (Heikkila *et al.*, 1984; Youdim & Bakhle, 2006). Similar results were obtained with phenylethylamine, another MAO-B substrate. This effect of phenylethylamine was attributed to competition at the active site of MAO-B, thus preventing oxidation of MPTP to the toxic metabolite, MPP^+ (Melamed & Youdim, 1985). For a MAO inhibitor to be effective in PD, it must increase the levels of dopamine at its receptor sites in the striatum (Youdim & Bakhle, 2006).

2.1.6. Substrates of MAO-B

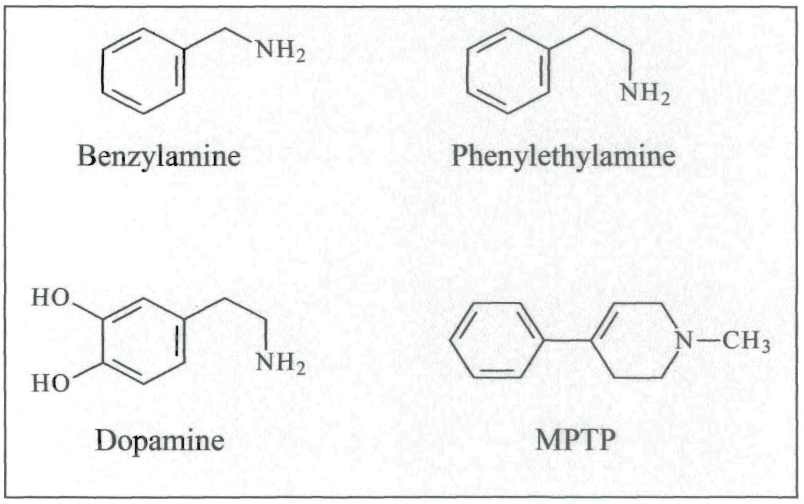

Figure 12. *Different substrates of MAO-B. Benzylamine, phenylethylamine and MPTP are good substrates for MAO-B, whereas dopamine is a substrate for both MAO-B and MAO-A (Binda et al., 2002a).*

Benzylamine, MPTP and phenylethylamine are all good substrates of MAO-B, although they are oxidized by MAO-A at a much slower rate (figure 12). Serotonin is a specific substrate for MAO-A, whereas dopamine and epinephrine are oxidized similarly by both MAO-A and MAO-B (Binda *et al.*, 2002a).

The reaction mechanism of MAO (figures 13 and 14) involves the oxidative deamination of primary, secondary and tertiary amines to the corresponding aldehyde and free amine, with hydrogen peroxide formed as reaction product (Youdim & Bakhle, 2006). This reaction occurs via an oxidative cleavage of the α-CH bond of the substrate to form an imine product and the concomitant reduction of the flavin cofactor (Binda *et al.*, 2002b). The imine product is then hydrolyzed (non-enzymatically) to the corresponding aldehyde and ammonia (or amine for secondary and tertiary amine substrates) (Binda *et al.*, 2002b). The aldehyde is rapidly metabolized by aldehyde dehydrogenase to acidic metabolites, which are used for the measurement of MAO activity *in vitro* and *in vivo* (Youdim & Bakhle, 2006). The reduced flavin coenzyme reacts with oxygen to yield hydrogen peroxide and the oxidized form of the flavin to complete the catalytic cycle (molecular oxygen is the general electron acceptor) (Binda *et al.*, 2002b).

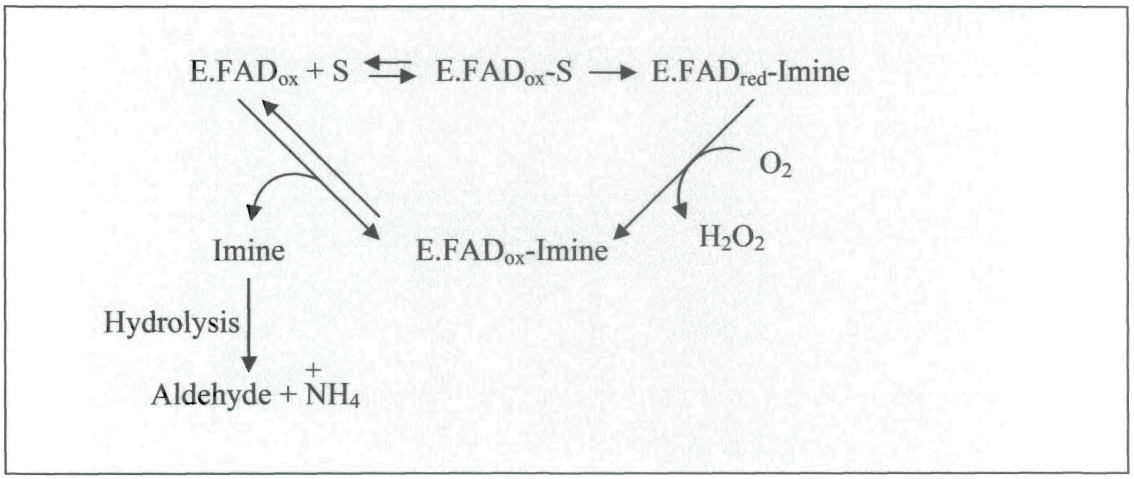

Figure 13. *Overall oxidative deamination reaction catalyzed by MAOs. Oxidation of the amine substrate leads to the reduction of FAD. The reduced enzyme-imine complex is reoxidized by molecular oxygen and generates hydrogen peroxide.*

The imine dissociates from the enzyme and the imine product is hydrolyzed in a nonenzymatic process (Adapted from Binda et al., 2002a). In the case of phenylethylamine the imine product dissociates from the reduced enzyme prior to the reoxidation of the FAD by oxygen (Walker & Edmondson, 1994).

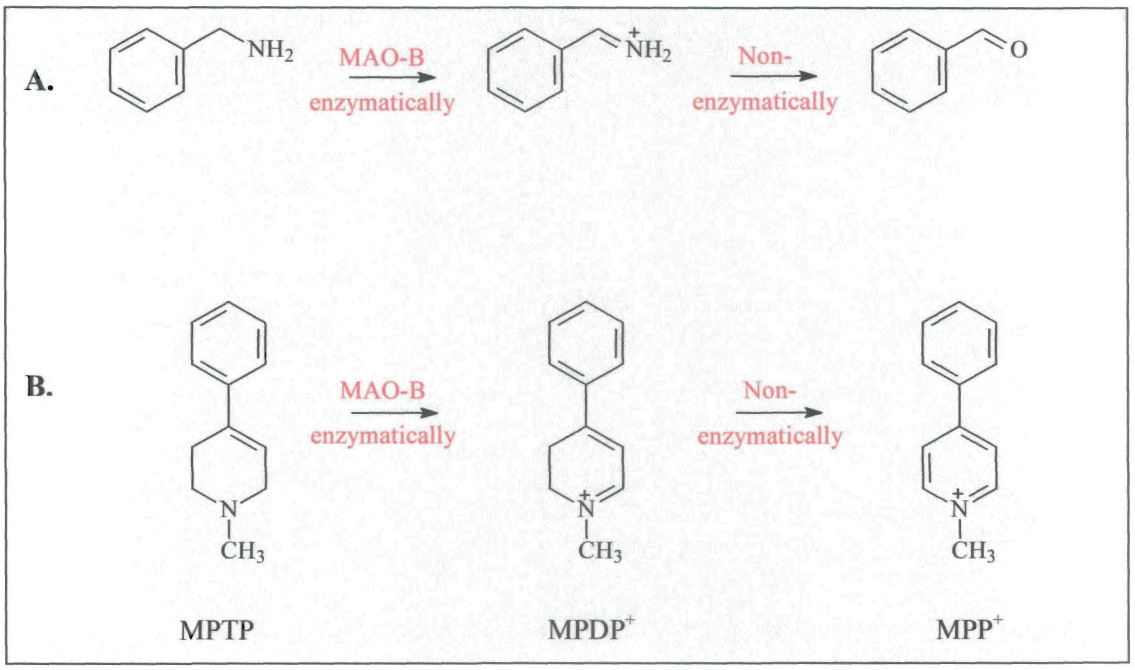

Figure 14. ***A.*** *The oxidation of benzylamine by MAO-B. The reaction products of phenylethylamine and dopamine will be similar to the products of benzylamine (Binda et al., 2002a).* ***B.*** *MPTP metabolism by MAO-B. MPTP is oxidized by MAO-B to $MPDP^+$ and then non-enzymatically converted to the ultimate neurotoxin MPP^+ (adapted from Loots et al., 2004).*

2.1.6.1. The neurotoxic MAO-B substrate, MPTP

The neurotoxic action of MPTP was discovered when drug users self-administered 1-methyl-4-phenyl-propionoxypiperidine (MPPP), an analogue of the narcotic meperidine, and developed a rapidly progressive parkinsonian syndrome due to MPTP that was present as a contaminant. MPTP caused extensive toxicity to dopaminergic neurons and

neurological injury to the substantia nigra (Langston *et al.*, 1983; Dauer & Przedborski, 2003), and produces an irreversible and severe parkinsonian syndrome (Irwin *et al.*, 1993). It also produces all the characteristics of PD, including temor, rigidity, and slowness of movement, postural instability, and freezing (Irwin *et al.*, 1993). Similarities to PD include the beneficial response to L-DOPA, the development of long-term motor complications to medical therapy, and the susceptibility which increases with age (Irwin *et al.*, 1993) Therefore, MPTP is an adequate model to study Parkinson's disease (PD) and other related neurodegenerative diseases (Irwin *et al.*, 1993).

A low-dose treatment of MPTP in monkeys caused preferential degeneration of dopaminergic nerve terminals in the putamen (Moratalla *et al.*, 1992), and is responsible for greater cell loss of neurons in the ventral and lateral segments of the SNpc, a regional pattern also found in MPTP-treated mice (Varastet *et al.*, 1994; Muthane *et al.*, 1994). Two important PD characteristics are absent in the monkey MPTP model. First, neurons are not consistently lost from other monaminergic nuclei and second, classical LBs have not been described convincingly in the brains of MPTP-intoxicated patients or monkeys (Forno *et al.*, 1993). Dopaminergic neurons that contain neuromelanin are more susceptible to MPTP-induced degeneration and may contribute to neurodegeneration by catalyzing reactive oxygen species (ROS) formation through an interaction with iron. A variety of organic molecules (including pesticides, MPTP, and MPP^+) interact with neuromelanin, so it may contribute to susceptibility of pigmented neurons by acting as a depository for toxic compounds (Zecca *et al.*, 2001; Herrero *et al.*, 1993).

2.1.6.2. The mechanism of MPTP toxicity

The highly lipophilic MPTP readily crosses the blood-brain barrier (BBB) (figure 15) (Markey *et al.*, 1984; Dauer & Przedborski, 2003), where MAO-B in glial cells and serotonergic neurons converts the proneurotoxin, MPTP, to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) (Dauer & Przedborski, 2003). $MPDP^+$ is non-enzymatically and spontaneously oxidized to the ultimate neurotoxin, 1-methyl-4-phenylpyridinium, MPP^+ (a high-affinity substrate for DAT, norepinephrine and serotonin transporters).

MPP^+ is released into the extracellular space by an unknown mechanism and enters neuronal cells via membrane carriers (Mayer *et al.*, 1986; Dauer & Przedborski, 2003).

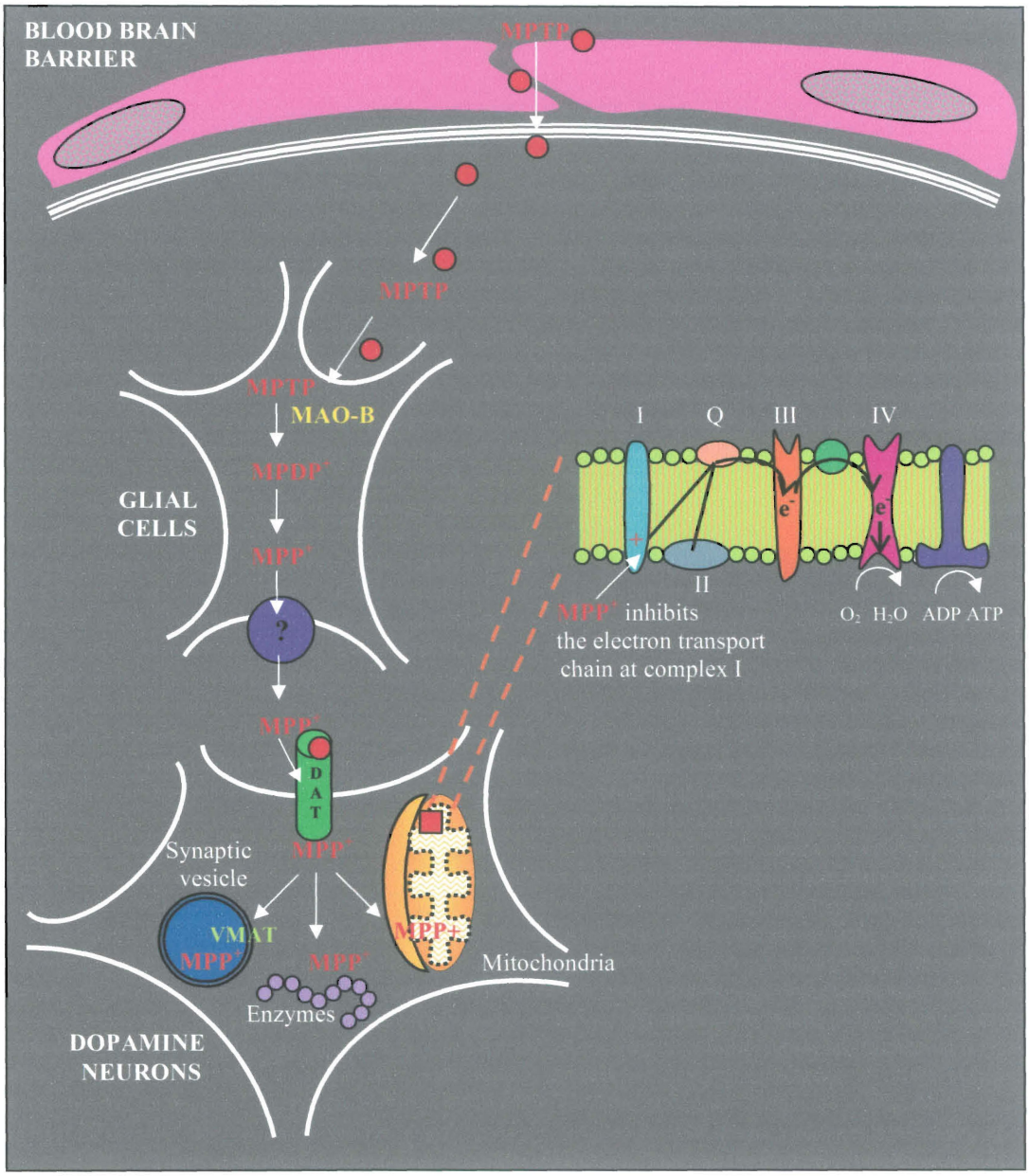

Figure 15. *MPTP metabolism and intracellular MPP^+ pathways. MPTP crosses the BBB and is converted to $MPDP^+$ by MAO-B and then to MPP^+ in the glail cells. MPP^+ is released into the extracellular space and concentrated in the dopamine neurons via the dopamine transporter (DAT) where it can follow*

one of three routes: (1) It can be actively transported into the mitochondria where it inhibits complex I of the electron transport chain, which interrupts the transfer of electrons from complex I to ubiquinone (Q). This leads to increased production of reactive oxygen species and decreased ATP production; (2) interact with cytosolic enzymes; (3) transported into synaptic vesicles via the vesicular monoamine transporters (VMAT) (adapted from Dauer & Przedborski, 2003).

MPP^+ accumulates in the dopaminergic neurons and is actively transported into the mitochondria where it inhibits mitochondrial respiration at complex I of the electron transport chain (Soliman & Mazzio, 2003; Ebadi *et al.*, 2001). This blockade rapidly leads to decreased tissue ATP content in the regions most sensitive to MPTP (the striatum and ventral midbrain) (Fabre *et al.*, 1999; Dauer & Przedborski, 2003). Another effect of complex I inhibition may be oxidative stress, a process by which the flow of electrons through complex I is hindered by MPP^+, and the production of ROS, especially superoxide, is stimulated (Hasegawa *et al.*, 1990; Dauer & Przedborski, 2003). The production of ROS can also be indirectly stimulated by MPP^+ by activating DA outflow from synaptic vesicles to the cytosol, possibly due the inability of VMAT2 to maintain concentration gradients when cellular ATP is depleted. The released DA may be oxidized by MAO yielding hydrogen peroxide which may lead to oxidative stress (Johnson, 1988; Dauer & Przedborski, 2003).

After MPTP administration, alterations in energy metabolism and generation of ROS peak within hours, days before neuronal death has occurred (Jackson-Lewis *et al.*, 1995; Dauer & Przedborski, 2003). Consequently, these initial events are expected to cause downstream cellular events that ultimately lead to dopaminergic neuron loss, and not directly causing cell death (Vila *et al.*, 2001; Dauer & Przedborski, 2003). Prolonged administration of low to moderate doses of MPTP to mice leads to apoptosis of SNpc dopaminergic neurons (Dauer & Przedborski, 2003; Tatton & Kish, 1997) and accumulation and nitration of α-synuclein in the cytosol of SNpc dopaminergic neurons.

Ablation of α-synuclein in mutant mice prevents MPTP-induced dopaminergic neurodegeneration (Przedborski *et al.*, 2001; Dauer *et al.*, 2002).

2.1.6.3. Deuterated substrates of MAO-B

MPTP is the first reported cyclic tertiary allylamine that acts as a MAO-B substrate (Salach *et al.*, 1984; Ottoboni *et al.*, 1989). MAO-B catalyzes the ring α-carbon oxidation of MPTP to $MPDP^+$ at the allylic C-6 position. Metabolic switching to the C-2 position does not occur (Ottoboni *et al.*, 1989). MAO-catalyzed oxidation pathways are all regio- and stereoselective (Ottoboni *et al.*, 1989).

For the oxidation of MPTP by MAO-B, MPTP has a K_m value of 0.48 mM and a V_{max} value of 18.2 nmol/min (Ottoboni *et al.*, 1989). The kinetic deuterium isotope effect for the oxidation of MPTP to $MPDP^+$ by MAO-B is 3.55 on V_{max} and 8.01 on V_{max}/K_m with MPTP-6,6-d_2 as the deuterated substrate (Ottoboni *et al.*, 1989). Similar values were obtained with MPTP-2,2,6,6-d_4 and MPTP-CD_3-2,2,6,6-d_4 (figure 16) (Ottoboni *et al.*, 1989).

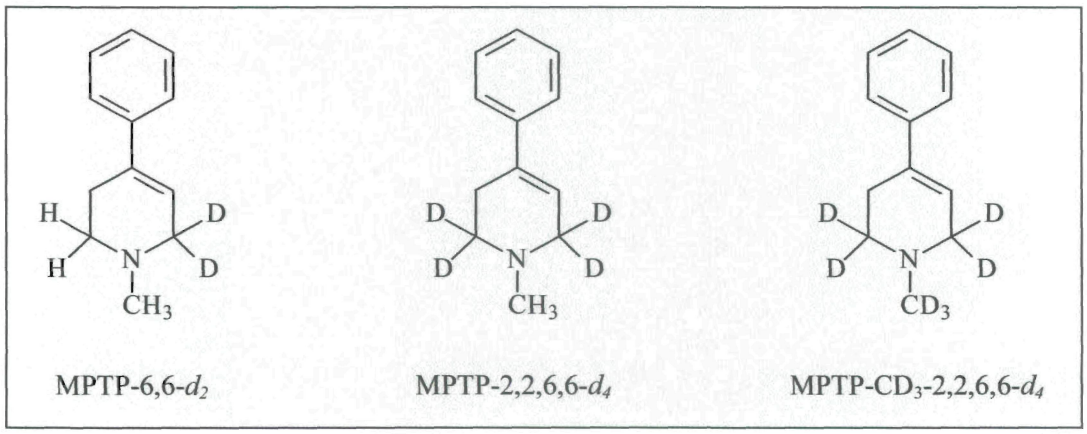

Figure 16. *The structures of the deuterated MPTP analogues.*

These isotope effect values were generated from noncompetitive, intermolecular kinetic measurements, and might not reflect the intrinsic isotope effects (Ottoboni *et al.*, 1989). $^DV_{max}$ suppression compared to $^D(V_{max}/K_m)$ suggests that the carbon-hydrogen bond

cleavage step is partially rate-determining because one complete MAO-B catalytic cycle is composed of 2 half-reactions (figure 17). The first reaction is the oxidation of the substrate, where the flavin moiety is reduced and the second reaction is where the product is released and the reduced flavin is reoxidized (Ottoboni *et al.*, 1989). While the first reaction, the carbon-hydrogen bond cleavage step is rate-determining, the rate of the second (reoxidative) half-reaction may be slow enough to affect the rate of the overall reaction (Ramsey *et al.*, 1987; Ottoboni *et al.*, 1989).

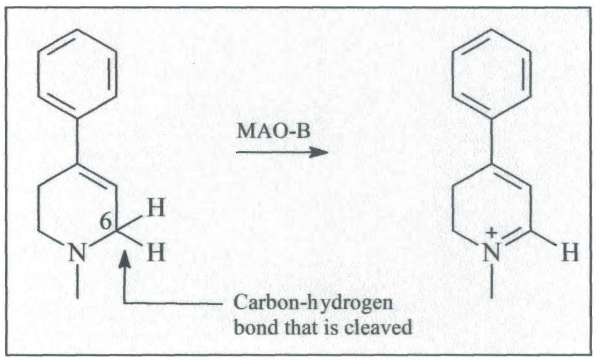

Figure 17. *The carbon-hydrogen bond cleavage step is rate-determining in the oxidation of MPTP at the C-6 α-carbon by MAO-B.*

The double bond in the tetrahydropyridine ring enhances the rate of hydrogen loss from the allylic position (Ottoboni *et al.*, 1989). The deuterium isotope effect observed with MPTP indicates that the carbon-hydrogen bond cleavage step is rate-determining and the transition state is highly symmetric (Ottoboni *et al.*, 1989). The oxidation of benzylamine by MAO-B also exhibits a large deuterium isotope effect on V_{max} ($^{D}V_{max}$ = 6.7) (Husain *et al.*, 1982; Ottoboni *et al.*, 1989). However, the oxidation of (R)-dopamine-α-d_1 had isotope effects of 3.1 on V_{max} and 2.8 on V_{max}/K_m (Yu *et al.*, 1986; Ottoboni *et al.*, 1989), and for the deamination of 2-phenylethylamine-1,1-d_2, 1.44 on V_{max} and 1.10 on V_{max}/K_m (Husain *et al.*, 1982; Ottoboni *et al.*, 1989). These lower isotope effect values might be consistent with the aminium radical pathway; therefore it may be possible that the MAO-B catalyzed oxidation of benzylic and allylic positions may proceed via an alternative pathway (Ottoboni *et al.*, 1989).

2.1.6.4. Saturated cyclic tertiary amine substrates of MAO-B

Cyclic 5- and 6-membered tertiary allylamines undergo two-electron α-carbon MAO-catalyzed oxidation to give cyclic, conjugated eniminium metabolites (Bissel *et al.*, 2008; Rimoldi *et al.*, 2005). As mentioned earlier, MPTP is oxidized at C-6 of the tetrahydropyridinyl ring by MAO-B to yield the conjugated eniminium metabolite, $MPDP^+$ (Weisman *et al.*, 1985). $MPDP^+$ then undergoes a second 2-electron oxidation to generate the ultimate neurotoxin MPP^+ (Chiba *et al.*, 1984; Ramsay *et al.*, 1991; Markey *et al.*, 1984). The presence of a 4,5-π bond of the heterocyclic moiety is an essential MAO-B substrate feature of MPTP (Bissel *et al.*, 2008). Since the corresponding saturated piperidinyl analogue of MPTP is not a MAO-B substrate (figure 18), it proves the necessity of the presence of the allylic π-bond for MAO-B substrate activity (Bissel *et al.*, 2008; Rimoldi *et al.*, 2005). Correspondingly, it is expected that while 3-pyrrolinyl derivatives are MAO-B substrates (figure 18), their saturated pyrrolidinyl analogues should not be (Rimoldi *et al.*, 2005). The ring α-carbon oxidation of 1-methyl-3-phenyl-3-pyrroline (a 3-pyrrolinyl derivative) by MAO-B results in the neutral 1-methyl-3-phenylpyrrole, an overall 2-electron process that most likely arises via the short-lived conjugate acid of the pyrrolyl product (Ogunrombi *et al.*, 2007).

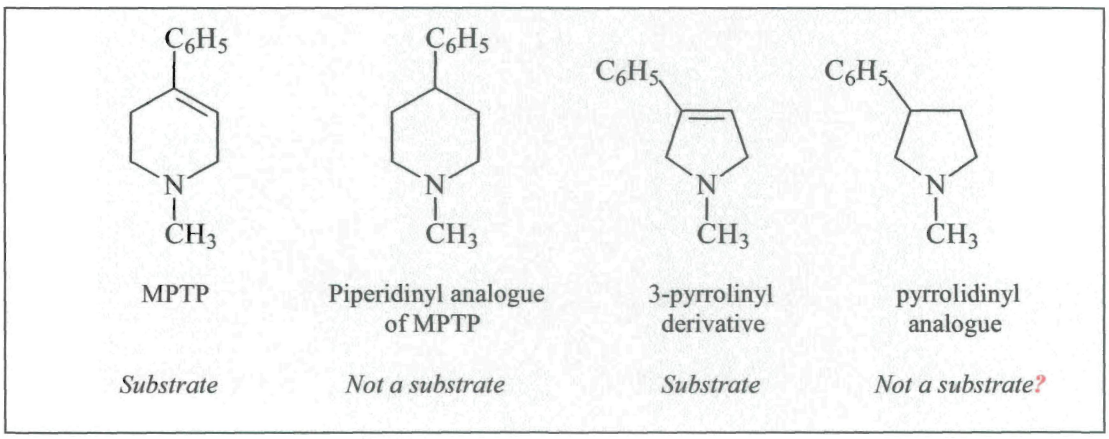

Figure 18. *The piperidinyl analogue of MPTP lacks MAO-B substrate properties due to the absence of the allylic π-bond (Bissel et al., 2008; Rimoldi et al., 2005). In this study the saturated pyrrolidinyl analogue will be evaluated as a possible MAO-B substrate.*

Furthermore, the observation that the α-carbon oxidation of MPTP and its 3,4-cyclopropyl analogue occurs regioselectively at C-6, suggests that the electronic properties of the allylic π-bond and cyclopropyl group may contribute to the stabilization of the corresponding α-carbon radicals that are thought to be intermediary in the catalytic mechanism of MAO-B (Rimoldi *et al.*, 2005).

3-Methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (figure 19) was the first reported saturated, cyclic tertiary amine with MAO-B substrate properties and is consistent with α-carbon radical stabilization as contributing factor in the catalytic pathway (Rimoldi *et al.*, 2005). Recent studies performed by Bissel and coworkers showed that racemic 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane, a fused 3,4-cyclopropyl analogue of MPTP, is catalyzed by MAO-B to form the 3-methyl-6-phenyl-3-aza-bicyclo[4.1.0]hept-2-enyliminiumyl analogue (Bissel *et al.*, 2008).

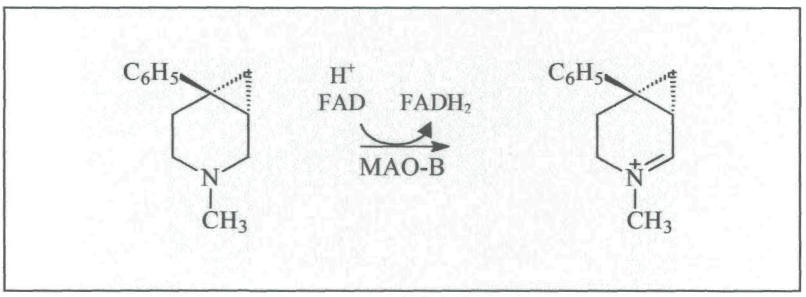

Figure 19. *The MAO-B catalyzed oxidation of 3-methyl-6-phenyl-3-azabicyclo[4.1.0]-heptane, to form the 3-methyl-6-phenyl-3-aza-bicyclo[4.1.0]hept-2-enyliminiumyl analogue (Bissel et al., 2008).*

Prochiral MAO substrates (including dopamine, benzylamine and l-aminoheptane) show α-carbon enantioselectivity, and all the conversions proceed via the selective loss of the pro-R proton (figure 20) (Bissel *et al.*, 2008). Substituents β to the nitrogen atom do not alter the stereochemical course of the reactions with selective loss of the α-pro-R proton (Bissel *et al.*, 2008).

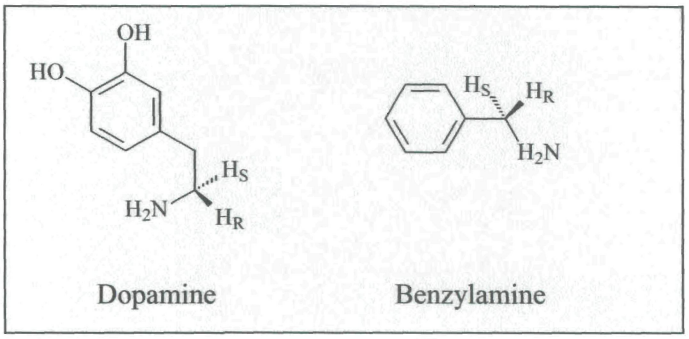

Figure 20. *The selective loss of the pro-R proton as a result of MAO-B oxidation of dopamine and benzylamine.*

2.2. Enzyme kinetics

Enzymes are protein molecules that manipulate other molecules (substrates). These target molecules bind to the active site of the enzyme and are transformed into products through a series of steps (figure 21) (the enzymatic mechanism) (Murray *et al.*, 2000).

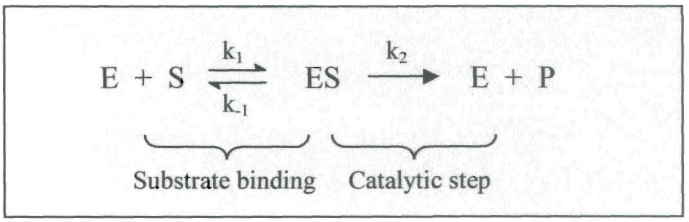

Figure 21. *An enzyme-catalyzed reaction, where k_1, k_{-1} and k_2 represent the rate constants for the individual steps. The substrate binds to the enzyme to form the enzyme-substrate complex after which the enzyme and the product(s) are released. There is typically one rate-determining enzymatic step that allows the mechanism to be modeled as a single catalytic step of rate constant k_2.*

Enzyme kinetics refers to the quantitative measurement of the rates of enzyme-catalyzed reactions and the factors that affect these rates. Kinetic analyses permit scientists to reconstruct the number and order of the individual steps by which enzymes transform substrates into products. The study of enzyme kinetics also represents the principal way

to identify potential therapeutic agents that selectively enhance or inhibit the rates of specific enzyme-catalyzed processes and can reveal details of the catalytic mechanisms. The kinetic theory (collision theory) states that for two molecules to react they must (1) approach within bond-forming distance of one another or collide and (2) must possess sufficient kinetic energy to overcome the energy barrier for reaching the transition state (Murray *et al.*, 2000).

2.2.1. The Michaelis-Menten Equation

The substrate concentration that produces half-maximal velocity (K_m or Michaelis constant, and has a dimension of molar concentration) is determined by graphing initial velocity, v_i, as a function of the substrate concentration [S] (see figure 22). When [S] is approximately equal to the K_m, v_i is very responsive to changes in [S], and the enzyme is working at half-maximal velocity (Murray *et al.*, 2000).

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

Equation 1. *The Michaelis-Menten equation.*

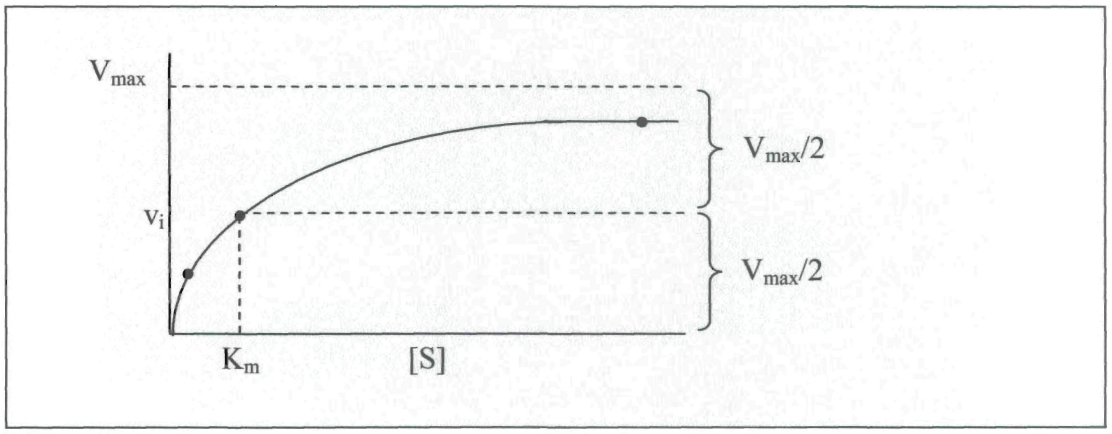

Figure 22. *The effect of substrate concentration on the velocity of an enzyme-catalyzed reaction.*

The Michaelis-Menten expression describes the behaviour of enzymes at different substrate concentrations (Murray *et al.*, 2000):

(1) **[S] is much less than K_m**, the initial velocity, v_i, depends upon the substrate concentration and [S] can be dropped from the denominator (point A in figure 22).

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

Equation 2. *The Michaelis-Menten equation when [S] is much less than K_m.*

(2) **[S] is greater than K_m**, the initial velocity, v_i, is maximal, V_{max}, and K_m can be dropped from the denominator (point C in figure 22).

$$v_i \approx \frac{V_{max}[S]}{[S]} \approx V_{max}$$

Equation 3. *The Michaelis-Menten equation when [S] is much greater than K_m.*

(3) **[S] is equal to K_m**, the initial velocity, v_i, is half-maximal (point B in figure 22).

$$v_i = \frac{V_{max}[S]}{[S]+[S]} = \frac{V_{max}}{2}$$

Equation 4. *The Michaelis-Menten equation when [S] is equal to K_m.*

2.2.2. K_m and V_{max} are determined by the linear form of the Michaelis-Menten equation

Linear representations of the Michaelis-Menten equation allow ready extrapolation of V_{max} and K_m from reaction velocities measured at less than saturating substrate concentrations, since the direct measurement of the numeric value of V_{max} often requires

very high concentrations of the substrate to achieve saturated conditions in the laboratory (Murray *et al.*, 2000).

The Michaelis-Menten reaction may be inverted and factored as follows (Murray *et al.*, 2000):

$$\frac{1}{v_i} = \frac{K_m + [S]}{V_{max}[S]}$$

Equation 5. *The inverted form of the Michaelis-Menten reaction.*

$$\frac{1}{v_i} = \frac{K_m}{V_{max}} \bullet \frac{1}{[S]} + \frac{[S]}{V_{max}[S]} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Equation 6. *The factored form of the Michaelis-Menten reaction.*

The equation for a straight line is:

$$y = mx + c$$

Equation 7. *The equation for a straight line.*

If y ($1/v_i$) is plotted as a function of x (1/[S]), the y intercept, c, is $1/V_{max}$ and the slope, m, is K_m/V_{max} (Murray *et al.*, 2000). The negative x intercept may be evaluated by setting y = 0, then (Murray *et al.*, 2000):

$$x = -\frac{c}{m} = -\frac{1}{K_m}$$

Equation 8. *The negative x intercept.*

K_m can be estimated from the Lineweaver-Burk or the double-reciprocal plot (reciprocal of v_i plotted against the reciprocal of [S]) using either the y intercept and the slope or the negative x intercept (see figure 23). The dimensions of K_m are molarity or moles per liter, since the [S] is expressed in molarity. Velocity, v_i, may be expressed in any units, since K_m is independent of the concentration of the enzyme. The Lineweaver-Burk or double-reciprocal plot requires relatively few points to define K_m, and is therefore most often used to determine K_m (Murray *et al.*, 2000).

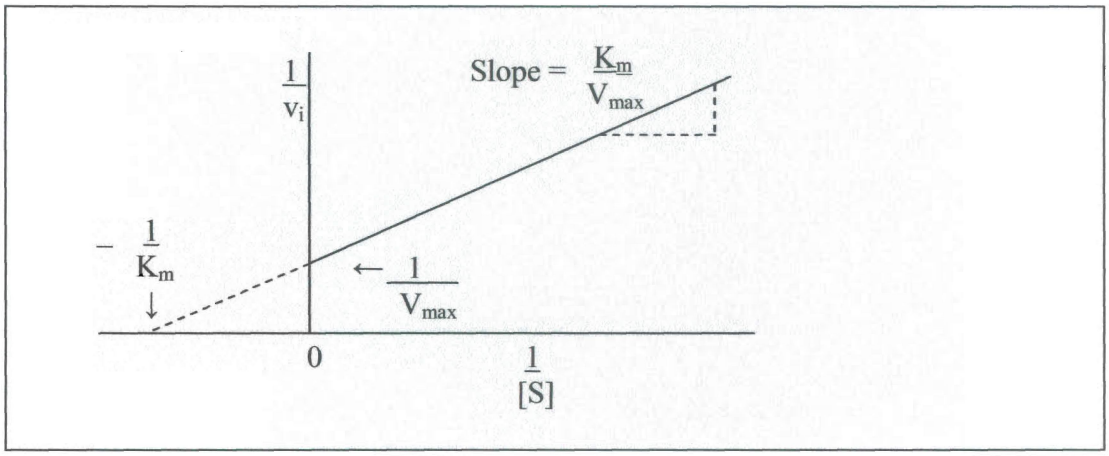

Figure 23. *Lineweaver-Burk or double-reciprocal plot of $1/v_i$ versus $1/[S]$ used to determine K_m and V_{max}.*

2.3. Measurements of MAO-B activity

MAO-B activity can be measured by various techniques and instruments. They are all based on the basic MAO catalyzed reaction (Holt *et al.*, 1997):

$RCH_2NR_1R_2 + H_2O + O_2 \rightarrow RCHO + NHR_1R_2 + H_2O_2$.

2.3.1. Measurement of product formation

Most MAO-B activity measurements are based on the measurement of one of the products generated in the enzymatic reaction. MAO-B activity can be measured continuous (usually spectrophotometric) or discontinuous (for example via HPLC

analysis). To determine K_m and V_{max} values, the initial rates of substrate oxidation by MAO-B are measured at various substrate concentrations, which should span at least two orders of magnitude (Ogunrombi *et al.*, 2007). The direct products of amine oxidation have been determined via spectrophotometry, HPLC and fluorescence analysis, depending on the chemical nature of the product (Holt *et al.*, 1997).

2.3.2. Measurement of the H_2O_2 production

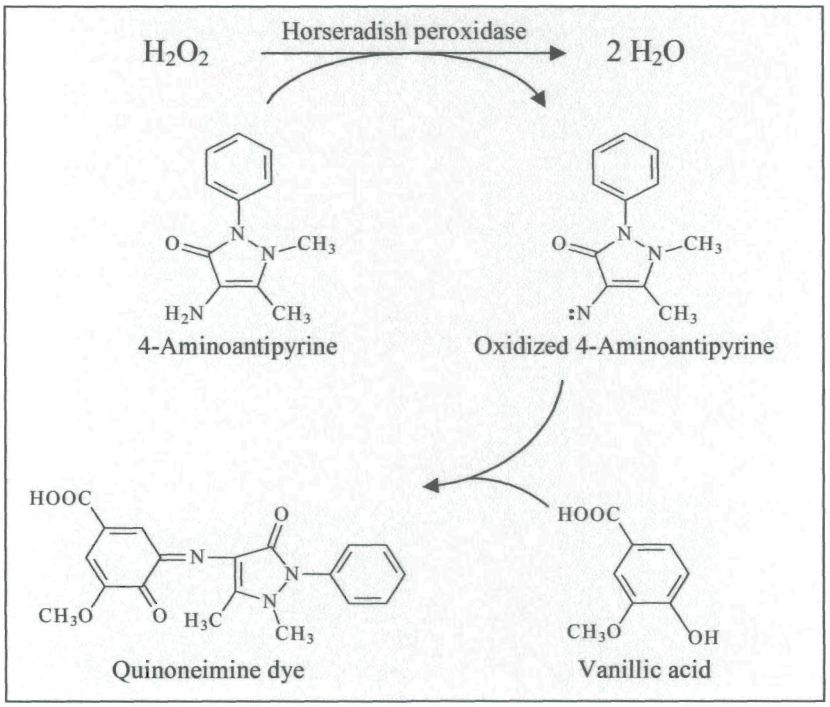

Figure 24. *The measurement of H_2O_2 in a peroxidase-linked reaction with 4-amino-antipyrine (Holt et al., 1997).*

This assay relies on H_2O_2 production rather than the direct product formation by MAO-B. It is a continuous, peroxidase-linked spectrophotometric assay which is suitable for monoamine oxidase, diamine oxidase and semicarbazide-sensitive amine oxidase activities measurements in tissue homogenates (Holt *et al.*, 1997). 4-Aminoantipyrine is oxidized and condenses with vanillic acid to give a red quinoneimine dye (figure 24) (Holt *et al.*, 1997). The absorbance (498 nm) is proportional to the amount of H_2O_2

released in the amine oxidase reaction (Holt *et al.*, 1997). This method is suitable for any amine oxidase substrate which has a higher oxidation-reduction potential than does 4-aminoantipyrine (Holt *et al.*, 1997). The concentration of the MAO-B generated H_2O_2 can also be determined via fluorescence in a coupled assay (Holt *et al.*, 1997).

2.3.3. Measurement of the radiolabeled aldehyde formation

This is a discontinuous, radiochemical assay which allows quantification of amine oxidase activities (Holt *et al.*, 1997). This assay relies on the formation of a radiolabeled aldehyde during incubation of an appropriately labeled amine substrate with MAO. Data obtained from this method can contain certain errors for example; workers fail to ensure that product formation proceeds linearly for the duration of the incubation period. In lieu of the radiochemical assay, some aldehydes can be detected directly by spectrophotometry. This technique is often unsuitable for use with crude tissue homogenates (Holt *et al.*, 1997).

2.3.4. Measurement of the ammonia production

The production of ammonia (NH_3) can be measured continuously with a coupled colorimetric assay. Primary amine substrates are oxidized to NH_3, by both the MAOs and semicarbazide-sensitive amine oxidase. The classical MAO enzymes can also metabolize secondary and tertiary amines (Holt *et al.*, 1997). Since the oxidation of the substrate does not lead to formation of NH_3, the measurement of ammonia product is not suitable for secondary and tertiary amines (Holt *et al.*, 1997).

2.3.5. Measurement of oxygen consumption

Oxygen consumption by MAO-B can be measured polarographically and is independent of the substrate used or of the enzyme classification (Holt *et al.*, 1997).

For the purpose of the second part of this study (see chapter 4) the production of H_2O_2 was used to determine MAO-B activity. This method has the advantage that the MAO-B activity measurement is independent of the nature of the substrate or products. To evaluate new substrates of MAO-B this method is especially useful when the direct product of amine oxidation is not available as reference standard.

Chapter 3

DEUTERIUM ISOTOPE EFFECTS FOR THE OXIDATION OF 1-METHYL-3-PHENYL-3-PYRROLINYL ANALOGUES BY MONOAMINE OXIDASE B

Accepted for publication 2 September 2008

(Published article in Annexure B)

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Graphical Abstract:

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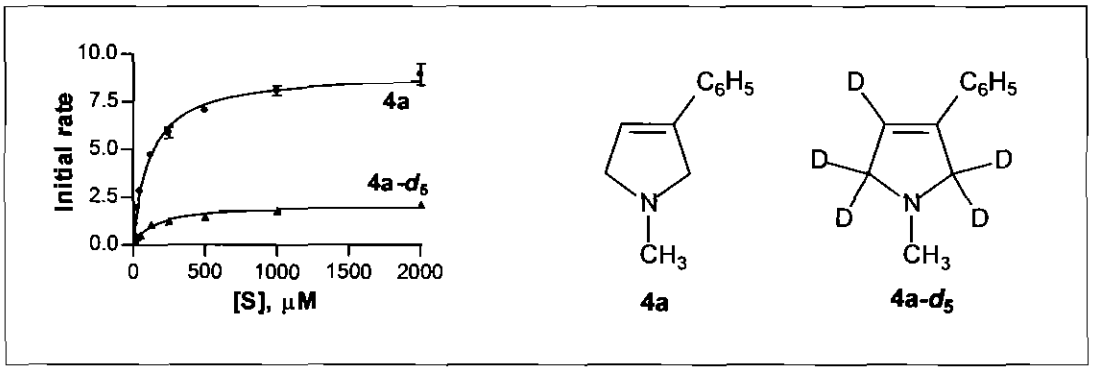

Deuterium isotope effects for the oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues by monoamine oxidase B

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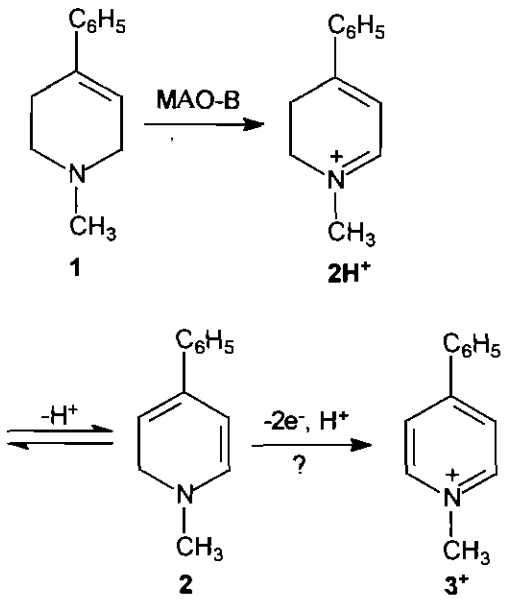
Abstract—The parkinsonian inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is a cyclic tertiary allylamine exhibiting good monoamine oxidase B (MAO-B) substrate properties. MAO-B catalyzes the ring α-carbon 2-electron bioactivation of MPTP to yield the 1-methyl-4-phenyl-2,3-dihydropyridinium species ($MPDP^+$). The corresponding 5-membered ring MPTP analogue, 1-methyl-3-phenyl-3-pyrroline, also undergoes MAO-B catalyzed oxidation to give the 2-electron oxidation product, 1-methyl-3-phenylpyrrole. Here we report the kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the steady-state oxidation of 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-fluorophenyl)-3-pyrroline by baboon liver MAO-B, using the corresponding pyrroline-2,2,4,5,5-d_5 analogues as the deuterated substrates. The apparent isotope effects for the two substrates were 4.29 and 3.98 on V_{max}, while the isotope effects on V_{max}/K_m were found to be 5.71 and 3.37, respectively. The values reported for the oxidation of MPTP by bovine liver MAO-B with MPTP-6,6-d_2, as deuterated substrate, are $^D(V_{max})$ = 3.55; $^D(V_{max}/K_m)$ = 8.01. We conclude that the mechanism of the MAO-B-catalyzed oxidation of pyrrolinyl substrates is similar to that of the tetrahydropyridinyl substrates and that a carbon-hydrogen bond cleavage step is, at least partially, rate-determining.

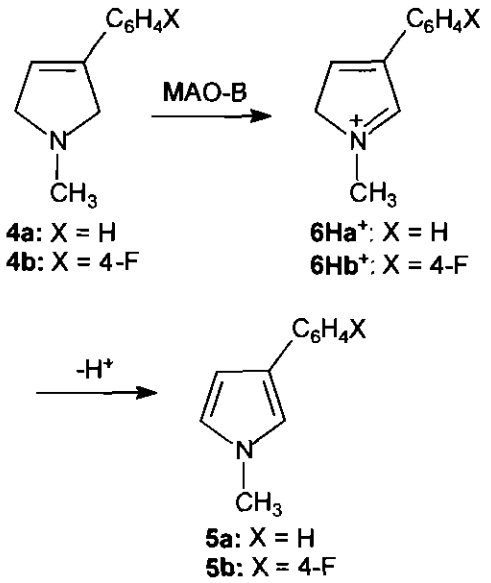
Keywords: Monoamine oxidase B; MPTP; 1-Methyl-3-phenyl-3-pyrroline; Kinetic isotope effect.

1. Introduction

The metabolic activation of the proneurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)], is catalyzed by the flavoenzyme, monoamine oxidase B (MAO-B) (Scheme 1),[1,2] to yield the α-carbon 2-electron oxidation product, the corresponding 1-methyl-4-phenyl-2,3-dihydropyridinium species, $MPDP^+$ ($\mathbf{2H^+}$). Following a second 2-electron oxidation, presumably via the corresponding free base **2**, the ultimate neurotoxic metabolite, 1-methyl-4-phenylpyridinium [MPP^+ ($\mathbf{3^+}$)] is generated.[1,3,4] MPP^+ is a mitochondrial toxin which selectively damages nigrostriatal neurons and induces a parkinsonian syndrome in humans[5,6] and other susceptible mammals.[7–9] MPTP is the first reported cyclic tertiary allylamine that acts as a good MAO-B substrate.[1] Several other tertiary aminyl substrates of MAO-B have since been described.[10,11] Among these, 1-methyl-3-phenyl-3-pyrroline (**4a**), the 5-membered ring analogue of MPTP, has MAO-B substrate properties comparable to those of MPTP.[12,13] The ring α-carbon oxidation of **4a** results in 1-methyl-3-phenylpyrrole (**5a**), an overall 2-electron process that most likely arises via $\mathbf{6Ha^+}$, the short-lived conjugate acid of **5a** (Scheme 2).

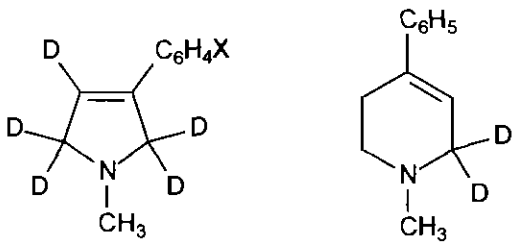


Scheme 1. The MAO-B-catalyzed α-carbon oxidation of MPTP (**1**) to yield the corresponding 2,3-dihydropyridinium product $MPDP^+$ ($\mathbf{2H^+}$). Further oxidation via an unknown pathway results in the pyridinium species MPP^+ ($\mathbf{3^+}$).



Scheme 2. The MAO-B-catalyzed α-carbon oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues (**4a** and **4b**) to yield intermediates $\mathbf{6Ha}^+$ and $\mathbf{6Hb}^+$. Deprotonation of $\mathbf{6Ha}^+/\mathbf{6Hb}^+$ results in the corresponding 1-methyl-3-phenylpyrrolyl species **5a** and **5b**.

In the present study we report the steady-state kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the oxidation of 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-fluorophenyl)-3-pyrroline (**4b**) by baboon liver mitochondrial MAO-B. For this purpose we have employed the corresponding 1-methyl-3-phenyl-3-pyrrolinyl-2,2,4,5,5-d_5 (**4a**-d_5) and 1-methyl-3-(4-fluorophenyl)-3-pyrrolinyl-2,2,4,5,5-d_5 (**4b**-d_5) analogues as deuterated substrates (Scheme 3). The apparent isotope effects for the two substrates were compared to the isotope effects previously reported for the oxidation of MPTP by bovine liver mitochondrial MAO-B using MPTP-**6**,6-d_2 (**1**-d_2) as deuterated substrate.[14] The results of this study are discussed with reference to the extent to which carbon-hydrogen bond cleavage is rate limiting during MAO-B catalyzed oxidation of pyrrolinyl substrates.



4a-d_5: X = H
4b-d_5: X = 4-F

1-d_2

Scheme 3. The structures of 1-methyl-3-phenyl-3-pyrroline-2,2,4,5,5-d_5 (**4a-d_5**), 1-methyl-3-(4-fluorophenyl)-3-pyrroline-2,2,4,5,5-d_5 (**4b-d_5**) and MPTP-6,6-d_2 (**1-d_2**).

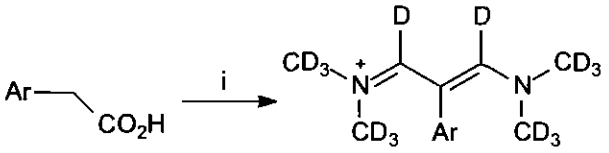
2. Results

2.1. Chemistry

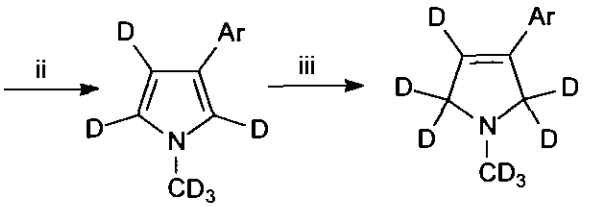
The 1-methyl-3-phenyl-3-pyrrolinyl analogues (**4a–4b**) examined here, were prepared by partial reduction of the corresponding pyrrolyl analogues **5a–b** as previously reported.[13,15,16] TLC and ^{1}H NMR analysis indicated that the 3-pyrrolines prepared in this manner were contaminated with the corresponding pyrrolidinyl derivatives formed by the over-reduction of the pyrrolyl substrates.[17] Also, HPLC analysis revealed trace amount of the pyrrolyl starting material present in samples of the free bases of **4a** and **4b**. In order to remove the volatile pyrrolidinyl contaminant from **4b**, the free base was subjected to high vacuum (see Experimental) while the pyrrolyl contaminant was removed by converting **4b** to the corresponding oxalate salt (**4b**·oxalate). For the purification of **4a**, both the corresponding pyrrolidinyl and pyrrolyl contaminants were removed via fractional recrystallization of the oxalate salt (**4a**·oxalate) as reported.[13]

In order to replace the methylene protons of the ring α-carbons (C-2 and C-5) of the pyrrolinyl analogues with deuterons, a similar synthetic approach was initially followed (Scheme 4). Because of the relative ease of purification of the 4-fluorophenyl substituted pyrrolinyl analogue **4b** compared to the unsubstituted analogue **4a** (see above), compound **4b-d_8** was chosen as the target 3-pyrroline. The key starting material, **7b-d_{14}** was prepared by reaction of 4-fluorophenylacetic acid with DMF-d_7 in the presence of $POCl_3$.[18] ^{1}H NMR analysis indicated that deuterium incorporation was >99% at the anticipated methyl and methinyl carbons (Scheme 4). Treatment of **7b-d_{14}** with a sodium methoxide solution (prepared with methanol-d_1) in dry pyridine yielded the pyrrolyl analogue **5b-d_6**.[19,20] While the deuterium incorporation at the N-CH_3 carbon was high (97.8%), proton-deuteron exchange occurred at the C-2, C-4 and C-5 of the

pyrrolyl heterocyclic system resulting in deuteron loss to the extent of 6–27%. However, upon reduction with zinc/DCl (to yield **4b-d_8**),[13,15,21] deuterium incorporation at C-2, C-4 and C-5 of the pyrrolinyl ring were again relatively high (>96%). This result indicated that the zinc/DCl reduction procedure could be accompanied by the exchange of pyrrolinyl protons for deuterons. It was possible to take advantage of this type of reaction to prepare both **4b-d_5** and the more difficult to purify **4a-d_5**, by simply reducing the corresponding pyrrolyl analogue with zinc/DCl. Accordingly, zinc/DCl reduction of **5a** and **5b** yielded **4a-d_5** and **4b-d_5**, respectively, with a relatively high degree (>98%) of deuterium incorporation at C-2, C-4 and C-5 of the pyrrolinyl ring (Scheme 5). No deuterium incorporation was observed at N-CH_3.

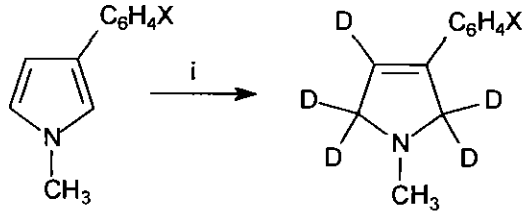


7b-d_{14}: Ar = 4-FC_6H_4



5b-d_6: Ar = 4-FC_6H_4 **4b-d_8**: Ar = 4-FC_6H_4

Scheme 4. Synthetic pathway to the deuterated pyrrolinyl substrate, **4b-d_8**. Key: (i) DMF-d_7, $POCl_3$, 80 °C; (ii) $NaOCH_3$ (from methanol-d_1), pyridine, reflux; (iii) Zn/DCl.



5a: X = H
5b: X = 4-F

4a-d_5: X = H
4b-d_5: X = 4-F

Scheme 5. Synthetic pathway to the deuterated pyrrolinyl substrates **4a-d_5** and **4b-d_5**. Key: (i) Zn/DCl.

2.2. General enzymology

In order to measure the rates of oxidation of the pyrrolinyl analogues **4a–4b** and **4a-d_5–4b-d_5**, the substrates were incubated with the enzyme for a fixed period of time at 37 °C and the corresponding pyrrolyl oxidation products were quantified by HPLC-UV analysis (Fig. 1) as described previously for **4a**.[13] HPLC-UV analysis was preferred to spectrophotometry since background interference in the near-UV wavelength range by the mitochondrial fractions used here as enzyme source, prevented accurate spectrophotometric measurements at 270 nm, the approximate wavelength of maximal absorption of the pyrrolyl products. The incubation time of the enzyme-catalyzed reactions were chosen to be 10 min since the oxidation of all the substrates evaluated here (**4a–4b** and **4a-d_5–4b-d_5**) was found to be linear (Fig. 2) for at least 15 min at substrate concentrations of 100 μM. Control incubations of the substrates carried out with no enzyme source added were used to verify the absence of the pyrrolyl products in the incubation samples.

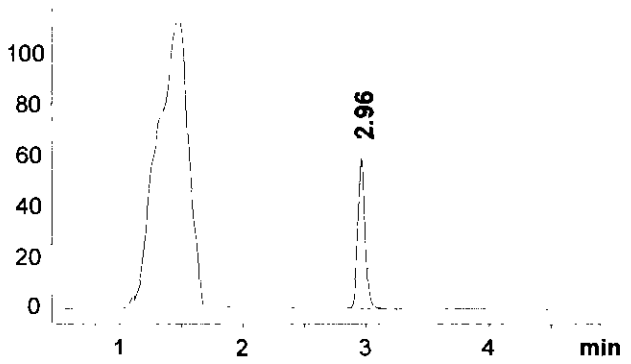


Figure 1. An HPLC-UV tracing showing the presence of 1-methyl-3-phenylpyrrole (**5a**) (retention time of 2.96 min) in an incubation of 1-methyl-3-phenyl-3-pyrroline (**4a**) with baboon liver MAO-B (0.15 mg protein/mL of the mitochondrial preparation). Following 10 min incubation, the reaction was terminated by the addition of trichlorocetic acid. After centrifugation, 50 μL of the supernatant was injected into the HPLC and the effluent was monitored at a wavelength of 270 nm.

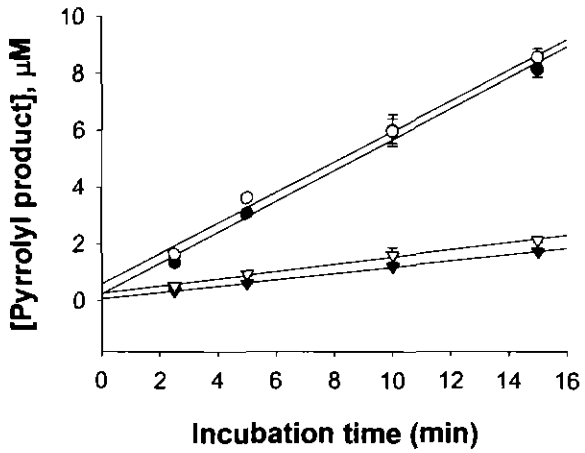


Figure 2. Linearity in the oxidation of pyrrolinyl analogues **4a** (filled circles), **4b** (open circles), **4a-d_5** (filled triangles) and **4b-d_5** (open triangles) by baboon liver mitochondrial MAO-B (0.15 mg protein/mL of the mitochondrial preparation). The concentration of pyrrolyl products was quantified via HPLC-UV analysis following termination of the enzyme catalyzed reaction at time points of 2.5, 5.0, 10.0 or 15.0 minutes. The concentration of the substrates used in this study was 100 µM.

To estimate the extent on non-MAO-B mediated oxidation of the pyrrolinyl substrates **4a** and **4a-d_5**, the baboon liver mitochondrial fractions that served as enzyme source were preincubated with the MAO-B selective inactivator (*R*)-deprenyl.[22] Following incubation of the test substrates (50 µM) with the mitochondria inactivated with (R)-deprenyl, only low concentrations of the pyrrolyl oxidation products were observed. For example, for substrate **4a** the yield of pyrrolyl oxidation product **5a** (0.54 ± 0.052 µM) with the inactivated mitochondrial preparation was only 8.4% of that formed with the fully active preparation (6.42 ± 0.18 µM). Similarly, when the deuterium labeled analogue **4a-d_5** was incubated with (*R*)-deprenyl pre-inactivated mitochondria only 0.037 ± 0.001 µM (about 2.6%) of the pyrrolyl oxidation product was observed compared to the amount formed (1.42 ± 0.05 µM) with the fully active preparation. These results document that oxidation of the pyrrolinyl substrates by the baboon liver mitochondrial fraction is MAO-B dependent. The residual activity not suppressed by (*R*)-deprenyl has been observed previously in experiments where tetrahydropyridines served as the MAO-B substrates.[22] The factors contributing to this residual activity remain to be identified. At least for baboon liver mitochondria the residual activity is not related to the action of the MAO-A isoform since the

baboon liver mitochondria are devoid of MAO-A activity.[22] Furthermore, 1-methyl-3-phenyl-3-pyrroline (**4a**) is reported to be an MAO-B selective substrate.[12]

2.3. Steady-state kinetic deuterium isotope effects on V_{max} and V_{max}/K_m

In order to estimate the values of the steady-state kinetic parameters (K_m and V_{max}) for the oxidation of the oxalate salts of the pyrrolinyl analogues (**4a–4b** and **4a-d_5–4b-d_5**) by MAO-B, initial rates were measured at eight substrate concentrations spanning at least two orders of magnitude. As illustrated by example with figure 3, the steady-state oxidation of the substrates (**4a–4b** and **4a-d_5–4b-d_5**) by MAO-B, followed Michaelis–Menten behavior. Also, large kinetic isotope effects on V_{max} were apparent from the graphs. The K_m and V_{max} values as well as the steady-state kinetic deuterium isotope effects obtained for the oxidation of the pyrrolinyl substrates by baboon liver mitochondrial MAO-B are summarized in Table 1. The apparent isotope effects on V_{max} for the oxidation of **4a** and **4b** were found to be 4.29 and 3.98 using **4a-d_5** and **4b-d_5**, respectively, as the deuterated substrates. These values are comparable to the $^D(V_{max})$ value of 3.55 previously reported for the oxidation of MPTP by bovine liver mitochondrial MAO-B with MPTP-6,6-d_2 as the deuterated substrate.[14] We have observed a smaller difference between the K_m value for **4a** and its deuterated analogue **4a-d_5** [$^D(K_m = 0.80)$] than the reported difference between the K_m values of MPTP and MPTP-6,6-d_2 [$^D(K_m = 0.45)$].[14] The K_m value of substrate **4b**, was found to be, within the range expected for experimental error, equal to that of its deuterared analogue, **4b-d_5** [$^D(K_m = 1.19)$].

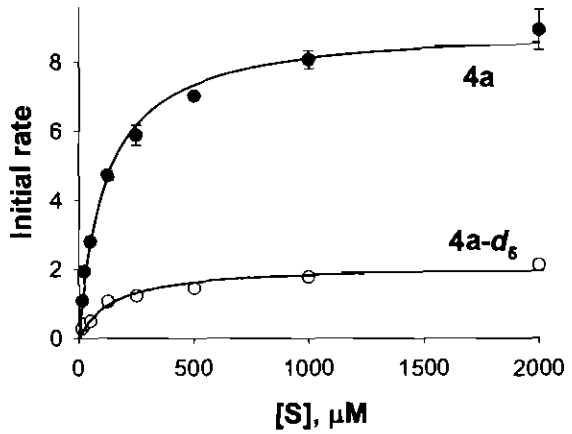


Figure 3. Determination of the K_m and V_{max} values for the oxidation of **4a** (filled circles) and **4a-d_5** (open circles) by baboon liver MAO-B (0.15 mg protein/mL of the mitochondrial preparation). The concentrations of the pyrrolyl oxidation products were measured by HPLC analysis

following a 10 min incubation at 37 °C. The rate data were fitted to the Michaelis–Menten equation. All measurements were conducted in triplicate and the concentration of the test substrates in the incubations ranged from 12.5 to 2000 μM. The initial rates are expressed as nmoles product formed/min-mg protein.

Table 1. Steady-state kinetic constants and deuterium isotope effects for the MAO-B catalyzed oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues

	V_{max}[a]	K_m (μM)	$^D(V_{max})$	$^D(K_m)$	$^D(V_{max}/K_m)$
4a	9.07±0.41	118±7.27			
4b	15.7±1.73	87.2±1.87			
4a-d_5	2.11±0.05	157±26.1	4.29±0.08	0.80±0.15	5.71±0.88
4b-d_5	3.96±0.52	73.8±4.46	3.98±0.09	1.19±0.10	3.37±0.20

[a]Values are expresses in nmol/min-mg mitochondria.

3. Discussion

In this study we have measured the kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the steady-state oxidation of 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-fluorophenyl)-3-pyrroline (**4b**) by baboon liver mitochondrial MAO-B. For this purpose the corresponding pyrroline-2,2,4,5,5-d_5 analogues (**4a-d_5** and **4b-d_5**) were employed as deuterated substrates. Moderately large apparent isotope effects of 4.29 and 3.98 on V_{max} were observed for the two substrates, respectively. The isotope effects on V_{max}/K_m were found to be 5.71 and 3.37 for the two substrates, respectively. Although these values may not reflect the intrinsic isotope effects, the V_{max} isotope effects suggest that a carbon-hydrogen bond cleavage step is at least partially rate determining.[23] The isotope effects on V_{max} for the two pyrrolinyl substrates are comparable to those reported for the oxidation of MPTP by bovine liver MAO-B [$^D(V_{max}) = 3.55$]. We conclude that the mechanism of the MAO-B-catalyzed oxidation of pyrrolinyl substrates is similar to that of the tetrahydropyridinyl substrates.

4. Experimental

4.1. Chemicals and instrumentation

All starting materials, unless otherwise stated, were obtained from Sigma-Aldrich and were used without purification. Deuterated solvents and reagents were obtained from Cambridge Isotope Laboratories. Proton and carbon NMR spectra were recorded on a Varian Gemini 300 spectrometer. ^{1}H-NMR spectra were recorded at a frequency of 300 MHz and ^{13}C-NMR spectra

at 75 MHz. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the NMR solvents, $CDCl_3$, DMSO-d_6 and CD_3OD. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), sep (septet) or m (multiplet) and the coupling constants (*J*) are given in hertz (Hz). Fast atom bombardment mass spectra (FAB-MS) were recorded with a VG 7070E mass spectrometer. Direct insertion electron impact ionization mass spectra (EI-HRMS) were obtained with a AutoSpec ETOF (Micromass). Melting points (mp) were determined with a Stuart SMP10 melting point apparatus; all melting points are uncorrected. HPLC analyses were performed with an Agilent 1200 HPLC system equipped with an Agilent 1200 series variable wavelength detector. Thin layer chromatography (TLC) was carried out using neutral aluminum oxide 60 (Merck) with UV_{254} fluorescent indicator.

4.2. Synthesis of *2-(4-Fluorophenyl)-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorate-d_{14}* (**7b-d_{14}**)

Compound **7b-d_{14}** was prepared in high yield from 4-fluorophenylacetic acid, N,N-dimethylformamide-d_7 (DMF-d_7) and phosphoryl chloride according the method described in literature.[18]

2-(4-Fluorophenyl)-3-(dimethylamino)allylidene(dimethyl)ammonium perchlorate-d_{14} (**7b-d_{14}**): yield 82.5%; mp 128–133 °C (from ethanol); ^{1}H NMR (DMSO-d_6) δ 7.22–7.29 (m, 2H), 7.31–7.38 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 38.45 (m), 47.57 (sep), 103.55, 115.27 (d), 128.74, 134.11 (d), 160.47, 162.66 (t), 163.74; FAB-MS *m/z*: 236 (MH^+).

4.3. Synthesis of 1-methyl-3-phenylpyrroles 5a–b and 5b-d_6

1-Methyl-3-phenylpyrroles **5a–b** were synthesized according to a modification[20] of the method described in the literature.[19] Compound **5b-d_6** was synthesized from **7b-d_{14}** according to the following procedure:[19,20] Sodium pieces (13 mmol) was reacted with methanol-d_1 (4.5 mL) and the resulting solution was added under an atmosphere of argon to a solution of **7b-d_{14}** (6 mmol) in 24 mL dry pyridine (distilled over CaH_2 and stored over 4 Å molecular sieves). The reaction was heated under reflux for 24 hours and most of the pyridine was removed via vacuum distillation to obtain a yellow pasty residue to which 25 mL D_2O was added. The resulting suspension was stirred for 30 min on an ice bath and filtered. The crude solid so obtained was dissolved in a minimum amount of ethylacetate and purified on a short column (30 × 60 mm) by neutral aluminum oxide chromatography (Fluka) with petroleum ether/ethylacetate, 90:10 as mobile phase to give a white solid. The melting points of previously reported **5a** and **5b** were as follows:

5a mp 46–48 °C (from petroleum ether), lit. mp 46–47 °C;[19] **5b** mp 100–101 °C (from methanol), lit. mp 98–100 °C;[20] The characterization of previously unreported **5b-d_6** is summarized below.

1-Methyl-3-(4-fluorophenyl)pyrrole-d_6 (**5b-d_6**) was synthesized from **7b-d_{14}** in a yield of 46.8%: mp 102–103 °C; ^{1}H NMR ($CDCl_3$) δ 6.37 (0.06H), 6.61 (0.01H), 6.82 (s, 0.27H), 6.97–7.04 (m, 2H), 7.39–7.46 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 35.67 (m), 106.11 (d), 115.25 (d), 118.19 (m), 122.67 (d), 124.09, 126.28 (d), 132.16 (d), 159.45, 162.68; EI-HRMS calcd. 181.11739, found 181.11819 (M^+).

4.4. Synthesis of 1-methyl-3-phenyl-3-pyrrolinyl analogues (4a–4b, 4a-d_5–4b-d_5 and 4b-d_8) and their respective oxalate salts

Compounds **4a–b** were synthesized from the corresponding pyrrolyl analogues, **5a–5b**, according to a modification[13] of the zinc reduction procedure reported in the literature.[15,16] Compounds **4a-d_5**, **4b-d_5** and **4b-d_8** were synthesized from **5a**, **5b** and **5b-d_6**, respectively, according to the following procedure: DCl (15 mL, 20%) was cooled on an ice bath (0 °C) and zinc dust (20 mmol) was carefully added followed by the appropriate pyrrolyl analogue (4 mmol) dissolved in 34 mL ethanol-d_1. The resulting suspension was stirred for 1 h at 0 °C and DCl (10 mL, 35%) was added. The reaction was allowed to return to room temperature and stirred for another 1 h. Another 2 portions of zinc dust (40 mmol) were added and the reaction was stirred for 2 h during which the reaction cleared. The remaining zinc was removed via filtration and washed with 5 mL D_2O. The residual ethanol-d_1 was removed from the filtrate under reduced pressure, the pH was adjusted to ~12 with NaOH (8 N) and the reaction was extracted to diethylether (3 × 30 mL). The organic phase was dried over $MgSO_4$, removed under reduced pressure to yield the product as a light yellow oil. The pyrrolinyl analogues were converted to their respective oxalic acid salts in diethyl ether. Compounds **4a** and **4a-d_5** were purified by recrystallizing their oxalate salts twice from methanol. Compounds **4b**, **4b-d_5** and **4b-d_8** were purified by placing the free bases in a vacuum oven at 40 °C for 24 hours. For previously described **4a**·oxalate the melting point was recorded as 151–153 °C (from methanol), lit. mp 152–154 °C.[13] The characterizations of compounds that were previously unreported are summarized below.

1-Methyl-3-phenyl-3-pyrroline-d_5 (**4a-d_5**) was synthesized from **5a** in a yield of 16.5% (oxalate salt): mp 152–155 °C; ^{1}H NMR (CD_3OD) δ 3.10 (s, 3H), 7.35–7.43 (m, 3H), 7.47–7.50 (m, 2H); ^{13}C NMR (CD_3OD) δ 41.68, 60.93 (m), 62.00 (m), 117.87 (m), 126.43, 129.37, 129.58, 132.21, 137.59, 166.28; EI-HRMS calcd. 164.13618, found 164.13641 (M^+).

1-Methyl-3-(4-fluorophenyl)-3-pyrroline (**4b**) was synthesized from **5b** in a yield of 29.5% (free base): mp 64–66 °C; ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 3H), 3.60–3.65 (m, 2H), 3.77–3.80 (m, 2H),

6.02 (m, 1H), 6.95–7.01 (m, 2H), 7.27–7.31 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 42.67, 62.38 (d), 115.30 (d), 121.93 (d), 127.00 (d), 130.67 (d), 138.97, 160.52, 163.79; EI-HRMS calcd. 177.09538, found 177.09629 (M^{+}).

1-Methyl-3-(4-fluorophenyl)-3-pyrroline-d_5 (**4b-d_5**) was synthesized from **5b** in a yield of 36.3% (free base): mp 63–66 °C; ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 3H), 6.94–7.00 (m, 2H), 7.26–7.30 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 42.66, 61.95 (m), 115.28 (d), 121.90 (m), 126.99 (d), 130.65 (d), 138.79, 160.50, 163.77; EI-HRMS calcd. 182.12676, found 182.12585 (M^{+}).

1-Methyl-3-(4-fluorophenyl)-3-pyrroline-d_8 (**4b-d_8**) was synthesized from **5b-d_6** in a yield of 48.0% (free base): mp 64–68 °C; ^{1}H NMR ($CDCl_3$) δ 6.94–7.02 (m, 2H), 7.24–7.32 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 41.90 (m), 61.95 (m), 115.30 (d), 121.90 (m), 127.01 (d), 130.69 (d), 138.83, 160.52, 163.79; EI-HRMS calcd. 185.14559, found 185.14566 (M^{+}).

4.5. Steady-state MAO-B activity measurements

Baboon liver mitochondria were isolated as described in the literature[24] and stored at –70 °C. The mitochondrial isolates were suspended in 1 volume sodium phosphate buffer (100 mM, pH 7.4) containing 50% glycerol (w/v) and the protein concentrations were determined by the method of Bradford.[25] Since baboon liver is devoid of MAO-A activity, inactivation of MAO-A was deemed unnecessary.[22] In order to estimate the K_m and V_{max} values for the oxidation of the oxalate salts of the pyrrolinyl analogues **4a–4b** and **4a-d_5–4b-d_5** by MAO-B, initial rates were measured at eight substrate concentrations spanning at least two orders of magnitude (12.5–2000 μM). The reactions were carried out in a final volume of 500 μL (in 100 mM sodium phosphate buffer, pH 7.4) and the enzyme concentration used was 0.15 mg mitochondrial protein/mL. Following incubation at 37 °C for a period of 10 min, the reactions were terminated by the addition of 20 μL trichloroacetic acid (1g/mL, w/v). A volume of 100 μL acetonitrile was added, and the resulting samples were centrifuged at 16000*g* for 10 min. The supernatant fractions were removed and the concentrations of the MAO-B-generated pyrrolyl products were measured by HPLC analysis with UV detection as described before.[13] The elution of the pyrrolyl oxidation products were monitored at a wavelength of 270 nm and quantitative estimations were made by means of linear calibration curves ranging from 1.5 to 25 μM of **5a** and **5b**. The steady-state kinetic data (initial rates as a function of substrate concentration) were fitted to the Michaelis–Menten equation using the one site binding model incorporated into the GraphPad Prism software package (GraphPad Software Inc.). All measurements were conducted in triplicate and the K_m and V_{max} values were expressed as means ± standard error of the mean (SEM).

4.6. Linearity of oxidation of pyrrolinyl substrates

In order to determine the time interval for which MAO-B catalyzed substrate oxidation remains linear, 100 μM of the oxalate salts of the test substrates (**4a**–**4b** and **4a**-d_5–**4b**-d_5) were incubated with 0.15 mg protein/mL of the baboon liver mitochondrial fraction. At time points 2.5, 5, 10 and 15 min, the reactions were terminated with the addition of 20 μL trichloroacetic acid (1g/mL, w/v) and the MAO-B-generated pyrrolyl products were measured by HPLC analysis as described above. All measurements were conducted in triplicate and the concentrations of the pyrrolyl products were expressed as means ± SEM.

4.7. (*R*)-Deprenyl studies

In order to estimate the degree to which the oxidation of the pyrrolinyl substrates (**4a** and **4a**-d_5) was dependent upon MAO-B, baboon liver mitochondria (0.3 mg protein/mL) were preincubated with (*R*)-deprenyl·HCl (3×10^{-6} M) at 37 °C for 30 min in 100 mM sodium phosphate buffer, pH 7.4. The pre-inactivated mitochondrial fractions were then added to the test substrates to yield final concentrations of 0.15 mg mitochondrial protein/mL. The volume of these incubation mixtures were 500 μL and the final concentration of the oxalate salts of the test substrates was 50 μM. The reactions were incubated for 10 min at 37 °C and terminated with the addition of 20 μL trichloroacetic acid (1g/mL, w/v). Control incubation reactions were carried out following the same procedure with the exception that the preincubations were conducted in the absence of (*R*)-deprenyl. The concentrations of the pyrrolyl products in the enzymatic reactions were measured by HPLC analysis as described above. All measurements were conducted in triplicate and the concentrations of the pyrrolyl products were expressed as means ± SEM.

Acknowledgements

We are grateful to Jan du Preez and the staff of the Analytical Technology Laboratory, North-West University for their support. The NMR and MS spectra were recorded by André Joubert, Johan Jordaan and Louis Fourie of the SASOL Centre for Chemistry, North-West University. This work was supported by grants from the National Research Foundation and the Medical Research Council, South Africa.

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Chapter 4

INTERACTIONS OF 1-METHYL-3-PHENYL-PYRROLIDINE AND 3-METHYL-1-PHENYL-3-AZABICYCLO[3.1.0]HEXANE WITH MONOAMINE OXIDASE B

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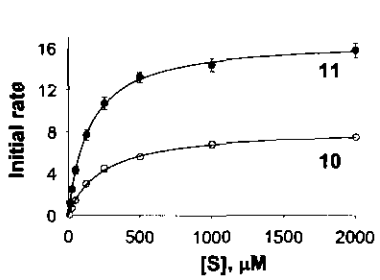
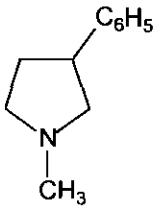
Interactions of 1-methyl-3-phenylpyrrolidine and 3-methyl-1-phenyl-3-azabicyclo-[3.1.0]hexane with monoamine oxidase B

Anél Pretorius,[a] Modupe O. Ogunrombi,[a] Neal Castagnoli, Jr.,[b] Jacobus J. Bergh,[a] and Jacobus P. Petzer[a]





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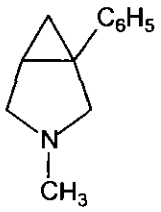

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Interactions of 1-methyl-3-phenylpyrrolidine and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane with monoamine oxidase B

Anél Pretorius,[a] Modupe O. Ogunrombi,[a] Neal Castagnoli, Jr.,[b] Jacobus J. Bergh,[a] and Jacobus P. Petzer[a]

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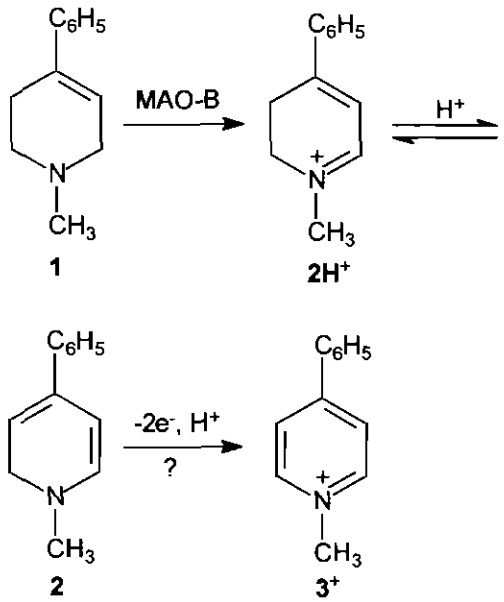
Abstract—The parkinsonian inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its corresponding 5-membered analogue, 1-methyl-3-phenyl-3-pyrroline, are cyclic tertiary allylamines that act as good substrates for monoamine oxidase B (MAO-B). The MAO-B catalyzed α-carbon 2-electron oxidation of this class of substrates appears to be dependent on the presence of the allylic π-bond, since the corresponding saturated piperidinyl analogue of MPTP is not an MAO-B substrate. The only saturated, cyclic tertiary amine known to act as an MAO-B substrate, is the 3,4-cyclopropyl analogue of MPTP, 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane. As part of our ongoing investigation into the MAO-B substrate properties of 5-membered azacyclic systems, we have examined the interactions of the corresponding saturated pyrrolidinyl (1-methyl-3-phenylpyrrolidine) and 3,4-cyclopropyl (3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane) analogues of 1-methyl-3-phenyl-3-pyrroline with MAO-B. The results document that both the pyrrolidinyl [K_m = 234 μM; V_{max} = 8.37 nmol/(min-mg mitochondrial protein)] and the 3,4-cyclopropyl analogues [K_m = 148 μM; V_{max} = 16.9 nmol/(min-mg mitochondrial protein)] are efficiently oxidized by baboon liver MAO-B. We have also examined the possibility that these saturated substrates may act as neurotoxins in the C57BL/6 mouse. Since striatal dopamine depletion was not observed while analogous treatment with MPTP resulted in 64–73% depletion, it can be concluded that, unlike MPTP, the cyclic tertiary amines examined here are not nigrostriatal toxins.

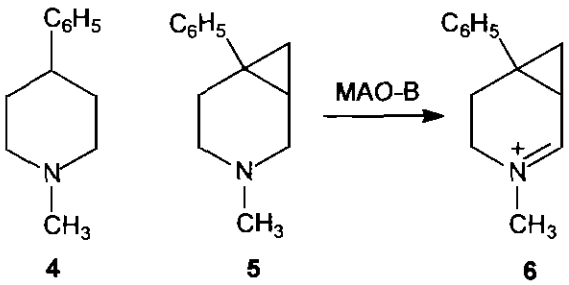
Keywords: Monoamine oxidase B; Saturated substrate; 1-Methyl-3-phenylpyrrolidine; 3-Methyl-1-phenyl-3-azabicyclo[3.1.0]hexane; 1-Methyl-3-phenyl-3-pyrroline.

1. Introduction

The flavoenzyme, monoamine oxidase B (MAO-B), catalyzes the α-carbon 2-electron oxidation of a variety of endogenous and exogenous arylalkylamines such as dopamine, adrenaline and benzylamine.[1] Also among its substrates is the parkinsonian inducing neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP, (**1**)] (Scheme 1).[2] MPTP is a member of the cyclic tertiary allylamine class of MAO-B substrates and is oxidized at C-6 of the tetrahydropyridinyl ring to yield the conjugated eniminium metabolite, 1-methyl-4-phenyl-2,3-dihydropyridinium [$MPDP^+$, ($\mathbf{2H^+}$)].[3] Presumably via the corresponding conjugate base **2**, the dihydropyridinium species undergoes a second 2-electron oxidation to generate the 1-methyl-4-phenylpyridinium metabolite, MPP^+ ($\mathbf{3^+}$), which is believed to act as the ultimate neurotoxin.[2,4,5] Inhibitors of MAO-B prevent the central conversion of MPTP to MPP^+ and protect experimental animals against the neurotoxic action of MPTP.[6] The MAO-B substrate properties of the cyclic tertiary aminyl class of substrates are thought to be dependent on the presence of the allylic π-bond, since the corresponding saturated piperidinyl analogue (**4**) of MPTP is not an MAO-B substrate (Scheme 2).[7,8] The only saturated, cyclic tertiary amine known to act as an MAO-B substrate is the 3,4-cyclopropyl analogue of MPTP, 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (**5**).[9] Compound **5** is oxidized by MAO-B to yield the corresponding iminium metabolite **6**. The π-bond characteristics of the cyclopropyl group may be responsible for **5** being a good substrate, while **4** is devoid of MAO-B substrate properties.[9]



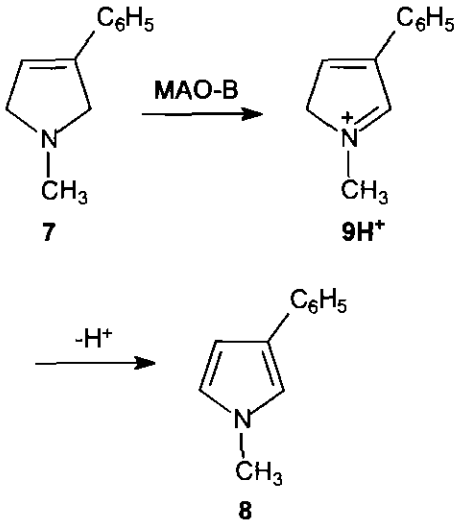
Scheme 1. The MAO-B-catalyzed oxidation of MPTP (**1**) at C-6 of the tetrahydropyridinyl ring to yield the corresponding conjugated eniminium metabolite $MPDP^+$ ($\mathbf{2H^+}$). Via an unknown pathway, $MPDP^+$ is oxidized further to yield the pyridinium MPP^+ ($\mathbf{3^+}$).



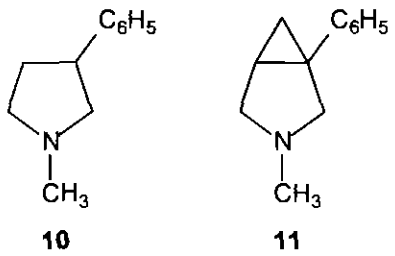
Scheme 2. The structures of 1-methyl-4-phenylpiperidine (**4**) and 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (**5**). The MAO-B catalyzed oxidation of **5** yields iminium **6**.

Another member of the cyclic tertiary allylamine class of MAO-B substrates is the 5-membered ring MPTP analogue, 1-methyl-3-phenyl-3-pyrroline (**7**) (Scheme 3).[10-12] MAO-B catalyzes the ring α-carbon oxidation of **7** to yield 1-methyl-3-phenylpyrrole (**8**) as the final product. This 2-electron oxidation process most likely proceeds via $\mathbf{9H^+}$, the short lived conjugate acid of the pyrrolyl product **8**.[13] As part of our ongoing investigation into the MAO-B substrate properties of 5-membered azacyclic systems, we have examined the interactions of the corresponding saturated pyrrolidinyl [1-methyl-3-phenylpyrrolidine (**10**)] and 3,4-cyclopropyl [3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**11**)] analogues of 1-methyl-3-phenyl-3-pyrroline with MAO-B

(Scheme 4). In view of the π-bond characteristics of the cyclopropyl group it is expected that **11** would, analogous to the 3,4-cyclopropyl analogue (**5**) of MPTP, act as a good MAO-B substrate. On the other hand, the pyrrolidinyl **10**, analogous to the piperidinyl **4**, would be expected to be devoid of MAO-B substrate properties.



Scheme 3. The MAO-B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrroline (**7**) at C-2 of the azacycle to yield intermediate $\mathbf{9H^+}$. Deprotonation of $\mathbf{9H^+}$ results in 1-methyl-3-phenylpyrrole (**8**).



Scheme 4. The structures of 1-methyl-3-phenylpyrrolidine (**10**) and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**11**).

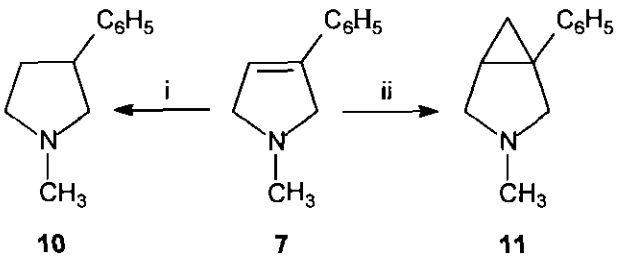
We have also examined the possibility that the saturated substrates **10** and **11** may act as neurotoxins in the C57BL/6 mouse.[14] The neurotoxic action of MPTP is dependent upon its metabolic conversion by MAO-B to yield the charged pyridinium species, MPP^+ ($\mathbf{3^+}$).[2] MPP^+, the ultimate neurotoxin[6,15] is thought to act by inhibiting complex I of the mitochondrial respiratory chain, only after accumulating in the inner mitochondrial membrane of the nigrostriatal nerve

terminals. This process is driven by the transmembrane electrochemical gradient[16,17] which appears to rely upon the presence of the permanent iminium charge of MPP^+. Since the MAO-B catalyzed 2-electron oxidations of substrates **10** and **11** may yield charged intermediates and end products, the possibility exists that these substrates could, similar to MPTP, be neurotoxic. In this study we have tested this hypothesis by measuring ex vivo striatal dopamine concentrations in C57BL/6 mice 7 days after administration of **10** and **11**.[13]

2. Results

2.1. Chemistry

Both substrates **10** and **11**, examined here, were synthesized from 1-methyl-3-phenyl-3-pyrroline (**7**)[13] according to the reaction pathways illustrated in Scheme 5. Atmospheric pressure hydrogenation of **7** in the presence of PtO_2 gave **10** in high yield (70.95%).[18] In a reaction similar to that previously described for the cyclopropylation of MPTP,[9] treatment of **7** with an excess of CH_2I_2 and diethylzinc yielded **11** (24.36%). Both substrates were converted to their respective oxalate salts. Compounds **10**[19] and **11**[20] have been previously reported, but have been prepared according to different synthetic pathways.



Scheme 5. Synthetic pathway to compounds **10** and **11** Key: (i) H_2 (atm), PtO_2, rt; (ii) $(C_2H_5)_2Zn$, CH_2I_2.

2.2. General enzymology

In the present study we have examined the MAO-B substrate properties of the saturated cyclic tertiary amines **10** and **11**. As enzyme source we have employed the mitochondrial fraction obtained from baboon liver tissue since it exhibits a high degree of MAO-B catalytic activity.[21] Also, based on the similarity of steady-state kinetic parameters (K_m and V_{max}) between baboon and human MAO-B, the interactions of substrates with MAO-B obtained from baboon liver tissue appears to be similar to the interaction with the human form of the enzyme.[21] The rates of oxidation of **10** and **11** by baboon liver mitochondrial MAO-B were measured by monitoring the

amount of H_2O_2 generated by the enzyme. In the catalytic cycle of MAO-B, one mole of O_2 is reduced to H_2O_2 for each mole of substrate oxidized. The concentration of H_2O_2 in the enzyme reactions can be conveniently measured by the peroxidase-coupled spectrophotometric assay system described previously.[22] For the purpose of this study we have chosen a discontinuous assay protocol with an incubation time of 20 min (for substrate **10**) or 15 min (for substrate **11**). As illustrated in figure 1, the H_2O_2 generated as a result of the oxidation of **10** and **11** by baboon liver mitochondrial MAO-B remained linear for at least 20 min at a substrate concentrations of 500 μM and 250 μM, respectively. Control incubations carried out in the absence of the test substrates documented that the background H_2O_2 generation by the mitochondrial fractions were less than 0.5 μM. This is less than 3% of the H_2O_2 measured in 20 min incubations of substrate **10** (21–24 μM) and in 15 min incubations of substrate **11** (33–39 μM) with the baboon liver mitochondrial fraction at V_{max} concentrations. This result verifies that the H_2O_2 generated (>97%) by the mitochondrial fractions are dependent upon the presence of the test substrates.

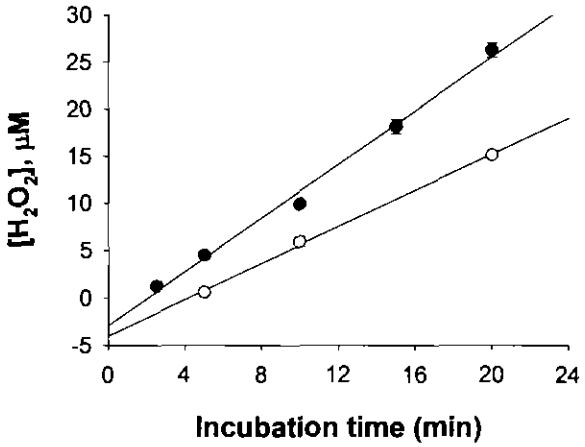


Figure 1. The interval for which MAO-B catalyzed oxidation of substrates **10** (500 μM; open circles) and **11** (250 μM; filled circles) remains linear. The enzyme source was baboon liver mitochondrial MAO-B (0.15 mg protein/mL of the mitochondrial preparation) and the enzyme generated H_2O_2 concentrations were determined by the peroxidase-coupled spectrophotometric assay system described previously.[22] The reactions were terminated at the indicated time points.

To estimate the extent of the H_2O_2 produced by non-MAO-B mediated oxidation of substrates **10** and **11**, the baboon liver mitochondrial MAO-B were pre-inactivated with the MAO-B selective inactivator (*R*)-deprenyl.[21] Following incubation of substrate **11** (250 μM) with the (*R*)-deprenyl pre-inactivated mitochondria, only low concentrations of H_2O_2 were observed. For example, the

H_2O_2 concentration (1.79 ± 0.11 μM) with the inactivated mitochondrial preparation was only 6.56% of that formed with the fully active preparation (27.3 ± 0.53 μM). Similarly, when substrate **10** (500 μM) was incubated with the (*R*)-deprenyl pre-inactivated mitochondria only 1.92 ± 0.06 μM of H_2O_2 was observed compared to the amount formed (20.6 ± 0.08 μM) with the fully active preparation. These results show that oxidation of **10** and **11** by the baboon liver mitochondrial fraction used here as enzyme source, is MAO-B dependent. The oxidation activity not suppressed by (*R*)-deprenyl has been observed previously where tetrahydropyridinyl analogues served as the MAO-B substrates.[21] At least for baboon liver mitochondria the residual activity is not related to the action of the MAO-A isoform since the baboon liver mitochondria are reported to be devoid of MAO-A activity.[21]

2.3. Steady-state MAO-B substrate properties of 10 and 11

The values of the steady-state kinetic parameters (K_m and V_{max}) for the MAO-B catalyzed oxidation of the test substrates **10** and **11** were determined by measuring the initial rates of H_2O_2 production[22] at eight substrate concentrations spanning at least two orders of magnitude. As illustrated in figure 2, the steady-state oxidation of **10** and **11** followed Michaelis–Menten behavior. The K_m and V_{max} values obtained for the oxidation of **10** and **11** by baboon liver MAO-B are presented in Table 1. Also listed are the corresponding kinetic values reported for the oxidation of MPTP (**1**) and 1-methyl-3-phenyl-3-pyrroline (7) by baboon liver MAO-B.[13] The K_m and V_{max} values (K_m = 148 μM; V_{max} = 16.9 nmol/min-mg protein) that were recorded for substrate **11** are comparable those of MPTP and 7. This documents that **11** acts as a good substrate of baboon liver MAO-B. Based on the V_{max}/K_m values it can be stated that **11** [V_{max}/K_m = 0.11 (min-mg protein)$^{-1}$] is a slightly weaker substrate than 7 [V_{max}/K_m = 0.17 (min-mg protein)$^{-1}$] while being a better substrate than MPTP [V_{max}/K_m = 0.039 (min-mg protein)$^{-1}$]. Considering that the steady-state kinetic parameters that are listed for of MPTP, 7 and **11** (Table 1) were measured with the same baboon liver mitochondrial preparation (and therefore the same MAO-B concentration) in our laboratory[13], the higher V_{max} value recorded for substrate **11** shows that it has a considerable higher turnover (k_{cat}) than the allylamine 7.

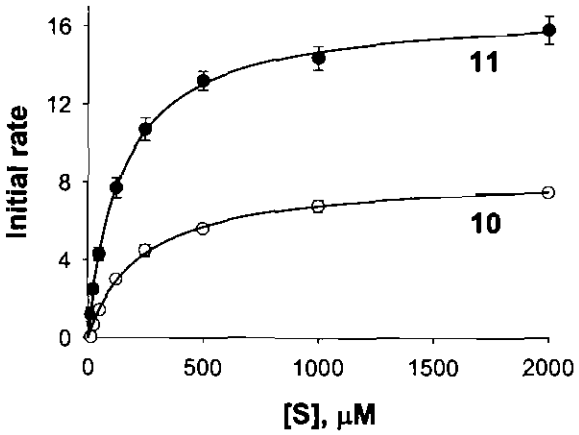


Figure 2. Determination of the values of the steady-state kinetic parameters (K_m and V_{max}) for the MAO-B catalyzed oxidation of test substrates **10** (open circles) and **11** (filled circles). The initial rates the oxidation by baboon liver MAO-B (0.15 mg protein/mL mitochondrial preparation) were measured by monitoring the amount of H_2O_2 generated by the enzyme following a 20 and 15 min incubation period (at 37 °C) for the two substrates, respectively. The rate data were fitted to the Michaelis–Menten equation and all measurements were conducted in triplicate. The initial rates are expressed as nmoles H_2O_2 formed/min-mg protein.

Table 1. Values of the steady-state kinetic parameters for the MAO-B catalyzed oxidation of cyclic tertiary aminyl substrates

	V_{max}[a]	K_m (μM)	V_{max}/K_m[b]
10	8.37±0.25	234±23.5	0.036
11	16.9±0.60	148±7.36	0.11
MPTP (**1**)	6.7[c]	173[c]	0.039[c]
7	9.2[c]	54.6[c]	0.17[c]

[a]Values are expresses in nmol/min-mg mitochondria.
[b]Values are expresses in (min-mg protein)$^{-1}$.
[c]Values obtained from reference.[13]

Substrate **10** was also found to be a relatively good substrate for baboon liver mitochondrial MAO-B with K_m and V_{max} values of 234 μM and 8.37 nmol/min-mg protein, respectively (Table 1). These values are also comparable to those reported for MPTP and 7 (Table 1). Based on the V_{max}/K_m values it can be stated that **10** [V_{max}/K_m = 0.036 (min-mg protein)$^{-1}$] is approximately as good a MAO-B substrate as is MPTP [V_{max}/K_m = 0.039 (min-mg protein)$^{-1}$]. Since the V_{max} values

of **10** (8.37 nmol/min-mg protein) and the corresponding alylamine **7** (9.2 nmol/min-mg protein) are within the same range, the turnover (k_{cat}) of these two substrates by baboon liver MAO-B are approximately equal.

2.4. Neurotoxicity studies

It has been documented that the administration of MPTP to C57BL/6 mice causes degeneration of the nigrostriatal neurons.[14] The extent of neuronal damage induced by the toxic action of MPP^+ can be estimated by measuring the degree of dopamine depletion in the striatum, 7–10 days following treatment. In a frequently used protocol, aged mice are treated with single intraperitoneal injections of MPTP (95–238 μmol/kg).[23,24] In this study we investigated whether similar treatment of C57BL/6 mice with **10** and **11** also results in nigrostriatal injury as indicated by a loss of dopamine in the striatum. For each of the test substrates a control group (n=10 mice), which received saline (0.20 ml /30g mouse), and a MPTP group (n=10 mice), which received the hydrochloric acid salt of MPTP (167 μmol/kg), were included. The third group (n=10 mice) was treated with the respective oxalate salts of substrates **10** and **11** at a dose of 238 μmol/kg. The animals were sacrificed 7 days after treatment, and the dopamine concentrations in the dissected striata were determined by HPLC-ECD analysis as described in the Experimental.[25] In accordance with expectation (Fig. 3) MPTP treatment significantly ($p < 0.01$) depleted the dopamine levels to 36% (Fig. 3, Top) and 27% (Fig. 3, Bottom) of the control values. In contrast, neither **10** nor **11** treatment depleted striatal dopamine with the measured dopamine concentrations at 99% (Fig. 3, Top) and 111% (Fig. 3, Bottom) of the control values, respectively.

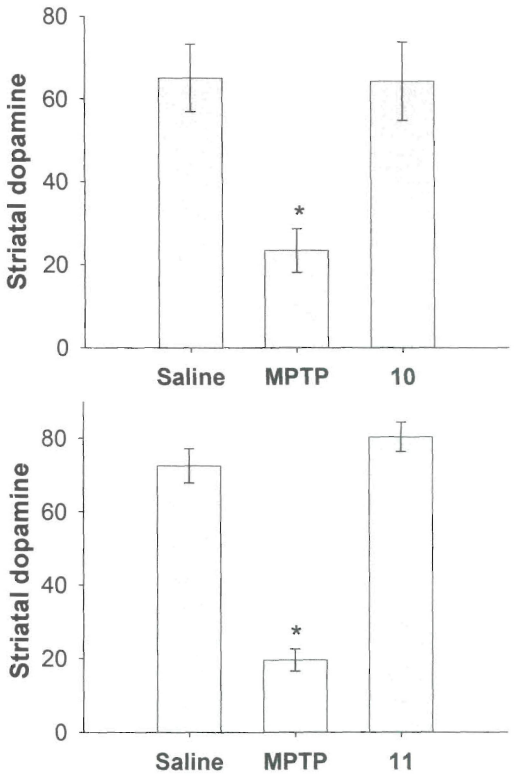


Figure 3. Striatal dopamine levels of C57BL/6 male mice (n=10 mice/group) measured seven days after treatment (ip) with saline, MPTP•HCl (167 μmol/kg), **10**•oxalate (238 μmol/kg) (Top) or **11**•oxalate (238 μmol/kg) (Bottom). Dopamine levels are expressed as pmol/mg tissue. *Significantly different ($p < 0.01$) from the saline treated group.

3. Discussion

The results document that the 3,4-cyclopropyl analogue **11** acts as an efficient substrate for baboon liver MAO-B with substrate properties comparable to that of the allylamine **7**. This result is expected since the 3,4-cyclopropyl analogue (**5**) of MPTP is also reported[9] to be a good MAO-B substrate. As mentioned in the introduction, the π-bond characteristics of the cyclopropyl group may be responsible for the observation that the saturated substrates **5** and **11** are approximately as good MAO-B substrates as their corresponding allylamine analogues MPTP (**1**) and **7**, respectively.[9] The pyrrolidinyl analogue **10** was also shown to be a relatively good substrate for baboon liver MAO-B, with substrate properties comparable to that of MPTP. This is in contrast to expectation since the saturated piperidinyl analogue (**4**) of MPTP is not a MAO-B substrate.[7,8]

This result therefore suggests that presence of an allylic π-bond or 3,4-cyclopropyl-fused system is not a prerequisite for all cyclic tertiary aminyl analogues to act as MAO-B substrates.

Also noteworthy is the observation that the α-carbon oxidation of MPTP and its 3,4-cyclopropyl analogue **5** occurs regioselectively at C-6.[9] This suggests that the electronic properties of the allylic π-bond and cyclopropyl group may contribute to the stabilization of the corresponding α-carbon radicals that are thought to be intermediary in the catalytic mechanism of MAO-B.[9] This could explain why piperidinyl **4** is stable in the presence of MAO-B. The finding of this study that the pyrrolidinyl analogue **10** acts as a relatively good MAO-B substrate, however, does not support this hypothesis. In addition to the electronic properties of the substrates, their three dimensional structures and binding orientations within the active site may also be determinants of the substrate properties and the regioselectivity of oxidation. The factors that determine the substrate properties of cyclic tertiary amines remain to be identified.

The neurotoxicity of MPTP is thought to be the consequence of its MAO-B-catalyzed metabolism in the brain that ultimately yields the mitochondrial toxin, MPP^+.[2,6] Evidence suggests that the mitochondrial toxicity and subsequent neurotoxic action of MPP^+ relies on it being permanently charged.[16,17] Accordingly, 1-methyl-3-phenyl-3-pyrroline (**7**), which is metabolized to the neutral pyrrolyl species, is not neurotoxic.[13] Since **10** and **11** may be metabolized to charged intermediates and end product by centrally located MAO-B, the possibility exists that they may, similar to MPTP, act as neurotoxins. Because of safety considerations we have in this investigation evaluated **10** and **11** as potential nigrostriatal neurotoxins. The results document that unlike MPTP, these cyclic tertiary amines are not neurotoxins. While the reasons for this lack of neurotoxicity are not known, an explanation may be that the pharmacokinetic properties and in vivo molecular interactions of **10** and **11** and their respective MAO-B-generated products are different from that of MPTP and MPP^+. Among the pharmacokinetic factors important for the neurotoxic action of MPTP are a high degree of blood–brain barrier permeability of MPTP and slow clearance of MPP^+ from the brain.[26] Among the molecular interactions that lead to neuronal death are the accumulation of MPP^+ in the nigrostriatal nerve terminals via the plasma membrane dopamine transporter (DAT),[27,28] the energy-dependent accumulation of MPP^+ within the inner mitochondrial membrane [16] and inhibition of complex I of the mitochondrial respiratory chain.[15]

4. Experimental

Caution. MPTP is a nigrostriatal neurotoxin and should be handled using disposable gloves and protective eyewear. Procedures for the safe handling of MPTP have been described previously.[29]

4.1. Chemicals and instrumentation

All starting materials, unless otherwise stated, were obtained from Sigma-Aldrich and were used without purification. MPTP•HCl was purchased from Sigma-Aldrich and 1-methyl-3-phenyl-3-pyrroline (7) was prepared as described previously[13] Petroleum ether used in this study had a distillation range of 40–60 °C. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Varian Gemini 300 spectrometer at frequencies of 300 MHz and 75 MHz, respectively. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the deuterated methanol (CD_3OD) or DMSO-d_6. Spin multiplicities are given as s (singlet) or m (multiplet). Fast atom bombardment mass spectra (FAB-MS) were recorded with a VG 7070E mass spectrometer while direct insertion electron impact ionization mass spectra (EI-HRMS) were obtained with an AutoSpec ETOF (Micromass). Melting points (mp) were determined on a Stuart SMP10 melting point apparatus and are uncorrected. UV-Vis spectra were recorded on a Shimadzu UV-2100 double-beam spectrophotometer. Column chromatography was carried out with Fluka aluminum oxide (Brockmann Activity I).

4.2. Synthesis of the oxalic acid salt of 1-methyl-3-phenylpyrrolidine (10)

1-Methyl-3-phenyl-3-pyrroline (7) (12.04 mmol, 3 g) and PtO_2 (1.32 mmol, 0.3 g) in 60 mL methanol was stirred at room temperature in an atmosphere of hydrogen.[18] After 14 hours of stirring the catalyst was removed via filtration and the solvent was evaporated to yield the free base **10** as a light yellow oil (70.95%). Compound **10** was converted into its oxalic acid salt in diethyl ether and recrystallized from boiling methanol to give white powdery crystals: yield 35.60%; mp 148–149 °C; ^{1}H NMR (DMSO-d_6) δ 2.05 (m, 1H), 2.37 (m, 1H), 2.85 (s, 3H), 3.23 (m, 1H), 3.41 (m, 2H), 3.63 (m, 2H), 7.23–7.34 (m, 5H); ^{13}C NMR (DMSO-d_6) δ 31.98, 40.61, 42.17, 54.76, 59.95, 126.99, 127.25, 128.63, 140.20, 165.02; EI-HRMS *m/z*: calcd. 161.12045, found 161.12007 ($M^{+\cdot}$).

4.3. Synthesis of the oxalic acid salt of 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (11)

Compound **11** was synthesized from 1-methyl-3-phenyl-3-pyrroline (**7**)[13] according to the procedure previously reported for the cyclopropylation of MPTP.[9] The crude product (24.36%) was purified by aluminum oxide chromatography (petroleum ether/ethyl acetate 50:50) and converted into its oxalic acid salt in diethyl ether. Compound **11** has been previously reported as

the hydrochloric acid salt[20]: yield 8.36%; mp 141–143 °C; ^{1}H NMR (CD_3OD) δ 1.22 (m, 1H), 1.37 (m, 1H), 2.16 (m, 1H), 2.96 (s, 3H), 3.59 (m, 2H), 3.82 (m, 1H), 4.01 (m, 1H), 7.23–7.37 (m, 5H); ^{13}C NMR (CD_3OD) δ 24.40, 32.37, 41.09, 49.47, 58.62, 61.54, 128.07, 128.27, 129.81, 139.75, 166.50; FAB-MS *m/z*: 174 (MH^+); EI-HRMS calcd. 173.12045, found 173.11993 (M^+).

4.4. Steady-state MAO-B activity measurements

Baboon liver mitochondria were isolated according to the previously reported procedure and stored at –70 °C.[30] To the mitochondrial isolates were added 1 volume sodium phosphate buffer (100 mM, pH 7.4) containing 50% glycerol (w/v) and the protein concentrations were determined by the method of Bradford.[31] The K_m and V_{max} values for the MAO-B catalyzed oxidation of the oxalate salts of the test substrates **10** and **11** were determined by measuring the initial rates of H_2O_2 generation at eight substrate concentrations spanning at least two orders of magnitude (12.5–2000 μM). The reactions contained 85 μL of the chromogenic solution[22] and were carried out in a final volume of 500 μL (in 100 mM sodium phosphate buffer, pH 7.4). The chromogenic solution was prepared in 100 mM sodium phosphate buffer (pH 7.4). The enzyme concentration used was 0.15 mg mitochondrial protein/mL. Following incubation at 37 °C for a period of 20 min (substrate **10**) or 15 min (substrate **11**), the reactions were terminated by the addition of 10 μL (*R*)-deprenyl (5 mM) and placing the reactions on ice. The samples were centrifuged at 16000*g* for 10 min and the H_2O_2 concentrations were measured by the peroxidase-coupled spectrophotometric assay system described previously.[22] Quantitative estimations were made by means of a linear calibration curve that ranged from 11 to 88 μM of H_2O_2. The initial rates as a function of substrate concentration were fitted to the Michaelis–Menten equation using the one site binding model incorporated into the GraphPad Prism software package (GraphPad Software Inc.). All measurements were carried out in triplicate and the K_m and V_{max} values were expressed as means ± standard error of the mean (SEM).

4.5. Linearity of oxidation of pyrrolinyl substrates

To determine the time interval for which MAO-B catalyzed production of H_2O_2 remains linear, the oxalate salts of test substrates **10** (500 μM) and **11** (250 μM) and 85 μL of the chromogenic solution[22] were incubated at 37 °C with 0.15 mg protein/mL of the baboon liver mitochondrial fraction. The reactions were carried out in 100 mM sodium phosphate buffer (pH 7.4) to a final volume of 500 μL. At the required time points (2.5–45 min), the reactions were terminated with the addition of 10 μL (*R*)-deprenyl (5 mM) and the MAO-B-generated H_2O_2 were measured as

described previously.[22] All measurements were conducted in triplicate and the concentrations of H_2O_2 were expressed as means ± SEM.

4.6. (*R*)-Deprenyl studies

To estimate the extent of the H_2O_2 produced by non-MAO-B mediated oxidation of substrates **10** and **11**, baboon liver mitochondria (0.3 mg protein/mL) were preincubated with the hydrochloric acid salt of the MAO-B selective inactivator (*R*)-deprenyl (3×10^{-6} M). These preincubations were carried out at 37 °C for 30 min in sodium phosphate buffer (100 mM, pH 7.4).[21] The pre-inactivated mitochondrial fractions were then added to the test substrates **10** (500 μM) and **11** (250 μM) to yield final concentrations of 0.15 mg mitochondrial protein/mL. The volume of these incubation mixtures were 500 μL and contained 85 μL of the chromogenic solution.[22] The reactions were incubated for 15 min at 37 °C and terminated with the addition of 10 μL (*R*)-deprenyl (5 mM). Control incubation reactions with the fully active enzyme preparation were carried out following the same procedure with the exception that for the preincubations the addition of (*R*)-deprenyl was omitted. The concentrations of the MAO-B-generated H_2O_2 were measured as described previously.[22] All measurements were conducted in triplicate and the concentrations of the pyrrolyl products were expressed as means ± SEM.

4.7. Animal studies and striatal dopamine measurements

Male C57BL/6 mice (30–35 g, 9–11 months of age) were provided by the Laboratory animal center of the Potchefstroom campus. The protocols for all animal trials were reviewed and approved by the Research Ethics Committee of the North-West University. Five animals were housed per cage in a temperature (21±0.5 °C) and humidity (50±5% relative humidity) controlled room on a 12 h light–12 h dark cycle with free access to food and water. All injections were intraperitoneal (ip) in a volume of 0.2 ml per 30 g mouse. Sterile saline was used as vehicle for all of the test compounds. Mice were sacrificed by rapid cervical dislocation. The dopamine concentrations in the dissected mouse striata were determined as described previously,[25] and were expressed as means ± SEM.

Acknowledgements

We are grateful to Jan du Preez and the staff of the Analytical Technology Laboratory, North-West University for their support. The NMR and MS spectra were recorded by André Joubert, Johan Jordaan and Louis Fourie of the SASOL Centre for Chemistry, North-West University.

This work was supported by grants from the National Research Foundation and the Medical Research Council, South Africa.

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Chapter 5

CONCLUSION

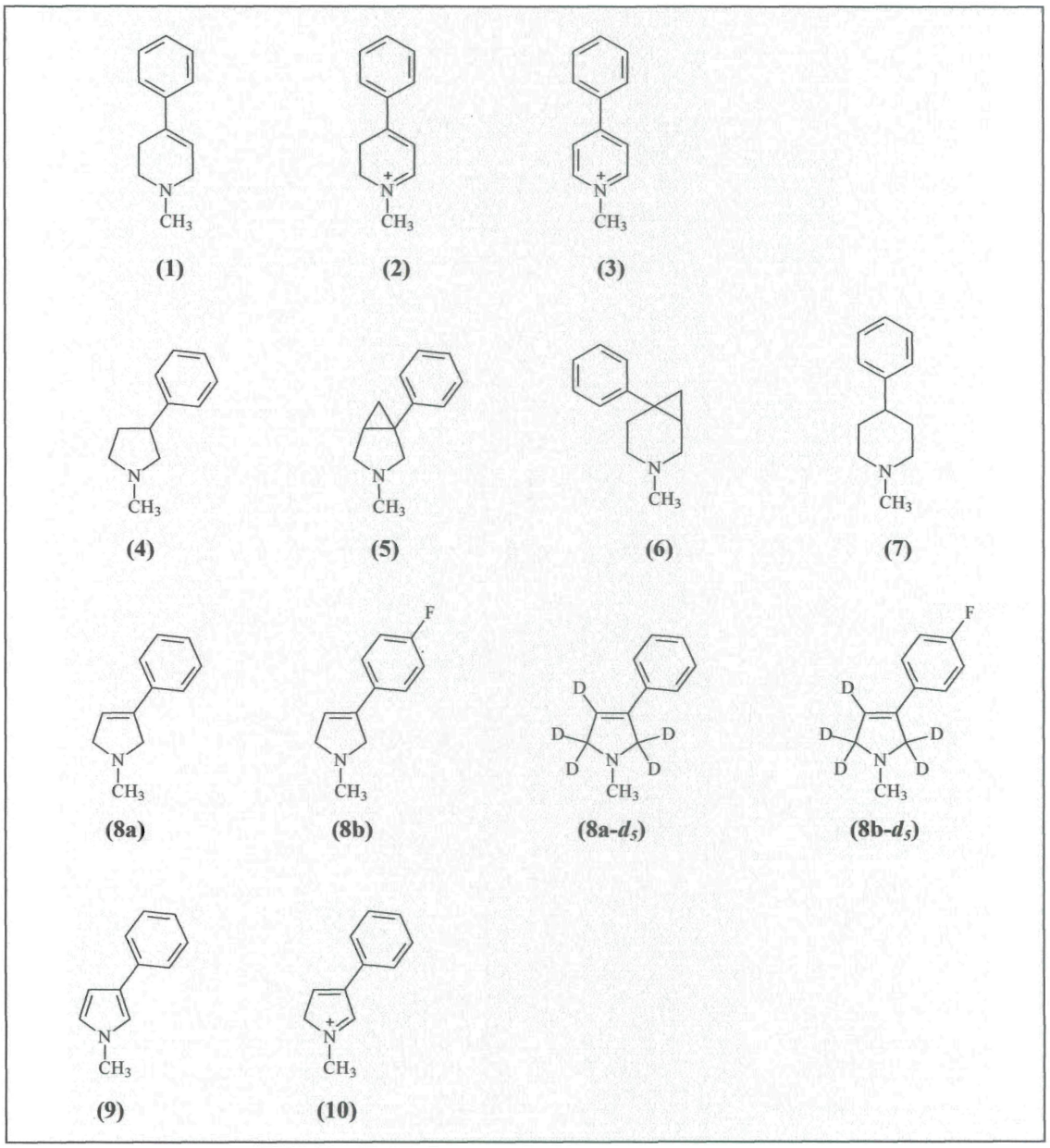

Figure 25. *The structures of the compounds mentioned in this chapter.*

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (**1**) is catalyzed by the flavo-enzyme MAO-B to yield the α-carbon 2-electron oxidation product, 1-methyl-4-phenyl-2,3-dihydropyridinium species ($MPDP^+$) (**2**), which undergoes a second 2-electron oxidation to generate the ultimate neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+) (**3**) (Chiba *et al.*, 1984; Ramsay *et al.*, 1991; Markey *et al.*, 1984). MPTP was the first cyclic tertiary allylamine reported to be a good MAO-B substrate (Chiba *et al.*, 1984), however several other tertiary aminyl substrates of MAO-B have since been described (Kalgutkar & Castagnoli, 1992; Rimoldi *et al.*, 2005). The 5-membered ring analogue of MPTP, 1-methyl-3-phenyl-3-pyrroline (**8a**), undergoes ring α-carbon oxidation to form 1-methyl-3-phenylpyrrole (**9**), an overall 2-electron process that most likely arises via the short-lived conjugate acid of 1-methyl-3-phenylpyrrole (**10**). 1-Methyl-3-phenyl-3-pyrroline (**8a**) has MAO-B substrate properties comparable to that of MPTP (**1**) (Wang *et al.*, 1998; Ogunrombi *et al.*, 2007).

In this study the kinetic deuterium isotope effects on V_{max} and V_{max}/K_m were measured for the steady-state oxidation of 1-methyl-3-phenyl-3-pyrroline (**8a**) and 1-methyl-3-(4-fluorophenyl)-3-pyrroline (**8b**) by baboon liver mitochondrial MAO-B using the corresponding 1-methyl-3-phenyl-3-pyrroline-d_5 (**8a-*d*$_5$**) and 1-methyl-3-(4-fluorophenyl)-3-pyrroline-d_5 (**8b-*d*$_5$**) analogues as deuterated substrates. The V_{max} isotope effects on the two substrates were 4.29 and 3.98, respectively, which are moderately large isotope effects. The isotope effects on V_{max}/K_m were found to be 5.71 and 3.37 for the two substrates, respectively. We conclude this part of the study with (1) although these values may not reflect the intrinsic isotope effects, the V_{max} isotope effects suggest that a carbon-hydrogen bond cleavage step is at least partially rate-determining, (2) that the V_{max} for the two pyrrolinyl substrates are comparable to those reported for the oxidation of MPTP by bovine liver MAO-B [$^D(V_{max}) = 3.55$], and (3) that the mechanism of the MAO-B-catalyzed oxidation of pyrrolinyl substrates is similar to that of the tetrahydropyridinyl substrates.

As previously mentioned, MPTP (**1**) is a member of the cyclic tertiary allylamine class of MAO-B substrates and is oxidized at C-6 of the tetrahydropyridinyl ring to yield the

conjugated eniminium metabolite, $MPDP^+$ (**2**) (Weisman *et al.*, 1985). $MPDP^+$ (**2**) undergoes a second 2-electron oxidation to generate MPP^+ (**3**) (Chiba *et al.*, 1984; Ramsay *et al.*, 1991; Markey *et al.*, 1984). 3-Methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (**6**) (the 3,4-cyclopropyl analogue of MPTP) is the only saturated, cyclic tertiary amine known to act as a MAO-B substrate (Rimoldi *et al.*, 2005), and is oxidized by MAO-B to yield the corresponding iminium metabolite. As already mentioned, 1-methyl-3-phenyl-3-pyrroline (**8a**), the 5-membered ring analogue of MPTP, is another member of the cyclic tertiary allylamine class of MAO-B substrates (Wang *et al.*, 1998; Williams & Lawson, 1998; Williams & Lawson, 1999), and is catalyzed by MAO-B at the ring α-carbon (a 2-electron oxidation process) to yield 1-methyl-3-phenylpyrrole (**9**) as the final product (Ogunrombi *et al.*, 2007).

The result that 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**5**) (a 3,4-cyclopropyl analogue) is an efficient substrate of baboon liver MAO-B with substrate properties comparable with that of the allylamine, 1-methyl-3-phenyl-3-pyrroline (**8a**), is in accordance with the finding that 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (**6**) is also a good MAO-B substrate (Rimoldi *et al.*, 2005). The π-bond characteristics of the cyclopropyl group may be responsible for the observation that the saturated substrates 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (**6**) and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**5**) are approximately as good MAO-B substrates as their corresponding allylamine analogues, MPTP and 1-methyl-3-phenyl-3-pyrroline (**8a**), respectively (Rimoldi *et al.*, 2005). The pyrrolidinyl analogue, 1-methyl-3-phenylpyrrolidine (**4**), was also shown to be a relatively good substrate for baboon liver MAO-B, with substrate properties comparable to that of MPTP. This is in contrast to expectation since the saturated piperidinyl analogue (**7**) of MPTP is not a MAO-B substrate (Heikkila *et al.*, 1985; Langston *et al.*, 1984). Therefore, we can conclude that the presence of an allylic π-bond or 3,4-cyclopropyl-fused system is not a requirement for all cyclic tertiary aminyl analogues to act as MAO-B substrates.

The α-carbon oxidation of MPTP and its 3,4-cyclopropyl analogue, 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane (**6**), occurs regioselectively at C-6 (Rimoldi *et al.*, 2005). This

suggests that the electronic properties of the allylic π-bond and cyclopropyl group may contribute to the stabilization of the corresponding α-carbon radicals which are thought to be intermediary in the catalytic mechanism of MAO-B (Rimoldi *et al.*, 2005). This could explain why the saturated piperidinyl analogue (**7**) of MPTP is stable in the presence of MAO-B. The finding of this study, that the pyrrolidinyl analogue, 1-methyl-3-phenylpyrrolidine (**4**), acts as a relatively good MAO-B substrate, however, does not support this hypothesis. In addition to the electronic properties of the substrates, their three dimensional structures and binding orientations within the active site may also be determinants of the substrate properties and the regioselectivity of oxidation. The factors that determine the substrate properties of cyclic tertiary amines remain to be identified.

The neurotoxic action of MPTP is dependent upon its metabolic conversion by MAO-B to yield the charged pyridinium species, MPP^+ (**3**) (Chiba *et al.*, 1984), which is the ultimate neurotoxin (Heikkila *et al.*, 1984; Singer *et al.*, 1988). Neuronal death is caused by the accumulation of MPP^+ in the inner mitochondrial membrane of the nigrostriatal nerve terminals, a process driven by the transmembrane electrochemical gradient (Sayre *et al.*, 1990; Rollema *et al.*, 1990). This process appears to rely upon the presence of the permanent iminium charge of MPP^+. 1-Methyl-3-phenyl-3-pyrroline (**8a**), however, is metabolized to the neutral pyrrolyl species, and is therefore not neurotoxic (Ogunrombi *et al.*, 2007). The possibility exists that the MAO-B catalyzed 2-electron oxidations of substrates 1-methyl-3-phenylpyrrolidine (**4**) and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]-hexane (**5**) may yield charged intermediates and end products, and that these substrates could be neurotoxic, similar to MPTP (Ogunrombi *et al.*, 2007).

The results established that these cyclic tertiary amines [1-methyl-3-phenylpyrrolidine (**4**) and 3-methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**5**)] are not neurotoxins like MPTP (**1**). A possible explanation may be that the pharmacokinetic properties [such as a high degree of blood–brain barrier permeability of MPTP (1) and slow clearance of MPP^+ (**3**) from the brain] (Castagnoli *et al.*, 1997) and *in vivo* molecular interactions of MPTP (**1**) and MPP^+(**3**) may be different than those of 1-methyl-3-phenylpyrrolidine (**4**) and 3-

methyl-1-phenyl-3-azabicyclo[3.1.0]hexane (**5**) and their respective MAO-B-generated products.

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ANNEXURE A

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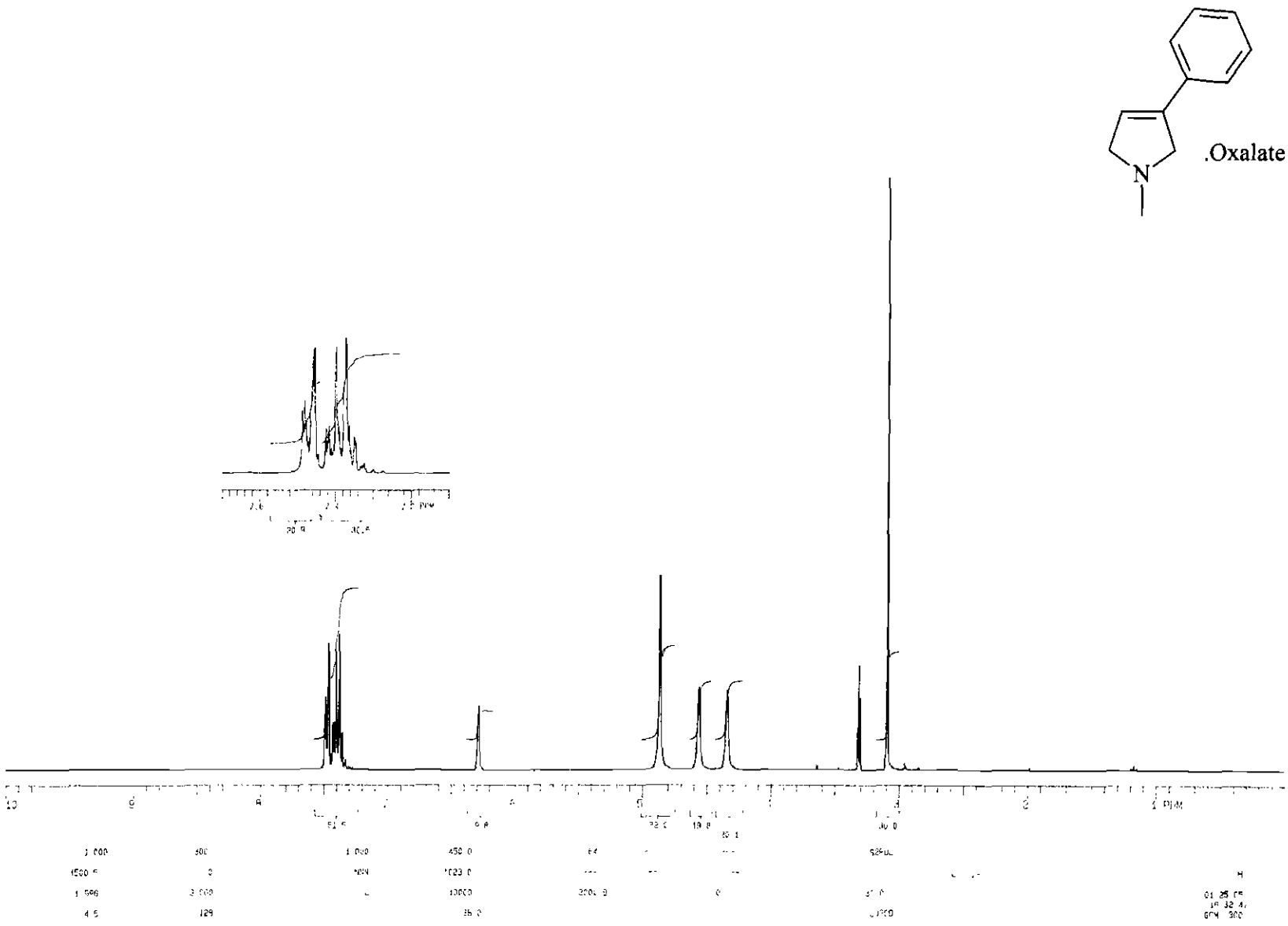
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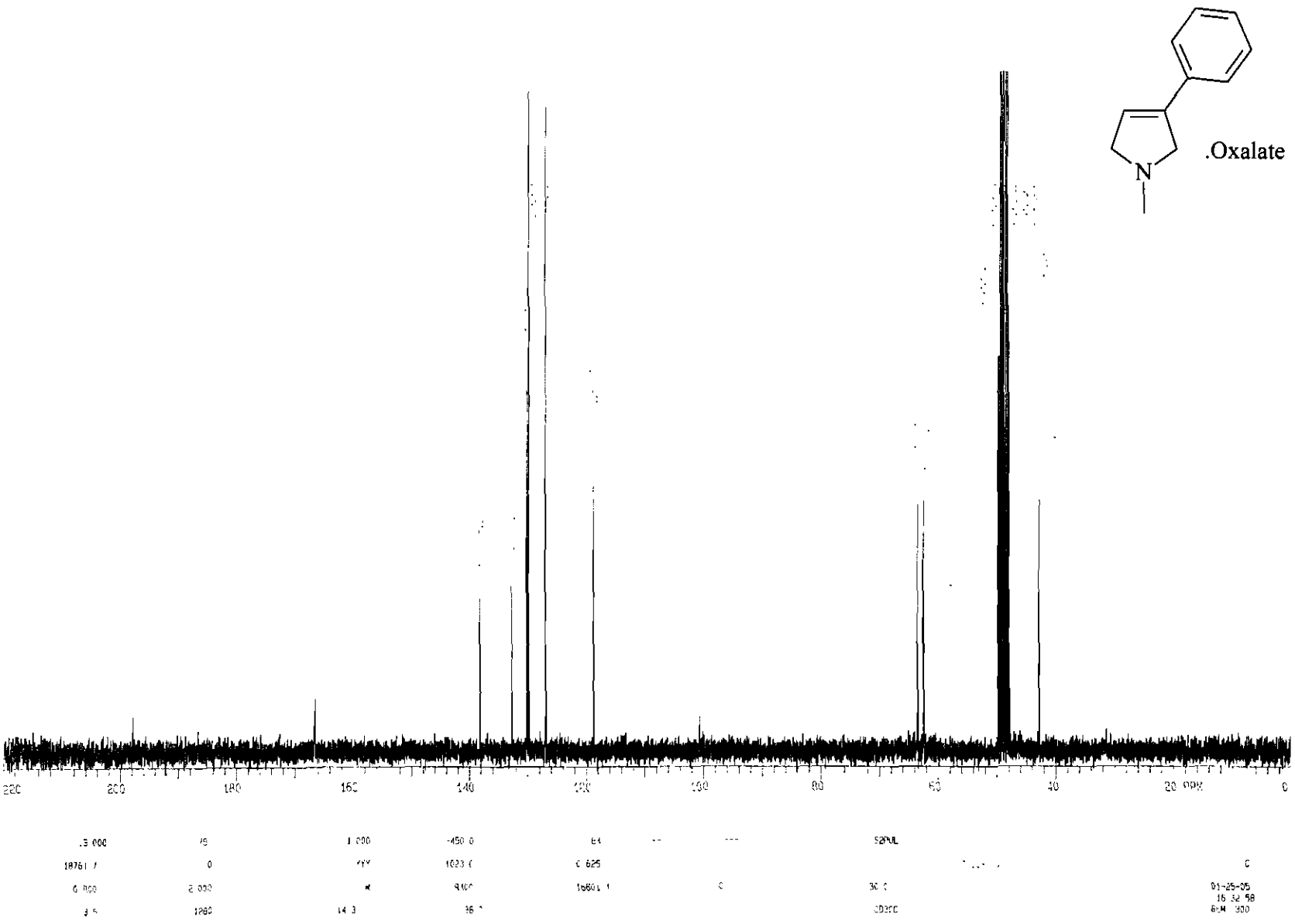
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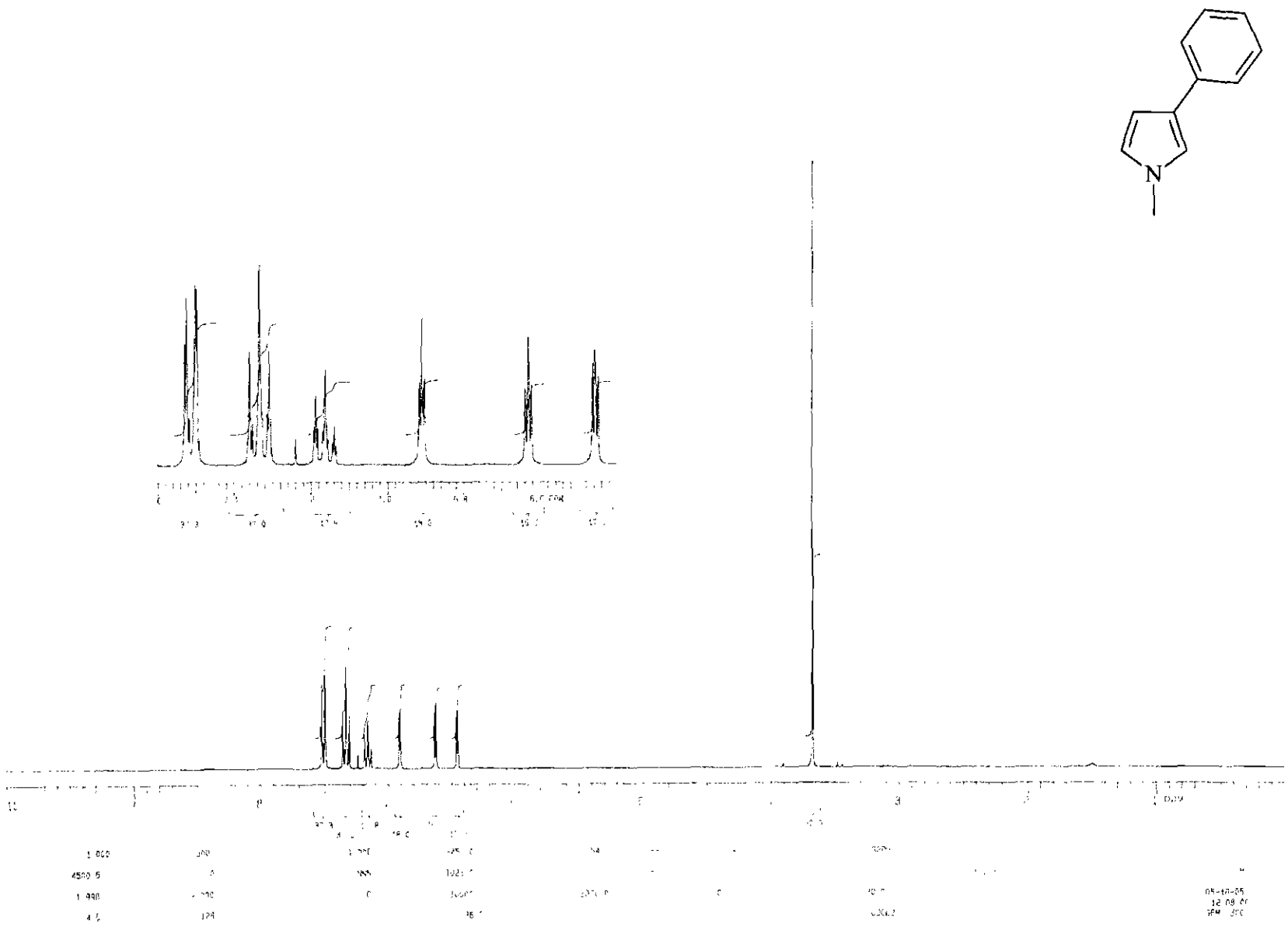
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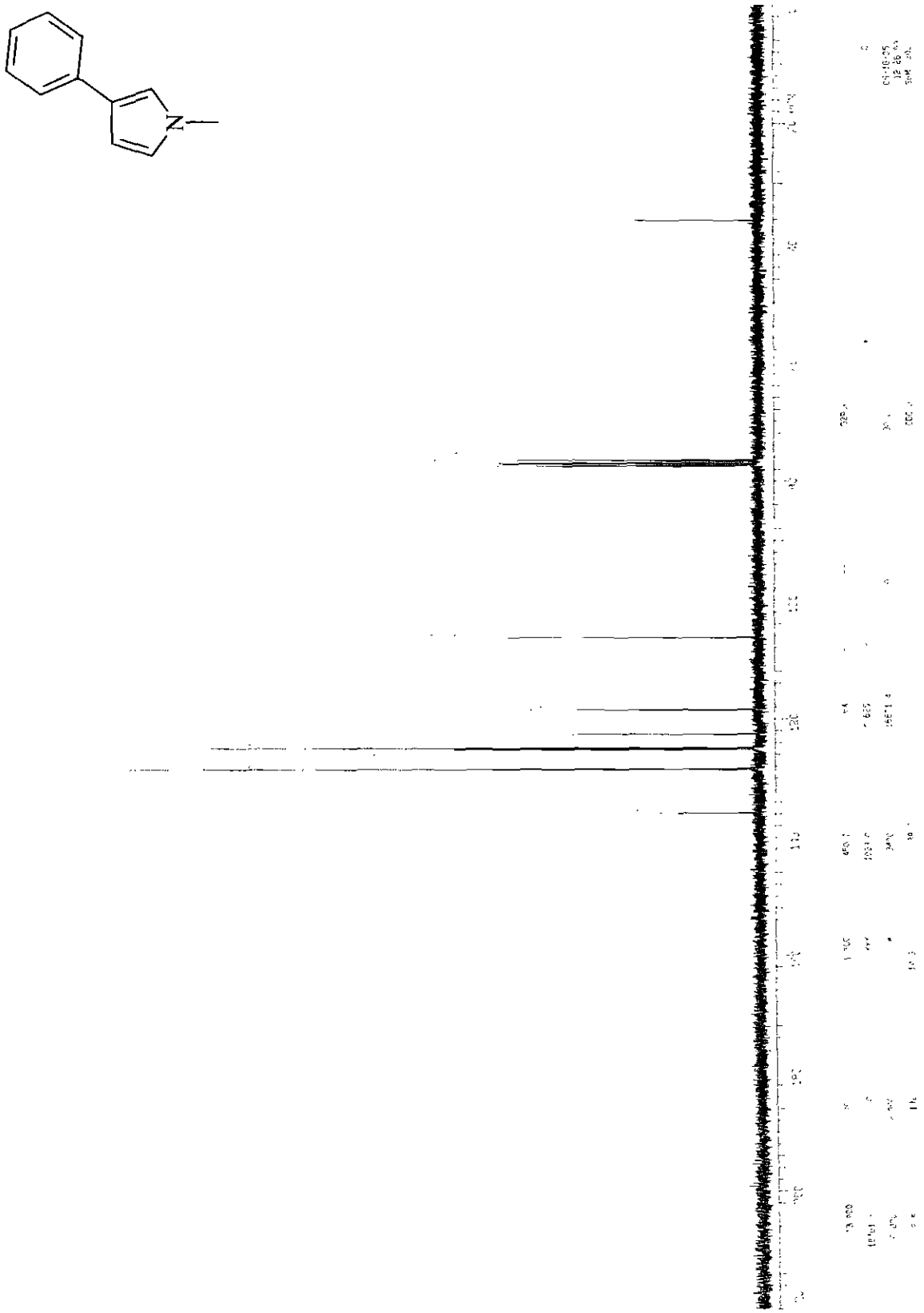

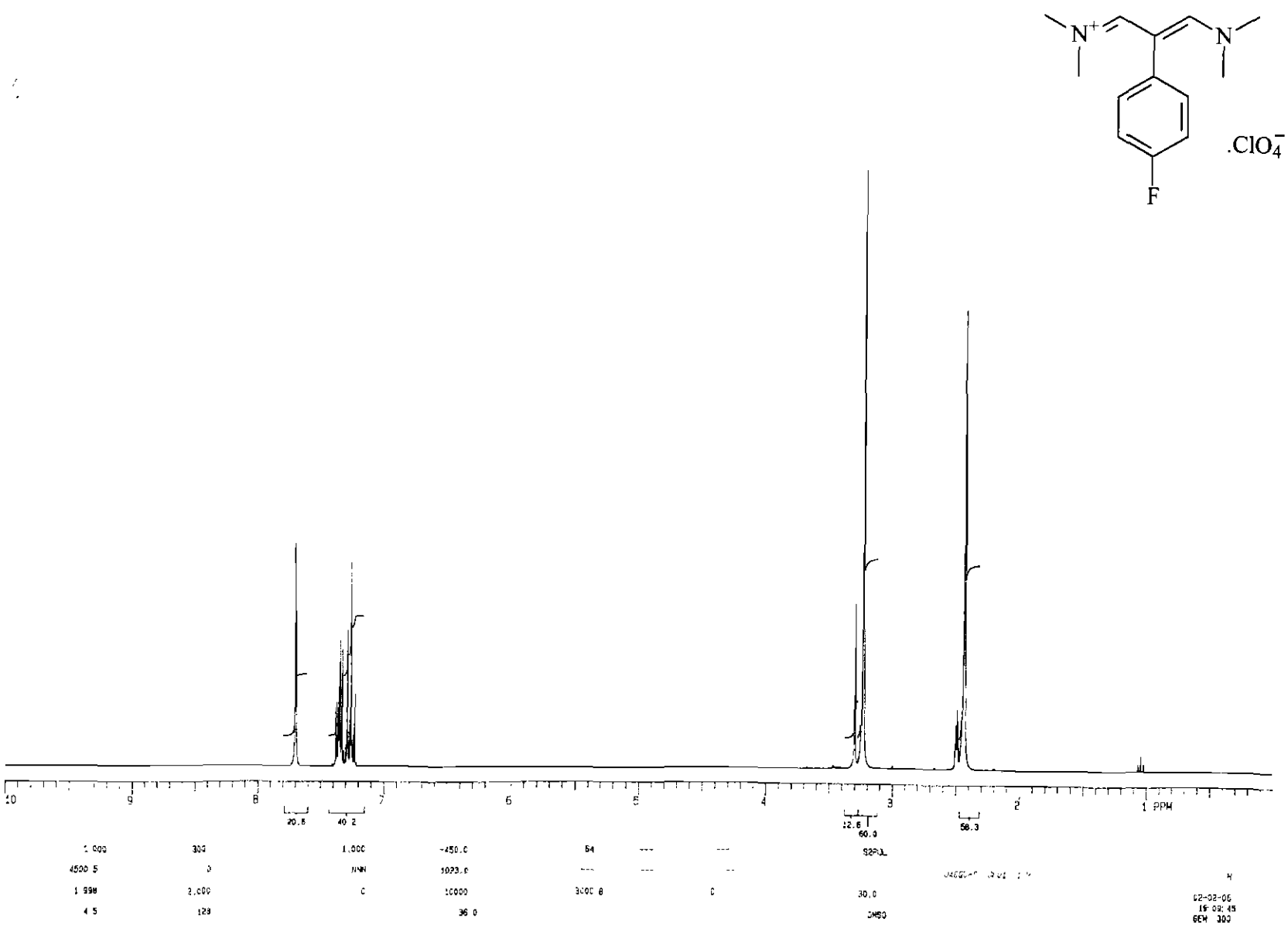
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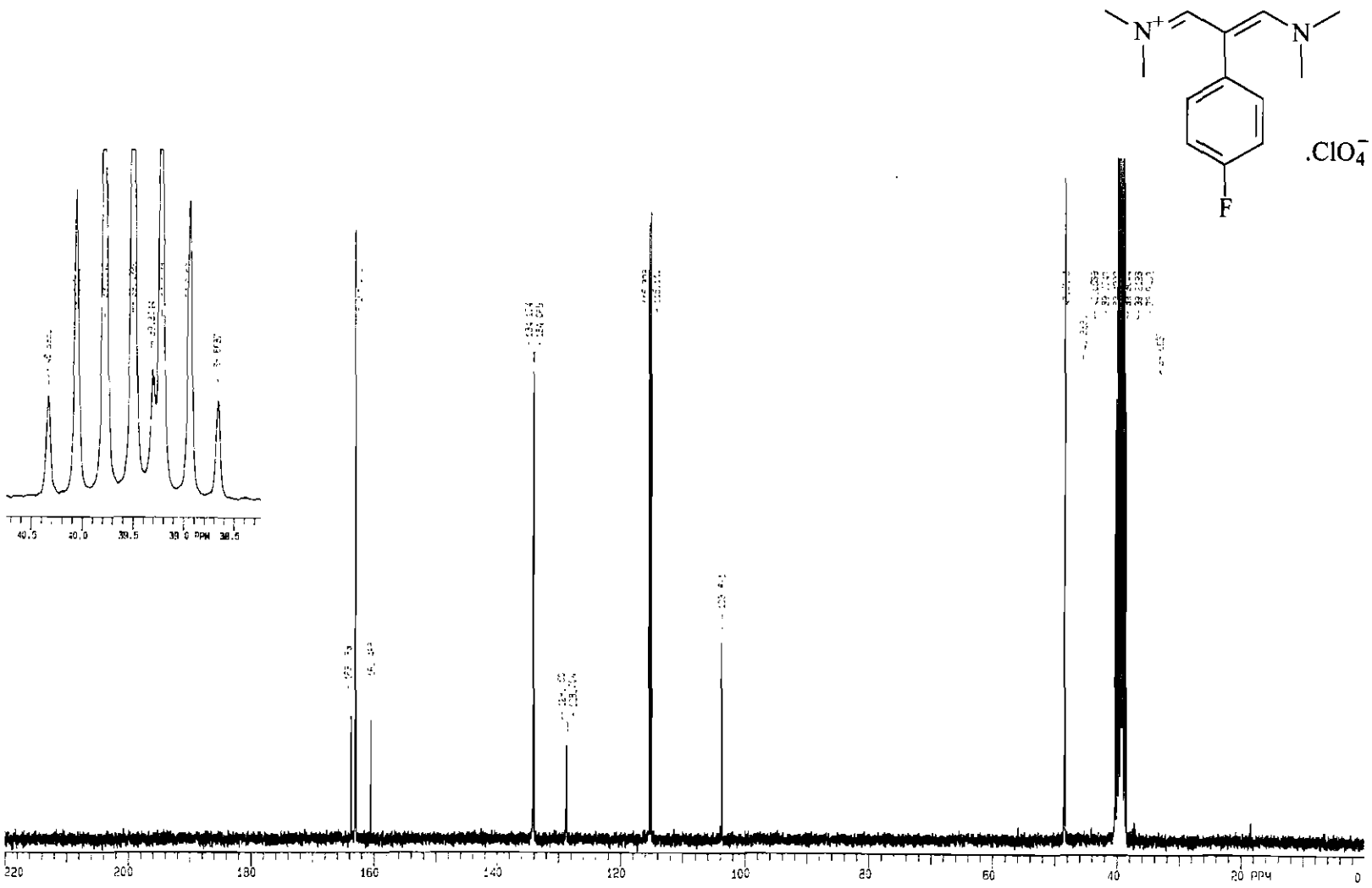
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38.5
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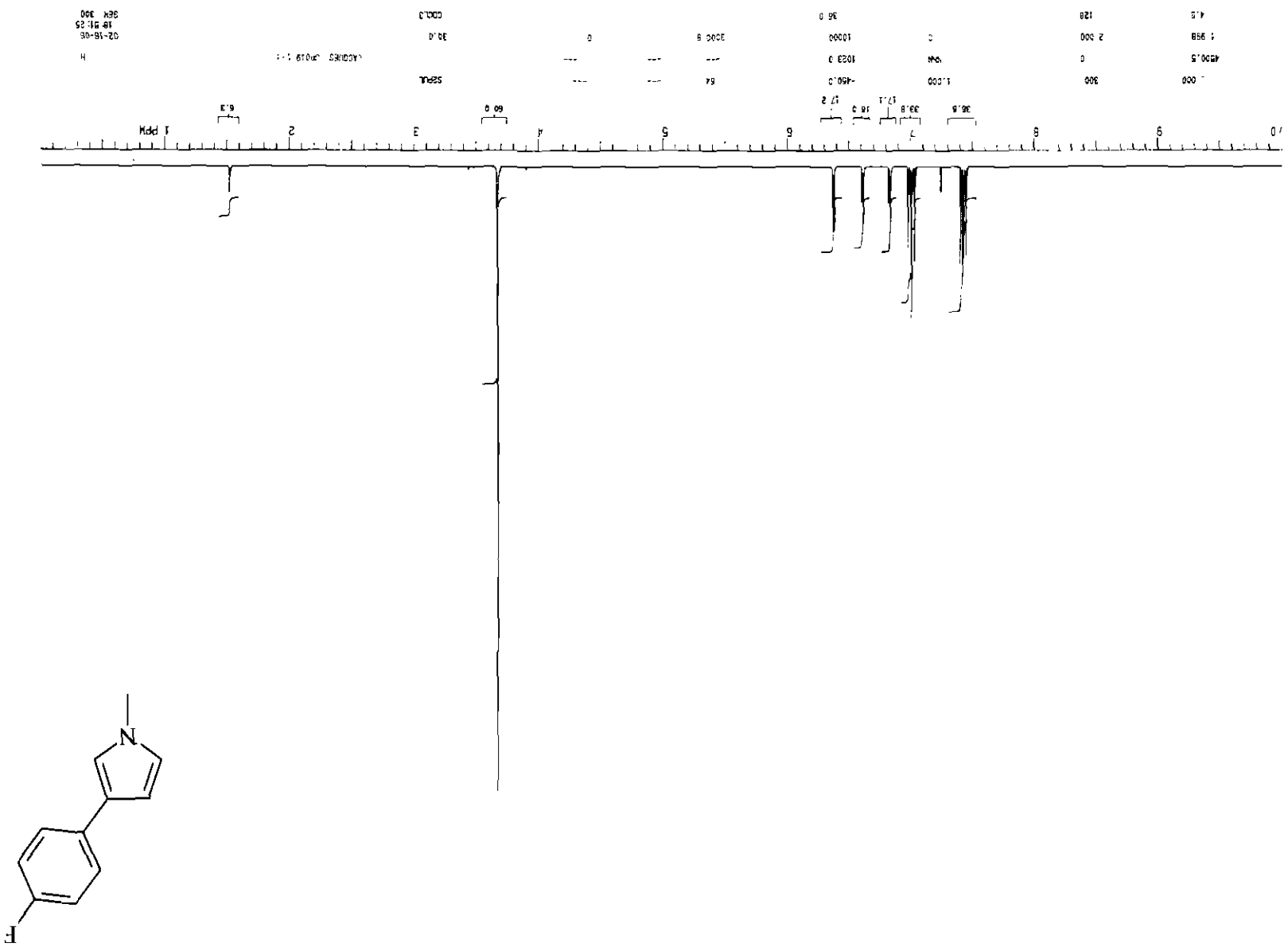
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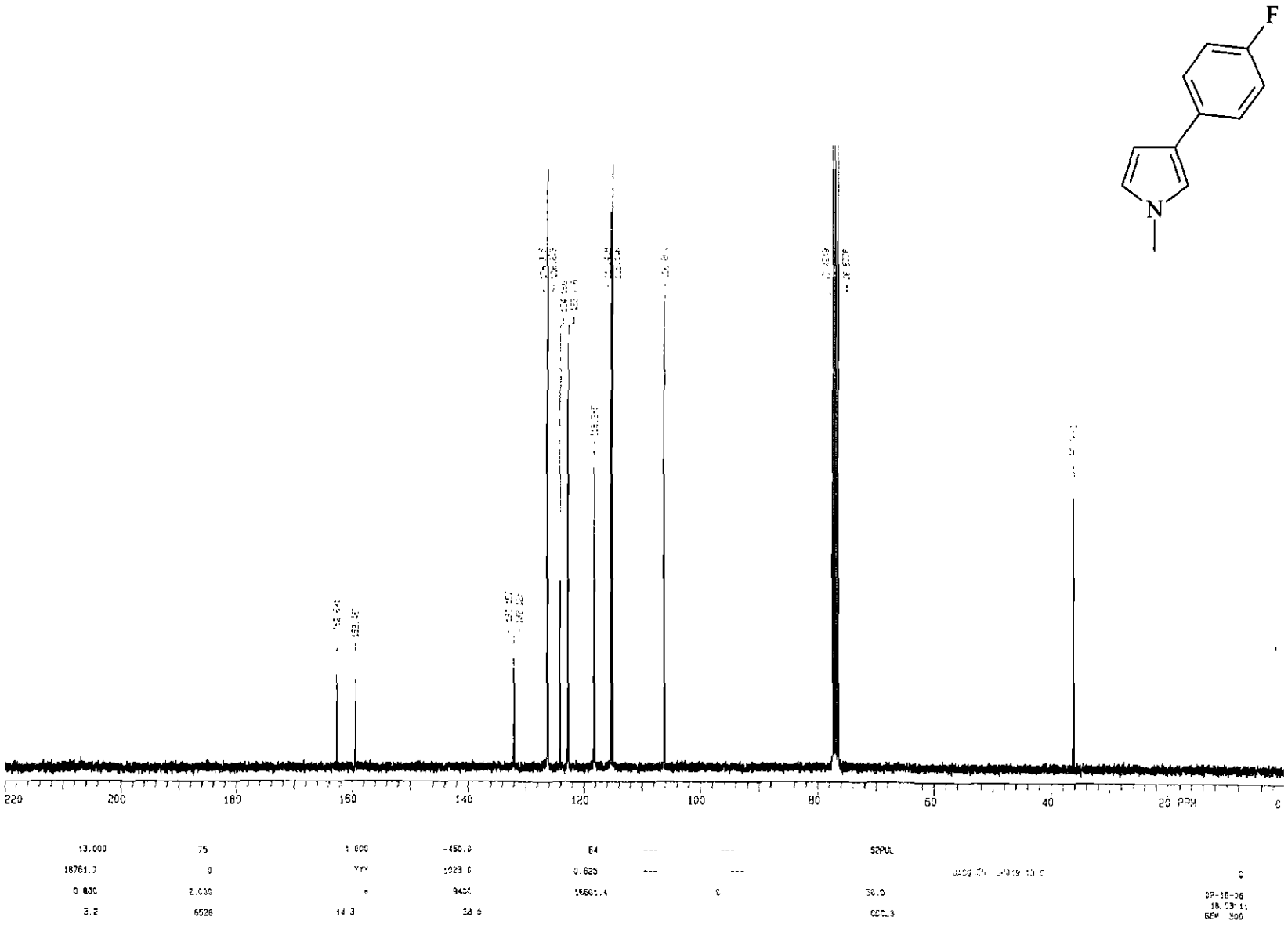
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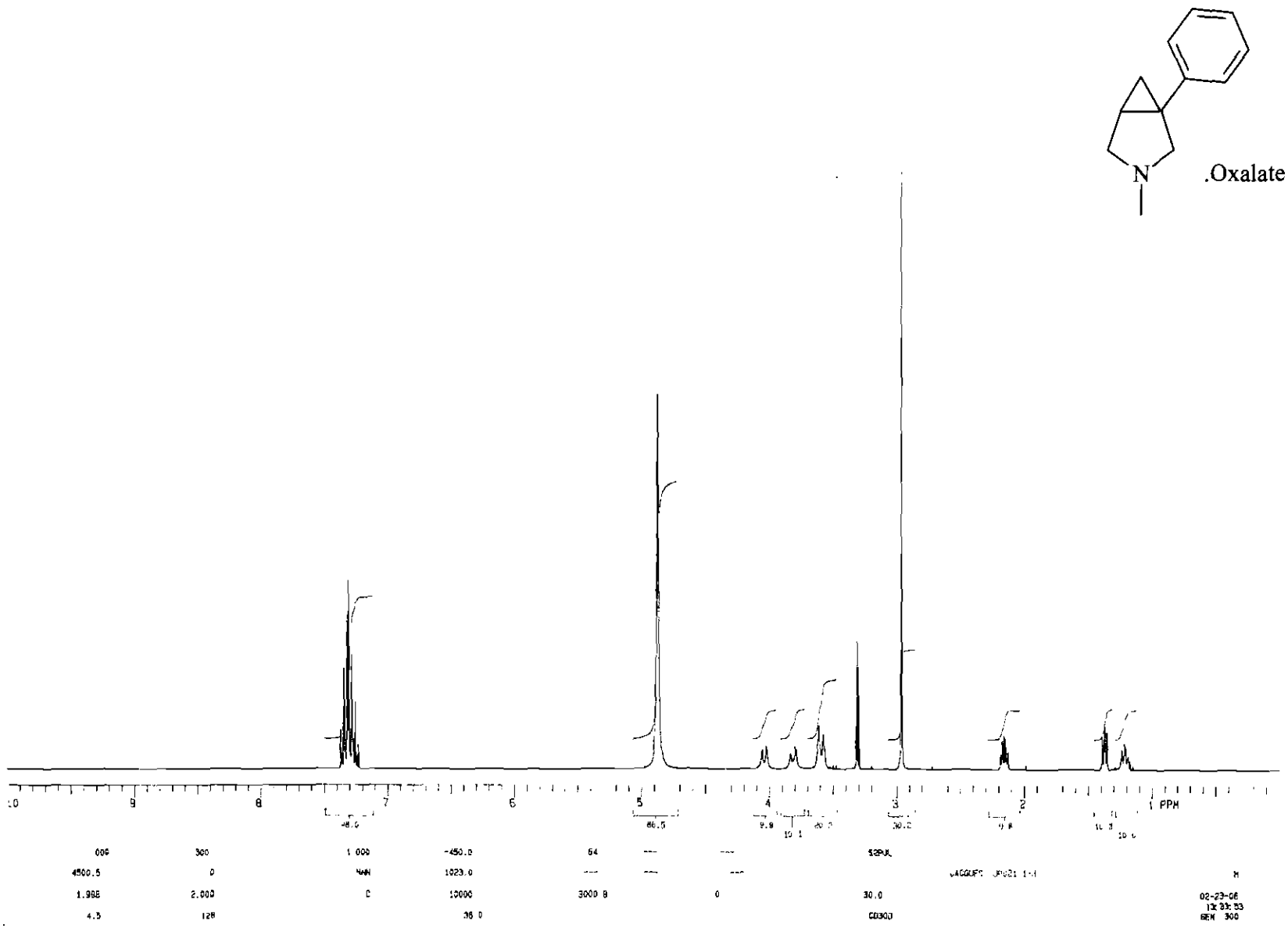
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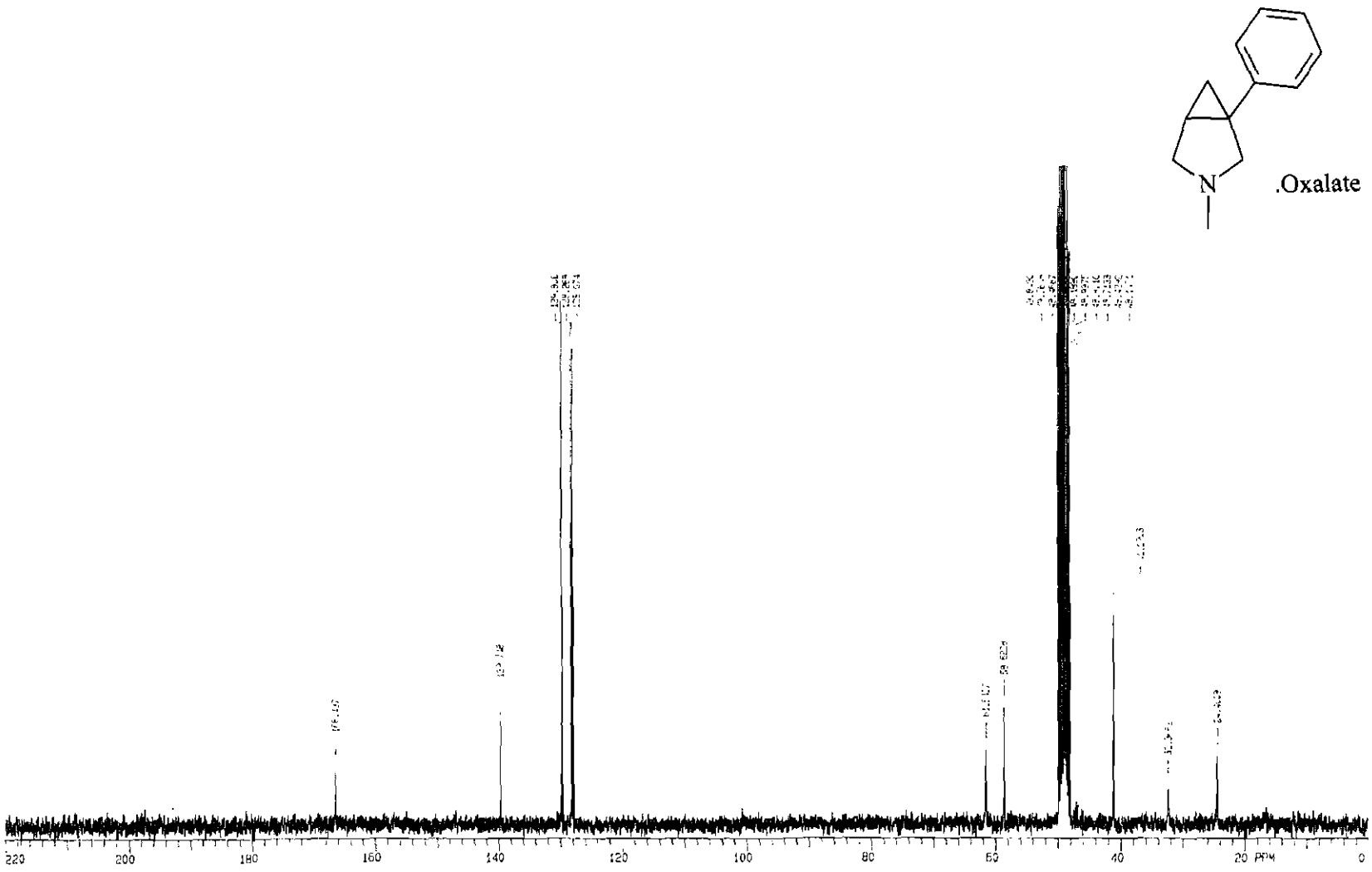
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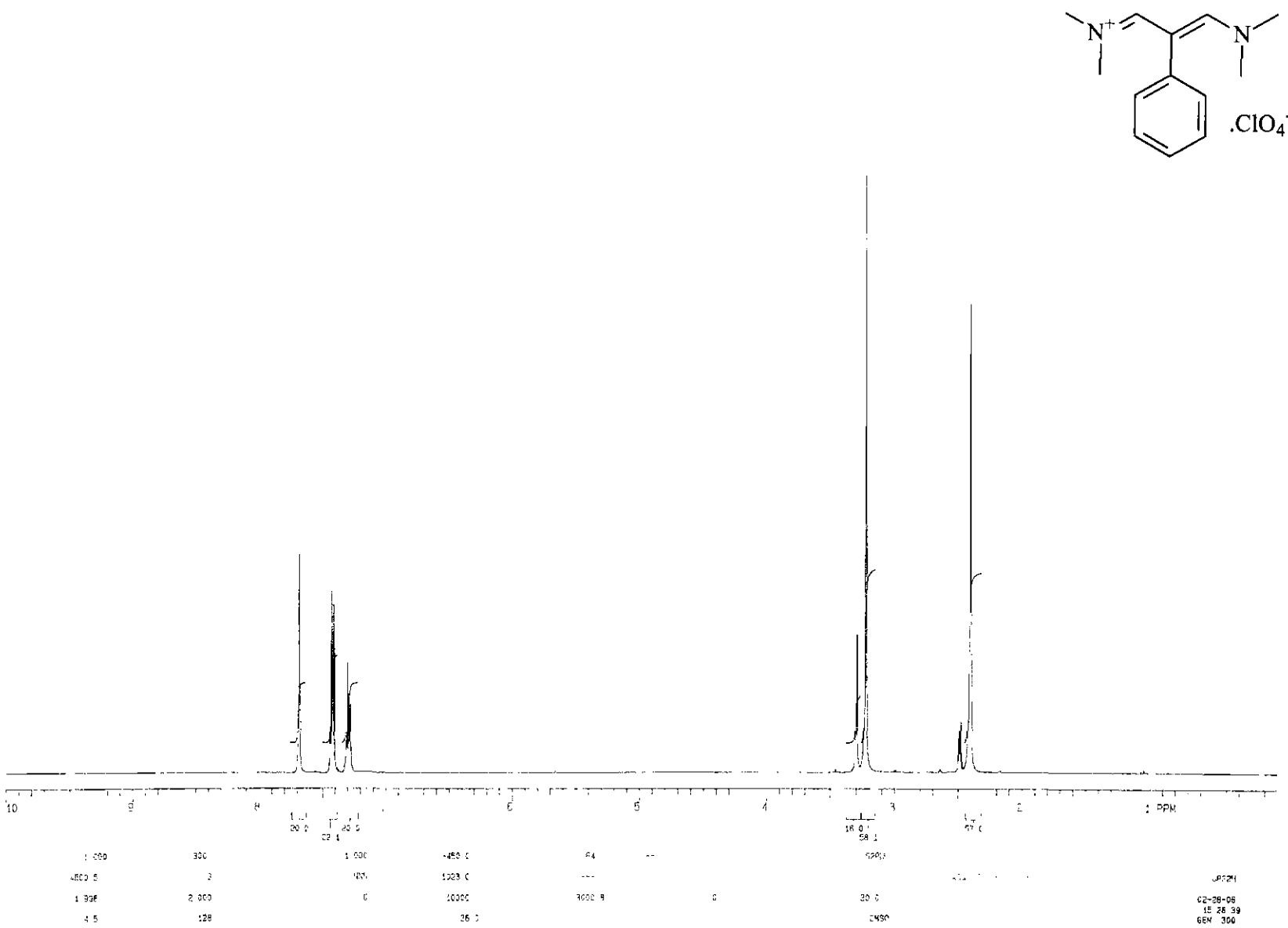
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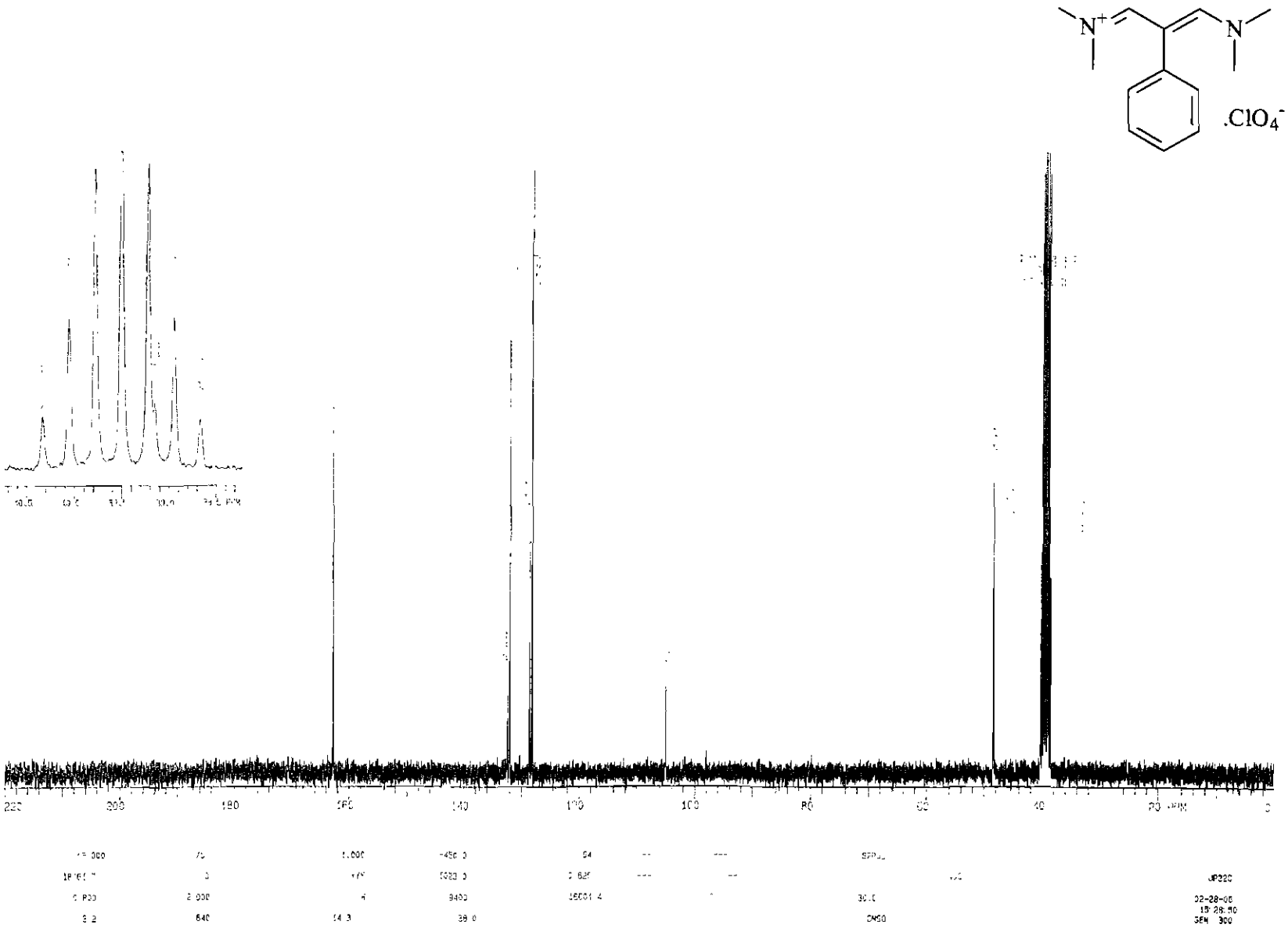
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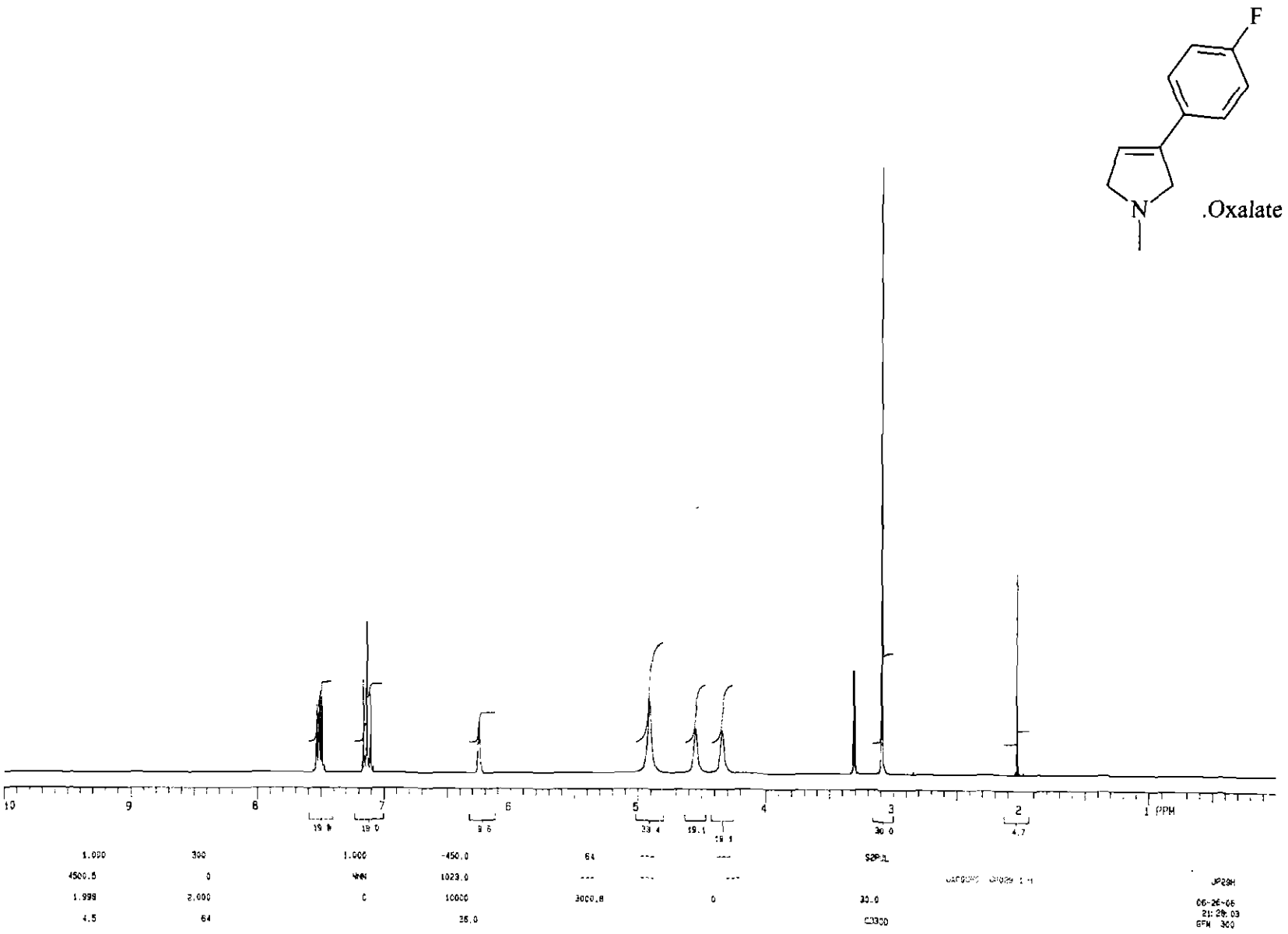
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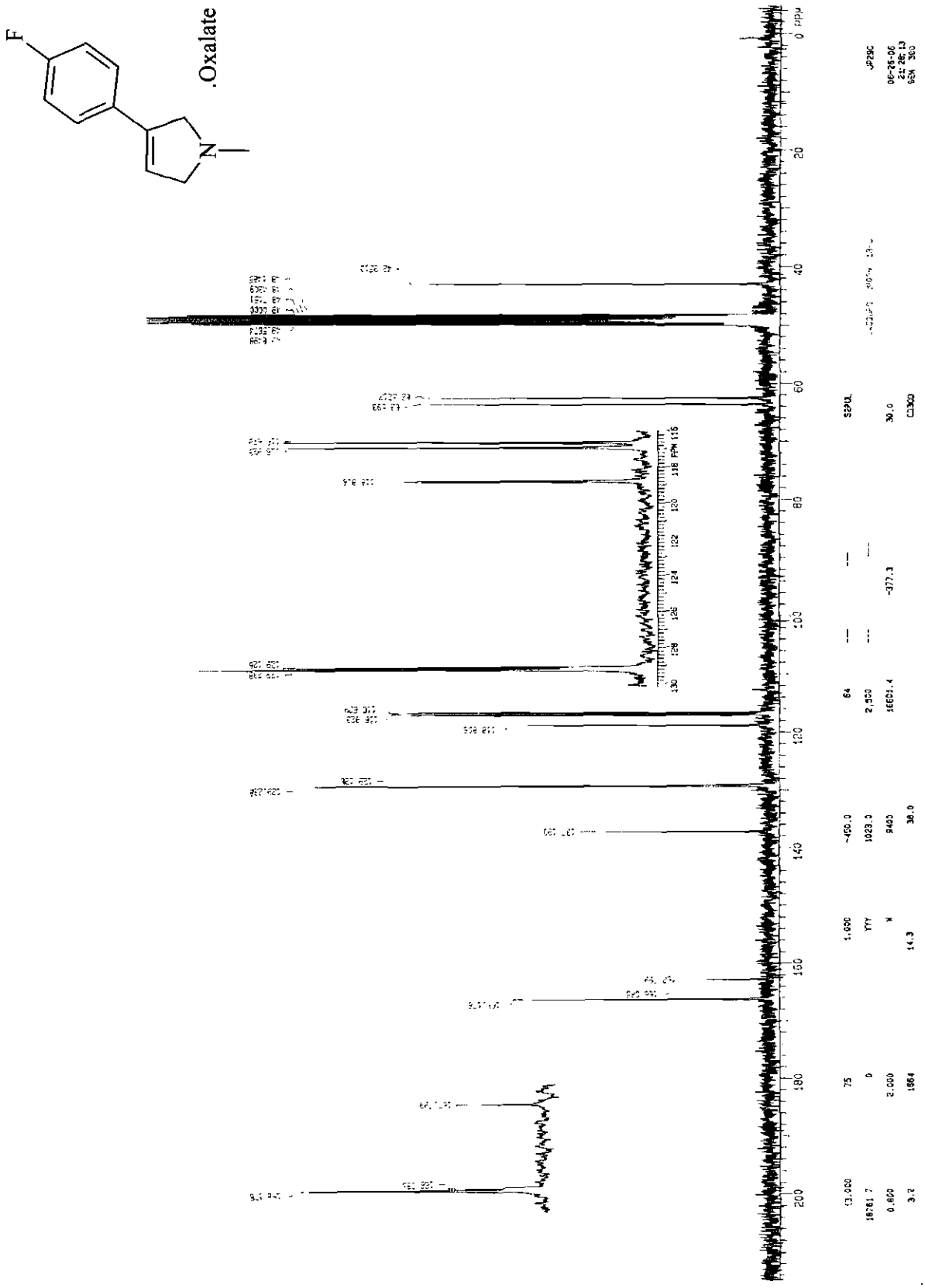
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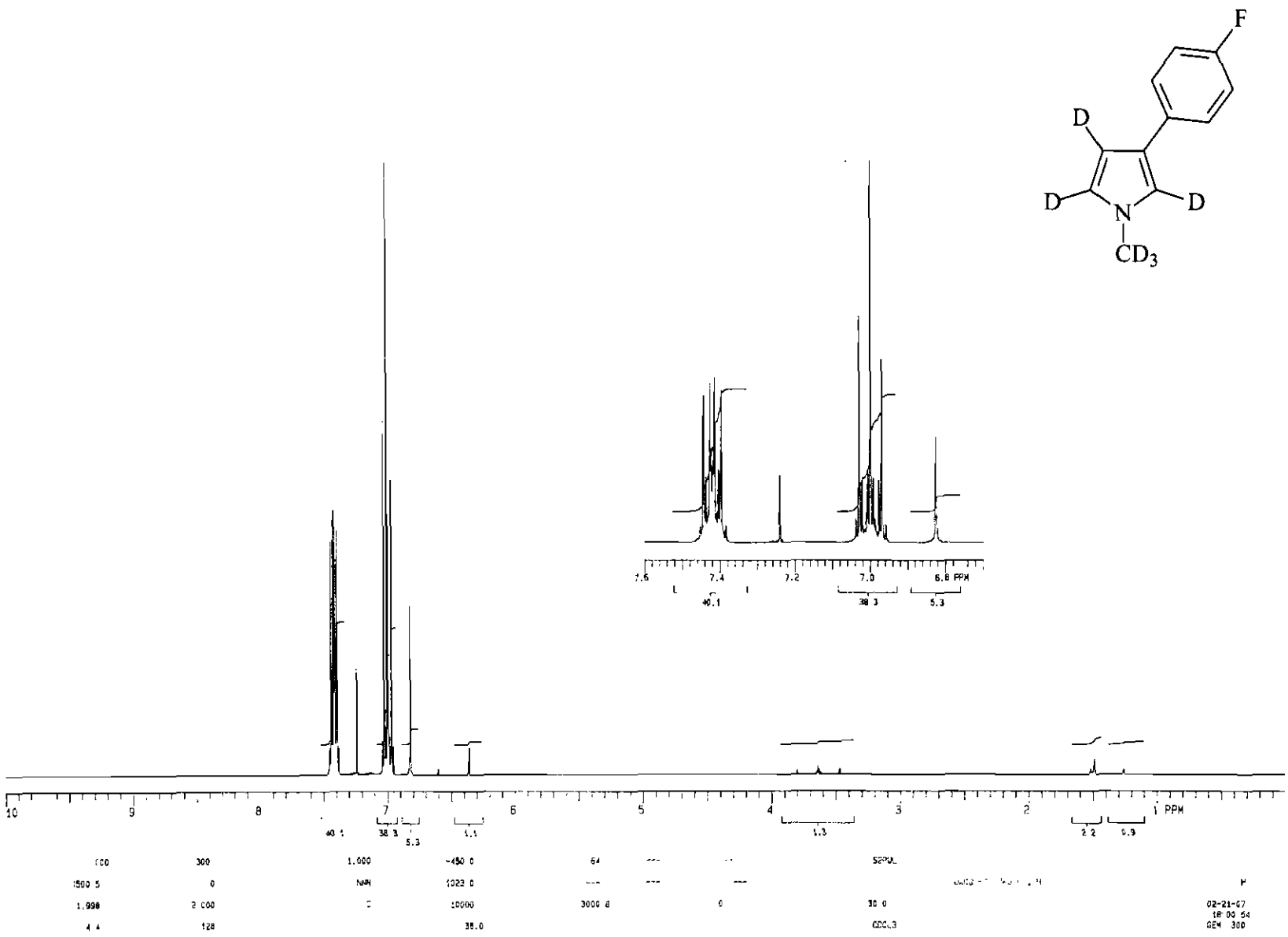
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10
9
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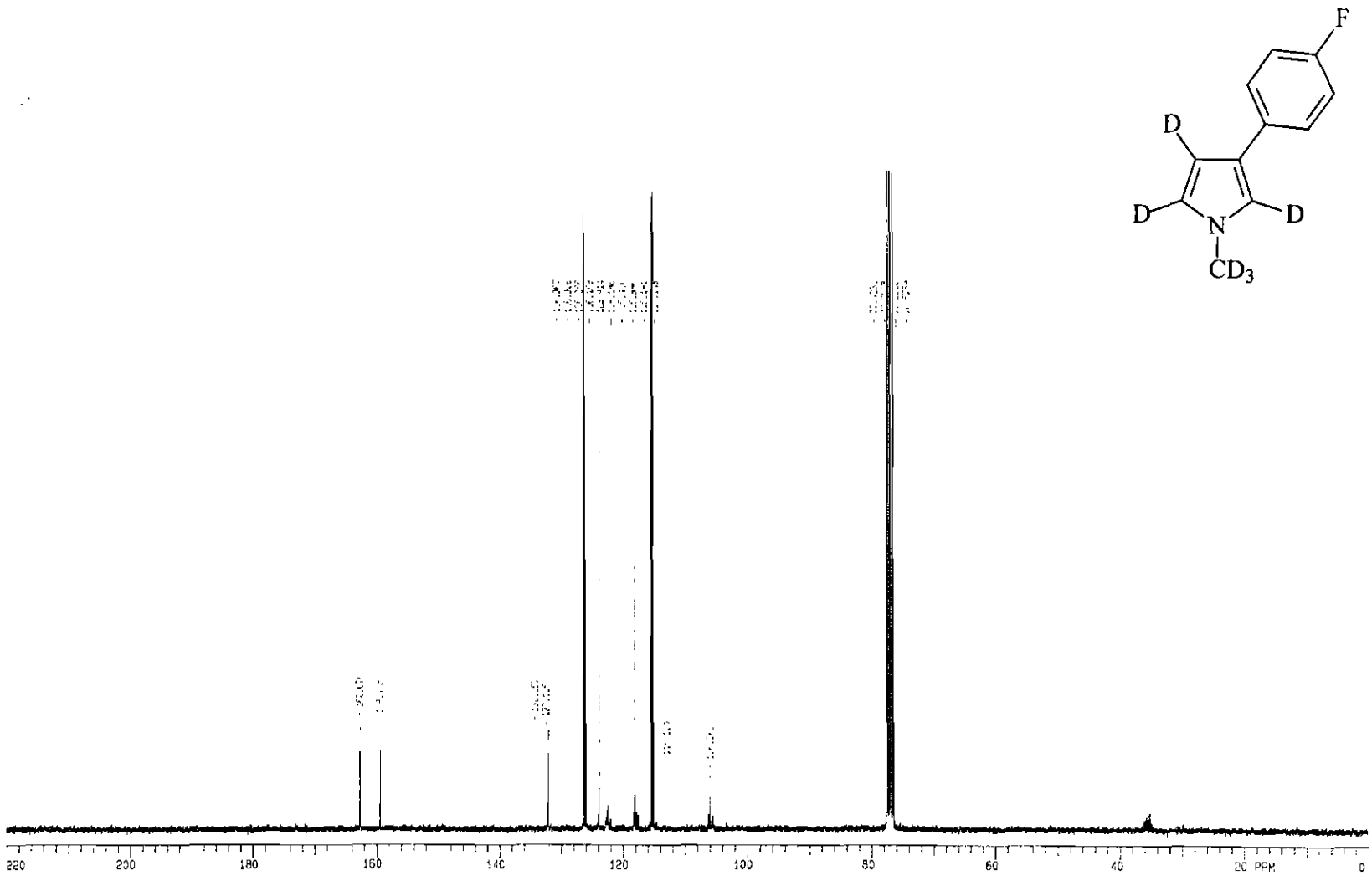
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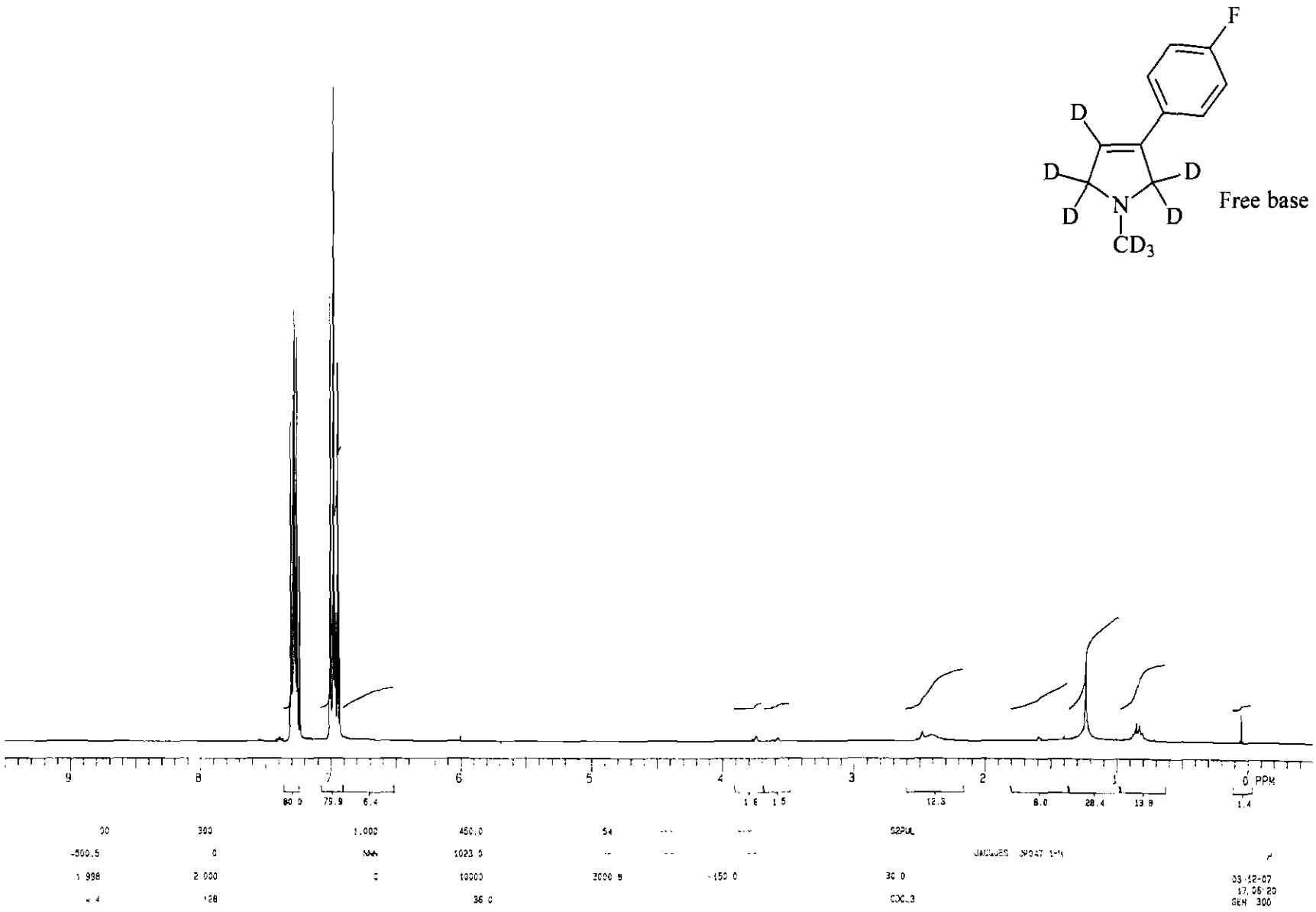
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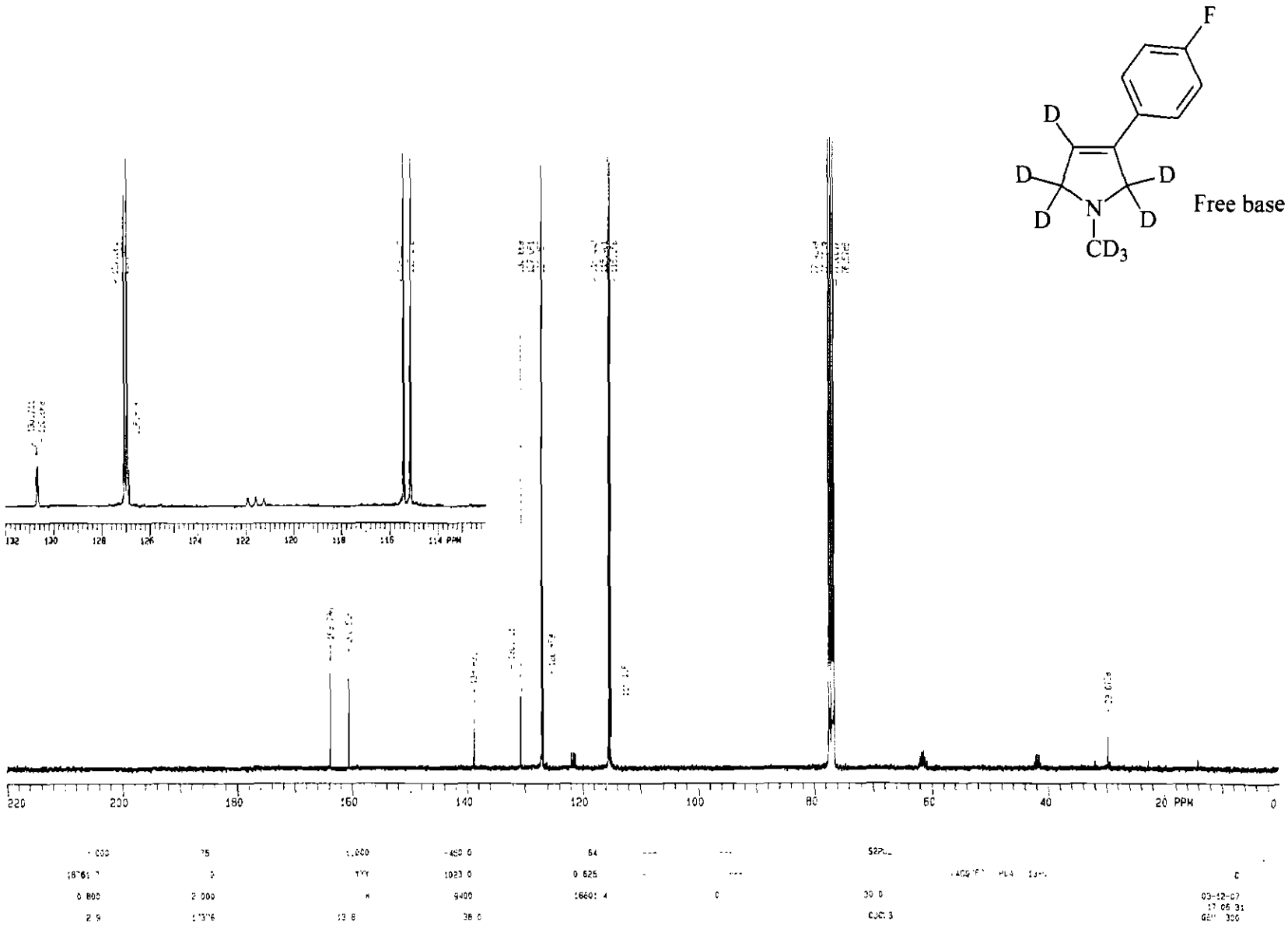
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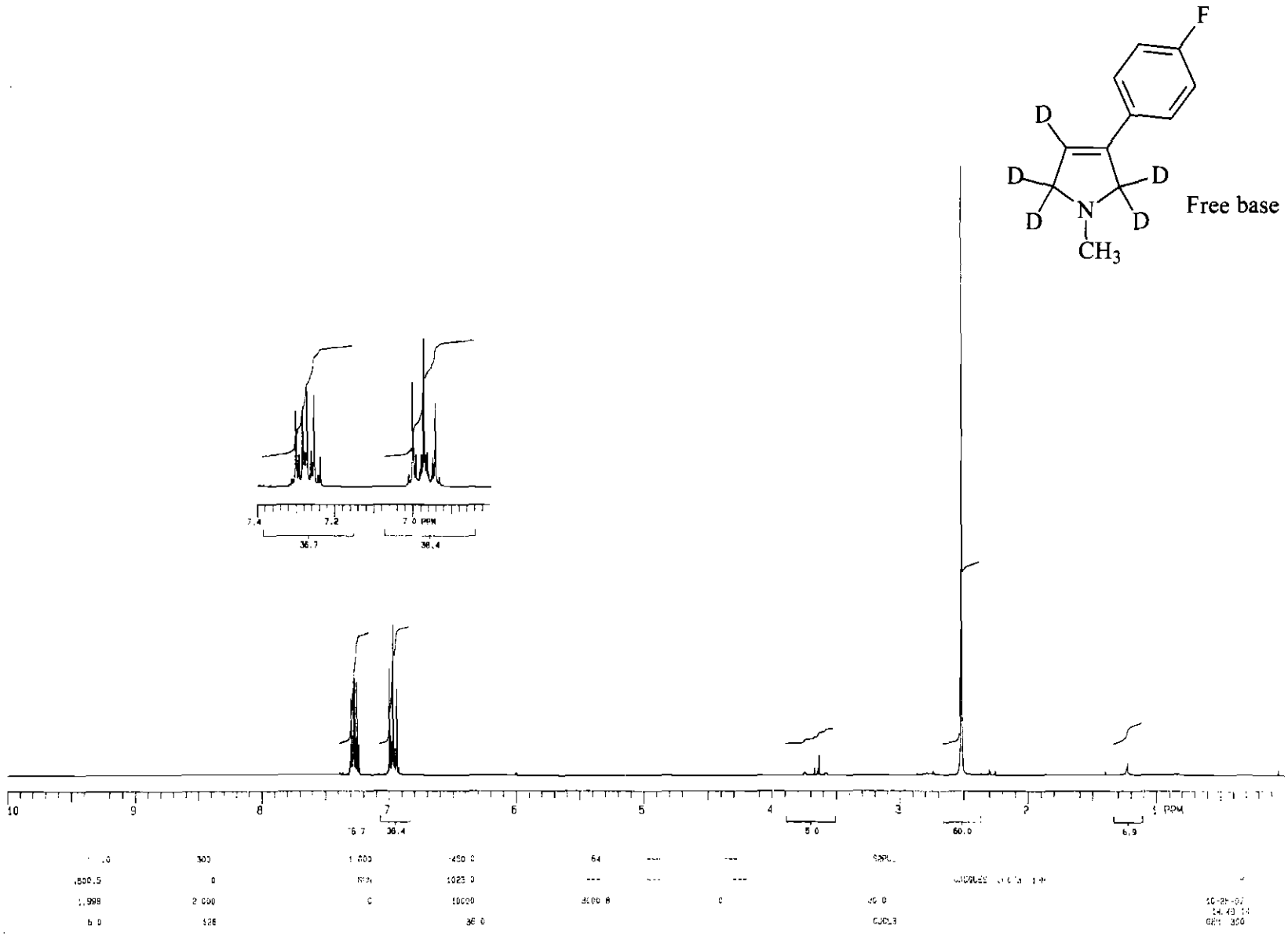
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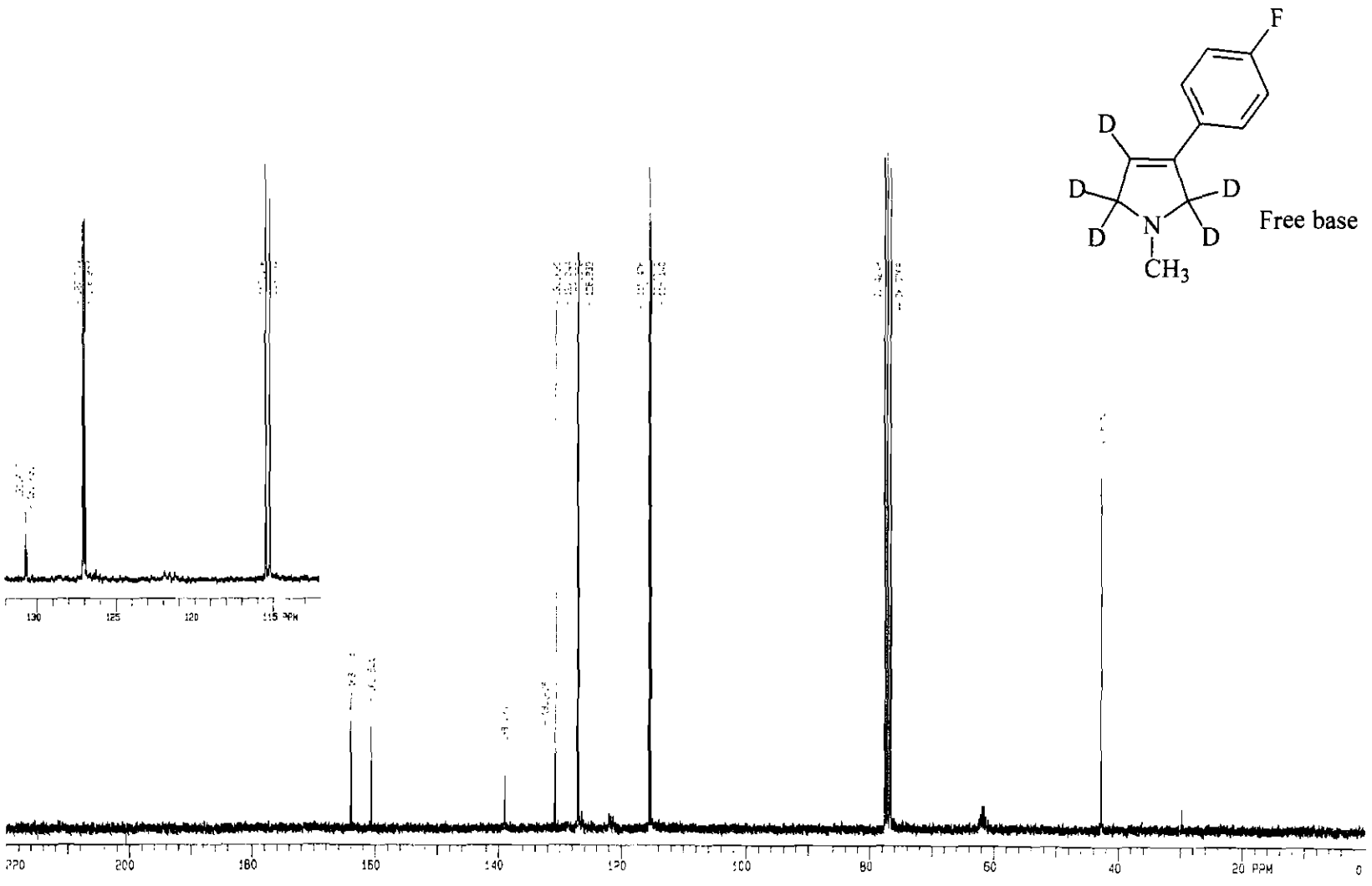
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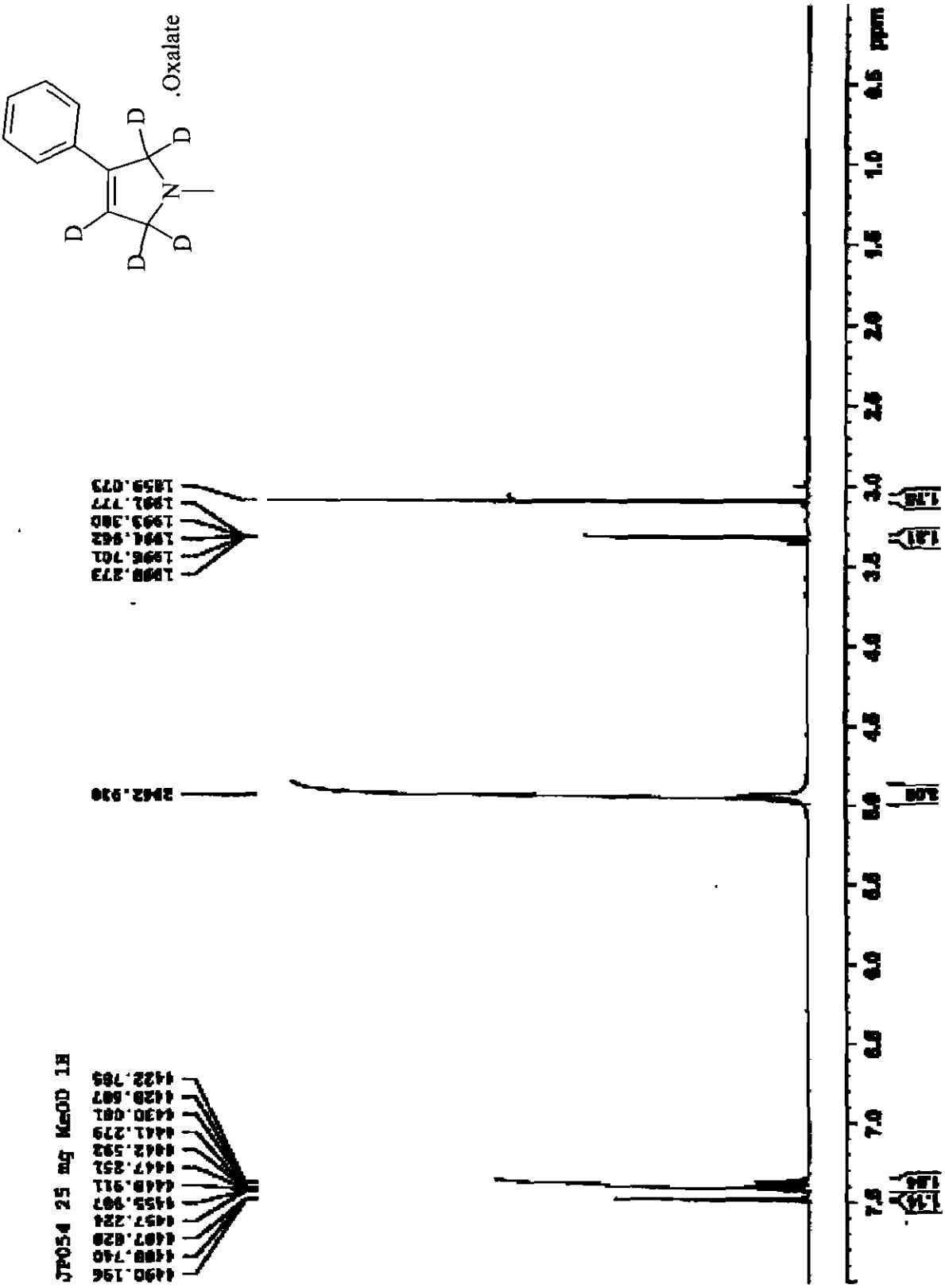
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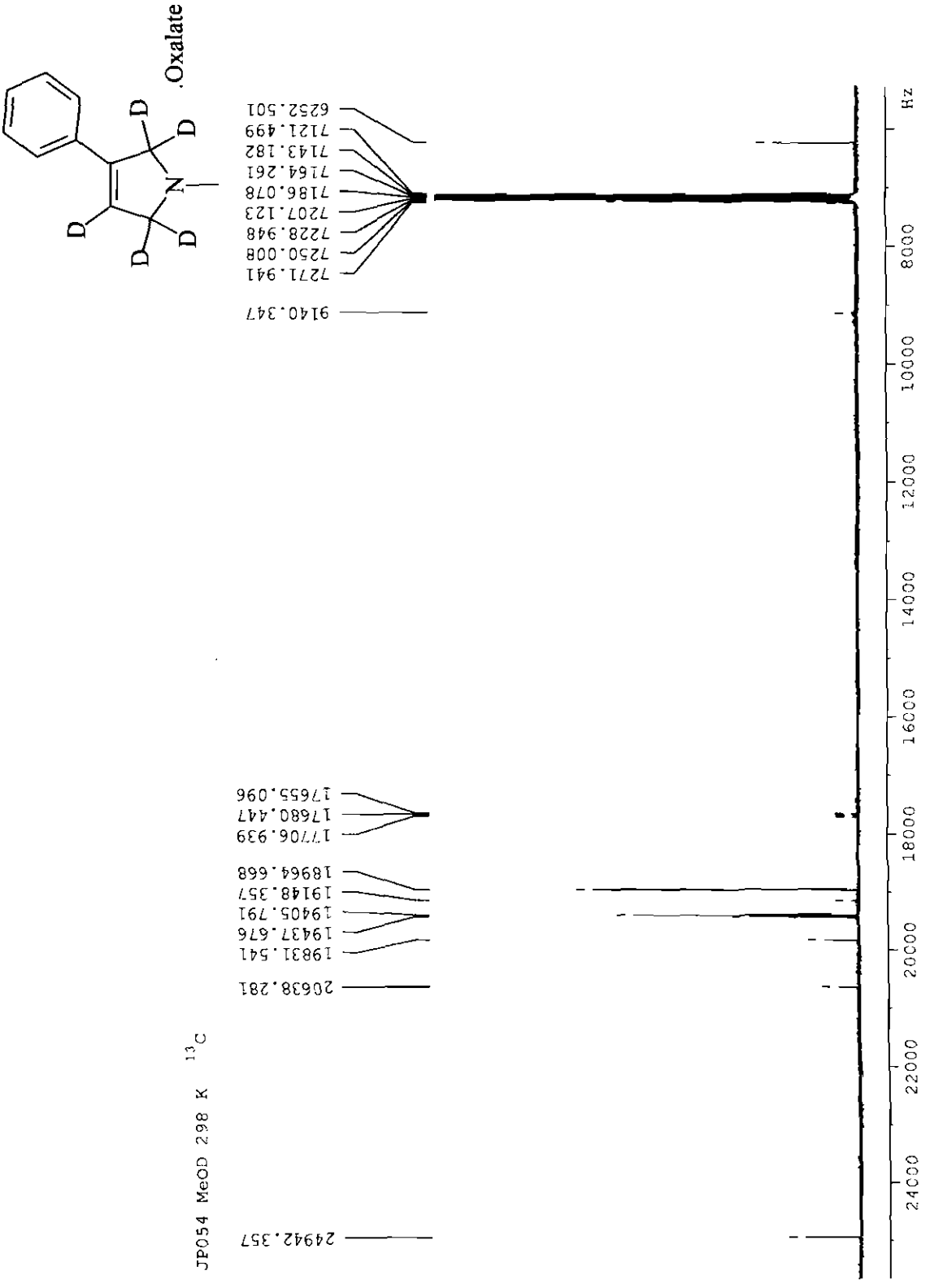
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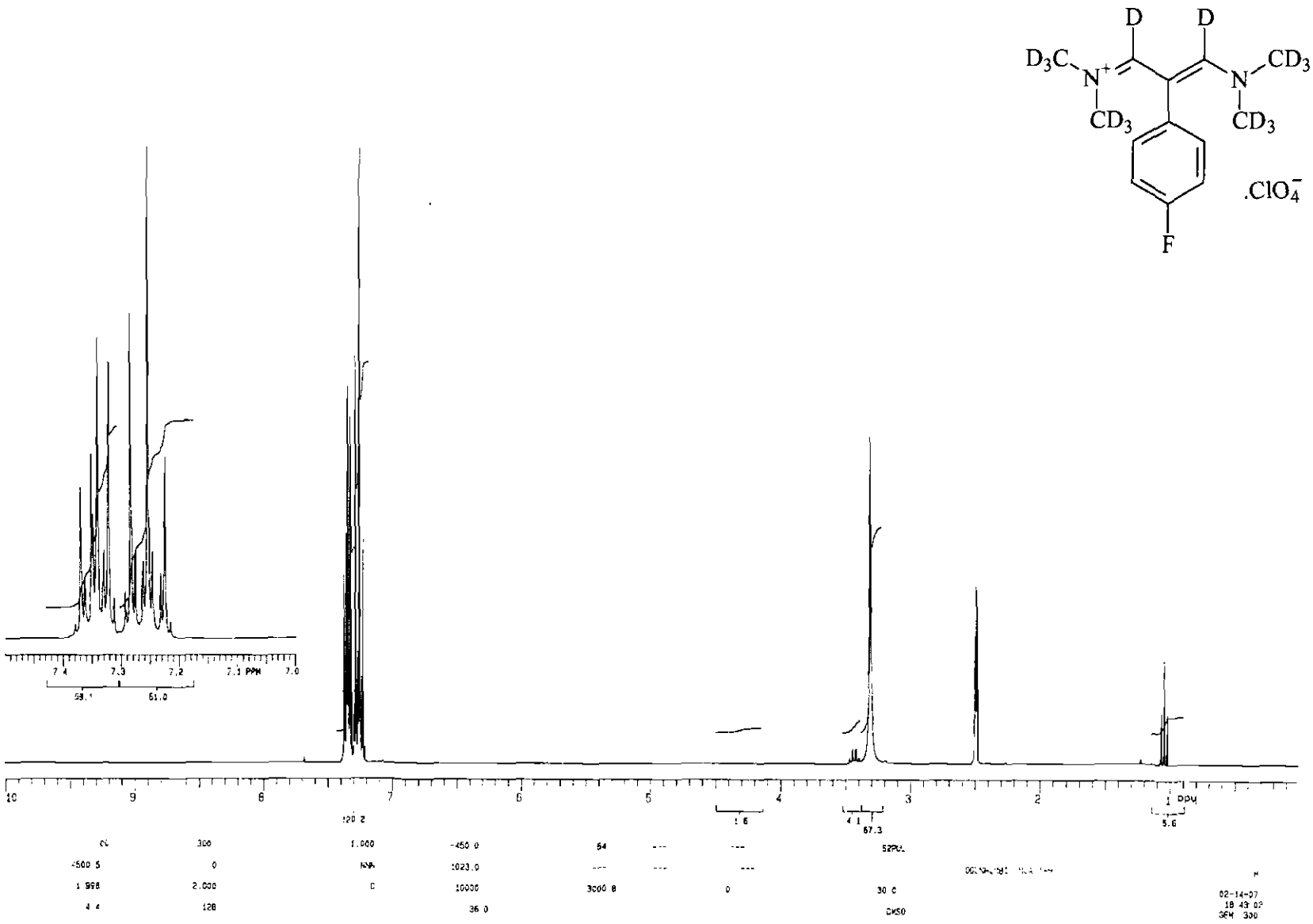
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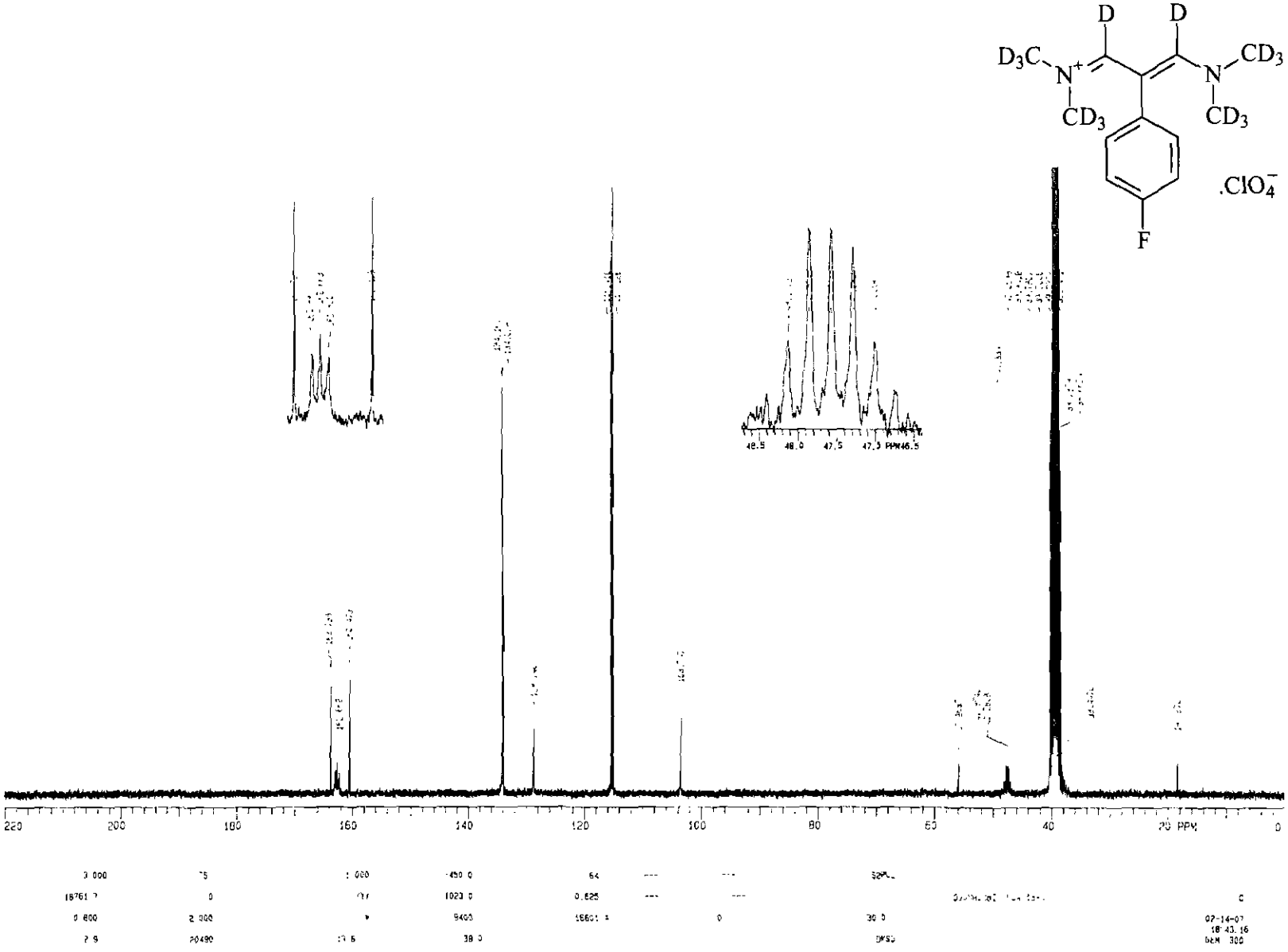
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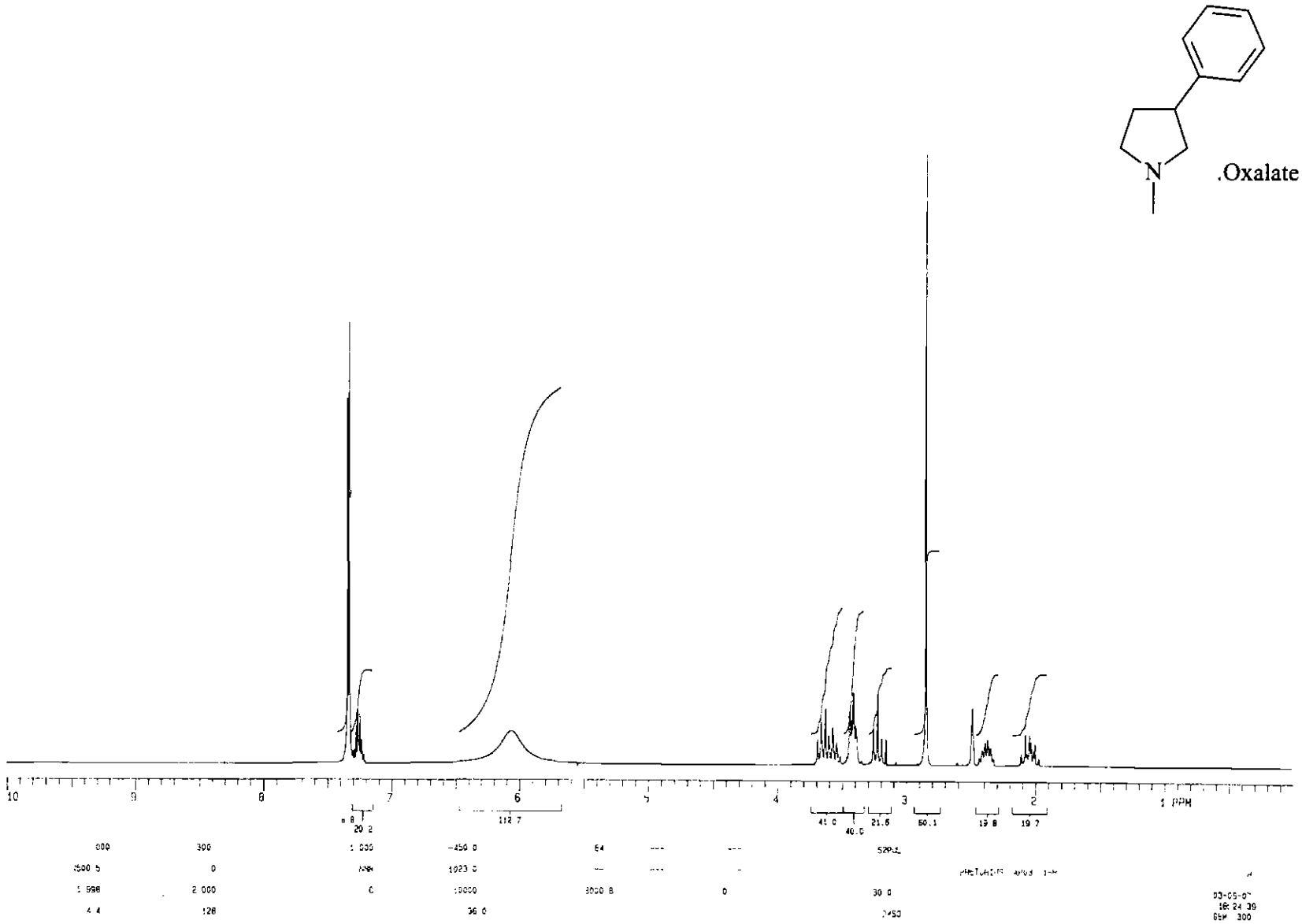
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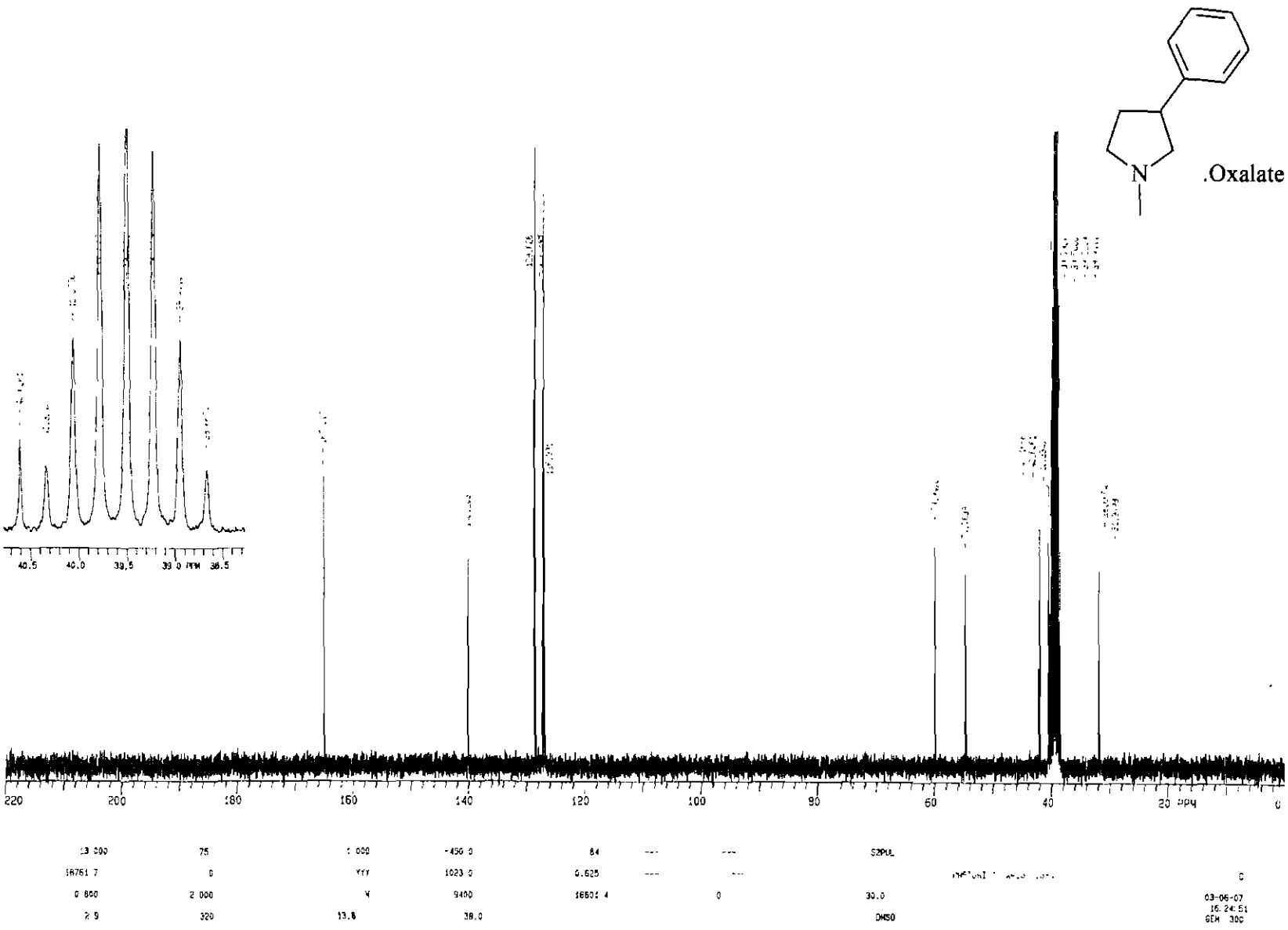
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0
DMSO

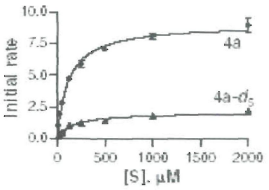
ANNEXURE B

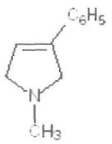
Published article

Graphical abstract

Deuterium isotope effects for the oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues by monoamine oxidase B pp xxx–xxx

Anél Pretorius, Modupe O. Ogunrombi, Gisella Terre'Blanche, Neal Castagnoli Jr., Jacobus J. Bergh, Jacobus P. Petzer *





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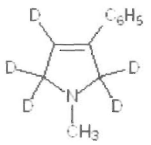

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Bioorganic & Medicinal Chemistry xxx (2008) xxx–xxx

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Bioorganic & Medicinal Chemistry

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Deuterium isotope effects for the oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues by monoamine oxidase B

Anél Pretorius [a], Modupe O. Ogunrombi [a], Gisella Terre'Blanche [a], Neal Castagnoli Jr. [b], Jacobus J. Bergh [a], Jacobus P. Petzer [a,*]

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ARTICLE INFO

Article history:
Received 6 June 2008
Accepted 2 September 2008
Available online xxxx

Keywords:
Monoamine oxidase B
MPTP
1-Methyl-3-phenyl-3-pyrroline
Kinetic isotope effect

ABSTRACT

The parkinsonian inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is a cyclic tertiary allylamine exhibiting good monoamine oxidase B (MAO-B) substrate properties. MAO-B catalyzes the ring α-carbon 2-electron bioactivation of MPTP to yield the 1-methyl-4-phenyl-2,3-dihydropyridinium species (MPDP$^+$). The corresponding 5-membered ring MPTP analogue, 1-methyl-3-phenyl-3-pyrroline, also undergoes MAO-B-catalyzed oxidation to give the 2-electron oxidation product, 1-methyl-3-phenylpyrrole. Here we report the kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the steady-state oxidation of 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-fluorophenyl)-3-pyrroline by baboon liver MAO-B, using the corresponding pyrroline-2,2,4,5,5-d_5 analogues as the deuterated substrates. The apparent isotope effects for the two substrates were 4.29 and 3.98 on V_{max}, while the isotope effects on V_{max}/K_m were found to be 5.71 and 3.37, respectively. The values reported for the oxidation of MPTP by bovine liver MAO-B with MPTP-6,6-d_2, as deuterated substrate, are $^D(V_{max}) = 3.55$; $^D(V_{max}/K_m) = 8.01$. We conclude that the mechanism of the MAO-B-catalyzed oxidation of pyrrolinyl substrates is similar to that of the tetrahydropyridinyl substrates and that a carbon–hydrogen bond cleavage step is, at least partially, rate determining.

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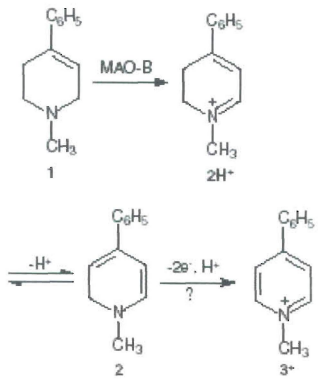
1. Introduction

The metabolic activation of the proneurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)], is catalyzed by the flavoenzyme, monoamine oxidase B (MAO-B) (Scheme 1),[1,2] to yield the α-carbon 2-electron oxidation product, the corresponding 1-methyl-4-phenyl-2,3-dihydropyridinium species, MPDP$^+$ (**2H$^+$**). Following a second 2-electron oxidation, presumably via the corresponding free base **2**, the ultimate neurotoxic metabolite, 1-methyl-4-phenylpyridinium [MPP$^+$ (**3$^+$**)] is generated.[1,3,4] MPP$^+$ is a mitochondrial toxin which selectively damages nigrostriatal neurons and induces a parkinsonian syndrome in humans[5,6] and other susceptible mammals.[7–9] MPTP is the first reported cyclic tertiary allylamine that acts as a good MAO-B substrate.[1] Several other tertiary aminyl substrates of MAO-B have since been described.[10,11] Among these, 1-methyl-3-phenyl-3-pyrroline (**4a**), the 5-membered ring analogue of MPTP, has MAO-B substrate properties comparable to those of MPTP.[12,13] The ring α-carbon oxidation of **4a** results in 1-methyl-3-phenylpyrrole (**5a**), an overall 2-electron process that most likely arises via **6Ha$^+$**, the short-lived conjugate acid of **5a** (Scheme 2).



Scheme 1. The MAO-B-catalyzed α-carbon oxidation of MPTP (**1**) to yield the corresponding 2,3-dihydropyridinium product MPDP$^+$ (**2H$^+$**). Further oxidation via an unknown pathway results in the pyridinium species MPP$^+$ (**3$^+$**).

In the present study, we report the steady-state kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the oxidation of 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-fluoro-

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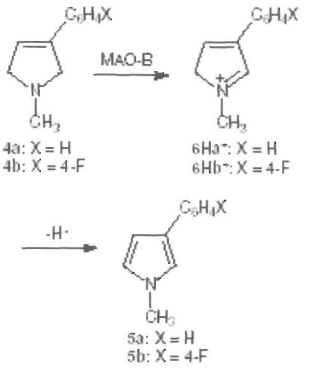
0968-0896/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.bmc.2008.09.001

Please cite this article in press as: Pretorius, A. et al., Bioorg. Med. Chem. (2008), doi:10.1016/j.bmc.2008.09.001



Scheme 2. The MAO-B-catalyzed α-carbon oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues (**4a** and **4b**) to yield intermediates **6Ha+** and **6Hb+**. Deprotonation of **6Ha+**/**6Hb+** results in the corresponding 1-methyl-3-phenylpyrrolyl species **5a** and **5b**.

phenyl)-3-pyrroline (**4b**) by baboon liver mitochondrial MAO-B. For this purpose we have employed the corresponding 1-methyl-3-phenyl-3-pyrrolinyl-2,2,4,5,5-d_5 (**4a**-d_5) and 1-methyl-3-(4-fluorophenyl)-3-pyrrolinyl-2,2,4,5,5-d_5 (**4b**-d_5) analogues as deuterated substrates (Scheme 3). The apparent isotope effects for the two substrates were compared to the isotope effects previously reported for the oxidation of MPTP by bovine liver mitochondrial MAO-B using MPTP-6,6-d_2 (**1**-d_2) as deuterated substrate.[14] The results of this study are discussed with reference to the extent to which carbon–hydrogen bond cleavage is rate limiting during MAO-B-catalyzed oxidation of pyrrolinyl substrates.

2. Results

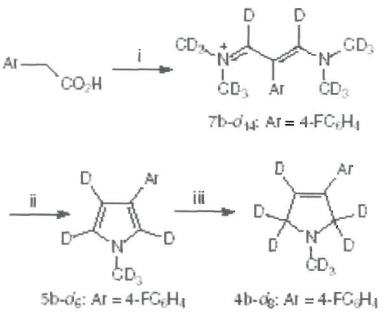
2.1. Chemistry

The 1-methyl-3-phenyl-3-pyrrolinyl analogues (**4a**–**4b**) examined here, were prepared by partial reduction of the corresponding pyrrolyl analogues **5a**–**b** as previously reported.[13,15,16] TLC and ^{1}H NMR analysis indicated that the 3-pyrrolines prepared in this manner were contaminated with the corresponding pyrrolidinyl derivatives formed by the over-reduction of the pyrrolyl substrates.[17] Also, HPLC analysis revealed trace amount of the pyrrolyl starting material present in samples of the free bases of **4a** and **4b**. In order to remove the volatile pyrrolidinyl contaminant from **4b**, the free base was subjected to high vacuum (see Section 4) while the pyrrolyl contaminant was removed by converting **4b** to the corresponding oxalate salt (**4b**-oxalate). For the purification of **4a**, both the corresponding pyrrolidinyl and pyrrolyl contaminants were removed via fractional recrystallization of the oxalate salt (**4a**-oxalate) as reported.[13]

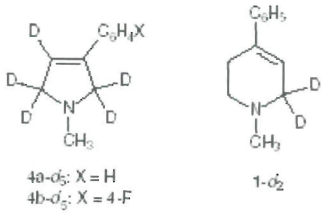
In order to replace the methylene protons of the ring α-carbons (C-2 and C-5) of the pyrrolinyl analogues with deuterons, a similar synthetic approach was initially followed (Scheme 4). Because of the relative ease of purification of the 4-fluorophenyl substituted pyrrolinyl analogue **4b** compared to the unsubstituted analogue **4a** (see above), compound **4b**-d_8 was chosen as the target 3-pyrroline. The key starting material, **7b**-d_{14} was prepared by reaction of 4-fluorophenylacetic acid with DMF-d_7 in the presence of $POCl_3$.[18] ^{1}H NMR analysis indicated that deuterium incorporation was >99% at the anticipated methyl and methinyl carbons (Scheme 4). Treatment of **7b**-d_{14} with a sodium methoxide solution (prepared with methanol-d_1) in dry pyridine yielded the pyrrolyl analogue **5b**-d_5.[19,20] While the deuterium incorporation at the N–CH_3 carbon was high (97.8%), proton–deuteron exchange occurred at the C-2, C-4, and C-5 of the pyrrolyl heterocyclic system resulting in deuteron loss to the extent of 6–27%. However, upon reduction with zinc/DCl (to yield **4b**-d_8),[13,15,21] deuterium incorporation at C-2, C-4, and C-5 of the pyrrolinyl ring were again relatively high (>96%). This result indicated that the zinc/DCl reduction procedure could be accompanied by the exchange of pyrrolinyl protons for deuterons. It was possible to take advantage of this type of reaction to prepare both **4b**-d_5 and the more difficult to purify **4a**-d_5, by simply reducing the corresponding pyrrolyl analogue with zinc/DCl. Accordingly, zinc/DCl reduction of **5a** and **5b** yielded **4a**-d_5 and **4b**-d_8, respectively, with a relatively high degree (>98%) of deuterium incorporation at C-2, C-4, and C-5 of the pyrrolinyl ring (Scheme 5). No deuterium incorporation was observed at N–CH_3.

2.2. General enzymology

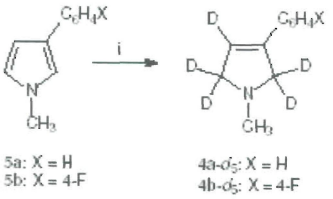
In order to measure the rates of oxidation of the pyrrolinyl analogues **4a**–**4b** and **4a**-d_5–**4b**-d_5, the substrates were incubated with



Scheme 4. Synthetic pathway to the deuterated pyrrolinyl substrate **4b**-d_8. Reagents and conditions: (i) DMF-d_7, $POCl_3$, 80 °C; (ii) $NaOCH_3$ (from methanol-d_1), pyridine, reflux; (iii) Zn/DCl.



Scheme 3. The structures of 1-methyl-3-phenyl-3-pyrroline-2,2,4,5,5-d_5 (**4a**-d_5), 1-methyl-3-(4-fluorophenyl)-3-pyrroline-2,2,4,5,5-d_5 (**4b**-d_5), and MPTP-6,6-d_2 (**1**-d_2).



Scheme 5. Synthetic pathway to the deuterated pyrrolinyl substrates **4a**-d_5 and **4b**-d_5. Reagent: (i) Zn/DCl.

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the enzyme for a fixed period of time at 37 °C and the corresponding pyrrolyl oxidation products were quantified by HPLC-UV analysis (Fig. 1) as described previously for **4a**.[13] HPLC-UV analysis was preferred to spectrophotometry since background interference in the near-UV wavelength range by the mitochondrial fractions used here as enzyme source, prevented accurate spectrophotometric measurements at 270 nm, the approximate wavelength of maximal absorption of the pyrrolyl products. The incubation time of the enzyme-catalyzed reactions were chosen to be 10 min since the oxidation of all the substrates evaluated here (**4a**–**4b** and **4a**-d_5–**4b**-d_5) was found to be linear (Fig. 2) for at least 15 min at substrate concentrations of 100 μM. Control incubations of the substrates carried out with no enzyme source added were used to verify the absence of the pyrrolyl products in the incubation samples.

To estimate the extent on non-MAO-B mediated oxidation of the pyrrolinyl substrates **4a** and **4a**-d_5, the baboon liver mitochondrial fractions that served as enzyme source were preincubated with the MAO-B selective inactivator (*R*)-deprenyl.[22] Following incubation of the test substrates (50 μM) with the mitochondria inactivated with (*R*)-deprenyl, only low concentrations of the pyrrolyl oxidation products were observed. For example, for substrate **4a** the yield of pyrrolyl oxidation product **5a** (0.54 ± 0.052 μM) with the inactivated mitochondrial preparation was only 8.4% of that formed with the fully active preparation (6.42 ± 0.18 μM). Similarly, when the deuterium labeled analogue **4a**-d_5 was incubated with (*R*)-deprenyl pre-inactivated mitochondria only 0.037 ± 0.001 μM (about 2.6%) of the pyrrolyl oxidation product was observed compared to the amount formed (1.42 ± 0.05 μM) with the fully active preparation. These results document that oxidation of the pyrrolinyl substrates by the baboon liver mitochondrial fraction is MAO-B dependent. The residual activity not suppressed by (*R*)-deprenyl has been observed previously in experiments where tetrahydropyridines served as the MAO-B substrates.[22] The factors contributing to this residual activity remain to be identified. At least for baboon liver mitochondria the residual activity is not related to the action of the MAO-A isoform since the baboon liver mitochondria are devoid of MAO-A activity.[22] Furthermore, 1-methyl-3-phenyl-3-pyrroline (**4a**) is reported to be an MAO-B selective substrate.[12]

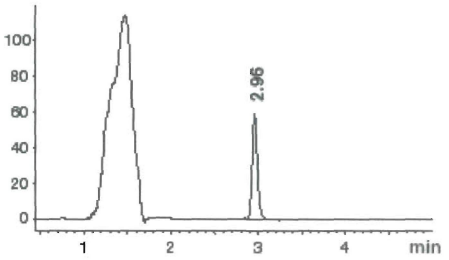

Figure 1. An HPLC-UV tracing showing the presence of 1-methyl-3-phenylpyrrole (**5a**) (retention time of 2.96 min) in an incubation of 1-methyl-3-phenyl-3-pyrroline (**4a**) with baboon liver MAO-B (0.15 mg protein/mL of the mitochondrial preparation). Following 10 min incubation, the reaction was terminated by the addition of trichloroacetic acid. After centrifugation, 50 μL of the supernatant was injected into the HPLC and the effluent was monitored at a wavelength of 270 nm.

2.3. Steady-state kinetic deuterium isotope effects on V_{max} and V_{max}/K_m

In order to estimate the values of the steady-state kinetic parameters (K_m and V_{max}) for the oxidation of the oxalate salts of the pyrrolinyl analogues (**4a**–**4b** and **4a**-d_5–**4b**-d_5) by MAO-B, initial rates were measured at eight substrate concentrations spanning at least two orders of magnitude. As illustrated by example with Figure 3, the steady-state oxidation of the substrates (**4a**–**4b** and **4a**-d_5–**4b**-d_5) by MAO-B, followed Michaelis–Menten behavior. Also, large kinetic isotope effects on V_{max} were apparent from the graphs. The K_m and V_{max} values as well as the steady-state kinetic deuterium isotope effects obtained for the oxidation of the pyrrolinyl substrates by baboon liver mitochondrial MAO-B are summarized in Table 1. The apparent isotope effects on V_{max} for the oxidation of **4a** and **4b** were found to be 4.29 and 3.98 using **4a**-d_5 and **4b**-d_5, respectively, as the deuterated substrates. These values are comparable to the $^D(V_{max})$ value of 3.55 previously reported for the oxidation of MPTP by bovine liver mitochondrial MAO-B with MPTP-6,6-d_2 as the deuterated substrate.[14] We have observed a smaller difference between the K_m value for **4a** and its deuterated analogue **4a**-d_5 [$^D(K_m = 0.80)$] than the reported difference between the K_m values of MPTP and MPTP-6,6-d_2 [$^D(K_m = 0.45)$].[14] The K_m value of substrate **4b**, was found to be, within the range

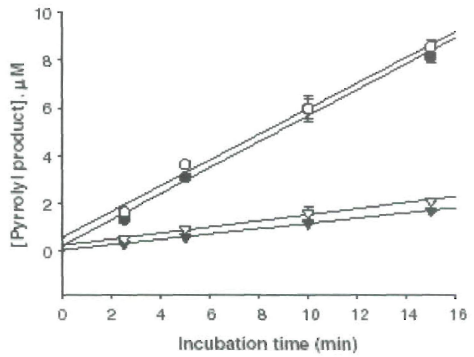

Figure 2. Linearity in the oxidation of pyrrolinyl analogues **4a** (filled circles), **4b** (open circles), **4a**-d_5 (filled triangles) and **4b**-d_5 (open triangles) by baboon liver mitochondrial MAO-B (0.15 mg protein/mL of the mitochondrial preparation). The concentration of pyrrolyl products was quantified via HPLC-UV analysis following termination of the enzyme-catalyzed reaction at time points of 2.5, 5.0, 10.0 or 15.0 min. The concentration of the substrates used in this study was 100 μM.

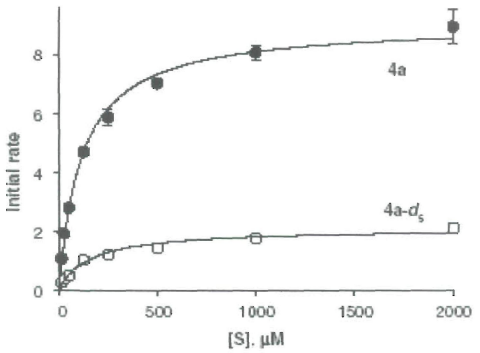

Figure 3. Determination of the K_m and V_{max} values for the oxidation of **4a** (filled circles) and **4a**-d_5 (open circles) by baboon liver MAO-B (0.15 mg protein/mL of the mitochondrial preparation). The concentrations of the pyrrolyl oxidation products were measured by HPLC analysis following a 10 min incubation at 37 °C. The rate data were fitted to Michaelis–Menten equation. All measurements were conducted in triplicate and the concentration of the test substrates in the incubations ranged from 12.5 to 2000 μM. The initial rates are expressed as nanomoles product formed/min-mg protein.

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Table 1
Steady-state kinetic constants and deuterium isotope effects for the MAO-B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues

Compound	V_{max}[a]	K_m (μM)	$^D(V_{max})$	$^D(K_m)$	$^D(V_{max}/K_m)$
4a	9.07 ± 0.41	118 ± 7.27			
4b	15.7 ± 1.73	87.2 ± 1.87			
4a-d_5	2.11 ± 0.05	157 ± 26.1	4.29 ± 0.08	0.80 ± 0.15	5.71 ± 0.88
4b-d_5	3.96 ± 0.52	73.8 ± 4.46	3.98 ± 0.09	1.19 ± 0.10	3.37 ± 0.20

[a] Values are expresses in nmol/min–mg mitochondria.

expected for experimental error, equal to that of its deuterated analogue, **4b**-d_5, [$^D(K_m$ = 1.19)].

3. Discussion

In this study we have measured the kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the steady-state oxidation of 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-fluorophenyl)-3-pyrroline (**4b**) by baboon liver mitochondrial MAO-B. For this purpose the corresponding pyrroline-2,2,4,5,5-d_5 analogues (**4a**-d_5 and **4b**-d_5) were employed as deuterated substrates. Moderately large apparent isotope effects of 4.29 and 3.98 on V_{max} were observed for the two substrates, respectively. The isotope effects on V_{max}/K_m were found to be 5.71 and 3.37 for the two substrates, respectively. Although these values may not reflect the intrinsic isotope effects, the V_{max} isotope effects suggest that a carbon–hydrogen bond cleavage step is at least partially rate determining.[23] The isotope effects on V_{max} for the two pyrrolinyl substrates are comparable to those reported for the oxidation of MPTP by bovine liver MAO-B [$^D(V_{max})$ = 3.55]. We conclude that the mechanism of the MAO-B-catalyzed oxidation of pyrrolinyl substrates is similar to that of the tetrahydropyridinyl substrates.

4. Experimental

4.1. Chemicals and instrumentation

All starting materials, unless otherwise stated, were obtained from Sigma–Aldrich and were used without purification. Deuterated solvents and reagents were obtained from Cambridge Isotope Laboratories. Proton and carbon NMR spectra were recorded on a Varian Gemini 300 spectrometer. ^{1}H NMR spectra were recorded at a frequency of 300 MHz and ^{13}C NMR spectra at 75 MHz. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the NMR solvents, $CDCl_3$, DMSO-d_6, and CD_3OD. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), sep (septet) or m (multiplet) and the coupling constants (*J*) are given in hertz (Hz). Fast atom bombardment mass spectra (FAB-MS) were recorded with a VG 7070E mass spectrometer. Direct insertion electron impact ionization mass spectra (EI-HRMS) were obtained with a AutoSpec ETOF (Micromass). Melting points (mp) were determined with a Stuart SMP10 melting point apparatus; all melting points are uncorrected. HPLC analyses were performed with an Agilent 1200 HPLC system equipped with an Agilent 1200 series variable wavelength detector. Thin layer chromatography (TLC) was carried out using neutral aluminum oxide 60 (Merck) with UV_{254} fluorescent indicator.

4.2. Synthesis of 2-(4-fluorophenyl)-3-(dimethylamino)-allylidene(dimethyl)ammonium perchlorate-d_{14} (**7b**-d_{14})

Compound **7b**-d_{14} was prepared in high yield from 4-fluorophenylacetic acid, *N*,*N*-dimethylformamide-d_7 (DMF-d_7), and phosphoryl chloride according the method described in the literature.[18]

4.2.1. 2-(4-Fluorophenyl)-3-(dimethylamino)allylidene(dimethyl)-ammonium perchlorate-d_{14} (**7b**-d_{14})

Yield 82.5%; mp 128–133 °C (from ethanol); ^{1}H NMR (DMSO-d_6) δ 7.22–7.29 (m, 2H), 7.31–7.38 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 38.45 (m), 47.57 (sep), 103.55, 115.27 (d), 128.74, 134.11 (d), 160.47, 162.66 (t), 163.74; FAB-MS *m/z*: 236 (MH^+).

4.3. Synthesis of 1-methyl-3-phenylpyrroles **5a–b** and **5b**-d_6

1-Methyl-3-phenylpyrroles **5a–b** were synthesized according to a modification[23] of the method described in the literature.[19] Compound **5b**-d_6 was synthesized from **7b**-d_{14} according to the following procedure[19,20]: sodium pieces (13 mmol) was reacted with methanol-d_1 (4.5 mL) and the resulting solution was added under an atmosphere of argon to a solution of **7b**-d_{14} (6 mmol) in 24 mL dry pyridine (distilled over CaH_2 and stored over 4 Å molecular sieves). The reaction was heated under reflux for 24 h and most of the pyridine was removed via vacuum distillation to obtain a yellow pasty residue to which 25 mL D_2O was added. The resulting suspension was stirred for 30 min on an ice bath and filtered. The crude solid so obtained was dissolved in a minimum amount of ethylacetate and purified on a short column (30 × 60 mm) by neutral aluminum oxide chromatography (Fluka) with petroleum ether/ethylacetate, 90:10 as mobile phase to give a white solid. The melting points of previously reported **5a** and **5b** were as follows: **5a** mp 46–48 °C (from petroleum ether), lit. mp 46–47 °C[19]; **5b** mp 100–101 °C (from methanol), lit. mp 98–100 °C[20]. The characterization of previously unreported **5b**-d_6 is summarized below.

4.3.1. 1-Methyl-3-(4-fluorophenyl)pyrrole-d_6 (**5b**-d_6)

The title compound was synthesized from **7b**-d_{14} in a yield of 46.8%; mp 102–103 °C; ^{1}H NMR ($CDCl_3$) δ 6.37 (0.06H), 6.61 (0.0+1H), 6.82 (s, 0.27H), 6.97–7.04 (m, 2H), 7.39–7.46 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 35.67 (m), 106.11 (d), 115.25 (d), 118.19 (m), 122.67 (d), 124.09, 126.28 (d), 132.16 (d), 159.45, 162.68; EI-HRMS calcd. 181.11739, found 181.11819 (M^+).

4.4. Synthesis of 1-methyl-3-phenyl-3-pyrrolinyl analogues (**4a–4b**, **4a**-d_5–**4b**-d_5 and **4b**-d_8) and their respective oxalate salts

Compounds **4a–b** were synthesized from the corresponding pyrrolyl analogues, **5a–5b**, according to a modification[13] of the zinc reduction procedure reported in the literature.[15,16] Compounds **4a**-d_5, **4b**-d_5, and **4b**-d_8 were synthesized from **5a**, **5b**, and **5b**-d_6, respectively, according to the following procedure: DCl (15 mL, 20%) was cooled on an ice bath (0 °C) and zinc dust (20 mmol) was carefully added followed by the appropriate pyrrolyl analogue (4 mmol) dissolved in 34 mL ethanol-d_1. The resulting suspension was stirred for 1 h at 0 °C and DCl (10 mL, 35%) was added. The reaction was allowed to return to room temperature and stirred for another 1 h. Another 2 portions of zinc dust (40 mmol) were added and the reaction was stirred for 2 h during which the reaction cleared. The remaining zinc was removed via filtration and washed with 5 mL D_2O. The residual ethanol-d_1 was removed from the filtrate under reduced pressure, the pH was adjusted to ~12 with NaOH (8 N) and the reaction was extracted to diethylether (3 × 30 mL). The organic phase was dried over $MgSO_4$, removed under reduced pressure to yield the product as a light yellow oil. The pyrrolinyl analogues were converted to their respective oxalic acid salts in diethyl ether. Compounds **4a** and **4a**-d_5 were purified by recrystallizing their oxalate salts twice from methanol. Compounds **4b**, **4b**-d_5 and **4b**-d_8 were purified by placing the free bases in a vacuum oven at 40 °C for 24 h. For previously described **4a**-oxalate the melting point was recorded as 151–153 °C (from methanol), lit. mp 152–154 °C.[13] The characterizations of compounds that were previously unreported are summarized below.

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4.4.1. 1-Methyl-3-phenyl-3-pyrroline-d_5 (4a-d_5)

The title compound was synthesized from **5a** in a yield of 16.5% (oxalate salt): mp 152–155 °C; ^{1}H NMR (CD_3OD) δ 3.10 (s, 3H), 7.35–7.43 (m, 3H), 7.47–7.50 (m, 2H); ^{13}C NMR (CD_3OD) δ 41.68, 60.93 (m), 62.00 (m), 117.87 (m), 126.43, 129.37, 129.58, 132.21, 137.59, 166.28; EI-HRMS calcd. 164.13618, found 164.13641 ($M^{+\cdot}$).

4.4.2. 1-Methyl-3-(4-fluorophenyl)-3-pyrroline (4b)

The title compound was synthesized from **5b** in a yield of 29.5% (free base): mp 64–66 °C; ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 3H), 3.60–3.65 (m, 2H), 3.77–3.80 (m, 2H), 6.02 (m, 1H), 6.95–7.01 (m, 2H), 7.27–7.31 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 42.67, 62.38 (d), 115.30 (d), 121.93 (d), 127.00 (d), 130.67 (d), 138.97, 160.52, 163.79; EI-HRMS calcd. 177.09538, found 177.09629 ($M^{+\cdot}$).

4.4.3. 1-Methyl-3-(4-fluorophenyl)-3-pyrroline-d_5 (4b-d_5)

The title compound was synthesized from **5b** in a yield of 36.3% (free base): mp 63–66 °C; ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 3H), 6.94–7.00 (m, 2H), 7.26–7.30 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 42.66, 61.95 (m), 115.28 (d), 121.90 (m), 126.99 (d), 130.65 (d), 138.79, 160.50, 163.77; EI-HRMS calcd. 182.12676, found 182.12585 ($M^{+\cdot}$).

4.4.4. 1-Methyl-3-(4-fluorophenyl)-3-pyrroline-d_8 (4b-d_8)

The title was synthesized from **5b**-d_5 in a yield of 48.0% (free base): mp 64–68 °C; ^{1}H NMR ($CDCl_3$) δ 6.94–7.02 (m, 2H), 7.24–7.32 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 41.90 (m), 61.95 (m), 115.30 (d), 121.90 (m), 127.01 (d), 130.69 (d), 138.83, 160.52, 163.79; EI-HRMS calcd. 185.14559, found 185.14566 ($M^{+\cdot}$).

4.5. Steady-state MAO-B activity measurements

Baboon liver mitochondria were isolated as described in the literature[24] and stored at −70 °C. The mitochondrial isolates were suspended in 1 volume sodium phosphate buffer (100 mM, pH 7.4) containing 50% glycerol (w/v) and the protein concentrations were determined by the method of Bradford.[25] Since baboon liver is devoid of MAO-A activity, inactivation of MAO-A was deemed unnecessary.[22] In order to estimate the K_m and V_{max} values for the oxidation of the oxalate salts of the pyrrolinyl analogues **4a**–**4b** and **4a**-d_5–**4b**-d_5 by MAO-B, initial rates were measured at eight substrate concentrations spanning at least two orders of magnitude (12.5–2000 μM). The reactions were carried out in a final volume of 500 μL (in 100 mM sodium phosphate buffer, pH 7.4) and the enzyme concentration used was 0.15 mg mitochondrial protein/mL. Following incubation at 37 °C for a period of 10 min, the reactions were terminated by the addition of 20 μL trichloroacetic acid (1 g/mL, w/v). A volume of 100 μL acetonitrile was added, and the resulting samples were centrifuged at 16,000*g* for 10 min. The supernatant fractions were removed and the concentrations of the MAO-B-generated pyrrolyl products were measured by HPLC analysis with UV detection as described before.[13] The elution of the pyrrolyl oxidation products were monitored at a wavelength of 270 nm and quantitative estimations were made by means of linear calibration curves ranging from 1.5 to 25 μM of **5a** and **5b**. The steady-state kinetic data (initial rates as a function of substrate concentration) were fitted to Michaelis–Menten equation using the one site binding model incorporated into the GraphPad Prism software package (GraphPad Software Inc.). All measurements were conducted in triplicate and the K_m and V_{max} values were expressed as means ± standard error of the mean (SEM).

4.6. Linearity of oxidation of pyrrolinyl substrates

In order to determine the time interval for which MAO-B-catalyzed substrate oxidation remains linear, 100 μM of the oxalate salts of the test substrates (**4a**–**4b** and **4a**-d_5–**4b**-d_5) were incubated with 0.15 mg protein/mL of the baboon liver mitochondrial fraction. At time points 2.5, 5, 10, and 15 min, the reactions were terminated with the addition of 20 μL trichloroacetic acid (1 g/mL, w/v) and the MAO-B-generated pyrrolyl products were measured by HPLC analysis as described above. All measurements were conducted in triplicate and the concentrations of the pyrrolyl products were expressed as means ± SEM.

4.7. (*R*)-Deprenyl studies

In order to estimate the degree to which the oxidation of the pyrrolinyl substrates (**4a** and **4a**-d_5) was dependent upon MAO-B, baboon liver mitochondria (0.3 mg protein/mL) were preincubated with (*R*)-deprenyl-HCl (3×10^{-6} M) at 37 °C for 30 min in 100 mM sodium phosphate buffer, pH 7.4. The pre-inactivated mitochondrial fractions were then added to the test substrates to yield final concentrations of 0.15 mg mitochondrial protein/mL. The volume of these incubation mixtures were 500 μL and the final concentration of the oxalate salts of the test substrates was 50 μM. The reactions were incubated for 10 min at 37 °C and terminated with the addition of 20 μL trichloroacetic acid (1 g/mL, w/v). Control incubation reactions were carried out following the same procedure with the exception that the preincubations were conducted in the absence of (*R*)-deprenyl. The concentrations of the pyrrolyl products in the enzymatic reactions were measured by HPLC analysis as described above. All measurements were conducted in triplicate and the concentrations of the pyrrolyl products were expressed as means ± SEM.

Acknowledgments

We are grateful to Jan du Preez and the staff of the Analytical Technology Laboratory, North-West University for their support. The NMR and MS spectra were recorded by André Joubert, Johan Jordaan, and Louis Fourie of the SASOL Centre for Chemistry, North-West University. This work was supported by grants from the National Research Foundation and the Medical Research Council, South Africa.

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Please cite this article in press as: Pretorius, A. et al., *Bioorg. Med. Chem.* (2008), doi:10.1016/j.bmc.2008.09.001