

Chapter 2. Metabolism of Cyclic Tertiary Amines

2.1. MPTP - A Parkinsonian Inducing Agent

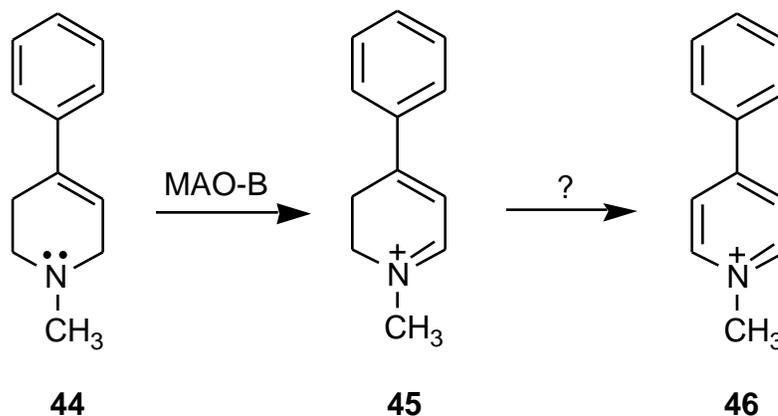
Parkinson's disease (PD) is a chronic and progressive neurological disorder that affects the motor system and is characterized by tremor, rigidity, bradykinesia, or slowness of movement, and impaired balance and coordination. PD occurs when dopaminergic neurons of the substantia nigra die or become impaired. Loss of dopamine causes the nerve cells of the striatum to fire out of control, leaving patients unable to direct or control their movements in a normal manner. The cause of cell death is unknown but many researchers believe that a combination of oxidative damage, environmental toxins, genetic predisposition and accelerated aging may be shown to cause the disease.

Motor deficits similar to those observed in Parkinson's disease were reported in young drug addicts following self-administration of a home-made street drug found to be contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **44**).⁶⁴ Langston has reported a striking similarity of the parkinsonian syndrome induced in human drug addicts with the syndrome observed in idiopathic Parkinson's disease.⁶⁵ MPTP has been effective in producing a non-human primate model of the disease in which selective nerve cell loss in the substantia nigra compacta, irreversible decreased dopamine levels in the striatum, and chronic impairment of extrapyramidal motor function are observed.^{66,67} Many researchers have investigated the mechanism of action of this neurotoxin, its metabolism, and have exploited its potential as a model of Parkinson's disease.

2.1.1. Mechanism of Action of MPTP

The mechanism of neurotoxicity of MPTP has been studied extensively. The toxication of MPTP is mediated by monoamine oxidase, principally type B, and to a lesser extent type A.⁶⁸ The enzyme catalyzes a two electron oxidation of MPTP to the unstable 2,3-dihydro-1-methyl-4-phenylpyridinium species MPDP⁺ (Scheme 11, **45**).⁶⁹⁻⁷¹ MPDP⁺ undergoes a further two electron oxidation by a pathway that may be enzyme mediated or by a disproportionation mechanism to result in the neurotoxic 1-methyl-4-phenylpyridinium species MPP⁺ (**46**).⁷²⁻⁷⁴ MPP⁺ is actively transported into the dopaminergic neurons by the dopamine uptake system.^{75,76} It is concentrated in the matrix of the mitochondria⁷⁵ where it inhibits NADH dehydrogenase^{77,78} and mitochondrial electron transport leading to cessation of oxidative phosphorylation, ATP depletion and neuronal death.⁷⁹

Scheme 11. MAO Catalyzed Oxidation of MPTP



The neurotoxicity of MPTP is blocked by (*R*)-deprenyl (**2**), a potent and selective inhibitor of MAO-B. MAO-B purified from beef liver catalyzes the oxidation of MPTP 38% as fast as benzylamine (the preferred MAO-B substrate) with a comparable K_m value (Table 2).⁸⁰ MAO-A, isolated from human

placenta, catalyzes the oxidation of MPTP to the same dihydropyridinium product at about 12% the rate of kynuramine, again with a comparable K_m value. This reaction is blocked by the MAO-A selective inhibitor clorgyline (**1**). This finding that MAO is responsible for the catalysis of MPTP oxidation was quite a surprise because no other cyclic tertiary amine had been reported to display substrate properties toward MAO.

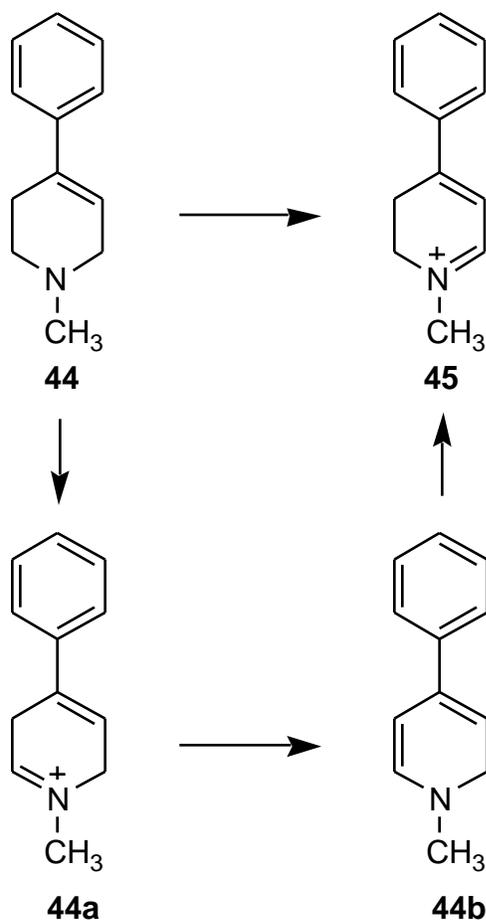
Table 2. Kinetic Parameters for the MAO Catalyzed Oxidation of Select Substrates

	MAO-A		MAO-B	
	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat} (min ⁻¹)	K_m (mM)
Kynuramine (9)	146	0.17		
Benzylamine (8)			283	0.29
MPTP (44)	20	0.14	204	0.39

2.1.2. Metabolism of MPTP

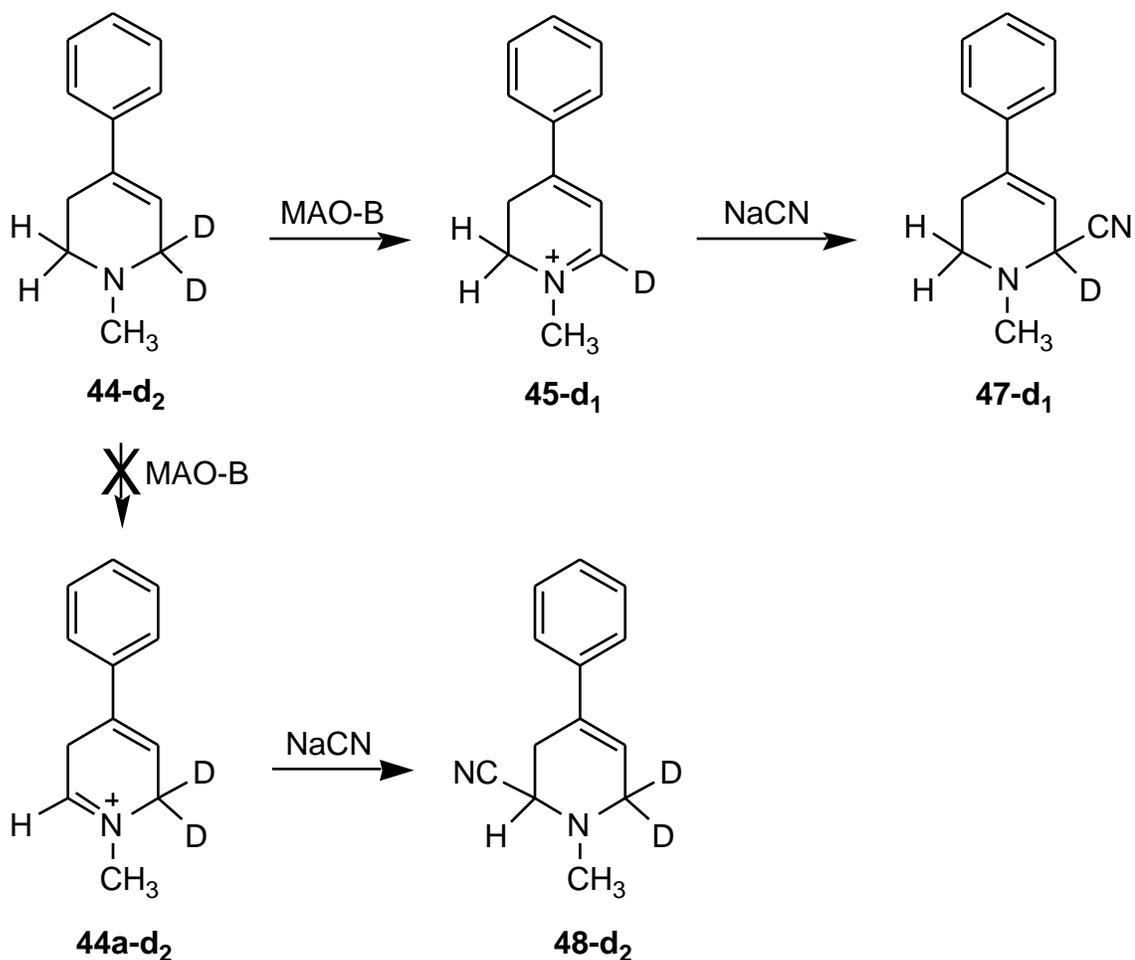
It was initially proposed that oxidation of MPTP to the dihydropyridinium metabolite (**45**) could result from direct attack at C-6 or from initial attack at C-2 followed by rearrangement of the isomeric 2,5-dihydropyridinium intermediate **44a** to **45** via the free base **44b** (Scheme 12).⁸¹

Scheme 12. Pathway for MPDP⁺ Formation



However, incubation of MPTP-6,6-d₂ (**44-d₂**) with MAO-B followed by NaCN treatment resulted in formation of **47-d₁** (Scheme 12a). Since no significant amounts of **48-d₂** were found, researchers ruled out a pathway proceeding via **44a**.⁸¹ They concluded that the formation of MPDP⁺ occurs exclusively from oxidation of MPTP at the allylic C-6 position. Consequently, isotope effect measurements will not be complicated by metabolic switching to the C-2 position.

Scheme 12a. Treatment of MPDP⁺ with NaCN



It has been suggested that the α -carbon oxidation of MPTP is stereoselective. Previous studies showed that incubations of MPTP-6-d₁ with MAO-B gave a d₁/d₀ product ratio that decreased with time.⁸¹ These results suggest that the enantiomeric composition of the substrate changes with time due to the stereoselective loss of one of the enantiotopic hydrogen/deuterium atoms. Other MAO catalyzed reactions are known to be stereoselective such as the oxidation pathways of tyramine,⁸² dopamine⁸³ and benzylamine.⁸³

In addition to the discovery of MPTP's good MAO-B substrate properties, it was found that MPTP shows progressive and irreversible inactivation of both forms of MAO.^{84,85} The inactivation process followed time-dependent, first order kinetics. Furthermore, the activity of MAO-B is not significantly regenerated following gel exclusion chromatography suggesting the formation of a covalent adduct with the enzyme. These results suggest that MPTP follows the criteria associated with a mechanism-based inactivator (see section 1.2.6.). It was reported by Singer et al.⁸⁶ that MPTP, MPDP⁺ and MPP⁺ are also reversible, competitive inhibitors of both MAO-A and MAO-B. The order of inhibition for the A form of the enzyme is MPDP⁺ > MPP⁺ > MPTP while for the B form the order shifts to MPTP > MPDP⁺ > MPP⁺. From these studies the first order rate constants were determined for MPTP and its bioactivated products, MPDP⁺ and MPP⁺, as regards to their time and concentration dependent inhibition of MAO-B (Table 3).

Table 3. First Order Rate Constants for the Inhibition of MAO-B.

Compound	Concentration (mM)	k (min ⁻¹)
MPTP (44)	0.5	3.1 x 10 ⁻³
	1	1.1 x 10 ⁻²
	2	1.4 x 10 ⁻²
	3	2.3 x 10 ⁻²
	5	3.4 x 10 ⁻²
MPDP ⁺ (45)	2.9	2.2 x 10 ⁻²
MPP ⁺ (46)	5	0

The K_i values for the competitive inhibition of both forms of the enzyme by MPTP, MPDP⁺ and MPP⁺ are shown in Table 4.

Table 4. Inhibition of MAO-A and MAO-B by MPTP and its Metabolites

Enzyme	Substrate	K_i (μ M) at 30 °C		
		MPTP	MPDP ⁺	MPP ⁺
MAO-A	Kynuramine	18	2.4	3.0
MAO-B	Benzylamine	100	200	230

The data shown in Table 3 indicate that the rates of inactivation are slow compared to the rates of oxidation of MPTP (Table 2). This suggests a large partition coefficient in favor of substrate turnover and MPDP⁺ formation. Of the two forms, MAO-B was more sensitive to irreversible inactivation.

Interest in the interactions of MPTP with MAO-B has been intense since inhibition of this biotransformation blocks formation of **46**, the putative ultimate nigrostriatal toxin,⁷² and protects susceptible animals against MPTP's neurotoxic properties.⁸⁷⁻⁸⁹ The possibility that the etiology of idiopathic Parkinson's disease may involve MPTP-type endogenous⁹⁰ or environmental substances⁹¹ has also prompted studies to glean a better understanding of MAO. In addition, MAO-B is linked to Parkinson's disease as a result of its degradation of brain dopamine.⁹² Consequently, compounds that selectively inhibit or inactivate MAO-B have been shown to be useful in the treatment of Parkinson's disease.⁹³ The elucidation of the mechanism of MAO may aid in the design of potential antiparkinsonian drugs.

2.2. MPTP Analogs - Structural Requirements

A vast area of research has been directed toward gaining knowledge about the structural features that are required for MAO activity. A number of MPTP analogs have been prepared and examined for their MAO substrate properties. These findings include the following:

- 1) The 4-5 double bond of the tetrahydropyridine ring is essential for compounds to be MAO substrates.⁹⁴
- 2) Only substituents at the C-4 and N-1 position are allowed. Placement of an alkyl group anywhere else in the tetrahydropyridine ring diminishes reactivity towards MAO.⁹⁵
- 3) Substitution at the N-1 position is limited to small substituents. The N-methyl group appears to be the ideal size while substituents such as N-H, N-methyl, N-ethyl and N-*n*-hydroxy ethyl are less favorable.⁹⁶
- 4) The phenyl ring is not necessary for compounds to be MAO substrates; replacement of the phenyl ring by a 1-methyl-2-pyrrolyl,⁹⁷ a benzyl⁹⁶ or a phenoxy group⁹⁸ enhances MAO reactivity. The 4-cyclohexyl analog is as good a substrate as MPTP.⁹⁶
- 5) *Para*-substituents on the phenyl ring produce steric hindrance unfavorable to reactivity, while *ortho*- and *meta*-substituents may have stabilizing interactions within the active site increasing reactivity.⁹⁹

Because no X-ray crystal structure has been obtained for MAO, our knowledge of the active site and its interactions with potential substrates and inhibitors are dependent on structure-activity relationship studies (SAR). Quantitative SAR (QSAR) studies have identified lipophilicity, sterics and electronics as important determinants for MAO activity.⁹⁹ A recent study has

examined the MAO-B substrate properties of a variety of tetrahydropyridine derivatives and distance measurements along the N₁-C₄ axis to define the maximum size that can be accommodated by the MAO-B active site.¹⁰⁰ It was found that only those compounds measuring less than 14 Å displayed significant MAO-B substrate properties.

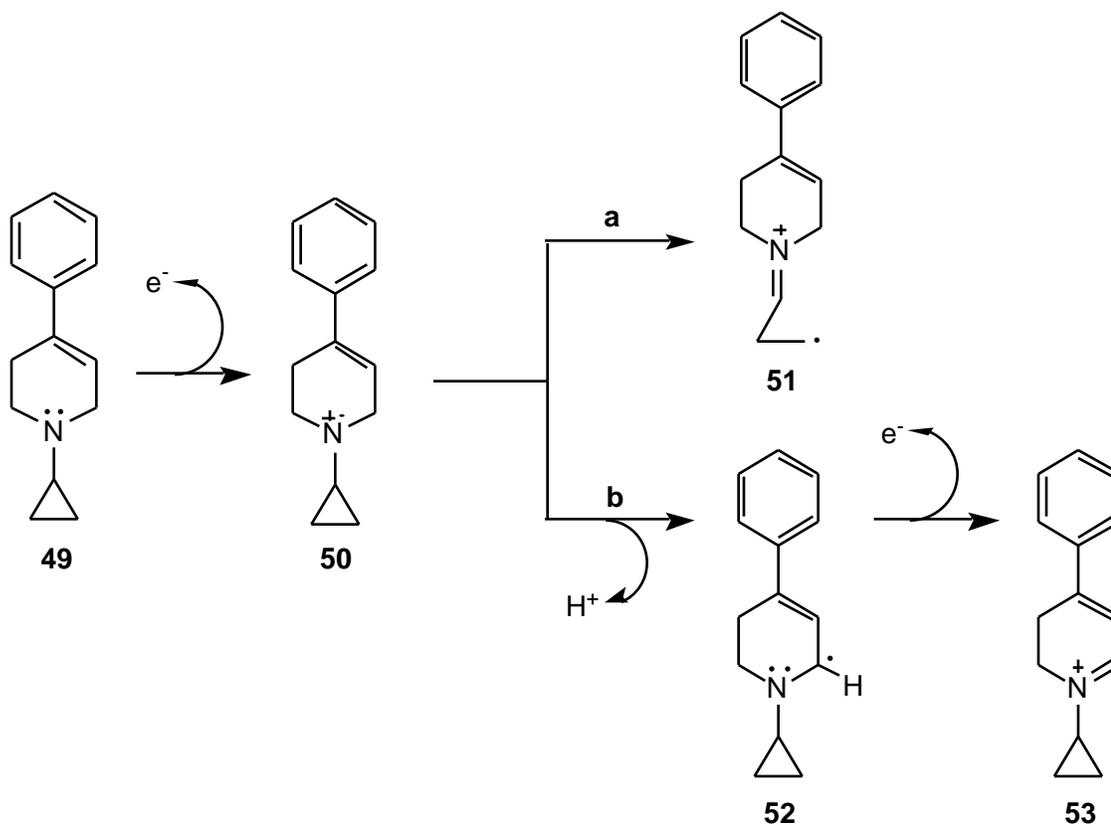
2.3. 1-Cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine

Previous studies have shown that 1° and 2° amine substrates can be converted to good mechanism-based inactivators by replacement of a N-H or N-CH₃ group with a cyclopropyl group (see section 1.4.1.). The underlying concept is that good substrate properties, when properly exploited, may lead to good inactivator properties, i.e. the enzyme may catalyze the formation of a reactive intermediate which prior to dissociating to product (like a substrate) could covalently bind to the enzyme (like an inactivator). In an effort to gain better knowledge about the interactions of MAO with cyclic tertiary amines, the N-cyclopropyl analog of MPTP was examined.

1-Cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine (**49**) proved to be a good time and concentration dependent inhibitor of MAO-B ($k_{\text{inact}}/K_{\text{I}} = 1.0 \text{ min}^{-1} \text{ mM}^{-1}$).¹⁰¹ The evidence that **49** is a mechanism-based inactivator of MAO implies that the enzyme processes the compound as a substrate but that an intermediate formed in the catalytic process inactivates the enzyme prior to escaping from the active site. A radical reaction pathway (Scheme 13) has been postulated analogous to that proposed by Silverman for N-benzylcyclopropylamine (Scheme 5). Initial one-electron transfer from **49** to FAD would generate the aminium radical **50**. This intermediate would have the option to ring open to the reactive primary radical **51** (pathway a) leading to

enzyme inactivation, or to lose an α -proton to give the secondary carbon radical **52**, which subsequently undergoes a second one-electron loss to generate the dihydropyridinium intermediate **53** (pathway b). The absence of detectable levels of the dihydropyridinium metabolite **53** suggests that the deprotonation of **51** to form **52** (pathway b) does not compete effectively with the ring opening pathway (a). These results were not surprising since cyclopropyl groups attached to a radical bearing atom undergo very rapid ring opening. For example, the cyclopropylmethylcarbinyl radical¹⁰² ring opens with a rate constant of $1.2 \times 10^8 \text{ sec}^{-1}$ while rates of ring opening of cyclopropylaminyl radicals have been reported at $7.2 \times 10^{11} \text{ sec}^{-1}$.¹⁰³

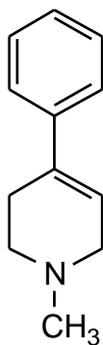
Scheme 13. Proposed SET Pathway for the MAO-B Catalyzed Oxidation of 1-Cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine



2.4. 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine

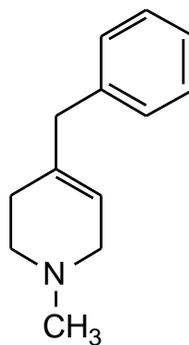
As part of an ongoing study to characterize the catalytic mechanism of MAO and its potential role in the bioactivation of protoxins capable of causing neuronal degeneration, the interactions of 4-benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine were examined with MAO-B. It was of interest to compare the inhibition properties of the 4-benzyl analog **54** with those of the 4-phenyl analog **49** because of the significantly improved substrate properties obtained when the phenyl group of MPTP is substituted with the benzyl group to give **55**.⁹⁶ The k_{cat}/K_m value for the MAO-B catalyzed oxidation of MPTP at 30 °C is

523 min⁻¹mM⁻¹ while the corresponding value for **55** is 1250 min⁻¹mM⁻¹. It was anticipated that substitution of the phenyl group present in **49** with the benzyl group present in **54** would lead to a more efficient inactivator.



44

$$k_{\text{cat}}/K_m = 523 \text{ min}^{-1}\text{mM}^{-1}$$



55

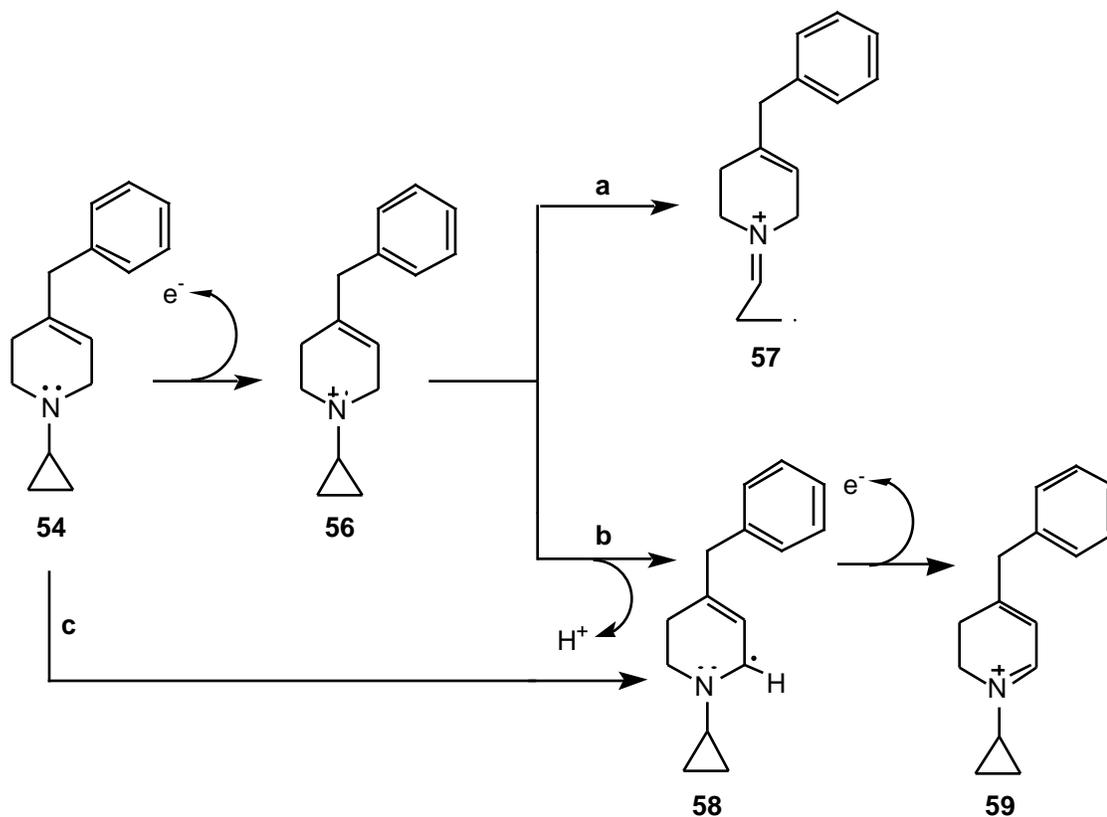
$$k_{\text{cat}}/K_m = 1250 \text{ min}^{-1}\text{mM}^{-1}$$

The 4-benzyl-1-cyclopropyl analog **54** proved to be a good time and concentration dependent inhibitor of MAO-B.¹⁰⁴ Attempts to determine the kinetic parameters k_{inact} and K_{I} were unsuccessful, however, because the inactivation process displayed nonlinear kinetic behavior. Unexpectedly compound **54** proved to be an excellent MAO-B substrate. Incubation of **54** with MAO-B showed a time and concentration dependent formation of the dihydropyridinium intermediate **59** (Scheme 14). The value for k_{cat}/K_m at 37 °C (1500 min⁻¹mM⁻¹) compares favorably with the corresponding values of the N-methyl analog **55** (2700 min⁻¹mM⁻¹) and MPTP (1400 min⁻¹mM⁻¹).

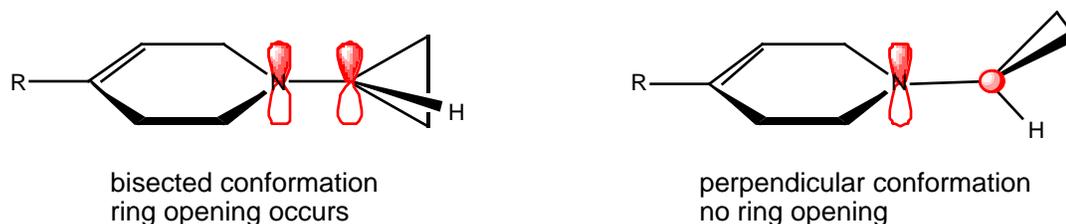
The exceptionally good substrate properties of **20** were a surprise since rates of ring opening of cyclopropylaminyl radical cations have been reported as too fast to measure.²⁹ The partition ratio, the ratio of product release to inactivation, for the MAO-B catalyzed oxidation of the 4-benzyl-1-cyclopropyl derivative **54** was estimated to be greater than 1000. Silverman has reported

partition ratios of 0 for some of the 1° and 2° cyclopropylamine inactivators, meaning every turnover of inactivator produces inactivated enzyme.¹⁰⁵ A partition ratio of 1000 clearly suggests that dihydropyridinium formation is the preferred pathway over enzyme inactivation. According to the SET mechanism, the intermediate cyclopropylaminyl radical cation **56** must partition between pathway **a** (Scheme 14), leading to the primary carbon centered radical **57** and enzyme inactivation, and pathway **b**, leading to the allylic radical intermediate **58** and product formation (**59**). The rates of ring opening of cyclopropylamine radical cations such as **56** have not been estimated experimentally, but based on the behavior of many -radical bearing cyclopropyl systems, it was anticipated that the cyclopropylaminyl radical cation **56** would favor the ring opening pathway (**a**) over the proton loss pathway (**b**). However, these considerations argue either that opening of the cyclopropyl ring of the radical cation **56** is slower than proton loss at C-6 or that dihydropyridinium (**59**) formation does not proceed via the radical cation **56**.

Scheme 14. Proposed SET Pathway for the MAO-B Catalyzed Oxidation of
4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine



It is possible that the favored partitioning of **54** to yield **59** rather than **57** may be explained by conformational constraints that would lead to poor overlap of the half-filled p-orbital on the nitrogen radical cation and the p-type orbitals of the cyclopropyl group. This restriction should retard the ring opening reaction leading to enzyme inactivation and therefore would favor the proton loss step leading to the dihydropyridinium product **59**.



An alternative pathway that could account for the good substrate properties of **54** involves a direct hydrogen atom transfer reaction (Scheme 14, pathway **c**). This pathway relies on homolytic cleavage of the relatively weak allylic carbon-hydrogen bond which might be effected by an enzyme bound radical proposed to be present in MAO-B purified from beef liver.⁶³

2.5. Rational for Proposed Research

Initial electron transfer vs hydrogen atom transfer in enzyme-catalyzed oxidations of amines has been a topic of debate. Although the SET pathway has enjoyed much favor, it also has been challenged. Results from rapid-scan stopped flow and magnetic field effect studies on the MAO-B catalyzed oxidation of [, -²H]benzylamine have led Edmondson to argue against the SET pathway since he could find no evidence of the formation of a flavin radical pair intermediate in this reaction.¹⁰⁶ Edmondson also has suggested that single electron transfer from an amine to the flavin is thermodynamically and kinetically improbable since the energy barrier for electron transfer is greater than the energy barrier for the reduction of FAD to FADH₂.⁴⁷ A similar debate concerns the cytochrome P450 catalyzed oxidations of tertiary amines.^{51,107-110} Energy estimates derived from chemical models and the enzyme-catalyzed oxidation of *N,N*-dimethylaniline derivatives again suggest that, even in polar solvents that might stabilize the radical cation, hydrogen atom transfer is energetically preferred over the SET pathway by several kcal/mol.⁵² Similar conclusions based on isotope effects and stereochemical arguments have been reached in the case of the cytochrome P450 catalyzed α -carbon oxidation of (S)-nicotine.¹⁰⁷

The unexpected substrate properties of **54** have prompted us to consider the possibility that the cyclopropylaminyl radical cation may not be an obligatory intermediate in the MAO-B catalytic pathway of 1,4-disubstituted tetrahydropyridines. This consideration has provoked new inquiry into the mechanism of catalysis of MAO-B. We would like to examine further the intermediacy of the aminium radical cation. In addition, we want to explore the possibility of another operative mechanism, specifically, a hydrogen atom transfer mechanism. The major effort of this research is to better define the mechanism of catalysis of MAO-B.

We have elected to examine these questions using various 1,4-disubstituted-1,2,3,6-tetrahydropyridine derivatives as probes. In an attempt to gain additional insight into the interactions of the 4-benzyl-1-cyclopropyl derivative with MAO-B, we have examined the deuterium isotope effects on the rates of substrate turnover and enzyme inactivation. Kinetic isotope effect studies are often used in studying questions of mechanism in order to establish the rate determining step. In addition, because there is a partitioning between two pathways, one leading to enzyme inactivation and the other leading to product formation, we wanted to examine the influence of isotopic substitution on these pathways. In order to establish that the rate determining step does not change with various substrates, we have examined the deuterium isotope effects with additional 1,4-disubstituted tetrahydropyridines.

We are particularly interested in the partitioning of substrates between product formation and enzyme inactivation. One possible explanation for the good substrate properties and poor inactivator properties of the 4-benzyl-cyclopropyl derivative (**54**) relative to the 4-phenyl analog (**49**) could be steric

constraints imposed by the active site that prevent proper orbital alignment required for ring opening. We have examined the MAO-B substrate and inactivator properties of a series of 1-methyl- and 1-cyclopropyltetrahydropyridine derivatives bearing C-4 heteroaromatic substituents. Since all of these compounds are 4-aryltetrahydropyridine derivatives with the potential to assume similar conformations with the active site of the enzyme, we speculated that the inactivation properties of the N-cyclopropyl derivatives would not be so influenced by differences in the extent to which the orbitals may align for ring opening as might be encountered with the benzyl analog. On the other hand, the putative allylic radical intermediates should be stabilized by electron rich heterocyclic aromatic groups at C-4, in which case the N-methyl analogs might be expected to display a relatively wide range of substrate properties. We anticipated that the results from these types of comparative studies would help to assess the intermediacy of allylic radicals in these α -carbon oxidation reactions.

QSAR studies using a series of MPTP analogs bearing various substituents on the phenyl ring have provided a more in depth analysis of the physicochemical parameters influencing the substrate properties. Lipophilicity was established as a primary determinant. In order to explore further the effects of polar substituents in the MAO-B catalyzed oxidation of MPTP analogs, we have carried out a study involving a variety of polar aromatic groups in which a nitrogen is directly attached to the C-4 position. The substrate/inactivation properties of the N-cyclopropyl derivatives were also examined.

In an attempt to gain additional information regarding the MAO-B catalytic pathway, we have exploited two chemical models that will allow us to induce a

single electron transfer reaction or a hydrogen atom transfer reaction on various tetrahydropyridine substrates. We have compared the rates of dihydropyridinium formation and rates of enzyme inactivation of the enzymatic reactions to the rates we obtain with the chemical model reactions.