

**SYNTHETIC AND METABOLIC STUDIES ON 1-METHYL-4-(1-METHYLPYRROL-2-YL)-1,2,3,6-TETRAHYDROPYRIDINE,  
A NEUROTOXIC ANALOG OF THE PARKINSONIAN  
INDUCING AGENT MPTP**

by

Hong Bai

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

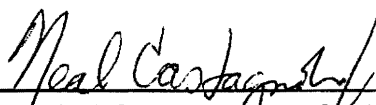
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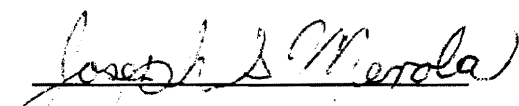
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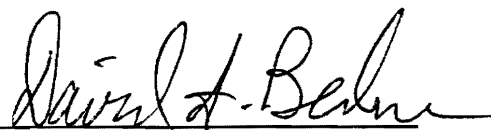
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Chemistry

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(ABSTRACT)

1-Methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP) is a neurotoxic analog of the parkinsonian inducing agent MPTP. TMMP and its putative metabolites 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium (MMDP<sup>+</sup>) and 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium (MMP<sup>+</sup>) were synthesized and fully characterized.

Substrate/inactivation properties of TMMP and its analog N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine with MAO-B were investigated. Kinetic data was obtained, including  $K_m$  and  $V_{max}$  for TMMP as an MAO-B substrate, and  $K_i$  and  $k_{inact}$  values for N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine.

The metabolic studies of TMMP and MMDP<sup>+</sup> were conducted with an

HPLC diode array assay. Both in-vivo and in-vitro metabolic studies showed that TMMP is oxidized to its dihydropyridinium species (MMDP<sup>+</sup>) in a reaction catalyzed by MAO-B. MMDP<sup>+</sup> undergoes autoxidation to form the pyridinium species (MMP<sup>+</sup>), the mechanism of this conversion is not clear. In-vitro studies show that MAO-B is not responsible for this conversion and the oxidation of MMDP<sup>+</sup> to MMP<sup>+</sup> is likely to be enzyme catalyzed.

Toxicity investigations include dopamine depletion studies of TMMP and MMDP<sup>+</sup>, mitochondrial respiration and microdialysis studies of MMDP<sup>+</sup> and MMP<sup>+</sup>. The above studies show that TMMP is an MPTP-type neurotoxin.

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# **Chapter 1**

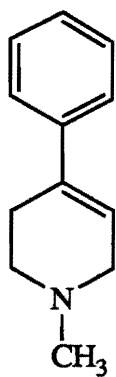
## **Introduction and Literature Review**

### **1.1 Parkinson's Disease**

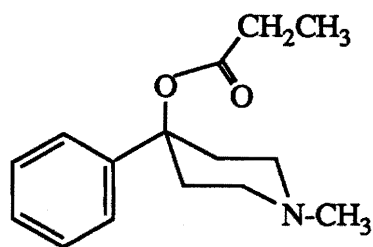
Parkinson's disease is a slowly progressive central neurodegenerative disorder disease affecting body movement. This disease, affecting middle aged humans, is characterized by hypokinesia, rigidity, and tremor due to a primary loss of neurons in zona compacta of substantia nigra.<sup>1-3</sup> The dopaminergic neurons in the substantia nigra degenerate irreversibly, generally over a long period of time to reach a critically low level. Patients suffering from this disease have either a lower initial number of dopaminergic neurons or an increased rate of loss of these neurons. Three hypotheses of Parkinson's disease have been made which implicate genetic factors, aging of the central nervous system, and infections or toxic factors.<sup>4,5</sup> None has been proven. It is likely that hereditary factors are not important and therefore many investigators have considered exposure to environmental agents or the generation of endogenous toxins as potentially important factors in the etiology of Parkinson's disease.<sup>6</sup>

## 1.2 MPTP

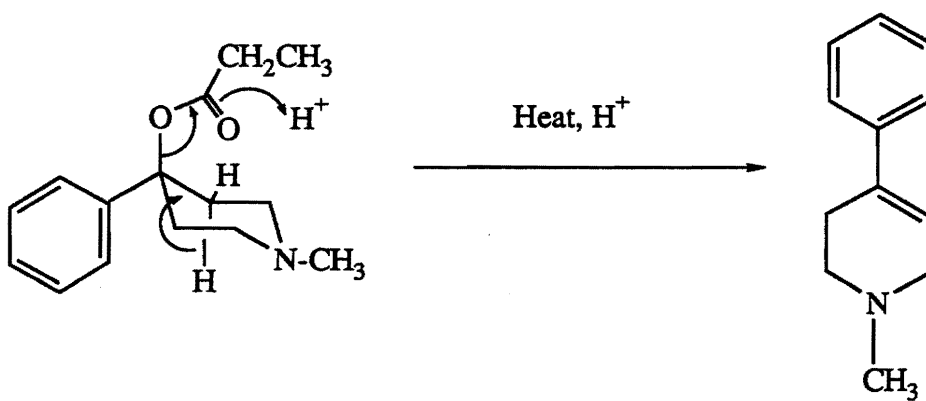
It is now well established that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 1) causes a motor disorder which resembles Parkinson's disease in some animals and humans. The biological properties of MPTP first were examined in the 1950's<sup>7</sup> when it was tested as a potential antiparkinsonian agent.<sup>8,9</sup> Although its parkinsonian inducing properties were observed, this observation was never published.<sup>10</sup> MPTP next came to the attention of the scientific community in the later 1970's.<sup>11</sup> A young college student rapidly developed rigidity and some tremor after self-administration of what proved to be an impure preparation of 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP, 2), a street drug known as "synthetic heroin". This preparation proved to be contaminated with MPTP (1) presumably because of the ease with which the propionoxy group at the 4-position undergoes elimination (shown in Scheme 1). After self-administration of several doses of the contaminated MPPP (2), he developed parkinsonian symptoms. He was initially treated for a possible psychological disorder but failed to respond to the treatment. Subsequently he was treated successfully with drugs normally administered to relieve parkinsonian symptoms. The glassware he had used in the synthesis of MPPP (2) was examined and found to contain MPTP (1) as a major contaminant.<sup>12</sup> However, because of the complex drug history of this young man, no further testing of MPPP (2) and MPTP (1) were performed except to attempt to demonstrate toxicity in the rodent which failed.



MPTP (1)



MPPP (2)



Scheme 1. Formation of MPTP from MPPP

Further investigation of MPTP (1) did not begin until several years later. Some young drug abusers also developed parkinsonian symptoms after taking contaminated MPPP (2) preparations. They were noted to have the same symptoms as the early case. MPTP (1) was proposed as the chemical cause of the parkinsonism symptoms and the death of nigrostriatal neurons.<sup>13</sup> Burns et al. established the neurotoxicity of MPTP (1) in the rhesus monkey.<sup>14</sup> Intravenous administration of MPTP (1) to rhesus monkeys in various doses produced a parkinsonism disorder. Not only were the characteristic tremors and rigidity observed but also cell loss was noted in the pars compacta of the substantia nigra. A marked dopamine depletion in the corpus striatum also was observed.

Squirrel monkeys also were used to study the effects of MPTP (1) in the Langston research group. Several repeated doses of MPTP (1) were administered via the intraperitoneal route to squirrel monkeys. The results these investigators found were essentially the same as those observed in the rhesus monkeys.<sup>15</sup> After the parkinsonian symptom had developed, the animals were given L-dopa, the accepted therapy for parkinson's disease. The activity level of the animals returned to near normal after their treatment. Their medication was essential to maintain normal behavior. Discontinuing the medication led to the return of the motor disturbances. The loss of nigral cells also was observed.

Chiueh et al. examined the neurotoxicity of MPTP (1) in rats, guinea pigs, and rhesus monkeys. Only the rhesus monkey produced persistent parkinsonism while rats and guinea pigs showed neither permanent dopamine deficiencies in the striatal nor locomotor disturbances.<sup>16,17</sup>



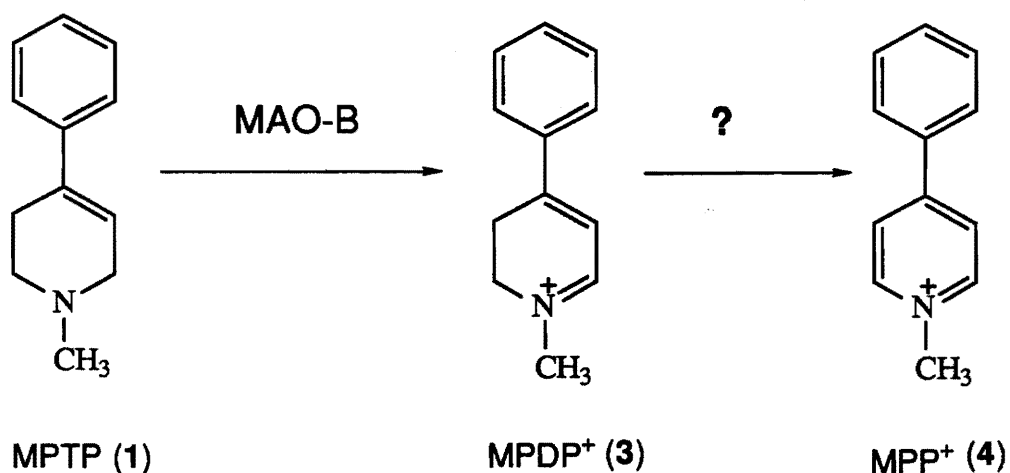
Parisi et al. extended the animal model studies to other species including beagle dogs.<sup>18</sup> The dogs were administered single or multiple doses of MPTP (1). Degeneration of the nigrostriatal track was found to be similar to that reported previously in the primate studies. Several researchers reported that MPTP (1) injected into rats was not toxic to striatal dopamine neurons<sup>19</sup> while some reported that a high dosage of MPTP (1) administered to mice, particularly the C57 BL/6J mice striatum, is toxic.<sup>20-22</sup>

It is apparent that difference species and tissues have different susceptibility to the toxicity of MPTP (1). Primates are more susceptible than rodents. Dopamine depletion resulting from MPTP (1) administration through various routes (interperitoneal, intravenous, subcutaneous, and intranigral) in mice was noted but no rigidity or tremors were found as is the case of men, monkeys, and dogs.<sup>23</sup> It was noted that much higher doses of MPTP (1) are required to induce neurotoxicity in mice than in primates or dogs.<sup>24</sup>

As the studies of MPTP toxicity continued it was found that MPTP itself was not toxic. Rather MPTP must be bioactivated to species which mediated the neurotoxic effects. Early evidence to this effect was found in squirrel monkeys injected with MPTP. The brain tissue was examined and a compound, which was shown to be MPP<sup>+</sup> (4), was isolated.<sup>25</sup> Later, radiolabelled MPTP was used to show that MPP<sup>+</sup> (4) accumulated in the nigrostriatal neurons of the monkey brain.<sup>26</sup> A study using dopaminergic neuron cultures has proved that MPTP (1) does not destroy the cells but MPP<sup>+</sup> (4) does.<sup>27</sup> Being a charged molecule, MPP<sup>+</sup> (4) can not cross the blood-brain barrier. Therefore, it is concluded that MPP<sup>+</sup> (4) must be formed in the brain.

Several research groups have shown that MPTP (1) is converted to an intermediate, the 1-methyl-4-phenyl-2,3-dihydropyridinium species (MPDP<sup>+</sup>, 3) in a reaction catalyzed by monoamine oxidase B (MAO-B) (Scheme 2).<sup>28-32</sup> MPDP<sup>+</sup> (3) is not stable and is oxidized further to form MPP<sup>+</sup> (4). The pathway involved in the oxidation of MPDP<sup>+</sup> (3) to MPP<sup>+</sup> (4) is not clear.

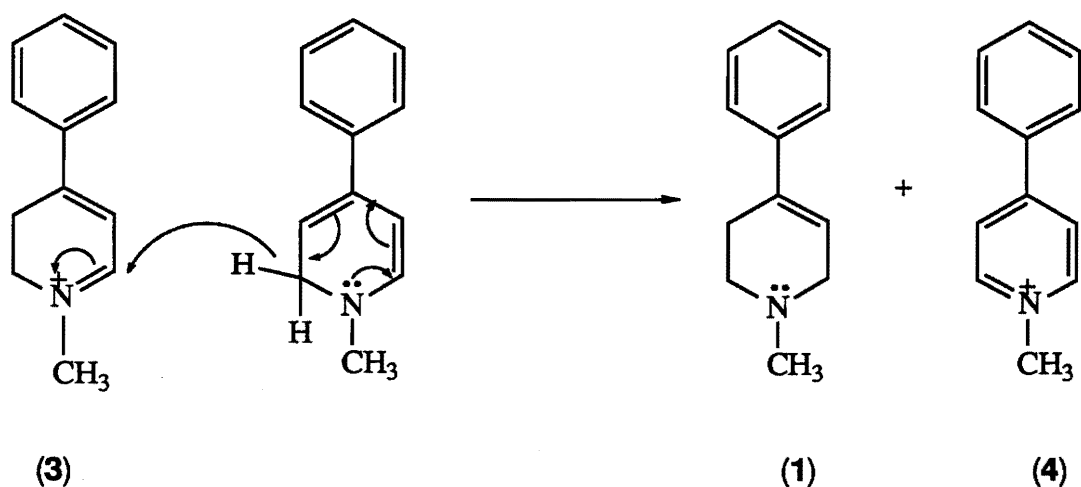
MPTP is a substrate of both the A and B forms of monoamine oxidase although it is a much better substrate of MAO-B. This is somewhat surprising since most substrates of MAO are primary or secondary amines such as dopamine and epinephrine. Since MAO-B is located primarily outside the dopaminergic neurons, the bioactivation of MPTP (1) occurs primarily in the glial cells and/or serotonergic neurons.<sup>33</sup>



Scheme 2: MPTP Metabolism

Kinetic studies on the MAO-B catalyzed oxidation of MPTP (1) established the  $K_m$  (300 $\mu$ M) and  $V_{max}$  (260 nmol/min/U enzyme) for this reaction.<sup>34</sup> It was also found that MPTP (1) was oxidized in the presence of MAO-A at a significant rate although not nearly as rapidly as with the B form. The oxidation product of MAO-A with MPTP (1) as substrate gave a spectrum identical to that observed with the B form. Thus, the role of MAO-A in the CNS in producing metabolites of MPTP which are presumed to be toxic can not be ignored.

MPDP<sup>+</sup> (3) is not stable at pH 7.4. This dihydropyridinium species undergoes both autoxidation to MPP<sup>+</sup> (4)<sup>35</sup> and disproportionation to form MPP<sup>+</sup> (4) and MPTP (1) as shown in Scheme 3.<sup>36</sup> The half time of 1 mM MPDP<sup>+</sup> (3) is 60 minutes. At lower concentrations, such as at 50  $\mu$ M, only autoxidation occurs (half time of 240 minutes).<sup>37</sup> It has been reported that pure MAO-B can increase the oxidation rate of MPDP<sup>+</sup> (3).<sup>38</sup> This conclusion is complicated since various mouse brain fractions, even after treatment with pargyline (an MAO-A/B inactivator) can accelerate the rate of oxidation of MPDP<sup>+</sup> (3) to MPP<sup>+</sup> (4).<sup>37</sup> Consequently, the details relating to the in vivo oxidation of MPDP<sup>+</sup> (3) to MPP<sup>+</sup> (4) remain poorly documented.



Scheme 3: Disproportionation pathway of MPDP<sup>+</sup> to form MPP<sup>+</sup> and MPTP

It was mentioned earlier that MPP<sup>+</sup> (4) but not MPTP (1) is the neurotoxin which causes cell death. Irwin et al. demonstrated that MPP<sup>+</sup> (4) accumulated only in the substantia nigra in primates and proposed that the dopamine uptake system might be responsible for the accumulation.<sup>39</sup> Snyder et al. have proved that the dopamine uptake system, which located on the dopaminergic nerve terminals, has the same affinity for MPP<sup>+</sup> (4) as for dopamine.<sup>40</sup> The above conclusion was supported by studies which used dopamine uptake blockers such as cocaine and mazindol. The results showed that dopamine uptake system blockers protected against MPP<sup>+</sup> (4) and MPTP (1) toxicity. The accumulation of MPP<sup>+</sup> (4) in dopaminergic neurons is required for its toxic effects.<sup>40-44</sup>

It also has been shown that MPP<sup>+</sup> (4) damages mitochondria.<sup>45-47</sup> Nicklas et al. proposed that MPP<sup>+</sup> (4) inhibites the oxidation of the reduced

nicotinamide-adenine dinucleotide (NADH)-linked substrate. Succinate oxidation is not affected.<sup>48</sup> Ramsay et al. proposed that MPP<sup>+</sup> (4) may accumulate in the mitochondria of the nerve terminals via an energy dependent uptake system which leads to a high concentration of MPP<sup>+</sup> (4) inside the mitochondria.<sup>49</sup> Trevor et al. proposed that MPP<sup>+</sup> (4) may inhibit oxidative phosphorylation by preventing electron transfer between NADH dehydrogenase and coenzyme Q.<sup>31</sup> Mizuno et al. provided evidence for the inhibition of ATP synthesis by MPP<sup>+</sup> (4) and concluded that  $\alpha$ -ketoglutarate dehydrogenase complex and complex 1 inhibition were the cause of mitochondrial respiration inhibition.<sup>50</sup>

### **1.3 Structure and Activity Relationships**

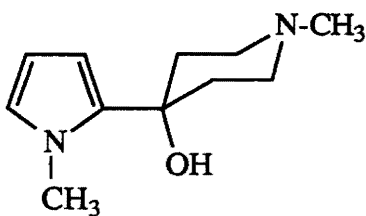
Several MPTP analogs have been prepared and tested for their neurotoxicity in order to determine the basic structural features responsible for causing neurotoxicity. Analysis of the available data leads to the following generalizations:

- The 4-5 double bond is essential for the activity of MPTP and its analogs.<sup>51</sup>
- The N-methyl group is essential for the activity.<sup>52</sup> Replacement with either H or large alkylgroup abolishes activity.<sup>53,54</sup>
- Substitution of alkyl groups in the tetrahydropyridine lead to loss of activity.<sup>54</sup>

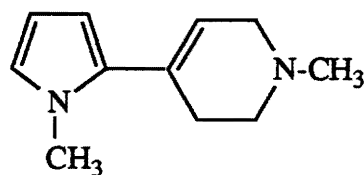
- The aromatic ring is not necessary for a compound to be an MAO substrate but a second ring, aromatic or not, may be required for neurotoxicity.<sup>55,56</sup>
- The second ring must be in position 4 of the tetrahydropyridine ring.<sup>57</sup>
- A number of phenyl substituents, such as 2-Et, 3-Me, 4-Me, 3-F, 3-Cl, 4-Cl, 2-NH<sub>2</sub>, 3-NH<sub>2</sub>, 4-NH<sub>2</sub>, may decrease or abolish neurotoxicity while others, such as 2-Me, 2-F, 2-CF<sub>3</sub>, will maintain or even enhance neurotoxicity.

#### **1.4 TMMP**

The tertiary alcohol 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol (MMPP, **5**) and its dehydration product 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP, **6**) are two analogs of MPTP (**1**) which have been examined for neurotoxicity. Although the structure of TMMP (**6**) satisfies the basic requirements for an MPTP (**1**) type neurotoxin, the initial studies conducted by Wilkening et al. indicated that only MMPP (**5**) was a neurotoxin.<sup>58</sup> After MMPP (**5**) was administered orally to monkeys, parkinsonian symptoms and lesions of neurons in the substantia nigra were observed. However, MMPP (**5**) had no effect when administered orally to dogs or rats. When MMPP (**5**) was administered orally to dogs, decreased spontaneous motor activity and tremor were observed. However, all the dogs administered MMPP (**5**) recovered completely within 20 days after dosing. When MMPP (**5**) was administered orally to rats, no neurologic damage signs could be found.



MMPP (5)



TMMP (6)

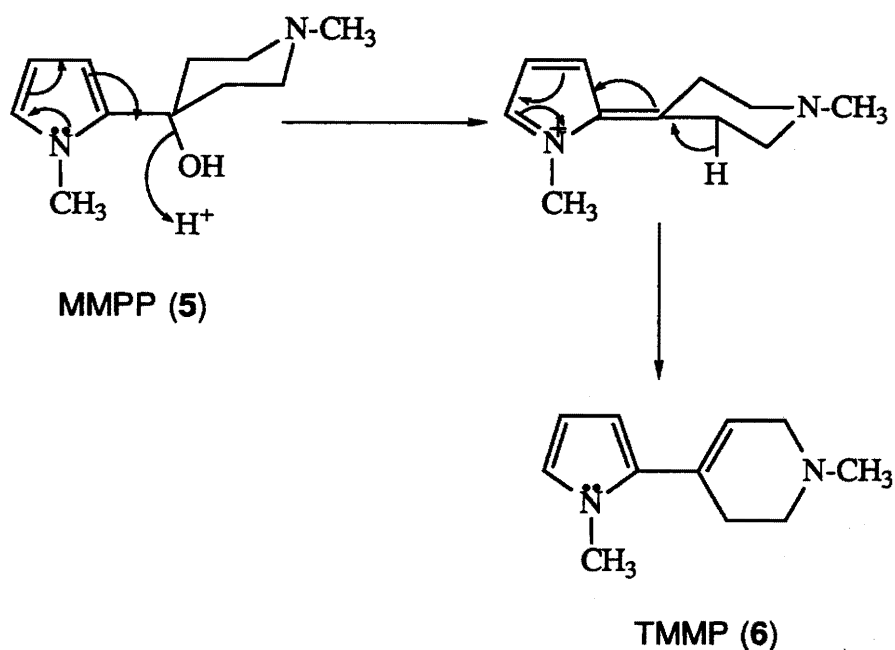
Subsequent experiments conducted by Fuller and Hemrick-Luecke<sup>59</sup> in mice showed results which contrasted to those reported by Wilkenling. Fuller et al. found that the route of administration affected the neurotoxicity of MMPP (5) and TMMP (6). It was found in their experiments that TMMP (6) could cause persistent depletion of striatal dopamine when injected subcutaneously to mice while MMPP (5) produced no depletion of dopamine under identical conditions. None of the three compounds (MPTP, TMMP, MMPP) affected dopamine or its metabolites when administered orally to mice. They also found that TMMP (6) could be oxidized by MAO-B in-vitro more rapidly than MPTP (1) while MMPP (5) was a very poor substrate.

The observations of Finnegan et al.<sup>60</sup> confirmed the neurotoxicity of TMMP (6) but failed to identify MMPP (5) as a neurotoxin. Their results showed that high doses of MPTP and TMMP could elicit neurotoxicity when administered orally to male C57BL/6J mice. They found that only TMMP (6) would cause persistent depletion of striatal dopamine and induce histologic evidence of nerve terminal degeneration when administered interperitoneally in mice. TMMP (6) produced larger dopamine depletions than MPTP (1) when mice were given the same dose. These workers also have demonstrated that TMMP (6) is preferentially oxidized by mouse brain monoamine oxidase B to a

water-soluble compound, presumably the pyridinium species. Their results also showed that pretreatment of mice with either the monoamine oxidase inhibitor pargyline or the dopamine reuptake inhibitor bupropion blocked the ability of TMMP (6) to deplete striatal dopamine.

Bembenek et al.<sup>61</sup> have reported that human monoamine oxidase A and B (MAO-A and MAO-B) can catalyze the oxidation of TMMP (6) and MMPP (5). Their results showed that TMMP (6) was a substrate of both forms of MAO (i.e. MAO-B,  $K_{m,app} = 52 \mu\text{M}$  and  $V_{max, app} = 6.63 \times 10^{-2}$  absorbance units/min) while MMPP (5) was a poor substrate of only MAO-B. Their work is not complete because of they lack of standard dihydropyridinium compound (rate unit in absorbance/min). Additional studies revealed that acid treatment of MMPP (5) led to the formation of a product that could be readily oxidized by both MAO-A and MAO-B. Similar acid pretreatment of TMMP (6) yielded an unidentified product which was a much poorer substrate for MAO-B than the parent compound. These results may partially explain why MMPP (5) when administered orally in monkeys produces neurotoxicity while TMMP (6) fails to induce chemical parkinsonism. Acid treatment of MMPP (5) may lead to the dehydration of MMPP (5) via a intermediate to form TMMP as shown in Scheme 4. TMMP (6), an electron-rich system, is unstable in acid, so that acidic conditions may cause its subsequent decomposition. When MMPP (5) is administered orally to monkeys TMMP (6) may be formed in the acidic environment of the monkey stomach and be absorbed into the blood system.





Scheme 4: Formation of TMMP From MMPP under acidic conditions

## 1.5 Research Proposal

The neurotoxicity of TMMP (6) has been demonstrated by several research groups but many unanswered questions still remain. Although Finnegan et al. have suggested that the metabolism of TMMP (6) may lead to a pyridinium species, they did not characterize the metabolites on the metabolic pathway since they did not have synthetic standards. Bembenek et al. have provided some kinetic data on the metabolism of TMMP (6). However, without the synthetic dihydropyridinium species, their results have no quantitative significance. Although TMMP (6) is an analog of MPTP (1), its mechanism of toxicity and its metabolism pathway still remain poorly defined. It is apparent

that the toxic mechanism of TMMP (6) and therefore MPTP (1) may be better understood if the bioactivation pathway of TMMP (6) is better established. This may be realized by having available synthetic TMMP (6) and its putative metabolites.

The objective of this research is to investigate the similarities and differences between the pathways mediating the neurotoxic properties of TMMP (6) and MPTP (1). The specific aims of this thesis are (1) to synthesize the putative dihydropyridinium and pyridinium metabolite of TMMP (6) and to examine their neurotoxic potential, (2) to establish the MAO-B substrate/inactivation properties of TMMP (6) and its putative dihydropyridinium metabolite, (3) to evaluate the in-vivo and in-vitro metabolic fate of TMMP (6), and (4) to evaluate the neurotoxicity of the dihydropyridinium and pyridinium species derived from TMMP (6).

Chapter 2 of this thesis describes the syntheses of TMMP (6) and its putative metabolites. Chapter 3 summarizes the results of the in-vivo and in-vitro metabolic studies and toxicity studies carried out on TMMP (6) and the corresponding dihydropyridinium and pyridinium derivatives. Chapter 4 lists the conclusions and recommendations.

## Chapter 2

### Syntheses

#### 2.1: Introduction

The cyclic tertiary amines 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol (MMPP, **5**) and 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP, **6**) were developed as potential antidepressant drugs but they were never used in clinical trials.<sup>62</sup> After MPTP was found to be neurotoxic, these compounds became of great interests. As reviewed in Chapter 1 it was found that like MPTP (**1**), TMMP (**6**) caused persistent nigrostriatal dopamine depletion in mice. It was also shown that TMMP (**6**) could be oxidized in the presence of mouse mitochondrial fractions and that it was a substrate of both forms of MAO but a much better MAO-B substrate.<sup>59-61</sup>

It is well established that MAO-B catalyzes the oxidation of MPTP (**1**) to form the corresponding dihydropyridinium species (**3**) and that the dihydropyridinium intermediate (**3**) undergoes further oxidation to form the pyridinium species (**4**). We postulated that TMMP (**6**) would be processed in a similar way by MAO-B to form the two electron oxidation product 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium (MMDP<sup>+</sup>, **11**) and that this dihydropyridinium intermediate could undergo further oxidation to form the four electron oxidation product, the 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium species MMP<sup>+</sup> (**16**). In

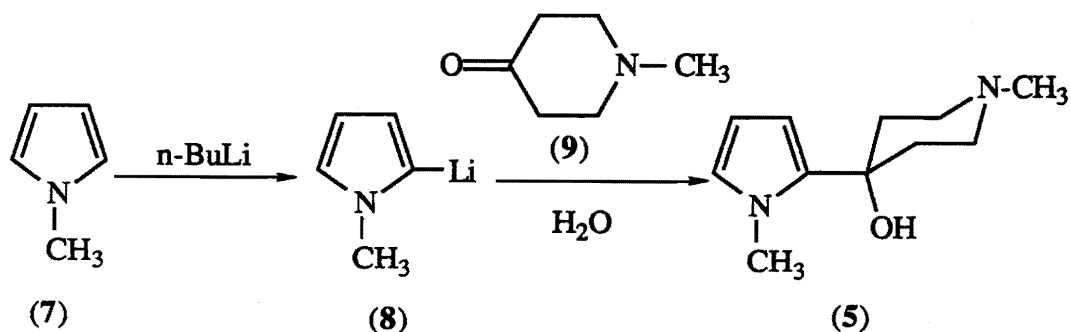
order to evaluate this postulate, we undertook the syntheses of MMDP<sup>+</sup> (11) and MMP<sup>+</sup> (16) which were required for our proposed metabolic studies.

In addition to its substrate properties, MPTP (1) is also an inhibitor of MAO-B.<sup>63</sup> The MPTP (1) derivative, 1-propargyl-4-phenyl-1,2,3,6-tetrahydropyridine has been shown to be a relative effective MAO-B inactivator.<sup>66</sup> Consequently, we also undertook the syntheses of 1-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (19) to extend the existing structure activity data on the interaction of the tetrahydropyridine derivative with MAO-B.

## **2.2: Results and Discussion**

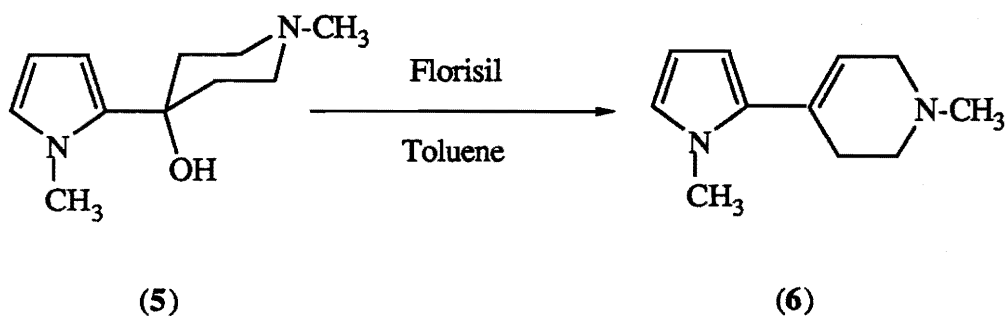
### **2.2.1: Synthesis of 1-Methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol (MMPP, 5) and 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP, 6)**

The synthesis of MMPP (5) was approached by following the procedure outlined in a chemical patent as shown in Scheme 5.<sup>62</sup> The reaction of *n*-butyllithium with *N*-methylpyrrole (7) proceeded readily under anhydrous and anaerobic conditions. The subsequent condensation of the lithiated pyrrole derivative 8 with 1-methyl-4-piperidone (9) yielded the desired MMPP (5). Recrystallization from heptane gave a white crystalline solid in 44% yield which was characterized by GC/MS and <sup>1</sup>H NMR see below.



Scheme 5: Synthetic Pathway to MMPP (5)

The dehydration of MMPP (5) required mild acidic conditions in order to avoid decomposition of the acid labile pyrrole system. This reaction was carried out by heating MMPP (5) in toluene under reflux in the presence of florisil which served as a dehydrating agent (Scheme 6). GC/MS and  $^1\text{H}$  NMR analysis of TMMP (6) free base are shown in Figs. 3 and 4. TMMP (6) also was converted to its HCl salt.



Scheme 6: Formation of TMMP (6) from MMPP (5)

The GC/EI mass spectrum of **5** (Fig. 1) displayed a molecular ion at  $m/z = 194$  and a diagnostic fragment ion resulting from loss  $H_2O$  from the molecular ion ( $m/z = 176$ ). The structure of **5** was confirmed by  $^1H$  NMR analysis (Fig. 2). The multiplets centered at 6.6 ppm (C-5') and 6.0 ppm (C-3' and C-4') were assigned to the pyrrole ring protons. The singlets at 3.85 ppm (N-CH<sub>3</sub>') and 2.35 ppm (N-CH<sub>3</sub>) were assigned to the pyrrole ring N-methyl group and piperidine ring N-methyl group, respectively. The triplets centered at 2.7 ppm and 2.5 ppm were assigned to the piperidine ring C-2 and C-6 protons. The multiplet centered at 2.2-2.0 ppm was assigned to the C-3 and C-5 four protons.

The base peak in the GC EI mass spectrum of **6** (Fig. 3) was the molecular ion at  $m/z = 176$ . The fragment at  $m/z = 94$  corresponded to loss of the N-methyl-pyrrole moiety. The  $^1H$  NMR (Fig. 4) analysis of **6** confirmed the structure. The spectrum of the free base was assigned with the aid of decoupling experiments. The multiplets centered at 6.6 ppm (C-5'), 6.1 ppm (C-3' and C-4') were assigned to the pyrrole ring protons. The multiplet centered at 5.7 ppm was assigned to the olefin C-5 proton. The singlets at 3.7 ppm (N'-CH<sub>3</sub>) and 2.4 ppm (N-CH<sub>3</sub>) were assigned to the pyrrole ring N-methyl group and piperidene ring N-methyl groups. The multiplets centered at 3.15 ppm (C-6), 2.7 ppm (C-2), and 2.5 ppm (C-3) were assigned to the remaining piperidene ring protons. The UV spectrum of **6** (Fig. 5) displays a single absorbance peak with  $\lambda_{max} = 266$  nm.

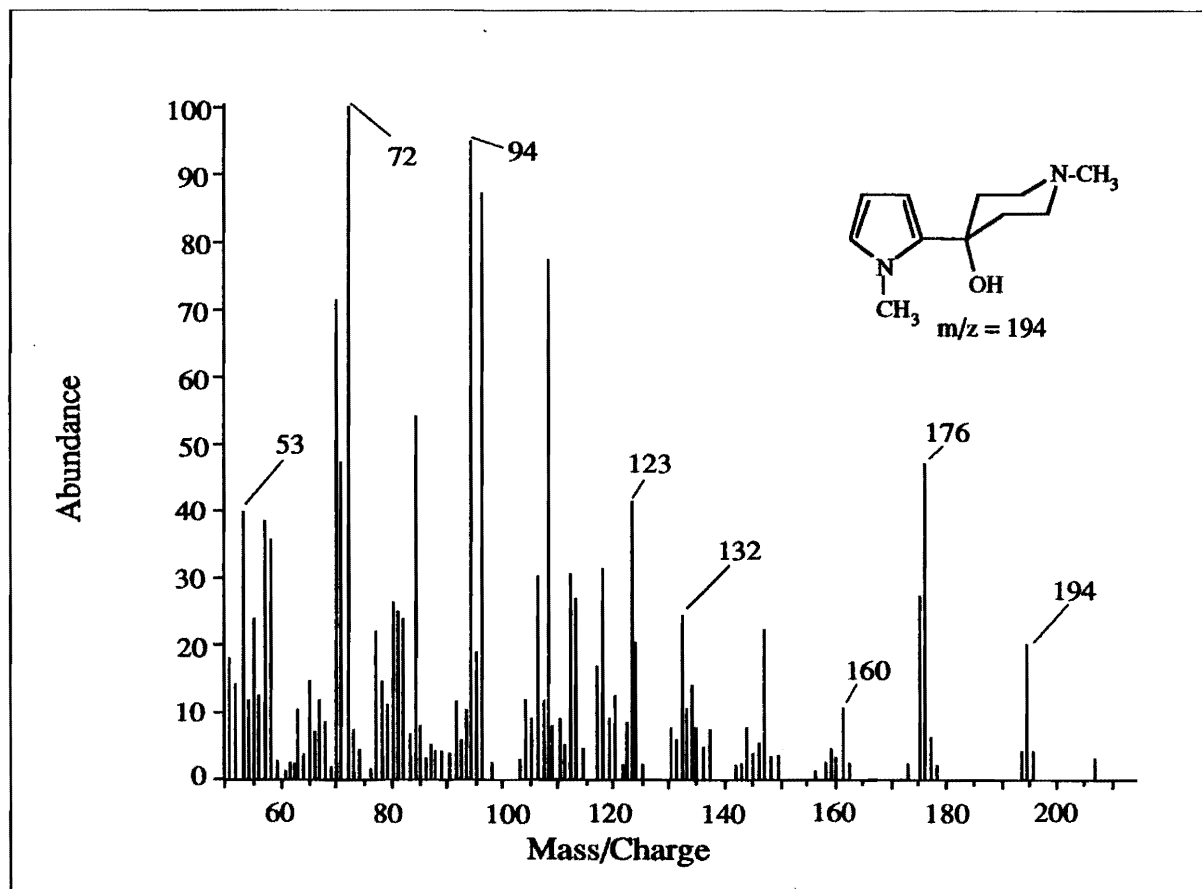


Figure 1: GC/EIMS of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol

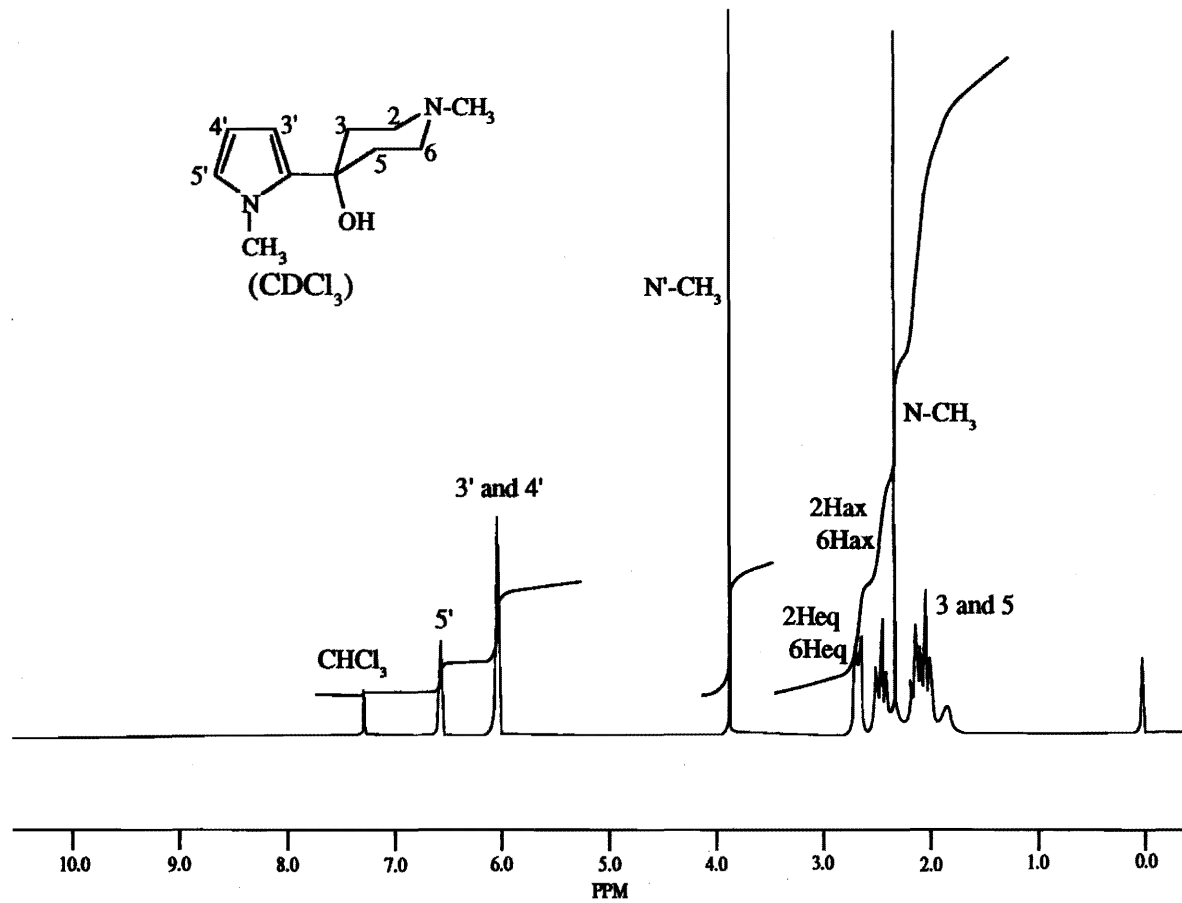


Figure 2:  $^1\text{H}$  NMR spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol



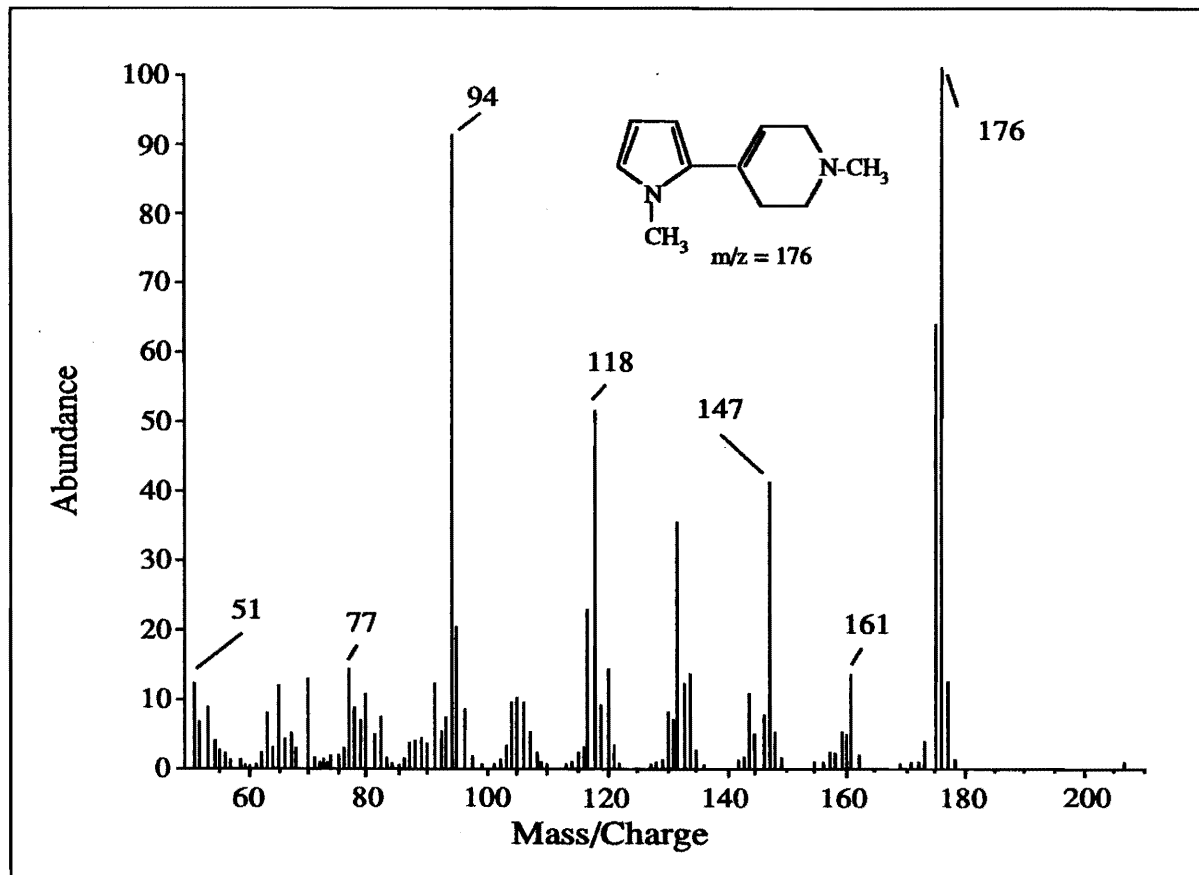


Figure 3: GC/EIMS of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine

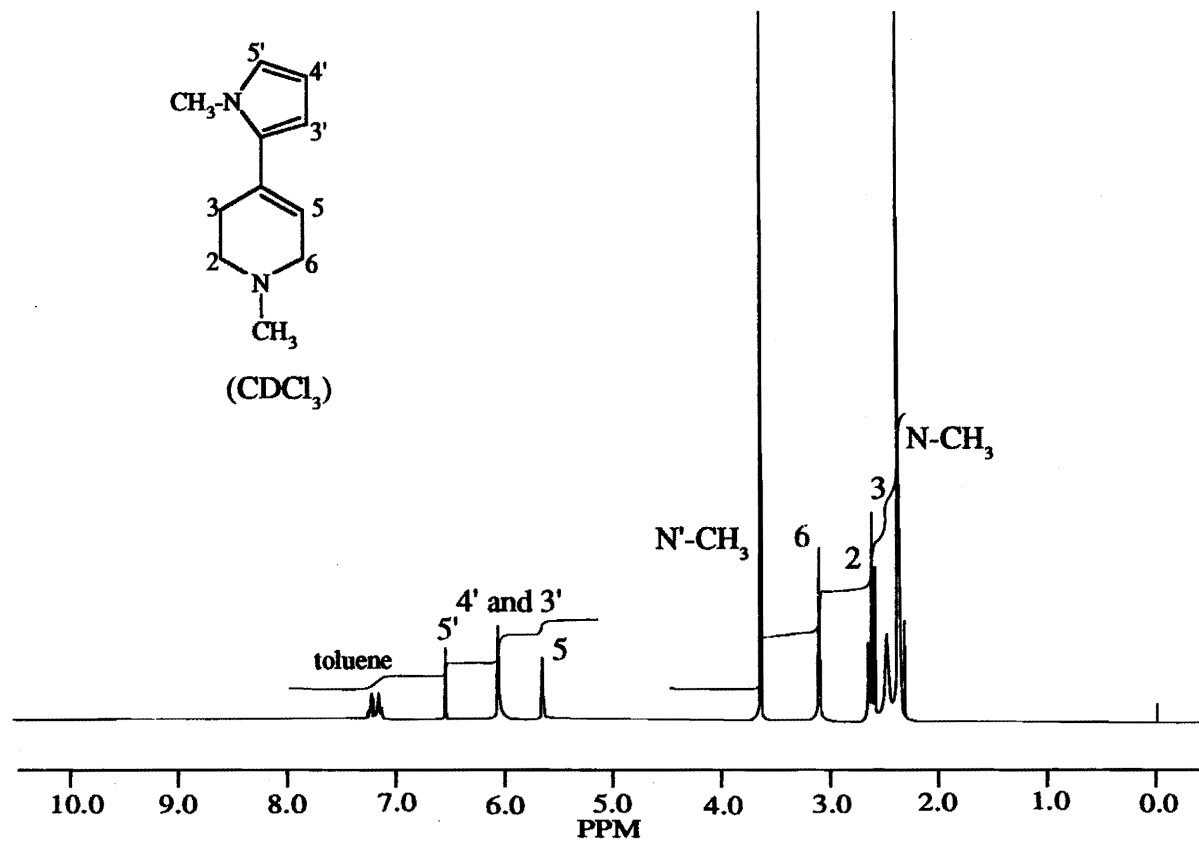


Figure 4:  $^1\text{H}$  NMR spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine

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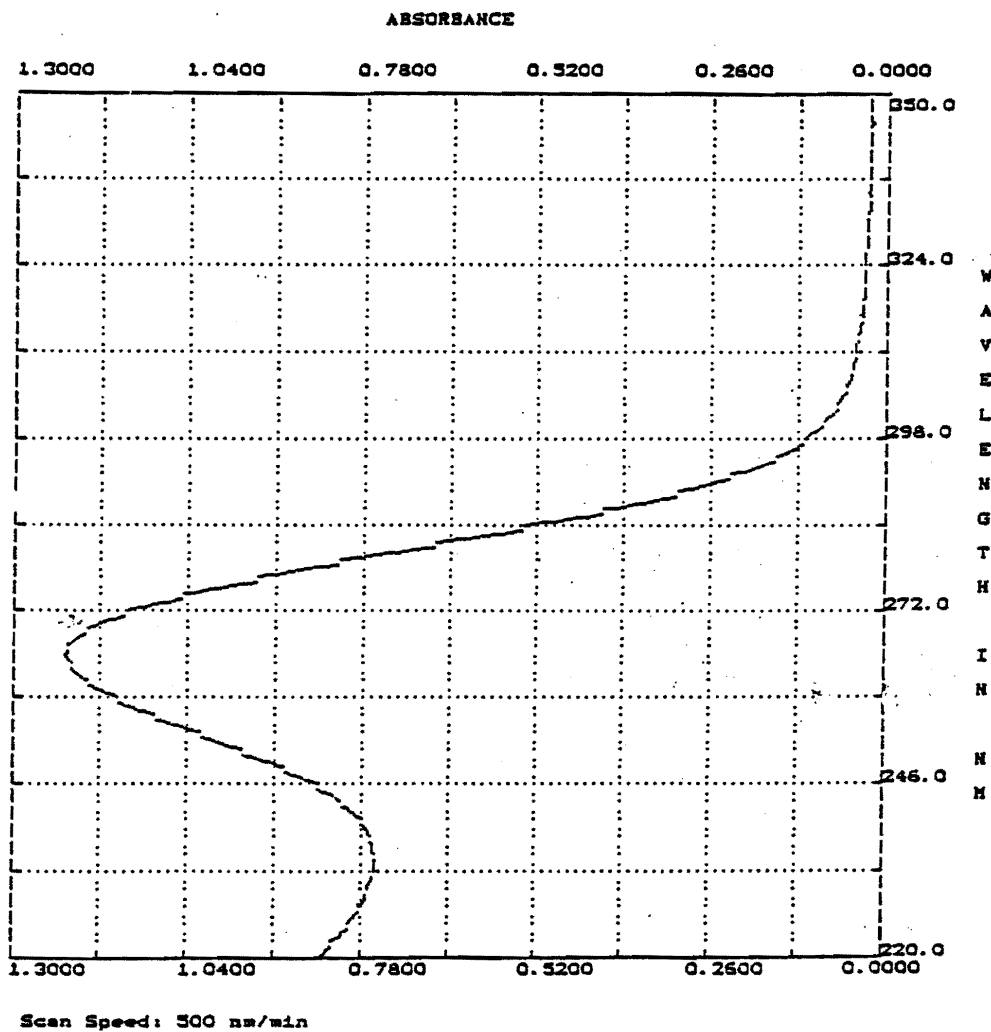


Figure 5: UV Spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP)

### 2.2.2: Synthesis of 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium (11)

The expected MAO-B generated metabolite of TMMP (6) is the 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium (MMDP<sup>+</sup>, 11) species. Since we had experience in the synthesis of MPDP<sup>+</sup> (3) from the corresponding N-oxide by the action of trifluoroacetic anhydride (TFAA), we approached the synthesis of 11 in the same way. Consequently, the first compound required was the intermediate 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol N-oxide (10).

The synthetic pathway leading to 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol N-oxide (10) is illustrated in Scheme 7. This intermediate was prepared by the reaction of adding *m*-chloroperoxybenzoic acid (MCPBA) in methylene chloride with MMPP (5). The crude N-oxide was isolated as its *m*-chlorobenzoate salt. The free base could be obtained by chromatography on basic alumina. The overall yield was 52%. This product was characterized by <sup>1</sup>H NMR (Fig. 6) and elemental analysis.

The <sup>1</sup>H NMR spectrum of 10 was assigned with the aid of decoupling experiments. The down field multiplets centered at 6.6 ppm (C-5'), 6.0 ppm (C-3') and 5.9 ppm (C-4') were assigned to the pyrrole ring protons. The singlets at 3.9 ppm (N'-CH<sub>3</sub>) and 3.2 ppm (N-CH<sub>3</sub>) were assigned to the pyrrole ring N-methyl group and piperidine ring N-methyl groups, respectively. The up field multiplets centered at 3.7 ppm and 3.1 ppm were assigned to the C-2 and C-6 methylene protons. The multiplet centered at 2.7 ppm and the doublet at 2.0 ppm were assigned to the C-3 and C-5 methylene protons. The UV spectrum of

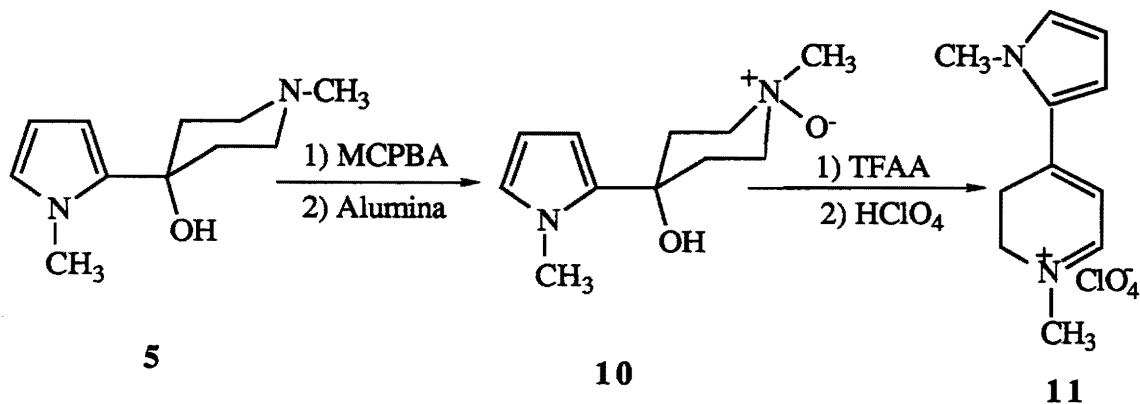
**10** (Fig. 7) displayed a single absorbance peak with  $\lambda_{\text{max}} = 220$  nm.

The synthesis of 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium perchlorate (MMDP<sup>+</sup>, **11**) was initially approached by adding TFAA to a solution of **10** in methylene chloride. The reaction was monitored by UV. A peak absorbing at 220 nm ( $\epsilon = 8400$ ) corresponding to the N-oxide (**10**) was initially observed. After adding TFAA, the color of the solution changed to yellow. A peak absorbing at 453 nm corresponding to the dihydropyridinium compound was observed this time. Attempts to obtain the perchlorate salt from methanolic HClO<sub>4</sub> as had been achieved with **3**, however, failed. At least part of the problem appeared to be due to the sensitivities of the pyrrole system to the acid.

Various solvents and acids were examined to isolate this product. A red solid could be obtained with anhydrous ethercal HCl. The <sup>1</sup>H NMR spectrum of this solid was encouraging (see below). Furthermore, the compound displayed the UV characteristics ( $\lambda=421\text{nm}$  with  $\epsilon=24000$ ) expected for **11**. However, C, H, N analysis showed that the product was not pure. It appeared to contain some inorganic material which might have come from the alumina column used in the purification of the N-oxide (**10**). The N-oxide (**10**) was then recrystallized. But no matter what we tried, we still could not obtain an analytically pure sample of the dihydropyridinium species.

Finally, **11** was obtained by the same approach discussed above, except the co-solvent ethanol was employed after the methanolic HClO<sub>4</sub>. Elemental analysis of the yellow crystalline solid showed that the product was pure and <sup>1</sup>H NMR spectrum confirmed that the product was the desired dihydropyridinium

compound (11).



Scheme 7: Syntheses of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol N-oxide and 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium (MMDP<sup>+</sup>)

The <sup>1</sup>H NMR spectrum (Fig. 8) of 11 was assigned with the aid of decoupling experiments and by comparison with the spectrum of 10. The down field doublets centered at 8.2 ppm (C-6) and 6.5 ppm (C-5) were assigned to the aromatic protons of the dihydropyridinium ring. The multiplets centered at 7.3 ppm (C-5'), 7.1 ppm (C-3'), and 6.2 ppm (C-4') were assigned to the pyrrole ring protons. The singlets at 3.95 ppm (N'-CH<sub>3</sub>) and 3.6 ppm (N-CH<sub>3</sub>) were assigned to the pyrrole ring N-methyl group and dihydropyridinium ring N-methyl group. The triplets centered at 3.85 ppm (C-2) and 3.2 ppm (C-3) were assigned to the remaining methylene groups. The UV spectrum of 11 displayed a single peak with  $\lambda_{\text{max}} = 421 \text{ nm}$  ( $\epsilon = 25000$ ).

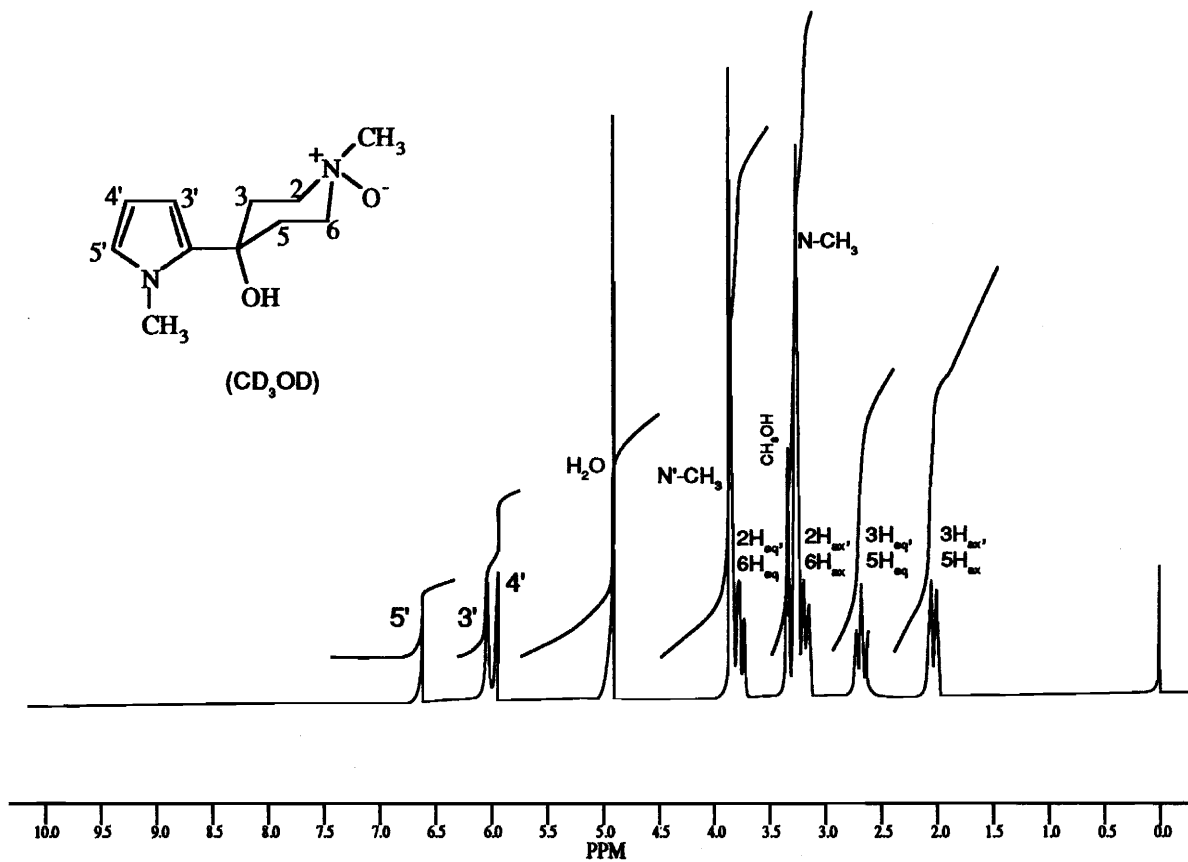


Figure 6:  $^1\text{H}$  NMR spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol N-oxide

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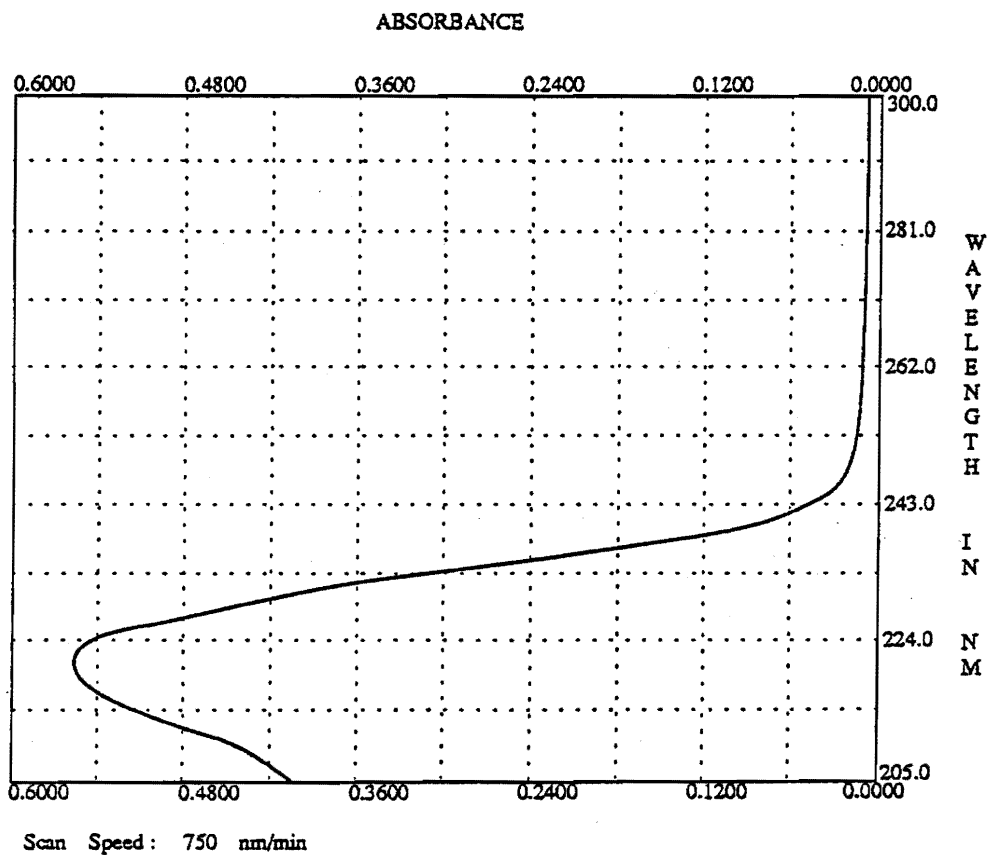


Figure 7: UV Spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol N-oxide



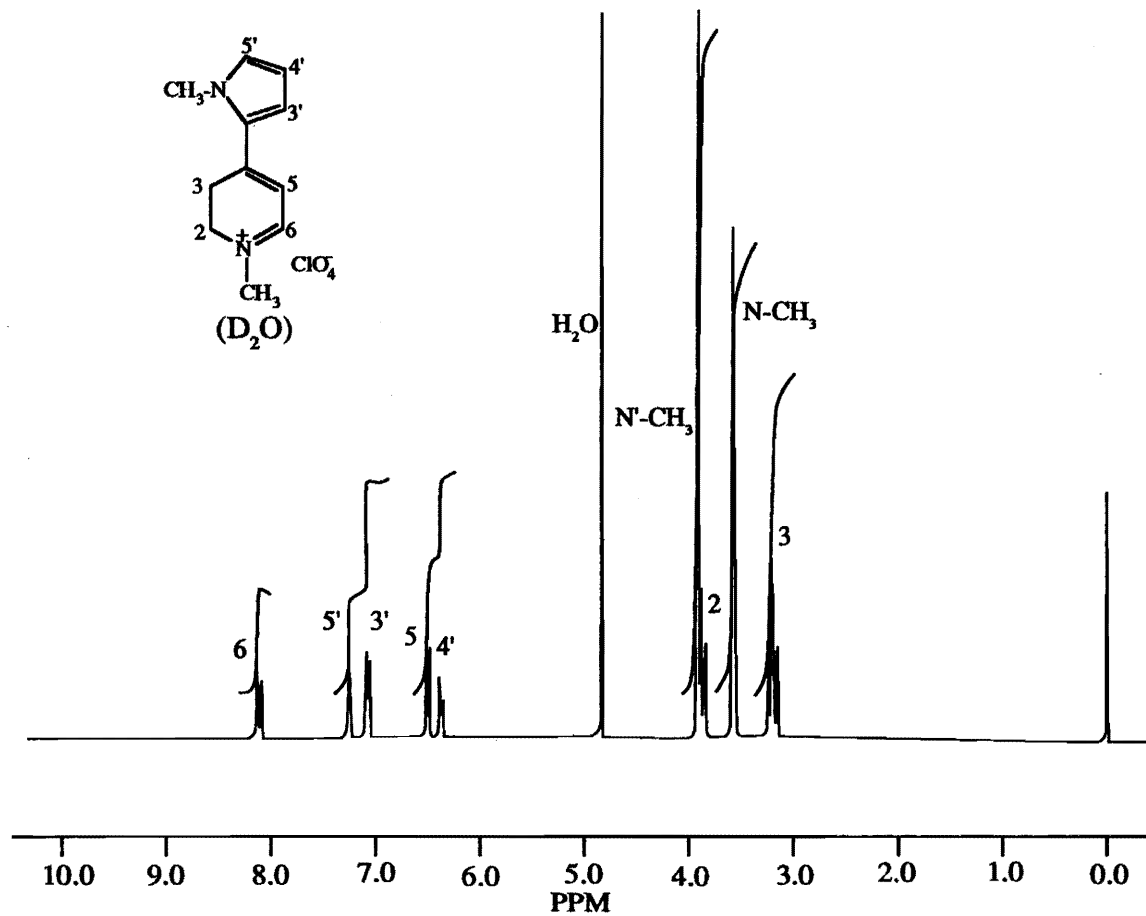


Figure 8:  $^1\text{H}$  NMR spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium perchlorate

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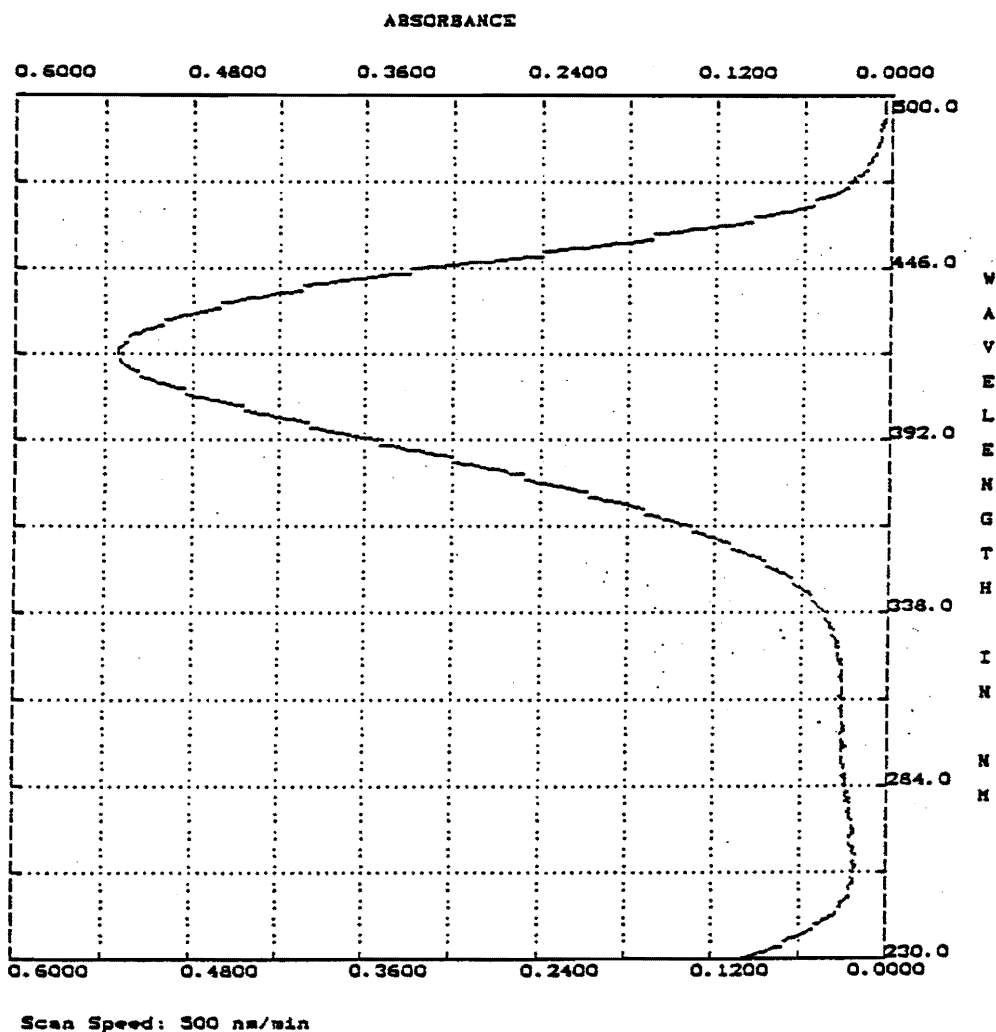


Figure 9: UV Spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium perchlorate

#### 2.2.4: Synthesis of 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium iodide (16)

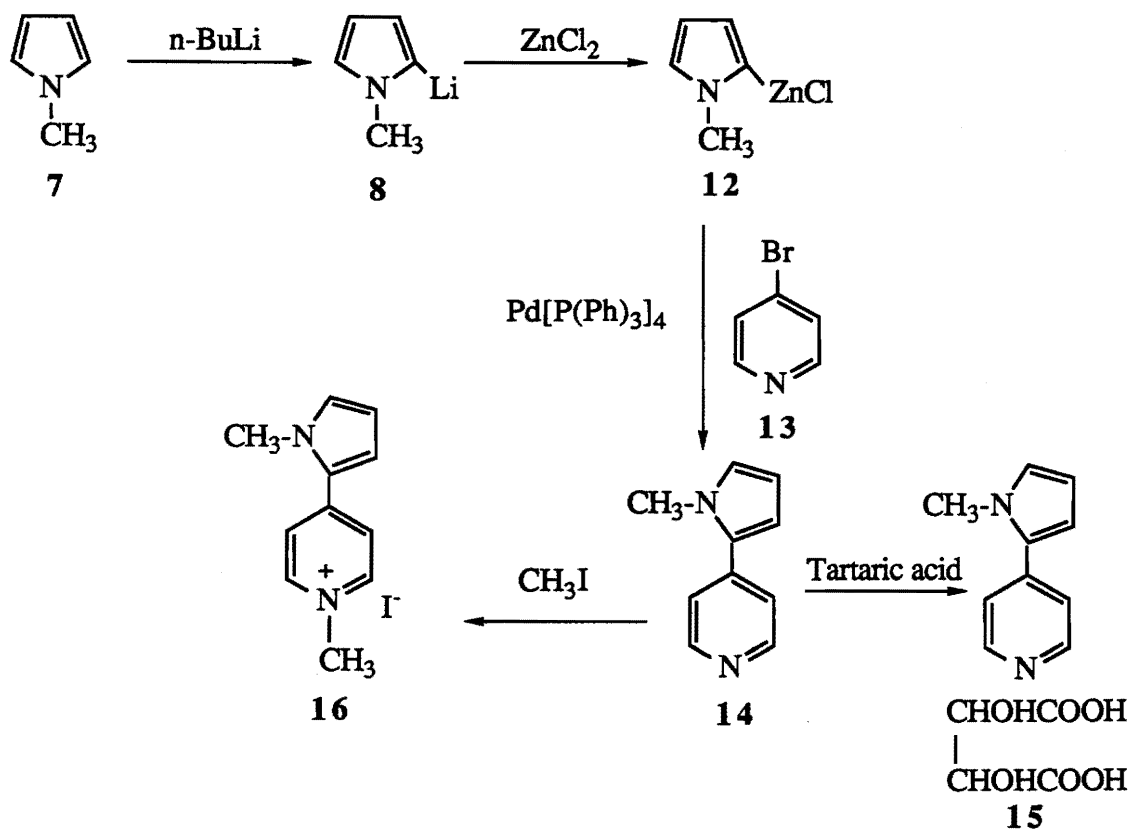
Synthesis of the title biaryl system was approached by a cross coupling reaction that requires a good catalyst in order to cope with the high activation energy involved. Palladium has generally been used as the catalyst for such cross-coupling reactions. Literature reports include description of cross-coupling reactions which yielded 2-arylfurans, 2-benzylfuran and 2-cinnamylfuran in which the organo halides were allowed to react with 2-lithiofuran and 2-furylzinc chloride in the presence of tetrakis(triphenylphosphine) palladium.<sup>64</sup> The products were obtained in a good yield. The coupling reaction between 4-bromopyridine (13) and 2-pyrrolylzinc chloride (12), catalyzed by the palladium reagent, was carried out successfully in high yield by following the approach used to make 2-phenylfuran mentioned above.<sup>64</sup> The synthetic pathway is illustrated in Scheme 8.

GC/EIMS analysis of the crude reaction mixture (Fig. 10) showed four peaks ( $M^+ = 158$ ,  $M^+ = 160$ ,  $M^+ = 158$ , and  $M^+ = 262$ ) corresponding to 4-bromopyridine, N,N'-dimethylbipyrrole, 4-(1-methylpyrrol-2-yl)pyridine (expected product), and triphenylphosphine. The main peak was the expected product 4-(1-methylpyrrol-2-yl)pyridine (14) which was obtained in 80% yield. The GC/EIMS spectrum of 14 (Fig. 11) displayed the molecular ion at  $m/z = 158$ . The final product could be obtained free of 4-bromopyridine if the 4-bromopyridine was used in a ratio of 2/3 relative to pyrrole. The N,N'-dimethylbipyrrole and triphenylphosphine could be removed after the 4-(1-methylpyrrol-2-yl)pyridine (14) was converted either to its tartaric acid salt (15)

or the methiodide (**16**).

The final product (**14**) was a liquid. It was purified as its tartaric salt **15** which was recrystallized from 95% ethanol.  $^1\text{H}$  NMR analysis confirmed the formation of this salt. The  $^1\text{H}$  NMR spectrum (Fig. 12) of **15** was assigned as follows: The two doublets centered at 8.5 ppm (C-2 and C-6) and 8.0 ppm (C-3 and C-5) were assigned to the pyridine ring protons. The multiplets centered at 7.2 ppm (C-5'), 7 ppm (C-3'), and 6.4 ppm (C-4') were assigned to the pyrrole ring protons. The singlet at 4.5 ppm was assigned to the tartaric acid protons and the singlet at 3.9 ppm (N-CH<sub>3</sub>') was assigned to the pyrrole ring N-methyl group. The UV spectrum of **15** (Fig. 13) displayed a single peak with  $\lambda_{\text{max}} = 310$  nm ( $\epsilon = 16500$ ).

Methylation of 4-(1-methylpyrrol-2-yl)pyridine (**14**) in distilled acetone gave the corresponding methiodide salt (**16**) as shown in Scheme 8. Recrystallization from water gave the pure compound in a 73% yield.  $^1\text{H}$  NMR (Fig. 14) analysis confirmed the structure. The two doublets centered at 8.7 ppm (C-2 and C-6) and 8.05 ppm (C-3 and C-5) were assigned to the pyridinium ring protons. The multiplets centered at 7.2 ppm (C-5'), 7.0 ppm (C-3') and 6.35 ppm (C-4') were assigned to the pyrrole ring protons. The two singlets at 4.25 ppm (N-CH<sub>3</sub>) and 4.0 ppm (N'-CH<sub>3</sub>) were assigned to the two methyl groups. The UV spectrum of **16** (Fig. 15) displayed a peak with  $\lambda_{\text{max}} = 374$  nm and molar extinction coefficient of 28222.



Scheme 8: The formation of 4-(1-methylpyrrol-2-yl)pyridine and 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium iodide

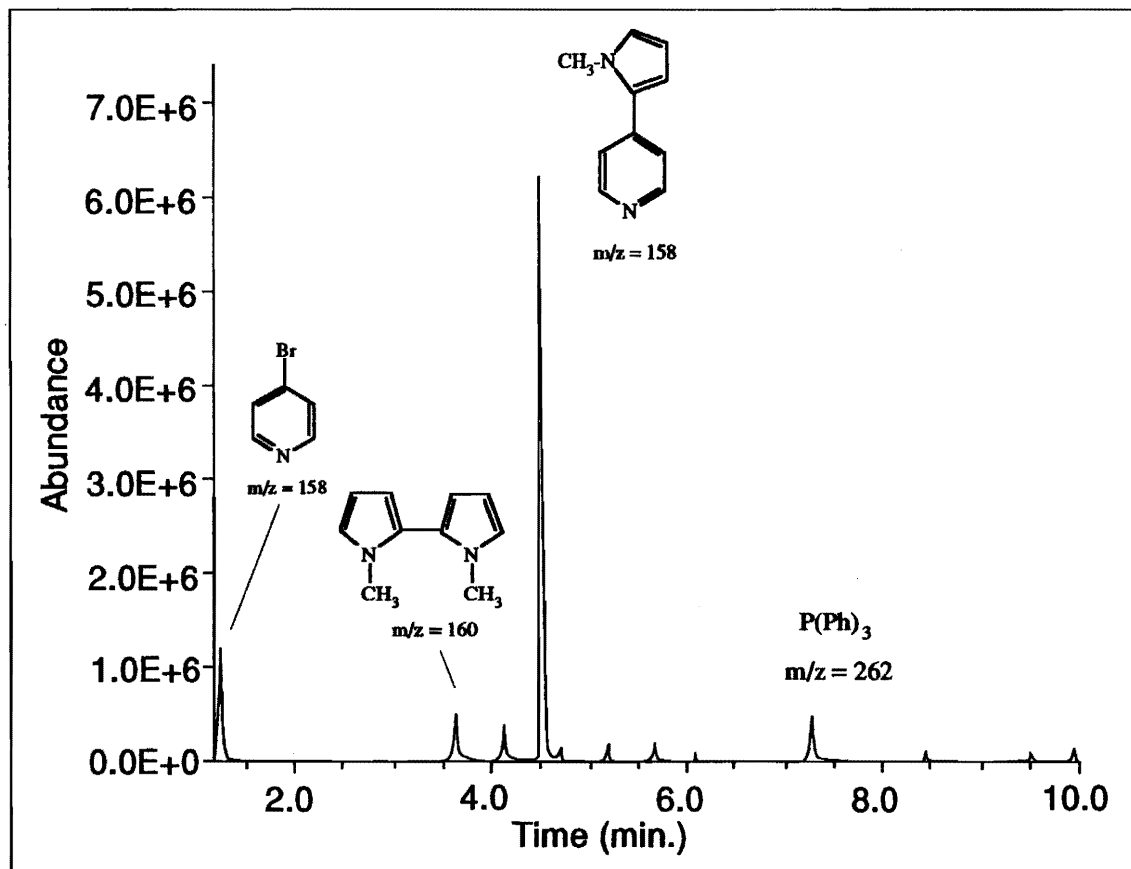


Figure 10: The total ion current chromatogram of 4-(1-methylpyrrol-2-yl)pyridine

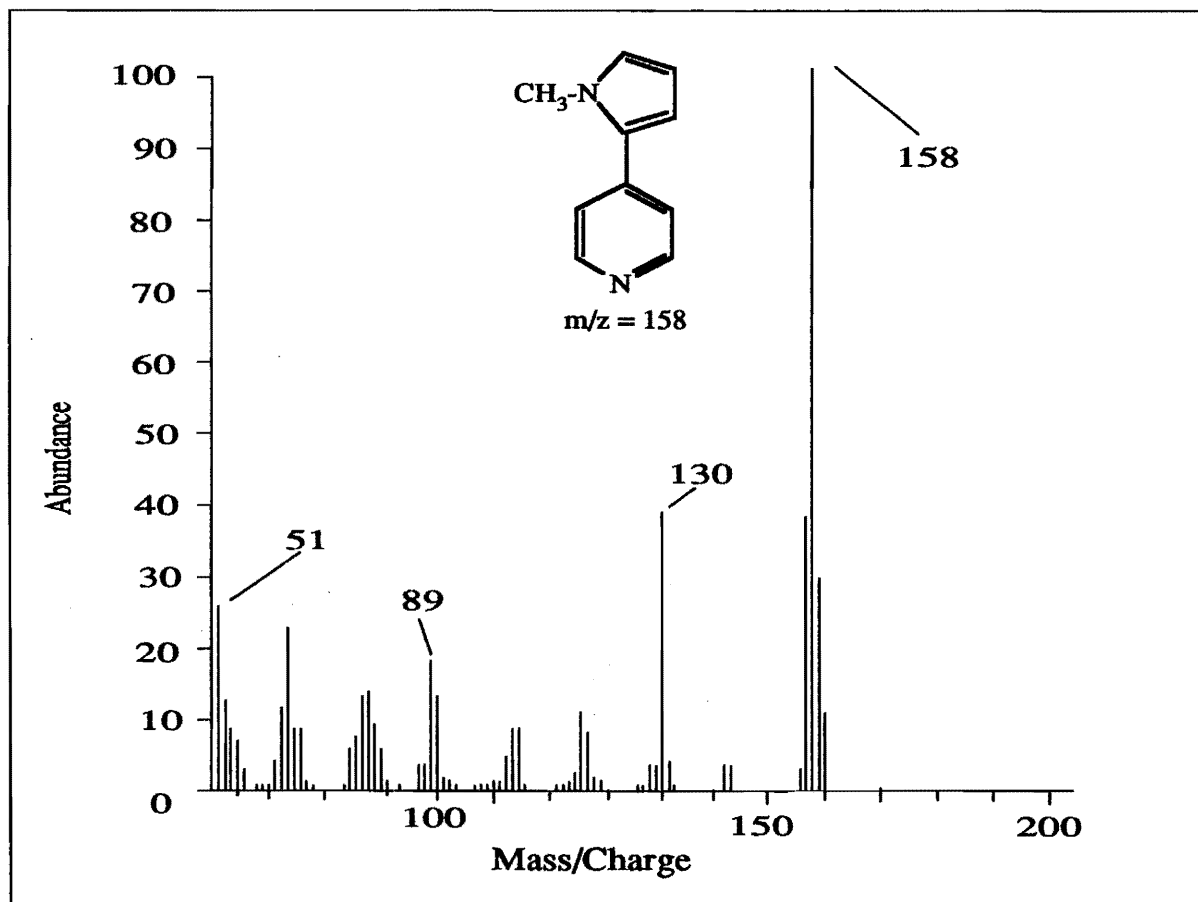


Figure 11: GC/EIMS of 4-(1-methylpyrrol-2-yl)pyridine

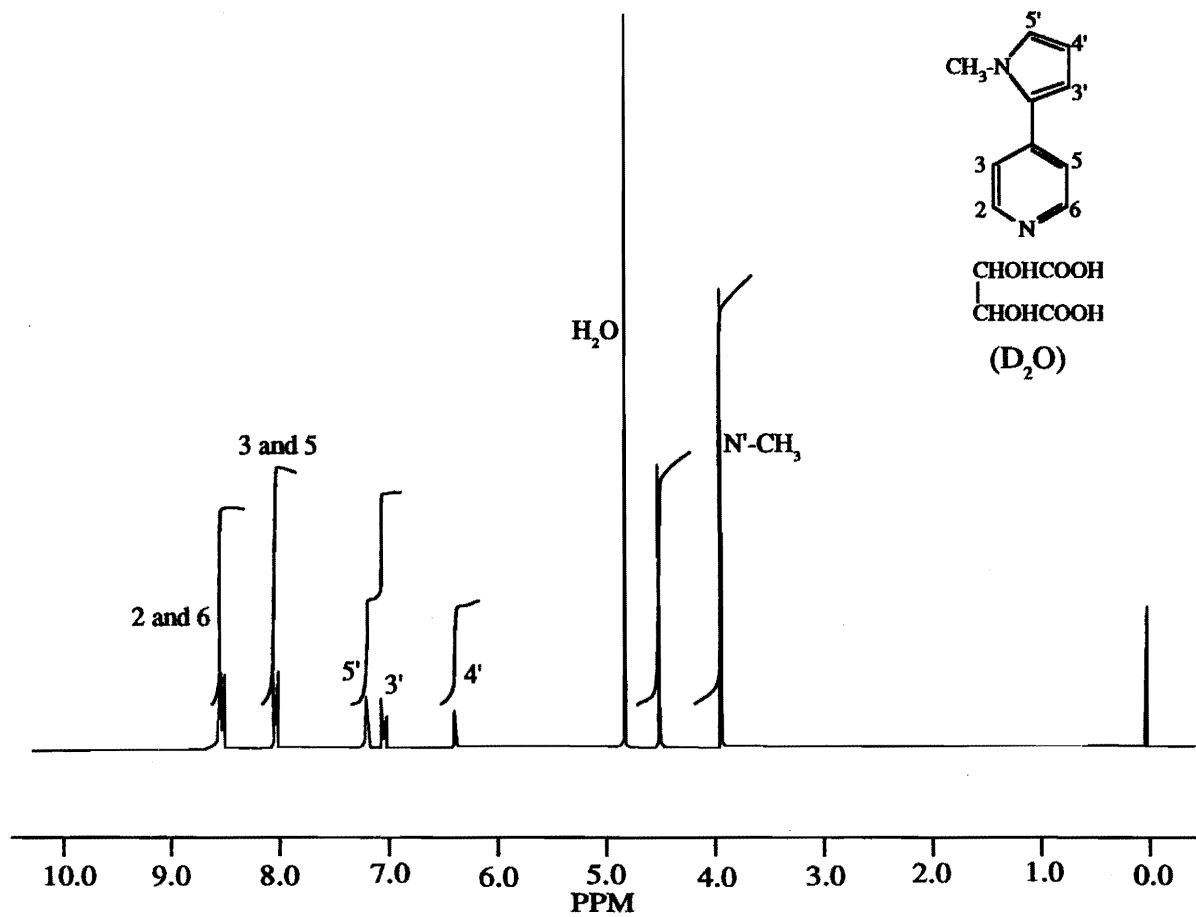


Figure 12:  $^1\text{H}$  NMR spectrum of 4-(1-methylpyrrol-2-yl)pyridine tartrate



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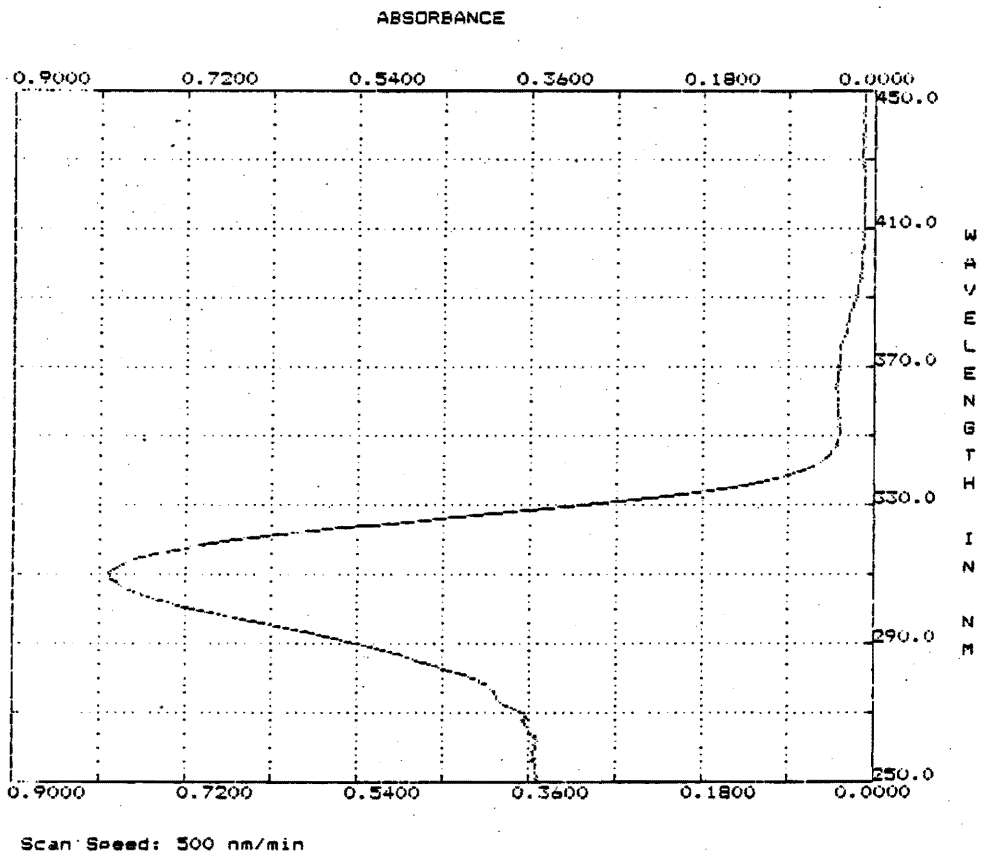


Figure 13: UV Spectrum of 4-(1-methylpyrrol-2-yl)pyridine tartrate

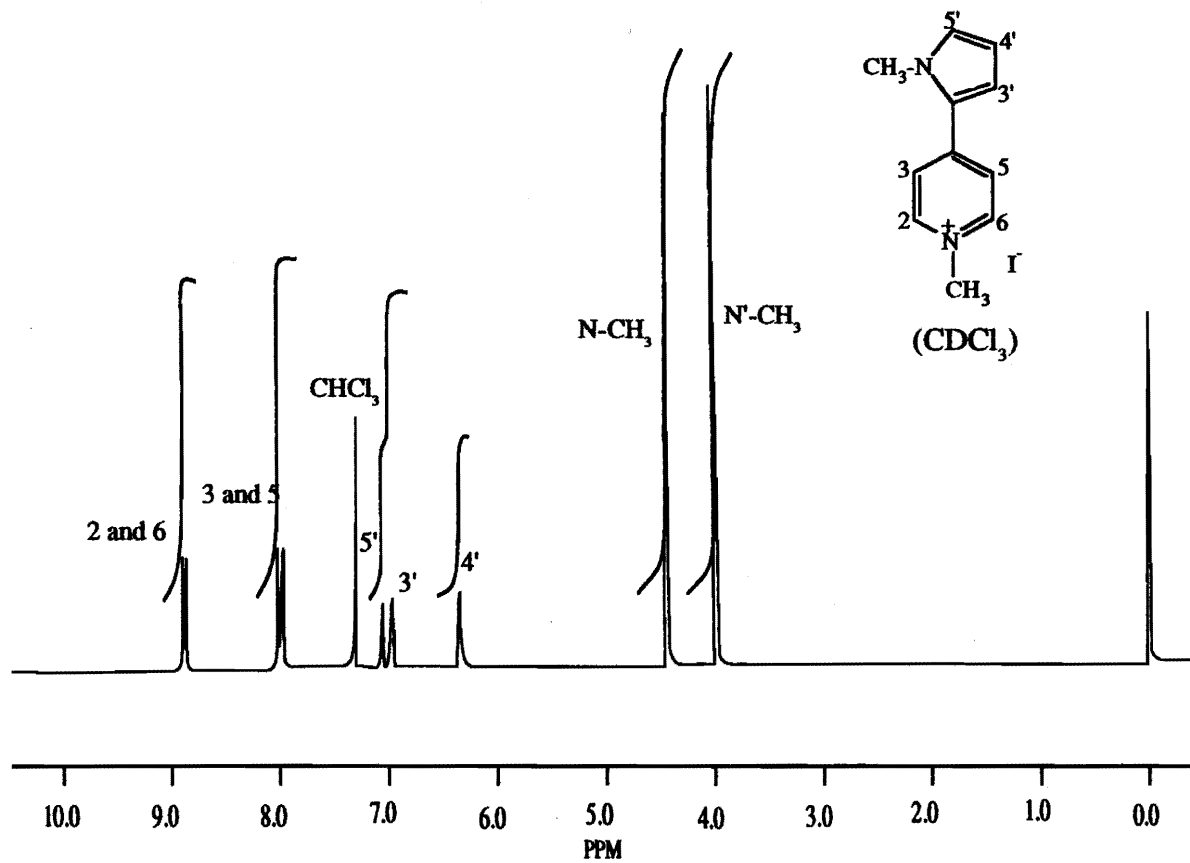


Figure 14:  $^1\text{H}$  NMR spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium iodide

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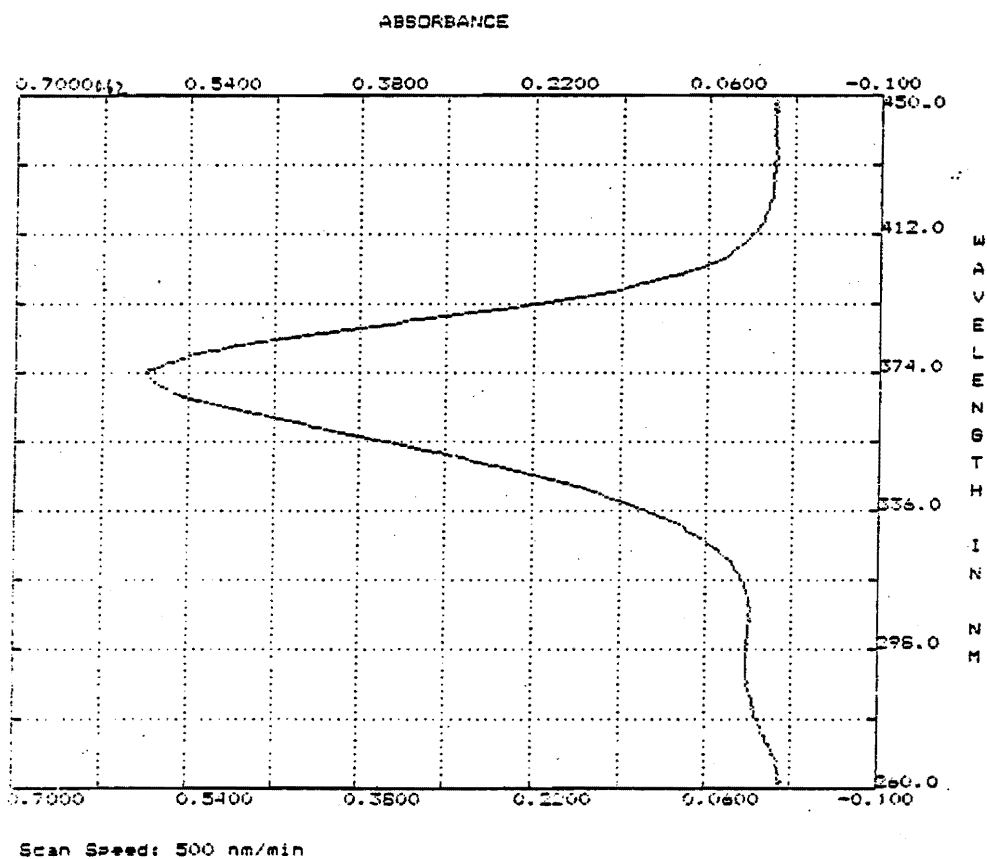


Figure 15: UV Spectrum of 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium  
Iodide

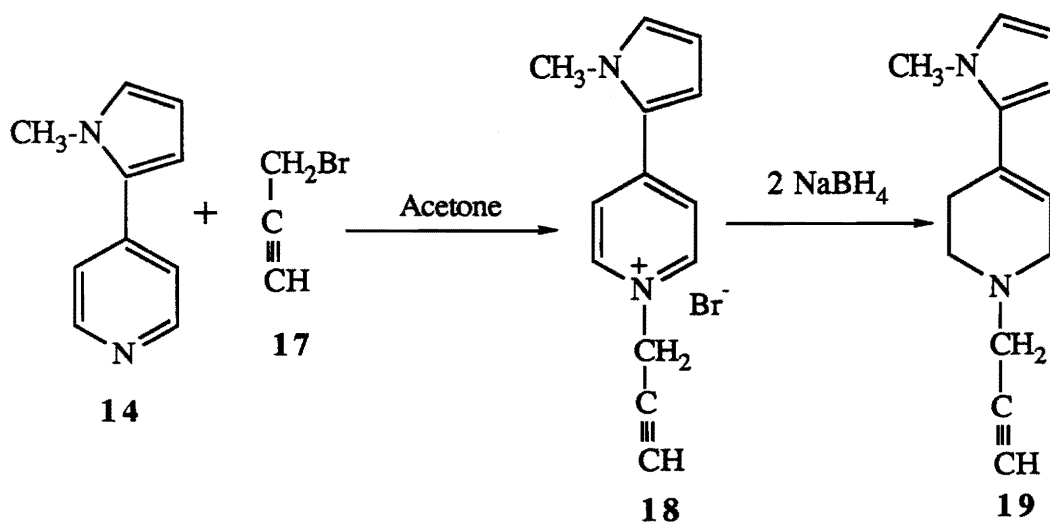
### **2.2.5: N-Propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydro-pyridine oxalate (19)**

In order to obtain N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydro-pyridine (**19**), the N-propargyl-4-(1-methylpyrrol-2-yl)pyridinium species (**18**) must be in hand. Then a simple reduction should yield the desired product.

N-Propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (**19**) was made in two steps as illustrated in Scheme 9. First, N-propargyl-4-(1-methylpyrrol-2-yl)pyridinium bromide (**18**) was made by adding propargyl bromide (**17**) to 4-(1-methylpyrrol-2-yl)pyridine (**14**) in acetone to yield the crude product. The crude product following recrystallization from methanol/ether provided a good  $^1\text{H}$  NMR spectrum and C,H,N analysis. The  $^1\text{H}$  NMR spectrum of **18** (Fig. 16) was assigned as follows. The two doublets centered at 8.7 ppm (C-2 and C-6) and 8.05 ppm (C-3 and C-5) were assigned to the pyridinium ring protons. The multiplets centered at 7.25 ppm (C-5'), 7.1 ppm (C-3') and 6.42 ppm (C-4') were assigned to the pyrrole ring protons. The singlet at 5.4 ppm was assigned to the methylene group and the singlet at 4.0 ppm (N'-CH<sub>3</sub>) was assigned to the N-methyl group of the pyrrole ring. The UV spectrum of **18** (Fig. 17) displayed a single peak with  $\lambda_{\text{max}} = 380 \text{ nm}$  ( $\epsilon = 31180$ ).

The reduction of N-propargylpyridinium bromide (**18**) was carried out by adding two equivalents of sodium borohydride to a solution of the pyridinium compound in methanol. Water was added and ether was used to extract the reduction product. GC/MS total ion current chromatography of this extraction showed a single peak and the base peak in the GC/EI mass spectrum (Fig. 18)

was the molecular ion at  $m/z = 200$ . The fragment ion at  $m/z = 161$  corresponding to loss N-propargyl moiety. The product was purified as its oxalate salt.  $^1\text{H}$  NMR (Fig. 2.20) and C,H,N analysis confirmed the structure. The  $^1\text{H}$  NMR spectrum of **19** (Fig. 19) was assigned as follows: The multiplets centered at 6.7 ppm (C-5'), 6.2 ppm (C-3') and 6.05 ppm (C-4') were assigned to the pyrrole ring protons. The multiplet centered at 5.8 ppm (C-5) was assigned to the olefin proton. The singlet at 4.1 ppm was assigned to the methylene group. The singlet at 3.7 ppm (N'-CH<sub>3</sub>) was assigned to the N-methyl group. The multiplet centered at 4.0 ppm (C-6), triplet centered at 3.6 ppm (C-2), and multiplet centered at 2.8 ppm (C-3) were assigned to the remaining piperidene ring protons. The UV spectrum of **19** (Fig. 20) displayed a peak with  $\lambda_{\text{max}} = 261$  nm and  $\epsilon = 10090$ .



Scheme 9. Formation of N-propargyl-4-(1-methylpyrrol-2-yl)pyridinium and N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine

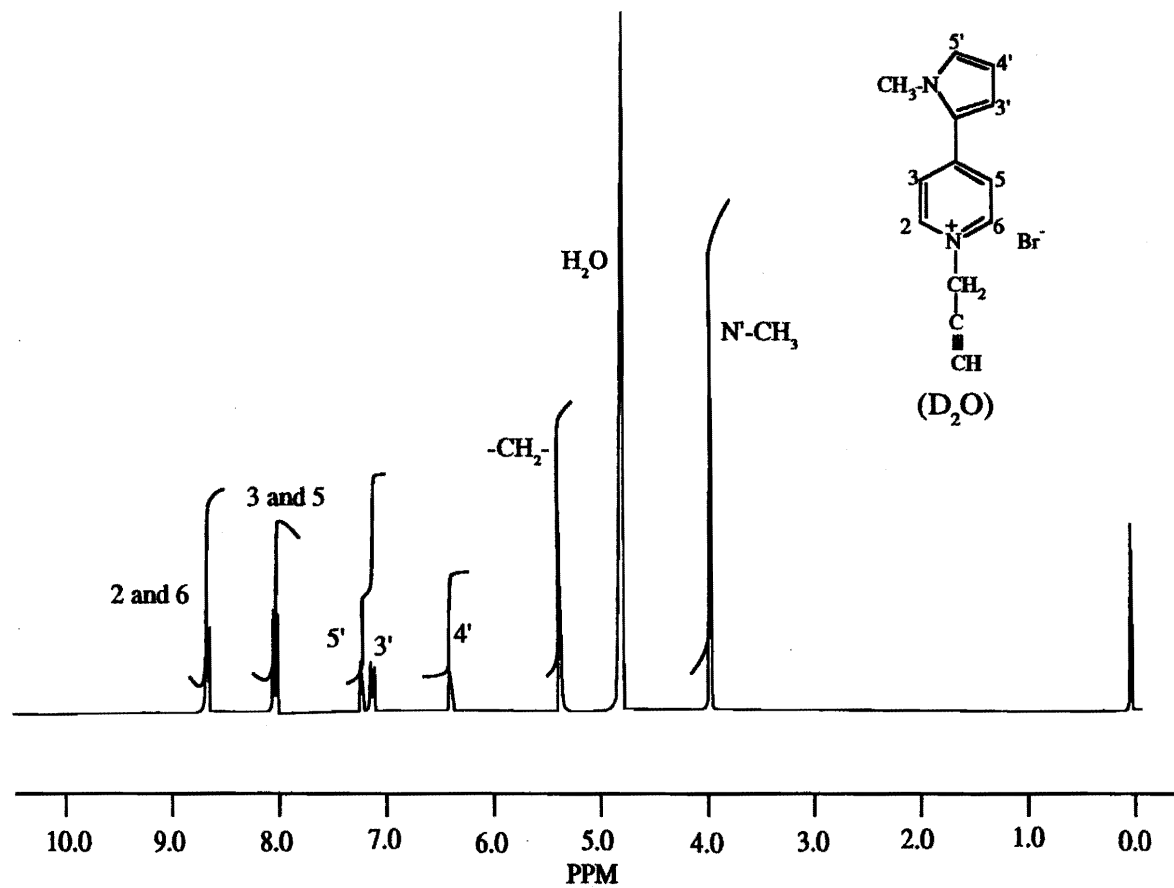


Figure 16:  $^1\text{H}$  NMR spectrum of N-propargyl-4-(1-methylpyrrol-2-yl)pyridinium bromide

BECKMAN  
DU-50 SPECTROPHOTOMETER

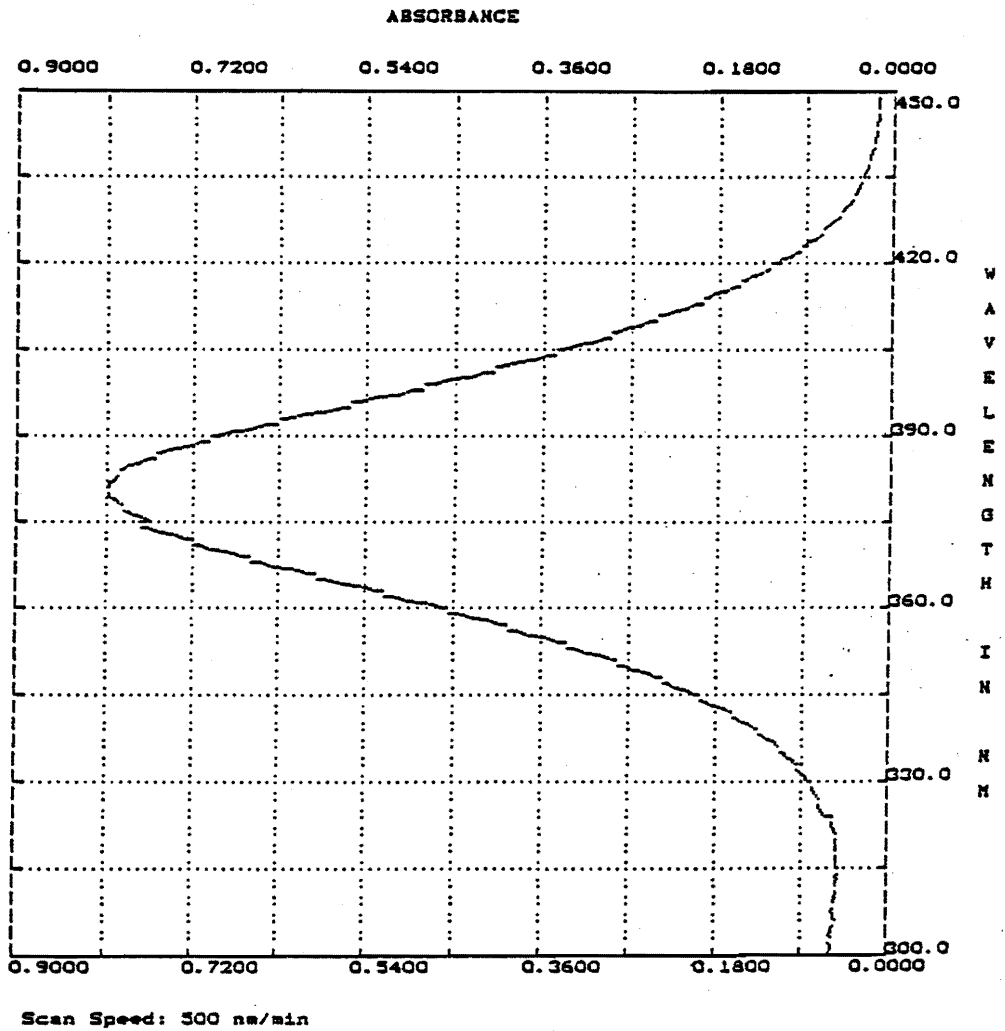


Figure 17: UV Spectrum of N-propargyl-4-(1-methylpyrrol-2-yl)pyridinium  
bromide

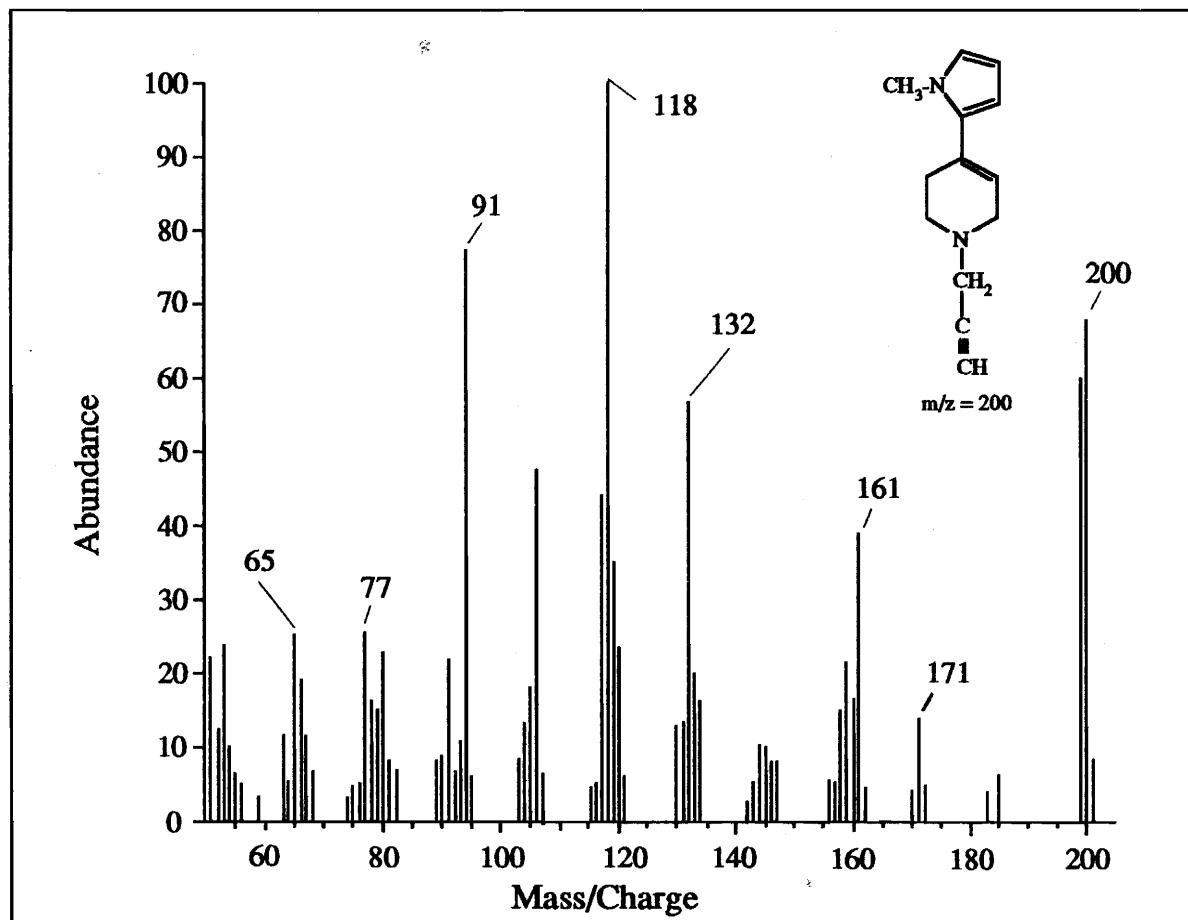


Figure 18: GC/EIMS of N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine



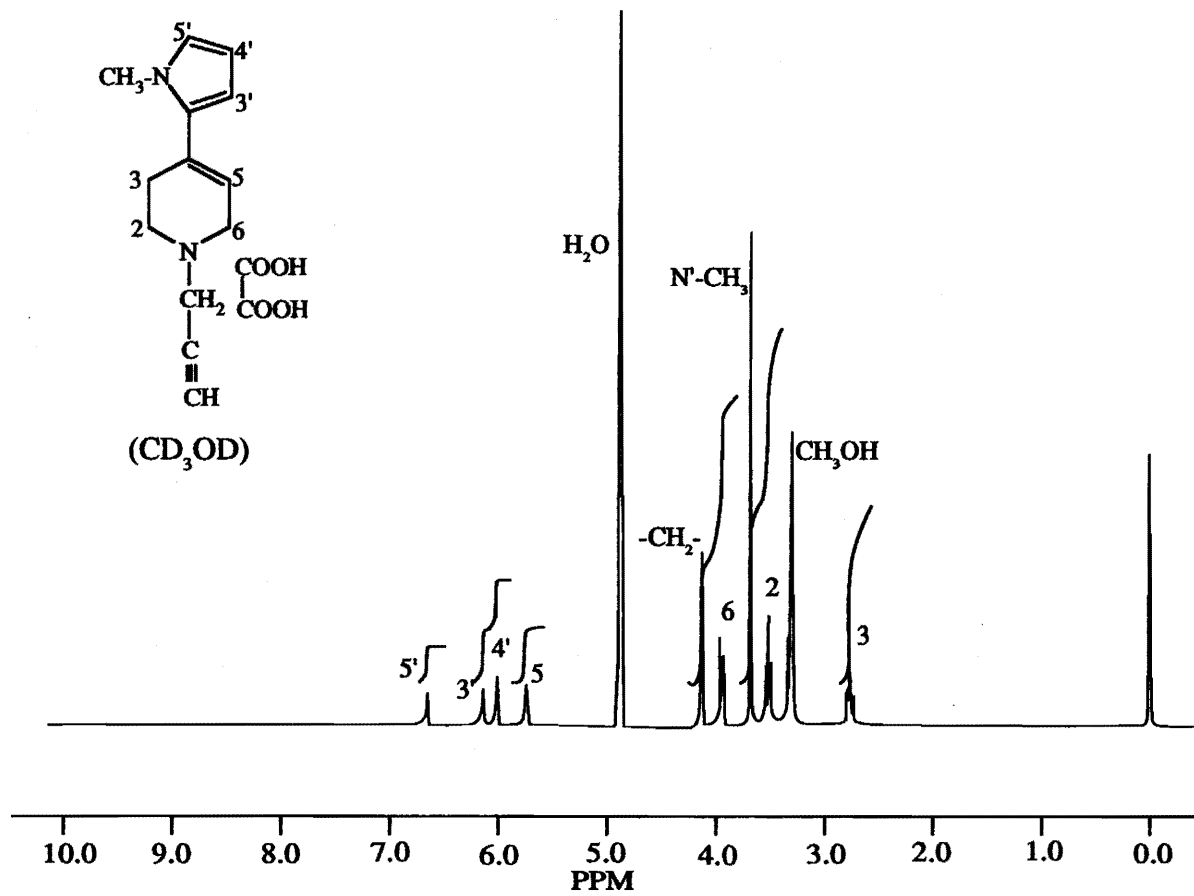


Figure 19:  $^1\text{H}$  NMR spectrum of N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine oxalate

BECKMAN  
DU-50 SPECTROPHOTOMETER

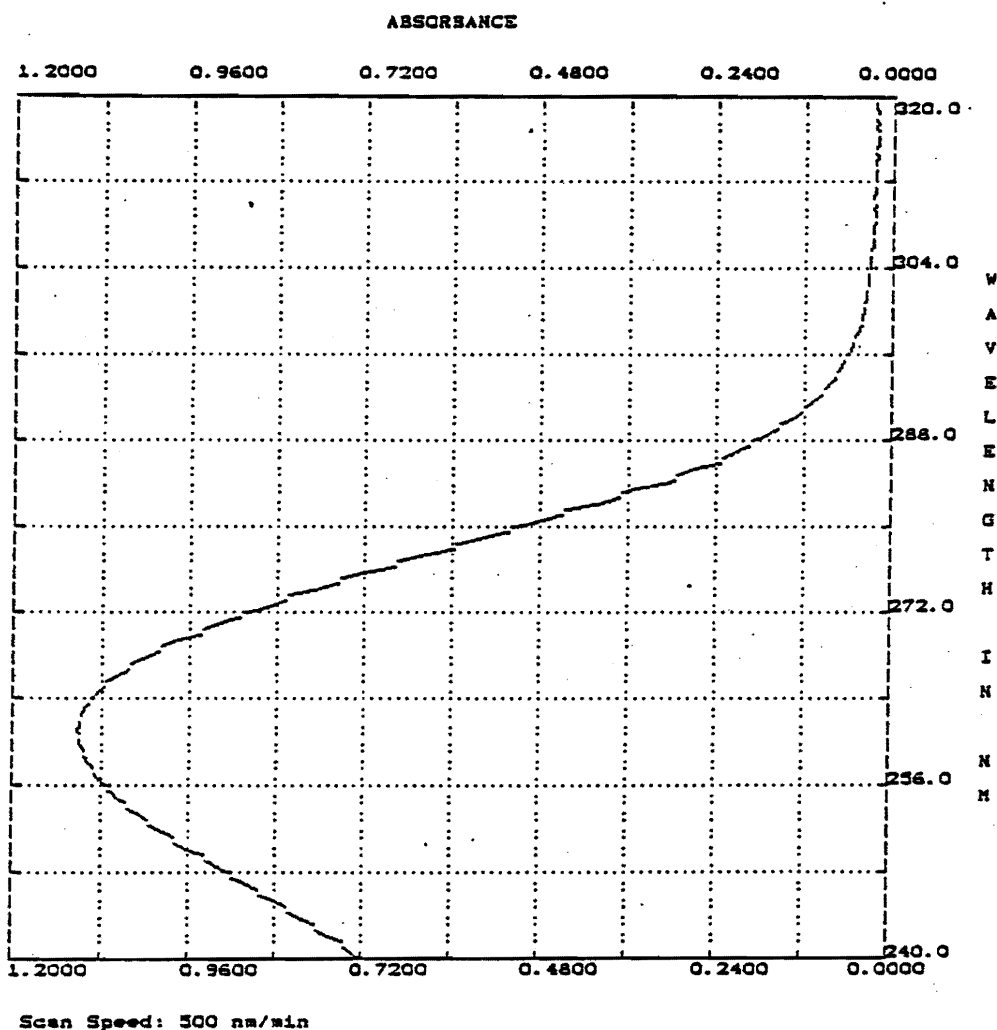


Figure 20: UV Spectrum of N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine oxalate

## 2.3: Experimental details

**General methods:** All chemicals used in the syntheses were reagent or HPLC grade and were obtained from Aldrich or Fisher. Melting points were measured on a Thomas-Hoover melting point apparatus and are uncorrected.  $^1\text{H}$  NMR spectra were recorded with a Bruker WP 270 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) with respect to the internal standard TMS except where noted. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), and m (multiplet). Gas chromatography/mass spectrometry (GC/MS) analysis was performed using a Hewlett Packard 5890 capillary GC coupled with a Hewlett Packard 5970 mass selective detector. Data were acquired with a Hewlett Packard 59970 MS chem station. The capillary column used in all cases was an HP-1 (12 m X 200  $\mu\text{m}$  X 0.33  $\mu\text{m}$  film thickness). UV spectral analyses were performed on a Beckman Model 50 spectrophotometer. Microanalyses were performed by the Atlantic Microlab, Inc., Norcross, GA.

**1-Methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol (5).** A 1.6 M solution of n-butyllithium in hexane (210 mL, 0.33 mol) was added dropwise at room temperature to a solution of N-methylpyrrole (24.2 g, 26.5 mL, 0.3 mol) in 250 mL of anhydrous diethyl ether contained in a reaction flask under a nitrogen blanket. After the addition was completed, the resulting mixture was heated under reflux with stirring for 16-20 hours and then cooled to 0°C. To this mixture was added 1-methyl-4-piperidone (9) (34 g, 0.3 mol) dropwise from an addition funnel while maintaining the temperature at 0°C. The reaction flask was cooled with a dry ice-acetone bath during the addition. The reaction

mixture then was stirred at room temperature for 3 hours and quenched in ice water to yield 22.5 g (40.8%) of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol (5) in crystalline form. Recrystallization from heptane yielded white prisms: mp 122-123°C (lit.<sup>62</sup> mp 122.5-123°C); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.8-2.2 [m, 4H, C3 and C5], 2.3 [s, 3H, N'-CH<sub>3</sub>], 2.4-2.5 [m, 2H, C2 or C6], 2.6-2.8 [d, 2H, C2 or C6], 4.35 [s, 3H, N-CH<sub>3</sub>], 6.0 [m, 2H, C3',C4'], 6.5 [s, 1H, C5']; GC/EIMS M<sup>+</sup> = 194.

**Hydrochloride salt of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (6).** A mixture of 3 g of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol, 3 g florisil, and 50 mL of toluene was heated under reflux for 7-8 hours, then cooled to room temperature, decanted, and evaporated to leave a heavy oil. This heavy oil was dissolved in 20 mL of anhydrous diethyl ether. Gaseous HCl was bubbled through the solution to yield 3.45 g (92%) of the title compound in crystalline form: mp 217-223°C [lit.<sup>62</sup> 222-230°C]; <sup>1</sup>H NMR of free base (CDCl<sub>3</sub>) δ 2.4 [s, 3H, N1CH<sub>3</sub>], 2.5 [m, 2H, C3], 2.6-2.7 [m, 2H, C2], 3.1 [m, 2H, C6], 3.6 [s, 3H, N1'CH<sub>3</sub>], 5.7 [m, 1H, C5], 6.1 [m, 2H, (C3', C4')], 6.6 [m, 1H, C5']; UV (sodium phosphate buffer, pH = 7.4) λ<sub>max</sub> = 266 nm (ε = 7325); GC/EIMS M<sup>+</sup> = 176.

**1-Methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol N-oxide (10).** *m*-Chloroperoxybenzoic acid (3.3 g, 19.1 mmol) in 50 mL CH<sub>2</sub>Cl<sub>2</sub> was added dropwise to a solution of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol (3 g, 15.5 mmol) in 10 mL CH<sub>2</sub>Cl<sub>2</sub> at 0°C. After the addition, the mixture was stirred for 1 hour at 0°C. About 80% of the solvent was removed in vacuo. Anhydrous ether (50 mL) was then added to the residue. The reaction mixture was again stirred at 0°C to yield a white precipitate (the benzoic acid salt of the N-oxide).

The salt was dissolved in 2 mL CH<sub>3</sub>OH and filtered through a basic alumina column (160 g). Then the product eluted with 5% CH<sub>3</sub>OH in CH<sub>2</sub>Cl<sub>2</sub> to yield 1.7 g (52%) of the free base of 1-methyl-4-(1-methylpyrrol-2-yl)-4-piperidinol N-oxide (**10**): mp 240°C (decompose); <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 1.9-2.1 [ d, 2H, (C3 or C5) ], 2.6-2.8 [m, 2H, (C3 or C5) ], 3.1-3.2 [ d, 2H, (C2 or C6 ) ], 3.22 [s, 3H, N1CH<sub>3</sub> ], 3.6-3.7 [m, 2H, (C2 or C6 ) ], 3.82 [s, 3H, N1'CH<sub>3</sub> ], 5.9 [m, 1H, C4' ], 6.0 [ m, 1H, C3' ], 6.5-6.6 [m, 1H, C5' ]; UV (C<sub>2</sub>H<sub>5</sub>OH) λ<sub>max</sub> = 220 nm (ε = 8400). Anal. Calcd. for C<sub>11</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub>: C, 62.85%; H, 8.57%; N, 13.33%. Found: C, 62.6%; H, 8.59%; N, 13.29%.

#### **1-Methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium**

**chloride.** Trifluoroacetic anhydride 0.63 mL (4.5 mmol) was added to a solution of 185 mg (0.88 mmol) N-oxide in 20 mL anhydrous ether at 0°C under nitrogen. The mixture was stirred at 0°C for 30 minutes. Hydrochloride gas was then bubbled into the solution to form a cloudy yellow liquid. The cloudy solution was put in the refrigerator for two days to yield a red colored solid. The red colored solid was collected and washed with acetone to obtain a hygroscopic solid in 20% yield: mp 158-160°C; <sup>1</sup>H NMR (D<sub>2</sub>O) δ 3.15-3.22 [t, 2H, C<sub>5</sub>], 3.56 [s, 3H, N'-CH<sub>3</sub>], 3.82-3.92 [t, 5H, N-CH<sub>3</sub> and C<sub>6</sub>], 6.35-6.4 [m, 1H, C<sub>4</sub>'], 6.45-6.51 [d, 1H, C<sub>3</sub>'], 7.04-7.1 [m, 1H, C<sub>5</sub>'], 7.25 [s, 1H, C<sub>3</sub>], 8.08-8.13 [d, 1H, C<sub>2</sub>]; UV (pH = 7.4 sodium phosphate buffer) λ<sub>max</sub> = 420 nm (ε = 24000). Anal. Calcd. for C<sub>11</sub>H<sub>15</sub>N<sub>2</sub>Cl: C, 62.7%; H, 7.18%; N, 13.3%; Cl, 16.82%. Found: C, 59.98%; H, 6.73%; N, 12.45%; Cl, 15.62%.

#### **1-Methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium**

**perchlorate (11).** The N-oxide (105 mg, 0.5 mmol) in 2mL CH<sub>2</sub>Cl<sub>2</sub> was

placed in a round bottom flask and 0.5 mL (2.5 mmol) TFAA in 1 mL CH<sub>2</sub>Cl<sub>2</sub> was added dropwise at 0°C. The resulting solution was stirred for 30 minutes at 0°C and then the solvent was removed in vacuo. To the residue was added 1 mL 10% HClO<sub>4</sub> in CH<sub>3</sub>OH followed by 2 mL ethanol. The resulting solution was stirred for 2 hours at 0°C to yield a yellow solid (50 mg, 36%) which was collected by suction filtration: mp 133-137°C; <sup>1</sup>H NMR (D<sub>2</sub>O) δ 3.2-3.28 [t, 2H, C<sub>3</sub>], 3.6 [s, 3H, N'-CH<sub>3</sub>], 3.85-3.93 [t, 2H, C<sub>2</sub>], 3.95 [s, 3H, N-CH<sub>3</sub>], 6.22-6.27 [m, 1H, C<sub>4</sub>'], 6.54 [d, 1H, C<sub>5</sub>], 7.12 [m, 1H, C<sub>3</sub>'], 7.3 [t, 1H, C<sub>5</sub>'], 8.2 [d, 1H, C<sub>6</sub>]; UV (pH = 7.4 sodium phosphate buffer) λ<sub>max</sub> = 420 nm (ε = 25000). Anal. Calcd. for C<sub>11</sub>H<sub>15</sub>N<sub>2</sub>ClO<sub>4</sub>: C, 48.1%; H, 5.5%; N, 10.2%; Cl, 12.91%. Found: C, 48.0%; H, 5.5%; N, 10.18%; Cl, 12.98%.

**4-(1-Methylpyrrol-2-yl)pyridine (14).** A solution of 4-bromopyridine hydrochloride (2.0 g, 10 mmol) in 5 mL water was treated with saturated K<sub>2</sub>CO<sub>3</sub> solution to adjust its pH to 9. Ether (3 x 40 mL) was used to extract the aqueous layer. The organic layers were combined and dried over MgSO<sub>4</sub> and the solvent was removed. It was found that 4-bromopyridine free base is very unstable; at room temperature the colorless liquid would change to a red solid rapidly, presumably due to self condensation reactions.

A hexane solution of n-butyllithium (12.5 mL, 15 mmol) was added from an addition funnel to a stirred solution of N-methylpyrrole (1.35 mL, 15 mmol) in 10 mL dry THF at room temperature under nitrogen. This mixture was allowed to stir for 3 hours at room temperature and then was transferred by a double-ended needle to a stirred solution of dry zinc chloride (2.0 g, 15 mmol) in 10 mL THF at room temperature. This mixture was stirred for 1 hour. Pd[P(Ph)<sub>3</sub>]<sub>4</sub>

(0.057 g, 0.055 mmol) was weighed into a 100 mL round bottomed flask under  $N_2$  and dissolved in 25 mL THF. The solution of 4-bromopyridine was added with a syringe to the stirred catalyst solution followed by the 1-methyl-2-pyrrolyzinc chloride solution. Then the temperature was allowed to raise to  $50^\circ C$  following which the reaction mixture was stirred for 24 hours. The THF solution was diluted with 70 mL ether and then washed with water (50 mL X 2). The aqueous layer was saturated with NaCl and then extracted with 100 mL ether. The organic layers were combined, dried over  $MgSO_4$ , and then removed in vacuo. A yellow oil was left in the flask which is the crude product: GC/MS  $M^+ = 158$ .  $^1H$  NMR ( $CDCl_3$ ).  $\delta$  3.8 [s, 3H,  $N'$ - $CH_3$ ], 6.25 [m, 1H,  $C_4'$ ], 6.52 [m, 1H,  $C_3'$ ], 6.85 [t, 1H,  $C_5'$ ], 7.46 [d, 2H,  $C_3$  and  $C_5$ ], 8.7 [d, 2H,  $C_2$  and  $C_6$ ]. Tartaric acid (1.5 g, 10 mmol) was dissolved in 20 mL 95% ethanol and the crude 4-(1-methylpyrrol-2-yl)pyridine, which was dissolved in 20 mL 95% ethanol, was added. After standing over night the solid product was collected to give 2.3 g (73%) crude 4-(1-methylpyrrol-2-yl)pyridine tartrate (15). This compound was recrystallized from 35 mL 95% ethanol: mp  $172-173^\circ C$ ; UV (MeOH)  $\lambda_{max} = 310$  nm ( $\epsilon = 16500$ );  $^1H$  NMR ( $D_2O$ ),  $\delta$  3.9 [s, 3H,  $N-CH_3$ ], 4.5 [s, 2H,  $-CH-CH-$ ], 6.4 [m, 1H,  $C_4'$ ], 7.03 [d, 1H,  $C_3'$ ], 7.2 [s, 1H,  $C_5'$ ], 8.0 [d, 2H,  $C_3$  and  $C_5$ ], 8.5 [d, 2H,  $C_2$  and  $C_6$ ]; Anal. Calc. for  $C_{14}H_{16}N_2O_6 \cdot 0.78 H_2O$ : C, 52.12%; H, 5.45%; N, 8.69%. Found: C, 52.08%, H, 5.48%, N, 8.47%.

**1-Methyl-4-(1-methylpyrrol-2-yl)pyridinium Iodide (16).** The crude 4-(1-methylpyrrol-2-yl)pyridine was dissolved in 20 mL distilled acetone and  $CH_3I$  (3 mL, 50 mmol) was added. The solid product separated within several minutes. The crude solid (1.3 g, 73%) was recrystallized from water to yield yellow needles: mp  $202-203^\circ C$ ; UV (95% ethanol)  $\lambda = 374$  nm,  $\epsilon =$

28222;  $^1\text{H NMR}$  ( $\text{CD}_3\text{OD}$ ),  $\delta$  4.0 [s, 3H, N'-CH<sub>3</sub>], 4.25 [s, 3H, N-CH<sub>3</sub>], 6.35 [m, 1H, C<sub>4</sub>'] 7.1 [m, 1H, C<sub>3</sub>'], 7.2 [m, 1H, C<sub>5</sub>'], 8.0-8.1 [d, 2H, C<sub>3</sub> and C<sub>5</sub>], 8.6-8.65 [d, 2H, C<sub>2</sub> and C<sub>6</sub>]. Anal. Calc. for C<sub>11</sub>H<sub>13</sub>N<sub>2</sub>I: C, 44.02%; H, 4.37%; N, 9.33%. Found: C, 43.96%; H, 4.36%; N, 9.27%.

**4-(1-Methylpyrrol-2-yl)-1-propargylpyridinium bromide (18).**

The crude 4-(1-methylpyrrol-2-yl)pyridine (0.5 g, 3 mmol) was dissolved in 10 mL acetone and 1 mL (1.06g, 8.4 mmol) of propargyl bromide was added under nitrogen. The mixture was stirred for about 4 hours to yield a brown precipitate. The solid was separated by suction and recrystallized from MeOH/ether to give the yellow crystalline product (60% yield): mp 181°C (dec.); UV (MeOH)  $\lambda_{\text{max}}$  = 380 nm ( $\epsilon$  = 31180);  $^1\text{HNMR}$  ( $\text{D}_2\text{O}$ )  $\delta$  3.95 [s, 3H, N-CH<sub>3</sub>], 5.39 [s, 2H, -CH<sub>2</sub>-], 6.42 [m, 1H, C<sub>4</sub>'], 7.1-7.15 [m, 1H, C<sub>3</sub>'], 7.24 [s, 1h, C<sub>5</sub>'], 8.0-8.06 [d, 2H, C<sub>3</sub> and C<sub>5</sub>], 8.65-8.70 [d, 2H, C<sub>2</sub> and C<sub>6</sub>]. Anal. Calcd. for C<sub>13</sub>H<sub>13</sub>N<sub>2</sub>Br · 0.2 H<sub>2</sub>O: C, 55.61%; H, 4.81%; N, 9.98%. Found: C, 55.69%; H, 4.72%; N, 9.78%.

**N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine oxalate (19).** N-propargylpyridinium bromide (450 mg, 1.6 mmol) in 15 mL CH<sub>3</sub>OH was placed in a flask, NaBH<sub>4</sub> (123 mg, 3.2 mmol) was added pinch by pinch at 0°C, and then the solution was stirred at room temperature for one hour. The methanol was removed under vacuum and water (15 mL) was added to the residue. The product was extracted into (3 x 20 mL) of ether. The combined ether extracts were dried over MgSO<sub>4</sub> and filtered. Oxalic acid (140 mg, 1.6 mmol) in 5 mL ether was added to precipitate 200 mg (43%) of the product: mp 75-80°C; UV (methanol)  $\lambda_{\text{max}}$  = 261 nm ( $\epsilon$  = 10090);  $^1\text{HNMR}$  ( $\text{CD}_3\text{OD}$ )  $\delta$  2.75 [m, 2H, C<sub>3</sub>], 3.4-3.46 [t, 2H, C<sub>2</sub>], 3.68 [s, 3H, N-CH<sub>3</sub>], 3.83-3.88



[m, 2H, C<sub>6</sub>], 4.04 [d, 2H, -CH<sub>2</sub>-], 5.75 [m, 1H, C<sub>5</sub>], 6.0-6.03 [m, 1H, C<sub>4</sub>'], 6.1-6.14 [m, 1H, C<sub>3</sub>'], 6.64-6.67 [t, 1H, C<sub>5</sub>']. Anal. Calcd. for C<sub>15</sub>H<sub>18</sub>N<sub>2</sub>O<sub>4</sub> · 0.3 H<sub>2</sub>O: C, 60.92%; H, 6.34%; N, 9.47%. Found: C, 60.86%; H, 6.28%; N, 9.30%.

## Chapter 3

### Biology

#### 3.1 Enzyme studies

##### 3.1.1 Introduction

It is well established that MPTP (**1**) is bioactivated by MAO-B in the brain to form the dihydropyridinium species (MPDP<sup>+</sup>, **3**) with  $K_m = 330 \mu\text{M}$  and  $V_{\text{max}} = 260 \text{ nmol/min/U enzyme}$ .<sup>34</sup> The dihydropyridinium species (MPDP<sup>+</sup>, **3**) is chemically unstable and undergoes further oxidation to form the pyridinium species (MPP<sup>+</sup>, **4**). This pyridinium species is the ultimate neurotoxin which inhibits mitochondrial respiration leading to cell death. Bioactivation of tetrahydropyridine derivatives by MAO-B, therefore, is a requirement for MPTP-type neurotoxins. Since 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP, **6**) is an analog of MPTP, we undertook experiments to compare its MAO-B substrate properties with those of MPTP.

Several research groups have reported that TMMP (**6**) is a substrate of MAO-B and presumed that the metabolite derived from TMMP (**6**) is the dihydropyridinium species MMDP<sup>+</sup> (**11**).<sup>59-61</sup> No definitive kinetic data have been reported because of the lack of the standard dihydropyridinium compound, the expected metabolite. In the present work, we confirmed that the

metabolite of TMMP (6) formed by the oxidative catalysis of MAO-B is the dihydropyridinium species MMDP<sup>+</sup> (11) and established the enzyme substrate kinetic parameters of TMMP (6) with purified MAO-B by using a spectrophotometric based assay and by an HPLC diode array based assay.

As stated in the introduction section, MAO is localized in the outer mitochondrial membrane and exists in two forms: A and B. Although these isozymes are similar, certain selectivities for substrates and inhibitors have been established. It has been shown that MAO-B inhibitors (pargyline and deprenyl) but not the MAO-A inhibitors clorgyline protect against MPTP (1) toxicity.<sup>26,28,41</sup> Some inactivation studies have been carried out. It was found that MPTP (1) behaves as a time-dependent, irreversible inhibitor as well as a substrate of MAO-B.<sup>34,63,65</sup> In order to develop structure activity relationships, some N-1 and C-4 substituted analogs of MPTP (1) have been made and tested for their substrate/inhibitor properties. One derivative of MPTP (1) is N-propargyl-4-phenyl-1,2,3,6-tetrahydropyridine which was synthesized and was shown to be an inactivator of MAO-B with  $K_I = 172 \mu\text{M}$  and  $k_{\text{inact}} = 0.24 \text{ min}^{-1}$ .<sup>66</sup> Therefore, TMMP (6) and its N-propargyl analog 19 have been examined for their MAO-B inactivation properties.

### **3.1.2 Results and discussion**

#### **1-Methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP, 6) as an MAO-B substrate**

TMMP (6) was incubated with purified MAO-B in a sodium phosphate buffer (pH = 7.4). The incubation mixture was placed in a spectrometer which

was maintained at 37°C. A compound absorbing at 420 nm (which is the  $\lambda_{\text{max}}$  of the synthetic dihydropyridinium MMDP<sup>+</sup>) formed after several minutes and this compound was stable during the incubation. To confirm that the MAO-B catalyzed metabolite of TMMP (6) is the dihydropyridinium species 11, synthetic 11 and the metabolite derived from the incubation were analyzed by HPLC with a diode array detector using an SCX column. The results showed identical spectra and chromatograms for the two samples. This proved that the metabolite of TMMP (6) formed via MAO-B was the dihydropyridinium species MMDP<sup>+</sup> (11). The stability of 11 was not expected in view of the reported instability of MPDP<sup>+</sup> in the presence of MAO-B.<sup>35-37</sup>

Kinetic studies were carried out with MAO-B using various concentrations of TMMP (6). The rate of product formation was monitored at 420 nm. A series of initial rate (absorbance/min) values were obtained and were expressed as nmol/min/U enzyme by using the dihydropyridinium extinction coefficient ( $\epsilon = 25000$ ) for MMDP<sup>+</sup>. The results are shown in Table 1. A double reciprocal plot was made by using  $1/V$  vs.  $1/[S]$  (Fig. 21). It was found from the plot that  $K_m = 201 \mu\text{M}$  and  $V_{\text{max}} = 250 \text{ nmol/min/U enzyme}$  which are similar to those of MPTP ( $K_m = 330 \mu\text{M}$  and  $V_{\text{max}} = 260 \text{ nmol/min/U enzyme}$ ).

Table 1: The initial rates for the conversion of TMMP to MMDP<sup>+</sup> in MAO-B incubation mixture

Experiment	1/[substrate] mM <sup>-1</sup>	rate absorbance/min	rate nmol/min/U
A	8	0.1205	96.4
B	6	0.1474	117.92
C	4	0.1779	142.32
D	2	0.2115	169.2
E	1	0.2879	230.32

### MAO-B Catalyzed Oxidation of TMMP

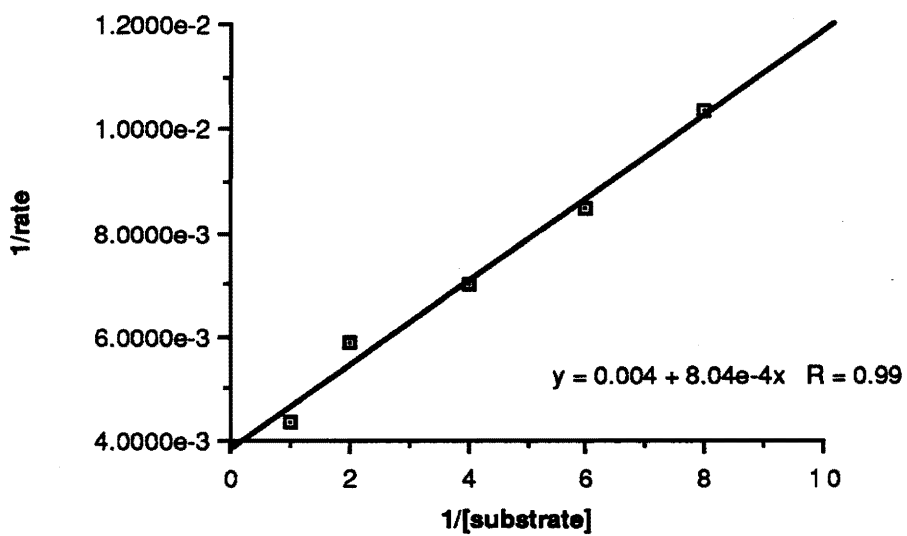


Figure 21: Double reciprocal plot for the MAO-B catalyzed oxidation of TMMP

## **N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (19) as an MAO-B inactivator**

In the presence of different concentrations of **19** the remaining enzyme activity (MAO-B) was determined at various incubation times by measuring the rate of oxidation of 5 mM MPTP to MPDP<sup>+</sup> (**3**). The formation of the dihydropyridinium species was monitored at 345 nm. A series of initial rate values was obtained and the percentage of the enzyme activity remaining was calculated. A plot was made by using  $\ln$  (% enzyme activity remaining) vs time (shown in Fig. 22) and a series of  $k_{obs}$  values was obtained from the slopes. A double-reciprocal plot (Fig. 23) was made by using  $1/k_{obs}$  vs.  $1/[I]$ ,  $K_I$  and  $k_{inact}$  were determined and were found to be 831  $\mu\text{M}$  and 1.11  $\text{min}^{-1}$  respectively, from the analysis of the double reciprocal plot.

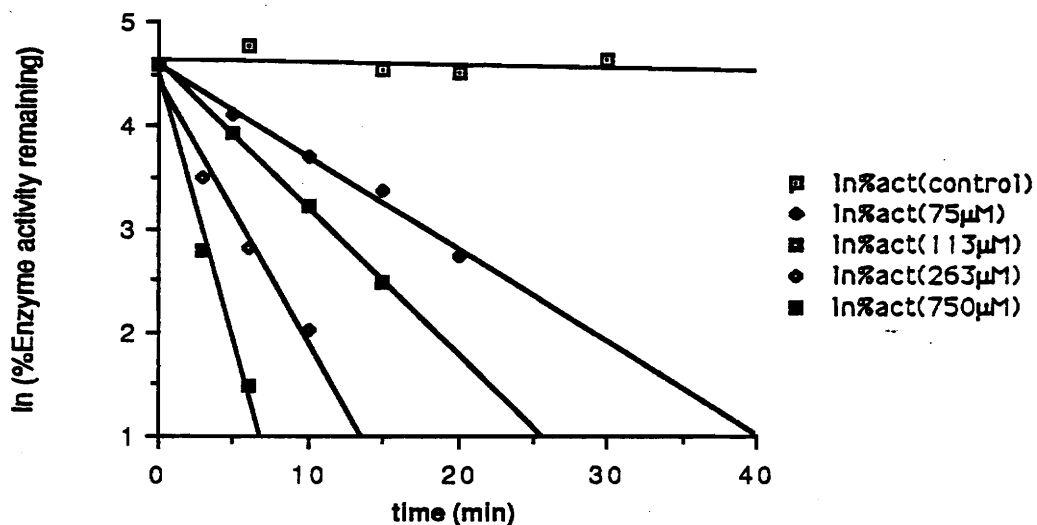


Figure 22: ln (% enzyme activity remaining) vs. time

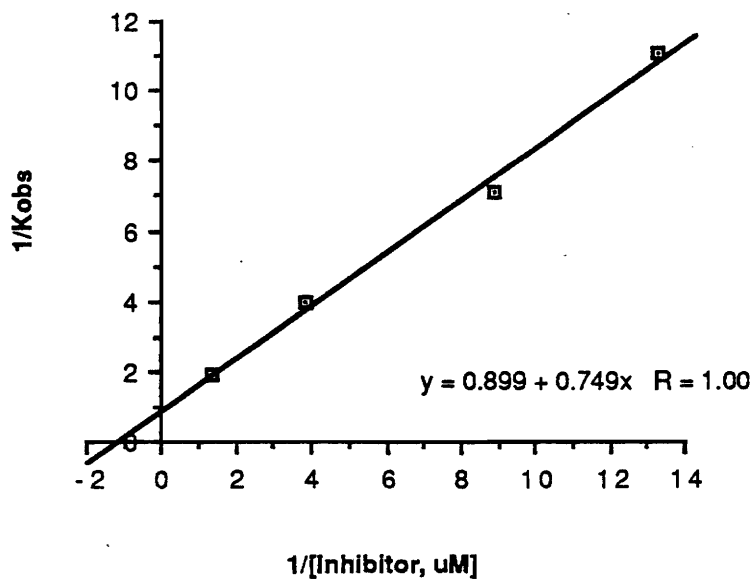


Figure 23: The double reciprocal plot of N-propargyl(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine as an MAO-B inactivator



## 3.2 Metabolic Studies

### 3.2.1: Introduction

MPTP (1) is metabolized in the presence of monoamine oxidase B (MAO-B) to form the dihydropyridinium species (MPDP<sup>+</sup> 3).<sup>28</sup> This intermediate undergoes further oxidation to the chemically stable product MPP<sup>+</sup> (4).<sup>32</sup> Although the MAO-B catalyzed oxidation of MPTP (1) is now well documented, relatively little is known about what is actually responsible for the oxidation of MPDP<sup>+</sup> (3) to MPP<sup>+</sup> (4) in the brain. Since MPP<sup>+</sup> is the ultimate neurotoxin, what causes the conversion of the dihydropyridinium to the pyridinium is especially important.

It has been reported that MAO-B can catalyze the oxidation of MPDP<sup>+</sup> to MPP<sup>+</sup>, although this reaction can also occur non-enzymatically.<sup>35-38</sup> It was reported by Wu et al. that 5 mM MPDP<sup>+</sup> (3) in sodium phosphate buffer (pH = 7.6) at 37°C undergoes quantitative conversion via disproportionation and autoxidation to yield a 3 to 1 mixture of MPP<sup>+</sup> (4) and MPTP (1) with a half time of product formation of about 25 minutes. At 50 μM MPDP<sup>+</sup> (3) only autoxidation occurs yielding MPP<sup>+</sup> (4) with a half time of about 240 minutes. Kinetic studies on the metabolism of MPDP<sup>+</sup> (3) in mouse brain incubation mixtures were conducted at a substrate concentration of 50 μM in order to minimize the contribution of spontaneous chemical transformations. The results showed that MPDP<sup>+</sup> (3) was converted exclusively to MPP<sup>+</sup> (4) in these mouse brain incubation mixtures. Whole brain homogenates and all brain which contained membrane significantly increased the rate of this oxidation (half time

ca. 60 min.) compared to incubations in buffer (half time 240 min).<sup>37</sup>

From our studies it is known that TMMP (6) can be converted to the dihydropyridinium species (MMDP<sup>+</sup>, 11) via MAO-B, but the metabolic fate of MMDP<sup>+</sup> (11) was unknown. Since MMDP<sup>+</sup> (11) is chemically stable as we observed in our enzyme studies, it gave us a chance to study the metabolism of this compound which is difficult to do with MPDP<sup>+</sup> (3) because of its chemical instability. In our experiment an HPLC system was utilized to monitor the metabolism of TMMP and MMDP<sup>+</sup>. We examined the fate of MMDP<sup>+</sup> in buffer and in the presence of purified MAO-B since MPDP<sup>+</sup> (3) was a MAO-B substrate. The metabolic fate of MMDP<sup>+</sup> with mouse brain homogenate also was examined in order to examine whether an enzyme system catalyzes this conversion. In-vivo studies on the metabolic fate of TMMP (6) were also carried out following i.p. administration to C57 BL/6J mice. The brain samples were analyzed by HPLC with diode array detection.

### **3.2.2: Results and discussion**

#### **HPLC assay**

The metabolic studies initially were monitored on a Beckman DU 50 UV-VIS spectrophotometer, but because of the overlap of MMDP<sup>+</sup> (11) and MMP<sup>+</sup> we could not obtain clear data. Therefore, an HPLC assay was developed on a Beckman 114 M chromatography system employing an HP Model 1040 A diode array detector using an SCX (10  $\mu$ m, 4.6 mm X 25 cm) column. The mobile phase was 75.3% H<sub>2</sub>O, 23.7% acetonitrile, 0.8% acetic acid, and 0.12% TEA (triethylamine) with the pH adjusted to 4.0. The flow rate was 1 mL/min. This

mobile system can successfully separate synthetic tetrahydropyridine (TMMP, **6**), the dihydropyridinium (MMDP<sup>+</sup>, **11**) and the pyridinium (MMP<sup>+</sup>, **16**) with retention times of 8.0 min., 14.0 min. and 16.5 min, respectively as can be seen in Fig. 24. The calibration curve of the concentrations of TMMP, MMDP<sup>+</sup> and MMP<sup>+</sup> vs. the corresponding peak area integrations of the HPLC chromatograms were made by using synthetic TMMP, MMDP<sup>+</sup> and MMP<sup>+</sup> as shown in Figs. 25, 26 and 27. Utilizing this HPLC assay, in-vivo and in-vitro metabolic studies of TMMP and MMDP<sup>+</sup> were performed and a series concentration of metabolites vs. time plots were made as will be discussed later.

#### **In-vitro studies of MMDP<sup>+</sup> (11)**

Since MMDP<sup>+</sup> (**11**) was chemically stable as in enzyme studies previously discussed, it provides us an opportunity to study the mechanism of the oxidation of this dihydropyridinium to the pyridinium species. To do so, we needed to determine the stability of this dihydropyridinium species in sodium phosphate buffer (pH = 7.4) and examine the substrate properties of **11** with MAO-B, as well as investigate the behavior of this compound with mouse brain homogenates. The results of these studies are summarized below.

The stability studies of MMDP<sup>+</sup> (**11**) were carried out by incubating 1 mM MMDP<sup>+</sup> (**11**) in 100 mM sodium phosphate buffer (pH = 7.4) at 37°C; the reaction was monitored by UV. It was found that only one compound was formed which has a  $\lambda_{\max}$  at 366 nm (which is also the  $\lambda_{\max}$  of the synthetic pyridinium MMP<sup>+</sup>, **16**). To confirm that this product was the pyridinium species (MMP<sup>+</sup>), synthetic MMP<sup>+</sup> and the metabolite derived from these incubations were analyzed by HPLC as described above. The results showed that the

spectra and the chromatograms of the two samples are identical. It was also found that at high concentrations of MMDP<sup>+</sup>, disproportionation do not occur within our HPLC detection limits, since no TMMP was detected from the incubation of 1 mM MMDP<sup>+</sup> (Fig. 28).

The autoxidation rate of MMDP<sup>+</sup> to MMP<sup>+</sup> was determined by incubating 100 μM MMDP<sup>+</sup> in 100 mM sodium phosphate buffer (pH = 7.4) at 37°C. An aliquot was removed from the incubation mixture at various time points and was analyzed by HPLC. A plot was made by using concentrations of MMDP<sup>+</sup> and MMP<sup>+</sup> vs time (Fig. 29). From Fig. 29, it was found that during the first 7 hours no reaction occurred and there is a lag time. After that lag time the reaction commenced and proceeded smoothly. This may be due to a radical based mechanism, but this is unknown at this time. Also, It was found that almost 100% of the MMDP<sup>+</sup> converted to MMP<sup>+</sup>.

MMDP<sup>+</sup> (100 μM) or TMMP (100 μM) was incubated with mouse whole brain homogenates in presence and absence of pargyline at 37°C. An aliquot was removed from the incubation mixture and analyzed. The concentration of metabolites vs time are shown in Figs. 30, 31, 32 and 33. As shown in Fig. 30, TMMP was converted to MMDP<sup>+</sup> very rapidly (in one hour), but MMDP<sup>+</sup> was oxidized to MMP<sup>+</sup> as a relatively slower rate. From Fig. 30 it can also be seen that only about 50% of the TMMP was converted to MMP<sup>+</sup>, suggesting the possibility that other metabolites may be formed perhaps via MMDP<sup>+</sup> as no TMMP remained after 1 hour incubation. As shown in Fig. 31, only TMMP was detectable when pargyline was added to mouse brain homogenate to inactivate MAO-B. In this case, TMMP decreases by only 20% after 15 hours incubation.

Fig. 32 and Fig. 33 shown the results of the incubation of MMDP<sup>+</sup> with mouse whole brain homogenates in presence and absence of pargyline, the results are similar to each other.

MMDP<sup>+</sup> or TMMP was incubated with purified MAO-B with or without pargyline inactivation at 37°C. Aliquots were removed from the incubation mixtures and analyzed using HPLC assay. Plots were made by graphing concentrations of metabolites vs time (Fig. 34, 35, 36, 37). Fig. 34 shows the formation of MMDP<sup>+</sup> and MMP<sup>+</sup> from TMMP incubated with MAO-B and Fig. 35 shows the results of incubating TMMP with MAO-B after pargyline inactivation. Fig. 36 and Fig. 37 show the results of the incubation of MMDP<sup>+</sup> with MAO-B in presence and absence of pargyline, the results are similar to each other.

The half time of the formation of MMP<sup>+</sup> from MMDP<sup>+</sup> or TMMP in various biological matrices was investigated and the results were summarized in Table 2. As shown in Table 2 and in Figs. 29 - 37, it was found that TMMP can be oxidized either by MAO-B or mice brain homogenate to form MMDP<sup>+</sup> very rapidly and 30 μM pargyline can completely block this effect. These confirmed that MAO-B catalyzed the oxidation reaction from TMMP to MMDP<sup>+</sup>. The formation of MMP<sup>+</sup> from TMMP is about 50%, this may due to the fact that some other metabolites were formed during the incubation. The reaction from MMDP<sup>+</sup> to form MMP<sup>+</sup> was much slower and was a 100% conversion. Both mouse brain homogenates and pure MAO-B significantly increase the rate of the latter oxidation reaction (compared with autoxidation in buffer), however pargyline does not block this effect. Consequently, MMDP<sup>+</sup> is not an MAO-B substrate and MAO-B is not responsible for this second oxidation reaction. The

catalyst of the second reaction may be due to the influence of a trace metal (in the case of purified MAO-B) or another enzyme system (in the case of the whole brain homogenate).

Table 2: Half times for the formation of MMP+ from MMDP+ or TMMP under various conditions

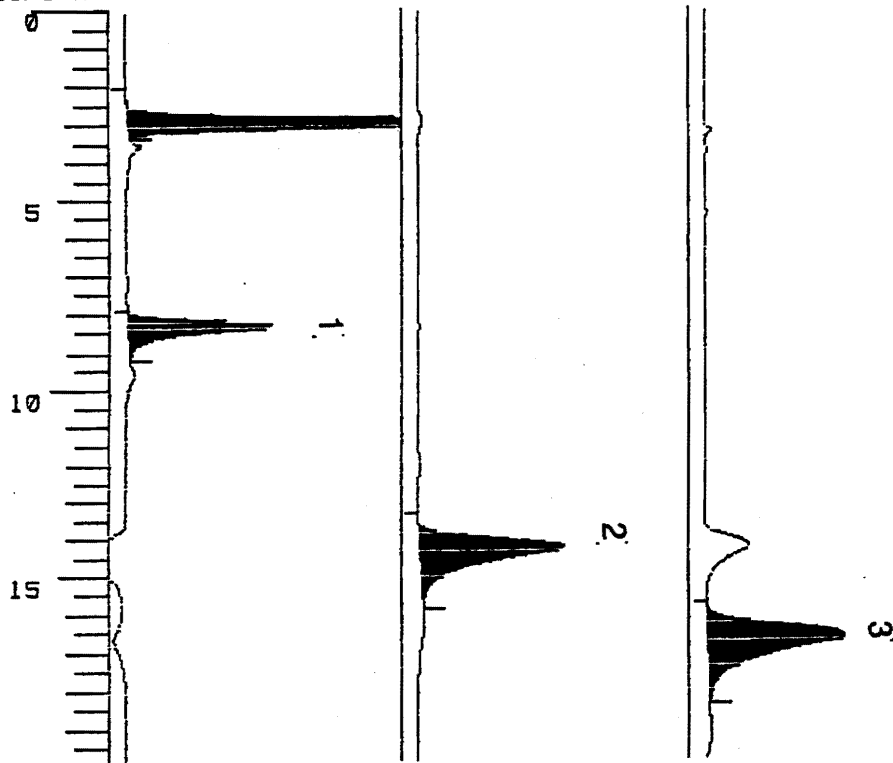
Substrate	Medium	Half time for the formation of MMP+(hours)
MMDP+	buffer only	25
TMMP	homogenate* (5 mg protein/mL)	8
TMMP	homogenate (5 mg protein/mL) pretreated with 30 $\mu$ M pargyline	ND*
MMDP+	homogenate (5 mg protein/mL)	8
MMDP+	homogenate (5 mg protein/mL) pretreated with 30 $\mu$ M pargyline	8
TMMP	MAO-B (0.05 U/mL)	11
TMMP	MAO-B (0.05 U/mL) pretreated with 30 $\mu$ M pargyline	ND
MMDP+	MAO-B (0.05 U/mL)	11
MMDP+	MAO-B (0.05 U/mL) pretreated with 30 $\mu$ M pargyline	11

\* ND means not detectable by the HPLC assay

\* Homogenate refers to whole mouse brain

Offline plot from DPU memory

Signal	A	B	C
S wl,bw	266,25	366,40	420,60
Range	40	50	10
Zero %	5	5	5



(1) TMMP; (2) MMP+; (3) MMDP+

Figure 24: HPLC tracings of the synthetic TMMP, MMDP+ and MMP+



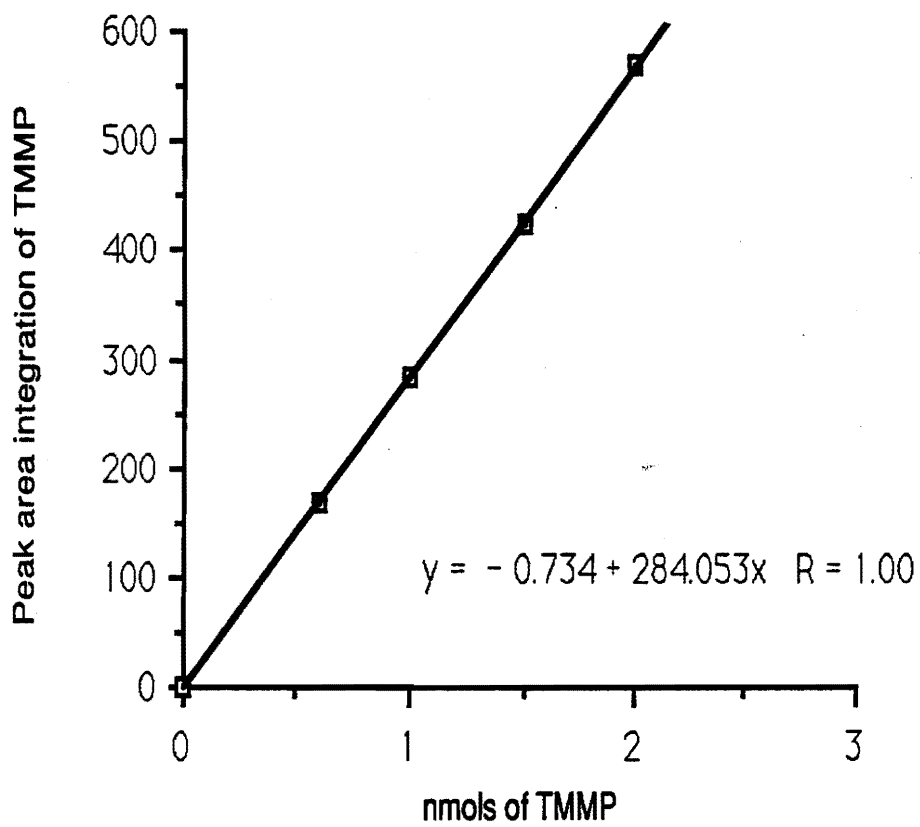


Figure 25: Calibration curve of TMMP

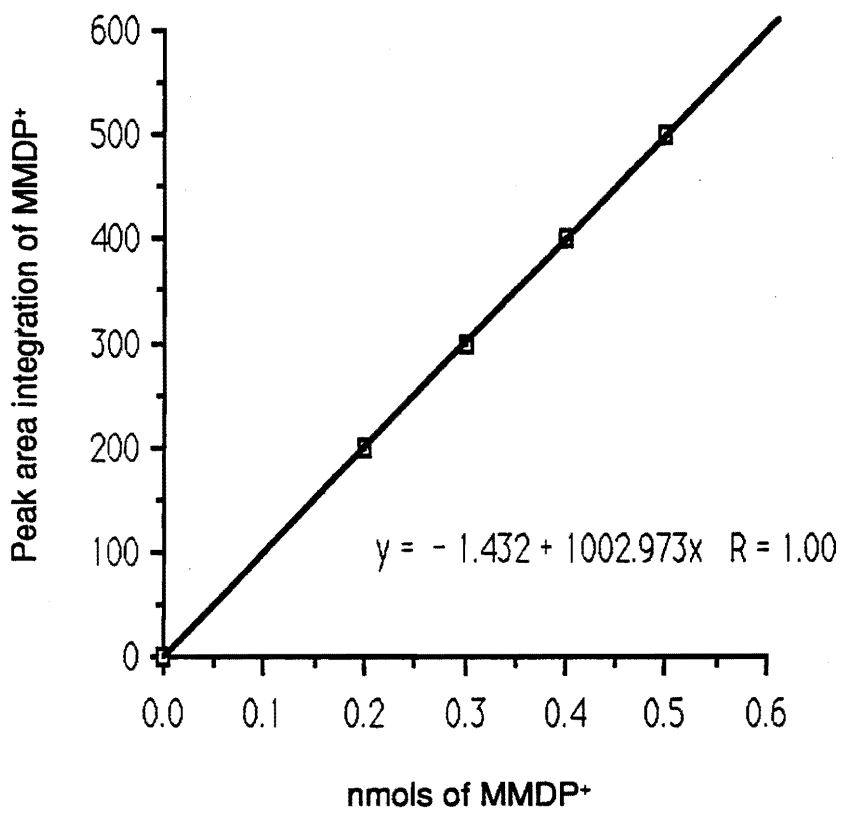


Figure 26: Calibration curve of MMDP+

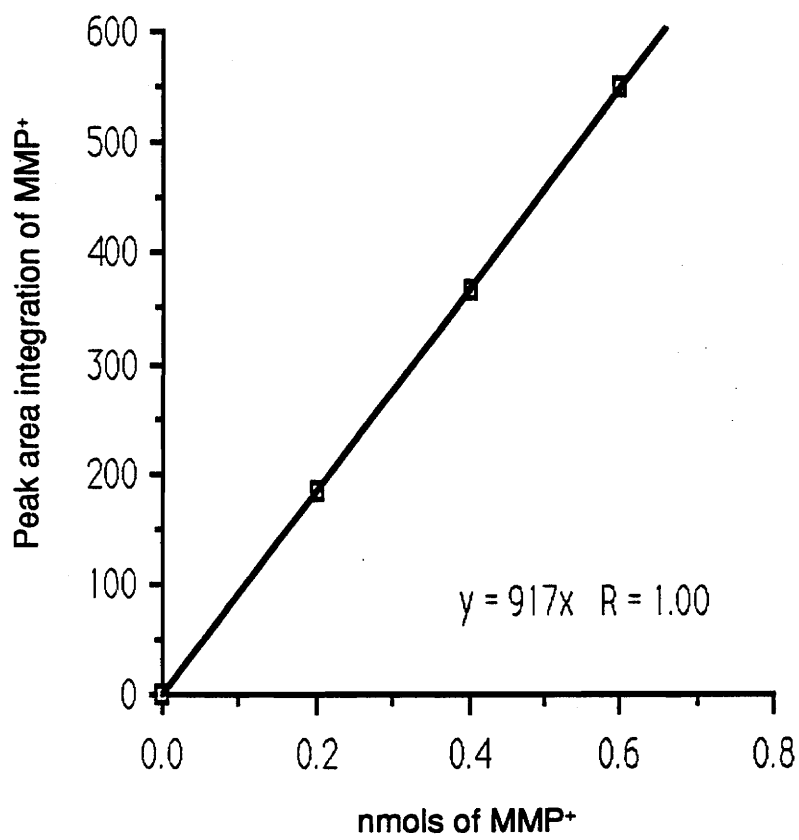


Figure 27: Calibration curve of MMP+

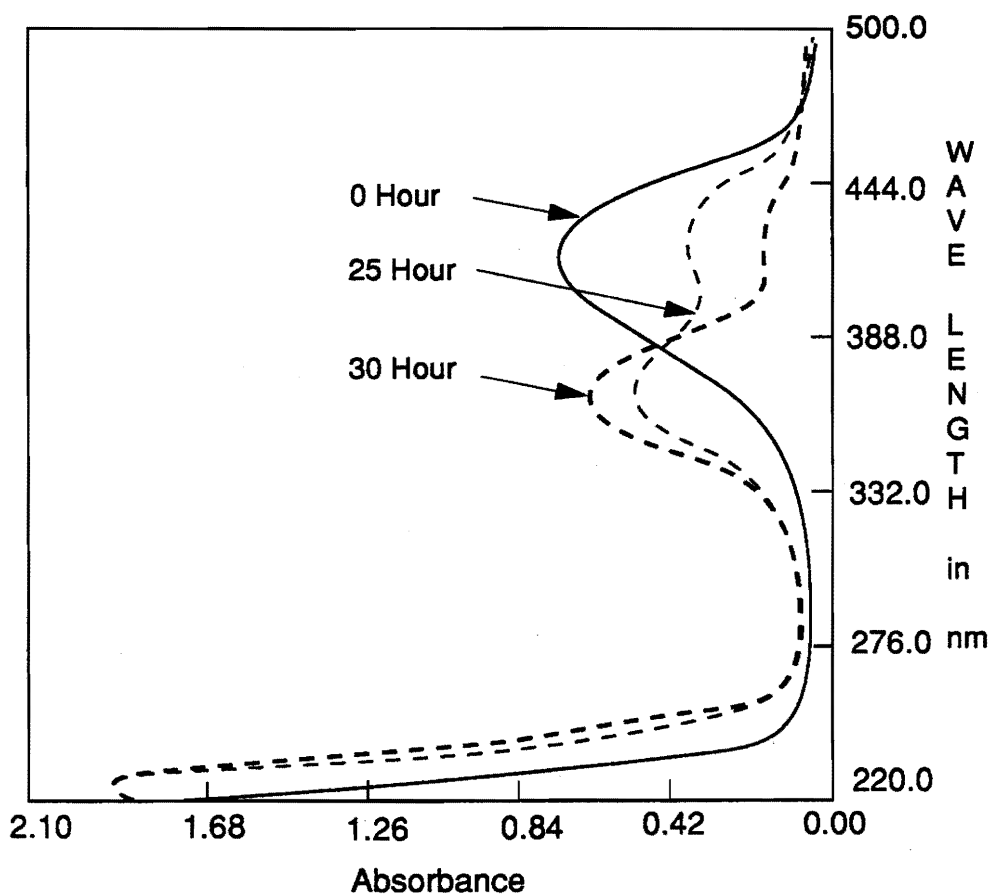


Figure 28: UV Spectra of the autoxidation of 1 mM MMDP<sup>+</sup> to form MMP<sup>+</sup> in 100 mM sodium phosphate buffer (pH = 7.4) at 37°C

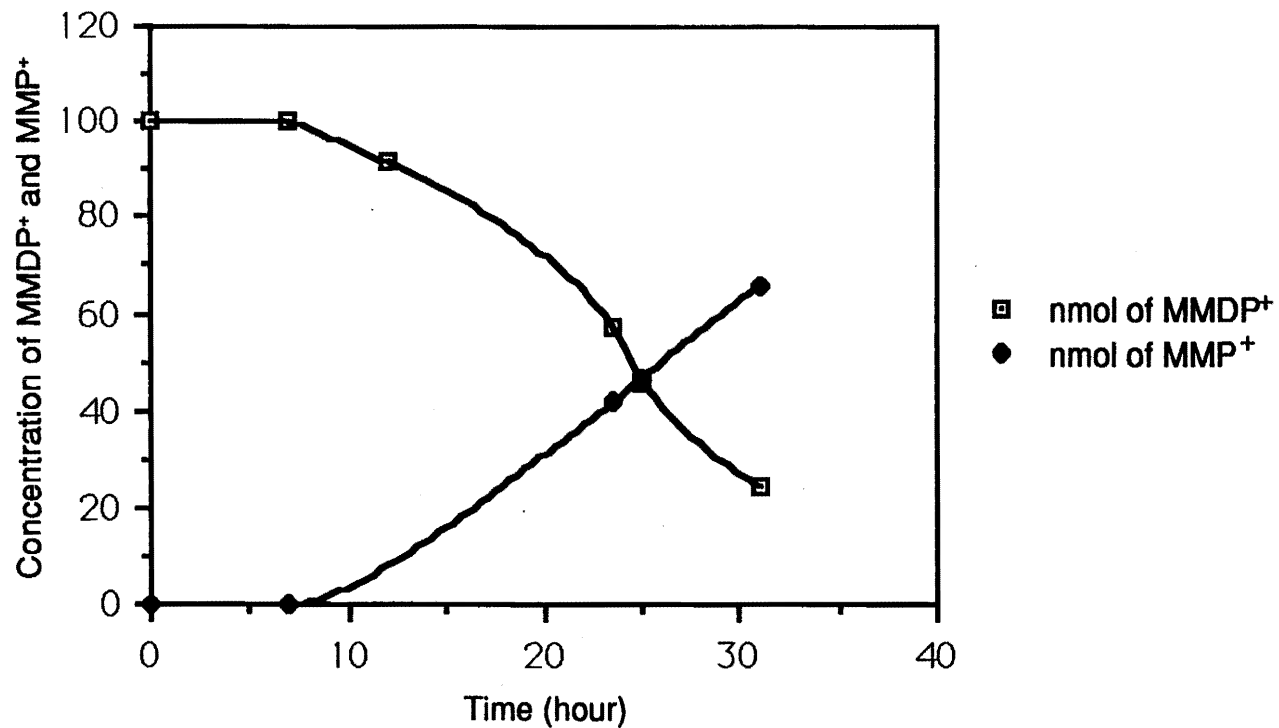


Figure 29: Autoxidation of 100  $\mu$ M MMDP<sup>+</sup> in 100 mM sodium phosphate buffer (pH = 7.4) at 37°C as determined by HPLC analysis

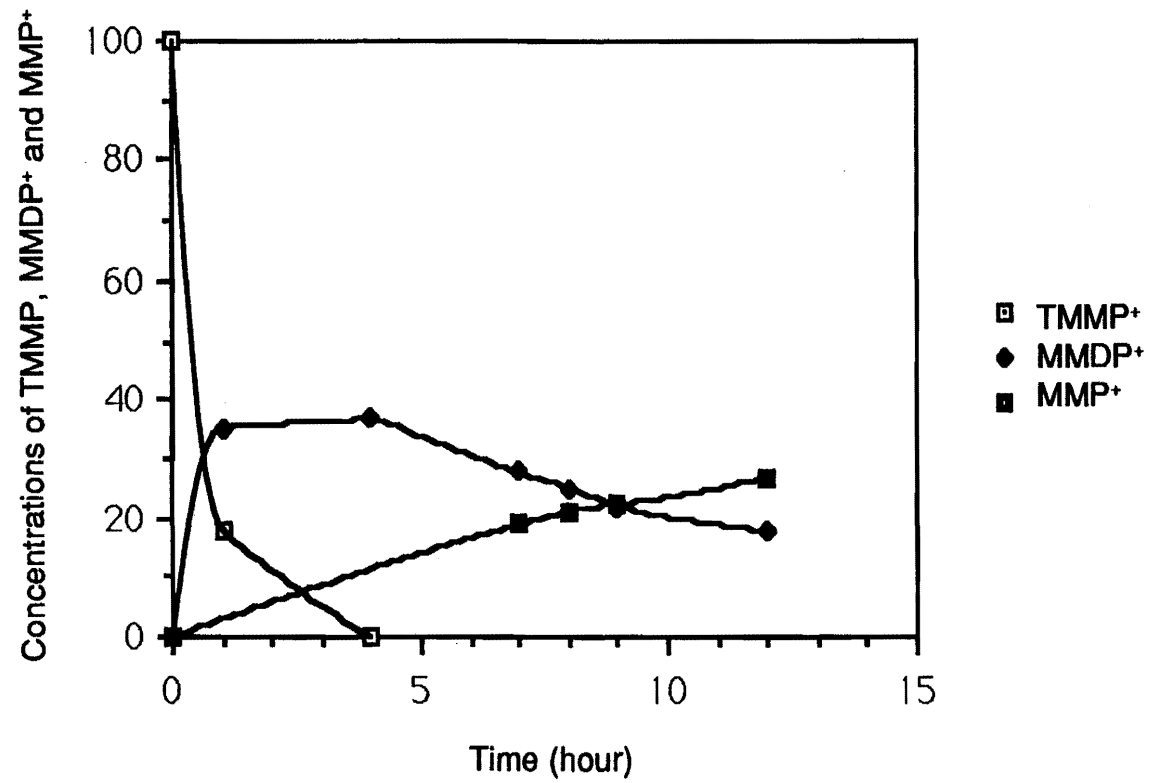


Figure 30: Metabolism of 100  $\mu$ M TMMP in mouse whole brain homogenates

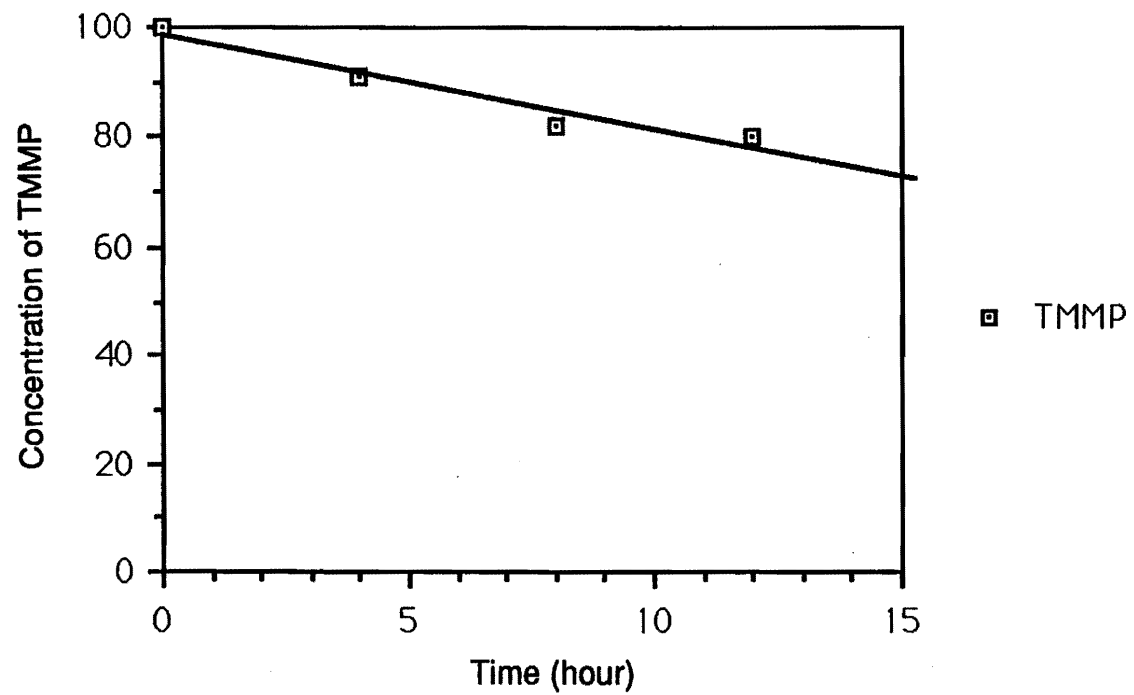


Figure 31: Metabolism of 100  $\mu$ M TMMP with mouse whole brain homogenates pre-treated with pargyline

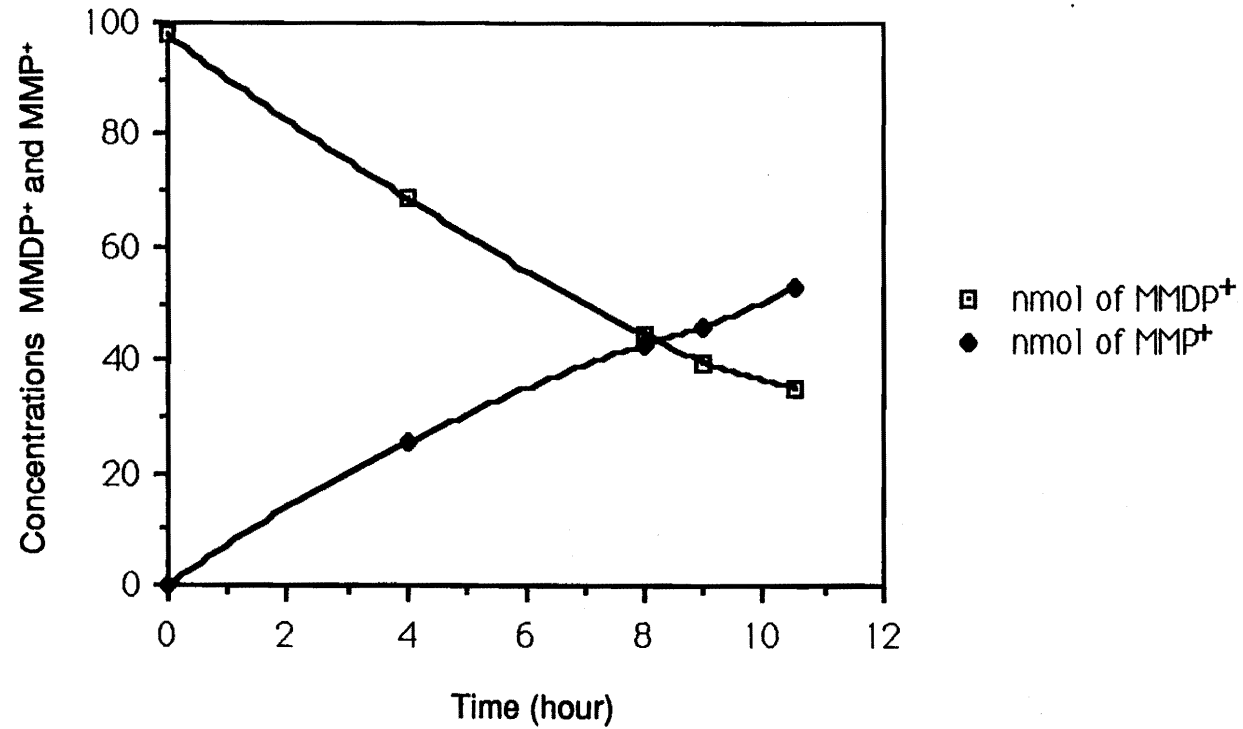


Figure 32: Metabolism of 100  $\mu$ M MMDP<sup>+</sup> with mouse whole brain homogenates



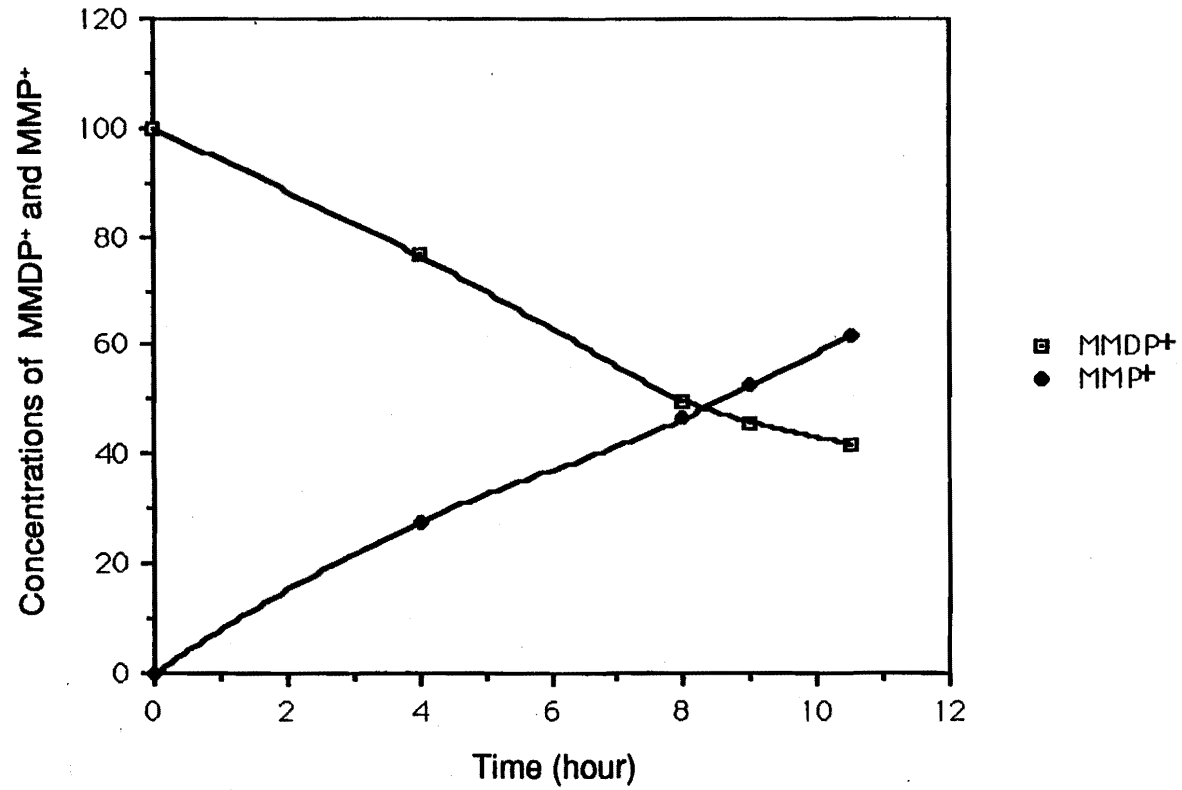


Figure 33: Metabolism of 100  $\mu$ M MMDP+ with mouse whole brain homogenates pre-treated with pargyline

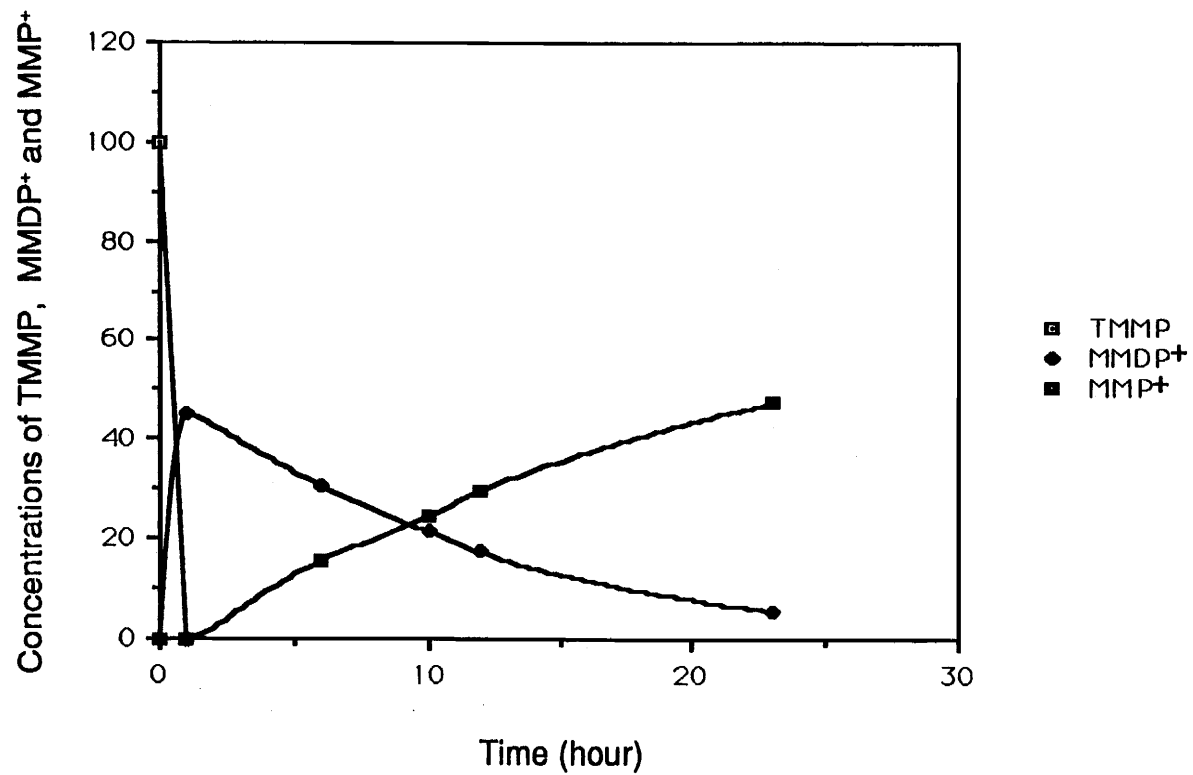


Figure 34: Metabolism of 100  $\mu$ M TMMP with purified MAO-B

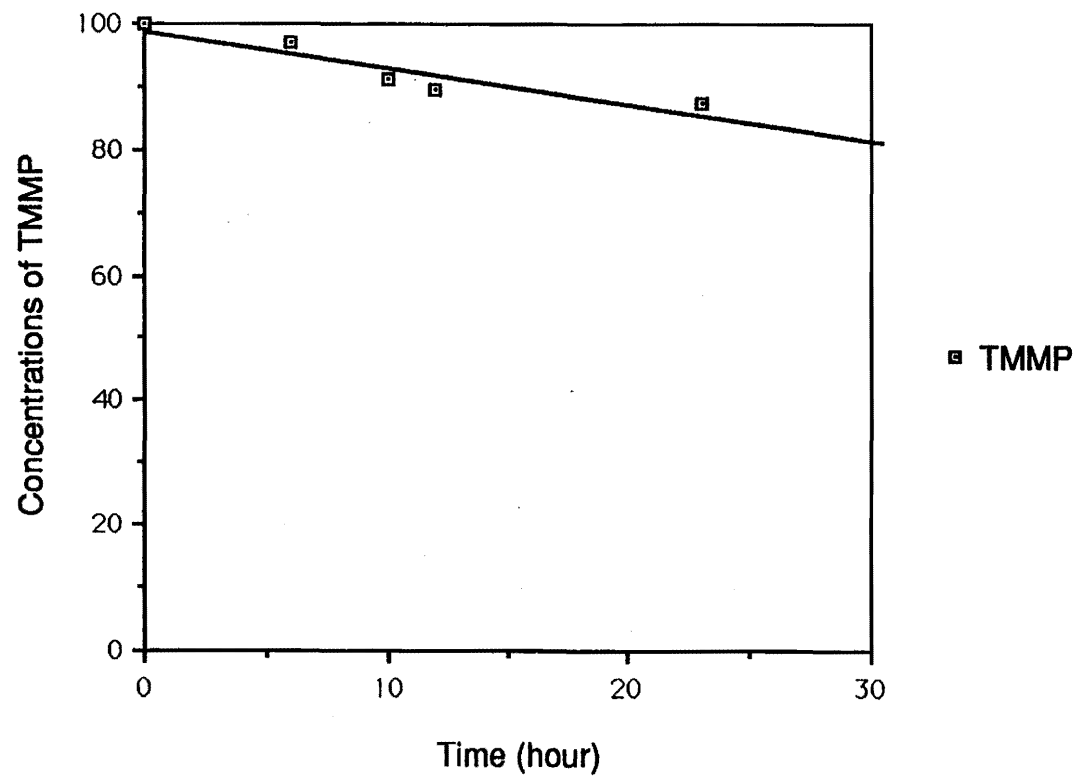


Figure 35: Metabolism of 100  $\mu$ M TMMP with MAO-B pretreated with pargyline

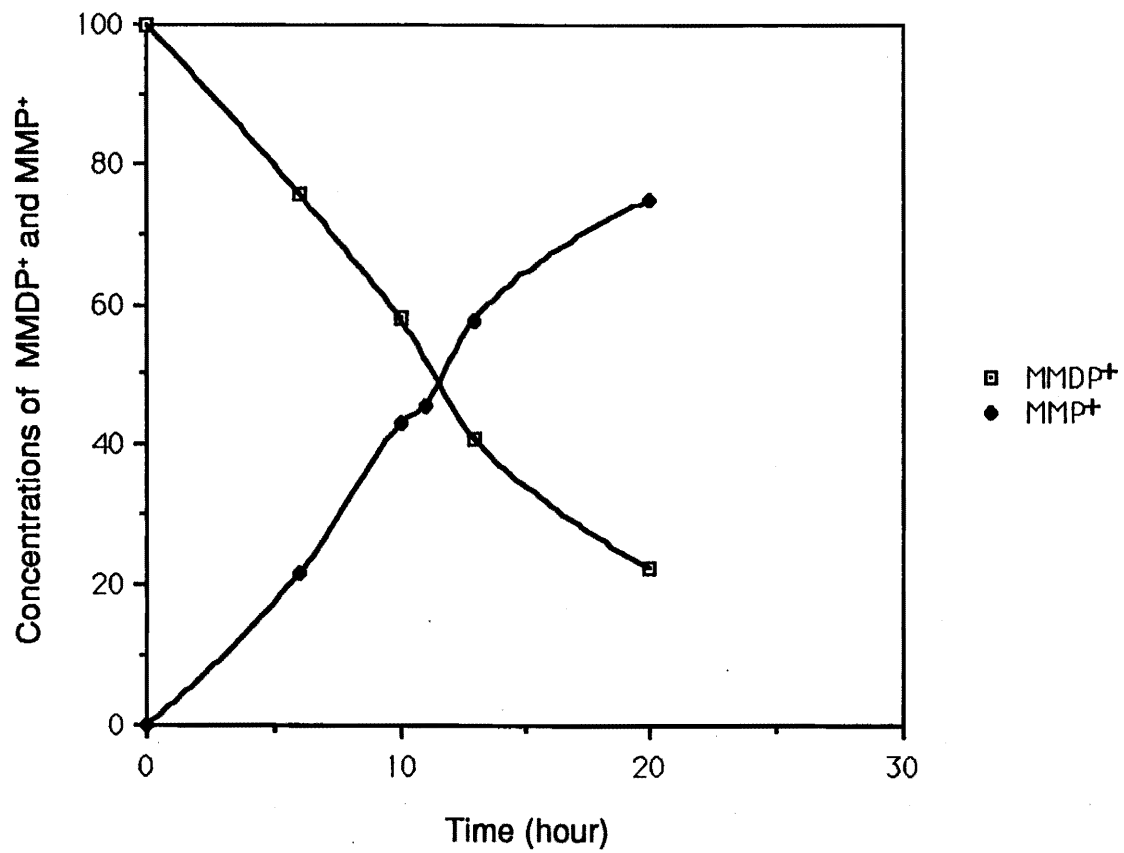


Figure 36: Metabolism of 100  $\mu$ M MMDP<sup>+</sup> with purified MAO-B

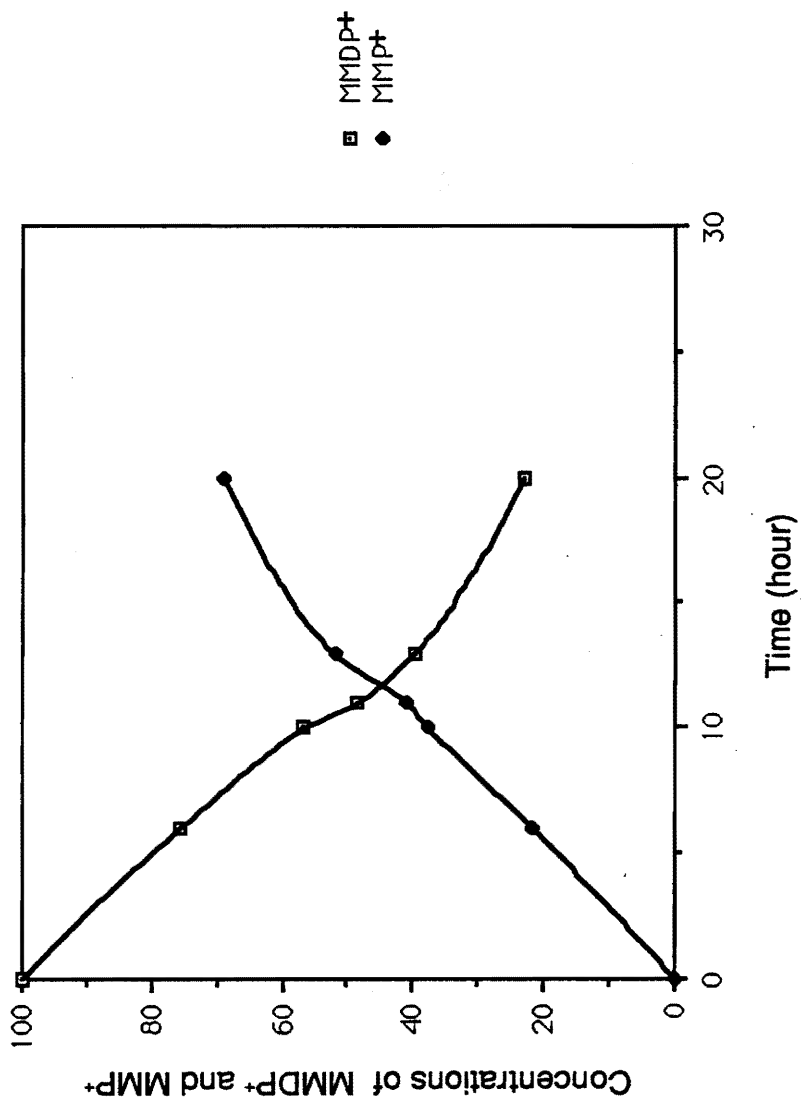


Figure 37: Metabolism of 100  $\mu$ M MMDP+ with MAO-B pretreated with pargyline

### **In-vivo studies of TMMP (6)**

From in-vitro metabolic studies of TMMP and MMDP<sup>+</sup>, it was found that the oxidation reaction of MMDP<sup>+</sup> to MMP<sup>+</sup> was slow and likely enzyme catalyzed, but not by MAO-B. The following in-vivo studies were carried out in order to investigate the mechanism of this conversion.

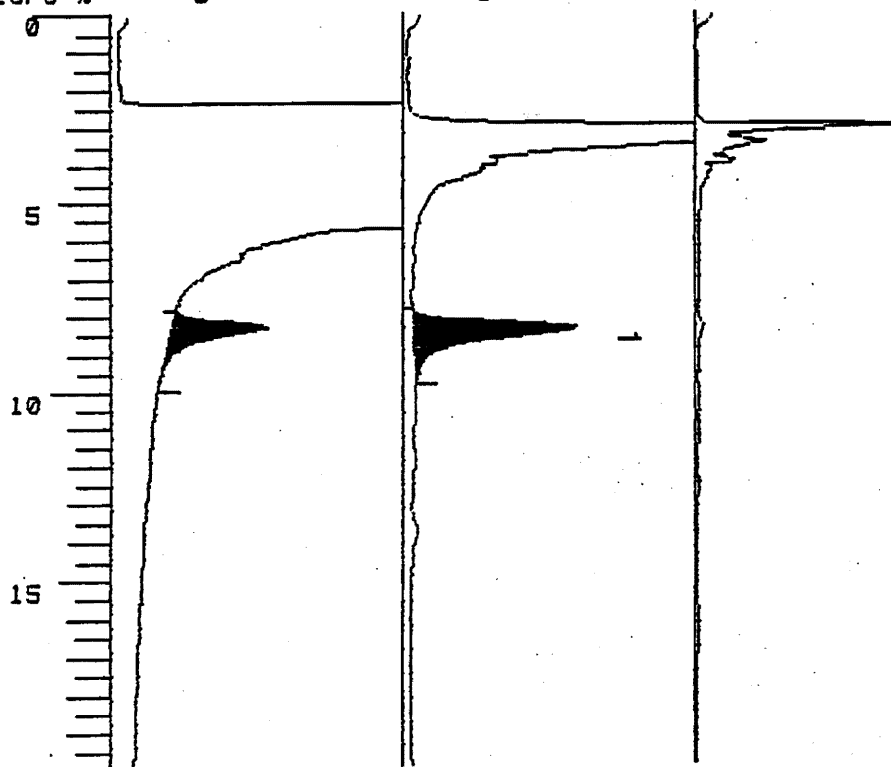
C57 BL/6J mice were injected with one dose of TMMP (15 mg/kg). At different time points after the injection, the mice were killed by cervical dislocation and the mice brains were isolated, 4-(1-methylpyrrol-2-yl)pyridine was added as a internal standard, and the brains then homogenized and extracted with methanol. One group of mice served as control and received no injections. The tissues were extracted and analyzed using the HPLC assay. The concentrations of MMDP<sup>+</sup> and MMP<sup>+</sup> in the brain tissue were estimated by comparison with the calibration curve as described. A plot was made by using concentrations of MMDP<sup>+</sup> and MMP<sup>+</sup> in the brain tissue vs time after injection (Fig.42).

From HPLC chromatograms of the metabolites (Fig. 38, control; Fig. 39, 30 min.; Fig. 40, 1 hour and Fig. 41, 2 hours), it was found that the biological background and the metabolites of interest were well separated. The peak occurring at a retention time of 9 min. with  $\lambda_{\max} = 256$  nm is biological background; not TMMP, because the control sample displays the same eluding identical peak. It also was found that TMMP (6) was converted to the dihydropyridinium species very rapidly and the parent compound could not be detected because of the fast conversion. The dihydropyridinium species

(MMDP<sup>+</sup>) reached a maximum concentration at 30 minutes and then was converted to the pyridinium species much faster than observed in the in-vitro studies. The pyridinium species reached a maximum concentration at 1 hour and then decreased slowly; being still detectable in the brain sample after 6 hours following i.p. injection. This fast conversion of MMDP<sup>+</sup> to MMP<sup>+</sup> in mouse brain may imply that some presently undetermined enzyme system in the mouse brain may catalyze this reaction. Again it was found that in the conversion of MMDP<sup>+</sup> to MMP<sup>+</sup>, only 50% of the possible MMP<sup>+</sup> was formed which is the same result we observed in the in-vitro studies. This may suggest the possibility that other metabolites are formed from MMDP<sup>+</sup> or that MMDP<sup>+</sup> was wash out from the mouse brain.

Offline plot from DPU memory

Signal	A	B	C
S wl,bw	266,25	366,40	420,60
Range	20	10	10
Zero %	5	5	5



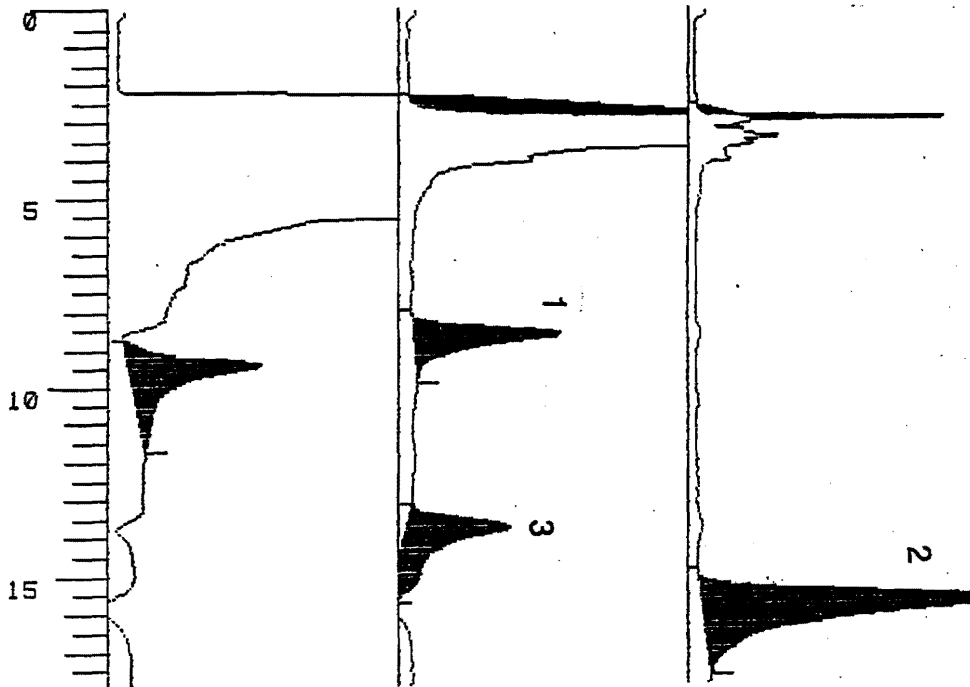
(1) 4-(Methylpyrrol-2-yl)pyridine (internal standard)

Figure 38: An HPLC chromatogram from C57 BL/6J mice receiving no injections



Offline plot from DPU memory

Signal	A	B	C
S wl,bw	266,25	366,40	420,50
Range	20	10	10
Zero %	5	5	5

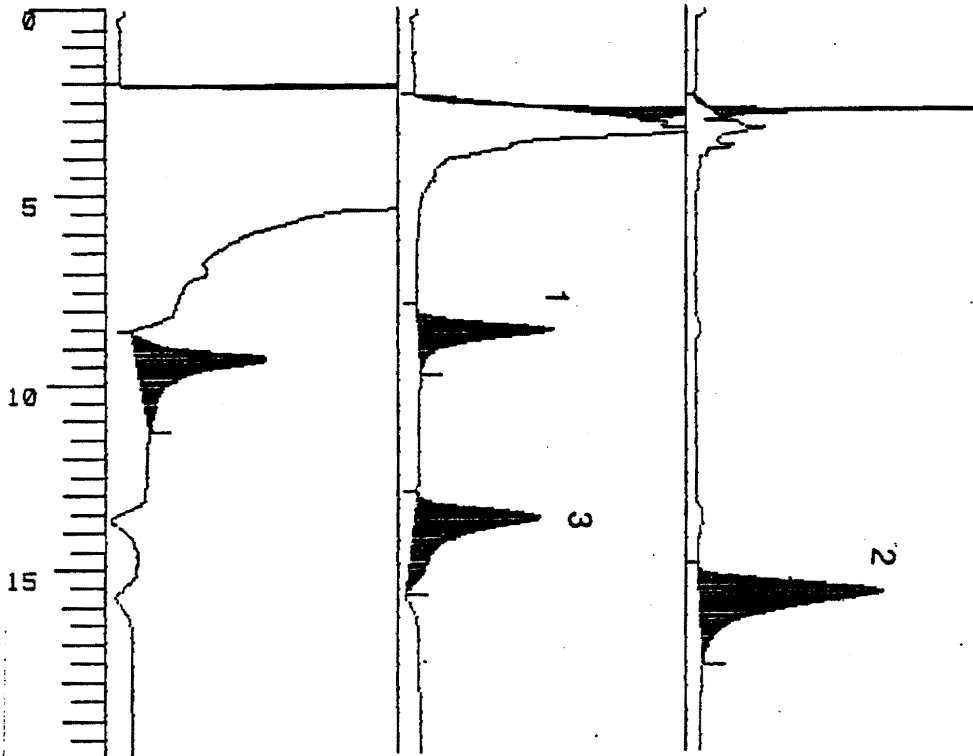


(1) 4-(Methylpyrrol-2-yl)pyridine (internal standard); (2) MMDP<sup>+</sup>; (3) MMP<sup>+</sup>

Figure 39: HPLC tracing of brain extract 30 minutes following i.p. administration of TMMP

Offline plot from DPU memory

Signal	A	B	C
S wl,bw	266,25	366,40	420,60
Range	20	10	10
Zero %	5	5	5

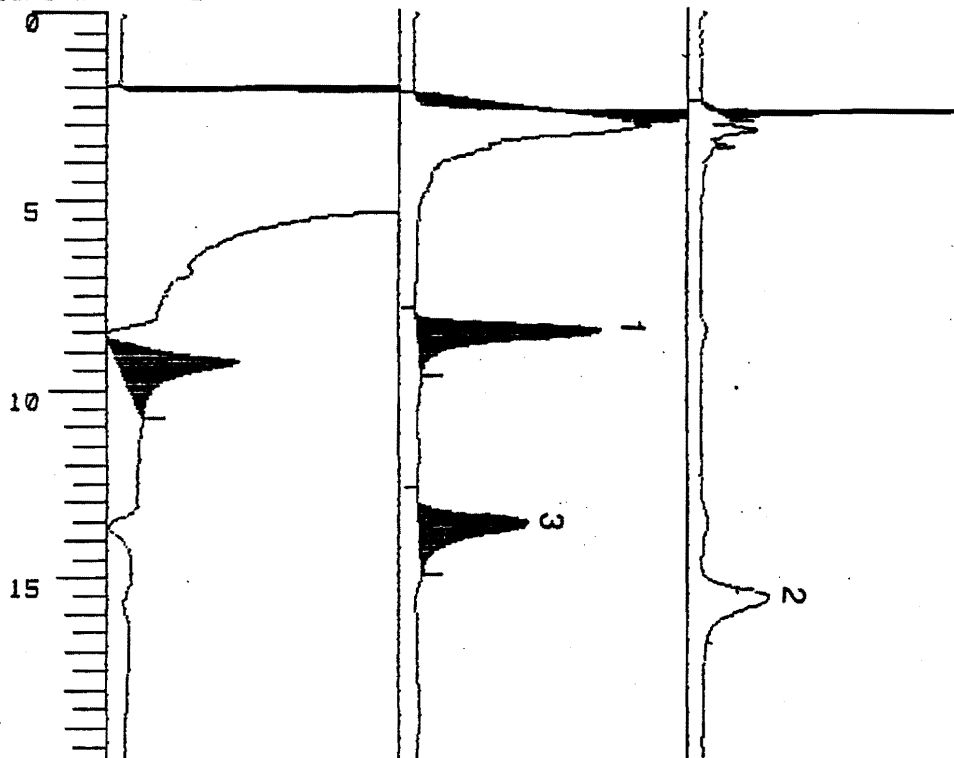


(1) 4-(Methylpyrrol-2-yl)pyridine (internal standard); (2) MMDP<sup>+</sup>; (3) MMP<sup>+</sup>

Figure 40: HPLC tracing of brain extract 1 hour following i.p. administration of  
TMMP

Offline plot from DPU memory

Signal	A	B	C
S wl,bw	266,25	366,40	420,60
Range	20	10	10
Zero %	5	5	5



(1) 4-(Methylpyrrol-2-yl)pyridine (internal standard); (2) MMDP+; (3) MMP+

Figure 41: HPLC tracing of brain extract 2 hours following i.p. administration of  
TMMP

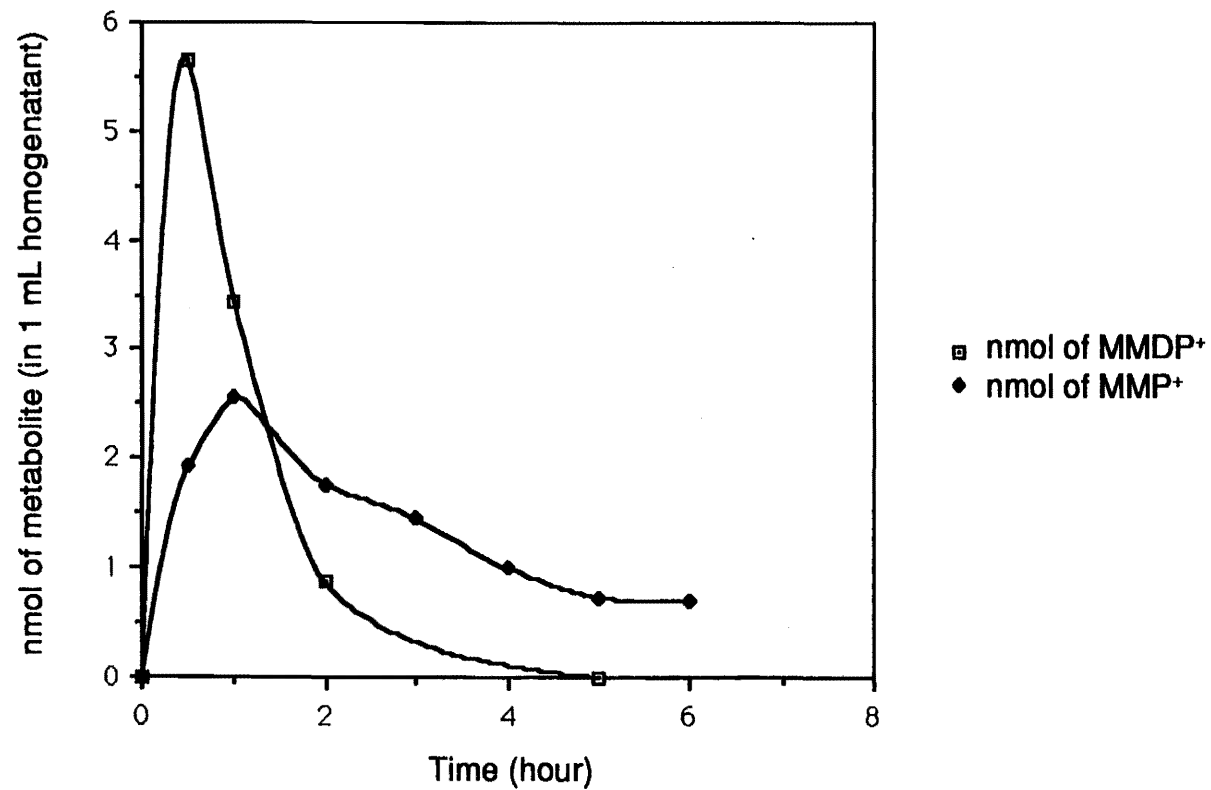


Figure 42: Time course for the brain metabolism of TMMP in the C57 BL/6 mouse following i.p. administration

## Metabolic studies of MMDP<sup>+</sup> with H<sub>2</sub>O<sub>2</sub>

Another product of the MAO-B catalyzed oxidation reaction of the tetrahydropyridine is hydrogen peroxide. It was reported that MPDP<sup>+</sup> can be converted to a lactam structure by aldehyde oxidase.<sup>37</sup> As we mentioned earlier that the detectable metabolites of the MAO-B catalyzed oxidation reaction of TMMP represented only 40 - 50% conversion, this suggested the possibility that other metabolite might be formed during the incubation which are undetectable by our HPLC diode-array assay techniques. We speculated that hydrogen peroxide might be responsible for the formation of this putative metabolite and the expected product would be a lactam. To investigate this possibility, the following experiment was performed.

MMDP<sup>+</sup> (100 μM) was incubated with H<sub>2</sub>O<sub>2</sub> at 37°C. After 1 hour and 4 hours an aliquot was removed and examined by UV and GC/MS. It was found from the incubation mixture that MMDP<sup>+</sup> was completely gone at 4 hours and a metabolic product with  $\lambda_{\text{max}}$  = at 293 nm was formed (Fig. 43). The remaining incubation mixture was extracted with methylene chloride and the extract was examined on GC/MS. Unfortunately, the GC/MS shows complex results, at least 10 peaks were present in the total ion chromatogram. The products of the reaction of MMDP<sup>+</sup> with H<sub>2</sub>O<sub>2</sub> remains therefore as a question.

BECKMAN  
DU-50 SPECTROPHOTOMETER

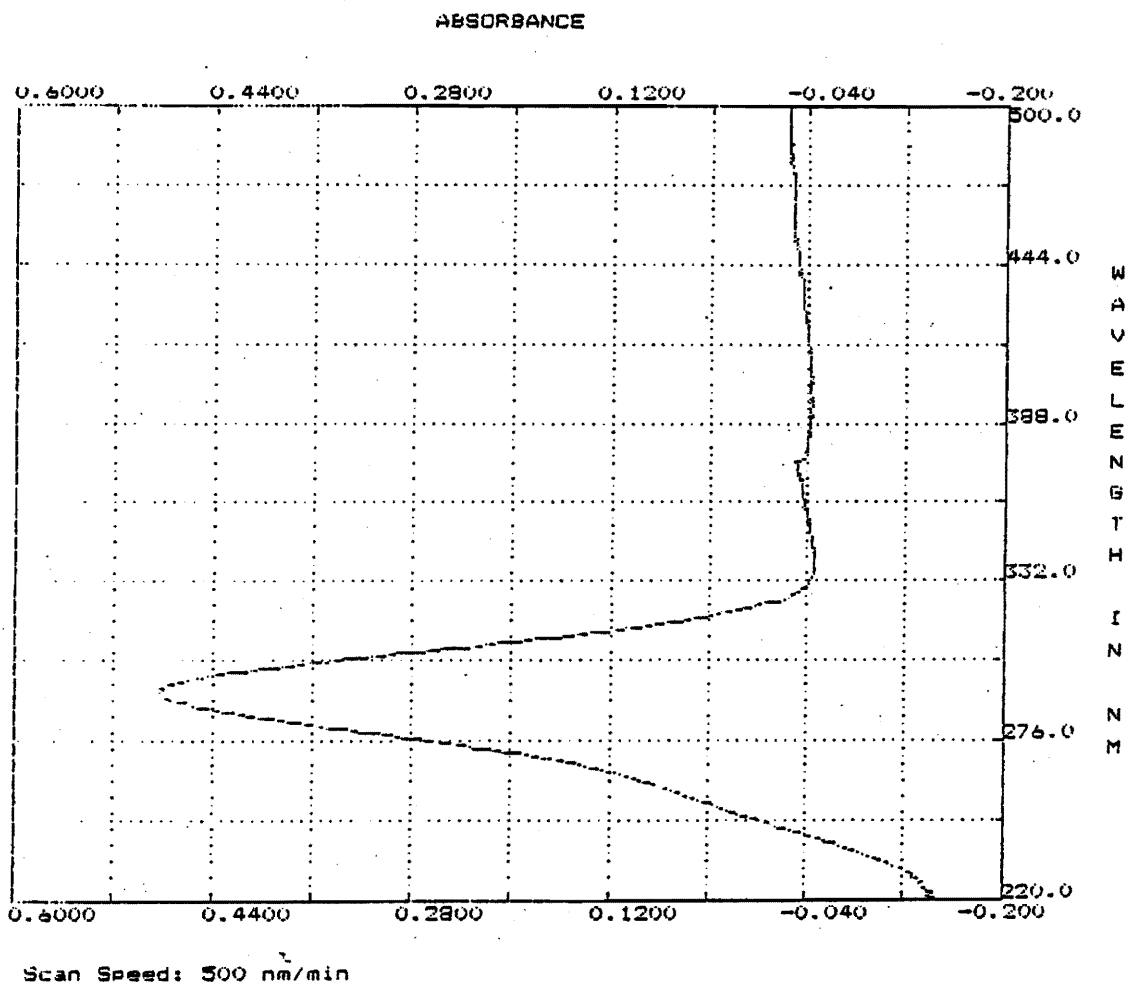


Figure 43: UV spectrum of the metabolism of MMDP+ with H<sub>2</sub>O<sub>2</sub>

## **3.3: Toxicity Studies**

### **3.3.1: Introduction**

As we discussed earlier, MPTP (1) causes degeneration of nigrostriatal dopaminergic neurons in humans and sub-human primates and induces a neurologic syndrome highly reminiscent of parkinson's disease. TMMP (6), an analog of MPTP, was found to cause persistent depletion of striatal dopamine and to induce histologic evidence of nerve terminal degeneration in mice.<sup>59,60</sup> To confirm and extend results we have evaluated the effects of TMMP (6) on striatal dopamine levels in the C57 BL/6J mouse brain with and without pretreatment with deprenyl (an inhibitor of MAO-B).

The MPTP type neurotoxin must be bioactivated in the brain by MAO-B to form the pyridinium species. Additional requirements for toxicity include the active transport of the corresponding pyridinium metabolite first into the striatal nerve terminals via the dopamine (DA) uptake system<sup>42</sup> and then into the inner mitochondrial membrane<sup>49</sup> where it must inhibit mitochondrial respiration.<sup>69</sup> To evaluate further the MPTP-type neurotoxic properties of TMMP, we have examined toxicity of its pyridinium (MMP<sup>+</sup>, 16) and dihydropyridinium (MMDP<sup>+</sup>, 11) metabolites by microdialysis. We also have estimated their mitochondrial toxicity by assessing their ability to inhibit mitochondrial respiration. The intracerebral microdialysis assay was performed with rats.<sup>70,71</sup> The rat is anesthetized and the skull exposed. Two holes are drilled in the skull and a cannula is inserted into each striatum. The two cannulas are secured by a metal plate attached to the skull. After the animal has recovered from the

surgery (usually after 14 hours). The basal dopamine release level is determined by analyzing the perfusate for dopamine by an HPLC assay with electrochemical detection (HPLC/EC). Then the brain is perfused with the test compound for a period of time. Aliquots of the brain perfusates are analyzed by HPLC/EC for dopamine. Neurotoxic compounds such as MPP<sup>+</sup> cause an immediate release of large amounts of dopamine. Twenty-four hours later an MPP<sup>+</sup> challenge dose is perfused through the brain. If irreversible damage has been done to the nerve terminals, the challenge dose will cause no further release of dopamine. ED<sub>90</sub> values, that is the time required for the test compound to cause a 90% destruction of the nerve terminals as determined by an MPP<sup>+</sup> challenge, can then be calculated.

### **3.3.2: Results and discussion**

#### **Dopamine depletion studies in the C57 BL/6J mouse**

The first experiment used a rather low dose TMMP (12.5 mg/kg i.p. injection). Group 1 was used as control and received no injections. We have previously evaluated control mice receiving injections of vehicle only vs no injections and find no differences in their striatal dopamine levels. Therefore, we do not inject the control mice. Group 2 received deprenyl (an MAO-B inhibitor) pretreatment before receiving TMMP-HCl (see experimental for the injection routine). Group 3 received TMMP-HCl only. The results are shown in Table 3. At this dose of TMMP, there was only a small decrease (10%) in striatal dopamine levels (group 3). Pretreatment with deprenyl (group 2) completely blocked the TMMP effects. Since the decreases in the dopamine levels are not large, another experiment was performed with a higher dose of



## TMMP.

The second experiment using a higher dose of TMMP was performed on four groups C57 BL/6J mice. Group 1 was used as control and received no injections. Group 2 received an deprenyl pretreatment before receiving TMMP (see experimental for the injection routine). Group 3 received the high dose of TMMP. Group 4 received a low dose of TMMP four times. The results of these experiments are shown in Table 4. Low multiple doses of TMMP led to a 10% decrease of striatal dopamine level (group 4 mice). High doses of TMMP (40 mg/kg) led to a large decrease of striatal dopamine levels (86%) (group 3 mice). Deprenyl blocked this effect (group 2 mice). It should be noted that two mice in group 2 who received a 40 mg/Kg dose of TMMP (6), died within 24 hours following the injection. The third mouse in the set received a dose of TMMP somewhat less than that described in the protocol. Two of four mice in group 3 died within 24 hours of injection; this may be due to a general toxicity of TMMP. The remaining mice appeared shaky the day following injection. All mice in group 4 survived until sacrificed two weeks later.

The third experiment using dihydropyridinium MMDP<sup>+</sup> was performed on three groups of C57 BL/6J mice. Group 1 used as control and received no injections. Group 2 received two injections of MMDP<sup>+</sup> (40 mg/kg i.p.) and group 3 received only one injection (see experimental for injection routine). The results of the above experiments are summarized in Table 5. The dihydropyridinium (MMDP<sup>+</sup>, 11) caused a 25% striatum dopamine depletion. Since MMDP<sup>+</sup> (11) is a charged ion, it should be unable to cross the blood-brain barrier but its uncharged form, the dihydropyridine, may cross the blood-brain

barrier and cause the dopamine depletion.

Table 3: Dopamine depletion studies using TMMP (low dose)

Group ID	Pk ht ratio DA/IS	Percent of Control
Group 1	0.191 +/- 0.026 CV=13.6%	100
Group 2	0.197 +/- 0.020 CV = 10.1%	103
Group 3	0.173 +/- 0.029 CV = 16.8%	90.5

Table 4: Dopamine depletion studies using TMMP (high dose)

Group ID (n = number of striata)	Pk ht ratio DA/IS	Percent of control
Group 1 (n = 6)	0.311 +/- 0.096 CV = 30.8%	100
Group 2 (n = 2)	0.346 +/- 0.134 CV = 38.7%	111
Group 3 (n = 4)	0.044 +/- 0.019 CV = 42.9%	14.1
Group 4 (n = 6)	0.298 +/- 0.067 CV = 22.4%	95.8

Table 5: Evaluation of dopamine deletion using MMDP+

Group ID (n = number of striata)	Pk ht ratio DA/IS	Percent of Control
Group 1 Controls (n = 7)	0.47 +/- 0.10 CV = 21%	100
Group 2 2 x 40 mg/kg i.p. (n = 8)	0.36 +/- 0.04 CV = 10%	75
Group 3 1 x 40 mg/kg i.p. (n = 6)	0.36 +/- 0.11 CV = 30%	75

## **Mitochondrial respiration**

As previously mentioned, MPP<sup>+</sup> effects its toxicity by inhibiting mitochondrial respiration within the dopaminergic neuron. In order to assess the toxicity of the 4-methylpyrrole system, mitochondrial respiration from mouse brain were examined and the inhibition of mitochondrial respiration was evaluated. IC<sub>50</sub> values were determined for this series of compound. IC<sub>50</sub> is the concentration of the test compound at which 50% of the rate of the O<sub>2</sub> uptake by intact mitochondria is inhibited. Relatively large IC<sub>50</sub> values indicate less toxicity and small IC<sub>50</sub> values mean greater toxicity. The IC<sub>50</sub> values of MMDP<sup>+</sup>, MMP<sup>+</sup> and 4-(methylpyrrol-2-yl)pyridine were determined and the results are summarized in Table 6. The IC<sub>50</sub> for MMP<sup>+</sup> is 197 μM which is close to that of MPP<sup>+</sup> (153 μM), the MMDP<sup>+</sup> and 4-(methylpyrrol-2-yl)pyridine have much larger IC<sub>50</sub> values. This implies that MMP<sup>+</sup> is neurotoxic while the dihydropyridinium MMDP<sup>+</sup> and the 4-methylpyrrolepyridine compound are not.

Table 6: IC<sub>50</sub> values of MMDP<sup>+</sup> and MMP<sup>+</sup>

Test compound	IC <sub>50</sub> (μM)	n = number of experiment
MPP <sup>+</sup>	153	4
4-phenylpyridine	195	2
MMP <sup>+</sup>	197	3
MMDP <sup>+</sup>	360	4
4-(methylpyrrol-2yl)pyridine	540	2

## Microdialysis

Microdialysis studies used male Sprague-Darley rats. ED<sub>90</sub> values of MMP<sup>+</sup> and MMDP<sup>+</sup> were determined by the method as described in the introduction and are summarized in Table 7. It was found that MMP<sup>+</sup> has a very low ED<sub>90</sub> value (12.7 min.) which is similar to MPP<sup>+</sup> (11.2 min.). MMDP<sup>+</sup> has a much high ED<sub>90</sub> value (120 min.).

Table 7: ED<sub>90</sub> values of MMP<sup>+</sup> and MMDP<sup>+</sup>

	MPP <sup>+</sup>	MMP <sup>+</sup>	MMDP <sup>+</sup>
ED <sub>90</sub> (min.)	11.2	12.7	120

Overall, it appears that TMMP is an MPTP type neurotoxin since (a) it is a good MAO-B substrate, and also the metabolic pathway appears to be the same (i.e. conversion to the dihydropyridinium and further oxidation to the pyridinium), (b) the pyridinium metabolite is neurotoxic both in terms of results from microdialysis studies and in terms of its ability to inhibit mitochondrial respiration, and (c) in the C57 BL/6J mouse model, TMMP causes depletion of striatal dopamine. These properties are not shared by the dihydropyridinium metabolite and paralleling the MPTP story, the dihydropyridinium (MMDP<sup>+</sup>) is not itself neurotoxic in these systems.

### **3.4: Experimental**

#### **Monoamine oxidase isolation and purification**

The isolation and purification of MAO-B from beef liver were carried out using a slight modification of the methodology reported earlier by Salach.<sup>67</sup> Fresh calf beef liver was obtained and processed at 4°C. Protein content was determined using the Sigma diagnostic Lowry protein assay kit.<sup>68</sup> About 1500 g of connective tissue-free liver yielded approximately 240 units of MAO-B.

#### **TMMP (6) as a MAO-B substrate**

TMMP was synthesized as described in chapter 2. The enzyme kinetic studies were performed on a Beckman-50 spectrophotometer. Initial reaction rates were monitored at 420 nm (which is the  $\lambda_{\max}$  of synthetic dihydropyridinium) in the presence of MAO-B during the first 3 minutes. Sodium phosphate buffer (100 mM, pH = 7.4) was used to prepare 125  $\mu$ M, 167  $\mu$ M, 250  $\mu$ M, 400  $\mu$ M, and 1000  $\mu$ M stock solution of TMMP (6). The enzyme was homogenized before use with a Teflon/glass homogenizer. A 0.5 mL aliquot of the appropriate stock solution was placed in a curvet and 25  $\mu$ L (0.025 U) of MAO-B were added. The mixture was then incubated at 37°C and the initial rate was measured for each concentration of TMMP.  $K_m$  and  $V_{\max}$  values were determined from the Lineweaver-Burke double-reciprocal plot.

#### **N-propargyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (19) as a MAO-B inactivator**

The remaining enzyme following timed incubation with 19 was estimated



with 5 mM MPTP as substrate. During the kinetic analysis, the formation of MPDP<sup>+</sup> (**3**) was monitored at 345 nm with the spectrophotometer. Data points were acquired every 3 seconds for 2 minutes.

Sodium phosphate buffer (100 mM, pH = 7.4) was used to prepare 100  $\mu$ M, 150  $\mu$ M, 350  $\mu$ M, 1000  $\mu$ M stock solutions of **19**. The enzyme was homogenized before use with a glass/teflon tube. A 300  $\mu$ L aliquot of the appropriate stock solution (or buffer for control analysis) was placed in a glass vial and MAO-B (100  $\mu$ L, 0.2 U) was added. The solution was incubated at 37°C and at time = 0, 5, 10, 15, 20 minutes, an aliquot (60  $\mu$ L) was removed and added to a curvet containing MPTP (5 mM, 0.5 mL). The absorbance at 345 nm was then monitored. The initial rate of the formation of MPDP<sup>+</sup> (**3**) for each aliquot at different time point was obtained from the spectrophotometer as a slope of the kinetic data. The  $k_{obs}$  was obtained as the slope of the plot of ln (% of the remaining enzyme activity) vs time.  $K_i$  and  $k_{inact}$  were obtained from analysis of double reciprocal plot.

### **General method for metabolic studies in-vitro**

All solvents were HPLC grade. Incubations were run in 100 mM sodium phosphate buffer pH = 7.4 and a Piltier temperature controller was used to control the temperature at 37°C. The analyses of the incubation samples were performed either on a Beckman Model DU-50 spectrophotometer or HPLC with a diode array detector. Quantitative and qualitative HPLC analyses were performed on a Beckman 114 M chromatograph system employing an HP Model 1040 A diode array detector using an SCX (10  $\mu$ m, 4.6 mm X 25 cm) column. The mobile phase was 75.3% H<sub>2</sub>O, 23.8% acetonitrile, 0.8% acetic

acid, and 0.12% TEA (triethylamine) with pH was adjusted to 4.0. The flow rate used was 1 mL/min. This HPLC assay can successfully separate synthetic tetrahydropyridine (TMMP, **6**), dihydropyridinium (MMDP<sup>+</sup>, **11**) and pyridinium (MMP<sup>+</sup>, **16**) with the retention time as 8.0 min., 14.0 min. and 16.5 min, respectively.

The TMMP, MMDP<sup>+</sup>, and MMP<sup>+</sup> were synthesized as described in Chapter 2. Pargyline (30  $\mu$ M) was used as an inhibitor of MAO by preincubating with purified MAO-B or mouse brain homogenates at 37°C for 10 min to block the enzyme effect. All the incubations were carried out in qorpak bottles at 37°C in a shaking water bath. All the incubation mixtures were analyzed on the ion exchange column by the HPLC diode-array assay except as noted. All the incubations were performed at least twice. All samples were stored at -10°C until analysis, the storage time less than 2 days.

### **Mice brain preparation**

The male C57 BL/6J mice (20-30g) were used for the preparation of tissue fractions. The mouse brain were minced and homogenized in 3 volumes of buffer (0.25 M sucrose-0.05 M Tris buffer, pH = 7.4) with a Teflon-glass homogenizer. The resulting homogenates were centrifuged at 700 x g for 10 minutes and the supernatants were used as the whole brain fraction. Protein concentrations were measured by the method of Lowry et al.<sup>68</sup>

### **Metabolic incubations (in-vitro)**

The following summarized the procedure followed for the various groups of experiments.

Group 1. MMDP<sup>+</sup> (1mM) in 100 mM sodium phosphate buffer (pH = 7.4) 2 mL in a qorpak bottle was incubated at 37°C. The reaction was monitored by UV. At times 0, 25 hours and 30 hours, a 15 µL aliquot of the incubation mixture was analyzed in 0.5 mL of the same buffer. The diluted solution was scanned from 220 nm to 500 nm. The results are shown in Fig. 28.

Group 2. MMDP<sup>+</sup> (100 µM) in 100 mM sodium phosphate buffer (pH = 7.4) 2 mL in a qorpak bottle was incubated at 37°C. At times 0, 8, 12, 24 and 25 hours, a 200 µL aliquot was removed and 100 µL cold buffer was added. A 60 µL aliquot of the resulting solution was analyzed using the HPLC diode-array assay as before.

Group 3. TMMP (100 µM) or MMDP<sup>+</sup>(100 µM) was incubated in mouse brain homogenate (5 mg protein/mL) or the corresponding homogenate which had been pretreated with 30 µM pargyline in 2 mL sodium phosphate buffer (pH = 7.4) as shown in Table 2. At times 0, 1, 4, 7, 8, 9, and 12 hours, a 200 µL aliquot was removed and the reaction was terminated with 100 µL ethanol. Trichloroacetic acid (TCA) could not be used because of the acid sensitivities of TMMP. After standing on ice for 1 hour, the mixture was centrifuged at 11,000 x g for 10 minutes and the resulting supernatant fraction was filtered through a 0.45 µm nylon filter. A 50 - 120 µL aliquot of each filtrate was analyzed on the ion exchange column by the HPLC diode array assay.

Group 4. TMMP (100 µM), or MMDP<sup>+</sup> (100 µM) was incubated with MAO-B (0.1U enzyme/mL incubation) with or without pretreatment with pargyline (30 µM pargyline preincubated for 10 min. at 37°C) in 2 mL 100 mM

sodium phosphate buffer (pH = 7.4). At times 0, 1, 6, 8, 10 and 11 hours, a 200  $\mu$ L aliquot was removed and mixed with 100  $\mu$ L ethanol to terminate the reaction. After standing on ice for 1 hour, each mixture was centrifuged at 11,000 x g for 10 min. and the resulting supernatant fraction was filtered through a 0.45  $\mu$ m nylon filter. A 60 - 120  $\mu$ L aliquot of each filtrate was analyzed on the ion exchange column by the HPLC diode-array assay as discussed earlier.

### **Metabolic in-vivo studies**

The mice used in this experiment were C57 BL/6J mice. Seven groups mice (n = 2) were injected with one dose of TMMP-HCl (15 mg/kg weight i.p.) at time t = 0. Then at time t = 0.5, t = 1, t = 2, t = 3, t = 4, t = 5 and t = 6 hours, the mice were sacrificed by cervical dislocation and the brain were isolated and homogenized with 1.5 times methanol (v:w) containing 0.7  $\mu$ g/mL 4-(methylpyrrol-2-yl)pyridine as internal standard. Two mice were used as control had no injections and received the same treatment as described. The homogenates were centrifuged at 11,000 x g for 10 min. and the supernatant fractions were filtrated through a 0.45  $\mu$ m nylon filter. The filtrates then were analyzed using HPLC diode array assay as discussed earlier. The results are summarized in Fig. 42.

### **Metabolic studies of MMDP<sup>+</sup> with H<sub>2</sub>O<sub>2</sub>**

MMDP<sup>+</sup> (100  $\mu$ M) was incubated with H<sub>2</sub>O<sub>2</sub> (30  $\mu$ L) in 2 mL 100 mM sodium phosphate buffer (pH = 7.4) at 37°C. At time t = 1 hour and 4 hours, a 200  $\mu$ L aliquot of the incubation mixture was removed and added to 0.4 mL same buffer. The diluted solution was scanned from 220 nm to 500 nm. The

results shown in Fig. 43. The incubation mixture was extract with  $\text{CH}_2\text{Cl}_2$ , the extraction was then analyzed on GC/MS.

### **Evaluation of dopamine depletion**

The toxicity of TMMP (6) was determined by systemic administration interperitoneally in retired breeder male C57 BL/6J mice (Supplied by Harland lab). Animals were housed 1 to a cage with food and water freely available. Dihydropyridinium (MMDP<sup>+</sup>, 11) also was administered to the mice to determine its toxicity. Mice from all groups were sacrificed by cervical dislocation two weeks after drug administration. The striata were dissected out, weighed and homogenized with 20  $\mu\text{L}/\text{mg}$  wet tissue 5% TCA (w:v) containing dihydroxybenzylamine (DHBA) as the internal standard. The samples were centrifuged for 1 minute at 14,000 rpm using an Eppendorf microcentrifuge. The resulting supernatant was analyzed for dopamine levels by an HPLC system with a Beckman-Altex pump, a BAS electrochemical detector (for the oxidation of dopamine and DHBA), a C-18 column, and a Kipp and Zonen strip chart recorder. Appropriate standards of dopamine and DHBA were analyzed in order to prepare a calibration curve from which the amount of dopamine in the striatal samples could be quantitatively estimated.

### **Protocol**

The experiment was divided into three sections and are described below. The results are summarized in Table3, Table 4 and Table 5.

#### **Experiment one**

Group 1: Three mice were used as controls and received no injections.

Group 2: Three mice were injected with 20 mg/Kg i.p. deprenyl hydrochloride on day 1 at t = 0. At t = 30 min., these mice were injected with 12.5 mg/Kg i.p. TMMP-HCl.

Group 3: Three mice were injected with 12.5 mg/Kg i.p. TMMP-HCl on day 1 at t = 0.

#### Experiment two

Group 1: Control mice, no injections.

Group 2: Two mice were injected with deprenyl hydrochloride (20 mg/Kg i.p.) on day 1 at t = 0, followed by injection of TMMP-HCl (40 mg/Kg i.p.) at t = 30 min.

Group 3: Four mice were injected with TMMP-HCl (40 mg/Kg i.p.) on day 1 at t = 0.

Group 4: Six mice were injected with TMMP-HCl (12.5 mg/Kg i.p.) on days 1, 2, 3, and 4 at t = 0.

#### Experiment three

Group 1: Four mice were used as control, no injections.

Group 2: Five mice were injected with MMDP<sup>+</sup> (11) as follows: 3 x 40.2 mg/kg = 191  $\mu$ moles/kg i.p. at 0, 1, and 2 h.

Group 3: Five mice were injected with MMDP<sup>+</sup> (11) as follows: 2 x 40.2 mg/kg i.p. at 0 and 1 h.

Unfortunately, problems with apparent general toxicity were encountered in experiment 3. In group 2, all five mice were given one injection followed by a second injection one hour later. One mouse died 50 minutes after the second injection, therefore it was decided not to inject the remaining 4 mice with a third dose. In group 3, one mouse died immediately after the second injection. Another mouse following administration of the first dose became noticeably depressed, had excess salivation, and the urine became discolored. This mouse did not receive a second injection. It was decided therefore, not to inject the remaining three mice with a second dose.

### **Mitochondrial Respiration**

The mitochondrial suspension was prepared from male ICR mice. The respiration activity of the mitochondria can be determined by measuring the rate of the reduction of the oxygen concentration in an air saturated medium. Mitochondrial oxygen consumption was measured with a Clark-type oxygen electrode using a 1 mL chamber maintained at 30°C. Plots of the percentage of inhibition versus concentration (log scale) were constructed and the IC<sub>50</sub> values were interpolated from these plots. The IC<sub>50</sub> values of the MMDP<sup>+</sup> (11), MMP<sup>+</sup> (16) and 4-(methylpyrrol-2-yl)pyridine (14) were determined and compared to those values of MPTP. These results are summarized in Table 6.

## **Microdialysis**

The dihydropyridinium (MMDP<sup>+</sup>, **11**) and pyridinium (MMP<sup>+</sup>, **16**) metabolites of TMMP (**6**) were directly delivered to the rat brain striatum by perfusion using Microdialysis as described earlier. A solution of the test compound was perfused through the brain cannula for various timed periods. The brain dopamine release was measured by HPLC/EC as described in the literature.<sup>70,71</sup> The ED<sub>90</sub> values of MMDP<sup>+</sup> (**11**) and MMP<sup>+</sup> (**16**) were determined and are summarized in Table 7.



## Chapter 4

### Conclusions

1) The MPTP analog, 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (TMMP, **6**), 1-methyl-4-(1-methylpyrrol-yl)-2,3-dihydropyridinium (MMDP<sup>+</sup>, **11**), 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium (MMP<sup>+</sup>, **16**) and N-propagyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine were synthesized and fully characterized. Except the TMMP (**6**), none of the above has been reported previously.

2) With the aid of a spectrophotometric assay of MAO-B enzyme, kinetic data were obtained including  $K_m$  and  $V_{max}$  for TMMP ( $K_m = 201 \mu M$ ,  $V_{max} = 250$  nmol/min/U enzyme) as an MAO-B substrate,  $K_i$  and  $k_{inact}$  values were obtained for N-propagyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine ( $K_i = 831 \mu M$ ,  $k_{inact} = 1.11 \text{ min}^{-1}$ ) as a MAO-B inactivator.

3) We developed an HPLC system which can successfully separate TMMP (**6**), MMDP<sup>+</sup> (**11**) and MMP<sup>+</sup> (**16**). By using this HPLC system in-vivo and in-vitro metabolic studies were performed and the results showed that TMMP (**6**) is oxidized rapidly to the dihydropyridinium compound (MMDP<sup>+</sup>, **11**) in a reaction catalyzed by MAO-B.

4)  $\text{MMDP}^+$  (11) undergoes autoxidation to form the corresponding pyridinium compound, details of the mechanism of this conversion are not clear. In-vitro studies show that MAO-B is not responsible for this reaction. In-vivo studies show that the oxidation of  $\text{MMDP}^+$  (11) to  $\text{MMP}^+$  (16) is likely to be enzyme catalyzed.

5) Toxicity studies showed that  $\text{TMMP}$  (6) is an MPTP-type neurotoxin which causes irreversible striatal dopamine depletion. As with MPTP, this toxicity is likely to be mediated by the corresponding pyridinium metabolite 16.

Since the neurotoxin is the pyridinium species, the mechanism of the oxidation of the dihydropyridinium intermediate to the pyridinium species is very important. So far, this mechanism is not clear for this pyrrole derivative. From our in-vivo studies, we expect that this reaction is enzyme catalyzed. Other potential catalysts for the transformation need to be examined.

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Hong Bai