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Studies on the bioactivation and neurotoxicity of the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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Studies on the Bioactivation and Neurotoxicity of the Nigrostriatal Toxin 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

by
Raymond George Booth

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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in the

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of the

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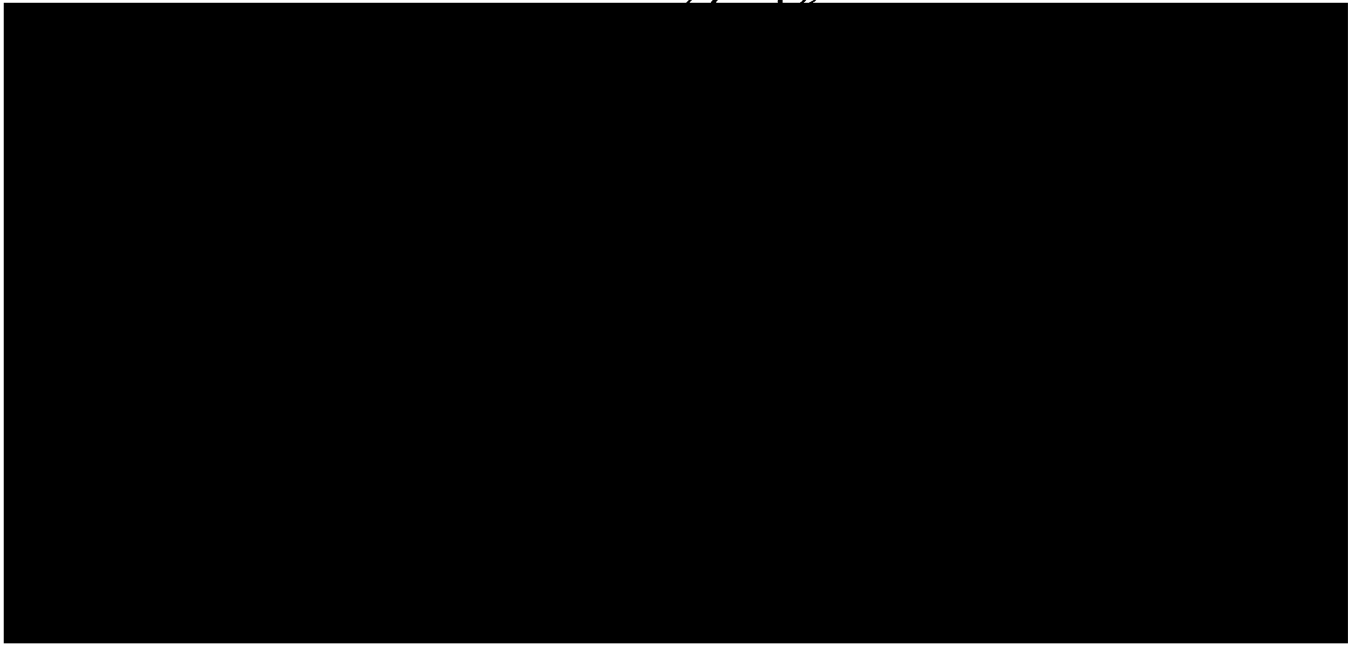

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Studies on the Bioactivation and Neurotoxicity of the Nigrostriatal Toxin 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

Raymond George Booth

Abstract of the Dissertation

The cyclic tertiary allylamine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a parkinsonian syndrome with concomitant dopamine depletion in humans and certain laboratory animals. In susceptible species, MPTP specifically lesions the cell bodies and/or nerve terminals of the nigrostriatum and the locus ceruleus while sparing other central and peripheral tissues. In a reaction catalyzed by monoamine oxidase-B (MAO-B), MPTP is oxidized to the 1-methyl-4-phenyl-2,3-dihydropyridinium species ($MPDP^+$), which subsequently undergos oxidation to the 1-methyl-4-phenylpyridinium species (MPP^+). MPTP, $MPDP^+$, and MPP^+ competitively inhibit MAO-B; MPTP is also a mechanism-based inactivator of the enzyme. Although the neurotoxicity of MPTP is dependent on its bioactivation by MAO-B, the mechanism of cellular toxicity induced by the MAO-B derived metabolites has not been established. It has been suggested that MPTP-like compounds present in the environment and/or produced endogenously may be etiological factors in idiopathic Parkinson's disease.

The work described in this thesis is concerned with the characterization of the structural features of MPTP-like tertiary allylamines and related molecules which are required for their MAO-B catalyzed bioactivation to neurotoxic products. Specifically, the degree of coplanarity of the phenyl and piperidiene rings and the extent of delocalization of the allylic bond in conjugation with the phenyl ring are addressed. Candidate compounds which probe these questions and/or have etiological relevance in that they may be found *in vivo* or in the environment are considered. Tetrahydroindenopyridine, tetrahydro-β-carboline, tetrahydroisoquinoline, and tetrahydroquinoline derivatives structurally related to MPTP have been synthesized and evaluated for their MAO-B substrate properties.

Although the tetrahydroindenopyridine derivatives did inhibit MAO-B both competitively and irreversibly in a manner similar to MPTP, none of the compounds was oxidized at a rate comparable to MPTP. It is concluded that incorporation of the MPTP moiety into fused ring structures is unlikely to produce molecules with good MAO-B substrate properties. However, poor MAO-B substrates such as the tetrahydro-β–carbolines and tetrahydroisoquinolines should be considered as possible nigrostriatal toxins in view of their possible *in vivo* formation.

These studies also assessed the neurotoxicity of the proposed MAO-B substrates and their corresponding MAO-B derived oxidation products. The lipophilic tetrahydro compounds were injected intraperitoneally in C-57 black mice to assess their ability to deplete striatal dopamine, one measure of MPTP induced neurotoxicity. The tetrahydroindenopyridine test compounds were not neurotoxic in this assay, presumably due to their poor MAO-B substrate properties. Analogs related to $MPDP^+$ and MPP^+ which will not cross the blood-brain barrier were administered to rats using an intracerebral microdialysis procedure that allows direct perfusion of the striata with the test compound. While none of the compounds tested were more potent than MPP^+, several of the indenopyridinium, β-carbolinium, isoquinolinium, and quinolinium species tested did demonstrate MPP^+-like neurotoxicity. The demonstration of striatal toxicity by the putative MAO-B derived aromatized metabolites of the nontoxic parent tetrahydro compounds confirm the obligatory role of MAO-B in the development of MPTP-type neurotoxicity. These results are important in view of the possible *in vivo* formation of these aromatic species via slow MAO-B catalyzed oxidation of their corresponding tetrahydro derivatives and should be considered as potential etiological factors in the cumulative and chronic degeneration of the nigrostriatal system in Parkinson's disease.

Neal Castagnoli

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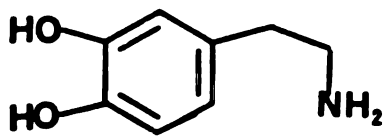
Chapter I

Introduction

Parkinson's disease, first described by James Parkinson in 1817, affects approximately one-half million people in the United States.[1] In 90% of the cases, the disease becomes manifest after age 55 and presents clinically as resting tremor, bradykinesia, rigidity, and a disturbance of posture.[1] Neuropathlogically, Parkinson's disease is a slowly progressive neurodegenerative disorder which is characterized by the destruction of cells from the substantia nigra.[2] Degenerative changes in the pigmented nuclei of the noradrenergic locus ceruleus are also typical as well as the appearance of intraneuronal inclusions called Lewy bodies.[2] Biochemically, the dopamine (**1**) deficiency resulting from the nigrostriatal lesion accounts for the major symptoms of the disease; thus treatment involves replacement therapy with the α-amino acid L-dopa (**2**), the precursor of dopamine.[3]



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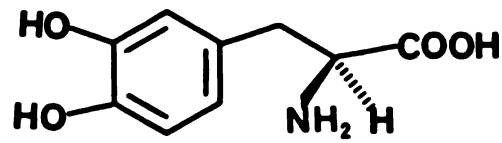

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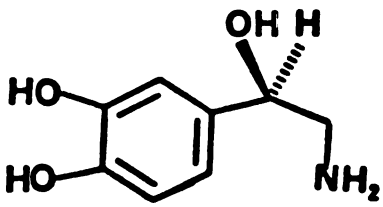

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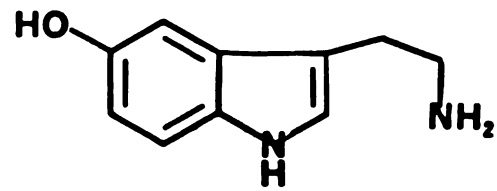

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The etiology of the neuronal degeneration in Parkinson's disease remains unclear. Dopamine neurons degenerate with advancing age and in normal adults, dopamine levels in the corpus striatum decline about 13% per decade.[4] Parkinsonian symptoms usually become apparent when striatal dopamine levels decline by about 80%.[5] It has been postulated that the condition might be the consequence of the effects of normal aging superimposed on a lesion in the substantia nigra that took place earlier in life.[6] Previous studies have suggested that the disease may be hereditary in nature.[7] More recent studies however, utilizing both identical and heterozygous twins have provided strong evidence that Parkinson's disease is not a genetic disorder.[8] There is also little evidence that the disease is autoimmune in nature.[7] Futhermore, studies by Gajdusek and Salzar[9] on a form of Parkinson's disease in villages in west New Guinea indicate that neither a genetic nor a communicable infectious etiology was involved.

The possiblility that an environmental neurotoxin might be involved in the etiology of Parkinson's disease has been discussed for years. For instance, manganese miners in South America are at a higher risk for a parkinsonian syndrome characterized by tremor, bradykinesia, postural difficulties, dystonia, and psychic disturbances.[10] And in the major agricultural region of the Quebec province of Canada, Barbeau *et al* [11] showed there was a remarkably high correlation between the incidence of Parkinson's disease and the sales of pesticides (Spearman rank coefficient = 0.967).

The idea that endogenously formed substances might be etiologic factors in Parkinson's disease is also not novel. In fact, dopamine itself has been implicated in the disease process through production of chemically reactive oxidation products.[3,12] The oxidative deamination of dopamine *in vivo* is catalyzed by monoamine oxidase (MAO, E.C. 1.4.3.4). MAO is a flavin-containing enzyme bound to the outer mitochondrial membrane that exists in two forms, A and B, which are thought to be two different proteins with different substrate specificities.[13] The MAO catalyzed oxidation of endogenous monamines [i.e., dopamine, norepinephrine (**3**), 5-hydroxytryptamine (**4**)] by MAO

generates hydrogen peroxide[13] (see equation 1) which in the Haber Weiss reaction[14], can undergo a redox reaction with superoxide to form the extremely cytotoxic hydroxy radical[3,15]; see equation 2.

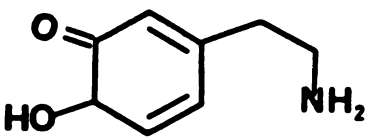
$$RCH_2CH_2NH_2 + O_2 + H_2O \longrightarrow RCH_2CHO + NH_3 + H_2O_2 \qquad \text{Equation 1}$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + OH^- + \cdot OH \qquad \text{Equation 2}$$

The autoxidation of dopamine to the corresponding semiquinone **5** and quinone **6** also has received attention since these oxidation products are toxic to neuroblastoma cells.[12] Manganese ion has been shown to catalyze this oxidation of dopamine and the resulting production of semiquinone and quinone species has been implicated in manganese neurotoxicity.[3,16,17] The autoxidation of dopamine also leads to the formation of the polymeric black pigment neuromelanin.[18] The physiological role of neuromelanin is poorly understood but the pigment is increasingly deposited into catecholaminergic neurons with advancing age and it is suggested that its accumulation in nigral neuronal cells eventually causes cell death.[2,19]

The discovery that 6-hydroxydopamine (**7**) can selectively lesion dopaminergic neurons in the substantia nigra and noradrenergic neurons in the locus ceruleus[20,21,22,23] gave investigators the chance to mimic the lesion of Parkinson's disease. It has never been suggested that 6-hydroxydopamine might be an etiologic factor in Parkinson's disease as there is no evidence that the compound is present in the environment. Also, like dopamine, 6-hydroxydopamine is a primary amine (pKa approximately 9) which is positvely charged at physiological pH and thus does not cross the blood brain barrier. Although the compound was detected in rat brain following a single large dose of

methamphetamine[24] (also a nigrostriatal neurotoxin in rats[25]), it has never been reported to be present under normal physiological conditions. Furthermore, 6-hydroxydopamine destroys sympathetic nerve endings in the periphery[20] which is not a classic feature of Parkinson's disease. Nevertheless, although the compound is not likely to be an etiologic factor itself, 6-hydroxydopamine has been useful as a tool in the study of catecholaminergic neuronal degeneration. Its mechanism of action has not yet been fully elucidated, however it is known that the compound must first be accumulated by catecholaminergic neurons through their high affinity uptake systems to achieve a critical intraneuronal toxic concentration.[3] It has also been suggested that electrophilic oxidation products of 6-hydroxydopamine (i.e., its semiquinone and quinone) react with nucleophilic functionalities present on neuronal structures[26,27] and a study confirmed that the extent of covalent binding of oxidation products correlated with toxicity.[28,29] In primates, only technically difficult intranigral administration of 6-hydroxydopamine has been shown to produce a parkinsonian behavioral syndrome with discrete nigral lesions.[3] However, a more promising primate model of Parkinson's disease has been developed since the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **9**), as will be described.



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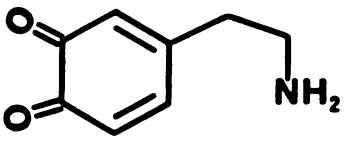

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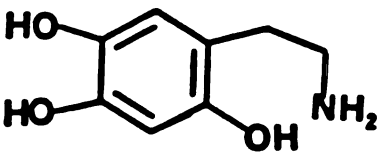

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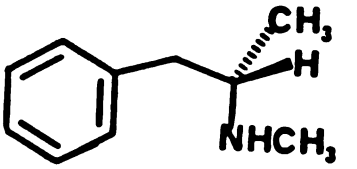

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The cyclic tertiary allylamine MPTP (**9**) induces a form of parkinsonism in humans[30,31] and monkeys[32] that is similar in neuropathology and motor abnormalities to those observed in idiopathic Parkinson's disease. The clinical and biochemical profiles of MPTP-induced and idiopathic Parkinson's disease are in fact, nearly identical.[30,32,33,34] MPTP not only produces all the major features of Parkinson's disease, but also many of the more subtle features such as kinesia paradoxia and seborrhea.[3,33] Most importantly, MPTP-induced parkinsonism produces the hallmark neuropathological features of the idiopathic disease; the specific degeneration of nigrostriatal[30,32,34] and locus ceruleus neurons[35] and the presence of eosinophillic inclusion bodies similar to Lewy bodies.[35]

MPTP was discovered as a contaminant in batches of the illicit "designer drug" 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP,**10**) which is the reverse ester of the narcotic meperidine (**11**).[30,31] The ester function in MPPP undergoes elimination at low pH or elevated temperature to form MPTP as shown in Figure 1. Drug abusers who self-administered pure MPTP or MPTP-contaminated MPPP developed clinically diagnosed parkinsonism that was responsive to L-dopa therapy.[30,31] The autopsy of one addict who died revealed extensive destruction of the substantia nigra.[30]

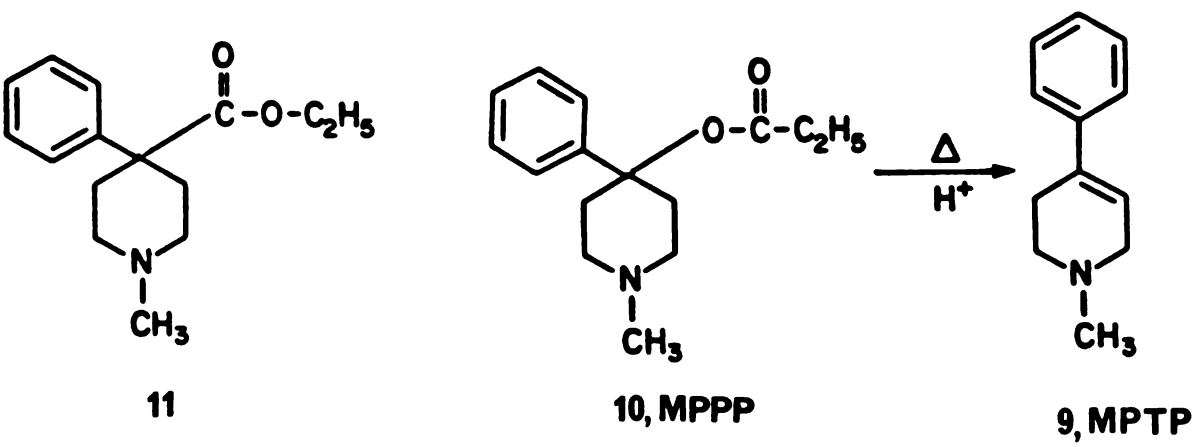
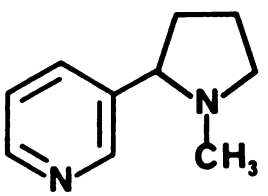


Figure 1. Formation of MPTP from MPPP.

Consideration of the chemical structure of MPTP would suggest the compound to be chemically inert as no highly reactive functional group is present. Almost immediately it was recognized that MPTP might undergo some type of "metabolic activation" to a more reactive metabolite analogous to the initial event in the chemical-induced toxicity of a wide variety of chemically inert substances.[36,37] Often, reactive metabolites are formed by the enzymes involved in drug metabolism.[38] The biotransformation of most nonopolar xenobiotics is carried out by the cytochrome P-450 family of isozymes.[39] The formation of reactive electrophilic iminium ions (i.e., of structure **12**) from the P-450 catalyzed oxidation of several cyclic tertiary amines including nicotine[40] (**13**), 1-benzylpyrrolidine[41] (**14**), methapyrilene[42] (**15**), and phencyclidine[43,44,45] (PCP, **16**) has been described.



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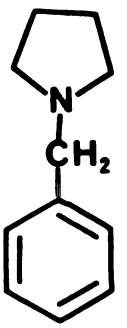

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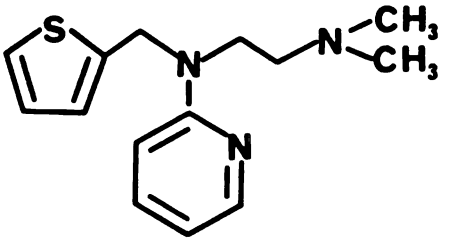

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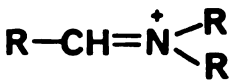

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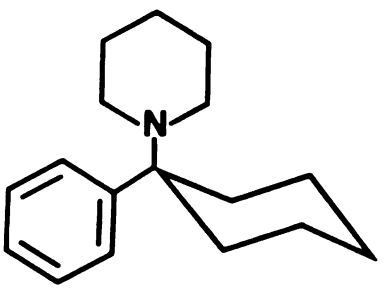
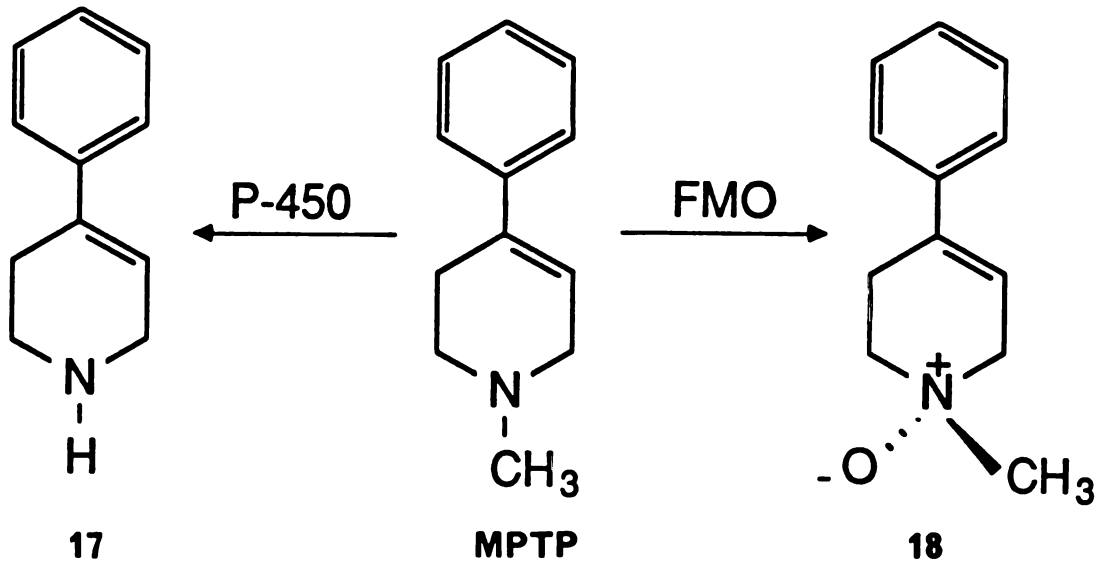


16, PCP

Electrophilic iminium species can be deleterious to biological systems because they have the potential to interact covalently with cellular nucleophiles (Overton, Hoag 1984, 1987).[44,45,46] Thus we speculated that MPTP might undergo P-450 catalyzed α-carbon oxidation to generate an electrophilic iminium species which would have the potential to react with nucleophilic components of nigrostriatal neurons. It is now known that liver microsomal P-450 and flavin monoxygenase predominately catalyze the N-demethylation and N-oxygenation of MPTP to yield 4-phenyl-1,2,3,6-tetrahydropyridine (PTP, **17**) and MPTP-N-oxide (**18**), respectively (see Figure 2).[47,48] Recently however, there has been a suggestion that cytochrome P-450 isozymes or related oxidases may oxidize MPTP at the allylic α-carbon position.[49]



17 **MPTP** **18**

Figure 2. Metabolic pathways for MPTP.

It was Chiba[50] who first discovered that crude brain mitochondrial preparations catalyzed the oxidation of MPTP by a process that was inhibited by pargyline (an MAO-B inhibitor) but not by clorgyline (a selective MAO-A inhibitor). The two-electron allylic α-carbon oxidation product of MPTP, the 1-methyl-4-phenyl-2,3-dihydropyridinium species ($MPDP^+$, **19**) was subsequently identified as the immediate MAO-B derived metabolite.[51] $MPDP^+$ undergos a further two-electron oxidation to the 1-methyl-4-phenylpyridinium species (MPP^+, **20**) via autoxidation and disproportionation[52] as well as MAO-catalyzed[53,54] and neuromelanin-catalyzed[55] oxidations. Figure 3 shows the overall 4-electron oxidation of MPTP to MPP^+. Purified MAO-B catalyzes the oxidation of MPTP at a rate of 40% of that observed with benzylamine, the most rapidly oxidized substrate for the enzyme.[56] The rate of oxidation of MPTP to $MPDP^+$ catalyzed by purified MAO-A is about 7% of the rate for purified MAO-B.[56]

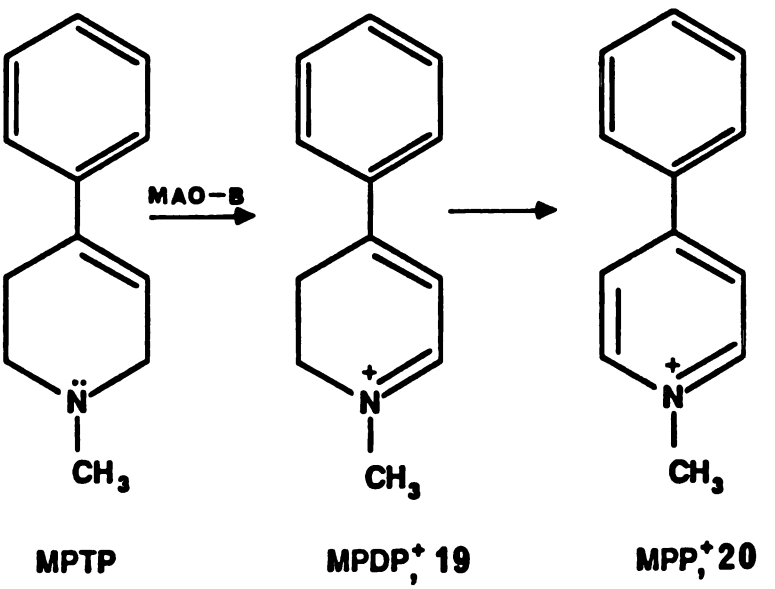


Figure 3. MAO-B catalyzed oxidation of MPTP.

These rates of oxidation are impressive since tertiary amines in general are only very slowly oxidized in the presence of MAO.[57] Thus, MPTP-like compounds have become a tool to probe the substrate specificity and catalytic mechanism of MAO as will be described in Part II of this thesis. *In vitro* studies also showed that MPTP, $MPDP^+$, and MPP^+ inhibit MAO-A and MAO-B competitively.[56] Additionally, MPTP and $MPDP^+$ mechanism-based[58] are irreversible inactivators of both types of MAO.[56]

Since MAO-B inhibitors prevent MPTP-induced parkinsonism and nigral cell death in primates[59,60] as well as dopamine depletion in rodents[61,62], it is apparent that the MAO-B derived metabolite(s) of MPTP (i.e., $MPDP^+$ and/or MPP^+) is (are) the true neurotoxin(s). Although a role for the chemically reactive dihydropyridinium species should not be overlooked (and will be examined in this thesis using a more stable tricyclic semirigid analog), most of the literature pertaining to MPTP neurotoxicity focuses on the MPP^+ metabolite. These results include those obtained from *in vivo* studies which have shown that MPP^+ and proposed endogenous MPP^+-like compounds cause irreversible lesions of striatal dopaminergic neurons when perfused intrastriatally into rats[63,64,65], see Part III.

One theory which may account for the remarkable selectivity of MPTP for the nigrostriatal dopaminergic system concerns the selective uptake into and binding of MPP^+ in dopaminergic neurons. It has been shown that MPP^+ (but not MPTP) is taken up by the dopamine uptake system with the same affinity as dopamine itself.[66,67] This sequestration of MPP^+ in dopaminergic neurons may be enhanced by neuromelanin which has been shown to bind MPP^+.[55,68] This sequestering hypothesis takes on added importance in view of the fact that the generation of MPP^+ from the MAO-B catalyzed oxidation of MPTP must occur extraneuronally since the dopamine cells of the nigrostriatal system contain little or no MAO-B. Apparently, MAO-B is localized in glial cells and serotonergic neurons in the brain.[69]

A clue to the molecular mechanism by which MPP^+ may exert its toxic effect on neuronal cells came from observations by Nicklas *et al.*[9] that MPP^+ inhibits NADH dehydrogenase in the mitochondrial respiratory chain. This observation was later confirmed by Ramsay and coworkers[71] who found that a variety of pyridine derivatives were capable of inhibiting NADH dehydrogenase.[72] Furthermore, MPP^+ has been reported to be accumulated rapidly by mitochondria via an energy-dependent carrier that concentrates the compound well in excess of the concentration required for complete inhibition of NADH dehydrogenase.[73] Recently, this inhibition of mitochondrial respiration has been demonstrated *in vivo* in the rat via the detection of enhanced lactic acid production after intrastriatal perfusion of MPP^+ and proposed endogenous MPP^+-like compounds[74], see Part III.

The results summarized above clearly establish that the lipophilic tertiary amine MPTP is biotransformed to neurotoxic metabolites by MAO-B. Although MPP^+ appears to be the putative neurotoxin, its mechanism of toxicity has not been established. The possibility that the exposure to lipophilic organic molecules such as MPTP might be involved in the etiology of Parkinson's disease has stimulated interest in searching for environmental and/or endogenous neurotoxins related to MPTP and/or MPP^+. The studies to be described in this thesis are concerned with the characterization of the structural features of molecules related to MPTP which are required for the MAO-catalyzed bioactivation of tertiary allylamines to neurotoxic products. Rather than initiate a "hit or miss" approach, the studies focus on the steric and electronic features of the MPTP moiety. Specifically, the steric role of the orientation of the phenyl and piperidiene rings and the electronic role of the allylic bond in conjugation with the phenyl ring will be assessed. Candidate compounds which probe these questions and/or which have etiological relevance in that they may be found *in vivo* or in the environment are considered.

The neurotoxicity of suitable MAO-B substrates will be assessed following their intraperitoneal injection into C-57 black mice, a species known to be susceptible to MPTP-

induced depletion of striatal dopamine with parallel nigral cell damage.[75] For those compounds which lack sufficient MAO-B substrate properties and fail to demonstrate neurotoxicity in the mouse model, the synthesis and toxicological properties of their putative metabolites will be studied in an effort to establish the pivotal role of MAO-B in the development of MPTP-induced nigrostriatal toxicity. Analogs of $MPDP^+$ and MPP^+ which do not cross the blood barrier, will be administered to rats using an intracerebral microdialysis procedure that allows direct perfusion of the striata with the test compound.[63,64,65]

Chapter II

Studies on the molecular mechanism of bioactivation of MPTP by MAO-B using indenopyridine, β-carboline, isoquinoline, and quinoline analogs of MPTP.

Introduction

As described in the Introduction, chapter I, MPTP undergos a two-electron oxidation to yield $MPDP^+$ in a reaction catalyzed by MAO-B and, to a lesser extent, MAO-A. In view of the fact that few tertiary amines are MAO substrates and none is turned over as rapidly as MPTP, the electronic, steric, and/or hydrophobic parameters of this compound are of interest in that MPTP-type molecules can serve as specific probes of the catalytic mechanism of MAO oxidation.

Previously, Silverman *et al.*[76,77] have provided evidence to support a catalytic mechanism for MAO that involves two successive one-electron transfers from the amine substrate to the flavoprotein of the enzyme. At the heart of this proposal is the necessary formation of the aminium radical cation intermediate **21** (see Figure 4)and removal of the most acidic α-proton of the amine substrate.[78] Silverman modeled the MAO proposal on the generally accepted mechanism for the electrochemical oxidation of amines[79,80,81] shown in Figure 4. In this scheme, the formation of the aminium radical cation **21** renders the α-proton much more acidic, thus facilitating proton loss.

Using the MAO mechanism-based inactivator N-cyclopropylbenzylamine (**22**, radiolabeled as ^{14}C in the phenyl ring or ^{3}H on the cyclopropyl carbon atom adjacent to the nitrogen atom) Silverman and collegues[77] found independent oxidation of both carbon atoms α to the nitrogen (see Figure 5). The group determined that 4 benzyl methylene carbon oxidations occur for each N-cyclopropyl carbon oxidation. As Figure 5 shows, the fact that both α-carbon atoms were oxidized indicated the formation of a nitrogen radical

cation intermediate species **23** that could partition between removal of the cyclopropyl carbon proton to give product **24** and the benzyl methylene proton to give product **25**. The nitrogen radical cation **23** was proposed to arise as the result of a one-electron transfer from the amine nitrogen to the flavin moiety of MAO as shown in Figure 6). When N-cyclopropylbenzylamine (radiolabeled as above) was oxidized electrochemically, the ratio of benzyl methylene carbon to cyclopropyl carbon oxidation was found to be similar to the MAO-catalyzed reaction ratio. This suggested a strong similarity between the mechanism of MAO-catalyzed amine oxidation of N-cyclopropybenzylamine and electrochemical amine oxidation. In subsequent studies, Silverman and Zieske[82] showed that 1-phenylcyclobutylamine (**26**) also inactivated MAO in an irreversible time-dependent fashion (i.e., mechanism-based inhibition). The inactivated enzyme-substrate complex and the metabolites were characterized to reveal that ring opening had occurred, possible only via the formation of a radical intermediate analogous to structure **23**.

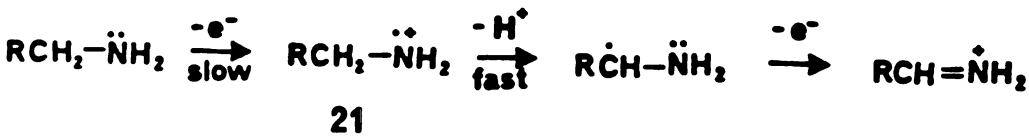
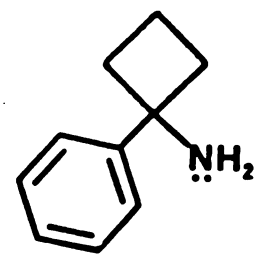


Figure 4. Proposed mechanism for electrochemical oxidation of amines.



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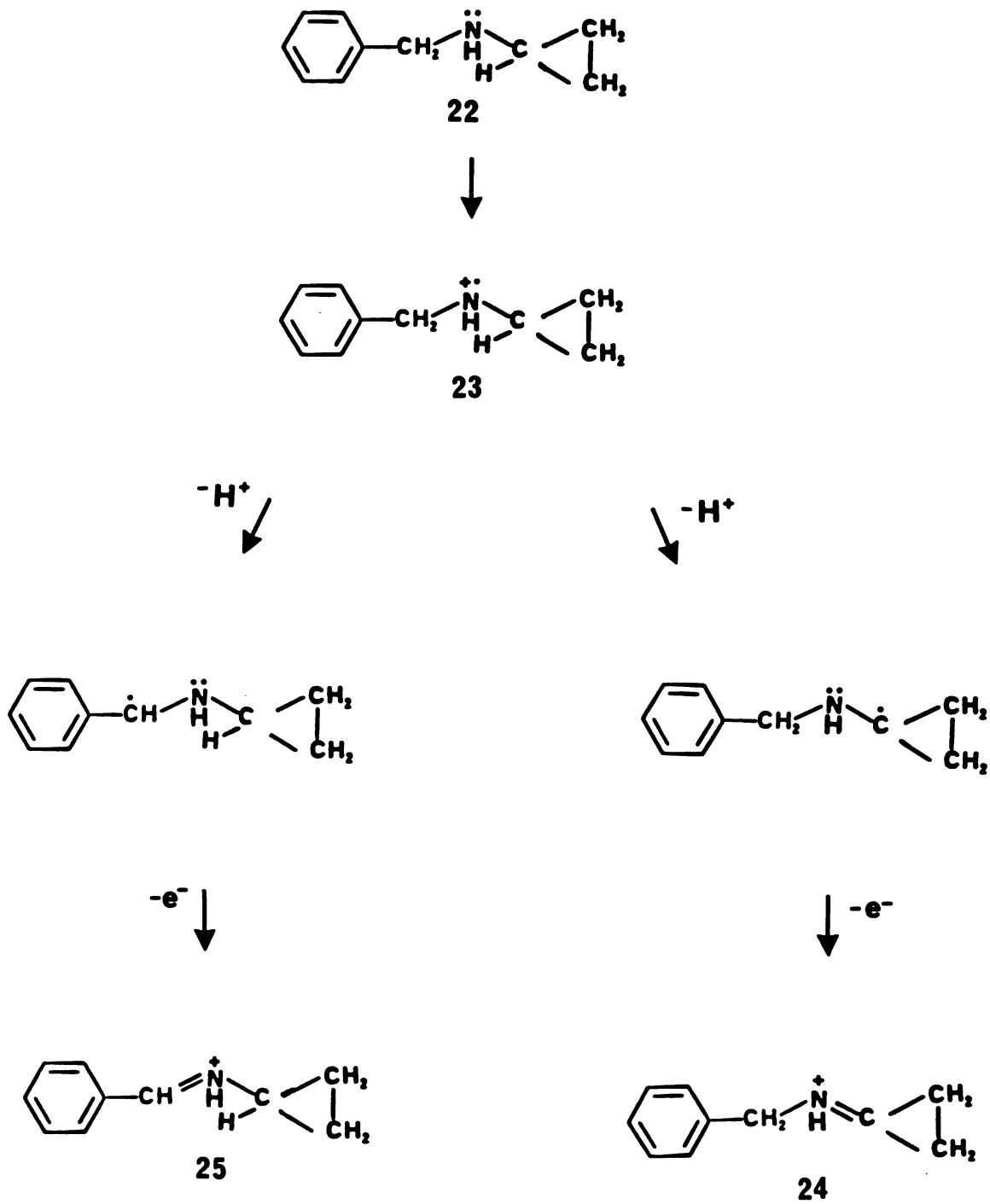


Figure 5. Proposed pathway for MAO catalyzed oxidation of N-cyclopropylbenzylamine (22).

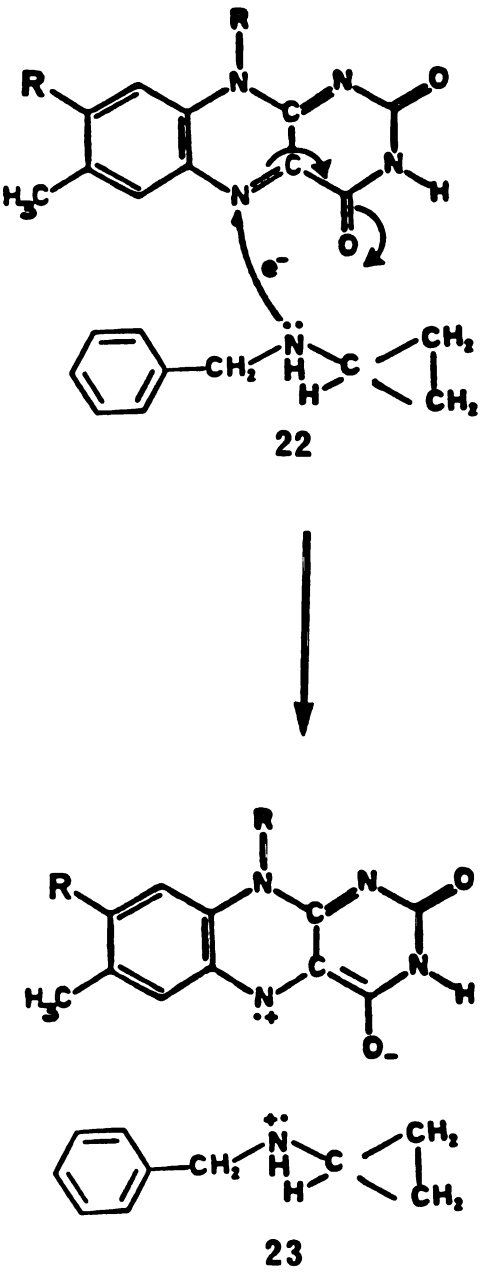
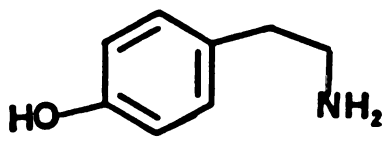


Figure 6. Proposed interaction of the flavin moiety of MAO with **22**.

Silverman *et al.*[77] proposed that similar to the electrochemical oxidation, the first electron transfer from the amine nitrogen to the flavin is rate determining. This proposal was substantiated by the observation that there was no kinetic deuterium isotope effect on the inactivation of MAO by deuterated-cyclopropylbenzylamine (radiolabeled on the cyclopropyl carbon).[77] However, other investigators[83] have found small (k_H/k_D = 1.2) isotope effects in experiments using tyramine (**27**) and kynuramine (**28**) substrates which suggests that although the first one-electron transfer from the nitrogen to the flavin may be rate limiting, it is probably reversible (see Figure 7).



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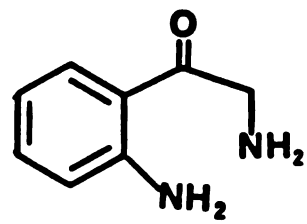

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Evidently, the loss of the α-proton and second electron in the subsequent steps are not much faster than the reverse reaction of step one. As shown in Figure 8, a rate determining step may be reversible as is the case in electrophilic sulfonation reactions.[84] Analogous to the sulfonation reaction in Figure 8, the energy barrier on either side of the aminium cation radical (Figure 7) could be about the same, thus some aminium radicals would go forward to lose the α-proton while others would accept the electron back from the flavoprotein of MAO to reform the starting amine.

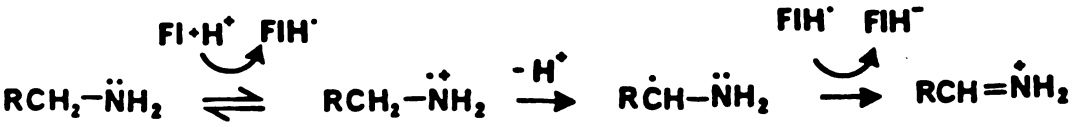
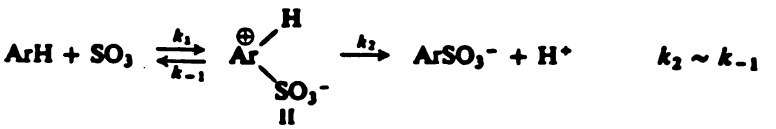


Figure 7. Proposed MAO catalyzed oxidation of amines.



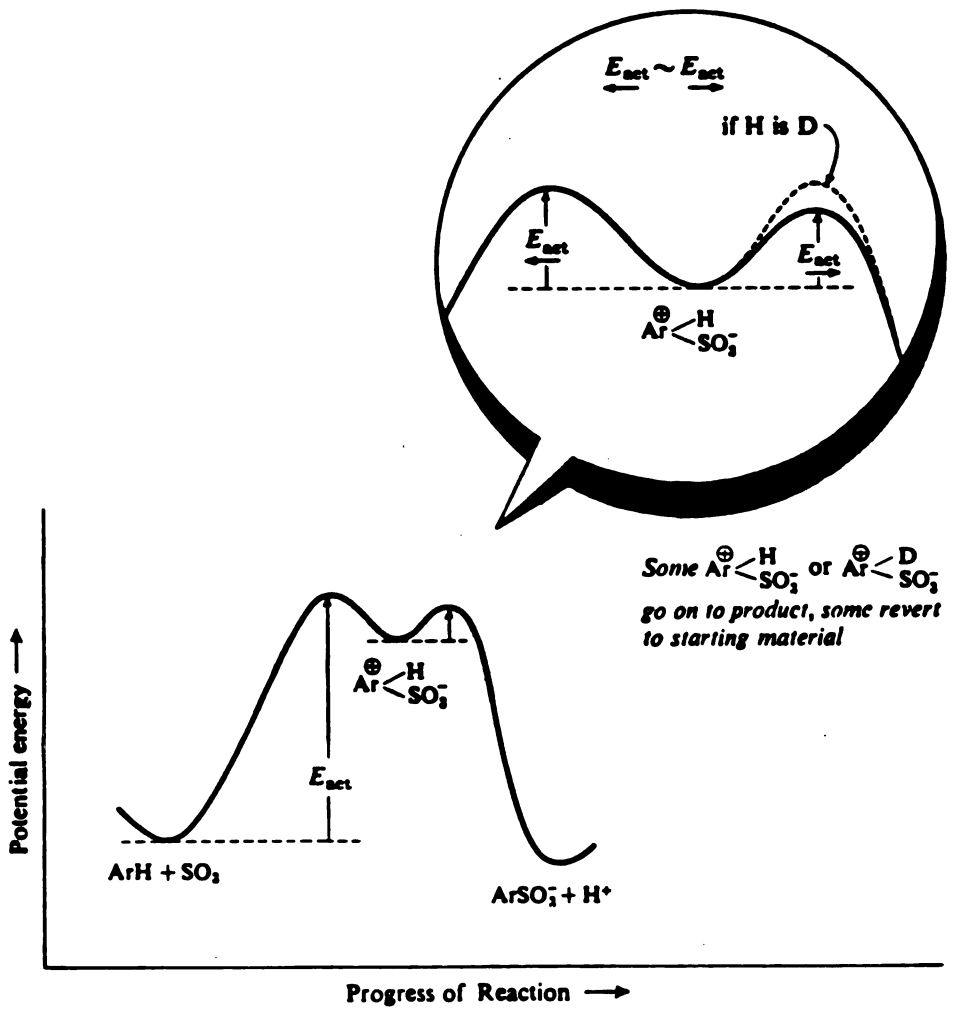
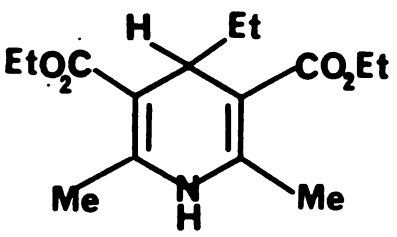


Figure 8. Proposed pathway of sulfonation.

In accordance with Silverman's work described above,we proposed that the MAO-catalyzed oxidation of MPTP might also involve a radical mechanism which may have bearing on its neurotoxic properties. It was proposed that an MPTP-aminium cation radical intermediate (**29**, Figure 9) would form by the transfer of an electron from the nitrogen lone pair to the flavin moiety of MAO-B. Further support for the formation of this species comes from studies of the electrochemical oxidation of tertiary amines wherein it has been shown that the transition state in the potential-determining step resembles the aminium radical.[81] Also, this successive one-electron transfer mechanism of biological oxidation has been suggested to be operative for other oxidative enzymes including cytochrome P-450 wherein spin trapping experiments have provided evidence for one-electron oxidation of 1,4-dihydropyridine substrates (i.e., of structure **30**).[85]



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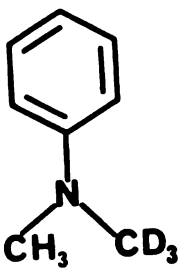

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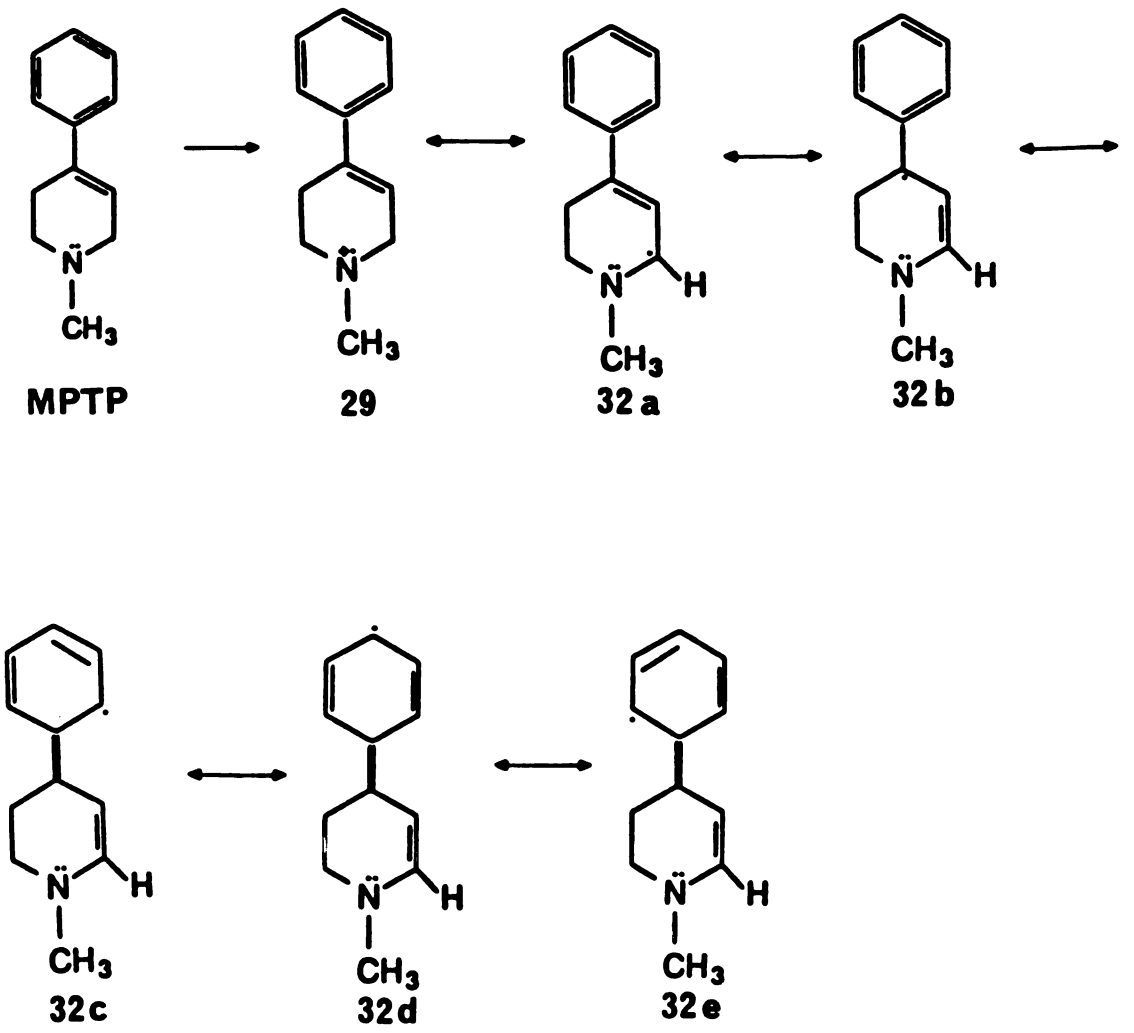
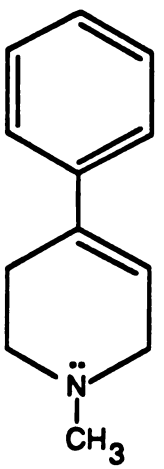


Figure 9. Proposed MAO-B catalyzed oxidation pathway for MPTP.

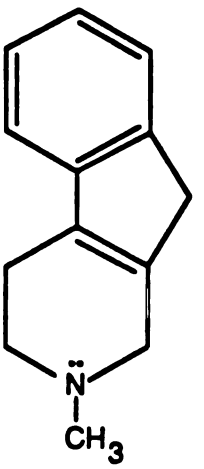
Given that the first one-electron transfer in MAO-catalyzed oxidation of amines may be reversible, one would expect large kinetic isotope effects ($k_H/k_D>3.6$)[86] if α-carbon-hydrogen abstraction were to occur as the rate determining step following aminium cation radical formation in the MAO catalytic sequence. However, both Silverman[77] and Belleau[83] observed only small isotope effects (k_H/k_D,1.2) indicating that spontaneous α-carbon deprotonation occurs as opposed to hydrogen atom abstraction.[86] This has also been shown to be the case for cytochrome P-450 and chloroperoxidase catalyzed oxidation of N-methyl-N-trideuteriomethylaniline (**31**).[86] Thus we hypothesized that following the formation of the MPTP aminium cation radical species **29**, α-carbon deprotonation and electron rearrangement to the neutral α-carbon centered radical **32a** (Figure 9) would occur. The formation of this intermediate was believed to be an important feature in the overall mechanism of the MAO catalyzed oxidation of MPTP. Previously, it had been established that the allylic double bond is required for MAO-B substrate activity[87,88] and that only the allylic position undergoes oxidative attack.[52] The presence of the double bond allylic to the nitrogen in MPTP allows for the delocalization of the α-carbon centered radical to the C(4)-position of the tetrahydropyridine ring (**32b**, Figure 9) and to the benzene ring to form the resonance structures **32**, **c-e** (Figure 9). In total, five resonance structures (**32**, **a-e**) contribute to the delocalization of the unpaired electron. The resonance stabilization of the carbon centered radical via the allylic bond was proposed to contribute to MPTP's exceptionally good MAO substrate properties. Following loss of the first electron, the two-electron oxidation product, $MPDP^+$ (**19**) forms via a second one-electron transfer to the flavin moiety of MAO. Electrochemical studies have shown that transfer of the second electron in amine oxidation occurs rapidly.[81]

In an effort to provide documentation of the structural features required of compounds with potential MPTP-like activity, we elected to focus our attention on the tetrahydroindenopyridine derivative 1,2,3,4-tetrahydro-2-methyl-9-H-indeno[2,1,-C]pyridine (**33**) and the tetrahydro-β-carboline derivative 2-methyl-1,2,3,4-tetrahydro-β-

carboline (**34**). These semirigid tricyclic compounds enabled us to examine the effect of locking the piperidiene ring into a configuration which is nearly coplanar with that of the benzene ring. This is in contrast to MPTP where in solution and/or at the surface of MAO-B the molecule may adopt a full range of rotamers with respect to the phenyl and piperidiene rings about the C(4) axis. It should be noted however, that the solid state crystal structure of MPTP is planar.[89] These semirigid analogs also provided us with an opportunity to probe the steric effects of an additional methylene or nitrogen substituent at the C(5) position of MPTP.



MPTP



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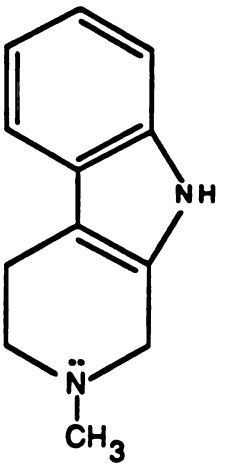
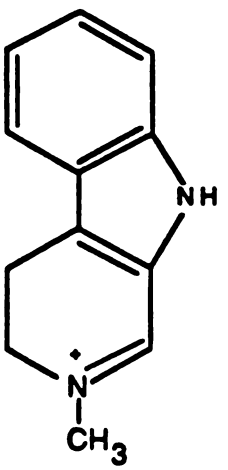


34

Since the 4a-9a bond of the tetrahydro-β-carboline **34** possesses aromatic character as opposed to purely allylic character, this compound provided an opportunity to further assess the importance of the allylic double bond in MPTP-type molecules. If one assumes that the formation and stabilization of an α-carbon centered radical is a crucial step in the MAO-catalyzed oxidation of tertiary allylamines, then the true allylic character of the 4a-9a bond of the tetrahydroindenopyridine **33** would allow for delocalization of a carbon

centered unpaired electron following formation of an aminium radical cation (see structures **35** and **36 a-c**, Figure 10) analogous to MPTP oxidation. However, in the tetrahydro-β-carboline **34**, such resonance stabilization of the carbon centered radical via an allylic bond is compromised by the aromatic character of the 4a-9a bond in this system. Thus we anticipated that the tetrahydroindenopyridine **33** might be a somewhat better MAO-B substrate than the tetrahydro-β-carboline **34**.

The tetrahydro-β-carboline **34** has neurotoxicological relevance in that this compound has been detected in the brains of normal laboratory rats[90] and is believed to form *in vivo* via a Pictet-Spengler type condensation between N-methyltryptamine and formaldehyde (see Figure 11).[91] Although this indole derivative may lower striatal levels of dopamine in mice[92,93], it does not appear to be neurotoxic in monkeys.[94,95] Based on results obtained with colorimetric assays and rat mitochrondrial preparations, Gibb *et al.*[96] suggested that **34** is not an MAO-A or MAO-B substrate. Therefore, the lack of MPTP-like neurotoxicity observed in the primate studies might reflect insufficient production of the dihydro-β-carbolinium and β-carbolinium species **37** and **38**, respectively. The neurotoxicity of the β-carbolinium **38** will be discussed in part III of this thesis.



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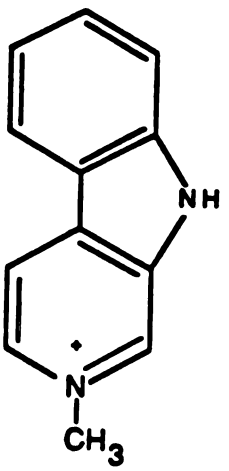

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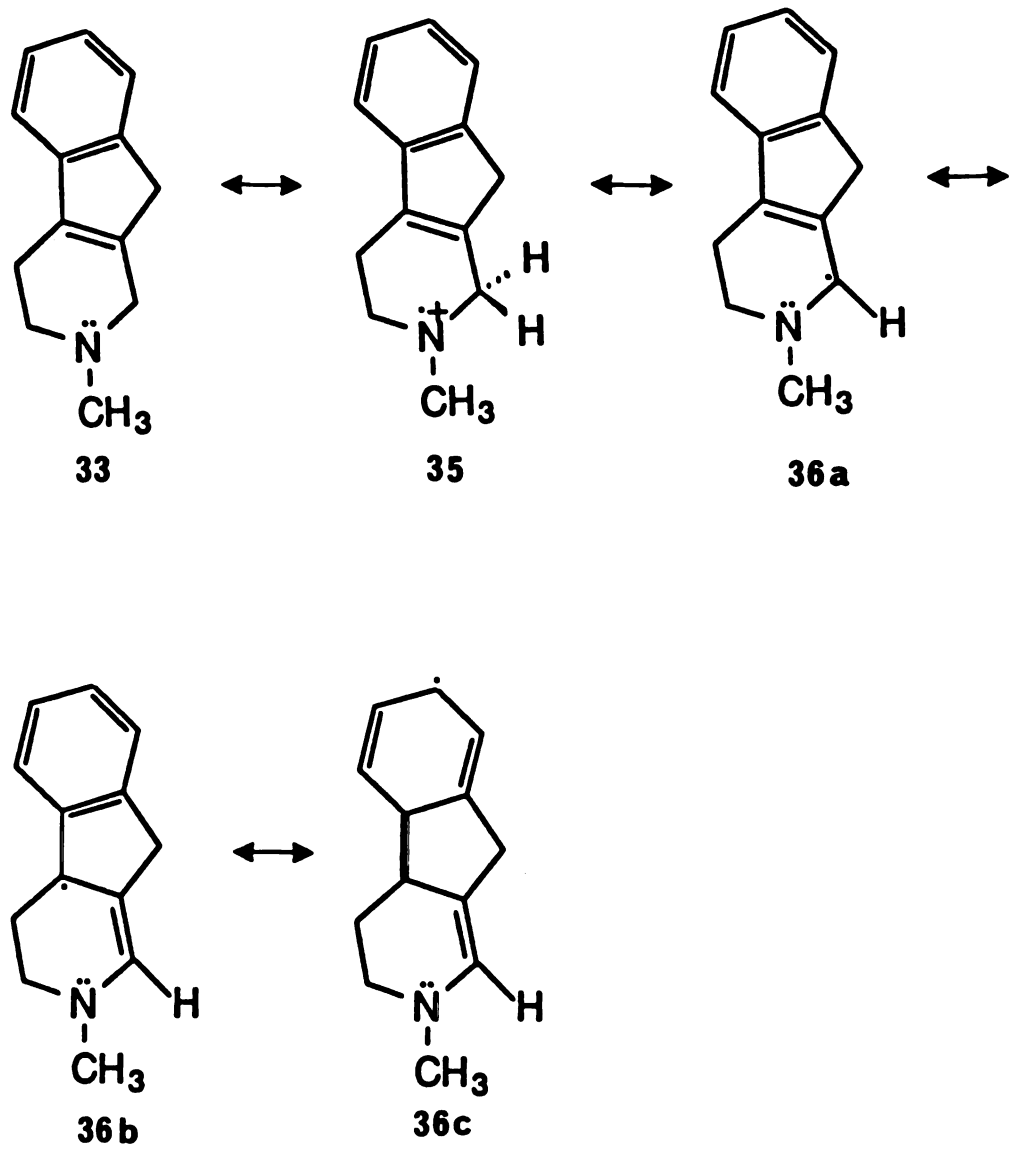


Figure 10. Proposed MAO-B catalyzed oxidation pathway for tetrahydroindenopyridine **33**

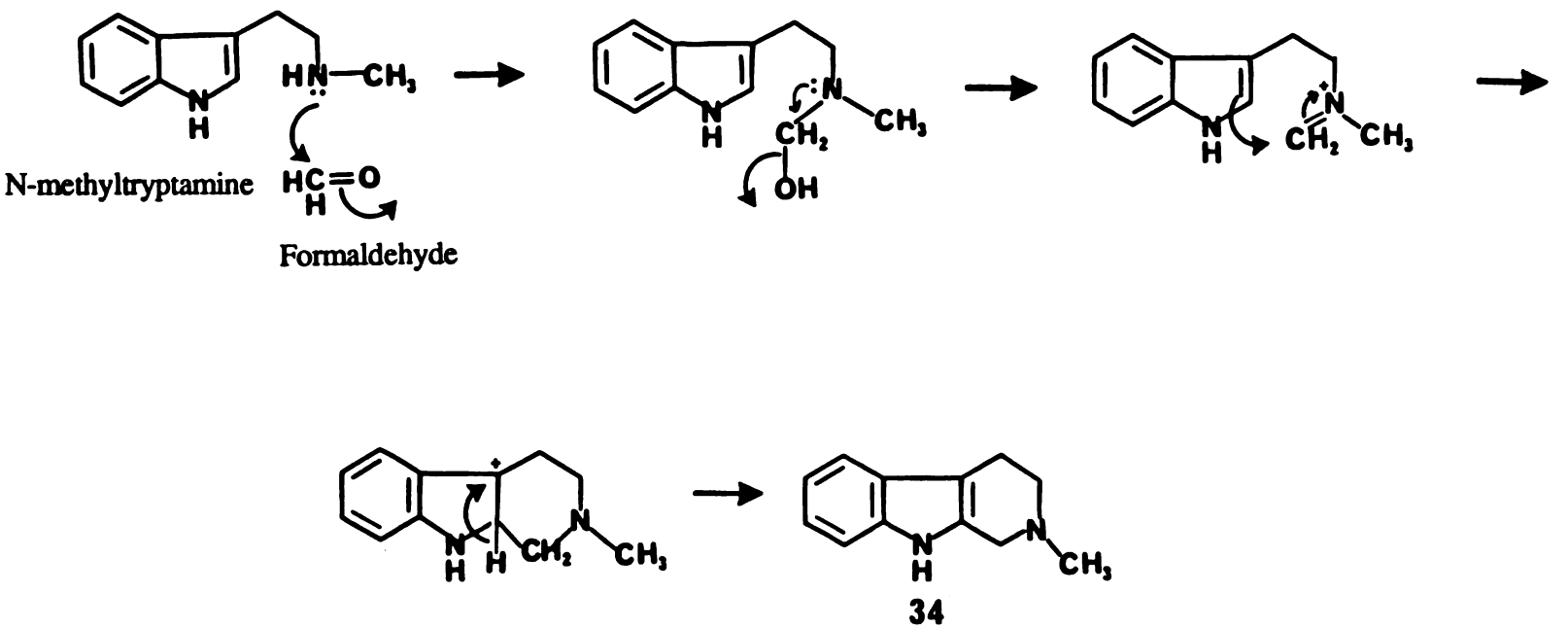
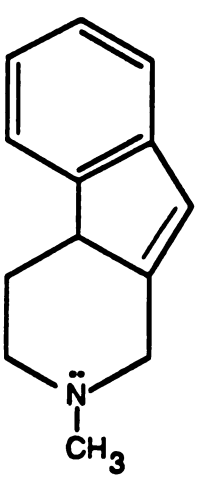


Figure 11. Proposed *in vivo* formation of tetrahydro-β-carboline **34** from N-methyltryptamine.

In view of the recent report that tetrahydroisoquinoline (**39**), an amine detected in human brain tissue[97] produces a parkinsonian syndrome in marmosets[98], we also elected to study the MAO-B substrate activity of **39**, its N-methyl analog **40**, and the corresponding tetrahydroquinolines **41** and **42** (For structures, see figure 12). We noted that in **39** and **40**, the C(1) α-carbon atom is benzylic and therefore by analogy with allylic position of MPTP, these amines may be MAO-B substrates. While the MAO substrate properties of **39**, **40**, **41**, and **42** have not previously been examined, it has been reported that tetrahydroisoquinoline (**39**) and tetrahydroquinoline (**41**) do not inhibit MAO-A or B.[99]

The isomeric tetrahydroindenopyridine compound **49** was obtained as a side product during the synthesis of **33** (see Figure 13). Compound **49** allowed us to assess the effects on MAO-B substrate activity of a molecule bearing an allylic double bond positioned exocyclic with respect to the tetrahydropyridine ring system. Futhermore, in view of the MAO-B substrate and inhibitor activity of MPDP+[56], we also were interested in the interaction of the planar 3,4-dihydroindenopyridinium species **50** with the enzyme.



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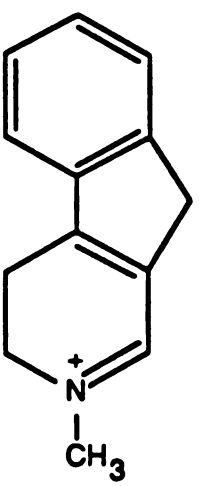

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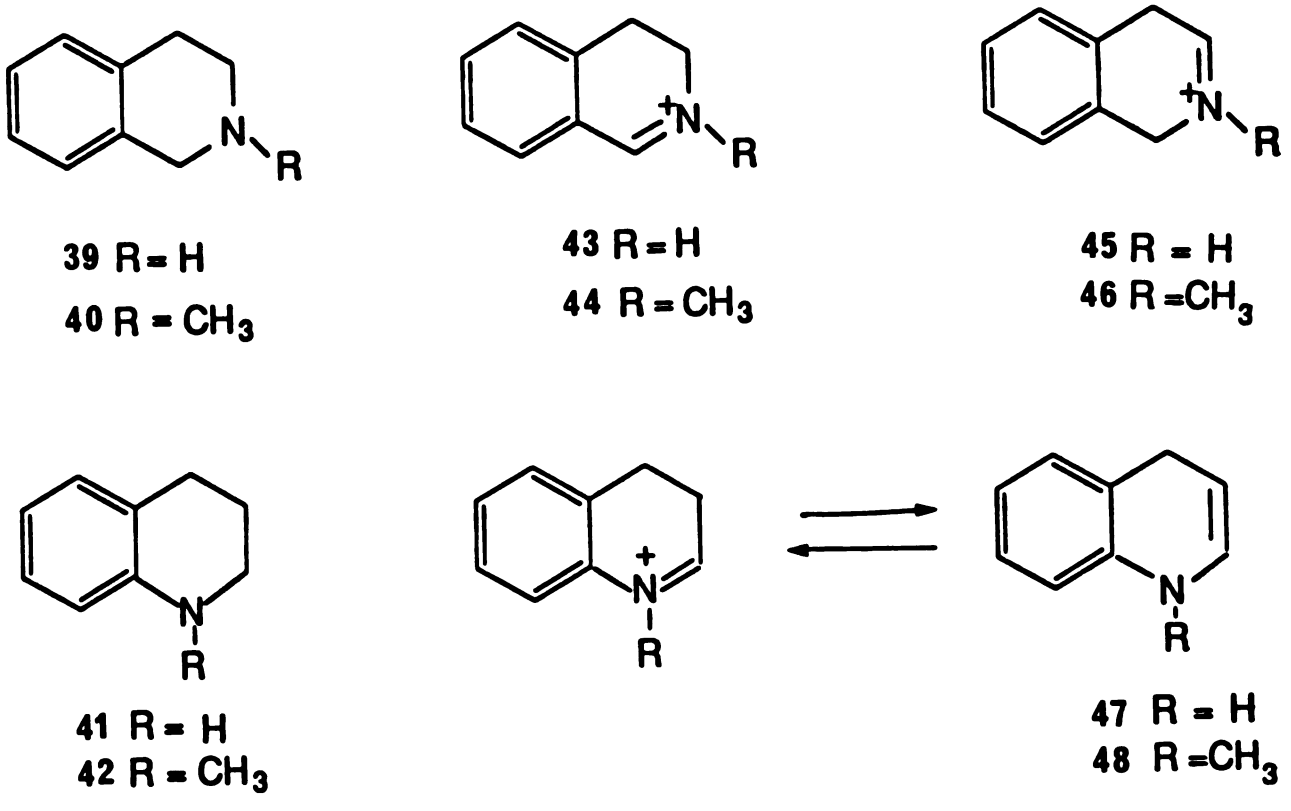


Figure 12. Structures of isoquinoline and quinoline derivatives.

Previous studies with MPTP (λ_{max} 245 nm) employed a UV-based spectrophotometric assay to monitor for the appearance of the MAO oxidation products $MPDP^+$ (λ_{max} 345 nm) and MPP^+ (λ_{max} 290 nm).[56] We anticipated that the tetrahydroindenopyridine **33** (as well as the isomer **49**) would have a UV absorbance similar to that of MPTP and that the corresponding MAO-derived oxidation products, the 3,4-dihydroindenopyridinium **50** and indenopyridinium **52** would have UV characteristics similar to $MPDP^+$ and MPP^+, respectively. Thus we proposed to monitor the MAO catalyzed metabolism of **33** using a UV method similar to the one described for MPTP. Similarly, the expected MAO-derived oxidation products of the tetrahydro-β-carboline **34** (λ_{max} 274 nm)[100], the 3,4-dihydro-2-methyl-β-carbolinium species (**37**, λ_{max} 360 nm)[101] and the N-methyl-β-carbolinium species (**38**, λ_{max} 305 nm)[100] could be monitored spectrophotometrically.

The tetrahydroisoquinoline and tetrahydroquinoline derivatives **39-42** do not have strong absorbance chromophores above 200 nm in the UV region. However, their 3,4-dihydro (**43** and **49**), 1,2-dihydro (**45** and **46**), and 1,4-dihdyro (**47** and **48**) oxidation products are known and have UV λ_{max} chromophores between 270-300 nm[102,103,104] and therefore should be easily detected in our proposed assay system.

Synthetic Work

Results and Discussion.

The synthetic scheme for the tetrahydroindenopyridine **33** (shown in Figure 13) followed a pathway reported previously by Plati *et al.*[105] for the preparation of 1,2,3,4-tetrahydro-2-methyl-9-phenyl-9-H-indeno[2,1-C]pyridine (**53**). Treatment of the free base of commercially available arecoline hydrobromide (**54** HBr) with phenylmagnesium bromide followed by distillation of the crude isolate yielded a yellow oil identified by its ^{1}H NMR spectrum (see Figure 14) as an approximately equimolar mixture of the methyl ester methyl(*cis, trans*)-1-methyl-4-phenyl-3-piperidinecarboxylate (**55**). In the interest of time and in consideration of the lack of geometric isomerism in the desired product **33**, the separation and characterization of the *cis* and *trans* isomers of **55** was not pursued. The hydrolysis of **55** with hydrochloric acid gave the hydrochloride salt of only one of the two possible epimeric 1-methyl-4-phenyl-3-piperidinecarboxylic acids **56**. It was not possible to establish the stereochemistry of this product on the basis of its ^{1}H NMR spectrum. Overall, this hydrolysis reaction proceeded quite slowly (8 hours) and gave a modest yield (40%). Longer or shorter reaction times resulted in even poorer yields. It seems reasonable to assume that the slow reaction rate is due to the formation of the intermediate dicationic species **57**. The energy barrier for formation of this intermediate is probably quite high due to the destabilizing effects of a doubly charged molecule. Cyclization of the piperidinylcarboxylic acid **56** with aluminum chloride under Friedel-Crafts conditions gave a single tricyclic ketone identified as 1,2,3,4,4a,9a-hexahydro-2-methyl-9-H-indeno[2,1C]pyridin-9-one (**58**). This product was obtained in 60% yield directly via cyclization of the methyl ester **55** with polyphosphoric acid according to the method described by Clarke.[106]

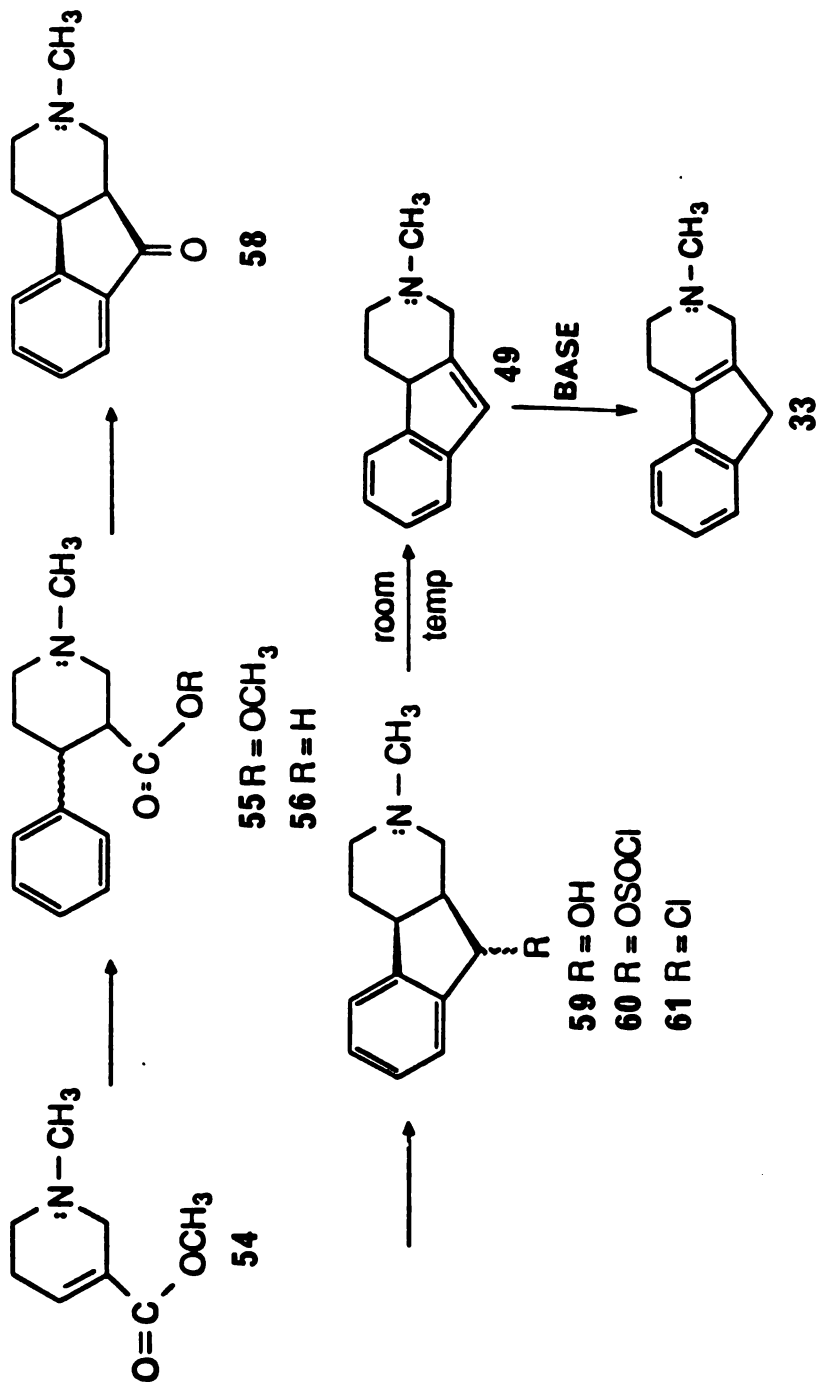


Figure 13. Synthetic pathway for tetrahydroindenopyridine 33.

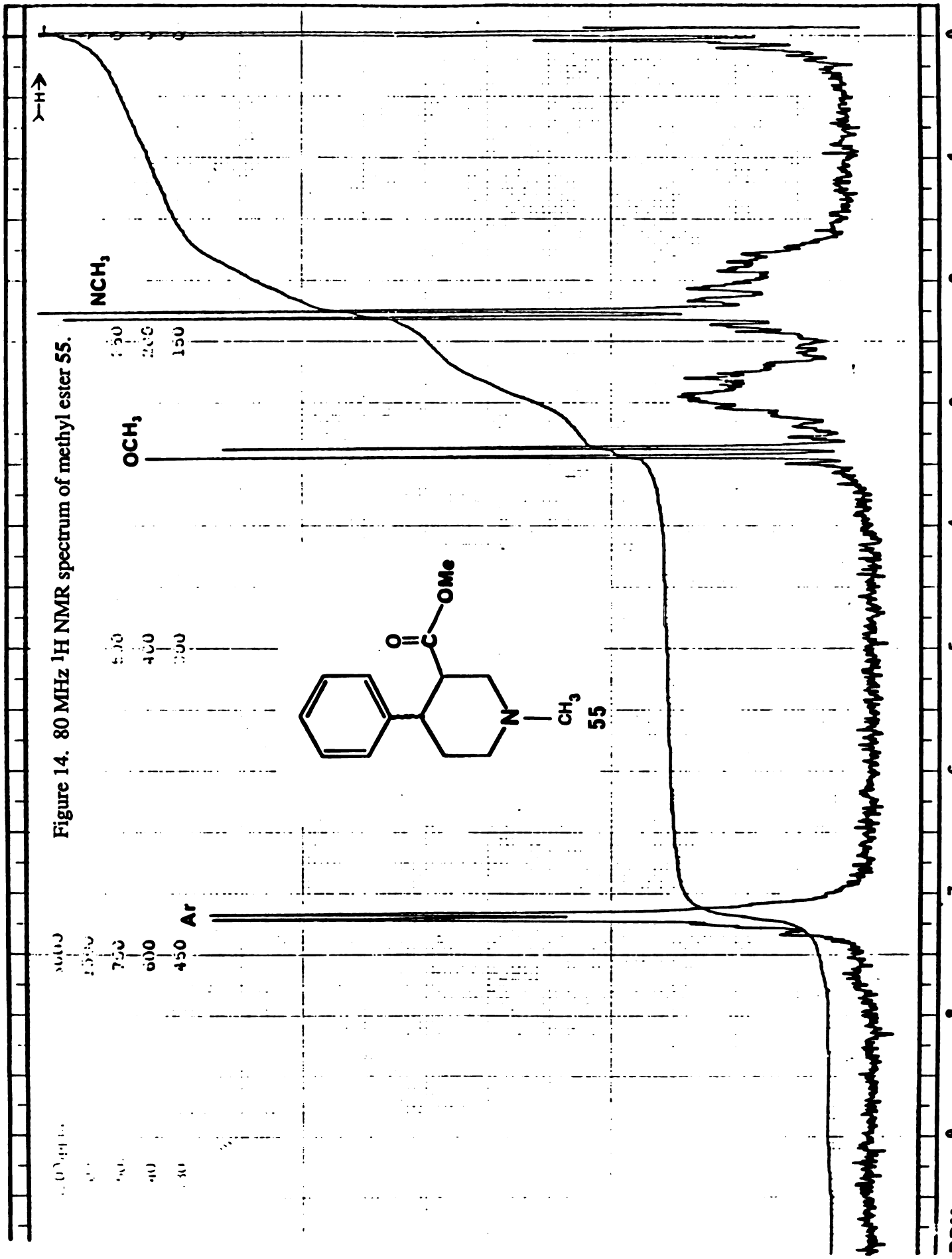
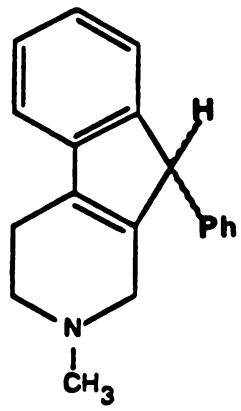


Figure 14. 80 MHz ^1H NMR spectrum of methyl ester **55**.



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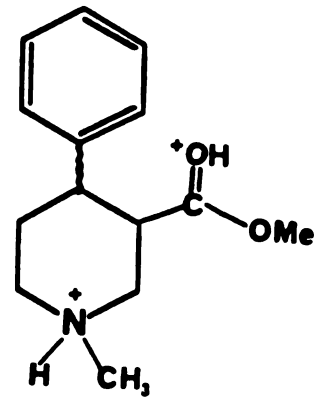

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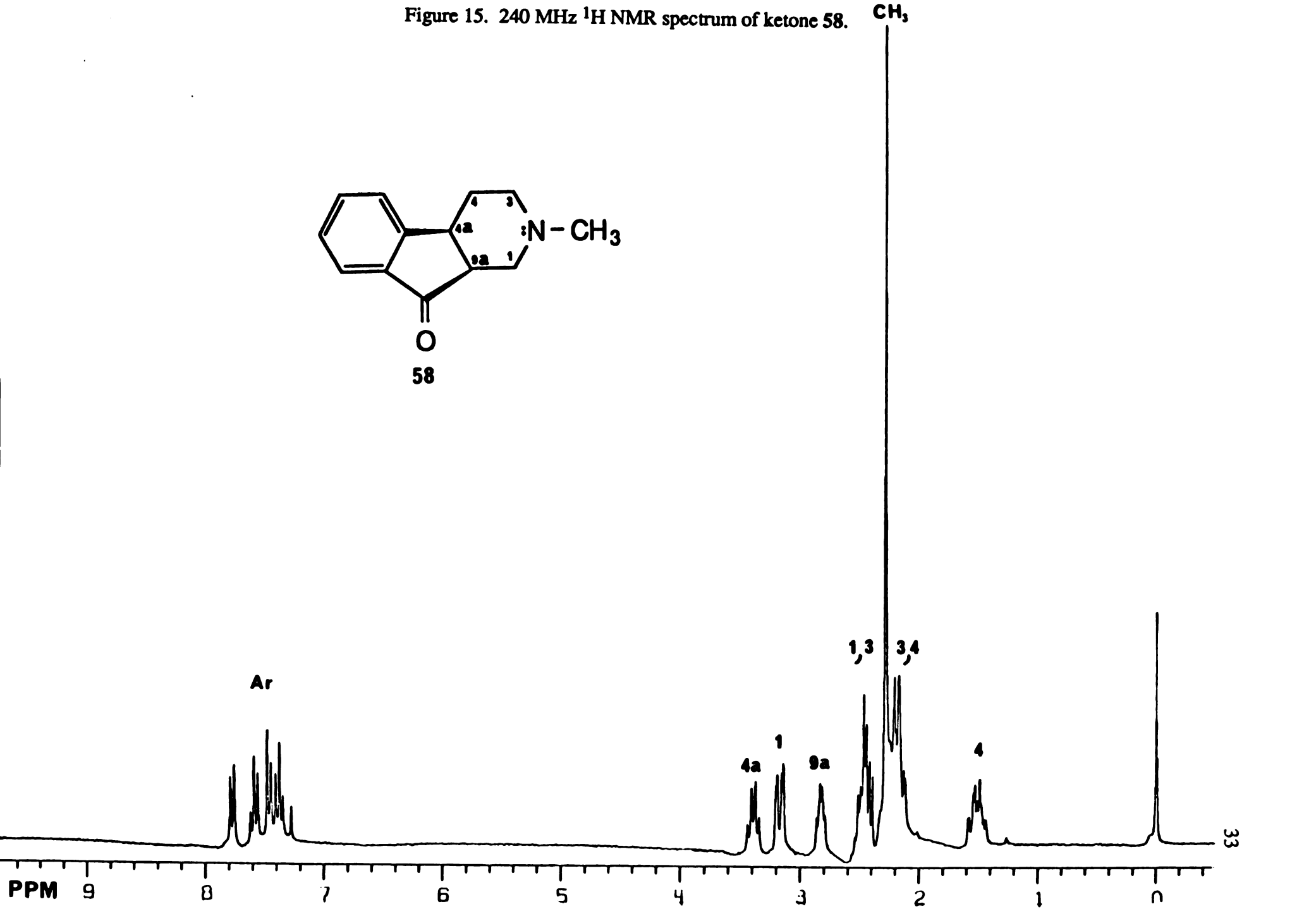
The assignment of the proton resonance signals in the 240-MHz ^{1}H NMR displayed in Figure 15 is consistent with the structure of ketone **58**. The *cis* stereochemistry of this product was established by double resonance experiments as follows: Irradiation of the signal from C(9a)-H at 2.8 ppm resulted in the collapse of the C(4a)-H quartet (3.4 ppm) into a symmetrical triplet, J = 7.2 Hz, corresponding to the couplings between C(4a)-H and each of the C(4)-methylene protons. Examination of Dreiding molecular models indicated that when the piperidine ring is in the chair conformation with the C(4a)-H in the axial position and the C(9a)-H in the equatorial position, the dihedral angles for C(4a)-H and C(4)-H*axial* and for C(4a)-H and C(4)-H*equatorial* are approximately 140° and 15°, respectively. Based on the Karplus relationship, this *cis* configuration is consistent with the observed symmetrical coupling constant of 7.2 Hz. If the stereochemistry had been *trans*-diequatorial, then the coupling between the C(4a)-H and C(4)-H*axial* would have been near 10 Hz (180° dihedral angle) and the coupling between the C(4a)-H and C(4)-H*equatorial* would have been near 4 Hz (45°

dihedral angle). Irradiation of the C(9a)-H signal also simplifies the signal at 3.15 ppm, assigned to one of the C(1) protons, to a doublet, J = 10.8 Hz corresponding to the coupling with the other C(1)-proton. This is in fact the Karplus gem coupling constant for the 112° angle between the C(1)-methylene protons observed in the molecular model. When the C(9a)-proton is allowed to couple with the C(1)-protons, the signal at 3.15 ppm is further split into a characteristic doublet of doublets, with symmetrical coupling constants of 3.6 Hz. This is in agreement with the Karplus correlation for an equatorial C(9)-H which is at an approximate 45°-dihedral angle with each of the C(1)-protons as observed in the *cis*-fused Dreiding model. Again, if the ring fusion had been *trans*-diequatorial, the coupling between the C(9a)-H and C(1)-H*axial* would have been about 10 Hz (180° dihedral angle) and the coupling between the C(9a)-H and C(1)-H*equatorial* would have been 1.5 Hz (60° dihedral angle). Therefore, in agreement with literature precedence[107,108] for *cis*-fusion of similar ring systems, we assign the stereochemistry of the ring fusion in ketone **58** as *cis* (4a-axial, 9a-equatorial).

Reduction of the ketone **58** with lithium aluminumhydride gave a mixture of diastereomeric alcohols **59** which could be separated by fractional crystallization. These isomers gave essentially identical FTIR and ^{1}H NMR spectra which precluded the possiblility of making stereochemical assignments. Clarke *et al.*[106] used sodium borohydride to reduce ketone **58** and obtained only one of the two possible epimeric alcohols which they identified as *cis*-**59** because its IR spectrum showed only sharp intramolecular hydrogen bonding (3500 cm^{-1}) when diluted to 5 mM. Although the solvent (CCl_4) and compounds were carefully dried, attempts to reproduce Clarke's IR results failed even with the use of the more sensitive FTIR which detected broad intermolecular hydrogen bonding (3300 cm^{-1}) that was still present at 1 mM (see Figure 16).

Figure 15. 240 MHz ^{1}H NMR spectrum of ketone 58.



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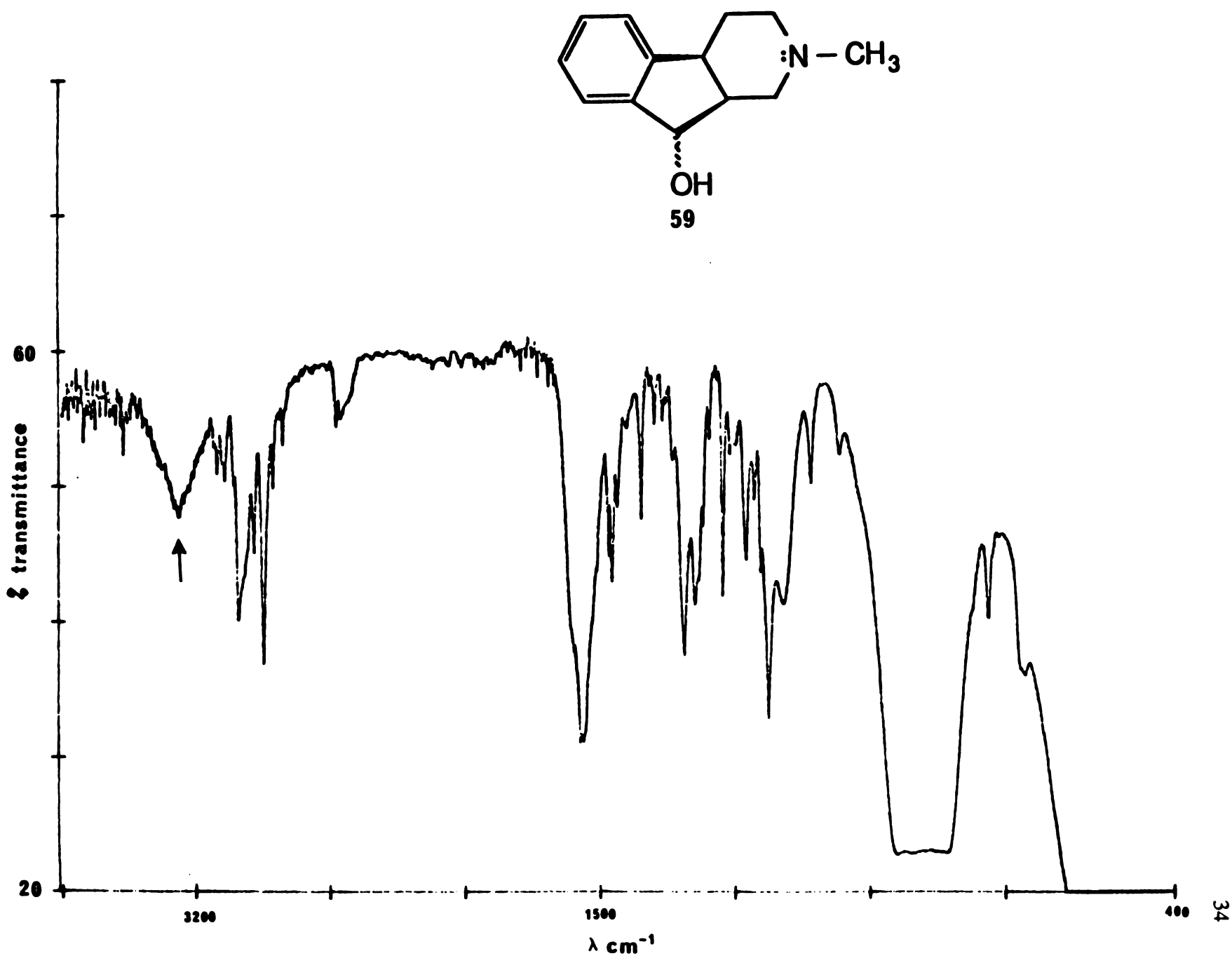
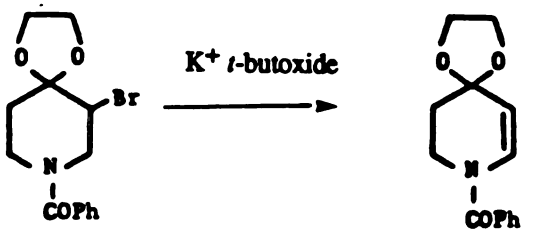


Figure 16. FTIR spectrum of alcohol **59** (1 mM in CCl_4).

It is possible that the intermolecular hydrogen bonds between the alcohol molecules are much stronger than the intramolecular hydrogen bonds within the molecules and thus are not diluted out at the concentration required for FTIR detection (1 mM). On the other hand, intramolecular hydrogen bonding could predominate over intermolecular hydrogen bonding in both *cis* and *trans*-**59** and thus one would expect no loss of absorbance in the 3300 cm^{-1} IR region even at high dilution. The only clue we have to the stereochemistry of **59** then is that the isomer with the lower melting point (124-125 °C) also had the higher r_f value on silica gel TLC ($r_f = 0.28$). This would seem to indicate intramolecular hydrogen bonding wherein the attractive forces within molecules are stronger than the attractive forces between molecules (intermolecular hydrogen bonding) thus providing a lower melting point. Futhermore, molecules that exhibit a greater degree of intramolecular hydrogen bonding would not interact with the hydroxyl groups of the silica gel as strongly and thus would have a higher r_f value. Based on these arguments then, the stereochemistry of the **59** isomer with the lower melting point and higher r_f value might be assigned as *cis*. This configuration would provide the minimal intramolecular distance between the hydroxyl and nitrogen groups and thus facilitate intramolecular hydrogen bonds. Clarke *et al.*[106] reported a melting point of 135-137 °C for the compound which they identified as *cis*-**59**; this melting point does not correspond to either of the isomers reported here. Clarke did not report spectral data other than IR nor did they report elemental analyses.

Room temperature treatment of the diastereomeric alcohols **59** with thionyl chloride (Figure 13) gave a mixture of compounds which following separation by column chromatography, yielded two crystalline compounds. The most polar compound was identified by its ^1H NMR, mass spectra, and elemental analysis as one of the two possible 9-chlorohexahydroindenopyridine derivatives **61**. Assignment of the stereochemistry of this intermediate was not pursued. The second product displayed a UV spectrum with λ_{max} 250 nm and mass spectral characteristics expected for a tetrahydroindenopyridine system. Proton NMR analysis (see Figure 17) revealed a 1-proton singlet at δ 7.0 ppm

which identified this compound as the $\Delta^{9,9a}$ olefin **49** that would form via elimination of the thioester intermediate **60** (see Figure 13). Although ^{1}H NMR analysis confirmed that compound **49** could rearrange to the desired product **33** (loss of the olefinic signal, see Figure 18) under relatively mild basic conditions (10% sodium hydroxide in methanol, room temperature), a method was desired to convert the 9-chloro derivative **60** directly to **33** and avoid the need of column purification. Schell and Williams[109] had shown that N-benzoyl-3-bromo-4-piperidone ethylene ketal (**62**) could be dehydrohalogenated to N-benzoyl-4-oxo-1,2,3,4-tetrahydropyridine ethylene ketal (**63**) with ease using potassium *tert*-butoxide in anhydrous tetrahydrofuran.



62 **63**

Thus, the tetrahydroindenopyridine **33** was prepared more efficiently by heating the crude mixture obtained in the thionyl chloride reaction with potassium *tert*-butoxide followed by purification (recrystallization) of the allylamine as its hydrochloride salt. As required, no olefinic proton signal was observed in the ^{1}H NMR spectrum of this compound (see Figure 18).

Figure 17. 240 MHz ^{1}H NMR spectrum of tetrahydroindenopyridine **49**.

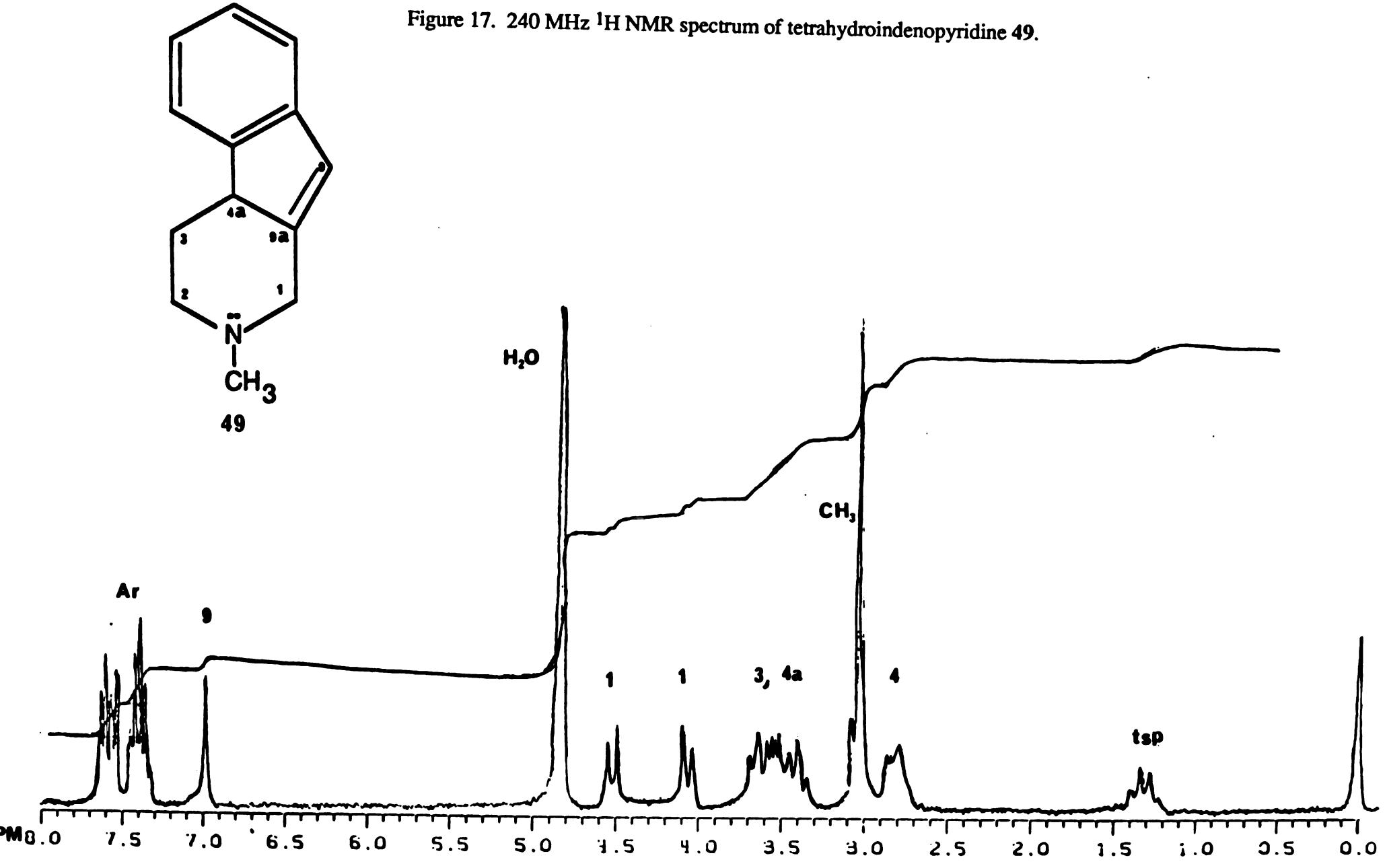
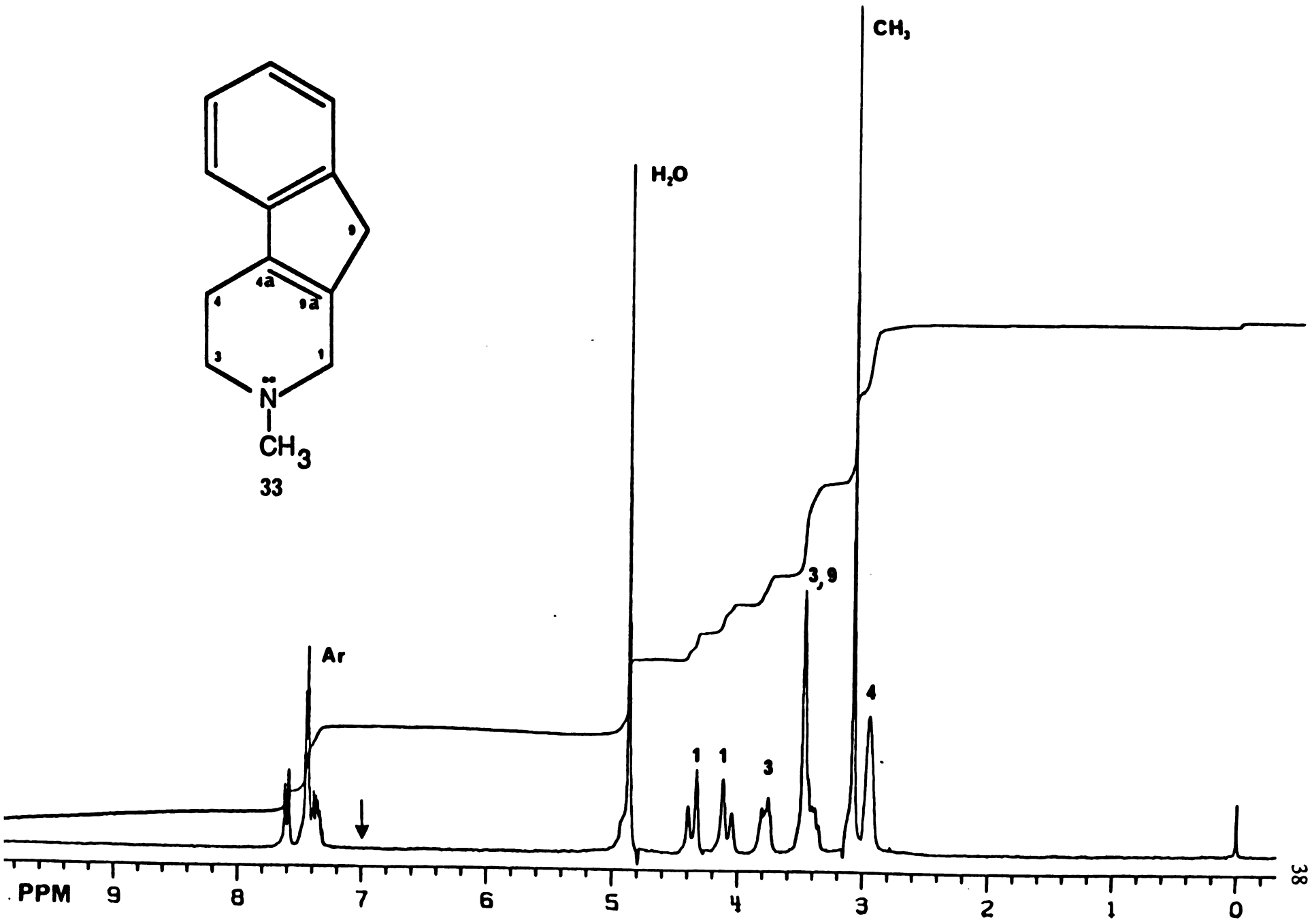


Figure 18. 240 MHz ^{1}H NMR spectrum of tetrahydroindenopyridine 33.



33

The scheme for the synthesis of the dihydroindenopyridinium species **50** is shown in Figure 19. The tetrahydroindenopyridine **33** was oxidized to the corresponding N-oxide **64** by heating the free base **33** under reflux with hydrogen peroxide and subsequent crstallization from benzene according to the method of Weissman *et al* [47]. Treatment of **64** with trifluoroacetic anhydride (TFAA) led to the formation of a product which displayed UV spectral characteristics (λ_{max} 360 nm) expected for the dihydroindenopyridinium species **50**. The crude product was isolated via its cyano adduct **65** which upon treatment with ethanolic perchloric acid[47,51] yielded the crystalline perchlorate salt of **50**. Unlike $MPDP^+$ (**19**) which undergoes autoxidation[52] and, at concentrations greater than 100 μM, disproportionation[52], the corresponding methylene-bridged dihydroindenopyridinium species **50** proved to be remarkably stable. UV scans (see Figure 20) of a 100 μM solution of **50** in pH 7.2 phosphate buffer showed no evidence of decomposition at 25 °C for at least 15 hours and a 10 mM solution was stable for at least 4 hours.

The oxidation of the dihydroindenopyridinium **50** to the indenopyridinium compound **52** proceeded with some difficulty. Gessner and Brossi[110] reported that $MPDP^+$ could be oxidized to MPP^+ in 4 hours in the presence of air and platinum catalyst in benzene. However, even after stirring and heating **50** with platinum and oxygen in benzene for 24 hours, UV spectroscopy indicated no formation of a chromophore that would correspond to the indenopyridinium species **52**. Previously, Buckley *et al.*[111] had reported the oxidation of **66** to **67** in benzene using chloranil (**68**) as the oxidizing agent which is reduced to the hydroquinone product **69** (see Figure 21). Following this method (see Figure 22), the dihydroindenopyridinium species **50** was stirred with chloranil in benzene at room temperature and monitored by UV spectroscopy for formation of the indenopyridinium species **52**. The oxidation proceeded quite slowly and was stopped after a period of 70 hours. Aqueous hydrochloric acid was added and the mixture was extracted with ether to remove remaining chloranil and the hydroquinone reaction product.

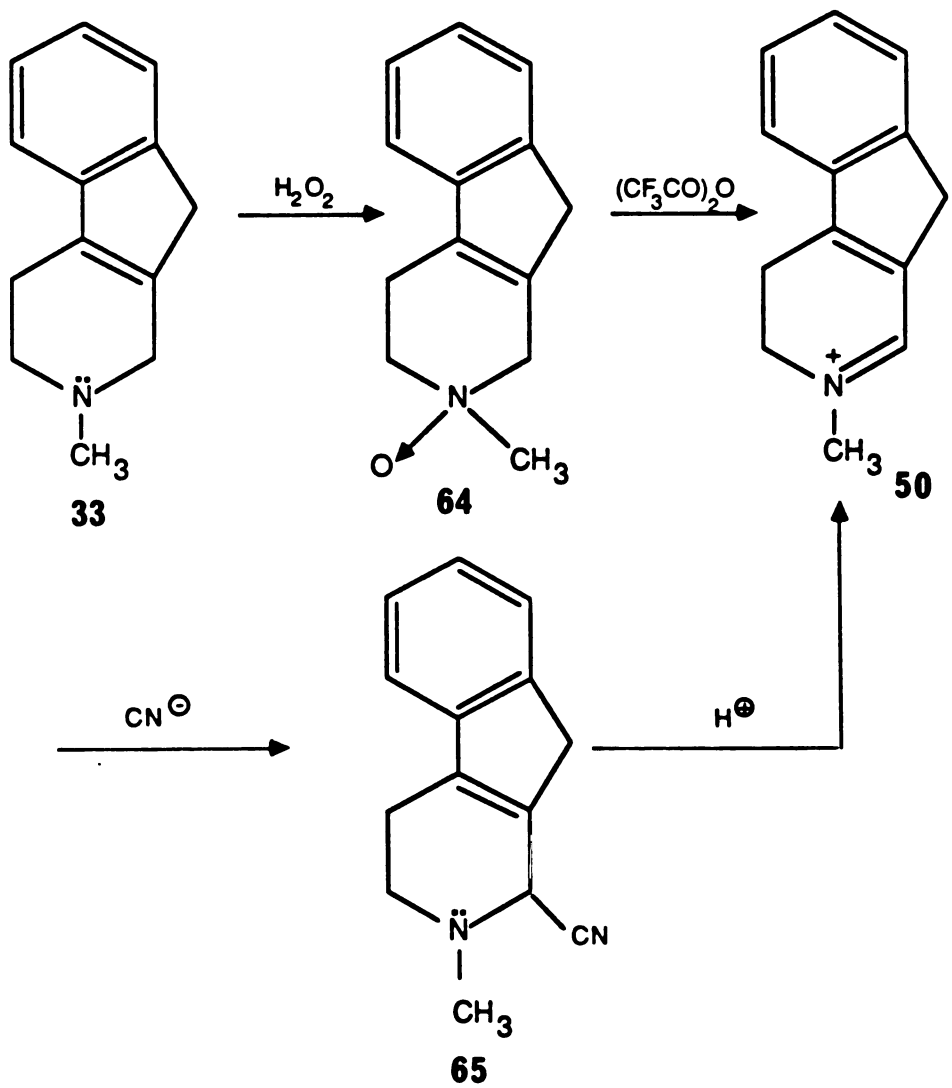


Figure 19. Synthetic pathway for dihydroindenopryidinium species **50**.

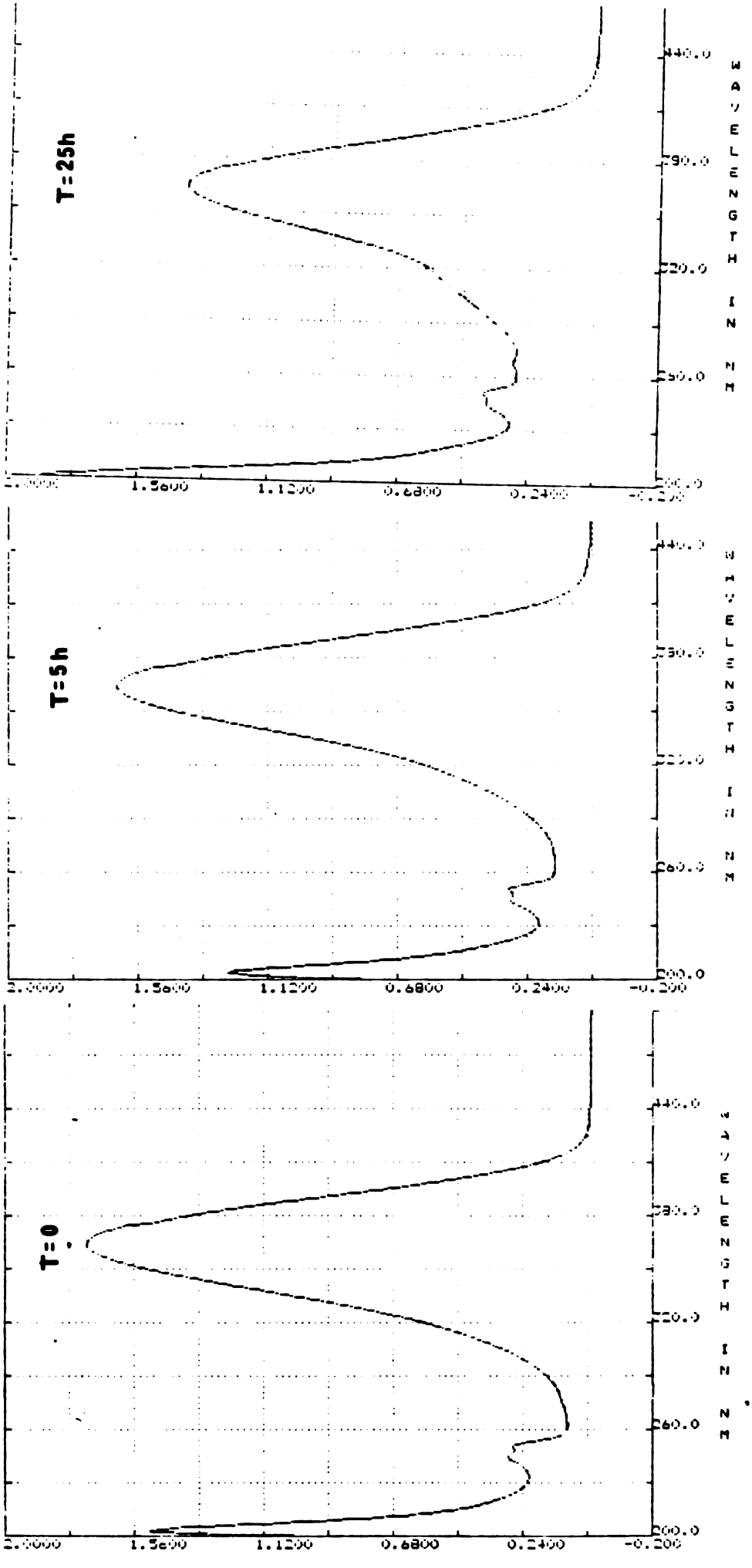


Figure 20. UV spectrum over time of 100 μM dihyroindenopyridinium 50, pH 7.2.

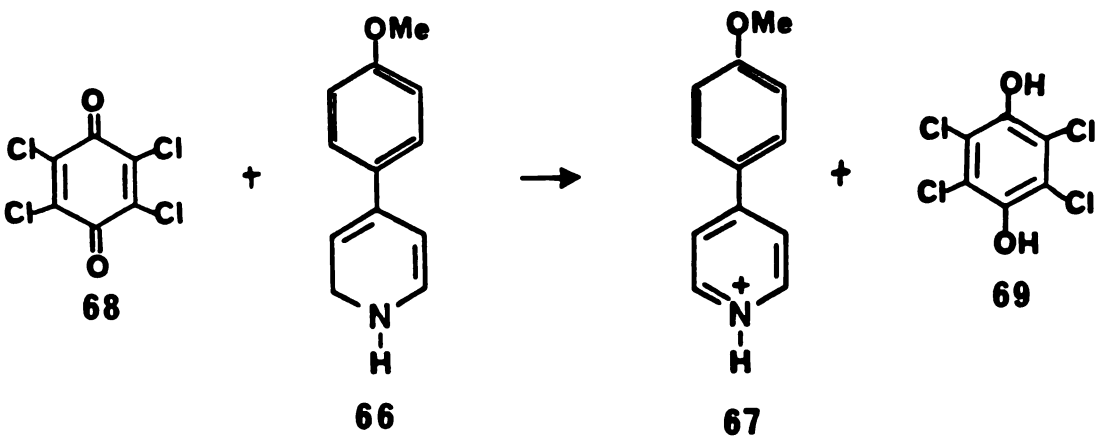


Figure 21. Oxidation of dihydropyridine **66** by chloranil (**68**).

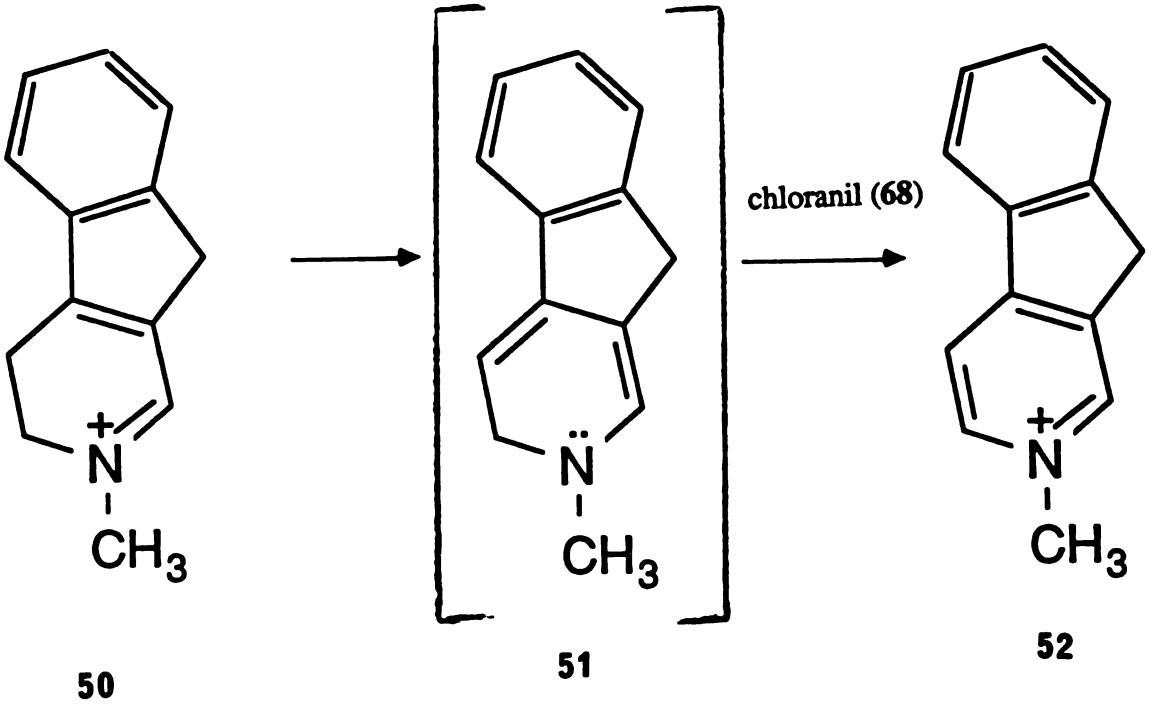


Figure 22. Synthesis of the indenopyridinium species **52** from the dihydroindenopyridinium species **50**.

After lyophilization, the solid was dissolved in water, brought to pH 10, and then extracted with benzene to remove unreacted traces of the dihydro free base **51**. A second lyophylization provided the indenopyridinium species **52** as a purple solid which was crystallized from acetone. Since the oxidation of dihyropyridinium compounds is believed to involve the intermediate formation of a dihydropyridine free base[111,112] and Dreiding molecular models indicate that the dihydroindenopyridine free base **51** (Figure 22) is a strained ring system, we believe that this may explain the long reaction time required for oxidation of **50**. The enhanced stability and resistance to oxidation of the dihydroindenopyridinium **50** compared to $MPDP^+$ (**19**) also may in part be due to the ring strain present in the free base **51.**

The β-carbolinium compound **38** was prepared by reaction of norharman (**70**) with methyl iodide[100] (see Figure 23). The reduction of **38** with sodium borohydride provided the free base tetrahydro-β-carboline **34** (Figure 24) which was converted to its hydrochloride salt.

The N-methylisoquinolinium and N-methylquinolinium species **72** and **76** were obtained by treating isoquinoline (**71**) and quinoline (**74**) with methyliodide[113,114], repectively (see Figure 24). As expected, **72** is easily reduced to its corresponding tetrahydro analog **40** with sodium borohydride.[115,116] Mechanistically, the sodium borohydride reduction of **72** leads first to the intermediate 1,2-dihydroisoquinoline species **73** which is subsequently protonated by the solvent to the 1,4-dihydrosioquinoline species **46** and reduced further to the tetrahydroisoquinoline **40** (see Figure 24).[115,116] In contrast, the reduction of quinoline by sodium borohydride does not lead directly to 1,2,3,4-tetrahydroquinoline.[116] Instead, quinoline (**74**) is reduced to 1,2-dihydroquinoline (**75**) or to a mixture of 1,2-dihydroquinoline and 1,2,3,4-tetrahydroquinoline (**41**) (see Figure 24). Presumably this path is followed because the protonation of 1,2-dihydroquinoline **75** is more difficult than the protonation of the 1,2-dihydroisoquinoline

dihydroisoquinoline intermediate **73** due to the less basic nature of quinolines (pKa ~ 4.9) compared to isoquinolines (pKa ~ 5.1).[117] Thus, in light of the difficulties involved in reducing quinolines to tetrahydroquinolines using sodium borohydride, N-methyltetrahydroquinoline (**42**) was synthesized here as its hydroiodide salt by catalytic hydrogenation of N-methylquinolinium iodide (**76**)[113] using the method of Uhle and Jacobs[164] (see Figure 24).

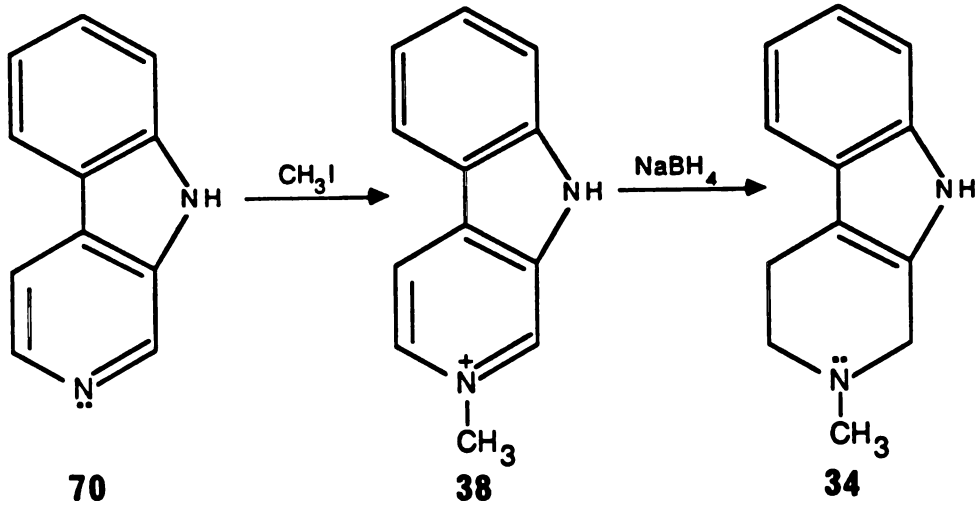


Figure 23. Synthetic pathway for the tetrahydro-β-carboline **34** and the β-carbolinium species **38**.

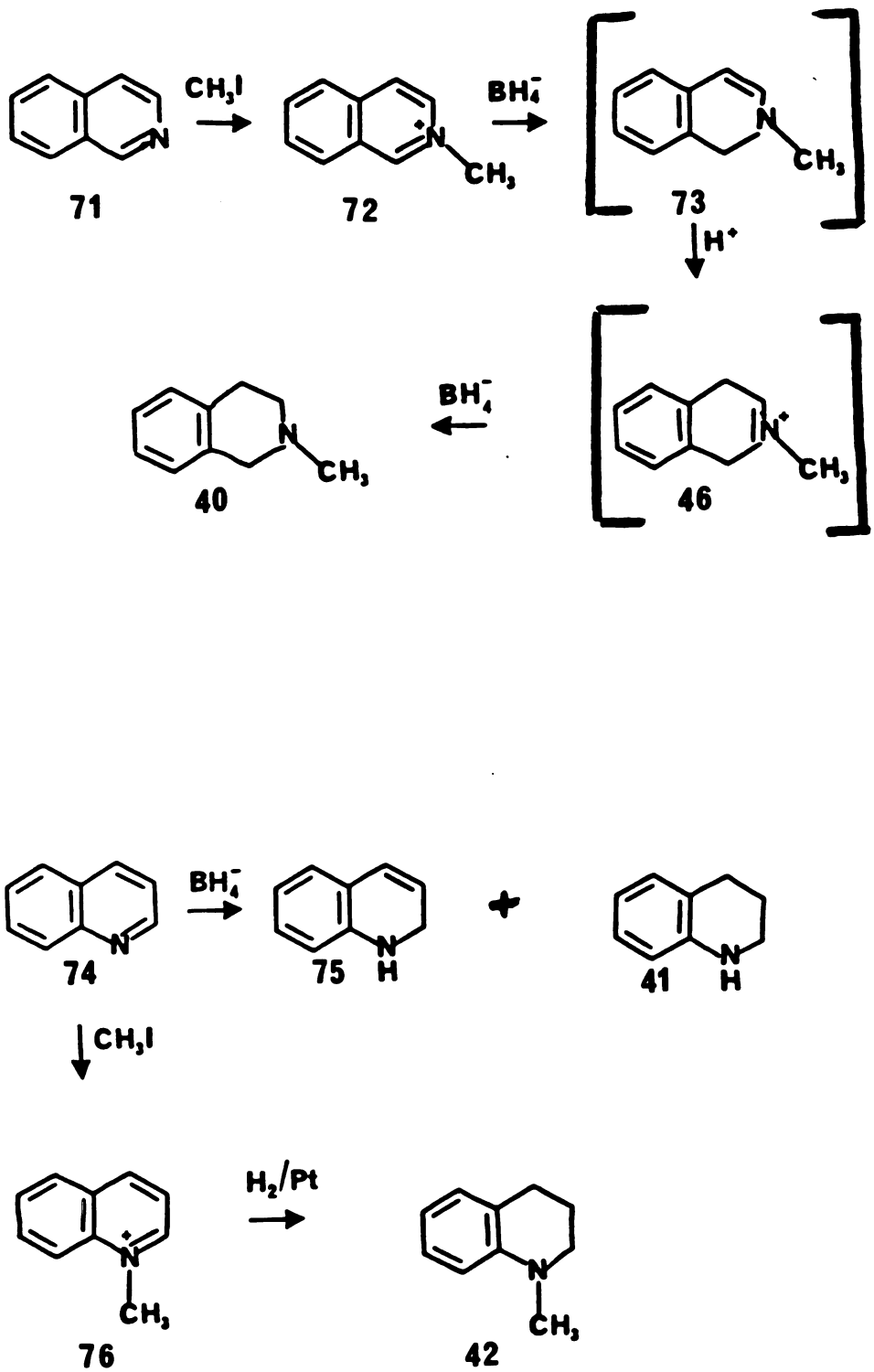


Figure 24. Synthetic pathway for isoquinoline and quinoline derivatives.

Enzymatic Studies

Results - Substrate Properties.

Purification of MAO-B from beef liver mitochondria by the method of Salach[118] provides a relatively stable enzyme with a specific activity of about 2 units/mg, a 100-fold purification. When the enzyme was incubated with 100 μM of tetrahydroindenopyridine, tetrahydro-β-carboline, or tetrahydroisoquinoline substrate (see below) in phosphate buffer, pH 7.2, at room temperature, the rate of oxidation was constant for up to about 48 hours.

When the tetrahydroindenopyridine **33** (100 μM) was incubated with MAO-B, a time-dependent loss of absorbance due to its chromophore at 250 nm corrresponded to an increase in absorbance at 360 nm (see Figure 25). The formation of the chromophore at 360 nm was inhibited when 10^{-5} M pargyline, an MAO-B inhibitor, was included in the incubation mixture. Thus, we attributed the absorbance at 360 nm to the chromophore of the MAO-B derived two-electron oxidation product of **33**, the 3,4-dihydroindenopyridinium species **50** (λ_{max} 360 nm). We ruled out the 1,4-dihydroindenopyridinium structure **77** as the oxidation product based on the results of Peterson *et al.*[52] which indicated exclusive allylic α-carbon oxidation of MPTP by MAO-B. Furthermore, the $\Delta^{2,3}$ double bond in **77** is not in conjugation with the benzene or indene systems and thus it is unlikely that this structure would absorb at such a high wavelength in the UV region.

The rate of oxidation of **33** to **50** was linear over a 48 hour period (100 μM **33**, 0.2 units MAO-B) and the rate could be increased by increasing the concentration of substrate and/or enzyme. This indicated that the reaction followed Michaelis-Menton enzyme kinetics.[119]

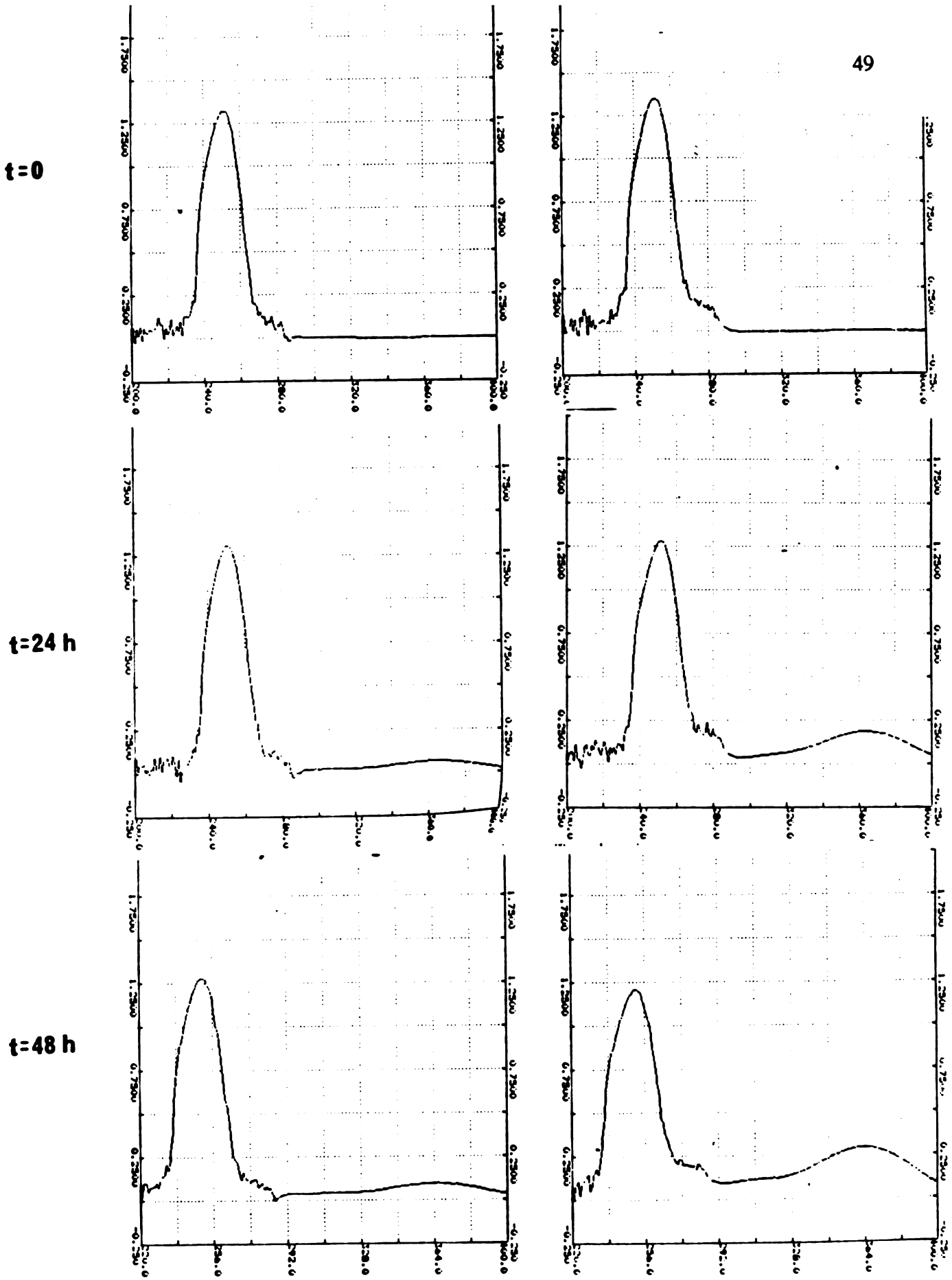
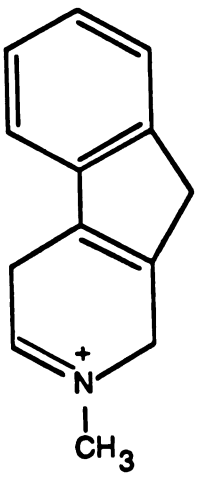


Figure 25. UV spectrum over time of 100 μM tetrahydroindenopyridine 33, pH 7.2, with 0.2 units MAO-B (right) and with MAO-B plus 10^{-5}M pargyline (left).



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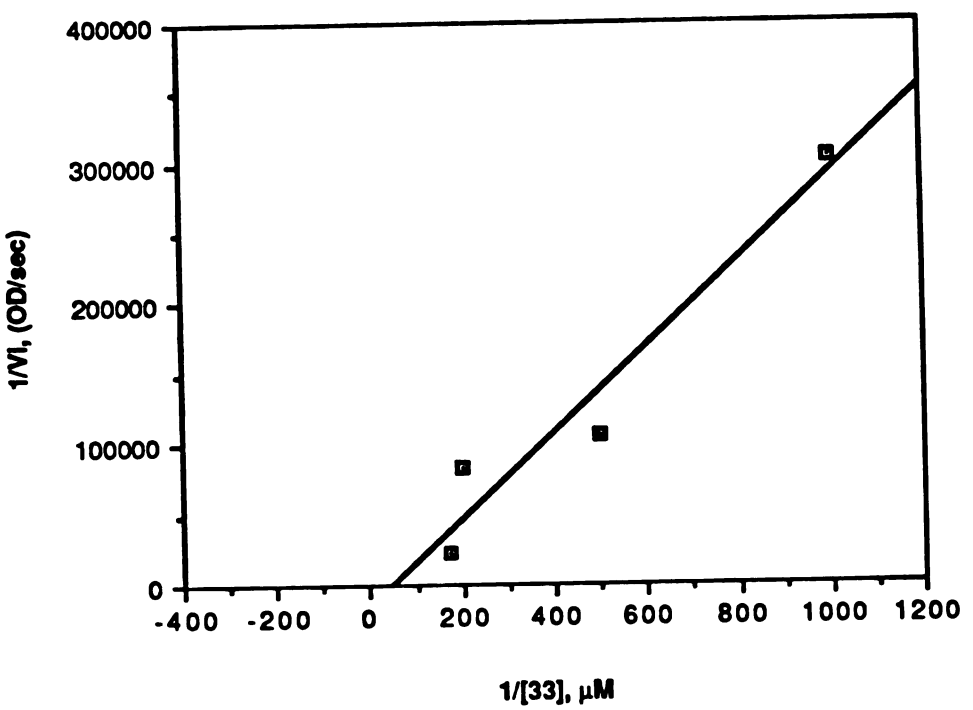


Figure 26. Lineweaver-Burk plot of the rate of oxidation of 100 μM tetrahydroindenopyridine 33 in the presence of 0.2 units MAO-B.

A Lineweaver-Burk plot of inverse substrate concentrations vs inverse initial velocities was made to determine Km and Vmax (Figure 26) so that we could quantify the binding affinity and rate of turnover of **33**. As the Lineweaver-Burk plot in Figure 26 shows, the curve does not intersect the Y-axis and therefore the Vmax was not reached even at a substrate concentration of 6 mM. Thus, the Km (curve intersection at the X-axis) can only be estimated to be much larger than 6 mM. Unable to obtain kinetic constants, we decided to express the rate of oxidation of **33** by comparison to MPTP as well as to the other semirigid tetrahydro analogs, **34** and **47**.

Similar to the tetrahydroindenopyridine **33**, when the tetrahydro-β-carboline **34** (λ_{max} 274) was incubated with MAO-B (100 μM **33**, 0.2 units MAO-B) and scanned over a 48 hour period, there was a loss of absorbance at 274 nm which corresponded to an increase in absorbance at 360 nm, the λ_{max} expected for the 3,4-dihydro-β-carbolinium species **37**. As required for MAO metabolism, 10^{-5} M pargyline inhibited this oxidation. The UV scans were similar to those shown in Figure 25 for MAO catalyzed oxidation of **33**.

Figure 27 shows that at the end of 48 hours, the tetrahydroindenopyridine **33** was about 30% metabolized to the dihydroindenopyridinium species **50** while the tetrahydro-β-carboline **34** was about 15% metabolized to the dihydro-β-carolinium **37**. The $\Delta^{9,9a}$ isomeric tetrahydroindenopyridine **49** however, was stable over the 48 hour incubation period.

As previously discussed, the tetrahydroisoquinoline and tetrahydroquinoline derivatives **39-42** (see Figure 28) do not have strong absorbance chromophores in the UV spectral region. However, the corresponding dihydro derivatives **43-48** (Figure 28) do have characteristic absorbances at 270-300 nm and thus we were able to monitor the formation of these metabolites spectrophotometrically. The results are shown schematically in Figure 28.

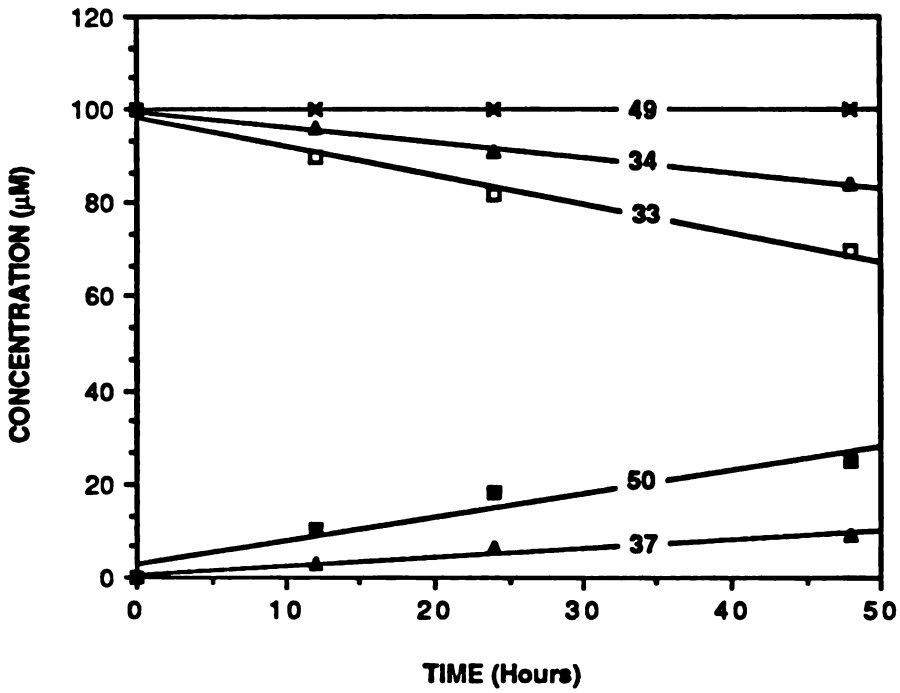


Figure 27. Rate of oxidation of 100 μM tetrahydroindenopyridines **33** and **49** and tetrahydro-β-carboline **34** in the presence of 0.2 units MAO-B. The rate of appearance of the dihydro oxidation products **37** and **50** corresponded to the disappearance of the substrates **33** and **34**.

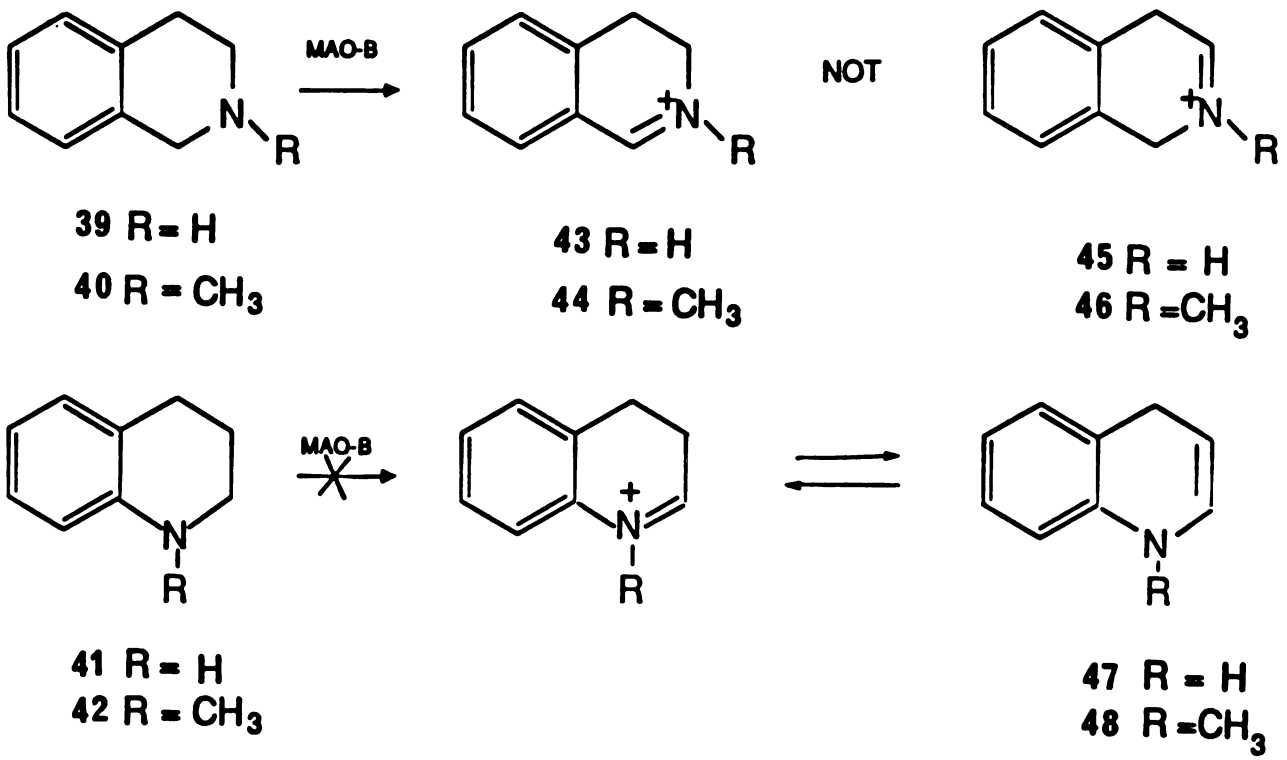


Figure 28. Oxidation of tetrahydroisoquinolines **39** and **40** and tetrahydroquinolines **41** and **42** in the presence of 0.2 units MAO-B.

Incubation mixtures containing 100 μM **39**, **40**, **41**, or **42** and 0.2 units MAO-B with and without 10^{-5} M pargyline were scanned over a 48 hour period. The incubation mixture containing tetrahydroisoquinoline (**39**) showed a time dependent increase in a chromophore corresponding to that of the 3,4-dihydroisoquinolinium species **43** (λ_{max} 274 nm) that was inhibited by 10^{-5} M pargyline. No absorbance corresponding to the isomeric 1,2-dihydro oxidation product **45** (λ_{max} 330 nm) was observed. Similarily, incubation of N-methyltetrahydroisoquinoline (**40**) with MAO-B resulted in the pargyline sensitive formation of a chromophore corresponding to the 3,4,-dihydroisoquinolinium product **44** (λ_{max} 284 nm) with no evidence of formation of the 1,2-dihydroquinoline compound **46** (λ_{max} 328 nm). Figure 29 shows that at the end of 48 hours, tetrahydroisoquinoline (**39**) was about 25% metabolized to **43** while N-methyltetrahydroisoquinoline (**40**) was about 45% metabolized to **44**. Neither tetrahydroquinoline (**41**) nor N-methyltetrahydroquinoline (**42**) were MAO-B substrates in this assay system as there was no pargyline-sensitive increase in absorbance above 300 nm corresponding to the λ_{max} of the corresponding dihydroquinolines **47** and **48**. There was, however, a weak absorbance (< 0.2 absorbance units at 48 hours) noted in the 230-260 nm region for both **41** and **42** which may have been due to decomposition products since it was observed also in the presence of pargyline. It is unlikely that the new chromophore corresponded to the absorbance of autoxidation products since 1,2-dihydroquinolines have λ_{max} values of about 340 nm[104] and 1,4-dihydroquinolines have λ_{max} values of about 300 nm[104], neither of which was observed.

Table I compares the initial rates of MAO-B catalyzed oxidation of the tricyclic analogs **33** and **34** with the isoquinoline analogs **39** and **40** and MPTP. The tetrahydroindenopyridine **33** is oxidized at about 1% the rate observed for the corresponding conversion of MPTP to $MPDP^+$. The tetrahydro-β-carboline **34** is oxidized at about 50% the rate observed for **33** while the tetrahydroisoquinolines **39** and **40** are metabolized to their dihydro analogs at about 3% the rate observed for MPTP oxidation.

40 are metabolized to their dihydro analogs at about 3% the rate observed for MPTP oxidation.

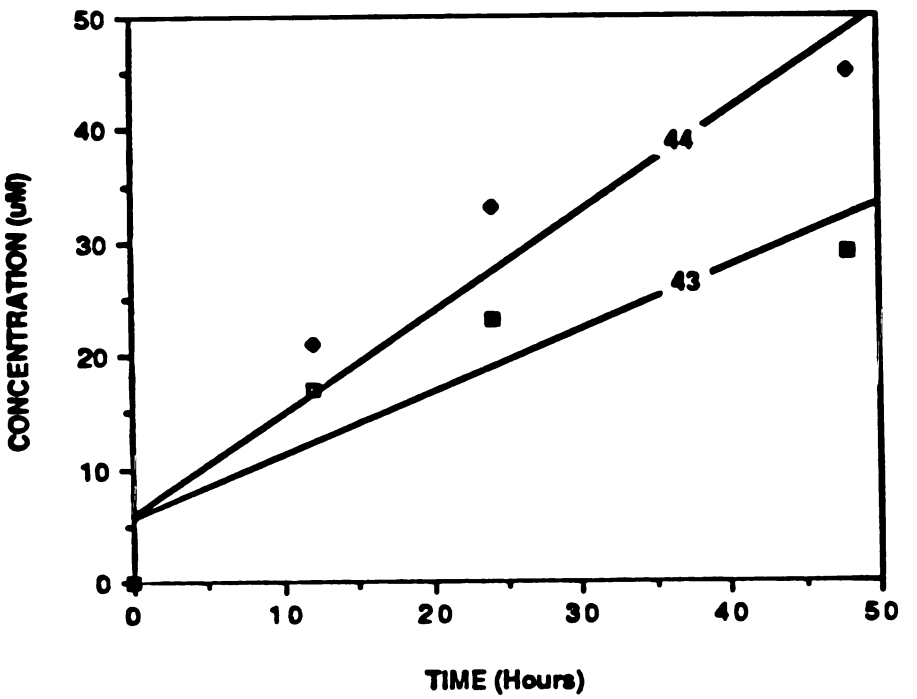


Figure 29. Rate of formation of dihydroisoquinilinium species **43** and **44** from the incubation of 100 μM tetrahydroisoquinolines **39** and **40** with 0.2 units MAO-B.

TABLE I. Oxidation of Tertiary Amine Substrates in the Presence of MAO-B

Compound	MPTP	**33**	**34**	**39**	**40**
Rate of Oxidation (nmol/min-mL)[a]	25	0.2	0.1	0.6	0.9

[a] Incubation mixtures consisting of 3 mM substrate and 0.1 units MAO-B were monitored spectrophotometrically at the λ_{max} corresponding to the dihydro product.

The dihydroindenopyridinium compound **50** also was incubated with MAO-B and its conversion to the corresponding indenopyridinium species **52** (λ_{max} 305 nm) was monitored spectrophotometrically. Figure 30 shows that at a concentration of 0.5 mM, there is a slow increase in absorbance at 305 nm and that the rate is faster at higher pH. This transformation however does not appear to be MAO-B catalyzed since the rate of oxidation is essentially identical in the presence and absence of enzyme and pargyline. An incubation mixture (pH 7.2) containing 100 μM **50** and 0.2 units MAO-B was scanned every 2 hours for 12 hours. There was no change observed in the chromophore (λ_{max} 360 nm) of **50** during this period. Thus, it appears that similar to the nonenzymatic disproportionation[52] and autoxidation[52] of $MPDP^+$ to MPP^+, the dihydroindenopyridinium species **50** may disproportionate and/or autoxidize to the indenopyridinium species **52** at concentrations greater than 100 μM. However, attention should be drawn to the fact that pargyline has no effect on the oxidation of **50** in the presence of MAO-B indicating that **50** is not a substrate for the enzyme. In contrast, the rate of oxidation of $MPDP^+$ in the presence of MAO-B is reduced by MAO-B inhibitors indicating that the enzyme catalyzes this reaction.[56]

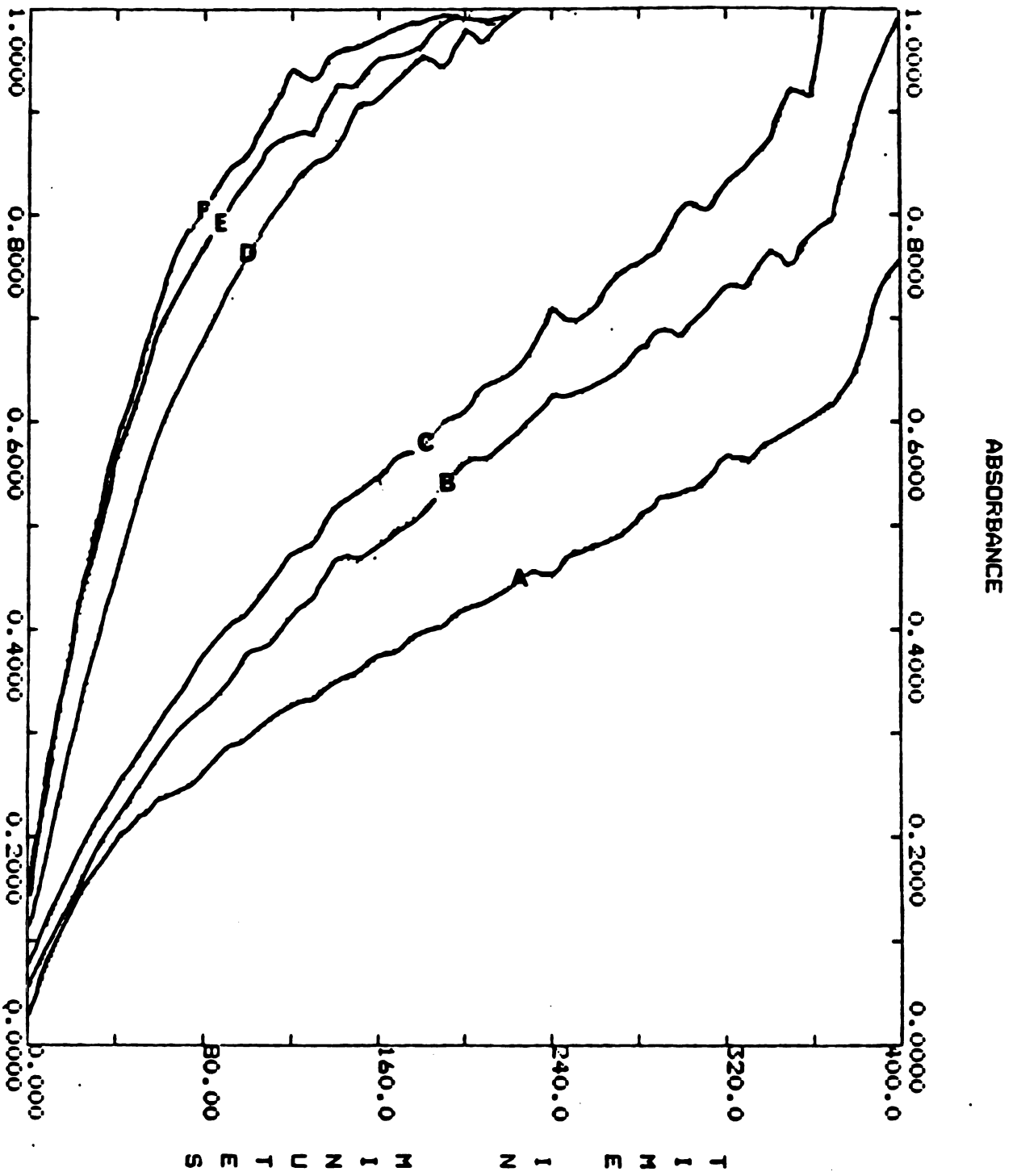


Figure 30. Formation of the indenopyridinium species 52 from the dihydroindenopyridinium compound 50.

pH 7.2	pH 8.9
A. 0.5 mM 50	D. 0.5 mM 50
B. A + 0.2 units MAO-B	E. D + 0.2 units MAO-B
C. B + 10^{-5}M pargyline	F. E + 10^{-5}M pargyline

Results - Inhibitor Properties.

As part of our studies to characterize the interaction of the tetrahydroindenopyridine and tetrahydro-β-carboline derivatives with MAO-B, we initiated a series of UV-based experiments to determine the inhibitory activity of these compounds on MAO-B. Previously, using benzylamine as the substrate, it was found that MPTP, $MPDP^+$, and MPP^+ inhibit the MAO-B catalyzed oxidation of benzylamine to benzaldehyde competitively.[56] Furthermore, MPTP and $MPDP^+$ are mechanism-based[58] irreversible inhibitors which inactivate the enzyme in a time dependent fashion.[56]

When the tetrahydroindenopyridine compounds **33** or **49** were incubated with benzylamine and MAO-B, the rate of formation of benzaldehyde was inhibited to an extent that depended on the concentration of the tetrahydroindenopyridine and/or benzylamine. A competitive type inhibition[119] was confirmed via the Lineweaver-Burk plot displayed in Figure 31. Figure 31 shows that at a given concentration of benzylamine, increasing concentrations of compound **33** increase the Km of benzylamine for MAO-B as is required for competitive inhibition that obeys Michaelis-Menton kinetics.[119] Also according to Michaelis-Menton kinetics, a plot of the slope from the Lineweaver-Burk plot versus inhibitor concentration gives a straight line that provides the competitive inhibition constant (Ki) at the X-axis intersection. Such a plot for **33** is shown in Figure 32. The Ki is 75 μM. Similarily, the isomeric tetrahydroindenopyridine **49** was found to have a Ki of 65 μM (see Figure 33).

Using a crude assay preparation, Meller *et al.*[120] have reported that **34** is a competitive inhibitor of MAO-B (IC_{50} of 650 μM). We obtained a Ki of 400 μM for the inhibition of pure MAO-B by tetrahydro-β-carboline **34** (see Figure 34) which is in reasonable agreement with the Meller results considering the differences in the purity of the enzyme preparations.

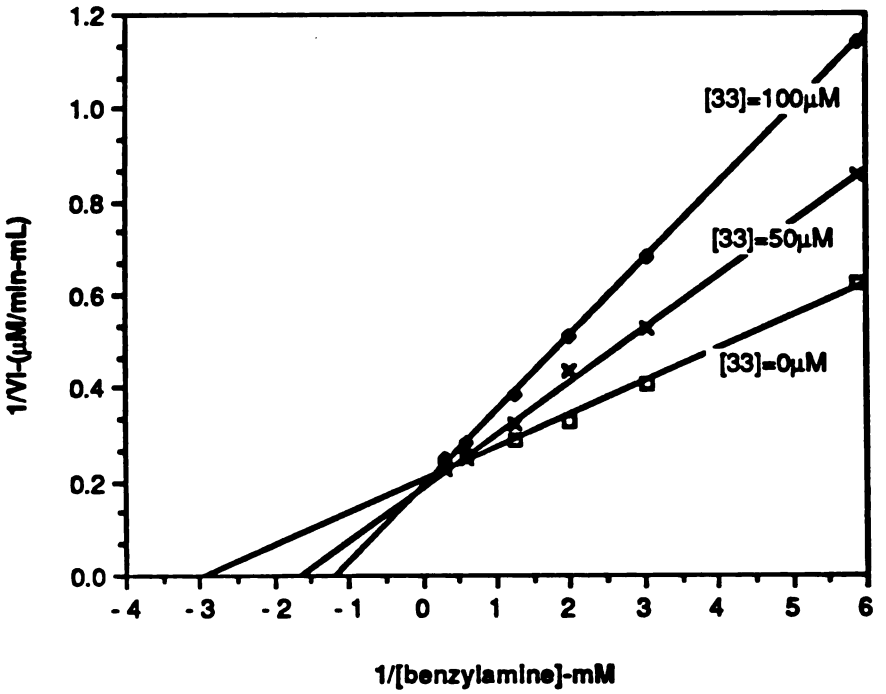


Figure 31. Lineweaver-Burk plot of the inhibition of the MAO-B catalyzed oxidation of benzylamine by tetrahydroindenopyridine 33.

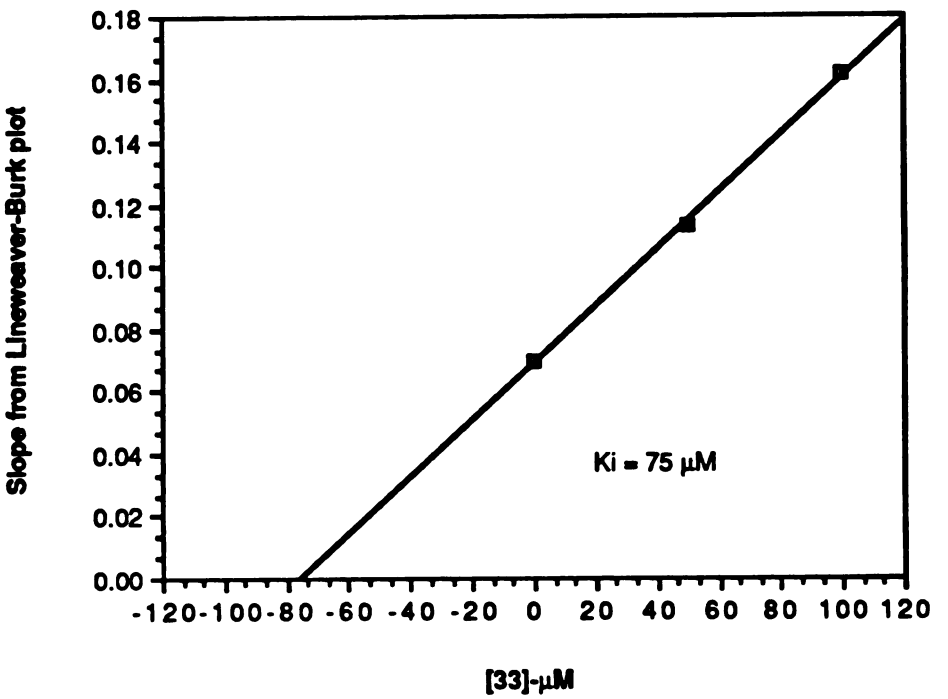


Figure 32. Plot of the slope from the Lineweaver-Burk plot of the tetrahydroindenopyridine **33** (Figure 31) vs the concentration of **33**.

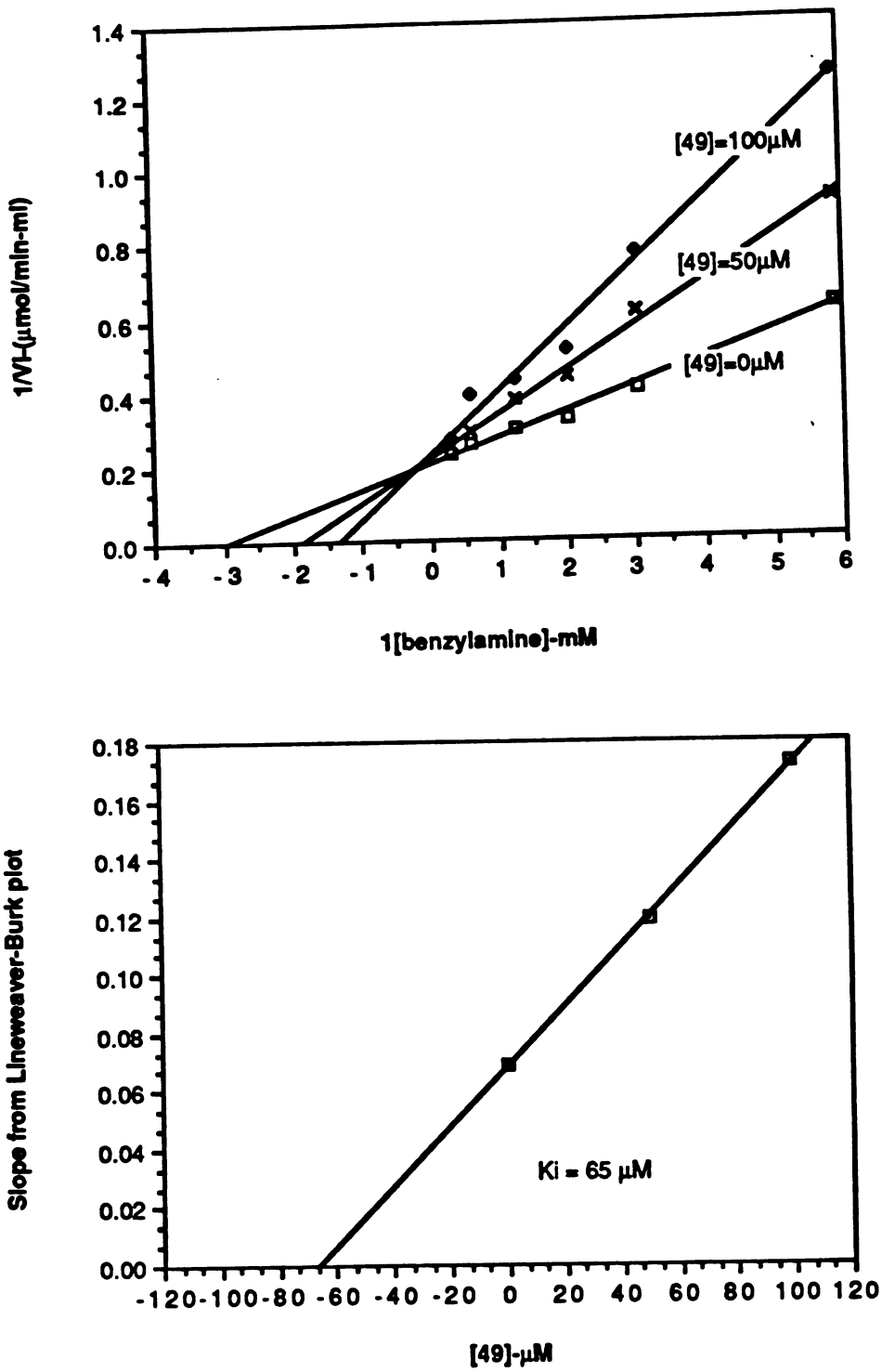


Figure 33. Lineweaver-Burk plot for inhibition of the MAO-B catalyzed oxidation of benzylamine by tetrahydroindenopyridine **49** and plot of slope vs concentration of **49**

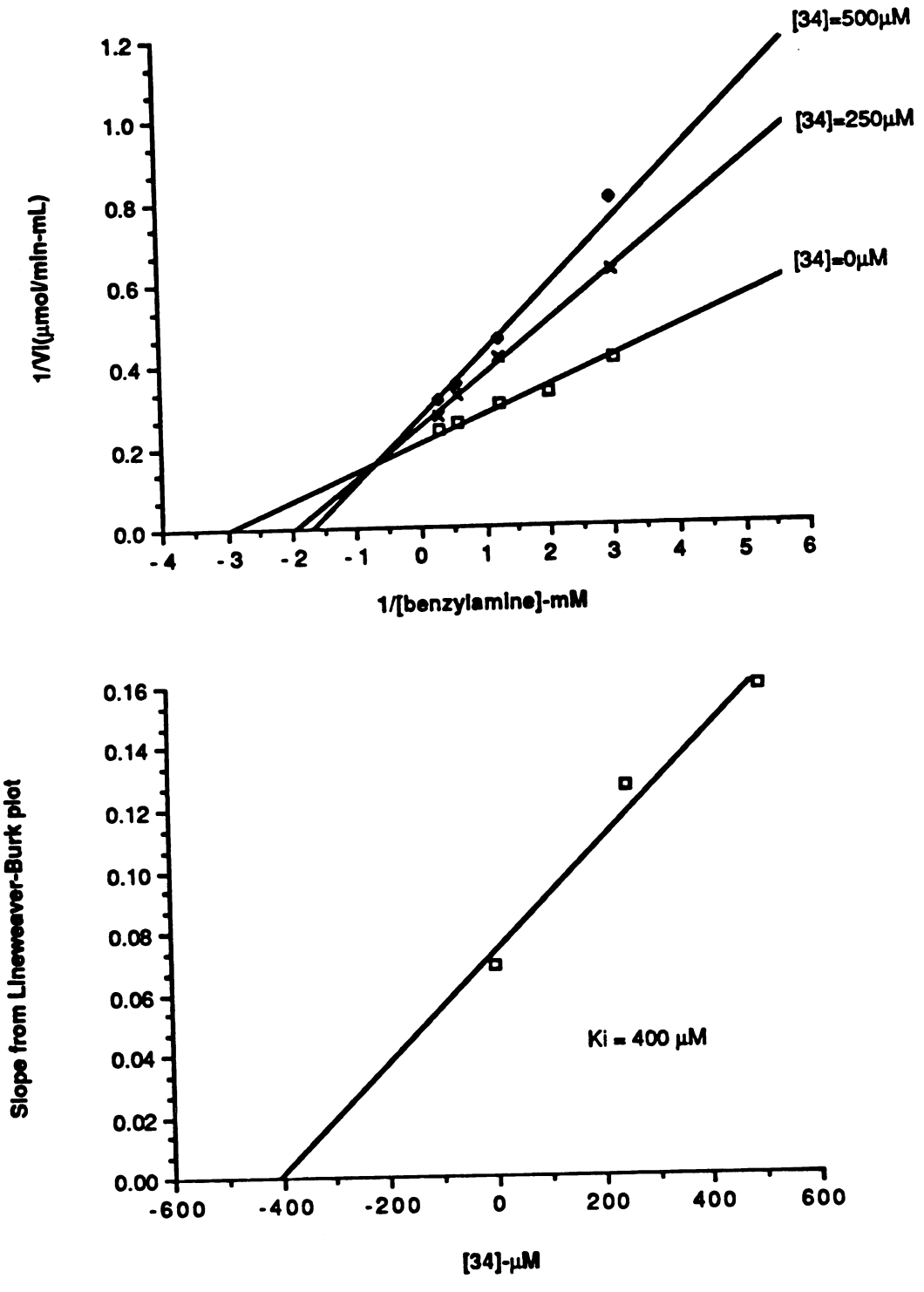


Figure 34. Lineweaver-Burk plot for inhibition of the MAO-B catalyzed oxidation of benzylamine by tetrahydro-β-carboline **34** and plot of slope vs concentration of **34**.

It should be noted however, that Meller used phenylethylamine as a substrate (as opposed to benzylamine) and thus the two results should only be compared qualitatively.

Table II lists the Ki values for the competitive inhibition of MAO-B by the semirigid tricyclic analogs and by MPTP. Similar to MPTP, which competitively inhibits MAO-A at about 2 orders of magnitude more potently than MAO-B [121], preliminary data from Kathy McKeown (V.A. Medical Center, San Francisco) indicate that the Ki values for the inhibition of MAO-A is about 4 μM for both tetrahydroindenopyridines **33** and **49**. Although the scarcity of purifed MAO-A prevented extensive studies to determine the Ki value for the tetrahydro-β-carboline **34** , Meller *et al.*[120] reported an IC_{50} value of 3.0 μM for the inhibition of crude MAO-A by **34** using serotonin as a substrate. Although Naoi and Nagatsu[122] reported slight competitive inhibition of a brain synaptosomal mitochondrial preparation of MAO-B by quinoline (Ki ~ 1.3 mM), they reported no inhibition by tetrahydroisoquinoline (**39**) and tetrahydroquinoline (**41**). Thus the isoqinoline and quinoline derivatives **39-42** were not examined further here in view of limited supplies of enzyme.

TABLE II. Competitive Inhibition of MAO-B by Tertiary Amine Substrates

Compound	MPTP[b]	**33**	**34**	**49**
Ki (μM)[a]	100	75	400	65

[a]Refers to the inhibition of the MAO-B catalyzed oxidation of benzylamine (0.17-3.3. mM) by test compound (0-0.5 mM).
[b]Reference 121.

We also examined the MAO-B inhibitory properties of the dihydroindenopyridinium compound **50**, a chemically stable analog of the irreversible inhibitor, $MPDP^+$.[56] Thus, 100 μM **50** was preincubated with MAO-B for 0 to 60 min. It was shown in the previous section that this concentration of **50** is stable in the presence of MAO-B for up to 12 hours. To these timed incubation mixtures was added 3.3 mM benzylamine and the formation of benzaldehyde was monitored at 250 nm. Table III summarizes the rate of benzylamine oxidation in the presence of the dihydropyridinium species **50**. There appears to be a time-dependent inactivation of the enzyme as reflected by a time-dependent decrease in the turnover of benzylamine. The immediate inhibition may indicate a preliminary reversible type inhibition of the enzyme as is seen with $MPDP^+$.[56]

TABLE III. Time-dependent inhibition of MAO-B by the dihydroindenopyridinium species **50**

[**50**] (μM)	Preincubation time with MAO-B (min)	Benzylamine oxidation (% of control rate)
0.0	0.0	100
0.0	10	98
0.0	15	98
0.0	30	92
0.0	60	80
100	0.0	87
100	10	78
100	60	59

All incubation mixtures contained 0.02 units MAO-B and 3.3 mM benzylamine

The data in Table III were plotted to give the graph shown in Figure 35. When benzylamine oxidation as percent of control was converted to MAO-B activity (units), the first order rate constant for inactivation (k_{inact}) of MAO-B by the dihydroindenopyridinium species **50** was calculated to be 0.013 units MAO-B inactivated/min. This value is very similar to the k_{inact} by $MPDP^+$, 0.022 units/min.[56] Since we have shown above that **50** is not metabolized by MAO-B, the inactivation by this dihydroindenopyridinium compound cannot be a mechanism-based type.[58] It appears then that there may be a simple covalent attachment by the electrophilic **50** to a nucleophilic group on the enzyme. On the other hand, there is the possibility that **50** may inactivate MAO-B with a partition coefficient of 1. In this case, for each molecule of **50** turned over by the enzyme, one molecule would be covalently attached to the enzyme. Thus, the substrate properties of the compound would be effectively masked. The possibility that a radical mechanism may be involved as has been suggested for $MPDP^+$[122] seems unlikely in view of the relative stability to oxidation of **50** both chemically and presumably in the presence MAO-B.

Similar irreversible inhibition studies using the tetrahydroindenopyridine **33** were performed by Kathy Mckeown (San Francisco VA Hospital) who obtained a $k_{inact} = 0.008$ units/min for the time-dependent inactivation of MAO-B by this compound. This rate of inactivation is about half of the observed rate for the mechanism-based inactivation by MPTP (0.034 units/min).[56] If, as with MPTP, a mechanism-based inactivation is operative, then the slower rate of inactivation of MAO-B by **33** as compared to MPTP may correspond to the slower rate of oxidation of **33** to its electrophilic metabolite **50** which presumably inactivates the enzyme by covalent attachment as described above. In other experiments, Ms. Mckeown has determined that the tetrahydroindenopyridine **33** is not oxidized by MAO-A and not unexpectedly then, MAO-A is not irreversibly inactivated by **33.**

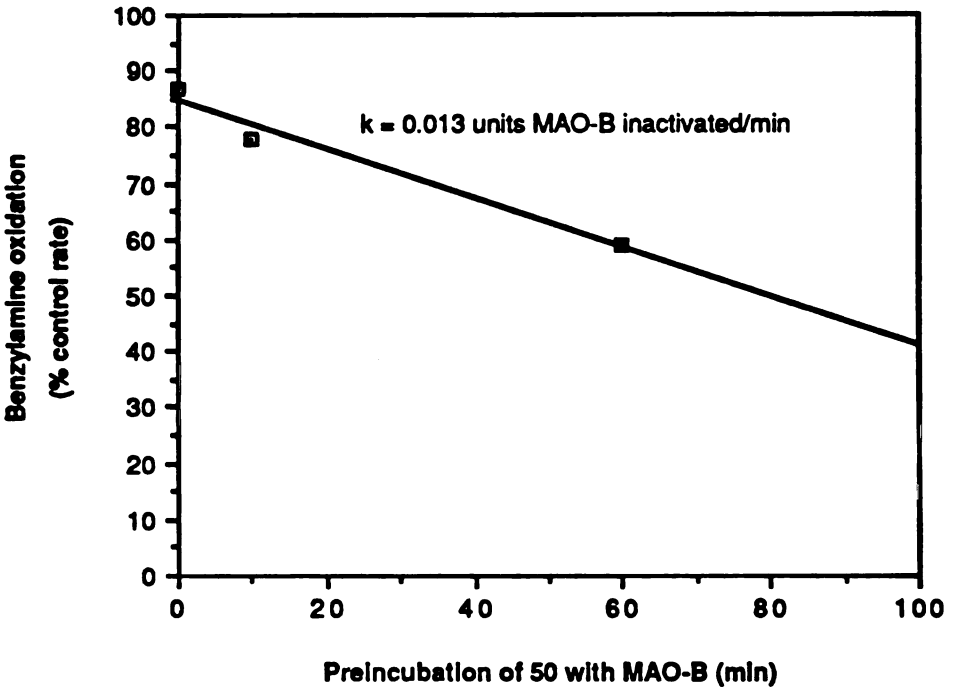
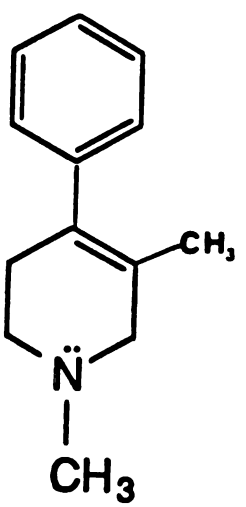


Figure 35. Rate of inactivation of 0.02 units MAO-B by 100 μM dihydroinenopyridinium species **50**.

Discussion

Subsequent to our studies described in this thesis, it was determined that the oxidation of the 5-methyl analog **78** of MPTP is not catalyzed by MAO-B[124] presumably due to steric interference of the 5-methyl substituent. Therefore, the steric bulk of the methylene and nitrogen substituents at C(5) of the tetrahydroindenopyridine **33** and tetrahydro-β-carboline **34** are likely to have hindered effective binding to the enzyme, thus compromising their substrate properties. The negative steric influence of the C(5) substituent in **78** should not be attributed to its effect of forcing noncoplanarity of the phenyl and piperidiene rings (as observed in Dreiding models) since the 2'-CH_3[125] and 2'-CH_2CH_3[126] analogs **79** and **80**, also nonplanar molecules, have recently been shown to be excellent MAO-B substrates, comparable to MPTP.



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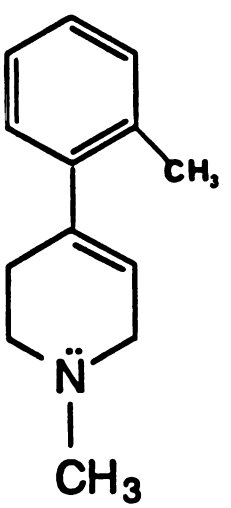

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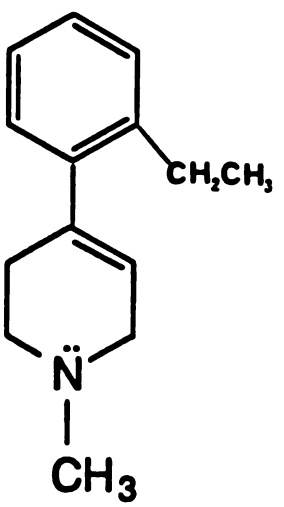
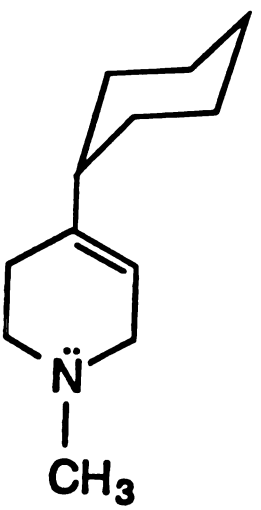


80

In view of the fact that the test compounds **33** and **34** have a C(5) substituent in addition to being relatively planar molecules, we are unable to assess which of these steric features is detrimental to MAO-B substrate activity. As demonstrated with compounds **79** and **80**, noncoplanarity is unlikely to influence substrate activity.

As described in the introduction to this chapter, it was proposed that the unexpected and exceptionally good MAO-B substrate properties of MPTP may be related to the stabilization of the α-carbon radical intermediate **30** via delocalization of the unpaired electron as in resonance structures **30, a-e** (see Figure 10). This proposal was consistent with the observations that the allylic double bond is required for substrate activity[87,88] and that only the allylic position undergoes oxidative attack.[52] Recently however, it has been determined that the MAO-B substrate properties of 1-methyl-4-cyclohexyl-1,2,3,6-tetrahydropyridine (**81**) are about the same as those of MPTP.[127] This compound also is a potent dopaminergic neurotoxin.[127] Futhermore, 1-methyl-4-benzyl-1,2,3,6-tetrahydropyridine (**82**) is oxidized at an even faster rate than MPTP, although this compound is not neurotoxic.[128]



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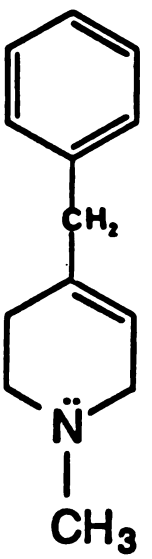

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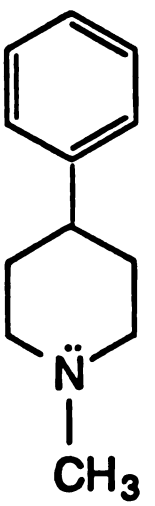


83

In both **81** and **82**, the C(4) substituent is unable to contribute to the delocalization of an unpaired electron through resonance and thus can play no electronic role in the stabilization of a radical intermediate. Thus, it appears that the phenyl group in conjugation with the allylic double bond in MPTP may not be electronically crucial for substrate activity and that the stabilization of an α-carbon centered radical in this system may not neccessarily be effected through resonance with the benzene ring. Such resonance may not be involved in the mechanism of tertiary allylamine oxidation by MAO-B. The question of whether or not the $\Delta^{4a,9a}$ bond in the tetrahydro-β-carboline **34** is less allylic in character than the corresponding double bond in the tetrahydroindenopyridine **33** may have only a small effect on their substrate properties. The less than 2-fold difference in oxidation rates between **33** and **34** is consistent with this view.

It is important to note that both the tetrahydroindenopyridine **33** and the tetrahydro-β-carboline **34** as well as the tetrahydroisoquinolines **39** and **40** were all exclusively oxidized at the allylic α-carbon position, similar to MPTP. Futhermore, the fully saturated analog **83** is not oxidized by MAO-B).[87,88] This suggests that the allylic bond plays some role in substrate activity. For example, there may be a steric requirement for sp^2 hybridization of the C(4) - C(5) positions of MPTP-type compounds. The observation that the $\Delta^{9,9a}$-tetrahydroindenopyridine **49** was not metabolized by MAO-B while the isomeric $\Delta^{4a,9a}$-isomer **33** is oxidized indicates a preference of the enzyme for the allylic bond to be endocyclic with respect to the heterocyclic ring. This may be due to a subtle steric phenomenon involving the differences in hybridization of the C(4a) and C(9) carbons between the two compounds. Finally, although extended delocalization of an unpaired electron may not be required, the resonance delocalization between the α-carbon and C(4)-centered radical via the allylic bond (i.e., structures **34a** and **34b**, Figure 10) may be all that is required to orient oxidative attack at the allylic α-carbon. In this regard, Dreiding molecular models show the C(4) radical species **34b** to be a highly strained tricyclic system, the formation of which would not be favored. The poor substrate properties of **33**

(and by analogy, **34**) then may reflect the loss of resonance stabilization between the carbon centered radical species **34a** and **34b**.

The exclusive oxidation of the tetrahydroisoquinolines **39** and **40** to the 3,4-dihydro oxidation products **43** and **44** (see Figure 28) implies that the C(1) α-carbon is sterically and/or electronically more subject to oxidative attack by MAO-B. This C(1) position is both allylic and benzylic and an unpaired electron at this position would certainly be stabilized through resonance with the benzene ring. The lack of an analogous "electronically equivalent" carbon center in the corresponding tetrahydroquinolines **41** and **42** may in part explain their lack of MAO-B substrate activity. Furthermore, as described in the synthesis section of this chapter, the nitrogen atom in tetrahydroquinoline is less basic than in tetrahydroisoquinoline and thus the less available lone pair electrons of the tetrahydroquinolines may make them less prone to oxidation by MAO-B. Of course steric differences between the tetrahydroisoquinolines and tetrahydroquinolines in the positions of the nitrogen and α-carbon atoms may also influence differences in substrate activity. .

An alternative general conclusion regarding the substrate studies may be that there is no significant electronic role for the allylic double bond in the MAO-B oxidation pathway of tertiary allylamines and the formation of an α-carbon centered radical species may be unneccessary. Figure 36 , pathways A + D, depict an oxidative pathway where hydrogen atom abstraction follows the formation of the aminium cation radical species **21** to give directly the iminium species **12**. This scheme contrasts with the proton abstraction mechanism presented in the introduction and depicted here in pathways A + C. Chemical studies have demonstrated that the intrinsic isotope effects associated with the deprotonation of aminium radicals are of relatively low magnitude ($k_H/k_D < 3.0$), while those associated with hydrogen atom abstraction are large ($k_H/k_D > 7$).[86] Preliminary studies in this laboratory have indicated that the kinetic isotope effect [$(V_{max}/Km)^H$ / $(V_{max}/Km)^D$] for the MAO-B catalyzed oxidation of MPTP vs 6,6-deuterated MPTP is about 8.0.[129]

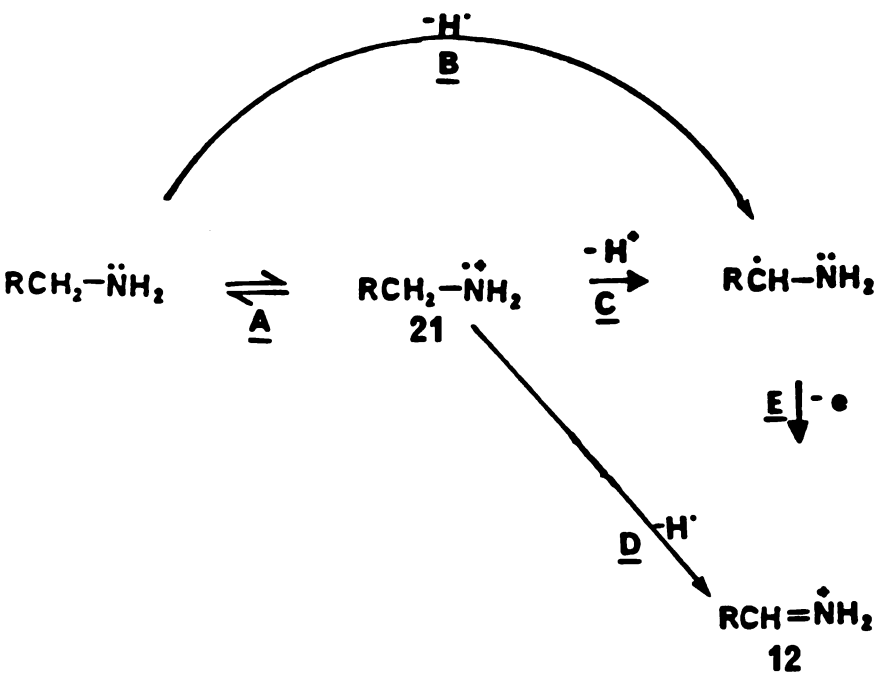


Figure 36. Proposed pathways for MAO catalyzed oxidation of amines.

This large isotope effect indicates carbon-hydrogen bond breaking associated with a rate determining step and suggests a hydrogen atom abstraction mechanism as depicted in Figure 36, pathways A + D and/or B + E. The latter pathway involves α-carbon centered radical formation and the discussion above regarding the allylic double bond may apply. However in pathway A + D, no intermediate carbon centered radical is formed and the electronic effects of an allylic bond become less important.

The stability of the dihydroindenopyridinium **50** in the presence of MAO-B indicates that, as in chemical oxidation, the MAO-B catalyzed oxidation may proceed via the dihydro free base as has been proposed for $MPDP^+$.[123] The ring strain apparent in the molecular model of the dihydroindenopyridine free base **51** makes **50** a weaker conjugate acid and therefore more stable to oxidation. The steric features of this molecule are also likely to contribute to its lack of MAO-B substrate properties. This conclusion assumes that the partition coefficient for inactivation of MAO-B per molecule of **50** is not 1, as discussed previouly.

The results of the inhibition studies add evidence to indicate that the tetrahydroindenopyridine **33** has steric access to the MAO-B active site as it is both a competitive inhibitor and mechanism-based inactivator. In view of the scarcity of pure MAO-B and the lack of substrate properties of the isomeric tetrahydroindenopyridine **49**, this compound was not tested as a mechanism-based inactivator of the enzyme. The results of the competitive inhibition studies however are similar to those for the isomer **31**. This suggests that **49** has access to the active site in a manner similar to **31** but that subtle steric effects do not allow for proper substrate orientation of **49**.

Chapter III

Neurotoxicity Studies

Introduction

While the requirement of MAO-B has been established for the expression of MPTP-type neurotoxicity, the biochemical events that take place prior to and subsequent to the bioactivation step as well as the molecular mechanism of toxicity remain obscure. The neuroglial cells and the endothelial cells surrounding the brain capillaries form tight junctions which severely restrict exchange of water soluble molecules into the central nervous system from the periphery.[130] The result is a blood-brain barrier that allows only slow diffusion of organic acids and bases into the brain and effectively keeps permanently charged species such as quaternary amines (i.e., MPP^+) out of the central nervous system. However, cerebral blood flow is the only limitation to permeation of the brain by lipophilic molecules such as the free base form of MPTP. Thus, it was determined early on that MPP^+ observed in the substantia nigra of patients and monkeys with an MPTP-induced parkinsonian syndrome had to have been generated within the brain itself. More importantly perhaps, once formed, MPP^+ is trapped within the central nervous system and this, at least in part, may account for its long half-life in the brain (approximately 10 days in monkeys).[131] In primates, MPP^+ accumulates in the cells that are most susceptible to MPTP toxicity; the nigrostriatal cells.[3,131] However, high concentrations of MPP^+ also accumulate without producing damage in the hypothalamus, nucleus accumbens, and adrenal gland.[131] Since MAO-B is predominately localized to glial cells and serotonergic neurons in the brain[69], it became apparent that MPP^+ was produced extraneuronally and transported into nigrostriatal neurons. It was subsequently determined that MPP^+ is taken up by dopamine uptake systems with the same affinity as dopamine itself.[66,67]

Interestingly though, dopamine uptake blockers have failed to protect primates against the neurotoxicity of MPTP.[3] Although it has been shown that MPP^+ binds with high affinity to neuromelanin which is found in the nigrostriatal cell bodies[68], this does not explain the striatal nerve terminal damage documented in rats after intrastriatal microdialysis perfusion of MPP^+ and its analogs.[65] Indeed, there are still many unanswered questions regarding the selectivity of MPTP's neurotoxicity towards nigrostriatal cells. It is particularly perplexing that the MAO-B catalyzed oxidation of MPTP occurs in many peripheral tissues as well as the neuroglial cells without producing irreversible lesions in these tissues.[3,75]

MPP^+ causes dopamine depletion and certain behavioral effects associated with parkinsonism.[132,133] Futhermore, MPP^+ is cytotoxic to cultured neuronal cells.[134] However, the mechanism of MPP^+-induced cell death remained obscure until Nicklas *et al.* [70] reported that 0.5 mM MPP^+ inhibits the oxidation of NAD^+-linked substrates in liver and brain mitochondria. Although this result provided a lead, questions still remained because the concentration of MPP^+ found in the nigrostriatal cells of MPTP-treated C-57 black mice is only 30 μM.[135,136] Furthermore, a concentration of 10 mM MPP^+ was required to inhibit the NADH dehydrogenase of inverted inner mitochondrial membrane preparations.[71] It was subsequently discovered that rat brain and liver mitochondria accumulate MPP^+ via an active energy-dependent uptake system.[73] The activity of the carrier is high enough to raise a concentration of 30 μM MPP^+ outside the mitochondria to several millimolar within the mitochondria in 30 minutes and thus inhibit NADH dehydrogenase extensively.[73]

The *in vitro* results described above form a biochemical sequela that may be regarded as discrete events in the development of the neurotoxic syndrome produced by MPTP in man and certain laboratory animals. In summary, MPTP administered peripherally must diffuse into the brain where it is oxidized in a reaction catalyzed by MAO-B to $MPDP^+$ and subsequently to MPP^+. Since the MAO-B step occurs extraneuronally, the metabolite(s) must then be taken up by and concentrated within the nigrostriatal

neurons. Presumably, the MPTP oxidation products are then accumulated by mitochondria within the target nigrostriatal cells and exert their toxic effect by inhibiting the respiratory chain, thus depleting cellular ATP. Each step in this scheme may carry with it a set of optimal structural and physicochemical properties required of MPTP-like compounds. Although candidate compounds might be screened according to their efficacy in any individual step, the ultimate criterion for identifying possible environmental and/or endogenous MPTP-like neurotoxins is the demonstration of frank *in vivo* MPTP-like neurotoxicity.

The development of suitable animal models of Parkinson's disease is perhaps one of the more important developments to come from the discovery of the neurotoxic properties of MPTP. All species of subhuman primates tested so far (rhesus, squirrel, and cynomologus monkeys, marmosets, baboons) develop a parkinsonian syndrome within several days after MPTP injections of 1 to 10 mg/Kg.[75] The acute motor impairment effects of MPTP last less than 24 hours after which the disorder then gradually increases in severity for several weeks perhaps owing to the long half-life of the major metabolite, MPP^+.[75] Substantia nigra cell death has been characterized in MPTP-treated monkeys[34,137] and the specificity of the dopaminergic lesion has also been confirmed by extensive measures of both neurotransmitters and their synthetic enzymes.[75] During the first 10 days after MPTP treatment, monkeys have an initial increase in dopamine in the caudate and putamen, but these levels drop markedly (to less than 10% of normal values) by the eighth week, coincident with the disappearance of cell bodies in the substantia nigra.[32,35]

Although monkeys are sensitive to relatively low doses of MPTP, much higher doses are required to cause a long lasting (5 months) depletion of dopamine in mouse brain.[138,139] Heikkila *et al.* [138] reported that mice adminsitered 30 mg/Kg/day of MPTP for 10 days showed marked cell loss in the substantia nigra, comparable to that observed in primates. However, Hallaman *et al.* [140] failed to reproduce the findings of the Heikkila

group. Subsequently, Ricuarte and coworkers[139] determined that in the young, mature mouse (6-8 weeks old), MPTP destroys a large number of striatal dopaminergic nerve terminals, but for the most part, does not affect dopaminergic cell bodies in the substantia nigra. The Ricuarte group used the same dose, route, and schedule of MPTP administration, as well as the same mouse strain as the Heikkila group. The differences in experimental results appear to be related to the age of the mice used as Ricuarte *et al.* [138,141] reported that older mice (8-12 months of age) do develop substantia nigra cell loss. The effects of MPTP on dopaminergic nerve terminals in young mature mice appear to be transient, as dopamine levels in the mouse striatum gradually increase begining about 3 weeks after MPTP treatment.[139] This may be due to regeneration of the dopaminergic nerve fiber projection from the cell body.

There is unanimous agreement in the literature that MPTP does not cause selective destruction of dopamine neurons in the rat.[142,143] The chronic administration of MPTP does not reduce striatal dopamine levels and even intranigral administration does not produce lesions of the nigrostriatal system in this rodent.[142] However, when rats received intrastriatal injections of MPP^+, a significant reduction of striatal dopamine was observed.[144] Futhermore, when MPP^+ and proposed endogenous MPP^+ analogs were infused into rat striata via a microdialysis technique, irreversible striatal nerve terminal damage with concomitant depletion of striatal dopamine resulted, as will be described.[63,64,65] Recent studies have demonstrated that rat cerebral microvessels oxidize MPTP much faster than human or mouse microvessels (30 and 10-fold faster, respectively).[145] This proposed detoxification mechanism may in part explain the lack of neurotoxicity of MPTP in this rodent and underscores the crucial role of MAO-B derived metabolites in the development of MPTP-induced nigrostriatal toxicity.

The susceptibility of other animal species to MPTP-like neurotoxicity has been explored but for our purposes, most species are probably less useful and less convenient for study than the mouse. While beagle dogs exhibit the same extensive and specific loss

of nigral neurons as primates at similar MPTP dose levels, cats, guinea pigs, and frogs are all considerably less affected.[75] Thus we have limited our *in vivo* neurotoxicity studies to intraperitoneal administration to mice and intrastriatal administration to rats.

Particularly, it is the black C-57strain of mice that is most sensitive to MPTP[138,140] and we elected to use this mouse model to asess the neurotoxicity of the tetrahydroindenopyridine analogs **33** and **49**. Previously it had been reported that the tetrahydro-β-carboline **34** lowered striatal levels of dopamine in mice[92] although it did not appear to be neurotoxic in monkeys.[94,95] Presumably, the lack of MPTP-like neurotoxicity observed in the primate studies was a result of insufficient production of the MAO-B derived metabolites, the dihydro-β-carbolinium and/or β-carbolinium species, **37** and **38**.

The dosage of the tetrahydro-β-carboline **34** used by Ung-Chun *et al* [92] to deplete striatal dopamine levels in C-57 black mice was extremely high (240 mg/Kg/day IP for 5 days). In contrast, an MPTP dosage regimen of 80 mg/Kg/day IP for 1 day has been shown to reduce striatal dopamine levels in the mouse for at least 5 months.[139] In order then to assess the neurotoxicity of the tetrahydroindenopyridine moiety as compared to MPTP, we elected to follow the established literature protocol for MPTP administration to C-57 black mice.[139]

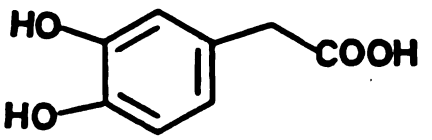
While the mouse model offers an acceptable means of assessing the neurotoxicity of test compounds relative to MPTP after peripheral administration, there are both metabolic and pharmacokinetic limitations to this assay method. Particularly, those tetrahydro test compounds which are not oxidized to their corresponding dihydro and/or fully oxidized metabolites at an appreciable rate would not be expected to display frank MPTP-type neurotoxicity. This argument has already been proposed to account for the lack of striatal dopamine depletion observed in monkeys following peripheral administration of the tetrahydro-β-carboline **34**. Thus we speculated that the poor MAO-B substrate properties of the tetrahydroindenopyridine system might render these compounds nonneurotoxic in

the mouse model. On the other hand, striatal depeletion of dopamine due to oxidative metabolites of the tetrahydroindenopyridines formed via non-MAO mediated pathways would also be detected using this assay method.

In an effort to determine the structural requirements of MPTP/MPP+-induced nigrostriatal toxicity as well as to demonstrate the pivotal role of MAO-B in the development of MPTP-like neurotoxicity, we required a method of administering the MAO-B derived polar metabolites of the tetrahydroindenopyridine, tetrahydro-β-carboline, and tetrahydroisoquinoline derivitives directly into the brains of experimental animals. Previously, Bradbury *et al.*[133] established the neurotoxicity of MPP+ in mice by intrastriatal infusion of the compound via an in-dwelling guide cannula. Futhermore, this group showed that MPP+ reduced striatal dopamine levels by 73% in rats when infused into the substantia nigra via an in-dwelling cannula. Heikkila *et. al.*[144] also demonstrated striatal dopamine depletion by MPP+ after injection into the rat striatum. Interestingly, and in line with the requirement for MAO-B oxidation, MPTP had no effect on dopamine when infused directly into the substantia nigra of rats.[133]

In contrast to the technique of injection, dissection, and analysis described above, brain dialysis is a sophisticated technique which allows the intracerebral administration of known amounts of compounds for specified time periods and the simultaneous monitoring of their effect on neurotransmitters in discrete brain areas.[146,147] *In vivo* brain dialysis involves the stereotaxic implantation of a suitable dialysis probe in the target brain area which is perfused with Ringer's solution. This administration setup is combined with an analytical method for the measurement of endogenous compounds in the perfusate. Rollema *et al.* [64] have used a U-shaped bilateral cannula with a cellulose ester membrane implanted into rat striata to measure dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC, **84**) and homovanillic acid (HVA, **85**) by on-line HPLC with electrochemical detection (HPLC-EC). After intrastriatal perfusion with MPTP (10 mM for 60 min) or MPP+ (0.1 mM for 60 min) in rats, there is a pronounced increase over basal

levels in the concentration of dopamine in the perfusate demonstrating that both compounds are potent striatal dopamine releasers comparable to (*S*)-amphetamine (**86**).[64]



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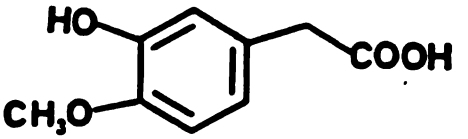

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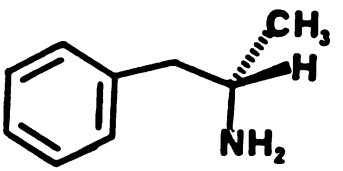

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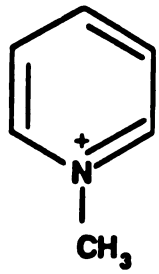
At 10 mM for 15 min, MPP^+ causes an instantaneous massive release of dopamine that is not only much higher than expected for dopamine releasing drugs (i.e., (*S*)-amphetamine), but is also irreversible as a second MPP^+ "challenge" perfusion (10 mM, 15 min) one day later fails to provoke any release of dopamine, indicating irreversible striatal dopamine depletion.[64] Furthermore, the concomitant decrease in the output of DOPAC and HVA is persistent with both metabolites being present in the perfusate at less than 5% of basal values. These effects bear a striking resemblance to those seen after sacrificing a rat by cervical dislocation and indicate that MPP^+ causes almost immediate and irreversible striatal nerve terminal damage.[64] In contrast, the effects of other dopamine releasing compounds such as MPTP and (*S*)-amphetamine are reversible since 1 day after their perfusion,

administration of an MPP^+ challenge perfusion causes the same massive release of dopamine seen in control rats.[64,65] Rollema *et al.*[63,64,65] have found that the MPP^+-induced damage of striatal nerve terminals is not due to a nonspecific effect of quaternary compounds. Compounds structurally related to MPP^+ such as the N-methylpyridinium species (**87**) and the herbicide paraquat (**88**, see Figure 37) are devoid of dopamine releasing effects and do not cause nerve terminal damage since the MPP^+ challenge perfusion provokes the same large increase in dopamine release seen in control rats.[64,65]



87

The results with paraquat are especially significant since MPP^+ has been proposed to participate in a redox-cycling pattern similar to that proposed for paraquat.[3,148] The one-electron reduction of paraquat by NADPH-cytochrome P-450 reductase produces the resonance stabilized paraquat radical cationic species **89** which in turn may reduce oxygen (O_2) to superoxide ($O_2^{\cdot -}$) as shown in figure 37.[149,150] As shown in equation 2 (see Introduction chapter) superoxide can undergo a Haber-Weiss redox reaction with hydrogen peroxide to form the extremely cytotoxic hydroxy radical. However, Frank *et al.*[151] determined that the one-electron reduction potential for MPP^+ to its radical cationic species **90** is -1.2 V which is beyond the capability of biological reductants. Furthermore, the Frank group reported the failure of MPP^+ to generate superoxide in the presence of NADPH-cytochrome P-450 reductase and oxygen. These results do not support a paraquat-like model of toxicity of MPP^+.

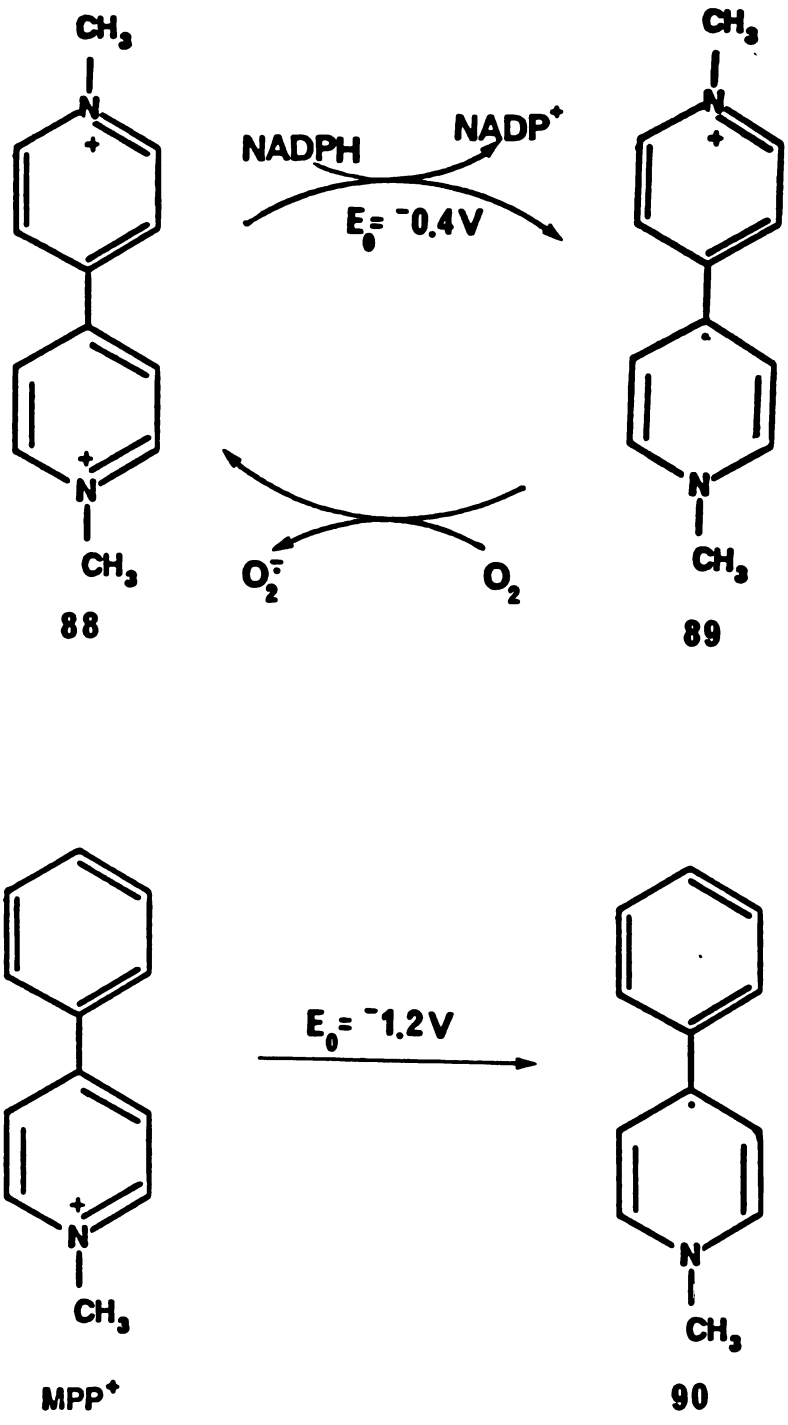


Figure 37. *In vivo* one-electron reduction of paraquat by NADPH-cytochrome P-450 reductase to the radical cationic species 89 with concomitant formation of superoxide anion. Also shown is the one-electron reduction potential for formation of the MPP^+ radical cationic species 90 from MPP^+

The *in vivo* brain dialysis technique has added evidence to support the proposal that the mechanism of cell death caused by MPP^+ involves the direct inhibition of mitochondrial respiration. MPP^+ has been shown to block mitochondrial respiration *in vitro* via inhibition of NADH dehydrogenase.[70,71,152] *In vivo*, the major consequence of this inhibition is interruption of ATP production and since the storage of dopamine is ATP-dependent, this results in the explosive release of dopamine observed when rat striata are perfused with 1 mM MPP^+.[64] Futhermore, *in vitro* studies also demonstrated an increase in lactate production as the result of anaerobic glycolysis stimulation in response to inhibition of aerobic glycolysis by MPP^+.[153] *In vivo* dialysis with an on-line lactate assay has confirmed that MPP^+ (10 mM, 1 min) induces a 4-fold increase (over basal levels) in lactate output from rat striatum whereas N-methylpyridinium (10mM, 120 min) had no effect.[64,154] Furthermore, the time courses of the MPP^+-induced efflux of dopamine and efflux of lactate are similar which suggests that both effects are consequences of inhibition of aerobic glycolysis by MPP^+. [154]

Thus, the *in vivo* brain dialysis technique, well described for the study of MPP^+ neurotoxicity, provided us with a means for assessing the potential neurotoxicity of the MAO-B derived polar metabolites as well as the parent compounds of the tetrahydroindenopyridine, tetrahydro-β-carboline, and tetrahydroisoquinoline systems.

Results and Discussion

A. Striatal dopamine levels in mice after intraperitoneal injection with test compound.

The effects of acute intraperitoneal administration of MPTP and the tetrahydroindenopyridines **33** and **49** on striatal dopamine levels in mice are summarized in Table IV and Figure 38. Consistent with several reports[138,139], significant reductions in dopamine were observed at 24 hours, 1 week, and 1 month after the last injection of MPTP. In contrast, the isomeric tetrahydroindenopyridine analogs **33** and **49** produced a transient rise in dopamine levels at 24 hours followed by a return to control values at the 1-week and 1-month time points.

In view of the relatively slow rate that the tetrahydroindenopyridine **33** undergos MAO-B catalyzed oxidation (1% of the MPTP rate, see Table I in Chapter II.) and the lack of oxidation of the isomer **49**, it is not surprising that these compounds do not display MPTP-type neurotoxic properties in the mouse model. These results agree with the lack of toxicity of the tetrahydro-β-carboline **34** in the mouse reported by Perry.[93] The dosage of **34** administered to C-57 black mice by the Perry group (280 mg/Kg cumulative dose over 4 days) was considerably lower than the amount Ung-Chun[92] administered which lowered mouse striatal dopamine levels (1200 mg/Kg cumulative dose over 5 days). Based on these results, it is reasonable to propose that higher doses of the tetrahydroindenopyridine **33** may have shown striatal toxicity, presumably as a result of MAO-B metabolism to the dihydroindenopyridinium species **50** and subsequent oxidation to the indenopyridinium species **52**. However, at a total daily dose of 100 mg/Kg of **33**, 50% of the mice injected died within 24 hours. Thus we were forced to limit our dosage to 80 mg/Kg/day. Indeed,

it is suprising that mice tolerated the 200 mg/Kg daily dose of **34** that was administered by Ung-Chun.

The initial increase in striatal dopamine levels observed with the tetrahydro-indenopyridines **33** and **49** may reflect their *in vivo* MAO inhibitory properties. As will be described below, these tetrahydroindenopyridines, like MPTP, are dopamine releasing agents *in vivo* and this may also account for the initial increase in striatal dopamine levels observed here.

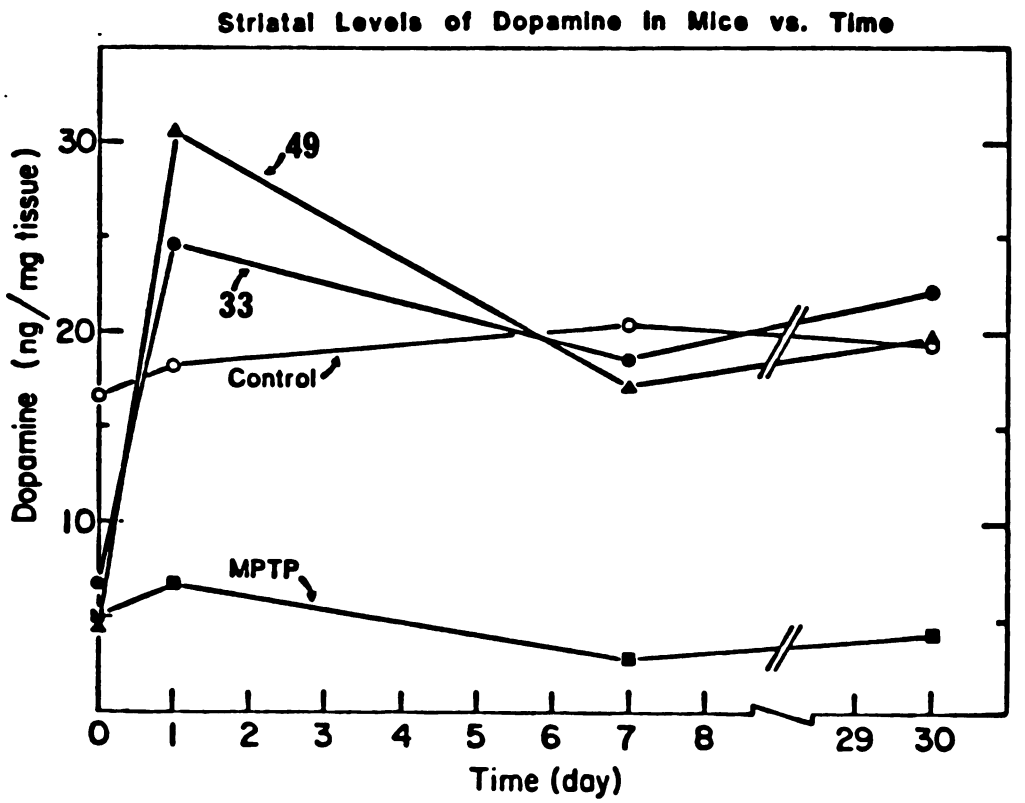
The results of these studies indicate that MPTP-like tricyclic compounds which are not oxidized at an appreciable rate by MAO-B will not deplete striatal dopamine when injected peripherally into the mouse at an MPTP dose that is neurotoxic. However, these results do not directly assess the neurotoxicity of the MAO-B derived metabolites which may deplete striatal dopamine at higher concentrations than can be achieved by peripheral administration of the tetrahydro precursor.

TABLE IV. Striatal Levels of Dopamine in Mice (ng/mg tissue)[a]

Time after last injection	Control (NaCl)	MPTP	33	49
4 hours	16.3 ± 1.8	4.8 ± 0.5	4.3 ± 0.4	6.5 ± 1.3
24 hours	18.0 ± 3.6	6.5 ± 1.0	24.5 ± 4.8	30.3 ± 1.8
1 week	20.1 ± 2.3	2.7 ± 1.7	18.2 ± 1.7	16.4 ± 2.7
1 month	19.0 ± 3.6	5.0 ± 1.3	21.9 ± 3.8	19.2 ± 4.6

[a]C-57 black mice (6 per data point) were injected intraperitoneally with the test compound (100 μmol/Kg) in 50 μL of normal saline every hour for 4 hours. Control values were obtained with vehicle only.

Figure 38.



B. Striatal dopamine levels in rats after dialysis perfusions of test compound*.

The results of the effects on dopamine release by the tetrahydroindenopyridine **33**, the dihydropyridinium **50**, and the indenopyridinium **52** are shown in Figure 39. MPP^+ and the N-methylpyridinium species **87** (MP^+) data are also included in this graph for comparison.[65] While all of the indenopyridine derivatives were found to have dopamine releasing properties, the indenopyridinium species **52** had the most pronounced effect. This compound also proved to be the most potent neurotoxin in this series as one day after the initial 10 mM, 60 min perfusion of **52**, the MPP^+ challenge perfusion provoked only a 4000% increase in basal release of striatal dopamine. In control rats, MPP^+ induces an increase in the basal release of dopmamine of over 23,000%.[65] After a 10 mM, 15 min perfusion of MPP^+, the challenge dose of MPP^+ on day later causes only a 200% increase over basal release of striatal dopamine. Thus, the striatal neurotoxicity of **52** is at least some 20-fold less potent at causing irreversible striatal nerve terminal damage than MPP^+. The tetrahydroindenopyridine **31** was not neurotoxic after a 10 mM, 60 min perfusion as the MPP^+ challenge perfusion one day later caused a 27,000% increase in the release of striatal dopamine. An essentially identical result is obtained with perfusions of MPTP[65] and other cyclic tertiary allylamines (see below). When perfused at 10 mM for 60 min, the neurotoxic effects of the dihydroindenopyridiniuum compound **50** were intermediate between the tetrahydro congener **31** and the fully aromatized compound **52**. However, since this bridged $MPDP^+$ analog is stable at 10 mM for at least 5 hours (see Chemistry Results and Discussion, Chapter II) it provided us with a means to assess the neurotoxicity of the $MPDP^+$ moiety at longer perfusion times.

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*The author wishes to acknowledge that a large portion of this work was performed with the help of and under the supervision of Dr. Hans Rollema.

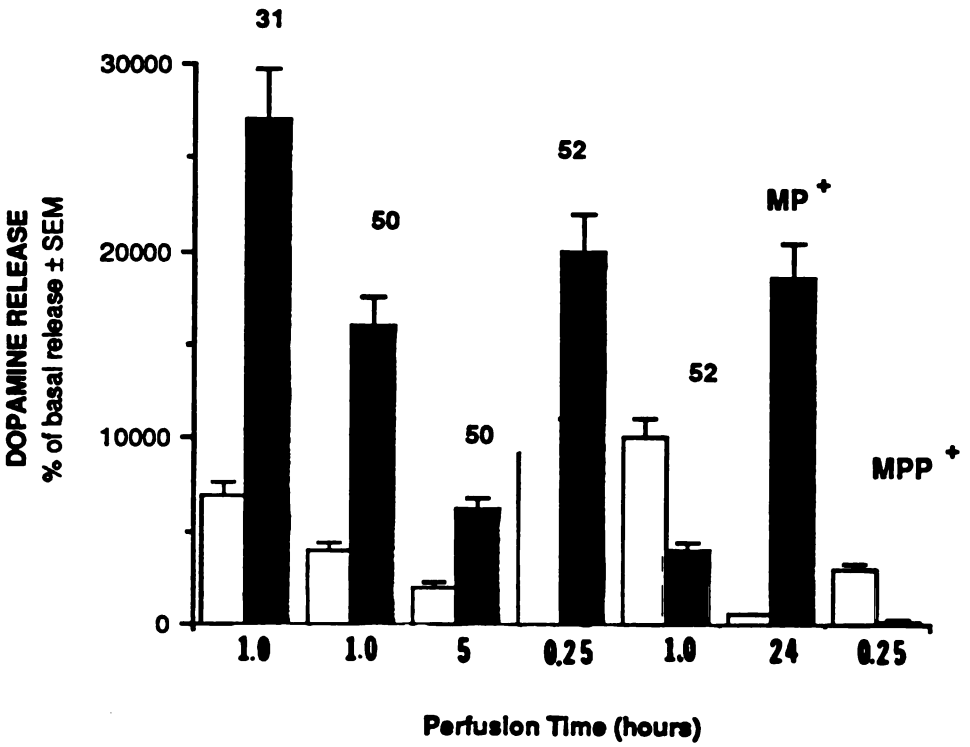


Figure 39. Effect of intrastriatal perfusions with 10 mM indenopyridine derivative test compound on the release of DA from rat striatum *in vivo*. Open bars: Maximal DA release after perfusion with the test compound on day 1. Solid bars: Maximal DA release after the challenge perfusion with MPP^+ on day 2. The effects of a 24 hour perfusion with 10 mM MP^+ and a 15 min perfusion with 10 mM MPP^+ are given for comparison. Increase in DA release is given as percentage of the initial basal release, which was measured in each rat before perfusion with the test compound.

31. tetrahydroindenopyridine compound
50. dihydroindenopyridinium compound
52. indenopyridinium compound

Although more potent than a 10 mM, 60 min perfusion, a 10 mM, 5 hour perfusion, of the bridged $MPDP^+$ analog **50** still did not compare to the neurotoxicity of a 10 mM, 15 min perfusion of MPP^+. In fact, at 5 hours perfusion time the dihydroindenopyridinium species **50** was still somewhat less neurotoxic than a 1 hour perfusion of the indenopyridinium species **52**.

It may only be a coincidence that the indenopyridinium compound **52** was the most potent dopamine releasing compound as well as the most neurotoxic since the tetrahydroindenopyridine **31** also proved to be a potent dopamine releasing agent but was devoid of irreversible neurotoxicity. Another indication that dopamine releasing effects and irreversible striatal nerve terminal toxicity may not be related is seen by comparing the 15 min and 1 hour perfusions of the indenopyridinium **52**. After a 15 min perfusion, there is no irreversible neurotoxicity observed and the maximal dopamine release is about a 11,000% increase over basal levels. Likewise, after a 1 hour (neurotoxic) perfusion of **52**, the induced dopamine release is still only about 10,000%. The two perfusions of **52** induced the same initial dopamine release, but only the longer perfusion time resulted in nerve terminal damage. Thus it appears that the initial dopamine release and nerve terminal damage may not be related. A similar case can be made regarding the dopamine releasing effects and striatal toxicity of the dihydroindenopyridinium **50**.

The indenopyridine derivatives also caused a decrease in the output of striatal DOPAC which was commensurate with their striatal neurotoxic potency. Table V shows DOPAC levels as percent of basal release on the day following perfusion of the test compound but before the MPP^+ challenge perfusion. A 60 min perfusion of the indenopyridinium compound **52** and a 5 hour perfusion of the dihydroindenopyridinium compound **50**, both of which cause irreversible striatal nerve terminal damage, reduce DOPAC levels to about 25% of control values. In contrast, DOPAC levels remain at about 70% of control values after administration of the nontoxic tetrahydroindenopyridine **33**.

These effects on DOPAC are no doubt at least in part due to the MAO-B inhibitory effects of the indenopyridine derivatives (see MAO-B inhibitors, Chapter II) as well as to irreversible dopamine depletion in the case of **50** and **52**.

A longterm perfusion (24 hours) of the N-methylpyridinium species **87** (MP+) caused no irreversible effects on dopamine nerve terminal function since the MPP^+ challenge dose one day later caused a massive release of dopamine comparable to control rats. Thus, the irreversible striatal neurotoxicity demonstrated here by MPP^+ and its analogs is apparently not due to nonspecific damage resulting from long term exposure to charged quaternary systems. Although **87** is a modest releaser of dopamine, it causes no long lasting reduction of DOPAC since levels of this metabolite are back to nearly 80% one day after the perfusion. This result is in agreement with its lack of striatal toxicity.

The results of these studies with the indenopyridine system lead us to several important conclusions regarding MPTP-type neurotoxicity. First, the role of MAO-B has been established here as a requirement in the bioactivation of non-neurotoxic tetrahydro tertiary allylamines to neurotoxic oxidation products, i.e., the tetrahydroindenopyridine and related compounds are not toxic under the same conditions that their MAO-B oxidation products are. Second, the dihydropyridinium-type species may be somewhat neurotoxic, but at least in the case of **50**, are likely to be less so than the fully aromatized compounds. If these results may be extrapolated to the MPTP-system, we may conclude that the majority of the *in vivo* neurotoxic effects observed after MPTP administration cannot be attributed to the $MPDP^+$ metabolite. Although this would seem to implicate MPP^+ as the ultimate neurotoxin, *in vivo* roles for radical intermediates derived from the one-electron oxidation and/or reduction of $MPDP^+$[123] cannot be ruled out by these experiments. In fact, it is the chemical instability of the $MPDP^+$ moiety (as opposed to the more stable dihydroindenopyridinium **50**) that keeps this compound viable as a candidate neurotoxic MPTP metabolite.

Table V. DOPAC levels one day after perfusion of test compound

Compound	Perfusion Time	DOPAC (% of basal release ± SEM, n=3)
MPP^+	15 min	19 ± 10
87	24 hours	80 ± 5
33	60 min	70 ± 10
50	5 hours	25
52	60 min	25 ± 6
34	24 hours	41 ± 7
38	10 hours	21 ± 6
39	10 hours	68 ± 5
40	10 hours	59 ± 8
71	10 hours	76 ± 4
72	150 min	28 ± 6
41	10 hours	76 ± 11
42	10 hours	58
74	10 hours	72 ± 5
76	150 min	42 ±12

Lastly, it appears that incorporating the MPP^+ moiety into a fused ring system is unlikely to produce molecules with potent MPP^+-like neurotoxicity. This structure-activity generalization is also supported by results summarized below which show that the β-carbolinium **38** is not a potent striatal neurotoxin.

Figure 40 shows the effects of the tetrahydro-β-carboline **34** and the β-carbolinium **38** on striatal dopamine release. For comparison, MPP^+ data are also included in this graph. These results show that a 10 mM, 5 hour perfusion of the the β-carbolinium **31** is nontoxic to striatal nerve terminals as the MPP^+ challenge dose one day later causes the same massive release of dopamine seen in control rats. However, a 10 mM, 10 hour perfusion of **38** demonstrates roughly the same irreversible striatal toxicity as a 1 mM, 150 min perfusion of MPP^+. Although the β-carbolinium is about 100-fold less potent than MPP^+, these results are interesting in view of the potential endogenous formation of this compound. As already described (see MAO-B substrates, Chapter II), the tetrahydro-β-carboline **34** has been identified *in vivo* in rat brain[90] and is oxidized by MAO-B *in vitro* to the β-carbolinium **38**.[155] However, in view of the relatively poor MAO-B substrate properties of this compound, it is not surprising that the tetrahydro compound **34** itself is not a potent striatal neurotoxin in this assay system. Even after a 24 hour perfusion of **34**, the large amount of dopamine released by MPP^+ one day later indicates little damage to striatal nerve terminals. This result is in general agreement with Perry *et al.*[93,95] who observed no striatal dopamine depletion in mice or monkeys that were treated with **34**. It is noted however that this striatal dopamine release is some 25% lower than control rats that did not receive a perfusion of **34**. This may indicate some *in vivo* formation of the neurotoxic β-carbolinium **38** during the 24 hour perfusion. These results are consistent with those of Ung-Chun[92] who did observe dopamine depletion after massive repeated doses of **34** were administered to mice.

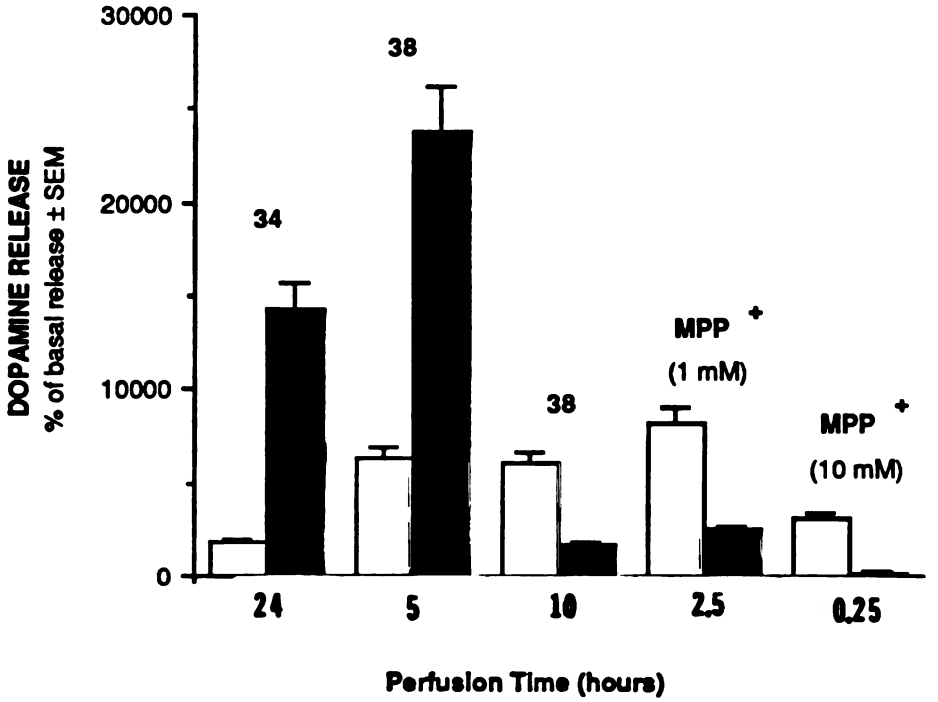


Figure 40. Effect of intrastriatal perfusions with 10 mM β-carboline derivative test compound on the release of DA from rat striatum *in vivo*. Open bars: Maximal DA release after perfusion with the test compound on day 1. Solid bars: Maximal DA release after the challenge perfusion with MPP^+ on day 2. The effects of a 150 min perfusion with 1 mM MPP^+ and a 15 min perfusion with 10 mM MPP^+ are given for comparison. Increase in DA release is given as percentage of the initial basal release, which was measured in each rat before perfusion with the test compound.

34. tetrahydro-β-carboline compound

38. β-carbolinium compound

Table V shows that DOPAC levels return to only 20% of basal values after a perfusion with a toxic dose of **38**. After perfusion of the tetrahydro-β-carboline **34**, DOPAC levels remain at about 40% of basal values (Table V). It is unlikely that this decrease in DOPAC output is due to MAO-B inhibition since **34** is a very poor inhibitor of MAO-B (see MAO-B inhibitor studies, Chapter II). However, dopamine is predominantly oxidized by MAO-A in rat brain[156,157] and **34** has been shown to be a potent inhibitor of this enzyme.[120] On the other hand, as suggested above, there may have occurred some nerve terminal damage after perfusion of **34** (presumably due to conversion to the β-carbolinium **38**) resulting in decreased dopamine formation with concomitant decrease in its metabolite, DOPAC.

Again, since both toxic (10 hours) and nontoxic (5 hours) perfusions of the β-carbolinium **38** have the same effect on dopamine release, it does not appear that neurotoxicity is dependent upon the ability of MPP^+ analogs to release dopamine. This indicates that the striatal dopamine depletion observed after a challenge dose of MPP^+ is not due merely to intense and prolonged dopamine release, but rather to nerve terminal damage.

Figures 41 and 42 show the effect of the isoquinoline and quinoline derivatives on striatal dopamine release.[158] MPP^+ data is included for comparison. All of these test compounds were found to have dopamine releasing properties and to decrease the output of DOPAC (see Table V). However, the maximal increases in the release of dopamine varied widely (Figures 41 and 42). After 10 hour perfusions with 10 mM isoquinoline or quinoline, the release of dopamine was increased 5-fold while the tetrahydro derivatives **39-42** (see Figure 12 for structures) caused a 15-fold increase (Figure 41). The tetrahydro test compounds and the aromatic compounds isoquinoline and quinoline, did not show dopaminergic neurotoxicity as the MPP^+ challenge dose one day later still caused a massive release of dopamine similar to control rats (Figure 41). In contrast, during a 2.5 hr perfusion, the N-methyl quaternary salts **72** and **76** (10 mM, see figure 24 for structures) induced a 200-fold increase in dopamine release (Figure 42) which is similar to that seen

after a 15 min perfusion with 10 mM MPP^+. The challenge perfusion with MPP^+ one day after administration of these quaternary salts caused only a small release of dopamine (Figure 42), confirming that both **72** and **76** are MPP^+-like neurotoxins. These compounds are less toxic than MPP^+ since irreversible dopamine depletion is not observed following the 15 min perfusion and only intermediate effects are observed after the 60 min perfusion. As expected, only the neurotoxic quaternary analogs **72** and **76** cause significant lowering of striatal DOPAC levels one day after their perfusion (see Table V) indicating depletion of striatal dopamine. Both **65** and **69** are also fairly potent competitiive inhibitors of MAO-A (Ki ~ 30 μM)[159] and this may contribute to the observed reduction in DOPAC levels. Quinoline (**74**) also inhibits MAO-A competitively (Ki ~ 30 μM)[122] but one day after perfusion, DOPAC levels are back to 70% of control values. Thus it appears that MAO inhibition may not significantly influence DOPAC levels in our assay system.

The lack of dopaminergic neurotoxicity of the tetrahydro compounds here is consistent with the results obtained above with intrastriatal perfusions of the tetrahydroindenopyridine **33**, the tetrahydro-β-carboline **34**, and MPTP. Furthermore, these studies are in agreement with the results of Perry *et al.* [160] who found that tetrahydroisoquinoline (**39**) was nontoxic when administered subcutaneously in C-57 black mice. Perry[95] also found N-methyltetrahydroisoquinoline (**40**) to be nontoxic when injected subcutaneously into marmosets. Although aromatic, both isoquinoline and quinoline do not contain a permanently charged quaternary moiety and are also not neurotoxic in our assay. Apparently, a quarternized nitrogen atom imparts essential electronic and/or steric properties which are associated with this type of neurotoxicity.

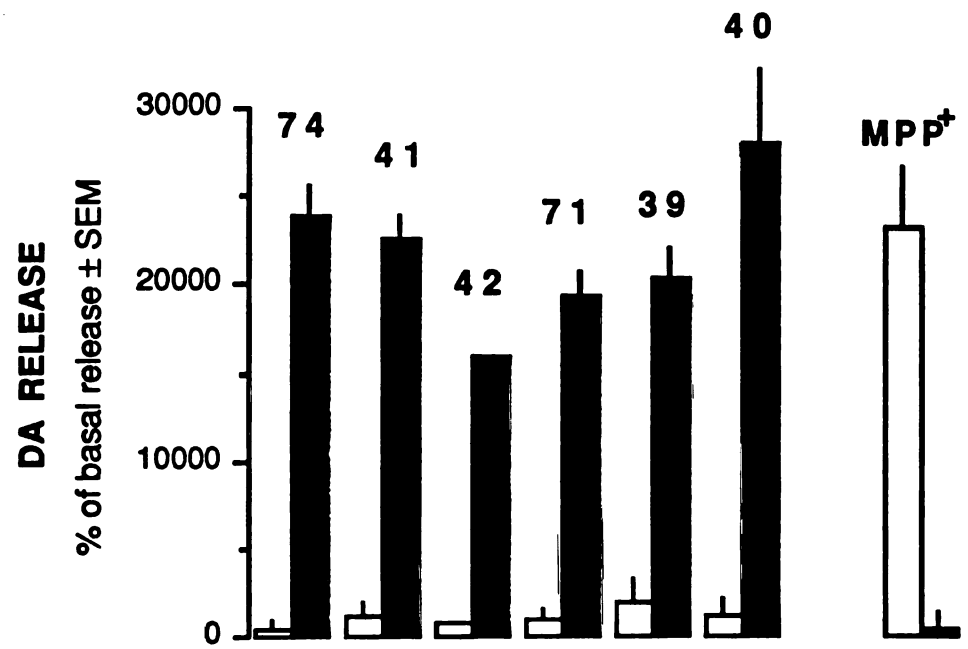


Figure 41. Effect of intrastriatal perfusions with 10 mM test compound for 10 hours on the release of DA from rat striatum *in vivo*. Open bars: Maximal DA release after perfusion with the test compound on day 1. Solid bars: Maximal DA release after the challenge perfusion with MPP^+ on day 2. The effects of a 15 min perfusion with 10 mM MPP^+ are given for comparison. Increase in DA release is given as percentage of the initial basal release, which was measured in each rat before perfusion with the test compound.

74. quinoline
41. tetrahydroquinoline
42. N-methyltetrahydroquinoline
71. isoquinoline
39. tetrahydroisoquinoline
40. N-methyltetrahydroisoquinoline

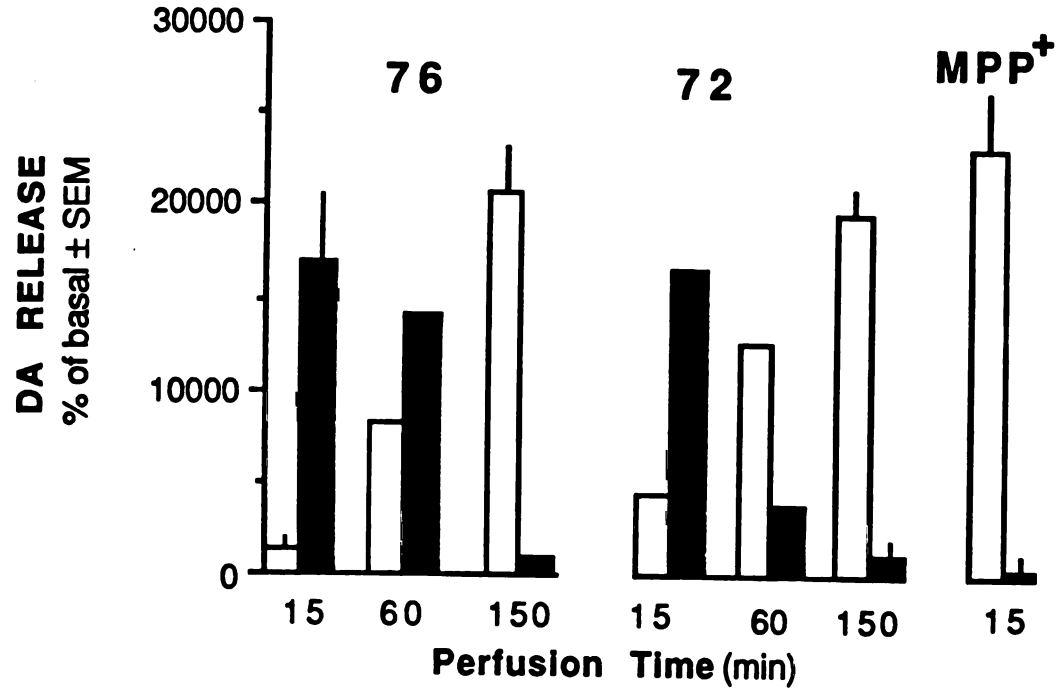


Figure 42. Effect of intrastriatal perfusions with 10 mM test compound for 15, 60 and 150 min on the release of DA from rat striatum *in vivo*. Open bars: Maximal DA release after perfusion with the test compound on day 1. Solid bars: Maximal DA release after the challenge perfusion with MPP^+ on day 2. The effects of a 15 min perfusion with 10 mM MPP^+ are given for comparison. Increase in DA release is given as percentage of the initial basal release, which was measured in each rat before perfusion with the test compound.

76. N-methylquinolinium species
72. N-methylisoquinolinium species

Despite the lack of neurotoxicity of tetrahydroisoquinoline (**39**) demonstrated here, it has been reported[98] that irreversible parkinsonism was observed in marmosets after intraperitoneal administration of this compound. It is possible that the neurotoxicity observed by Nagatsu was due to the *in vivo* formation of the neurotoxic N-methylisoquinolinium species **72** which could arise *in vivo* via the MAO-B catalyzed oxidation (see MAO-B substrates, Chapter II) of N-methyltetrahydroisoquinoline (**40**). This compound could be formed via N-methylation of tetrahydroisoquinoline (**39**) by brain methyltransferases analogous to the reported formation of MPTP from 4-phenyl-1,2,3,6-tetrahydropyridine[161] (see Figure 43). Alternatively, tetrahydroisoquinoline (**39**), also an MAO-B substrate, could be oxidized *in vivo* to isoquinoline followed by N-methylation to the N-methylisoquinolinium species **72** via brain methyltransferases which have been shown to methylate similar heterocyclic systems including 4-phenylpyridine[161] (see figure 43).

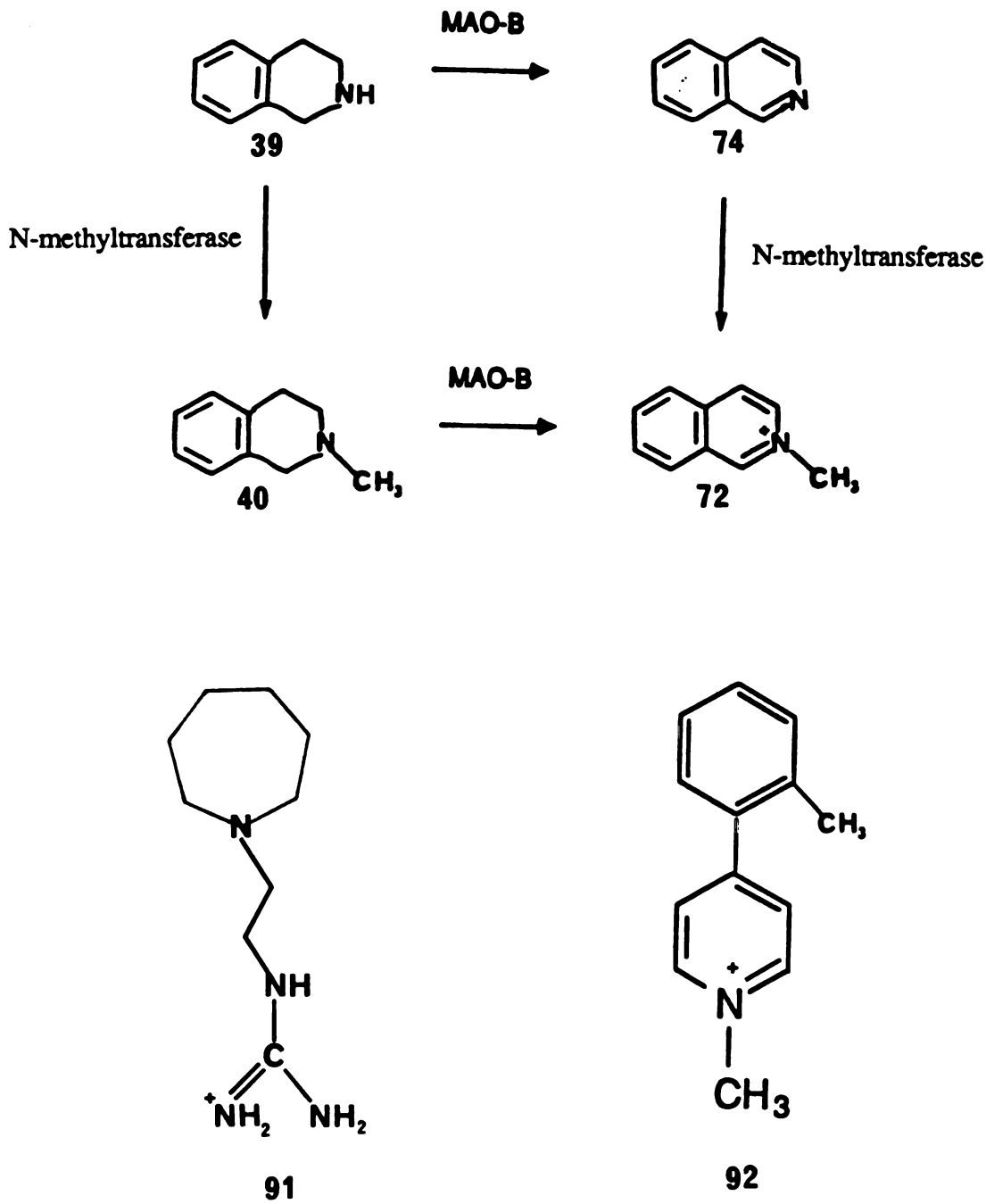
The results of these studies firmly establish the role of MAO-B in the development of MPTP-like neurotoxicity. The tetrahydroindenopyridine, tetrahydro-β-carboline, and the tetrahdyroisoquinoline systems are all relatively poor substrates for MAO-B when compared to MPTP. These tetrahydro compounds are not neurotoxic when administered acutely in the mouse via intraperitoneal injection nor do they cause irreversible striatal neurotoxicity when administered to rats via intrastriatal microdialysis. However, their corresponding dihydro and fully aromatized oxidation products are striatal neurotoxins in the rat when administered via striatal microdialysis perfusions for specific lengths of time. Thus, although *in vitro* data support the oxidation of the tetrahydro compounds by MAO-B (see chapter on MAO-B substrates), apparently these poor substrates are not oxidized at an effective rate *in vivo*.

It also is evident that the potent striatal toxicity of MPP^+ is unique. Although a quaternized amine group appears to be a crucial structural requirement, the steric,

hydrophobic, and electronic parameters of the candidate compound must also be considered when attempting to predict MPP^+-like neurotoxicity. For instance, while the N-methylpyridinium species **87** is devoid of MPP^+-like neurotoxicity, guanethidine (**91**) is about equipotent with the β-carbolinium **38**.[162] While the MPP^+ analogs tested here, as well as many others tested by Dr. Hans Rollema[162], cause MPP^+-like irreversible striatal nerve terminal damage, most are one to two orders of magnitude less potent than MPP^+ itself. The 2'-methyl MPP^+ analog **92**, about half as potent as MPP^+ itself, is the most potent analog of about 3 dozen compounds tested for *in vivo* striatal toxicity so far.[162]

It appears that the structure - activity relationship for MPP^+-like neurotoxicity may involve several prerequisite steps for each of which MPP^+ seems to have the ideal structural features. Specifically, it is proposed that a candidate MPP^+-like neurotoxin must be a suitable substrate for the dopamine uptake system in nigrostriatal neurons and also must inhibit mitochondrial respiration. Preliminary work to correlate these events with striatal neurotoxicity has been undertaken in our laboratory. Anne Johnson and Ellen Wu[163] have found that the *in vivo* neurotoxic potencies of the dihydroindenopyridinium species **50**, the indenopyridinium species **52**, and the β-carbolinium compound **38** (as well as several other MPP^+ analogs) correlate well with their potencies to inhibit synaptosomal uptake of ^{3}H-dopamine. Furthermore, Hans Rollema[162] has determined that there is a correlation between striatal toxicity and *in vivo* inhibition of mitochondrial respiration for MPP^+, the N-methylisoquinolinium species **72**, the N-methylquinolinium species **76**, and the 2'-methyl MPP^+ analog **92**.

Figure 43. Proposed pathways for the formation of endogenous neurotoxins.



Chapter IV

Thesis Conclusions

The results described in this thesis help to define the structural features required of molecules to demonstrate MAO-B substrate activity. Tricyclic planar systems such as the tetrahydroindenopyridines and tetrahydro-β-carbolines are relatively poor substrates for the enzyme when compared to MPTP. As discussed, both steric and electronic effects may account for these differences. The tetrahydroisoquinoline and tetrahydroquinoline systems are also poor MAO-B substrates, again presumably due to negative steric and electronic influences of the fused heterocyclic and phenyl rings.

The tetrahydro compounds above did not deplete striatal dopamine when injected peripherally into the mouse or infused intrastriatally into the rat. However, their identified or proposed MAO-B derived metabolites did demonstrate MPP^+-like neurotoxicity when administered intrastriatally to rats. This firmly establishes the pivotal role MAO-B plays in the development of MPTP-type neurotoxicity.

The structural features required of molecules to demonstrate MPP^+-like neurotoxicity appear to be less strict than those required for MAO-B substrate activity. However, it should be noted that no compound tested here nor any compound reported in the literature compares in neurotoxic potency to MPP^+. This may be due to a unique series of biochemical steps which are required to localize MPTP/MPP^+-like neurotoxins at nigrostriatal dopaminergic neurons, the site of the primary lesion. Apparently, the structural features of MPTP and MPP^+ are ideal with regard to the oxidation step by MAO-B, the proposed uptake into dopaminergic neurons, the proposed uptake into mitochondria and subsequent inhibition of mitochondrial respiration.

Although the β-carboline and isoquinoline derivatives described were not particularly good MAO-B substrates, they must be considered in regard to their presence *in vivo*. The cumulative and chronic degenerative nature of Parkinson's disease would dictate

that candidate endogenous neurotoxins induce more subtle neurotoxicity than the acutely devastating effects of MPTP. The corollary to this observation is that the MAO-B derived metabolites must also be less dramatically neurotoxic than MPP^+. In this context then, the β-carboline and isoquinoline derivatives described become viable candidates as endogenously produced MPTP/MPP^+-like neurotoxins which may be etiological factors in the development of idiopathic Parkinson's disease. Futhermore, based on these results, it is not unlikely that other amines which are present in the environment and share chemical and biological properties similar to MPTP might also be caustive agents of Parkinson's disease.

Chapter V

Experimental

General.

All synthetic reactions were carried out under a nitrogen atmosphere. The following items were purchased from Sigma Chemical, St. Louis, MO: Arecoline HBr, phospholipase C (type I), benzylamine, polyethylene glycol (PEG) 6000, Dextran-250, Ficol (400,000 MW), dopamine HCl, and dihydroxyphenylacetic acid (DOPAC). Sodium 1-heptanesulfonate (HSA) was obtained from Eastman Kodak, Rochester, NY. MPTP HCl was purchased from Research Biochemicals, Natick, MA. All other chemicals were of reagent grade or HPLC grade and were purchased from Aldrich Chemical, Milwaukee, WI. Proton NMR spectra were obtained at 80 MHz on a Varian FT-80 instrument, at 240 MHz on a custom-built instrument linked to a Nicolet 1180 computer, or at 500 MHz on a General Electric instrument. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) in $CDCl_3$ or 3-(tetramethylsilyl)propionic-2,3,3,3-d4 acid, sodium salt (TSP) in the case of D_2O and CD_3OD. Spin multiplicity is given as (s) singlet, (d) doublet, (t) triplet, (q) quartet, or (m) multiplet. Chemical ionization mass spectra (CIMS) were obtained on a modified AEI MS 902S at 8kV with isobutane (ca. 1 torr) as reagent gas and gas chromatography-electron ionization mass spectra (GC/EI-MS) on a Kratos MS 25S. Liquid secondary ion mass spectra (LSIMS) were obtained on a Kratos MS 50S. Mass spectral analyses were provided by the UCSF Mass Spectrometry Facility. Ultraviolet (UV) spectra were recorded using a Beckman DU-50 spectrophotometer connected to an AT&T PC 6300 computer. Infrared spectra were obtained on a Nicolet-5DX FTIR instrument. Melting points were obtained on a Thomas-Hoover or Fisher-

Johns apparatus and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, University of California, Berkeley, CA.

Synthetic Work.

Methyl (*cis, trans*)-1-methyl-4-phenyl-3-piperidecarboxylate (**55**). To 50 g (0.2 mol) arecoline hydrobromide (**54** HBr) was added 100 mL of 50% (by weight) potassium carbonate solution. The mixture was extracted with 5x100 mL benzene. The combined benzene extracts were dried over sodium sulfate and evaporated *in vacuo*. A total of 31 g (0.2 mol) of **54** (free base) was obtained as a colorless oil (one spot on silica gel TLC). The free base **54** was dissolved in 100 mL of ether and 0.3 mol phenylmagnesium bromide was added (100 mL of a 3 M etheral solution) dropwise with stirring over 1 hour. The course of the reaction was monitored by the loss of starting material **54** on silica gel TLC [$CHCl_3$: CH_3OH, (8 : 2)]. After 1 hour, the mixture was treated with 200 mL of cold 6 *N* hydrochloric acid. The aqueous layer was separated, made basic (pH 9) with 300 mL of 50% potassium carbonate, and then extracted exhaustively with ether. The combined ether extracts were dried over sodium sulfate and evaporated *in vacuo* to a dark yellow oil weighing 43 g. This oil was distilled *in vacuo* to obtain 18 g (0.08 mol, 40%) **55** as a light yellow oil: bp 120-130 °C at 0.5 mm Hg (lit.[105] bp 124-128 °C at 5 mm Hg); 80 MHz ^1H NMR ($CDCl_3$) δ 2.2-2.3 (d, 6H, NCH_3), 3.3-3.4 (d, 6H, OCH_3), 7.1-7.3 (d, 8H, ArH).

1-Methyl-4-phenyl-3-piperidinecarboxylic HCl (**56**). To the methyl ester **55** obatined above (18 g, 0.08 mol) was added 100 mL of 6 *N* hydrochloric acid and the mixture was concentrated at 50 °C, 10 mm Hg over an 8 hour period. The remaining

solvent (50 mL) was removed *in vacuo* to yield 12 g of a whitish residue. This material was recrystallized from a 1:1 mixture of ethanol and ether to yield 7.5 g (0.03 mol, 40%) of white crystalline **56**: mp 217-218 °C (lit.[105] mp 214-216 °C), 80 MHz ^{1}H NMR (D_2O) δ 2.05-2.20 [m, 2H, C(5)-H], 2.85 (s, 3H, CH_3), 7.25 (s, 4H, ARH).

Cis-1,2,3,4,4a,9a-Hexahydro-2-methyl-9-H-indeno[2,1-C]pyridin-9-one (**58**) Via Friedel-Crafts cyclization of **56**. To 5 g (0.02 mol) of **56** prepared as above, as added 75 mL of thionyl chloride. After stirring the solution at room temperature for 2 hours, the thionyl chloride was removed *in vacuo* (50 °C, 20 mm Hg). To the dark orange residue was added 100 mL of anhydrous tetrachloroethane. The resulting orange-colored solution was distilled (50 °C, 20 mm Hg) until 50 mL of distillate was collected. To the residual solution was added 50 mL more of tetrachloroethane. The solution was then cooled to 40 °C and 6 g (0.05 mol) of anhydrous aluminum chloride was added. After stirring for several seconds, the evolution of hydrogen chloride gas was detected by litmus paper and visible bubbling inside the reacton vessel. The mixture was stirred at 40 °C for 3 hours and then poured into 300 g cracked ice containing 25 mL of concentrated hydrochloric acid. After standing overnight, the organic and aqueous layers were separated. The aqueous layer was made strongly alkaline (pH 12) wiht 50% sodium hydroxide and the free base amine was extracted with 5 x 200 mL of ether. The combined ether extracts were dried over sodium sulfate and evaporated *in vacuo* to yield 1.9 g of a brown residue. This material was sublimed to give 1.83 g (0.01 mol, 50%) **58** as tan crystals; mp 62-64 °C (lit.[105] mp 64.5-65.5 °C). Via polyphosphoric acid dehydration of **55**. To 70 g (0.45 mol) of the methyl ester **55** was added 500 mL polyphosphoric acid and the mixture was stirred at 150 °C for 6 hours. Subsequently, the mixture was made strongly alkaline (pH 12) by addition of concentrated ammonium hydroxide and the product was extracted with ether. The ethereal extract was dried over sodium sulfate and evaporated *in vacuo* to leave 60 g of a brown solid that melted at 52-55 °C. Two recrystallizations from pentane provided 55 g

provided 55 g (0.21 mol, 61%) **58** as a tan powder: mp 63-65 °C; CIMS 202 (MH^+, 100); 240-MHz ^1H NMR analysis in $CDCl_3$: δ 1.5 [m, 1H, C(4)-H], 2.15 [m, 2H, C(4)-H, C(5)-H], 2.25 (s, 3H, CH_3), 2.45 [m, 2H, C(3)-H, C(1)-H], 2.8 [q, 1H, C(9a)-H], 3.15 [q, 1H, C(1)-H], 3.4 [q, 1H, C(4a)-H], 7.4-7.8 (t, d, t, d 4H ArH).

Cis- and *trans*- 1,2,3,4,4a,9a-hexahydro-2-methyl-9-H-indeno[2,1-C]pyridin-9-ol (**59**). An ethereal solution of ketone **58** (14 g, 70 mmol) was added slowly to a suspension of $LiAlH_4$ (6 g, 160 mmol) in ether with stirring and the resulting mixture was heated under reflux for 3 hours. The reaction was quenched by the addition of 6 mL water followed by 6 mL 15% sodium hydroxide and 18 mL water after which the pH was adjusted to 10 with 15% sodium hydroxide. Ether extraction provided 12.9 g (91%) of a white powder (mp 110-140 °C) that gave two spots on silica gel TLC [$CHCl_3$ - CH_3OH (1:1), r_f = 0.15 and 0.18]. Crystallization from ethyl acetate gave one of the isomeric alcohols as white crystals (mp 144-145 °C; r_f = 0.15) while concentration of the mother liquors yielded the second as large white prisms (mp 124-125; r_f = 0.28). The 240-MHz ^1H NMR ($CDCl_3$) spectra of these compounds were indistinguishable--δ 1.8 (m, 1H), 2.1 (m, 2H), 2.25 (s, 3H, CH_3), 2.5 (m, 2H), 2.7 (m, 1H), 3.0 (m, 2H), 5.08 [d, 1H, C(9)-H], 7.2-7.5 (m, 4H, ArH). Both compounds also gave identical CI mass spectra--204 (MH^+, 100), 186 (43) --and FTIR spectra (15 mM and 1 mM CCl_4)--3300 cm^{-1} (broad, intermolecular bonded OH). Anal. Calcd for $C_{13}H_{17}NO$: C, 76.81; H, 8.43; N, 6.89. Found: C, 76.79; H, 8.41; N, 6.86 (for mp 125 °C), C, 76.95; H, 8.45; N, 6.83 (for mp 144 °C).

Reaction of alcohols **59** with thionyl chloride. A mixture of alcohols **59** (2.0 g, 9.9 mmol) and 25 mL thionyl chloride was stirred at room temperature for 15 min following which the thionyl choride was removed under reduced pressure to yield a hard white mass. Chromatography on 50 g of silica gel (70-230 mesh) using chloroform with a methanol

gradient gave three fractions. The first fraction (0.3 g, 200 mL $CHCl_3$:CH_3OH, 9.5:0.5) yielded a white solid melting at 227-230 °C (dec). Recrystallization from a 1:1 mixture of methanol and ethanol provided the pure 1,2,3,4,4a,9a-hexahydro-9-chloro-2-methyl-9-H-indeno[2,1-C]pyridine (**61**) as its hydrochloride salt: mp 294-297 °C (dec); 80-MHz ^1H NMR (CH_3OD) δ 2.82 (s, 3H, CH_3), 3.0-3.7 (m, 8H), 5.3 [d, 1H, C(9)-H], 7.3 (m, 4H, ArH); CIMS 224/222 (MH^+, 33/100), 186 (100). Anal. Calcd for $C_{13}H_{17}NCl_2$: C, 60.47; H, 6.63; N, 5.43; Cl, 27.46. Found: C, 60.56; H, 6.63; N, 5.38; Cl, 27.61. The second fraction (100 mL $CHCl_3$:CH_3OH, 9:1) gave 0.2 g of a dark green oil with ^1H NMR and CI mass spectral characteristics corresponding to a mixture of the two possible diastereomeric 9-chloro compounds **61**. The third fraction (200 mL $CHCl_3$:CH_3OH, 9:1) yielded 0.6 g of a white powder melting at 260-265 °C (dec.). Recrystallization from a 1:1 mixture of methanol and ethanol provided the pure 2,3,4,4a-tetrahydro-2-methyl-1-H-indeno[2,1-C]pyridine (**49**) as its hydrochloride salt: mp 288-291 °C (dec.); 240-MHz ^1H NMR (D_2O) δ 2.8-2.9 [m, 2H, C(4)-H_2], 3.0 (s, 3H, CH_3), 3.4-3.7 [m, 3H, C(4a)-H, C(3)-H_2], 4.1 [d, 1H, C(1)-H], 4.5 [d, 1H, C(1)-H], 7.0 [s, 1H, C(9)-H], 7.4 (m, 2H, ArH), 7.6 (m, 2H, ArH); CIMS 186 (MH^+, 100); UV (CH_3OH) λ_{max} 250 nm (ε 11,000). Anal. Calcd for $C_{13}H_{16}NCl$: C, 70.42; H, 7.27; N, 6.32. Found: C, 70.22; H, 7.25; N, 6.12.

1,2,3,4-Tetrahydro-2-methyl-9-H-indeno[2,1-C]pyridine hydrochloride (**33** HCl). The mixture of diastereomeric alcohols (**59**, 1.0 g, 4.9 mmol) was stirred at room temperature in 15 mL thionyl chloride for 15 min. The white powder that remained after removal of the thionyl chloride was heated at 50 °C for 18 hours in 50 mL of anhydrous THF containing potassium *tert*-butoxide (2.0 g, 17.8 mmol). The reaction mixture was poured into 1.0 L of water and extracted exhaustively with chloroform. The combined extracts were dried over anhydrous sodium sulfate and the solvent was removed to give 0.7 g of a brown residue. Treatment of this residue in ether with HCl gas precipitated a white

powder (0.63 g.) which was recrystallized from methanol to yield the desired tetrahydroindenopyridine **33** as its hydrochloride salt: mp 286-290 °C (dec.); UV (CH_3OH) λ_{max} 250 nm (ε 11,000); 240-MHz ^1H NMR (D_2O) δ 2.9 [bs, 2H, C(4)-H_2], 3.1 (s, 3H, CH_3), 3.4 [m, 3H, C(3)-H, C(9)-H_2], 3.8 [bd, 1H, C(3)-H], 4.1 [d, 1H, C(1)-H], 4.4 [d, 1H, C(1)-H], 7.4-7.6 (m, 4H, ArH); GC/EIMS 185 ($M^{\cdot+}$, 88), 184 (100), 142 (57). Anal. Calcd for $C_{13}H_{16}NCl$: C, 70.42; H, 7.27; N, 6.32. Found: C, 70.36; H, 7.28; N, 6.22.

1,2,3,4-Tetrahydro-2-methyl-9-H-indeno[2,1-C] pyridine N-oxide bishydrate (**64** $2H_2O$). A solution of **33** (3.2 g, 17.3 mmol) in 25 mL of ethanol and 25 mL dichloromethane containing 5 mL of 30% hydrogen peroxide was heated under reflux for 22 hours and, after the addition of 0.3 g 10% Pd/C, for an additional 12 hours. The reaction mixture was filtered through celite and the filtrate was dried by azeotropic distillation *in vacuo* three times using 4 volumes of anhydrous ethanol and three times using 4 volumes of anhydrous benzene. The light-yellow solid thus obtained was crystallized from benzene to give 2 g (10 mmol, 57%) of pure **64** as its bishydrate: mp 141-143 °C; UV (CH_3OH) λ_{max} 255 nm (ε 9000); 500-MHz ^1H NMR (D_2O) δ 2.9 [bs, 2H, C(4)-H_2], 3.3 (s, 3H, CH_3), 3.4 [d, 2H, C(9)-H_2], 3.6 [t, 2H, C(3)-H_2], 4.2 [d, 1H, C(1)-H], 4.5 [d, 1H, C(1)-H], 7.3-7.6 (m, 4H, ArH); EIMS 201 ($M^{\cdot+}$,7), 184 (85), 142 (88); LSIMS 202 (MH^+). Anal Calcd for $C_{13}H_{15}NO.2H_2O$: C, 67.07; H, 7.94; N, 6.01. Found: C, 67.03; H, 7.82; N, 5.93.

3,4-Dihydro-2-methyl-9-H-indeno[2,1-C]pyridinium perchlorate (**50** ClO_4^-). The above N-oxide (**64**, 0.4 g, 2 mmol) in 50 mL of dichloromethane was stirred with trifluoroacetic anhydride (1 mL, 0.7 mmol) under nitrogen for 1 hour at 0 °C and an additional 30 min at room temperature. Removal of the solvent produced a yellow oil which was dissolved in 10 mL of water and treated with potassium cyanide (0.5 g, 4.4

mmol). The reaction mixture was stirred vigorously and the pH was adjusted quickly to 4 by addition of 10% aqueous sodium carbonate. After stirring for an additional 20 min the pH was adjusted to 10 with aqueous sodium carbonate and the organic layer was separated and discarded. The remaining aqueous phase was extracted with 2 x 25 mL methylene chloride and the combined organic extracts were dried over sodium sulfate, filtered and evaporated to a dark green oil. This oil in 30 mL of ethanol was stirred at room temperature with 1 mL of 70% perchloric acid which caused a light-brown precipitate to form. The product (400 mg) was collected 20 hours later and melted at 74-84 °C. Recrystallization 2 times from methanol provided orange prisms of the perchlorate salt of **50** (100 mg, 0.4 mmol, 20%): mp 142-145 °C; UV (CH_3CN) λ_{max} 360nm (ε 16,700); 500-MHz ^1H NMR (CD_3CN) δ 3.26 [m, 2H, C(4)-H_2], 3.6 (s, 3H, CH_3), 3.8 [2H, C(9)-H_2], 4.0 [m, 2H, C(3)-H_2], 7.55 (m, 2H, ArH), 7.75 (m, 2H, ArH), 8.4 [s, 1H, C(1)-H]; LSIMS 184 (M^+). Anal. Calcd for $C_{13}H_{14}NO_4Cl$: C, 55.03; H, 4.97; N, 4.93; Cl, 12.51. Found: C, 54.86; H, 4.99; N, 4.85; Cl, 12.60.

2-Methyl-9-H-indeno[2,1-C]pyridinium chloride hydrate (**52** $Cl^- H_2O$). In a separate reaction the crude dihydroindenopyridinium compound obtained as above from 200 mg (1 mmol) of N-oxide **64** was dissolved in 100 mL of benzene containing 1.2 g (5 mmol) of chloranil. The solution was stirred in the dark at room temperature for a total of 70 hours. The reaction mixture was extracted with 25 mL 6 N HCl and the aqueous layer was washed exhaustively with ether to remove remaining chloranil and the corresponding hydroquinone reaction product. Lyophilization of the aqueous layer yielded a yellow-brown powder which was dissolved in 10 mL of water. After adjusting the pH to 10 with concentrated ammonium hydroxide, the resulting purple solution was extracted with benzene to remove traces of organic bases. The aqueous layer was lyophilized to a light purple powder which was dissolved in chloroform and filtered to remove inorganic salts. The purple residue obtained after removal of the chloroform was dissolved in methanol and

passed through a 10 g column of Bio-Rad AG 1-X8, 50-100 mesh, chloride form. The methanol was evaporated to leave a purple crystalline material which was recrystallized from acetone. The product, **52** Cl^- (50 mg, 0.23 mmol, 23%), was somewhat hydroscopic: mp 138-141 °C; UV(CH_3OH) λ_{max} 305 (ε 10,000); 500-MHz ^1H NMR (D_2O) δ 4.17 [s, 2H, C(9)-H_2], 4.36 (s, 3H, CH_3), 7.6 [t, 1H, C(6)-H], 7.7 [t, 1H, C(7)-H], 7.77 [d, 1H, C(8)-H], 8.1 [d, 1H, C(5)-H], 8.25 [d, 1H, C(4)-H], 8.6 [d, 1H, C(3)-H], 8.77 [s, 1H, C(1)-H]; EIMS 181 ($M^{\cdot+}$, free base resulting from loss of a C-9 methylene proton,100), 166 (77), 139 (31), LSIMS 182 (M^+). Anal. Calcd for $C_{13}H_{12}NCl.H_2O$: C,70.42; H, 7.27; N, 6.32. Found: C, 70.36; H, 7.28; N, 6.22.

2-Methyl-β-carbolinium iodide (**38** I^-). Norharman (**70**, 2 g, 11.9 mmol) was dissolved in 75 mL of methanol and 3.5 g (24 mmol) of methyl iodide was added slowly with stirring at room temperature. After heating under reflux for 3.5 hours, TLC analysis [silica gel, $CHCl_3$-CH_3OH (9:1)] indicated complete conversion of the starting material and the solution was filtered. As the solution cooled to room temperature, a massive precipitation of yellow crystals occurred. Recrystallization from methanol provided 3.1 g (10 mmol, 84%) of pure **38** I^- : mp 230-232 °C (lit.[100] mp 234-236 °C); UV (CH_3OH) λ_{max} 305 nm (ε 15,000); 500-MHz ^1H NMR (D_2O) δ 4.3(s, 3H, CH_3), 7.33[t, 1H, C(7)-H], 7.45[d, 1H, C(5)-H], 7.7[t, 1H, C(6)-H], 7.9[d, 1H, C(8)-H], 8.05[m, 2H, C(3)-H, C(4)-H], 8.5[s,1H, C(1)-H].

2-Methyl-1,2,3,4-tetrahydro-β-carboline (**34**). To a solution of the above β-carbolinium **38** I^- (2.5 g, 8 mmol) in methanol (75 mL) was added sodium borohydride (0.6 g, 16 mmol) and the solution was heated under reflux for 1 hour. The solution was then cooled to room temperature and white crystals precipitated. Recrystallization from methanol gave 0.5 g (2.7 mmol, 34%) **34**: mp 209-211 (lit.[100] mp 216-217 °C); UV(CH_3OH) λ_{max} 274nm (ε 5,500).

2-Methyl-1,2,3,4-tetrahydro-β-carboline hydrochloride (**34** HCl). The free base **34** was dissolved in ethanol (100%) and HCl gas was introduced which caused a white precipitate to develop. Evaporation of the solvent gave **34**-HCl in quantitative yield: mp 239-241 ° C. Anal. Calcd for $C_{12}H_{15}N_2Cl$: C, 64.71; H, 6.79; N, 12.57; Cl, 15.94. Found: C, 64.70; H, 6.79; N, 12.48; Cl, 15.84.

2-Methylisoquinolinium idodide (**76** I^-). To 45 mL (0.4 mol) isoquinoline in 250 mL of isopropanol was added 50 mL (0.8 mol) methyl iodide with stirring at room temperature. After 5 min the yellow crystalline mass that precipitated was collected and recrystallized from 1:1 ethanol-benzene to give 81 g (0.3 mol, 76%) **76** I^-: mp 160-162 °C (lit.[114] mp 160-162); UV (CH_3OH) λ_{max} 330 (ε 4000).

2-Methyl-1,2,3,4-tetrahydroisoquinoline hydrochloride (**40** HCl). To a solution of **76** I^- (10 g, 37 mmol) in 100 mL of methanol was added 3 g (80 mmol) sodium borohydride and the mixture was stirred at room temperature for 30 min. At the end of this time silica gel TLC analysis [$CHCl_3$-CH_3OH (7:3)] showed that starting material was still present in addition to a new, less polar compound. The mixture was then heated under reflux for 3.5 hours after which time the same TLC analysis indicated complete conversion of starting material. After evaporation *in vacuo*, a pinkish residue remained that was taken up with 200 mL of water and extracted with 2 x 100 mL of ether. The combined ether extracts were dried over sodium sulfate and hydrogen chloride gas was introduced which caused the precipitation of white crystals. The crystals were collected and recrystallized from ethanol to give 0.7 g (3.8 mmol, 10%) **40** as its hydrochloride salt: mp 222-223 °C (lit.[114] mp 225-226).

1-Methylquinolinium iodide (**72** I^-). Quinoline (10 mL, 78 mmol) was dissolved in 25 mL of acetone and 10 mL (176 mmol) of methyl iodide were added. The mixture was

stirred at room temperature for 20 min and then heated under reflux for 20 min after which time TLC analysis (as above) indicated complete conversion of starting material. As the mixture cooled to room temperature, yellow crystals precipitated. The crystals were collected and recrystallized from ethanol to give 10 g 37 mmol, 50%) **72** I^-: mp 142-144 (lit.[113] mp 142-144); UV (CH_3OH) λ_{max} 315 (ϵ 7000).

1-Methyl-1,2,3,4-tetrahydroquinoline hydroiodide (**42** HI^-). A mixture of 300 mg (1.1 mmol) **72** I^- and 25 mg platinum (IV) oxide was prepared in 30 mL of absolute methanol. The mixture was shaken under an atmophere of hydrogen (16 psi) for 2 hours during which time 2.2 mmol of hydrogen were consumed. The mixture was filtered through celite and evaporated *in vacuo*. The yellowish residue that remained was recrystallized from ethanol to yield 100 mg (0.4 mmol, 33%) **42** HI^-: mp 165-167 (lit.[164] mp 167-168).

Enzymology.

Preparation of MAO-B from beef liver mitochondria.[118] Beef liver mitochondrial preparation and phospholipase A were provided by Jim Salach, VA Hospital, San Francisco. Unless otherwise noted, all homogenizations were performed with a Teflon-glass homogenizer. All protein concentrations were determined by the Biuret method. The activity of the enzyme is determined spectrophotometrically by initial rate measurements (30 to 120 sec reaction periods) of the oxidation of benzylamine to benzaldehyde (λ_{max} 250 nm) at 30 °C in 50 mM sodium phosphate buffer, pH 7.2. One unit of MAO-B is that amount of enzyme that oxidizes 1.0 μmole of benzylamine to benzaldehyde per minute at 30 °C, pH 7.2.

The beef liver mitochondrial preparation (4.5 Kg) was homgenized into 1 L of cold H_2O. The protein concentration was determined to be 23.9 mg/mL with a specific activity of 0.023 units/mg. The volume of the homogenized mixture was adjusted to 2500 mL and centrifuged at 41,000g for 15 min. The pellet was dissolved into 600 mL of 0.1 M triethanolamine (TEA) buffer, pH 7.2, and homogenized. The volume of the homogenized mixture was adjusted to 1 L with TEA buffer and calcium chloride was added to give a 25 mM final concentration followed by 50 gm of phospholipase C and 83 mg of phospholipase A. The mixture was stirred at 30 °C for 1 hour, maintaining pH at 7.2 with NH_4OH. The suspension was then centrifuged at 41,000 g for 15 min and the sedimented particles were suspended into TEA buffer (room temperature) and homogenized. The volume was adjusted to 1L with TEA buffer(10-15 mg protein/mL). A total of 8 g of Triton X-100 (as a 20% solution) was added and the mixture was homogenized in a Waring blender for 25 min at room temperature followed by centrifugation at 41,000g for 15 min. The supernatant was collected (1535 mL) and to this was added the following: 169 g Dextran-250, 184 g Ficol, 122 g PEG, and 292 mL water. The volume of the mixture was adjusted to 2600 mL with TEA to provide 3 mg Triton X-100/mL. The suspension was stirred at room temperature until all the solids had dissolved and then centrifuged for 20 min at 10,000g. The emulsion thereby separated into two transparent polymer phases with a protein interface. The semi-solid interface was isolated (0-4 °C) by decanting and aspirating the polymer phases. The protein was homogenized into 460 mL of cold TEA buffer to provide a protein concentration of 10 mg/mL. This mixture was centrifuged at 41,000 g for 20 min. The resulting supernatant solution was stored overnight at 0-4 °C and then centrifuged as above. The clear supernatant solution that was obtained was recentrifuged at 252,000 g for 90 min at 0-4 °C. The resulting pellets were harvested into 5 mL of 50% glycerol, 50 mM sodium phosphate buffer, pH 7.2 and stored at 0-4 °C.

The starting protein concentration of the crude homogenate (1 L) from the mitochondrial preparation was 24 mg/mL with a specific activity of 0.023 units/mg (550 units). The protein concentration after the last purification step (252,000 g spin) was 32 mg/mL (5 mL) with a specific activity of 2.05 units/mg (300 units). Thus the yield of MAO-B from the beef liver mitochondrial preparation was 55%. The specific activity of 2.05 units/mg obtained in this preparation is comparable to values in literature reports wherein the enzyme was judged pure by electrophoresis on polyacrylamide.[118]

Enzyme assays. All assay mixtures (1.0 mL) contained 50 mM sodium phosphate buffer, pH 7.2 with 0.2% (w/v) Triton X-100 (reduced form). For substrate studies, incubation mixtures (room temperature) containing 0.2 units MAO-B and 100 μM **33**, **34**, **39-42**, or **49** were scanned spectrophotometrically (500-200 nm) every 12 hours for 48 hours. Initial rates of formation of dihydro compounds from 3 mM tetrahydro substrate and 0.1 units MAO-B were monitored at the λ_{max} for the dihydro compound. The formation of the indenopyridinium compound **52** from the dihydroindenopyridinium species **50** in the presence of 0.2 units MAO-B was monitored at 305 nm. All assays were compared to two controls -- one contained test compound alone and another contained test compound, enzyme, and 10^{-5} M pargyline. Competitive inhibition assays were performed using benzylamine as substrate (0.17-3.3 mM, Km = 0.4 mM) and 0.02 units MAO-B. Test compound concentrations ranged from 0.0 to 0.5 mM. Irreversible inhibition assays were performed by preincubating 100 μM **50** with 0.02 units MAO-B for 0 to 60 min and then measuring the rate of formation of benzaldehyde (λ_{max} 250 nm) from 3.3 mM benzylamine subsequently added to the mixture.

Neurotoxicity Studies.

Striatal dopamine levels in mice. Eight to 10 week old male C-57 black mice weighing 25 - 30 g were housed 6 per cage with free access to food and water in an animal colony room maintained at 23 °C with controlled lighting (lights on 06:00 - 18:00 hours). Treated mice were administered the test compounds MPTP, **33**, or **49** (100 μmols/kg/hour x 4 hours) in 50 μL normal saline. Control mice were injected with 50 μL normal saline. Following decapitation, the striata were dissected according to the method of Ricuarte *et al.*[139] and immediately stored at -80 °C. All samples were assayed within 2 weeks. Dopamine was determined by an HPLC-electrochemical detection method[165,166] as follows: Tissue samples were spiked with 3 ng/mg of 3,4-dihydroxybenzylamine (DHBA) as an internal standard and then were homogenized with 1.0 mL of cold 0.2 M $HClO_4$. After centrifugation at 13,000 rpm for 15 min, a 500 μL aliquot of supernatant was added to 20 mg of acid-washed Al_2O_3 (pH 3.4) in a 1.5 mL polypropylene tube. To this was added 1.0 mL of 1.0 M Tris buffer (pH 8.6) and the mixture was vortexed for 5 seconds and then shaken mechanically for 15 min. The supernatant was discarded and the Al_2O_3 was washed with 1.5 mL of cold H_2O. The catechols were eluted by addition of 1.0 mL of cold 1.0 M $HClO_4/NaClO_4$ followed by vortexing for 15 seconds. The supernatant was filtered through a 3 mm Cameo nylon membrane before injection onto the HPLC column. Samples (10-100 μL) were injected onto an analytical Alltech C-18, 5 mm column. A single piston Altex LC pump, connected to a Bioanalytical System LC-3 amperometric detector (sensitivity set at 5 nA/V), and Shimadzu integrator/printer were used for all assays. The mobile phase (0.06 M methanesulfonic acid, 0.03 M phosphoric acid, 0.05

mM EDTA, pH 3.6) was maintained at a flow rate of 1.5 mL per min. Catechols were oxidized at an applied potential of +750 mV at room temperature. The concentrations of dopamine were determined by peak height ratio analysis (internal standard/dopamine) using linear standard calibration curves prepared from spiked samples (0 to 150 ng dopamine/mL) in buffer Recoveries averaged 20%.

Microdialysis. In vivo brain microdialysis was performed essentially as described by Rollema.[63,64,65] In brief, U-shaped cannulae equipped with cellulose dialysis membranes were implanted into the striata of chloral hydrate anaesthetized male Sprague-Dawley rats (200-240 g, Bantin and Kingman, Fremont, USA) with the aid of a stereotaxic frame. All experiments were performed one day after surgery. The striata were perfused (4.0 µl/min) with Ringer's solution using a Micro Perpex 2132 pump (LKB, Washington, USA). The perfusate was delivered directly into a 50 ml loop of an automated injection valve (Valco Instruments Co, Houston, USA). Dopamine and DOPAC were measured by HPLC-EC using an Ultrasphere 5 mm ODS column, 25 x 0.46 cm (Beckman, USA) and a BAS LC-3 amperometric detector with a carbon paste electrode set at 650 mV (Bioanalytical Systems, Lafayette, USA). The mobile phase, consisting of 0.05 M sodium acetate buffer with 2mM HSA and 10% methanol, pH 3.7, was delivered at a flow rate of 1.0 ml/min with an Altex 110A pump.

Perfusion studies. Once the basal levels of dopamine and DOPAC had stabilized, the striatum was perfused with a Ringer's solution of the test compound for 15 min to 10 hours. Dialysate concentrations of dopamine and DOPAC were measured at 20 min intervals. One day after the end of the drug perfusion (Day 2), the new basal striatal levels of dopamine and DOPAC were measured. The extent of nerve terminal damage caused by the initial drug treatment was then assessed the same day by perfusing the brain with 10 mM MPP^+ for 15 min and measuring once again dopamine and DOPAC levels. Irreversible nerve terminal damage is indicated if this MPP^+ challenge dose does not cause a large release of DA.[64]

Chapter VI

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