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Model studies on mechanisms of selected chemically-induced neurological disorders

Singh, Malvinder Pal, Ph.D.
Case Western Reserve University, 1990

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MODEL STUDIES ON MECHANISMS OF SELECTED CHEMICALLY-INDUCED NEUROLOGICAL DISORDERS

by

MALVINDER PAL SINGH

Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Thesis Advisor: Dr. Lawrence M. Sayre

Department of Chemistry
CASE WESTERN RESERVE UNIVERSITY
May 1990
CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

MALVINDER PAL SINGH

candidate for the Ph.D.
degree.*

Signed:

F. J. Wrona (Chairman)

John Stuehr

R. Kalenia

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Mahinder Pal Singh
MODEL STUDIES ON MECHANISMS OF SELECTED
CHEMICALLY-INDUCED NEUROLOGICAL DISORDERS

Abstract

by

MALVINDER PAL SINGH

Some structurally simple chemicals are known to induce neurologic defects, and elucidation of the mechanisms of their action is expected to provide crucial leads regarding the pathogenesis of naturally occurring disorders. The mechanisms for three selected examples of chemically-induced neurological disorders are explored in this dissertation through chemical model studies.

1. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-Induced Parkinsonism.

This study has taken a structure-activity approach to elucidate the mechanisms for the various biochemical events associated with dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its metabolite, 1-methyl-4-phenylpyridinium cation (MPP⁺). Current consensus holds that MPP⁺, produced by monoamine oxidase (MAO)-mediated oxidation of MPTP, causes eventual cell death due to inhibition of Complex I of the respiratory chain. Such efficient inhibitory
activity of MPP⁺ is largely due to its concentration inside the mitochondria by an energy-dependent process. This work explored the possible structural requirements that govern the charge-based accumulation of MPP⁺ through evaluation of several analogs for inhibition of respiration in the intact mitochondria (Mₘ) and in electron transport particles (ETP). Replacement of the 4-phenyl ring of MPP⁺ by a variety of aromatic and non-aromatic rings preserves the inhibitory patterns of MPP⁺. The results support our theory that the enhancement of inhibition observed in Mₘ is a result of a Nernstian passive diffusion of the pyridinium cations across the mitochondrial inner membrane in response to the transmembrane potential (ΔѰ, negative inside), and not due to a carrier-mediated transport. The confirmation of these results was sought through the design, synthesis, and biological evaluation of bis-pyridine analogs in neutral, monocationic, and dicationic forms. In contrast to the neutrals, which were potent inhibitors on ETP, the monocations and particularly the dication showed reduced inhibition in ETP, but the activity in Mₘ was enhanced, consistent with their accumulation in the mitochondrial matrix. In the two cases, where the dications possessed greater lipophilicities, they exhibited a greater accumulation-dependent enhancements
of inhibitory activity than the corresponding monocations.

The MAO-mediated dehydrogenation of MPTP is the first necessary event in the expression of neurotoxicity, and some structural requirements for this process were also explored. Four compounds were observed to be better substrates for MAO than MPTP. One such case, namely 1-methyl-4-benzyl-1,2,3,6-tetrahydropyridine (MBnTP), is a good substrate for MAO, but this does not lead to the corresponding 1-methyl-4-benzylpyridinium (MBnP⁺) species, which was shown independently to be toxic. We believe that the "discrepancy" in this case is due to a non-productive deprotonation of the initially generated intermediate, 1-methyl-4-benzyl-2,3-dihydroxypyridinium (MBnDP⁺) to give an exocyclic diename, rather than the endocyclic diename required for producing MBnP⁺.

2. γ-Diketone-Induced Peripheral Axonopathies.

The neurotoxicity of 2,5-hexanedione (2,5-HD) and related γ-diketones is characterized by massive focal accumulation of neurofilaments (NF) in peripheral axons. The accumulation of NF is believed to be initiated through Paal-Knorr condensation of the γ-diketone with the primary amine side-chain residues of critical lysine
residues of NF, resulting in the formation of pyrroles. However, it is not known whether pyrrole formation itself is responsible for neurotoxicity, or if subsequent pyrrole autoxidation giving protein cross-links is required. The current study has attempted to achieve a mechanistic distinction between the two theories through the use of two fluorinated hexanones, 1,1,1-trifluoro-2,5-hexanedione (F₃-HD) and 3-(trifluoromethyl)-2,5-hexanedione (TFM-HD). The latter was found to form pyrroles more rapidly than 2,5-HD with model primary amines, and the resulting pyrroles were found to be resistant toward oxidation. The mechanism of the Paal-Knorr condensation for this and other unsymmetrical diketones was probed by multinuclear NMR kinetics.

3. Mechanism-Based Inhibition of Copper Amine Oxidases by β-Aminopropionitrile and Cyclopropylamines.

The naturally occurring lathyrogen β-aminopropionitrile (BAPN) and cyclopropylamine are time-dependent irreversible inhibitors of various pyrroloquinoline quinone (PQQ)-containing copper amine oxidase. This class of enzymes oxidatively deaminates primary amines via a pyridoxal-like transamination pathway mediated by the PQQ cofactor. Using 3,5-di-t-butyl-1,2-benzoquinone (DTBQ) and 4,7-phenanthroline-5,6-dione (PHAN) as models for PQQ, this study shows that the above amine inactiva-
tors, as well as other related amines such as 1-phenyl-cyclopropylamine (PCPA), result in a rechanneling of the normal transamination mechanism to give stable or metastable adducts of the o-quinone. A hydrolytically metastable β-cyanoenamine is generated from BAPN, and ring-opened adducts are formed in the case of the cyclopropylamines. The factors which govern the occurrence of these and other competing reaction pathways (e.g. benzoxazole production) were traced to the solvent effects and the relative oxidizing capability of the two model quinones.
Dedication

This thesis is dedicated to the fond memory of my late brother, Captn. A. P. Singh of Indian Army, prematurely deceased in March, 1984.
ACKNOWLEDGEMENTS

I wish to thank my parents, R. S. Hansra and Harcharan Hansra, and my sister Parminder for their constant affection, motivation and support, without which none of this would have been likely.

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I also thank Department of Chemistry, Case Western Reserve University for the financial support in form of a Graduate Alumni Award for the academic year 1987-88.

Dr. Subir V. Gokarn deserves a special word of thanks for his unfailing friendship and constant encouragement in times of frustration and low "spirits". Many thanks are also due to Drs. C.L. Hoppel, S.I. Harik, N.J. Riachi, P.K. Arora, and F. Wang for their participation in the project relating to MPTP neurotoxicity.

Lastly, I wish to express my sincere gratitude -- all the faculty members and technical staff at Chemistry Department for their invaluable help and guidance at various steps along my graduate studies.
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<td>BAPN</td>
<td>β-aminopropionitrile</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopropylamine</td>
</tr>
<tr>
<td>Cu-AO</td>
<td>copper amine oxidase</td>
</tr>
<tr>
<td>DMAB</td>
<td>4-(dimethylamino)benzaldehyde</td>
</tr>
<tr>
<td>3,3-DMHD</td>
<td>3,3-dimethyl-2,5-hexanediione</td>
</tr>
<tr>
<td>3,4-DMHD</td>
<td>3,4-dimethyl-2,5-hexanediione</td>
</tr>
<tr>
<td>DTB-AP</td>
<td>4,6-di-β-butyl-2-aminophenol</td>
</tr>
<tr>
<td>DTBQ</td>
<td>3,5-di-β-butyl-1,2-benzoquinone</td>
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<tr>
<td>ETP</td>
<td>electron transport particles</td>
</tr>
<tr>
<td>F3-HD</td>
<td>1,1,1-trifluoro-2,5-hexanediione</td>
</tr>
<tr>
<td>GC</td>
<td>glassy carbon</td>
</tr>
<tr>
<td>2,5-HD</td>
<td>2,5-hexanediione</td>
</tr>
<tr>
<td>IC50</td>
<td>concentration required for 50% inhibition</td>
</tr>
<tr>
<td>IDPN</td>
<td>β,β-iminodipropionitrile</td>
</tr>
<tr>
<td>K_m</td>
<td>the Michaelis constant (the substrate concentration at which the reaction is half the maximal rate)</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MBnDP⁺</td>
<td>1-methyl-4-benzyl-2,3-dihydropyridinium cation</td>
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<td>MBnP⁺</td>
<td>1-methyl-4-benzylpyridinium cation</td>
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<td>MChTP</td>
<td>1-methyl-4-cyclohexyl-1,2,3,6-tetrahydropyridine</td>
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<td>MCpTP</td>
<td>1-methyl-4-cyclopentyl-1,2,3,6-tetrahydropyridine</td>
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<td>Acronym</td>
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<td>3-MHD</td>
<td>3-methyl-2,5-hexanedione</td>
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<td>MnBK</td>
<td>methyl n-butyl ketone</td>
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<td>MPDP⁺</td>
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<td>1-methyl-4-phenyl-1,2-dihydropyridine</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>Mᵊ</td>
<td>intact mitochondria</td>
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<td>NF</td>
<td>neurofilament</td>
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<td>NOE</td>
<td>nuclear Overhauser effect</td>
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<td>Py⁺</td>
<td>pyridinium</td>
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<tr>
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<td>saturated calomel electrode</td>
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<td>TFM-HD</td>
<td>3-(trifluoromethyl)-2,5-hexanedione</td>
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<td>TPB⁻</td>
<td>tetraphenylborate anion</td>
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<tr>
<td>V_max</td>
<td>the maximal velocity for an enzymatic reaction</td>
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CHAPTER I.

Model Studies on

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -

Induced Parkinsonism.
INTRODUCTION:

Since the initial discovery\(^1\) in 1983, it is now well known that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPP) induces a Parkinsonian condition in humans\(^1,2\) and other experimental animals.\(^3-6\) Systemic administration of MTPP to test animals leads to the appearance of essentially all biochemical and histopathologic hallmarks of Parkinson's Disease (PD) viz. dopamine deficiency and destruction of the pars compacta region of the substantia nigra in the brain.\(^1-6\) Intense research efforts aimed at elucidating the mechanism(s) for MTPP-induced neurotoxicity have therefore been expected to provide important leads regarding the pathogenesis of PD, normally an age-related disorder.\(^6-8\)

Studies directed at understanding MTPP neurotoxicity have revealed a quite complex sequence of events associated with MTPP and its metabolites.\(^9-12\) Thus MTPP, a cyclic tertiary allylic amine can cross the blood-brain barrier in its neutral form upon systemic administration. Once in the brain MTPP is metabolized initially to its 2-electron oxidation product 1-methyl-4-phenyl-2,3-dihydropyridinium ion (MPDP\(^+\))\(^13,14\) which is metastable but is further converted to 1-methyl-4-phenylpyridinium cation (MPP\(^+\))\(^13,14,19\) the ulti-
mate 4-electron oxidized product that has been detected in the brain of rodents and primates.\textsuperscript{15-17}

The first step in the metabolic activation, i.e., two-electron oxidation of MPTP to MPDP\textsuperscript{+}, is now widely accepted to be mediated by the enzyme monoamine oxidase B (MAO-B).\textsuperscript{13,18} Direct evidence for the intermediacy of MPDP\textsuperscript{+} comes from the isolation of its cyano adduct obtained by trapping the iminium species \textit{in vitro} with NaCN.\textsuperscript{14,19,20} The crucial involvement of MAO-B in the metabolism of MPTP has been confirmed by studies which show that selective MAO-B inhibitors which block the metabolism of MPTP also provide protection against its neurotoxicity.\textsuperscript{21-25} The second step i.e MPDP\textsuperscript{+} $\rightarrow$ MPP\textsuperscript{+} presumably occurs through the deprotonated neutral dienamine form of MPDP\textsuperscript{+} (Figure 1). The exact site and nature of this conversion has not been elucidated thus far.

![Diagram](image_url)

\textbf{Figure 1.} Pathway for the metabolic activation of MPTP by monoamine oxidase.
Early hypotheses regarding the mechanisms for MPTP-induced neuronal degeneration focused on the two positively charged metabolites MPDP⁺ and MPP⁺. Thus MPDP⁺, bearing a chemically reactive α,β-unsaturated iminium functionality was speculated to be capable of alkylating biomacromolecules and/or directly oxidizing dopamine to dopaquinone.²⁶-²⁸ Also, based on its structural similarity to a known herbicide and lung toxin paraquat, MPP⁺ was speculated to operate through paraquat-like toxicity mechanisms²⁹,³⁰ which involve catalytic production of active oxygen radicals.³¹ Sentiment for such a mechanism arises in part from a commonly held belief that free-radical and oxidant stress mechanisms contribute to the normal age-related loss of nigral neurons.³² All these mechanisms have been subsequently shown to be irrelevant, based on electrochemical measurements³³,³⁴ and chemical model studies,³³ and thereafter the attention has focused on alternate explanations for the contributions of these cations in the expression of MPTP toxicity.

Most studies have focused on the end-product of metabolism, MPP⁺, since rapid neurologic damage is produced upon directly injecting it into the brain.³⁵-⁴⁰ MPP⁺ has been shown to be selectively concentrated in the nigrostriatal dopamine-containing neurons,¹⁵,²⁹,³⁰
and the relatively selective toxicity of MPP⁺ to the
dopaminergic neurons is attributed to its active accumu-
lation via the high affinity dopamine reuptake
pathway.⁴¹-⁴⁴

The crucial turning point in the MPTP story was
the key observation that MPP⁺ is a potent inhibitor of
mitochondrial respiration.⁴⁵-⁵⁰ This inhibition, re-
sulting in a compromise of cellular energy production,
is now believed to be the primary reason for cell death
associated with MPTP neurotoxicity.⁴⁵,⁴⁶ Inhibition of
respiration by MPP⁺ has been localized to an inhibition
of complex I,⁴⁹,⁵⁰ the segment of the respiratory chain
which constitutes the enzyme NADH: ubiquinone
reductase.⁵¹ A number of chemically unrelated compounds
have been shown in the past to be effective potent
inhibitors of complex I and have been used as tools for
studying the numerous sites within this complex.⁵²
Direct evidence for the relationship between MPP⁺-in-
duced inhibition of mitochondrial respiration and the
level of cell death associated with MPTP intoxication
is the demonstration by Heikkila’s group³⁶ both in vivo
and in vitro, that MPTP induces lactate accumulation and
other biochemical hallmarks of cells on the verge of
dying from energy depletion.

A crucial aspect of MPP⁺ induced inhibition of
mitochondrial respiration is that it is a weak intrinsic Complex I inhibitor but is an effective inhibitor on intact mitochondria because it is accumulated inside the organelle. Such an accumulation allows MPP⁺ to reach substantial concentration levels in the mitochondrial matrix, to which the respiratory chain is exposed. It has been shown that the mitochondrial accumulation of MPP⁺ is an energy-dependent process which relies on MPP⁺ being permanently charged. This charge-based accumulation of MPP⁺ is consistent with the finding that 4-phenylpyridine, the corresponding neutral analog, which is a stronger inhibitor of complex I in the isolated respiratory chain, is not concentrated inside the mitochondria and therefore is not a sufficiently effective inhibitor on intact mitochondria to exhibit any toxicity.

At the outset of the present study it had been proposed that the concentration of MPP⁺ across the mitochondrial membranes arises from a carrier-mediated transport, based mainly on two observations: (i) that active uptake of MPP⁺ exhibits an apparent temperature- and concentration-dependent saturation, and (ii) that various MPP⁺ analogs exert a concentration-dependent competition against uptake of [³H]MPP⁺. It was, however, not clear as to what the structural re-
quirements for such a carrier were. In order to determine the structure-activity relationships for an active transport of MPP\(^+\)-like molecules, we desired to compare the inhibitory potencies of a set of neutral pyridines and their respective N-methylated pyridinium derivatives. Our initial results\(^5\) indicated no apparent stringent relationship between structure and the ability to shut down mitochondrial respiration, which was not the expected finding were a carrier involved. Instead, we proposed that the accumulation of MPP\(^+\) inside mitochondria occurred by a passive Nernstian transport in response to the transmembrane potential gradient (\(\Delta \Psi\), positive outside and negative inside),\(^4\) in an analogous manner to what is known for tetraphenylphosphonium and other "permeant" organic cations.\(^5\)–\(^6\)

The mitochondrial electron transport chain is asymmetrically distributed in the membrane in such a way that transfer of electrons from NADH to oxygen results in translocation of H\(^+\) from one side of the membrane to the other. The membrane is also impermeable to H\(^+\), and the H\(^+\) translocation by the electron transport chain sets up a [H\(^+\)] gradient as a potential energy store used for the synthesis of ATP. This electrochemical gradient has two components: an electrical component (the membrane potential, \(\Delta \Psi\)) and a chemical component (the pH
gradient, $\Delta pH$). The membrane potential $\Delta \Psi$, maintained by actively respiring mitochondria, will drive the accumulation of lipophilic cations (of charge $Z$) across the membrane to an extent which is given by the following Nernst equation:

$$\Delta \Psi = R T/F \ln [(\text{cation}_{\text{inside}})/(\text{cation}_{\text{outside}})]$$

Consistent with the Nernstian based concentration of MPP$^+$ are the recent findings$^{61-63}$ that a permanently charged lipophilic anion tetraphenylborate (TPB$^-$) accelerates the mitochondrial accumulation of MPP$^+$ and thereby enhances the onset of respiratory inhibition by MPP$^+$ on intact mitochondria. TPB$^-$ is widely known to accelerate the accumulation of other permeant cations.$^{57}$ At the time of writing this dissertation a report has appeared which indicates an overall potentiation of MPTP neurotoxicity by TPB$^-$ in vivo.$^{64}$ This is consistent with the above findings and also with the fact that inhibition of respiration by MPP$^+$ is a crucial and necessary step in the manifestation of MPTP-like neurotoxicity.

In order to obtain further evidence for the passive Nernstian concentration mechanism for MPP$^+$, we have tested a variety of rationally designed structural
analogs. Additionally, we sought extended aromatic structures containing 4-substituted pyridines at either end, so that they could be mono- and di-quaternized. For a Nernstian accumulation mechanism, the dications are theoretically expected to be accumulated to a concentration gradient that is the square of that for the monocations.\textsuperscript{65}

Referring back to the initial reports\textsuperscript{13,14,18,24} on the role of MAO in the bioactivation of MPTP, several groups of investigators have directed their efforts to characterize the structural requirements for substrate activity for this enzyme. These studies have led to an identification of several analogs\textsuperscript{66-68} which exhibit MPTP-like neurotoxic properties and confirm the crucial requirement of oxidation by monoamine oxidase\textsuperscript{68a} for the manifestation of their neurotoxicity. We have also been interested in this aspect, and the present study describes 7 such compounds designed to test certain structural variations.

First, the 4-phenyl substituent was replaced by cyclopentyl and cyclohexyl groups in order to determine if the electronic or steric nature of the 4-substituent was more important. Another two examples separate the 4-phenyl ring from the heterocycle by a one-carbon unit. In one of them the position of the double bond has been
switched so that it is homoallylic to nitrogen. Third, a furanyl substituent was chosen to replace the 4-phenyl group in analogy to previous reports on the 4-thienyl$^{68c}$ and 4-pyrroly$^{68a,68d}$ analogs. Lastly, an additional methyl substituent was introduced at the 5-position of the tetrahydropyridine ring (5-Me-MPTP) in order to explore the steric requirement close to the reactive tertiary amine center.

Of these MPTP analogs, the 4-benzyl derivative was of particular interest because it was reported to be a better monoamine oxidase substrate than MPTP itself, yet it is not neurotoxic.$^{9,67}$ For this reason we synthesized its expected 2-electron oxidized product, 1-methyl-4-benzyl-2,3-dihydropyridinium cation (MBnDP$^+$) and studies were carried out to investigate the possibility that its inability to undergo efficient conversion to a pyridinium product might be responsible for the absence of neurotoxicity.

Taken together, the studies presented in this chapter reflect an interdisciplinary structure-activity approach to a rather complex toxic activation problem, with a focus on mechanism. In general, the mitochondria-directed cytotoxicity associated with the wide variety of structures described in this study, and the fact that respiratory inhibition by MPP$^+$ is responsible
for MPTP neurotoxicity, suggests a possible role of lipophilic organic cations in other toxicologic contexts.
CHEMICAL SYNTHESIS:

Preparation of 4-substituted pyridines has been reported in the past from direct reaction of pyridine with nucleophilic reagents, followed by oxidation of the intermediate dihydropyridine. However both C₂ and C₄ positions in pyridine are electrophilic in nature and therefore a mixture of 2- and 4-substituted pyridines results. Quarternary pyridinium salts are usually more reactive towards nucleophilic addition, and the choice of a good leaving group on pyridine nitrogen permits a facile fragmentation of the intermediate N-substituted dihydropyridine. Exclusive attack at C₂ however renders this method inefficient for production of 4-pyridines. Similar results have also been reported with the use of N-acyl pyridiniums, except when special cumbersome procedures (e.g., preparation of cuprates) were used. Recently two groups have reported remarkable success in switching the regioselectivity for nucleophilic attack on pyridinium salts by using novel N-substituents. Thus the 2,6-dimethyl-4-oxopyridin-1-yl group has been used to both activate the pyridine ring and serve as a good leaving group, and also provide a steric congestion around the α-positions, thereby directing the nucleophiles to the γ-position of the pyridine. This procedure however requires special preparation of a pyridi-
nopyridone as the starting material. The use of the tert-butyldimethylsilyl group is equally successful for the same purpose as demonstrated by Akiba et al.\textsuperscript{75} This method was used by us for the several MPP\textsuperscript{+} analog precursors required in our study: 3-methyl-4-phenylpyridine (4), 4-cyclopentylpyridine (21), and bispyridines 35 and 37 (Figures 2a and 2b).

γ-Diketones are known to readily condense with primary alkyl amines to give pyroles and also undergo an acid-catalyzed cyclocondensation reaction to lead to the formation of furan derivatives.\textsuperscript{70} We made use of these two reactions to prepare the 4-heteroaryl pyridine analogs 14 and 16 employed in this study. The corresponding pyridine-substituted diketone precursor was obtained by the Stetter reaction\textsuperscript{76} which is based upon addition of "unpoiled" aldehydes to α,β-unsaturated ketones. Thus, CN\textsuperscript{-}-catalyzed addition of pyridine-4-carboxaldehyde to methyl vinyl ketone gave 1-(4-pyridinyl)-1,4-pentanedione (13), which upon condensation with methylamine gave 14, while dehydrative ring closure catalyzed by HCl provided 16 (Figure 3).
Figure 2(a). Reaction schemes for synthesis of 4 and 21.

Figure 2(b). Reaction schemes for synthesis of bis-pyridine analogs 35 and 37.

Figure 3. Reaction schemes for preparation of 4-pyrylyl- (14) and 4-furanyl- (16) pyridine analogs.
The Stetter reaction has also been achieved with Mannich bases, which serve as the source of $\alpha,\beta$-unsaturated ketones in situ.$^{77}$ We employed a Mannich base from acetophenone for preparing 1-phenyl-4-(4-pyridinyl)-1,4-butanedione (31) and further extended this method to the synthesis of 38 from the bis Mannich base obtained from diacetylbenzene (Figure 4). The other dimeric pyridine analog 36 was prepared in analogy to a reported procedure for synthesis of a structurally related 2,2'-bispyridine (Figure 5).$^{78}$

Having obtained the various 4-substituted pyridines by these miscellaneous routes, the corresponding methylated pyridinium cations were prepared by treatment with CH$_3$I. In the case of the 4-cyclopentyl, 4-cyclohexyl, and 4-benzyl analogs, we encountered difficulty with the methylation reaction, which we have traced to an apparent iodide-mediated benzylic oxidation. In the case of the benzyl derivative, oxidation to 1-methyl-4-benzoylpyridinium was found to have been reported earlier.$^{79}$ Therefore in these cases methyl-$p$-toluenesulfonate was employed as the methylating agent. The quaternary tosylates were stable under normal experimental conditions of isolation and biological testing.

The tetrahydropyridine analogs required for evalu-
ation of their ability to serve as MAO substrates were obtained by NaBH₄ reduction of the corresponding pyridinium derivatives. Compounds 42 and 44 were prepared via a Wittig reaction and direct condensation of the mono-Grignard reagent from dibromobenzene with 1-methyl-4-piperidone, followed by an acid-catalyzed dehydration of the tertiary alcohol formed.
Figure 4. Reaction scheme for synthesis of bis-oxindole analog 38.

Figure 5. Reaction scheme for preparation of bis-pyridine analog 36.
BIOLOGICAL DATA ON INHIBITION OF MITOCHONDRIAL RESPIRATION.

The respiratory inhibition potencies of various MPP⁺ analogs were determined in the labs of Prof. C. L. Hoppel (Department of Pharmacology and Medicine) both on intact mitochondria (Mₜ)⁵⁶ and inverted membrane preparations (submitochondrial particles, SMP or electron transport particles, ETP)⁵⁶ through measurement of the concentrations required to reduce (by 50%) the rate of O₂ consumption. These I₅₀ values are presented in Tables I-III for various neutral and charged MPP⁺ analogs. For the charged compounds, the measurements were made 10 min after introducing the compound, in order to allow for its accumulation across the mitochondrial membrane. The accumulation of MPP⁺ itself is known to be time-dependent from previous studies, which have also shown that the 10 min interval is sufficient to produce maximal inhibition.⁴⁷ Although this may not be the case for all the charged compounds in Tables I-III, we nonetheless used the 10 min I₅₀ values as a way of comparing inhibitory efficiency.

In analogy to the observed inhibitory characteristics of both MPP⁺ and 4-phenylpyridine,⁴⁹ the compounds used in the present study are also presumed to be acting on Complex I of the respiratory chain. This was con-
firmed by the observation that mitochondria incubated with a 90% inhibitory (I90) dose of each compound restored full respiration upon subsequent addition of 10 mM succinate, a respiratory substrate which bypasses complex I and provides reducing equivalents directly through complex II of the electron transport chain.

Table I shows the results on a series of cationic MPP⁺ analogs and the corresponding neutral pyridines. In general, the cationic compounds showed an enhanced potency on intact mitochondria compared to electron transport particles, indicating that they are concentrated inside mitochondria, as observed for MPP⁺. The ratios of I50 values on SMP and Mₔ, i.e. (SMP/Mₔ) were calculated in order to provide a quantitative measure of this accumulation which is independent of intrinsic inhibitory potency.

Also evident from the results of Table I is the fact that the neutral unmethylated versions are all much better inhibitors of electron transport (SMP assays) than their respective quarternary pyridinium partners. Their inhibitory activities are not enhanced on Mₔ since they are not actively concentrated. In fact, their I50 values on Mₔ are 2.5 times higher than on SMP, apparently on account of some limitation on the diffusion of the uncharged analogs to the inhibitory site in the intact
organelle. This inhibitory activity of the neutral pyridines is surprisingly insensitive to structural variations. Taken together, these results are indicative of a rather "soft" structure-activity dependence for the Complex I inhibition.
Table 1. Inhibition of respiration (IC₅₀, mM) by neutral and charged pairs of MPP⁺ analogs on submitochondrial particles (SMP) prepared from rat heart mitochondria and on intact rat liver mitochondria (Mₜ).

<table>
<thead>
<tr>
<th>Entry no.</th>
<th>IC₅₀ SMP</th>
<th>IC₅₀ Mₜ</th>
<th></th>
<th>Entry no.</th>
<th>IC₅₀ SMP</th>
<th>IC₅₀ Mₜ</th>
<th>Relative Activity (%)</th>
<th>IC₅₀ Ratio SMP/Mₜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.09</td>
<td>0.2</td>
<td>MPP⁺</td>
<td>2.3</td>
<td>0.09</td>
<td></td>
<td>100</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>0.08</td>
<td>0.5</td>
<td></td>
<td>3.3</td>
<td>0.14</td>
<td></td>
<td>64</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>0.90</td>
<td>2.0</td>
<td></td>
<td>0.67</td>
<td>&gt; 10</td>
<td>&lt; 1</td>
<td>&lt; 0.07</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.07</td>
<td>0.18</td>
<td></td>
<td>2.1</td>
<td>0.26</td>
<td></td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>0.13</td>
<td>0.2</td>
<td></td>
<td>3.7</td>
<td>0.33</td>
<td></td>
<td>27</td>
<td>11.2</td>
</tr>
</tbody>
</table>
In general the charged compounds, 1,3-dimethyl-4-phenylpyridinium (5), 1-methyl-4-benzylpyridinium (MBnP⁺) (9) and 1-methyl-4-(4-hydroxyphenoxy)pyridinium (11) were all found to behave like MPP⁺, albeit with lower potencies in both SMP and \( M_w \) assays. One interesting exception noted however was 1-methyl-4-phenyl-1,3-pyrimidinium cation (7), which was more potent on SMP than MPP⁺, but was ineffective on intact mitochondria. This implies its inability to be transported across the membrane, a possible explanation being the loss of its cationic property and consequently its ability to be actively concentrated, due to the formation of a neutral pseudobase. The \( pK_a \) for the pseudobase formation from the pyrimidinium species is expected to be much lower than that for pyridinium, due to a higher electrophilicity of the C-2 center. \(^{81}\)

Table II shows respiratory inhibition data for additional charged analogs of MPP⁺. Once again the \( I_{50} \) values on \( M_w \) are low compared to those on the inverted membrane preparation (ETP in this case), indicative of their concentration inside the mitochondria. The \( I_{50} \) ratios are quite large for many of the resonance-delocalized cations, with no apparent stringent structural requirements, viz., the 1-methoxy (12), 4-heteroaryl (15, 17), 4-biaryl (18) or 4-cycloalkyl (22, 23) analogs
were all found to exhibit an $I_{50}$ ratio $> 10$. These ratios were, however, low for compounds 26-28 (the 4-arylalkyl-pyridiniums containing an alkyl bridge). This is due not to an increased inhibition on ETP, but rather to a diminished activity on $M_w$, reflecting a decreased ability to be accumulated across the membrane. Compounds containing a polar hydroxyl substituent in the structure also exhibited lower inhibition on $M_w$, and thereby lower $I_{50}$ ratios compared to their non-hydroxylic counterpart (e.g. compare 11 vs 24).

As described in the introduction a Nernstian accumulation mechanism predicts that dications should theoretically be accumulated to a concentration gradient that is the square of that for the monocations. The enhancement of inhibitory potency in $M_w$ relative to ETP ($I_{50}$ ratio) should therefore be much larger for dications than for monocations of similar structure and physicochemical properties. In order to test this prediction, we synthesized four sets of MPP$^+$-like pyridine-based dimers in their neutral, monocationic and dicationic forms, and compared their ability to inhibit respiration in ETP and $M_w$.

Respiratory inhibition data on these analogs is given in Table III in comparison to MPP$^+$. The inhibitory activities for the neutrals (35-38) in ETP are in the
expected range in comparison to the I$_{50}$ of 0.12 mM for 4-phenylpyridine. Further, inhibitory potency in ETP for the monocations and dications is much lower than for the corresponding neutrals (by factors of 16-120 and 30-3600 respectively). This is not surprising since MPP$^+$ is 79 times weaker than 4-phenylpyridine in ETP. The lower activity of monocations, and particularly the dications (in ETP) is also a reflection of the low lipophilicities of these charged compounds. The partition coefficients (measured by Dr. F. Wang in our group) for the cationic compounds are also listed in Table III, and understandably the dications are observed to have much lower lipophilicity than the corresponding monocations.

For the first series of "dimers" 35 (Table III) the inhibitory potencies for both the monocation and the dication on M$_w$ are only slightly enhanced compared to the inhibition displayed on ETP. Furthermore contrary to our expectation, the I$_{50}$ ratio of dication 35b is lower than that of monocation 35a.

For the second and third sets of "dimers" (36 and 37), the inhibition is enhanced on M$_w$ for both monocationic and the dicationic derivatives, and in these cases, the dications appear to be accumulating to a greater extent than the monocations (the I$_{50}$ ratio for
dication 37b is 10 times greater than for monocation 37a). This observation is consistent with the expected magnification of the Nernstian based concentration factor for dications due to the overall +2 charge.

The fourth set of dimers (38) employed the use of a bis(furanyl)benzene spacer group, which adds both to the lipophilicity of the molecules and to the flexibility in terms of rotational isomers. The results (Table III) show that both monocation and dication in this case are essentially equipotent on intact mitochondria and that the dication is accumulated to a greater extent than the monocation.
TABLE II. Inhibition of respiration (IC50, mM) by a variety of MPP⁺ analogs on electron transport particles (ETP) prepared from rat heart mitochondria or intact rat liver mitochondria (Mₑ).

<table>
<thead>
<tr>
<th>Entry no.</th>
<th>ETP</th>
<th>Mₑ</th>
<th>Relative Activity (%)</th>
<th>IC50 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MPP⁺)</td>
<td>9.5</td>
<td>0.08</td>
<td>100</td>
<td>125</td>
</tr>
<tr>
<td>12</td>
<td>3.8</td>
<td>0.12</td>
<td>75</td>
<td>32</td>
</tr>
<tr>
<td>15</td>
<td>6.8</td>
<td>0.04</td>
<td>200</td>
<td>170</td>
</tr>
<tr>
<td>17</td>
<td>2.0</td>
<td>0.04</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>18</td>
<td>5.0</td>
<td>0.075</td>
<td>107</td>
<td>67</td>
</tr>
<tr>
<td>20</td>
<td>16.8</td>
<td>1.8</td>
<td>&lt;5</td>
<td>9.3</td>
</tr>
<tr>
<td>22</td>
<td>10.4</td>
<td>0.128</td>
<td>63</td>
<td>81</td>
</tr>
<tr>
<td>23</td>
<td>0.75</td>
<td>0.07</td>
<td>114</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Structure</td>
<td>ETP</td>
<td>$M_w$</td>
<td>Relative Activity (%)</td>
</tr>
<tr>
<td>---</td>
<td>-----------</td>
<td>-----</td>
<td>-------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>24</td>
<td><img src="image1" alt="Structure" /></td>
<td>10</td>
<td>0.155</td>
<td>48</td>
</tr>
<tr>
<td>11</td>
<td><img src="image2" alt="Structure" /></td>
<td>3.7</td>
<td>0.33</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td><img src="image3" alt="Structure" /></td>
<td>1.3</td>
<td>0.12</td>
<td>66</td>
</tr>
<tr>
<td>26</td>
<td><img src="image4" alt="Structure" /></td>
<td>12.5</td>
<td>4.85</td>
<td>&lt;5</td>
</tr>
<tr>
<td>27</td>
<td><img src="image5" alt="Structure" /></td>
<td>14.5</td>
<td>5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>28</td>
<td><img src="image6" alt="Structure" /></td>
<td>35</td>
<td>11</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Molecule</td>
<td>ETP IC₅₀ (mM)</td>
<td>Mₜ IC₅₀ (mM)</td>
<td>Relative Activity (%)</td>
<td>ETP/Mₜ Ratio (10 min)</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>---------------</td>
<td>-----------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>9.5</td>
<td>0.1</td>
<td>0.08</td>
<td>100</td>
</tr>
<tr>
<td>35</td>
<td>0.017</td>
<td>0.07</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>35a</td>
<td>2.0</td>
<td>0.225</td>
<td>0.12</td>
<td>67</td>
</tr>
<tr>
<td>35b</td>
<td>4.5</td>
<td>2.6</td>
<td>1.25</td>
<td>6.4</td>
</tr>
<tr>
<td>36</td>
<td>0.10</td>
<td>0.045</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>36a</td>
<td>0.44</td>
<td>0.41</td>
<td>0.22</td>
<td>36</td>
</tr>
<tr>
<td>36b</td>
<td>6.0</td>
<td>5.4</td>
<td>2.8</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Structure</td>
<td>ETP $I_{50}$ (mM)</td>
<td>$M_0$ $I_{50}$ (mM)</td>
<td>Relative Activity (%)</td>
</tr>
<tr>
<td>---</td>
<td>-----------</td>
<td>------------------</td>
<td>--------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>37</td>
<td><img src="image1" alt="Structure" /></td>
<td>0.001</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>37a</td>
<td><img src="image2" alt="Structure" /></td>
<td>0.087</td>
<td>0.029</td>
<td>0.015</td>
</tr>
<tr>
<td>37b</td>
<td><img src="image3" alt="Structure" /></td>
<td>3.2</td>
<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>38</td>
<td><img src="image4" alt="Structure" /></td>
<td>0.003</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>38a</td>
<td><img src="image5" alt="Structure" /></td>
<td>0.048</td>
<td>0.082</td>
<td>0.041</td>
</tr>
<tr>
<td>38b</td>
<td><img src="image6" alt="Structure" /></td>
<td>0.09</td>
<td>0.085</td>
<td>0.042</td>
</tr>
</tbody>
</table>

Table III. contd
MAO substrate activity for a few MPTP analogs:

Seven MPTP analogs were evaluated, in collaboration with Drs. N. Riachi and S. I. Harik, for their ability to be oxidized by rat liver mitochondrial preparations enriched in monoamine oxidase.\textsuperscript{68b} The assay for MAO utilized a colorimetric measurement of the H\textsubscript{2}O\textsubscript{2} by-product produced upon incubation with the mitochondrial preparation at 37°C.\textsuperscript{85} The oxidation of each analog was found to be blocked by 0.1 mM pargyline, a known selective inhibitor of monoamine oxidase.\textsuperscript{24,25} The kinetic parameters $V_{\text{max}}$ and $K_m$ for the oxidation of these compounds are presented in Table IV. Three compounds, namely MCpTP (39), MChTP (40), and MBnTP (41) exhibited $V_{\text{max}}$ values larger than did MPTP itself, but their relative affinities for the enzyme ($K_m$ values) also varied.

The substrate specificity for an enzyme is best described as the $V_{\text{max}}/K_m$ term. A substrate is considered better than another if it either binds better (lower $K_m$) or reacts faster when it is bound (higher $V_{\text{max}}$). As is evident from the $V_{\text{max}}/K_m$ values, the three aforementioned analogs are better substrates than MPTP itself.
Table IV. Kinetic parameters for MAO oxidation of MPTP analogs

<table>
<thead>
<tr>
<th>Compound #, and structure</th>
<th>$V_{\text{max}}$ (nmol $\text{H}_2\text{O}_2/\text{mg protein/ hr}$)</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}/K_m$</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPTP</td>
<td>227 ± 22</td>
<td>78 ± 13</td>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>39</td>
<td>733 ± 35</td>
<td>124 ± 04</td>
<td>5.9</td>
<td>196</td>
</tr>
<tr>
<td>40</td>
<td>318 ± 27</td>
<td>59 ± 12</td>
<td>5.4</td>
<td>180</td>
</tr>
<tr>
<td>41</td>
<td>516 ± 41</td>
<td>85 ± 17</td>
<td>6.07</td>
<td>202</td>
</tr>
<tr>
<td>42</td>
<td>52 ± 19</td>
<td>79 ± 12</td>
<td>0.66</td>
<td>22</td>
</tr>
<tr>
<td>43</td>
<td>50 ± 16</td>
<td>245 ± 66</td>
<td>0.2</td>
<td>7</td>
</tr>
<tr>
<td>44</td>
<td></td>
<td></td>
<td>8% activity compared to MPTP at 1 mM</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td></td>
<td></td>
<td>No detectable activity at 1mM</td>
<td></td>
</tr>
</tbody>
</table>


The iso-benzyl analog 42 is a good substrate in terms of its binding capacity ($K_m$ is equal to that of MPTP), but it is oxidized at a relatively slow rate, so that the overall activity is 22% that of MPTP. The furanyl (43) and the $p$-bromophenyl (44) analogs are both found to exhibit very low activity (<10%), and the former also binds poorly to the enzyme. The last compound (45) with a methyl substituent on the pyridine ring also shows a very low activity, again owing to poor binding.

The 4-benzyl derivative is of particular interest since although it is a better MAO substrate than MPTP, it is reported to be non-neurotoxic. In analogy to the formation of MPDP$^+$ from MPTP, the 4-benzyl analog is presumably converted to MBnDP$^+$ by MAO. However, subsequent deprotonation can lead to the formation of the exocyclic dienamine instead of the endocyclic dienamine structure required for formation of a pyridinium compound. Indeed, synthetic MBnDP$^+$ (48), under kinetic deprotonation conditions was observed to give the exocyclic dienamine 50 (see Figure 6), the structure of which was deduced (vide infra) from its $^1$H NMR spectral characteristics, NOE experiments, analogous results on deprotonation of MBnP$^+$ (9) (vide infra), and recently published results on 4-benzylpyridine anion.
Figure 6. Scheme for generation of macrocyclic dienamine 50 (E configuration)
Studies on deprotonation of benzyl analogs of MPDP$^+$ and MPP$^+$:

In order to obtain a frame of reference for the eventual structural assignment of the dienamine $50$ derived from MBnDP$^+$, we first studied the deprotonation of MBnP$^+$ which can give only a single neutral trienamine ($51$).

The $^1$H NMR spectrum of the material ($51$) obtained by CHCl$_3$ extraction after treatment of MBnP$^+$ ($9$) with base, is shown in Figure 7. This shows a non-equivalence of H(3) and H(5) and also of H(2) and H(6) of the pyridine ring and suggests a hindered rotation around the bond linking the methylene center with C(4) of the pyridine ring. The assignment of the $^1$H spectrum is based on decoupling experiments and NOE measurements. The two NOE difference spectra are also shown in Figure 7. The signals at $\delta$ 6.04 and 6.13 were identified as H-2 and H-6 based on decoupling experiments and NOE's of 13% and 15% respectively, upon saturation of the N-CH$_3$ signal ($\delta$ 3.07) (Figure 7(b)). Similarly, saturation of the $\delta$ 5.28 signal (CH) resulted in NOE's of 12% and 5% respectively for the signals at $\delta$ 5.69 and 6.34; this NOE data along with the decoupling experiments confirmed these signals as due to H-3 and H-5 (Figure 7(c)). The smaller NOE of the signal at $\delta$ 6.34 led us to assign
this as H-5 (the inner proton). This assignment is consistent with the spectral characteristics of 4-benzylpyridine anion, where a similar chemical shift non-equivalence of inner and outer protons was observed and attributed to phenyl ring current effects. Furthermore, the exocyclic double bond character was claimed to force a mutual twisting of the two rings in this case.

The trienamine 51 also exhibited two unusual proton-proton couplings: H(2) and H(6) and also H(3) and H(5) were seen to be coupled (J_{2,6} = 2.0 Hz and J_{3,5} = 2.6 Hz), which we attribute to a staggered W-conformation of the two sets of protons. Such "W-coupling" is well known through C centers, but we are not aware of such coupling through a N center. Furthermore, a long-range coupling of 1 Hz was observed between H(6) and the methylene hydrogen, which falls into an extended W conformation category. These assignments and arguments helped us analyze the spectrum of the exocyclic dienamine 50.

Kinetic deprotonation of MBnDP+ (48) by adding it as a dilute solution to cold aqueous alkali provided 50, which was extracted with CHCl₃ for its spectral characterization. Consistent with the exocyclic dienamine structure, the ¹H spectrum displayed two coupled doublets for the C-5 and C-6 methine protons at δ 5.59 and
6.08, along with two coupled triplets for the C-2 and C-3 methylene protons at 2.66 and 3.12 δ; a feature which would not be expected from the endocyclic diamine structure.
Figure 7 (a) $^1$H-NMR spectrum of S1
(b) NOE difference spectrum upon saturating N-CH$_3$
(c) NOE difference spectrum upon saturation of -CH-Ph
The signals observed for the vinylic positions in this compound correspond to an E-configuration (Figure 6). Thus, H(3) was upfield of H(2), in analogy to the outer protons in 4-benzylpyridine anion and the exocyclic trienamine described above. The effect of ring current is demonstrated by the shift in the position of C(3)-H$_2$ triplet, which is at lower field compared to C(6)-H$_2$. The structure is consistent with the NOE of 17% observed for H(2) upon saturation of N-CH$_3$ signal. No NOE was observed however for the triplet signal at $\delta$ 2.66, presumably because the irradiation frequency is too close. For this same reason, there was no NOE observed for the signal at $\delta$ 5.59 (H-3) when the methylene CH was irradiated. The signal at $\delta$ 3.12 also was not affected in this same experiment, consistent with the structure shown (Figure 6).

The $^{13}$C NMR spectra for the two exocyclic enamine structures (50 and 51) were also recorded. However, a conclusive assignment of the structure would have required more advanced and longer COSY experiments, which posed great difficulty in our case because both compounds slowly but irreversibly degrade to unknown products. Thus additional spectral experiments were halted.
DISCUSSION:

Inhibition of mitochondrial respiration by MPP⁺-analogs:

Since the initial reports on the Parkinsonian symptoms induced by MPTP,¹⁻⁶ significant progress has been made in identifying the individual steps involved in the toxic activation mechanism.⁹⁻¹² The rapid progress made owes, in part, to the expectation that the MPTP mechanism might provide clues about the pathogenesis of this incurable neurologic disease.⁶⁻⁸ Substantial evidence has now accumulated that MPTP induced neurotoxicity arises largely from the cytotoxicity associated with MPP⁺,⁹⁻¹¹,¹⁵ the end-product of MAO-mediated metabolism of MPTP.¹³,¹⁴,¹⁹ Furthermore there is now a general consensus that MPP⁺ cytotoxicity arises on account of its effective inhibition of mitochondrial respiration.⁴⁵⁻⁵⁰,⁵⁶,⁶³ Thus MPP⁺ has been shown to be an effective site 1 inhibitor in isolated mitochondria⁸² as well as in rat striatal slices.⁸³ MPP⁺ induces metabolic changes (ATP depletion, lactate accumulation or overproduction, and increased glucose utilization) both in vivo and in vitro, which is consistent with a cellular energy crisis.³⁶

Although by itself a weak inhibitor on the elec-
tron transport chain, MPP\(^+\) is a potent inhibitor on intact mitochondria due to the fact that it is concentrated across the inner membrane by an energy-dependent process.\(^{47}\) That the accumulation is driven by the transmembrane electrochemical gradient and not by the proton gradient is evident from the fact that valinomycin along with K\(^+\) stops the MPP\(^+\) uptake (by collapsing the electrochemical gradient), whereas agents which abolish the proton gradient do not affect MPP\(^+\) uptake.\(^{47}\) It was originally speculated\(^{10,53,55}\) that MPP\(^+\) transport was a carrier-mediated phenomenon. Such an unprecedented existence of a carrier for organic cations has however not been supported by any conclusive evidence to date.

Our initial finding that a host of MPP\(^+\) analogs exhibit an accumulation-dependent enhancement of respiratory inhibition independent of structure is not supportive of carrier-mediation.\(^{56}\) Rather, we proposed a passive diffusion of these positively charged lipophilic cations across the mitochondrial membrane in analogy to the well-known fact that lipophilic cations, in response to the transmembrane potential, undergo a passive Nernstian concentration inside the mitochondria.\(^{57-60}\) Furthermore the rate of such Nernstian accumulation of permeant cations is enhanced by the presence of permeant
anions like tetraphenylborate. A similar stimulation of the onset of MPP⁺ inhibition on intact mitochondria by TPB⁻ has now been confirmed by work in our laboratory and others. This is not expected for a carrier-mediated accumulation mechanism.

A Nernstian accumulation mechanism predicts that any permanently charged cation with sufficient lipophilicity and delocalization of charge, which also has reasonable complex I inhibitory capability, should show an energy-dependent potentiation of inhibition on intact mitochondria. This point is substantiated by the observation that the inhibitory activity of MPP⁺ is preserved upon replacing the Ph group with other aromatic groups (substituted phenyl and heterocyclic) and even with cycloalkyl groups. These results further support a passive rather than carrier-mediated transport mechanism for mitochondrial uptake of MPP⁺.

We have assessed the energy-dependent accumulation of MPP⁺ analogs across the membrane indirectly by determining the inhibitory enhancement on Mₑ compared to ETP or SMP (the I₅₀ ratio). According to our Nernstian accumulation mechanism, this factor should be relatively constant assuming that equilibrium accumulation levels are attained during the 10 min incubation period which we have used for our measurements. In actuality, howev-
er, this ratio is observed to vary over a fairly large range (Tables I-III). Thus for MPP$^+$ and its close biaryl mimics, the ratio is large (>10) while (i) a hydroxyl substituent in the 4-aryl ring (compare 11 to MPP$^+$ and 24) and (ii) introduction of an alkyl bridge between the pyridinium and aryl ring (compounds 26-28), all showed a diminished ratio, primarily because of an unusually low activity in $M_w$. An explanation for this variation in $I_{50}$ ratio is discussed in the next section dealing with the bis-pyridine "dimers".

The findings that the neutral pyridines are much better inhibitors of complex I than are the corresponding pyridinium cations indicate that the neutral pyridine ring is recognized at the locus of inhibition with greater preference over the charged pyridinium groups. This suggests that the design of superior inhibitors for intact mitochondria should preserve the overall positive charge (in order to ensure a Nernstian accumulation) but in a way that preserves a neutral pyridine recognition element. We sought to achieve this in two ways: (i) through extensive delocalization of the positive charge and/or (ii) by locating the delocalized positive charge at a position removed from the pyridine recognition element. This goal was best achieved by the monocationic versions of our bis-pyridine "dimers" which look like
MPP\(^+\) at one end and like the neutral 4-phenylpyridine at the other end, discussed next. At this point, the results described so far confirm our assertion that there is no strict dependence of the complex I inhibition on the structural and electronic properties of MPP\(^-\)-like inhibitors.

A final test for the validity of a Nernstian based accumulation mechanism was sought through a comparison of the inhibitory activities of physicochemically similar mono- and di-cationic compounds, wherein the latter are expected to accumulate to an extent which is the square of that occurring for the former.\(^65\) However, experimental verification of this prediction has not been found, presumably owing to the fact that any dication is inherently less lipophilic than its corresponding monocation, and thereby less capable of traversing biological membranes. Nonetheless, the comparison of several related sets of bis-pyridines in both monocationic and dicationic forms was expected to help clarify the criteria for the manifestation of enhanced inhibition by dications over monocations, owing to a greater Nernstian accumulation factor.

As indicated by the relatively low ETP/M\(_W\) \(I_{50}\) ratios observed (Table III), the monocations and especially the dications are transported across mitochondria
less efficiently than MPP\(^+\) itself. We have already seen that for a wide variety of monocationic analogs of MPP\(^+\) all of which should accumulate equally at equilibrium, that the actual degree of accumulation, based on the enhanced inhibition in \(M_w\) relative to ETP, varies considerably; being smaller for the analogs with low lipophilicity. This is presumably because the expected Nernstian accumulation is denied due to limited membrane permeability which prevents attainment of electrochemical equilibrium. Although poor transport of dications can be understood in terms of very low lipophilicity, the limiting factor for the monocations is not entirely clear since, for example, the partition coefficient for 37a is 21 times greater than that for MPP\(^+\).\(^{84}\) This points towards the existence of an additional factor limiting transport which may be related to the flexible and extended molecular dimensions of the pyridine-base dimers, compared to the more rigid and shorter structure of MPP\(^+\).

Another factor which may prevent achievement of the predicted Nernstian accumulations is that the inhibitory effect exerted by that compound already accumulated can reduce the transmembrane potential driving this same accumulation. For MPP\(^+\), accumulation inside the mitochondria occurs very rapidly such that the predicted
Nernstian level is reached before the onset of significant inhibition. In contrast, for the slowly transported analogs, inhibition starts to appear at an early stage of the accumulation. This "negative feedback" effect is apparently particularly pronounced for the dicaticonic bis-pyridine "dimers", where studies conducted by Dr. Wang in our group indicate that a trade-off between inhibition and accumulation develops before there is a chance for enough accumulation to produce complete inhibition of respiration (i.e. O₂ consumption does not cease but plateaus at some low level). It should be kept in mind that the I₅₀ ratios we report are only indirect measures of the accumulation factor. A direct measurement, however, would require the use of radiolabeled derivatives, which is impractical in our case.

The above arguments adequately rationalize that while MPP⁺ shows a large ETP/Mᵦ I₅₀ ratio indicative of an efficient Nernstian accumulation, the same ratios for the slowly transported MPP⁺ analogs and especially the bis-pyridine "dimers" substantially underestimate the theoretical Nernstian accumulation factor. Since this underestimation is much greater for the dications than for the monocations, the observation for the series 37a/37b and 38a/38b that the I₅₀ ratio is still larger
for the dication than monocation supports our contention of a greater theoretical Nernstian accumulation of the dications. The lack of an enhanced effect for dications 35b and 36b is presumably due to their minimal lipophilicity (i.e. the extremely low partition coefficient for 35b). Only in the case of 37a/b is the prediction of a squared accumulation factor for the dication borne out in the observed I_{50} ratios. In general, a convincing confirmation of this prediction would require the use of monocation/dication pairs possessing similar physico-chemical characteristics, a situation which will be hard to realize.

In conclusion the compounds used in this study constitute a class of structurally simple yet potent inhibitors of mitochondrial respiration. For the bis(pyridine) "dimers", the monocationic forms, which combine a neutral arylpyridine component with the delocalized cationic charge crucial to Nernstian concentration, are also observed to exhibit increased inhibitory activity on intact mitochondria. In fact, the compound 37a, which looks like 4-phenylpyridine at one end and mimics MPP⁺ at the other, exhibits the most potent inhibition on Mₙ yet known for MPP⁺ like molecules.

Finally an interesting proposal emerges out of our studies which relates to a possible application of these
compounds in medicine. We wonder if these charged compounds, especially the ones which are found to have a greater inhibitory potency than MPP+, could be of therapeutic potential in mitochondrial based chemotherapy. We are aware of one such approach to antitumor therapy based on a selective accumulation of lipophilic organic cations in tumor cell mitochondria,60,65 which are known to possess greater transmembrane electrochemical potentials and are thereby capable of accumulating cationic compounds to a greater extent than normal cell mitochondria.

MAO activity of MPTP analogs:

The role of monoamine oxidase in MPTP induced neurotoxicity became apparent from the early report13,14 that MPTP is a good substrate for this enzyme in vitro, and is initially converted to MPDP+. This was subsequently confirmed by the observations that MAO inhibitors provide a protection against the neurotoxic effects of MPTP in experimental animals.21-25 Although the detailed mechanism for the oxidation of MPTP by MAO is unclear, the formation of MPDP+ is analogous to the generation of imines from primary and secondary amines by MAO.85 In view of these observations, there has been a considerable effort to elucidate substrate-structural
requirements for oxidation by MAO.

Our contribution to this area has been to obtain MAO data on seven MPTP analogs. Thus, we find that the 4-phenyl substituent in MPTP can be replaced by cycloalkyl groups (compounds 39 and 40) and the compounds still retain their ability to be oxidized by MAO. During the course of this study, others have reported similar results with the 4-cyclohexyl analog. Systemic administration of this compound has been shown to produce neurotoxicity in mice in a manner similar to MPTP. Although we have not tested the 4-cyclopentyl analog for its neurotoxicity, we feel that this compound should also be neurotoxic.

A possible deleterious steric effect on the tetrahydropyridine ring is indicated by the relative inability of the 1,5-dimethyl-4-phenyl-1,2,3,6-tetrahydropyridine (45) to be oxidized under the conditions used. Fries et al have shown this compound to be non-neurotoxic in mice. Replacement of the phenyl group by heterocyclic rings, 4-thienyl and 4-(1-methylpyrrol-2-yl) has previously been shown to be tolerated in terms of substrate requirements for MAO. The 4-(5-methylfuran-2-yl) analog (43) used in this study, however, shows a lowered MAO activity. This appears to result from the presence of the methyl sub-
stituent on the furan ring, since it has been shown that the substitution pattern on the 4-phenyl ring of MPTP follows the MAO substrate activity order ortho > meta > para, suggesting that the lengthening in the para direction is disadvantageous.\textsuperscript{11, 66-69} Another observation consistent with this general pattern is that the 4-(4-bromophenyl) analog (44), bearing a rather large Br group at the para position, is also not a MAO substrate.

Two other MPTP analogs used in this investigation are of particular interest. The "isobenzyl" analog (42), contains an exocyclic double bond, i.e. it is a homoallylic amine in contrast to MPTP which is an allylic amine. The finding that this compound is oxidized by MAO is surprising. The lack of MAO substrate activity of 1-methyl-4-phenylpiperidine previously led to the hypothesis\textsuperscript{88} that a double bond in the 4-5 position is required for both MAO oxidation and also for the expression of neurotoxicity. The latter appeared to be a strict requirement since moving the C=C to an exocyclic position while retaining the allylic character (1-methyl-3-benzylideneepiperidine) was accompanied by a loss of MAO activity.\textsuperscript{89} Compound 42 thus presents the first example of an MPTP analog with an alteration in the position of unsaturation, which retains its ability to serve as a substrate for MAO. This is also interesting
from the enzymologic point of view, since most tertiary amines are rather poor substrates for MAO, and it has always been puzzling as to why MPTP itself is such a good substrate.

The 4-benzyl analog (41) also shows good MAO activity, in fact much better than MPTP itself. This has been observed by others also, a noteworthy finding in view of the fact that this compound is not neurotoxic. The greater substrate activity of compounds 39-41 clearly shows that the conjugated 4-phenyl group of MPTP is not an important requirement for substrate activity. However some compact lipophilic group is nonetheless important for an apparent "hydrophobic anchoring" to the active site of MAO.

Non-neurotoxicity of MBnTP:

Up until the current study there were essentially four features recognized as criteria for a structural analog of MPTP to exhibit neurotoxicity. These are: (1) the compound should be a substrate for the enzyme MAO; (2) the pyridinium product presumed to be ultimately formed (e.g., MPP+ from MPTP) should be an effective substrate for the dopamine reuptake pathway; (3) the pyridinium compound should further be accumulated across the mitochondrial membrane, where it should inhibit
complex I of the respiratory chain.

The 4-benzyl analog is thus quite interesting in that although it is a much better substrate for MAO than MPTP itself, it does not produce neurologic defects in mice.\(^{67}\) We have found that the corresponding pyridinium analog 9 (MBnP\(^+\)) does show in vivo dopaminergic toxicity as determined by its intranigral infusion into rat substantia nigra.\(^{90}\) At the same time 9 is also a potent respiratory inhibitor, as shown in the earlier part of this dissertation. Thus were MBnP\(^+\) to form from MAO oxidation of MBnTP, one would have predicted MBnTP to be neurotoxic. The suggestion is then that MBnP\(^+\) is in fact not generated physiologically, a conclusion consistent with the inability of others to detect MBnP\(^+\) following oxidation of MBnTP by mouse brain mitochondria.\(^{67,91}\) Our premise was that MBnTP was being oxidized normally by MAO to the corresponding dihydropyridinium MBnDP\(^+\) but that the latter was not undergoing efficient physiologic conversion to MBnP\(^+\). Since the pyridinium species are thought to arise from autoxidation of the endocyclic dienamine forms of the initial dihydropyridinium MAO products, we further postulated that the inability of MBnDP\(^+\) to be oxidized to MBnP\(^+\) arose from the fact that it could be deprotonated to the exocyclic dienamine 50 rather than the endocyclic diena-
mine (Figure 6). This was in fact experimentally demonstrated by the formation of exocyclic diename 50 upon deprotonation of synthetic sample of MBnDP⁺. As is evident from the structure, two geometrical E and Z forms are possible owing to the asymmetry in the molecule and a restricted rotation around the bond linking the C-4 of the pyridine ring and the methylene carbon. There was, however, only one isomer detected in our hands, and was unambiguously determined to be the E-isomer, based on its NMR spectral analysis and NOE experiments.

The preferential deprotonation to yield the exocyclic diename appeared to be consistent with the reported undetectability of MBnP⁺. In our hands, autoxidation at this stage under model physiologic conditions led to the formation of a complex mixture of degradation products, the exact nature of which could not be determined by us, but which contained only small amounts of MBnP⁺.

These results further extend our understanding of the mechanism(s) associated with various events involved in the expression of neurotoxicity by MPTP. Thus, the ability of MPTP analogs to be good substrates for the enzyme MAO appears to be an essential step. However, being a good substrate for MAO does not ensure neurotox-
icity, since the oxidation may not always lead to the formation of the corresponding MPP⁺ analog.
EXPERIMENTAL:

General Comments. MPTP (1), 4-phenylpyridine (2) and MPP⁺ (3) were prepared in earlier studies from our laboratory. 4-Phenylpyrimidine (6) and 4-benzylpyridine (8) were obtained from Aldrich Chemical Co. and converted to their hydrochloride salts by treatment with ethanolic HCl, followed by recrystallization from EtOH-ether. 1-Methyl-4-(5-phenyl-2-oxazolyl)pyridinium p-toluenesulfonate (18) was obtained from Aldrich Chemical Co. and was used as such. The remaining compounds were synthesized as described below. No attempts were made to optimize the yields in the synthetic procedures. The procedures for safe handling of the potentially neurotoxic MPTP and MPP⁺ analogs were followed according to published reports.

In the synthetic procedures described below, all evaporations were conducted in vacuo using a rotary evaporator. The reactions involving the use of organometallic reagents were carried out strictly under a N₂ atmosphere in flame-dried glass assemblies. All starting organic chemicals were obtained from Aldrich Chemical Co., unless noted otherwise. Solvents used were of analytical or HPLC grade. Anhydrous ether and THF were distilled immediately before use from sodium benzophe-
none ketyl. Pyridine was distilled from BaO prior to use. Anhydrous DMF was obtained by distillation from BaO and was stored over molecular sieves. Melting points were recorded with a capillary melting point apparatus and are uncorrected. The $^1$H- and $^{13}$C- NMR spectra were obtained with a Varian XL-200 multinuclear instrument operating at 200 MHz for proton and 50.31 MHz for carbon. All $^1$H chemical shifts are reported relative to (CH$_3$)$_4$Si as internal standard when CDCl$_3$ or DMSO-$d_6$ was used as the solvent. In cases where D$_2$O was used, the residual HOD signal ($\delta$ 4.67) was taken as the reference. All $^{13}$C chemical shifts are relative to CDCl$_3$ ($\delta$ 77 ppm). Apparent coupling constants (J) are given in Hz. The following notations for expressing spin multiplicities are used: br = broad, d = doublet, dd = doublet of doublets, m = multiplet, q = quartet, s = singlet, t = triplet. High resolution mass spectra were determined with a Kratos MS-25 spectrometer. All final products used for the biological experiments were estimated to be at least 95% pure by the absence of any unaccountable NMR signals at 200 MHz, and ran as a single spot on thin layer chromatography.

For assigning the $^1$H and $^{13}$C signals for the pyridine containing compounds, we have used a numbering scheme which refers to the nitrogen of the pyridine or
pyridinium ring as position 1. In cases where both pyridine and pyridinium rings are present, the distinction is made by referring to Py and Py\textsuperscript{+} respectively. For determination of hydrogen substitution on C atoms, APT (attached proton test) $^{13}$C NMR experiments on fully decoupled spectra were performed. The (+) and (-) signs refer to the signals above (C and CH\textsubscript{2}) and below (CH and CH\textsubscript{3}) the baseline line respectively.

NOE experiments were performed using the 2-pulse sequence described in the Varian manual for the advanced experiments\textsuperscript{110}. The calculations were done using the NOE calculation subroutine program supplied. In brief, the NOE difference spectra were obtained at 25°C. The signal of interest was irradiated for 20 s, and then a transient acquired with the decoupler off using an acquisition time of 2 s. The signal was irradiated during the 20-s delay with sufficient power to just saturate the signal. This process was repeated with the decoupler set far off resonance, and then the total procedure was repeated until 64 transients were collected at each decoupler setting. The resulting FID's were subtracted, and the spectra obtained after Fourier transformation were displayed as the NOE difference spectra. Quantitation was obtained by measuring the integral of the enhanced signals and dividing by the
integral of the signal being saturated.

General methods for preparation of pyridinium compounds from the corresponding pyridine analogs:

Method A: A typical procedure involved dissolution of 1 mmol of the pyridine compound in 20 mL acetone followed by cooling the solution to 0-5°C. An excess of CH₃I (1 mL, 16 mmol), normally stored at < 5°C, was then added and the resulting solution allowed to stand at the same temperature. Usually the product crystallized out of this solution upon standing for a period of 5-6 h. The crystalline solid was then collected by filtration and further recrystallized from an appropriate solvent as mentioned for each compound individually. In cases where the product did not crystallize from the reaction mixture, the solvent was evaporated after overnight standing (usually the TLC analysis showed complete reaction at this time), and the resulting residue was recrystallized to obtain the pyridinium product.

Method B: This procedure was used for the controlled mono-methylation of the dimeric bis-pyridine analogs to obtain the monocationic compounds. Thus, typically a solution of 2 mmol of the bis-pyridine analog was prepared in CH₂Cl₂ (20 mL) and cooled to 5°C. Water (20 mL) was then added and the resulting biphasic
solution vigorously stirred at 0-5°C. A dilute solution of 0.1 M CH₃I (20 mL, 2 mmol) in CH₂Cl₂ was then added dropwise over a period of 20 min, and stirring was continued for 3 h at the same temperature. The upper water layer was then separated and washed thoroughly with ether. The organic extracts were combined and evaporated to dryness to recover any unreacted starting material. Evaporation of water under reduced pressure afforded a solid which was crystallized from an appropriate solvent, as mentioned for each product, to obtain pure monomethiodides.

3-Methyl-4-phenylpyridine⁷⁴ (4) was prepared via a recently reported general method⁷⁵ for regioselective preparation of 4-substituted pyridines. Thus TBDMS triflate (9.2 mL, 40 mmol) was added dropwise to a solution of 3-picoline (3.72 g, 40 mmol) in 20 mL anhydrous THF with stirring under N₂ at -70°C, yielding a white suspension, which was allowed to warm to 25°C and stirred further for 30 min. The mixture was cooled again to -70°C and a PhMgBr solution (13 mL of a 3.1 M solution in Et₂O, 40.3 mmol) was added dropwise over a period of 15 min. After complete addition, the reaction mixture was stirred at 45°C for 2 h, cooled to 0°C and poured into a 20% aqueous NH₄Cl solution, which was then extracted with ether. Evaporation of the ether layer
afforded the intermediate N-silylated dihydropyridine derivative in the form of an oil.

The crude oil was dissolved in 40 mL CH₂Cl₂ and exposed to O₂ bubbling for 10 h. This solution was then shaken with dilute aqueous HCl (to pH 2), and the organic layer was discarded. The aqueous layer was made basic (to pH 9) with 10% aqueous NaOH and extracted with CH₂Cl₂. The organic layer was washed with water, dried (Na₂SO₄), and evaporated to dryness to obtain the crude product as an oil, which was subjected to silica gel flash chromatography (EtOAc) to afford pure 3-methyl-4-phenylpyridine (4.06 g, 60% isolated yield). The HCl salt was prepared by treatment with ethanolic HCl, with recrystallization from EtOH-ether; m.p. 190°C; ¹H-NMR (D₂O) δ 2.30 (s, 3H, CH₃), 7.36-7.48 (m, 5H, Ph-H), 7.75 (d, 1H, J = 6.0 Hz, C₅-H), 8.46 (d, 1H, J = 6.0 Hz, C₆-H), 8.51 (s, 1H, C₂-H).

1,3-Dimethyl-4-phenylpyridinium iodide (5) was prepared from 3-methyl-4-phenyl pyridine (4) (400 mg, 2.36 mmol) using method A described above. The crystalline solid that appeared was recrystallized from EtOH-ether to afford 660 mg of the title compound (90% yield); m.p. 137-38°C (lit. 94 140-141°C); ¹H-NMR (D₂O) δ 2.28 (s, 3H, CH₃), 4.27 (s, 3H, N-CH₃), 7.4-7.55 (m, 5H, Ph-H), 7.70 (d, 1H, J = 7.0 Hz, C₅-H), 8.48 (d, 1H,
\[ J = 7.6 \text{ Hz, C}_6\text{-H}, 8.57 (s, 1H, C_2\text{-H}); \text{ EIMS (70 eV)} 169 (\text{[M-CH}_3]^+, 100\%). \]

1-Methyl-4-phenyl-1,3-pyrimidinium iodide (7) was obtained from commercially available 4-phenyl-1,3-pyrimidine (156 mg, 1 mmol) using method A for the methylation reaction. The final purification was accomplished by recrystallization from EtOH (92\% yield): m.p. 168°C; \(^1\text{H-NMR (D}_2\text{O)} \delta 4.36 (s, 3H, N-CH}_3\text{), 7.62-7.94 (m, 3H, Ph-H), 8.34 (d, 2H, J = 8.6 Hz, C}_2\text{-/C}_6\text{-H), 8.54 (d, 1H, J = 8.0 Hz, C}_5\text{-H), 9.1 (d, 1H, J = 8.0 Hz, C}_6\text{-H), 9.5 (s, 1H, C}_2\text{-H).} \]

1-Methyl-4-benzylpyridinium \( p \)-toluenesulfonate (9). A mixture of 4-benzylpyridine (3.4 g, 20 mmol) and methyl \( p \)-toluenesulfonate (4.3 g, 23 mmol) in 60 mL benzene was heated under \( \text{N}_2 \) at a gentle reflux. TLC analysis showed conversion to a single product after 2 h (\( R_f = 0.35; \text{EtOAc/MeOH/AcOH/water 2:6:1:1} \)). The reaction mixture was then allowed to cool and was then partitioned between water and ether. The aqueous layer was washed several times with ether and evaporated in vacuo to afford the desired product in 80\% yield after recrystallization from EtOH: m.p. 99-101°C; UV/VIS (H\(_2\text{O}) \lambda_{\text{max}} 217 \text{ nm (} \epsilon 20650 \text{ M}^{-1}\text{cm}^{-1}), 254 \text{ nm (} \epsilon 5640 \text{ M}^{-1}\text{cm}^{-1}); \(^1\text{H-NMR (D}_2\text{O)} \delta 2.18 (s, 3H, OTs-CH}_3\text{), 4.08 (s, 5H, N-CH}_3\text{ and PhCH}_2\text{), 7.13 and 7.47 (2d, 2H each, J = \)
8.0 Hz, OTs Ar-H), 7.23 (m, 5H, Ph-H), 7.61 (d, 2H, J =
6.6 Hz, C3/C5-H), 8.36 (d, 2H, J = 6.6 Hz, C2/C6-H).
Note: A mixture of 9 (36 mg, 0.1 mmol) and sodium iodide
(16 mg, 0.1 mmol) in 5 mL water was stirred for 5 h at
30°C, and the mixture was evaporated to dryness to
afford a yellow solid which was recrystallized from
EtOH-ether to obtain a compound which was identical to
1-methyl-4-benzoylpyridinium iodide! as determined by
the melting point, TLC and spectral analysis: 1H-NMR
(D2O) δ 4.42 (s, 3H, N-CH3), 7.50-7.71 (2m, 2H + 3H,
Ph-H), 8.17 (d, 2H, J = 6.4 Hz, C3/C5-H), 8.90 (d, 2H, J
= 6.4 Hz, C2/C6-H); UV/VIS (H2O) λmax 224 nm (ε 23100
M⁻¹cm⁻¹), 270 nm (ε 8470 M⁻¹cm⁻¹).

4-(4-Hydroxyphenoxy)pyridine (10). A mixture of
hydroquinone (220 mg, 2 mmol), 4-bromopyridine hydro-
chloride (496 mg, 2.1 mmol), and 1.5 mL of a 2M aqueous
NaOH solution (3 mmol) in 15 mL DMSO was heated under N2
at 90°C for 5 h. The mixture was diluted with water and
extracted with ether. The ether extracts were washed
thoroughly with water to remove excess DMSO, dried
(Na2SO4), and evaporated to afford a mixture containing
the desired product and minor amounts of the bis-coupled
product (36). The separation was achieved through
silica gel flash chromatography (MeOH eluant). The
slower eluting material (Rf = 0.25) corresponded to the
structure of the title compound, as determined by its spectral analysis (isolated yield 200 mg, 51%): m.p. 190°C; $^1$H-NMR (CDCl$_3$) δ 6.82 (d, 2H, J = 6.4 Hz, C$_3$/C$_5$-H), 6.86-7.00 (m, or q, 4H, Ar-H), 8.45 (d, 2H, J = 6.4 Hz, C$_2$/C$_6$-H), 9.11 (br s, 1H, OH); HRMS (40 eV) calcd for C$_{11}$H$_9$NO$_2$ m/z 187.0634, found 187.0633 (M$^+$, 100%).

1-Methyl-4-(4-hydroxyphenoxy)pyridinium iodide (11). 4-(4-Hydroxyphenoxy)pyridine (10) (187 mg, 1 mmol) was treated with CH$_3$I according to method A and the solid obtained was recrystallized from MeOH-ether to afford 240 mg of the title compound (70% yield): m.p. >260°C (dec); $^1$H-NMR (D$_2$O) δ 4.07 (s, 3H, N-CH$_3$), 6.88 and 7.01 (2d, 2H each, J = 9.0 Hz, Ar-H), 7.24 (d, 2H, J = 7.5 Hz, C$_3$/C$_5$-H), 8.40 (d, 2H, J = 7.5 Hz, C$_2$/C$_6$-H).

1-Methoxy-4-phenylpyridinium perchlorate (12). This perchlorate salt was prepared from the corresponding methylsulfate salt.$^{95}$ Thus a mixture of 4-phenylpyridine-1-oxide (1.4 g, 8.17 mmol) and dimethylsulfate (1.04 g, 8.17 mmol) was heated under N$_2$ at 100°C for 3 h. The mixture was then dissolved in 4 mL EtOH, and then 70% HClO$_4$ (1.2 mL, 8.17 mmol) was added with ice-cooling. Addition of 30 mL EtOAc led to the formation of a precipitate which was collected by filtration and recrystallized from MeOH to afford 1.7 g of the
title compound (73% yield): m.p. 98-99°C; \textsuperscript{1}H-NMR (D\textsubscript{2}O) δ 4.28 (s, 3H, OCH\textsubscript{3}), 7.48-7.76 (m, 5H, Ph-H), 8.20 (d, 2H, J = 6.4 Hz, C\textsubscript{3}/C\textsubscript{5}-H), 8.93 (d, 2H, J = 6.4 Hz, C\textsubscript{2}/C\textsubscript{6}-H).

1-(4-Pyridinyl)-1,4-pentanedione (13) was prepared via the general method of Stetter.\textsuperscript{76} NaCN (500 mg, 10 mmol) was suspended in 25 mL anhydrous DMF in a dry three-neck r.b. flask equipped with an addition funnel, mechanical stirrer and a reflux condenser fitted with a CaCl\textsubscript{2} drying tube. The assembly was flushed with N\textsubscript{2} and a solution of freshly distilled pyridine-4-carboxaldehyde (10.7 g, 100 mmol) in 25 mL DMF was then added dropwise, with vigorous stirring at a rate so as to maintain the reaction temperature at 35-40°C. After complete addition, the resulting dark red suspension was further stirred vigorously for 15 min, followed by dropwise addition of 50 mL of a DMF solution containing methyl vinyl ketone (5.3 g, 75 mmol) at a rate which maintained the reaction temperature under 40°C. The resulting orange-red solution was then stirred under N\textsubscript{2} for 5 h, followed by dilution with 200 mL water and extraction with four 200 mL portions of CHCl\textsubscript{3}. The organic extracts were combined, repeatedly washed with water to remove excess DMF, dried (Na\textsubscript{2}SO\textsubscript{4}), and finally evaporated to obtain the title compound, which was
purified by crystallization from i-ProOH (final yield 10.6 g, 80% based on methyl vinyl ketone): m.p. 92°C; 
^1\text{H-NMR (CDCl}_3\text{)} \delta 2.27 (s, 3H, CH_3), 2.93 (t, 2H, J = 6.2 Hz, \text{CH}_3\text{COCH}_2\text{), 3.25 (t, 2H, J = 6.2 Hz, PyCOCH}_2\text{), 7.76 (d, 2H, J = 6.2 Hz, C}_3\text{/C}_5\text{-H), 8.81 (d, 2H, J = 6.2 Hz, C}_2\text{/C}_6\text{-H). Note: Further characterization of this compound was by conversion to 14 and 16.}

4-(1,5-Dimethylpyrrol-2-yl)pyridine (14) was prepared by the general methods for condensation of \gamma\text{-diketones with primary alkylamines to give pyrroles. A mixture of 1-(4-pyridinyl)-1,4-pentanedione (13) (440 mg, 2.5 mmol) and methylamine hydrochloride (338 mg, 5 mmol) in 15 mL EtOH was heated under reflux for 15 h. The solvent was then removed by evaporation, and the residue was dissolved in 30 mL water. The solution was made basic (to pH 10) by adding 10% aqueous NaOH, yielding a precipitate, which was extracted with CHCl_3. The CHCl_3 extract was washed with water, dried (\text{Na}_2\text{SO}_4), and evaporated to afford the title compound, which was purified by silica gel flash chromatography (EtOAc eluant) (yield 350 mg, 82%): m.p. 78°C; ^1\text{H-NMR (CDCl}_3\text{)} \delta 2.31 (s, 3H, CH_3), 3.60 (s, 3H, N-CH_3), 6.00 (d, 1H, J = 3.65 Hz, C_4'-H), 6.33 (d, 1H, J = 3.65 Hz, C_3'-H), 7.28 (d, 2H, J = 6.1 Hz, C_3/C_5-H), 8.57 (d, 2H, J = 6.1 Hz, C_2/C_6-H); HRMS (40 eV) calcd for m/z C_{11}H_{12}N_2
172.1001, found 172.1001 (M+ 100%).

1-Methyl-4-(5-dimethylpyrrol-2-yl)pyridinium iodide (15) was obtained from the preceding pyridine analog (14) (200 mg, 1.16 mmol) using method A described above. The solid obtained was purified by crystallization from acetone-ether (yield 325 mg, 90%); m.p. 233°C (dec); 1H-NMR (D2O) δ 2.34 (s, 3H, C5'-CH3), 3.74 (s, 3H, N1'-CH3), 4.18 (s, 3H, N1-CH3), 6.20 and 6.94 (2d, 1H each, J = 4.0 Hz, C3'/C4'-H), 7.82 (d, 2H, J = 6.3 Hz, C3/C5-H), 8.39 (d, 2H, J = 6.3 Hz, C2/C6-H).

4-(5-Methylfuran-2-yl)pyridine (16) was prepared via the general method70 for acid catalyzed cyclocondensation of γ-diketones to furans. A mixture of 1-(4-pyridinyl) pentane-1,4-dione (13) (177 mg, 1 mmol) in 5 mL of concentrated HCl (5 mL) was stirred under N2 at 25°C for 2 h. The solution was then evaporated to dryness, and the residue was redissolved in 10 mL water before basification to pH 10 with 10% aqueous NaOH. The precipitate obtained was isolated by extraction with CHCl3. The organic extract was washed with water, dried (Na2SO4), and evaporated to afford 120 mg (76%) of the title compound, which was of sufficient purity as analyzed by 1H NMR to be used directly in the next step. An analytical sample for characterization was obtained by preparative TLC (CHCl3 mobile phase, Rf = 0.38):
m.p. 65°C; 1H-NMR (CDCl₃) δ 2.38 ( s, 3H, CH₃), 6.11 (d, 1H, J = 3.3 Hz, C₆'-H), 6.76 (d, 1H, J = 3.3 Hz, C₃'-H), 7.45 (d, 2H, J = 6.2 Hz, C₃/C₅-H), 8.55 (d, 2H, J = 6.2 Hz, C₂/C₆-H); HRMS (40 eV) calcd for C₁₀H₉NO m/z 159.0684, found 159.0682 (M⁺, 68%).

1-Methyl-4-(5-methylfuran-2-yl)pyridinium iodide (17). The preceding pyridine analog (16) (120 mg, 0.75 mmol) was methylated using method A described above, with recrystallization from EtOH-ether, to afford 175 mg of pure quarternary salt (77% yield): m.p. 308-209°C (dec); 1H-NMR (D₂O) δ 2.45 (s, 3H, CH₃), 4.23 (s, 3H, N-CH₃), 6.43 (d, 1H, J = 3.5 Hz, C₆'-H), 7.45 (d, 1H, J = 3.5 Hz, C₃'-H), 8.02 (d, 2H, J = 6.8 Hz, C₃/C₅-H), 8.52 (d, 2H, J = 6.8 Hz, C₂/C₆-H).

4-(2,5-Dimethylpyrrol-1-yl)pyridine (19). A mixture of 4-aminopyridine (940 mg, 10 mmol) and 2,5-hexanedione (1.14 g, 10 mmol) was stirred at 100°C under N₂ for 8 h. The mixture was cooled, partitioned between water and ether, and the ether extracts were evaporated to afford a residue which was purified by silica gel flash chromatography (EtOAc eluant) to obtain 510 mg of the title compound (30% isolated yield): m.p. 97-98°C (lit. 96 m.p. 101°C); 1H-NMR (CDCl₃) δ 2.09 (s, 6H, CH₃), 5.95 (s, 2H, C₃'/C₄'-d), 7.18 (d, 2H, J = 5.2 Hz, C₃/C₅-H), 8.73 (d, 2H, J = 6.2 Hz, C₂/C₆-H); HRMS (40
eV) calcd for C_{11}H_{12}N_{2} m/z 172.1001, found 172.0996 (M^+, 69%), 171.0969 (M-1, 100%), 156.0755 (M-16, 38%).

1-Methyl-4-(2,5-dimethylpyrrol-1-yl)pyridinium iodide (20). Treatment of the preceding compound (19) (60 mg, 0.35 mmol) with CH_{3}I using method A provided a solid which was recrystallized from EtOH-ether to obtain 100 mg of the title compound (91% yield): m.p. 237°C (dec); \textit{^1}H-NMR (D_{2}O) δ 2.20 (s, 6H), 4.40 (s, 3H, N-CH_{3}), 6.14 (s, 2H, C_{3}-CH_{3}), 7.93 (d, 2H, J = 7.0 Hz, C_{3}/C_{5}-H), 8.86 (d, 2H, J = 7.0 Hz, C_{2}/C_{6}-H).

4-Cyclopentylpyridine (21). To a stirred suspension of N-(t-butyldimethylsilyl)pyridinium triflate, 75 prepared from pyridine (3.16 g, 40 mmol) and TBDMS triflate (9.2 mL, 40 mmol), and cooled to -78°C, a solution of cyclopentyl magnesium bromide (20 mL of 2M solution in Et_{2}O, 40 mmol) was added dropwise over 20 min. The resulting pale green solution was stirred for 30 min at 0°C and for an additional 3 h at 25°C. The reaction was quenched by pouring into 100 mL ice-cold 5% aqueous NaHCO_{3}, and the mixture was extracted with ether. The ether extracts were washed with water, dried (Na_{2}SO_{4}) and evaporated under reduced pressure to afford the N-(t-butyldimethylsilyl)dihydropyridine intermediate in the form of a yellow oil.

The crude oil was stirred in a stream of O_{2} and
the progress of the reaction monitored by TLC on ali-quot withdrawn periodically. TLC analysis (EtOAc) after 5 h showed complete conversion of the $R_f = 0.88$ dihydropyridine to a single product with $R_f = 0.51$. 1N HCl was then added followed by extraction with CH$_2$Cl$_2$ (pH of aqueous layer was 2). The aqueous layer was basified to pH 10 with 10% aqueous NaOH and then re-extracted with CH$_2$Cl$_2$. The organic extracts were washed with water, dried over Na$_2$SO$_4$, and evaporated to dryness to obtain 4-cyclopentylpyridine which was purified by flash chromatography ($R_f = 0.38$, EtOAc eluant) (overall isolated yield 2.3 g, 40%): $^1$H-NMR (CDCl$_3$) $\delta$ 1.5-2.15 (m, 8H), 2.98 (p, 1H, $J = 8.0$ Hz, cyclopentyl CH), 7.15 (d, 2H, $J = 6.0$ Hz, C$_3$/C$_5$-H), 8.48 (d, 2H, $J = 6.0$ Hz, C$_2$/C$_6$-H); $^1$H-NMR (of HCl salt in D$_2$O) $\delta$ 1.45-1.72 (m, 6H), 1.96-2.03 (m, 2H), 3.17 (p, 1H, $J = 8$ Hz, cyclopentyl CH), 7.77 (d, 2H, $J = 5.4$ Hz, C$_3$/C$_5$-H), 8.43 (d, 2H, $J = 5.4$ Hz, C$_2$/C$_6$-H). Note: This compound has been synthesized previously by other routes, 101 but without spectral characterization.

1-Methyl-4-cyclopentylpyridinium $p$-toluenesulfonate (22). A solution of 4-cyclopentylpyridine (21) (1.08 g, 7.35 mmol) and methyl $p$-toluenesulfonate (2.62 g, 8.7 mmol) in 20 mL benzene was refluxed under N$_2$ for 3 h. The reaction mixture was then cooled and extracted
with water. aqueous layer was further washed with ether and evaporated to afford a white solid which was crystallized twice EtOH-ether to obtain 2.9 g (91% yield) of pure p-tosylate salt. TLC (EtOAc/MeOH/AcOH/water 2:6:1:1; Rf = 0.35): m.p. 133-34°C; ¹H-NMR (D₂O) δ 1.41-1.66 (m, 6H), 1.95-2.02 (m, 2H), 2.21 (s, 3H, OTs-CH₃), 3.12 (p, 1H, J = 8.0 Hz, cyclopentyl CH), 4.10 (s, 3H, N-CH₃), 7.19 and 7.50 (2d, 2H each, J = 8.2 Hz, OTs Ar-H), 7.69 (d, 2H, J = 6.7 Hz, C₃/C₅-H), 8.37 (d, 2H, J = 6.7 Hz, C₂/C₆-H).

1-Methyl-4-cyclohexylpyridinium p-toluenesulfonate (23) was obtained by the reaction of 4-cyclohexylpyridine¹⁰² (332 mg, 2 mmol) with methyl p-toluenesulfonate (430 mg, 2.2 mmol) using the same method as described for 22. Recrystallization from EtOH-ether gave pure 23 (530 mg, 76% yield): m.p. 152-154°C; ¹H-NMR (D₂O) δ 1.20-1.30 and 1.60-1.75 (2m, 5H each), 2.2 (s, 3H, OTs-CH₃), 2.65 (m, 1H, cyclohexyl CH), 4.08 (s, 3H, N-CH₃), 7.16 and 7.48 (2d, 2H each, J = 8.0 Hz each, OTs Ar-H), 7.65 (d, 2H, J = 6.0 Hz, C₃/C₅-H), 8.36 (d, 2H, J = 6.0 Hz, C₂/C₆-H).

1-Methyl-4-phenoxypyridinium iodide (24). 4-Phenoxyppyridine⁹⁷ (100 mg, 0.58 mmol), prepared from the reaction of 4-pyridinylpyridinium dichloride⁹⁸ with sodium phenolate, was treated with CH₃I as per method A
given earlier. The product was recrystallized from MeOH (isolated yield 154 mg, 85%): m.p. 221-223°C; \(^1\)H-NMR (D\(_2\)O) \(\delta\) 4.05 (s, 3H, N-CH\(_3\)), 7.12 (d, 2H, J = 8.2 Hz, C\(_2\)/C\(_6\)-H), 7.24 (d, 2H, J = 7.2 Hz, C\(_3\)/C\(_5\)-H), 7.30-7.44 (m, 3H, C\(_3\)/C\(_4\)/C\(_5\)-H), 8.39 (d, 2H, J = 7.2 Hz, C\(_2\)/C\(_6\)-H).

4-(2,5-Dimethylpyrrolylmethyl)pyridine (25). A mixture of 4-(aminomethyl)pyridine (1.3 g, 12 mmol) and 2,5-hexanediol (1.14 g, 10 mmol) in 30 mL EtOH containing a catalytic amount of acetic acid was stirred under N\(_2\) at 40°C for 10 h.\(^{99}\) The solvent was evaporated off, and the residue was partitioned between 2% aqueous NaHCO\(_3\) and ether. The ether extract was washed several times with water to remove unreacted diketone, dried (Na\(_2\)SO\(_4\)), and concentrated to afford a 1.5 g of a residue (crude yield 80%) which was purified by column chromatography (alumina; EtOAc eluant): m.p. 71°C; \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 2.21 (s, 6H), 5.00 (s, 2H, CH\(_2\)), 5.89 (s, 2H, C\(_3\)/C\(_4\)-H), 6.80 (d, 2H, J = 6.2 Hz, C\(_3\)/C\(_5\)-H), 8.53 (d, 2H, J = 6.2 Hz, C\(_2\)/C\(_6\)-H); \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\) 12.13 (\(-,\ CH_3\)), 45.57 (\(+,\ CH_2\)), 105.75 (\(-,\ C_3/C_4\)), 120.55 (\(-,\ C_3/C_5\)), 127.55 (\(+,\ C_2/C_5\)), 147.52 (\(+,\ C_4\)), 149.97 (\(-,\ C_2/C_6\)); HRMS (40 eV) calcd for C\(_{12}\)H\(_{14}\)N\(_2\) m/z 186.1158, found 186.1151 (M\(^+\), 100%).

1-Methyl-4-(2,5-dimethylpyrrolylmethyl)pyridinium
iodide (26). The preceding compound (25) (186 mg, 1 mmol) upon reaction with CH3I using method A gave a solid which gave 310 mg of the title compound (95% yield) after recrystallization from EtOH-ether: m.p. 182°C; UV/VIS (H2O) $\lambda_{max}$ 254 nm ($\epsilon$ 4700 M$^{-1}$cm$^{-1}$); 1H-NMR (D2O) $\delta$ 2.09 (s, 6H, CH3), 4.33 (s, 3H, N-CH3), 5.41 (s, 2H, CH2), 5.98 (s, 2H, C3/C4-H), 7.50 (d, 2H, J = 6.6 Hz, C3/C5-H), 8.67 (d, 2H, J = 6.6 Hz, C2/C6-H).

1-Methyl-4-(pyrrolylmethyl)pyridinium iodide (27) was prepared from 4-(pyrrolylmethyl)pyridine$^{100}$ (158 mg, 1 mmol) using method A. Recrystallization from acetone-ether afforded 270 mg of the title compound (90% yield): m.p. 160°C (dec); UV/VIS (H2O) $\lambda_{max}$ 254 nm ($\epsilon$ 4850 M$^{-1}$cm$^{-1}$); 1H-NMR (D2O) $\delta$ 4.17 (s, 3H, N-CH3), 5.5 (s, 2H, CH2), 6.17 (t, 2H, J = 2.0 Hz, pyrrole H$_2$), 6.75 (t, 2H, J = 2.0 Hz, pyrrole H$_3$), 7.44 (d, 2H, J = 6.4 Hz, C3/C5-H), 8.51 (d, 2H, J = 6.4 Hz, C2/C6-H).

1-Methyl-4-(2-(4-pyridinyl)ethyl)pyridinium iodide (28). The mono-$m$-methylation of commercial 1,2-bis[(4-pyridinyl)ethane] (368 mg, 2 mmol) was accomplished using method B described above, with the final purification by recrystallization from MeOH to obtain 350 mg (53% yield) of 28: m.p. 160°C; UV/VIS(H2O) $\lambda_{max}$ 223 nm ($\epsilon$ 28900 M$^{-1}$cm$^{-1}$), 254 nm ($\epsilon$ 8100 M$^{-1}$cm$^{-1}$); 1H-NMR (D2O) $\delta$ 2.97 and 3.14 (2x, 2H each, J = 7.0 Hz, CH$_2$CH$_2$),
4.12 (s, 3H, N-CH₃), 7.10 and 8.22 (2d, 2H each, J = 5.0 Hz, Py C₃/C₅-H and C₂/C₆-H), 7.63 and 8.41 (2d, 2H each, J = 6.4 Hz, Py⁺ C₃/C₅-H and C₂/C₆-H).

3-Dimethylamino-1-phenyl-1-propanone (29) was prepared via published procedure.¹⁰⁵: ¹H-NMR (CDCl₃) δ 2.29 (s, 6H, N-CH₃), 2.76 (t, 2H, COCH₂), 3.16 (t, 2H, CH₂-N), 7.43-7.57 (m, 3H), 7.95-7.99 (m, 2H).

1-Phenyl-4-(4-pyridinyl)-1,4-butanedione (30). To a solution of 3-dimethylamino-1-phenyl-1-propanone (1.77 g, 10 mmol) in anhydrous DMF (50 mL) containing NaCN (50 mg, 1 mmol), was added dropwise a 20 mL DMF solution of freshly distilled pyridine-4-carboxaldehyde (1.3 g, 12 mmol) under N₂, while maintaining the reaction temperature at 45°C. After the addition was complete, the mixture was stirred at 45°C for 5 h, and then was diluted with 3 volumes of water. The mixture was extracted with CHCl₃, and the CHCl₃ extracts were washed successively with 5% aqueous NaHCO₃ and water, dried (Na₂SO₄), and evaporated to afford a yellow solid which was recrystallized from aqueous MeOH to obtain 1.2 g of the title compound (50% yield): ¹H-NMR (CDCl₃) δ 3.44-3.51 (m, 4H, CH₂), 7.49-7.57 (m, 3H, Ph-H), 7.82 (d, 2H, J = 6.0 Hz, C₃/C₅-H), 8.04 (d, 2H, J = 9.0 Hz, Ph ortho-H), 8.34 (d, 2H, J = 6.0 Hz, C₂/C₆-H); HRMS (40 eV) calcd for C₁₅H₁₃N₂ O₂ m/z 239.0947, found 239.0952 (M⁺, 7%).
106.0260 (M-133, 100%).

(5-Phenylfuran-2-yl)pyridine (31) was prepared from the preceding diketone 30 (480 mg, 2 mmol) by stirring for 10 h with 3 mL conc. HCl. The reaction mixture was diluted with water and basified to pH 10 with 1M NaOH solution, followed by extraction with CHCl₃. The CHCl₃ extracts were washed with brine and evaporated to afford the title compound (340 mg, 76% yield), which was recrystallized from acetone-ether: m.p. 89-91°C; ¹H-NMR (CDCl₃) δ 6.80 and 6.98 (2d, J = 3.6 Hz, furan CH), 7.35-7.51 (m, 3H, Ph-H), 7.59 and 8.64 (2d, 2H each, J = 6.2 Hz, C₃/C₅-H and C₂/C₆-H), 7.76-7.82 (m, 2H, Ph-H); HRMS (40 eV) calcd for C₁₅H₁₁NO m/z 221.0841, found 221.0845 (M⁺, 100%).

1-Methyl-(5-phenylfuran-2-yl)pyridinium iodide (32): prepared in 84% yield (300 mg) from 31 (220 mg, 1 mmol) using method A. The title compound was purified by recrystallization from MeOH-ether: m.p. 164°C (dec); ¹H-NMR (D₂O) δ 4.26 (s, 3H, N-CH₃), 7.38 and 8.15 (2d, 1H each, J = 3.6 Hz, furan CH), 7.88-8.01 (m, 5H, Ph-H), 8.46 and 8.92 (2d, 2H each, J = 6.8 Hz, C₃/C₅ and C₂/C₆-H).

1,1'-(1,4-Phenylene)bis-[3-(dimethylamino)-1-propanone] (33) was prepared via a reported Mannich reaction. Thus a mixture of 1,4-diacetylbenzene (4.05
g, 25 mmol), dimethylamine hydrochloride (5.27 g, 65 mmol) and paraformaldehyde (1.98 g, 22 mmol) in 50 mL ethanol, containing 0.5 mL conc. HCl was heated under reflux for 20 h. The solid material obtained upon cooling the reaction mixture was filtered and redisolved in 100 mL water. This solution was cooled to 5°C and basified to pH 10 with ice-cold aqueous 10% NaOH. The heterogeneous mixture was then extracted with CHCl₃. The CHCl₃ extracts were washed with water, dried (Na₂SO₄) and evaporated to dryness. The residue obtained was used for the next step without further purification: ¹H-NMR (CDCl₃) δ 2.29 (s, 12H, N-CH₃), 2.76 (t, 4H, J = 7.3 Hz, CH₂), 3.18 (t, 4H, J = 7.3 Hz, COCH₂), 8.05 (s, 4H, Ar-H).

1,1’-(1,4-Phenylene)bism-[4-(4-pyridinyl)-1,4-butanedione] (34) was prepared by a modified Stetter reaction⁷⁷ which involved dropwise addition of freshly distilled pyridine-4-carboxaldehyde (1.28 g, 12 mmol) to the above prepared bis Mannich base 33 (1.38 g, 5 mmol), dissolved in 20 mL anhydrous DMF containing a catalytic amount of NaCN (50 mg, 1 mmol). After the addition was complete, the reaction mixture was further stirred at 45°C for 12 h. The work-up involved dilution with 200 mL water followed by extraction with CHCl₃. The title compound was obtained upon evaporation of CHCl₃ as a
yellowish-orange solid, which was purified by crystallization from 1-PrOH-butane (isolated yield 700 mg, 35%). $^1$H-NMR (CDCl$_3$) $\delta$ 3.47-3.53 (m, 8H, CH$_2$), 7.83 (d, 4H, J = 6.0 Hz, C$_3$/C$_5$-H), 8.14 (s, 4H, Ar-H), 8.58 (d, 4H, J = 6.0 Hz, Py C$_2$/C$_6$-H): HRMS (40 eV) calcd for C$_{24}$H$_{20}$N$_2$O$_4$ m/z 400.1424, found 400.1422 (M$^+$, 23%), 266.0948 (90%), 106.0350 (100%).

4,4'-{(1,4-Phenylene)bispyridine (35). To a stirred suspension of N-((t-butyldimethyl)silyl)pyridinium triflate, prepared from freshly distilled pyridine (4.12 g, 52.15 mmol) and t-butyldimethylsilyl triflate (12 mL, 52.25 mmol) in anhydrous THF under N$_2$, was added, at -20°C, through a U-shaped tube, a suspension of the di-Grignard reagent, obtained from $p$-dibromo-mobenzene (6 g, 25.4 mmol) and Mg (2.45 g, 100 mmol) in THF. The reaction mixture was heated under reflux for 10 h, and then quenched by pouring over 50 mL of a water-ice mixture. The mixture was extracted with ether. The ether extracts were washed with water, dried (Na$_2$SO$_4$), and evaporated to dryness to afford the bis(N-silyldihydropyridine) derivative which was used without purification for the next step.

The crude residue was dissolved in 20 mL CH$_2$Cl$_2$, and O$_2$ was bubbled through the solution for 15 h to ensure complete oxidation of the bis(dihydropyridine) to
the bis(pyridine). 1N HCl was then added, and the aqueous layer was separated after vigorous shaking, basified to pH 10, and extracted with CHCl₃. The CHCl₃ extracts were washed with water, dried (Na₂SO₄), and evaporated to give 2.8 g (48%) of the crude product. Final purification of 1 g of crude material was accomplished by silica gel flash chromatography (Rf = 0.28, EtOAc eluent): m.p. 181-183°C; ¹H-NMR (CDCl₃) δ 7.56 (d, 4H, J = 6.2 Hz, C₃/C₅-H), 7.78 (s, 4H, Ar-H), 8.71 (d, 4H, J = 6.2 Hz, C₂/C₆-H); ¹³C-NMR (CDCl₃) δ 121.28 (C₃/C₅), 127.51 (C₂/C₃/C₅/C₆), 138.52 (+, C₄), 147.09 (+, C₆), 150.22 (-, C₂/C₆); HRMS (40 eV) calcd for C₁₆H₁₂N₂ m/z 232.1001, found 232.1009 (M⁺, 100%).

4,4’-(1,4-Phenylene)bis(pyridine) monomethiodide (35a) was prepared from 35 (464 mg, 2 mmol) using the controlled methylation conditions described above (method B). The monomethiodide obtained was further purified by recrystallization from acetone-ether (isolated yield 430 mg, 58%): m.p. >280°C (dec); UV/VIS (H₂O) λ_max 222 nm (ε 24620 M⁻¹cm⁻¹), 310 nm (ε 34740 M⁻¹cm⁻¹); ¹H-NMR (DCl₃) δ 4.35 (s, 3H, N-CH₃), 7.85 (d, 2H, J = 6.2 Hz, C₃/C₅-H), 8.10 (d, 2H, J = 8.6 Hz, C₃/C₅-H), 8.25 (d, 2H, J = 8.6 Hz, C₂/C₆-H), 8.60 (d, 2H, J = 6.8 Hz, Py⁺ C₃/C₅-H), 8.72 (d, 2H, J = 6.2
Hz, C$_2$/C$_6$-H), 9.05 (d, 2H, J = 6.8 Hz, Py$^+$ C$_2$/C$_6$-H).

4,4'-((1,4-Phenylene)bispyridine dimethiodide (35b) was obtained by methylation of the bis-pyridine analog 35 (232 mg, 1 mmol) according to method A, using instead a 10-fold molar excess of CH$_3$I and MeOH as the reaction solvent. The solid obtained was recrystallized from MeOH-acetone to afford 450 mg of the title compound (88% yield): m.p. >320°C (dec); UV/VIS (H$_2$O) $\lambda_{\text{max}}$ 221 nm ($\varepsilon$ 31420 M$^{-1}$cm$^{-1}$), 314 nm ($\varepsilon$ 35440 M$^{-1}$cm$^{-1}$); $^1$H-NMR (D$_2$O) $\delta$ 4.38 (s, 6H, N-CH$_3$), 8.36 (s, 4H, Ar-H), 8.66 (d, 4H, J = 7.0 Hz, C$_3$/C$_5$-H), 9.10 (d, 4H, J = 7.0 Hz, C$_2$/C$_6$-H).

1,4-Di-(4-pyridinyloxy)benzene (36) was prepared via a reported procedure for the preparation of a related structural analog. A 2-neck r.b. flask equipped with a N$_2$ inlet and a reflux condenser was charged with 4-bromo-pyridine hydrochloride (970 mg, 4.1 mmol), hydroquinone (220 mg, 2 mmol) and 30 mL DMSO. An aqueous solution of 2M NaOH (3.1 mL, 6.2 mmol) was then added, and the resulting mixture was heated under N$_2$ at 90°C for 5 h. The reaction mixture was cooled to room temperature, diluted with three volumes of water, and extracted with ether. The ether extracts were washed thoroughly with water to remove DMSO, dried (Na$_2$SO$_4$), and concentrated to dryness to afford a solid residue. $^1$H NMR analysis of the residue showed the presence of
4-(4'-hydroxyphenoxy)-pyridine and the desired bispyridine compound which were separated by silica gel flash chromatography (MeOH eluant). The faster compound with $R_f = 0.4$ corresponded to the desired structure as determined by its $^1$H NMR spectrum and MS analysis (isolated yield 250 mg, 47%): m.p. 132-134°C; $^1$H-NMR (CDCl$_3$) $\delta$ 6.88 (d, 4H, $J = 6.2$ Hz, C$_3$/C$_5$-H), 7.17 (s, 4H, Ar-H), 8.51 (d, 2H, $J = 6.2$ Hz, C$_2$/C$_6$-H); $^{13}$C-NMR (CDCl$_3$) $\delta$ 111.90 (–, C$_2$/C$_3$/C$_5$/C$_6$), 122.20 (+, C$_4$), 122.34 (–, C$_3$/C$_5$), 151.28 (–, C$_2$/C$_6$), 164.39 (+, C$_1$/C$_4$); HRMS (40 eV) calcd for C$_{16}$H$_{12}$N$_2$O$_2$ m/z 264.0898, found 264.0892 (M$^+$, 100%).

1,4-Di-(4-pyridinyloxy)benzene monomethiodide (36a) was prepared from 36 (265 mg, 1 mmol) using method B. The final purification step involved recrystallization from acetone-ether (yield 250 mg, 63%): m.p. 98°C; UV/VIS (H$_2$O) $\lambda_{max}$ 225 nm ($\varepsilon$ 45500 M$^{-1}$cm$^{-1}$); $^1$H-NMR (DMSO-d$_6$) $\delta$ 4.23 (s, 3H, N-CH$_3$), 7.04 (d, 2H, $J = 6.0$ Hz, Py C$_3$/C$_5$-H), 7.38-7.48 (m, 4H, Ar-H), 7.64 (d, 2H, $J = 7.4$ Hz, Py$^+$ C$_3$/C$_5$-H), 8.51 (d, 2H, $J = 6.0$ Hz, Py$^+$ C$_2$/C$_6$-H), 8.83 (d, 2H, $J = 7.4$ Hz, Py$^+$ C$_2$/C$_6$-H).

1,4-Di-(4-pyridinyloxy)benzene dimethiodide (36b) was prepared from 36 (135 mg, 0.5 mmol) according to method A with the minor modifications described earlier for 35b. The solid residue obtained was recrystallized
from acetone-MeOH to afford pure 36b (200 mg, 73% yield): m.p. 252°C (dec); UV/VIS (H₂O) λ_max 225 nm (ε 35140 M⁻¹cm⁻¹); ¹H-NMR (DMSO-d₆) δ 4.25 (s, 6H, N-CH₃), 7.57 (s, 4H, Ar-H), 7.69 (d, 4H, J = 7.4 Hz, C₃/C₅-H), 3.87 (d, 4H, J = 7.4 Hz, C₂/C₆-H).

1,1'‐Oxybis-[4-(4-pyridinyl)benzene] (37) was prepared from N-(t-butyldimethylsilyl)pyridinium triflate, obtained from freshly distilled pyridine (1.03 g, 13 mmol) and TBDMS triflate (3 mL, 13.1 mmol), using the procedure described above for 35, and employing instead the di-Grignard reagent from 4,4'-dibromodi- phenyl ether (2.08 g, 6.3 mmol) and Mg (615 mg, 25 mmol) in anhydrous THF. The final product obtained after oxidation and work-up was purified by silica gel flash chromatography (EtOAc:Acetone 3:1) (overall isolated yield 585 mg, 30%): m.p. 141°C; ¹H-NMR (CDCl₃) δ 7.19 (d, 4H, J = 9.2 Hz, C₃′/C₅′-H), 7.53 (d, 4H, J = 6.7 Hz, C₃/C₅-H), 7.68 (d, 4H, J = 9.2 Hz, C₂′/C₆′-H), 8.68 (d, 4H, J = 6.7 Hz, C₂/C₆-H); ¹³C-NMR (CDCl₃) δ 119.51 (‐, C₃′/C₅′), 121.31 (‐, C₃/C₅), 128.57 (‐, C₂′/C₆′), 133.41 (+, C₁′), 147.45 (+, C₄), 150.25 (‐, C₂/C₆), 157.75 (+, C₄'); HRMS (40 eV) calcd for C₂₂H₁₆N₂O m/z 324.1363, found 324.1508 (M⁺, 100%).

1,1'‐Oxybis-[4-(4-pyridinyl)benzene] monomethiodide (37a) was prepared from 37 (320 mg, 1 mmol) using
method B described earlier. Recrystallization from MeOH-H₂O afforded 270 mg of pure 37a (58% yield): m.p. 192°C; UV/VIS (H₂O) λmax 268 nm (ε 14640 M⁻¹cm⁻¹); 322 nm (ε 17440 M⁻¹cm⁻¹); ¹H-NMR (DMSO-d₆) δ 4.31 (s, 3H, N-CH₃), 7.28 and 7.93 (2d, 2H each, J = 8.6 Hz, C₃/C₅,–H and C₂/C₆,–H next to Py), 7.30 and 8.17 (2d, 2H each, J = 8.6 Hz, C₃/C₅,–H and C₂/C₆,–H next to Py⁺), 7.74 and 8.65 (2d, 2H each, J = 6.0 Hz, Py C₃/C₅–H and C₂/C₆–H), 8.48 and 8.98 (2d, 2H each, J = 6.8 Hz, C₃/C₅–H and C₂/C₆–H of Py⁺).

1,1′-Oxybis-[4-(4-pyridinyl)benzene] dimethiodide (37b) was obtained in 76% yield (230 mg) from 37 (160 mg, 0.5 mmol) by the modified method A as described for the preparation of 35b, with the final purification by recrystallization from H₂O: m.p. 180°C; UV/VIS (H₂O) λmax 222 nm (ε 44600 M⁻¹cm⁻¹), 327 nm (ε 40500 M⁻¹cm⁻¹); ¹H-NMR (DMSO-d₆) δ 4.32 (s, 6H, N-CH₃), 7.36 and 8.20 (2d, 4H each, J = 8.7 Hz, C₃/C₅,–H and C₂/C₆,–H), 8.50 and 9.00 (2d, 4H, J = 6.7 Hz, C₃/C₅–H and C₂/C₆–H).

2,2′-(1,4-Phenylene)bis-[5-(4-pyridinyl)furan] (38) was prepared by cyclocondensation of tetracarbonyl compound 34 (640 mg, 1.6 mmol) by stirring with conc. HCl (5 mL) at 90°C for 10 h. At the completion of the reaction, the mixture was diluted with 100 mL water, and the solution was neutralized to pH 7 with aqueous
alkali followed by extraction with CHCl₃. The CHCl₃ layer was separated, washed with water, dried (Na₂SO₄) and evaporated to afford a solid residue which was purified by recrystallization from acetone (420 mg, 72% yield): m.p. 120°C (dec); ¹H-NMR (CDCl₃) δ 6.88 and 7.04 (2d, 2H each, J = 3.6 Hz, furan CH), 7.63 (d, 4H, J = 6.2 Hz, C₃/C₅-H), 7.85 (s, 4H, Ar-H), 8.66 (d, 4H, J = 6.2 Hz, C₂/C₆-H); HRMS (40 eV) calcd for C₂₄H₁₆N₂O₂ m/z 364.1212, found 364.1220 (M⁺, 100%), 365.1247 (M+1, 33%), 258.0808 (M-106, 28%).

2,2’-(1,4-Phenylene)bis-[5-(4-pyridinyl)furan] monomethiodide (38a) was obtained from 38 (182 mg, 0.5 mmol) using method B. The product was recrystallized from acetone-ether for purification (yield 65 mg, 26%): m.p. 163°C; UV/VIS (H₂O) λ_max 423 nm (ε 21080 M⁻¹cm⁻¹); ¹H-NMR (DMSO-d₆) δ 4.28 (s, 3H, N-CH₃), 7.39 and 7.48 (2 d, 1H each, J = 3.8 Hz, furan CH’s next to Py), 7.52 and 8.00 (2d, 1H each, J = 3.8 Hz, furan CH’s next to Py⁺), 7.87 and 8.66 (2d, 2H each, J = 6.1 Hz, Py C₃/C₅-H and C₂/C₆-H), 8.12 (m, 4H, Ar-H), 8.46 and 8.93 (2d, 2H each, J = 6.8 Hz, Py⁺ C₃/C₅-H and C₂/C₆-H).

2,2’-(1,4-Phenylene)bis-[5-(4-pyridinyl)furan] dimethiodide (38b) was prepared from 38 (182 mg, 0.5 mmol) using the procedure described earlier for 35b. The final purification was accomplished by recrystall-
lization from acetone-EtOH (yield 160 mg, 52%): m.p. >198°C (dec); UV/VIS (H2O) λmax 397 nm (ε 5660 M⁻¹cm⁻¹); ¹H-NMR (DMSO-d₆) δ 4.29 (s, 6H, N-CH₃), 7.58 (d, 2H, J = 3.7 Hz, furan CH), 8.03 (d, 2H, J = 3.7 Hz, furan CH), 8.19 (s, 4H, Ar-H), 8.47 (d, 4H, J = 6.8 Hz, C₃/C₅-H), 8.95 (d, 4H, J = 5.8 Hz, C₂/C₆-H).

Preparation of tetrahydropyridines:

General procedure¹⁰⁶ for NaBH₄ reduction of pyridinium analogs to their corresponding tetrahydropyridine derivatives. To a stirred suspension of NaBH₄ (320 mg, 8.4 mmol) in 20 mL EtOH under N₂ at 0°C, a 20 mL EtOH solution of the pyridinium compound (4 mmol) was added dropwise over 15 min. The reaction mixture was stirred under N₂ for 30 min at 25°C and then poured into ice-cold 1N HCl (50 mL) followed by extraction of the mixture with ether. The ether extracts were discarded and the aqueous layer was brought to pH 11 with 10% aqueous NaOH. The resulting heterogeneous solution was extracted with ether, and the organic extracts were separated, washed with water and dried (Na₂SO₄) before evaporation to afford a residue which was treated with ethanolic HCl to yield the tetrahydropyridine HCl salt in pure form upon recrystallization from EtOH-ether.

1-Methyl-4-cyclopentyl-1,2,3,6-tetrahydropyridine
hydrochloride (39) was obtained in 96% yield (0.85 g) from 1-methyl-4-cyclopentyl pyridinium tosylate 22 (1.47 g, 4.38 mmol) using the procedure described above: m.p. 156-158°C (dec); 1H-NMR (free base in CDCl₃) δ 1.36-1.78 (m, 8H, cyclopentyl CH₂'s), 2.11-2.17 (m, 2H, C₃-H), 2.33 (s, 3H, N-CH₃), 2.34-2.35 (m, 1H, cyclopentyl CH), 2.51 (t, 2H, J = 5.8 Hz, C₂-H), 2.87-2.92 (m, 2H, C₆-H), 5.37-5.40 (m, 1H, C₅-H); HRMS (40 eV) calcd for C₁₁H₁₉N m/z 165.1519, found 165.1515 (M⁺, 70%), 164.1430 (M-1, 90%), 122.0972 (M-43, 100%), 107.0763 (M-58, 45%).

1-Methyl-4-cyclohexyl-1,2,3,6-tetrahydropyridine hydrochloride (40) was similarly obtained in 76% yield (164 mg) from 23 (350 mg, 1 mmol): m.p. 162°C; 1H NMR (free base in CDCl₃) δ 1.10-1.30 and 1.55-1.81 (2m, 10H, cyclohexyl CH₂'s), 2.10-2.27 (m, 2H, C₃-H), 2.28-2.32 (m, 1H, cyclohexyl CH), 2.33 (s, 3H, N-CH₃), 2.50 (t, 2H, J = 5.8 Hz, C₂-H), 2.90 (dd, 2H, J₁ = 2.4 Hz, J₂ = 5.2 Hz, C₆-H), 5.34-5.36 (m, 1H, C₅-H); HRMS (40 eV) calcd for C₁₂H₂₁N m/z 179.1675, found 179.1676 (M⁺, 67%), 178.1627 (M-1, 100%), 136.1144 (M-43, 20%), 122.0934 (M-47, 55%), 107.0886 (M-72, 40%).

1-Methyl-4-benzyl-1,2,3,6-tetrahydropyridine hydrochloride (41) was obtained in 79% yield (1.8 g) using the reduction procedure described above from 1-methyl-4-benzylpyridinium tosylate 9 (3.6 g, 10.2 mmol):
m.p. 147°C (lit. 143-145°C); 1H-NMR (free base in CDCl₃) δ 2.06 (br s, 2H, C₃-H), 2.31 (s, 3H, N-CH₃), 2.47 (t, 2H, J = 5.7 Hz, C₂-H), 2.90 (br s, 2H, C₆-H), 3.28 (s, 2H, PhCH₂), 5.39 (br s, 1H, C₅-H), 7.2 (m, 5H, Ph-H); HCl salt HRMS (40 eV) calcd for C₁₃H₁₇N m/z 187.1362, found 187.1366 (M⁺, 30%).

1-Methyl-4-benzylidine-piperidinium hydrochloride (42). To a stirred suspension of benzyltriphenylphosphonium bromide (3.57 g, 10 mmol) in 50 mL anhydrous benzene was added dropwise a solution of n-BuLi (6.25 mL of a 1.6 M solution in hexanes, 10 mmol) under N₂ at -78°C. The resulting slurry was allowed to warm to 25°C and stirred further for 3 h. The mixture was then evaporated to dryness in vacuo and 25 mL anhydrous THF was added. The resulting solution containing the phosphonium ylid was then cooled again to -78°C and freshly distilled 1-methyl-4-piperidone (1.15 g, 10 mmol) was added all at once. The mixture was then stirred under N₂ for 6 h at 60°C, followed by cooling and treatment with ice-cold aqueous 10% Na₂CO₃ solution. Extraction of the mixture with CHCl₃ and evaporation of CHCl₃ gave a residue which was dissolved in MeOH and passed through a short silica gel column (MeOH eluant). The MeOH solution was concentrated to dryness and the residue was treated with ethanolic HCl to afford 720 mg.
of the title hydrochloride salt (32% yield): m.p. 207°C; \(^1\)H-NMR (free base in CDCl\(_3\)) \(\delta\) 2.33 (s, 3H, N-CH\(_3\)), 2.37-2.58 (m, 8H, C\(_2\)/C\(_3\)/C\(_4\)/C\(_5\)-H), 6.31 (br s, 1H, vinyl CH), 7.21-7.38 (m, 5H, Ph-H); HRMS (40 eV) calcd for C\(_{13}\)H\(_{17}\)N m/z 187.1362, found 187.1372 (M\(^+\), 100%).

1-Methyl-4-(5-methylfuran-2-yl)-1,2,3,6-tetrahydropyridine hydrochloride (43) was prepared from 17 (600 mg, 2 mmol) in 86% yield (370 mg), using the above mentioned reduction procedure: m.p. 180-182°C (darkens); \(^1\)H NMR (free base in CDCl\(_3\)) \(\delta\) 2.29 (s, 3H, CH\(_3\)), 2.39 (s, 3H, N-CH\(_3\)), 2.44 (m, 2H, C\(_3\)-H), 2.61 (t, 2H, C\(_2\)-H), 3.10 (q, 2H, C\(_6\)-H), 5.93 and 6.09 (2d, 1H each, J = 3.2 Hz, furan CH), 6.11 (m, 1H, C\(_5\)-H).

1-Methyl-4-(4-bromophenyl)-1,2,3,6-tetrahydropyridine hydrochloride (44). 1,4-Dibromobenzene (2.36 g, 10 mmol) was treated with 1 equiv of Mg (240 mg, 10 mmol) in 80 mL anhydrous ether under N\(_2\), to afford the mono-Grignard reagent. The solution of this Grignard reagent was then added dropwise to a solution of 1-methyl-4-piperidone (1.13 g, 10 mmol) in 20 mL ether under N\(_2\) at -78°C. The resulting mixture was then allowed to warm to 25°C and stirred further for 5 h. An ice-cold 20% aqueous NH\(_4\)Cl solution was then added and the mixture extracted with CHCl\(_3\). The organic layer was separated, dried (Na\(_2\)SO\(_4\)) and evaporated to afford 1-
methyl-4-(4-bromophenyl)piperidin-4-ol as an oily residue, which was used for the next step without spectral characterization or purification.

The residue was dissolved in 30 mL conc. HCl and the solution was heated under reflux for 10 h. The reaction mixture was then cooled to room temperature and diluted with 100 mL water, followed by basification to pH 10 with aqueous 10% NaOH solution. The turbid solution obtained was extracted with ether, and the ether extracts were washed with water, dried (Na₂SO₄), and evaporated to yield an oily residue which solidified upon cooling. The residue was treated with ethanolic HCl with ice-cooling to afford a white solid, which was collected by filtration and recrystallized from EtOH-ether to yield 1.5 g of the title compound (52% overall yield): m.p. 180-132°C; ¹H NMR (free base in CDCl₃) δ 2.41 (s, 3H, N-CH₃), 2.54-2.58 (m, 2H, C₂-H), 2.67 (t, 2H, J = 5.4 Hz, C₂-H), 3.10 (dd, 2H, J₁ = 2.6 Hz, J₂ = 5.8 Hz, C₆-H), 6.06-6.08 (m, 1H, C₅-H), 7.25 and 7.44 (2d, 2H each, J = 8.2 Hz, Ar-H); HRMS (40 eV) calcd for C₁₂H₁₄BrN m/z 251.0310, found 251.0304 (M⁺, 100%).

1,5-Dimethyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (45) was obtained in 85% yield (380 mg) from 1,3-dimethyl-4-phenylpyridinium iodide 5 (620 mg, 2 mmol) using the above mentioned reduction procedure:
m.p. 186-188°C (lit. 109 189-190°C); ¹H-NMR (free base in CDCl₃) δ 1.58 (s, 3H, C₅-CH₃), 2.40 (s, 3H, N-CH₃), 2.46 (m, 2H, C₃-H), 2.62 (t, 2H, J = 6.0 Hz, C₂-H), 2.94 (br s, 2H, C₆-H), 7.16-7.35 (m, 5H, Ph-H).

1-Methyl-4-benzyl-1,2,3,6-tetrahydropyridine-1-oxide (46). To a solution of the free base of 41 (1.7 g, 9.1 mmol) in 20 mL EtOH was added 30% H₂O₂ (2 mL) and the mixture was stirred under N₂ at 50°C for 2 h. An additional 1.5 mL of 30% H₂O₂ was then added, and stirring was continued for 10 h. The reaction mixture was then cooled to 0°C and 300 mg of 10% Pd/C was added to destroy excess peroxide. The catalyst was removed by filtration through Celite, and the filtrate was evaporated to dryness, affording the N-oxide as an oil (crude yield 1.6 g, 87%), which was purified by silica gel flash chromatography (EtOAc-MeOH 1:1 eluant): ¹H-NMR (CDCl₃) δ 2.23 and 2.50 (2m, 2H, C₃-H), 3.18 (s, 3H, N-CH₃), 3.35-3.42 (m, 4H, C₂-H and PhCH₂), 3.92 (br s, 2H, C₆-H), 5.41 (br m, 1H, C₅-H), 7.18-7.42 (m, 5H, Ph-H).

1-Methyl-4-benzylidinane-piperidinium-1-oxide (47) was similarly prepared in 74% yield (300 mg) from 42 (450 mg, 2 mmol) using the same method as described above for 46: ¹H-NMR (CDCl₃) δ 2.35 (br d, 1H, C₃-H), 2.77 (m, 1H, C₃-H), 3.17 (m, 2H, C₅-H), 3.35-3.67 (2m, 4H, C₂/₆-H) 3.39 (s, 3H, N-CH₃), 6.48 (s, 1H, vinyl
CH), 7.16-7.39 (m, 5H, Ph-H).

1-Methyl-4-benzyl-2,3-dihydropyridinium trifluoroacetate (48). The preceding N-oxide 46 (400 mg, 2 mmols) was dissolved in 20 mL CH₂Cl₂ and the solution cooled to 0°C. A slight molar excess of freshly distilled trifluoroacetic anhydride (0.3 mL, 2.1 mmols) was then added, and the solution was stirred at 0°C for 1 h. The solvent was then evaporated under high vacuum at 0°C to obtain 48 in form of a reddish oil in essentially quantitative yield as determined by NMR analysis:

UV/VIS (H₂O) λ max 290 nm (ε 53200 M⁻¹cm⁻¹);

¹H-NMR (CDCl₃) δ 2.73 (t, 2H, J = 9.5 Hz, C₃-H), 3.60 (s, 3H, N-CH₃), 3.68 (s, 2H, Ph-CH₂), 3.83 (t, 2H, J = 9.5 Hz, C₂-H), 6.13 (d, 1H, J = 4.0 Hz, C₅-H), 7.12-7.38 (m, 5H, Ph-H), 8.12 (d, 1H, J = 4.0 Hz, C₆-H). Note: Treatment of the N-oxide 47 with trifluoroacetic anhydride under the same conditions described above afforded a material which exhibited an identical ¹H-NMR spectrum as that for 48. Further characterization of 48 was by its conversion to 49.

1-Methyl-4-benzyl-6-cyano-1,2,3,6-tetrahydropyridine (49) was prepared by dropwise addition of an aqueous solution (10 mL) of NaCN (60 mg, 1.2 mmols), under N₂, to a solution of 48 (300 mg, 1 mmol) in 15 mL MeOH with ice-cooling (5°C). The reaction mixture was al-
lowed to stir at the same temperature for 3 h and then extracted with EtOAc. The residue obtained upon evaporation of the organic extracts was purified by silica gel chromatography (EtOAc eluant) to obtain 136 mg of the title compound (64% yield): $^1$H-NMR (CDCl$_3$) $\delta$ 1.95 and 2.20 (2m, 1H each, C$_3$-H), 2.5-2.75 (m, 2H, C$_2$-H), 3.33 (s, 2H, Ph-CH$_2$), 4.07 (br d, 1H, C$_6$-H), 5.41 (br d, 1H, C$_5$-H), 7.15-7.39 (m, 5H, Ph-H).

1-Methyl-4-benzylidene-1,2,3 4-tetrahydropyridine (50). The compound 48 (300 mg, 1 mmol) was dissolved in 700 mL of ice-cold water, purged with N$_2$ for 30 min and then added dropwise over 3 h to a 5% aqueous NaOH solution (30 mL) with vigorous stirring under N$_2$. The temperature of the solutions was maintained at 5°C during the addition. After the addition was complete, the solution was extracted with CDCl$_3$ for $^1$H-NMR analysis: $^1$H-NMR (CDCl$_3$) $\delta$ 2.66 (dt, 2H, J = 1.2 and 6.7 Hz, C$_2$-H), 2.77 (s, 3H, N-CH$_3$), 3.12 (t, 2H, J = 6.7 Hz, C$_3$-H), 5.59 (d, 1H, J = 8.3 Hz, C$_5$-H), 5.76 (s, 1H, Ph-CH$_2$), 6.08 (dd, 1H, J = 1.2 and 8.3 Hz, C$_6$-H), 7.13-7.46 (m, 5H, ArH).

In NOE experiments, saturation of signal at 2.77 (s, N-CH$_3$) led to a 17% NOE of more downfield dd signal at 6.08 ppm which was assigned as C$_6$-H. In the same experiment we could not obtain a true measurement of NOE
in any of the two aliphatic triplets due to the fact that the signals are too close to the radiation frequency.

Similarly saturation of the signal corresponding to methine proton at 5.76 ppm (C<sub>α</sub>H) did not show any NOE of any of the triplets which leads us to believe that the CH is away from the aliphatic carbons C<sub>2</sub> and C<sub>3</sub>.

1-Methyl-4-benzylidene-1,4-dihydropyridine (51). A solution of pyridinium 9 (177 mg, 0.5 mmols) in 50 mL water was purged with N<sub>2</sub> for 30 min and cooled to 5°C. 10 mL of a N<sub>2</sub> purged 10% aqueous NaOH solution was then added under N<sub>2</sub>. An equal volume of CHCl<sub>3</sub> was then added and the resulting mixture stirred vigorously before separating the organic layer which was evaporated to dryness and redissolved in N<sub>2</sub> flushed CDCl<sub>3</sub> for NMR analysis: ¹H-NMR (CDCl<sub>3</sub>) δ 3.07 (s, 3H, N-CH<sub>3</sub>), 5.28 (s, 1H, Ph-CH=), 5.69 (dd, J = 2.6 and 7.6 Hz, C<sub>3</sub>-H), 6.04 (dd, J = 2.0 and 7.6 Hz, C<sub>2</sub>-H), 6.13 (ddd, J = 1.0, 2.0 and 8.0 Hz, C<sub>6</sub>-H), 6.34 (dd, J = 2.6 and 8.0 Hz, C<sub>5</sub>-H), 6.96 (m, 1H, Ph-H), 7.21-7.25 (m, 4H, Ph-H); ¹³C NMR (CDCl<sub>3</sub>) δ 41.11 (-, N-CH<sub>3</sub>), 102.82 (-), 106.17 (-), 114.29 (-), 122.97 (-), 126.06 (-), 128.10 (-), 131.89 (-), 133.17 (+), 134.23 (-), 140.27 (+) (total 11 signals).
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CHAPTER II.

Model Studies on γ-Diketone-Induced Peripheral Axonopathies.
INTRODUCTION:

Chronic exposure to hexane and methyl n-butyl ketone (MnBK) results in a neurotoxic condition characterized by the massive focal accumulation of neurofilaments (NF) at prenodal sites in peripheral axons. These neurological deficits are similar to inherited or acquired human neuropathies. The toxicity of hexane and MnBK is now known to be associated with their common metabolite 2,5-hexanedione. This and related γ-diketones constitute one class of chemicals now widely used as an experimental model to study the pathogenesis of neurofilamentous axonopathies. It is also noteworthy that some other chemically unrelated non-ketonic compounds, e.g. acrylamide, IDPN (β,β'-iminodipropionitrile), CS$_2$ and aluminum salts etc., have also been found to produce morphologically similar effects.

For γ-diketones, the accumulation of NF is believed to be initiated through Paal-Knorr condensation with the primary amine side-chains of critical lysine residues of NF or related cytoskeletal proteins, resulting in the formation of pyrroles. Such modification itself could account for the NF accumulation by altering the noncovalent interactions crucial to the supramolecular organization of the NF-microtubule meshwork. Alternative-
ly, since alkyl-pyrroles are known to undergo autoxidation to reactive electrophiles,\textsuperscript{7-9} some researchers favor an autoxidation-dependent protein cross-linking occurring subsequent to pyrrole formation as the primary event responsible for the initiation of NF accumulation.\textsuperscript{10,11}

Regarding the structure-activity relationships for \(\gamma\)-diketone induced neuropathies, it has been observed that while 2,5-HD induces NF-containing enlargements in the distal regions of the axon, 3,4-dimethyl-2,5-hexanedione (3,4-DMHD) is 25 times more neurotoxic and induces proximal axonal enlargements (near the perikarya), often at the first node of Ranvier.\textsuperscript{12} The monomethyl analog, 3-methyl-2,5-hexanedione (3-MHD), a structural average between the parent 2,5-HD and the dimethyl analog 3,4-DMHD, has been found to produce NF accumulation at midway axonal regions with an intermediate level of neurotoxicity.\textsuperscript{13}

The enhanced neurotoxic potency of 3,4-DMHD could be accounted for by considering the effect on either of the two steps mentioned above, i.e. the formation of pyrroles or their subsequent autoxidation. Since the vicinal dimethyl substitution in 3,4-DMHD accelerates both pyrrole formation and the subsequent autoxidation,\textsuperscript{14} this compound does not permit a mecha-
nistic distinction between the events primarily responsible for initiating the axonal accumulation of NF. Also, the fact that 3,4-DMHD exists as meso and dl diastereomers complicates the structure-activity correlations.\textsuperscript{12}

The question of different diastereomers of 3,4-DMHD was, in fact, addressed in recent studies, which show that the \textit{dl}-isomer forms the pyrrole faster than \textit{meso}-DMHD and is also more neurotoxic.\textsuperscript{15} This suggests that the formation of pyroles is an important pathogenic step. However the rates of pyrrole formation with \textit{meso}-DMHD and 2,5-HD are essentially the same, while \textit{meso}-DMHD is more neurotoxic than 2,5-HD.\textsuperscript{15} This realization was cited as evidence that autooxidation-mediated crosslinking may also contribute to neurotoxicity, since the tetramethylpyrrole obtained from \textit{meso}-DMHD (which is the same as that from \textit{dl}-DMHD) is more susceptible to oxidation than the dimethylpyrrole formed from 2,5-HD.\textsuperscript{15}

Nonetheless, these observations do not provide a clear picture regarding the relative importance of the various steps responsible for the neurologic damage, and it is therefore important to investigate additional \textit{γ}-diketones which would help to distinguish between the pyrrole formation and pyrrole autooxidation reactions.

In the present study we sought to investigate \textit{γ}-
diketone analogs where pyrroles could form as rapidly or more rapidly than in the case of 2,5-HD, but which would then be less susceptible to oxidation. Biological evaluation of such diketones should provide the information required for distinguishing the pyrrole formation per se hypothesis from the proposal of a required autoxidation-induced protein cross-linking. 1-Substituted and 3-monomesubstituted analogs were employed in order to avoid complications due to diastereoisomerism.

In particular, we prepared two fluorinated analogs of 2,5-HD, namely 1,1,1-trifluoro-2,5-hexanedione (F₃-HD) and 3-trifluoromethyl-2,5-hexanedione (TFM-HD). Condensations of these analogs with a protein-based primary amine would occur as shown below (Figure 1). α-Fluoro carbonyl compounds are known to be very susceptible to nucleophilic attack due to electronic effects. Thus, F₃-HD is expected to condense with primary amines more rapidly than 2,5-HD itself. The resulting pyrrole should not be too dissimilar in physicochemical characteristics to the pyrrole formed from 2,5-HD. However, the electron-withdrawing CF₃ group should greatly suppress the rate of pyrrole autoxidation, a reaction which is initiated by electrophilic attack of O₂.¹⁶ Similarly, the other analog TFM-HD should be comparable to 3-MHD in terms of pyrrole formation rate, but the resulting
trifluoromethyl-pyrrole should exhibit a reduced autoxidation tendency.

![Diagram showing reaction scheme for basis of selecting two fluorinated diketones in this study.](image-url)
Thus both fluorinated analogs should permit a dissociation between the effects of pyrrole formation and pyrrole autoxidation in governing the formation and position of NF accumulations. This dissertation is also the first report on the synthesis of the two aforementioned fluorinated diketones.

A recent publication by Katritzky describes the use of NMR to obtain mechanistic information on the condensation reaction of 2,5-hexanediol with a variety of primary amines. In particular, the authors provide evidence for the intermediacy of imines on the reaction pathway leading to the formation of pyrroles. We found such experiments extremely useful for obtaining additional mechanistic details on the Paal-Knorr reaction as well as for assessing relative rates of pyrrole formation from various diketones. In the case of the fluorinated analogs used in this study, access to $^{19}$F-NMR provided for an independent check on the mechanism of the Paal-Knorr reaction through a more facile data interpretation.

Pyrroles are electron rich heterocycles and are known to be readily oxidized. Although the nature of products from their reaction with molecular oxygen is not known, the rate-limiting step for the autoxidation of alkylpyrroles has been proposed to involve a one-
electron transfer to molecular oxygen. Furthermore a direct correlation has been found to exist between the rate of oxidation of pyrroles and their anodic oxidation potentials as determined by electrochemical measurements. Increased electron density due to increased substitution by alkyl groups is found to lower the anodic oxidation potentials, whereas electron-withdrawing substituents are known to lead to increased anodic oxidation potentials. Thus, in an effort to assess the relative susceptibility of the pyrroles considered here to undergo physiological autoxidation, we measured the oxidation potentials by cyclic voltammetry of model pyrroles obtained from condensation of a selected primary amine with the various γ-diketones employed in this study. We have also compared the relative propensity of these pyrroles to undergo chemical oxidation with potassium peroxodisulfate.
RESULTS:

Preparation of fluorinated ketones.

3-(Trifluoromethyl)-2,5-hexanedione (TFM-HD) was prepared via a Michael addition strategy shown in Figure 3. Thus 1,4-addition of nitroethane carbanion to 5,5,5-trifluoropent-3-en-2-one, prepared by a modification of a published procedure,20 afforded a diastereomeric mixture of nitro-ketone 2, which under mild "Nef" reaction conditions21 was oxidized to the title compound. We also attempted to prepare TFM-HD via traditional coupling methods for the preparation of unsymmetrical 7-diketones, employing 4,4,4-trifluoro-2-butanone prepared electrochemically according to the procedure of Miller.22 However, no synthetically useful coupling routes were found. For example, two methods based on the use of enol-silyl ether were attempted. The enol-silyl ether, obtained from 4,4,4-trifluorobutanone by reaction with triethylamine and trimethylsilyl chloride, upon addition to 2-nitropropene did not yield the expected 1,4-addition product.40 Similarly its reaction with chloroacetone also failed.
Figure 2. Reaction scheme for synthesis of TFM-HD (3).

Figure 3. Reaction scheme for synthesis of F2-HD (8).
1,1,1-Trifluoro-2,5-hexanedione (F$_3$-HD) was synthesized from commercially available levulinic acid by the multistep sequence summarized in Figure 3. Levulinic acid was converted to its benzyl ester, followed by protection of the keto group in the form of a ketal. The NaH mediated Claisen condensation with benzyl trifluoroacetate$^{28}$ followed by deprotection of the keto group afforded the diketo-ester containing the trifluoroacetyl group. Removal of the benzyl group by catalytic hydrogenation occurred with concomitant decarboxylation, giving the desired product, which was purified by fractional distillation.

**Pyrrole formation studies.**

Pyrrole formation was studied by following the reactions of the various γ-diketones used in this study with a variety of primary amines by $^1$H, $^{13}$C and also $^{19}$F NMR in the case of the fluorinated compounds. The combined NMR methods permitted not only a measure of the relative rates of γ-diketone disappearance and pyrrole formation, but also a measure of the formation and decay of the initial imine and other intermediates. Three different primary amines of structure RCH$_2$NH$_2$ were used for monitoring the reaction rates with 2,5-HD, 3-MHD,
3,4-DMHD, and TFM-HD. In each case the diketone and the model amine were mixed together in CDCl$_3$ in a constant 1:1 molar ratio, and $^1$H NMR spectra were recorded at regular time intervals. In each of these reactions it was most informative to monitor the $\text{N-CH}_2$ methylene signals, which exhibited a chemical shift difference of 1-1.2 ppm (downfield) upon going from amine to pyrrole. This characteristic change has been attributed to the diatropic ring current of the pyrrole. The $\gamma$-diketone-derived signals were gradually replaced by those arising from the respective pyroles.

There are several advantages that this NMR-based method offers over other techniques used previously for evaluating the rates of pyrrole formation. One method reported was based on the DMAB (dimethylaminobenzaldehyde) assay for detection of pyrroles.$^{14,23}$ Although the mechanism of the reaction of unsubstituted pyrroles with DMAB is fairly well understood,$^{24}$ it is not clear as to how fully substituted pyrrole derivatives lead to formation of chromophores and therefore whether the method in question gives an accurate evaluation of pyrrole formation rates. Another group has recently reported an HPLC-based method for quantifying pyrroles.$^{25}$ However this method involves tedious and cumbersome manipulations, and was carried out under
pseudo-first order conditions with the model amine in a large excess over the diketones. Although it is a common practice to use such pseudo-order conditions for kinetic studies, we have observed additional intermediates in the reaction when [amine] > [diketone] compared to the situation where [amine] = [diketone], as determined by the $^{19}$F NMR study on the reaction between TFM-HD and iso-butylamine (see below). This different behaviour is most likely arising from a possible change in the mechanism of Paal-Knorr reaction under pseudo-first order conditions. Our NMR studies therefore permitted a kinetic analysis as well as a direct observation of the intermediates and products of the reactions.
<table>
<thead>
<tr>
<th></th>
<th>R = -CH₂CH₂(CH₃)₂</th>
<th>-CH₂C(CH₃)₂</th>
<th>-CH₂(C₆H₅N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5-HD</td>
<td>800</td>
<td>5400</td>
<td>200</td>
</tr>
<tr>
<td>3-MHD</td>
<td>50</td>
<td>264</td>
<td>&gt;2</td>
</tr>
<tr>
<td>TFM-HD</td>
<td>40</td>
<td>227</td>
<td>18</td>
</tr>
<tr>
<td>3,4-DMHD</td>
<td>&lt;30*</td>
<td>&lt;54</td>
<td>&lt;10*</td>
</tr>
</tbody>
</table>

* data for the mixture of d- and meso-diastereomers.
The half-lives of pyrrole formation as determined by \(^1\)H NMR are collected together in Table I. Since pyrrole formation involves multiple (rate-limiting) steps,\(^17,26\) we used the half-lives of formation of the final product to assess the substituent effects on the overall reaction rate. As can be seen, the relative rate of pyrrole formation follows the order

\[
3,4-\text{DMHD} > \text{TFM-HD} > 3-\text{MHD} > 2,5-\text{HD}
\]

Although \(^1\)H NMR was useful for monitoring pyrrole formation, the nature of the (imine) intermediates could not be ascertained due to overlap of signals with those of the starting materials. Thus, we followed the reactions by \(^1\)H NMR, in which case signals due to intermediates were expected to be well resolved from those of starting materials and the pyrrole product. For the identification of intermediates based on the \(^1\)H chemical shift values, we have relied on the analysis and interpretations made in the earlier report on Paal-Knorr reaction by Katritzky and co-workers. The \(^1\)H NMR study of reactions with various diketones revealed characteristic signals for the imine intermediates, which appeared in the initial phase of the reactions, grew to a maximum and then were replaced by the characteristic
peaks for the pyrrole products. Assignments of the new signals to the imines was made on the basis of the chemical shift values, the most characteristic being the C-N carbon (in the range 160-170 ppm)\(^{27}\) and the N-CH\(_2\) carbon\(^{17}\) which in each case came 5-10 ppm downfield from the starting amine. In the case of the two unsymmetrical diketones, 3-MHD and TFM-HD, two sets of such characteristic peaks were observed, which are attributed to the two distinct imine intermediates. The spectra obtained at the completion of the reactions were ascribed to the pyrrole products by comparison to authentic samples.

In order to permit a distinction between the two imine intermediates in the case of TFM-HD, a \(^{19}\)F NMR study was undertaken. In one experiment a 1:1 mixture of TFM-HD and isobutylamine showed, similar to the observations made with earlier NMR experiments, an initial build-up of two imines, identified by the appearance of two new doublets (-67.28 and -68.35 ppm) at the expense of the doublet due to starting diketone (Figure 4). Although two geometrical forms (syn and anti) are conceivable for each of the regioisomeric imine intermediates, we assume that the rather large difference in the observed \(^{19}\)F chemical shifts are due to the regioisomeric imines. As depicted in Figure 7,
these two imines have the CF₃ group in non-equivalent positions, α- or β- to the imine functionality. In order to make definitive assignments of the ¹⁹F signals to these imines, we used 4,4,4-trifluorobutan-2-one²² as a model, and observed that the generation of imine with iso-butylamine in this case resulted in a upfield shift of 1.1 ppm. This value was then assumed to be a diagnostic ¹⁹F chemical shift difference for the formation of α-CF₃-substituted imines. Thus, the more downfield signal in the reaction of TFM-HD corresponded to the imine I 1 (see Figure 7), and interestingly this intermediate formed faster and also transformed to the pyrrole product faster than the other imine, probably because of the stereoelectronic effects at some stage of the reaction.
Figure 4. $^{19}$F NMR changes during the reaction of TFH-ID with isobutylamine (1:1 molar ratio).

158 min

72 min

42 min

17 min

2 min

8 ppm upfield of CFCl$_3$
Figure 5. $^{19}$F NMR changes during the reaction of TFM-HD with isobutylamine (excess)

205 min

105 min

18 min

5 min

1.5 min

6 ppm upfield of CFCl$_3$
In another experiment the reaction of TFM-HD with a 6-fold molar excess of iso-butylamine was observed to lead to a rapid disappearance of the starting diketone. We observed additional intermediates in this case as shown by the changes in the $^{19}$F spectra (Figure 5). In addition to the signals due to the initial mono-imine intermediates, there was a build-up of two new signals (a doublet at -68.6 and a singlet at -55.25 ppm) which we attribute to the mono-enamines of bis-Schiff base adducts. This assignment is not unambiguous, since there are other species which could show similar characteristic multiplicites e.g. cis/trans isomers of enamo-ketones arising from the two initially formed mono-imines (Figure 7). Nevertheless, as pointed out earlier these results demonstrate that the use of pseudo-first order conditions in assessing the rate of Paal-Knorr reaction is not appropriate on account of a possible change in the reaction mechanism.

The reaction of F$_3$-HD, the other fluorinated diketone prepared by us, did not give a pyrrole product with amines. Instead multiple products (more than four) containing CF$_3$ group (as revealed by a number of singlet peaks in $^{19}$F NMR spectrum) were obtained in these reactions. Till this point these products could not be individually isolated or characterized by us.
Pyrrole oxidation.

Cyclic voltammetry was performed on pyrroles obtained from isobutylamine and the various diketones mentioned in this study, in order to determine the relative ease of one-electron oxidation of N-isobutylpyrroles. These potentials (Table II) were measured under anaerobic conditions and reveal the clear facilitation of oxidation by increasing the number of methyl substituents, as well as the very large retarding influence of the strongly electron-withdrawing CF₃ substituent.

In each case, the voltammogram was typical of that expected for an irreversible one-electron abstraction, presumably because of the highly reactive nature of the resulting pyrrole π-cation radical. Only a single sweep was performed owing to the irreversibility of the oxidation and the values for the peak oxidation potentials were directly read from the charts.

Chemical oxidation of the neopentylamine-derived pyrroles by persulfate was monitored spectrophotometrically at pH 6.85. The half-lives for the formation of characteristic chromophore in each case were determined from the first-order plots and are presented in Table II. Under these conditions the relative rates of oxidation with K₂S₂O₈ were found to show the same rank-order
as the ease of one-electron oxidation, determined by cyclic voltammetry; the pyrrole derived from TFM-HD was in fact found to react very slowly with $\text{S}_2\text{O}_8^{2-}$. 
Table II. Summary of oxidation data on pyroles.

<table>
<thead>
<tr>
<th>Pyrole</th>
<th>Diketone precursor</th>
<th>$E_{p} (V)^a$ (vs. SCE)</th>
<th>$t_{1/2} (\text{min})$ for $K_2S_2O_8$ oxidation$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Pyrole Structure" /></td>
<td>3,4-DMHD</td>
<td>+ 0.62</td>
<td>104</td>
</tr>
<tr>
<td><img src="image" alt="Pyrole Structure" /></td>
<td>3-MHD</td>
<td>+ 0.70</td>
<td>188</td>
</tr>
<tr>
<td><img src="image" alt="Pyrole Structure" /></td>
<td>2.5-HD</td>
<td>+ 0.84</td>
<td>244</td>
</tr>
<tr>
<td><img src="image" alt="Pyrole Structure" /></td>
<td>TFM-HD</td>
<td>+ 1.43</td>
<td>300</td>
</tr>
</tbody>
</table>

$^a$ from cyclic voltammetry for $R = \text{CH}_2\text{CH(CH}_3)_2$; data measured in CH$_2$CN solutions containing 0.1 M tetrabutylammonium perchlorate and 2 x 10$^{-3}$ M pyrole; Glassy carbon disk electrode, scan rate 100 mV/s.

$^b$ oxidation of 2 x 10$^{-6}$ M pyroles (R = CH$_2$C(CH$_3$)$_3$) with 2 x 10$^{-3}$ M $K_2S_2O_8$ in MeOH-H$_2$O at 30$^\circ$C, pH 6.85.
DISCUSSION:

Pyrrole formation in NF proteins has been implicated as a critical step in the mechanism of induction of axonopathies by γ-diketones.⁴-⁶ Although pyrrolylation of proteins upon exposure to 2,5-HD has been demonstrated both in vivo and in vitro,³⁰,³¹ several aspects of this reaction remain obscure. These uncertainties involve the identification of rate-limiting steps and intermediates in the reaction, the possible formation of non-pyrrole adducts,³² and the role of autoxidation derived products from the pyrroles in induction of neurotoxicity.¹⁰,¹¹

That pyrrole formation may be the critical initiating event in γ-diketone neuropathy, stems from the observations that diketone neuropathy is restricted to γ-isomers vis-a-vis β- or δ-diketones.³³ This is consistent with the fact that only γ-diketones can condense with primary amines to give the five-membered pyrrole nucleus. Further evidence for the critical role of pyrroles comes from recent observations with 2,5-HD isomers substituted at the three and/or four positions. Thus, the finding that 3-MHD and especially 3,4-DMHD are more neurotoxic than the parent 2,5-HD,¹² is consistent
with the finding that 3-MHD (this study) and 3,4-DMHD condense with model primary amines to form pyrroles faster than does 2,5-HD. A second piece of evidence supporting the pyrrole mechanism comes from studies reported previously from our lab regarding the lack of neurotoxicity of 3,3-dimethyl-2,5-hexanedione (3,3-DMHD). This analog would be capable of condensing with primary amines but the final step (the dehydration step in Figure 6) is blocked due to the presence of gem-dimethyl substitution, and thus pyrrole cannot be formed. A third piece of evidence for the pyrrole theory is that 2,5-HD-d_{10} which forms pyrroles more slowly than does 2,5-HD on account of the isotope effect, is also much less neurotoxic.

Correlation between pyrrole formation rate and neurotoxic potency seems clear, but the overall situation is complicated by morphological differences. Unlike the NF enlargements produced by 2,5-HD in the distal regions of the axon, 3,4-DMHD produces them in proximal regions. This observation is difficult to account for only on the basis of the increased rate of pyrrole formation, since the secondary pyrrole autoxidation reaction (the other event suggested to be responsible for cross-linking and therefore expression of neurotoxicity) is also subject to acceleration due to increased
electron density of the pyrrole nucleus afforded by the two additional methyl substituents.19

The arguments in favor of key role of autoxidation-induced cross-linking in the neurotoxic process are (1) the elevated levels of neurotoxicity by 2,5-HD in the presence of hyperbaric oxygen,36 and (2) the enhanced neurotoxicity of meso-DMHD compared to 2,5-HD;15 the pyroles are formed at essentially same rates but the pyrrole product for the former is more reactive towards oxidation.

On the contrary, we favor a neurotoxicity mechanism involving pyrrole formation without a requisite secondary autoxidation-cross-linking process on the basis (1) of the similar nature of neuropathies induced by a variety of chemicals4 which are not capable of leading to any obvious cross-links, and (2) that 2,5-HD and 3-MHD actually accelerate the transport of neurofilaments along the axon,37 instead of slowing it down as would be expected for a cross-link based mechanism.

The exact mechanism of pyrrole formation (Paal-Knorr reaction) has recently been explored by Katritzky et al. under organic reaction conditions through 1H-, 13C- and 15N-NMR spectroscopy.17 Two rate-determining steps (Figure 6) have been identified: (i) the initial nucleophilic attack by the amine nitrogen on the carbo-
nyl group, and, subsequent to a rapid imine-enamine tautomerization, (ii) intramolecular attack by the enamine nitrogen on the second carbonyl. The N-substituted imine intermediates were identified in the reaction mixtures and the possible intermediacy of a dihydroxypyrrolidine was ruled out by Katritzky and co-workers. A similar mechanism has been demonstrated under aqueous conditions.32

In the present investigations we have extended the same approach to the study of the Paal-Knorr reaction mechanism for the unsymmetrical diketone (3-MHD) and the "more reactive" fluorinated analogs. We have also measured the rates of various steps in the reaction scheme and compared the relative accelerations for the differently substituted γ-diketone analogs. In addition, the use of 19F-NMR spectroscopy has allowed for a clean observation of various intermediates in the case of TFM-HD.

Thus for the unsymmetrical γ-diketones 3-MHD and TFM-HD, two of the 4 possible initial monoimines could be detected and were found to convert to pyrrole at different rates. The enamine intermediate could not be detected by Katritzky et al., presumably due to the unfavorable equilibrium for its formation. In the case of TFM-HD, however, the mono enamines of the bis-Schiff
adducts could be observed when [amine] >> [diketone] although we do not know whether these species (Figure 7) are on the reaction pathway leading to pyrrole. Also in this case the study allowed the independent measurements of the rate of formation and decay of both possible imine-enamine isomers (see Figure 7). When [amine]₀ = [diketone]₀, only the initial monoimine intermediates were observed to build up in solution.
Figure 6. Various steps involved in the Paal-Knorr reaction of 2,5-HD with primary alkylamine to form pyroles (from Ref 17).
Figure 7. Possible reaction pathways for Paal-Knorr condensation of TFM- HD with primary amines.
Interestingly, in the case of F3-HD, a pyrrole was not formed, and instead we observed multiple products which have not been fully characterized thus far (Figure 8). The lack of pyrrole formation in this case can be explained as: the nucleophilic attack on the trifluoro-carbonyl group is known to proceed with great ease and the resulting hydrates (with water as nucleophile) or carbinolamines (in the case of amines) are relatively resistant to further dehydration, due to the unfavorable development of partial positive charge adjacent to CF3 group and due to the unfavorable electronic effect on the sp3-to-sp2 rehybridization.
Figure 8. Possible products from reaction of $F_3$-HD with primary amine and water.
Figure 9. Scheme for the reaction of 3,4-DMHD with a primary alkylamine with possible rate limiting steps.
In view of our goal of selecting γ-diketone analogs to distinguish the primary chemical events responsible for neurotoxicity, it is important to understand in detail the structural factors which affect pyrrole formation rates. In the present study, we confirmed the report by Graham and co-workers that 3,4-DMHD reacts faster than 2,5-HD.\textsuperscript{29} Additionally their work with the individual diastereomers of 3,4-DMHD shows that the di-diastereomer reacts faster than meso. The authors attribute this to the "attainment of the planar conformation required for the ring formation"\textsuperscript{29} (the earlier interpretation of this difference in terms of a "gem-dimethyl" effect\textsuperscript{14} was nonsense because 3,4-DMHD has 2 vicinal methyl substituents so that such an interpretation is totally incorrect). However, an influence on the cyclization step cannot account for such a rate distinction because the meso vs. dl stereochemistry has been lost at the stage of the enamine which undergoes cyclization (Figure 9). Instead, an observed rate difference between the two diastereomers requires either that there be a rate difference in (i) the initial attack of amine on diketone, (ii) the dehydration of first formed carbinolamine to imine, and/or (iii) the imine-enamine tautomerization. Since step (ii) is fast (carbinolamine intermediates are not observed), the
rate-limiting differences must be steps (i) and/or (iii). As far as step (i) is concerned, we propose that there is some assistance from one carbonyl group on nucleophilic attack by the amine on the other carbonyl group, particularly in the dl isomer where the two carbonyls are gauche to each other.

Such an explanation, based on stereoelectronic effects, is also consistent with the observation that in the case of unsymmetrical diketones, TFM-HD and 3-MHD, there are two distinct initial mono-imine intermediates formed and that one of them appears faster than the other. This same fast forming imine is also converted to the pyrrole product faster than the other. This may be explained if either (a) there is neighboring group participation from one carbonyl group to the other, or (b) the imine-enamine tautomerization step is influenced by the effects of the substituent on α-deprotonation.

dl-3,4-DMHD

meso-3,4-DMHD
The second aspect of our plan to distinguish between the pyrrole formation and their subsequent autoxidation as the crucial factor governing neurotoxicity, involved the expected differential propensity of the pyrroles obtained from the aforementioned diketones to undergo autoxidation. We did not attempt to assess autoxidation rates directly because it was not clear what conditions would be most relevant to the physiological autoxidation phenomenon. Instead, we resorted to electrochemical and chemical oxidative reactions because it has been shown that model systems that the rate of oxidation (with molecular O₂) correlates well with the one-electron oxidation capacity of pyrroles.¹⁸

Various studies on anodic oxidation of pyrroles have been described.¹⁹,²⁹ One-electron anodic oxidation of simple pyrroles is known to be irreversible with the resulting π-cation radical undergoing further reactions to give a mixture of products.²⁹

Our cyclic voltammetry experiments reveal the clear facilitation of pyrrole oxidation upon increasing the number of methyl substituents, as well as the very large retarding influence of the strongly electron-withdrawing CF₃ substituent. These Ep values correlate qualitatively with the relative rates for the chemical oxidation of pyrroles by persulfate at pH 6.85. At low pH, relative
rate comparisons are complicated by substantial pK_a differences arising from the particular substitution pattern on the pyrrole. Thus, at higher pH values the relative rates of oxidation were found to parallel the ease of one-electron oxidation as revealed by cyclic voltammetry; 2,5-dimethyl-3-(trifluoromethyl)-pyrrole was in fact found to be rather sluggish.

Our results indicate that TFM-HD would be a good candidate for distinguishing between the two processes being considered in the development of γ-diketone neuropathies since it would form a pyrrole faster than 2,5-HD, but this pyrrole would be essentially inert to autoxidation. If pyrrole formation itself is the crucial event, TFM-HD should be more neurotoxic than 2,5-HD and should induce more proximal axonal enlargements. On the other hand, if there is a requirement for autoxidation and then cross-linking subsequent to pyrrole formation, then TFM-HD should display little if any neurotoxicity. It is unclear what F3-HD may do since it can form non-pyrrole adducts with protein-based amines, but the stability of such adducts is not known. Biological evaluations of the two fluorinated analogs for neurotoxic effects are now underway and are expected to reveal the actual event responsible for the onset of NF accumulations.
The above mentioned studies have also permitted the elucidation of additional mechanistic features of the Paal-Knorr reaction, and also highlight the use of fluoro-organics in chemical mechanistic studies.
EXPERIMENTAL.

General comments. All evaporation were conducted under reduced pressure using a rotary evaporator. Reactions requiring anhydrous conditions were performed in flame-dried glassware under a positive pressure of N₂. ¹H, ¹³C, and ¹⁹F NMR were recorded on a Varian-XL 200 multinuclear instrument operating at 200 MHz for proton, 50.31 MHz for carbon, and 188.22 MHz for fluorine. ¹⁹F spectra were recorded in CDCl₃ with either CFCI₃ or PhCF₃ as the internal standards; the chemical shift values are reported in ppm upfield of CFCl₃ taken as 0). For other general methods refer to experimental section under chapter I.

5,5,5-Trifluoro-3-penten-2-one (1) was prepared by following the procedure patterned after the method of Ogoshi et al.²⁰ Minor modifications involved the use of a positive pressure of N₂ to sweep the gaseous CF₃CHO generated in one assembly through a reflux condenser packed with dry CaCl₂ into the reaction flask containing a suspension of 1-triphenylphosphoranylidene-2-propanone in anhydrous ether. The title product was purified by fractional distillation. The fraction collected at 93°C
(lit.\textsuperscript{20} b.p. 89-91°C) was used for the next step without any attempt at separating the geometric isomers: \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \( \delta \) 2.37 (s, 3H, CH\text{3}), 6.59-6.66 (m, 2H, olefin CH); \textsuperscript{13}C-NMR (CDCl\textsubscript{3}) \( \delta \) 28.43 (CH\text{3}), 122.46 (q, \textsuperscript{1}J\text{C-F} = 270 Hz, CF\text{3}), 129.2 (q, \textsuperscript{2}J\text{C-F} = 35 Hz, C\text{4}), 134.98 (q, \textsuperscript{3}J\text{C-F} = 5.8 Hz, C\text{3}), 196.04 (C=O); \textsuperscript{19}F-NMR (CDCl\textsubscript{3}) \( \delta \) -65.95 ppm (d, \textsuperscript{1}J\text{H-F} = 5.3 Hz); HRMS (40 eV) calc'd for C\textsubscript{5}H\textsubscript{5}F\textsubscript{3}O m/z 138.089, found 139.0325 (M+1, 15%), 138.0297 (M\textsuperscript{+}, 12%), 123.0071 (M-15, 100%).

5-Nitro-4-(trifluoromethyl)-2-hexanone (2) was prepared from trifluoropentenone (1) in analogy to its known reactivity with a variety of nucleophiles.\textsuperscript{20} To a solution of 1 (14 g, 100 mmol) in 100 mL CH\textsubscript{3}CH\textsubscript{2}NO\textsubscript{2} was added 30 mL of aqueous Na\textsubscript{2}CO\textsubscript{3} (1.6 g, 15 mmol) solution, and the resulting mixture was heated at reflux for 6 h under N\textsubscript{2}. The reaction mixture was then cooled, and was partitioned between water and CHCl\textsubscript{3}. The organic extracts were washed with water, dried (Na\textsubscript{2}SO\textsubscript{4}), and evaporated to afford an oil. NMR (\textsuperscript{1}H, \textsuperscript{13}C, and \textsuperscript{19}F) analysis of this oil showed the presence of the title compound in the form of a 1:1 mixture of diastereomers, which was used for the next step without separation or further purification. An analytical sample for spectral characterization was obtained by silica gel flash chromatography (CH\textsubscript{2}Cl\textsubscript{2}-EtOAc 1:1 eluant): \textsuperscript{1}H-NMR
(diastereomeric mixture in CDCl₃) δ 1.64 and 1.67 (2d, 3H, CH₃), 2.31 (s, 3H, CH₃CO), 2.87 and 2.90 (2d, 2H, CH₂), 3.65-3.9 (2m, 1H, CF₃CH), 4.9 (m, 1H, CH); ¹³C-NMR (CDCl₃) δ 15.57 and 16.27 (C₆), 29.86 and 29.89 (C₁), 37.45 and 38.1 (C₃), 41.50 and 42.25 (q each, ²JC₁-F = 25 Hz, C₄), 79.75 and 80.12 (C₅), 126.01 and 126.41 (q each, ¹JC₂-F = 280 Hz, CF₃), 202.74 and 203.09 (C₂); ¹⁹F-NMR (CDCl₃) δ -68.91 ppm (d, JH-F = 8.9 Hz), and -69.42 ppm (d, JH-F = 7.6 Hz); HRMS (40eV) calcd for C₇H₁₀F₃NO₃ m/z 213.0661, found 214.0684 (M+). 

3-(Trifluoromethyl)-2,5-hexanedione (3) was prepared using a general procedure ("Nef" reaction)²¹ for oxidation of nitroalkanes to carbonyl compounds. Thus nitroketone 2 (10.7 g, 50 mmol) was dissolved in 350 mL MeOH and the solution was cooled to 5°C. A freshly prepared solution of KOH (3.08 g, 55 mmol) in 500 mL MeOH was then added dropwise over 2 h with vigorous stirring under N₂, while maintaining the reaction temperature at < 5°C. After the addition was complete, a solution of KN₃O₄ (6.32 g, 40 mmol) and MgSO₄ (6.98 g, 58 mmol) in 700 mL water was added dropwise, while maintaining the reaction temperature at 0-2°C. The brown solution was then stirred for 3 h at 0-5°C and then filtered through Celite. The filtrate was transferred to a separatory funnel and shaken with CHCl₃.
The CHCl₃ extracts were washed with brine, dried (Na₂SO₄), and evaporated to obtain an oily residue which was distilled under reduced pressure to afford 6.3 g of the title compound (isolated yield 69%): b.p. 53-55°C/7 mm Hg; ¹H-NMR (CDCl₃) δ 2.20 and 2.42 (2s, 3H each, CH₃), 2.76 (dd, 1H, J = 2.8 and 18.4 Hz), 3.32 (dd, 1H, J = 11.0 and 18.4 Hz), 3.81 (m, 1H); ¹³C-NMR (CDCl₃) δ 29.30, 31.39, 39.76, 50.34 (q, ²J_C-F = 25 Hz, C₃), 124.69 (q, ¹J_C-F = 279 Hz, CF₃), 200.69 and 204.55; ¹⁹F-NMR (CDCl₃) δ -67.12 ppm (d, J_H-F = 8.6 Hz); HRMS (40eV) calcd for C₇H₉F₃O₂ m/z 182.0555, found 182.0552 (M⁺, 11%), 167.0355 (M-15, 33%), 147.0550 (M-35, 100%).

4-Oxo-1-pentanoic acid phenylmethyl ester (4): A mixture of levulinic acid (116 g, 1 mol), benzyl chloride (126.5 g, 1 mol) and Et₃N (201 g, 2 mol) in anhydrous toluene (250 mL) was heated, under N₂, at reflux for 10 h. The mixture was then cooled, and the solid (Et₃N.HCl) that appeared was removed by filtration. The organic filtrate was washed with water, and was evaporated to afford 152 g (74% yield) of the title ester after purification by fractional vacuum distillation: b.p. 90°C/0.2 mm Hg; ¹H-NMR (CDCl₃) δ 2.13 (s, 3H, CH₃), 2.57 and 2.71 (2t, 2H each, J = 7.6 Hz, CH₂), 5.07 (s, 2H, PhCH₂), 7.26-7.34 (m, 5H, Ph-H).

2-Methyl-1,3-dioxolane-2-propanoic acid phenyl-
methyl ester (5). A mixture of 4 (69.3 g, 0.34 mol) and ethylene glycol (22.9 g, 0.37 mol) in benzene, containing a catalytic amount of p-toluenesulfonic acid (300 mg), was heated under reflux, with azeotropic removal of water using a Dean-Stark trap. After the calculated amount of water (ca 6 mL) had collected, the reaction mixture was cooled, and was washed successively with 10% aqueous NaHCO₃ and brine. The organic layer was dried (Na₂SO₄) and then evaporated to obtain the title compound, which was purified by fractional distillation under reduced pressure (68 g, 80% isolated yield): b.p. 132°C/0.25 mm Hg; ¹H-NMR (CDCl₃) δ 1.31 (s, 3H, CH₃), 2.04 and 2.45 (2t, 2H each, J = 7.6 Hz, CH₂'s), 3.86-3.91 (m, 4H, 0CH₂CH₂O), 5.11 (s, 2H, PhCH₂), 7.32-7.36 (m, 5H, Ph-H).

2-Methyl-1,3-dioxolane-2-(2-trifluoroacetyl)propanoic acid phenylmethyl ester (6). An assembly consisting of a flame-dried three-neck r.b. flask equipped with a reflux condenser, an addition funnel and a N₂ inlet, was set up. A suspension of oil-free NaH (19.7 g, 0.82 mol) in 200 mL anhydrous benzene was transferred to the flask under N₂ and the addition funnel was charged with a 200 mL benzene solution containing 5 (51.35 g, 0.21 mol) and benzyl trifluoroacetate²⁸ (50.25 g, 0.25 mol). A catalytic amount of benzyl alcohol (0.5 mL) was added to
the flask, and the solution in the addition funnel was added dropwise over 2 h at 10°C. After the addition was complete, the reaction mixture was stirred under N₂ for 3 h at 80°C. The mixture was then cooled, and unreacted NaH was destroyed by cautiously pouring the reaction solution into ice. The organic layer was separated and the aqueous layer was extracted with ether. The organic extracts were combined, washed with brine, and evaporated to afford 38 g (crude yield 52%) of a yellow oil which was distilled under reduced pressure to afford the title compound: b.p. 140-142°C/0.2 mm Hg; ¹H-NMR (CDCl₃) δ 1.32 (s, 3H, CH₃), 2.49 (dd, 1H, J = 4.0 and 14.0 Hz), 2.67 (dd, 1H, J = 10.0 and 14.0 Hz), 3.82-3.92 (m, 4H, OCH₂CH₂O), 4.18 (dd, 1H, J = 4.0 and 10.0 Hz), 5.15-5.18 (m, 2H, PhCH₂), 7.29-7.40 (m, 5H, Ph-H).

4-Oxo-2-(trifluoroacetyl)-1-pentanoic acid phenylmethyl ester (7) was prepared by deprotection of the ketal group of 6. Thus a solution of 6 (16.75 g, 48 mmol) in 80 mL of an acetone-water (2:1) solvent mixture, containing 100 mg p-toluenesulfonic acid, was heated under reflux for 10 h. The mixture was cooled and evaporated to dryness to afford an oily residue, which was partitioned between water and CHCl₃. The CHCl₃ extracts were dried (Na₂SO₄) and evaporated to afford 13 g (89%) of the crude product, which was used
for the next step without purification. An analytical sample for spectral characterization was obtained by silica gel flash chromatography (EtOAc-Hexane 1:1 eluant): $^1$H-NMR (CDCl$_3$) $\delta$ 2.14 (s, 3H, CH$_3$), 3.11 (dd, 1H, J = 4.2 and 16 Hz), 3.35 (dd, 1H, J = 10.8 and 16 Hz), 4.27 (dd, 1H, J = 4.2 and 10.8 Hz, CH), 5.10 (s, 2H, CH$_2$), 7.30-7.38 (m, 5H, Ph-H).

1,1,1-Trifluoro-2,5-hexanodione (8). To a solution of compound 7 (13 g, 43 mmol) in 50 mL EtOAc was added 10% Pd/C (1.8 g), and H$_2$ was bubbled through the mixture for 8 h at 65°C. The progress of the reaction was monitored by TLC examination on aliquots drawn periodically (conversion of $R_f$ = 0.6 to $R_f$ = 0.4, CH$_2$Cl$_2$ eluant). At the completion of reaction, the mixture was filtered through Celite to remove the catalyst, and the filtrate concentrated to ca 10 mL by evaporation at 25°C. The residue obtained was distilled under reduced pressure, and the fraction collected at 48-50°C (5 mm Hg) corresponded to the title compound as revealed by $^1$H and $^{19}$F NMR (isolated yield 4.3 g, 60%): $^1$H-NMR (CDCl$_3$) $\delta$ 2.22 (s, 3H, CH$_3$), 2.89 and 3.02 (2t, 2H each, J = 7 Hz, CH$_2$); $^{19}$F-NMR (CDCl$_3$) $\delta$ -80 ppm (s).

3,3,3-Trifluoro-2-butanone (9) was obtained by a reported electrochemical procedure of Miller.$^{22}$

3-Methyl-2,5-hexanodione was from a previous study
in our lab, and was distilled before use: \(^{13}\text{C}-\text{NMR (CDCl}_3\) \(\delta 16.11\) (C\(_3\)-CH\(_3\)), 28.11 and 29.56 (C\(_1\) and C\(_6\)), 41.34 (C\(_4\)), 45.95 (C\(_3\)), 206.92 and 211.05 (C\(_2\) and C\(_5\)).

**General procedure for preparation of pyrroles from diketones:** Pyrrole derivatives were prepared by the following reported general procedure\(^{39}\) for condensation of \(\gamma\)-diketones with primary alkylamines. Typically, a solution of the appropriate diketone (2 mmol) in 10 mL EtOH was mixed with an ethanolic solution of the required amine (2.1 mmol in 10 mL), and the resulting mixture was stirred under N\(_2\) at 50°C for 10 h. The solvent was then evaporated, and the residue obtained was partitioned between water and ether. The ether extracts were washed several times with water, dried (Na\(_2\)SO\(_4\)), and evaporated to afford the desired pyrrole product, which was dissolved in 50 mL EtOAc, and passed through a short column packed with alumina. Evaporation of EtOAc then provided the pure pyrrole products in the form of colorless oils. *Storage of these products, even under N\(_2\) and low temperatures, led to darkening, and thus the pyrroles were prepared immediately prior to use for the chemical and electrochemical oxidation studies.*

\(^N\)-(2-Methylpropyl)-2,5-dimethylpyrrole (10a). \(^1\text{H}-\text{NMR (CDCl}_3\) \(\delta 0.90\) (d, 6H, J = 6.8 Hz, CH\(_3\)), 1.97 (m, 1H, CH), 2.20 (s, 6H, CH\(_3\)), 3.52 (d, 2H, J = 7.6 Hz,
CH\textsubscript{2}), 5.77 (s, 2H, pyrrole CH); \textsuperscript{13}C-NMR (CDCl\textsubscript{3}) \(\delta\) 12.20, 19.46, 29.32, 50.32 (N-CH\textsubscript{2}), 104.52 (pyrrole C\textsubscript{β}), 127.01 (pyrrole C\textsubscript{α}).

N-(4-Pyridinylmethyl)-2,5-dimethylpyrrole (10b). \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) 2.21 (s, 6H, CH\textsubscript{3}), 5.00 (s, 2H, CH\textsubscript{2}), 5.89 (s, 2H, pyrrole CH), 6.80 and 8.53 (2d, 2H each, J = 6.2 Hz, C\textsubscript{3}/C\textsubscript{5}-H and C\textsubscript{2}/C\textsubscript{6}-H); \textsuperscript{13}C-NMR (CDCl\textsubscript{3}) \(\delta\) 12.16 (CH\textsubscript{3}), 45.60 (N-CH\textsubscript{2}), 105.78 (pyrrole C\textsubscript{β}), 120.58 (pyridine C\textsubscript{3}/C\textsubscript{5}), 127.57 (pyrrole C\textsubscript{α}), 147.55 (pyridine C\textsubscript{4}), 149.99 (pyridine C\textsubscript{2}/C\textsubscript{6}).

N-(2,2-Dimethylpropyl)-2,5-dimethylpyrrole (10c). \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) 0.97 (s, 9H, CH\textsubscript{3}), 2.23 (s, 6H, CH\textsubscript{3}), 3.75 (s, 2H, CH\textsubscript{2}), 5.80 (s, 2H, pyrrole CH).

N-(2-Methylpropyl)-2,3,5-trimethylpyrrole (11a). \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) 0.90 (d, 6H, J = 6.8 Hz, CH\textsubscript{3}), 1.98 and 1.99 (m and s, 4H, CH and CH\textsubscript{3}), 2.10 and 2.17 (2s, 3H each, CH\textsubscript{3}), 3.49 (d, 2H, J = 7.6 Hz, CH\textsubscript{2}), 5.80 (s, 1H, pyrrole CH); \textsuperscript{13}C-NMR (CDCl\textsubscript{3}) \(\delta\) 9.98, 11.06, 12.39, 19.95, 29.88, 49.94 (N-CH\textsubscript{2}), 106.91 (pyrrole C\textsubscript{4}), 112.80 (pyrrole C\textsubscript{3}), 123.34 (pyrrole C\textsubscript{2}), 126.08 (pyrrole C\textsubscript{5}).

N-(4-Pyridinylmethyl)-2,3,5-trimethylpyrrole (11b). \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) 2.01 (s, 3H, CH\textsubscript{3}), 2.02 (s, 3H, CH\textsubscript{3}), 2.09 (s, 3H, CH\textsubscript{3}), 4.96 (s, 2H, CH\textsubscript{2}), 5.78 (s, 1H, pyrrole CH), 6.80 (d, 2H, J = 6.2 Hz, C\textsubscript{3}/C\textsubscript{5}-H), 8.52 (d, 2H, J = 6.2 Hz, C\textsubscript{2}/C\textsubscript{6}-H); \textsuperscript{13}C-NMR (CDCl\textsubscript{3}) \(\delta\) 9.53, 11.05,
11.81, 45.71 (N-CH₂), 107.75 (pyrrole C₄), 113.81 (pyrrole C₃), 120.63 (pyridine C₃/C₅), 123.36 (pyrrole C₂), 126.22 (pyrrole C₅), 147.89 (pyridine C₄), 149.89 (pyridine C₂/C₆).

**N-(2,2-Dimethylpropyl)-2,3,5-trimethylpyrrole**

(11c). ¹H-NMR (CDCl₃) δ 0.98 (s, 9H, CH₃), 2.10, 2.12, and 2.19 (3s, 3H each, CH₃), 3.58 (s, 2H, CH₂), 5.84 (s, 1H, pyrrole CH).

**N-(2-Methylpropyl)-2,3,4,5-tetramethylpyrrole**

(12a). ¹H-NMR (CDCl₃) δ 0.90 (d, 6H, J = 6.8 Hz, CH₃), 1.94 and 2.11 (2s, 6H each, CH₃), 1.99 (m, 1H, CH), 3.49 (d, 2H, J = 7.6 Hz, CH₂); ¹³C-NMR (CDCl₃) δ 88.4, 9.59, 19.61, 29.64, 50.43 (N-CH₂), 112.08 (pyrrole C₇), 121.85 (pyrrole C₈).

**N-(4-Pyridinylmethyl)-2,3,4,5-tetramethylpyrrole**

(12b). ¹H-NMR (CDCl₃) δ 1.97 and 2.02 (2s, 6H each, CH₃), 4.96 (s, 2H, CH₂), 6.80 (d, 2H, J = 6.2 Hz, C₃/C₅-H), 8.51 (d, 2H, J = 6.2 Hz, C₂/C₆-H); ¹³C-NMR (CDCl₃) δ 9.30 and 9.54 (CH₃), 45.71 (N-CH₂), 113.62 (pyrrole C₇), 120.69 (pyridine C₃/C₅), 122.26 (pyrrole C₈), 148.30 (pyridine C₄), 149.82 (pyridine C₂/C₆).

**N-(2,2-Dimethylpropyl)-2,3,4,5-tetramethylpyrrole**

(12c). ¹H-NMR (CDCl₃) δ 0.99 (s, 9H, CH₃), 1.99 and 2.17 (2s, 6H each, CH₃), 3.6 (s, 2H, CH₂).

**N-(2-Methylpropyl)-2,5-dimethyl-3-(trifluorometh-**
(13a). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 0.89 (d, 6H, J = 6.8 Hz, CH\(_3\)), 1.95 (m, 1H, CH), 2.16 and 2.25 (2s, 3H each, CH\(_3\)), 3.52 (d, 2H, J = 7.6 Hz, CH\(_2\)), 5.98 (s, 1H, pyrrole CH); \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\) 10.38 and 12.07 (CH\(_3\)), 19.50 (i-Pr CH\(_3\)), 29.37 (CH), 50.57 (CH\(_2\)), 103.62 (pyrrole C\(_4\)), 105.17 (q, \(^2\)J\(_{C-F}\) = 35.6 Hz, pyrrole C\(_3\)), 121.59 (pyrrole C\(_2\)), 127.42 (pyrrole C\(_5\)), 129.85 (q, \(^1\)J\(_{C-F}\) = 264.8 Hz, CF\(_3\)); \(^1\)F-NMR (CDCl\(_3\)) \(\delta\) -55.75 ppm (s).

N-(4-Pyridinylmethyl)-2,5-dimethyl-3-(trifluoromethyl)pyrrole (13b). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 2.10 and 2.20 (2s, 3H each, CH\(_3\)), 5.02 (s, 2H, CH\(_2\)), 6.12 (s, 1H, pyrrole CH), 6.82 and 8.54 (2d, 2H each, J = 6.2 Hz each, C\(_3\)/C\(_5\)-H and C\(_2\)/C\(_6\)-H); \(^1\)F-NMR (CDCl\(_3\)) \(\delta\) -54.6 ppm (s).

N-(2,2-Dimethylpropyl)-2,5-dimethyl-3-(trifluoromethyl)pyrrole (13c). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 0.98 (s, 9H, CH\(_3\)), 2.21 and 2.27 (2s, 3H each, CH\(_3\)), 3.64 (s, 2H, N-CH\(_2\)), 5.94 (s, 1H, pyrrole CH).

\(^1\)H NMR Kinetics. General procedures. All kinetic runs were performed under N\(_2\) in thermostatted probe maintained at 25 ± 0.1°C. Standard stock solutions (0.2 M) of 4-(aminomethyl)pyridine, isobutylamine and neopenetylamine were prepared in N\(_2\)-flushed CDCl\(_3\) and stored under N\(_2\). The stock solutions (0.2 M) of \(\gamma\)-diketones
were similarly prepared in N₂-flushed CDCl₃. The required amounts of amine and γ-diketone solutions were pre-equilibrated to 25°C, mixed, and immediately transferred to a 5-mm NMR tube. Data was accumulated at regular intervals until the integration ratios showed a > 50% conversion of the starting amine to the pyrrole product. Acquisition parameters: number scans, 64; acquisition time, 1 s/scan; relaxation delay, 6 s/scan. For estimating the half-lives, the plots of integration ratios of N-CH₂ signals (amine vs pyrrole) vs time were constructed; the half-life corresponded to the time when this ratio was ~ 1.

¹³C NMR experiments were done at 22°C under N₂ in 10 mm NMR tubes. In a typical run, 3 mmol of γ-diketone was dissolved in 0.45 mL of N₂ purged CDCl₃ and equilibrated to 22°C. A CDCl₃ solution (0.45 mL) containing the desired amine (3 mmol) was then added and the spectra were recorded at regular time intervals. At the completion of reactions, indicated by the disappearance of the signals corresponding to the starting materials, aliquots were analyzed for pyrrole products by their chromatographic and ¹H NMR characteristics. In the case of TFM-HD additional confirmation was afforded by ¹⁹F NMR.

¹⁹F NMR experiments were performed at 25°C under
N₂. A 0.2 mL CDCl₃ solution containing 0.02 mmol of TFM-HD was mixed with a 0.2 mL solution of 0.02 mmol isobutylamine in a 5 mm NMR tube and the ¹⁹F NMR spectra were recorded at regular time intervals. PhCF₃ (-63.23 ppm, upfield of CFCl₃) was used as the internal reference. The reaction was followed until complete disappearance of starting diketone. The reaction of trifluorobutanone (9) with isobutylamine was followed similarly under the same conditions. The experiment with excess iso-butylamine (0.12 mmol) was done under identical conditions and acquisition parameters.

Electrochemical measurements. Cyclic voltammetric measurements were made with a PAR Model 173 potentiostat/galvanostat and a PAR Model 175 programmer attached to a PAR Model EG&G XY recorder. The measurements were made in a glass cell with three compartments for the working electrode, a Pt foil counter electrode, and SCE reference electrode. The working electrode was a glassy carbon disk (Pine Instrument Co.) with an area of 0.46 cm². The glass cell was initially degreased in 6M KOH for a day followed by thorough washings with ultrapure water (reverse osmosis + distillation). The cell was then filled with a 1:1 volume mixture of conc. H₂SO₄ and HNO₃ for several days. The cell was drained and repeatedly rinsed with deionized water. Before each electro-
chemical run, the cell was steamed for 1-2 h and then used directly after cooling. The working electrode was repeatedly mechanically polished with α-Al₂O₃ before every use. Sample solutions consisted of freshly prepared 2 mM pyrrole (10a-13a) in CH₃CN containing 0.1 M tetra-n-butylammonium perchlorate, and the solutions were purged with Ar for 30 min before each run. Voltamograms were recorded at a scan rate of 100 mV/s.

Kinetic measurements on oxidation of pyrroles (10c-13c) by K₂S₂O₈. Kinetic experiments were carried out in 1 cm quartz cells in the thermostatted cell compartment of a Perkin-Elmer Lambda 3B spectrophotometer. The temperature was maintained at 30°C. The kinetic runs were made under pseudo-first order conditions with a large excess of the oxidant. The reactions were initiated by addition of freshly prepared MeOH solutions of pyrrole derivatives (10-13c) to thermally equilibrated aqueous K₂S₂O₈ solutions containing constant buffer concentrations. The absorption spectra were then recorded at regular time intervals in the appropriate wavelength ranges. The rank order for the relative rates of oxidation was obtained under the standard conditions of: solvent system 1:2 v/v MeOH-H₂O; [pyrrole] = 2 x 10⁻⁴ M; [S₂O₈²⁻] = 2 x 10⁻² M (in DD water); pH 6.85 buffer: 6.66 x 10⁻² M [KH₂PO₄]; 30°C.
The reaction in each instance was monitored by the increase in absorbance at the following characteristic $\lambda_{\text{max}}$ values: 2,5-dimethylpyrrole (10c), 467 nm; 2,3,5-trimethylpyrrole (11c), 490 nm; 2,3,4,5-tetramethylpyrrole (12c), 507 nm; and 2,5-dimethyl-3-(trifluoromethyl)pyrrole (13c), 292 nm.
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CHAPTER III.

Model Studies on
Mechanism-Based Inhibition of Copper Amine Oxidases
by
β-Aminopropionitrile (BAPN) and Cyclopropylamines.
**INTRODUCTION.**

Copper amine oxidases (Cu-AO) are enzymes that are widely distributed in microorganisms, plants, and mammals.\(^1\) The common feature of these enzymes is the overall stoichiometry for oxidative deamination of primary amines (eq.1).\(^1,2\) All these proteins are highly reactive towards carbonyl reagents such as semicarbazide and phenylhydrazine, and it was clear early on that these enzymes utilized a pyridoxal-like transamination mechanism for conversion of amines to aldehydes. However, pyridoxal phosphate (PLP) itself appeared not to be the actual cofactor which was known to be nondissociable from the enzymes.\(^2\)

\[
\text{RCH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \quad \rightarrow \quad \text{RCH}=\text{O} + \text{NH}_3 + \text{H}_2\text{O}_2
\]

The enzyme lysyl oxidase\(^4-7\) belongs to the general class of Cu-AO's and has a crucial role in mediating the formation of cross-links which stabilize the connective tissue proteins collagen and elastin.\(^6\) These cross-links arise from the oxidative deamination of lysine residues to peptidyl α-amino adipic-6-semialdehyde, which is further involved in aldol condensation reactions and/or Schiff base adduct formation.\(^6\)
Figure 1. Structures of various compounds described in text.
A toxic condition called lathyrisrn is known to result from ingestion, by man (mainly in India) and animals, of various species of the lathyrus genus of leguminous plants. Osteolathyrisrn is a connective tissue disorder attributed to deficient levels of the lysyl oxidase-derived crosslinkages, which results in increased fragility of all connective tissues when lathyrus seeds are ingested by growing animals.

Nearly all the copper-dependent "semicarbazide-sensitive" amine oxidases, including lysyl oxidase, have now been proposed to contain PQQ(4,5-dihydro-4,5-dioxo-1H-pyrrolo-[2,3-f]quinoline-2,7,9-tricarboxylic acid) (see Figure 1 for structure) as the covalently-bound organic prosthetic group. The presence of PQQ rationalizes the sensitivity of the enzymes to carbonyl reagents like hydrazines, hydrazides etc. PQQ contains a carbonyl group (at C-5) in a position electronically equivalent to that in PLP, and has itself been shown to mediate pyridoxal-like transamination.

Certain prokaryotic alcohol dehydrogenases are also known to contain PQQ as the organic cofactor. The existing chemical literature on PQQ and other related model quinones indicates an unusually high electrophilicity of the C-5 carbonyl group, and the formation of covalent adducts at this position has been consistently
considered in the mechanistic discussions of PQQ-mediated substrate oxidations.\textsuperscript{13} Regarding this point, a rather simple mechanism can be envisioned for the oxidation of alcohols by PQQ.\textsuperscript{13} As depicted in eq. 2, this involves the initial formation of a hemiketal followed by a 1,5-prototropic shift (retro-ene fragmentation) to yield directly the reduced quinol form of PQQ.

\begin{align*}
\text{RCH}_2\text{OH} & \quad \xrightarrow{\text{RCH}_2\text{OH}} \quad \text{RCH}_2\text{OH} \quad \text{(2)} \\
\text{RCH}_2\text{NH}_2 & \quad \xrightarrow{\text{H}_2\text{O}} \quad \text{RCH}_2\text{H} \quad \text{(3)} \\
\text{RCH}_2\text{H} & \quad \xrightarrow{\text{H}_2\text{O}} \quad \text{RCH}_2\text{NH}_2 \quad \text{(4)}
\end{align*}
An analogous mechanism for amine oxidation has been proposed,\textsuperscript{16} which is consistent with the observation of the formation of quinol under anaerobic conditions,\textsuperscript{15,16} eq 3. The fundamental distinction between these mechanisms involving hemiketal and carbinolamine intermediates arises from the potential for the latter to dehydrate further to generate stable Schiff base or imine intermediates. In this case subsequent 1,5-prototropic shift followed by hydrolysis gives the aminophenol form of the reduced PQQ (eq 4).\textsuperscript{14} Although this pathway was earlier reported as only a minor one,\textsuperscript{115,16} more recent detailed investigations with model compounds demonstrate that this indeed is the major reaction at physiologically relevant pH values.\textsuperscript{17}

As an alternative to the formation of covalent adducts, one can consider a direct 1-electron transfer or a hydride transfer from the substrate to PQQ.\textsuperscript{16} The former possibility can, however, be ruled out on the basis of a relatively low oxidation potential of PQQ ($E_{1/2} = 90 \text{ mV}$).\textsuperscript{18} It has also been observed that the order of reactivity of amines with PQQ is primary $>$ secondary $>$ tertiary, whilst either electron- or hydride-transfer mechanisms would predict an opposite rank order for amines based on electronic effects.\textsuperscript{16}

Although the exact mechanistic details involving
each step of the catalytic cycle of Cu-AO's are still awaited, a currently accepted overall mechanism consistent with the stoichiometry and the known "ping-pong" order of substrate/product binding is summarized in Figure 2. Thus the net reaction is composed of two half-reactions catalyzed by the enzyme: (i) first, the condensation of the amine with PQQ at the more reactive C-5 carbonyl group provides activation of $\text{C}_\alpha-\text{H}$, and the initially formed Schiff base is converted to the tautomeric Schiff base, which then hydrolyzes to aldehyde product and the reductively aminated form of PQQ, followed by (ii) reoxidation of the PQQ-derived aminophenol by molecular oxygen with a probable involvement of copper to yield PQQ, ammonia and hydrogen peroxide.
Figure 2. Catalytic cycle for PQQ-mediated oxidative deamination of primary amines.
The chemical agent in *Lathyrus* that is responsible for the development of osteolathyrism is \( \beta \)-aminopropionitrile (BAPN).\(^4\) The lathyrogenic activity of BAPN has been attributed to its ability to cause irreversible inhibition of lysyl oxidase.\(^4\)

Based on the normal transamination of primary amines by PQQ, one can propose the formation of cyanoacetalddehyde as the expected product of oxidative deamination. Indeed cyanoacetic acid is a major metabolite of BAPN, and is presumed to result from the subsequent oxidation of cyanoacetalddehyde.\(^8,\,25\) It was originally proposed that the generation of this reactive electrophile i.e. cyanoacetalddehyde, might be responsible for BAPN toxicity.\(^20\) However BAPN-induced lathyrisim is now known to arise from a direct action of BAPN as a mechanism-based inhibitor of lysyl oxidase.\(^21,\,22\) In vitro, irreversible inhibition of lysyl oxidase by BAPN at micromolar concentrations shows first-order time dependence and other characteristics consistent with classifying the agent as a suicide inactivator.\(^23\) The mechanism for BAPN-induced inactivation of lysyl oxidase has not been ascertained, however. In an attempt to probe the structural requirements for the observed inactivation of lysyl oxidase, Kagan and co-workers found that 2-nitroethylamine, 2-bromoethylamine and
ethylenediamine also inactivate this enzyme.\textsuperscript{24,50} Consistent with the universal presence of PQQ as the cofactor in Cu-AO's, BAPN inhibits other enzymes besides lysyl oxidase, albeit at higher concentrations. Interestingly in some of these cases (plasma benzylamine oxidase, rat aorta amine oxidase, and bovine aorta benzylamine oxidase) the inhibition appears to be reversible.\textsuperscript{25,26}

![Figure 3. Kagan's Ketenimine Proposal for Suicide Inactivation of Lysyl Oxidase by BAPN (ref 29).](image-url)
An obvious possibility considered for BAPN toxicity was in regard to the known release of cyanide from organonitriles.\textsuperscript{27,28} This has been ruled out by an elegant double labeling experiment by Kagan and coworkers, which showed that both the cyano carbon and the ethylene carbons bind to the enzyme to equivalent extents.\textsuperscript{29} These workers tentatively proposed a ketenimine mechanism for the suicide inactivation (Figure 3), based on earlier proposals for the inhibition induced by aminoacetonitrile.\textsuperscript{30} However, these arguments were not convincing to us, and thus we have attempted to re-evaluate the mechanism by studying the reactivity of BAPN with model quinones, namely 3,5-di-\textit{t}-butyl-1,2-benzoquinone (DTBQ) and 4,7-phenanthroline-5,6-quinone (PHAN) (see structures in Figure 1).

DTBQ was used earlier by Corey as an unusually efficient catalyst for oxidative deamination of sec-alkyl primary amines to ketones.\textsuperscript{31} As noted by these workers, the reaction was not useful for generating aldehydes from unbranched primary amines owing to the formation of benzoxazoles. The main criterion in our choice of these models was the presence of an electronically-activated carbonyl group in a configuration which permits a facile isomerization of the initially formed Schiff base adduct, driven by aromatization to an N-
substituted aminophenol. The presence of t-butyl substituents in DTBQ avoids complications due to Michael addition of amine, and directs nucleophilic attack of amine at a single carbonyl group.

Our initial aim was to investigate the possibility that transamination on BAPN would be followed immediately by a second tautomerization to a resonance-stabilized cyanoenamine. Relative resistance of the latter to hydrolysis could then account for the observed inhibition of PQQ-containing enzymes. In the process of this investigation, we uncovered several mechanistic details and recorded observations that might be relevant to the mechanism of lathyrogenic action of BAPN.

In a related study, we have investigated the general reactivity of the model quinones towards cyclopropylamines. Cyclopropylamines have been used extensively by Silverman and co-workers for their ability to inhibit another class of amine oxidases namely the flavin-dependent mitochondrial enzyme.32,33 In these studies, Silverman observed the formation of ring-cleaved covalent adducts with the flavin or an active site amino-acid residue, and cited this as evidence for a direct one-electron-transfer mechanism for the oxidation of amines by this enzyme.33 Our results show that these strained cyclopropylamines undergo ring-cleavage reac-
tions with PQQ models involving heterolytic or concerted mechanisms.
RESULTS:

Reaction of DTBQ with primary amines under aqueous conditions:

The reaction of DTBQ with three different ethyl amines, including BAPN, under aerobic aqueous conditions (3:1 CH$_3$CN:H$_2$O solvent mixture at pH 8) led to the formation of two main products i.e. the substituted benzoxazole (4a-c) and di-t-butylcatechol (DTBQH$_2$). These results are best summarized in terms of reaction scheme shown in Figure 4. The formation of benzoxazole is consistent with the earlier Corey report on the oxidative deamination of unbranched primary amines by DTBQ. However, these authors did not provide a detailed mechanism of the reaction nor was the pathway leading to benzoxazole discussed. As shown in this scheme, the production of benzoxazole might occur by a multistep process involving initial imine (1) formation followed by a prototropic rearrangement to the corresponding aminophenol Schiff base 2, which upon intramolecular cyclization to 3 and subsequent oxidation leads to the product 4. Experimental evidence for the involvement of DTBQ in the terminal step is derived from the following observations: (i) essentially quantitative yields of benzoxazoles, based on the starting
amines, were obtained when the reaction was carried out with 2 equivalents of DTBQ, and (ii) the product distribution in cases of either 1 or 2 equivalents of DTBQ was the same whether an O₂ or N₂ atmosphere was maintained, ruling out the ability of O₂ to effect conversion to benzoxazole. Additional evidence was obtained by following the reaction of the DTBQ-derived aminophenol (DTB-AP) with phenylacetaldehyde by ¹H NMR (DMSO-d₆), which showed a build-up of dihydrobenzoxazole 3a, even under O₂. In this case, conversion to benzoxazole 4a occurred only on addition of DTBQ.
Figure 4. Reaction scheme for generation of benzoxazoles in oxidation of amines with DTBQ.
Reaction of DTBQ with amines under anhydrous conditions:

Owing to our primary interest in BAPN, we undertook a \(^1\)H NMR time study (in DMSO-\(d_6\)) of its reaction with DTBQ in order to follow the stepwise mechanism through the detection of any intermediates. For comparative purposes we also followed the reactions with PhCH\(_2\)CH\(_2\)NH\(_2\) and p-NO\(_2\)C\(_6\)H\(_4\)CH\(_2\)CH\(_2\)NH\(_2\) under similar conditions. In the latter two cases, the reactions in DMSO-\(d_6\) were observed to result in generation of benzoxazole, as had been observed in CH\(_3\)CN-H\(_2\)O.

In contrast to the phenethylamines, the reaction of BAPN followed an alternate pathway (Figure 5) in dry DMSO-\(d_6\) (vs. aqueous conditions described earlier). The \(^1\)H NMR spectrum exhibited a parallel attenuation of signals for the aromatic quinonoid protons and starting amine, accompanied by the transient appearance of a new set of triplet signals (at \(\delta\) 2.2 and 3.8) which we tentatively assign to the anti form (5b) of the initial imine. The final spectrum of the reaction mixture showed two products which were identified as the E and Z forms (7a and 7b) of the cyanoenamine adduct, confirmed through its independent generation by an alternate route (Figure 6).
Figure 5. Reaction scheme for β-cyanoamine generation from DTBO and BAPN.
Figure 6. Reaction scheme for independent generation of cyanoenamines 7a and 7b.
Since the benzoxazole (4c) product was not formed in the case of the BAPN reaction run in dry DMSO-\(d_6\), we carried out some additional experiments in an effort to elucidate the apparent solvent effect on the course of reaction. We observed that although the addition of excess (1 equivalent) DTBQ to the NMR sample containing the cyanoenamine adduct did not produce any spectral changes, addition of 1 equivalent DTBQ along with water (\(D_2O\)) afforded the same benzoxazole product as was formed under the mixed solvent (\(CH_3CN:H_2O\)) conditions. Thus there appears to be a crucial role of water in determining the reaction course, the reason for which is not clear. One possibility is that formation of the benzoxazole precursor 3c from either imine cyclization or internal Michael cyclization of the cyanoenamine 7 (Figure 5) occurs only in the presence of water. Alternatively, quinone oxidation of 3 to 4 may require the presence of water.

Reaction of BAPN with PHAN:

At this point we desired to extend the study to another model quinone, namely PHAN, which has previously been shown to be a more relevant model for PQQ,\(^{34}\) because of a similarity of redox potentials. Surprisingly, the reaction of BAPN with PHAN, unlike that with DTBQ, under both aqueous and anhydrous (NMR) conditions fol-
ollowed the cyanoenamine pathway (Figure 7). In the NMR experiment (DMSO-$d_6$), there was observed an initial generation of the syn and anti imines (9a and 9b) corresponding to two sets of triplet peaks ($\delta$ 2.81 and 4.60; and $\delta$ 3.06 and 4.09). Unambiguous assignments of these signals could not be made due to the short lifetimes of these intermediates. Nevertheless, further monitoring showed a preferential loss of the former set of signals followed by the appearance of an isomeric mixture of cyanoenamine adducts. The latter are characterized by diagnostic upfield vinyl signals $\alpha$ to the cyano substituent (two doublets at 4.43 and 4.93 with characteristic coupling constants of 8.4 and 13.6 Hz for E and Z forms, 10a and 10b).

Carrying out the reaction in CH$_3$CN-H$_2$O also led to the cyanoenamines (10a and 10b) under non-acidic work-up conditions, along with 3-(2-cyanoethylamino)propenenitrile (dehydro-IDPN), which was identified by comparison to an authentic specimen$^{35}$ (characteristic $^1$H-NMR vinyl resonances at ca 3.9 and 6.8 $\delta$). Dehydro-IDPN apparently arises from a trans-enamination reaction between the cyanoenamine adduct 10 and the starting amine (BAPN).$^{35}$
Figure 7. Scheme for the reaction of PHAN with BAPN.
Reaction of cyclopropylamines with DTBQ:

We focused on reactions of DTBQ with two cyclopropylamines, namely cyclopropylamine (CPA) and 1-phenylcyclopropylamine (1-PCPA). The reaction of DTBQ with CPA followed by \(^1\text{H NMR}\) showed that the characteristic \(^1\text{H multiplet}\) for the free amine at 2.34 \(\delta\) and quinone signals (\(^1\text{H doublets}\) at 6.27 and 7.11 \(\delta\)) are replaced by a new \(^1\text{H multiplet}\) at 3.72 \(\delta\) and new \(^1\text{H doublets}\) at 6.92 and 7.20 \(\delta\). The new signals were then gradually replaced by other multiple complex patterns which could not be ascribed definitively to any obvious structures. The initial NMR spectral changes were attributed to the formation of the initial orthoquinoneimine intermediate (see structures in Figure 8), presumably the less crowded \(\text{anti} (E)\) isomer. In the reactions of DTBQ with simple primary amines, such intermediates have not been observed, and are expected to have very short lifetimes, due to their rapid tautomerization (transamination). In the case of CPA, however, such isomerization is disfavored on account of geometrical strain, rendering the quinoneimine metastable. Multiple products were obtained at the completion of the reaction of DTBQ with CPA, the structures of which have yet to be fully characterized.
(a) α-quinoneimines from DTBQ and CPA

syn isomer

anti isomer

(b) Two products formed in the reaction of DTBQ and 1-PCPA

14

7,9-Di-t-butyl-4-phenyl-4,5-dehydro-1,5-benzoazepine

15

2-Phenyl-7,9-di-t-butyl-1-azaspiro[4.5]deca-1,7,9-triene-6-one

Figure 8. Structures of products formed in the reactions of DTBQ with (a) CPA; (b) 1-PCPA.
In the reaction of DTBQ with 1-PCPA under aqueous conditions two products formed, which were separated by chromatography and spectrosopically identified as benzoxazepine 14 and spirocyclic compound 15 (2-phenyl-7,9-di-t-butyl-1-azaspiro[4.5]deca-1,7,9-triene-6-one) (see structures in Figure 8 and 9). The $^1$H-NMR analysis of an aliquot from the completed reaction showed their presence in a ca 1:1 ratio based on the integration ratio of various characteristic signals. Compound 14 was characterized by an $A_2B_2$ pattern in the $^1$H-NMR, by an imine carbon (C=N) signal at 167.7 ppm (expected: 160-170 ppm$^{37}$) in the $^{13}$C-NMR, and by HRMS. As depicted in Figure 9, the reaction is proposed to proceed through initial formation of the quinoneimine which undergoes further ring-opening steps.

In order to examine the possible involvement of ring-cleaved imine intermediate 12b (Figure 9, pathway A/D) on the reaction pathway to the products 14 or 15, we carried out the reaction of DTBQ with 1-phenyl-2-propenamine and observed the formation of 14 as the sole product in essentially quantitative yields. Based on the chemistry discussed thus far, the generation of 14 can be rationalized by pathway E (Figure 9). Most importantly the spirocyclic compound (15) was not detected in this case, suggesting that 15 forms via a
pathway independent of 12b.
Figure 9. Mechanism for reaction of 1-PCPA with DTBQ.
DISCUSSION:

Mechanism of quinone-mediated oxidation of BAPN:

The lathyrogenic activity of a naturally occurring toxin BAPN is now known to arise from its ability to inactivate the enzyme lysyl oxidase which is crucial in providing cross-links in connective tissues.21,22 Kagan's studies with radioactive BAPN,29 demonstrated that the entire three-carbon unit of BAPN binds to the enzyme, and that enzyme inactivation is not caused by release of cyanide. The extent of covalent binding with BAPN is also reduced upon treatment with carbonyl reagents which suggests the involvement of a Schiff base complex on the inactivation pathway.

At the time of Kagan's study the presence of PQP as the cofactor in lysyl oxidase was not known, and a possible mechanism for the suicide inactivation was proposed (Figure 3) in analogy to mechanisms given for inactivation of pyridoxal enzymes by acetylenic amines and aminoacetonitrile.30,38 This hypothesis invoked a rearrangement of the initial imine to intermediate E1, followed by a base-catalyzed tautomerization to ketenimine E2, which was thought to acylate a nucleophilic group in or around the active site. However, the ketenimine proposal cannot be extended to the observed inactivation by other inhibitors, 2-nitroethylamine in
particular.24

Based on our model studies, we present evidence that the processing of BAPN by lysyl oxidase and other FQQ-containing enzymes would result in a hydrolytically stable cyanoenamine which can explain the BAPN-induced inhibition of these enzymes owing to covalent modification of the organic cofactor.

The mechanism of reaction between BAPN and model ortho-quinones is summarized for two cases in Figures 5 and 7. Our study is also the first report on the detection of o-quinoneimines in the oxidation of amines by c-quinones. The tautomerization, subsequent to the initial imine formation, depicted in Figures 5 and 7, appears to be an electrocyclic (1,5-sigmatropic) process on the basis of the sluggishness of the similar reaction for para-DTBQ39 and the fact that one isomer, presumably syn, of the initial quinoneimine is selectively removed. The anti isomer, which cannot undergo the electrocyclic process, apparently reacts more slowly, probably through initial isomerization to the syn quinoneimine.

The second tautomerization step involving an imine-enamine equilibrium in the reactions of BAPN with our model quinones is evidently due to the strongly electron withdrawing nature of the cyano group and is driven by the formation of resonance-stabilized cyanoe-
namines. Imine-enamine equilibration of this type is well known but the generation of β-cyanoenamines in the transamination of β-cyanoamines has not been previously reported.

Our results highlight a clear distinction between the outcome of transaminative oxidation using two o-quinones DTBQ and PHAN. The former, being a relatively strong oxidant ($E_0 = +700 \text{ mV}$), is more likely to participate in the oxidation reaction leading to the formation of stable benzoxazole products ($E_{1/2}$ for PHAN = -0.25 V vs. NHE). Particularly instructive is a comparison of the quinone reactivities with BAPN under aqueous conditions. The fact that the cyanoenamine is formed exclusively with apparently no formation of the cyanomethyl benzoxazole in the case of PHAN shows (i) that the dihydrobenzoxazole intermediate (benzoxazole precursor) is not formed in this case, or (ii) if formed, it is relatively resistant to oxidation, and is in equilibrium with the imine which is eventually converted completely to cyanoenamine. Certainly the reactivity of PHAN is more relevant from the point of view of the enzyme inhibition, since PHAN more closely shares the redox properties of PQQ, and benzoxazole formation is not biologically relevant anyway since its formation requires a participation of 2 moles of quinone. In fact,
the propensity of DTBQ to generate benzoxazoles limits its utility as a model for PQQ-enzymes.

The relative resistance of cyanoenamines towards hydrolysis is consistent with the cyanoenamine formation being a potential pathway of persistent enzyme inhibition. The product of hydrolysis would be cyanoacetaldehyde, a reactive electrophile, and it is also possible that covalent binding of this molecule to the enzyme contributes to the inactivation.\textsuperscript{25,26} Either hypothesis is consistent with the observation that BAPN is only a reversible inhibitor of other PQQ containing enzymes, where the release of cyanoacetaldehyde has been observed.\textsuperscript{25,26} Another interesting property of aliphatic cyanoenamines was reported in an earlier study from our lab, which demonstrated their rapid transamination (or transenamination) reactions (relative to hydrolysis) with amines at pH 7, converting the latter to the same cyanoenamine derivatives that would form from reaction with cyanoacetaldehyde.\textsuperscript{35} The formation of dehydro-IDPN from the PHAN-derived cyanoenamine adduct is consistent with this chemistry.

It should also be noted that if Kagan's proposal of ketenimine generation were operative, two products are likely to be formed in the reaction of BAPN with quinones (Figure 10). None of these products were detected
in our reactions, and we therefore rule out the possibility of ketenimine generation during processing of BAPN by o-quinone.

Figure 10. Reaction scheme for possible products from DTBO and BAPN, based on Kagan's ketenimine proposal.
In conclusion, we believe that the generation of cyanoenamine from BAPN is more relevant than Kagan's ketenimine proposal, which cannot directly account for the observed inhibition with 2-nitroethylamine. In contrast our enamine-based mechanism can readily be extended to the formation of nitroenamines in the case of the nitro inhibitor.

**Mechanisms of quinone-mediated oxidative ring-opening of cyclopropylamines:**

There is growing popularity for the use of ring-opening reactions of cyclopropanes as probes for enzyme mechanisms. In particular cyclopropylamines have been used in the past as inhibitors of flavin-containing amine oxidases. The observation of ring-opened products has been attributed to an initial one-electron transfer from the amines to the flavin cofactor. However, cyclopropylamine is also an inactivator of the PQQ-containing plasma amine oxidase. In this case the most likely scenario is not electron-transfer but Schiff base formation with PQQ. We considered that subsequent heterolytic reactions of the initial quinoneimine might lead to ring-opening and thus might explain the observed inactivation. This hypothesis was studied using DTBQ and two cyclopropylamines. In both cases we observed the generation of ring opened
adducts.

Our proposed mechanism for the formation of the products formed from 1-PCPA is shown in Figure 9. This hypothesis is based on a nitrenium-like ring-expansion\(^{44-46}\) to intermediate (13) for which we have no direct evidence other than the fact that it can explain the formation of both products obtained in the reaction. Thus, iminium intermediate 13 arising from cyclopropyl ring-cleavage, can then follow two separate intramolecular steps to give the observed products.

Another possible mechanism for the formation of 14 from 1-PCPA can be envisioned as involving a two-step fragmentation-Michael cyclization via intermediate 12a (Figure 9, Paths D/E). However, this pathway does not explain the formation of the spirocyclic compound, since the reaction of phenylallyl amine with DTBQ, which must proceed through 12b, leads exclusively to the benzoxazepine 14. We recognize that 14 and spirocyclic 15 could be arising from independent routes, but a related study by Dr. P.B.Kokil in our group on the corresponding series of amines without the Ph group (CPA vs. allylamine) has ruled out the possible involvement of a 12b-like intermediate in the CPA-derived products (since even the product corresponding to 14 too is not formed in this case).
We are proposing that the ring-cleaved products arise from the heterolytic chemistry discussed above and not from electron transfer chemistry (vide infra). Theoretically, the cleavage products observed could arise from one-electron transfer from amine to quinone (to produce aminium radical cation) followed by radical combination. However, such a mechanism is much less attractive for several reasons. The UV/VIS spectrophotometry studies (P.B. Kokil and L.M. Sayre, unpublished results) in our lab have shown a lower reactivity of the tertiary amine 1-phenyl-N,N-dimethylcyclopropylamine with DTBQ compared to 1-PCPA. If a 1-e⁻ oxidation process were involved, a faster reaction of the tertiary amine would have been expected (as is observed in the case of [Fe(CN)₆]³⁻, a standard one-electron oxidant). Secondly, the formation of imine is evident by ¹H NMR (this study), at least in the case of CPA. Our proposal for ring-opening subsequent to the imine formation clearly rationalizes the higher reactivity of primary amine vs. the tertiary amine.

Thus the aforementioned evidence towards non-homolytic oxidative cleavage of cyclopropylamines may be relevant to enzymes and should be viewed alongside other reports of enzymatic cyclopropane cleavages which appear to proceed through heterolytic mechanisms. It should
also be borne out, in regards to the recently growing popularity of utilizing cyclopropyl analogs as probes of mechanism, that although an initial one-electron oxidation of strained ring amines is often associated with ring opening reactions, the latter is not an unambiguous diagnostic of mechanism.

EXPERIMENTAL:

General Comments. PHAN was a generous gift from Ciba-Geigy Co. (Switzerland). L-PCPA was prepared by Dr. P. B. Kokil in our group by a literature procedure. All other chemicals were purchased from Aldrich Chemical Co. and were used as obtained. Other general procedures are described in the experimental section for chapter I.

General method for oxidation of primary amines with DTBQ under aqueous conditions. The reactions of amines with DTBQ were carried out via a variation of Corey's procedure, by mixing 15 mL of an aqueous solution of amine (1 mmol) with 45 mL of a CH₃CN solution of DTBQ (220 mg, 1 mmol). The pH of the mixture was then adjusted to 8 with 1% aqueous NaOH, and the reaction solution was stirred for 16 h at 25°C under N₂. The mixture was then diluted with 2 volumes of water and
extracted with CHCl₃. The CHCl₃ extracts were washed with water, dried (Na₂SO₄), and evaporated to afford a mixture of substituted benzoazole and di-tert-butyl-catechol, which were separated by preparative TLC (CHCl₃ mobile phase).

5,7-Di-tert-butyl-2-phenylmethyl-1,3-benzoazole (4a) was thereby obtained from PhCH₂CH₂NH₂: ¹H-NMR (CDCl₃) δ 1.24 and 1.31 (2s, 9H each, tert-Bu), 4.29 (s, 2H, PhCH₂), 7.16 and 7.51 (2d, 1H each, J = 2.2 Hz, Ar-H), 7.18-7.51 (m, 5H, Ph-H).

5,7-Di-tert-butyl-2-(4-nitrophenylmethyl)-1,3-benzoazole (4b) was thereby obtained from p-nitrophenyylethylamine: ¹H-NMR (DMSO-d₆) δ 1.21 and 1.34 (2s, 9H each, tert-Bu), 4.36 (s, 2H, Ar-CH₂), 7.35-7.80 (m, 6H).

2-Cyanomethyl-5,7-di-tert-butyl-1,3-benzoazole (4c) was thereby obtained from BAPN: ¹H-NMR (CDCl₃) δ 1.37 and 1.42 (2s, 9H each, tert-Bu), 4.11 (s, 2H, CH₂CN), 7.34 and 7.59 (2d, 1H each, J = 2.0 Hz, Ar-H).

2-(2-Cyanoethyl)amino-4,6-dimethylphenol (8). A mixture of 2-amino-4,6-di-tert-butylphenol (220 mg, 1 mmol) and excess acrylonitrile (10 mL) was heated under N₂ at reflux for 6 h. Unreacted acrylonitrile was then distilled from the reaction mixture, and the residue from the distillation was further dried under high vacuum to afford a white solid. TLC and ¹H NMR analysis
of the solid revealed a mixture of momo- and bis-adducts, which were separated by silica gel flash chromatography (hexane-EtOAc 2:1 eluant). The desired monoadduct had m.p. 128°C; \( ^1H \)-NMR (DMSO-\( d_6 \)) \( \delta \) 1.23 (2s, 9H each, t-Bu), 2.75 (t, 2H, \( J = 6.6 \) Hz, CH\(_2\)-CN), 3.35 (m, 2H, N-CH\(_2\)-), 4.86 (t, 1H, \( J = 7.4 \) Hz, exchangeable, NH), 6.52 and 6.58 (2d, 1H each, \( J = 2.2 \) Hz, Ar-H), 7.41 (s, 1H, OH); HRMS (40 eV) calcd for \( \text{C}_{17}\text{H}_{26}\text{N}_2\text{O} \) m/z 274.2047, found 274.2052 (M\(^+\), 17%), 216.1435 (M-58, 100%).

3-(3,5-Di-t-butyl-2-hydroxyphenylamino)propenenitrile (7) was prepared in an NMR tube experiment by adding chloranil (12 mg, 0.05 mmol) directly to a solution of the 8 (14 mg, 0.05 mmol) in 0.5 mL DMSO-\( d_6 \). A 4:1 mixture of E (7b) and Z (7a) forms was obtained:

7a (Z isomer) \( ^1H \)-NMR: \( \delta \) 4.24 (d, \( J = 8.6 \) Hz, CH next to CN), 7.41 (dd, \( J_1 = 8.6 \) Hz, \( J_2 = 13.0 \) Hz, CH next to NH), 8.25 (d, \( J = 13.0 \) Hz, NH).

7b (E isomer) \( ^1H \)-NMR: \( \delta \) 4.55 (d, \( J = 13.6 \) Hz, CH next to CN), 7.71 (dd, \( J_1 = 11.6 \) Hz, \( J_2 = 13.6 \) Hz, CH next to NH), 8.83 (d, \( J = 11.6 \) Hz, NH).

*Note: The NMR spectrum obtained was extensively analyzed through decoupling experiments, with irradiation of each signal to observe the changes produced in the spectra. These experiments helped in determining the apparent coupling constants and in identifying the*
individual isomers.

General procedure for $^1$H-NMR monitoring of reactions of amines with quinones. Stock solutions of DTBQ (22 mg, 0.1 mmol) and PHAN (21 mg, 0.1 mmol) were prepared in DMSO-$d_6$ (0.4 mL). For each reaction 0.2 mL of quinone solution was transferred to a 5 mm NMR tube, followed by the addition of a 0.2 mL of DMSO-$d_6$ solution containing 0.05 mmol of the amine. Immediately after mixing, the tube was flushed with $N_2$ and the spectra were recorded at regular time intervals. For experiments involving further addition of DTBQ, the calculated quantity was weighed out and added directly to the NMR samples.

Reaction of DTBQ with BAPN led to the initial appearance of two characteristic triplet signals at $\delta$ 2.10 and 3.8, and the NMR at the completion of reaction showed the presence of 7a and 7b with spectral characteristics given above.

Reaction of PHAN with BAPN led to the initial appearance of two sets of triplet signals: (i) $\delta$ 2.81 and 4.60 ($J = 6.8$ Hz), and (ii) $\delta$ 3.06 and 4.09 ($J = 6.6$ Hz). The NMR at the end showed a mixture of E/Z isomers of 5-hydroxy-6-(2-cyanoethyl)amino-4,7-phenanthroline (10): $^1$H-NMR (DMSO-$d_6$) E-isomer (10a): $\delta$ 4.43 (d, $J = 8.4$ Hz, CH next to CN), 8.48 (dd, $J_1 = 8.4$ Hz, $J_2 = 13.6$ Hz).
Hz, CH next to NH); **Z-isomer (10b)**: $\delta$ 4.93 (d, $J = 13.6$ Hz, CH next to CN), 8.45 (dd, $J_1 = 12.8$ Hz, $J_2 = 13.6$ Hz, CH next to NH).

Reaction of DTB-AP with phenylacetaldehyde was carried out in a similar fashion as outlined in the general procedure for NMR experiments, with equimolar concentrations of DTB-AP and PhCH$_2$CHO (0.05 mmol). NMR of the reaction mixture showed the presence of dihydro-benzoxazole 3a: $\delta$ 1.19 and 1.32 (2s, $\tau$-Bu), 3.05 (m, 2H, PhCH$_2$), 5.94 (m, 1H), 6.81 and 6.96 (2d, $J = 2.2$ Hz), 7.15-7.42 (m, 5H, Ph-H); **Note**: addition of 1 equivalent of DTBQ to this sample gave a product which was identical to 4a.

Oxidation of 1-phenylcyclopropylamine (1-PCPA) with DTBQ was carried out according to the general method above. After 16 h, a solid was present, which was filtered and recrystallized from EtOH-H$_2$O to afford an analytically pure compound identified as 7,9-di-$\tau$-butyl-4-phenyl-2,3-dihydro-1,5-benzoxazepine (14): m.p. 108°C; $^1$H-NMR (CDCl$_3$) $\delta$ 1.33 and 1.40 (2s, 9H each, $\tau$-Bu), 3.11 (t, 2H, $J = 6.2$ Hz), 4.67 (t, 2H, $J = 6.2$ Hz), 7.18 and 7.22 (2d, 1H each, $J = 2.4$ Hz), 7.44-7.48 (m, 3H, Ph-H), 7.96-8.00 (m, 2H, Ph-H); $^{13}$C-NMR 30.90 (-), 31.53 (-), 32.01 (+), 34.56 (+), 35.39 (+), 76.24 (+), 121.38 (-), 123.42 (-), 126.99 (-), 128.49
(-), 130.34 (-), 138.91 (+), 141.71 (+), 142.12 (+),
145.15 (+), 145.73 (+), 167.76 (+); HRMS (40 eV) calcd
for C_{23}H_{29}NO m/z 335.2250, found 335.2259 (M^+, 82%).
The filtrate obtained in this reaction was evaporated to
afford a residue containing 2-phenyl-7,9-di-t-butyl-1-
aza-spiro[4.5]deca-1,7,9-triene-6-one (15) and a small
amount of 14. The mixture was separated by silica gel
flash chromatography (CHCl₃ eluant) to afford a pure
sample of 15: ¹H-NMR (CDCl₃) δ 1.15 and 1.24 (2s, 9H
each, t-Bu), 1.86-1.99 (complex m, 1H), 2.31-2.39
(complex m, 1H), 3.12-3.23 (complex m, 2H), 5.96 and
6.99 (2d, 1H each, J = 2.4 Hz), 7.32-7.50 (m, 3H, Ph-H),
7.87-7.92 (m, 2H, Ph-H); HRMS (40 eV) calcd for
C_{23}H_{29}NO m/z 335.2250, found 335.2249 (M^+, 22%).

Reaction of DTBQ with 1-phenyl-2-propenamine ac-
cording to general method described above led to the
appearance of a solid after stirring under N₂ for ~0 h.
The solid was collected by filtration and recrystallized
from EtOH to afford a material which was identical to
benzoxazepine 14 in all respects.
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CHAPTER I.

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CHAPTER II.


CHAPTER III.


