STUDIES ON THE MONOAMINE OXIDASE SUBSTRATE/INACTIVATOR PROPERTIES OF PIPERIDINE ANALOGS OF THE NEUROTOXIN MPTP

by
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(Abstract)

The unexpected monoamine oxidase (MAO) substrate properties of 1-cyclopropyl-4-substituted-1,2,3,6-tetrahydropyridines have been interpreted in terms of partitioning of these tertiary cyclic allylamines between substrate turnover and ring opening inactivation processes. To evaluate further this proposal, we examined the bioactivities of the related saturated analogs. Several 1,4-disubstituted piperidine derivatives were synthesized and their interactions with MAO-A and MAO-B were characterized. These compounds displayed poor substrate properties toward MAO-A and MAO-B and led to the expected α-carbon oxidized metabolites which were fully characterized.

Both the N-methyl and N-cyclopropyl derivatives were good inactivators of MAO-B, suggesting that some species other than the radical resulting from cyclopropyl ring opening is responsible for the inactivation. Both the N-methyl and N-cyclopropyl derivatives also inactivated MAO-A. In this instance, the N-cyclopropyl analogs were much more potent inactivators than the N-methyl analogs. These results suggest that the radical derived from cyclopropyl ring opening may be involved in this inactivation process.

The MAO substrate/inactivator properties of these piperidine analogs are

discussed in terms of current proposed mechanisms for the MAO catalyzed oxidation of amines.

This thesis is dedicated to my parents for their endless love, support and encouragement through all of these years.

Acknowledgments

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Contents

List of Schemes

List of Figures

List of Tables

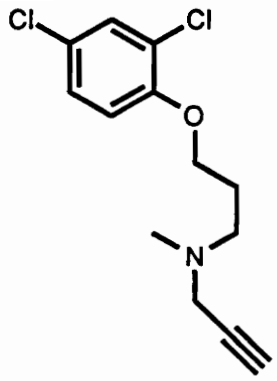
Chapter I. Introduction

1.1 Monoamine Oxidase

1.1.1. Biological Background

Amine oxidases are divided into the monoamine oxidases (MAOs), diamine oxidases (DAOs) and polyamine oxidases (PAOs) according to their ability to catalyze the oxidation of monoamines, diamines, or polyamines, respectively (*1*). Monoamine oxidase, which is the focus of this thesis, was first characterized by Mary Hare in 1928 as tyramine oxidase (*2*) and was later called monoamine oxidase by Zeller (*3*).

MAO is an integral protein of the mitochondrial outer membrane of neuronal, glial and other cells. The classification of MAO into two forms, MAO-A and MAO-B, was first suggested by Johnston in 1968 (*4*) on the basis of substrate preference and inhibitor sensitivity. MAO-A is inhibited by clorgyline (**1**) at nanomolar concentrations (*4*) while MAO-B is inhibited selectively by R-deprenyl (**2**) at nanomolar concentrations (*5*). Serotonin (**3**) is mainly an MAO-A substrate while ß-phenylethylamine (**4**) is mainly an MAO-B substrate. In humans, most tissues express both MAO-A and MAO-B. Tissues which express one form of MAO are placenta, containing predominantly MAO-A, and platelets and lymphocytes, expressing only MAO-B. Both forms of the enzyme are present in most regions of the brain.



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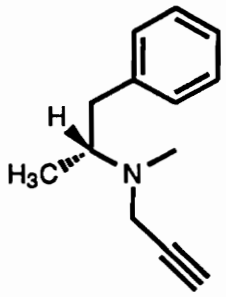

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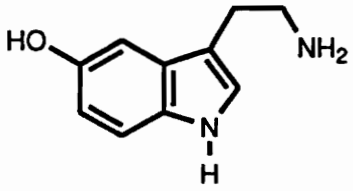

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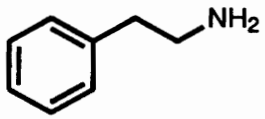

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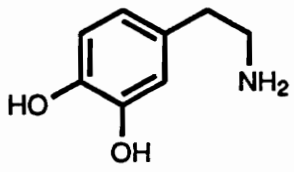
Problems with solubilizing proteins from mitochondrial membranes, together with aggregation and possible interspecies differences have made difficult in estimation of the molecular weight of the MAOs. On the basis of flavin content, bovine liver MAO has a molecular weight of 100,000 Da. The molecular weight of the MAO-A subunit isolated from human placenta has been reported to be 67,000 Da by Brown (*5*) and 63,000 Da by Cawthon *et al.* (*6*). An MAO-B subunit from human platelet has a molecular weight of 63,000 Da according to Brown (*5*) and 60,000 Da according to Cawthon (*6*). The primary amino acid sequences for the two forms are known, and it has been found that the two forms share much similarity. The structures of the enzyme active sites have not yet been characterized due to the lack of x-ray structural data.

1.1.2. MAO Substrates

The major endogenous substrates for MAO are dopamine (**5**), norepinephrine (**6**), epinephrine (**7**), serotonin (**3**), and ß-phenylethylamine (**4**). In addition to the biogenic amines, Several exogenous amines from various sources have been found to be substrates of MAO. The parkinsonian syndrome inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (**8**) and its analogs, of particular interest to us, show good MAO substrate properties (for details see section 1.3.).



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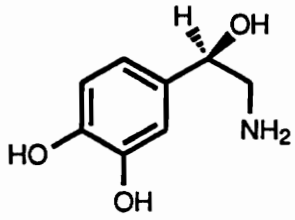

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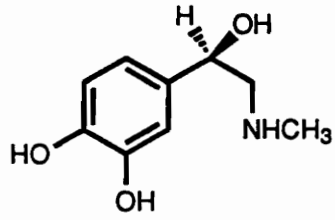

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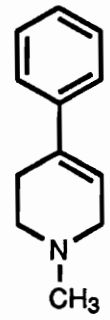
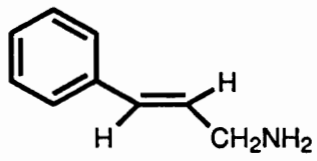


MPTP 8

A variety of studies on substrate selectivity for the two forms shows that

primary and secondary amines are indiscriminately deaminated by both forms of MAO while compounds such as N,N-diethylaminoalkylindoles are MAO-B selective substrates (*7*). This may suggest that MAO-A is more sensitive to steric interactions than MAO-B. Lipophilicity is another factor involved in substrate recognition. Straight chain aliphatic amines containing 5 to 10 carbon atoms have been shown to be selective substrates of MAO-B (*8*), possibly because of their lipophilic properties which appear to be a common property of typical MAO-B substrates.

The ability of MAO-B to oxidize E- and Z-1-methylcinnamylamines (**9a** and **9b**) with almost equal efficiency suggests that, in addition to a binding site for the amino group, MAO has a hydrophobic site that binds the aromatic residue of the substrates (*9*). If such a site exists, the high selectivities of cinnamylamine isomers, benzylamine (**10**), phenylalkylamines and aliphatic amines for MAO-B and that of 5-hydroxytryptamine (**3**) for MAO-A, suggests that MAO-B must have a more extensive hydrophobic binding site than does MAO-A.



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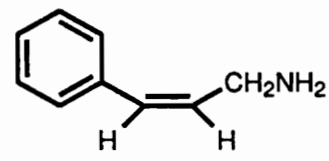

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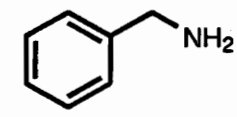


10

Substrate recognition of the tetrahydropyridine system will be discussed in section 1.3.

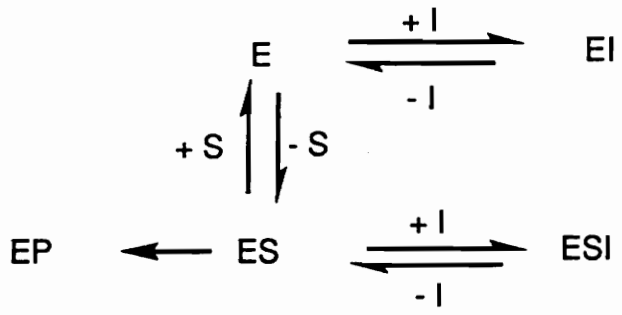
1.1.3. MAO Inhibitors

Substances that decrease the rates of the enzyme catalyzed reactions when present in the reaction mixtures are called inhibitors. Kinetic studies on the inhibitory effects of such compounds can provide valuable information on their potency and mechanism of action. Inhibition can arise in a variety of ways. Generally inhibitors can be classified into reversible and irreversible inhibitors based on the binding characteristics of enzyme-inhibitor.

Reversible Inhibitors

Reversible inhibitors are compounds that have structures similar to substrates or products of the target enzymes and which bind at the substrate binding sites, thereby blocking substrate access as illustrated in Scheme 1. The reversible inhibitor binds with the enzyme forming an EI complex which can also dissociate back to free inhibitor and enzyme. The inhibitor can also bind together with the substrate to form an ESI complex in an equilibrium process.

Scheme 1. Enzyme Inhibition by Reversible Inhibitors

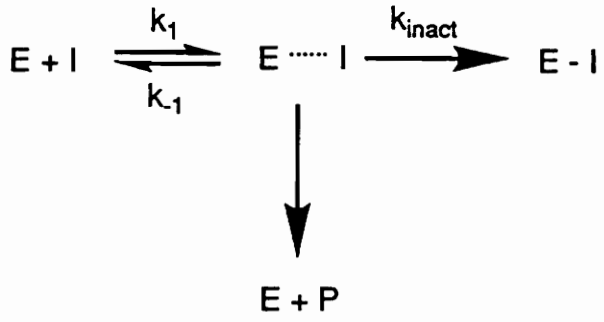


The reversible inhibitor binding process can be studied through kinetic analysis which reports effects on the velocity of an enzyme-catalyzed reaction. Binding studies also can be carried out directly by determining the amount of complex formed when the enzyme has been equilibrated with a fixed concentration of inhibitor by using techniques such as equilibrium dialysis, flow dialysis, gel filtration or ultrafiltration. Alternatively they may be carried out indirectly by observing changes in a property of the enzyme or inhibitor, such as fluorescence absorbence, optical rotatory dispersion or electron spin resonance. When binding occurs in these cases, however, it is necessary to assume that the change observed is proportional to the degree of saturation of the enzyme's active site (*10*).

Irreversible Inhibitors

Irreversible inhibition can be represented by the equation shown in Scheme 2 in which the inhibitor binds to the enzyme via a covalent bond resulting in a binding product (E - I) that will not dissociate back to the free enzyme and inhibitor.

Scheme 2. Enzyme Inhibition by Irreversible Inhibitors



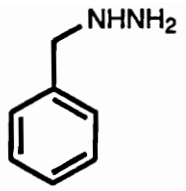
A very important class of irreversible inhibitors the mechanism-based inhibitors. These are compounds which have a structure similar to the substrate or product of the enzyme. A mechanism-based inhibitor itself is not active but acts initially like a true substrate. It is converted by the catalytic mechanism of the enzyme into products. However, the product of the enzyme-mediated reaction is generally a reactive species that then becomes attached covalently to the enzyme. The important event is that the conversion to the activated form is expected to be initiated by the same catalytic steps involved in the reaction with normal substrates, only the products happen to be more reactive than those generated from normal substrates. Therefore the mechanistic information that can be deduced from inactivation studies is directly related to the catalytic mechanism of the enzyme .

In order to characterize a mechanism-based inactivator, the following criteria have to be fulfilled:

- A time dependent loss of enzyme activity is observed.
- The rate of inactivation is proportional to the inhibitor concentration at low concentrations but independent of the inhibitor concentration at high concentrations (saturating conditions).
- The rate of inactivation is slower in the presence of substrate than its absence.
- Enzyme activity does not return upon dialysis or gel filtration.
- A 1:1 stoichiometry of radio-labeled inactivator to active site usually results after inactivation followed by dialysis or gel filtration.

MAO has been studied with a wide variety of inhibitors (*11*). The α-methylmonoamines reversible inhibitions of MAO were described as early as 1937 (*12*). Subsequently, harmala and vinca alkaloids (*13-15*), tetrahydro-ß-carbolines (*16,17*), oxazolidinone derivatives (*18,19*), and xanthones (*20*) have been shown to be reversible, competitive MAO inhibitors. Irreversible MAO inhibitors include basically three classes of compounds: hydrazines (*21,22*), propargylamines (*23*), and cyclopropylamines (*24*).

The early MAO inhibitors (MAOIs) were used clinically in the late 1950s to early 1960s for the treatment of depression (*25*). But many MAOIs were found to be hepatotoxic. Furthermore, the well-documented "cheese effect", a phenomenon which gives rise to a hypertensive crisis caused by the tyramine found in certain food which remains unmetabolized after MAO inhibition (*26,27*), led to the withdrawal of these types of drugs in human therapy in the 1970s. At present only a few drugs, including phenelzine (**11**), tranylcypromine (**12**), isocanboxazid (**13**), and pargyline (**14**), remain approved for clinical use in certain European countries. The emergence of the MAO-A selective inhibitor clorgyline (**1**) has provided a little encouragement but it is also prone to have the cheese effect observed with nonselective MAOIs. Renewed interest in the selective inhibition of MAO by (R)-deprenyl (**2**), also known as selegiline, has emerged recently. (R)-deprenyl is a powerful, irreversible inhibitor of MAO-B. Pharmacologically this MAO-B inhibitor appears not to cause the cheese effect (*28*).



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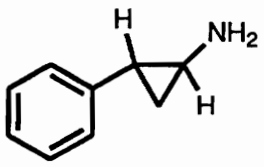

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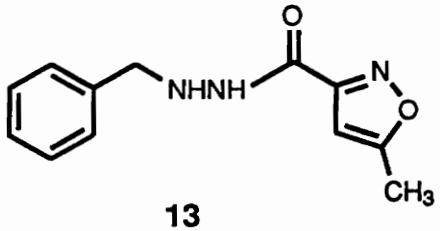

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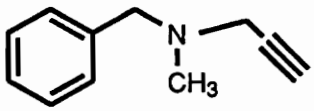

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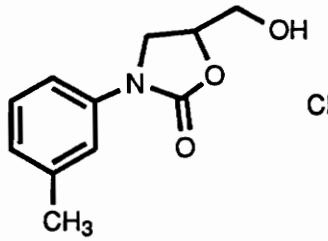

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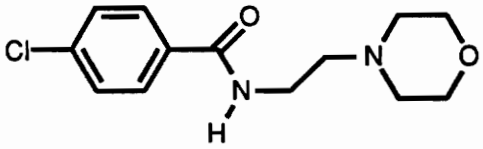

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Toloxatone (**15**) and moclobemide (**16**) have been reported to be selective and short-acting MAO-A inhibitors (*29,30*). They have been used as antidepressants in the clinic and were found to have no cheese effect (*29*).

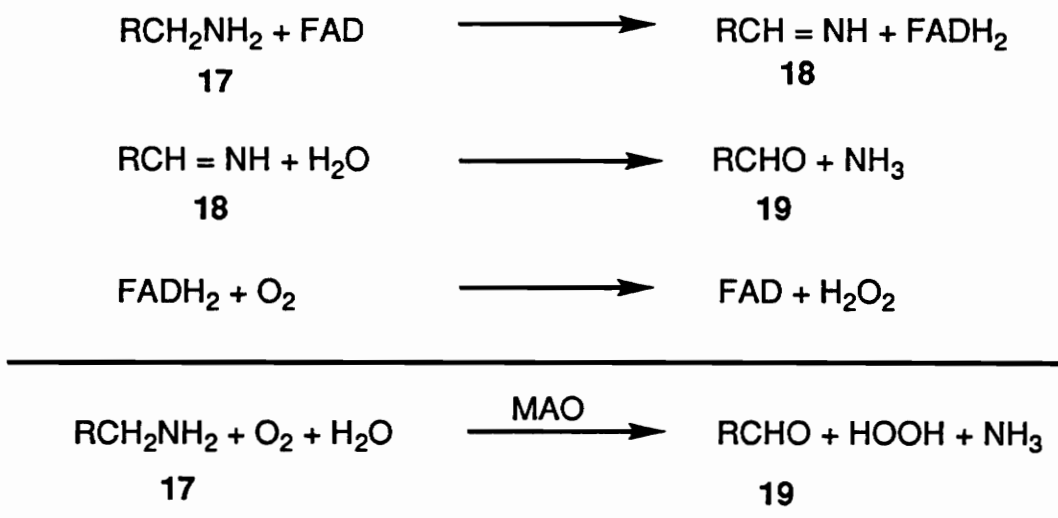
A revival of interest in MAO inhibitors due to the availability of a new generation of molecules may lead to full rehabilitation of this class of central nervous system agents. Furthermore, a promising application of MAO inhibitors may be the prevention of oxidative stress, resulting from metabolism of monoamines under pathophysiological conditions where they are produced in excess.

1.2. Mechanisms of Monoamine Oxidase Catalysis

The stoichiometry of monoamine oxidase mediated oxidations of amines is as shown in Scheme 3 in which the substrate amine (**17**) is oxidized by flavin (FAD) to the imine (**18**) which is then further hydrolyzed to aldehyde (**19**) and ammonia. Coupled to the substrate oxidation is the reduction of FAD to $FADH_2$ which is then reoxidized by the dioxygen to FAD with the production of hydrogen peroxide.

The exact catalytic pathway is not fully understood. Many mechanistic studies dedicated to unraveling the MAO catalytic pathway have lead to the following three main proposals: (1) a single electron transfer (SET), (2) hydrogen atom transfer (HAT) and (3) a polar addition-elimination pathway.

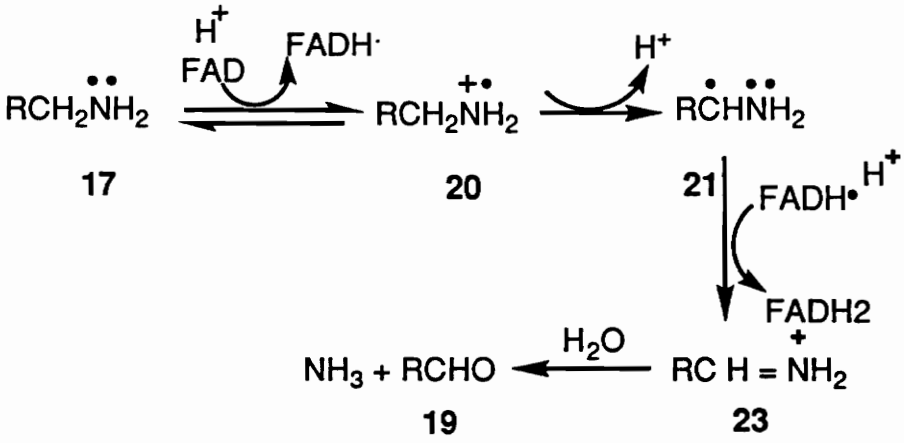
Scheme 3. MAO Catalyzed Amine Oxidation



1.2.1. Single Electron Transfer Pathway

The single electron transfer (SET) pathway proposed by Silverman and his coworkers (Scheme 4) is based on studies with mechanism-based inactivators derived from cyclopropylamines, cyclobutylamines and, later on, silylamines (*31*). It is suggested that the oxidation process starts with an initial single electron transfer from the lone pair of nitrogen of the amine substrate (**17**) to the oxidized form of the flavin (FAD), generating an aminyl radical cation (**20**) and a flavin radical (FADH·). The highly reactive intermediate **20** undergoes α-proton loss to form the radical **21** which goes through another electron transfer to yield the iminium species **22** which is hydrolyzed to the aldehyde **19** and ammonia.

Scheme 4. Proposed Single Electron Transfer Pathway

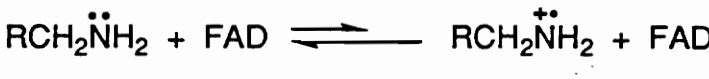


More direct evidence for a radical intermediate in MAO-catalyzed amine oxidations was sought by carrying out the reaction of MAO with poor substrates such as cyclobutylamines in the presence of a radical spin trap in an ESR tube

(*32*). Spectra supported the existence of radical species. The failure to observe the radical intermediates by ESR with cyclopropylamines and benzylamine was explained in terms of the high reactivity of the corresponding radicals (*32*).

Edmondson, based on his work with substituted benzylamines, questioned the SET hypothesis (*33*). The $\Delta G^{\neq}$ value (the activation energy for electron transfer from the amine to FAD) estimated for the limiting reduction rate of MAO-B by the *meta*-substituted benzylamine analogs using the Eyering equation is 12.3 kcal/mol whereas the ΔG_{et} value (the free energy of electron transfer from amine to flavin) calculated from the difference in flavin-amine one-electron couples is in the range of 20-30 kcal/mol. This means that the ΔG_{et} value is larger than $\Delta G^{\neq}$, which suggests that the initial single electron transfer step in SET is thermodynamically improbable. Redox potentials for the electron transfer step have also been measured. The one electron oxidation potentials of primary amines are in the range of ~ 1.5 V vs NHE (*34*). The one electron reduction of the covalent flavin in MAO-B has not been determined but on the basis of analogy with other flavoenzymes, it should be in the range of 0.0 - 0.2 V vs NHE (*35*). This means that the equilibrium constant calculated for a reversible one electron transfer between amine substrate and flavin (Scheme 5) would be in the range of 10^{-15}-10^{-25}. Thus redox potential considerations make one electron oxidation of the amines by the MAO flavin very unlikely.

Scheme 5. Single Electron Transfer From Amine to Flavin



17 **20**

1.2.2. Hydrogen Atom Transfer Pathway

Back in 1967, Hull *et al.* (*36*) performed studies on primary amine oxidations by ClO_2 and demonstrated that, in addition to an electron transfer mechanism, hydrogen atom abstraction occurs from carbon to generate the carbon centered radical **21** (Scheme 6). The radical undergoes electron transfer to yield the iminium species **22**. For a hydrogen atom abstraction mechanism to be operative, an acceptor of H· would be involved such that the bond dissociation energy of HX (X, H· acceptor) would be greater than the bond dissociation energy of α-carbon hydrogen of the amine. There is no evidence for such an hydrogen atom abstraction species in the vacuity of the enzyme's active site.

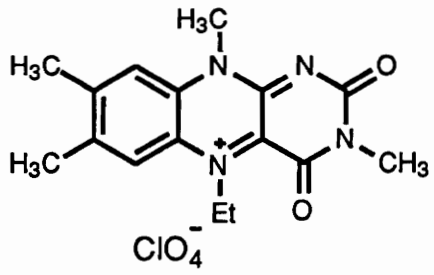
Scheme 6. Proposed Hydrogen Atom Transfer Pathway

17 **21** **22**

1.2.3. Polar Addition-Elimination Pathway

The flavin analog 3-methyl-5-ethyllumiflavinium perchlorate (**23**) was used by Mariano (*37,38*) as a chemical model to study three major MAO inactivators — cyclopropylamines, α-silylamines and hydrazines. From the results of these studies the mechanism shown in Scheme 7 was proposed. Amine **17** acting as a nucleophile attacks the activated flavin to form an adduct

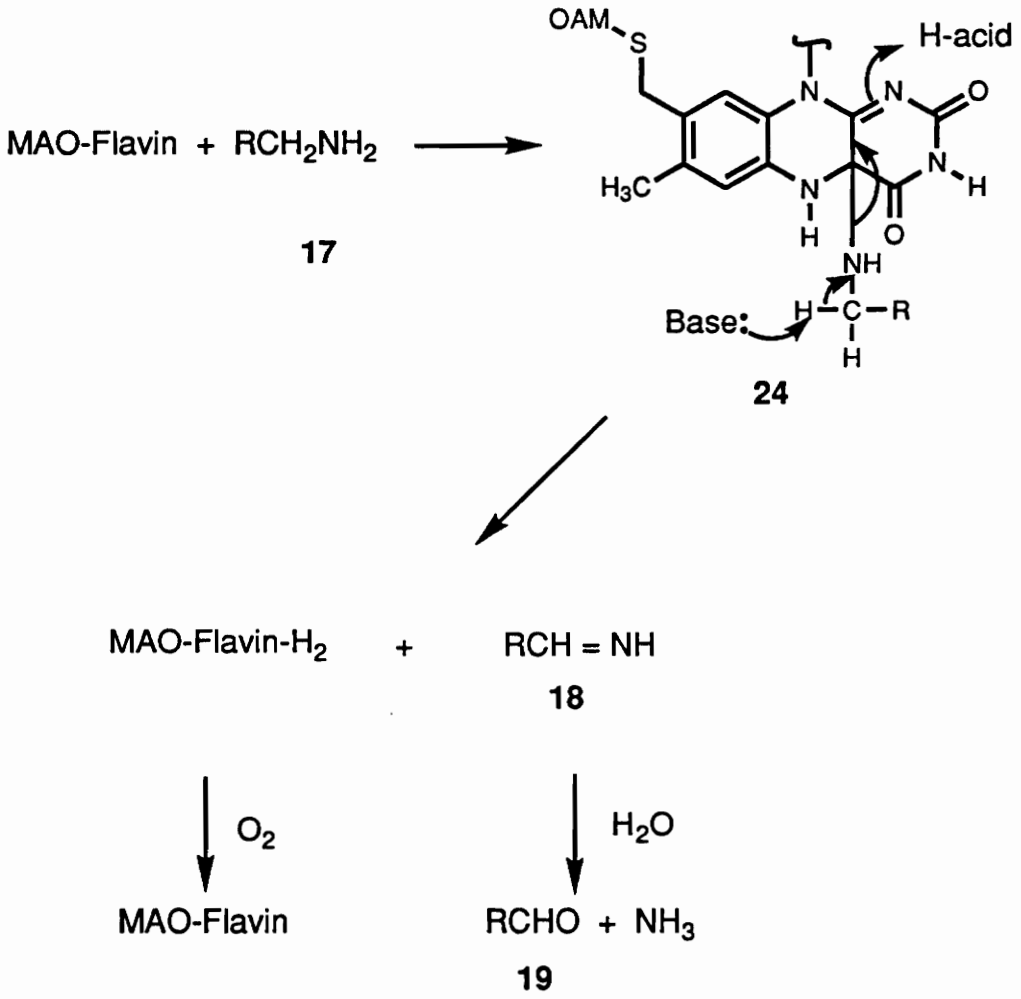
24. In a concerted step, the resulting adduct **24** undergoes cleavage to release the imine **18** and reduced flavin. Further hydrolysis of **18** leads to aldehyde **19** and ammonia.



23

This study demonstrated that activated flavins such as **23** (i.e. those with high reduction potentials and electrophilicity) promote ground state oxidative deamination reactions of primary and secondary amines. Tertiary amines have not been examined experimentally but it seems that tertiary amines would be too bulky to form an adduct like species **24**. This chemical model study was carried out in an organic solvent system and the reactions require heat and a long period of reaction time. These characteristics are quite different from the enzyme catalyzed process.

Scheme 7. Proposed Polar Addition-Elimination Mechanism



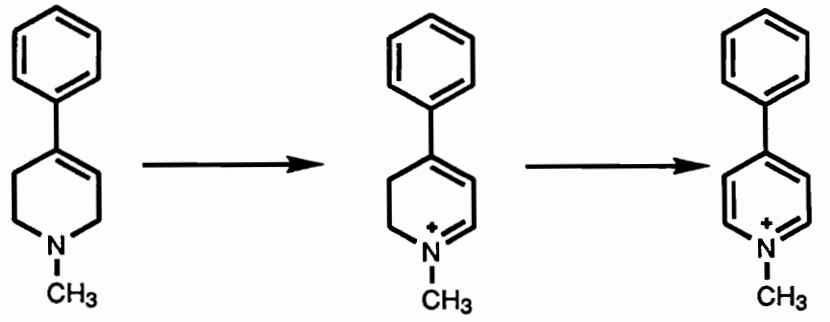
1.3. 1,2,3,6-Tetrahydropyridine Derivatives

1.3.1. Background

The cyclic tertiary amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (**8**) was first examined as a potential antiparkinsonian agent in the 1950s (*39*). Instead, parkinsonian inducing properties were observed. Nevertheless, it was not until the late 1970s that this compound invoked great interest. The event which led to this renewed interest was when a young drug user, after self-administration of an MPTP contaminated street drug known as "synthetic heroin", was found to develop a parkinsonian syndrome (*40*).

It has been demonstrated that MPTP itself is not neurotoxic. Mediated by MAO, this species is converted to the dihydropyridium species $MPDP^+$ (**25**) which is thought to undergo auto oxidation to form the pyridinium species MPP^+ (**26**), the actual neurotoxin (Scheme 8) (*41-46*). This oxidation process happens extraneuronally in astrocytes. Then MPP^+ is actively transported to the dopaminergic neurons by the dopamine uptake system (*47,48*). MPP^+ is concentrated in the matrix of the mitochondria where it inhibits NADH oxidase (*49*) and mitochondrial electron transport leading to cessation of oxidative phosphorylation (*43*), ATP depletion and neuronal death (*50,51*). MPTP and its analogs are so far the only class of cyclic, tertiary amines reported to be good MAO substrates. Consequently they provide a good opportunity to study the MAO catalytic pathway and the MAO mediated bioactivation of neurotoxins.

Scheme 8. MAO-B Catalyzed Oxidation of MPTP



MPTP **MPDP$^+$** **MPP$^+$**

8 **25** **26**

1.3.2. General Structure–Activity Relationships

Quantitative structure–activity relationship (QSAR) studies are very useful tools to probe the enzyme active site and to construct drug candidates. QSAR studies on MPTP analogs demonstrated that some structural features are essential for these types of compounds to be MAO substrates (*52*) :

- The 4-5 double bond is essential for compounds to be MAO substrates (*53*).
- The N-H, N-ethyl, and N-ethanol analogs are less active than MPTP; in general, longer alkyl chains abolish MAO reactivity (*54-56*).
- The phenyl ring is not necessary for substrate activity; replacement of the phenyl ring, for example, by a benzyl group enhances the activity (*55,56*); the 4-cyclohexyl analog is as good substrate as is MPTP

(*55,56*); the 4-ethyl derivative is less active than MPTP (*56*).

- The open-ring analog of MPTP is a good MAO substrate (*57*).
- The substituent has to be in position 4 of the tetrahydropyridine ring (*55*).

In our laboratory, QSAR studies merged with computational chemistry provided additional insights leading to models of the MAO-A and B enzyme active sites. The principal findings are as follows (*58*):

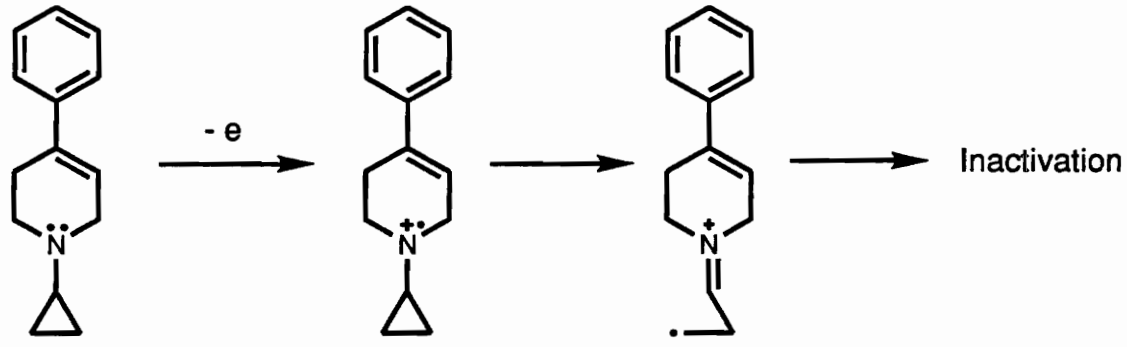
- The dihedral angle between the 4-5-position in the tetrahydropyridine ring and the C-4 substituent is a very important determinant relating to substrate properties.
- The absolute length of the molecule can not be longer than 12 Å.
- The dipole moment and polarity of the molecule also play important roles.

Chapter II. Research Proposal

2.1. Unusual Findings

In an effort to use inhibitors to probe the mechanism of MAO catalysis, we have designed and synthesized a series of 1-cyclopropyl-4-substituted-1,2,3,6-tetrahydropyridine derivatives (*59-62*). The inactivator properties of these compounds have been characterized. For example, 1-cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine (**27**) is a very good inhibitor for MAO-B (*60*). This observation seems consistent with Silverman's SET proposal (Scheme 9) which assumes that the primary radical (**29**), resulting from the rapid cyclopropyl ring opening, is responsible for the inactivation. Surprisingly, however, when the substituent at C-4 is benzyl (*62*) or 1-methyl-2-pyrryl (*63*), both inactivator and substrate properties are observed for MAO-B. More dramatically, when the C-4 position bears the phenoxy, 3-chlorophenoxy, 4-nitrophenoxy, thiophenoxy, or 2-methylphenyl substituents, the compounds are only MAO-B substrates with no inactivating properties (*59*).

Scheme 9. Possible Mechanism of the MAO-B Catalyzed Inactivation of 1-Cyclopropyl-4-phenyl-1,2,3,6-tetrahydropyridine

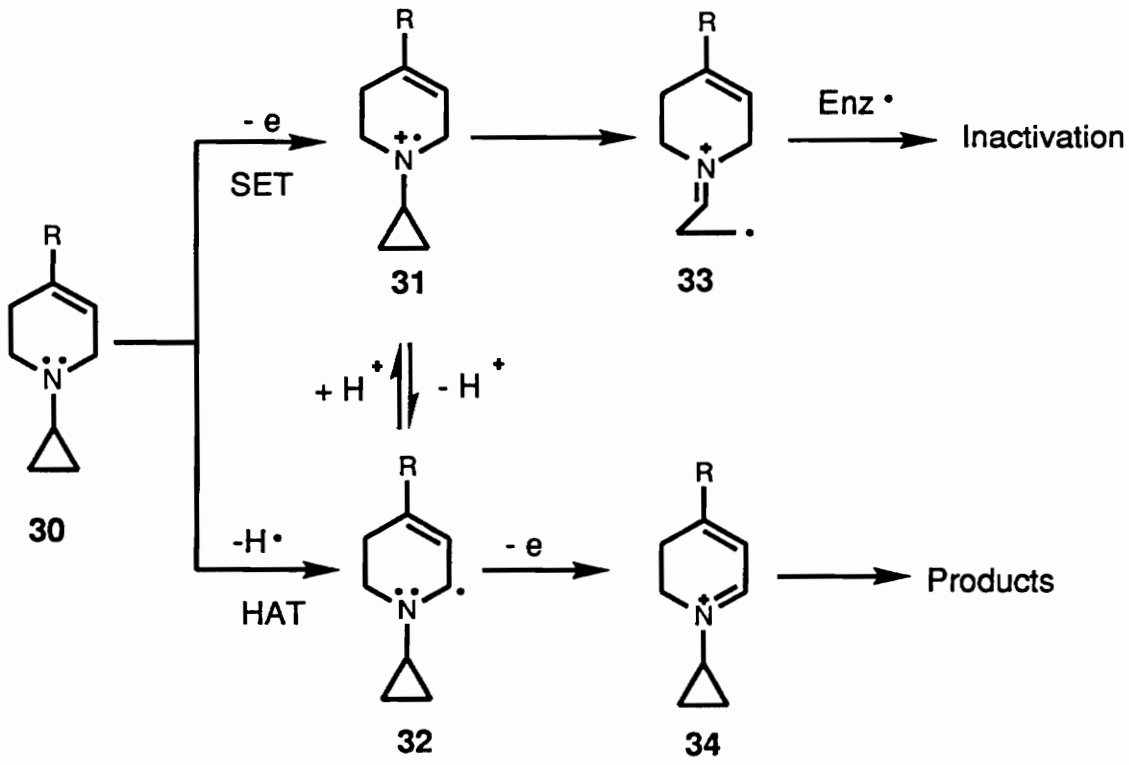


27 **28** **29**

In order to explain the substrate properties observed with the 1-cyclopropyl compounds **30**, one has to go through the deprotonation process (Scheme 10) from **31** to form the carbon centered radical **32** which subsequently can go to product via an electron loss. The rate of ring opening of the cyclopropyl aminyl radical cation **31** is so fast that attempts to measure it have failed (*64*). The rates of ring opening of cyclopropyl carbinyl radicals have been measured to be 10^8 - 10^{10} s^{-1} (*65*). Therefore, the deprotonation process, which leads to product formation, has to be compatible with the fast cyclopropyl ring opening process. An alternative pathway is hydrogen atom transfer (HAT) which involves a hydrogen atom loss directly from the C-6 position to generate **32** which results in substrate turnover. The attractive feature of this mechanism, especially in this tetrahydropyridine system, is the weak α C-H bond dissociation energy due to the fact that it is allylic and next to an electronegative atom nitrogen. To accommodate the inactivation by these

systems, however, generation of the radical cation **31** has to go through a protonation step from the carbon centered radical **32**. No evidence of such a protonation reaction has been reported so far. Another possibility is that both SET and HAT pathways operate in concert by partitioning through the substrate molecule **30**.

Scheme 10. Possible Catalytic Pathways for the MAO Catalyzed Oxidation of 1-Cyclopropyl-4-substituted-1,2,3,6-tetrahydropyridines



2.2. Research Proposal

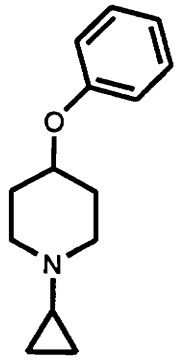
If we assume that species **33** is responsible for the inactivation and

radical **32** is the key intermediate leading to turnover, then we can see that the double bond at the 4-5 position is very important in terms of the stabilization of the radical **32**. Increased radical stabilization, therefore, should favor product formation. QSAR and proton NMR studies on a series of tetrahydropyridines show that electron-donating groups at C-4 do enhance the substrate properties of these types of compounds (*63*). On the other hand, since the double bond would not be expected to effect the stability of the cation radical **31** to any great extent, the inactivating properties of the molecules should not be effected by the double bond at the 4-5 position or electronic effects of the C-4 substituents.

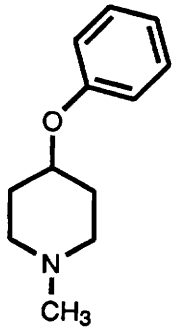
The importance of the double bond at the 4-5 position in the tetrahydropyridine ring has been discussed by other workers (*52*). However, no studies detailing the role of the double bond (through radical stabilization or geometry preference) on the substrate properties or on the inhibiting properties of this ring system have been reported. Therefore, it should be informative to study the bioactivity of the piperidine system in detail in order to understand better the role of the double bond in this class of compounds.

Consequently, we have elected to examine the interactions of various piperidine derivatives with MAO-A and MAO-B. As discussed before, 1-cyclopropyl-4-phenoxy-1,2,3,6-tetrahydropyridine (**35**) displays MAO-B substrate properties and no inactivator properties. The explanation for this could be that product formation is favored by geometry and/or stabilization of the putative radical intermediate (like **32**) such that opening of the cyclopropyl ring becomes less competitive. Removing the double should disfavor substrate

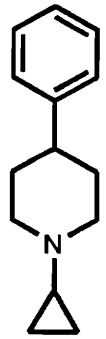
turnover, in which case the inactivation process would dominate. We also elected to study 1-methyl-4-phenoxypiperidine (**36**) in order to compare the substrate properties with those of the corresponding 1-methyl tetrahydropyridine derivative which is an excellent substrate for MAO-B. To evaluate the inactivator properties further, 1-cyclopropyl-4-phenylpiperidine (**37**) was chosen. The substrate properties of the related 1-methyl-4-phenylpiperidine (**38**) also was included for comparison. Interest in checking the reactivities of 1-cyclopropyl-4-benzylpiperidine (**39**) is based on the corresponding tetrahydropyridine which shows both substrate and inactivator properties. The 1-methyl-4-(3-phenylphenoxy)piperidine compound **40** was selected since the corresponding tetrahydropyridine is an excellent MAO-A but poor MAO-B substrate.



35



36



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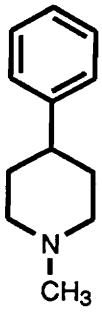

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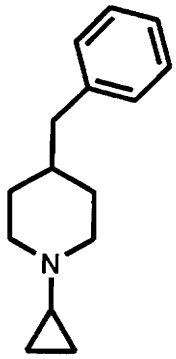

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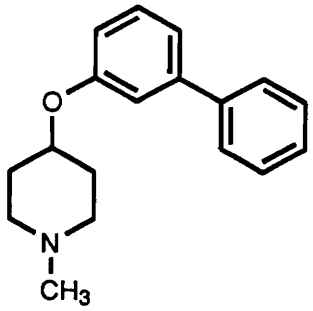

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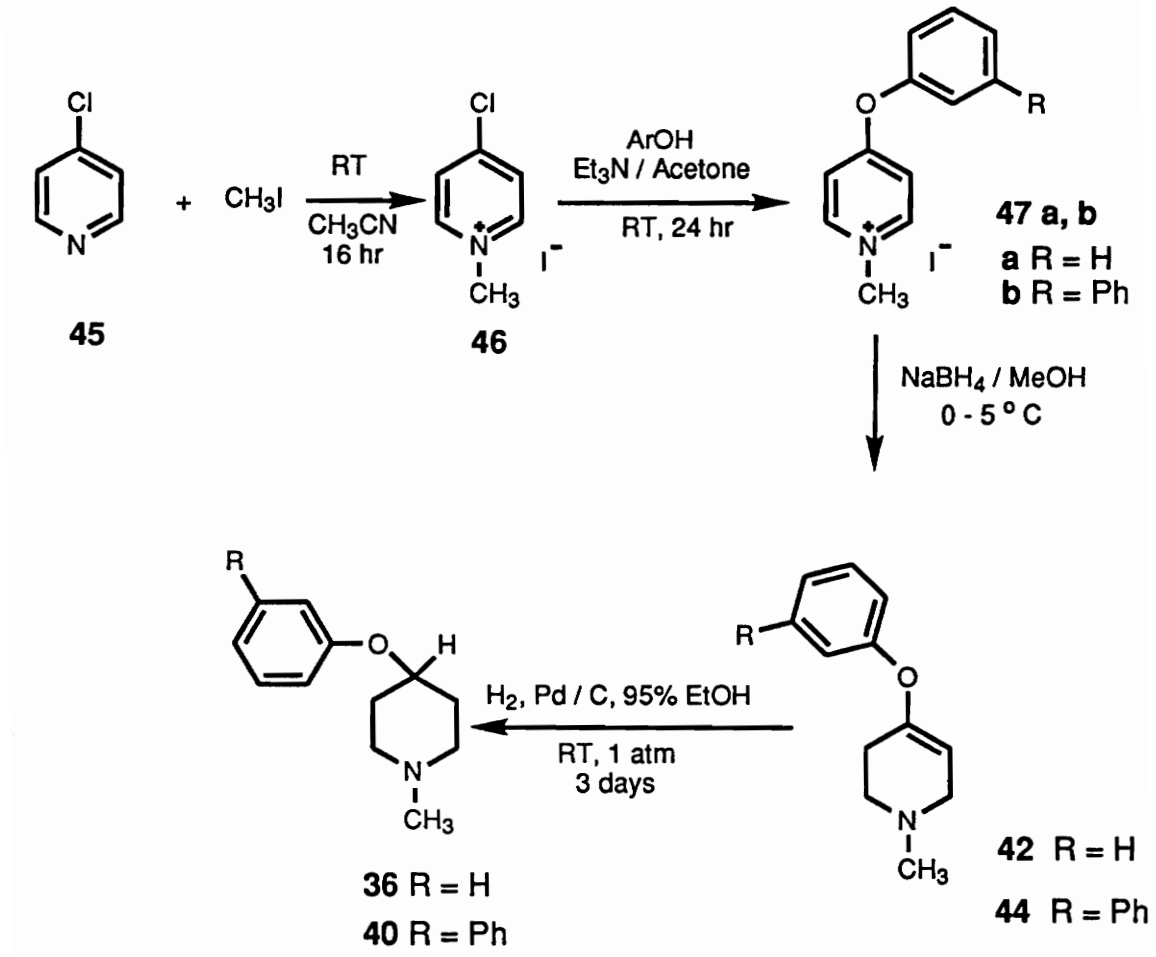
Chapter III. Results and Discussion

3.1. Chemistry

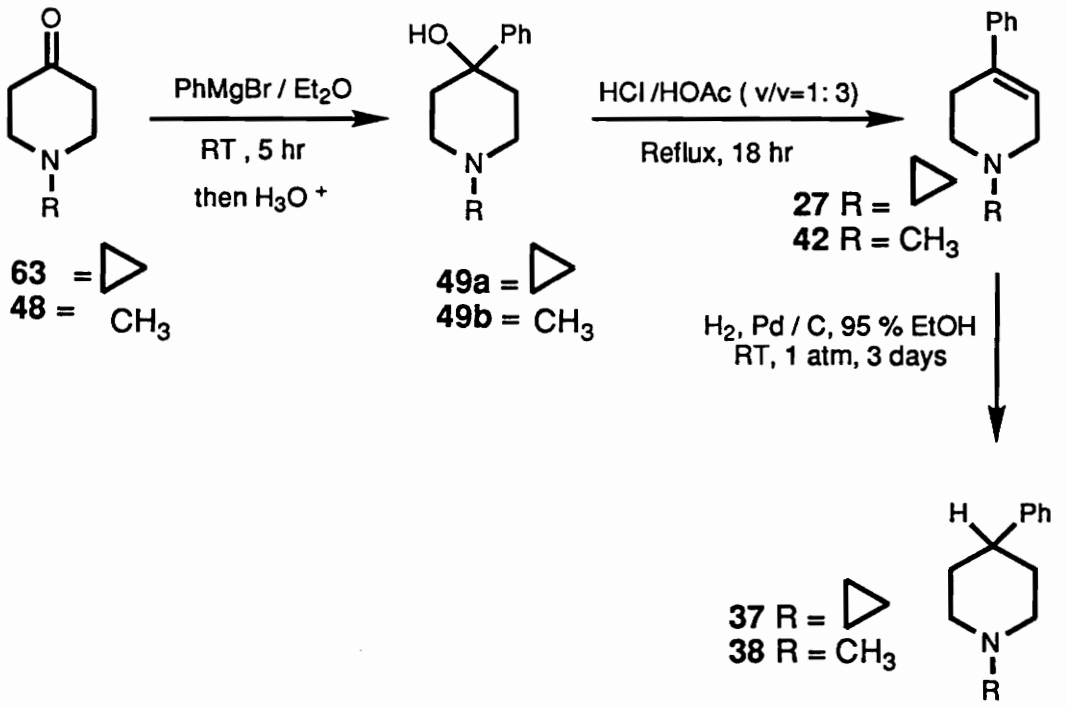
With the exception of 1-cyclopropyl-4-phenoxypiperidine (**35**), all of the piperidine derivatives **36, 37, 38, 39, 40** were obtained through hydrogenation of the corresponding tetrahydropyridines **41, 27, 8, 43, 44** (Scheme 11, 12, 13) on 10% Pd/C in 95% ethanol at room temperature and atmospheric pressure. The synthesis of the tetrahydropyridine intermediates were accomplished by following the methodologies already established in this laboratory (*59,62*). Every known intermediate was checked against the literature.

The catalytic hydrogenation normally took 3 days and gave yields as high as 90%. Palladium was chosen to be the catalyst. We used 95% ethanol instead of absolute ethanol because the more polar solvent can enhance the solubility of hydrogen and therefore to improve the rate of the reaction.

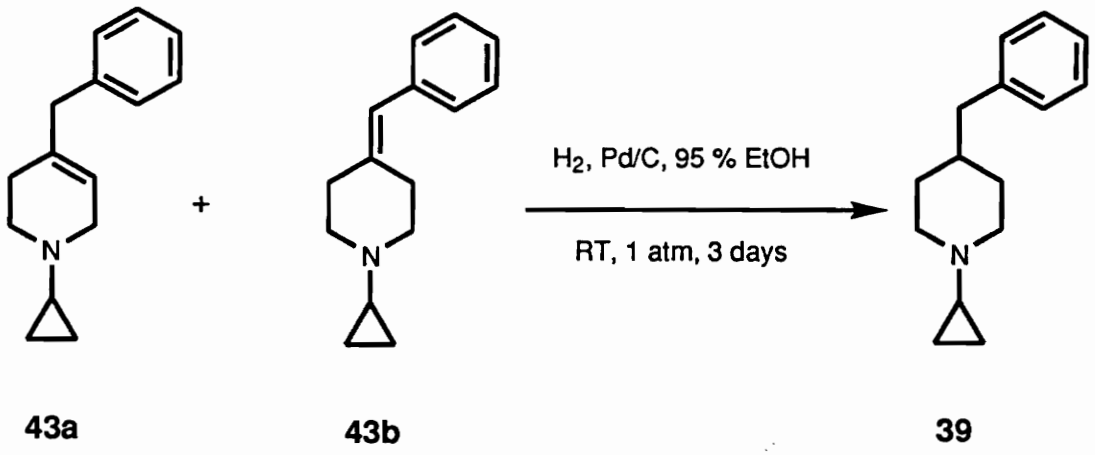
Scheme 11. Synthesis of Phenyl-substituted 1-Methyl-4-phenoxypiperidines



Scheme 12. Synthesis of 1-Substituted 4-phenylpiperidines



Scheme 13. Synthesis of 1-Cyclopropyl-4-benzylpiperidine



43a 43b 39

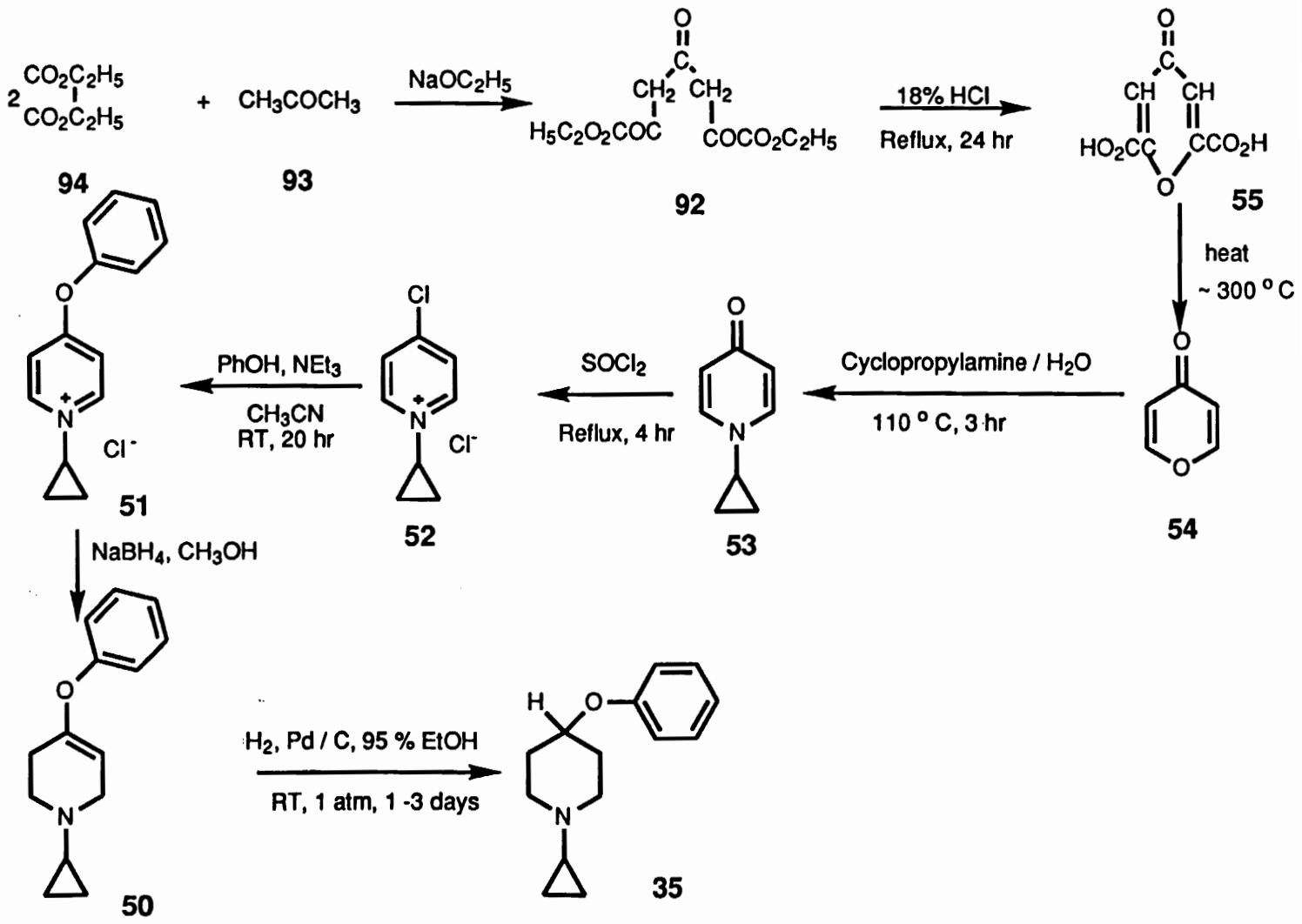
Gas chromatography electron-impact mass (GC/EIMS) spectra of the piperidine compounds **36**, **37**, **38**, **39**, **40** gave the expected parent ions (Fig. 1*, Fig. 2*, Fig. 3*, Fig. 4*, Fig. 5*), respectively. The M-15 fragmentation in N-cyclopropyl piperidine derivatives (Fig. 2*, Fig. 4*) was due to the loss of methyl group after the cyclopropyl ring opened (*66*). While the M-29 fragmentation in the cyclopropyl compounds can not be explained. In the ^{1}H NMR spectra of the piperidines (Fig. 6*, Fig. 7*, Fig. 8*, Fig. 9*, Fig. 10*), as expected, the diagnostic signals for the allylic protons of tetrahydropyridine derivatives were absent and two more protons were present. As an example, the signals in the ^{1}H NMR spectrum of compound **36** (Fig. 6*) are assigned as follows: multiplets in the region δ 7.31- 6.92 are assigned to the aromatic protons. The C-4 proton signal appears as a broad singlet centered near δ 4.60. The two symmetric multiplets at δ 3.21 and 3.09 are assigned to the equatorial and axial protons at C-3 and C-5, respectively. The C-2 and C-6 equatorial proton signals appear at δ 2.07 as broad multiplets while the axial proton signals are located at δ 1.88. The methyl proton signal appears as a singlet at δ 2.71. The structure of compound **36** is also confirmed by its ^{13}C NMR spectrum (Fig. 11*): δ 156.7, 116.4, 130.1 and 121.5 (aromatic carbons), δ 68.7 (C-4), δ 50.2 (C-2, C-6), δ 43.0 (C-3, C-5), δ 27.9 (CH_3). The ^{1}H NMR spectrum (Fig. 12*) for the 1-cyclopropyl analog **35** was similar to that of the 1-methyl analog **36** except that the signals for the methyl group were replaced by the corresponding signals for the cyclopropyl group [multiplets between δ 0.71 and δ 0.61 (CH_2CH_2) and multiplet centered at δ 2.39 (CH)]. Similarities in the ^{13}C NMR spectra between **35** (Fig. 13*) and **36** (Fig. 11*) were found. The methyl carbon signal in Fig. 13* was replaced by the

* All Figures with an asteris are located in Appendix 1.

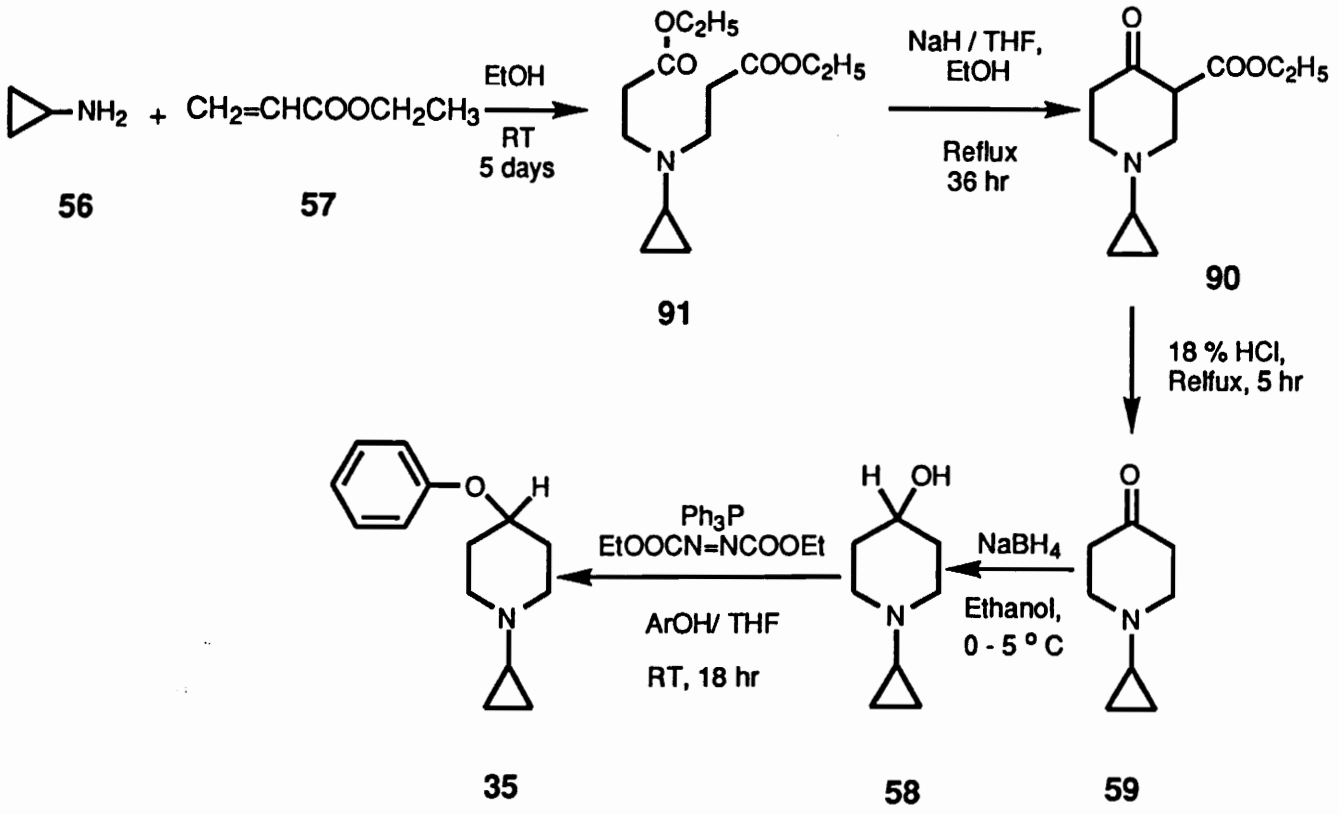
signal for the methine carbon of the cyclopropyl ring (δ 28.4) and the signal for the methylene carbons of the cyclopropyl ring (δ 4.4). Finally the microanalytical data were in accordance with the calculated values for C, H, and N. ^{1}H NMR spectra of compounds **37** and **38** (Fig. 7* and Fig. 8*) were similar to the spectra of **35** and **36**, respectively. Fig. 9* shows the ^{1}H NMR spectrum for the 4-benzyl-1-cyclopropyl derivative **39**, in which the C-4 methine proton signal overlaps with the C-3 and C-5 methylene equatorial proton signals. The signals for the benzylic proton and methine proton of cyclopropyl ring are hidden under the DMSO (NMR solvent) peak. Compound **40** gives a ^{1}H NMR spectrum which is distinguished by signals in the region δ 7.67-7.00 which are due to the protons of the two aromatic groups.

The preparation of 1-cyclopropyl-4-phenoxypiperidine (**35**) was attempted by hydrogenation of tetrahydropyridine **50**, which was synthesized through an established method (Scheme 14) (*59*). The γ-pyrone intermediate **54** was obtained through the thermal decarboxylation of chelidonic acid (**55**) at high temperature (300 °C) in only a 25% yield. An alternative synthesis was approached. As shown in scheme 15, starting with cyclopropylamine (**56**) and ethyl acrylate (**57**), the 1-cyclopropyl-4-piperidinol (**58**) can be readily obtained from sodium borohydride reduction of 1-cyclopropyl-4-piperidone (**59**) which was available by an established route (*62*). Direct introduction of the phenoxy group to obtain compound **35** was pursued through a Mitsunobu reaction (Scheme 15) (*67*). Intermolecular nucleophilic substitution of the alcohol group of 1-cyclopropyl-4-piperidinol (**58**) with the phenoxy substituent was carried out

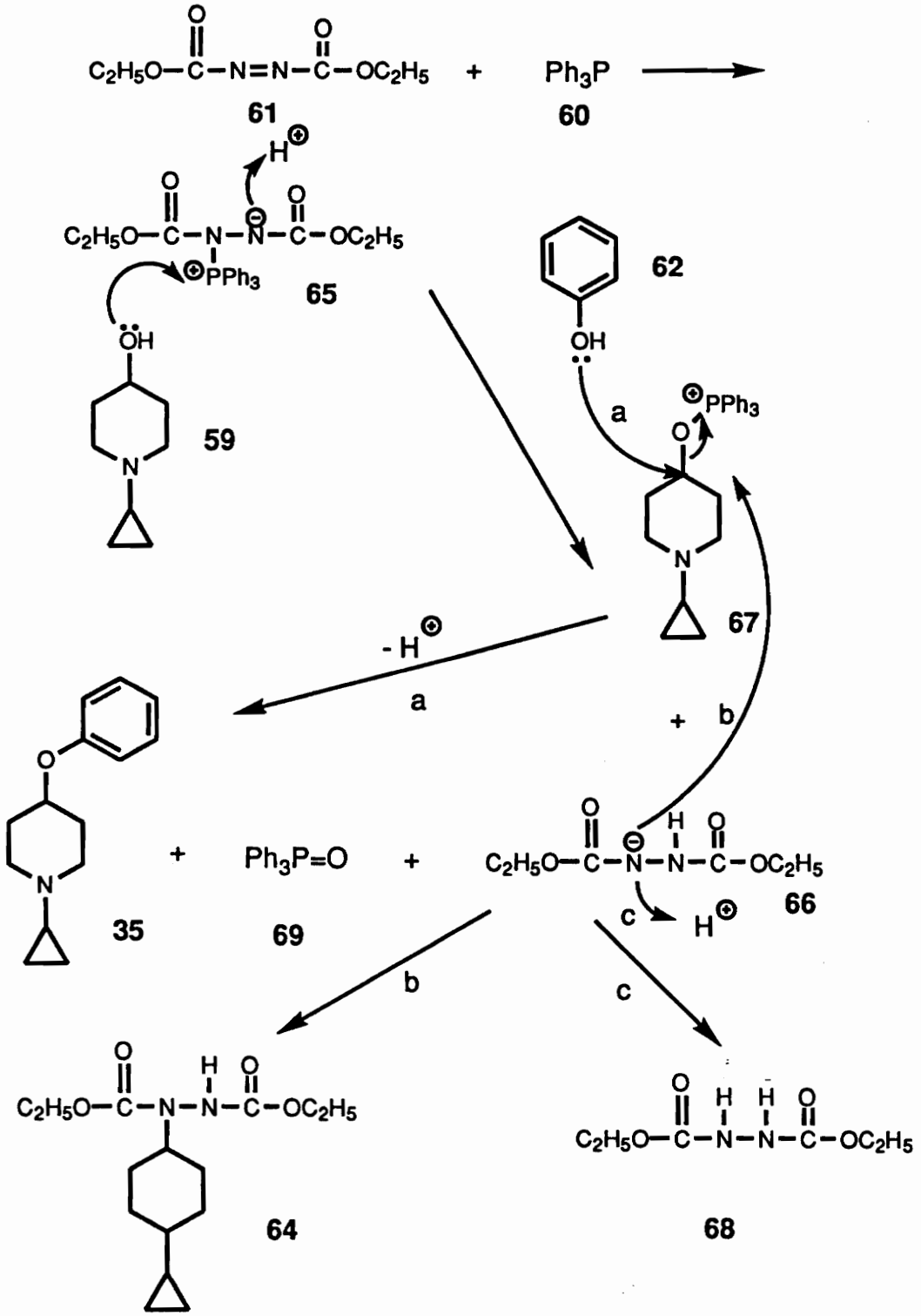
Scheme 14. Synthesis of 1-Cyclopropyl-4-phenoxypiperidine



Scheme 15. Alternative Synthetic Pathway of 1-Cyclopropyl-4-phenoxypiperidine



Scheme 16. Mitsunobu Reaction Pathways



in the presence of triphenylphosphine (**60**) and diethylazodicarboxylate (DEAD) (**61**) with phenol (**62**) as the nucleophile and THF as solvent. The first trial with all of the reagents mixed together gave two major products, one of which was the desired product. The other product gave a mass spectrum consistent with structure **64** (Scheme 16). The possible pathway of generating **64** as follows: the activated triphenylphosphine **65**, undergoes nucleophilic attacked by the alcohol **58** to yield the reduced diethyl hydrazindicarboxylate anion **66** and the alkoxyphosphonium intermediate **67**. The phosphonium intermediate **67** then can undergo nucleophilic attack by phenol (**62**) and the hydrazindicarboxylate anion **66**, to generate the two products **35** and **64**, respectively. The hydrazindicarboxylate anion **66** also can be protonated, giving a by-product hydrazindicarboxylate (**68**) . To avoid the side reaction of generation of **64**, DEAD (**61**) was added dropwise to a mixture of all other reagents with stirring. The reaction gave a yield of 52% after silica gel column purification.

3.2. Enzymology

3.2.1. Substrate Studies

Substrate Study with MAO-B

Substrate studies of the piperidine derivatives can not be conducted as conveniently as with the tetrahydropyridine system because these compounds do not have useful chromophores to monitor. Consequently, we developed a

GC-EIMS assay in which the disappearance of the starting material or/and the appearance of the metabolite(s) was monitored. To each aliquot of the incubation mixture of the substrate and the purified MAO-B preparation, an internal standard in sodium phosphate buffer solution was added just prior to work up in order to obtain the quantitative information about the MAO catalyzed oxidation process. A 10% Na_2CO3 solution was added to the resulting solution to destroy the enzyme and basify the solution for extraction. The analytes were partitioned into an organic solvent from the incubation mixture. Analysis was by GC-MS. The criteria used for selection of the internal standard are as follows: 1) it should have similar physical properties to the analyte; 2) it should be stable through the assay; and 3) it must have a different retention time from that of the analyte. Consequently, the internal standards were selected within the prepared piperidine derivatives (see Appendix 2).

The first set of substrate studies were conducted under the conditions reported for the tetrahydropyridines in which the enzyme to substrate molar ratios ranged from 1:2,200 to 1:22,000. As shown in Table 1, for all of the compounds examined the ratios of substrate vs internal standard did not change during the 90 minute incubation period. This indicated that none of the compounds were substrates of the enzyme (MAO-A and MAO-B). Since it was possible that the rates of MAO catalyzed oxidation of these piperidines were too slow to be observed under these low enzyme concentrations, a second set of experiments with compounds **35–38** was conducted at higher enzyme concentrations.

Table 1. Substrate Study on Selected Piperidines at Low Enzyme Concentrations

Compound	MAO-A (Substrate/Standard)						MAO-B (Substrate/Standard)					
	T (min)	0	15	30	60	90	T (min)	0	15	30	60	90
35	2.5 mM	0.88	0.89	0.88	0.94	0.93	2.5 mM	0.92	0.90	0.79	0.70	0.77
38	0.5 mM	3.74	4.80	6.96	5.65	5.19	0.5 mM	5.13	5.37	6.21	4.64	5.70
	0.5 mM	0.64	0.56	0.58	0.53	0.55						
	2.5 mM	1.43	1.41	1.60	1.56	1.65						
39	1.3 mM	0.69	0.63	0.70	0.74	0.74	1.3 mM	0.68	0.76	0.83	0.83	0.68
40	0.5 mM	0.71	0.67	0.59	0.44	0.46	0.5 mM	0.63	0.77	0.63	0.56	0.63
	2.5 mM	1.00	1.09	0.82	1.05	0.95						

35: 1-cyclopropyl-4-phenoxypiperidine; **38**: 1-methyl-4-phenylpiperidine;
39: 1-cyclopropyl-4-benzylpiperidine; **40**: 1-methyl-4-(3-phenylphenoxy)piperidine.

In the second set of experiments, the molar ratio of enzyme to substrate was 9:2,000 (except for compound **36** which was 9:1,000). Under these conditions the time dependent loss of the substrate was observed. Fig. 14 illustrates typical plots of substrate study by the GC-MS assay. Fig. 14a shows the remaining substrate [1-methyl-4-phenoxypiperidine (**36**)] (vs standard) at a starting concentration of 0.5 mM vs time. It is clear that the substrate disappeared with time. After 90 minutes, no substrate was detected. A semilog plot of the amount of remaining starting material vs time (Fig. 14b) gave a straight line, suggesting that this MAO-B catalyzed oxidation process is a first order process. The rate of the oxidation, calculated from the slope of the line in Fig. 14b, was found to be 20 min^{-1} at 0.5 mM substrate. This rate ($k_{0.5\ mM}$ = 20 min^{-1}) is relatively slow when compared with the rate of oxidation of the corresponding tetrahydropyridine **41** ($k_{0.5\ mM}$ = 240 min^{-1}). This slow rate also

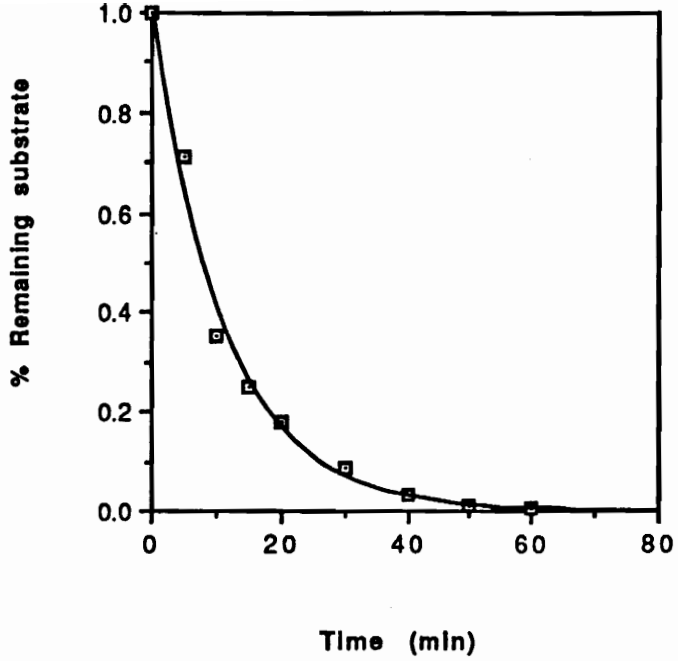


Fig. 14a

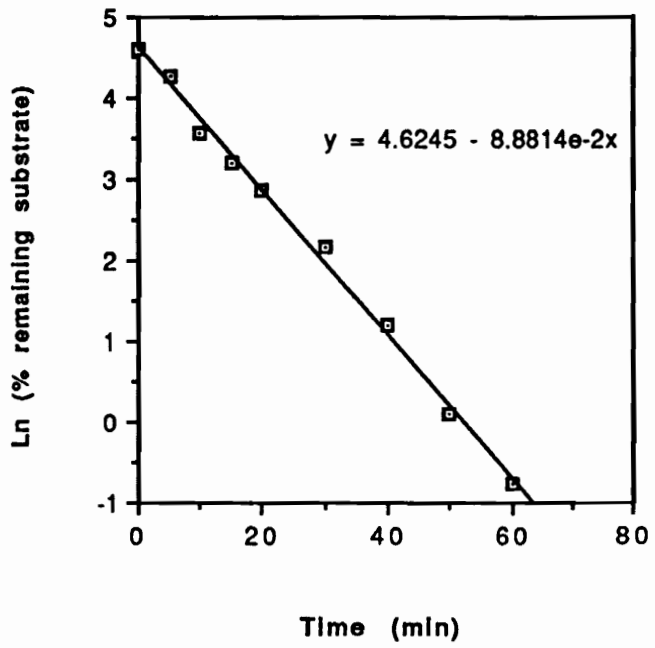


Fig. 14b

Fig. 14. MAO-B Substrate Study on1-Methyl-4-phenoxypiperidine (0.5 mM) - GC/EIMS Method

Fig. 14a (left panel) shows the % remaining substrate vs time

Fig. 14b (right panel) demonstrates the oxidation is first order reaction; the rate constant of the oxidation at 0.5 mM substrate is derived from the slope

explains why we did not observe the oxidation at lower enzyme concentrations. The control experiment, in which MAO-B was preincubated with 10^{-5} M deprenyl (an MAO-B selective inhibitor) for 20 minutes showed that the ratio of substrate (compound **36**) to the internal standard did not change during the 90 minutes incubation period (Table 2). This confirmed that the phenomenon observed was enzyme mediated.

Table 2. Control Experiment with MAO-B on 1-Methyl-4-phenoxypiperidine (**36**)

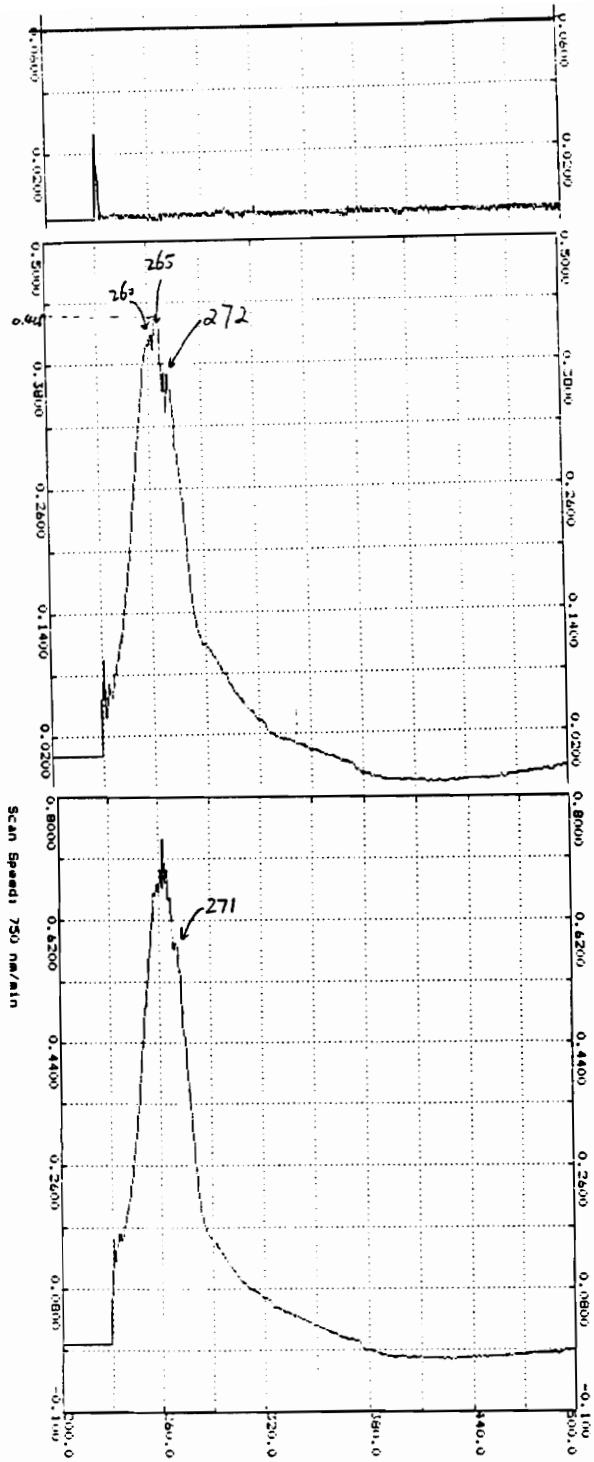
		Substrate/Standard (selected ions 98/77)
T (min)	0	0.88
	90	0.93

4-Ethylphenol was used as internal standard.

Even though we observed the disappearance of **36** by GC-MS, no metabolite(s) was detected in the GC-MS tracing (pH = 11 extract). UV spectral analysis, however, did reveal a possible metabolite. As shown in Fig. 15 (the UV spectrum was difference spectrum by blanking on the substrate sodium phosphate buffer solution), an absorption centered at 265 nm with a shoulder at 271 nm increased in intensity with time. A consideration of the potential reaction pathway for **36** led us to speculate that this chromophore was due to the product of phenol (**62**, Scheme 17). Phenol has a λ_{max} = 270 nm (*68*). It was not surprising that phenol was not be detected in the GC-MS tracing when the incubation mixture was worked up at pH = 11 since at this pH the phenol exists as phenolate anion which would not be extracted into an organic phase.

GC-MS analysis of an extract of the incubation mixture at pH = 2 ~ 3 led to the detection of a peak with a parent ion at m/z 94 and major fragment ion peaks at m/z 74, 66, 39 and 28. The retention was time 4.78 minutes. Under the same GC-MS conditions, commercial phenol gave the same spectrum and retention time (Fig. 16).

Commercial phenol in sodium phosphate buffer solution gave a UV spectrum with a λ_{max} = 271 nm (Fig. 17), which corresponds to the shoulder in the spectrum of the incubation solution. Further confirmation of the presence of the phenol in the incubation mixture was obtained based on the fact that phenol is transformed to the phenolate anion at basic condition (pH > 10) with a λ_{max} shift from 271 nm to 287 nm (*68*). As shown in Fig. 17, the spectra of the basified incubation mixture and the commercial phenol solution gave the same bathochromic shift.



T (min) = 0

Fig. 15a

T (min) = 30

Fig. 15b

T (min) = 60

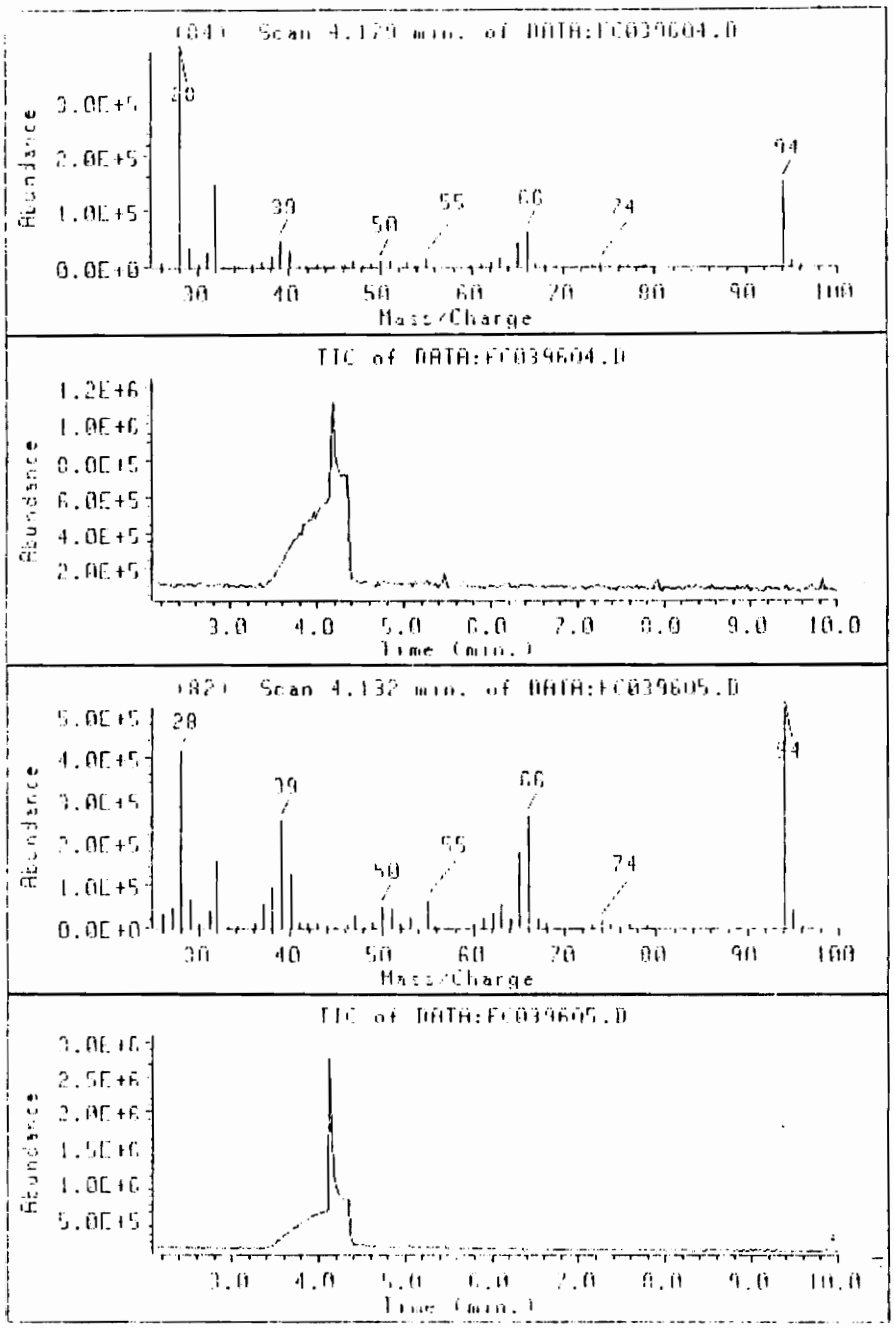
Fig. 15c

Fig. 15. Metabolites Formation Monitoring - UV

Fig. 15a UV spectrum of the incubation mixture at initial time

Fig. 15b UV spectrum of the incubation mixture after 30 min

Fig. 15c UV spectrum of the incubation mixture after 60 min



Extract at pH = 2 ~ 3

Fig. 16a

Commercial Phenol

Fig. 16b

Fig. 16. Phenol Metabolite Tracing - GC/EIMS

Fig. 16a GC/EIMS of extract of the incubation solution at pH = 2 ~ 3

Fig. 16b GC/EIMS of the commercial phenol

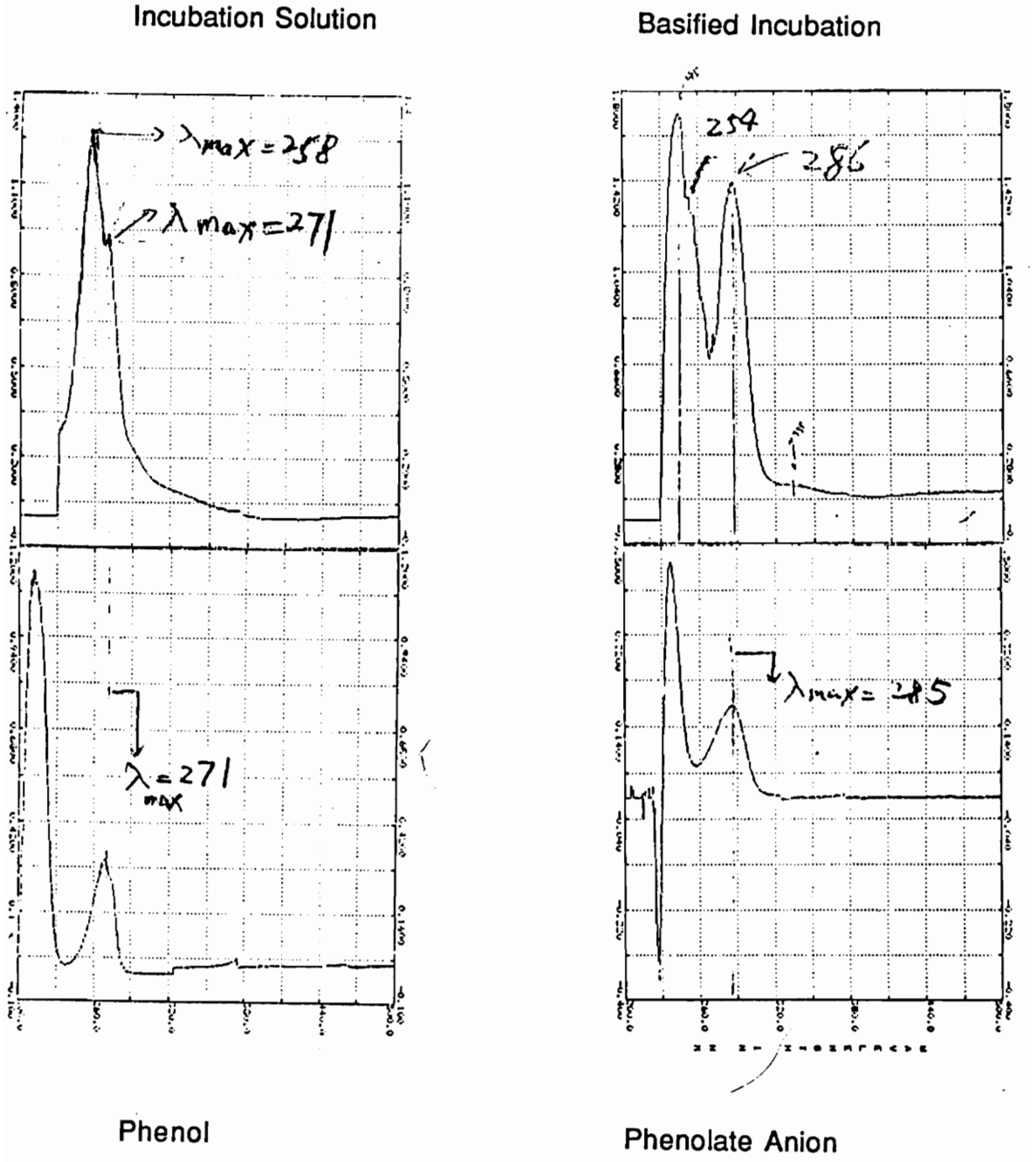
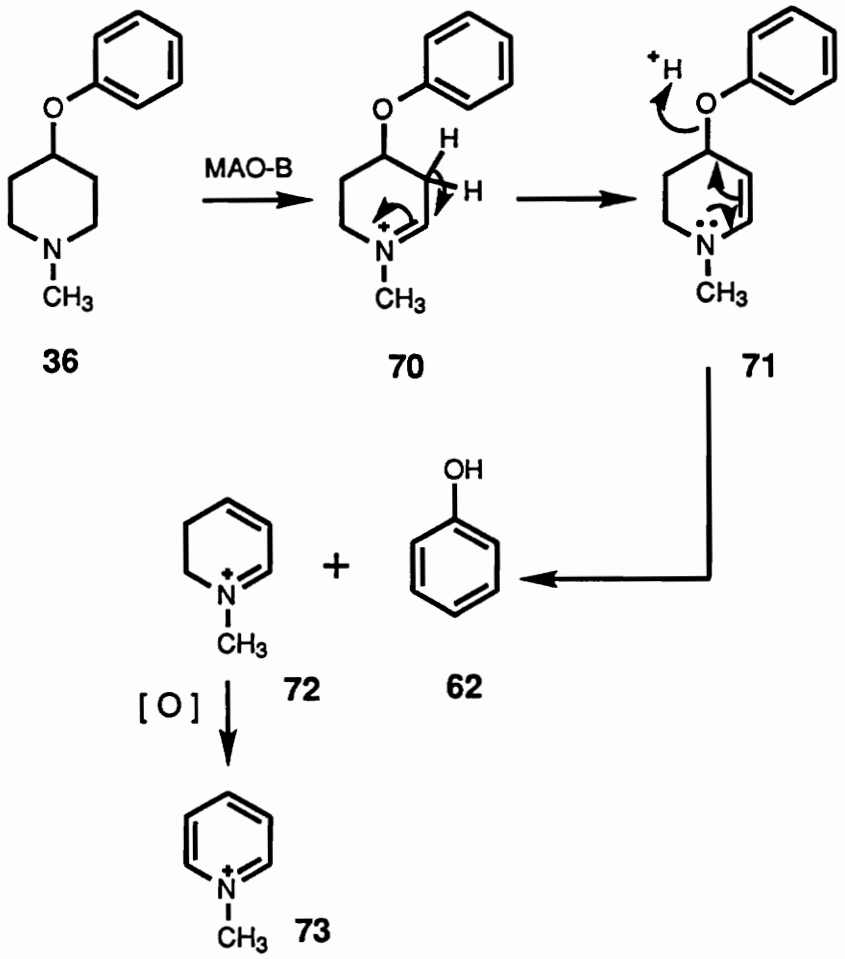


Fig. 17. Phenol Metaboite - UV

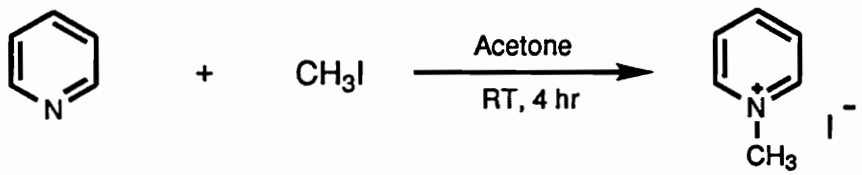
Scheme 17. Proposed Pathway for Metabolite Formation



A possible pathway for the MAO-B catalyzed oxidation of **36** is shown in Scheme 17. The substrate **36** first is oxidized to the tetrahydropyridinium intermediate **70** which undergoes deprotonation at C-3 to yield 1-methyl-4-phenoxy-1,2,3,4-tetrahydropyridine (**71**). Subsequent loss of phenol (**62**) produces the unstable dihydropyridinium species **72** which would be expected to autoxidize to yield the 1-methylpyridinium species **73**.

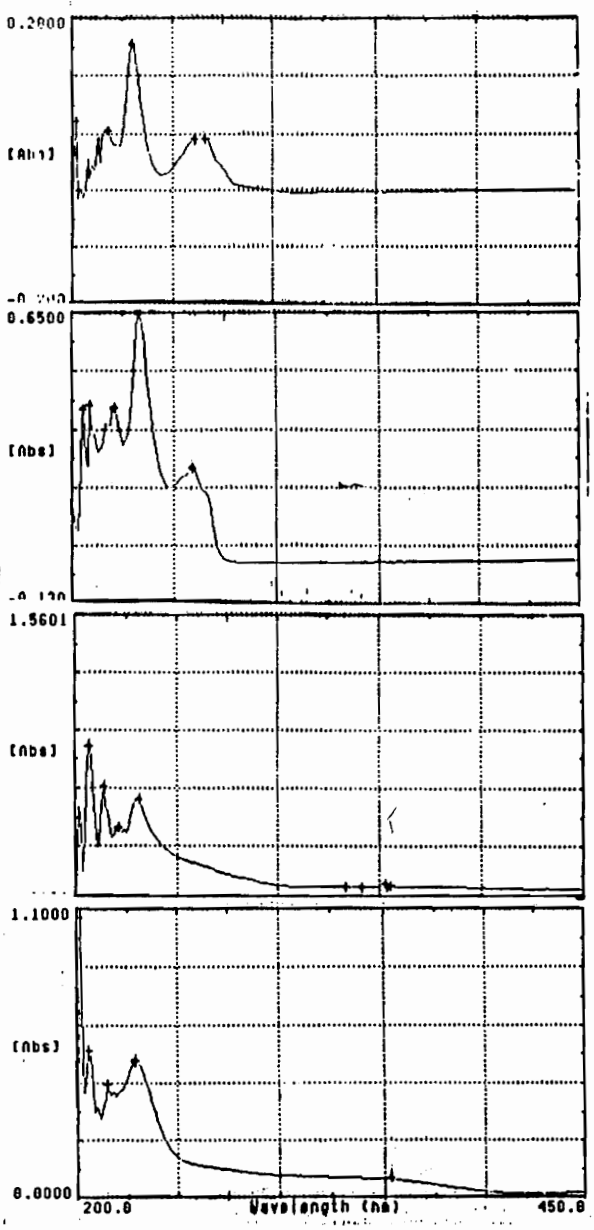
Attempts to detect the proposed 1-methylpyridinium metabolite **73** were pursued by first extracting the post incubation mixture at pH 10 to remove unchanged substrate. The pH was then adjusted to 2 ~ 3 and extracted again to remove the phenol. UV analysis of the remaining aqueous layer demonstrated the spectrum (Fig. 18a) with two bands, λ_{max} = 230 nm and λ_{max} = 263 nm. The 1-methylpyridinium iodide (**73·I**) was obtained by treating pyridine (**74**) with methyl iodide (**75**) at room temperature in acetone (Scheme 18). The synthetic pyridinium product at the same conditions as the incubation mixture gave the same UV spectrum as the metabolite (Fig. 18b). Further confirmation of this structure was obtained by sodium borohydride reduction of both the incubation mixture and the synthetic compound. Disappearance of the characteristic band λ_{max} = 260 nm for both solutions (Fig. 18c, d) confirmed the pyridinium to be present in the incubation mixture and supported the proposed pathway (Scheme 17).

Scheme 18. Synthesis of 1-Methylpyridinium Iodide Salt



74 **75** **73·I**

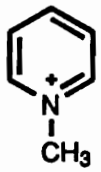
The substrate properties for compounds **35**, **37** and **38** were studied by using the same GC-MS assay. It was found that all of these compounds were metabolized by first order reaction processes. The rate constants at 1 mM substrate were found to be as follows: $k_{(1mM)}$ = 4 min^{-1} (**35**); $k_{(1mM)}$ = 3 min^{-1}



Incubation mixture following extraction of substrate and phenol

A *Fig. 18a*

Synthetic



B *Fig. 18b*

A + $NaBH_4$

Fig. 18c

B + $NaBH_4$

Fig. 18d

Fig. 18. 1-Methylpyridinium Metabolite Identification - UV

Fig. 18a UV spectrum of incubated mixture following extraction of substrate and phenol

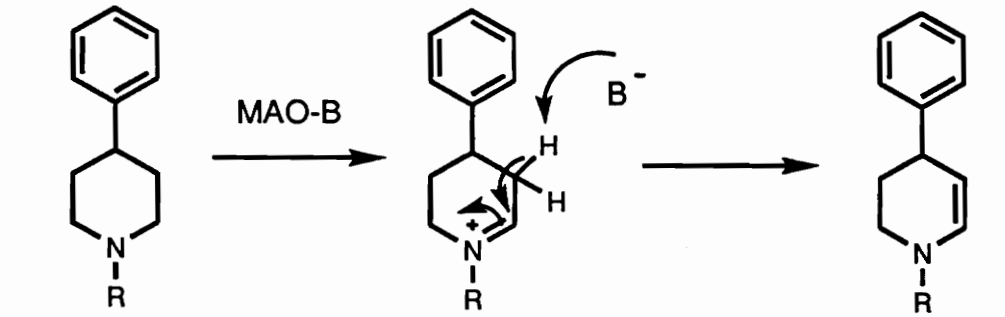
Fig. 18b UV spectrum of synthetic 1-methylpyridinium in sodium buffer solution

Fig. 18c UV spectrum of incubation mixture A after treating with $NaBH_4$

Fig. 18 d UV spectrum of synthetic 1-methylpyridinium B after treating with $NaBH_4$

(**37**); $k_{(1mM)}$ = 4 min^{-1} (**38**). The corresponding tetrahydropyridine analogs have the following rates of oxidation: $k_{(1mM)}$ = 225 min^{-1} (**50**); $k_{(1mM)}$ = 30 min^{-1} (**27**); $k_{(1mM)}$ = 260 min^{-1} (**8**). For compound **35**, phenol was detected by GC-MS as a metabolite. The metabolic pathway is thought to be the same as that proposed for 1-methyl-4-phenoxypiperidine (**36**). For compounds **37** and **38**, the metabolites observed by GC-MS were 1-cyclopropyl-4-phenyl-1,2,3,4-tetrahydropyridine (**76**) and 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (**77**). Product formation presumably proceeds through the same pathway as shown in Scheme 17 except that the process stops at the tetrahydropyridine species since no leaving group is present (Scheme 19).

Scheme 19. Proposed Pathway for the 4-Phenyl Metabolite formation



Of the four piperidine compounds (**35**, **36**, **37** and **38**), the 1-methyl-4-phenoxy analog **36** is the best MAO-B substrate. Interests in pursuing further the kinetic study in order to provide additional valuable information about the catalytic process and binding character on compound **36** led us to estimate the

k_{cat} and K_M values for the conversion.

Attempts to monitor the rate of phenol formation failed because the molar absorptivity is low, [ε_{max} = 1,450 (λ_{max} = 271 nm)] (*68*) and the oxidation process also is very slow. Additionally, the phenol band at 271 nm overlaps with other bands (Fig. 15). Since the enzyme concentration was higher (0.9 μM), the absorbance due to the enzyme (λ_{max} = 274 nm) was significant.

Phenol at pH > 10 is transformed to the phenolate anion which has λ_{max} = 288 nm and ε_{max} = 2,600 (*68*). The higher λ_{max} and ε values improved the accuracy of the assay considerably. From the GC-MS substrate study it was known that the oxidation process is a first order reaction. Therefore by monitoring the rate of phenolate anion formation, the rate of oxidation at various substrate concentrations could be derived. Fig. 19a illustrates the amount of phenolate formed during a certain period of time (30-40 minutes) at various starting substrate concentrations. The rate constants (k_{obs}) of the MAO-B catalyzed oxidation of 1-methyl-4-phenoxypiperidine (**36**) were calculated for each substrate concentration. A lineweaver-Burke plot (Fig. 19b) gave k_{cat} and K_M values 13.8 min^{-1} and 0.9 mM, respectively. Comparison of the kinetic data of **36**, the best MAO-B substrate of the four tested compounds, with those of the corresponding tetrahydropyridine **50** (k_{cat} = 240 min^{-1}; K_M =0.058 mM; k_{cat}/K_M = 4138 $min^{-1}mM^{-1}$ for MAO-B) led to the conclusions that the piperidines are poor substrates for MAO-B. The high K_M value for compound **36**, compared to good MAO-B substrates (K_M ranging from 0.01-0.5 mM), may indicate that the change in geometry has a negative effect on the binding of the piperidine molecule to

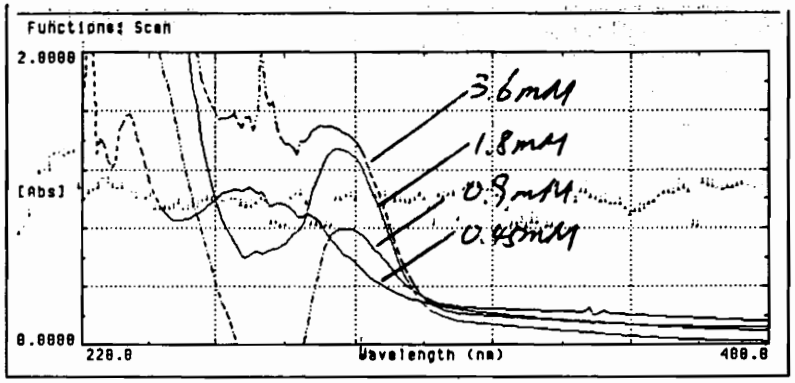


Fig. 19a

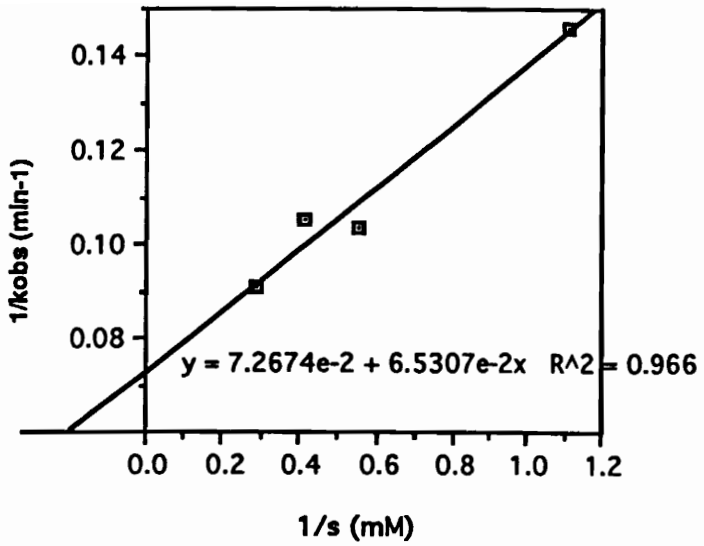


Fig. 19b

Fig. 19. MAO-B Substrate Study on 1-Methyl-4-phenoxypiperidine - UV Method

Fig. 19a UV spectrum trace of amount of phenolate formed during 30-40 min with various starting concentrations of 1-methyl-4-phenoxypiperidine

Fig. 19b Lineweaver-Burke plot constructed from rate constants at different substrate concentrations

the MAO-B active site.

Substrate Study with MAO-A

MAO-A substrate studies with **35**, **38**, **39** and **40** showed that these compounds were not MAO-A substrates, at least at the 0.1 μM enzyme concentration examined (Table 1). The MAO-A substrate properties of 1-methyl-4-phenoxypiperidine (**36**) were also examined at higher enzyme concentrations. The results (Table 3) confirmed that even under these conditions, MAO-A did not catalyze the oxidation of **36**. Limited amount of MAO-A prevented additional experiments.

Table 3. MAO-A Substrate Study on 1-Methyl-4-phenoxypiperidine (**36**)

	36 (selected ions 98/77)	Control (selected ions 98/77)
T (min) = 0	0.51	0.57
= 90	0.51	0.50

4-Ethylphenol was used as the internal standard.

That all of these piperidine analogs have poor substrate properties for both forms of MAO meets our expectations. These results suggest that the $C_{4\text{-}5}$ double bond plays an important role in the catalytic process. Our interpretation of these results will be considered in greater detail following the presentation of the results of our inhibition studies.

3.2.2. Inhibition Studies

Inhibition Study with MAO-B

The MAO-B inhibiting properties of the piperidine derivatives prepared in these studies were investigated by assessing the rate of loss of enzyme activity (as measured by the initial rate of oxidation of 5 mM MPTP) vs time at different concentrations of the potential inhibitor. Fig. 21a and Fig. 20b present the data for 1-cyclopropyl-4-phenoxypiperidine (**35**). From Fig. 20a, the observed rate of loss of enzyme activity (k_{obs}) could be calculated for each concentration of the inhibitor. It is clear that the rate of loss of enzyme activity is time dependent and increases as the concentration of inhibitor **35** increases. These characteristics of time and concentration dependent inhibition of enzyme activity are typical of those associated with mechanism based inactivators that lead to irreversible enzyme inhibition. The double reciprocal plot of $1/k_{obs}$ vs $1/_{\text{inhibitor concentration}}$ (Fig. 20b) provided estimates of k_{inact} (the maximum rate of inactivation) and K_I (the concentration of inhibitor that gives 50% maximal rate of inactivation).

Compounds **37** and **38** were also found to be time and concentration dependent MAO-B inhibitors. The k_{inact} and K_I data are summarized in Table 4. However, compound **36** was found to be linear time dependent inhibitor only during the first initial 10 minutes of the study. The observed rates of loss of enzyme activity vs time plots for **36** are shown as Fig. 21. The rate of inhibition was fast at initial time and then slowed. One possible reason for this behavior may be that compound **36** is a moderate substrate for MAO-B. In the presence of substrate (which is the molecule itself in this case), that the rate of inhibition is

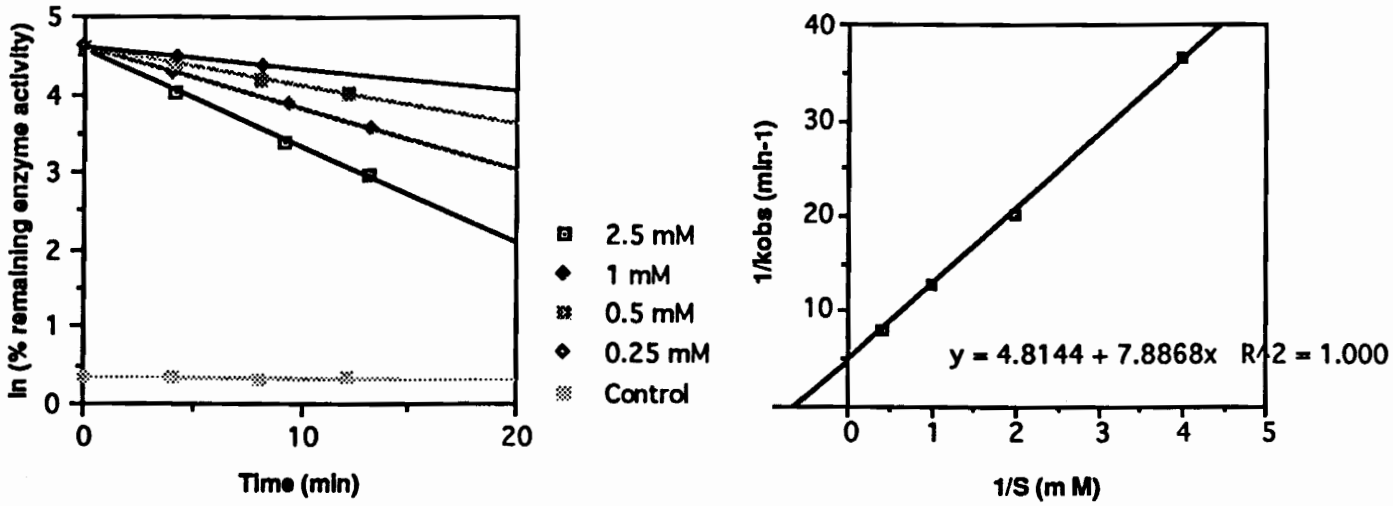


Fig. 20a

Fig. 20b

Fig. 20. MAO-B Inhibition Study on 1-Cyclopropyl-4-phenoxypiperidine

Fig. 20a (left panel) shows the time and concentration dependent inactivation curves
Fig. 20b (right panel) is a double reciprocal plot constructed from the linear semilog plots shown in Fig. 21a

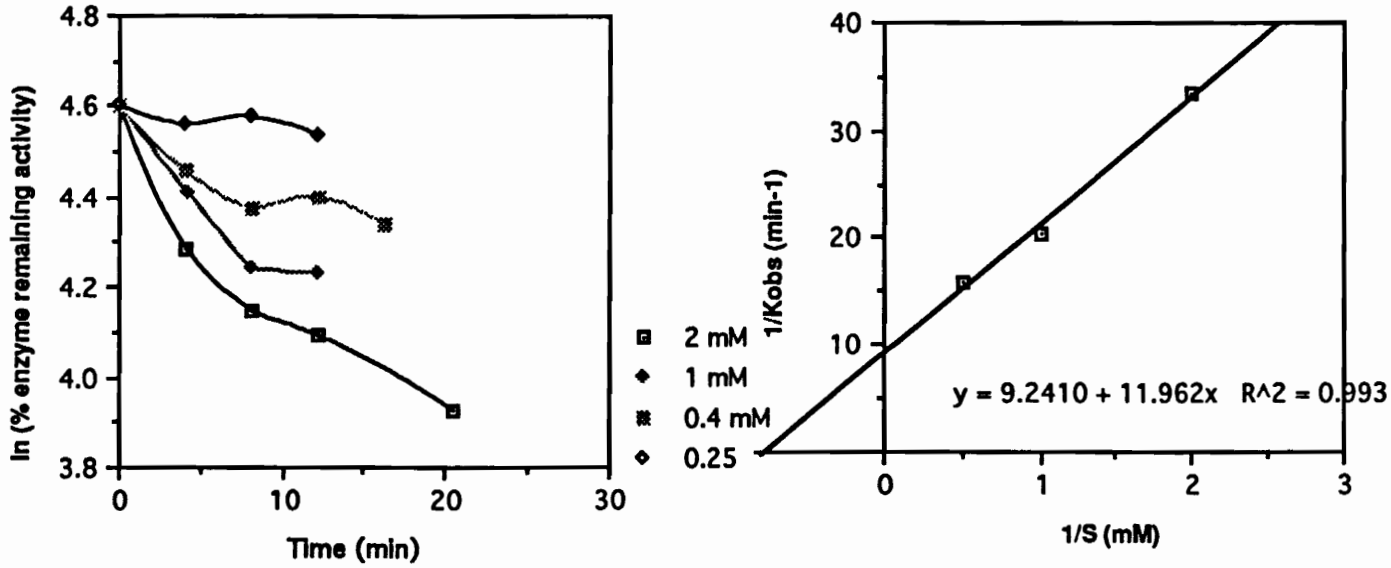


Fig. 21a

Fig. 21b

Fig. 21. MAO-B Substrate Study on 1-Methyl-4-phenoxypiperidine

Fig. 21a (left panel) shows the inactivation curve vs. time at different concentrations of the inhibitor
Fig. 21b (right panel) is double reciprocal plot (rate constants are from the initial period)

slowed down is a character of an mechanism based inactivator. This behavior was also observed in the irreversibility experiment in which the time needed to completely inactivate the MAO-B was much longer than the calculated time (for detailed discussion see the irreversibility part). The k_{inact} and K_I values for compound **36** were calculated based on the initial four minutes.

Table 4. MAO-B Inhibition Study

Compound	N	C-4	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I ($min^{-1}mM^{-1}$)
35	c	OPh	0.21	1.6	0.13
36	m	OPh	0.11	1.3	0.08
37	c	Ph	0.11	1.3	0.08
38	m	Ph	0.04	0.4	0.09
39	c	CH_2Ph	0.13	0.8	0.15
40	c	(3'-Ph)OPh	very poor		

c: cyclopropyl; m: methyl

Table 4 summarizes the kinetic data of all the compounds for the inhibition of MAO-B. The 1-cyclopropyl analogs show reasonably good MAO-B inhibitor properties. Surprisingly, the k_{inact}/K_I values for the 1-methyl derivatives are also around 0.1 $min^{-1}mM^{-1}$, the same as those for 1-cyclopropylpiperidines. These results indicates that both N-methyl and N-cyclopropyl derivatives have similar MAO-B inactivation properties.

Inhibition Study with MAO-A

The MAO-A inhibiting properties of these piperidine derivatives were determined in the same way as with MAO-B except that the MAO-A substrate used was 1 mM 1-methyl-4-phenoxy-1,2,3,6,-tetrahydropyridine. The kinetic data are summarized in Table 5 which also includes the data for the corresponding tetrahydropyridines for comparison. From Table 5, we notice that there are two types of inhibition profiles for the 1-cyclopropylpiperidines when compared with the corresponding tetrahydropyridines. In one case the 1-cyclopropyl piperidine derivative show less inhibiting ability than the corresponding tetrahydropyridine. For example, piperidine **37** is a less powerful inhibitor than the corresponding tetrahydropyridine **27**. The k_{inact} values decreased and K_I values increased for these piperidines. This means that the inhibiting properties for MAO-A of these compounds decrease after removing the double bond. Also note that the tetrahydropyridines **27** and **43** are non MAO-A substrates. On the other hand, 1-cyclopropyl-4-phenoxypiperidine (**35**) is a very good inactivator (the inactivation was too fast to obtain kinetic data) while the 1-cyclopropyl-4-phenoxy-1,2,3,6-tetrahydropyridine (**50**) shows neither inhibitor nor substrate properties toward MAO-A. If the previous case can be explained by the assumption that the geometry change from that of a tetrahydropyridine to piperidine decreases the MAO-A inactivation, then the later case is just the opposite. This may indicate that it is not the planar feature of the 4-5 position in the ring that plays a role in the inhibitor recognition (more specifically, binding recognition), but the preference of the geometry of the whole molecule that is more important.

Table 5. MAO-A Inhibition Study

Compound	N	C-4	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I ($min^{-1}mM^{-1}$)
35^P	c	OPh	very high	very low	
50^T	c	OPh		not inhibitor	
36^P	m	OPh	0.08	0.61	0.14
41^T	m	OPh		not inhibitor	
37^P	c	Ph	0.19	0.07	2.56
27^T	c	Ph	0.52	0.015	34.54
38^P	m	Ph		not inhibitor	
8^T	m	Ph		no data (not inhibitor)	
39^P	c	CH_2Ph	0.15	0.04	3.92
43^T	c	CH_2Ph	very high	very low	
40^P	m	(3'-Ph)OPh	0.34	2.72	0.12
44^T	m	(3'-Ph)OPh		no data (not inhibitor)	

P: piperidine; T: tetrahydropyridine; c: cyclopropyl; m: methyl

The 1-methylpiperidine compounds **36** and **40** gave the same surprising results as those of the MAO-B inhibition study. The inhibitor properties associated with 1-methyl compounds have been observed with some of 1-methyltetrahydropyridines also (*69,70*). It was reported by Singer *et al.* (*69*) that MPTP (**8**) is a time and concentration dependent inactivator with k_{inact} = 0.034 min^{-1} at an inhibitor concentration of 5 mM. Normally, because MPTP is such a good MAO-B substrate, its inactivator properties are ignored. The inhibition properties of 1-methyl-tetrahydropyridines **41** and **44**, which are good MAO-A

substrates, have not been fully examined. Like MPTP, even though **41** and **44** may be inactivators, these inhibiting properties may be overwhelmed by their substrate properties.

Reversibility Study of MAO-B Inhibition with Piperidines **35** and **36**

Attempts to answer mechanistic questions associated with the observation that 1-cyclopropyl and 1-methylpiperidines have the same MAO-B inhibition properties led us to examine the reversibility of the MAO-B inhibition by compounds **35** and **36**. The reversibility of the MAO-B inhibition by the 1-cyclopropyl piperidine **35** and 1-methyl analog **36** was studied by gel filtration. The activity of the inactivated enzyme was examined before and after the incubation solution was passed through a Sephadex G-25 column. Control incubations, which contained no potential inhibitor, also were run.

Table 6. Reversibility of the Inhibition of MAO-B by 1-Cyclopropyl and Methyl-4-phenoxypiperidines (**35**) and (**36**)

	35		**36**		Control	
	Before	After	Before	After	Before	After
MAO-B activity	0.064	0.057	0.140	0.131	0.313	0.301
% vs control	20	19	42	44	100	100

As shown in Table 6, in the case of compound **35**, the preincubated enzyme had the same activities before (20% vs control) and after (19% vs

control) passing through the Sephadex column (the enzyme activity was indicated by the rate of oxidation of MPTP). Since that the enzyme activity did not return, the inhibitor is probably covalently (strongly) bound with the enzyme and the inhibition is irreversible. Compound **36** is also an MAO-B irreversible inhibitor.

We noticed that the time needed for compound **35** to completely inactivate the enzyme was much longer than the time calculated by using the first order reaction rate equation $[A]_t = [A]_o\ e^{-kt}$. For 1-methyl-4-phenoxypiperidine (**36**) the time prolongation was even longer. This may be explained by the fact that these compounds are not only inhibitors, but also substrates for MAO-B. As shown in Scheme 2, for better substrates, the partition between the enzyme and substrate/inhibitor binding species (E ···· I) will be more favorable for substrate turnover and therefore the inhibition process will be retarded. This is behavior characteristic of irreversible inhibitors.

For compound **36**, after a 6 hour incubation period, 40% enzyme activity still remained. This is because the compound had been complete consumed by the enzyme before the enzyme was complete inactivated. Compound **36** is a better substrate than **35**, with $k_{(1mM)} = 14\ min^{-1}$. The calculated time needed to convert all of the 0.5 μmol of compound **36** by the 2.25 nmol of the enzyme is 16 min (assuming no inhibition). The actual time required to convert all the starting material was 2 hours since the compound is also an inactivator.

Chapter IV. Conclusions

The objective of this exercise was to evaluate the importance of the double bond on the substrate and inhibitor properties of cyclic tertiary amines and to understand better the mechanism of the MAO catalyzed oxidation of these types of amines.

Table 7 summarizes the kinetic data. K_M and K_I are the parameters that describe the binding of the substrate and inhibitor to the enzyme's active site. Almost all of the K_M and K_I values of the piperidines for both forms of the enzymes are higher than those of the corresponding tetrahydropyridines. Exceptions are the K_I for 1-cyclopropyl-4-phenoxypiperidine (**35**) with MAO-A and 1-cyclopropyl-4-benzylpiperidine (**39**) with MAO-B. This may indicate that binding of piperidines to the enzyme is not as effective as for tetrahydropyridines. The QSAR study carried out in our laboratory on the tetrahydropyridine system demonstrates that the dihedral angle between the tetrahydropyridine ring and the ring at C-4 should be within a certain range to be accommodated by the enzyme active site (*63*) The trend of the higher K_M and K_I values of the piperidine may reflect that the greater flexibility at 4-5 position changes the dihedral angle between the two rings to an unfavorable conformation. In contrast, for piperidine derivative **35**, the inhibiting activity toward MAO-A is so high that the K_I could not be accurately measured by the method used in our laboratory. The corresponding tetrahydropyridine **50** is neither an inhibitor nor a substrate. The piperidine derivative **39** seems to bind to MAO-B better than the corresponding tetrahydropyridine **43** [compare the K_I

Table 7. Kinetic Data Summary

Comp.	N	C-4	MAO-A k_{cat} or k_{obs} (min^{-1})	MAO-A K_M or M (mM)	MAO-A k_{inact} (min^{-1})	MAO-A K_I mM	MAO-A K_{inact}/K_I (min^{-1} mM^{-1})	MAO-B k_{cat} or k_{obs} (min^{-1})	MAO-B K_M or M (mM)	MAO-B k_{inact} (min^{-1})	MAO-B K_I (mM^{-1})	MAO-B k_{inact}/K_I (min^{-1} mM^{-1})
19P	c	OPh	No test		high	low		4a	1a	0.21	1.6	0.13
25T	c	OPh	None			None		215	0.13		None	
20P	m	OPh	None		0.08	0.61	0.14	14.3	0.92	0.11	1.3	0.08
26T	m	OPh	130	0.055		none		241	0.058		poor	
21P	c	Ph	poor		0.19	0.075	2.58	3a	1a	0.11	1.3	0.08
27T	c	Ph	None		0.52	0.015	34.5	poor		0.7	0.18	3.85
22P	m	Ph	poor			None		3a	1a	0.04	0.43	0.09
8T	m	Ph	31	0.65		None		273	0.19		poor	
23P	c	b	poor		0.15	0.04	3.9	poor		0.13	0.85	0.15
29T	c	b	None		high	low		637	0.41	0.70	1.5	0.47
24P	c	d	poor		0.34	2.72	0.12	poor			poor	
30T	c	d	1292	0.23		None		220	0.35		None	

P: piperidine; T: tetrahydropyridine; c: cyclopropyl; m: methyl; b: benzyl; d: (3'-phenyl)phenoxy. a: k_{obs}, M data.

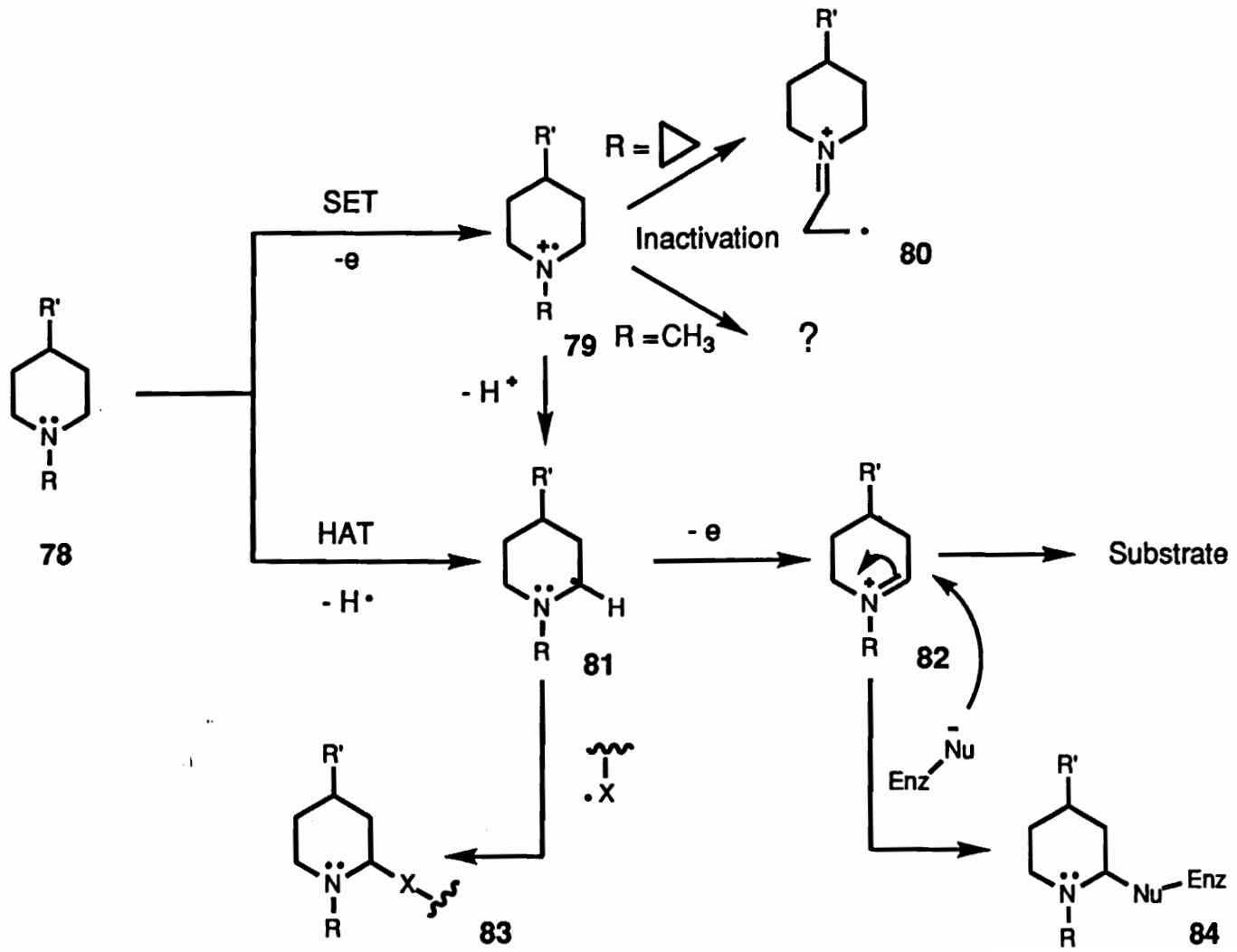
values (Table 7)]. This may suggest that the absence of the double bond in the case of **39** and **43** change the geometry of the molecule to a better conformation that can be accommodated better by the enzyme's active site. It appears that the double bond is not necessary for effective binding.

By comparing the K_I values of the piperidines for MAO-A with those for MAO-B, we notice that the K_I values for MAO-A are always lower than those for MAO-B. The K_I for 1-methyl-4-phenoxypiperidine (**36**), for example for MAO-A is 0.61 mM and for MAO-B the value is 1.29 mM, twice of the value for MAO-A. This indicates that the MAO-A active site is less sensitive to the geometry change (or steric effect) than the MAO-B active site.

The kinetic results demonstrate that all of the piperidines examined are very poor MAO substrates but relatively good inhibitors for both forms of the enzymes. These results are not consistent with the polar addition-elimination pathway since tertiary amines are unlikely to form an adduct with FAD due to steric crowding.

According to the SET pathway, rapid ring opening of the aminyl cation radical **79** (Scheme 20) occurs after the substrate looses one electron from lone pair of the nitrogen of the substrate. The similar inhibition properties of both the 1-cyclopropyl- and 1-methylpiperidines are hard to explain in terms of this pathway. First of all, if the radical **80** resulting from the cyclopropyl ring opening is the inactivating species, then what is the corresponding species in the 1-methyl series? Secondly, the same inhibiting properties among all of the

Scheme 20. Possible Pathways for the MAO-B Catalyzed Oxidation of Piperidines

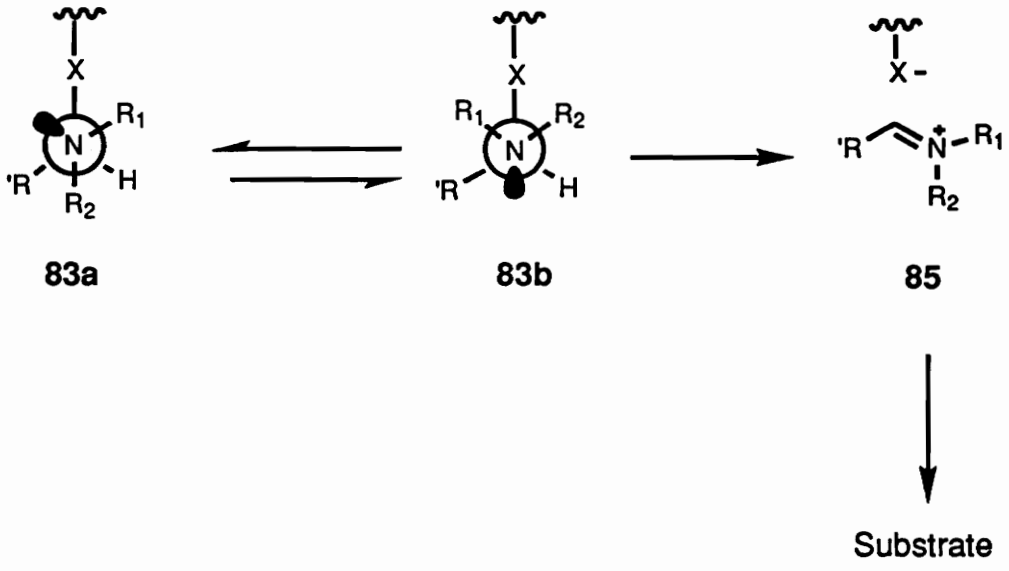


piperidines examined suggests that the inactivating species is independent of the N-substituent. The carbon centered radical **81** could be the inactivating species which can bind with a radical residue at the enzyme active site. Earlier evidence (*71*) indicates that the group on the enzyme that binds the radical is a cysteinyl residue. A cysteinyl radical could be generated by hydrogen atom transfer from the cysteinyl thiol to the flavin semiquinone radical anion. A nucleophilic species on the enzyme also could attack the iminium species **82** to inactivate the enzyme. A similar species was proposed to explain the MAO-B inactivation caused by 3,3-dimethyl-MPTP (*72*). The formation of iminium species like **82**, which presumably are formed from radical intermediates like **81** via electron transfer, has been documented in our metabolic profiling work.

To rationalize the observation that 1-methylation or N-,N-dimethylation of primary amines converts MAO-B substrates inactivators of the enzyme, Silverman *et al.* (*73*) proposed an α-carbon centered radical to be the responsible inactivation species. This proposal is similar to the our proposal in that the inactivation is a result of the radical **81** binding to an X· residue on the enzyme. According to Silverman, the cause for enzyme inactivation is the stabilization of the covalent intermediate **83** in the reaction as a result of the inductive effect of the atom at the C-4 position and a stereoelectronic effect as a result of hindered rotation of an active site covalent adduct. In the case of a tertiary amine, the conformation **83a** (Scheme 21) is preferred since it minimizes gauche interactions. This conformation orients the orbital containing the nonbonded electrons orthogonal to the C-X bond and, therefore, expulsion of the leaving group (the active site residue) becomes more difficult. An

inductive effect from the R' group when the C-4 group is electron withdrawing makes the iminium species **85** less stable and therefore the adduct **83** is less likely to dissociate to the iminium ion **85**. In the case when R_1 and R_2 are small groups like H, the Newman projection adopts conformation **83b** to take advantage of the anomeric stabilizing effect with the nonbonded electrons of the nitrogen atom antiperiplanar to the C-X bond. This conformation readily expels the leaving group (the active site amino acid residue) to give the iminium ion **85** and reduced enzyme. The inductive effect when the C-4 group is electron-donating can assist in the formation of the iminium ion **85**.

Sheme 21. Newman Projections of the Adduct **83**



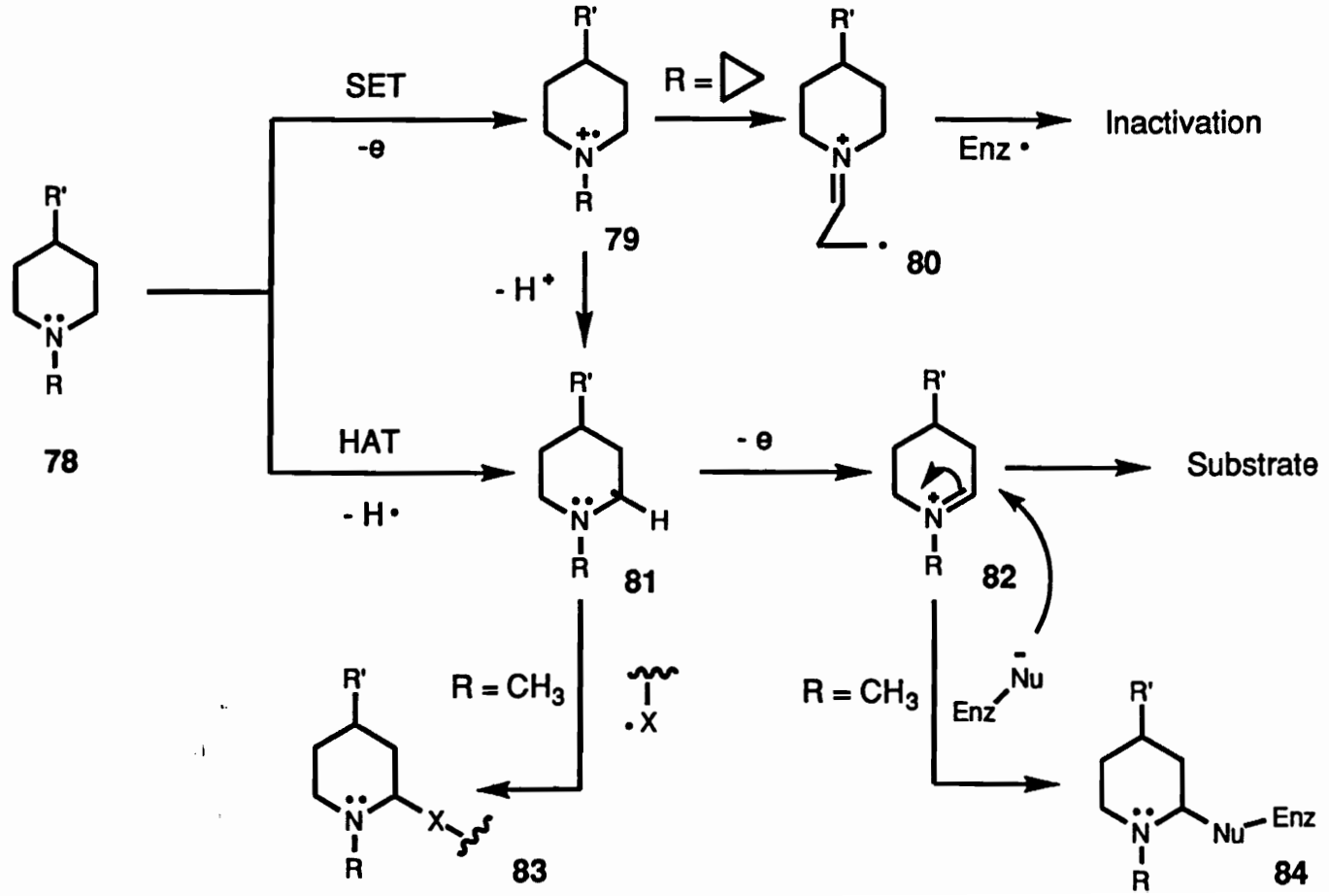
Based in the above discussion, we should expect, in the case of tertiary cyclic amines when the C-4 position bears an electron withdrawing group, that the molecule would be more likely to be an irreversible inactivator. The

reversibility of the MAO-B inhibition by 1-cyclopropyl-4-phenoxypiperidine (**35**), and 1-methyl-4-phenoxypiperidine (**36**) demonstrate that both compounds are irreversible inhibitors of MAO-B. This partially supports the above hypothesis. The poor substrate properties of the piperidines can be explained in terms of the lack of stabilization of radical species **81** (Scheme 20). Therefore the oxidation of **78** to radical **81** is slow.

In the case of MAO-A, the inactivation properties of the 1-cyclopropyl and 1-methyl derivatives are dramatically different. The 1-cyclopropyl-4-substituted piperidines are much more potent inhibitors than the 1-methyl derivatives. For example, 1-cyclopropyl-4-phenoxypiperidine (**35**) is an excellent inactivator of MAO-A with a k_{inact} too fast to be measured and an K_I value too low to be estimated by the methods we used in our laboratory. On the other hand, the 1-methyl derivative **36** is only a good inactivator with K_{inact} = 0.08 min^{-1} and K_I = 0.61 mM. These results may indicate that reactive species derived from the 1-cyclopropylpiperidine derivatives are different from those derived from the 1-methylpiperidine derivatives. Radical **80** (Scheme 22) resulting from the opening of the cyclopropyl ring, may be responsible for the fast inactivation. The MAO-A inactivation properties of 1-methylpiperidines could be the same as for MAO-B in which case radical **81** or iminium **82** would be the inactivating species. The observation that the k_{inact}/K_I values for 1-methyl-4-phenoxypiperidine (**36**) and 1-methyl-4-(3-phenylphenoxy)piperidine (**40**) are around 0.1 $min^{-1}mM^{-1}$ (Table 7) support this view.

None of the piperidines displayed MAO-A substrate properties. This may

Scheme 22. Possible Pathways for the MAO-A Catalyzed Oxidation of Piperidines



be due to the lack of stabilization of radical **81** (Scheme 22). Or the other possibility is that the active site can not accommodate to these compounds in a catalytically effective way.

In summary, studies on the piperidine system have provided useful information on the differences of the MAO-A and MAO-B active sites, the role of the double bond at the 4-5 position in the tetrahydropyridine ring, and also some insight into the mechanism of MAO catalyzed oxidation of cyclic tertiary amines. In terms of mechanism, the cyclopropyl ring opening pathway may operate with these compounds in the case of MAO-A. In the case of MAO-B, the conformation of the molecule at the enzyme's active site may prevent ring opening in which case a radical or iminium species may be the responsible inactivating species. The double bond at 4-5 position in the tetrahydropyridine ring may not only set the geometry of the molecule but also may stabilize the radical leading to substrate turnover.

This work supports the substrate partition proposal in explaining the substrate properties observed with some 1-cyclopropyl-4-substituted tetrahydropyridines. In the absence of stabilization of the double bond, radical **81** (Scheme 20 or 22) is unstable and therefore the substrate properties are diminished or abolished. On the other hand, the cyclopropyl ring opening is not effected by the absence of the double bond.

Future Studies

Comparing the isotope effects for the inactivation of MAO-A by

appropriately deuterated 1-cyclopropylpiperidine and tetrahydropyridine derivatives could lead to some useful information. Assuming that the initial single electron transfer is rate determining step for SET and the hydrogen atom loss is the rate limiting step for HAT, if SET operates only, then the isotope effects observed for the piperidine and tetrahydropyridine analogs should be the same. If HAT is the main pathway, the primary isotope effects should be different between the piperidine and tetrahydropyridine analogs as reflected by the differences in the α C-H bond dissociation energies.

To further investigate the species responsible for the inactivation, kinetic isotope effect studies with selected deuterated piperidine derivatives towards both forms of the enzymes may be informative. If the radical like **81** or **82** (Scheme 20 or 22) (generated by either SET or HAT) is responsible to the inactivation, then there should be a primary isotope effect (HAT) or no isotope effect (SET). If the cyclopropyl ring opened radical is the inactivating species, no isotope effect should be observed (SET pathway) or a reverse isotope effect should be observed (HAT).

Chapter V. Experiment

5.1. Chemistry

General. Reagents and starting materials were obtained from commercial suppliers and were used without further purification. Tetrahydrofuran and diethyl ether were distilled from sodium/benzophenone ketyl. Reactions were conducted using flame dried glassware under an atmosphere of dry nitrogen. Chromatography refers to flash column chromatography on silica gel unless otherwise noted. Melting points were performed on a Thomas-Hoover melting point apparatus and are uncorrected. Microanalyses were performed by Atlantic Microlab, Inc., Norcross, GA. Proton and carbon spectra were recorded on a Bruker WP 270-MHz, or Varian 400-MHz spectrometer. Chemical shifts are expressed in ppm downfield from internal tetramethylsilane ($\delta = 0$). Spin multiplicities are given as s (singlet), bs (broad singlet), d (doublet), t (triplet) or m (multiplet). Coupling constants (J) values are given in hertz (Hz). Gas chromatography-electron ionization mass spectrometry (GC-EIMS) was performed on a Hewlett Packard 5890 GC fitted with an HP-1 capillary column which was coupled to a Hewlett Packard 5870 mass-selective detector. Data were acquired using an HP 5970 Chemstation. The following GC temperature program was employed unless otherwise noted: 125 °C for 2 minutes, followed by a ramp of 20 °C for 10 minutes. Normalized peak heights are reported as a percentage of the base peak.

General Procedures for Synthesis the Oxalate Salts of 1,4-Substituted piperidines 36, 37, 38, 39 and 40. The appropriate 4-substituted-1,2,3,6-tetrahydropyridine (1 mmol) was dissolved in 95% ethanol (30 mL). 10% Pd on carbon (5% of the weight of the tetrahydropyridine) was added to the solution. The resulting mixture was stirred under hydrogen at 25 °C, 1 atm for 3 days. The reaction mixture was filtered through celite, giving a clear solution. The solvent was removed to yield the crude product. The oxalate salt was made by adding an ethereal solution of oxalic acid (1.5 eq.) to the tetrahydropyridine in ether. The analytical sample was recrystallized from the indicated solvent system.

1-Methyl-4-phenoxypiperidine-$(COOH)_2$ (36). Recrystallized from ethanol (0.24 g, 93%): mp 157-158 °C; ^{1}H-NMR (DMSO-d_6) δ 7.29 (m, 2H), 6.97 (m, 3H), 4.60 (m, 1H), 3.25 (m, 4H), 3.05 (s, 3H), 1.97 (m, 4H); ^{13}C-NMR (DMSO-d_6) 165.1, 167.1, 130.1, 121.5, 116.4, 68.7, 50.2, 43.0, 27.9; MS(EI) m/z (%) 191 (34), 163 (4), 114 (3), 98 (100), 70 (34), 55 (42), 42 (46). Anal. Calcd. for $C_{14}H_{19}NO_5$: C, 59.78; H 6.81; N 4.98. Found: C, 59.80; H 6.85; N 4.92.

1-Cyclopropyl-4-phenylpiperidine-$(COOH)_2$ (37). Recrystallized from methanol/ether (0.2 g, 91%): mp 178 - 178.5 °C (decomposed); ^{1}H-NMR (DMSO-d6, 400 MHz) δ 7.28 (m, 2H), 7.19 (m, 3H), 3.34 (m, 2H), 2.69 (m, 2H), (2.58, 1H), 2.42 (m, 1H), 1.87 (m, 2H), 1.76 (m, 2H), 0.67 (m, 4H); MS(EI) m/z (%) 210 (21), 186 (30), 172 (100), 129 (6), 115 (11), 91 (15), 82 (52), 68 (18). Anal. Calcd. for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.27; N, 4.81. Found: C, 65.94; H,

7.25; N, 4.75.

1-Methyl-4-phenylpiperidine-(COOH)$_2$ (38). Recrystallized from methanol/ether (0.23 g, 93%): mp 144-144.5 °C; ^{1}H-NMR (CD_3OD) δ 7.18 (m, 5H), 3.50 (m, 2H), 3.04 (m, 2H), 2.81 (s, 3H), 2.72 (m, 1H), 1.97 (m, 4H); MS (EI) m/z (%) 175 (96), 174(100), 160 (5), 115(12), 97 (31), 77 (20), 70 (100), 57 (43). ANal. Calcd. for $C_{14}H_{19}NO_4$: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.32; H, 7.17; N, 5.23.

1-Cyclopropyl-4-benzylpiperidine-(COOH)$_2$ (39). Recrystallized form methanol/ether (0.14 g, 91%): mp 152-152.5 °C; ^{1}H-NMR (DMSO-d_6) δ 7.27 (m, 2H), 7.17 (m, 3H), 3.31 (m, 2H), 2.49 (m, 3H), 1.69 (m, 3H), 1.34 (m, 2H), 0.83 (m, 2H), 0.66 (m, 2H); MS(EI) m/z (%) 215 (48), 200 (100), 186 (54), 124 (31), 108 (33), 91 (94), 82 (55), 55 (48). Anal. Cald. for $C_{17}H_{23}NO_4$: C, 66.87; H, 7.59; N, 4.59. Found: C, 66.72; H, 7.56; N, 4.57.

1-Methyl-4-(3-phenylphenoxy)piperidine-(COOH)$_2$ (40). Recrystallized from methanol/ether (0.14 g, 89%): mp 168-169 °C; ^{1}H-NMR (DMSO-d_6) δ 7.66 (m, 2H), 7.43 (m, 4H), 7.25 (m, 2H), 7.02 (d, J = 8.2 Hz, 1H), 4.73 (m, 1H), 3.19 (m, 4H), 2.74 (s, 3H), 2.00 (m, 4H); MS(EI) m/z (%) 267 (24), 239 (3), 141 (9) 115 (12), 98 (100), 60 (30), 55 (46). Anal. Cald. for $C_{20}H_{23}NO_5$: C, 67.21; H, 6.49; N, 3.92. Found: C, 67.11; H, 6.51; N, 3.87.

1-Cyclopropyl-4-piperidinol (58). A stirred solution of 1-cyclopropyl-4-piperidone (**59**) (*62*) (0.278 g, 2 mmol) in absolute ethanol (50 mL) was

treated at 0 °C portionwise with $NaBH_4$ (0.076 g, 2 mmol). After stirring for an additional 4 hours at room temperature, the solvent was removed. The residue was treated with water and the mixture was acidified to pH = 1-2 with dilute hydrochloric acid. Then the pH of the solution was adjusted 9-10 by Na_2CO_3 solution. The resulting solution was extracted with ethyl acetate (4 x 40 mL). The organic layers were combined and dried over $MgSO_4$, and evaporated to yield the crude product. ^{1}H-NMR ($CDCl_3$) δ 3.65 (m, 1H), 2.90 (m, 2H), 2.32 (m, 2H), 1.83 (m, 2H), 1.56 (m, 3H), 0.43 (m, 4H); ^{13}C-NMR ($CDCl_3$) δ 67.8, 51.3, 38.3, 34.2, 5.9; MS (EI) m/z (%) 141 (48), 126 (94), 112 (90), 96 (31), 82 (95), 68 (73), 41 (100), 28 (93).

1-Cyclopropyl-4-phenoxypiperidine-$(COOH)_2$ (35). Diethyl azodicarboxylate (2 mmol) in THF (10 mL) was added into a stirred solution of 1-cyclopropyl-4-piperidinol (**58**, 2 mmol), phenol (2 mmol) and triphenylphosphine (2 mmol) in 50 mL anhydrous THF. After stirring at room temperature for 24 hr, the reaction mixture was treated with dilute hydrochloric acid, to adjust the pH to 1-2, followed by extracting with diethyl ether (4 x 40 mL). The aqueous solution was basified to pH = 9-10 with saturated potassium carbonate solution, then extracted with ether (4 x 40 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to yield the crude product. The oxalate salt was made by adding oxalic acid (1.2 eq.) in ether to the free base of the crude product in ether. The Crude salt was recrystallized from methanol (0.12 g , 52%): mp 176.5-177.5 °C; ^{1}H-NMR (DMSO-d_6) δ 7.28 (m, 2H), 6.96 (m, 3H), 4.53 (m, H), 3.17 (m, 2H), 2.96 (m, 2H), 2.39 (m, 1H), 2.00 (m, 2H), 1.77 (m, 2H), 0.64 (m, 4H); ^{13}C-NMR (DMSO-d_6) δ 163.4, 156.8, 129.6,

120.9, 15.9, 68.5, 49.8, 38.2, 28.4, 4.4. Anal. Calcd. for $C_{16}H_{21}NO_5$: C,62.53; H, 6.89; N, 4.56. Found: C, 62.50, H, 6.95, N 4.51.

5.2. Enzymology

The isolation and purification of MAO-A from human placenta and MAO-B from beef liver were carried out using the procedures reported by Salach (*74*) with the following modifications. The phospholipase A used in our preparation was obtained commercially (Sigma, St. Louis, MO) rather than from the crude venom. We did not subject the MAO-A preparation to the Sephadex purification or the MAO-B preparation to the glucose gradient purification step. In both cases, however, we obtained highly active preparations. The specific activity of MAO-A (7 nmol/mL) was established with kynuramine as substrate at 30 °C according to Salach (*74*). The specific activity of MAO-B (9 nmol/mL) was established with MPTP as substrate at 30 °C as reported earlier (*75*). The MAO-B preparation was found to be stable when stored at -15 °C. The MAO-A preparation was less stable and its specific activity had to be estimated on a biomonthly basis. Because of the viscosity, the MAO-A preparation was diluted with 2 volumes of phosphate buffer just prior to analysis.

5.2.1. Substrate Study

MAO substrate studies were conducted using gas chromatography

electron-impact mass spectrometer (GC/EIMS). The following GC temperature program was employed unless otherwise noted: 50 °C for 3 minutes, followed by a ramp of 25 °C for 10 minutes. The prepared oxalate salts of piperidines were chosen as internal standards unless otherwise noted (listed in Appendix 2). The integration of the selected ion peaks of analytes and internal standards were obtained. No standard curve was performed. The selected ions used are listed in the appendix 2.

Substrate studies on these compounds at low enzyme concentrations [as used in the studies with the tetrahydropyridine system (*59*), in which the enzyme to substrate molar ratios ranged from 1:2,200 to 1:22,000] indicated that they are not MAO substrates. Therefore, studies at a higher enzyme concentration were conducted: A 50 μL solution of the substrate (2 mM, except for **35**, 1 mM) sodium phosphate buffer (100 mM, pH = 7.4) was incubated with the MAO-B preparation (9 μM, 50 μL). The resulting mixture was incubated with gentle agitation in a water bath at 37 °C. An aliquot (15-20 μL, varied from compound to compound, depending on the compound's response on GC/EIMS) of each incubation mixture was taken at 0, 15, 30, 60, 90 minuted and stored in the freezer. The aliquot was treated with 10% Na_2CO_3 solution (5 μL or 10 μL), followed by adding a certain amount of internal standard. The amount was fixed for each set of experiments. And the oxalate salt of the internal standard in sodium phosphate buffer solution was used. For the details see Appendix 2. The resulting mixture then was extracted with ether (200 μL x 3). The total (360 μL) extract was transferred to another microcentrifuge tube. After being dried over anhydrous Na_2SO_4, the ether solution was transferred again and

combined with washing solution of the drying agent. Ether was evaporated by using sample concentrator, then 20 μL methanol was added to each tube and 1 μL of the methanol solution was injected into GC/EIMS. Integration of GC peaks or selected ion peaks was obtained. The ratios of substrate/standard were determined.

Note: 1) For the substrate studies at low enzyme concentrations, the internal standard was N-methyl-4-piperidone, except that for the N-cyclopropyl-4-phenoxypiperidine, the internal standard was 4-phenylpyridine. The procedures for working up the incubation solutions were slightly different from the above: the internal standard was added to the analyte solutions just prior to the GC/EIMS analysis.

2) In the experiments when 4-ethylphenol was used as the internal standard. It was added just prior to the GC/EIMS analysis.

5.2.2. Inhibition Study

MAO inactivation studies were conducted using a Beckman DU Series 50, or Beckman DU Series 7000 spectrophotometer as follows: different solutions of substrates (ranging from 100 to 5000 μM) in sodium phosphate buffer (100 mM, pH = 7.4) were prepared. Each solution (50 μL) was mixed with 50 μL of the stock MAO enzyme preparation and the resulting mixtures were incubated with gentle agitation in water bath at 37 °C. A 10 μL aliquot of each incubation mixture taken at 0, 4, 8, 12 minutes was added to a sample cuvette

containing 490 µl of a 5 mM solution of MPTP for MAO-B or a 1 mM 1-methyl-4-phenoxy-1,2,3,6 tetrahydropyridine for MAO-A in sodium phosphate buffer (100 mM, pH = 7.4). The rate of oxidation was determined at 37 °C by monitoring the absorbance at 343 nm for MAO-B studies and 324 nm for MAO-A kinetics under UV spectrophotometer every 3 seconds for 2 minutes.

5.2.3. Determination of k_{cat} and K_M Values for the MAO-B Catalyzed Oxidation of 1-Methyl-4-phenoxypiperidine

This kinetic study was carried out using a Beckman DU-7000 spectrophotometer. Solutions of the 1-methyl-4-phenoxypiperidine (**36**) (final volume 500 µl, final substrate concentrations 0.9 – 3.6 mM) in 100 mM sodium phosphate solution (pH = 7.4) were incubated in the presence of 0.9 µM MAO-B at 37 °C for 30 - 50 minutes. The rate of oxidation was obtained by monitoring the formation of the phenolate anion at 288 nm during the incubation period after the incubation solution had been treated with 3 drops of 10% sodium carbonate solution. The molar absorptivity (ε_{max} = 2,600 M^{-1}) was used for rate calculations. The K_{cat} and K_M values were calculated from double-reciprocal plots.

5.2.4. Reversibility of the MAO-B inhibition by 1-Substituted-4-phenoxypiperidines

The reversibility of the MAO-B inhibition by 1-cyclopropyl and 1-methyl-4-

phenoxypiperidines (**35** and **36**) at 37 °C was examined by gel filtration.

A pretest determined that the time needed to completely inactivate MAO-B was 6 hours. Sephadex G-25 column was packed after 3 g of Sephadex G-25 was stirred with 20 mL of sodium phosphate buffer solution (100 mM, pH = 7.4) for 3 hours. The amount of the enzyme can be monitored by using its characteristic band (MAO-B preparation used had characteristic λ_{max} = 274 nm). Standard enzyme concentration vs absorbance curve (Fig. 22*) was plotted by a series of dilution of stored MAO-B enzyme preparation in sodium buffer solution.

Solutions of the test compounds (250 µL, 2 mM) in 100 mM sodium phosphate (pH = 7.4) were incubated with 250 µL MAO-B (9 µM) for 6 hours. The control sample (250 µl sodium phosphate buffer solution contained 250 µL of a MAO-B preparation) also was incubated for 6 hours. The remaining MAO-B activity of each incubation mixture was established with MPTP as substrate at 37 °C as reported earlier (*75*). Then each incubation mixture was mixed with 6 drops of blue dextran (as an indicator). The resulting solutions were passed through the Sephadex column by using sodium buffer solution (100 mM, pH = 7.4) as the eluting solvent. Fractions were collected. The enzyme concentration for each fraction was determined against the standard enzyme concentration vs absorbance curve (Fig. 22*). The recovered enzyme activity of each fraction was measured and was compared with the enzyme activity before passing the column.

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Appendices

Appendix 1. Figure 1-13 and Figure 22.

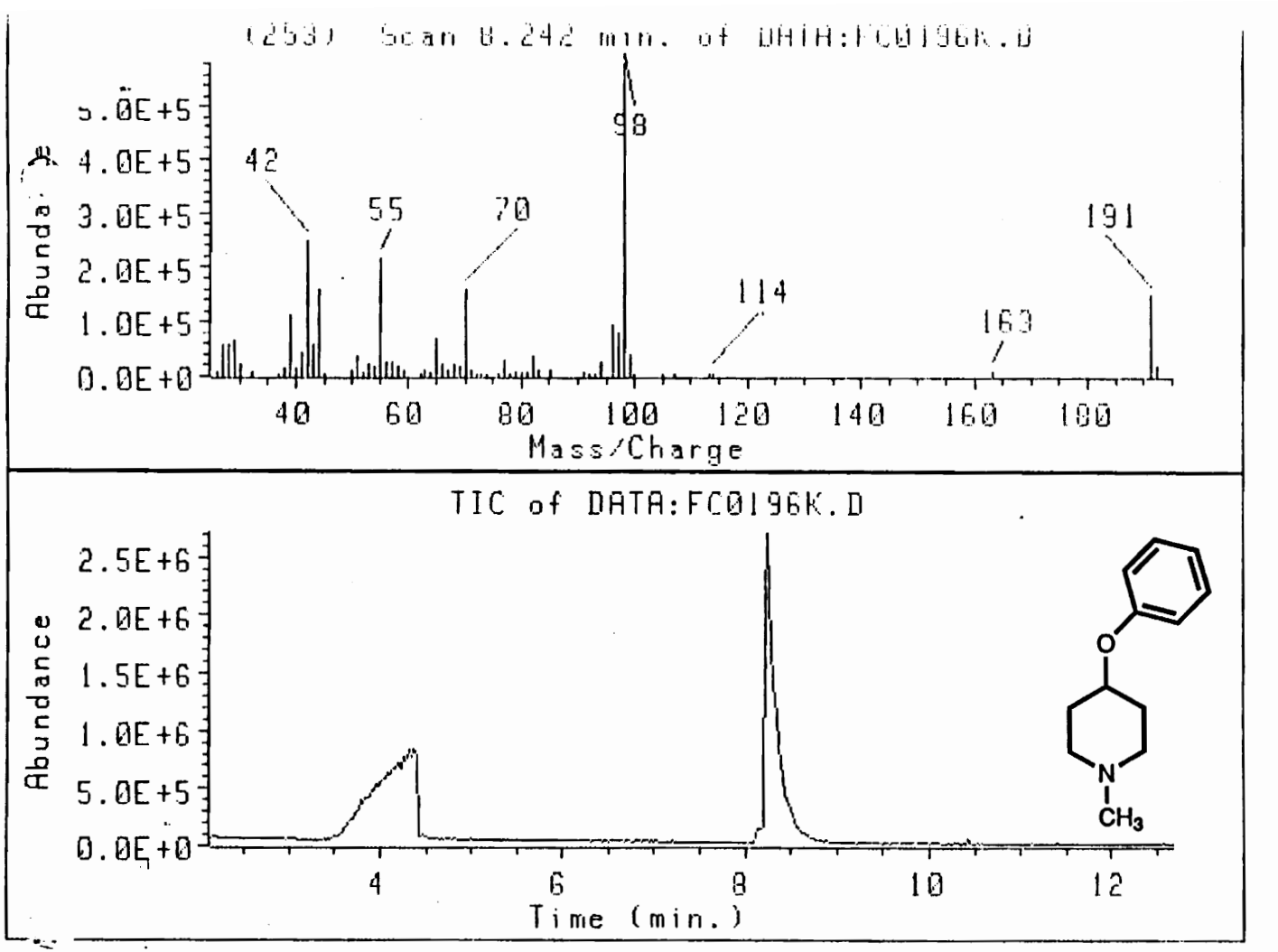

Fig. 1. GC/EIMS Spectrum of 1-Methyl-4-phenoxypiperidine

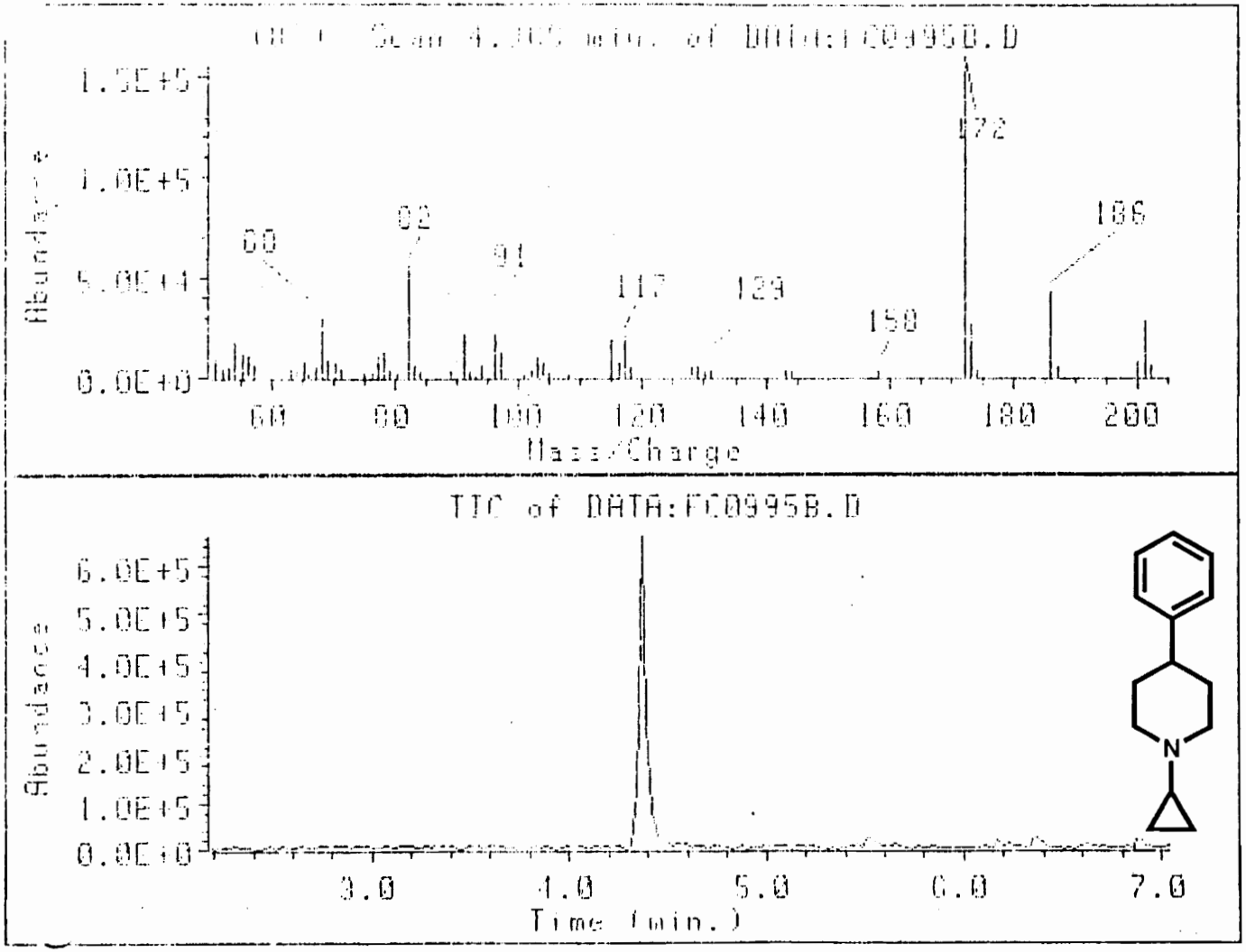

Fig. 2. GC/EIMS Spectrum of 1-Cyclopropyl-4-phenylpiperidine

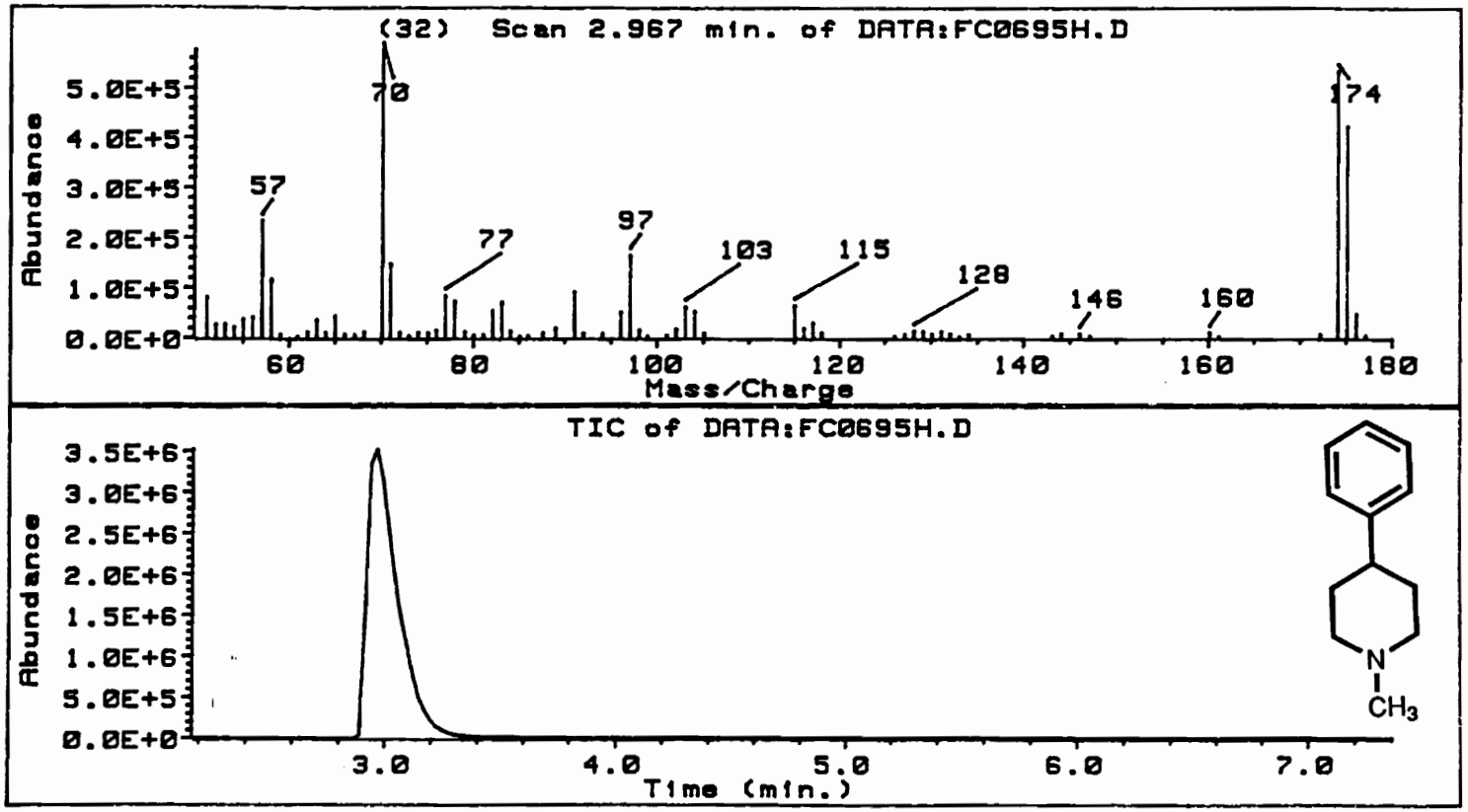

Fig. 3. GC/EIMS Spectrum of 1-Methyl-4-phenylpiperidine

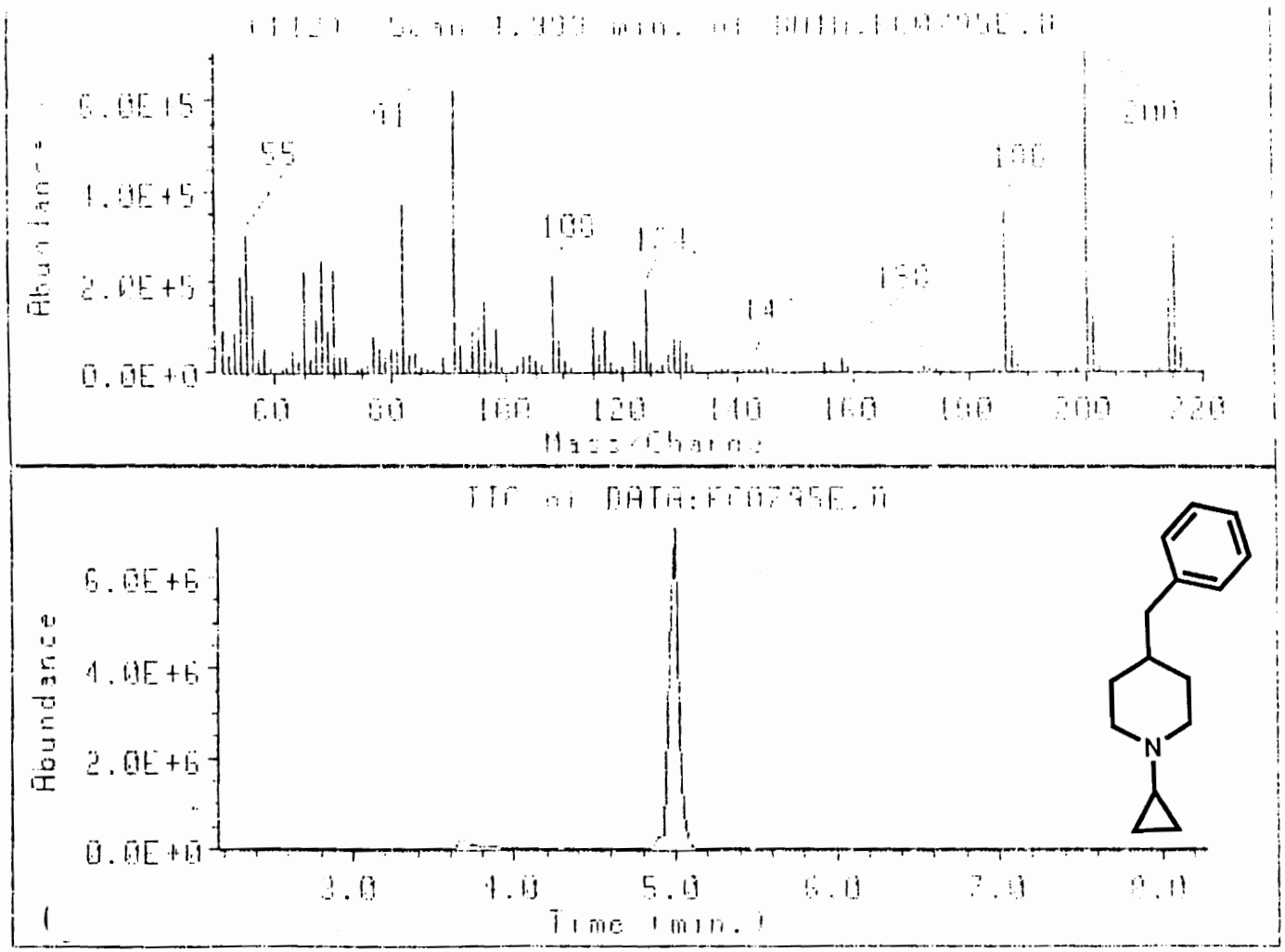

Fig. 4. GC/EIMS Spectrum of 1-Cyclopropyl-4-benzylpiperidine

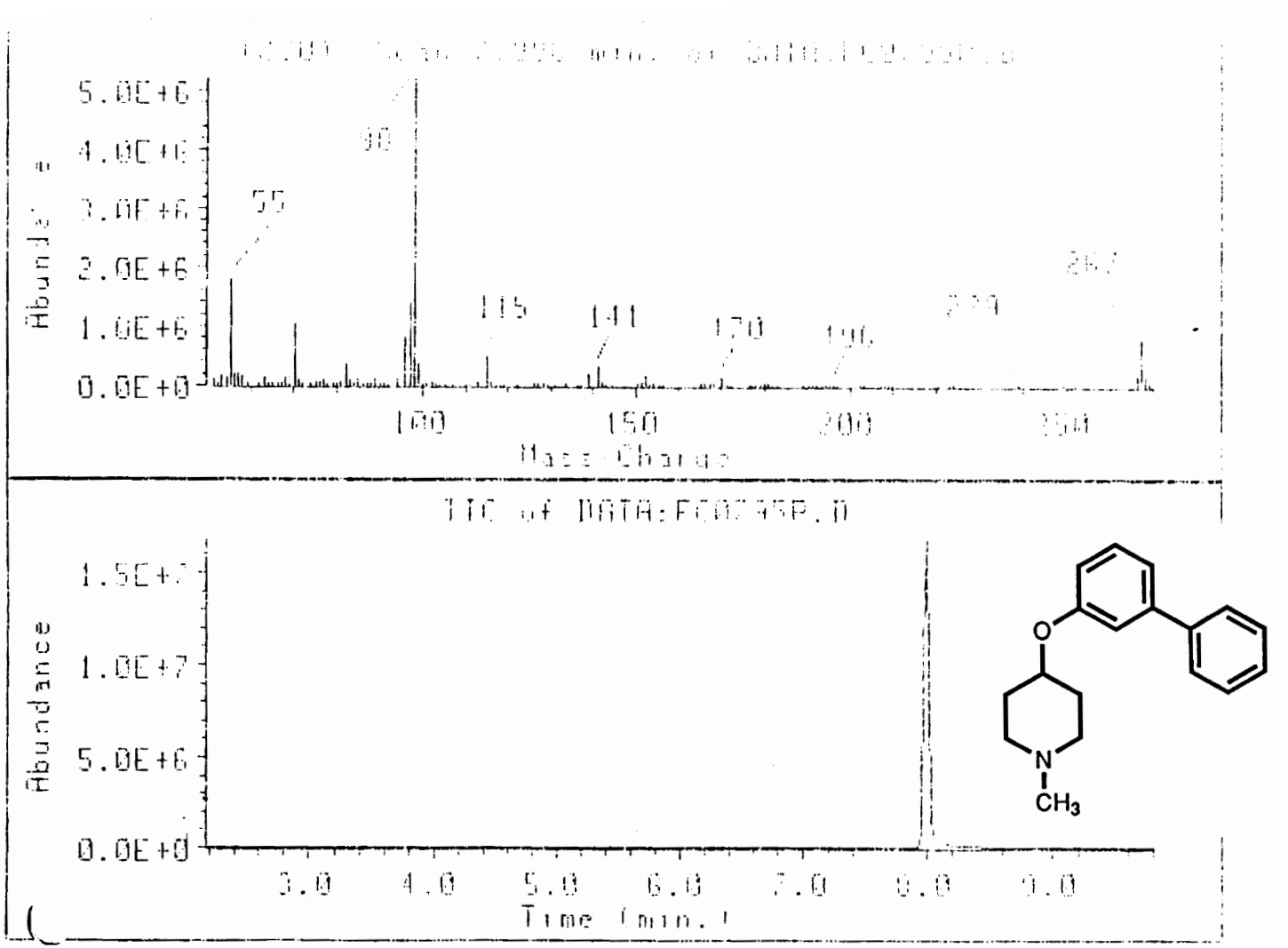

Fig. 5. GC/EIMS Spectrum of 1-Methyl-4-(3'-phenyl)phenoxypiperidine

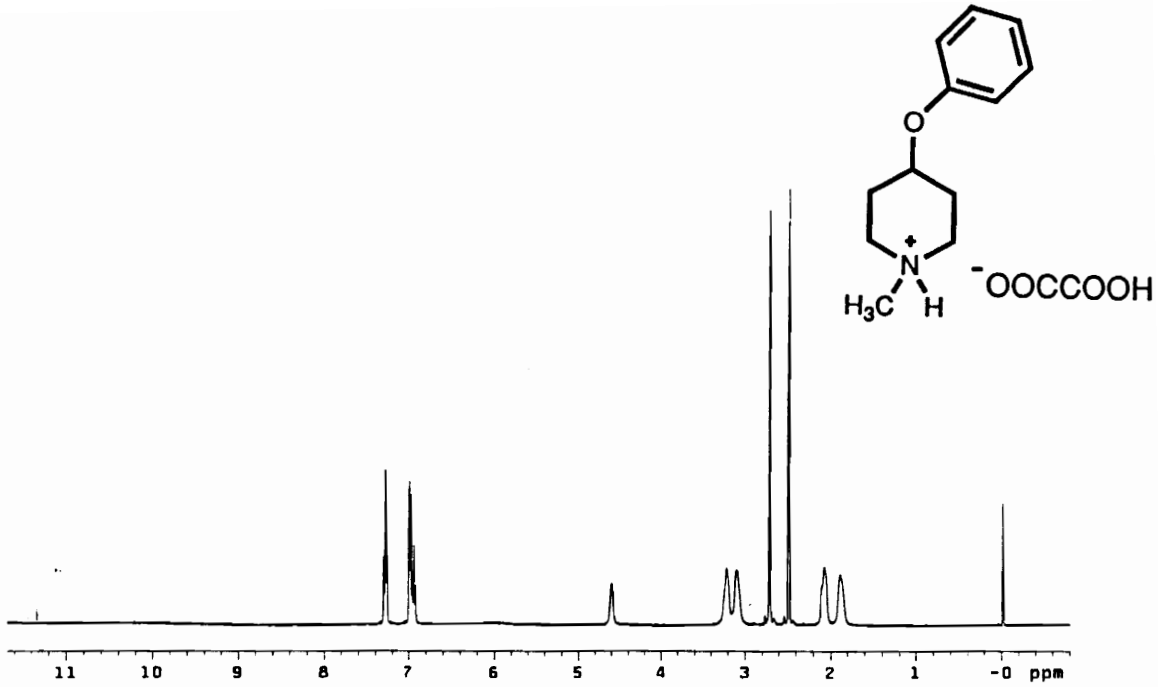

Fig. 6. ^{1}H NMR Spectrum (DMSO-d_6) of 1-Methyl-4-phenoxypiperidine-Oxalate Salt

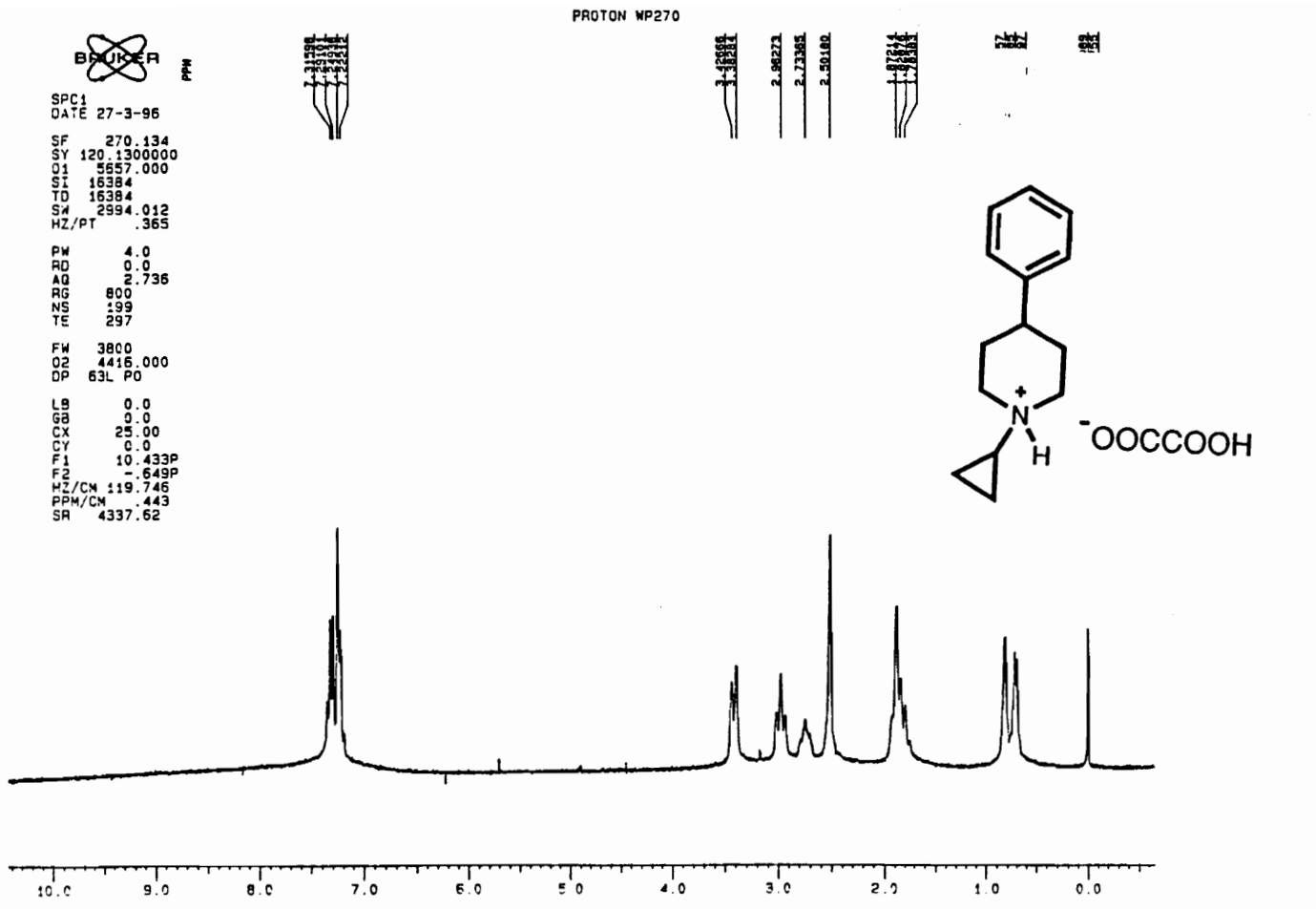

Fig. 7. ^1H NMR Spectrum (DMSO-d_6) of 1-Cyclopropyl-4-phenxypiperidine-Oxalate Salt

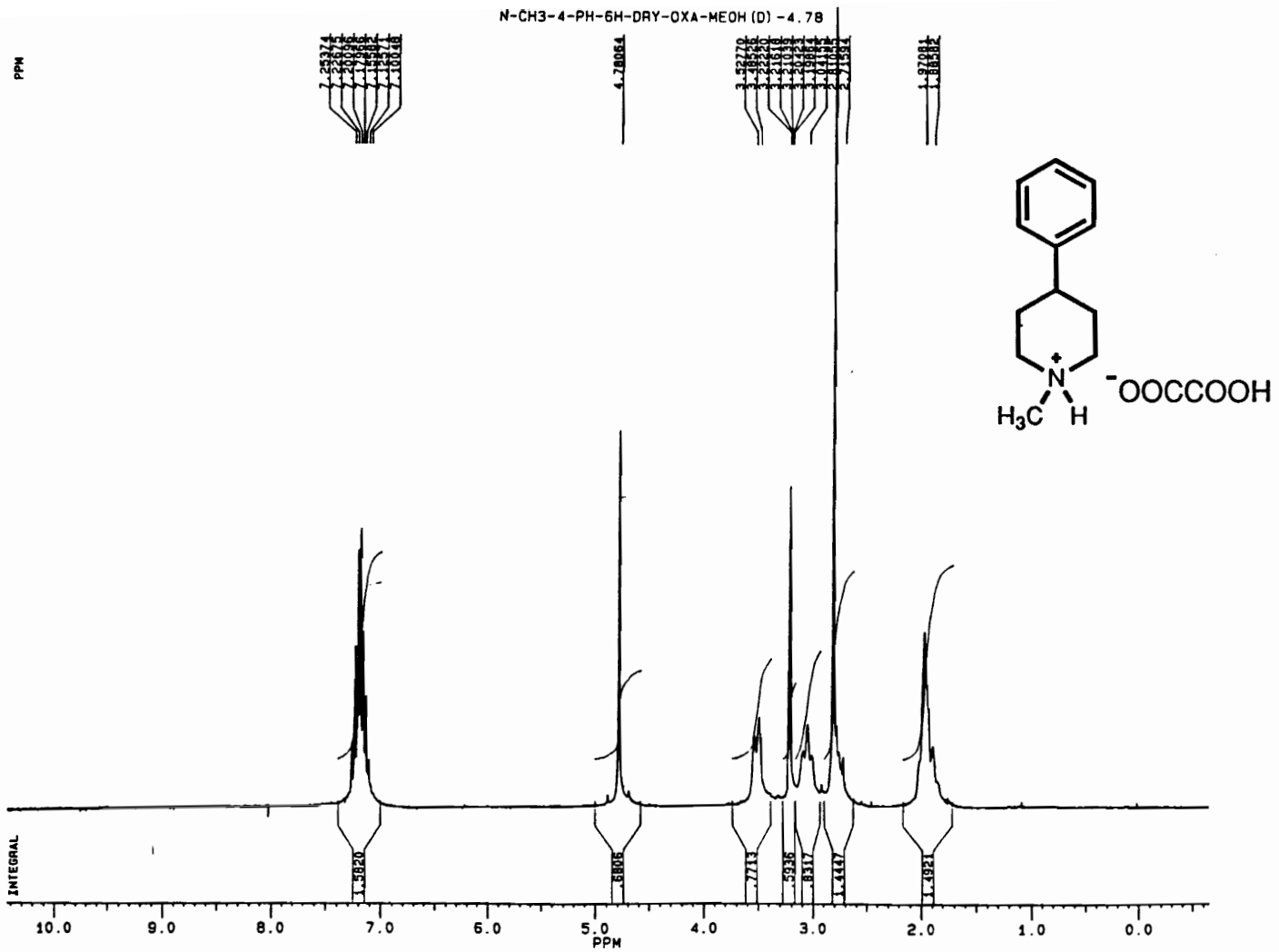

Fig. 8. ^{1}H NMR Spectrum (CD_3OD) of 1-Methyl-4-phenylpiperidine-Oxalate Salt

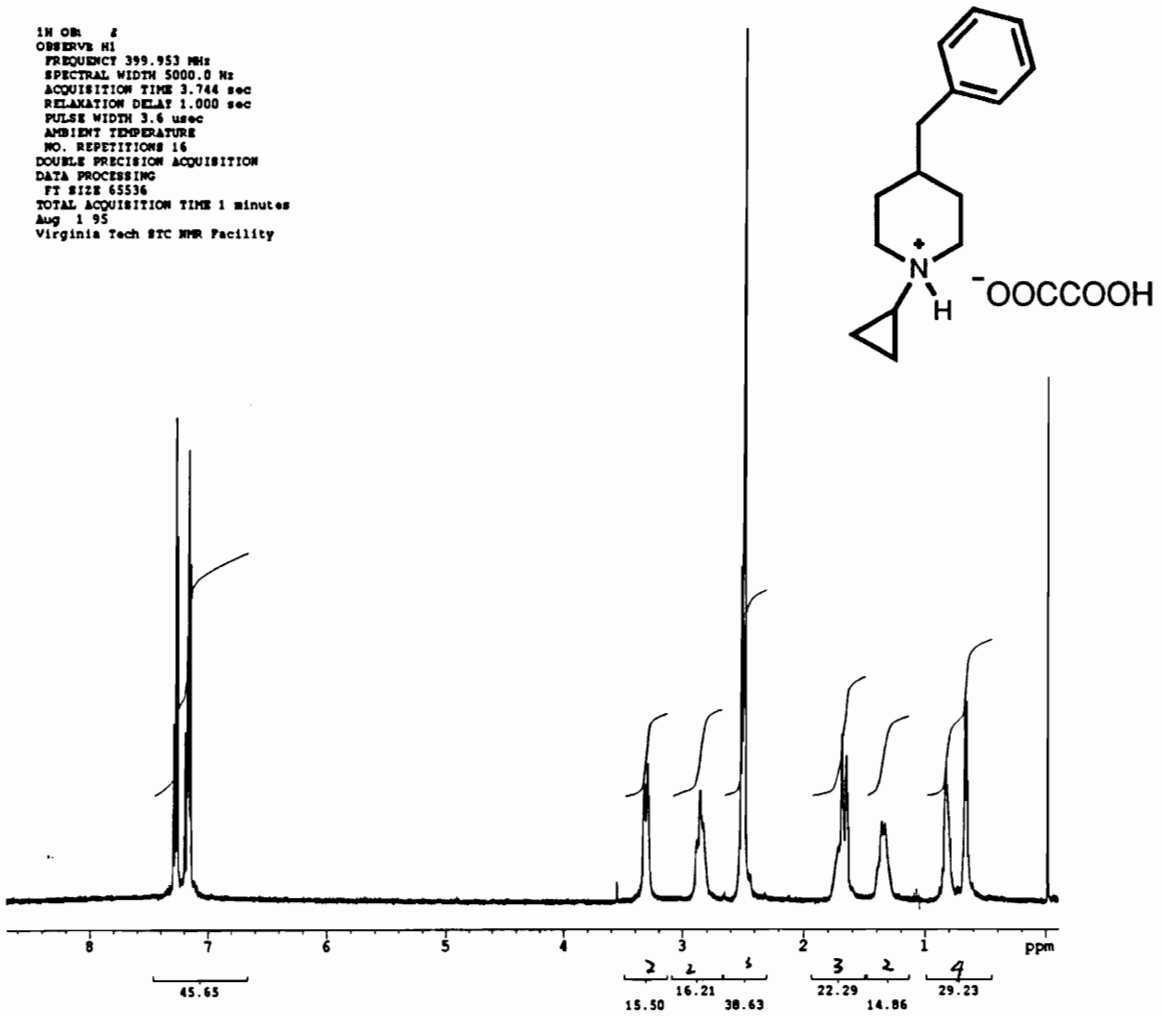

Fig. 9. ^{1}H NMR Spectrum (DMSO-d_6) of 1-Cyclopropyl-4-benzylpiperidine-Oxalate Salt

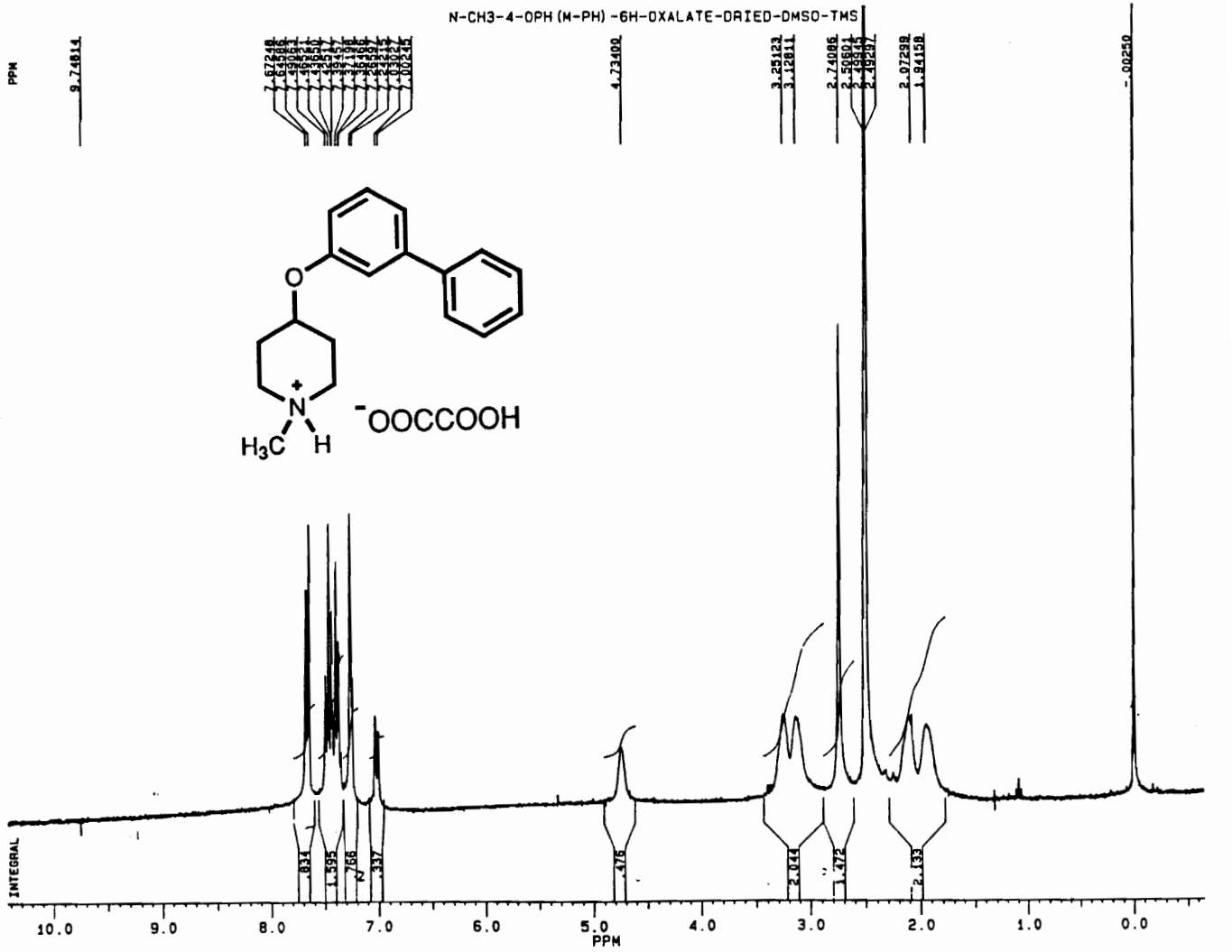

Fig. 10. ^1H NMR Spectrum (DMSO-d_6) of 1-Methyl-4-(3'-phenyl)phenoxypiperidine-Oxalate Salt

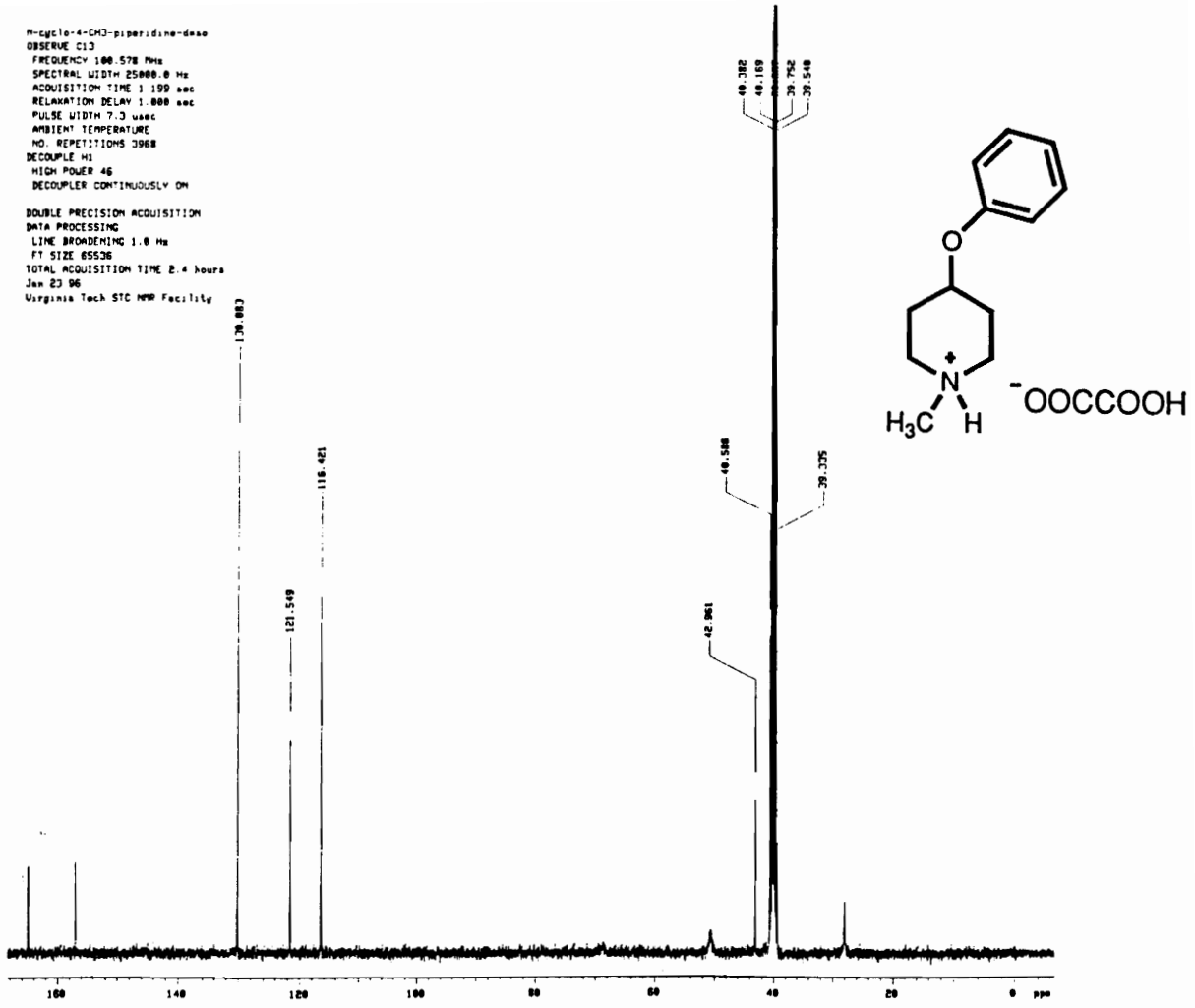

Fig. 11. ^{13}C NMR Spectrum (DMSO-d_6) of 1-Methyl-4-phenoxypiperidine-Oxalate Salt

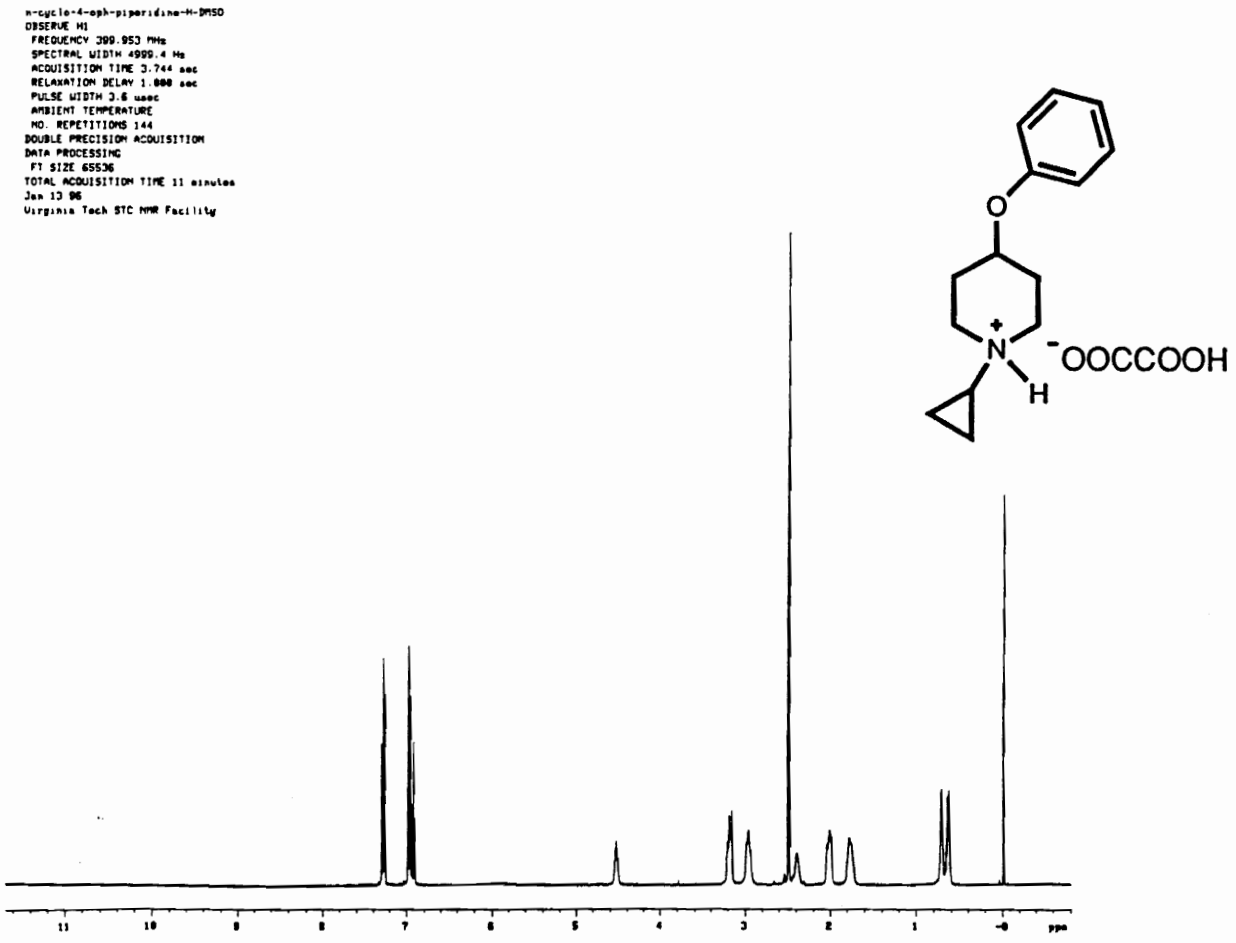

Fig. 12. ^1H NMR Spectrum (DMSO-d_6) of 1-Cyclopropyl-4-phenoxypiperidine-Oxalate Salt

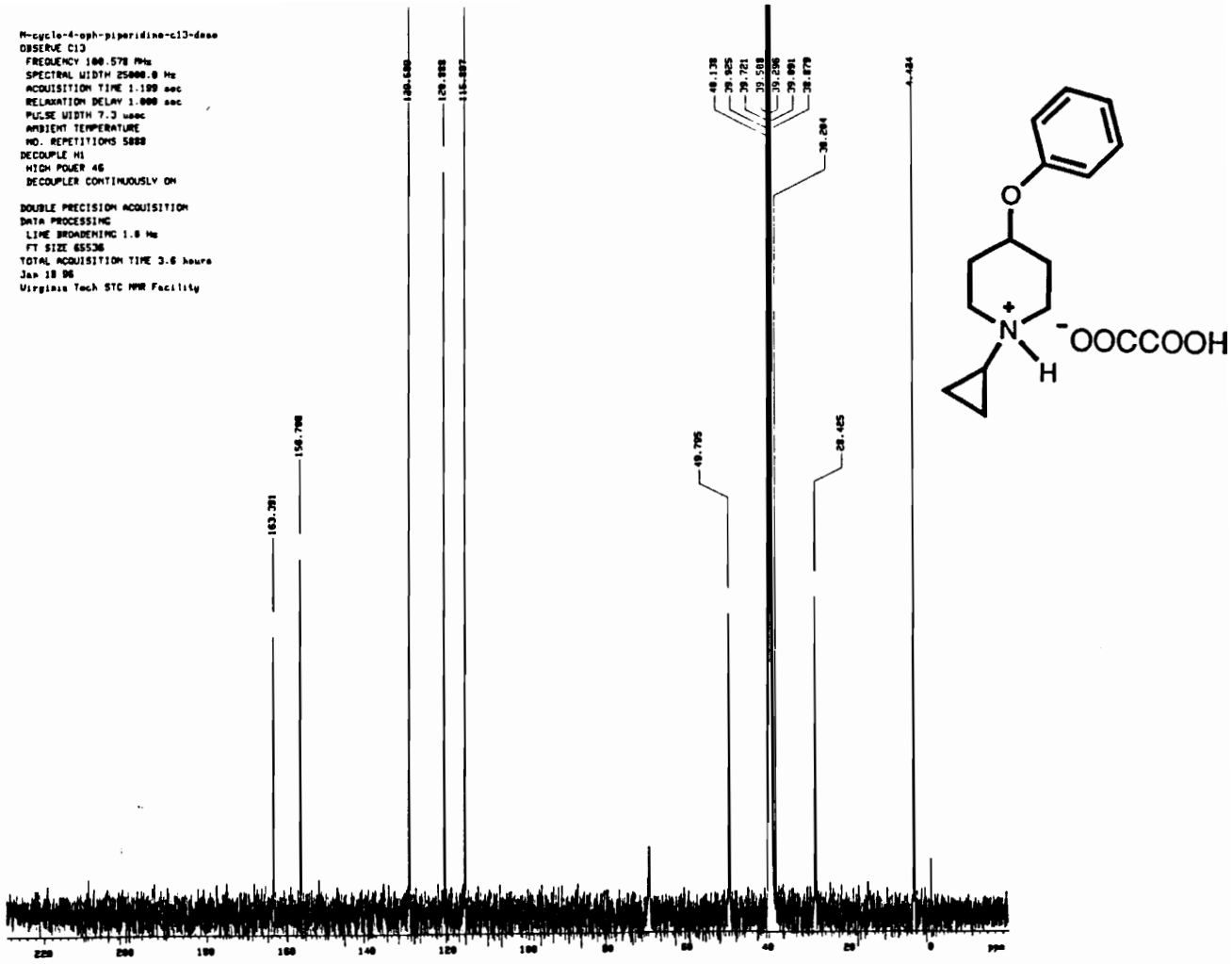

Fig. 13. ^{13}C NMR Spectrum (DMSO-d_6) of 1-Cyclopropyl-4-phenoxypiperidine-Oxalate Salt

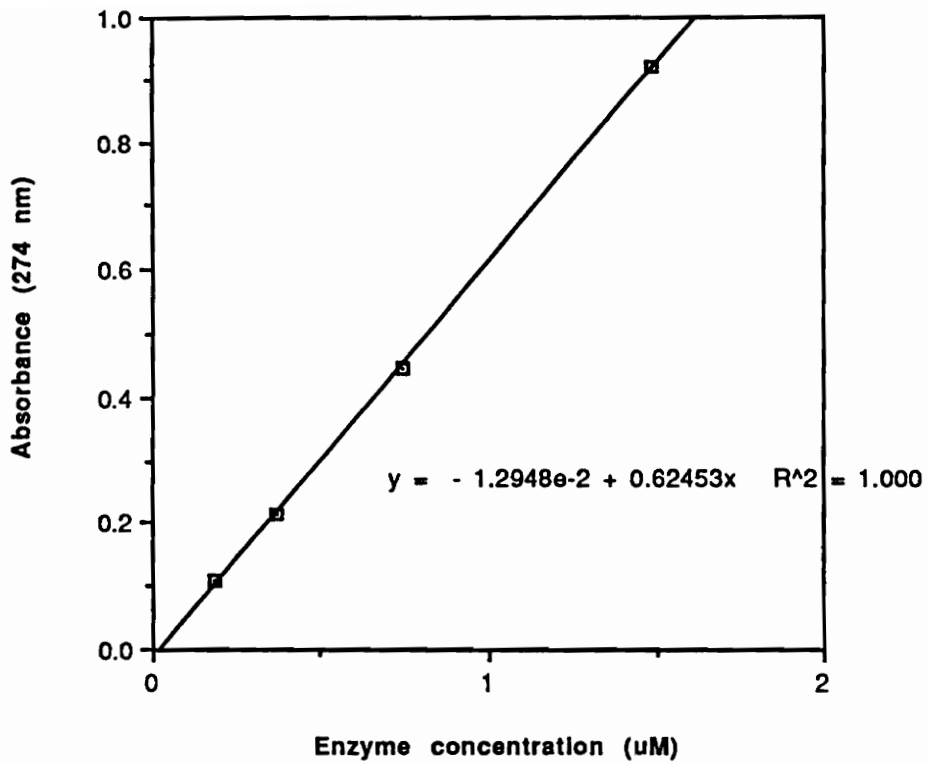

Fig. 22. Enzyme Concentration vs Enzyme Absorbance at 274 nm Standard Curve

Appendix 2. Piperidine Internal Standards

	Analyte	Internal Standard
Compund Name	N-cyclopropyl-4-phenoxypiperidine **35**	N-cyclopropyl-4-phenylpiperidine **37**
Volume, Concentration	(15 μL, 2 mM)	(7.5 μL, 2 mM)
Selected Ion Used in the Integration	188 AMU	172 AMU
Compund Name	N-methyl-4-phenoxypiperidine **36**	N-methyl-4-phenylpiperidine **38**
Volume, Concentration	(15 μL, 0.5 mM)	(25 μL, 0.22 mM)
Selected Ion Used in the Integration	98 AMU	175 AMU
Compund Name	N-cyclopropyl-4-phenylpiperidine **37**	N-cyclopropyl-4-phenoxypiperidine **35**
Volume, Concentration	(30 μL, 1 mM)	(10 μL, 2mM)
Selected Ion Used in the Integration	172 AMU	188 AMU
Compund Name	N-methyl-4-phenylpiperidine **38**	N-methyl-4-phenoxypiperidine **36**
Volume, Concentration	(15 μL, 1 mM)	(20 μL, 0.2 mM)
Selected Ion Used in the Integration	175 AMU	98 AMU

Vita

Feng Chi was born on April 10, 1969, as an Arise girl in Sichuan, China. Growing up in a family of chemists, with the thought that the only thing interesting on earth was chemistry, she went to Jilin University in Changchun to start the chemistry dream. After receiving her Bachelor of Science degree in Chemistry from Jilin University in 1991, she did her graduate study at the same school for one and half years. In the fall of 1993, she came to Virginia Polytechnic Institute and State University to pursue her Master's degree in Chemistry. Under the guidance of Dr. Neal Castiganoli, Jr., her research focused on the synthesis and bioactivity studies on the MPTP analogs. She received the Master of Science in Chemistry from Virginia Polytechnic Institute and State University in July, 1996.

Feng Chi