Chapter 3. 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d_4

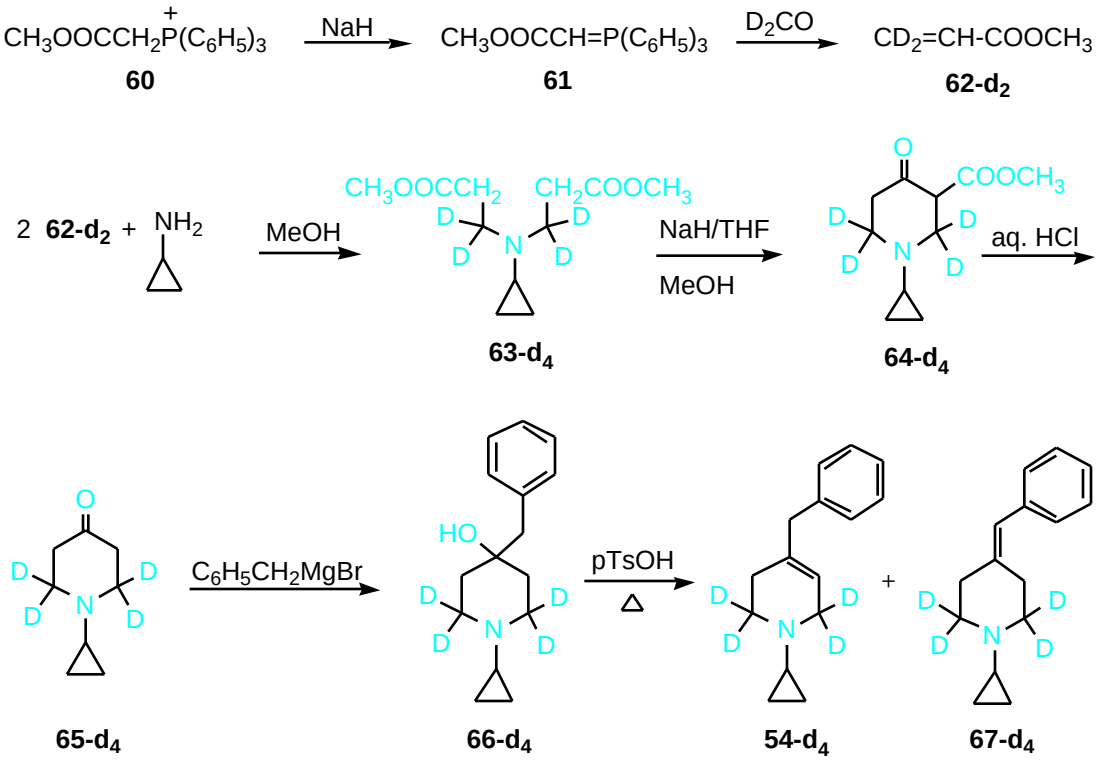
3.1. Synthesis of the d_4 Analog

The synthesis of 4-benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d_4 (**54-d_4**, Scheme 15) was carried out following the methodology reported earlier for the d_0 analog.[104] The key intermediate, methyl acrylate-d_2, (**62**) was achieved via a Wittig reaction.[111] The reaction of carbomethoxymethyltriphenylphosphonium bromide (**60**) with NaH yielded the ylide (**61**) which was treated with cracked paraformaldehyde-d_2. In order for the reaction to proceed, the paraformaldehyde must first be "cracked" in a separate flask that is heated to 200 °C. The vapors condense in the reaction flask. The dideutero product was collected by vacuum distillation with the receiver at -78 °C.

Condensation of methyl acrylate-d_2 (**62**) with cyclopropylamine afforded the *bis*-Michael adduct **63.** Treatment of **63** with NaH in THF gave the cyclic β-ketoester **64** which, when heated in 18% HCl, provided 1-cyclopropyl-4-piperidone-d_4 (**65**). Reaction of **65** with benzylmagnesium chloride afforded the tertiary carbinol **66** which underwent acid catalyzed dehydration to yield a mixture of isomeric olefins **54** and **67** (see below) in an approximate ratio of 3:1, respectively, as determined by GC-EIMS. In a previous study, one equivalent of acid was used for 1 equivalent of the oxalate salt of **54** for the dehydration reaction**.** When the free base itself was ran, two equivalents of acid were required. The first equivalent protonates the amine and the second equivalent promotes the dehydration step.

The corresponding mixture of oxalate salts was recrystallized several times from acetone which led to the isolation of the major isomer that proved to be the desired tetrahydropyridine **54**. The characteristic signals for the C-5 olefinic proton (s at 5.34 ppm) and the benzylic methylene protons (s at 3.31 ppm) readily distinguished this endocyclic olefin (**54**) from **67** which displayed its olefinic proton signal at 6.28 ppm and no benzylic proton signals. The endo (**54**) and exo (**67**) isomers showed distinct retention times by GC-EIMS analysis of 4.140 min and 4.269 min, respectively. The endo isomer displayed a large fragmentation ion corresponding to the tropylium ion (m/z = 91).

Scheme 15. Synthetic Pathway for the Preparation of 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d_4



3.2. Enzymology

UV scans of a 1 mM solution of **54-d$_4$** in the presence of 0.08 μM MAO-B revealed the time-dependent formation of a chromophore with maximal absorption at 296 nm as expected for the dihydropyridinium metabolite **59-d$_3$** (Scheme 16). The absorption maximum of the incubation mixture shifted slowly from 296 nm to 342 nm during the course of a 24 minute study. Upon adjusting the pH to 1, however, the λ_{max} reverted to 296 nm. At pH 10, the 342 nm absorbing chromophore reappeared (Scheme 2a). The same behavior had been observed with the d_0 analog (**54-d$_0$**).[104]

Figure 2. UV Scans of an Incubation Mixture Containing 1 mM **54-d$_4$** and 0.08 μM MAO-B

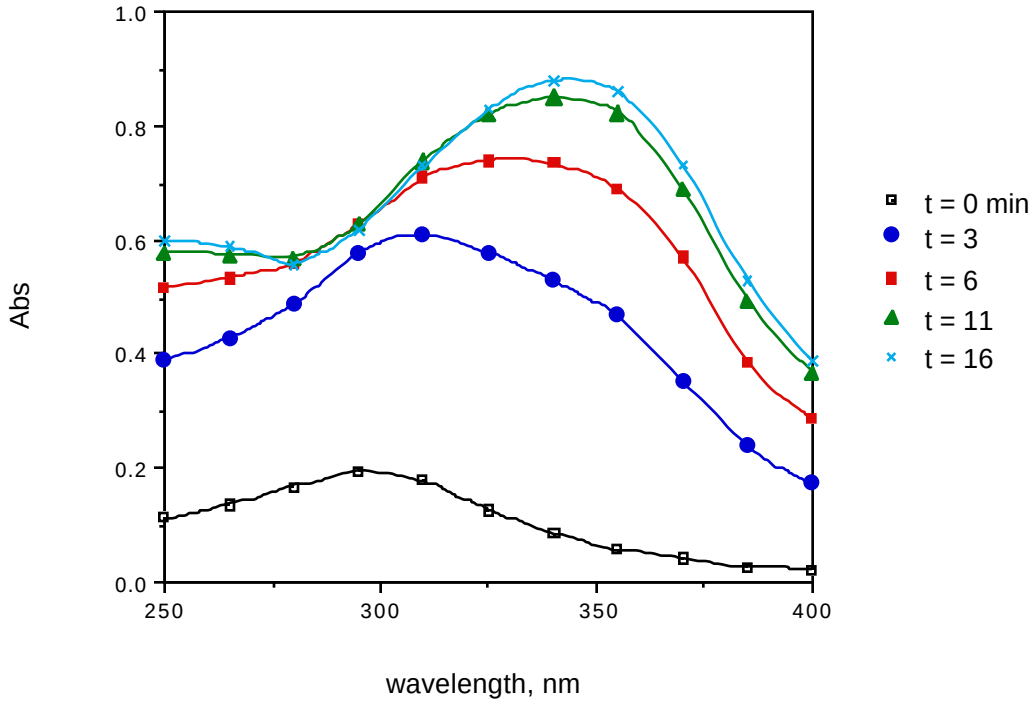
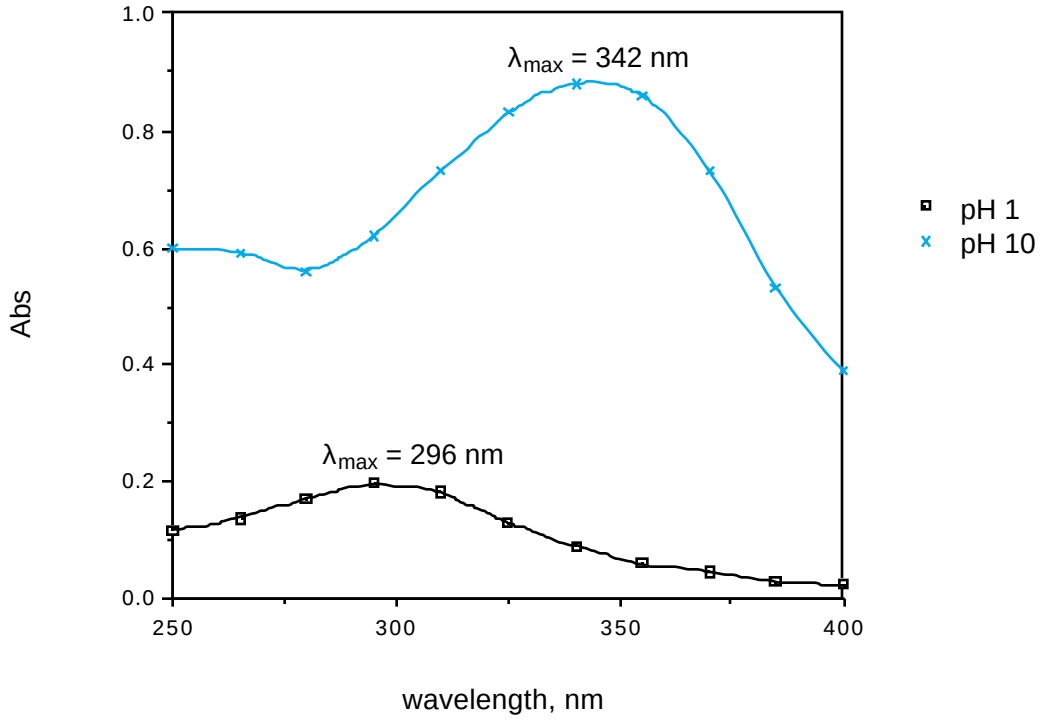


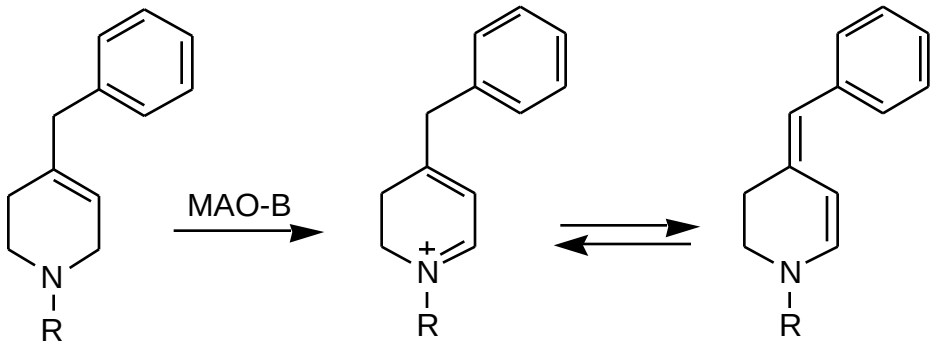
Figure 2a. Influence of pH on the Behavior of Dihydropyridinium Intermediate **59-d$_3$**



The spectroscopic behavior of an incubation mixture containing MAO-B and the corresponding 1-methyl analog **55** was examined as a model for the proposed transformation. The resulting dihydropyridinium metabolite **68** also absorbed maximally at 296 nm but was stable under the incubation conditions. This chromophore, however, did shift to 342 nm at pH 10 and back to 296 nm at pH 1. Furthermore, the synthetic perchlorate salt of the N-methyldihydropyridinium species[135] **68** displayed identical UV spectral behavior as that observed for the enzyme generated metabolite.

These results lead us to conclude that the MAO-B generated products derived from the 4-benzyltetrahydropyridine substrates **54** and **55** are the corresponding dihydropyridinium species **59** and **68**, respectively, and that these 4-benzyl-2,3-dihydropyridinium metabolites (λ_{max} 296) behave as weak carbon acids which may undergo reversible conversion to the corresponding 1,2-dihydropyridine free bases **69** and **70** (λ_{max} 342 nm). The N-cyclopropyldihydropyridinium analog **54** may be a somewhat stronger acid than the N-methyl analog **55**, which would account for its conversion to the conjugate base **69** at a lower pH than that observed with the N-methyl analog **70**.

Scheme 16. Metabolic Fate of the 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine derivatives **54** and **55**



54: R = △ **59**: R = △ **69**: R = △

55: R = CH_3 **68**: R = CH_3 **70**: R = CH_3

The kinetic deuterium isotope effects on V_{max} and K_m for the MAO-B catalyzed oxidation of **54-d$_0$** vs **54-d$_4$** were estimated by measuring the rates of formation of the corresponding dihydropyridinium metabolites **59-d$_0$** and

59-d$_3$ spectrophotometrically at 296 nm for two minutes. The λ_{max} at 296 nm was stable during this incubation period. A substrate concentration range of 150 to 1000 μM was examined in the presence of a fixed concentration (0.08 μM) of MAO-B. At the higher substrate concentrations the plots of time vs concentration of dihydropyridinium metabolites were curvilinear. Since only a small percentage of the substrate would have been consumed in these experiments during the 2 minute data collection periods, the slowing down of the rate of product formation was assumed to be a consequence of the inactivation of the enzyme. Despite this problem, the double reciprocal plots generated from these rate data gave straight lines ($r^2 > 0.97$) for both the d_0 (Figure 3) and the d_4 (Figure 4) substrates. The V_{max} and K_m values estimated for the d_0 substrate (810 ± 78 min^{-1} and 0.4 ± 0.08 mM, respectively) compared well with the corresponding values (637 min^{-1} and 0.4 mM) reported previously.[104] The corresponding analyses for **54-d$_4$** gave values of 530 ± 65 min^{-1} for V_{max} and 0.4 ± 0.07 mM for K_m. Based on these data, the deuterium kinetic isotope effect on V_{max}/K_m is 1.4 and on V_{max} 1.5. This intermolecular isotope effect argues that cleavage of the allylic carbon-hydrogen bond is at least partially rate determining in the MAO-B catalyzed oxidation of **54** to the corresponding dihydropyridinium metabolite **59**.

Figure 3. Lineweaver-Burke Plot for the MAO-B Catalyzed Oxidation of 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine (**54-d$_0$**)

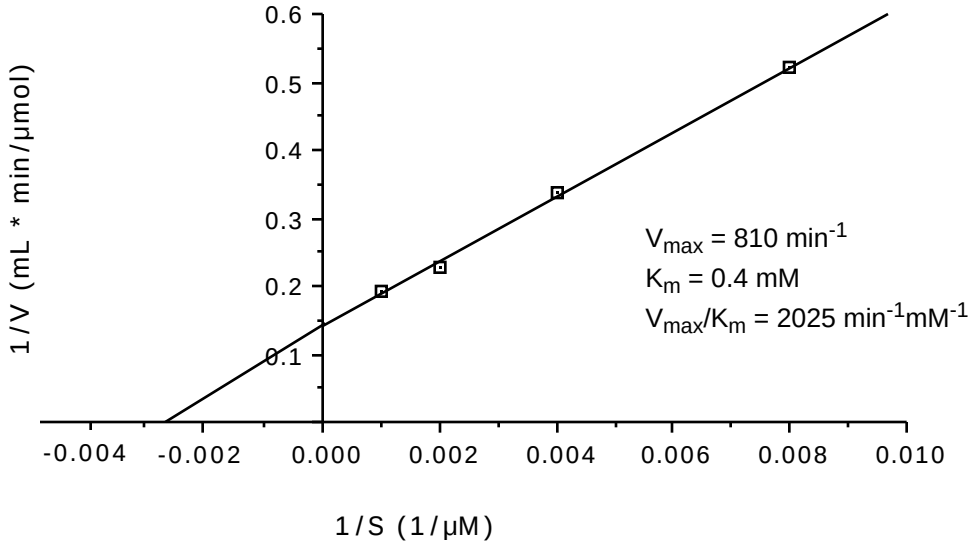
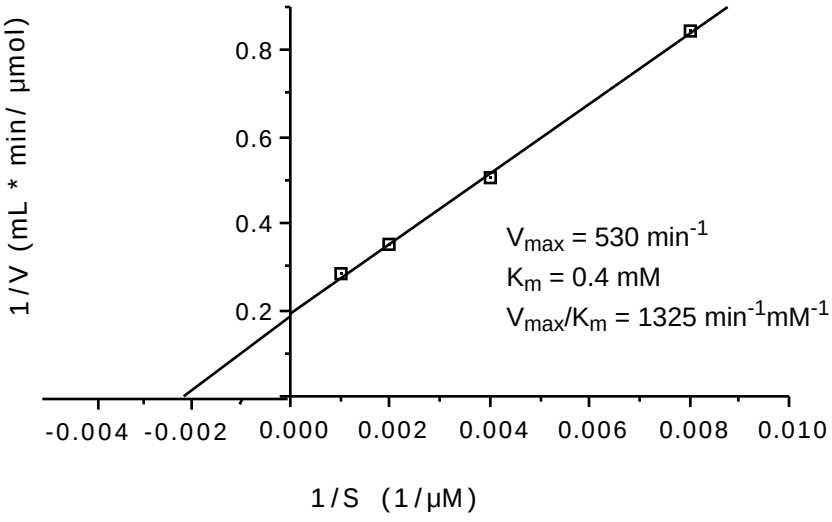
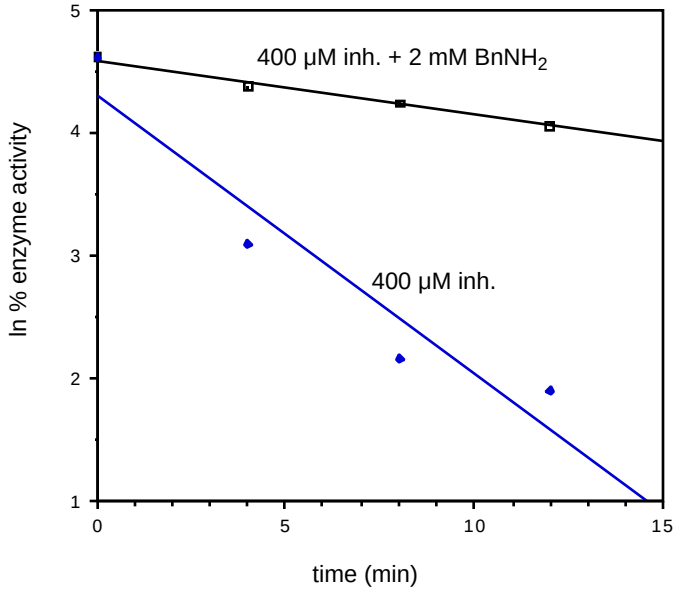


Figure 4. Lineweaver-Burke Plot for the MAO-B Catalyzed Oxidation of 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d$_4$ (**54-d$_4$**)



The previously observed concentration and time dependent MAO-B inhibition properties of **54** were interpreted as being consistent with a mechanism-based inactivation pathway.[112] We have obtained additional evidence to support this tentative conclusion. In order to characterize a compound as a mechanism-based inactivator, certain criteria must be established (see section 1.2.6.). One of these criteria is substrate protection. To show that inactivation is occurring at the active site of the enzyme, the inactivation experiment is repeated under identical conditions in the presence and absence of a known substrate for the enzyme. Because both the inactivator and the substrate must bind at the active site, the presence of the substrate will prevent the binding of the inactivator and, therefore, the rate of inactivation will decrease compared with the rate in the absence of substrate. As expected, the preferred MAO-B substrate benzylamine (2 mM) protected against loss of enzyme activity mediated by **54-d$_0$** (500 μM). After an 8 minute incubation period only 10% of the original activity remained in the absence of benzylamine while 68% remained in the presence of benzylamine (Figure 5).

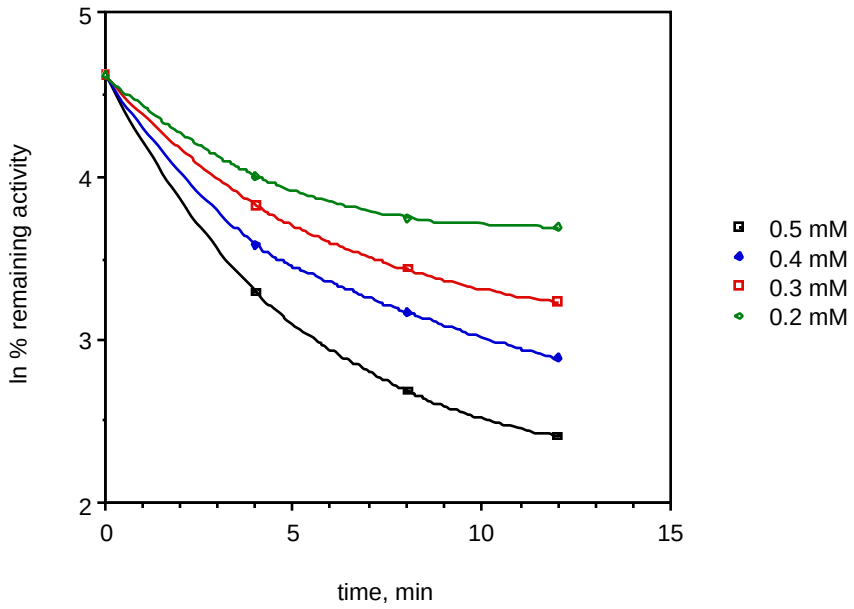
Figure 5. Substrate Protection by Benzylamine from the Inactivation of MAO-B Mediated By 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine (**54**)



Another criterion that should be met is irreversibility of the enzyme inhibition. Most cases of mechanism-based inactivation results in the formation of covalent, irreversible adducts. Consequently, dialysis or gel filtration does not restore enzyme activity. We examined the reversibility of the inhibition of MAO-B by **54-d$_0$**. MAO-B (2.3 μM) was completely inhibited following incubation with **54-d$_0$** (500 μM) for 180 minutes. The enzyme recovered following passage through Sephadex G-25 showed no recovery of activity. In a control experiment (buffer only) over 90% of the original activity was recovered. Consequently, it is reasonable to assume that **54** is a classical mechanism-based inactivator that inhibits MAO-B through covalent bond formation between an enzyme generated reactive species and an active site functionality.

Previous attempts to estimate k_{inact} and K_I for the inactivation of MAO-B by **54-d$_0$** failed, presumably because the combination of the good substrate and good inactivator properties of this compound precludes the possibility that the kinetic behavior will meet the behavior required for Michaelis-Menton analysis. In a typical inactivation experiment, varying concentrations of inhibitor are incubated with a fixed enzyme concentration. Aliquots of the incubation mixture are removed at timed intervals and assayed for remaining enzyme activity. One expects to observe a linear time dependent loss of enzyme activity. However, kinetic analysis of the inactivation properties of **54-d$_0$** and **54-d$_4$** revealed nonlinear rates. Analysis of incubation mixtures of **54-d$_4$** (0.2-0.5 mM) and 4.5 μM MAO-B showed biphasic kinetic behavior (Figure 6). The initial, rapid phase had a k_{obs} = 0.06 min^{-1} and the second, slow phase had a k_{obs} = 0.005 min^{-1}.

Figure 6. Inactivation of MAO-B by 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine-2,2,6,6-d_4 (**54-d_4**)

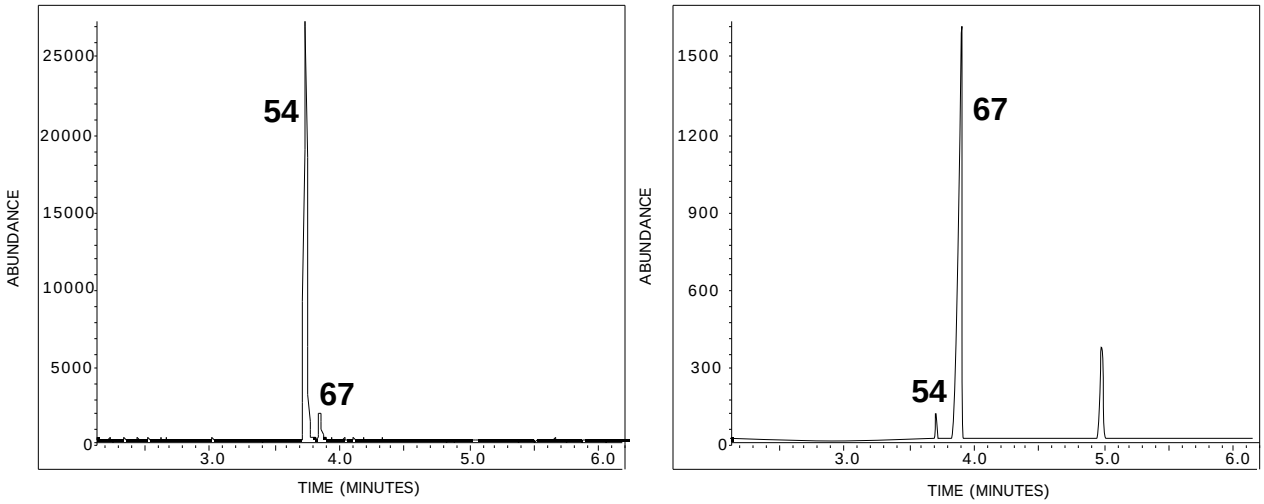


A further complication in characterizing the inactivation of MAO-B by **54** was identified when we considered the time course observed for enzyme inactivation vs the expected concentration of remaining substrate. For example, estimates based on V_{max} and K_m suggested that the concentration of remaining substrate following a two minute incubation period of a solution containing 500 μM **54** and 4.5 μM MAO-B should be essentially zero. The V_{max} value reveals that 810 nmoles of substrate are converted to the dihydropyridinium metabolite per nmole of enzyme in one minute. There are 0.5 nmoles of enzyme present in the incubation mixture. Therefore, under maximal velocity, 405 nmoles of tetrahydropyridine should be converted to the dihydropyridinium product in one minute. However, there are only 250 nmoles of substrate initially present in the incubation mixture. Under these incubation conditions, all of the

tetrahydropyridine substrate (**54**) should be converted to the dihydropyridinium metabolite **59**. On the other hand, 54 ± 8% (average of 4 experiments) of the enzyme activity was still present at this time whereas only 17 ± 2% of the initial activity was present after eight minutes. Even though our calculations suggest that all of the substrate had been depleted in two minutes, we continued to observe a decline in enzyme activity past eight minutes.

We developed a GC-EIMS assay to measure the actual concentration of remaining substrate in this experiment. Figure 7 (left panel) shows the total ion current chromatogram of an extract of an incubation mixture containing 500 μM **54-d_0** and 4.5 μM MAO-B at t = 0. The major peak eluting at 3.74 minutes can be assigned to the starting tetrahydropyridine **54** while the minor peak eluting at 3.85 minutes is due to the double bond isomeric species **67**. GC-EIMS analysis of this incubation mixture 2 minutes later gave the chromatogram shown in Figure 7 (right panel). The concentration of remaining substrate at this time is only 2 μM, a concentration which would show no detectable enzyme inhibition at these enzyme concentrations. The isomeric species **67** now dominates the tracing. The differences in ion current intensities reflect the differences in amounts of the analytes present in the sample. The concentration of **67** does not change with time establishing that the exo isomer is not metabolized by MAO-B. Therefore, the slight contamination of **67** in the incubation mixture should not affect the kinetic results.

Figure 7. GC-EIMS Analysis of an Incubation Mixture Containing 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine and MAO-B



t = 0 min incubation

t = 2 min incubation

Consequently, it appears that the continued loss in enzyme activity following depletion of the substrate may be mediated by a metabolite derived from **54**, possibly the dihydropyridinium species **59**. This postulation is consistent with literature reports that non-pseudo first order kinetics would be observed if a product generated from the enzymatic reaction is a good inhibitor of the enzyme.[112] Unfortunately, attempts to synthesize the dihydropyridinium species **59** were not successful. Earlier studies on the time and concentration dependent inhibition of MAO-B by MPTP have shown that the corresponding dihydropyridinium metabolite inhibits the enzyme with equal facility to that of MPTP itself.[84] Indeed, the excellent substrate properties and poor inactivating properties of MPTP preclude the possibility that the parent compound is the

immediate precursor to the species responsible for enzyme inactivation. A similar phenomenon may be operating in the case of **54**. The extent to which **59** or a reactive species derived from **59** may contribute to the inactivation of MAO-B remains to be determined. However, the inactivation must also be irreversible since no enzyme activity is recoverable following sephadex chromatography.

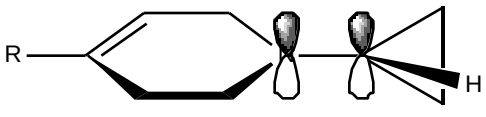
We made an additional attempt to collect useful inactivation kinetic data by sampling the incubation mixture for remaining enzyme activity every one minute rather than every four minutes. During this period, the inactivation process was linear. Even under these conditions, however, the kinetics of inactivation still did not follow Michaelis-Menton behavior, i.e. the double reciprocal plot of k_{obs} vs inhibitor concentration gave an X-intercept greater than zero. These preliminary data suggest that the K_I value for this transformation is high. In order to obtain good inactivation data, the inhibitor concentrations examined should fall on both sides of K_I. However, due to the limited solubility properties of **54**, we were not able to examine a greater concentration range which might have provided more reliable data.

In an effort to obtain a useful estimate of the influence of deuterium substitution on the relative rates at which **54-d$_0$** and **54-d$_4$** inactivate MAO-B, we determined the nmoles of enzyme inactivated at 1 minute in an incubation mixture containing 500 μM **54-d$_0$** or **54-d$_4$** and 0.1 μM MAO-B, that is, under incubation conditions in which less than 10% of the substrate would be consumed at the time of the measurement. The experiment was repeated three times and gave values of 32.0 ± 0.6 x 10^{-3} nmol MAO-B inactivated/mL-min for **54-d$_0$** and 35.0 ± 1.9 x 10^{-3} nmol MAO-B inactivated/mL-min for **54-d$_4$**. From

these data an isotope effect of 0.92 (0.85 to 0.98) was calculated for the rate of inactivation of 0.1 μM MAO-B by 500 μM **54**. The rates of substrate turnover under the same conditions gave values of 40.0 ± 2.5 nmol of dihydropyridinium formed/mL-min for **54-d$_0$** and 28.0 ± 1.7 nmol dihydropyridinium formed/mL-min for **54-d$_4$**. The partition ratio was calculated by dividing the nmoles of product formed/mL-min by the nmoles of enzyme inactivated/mL-min. The values are 1250 (**54-d$_0$**) and 800 (**54-d$_4$**) nmol dihydropyridinium formed/nmolMAO-B inactivated and the isotope effect on the partition ratio is 1.6.

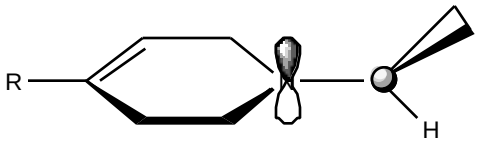
3.3. Discussion

The unexpected substrate properties of **54** have prompted us to consider the possibility that the cyclopropylaminyl radical cation may not be an obligatory intermediate in the MAO-B catalytic pathway of 1,4-disubstituted-1,2,3,6-tetrahydropyridine derivatives. Opening of the cyclopropyl ring via a conformation which allows overlap of the half filled p-orbital of the radical cation with the p-like orbitals of the cyclopropyl carbon-carbon bonds [bisected confomation (**A**)] is considered to be an energetically favored process because of the release of ring strain.[25]



bisected conformation
ring opening occurs

A



perpendicular conformation
no ring opening

B

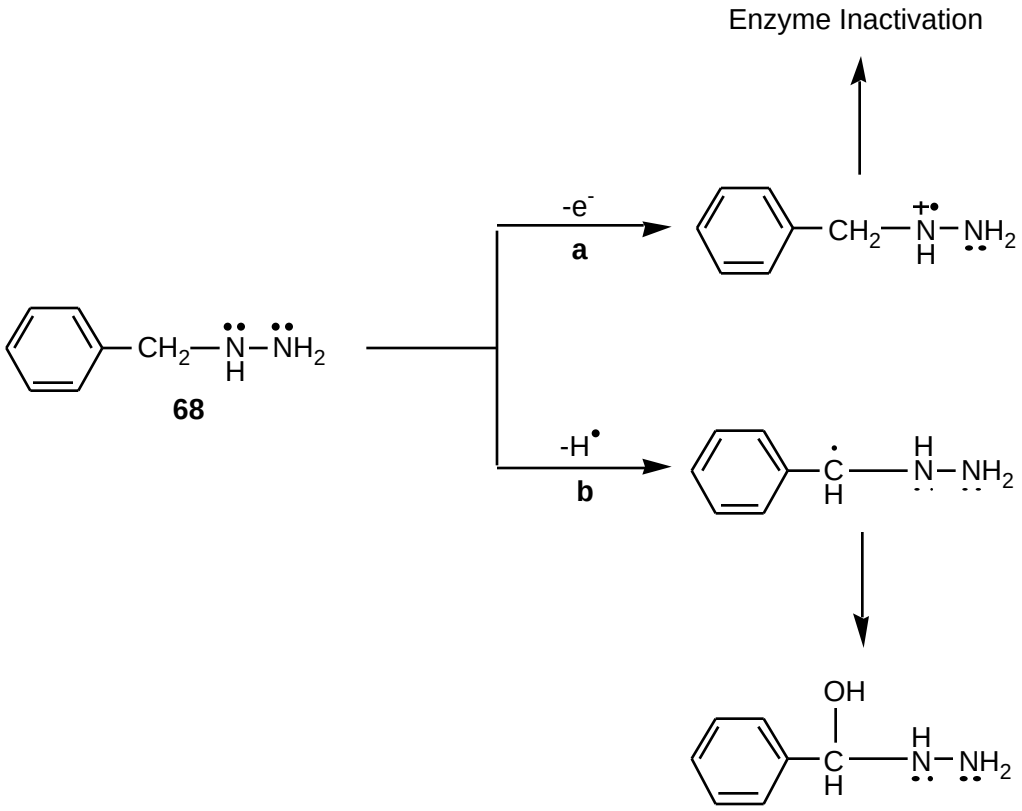
Nevertheless, conformational constraints imposed by the active site on the conformation of the tetrahydropyridine substrate could restrict orbital alignments such that cyclopropyl ring opening is slowed due to poor orbital overlap. If the substrate is locked into a perpendicular conformation (**B**) where orbital overlap does not occur, the cyclopropyl ring may remain intact. In this case an alternative reaction, proceeding through an active site base promoted deprotonation at the allylic C-6 position, might compete kinetically with the ring opening reaction. In this case formation of the dihydropyridinium product could dominate the reaction as, in fact, is observed with the MAO-B catalyzed oxidation of **54**. On the other hand, one also could account for the observed substrate and inactivation properties of **54** if partitioning were to occur between an electron transfer pathway and a pathway proceeding via direct carbon hydrogen bond cleavage (see below).

Fitzpatrick and Villafranca have proposed such a partitioning for mixed substrate/inactivators of dopamine β-hydroxylase such as benzylhydrazine (**68**).[113] These investigators observed a normal deuterium isotope effect on product formation (Scheme 17, pathway b), an inverse deuterium isotope effect on enzyme inactivation (pathway a) and a normal isotope effect on the partition ratio. The observed isotope effect on product formation and the partition ratio were identical. They reported that these results are most consistent with a partitioning that occurs at the point of C-H bond cleavage and that this bond cleavage is involved only in product formation and not in inactivation.

Studies have revealed the power of deuterium isotope effect studies on determining the point in the catalytic pathway at which a mechanism-based inactivator partitions between product formation and inactivation of the

enzyme.[139] The absence of an isotope effect on the partition ratio and on V_{max}, but the same isotope effect on k_{inact}/K_I and V_{max}/K_I indicates that inactivation is occuring from a species in which C-H bond cleavage has already occured. Both product formation and inactivation occur from a common intermediate. On the other hand, the absence of an isotope effect on the partition ratio but a different effect on V_{max} than on k_{inact} demonstrates that product formation and inactivation occur from different species and both pathways involve C-H bond cleavage. Similar to the results observed by Fitzpatrick and Villafranca, a normal isotope effect on the partition ratio, an inverse isotope effect on k_{inact}/K_I and a normal isotope effect on V_{max}, indicates that partitioning occurs at the point of C-H bond cleavage. This bond breakage is involved only in product formation, not in inactivation.

Scheme 17. Proposed Metabolic Pathway of Benzylhydrazine



Although we have not been able to obtain k_{inact} and K_I values, the results of our kinetic studies suggest a weak inverse isotope on the inactivation of MAO-B by **54** and a normal isotope effect on product formation. The partition ratio also is analogous to the kinetic pattern reported by Fitzpatrick and Villafranca. Consequently, although partitioning following electron transfer cannot be ruled out, we interpret the results reported here as consistent with a pathway involving the partitioning of **54** between the single electron transfer product **56** and the hydrogen atom transfer product **58** (Scheme 18). Such a process presumably would involve the participation of a protein derived radical species (Enz-X·) that would generate the allylic radical intermediate **58**. One electron oxidation of **58** by FAD would result in product **59** formation. Finally,

the enzyme bound radical would be regenerated in a reaction coupled to the reduction of FADH· to $FADH_2$. An inverse isotope effect on inactivation would be expected since the normal isotope effect on hydrogen atom abstraction would increase the probability of the one electron oxidation of **54** to **56**. This pathway is attractive in that it obviates the need to invoke a kinetic preference for proton loss over ring opening of a cyclopropylaminyl radical cation and is consistent with the direct formation of a highly resonance stabilized (by the nitrogen lone pair and the styryl system) carbon radical.

Scheme 18. Proposed Pathway for the MAO-B Catalyzed Oxidation of 4-Benzyl-1-cyclopropyl-1,2,3,6-tetrahydropyridine