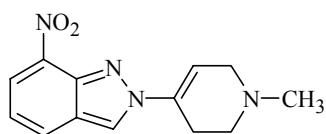


## 5. BIOLOGY

### 5.1. INVESTIGATION OF SUBSTRATE PROPERTIES OF THE 7-NI PRODRUG

The potential prodrug 1-methyl-4-(7-nitroindazol-2-yl)-1,2,3,6-tetrahydropyridine (**86**) is expected to release 7-NI via the mechanism proposed in Scheme 27. The first step in this mechanism is the MAO-B catalyzed  $\alpha$ -carbon oxidation of the prodrug to the corresponding dihydropyridinium species which is followed by subsequent hydrolysis and cleavage to release 7-NI. In order to investigate the feasibility of this metabolic pathway we tested the MAO-B substrate properties of the prodrug **86**.

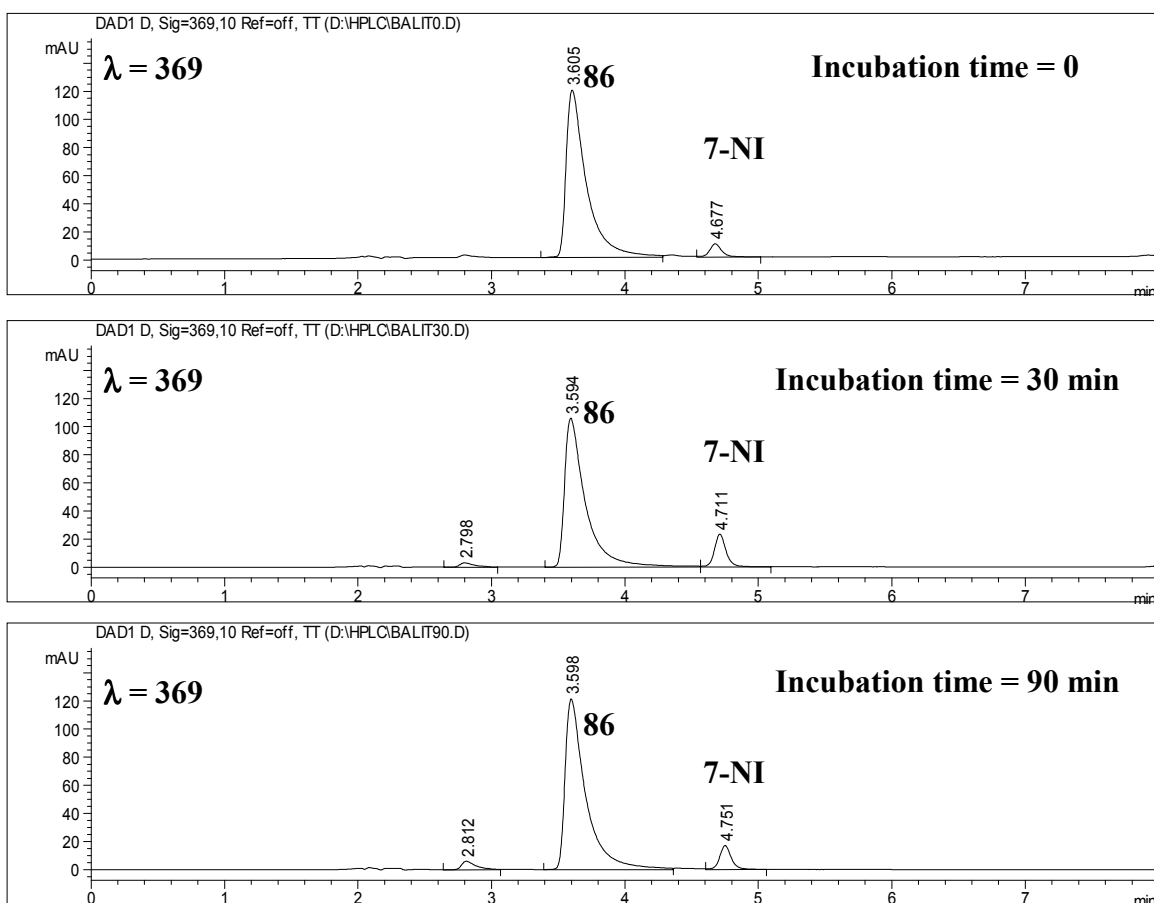


**86**

Baboon liver mitochondria, which express only the B form of the enzyme,<sup>176</sup> were used as the enzyme source. Incubations were carried out at 37 °C with a prodrug concentration of 100  $\mu$ M and a protein concentration of 1 mg/mL. Incubations were stopped by adding acetonitrile at 15, 30, 60, 90 minutes. The HPLC tracings shown in Figure 38 were obtained.

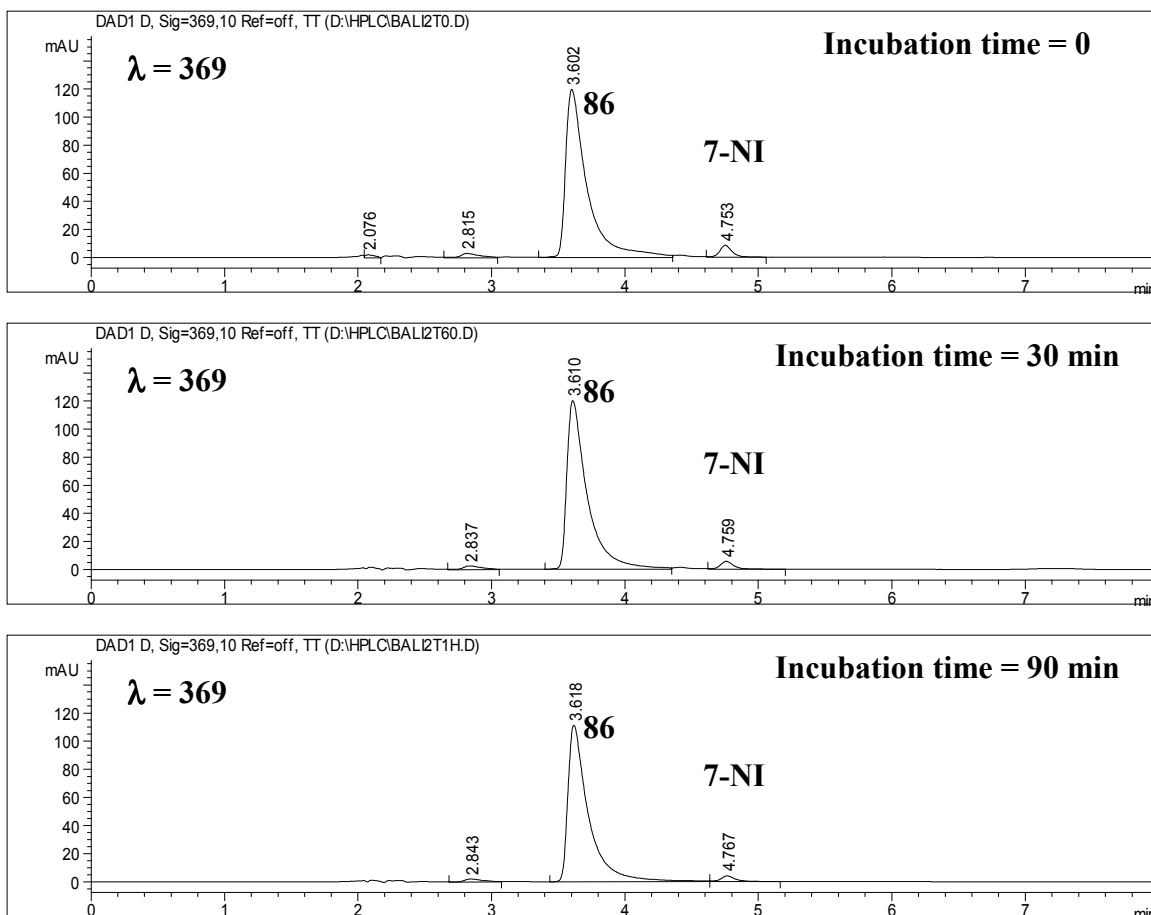
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<sup>176</sup> Inoue, H., Castagnoli, K., Van Der Schyf, C., Mabic, S., Igarashi, K., Castagnoli, N. Jr. (1999) Species-dependent differences in monoamine oxidase A and B-catalyzed oxidation of various C4 substituted 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinyl derivatives. *J. Pharmacol. Exp. Ther.* **291**, 856-864.



**Figure 38. HPLC analysis of supernatant from incubation mixtures containing 86 and MAO-B.**

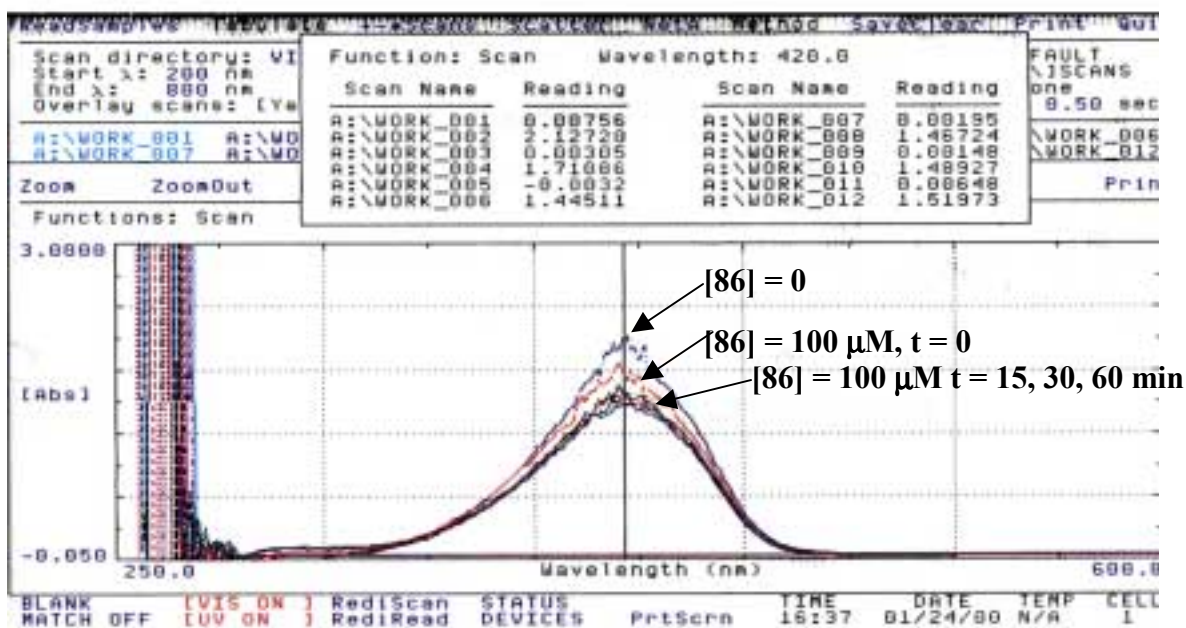
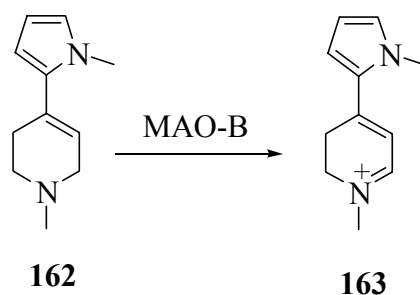
The above tracings showed an increase in the intensity of the peak corresponding to 7-NI which seemed to slow after 30 minutes, consistent with MAO-B inhibition by 7-NI. The same incubation was carried out a second time but no significant formation of 7-NI was observed (Figure 39) this time showing that the presence of 7-NI is not due to metabolism of the “prodrug”. Since the “prodrug” was obtained as the pure compound, the source of 7-NI in “prodrug” solutions can not be explained at this point. We conclude, however, **86** is not and MAO-B substrate. Its MAO-A substrate properties still need to be examined.



**Figure 39. HPLC analysis of supernatant from incubation mixtures containing 86 and MAO-B (2<sup>nd</sup>. attempt).**

According to the prodrug approach we have pursued, release of the active drug, 7-NI, would be followed by inhibition of MAO-B. Therefore, the extent of inhibition will increase as more prodrug is metabolized leading to the time dependent inhibition of the enzyme. An assay was conducted to determine if MAO-B underwent time dependent loss of activity in the presence of **86**. The enzyme was preincubated with the **86** for 15, 30 and 60 minutes. The N-methylpyrrolyl analog **162** of MPTP was added as the MAO-B substrate to the enzyme/prodrug mixture following the preincubation. Conversion of **156** to the dihydropyridinium species **163** (Scheme 68) was monitored by UV spectrophotometry ( $\lambda_{\text{max}} = 420 \text{ nm}$ ).

**Scheme 68. Conversion of 1-methyl-4-(1-methyl-2-pyrrolyl)-1,2,3,6-tetrahydropyridine (162) to the corresponding dihydropyridinium species 163.**



**Figure 40. UV analysis of the supernatant from the incubation mixtures containing no 86 and 100  $\mu\text{M}$  of 86 pre-incubated for 0, 15, 30, 60 minutes.**

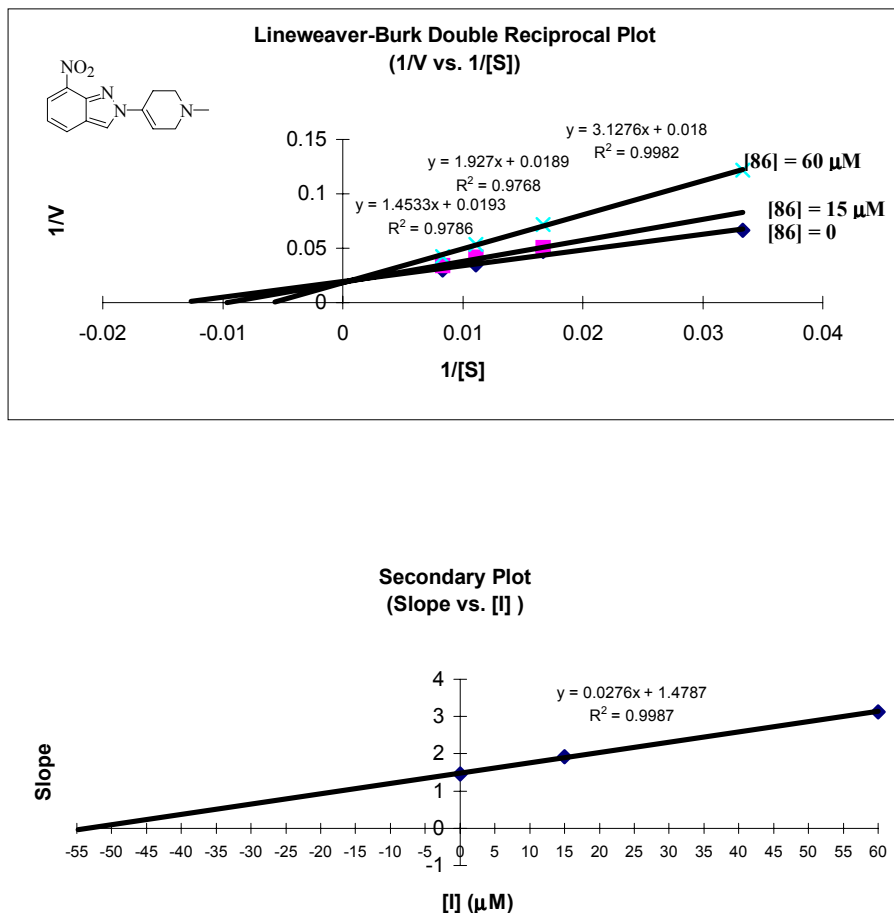
There was not any significant decrease in the absorbance values with the preincubation time (Figure 40). Consequently there was no time dependent inhibition of MAO-B.

## 5.2. INHIBITORY PROPERTIES OF THE 7-NI PRODRUG

We examined the MAO-B inhibitory properties of **86** to provide information on the active site of the enzyme that may be useful for the design of soluble indazole containing inhibitors of MAO-B and, possibly, nNOS.

Inhibition studies were carried out using baboon liver mitochondria as the enzyme source and the N-methylpyrrolyl analog **162** of MPTP as the substrate. Oxidation of **162** (30, 60, 90, 120  $\mu\text{M}$ ) to the corresponding dihydropyridinium species **163** was monitored in the presence of varying prodrug concentrations (0, 15, 30, 60  $\mu\text{M}$ ) by UV spectrophotometry at 420 nm (Scheme 70). A decrease in the absorbance values at 420 nm ( $\lambda_{\text{max}}$  for the dihydropyridinium metabolite) was expected if **86** had inhibitor properties.

In fact, **86** displayed concentration dependent inhibition properties. The double reciprocal plots of 1/rate of formation of metabolite [**(163)** (1/V)] vs 1/[**162**] were constructed at each concentration of **86**. The  $K_i$  value (-x when  $y = 0$ ) was obtained from the replot where the values of the slopes obtained from the double-reciprocal plots were used as the y axis values and the concentration of the prodrug as the x-axis (Figure 41).



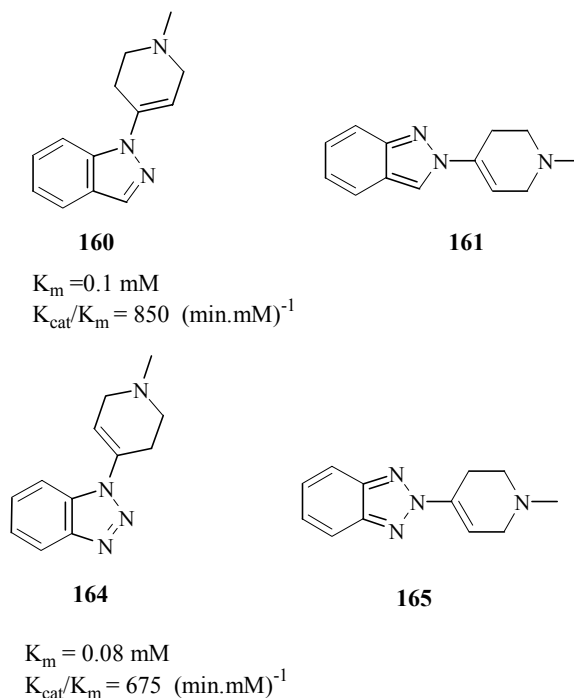
**Figure 41. 1. Double reciprocal (1/V vs 1/[S]) plots of the rate of formation of the dihydropyridinium species 163 with varying concentrations of 86. 2.Replot of the slopes vs [86].**

The prodrug had a  $K_i$  value of 54  $\mu\text{M}$  which is comparable to the  $K_i$  value obtained for 7-NI. Intersection of the straight lines in the double reciprocal plot at a common point above the x-axis suggests that the inhibition is competitive rather than noncompetitive (intersection on the x-axis) or uncompetitive (parallel lines).<sup>177</sup> These results suggested the possibility that the enzyme inhibitory properties of **86** may mask any substrate properties.

<sup>177</sup> Copeland, R.A. (1996) Reversible inhibitors. In *Enzymes. A Practical Introduction to Structure, Mechanism, and Data Analysis*, pp. 187-223, VCH Publishers, New York.

### 5.3. STRUCTURE ACTIVITY RELATIONSHIP STUDIES FOR PREVIOUSLY INVESTIGATED 4-AZAARYL-1-METHYL-1,2,3,6-TETRAHYDROPYRIDINE DERIVATIVES AS MAO-B SUBSTRATES

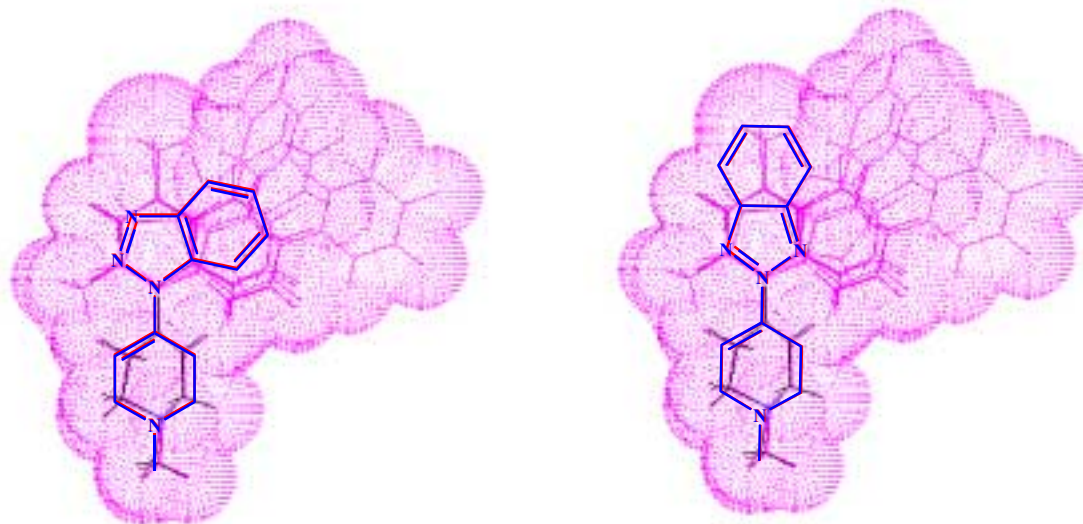
Previously several 4-azaaryl-1-methyl-1,2,3,6-tetrahydropyridinyl derivatives having structural features similar to those of the 7-NI prodrug were synthesized and their MAO-B substrate properties were investigated by Castagnoli's group.<sup>178</sup> As in the case of 7-NI, some of these tetrahydropyridinyl derivatives may exist as more than one isomer which may have different substrate properties (Scheme 76). 1-Methyl-4-(indazol-1-yl)-1,2,3,6-tetrahydropyridine (**160**) was shown to have good MAO-B substrate properties. However the regioisomer, 1-methyl-4-(indazol-2-yl)-1,2,3,6-tetrahydropyridine (**161**) was not an MAO-B substrate. Among the two possible isomers of the structurally similar benzotriazolyl compounds **164** and **165**, the *1H* isomer (**164**) was a good MAO-B substrate whereas the *2H* isomer<sup>179</sup> was not an MAO-B substrate.



<sup>178</sup> Nimkar, S.K., Mabic, S., Anderson, A.H., Palmer, S.L., Graham, T.H., De Jonge, M., Hazelwood, L., Hislop, S.J., Castagnoli, N. Jr. (1999) Studies on the monoamine oxidase-B-catalyzed biotransformation of 4-azaaryl-1-methyl-1,2,3,6-tetrahydropyridine derivatives. *J. Med. Chem.* **42**, 1828-1835.

<sup>179</sup> The data for the MAO-B substrate properties of the *2H* isomer were obtained from the unpublished results by Millie de Jonge.

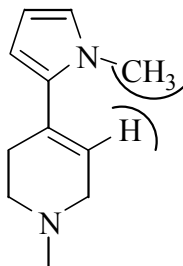
According to the MAO-B active site model (crystal structure of the enzyme is not available yet) developed by Castagnoli's group,<sup>180</sup> the *1H* isomers **160** and **164** are more likely to fit into the active site and be MAO-B substrates than are the *2H* isomers **161** and **165** (Figure 42).



**Figure 42. Compounds 160 and 164 fit in the active site model (left). Phenyl moieties of the compounds 161 and 165 lie outside the active site (right).**

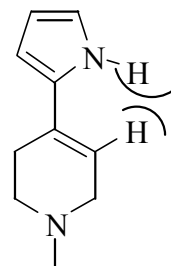
Another factor is the sterically controlled differences in the conformation of the tetrahydropyridinyl moiety. The most striking evidence to support the importance of geometry in SAR though comes from the pyrrolyl substituted tetrahydropyridines **162** and **166**. The 1-methyl analog **162** has a turnover number which is 40 fold larger than that of compound **166**.

<sup>180</sup> Palmer, S.L., Mabic, S., Castagnoli, N., Jr. (1997) Probing the active sites of monoamine oxidase A and B with 1,4-disubstituted tetrahydropyridine substrates and inactivators. *J. Med. Chem.* **40**, 1982-1989.

**162**

$$K_m = 0.2 \text{ mM}$$

$$K_{\text{cat}}/K_m = 1800 \text{ (min.mM)}^{-1}$$

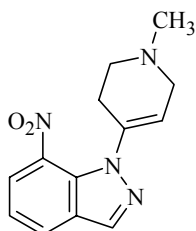
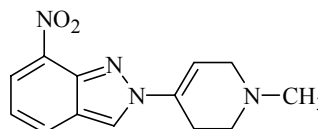
**166**

$$K_m = 1.80 \text{ mM}$$

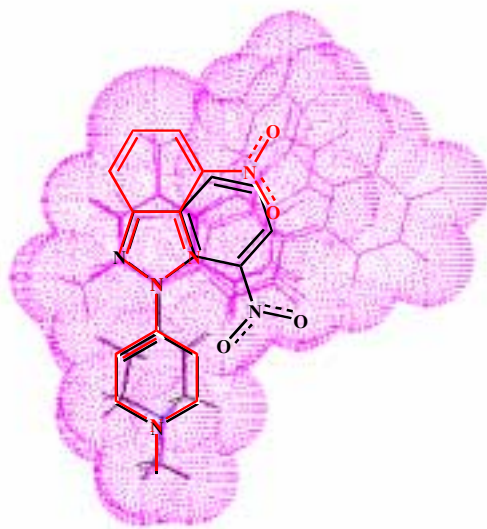
$$K_{\text{cat}}/K_m = 46 \text{ (min.mM)}^{-1}$$

The presence of the methyl group causes the deviation from the co-planarity due to the interaction with the vinylic hydrogen atoms (or the C3 methylene hydrogen atoms) on the tetrahydropyridinyl moiety which is not as effective in the case of **166** where the N-methyl group is replaced by a hydrogen atom. Previously investigated compounds suggest that it is possible that this deviation from planarity may play a role in increasing MAO-B substrate properties of the 4-azaaryl substituted tetrahydropyridines.<sup>181</sup>

These data are in agreement with our experimental results for 1-methyl-4-(7-nitroindazol-2-yl)-1,2,3,6-tetrahydropyridine (**86**). Unlike the *1H* isomer, **86** is close to being planar. In addition to this steric argument, the *1H* prodrug of 7-NI (**86**) is more likely to fit into the active site and be an MAO-B substrate than is the *2H* isomer (**85**) (Figure 43).

**85****86**

<sup>181</sup> Reference 178



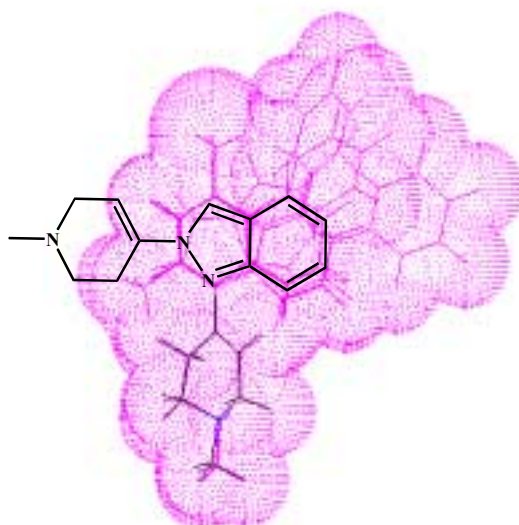
**Figure 43. Overlap of the compounds 85 and 86 on the active site model.**

Although the *2H* isomer (**86**) turned out not to be an MAO-B substrate, its MAO-B inhibiting properties attracted our attention. Due to the favorable interaction with the flavin moiety at the active site, many of the inhibitors of the enzyme were reported to be planar compounds.<sup>182</sup> Therefore, according to these observations, compound **161**, which is planar and is not an MAO-B substrate, is likely to be an MAO-B inhibitor. If **161** is a good MAO-B inhibitor, this could lead to a better appreciation of the SAR including the properties of the planar 4-azaaryl tetrahydropyridines which were studied for their substrate properties but not for their inhibitory properties. This may lead to the reinvestigation of these compounds for their MAO-B inhibition properties which will complete the studies involving their interactions with MAO-B.

Inhibition studies were carried out as previously described. The indazolyl analog 1-methyl-4-(indazol-1-yl)-1,2,3,6-tetrahydropyridine (**161**) was shown to be a competitive MAO-B inhibitor with a  $K_i$  value of 50  $\mu\text{M}$ . Since this compound was not a substrate, the tetrahydropyridinyl moiety may be located outside the active site of the

<sup>182</sup> Altomare, C., Cellamare, S., Summo, L., Catto, M., Carotti, A., Thull, U., Carrupt, P., Testa, B., Evans, H.S. (1998) Inhibition of monoamine oxidase-B by condensed pyridazines and pyrimidines: Effects of lipophilicity and structure-activity relationships. *J. Med. Chem.* **41**, 3812-3820.

enzyme in which case the indazolyl moiety would be responsible for the inhibitory effects (Figure 44).



**Figure 44. The 2*H* isomer may be located in a different conformation with respect to the active site causing the inhibition of the enzyme.**

We also carried out inhibition studies on indazole. Indazole also was found to be a competitive inhibitor of MAO-B with a  $K_i$  value of 72  $\mu\text{M}$  which is comparable to that found for compound **161**. This result supports the idea that planar 4-azaaryl-tetrahydroimidines, where the tetrahydropyridinyl moiety is located outside the active site, are inhibitors but not substrates of MAO-B.

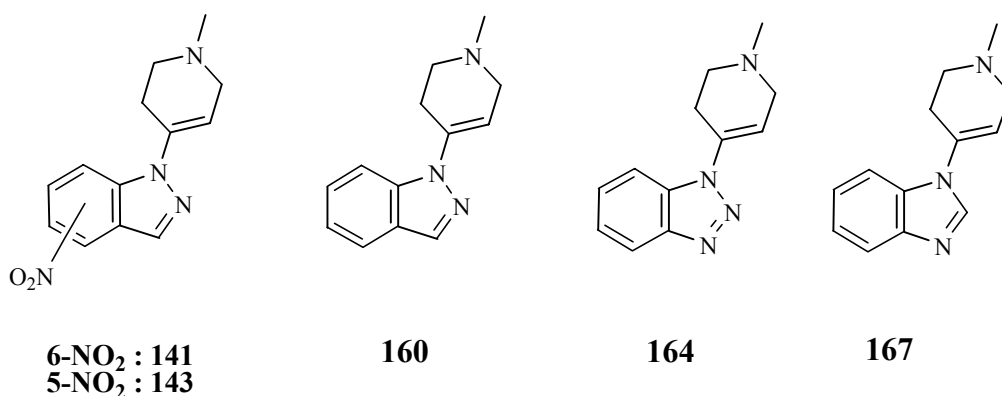
Besides giving information on the active site and clues for the design of potent MAO-B inhibitors, salts of the above mentioned compounds (**86** and **161**) are highly soluble in water at pH 7.4 which is a useful property for biological studies.

#### **5.4. INVESTIGATION OF THE MAO-B INHIBITION PROPERTIES OF 6- AND 5-NI AND THE INTERACTION OF THEIR PRODRUGS WITH MAO-B**

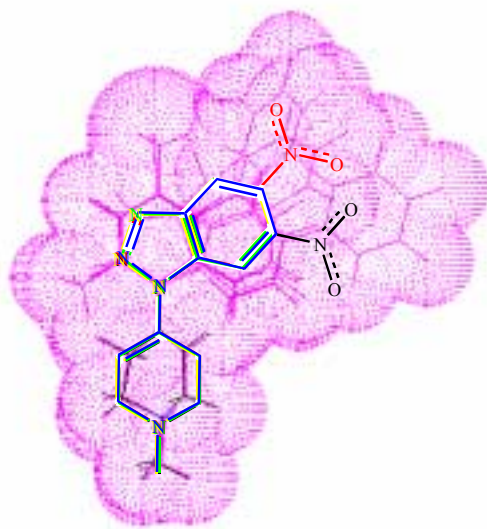
We have previously mentioned that 6- and 5-NI were inhibitors of nNOS like 7-NI but less potent than 7-NI. Although 7-NI had been investigated also for its MAO-B inhibition properties, this information is not available for 6- and 5-NI. Therefore we first carried out the inhibition studies on these compounds. Both compounds produced competitive inhibition of MAO-B.  $K_i$  values obtained from secondary plots were 12  $\mu\text{M}$

and 5  $\mu\text{M}$ , respectively for **122** and **123**. These values showed that these compounds were as potent MAO-B inhibitors as 7-NI, the  $K_i$  value of which was reported to be 40  $\mu\text{M}$  in purified beef liver MAO-B and 4  $\mu\text{M}$  in mouse brain mitochondrial preparations.<sup>183</sup> For a more accurate comparison, we carried out the incubations with 7-NI using baboon liver mitochondria as the enzyme source. These incubations gave a  $K_i$  value of 14  $\mu\text{M}$  which suggested an increasing potency as we change the position of the nitro group from C7 towards C5.

We extended our studies to include the “prodrugs” of 6-NI and 5-NI, **141** and **143**, respectively. According to our previously constructed MAO-B active site model, both **141** and **143** are predicted to be substrates. When we overlap **141** and **143** on the active site together with 1-methyl-4-indazol-1-yl (**160**), 1-methyl-4-benzotriazol-1-yl- (**163**) and 1-methyl-4-benzimidazolyl- (**167**) tetrahydropyridines, we can see that the tetrahydropyridinyl moieties all align and occupy the lower site of the active site. The azaarenyl substituents also align with respect to each other and occupy the upper right site of the active site (Figure 45). This orientation of the compounds with the active site may be a key factor in defining substrate molecules.

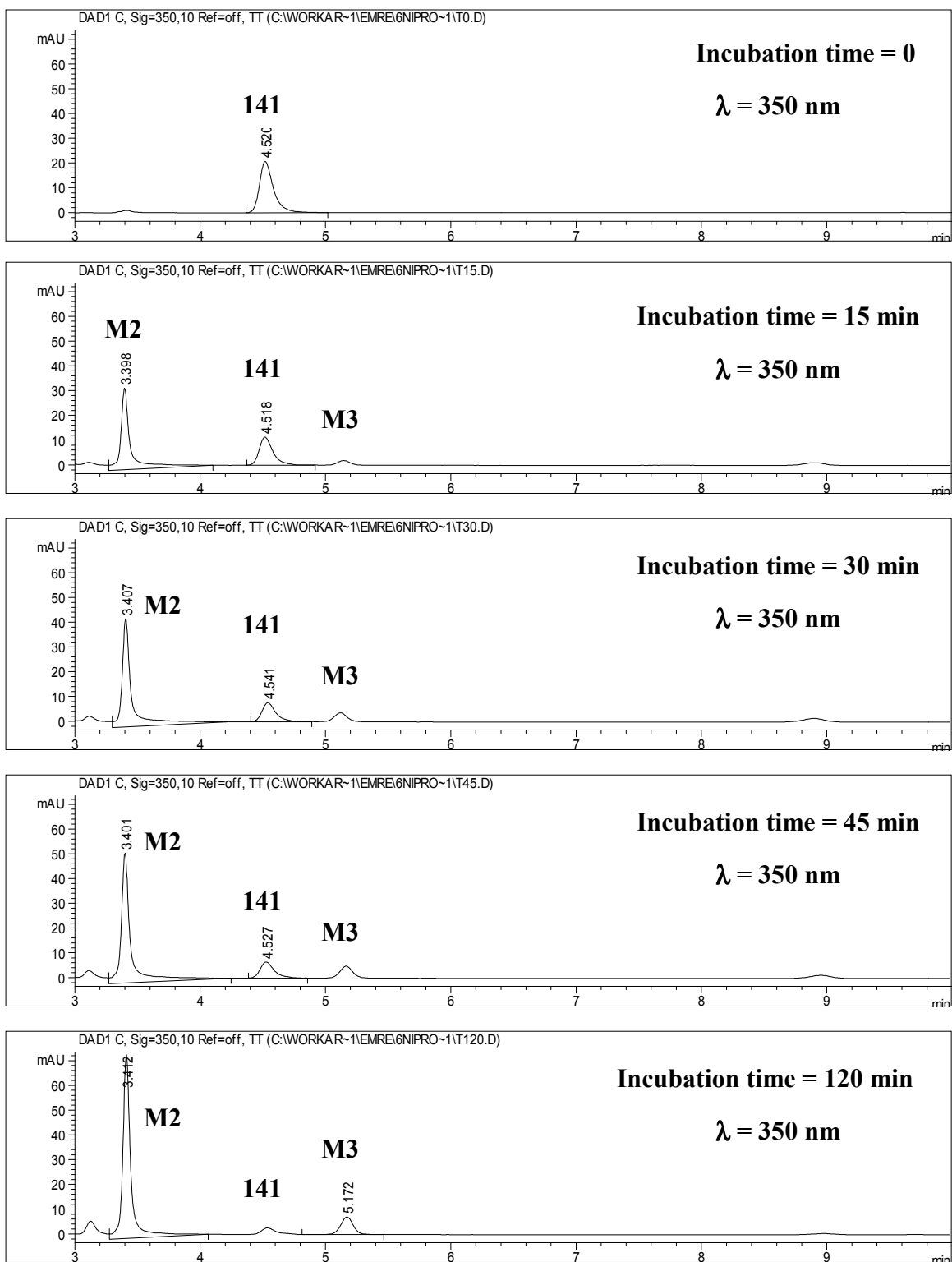


<sup>183</sup> Castagnoli, K., Palmer, S., Anderson, A., Bueters, T., Castagnoli, N. Jr. (1997) The neuronal nitric oxide synthase inhibitor 7-nitroindazole also inhibits the monoamine oxidase-B –catalyzed oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Chem. Res. Toxicol.* **10**, 364-368.

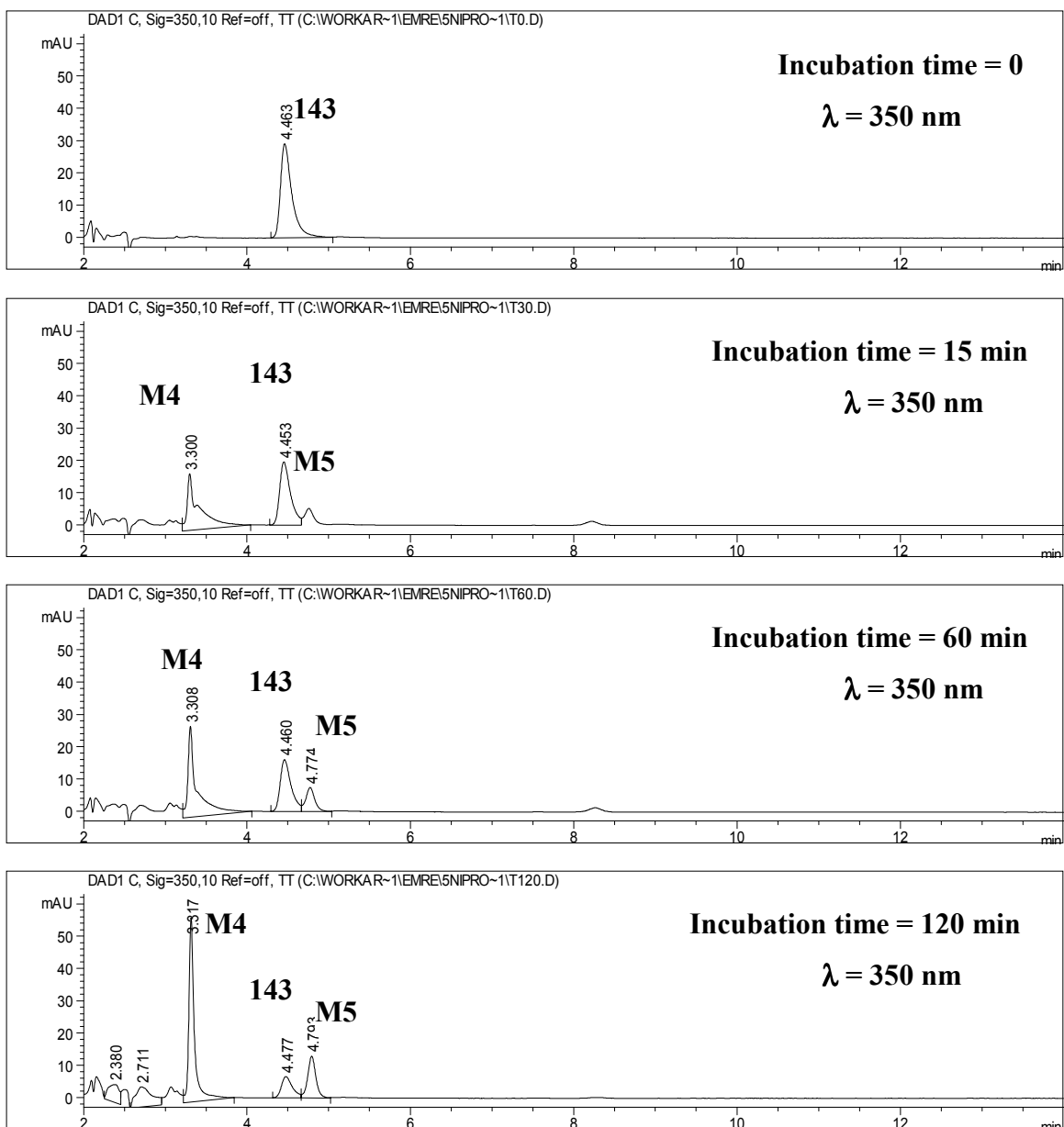


**Figure 45. Overlap of compounds 141, 143, 160, 164 and 167 on the active site model.**

We then examined the MAO-B substrate properties of **141** and **143**. We incubated 40  $\mu\text{M}$  solutions of each compound in pH 7.4, 0.1M phosphate buffer with baboon liver mitochondria (0.15 mg of protein/mL) as the enzyme source for 0, 15, 30, 45, 60, 120 minutes with gentle agitation at 37  $^{\circ}\text{C}$  in a water bath. Incubations were stopped by adding 500  $\mu\text{L}$  of acetonitrile. The denatured protein was sedimented by centrifugation at 10,000 g for 6 minutes. The supernatant fractions were analyzed by HPLC/DA. For both compounds, we observed the disappearance of the substrate peak, the appearance of a peak with a shorter retention time and another minor peak with a longer retention time (Figures 46 and 47).



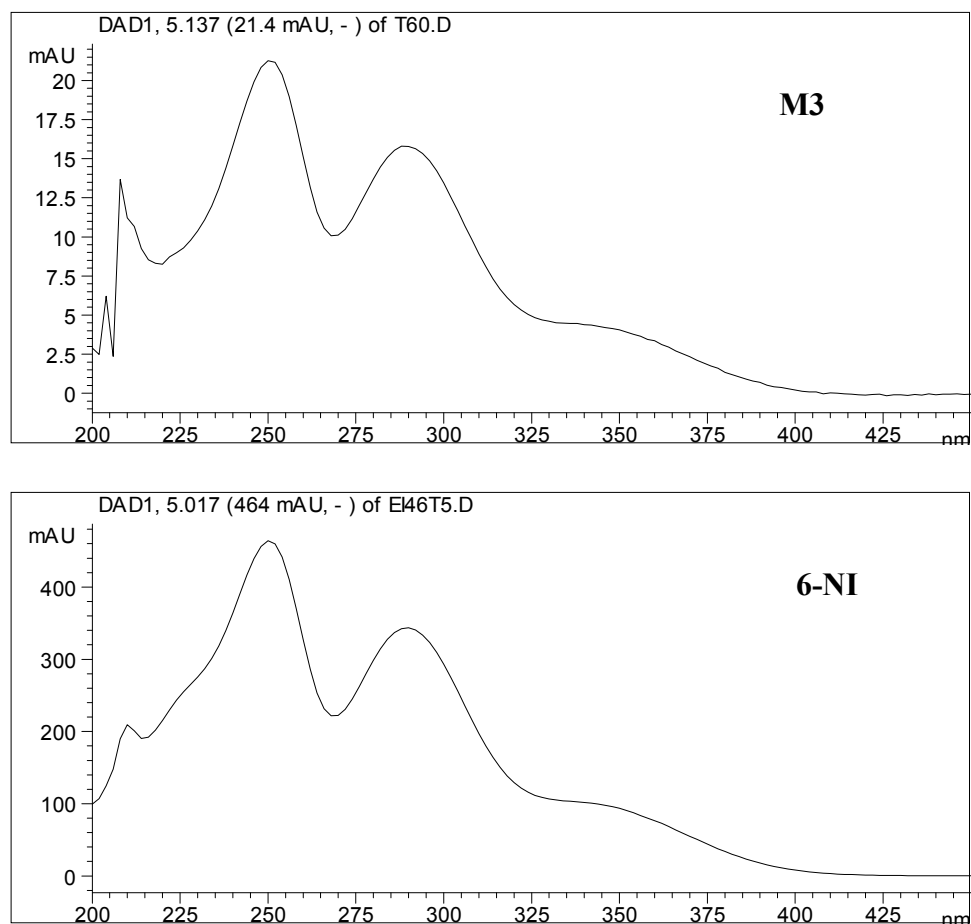
**Figure 46. HPLC analysis of supernatants from incubation mixtures containing 141 and MAO-B.**



**Figure 47. HPLC analysis of supernatants from incubation mixtures containing 143 and MAO-B.**

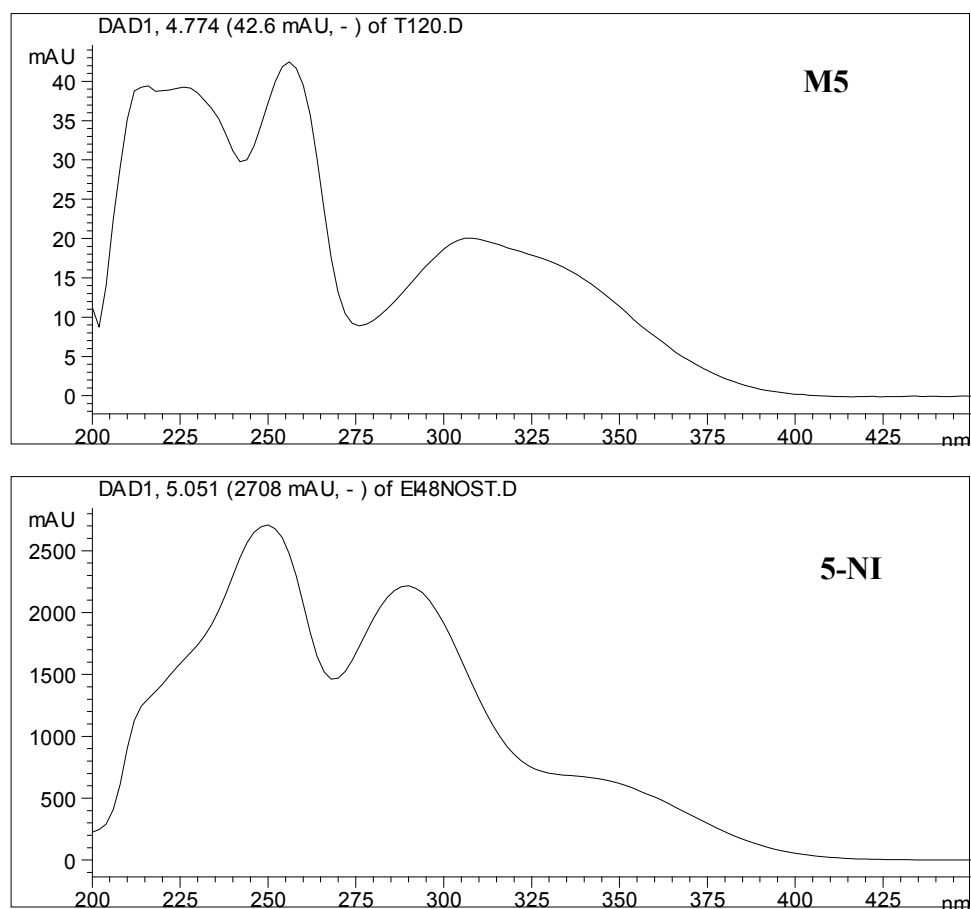
According to our proposed bioactivation pathway (Scheme 27), “prodrugs” are expected to release the parent compounds, 6-NI and 5-NI, respectively, in this case. The UV spectrum and the retention time of the minor metabolite peak (**M3**) derived from the

6-NI compound **141** indeed resembled that of 6-NI (Figure 48). Although it was not the predominant metabolite, the release of 6-NI was observed.



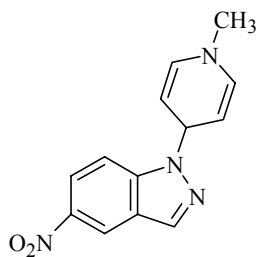
**Figure 48. UV spectra of M3 and commercial 6-NI.**

In the case of the 5-NI prodrug **143**, the UV spectrum of the minor metabolite (**M5**) was different from that of 5-NI (Figure 49).

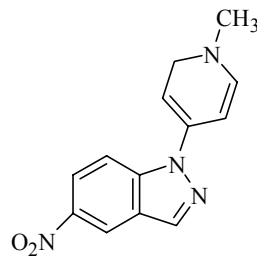


**Figure 49. UV spectra of M5 and commercial 5-NI.**

At this point, we have not characterized **M5**. But one suggested metabolite is the dihydropyridine **168** or **169** which may be formed by the MAO-B catalyzed oxidation of the substrate **143**.



**168**



**169**

The shorter retention times of the major metabolite peaks **M2** and **M4** (from **141** and **143** respectively) suggested the formation of a more polar species than the substrates (**141** or **143**) in both cases.

We then turned our attention to two other possible sets of metabolites, one being the dihydropyridinium species **169** and **170** which will be formed by the MAO-B catalyzed two-electron oxidation of the corresponding tetrahydropyridines **141** and **143**. The second set of metabolites are the stable pyridinium species **145** and **152** which will be formed by the further oxidation of the corresponding dihydropyridinium intermediates (Scheme 68) as observed in the case of MPTP (Scheme 7).

Comparison of the UV spectrum of the metabolite **M2** with our synthetic pyridinium compound **145** and **M4** with synthetic **152** showed that they were identical and that these metabolites were indeed the pyridinium species (Figures 50 and 51).

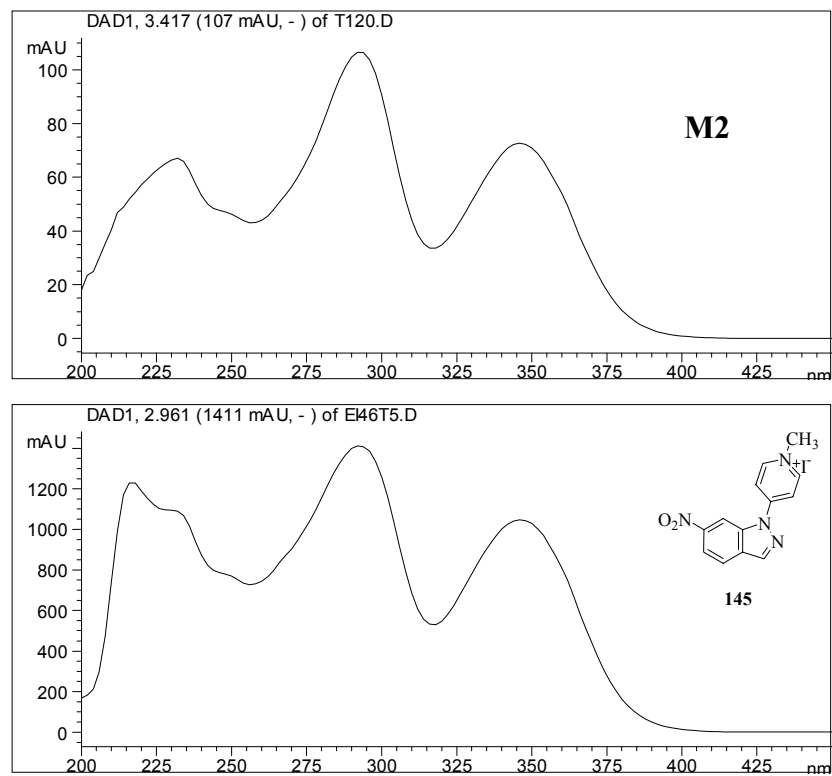


Figure 50. UV spectra of M2 and synthetic 145.

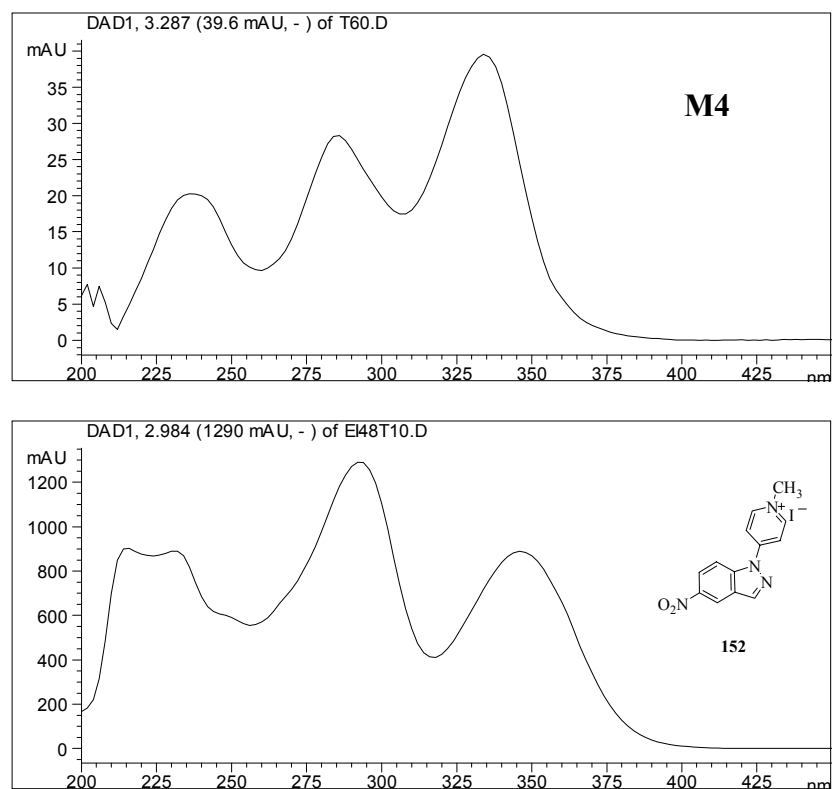
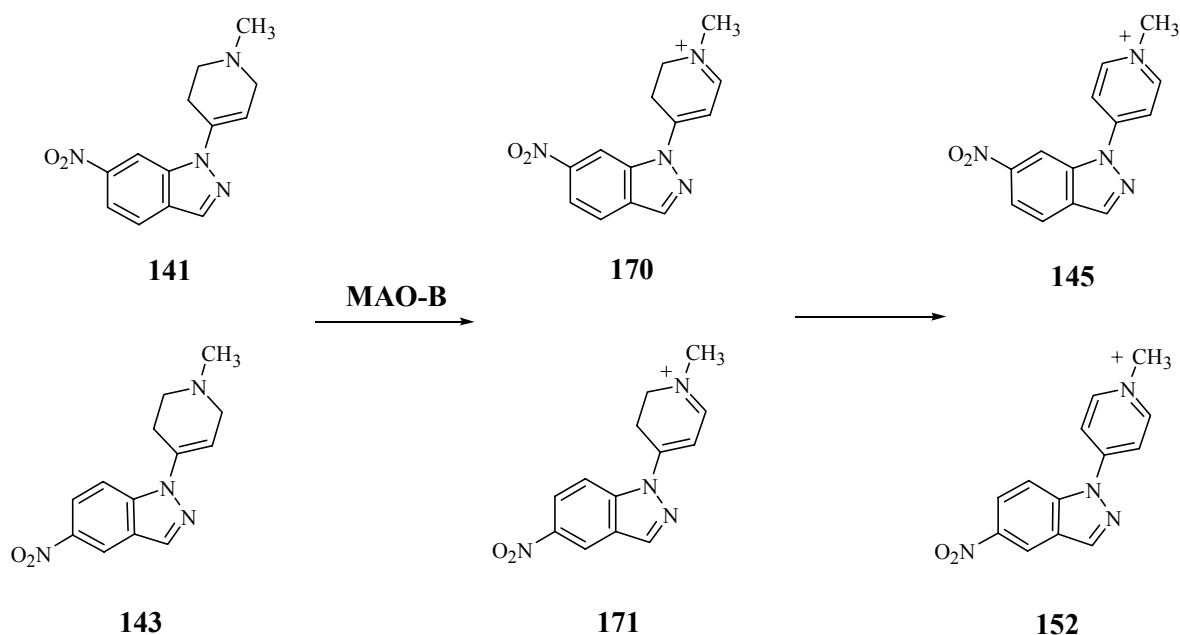


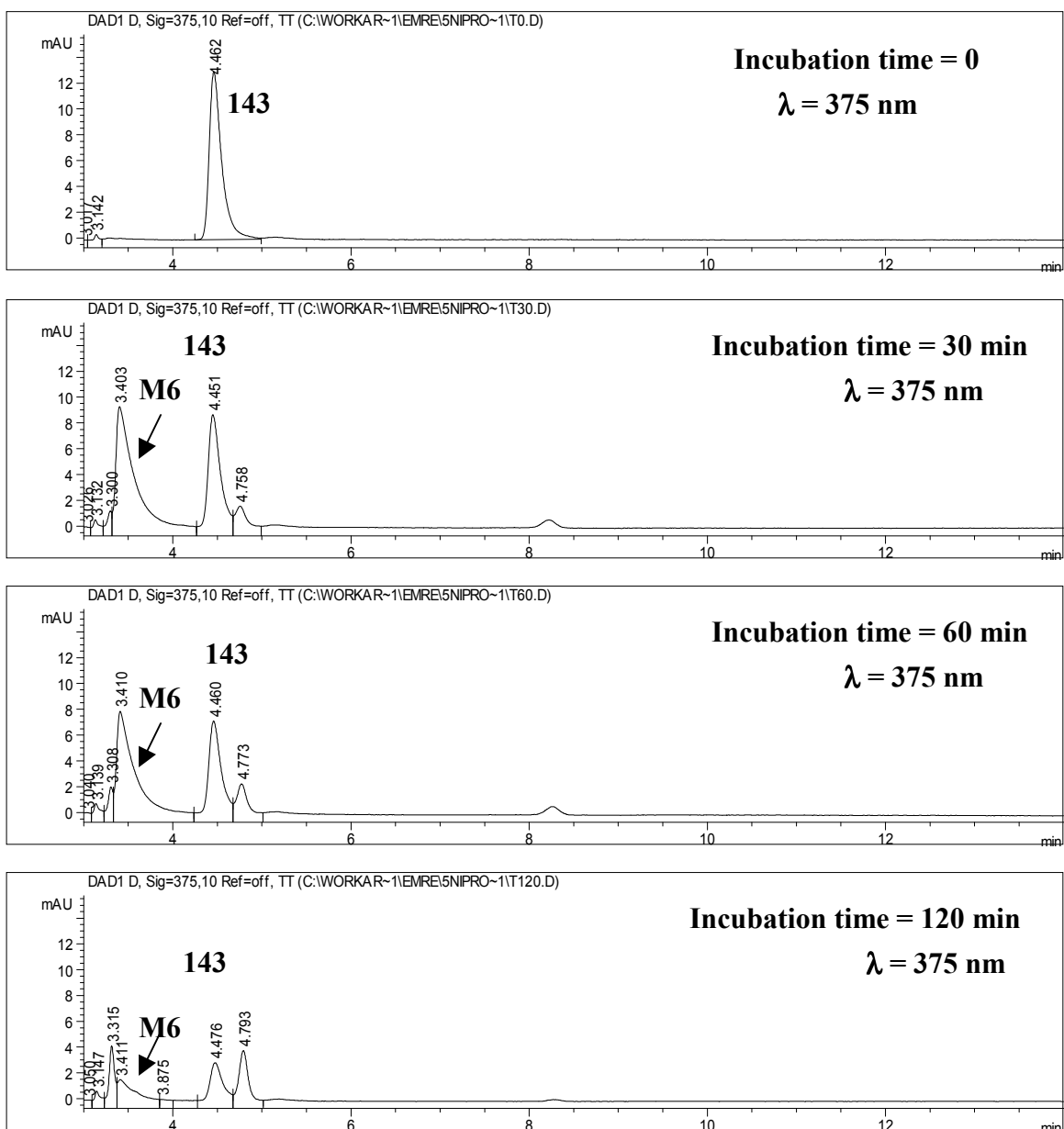
Figure 51. UV spectra of M5 and synthetic 152.

As mentioned above, the first step in the metabolic pathway yielding to the pyridinium species **145** and **152** is the MAO-B catalyzed two electron oxidation of the tetrahydropyridines **141** and **143** to the corresponding dihydropyridinium species **170** and **171** (Scheme 69).

**Scheme 69. MAO-B Catalyzed 2 electron oxidation of 141 and 143 yielding 170 and 171 followed by further oxidation to the pyridinium species 145 and 152**



When we examine the HPLC tracings obtained from MAO-B incubations of the 5-NI prodrug **143** at a longer wavelength (375 nm), we were able to see a minor peak (M6) at 3.4 minutes which disappears with time (Figure 52). This peak was proposed to be the dihydropyridinium species **171** which is initially formed but further oxidized to the stable pyridinium species **152**. The synthetic standard is not available for **171**. However, the shift of the chromophore to longer wavelengths suggests that this peak may be the dihydropyridinium species based on comparison with the UV behavior of related systems.

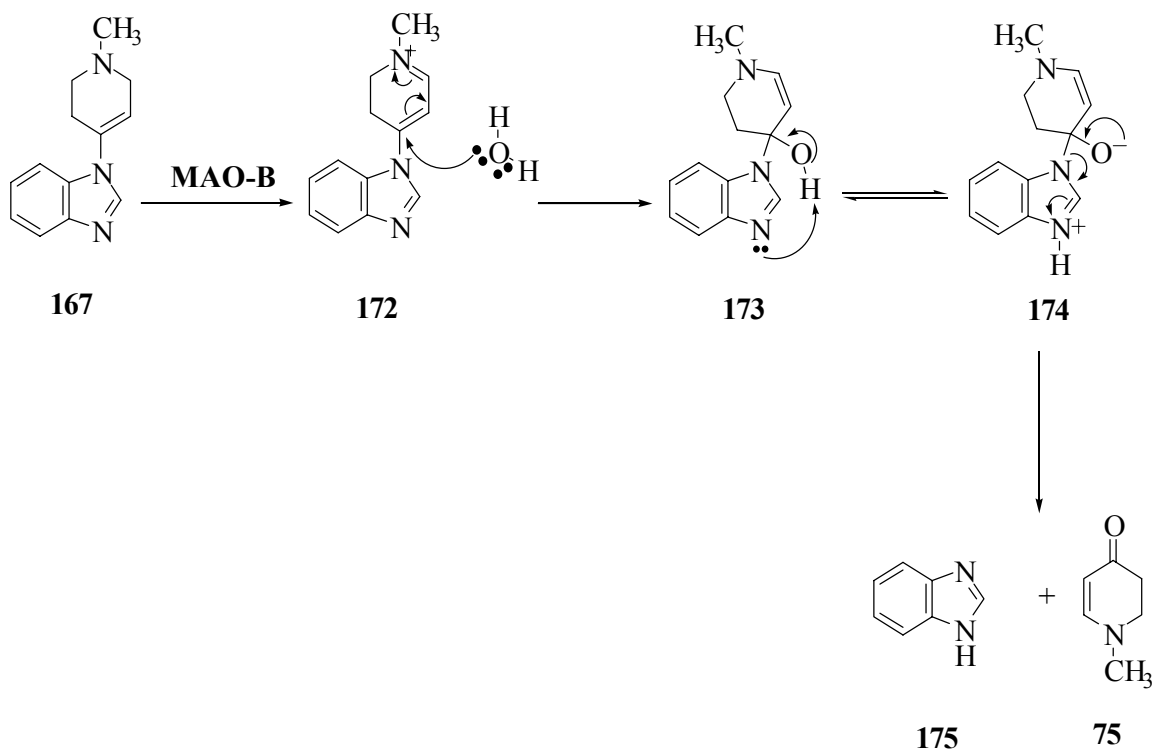


**Figure 52. HPLC analysis of supernatants from incubation mixtures containing 143 and MAO-B at 375 nm.**

These findings prompted the question: Why are the dihydropyridinium species **170** and **171** not undergoing the anticipated hydrolysis to release 6-NI and 5-NI, respectively, as the major metabolites. This hydrolysis had been observed in the case of a structurally related compound 1-methyl-4-benzimidazol-1-yl-1,2,3,6-tetrahydropyridine [(**166**) Scheme 70].<sup>184</sup>

<sup>184</sup> See reference 144

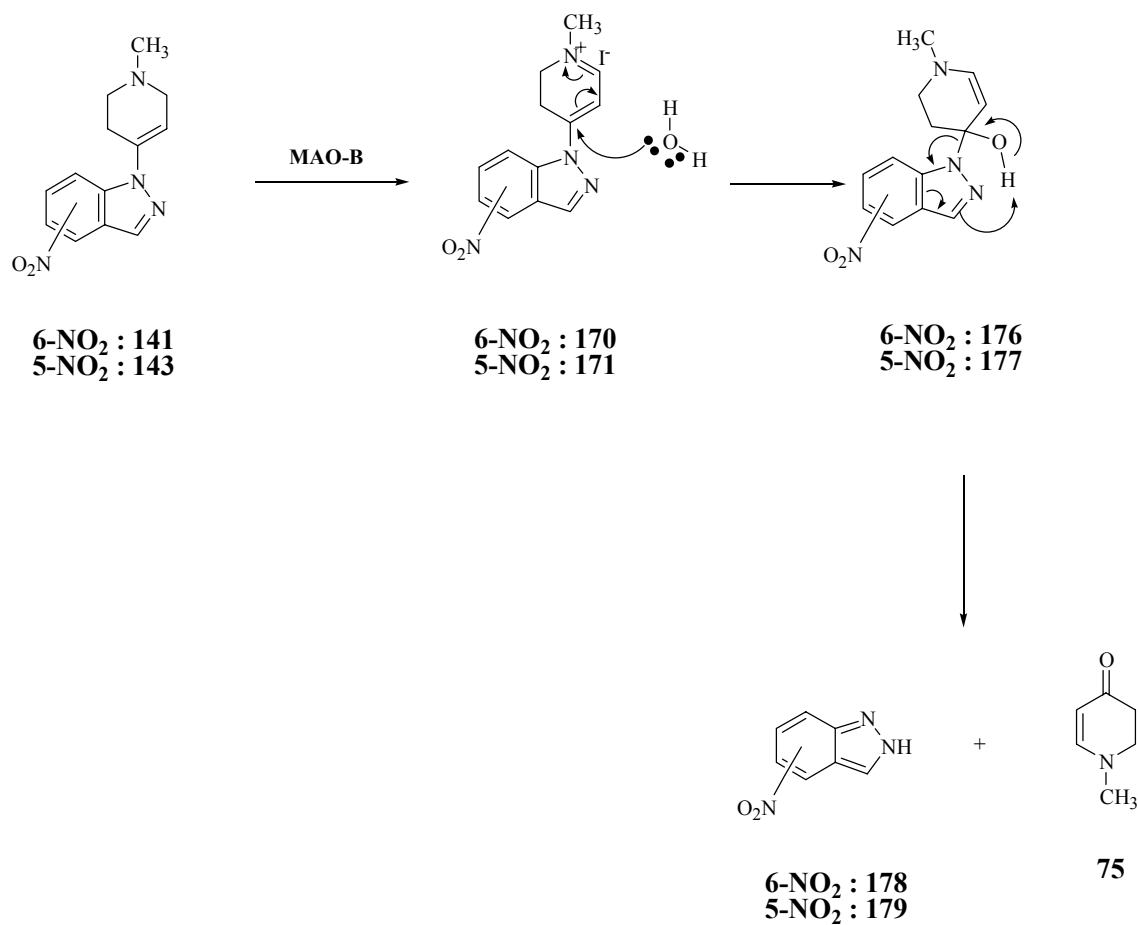
**Scheme 70. Release of benzimidazole (175) via the MAO-B catalyzed bioactivation of the corresponding “prodrug” 167.**



In this case, hydrolysis of the metabolite **172** results in the formation of the stable benzimidazole nucleus **175**. However, in the case of **170** and **171**, hydrolytic cleavage would lead initially to the less stable *2H* tautomers **178** and **179**. The *2H* tautomer lacks the benzenoid structure and thus should not be as stable as either the *1H* tautomer or benzimidazole.<sup>185</sup> This makes the oxidation to pyridinium species **145** and **152** more favored over the hydrolysis reaction yielding to the parent compounds **178** and **179** (Scheme 71).

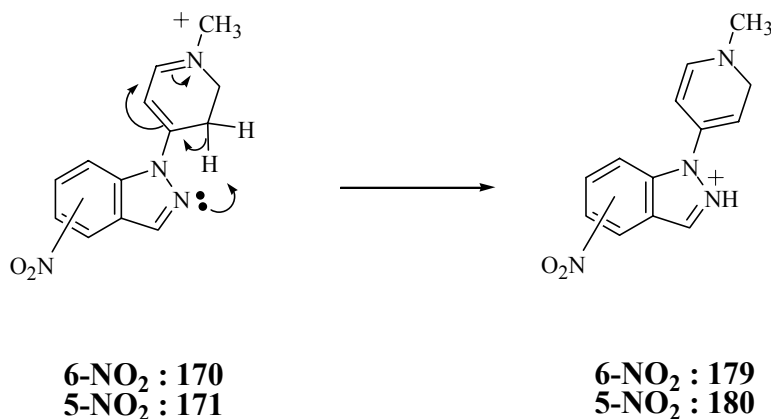
<sup>185</sup> See page 38 for a detailed discussion of the tautomerization of indazole

**Scheme 71. Release of 6-NI and 5-NI respectively from 141 and 143 upon hydrolysis of the dihydropyridinium species 169 and 170 followed by cleavage.**



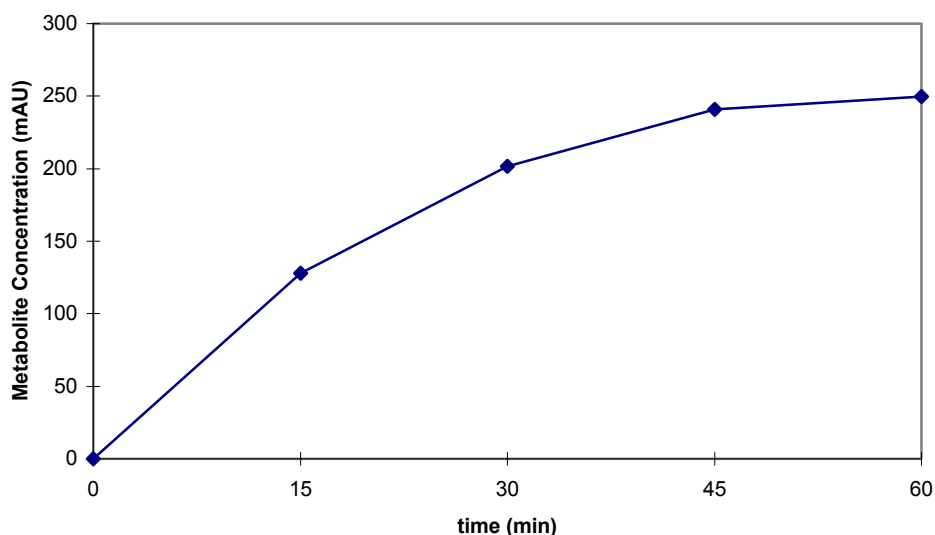
Another factor favoring the oxidation of **170** and **171** to **145** and **152** is the participation of N2 as an internal base facilitating the formation of the dihydropyridines **179** and **180**, intermediates leading to the pyridinium species (Scheme 72). This factor is not present in the case of **166** where the hydrolysis is favored over the second oxidation reaction.

**Scheme 72. N2 can act as an internal base to facilitate the oxidation to the pyridinium species.**



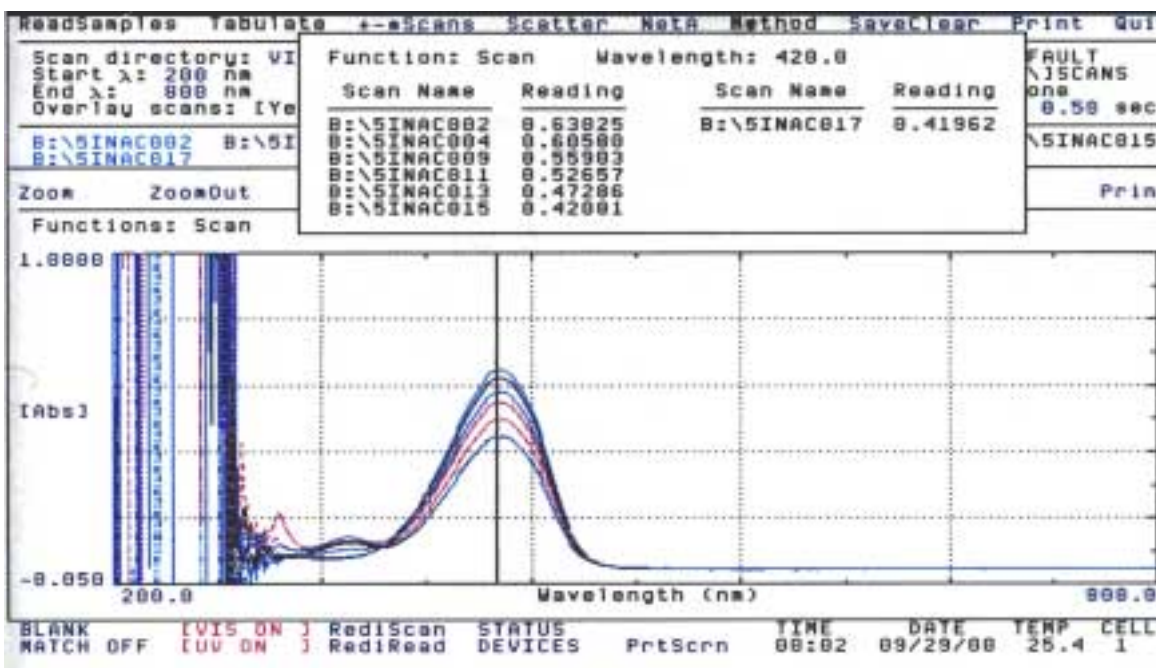
In order to obtain a quantitative measure of the substrate properties of **143**, we also estimated the  $K_m$  values for the reaction. We determined the initial rate of metabolite (**M4**) formation ( $V_i$ ) by measuring and plotting the increase in metabolite concentration versus time at different substrate concentrations using HPLC-DA as well as UV spectrophotometry. The  $K_m$  values ( $-1/x$  when  $y = 0$ ) were determined from the double-reciprocal plots of  $1/(V_i)$  versus  $1/(\text{substrate concentration})$ . The  $K_m$  value for **143** was estimated to be 50  $\mu\text{M}$  (The results obtained by HPLC-DA assay was in agreement with UV spectrophotometry assay). The  $K_m$  value of the **141** remains to be determined.

Plots of metabolite concentration versus time showed that the metabolite formation slows down after 30 minutes (Figure 53). One possibility to account for the decrease in rate of metabolite formation is the loss of enzyme activity. This possibility led us to investigate the enzyme inhibitory properties of **141** and **143**.



**Figure 53. Metabolite concentration versus time plot for 143 obtained by HPLC-DA analysis ( $\lambda = 269$  nm) of the supernatants of the incubation mixtures containing 40  $\mu$ M of 143 and MAO-B.**

Both compounds **141** and **143** were found to inhibit MAO-B. The  $K_i$  values were estimated to be 12  $\mu$ M and 4  $\mu$ M, respectively. The mode of inhibition was determined to be competitive. However, at this point it is not possible to determine whether the substrate molecules **141** and **143**, themselves, or the metabolites derived from them were responsible for the observed inhibition. Therefore, we also carried out a time-dependent inhibition assay where we pre-incubated 40  $\mu$ M solutions of **143** with MAO-B for 5, 10, 15, 20, 25 and 30 minutes. Following the pre-incubations, 2 mM **162** was used to assay remaining enzyme activity. After 30 minutes the incubations were stopped by adding acetonitrile, the protein was sedimented by centrifugation at 10,000 g for 6 minutes and the supernatants were analyzed by UV (Figure 54).



**Figure 54. UV analysis of the supernatant from the incubation mixtures containing MAO-B and post-incubated with 162 (top to bottom) : 1. [143] = 0 2. [143] =40  $\mu\text{M}$   $t_{\text{pre}} = 0$  minutes 3. [143] =40  $\mu\text{M}$   $t_{\text{pre}} = 10$  minutes 4. [143] =40  $\mu\text{M}$   $t_{\text{pre}} = 15$  minutes 5. [143] =40  $\mu\text{M}$   $t_{\text{pre}} = 20$  minutes 6. [143] =40  $\mu\text{M}$   $t_{\text{pre}} = 25$  and 30 minutes.**

The results showed the time dependent loss of enzyme activity which suggests that pyridinium metabolites are responsible for the inhibition. This was not unexpected since inhibition of MAO-B by pyridinium metabolites had been previously reported.<sup>186</sup> Evidence obtained so far, however, is not sufficient to determine whether the loss of enzyme activity is due to the mechanism based inactivation of the enzyme or competitive inhibition resulting from pyridinium production. However, in the case of inactivation more drastic loss of enzyme activity is expected over time. This suggests that the competitive inhibition by the pyridinium metabolites is a more likely possibility. In order to be able to reach to a more definitive conclusion,  $K_i$  values for pyridinium compounds **145** and **152** should be determined and compared with the  $K_i$  values obtained for **141** and **143**.

<sup>186</sup> Fang, J., Yu, P.H., Gorrod, J.W., Boulton, A.A. (1995) Inhibition of monoamine oxidases by haloperidol and its metabolites: pharmacological implications for the chemotherapy of schizophrenia. *Psychopharmacology (Berl)* **118**, 206-212.

## 6. CONCLUSIONS

The possible relationship between the inhibition of nNOS and MAO-B and neuroprotective processes is a topic of current interest both in terms of fundamental biological issues and the potential to develop neuroprotective agents. Many MAO-B inhibitors and a few nNOS inhibitors have been evaluated for their neuroprotective properties in the MPTP mouse model of neurodegeneration. One such compound is 7-NI (**68**). This compound has the interesting property of inhibiting both enzymes. However, its limited solubility complicates *in vivo* experimentation. In this work we initially designed a potential “prodrug” of 7-NI where a tetrahydropyridinyl moiety is used as a carrier. This prodrug was designed to undergo bioactivation via the brain MAO-B catalyzed  $\alpha$ -carbon oxidation pathway followed by hydrolysis of the resulting dihydropyridinium intermediate to release 7-NI in the brain. Because indazole and its derivatives are ambident nucleophiles, the reaction pathways, which involved nucleophilic aromatic substitution reactions, could lead to one or both regioisomers.

Previous attempts to synthesize the “prodrug” precursors **119** and **120** had yielded a single product that could be converted to one of the two possible regioisomers. The yields were low and, during the course of this high temperature reaction between 4-chloro-1-methylpyridinium iodide (**115**) and 7-nitroindazole, the initially formed condensation product underwent demethylation to give the corresponding pyridinyl species **108** or **110**. The structure had been tentatively assigned to be the *IH* isomer **108**. Regiochemical issues led us to undertake a DPGSE-NOE  $^1\text{H}$  NMR experiment to establish the structure of this product. Unambiguous evidence obtained from the DPGSE-NOE  $^1\text{H}$  NMR experiment proved the regiochemistry of the compound to be the *2H* isomer **110**. This assignment was confirmed by through space NOE  $^1\text{H}$  NMR experiments performed on the corresponding amino derivative **124**. Attempts to repeat this reaction led to similar results although it was possible to isolate small quantities of the *IH* isomer **108** and to detail the differences between the  $^1\text{H}$  NMR and mass spectral feature of the two isomers.

The progress of the nucleophilic aromatic substitution reaction between 4-chloro-1-methylpyridinium iodide (**115**) with 7-NI was examined to gain a better understanding of the reaction pathway and to explore the possibility of synthesizing the *IH* isomer. This

study led to several important observations. Evidence was obtained indicating that the product of the reaction was decomposing at the injection port of the GC-EIMS to give 7-NI. Consequently, the earlier attempts to monitor the reaction by GC-EIMS were providing misleading data in that it appeared that the reaction was not progressing. It was not possible to recognize this problem under high temperature reaction conditions which led to the irreversible demethylation of the initially formed N-methylpyridinium products **127** and **128**. When monitored by HPLC, however, it became clear that the reaction was proceeding efficiently at room temperature to form the stable N-methylpyridinium species in high yield. Work up of the reaction led to the isolation of a single nitroindazolyl product which was shown to be the *2H* isomer. Evidence suggesting the possibility that the *2H* and *1H* isomers are in equilibrium was obtained. Therefore, attempts were made to convert the *2H* isomer to the *1H* isomer, which, based on molecular modeling calculations at the semi-empirical level, was expected to be slightly more stable than the *2H* isomer. However, heating the *2H* isomer led only to its decomposition to 7-NI and 4-iodo-1-methylpyridinium iodide. Under these conditions, iodomethylpyridinium iodide (and, perhaps, the indazolylpyridinium product) underwent demethylation. These reversible events were followed by the irreversible capture of the resulting iodomethane by the 2,2,6,6-tetramethylpiperidine (to give the pentamethyl product detected by GC-EIMS analysis) that was added in this reaction since it was used as the base in the condensation reaction. The possible reaction of 4-iodopyridine with 7-nitroindazole at high reaction temperatures may explain the formation of both the *1H* and the *2H* isomers under reflux. However due to the low reactivity of 4-iodopyridine and the steric hindrance caused by the nitro group, the extent of reaction was limited resulting in very low yields of the *1H* isomer **108**.

The success of the nucleophilic aromatic substitution reaction between 4-chloro-1-methylpyridinium iodide and 7-NI prompted additional studies to examine the generality of this synthetic approach to potential “prodrugs”. Two other commercially available nitroindazoles, 6-nitroindazole (**122**) and 5-nitroindazole (**123**), were examined. Both compounds have been reported to be selective inhibitors of nNOS. The synthetic efforts to obtain the “prodrugs” of these compounds led to important insights into the mechanism of the reaction and the regiochemistry involved. The nucleophilic aromatic

substitution reaction between 4-chloro-1-methylpyridinium iodide (**115**) and 6-NI gave the corresponding pyridinium species in the presence of base at room temperature. The yields were satisfactory and the product was isolated as a single isomer. The evidence obtained from DPGSE-NOE  $^1\text{H}$  NMR experiment showed the regiochemistry of the product to be the *IH* isomer **145**. The analogous reaction with 5-NI also resulted in the formation of a single isomer which was determined to be the *IH* compound **152**. This regiochemical outcome shows that the more stable *IH* isomer is favored when the steric hindrance caused by 7-NI is removed as the nitro group is placed at C5 or C6. Studies with 6-NI led to the investigation of the reversibility of the reaction. It was possible to demonstrate clearly the reversibility of the reaction with the aid of the good nucleophile, cyanide. The *IH* compound **145**, in the presence of cyanide, yielded 6-NI and presumably 4-cyano-1-methylpyridinium iodide via the capture of 4-iodo-1-methylpyridinium iodide by cyanide.

In order to gain a more thorough understanding of the reaction mechanism and to examine the effect of the nitro group on the reaction, we extended our studies to the parent compound indazole. Investigation of the nucleophilic aromatic substitution reaction under various conditions showed that it was possible to take advantage of two pathways to form both of the regioisomers **155** and **156**. The *IH* isomer was obtained as the major product when the reaction was carried out in the presence of base at room temperature. In the absence of base but at higher temperature the only product was the *2H* isomer. These results were rationalized by the higher energy of the intermediate leading to the *IH* isomer, compared to the *2H* isomer, that would form in the absence of base. When base was present, this energy difference was lower. Consequently the thermodynamically more stable *IH* isomer **155** was formed. Consistent with these energy considerations, the isomerization of the less stable *2H* isomer to the stable *IH* isomer proceeded in the presence of base at high temperatures. The *IH* isomer **155**, however, was stable under these conditions.

After obtaining the pyridinium compounds **120**, **145**, **152**, **155** and **156**, they were reduced to the corresponding tetrahydropyridinyl species **86**, **141**, **143**, **160** and **161**, respectively. Interaction of these “prodrugs” with the MAO-B were investigated *in vitro*. The “prodrug” of 7-NI, **86**, did not show any MAO-B substrate properties. However, it

turned out to be a competitive inhibitor of MAO-B. Inhibition of the enzyme did not show any time dependency and therefore the inhibition was due to the parent compound and not to metabolically generated 7-NI.

The observation that the *2H* analog of 7-NI is an inhibitor but not a substrate is noteworthy since, in the case of indazole, only the *1H* isomer **160** had displayed substrate properties whereas the *2H* isomer **161** had not. However, the inhibitory properties of the *2H* isomer **161** had not been investigated. Compound **161** proved to be a competitive inhibitor of MAO-B with a  $K_i$  value comparable to that of the parent compound indazole.

The *1H* indazolyltetrahydropyridinyl analogs obtained with 6-NI and 5-NI, compounds **141** and **143**, were tested for their MAO-B substrate properties. Compound **143** was a substrate which displayed a  $K_m$  value of 50  $\mu\text{M}$ . The  $K_m$  value of compound **141**, also a substrate remains to be determined. These results were in agreement with our hypothesis that the *1H* isomers are more likely to fit in the active site of the enzyme and display substrate properties than the *2H* isomers. The *2H* isomers, which do not display substrate properties as in the case of the “prodrug” of indazole, may be assuming a different orientation with respect to the active site of the enzyme and act as inhibitors of the enzyme.

Although these proposed “prodrugs” showed substrate properties, the dihydropyridinium species formed after the initial MAO-B catalyzed 2 electron oxidation did not undergo the expected hydrolysis to release the parent compounds. Instead, a second oxidation took place leading to the pyridinium metabolites **145** and **152**. Structural analysis of the dihydropyridinium species suggest that both the instability of the non-benzenoid products that would be formed and the possibility of intramolecular proton transfer to form the dihydropyridine intermediate leading to the pyridinium metabolites may be responsible for this behavior. The formation of the pyridinium species in these reactions also may account for the observed time-dependent slowing of the rate of the MAO-B catalyzed oxidation of the parent tetrahydropyridinyl substrates.

The 6-NI and the 5-NI “prodrugs” **141** and **143** also proved to be competitive inhibitors of MAO-B as did the parent compounds 6-NI (**122**) and 5-NI (**123**). The  $K_i$  values estimated for 6-NI and 5-NI were comparable to that of 7-NI. This finding may be

of interest since *in vivo* studies with these compounds may reveal important information on the role of MAO-B versus. nNOS inhibition in neuroprotection.

Overall, it may be concluded that the initial concept of a tetrahydropyridinyl carrier for the construction of nitroindazole prodrugs is unlikely to be successful. The results of these studies, however, have provided new insights into the chemistry of indazole and should be valuable in future synthetic undertakings with this diazaarene. Additionally these studies have provided useful information on the active site of MAO-B. The regioisomers interact differently and predictably with the active site. Whether the compound will act as a substrate or an inhibitor can be predicted with the aid of the previously constructed model of the active site. This information may be useful in efforts to design inhibitors as well as substrates of MAO-B. Finally, the compounds prepared in this study should be studied *in vivo* for their neuroprotective effects in animal models. Their water solubility properties should facilitate such studies.

## 7.EXPERIMENTAL

### 7.1.CHEMISTRY

**General methods.** All reactions were carried out using glassware that had been flame dried under an inert atmosphere of dry nitrogen. All chemicals were reagent or HPLC grade. General Melting points were determined using a Thomas-Hoover melting point apparatus. The  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra were recorded with a Bruker AM-360-MHz spectrometer DPFGE-NOE  $^1\text{H}$  NMR experiments were performed on a Jeol 500 MHz spectrometer.  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to the internal standard tetramethylsilane (TMS). Spin multiplicities are given as s(singlet), d(doublet), t(triplet), q(quartet). Coupling constants (J) are given in hertz (Hz). UV-Vis absorption spectra were recorded on a Beckman DU Series 7400 spectrophotometer. Gas chromatography-electron ionization mass spectrometry (GC-EIMS) was performed on a Hewlett Packard (HP) Model 6890 gas chromatography fitted with a HP-5MS column(30 m x 0.25 mm x 0.25  $\mu\text{m}$  thickness) which was coupled to a HP5973 mass selective detector. Helium was the carrier gas (1 ml./min/). All GC-EIMS data were obtained using an initial oven temperature of 60  $^\circ\text{C}$  ramping at 20  $^\circ\text{C}/\text{min}$  to a final temperature of 290  $^\circ\text{C}$  with a solvent delay of 3.2 min and injection port temperature of 220  $^\circ\text{C}$ . Normalized peak heights are reported as a percentage of the base peak. High pressure liquid chromatography (HPLC) was performed on a HP model 1100 HPLC system equipped with quaternary pump, degasser, diode array detector and Zorbax C8 column.

**7-Nitroindazole (26):** Prepared according to the literature.<sup>187</sup> A solution of 2-methyl-6-nitroaniline (3.04g, 20.0 mmole) in 50 mL of AcOH was cooled in an ice bath while stirring.  $\text{NaNO}_2$  (1.38g, 20.0 mmole) was dissolved in as less  $\text{H}_2\text{O}$  as possible and was added to solution at one time. The mixture was stirred for 15 minutes at 0  $^\circ\text{C}$ , and then 14 h. at 25  $^\circ\text{C}$ . The solution was concentrated to 1/3 of its volume. To the residue, 1/4 of the volume  $\text{H}_2\text{O}$  was added. After warm filtration, the compound was crystallized from the solvent. After filtration, the compound was recrystallized from 100% EtOH to yield 2.35g (14.4 mmole, 72%) of an orange solid: mp 185-186  $^\circ\text{C}$ . Known compound

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<sup>187</sup> Barbet, O. , Minjat, M., Petavy, A., Paris, J. (1986) Phenyl urees derivees de l'indazole;recherche de l'activite anthelminthique, effet des substituants. *Eur. J. Med. Chem. - Chim. Ther.* **21**, 359-362.

(mp 185-186 °C); <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz): δ 8.4 (dd, J=7.9 Hz, J= 2Hz, 1H), δ 8.3 (s, 1H), δ8.2 (dd, J=7.9 Hz, J=0.2 Hz, 1H), δ7.3 (t, J=7.9 Hz, 1H); <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 100 MHz) δ135.8, 132.0, 131.9, 130.0, 127.2, 123.5, 120.3; GC-EIMS m/z (%) 163 (M<sup>+</sup>, 100), 147 (2), 133 (26), 117 (19), 105 (19), 90 (72), 78 (11), 63 (49); UV (nm, MeOH) 207, 228, 335.

**1-Methyl-4-chloropyridinium iodide (36).** Prepared according to the literature.<sup>188</sup> 4-chloropyridine hydrochloride salt (6.03g, 40.2 mmole) was converted into to the free base by dissolving in H<sub>2</sub>O and adding saturated K<sub>2</sub>CO<sub>3</sub> dropwise until pH 9, and extracting with CH<sub>2</sub>Cl<sub>2</sub>. CH<sub>2</sub>Cl<sub>2</sub> was dried and the solvent was evaporated. Methyl iodide (22.8g, 160.6 mmole) was added to the free base dropwise at 0 °C over 15 minutes while stirring. The reaction was kept at 0 °C over night in the dark. The product was filtered and crystallized from MeOH/Ether to yield 7.71g. (30.2 mmole, 74 %) of a nearly white solid; mp 160-162 °C. Known compound (mp:161-163 °C); <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 360 MHz): δ 9.0 (m, 2H), δ 8.4 (m, 2H), δ4.3 (s, 3H); <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 100 MHz) δ152.2, 146.8, 127.8, 47.6; UV (nm, MeOH) 225, 261.

**1-Methyl-4-(7-nitroindazol-1-yl)pyridine (108).** To a solution of 7-nitroindazole (**26**) (163 mg, 1 mmole) in N,N-dimethylformamide, 2,2,6,6-tetramethylpiperidine (0.17 mL, 1 mmole) was added. After stirring for 15 minutes, 4-chloro-1-methylpyridinium iodide (**36**) (256 mg, 1 mmole) was added at one time. After stirring for 24 hours under reflux, the reaction mixture was cooled to room temperature. To the reaction mixture H<sub>2</sub>O was added and extracted with ethylacetate. Organic extracts were combined, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the solvent was evaporated under vacuum. The crude product was subjected to column chromatography using silica gel as the stationary phase. First, 7-NI was removed by eluting with hexane/EtOAc. The two possible isomeric products were isolated by first eluting with EtOAc/hexane (6:4), then eluting with EtOAc/hexane (8:2). The more polar *IH* isomer was further purified by preparative TLC using EtOAc/hexane (8:2) as the mobile phase to give 6 mg (0.025mmole, 2.5 %) of a light yellow solid: <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz): δ 8.7 (m, 2H), δ 8.4 (s, 1H), δ8.2 (dd, J=8.2 Hz, J=1.2 Hz, 1H), δ8.1 (dd, J=8.0, J=1.2 Hz, 1H), δ7.4 (dd, J=8.2, J=8.1, 1H),

<sup>188</sup> Sprague, R.H., Brooker, L.G.S. (1937) Studies in the cyanine dye series. IX. 4,4'-pyridocyanines and 4-pyrido-4'-cyanines. *J. Am. Chem. Soc.* **59**, 2697-2699.

$\delta$ 7.3(dd,  $J=7.5$  Hz,  $J=6.8$ , 2H);GC( $t_R = 11.83$  min)-EIMS  $m/z$  (%)240 ( $M^+$ , 73), 223 (67), 185 (43), 166 (23), 155 (43), 140 (92), 128 (52), 113 (39), 102 (25), 89 (21), 78 (33), 63 (36), 51 (100).

**1-Methyl-4-(7-nitroindazol-2-yl)pyridine (110).** The less polar isomer was isolated as the *2H* isomer from the above reaction and recrystallized from MeOH to give 100 mg of a yellow solid (0.42 mmole, 42 %) mp 257-259 °C;  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ , 360 MHz):  $\delta$  8.8 (m, 2H),  $\delta$  8.7 (s, 1H),  $\delta$ 8.4(dd,  $J=6.8$  Hz,  $J=1.0$  Hz, 1H),  $\delta$ 8.1 (dd,  $J=7.5$ ,  $J=0.8$  Hz, 1H),  $\delta$ 8.0(dd,  $J=7.5$ ,  $J=7.8$ , 2H),  $\delta$ 7.3(dd,  $J=7.5$  Hz,  $J=6.8$ , 1H);  $^{13}\text{C-NMR}$  ( $\text{DMSO-d}_6$ , 100 MHz)  $\delta$ 162.0, 149.8, 147.7, 137.2, 130.9, 128.9, 127.8, 126.3, 122.8, 117.3, 47.4; GC( $t_R = 14.02$  min)-EIMS  $m/z$  (%) 240 ( $M^+$ , 100), 210 (13), 193 (20), 182 (62), 167 (13), 155 (9), 140 (34), 78 (42), 51 (61) Anal. ( $\text{C}_{12}\text{H}_{11}\text{N}_4\text{O}_2$ ) C, H, N.

**1-Methyl-4-(7-nitroindazol-2-yl)pyridinium iodide (120).** To a solution of 7-nitroindazole (1.63g, 10 mmole) in 15 mL *N,N*-dimethylformamide, 2,2,6,6-tetramethylpiperidine (1.69 mL, 10 mmole) was added. After stirring for 15 minutes, 4-chloro-1-methylpyridinium iodide (2.56 g., 10 mmole) was added at one time. After stirring for 24 hours at room temperature, reaction mixture was filtered to give 2.26g (5.9 mmole, 58 %) of an orange solid. It was recrystallized from boiling MeOH.: mp 261-262 °C;  $^1\text{H-NMR}$  ( $\text{DMSO-d}_6$ , 400 MHz):  $\delta$  9.3 (s, 1H),  $\delta$  8.8(d,  $J=7.4$ , 2H),  $\delta$ 8.5(d,  $J=7.7$ , 2H),  $\delta$ 8.4 (dd,  $J=7.4$ ,  $J=0.8$ , 1H),  $\delta$ 8.2(dd,  $J=8.4$  Hz,  $J=0.8$  Hz, 1H),  $\delta$ 7.4(dd,  $J=8.4$  Hz,  $J=7.4$  Hz, 1H),  $\delta$ 4.3(s, 3H);  $^{13}\text{C-NMR}$  ( $\text{DMSO-d}_6$ , 100 MHz)  $\delta$ 162.0, 149.8, 147.7, 137.2, 130.9, 128.9, 127.8, 126.3, 122.8, 117.3, 47.4; GC-EIMS  $m/z$  (%) 240 ( $M^+$ , 100), 210 (12), 193 (19), 182 (61), 167 (14), 140 (33); UV (nm, MeOH) 221, 257, 326, 377. Anal. ( $\text{C}_{13}\text{H}_{11}\text{N}_4\text{O}_2\text{I}$ ) C, H, N.

**1-Methyl-4-(7-nitroindazol-2-yl)-1,2,3,6-tetrahydropyridine (86).** Sodium borohydride (40 mg, 1.1 mmole) was added in small portions to a suspension of 1-methyl 4-(7-nitroindazol-2-yl)pyridinium iodide (**120**, 190 mg, 0.5 mmole) in methanol (5 mL) at room temperature while stirring. After 24 hours methanol was evaporated. The residue was purified by column chromatography on basic alumina, eluting with ethylacetate/hexane (9:1). The solid was recrystallized from absolute ethanol to give 106 mg (0.41 mmole, 82 %) of the desired product (**86**): mp 90-91°C;  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ , 360 MHz)  $\delta$ 8.3 (dd,  $J=0.9$  Hz,  $J=7.5$  Hz, 1H),  $\delta$ 8.3 (s, 1H),  $\delta$ 8.0 (dd,  $J=0.9$  Hz,  $J=8.3$  Hz,

1H),  $\delta$ 7.2 (dd,  $J=7.8$  Hz,  $J=0.4$  Hz, 1H),  $\delta$ 6.6 (t,  $J=1.4$  Hz, 1H),  $\delta$ 3.2 (m, 2H),  $\delta$ 2.9 (m, 2H),  $\delta$ 2.8 (m, 2H),  $\delta$ 2.4 (s, 3H); GC-EIMS  $m/z$  (%) 240 ( $M^+$ , 100), 210 (33), 194 (19), 167 (24), 140 (45); UV (nm, MeOH) 212, 237, 319, 372. Anal. ( $C_{13}H_{15}N_4O_2Cl$ ) C, H, N.

**1-Methyl-4-(7-aminoindazol-2-yl)pyridine (124).** A mixture of 1-methyl-4-(7-nitroindazol-2-yl)pyridine (**110**) (120 mg, 0.5 mmole) and  $SnCl_2 \cdot 2H_2O$  (565 mg, 2.5 mmole) in 10 mL of absolute ethanol was stirred at 75 °C. After 2 hours, the solution was allowed to cool down and poured onto ice. The pH was made slightly basic (pH 7-8) by addition of 5 % aqueous sodium bicarbonate and extracted with ethylacetate. The organic phase was thoroughly washed with brine and dried over anhydrous  $Na_2SO_4$ . The solvent was evaporated in vacuo. The crude product was subjected to column chromatography using silica gel as the stationary phase and first eluting with EtOAc/hexane (6:4) to remove the starting material and then with EtOAc/hexane (9:1) to isolate 8 mg of **124** (0.04 mmole, 8 %).  $^1H$ -NMR ( $DMSO-d_6$ , 400 MHz):  $\delta$  9.0 (s, 1H),  $\delta$  8.7(d,  $J=7.4$ , 2H),  $\delta$ 8.0(d,  $J=7.7$ , 2H),  $\delta$ 6.8 (dd,  $J=8.4$ ,  $J=1.2$ , 1H),  $\delta$ 6.8(dd,  $J=8.4$  Hz,  $J=6.8$ , 1H),  $\delta$ 6.3(dd,  $J=6.8$  Hz,  $J=1.2$  Hz, 1H; GC( $t_R$  = 13.10 min)-EIMS  $m/z$  (%) 210 ( $M^+$ , 100), 182 (6), 132 (21), 105 (25), 78 (24), 51 (26).

**1-Methyl-4-(6-nitroindazol-1-yl)pyridinium iodide (145).** To a solution of 6-nitroindazole (**122**) (163 mg, 1 mmole) in 3 mL of *N,N*-dimethylformamide, 4-chloro-1-methylpyridinium iodide (**36**) (256 mg, 1 mmole) was added. To the solution, 2,2,6,6-tetramethylpiperidine (0.17 mL, 1 mmole) was added at one time. After stirring for 2 hours at room temperature, the reaction mixture was filtered to give 320 mg of yellow solid (0.83 mmole and 83 %). The product was crystallized from boiling methanol/water (50:50): mp 283-284 °C;  $^1H$ -NMR ( $D_2O$  500 MHz):  $\delta$  9.0 (ddd,  $J=0.9$  Hz,  $J=1.9$  Hz,  $J=1.4$  Hz, 1H),  $\delta$  8.9 (d,  $J=7.3$  Hz, 2H),  $\delta$ 8.7 (d,  $J=0.9$  Hz, 1H),  $\delta$ 8.5 (d,  $J=7.3$  Hz, 2H),  $\delta$ 8.3(dd,  $J=8.8$  Hz,  $J=1.9$  Hz, 1H),  $\delta$ 8.2 (dd,  $J=8.8$  Hz,  $J=0.9$  Hz, 1H),  $\delta$ 4.4(s, 3H);  $^{13}C$ -NMR ( $DMSO-d_6$ , 125 MHz)  $\delta$ 150.6, 148.6, 147.7, 142.1, 138.0, 131.1, 124.5, 120.0, 117.3, 109.6, 56.4; GC ( $t_R$  = 13.24 min)-EIMS  $m/z$  (%) 240 ( $M^+$ , 100), 210 (21), 194 (30), 167 (29), 140 (49); UV (nm, MeOH) 221, 293, 344. Anal. ( $C_{13}H_{11}N_4O_2I$ ) C, H, N.

**1-Methyl-4-(5-nitroindazol-1-yl)pyridinium iodide (152).** To a solution of 5-nitroindazole (**123**) (163 mg, 1 mmole) in 3 mL of *N,N*-dimethylformamide, 4-chloro-1-methylpyridinium iodide (**36**) (256 mg, 1 mmole) was added. To the solution, 2,2,6,6-

tetramethylpiperidine (0.17 mL, 1 mmole) was added at one time. After stirring for 2 h at room temperature, the reaction mixture was filtered to give 330 mg of yellow solid (0.86 mmole and 86 %). The product was crystallized from boiling methanol-water (50:50): mp 280-281° C; <sup>1</sup>H-NMR (D<sub>2</sub>O 500 MHz): δ 9.0 (d, J=2.3 Hz, 1H), δ 8.9 (d, J=7.6 Hz, 2H), δ 8.8(s, 1H), δ 8.6 (dd, J=9.4Hz, J=2.3 Hz 1H), δ 8.5(d, J=7.3 Hz, 2H), δ 8.3 (d, J=9.4 Hz, 1H), δ 4.4(s, 3H); <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 125 MHz) δ 150.7, 147.7, 144.5, 143.5, 141.2, 127.6, 124.6, 120.2, 117.3, 114.2, 56.3; GC (t<sub>R</sub> = 13.64 min)-EIMS m/z (%) 240 (M<sup>+</sup>, 100), 210 (33), 194 (19), 167 (24), 140 (45); UV (nm, MeOH) 218, 284, 335. Anal. (C<sub>13</sub>H<sub>11</sub>N<sub>4</sub>O<sub>2</sub>) C, H, N.

**Oxalate salt of 1-methyl-4-(6-nitroindazol-1-yl)-1,2,3,6-tetrahydropyridine (141).** Sodium borohydride (80 mg, 2 mmole) was added in small portions to a suspension of 1-methyl-4-(6-nitroindazol-1-yl)pyridinium iodide (**145**) (190 mg, 0.5 mmole) in methanol (5 mL) at room temperature while stirring. After 2 hours methanol was evaporated. The residue was extracted with dichloromethane and saturated NaCl solution. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the solvent was evaporated in vacuo to give the free base **141**. The oxalate salt of **141** was prepared by adding dropwise a saturated ethereal solution of oxalic acid to the tetrahydropyridine dissolved in ether. The oxalate salt was crystallized from boiling water/acetonitrile (50:50) to give 165 mg (0.48 mmole, 96 %) of **141**: mp 215-216 °C; <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 500 MHz) δ 8.6 (ddd, J=0.9 Hz, J=1.9 Hz, J=1.4 Hz, 1H), δ 8.2 (d, J=0.9 Hz, 1H), δ 8.0 (dd, J=8.8 Hz, J=1.9 Hz, 1H), δ 7.8 (dd, J=8.8 Hz, J=0.9 Hz, 1H), δ 6.1 (t, J=1.4 Hz, 1H), δ 3.3 (m, 2H), δ 2.9 (m, 2H), δ 2.8 (m, 2H), δ 2.5 (s, 3H); <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 125 MHz) δ 163.8, 146.6, 136.7, 135.3, 133.5, 127.6, 122.7, 116.2, 122.7, 107.7, 50.6, 49.7, 42.2; GC (t<sub>R</sub> = 14.17 min)-EIMS m/z (%) 258 (M<sup>+</sup>, 24), 230 (8), 202 (8), 168 (19); 94(28) 70 (100); UV (nm, MeOH) 205, 221, 251, 288, 361. Anal. (C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>O<sub>4</sub>) C, H, N.

**Oxalate salt of 1-methyl-4-(5-nitroindazol-1-yl)-1,2,3,6-tetrahydropyridine (143).** Sodium borohydride (80 mg, 2 mmole) was added in small portions to a suspension of 1-methyl-4-(6-nitroindazol-1-yl)pyridinium iodide (**152**) (190 mg, 0.5 mmole) in methanol (5 mL) at room temperature while stirring. After 2 h. methanol was evaporated. The residue was extracted with dichloromethane and saturated NaCl solution.

The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the solvent was evaporated in vacuo to give the free base **143**. The oxalate salt of **143** was prepared by adding dropwise a saturated ethereal solution of oxalic acid. The oxalate salt was crystallized from boiling water/acetonitrile (50:50) to give 160 mg (0.46 mmole, 92 % ) of **143**: mp 207-208 °C; <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 500 MHz) δ8.9 (d, J=2.1 Hz 1H), δ8.6 (s, 1H), δ8.3 (dd, J=2.1 Hz, J=9.3 Hz 1H), δ8.0 (d, J=9.3 Hz, 1H), δ6.2 (t, J=1.4 Hz, 1H), δ3.8 (m,2H), δ3.3 (m, 2H), δ3.0 (m, 2H), δ2.8 (s, 3H); <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 125 MHz) δ163.8, 142.2, 139.8, 137.6, 133.4, 123.9, 121.9, 119.2, 112.7, 112.0, 50.1, 49.6, 42.2; GC (t<sub>R</sub> = 13.42 min)-EIMS m/z (%) 258 (M<sup>+</sup>, 65), 243 (14), 94 (34),70 (100) UV (nm, MeOH) 205, 229, 265, 311, 335. Anal. (C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>O<sub>4</sub>) C, H, N.

**4-(Indazol-1-yl)-1-methylpyridinium iodide (155)**. To a solution of indazole (**89**) (120 mg, 1 mmole) in 3 mL of N,N-dimethylformamide, 4-chloro-1-methylpyridinium iodide (**36**) (260 mg, 1 mmole) was added. To the solution, 2,2,6,6-tetramethylpiperidine (0.17 mL, 1 mmole) was added at one time. After stirring for 2 hours at room temperature, the reaction mixture was filtered and the product was recrystallized from boiling methanol to give 195 mg of yellow solid (0.58 mmole and 58 %). mp 261-263 °C; <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 360 MHz): δ 8.94 (m, 2H), δ 8.77 (d, J=0.9 Hz, 1H), δ8.53 (m, 2H), δ8.36 (dddd, J=0.9 Hz, J=0.9 Hz, J=0.9 Hz, J= 8.6 Hz, 1H), δ8.02 (ddd, J=0.9 Hz, J=0.9 Hz, J=8.0 Hz, 1H), δ7.73 (ddd, J=0.9 Hz, J=7.1 Hz, J=8.5 Hz, 1H), δ7.49 (ddd, J=0.9 Hz, J=7.2 Hz, J=8.0 Hz, 1H), δ4.3 (s, 3H); <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 360 MHz) δ150.9, 146.6, 142.1, 138.2, 129.8, 127.2, 124.6, 122.7, 115.2, 112.8, 46.6; GC (t<sub>R</sub> = 11.43 min)-EIMS m/z (%) 195 (M<sup>+</sup>, 100), 168 (42), 140 (13), 78 (8); 67(9) 51 (26); UV (nm, MeOH) 209, 224, 266, 335. Anal. (C<sub>13</sub>H<sub>12</sub>N<sub>3</sub>I) C, H, N.

**4-(Indazol-2-yl)-1-methylpyridinium iodide (156)**. To a solution of indazole (**89**) (120 mg, 1 mmole) in 3 mL of N,N-dimethylformamide, 4-chloro-1-methylpyridinium iodide (**36**) (260 mg, 1 mmole) was added. After stirring for 24 h at 100 °C, the reaction mixture was cooled to room temperature to induce crystal formation, filtered and the product was recrystallized from boiling methanol to give 180 mg of yellow solid (0.53 mmole and 53 %): mp 241-243 °C; <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 360 MHz): δ 9.55 (d, J=1.0 Hz, 1H), δ 9.13 (m, 2H), δ8.77 (m, 2H), δ7.83 (ddd, J=1.1 Hz, J=1.1 Hz,

$J = 8.7$  Hz, 1H),  $\delta 7.74$  (dddd,  $J = 1.1$  Hz,  $J = 1.1$ ,  $J = 1.1$  Hz,  $J = 8.9$  Hz, 1H),  $\delta 7.44$  (ddd,  $J = 1.1$  Hz,  $J = 6.5$  Hz,  $J = 8.9$  Hz, 1H),  $\delta 7.20$  (ddd,  $J = 1.1$  Hz,  $J = 6.5$  Hz,  $J = 8.7$  Hz, 1H),  $\delta 4.4$  (s, 3H);  $^{13}\text{C-NMR}$  (DMSO- $d_6$ , 360 MHz)  $\delta 151.0$ , 150.2, 147.3, 129.8, 124.7, 124.3, 123.4, 121.5, 117.8, 116.4, 47.1; GC ( $t_R = 11.73$  min)-EIMS  $m/z$  (%) 195 ( $M^+$ , 100), 168 (24), 140 (13), 118 (17); 78(21), 63 (11), 51 (36); UV (nm, MeOH) 205, 219, 257, 327. Anal. ( $\text{C}_{13}\text{H}_{12}\text{N}_3\text{I}$ ) C, H, N.

**Oxalate salt of 1-methyl-4-indazol-1-yl-1,2,3,6-tetrahydropyridine (160).** Sodium borohydride (80 mg, 2 mmole) was added in small portions to a suspension of 1-methyl-4-indazol-1-ylpyridinium iodide (**155**) (170 mg, 0.5 mmole) in methanol (5 mL) at room temperature while stirring. After 2 hours methanol was evaporated in vacuo. The residue was extracted with dichloromethane and saturated NaCl solution. The organic layer was dried over anhydrous  $\text{MgSO}_4$  and the solvent was evaporated in vacuo to give the free base **160**. The oxalate salt of **160** was prepared by adding dropwise a saturated ethereal solution of oxalic acid. The oxalate salt was crystallized from boiling water/acetonitrile (50:50) to give 130 mg (0.42 mmole, 84 % ) of **160**: mp 174-175 °C;  $^1\text{H-NMR}$  (DMSO- $d_6$ , 360 MHz)  $\delta 8.3$  (d,  $J = 0.9$  Hz 1H),  $\delta 7.8$  (m, 2H),  $\delta 7.5$  (ddd,  $J = 1.2$  Hz,  $J = 6.9$  Hz,  $J = 8.0$  Hz, 1H),  $\delta 7.2$  (ddd,  $J = 0.6$  Hz,  $J = 6.9$  Hz,  $J = 7.9$  Hz, 1H),  $\delta 6.2$  (d,  $J = 3.3$  Hz, 1H),  $\delta 3.8$  (m, 2H),  $\delta 3.4$  (m, 2H),  $\delta 3.0$  (m, 2H),  $\delta 2.8$  (s, 3H);  $^{13}\text{C-NMR}$  (DMSO- $d_6$ , 125 MHz)  $\delta 164.1$ , 137.8, 134.9, 134.0, 127.3, 124.8, 121.7, 121.4, 111.3, 108.8, 50.3, 49.5, 41.9; GC-EIMS  $m/z$  (%) 213 (40), 185 (24), 157 (36), 144 (18), 130 (19), 96 (27), 77 (11), 70(100) UV (nm, MeOH) 208, 244, 301; Anal. ( $\text{C}_{15}\text{H}_{16}\text{N}_4\text{O}_4$ ) C, H, N.

**1-Methyl-4-indazol-2-yl-1,2,3,6-tetrahydropyridine (161).** Sodium borohydride (80 mg, 2 mmole) was added in small portions to a suspension of 1-methyl-4-(6-nitroindazol-1-yl)pyridinium iodide (**156**) (170 mg, 0.5 mmole) in methanol (5 mL) at room temperature while stirring. After 2 h. methanol was evaporated in vacuo. The residue was extracted with dichloromethane and saturated NaCl solution. The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and the solvent was evaporated in vacuo to give 115 mg (0.46 mmole, 92 % ) of the free base **161**: mp 101-103 °C;  $^1\text{H-NMR}$  (DMSO- $d_6$ , 360 MHz)  $\delta 8.6$  (d,  $J = 0.7$  Hz 1H),  $\delta 7.7$  (ddd,  $J = 1.1$  Hz,  $J = 1.1$  Hz,  $J = 8.5$  Hz, 1H),  $\delta 7.6$  (dddd,  $J = 1.0$  Hz,  $J = 1.0$  Hz,  $J = 1.0$  Hz,  $J = 8.8$  Hz 1H),  $\delta 7.0$  (ddd,  $J = 0.9$  Hz,  $J = 6.6$  Hz,  $J = 8.4$  Hz, 1H),  $\delta 6.5$  ( $\mu$ , 1H),  $\delta 3.1$  (m, 2H),  $\delta 2.8$  (m, 2H),  $\delta 2.6$  (t,  $J = 5.3$  Hz, 2H),  $\delta 2.3$  (s, 3H);

$^{13}\text{C}$ -NMR (DMSO- $d_6$ , 100 MHz)  $\delta$ 148.0, 134.9, 126.3, 121.5, 121.4, 120.7, 120.4, 117.1, 114.5, 52.8, 51.0, 44.9, 26.2; GC-EIMS  $m/z$  (%) 258 ( $\text{M}^+$ , 2), 212 (65), 185 (73), 169 (54), 157 (69), 131 (26), 94 (100), 53 (39) UV (nm, MeOH) 207, 229, 297.

**1-Methyl-4-cyanopyridinium iodide (150).** To a solution of 4-cyanopyridine (**151**) (1.04 g, 10.0 mmole) in 10 mL of acetone, methyl iodide (4.26 g, 30 mmole) was added dropwise at 0 °C over 15 minutes while stirring. The reaction was warmed to room temperature. After stirring for 24 hours at room temperature the product was isolated by filtration and crystallized from MeOH/Ether to yield 1.20 g. (47.0 mmole, 47 %) of a yellow solid; mp 195-196 °C.  $^1\text{H}$ -NMR (DMSO- $d_6$ , 500 MHz):  $\delta$  9.2 (d,  $J=6$  Hz, 2H),  $\delta$  8.6 (d,  $J=6$  Hz, 2H),  $\delta$ 4.4 (s, 3H);  $^{13}\text{C}$ -NMR (DMSO- $d_6$ , 100 MHz)  $\delta$ 147.6, 131.0, 127.2, 115.4, 49.5; GC ( $t_R = 5.82$  min)-EIMS  $m/z$  (%) 104 (100), 77 (54), 64 (12), 50 (22).

**1,2,2,6,6-Pentamethylpiperidine (140).** To a solution of 2,2,6,6-tetramethylpiperidine (1.41 g, 10 mmole) in 10 mL of N,N-dimethylformamide, methyl iodide (4.26 g, 30 mmole) was added dropwise at 0 °C. After an exothermic reaction, white crystals formed rapidly. After 30 minutes the product, hydroiodide salt of 1,2,2,6,6-pentamethylpiperidine was isolated by filtration. The crystals were washed with ether, dissolved in 10 mL of water and extracted with 3x10 mL of diethyl ether to remove remaining starting material. The aqueous layer was made basic by adding saturated aqueous  $\text{K}_2\text{CO}_3$  to form the free base 1,2,2,6,6-pentamethylpiperidine (**140**). The basic solution was extracted with 3x10 mL of diethylether. The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and the solvent was evaporated in vacuo to give 0.98 g of **140** as a yellow liquid (63 mmole, 63 %).  $^1\text{H}$ -NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$ 2.2 (s, 2H),  $\delta$ 1.5 (m, 2H),  $\delta$ 1.4 (m, 4H),  $\delta$ 1.0 (s, 12H);  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ , 125 MHz)  $\delta$ 41.1, 31.2, 28.6, 26.3, 17.9; GC ( $t_R = 7.1$  min)-EIMS  $m/z$  (%) 155 ( $\text{M}^+$ , 10), 140 (100), 84 (55), 72 (23), 56 (22).

**Formation of 7-nitroindazolyl anion from 7-nitroindazole in presence of base.**

To a 0.1 mM solution of 7-NI in MeOH, excess amount of base (TMP or concentrated NaOH solution) was added. UV-Vis absorption spectra were recorded on a Beckman DU Series 7400 spectrophotometer. The effect of the added base on the UV chromophore of 7-NI was examined where a shift to higher wavelengths indicated the formation of the indazolyl anion.

**Investigation of the role of base in coupling reaction between 7-NI (26) and 4-chloro-1-methylpyridinium iodide (36).**

All reactions designed to investigate the reaction mechanism were carried out in conical micro reaction vials equipped with caps containing septa to allow aliquot taking using a micro syringe. To a solution of 7-NI (26) (50 mg, 0.3 mmole) in 0.3 mL N,N-dimethylformamide, 36 (75 mg, 0.3 mmole) was added. The reaction mixture was stirred at room temperature for 1 hour and 10  $\mu$ L aliquots were taken at 0, 30 and 60 minutes. Then the temperature was increased to 100  $^{\circ}$ C. The reaction mixture was stirred for 3 hours at 100  $^{\circ}$ C and again 10  $\mu$ L aliquots were taken every 30 minutes. These aliquots were diluted in 0.5 mL of MeOH and samples (1  $\mu$ L) were subjected to GC-EIMS analysis.

**Investigation of the role of temperature in coupling reaction between 7-NI (26) and 4-chloro-1-methylpyridinium iodide (36).**

To a solution of 7-NI (26) (50 mg, 0.3 mmole) in 0.3 mL N,N-dimethylformamide, TMP (5  $\mu$ L, 0.3 mmole) and 36 (75 mg, 0.3 mmole) was added. The reaction mixture was stirred at room temperature for 1.5 hours and 10  $\mu$ L aliquots were taken at 0, 30, 60, 90 minutes. Then the temperature was first increased to 50  $^{\circ}$ C. The reaction mixture was stirred for 30 minutes at 50  $^{\circ}$ C and a 10  $\mu$ L aliquot was taken after 30 minutes. These aliquots were diluted in 0.5 mL of MeOH and samples (1  $\mu$ L) were subjected to GC-EIMS analysis. Since no product formation was observed the reaction mixture was heated to 100  $^{\circ}$ C and was stirred for 60 minutes taking 10  $\mu$ L aliquots at 30 and 60 minutes. Aliquots were diluted in 0.5 mL MeOH and samples (1  $\mu$ L) were subjected to GC-EIMS analysis.

**Monitoring the progress of the coupling reaction between 7-NI (26) and 4-chloro-1-methylpyridinium iodide (36) in the presence of TMP by HPLC-DA.**

To a solution of 7-NI (26) (50 mg, 0.3 mmole) in 0.3 mL N,N-dimethylformamide, TMP (5  $\mu$ L, 0.3 mmole) and 36 (75 mg, 0.3 mmole) was added. The reaction mixture was stirred at room temperature for 3 hours and 10  $\mu$ L aliquots were taken at 0, 30, 60, 90, 120, 180 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN and were subjected to HPLC-DA analysis. HPLC was performed on a Hewlett Packard

1100 HPLC system equipped with a UV/VIS diode array detector (G1315A0), a Rheodyne 7725I injector and a quaternary pump (GB11A). Chromatography was carried on a 250 mm x 4.6 mm Zorbax SB-C8 5  $\mu$ m column (reverse phase) with an in-line pre-column filter (2 $\mu$ M, Upchurch ScientificInc.) using isocratic condition at 50 % acetonitrile and 50 % aqueous buffer (pH 4.7) containing 0.6 % acetic acid and 1 % triethylamine. The sample volume was 10  $\mu$ L. The monitoring wavelength was 375 nm.

**Monitoring the thermal decomposition of **120** by HPLC-DA.**

A solution of **120** (115 mg, 0.3 mmole) in 0.3 mL of N,N-dimethylformamide was heated to 100 °C and stirred for 60 minutes at 100 °C and 10  $\mu$ L aliquots were taken at 0, 30 and 60 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN and subjected to HPLC-DA analysis. HPLC analysis was performed under conditions described above. Two simultaneous monitoring wavelengths were chosen as 269 nm and 369 nm. No change was observed in the intensity of the peak corresponding to **120** ( $t_R$  = 3.41 minutes). After 60 minutes, the reaction mixture was cooled to room temperature and TMP (5  $\mu$ L, 0.3 mmole) was added. The reaction mixture was stirred at 80 °C for 60 minutes and aliquots were taken at 0, 30 and 60 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN subjected to HPLC-DA analysis.

**Monitoring the progress of the coupling reaction between 6-NI (**122**) and 4-chloro-1-methylpyridinium iodide (**36**) in the presence of TMP by HPLC-DA.**

To a solution of 6-NI (**122**) (50 mg, 0.3 mmole) in 0.3 mL N,N-dimethylformamide, TMP (5  $\mu$ L, 0.3 mmole) and **36** (75 mg, 0.3 mmole) was added. The reaction mixture was stirred at room temperature for 60 minutes and 10  $\mu$ L aliquots were taken at 0, 5, 10, 15, 30, 60 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN and were subjected to HPLC-DA analysis. HPLC was performed as described above. The monitoring wavelength was 375 nm.

**Monitoring the progress of the coupling reaction between 5-NI (**125**) and 4-chloro-1-methylpyridinium iodide (**36**) in the presence of TMP by HPLC-DA.**

To a solution of 5-NI (**125**) (50 mg, 0.3 mmole) in 0.3 mL N,N-dimethylformamide, TMP (5  $\mu$ L, 0.3 mmole) and **36** (75 mg, 0.3 mmole) was added. The reaction mixture was stirred at room temperature for 60 minutes and 10  $\mu$ L aliquots were taken at 0, 5, 10, 15, 30, 60 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN

and were subjected to HPLC-DA analysis. HPLC was performed as described above. The monitoring wavelength was 290 nm.

**Investigation of the reversibility of the coupling reaction between 6-NI (125) and 4-chloro-1-methylpyridinium iodide (36) in the presence of KCN by HPLC-DA.**

To a suspension of **120** (115 mg, 0.3 mmole) in 0.3 mL of N,N-dimethylformamide an excess amount of KCN was added. The reaction mixture was stirred for 2 hours at room temperature and 10  $\mu$ L aliquots were taken at 0, 5, 15, 30, 60, 90 and 120 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN and subjected to HPLC-DA analysis. HPLC analysis was performed under conditions described above. The monitoring wavelength was chosen as 350 nm.

**Monitoring the progress of the coupling reaction between indazole (89) and 4-chloro-1-methylpyridinium iodide (36) in the presence of TMP by HPLC-DA.**

To a solution of indazole (**89**) (35 mg, 0.3 mmole) in 0.3 mL N,N-dimethylformamide, TMP (5  $\mu$ L, 0.3 mmole) and **36** (75 mg, 0.3 mmole) was added. The reaction mixture was stirred at room temperature for 2 hours and 10  $\mu$ L aliquots were taken at 0, 5, 10, 15, 30, 60, 90, 120 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN and were subjected to HPLC-DA analysis. HPLC was performed as described above. The monitoring wavelength was 269 nm.

**Monitoring the progress of the coupling reaction between indazole (89) and 4-chloro-1-methylpyridinium iodide (36) in the absence of TMP by HPLC-DA.**

To a solution of indazole (**89**) (35 mg, 0.3 mmole) in 0.3 mL N,N-dimethylformamide and **36** (75 mg, 0.3mmole) was added. The reaction mixture was stirred first at room temperature for 30 minutes and 10  $\mu$ L aliquots were taken at 0, 15, 30 minutes. Then the reaction mixture was heated to 60 °C and was stirred at 60 °C for another 30 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN and were subjected to HPLC-DA analysis. HPLC was performed as described above. The monitoring wavelength was 269 nm. No change was observed for the intensity of the peaks corresponding to the starting materials, **36** ( $t_R$ = 2.65 minutes) and indazole ( $t_R$ = 4.40 minutes). The temperature was increased to 100 °C and the reaction mixture was stirred at 100 °C for 60 minutes taking 10  $\mu$ L aliquots at 0, 15, 30, 60 minutes. These aliquots were diluted in 0.5 mL of CH<sub>3</sub>CN and were subjected to HPLC-DA analysis.

**Monitoring the isomerization of 4-(indazol-2-yl)-1-methylpyridinium iodide (156) to 4-(indazol-1-yl)-1-methylpyridinium iodide (155) by HPLC-DA.**

To a suspension of **156** (100 mg, 0.3 mmole) in 0.3 mL of N,N-dimethylformamide, 2,2,6,6-tetramethylpiperidine (5  $\mu$ L, 0.3 mmole) was added. The reaction mixture was stirred at 60 °C for 48 hours and 10  $\mu$ l aliquots were taken at 0, 15, 60, 120 minutes and 48 hours. Aliquots were diluted in 0.5 mL in acetonitrile. The diluted aliquots (10  $\mu$ l) were applied to HPLC-DA. HPLC was performed as described above. The monitoring wavelength was 350 nm. The intensity of the signal ( $t_R$  = 3.14 minutes) corresponding to **156** began to decrease and the intensity of the signal ( $t_R$  = 3.28 minutes) corresponding to **155** began to appear during the time course of the reaction. After 48 hours, the signal corresponding to **156** completely disappeared and only one signal was present with a retention time of 3.28 minutes which corresponds to **155**.

## **7.2.BIOLOGY**

### **7.2.1.MAO-B SUBSTRATE PROPERTIES**

Substrate properties of test compounds were studied using HPLC-DA assay in which baboon liver mitochondrial preparations were used as the enzyme source.

#### **Preliminary experiments:**

Preliminary experiments were carried out to determine if the test compounds display MAO-B substrate properties. A stock solution of the test compound (2mM) was prepared in pH 7.4 0.1 M sodium phosphate buffer. Enzyme (25  $\mu$ L, 3 mg protein/mL) was added to incubation mixtures (pre-equilibrated at 37 °C) consisting of pH 7.4 0.1 M phosphate buffer (465  $\mu$ L) and the test compound (10  $\mu$ L) to yield a final concentration of 40  $\mu$ M. The final volume of the incubation mixtures was 500  $\mu$ L and the final protein concentration was 0.15 mg/ml. These mixtures were incubated with gentle agitation in a water bath at 37 °C for 0, 30, 60, 120 minutes. Acetonitrile (500  $\mu$ L) was added and the resulting mixture was vortex agitated. The denatured protein was sedimented by centrifugation at 10,000 g for 6 minutes. The supernatants (50  $\mu$ L) were applied to HPLC/DA. Control incubations were conducted in the absence of the substrate or enzyme and no metabolite formation was detected.

### Kinetic Analysis

Stock solutions (1, 2, 3, 4 mM) were prepared in pH 7.4 0.1 M phosphate buffer. Enzyme (25  $\mu$ L, 3 mg protein/mL) was added to incubation mixtures (pre-equilibrated at 37 °C) consisting of pH 7.4 0.1 M phosphate buffer (465  $\mu$ L) and the test compound (10  $\mu$ L) to yield a final concentration of 20, 40, 60, 80  $\mu$ M. The final volume of the incubation mixtures was 500  $\mu$ L and the final protein concentration was 0.15 mg/mL. These mixtures were incubated with gentle agitation in a water bath at 37 °C for 0, 15, 30, 60 minutes. Acetonitrile (500  $\mu$ L) was added and the resulting mixture was vortex agitated. The denatured protein was sedimented by centrifugation at 10,000 g for 6 minutes. The supernatants (50  $\mu$ L) were applied to HPLC-DA. Initial rates of oxidation (V) of the test compounds were estimated at four different concentrations by monitoring the formation of the metabolite using HPLC-DA. Plots were constructed where the metabolite concentration was plotted versus time and the slopes were determined for the linear part of the graphs. The  $K_m$  ( $-1/x$  when  $y=0$ ) values were determined from the double reciprocal plots in which the values of the slopes (V) (obtained from the first plots of the metabolite concentration versus time) are plotted against the concentration of the test compound.

### 7.2.2.MAO-B INHIBITION STUDIES

The inhibition properties were determined using a spectrophotometric assay in which baboon liver mitochondrial preparations were used as the enzyme source. Baboon liver mitochondrial MAO-B catalyzed oxidation of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (**156**) to the corresponding dihydropyridine **157** was employed to determine the  $K_i$  values. Standard solutions of **156** (300, 600, 900, 1200  $\mu$ M) and test compounds (300, 600, 1200  $\mu$ M) were prepared in pH 7.4 0.1 M sodium phosphate buffer. Enzyme was added (25  $\mu$ L, 3 mg of protein/mL) to incubation mixtures (pre-equilibrated at 37 °C) consisting of 0.1 M sodium phosphate buffer (pH 7.4, 400  $\mu$ L), **156** (50  $\mu$ L) to yield final concentrations of 30, 60, 90, 120  $\mu$ M (bracketing the  $K_m$  value for **156** is previously determined to be 60  $\mu$ M<sup>189</sup>) and test compound (25  $\mu$ L) to yield a final

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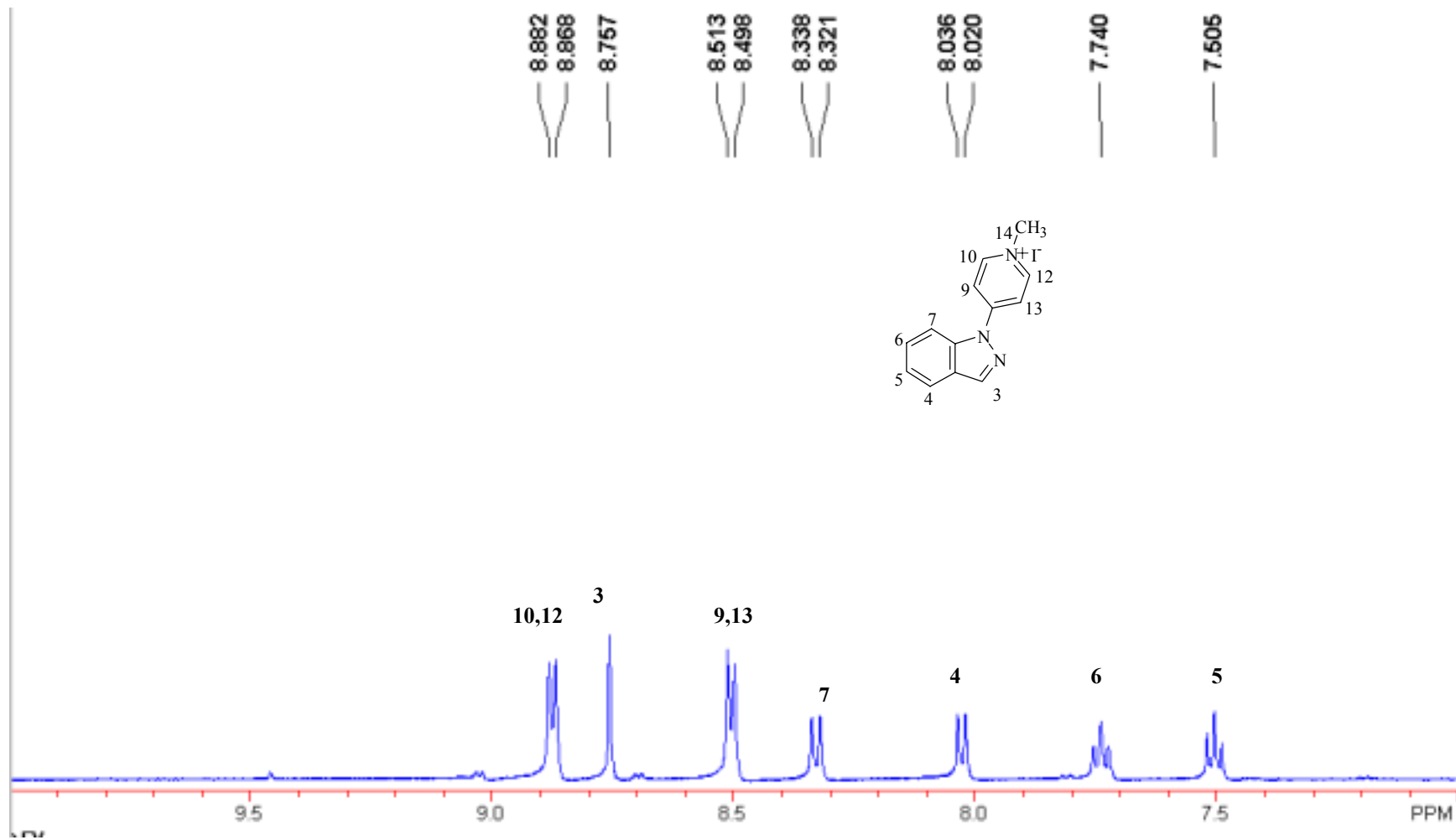
<sup>189</sup> See reference 176

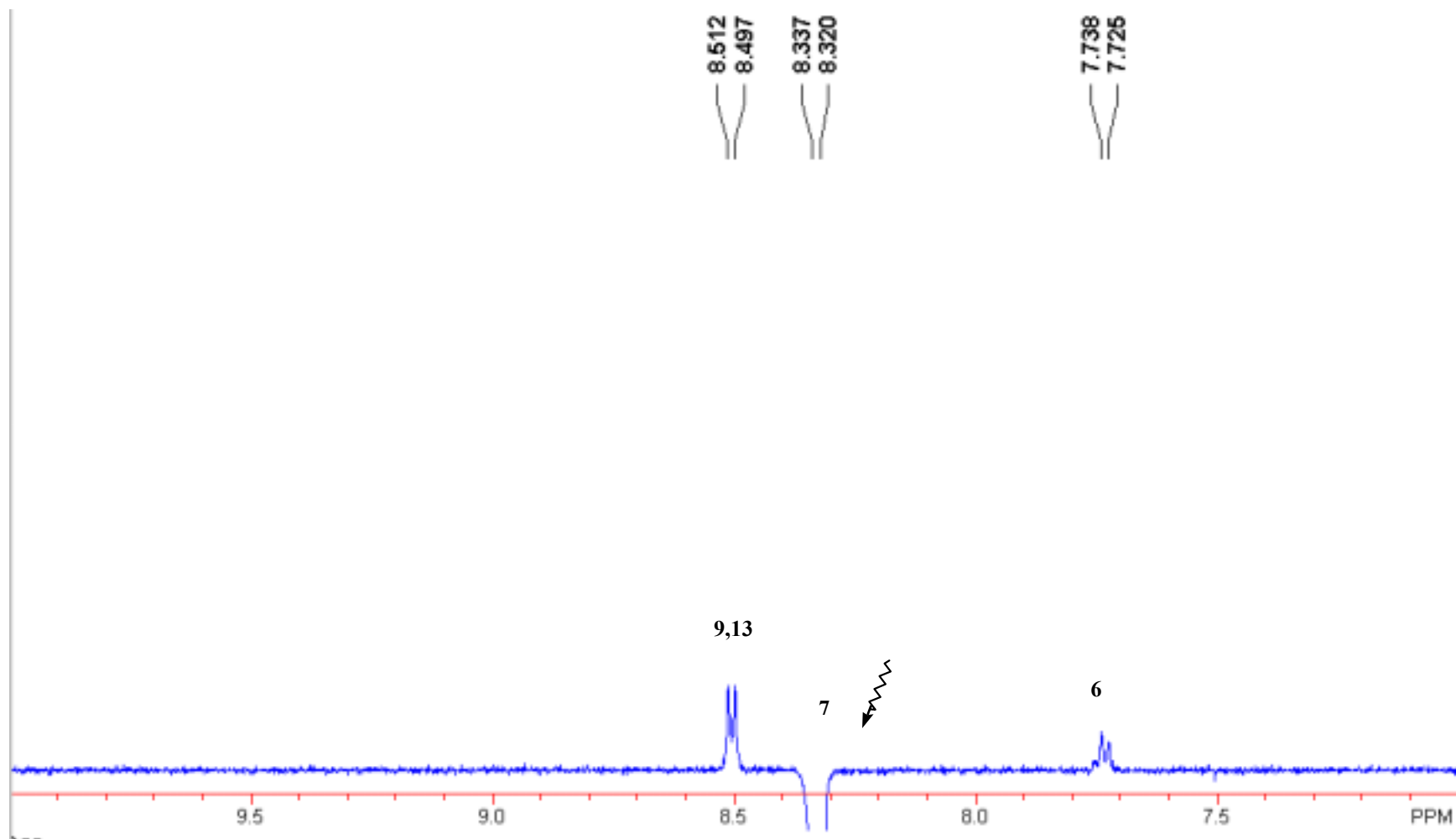
concentration of 15, 30, 60  $\mu\text{M}$ . The final volume of the incubation mixtures was 500  $\mu\text{L}$  and the final protein concentration was 0.15 mg/mL. These mixtures were incubated with gentle agitation in a water bath at 37  $^{\circ}\text{C}$  for 15 minutes. Acetonitrile (500  $\mu\text{L}$ ) was added and the resulting mixture was vortex agitated. The denatured protein was sedimented by centrifugation at 10,000 g for 6 minutes and the supernatant was scanned from 600 to 250 nm on a Beckman DU-7400 UV spectrophotometer. The absorbance at 420 nm was used to estimate the concentration of **157**. The  $K_i$  value ( $-x$  when  $y=0$ ) was estimated from the replot in which the values of the slopes obtained from the double-reciprocal plots of  $1/V$  versus  $1/[S]$  with increasing concentrations of the test compound are plotted against the concentration of the test compound.

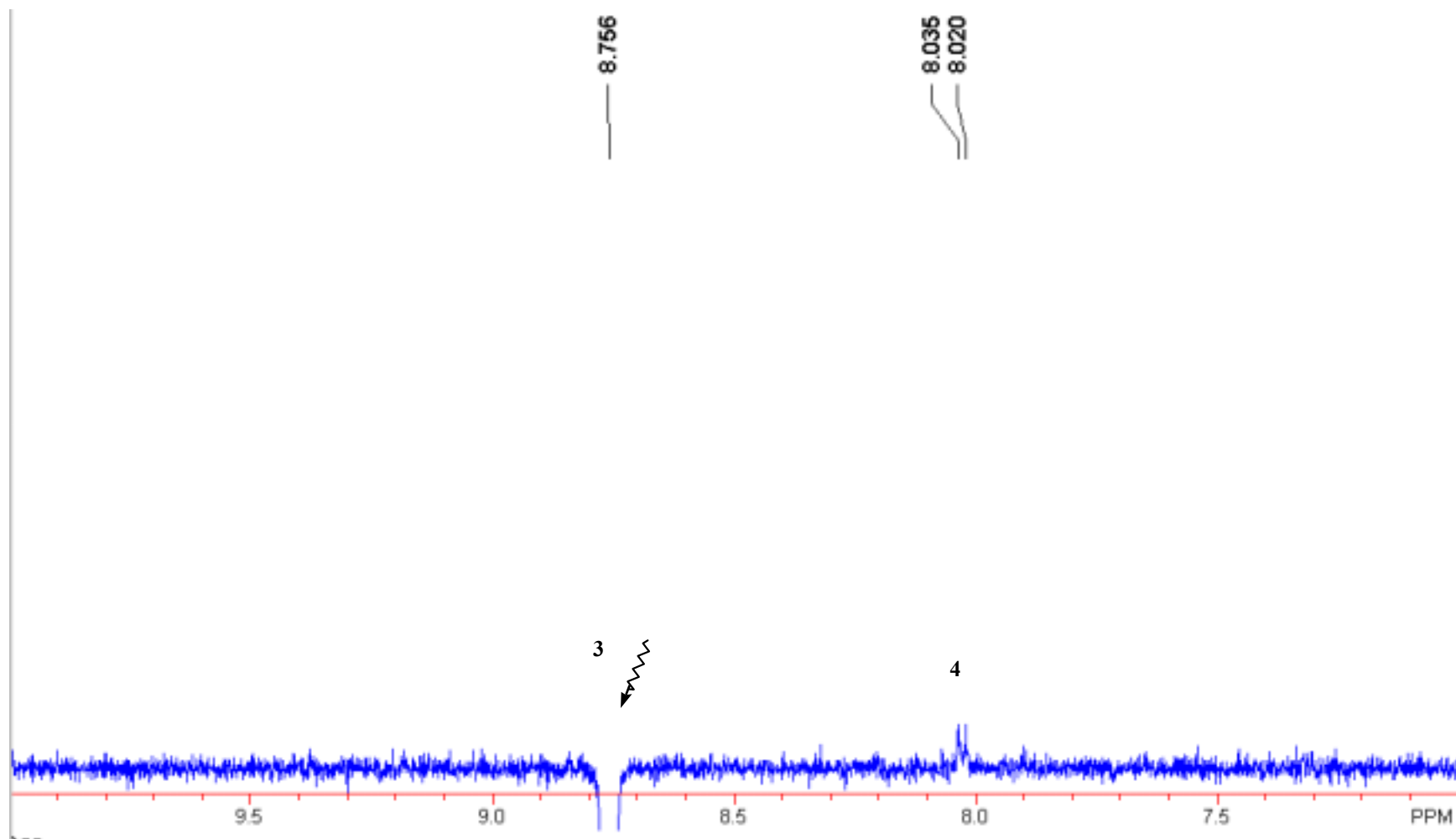
#### **Time dependent inhibition studies:**

Inhibitory properties of the test compounds were investigated as a function of time. Standard solutions of **156** (4 mM) and test compounds (2 mM) were prepared in pH 7.4 0.1 M sodium phosphate buffer. Enzyme was added (25  $\mu\text{L}$ , 3 mg of protein/mL) to the incubation mixtures consisting of the test compound (10  $\mu\text{L}$ , total concentration of 40  $\mu\text{M}$ ) and pH 7.4 0.1 M sodium phosphate buffer (265  $\mu\text{L}$ ). These incubation mixtures were pre-incubated for 5, 10, 15, 20, 15, 30 minutes. Following the pre-incubation **156** (250  $\mu\text{L}$ , total concentration of 2mM) was added and the mixtures were incubated for 30 minutes. Acetonitrile (500  $\mu\text{L}$ ) was added and the resulting mixture was vortex agitated. The denatured protein was sedimented by centrifugation at 10,000 g for 6 minutes and the supernatant was scanned from 600 to 250 nm on a Beckman DU-7400 UV spectrophotometer. The absorbance at 420 nm was used to estimate the concentration of **157**.

## APPENDIX







## VITA

Emre M. Işın was born on July 20, 1975 in Istanbul, Turkey. He finished Kadiköy Anatolian High School in 1993 and received his Bachelor of Science degree in Chemistry from Boğaziçi (Bosphorous) University in 1997. He started his graduate studies at Virginia Polytechnic Institute and State University Chemistry Department under the direction of Prof. Neal Castagnoli, Jr. in the Fall of 1997. As a graduate teaching assistant, he taught undergraduate organic chemistry laboratories for two years and organic synthesis and techniques laboratory for two semesters. He received the graduate assistant teaching award in Spring 2000.

He completed his studies leading to the Master of Science degree in Chemistry in the Fall of 2000. Emre M. Işın will start the Doctor of Philosophy program in the Chemistry Department at VPI&SU in Spring 2001 under the direction of Prof. Neal Castagnoli, Jr.