Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3031–3041

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Biological Evaluation of MAO-A Selective 1,4-Disubstituted-1,2,3,6-tetrahydropyridinyl Substrates

Philippe Bissel, Mark C. Bigley, Kay Castagnoli and Neal Castagnoli Jr.*

Department of Chemistry, Virginia Polytechnic Institute and State University, 107 Davidson Hall, Blacksburg, VA 24061-0212, USA

Received 12 November 2001; accepted 19 February 2002

Abstract—Many mammalian tissues express both the A and B forms of monoamine oxidase (MAO), flavoenzymes that catalyze the oxidative deamination of a variety of endogenous and exogenous amines and the ring α-carbon oxidative bioactivation of neurotoxic 1,4-disubstituted-1,2,3,6-tetrahydropyridinyl derivatives. Substrates selective for MAO-A that display good kinetic and spectroscopic properties would be of value for developing quantitative assays for MAO-A in tissues that express both the A and B forms of the enzyme. This paper describes the synthesis of several 1-substituted-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridinyl derivatives. Kinetic parameters and MAO-A selectivity indicate that 1-allyl- and 1-propyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine should be good candidates to develop a robust spectrophotometric-based assay that is selective for MAO-A. © 2002 Published by Elsevier Science Ltd.

Introduction

The monoamine oxidases A and B (MAO-A and MAO-B) are mitochondrial flavoenzymes that catalyze the α-carbon oxidation of a variety of amines. Examples (Fig. 1) include the oxidative deamination of endogenous neurotransmitter substances, such as serotonin (**1**),[1–3] and dietary amines, such as the pressor amine tyramine (**2**). The resulting iminium products, **3** and **4**, undergo hydrolysis to the aldehydes **5** and **6**, respectively.[4,5] The parkinsonian inducing amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**7**)] is a member of a special class of cyclic tertiary allylamines that are good MAO-B substrates.[6–9] The MAO-B catalyzed allylic α-carbon oxidation of **7** generates the dihydropyridinium metabolite **8**. Since the hydrolysis of **8** is reversible, its ultimate fate involves a second 2-electron oxidation, presumably via the free base **9**, to form the pyridinium species MPP^+ (**10**), a mitochondrial toxin that causes the selective destruction of nigrostriatal neurons.[10–13]

Human blood platelets and human placentas express exclusively MAO-B[14,15] and MAO-A,[16,17] respectively. Most human tissues, however, express both forms of the enzyme. For example, the ratios of MAO-B to MAO-A activity per mg protein in human gut and liver

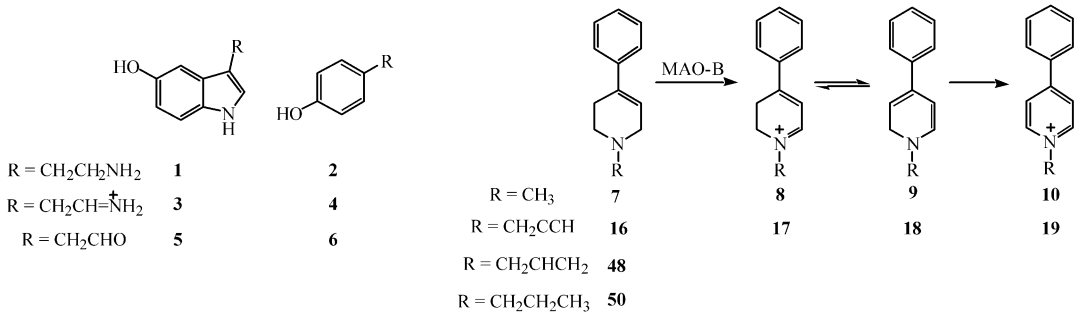


Figure 1. Examples of MAO catalyzed α-carbon oxidations.

*Corresponding author. Tel.: +1-540-231-8202; fax: +1-540-231-8890; e-mail: ncastagn@vt.edu

0968-0896/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0968-0896(02)00136-0

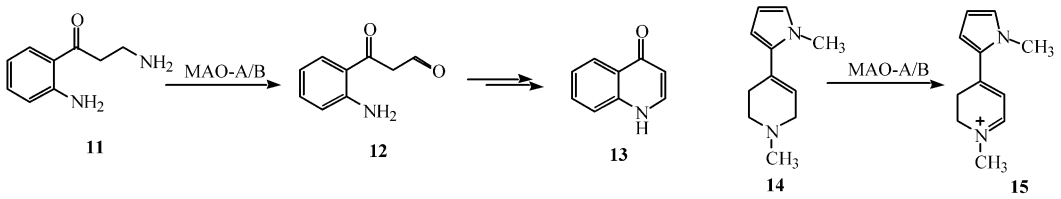
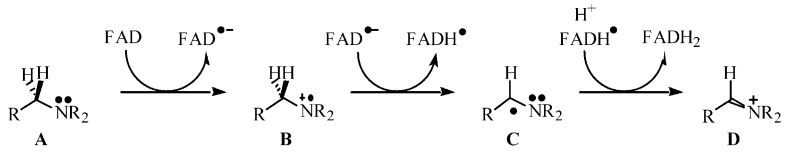


Figure 2. The MAO-A/B catalyzed oxidations of kynuramine (**11**) and 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (**14**).



Scheme 1.

mitochondrial preparations are about 1 to 1[18] and 4 to 1,[19,20] respectively. Inhibition of MAO-A can result in high circulating levels of diet derived tyramine, an MAO-A selective substrate.[21,22] Such high levels of this pressor amine can lead to severe hypertension.[23–25] The present studies were undertaken in an effort to develop MAO-A selective substrates to be used in assays to characterize the MAO-A inhibiting properties of drug candidates in tissues that express both forms of the enzyme.

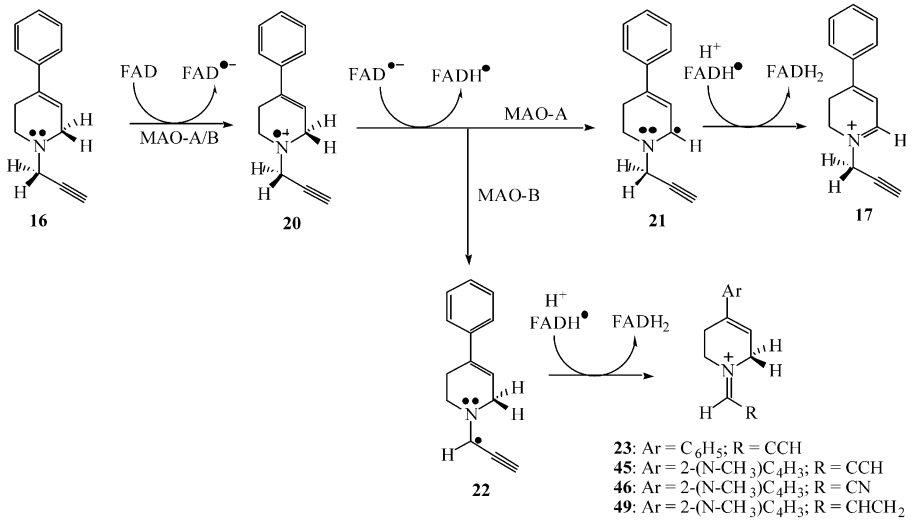
The selective MAO-A substrate serotonin has been used to measure MAO-A activity but the aldehyde metabolite **5** does not possess a good chromophore and therefore these assays depend on radioactivity measurements.[26] As shown in Figure 2, kynuramine (**11**), via the MAO generated aldehyde **12**, gives 4-quinolone (**13**). This reaction has been used to estimate MAO-A activity mainly by fluorescence-based methods.[27] We have developed an ultraviolet–visible (UV–vis) spectrophotometric-based assay for MAO using 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (**14**) as substrate.[28] The λ_{max} for the MAO generated dihydropyridinium metabolite **15** is 420 nm, far from the region where most biological materials absorb. Furthermore, unlike the dihydropyridinium metabolite **8** derived from MPTP,[29] **15** is stable under prolonged incubation conditions.[30] Studies with semi-purified human placenta MAO-A and beef liver MAO-B have established that this compound, like kynuramine, is a mixed MAO-A/MAO-B substrate.[28,31] The utility of these substrates is limited because they require the inhibition of MAO-B when attempting to estimate MAO-A activity in tissues such as human gut and liver.

This report describes the results of studies designed to develop selective MAO-A substrates that will generate stable metabolites with good UV absorbing properties. Past efforts have been only marginally successful because of problems with selectivity and solubility of the substrates and instability of the dihydropyridinium metabolites.[32] The observation that the time and concentration dependent MAO-B inhibitor 4-phenyl-1-propargyl-1,2,3,6-tetrahydropyridine (**16**, Fig. 1)[33] is a substrate, albeit a poor substrate, for MAO-A,[34] was the starting point for the present study.

Results and Discussion

Although controversial,[35,36] MAO catalysis is thought by many to proceed via the single electron transfer (SET) pathway illustrated in Scheme 1.[37,38] According to this mechanism, one electron from the nitrogen lone pair of the substrate **A** is transferred to oxidized flavin (FAD) to generate the aminyl radical cation **B** and $FAD^{\cdot -}$. Loss of a proton from **B** gives the radical intermediate **C** that undergoes a second 1-electron oxidation to yield the final iminium product **D** and reduced flavin ($FADH_2$).

Tertiary propargylamines are thought to inactivate MAO via covalent bond formation between a nucleophilic group present on the enzyme and the enzyme generated electrophilic ethynyliminium oxidation product.[39] Consistent with this pathway, the *N*-propargyl analogue **16** of the MAO-B selective substrate MPTP was found to be a good inactivator of beef liver MAO-B.[33] Unexpectedly, however, **16** proved to be a modest substrate for human placental MAO-A.[34] This behavior may be rationalized by the pathways shown in Scheme 2. The propargylaminyl radical cation **20** formed from **16** undergoes deprotonation to form either the propargylic radical **21** (MAO-A pathway) or the propargylic radical **22** (MAO-B pathway). Loss of a second electron from **21** yields the dihydropyridinium metabolite (substrate turnover) while loss of the second electron from **22** gives the electrophilic ethynyliminium species **23** that undergoes a Michael addition reaction with a nucleophilic group on the enzyme (enzyme inactivation). It should be noted that **16** also is a moderately effective inactivator of MAO-A. In this case, however, the partition ratio favors substrate turnover. The factors that are responsible for the different fates of the putative propargylaminyl radical cation **20** remain to be elucidated.



Scheme 2.

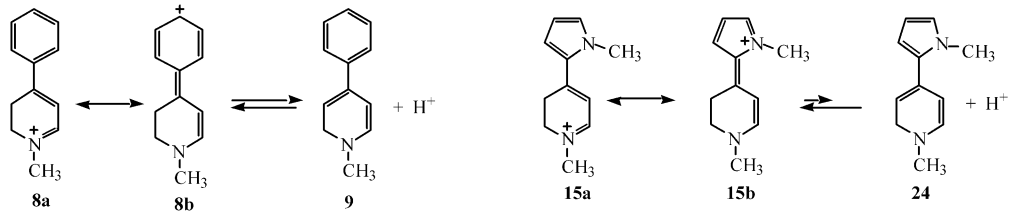
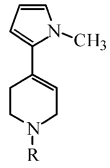
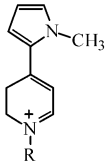


Figure 3. Acid-base equilibria for the dihydropyridinium species **8** and **15**.

The MAO-A generated dihydropyridinium metabolite **17** proved to be very unstable. This was apparent from the hypsochromic shift observed in the UV tracings during the first few min following initiation of the metabolic reaction. This shift is likely to be due to oxidation of **17** (λ_{max} 343 nm) to the pyridinium species **19** (λ_{max} 290 nm). Since the conversion of **17** to **19** (Fig. 1) involves a net loss of two electrons, it is reasonable to speculate that this reaction proceeds via the corresponding dihydropyridinyl free base **18**. Due to the electronegativity of the propargyl group, the equilibrium between **17** and **18** will be shifted to the right relative to that of the *N*-methyl analogue **8**. The resulting higher concentration of the free base **18** compared to **9** could account for the more rapid oxidation of **17** to **19**. This view is supported by the greater stability of the dihydropyridinium metabolite **15** (stable chromophore for at least 2 h), formed from the pyrrolyl analogue **14**, compared to the corresponding dihydropyridinium metabolite **8**. The more extensive resonance stabilization of the pyrrolyl species (**15a↔15b)** compared with the phenyl species (**8a↔8b**) makes **15** a weaker acid than **8**. The resulting lower equilibrium concentrations of the free base **24** versus **9** should slow the rate of oxidation of **15** compared to that of **8** (Fig. 3).

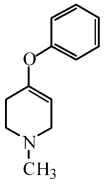


25: R = CH_2CCH
26: R = CH_2CN
27: R = CH_2CHCH_2
28: R = $CH_2CH_2CH_3$
29: R = $CH(CH_3)_2$



30: R = CH_2CCH
31: R = CH_2CN
32: R = CH_2CHCH_2
33: R = $CH_2CH_2CH_3$
34: R = $CH(CH_3)_2$

This analysis plus the excellent MAO-A substrate properties of **14** prompted us to examine the efficiencies with which MAO-A and MAO-B catalyze the oxidation of a series of 1-substituted-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridinyl derivatives (**25**–**29**) to the corresponding dihydropyridinium metabolites (**30**–**34**). We anticipated that these compounds would display MAO-A selective substrate properties, as observed with the 4-phenyl-1-propargyl derivative **16**, but would yield stable dihydropyridinium metabolites, as observed with the pyrrolyl analogue **14**.



35

In order to approximate more closely the environment in which these enzymes function in vivo, we utilized mitochondrial preparations rather than the semi-purified enzymes in these studies. Estimates of the concentrations of MAO-A in human placental mitochondria (which express MAO-A activity only) and MAO-B in beef liver mitochondria (which express MAO-B activity only) were obtained as follows. The turnover number (nmol metabolite formed/nmol enzyme-min or min^{-1}) for the semi-purified human placental MAO-A catalyzed oxidation of 1-methyl-4-phenoxy-1,2,3,6-tetrahydropyridine

Table 1. The MAO-A and MAO-B substrate properties of selected 1,4-disubstituted-1,2,3,6-tetrahydropyridinyl derivatives

		MAO-A			MAO-B			
		K_m[a]	V_{max}[b]	V_{max}/K_m[c]	K_m[a]	V_{max}[b]	V_{max}/K_m[c]	SC_{MAO-A}
7 MPTP	Avg	32[d]	11	338	227[e]	169	748	0.5
	SD, CV	3, 9	2, 21	74, 22	42, 19	28, 17	37, 5	
14 N–CH_3	Avg	45[f]	135	3010	100[f]	361	3740	0.8
	SD, CV	14, 31	38, 28	227, 8	16, 16	97, 27	1285, 34	
25 CH_2CCH	Avg	163[f,g]	135	831	ND	ND	ND	—
	SD, CV	22, 13	21, 16	97, 12	—	—	—	
26 CH_2CN	Avg	172[g]	62	371	576[h]	149	253	1.5
	SD, CV	47, 27	10, 17	49, 13	90, 16	51, 34	56, 22	
27 CH_2CHCH_2	Avg	48[f]	92	1890	133[i]	12	90	21.0
	SD, CV	5, 11	14, 15	78, 4	10, 7	0.6, 5	3, 3	
28 $CH_2CH_2CH_3$	Avg	18[j]	81	4490	228[i]	52	230	19.5
	SD, CV	2, 9	9, 11	545, 12	24, 11	3, 6	14, 6	

Average of three determinations; SD, standard deviation; CV, coefficient of variation; ND, not determined. See text for explanation; SC_{MAO-A}, selectivity coefficient.
[a]μM.
[b]nmol metabolite/min-nmol enzyme.
[c]nmol metabolite formed/min-nmol enzyme mM.
Substrate concentration range:
[d]50–300 μM.
[e]100–600 μM.
[f]25–200 μM.
[g]100–400 μM.
[h]200–1000 μM.
[i]50–300 μM.
[j]20–80 μM.

(**35**) at 37 °C has been reported to be 130 min^{-1}.[40] We therefore estimated the number of nmol MAO-A/mg human placental mitochondrial protein, by determining the initial rate of oxidation of 4 mM **35** (V_{max} conditions and linear rate of oxidation) by human placental mitochondria as described previously.[40] The observed rates of triplicate determinations (16.3, 15.1 and 16.3 nmol metabolite formed/min-mg protein) gave an estimate of 0.12 nmol MAO-A/mg protein. The concentration of MAO-B in mitochondria prepared from beef liver was estimated with 5 mM MPTP and the reported turnover number for the purified beef liver MAO-B catalyzed oxidation of 5 mM MPTP (204 min^{-1}) at 30 °C.[41] Duplicate determinations of this rate (9.6 and 9.3 nmol metabolite formed/mg protein-min) gave an estimate of 0.05 nmol MAO-B/mg protein. These values have been used to express the rates of oxidation of the substrates examined in this study in units of nmoles dihydropyridinium metabolite formed/min-nmol enzyme or min^{-1}.

The spectrophotometric assay used in these studies involves the treatment of post mitochondrial incubation mixtures with $HClO_4$ to precipitate the macromolecular fraction before scanning the spectrum to determine the amount of dihydropyridinium metabolite formed. All assays for K_m and V_{max} were performed in triplicate (see Table 1). The averaged values of these determinations are presented in the discussion that follows. With the exception of the 1-propargyl analogue **25** (see below), the rates of metabolite formation for all substrates at K_m were constant over at least a 30-min incubation period.

We first examined the interactions of human placental and beef liver mitochondrial preparations with the *N*-methyl analogue **14**, a known MAO-A/MAO-B mixed substrate for which we had a synthetic standard of the dihydropyridinium metabolite **15**.[42] Figure 4 shows a series of scans obtained with 10-min incubation mixtures containing **14** (25–200 μM) and human placental mitochondria (equivalent to 18 nM MAO-A, Fig. 4a) and beef liver mitochondria (equivalent to 7.5 nM MAO-B Fig. 4b). The inserts show the double reciprocal plots from which we calculated the V_{max} and K_m values for the two reactions. The MAO-A selectivity at V_{max} [V_{max}(MAO-A)/V_{max}(MAO-B)] for this substrate is 135/361 = 0.37. The corresponding value for MPTP, determined under these conditions, is only 11/169 = 0.07. Consequently, replacement of the 4-phenyl substituent with the 4-(1-methylpyrrol-2-yl) substituent increases the selectivity for MAO-A by a factor of 5. This difference reflects the very poor V_{max} value (11 min^{-1}) for the MAO-A catalyzed oxidation of MPTP. When measured in terms of the V_{max}/K_m, the overall MAO-A selectivity coefficient [$SC_{MAO-A} = V_{max}/K_m$(MAO-A)/V_{max}/K_m(MAO-B] for the pyrrolyl compound (0.8), however, is similar to the corresponding value for MPTP (0.5) due to the low K_m value (32 μM) for the MAO-A catalyzed oxidation of MPTP.

The synthetic route to the targeted tetrahydropyridinyl derivatives is shown in Scheme 3.[43] Sequential reactions of 1-methylpyrrole (**36**) with *s*-butyllithium followed by $ZnCl_2$ gave the zinc reagent **37** that underwent a cross coupling reaction with 4-bromopyridine in the presence of $Pd(PPh_3)_4$. The resulting pyridinylpyrrole **38** was treated with the required alkyl bromide to yield the pyridinium intermediates **39**–**43** that were reduced to the desired tetrahydropyridinyl products. Special features for the preparation of specific analogues are discussed separately.

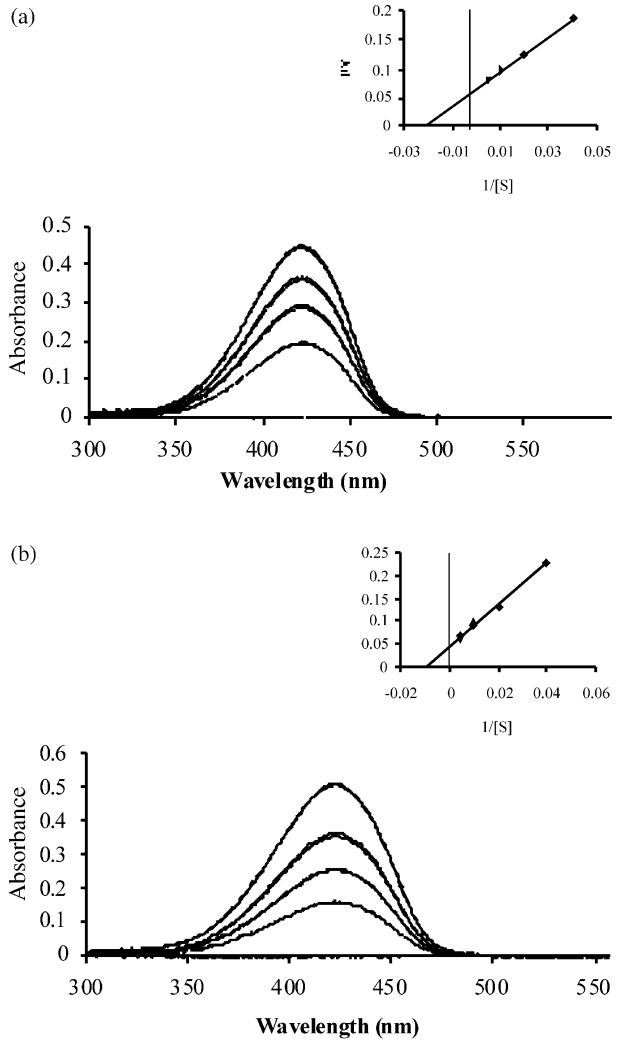
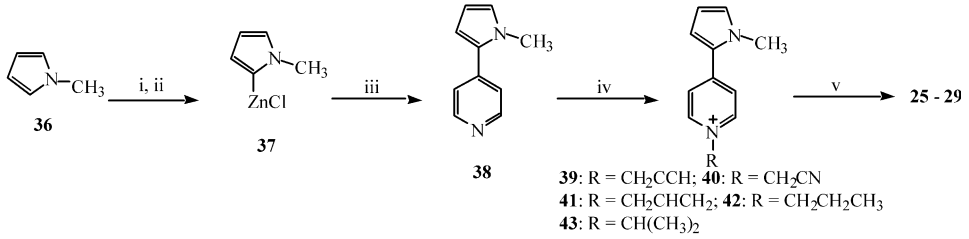


Figure 4. (a) UV–vis scans (duplicate assays) of 10 min-incubation mixtures (after precipitation of proteins with $HClO_4$) containing 25–200 μM 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (**14**) and human placental mitochondria (equivalent to 18 nM MAO-A). (b) The corresponding tracings with beef liver mitochondria (equivalent to 7.5 nM MAO-B). The inserts show the double reciprocal plots derived from the tracings.

Aqueous solutions of the oxalate salt of the propargyl analogue **25** (pK_a 6.4) turned yellow upon standing. We speculated that oxalic acid (first pK_a 1.23) is too weak an acid to protonate **25** fully and that the free base was undergoing autoxidation. Attempts were made to prepare a more stable salt with $HClO_4$ but **25** underwent rapid decomposition to highly colored products when treated with this strong acid. The HCl salt of **25** proved to be easy to prepare and was adequately stable in solution for enzyme kinetic studies. However, upon standing for a prolonged period, this HCl salt underwent slow decomposition.

Studies with beef liver mitochondria established that **25** is not an MAO-B substrate (absence of a chromophore corresponding to the dihydropyridinium metabolite **30**). This behavior was expected since an earlier study had established that **25** inactivates semi-purified beef liver MAO-B.[44] The estimated k_{inact}/K_I value [1.3 (min-mM)$^{-1}$] is similar to that found for the inactivation of semi-purified beef liver MAO-B by the phenyl analogue **16** [$(k_{inact}/K_I = 1.4$ (min-mM)$^{-1}$].[33] In the present study, we examined the inactivation of beef liver mitochondrial MAO-B (equivalent to 75 nM MAO-B) by 400 μM **25**. The remaining enzyme activity was measured using 4 mM **14**. MAO-B activity was completely inhibited by 20 min.

Similar to the behavior of the phenyl analogue **16**, this propargyl derivative proved to be a human placental mitochondrial MAO-A substrate. In contrast to the other pyrrolyl analogues examined in this study, the plot of the concentration of the dihydropyridinium metabolite **30** generated with 2 mM **25** (V_{max} conditions) versus time was curvilinear (Fig. 5). We conclude, therefore, that **25** is a relatively weak inactivator of MAO-A. No shift in the λ_{max} value corresponding to the dihydropyridinium metabolite **30** was observed in the tracings over a period of 40 min. This result confirms our expectations, based on resonance considerations discussed above (Fig. 3), that the pyrrolyldihydropyridinium metabolite **30** would be stable under these incubation conditions. Experiments (10-min incubation periods) to determine the substrate concentration dependent rates of dihydropyridinium metabolite formation established the V_{max} (125 min^{-1}) and K_m (162



Scheme 3. Synthetic route to 4-(1-methylpyrrol-2-yl)-1-substituted-1,2,3,6-tetrahydropyridines **25**–**29**: reagents: (i) *sec*-butyllithium in THF; (ii) $ZnCl_2$; (iii) 4-bromopyridine/$Pd(PPh_3)_4$/THF; (iv) alkyl bromide/acetone; (v) $NaBH_4$/MeOH or $NaCNBH_3$/MeOH.

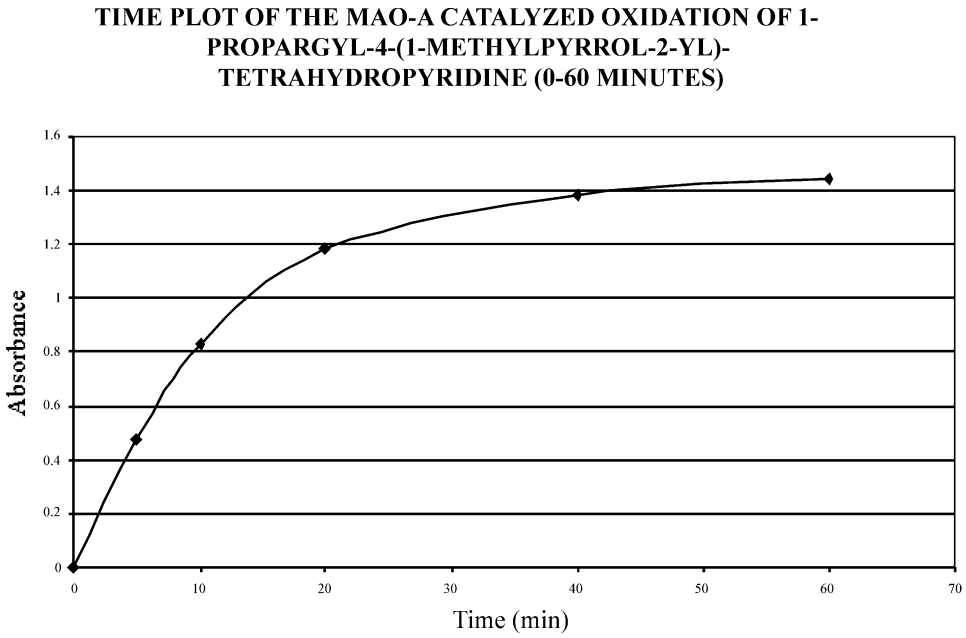
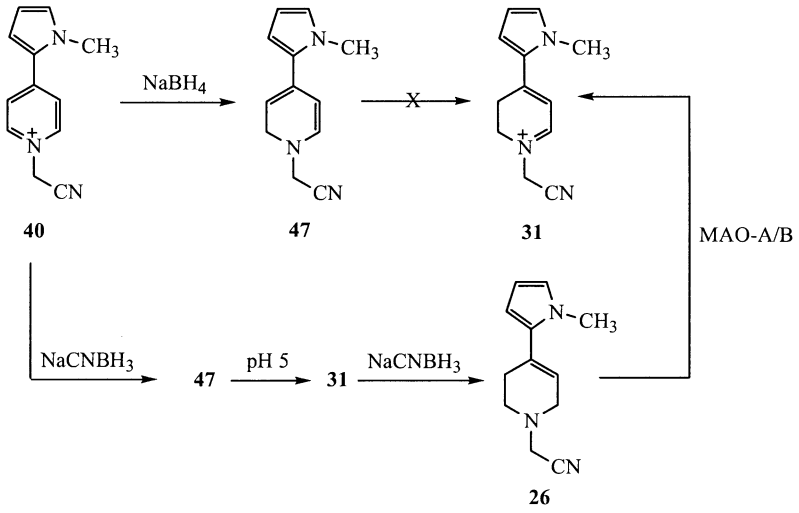


Figure 5. A plot of the UV absorbance (420 nm) versus time obtained from incubation mixtures of human liver mitochondria containing 2 mM **25** and 18 nM MAO-A.



Scheme 4.

μM) values for this substrate.[44] The corresponding values for the 4-phenyl analogue **16** could not be determined because of its relatively potent MAO-A inactivating properties. Thus, the *N*-propargyl analogues **16** (phenyl) and **25** (pyrrolyl) both inactivate beef liver MAO-B efficiently. By contrast, the phenyl analogue is a very poor human placental mitochondrial MAO-A substrate (the rate too slow to measure) while the pyrrolyl analogue is a good MAO-A substrate [$V_{max}/K_m = 831$ (min-mM)$^{-1}$].

The inactivation of MAO-B by the propargylamine **25** presumably proceeds according to the mechanism shown in Scheme 2 for the phenyl analogue **16** with the ethynyliminium species **45** serving as the bioalkylating agent. An analogous pathway should not operate with the cyanomethyl analogue **26** since the cyanoiminium species **46**, if formed, will not alkylate the enzyme.

The synthesis of **26** (Scheme 4) was pursued according to the route outlined in Scheme 3. Attempted reduction of the cyanomethylpyridinium intermediate **40** with $NaBH_4$ in ethanol, however, failed to yield the expected tetrahydropyridinyl product. UV analysis of the reaction mixture indicated that all of the starting material had been consumed within 5 min after the addition of the $NaBH_4$. It appeared, therefore, that the second reductive step, via the dihydropyridinium species **31**, was not proceeding. Since the pK_a of **26·HCl** is only 3.5 (compared to 9.5 for **14·HCl**), we speculated that the initially formed *N*-cyanomethyldihydropyridine **47** is too weak a base to undergo protonation in methanol.

Consistent with this view, when the reaction was carried out with $NaCNBH_3$ at pH 5, the tetrahydropyridine **26**, as its HCl salt, was obtained in 43% yield.

Compound **26** was found to be a good MAO-A [$K_m = 172$ μM; $V_{max} = 62$ min^{-1}; $V_{max}/K_m = 371$ min-mM)$^{-1}$] and MAO-B [$K_m = 576$ μM; $V_{max} = 149$ min^{-1}; $V_{max}/K_m = 253$ (min-mM)$^{-1}$] substrate. As expected, evidence for the time dependent loss in enzyme activity was not observed with either enzyme.

We next turned our attention to the corresponding *N*-allyl analogue **27** that was prepared by $NaBH_4$ reduction of the pyridinium species **32**. The corresponding 4-phenyl analogue **48** (Fig. 1) is neither an MAO-A nor an MAO-B substrate.[34] Kinetic studies with mitochondrial human placental MAO-A established the K_m and V_{max} values for **27** to be 48 μM and 92 min^{-1}, respectively. This compound proved to be a poor MAO-B substrate. Because of its poor MAO-B substrate properties, the kinetic values reported here ($K_m = 133$ μM and $V_{max} = 12$ min^{-1}) had to be obtained with a concentration of MAO-B 10 times higher than that required for *N*-methyl (**14**) and *N*-cyanomethyl (**26**) analogues. The $SC_{MAO\text{-}A}$ for the *N*-allyl analogue **27** was calculated to be 21.0. The resulting dihydropyridinium metabolite **32** was stable over a 60-min incubation period. Furthermore, there was no evidence of loss of MAO-A or MAO-B activity in the presence of 2 mM **27** over time. Consequently either the eneiminium species **49** (Scheme 2) is not formed or is not an effective alkylating agent.

The 1-propyl analogue **28** proved to be an equally poor MAO-B substrate (228 μM and 52 min^{-1}). The MAO-B substrate properties of the corresponding phenyl analogue **50** (Fig. 1) are too poor to yield useful kinetic data.[33] Compound **28**, however, is an excellent MAO-A substrate ($K_m = 18$ μM; $V_{max} = 81$ min^{-1}). No evidence of enzyme inactivation was observed with either enzyme and the dihydropyridinium metabolite **33**, like the corresponding *N*-methyldihydropyridinium species **15**, was stable for at least 2 h. In view of these excellent MAO-A substrate characteristics of the *N*-propyl analogue, we were somewhat surprised to observe that the corresponding *N*-isopropyl analogue **29** is neither an MAO-A nor an MAO-B substrate. The exact features of the active site that lead to such dramatic differences in substrate properties remain to be investigated.

The results of these studies are summarized in Table 1. With respect to MAO-B, it is apparent that, with the exception of the propargyl analogue **25,** replacement at C4 of the phenyl substituent with the 1-methylpyrrol-2-yl substituent leads to enhanced MAO-B substrate properties. Thus, in the 4-phenyl series, only MPTP is a good MAO-B substrate [$V_{max}/K_m = 748$ (min-mM)$^{-1}$]. The rates of oxidation of the *N*-propargyl, *N*-allyl and *N*-propyl analogues of MPTP all are too slow to measure even when high (mM) substrate concentrations are used. In the 4-pyrrolyl series examined in this study, the V_{max}/K_m value for the MAO-B catalyzed oxidation for the *N*-propargyl analogue **25** could not be measured. The four other analogues had V_{max}/K_m values that ranged from a low of 90 (min-mM)$^{-1}$ for the *N*-allyl analogue **27** to a high of 3740 (min-mM)$^{-1}$ for the *N*-methyl analogue **14**. The time and concentration dependent MAO-B inhibiting properties of the propargyl analogue **25** are remarkable when contrasted with its very good MAO-A substrate properties [$V_{max}/K_m = 831$ (min-mM)$^{-1}$].

The very good to excellent MAO-A substrate properties [$V_{max}/K_m = 371$ to 4490 (min-mM)$^{-1}$] for the 5 pyrrolyl analogues **14** and **25**–**28** examined here are of particular interest. The principal goal of this effort has been to identify selective MAO-A substrates that may be useful for the analysis of MAO-A activity in tissues that express both the A and B forms of the enzyme. In this regard, the $SC_{MAO\text{-}A}$ values of approximately 20 for the *N*-allyl analogue **27** and *N*-propyl analogue **28** recommend these compounds as candidates for further development. The stability of the corresponding MAO-A generated dihydropyridinium metabolites **32** and **33** and the absence of any evidence for loss of MAO-A activity with time also are desirable properties for assay development. Although the MAO-A selectivity of the *N*-propargyl analogue **25** is even better than the *N*-allyl and *N*-propyl analogues due to its MAO-B inactivating properties, the apparent loss of MAO-A activity over time (Fig. 5) makes this compound less attractive for assay development.

Current efforts are being directed to examining the substrate properties of these compounds in human gut and liver mitochondrial preparations and to evaluate further their utility for selective analysis of MAO-A activity. Additional studies are underway to expand our understanding of the structure–activity relationships that govern MAO-A and MAO-B selectivity of 1,4-disubstituted-1,2,3,6-tetrahydropyridinyl derivatives.

Experimental

Important notice: Some 1,4-disubstituted-1,2,3,6-tetrahydropyridines are known nigrostriatal neurotoxins and should be handled using disposable gloves in a properly ventilated hood following good laboratory practices. Detailed procedures for the safe handling of MPTP have been reported.[45]

Chemistry

General methods. Unless otherwise noted, reagents and starting materials were obtained from commercial suppliers and were used without purification. THF was distilled over sodium and benzophenone. $ZnCl_2$ was flame dried before use. All reactions were carried out using flame-dried glassware under an atmosphere of argon. Proton and carbon NMR spectra were recorded on a Bruker AM 360-MHz spectrometer. Chemical shifts are reported in ppm downfield from internal tetramethylsilane. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), sext (sextet) or m (multiplet). Coupling constants (J) are given in hertz (Hz). All

UV–vis spectra were recorded on a Beckman DU 7400 spectrophotometer.

pK_a Measurements. Estimations of the pK_as of salts of the 1-substituted-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridinyl derivatives (1 mM solution in water, 20 mL) were made using a Corning 320 pH meter and a Corning '3 in 1' Combo w/RJ electrode. The compounds were titrated potentiometrically at 23 °C with standard aqueous 2.02 mM NaOH (prepared from a 0.1009 M volumetric standard purchased from Aldrich). The calibration of the electrode was made using two buffer solutions at pH 4.00 (potassium phosphate monobasic-sodium hydroxide buffer, 0.05 M) and at pH 7.00 (potassium biphthalate buffer, 0.05 M) purchased from Fisher. All pK_a determinations were performed in duplicate.[46]

Oxalate salt of 4-(1-methylpyrrol-2-yl)pyridine [38·(COOH)$_2$]. The free base **38** has been reported previously.[47] A solution of 1-methylpyrrole (**36**, 19.5 mL, 0.22 mol) in THF (200 mL) was treated with *sec*-BuLi (170 mL of a 1.3 M solution in cyclohexane, 0.22 mol) at 0 °C. The mixture was stirred for 1 h at room temperature and then added to a solution of $ZnCl_2$ (33.2 g, 0.24 mol) in THF (400 mL) at room temperature. After 1 h, this mixture was added to a solution of 4-bromopyridine (18.4 g, 0.12 mol) and $Pd(PPh_3)_4$ (1.39 g, 1.2 mmol) in THF (200 mL). After stirring under reflux for 2 h and at room temperature for 24 h, the mixture was treated with a saturated aqueous solution of NH_4Cl (300 mL). The pH was adjusted to 10 with aq NaOH, the mixture was filtered and most of the THF was removed under reduced pressure. The remaining aqueous phase was extracted with AcOEt (2×150 mL). The combined organic phases were washed with water (2×150 mL) followed by brine (150 mL) and were dried over $MgSO_4$. The isolated crude solid was dissolved in ether and a saturated solution of oxalic acid in ether was added with stirring. After filtration, the solid was recrystallized from CH_3OH/ether to give the oxalate salt of **38** as light-yellow crystals (21.6 g, 73%): mp 143–145 °C; ^{1}H NMR (D_2O) δ 3.72 (s, 3H), 6.19 (m, 1H), 6.82 (m, 1H), 6.99 (m, 1H), 7.73 (m, 2H), 8.32 (m, 2H); ^{13}C NMR (D_2O) δ 25.4, 99.0, 106.4, 109.6, 116.8, 122.2, 128.5, 136.7, 153.6. Anal. calcd for $C_{12}H_{12}N_2O_4 \cdot 0.18\ H_2O$ (251.44): C, 57.27; H, 4.91; N, 11.13. Found: C, 57.27; H, 5.04; N, 10.95; FAB-HRMS: Calcd for $C_{10}H_{11}N_2^+$: 159.0922. Found: 159.0916.

4-(1-Methylpyrrol-2-yl)-1-propargylpyridinium bromide (39·Br). Propargyl bromide (2.9 mL, 26 mmol) was added to a solution of free base **38** (1 g, 6.4 mmol) in acetone (20 mL). The mixture was stirred for 20 h at room temperature and the resulting precipitate was collected and crystallized from EtOH to give **39·Br** as yellow crystals (1.52 g, 86%): mp 213–215 °C (dec.); UV (H_2O) λ_{max} 376 nm (ε 26,300); ^{1}H NMR (CD_3OD) δ 3.49 (t, J = 2.5 Hz, 1H), 4.01 (s, 3H), 5.42 (d, J = 2.5 Hz, 2H), 6.35 (m, 1H), 7.17 (m, 1H), 7.24 (m, 1H), 8.11 (m, 2H), 7.83 (m, 2H), 8.74 (m, 2H); ^{13}C NMR (CD_3OD) δ?37.9, 49.7, 75.8, 80.8, 112.1, 120.6, 123.0, 129.6, 135.8, 144.2, 149.4. Anal. calcd for $C_{13}H_{13}BrN_2$ (277.15): C, 56.33; H, 4.73; N, 10.11. Found: C, 56.17; H, 4.80; N, 10.10.

4-(1-Methylpyrrol-2-yl)-1-propargyl-1,2,3,6-tetrahydropyridine hydrochloride (25·HCl). To a stirred suspension of **39·Br** (1 g, 3.6 mmol) in EtOH (30 mL) was added $NaBH_4$ (0.56 g, 15 mmol). After stirring at room temperature for 2 h, the reaction mixture was treated with water (20 mL) and most of the EtOH was removed under reduced pressure. The aqueous layer was extracted with AcOEt (3×20 mL) and the combined organic phases were dried over $MgSO_4$ and the solvent removed under reduced pressure. The residue as the free base was purified by silica gel chromatography (AcOEt). A methanolic solution of HCl (1.5 equiv) was added at 0 °C to a solution of the free base in EtOH (2 mL) and the resulting precipitate was recrystallized from EtOH to give **25·HCl** as light-yellow crystals (406 mg, 58%): mp 174–176 °C; UV (0.1 M Na_2HPO_4/NaH_2PO_4 buffer, pH 7.4) λ_{max} 261 nm (ε 10,047); ^{1}H NMR (D_2O) δ 2.81 (m, 2H), 3.15 (t, J = 2.5 Hz, 1H), 3.68 (s, 3H), 2.70–4.10 (m, 4H), 4.17 (d, J = 2.5 Hz, 2H), 5.82 (m, 1H), 6.19 (m, 1H), 6.27 (m, 1H), 6.82 (m, 1H); ^{13}C NMR (CD_3OD) δ 27.2, 36.3, 45.9, 50.1, 51.3, 73.2, 81.4, 108.4, 110.2, 114.8, 126.8, 129.8; 132.2. Anal. calcd for $C_{13}H_{17}ClN_2$ (236.73): C, 65.96; H, 7.24; N, 11.83. Found: C, 65.94; H, 7.38; N, 11.68.

1-Cyanomethyl-4-(1-methylpyrrol-2-yl)pyridinium bromide (40·Br). Reaction of bromoacetonitrile (1.74 mL, 25 mmol) and the free base **38** (1 g, 6.3 mmol) in acetone (25 mL) for 4 h at room temperature gave a solid that was crystallized from MeOH/H_2O to give pure **40·Br** as yellow needles (1.53 g, 87%): mp 235–238 °C (dec.); UV (H_2O) λ_{max} 387 nm (ε 32710); ^{1}H NMR (CD_3OD) δ 4.03 (s, 3H), 4.87 (s, 2H), 6.38 (m, 1H), 7.26 (m, 1H), 7.29 (m, 1H), 8.14 (m, 2H), 8.70 (m, 2H); ^{13}C NMR (D_2O) δ 37.8, 46.5, 111.7, 114.2, 120.5, 122.0, 128.4, 136.2, 143.6, 148.4. Anal. calcd for $C_{12}H_{12}BrN_3$ (278.14): C, 51.82; H, 4.35; N, 15.11. Found: C, 51.98; H, 4.45; N, 15.18.

1-Cyanomethyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine hydrochloride (26·HCl). To a suspension of **40·Br** (500 mg, 1.8 mmol) in EtOH (20 mL) was added $NaCNBH_3$ (0.57 g, 9 mmol). The pH of the reaction mixture was adjusted approximately to 5 with aq HCl. The mixture was stirred at room temperature for 24 h at which time 10% aq HCl (10 mL) was added. This mixture was stirred for an additional 15 min and then was treated with aqueous KOH (pH 8.5) and extracted with ether (3×20 mL). The combined organic phases were dried over $MgSO_4$, filtrated through basic alumina and the solvent removed under reduced pressure. A methanolic solution of HCl (1.5 equiv) was added at 0 °C to a solution of the free base in EtOH (2 mL). The precipitate was collected and crystallized (EtOH/MeOH) to give pure **26·HCl** as light-orange crystals (184 mg, 43%): mp 178–180 °C (dec.); UV (H_2O) λ_{max} 263 nm (ε 9633); ^{1}H NMR (DMSO-d_6) δ 2.66 (m, 2H), 3.29 (m, 2H), 3.63 (s, 3H), 3.74 (m, 2H), 4.46 (m, 2H), 5.77 (m, 1H), 5.97 (m, 1H), 6.10 (m, 1H), 6.77 (m, 1H); ^{13}C NMR (D_2O) δ 26.1, 35.9, 43.1, 50.6, 51.5, 107.4, 108.9,

112.5, 114.9, 126.7, 128.1, 132.1. Anal. calcd for $C_{12}H_{16}ClN_3$ (237.73): C, 60.63; H, 6.78; N, 17.68. Found: C, 60.36; H, 6.95; N, 17.45.

1-Allyl-4-(1-methylpyrrol-2-yl)pyridinium bromide (41·Br). Treatment of the free base **38** (1 g, 6.3 mmol) with allyl bromide (2.2 mL, 25 mmol) according to the previous description gave 1.65 g (94%) of the corresponding allylpyridinium bromide **41·Br** as yellow needles from EtOH: mp 206–208 °C; UV (H_2O) λ_{max} 372 nm (ε 28,768); ^{1}H NMR (CD_3OD) δ 3.99 (s, 3H), 5.10 (m, 2H), 5.51 (m, 2H), 6.18 (m, 1H), 6.34 (m, 1H), 7.13 (m, 1H), 7.21 (m, 1H), 8.08 (m, 2H), 8.61 (m, 2H); ^{13}C NMR (CD_3OD) δ 37.9, 62.9, 111.9, 120.0, 123.1, 123.2, 129.7, 132.4, 135.3, 144.8, 148.8. Anal. calcd for $C_{13}H_{15}BrN_2$ (279.17): C, 55.93; H, 5.42; N, 10.03. Found: C, 55.96; H, 5.53; N, 9.88.

1-Allyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine hydrochloride (27·HCl). The free base of this compound was prepared according to the procedure described previously for the corresponding free base of the propargyl derivative **25**. A methanolic solution of HCl (1.5 equiv) was added at 0 °C to a solution of the free base in MeOH (2 mL). After standing overnight in the freezer, pure **27·HCl** (59%) was obtained: mp 196–198 °C; UV (H_2O) λ_{max} 268 nm (ε 11,376); ^{1}H NMR (DMSO-d_6) δ 2.55–2.74 (m, 2H), 3.15 (m, 1H), 3.52 (m, 1H), 3.64 (s, 3H), 3.71 (m, 1H), 3.78–3.82 (m, 3H), 5.54 (m, 2H), 5.76 (m, 1H), 5.98–6.13 (m, 3H) 6.77 (m, 1H), 10.97 (bs, 1H); ^{13}C NMR (CD_3OD) δ 27.3, 36.3, 50.0, 51.3, 59.4, 108.4, 110.1, 115.3, 126.6, 127.0, 127.8; 129.9. Anal. calcd for $C_{13}H_{19}ClN_2$ (238.75): C, 65.40; H, 8.02; N, 11.73. Found: C, 65.37; H, 8.08; N, 11.58.

4-(1-Methylpyrrol-2-yl)-1-propylpyridinium bromide (41·Br). A mixture of the free base **38** (1 g, 6.3 mmol) and propyl bromide (2.3 mL, 25 mmol) was stirred for 48 h under reflux. After cooling, the precipitate was collected and recrystallized from EtOH to give pure **41·Br** as light-yellow crystals (1.7 g, 96%): mp 222–224 °C; UV (H_2O) λ_{max} 370 nm (ε 25,352); ^{1}H NMR (CD_3OD) δ 1.02 (t, J = 7.6 Hz, 3H), 2.03 (sext, J = 7.6 Hz, 2H), 3.99 (s, 3H), 4.42 (t, J = 7.6 Hz, 2H), 6.33 (m, 1H), 7.13 (m, 1H), 7.20 (m, 1H), 8.06 (m, 2H), 8.65 (m, 2H); ^{13}C NMR (CD_3OD) δ 11.0, 25.7, 37.8, 62.7, 111.8, 119.8, 123.2, 129.7, 135.0, 144.8, 148.6. Anal. calcd for $C_{13}H_{17}BrN_2$ (281.18): C, 55.53; H, 6.09; N, 9.96. Found: C, 55.44; H, 6.09; N, 9.96.

4-(1-Methylpyrrol-2-yl)-1-propyl-1,2,3,6-tetrahydropyridine hydrochloride (28·HCl). The free base **28**, prepared as described for the propargyl analogue **25**, was converted to its HCl salt in methanol at 0 °C. Crystallization from EtOH gave pure **28·HCl** in 43% overall yield: mp 205–208 °C (dec.); UV (H_2O) λ_{max} 267 nm (ε 10,093); ^{1}H NMR (CD_3OD) δ 1.05 (t, J = 7.2 Hz, 3H), 1.84 (m, 2H), 2.78 (m, 2H), 3.19 (m, 2H), 3.35–3.65 (m, 2H), 3.69 (s, 3H), 3.94 (m, 2H), 5.75 (m, 1H), 6.03 (m, 1H), 6.17 (m, 1H), 6.69 (m, 1H); ^{13}C NMR (CD_3OD) δ 11.4, 18.9, 27.3, 36.3, 50.7, 51.7, 58.9, 108.4, 110.1, 115.2, 126.6, 129.7, 128.6. Anal. calcd for $C_{13}H_{21}ClN_2$ (240.77): C, 64.85; H, 8.79; N, 11.93. Found: C, 64.70; H, 8.77; N, 11.44.

1-Isopropyl-4-(1-methylpyrrol-2-yl)pyridinium bromide (43·Br). A mixture of the free base **38** (1 g, 6.3 mmol) and isopropyl bromide (5 mL) was stirred for 24 h under reflux. The resulting solid was collected and washed with ether to give analytically pure **43·Br** as yellow crystals (0.63 g, 42%): mp 155–157 °C; UV (H_2O) λ_{max} 368 nm (ε 25,500); ^{1}H NMR (CD_3OD) δ 1.66 (d, J = 6.8 Hz, 6H), 3.99 (s, 3H), 4.86 (m, 1H), 6.33 (m, 1H), 7.10 (m, 1H), 7.20 (m, 1H), 8.06 (m, 2H), 8.72 (m, 2H); ^{13}C NMR (CD_3OD) δ 23.4, 38.2, 64.5, 111.8, 119.7, 123.5, 129.6, 135.0, 143.1, 148.4. Anal. calcd for $C_{13}H_{17}BrN_2$ (281.18): C, 55.53; H, 6.09; N, 9.96. Found: C, 55.72; H, 6.12; N, 9.91.

Oxalate salt of 1-isopropyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine [29·(COOH)$_2$]. An ether solution of the free base **29**, prepared as described above for the propargyl analogue **25**, was filtrated through basic alumina. The filtrate was treated with an etheral solution of oxalic acid (1.0 equiv) to yield the analytically pure oxalate salt of **29** (43%) as a light yellow solid: mp 138–140 °C; UV (H_2O) λ_{max} 266 nm (ε 10,350); ^{1}H NMR (CD_3OD) δ 1.41 (d, J = 6.5 Hz, 6H), 1.80 (m, 2H), 3.49 (m, 2H), 3.63 (m, 1H), 3.69 (s, 3H), 3.91 (m, 2H), 5.77 (m, 1H), 6.03 (m, 1H), 6.15 (m, 1H), 6.68 (m, 1H); ^{13}C NMR (CD_3OD) δ 17.2, 28.0, 36.2, 47.4, 47.8, 58.9, 108.4, 109.2, 116.0, 126.5, 129.9, 132.8, 166.8. Anal. calcd for $C_{15}H_{22}N_2O_4$ (294.35): C, 61.21; H, 7.53; N, 9.52. Found: C, 60.88; H, 7.53; N, 9.35.

Enzymology

General methods. All collections of human placenta were approved by the Internal Review Boards of Montgomery County Hospital and Virginia Tech, and the collection of beef liver was approved by the Animal Care Committee of Virginia Tech. Human placenta and beef liver mitochondrial homogenates were prepared using the methodology reported earlier by Salach with minor modifications and were stored at −70 °C prior to use.[48] Phosphate buffer was prepared using Na_2HPO_4 and NaH_2PO_4. For determinations of the protein concentrations (triplicate samples) using the Bradford assay, the mitochondrial preparations initially were diluted approximately 1:2 with phosphate buffer containing 50% (w/v) glycerol.[49] A UV–vis background subtraction was performed for each sample as follows: Background samples were prepared following the same procedures as those for the corresponding samples used for the analysis of substrate and inhibitor properties of the test compounds. At t = 0 min 70% aq $HClO_4$ was added to each background sample and the resulting mixture was centrifuged. The UV–vis signal from the supernatant fraction was subtracted from each corresponding sample signal.

Enzyme concentration determinations. Studies to estimate enzyme concentrations (nmol enzyme/mg mitochondrial protein) were conducted as follows: The rate of the MAO-A catalyzed oxidation of 1-methyl-4-phenoxy-1,2,3,6-tetrahydropyridine (**35**) was determined using a 4 mM stock solution of the substrate in 0.1 M phosphate buffer (pH 7.2–7.4, 475 μL) preincubated for

10 min with gentle agitation in a water bath incubator at 37 °C. To each sample was added 25 μL of a well mixed suspension of human placental mitochondria (3.0 mg protein/mL in phosphate buffer containing 50% w/v glycerol). The resulting mixtures were incubated for 0, 2, 4, 6, 8, 10 min at 37 °C with gentle agitation in a water bath. The reactions were quenched with 70% aq $HClO_4$ (20 μL) and the resulting mixtures were centrifuged at 16,000*g* for 5 min. The supernatants (470 μL) in sample cuvettes were scanned from 250 to 600 nm and the absorbances were measured at 314 nm, the λ_{max} for the dihydropyridinium metabolite of **35**. An analogous procedure for MAO-B was followed using 5 mM 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**7**) as substrate with beef liver mitochondrial homogenates (3.0 mg protein/mL in phosphate buffer containing 50% w/v glycerol) at 30 °C with absorbance readings taken at 347 nm, the λ_{max} for **8**. In each case a plot of absorbance versus time provided an estimate of the initial rate of the enzyme catalyzed oxidation. The concentrations of the enzymes then were calculated using published turnover numbers for the human placental semipurified MAO-A catalyzed oxidation of **35** (130 min^{-1})[40] and the beef liver purified MAO-B catalyzed oxidation of **7** (204 min^{-1}).[41]

Substrate studies with MAO-A and MAO-B. Initial studies were carried out with the test compounds (2 mM) and human placental mitochondrial MAO-A and beef liver mitochondrial MAO-B. Stock solutions (4 mM) of the compounds were prepared in phosphate buffer solution (0.1 M, pH 7.2–7.4). Aliquots (250 μL) of each compound diluted with additional phosphate buffer (225 μL) were preincubated in Eppendorf tubes for 10 min with gentle agitation in a water bath incubator at 37 °C. Suspensions of the previously diluted mitochondria (25 μL of 3.0 mg protein/mL in phosphate buffer containing 50% w/v glycerol) were added to give a final enzyme concentration of 18 nM for MAO-A and 7.5 nM for MAO-B. The mixtures were allowed to incubate for 10, 20 and 30 min at which times the reactions were quenched by the addition of 70% aq $HClO_4$ (20 μL). The homogenates were centrifuged at 16,000*g* for 5 min, the resulting supernatants (470 μL) were added to sample cuvettes and the concentrations of the dihydropyridinium metabolites were determined by measurements of the absorbance at the λ_{max} for each metabolite [**15** (420 nm); **32** and **33** (424 nm); **31** (422 nm) and **30** (435 nm)] obtained from the 250–600 nm UV–vis scans. The results of these studies provided initial estimates of the MAO-A and MAO-B substrate properties of the test compounds and allowed for evaluation of the linearity of metabolite production versus time and the stability of the dihydropyridinium metabolites.

Studies to determine the K_m and V_{max} values for each substrate were performed in triplicate in an analogous fashion. The preliminary studies gave a rough estimate of K_m from which four substrate concentrations were selected to bracket K_m (0.5, 1.0, 1.5 and 2.0 K_m). The K_m and V_{max} values were calculated from double reciprocal plots (1/V vs 1/[S]). The absorbance measurements for the MAO-B catalyzed oxidation of the allyl and propyl analogues were quite low and could not be measured accurately. Therefore, the final enzyme concentration in these samples was increased to 75 nM MAO-B.

MAO-A and MAO-B inhibition studies. An aliquot (80 μL) of a 2 mM stock solution of the test compound in 0.1 M phosphate buffer was diluted with 0.1 M phosphate buffer (120 μL) and the resulting mixture was preincubated for 15 min with gentle agitation in a water bath incubator at 37 °C. The MAO-A or MAO-B containing mitochondrial preparation (a 200 μL aliquot containing 3.0 mg protein/mL in 0.1 M phosphate buffer) was added. The final enzyme concentration was 180 nM (MAO-A) or 75 nM (MAO-B) and final inhibitor concentration was 400 μM. This mixture was incubated at 37 °C and aliquots (25 μL), taken at 0, 5, 10, 15 and 20 min, were added to a solution of 225 μL 0.1 M phosphate buffer and 250 μL of 4 mM **14** (in phosphate buffer) as substrate. This sample was incubated for an additional 15 min at 37 °C at which time 70% aqueous $HClO_4$ (20 μL) was added. The homogenate was centrifuged at 16,000*g* for 5 min. The supernatant (470 μL) was added to a sample cuvette and the concentration of the dihydropyridinium metabolite **15** was determined from the absorbance at 420 nm.

Acknowledgements

This work was supported by a grant from Pharmacia Corporation and the Harvey W. Peters Research Center.

References and Notes

1. Dosert, P.; Strolin Benedetti, M.; Tipton, K. F. *Med. Res. Rev*. **1989**, *9*, 45.
2. Kuhn, D. M.; Murphy, D. L.; Youdim, M. B. H. In *Physiological and Clinical Aspects of Monoamine Oxidase. Structure and Function of Amine Oxidases*; Kuhn, D. M., Murphy, D. L., Youdim, M. B. H., Eds.; CRC: Boca Raton, 1985; Vol. 22, p 231.
3. Green, A. R.; Mitchell, B.; Tordoff, A.; Youdim, M. B. H. *Br. J. Pharmacol*. **1977**, *60*, 343.
4. Valoti, M.; Moron, J. A.; Benocci, A.; Sgaragli, G.; Unzeta, M. *J. Biochem. Pharmacol*. **1998**, *55*, 37.
5. Strolin Benedetti, M.; Boucher, T.; Carlsson, A.; Fowler, C. J. *J. Biochem. Pharmacol*. **1983**, *32*, 47.
6. Youngster, S. K.; McKeown, K. A.; Jin, Y. Z.; Ramsay, R. R.; Heikkila, R. E.; Singer, T. P. *J. Neurochem*. **1989**, *53*, 1837.
7. Singer, T. P.; Castagnoli, N., Jr.; Trevor, A. *Neurol. Neurobiol. 42A (Progr. Catecholamine Res., Pt. A)* **1988**, 75.
8. Singer, T. P.; Salach, J. I.; Crabtree, D. *Biochem. Biophys. Res. Commun*. **1985**, *127*, 707.
9. Salach, J. I.; Singer, T. P.; Castagnoli, N., Jr.; Trevor, A. *Biochem. Biophys. Res. Commun*. **1984**, *125*, 831.
10. Chiba, K.; Trevor, A.; Castagnoli, N., Jr. *Biochem. Biophys. Res. Commun*. **1984**, *120*, 574.
11. Youdim, M. B. H. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **1989**, *13*, 363.
12. Glover, V.; Gibb, C.; Sandler, M. *Neurosci. Lett*. **1986**, *64*, 216.
13. Cohen, G. *Ann. N. Y. Acad. Sci*. **2000**, *899*, 112.

14. O'Carroll, A. M.; Anderson, M. C.; Tobbia, I.; Phillips, J. P.; Tipton, K. F. *J. Biochem. Pharmacol.* **1989**, *38*, 901.
15. Szutowicz, A.; Tomaszewicz, M.; Orsulak, P. *J. Biol. Chem.* **1989**, *264*, 17660.
16. Riley, L. A.; Waguespack, M. A.; Denney, R. M. *Mol. Pharmacol.* **1989**, *36*, 54.
17. Weyler, W.; Salach, J. I. *J. Biol. Chem.* **1985**, *260*, 13199.
18. Saura, J.; Nadal, E.; van den Berg, B.; Vila, M.; Bombi, J. A.; Mahy, N. *Life Sci.* **1996**, *59*, 1341.
19. Inoue, H.; Castagnoli, K.; Van der Schyf, C.; Mabic, S.; Igarashi, K.; Castagnoli, N., Jr. *J. Pharma. Exp. Ther.* **1999**, *291*, 856.
20. Riley, L. A.; Denney, R. M. *Biochem. Pharmacol.* **1991**, *42*, 1953.
21. Hauptmann, N.; Grimsby, J.; Shih, J. C.; Cadenas, E. *Arch. Biochem. Biophys.* **1996**, *335*, 295.
22. Finberg, J. P. M.; Youdim, M. B. H. *Br. J. Pharmacol.* **1985**, *85*, 541.
23. Bieck, P. R.; Antonin, K.-H.; Schmidt, E. *Clinical Neuropharmacol* **1993**, *16*, 534.
24. Brown, C.; Taniguchi, G.; Yip, K. *J. Clin. Pharmacol.* **1989**, *29*, 529.
25. McCabe, B. J. *J. Am. Diet. Assoc.* **1986**, *86*, 1059.
26. Tsuiki, K.; Takada, A.; Grdisa, M.; Diksic, M. *Neurochem. Int.* **1994**, *24*, 231.
27. Soto-Otero, R.; Mendez-Alvarez, E.; Hermida-Ameijeiras, A.; Sanchez-Sellero, I.; Cruz-Landeira, A. L.; Lopez-Rivadulla, M. *Life Sci.* **2001**, *69*, 879.
28. Flaherty, P.; Castagnoli, K.; Wang, Y.-X.; Castagnoli, N., Jr. *J. Med. Chem.* **1996**, *39*, 4756.
29. Chiba, K.; Peterson, L. A.; Castagnoli, K.; Trevor, A. J.; Castagnoli, N., Jr. *Drug Metab. Disp* **1985**, *13*, 342.
30. Zhao, Z.; Dalvie, D.; Naiman, N.; Castagnoli, K.; Castagnoli, N., Jr. *J. Med. Chem.* **1992**, *35*, 4473.
31. Dhingra, N. K.; Raju, T. R.; Meti, B. L. *Brain Res.* **1997**, *758*, 237.
32. Palmer, S. L.; Mabic, S.; Castagnoli, N., Jr. *J. Med. Chem.* **1997**, *40*, 1982.
33. Kalgutkar, A. S.; Castagnoli, N., Jr. *J. Med. Chem.* **1992**, *35*, 4165.
34. Kalgutkar, A. S. PhD Thesis, Virginia Tech, 1993; Chapter 4, p 202.
35. Walker, M. C.; Edmondson, D. E. *Biochemistry* **1994**, *33*, 7088.
36. Silverman, R. B.; Zhou, J. P.; Eaton, P. E. *J. Am. Chem. Soc.* **1993**, *115*, 8841.
37. Silverman, R. B. *Acc. Chem. Res.* **1995**, *28*, 335.
38. Glass, R. S.; Baciocchi, E.; Kalyanaraman, B.; Cavalieri, E. L.; Rogan, E. G.; Guengerich, F. P.; Okasaki, O.; Seto, Y.; MacDonald, T. L.; Karki, S. B.; Dinnocenzo, J. P.; Dunford, H. B.; Edmondson, D. E.; Stack, D. E.; Cremonesi, P.; Hanson, A.; Doerge, D. R.; Divi, R. L.; Sayre, L. M.; Engelhart, D. A.; Nadkarni, D. V.; Babu, M. K.; Klein, M. E.; McCoy, G. *Xenobiotica* **1995**, *25*, 637.
39. Yu, P. H.; Davis, B. A.; Boulton, A. A. *Adv. Exp. Med. Biol.* **1995**, *363*, 17.
40. Wang, Y.-X.; Castagnoli, N., Jr. *J. Med. Chem.* **1995**, *38*, 1904.
41. Youngster, S. K.; McKeown, K. A.; Jin, Y.-Z.; Ramsay, R. R.; Heikkila, R. E.; Singer, T. P. *J. Neurochem.* **1989**, *53*, 1837.
42. The approximate ε value for all dihydropyridinium metabolites was taken as 24,000 M^{-1} (Bai, H. MS Thesis, Virginia Tech, 1991; Chapter 2, p 49) the experimentally determined ε value for synthetic 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium perchlorate.
43. Nimkar, S. K.; Anderson, A. H.; Rimoldi, J. M.; Stanton, M.; Castagnoli, K.; Mabic, S.; Wang, Y.-X.; Castagnoli, N., Jr. *Chem. Res. Tox* **1996**, *9*, 1013.
44. Bai, H. MS Thesis, Virginia Tech, 1991; Chapter 3, p 59.
45. Pitts, S. M.; Markey, S. P.; Murphy, D. L.; Weisz, A. In *Recommended Practices for the Safe Handling of MPTP*; Markey, S. P., Castagnoli, N., Jr., Trevor, A. J., Kopin, I. J., Eds.; Academic: New York, 1995, p 703.
46. Koppel, I. A.; Koppel, J. B.; Muuga, L. I.; Pihl, V. O. *Organic Reactivity* **1988**, *25*, 131.
47. Shiao, M.-J.; Shih, L.-H.; Chia, W.-L.; Chau, T.-Y. *Heterocycles* **1991**, *32*, 2111.
48. Salach, J. I.; Weyler, W. In *Methods in Enzymology*; Kaufman, S., Ed.; Academic: London, 1987; Vol. 142, p 627.
49. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.