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Deuterium isotope effects for the oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues by monoamine oxidase B

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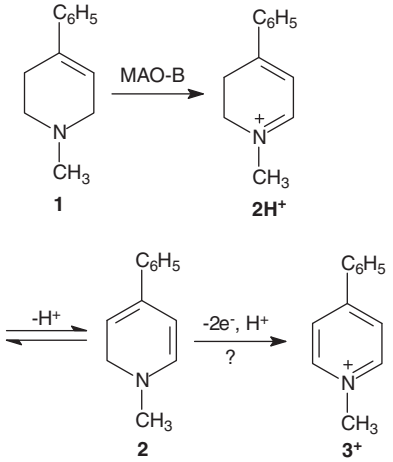
ABSTRACT

The parkinsonian inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is a cyclic tertiary allylamine exhibiting good monoamine oxidase B (MAO-B) substrate properties. MAO-B catalyzes the ring α-carbon 2-electron bioactivation of MPTP to yield the 1-methyl-4-phenyl-2,3-dihydropyridinium species ($MPDP^+$). The corresponding 5-membered ring MPTP analogue, 1-methyl-3-phenyl-3-pyrroline, also undergoes MAO-B-catalyzed oxidation to give the 2-electron oxidation product, 1-methyl-3-phenylpyrrole. Here we report the kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the steady-state oxidation of 1-methyl-3-phenyl-3-pyrroline and 1-methyl-3-(4-fluorophenyl)-3-pyrroline by baboon liver MAO-B, using the corresponding pyrroline-2,2,4,5,5-d_5 analogues as the deuterated substrates. The apparent isotope effects for the two substrates were 4.29 and 3.98 on V_{max}, while the isotope effects on V_{max}/K_m were found to be 5.71 and 3.37, respectively. The values reported for the oxidation of MPTP by bovine liver MAO-B with MPTP-6,6-d_2, as deuterated substrate, are $^D(V_{max}) = 3.55$; $^D(V_{max}/K_m) = 8.01$. We conclude that the mechanism of the MAO-B-catalyzed oxidation of pyrrolinyl substrates is similar to that of the tetrahydropyridinyl substrates and that a carbon–hydrogen bond cleavage step is, at least partially, rate determining.

1. Introduction

The metabolic activation of the proneurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)], is catalyzed by the flavoenzyme, monoamine oxidase B (MAO-B) (Scheme 1),[1,2] to yield the α-carbon 2-electron oxidation product, the corresponding 1-methyl-4-phenyl-2,3-dihydropyridinium species, $MPDP^+$ ($\mathbf{2H^+}$). Following a second 2-electron oxidation, presumably via the corresponding free base **2**, the ultimate neurotoxic metabolite, 1-methyl-4-phenylpyridinium [MPP^+ ($\mathbf{3^+}$)] is generated.[1,3,4] MPP^+ is a mitochondrial toxin which selectively damages nigrostriatal neurons and induces a parkinsonian syndrome in humans[5,6] and other susceptible mammals.[7–9] MPTP is the first reported cyclic tertiary allylamine that acts as a good MAO-B substrate.[1] Several other tertiary aminyl substrates of MAO-B have since been described.[10,11] Among these, 1-methyl-3-phenyl-3-pyrroline (**4a**), the 5-membered ring analogue of MPTP, has MAO-B substrate properties comparable to those of MPTP.[12,13] The ring α-carbon oxidation of **4a** results in 1-methyl-3-phenylpyrrole (**5a**), an overall 2-electron process that most likely arises via $\mathbf{6Ha^+}$, the short-lived conjugate acid of **5a** (Scheme 2).



Scheme 1. The MAO-B-catalyzed α-carbon oxidation of MPTP (**1**) to yield the corresponding 2,3-dihydropyridinium product $MPDP^+$ ($\mathbf{2H^+}$). Further oxidation via an unknown pathway results in the pyridinium species MPP^+ ($\mathbf{3^+}$).

In the present study, we report the steady-state kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the oxidation of 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-fluoro-

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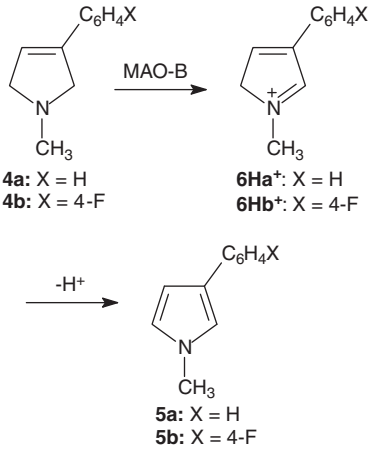


Scheme 2. The MAO-B-catalyzed α-carbon oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues (**4a** and **4b**) to yield intermediates **6Ha**$^+$ and **6Hb**$^+$. Deprotonation of **6Ha**$^+$/**6Hb**$^+$ results in the corresponding 1-methyl-3-phenylpyrrolyl species **5a** and **5b**.

phenyl)-3-pyrroline (**4b**) by baboon liver mitochondrial MAO-B. For this purpose we have employed the corresponding 1-methyl-3-phenyl-3-pyrrolinyl-2,2,4,5,5-d_5 (**4a**-d_5) and 1-methyl-3-(4-fluorophenyl)-3-pyrrolinyl-2,2,4,5,5-d_5 (**4b**-d_5) analogues as deuterated substrates (Scheme 3). The apparent isotope effects for the two substrates were compared to the isotope effects previously reported for the oxidation of MPTP by bovine liver mitochondrial MAO-B using MPTP-6,6-d_2 (**1**-d_2) as deuterated substrate.[14] The results of this study are discussed with reference to the extent to which carbon–hydrogen bond cleavage is rate limiting during MAO-B-catalyzed oxidation of pyrrolinyl substrates.

2. Results

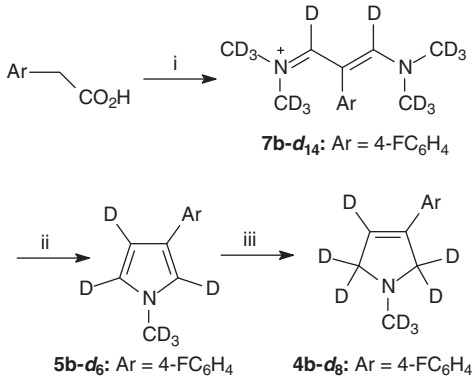
2.1. Chemistry

The 1-methyl-3-phenyl-3-pyrrolinyl analogues (**4a**–**4b**) examined here, were prepared by partial reduction of the corresponding pyrrolyl analogues **5a**–**b** as previously reported.[13,15,16] TLC and ^{1}H NMR analysis indicated that the 3-pyrrolines prepared in this manner were contaminated with the corresponding pyrrolidinyl derivatives formed by the over-reduction of the pyrrolyl substrates.[17] Also, HPLC analysis revealed trace amount of the pyrrolyl starting material present in samples of the free bases of **4a** and **4b**. In order to remove the volatile pyrrolidinyl contaminant from **4b**, the free base was subjected to high vacuum (see Section 4) while the pyrrolyl contaminant was removed by converting **4b** to the corresponding oxalate salt (**4b**·oxalate). For the purification of **4a**, both the corresponding pyrrolidinyl and pyrrolyl contaminants were removed via fractional recrystallization of the oxalate salt (**4a**·oxalate) as reported.[13]

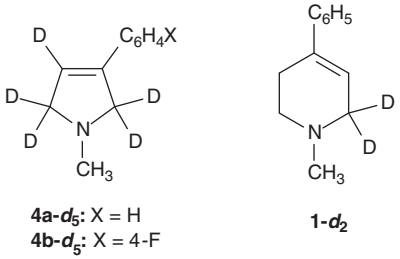
In order to replace the methylene protons of the ring α-carbons (C-2 and C-5) of the pyrrolinyl analogues with deuterons, a similar synthetic approach was initially followed (Scheme 4). Because of the relative ease of purification of the 4-fluorophenyl substituted pyrrolinyl analogue **4b** compared to the unsubstituted analogue **4a** (see above), compound **4b**-d_8 was chosen as the target 3-pyrroline. The key starting material, **7b**-d_{14} was prepared by reaction of 4-fluorophenylacetic acid with DMF-d_7 in the presence of $POCl_3$.[18] ^{1}H NMR analysis indicated that deuterium incorporation was >99% at the anticipated methyl and methinyl carbons (Scheme 4). Treatment of **7b**-d_{14} with a sodium methoxide solution (prepared with methanol-d_1) in dry pyridine yielded the pyrrolyl analogue **5b**-d_6.[19,20] While the deuterium incorporation at the N–CH_3 carbon was high (97.8%), proton–deuteron exchange occurred at the C-2, C-4, and C-5 of the pyrrolyl heterocyclic system resulting in deuteron loss to the extent of 6–27%. However, upon reduction with zinc/DCl (to yield **4b**-d_8),[13,15,21] deuterium incorporation at C-2, C-4, and C-5 of the pyrrolinyl ring were again relatively high (>96%). This result indicated that the zinc/DCl reduction procedure could be accompanied by the exchange of pyrrolinyl protons for deuterons. It was possible to take advantage of this type of reaction to prepare both **4b**-d_5 and the more difficult to purify **4a**-d_5, by simply reducing the corresponding pyrrolyl analogue with zinc/DCl. Accordingly, zinc/DCl reduction of **5a** and **5b** yielded **4a**-d_5 and **4b**-d_5, respectively, with a relatively high degree (>98%) of deuterium incorporation at C-2, C-4, and C-5 of the pyrrolinyl ring (Scheme 5). No deuterium incorporation was observed at N–CH_3.

2.2. General enzymology

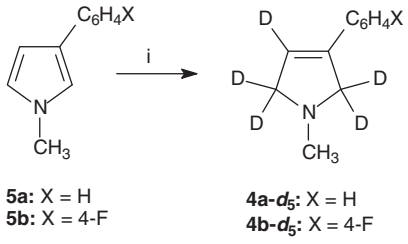
In order to measure the rates of oxidation of the pyrrolinyl analogues **4a**–**4b** and **4a**-d_5–**4b**-d_5, the substrates were incubated with



Scheme 4. Synthetic pathway to the deuterated pyrrolinyl substrate, **4b**-d_8. Reagents and conditions: (i) DMF-d_7, $POCl_3$, 80 °C; (ii) $NaOCH_3$ (from methanol-d_1), pyridine, reflux; (iii) Zn/DCl.



Scheme 3. The structures of 1-methyl-3-phenyl-3-pyrroline-2,2,4,5,5-d_5 (**4a**-d_5), 1-methyl-3-(4-fluorophenyl)-3-pyrroline-2,2,4,5,5-d_5 (**4b**-d_5), and MPTP-6,6-d_2 (**1**-d_2).



Scheme 5. Synthetic pathway to the deuterated pyrrolinyl substrates **4a**-d_5 and **4b**-d_5. Reagent: (i) Zn/DCl.

the enzyme for a fixed period of time at 37 °C and the corresponding pyrrolyl oxidation products were quantified by HPLC-UV analysis (Fig. 1) as described previously for **4a**.[13] HPLC-UV analysis was preferred to spectrophotometry since background interference in the near-UV wavelength range by the mitochondrial fractions used here as enzyme source, prevented accurate spectrophotometric measurements at 270 nm, the approximate wavelength of maximal absorption of the pyrrolyl products. The incubation time of the enzyme-catalyzed reactions were chosen to be 10 min since the oxidation of all the substrates evaluated here (**4a**–**4b** and **4a**-d_5–**4b**-d_5) was found to be linear (Fig. 2) for at least 15 min at substrate concentrations of 100 μM. Control incubations of the substrates carried out with no enzyme source added were used to verify the absence of the pyrrolyl products in the incubation samples.

To estimate the extent on non-MAO-B mediated oxidation of the pyrrolinyl substrates **4a** and **4a**-d_5, the baboon liver mitochondrial fractions that served as enzyme source were preincubated with the MAO-B selective inactivator (*R*)-deprenyl.[22] Following incubation of the test substrates (50 μM) with the mitochondria inactivated with (*R*)-deprenyl, only low concentrations of the pyrrolyl oxidation products were observed. For example, for substrate **4a** the yield of pyrrolyl oxidation product **5a** (0.54 ± 0.052 μM) with the inactivated mitochondrial preparation was only 8.4% of that formed with the fully active preparation (6.42 ± 0.18 μM). Similarly, when the deuterium labeled analogue **4a**-d_5 was incubated with (*R*)-deprenyl pre-inactivated mitochondria only 0.037 ± 0.001 μM (about 2.6%) of the pyrrolyl oxidation product was observed compared to the amount formed (1.42 ± 0.05 μM) with the fully active preparation. These results document that oxidation of the pyrrolinyl substrates by the baboon liver mitochondrial fraction is MAO-B dependent. The residual activity not suppressed by (*R*)-deprenyl has been observed previously in experiments where tetrahydropyridines served as the MAO-B substrates.[22] The factors contributing to this residual activity remain to be identified. At least for baboon liver mitochondria the residual activity is not related to the action of the MAO-A isoform since the baboon liver mitochondria are devoid of MAO-A activity.[22] Furthermore, 1-methyl-3-phenyl-3-pyrroline (**4a**) is reported to be an MAO-B selective substrate.[12]

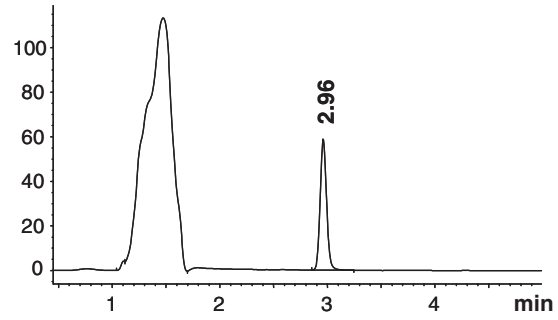


Figure 1. An HPLC-UV tracing showing the presence of 1-methyl-3-phenylpyrrole (**5a**) (retention time of 2.96 min) in an incubation of 1-methyl-3-phenyl-3-pyrroline (**4a**) with baboon liver MAO-B (0.15 mg protein/mL of the mitochondrial preparation). Following 10 min incubation, the reaction was terminated by the addition of trichloroacetic acid. After centrifugation, 50 μL of the supernatant was injected into the HPLC and the effluent was monitored at a wavelength of 270 nm.

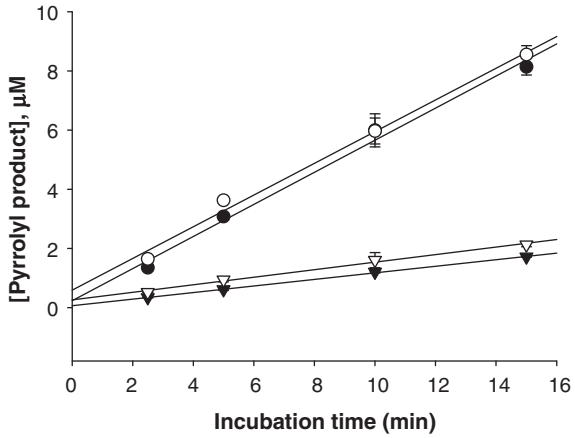


Figure 2. Linearity in the oxidation of pyrrolinyl analogues **4a** (filled circles), **4b** (open circles), **4a**-d_5 (filled triangles) and **4b**-d_5 (open triangles) by baboon liver mitochondrial MAO-B (0.15 mg protein/mL of the mitochondrial preparation). The concentration of pyrrolyl products was quantified via HPLC-UV analysis following termination of the enzyme-catalyzed reaction at time points of 2.5, 5.0, 10.0 or 15.0 min. The concentration of the substrates used in this study was 100 μM.

2.3. Steady-state kinetic deuterium isotope effects on V_{max} and V_{max}/K_m

In order to estimate the values of the steady-state kinetic parameters (K_m and V_{max}) for the oxidation of the oxalate salts of the pyrrolinyl analogues (**4a**–**4b** and **4a**-d_5–**4b**-d_5) by MAO-B, initial rates were measured at eight substrate concentrations spanning at least two orders of magnitude. As illustrated by example with Figure 3, the steady-state oxidation of the substrates (**4a**–**4b** and **4a**-d_5–**4b**-d_5) by MAO-B, followed Michaelis–Menten behavior. Also, large kinetic isotope effects on V_{max} were apparent from the graphs. The K_m and V_{max} values as well as the steady-state kinetic deuterium isotope effects obtained for the oxidation of the pyrrolinyl substrates by baboon liver mitochondrial MAO-B are summarized in Table 1. The apparent isotope effects on V_{max} for the oxidation of **4a** and **4b** were found to be 4.29 and 3.98 using **4a**-d_5 and **4b**-d_5, respectively, as the deuterated substrates. These values are comparable to the $^D(V_{max})$ value of 3.55 previously reported for the oxidation of MPTP by bovine liver mitochondrial MAO-B with MPTP-6,6-d_2 as the deuterated substrate.[14] We have observed a smaller difference between the K_m value for **4a** and its deuterated analogue **4a**-d_5 [$^D(K_m = 0.80)$] than the reported difference between the K_m values of MPTP and MPTP-6,6-d_2 [$^D(K_m = 0.45)$].[14] The K_m value of substrate **4b**, was found to be, within the range

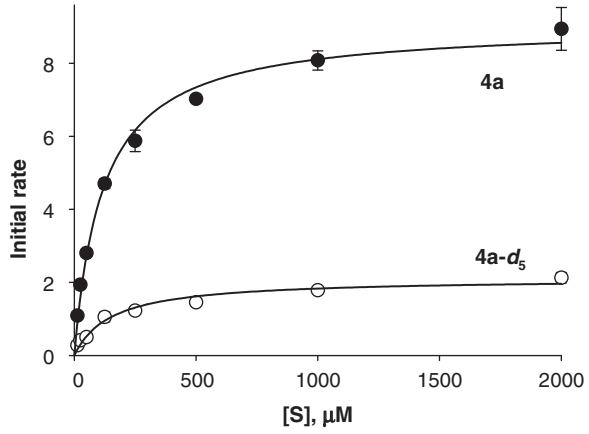


Figure 3. Determination of the K_m and V_{max} values for the oxidation of **4a** (filled circles) and **4a**-d_5 (open circles) by baboon liver MAO-B (0.15 mg protein/mL of the mitochondrial preparation). The concentrations of the pyrrolyl oxidation products were measured by HPLC analysis following a 10 min incubation at 37 °C. The rate data were fitted to the Michaelis–Menten equation. All measurements were conducted in triplicate and the concentration of the test substrates in the incubations ranged from 12.5 to 2000 μM. The initial rates are expressed as nanomoles product formed/min-mg protein.

Table 1
Steady-state kinetic constants and deuterium isotope effects for the MAO-B-catalyzed oxidation of 1-methyl-3-phenyl-3-pyrrolinyl analogues

Compound	V_{max}[a]	K_m (μM)	$^D(V_{max})$	$^D(K_m)$	$^D(V_{max}/K_m)$
4a	9.07 ± 0.41	118 ± 7.27			
4b	15.7 ± 1.73	87.2 ± 1.87			
4a-d_5	2.11 ± 0.05	157 ± 26.1	4.29 ± 0.08	0.80 ± 0.15	5.71 ± 0.88
4b-d_5	3.96 ± 0.52	73.8 ± 4.46	3.98 ± 0.09	1.19 ± 0.10	3.37 ± 0.20

[a] Values are expresses in nmol/min-mg mitochondria.

expected for experimental error, equal to that of its deuterated analogue, **4b**-d_5 [$^D(K_m = 1.19)$].

3. Discussion

In this study we have measured the kinetic deuterium isotope effects on V_{max} and V_{max}/K_m for the steady-state oxidation of 1-methyl-3-phenyl-3-pyrroline (**4a**) and 1-methyl-3-(4-fluorophenyl)-3-pyrroline (**4b**) by baboon liver mitochondrial MAO-B. For this purpose the corresponding pyrroline-2,2,4,5,5-d_5 analogues (**4a**-d_5 and **4b**-d_5) were employed as deuterated substrates. Moderately large apparent isotope effects of 4.29 and 3.98 on V_{max} were observed for the two substrates, respectively. The isotope effects on V_{max}/K_m were found to be 5.71 and 3.37 for the two substrates, respectively. Although these values may not reflect the intrinsic isotope effects, the V_{max} isotope effects suggest that a carbon–hydrogen bond cleavage step is at least partially rate determining.[23] The isotope effects on V_{max} for the two pyrrolinyl substrates are comparable to those reported for the oxidation of MPTP by bovine liver MAO-B [$^D(V_{max}) = 3.55$]. We conclude that the mechanism of the MAO-B-catalyzed oxidation of pyrrolinyl substrates is similar to that of the tetrahydropyridinyl substrates.

4. Experimental

4.1. Chemicals and instrumentation

All starting materials, unless otherwise stated, were obtained from Sigma–Aldrich and were used without purification. Deuterated solvents and reagents were obtained from Cambridge Isotope Laboratories. Proton and carbon NMR spectra were recorded on a Varian Gemini 300 spectrometer. ^{1}H NMR spectra were recorded at a frequency of 300 MHz and ^{13}C NMR spectra at 75 MHz. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the NMR solvents, $CDCl_3$, DMSO-d_6, and CD_3OD. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), sep (septet) or m (multiplet) and the coupling constants (J) are given in hertz (Hz). Fast atom bombardment mass spectra (FAB-MS) were recorded with a VG 7070E mass spectrometer. Direct insertion electron impact ionization mass spectra (EI-HRMS) were obtained with a AutoSpec ETOF (Micromass). Melting points (mp) were determined with a Stuart SMP10 melting point apparatus; all melting points are uncorrected. HPLC analyses were performed with an Agilent 1200 HPLC system equipped with an Agilent 1200 series variable wavelength detector. Thin layer chromatography (TLC) was carried out using neutral aluminum oxide 60 (Merck) with UV_{254} fluorescent indicator.

4.2. Synthesis of 2-(4-fluorophenyl)-3-(dimethylamino)-allylidene(dimethyl)ammonium perchlorate-d$_{14}$ (7b-d_{14})

Compound **7b**-d_{14} was prepared in high yield from 4-fluorophenylacetic acid, *N*,*N*-dimethylformamide-d_7 (DMF-d_7), and phosphoryl chloride according the method described in the literature.[18]

4.2.1. 2-(4-Fluorophenyl)-3-(dimethylamino)allylidene(dimethyl)-ammonium perchlorate-d_{14} (7b-d_{14})

Yield 82.5%; mp 128–133 °C (from ethanol); ^{1}H NMR (DMSO-d_6) δ 7.22–7.29 (m, 2H), 7.31–7.38 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 38.45 (m), 47.57 (sep), 103.55, 115.27 (d), 128.74, 134.11 (d), 160.47, 162.66 (t), 163.74; FAB-MS m/z: 236 (MH^+).

4.3. Synthesis of 1-methyl-3-phenylpyrroles 5a–b and 5b-d_6

1-Methyl-3-phenylpyrroles **5a**–**b** were synthesized according to a modification[20] of the method described in the literature.[19] Compound **5b**-d_6 was synthesized from **7b**-d_{14} according to the following procedure[19,20]: sodium pieces (13 mmol) was reacted with methanol-d_1 (4.5 mL) and the resulting solution was added under an atmosphere of argon to a solution of **7b**-d_{14} (6 mmol) in 24 mL dry pyridine (distilled over CaH_2 and stored over 4 Å molecular sieves). The reaction was heated under reflux for 24 h and most of the pyridine was removed via vacuum distillation to obtain a yellow pasty residue to which 25 mL D_2O was added. The resulting suspension was stirred for 30 min on an ice bath and filtered. The crude solid so obtained was dissolved in a minimum amount of ethylacetate and purified on a short column (30 × 60 mm) by neutral aluminum oxide chromatography (Fluka) with petroleum ether/ethylacetate, 90:10 as mobile phase to give a white solid. The melting points of previously reported **5a** and **5b** were as follows: **5a** mp 46–48 °C (from petroleum ether), lit. mp 46–47 °C[19]; **5b** mp 100–101 °C (from methanol), lit. mp 98–100 °C[20]; The characterization of previously unreported **5b**-d_6 is summarized below.

4.3.1. 1-Methyl-3-(4-fluorophenyl)pyrrole-d_6 (5b-d_6)

The title compound was synthesized from **7b**-d_{14} in a yield of 46.8%: mp 102–103 °C; ^{1}H NMR ($CDCl_3$) δ 6.37 (0.06H), 6.61 (0.0+1H), 6.82 (s, 0.27H), 6.97–7.04 (m, 2H), 7.39–7.46 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 35.67 (m), 106.11 (d), 115.25 (d), 118.19 (m), 122.67 (d), 124.09, 126.28 (d), 132.16 (d), 159.45, 162.68; EI-HRMS calcd. 181.11739, found 181.11819 ($M^{\cdot+}$).

4.4. Synthesis of 1-methyl-3-phenyl-3-pyrrolinyl analogues (4a–4b, 4a-d_5–4b-d_5 and 4b-d_8) and their respective oxalate salts

Compounds **4a**–**b** were synthesized from the corresponding pyrrolyl analogues, **5a**–**5b**, according to a modification[13] of the zinc reduction procedure reported in the literature.[15,16] Compounds **4a**-d_5, **4b**-d_5, and **4b**-d_8 were synthesized from **5a**, **5b,** and **5b**-d_6, respectively, according to the following procedure: DCl (15 mL, 20%) was cooled on an ice bath (0 °C) and zinc dust (20 mmol) was carefully added followed by the appropriate pyrrolyl analogue (4 mmol) dissolved in 34 mL ethanol-d_1. The resulting suspension was stirred for 1 h at 0 °C and DCl (10 mL, 35%) was added. The reaction was allowed to return to room temperature and stirred for another 1 h. Another 2 portions of zinc dust (40 mmol) were added and the reaction was stirred for 2 h during which the reaction cleared. The remaining zinc was removed via filtration and washed with 5 mL D_2O. The residual ethanol-d_1 was removed from the filtrate under reduced pressure, the pH was adjusted to ~12 with NaOH (8 N) and the reaction was extracted to diethylether (3× 30 mL). The organic phase was dried over $MgSO_4$, removed under reduced pressure to yield the product as a light yellow oil. The pyrrolinyl analogues were converted to their respective oxalic acid salts in diethyl ether. Compounds **4a** and **4a**-d_5 were purified by recrystallizing their oxalate salts twice from methanol. Compounds **4b**, **4b**-d_5 and **4b**-d_8 were purified by placing the free bases in a vacuum oven at 40 °C for 24 h. For previously described **4a**·oxalate the melting point was recorded as 151–153 °C (from methanol), lit. mp 152–154 °C.[13] The characterizations of compounds that were previously unreported are summarized below.

4.4.1. 1-Methyl-3-phenyl-3-pyrroline-d_5 (4a-d_5)

The title compound was synthesized from **5a** in a yield of 16.5% (oxalate salt): mp 152–155 °C; ^{1}H NMR (CD_3OD) δ 3.10 (s, 3H), 7.35–7.43 (m, 3H), 7.47–7.50 (m, 2H); ^{13}C NMR (CD_3OD) δ 41.68, 60.93 (m), 62.00 (m), 117.87 (m), 126.43, 129.37, 129.58, 132.21, 137.59, 166.28; EI-HRMS calcd. 164.13618, found 164.13641 ($M^{\cdot+}$).

4.4.2. 1-Methyl-3-(4-fluorophenyl)-3-pyrroline (4b)

The title compound was synthesized from **5b** in a yield of 29.5% (free base): mp 64–66 °C; ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 3H), 3.60–3.65 (m, 2H), 3.77–3.80 (m, 2H), 6.02 (m, 1H), 6.95–7.01 (m, 2H), 7.27–7.31 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 42.67, 62.38 (d), 115.30 (d), 121.93 (d), 127.00 (d), 130.67 (d), 138.97, 160.52, 163.79; EI-HRMS calcd. 177.09538, found 177.09629 ($M^{\cdot+}$).

4.4.3. 1-Methyl-3-(4-fluorophenyl)-3-pyrroline-d_5 (4b-d_5)

The title compound was synthesized from **5b** in a yield of 36.3% (free base): mp 63–66 °C; ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 3H), 6.94–7.00 (m, 2H), 7.26–7.30 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 42.66, 61.95 (m), 115.28 (d), 121.90 (m), 126.99 (d), 130.65 (d), 138.79, 160.50, 163.77; EI-HRMS calcd. 182.12676, found 182.12585 ($M^{\cdot+}$).

4.4.4. 1-Methyl-3-(4-fluorophenyl)-3-pyrroline-d_8 (4b-d_8)

The title was synthesized from **5b**-d_6 in a yield of 48.0% (free base): mp 64–68 °C; ^{1}H NMR ($CDCl_3$) δ 6.94–7.02 (m, 2H), 7.24–7.32 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 41.90 (m), 61.95 (m), 115.30 (d), 121.90 (m), 127.01 (d), 130.69 (d), 138.83, 160.52, 163.79; EI-HRMS calcd. 185.14559, found 185.14566 ($M^{\cdot+}$).

4.5. Steady-state MAO-B activity measurements

Baboon liver mitochondria were isolated as described in the literature[24] and stored at −70 °C. The mitochondrial isolates were suspended in 1 volume sodium phosphate buffer (100 mM, pH 7.4) containing 50% glycerol (w/v) and the protein concentrations were determined by the method of Bradford.[25] Since baboon liver is devoid of MAO-A activity, inactivation of MAO-A was deemed unnecessary.[22] In order to estimate the K_m and V_{max} values for the oxidation of the oxalate salts of the pyrrolinyl analogues **4a**–**4b** and **4a**-d_5–**4b**-d_5 by MAO-B, initial rates were measured at eight substrate concentrations spanning at least two orders of magnitude (12.5–2000 μM). The reactions were carried out in a final volume of 500 μL (in 100 mM sodium phosphate buffer, pH 7.4) and the enzyme concentration used was 0.15 mg mitochondrial protein/mL. Following incubation at 37 °C for a period of 10 min, the reactions were terminated by the addition of 20 μL trichloroacetic acid (1 g/mL, w/v). A volume of 100 μL acetonitrile was added, and the resulting samples were centrifuged at 16,000g for 10 min. The supernatant fractions were removed and the concentrations of the MAO-B-generated pyrrolyl products were measured by HPLC analysis with UV detection as described before.[13] The elution of the pyrrolyl oxidation products were monitored at a wavelength of 270 nm and quantitative estimations were made by means of linear calibration curves ranging from 1.5 to 25 μM of **5a** and **5b**. The steady-state kinetic data (initial rates as a function of substrate concentration) were fitted to Michaelis–Menten equation using the one site binding model incorporated into the GraphPad Prism software package (GraphPad Software Inc.). All measurements were conducted in triplicate and the K_m and V_{max} values were expressed as means ± standard error of the mean (SEM).

4.6. Linearity of oxidation of pyrrolinyl substrates

In order to determine the time interval for which MAO-B-catalyzed substrate oxidation remains linear, 100 μM of the oxalate salts of the test substrates (**4a**–**4b** and **4a**-d_5–**4b**-d_5) were incubated with 0.15 mg protein/mL of the baboon liver mitochondrial fraction. At time points 2.5, 5, 10, and 15 min, the reactions were terminated with the addition of 20 μL trichloroacetic acid (1 g/mL, w/v) and the MAO-B-generated pyrrolyl products were measured by HPLC analysis as described above. All measurements were conducted in triplicate and the concentrations of the pyrrolyl products were expressed as means ± SEM.

4.7. (*R*)-Deprenyl studies

In order to estimate the degree to which the oxidation of the pyrrolinyl substrates (**4a** and **4a**-d_5) was dependent upon MAO-B, baboon liver mitochondria (0.3 mg protein/mL) were preincubated with (*R*)-deprenyl·HCl (3×10^{-6} M) at 37 °C for 30 min in 100 mM sodium phosphate buffer, pH 7.4. The pre-inactivated mitochondrial fractions were then added to the test substrates to yield final concentrations of 0.15 mg mitochondrial protein/mL. The volume of these incubation mixtures were 500 μL and the final concentration of the oxalate salts of the test substrates was 50 μM. The reactions were incubated for 10 min at 37 °C and terminated with the addition of 20 μL trichloroacetic acid (1 g/mL, w/v). Control incubation reactions were carried out following the same procedure with the exception that the preincubations were conducted in the absence of (*R*)-deprenyl. The concentrations of the pyrrolyl products in the enzymatic reactions were measured by HPLC analysis as described above. All measurements were conducted in triplicate and the concentrations of the pyrrolyl products were expressed as means ± SEM.

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