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A novel and selective monoamine oxidase B substrate

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Abstract—Cyclic five- and six-membered tertiary allylamines constitute a unique class of monoamine oxidase substrates that undergo a net two-electron α-carbon oxidation to form the cyclic, conjugated eniminium metabolites. The corresponding saturated pyrrolidinyl and piperidinyl systems are not substrates for this flavoenzyme system. In an attempt to evaluate possible contributions that π-orbital stabilization of the putative α-carbon radical intermediates may play in the catalytic pathway, we have examined the substrate properties of 3-methyl-6-phenyl-3-aza-bicyclo[4.1.0]heptane, the 3,4-cyclopropyl analog of the selective monoamine oxidase B substrate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The results, which document the first reported example of a saturated, cyclic tertiary amine with monoamine oxidase substrate properties, are consistent with α-carbon radical stabilization as a contributing factor in the catalytic pathway.

1. Introduction

The outer mitochondrial membrane flavoenzymes monoamine oxidase A and B (MAO-A and MAO-B) catalyze the two-electron α-carbon oxidation of a variety of primary and secondary amines including the biogenic amine neurotransmitters.[1] Acyclic tertiary amine substrates are rare[2] and, with the exception of the class of compounds under consideration in this study, no cyclic tertiary amine has been unambiguously described as an MAO substrate.[3] This exceptional class of compounds is a group of cyclic tertiary allylamines that undergo MAO-catalyzed oxidation to give the corresponding cyclic, conjugated eniminium metabolites.[4] This pathway is illustrated in Scheme 1 by the MAO-B-catalyzed oxidation of the parkinsonian inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)] that yields the corresponding dihydropyridinium metabolite $\mathbf{3H}^+$.[5] The MAO catalytic pathway is thought to proceed via α-carbon radical intermediates, such as **2**$^{\cdot}$, that may be generated either directly via a hydrogen atom transfer (HAT)[6] step or via an initial single electron transfer (SET)[7] step and subsequent α-carbon deprotonation of the resulting aminyl radical cation $\mathbf{1}^{\cdot+}$. A second 1-electron transfer then generates the metabolite and the reduced flavin $FADH_2$. In the case of **1**, conversion to the homoallylic radical **4**$^{\cdot}$ does not take place to any significant extent since the isomeric dihydropyridinium species $\mathbf{5H}^+$ is not observed as a primary metabolite although it is formed by rearrangement of $\mathbf{3H}^+$.[8]

The unique MAO substrate properties of this class of compounds are dependent on the presence of the 4,5 π-bond of the heterocyclic moiety. Contrary to an earlier report,[9] the corresponding piperidinyl analog **6** of MPTP is not an MAO-B substrate.[10] Similarly, 3-pyrrolinyl derivatives,[11,12] such as **7**, are good MAO-B substrates; the corresponding pyrrolidinyl analog **8**, however, is stable in the presence of the enzyme.[12] Also noteworthy is the C(6)-regioselectivity of the α-carbon oxidation of MPTP mentioned in Scheme 1.[8] These observations suggest that stabilization of the putative α-carbon radical intermediates, such as **2**$^{\cdot}$, may contribute to the unique substrate properties of this class of compounds and have prompted efforts to examine the effects on substrate properties of molecules bearing other α-carbon radical stabilizing groups.

The cyclopropyl group shares some of the electron-donating properties of the π-bond.[13] For example, the

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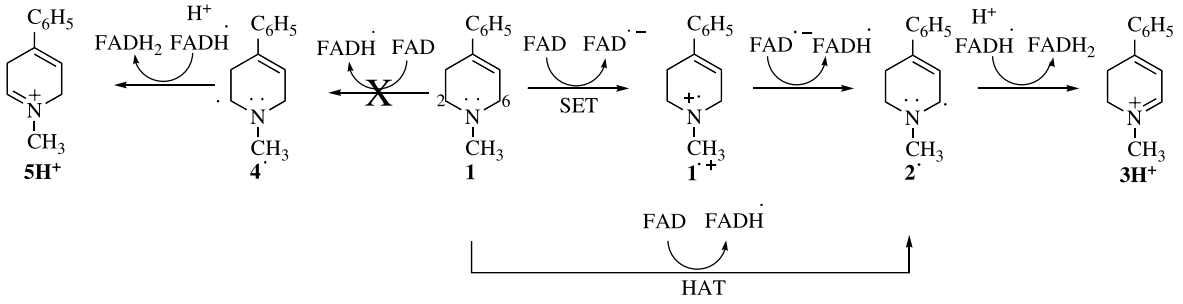
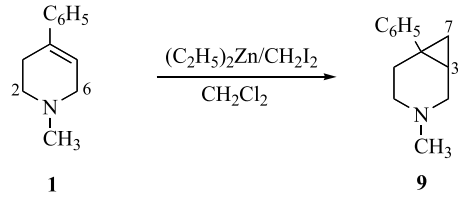


Scheme 1.

λ_{max} of the cyclopropyltropylium system is shifted to longer wavelengths [247 nm (ε 29,800) and 327 nm (ε 12,300)] compared to the isopropyl analog [λ_{max} of 225 nm (ε 32,000) and 274 nm (ε 14,400)].[14] The calculated carbon–heteroatom bond rotational energy barriers for vinylborane versus cyclopropylborane versus isopropylborane are 7.1 versus 7.2 versus 0.7 kcal/mol, respectively; the corresponding values for vinylamine versus cyclopropylamine versus isopropylamine are 4.9 versus 4.9 versus 3.1 kcal/mol.[15] Additionally, the optimized length for the vinyl–cyclopropyl C–C bond, 1.475 Å,[16] is considerably shorter than that of the vinyl–methyl C–C bond, 1.503 Å.[17] In view of these π-bond characteristics of the cyclopropyl group, studies have been initiated to examine the MAO enzyme substrate properties of 3-methyl-6-phenyl-3-aza-bicyclo[4.1.0]heptane (**9**), a structurally rigid, chiral aminomethylcyclopropyl analog of the aminomethylvinyl derivative MPTP. A literature search identified a report describing the MAO-B substrate properties of *trans*, *trans*-1-(aminomethyl)-2-methoxy-3-phenylcyclopropane (**10**), a primary amine and somewhat distant structural analog of **9**. Beef liver MAO-B catalyzed the conversion of this compound (K_m = 10.8 mM) to a pyrrolyl metabolite via a complex reaction sequence.[18]

2. Results and discussion

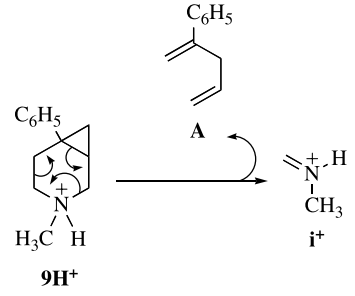
The synthesis of **9** via a direct Simmons–Smith cyclopropylation reaction (Et_2Zn, CH_2I_2) of MPTP (**1**) in CH_2Cl_2[19] proceeded in 57% yield (Scheme 2). Strictly anhydrous conditions and at least 6 equiv. of both diethylzinc and diiodomethane were necessary for the reaction to proceed effectively.[20] The proton ^{1}H NMR spectrum documented the replacement of the signal for the C(5)-olefinic proton (δ 5.86 ppm) of the starting material (**1H$^+$** · **Cl$^-$**) with three signals [δ 1.60 (m, 1H), 1.24 (m, 1H), and 1.14 (m, 1H)] corresponding to the methide proton at C(3) and the methylene protons at C(7) of the newly introduced cyclopropyl group.



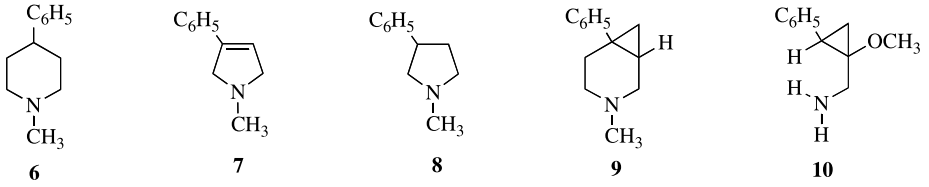
Scheme 2.

The ESI/MS product ion spectrum of **9H$^+$** (MH$^+$ 188) displayed a single fragment ion at *m/z* 44 that was assigned structure **i$^+$**. The formation of **i$^+$** is likely to proceed via a reverse [2pi + 2pi + 2pi] cycloaddition pathway with 2-phenyl-1,4-pentadiene (**A**) as the neutral loss species (Scheme 3).

The substrate properties of **9** were examined with baboon liver mitochondrial MAO-B and human placental mitochondrial MAO-A.[21] Spectrophotometric analysis of the incubation mixtures showed the time-dependent formation of a new chromophore (λ_{max} 253 nm) with the baboon liver preparations but not with the human placental preparations demonstrating that **9**, like MPTP,[22] is an MAO-B selective substrate. (*R*)-deprenyl, a potent and selective mechanism-based inactivator of MAO-B,[23] completely inhibited the reaction.



Scheme 3.



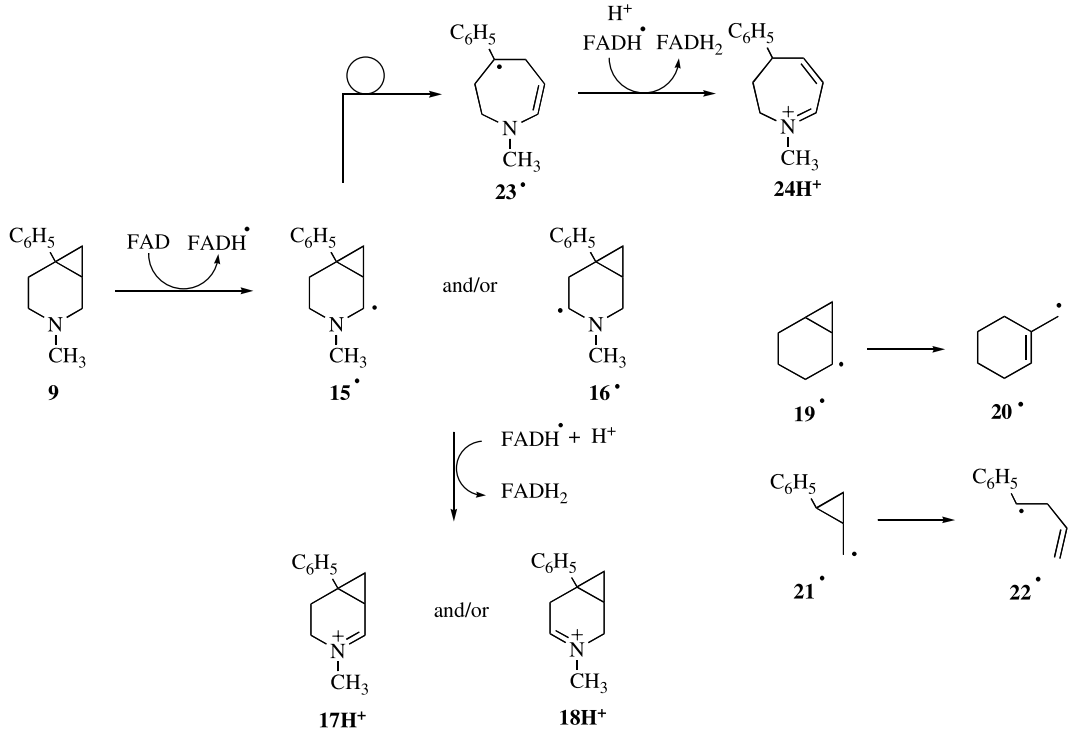
A plot of the absorbance at 253 nm versus time was linear for the first 15 min (0.048 OD units/min).

The LC-ESI/MS total ion current tracing of the post-incubation mixture showed two major peaks, one containing the starting material **9** and the second containing a compound with MH^+ 186 Da, consistent with a metabolite structure at an oxidation state two-electrons higher than that of the substrate (MH^+ 188). The two possible α-carbon radical intermediates anticipated in this biotransformation, **15**$^{\bullet}$ and **16**$^{\bullet}$, will lead to the corresponding iminium metabolites **17H**$^+$ and **18H**$^+$ following the second one-electron oxidation (Scheme 4). According to the proposed α-carbon radical stabilization by the cyclopropyl group, the cyclopropylcarbinyl radical **15**$^{\bullet}$ would be the preferred structure. Carbocyclic radicals analogous to **15**$^{\bullet}$ are reported to undergo spontaneous rearrangements to ring-opened products, a reaction that is promoted by release of ring strain. For example, the bicyclo[4.1.0]heptyl radical **19**$^{\bullet}$ is reported to rearrange to the cyclohexylmethyl radical **20**$^{\bullet}$ at a rate of 2×10^8/s[24] and the *trans*-2-phenylcyclopropylmethyl radical **21**$^{\bullet}$ to the open-chain benzylic radical **22**$^{\bullet}$ at a rate of 1.9×10^{11}/s.[25] Consequently, **15**$^{\bullet}$ also could undergo a rearrangement reaction that most likely would lead to the benzylic radical **23**$^{\bullet}$. Subsequent oxidation of this intermediate would lead to a dihydroazepinium system with the 2,3-dihydro-1*H*-azepinium species **24H**$^+$ as the thermodynamically preferred product.[26]

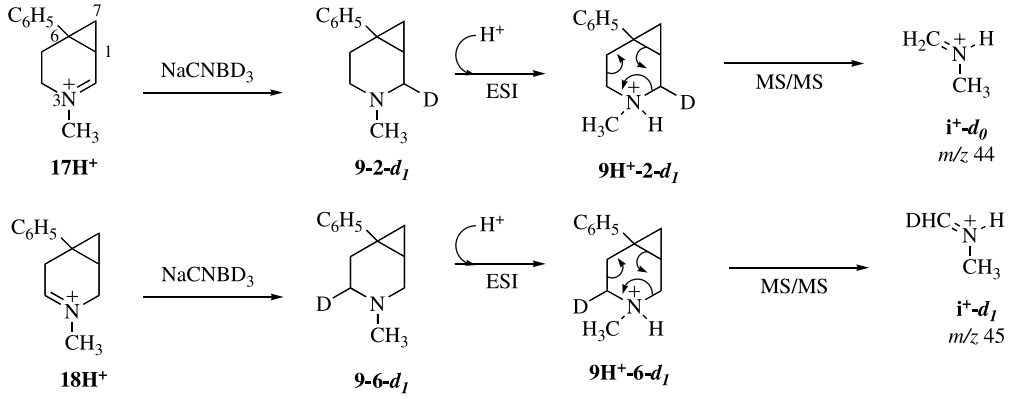
By analogy with the shift observed with the cyclopropyltropylium system,[14] the UV spectrum of 'pseudoallylic' structure **17H**$^+$ would be expected to display a bathochromic shift relative to the starting amine due to the conjugation between the iminium and cyclopropyl groups. Therefore, the observed λ_{max} 253 nm of the metabolite argues against the 'pseudohomoallylic' structure **18H**$^+$ since this compound should have a chromophore similar to that of the starting material (λ_{max} 211 nm, ε 5945). This chromophore of the metabolite, however, does not rule out the dihydroazepinium structure **24H**$^+$.

This polar metabolite proved to be quite stable and could be isolated in small quantities by preparative TLC. GC-EI/MS analysis of the purified metabolite displayed two isomeric peaks with qualitatively similar fragmentation patterns: Peak 1 (t_R 10.0 min, major product) 185 ($M^{\bullet+}$, 100%), 184 (20%), 157 (20%), 142 (80%), 141 (60%), 128 (15%), 115 (25%), and 82 (90%); Peak 2 (t_R 9.2 min, minor product) 185 ($M^{\lambda+}$ 40%), 184 (100%) 157 (10%), 142 (10%), 141 (15%), 128 (10%), 115 (15%), and 82 (5%). These compounds could be a pair of isomeric amines derived following thermal deprotonation of a cationic (iminium) metabolite. The complex fragmentation patterns did not help to differentiate amongst the proposed structures. The LC-ESI/MS total ion current tracing of this isolate showed a single peak at t_R 11 min. The product ion spectrum of the MH^+ ion also was complicated: [*m*/*z* 186 (100%), 171 (20%), 157 (40%), 155 (50%), 143 (50%), 129 (50%), 128 (50%), 105 (50%), 94 (80%), 91 (100%)] and again did not help to distinguish amongst the proposed structures.

The partially purified metabolite was treated with $NaCNBD_3$ and the crude product was analyzed by mass spectrometry. The GC-EI/MS of the product ($M^{\bullet+}$



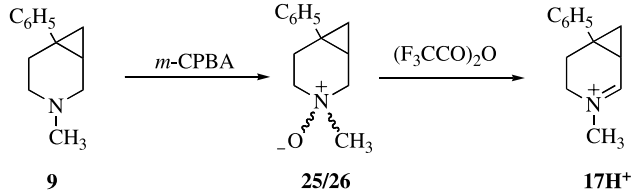
Scheme 4.



Scheme 5.

188) had the same retention time (9.0 min) and fragmentation pattern [188 ($M^{\cdot+}$, 40%), 159 (60%), 144 (65%), 130 (70%), 129 (70%), 115 (20%), 103 (30%), and 58 (100%)] as the starting amine **9** [187 ($M^{\cdot+}$, 40%), 159 (60%), 143 (65%), 130 (70%), 129 (100%), 115 (20%), 103 (30%), and 57 (100%)] except for mass shifts due to the presence of a deuterium atom. The LC-ESI/MS (MH^+ 189) also displayed a single species with the same retention time (12 min) as the starting amine. These data eliminate the dihydroazepinium **24H**$^+$ as a possible structure but do not distinguish between the two possible iminium species **17H**$^+$ and **18H**$^+$. The product ion spectrum of MH^+ (189 Da), however, led to a more definitive outcome. The spectrum showed a single fragment ion at *m/z* 44. A consideration of the reverse cycloaddition pathway proposed to account for the fragmentation observed with the starting amine **9H**$^+$ provides convincing evidence that the MAO-B-catalyzed oxidation of **9** proceeds exclusively to give the 3-methyl-6-phenyl-3-azabicyclo[4.1.0]hept-2-enyl species **17H**$^+$. Reduction of **17H**$^+$ with $NaCNBD_3$ gives **9-2-*d*$_1$**that, following collision-induced dissociation, will yield fragment ion **i**$^+$**-*d*$_0$** with a mass of 44 Da. The corresponding reduction of the isomeric 3-methyl-6-phenyl-3-azabicyclo[4.1.0]hept-3-enyl species **18H**$^+$ will yield **9-6-*d*$_1$**. The reverse cycloaddition fragment ion (**i**$^+$**-*d*$_1$**) that would be obtained in the product ion spectrum of **9-6-*d*$_1$** would have a mass of 45 Da rather than the observed mass of 44 Da (Scheme 5). Based on these data, a tentative assignment of **17H**$^+$ was made for the structure of the MAO-B generated metabolite of **9**.

The synthesis of **17H**$^+$ (Scheme 6) was achieved via a reaction sequence used in the past to prepare 2,3-dihydropyridinium derivatives such as **3H**$^+$.[27] The diastereomeric mixture of N-oxides **25/26** obtained by treatment of **9** with *m*-chloroperoxybenzoic acid (*m*-CPBA), when



Scheme 6.

subjected to Polonovskii reaction conditions (trifluoroacetic anhydride in $CHCl_3$), generated a product that was obtained in pure form as a stable perchlorate salt. The ^1H NMR spectrum of this product in DMSO-d_6 displayed a complex signal centered at δ 8.96 ppm that was assigned to the C(2) proton of **17H**$^+$. The other signals also were consistent with this structure (see Section 3). The product ion spectrum of *m/z* 186 (MH^+) was identical to that observed with the metabolite generated in the MAO-B-catalyzed oxidation of **9**. Finally, reduction of this synthetic product with $NaCNBD_3$ led to the same monodeuterated reduction product, **9-2-*d*$_1$**, that was obtained with the metabolite.

The results of these studies provide the first example of a saturated, cyclic tertiary amine with MAO substrate properties. Furthermore, the substrate properties of this bicyclic compound parallel those of the corresponding cyclic allylamine analog MPTP in terms of the MAO-B selectivity and regiospecificity. We speculate that the regiospecificity observed with these two compounds may reflect stabilization of the corresponding α-carbon radicals that are thought to be intermediates in the catalytic pathways. Kinetic studies are underway to evaluate this proposal and also to characterize the potential enantioselectivity and the C(2)-diastereoselectivity of this biotransformation.

3. Experimental

Note: 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.[28]

3.1. Chemistry

3.1.1. General methods. Unless otherwise noted, reagents and starting materials were obtained from commercial suppliers and were used without purification. Diethyl ether was distilled over sodium and benzophenone. CH_2Cl_2 and $CHCl_3$ were dried over Linde type 3 Å molecular sieves. All reactions were carried out using flame-dried glassware under an atmosphere of argon.

Proton and carbon NMR spectra were recorded on a JEOL 500 MHz spectrometer. Chemical shifts are reported in ppm downfield from internal tetramethylsilane. Spin multiplicities are given as s (singlet), bs (broad singlet), or m (multiplet). All UV–vis spectra were recorded on a Beckman DU 7400 spectrophotometer. The high-resolution FAB mass spectra were obtained on a JEOL HX-110 instrument.

3.1.2. Hydrochloride salt of 3-methyl-6-phenyl-3-azabicyclo[4.1.0]heptane ($9H^+ \cdot Cl^-$). Diiodomethane (9.3 g, 35 mmol) was added dropwise to a solution of diethylzinc (15% solution in toluene, 29 mL, 35 mmol) in CH_2Cl_2 (10 mL) at 0 °C under argon. After stirring for 0.5 h, a solution of amine 1 (1.0 g, 5.8 mmol) in CH_2Cl_2 (5.0 mL) was added dropwise at 0 °C. The reaction mixture was then stirred at room temperature for 18 h. Aqueous saturated ammonium chloride (50 mL) was added at 0 °C. The inorganic salts were filtered and washed with CH_2Cl_2(20 mL). The organic layer was washed with water (3×30 mL), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by alumina chromatography (Hexanes/EtOAc, 50:50) to give the free base **9** (0.62 g, 57%) as a colorless oil. To a solution of the free base **9** (0.5 g, 2.7 mmol) in diethyl ether (15 mL), an etheral solution of HCl (excess) was added at 0 °C. The precipitate was filtered to give an analytical pure sample of $9H^+ \cdot Cl^-$ as a white solid (0.54 g, 92%): mp 286 °C (dec); ^{1}H NMR (500 MHz, CD_3OD): δ 1.14 (m, 1H), 1.24 (m, 1H), 1.60 (m, 1H), 2.43 (m, 2H), 2.81 (s, 3H), 2.90 (m, 1H), 3.11 (m, 1H), 3.35 (m, 1H), 4.00 (m, 1H), 7.21 (m, 1H), 7.31 (m, 2H), 7.40 (m, 2H); ^{13}C NMR (125.8 MHz, CD_3OD): δ 15.0, 17.5, 22.9, 28.5, 42.0, 49.2, 53.9, 126.6, 127.6, 128.4, 145.0; ESI/MS (MH^+) 188 Da; UV (MeOH) λ_{max} 211 nm (ε 5945); FAB-HRMS: Calcd. for $C_{13}H_{18}N^+$: 188.1439. Found: 188.1441. Anal. Calcd for $C_{13}H_{18}NCl \cdot 0.24\ H_2O$ (228.08): C, 68.46; H, 8.10; N, 6.14. Found: C, 68.46; H, 8.08; N, 6.17.

3.1.3. 3-Methyl-6-phenyl-3-azabicyclo[4.1.0]hept-2-enyl perchlorate ($17H^+ \cdot HClO_4^-$). A solution of *m*-CPBA (1.0 g, 70%, 4.1 mmol) in CH_2Cl_2 (10 mL) was added dropwise to a solution of **9** (0.55 g, 2.9 mmol) in CH_2Cl_2(10 mL) at 0 °C. After 4 h at room temperature, the solvent was removed under reduced pressure to give the crude diastereomeric N-oxides **25/26**. The N-oxides were used for the next step without purification. A solution of TFAA (4.2 mL) in $CHCl_3$ (7.0 mL) was added dropwise to a solution of **25/26** (0.70 g, 3.4 mmol) in $CHCl_3$(5.0 mL) at room temperature. After 1 h, the solvent was removed under reduced pressure. The residue was purified by alumina chromatography ($CHCl_3$/MeOH, 95:5). The oil was warmed in 3 mL of 10% methanolic $HClO_4$, cooled to 0 °C and diethyl ether was added. The precipitate was filtered and recrystallized from $CHCl_3$/diethyl ether to give $17H^+ \cdot HClO_4^-$ as white crystals (0.31 g, 32%): mp 142 °C; UV (MeOH) λ_{max} 253 nm (ε 4,700), 205 (4,080); ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.00 (bs, 1H), 2.15 (m, 1H), 2.47 (bs, 3H), 3.56 (s, 3H), 3.74 (m, 2H), 7.29–7.42 (m, 5H), 8.96 (m, 1H); ^{13}C NMR (125.8 MHz, DMSO-d_6): δ 23.1, 23.3, 25.5, 35.0, 49.1, 127.9, 128.0, 129.0, 140.9, 178.4; FAB-HRMS: Calcd for $C_{13}H_{16}N^+$: 186.1283. Found: 186.1286. Anal. Calcd for $C_{13}H_{16}NClO_4 \cdot 0.14\ H_2O$ (288.45): C, 54.13; H, 5.64; N, 4.86. Found: C, 54.13; H, 5.25; N, 4.80.

3.2. Enzymology

3.2.1. General methods. Collections of human placenta were approved by the Internal Review Boards of Montgomery County Hospital and Virginia Tech and the collections of baboon liver were approved by the Animal Care Committee of Virginia Tech. Human placenta and baboon liver mitochondrial homogenates were prepared using the methodology reported earlier by Salach and were stored at −70 °C prior to use.[29] The MAO-A and MAO-B substrate properties of $9H^+ \cdot Cl^-$ were examined on a Molecular Devices Spectra Max Plus 384 microplate spectrophotometer with the Softmax Pro 4.7.1 software.

3.2.2. MAO-B and MAO-A substrate properties of $9H^+ \cdot Cl^-$. Mixtures of $9H^+ \cdot Cl^-$ (2 mM) and 0.06 mg baboon liver mitochondrial protein (MAO-B) or human placental mitochondrial protein (MAO-A) in phosphate buffer (100 mM, pH 7.4) in a final volume of 200 μL were incubated at 37 °C in the microplate spectrophotometer. Scans of these mixtures (200–600 nm) established the formation of a new chromophore (λ_{max} 253 nm) in the baboon liver preparation only. A plot of absorbance versus time established that the increase in absorbance at 253 nm was linear for 15 min.

3.2.3. Studies on the regioselectivity of the MAO-B-catalyzed oxidation of $9H^+ \cdot Cl^-$. A total of 9.6 mg of $9H^+ \cdot Cl^-$ [eight flasks each containing substrate (1.2 mg) and baboon liver mitochondria (0.3 mg protein) in phosphate buffer (10 mL, pH 7.4)] were incubated for 60 min at 37 °C with gentle agitation in a water bath. The pH of the combined incubation mixtures was adjusted to 10 with saturated aqueous K_2CO_3 and the resulting mixture was extracted with EtOAc (3×50 mL). The organic layer was dried over anhydrous $Na_2SO_4$and the solvent removed under reduced pressure. Preparative TLC on silica [EtOAc/$CHCl_3$(60:40) containing a few drops of NH_4OH] separated the starting material $9H^+$ (R_f 0.8) from the iminium metabolite $17H^+$ (R_f 0.2). The metabolite, which was recovered from the silica gel with MeOH, was shown by LC-ESI/MS to be free of starting material. A solution of this isolate in MeOH (1 mL) was treated with excess of $NaCNBD_3$ at room temperature for 15 min. An equal volume of water was added and the resulting mixture was extracted with CH_2Cl_2 (3×1 mL), the combined extracts dried over Na_2SO_4 and the solvent removed under reduced pressure. GC-EI/MS, ESI/MS, and ESI/MS/MS established the product to be the $9H^+$-**2-*d*$_1$**.

Acknowledgments

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