Isolation and Characterization of a Monoamine Oxidase Inhibitor from Tobacco Leaves

Ashraf A. Khalil,† Salome Steyn, and Neal Castagnoli, Jr.*

Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0212

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Recent positron emission tomography imaging studies have demonstrated a significant decrease in both monoamine oxidase A and B (MAO-A and MAO-B) activities in the brains of smokers. Normal levels of activity are observed in former smokers, suggesting the presence of one or more compounds in tobacco smoke that may inhibit these enzymes. In this paper, we report the results of efforts to identify compounds present in flue-cured tobacco leaves that inhibit MAO. The isolation procedure was guided by estimating the inhibitory properties of tobacco leaf extracts on the liver mitochondrial MAO-B-catalyzed oxidation of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine to the corresponding dihydropyridinium metabolite. Fractionation of extracts from flue-cured tobacco leaves led to the isolation of a competitive inhibitor of human MAO-A ($K_i = 3\ \mu M$) and MAO-B ($K_i = 6\ \mu M$), the structure of which could be assigned by classical spectroscopic analysis and confirmed by synthesis. This information may help to provide insights into some aspects of the pharmacology and toxicology of tobacco products.

Introduction

The flavoenzymes monoamine oxidase A and B (MAO-A[1] and MAO-B, respectively) catalyze the oxidative deamination of the biogenic amine neurotransmitters and a variety of xenobiotic amines (*1*). The development of drugs which lead to the selective, reversible inhibition of MAO-A is a subject of considerable interest since the resulting increase in dopaminergic activity in the central nervous system is thought to have antidepressant effects (*2*). The selective inhibition of MAO-B also has been of interest due to the reported neuroprotective properties of (*R*)-deprenyl (*3*), a well-characterized and highly selective mechanism-based inactivator of this form of the enzyme (*4*) which is used to treat Parkinson's disease (*5*). Consequently, the recent findings that the activities of both MAO-A (*6*) and MAO-B (*7*) are significantly reduced in the brains of smokers have raised the possibility that tobacco may contain a substance or substances with antidepressant and/or neuroprotective properties. Antidepressant effects could contribute to the addiction liability of tobacco, and neuroprotective effects could be linked to the reported reduced risk that smokers have for developing Parkinson's disease (*8*−*14*).

More than 2200 compounds have been identified in the tobacco plant and/or in tobacco smoke (*15*). The possibility that (*S*)-nicotine, the principal pharmacologically active substance present in tobacco, may be responsible for the lowered MAO-A and MAO-B activity in the brains of smokers has been ruled out (*16*). The suggestion that these effects may be caused by the 4-phenylpyridine and/or hydrazine present in tobacco smoke (*17*) appears unlikely since these compounds have no effects on MAO activity or on dopamine levels in rodent brains (*18*).

We have initiated a systematic study to identify compounds present in tobacco leaf extracts that inhibit MAO. These studies have been aided by a robust spectrophotometric assay in which baboon liver mitochondrial preparations[2] were used as the enzyme source and 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (**1**) was used as the substrate (Scheme 1). The MAO-generated metabolite, the 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium species **2**, absorbs maximally at 420 nm (*19*), which is distant from commonly encountered biological background absorbances. Since baboon liver expresses essentially 100% MAO-B (*20*), this screening assay did not report on the MAO-A inhibiting properties of the extracts. Studies on the inhibition of human MAO-A employed the same substrate and mitochondria prepared from gastrointestinal mucosa which expresses principally the A form of the enzyme.[3] The human liver mitochondria-catalyzed oxidation of the MAO-B selective substrate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**3**) to the corresponding 1-methyl-4-phenyl-2,3-dihydropyridinium metabolite **4** ($\lambda_{max} = 343$ nm) (*21*) was used to estimate the MAO-B inhibiting properties of the active compound isolated from tobacco.

Experimental Procedures

Caution: *1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**3**) is a known nigrostriatal neurotoxin and should be handled using disposable gloves in a properly ventilated hood.*

* To whom correspondence should be addressed.

† Current address: Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia.

[1] Abbreviations: GC/EIMS, gas chromatography/electron ionization mass spectrometry; HMBC, homonuclear magnetic bond coordination; HMQC, homonuclear magnetic quadrupole coordination; MAO, monoamine oxidase; SAR, structure−activity relationships; TIC, total ion current.

[2] The availability of large quantities of baboon liver mitochondria (see ref *20*) made this a convenient preparation for our screening studies.

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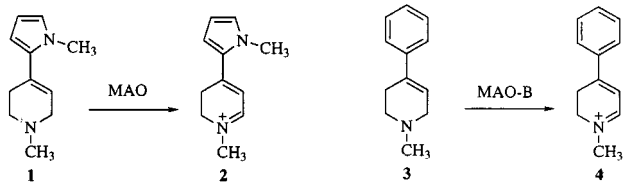
Scheme 1. MAO-Catalyzed Oxidation of the 1-Methyl-4-aryl-1,2,3,6-tetrahydropyridinyl Substrates 1 and 3



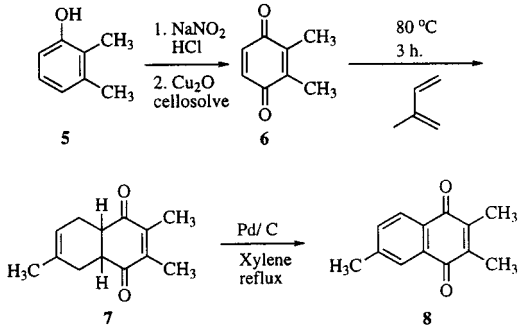
General Methods. Synthetic starting materials and analytical standards not described elsewhere were purchased from Aldrich Chemical Co. (Milwaukee, WI). Enzyme grade mono and dibasic sodium phosphate were obtained from Fisher Scientific (Pittsburgh, PA). Water was obtained from a Waters Milli-Q system (Milford, MA). The pyrrolyltetrahydropyridine **1** was synthesized as described previously (*19*); the corresponding phenyl analogue **3** was purchased from Research Biochemicals Inc. (Natick, MA). The molar extinction coefficients of 24 000 M^{-1} cm^{-1}, for the dihydropyridinium metabolite **2** (*19*), and 16 000 M^{-1} cm^{-1}, for the dihydropyridinium metabolite **4** (*21*), are those reported in the cited literature. The human liver sample was provided by F. P. Guengerich (Vanderbilt University, Nashville, TN) and the gastrointestinal mucosa by K. Thummel (University of Washington, Seattle, WA). The tissues were processed to yield mitochondrial fractions according to the procedure described by Salach and Wyler (*22*). All protein concentrations were determined by the method of Bradford (*23*). NMR spectra were recorded on a Varian Unity NMR instrument at 399.951 MHz for ^1H and 100.578 MHz for ^{13}C. Standard Varian pulse sequence programs were used. Gas chromatography/electron ionization mass spectrometry (GC/EIMS) was performed on a Hewlett-Packard 5890 gas chromatograph fitted with an HP-1 capillary column (15 m × 0.2 mm i.d., 0.33 mm film thickness) which was coupled to a Hewlett-Packard 5870 mass selective detector. Data were acquired using an HP 5970 Chemstation. The GC-chemical ionization spectrum was obtained on a CE-Fisons 8000 gas chromatograph coupled to a VG Quattro mass spectrometer.

Bioassay-Guided Fractionation of Tobacco Leaf Extracts. Shredded, flue-cured, and burley tobacco leaves were kindly provided by Murty Pharmaceuticals (Lexington, KY). Both types of tobacco (100 g) were stirred at room temperature with hexane (2 × 1 L). The solid pack obtained after filtering off the hexane was stirred with methanol (1 × 1 L) and, again after filtration, with water (1 × 1 L). The assay used to monitor the extracts was as follows. A mixture (500 μL, final volume) composed of 0.1 M sodium phosphate buffer (pH 7.4, 350 μL), the baboon liver mitochondrial preparation (25 μL, 3.0 mg of protein/mL), 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (**1**) in 0.1 M sodium phosphate buffer (pH 7.4, 100 μL, 250 μM), and tobacco leaf extracts (25 μL containing final concentrations of tobacco-derived materials ranging from 50 to 200 μg/mL based on the stage of purification) in DMSO (final concentration of DMSO held constant at 5%) was incubated with gentle agitation in a water bath at 37 °C for 30 min. A solution of 70% aqueous $HClO_4$ (20 μL) then was added, and the resulting mixture was vortex agitated. The denatured protein was sedimented at 14 000 rpm for 5 min, and the supernatant was scanned from 600 to 250 nm on a Beckman DU-7400 spectrophotometer. The absorbance at 420 nm was used to estimate the concentration of **2**.

Subsequently, 1 kg of flue-cured tobacco was extracted with hexane (3 × 2 L), and after being concentrated to a final volume of 500 mL, the hexane extract was partitioned with 80% aqueous methanol (3 × 1 L). The aqueous methanol fraction, which contained the majority of the activity, was diluted with water (500 mL), and the active components were extracted into chloroform (4 × 500 mL). The residue obtained after removing the solvent (7.3 g) was chromatographed on silica gel (220 g) using chloroform (1 L) followed by chloroform/methanol (9:1, 1 L). The most active fraction (fraction 2, 94 mg) was rechromatographed on silica gel (10 g) using a gradient elution starting with chloroform/hexane (70:30) and ending with pure chloroform. Fractions of 15 mL were collected. The most active fraction, fraction 4 (15 mg), when subjected to preparative TLC (500 μM neutral alumina and 40% hexane in chloroform) led to the isolation of 2 mg of highly active material which, upon rechromatography on neutral alumina, yielded 0.8 mg of a pure, active compound.

[3] The characterization of the MAO activity present in human gastrointestinal mucosa will be reported separately.

Scheme 2. Synthetic Route to 2,3,6-Trimethyl-1,4-naphthoquinone (8)



2,3,6-Trimethyl-1,4-naphthoquinone (8). All reactions were monitored by GC/EIMS. A solution of 2,3-dimethyl-1,4-benzoquinone (**6**, 1.0 g, 7 mmol), prepared by oxidation of 2,3-dimethylphenol as described previously (*24*), and isoprene (0.57 g, 8.4 mmol) in ethanol (2 mL) was heated in a sealed vial at 80 °C for 3 h. The mixture was evaporated under reduced pressure to yield 2,3,6-trimethyl-4a,5,8,8a-tetrahydro-1,4-naphthoquinone (**7**, Scheme 2). The crude tetrahydronaphthoquinone (1.1 g) was heated under reflux in xylene (30 mL) containing 10% Pd/C (800 mg) for 7 h. The cooled reaction mixture was filtered, the solvent removed, and the residue purified by passage through a column of neutral Al_2O_3 (50 g) using a gradient elution (hexane to 30% $CHCl_3$ in hexane). The fractions containing the desired 2,3,6-trimethyl-1,4-naphthoquinone (**8**) were crystallized from methanol to yield 0.75 g (51%) of pure **8**: mp 97 °C [lit. mp 103 °C (*25*)]. UV, IR, NMR, and GC/EIMS properties were identical to those reported in ref *25*.

Determination of the Mode of Inhibition of MAO-B by 8. The human liver mitochondrial preparation (67 μL, 3 mg of protein/mL) was incubated in 408 μL of 0.1 M sodium phosphate buffer (pH 7.4) containing 25 μL of 1 mM **8** [final inhibitor concentration of 50 μM in DMSO (5% final concentration); final protein concentration of 3 mg/mL] for 0, 15, 30, and 60 min. Each enzyme preparation was added at the specified time to a mixture of 450 μL of 0.1 M sodium phosphate buffer (pH 7.4) and 50 μL of 100 mM **3** (final substrate concentration of 5 mM) which had been pre-equilibrated to 37 °C. The resulting mixtures were incubated for 45 min with gentle agitation in a water bath at 37 °C and workup as described above for the screening assay. The concentration of the dihydropyridinium metabolite (**4**) was estimated at $\lambda_{max} = 343$ nm by scanning the supernatant from 600 to 250 nm.

Inhibition of Human Liver Mitochondrial MAO-B and Human Gastrointestinal Mitochondrial MAO-A by 8. All kinetic studies were performed in duplicate, and the resulting values varied by less than 5%.

Due to the limited solubility of the naphthoquinone in aqueous solutions, methanol was used as a cosolvent. The final methanol concentration in all incubations was kept at 4%. It was necessary to evaluate the effects of methanol on the K_m values for the substrate molecules before starting the inhibition studies. In the case of the MAO-B selective substrate **3**, an aliquot of the human liver mitochondrial preparation was preincubated with 10^{-8} M clorgyline at 37 °C for 15 min. This

preparation (25 μL, 12 mg of protein/mL) was added to a mixture consisting of 0.1 M sodium phosphate buffer (pH 7.4, 355 μL) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**3**) in 0.1 M sodium phosphate buffer (pH 7.4, 100 μL) to yield final concentrations of 40, 80, 160, 240, 320, and 640 μM. These mixtures were incubated for 15 min with gentle agitation in a water bath at 37 °C and worked up as described for the screening assay. The absorbance at 343 nm was used to estimate the concentration of the dihydropyridinium metabolite (**4**). The K_m value ($-1/x$ when $y = 0$) for **3** was determined from the double-reciprocal plot of 1/(rate of formation of **4**) (i.e., $1/V$) versus 1/[**3**]. The inhibition studies of the effect of **8** on MAO-B activity were performed following the procedure described above except in the presence of varying final concentrations of **8** (0, 4, 8, 16, and 32 μM). The final methanol concentration was kept at 4%. The K_i value ($-x$ when $y = 0$) was estimated from the replot in which the values of the slopes obtained from the double-reciprocal plots of $1/V$ versus 1/[S] with increasing concentrations of **8** are plotted against the concentration of **8** (see Figure 6).

A parallel study was conducted with mitochondria prepared from human gastrointestinal mucosa using the pyrrolyl substrate **1**. The good MAO-B substrate properties of **1** (*19*) required that this form of the enzyme be inactivated prior to examining the effects of **8** on the MAO-A-catalyzed oxidation of **1**. The mitochondria, therefore, were preincubated with 3×10^{-7} M (*R*)-deprenyl for 15 min to inactivate MAO-B.[4] The K_m value for **1** (10, 20, 40, 60, 80, and 160 μM) in the presence of 4% methanol was determined from the same type of study described above for human liver mitochondrial MAO-B. The MAO-B-inactivated preparations were then examined as described above to estimate the K_i value for the inhibition of MAO-A by the naphthoquinone **8** using the same type of replot described above (see Figure 6).

Results and Discussion

Isolation and Structure Analysis. The fractionation procedure was based on the partitioning of the tobacco leaf components between polar and nonpolar solvents to avoid loss of activity due to exposure to acids and bases. It should be noted that activity (as measured by the rate of oxidation of the pyrrolyltetrahydropyridine substrate **1** by baboon liver mitochondria) did not localize in one fraction. Consequently, more than one compound may be present in the tobacco plant with MAO inhibiting properties. Results from the initial partitioning experiments demonstrated that the components of interest were lipophilic in nature since activity could be extracted from the plant into hexane but not into water. Alkaloid-type structures that should react with Dragendorff's reagent were not present in this hexane extract.

A series of liquid−liquid partitionings led to the isolation of 7 g of active material (200 μg/mL resulting in 23% inhibition) that subsequently was subjected to column chromatography on silica gel to yield 94 mg (200 μg/mL resulting in 71% inhibition). The total ion current (TIC) chromatogram of this fraction obtained by GC/EIMS is shown in Figure 1.

The complexity of this tracing led us to rechromatograph this material on a column of silica gel, which led to a 2-fold increase in activity. Preparative silica gel TLC of this material (15 mg) gave 11 fractions, two of which [fraction 2 (2 mg) and fraction 3 (4 mg)] exhibited similar GC-TIC reacings (Figures 2 and 3, respectively). Fraction

[4] The reader is referred to our recent publication (*20*) that describes how the conditions for selectively inhibiting MAO-B activity were established.

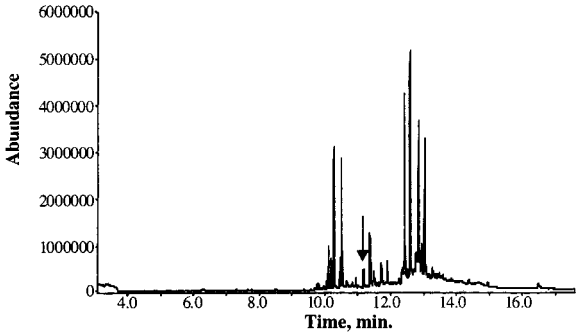


Figure 1. GC/EIMS TIC chromatogram of the active fraction.

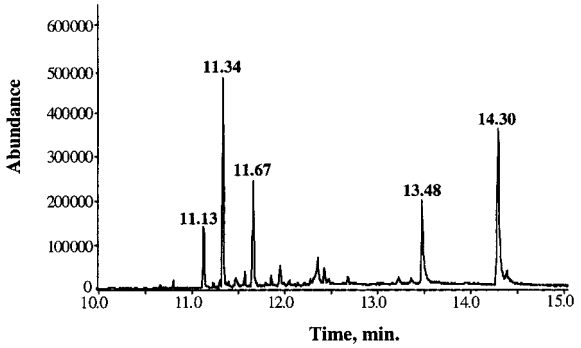


Figure 2. GC/EIMS TIC chromatogram of fraction 2.

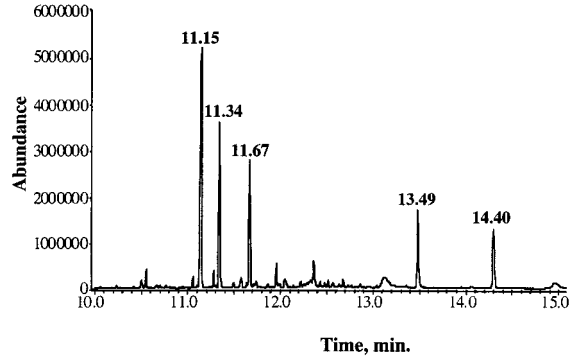


Figure 3. GC/EIMS TIC chromatogram of fraction 3.

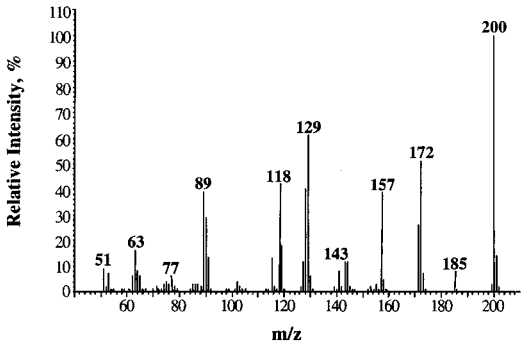


Figure 4. GC-EI mass spectrum of the peak eluting at 11.15 min.

3 inhibited MAO-B activity (50 μg/mL resulting in 87% inhibition) about 4 times more effectively than did fraction 2. The same 4:1 ratio was observed for the peak eluting at 11.14 min in the two chromatograms. The arrow in Figure 1 indicates the retention time corresponding to this peak in the more crude isolate.

The EI mass spectrum of this peak (Figure 4) indicated the presence of a single compound with a molecular mass of 200 Da. The corresponding GC-chemical ionization

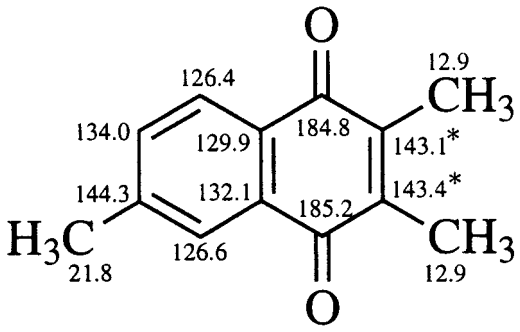


Figure 5. ^{13}C NMR chemical shift assignments (parts per million) of compound **8**. The asterisks denote interchangeable assignments.

mass spectrum (not shown) gave a parent ion (MH^+) at *m*/*z* 201, confirming the molecular mass assignment. An MS library search (Wiley library) resulted in 20 hits, one of which [2,3,6-trimethyl-1,4-naphthoquinone (**8**)] had a fragmentation pattern essentially identical to that of the isolate. A literature search established that this naphthoquinone is present in tobacco smoke (*26*, *27*). However, this would be the first report of the presence of **8** in the tobacco plant itself.

The synthesis of **8** was pursued in an effort to confirm this assignment and to provide larger quantities for biological studies. Older synthetic routes to **8** were unattractive. The first reported synthetic pathway is based on the oxidation of 2,3,6-trimethylnaphthalene that was derived from the fractional distillation of petroleum (*28*). The second route includes oxidation of the commercially available 2,6-dimethylnaphthalene followed by the introduction of the C-3 methyl group with diazomethane, a low-yield reaction (*29*). Our approach proceeded via 2,3-dimethylbenzoquinone (**6**) which was obtained by oxidation of commercially available 2,3-dimethylphenol (**5**) (*24*). Reaction of **6** with isoprene in a sealed vial gave the Diels−Alder adduct **7** which underwent dehydrogenation when heated in the presence of Pd/C to yield **8**. GC/EIMS and TLC analyses established that synthetic **8** and the product isolated from the tobacco plant were identical. Because of our interest in conducting SAR studies, the structure of **8** was fully characterized by NMR spectroscopy. The assignments of the ^{13}C NMR chemical shifts (Figure 5) were achieved with the aid of two-dimensional NMR (HMQC and HMBC).

Enzyme Studies. The MAO-B inhibiting properties of **8** observed in the baboon liver mitochondrial assay were qualitative in nature only. More quantitative studies on the interactions of synthetic **8** with both MAO-A and MAO-B were pursued with human enzymes. The effect of the MAO-B inhibitory properties of **8** on the oxidation of the tetrahydropyridine **3** was investigated first as a function of time to determine if **8** was a time-dependent inactivator of MAO-B. When the human liver mitochondrial preparation was preincubated with **8** (50 μM) for 0, 15, 30, and 60 min, the rate of MAO-B-catalyzed oxidation of **3** (5 mM) was unchanged. These studies established that the MAO-B catalytic activity of the human liver mitochondria was stable over time and that the inhibitory properties of **8** were not time-dependent.

The human gastrointestinal mitochondrial MAO-A-catalyzed oxidation of the pyrrolyl substrate **1** and the human liver mitochondrial MAO-B-catalyzed oxidation of the phenyl substrate **3** were employed to estimate the

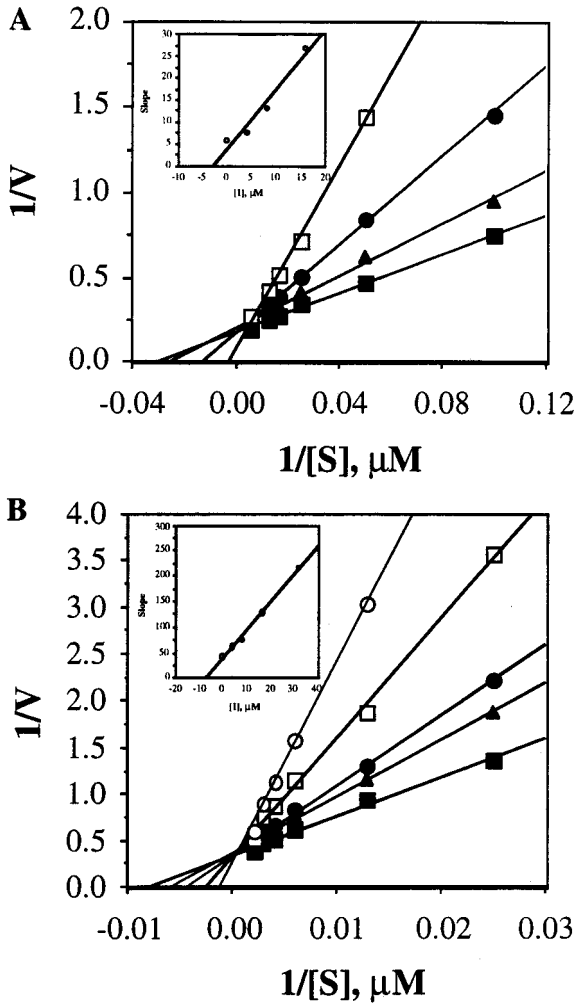


Figure 6. Double-reciprocal (1/*V* vs 1/[S]) plots of the rates of formation of the dihydropyridinium species **2** with varying concentrations of substrate **1** carried out in the presence of varying concentrations of inhibitor **8**. The insets are the replots of the slopes vs inhibitor concentrations ($K_i = -x$ when $y = 0$). Panel A depicts data for the human gastrointestinal MAO-A-catalyzed oxidation of 1-methyl-4-(1-methyl-2-pyrrolyl)-1,2,3,6-tetrahydropyridine (**1**), while panel B depicts the data for the human liver MAO-B-catalyzed oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**3**).

K_i values for the inhibition of these enzymes by **8**. The K_m value was for the oxidation of **1** by human gastrointestinal mitochondrial MAO-A in the presence of 10^{-7} M (*R*)-deprenyl was estimated to be 34 μM from the plot of the reciprocal of the velocities of this reaction versus the reciprocal of the substrate concentrations. A similar analysis of the oxidation of **3** by human liver mitochondrial MAO-B in the presence of 10^{-8} M clorgyline gave a K_m value of 128 μM.

The extent of inhibition of these reactions by various concentrations of **8** (4, 8, 16, and 32 μM) was examined at substrate concentrations that bracketed the above K_m values. Figure 6A shows the double-reciprocal plots for the oxidation of **1** by human gastrointestinal mitochondria in the presence of varying concentrations of **8**. This is a classical plot for a competitive inhibitor (*30*). The secondary plot of the slopes of the lines against the concentration of **8** (see the inset) provided an estimate for K_i of 3 μM (intercept of the *x*-axis with the *y* origin). A similar study gave a K_i value of 6 μM for the inhibition by **8** of the oxidation of **3** by human liver mitochondrial

MAO-B (Figure 6B). The straight lines in the double-reciprocal plots for MAO-A and -B (panels A and B of Figure 6, respectively) show common intersections above the *x*-axis which suggests that this inhibition is competitive rather than noncompetitive (intersection on the *x*-axis) or uncompetitive (parallel lines). On the basis of these values, the purification factor starting from the active fraction (7 g isolated from 1000 g of tobacco) can be estimated to be 500-fold.

The potential biological significance of these findings is being studied. We are particularly interested in determining the in vivo activities of **8** on the activities of brain MAO-A and MAO-B. A positive finding from these experiments may provide useful information about the effects of smoking on human behavior and a possible rationale for helping to explain the lowered risk of smokers to developing Parkinson's disease.

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