

# Isolation and characterization of a monoamine oxidase B selective inhibitor from tobacco smoke

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**Abstract**—It is well established that tobacco smokers have reduced levels of monoamine oxidase activities both in the brain and peripheral organs. Furthermore, extensive evidence suggests that smokers are less prone to develop Parkinson's disease. These facts, plus the observation that inhibition of monoamine oxidase B protects against the parkinsonian inducing effects of the nigrostriatal neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, have prompted studies to identify monoamine oxidase inhibitors in the tobacco plant and tobacco cigarette smoke. Our previous efforts on cured tobacco leaf extracts have led to the characterization of 2,3,6-trimethyl-1,4-naphthoquinone, a non-selective monoamine oxidase inhibitor, and farnesylacetone, a selective monoamine oxidase B inhibitor. We now have extended these studies to tobacco smoke constituents. Fractionation of the smoke extracts has confirmed and extended the qualitative results of an earlier report [J. Korean Soc. Tob. Sci. 1997, 19, 136] demonstrating the inhibitory activity of the terpene *trans,trans*-farnesol on rat brain MAO-B. In the present study,  $K_i$  values for the inhibition of human, baboon, monkey, dog, rat, and mouse liver MAO-B have been determined. Noteworthy is the absence of inhibitory effects on human placental MAO-A and beef liver MAO-B. A limited structure–activity relationship study of analogs of *trans,trans*-farnesol is reported. Although the health hazards associated with the use of tobacco products preclude any therapeutic opportunities linked to smoking, these results suggest the possibility of identifying novel structures of compounds that could lead to the development of neuroprotective agents.

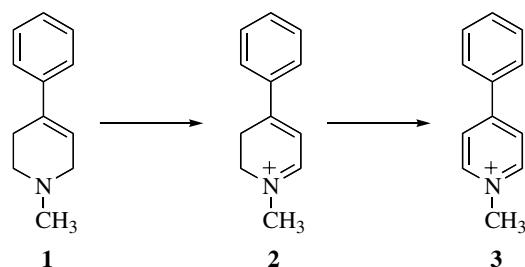
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## 1. Introduction

Interest in possible relationships between tobacco smoking, brain monoamine oxidase (MAO) activity, and the Parkinson's disease<sup>1</sup> has been prompted by a number of critical observations. First, several epidemiological studies have documented that the frequency of Parkinson's disease is lower in smokers.<sup>2</sup> A second important finding established that the neurotoxicity of the parkinsonian inducing compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP (**1**)] is dependent on its MAO-B catalyzed bioactivation to give the dihydropyridinium (**2**) and pyridinium (**3**) species (Scheme 1), the ultimate toxin.<sup>3</sup> Treatment with an MAO-B inhibitor protects susceptible animals against the neurodegenerative properties of MPTP.<sup>4</sup> The third critical observation was the finding that brain MAO-A and MAO-B activities are dramati-

ly lower in smokers.<sup>5</sup> These results, obtained from positron emission tomography (PET) images, had been anticipated by several earlier studies showing that MAO-B activity is lower in blood platelets isolated from smokers compared to non-smokers.<sup>6</sup> A recent publication provides additional dramatic PET images of the decreased MAO-B activity in peripheral organs (heart, lung, and kidney) as well as brain in smokers.<sup>7</sup>

MAO-A and B are flavoenzymes that catalyze the oxidative deamination of the biogenic amine neurotransmit-



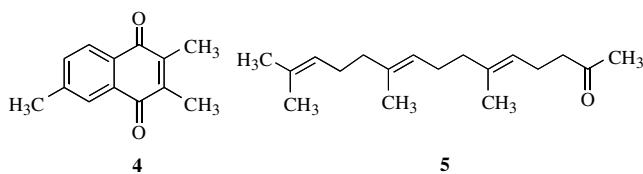
Scheme 1.

**Keywords:** *trans,trans*-Farnesol; Monoamine oxidase inhibition; Tobacco smoke isolate.

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ters and some xenobiotic amines.<sup>8</sup> Both forms of the enzyme catalyze the oxidative deamination of dopamine, the brain neurotransmitter that is depleted in Parkinson's disease.<sup>9</sup> Inhibitors of these enzymes are used therapeutically<sup>10</sup> or show clinical potential<sup>11</sup> to treat depression (MAO-A)<sup>12</sup> and to provide symptomatic relief from Parkinson's disease (MAO-B).<sup>13</sup> Human MAO-A and B are composed of 527 and 520 amino acids, respectively, and have a sequence identity of 70%.<sup>14</sup> The specific structural features responsible for the substrate and inhibitor selectivities of these enzymes are under intense investigation.<sup>15,16</sup>

Although the health hazards associated with the use of tobacco products preclude any therapeutic opportunities linked to smoking, possible relationships between smoking, MAO activity, and the incidence of Parkinson's disease have prompted studies to identify tobacco-derived compounds that inhibit MAO as possible new leads for the design of potential neuroprotective agents. These studies have led to the isolation of 2,3,6-trimethyl-1,4-naphthoquinone [TMN (**4**)] from tobacco leaf extracts.<sup>17</sup> This analog of menadione is a competitive inhibitor of both human MAO-A ( $K_i = 3 \mu\text{M}$ ) and MAO-B ( $K_i = 6 \mu\text{M}$ ). Consistent with its MAO-B inhibiting properties, treatment with TMN protects against the MPTP-induced depletion of neostriatal dopamine levels in the C57 BL/6 mouse model of neurodegeneration.<sup>18</sup> A second MAO inhibitor, farnesylacetone (**5**), also was characterized in tobacco leaf extracts.<sup>19</sup> Unlike TMN, farnesylacetone was a selective MAO-B inhibitor of baboon liver mitochondrial MAO-B; it was without activity against human placental MAO-A.

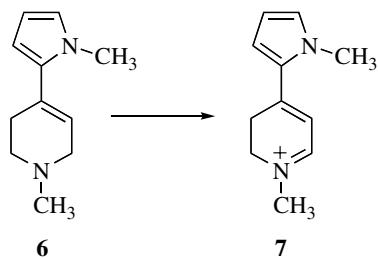


The present paper reports the results of our ongoing efforts to isolate and characterize potential neuroprotective MAO-B inhibitors in tobacco. In these studies, we have focused on extracts of cigarette tobacco smoke, a complex mixture containing over 6000 compounds.<sup>20</sup> As in our earlier work, these fractionation studies were guided by a robust spectrophotometric assay that employs the inhibition of the formation of the dihydropyridinium species **7** (Scheme 2), the metabolite resulting from the MAO catalyzed oxidation of 1-methyl-4-(1-methyl-2-pyrrolyl)-1,2,3,6-tetrahydropyridine (**6**).<sup>21</sup> Compound **6** is a mixed MAO-A/B substrate and therefore we have used baboon liver mitochondrial preparations in these assays since baboon liver expresses only MAO-B activity.<sup>21</sup>

## 2. Results and discussion

### 2.1. Fractionation and structure analysis

A series of solvent traps (pH 7 phosphate buffer, methanol, ethyl acetate, chloroform, and hexane) was



Scheme 2.

examined to determine the efficiency with which inhibitors of MAO were extracted. Hexane proved to be the most effective solvent. The residue obtained by passing the cigarette smoke through hexane was extracted with various solvents of differing polarity. The hexane extract of the residue showed the highest inhibitory activity in the MAO assay [33% inhibition at 50 µg/mL]. This solution was washed with sodium phosphate buffer (pH 5.0) to separate basic from non-basic components. The MAO inhibitory activity (37% inhibition at 50 µg/mL) was concentrated in the neutral fraction. Column chromatography of this fraction on Sephadex LH-20 yielded a more active isolate (86% inhibition at 50 µg/mL). Further concentration of the active species was achieved by chromatography on neutral alumina (three sequential columns) to yield 2 mg of an isolate with a specific inhibitory activity of 75% at 6 µg/mL.

An MS library search (Wiley library) indicated that one of the minor components present in this isolate had the same GC-EIMS characteristics as those of *trans,trans*-farnesol (**8**). Figure 1 presents the GC-EIMS spectrum of the isolate which is identical to that of an authentic, commercial sample of *trans,trans*-farnesol. Since our earlier work had indicated that farnesylacetone had MAO-B inhibitory properties, the possibility that *trans,trans*-farnesol was contributing to the observed MAO-B inhibitory properties of the active fraction was explored. The specific inhibitory activity of *trans,trans*-farnesol was estimated to be 86% at 2.2 µg/mL (10 µM) when analyzed in the baboon liver mitochondrial assay. Consequently, we conclude that at least part of the MAO-B

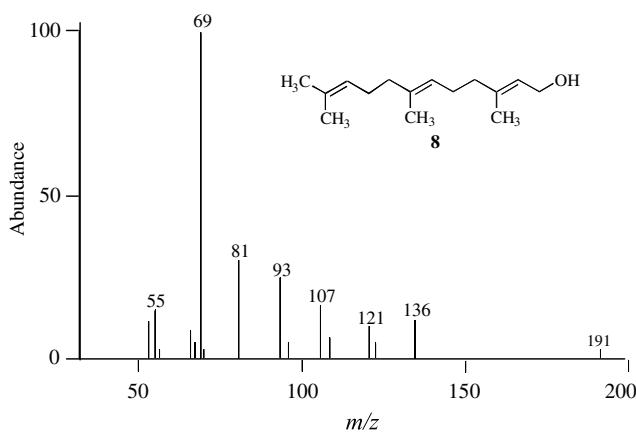


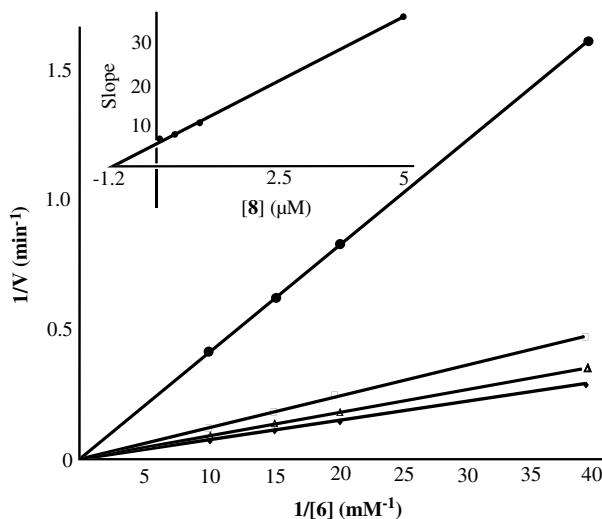
Figure 1. GC-EIMS of *trans,trans*-farnesol isolated from a tobacco smoke extract.

inhibitory properties of this highly active fraction is mediated by *trans,trans*-farnesol.

Following completion of this work, a literature search revealed that *trans,trans*-farnesol had been described previously as a component of tobacco smoke and as an inhibitor of rat brain MAO-B.<sup>22</sup> In addition to its well-known role as a precursor to plant sterols, sesquiterpenes, and mammalian cholesterol,<sup>23</sup> *trans,trans*-farnesol also has been reported to modulate neuronal voltage-gated calcium channels,<sup>24</sup> to have antibacterial activity against *Staphylococcus aureus*,<sup>25</sup> and to have anti-inflammatory activity.<sup>26</sup>

## 2.2. Enzyme studies

A more complete characterization of the MAO-B inhibitory properties of *trans,trans*-farnesol was undertaken. The possibility that this terpene was a time-dependent inhibitor of MAO-B was examined by estimating the remaining enzyme activity of a mixture of the baboon liver mitochondrial preparation when preincubated in the presence of *trans,trans*-farnesol (2  $\mu$ M, final concentration) for 0, 30, and 60 min. The rate of oxidation of the test substrate **6** (50  $\mu$ M, the  $K_m$  for this substrate) did not decrease with time and therefore we conclude that *trans,trans*-farnesol is not a time-dependent baboon liver MAO-B inhibitor.



**Figure 2.** Double-reciprocal ( $1/V$  vs  $1/[S]$ ) plots of the rates of formation of the dihydropyridinium species **7** generated by baboon liver mitochondrial MAO-B with varying concentrations of substrate **6** carried out in the presence of varying concentrations of the inhibitor *trans,trans*-farnesol (**8**). The inset is the replot of the slopes versus inhibitor concentrations.

The  $K_i$  value for the inhibition of baboon liver mitochondrial MAO-B by *trans,trans*-farnesol was determined by a classical inhibitor concentration and substrate concentration analysis of reaction rates. Figure 2 shows the double-reciprocal plots for the oxidation of **6** (25, 50, 67, and 100  $\mu$ M) by baboon liver mitochondria (0.15 mg protein/mL) in the presence of *trans,trans*-farnesol (0, 1, 2, and 5  $\mu$ M). The secondary plot of the slopes of the lines against the concentration of *trans,trans*-farnesol (see the inset) provided a  $K_i$  value of 676 nM (intercept of the  $x$ -axis with the  $y$ -origin).

The inhibitory activity of *trans,trans*-farnesol also was evaluated against human placental mitochondrial MAO-A<sup>21</sup> and human liver mitochondrial MAO-B. The mixed MAO-A/MAO-B substrate **6** also was used in these studies. Since human liver expresses both MAO-A and MAO-B,<sup>21</sup> MAO-A activity was inhibited with clorgyline prior to the MAO-B assay. The results of these assays revealed that *trans,trans*-farnesol is a potent inhibitor of human liver MAO-B activity ( $K_i = 800 \text{ nM}$ ) but is inactive against human placenta MAO-A at 10  $\mu$ M.

The inhibitory activity of *trans,trans*-farnesol also was examined with liver mitochondria prepared from beef, Cynomolgus monkey, Sprague-Dawley rat, Beagle dog, and C-57 BL/6 mouse (Table 1). Since rat liver expresses both MAO-A and MAO-B,<sup>21</sup> this preparation also was pretreated with clorgyline prior to the MAO-B assay. *trans,trans*-Farnesol proved to be a potent inhibitor of monkey liver mitochondrial MAO-B (544 nM) and a good inhibitor of dog (3.0  $\mu$ M), rat (5.0  $\mu$ M), and mouse (2.4  $\mu$ M) liver mitochondrial MAO-B. On the other hand, it showed no inhibitory activity against beef liver MAO-B at 10  $\mu$ M.

The absence of an inhibitory activity of *trans,trans*-farnesol on human MAO-A and beef liver MAO-B and the excellent inhibitory properties of *trans,trans*-farnesol against the MAO-B of the other species examined here are of particular interest. The recently reported X-ray structures of human MAO-B that had been crystallized following inactivation with pargyline<sup>16a</sup> or tranylcypromine or following inhibition with various competitive inhibitors<sup>16b</sup> show that the active site of this enzyme consists of a  $420 \text{ \AA}^3$  hydrophobic active site chamber which is connected to an entrance chamber of  $290 \text{ \AA}^3$ .<sup>16a</sup> Structural data show that the MAO-B specific inhibitor 1,4-diphenyl-2-butene occupies both the entrance and substrate chambers of human MAO-B.<sup>16b</sup> We suspect that *trans,trans*-farnesol may bind in a similar manner to human MAO B. Sequence/structural analysis of the human enzymes shows that the only active site residues that differ between the A and B forms

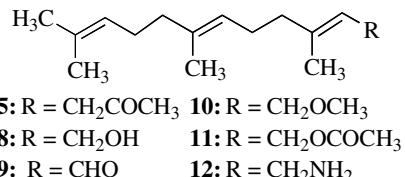
**Table 1.**  $K_i$  values (single determinations) for the *trans,trans*-farnesol mediated inhibition of the oxidation of the pyrrolyl substrate **6** by various mitochondrial MAO preparations

Enzyme source	Human liver (B)	Human placental (A)	Baboon liver (B)	Monkey liver (B)	Dog liver (B)	Rat liver (B)	Mouse liver (B)	Beef liver (B)
$K_i$ ( $\mu$ M)	0.80	No effect at 10 $\mu$ M	0.67	0.54	3.0	5.0	2.4	No effect at 10 $\mu$ M

are located between the entrance and substrate chambers. These sequence differences are conserved in the dog, rat, mouse and, possibly, also in the baboon and monkey. The sequence of beef liver MAO-B differs from those of other MAO-Bs in this region. These sequence alterations in the region between the substrate and entrance chambers may contribute to the absence of inhibitory activity of *trans,trans*-farnesol against human placental MAO-A and to beef liver MAO-B, and raise interesting possibilities with regard to how specific sequences may influence inhibitor and, possibly, substrate interactions with various forms of the enzyme. Investigations into the molecular details of that specificity are currently underway in collaboration of this laboratory with investigators at Emory University and the University of Pavia.

### 2.3. Structure–activity relationship study

Even though shown as the all trans isomers below for convenience, GC–EIMS analysis showed that commercially available farnesylacetone (**5**), farnesal (**9**), and farnesyl methyl ether (**10**) are mixtures of three (presumably double bond) isomers that are present in roughly comparable amounts. These compounds were used without further purification. *trans,trans*-Farnesol (**8**) and *trans,trans*-farnesyl acetate (**11**) were available as the pure *trans,trans*-isomers. The preliminary screening of these analogs at 10 µM final concentration revealed that the *trans,trans*-farnesol, farnesal, and farnesyl acetate analogs (86%, 58%, and 56% inhibition, respectively) are more potent inhibitors of baboon liver mitochondrial MAO-B than are farnesylacetone (28%) and farnesyl methyl ether (3%). The availability of the all trans form of farnesyl acetate allowed us to determine its  $K_i$  value that proved to be 3.0 µM, about five times less potent than *trans,trans*-farnesol. Since farnesal, farnesylacetone, and farnesyl methyl ether used in this study are mixtures of isomers, only a very rough comparison of their inhibitor properties is justified. These limited data lead us to speculate that the hydroxy group of *trans,trans*-farnesol may be an important structural feature involved in the binding with forms of MAO-B. Should this be the case, then we anticipate that *trans,trans*-farnesylamine (**12**) will either display good MAO-B selective substrate or inhibitor properties. Studies directed to a broader structure–activity analysis of terpenoid type compounds and their nitrogen containing analogs are in progress (Table 2).



**Table 2.** Inhibition of the baboon liver mitochondrial MAO-B catalyzed oxidation of **6** by *trans,trans*-farnesol and select analogs all at 10 µM

Compound	<i>trans,trans</i> -Farnesol ( <b>8</b> )	Farnesal ( <b>9</b> ) (mixture)	<i>trans,trans</i> -Farnesyl acetate ( <b>11</b> )	Farnesylacetone ( <b>5</b> ) (mixture)	Farnesyl methyl ether ( <b>10</b> ) (mixture)
% Inhibition	86	58	56	28	3

### 3. Experimental

*Caution!* 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (**1**) and some of its analogs are nigrostriatal neurotoxins and should be handled using disposable gloves in a properly ventilated hood.<sup>27</sup>

#### 3.1. General methods

*Chemicals:* *trans,trans*-Farnesol and farnesylacetone, farnesal, and farnesyl methyl ether (mixtures of isomers) were purchased from Fluka Chemical Co. (Milwaukee, WI); *trans,trans*-farnesyl acetate was purchased from Aldrich (Milwaukee, WI). Enzyme grade mono and dibasic sodium phosphate were obtained from Fisher Scientific (Pittsburgh, PA). Clorgyline was purchased from Sigma (St. Louis, MO). Water was produced from a Waters Milli-Q system (Milford, MA). The pyrrolytetrahydropyridine (**6**) was synthesized as described previously.<sup>28</sup> The liver sample was provided by Professor F. P. Guengerich (Vanderbilt, University). All tissues were processed to yield mitochondrial fractions according to the procedure described by Salach and Wyler.<sup>29</sup> Protein concentrations were determined by the method of Bradford.<sup>30</sup> Cigarette smoke was collected using a Borgwaldt smoking apparatus. GC–EIMS was performed on a Hewlett Packard 6890 gas chromatograph fitted with an Agilent capillary column (122-5562, DB-5MS 5% phenyl 95% methyl, 15 m × 0.20 mm i.d., 0.33 µm film thickness) which was coupled to a Hewlett Packard 5870 mass-selective detector. The gas flow was 1 mL/min. The inlet temperature was maintained at 275 °C. The initial column temperature was 60 °C (3 min hold), while the final temperature was 275 °C (5 min hold); the temperature ramp was 25 °C/min. The solvent delay was 4.2 min. Data were acquired using an HP 5970 Chemstation.

#### 3.2. Bioassay-guided fractionation of tobacco smoke extracts

The smoke of 390 cigarettes (13 × 30 Marlboro cigarettes) was generated. The smoke collected from each run was passed by a slight negative pressure through a glass tube that terminated with a sintered disk located at the bottom of a 30 × 7 cm radius column of hexane (500 mL). The residue (3.51 g) obtained after removing the hexane under vacuum was extracted first with 100 mL hexane followed by 100 mL EtOAc and finally 100 mL MeOH. Following removal of each solvent under reduced pressure, residues weighing 1.58, 1.10, and 0.83 g, respectively, were obtained. The three extracts were screened for their MAO inhibitory activity. The following assay was used to monitor the activity of the extract: a mixture (500 µL, final volume) composed of 0.1 M sodium phosphate buffer, pH 7.4 (355 µL), the baboon liver mitochondrial

preparation (25  $\mu$ L, 3.0 mg protein/mL), 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (**6**) in 0.1 M sodium phosphate buffer, pH 7.4 (100  $\mu$ L, 250  $\mu$ M), and tobacco smoke extracts (20  $\mu$ L containing final concentrations of tobacco-derived materials ranging from 50 to 200  $\mu$ g/mL based on the stage of purification) in DMSO (final concentration of DMSO constant at 4%) was incubated with gentle agitation in a water bath at 37 °C for 30 min. A solution of 70% aqueous HClO<sub>4</sub> (20  $\mu$ L) then was added and the resulting mixture was vortex agitated. The denatured protein was sedimented in an Eppendorf centrifuge at 14,000 rpm for 6 min and the supernatant was scanned from 600 to 250 nm on a Beckman DU-7400 spectrophotometer. Each sample was scanned against its own background, which was prepared following the same procedure except that the solution of 70% aqueous HClO<sub>4</sub> (20  $\mu$ L) was added before the addition of the enzyme for immediate protein precipitation. The absorbance at 420 nm [ $\lambda_{\text{max}}$ ,  $\epsilon$  24,000 M<sup>-1</sup> cm<sup>-1</sup>]<sup>28</sup> was used to estimate the concentration of the dihydropyridinium metabolite **7**.

### 3.3. Separation of the alkaloidal from non-alkaloidal components

The hexane extract residue (1.58 g) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (150 mL) and the resulting solution was extracted with 150 mL sodium phosphate buffer (pH 5.0). The organic layer was separated, washed with water (1 × 150 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and evaporated under reduced pressure to yield 1.35 g of the non-alkaloidal fraction. The aqueous layer was alkalinized with 10% aqueous NaOH to pH 10 and then was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 × 50 mL). The organic layer was separated, washed with water (2 × 50 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and evaporated under reduced pressure to give 0.14 g of the alkaloidal fraction. Both fractions were screened for their inhibitory activity as described above.

### 3.4. Fractionation of the non-alkaloidal components

The non-alkaloidal fraction (1.35 g) was fractionated on a pre-conditioned Sephadex LH-20 (40 g) column using a gradient starting with CH<sub>2</sub>Cl<sub>2</sub> and ending with EtOAc. A total of five fractions were collected. Fractions 1–3 were eluted using CH<sub>2</sub>Cl<sub>2</sub> (20, 50, and 30 mL, respectively), fraction 4 was eluted using 10% EtOAc in CH<sub>2</sub>Cl<sub>2</sub> (100 mL), while fraction 5 was eluted with EtOAc (100 mL). The fractions were evaporated under reduced pressure (100% recovery). The most active fraction (fraction 3, 290 mg) was rechromatographed on neutral alumina column (30 g) using a gradient elution starting with hexane:CH<sub>2</sub>Cl<sub>2</sub> (10:90) and ending with MeOH:EtOAc (3:97). Eight fractions of 50 mL each were collected. Based on GC–EIMS analyses, fractions 2, 4, and 5 were combined (90.1 mg). Fraction 3 was excluded since it contained a large amount of an inactive compound. The combined fractions were rechromatographed on neutral alumina column (10 g) using a gradient elution starting with CH<sub>2</sub>Cl<sub>2</sub>:hexane (50:50) and ending with MeOH:EtOAc (3:97). Eight fractions of 30 mL each were collected. The most active fraction, fraction 4, was rechromatographed on a neutral alumina

column (0.8 g) using a gradient elution starting with hexane:CH<sub>2</sub>Cl<sub>2</sub> (75:25) and ending with EtOAc:CH<sub>2</sub>Cl<sub>2</sub> (40:60). Five fractions of 20 mL each were collected. GC-EIMS analysis of the most active fraction indicated the presence of a peak at 10.05 min which proved to have a retention time and fragmentation pattern identical to those obtained with an authentic sample of *trans,trans*-farnesol (**8**).

### 3.5. Determination of the mode of inhibition of MAO-B by *trans,trans*-farnesol

Due to the limited solubility of *trans,trans*-farnesol in aqueous solutions, DMSO was used as a co-solvent. The final DMSO concentration in all incubations was kept at 4% and the final volume of all samples was 500  $\mu$ L. The baboon liver mitochondrial preparation (25  $\mu$ L, 3 mg protein/ $\mu$ L) was incubated in 375  $\mu$ L of 0.1 M sodium phosphate buffer (pH 7.4) containing 20  $\mu$ L of 50  $\mu$ M *trans,trans*-farnesol in DMSO (final inhibitor concentration 2  $\mu$ M; final protein concentration 0.15 mg/mL) for 0, 30, and 60 min. At the specified time, 100  $\mu$ L of 250  $\mu$ M **6** (final substrate concentration 50  $\mu$ M), which had been pre-equilibrated to 37 °C, was added. The resulting mixtures were incubated for 30 min with gentle agitation in a water bath at 37 °C, and worked up and analyzed spectrophotometrically as described above for the screening assay.

### 3.6. Inhibition of baboon liver mitochondrial MAO-B by *trans,trans*-farnesol

The experimental details for these assays are as follows: a total of 25 solutions (475  $\mu$ L each) were prepared in 1.5 mL Eppendorf tubes. The first series of four tubes contained 0  $\mu$ M *trans,trans*-farnesol (DMSO, 20  $\mu$ L was added) and quantities of substrate **6** to give the following final concentrations: 25, 50, 75, and 100  $\mu$ M. The second through fifth series were the same except that the concentrations of *trans,trans*-farnesol (series 2–5, respectively) were as follows: 0.5, 1.0, 3.0, and 6.0  $\mu$ M. The concentration of DMSO was constant at 4%. One additional series that served as a background for the spectroscopic assay was prepared. This series consisted of five Eppendorf tubes each containing 100  $\mu$ M substrate **6** and concentrations of *trans,trans*-farnesol of 0, 0.5, 1.0, 3.0, and 6.0  $\mu$ M. In the case of the background samples, 70% HClO<sub>4</sub> (20  $\mu$ L) was added and the mixture was vortex agitated. To all samples, the baboon liver mitochondrial preparation (25  $\mu$ L, 0.15 mg protein/mL, final concentration) was added and the resulting mixtures were incubated for 30 min with gentle agitation in a 37 °C water bath. With the exception of the background samples, the incubation mixtures were treated with 70% HClO<sub>4</sub> (20  $\mu$ L) and all samples were worked up as described for the screening assay. The absorbance at 420 nm was used to estimate the concentration of the dihydropyridinium metabolite (**7**). 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 *trans,trans*-farnesol are plotted against the concentration of *trans,trans*-farnesol (see Fig. 2).

### 3.7. Species-dependent inhibition of mitochondrial MAO-B by *trans,trans*-farnesol

The  $K_i$  values of *trans,trans*-farnesol against Cynomolgus monkey, Beagle dog, and C-57 BL/6 mouse liver mitochondrial MAO-B were determined following the same procedure described for the baboon liver mitochondrial MAO-B. Unlike the above-named species, human and rat liver mitochondria express both forms of MAO.<sup>21</sup> Therefore, these enzyme preparations were pretreated with clorgyline prior to adding substrate to inhibit MAO-A activity as follows: The mitochondria (from 345  $\mu$ L of a stock suspension of 6 mg of protein/ $\mu$ L) were preincubated in 6.56  $\mu$ L of a solution of  $3.32 \times 10^{-8}$  M clorgyline for 15 min at 37 °C with gentle agitation in a water bath to inhibit the MAO-A activity. Otherwise, the experimental details for these assays are the same as those described above except that the initial samples containing different concentrations of inhibitor and substrate were prepared in a volume of 250  $\mu$ L. After vortex mixing to insure homogeneity, a 250  $\mu$ L aliquot of the clorgyline pretreated mitochondrial preparation was added to each of the solutions containing substrate and inhibitor. The final protein concentration was 0.15 mg/mL. Again, in this experiment, the background samples were treated with 70% HClO<sub>4</sub> (20  $\mu$ L) prior to addition of the enzyme. These mixtures were incubated for 30 min with gentle agitation in a 37 °C water bath and worked up as before. In all experiments, 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 *trans,trans*-farnesol are plotted against the concentration of *trans,trans*-farnesol.

### 3.8. Inhibition of human placental mitochondrial MAO-A by *trans,trans*-farnesol

The experimental details for this assay are the same as described in the screening assay except that *trans,trans*-farnesol was used at 10  $\mu$ M final concentration. The final concentration of human placenta mitochondria was 0.15 mg protein/mL.

### 3.9. Structure–activity relationship (SAR) study

The inhibitory activities (10  $\mu$ M) of farnesylacetone (**5**), *trans,trans*-farnesol (**8**), farnesal (**9**), *trans,trans*-farnesyl acetate (**10**), and farnesyl methyl ether (**11**) against baboon liver mitochondrial MAO-B were compared by the assay described above. The resulting values are approximate for all compounds except for *trans,trans*-farnesol and *trans,trans*-farnesyl acetate since only these compounds were available in the all *trans* form. The  $K_i$  value of *trans,trans*-farnesyl acetate against baboon liver mitochondrial MAO-B also was determined using the procedure described above.

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