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# Inactivation of Mitochondrial Monoamine Oxidase B by Methylthio-Substituted Benzylamines

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**Abstract**—Mitochondrial monoamine oxidase was inactivated by *o*-mercaptobenzylamine (**1**) and *o*- (**2**) and *p*-methylthiobenzylamine (**5**). Experiments were carried out to provide evidence for possible mechanisms of inactivation. The corresponding *o*- (**3**) and *p*-hydroxybenzylamine (**4**) are not inactivators. Four radiolabeled analogues of **2** and **5**, having radioactivity at either the methyl or benzyl groups, were synthesized, and all were shown to incorporate multiple equivalents of radioactivity into the enzyme. Inactivation in the presence of an electrophile scavenger decreased the number of molecules incorporated, but still multiple molecules became incorporated; catalase did not further reduce the number of inactivator molecules bound. Two inactivation mechanisms are proposed, one involving a nucleophilic aromatic substitution ( $S_NAr$ ) mechanism and the other a dealkylation mechanism. Evidence for both mechanisms is that inactivation leads to reduction of the flavin (oxidation of the inactivator), but upon denaturation the flavin is reoxidized, indicating that attachment is not at the flavin. A cysteine titration indicates the loss of four cysteines after inactivation and denaturation. Support for the  $S_NAr$  mechanism was obtained by showing that *o*- and *p*-chlorobenzylamine also inactivate MAO. Chemical model studies were carried out that also support both  $S_NAr$  and dealkylation mechanisms.

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## Introduction

Mitochondrial monoamine oxidase (MAO; EC 1.4.3.4) is one of the enzymes responsible for the catabolism of various monoamine neurotransmitters, such as tryptamine, dopamine, norepinephrine, epinephrine, and serotonin, and therefore plays an important role in the regulation of the intracellular concentrations of these compounds.<sup>1</sup> These amines mediate arousal and emotion and influence temperature regulation, eating behavior, behavioral aspects of sexuality and aggression, sleep and consciousness, and pituitary regulation.<sup>2</sup> It has been found that compounds that inhibit monoamine oxidase have many important pharmacological properties, especially as antidepressant agents<sup>3</sup> or anti-parkinsonian agents.<sup>4</sup> In 1968, it was found that MAO exists in two different isozymic forms (MAO A and MAO B) in humans;<sup>5</sup> MAO A selectively oxidizes nor-

epinephrine and serotonin, and MAO B selectively oxidizes dopamine.

The primary sequences of human liver MAO A and MAO B were deduced from the corresponding cDNA clones.<sup>6</sup> The A and B forms of MAO are homodimers with subunit molecular weights of 59,700 and 58,800, respectively. The two isozymes are derived from separate genes but have about 70% sequence homology. Both isozymes have been expressed in mammalian cells and are localized to the mitochondrial outer membrane. The two subunits were shown to be identical,<sup>7</sup> and each subunit contains one flavin adenine dinucleotide (FAD) coenzyme<sup>8</sup> covalently bound via its 8 $\alpha$ -methyl group to an active-site cysteine residue.<sup>9</sup> It is known that the flavin coenzyme must be in its oxidized form for the reaction to take place, and upon oxidation of the monoamine, the flavin is converted to its reduced form.<sup>10</sup> As is true for all flavin oxidases, molecular oxygen is then required to convert the reduced flavin back into its oxidized form with production of hydrogen peroxide (Scheme 1).

Much experimental evidence supports a single-electron transfer mechanism for MAO (Scheme 2).<sup>11</sup> Because of

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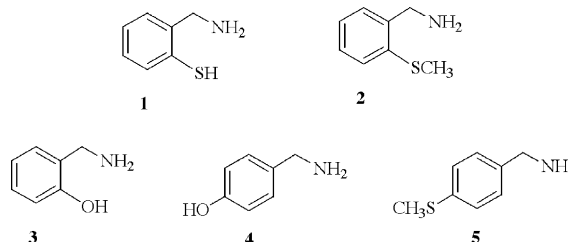
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the support for an electron-transfer mechanism and the evidence for the formation of radical intermediates, it was thought that a novel approach for the inactivation of MAO would be by an intramolecular hydrogen atom transfer mechanism as shown in Scheme 3 for *o*-mercaptobenzylamine (**1**). Initially, it was thought that attachment of the thiyl radical could occur either at the flavin or at the previously-proposed active site cysteine residue.<sup>12</sup> However, the crystal structure of MAO B has shown that there are no cysteine residues in the active site.<sup>13</sup> Compound **1** was, in fact, found to be an inactivator, but so was the corresponding *o*-methylthiobenzylamine (**2**). In this paper, we describe experiments to elucidate the mechanism for the unexpected inactivation of MAO B by **2** and related compounds.

## Results and Discussion

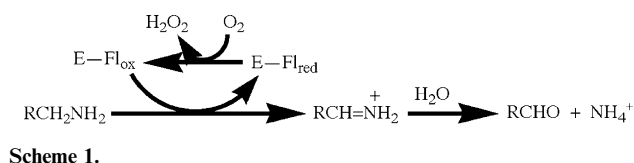
*o*-Mercaptobenzylamine (**1**) was found to be a concentration- and time-dependent inactivator of mitochondrial MAO B with inactivation kinetic constants  $K_I = 350 \mu\text{M}$  and  $k_{\text{inact}} = 0.13 \text{ min}^{-1}$ . To gain support for the proposed inactivation mechanism shown in Scheme 3, the corresponding phenol **3** was prepared, which was shown to exhibit no inactivation of MAO B. Abstraction of a hydrogen atom from a phenol is a much higher energy process than abstraction from a mercaptan. Likewise, *p*-hydroxybenzylamine (**4**) also was not an inactivator. Further support for the mechanism in Scheme 3 was sought using *o*-methylthiobenzylamine (**2**). In this case, a methyl radical would have to be transferred, which is a much less likely event. However,

to our surprise, **2** also was a concentration- and time-dependent inactivator of MAO B with  $K_I = 25 \text{ mM}$  and  $k_{\text{inact}} = 0.47 \text{ min}^{-1}$ . To test whether intramolecular methyl radical transfer was responsible for inactivation, the corresponding isomer, *p*-methylthiobenzylamine (**5**), was used, and it too was found to cause inactivation with a  $K_I = 27 \text{ mM}$  and  $k_{\text{inact}} = 0.38 \text{ min}^{-1}$ . It is apparent that the mechanism in Scheme 3 does not apply to inactivation of MAO B by the methylthiobenzylamines.

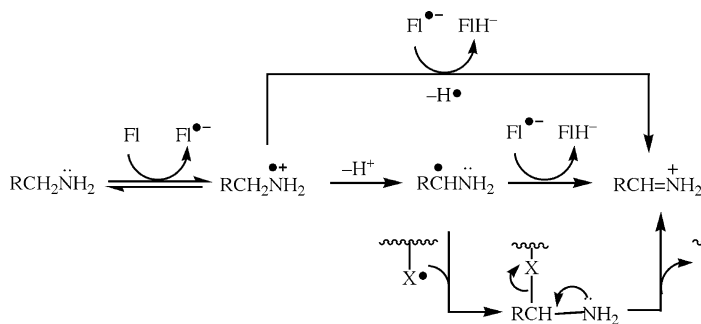


To determine the fate of the methyl and benzyl groups during inactivation by **2** and **5**, four radioactively labeled analogues were synthesized, [methyl-<sup>14</sup>C]-**2**, [methyl-<sup>14</sup>C]-**5** (Scheme 4), [benzyl methylene-<sup>3</sup>H]-**2**, and [benzyl methylene-<sup>3</sup>H]-**5** (Scheme 5). The results of the inactivation experiments with these radiolabeled inactivators are shown in Table 1. Multiple molecules of inactivator are incorporated into the enzyme, even after denaturation, suggesting that an electrophilic species is generated which indiscriminantly attaches to enzyme nucleophiles. This is reminiscent of the inactivation of MAO B by allylamine,<sup>14</sup> which is oxidized to acrolein (propenal), a highly electrophilic product that continued to react with the enzyme even after all activity was lost.

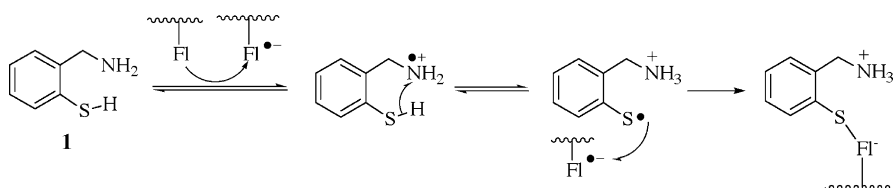
In order for the inactivator to accumulate in MAO B, an electrophilic species must be released and either attach to a nucleophile on the way out or re-enter after release. When the inactivation was carried out in the presence of a large excess of  $\beta$ -mercaptoethanol to trap released electrophiles, there was a decrease in the number



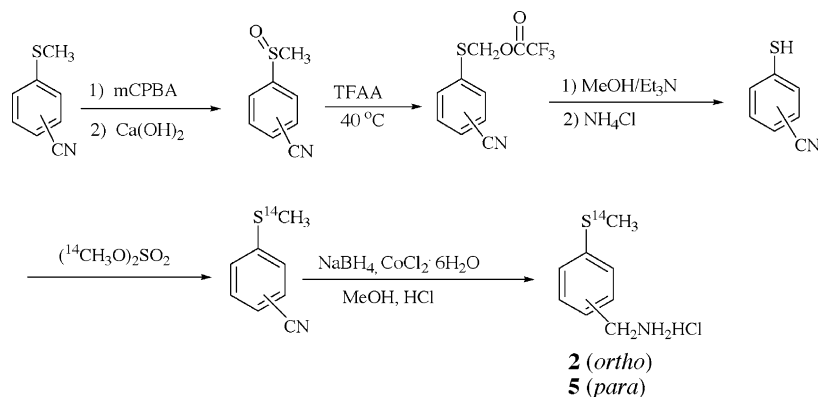
Scheme 1.



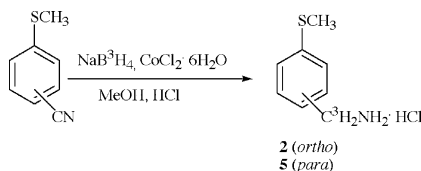
Scheme 2.



Scheme 3.



Scheme 4.



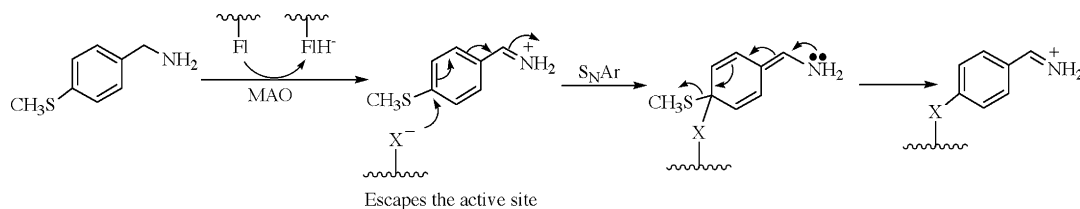
Scheme 5.

of inactivator molecules attached to the enzyme (Table 1), but still five molecules of the benzyl part of the compound remained bound to the dimeric enzyme and 14 or 15 molecules derived from the labeled methyl group remained bound to the dimer. Although there are seven free cysteine residues in each subunit (14 for the dimer), nucleophiles other than cysteine may be involved in the modification event, again similar to what was observed with acrolein as an inactivator.<sup>14</sup> There was little or no change when catalase was added to the inactivation mixture, indicating that hydrogen peroxide generated from normal turnover of amines is not responsible for the electrophilic species.

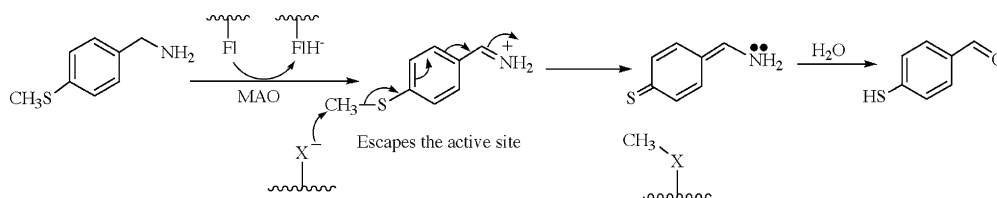
The mechanisms shown in Schemes 6 and 7 are consistent with the observation that radioactivity from both the benzyl group as well as from the methyl group, respectively, is incorporated into the enzyme (note that because these experiments provide no support for any

particular mechanism of oxidation to the iminium ion, it is not specified in these schemes). Evidence for the mechanisms in Schemes 6 and 7 was obtained by demonstrating that inactivation of MAO B with *p*-methylthiobenzylamine leads to reduction of the flavin, but denaturation of the inactivated enzyme results in reoxidation of the flavin (data not shown), despite the fact that multiple inactivator molecules are still bound (see Table 1). This indicates that the inactivator becomes oxidized during inactivation and attachment of the inactivator does not occur at the flavin. A titration of cysteine residues with DTNB indicated that after inactivation and dialysis only three cysteine residues remained (the control without inactivator contained 6.9 cysteine residues), yet all of the enzyme activity returned. This supports a random modification of enzyme nucleophiles, including cysteine residues, which have little effect on the active site.

The mechanism in Scheme 6 is nucleophilic aromatic substitution. If this is relevant, then it suggests that the methylthio group is simply acting as a leaving group, and other leaving groups could be substituted. Consequently, a series of *ortho*- and *para*-substituted halo-benzyl amines was tested for inactivation. In fact, both *ortho*- and *para*-chlorobenzylamine were time-dependent irreversible inhibitors of MAO B with  $K_I = 65 \text{ mM}$  and  $k_{\text{inact}} = 0.09 \text{ min}^{-1}$  for the *ortho*-isomer and



Scheme 6.



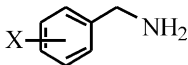
Scheme 7.

$K_I = 23$  mM and  $k_{\text{inact}} = 0.09 \text{ min}^{-1}$  for the *para*-isomer. However, when either the *ortho*- or *para*-positions were substituted with fluorine or bromine, no time-dependent inactivation occurred. It is not clear why these compounds do not inactivate the enzyme, although all of the halo-substituted benzylamines are substrates for MAO B (Table 2), and possibly the inactivation pathway for the chloro-substituted analogues is somewhat competitive with turnover, but not in the case of the other two.

Chemical model reactions (Scheme 8) were run to test for the feasibility of the reactions depicted in Schemes 6 and 7. As a model for the unstable *para*-methylthiobenzylimine, *para*-methylthiobenzaldehyde (**6**) and ethyl *para*-methylthiobenzoate (**9**) were used. As a mimic for an enzyme cysteine residue, sodium ethylthiolate was used as the nucleophile. Although these reactions are run under non-biological conditions, the enzyme has entropic and enthalpic advantages that can allow these reactions to proceed under more mild conditions. The reaction with **6** gave *para*-ethylthiobenzyl alcohol (**7**,

30% yield) and *para*-ethylthiobenzoic acid (**8**, 22% yield), indicating that a  $S_NAr$  reaction took place. The conversion of the aldehyde to an approximately equal amount of both the alcohol and carboxylic acid is reminiscent of a Cannizzaro reaction, which generally is carried out with hydroxide as the nucleophile, but has been reported also to occur with thiols.<sup>15</sup> However, substitution of the methylthiol group by ethylthiolate does not indicate whether the reaction was a direct  $S_NAr$  with ethanethiolate or if it proceeded via initial demethylation to the *para*-mercapto analogue (as in Scheme 8, pathway B) followed by  $S_NAr$  reaction of that product with ethanethiolate (with loss of  $\text{HS}^-$ ) or both. To determine if demethylation had occurred, GC–MS of the reaction products was carried out (Fig. 1). The results are summarized in Scheme 9. Direct demethylation of **9** by ethylthiolate gives ethyl methyl sulfide

**Table 2.** Kinetic constants for *ortho*- and *para*-substituted benzylamines

|  |            |                                        |            |                                          |
|-------------------------------------------------------------------------------------|------------|----------------------------------------|------------|------------------------------------------|
| X                                                                                   | $K_m$ (mM) | $k_{\text{cat}}$ ( $\text{min}^{-1}$ ) | $K_I$ (mM) | $k_{\text{inact}}$ ( $\text{min}^{-1}$ ) |
| 2-MeS                                                                               | 0.56       | 39                                     | 25         | 0.50                                     |
| 4-MeS                                                                               | 6.6        | 38                                     | 27         | 0.38                                     |
| 2-Cl                                                                                | 0.98       | 11.5                                   | 65         | 0.09                                     |
| 4-Cl                                                                                | 0.18       | 3.1                                    | 23         | 0.09                                     |
| 2-F                                                                                 | 0.88       | 19                                     | a          | a                                        |
| 4-F                                                                                 | 50         | 51                                     | a          | a                                        |
| 2-Br                                                                                | b          | b                                      | a          | a                                        |
| 4-Br                                                                                | 23.1       | 81                                     | a          | a                                        |

<sup>a</sup>Not an inactivator.

<sup>b</sup>Not a substrate.

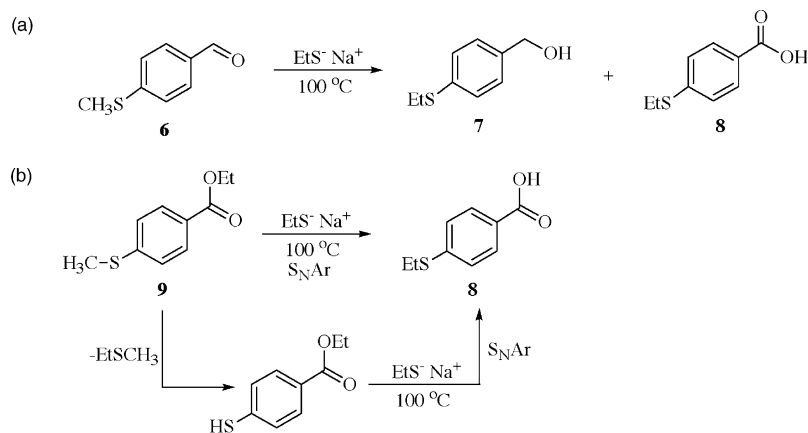
**Table 1.** Equivalents of radioactivity bound to MAO B upon inactivation by radiolabeled methylthio-substituted benzylamines

| Compd                                       | Conditions A <sup>a</sup> | Conditions B <sup>b</sup> | Conditions C <sup>c</sup> |
|---------------------------------------------|---------------------------|---------------------------|---------------------------|
| [methyl- <sup>14</sup> C]- <b>5</b>         | 27                        | 15                        | —                         |
| [methyl- <sup>14</sup> C]- <b>2</b>         | 49                        | 14                        | 13                        |
| [benzylmethylene- <sup>3</sup> H]- <b>5</b> | 8                         | 5                         | —                         |
| [benzylmethylene- <sup>3</sup> H]- <b>2</b> | 8                         | 5                         | —                         |

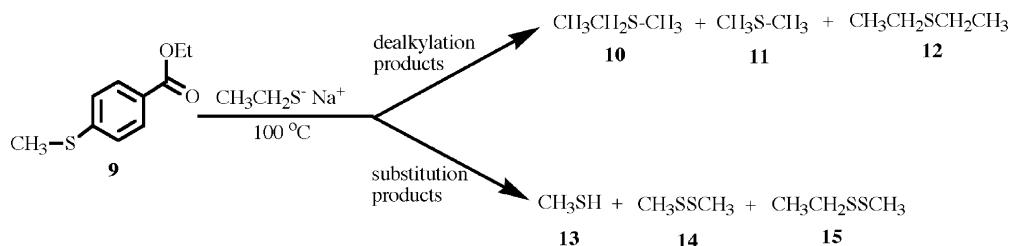
<sup>a</sup>Compound (40 mM) was incubated with MAO B (17.5  $\mu\text{M}$ ) at pH 7.3 until inactive, then dialyzed.

<sup>b</sup>Same as Conditions A, except with the addition of 0.2 mM 2-mercaptoethanol, followed by urea denaturation.

<sup>c</sup>Same as Conditions B, except with the addition of 500  $\mu\text{g}$  of catalase.



**Scheme 8.**



**Scheme 9.**

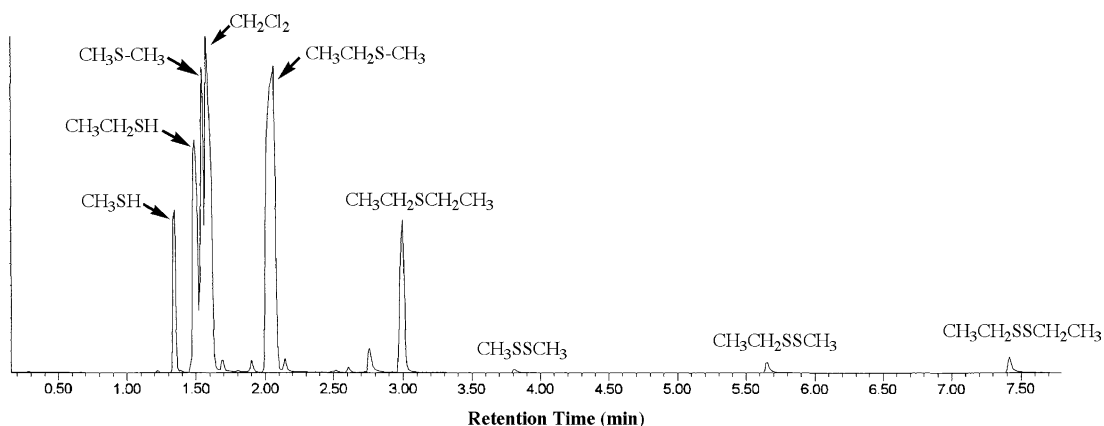
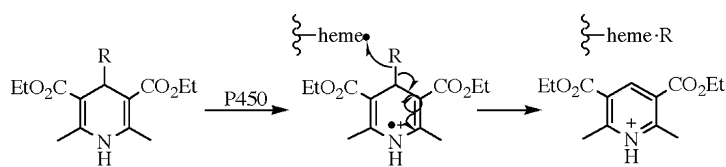
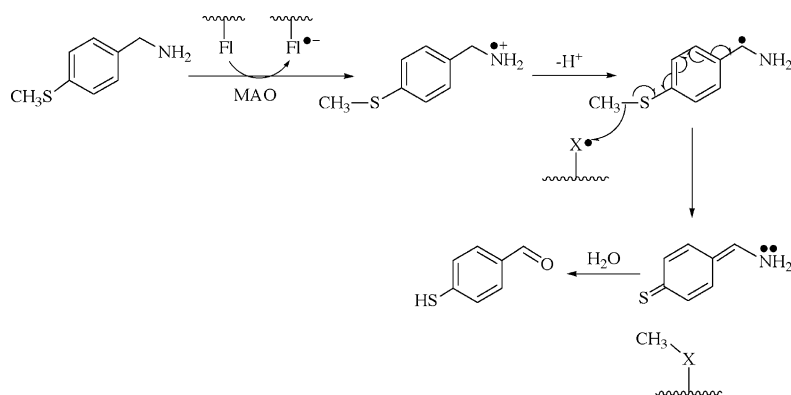


Figure 1.



Scheme 10.



Scheme 11.

(10). Dimethyl sulfide (11) is the product of initial  $S_NAr$  displacement of methylthiolate by ethylthiolate followed by methylthiolate demethylation of 9. Diethyl sulfide (12) could arise from initial  $S_NAr$  displacement of methylthiolate (13) to give ethyl 4-ethylthiobenzoate followed by ethylthiolate deethylation. The disulfide products (14 and 15) are oxidation products of methylthiolate and ethylthiolate, respectively (diethyldisulfide also was produced, but that would derive from direct oxidation of ethylthiolate).

Both the radioactive inactivator studies with MAO B and the model studies support the proposed mechanisms shown in Schemes 6 and 7 as reasonable possibilities. An alternative to Scheme 7 that should be mentioned is a one-electron mechanism, related to the proposed mechanism of inactivation of cytochrome P450 by dihydropyridines (Scheme 10).<sup>16</sup> This mechanism involves one-electron oxidation of the pyridine

nitrogen, which activates the 4-alkyl group for homolytic cleavage by the active site heme radical. The corresponding mechanism for MAO B is shown in Scheme 11. The problem with this mechanism stems from the elucidation of the active site as shown in the crystal structure,<sup>13</sup> namely, there are no cysteine residues in the active site, the most likely protein source of radicals. Also, if  $X\cdot$  in Scheme 11 is the flavin semiquinone, then alkylation of the flavin would result; however, denaturation of inactivated enzyme leads to reoxidation of the flavin. Other inactivators that we have reported to attach to the flavin produce stable flavin adducts that remain reduced even upon denaturation.<sup>17</sup>

These enzyme and chemical model results support the mechanisms shown in Schemes 6 and 7. Although the compounds are not very potent inactivators, their mechanisms of inactivation appear to be unique for monoamine oxidase.

## Experimental

### Chemicals and analytical methods

NMR spectra were recorded on either a Varian 300-MHz or Varian Unity Plus 400-MHz spectrometer. Chemical shifts are reported as  $\delta$  values in parts per million down field from Me<sub>4</sub>Si as the internal standard in CDCl<sub>3</sub>. Thin-layer chromatography was performed on EM/UV silica gel plates with a UV indicator. Mass spectra were obtained on a VG instruments VG70-250SE high-resolution spectrometer. GC–MS spectra were recorded on a HP 6890 GC system with mass selective detector. UV spectra were recorded on a Perkin-Elmer Lambda 10UV/Vis spectrometer. Radioactivity was measured on a TRI-CARB 2100 TR Liquid Scintillation Analyzer. Column chromatography was performed with Merck silica gel (230–240 mesh). [<sup>14</sup>C]-Dimethyl sulfate and [<sup>3</sup>H] sodium borohydride were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Other chemicals were purchased from Aldrich Chemical Co. Biochemicals and enzymes were purchased from Sigma Chemical Co.

**2-Methylthiobenzylamine hydrochloride (2).**<sup>18</sup> To a suspension of 2-methylthiobenzaldehyde (3.04 g, 20 mmol) in 95% ethanol (120 mL) and sodium carbonate (2.44 g, 23 mmol) in water (24 mL) was dropwise added hydroxylamine hydrochloride (2.1 g, 30 mmol) in water (8 mL) over a period of 0.5 h. After addition was complete the reaction mixture was stirred for 24 h followed by filtration to remove the inorganic salts and evaporation of ethanol. To the residue was added water to precipitate the oxime which was filtered, washed with water and dried overnight under vacuum, yielding 3 g (90%) of the oxime; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  8.17 (s, 1H), 7.52 (d, 2H), 7.26 (d, 2H), 2.44 (s, 3H).

To a solution of the oxime (3 g, 18 mmol) in 95% ethanol (150 mL) was added zinc powder (13 g, 200 mmol) with vigorous stirring. The mixture was brought to reflux and 10% hydrochloric acid (70 mL) was added over 2 h. The mixture was refluxed overnight followed by filtration of excess zinc and removal of ethanol from the filtrate. To the resulting white emulsion was added 20% sodium hydroxide (30 mL) followed by filtration of the resulting zinc hydroxide. The filtrate was saturated with sodium chloride, and the filter cake was triturated with chloroform. The chloroform triturate was used to extract the filtrate saturated with sodium chloride. The combined chloroform extracts were washed with brine. The chloroform solution was extracted with 3% hydrochloric acid followed by evaporation under the vacuum, and the residue was recrystallized from ethanol-ether to give **2** as a white solid (1.07 g, 40%); <sup>1</sup>H NMR (D<sub>2</sub>O)  $\delta$  7.10–7.28 (m, 4H); 4.10 (s, 2H); 2.34 (s, 3H). Anal. calcd for C<sub>8</sub> H<sub>12</sub> ClNS: C, 50.65; H, 6.33; N, 7.39. Found: C, 50.75; H, 6.41; N, 7.33.

**4-Methylthiobenzylamine hydrochloride (5).** 4-Methylthiobenzylamine hydrochloride (**5**) was prepared by the same procedure as 2-methylthiobenzylamine hydrochloride described above. The yield was 510 mg (19%); <sup>1</sup>H NMR (D<sub>2</sub>O)  $\delta$  7.35 (q, 4H), 4.37 (s, 2H), 2.46 (s,

3H); HRMS calcd for C<sub>8</sub>H<sub>11</sub>SN 153.0612, Found 153.0607. Anal. calcd for C<sub>8</sub> H<sub>12</sub> ClNS: C, 50.65; H, 6.33; N, 7.39. Found: C, 50.45; H, 6.25; N, 7.28.

**4-Methylthiobenzyl[methylene-<sup>3</sup>H]amine.** This compound was synthesized by a literature procedure.<sup>19</sup> 4-Methylthiobenzonitrile (149 mg, 1 mmol) and cobalt chloride hexahydrate (476 mg, 2 mmol) were dissolved in 99% methanol (4 mL), and a solution of [<sup>3</sup>H]sodium borohydride (19.5 mCi, 170 mCi/mmol) and sodium borohydride (380 mg) in 4 mL methanol was added in portions with stirring at 20 °C. Evolution of hydrogen gas was observed. When the addition was completed, stirring was continued for 1 h at 20 °C. 3 N Hydrochloric acid (2 mL) was poured into the reaction mixture and stirred until the black precipitate dissolved. After removal of methanol by evaporation, the unreacted 4-methylthiobenzonitrile was extracted with ether. The aqueous layer was made alkaline with ammonium hydroxide solution and then was extracted with ether (3 × 10 mL). The combined extracts were washed with saturated sodium chloride solution, and the organic phase was extracted with 1 N hydrochloric acid (3 × 3 mL) and evaporated. The residue was recrystallized from ethanol-ether to give the product as a white solid (114 mg, 60%); specific radioactivity: 2.8 × 10<sup>9</sup> dpm/mmol; radiopurity: 96% (by TLC).

**2-Methylthiobenzyl[methylene-<sup>3</sup>H]amine.** This compound was synthesized by the same procedure used to make 4-methylthiobenzyl[methylene-<sup>3</sup>H]amine described above, but the yield was 88 mg (50%); specific radioactivity: 2.0 × 10<sup>9</sup> dpm/mmol; radiopurity: 95%.

**4-[methyl-<sup>14</sup>C]Methylthiobenzylamine.** 4-Mercaptobenzonitrile was synthesized by a literature procedure.<sup>20</sup> 4-Methylthiobenzonitrile (745 mg, 5 mmol) in chloroform (15 mL) was oxidized with *meta*-chloroperoxybenzoic acid at 0 °C for 2 h. Then the mixture was stirred with Ca(OH)<sub>2</sub> (555 mg, 7.5 mmol) at 20 °C for 20 min followed by filtration and evaporation to give the essentially pure sulfoxide, which was heated in trifluoroacetic anhydride (10 mL) at reflux for 30 min. The volatile components were removed by evaporation, the residue was dissolved in methanol–triethylamine (1:1, 100 mL) and evaporated to dryness. The residue was dissolved in chloroform, washed with NH<sub>4</sub>Cl (saturated), dried over sodium sulfate, and evaporated to dryness. The residue was purified by silica gel chromatography to give mercaptobenzonitrile (219 mg, 32%).

To a solution of 4-mercaptobenzonitrile (203 mg, 1.5 mmol) in dry THF (5 mL) was added sodium hydride (43 mg, 1.8 mmol) and stirred for 3 h at room temperature. To this solution was added [<sup>14</sup>C] dimethyl sulfate (0.3 mCi, 0.4 mCi/mmol) in THF (2 mL) followed by dimethyl sulfate (150  $\mu$ L, 1.6 mmol). The solution was stirred overnight at room temperature, acidified with 3 N hydrochloric acid, and extracted with ether (3 × 15 mL). The combined extracts were dried over MgSO<sub>4</sub> and evaporated. The residue was reduced with sodium borohydride and cobaltous chloride hexahydrate, as was described above for 4-methylthiobenzyl[methylene-<sup>3</sup>H]amine. The

reduced product was recrystallized from ethanol–ether to give 4-[ $^{14}\text{C}$ ]-methylthiobenzylamine as a white solid (80 mg, 30%); specific radioactivity:  $6.9 \times 10^7$  dpm/mmol; radiopurity 95%.

**2-[methyl- $^{14}\text{C}$ ]Methylthiobenzylamine.** This compound was synthesized by the same method as for 4-[methyl- $^{14}\text{C}$ ]methylthiobenzylamine, but in a yield of 8% (22 mg); specific radioactivity:  $6.9 \times 10^7$  dpm/mmol; radiopurity 99%.

#### The reaction of 4-methylthiobenzaldehyde and ethyl 4-methylthiobenzoate with sodium ethanethiolate

A solution of 4-methylthiobenzaldehyde (304 mg, 2 mmol) and sodium ethanethiolate (840 mg, 10 mmol) in 1-methyl-2-pyrrolidinone (NMP) was heated at 100 °C for 14 h. The solution was acidified with 2 N HCl (10 mL) to pH 2 and extracted with ether ( $3 \times 10$  mL). The combined extracts were washed with 10% sodium carbonate solution. The ether solution was then dried and evaporated. The residue was purified by silica gel chromatography. The product was identified by  $^1\text{H}$  NMR and GC–MS as 4-ethylthiobenzyl alcohol (100 mg, 30%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.25 (m, 4H); 4.60 (s, 2H); 2.91 (q, 2H); 1.27 (t, 3H).

The combined sodium carbonate solutions were acidified with 2 N HCl to pH 2, then it was extracted with ether ( $3 \times 10$  mL). The extracts were dried and evaporated to dryness. The residue was identified as 4-ethylthiobenzoic acid (81 mg, 22%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.95 (dd, 2H); 7.28 (dd, 2H); 3.00 (q, 2H); 1.35 (t, 3H).

As a control reaction, benzaldehyde was treated with sodium ethanethiolate under the same conditions as the reaction above with 4-methylthiobenzaldehyde. The products, benzyl alcohol and benzoic acid, were identified by  $^1\text{H}$  NMR and GC–MS.

A solution of ethyl 4-methylthiobenzoate and sodium ethanethiolate in the NMP was heated under the same conditions as above. After workup, the product was identified by  $^1\text{H}$  NMR and GC–MS as 4-ethylthiobenzoic acid;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.99 (dd, 2H); 7.31 (dd, 2H); 3.03 (q, 2H); 1.38 (t, 3H).

#### Enzyme and assay

Bovine liver MAO B was isolated and assayed as previously reported.<sup>21</sup>

#### Determination of $K_m$ and $k_{\text{cat}}$

The method of Szutowicz et al.<sup>22</sup> as previously modified<sup>23</sup> was used to assay formation of hydrogen peroxide. Solutions of the benzylamine analogues were prepared (0.1, 0.2, 0.4, 0.7, 1.0, 1.5 and 2.0 mM in 0.1 M sodium phosphate, pH 7.0 buffer with 10  $\mu\text{M}$  sodium azide). An assay solution was prepared containing horseradish peroxidase (0.05 mg/mL) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 18 mM) in phosphate–citrate buffer

(0.5 M, pH 4.0). Dilute MAO B (20  $\mu\text{M}$ ) was prepared by mixing stock MAO B (110  $\mu\text{M}$ ) with the sodium phosphate buffer (pH 7.0 containing 10  $\mu\text{M}$   $\text{NaN}_3$ ). All solutions and the spectrometer were kept at 25 °C with the use of a temperature controlled water bath/recirculator and a block heater. The substrate solutions and control without substrate (90  $\mu\text{L}$ ) were incubated with diluted MAO (10  $\mu\text{L}$ ) for 1, 2, 3, 4, or 5 min (depending on activity), then a 10- $\mu\text{L}$  aliquot was mixed with the assay solution (490  $\mu\text{L}$ ) and the absorbance was measured at 414 nm versus a blank of assay solution (500  $\mu\text{L}$ ), dilute MAO (10  $\mu\text{L}$ ), and buffer (0.1 M sodium phosphate, pH 7.0 buffer with 10  $\mu\text{M}$  sodium azide). The reported extinction coefficient of 24,600  $\text{mol}^{-1} \text{cm}^{-1}$  for the ABTS dye was used to convert the absorbance measurements into concentration units. The concentration of hydrogen peroxide was calculated from the calibration curve, and the data were then plotted in a double reciprocal plot (Lineweaver–Burke) for approximate  $k_{\text{cat}}$  and  $K_m$  determination.<sup>24</sup>

#### Inactivation of MAO B by compounds 3, 4, 5 and halogenated benzylamine

A solution of the benzylamine analogues (10–50 mM) in sodium phosphate buffer (0.2 M, pH 7.2, 95  $\mu\text{L}$ ) and MAO B (130  $\mu\text{M}$ , 5  $\mu\text{L}$ ) in sodium phosphate (50 mM, pH 7.2) was incubated at room temperature. Aliquots of the incubation solution (10  $\mu\text{L}$ ) were assayed periodically with a cinnamylamine solution (1.01 mM, 490  $\mu\text{L}$ ) in Tris buffer (20 mM, pH 9.0). The rate of oxidation was determined at 290 nm, the wavelength corresponding to the formation of cinnamaldehyde. A control without inactivator also was assayed. The inactivation rate corresponding to each concentration of the inactivator was obtained, and the  $K_i$  and  $k_{\text{inact}}$  values were determined by the method of Kitz and Wilson.<sup>25</sup>

#### Incorporation of radioactivity into MAO B by [ $^3\text{H}$ ]- or [ $^{14}\text{C}$ ]-labeled methylthiobenzylamines

MAO B (112  $\mu\text{M}$ , 50  $\mu\text{L}$ ) was incubated with [ $^3\text{H}$ ]- or [ $^{14}\text{C}$ ]-labeled compounds (50 mM) in 200 mM sodium phosphate buffer (500  $\mu\text{L}$ , containing 30% DMSO), pH 7.3 at 25 °C. A control without inactivator was run simultaneously at one-fifth the scale. MAO B treated with inactivator was devoid of activity when checked after 2 days. To the inactivated enzyme solution was added 480 mg of urea, and the solution was heated at 50 °C overnight to denature the enzyme. This solution was dialyzed at 4 °C versus 8 M urea in 50 mM sodium phosphate buffer (500 mL). The dialysis buffer was changed four times over 24 h, during which time the radioactivity of the denatured enzyme was monitored. The denatured enzyme solution was assayed for radioactivity and protein concentration, from which the number of equivalents of radioactivity attached per MAO B molecule was calculated.

#### Cysteine titration of MAO B before and after inactivation with 2-methylthiobenzylamine

The procedure of Silverman and Zieske<sup>26</sup> was followed. The number of enzyme sulfhydryl groups was determined

by the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) assay of Fernandez Diez et al.<sup>27</sup> MAO B (140  $\mu$ M, 20  $\mu$ L) in sodium phosphate buffer (50 mM, pH 7.2) was added to 2-methylthiobenzylamine (33 mM, 880  $\mu$ L), and the solution was incubated for two days. The inactivated enzyme solution was dialyzed against sodium phosphate buffer (66 mM, pH 7.0, 4  $\times$  500 mL) to remove the excess inactivator. The MAO B activity returned to the same activity of the control without inactivator. To the enzyme solution was added a solution of 300  $\mu$ L of 20% NaDodSO<sub>4</sub> in water containing EDTA (1 mg/mL) and DTNB (40 mg/mL, 20  $\mu$ L). Two controls, one without MAO B and one without DTNB, were carried out as well. The absorbance at 412 nm was recorded for the three solutions from which the number of sulfhydryl groups could be determined.

#### Flavin difference spectra of 4-methylthiobenzylamine-inactivated MAO B

The procedure of Silverman and Banik was followed.<sup>28</sup> A solution of 4-methylthiobenzylamine (10 mM) in Tris buffer (0.1 M, pH 7.0, 500  $\mu$ L) and MAO B (110  $\mu$ M) in sodium phosphate buffer (50 mM, pH 7.0, 20  $\mu$ L) was incubated at room temperature for 5 h. A reaction without inactivator also was run as a control. The enzyme activity was assayed periodically. The enzyme with inactivator was found to be devoid of activity. A flavin difference spectrum was recorded, and then the enzymes were denatured by the addition of sodium dodecyl sulfate (20%, 400  $\mu$ L), and the flavin difference spectrum was again recorded.

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#### References and Notes

- Haefely, W. E.; Burkard, W. P.; Cesura, A. M.; Kettler, R.; Lorez, H. P.; Martin, J. P.; Richards, J. G.; Scherschlicht, R.; Da Prada, M. *Psychopharmacology* **1992**, 106 (Suppl.), 6.
- Barchas, J. D.; Ciaranello, R. D.; Stolk, J. M.; Brodie, H. K. H.; Hamburg, D. A. In *Hormones and Behavior*; Levine, S., Ed.; Academic: New York, 1972; p 235.
- Ives, J. L.; Heym, J. *Annu. Rep. Med. Chem.* **1989**, 24, 21.
- Tetrad, V. W.; Langston, J. W. *Science* **1989**, 245, 519.
- Johnston, J. P. *Biochem. Pharmacol.* **1968**, 17, 1285.

- Bach, A. W. J.; Lan, N. C.; Johnson, D. L.; Abell, C. W.; Bembenek, M. E.; Kwan, S.-W.; Seeburg, P. H.; Shih, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, 85, 4934.
- Lan, N. C.; Chen, C.; Shih, J. C. *J. Neurochem.* **1989**, 52, 1652.
- Weyler, W. *Biochem. J.* **1989**, 260, 725.
- (a) Kearney, E. B.; Salach, J. I.; Walker, W. H.; Seng, R. L.; Singer, T. P. *Biochem. Biophys. Res. Commun.* **1971**, 42, 490. (b) Kearney, E. B.; Salach, J. I.; Walker, W. H.; Seng, R. L.; Kenney, W.; Zeszotek, E.; Singer, T. P. *Eur. J. Biochem.* **1971**, 24, 321.
- Hellerman, L.; Chuang, H. Y. K.; DeLuca, D. C. *Adv. Biochem. Psychopharmacol.* **1972**, 5, 327.
- (a) Silverman, R. B. *Acc. Chem. Res.* **1995**, 28, 335. (b) Lu, X.; Rodriguez, M.; Ji, H.; Silverman, R. B.; Vintém, A. P. B.; Ramsay, R. R. In *Flavins and Flavoproteins 2002: Proceedings of the 14th International Symposium*; Chapman, S. K., Perham, R. N., Scrutton, N. S., Eds.; Rudolf Weber: Berlin, Germany, 2002; p 817.
- Zhong, B.; Silverman, R. B. *J. Am. Chem. Soc.* **1997**, 119, 6690.
- Binda, C.; Newton-Vinson, P.; Hubálek, F.; Edmondson, D. E.; Mattevi, A. *Nat. Struct. Biol.* **2002**, 9, 22.
- Silverman, R. B.; Hiebert, C. K.; Vazquez, M. L. *J. Biol. Chem.* **1985**, 260, 14648.
- Franzen, V. *Chem. Ber.* **1955**, 88, 1361. (b) Franzen, V. *Chem. Ber.* **1957**, 90, 623.
- (a) Augusto, O.; Beilan, H. S.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1982**, 257, 11288. (b) Ortiz de Montellano, P. R.; Beilan, H. S.; Kunze, K. L. *J. Biol. Chem.* **1981**, 256, 6708. (c) DeMatteis, F.; Hollands, C.; Gibbs, A. H.; deSa, N.; Rizzardini, M. *FEBS Lett.* **1982**, 145, 87.
- (a) Silverman, R. B.; Yamasaki, R. B. *Biochemistry* **1984**, 23, 1322. (b) Silverman, R. B. *Biochemistry* **1984**, 23, 5206. (c) Silverman, R. B.; Zieske, P. A. *Biochemistry* **1985**, 24, 2128.
- Euerby, M. R.; Waigh, R. D. *J. Chem. Res. Miniprint* **1982**, 9, 2417.
- Satoh, T.; Suzuki, S.; Suzuki, Y.; Miyaji, Y.; Imai, Z. *Tetrahedron Lett.* **1969**, 4555.
- Young, R. N.; Gauthier, J. Y.; Coombs, W. *Tetrahedron Lett.* **1984**, 25, 1753.
- Weyler, W.; Salach, J. I. *Arch. Biochem. Biophys.* **1981**, 212, 147.
- Szutowicz, A.; Kobes, R. D.; Orsulak, P. H. *Anal. Biochem.* **1984**, 138, 86.
- Silverman, R. B.; Hawe, W. P. *J. Enz. Inhib.* **1995**, 9, 203.
- Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, 56, 658.
- Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, 237, 3245.
- Silverman, R. B.; Zieske, P. A. *Biochem. Biophys. Res. Commun.* **1986**, 135, 156.
- Fernandez Diez, M. J.; Osuga, D. T.; Feeney, R. E. *Arch. Biochem. Biophys.* **1964**, 107, 449.
- Silverman, R. B.; Banik, G. M. *J. Am. Chem. Soc.* **1990**, 112, 4499.