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# Inactivation of Monoamine Oxidase B by *cis*- and *trans*-5-Aminomethyl-3-(4-methoxyphenyl)dihydrofuran-2(3*H*)-ones

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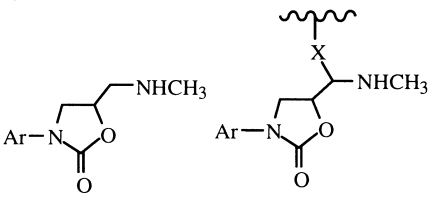
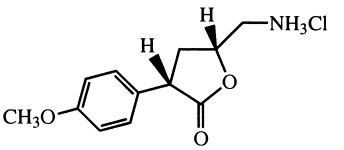
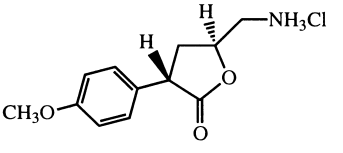
**Abstract**—Monoamine oxidase B was previously shown to be inactivated by *cis*- (**3**) and *trans*-5-(aminomethyl)-3-(4-methoxyphenyl)dihydrofuran-2(3*H*)-one hydrochloride (**4**) in a time-dependent manner (Ding, Z.; Silverman, R. B. *J. Med. Chem.* **1992**, *35*, 885) and to catalyze its oxidative decarboxylation (Silverman, R. B.; Zhou, J. J. P.; Ding, C. Z.; Lu, X. *J. Am. Chem. Soc.* **1995**, *117*, 12895). By [$^{14}$C]-labeling of the aryl methoxyl groups of these two inactivators, it is shown that this is not a mechanism-based inactivation and that multiple enzyme residues are labeled. 

## Introduction

Monoamine oxidase (EC 1.4.3.4; MAO) is a flavoenzyme that is important in the degradation of a variety of biogenic amines. Compounds that inhibit MAO exhibit either antidepressant activity, if they inhibit the A isozyme,[1] or antiparkinsonian activity, if they inhibit the B isozyme.[2] 5-(Methylaminomethyl)-3-aryl-2-oxazolidinones (**1**) were reported to be selective inactivators of MAO B that may have potential as adjuncts to L-dopa treatment for Parkinson's disease.[3] Several years ago we investigated the mechanism of inactivation of MAO B by **1** and concluded that inactivation resulted from enzyme-catalyzed oxidation of the methylaminomethyl group and attachment of an active-site residue to the α-position (**2**). The stability of this enzyme adduct was attributed to the electron-withdrawing effect of the heteroatoms in the oxazolidinone ring.[4] To support that hypothesis *cis*- (**3**) and *trans*-5-(aminomethyl)-3-(4-methoxyphenyl)dihydrofuran-2(3H)-one hydrochloride (**4**), were designed, prepared, and also shown to be effective inactivators.[5] Incorporation of a $^{14}$C label at the carbonyl carbon of the lactone ring resulted in some loss of the radioactive label as $^{14}CO_2$, which supported a radical mechanism to give the α-carbon radical; loss of $^{14}CO_2$ can only come from the formation of this radical.[6] However, radioactivity was found attached to the enzyme,[7] indicating that the pathway that produces carbon dioxide is different from that which inactivates the enzyme. In this paper, we report the synthesis of analogues of **3** and **4** that are $^{14}$C-labeled at the methoxyl group, and an investigation of their inactivation of MAO B.




Key words: Monoamine oxidase; inactivation; dihydrofuranone; mechanism-based inactivation; 2-mercaptoethanol.
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## Results

### *cis*- (5) and *trans*-5-Aminomethyl-3-[methoxyl-$^{14}$C]-(4-methoxyphenyl)dihydrofuran-2(3*H*)-one hydrochloride (6)

The syntheses of *cis*- (**5**) and *trans*-5-aminomethyl-3-[methoxyl-$^{14}$C]-(4-methoxyphenyl)dihydrofuran-2(3*H*)-one hydrochloride (**6**) were carried out by the route shown in Scheme 1, which is closely related to the syntheses of *cis*- and *trans*-[2-$^{14}$C]-5-aminomethyl-3-(4-methoxyphenyl)dihydrofuran-2(3*H*)-one previously reported.[5] The principal difference is that the $^{14}C$-labeled 4-methoxyphenylacetonitrile (**7**) was synthesized from 4-hydroxyphenylacetonitrile and [$^{14}$C]-dimethyl sulfate. Compound **7** was then carried through the earlier synthesis as reported.[5]

### Cysteine titration of MAO B following inactivation by 4

Following inactivation of MAO B by **4**, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) titration showed 4.7 cysteines remaining; the uninactivated control had 7.4 cysteine residues.

### Effect of 2-mercaptoethanol on the rate of inactivation of MAO-B by 3 and 4

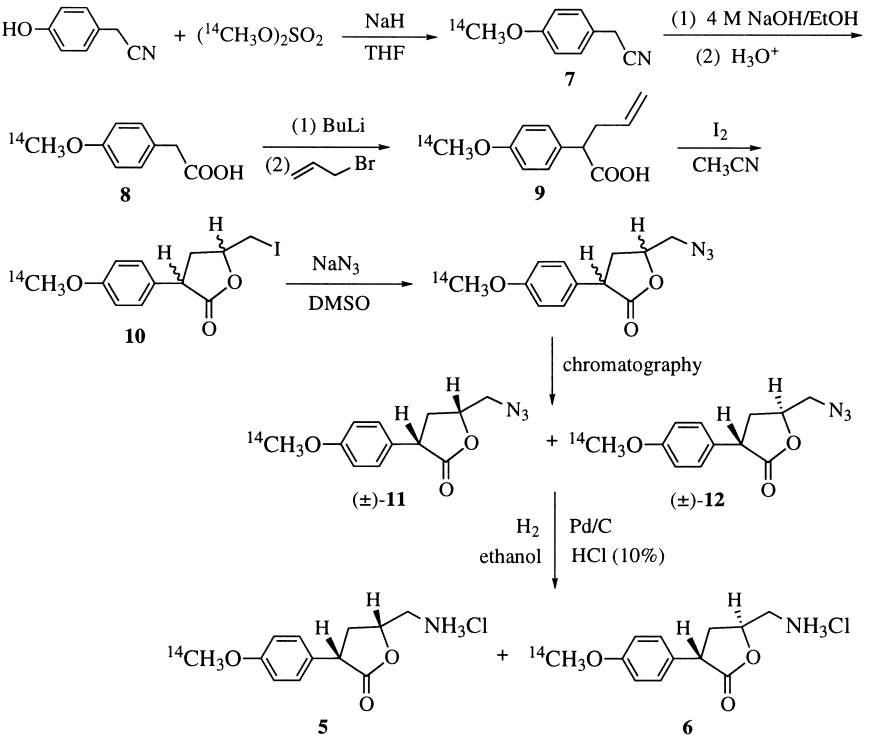
As depicted in Figure 1, the rate of inactivation of MAO B by either **3** or **4** was strongly retarded by the presence of 5 mM 2-mercaptoethanol. 2-Mercaptoethanol had no effect on MAO.

### Equivalents of 5 and 6 bound to MAO B after inactivation

After incubation of MAO B with **5** for two days (10% enzyme activity remaining) followed by denaturation, 4.9 equiv of radioactivity were attached per MAO B molecule. In the presence of 5 mM 2-mercaptoethanol after 2 h (78% enzyme activity remaining), 2.6 equiv of radioactivity remained bound after denaturation. A similar experiment with **6** gave 12.5 equiv of radioactivity bound after five days of incubation (5% enzyme activity remaining) and only 4.0 equiv bound in the presence of 5 mM 2-mercaptoethanol (3 h incubation; 76% enzyme activity remaining).

## Discussion

The original report of these inactivators indicated that inactivation in the presence of 2-mercaptoethanol had no effect on the rate of inactivation,[5] suggesting a mechanism-based inactivation.[8] To determine if the inactivation is a mechanism-based inactivation several experiments were carried out. A DTNB cysteine titration of native MAO B compared with MAO B that was inactivated by **4** showed that 2.7 cysteine residues were




**Scheme 1.**

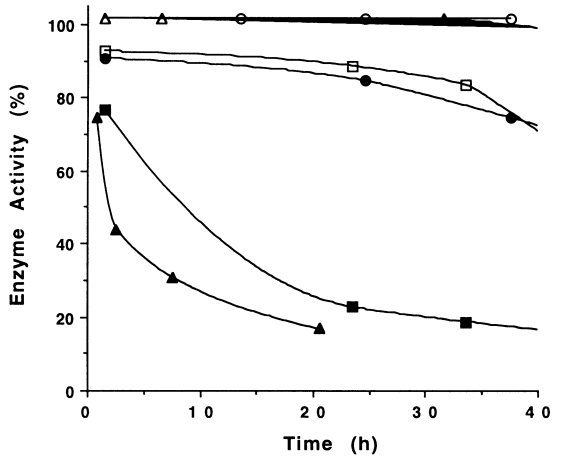
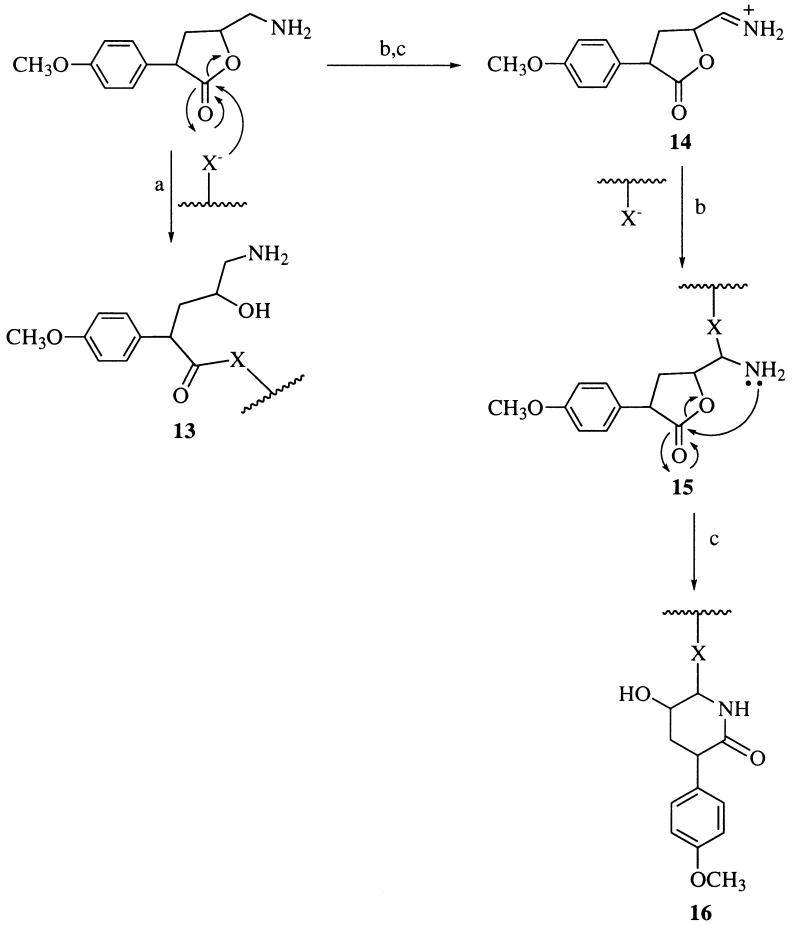



**Figure 1.** Effect of 2-mercaptoethanol on the inactivation of MOA B by **3** or **4** (Δ), buffer alone; (○), buffer containing 5 mM 2-mercaptoethanol; (□), **3** (17.5 mM) containing 2-mercaptoethanol (5 mM); (●), **4** (17.5 mM) containing 2-mercaptoethanol (5 mM); (■), **3** (17.5 mM); (▲), **4** (17.5 mM).

modified after extended inactivation times. Since mechanism-based enzyme inactivation involves stoichiometric modification of the enzyme, this is not a mechanism-based type inactivator.

Furthermore, the rates of inactivation of MAO B by **3** and **4** were diminished by the presence of 2-mercaptoethanol in the preincubation mixture (Fig. 1). 2-Mercaptoethanol did not completely block inactivation by **3** and **4**, and with time, the rate of inactivation slowly increased (Fig. 1). This inactivation-rate increase at latter time points may be the result of oxidation of the 2-mercaptoethanol. When additional fresh 2-mercaptoethanol was added, the rate of inactivation again leveled off (data not shown), supporting the suggestion that the 2-mercaptoethanol was being oxidized. This is reasonable since 2-mercaptoethanol is readily oxidized in oxygenated buffers, and also one of the by-products from oxidation of **3** and **4** is hydrogen peroxide, which even more rapidly than oxygen oxidizes mercaptans to disulfides.




**Scheme 2.**

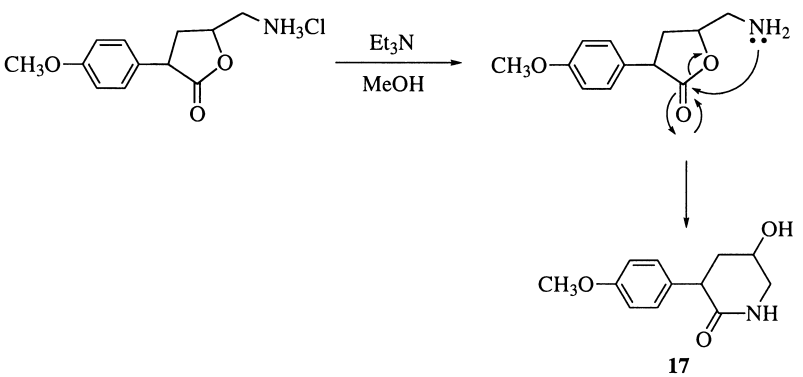
Protection against inactivation by a thiol generally suggests that the actual inactivating species is an electrophile that escapes from the active site prior to inactivation, then returns to cause inactivation. In the presence of 2-mercaptoethanol, the released electrophile can be trapped prior to its return to the enzyme; thereby, preventing inactivation from occurring. It is not known why this result differed from that in the original report, unless the 2-mercaptoethanol used originally was already mostly in an oxidized form.[5]

Confirmation of these results is found in the observation that multiple equiv of inactivator are incorporated into the enzyme during inactivation: the *cis*-isomer (**5**) leads to the incorporation of 4.9 equiv of radioactivity per enzyme molecule after 2 days of incubation, and the *trans*-isomer (**6**) results in the incorporation of 12.5 equiv after 5 days of incubation. To show that incorporation of radioactivity from **5** or **6** does not correlate with enzyme inactivation, incubation of MAO B with **5** and **6** was carried out in the presence of 2-mercaptoethanol and was quenched after a relatively short period of time. With **5**, after 2 h there was still 78% of enzyme activity remaining (relative to a noninactivated control), but 2.6 equiv of radioactivity was incorporated into the enzyme. Likewise, after 3 h of incubation with **6** in the presence of 2-mercaptoethanol there was 76% of enzyme activity remaining but 4.0 equiv of radioactivity were incorporated into the enzyme. It is apparent that multiple sites are reacting with a product of oxidation, but modification of most of the sites does not affect enzyme activity. MAO B is known from the cDNA sequence to contain nine cysteine residues (one is attached to the flavin);[9] however, site-directed mutagenesis of each of the cysteine residues showed that only two (in addition to the cysteine to which the flavin coenzyme is attached) were important to enzyme activity.[10] This is consistent with the incorporation of multiple inactivator molecules (possibly by attachment to the nonessential cysteine residues) without inactivation of the enzyme. Because of the large amount of nonspecific incorporation of radioactivity into the enzyme, it was not possible to determine the structure of the actual active-site inactivating species.

There are at least three mechanisms that could account for the incorporation of radioactivity into MAO B by **5** and **6** (Scheme 2, pathways a–c, leading to **13**, **15**, and **16**). Pathway a, leading to **13**, depicts a simple acylation of active site nucleophiles (affinity labeling): γ-lactones are susceptible to nucleophile addition. Pathways b and c require initial oxidation of the inactivator to **14**, which reacts with an active site nucleophile to give **15** (pathway b) or further rearrangement of **15** to **16** (pathway c). This rearrangement was modeled by treatment of *trans*- (**4**) or a *cis*/*trans*-mixture of 5-aminomethyl-3-(4-methoxyphenyl)dihydrofuran-2(3*H*)-one·HCl with triethylamine at room temperature; both diastereomers of the corresponding rearrangement product, 5-hydroxy-3-(4-methoxyphenyl)tetrahydropyridin-2(3*H*)-one (**17**), were obtained (Scheme 3). When a *cis*/*trans*-mixture of 5-aminomethyl-3-(4-methoxyphenyl)dihydrofuran-2(3*H*)-one·HCl in pH 7.4 buffer was incubated with MAO B, two of the products observed were the corresponding rearranged compounds resulting from this nonenzymatic rearrangement.[11] Given the facility with which this rearrangement occurs in solution, it is a reasonable possibility inside the enzyme.

## Conclusion

From these studies it is apparent that **5** and **6** are not mechanism-based inactivators of MAO B. Given that they are substrates for MAO B as well as inactivators,[11] it is reasonable that one or more turnover products become attached to peripheral residues that do not affect enzyme activity greatly, but also eventually become attached to residues that are important to catalysis.




**Scheme 3.**

## Experimental

### Chemicals and analytical methods

NMR spectra were recorded on either a Varian 300-MHz or a Varian Unity Plus 400-MHz spectrometer. Chemical shifts are reported as δ values in parts per million down field from $Me_4Si$ as the internal standard in $CDCl_3$. 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. UV spectra were recorded on a Perkin Elmer Lambda 10 UV/Vis spectrometer. Radioactivity was measured on a TRI-CARB 2100TR Liquid Scintillation Analyzer. Column chromatography was performed with Merck Silica gel (230-400 mesh). [$^{14}$C]-Dimethyl sulfate was 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.

**[4-$^{14}$C]-Methoxyphenyl acetonitrile (7).** To a solution of 4-hydroxyphenyl acetonitrile (266 mg, 2 mmol) in dry THF (5 mL) was added sodium hydride (96 mg, 60% dispersion in mineral oil, 2.4 mmol) with stirring at room temperature. After 2 h, the reaction solution was checked by TLC (hexane/ethyl acetate, 2/1); no starting material was found. To the reaction solution was added [$^{14}$C]-dimethyl sulfate (2 mCi, 2.53 mCi/mmol), 126 μL of dimethyl sulfate, and 2 mL of THF used to wash the ampoules of [$^{14}$C]-dimethyl sulfate, with stirring at room temperature. After 3 h, TLC showed that only one spot was present. The resulting solution was acidified with 2 N HCl to pH 2, then evaporated to remove the THF. The mixture was extracted with ether (3×20 mL), and the extracts were dried over sodium sulfate. After being concentrated under vacuum, the residue (151 mg, 77%) was used directly in the next step.

**[4-$^{14}$C]-Methoxyphenyl acetic acid (8).** To the residue, which was collected from the last step, were added ethanol (12 mL) and a 20% sodium hydroxide solution (10 mL) with stirring. The mixture was then refluxed for 15 h, after which time the TLC showed that no starting material was present. The clear solution was treated with 10 mL of water, and the ethanol was removed by evaporation. The solution was washed with ether (10 mL) to purify the product. The aq layer was acidified with 3 N HCl to pH 2. The acidic solution was extracted with ether (3×20 mL): TLC showed that the product was the acid **8**. The extracts were dried, and after being concentrated under vacuum, the residue (150 mg, 90%) was directly used in the next step.

**2-([4-$^{14}$C]-Methoxyphenyl)-4-pentenoic acid (9).** The residue from the last step was dissolved in dry THF (5 mL), and *n*-butyllithium (2.5 M, 1.4 mL, 3.5 mmol) was added at 0 °C with stirring under nitrogen. The color changed from cloudy white to clear yellow. The solution was stirred at 0 °C for 2 h, then for 2 h at room temperature. To the resulting solution was added allyl bromide (600 μL, 6.9 mmol) at 0 °C via syringe. After being stirred overnight, the reaction was quenched by addition of an aqueous 10% HCl solution to pH 2 at 0 °C. The organic layer was isolated, and the aqueous layer was extracted with ethyl acetate (3×15 mL). The combined organic layers were dried over sodium sulfate. TLC analysis showed that the strong UV absorbant spot comigrated with **9**. After being concentrated in vacuo, the residue (154 mg, 82%) was directly used in the next step.

***cis*- and *trans*-5-(Iodomethyl)-3-([4-$^{14}$C]-methoxyphenyl)-dihydrofuran-2(3*H*)-ones (10).** Iodine (600 mg, 2.4 mmol) was added to a solution of the residue from the above step in acetonitrile (10 mL) at room temperature. The resulting purple solution was stirred under nitrogen at room temperature for 24 h. TLC analysis showed that the starting material was completely consumed. The reaction was quenched by addition of saturated sodium thiosulfate at 0 °C. The aqueous layer was extracted with ether (4×10 mL) and dried over magnesium sulfate. Removal of the solvent resulted in a yellowish oil (196 mg, 79%), which was identified by TLC analysis as a mixture of the *cis*- and *trans*-iodolactones (**10**) contaminated with some impurities.

***cis*- (11) and *trans*-5-(Azidomethyl)-3-([4-$^{14}$C]-methoxyphenyl)dihydrofuran-2(3*H*)-one (12).** To a solution of the diastereomeric iodolactones from above in anhydrous DMF (8 mL) at room temperature under nitrogen was added sodium azide (400 mg, 6.15 mmol). After the mixture was stirred at room temperature for 24 h, TLC analysis showed that starting material was gone. The mixture was diluted with ether (20 mL) and washed with water (5×10 mL). The combined washes were back extracted with ether (2×10 mL). The combined organic layers were dried over magnesium sulfate. After being concentrated in vacuo, a crude mixture of the diastereoisomeric azidolactones was separated by silica gel column chromatography, eluting with a mixture of hexane and ethyl acetate (2:1) to give two products: **11** (77.7 mg, 54%) and **12** (25.2 mg, 18%).

***cis*- (5) and *trans*-5-(Aminomethyl)-3-[methoxyl-$^{14}$C]-(4-methoxyphenyl)dihydrofuran-2(3*H*)-one hydrochloride (6).** Compounds **11** and **12** were separately dissolved in 10 mL of a mixture of ethanol and 10% aq HCl (9:1). After addition of 10% palladium on carbon (50–60 mg), the reaction mixture was hydrogenated at room temperature and atmospheric pressure overnight. TLC showed that no starting material was present. The mixture was filtered and the filtrate was concentrated in

vacuo, yielding each product (recrystallized from ethanol/ether) as a white solid; which were **5** (52 mg, 64%) and **6** (21 mg, 80%). Specific activity: $1.37\times10^6$ dpm/μmol; Radiopurity: ≥97%.

**5-Hydroxy-3-(4-methoxyphenyl)tetrahydropyridin-2(3*H*)-one (17).** This product was obtained in two ways: one in methanol and triethylamine, and the other under conditions of the enzymatic experiment. The products were identical by HPLC and mass spectrometry. The non-aqueous reaction allowed for isolation and complete characterization of the product.

To a stirred solution of compound **4** (150 mg, 0.7 mmol) in methanol (5 mL) at room temperature was added triethylamine (0.5 mL, 5 mmol). The reaction was stirred overnight and monitored by TLC (50% ethyl acetate, 1% formic acid in hexane). The solvent was removed under vacuum, and the residue was recrystallized from water to give **17** as a white solid (121 mg, 80%). Mp 182–183 °C; mass spectrum (*m*/*z*) 222 (M + 1), 204, 186, 176, 159, 147, 144, 133; $^1$H NMR (DMSO-$d_6$) δ 7.44 (s, 1H), 7.10 (AB, 2H), 6.86 (AB, 2H), 5.04 (d, 1H), 3.99 (t, 1H), 3.72 (s, 1H), 3.58 (q, 1H), 3.36 (d, 2H), 3.11(m, 1H), 1.98 (t, 1H); $^{13}$C NMR (DMSO-$d_6$) δ 171.1, 157.6, 134.0, 129.5, 113.4, 63.8, 61.2, 54.9, 48.4, 45.2, 42.6, 37.0; Anal. calcd for $C_{12}H_{15}O_3N$: C, 65.16; H, 6.79; N, 6.33. Found: C, 64.55; H, 6.59; N, 6.26.

**Enzyme and assays.** Bovine liver MAO B was isolated and assayed as previously reported.[12]

**Cysteine titration of MAO B before and after inactivation with 3 and 4.** The procedure of Silverman and Zieske[13] was followed.

**Effect of 2-mercaptoethanol on the rate of inactivation of MAO B by 3 or 4.** The following solutions (180 μL each) were prepared in sodium phosphate buffer (100 mM, pH 7.2) containing 10% DMSO at 25 °C: buffer alone, buffer containing 2-mercaptoethanol (5 mM), **3** or **4** (17.5 mM). To these six solutions was added MAO B (20 μL, 3 mg/mL). The solutions were incubated at 25 °C, and the MAO B activity was assayed periodically as described previously.[4] Over an extended period of time, it was found that the loss of enzyme activity stopped by the addition of fresh 2-mercaptoethanol (5 μL, 5 mM).

**Incorporation of radioactivity into MAO B by 5 or 6.** MAO B (140 μM, 100 μL) was incubated with compound **5** or **6** (17.5 mM) in 100 mM sodium phosphate buffer (2200 μL, containing 10% of DMSO), pH 7.2 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 960 mg of urea, and the solution was heated at 50 °C overnight to denature the enzyme. This solution was dialyzed at room temperature versus 8 M urea in 100 mM ammonium bicarbonate (500 mL). The dialysis buffer was changed three 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 equiv of radioactivity attached per MAO B molecule was calculated.

Another procedure for calculating the number of equiv of radioactivity bound to the enzyme was to precipitate the protein from the denatured and dialyzed enzyme solution (2 mL, about 1 mg of denatured protein) with trichloroacetic acid (200 mg). The precipitated protein was isolated by microfuge, then washed with acetone (2×100 μL) and water (2×100 μL). The precipitate was dissolved in guanidine (6 M, 150 μL), and the number of equiv of radioactivity attached per MAO B molecule was calculated from the protein concentration and its radioactivity.

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