A New Strategy for the Design of Monoamine Oxidase Inactivators. Exploratory Studies with Tertiary Allylic and Propargylic Amino Alcohols

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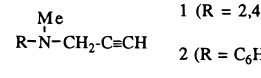
Abstract: A new strategy for the design of monoamine oxidase (MAO) inhibitors is proposed. The strategy is based on the premise that tertiary-amine containing MAO-inactivators which operate by alkylation of active site nucleophiles are activated *in situ* by single electron transfer (SET) to the MAO-flavin cofactor to form aminium cation radicals which undergo secondary fragmentation reactions to produce reactive electrophiles. The purpose of the current work was to assess the feasibility and applicability of this proposal for the design of new families of MAO-inactivators. Based on the documented retro-aldol type fragmentation reactivity of β-amino-alcohol cation radicals, tertiary β-allylic and -propargylic β-amino-alcohols were expected to serve as precursors of conjugated ketones in SET-promoted processes. Evidence supporting this hypothesis was gained from studies of model SET-photoreactions of members of this amino-alcohol family with 3-methyllumiflavin (3MLF). The efficient production of 4a- and 4a,5-flavin adducts in these excited-state reactions demonstrates that aminium radicals, arising by SET-oxidation of tertiary β-allylic and -propargylic β-amino-alcohols, fragment to generate α,β-unsaturated ketones which react rapidly with the simultaneously formed 3MLF-hydroflavin anion. The second feature of the MAO-inactivator design strategy pathway was tested by examining reactions of the MAOs with substances which contain electrophilic, conjungated enone and ynone moieties tethered to amine functions to ensure delivery to the enzyme active sites. The covalent modification of active site cysteine thiol residues by the unsaturated ketone groups in these substances was confirmed by demonstrating that they serve as active site-directed, time-dependent, nonredox based, inactivators of MAO-A and MAO-B. In the key test of the feasibility of the new MAO-inactivator design strategy, it was shown that selected tertiary β-allylic and -propargylic β-amino-alcohols undergo redox reactions in the MAO-A active site which result in inactivation of the enzyme via covalent modification of a single cysteine residue. The experimental results which support the conclusions stated above are presented and discussed in this paper.

Introduction

The monoamine oxidases MAO-A and MAO-B are two structurally homologous, redox active enzymes found in the mitochondrial membranes of most mammalian tissues.[1] The primary function of the MAOs is to regulate the concentrations of primary and secondary amines by catalyzing their oxidative deamination to produce amine and carbonyl products. Among the more important substrates of these enzymes are the neurologically active amines epinephrine, dopamine, and serotonin. As a consequence, the MAOs play key roles in regulating the level of these neurotransmitters in the human brain.[2] MAO-inhibitors cause an elevation in the levels of the neuroactive amines and are thus of medicinal importance. MAO-A which preferentially oxidizes serotonin is selectively and irreversibly inhibited by the tertiary propargylic amine clorgyline (**1**), an agent used in the treatment of depression.[4] On the other hand, MAO-B is preferentially inactivated by the inhibitor deprenyl (**2**) which is used in conjunction with L-DOPA to treat Parkinson's disease.[5]



Both MAO-A and MAO-B contain a flavin (FAD) cofactor, covalently bound through a cysteine thioether linkage to the 8-position of the isoalloxazine ring.[3] The chemical steps of

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(1) For comprehensive reviews of MAO-biochemistry, see: (a) Singer, T. P. *J. Neur. Trans.* **1987**, *Suppl. 23*, 1. (b) Singer, T. P. In *Chemistry and Biochemistry of Flavoenzymes*; Muller, F., Ed.; CRC Press: Boca Raton, FL, 1991; Vol. 3, pp 437−470. (c) Weyler, W.; Hsu, Y. P. P.; Breakfield, X. O. *Pharmac. Ther.* **1990**, *47*, 391.

(2) Schildkraut, J. T.; Kety, S. S. *Science* **1967**, *156*, 21.

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(4) For a general review, see: (a) Tripton, K. F.; Dostert, P.; Benedetti, M. S. *Monoamine Oxidase and Disease*; Academic Press: New York, 1984. (b) Dawson, J. H. *J. Nerual. Transm.* **1987**, *23*, 121.

(5) Youdim, M. B. H.; Finberg, J. P. *Advances in Neurology*; Yahr, M. D., Bergmann, J., Eds.; Raven Press: New York, 1986; Vol. 45, pp 127−136. Knoll, J. In *Advances in Neurology*; Yahr, M. D., Bergman, K. J., Eds.; Raven Press: New York, 1986; Vol. 45, pp 107−110.

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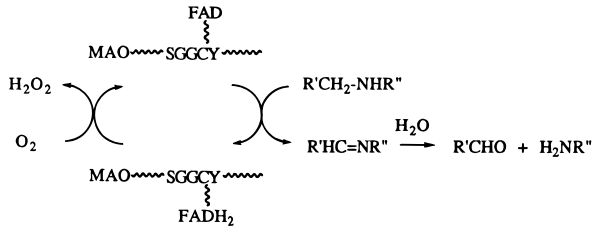
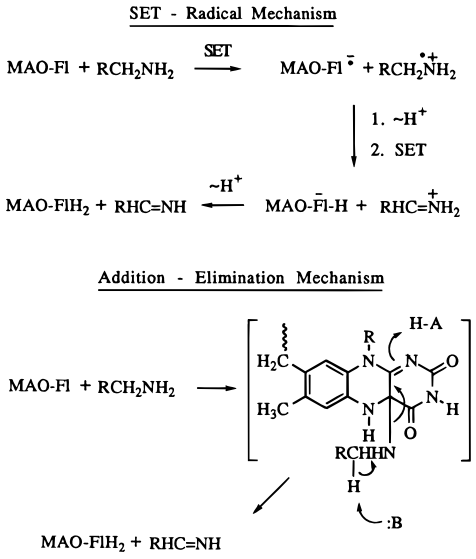
Scheme 1



Scheme 2

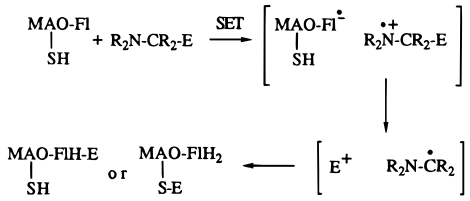


MAO catalysis, represented in Scheme 1, involve simultaneous oxidation of the amine substrate and reduction of the oxidized flavin cofactor to generate imine and 1,5-dihydroflavin products. Hydrolysis of the imine and flavin oxidation by molecular oxygen then complete the catalytic cycle.

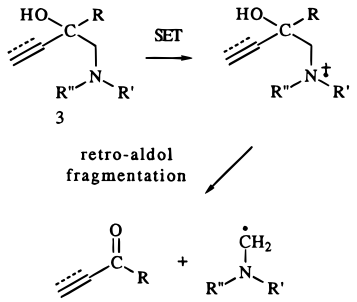
The first partial reaction, oxidation of the amine substrate, has been the focus of much mechanistic speculation. While some investigators favor a single electron transfer (SET)/radical mechanism[6] for the oxidation of primary and secondary amines, the polar addition−elimination mechanism originally proposed by Hamilton[7] is equally feasible[8] (Scheme 2). Tertiary amines, on the other hand, because of their reduced nucleophilicity and increased electron-donor ability, are perhaps better candidates for the SET initiated mechanistic pathway.[9]

Much of what is known about the chemical mechanism of the amine oxidation partial reaction catalyzed by MAO has derived from the results of studies of the mechanism(s) for inactivation of the enzyme by mechanism based inhibitors.[10,11] There seems to be agreement that the respective catalytic and inactivation processes involving the MAOs and tertiary amine substrates and inactivators are initiated by SET to the flavin

Scheme 3



Scheme 4



cofactor to generate aminium radical intermediates.[9] However, less is known about the events which lead to enzyme covalent adduct formation and, specifically whether this involves radical[12,13] or nucleophile−electrophile[8,14] coupling processes. We believe that a reasonable argument can be made for operation of a general route for MAO inactivation by tertiary amines which proceeds via amine cation radical fragmentation to form electrophilic intermediates which then alkylate a nucleohilic residue in the enzyme active site region (Scheme 3). *The reasoning embodied in this scheme leads to the general prediction that tertiary amines whose cation radicals have a high propensity to fragment to produce electrophilic intermediates should serve as effective MAO-inactivators.*

We have initiated studies to test this approach to the rational design of new tertiary amine containing MAO-inactivators. Based on the documented retro-aldol type fragmentation reactivity of β-hydroxy-aminium radicals,[15] we anticipated that tertiary β-allylic and β-propargylic β-amino alcohols of general structure **3** would serve as precursors of conjugated ketones in SET-promoted processes (Scheme 4) and, consequently, as MAO-inactivators.

A combination of photochemical and biological studies with selected members of this family of compounds have provided preliminary results which demonstrate that (1) SET-induced photochemical reactions of these substances with the model flavin, 3-methyllumiflavin, results in production of conjugated ketone intermediates, (2) conjugated ketones which are tethered to tertiary amine functions to ensure active site binding inactivate the MAOs by covalent modification of a single cysteine thiol residue, and (3) selected tertiary β-allylic and β-propargylic β-hydroxyamines inactivate MAO-A by a pathway that involves redox participation by the flavin cofactor and that results in alkylation of an active site cysteine thiol grouping.

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(8) Kim. J.-M.; Hogey, S. E.; Mariano, P. S. *J. Am. Chem. Soc.* **1994**, *116*, 1000.
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(14) Kim, J.-M.; Bogdan, M. A.; Mariano, P. S. *J. Am. Chem. Soc.* **1991**, *113*, 925.
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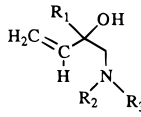
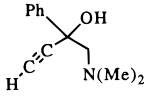
Results

SET-Photochemical Studies. It is well-recognized that the triplet excited state of 3-methyllumiflavin (3MLF), produced by excitation and intersystem crossing, is an excellent one-electron oxidizing agent owing to its large excited-state reduction potential (ca.+1.3 V).[12,16] As such, photoreactions of this flavin serve as useful models to explore SET-induced reactions of electron donors. To determine if the aminium radicals derived from tertiary β-allylic and β-propargylic β-hydroxyamines **3** do indeed undergo efficient retro-aldol type fragmentation reactions to generate electrophilic conjugated ketones, we have examined the SET-promoted photoreactions of the β-hydroxyamines **4**−**7** with 3-methyllumiflavin.



4 (R_1 = Ph, R_2 = Me, R_3 = Bn)
5 (R_1 = Ph, R_2 = R_3 = Me)
6 (R_1 = Ph, R_2 = R_3 = H)



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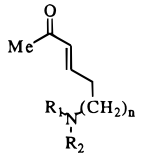
The synthetic sequences used to prepare **4**−**7** are provided in Supporting Information. Photoreactions were performed by irradiating N_2-purged MeOH solutions containing 3MLF and the allylic and propargylic amino alcohols with Uranium glass filtered-light (λ>320 nm, ensuring that 3MLF is the primary light absorbing species). The progress of each photoreaction was monitored by UV−visible spectroscopy, and irradiation was terminated when the absorbance at 450 nm associated with 3MLF reached ca. 20% of its original value. Product separation was accomplished by either silica gel TLC or precipitation (in the case of flavin 4a-adducts). The results in terms of products and yields are summarized in Schemes 5. The efficient formation of flavin adducts in these photoreactions is consistent with the proposed retro-aldol type reactivity of intermediate tertiary β-allylic and β-propargylic β-hydroxyaminium radicals which results in generation of conjugated ketone intermediates.

Determination of Enzymatic Inhibition and Inactivation Constants for Tethered Amino-Enones and Ynones. The known silylmethylamino-enone **11**[17] and -ynone **15**[18] and their homologues and non-silicon containing analogues **12**, **14**, and **16** were prepared (Supporting Information) in order to determine if, and how, active site-bound, conjugated ketones inactivate the MAOs. Accordingly, these substances were first tested as inhibitors of the MAOA-catalyzed oxidation reaction of kynuramine.[19] Double recipricol plots[20b] of the initial velocities versus kynuramine concentration at various inhibitor concentrations showed that **11**−**16** are all reversible, competitive inhibitors of this enzyme. The inhibition constants, K_i, determined[21] for these substances as well as for the saturated amino-ketones **17** and **18** and tertiary α-silylamine **19** are recorded in Table 1.

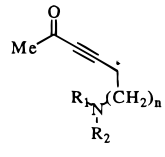
Table 1. Data for Competitive Inhibition and Inactivation of MAO-A by Aminoenones **11**−**14**, Aminoynones **15** and **16**, Related Substances **17**−**19**, and β-Hydroxyamines **5**−**7**

compd	K_i (mM)	K_{inact} (mM)	$k_{inact} \times 10^2$ (min^{-1})
11	4.8 ± 0.5	13.8 ± 0.3	9.5 ± 0.1
12	4.1 ± 0.5	7.6 ± 0.5	13.0 ± 0.3
13	1.5 ± 0.1	3.7 ± 0.1	1.2 ± 0.4
14	4.0 ± 0.6	6.0 ± 0.2	1.4 ± 0.4
15	2.8 ± 0.2	7.3 ± 0.2	1.9 ± 0.2
16	0.4 ± 0.3	3.7 ± 0.5	1.2 ± 0.3
17	1.0 ± 0.1	*a*	*a*
18	0.8 ± 0.2	*a*	*a*
19	1.9 ± 0.2	*a*	*a*
5	2.1 ± 0.2	13.9 ± 0.5	1.7
(+)-(*R*)-**5**	1.4 ± 0.1	11.8 ± 0.4	1.3
(−)-(*S*)-**5**	2.3 ± 0.4	2.5 ± 0.5	1.4 ± 0.1
6	0.4 ± 0.03	*a*	*a*
7	2.0 ± 0.3	2.6 ± 0.2	0.7 ± 0.08

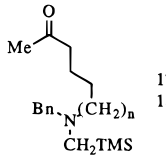
[a] Not determined.



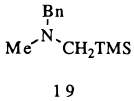
11 (n = 2, R_1 = Bn, R_2 = CH_2TMS)
12 (n = 1, R_1 = Bn, R_2 = CH_2TMS)
13 (n = 1, R_1 = Bn, R_2 = Me)
14 (n = 2, R_1 = Bn, R_2 = Me)



15 (n = 1, R_1 = Bn, R_2 = CH_2TMS)
16 (n = 1, R_1 = Bn, R_2 = Me)



17 (n = 2)
18 (n = 1)



19

The ability of the amino-enones and -ynones to serve as inactivators of MAO-A was examined next. MAO-A (50 mM) in Na_2HPO_4 buffer (pH 7.2) containing 0.2% Triton-X at 25 °C was independently reacted with **11**−**16**. Catalytic activity was monitored periodically by using the kynuramine assay.[19] As exemplified in Figure 1 for the case of amino-enone **11**, MAO-A inactivation occurs in a time and concentration dependent manner. Kitz-Wilson replots[20a] of the reciprocals of the apparent rate constants for the inactivation processes vs the reciprocals of the inactivator concentrations provide the rate constants for inactivation, k_{inact}, and the inactivation dissociation constants, K_{inact}, listed in Table 1. Significantly, the amino-ketones **17** and **18** and the tertiary α-silylamine **19** do not serve as efficient inactivators of MAO-A over the time periods in which their α,β-unsaturated carbonyl analogues bring about complete inactivation of the enzyme, suggesting that the reactions occurring between MAO-A and **11**−**16** must involve the respective enone or ynone functionalities.

To demonstrate that the MAO inactivation occurs while the tethered enones/ynones are bound to the MAO-A active site, inhibition of the inactivation processes by the potent MAO competitive inhibitor, (+)-(*S*)-amphetamine (K_i = 34 μM for MAO-A)[22] was tested. Solutions of MAO-A containing the inactivators **11** and **15** (6 mM) and (*S*)-amphetamine (1.6 mM) were incubated for ca. 2.5 h time periods with periodic monitoring of enzyme activity. MAO-A inactivation is completely prevented in the presence of (*S*)-amphetamine. Thus,

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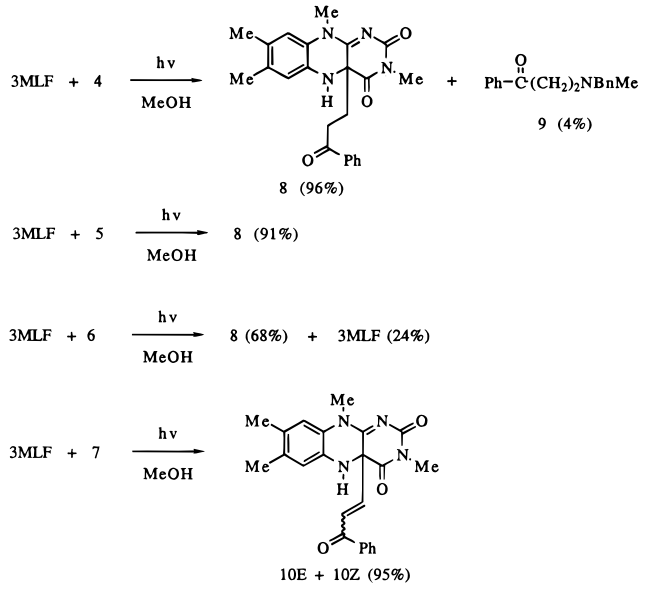
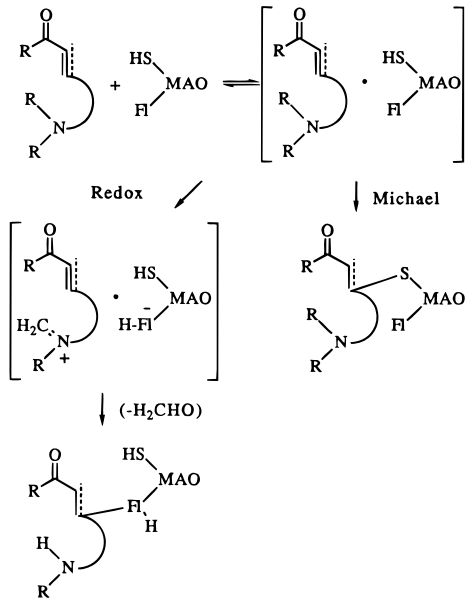
(22) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.

Scheme 5



Scheme 6



MAO-A inactivations by the amino-enones and -ynones depends on these compunds binding in the active site of the enzyme.

The amino-enones and -ynones also serve as affinity labeling reagents of MAO-B. For example, the amino-ynone **15**, is a reversible competitive inhibitor of MAO-B (K_i = 1.3 mM) as well as a time and concentration dependent inactivator (K_{inact} = 7.3 mM and $k_{inact} = 1.9 \times 10^{-2}$ min^{-1}). The inactivation of MAO-B by **15** is also prevented by (*S*)-amphetamine.

Determination of the Alkylation Site in MAO Inactivation by Tethered Amino-Enones and -Ynones. As shown in Scheme 6, two pathways are possible for the inactivation reactions of the tethered α,β-unsaturated ketoamines. One involves simple nucleophilic Michael-addition of the cysteine thiol group which has been previously characterized as being located in the MAO active site region.[23] The other route involves redox participation by the flavin cofactor and results in the generation of a covalent adduct containing a reduced flavine moiety. UV−visible spectroscopic methods were used to distinguish between the two mechanistic pathways for MAO-A inactivation. Accordingly, three deoxygenated, sealed curvettes containing MAO-A alone, MAO-A and the amino-enone **11**, and MAO-A and kynuramine were incubated for a 2 h time period leading to ca. 97% inactivation of the enzyme. The solution containing MAO-A and kynuramine, as expected, experiences a rapid decrease in the flavin absorbance in the 350−550 nm region as a result of reduction to the 1,5-dihydro form.[24] In contrast, the oxidized flavin spectrum of the solution containing MAO-A and **11** remains unchanged during this time period. Thus, the flavin cofactor in MAO-A is not redox active in the reaction with **11**.

Table 2. Free-Thiol Determinations on MAO-A and Its Derivatives Arising by Inactivations with Aminoenones **11** and **12**, Aminoynone **15**, and Hydroxyamines **5** and **7**

enzyme	no. of thiols[a]
MAO-A	6.8 ± 0.1
MAO-A + **11**	5.8 ± 0.1
MAO-A + **12**	5.9 ± 0.1
MAO-A + **15**	1.3 ± 0.1
MAO-A + **5**	5.7 ± 0.2
MAO-A + **7**	5.6 ± 0.1

[a] Determined by use of the DTNB titration method (ref 25) and three independent experiments each.

To demonstrate that amino-enone inactivation is a consequence of alkylation of an MAO cysteine thiol function, the number of free thiols within the enzyme before and following inactivation was determined by using the 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) titration method.[22] Accordingly, native MAO-A was found to contain seven free thiol functions in agreement with the seven known (*i.e.*, predicted from the gene sequence)[25] free cysteine residues of this enzyme (Table 2). In contrast, DTNB titrations of the enzymes, inactivated by the amino-enones **11** and **12**, showed that both contain ca. 6-titratable thiol moieties (Table 2). However, the amino-ynone **15** serves as a much less selective alkylating agent for MAO-A, indicated by the data in Table 2; complete inactivation of MAO-A by **15** results in a modified enzyme that contains ca. 1-free thiol only. The combined results demonstrate conclusively that the major, if not exclusive, process involved in MAO-inactivation by the amino-enones (and perhaps -ynones) is active site thiol alkylation.

Determination of MAO Binding Constants for the β-Allylic- and β-Propargylic-β-hydroxyamines. The β-hydroxyamines **5**−**7**, although lacking phenolic functionality, are structural analogues of the aryl-ethanolamine containing MAO inhibitors/substrates, amphetamine, epinephrine, and norepinephrine. Thus, modest binding affinities to the MAO active sites were expected. Indeed, the steady-state initial velocity data listed in Table 1 show that **5**−**7** are reversible competitive inhibitors of MAO-A, with dissociation constants which are of comparable magnitude to those of other tertiary amines (e.g., K_i = 7.1 mM for MAO-B by *N*,*N*-dimethyl-N-(2-phenylethyl)-amine)[26] and sterically crowded primary amines (e.g, K_i = 0.2 mM for MAO-B by 1-phenylcyclopropylamine).[27]

To determine if the individual enantiomers of these β-amino alcohols have different MAO-A binding affinities, resolution of **5** was carried out. Separation of the antipodes was performed

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(27) Silverman, R. B.; Zieske, P. A. *Biochemistry* **1985**, *24*, 2128.

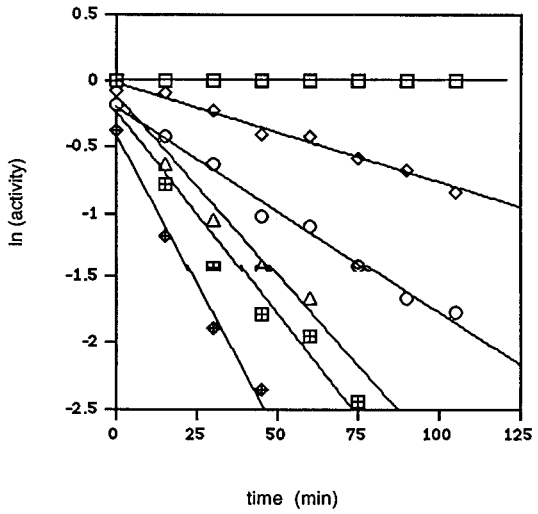
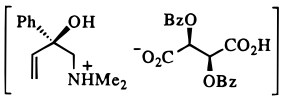


Figure 1. A plot of the natural log of the percent activity remaining vs time for inactivation of MAO-A by the amino-enone **11**. MAO-A (8.5 μm) was incubated with **11** (0, square; 1, diamond; 2.5, circle; 5.0, triangle; 7.5, filled square; 10.0, filled diamond mM) in 50 mM Na_2HPO_4 (pH 7.2) solutions containing 0.2% Triton-X at 25 °C. Aliquots (50 μL) were removed periodically and assayed for MAO-activity by use of the kynuramine procedure.

by HPLC through the use of a Chiralcel OJ column or by crystallization of and regeneration from the (+)-D-dibenzoyltartrate salt. Absolute stereochemical assignments to **(+)-5** and **(−)-5** as (*R*) and (*S*), respectively, were made based on X-ray crystallographic analysis of the tartrate salt **20** of **(−)-5** (Supporting Information). As indicated by the similar magnitudes of the inhibition constants exhibited by the (*R*)-enantiomer and (*S*)-enantiomer of **5** (Table 1), the enzyme displays little stereoselectivity in binding the allylic β-amino alcohol antipodes. This is not an unexpected result based on the observation[28] that MAO-A displays only a small binding preference for the (*R*)- (K_i = 0.1 mM) vs the (*S*)- (K_i = 0.11 mM) enantiomers of *N*-methylamphetamine.



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Determination of the Thermodynamic and Kinetic Constants for MAO Inactivation with the β-Allylic- and β-Propargylic-β-Hydroxyamines. The β-allylic and β-propargylic β-hydroxyamines **5**−**7** were tested as irreversible inhibitors of MAO-A by use of time dependent reactions in which catalytic activity was assayed by use of the kynuramine method.[19] The apparent rate constants for inactivation, derived from the slopes of plots of the natural log of activity remaining vs time (Figure 2 for **5**), were evaluated. From the Y-intercepts of double recipricol plots[20b] of apparent k_{inact} value vs inactivator concentrations, the k_{inact} value were derived, and from the X-intercepts, the dissociation constants, K_{inact}, for MAO-inactivatior were determined. The inactivation constants determined in this manner for **5** and **7** are recorded in Table 1. Interestingly, the primary β-amino alcohol **6** was found not to be an MAO-A inactivator.

To determine if MAO-A inactivation by the β-amino alcohols **5** and **7** is associated with covalent modification of the enzyme,

(28) Robinson, J. B. *Biochem. Pharmacol.* **1985**, *34*, 4105.

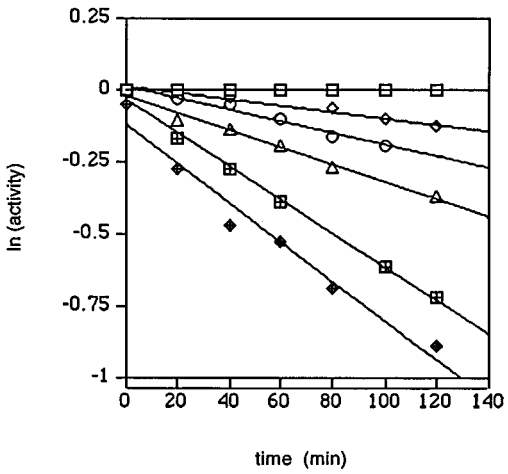


Figure 2. Plot of the natural log of the percent activity remaining vs time for inactivation of MAO-A by **5**. MAO-A (10 mM) was incubated with **5** (0, square; 1, diamond; 2, circle; 4, triangle; 8, filled square; 10 mM, filled diamond) in 50 mM Na_2HPO_4 buffer (pH 7.2) at 25 °C. MAO-A activity was monitored by use of the kynuramine assay.

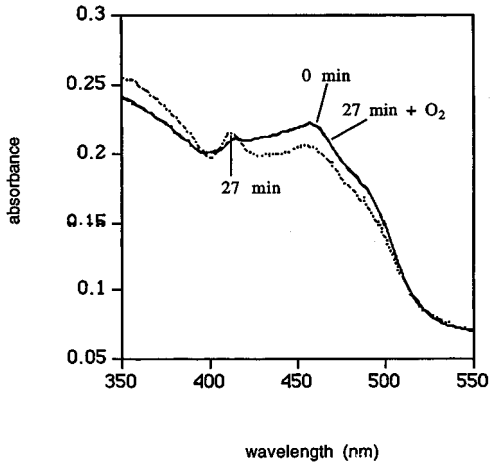
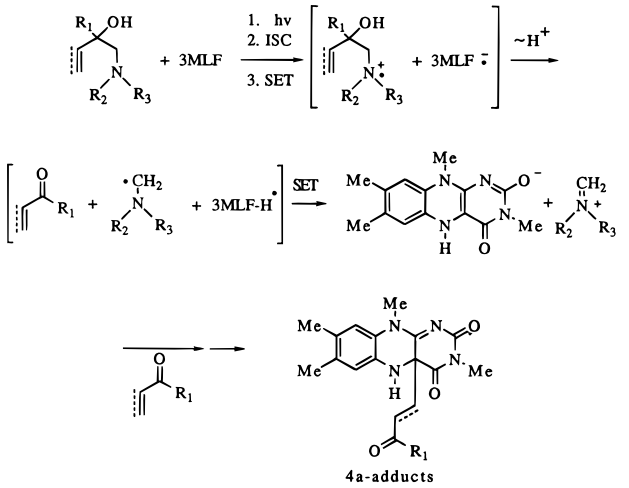


Figure 3. UV−visible spectra of the flavin-cofactor absorbance region of MAO-A during the course of its inactivation by the propargylic-β-amino alcohol **7**. MAO-A (25 μM) was incubated with **7** (23 mM) in 50 mM Na_2HPO_4 (pH 7.2) containing 0.2% Triton-X under anaerobic conditions. Spectra were recorded before addition of **7**, 27 min after addition of **7** under anaerobic conditions, and after admission of air to the reaction mixture.

active site protection experiments were carried out. Specifically, the inactivation reactions were carried out with 16 mM of each inactivator and in the presence or absence of saturating (+)-(*S*)-amphetamine (1.6 mM). As expected for mechanism-based inactivation, (+)-(*S*)-amphetamine completely blocks the reactions of **5** and **7** (16 mM) with this enzyme. In addition, application of the DTNB titration method shows that MAO-A inactivated by both the allylic- and propargylic-tertiary amino alcohols, **5** and **7**, contains one less cysteine-thiol function than the native enzyme (see Table 2).

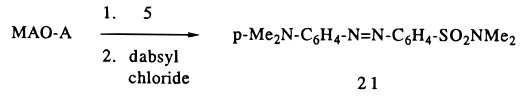
UV−visible spectroscopic monitoring of the reaction of MAO-A with β-amino alcohol **7** has provided information about the redox-participation of the flavin cofactor in the process. As shown by the spectra reproduced in Figure 3, anaerobic reaction of MAO-A with **7** is associated with a change from the typical enzyme oxidized flavin spectrum with a maximum at ca. 450 nm to one that contains a maximum at ca. 410 nm. This change, which is reversed by admission of oxygen into the reaction solution, is not consistent with complete reduction of the flavin residue in the enzyme to produce either the 1,5-dihydro form

Scheme 7



or a 4a- or N5-adduct.[24] In fact, previous efforts[29] have shown that the 410 nm absorption band is associated with an anionic flavin-semiquinone radical. Thus, this species might be a metastable product in the β-amino alcohol inactivation pathway (see below) suggesting that the flavin cofactor of MAO-A is involved in the mechanism for inactivation by **5** and **7**.

As shown in Scheme 4, fragmentation of aminium radicals derived from the allylic and propargylic β-amino alcohols **5** and **7** forms a dimethylaminomethyl radical which would be further transformed by oxidation and subsequent hydrolysis to *N*,*N*-dimethylamine. Dabsyl-chloride[30] treatment of the crude mixture obtained from reaction of MAO-A with amino-alcohol **5** leads to isolation of the dimethylamine derived sulfonamide **21**[30] characterized by TLC, HPLC, and ^{1}H NMR analyses.

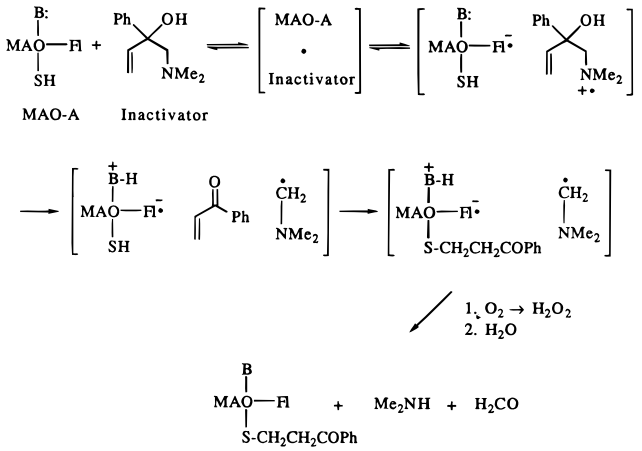


Finally, MAO-inactivation by the allylic- and propargylic-amino alcohols **5** and **7** appears to be selective for MAO-A. For example, although **5** is a modest competitive inhibitor of MAO-B (K_i = 2.7 mM), neither it nor its acetylene analogue **7** are inactivators of the B-enzyme.

Discussion

The results presented above demonstrate the validity of the proposed strategy for MAO-inactivator design. It is clear that SET-photochemical processes can serve as a predictive method for the identification of tertiary amines which are capable of reacting with the MAOs to generate electrophilic products in the enzyme active site region. The observations made in the current photochemical study confirm that tertiary aminium radicals arising from β-allylic- and β-propargylic-β-hydroxyamines undergo facile retro-aldol like fragmentation[15] to produce conjugated enone or ynone products. The operation of this process is revealed by the formation of 4a-adducts (e.g., **8**) as the major if not exclusive substances produced in these photoreactions. Accordingly, SET from the amine function in the β-amino alcohols to the triplet excited state of 3MLF generates an ion radical pair in which aminium radical retro-aldol-like fragmentation occurs with simultaneous proton transfer to the anion radical of 3MLF (Scheme 7). This fragmentation route dominates alternative decay reactions of the aminium radicals involving (*e.g.*, αCH-deprotonation)[31] especially in the cases of the α-aryl analogues.[15] α-Amino radicals are known to be exceptionally powerful reducing agents.[32] As a result, SET from these intermediates to the hydroflavin radical is facile and results in formation of the nucleophilic hydroflavin anion. Michael addition of the hydroflavin anion to the conjugated carbonyl products then produces the 4a-adducts.

Scheme 8



Observations made in our investigations with the tethered amino-enones and -ynones lend credence to suggested mechanisms for MAO-inactivation involving the generation and addition reactions of electrophilic intermediates. These substances serve as affinity labeling agents owing to the presence of both the amine functions which lead to active site binding and the α,β-unsaturated ketone groups which provide Michael addition reactivity. The active nucleophile in these inactivation processess is a cysteine thiol which earlier chemical studies[23] have identified as being required for catalytic activity.

The results point to the operation of mechanism based pathways for MAO-A inactivation by the β-hydroxyamines **5** and **7**. In a manner consistent with our proposal, the enzymatic sequence (Scheme 8) is most likely initiated by SET within the enzyme−inactivator complex which is then followed by retro-aldol fragmentation of the intermediate tertiary aminium radical. This step might be facilitated by the same basic residue that participates as a general base in the MAO-catalytic reaction. The α,β-unsaturated carbonyl product formed in this manner serves as a Michael acceptor in reaction with a cysteine thiol to produce the inactivated enzyme. In the presence of oxygen, both the anionic flavin semiquinone, possibly responsible for the 410 nm absorption band under anaerobic conditions, and the dimethylaminomethyl radical are converted to the respective oxidized flavin and the iminium cation precursor of dimethylamine.

A few additional observations made in this study are worthy of comment. The first concerns the fact that the primary amine **6**, although serving as a comparable reversible competitive inhibitor of MAO-A, is not an inactivator of this enzyme. While there are a number of possible reasons for the lack of reactivity of this substance, one of these might be related to the fact that

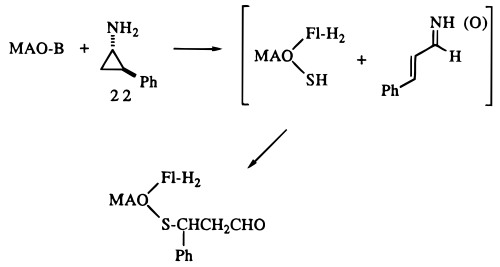
(29) Yue, K. T.; Bhattacharyya, A. K.; Zhelyaskov, V. R.; Edmondson, D. E. *Arch. Biochem. Biophys.* **1993**, *300*, 178.

(30) Lin, J. K.; Wang, C. H. *Anal. Chem.* **1980**, *52*, 630.

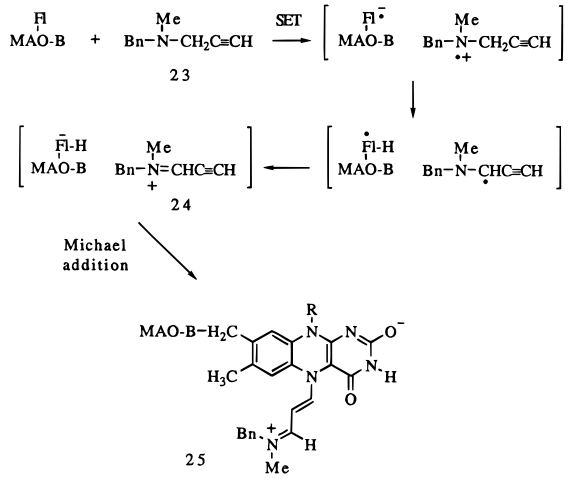
(31) Pienta, N. J. In *Photoinducd Electron Tranfer*; Fox, M. A.. Channon, M., Eds.; Elsevier: New York, 1988; Part C, pp 421−486. Lewis, F. D. *Acc. Chem. Res.* **1986**, *19*, 401.

(32) Wayner, D. D. M.; McPhee, D. J.; Griller, D. *J. Am. Chem. Soc.* **1988**, *110*, 132.

Scheme 9



Scheme 10



primary amines often have higher oxidation potentials than their tertiary analogues.[31,33] Indeed, the current photochemical studies suggest that the aminium radical derived from **6** undergoes ready retro-aldo like fragmentation. Thus, the inability of **6** to serve as an MAO-A inactivator may be a consequence of the fact that it, like other primary and secondary amine substrates and inactivators, does not serve as an effective SET-donor to the flavin group in the enzyme.

The possibility exists that several other well-known MAO inactivation reactions, previously discussed in terms of radical coupling mechanisms, may also follow routes involving generation of and alkylation by electrophilic intermediates. For example, Silverman[11] has shown that *trans*-2-phenylcyclopropylamine **22** reacts with MAO-B to yield a single covalent adduct via alkylation of the key cycteine residue. Bond formation in this process may well be a result of the formation[11] and Michael addition of cinnamaldehyde (or its imine) (see Scheme 9).

In a similar way, MAO-B inactivation by the tertiary propargylic amine, pargyline **23**[34] may be promoted by an initial SET step,[12] but the known flavocyanine adduct **25** might arise by Michael addition to the α,β-unsaturated iminium cation intermediate **38** (Scheme 10) rather than by a radical coupling mechanism.

In summary, a novel mechanism-based design strategy has been used to discover a new class of MAO-inactivators. Further efforts are necessary, however, to fully elucidate the interrelationships that may or may not exist between MAO-inactivation and catalytic reaction pathways and to refine the design of β-amino alcohol inactivators to yield candidates with higher binding affinities and inactivation rates.

(33) Yoon, U. C.; Mariano, P. S. *Acc. Chem. Res.* **1992**, *25*, 233.

(34) Maycock, A. L.; Abeles, R. H.; Salach, J. I.; Singer, T. P. *Biochemistry* **1976**, *15*, 114.

Experimental Section

General. All reactions were run under N_2 or Ar atmospheres unless otherwise noted and magnesium sulfate was used as a drying agent. All new compounds were obtained as oils in >90% purity (by ^{1}H and ^{13}C NMR) unless otherwise noted. ^{1}H NMR (200, 400, 500 MHz) and ^{13}C NMR (50 MHz) spectra were recorded on $CDCl_3$ solutions unless otherwise noted and chemical shifts are reported in parts per million relative to $CHCl_3$ (7.24 for ^{1}H NMR and 77.00 for ^{13}C NMR) as an internal standard. Coupling constants are given in Hertz (Hz). ^{13}C NMR resonance assignments were aided by use of the DEPT technique to determine number of attached hydrogens. Mass spectrometric data was obtained by use of either electron impact (EI) or chemical ionization (CI) techniques and fragments are recorded as *m*/*z* (relative intensity). Infrared (IR) spectroscopic data are recorded in units of cm^{-1}. Optical rotations were recorded by using the sodium D-line (589 nm). Thin-layer chromatography (TLC) was performed on 0.25 mm silica coated glass or plastic plates. Preparative TLC was performed on 20 × 20 cm plates coated with silica gel. Column chromatography was performed with the use of 230−400 mesh silica gel, 100−200 mesh fluorisil, or 80−200 mesh alumina. Unless otherwise noted, materials obtained from commercial sources were used without further purification. All distillations were performed under dry N_2 or Ar atmospheres unless otherwise noted. All reaction solvents were dried and distilled prior to use. The solvent used for photoreactions (MeOH) was of spectrograde quality.

Preparative photochemical reactions were conducted by using an apparatus consisting of a 450W Hanovia medium pressure, mercury lamp surrounded by a Uranium glass filter (λ > 330 nm) within a quartz, water cooled well immersed in the reaction solution (ca. 15 °C reaction temperature). In each case, the photolysis solution was purged with deoxygenated N_2 before and during irradiation.

MAO-A (K_M = 0.12 mM, k_{cat} = 35 min^{-1}) was purified[35] from the yeast *Saccharomyces cerevisiae* which was overexpresseed with human liver MAO-A (gift from Walter Weyler). MAO-B was obtained from beef liver, purchased fresh from a local slaughter house, and was purified by a minor modification of the procedure of Salach.[36a]

Irradiation of 3MLF and the Amino Alcohol 4. A solution (170 mL) of 77 mg (0.26 mmol) of 3MLF and 120 mg (0.45 mmol) of the amino alcohol **4** in MeOH was irradiated for 1 h. The photolyzate was concentrated in vacuo to give a residue which was dissolved in $CHCl_3$ and diluted with *n*-hexane. The resulting precipitate was collected by filtration to give 101 mg (96%) of the adduct **8**.[14] Concentration of the filtrate gave 5 mg (4%) of 3-(*N*-benzyl-*N*-methyl)-aminopropiophenone (**9**).

9: ^{1}H NMR 2.20 (s, 3H, N−CH_3), 2.83 (t, J = 7.0 Hz, 2H, H-2), 3.12 (t, J = 7.0, 2H, H-3), 3.49 (s, 2H, benzylic), 7.25, 7.38, 7.50, and 7.90 (m, 10H, aromatic); ^{13}C NMR 36.8 (C-2), 42.1 (N−CH_3), 52.4 (C-3), 62.3 (benzylic), 126.9, 128.0, 128.1, 128.5, 128.9, 132.8, 136.9, and 138.7 (aromatic Ph), 199.3, (C═O); IR 1682; EIMS 253 (M, 2), 176 (2), 162 (41), 134 (100), 105 (42), 91 (85), 77 (20); HRMS(EI) *m*/*e* 253.1478 ($C_{17}H_{19}NO$ requires 253.1467).

Irradiation of 3MLF and 4-(*N*,*N*-Dimethylamino)-3-phenylbut-1-en-3-o1 (5). A solution of 71 mg (0.26 mmol) of 3MLF and 86 mg (0.45 mmol) of the amino alcohol **5** in MeOH (150 mL) was irradiated for 1 h. The photolyzate was concentrated in vacuo to give a residue which was dissolved in 1 mL of $CHCl_3$. To this solution was added 30 mL of *n*-hexane, and the resulting precipitate was collected by filtration to give 98 mg of the 4a-adduct (91%) **8.**[14]

Irradiation of 3MLF and 4-Amino-1-buten-3-phenyl-3-ol (6). A solution of 71 mg (0.26 mmol) of 3MLF and 74 mg (0.46 mmol) of the amino alcohol **6** in MeOH (150 mL) was irradiated for 1 h. The photolyzate was concentrated in vacuo to give a residue which was

(35) Weyler, W.; Titlow, C. C.; Salach, J. I. *Biochem. Biolphys, Res. Commun.* **1990**, *173*, 1205 and ref 23b.

(36) (a) Salach, J. I.; Weyler, W. In *Methods in Enzymology*; Academic Press: New York, 1987; Vol. 142, pp 627−637. Salach, J. I.; Weyler, W. *Arch. Biochem. Biophys*. **1979**, *192*, 128. (b) Tabor, C. W.; Tabor, H. *J. Biol. Chem.* **1954**, *208*, 645.

(37) Kim, J. M.; Cho, J. S.; Mariano, P. S. *J. Org. Chem.* **1991**, *56*, 4943.

dissolved in ether. Filtration gave 17 mg (24%) of 3-MLF as the precipitate. Concentration of the filtrate in vacuo gave a residue which was subjected to preparative TLC on silica gel (ether) to provide 55 mg (68%) of the 4a-adduct **8**.[14]

Irradiation of 3MLF and 4-(*N*,*N*-Dimethylamino)-3-phenylbut-1-yn-3-ol (7). A solution of 50 mg (0.19 mmol) of 3MLF and 105 mg (0.56 mmol) of the amino alcohol **7** in 150 mL of MeOH was irradiated for 2 h. The photolyzate was concentrated in vacuo giving a residue which was mixed with ether, followed by filtration to give 26 mg of 3-MLF as the precipitate. The filtrate was concentrated in vacuo giving a residue which was subject to preparative TLC on silica gel (ether) to yield 27 mg of recovered amino alcohol **7**, 18 mg (50%) of the adduct **10E** (mp 229−231 °C), and 16 mg (45%) of the adduct **10Z** (mp 211−213 °C).

10E: ^{1}H NMR: 2.19 (s, 6H, C-7 and C-8), 3.31 (s, 3H, N-10), 3.67 (s, 3H, N-3), 5.08 (s, 1H, NH), 6.65 (d, 1H, J = 15.5, H-1′), 6.69 (s, 1H, C-6), 6.84 (s, 1H, C-9),6.92 (d, 1H, J = 15.5, H-2′), 7.37, 7.55, 7.66 (m, 5H, aromatic); ^{13}C NMR 19.4 (C-7 and C-8 CH_3), 28.4 (N-10 CH_3), 32.4 (N-3 CH_3), 65.8 (C-4a), 117.0 (C-1′), 117.7 (C-2′), 125.7 (C-8), 128.6, 128.7, 133.6, 136.6 (aromatic), 129.0 (C-7), 129.4 (C-9a), 130.2 (C-5a), 134.9 (C-6), 134.9 (C-9), 155.6 (C-10a), 160.0 (C-2), 167.0 (C-4), 188.3 (C-3′); IR 3294, 1713, 1678, 1654, 1560, 1063; EIMS 402 (M, 15), 345 (30), 240 (41), 105 (100), 149 (52), 77 (33), 51 (45); HRMS (EI) *m*/*z* 402.1702 ($C_{23}H_{22}N_4O_3$ requires 402.1692).

10Z: ^{1}H NMR: 1.76 (s, 3H, C-8), 2.05 (s, 3H, C-7), 3.29 (s, 3H, N-10), 3.46 (s, 3H, N-3), 5.34 (s, 1H, NH), 5.75 (d, 1H, J = 12.7, H-1′), 6.26 (s, 1H, C-6), 6.63 (d, 1H, J = 12.7, H-2′), 6.66 (s, 1H, C-9), 7.37, 7.55, 7.60 (m, 5H, aromatic); ^{13}C NMR 18.9 and 19.2 (C-7 and C-8 CH_3), 28.5 (N-10 CH_3), 32.3 (N-3 CH_3), 59.0 (C-4a), 117.2 (C-1′), 118.0 (C-2′), 125.6 (C-8), 128.4, 128.7, 134.1, 136.4 (aromatic), 128.6 (C-7), 130.0 (C-9a), 133.5 (C-6), 134.4 (C-5a), 134.8 (C-9), 155.8 (C-10a), 160.0 (C-2), 166.4 (C-4), 191.3 (C-3′); IR 3346, 2943, 1725, 1667, 1557, 1283, 1146; EIMS 402 (M, 19), 345 (100), 303 (22), 240 (60), 105 (38), 77 (27), 51 (30); HRMS (EI) *m*/*z* 402.1698 ($C_{23}H_{22}N_4O_3$ requires 402.1692).

Enzyme Assays. MAO-A was assayed by the method of Weissbach.[19] MAO-A in 50 mM sodium phosphate buffer (pH 7.2) containing 0.2% Triton X-100 at 25 °C was mixed with 1 mM kynuramine as the substrate. The rate of the kynuramine reaction to form 4-hyroxyquinoline was measured by monitoring the increase in absorbance at 314 nm. The enzyme activity of MAO-B was measured by use of a modified procedure of Tabor.[36b] MAO-B in 50 mM sodium phosphate buffer (pH 7.2) containing 0.2% Triton X-100 at 25 °C was mixed with benzylamine as the substrate. The rate of reaction to produce benzaldehyde was measured by monitoring the increase in absorbance at 250 nm. One unit of enzyme activity equals that amount needed to form 1 μmol of product per minute.

Inhibition of MAO-A Catalysis of Kynuramine Oxidation. Initial velocities for MAO-A catalyzed oxidation of kynuramine[19] were determined by monitoring the changes in absorbance at 314 nm. Reactions were initiated by adding MAO-A (final concentrations in the range of 5.95−10.4 μM) to solutions containing varying concentrations of kynuramine and the selected inhibitors in 50 mM sodium phosphate buffer at pH 7.2 containing 0.2% Triton X-100 at 25 °C. The K_i value for each inhibitor was determined by use of a Lineweaver−Burk[20b] plot of 1/ [kynuramine] vs 1/initial velocity and the Cleland enzyme kinetic computer analysis.[21] All K_i determinations were made in duplicate.

Time Dependent Inactivation of MAO-A. Aliquots of an MAO-A stock solution were added to solutions containing varying concentrations of the inactivators in 50 mM sodium phosphate buffer (pH 7.2), containing 0.2% Triton X-100. After mixing, the samples were incubated at 25 °C and periodically assayed for enzyme activity by use of the kynuramine assay procedure.[19] All inactivations were performed either in duplicate or triplicate. Plots of the natural log of the MAO-A activity remaining versus time in each case gave apparent inactivation rate constants from which the kinetic constants K_{inact} and k_{inact} were determined by the use of Kitz and Wilson plots.[20a]

Effect of Amphetamine on the Inactivation of MAO-A. Solutions of MAO-A in 50 mM sodium phosphate buffer at pH 7.2 with 0.2% Triton X-100 at 25 °C containing either amphetamine, amphetamine and the inactivator, or only inactivator were incubated at 25 °C. Aliquots were removed at various time intervals, and enzyme activity was assayed by use of the kynuramine assay procedure.

Sulfhydryl Titration of MAOs. Solutions of MAO A with and without added inactivator in 100 mM sodium phosphate buffer (pH 7.2) containing 10% glycerol and 0.2% Triton X-100 were incubated at 25 °C. When the enzyme activity was <5% of the control activity, the solutions were dialyzed for 4 h against three changes (500 mL) of 100 mM sodium phosphate buffer (pH 7.2), containing 10% glycerol and 0.2% Triton X-100. The dialyzed solutions were assayed for enzyme activity and protein content.

Thiol titrations with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) were performed according to a modification of the literature procedure.[25] A 200 μL aliquot of either the control or inactivated MAO A solution was added to a solution of 380 μL of deionized water, 200 μL of 100 mM sodium phosphate buffer (pH 8.0), and 100 μL of 20% sodium dodesyl sulfate ($NaDodSO_4$) with 1 mg/mL EDTA. The absorbance of each solution at 412 nm was recorded as an enzyme absorbance background reading. The blank absorbance was zeroed by using a solution of 380 μL deionized water, 200 μL of 100 mM sodium phosphate buffer (pH 8.0), 100 μL of 20% $NaDodSO_4$ with 1 mg/mL EDTA, and 200 μL of 10% glycerol in 100 mM sodium phosphate buffer (pH 7.2), containing 0.2% Triton X-100. A 20 μL aliquot of 4 mg/mL of DTNB in 100 mM sodium phosphate buffer (pH 8.0) was added to each enzyme solution, and the absorbance at 412 nm was measured over a 30 min period. A 20 μL aliquot of the DTNB solution was added to the solution used to prezero the spectrometer, and the absorbance was measured and used as a DTNB absorbance background. The total amount of free 5-mercapto-2-nitrobenzoate produced was calculated from the absorbance at 412 nm of the DTNB treated MAO-A solution minus the two background readings. The assay was performed in triplicate.

Changes in the Flavin UV−Visible Spectrum upon Inactivation of MAO-A. Solutions of MAO-A (25 μM) in 50 mM potassium phosphate buffer, at pH 7.2 containing 0.2% Triton X-100, were placed in individual cuvettes that were then sealed with rubber septa. These cuvettes were repeatedly, sequentially purged with argon and evacuated. A solution of each inactivator was then added by using an airtight syringe, and the UV−visible spectra were recorded periodically over 27 min time period. These experiments were performed in duplicate.

Dimethylamine Production in the Reaction of MAO-A with *β*-Hydroxyamine 5. MAO-A (100 μM) was inactivated with β-hydroxyamne **5** (25 mM) in 50 mM sodium phosphate buffer, at pH 7.2, containing 5% glycerol and 0.2% Triton X-100. The mixture was then made basic by addition of saturated $NaHCO_3$ and mixed with 1 mL of a 2 mg/mL solution of dabsyl chloride in acetone.[30] The solution was kept at 25 °C for 1 h and extracted with chloroform. The extracts were dried, and a 10 μL portion was removed for HPLC analysis (Beckman Ultrasphere ODS C-18 column (0.46 cm × 25 cm), eluted with hexane:2-propanol (80:20) at a flow rate of 1 mL/min). TLC (ether) was performed with dabsyl chloride (R_f = 0.76), β-amino alcohol **5** (R_f = 0.52), and *N*,*N*-dimethyldabsylamide (**21**) (R_f = 0.64).[30] The remaining solution was subjected to preparative TLC (ether). The band that comigrated with *N*,*N*-dimethyldabsylamide (**21**) was collected and subjected to ^{1}H NMR analysis. A control containing 25 mM **5** in 50 mM sodium phosphate buffer, at pH 7.2, containing 5% glycerol and 0.2% Triton X-100 was also treated with dabsyl chloride in the same manner.

Inhibition of MAO-B Catalyzed Oxidation of Benzylamine. Initial velocities for MAO-B catalyzed oxidation of benzylamine[36b] were determined by monitoring the changes in absorbance at 250 nm. Reactions were initiated by adding aliquots of MAO-B to solutions containing varying concentrations of benzylamine and the inhibitors in 50 mM sodium phosphate buffer at pH 7.2 containing 0.2% Triton X-100 at 25 °C. The K_i value for each inactivator was determined by use of the Lineweaver−Burk[20b] plot and Cleland enzyme kinetic computer analysis[21] methodologies. All K_i determinations were made in duplicate.

Time Dependent Inactivation of MAO-B. Aliquots of an MAO-B stock solution were added to solutions containing varying concentrations of inactivators in 50 mM sodium phosphate buffer (pH 7.2), containing 0.2% Triton X-100. The samples were incubated at 25 °C and periodically assayed for enzyme activity by use of the benzylamine assay procedure.[36b] All assays were performed in duplicate.

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Supporting Information Available: Synthetic procedures for the preparation of **4**−**7**, **12**−**14**, and **16**−**18** and a Chem 3D-plot and supporting data of the crystallographically determined structure of **20** (40 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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