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3-Pyrrolines Are Mechanism-Based Inactivators of the Quinone-Dependent Amine Oxidases but Only Substrates of the Flavin-Dependent Amine Oxidases

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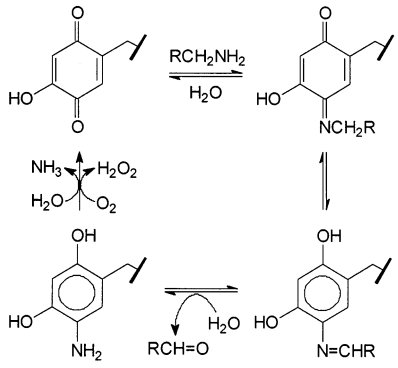
Abstract: We previously reported that 3-pyrroline and 3-phenyl-3-pyrroline effect a time-dependent inactivation of the copper-containing quinone-dependent amine oxidase from bovine plasma (BPAO) (Lee et al. *J. Am. Chem. Soc.* **1996**, *118*, 7241−7242). Quinone cofactor model studies suggested a mechanism involving stoichiometric turnover to a stable pyrrolylated cofactor. Full details of the model studies are now reported along with data on the inhibition of BPAO by a family of 3-aryl-3-pyrrolines (aryl = substituted phenyl, 1-naphthyl, 2-naphthyl), with the 4-methoxy-3-nitrophenyl analogue being the most potent. At the same time, the parent 3-phenyl analogue is a pure *substrate* for the flavin-dependent mitochondrial monoamine oxidase B from bovine liver. Spectroscopic studies (including resonance Raman) on BPAO inactivated by the 4-methoxy-3-nitrophenyl analogue are consistent with covalent derivatization of the 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor. The distinction of a class of compounds acting as an inactivator of one amine oxidase family and a pure substrate of another amine oxidase family represents a unique lead to the development of selective inhibitors of the mammalian copper-containing amine oxidases.

Introduction

The quinone-dependent copper amine oxidases mediate a pyridoxal phosphate (PLP)-like transamination mechanism for conversion of primary amines to aldehydes.[1] Of the several mammalian enzymes in this enzyme class, plasma amine oxidase (PAO) appears to be responsible for regulating the levels of blood-borne biogenic amines and for metabolizing exogenous amines, though the exact roles of this and other tissue-specific copper amine oxidases are only partially understood.

Substrate oxidation is initiated by Schiff base formation between primary amine and the 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor derived posttranslationally from an active-site tyrosine residue present in each monomer of these dimeric enzymes.[2] The resulting "substrate Schiff base" tautomerizes to a "product Schiff base", which in turn hydrolyzes to aldehyde product and a reductively aminated cofactor. The latter is oxidatively recycled to the starting quinone at the expense of reducing O_2 to H_2O_2 (Scheme 1). This mechanism has been reproduced by use of simple models for the TPQ cofactor.[3,4]

Scheme 1



Mechanism-based irreversible inactivators[5] are superior enzyme inhibitors because they result in long-acting inhibition in vivo (recovery of activity requires new protein synthesis) and because they offer a second level of selectivity: a given mechanism-based inhibitor may bind reversibly to and thus

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(1) (a) Knowles, P. F.; Dooley, D. M. *Metal Ions Biol. Sys.* **1994**, *30*, 361−403. (b) Anthony, C. *Biochem. J.* **1996**, *320*, 697−711. (c) Klinman, J. P. *Chem. Rev.* **1996**, *96*, 2541−2561.
(2) (a) Tanizawa, K. *J. Biochem. (Tokyo)* **1995**, *118*, 671−678. (b) Dooley, D. M. *J. Biol. Inorg. Chem.* **1999**, *4*, 1−11. (c) Klinman, J. P. *J. Biol. Chem.* **1996**, *271*, 27189−27192.
(3) (a) Wang, F.; Bae, J. Y.; Jacobson, A. R.; Lee, Y.; Sayre, L. M. *J. Org. Chem.* **1994**, *59*, 2409−2417. (b) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 3096−3105. (c) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 11823−11828.
(4) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 8707−8718.
(5) Silverman, R. B. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; pp 3−30.

competitively inhibit several enzymes but will have a long-acting effect only on the enzyme that suffers inactivation. Several mechanism-based inactivators for the copper amine oxidase family have been reported over the years. In most cases, the overall approach is to take advantage of the change in hybridization of the amino group-bearing carbon from sp^3 to sp^2 that occurs following transamination, in the same manner that Abeles and Maycock[6] pioneered for the development of amino acid-based inactivators of PLP enzymes. Thus, turnover of β-haloamines or β,γ-unsaturated amines results in α-haloaldehydes or α,β-unsaturated aldehydes, respectively, that may bind covalently to active-site nucleophiles, thereby affording irreversible inhibition. However, other enzymes that carry out the same overall stoichiometry of $RCH_2NH_2 \rightarrow RCH{=}O$, such as the flavin-dependent monoamine oxidase (MAO) associated with the outer mitochondrial membrane, would potentially also be irreversibly inactivated by such agents. Examples of compounds that inhibit both the quinone- and flavin-dependent amine oxidases have been reported.[7]

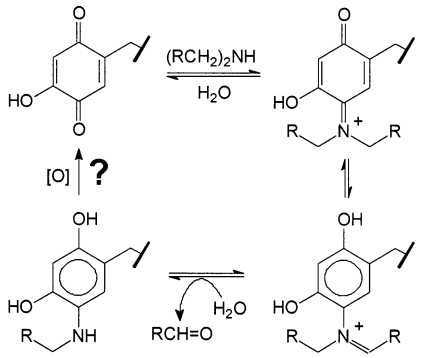
Substantial evidence has been accrued suggesting that MAO-A and -B oxidize their amine substrates by a mechanism initiated by electron transfer and involving radical intermediates.[8] Although other mechanisms for MAO have also been considered,[9−11] it is clear that these flavin-dependent reactions do not utilize an imine shift (transamination) mechanism. Thus, amines capable of effecting a *transamination-specific* irreversible modification should potentially inactivate only the quinone-dependent copper amine oxidase family. Such compounds would be superior probes for evaluating the physiological functions of these enzymes and may have therapeutic potential as selective inhibitors.

We previously reported that 3-pyrrolines inactivate bovine PAO (BPAO) and that model studies with a TPQ mimic suggest that inactivation reflects irreversible pyrrolylation of the enzyme quinone cofactor through a transamination-dependent mechanism.[12] This finding was of special interest in that it represented the first observation of processing of a secondary amine by this enzyme class. Although 3-pyrroline itself was a weak inactivator of BPAO, its 3-phenyl analogue exhibited improved inhibitory properties, a finding in line with this enzyme preferentially metabolizing *aryl*alkylamines rather than simple alkylamines. In this paper, we divulge full details of the inhibitory action of a variety of 3-aryl-3-pyrrolines on BPAO, as well as of model studies directed at elucidating their mechanism of action. In addition, we show that the parent 3-phenyl analogue is an excellent pure substrate (no inactivation) of MAO-B purified from beef liver.

Results and Discussion

Irreversible Inactivation of Bovine Plasma Amine Oxidase by the Secondary Amine 3-Pyrroline. Early studies on several members of the copper amine oxidase family indicated that simple secondary amines (e.g., *N*-methylbenzylamine) are not substrates. According to the transamination mechanism (Scheme 1), processing of a secondary amine (Scheme 2) would lead to an iminium substrate Schiff base and then an iminium product Schiff base that, upon hydrolysis, would *not* afford the normal reductively aminated cofactor; one alkyl arm of the secondary amine would still be attached. It is probable that the enzyme evolved in a manner to deliberately sterically exclude secondary amine substrates, so that it would not have to deal with oxidation of the *N-alkyl* reductively aminated cofactor. We postulate that steric exclusion, rather than the issue of forming quaternary Schiff base intermediates, is the main reason the copper amine oxidases do not normally metabolize secondary amines.

Scheme 2



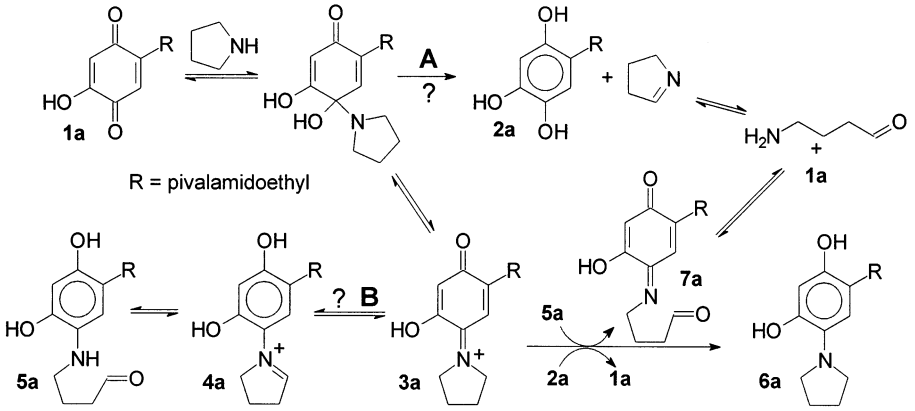
It was thus of great interest to find that, despite detection of no O_2 uptake when BPAO was incubated with up to 10 mM pyrrolidine or 3-pyrroline, preincubation with 3-pyrroline resulted in a concentration- and time-dependent irreversible pseudo-first-order loss of BPAO activity.[12] In this regard, it is clear that irreversible inactivation serves as a much more sensitive test of enzymatic processing than does substrate activity. Kitz and Wilson analysis of the first-order inactivation rate constants for 3-pyrroline at 30 °C, pH 7.2, yielded values of $K_i = 50$ mM and $k_{inact} = 0.3$ min^{-1}.[12] The rather high K_i reflects the expected very weak binding for this simple aliphatic amine. Furthermore, loss of the characteristic 480 nm absorption of the TPQ anion, and the absence of an increase at A_{440} normally observed upon derivatization of the quinone cofactor with phenylhydrazine, suggested complete loss of the cofactor quinone character.

Reaction of Pyrrolidine and 3-Pyrroline with TPQ Models. The reactivity of TPQ with pyrrolidine and 3-pyrroline was explored with TPQ models, as monitored by NMR spectroscopy in CD_3CN. After 2 days, the NMR tube reaction of quinone **1a** with pyrrolidine (3 equiv) was seen to generate (pyrrolidino)-resorcinol **6a** (Scheme 3) in 40% yield, based on integration, along with other unidentified compounds. The appearance of **6a** is understood in terms of generation of quinoniminium intermediate **3a**, and its reduction by a product of redox turnover, either **2a** arising from addition−elimination (Scheme 3, path A) or (alkylamino)resorcinol **5a** arising from transamination via **4a** (Scheme 3, path B).

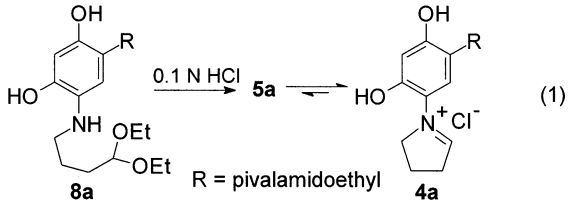
Operation of the transamination path B would mandate generation of **4a**/**5a**. When the reaction mixture of **1a** and pyrrolidine was acid-quenched (with HCl), evaporated, and analyzed by ^{1}H NMR in D_2O, signals corresponding to **4a** were observed, verified by comparison to the ^{1}H NMR spectrum of

(6) Abeles, R. H.; Maycock, A. L. *Acc. Chem. Res.* **1976**, *9*, 313−319.
(7) (a) Lyles, G. A.; Fitzpatrick, C. M. S. *J. Pharm. Pharmacol.* **1984**, *37*, 329−335. (b) Yu, P. H.; Zuo, D.-M. *Biochem. Pharmacol.* **1992**, *43*, 307−312. (c) Riceberg, L. L.; Simon, M.; Van Vunakis, H.; Abeles, R. H. *Biochem. Pharmacol.* **1975**, *24*, 119−125.
(8) Silverman, R. B. *Acc. Chem. Res.* **1995**, *28*, 335−342.
(9) Miller, J. R.; Edmondson, D. E. *Biochemistry* **1999**, *38*, 13670−13683.
(10) Kim, J.; Bogdan, M. A.; Mariano, P. S. *J. Am. Chem. Soc.* **1993**, *115*, 10591−10595.
(11) Anderson, A. H.; Kuttab, S.; Castagnoli, N., Jr. *Biochemistry* **1996**, *35*, 5−3340.
(12) Lee, Y.; Huang, H.; Sayre, L. M. *J. Am. Chem. Soc.* **1996**, *118*, 7241−7242.

Scheme 3



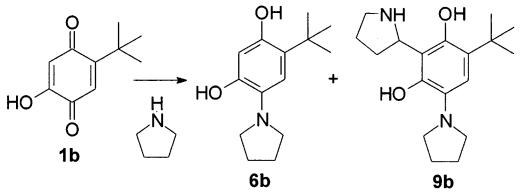
the authentic compound independently synthesized by acid deprotection of **8a** (eq 1). However, **4a**/**5a** could also arise via



(1)

path A if 4-aminobutanal, generated from 1-pyrroline, condensed with **1a** or **3a** to give **7a**, which was then reduced to **5a** by **2a**.

The reaction of pyrrolidine was repeated with the *tert*-butyl TPQ model **1b** to aid in identification of reaction products besides **6**. In addition to the pyrrolidinoresorcinol **6b**, a product **9b** was isolated (eq 2), formally representing condensation (electrophilic aromatic substitution) of **6b** with 1-pyrroline. Since



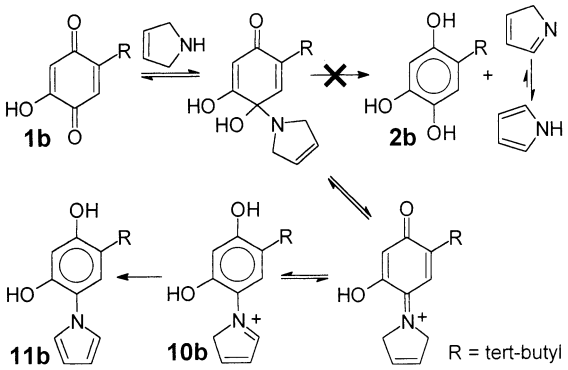
(2)

1-pyrroline can arise either from the addition−elimination mechanism (Scheme 3, path A) or from hydrolysis of **7** formed from oxidation of **5** (Scheme 3, path B), the discovery of **9b** does not help distinguish between operation of the two mechanisms considered in Scheme 3.

In contrast to pyrrolidine, reaction of 3-pyrroline with **1b** in CD_3CN was found to give **11b** quantitatively (Scheme 4) and not a trace of triol **2b**, pyrrole, or (3-pyrrolino)resorcinol analogous to **6**. The lack of side products is understood in terms of the irreversible deprotonation of **10b** giving **11b**, thereby averting generation of a reducing species analogous to **5a**. This result highlights the superb utility of 3-pyrroline as a mechanistic probe for a transamination mechanism. In addition, this observation suggests the dominance of transamination over addition−elimination also for pyrrolidine (Scheme 3), because partitioning of the initial carbinolamine intermediate between elimination and dehydration should hardly be affected by the presence or absence of the remote C═C in 3-pyrroline.

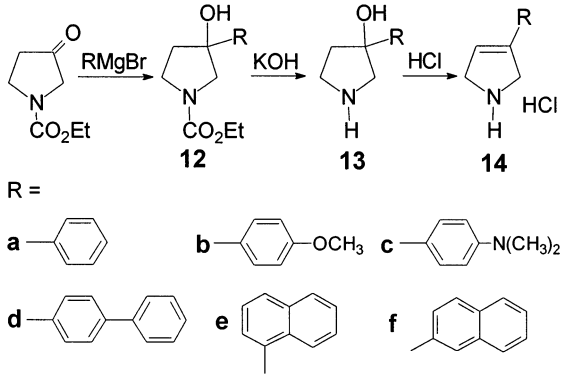
Synthesis of 3-Aryl-3-pyrrolines. Since the preferred substrates of BPAO are arylalkylamines, it was surmised that adding

Scheme 4



an aryl ring "recognition element" to 3-pyrroline would improve binding and thus inhibitory efficiency. A series of 3-aryl-3-pyrrolines **14a**−**f** was synthesized from *N*-(carboethoxy)-3-pyrrolidone by the Grignard/deprotection/dehydration sequence shown in Scheme 5.[13,14]

Scheme 5



To permit spectroscopic studies on interaction of the inhibitor with BPAO, a nitro-containing analogue was also sought. Attempts to prepare 3-(4-nitrophenyl)-3-pyrroline by direct nitration of **14a** or its *N*-acetyl analogue, under a variety of nitration conditions, all caused aromatization or destruction of the pyrroline ring. This failure raised concern that the desired 3-(4-nitrophenyl)-3-pyrroline might be unstable with respect to internal oxidation−reduction to give 3-(4-nitrosophenyl)pyrrole.

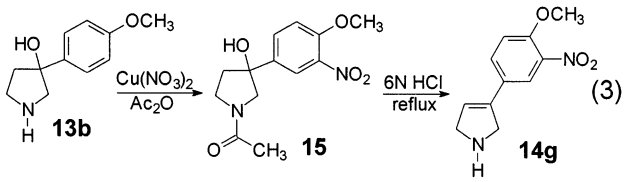
(13) Wu, Y.; Gould, W.; Lobeck, W. G., Jr.; Roth, H. R.; Feldkemp, R. F. *J. Med. Chem.* **1962**, *5*, 752−769.
(14) Gould, W. A.; Lish, P. M.; Wu, Y.-H.; Roth, H. R.; Lobeck, W. G., Jr.; Berdahl, J. M.; Feldkemp, R. F. *J. Med. Chem.* **1964**, *7*, 60−67.

Table 1. Inactivation of BPAO by 3-Aryl-3-pyrrolines (**14a**−**g**)

inactivator	concentration (mM)	$t_{1/2}$[a] (min)
14a	0.4	7
14b	0.4	10
14c	0.4	31
14d	0.1[b]	45
14e	0.4	13
14f	0.2	6
14g	0.015	3

[a] Incubation time required for 50% inactivation. [b] Limited solubility of **14d** precluded its evaluation at higher concentration.

Thus it was decided alternatively to prepare 3-(4-methoxy-3-nitrophenyl)-3-pyrroline (**14g**), presuming that the nitration of 4-methoxy analogue **14b** could be accomplished smoothly and that, with the nitro group being meta rather than para, the compound would be stable with respect to internal redox. Unfortunately, even very mild nitration of **14b** or its *N*-acetyl derivative again resulted in complete loss of the pyrroline ring, indicating that nitration would have to be effected prior to generation of the 3-pyrroline C═C. In this regard, nitration of alcohol **13b** in acetic anhydride with copper nitrate at room temperature successfully afforded the acetylated nitro product **15**; the latter was then heated at reflux with aqueous hydrochloric acid to give the target molecule **14g** (eq 3).



It was considered that the synthetic route could be shortened by one step if the carbethoxy precursor **12b** could be nitrated, and the carbamate could be hydrolyzed in the final acid dehydration step. Although nitration of **12b** proceeded smoothly, conditions could not be found for acid hydrolysis of the ethyl carbamate that preserved the C═C presumed to form quickly. In addition, attempted *basic* hydrolysis of the nitrated carbamate resulted in nucleophilic aromatic substitution (HO replacing CH_3O). Thus, the pathway summarized in eq 3 appears to be the best route to **14g**. Finally, although the successful nitration of alcohol **13b** suggested that we might have been able to prepare the originally sought 4-nitrophenyl analogue via nitration of **13a**, this attempt resulted in dehydration to the exclusion of nitration of the benzene ring.

Evaluation of 3-Aryl-3-pyrrolines as Inhibitors of Bovine Plasma Amine Oxidase. As was the case for the parent 3-phenyl analogue **14a**,[12] all the 3-aryl analogues were more potent time-dependent inhibitors of BPAO than 3-pyrroline itself. Table 1 lists inactivation half-life data for single concentrations of the 3-aryl-3-pyrrolines. The presence of electron-donating 4-methoxy (**14b**) and 4-(dimethylamino) (**14c**) substituents and the sterically bulky 1-naphthyl substituent (**14e**) resulted in less potent inactivators than the parent 3-phenyl analogue **14a**. On the other hand, the 2-naphthyl analogue **14f** and especially the 3-nitro-4-methoxyphenyl analogue **14g** were more potent than **14a**. The latter two compounds and the parent **14a** all displayed a pseudo-first-order loss of BPAO activity [Figure 1(top) and Supporting Information]. Kitz and Wilson replots of the inactivation rate data [Figure 1 (bottom) and

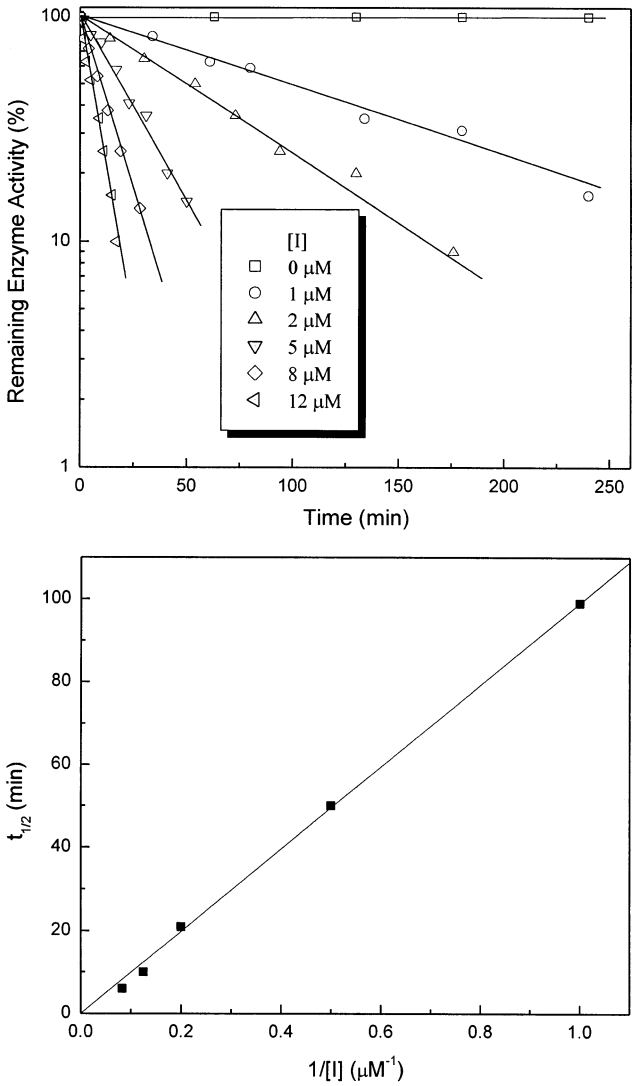


Figure 1. Time-dependent inhibition (top panel) of BPAO by 3-(4-methoxy-3-nitrophenyl)-3-pyrroline (**14g**) and Kitz and Wilson replot of the data (bottom panel).

Supporting Information] in all three cases came too close to the 0−0 origin to justify an attempt to estimate the intercepts. We suspect that the apparent lack of saturation[5,15] reflects a more efficient turnover (leading to inactivation) at the expense of a binding limited by the normal steric exclusion of secondary amines. For comparative purposes, inactivation by **14a**, **14f**, and **14g** can be quantified in terms of second-order rate constants, 230, 528, and 6970 M^{-1} min^{-1}, respectively, which can be compared to $k_{inact}/K_i = 6$ M^{-1} min^{-1} for 3-pyrroline itself.[12] The high potency of **14g** clearly reflects the electron-withdrawing nitro group. For all three cases, enzyme activity was not recovered by gel filtration, indicative of irreversible modification, and the absence of an increase in A_{440} upon treating the inactivated enzyme with excess phenylhydrazine is suggestive of irreversible modification of the quinone cofactor. The inactivation kinetics of the other analogues were not fully characterized, but they most likely follow the same irreversible behavior as do **14a**, **14f**, and **14g**.

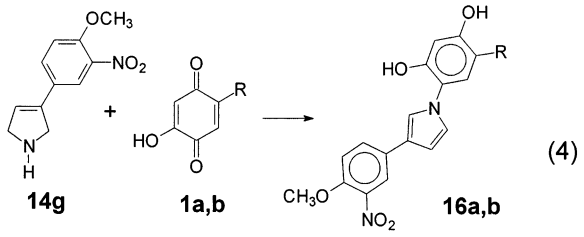
Additional evidence for modification of the quinone cofactor is provided by the nitroblue tetrazolium (NBT) redox cycling

(15) Saturation may become apparent at low temperature.

assay[16] carried out on a sample of purified BPAO that was inhibited by **14a** to the extent of 98.4% and then denatured. Compared to the color intensity of the control sample as representing 100% redox activity, the inhibited sample had no detectable redox cycling activity. On the basis of the accumulated enzymologic data (including the observed lack of O_2 uptake) and the model study results, the most likely explanation for the observed behavior of the 3-pyrrolines is that they induce a stoichiometric covalent modification of the quinone cofactor in a single turnover. To determine whether a native enzyme structure is required for the modification of the quinone cofactor to occur, the NBT assay was conducted on a sample of enzyme treated with the inhibitor *after* denaturation. In this case, the NBT staining was not totally eliminated, as it was when the incubation with inhibitor occurred prior to denaturation (data not shown). The finding that there was still about 60% reduction of the NBT staining observed for the control enzyme sample indicates that the denatured enzyme is still capable of reacting with **14a**, though not to the extent that occurs for the native enzyme. We interpret this difference to indicate the extent to which cofactor derivatization by **14a** is facilitated by the normal catalytic assistance of the active site.

Additional support for compounds **14** satisfying the criteria of mechanism-based inactivation is that substrate benzylamine effected a concentration-dependent (but incomplete) protection against inhibition by **14f** (see Experimental Section) in the presence of catalase used to prevent H_2O_2-dependent inactivation by the benzylamine.[17]

Spectroscopic Analysis of BPAO Inactivated by 3-(4-Methoxy-3-nitrophenyl)pyrroline (14g). The results of the model studies suggested that the mechanism of inactivation of BPAO by 3-pyrroline and its 3-aryl analogues involves condensation with the TPQ cofactor and transamination to give, following loss of proton, the corresponding pyrrolylated cofactor.[12] The availability of chromophoric inhibitor **14g** suggested that direct evidence for the nature of enzyme inactivation might be obtained by comparing the spectral properties of the **14g**-inactivated enzyme with those of small molecule adducts generated by reaction of **14g** with TPQ models. Thus, **14g** was condensed with quinone cofactor models **1a** and **1b** in buffered aqueous CH_3CN, yielding the corresponding model cofactor pyrrole derivatives **16a** and **16b** (eq 4). Absorption spectra of



(4)

a: R = $CH_2CH_2NHC(=O)C(CH_3)_3$
b: R = $C(CH_3)_3$

14g (0.96 mM, HCl salt) and **16b** (1.7 mM) in methanol are displayed in Figure 2. Compound **14g** has λ_{max} at 340 nm (355 in H_2O) with $\epsilon = 1960\ M^{-1}\ cm^{-1}$ (2160 in H_2O), while **16b**

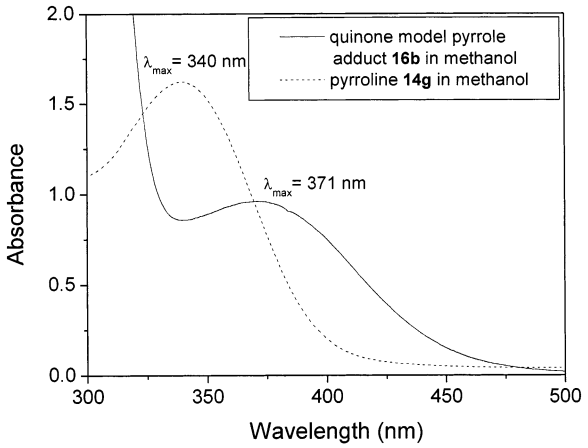


Figure 2. Spectra of **14g** (9.60×10^{-4} M) and **16b** (1.70×10^{-3} M).

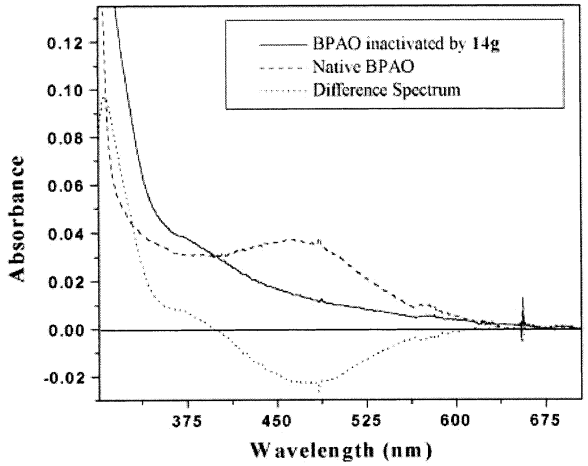


Figure 3. Spectrum of native BPAO (16.95 μM TPQ), spectrum of BPAO inactivated by 1 equiv of **14g** at 30 °C for 3.5 h with subsequent gel filtration and dilution to 17 μM of **14g**−BPAO monomer complex, and difference spectrum.[18]

has λ_{max} at 371 nm with $\epsilon = 570\ M^{-1}\ cm^{-1}$. From these data, one would expect that derivatization of the native cofactor to a pyrrole derived from reaction with **14g** should result in an inactivated enzyme spectrum with a loss of the native 480 nm quinone chromophore and a gain of absorbance near 370 nm.

In Figure 3 are shown spectra for native BPAO and for a sample of enzyme inactivated by **14g**. The inactivated enzyme spectrum exhibits the expected loss at 480 nm and a shoulder near 370 nm, clearly consistent with the proposed enzymatic oxidation of **14g** to give the respective TPQ−pyrrole derivative. Though discerning the shoulder at 370 nm is limited by the strong background absorbance in this region, the estimated difference spectrum,[18] also shown in Figure 3, shows the shoulder at 371 nm more prominently. Furthermore, the increased absorbance in the spectrum of the inactivated enzyme between 300 and 350 nm, as compared to native BPAO, is attributed to a strong absorbance of the TPQ−**14g** derivative

(16) Fluckiger, R.; Paz, M. A.; Gallop, P. M. *Methods Enzymol.* **1995**, *258*, 14−19.
(17) Lee, Y.; Shepard, E.; Smith, J.; Dooley, D. M.; Sayre, L. M. *Biochemistry* **2001**, *40*, 822−829.
(18) Calculation of the difference spectrum required precise knowledge of the concentration of both native and inactivated enzyme cofactor. Whereas this could be calculated in the former case from the known extinction coefficient of enzyme-bound TPQ, this was estimated for the BPAO−**14g** TPQ derivative by assuming that the shoulder at 370 nm represented the proposed pyrrole and that the displacement of the shoulder over the background rise in absorbance in this region could be converted by use of the same extinction coefficient as exhibited by model **16b** at 370 nm. The concentration estimated in this way agreed reasonably well with that based on dilution of the sample obtained following gel filtration.

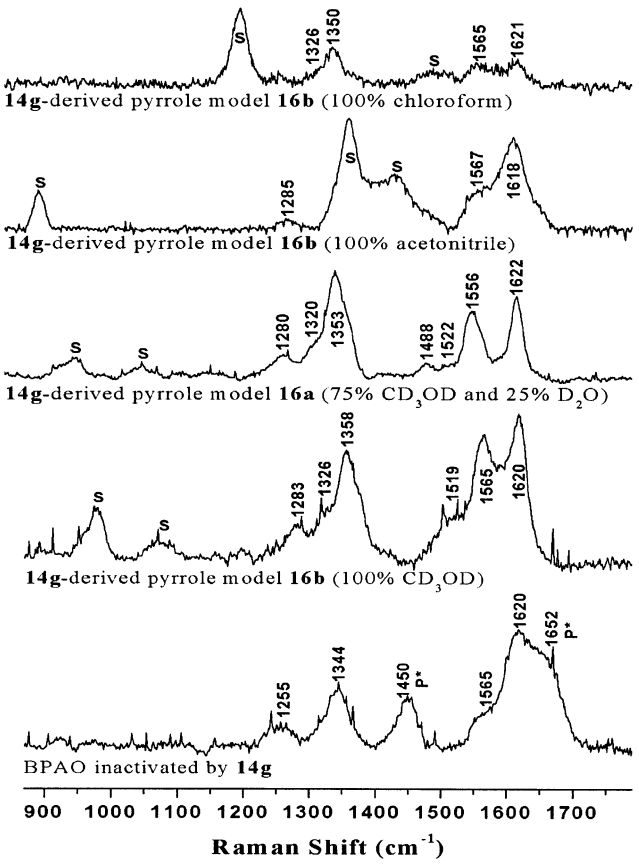


Figure 4. Resonance Raman spectra of **14g**-derived pyrrole model compounds (approximately 5 mM) and of BPAO inactivated by **14g** (~200 μM BPAO dimer). P* denotes protein mode.

chromophore in this region, as seen in the spectrum of model **16b** (Figure 2).

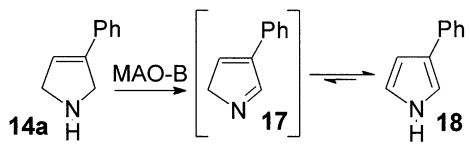
Additional evidence to support enzymatic transformation of **14g** to the TPQ−pyrrole derivative was obtained through comparison of resonance Raman (rR) data on the inactivated enzyme and respective **14g**-derived models **16a** and **16b** (Figure 4). In native BPAO, the region from 1450 to 1690 cm^{-1} is dominated by TPQ ring stretches, with the C5═O stretching mode at 1678 cm^{-1} and a protein mode at 1450 cm^{-1}.[19] Given the observation that the amide I vibration is between 1650 and 1660 cm^{-1},[20] we tentatively assign the 1652 cm^{-1} stretch as a protein mode, in agreement with previous reports.[21,22] Analysis of the rR data for the inactivated enzyme reveals the presence of the 1450 and 1652 cm^{-1} modes and the absence of the C5═O stretch. The latter suggests that the C5═O bond has been altered upon modification by **14g**.

Comparison of the rR spectra of the inactivated enzyme and respective model compounds reveals similar vibrational patterns, with the 1565 and 1620 cm^{-1} modes matching exactly. We propose that the 1344 cm^{-1} mode in the inactivated enzyme spectrum relates to the 1350−1358 cm^{-1} modes seen in the model rR data in solution. The variation of the latter mode over the range 1350−1358 cm^{-1} in the model spectra shown is presumably due to different solvent interactions. The data obtained for model **16b** in 63% CD_3OD and 37% D_2O (not shown) depict a shift from 1358 cm^{-1} (in 100% deuterated methanol) to 1355 cm^{-1}. Additionally, a shift from 1344 to 1350 cm^{-1} was observed in the rR spectrum of a denatured preparation of BPAO inactivated by **14g** (data not shown; see Experimental Section). Except for this shift, which we believe to be solely due to interactions with solvent, the rR spectrum for the denatured sample was identical to that shown in Figure 4 for the nondenatured enzyme. We can tentatively assign the 1344−1358 cm^{-1} frequency (inactivated enzyme and models) to the nitro aromatic stretch (aryl−NO_2) originating from **14g**, as stretching modes for such groups range from ~1330 to 1360 cm^{-1}.[20] This assignment is also in agreement with the 1357 cm^{-1} mode seen in the Raman spectrum for **14g** itself in H_2O (data not shown) and with our previous rR analysis of BPAO inactivated by 4-nitrobenzylamine, where a 1344 cm^{-1} mode was observed.[17]

Three or more coupled C═C stretches have a characteristic vibration between 1540 and 1620 cm^{-1},[20] so it is possible that the 1565 and 1620 cm^{-1} modes are due to the aryl conjugation present in the adduct. Since there are modes at 1570 and 1620 cm^{-1} in the Raman spectrum of **14g** (data not shown), it is plausible that the frequencies at 1565 and 1620 cm^{-1} are due to the coupled deformation of ring modes in the TPQ−**14g** derivative. It should be noted that the apparent shift of the mode at 1565−1567 cm^{-1} observed for model **16b** to 1556 cm^{-1} for **16a** in 75% CD_3OD and 25% D_2O is not a solvent shift and must reflect subtle differences between the two quinone models, since this mode in the rR spectrum of **16b** in 63% CD_3OD and 37% D_2O still occurs at 1565 cm^{-1}.

In the rR spectrum of the BPAO−methylamine adduct, an intense mode at 1616 cm^{-1} was assigned to the N═C stretch of the product Schiff base.[21] The absence of a stretching frequency at 1616 cm^{-1} in the spectrum of the inactivated enzyme is consistent with resonance delocalization in a pyrrolylated cofactor, rather than any derivative containing a localized imine. Overall, the similarities in the spectra of models **16a**/**16b** and the inactivated BPAO−**14g** derivative indicate that the pyrrole structure seen to form in the model reaction is a good candidate for the structure of the adduct formed enzymatically.

Interaction of 3-Phenyl-3-pyrroline (14a) with Bovine Liver Mitochondrial Monoamine Oxidase B. The reaction of **14a** with MAO-B was followed spectrophotometrically at 30 °C. Over a period of 1 h, there was observed a monotonic conversion of the spectrum of **14a** (λ_{max} 251 nm) to one exhibiting λ_{max} values at 225 and 277 nm with isosbestic points at 236 and 264 nm (difference spectrum is shown in Figure 5), at which point no further spectral change was seen. The final spectrum was identical with that of independently prepared 3-phenylpyrrole (**18**), indicating that **14a** is a substrate for clean dehydrogenation by MAO-B, presumably first giving isopyrrole **17**, which rapidly tautomerizes to **18** (eq 5). In addition,



(5)

(19) Nakamura, N.; Matsuzaki, R.; Choi, Y. H.; Tanizawa, K.; Sanders-Loehr, J. *J. Biol. Chem.* **1996**, *271*, 4718−4724.
(20) Lin-Vien, D.; Colthup, N.; Fately, W.; Grasselli, J. *The Handbook of Infrared and Raman Characteristic Frequencies of Organic Molecules*; Academic Press: Boston, 1991.
(21) Nakamura, N.; Moënne-Loccoz, P.; Tanizawa, K.; Mure, M.; Suzuki, S.; Klinman, J. P.; Sanders-Loehr, J. *Biochemistry* **1997**, *36*, 11479−11486.
(22) Lord, R. C.; Yu, N. T. *J. Mol. Biol.* **1970**, *50*, 509−524.

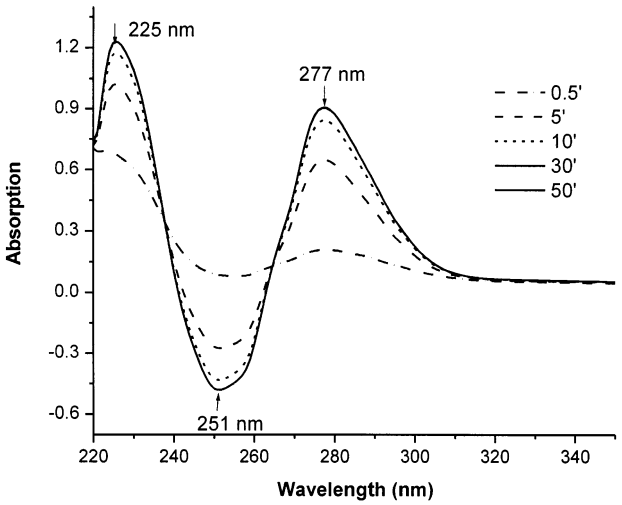


Figure 5. Time course of MAO-B-mediated oxidation of 3-phenyl-3-pyrroline (**14a**). The spectrophotometer was blanked on a solution of **14a** (100 μM) in 50 mM sodium phosphate, pH 7.2, at 30 °C. Then 25 nmol of MAO-B was added and the spectral changes were followed over time. The 50 min spectrum overlapped completely with the 30 min spectrum.

following incubation of **14a** with MAO-B for 1 h at room temperature, reverse-phase HPLC analysis of the evaporated ether extract of the reaction mixture indicated complete conversion of starting **14a** to a single product with identical retention time to that seen for authentic **18**. Although mass spectrometry was also confirmatory, exhibiting the expected strong molecular ion at *m*/*z* 143, it was found that starting **14a** also exhibited a *m*/*z* 143 mass spectrum, owing to apparently facile dehydrogenation in the mass spectrometer.

Steady-state MAO-B substrate kinetics (25 °C) for **14a**, where the ABTS−horseradish peroxidase coupled assay was used to monitor generation of the stoichiometric byproduct H_2O_2, afforded Michaelis parameters $K_m = 252$ μM and $k_{cat} = 13.9$ min^{-1}, indicating that **14a** is an excellent substrate for MAO-B. Tight binding of **14a** was also apparent by its evaluation as a reversible competitive inhibitor of the MAO-B-mediated oxidation of benzylamine. The Dixon plot of the data (30 °C) revealed a K_i of 35 μM for **14a**. When the benzylamine oxidase activity of MAO-B was followed over time (25 °C) in the presence of various concentrations of **14a**, concentration-dependent inhibition was observed during the first few hours, but no decrease in enzyme activity was observed at 20 h relative to the control incubation containing no **14a**. Thus, **14a** appears to be a pure substrate for MAO-B with no irreversible inhibitory properties.

The substrate activity of 3-phenyl-3-pyrroline (**14a**) for MAO-B is consistent with the recent report that the tertiary *N*-methyl derivatives of 3-pyrroline, 3-phenyl-3-pyrroline, and ring-substituted isoindolines are also substrates for MAO-B (presumably undergoing conversion to the corresponding pyrroles) whereas *N*-methylpyrrolidine is not.[23] Since the latter compound lacks an allylamine fragment, it appears that MAO-B substrate behavior for both the secondary and tertiary five-membered cyclic amines depends on the presence of allylic unsaturation, just as in the case where MAO-B substrate activity is seen for 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) but not for 1-methyl-4-phenylpiperidine.[24]

(23) Wang, Y.-X.; Mabic, S.; Castagnoli, N., Jr. *Bioorg. Med. Chem.* **1998**, *6*, 143−149.

Conclusions. Our studies have shown that the inactivation of bovine plasma amine oxidase by 3-pyrrolines generalizes to 3-aryl-3-pyrrolines with differing phenyl ring substituents. The fact that the 4-methoxy-3-nitrophenyl analogue is one of the most potent BPAO inactivators known (15 μM effects 50% inactivation in 3 min at 30 °C), is remarkable, considering that secondary amines were previously thought to be nonsubstrates for this enzyme. It appears that inactivation efficiency is greatly improved by electron-withdrawing groups that enhance amine C_α deprotonation, as has been seen for the substrate activity of substituted benzylamines.[25] Model studies, supported by spectroscopic studies on the inactivated enzyme, suggest a mechanism involving a unique conversion of the quinone cofactor to a reduced pyrrolylated form that cannot be reoxidized to the starting quinone. There is no evidence for competing productive turnover, and indeed, cofactor pyrrolylation would correspond to mechanism-based inactivation with a partition ratio of 0. In contrast, for bovine liver mitochondrial monoamine oxidase B, the parent 3-phenyl analogue **14a** is an excellent substrate for dehydrogenation to 3-phenylpyrrole. The action of a specific class of compounds as pure inactivators of one enzyme class and pure substrates for another enzyme class is extraordinary, permitting us to refer to the 3-pyrrolines as *transamination-specific* mechanism-based inactivators.

The finding that the 3-pyrrolines are selective inhibitors of the quinone-dependent amine oxidases does not preclude the ability of appropriately substituted 3-pyrrolines to exert selective inactivation of particular enzymes within this family. Indeed, preliminary studies on **14a** against a series of purified amine oxidases indicate that whereas 1 equiv (based on active sites) of **14a** effected ∼50% loss of activity of equine plasma amine oxidase (EPAO) and *Pichia pastoris* lysyl oxidase (PPLO) in addition to BPAO, 40 equiv was needed to effect ∼80% inhibition of *Arthrobacter globiformis* phenethylamine oxidase (AGAO), and 40 equiv effected only 13−16% inhibition of pea seedling amine oxidase (PSAO) and human kidney amine oxidase (HKAO).[26] Thus, in addition to selective inhibition of the quinone-dependent amine oxidases on the basis of mechanism, a further level of discrimination can be achieved by fine-tuning preferential recognition by the enzyme substrate binding cavity. Overall, the development of highly selective inhibitors should be possible, and such inhibitors should aid in unraveling the only partly understood physiological functions of the various mammalian quinone-dependent amine oxidases, as well as serving as potential drug candidates.

Experimental Section

General Methods. NMR spectra were obtained at 300 MHz (^{13}C NMR at 75 MHz), with chemical shifts referenced to the solvent peak. In the ^{13}C NMR spectra, attached proton test (APT) designations are given as (+) for CH_2 and C and (−) for CH_3 and CH. High-resolution mass spectra (HRMS), electron impact or fast atom bombardment (FAB), were obtained at 20−40 eV on a Kratos MS-25A instrument. UV−vis spectra were obtained on either Perkin-Elmer Lambda 3 (chemical studies) or Lambda 10 (kinetics studies) spectrophotometers fitted with a jacketed (temperature-controlled) cell compartment.

(24) Heikkila, R. E.; Manzino, L.; Cabbat, F. S.; Duvoisin, R. C. *J. Neurochem.* **1985**, *45*, 1049−1054.
(25) Hartmann, C.; Klinman, J. P. *Biochemistry* **1991**, *30*, 4605−4611.
(26) Purification of these enzymes and conduct of their activity assays is as described: Shepard, E. M.; Smith, J.; Elmore, B. O.; Kuchar, J. A.; Sayre, L. M.; Dooley, D. M. *Eur. J. Biochem.* **2002**, *269*, 3645−3658.

Absorption spectra were taken on a Hewlett-Packard 8453 photodiode array spectrophotometer. Raman spectra were collected on a Spex Triplemate 1877 (1800 groove setting), with a liquid-N_2 cooled Spex Spectrum One CCD detector and Coherent Ar ion laser. Doubly distilled water was used for all experiments. All solvents, reagents, and organic fine chemicals were the most pure available from commercial sources. Pyrrolidine was freshly distilled before use. Bovine plasma amine oxidase for kinetic studies (BPAO, 100 units/g of protein) and Sephadex G-25 were purchased from Sigma. The concentration of BPAO active monomer was estimated from the rate of benzylamine oxidation (1 unit oxidizes 1.0 μmol of benzylamine to benzaldehyde per minute at 25 °C), using an activity of 0.48 unit/mg of protein for the pure monomer of molecular weight 85 000[27] and $\Delta\epsilon_{250} = 12\,800\ M^{-1}\ cm^{-1}$ for benzaldehyde [corresponding to an A_{250} of 12.8 min^{-1} (unit of activity)$^{-1}$ for 1 mL volume]. BPAO used for spectroscopic studies was purified as described previously.[27] Bovine mitochondrial monoamine oxidase B was isolated and assayed as described previously[28] and stored as a concentrated solution (15−25 mg/mL) in 50 mM sodium phosphate buffer, pH 7.2, at 4 °C. O_2 uptake was monitored by a Yellow Springs Instruments 5300 biological oxygen meter. All evaporations were conducted at reduced pressure in a rotary evaporator.

Reaction of TPQ Model 1a with Pyrrolidine. Pyrrolidine (7.5 μL, 0.09 mmol) was added to a 5 mm NMR tube containing 2-hydroxy-5-(2-pivalamidoethyl)-1,4-benzoquinone (**1a**, 7.5 mg, 0.03 mmol) in degassed CD_3CN (0.6 mL). Integration of the ^1H NMR spectrum recorded after 2 days at room temperature indicated generation of **6a** in 40% yield (by comparison to the authentic compound synthesized by "redox cycling" reaction[3a] of triol **2a** with pyrrolidine), along with other unidentified compounds. The reaction mixture was quenched by pouring it into 0.1 N aqueous HCl (1 mL), and the resulting solution was evaporated under high vacuum to give a product mixture, which was dissolved in D_2O. In addition to the major product (HCl salt of **6a**), signals corresponding to the iminium salt **4a** (identified as discussed below) were seen in the ^1H NMR spectrum (~3% of the amount of **6a**).

Independent Generation of Iminium Salt 4a. To a 5 mm NMR tube containing triol **2a** (25.3 mg, 0.1 mmol) in CD_3CN (0.6 mL) was added 4-aminobutyraldehyde diethylacetal (90%, 19 μL, 0.1 mmol) via syringe. The ^1H NMR spectrum after overnight standing showed clean "redox cycling" conversion[3a] of the starting materials to 4-(4,4-diethoxybutanamino)-6-(2-pivalamidoethyl)resorcinol (**8a**): ^1H NMR (CD_3CN) δ 1.11 (s, 9H), 1.12 (t, $J = 6.99$ Hz, 6H), 1.60 (m, 4H), 2.63 (t, $J = 6.80$ Hz, 2H), 3.01 (m, 2H), 3.24 (m, 2H), 3.39−3.65 (4H), 4.46 (m, 1H), 6.36 (s, 2H), 6.76 (br t, $J = 4.38$ Hz, 1H); ^{13}C NMR (CD_3CN) δ 15.7 (2C), 25.5, 27.8 (3C), 30.6, 32.2, 39.1, 41.5, 45.6, 61.9 (2C), 103.6, 104.0, 115.4, 117.3, 131.4, 145.0, 147.5, 180.1. The solution of **8a** was poured into 0.1 N aqueous HCl (6 mL), and the resulting solution was concentrated to dryness under high vacuum to give iminium salt **4a** as a solid: ^1H NMR (D_2O) δ 1.03 (s, 9H), 2,42 (m, 2H), 2.71 (t, $J = 6.38$ Hz, 2H), 3.38−3.42 (4H), 4.58 (m, 1H), 6.54 (s, 1H), 7.21 (s, 1H), 9.02 (br s, 1H); ^{13}C NMR (D_2O) δ 18.9, 26.5 (3C), 28.4, 36.4, 38.3, 39.2, 61.7, 103.4, 117.3, 118.5, 125.8, 149.4, 157.4, 180.6, 182.0.

Reaction of TPQ Model 1b with Pyrrolidine. To a solution of 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone (**1b**, 5.4 mg, 0.03 mmol) in degassed CD_3CN (0.5 mL) was added pyrrolidine (7.5 μL, 0.09 mmol) via syringe. After 3 days, all volatiles were evacuated, and the remaining residue was redissolved in degassed CD_3CN (0.5 mL) for recording of spectral data. Integration of the ^1H NMR spectrum indicated a mixture of **6b** and another major product, 2-(pyrrolidin-2-yl)-4-(pyrrolidin-1-yl)-6-*tert*-butylresorcinol (**9b**), in a ratio of 3:4, along with traces of other side products. Separation of the air-sensitive product mixture was impractical; thus, identification of **9b** was made by acquisition of NMR and mass spectral data on the mixture and then subtracting out signals due to **6b**. **9b**: ^1H NMR (CD_3CN) δ 1.32 (s, 9H), 1.56 (m, 1H), 1.87 (m, 2H), 1.92 (m, 4H), 2.23 (m, 1H), 2.88 (m, 4H), 3.05 (m, 2H), 4.62 (dd, $J = 9.60$ and 7.11 Hz, 1H), 6.96 (s, 1H); ^{13}C NMR (CD_3CN) δ 25.1(2C), 26.1, 30.1(3C), 33.2, 35.1, 45.8, 54.7(2C), 57.6, 112.2, 119.8, 126.8, 128.2, 149.8, 156.7; HRMS calcd for $C_{18}H_{28}N_2O_2$ *m*/*z* (rel intensity) 304.2152, found 304.2100 (16.8%).

Reaction of TPQ Model 1b with 3-Pyrroline. To a solution of **1b** (18 mg, 0.10 mmol) in degassed CD_3CN (0.6 mL) was added 3-pyrroline (11.6 μL, 0.15 mmol) via syringe. The ^1H NMR spectrum was recorded periodically and no further change was seen after 2 h, at which point the only species present was 4-(pyrrol-1-yl)-6-*tert*-butylresorcinol (**11b**): ^1H NMR (CD_3CN) δ 1.34 (s, 9H), 4.83 (br s, OH, 2H), 6.18 (t, $J = 2.14$ Hz, 2H), 6.43 (s, 1H), 6.85 (t, $J = 2.14$ Hz, 2H), 6.98 (s, 1H); ^{13}C NMR (CD_3CN) δ 29.88 (3C), 34.61, 105.61, 109.13 (2C), 122.95 (2C), 125.49, 128.43, 128.84, 150.25, 155.87; HRMS calcd for $C_{14}H_{17}NO_2$ *m*/*z* (rel intensity) 231.1260, found 231.1260 (88.7%).

General Procedure for the Synthesis of Ethyl 3-Aryl-3-hydroxy-1-pyrrolidinecarboxylates 12. To a solution of ethyl-3-oxo-1-pyrrolidinecarboxylate[13] (25 mmol) in anhydrous ether (150 mL) was added dropwise a solution of the substituted aryl Grignard reagent (28 mmol) (prepared from either bromobenzene, 4-bromoanisole, 4-bromobiphenyl, 1-bromonaphthalene, or 2-bromonaphthalene) in anhydrous ether (100 mL) at room temperature. After the addition was completed, the reaction mixture was heated at reflux for 10 min and then cooled to room temperature and poured into 3 M aqueous ammonium chloride (100 mL). The ether layer was separated and dried over sodium sulfate to afford **12** after evaporation of solvent. In the case of **12c**, it was found necessary to replace the use of diethyl ether with THF as both Grignard (from 4-bromo-*N*,*N*-dimethylaniline) and reaction solvent. The products were purified by crystallization (white solids) or flash silica gel chromatography (oils), with isolated yields ranging from 60% to 80%. NMR analysis indicated that compounds **12** exist as mixtures of syn and anti carbamate isomers.

General Procedure for the Synthesis of 3-Aryl-3-pyrrolidinols 13. A mixture of 10 mmol of the ethyl 3-aryl-3-hydroxy-1-pyrrolidinecarboxylate **12**, *n*-propanol (5 mL), H_2O (5 mL), and KOH (3 g) was heated at reflux for 20 h.[14] After cooling, the alcoholic layer was separated and the aqueous layer was extracted with ether. The combined organic layer was dried over sodium sulfate and concentrated to give the corresponding 3-aryl-3-pyrrolidinols **13** as white solids, which were used in the next step without purification (for yields in excess of 80%) or recrystallized (for yields 60−80%).

General Procedure for the Synthesis of 3-Aryl-3-pyrroline Hydrochlorides 14a−f. A solution of 3-aryl-3-pyrrolidinol **13** (7 mmol) in 12 N hydrochloric acid (15 mL) was heated at reflux for 1 h. The crude residue obtained upon evaporation of solvent was recrystallized from ethanol or, in the case of **14b**, from ethanol−ether.

Time-Dependent Inactivation of BPAO by 3-Aryl-3-pyrrolines. Solutions (0.9 mL) of 3-aryl-3-pyrrolines (final concentration ranging from 0.05 to 1 mM for **14a−f** and from 1 to 20 μM for **14g**) in 100 mM potassium phosphate buffer, pH 7.2, were mixed with 0.1 mL of a 20 μM solution of BPAO, and the mixture was incubated at 30 °C. Aliquots (0.1 mL) were periodically withdrawn and diluted with 1.0 mL of benzylamine (10 mM in 50 mM sodium phosphate buffer, pH 7.2) in a 1 cm cuvette (1.5 mL). The rate of oxidation of benzylamine to benzaldehyde was measured by recording the increase in absorbance at 250 nm and compared to the rate of benzylamine oxidation in a companion control solution of enzyme lacking the inhibitor. Since the Kitz and Wilson plots (inactivation $t^{1/2}$ vs [inhibitor]$^{-1}$) went through the 0−0 origin within experimental error, the inactivation kinetics for **14a** were repeated at 5 °C, but the Kitz and Wilson plot still came too close to the 0−0 origin to permit a valid estimate of K_i and k_{inact}.

Irreversibility of BPAO Inactivation by 3-Pyrroline and 3-Aryl-3-pyrrolines. The irreversibility of the inhibition for 3-pyrroline,

(27) Janes, S. M.; Klinman, J. P. *Biochemistry* **1991**, *30*, 4599−4605.
(28) Banik, G. M.; Silverman, R. B. *J. Am. Chem. Soc.* **1990**, *112*, 4499−4507.

3-phenyl-3-pyrroline, and 3-(2-naphthyl)-3-pyrroline was verified by applying enzyme preparations that were 50−90% inhibited to a Sephadex G-25 column (1 × 10 cm) to separate noncovalently bound small molecules. Remeasurement of the enzyme activity and correcting for the volume change indicated no detectable regain of enzyme activity in up to 16 h. Furthermore, incubation of 90% inhibited enzyme (by 3-pyrroline) with phenylhydrazine following Sephadex G-25 chromatography failed to reveal the normal 450 nm complex chromophore.

Nitroblue Tetrazolium Redox Cycling Assay on BPAO Inactivated by 14a. Purified BPAO was incubated with 3.3 mM **14a** for 2 h at 30 °C, which achieved >98% inactivation. SDS−PAGE analysis of the inactivated, control, and phenylhydrazine-inactivated enzyme incubations were performed by standard methods on a polyacrylamide slab gel (6% acrylamide with 0.16% bisacrylamide). Inhibited and control enzyme samples (40 μL) were mixed with 20 μL of standard denaturing buffer (10% SDS with 0.5 M 2-mercaptoethanol) and the mixture was heated at 100 °C for 6 min before application in duplicate to two halves of the gel. After the gel was run (at 0.02 amp constant current, voltage near 200 V), it was cut in half and the two halves were stained with either Coomassie blue (0.25% Coomassie in 50% methanol and 7% acetic acid) or 0.24 mM nitroblue tetrazolium in 2 M potassium glycinate, pH 10, for 120 min (BPAO) in the dark.[16] No alteration of the electrophoretic properties of the enzyme or the intensity of Coomassie staining was observed.

Substrate Protection against BPAO Inactivation by 3-(2-Naphthyl)-3-pyrroline (14f). Incubation of BPAO (1.7 μM) with **14f** (0.1 mM) in 100 mM phosphate buffer, pH 7.2, for 20 min at 30 °C resulted in 35% remaining activity after Sephadex G-25 gel filtration. When the inactivation experiment described above was performed in the presence of varying amounts of benzylamine (4, 10, 20, and 30 mM) and catalase (670 units/mL), increased levels of remaining activity (42%, 46%, 56%, and 64%, respectively) were measured after Sephadex G-25 gel filtration of the corresponding incubation solution.

Spectroscopic Characterization of Pyrrole Models 16 and BPAO Modified by 14g. A solution of highly purified BPAO (~25 μM dimer, ~50 μM TPQ) was incubated with 1 or 4 equiv of **14g** based on TPQ content at 30 °C for 3.5 or 1 h, respectively. The protein sample was assayed and found to have no remaining activity. The sample was subsequently run over a Sephadex G-25M PD-10 column in 0.1 M phosphate buffer, pH 7.2, and then concentrated to ~400 μM TPQ, reassayed, and found to contain no activity. Optical spectra of appropriate dilutions of native and inactivated enzyme were recorded (Figure 3). Raman spectra on the undiluted samples (Figure 4) were collected at room temperature after 413.1 nm excitation. The collection times were 5 min (at 30 mW) for model **16b** in 100% acetonitrile, 8 min (at 70 mW) for model **16a** in 75% deuterated methanol and 25% D_2O, 15 min (at 30 mW) for model **16b** in 100% deuterated methanol, and 60 min (at 50 mW) for BPAO inactivated by **14g**. Model **16b** in 100% chloroform was only collected for 0.5 min (at 20 mW) due to the formation of a precipitate. Raman spectra were also collected for model **16b** in 63% CD_3OD and 37% D_2O (8 min at 75 mW), **14g** in H_2O (30 min at 20 mW), and a denatured sample of BPAO inactivated by **14g** (13 min at 28 mW). Preparation of the latter sample involved adding 55.5 μM **14g** to a solution of BPAO (52.6 μM in TPQ). Time point aliquots were removed and assayed for activity. At 3.5 h, 1.6% activity remained and the sample was subsequently run over a Sephadex column as described above. The concentrated sample (~400 μM TPQ) was reassayed and found to contain 2.1% activity. The sample was then thoroughly mixed with 0.45% SDS and 0.1 mM dithiothreitol before being placed into a thermocycler programmed to heat the sample to 55 °C for 6 min with subsequent cooling to 25 °C. Raman data were then immediately collected.

Substrate Activity of 3-Phenyl-3-pyrroline (14a) for MAO-B. For spectrophotometric studies, MAO-B (10 μM) was added to a solution of **14a** (0.1 mM) in sodium phosphate buffer, pH 7.2 (100 mM), in a 1 mL cuvette, and the reaction was monitored in the range of 200−400 nm at 30 °C for 2 h. Steady-state rates of oxidation of **14a** were determined at 25 °C by the horseradish peroxidase-coupled assay for the determination of released H_2O_2 with 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) as chromogen, according to a modification of the published method.[29] In brief, a calibration curve from serial dilutions of a standard H_2O_2 solution was first constructed, with monitoring of the ABTS oxidation product at 414 nm. Then, 5 μL aliquots of incubations of various concentrations of **14a** (0.1−1.2 mM) with MAO-B (2 μM) at 25 °C were removed at standard periods of time and diluted with 0.5 mL of a solution of horseradish peroxidase (10 units/mL) and ABTS (0.2 mM). The amounts of H_2O_2 formed at the various times were then read off the calibration plot, and these values were plotted vs time to obtain the velocity of H_2O_2 production. Steady-state kinetic parameters (K_m and V_{max}) were obtained graphically from a plot of $(\text{velocity})^{-1}$ vs $[\mathbf{14a}]^{-1}$. The value for V_{max} was converted to k_{cat} by dividing by [enzyme].

Inhibition of MAO-B-Mediated Metabolism of Benzylamine by 14a. The initial rates of oxidation of various concentrations of benzylamine (0−2 mM) by MAO-B (10 μM) in the presence of various concentrations of **14a** (0−80 μM) were determined by spectrophotometric monitoring of the production of benzaldehyde at 250 nm in 100 mM Tris buffer, pH 9.0, at 30 °C. A Dixon plot of the data $(\text{velocity})^{-1}$ vs [**14a**] was consistent with competitive inhibition, for which the reversible inhibition constant K_i could be calculated. To address the possibility of irreversible MAO-B inhibition by **14a**, the enzyme (100 μM) was incubated with various concentrations of **14a** (0−6 mM) in 100 mM phosphate buffer, pH 7.2, at 25 °C. At various time intervals up to 20 h, aliquots (20 μL) of the primary incubation mixture were removed and diluted with 0.5 mL of a solution of benzylamine (1 mM) in 100 mM Tris buffer, pH 9.0, at 30 °C, and the rate of production of benzaldehyde was determined spectrophotometrically.

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Supporting Information Available: Preparation and characterization of **6a**−**b**; characterization data on **12a**−**f**, **13a**−**f**, and **14a**−**f**; preparation and characterization of **15**, **14g**, **16a**−**b**, and **18**; activity vs time semilog plots and Kitz and Wilson plots for inactivation of BPAO by **14a** and **14f**; Lineweaver−Burk plot for substrate activity of **14a** for MAO-B; Dixon plot for inhibition of MAO-B-mediated oxidation of benzylamine by **14a**; activity vs time plot for MAO-B-mediated oxidation of benzylamine in the presence of various concentrations of **14a** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(29) Szutowicz, A.; Kobes, R. D.; Orsulak, P. J. *Anal. Biochem.* **1984**, *138*, 86−94.