J|A|C|S
ARTICLES
Published on Web 06/03/2004

Selective Inhibition of Bovine Plasma Amine Oxidase by Homopropargylamine, a New Inactivator Motif

Chunhua Qiao, Heung-Bae Jeon, and Lawrence M. Sayre*

Contribution from the Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

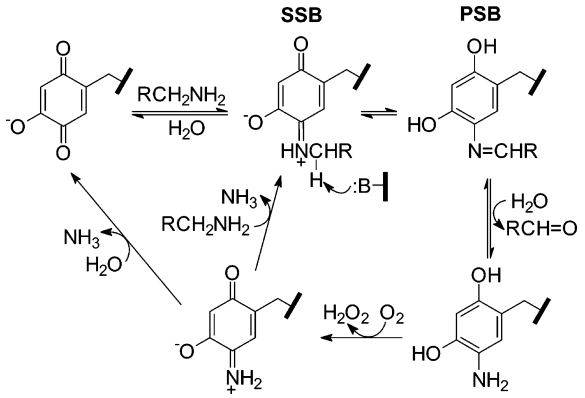
Received January 24, 2004; E-mail: lms3@case.edu

Abstract: Propargylic and activated allylic amines are known to inactivate the quinone-dependent plasma amine oxidases, possibly through active-site modification by the α,β-unsaturated aldehyde turnover products. Although homopropargylamine (1-amino-3-butyne, **1**) is a nonobvious candidate as a mechanism-based inhibitor, **1** was found to be an unusually potent time- and concentration-dependent irreversible inactivator of bovine plasma amine oxidase (BPAO), exhibiting a 30 min IC_{50} of 2.9 μM at 30 °C ([BPAO] = 1.2 μM). Preserved cofactor redox activity of the denatured inactivated enzyme indicates that inactivation by **1** involves either a cofactor modification that reverses upon enzyme denaturation or a modification of an active-site residue. Because inactivation by **1** may involve enzyme alkylation by the reactive 2,3-butadienal (**3**) tautomer of the 3-butynal turnover product of **1**, aldehyde **3** was prepared and was found to inactivate BPAO, but only at high concentration. In addition, whereas inhibition by **3** was blunted by the presence of mercaptoethanol, no such protection was observed against **1**. The amine whose turnover should lead directly to **3** was prepared (1-amino-2,3-butadiene, **4**) and was found to be an even more potent inactivator of BPAO than **1**, exhibiting a 5 min IC_{50} of 1.25 μM. Rat liver mitochondrial monoamine oxidase was also inactivated by **4**, as expected, but only very weakly by **1**. Potential mechanisms explaining the selective inhibition of BPAO by **1** are discussed.

Introduction

Oxidative deamination of primary amines by the copper-containing amine oxidases is known to be mediated by an active-site Tyr-derived 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor.[1] Following condensation of the substrate amine with TPQ to give the "substrate Schiff base" (SSB), a conserved active-site catalytic base (Asp) abstracts the C_α proton, inducing tautomerization to the "product Schiff base" (PSB), which hydrolyzes to aldehyde product and reductively aminated cofactor (an aminoresorcinol). The latter subsequently is reoxidized at the expense of reduction of O_2 to H_2O_2 with hydrolytic release of NH_3 or displacement of NH_3 by another substrate amine (Scheme 1). That amines bearing an unsaturated C−C bond at the β-position could inactivate bovine plasma amine oxidase (BPAO) was first reported by Abeles and co-workers in the 1970s.[2] Recently, inactivation of BPAO by various propargylamine and chloroallylamine analogues was studied extensively in this laboratory.[3] The structure-inhibitory profile for these analogues suggests that size and shape attributes can greatly modulate inactivation potency. These differences and the nature of the inactivating moiety can also control selectivity of inactivation among the large family of known copper amine oxidases.[4]

Scheme 1

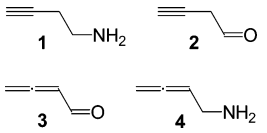


In terms of inactivation mechanism, present data on the propargylamines are consistent with alkylation of an active-site residue by the reactive RC≡C−CH═O product or the allenic tautomer RCH═C═C═O, either as free aldehyde or in imine linkage to the reduced aminoresorcinol form of the cofactor at the product Schiff base stage prior to hydrolytic release of aldehyde.[3] On the basis of such inactivation mechanism, amines bearing a γ- rather than β-C≡C bond are not obvious candidates as inactivators, since their predicted immediate turnover product aldehydes are not conjugated. Nonetheless, a preliminary study indicated that 1-amino-3-butyne (**1**) is in fact a highly potent inactivator of BPAO.[5] In considering

(1) Brown, D. E.; McGuirl, M. A.; Dooley, D. M.; Janes, S. M.; Mu, D.; Klinman, J. P. *J. Biol. Chem.* **1991**, *266*, 4049−4051.
(2) Abeles, R. H.; Maycock, A. L. *Acc. Chem. Res.* **1976**, *9*, 313−319.
(3) (a) Jeon, H. B.; Sun, G.; Sayre, L. M. *Biochim. Biophys. Acta* **2003**, *1647*, 343−354. (b) Jeon, H. B.; Sayre, L. M. *Biochem. Biophys. Res. Commun.* **2003**, *304*, 788−794. (c) Jeon, H. B.; Lee, Y.; Qiao, C.; Huang, H.; Sayre, L. M. *Bioorg. Med. Chem.* **2003**, *11*, 4631−4641.
(4) Shepard, E. M.; Smith, J.; Elmore, B. O.; Kuchar, J. A.; Sayre, L. M.; Dooley, D. M. *Eur. J. Biochem.* **2002**, *269*, 3645−3658.

10.1021/ja049568o CCC: $27.50

a possible mechanistic explanation based on the known facile tautomerization of the theoretical turnover product 3-butynal (**2**) to 2,3-butadienal (**3**), we also synthesized and evaluated the inhibition of BPAO by 1-amino-2,3-butadiene (**4**), which turns out to be one of the most potent inactivators of BPAO known to date. Full characterization of the inhibition of BPAO by these compounds is herein described, and selectivity was assessed by determining their ability to inhibit rat liver mitochondrial monoamine oxidase. Possible mechanisms of inactivation consistent with the data are discussed. Development of selective inhibitors is important in terms of the emerging physiological roles of the human forms of the quinone-dependent amine oxidases.



Results and Discussion

Irreversible Inactivation of BPAO by 1-Amino-3-butyne (1). We found that the known **1**[6] could be prepared by direct amination of the mesylate of commercially available 3-butyn-1-ol, without the need to go through the azide intermediate.[7] Amine **1** is a very potent time- and concentration-dependent inactivator of BPAO at 30 °C (benzylamine as substrate), with the loss of enzyme activity following pseudo-first-order kinetics (Figure 1A). The enzyme (1.2 μM) was half inactivated at 10 μM in 5 min and at 2.9 μM at 30 min, but the Kitz and Wilson replot[3c] ($t_{1/2}$ vs 1/[*I*]) of the data comes too close to the 0−0 origin to justify extrapolation of a K_I. In an attempt to observe saturation, we repeated the kinetics at 2 °C. Under these conditions, the 5 min IC_{50} was 62.2 μM and pseudo-first-order kinetics were again followed (Figure 1B), but the Kitz and Wilson replot of the data still came too close to the origin. The inactivation is thus best described in terms of bimolecular units (slope through the origin): 17 mM^{-1} min^{-1} at 30 °C and 4.8 mM^{-1} min^{-1} at 2 °C. The lack of measurable saturation does not require an interpretation that **1**, which has no intrinsic reactivity, is reacting in a bimolecular reaction with the enzyme, but only that the characteristic k_{inact} is fast compared to dissociation of the E·I complex. At 30 °C, even at the lowest concentration evaluated (2 μM), no plateau behavior was seen up to 2 h, suggesting that if there is any competing productive turnover, the partition ratio is very low.

No recovery of activity was seen either after gel filtration or after 24 h dialysis, indicating that **1** is an irreversible inactivator of BPAO, implicating a stable covalent adduct with BPAO. Phenylhydrazine forms an intensely yellow derivative (absorbing at 450 nm) of the quinone cofactor of BPAO,[8] and thus a phenylhydrazine spectral titration has been used to assess the state of the cofactor as a function of various enzyme modifications.[3] Treatment of BPAO inactivated by **1** with phenylhydrazine failed to show the characteristic 450 nm absorbance, indicating either that **1** modifies the cofactor or that **1** modifies

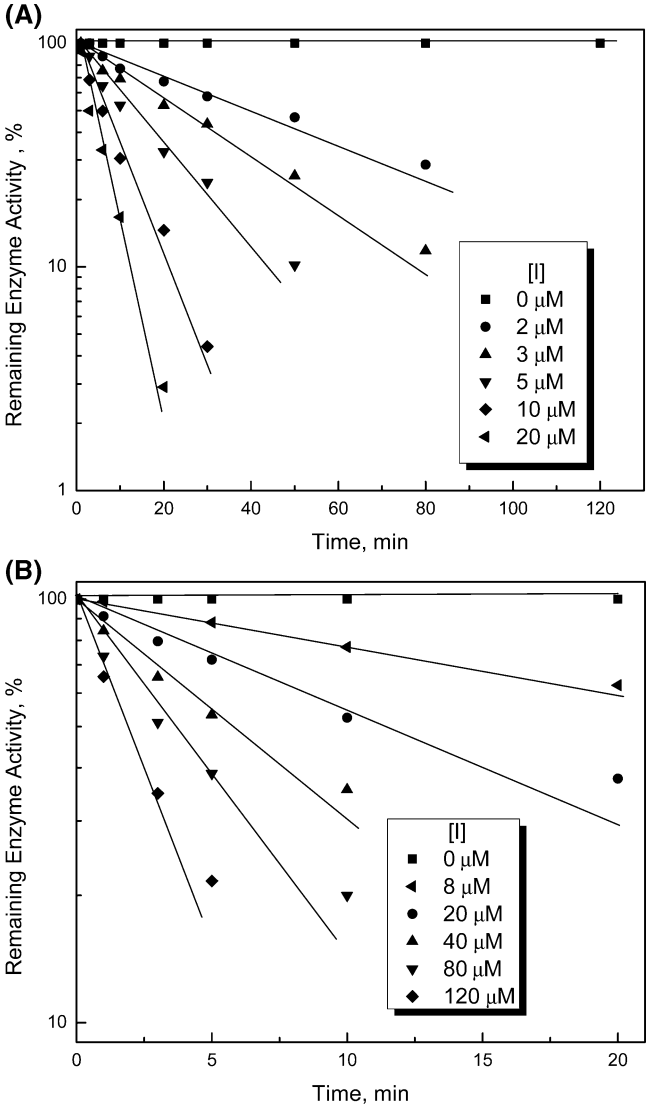


Figure 1. Inactivation of BPAO (1.2 μM) by 1-amino-3-butyne (**1**) in 100 mM, pH 7.2, phosphate buffer at 30 °C (panel A) and 2 °C (panel B).

an active-site residue in a manner that sterically blocks access of phenylhydrazine. Further information was gained by performance of the so-called redox cycling assay with nitroblue tetrazolium (NBT) on the denatured inactivated enzyme.[9] This assay determines the competency of the quinone cofactor to mediate the O_2-dependent chemical deamination of glycinate (reoxidation of the glycinate-reduced cofactor generates superoxide anion, which is detected by NBT) and is independent of steric accessibility issues. The NBT staining for denatured BPAO inactivated by **1** was indistinguishable from that for the denatured control enzyme, indicating complete quinone cofactor redox competence following denaturation of the inactivated enzyme. These results together demonstrate that **1** inactivates BPAO either by covalent binding to an active-site residue that blocks substrate access to the quinone cofactor or by tying up the cofactor in a form that reverses only during enzyme denaturation.

Attempted Preparation of 3-Butynal (2) and Enzymologic Evaluation of 2,3-Butadienal (3). In considering possible

(5) Jeon, H. B. Ph.D. Thesis, Case Western Reserve University, Cleveland, OH, 2001.
(6) Dumont, J. L.; Chodkiewicz, W.; Cadiot, P. *Bull. Soc. Chim. Fr.* **1967**, 588−596.
(7) Taylor, E. C.; Pont, J. L. *Tetrahedron Lett.* **1987**, *28*, 379−382.
(8) Janes, S. M.; Klinman, J. P. *Biochemistry* **1991**, *30*, 4599−4605.
(9) Paz, M. A.; Fluckiger, R.; Boak, A.; Kagan, H. M.; Gallop, P. M. *J. Biol. Chem.* **1991**, *266*, 689−692.

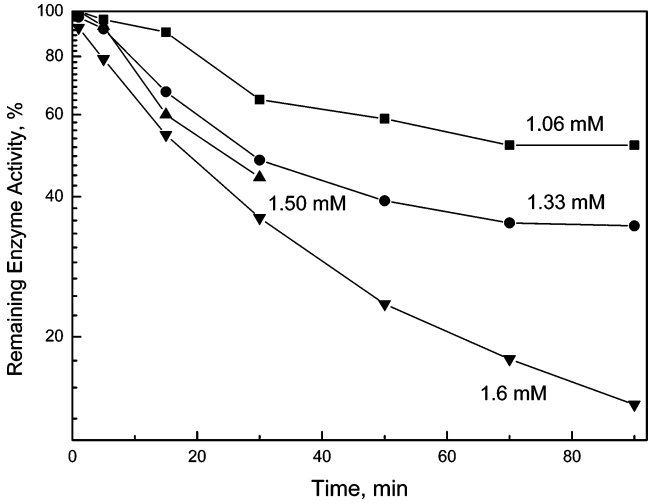


Figure 2. Inhibition of BPAO by 2,3-butadienal (**3**) at 30 °C, 100 mM, pH 7.2, phosphate buffer.

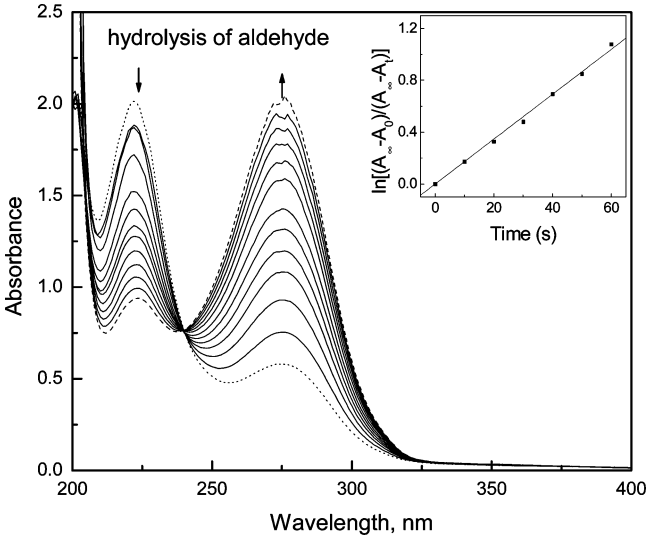
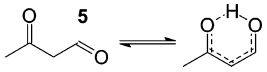


Figure 3. Hydrolysis of 2,3-butadienal (**3**) in 100 mM, pH 7.2, phosphate buffer at 30 °C showing isosbestic conversion to **5**. Time interval: 10 min. Inset: $\ln[(A_\infty - A_0)/(A_\infty - A_t)]$ vs time plot for the increase of absorbance at 276 nm due to **5**.

mechanisms for the inactivating effect of **1**, it was desirable to evaluate the effect of incubating the enzyme with the theoretical turnover product of **1**, 3-butynal (**2**). The acetal precursor, 4,4-diethoxy-1-butyne was successfully prepared by the reaction of propargyl bromide with triethyl orthoformate in the presence of aluminum amalgam as catalyst.[10] We knew that to avoid tautomerization of **2** to its allenic form, 2,3-butadienal (**3**), the subsequent acidic hydrolysis would have to be carried out under mild conditions. However, all efforts to generate **2** in good yield failed. Although many different acids were tried, either no hydrolysis occurred or, once there was sufficient acid strength to begin to convert the acetal, a substantial amount of **2** underwent tautomerization to **3**. Another route to aldehydes is the cleavage of dithioacetals by mercury(II), ferric(III), or cerium(III) salts under neutral conditions. 4,4-Diethoxy-1-butyne was successfully converted to 4,4-bis(ethylsulfanyl)-1-butyne,[11] but even mild oxidative cleavage conditions failed to afford the desired aldehyde **2**. A summary of these experiments confirms the high instability of 3-butynal with respect to its allenyl tautomer and is consistent with the fact that **2** has been reported in the literature only as a mixture with **3**.[12]

The facile tautomerization reaction of **2** to **3** suggests that if **2** were truly the initial direct product of metabolic turnover of **1**, it would soon be converted to **3**. Also, it is possible that the tautomerization could occur at the product Schiff base stage prior to hydrolytic release of aldehyde. In either case, enzyme inactivation could then be arising from alkylation of an active-site nucleophile by the highly reactive electrophile 2,3-butadienal (**3**). Thus, it seemed prudent to evaluate the interaction of BPAO with **3**, which was already in hand from attempted synthesis of **2**, but was prepared in excellent yield independently by a known procedure.[13] Data shown in Figure 2 demonstrate that **3** exerts time- and concentration-dependent inactivation of BPAO, *though only at relatively high concentrations.*

At lower concentrations of **3** ($\leq$1.3 mM), the loss of activity with time reached a plateau after 1.5 h. Assuming **3** inactivates BPAO by a simple bimolecular alkylation reaction (or via an E·I complex characterized by a high K_i), the cessation of further inactivation after some time suggests that little **3** remains at this point, even though its initial concentration was far in excess over enzyme. In fact, aldehyde **3** was expected to hydrolyze in aqueous solution to 3-oxobutanal (**5**).



The hydrolysis of **3** was monitored over time spectrophotometrically under the conditions used for the enzyme incubations (pH 7.2, 100 mM potassium phosphate buffer, 30 °C). As judged by the isosbestic behavior shown in Figure 3, consumption of **3** (λ_{max} 222 nm) occurs concomitant with the formation of **5** (λ_{max} 276 nm), which was shown by ^{1}H NMR to exist at least partly as the enol.[14] Under these conditions, **3** has a half-life of 38 min. The authentic compound **5** was prepared[15] and shown not to inhibit BPAO at a concentration up to 2 mM. Thus, the plateau behavior in Figure 2 at lower concentrations of **3** can be understood in terms of the predominance of the unimolecular hydrolysis reaction (converting inactivator **3** to noninactivator **5**) in competition with the bimolecular enzyme inactivation reaction. In contrast, at higher concentrations of **3**, the bimolecular inactivation reaction proceeds to near completion within the lifetime of **3**. The sensitivity of this competition to relatively small changes in concentration of **3** is remarkable.

Irreversible Inactivation of BPAO by 1-Amino-2,3-butadiene (4). The strong inactivation of BPAO by **1** coupled with the observation of enzyme inactivation by **3** prompted us to explore the activity of 1-amino-2,3-butadiene (**4**), since this is the amine that would theoretically give **3** as its normal turnover product. The known amine **4** was prepared[16] and was found to

(10) Vereshchagin, L. I.; Gavrilov, L. D.; Titova, E. I.; Vologdina, L. P. *Zh. Org. Khim.* **1973**, *9*, 247−252.
(11) Rothstein, E.; Whiteley, R. *J. Chem. Soc., Abstr.* **1953**, 4012−4017.
(12) Blankespoor, R. L.; Smart, R. P.; Batts, E. D.; Kiste, A. A.; Lew, R. E.; Van der Vliet, M. E. *J. Org. Chem.* **1995**, *60*, 6852−6859.
(13) (a) Shostakovskii, M. F.; Khomenko, A. K. *Bull. Acad. Sci. USSR, Div. Chem. Sci.* **1960**, *1022−1026*. (b) Burgers, P. C.; Holmes J. L.; Lossing, F. P.; Mommers, A. A.; Povel, F. R.; Terlouw, J. K. *Can. J. Chem.* **1982**, *60*, 2246−2255.
(14) George, W. O.; Mansell, V. G. *J. Chem. Soc., B* **1968**, 132−134.
(15) Vavilova, A. N.; Trofimov, B. A.; Volkov, A. N.; Keiko, V. V. *J. Org. Chem. USSR* **1981**, *17*, 809−812.
(16) Casara, P.; Jund, K.; Bey, P. *Tetrahedron Lett.* **1984**, *25*, 1891−1894.

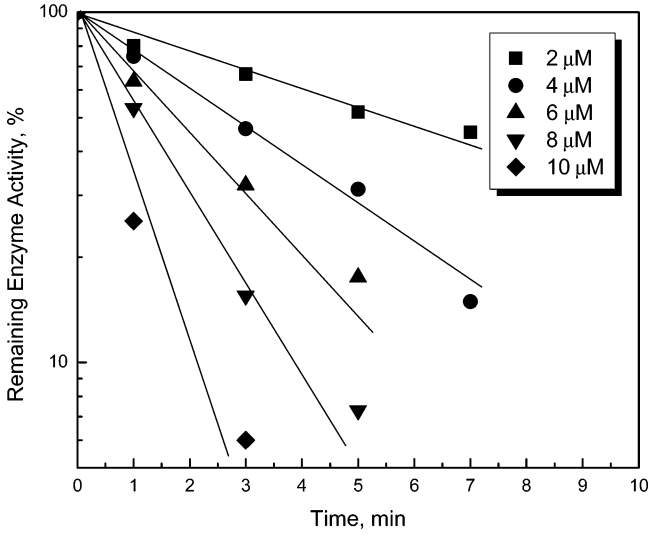


Figure 4. Time course of inactivation of BPAO by various concentration of 1-amino-2,3-butadiene (**4**) at 2 °C, 100 mM, pH 7.2, phosphate buffer.

be an even more potent time- and concentration-dependent inactivator of BPAO than **1**, exhibiting a 5 min IC_{50} of 1.25 μM at 30 °C. At 2 μM **4**, the enzyme lost more than 90% activity in 10 min. This makes **4** one of the most potent inactivators of BPAO reported to date.[3] A Kitz and Wilson replot of the activity time data at 30 °C was very close to the 0−0 origin. Inactivation data was also obtained at 2 °C (Figure 4), but even at this low temperature the Kitz and Wilson replot of the data failed to provide an intercept that was significantly different from the 0−0 origin. Thus, as with **1**, the kinetics are best described in apparent bimolecular terms: 7.1×10^2 mM^{-1} min^{-1} at 30 °C and 98 mM^{-1} min^{-1} at 2 °C.

No recovery of enzyme activity was observed after dialysis of BPAO inactivated by **4**, indicating that inactivation is irreversible. However, as revealed by an unaltered NBT redox cycling activity but a complete absence of derivatization by phenylhydrazine, **4** must be inactivating BPAO either by modifying an active-site residue that sterically interferes with substrate processing or by derivatizing the TPQ cofactor in a form that reverses during enzyme denaturation. As was true also for **1**, these two possibilities could conceivably be distinguished by using radiolabeled inhibitors, in that radiolabel would remain bound to protein during denaturation or would be released, respectively. However, the likelihood of adventitious covalent binding of **3** to surface residues discouraged our consideration of such experiments.

Substrate Protection against BPAO Inactivation by 1 and 4. Despite the lack of a demonstrable saturation phenomenon for **1** and **4**, the absence of any intrinsic chemical reactivity of these unsaturated amines leads to the conclusion that they are turnover-dependent (mechanism-based) inhibitors. A key criterion in this regard is that one should observe concentration-dependent substrate protection against the loss of activity. BPAO (1.2 μM) was incubated under standard conditions at 30 °C with a concentration of each inhibitor that resulted in ~5% remaining activity after PDX G.F.25 gel filtration. The same incubations were then performed in the presence of either 1 or 5 mM benzylamine and catalase (0.6 mg, 1200−3000 units/mL).[17]

(17) Lee, Y.; Shepard, E.; Smith, J.; Dooley, D. M.; Sayre, L. M. *Biochemistry* **2001**, *40*, 822−829.

Table 1. Protection by Substrate Benzylamine (BA) against Inactivation of BPAO by 1-Amino-3-butyne (**1**) and 1-Amino-2,3-butadiene (**4**)

	remaining enzyme activity (%) after 30 min		
inhibitor	[BA] = 0	[BA] = 1 mM	[BA] = 5 mM
1 (7 μM)	8.9	27.8	66.4
4 (1.5 μM)	8.2	23.5	32.2

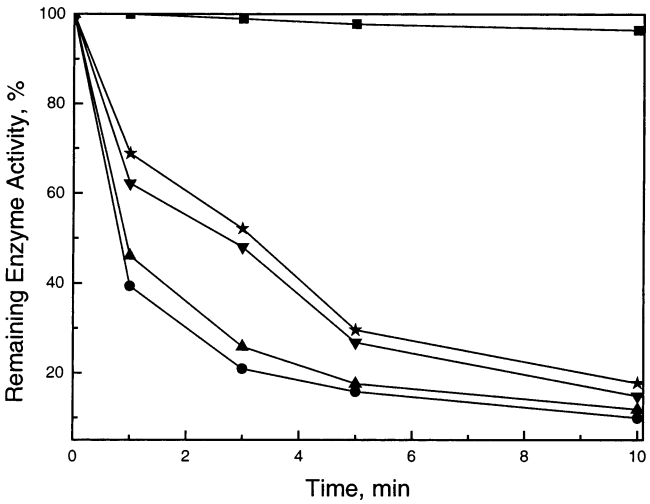


Figure 5. Inhibition of BPAO by 2 μM **4** (●) and protection by 20 μM (▲), 200 μM (▼), or 1 mM (★) thiocholine bromide. Thiocholine bromide alone at 5 mM (■).

Following gel filtration, comparison of the remaining enzyme activity in the presence versus the absence of substrate indicated a concentration-dependent substrate protection in both cases (Table 1).

Abatement of Inactivation by Thiocholine. Our preliminary study showed that some thiols, such as thiocholine (bromide) and mercaptoethanol, at concentrations that had an insignificant effect on benzylamine oxidase activity of BPAO, reduced the extent of inactivation caused by certain mechanism-based inhibitors.[5] Interestingly, mercaptoethanol protected in only rare cases, whereas thiocholine was effective against several of the inactivators studied. Mercaptoethanol appears to act by trapping a solvent-borne reactive intermediate. Whereas thiocholine can also function as a trapping agent, recent studies (C. Qiao and L. M. Sayre, unpublished work) suggested that the ability of thiocholine to inhibit inactivation was related to its ability to act as a competitive inhibitor toward processing of certain classes of substrates (including mechanism-based inhibitors) but not of benzylamine. This appears to involve interaction with a cation binding site in the enzyme.

Mercaptoethanol up to 10 mM had no effect on inactivation by **1** or **4** (data not shown). Also, thiocholine up to 10 mM had no effect on inhibition by **1**. However, as shown in Figure 5, thiocholine exerted a concentration-dependent (20−1000 μM) slowing of the rate of loss of activity caused by 2 μM **4**, mainly at early time points (1−5 min), with the ultimate level of inactivation (e.g., at 10 min) being relatively unaltered. Thiocholine alone had a very minor effect on enzyme activity even at 5 mM. Whereas benzylamine protected against inactivation by *both* **1** and **4**, and because the structures of **1** and **4** are nearly identical, the effect of thiocholine on inactivation by **4** but not **1** suggests that thiocholine is not behaving as a simple

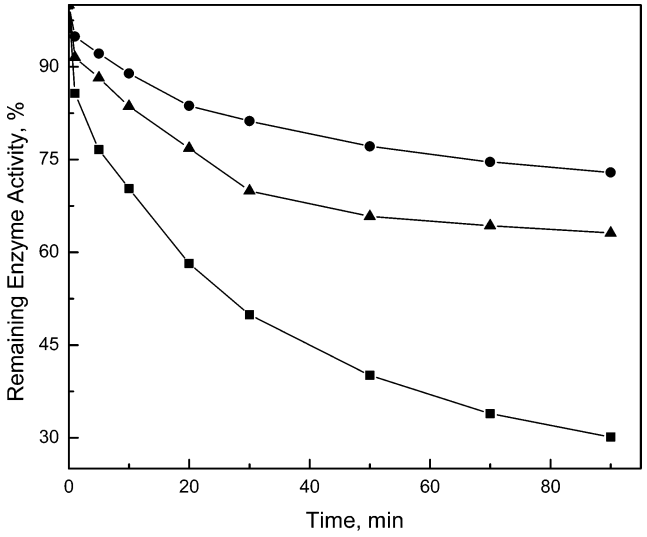


Figure 6. Time course of inactivation of BPAO by 1.3 mM **3** (■) and the blunting of inactivation by the co-presence of 1.3 mM thiocholine bromide (●) or 1.3 mM mercaptoethanol (▲).

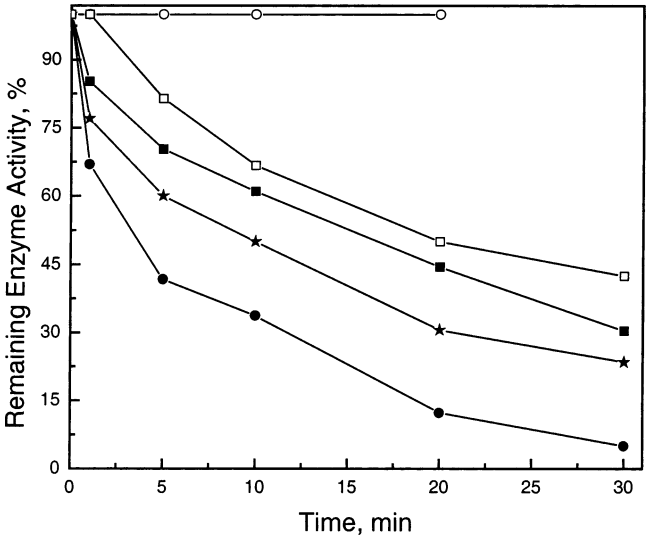


Figure 7. Inactivation of rat liver mitochondrial benzylamine oxidase activity by **1** at 0.2 (○) and 1 mM (□), and by **4** at 0.5 (■), 2 (★), and 5 μM (●).

competitor in the binding step. We consider instead that thiocholine exerts its effect at some stage along the inactivation pathway for **4** prior to the crucial alkylation event. Clearly, there are differences in the enzymatic processing of **1** and **4** following enzyme−substrate complex (or even substrate Schiff base) formation, at which point thiocholine could exert a differential effect.

In contrast to the above, both mercaptoethanol and thiocholine can distinctly protect against inhibition caused by 2,3-butadienal (**3**) at equivalent concentrations (Figure 6). This result undoubtedly reflects the straightforward bimolecular trapping of conjugated aldehyde by thiol in the bulk solution, which was demonstrated independently. Thus, in the presence of 1.3 mM thiocholine bromide or 1.3 mM mercaptoethanol, the half-life of 1.3 mM **3** in pH 7.2, 100 mM phosphate buffer at 30 °C is around 22 or 26 min, respectively, in marked contrast to the half-life (38 min) of the aldehyde under simple hydrolysis reaction conditions.

Inactivation of Rat Liver Mitochondrial Monoamine Oxidase by 1 and 4. The selectivity of **1** and **4** for BPAO was evaluated by determining whether these compounds also inhibit the benzylamine oxidase (mainly MAO-B) activity of rat liver mitochondria.[18] Allenylamines are well-known inactivators of MAO,[19] and thus, **4** was expected to be a fairly potent inactivator. The data in Figure 7 shows that whereas both compounds are concentration- and time-dependent inactivators of MAO-B, **4** displayed the expected high inhibitory potency, but **1** was a weak inactivator. Under these conditions, the approximate 20 min IC_{50} values are 0.5 μM for **4** and 1 mM for **1**. In neither case was activity recovered following dialysis, verifying that **1** and **4** are irreversible inactivators.

Using 1 mM **1** and 1 μM **4**, we found that the level of MAO activity lost in 30 min was not significantly effected by the co-presence of either mercaptoethanol or thiocholine at 1 mM (data not shown), indicating that inactivation does not appear to be due to generation of a solvent-borne reactive intermediate in either case. In summary, whereas the allenylamine **4** is a potent inactivator of both BPAO and MAO-B, homopropargylamine (**1**) is a weak MAO-B inactivator. Additional studies in progress on the inhibition by **1** of purified forms of a number of quinone-dependent copper amine oxidases (B. Hale, E. M. Shepard, D. M. Dooley, and L. M. Sayre, unpublished work) confirm the potent inactivation of BPAO by **1**, an approximately 10-fold weaker effect against human kidney diamine oxidase, an even weaker effect on pea seedling diamine oxidase, and very little effect on the amine oxidases from *Arthrobacter globiformis*, equine plasma, and *Pichia pastoris*. Thus, **1** can be considered to be a fairly selective inactivator of BPAO.

Model Studies To Reveal Possible Mechanisms of Inactivation of BPAO by 1-Amino-3-butyne (1). As in the case of other activated allylamines, inactivation by **4** can be readily justified by alkylation of an active-site residue by the α,β-unsaturated aldehyde turnover product or at the preceding α,β-unsaturated imine product Schiff base stage (see below). Since this is not the case for **1**, however, we decided to perform a model reaction with an appropriate TPQ analogue to elucidate intrinsic chemical features of transaminative processing of **1**. Because 5-*tert*-butyl-2-hydroxy-1,4-benzoquinone used in other recent studies[20,21] is not effective at transaminating unactivated amines, the more reactive 3,5-di-*tert*-butyl-1,2-benzoquinone (DTBQ) was chosen, despite its propensity for yielding benzoxazole side products with unbranched primary amines.[22]

An NMR tube-scale reaction of DTBQ and **1**·HCl (0.5 equiv) in DMSO-d_6, using diisopropylethylamine (0.5 equiv) to neutralize the HCl, was monitored by ^{1}H NMR. After mixing

(18) MAO-B is the principal isoform that metabolizes benzylamine in rat liver mitochondria: Kinemuchi, H.; Fowler, C. J.; Tipton, K. F. *Monoamine Oxidase Dis. [Proc. Conf.]*, Paris, Oct. 26−28, 1983; Tipton, K. F., Dostert, P., Strolin Benedetti, M.; Academic Press: London, 1984; pp 53−62.

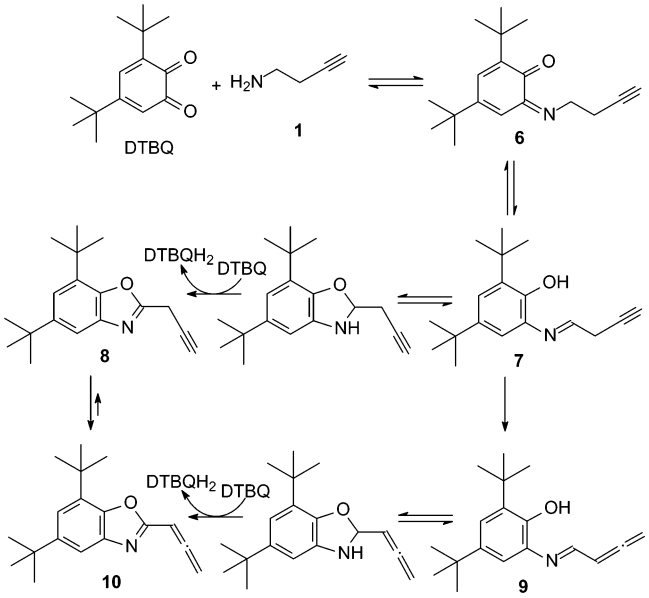
(19) (a) Halliday, R. P.; Davis, C. S.; Heotis, J. P.; Pals, D. T.; Watson, E. J.; Bickerton, R. K. *J. Pharm. Sci.* **1968**, *57*, 430−433. (b) Krantz, A.; Lipkowitz, G. S. *J. Am. Chem. Soc.* **1977**, *99*, 4156−4159. (c) White, R. L.; Smith, R. A.; Krantz, A. *Biochem. Pharmacol.* **1983**, *32*, 3661−3664. (d) Sahlberg, C.; Ross, S. B.; Fagervall, I.; Ask, A. L.; Claesson,; A. *J. Med. Chem.* **1983**, *26*, 1036−1042. (e) Smith, R. A.; White, R. L.; Krantz, A. *J. Med. Chem.* **1988**, *31*, 1558−1566.

(20) (a) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 3096−3105. (b) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 11823−11828. (c) Lee, Y.; Ling, K.-Q.; Lu, X.; Silverman, R. B.; Shepard, E. M.; Dooley, D. M.; Sayre, L. M. *J. Am. Chem. Soc.* **2002**, *124*, 12135−12143.

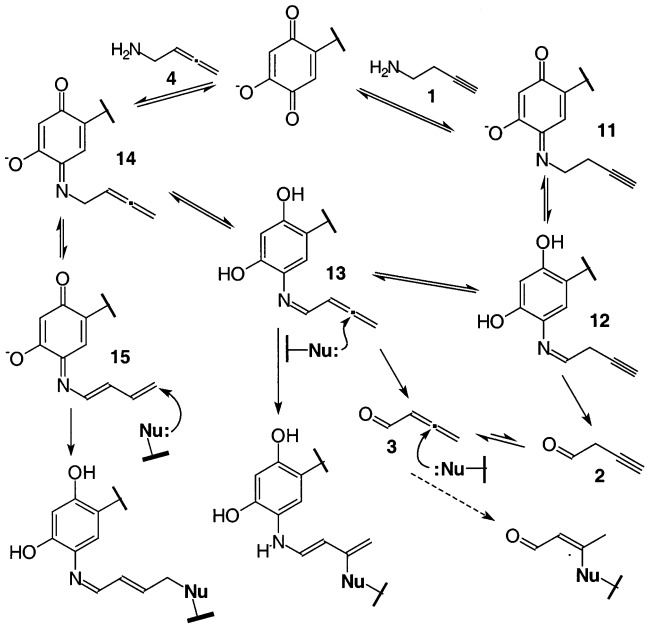
(21) (a) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 8698−8706. (b) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 8707−8718.

(22) Corey, E. J.; Achiwa, K. *J. Am. Chem. Soc.* **1969**, *91*, 1429−1432.

Scheme 2



Scheme 3



DTBQ and **1**·HCl, as soon as the base was introduced, the reaction proceeded very rapidly. The earliest spectrum recorded (2 min) showed that the starting DTBQ had decreased, resulting in the generation of two major products, one being the corresponding 3,5-di-*tert*-butylcatechol ($DTBQH_2$) and the other a benzoxazole derivative (characteristic signals at δ 7.49 and 7.24), exhibiting additional peaks at δ 5.67 (doublet) and δ 6.56 (triplet). At a later time, both reactants decreased further, with a corresponding increase in the catechol and benzoxazole derivative. After 2 h, **1** was completely consumed, and even after 7 h, the NMR spectrum still showed only the initially seen products. Isolation of the benzoxazole derivative by preparative TLC showed it to have the allenic structure **10**.

According to the proposed mechanism for generation of **10** (Scheme 2), initial condensation of **1** with DTBQ generates the substrate Schiff base **6**, which is transformed to the product Schiff base **7** in the key transamination step. The final product **10** can arise from **7** by two different pathways. One possibility is that the dihydrobenzoxazole in equilibrium with **7** is oxidized to **8** by the second equivalent of DTBQ, giving the observed catechol $DTBQH_2$, and then **8** is transformed rapidly to the allene **10** by tautomerization. The other possibility is that **7** is first tautomerized rapidly to its allene form **9** on account of the marked acidity of the methylene position between the C═N and C≡C, with subsequent ring closure and oxidation.

Although no intermediate (e.g., **8** or **9**) was detected in DMSO-d_6, when the same reaction was conducted in CD_3CN/D_2O (9:1), benzoxazole **8** was observed, distinguished from other possible intermediates by having typical benzoxazole aryl ^{1}H NMR signals and a methylene doublet (J = 2.0 Hz) at 4.08 ppm. The earliest spectrum recorded (4 min) showed that the starting DTBQ was still present, along with a mixture of $DTBQH_2$, intermediate **8**, and product **10**. At later reaction times, the starting DTBQ and **8** decreased, with a corresponding increase in $DTBQH_2$ and **10**. After 55 min, the spectrum showed only $DTBQH_2$ and **10** as major products.

Interestingly, when the same reaction was carried out in DMF-d_7, the spectrum exhibited peaks corresponding not to **8**, but instead to a different intermediate maximizing at short reaction time, characterized by an imine CH═N doublet at 8.36 ppm. This intermediate can be assigned to the tautomerized product Schiff base **9** rather than initial product Schiff base **7** on the basis that the CH═N ^{1}H NMR signal of the latter would be a triplet rather than a doublet. These results suggest that both pathways to **10** shown in Scheme 2 can operate, depending on the reaction conditions.

Proposed Mechanism of Inactivation of BPAO by 1 and 4 (see Scheme 3). The model study described above suggests that for 1-amino-3-butyne (**1**), the product Schiff base **12** (corresponding to **7**) may tautomerize to its allene form **13** (corresponding to **9**). The conjugated electrophile **13**, which would form *directly* from the processing of 1-amino-2,3-butadiene (**4**), could then alkylate an active-site residue as a common ultimate inactivating species arising from both **1** and **4**. It is in fact tempting to consider a common mechanism for inactivation of BPAO by these two amines, and the greater potency of **1** could presumably be rationalized on the basis that a second (active-site) base-assisted tautomerization is required in the case of **1**.

However, a common mechanism seems inconsistent with the finding that thiocholine slows down inactivation only by **4**. The action of thiocholine could conceivably be manifested as competitive inhibition in the initial binding step (k_{on}), but the structures of **1** and **4** are so similar (neglecting hydrogens, the only difference is the slightly different bond angle at C-2) that competitive binding is unlikely to reconcile such a pronounced difference in the effect of thiocholine (there is no effect on **1** even at a 500-fold greater concentration). In the same manner, formation of the substrate Schiff base (**11** or **14**) would appear not to be a reaction that could be differentially affected by thiocholine. The apparent modulation by thiocholine of the kinetics of inactivation by **4** can be manifested at any step prior to when the enzyme becomes irreversibly inactivated. Thus, a possible explanation is that thiocholine interferes, perhaps through a conformational perturbation, with the alkylation event. If both **1** and **4** inactivated through the same intermediate **13**,

one could again not easily explain the differential effect of thiocholine.

If product Schiff base **12** arising from **1** undergoes hydrolysis instead of tautomerizing to **13**, then 3-butynal (**2**) would be released and could subsequently tautomerize to the reactive alkylating agent 2,3-butadienal (**3**). If this tautomerization occurs within the active site, alkylation of an active-site residue by **3** could involve different active-site residues than are alkylated by **13** in the case of inactivation by **4**, thus providing a basis for the observed differential effect of thiocholine. However, it is unclear if tautomerization of **2** to **3** could occur during the time that **2** would reside in the active site. If **3** were generated only after **2** were released into bulk solvent, this reactive molecule would be trapped by any added thiol (BPAO was protected against inactivation by externally added **3** by both mercaptoethanol and thiocholine). Thus, although **3** inactivates BPAO at high concentrations, it is unclear to what extent, if any, inactivation of BPAO by **1** (or **4**) occurs through a pathway involving **3**.

A reasonable alternative explanation for the selectivity of thiocholine is that **1** and **4** do not in fact follow identical inactivation mechanisms. One possibility is that in the processing of **4**, C_α proton abstraction by the consensus Asp at the substrate Schiff base stage **14** results in its tautomerization to **15** rather than in cofactor reduction. In this case, cofactor reduction would occur when **15** is attacked by an active-site nucleophile. Because nucleophilic addition to **15** and to **13** (arising from **1**) could clearly reflect distinct spatial arrangements, it would be possible for thiocholine to interfere with one and not the other.

Conclusions

Both homopropargylamine (1-amino-3-butyne, **1**) and 1-amino-2,3-butadiene (**4**) are very potent irreversible inactivators of BPAO. Allylic amine **4** was expected to be an inactivator of this enzyme, and it also potently inhibits MAO-B. However, the selective inactivation of BPAO by **1** is unprecedented, and **1** appears also to selectively inactivate BPAO relative to other copper amine oxidases. Neither **1** nor **4** exhibit evidence of normal turnover, implying a very efficient mechanism of inactivation in both cases, suggestive of a covalent modification. However, the cofactor regains redox activity upon denaturation of the inactivated enzyme, and thus inactivation involves either alkylation of an active-site residue and/or the modification of the cofactor in a manner that reverses upon enzyme denaturation. Consistent with very efficient inactivation mechanisms, no saturation could be ascertained for either inhibitor even at 2 °C, indicating that k_{inact} is fast relative to binding rate constant k_{-1}. Although it is possible to rationalize inactivation in terms of a common allenyl product Schiff base intermediate, the finding that thiocholine at low concentration slows down inactivation by **4** but has no effect on **1** even at very high concentration makes it difficult to reconcile a common intermediate mechanism. Additional work will be needed to clarify this differential phenomenon, but one possibility is that the two inhibitors are processed by distinct mechanisms. These results speak to the role that different active-site residues may play in trapping reactive intermediates between two parent amine inhibitors that are so similar in structure. In any event, further analogue development in the 1-amino-3-butyne class may lead to efficient and selective inactivators of the quinone-dependent amine oxidase family of enzymes.

Experimental Section

General. ^{1}H NMR spectra were obtained on a Varian Gemini 200 (^{13}C NMR at 50 MHz) instrument, with chemical shifts being referenced to TMS or the solvent peak. In the ^{13}C NMR line listings, attached proton test (APT) designations are given as (+) or (−) following the chemical shift. High-resolution mass spectra (HRMS, electron impact) were obtained at 20−40 eV on a Kratos MS-25A instrument. UV−vis spectra were obtained using a jacketed (temperature-controlled) cell compartment and Perkin-Elmer PECSS software. Doubly distilled water was used for all enzyme experiments. All synthetic operations were carried out at ambient temperature, unless indicated otherwise. Thin-layer and preparative thin-layer chromatography were run on Merck silica gel 60 plates with 254 nm indicator. All column chromatography was run using flash-grade silica gel. All solvents, reagents, and organic fine chemicals were the purest available from commercial sources. BPAO (22.5 Tabor units = 0.0052 IU/mg of protein) was purchased from Worthington and PDX G.F.25 from Sigma. All evaporations were conducted at reduced pressure using a rotary evaporator.

1-Amino-3-butyne (1). A solution of 4-mesyloxy-1-butyne (3.1 g, 21 mmol) in 30 mL of EtOH and 70 mL of NH_4OH was stirred at 50 °C in a pressure bottle for 3 h. To the reaction mixture was added 3N HCl to adjust the solution pH to 2, and the solution was concentrated. To the residue was added 2N aqueous NaOH to bring the solution to pH 11, which was then extracted with diethyl ether (3 × 60 mL). The organic layer was separated, washed with brine (50 mL), and concentrated at low temperature. The residue was diluted with MeOH, acidified with concd HCl, and concentrated, and the residue was recrystallized from EtOH−Et_2O to afford the HCl salt. In another preparation, the residue was dissolved in 40 mL of CH_3CN-H_2O (1:1), and 4.8 g (22 mmol) of (t-BuOCO)$_2$O (t-Boc_2O) was added periodically while maintaining the solution pH at 10 by dropwise addition of 2N NaOH. The mixture was diluted with 30 mL of water and extracted with CH_2Cl_2 (3 × 60 mL), and the organic layer was dried (Na_2SO_4) and concentrated. The crude derivative was purified by silica gel flash chromatography (hexanes−EtOAc, 3:1 as eluent) and deprotected with ethanolic HCl (3N) for 1 h to afford 1.36 g (61%) of analytically pure **1**·HCl:[23] ^{1}H NMR (CD_3OD) δ 3.25 (t, 2H, J = 6.9 Hz), 2.70 (dt, 2H, J = 6.9 and 2.7 Hz), 2.52 (t, 1H, J = 2.7 Hz); ^{13}C NMR (CD_3OD) δ 79.25, 73.39, 47.28, 16.87.

1-Amino-2,3-butadiene (4). The t-Boc derivative of propargylamine was first prepared by treating the latter with 1.05 equiv of t-Boc_2O in CH_2Cl_2 in the presence of 2 equiv of Et_3N for 2 h. The reaction mixture was washed with brine, and the organic layer was dried (Na_2SO_4) and concentrated. According to a published method,[16] a dioxane solution of the t-Boc derivative, formaldehyde, and diisopropylamine, with freshly prepared[24] CuBr added, was heated for 12 h under Ar. Quenching with 1N HOAc, extraction with diethyl ether, drying (Na_2SO_4) and evaporation of the ether extract, and silica gel flash chromatography (hexanes−EtOAc, 4:1 as eluent) of the crude product afforded the t-Boc derivative of **4** in 45% yield: ^{1}H NMR ($CDCl_3$) δ 5.19 (m, 1H), 4.81 (m, 2H), 3.70 (m, 2H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 207.924 (+), 155.747 (+), 88.534 (−), 79.463 (+), 77.467 (+), 38.877 (+), 28.421 (−). A solution of 2.0 g of the t-Boc derivative dissolved in 10 mL of ethanol, and 3 mL of concd HCl was stirred for 1 h and concentrated to dryness to afford **4**·HCl: ^{1}H NMR (CD_3OD) δ 5.38 (p, 1H, J = 6.4 Hz), 5.05 (dt, 2H, J = 2.9 and 6.4 Hz), 3.54 (dt, 2H, J = 2.9 and 6.4 Hz); ^{13}C NMR (CD_3OD) δ 210.2 (+), 85.1 (−), 78.8 (+), 39.0 (+); HRMS (FAB) calcd for C_4H_8N (MH^+) m/z 70.0657; found, 70.0669.

(23) Doetz, K. H.; Schaefer, T. O.; Harms, K. *Synthesis* **1992**, 146.
(24) Hartwell, J. L. *Organic Syntheses*; Wiley & Sons: New York, 1955; *Collect. Vol. III*, 185−187.

Buta-2,3-dienal (3). According to the published procedure,[13a] dropwise addition of 1,4-dichloro-2-butyne to hot methanolic KOH, dilution of the cooled reaction mixture with water, saturation of the latter with NaCl and extraction with CH_2Cl_2, washing and drying of the combined extract, and careful fractional distillation through a 60 cm Vigreux column afforded *Z*-1-methoxy-but-1-en-3-yne in 26.3% yield: ^{1}H NMR ($CDCl_3$) δ 6.33 (dd, 1H, J = 0.9 and 6.5 Hz), 4.45 (dd, 1H, J = 2.4 and 6.5 Hz), 3.79 (s, 3H), 3.08 (dd, 1H, J = 0.9 and 2.4 Hz); ^{13}C NMR ($CDCl_3$) δ 158.12 (−), 84.29 (−), 80.67 (+), 78.27 (+), 60.87 (−). The latter liquid (2 g) was added to 10 mL of phosphoric acid (5 M) at 0 °C,[13b] and after 5 min, the crude aldehyde **3** (containing water, methanol, and traces of starting ether) was collected in a CO_2/acetone trap at 15 mm of Hg. Aldehyde **3** was purified by repetitive trap-to-trap distillations to give 0.75 g (20%) of **3**: ^{1}H NMR (CD_3CN) δ 9.52 (d, 1H, J = 7.0 Hz), 5.84 (dt, 1H, J = 6.4 and 7.0 Hz), 5.46 (d, 2H, J = 6.4 Hz); ^{13}C NMR (CD_3CN) δ 223.16, 192.69, 97.96, 80.66.

Reaction of 1 with DTBQ in DMSO-d_6. To a 5 mm NMR tube containing DTBQ (33 mg, 0.15 mmol) and **1**·HCl (8 mg, 0.075 mmol) in DMSO-d_6 (0.5 mL) was added diisopropylethylamine (13 μL, 0.075 mmol) via syringe. The ^{1}H NMR spectrum recorded after 2 min showed generation of $DTBQH_2$ and 5,7-di-*tert*-butyl-2-(1,2-propadienyl)benzoxazole (**10**) along with other unidentified compounds. After 7 h, DTBQ had disappeared completely, with **10** and $DTBQH_2$ as major compounds along with traces of side products. After 16 h, **10** was separated by preparative TLC using $CHCl_3$−hexanes (4:1) as eluent. **10**: ^{1}H NMR ($CDCl_3$) δ 1.37 (s, 9H), 1.47 (s, 9H), 5.42 (d, 2H, J = 6.8 Hz), 6.34 (t, 1H, J = 6.8 Hz), 7.25 (d, 1H, J = 1.9 Hz), 7.54 (d, 1H, J = 1.9 Hz); FAB HRMS calcd for $C_{18}H_{24}NO$ (MH^+) m/z 270.1858; found, 270.1832 (11%).

Reaction of 1 with DTBQ in CD_3CN/D_2O. To a 5 mm NMR tube containing DTBQ (33 mg, 0.15 mmol) and **1**·HCl (8 mg, 0.075 mmol) in CD_3CN/D_2O (0.45 mL/0.05 mL) was added diisopropylethylamine (13 μL, 0.075 mmol) via syringe. The earliest ^{1}H NMR spectrum recorded after 4 min showed the generation of $DTBQH_2$ and 5,7-di-*tert*-butyl-2-(2-propynyl)benzoxazole (**8**) as major compounds and **10** as a minor compound. Over the course of the reaction, signals corresponding to **10** and $DTBQH_2$ increased at the expense of **8**. Partial ^{1}H NMR signals deduced for **8**, not isolated: (CD_3CN/D_2O, 9:1) δ 2.71 (t, 1H, J = 2.0 Hz), 4.08 (d, 2H, J = 2.0 Hz), 7.47 (d, 1H, J = 1.3 Hz), 7.67 (d, 1H, J = 1.3 Hz).

Reaction of 1 with DTBQ in DMF-d_7. To a 5 mm NMR tube containing DTBQ (66 mg, 0.30 mmol) and **1**·HCl (16 mg, 0.15 mmol) in DMF-d_7 (1 mL) was added diisopropylethylamine (26 μL, 0.15 mmol) via syringe. The earliest ^{1}H NMR spectrum recorded after 2 min showed the remaining DTBQ and $DTBQH_2$ as major compounds with a trace of **10**, as well as some peaks (8.36 ppm, doublet; 5.67 ppm, doublet) that were assigned to the 2,3-butadienyl imine **9** of 2-amino-4,6-di-*tert*-butylphenol. The latter signals remained at trace levels, slowly decreasing over 30 min (along with starting DTBQ), concomitant with the increase in signals corresponding to $DTBQH_2$ and **10**. After 2 h, **10** and $DTBQH_2$ were seen as major compounds along with a trace of DTBQ.

Inactivation of BPAO. Determination of the effective concentration of BPAO, time-dependent inactivation of BPAO by candidate inhibitors, determination of irreversibility of the inhibition, determination of substrate protection, and phenylhydrazine titrations were carried out as previously described.[3a] All percent activities refer to ratios of measured benzaldehyde formation slopes to those obtained on a control incubation lacking inhibitor. This approach corrected for the slightly reduced activities observed when activity measurements were made in the first minute or two following dilution of the ice-cold enzyme stock into the primary incubation reaction at 30 °C. Enzyme activity did not deteriorate detectably during the time course of our measurements (up to several hours). To correct for possible errors in the concentration of candidate inhibitors arising, e.g., from solvent contamination of the final amine HCl salts, the concentrations of stock solutions were determined by ^{1}H NMR by combining a measured aliquot with an aliquot of an integration standard (usually fumaric acid) and then recording the integrated spectrum of the evaporated mixture in D_2O or CD_3OD.

NBT Assay of BPAO Inactivated by 1 and 4. A solution of purified BPAO[17] (final concentration 2.1 mg/mL) and either 2 mM **1** or 1 mM **4** in pH 7.2 phosphate buffer was incubated at 30 °C for 2 h, resulting in enzyme preparations that had <5% activity compared to a control sample incubated in the absence of inhibitor. Aliquots (40 μL) of control and inhibitor-treated solutions diluted with 20 μL denaturing solution (2% mecaptoethanol, 10% glycerol, 4% SDS, 0.03% bromphenol blue) were heated at 100 °C for 6 min. The denatured solutions were clarified by centrifugation before application of the supernatant in duplicate to two halves of a polyacryamide slab gel (6% acrylamide with 0.16% bis-acrylamide). The gel was run at a constant current of 0.02 amp (voltage near 200 V) and was stopped when the bromphenol blue dye reached the bottom. The gel was cut in half, and the two halves were stained with either Coomassie blue (0.25% Coomassie in 50% methanol, 7% acetic acid) or 0.24 mM nitroblue tetrazolium in 2 M pH 10.0 potassium glycinate for 120 min in the dark.

Isolation and Purification of Mitochondria. According to a general procedure,[25] male Sprague−Dawley rats (400−500 g weight) were euthanized by decapitation, and the liver was quickly removed and placed in ice-cold MSM buffer (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4). The liver was rinsed, blotted, weighed, finely minced, and washed with cold MSM buffer. A 10% liver tissue homogenate containing MSM with 2 mM EDTA was prepared using two strokes of a Potter-Elvehjem loose-fitting pestle. Mitochondria were extracted by differential centrifugation, washed twice with MSM buffer, and diluted to a concentration of 80−100 mg/mL. Protein concentration was determined by the biuret method with bovine serum albumin as standard.

Benzylamine Oxidase Activity of Rat Liver Mitochondria. Solutions of mitochondria (20 μL, 1.5 mg) and various concentrations of inhibitor in 50 mM, pH 7.5, sodium phosphate buffer (final volume 60 μL) with Triton X-100 added (0.1%) as detergent to achieve homogeneity were incubated at 30 °C. Aliquots (10 μL) of this primary incubation were removed periodically and diluted into 600 μL of 3 mM benzylamine in 50 mM, pH 7.5, sodium phosphate buffer, at 30 °C, and MAO activity was determined by spectrophotometric monitoring of the production of benzaldehyde at 245 nm. The initial oxidation rate of benzylamine (3 mM) was ΔA 0.012/min when the concentration of mitochondria was 250 μg/600 μL in the assay cuvette.

Irreversibility of MAO Inhibition. Solutions of 50 μL of 50 mM, pH 7.5, sodium phosphate buffer containing various concentrations of candidate inhibitor (**1** or **4**) and 10 μL (0.75 mg) of mitochondria were incubated for 30 min at 30 °C. A control solution without inhibitor was incubated at the same time. After 30 min, incubation with 2 mM **1** gave 94.3% inhibition, and incubation with 5 μM **4** gave 91.1% inhibition relative to the control incubation. The inhibited solutions were transferred to a Slide-A-Lyzer Mini Dialysis cup and dialyzed in 50 mM sodium phosphate buffer, pH 7.2, at room temperature for 24 h. No apparent recovery of activity was observed in either case.

Acknowledgment. We thank the National Institute of General Medical Sciences, NIH for support of this work through Grant GM 48812. We also thank David M. Dooley, Montana State University, for the sample of purified bovine plasma amine oxidase used in the NBT assays and Charles L. Hoppel, Case Western Reserve University and Louis Stokes Veterans Affairs Medical Center, for the sample of liver mitochondria used in the MAO assay.

JA049568O

(25) Hoppel, C. L.; Kerner, J.; Turkaly, P.; Turkaly, J.; Tandler, B. *J. Biol. Chem.* **1998**, *273*, 23495−23503.