Model Reactions for the Quinone-Containing Copper Amine Oxidases. Anaerobic Reaction Pathways and Catalytic Aerobic Deamination of Activated Amines in Buffered Aqueous Acetonitrile

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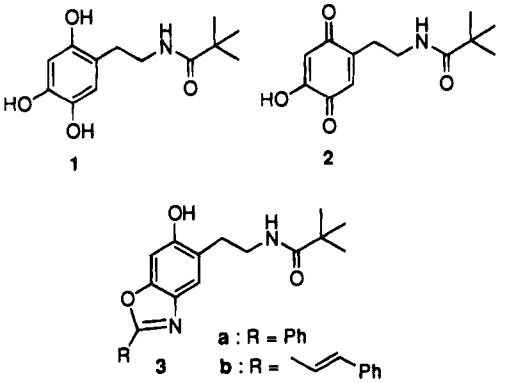
Abstract: The quinone form **2** of the *N*-pivaloyl derivative of 6-hydroxydopamine, developed as a model for the quinone form of the peptidyl 2,4,5-trihydroxyphenylalanine (TOPA) cofactor of the copper amine oxidases, is an effective catalyst for aerobic oxidative deamination of activated amines (e.g., benzylamine and cinnamylamine) in buffered aqueous acetonitrile. The reaction efficiency increases at higher pH and exceeds six turnovers for benzylamine at pH 10, where an apparent α-C deuterium kinetic isotope effect of 10 is observed. The yield is limited by the eventual conversion of **2** to the corresponding benzoxazoles (confirming nucleophilic attack of amine at the most electrophilic C-5 carbonyl) and other forms which are incapable of oxidative recycling. Copper(II) inhibits the reaction, in contrast to the free TOPA amino acid, which deaminates benzylamine only when Cu(II) is present. Under anaerobic "single turnover" conditions, the products of catalyst reduction are (alkylamino)resorcinols formed via redox cycling reactions of the initial reduced cofactor (either benzenetriol or aminoresorcinol) in the presence of excess amine. Unactivated amines exhibit low deaminative turnover and at high concentrations effect side-chain cleavage of the quinone catalyst induced by C-1 Michael addition of amine to the intermediate *N*-alkyl quinone imines.

Introduction

The copper amine oxidases utilize a covalently-bound "active carbonyl" cofactor to carry out a pyridoxal-like conversion of *primary* amines to aldehydes. The reduced cofactor thereby generated is reoxidized in a subsequent step concomitant with reduction of O_2 to H_2O_2.[1,2] The carbonyl cofactor has been shown to be the quinone form of an active-site-based 2,4,5-trihydroxyphenylalanine (TOPA) residue.[3,4] Quinone-mediated transamination of amines has precedent in the synthetically useful deamination of branched primary amines by 3,5-di-*tert*-butyl-1,2-benzoquinone (DTBQ)[5] and in the deamination of benzylamine by pyrroloquinolinequinone (PQQ),[6,7] though in the latter case, a carbinolamine β-elimination mechanism (giving $PhCH{=}NH$ from $PhCH_2NH_2$ and a quinol rather than aminophenol as reduced cofactor) competes with transamination under certain conditions.

We recently described a family of models for the reduced (TOPA) and oxidized 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor.[8] One of these, the hydantoin form, was also characterized independently by other workers.[9] The amino acid TOPA itself is commercially available but, like its descarboxy version, the well-known 6-hydroxydopamine, is of limited value for studying amine deamination because the free α-amino group undergoes cyclocondensation at the quinone stage.[10,11] In addition, the simple 1,2,4-benzenetriol/2-hydroxy-1,4-benzoquinone system is an unsuitable model because the absence of the C-1 alkyl substituent here results in oxidative coupling to 2,2′-bi-*p*-quinones.[12]

This article describes studies on the amine reactivity, under buffered conditions, of our recently developed pivalamidoethyl-based triol/quinol model system (**1**/**2**).[8] Catalytic aerobic deamination of activated amines is observed, and parallel NMR and UV−vis spectral studies have been performed anaerobically to evaluate the reaction course under single turnover conditions. Rapid equilibration between benzenetriol, aminoresorcinol, and (alkylamino)resorcinol forms of the cofactor prevent an assignment of the mechanism of deaminative turnover under these conditions. Nonetheless, important information has been ob-



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(1) Mondovi, B.; Riccio, R. *Adv. Inorg. Biochem.* **1985**, *6*, 225.
(2) Knowles, P. F; Yadav, K. D. S. In *Copper Proteins and Copper Enzymes*; Lontie, R., Ed.; CRC Press: Boca Raton, FL, 1984; Vol. II, p 103.
(3) Janes, S. M.; Mu, S.; Wemmer, D.; Smith, A. J.; Kaur, S.; Maltby, D.; Burlingame, A. L.; Klinman, J. P. *Science* **1990**, *248*, 981.
(4) Brown, D. E.; McGuirl, M. A.; Dooley, D. M.; Janes, S. M.; Mu, D.; Klinman, J. P. *J. Biol. Chem.* **1991**, *266*, 4049.
(5) Corey, E. J.; Achiwa, K. *J. Am. Chem. Soc.* **1969**, *91*, 1429.
(6) Ohshiro, Y.; Itoh, S. *Bioorg. Chem.* **1991**, *19*, 169.
(7) Rodriguez, E. J.; Bruice, T. C. *J. Am. Chem. Soc.* **1989**, *111*, 7947.
(8) Wang, F.; Bae, J.-Y.; Jacobson, A. R.; Lee, Y.; Sayre, L. M. *J. Org. Chem.* **1994**, *59*, 2409.
(9) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 7117.
(10) Chapman, R. F.; Percival, A.; Swan, G. A. *J. Chem. Soc. C* **1970**, 1664.
(11) Senoh, S.; Witkop, B. *J. Am. Chem. Soc.* **1959**, *81*, 6231.
(12) Corbett, J. F. *J. Chem. Soc. C* **1970**, 2101.

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Table 1. Catalytic Aerobic Deaminations Mediated by TPQ Model **2**[a]

substrate	pH[b]	buffer, conc (cat)	time (h)	yield[a] (%)
benzylamine	8.5	boric acid, 50 mM	5	250
			18	294
benzylamine	9.0	phosphate, 5 mM	4	290
			23	420
benzylamine	10.0	boric acid, 8 mM[c]	3	330
			10	410
benzylamine	10.0	phosphate, 5 mM[c]	3	350
			4	385
			9	470
			23	610
			48	630
benzylamine-d_2	10.0	phosphate, 5 mM[c]	4	30
			23	39
benzylamine-d_0/ benzylamine-d_2[d]	10.0	phosphate, 5 mM[c]	4	240
			23	412
benzylamine-d_1[e]	10.0	phosphate, 5 mM[c]	4	160
			23	350
benzylamine	10.0	phosphate, 5 mM (Cu^{2+})[f,g]	4	56
			23	99
benzylamine	9.0	phosphate, 5 mM (Cu^{2+})[f]	4	180
			23	240
cinnamylamine[h]	10.0	phosphate, 5 mM	14	180
dibenzylamine[i]	8.0	phosphate, 5 mM[c]	4	37
			23	146
dibenzylamine[i]	9.0	phosphate, 5 mM[c]	4	53
			23	147

[a] The reactions are 50 mM in amine and 1 mM in **2**, conducted at 26 °C in buffer−CH_3CN 7:3. Yields represent mol % of isolated (2,4-dinitrophenyl)hydrazine derivative of corresponding aldehyde on the basis of **2** present, and numbers shown are an average of two−five determinations (the data varied at most 10% from the average values). [b] Measured pH, not corrected for the mixed solvent system. [c] The yields shown are corrected for the amount of aldehyde formed as a consequence of cofactor-independent autoxidation. Such autoxidation for benzylamine itself was negligible at pH 9 and below. [d] Each amine was 25 mM. The 2,4-DNP derivative was a (10 ± 1):1 mixture of the PhCH=O to PhCD=O derivative at both reaction times. [e] The 2,4-DNP derivative was a (10 ± 1):1 mixture of the PhCD=O to PhCH=O derivative at both reaction times. [f] Presence of 1 mM $Cu(ClO_4)_2$ and 1 mM 2,2′-bipyridine. [g] The yields at 4 and 23 h were not altered within experimental error by using a 1:1.2 or 1.2:1 ratio of $Cu(ClO_4)_2$ to 2,2′-bipyridine, showing that the 1:1 results do not reflect action of a trace excess of "free" Cu(II). Reducing the mole fraction that (bipy)Cu(II) was of catalyst **2** (e.g., below 1 mM) reduced the degree of inhibition in a concentration-dependent fashion. The bipy ligand by itself (1 mM) had only a small inhibitory effect on its own. [h] The amine concentration was 25 mM. [i] The solvent used was buffer−CH_3CN 1:1 (the dibenzylamine was incompletely soluble in the 7:3 system). The corrected yields do not account for **2**-mediated production of PhCH=O which may arise from any benzylamine released in the non-**2**-dependent autoxidation of dibenzylamine.

tained about the various forms that the cofactor can take in reactions with amines, providing relevant insight into the enzyme reaction. Additionally, the side reactions which are responsible for limiting the catalytic turnover yields for our model have been fully characterized, providing guidelines to the development of improved models/catalysts.

Results and Discussion

Catalytic Deaminative Turnover. Table 1 lists data for the successful catalytic action of **2** in the O_2-dependent deamination of benzylamine (present in large excess) in buffered aqueous CH_3CN at 26 °C. This success is in marked contrast to the free amino acid TPQ[13,14] or corresponding amine (6-hydroxydopamine quinone),[13] which have been reported to be ineffective in deaminating amines on their own. Although the amine concentration we used was higher than the inorganic buffer concentration, the latter was found to be needed only in excess of the turnover stoichiometry in order to minimize the pH drop which otherwise occurs during the course of the reaction according to eq 1. Higher phosphate concentrations were found

$$2\ PhCH_2NH_3^+ + O_2 \rightarrow PhCH_2N{=}CHPh + H_2O_2 + H^+ + NH_4^+ \quad (1)$$

to create miscibility problems in the mixed solvent system required to keep the amine in solution at higher pH. At the high end of the pH range we studied, the amine itself becomes the principal buffer species.

NMR analysis of organic extracts of the reaction solution revealed that the PhCH=O product is generated in the form of the Schiff base $PhCH{=}NCH_2Ph$ under these reaction conditions (eq 1). Although the reaction can be quantified on this basis, the yield of PhCH=O is more easily determined by weighing the precipitated PhCH=O (2,4-dinitrophenyl)hydrazone obtained upon workup of the reaction with acidified (2,4-dinitrophenyl)-hydrazine (2,4-DNP) reagent.

In preliminary studies, we determined that the pH was the major factor controlling the deamination yield, not the identity of the buffering agent. Control studies showed that the amount of PhCH=O produced in the absence of **2** from simple autoxidation was negligible below pH 10 but required a correction to be made in the pH 10 yields. Table 1 shows that the **2**-dependent deamination yield increases with increasing pH over the range 8.5−10.0 and that deamination slows considerably over a period of several hours. The yields shown at the longest times represent essentially the t = infinity yields at each pH and in one case exceeds six turnovers of the catalyst. The catalysis by **2** ceases on account of its irreversible conversion into forms which are incapable of aerobic recycling. In the pH range we studied, **2** exists wholly as the resonance-stabilized anion,[8] and progress toward the ultimate fate of the catalyst is accompanied by bleaching of the 486 nm chromophore and growth of a strong, broad absorption in the 310−370 nm range, witnessed as a color change from deep red to weak yellow.

α-Deuteration is known to slow the deamination of primary amines by copper amine oxidases according to a primary kinetic isotope effect (DKIE).[15-17] The data in Table 1 show that deamination of benzylamine-d_2 is slowed considerably (by a factor of ≥10 at 4 h) relative to its d_0 partner at pH 10. An increase in the difference between PhCH=O and PhCD=O yields at longer reaction times is probably an artifact of kinetic partitioning effects[18] associated with side reaction(s) which remove the active form of the catalyst. When a 1:1 mixture of benzylamine-d_0 and -d_2 was reacted, the overall yields of the 2,4-DNP derivative at 4 and 23 h were intermediate between those obtained for the d_0 and d_2 cases, but ^1H NMR and high-resolution mass spectrometry (HRMS) indicated a 10:1 ratio of the PhCHO to PhCDO derivative (the same ratio of PhCH= to PhCD= derived benzylidenebenzylamines were seen by NMR prior to 2,4-DNP quench). Although relative yield data of this type can represent only a rough assessment of the intrinsic DKIE, it is noteworthy that the same apparent DKIE of 10 was

(13) Shah, M. A.; Bergethon, P. R.; Boak, A. M.; Gallop, P. M.; Kagan, H. M. *Biochim. Biophys. Acta* **1992**, *1159*, 311.
(14) Kano, K.; Mori, T.; Uno, B.; Goto, M.; Ikeda, T. *Biochim. Biophys. Acta* **1993**, *1157*, 324.
(15) (a) Palcic, M. M.; Klinman, J. P. *Biochemistry* **1983**, *22*, 5957. (b) Farnum, M.; Palcic, M.; Klinman, J. P. *Biochemistry* **1986**, *25*, 1898. (c) Grant, K. L.; Klinman, J. P. *Biochemistry* **1989**, *28*, 6597.
(16) Yu, P. H. *Biochem. Cell Biol.* **1988**, *66*, 853.
(17) Olsson, B.; Olsson, J.; Pettersson, G. *Eur. J. Biochem.* **1976**, *64*, 327.
(18) Thiblin, A.; Ahlberg, P. *Chem. Soc. Rev.* **1989**, *18*, 209.

indicated by an *intra*molecular isotope competition using $PhCHDNH_2$ (determining the ratio of PhCD=NCHDPh to PhCH=NCHDPh as well as of the resulting 2,4-DNP derivatives by ^1H NMR). This suggests to us that α-carbon–hydrogen bond cleavage is nearly completely rate-limiting under our model catalytic deamination conditions at pH 10.

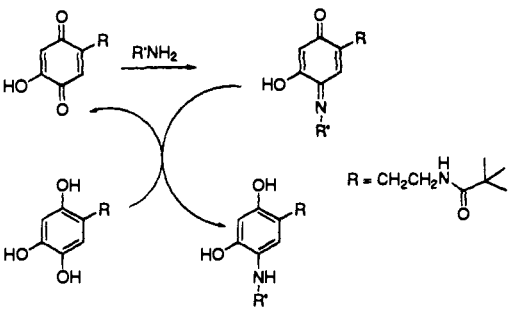
Compared to benzylamine, cinnamylamine also undergoes deamination (quantified in terms of the 2,4-DNP derivative of cinnamaldehyde), though the turnover yield is much lower on account of a more rapid deterioration of the catalyst (Table 1). In contrast to these two activated amines, neither *trans*-2-phenylcyclopropylamine (2-PCPA) nor butylamine undergoes any significant deamination. This is in marked contrast to DTBQ, which, being a stronger transaminating agent, effects reaction of non-benzylic as well as benzylic amines and induces oxidative ring cleavage of 2-PCPA.[5,19]

Curiously, the activated secondary amine dibenzylamine seems to undergo significant deamination under our model conditions. However, this amine is more susceptible to catalyst-independent autoxidation than benzylamine, and yield corrections (PhCH=O equivalents) were required at both pH 8 and 9. Moreover, these yield corrections cannot account for the PhCH=O produced by action of catalyst **2** on any $PhCH_2NH_2$ released in the dibenzylamine autoxidation. Thus, the values given in Table 1 must be considered as upper estimate limits for the PhCH=O arising specifically from the direct action of **2** on dibenzylamine—the *actual* specific yield values may be much smaller. In this regard, it should be recalled that secondary amines are not substrates for the enzymes themselves.

Catalyst Destruction under Turnover Conditions. One possibility we considered for the irreversible loss of turnover competence of quinone **2** was its decomposition by the H_2O_2 generated upon autoxidative recycling of the reduced form **1**. We found that addition of H_2O_2 to **2** anion at pH 8–10 caused a time-dependent but substoichiometric loss of the 486 nm chromophore in a manner which could be prevented by the presence of catalase in the reaction mixture. However, we found that the presence of catalase did not, within experimental error, alter at any time point the turnover yields with benzylamine from those listed in Table 1 (data not shown). Thus H_2O_2 was clearly not responsible for catalyst deterioration.

In previous studies on reactions of *o*-quinones with *unbranched* primary amines, we have had to worry about a benzoxazole-forming side reaction first documented for DTBQ-mediated transaminations[5] wherein the dihydrobenzoxazole tautomer of the product Schiff base generated upon transamination is oxidized by starting DTBQ[20,21] more rapidly than DTBQ is consumed by condensation with amine. In our catalytic system using **2**, an analysis of the neutral organic materials present at the point where deamination turnover for benzylamine and cinnamylamine had ceased resulted in the isolation of benzoxazole products **3**. NMR spectral analysis of the crude organic extract indicated that these benzoxazoles are the major end products accounting for 30–40% of the catalyst **2** in these cases, though other species are also present as evidenced by additional *tert*-butyl singlets appearing in the high-field ^1H NMR spectra. The more rapid catalyst deterioration which occurs in the case of cinnamylamine (thus resulting in inferior turnover yields) may reflect the greater ease of oxidation

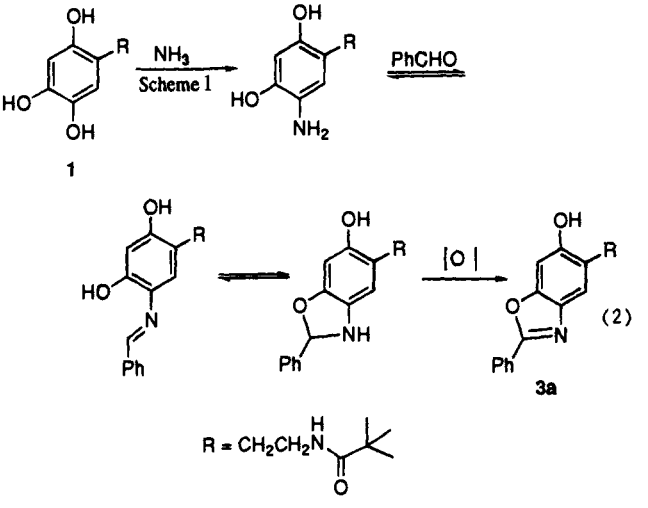
Scheme 1



at the dihydrobenzoxazole stage in this case (relative to the productive turnover pathway).

On the basis of the recognition that benzoxazole formation is a major cause of catalyst destruction, we guessed that improved deamination yields might be seen for the *branched* primary amine α-methylbenzylamine (1-phenethylamine), which gives excellent yields of acetophenone using DTBQ.[5] In the case of **2**, however, only poor yields of the 2,4-DNP derivative were seen in 24 h (less than one turnover). Analysis of the reaction mixture at 24 h revealed the persistence of unconverted **2**, suggesting that the limiting factor in this case may be steric slowing by the α-methyl group of one or more steps in the deamination mechanism rather than catalyst destruction. It is interesting to note that branched primary amines are also poor substrates for the copper amine oxidase enzymes, presumably for steric reasons.

The benzoxazole structure **3** implicates condensation of amine at the more electrophilic C-5 carbonyl of **2** (using the TOPA numbering scheme). The other possible benzoxazole isomer, which would have the N and O reversed, could not be excluded by any of the spectral data and would arise from some type of transamination mechanism initiated by reaction of amines at C-4. In order to prove that the benzoxazole has structure **3**, we carried out an independent synthesis in the case of the benzylamine-derived material **3a** (eq 2). We recently showed



that the reduced benzenetriol forms of TPQ models are readily converted to C-5 alkylamino-substituted derivatives by reaction of the triol with alkylamines.[8] The reaction involves the catalytic action of a trace of quinone present in the triol preparation, wherein the quinone imine formed from amine and the trace of quinone is reduced by triol to give (alkylamino)-resorcinol, regenerating the quinone catalyst (Scheme 1). The regiochemistry of this substitution was verified by a combination of NOE difference and long-range $^{13}C-^1H$ coupling data.[8] As

(19) Sayre, L. M.; Singh, M. P.; Kokil, P. K.; Wang, F. *J. Org. Chem.* **1991**, *56*, 1353.

(20) DTBQ and not O_2 is the oxidant: Singh, M. P.; Kokil, P. B.; Venkataraman, B.; Klein, M.; Sayre, L. M. *Abstracts of Papers*, 199th National Meeting of the American Chemical Society, Boston, April 22–27, 1990; American Chemical Society: Washington, DC, 1990; ORGN 429.

(21) A similar reaction occurs with amino acids: Vander Zwan, M. C.; Hartner, F. W.; Reamer, R. A.; Tull, R. *J. Org. Chem.* **1978**, *43*, 509.

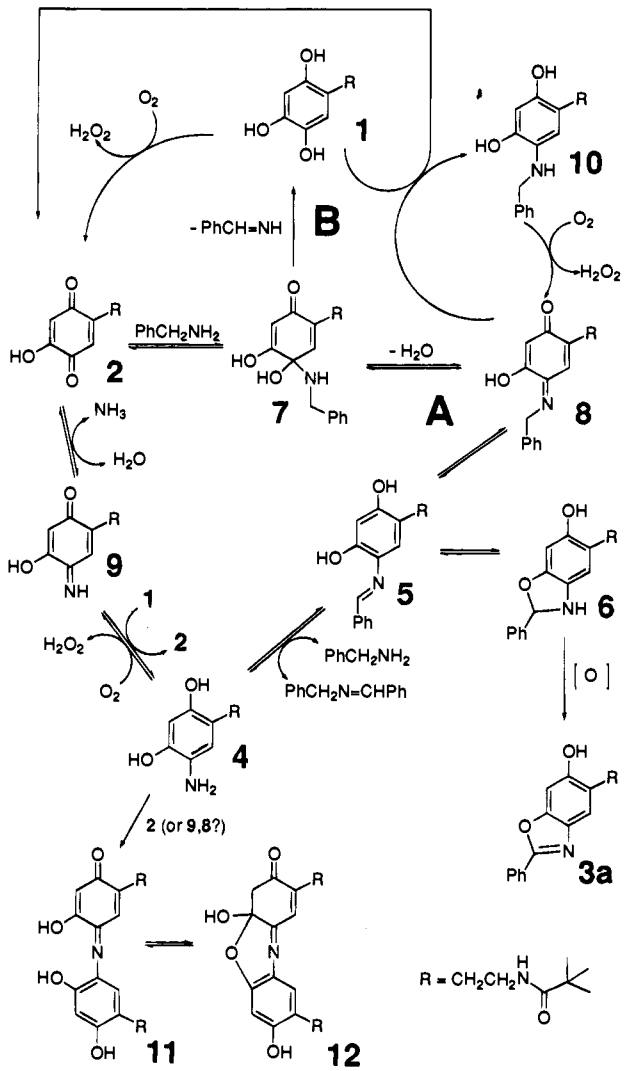
predicted by Scheme 1, bubbling of NH_3 through a solution of triol **1** in CH_3CN gave the aminoresorcinol **4**. Reaction of **4** with a 2-fold excess of PhCH=O in CH_3CN, followed by removal of the solvent (and excess PhCH=O) yielded the Schiff base **5** (by ^1H NMR, not isolated) which, upon exposure to air, was observed to be predominantly converted, presumably via oxidation of its cyclic tautomer **6**, to benzoxazole **3a** (eq 2).[22] The regiochemistry of Scheme 1 and assignment of the benzoxazole side-product structure as **3** confirm that the intrinsic chemical preference for reactions of *aliphatic amines* with TPQ is at the more electrophilic C-5 carbonyl, as previously[9] proposed and demonstrated in the case of aryl*hydrazines*.[8,9]

Effect of Cu(II) on Turnover Yield. In a previously studied catalytic aerobic deamination of benzylamine by the free amino acid TOPA,[23] the presence of (bipy)Cu(II) was reported to be an essential ingredient, seemingly consistent with the crucial role of copper in the enzymatic reaction. However, the data in Table 1 shows that (bipy)Cu(II) is inhibitory in *our* catalytic system,[24] with the inhibition being greater at higher pH. Since copper would be expected to aid in the autoxidative recycling of the reduced catalyst, the different effect of Cu(II) in our case compared to the free TOPA model case must arise from differences in the nature of coordination of Cu(II) at the amine processing stage. For our model, Cu(II) is most likely chelated by the oxo enolate portion of **2** anion. In the case of free TOPA, Cu(II) is coordinated to the free amino carboxylate end of the molecule.[23] The latter coordination likely prevents the well-known cyclization of the free amino group into the quinone nucleus, and we believe that this is the best explanation for why *free TOPA by itself* is ineffective in catalyzing aerobic deamination of benzylamine.[13,14] Since our model (which, like the enzyme, lacks the problematic free amino group) is active in deaminating benzylamine on its own, we believe it is inappropriate to relate the requirement for Cu(II) in the free TOPA case to the question of the catalytic role of copper in the enzymatic reaction.

The presence of Cu(II) in our model turnover reactions was observed to cause a more rapid quenching of the **2** anion color, implicating an accelerated destruction of the catalyst, consistent with the reduced turnover yields. The fate of the catalyst was found to be unaltered; benzoxazole **3a** was isolated in 60% yield from one of the pH 10 reactions. It is possible that coordination of Cu(II) in the reaction center accelerates the oxidation of the dihydrobenzoxazole precursor to **3a** at the expense of the turnover pathway; any aid by Cu(II) in autoxidative recycling of the reduced catalyst must be of lesser significance.

Mechanisms for Catalytic Deamination. For the copper amine oxidases, the finding that aldehyde can be produced under stoichiometric single-turnover (anaerobic) conditions, but that NH_3 is not released until the O_2-dependent cofactor reoxidation stage, has led to a description of the enzyme mechanism in terms of a transamination mechanism.[25] Nonetheless, an alternative mechanism involving β-elimination of aldimine at the carbinolamine preceding the quinoneimine has been documented to be a competing reaction for pyrroloquinolinequinone (PQQ) and its analogs.[6,7] Application of the two mechanisms to our present

Scheme 2



model in the case of benzylamine is shown in Scheme 2.[26] The transamination mechanism (path A) would involve tautomerization of the quinone imine **8** to give the PhCH=O Schiff base **5**. In the presence of high [$PhCH_2NH_2$], the $PhCH{=}NCH_2Ph$ product would undoubtedly arise from direct interception of **5** by $PhCH_2NH_2$ rather than via hydrolysis of **5** and recondensation of PhCH=O with $PhCH_2NH_2$. The reductively aminated catalyst **4** thereby released would be oxidized by O_2 to quinoneimine **9**, presumably in two steps via the corresponding semiquinone imine (not shown), with **9** in turn being converted to quinone **2** by hydrolysis and/or directly to **8** by transimination with $PhCH_2NH_2$ (not shown). Alternatively, the product Schiff base **5** could, in the form of its tautomer **6**, undergo oxidation to benzoxazole **3a**. According to this scenario, a key factor governing catalyst lifetime would be the partitioning of **5** between direct oxidation to **3a** and transimination with $PhCH_2NH_2$ prior to oxidation, so that an increased amount of PhCH=O product would be expected at higher [$PhCH_2NH_2$] (all other factors remaining unchanged).

If our model deaminations followed the alternative addition–elimination mechanism (Scheme 2, path B), the triol **1** formed upon β-elimination of PhCH=NH from carbinolamine **7** would

(22) We do not know whether this oxidation is effected directly by O_2 or by a catalytic amount of quinone **2** present in the reaction mixture (in which case the reduced form **1** would be reconverted to **2** in air[8]).

(23) Nakamura, N.; Kohzuma, T.; Kuma, H.; Suzuki, S. *J. Am. Chem. Soc.* **1992**, *114*, 6550.

(24) The use of 1 equiv of $Cu(ClO_4)_2$ by itself at pH 9 was slightly more inhibitory, but the reaction was cloudy, indicating precipitation of $Cu(OH)_2$. The use of an equimolar quantity of 2,2′-bipyridine was found to ensure homogeneity even at pH 10.

(25) Janes, S. M.; Klinman, J. P. *Biochemistry* **1991**, *30*, 4599.

(26) The quinone **2** exists wholly as an anion in the pH range of our studies, and there are conjugate acid/base issues for the amine substrates and most of the reaction intermediates as well. However, for the sake of clarity, we have chosen to depict all species in their neutral (stoichiometric) forms.

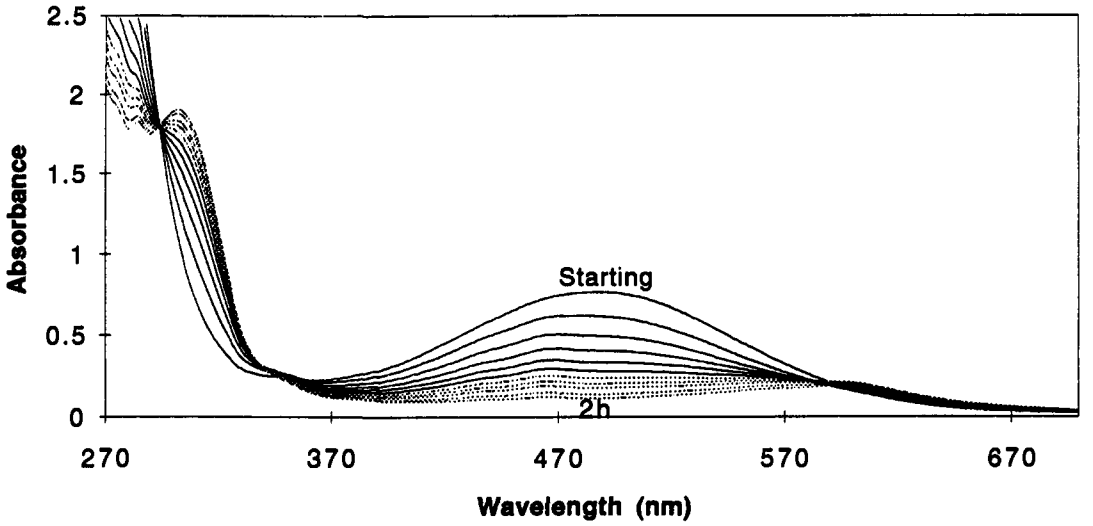


Figure 1. Progress of the reaction of **2** (5 mM) with excess benzylamine (50 mM) in 1:1 CH_3CN-H_2O (33 mM pH 10 phosphate buffer) followed in a 0.1 cm cuvet at 12 min intervals. Decay of the **2** anion absorption at 490 nm occurs isosbestically with appearance of a 300 nm absorption for **10**.

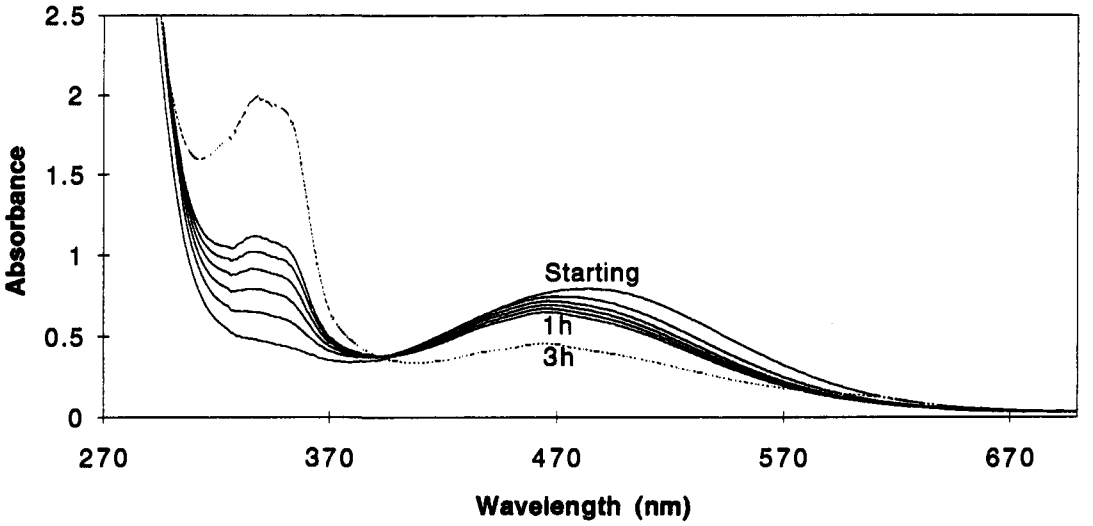
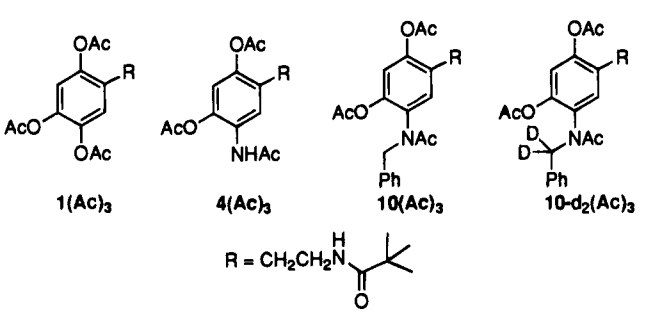


Figure 2. Progress of the reaction of **2** (5 mM) with excess benzylamine-d_2 (50 mM) in 1:1 CH_3CN-H_2O (33 mM pH 10 phosphate buffer) followed in a 0.1 cm cuvet at 12 min intervals. There is an initial gradual shift of the 490 nm **2** anion absorption to a $\lambda_{max} = 460$ nm characteristic of the quinoneimine **8**-d_2 anion, concomitant with growth of a strong, long-lived absorption with dual λ_{max} at 335/345 nm characteristic of the fragmentation product **18** (R′ = CD_2Ph). Similar spectra are obtained in the reactions of **2** with "unactivated" amines such as neopentylamine and *n*-propylamine.

be autoxidized back to quinone **2** under the reaction conditions,[8] presumably in two steps via the corresponding semiquinone (not shown).[14] In this case, the product $PhCH{=}NCH_2Ph$ would undoubtedly arise from direct reaction of $PhCH{=}NH$ with $PhCH_2NH_2$. If path B is the mechanism for deaminative turnover, dehydration of **7** to quinone imine **8** would constitute a side reaction which inevitably is responsible for "draining off" of active catalyst in the form of the isolated benzoxazole side-product **3a**.

Product Analysis under Anaerobic Conditions. Theoretically, the two mechanisms can be distinguished through determination of the product of quinone reduction in the absence of catalytic recycling, viz., when the reaction is conducted under anaerobic conditions; transamination (path A) results in aminoresorcinol **4**, whereas carbinolamine elimination (path B) results in triol **1**. Since both potential products are easily autoxidized, we presumed that a more practical approach than direct identification was to characterize the product after trapping it in the form of an air-stable derivative. In this manner, the final product mixture of anaerobic deamination of benzylamine was subjected to flash vacuum evaporation, and the residue was treated under argon with an excess of acetic anhydride to afford the triacetylated derivatives. The triacetyl forms of **1** and **4** were prepared independently for comparison purposes. The isolated product from benzylamine deamination was shown to be neither **1** nor **4**, and instead was exclusively the triacetylated derivative of (benzylamino)resorcinol **10**. The triacetyl compound constitutes an interesting example of atropisomerism-



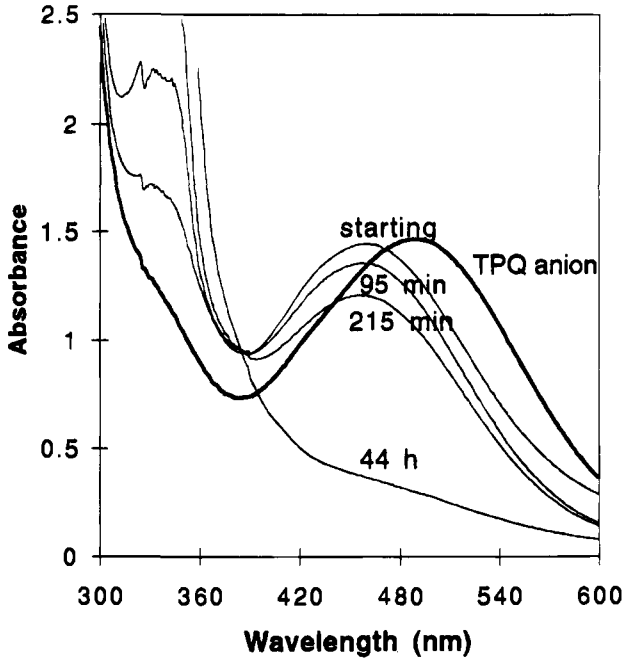


Figure 3. Progress of the reaction of **2** (1 mM) with excess methylamine (50 mM) in CH_3CN-H_2O 1:1 followed in a 1 cm cuvet. The first kinetic point shows that the 490 nm absorption of the quinone anion has already completely shifted to the 460 nm absorption characteristic of the quinoneimine anion (**14**, R′ = CH_3).

induced diastereotopicity (see Experimental Section).

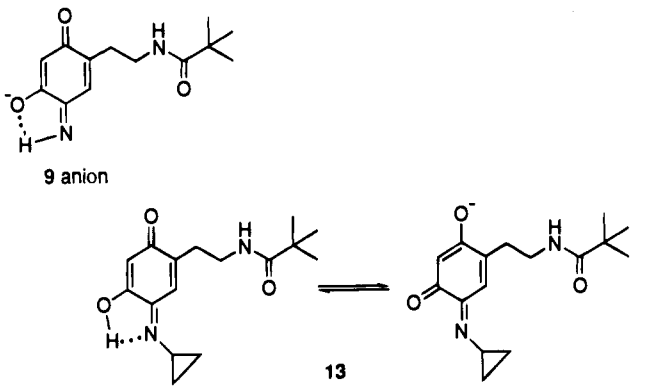
The finding of **10** as the product of quinone reduction can arise if the mechanism follows path B, since we already reported that triol **1** is converted to **10** in the presence of excess benzylamine (Scheme 1),[8] catalyzed by a trace of quinone **2**. Nonetheless, as might be predicted, we found that aminoresorcinol **4** is also converted to **10** in the presence of excess benzylamine. This reaction must be catalyzed by a trace of quinone imine **9** (or quinone **2**) because the observed conversion is immediately quenched upon addition of $NaBH_4$ to the reaction, as we found in the case of the triol **1** conversions. These results demonstrate that the *isolated* product expected from anaerobic deamination of benzylamine would be **10** irrespective of mechanism. This ambiguity highlights the difficulty of establishing mechanism in this model system. Nonetheless, our characterization of **10** is of interest in so far that this represents the product of reductive trapping of substrate-derived imine in the enzymatic processing of benzylamine.[28]

Anaerobic Reactions of Quinone 2 with Benzylamine. Another approach to elucidating the mechanism is to follow the anaerobic reactions simultaneously by ^1H NMR and UV–vis spectroscopy in an effort to distinguish pathway-specific intermediates. For this purpose, the large excess of amine used in the aerobic turnover experiments of Table 1 is not amenable to NMR studies. Using instead a 3–5-fold excess of benzylamine in CH_3CN-H_2O (with or without pH 10 phosphate buffer), the **2** anion absorption at 490 nm was seen to convert to the 300 nm absorption characteristic of **10** (~2.5-fold greater extinction), with isosbestic points at 290 and 350 nm (see Figure 1). The conversion of **2** to **10** was confirmed by ^1H NMR in CD_3CN-H_2O (no buffer added in this case). Our inability to detect intermediates during the conversion of **2** to **10** by either ^1H NMR or UV–vis spectroscopies implies that deamination is not sufficiently fast to compete with the conversion of the product of **2** reduction (either **4** or **1**) to **10**, thereby preventing an assignment of mechanism under these reaction conditions.

Interestingly, the anaerobic benzylamine reactions develop a broad absorption at 590 nm at long reaction time, accompanied by a blue-violet coloration of the solution. This observation immediately led us to suspect formation of the indophenol **11** (see Scheme 2), an assignment supported by our ability to prepare **11** by independent synthesis from condensing **4** with **2**. The ^1H and ^{13}C NMR spectra of the isolated neutral form of **11** in DMSO-d_6 indicated the existence of the compound in a tautomeric form. The appearance of (i) only three (rather than four) low-field ^{13}C and ^1H vinyl signals, (ii) diastereotopic splittings of one of the two CH_2CH_2 side chains, and (iii) an upfield AB 2H ^1H NMR signal in place of the missing vinyl H suggests to us that **11** exists as the cyclic hemiketal **12**. Although the formation of indophenol **11** implicates the generation of aminoresorcinol **4**, it is not clear if **4** is formed directly from a transamination mechanism (upon benzylamine-induced transimination) or from triol **1** and NH_3 (evolved upon condensing PhCH=NH with $PhCH_2NH_2$) according to the redox cycling mechanism of Scheme 1.

Quinoneimine Formation from Reaction of 2 with Ammonia and Cyclopropylamine. Since we expected that the reaction of **2** with unactivated amines might lead to detectable quinone imine intermediates, we first studied the conversion of **2** to quinone imine **9** by NH_3. Exposure of **2** to aqueous CH_3CN solutions of increasing NH_3 content resulted in an increasingly more rapid and complete isosbestic shift of the 490 nm (red) absorption for **2** anion to a λ_{max} 450 nm absorption (orange) with essentially the same extinction. The 450 nm absorption and chemical shift positions seen in the ^1H NMR spectrum suggested to us the existence of **9** as a (possibly intramolecularly hydrogen-bonded) *anion*.[29]

The reaction of **2** with excess cyclopropylamine in aqueous CH_3CN exhibited UV–vis (λ_{max} = 455 nm) and ^1H NMR (CD_3CN-H_2O) characteristics indicating generation of quinone imine **13** in *anionic* form. Consistent with its stability expected on



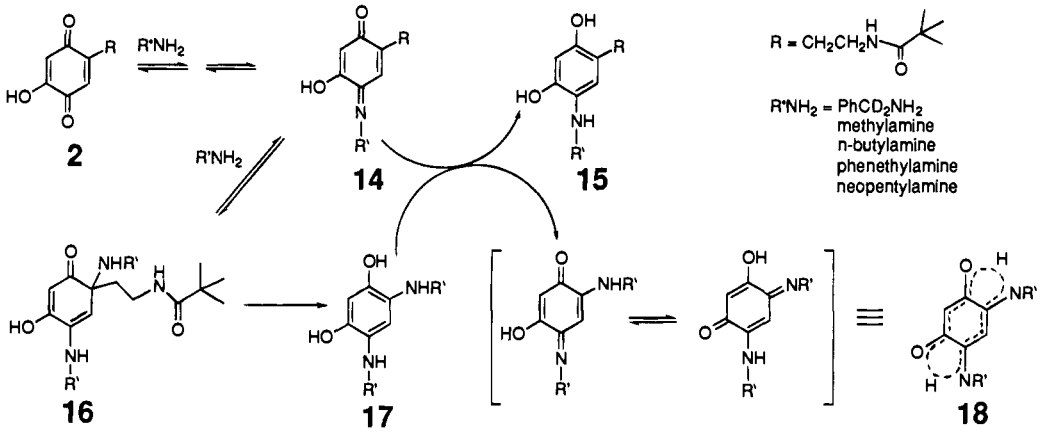
the basis of a combination of pseudo-vinyl resonance and inhibited transamination due to angle strain, **13** was found to be readily isolable (as the sparingly soluble cyclopropylammonium salt). When the isolated **13** salt was suspended in *anhydrous* CD_3CN, the soluble component exhibited UV–vis (λ_{max} = 350 nm) and ^1H NMR characteristics more consistent with the *neutral* quinone imine form. Recent rapid scan studies on the anaerobic reaction of bovine serum amine oxidase with benzylamine revealed a transient 340 nm absorption which was

(27) Turowski, P. N.; McGuirl, M. A.; Dooley, D. M. *J. Biol. Chem.* **1993**, *268*, 17680.

(28) Hartmann, C.; Klinman, J. P. *J. Biol. Chem.* **1987**, *262*, 962.

(29) A quinone imine with λ_{max} = 448 nm was reported to form in the reaction of a C1-ethyl TPQ model with NH_3,[9] but whether this represented the neutral or anionic form was not discussed.

Scheme 3



assigned to the quinone imine intermediate.[30] Our model results are consistent with the existence of such enzyme-based quinone imine in neutral form.

Anaerobic Reactions of Quinone 2 With "Unactivated" Primary Amines. The reactions of **2** in CH_3CN-H_2O (±pH 10 phosphate buffer) with a 3−10 fold excess of neopentylamine, α,α-dideuteriobenzylamine, methylamine, or *n*-propylamine were monitored anaerobically by NMR and UV−vis spectroscopy under identical conditions. In contrast to what was seen with benzylamine (see above), these "unactivated" amines resulted in an initial shift of the **2** anion chromophore at 490 nm to shorter wavelength (460−470 nm), followed by quenching of this absorption with isosbestic (at 395 nm) generation of a strong long-lived dual λ_{max} absorption at 335/345 nm and an even larger absorption at and below 300 nm (Figure 2). This spectral evolution is accompanied in the ^1H NMR spectrum (unbuffered CD_3CN-H_2O 9:1) by a steady reduction in the **2** anion vinyl singlets at δ 5.35 and 6.15 and appearance of new vinyl singlets, including a long-lived pair of signals in the range of δ 5.1−5.2 and 5.3−5.5. In the case of neopentylamine and $PhCD_2NH_2$, ^1H NMR aryl H singlets for the corresponding (alkylamino)resorcinols (at δ 6.31/6.37 and 6.31/6.34, respectively) were seen to grow in during this same time frame. Formation of (alkylamino)resorcinols **15**, which is responsible at least in part for the large absorbance increase at ~290 nm, is indicative of at least some deamination (Scheme 2, paths A or B) for these substrates and/or the generation of a side product which reduces the quinone imine (see Scheme 3 and below).

When using higher amine concentrations, especially with the very sluggishly deaminating amines *n*-propylamine and methylamine, the initial shift of λ_{max} from 490 to 460−470 could be discerned as a separate process proceeding to apparent completion prior to extensive evolution of the spectral changes occurring at shorter wavelength. The optimal conditions for studying this phenomenon were found to be the use of high concentrations of methylamine, in which case a (fast) discrete shift of λ_{max} from 490 to 460 nm (with equal extinction, Figure 3) occurred in the absence of any other significant reaction. At this time, the ^1H NMR spectrum exhibited new vinyl singlets at δ 6.74 and 5.29 (D_2O exchangeable) as well as a new methyl singlet at δ 3.79. The similarity of these UV−vis and ^1H NMR spectral characteristics to those seen upon exposure of **2** to NH_3 or cyclopropylamine in aqueous CH_3CN supports our assignment of the 460 nm absorbing species to the anionic forms of quinone imines **14**.

(30) Hartmann, C.; Brzovic, P.; Klinman, J. P. *Biochemistry* **1993**, *32*, 2234.

Decomposition of Catalyst 2 Induced by "Unactivated" Amines. In an effort to obtain ^{13}C NMR spectra for the quinone imines, we examined higher concentrations of **2**/amine mixtures in order to obtain sufficient ^{13}C NMR information within the short time frame mandated for these reactive intermediates. However, under these conditions (e.g., 80 mM quinone **2** and 16 equiv of methylamine in CD_3CN-H_2O), an altogether new spectral pattern was observed; a single species exhibiting ^1H NMR vinyl singlets at δ 4.47 and 5.09 and a UV−vis absorption at 340 nm was generated cleanly in 15 min. In 2−3 h, half of this species was converted into the previously observed long-lived species with characteristic vinyl singlets at δ 5.10 and 5.31 and UV−vis absorptions at 335/345 nm. Full $^1H/^{13}C$ NMR spectral analysis of the metastable species present at 15−30 min into the reaction could be obtained and implicated a 2:1 amine−quinone adduct (*two* methylamine-derived ^1H and ^{13}C methyl signals were seen). The appearance of large diastereotopic splitting of the ethylene side-chain ^1H NMR signals and the finding that one of the four ring quaternary ^{13}C signals appears upfield at δ 49 permits us to assign the species as the C-1 amine Michael adduct **16** of the *N*-methyl quinone imine. Interestingly, when the same reaction was conducted in CD_3CN-D_2O, the downfield vinyl ^1H singlet of the Michael adduct was found to be D-exchangeable, in contrast to the case of quinone **2** and quinone imine **9**, where the upfield vinyl ^1H singlet is D-exchangeable. Since it is the β-diketone-like H-3 which is subject to D-exchange, this result indicates that the H-6 of the Michael adduct **16** resonates at δ 4.58, a reasonable outcome for an enolic ^1H.

In the case of *n*-propylamine and $PhCD_2NH_2$ under the same conditions (80 mM **2** and 16 equiv of amine in CD_3CN-H_2O 9:1), the initial shift of the 490 nm absorption to 460 nm starts to merge with the appearance of the 335/345 nm species too fast to permit ^{13}C NMR analysis. Nonetheless, the characteristic vinyl signals for the proposed quinone imine Michael adducts **16** could be seen clearly in the ^1H NMR spectrum (δ 4.58 and 5.10 for *n*-propylamine).

For each amine, the species giving rise to the long-lived absorption at 335/345 nm and ^1H NMR resonances at δ 5.1−5.2 and 5.3−5.5 could be isolated as a stable end product and was identified as the two-electron oxidized form **18** of the fragmentation product **17** arising from quinone imine Michael adduct **16** (Scheme 3). Under these anaerobic conditions, the simultaneous generation of (alkylamino)resorcinols **15** discussed above can be rationalized in terms of the redox reaction between the quinone imines **14** and the strongly reducing species **17**, as shown. The existence of **18** as symmetrical resonance-stabilized

hydrogen-bonded tautomers has been previously described.[31] Although we have not identified the fate of the side chain in the fragmentation leading to **17**, we believe the reaction must be a base-induced β-elimination, rather than an intramolecular side-chain amide displacement, because (i) the 2-*tert*-butyloxazoline expected from the latter pathway is not seen and (ii) the hydantoin version of the TPQ model,[8] which cannot undergo cycloelimination, gives the fragmentation product more quickly than does **2**.

Compounds **18** could be independently accessed by oxidation of 1,2,4-benzenetriol in the presence of excess amine. We ultimately determined that the fragmentation products **18** constitute the main form of catalyst destruction which competes with productive deamination in the case of attempted aerobic turnover deamination of the "unactivated" amines.

Conclusion and Biochemical Significance. We have described a structurally relevant model system for the TPQ quinone cofactor of copper amine oxidases that is active in the catalytic aerobic deamination of benzylamine and certain other activated amines. This is the first TPQ model described[32] which is an effective deaminating catalyst on its own. The free amino acid TPQ can deaminate amines, but only in the presence of Cu(II),[13,23] which is needed in this case to prevent termination of catalytic recycling via cyclocondensation of the free α-amino group into the quinone nucleus, an event without relevance to the enzymatic reaction. Since there is good evidence for location of the enzyme copper near the C-2 oxygen of the quinone cofactor and for a role of this copper in catalyzing reoxidation of the reduced cofactor,[27] we expected that the addition of Cu(II) to our reaction system might improve turnover yields. Our finding of inhibition rather than stimulation suggests that Cu(II) interacts with the catalyst in our model reaction system in a mode which is prevented in the enzyme active site. Models containing ligands which coordinate the Cu(II) in a manner that mimics the active site structure may unmask an enzymologically relevant beneficial effect of Cu(II) in model deaminations.

The course of the reaction of our quinone model with both activated and unactivated amines was monitored by NMR and UV−vis spectroscopy under anaerobic conditions. These results coupled with product analyses on the aerobic reaction mixtures have led to an understanding of the factors which limit the turnover potential of our catalyst. A benzoxazole-forming side reaction under aerobic conditions could theoretically occur for the enzyme and should be kept in mind as a possible source of enzyme inactivation by certain amine substrates. On the other hand, the formation of quinoneimine C-1 Michael adducts at high [amine], and the fragmentation between quinoid nucleus and side chain thereby induced, are probably precluded in the enzyme case due to steric protection afforded by constraints of active site structure. More bulky C-1 alkyl substituents may thus provide access to models that are better *functional* mimics of the enzyme.

Although we can conclude that deamination must proceed following initial amine addition to the most electrophilic C-5 carbonyl, the conditions used here (excess amine in aqueous CH_3CN) are not amenable to a dissection of mechanism owing to the occurrence of rapid redox interchanges between the various oxidized and reduced quinone and quinone imine species. Certainly, the fact that efficient turnover in our model system is limited to *activated* primary amines points out an important feature of the enzyme mechanism which is not being recruited by our model, perhaps the involvement of intramolecular general base catalysis. Nonetheless, our work has succeeded in generating full spectral characterization of the intermediates likely to be involved in the reaction cycle, ultimately required for a complete evaluation of mechanism.

Experimental Section

General Methods. NMR spectra were obtained on a Varian Gemini 300 instrument (^{13}C NMR at 75 MHz), with chemical shifts being referenced to TMS or the solvent peak. For spectra taken in CD_3CN-H_2O, the CD_3CN ^1H and ^{13}C signals served as reference, and the water signal was suppressed. High-resolution mass spectra (HRMS, electron impact) were obtained at 20−40 eV on a Kratos MS-25A instrument. UV−visible spectra were obtained using a jacketed (temperature-controlled) cell compartment and Perkin-Elmer PECSS software. For anaerobic UV−vis and NMR experiments, the solvent was thoroughly degassed by freeze−pump−thaw prior to the admission of argon. Doubly distilled water was used for all experiments. Melting points are uncorrected. Thin-layer and preparative-layer chromatography were run on Merck silica gel 60 plates with a 254 nm indicator. All solvents, reagents, and organic fine chemicals were the most pure available from commercial sources. Amines were freshly fractionally distilled under N_2 from NaOH pellets. Benzylamine-d_2 was prepared from $LiAlD_4$ (98% atom D) reduction of benzamide. Benzylamine-d_1 was prepared via the intermediacy of benzyl alcohol-d_1 as described.[33] All evaporations were conducted at reduced pressure using a rotary evaporator. *N*-(2,4,5-Trihydroxyphenethyl)pivalamide (**1**) and 2-hydroxy-5-(2-pivalamidoethyl)-1,4-benzoquinone (**2**) were prepared as described previously.[8]

General Method for Aerobic Catalytic Deamination. A mixture of 5 mmol of benzylamine, 0.5 mmol of KH_2PO_4 (or 0.5 to 5 mmol of boric acid), and 0.1 mmol of **2** in 100 mL of 30% aqueous CH_3CN was adjusted to pH 10.0 (or some other pH, see Table 1) with KOH. The pH values listed in Table 1 are those read on a pH meter standardized with aqueous reference buffers and have not been corrected for the mixed solvent system. The solutions were magnetically stirred vigorously in an open 250 mL Erlenmeyer flask at 26 °C with monitoring of the pH, the reaction volume being maintained by periodic addition of CH_3CN, which evaporates somewhat at long reaction times. The pH remained at the initially set value during the first 4 h of the reactions and fell at most 0.10 pH unit over the course of 23 h. Two 50 mL aliquots were worked up at different reaction times by addition to each of 18 mL of standard (2,4-dinitrophenyl)hydrazine reagent (142 mM, in 2.7 N H_2SO_4 in 70% aqueous EtOH). After cooling to O °C for 1 h, the solution was filtered, and the precipitate was dried to constant weight to obtain the yield of the 2,4-DNP derivative of PhCH=O, the identity and purity of which were confirmed by TLC and ^1H NMR. Deamination of cinnamylamine was conducted in the same manner except that 2.5 mmol of the amine·HCl and 0.5 mmol of K_2HPO_4 were used. Deamination of dibenzylamine required the use of 50% aqueous CH_3CN as solvent. The 2,4-DNP derivatives of the corresponding aldehydes (benzaldehyde and cinnamaldehyde) were confirmed by TLC and ^1H NMR. Similar experiments on *n*-butylamine and *trans*-2-phenylcyclopropylamine·HCl did not yield any amine-derived carbonyl product.

Isolation of Phenyl- and Styrylbenzoxazoles 3. For the benzylamine and cinnamylamine reactions above, aliquots after 24 h were concentrated to half the original volume and extracted with EtOAc (2 × 40 mL). The organic layer was dried and evaporated, and the residue was subjected to silica gel chromatography (eluent EtOAc) to give, after recrystallization, analytical samples of the respective benzoxazoles. **6-Hydroxy-2-phenyl-5-(2-pivalamidoethyl)benzoxazole (3a)** (7% isolated yield): mp 208 °C; λ_{max} (CH_3CN-H_2O) 314 nm (sh 330 nm); ^1H NMR (DMSO-d_6) δ 1.05 (s, 9H), 2.77 (t, 2H, J = 7.1 Hz), 3.29 (m, 2H), 7.11 (s, 1H), 7.41 (s, 1H), 7.49 (bt, 1H, NH), 7.56−7.58 (m, 3H), 8.09−8.13 (m, 2H), 9.98 (s, 1H, OH); ^{13}C NMR (DMSO-d_6) δ

(31) Manthey, M. K.; Pyne, S. G.; Truscott, R. J. W. *Aust. J. Chem.* **1989**, *42*, 365.

(32) The work described here was presented in preliminary form: Lee, Y.; Sayre, L. M. *Abstracts of Papers*, 208th National Meeting of the American Chemical Society, Washington, DC, August 21−25, 1994; American Chemical Society: Washington, DC, 1994; ORGN 085.

(33) Kim, J.-M.; Bogdan, M. A.; Mariano, P. S. *J. Am. Chem. Soc.* **1993**, *115*, 10591.

27.4, 30.1, 37.9, 39.0, 96.4, 120.2, 124.3, 126.6, 126.8, 129.2, 131.1, 133.7, 149.6, 154.2, 160.3, 177.2; HRMS calcd for $C_{20}H_{22}N_2O_3$ *m/z* (rel intensity) 338.1632, found 338.1661 (M^+, 28). **6-Hydroxy-2-styryl-5-(2-pivalamidoethyl)benzoxazole (3b)** (15% isolated yield): mp 159 °C; λ_{max} (CH_3CN-H_2O) 342 nm; ^1H NMR (DMSO-d_6) δ 1.05 (s, 9H), 2.50 (t, 2H, J = 6.9 Hz), 3.28 (m, 2H), 7.06 (s, 1H), 7.23 and 7.66 (2d, 1H each, J = 16.4 Hz), 7.34 (s, 1H), 7.40 (m, 1H), 7.42 (m, 2H), 7.47 (bt, 1H, NH), 7.75 (m, 2H), 9.97 (bs, 1H, OH); ^{13}C NMR (DMSO-d_6) δ 27.4, 30.0, 37.9, 39.0, 96.2, 114.1, 120.1, 124.1, 127.5, 128.8, 129.4, 134.1, 135.1, 137.2, 149.2, 154.2, 160.5, 177.2; HRMS calcd for $C_{22}H_{24}N_2O_3$ *m/z* (rel intensity) 364.1788, found 364.1768 (M^+, 7).

4-Amino-6-(2-pivalamidoethyl)resorcinol (4). Through a solution of triol **1** (50.6 mg, 0.2 mmol, containing a trace of quinone **2** as evidenced by its slight yellow coloration) in 0.5 mL of *degassed* CH_3CN was bubbled NH_3 gas for 2 min. The mixture was allowed to stand for 2 h in a sealed system, and the solvent (and NH_3) was removed under high vacuum to leave an air-sensitive resin: ^1H NMR (CD_3CN) δ 1.09 (s, 9H), 2.57 (t, 2H, J = 6.7 Hz), 3.22 (m, 2H), 6.36 (s, 1H), 6.38 (s, 1H), 6.89 (b, >1H, NH); ^{13}C NMR (CD_3CN) δ 27.8, 30.2, 39.1, 41.7, 104.4, 117.3, 118.8, 128.7, 145.3, 148.5, 179.9; HRMS calcd for $C_{13}H_{20}N_2O_3$ *m/z* (rel intensity) 252.1469, found 252.1473 (17.1).

Independent Synthesis of 4-Benzimino-6-(2-pivalamidoethyl)-resorcinol (5) and Benzoxazole 3a from Aminoresorcinol 4. The aminoresorcinol **4** obtained from 0.2 mmol of **1** as above was dissolved in 1 mL of CH_3CN, to which was added benzaldehyde (40 μL, 0.4 mmol). After 30 min, the solvent and excess PhCHO was removed under high vacuum to afford, without further purification, the Schiff base **5**: λ_{max} (CH_3CN-H_2O) 370 nm; ^1H NMR (CD_3CN) δ 1.12 (s, 9H), 2.71 (t, 2H, J = 7.1 Hz), 3.32 (m, 2H), 6.47 (s, 1H), 6.75 (br t, 1H, NH), 7.18 (s, 1H), 7.44–7.47 (m, 3H), 7.92–7.96 (m, 2H), 8.66 (s, 1H, N=CH); ^{13}C NMR (CD_3CN) δ 27.8, 30.6, 39.2, 40.9, 102.8, 118.5, 119.1, 128.8, 129.4, 129.7, 131.8, 137.6, 153.4, 154.9, 157.3, 180.2; HRMS calcd for $C_{20}H_{24}N_2O_3$ *m/z* (rel intensity) 340.1788, found 340.1788 (23.5).

The residue **5** was taken up in 50 mL of CH_3CN-H_2O (1:1), which was adjusted to pH 10 and stirred for 16 h open to the air. The reaction mixture was concentrated to remove CH_3CN and extracted with EtOAc. The organic extract was dried (Na_2SO_4) and evaporated. The residue was shown by TLC (EtOAc) and NMR (DMSO-d_6) to contain benzoxazole **3a** as a major component.

General Method for Preparation of 4-(Alkylamino)-6-(2-(pival-amidoethyl)resorcinols 10 and 15. To a solution of triol **1** (containing a trace of quinone **2**) in *degassed* CH_3CN is added 1.05 equiv of amine. Evaporation of the solvent after several hours affords the desired product, for which NMR spectra can be obtained under argon without any noticeable decomposition. The phenethylamine analog was previously characterized.[8]

4-(Benzylamino)-6-(2-pivalamidoethyl)resorcinol (10): ^1H NMR (DMSO-d_6) δ 1.07 (s, 9H), 2.48 (t, 2H, J = 7.3 Hz), 3.12 (app q, 2H), 4.21 (s, 2H), 6.21 (s, 1H), 6.35 (s, 1H), 7.19–7.34 (m, 5H), 8.38 (br s, 1H, NH), 9.0 (br, 1H, OH); ^{13}C NMR (DMSO-d_6) δ 27.3, 29.5, 37.8, 39.9, 47.8, 102.7, 112.9, 115.3, 126.5, 127.2, 128.1, 129.7, 140.8, 143.2, 145.9, 177.2; HRMS calcd for $C_{20}H_{26}N_2O_3$ *m/z* (rel intensity) 342.1944, found 342.1935 (15.9).

4-[(Phenyldideuteriomethyl)amino]-6-(2-pivalamidoethyl)resorcinol (10-d_2): ^1H NMR δ 1.10 (s, 9H), 2.57 (t, 2H), 3.21 (app q, 2H), 5.0 (br, NH/OH), 6.35 (s, 1H), 6.40 (s, 1H), 6.75 (br t, 1H, NH), 7.19–7.34 (m, 5H); ^{13}C NMR (CD_3CN) δ 27.77 (CH_3), 30.56 (CH_2), 39.14 (C), 41.52 (CH_2), 104.11 (CH), 115.44 (CH), 117.20 (C), 127.75 (CH), 128.44 (CH), 129.32 (CH), 131.20 (C), 141.61 (C), 144.98 (C), 147.59 (C), 180.23 (C), deuterated C not seen; HRMS calcd for $C_{20}H_{24}D_2N_2O_3$ *m/z* (rel intensity) 344.2070, found 342.2071 (29.8).

4-(Methylamino)-6-(2-pivalamidoethyl)resorcinol (15, R′ = CH_3): ^1H NMR (DMSO-d_6) δ 1.06 (s, 9H), 2.53 (t, 2H, J = 7.4 Hz), 3.14 (app q, 2H), 6.14 (s, 1H), 6.29 (s, 1H), 7.39 (t, 1H, J = 5.1 Hz, NH), 8.30 (br, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 27.4 (CH_3), 29.5 (CH_2), 30.9 (CH_3), 37.8 (C), 40.0 (CH_2), 102.6 (CH), 111.7 (CH), 115.3 (C), 131.3 (C), 143.0 (C), 145.4 (C), 177.0 (C); HRMS calcd for $C_{14}H_{22}N_2O_3$ *m/z* (rel intensity) 266.1631, found 266.1617 (34.1).

4-(Neopentylamino)-6-(2-pivalamidoethyl)resorcinol (15, R′ = Me_3CCH_2): ^1H NMR (CD_3CN) δ 0.96 (s, 9H), 1.11 (s, 9H), 2.64 (t, 2H, J = 6.9 Hz), 2.79 (s, 2H), 3.26 (app q, 2H, CH_2NH), 6.38 (app s, 2H), 6.78 (br t, 1H, NH); ^{13}C NMR (CD_3CN) δ 27.81, 27.97, 30.54, 32.45, 39.15, 41.60, 58.09, 103.98, 115.20, 117.21, 132.45, 144.90, 147.23, 180.11; HRMS calcd for $C_{18}H_{30}N_2O_3$ *m/z* (rel intensity) 322.2258, found 322.2246 (21.8).

4-(Cyclopropylamino)-6-(2-pivalamidoethyl)resorcinol (15, R′ = c-C_3H_5): ^1H NMR (CD_3CN) δ 0.40 (m, 2H), 0.63 (m, 2H), 1.12 (s, 9H), 2.29 (m, 1H), 2.64 (t, 2H, J = 7.1 Hz), 3.25 (app q, 2H), 6.32 (s, 1H), 6.68 (s, 1H), 6.71 (br, 1H, NH); ^{13}C NMR (CD_3CN) δ 7.5, 26.6, 30.8, 39.1, 41.4, 103.8, 115.3, 117.2, 131.8, 144.2, 147.6, 180.0; HRMS calcd for $C_{16}H_{24}N_2O_3$ *m/z* (rel intensity) 292.1788, found 292.1787 (3.1).

General Method for Identification of the Products of Amine-Induced Anaerobic Reduction of Quinone Cofactor Model 2. There are three potential reduced products, the triol **1**, the aminoresorcinol **4**, and the (alkylamino)resorcinol (e.g., **10**). Since all are unstable in air, identification and quantitation are best accomplished by conversion to the corresponding air-stable triacetylated forms. For NMR and UV–vis spectral analysis of anaerobic versions of the aerobic turnover deamination reactions (Table 1), a solution of 0.01 mmol of **2** in 0.5 mL of CD_3CN-H_2O (9:1) was degassed, and 3–5 equiv of amine was added under argon via microliter syringe. At the end of the observation period, all volatiles were removed *in vacuo*. To this residue, or, to the independently-generated **1**, **4**, or **10** dissolved in a minimal amount of DMSO was added 50 equiv of triethylamine and then 50 equiv of acetic anhydride under argon. Utilization of a smaller excess of the reagent risks isolation of some diacetylated derivative. After 24 h, the mixture is taken to dryness at high vacuum, and the residue is partitioned between water and EtOAc. Drying of the organic extract (Na_2SO_4) and evaporation afforded the triacetylated derivatives, which can be purified by silica gel chromatography (EtOAc eluent) if necessary.

The *O,O,O-triacetylated derivative of* **1** displayed ^1H NMR ($CDCl_3$) δ 1.11 (s, 9H), 2.239/2.245/2.31 (3s, 3H each, CH_3C=O), 2.70 (t, 2H, J = 6.8 Hz), 3.41 (app q, 2H, CH_2NH), 5.84 (br t, 1H, NH), 6.97 (s, 1H), 7.02 (s, 1H). The *N,O,O-triacetylated derivative of* **4** displayed ^1H NMR ($CDCl_3$) δ 1.12 (s, 9H), 2.22/2.28/2.36 (3s, 3H each, CH_3C=O), 2.76 (t, 2H, J = 6.9 Hz), 3.45 (app q, 2H, CH_2NH), 5.83 (br t, 1H, NH), 7.09 (s, 1H), 7.11 (s, 1H). The *N,O,O-triacetylated derivative of* **10** exhibits large diastereotopic splittings apparently on account of restricted rotation (only a single atropisomer is seen): ^1H NMR ($CDCl_3$) δ 1.12 (s, 9H), 1.88/2.16/2.36 (3s, 3H each, CH_3C=O), 2.61 (m, 2H), 3.19 and 3.37 (2m of symmetric ABXY, 1H each), 4.30 and 5.21 (2d, 1H each, J = 14.3 Hz, $PhCH_2$), 5.62 (br t, 1H, NH), 6.76 (s, 1H), 7.01 (s, 1H), 7.17–7.27 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 20.69, 20.98, 22.26, 27.54, 29.49, 38.63, 39.38, 51.69, 118.30, 127.62, 128.47, 129.10, 129.81, 131.33, 132.49, 137.27, 145.67, 148.75, 168.30, 169.04, 170.79, 178.49; HRMS calcd for $C_{26}H_{32}N_2O_6$ 468.2262, found 468.2284.

The *N,O,O-triacetylated derivative of* **10**-d_2 exhibits similar diastereotopic splittings: ^1H NMR ($CDCl_3$) δ 1.11 (s, 9H), 1.87/2.15/2.34 (3s, 3H each, CH_3C=O), 2.61 (m, 2H), 3.19 and 3.36 (symmetric 2m of ABXY, 1H each), 5.63 (br t, 1H, NH), 6.76 (s, 1H), 7.01 (s, 1H), 7.17–7.27 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 20.68, 20.97, 22.25, 27.54, 29.48, 38.62, 39.39, 118.30, 127.64, 128.47, 129.10, 129.82, 131.34, 132.44, 137.16, 145.68, 148.75, 168.30, 169.04, 170.80, 178.51, (CD_2 not seen); HRMS calcd for $C_{26}H_{30}D_2N_2O_6$ *m/z* (rel intensity) 470.2487, found 470.2377 (87.8).

Independent Synthesis of Indophenol 11 From Aminoresorcinol 4 and Quinone 2. The aminoresorcinol **4** was prepared as described above from 0.23 mmol of **1** in degassed CH_3CN. After removal of the solvent, quinone **2** (0.23 mmol) was added to the residue dissolved in 0.5 mL of degassed DMSO-d_6. The NMR spectrum at this time indicated the presence of a new 1:1 adduct (coinciding with appearance of an absorption at 580 nm) along with trace amounts of **4** and triol **1**. Purification of **11** was accomplished by concentration at high vacuum and application of the residue to a preparative TLC plate, eluting with EtOAc:acetone (3:1). Extraction of the fast-moving yellow band (λ_{max} 365 nm) with EtOAc–acetone affords the neutral material, whereas extraction with MeOH–acetone affords the sodium salt of the anionic form (silica contains sodium ions) reponsible for the 580 nm absorption. The ^1H and ^{13}C NMR spectra of the neutral form in DMSO-d_6 indicated that the compound does not exist as a straightforward indophenol. The appearance of (i) only three (rather than four) low-field ^{13}C signals

and ^{1}H vinyl signals, (ii) diastereotopic splittings of one of the two CH_2CH_2 side chains, and (iii) an upfield AB 2H ^{1}H NMR signal to replace the missing vinyl H suggest to us that **11** exists as the cyclic hemiketal **12**: ^{1}H NMR (DMSO-d_6) δ 1.00 (s, 9H), 1.06 (s, 9H), 2.30 (m, 1H), 2.37 (m, 1H), 2.64 (t, 2H), 3.12 (AB, 2H), 3.22 (m, 4H), 6.46 (s, 1H), 6.94 (s, 1H), 7.11 (s, 1H), 7.3 (variable, br s, 1H, slowly exchanged OH), 7.48 (m, 2H, NH); ^{13}C NMR (DMSO-d_6) δ 27.36 (CH_3), 27.42 (CH_3), 28.99 (CH_2), 29.80 (CH_2), 36.87 (CH_2), 37.89 (C), 38.96 (CH_2), 49.90 (CH_2), 88.65 (C), 102.63 (CH), 120.72 (C), 126.29 (C), 129.44 (CH), 138.72 (CH), 142.15 (C), 143.66 (C), 151.54 (C), 157.00 (C), 177.17 (C), 177.43 (C), 194.31 (C); FAB HRMS calcd for $C_{26}H_{35}N_3O_6$ *m/z* (rel intensity) 485.2527, found 485.2488 (5).

Conversion of Quinone 2 to Quinone Imine 9. Exposure of solutions of **2** in CH_3CN-H_2O (1.0 mM) to excess NH_4OH results in a shift of the **2** anion chromophore at 490 nm to a 450 nm absorption of equal extinction, interpreted in terms of generation of the anion of **9**. For NMR spectral confirmation of this conversion, anhydrous NH_3 was bubbled for 1 min through a solution of **2** (0.1 mmol) in 0.5 mL of DMSO-d_6. After 2 h, excess NH_3 was removed through freeze–pump–thaw cycling at high vacuum. NMR indicated quantitative conversion to **9** anion: ^{1}H NMR (DMSO-d_6) δ 1.05 (s, 9H), 2.42 (t, 2H, J = 6.9 Hz), 3.13 (m, 2H), 4.93 (s, 1H), 6.50 (s, 1H), 7.60 (br t, 1H, J = 4.9 Hz, NH); ^{13}C NMR (DMSO-d_6) δ 27.4, 29.4, 37.8, 38.9, 100.0, 130.2, 145.1, 167.2, 169.1, 177.1, 184.3. Addition of a slight excess of $NaBH_4$ to samples of **9** results in an instantaneous appearance of the spectral properties exhibited by aminoresorcinol **4**.

Conversion of Quinone 2 to *N*-(Cyclopropyl) Quinone Imine 13. Dissolution of **2** (12.6 mg, 0.05 mmol) and cyclopropylamine (10 μL, 0.15 mmol) in CD_3CN resulted in precipitation of a yellow solid; filtration after 30 min afforded 8 mg (55%) of the cyclopropylamine salt of **13** anion: mp 179–180 °C; ^{1}H NMR (CD_3CN-H_2O 9:1) δ 1.08 (s, 9H), 1.10 (m, 2H), 1.20 (m, 2H), 2.51 (t, 2H, J = 6.3 Hz), 3.26 (app q, 2H), 3.58 (m, 1H), 5.54 (s, 1H), 7.09 (s, 1H), 6.88 (br s, 1H, NH), 7.09 (s, 1H); λ_{max} 455 nm (CH_3CN-H_2O). The spectra obtained in anhydrous CD_3CN (the salt is only slightly soluble) corresponds to the neutral form of imine **13**: ^{1}H NMR (CD_3CN) δ 1.07 (s, 9H), 1.15 (m, 2H), 1.26 (m, 2H), 2.54 (t, 2H, J = 6.3 Hz), 3.30 (app q, 2H), 3.64 (m, 1H), 5.76 (s, 1H), 6.39 (br s, 1H, NH), 7.19 (s, 1H); λ_{max} 348 nm (CH_3CN); HRMS calcd for $C_{16}H_{22}N_2O_3$ *m/z* (rel intensity) 290.1631, found 290.1633 (4.5).

Reaction of Quinone 2 with "Unactivated" Amines. An excess of methylamine (as a 40% aqueous solution), *n*-propylamine, *n*-butylamine, or neopentylamine is added under argon to a thoroughly degassed solution of quinone **2** in CH_3CN-H_2O (1:1). In the case of methylamine, UV–vis spectroscopy (1 mM **2**, 50 mM CH_3NH_2) indicates an immediate λ_{max} shift from 490 to 460 nm followed by a slow decay of this absorption with isosbestic increase at 335/345 nm (Figure 3). NMR spectra (5 mM **2**, 30 mM CH_3NH_2) taken within the first 30 min are consistent with the presence of the quinone imine **14** (R′ = CH_3): ^{1}H NMR (CD_3CN-H_2O 1:1) δ 1.08 (s, 9H), 2.53 (m, 2H), 3.30 (m, 2H), 3.46 (s, 3H), 5.33 (s, 1H, D_2O exchangeable), 6.79 (s, 1H).

The use of higher amine concentrations and lower H_2O solvent content permits observation of metastable Michael adducts **16**. Reaction of **2** (2.5 mg, 10 mM) with methylamine (8.6 μL, 100 mM) in 1 mL of CD_3CN-H_2O 9:1 gives after 5 min **16**, R′ = CH_3: ^{1}H NMR δ 1.07 (s, 9H), 1.60 and 1.73 (symmetric 2m of ABMN, 1H each), 1.97 (s, 3H), 2.64 (s, 3H), 2.95 and 3.08 (symmetric 2m of ABMN, 1H each), 4.51 (s, 1H), 5.13 (s, 1H), 7.08 (br s, 1H, NH); ^{13}C NMR δ 27.7 (+), 30.1 (+), 30.3 (+), 36.7 (−), 39.1 (−), 41.3 (−), 64.0 (−), 102.3 (+), 102.7 (+), 144.4 (−), 180.0 (−), 182.8 (−), 195.7 (−); λ_{max} 340 nm.

At longer reaction time, the principal species present (by ^{1}H NMR) when **2** is exposed to high concentrations of unactivated amines are the corresponding (alkylamino)resorcinols **15** (see above for independent synthesis and characterization) and the corresponding elimination products **18**. The latter sometimes crystallize selectively from solution. Compounds **18** can also be isolated (usually by silica gel chromatography, EtOAc eluent) from aerobic reactions of **2** in the presence of excess "unactivated" amines, since any (alkylamino)resorcinol formed is oxidatively turned over. A high yield in the case of **18** (R′ = CD_2Ph) was obtained in this way, and was fully characterized as described below. Additionally, analogs **18** can be prepared independently from 1,2,4-benzenetriol.

5-Hydroxy-*N*-(phenyldideuteriomethyl)-2-[(phenyldideuteriomethyl)amino]-1,4-benzoquinone Imine (18, R′ = CD_2Ph). Through a solution of **2** (37.5 mg, 0.15 mmol) and $PhCD_2NH_2$ (96 μL, 0.9 mmol) in 5 mL of CH_3CN-H_2O (9:1) was bubbled O_2 for 20 min. After 20 h, the precipitated solid was filtered, washed (CH_3CN), and dried to give 29 mg (60%): mp 182 °C; ^{1}H NMR (DMSO-d_6) δ 4.98 (s, 1H), 5.61 (s, 1H), 7.20–7.31 (m, 10 H), 9.58 (br s, 2H); ^{13}C NMR (DMSO-d_6) δ 82.7 (−), 97.7 (−), 127.5 (−), 127.7 (−), 128.5 (−), 136.4 (+), 156.2 (+), 172.1 (+); HRMS calcd for $C_{20}H_{14}D_4N_2O_2$ *m/z* (rel intensity) 322.1620, found 322.1615 (19.8).

***N*-Alkyl-2-(alkylamino)-5-hydroxy-1,4-benzoquinone Imines 18.** Through a solution of 1,2,4-benzenetriol (12.6 mg, 0.1 mmol) and 10 equiv of alkylamine in 0.5 mL of CH_3CN (CD_3CN) was bubbled O_2 for 10 min. The precipitated solid was filtered. From neopentylamine: ^{1}H NMR (CD_3CN) δ 0.99 (s, 18 H), 3.26 (d, 4H, J = 7.14 Hz), 5.09 (s, 1H), 5.51 (s, 1H), 8.32 (br s, 2H). From benzylamine: ^{1}H NMR (CD_3CN-H_2O 9:1) δ 4.57 (s, 4H), 5.19 (s, 1H), 5.44 (s, 1H), 7.25–7.35 (m, 10 H). From *n*-butylamine: ^{1}H NMR (DMSO-d_6) δ 0.89 (t, 6H, J = 7.3 Hz), 1.30 (m, 4H), 1.56 (m, 4H), 3.39 (t, 2H, J = 6.3 Hz), 4.96 (s, 1H), 5.47 (s, 1H), 9.12 (br s, 2H). The latter compound was reported previously.[31]

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