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Model Studies on the Quinone-Containing Copper Amine Oxidases. Unambiguous Demonstration of a Transamination Mechanism

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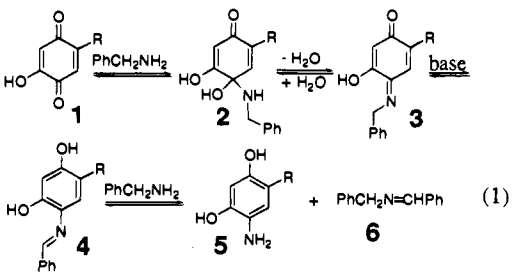
Abstract: Utilizing a pivalamidoethyl-based model for the 2,4,5-trihydroxyphenylalaninequinone cofactor of copper amine oxidases, we recently reported (*J. Am. Chem. Soc.* **1995**, *117*, 3096) catalytic aerobic deamination of activated amines and full characterization of the various possible intermediates which could be involved in either a transamination mechanism, employed by the enzymes, or an alternate addition–elimination mechanism shown to compete in other quinone model reactions. However, attempts to distinguish between these two mechanisms through product analysis under anaerobic single-turnover conditions were thwarted by the occurrence of redox interchange reactions that scrambled the initial cofactor reduction product. Utilizing a *tert*-butyl cofactor model, we here describe reaction conditions which permit a definitive conclusion of transamination in the case of benzylamine using either CH_3CN or DMSO as solvent. The interplay between the rate-limiting step in such conditions and the appearance of a primary kinetic isotope effect using $PhCD_2NH_2$ is discussed. Further, the use of 5-amino-2,4-cyclohexadienecarboxylic acid permits unambiguous mechanistic conclusions because the initial single-turnover products in this case tautomerize to aromatic moieties incapable of redox interchange. The reaction follows mainly transamination, though addition–elimination appears to compete somewhat for this branched primary amine.

Introduction

Since the report in 1990 that the active carbonyl cofactor used by copper amine oxidases is the quinone form (TPQ) of an active site 2,4,5-trihydroxyphenylalanine (TOPA) residue,[1] there has been renewed interest in mechanistic and model studies.[2-6] On the basis of our development of a pivalamidoethyl-based model (**1**, R = $CH_2CH_2NHC(=O)C(CH_3)_3$) which effected deamination of benzylamine,[2] we recently characterized the reactivity of this model for catalytic aerobic deamination of activated amines in buffered aqueous acetonitrile and investigated the mechanism under single-turnover (anaerobic) conditions.[3] A pyridoxal-like transamination mechanism (eq 1)



(1)

appears to be in force for the enzyme, since anaerobic single turnover results in release of aldehyde product, with NH_3 being

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(1) Janes, S. M.; Mu, S.; Wemmer, D.; Smith, A. J.; Kaur, S.; Maltby, D.; Burlingame, A. L.; Klinman, J. P. *Science* **1990**, *248*, 981.
(2) Wang, F.; Bae, J. Y.; Jacobson, A. R.; Lee, Y.; Sayre, L. M. *J. Org. Chem.* **1994**, *59*, 2409.
(3) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 3096–3105.
(4) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 7117.
(5) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 8707.
(6) Nakamura, N.; Kohzuma, T.; Kuma, H.; Suzuki, S. *J. Am. Chem. Soc.* **1992**, *114*, 6550.

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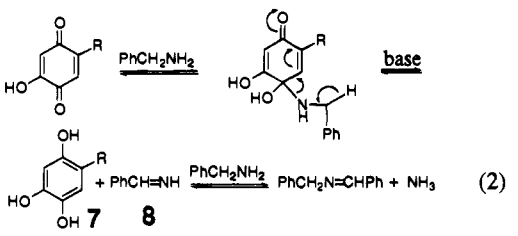
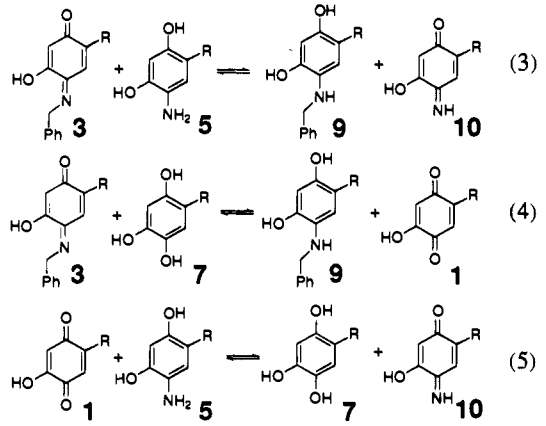
released only upon O_2-dependent reoxidation of the reduced cofactor. However, an alternative addition−elimination mechanism (eq 2) cannot *a priori* be ruled out for any given model



(2)

system because this pathway has been observed to compete with transamination in reactions of amines with pyrroloquinoline-quinone.[7,8]

The two mechanisms are *theoretically* readily distinguished through determination of the product of model cofactor reduction after single turnover; transamination (eq 1) predicts aminoresorcinol **5** or its benzaldehyde Schiff base derivative **4**, whereas addition−elimination (eq 2) predicts benzenetriol **7**. Unfortunately, we found that, under conditions where the products should be assignable unambiguously on the basis of NMR, redox interchange reactions[2] occur competitively with the deamination reaction, so that the actual observed product of model cofactor reduction was either entirely the unexpected (benzylamino)-resorcinol **9** or a scrambled mixture of **9** with **5** and **7**.[3] The production of **9** could be rationalized by *either* mechanism, arising from reduction by **5** (eq 3) or **7** (eq 4) of the "substrate imine" **3**, present in any event in equilibrium with **1**.[9] Moreover, although aminoresorcinol **5** or triol **7** would appear to arise from transamination (eq 1) or addition−elimination (eq 2), respectively, triol **7** could also form from transamination via reduction of starting quinone **1** by aminoresorcinol **5** (eq 5), whereas



aminoresorcinol **5** could also form from addition−elimination via triol **7** reduction (reverse of eq 5) of the quinone imine **10**, the latter derived from **1** and NH_3. In this paper, we now describe approaches found to circumvent these problems, and provide unambiguous evidence for a transamination pathway in a model reaction.[10]

Results and Discussion

The limitation of our previous attempts to use NMR to identify the *initial* product of cofactor reduction under single-turnover (strictly anaerobic) conditions was that such initial product did not have a sufficient lifetime under the reaction conditions to be observed prior to the point where redox "scrambling" made any assignment of mechanism ambiguous. Our aim here then was to find conditions which eliminated or delayed the onset of redox scrambling. The experiments described in this paper were all performed with the R = *tert*-butyl TPQ (topaquinone) model **1** recently described by Mure and Klinman.[5] Although less reactive, this model behaves identically to our previously described R = pivalamidoethyl model, and is advantageous in that the *tert*-butyl group sterically blocks the side-chain elimination side reaction we observed in cases involving high concentrations of "transaminatively unactivated" amines.[3]

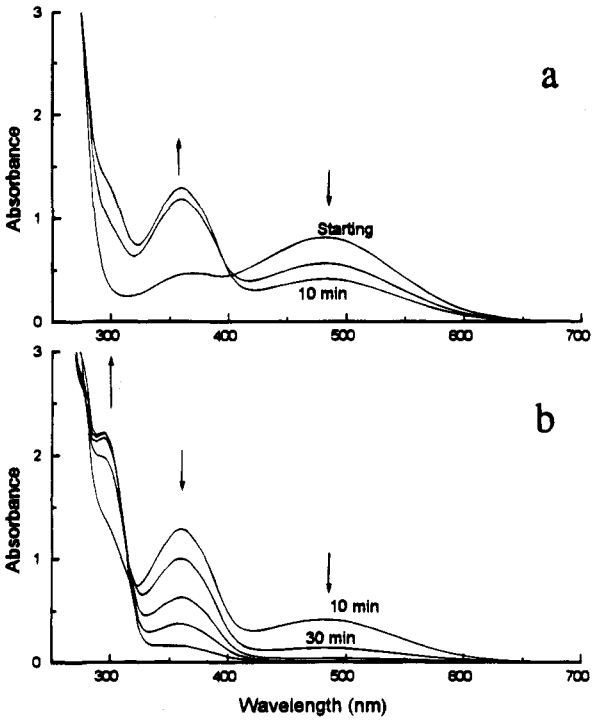


Figure 1. Progress of reaction of **1** (0.5 mM) with benzylamine (50 mM) in degassed CH_3CN under argon, 25.0 °C: (a) 0, 5, and 10 min; (b) 10, 30, 60, 120, and 300 min.

UV−Vis Evidence. Since all the redox interchanges involved bimolecular reactions of cofactor-derived species, one approach to minimizing their occurrence was to ensure low concentrations of all cofactor-derived species by choosing a low starting concentration of quinone **1**. In fact, we found that by dropping the concentration of **1** (R = *tert*-butyl) from 5 mM (used in our previous study[3]) to 0.5 mM (using 50 mM benzylamine in CH_3CN under strictly anaerobic conditions), the UV−vis spectral profile (25.0 °C) changed from one showing isosbestic conversion of **1** anion to **9**[3] to one showing two phases, an initial rapid partial decay of **1** anion at 490 nm with nearly isosbestic growth at 365 nm (peaking at 10 min), followed by a slower drop at 365 nm and a continued drop at 490 nm with growth at 296 nm (Figure 1). On the basis of our previous spectral assignments,[3] a consistent interpretation would involve intermediate production of "product imine" **4** (at 365 nm) and final production of aminoresorcinol **5** (at 296 nm), supportive of a transamination mechanism. Such a conclusion was reached independently by Mure and Klinman[5] utilizing reaction conditions very similar to those of Figure 1.

We did not believe, however, that a mechanistic conclusion could be made definitively on the basis of absorption spectroscopy alone, in large part because of overlapping chromophores

(7) Ohshiro, Y.; Itoh, S. *Bioorg. Chem.* **1991**, *19*, 169.
(8) Rodriguez, E. J.; Bruice, T. C. *J. Am. Chem. Soc.* **1989**, *111*, 7947.
(9) The quinone imine **10** formed from either eq 3 or eq 4 would be reconverted to **3** by reaction with benzylamine (releasing NH_3).
(10) This work was presented in part previously: Lee, Y.; Huang, H.; Sayre, L. M. *Abstracts of Papers*, 210th National Meeting of the American Chemical Society, Chicago, IL, Aug 20−24, 1995; American Chemical Society: Washington, DC, 1995; ORGN 439.

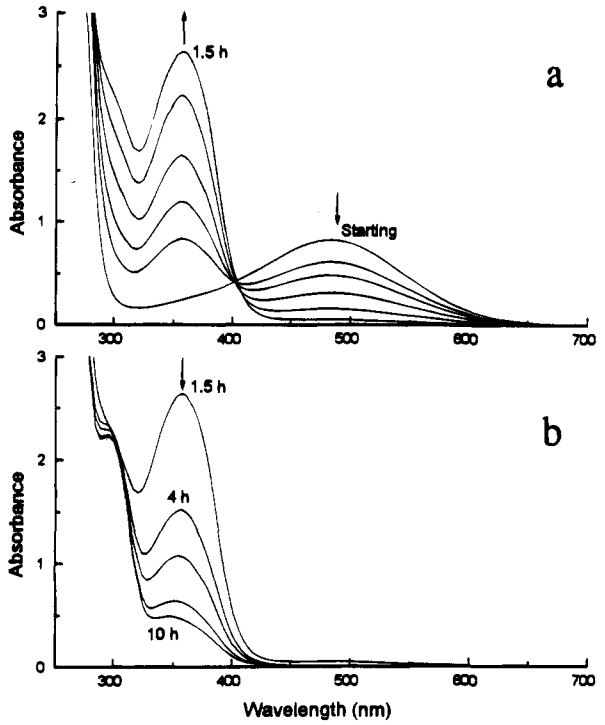
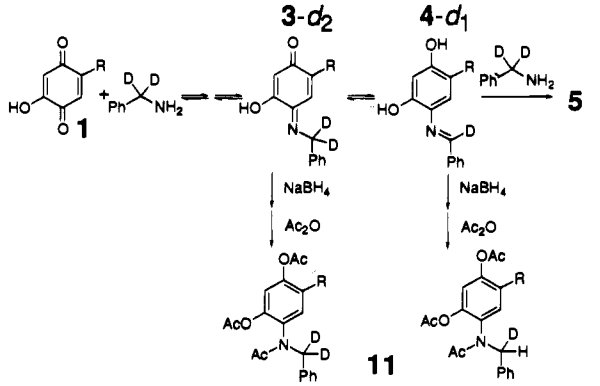


Figure 2. Progress of reaction of **1** (0.5 mM) with benzylamine-d_2 (50 mM) in degassed CH_3CN under argon, 25.0 °C: (a) 0, 5, 10, 20, 40, and 90 min; (b) 1.5, 4, 5, 7, and 10 h.

for the "substrate" and "product" imines **3** and **4**.[3,5] In fact, under the same reaction conditions as in Figure 1, benzylamine-d_2 displayed a very different spectral time profile (Figure 2): the isosbestic decrease at 490 nm with a rise at 360 nm lasted for 90 min (contrasted to 10 min), at which point no 490 nm absorbance remained at all, followed by a slower drop at 360 nm.[11] Since deuterium substitution was not expected to slow conversion of **4** to **5** as much as the transamination step (**3** to **4**),[12] we suspected that the much longer lifetime of the 360 nm absorption represented a persistence of the substrate imine **3** in this case rather than of the product imine **4**. According to this interpretation, the 365 nm absorption in Figure 1 and especially the 360 nm absorption in Figure 2 represented weighted composites of the product imine **4** (with λ_{max} = 370 nm)[3] and substrate imine **3** (with $\lambda_{max} \approx$ 350 nm).[13] In order to confirm our suspicion that the long-lived A_{360} in Figure 2 might represent more **3** than **4**, on account of a primary deuterium kinetic isotope effect, we quenched this reaction with $NaBH_4$ at the point of maximum A_{360} (90 min), followed by anaerobic acetylation and isolation of the triacetylated (benzylamino)resorcinol derivative

Scheme 1



as a stable form amenable to mass spectral analysis of the deuterium content (Scheme 1). We found a 68:32 mixture of **11**-d_2 and **11**-d_1, reflecting a 68:32 mixture of substrate imine **3**-d_2 and product imine **4**-d_1 at the point of the borohydride quench.[14] Our finding here is a good illustration of the limitation of absorption spectroscopy alone as a determinant of structural (and in turn mechanistic) assignments.

NMR Evidence. The low concentration of starting **1** used for the UV−vis experiments (0.5 mM) is too dilute to permit monitoring of the reaction by NMR, and the higher concentrations we used previously[3] have already been described to result in "redox scrambling" of the product. The ^1H NMR spectrum reported by Mure and Klinman (their Figure 6B)[5] is a good illustration of this problem. In their paper, they called attention to the predominant species seen at 30 min into the anaerobic reaction of **1** (R = *tert*-butyl) with $PhCH_2NH_2$ in CD_3CN, exhibiting singlets at δ 1.2, 4.25, 6.2, and 6.5, which they called **X**, but which was considered to be possibly the benzylamine addition product to the product Schiff base **4**.[15] If this were the case, their NMR spectrum, which also shows traces of the aminoresorcinol **5**, would support a transamination mechanism. However, through isolation and independent synthesis (see Experimental Section), we now know that their compound **X** is the (benzylamino)resorcinol **9** (the peaks listed above give the expected 9:2:1:1 integral ratio, and there is no additional downfield methine signal [PhCN(N−)$_2$] required by the putative benzylamine−**4** adduct). Moreover, ^1H NMR signals corresponding to the presence of traces of the triol **5** (vinyl singlets

(11) In contrast to our result, Mure and Klinman[5] reported that the reaction of **1** (R = *tert*-butyl) with $PhCD_2NH_2$ followed a spectral profile nearly identical to that seen for $PhCH_2NH_2$ (their Figure 8) under conditions which were nearly identical to those we employed. We have no explanation at present for this discrepancy. However, it is interesting to note that we also monitored the anaerobic reaction of **1** with the monodeuterio substrate $PhCHDNH_2$, which displayed a behavior intermediate between what we report for $PhCH_2NH_2$ (Figure 1) and what we report for $PhCD_2NH_2$ (Figure 2); viz., the adsorption at 360 nm grew to an intermediate maximal height (A_{max} = 1.8) over an intermediate duration (60 min) (data not shown). Detailed interpretation of these behaviors in terms of stepwise kinetic isotope effects will require further study.

(12) We reported an apparent primary deuterium kinetic isotope effect (KIE) of 10 (uncorrected for the secondary KIE) for the aerobic catalytic deaminative turnover of $PhCD_2NH_2$ relative to $PhCH_2NH_2$ for our pivalamidoethyl-based model in buffered (pH 10) aqueous CH_3CN.[3]

(13) We reported[3] that whereas the anionic form of **3** (from **1**, R = pivalamidoethyl, and cyclopropylamine) absorbed at λ_{max} = 455 nm in 90% aqueous CH_3CN, the neutral form absorbed at λ_{max} = 348 nm, apparently indicating that, in the present reaction conditions (100% CH_3CN), **3** exists predominantly in neutral form.

(14) Independent evidence for the persistence of substrate imine **3** in the reaction of $PhCD_2NH_2$ is that the intermediate could be observed directly by ^1H NMR during the initial stages of the reaction (e.g., at 5 min) using 20 mM **1** and 60 mM $PhCD_2NH_2$ in CD_3CN-H_2O, 9:1. In contrast, this intermediate could not be observed in the reaction of $PhCH_2NH_2$ under the same conditions. **3**-d_2 (*R* = *tert*-butyl) exhibited δ 1.27 (s, 9H), 5.62 (s, 1H), 7.05 (s, 1H), and 7.26−7.35 (m, should be 5H but overlaps with the aryl signal from $PhCD_2NH_2$), consistent with what we reported previously for **3** (R = pivalamidoethyl).[3]

(15) Evidence presented by Mure and Klinman[5] that their compound **X** might be the benzylamine−**4** adduct was that it could be seen to form (i) at *long times* from reaction of product imine **4** with $PhCH_2NH_2$ and (ii) in the reaction of aminoresorcinol **5** with $PhCH{=}NCH_2Ph$. Clearly, were the benzylamine−**4** adduct a stable species, it should also be accessible from addition of **5** to $PhCH{=}NCH_2Ph$. Since we are claiming that their **X** is actually **9**, it is incumbent on us to explain why **9** would form under the two conditions discussed by Mure and Klinman. First, the reaction of product imine **4** with excess $PhCH_2NH_2$ would initially give aminoresorcinol **5a** cleanly. Over time, however, traces of O_2 getting into the reaction would result in oxidation of **5** to quinone imine **10**, which would condense with the excess $PhCH_2NH_2$ present to give substrate imine **3**, which in turn would be reduced by **5** to give **9**. Second, the reaction of equimolar **5** with $PhCH{=}NCH_2Ph$ would initially reach an equilibrium, now also containing equimolar product imine 4 and $PhCH_2NH_2$. Although it is conceivable that some **4** undergoes retrotransamination to substrate imine **3** which would immediately be reduced by **5** to give **9**, it is more likely that a trace of O_2 oxidizes a fraction of **5** to **10**, which condenses, with the $PhCH_2NH_2$ being released in the reaction to form **3**.

Table 1. ^{1}H NMR Data for Reactions Conducted in CD_3CN

	tert-butyl signal (s, 9H)	vinyl signals (2s, 1H each)	benzylic signal	aryl signals
1 anion	1.27	5.22, 6.18		
7[a]	1.30	6.25, 6.66		
5[b]	1.30	6.20, 6.58		
9[c]	1.23	6.21, 6.46	4.25 (s, 2H)	7.20–7.38 (m, 5H)
4[d]	1.39	6.37, 7.26	8.72 (s, 1H)	7.30–7.50 (m, 3H) 7.99 (m, 2H)

[a] The ^{13}C NMR spectrum displayed (CD_3CN) δ 30.0, 34.5, 105.1, 115.2, 128.4, 137.5, 143.5, and 149.4. [b] Generated independently by redox cycling reaction[3] of **7** (containing an inevitable traces of **1**) with NH_3. The ^{13}C NMR spectrum displayed (CD_3CN) δ 30.4, 34.6, 105.2, 116.2, 127.5, 127.9, 144.8, and 149.6. This compound was characterized as its HCl salt in ref 5. [c] See the Experimental Section. [d] This compound was characterized in ref 5.

at δ 6.25 and 6.66; see Table 1) can also be seen in their spectrum. As discussed above, the predominance of **9**, accompanied by traces of both aminoresorcinol **5** and triol **7**, is exactly the "redox scrambled" result we described above to be consistent with either transamination or addition–elimination mechanisms.

Clearly, making a mechanistic conclusion on the basis of ^{1}H NMR required finding a "trick" to minimize the occurrence of redox scrambling under conditions where the starting quinone **1** concentration was high enough (2 mM) to ensure unambiguous distinction of intermediates. Presuming that the reaction did follow a transamination pathway, our approach was to find a way to speed production of **4** without also speeding generation of **5** (the reducing agent which gives rise to the redox interchange byproducts). We retained a relatively high concentration of benzylamine (40 mM) and, in addition, added 40 mM triethylamine as an ancillary base with the hope of speeding the transaminative conversion of **3** to **4** (and thereby reducing the chance of forming byproduct **9** of **3** reduction) without additionally speeding the conversion of **4** to reducing species **5** responsible for generation of byproduct **9**. The 40 mM benzylamine was found to be a compromise, since, although lowering this concentration reduces the "undesired" conversion of **4** to **5**,[16] it also slows the initial conversion of **1** to **3** and thus risks production of byproduct **7** of **1** reduction. Under these conditions (anhydrous CD_3CN, anaerobic; see Table 1), we could observe *at the earliest times*, the exclusive conversion of **1** (R = *tert*-butyl) to a mixture of **4** (major, becoming minor) and **5** (minor, becoming major) (see eq 1, step D), with redox-interchange byproducts **7** and **9** (trace amounts) showing up only near the point of ~75% conversion of **1** (see Figure 3 for examples of early and late spectra).

An even "cleaner" reaction course, exhibiting only a small amount of **7** at the end of the reaction and *none* of the confounding **9**, could be observed by reacting **1** (R = *tert*-butyl, 2 mM) with benzylamine (120 mM) anaerobically in anhydrous DMSO-d_6.[17] Again, at the first point of detectable conversion of **1**, production of product imine **4** could alone be seen, followed rapidly by the appearance of **5**, and then a growth of both species, with the latter rising more rapidly (see Figure 4 for examples of early, medium-late, and "final" spectra). This and the previous NMR study in CD_3CN *constitute the first report of direct observation of product imine 4 by ^{1}H NMR as*

(16) A reaction of 2 mM **1** (R = *tert*-butyl) with 100 mM benzylamine in CD_3CN was very rapid and at the time of the first ^{1}H NMR spectrum (5 min) already showed a 1:1 mixture of aminoresorcinol **5** and (benzylamino)-resorcinol **9** (in addition to a small amount of remaining **4** and considerable remaining **1**).

(17) When run under identical conditions (0.5 mM **1** and 50 mM amine), the reactions in DMSO were seen to be about 30 times slower than in CH_3CN, on the basis of the rate of disappearance of starting quinone **1**.

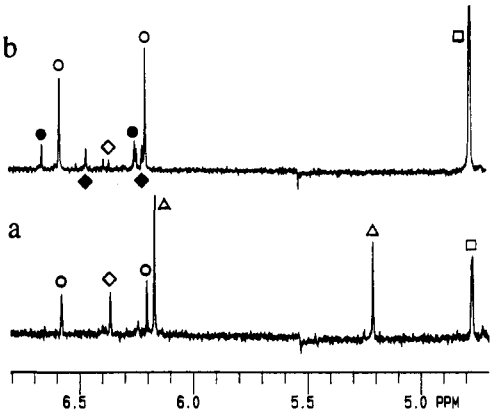


Figure 3. ^{1}H NMR spectra of the reaction of **1** (2 mM), benzylamine (40 mM), and triethylamine (40 mM) in degassed CD_3CN: (a) 45 min; (b) 8 h; (△) anion form of 1, (◇) **4**, (○) **5**, (●) **7**, (◆) **9**, (□) $PhCH{=}NCH_2Ph$.

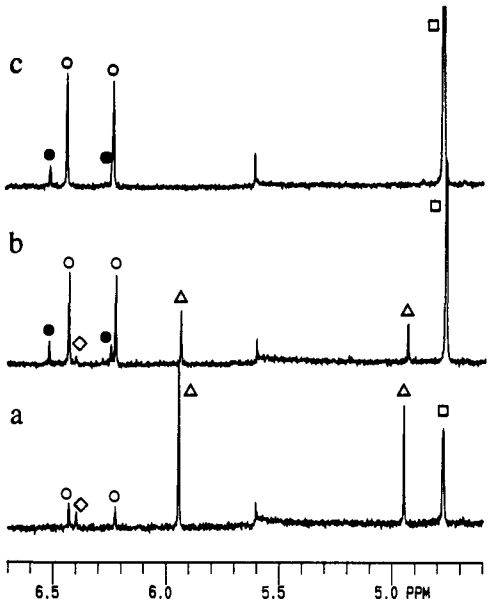


Figure 4. ^{1}H NMR spectra of the reaction of **1** (2 mM) and benzylamine (120 mM) in degassed DMSO-d_6: (a) 1 h; (b) 4 h; (c) 20 h; (△) anion form of **1**, (◇) **4**, (○) **5**, (●) **7**, (□) $PhCH{=}NCH_2Ph$.

the initially formed product prior to the occurrence of side reactions which confuse the mechanistic conclusion. Under these reaction conditions at least, it is clear that *the reaction follows a transamination rather than addition–elimination course.*

Interestingly, the complete absence of **9** at the end of the DMSO-d_6 reaction suggested that this solvent had minimized the lifetime of the precursor to **9**, namely, the substrate imine **3**, suggesting that the key imine shift step of the transamination mechanism had become a fast, non-rate-limiting one. If so, then one might not expect to see the manifestation of a primary kinetic isotope effect using $PhCD_2NH_2$. In fact, UV–vis spectral monitoring of the reaction under identical conditions using either $PhCH_2NH_2$ (Figure 5) or $PhCD_2NH_2$ (Figure 6) showed an initial isosbestic decrease at 510 nm (**1** anion in the solvent) with an increase at 370 nm of twice the extinction (and some growth at 296 nm), followed, after 40 min, by a decrease at 370 nm, a continuing decrease at 510 nm, and a continuing increase at 296 nm. This is consistent with the ^{1}H NMR information, assigning A_{370} to **4** and A_{296} to **5**. The only slight slowing of the progression of reaction in the case of $PhCD_2NH_2$ confirms that the transamination step has become non-rate-limiting under these conditions. This may result from a

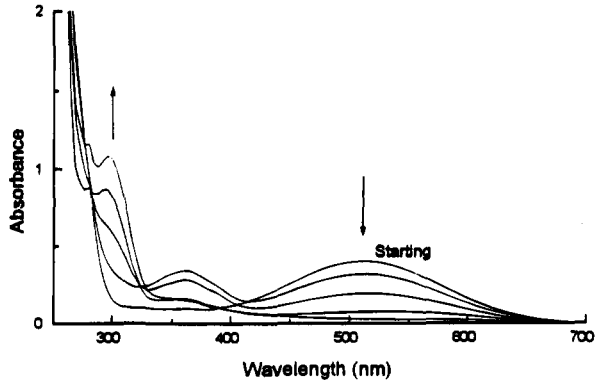


Figure 5. Progress of the reaction of **1** (2 mM) with benzylamine (120 mM) in degassed DMSO under argon, 25.0 °C, using a 1 mm path length cuvette: 0 min, 40 min, 2 h, 4 h, and 24 h.

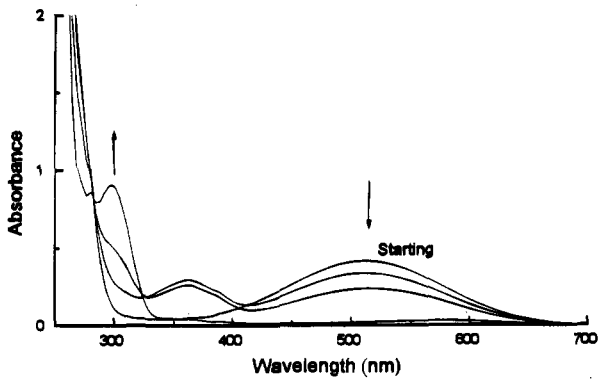
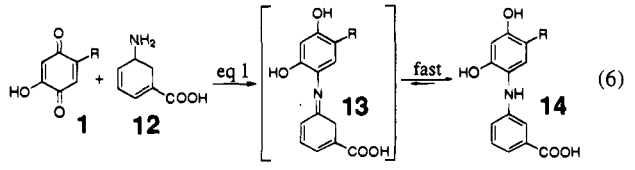


Figure 6. Progress of the reaction of **1** (2 mM) with benzylamine-d_2 (120 mM) in degassed DMSO under argon, 25.0 °C, using a 1 mm path length cuvette: 0 min, 40 min, 2 h, and 16 h.

selective slowing of the initial condensation step in DMSO relative to the imine shift step, compared to the case in CH_3CN, such that the disappearance of **1** becomes rate-limiting.[17] Whatever the explanation, our observation of a cleaner reaction outcome in DMSO may be a useful clue to the development of conditions for the efficient transamination of even *unactivated* amines.

A Diagnostic Substrate Probe for Transamination. For a completely independent strategy for conformation of mechanism, we resorted to the use of the mechanistic probe 5-amino-1,3-cyclohexadienecarboxylic acid (gabaculine, **12**), a fungal metabolite employed previously as a partition ratio 1 mechanism-based inactivator of the pyridoxal phosphate-dependent enzyme GABA transaminase.[18] As shown in eq 6, transamination (eq 1) is expected to result in product imine **13**, which should rapidly tautomerize to the 3-carboxyanilino derivative **14**. In contrast, the addition−elimination mechanism (eq 2) should result in the production of triol **7** and intermediate **15** (eq 7), the latter rapidly



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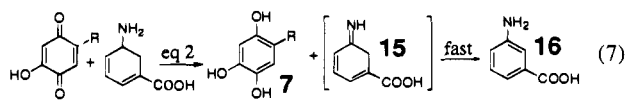
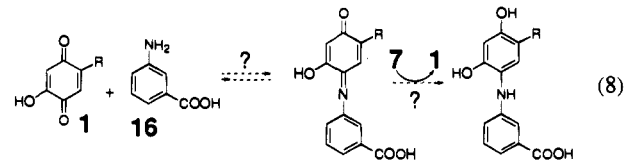


(7)

tautomerizing to 3-aminobenzoic acid (**16**). The strategy here was that the irreversible aromatizations would result in a product outcome which could not scramble according to the redox interchange problems described above. That is, were **16** to form, because it is an aniline rather than aliphatic amine, we would not expect it to condense with starting **1** to permit conversion to **14** according to eq 8. A control study was performed to confirm that **16** does not react with **1** in the presence of **7**.



(8)

The reaction of **12** with **1** (R = *tert*-butyl) was monitored by ^1H NMR in either CD_3CN-H_2O (9:1) or DMSO-d_6 anaerobically in a sealed tube (**12** is supplied as the HCl salt, and we used 3 equiv of *tert*-butylamine as a base to ensure that the amino group of **12** was in the free base form). Over a several day period, signals due to both **14** (isolated and characterized) and **16** (confirmed by "spiking" with the authentic compound) grew *at the same rate*, maintaining an approximately 3:1 integral ratio (more cleanly measured when the reaction was run at 40 °C in DMSO-d_6). The formation of some **16** (and **7**) indicated apparent competition of an addition−elimination mechanism with the main transamination pathway. However, *m*-hydroxybenzoic acid, which would be formed if either **13** or **15** suffered hydrolysis prior to aromatization, was not detected at all.

We wanted to make sure that **16** did not arise from **14**, possibly through oxidation by **1** to a quinone imine, followed by hydrolysis (though the constant production of **16** right from the beginning suggested it arose directly rather than via **14**). We found that a mixture of **14** and **1** under the reaction conditions led to no detectable generation of **16** in 3 days. Although our results force us to conclude that transamination is accompanied by a minor yet authentic addition−elimination pathway, further studies will be needed to determine whether this is a general occurrence for branched primary amines, since we found no evidence above for direct addition−elimination in the case of benzylamine. It should be recalled that the TPQ-containing amine oxidases are very ineffective at deamination of branched primary amines, so it is unclear if the minor extent of addition−elimination apparent here is enzymologically relevant.

Experimental Section

General Procedures. 5-*tert*-Butyl-1,2,4-benzenetriol **7** was prepared from 1,2,4-benzenetriol (Aldrich) by Friedel−Crafts alkylation (*tert*-butyl alcohol and H_2SO_4−HOAc),[19] and oxidized to the known 5-*tert*-butyl-2-hydroxy-1,4-benzoquinone[19,20] **1** by the $FeCl_3$ procedure we used previously.[3] Anaerobic reactions monitored by ^1H NMR spectrometry in degassed CD_3CN or DMSO-d_6 used at least five freeze−pump−thaw cycles on the solutions of **1** prior to initiation of reaction by addition of the amine (neat) under argon. Anaerobic reactions monitored by UV−vis spectroscopy in CH_3CN or DMSO involved argon bubbling for 20 min through solutions of **1** and the amine individually prior to mixing under argon. All NMR reactions were run at ambient temperature (averaging 20 °C) unless otherwise mentioned, using a Varian Gemini 300 MHz instrument. Chemical shifts were referenced to the residual proton peaks in the deuterated solvents, and ^{13}C APT (attached proton test) designations are given as (+) or (−). UV−vis spectra were recorded on a Perkin-Elmer Lambda 3B instrument using the PECSS software, with a constant temperature being maintained by a water-jacketed multiple cell holder. High-resolution mass spectra (HRMS) were obtained by electron impact

(18) Rando, R. R. *Biochemistry* **1977**, *16*, 4604.

(19) Flaig, W.; Ploetz, T.; Biergans, H. *Justus Liebigs Ann. Chem.* **1955**, *597*, 196.

(20) Musso, H.; Maassen, D. *Justus Liebigs Ann. Chem.* **1965**, *689*, 93.

ionization (20−40 eV) on a Kratos MS25RFA instrument. Amines were freshly fractionally distilled; $PhCD_2NH_2$ was prepared as in our previous study.[3]

4-(Benzylamino)-6-*tert*-butylresorcinol 9. Utilizing our previously described "redox cycling" strategy,[3] a solution of triol **7** (36 mg, 0.20 mmol) and 23 μL of $PhCH_2NH_2$ (0.21 mmol) in 1 mL of CH_3CN was kept in a closed tube at room temperature. The tube was opened briefly to the atmosphere four times over the course of 2 days. Evaporation of the solvent at this time left the desired **9** (contaminated by a trace of $PhCH{=}NCH_2Ph$) as a highly air-sensitive material which becomes less pure upon attempted purification: the ^1H NMR spectral data are given in Table 1; ^{13}C NMR (CD_3CN) δ 30.2 (+), 34.7 (−), 49.9 (−), 105.0 (+), 113.3 (+), 127.7 (+), 128.5 (+), 129.3 (+), 142.0 (−), 144.3 (−), 148.1 (−); HRMS (EI, 20 eV) for $C_{17}H_{21}NO_2$, calcd 271.1573, found 271.1584 (2.7%).

Reductive Analysis of the Reaction of 1 with $PhCD_2NH_2$. According to the conditions of UV−vis monitoring (Figure 2), 60 mL of a solution of **1** (0.5 mM) and $PhCD_2NH_2$ (50 mM) in CH_3CN (initially degassed by argon bubbling for 20 min) was held at 25 °C for 90 min. At this time $NaBH_4$ (50 mg) was added under an argon stream, followed 10 min later by the addition of triethylamine (2.5 mL) and acetic anhydride (2 mL). After stirring for 3 h under argon, all volatile components were removed at high vacuum, and the remaining residue was partitioned between water and $CHCl_3$. The organic layer was dried (Na_2SO_4) and concentrated, and the residue obtained upon evaporation of the solvent was purified by silica gel flash chromatography using EtOAc as eluant. The fractions showing the appropriate fast-moving spot on TLC were combined and evaporated, and the residue was analyzed by ^1H NMR and by HRMS (EI). The relative intensities for the peak corresponding to $C_{23}H_{26}DNO_5$ (calcd 398.1953) and $C_{23}H_{25}D_2NO_5$ (calcd 399.2016) were calculated to be 32:68 after correction for the ^{13}C contribution. The ^1H NMR spectrum was consistent with the predominance of **11**-d_2 by comparison to a spectrum of authentic **11**-d_2 obtained by acetylation of the product of redox cycling[3] between $PhCD_2NH_2$ and **7** (containing an inevitable trace of **1**): ^1H NMR ($CDCl_3$) δ 1.16 (s, 9H), 1.88 (s, 3H), 2.19 (s, 3H), 2.32 (s, 3H), 6.69 (s, 1H), 6.96 (s, 1H), 7.16−7.29 (m, 5H).

Reaction of 1 with 12. To a 5 mm NMR tube containing a solution of **1** (5.1 mg, 0.028 mmol) and **12** (5.0 mg, 0.028 mmol) in 0.5 mL of degassed DMSO-d_6 was added freshly distilled *tert*-butylamine (8.8 μL, 0.084 mmol) to initiate the reaction. The reaction tube was kept in a 40 °C oil bath, and the ^1H NMR spectrum was recorded periodically over a 4 day period. Signals due to both **14** and **16** grew at the same rate, maintaining a 3:1 molar ratio (by integration). After completion of the reaction, the presence of **7** and **16** was confirmed by spiking with the authentic compounds. An analytical sample of product **14** was isolated by silica gel flash chromatography using EtOAc as the eluant: ^1H NMR (DMSO-d_6) δ 1.28 (s, 9H), 6.43 (s, 1H), 6.62 (m, 1H), 6.87 (s, 1H), 6.97 (apparent t, 1H, J = 7.76 Hz), 7.11 (br d, 1H, J = 7.44 Hz), 7.29 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 29.7 (+), 33.6 (−), 104.5 (+), 114.5 (+), 117.8 (+), 120.1 (−), 122.9 (+), 125.7 (−), 127.4 (+), 127.5 (+), 139.8 (−), 146.8 (−), 149.6 (−), 152.4 (−), 170.6 (−); HRMS for $C_{17}H_{19}NO_4$, calcd 301.1315, found 301.1315 (100%).

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