Synthesis and Characterization of Models for the 2,4,5-Trihydroxyphenylalanine (TOPA)-Derived Cofactor of Mammalian Copper Amine Oxidases, and Initial Amine Reactivity Studies

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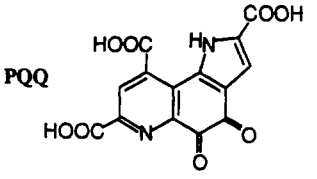
The mammalian copper amine oxidases effect the oxidative deamination of primary amines through utilization of an "active carbonyl" cofactor, shown recently to be the quinone form (TPQ) of a protein-based 2,4,5-trihydroxyphenylalanine (TOPA) residue. We synthesized three models for the cofactor in both reduced (benzenetriol) and oxidized (hydroxyquinone) forms, which differ in the nature of the alkyl substituent mimicking the connection to the protein backbone: hydantoinylmethyl, phthalimidoethyl, and pivalamidoethyl. The quinone forms were capable of deaminating benzylamine in aqueous CH_3CN both stoichiometrically and catalytically (in the presence of O_2), but incapable of deaminating non-benzylic amines. In order to clarify the various reactions potentially occurring during aerobic autorecycling deamination, we studied the pH-dependent benzenetriol → hydroxyquinone autoxidation as well as the possible reaction of amines with the benzenetriol forms. The latter undergo stoichiometric substitution with amines to give (alkylamino)resorcinols, not via cyclohexadienone tautomerization previously proposed, but via a redox cycling mechanism involving condensation of the amines with traces of hydroxyquinone present in the benzenetriol preparations. This observed substitution regiochemistry, as well as structural characterization of the hydroxyquinone arylhydrazine derivatives, confirms that amines react exclusively at the electrophilic C5 carbonyl position of TPQ models. Both the hydroxyquinone and benzenetriol forms were found to react with ethylenediamine in the presence of O_2 to give 6-hydroxy-7-(2-pivalamidoethyl)quinoxaline, consistent with the postulated generation of such moiety when lysyl oxidase is inactivated by ethylenediamine.

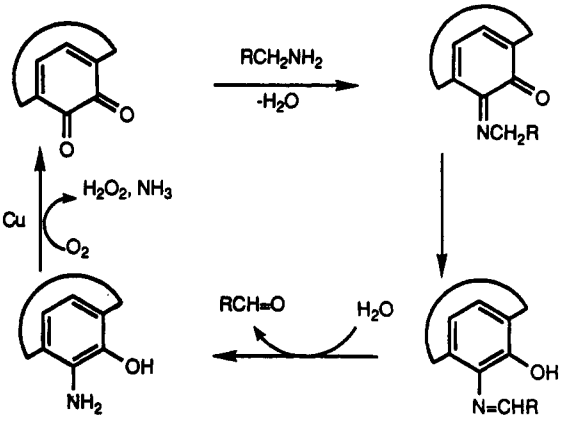
The copper amine oxidases constitute an important class of mammalian enzymes involved in endobiotic and exobiotic metabolism.[1] Various enzymes have been isolated from plasma (plasma or serum amine oxidase), kidney (diamine oxidase), and aorta (lysyl oxidase), as well as from the plant kingdom and from prokaryotes. In some cases there is confusion as to the relationship between what is referred to as "benzylamine oxidase" or "semicarbazide-sensitive" amine oxidase (SSAO) and the tissue-specific enzymes referred to above. However, all these enzymes utilize a covalently-bound "active carbonyl" cofactor to achieve a pyridoxal-like transamination of *primary* amines to aldehydes, the role of copper being to mediate the O_2-dependent reoxidation of the reductively aminated cofactor (H_2O_2 and NH_3 are byproducts), which occurs *subsequent* to hydrolytic release of the aldehyde product. The overall transformation catalyzed by this enzyme class is usually described in general terms as shown in Scheme 1.

During the 1980's, evidence accumulated that the cofactor for all the copper amine oxidases was universally pyrroloquinoline quinone (PQQ, methoxatin), a known *dissociable* cofactor for some bacterial dehydrogenases.[2,3]



Both Bruice and the Japanese group of Itoh and Ohshiro

Scheme 1



carried out extensive model studies on the mechanism of transamination of amines by PQQ and PQQ analogs,[4-7] both stoichiometrically,[4-6] and catalytically in the presence of O_2 and a micellar environment.[7] However, the identification of PQQ as cofactor for the copper amine oxidases turned out to be an artifact;[8-10] Klinman and co-workers demonstrated in 1990 that the actual cofactor of bovine

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(2) Hartmann, C.; Klinman, J. P. *BioFactors* **1988**, *1*, 41.
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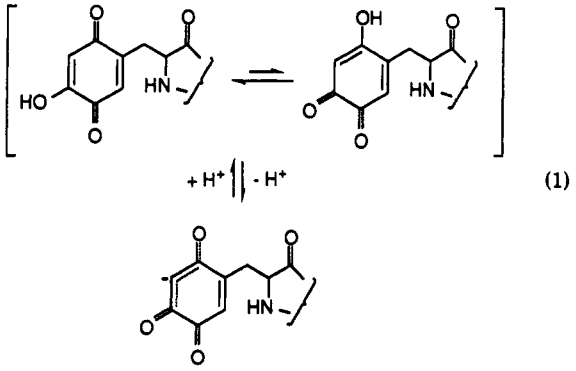
plasma amine oxidase (PAO) was the quinone form of an active-site-based 2,4,5-trihydroxyphenylalanine (TOPA) residue (see eq 1).[11] Soon after, the bacterial enzyme



(1)

methylamine dehydrogenase was shown to contain an active site tryptophan–tryptophylquinone (TTQ).[12] Although it is possible that distinct cofactor structures may in time be discovered for other enzymes in this class, the *mammalian* copper amine oxidases for the most part appear to contain TOPA quinone (TPQ) as cofactor.[8,13]

Previous model transamination studies carried out using PQQ undoubtedly delineated important quinone–amine reactivity patterns, but transamination induced by TTQ and especially TPQ might involve significant mechanistic differences. First, the transaminating potential and mechanism for PQQ has been shown to reflect both the peri pyridine nitrogen[14] and the pyrrolic NH moiety,[15] which are obviously absent in TPQ. Second, the presence of the hydroxy substituent in TPQ creates a vinylogous carboxylic acid, which will be completely dissociated to an anion at physiologic pH (the hydroxybenzoquinone moiety of the TPQ amino acid has a pK_a of 4.5).[16] In this regard, it is worth noting that although the *p*-benzoquinone (2-hydroxy) tautomer of *neutral* TPQ is thermodynamically favored over the *o*-benzoquinone (4-hydroxy) tautomer, its existence as a resonance-stabilized conjugate base at physiologic pH makes it senseless to argue whether TPQ reactivity should reflect that of a *p*-quinone or an *o*-quinone (eq 1). The hydroxy substituent results in lowering of the reduction potential: the $E_{1/2}$ (vs NHE) for both TPQ (free amino acid) and 2-hydroxy-*p*-benzoquinone has been reported to be −18 mV compared to +198 mV for *p*-benzoquinone in 0.1 M pH phosphate buffer.[17] The low reduction potential would in turn be expected to confer a weak transaminating strength. Third, whereas the structure of PQQ sterically directs amine nucleophilic attack at the quinone carbonyl, the reaction of amines with TPQ could in theory either occur at the most electrophilic carbonyl group or alternatively follow a Michael addition pathway (which does not necessarily preclude eventual transamination).[18]

Based on the above factors, it is clear that studies on 2-hydroxy-1,4-benzoquinones themselves are required to establish amine reactivity characteristics relevant to the TPQ cofactor. 2-Hydroxy-1,4-benzoquinone itself is commercially available in its reduced form (1,2,4-benzenetriol), but the absence of a C5 alkyl substituent is known to result in an oxidative intermolecular C–C coupling reaction, with ultimate formation of 2,2′-bi-*p*-quinones.[19] Also, some workers have utilized the commercially available TOPA *amino acid* to evaluate O_2- and Cu(II)-dependent reaction with amines.[17,20] An alternative possibility is the descarboxy version, the well-known neurotoxin 6-hydroxydopamine (6-OHDA).[21] However, neither of these two compounds is suitable for a rigorous chemical study because the free amino group undergoes cyclocondensation at the quinone stage.[22,23] Such cyclization, although not as prevalent as with dopaquinone, would always be a competitive side reaction in studies with external amine substrates. Furthermore, the mode of interaction of Cu(II) with TOPA/TPQ amino acid in aqueous solution is controlled by the free α-amino and -carboxy groups and thus may be irrelevant to how Cu(II) interacts with the cofactor within the enzyme, where these groups are part of the peptide backbone. This report describes our efforts to develop C-5 alkyl models which are more consistent with the structural presentation of TPQ found at the enzyme active site.[24]

Results and Discussion

Model Development. One model which closely reproduces the context of TPQ as it exists in the protein and which presumably eliminates the possibility of cyclocondensation, is the TPQ hydantoin **2**.[11] This we prepared via $FeCl_3$ oxidation of the triol **1** generated from debenzylation of the known tri-*O*-benzyl-protected hydantoin.[25,26] The properties of **2** were, as expected, similar to that reported for the quinone forms of TOPA and 6-OHDA, giving weak yellow solutions at low pH (λ_{max} = 370 nm), but forming deep red conjugate base anions (λ_{max} = 490–495 nm) at pH ≥ 5. It is noteworthy that the resting (oxidized) forms of mammalian copper amine oxidases display a characteristic 480-nm absorption.[1] There is little change in the ^1H NMR chemical shift (DMSO-d_6) of the C3 and C6 vinyl hydrogens between the triol **1** (δ 6.30 and

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(24) Some studies were reported at the 203rd National Meeting of the American Chemical Society, San Francisco, April 6–10, 1992, Abst ORGN 171. Remaining studies were reported at the 206th National Meeting of the American Chemical Society, Chicago, Aug. 22–27, 1993, Abst ORGN 317.
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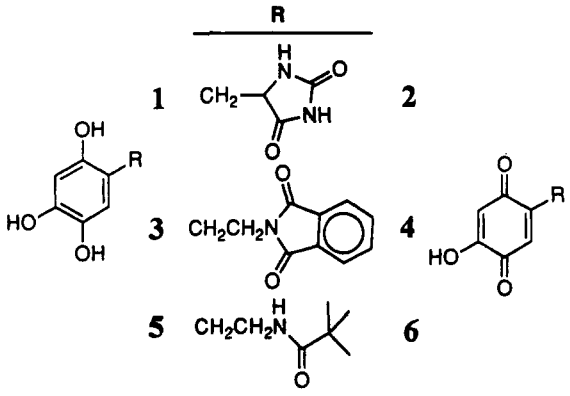
(26) While this manuscript was in review, a publication appeared which reported the synthesis and characterization of hydantoin models **1** and **2** described here: Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 7117.

6.44) and the quinone 2 (δ 6.06 and 6.69), though the *anion* of 2, which is the form present in all reactions with amines, exhibits marked upfield shifts of both vinyl signals (to δ 5.05 and 6.14).



Reaction of TPQ Hydantoin 2 with Benzylamine. We were encouraged to find that 2 was effective in deaminating benzylamine in both DMSO and buffered CH_3CN–H_2O (1:1) at 40–50 °C. In these reactions, benzylamine is used in excess, and thus the initial deamination product is trapped by $PhCH_2NH_2$ (probably without release of free PhCHO) to give $PhCH{=}NCH_2Ph$ as isolated product. Under turnover conditions (O_2 bubbling), we found that using 1 mM 2 in a CH_3CN–H_2O 3:5 solution of 50 mM $PhCH_2NH_2$, a 480% yield of $PhCH{=}NCH_2Ph$ was formed (based on 2) in 17 h at 25 °C.

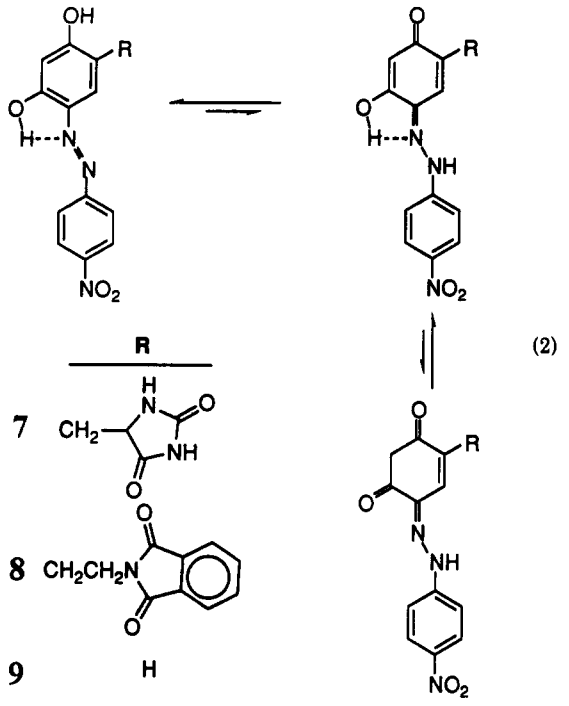
In an effort to obtain quantitative data for the successful deamination of benzylamine by 2, we carried out anaerobic kinetic studies for both $PhCH_2NH_2$ and $PhCD_2NH_2$ as a function of pH in buffered CH_3CN–H_2O 2:1 (40.0 °C, N_2) under pseudo-first-order conditions (10-fold excess of amine over quinone 2). Reactions were followed by monitoring the disappearance of the red conjugate base 2 anion at 490 nm, which is fully formed in the pH range (6–9) of our study. When 2 was mixed with excess amine, we observed a brief shift of the 490-nm absorption to a lower wavelength absorption (475–488 nm, depending on pH and identity of the amine), which then decayed in an isosbestic fashion. Crude rate information obtained from the first-order plots of the latter absorption decay (not shown) revealed a rate increase with increasing pH, but by a margin (21-fold from pH 6 to 9) less than expected if the rate reflected merely the fraction of amine substrate present in the free base form. Secondly, α-deuterium substitution (using $PhCD_2NH_2$ rather than $PhCH_2NH_2$) was seen to produce a pH-dependent rate slow down, ranging from ~20% at pH 6 to nearly 3-fold at pH 9.

Although it is tempting to extract mechanistic information from this data, the spectral profiles for the protio and deuterio benzylamines were surprisingly different. For $PhCH_2NH_2$, the decrease in A_{485} was accompanied by a small increase in A_{350} (isosbestic point at 365 nm). For $PhCD_2NH_2$, however, the increase in absorption at 340–350 nm was much larger than the A_{485} decrease, and remained "off scale" after complete bleaching of the long-wavelength absorption. Interestingly, the reaction of 2 with *non-benzylic* primary amines (e.g., phenethylamine) under the same conditions follows a nearly identical profile to that seen with $PhCD_2NH_2$. The 340–350 nm absorption may be ascribed to an initial quinoneimine intermediate, which would have a longer lifetime in the cases of non-benzylic amines and $PhCD_2NH_2$ relative to $PhCH_2NH_2$ on account of reduced α-H lability. This assignment is consistent with recent rapid-scan stopped-flow spectroscopic studies on the reaction of bovine plasma amine oxidase with amines, which identified a quinoneimine intermediate absorbing at 340 nm.[27] In any event, an interpretation of kinetic data in terms of mechanism, as well as the establishment of microscopic rather than overall observed kinetic isotope effects, will require a complete kinetic analysis of intermediates and possible rate-limiting step shifts. Studies along these lines are in progress and will be reported in due course.

Whereas bleaching of the red quinone anion in the case of benzylamine is accompanied by the generation of deamination product, reactions of 2 with non-benzylic amines in the same buffered aqueous CH_3CN system resulted in loss of the red chromophore without giving rise to aldehyde product. Complete transformation of starting materials was observed in refluxing CH_3CN, but complex product mixtures were obtained. In attempts to isolate (by preparative TLC) and characterize (by NMR) constituents of these mixtures, we observed the conversion of 2 in part to apparent amine adducts and in part to altered forms of 2 that did not contain the amine, leading us to suspect the possibility of base-mediated cyclization. In order to circumvent the latter potential side reaction(s), we decided to switch over to using the alternative TPQ models 4 and/or 6, prepared via modification of the 6-hydroxydopamine synthesis.[25] Since 4 lacks an NH (and cyclization through an imide oxygen would involve a seven-membered ring), whereas the NH and O in 6 are sterically hindered, these models were expected to eliminate the possibility of base-induced cyclization. Initial studies suggested that although both 4 and 6 gave less complex product mixtures with unactivated amines than did hydantoin 2, the phthalimide moiety of 4 suffered competitive reaction with $PhCH_2CH_2NH_2$ at long reaction times when the amine was used in excess. Thus, our current model studies are focusing on the pivalamide 6.

Reaction of TPQ Models with (4-Nitrophenyl)-hydrazine. In light of our inability at this time to establish the mechanism of benzylamine transamination or the structure of the intermediates involved, we felt it was useful to determine the structure of *stable* adducts of the TPQ models with arylhydrazines. The derivatization of 2 and 4 with (4-nitrophenyl)hydrazine gave as major products 7 and 8, arising from condensation at the more electrophilic carbonyl, a previously surmised outcome in the case of 2.[11] The structures of 7 and 8 were assigned on the basis of determining the structure of the product 9 obtained from reacting (4-nitrophenyl)hydrazine with oxidized 1,2,4-benzenetriol. The structure of 9 was proved by independent synthesis from 4-nitrophenyldiazotization of resorcinol (9 is in fact available commercially by this route). It has been reported that 9 exists in DMSO-d_6, acetone-d_6, and CD_3OD as the azobenzenediol rather than quinone hydrazone tautomer on the basis that the ^{13}C NMR chemical shifts of C1 (~165 ppm, relative to TMS) and C3 (153–155 ppm) are in the "benzenoid" range, whereas for analogs existing as quinone hydrazones, the chemical shift values of C1 (175–177 ppm) and C3 (184–186 ppm) are in the "carbonyl" range.[28]

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



The ^{13}C NMR spectral data for 8 in DMSO-d_6 is depicted in Figure 1 alongside the values reported[28] for 9 (in brackets) in the same solvent. There is very close correspondence of the chemical shift values except in the case of C6, where the alkyl substituent in 8 replaces the hydrogen in 9. The azobenzene tautomeric structure shown for 8 is evident from the observation that no chemical shift is below 165 ppm. Thus, the *p*-quinone hydrazone tautomer previously depicted for TPQ[11,29] appears not to predominate (at least in neutral solution), though it is clearly this equilibrium form which explains the D-for-H exchange which occurs specifically at C2 (eq 2) when the (4-nitrophenyl)hydrazine derivatives are exposed to D_2O (ref 11 for 7 and confirmed here for 7 and 8).[30]

Amination of the Reduced Triol (TOPA) Forms of TPQ Models. In the interest of clarifying potential reaction pathways which might occur during aerobic autorecycling deamination (turnover) mediated by TPQ models, we monitored the reactions of the benzenetriol TOPA model 5 under three conditions (amine + O_2, O_2 only, amine only) by ^1H NMR spectroscopy in DMSO-d_6. We were surprised to discover that the *reduced triols themselves* appeared to react anaerobically with amines. The same result was obtained in CD_3CN. In these reactions, we used 1 equiv of benzylamine, phenethylamine, neopentylamine, or cyclopropylamine in the presence of 1 equiv of *tert*-butylamine as a makeshift buffer and/or base catalyst, which itself was found to give no reaction. A gradual and, except in the case of benzylamine, complete transformation was evidenced by a shift of the C3 and C6 aryl-H singlets concomitant with a downfield shift of the amine C_α-H.

Although the reaction corresponds formally to a nucleophilic aromatic substitution of amine for one of the

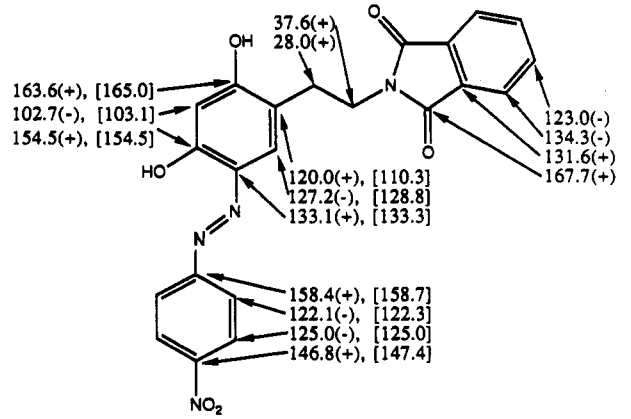
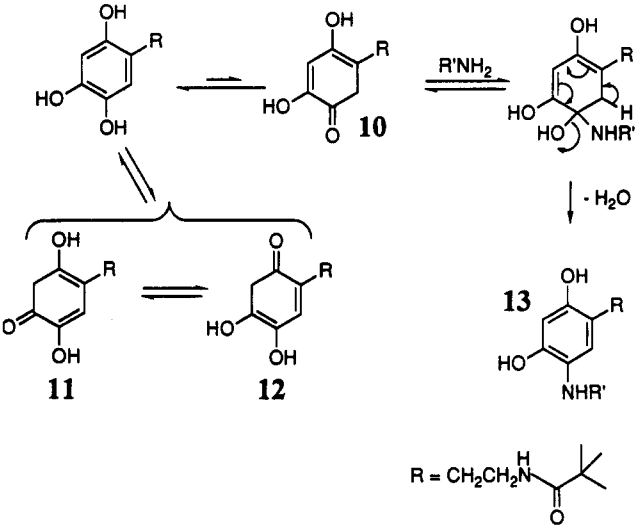


Figure 1. ^{13}C NMR chemical shifts (DMSO-d_6) and attached proton test (APT) designations for 8 and for 4-[(4-nitrophenyl)-azo]resorcinol (9, in brackets) as reported in ref 28.

Scheme 2



three ring hydroxyls, the substrate in our case is not activated for the typical addition–elimination mechanism (S_NAr). Nonetheless, literature precedent exists for the reaction of 1,2,4-benzenetriol with NH_3 and primary amines to give 4-aminoresorcinols.[31] The mechanistic explanation given by these workers was that benzenetriol exists to a significant extent in one or more cyclohexadienone tautomeric forms that could react with amine to give carbinolamine adducts, dehydration of which would afford aminobenzenediols. Selective replacement of the 4- rather than 1- or 2-hydroxyl group was claimed on the basis of comparing the NH_3-derived product to the aminoresorcinol obtained from reduction of 4-nitroresorcinol.[31] A postulated mechanism is shown in Scheme 2; the resonance-stabilized tautomers 11 and 12 should be more prevalent than 10, but reaction of the latter is required to yield the aminoresorcinols 13.

In every case we studied, a single isomer was obtained by ^1H NMR; nonetheless we endeavored to prove that the structures did correspond to aminoresorcinols 13 (rather than the isomeric aminocatechols or aminohydroquinones) by isolating and characterizing the product in the case of phenethylamine. A preparative-scale reaction of 5 and $PhCH_2CH_2NH_2$ (1:1) stoichiometrically afforded the substitution product, confirmed by mass spectral analysis.

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(30) The same structural conclusion was arrived at independently for the (nitrophenyl)hydrazine derivative 7 of TPQ hydantoin 2, using the 2D NMR arguments reported in ref 26.
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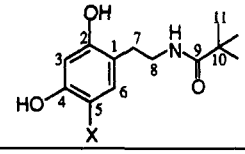
Table 1. ^{13}C NMR Chemical Shifts, Multiplicities, and Aryl Carbon Long-Range Couplings for 5 and 13 (R' = $NHCH_2CH_2Ph$) in DMSO-d_6[a]



	X = OH				X = $NHCH_2CH_2$- (12 13 14 15 16 aryl 17)			
carbon	δ	$^1J_{C-H}$	$^2J_{C-H}$	$^3J_{C-H}$	δ	$^1J_{C-H}$	$^2J_{C-H}$	$^3J_{C-H}$
1	115.5	-	m		115.6	-	m	
2	147.6	-	H3 d 3.0	H6 d 7.4 H7 t 1.2	146.0	-	H3 d 3.6	H6 d 9.1
3	103.7	d 154.9			103.0	d 154.8		
4	143.7	-	H3 d 3.6	H6 d 8.0	143.4	-	H3 d 3.4	H6 d 9.7
5	137.4	-	H6 d 3.3	H3 d 6.8	129.9	-	H3 d 6.1	
6	117.3	d 153.7		H3 t 5.3	113.0	d 151.9		H3 t 4.6
7	29.3	t 113.6			29.6	t 127.6		
8	40.0	t 136.5			40.3	t 141.1		
9	177.3	-			177.5	-		
10	38.0	-			38.0	-		
11	27.5	q 126.3			27.6	q 126.3		
12					45.9	t 135.3		
13					35.3	t 126.9		
14					140.1	-	m	
15					128.7	d 156.9		d,d,t 6.0[b]
16					128.4	d 159.4		H16 d 7.4
17					126.1	d 160.4		H15 t 7.2

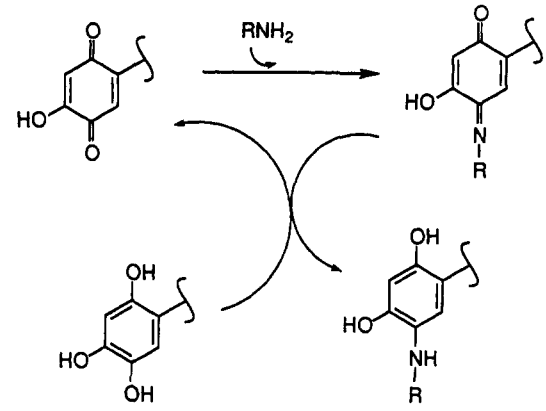
[a] Referenced to DMSO-d_6 at δ 37.50. [b] Apparent quintuplet with $^3J_{C-H17} \sim {}^3J_{C-H15} \sim {}^3J_{C-H13}$

Referring to the carbon positions depicted in Table 1 (X = $NHCH_2CH_2Ph$), NOE difference studies indicated that whereas irradiation of the C7 methylene at δ 2.49 produced equivalent enhancements in the C3 and C6 vinyl signals, irradiation of the C13 methylene at δ 2.80 produced a 3-fold greater enhancement in the C6 vinyl signal than in the C3 vinyl signal. Reciprocal NOE information from irradiation of the vinyl signals could not be obtained because of their close proximity, and NOE information from irradiation at the C12 methylene could not be obtained because of its overlap with the C8 methylene. In addition to the NOE evidence, our structural assignment is fully supported by the $^{13}C-^1H$ long-range coupling data summarized in Table 1, where the assignments shown were confirmed by single frequency proton irradiation, with correlation to the same experiment performed on the triol **5** (X = OH).

With knowledge that the sole apparent amine substitution product is the aminoresorcinol **13**, we next desired to confirm the mechanism of its formation. Scheme 2 predicts that carrying out the reaction in a deuterated solvent would result in exchange at C6 at a rate equal to or greater than the rate of product generation. We found however, that in a mixed DMSO-d_6/D_2O solvent system, (phenethylamino)resorcinol (**13**) was obtained cleanly without any washout of protium at C6. This result rules out the tautomerization-based substitution mechanism of Scheme 2.

The mechanism we now favor for this apparent substitution is the redox cycle shown in Scheme 3. Even purified preparations of 1,2,4-benzenetriols contain a trace of the oxidized hydroxyquinone, as evidenced by their slight coloration in the presence of amine bases. According to Scheme 3, Schiff base condensation of amine at the most electrophilic carbonyl (C5) of the hydroxyquinone **6** contaminant in **5** gives a hydroxyquinoneimine, which is subsequently reduced by benzenetriol **5**, affording the apparent substitution product **13** and regenerating the

Scheme 3



trace of hydroxyquinone **6**. Following the reactions by ^1H NMR reveals no evidence for involvement of either hydroxyquinone or hydroxyquinoneimine on account of their very low steady state concentration. Nonetheless, confirmation of this mechanism is that the rate of aminoresorcinol production increases proportional to increasing small amounts of **6** added to the reactions. Furthermore, the reaction does not proceed when a small amount of external reductant ($NaBH_4$) is added to a degassed and anaerobically sealed NMR tube containing the reactants (**5** and amine). Lastly, the incomplete nature of the reaction with benzylamine is explained similarly due to consumption of the small amount of quinone present via successful oxidative deamination which occurs in this case.

The driving force for the redox direction depicted in Scheme 3 cannot be large and actually appears opposite to expectations, i.e., one might expect reduction of hydroxyquinone by aminoresorcinol to be favored over reduction of hydroxyquinoneimine by benzenetriol. The switch in redox direction may arise because the hydroxy-

Table 2. Kinetic Data for the Autoxidation of Benzenetriol 5 (1 mM) in CH_3CN–H_2O (1:1), 100 mM Phosphate Buffer, 25.0 °C, First-Order k (min^{-1})

pH	without O_2 bubbling	with O_2 bubbling
5.0	0.015	0.021
6.0	0.076	0.089
7.0	0.39	–

quinone product forms a resonance-stabilized anion in presence of amine. Whatever the explanation, the reaction discussed here is important for two reasons. First, it indicates that the preferred site for reaction of TPQ model **6** with amines is at the electrophilic C5 position, as we found for phenylhydrazine derivatization. Second, it reveals the complexity of the various transformations which can plague a mechanistic interpretation of model quinone-mediated deamination reactions.

Autoxidation of the 1,2,4-Benzenetriol (Reduced) Forms of TPQ Models. Since the first effort to prepare TOPA hydantoin **1** resulted only in isolation of the quinone **2** (in the form of its arylhydrazones), it was surmised that autoxidation of the benzenetriol was a facile process.[11] However, in our larger scale syntheses, we routinely found that the benzenetriols generated upon H_2/Pd-mediated debenzylation could be isolated cleanly without requiring strict anaerobic workup, as long as basic solutions are avoided. Nonetheless, we felt it was important to establish the ease of autoxidation as a function of pH. First-order kinetic plots for autoxidations of **5** in CH_3CN–H_2O (1:1) at pH 5–7, 25.0 °C using O_2-equilibrated solvents were linear only to a little over one half-life, presumably on account of depletion of dissolved O_2. The rate constants given in Table 2 represent the initial linear portions. It can be seen that the increase in log k with increasing pH has a slope less than unity in this pH range. The slower reactions at pH 5 and 6 were repeated with bubbling of O_2 into the cuvette in between each 5-min kinetic point. The first-order plots for these latter reactions were linear to more than 4 half-lives, and the calculated rates were seen to be 20–30% faster (Table 2). We can say little about the mechanism of the oxidation at this time, except that the rate of oxidation at pH 6 was not affected by the addition of 1 equiv of H_2O_2 (1 mM) nor by the addition of 0.2 mM EDTA (to chelate possible trace metal ion catalysts).

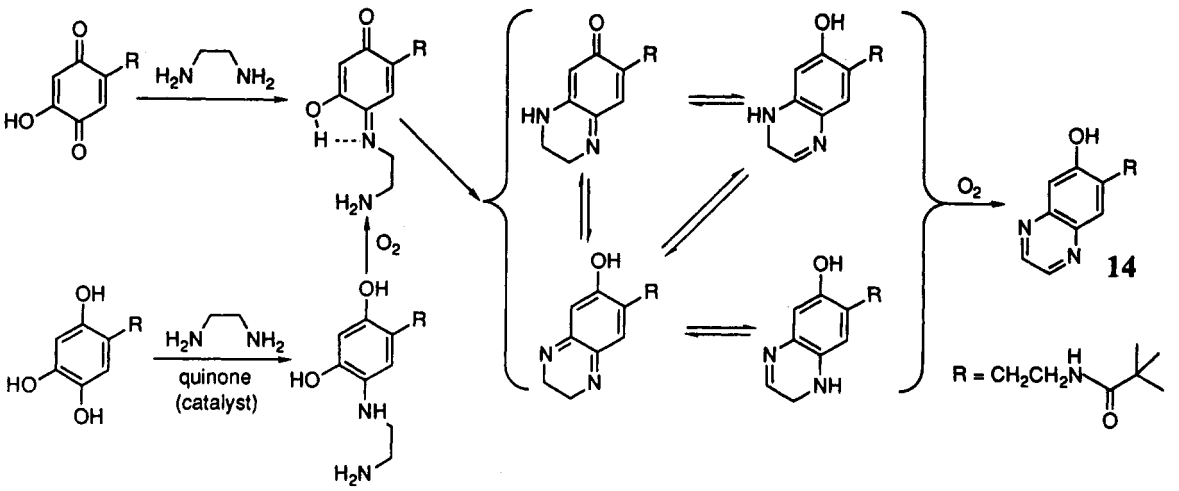
Reaction of TPQ Models with Ethylenediamine. In probing the structural requirements for inhibition of lysyl oxidase by primary amines, Kagan found that 1,2-diamines are potent inactivators.[32] This finding was rationalized on the basis of formation of a dehydrogenated pyrazine adduct,[33] presuming the cofactor for this enzyme was an *o*-quinone (or behaved like one). On the basis of the significance of lysyl oxidase in initiating the physiological intra- and intermolecular lysine-based cross-linking of connective tissue proteins, and the fact that its cofactor identity is still uncertain, we felt it was worthwhile to determine if the hydroxyquinone moiety of **6** would form a cyclic adduct with 1,2-diamines.

The reaction of quinone **6** with ethylenediamine (EDA), followed by ^1H NMR in DMSO-d_6, was seen to proceed *anaerobically* to a metastable intermediate, which, when exposed to O_2, ultimately afforded the cyclic aromatized quinoxaline **14** (see Scheme 4, characterized after isolation from a preparative-scale reaction in refluxing CH_3CN). When the reduced triol form **5** was exposed to EDA anaerobically, rapid amination took place giving **13** (R = $CH_2CH_2NH_2$) according to Scheme 3; subsequent exposure to O_2 resulted in the same quinoxaline **14** (Scheme 4). Although additional work would be needed to elucidate the structures of the various possible intermediates, our observation demonstrates that the cyclic (pyrazine) adduct proposed by Kagan to explain inactivation of lysyl oxidase by ethylenediamine, is consistent with TPQ being the cofactor for this enzyme.

Experimental Section

General Procedures and Materials. ^1H NMR spectra were obtained at 200 and 300 MHz; ^{13}C NMR spectra were obtained on 300- and 400-MHz instruments (operating at 75.46 and 100.63 MHz, respectively). ^1H NMR chemical shifts are reported as ppm downfield from tetramethylsilane (TMS) or, in D_2O, from sodium 3-(trimethylsilyl)-1-propanesulfonate. ^{13}C NMR chemical shifts were referenced to the solvent peak and were corrected with respect to TMS. The ^{13}C NMR assignments listed (CH_3, CH_2, CH, C) were made on the basis of attached proton test (APT) or DEPT experiments. NMR assignments to specific nuclei were achieved in some cases through the use of ^1H–^1H decoupling, long-range ^{13}C–^1H coupling (with noted augmentation by single frequency proton irradiation), NOE difference data, and/or D-exchange data. Coupled ^1H signals have been grouped together in the δ listings. UV-visible spectra were obtained using a jacketed (temperature-controlled) cell compartment, and doubly distilled water was used for all kinetics experiments. High-resolution electron impact mass spectra were recorded at 20–40 eV. Melting points are uncorrected. Thin-layer and preparative-

Scheme 4



layer chromatography were run on commercial silica gel 60 plates with 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. 2,4,5-Tris(benzyloxy)benzaldehyde was purchased from Regis Chemical Co. All evaporations were conducted at reduced pressure using a rotary evaporator.

5-[2,4,5-Tris(benzyloxy)benzyl]hydantoin. 5-[2,4,5-Tris(benzyloxy)benzylidene]hydantoin was prepared from condensation of 2,4,5-tribenzyloxybenzaldehyde (10 g, 0.024 mol) with hydantoin as described,[25] except that the solid obtained upon cooling after overnight reflux at 140 °C was washed with cold water and then with cold ethanol and recrystallized from $CHCl_3$ to yield 3.2 g (28%) of the yellow solid in the first crop. The previously unreported ^1H NMR spectrum: ($CDCl_3$) δ 5.09/5.21/5.26 (3s, 2H each, OCH_2), 6.62 and 6.71 (2s, 1H each, Ar H), 6.91 (s, 1H, vinyl), 7.40–7.54 (m, 15H), 7.66 (s, 1H, NH). The C=C was then reduced by Na(Hg) in aqueous dioxane as described,[25] except that the solution obtained after removal of Hg was evaporated to dryness, the residue was partitioned between H_2O and $CHCl_3$, and the $CHCl_3$ layer was dried and evaporated to yield the desired white hydantoin. The previously unreported ^1H NMR spectrum: ($CDCl_3$) δ 2.72 (dd, 1H, J = 8.2 and 14.3 Hz), 3.38 (dd, 1H, J = 4.3 and 14.3 Hz), 4.28 (dd, 1H, J = 4.3 and 8.2 Hz), 5.01/5.13/5.16 (3s, 2H each, $PhCH_2$), 5.34 (s, 1H, NH), 6.66 and 6.77 (2s, 1H each, ArH), 7.37–7.46 (m, 15H), 7.91 (br s, 1H, NH).

5-(2,4,5-Trihydroxybenzyl)hydantoin (1). 5-[2,4,5-Tris(benzyloxy)benzyl]hydantoin (2 g, 4 mmol) in 150 mL EtOAc containing 200 mg of 10% Pd/C was subjected to hydrogenolysis at 60 torr in a Parr shaker apparatus at 50 °C for 8 h. Filtration of the catalyst, evaporation of the solvent, and crystallization of the residue from CH_3CN yielded 0.88 g (94%), mp 263–265 °C dec; ^1H NMR (DMSO-d_6) δ 2.46 (dd, 1H, J = 9.0 Hz [other coupling indeterminable due to overlapping solvent signal], CHH), 2.93 (dd, 1H, J = 5.0 and 14.0 Hz), 4.19 (dd, 1H, J = 5.0 and 9.0 Hz), 6.30 and 6.44 (2s, 1H each, ArH), 7.58 (s, 1H, NH), 8.03/8.58/8.62 (3s, 1H each, OH), 10.48 (s, 1H, NH). ^1H NMR (CD_3OD) δ 2.57 (dd, 1H, J = 7.3 and 13.8 Hz), 3.14 (dd, 1H, J = 4.8 and 13.8 Hz), 4.28 (dd, 1H, J = 4.8 and 7.3 Hz), 6.29 and 6.53 (2s, 1H each, ArH); ^{13}C NMR (DMSO-d_6) δ 32.0 (CH_2), 58.0 (CH), 103.5 (CH), 112.2 (C), 118.0 (CH), 137.3 (C), 144.3 (C), 147.9 (C), 157.3 (C), 175.8 (C); HRMS calcd for $C_{10}H_{10}N_2O_5$ m/z 238.0590, found 238.0593 (M^+, 13%); base peak is m/z 139.04 for $C_7H_7O_3^+$ from benzylic C–C cleavage.

5-[(2-Hydroxy-1,4-dioxo-2,5-cyclohexadien-5-yl)methyl]hydantoin (2). This compound was prepared by oxidation of 1 either by stirring with 2.5 equiv of $FeCl_3 \cdot H_2O$ in H_2O–CH_3CN (1:1) or by extended exposure of its methanolic solution to O_2. Recrystallization was from wet CH_3CN: mp 244–246 °C dec; ^1H NMR (CD_3CN) δ 2.56 (dd, 1H, J = 9.5 and 14.2 Hz), 3.03 (dd, 1H, J = 4.8 and 14.2 Hz), 4.22 (ddd, 1H, J = 1.9, 4.8, and 9.5 Hz, NHCH), 6.06 and 6.69 (2s, 1H each, vinyls) 6.14 and 8.51 (2br s, 1H each, NH); ^{13}C NMR (DMSO-d_6) δ 31.55 (CH_2), 56.39 (CH), 108.67 (CH), 132.36 (CH), 144.23 (C), 157.16 (C), 157.54 (C), 174.93 (C), 183.52 (C), 187.25 (C); HRMS calcd for $C_{10}H_8N_2O_5$ m/z 236.0433, found 236.0431 (M^+, 32%); base peak is m/z 138.03 for $C_7H_6O_3^+$ arising from benzylic C–C cleavage.

If silica gel chromatography is used to purify this or the other hydroxyquinones 4 and 6 described below, the material which elutes from the column is the sodium salt. The anions are apparently sufficiently good chelators to extract the sodium counterion from the silicates present in silica gel.

Anion of 2: ^1H NMR (DMSO-d_6) δ 2.33 (dd, 1H, J = 9.1 and 14.4 Hz), 2.87 (dd, 1H, J = 4.8 and 14.4 Hz), 4.14 (dd, 1H, J = 4.8 and 9.1 Hz), 5.05 and 6.14 (2s, 1H each, vinyls), 7.7 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 33.0 (CH_2), 56.9 (CH), 103.2 (CH), 129.7 (CH), 148.0 (C), 157.3 (C), 171.3 (C), 175.4 (C), 182.1 (C), 188.9 (C).

"4-Nitrophenylhydrazone of 2", 5-[[1,3-Dihydroxy-4-[(4-nitrophenyl)azo]phenyl]methyl]hydantoin (7). To a solution of quinone 2 (25 mg, 0.10 mmol) in 0.3 mL of dioxane was added 1.0 mL (1.0 mmol) of a 1 M solution of (4-nitrophenyl)-hydrazine in dioxane–H_2O (2:3), acidified to pH 1 (HCl). The resulting precipitate was filtered, washed with 0.5 M aqueous HCl and then with H_2O, and dried in a vacuum dessicator: mp 204–206 °C dec; ^1H NMR (acetone-d_6) δ 2.72 (dd, 1H, J = 8.3 and 13.8 Hz), 3.22 (dd, 1H, J = 4.1 and 13.8 Hz), 4.39 (dd, 1H, J = 4.1 and 8.3 Hz), 6.13 and 7.39 (2s, 1H each, ArH), 7.84 and 8.32 (2d, 2H each, J = 8.5 Hz, $O_2NC_6H_4$). In D_2O the downfield signals are at δ 5.51 (s, 1H, exchanges, ArH), 6.89 (s, 1H, ArH), 7.47 and 8.26 (2d, 2H each, J = 9.1 Hz, $O_2NC_6H_4$).

2,4,5-Tris(benzyloxy)phenethylamine. Essentially as described,[25] a mixture of 5 g (11.8 mmol) of 2,4,5-tris(benzyloxy)-benzaldehyde, 0.45 g of NH_4OAc, and 60 mL of nitromethane was heated at reflux for 5 h under N_2. The yellow-orange precipitate obtained upon cooling was collected by filtration, washed with water, cold methanol, and hexane, and dried in a vacuum oven overnight, affording 4.6 g (88%) of 2,4,5-tris-(benzyloxy)-β-nitrostyrene, mp 139–140 °C (lit.[25] 138–140 °C). The previously unreported ^1H NMR spectrum: ($CDCl_3$) δ 5.06/5.10/5.15 (3s, 2H each, OCH_2), 6.58 and 6.98 (2s, 1H each, ArH), 7.31–7.44 (m, 15H), 7.67 and 8.08 (2d, 1H each, J = 14 Hz, vinyls). The latter material (4.6 g, 9.9 mmol) was reduced with $LiAlH_4$ in THF as described.[25] The solid obtained after aqueous workup was extracted twice with hot THF, and the combined THF extract was dried (Na_2SO_4) and evaporated to give 3.0 g (70%) of the white amine: mp 75–77 °C (lit.[25] 70–77 °C). The previously unreported ^1H NMR spectrum: ($CDCl_3$) δ 1.34 (br s, 2H, NH_2), 2.67 and 2.84 (2t, 2H each, J = 6.2 Hz), 4.93/5.08/5.09 (3s, 2H each, OCH_2), 6.60 and 6.76 (2s, 1H each, ArH), 7.31–7.40 (m, 15H).

5-(2-Phthalimidoethyl)-1,2,4-trihydroxybenzene (3). A mixture of 1.75 g (4.0 mmol) of 2,4,5-tris(benzyloxy)phenethylamine, 0.62 g (4.2 mmol) of phthalic anhydride, and 10 mL of DMF was heated at 115 °C for 16 h under N_2. The resulting mixture was cooled, concentrated to remove most of the DMF, diluted with 30 mL of EtOAc, and washed sequentially with dilute HCl, aqueous half-saturated $NaHCO_3$, and brine. The organic layer was dried (Na_2SO_4) and concentrated to give a residue which crystallized on standing. The solid (R_f 0.57, Et_2O–hexane 5:1) was triturated with MeOH, filtered, and washed with MeOH and then with Et_2O to yield 1.8 g (60%) of 5-(2-phthalimidoethyl)-1,2,4-tris(benzyloxy)benzene as a white solid: mp 122–124 °C; ^1H NMR ($CDCl_3$) δ 2.98 and 3.95 (2t, 2H each, J = 7.2 Hz), 4.92/4.98/5.08 (3s, 2H each, OCH_2), 6.60 and 6.80 (2s, 1H each, ArH), 7.30–7.47 (m, 15H), 7.60–7.67 and 7.73–7.79 (2m, 2H each, phthaloyl H). The latter material (0.8 g, 1.4 mmol), 0.24 g of 10% Pd/C, and 25 mL of ethanol were subjected to 60 psi H_2 in a Parr apparatus at 50 °C for 10 h. Filtration of the catalyst, evaporation of the solvent, and recrystallization of the residue from $CHCl_3$ yielded 0.36 g (86%) of 3 as a yellow solid: mp 204–206 °C dec; ^1H NMR (DMSO-d_6) δ 2.66 and 3.69 (2t, 2H each, J = 6.7 Hz), 6.21 and 6.34 (2s, 1H each, ArH), 7.83 (br s, 4H, phthaloyl H), 7.96/8.45/8.51 (3s, 1H each, OH); ^{13}C NMR (DMSO-d_6) δ 28.3 (CH_2), 38.2 (CH_2), 103.7 (CH), 114.3 (C), 117.3 (CH), 123.0 (CH), 131.7 (C), 134.3 (CH), 137.5 (C), 144.1 (C), 148.0 (C), 167.8 (C); HRMS calcd for $C_{16}H_{13}NO_5$ m/z 299.0794, found 299.0791 (M^+, 28%); major peaks at m/z 160.04 for $C_9H_6NO_2^+$ (57%) and 139.04 for $C_7H_7O_3^+$ (54%) arising from benzylic/α-heteroatom C–C cleavage and at m/z 152.04 for $C_8H_8O_3^+$ (51%) arising from phthalimide elimination; base peak is m/z 104.03 for $C_6H_4C{=}O^+$ arising from phthaloyl fragmentation.

2-Hydroxy-5-(2-phthalimidoethyl)-1,4-benzoquinone (4). To a solution of 0.14 g (0.5 mmol) of 3 in 10 mL of H_2O–CH_3CN (1:1) was added a solution of 0.316 g (1.2 mmol) of $FeCl_3 \cdot H_2O$ in 4 mL of H_2O with stirring and cooling in an ice bath. After the addition was complete, yellow crystals precipitated from the solution. The solid was collected by filtration and recrystallized from CH_3CN, yielding 0.12 g (87%) of yellow solid: mp 202–204 °C dec; ^1H NMR (DMSO-d_6) δ 2.64 and 3.74 (2t, 2H each, J = 5.7 Hz), 5.88 (s, 1H, exchanges upon adding D_2O, vinyl), 6.59 (s, 1H, vinyl), 7.82 (br s, 4H); ^{13}C NMR (DMSO-d_6) δ 28.4 (CH_2), 36.5 (CH_2), 108.7 (CH), 123.1 (CH), 130.9 (CH), 131.5 (C), 134.4 (CH), 146.8 (C), 157.3 (C), 167.8 (C), 183.6 (C), 187.3 (C); HRMS

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calcd for $C_{16}H_{11}NO_5$ m/z 297.0637, found 297.0632 (M^+, 5%); base peak is m/z 160.04 for $C_9H_6NO_2^+$ arising from benzylic/α-hetero C–C fragmentation.

"4-Nitrophenylhydrazone of 4", 4-[(4-Nitrophenyl)azo]-6-(2-phthalimidoethyl)benzene-1,3-diol (8). To a solution of quinone 4 (25 mg, 0.085 mmol) in 0.3 mL of dioxane was added 0.85 mL (0.85 mmol) of a 1 M solution of (4-nitrophenyl)hydrazine in dioxane–H_2O (2:3), acidified to pH 1 (HCl). The resulting precipitate was filtered, washed with 0.5 M aqueous HCl and then with H_2O, and dried in a vacuum dessicator: mp 195–198 °C dec; ^{1}H NMR (acetone-d_6) δ 3.05 and 3.99 (2t, 2H each, J = 6.7 Hz), 6.42 and 7.63 (2s, 1H each, ArH), 7.81 (s, 4H), 8.01 and 8.38 (2d, 2H each, J = 8.2 Hz, $O_2NC_6H_4$), 10.10 (br s, 1H, OH), 13.13 (s, 1H, intramolecular H-bonded[34] OH); ^{13}C NMR (DMSO-d_6) see Figure 1.

Preparation of the "4-Nitrophenylhydrazone of 2-Hydroxy-1,4-benzoquinone", 4-[(4-Nitrophenyl)azo]-1,3-benzenediol (9). (a) From 1,2,4-benzenetriol and (4-Nitrophenyl)hydrazine: To a solution of 1,2,4-benzenetriol (50 mg, 0.40 mmol) in dioxane was added 0.4 mL (4.0 mmol) of a 1 M *solution of (4-nitrophenyl)hydrazine in dioxane–H_2O (2:3) acidified* to pH 1 (HCl), followed by 107 mg (0.40 mmol) of $K_2S_2O_8$. After 4 h, the solution was filtered, and the derivative was washed with 0.5 M aqueous HCl and then with H_2O and dried overnight in a vacuum dessicator. **(b) From resorcinol:** A solution of 0.50 g (4.5 mmol) resorcinol in 4 mL of 10% aqueous NaOH was added at 0 °C to a solution of $NaNO_2$ (0.35 g, 5 mmol) in 2 mL of H_2O. This solution was then added at 0 °C to a solution of 4-nitroaniline (0.75 g, 5.4 mmol) in 1.5 mL of H_2O and 2 mL of concd HCl. After the mixture was stirred and allowed to come to room temperature, the solid was collected by filtration, washed with 0.5 M aqueous HCl and then with H_2O, and dried in a vacuum dessicator. The deep red product from both methods was identical as per mp (181–183 °C, dec),[35] TLC (R_f = 0.70, Et_2O), and ^{1}H NMR: (acetone-d_6) δ 6.42 (d, 1H, J = 2.5 Hz), 6.68 (dd, 1H, J = 2.5 and 8.9 Hz), 7.81 (d, 1H, J = 8.9 Hz), 8.09 and 8.41 (2d, 2H each, J = 9.1 Hz, $O_2NC_6H_4$), 9.83 (br s, 1H, OH), 13.11 (s, 1H, intramolecular H-bonded[34] OH); HRMS (20 eV) m/z calcd for $C_{12}H_9N_3O_4$ 259.0593, found 259.0600 (M^+, 18%).

***N*-(2,4,5-Trihydroxyphenethyl)pivalamide (5).** A solution of 0.7 g (5.8 mmol) of pivaloyl chloride in 10 mL of dry ether was added dropwise with stirring to a solution of 2.4 g (5.8 mmol) of 2,4,5-tris(benzyloxy)phenethylamine and 0.88 g (8.7 mmol) of triethylamine in 125 mL of dry ether. After 1 h at room temperature, the white solid which had precipitated was filtered and extracted with ether. The combined ether layers were washed with 40 mL of water, dried (Na_2SO_4), and evaporated to give 2.8 g (97%) of *N*-[(2,4,5-tris(benzyloxy)phenethyl]pivalamide as a white solid: mp 79–80 °C; ^{1}H NMR ($CDCl_3$) δ 1.05 (s, 9H), 2.76 (t, 2H, J = 6.0 Hz), 3.40 (q, 2H, J = 6.0 Hz, NHCH_2), 4.95/5.06/5.10 (3s, 2H each, OCH_2), 6.62 and 6.78 (2s, 1H each, ArH), 7.25–7.44 (m, 15H). A solution of 2.4 g (4.6 mmol) of the latter material in 70 mL of EtOH containing 0.70 g of 10% Pd/C was subjected to H_2 at 60 psi in a Parr apparatus at 40 °C for 3 h. The catalyst was removed by filtration, the filtrate was evaporated, and the residue was washed with $CHCl_3$ (3 × 15 mL), yielding 1.3 g (81%) of **5** as a light-brown solid: mp 58–60 °C; ^{1}H NMR (DMSO-d_6) δ 1.05 (s, 9H), 2.47 (t, 2H, J = 7.4 Hz), 3.09 (br q, 2H, NHCH_2), 6.26 and 6.38 (2s, 1H each, ArH), 7.38 (t, 1H, J = 5.2 Hz, NH), 7.97 (s, 1H, OH), 8.51 (s, 2H, OH); ^{13}C NMR (DMSO-d_6) see Table 1; HRMS calcd for $C_{13}H_{19}NO_4$ m/z 253.1315, found 253.1314 (M^+, 13%); base peak is m/z 152.04 for $C_8H_8O_3^+$ arising from pivalamido elimination.

2-Hydroxy-5-(2-pivalamidoethyl)-1,4-benzoquinone (6). To a solution of 0.6 g (2.4 mmol) of **5** in 6 mL CH_3CN was added 1.6 g (6.0 mmol) of $FeCl_3 \cdot H_2O$ in 6 mL of H_2O with stirring and cooling in an ice bath. After the addition was complete, the mixture was extracted with CH_2Cl_2 (3 × 60 mL). The combined organic layer was dried (Na_2SO_4) and evaporated, leaving a dark-brown residue which was recrystallized from 5 mL of CH_3CN to give 0.33 g (55%) of a yellow solid: mp 138–139 °C dec; UV (CH_3CN) ϵ = 714 at λ_{max} 370 nm; UV (of anion, CH_3CN containing excess Et_3N) ϵ = 1750 at λ_{max} 490 nm; ^{1}H NMR (DMSO-d_6) δ 1.00 (s, 9H), 2.47 (t, 2H, J = 6.0 Hz), 3.16 (br q, 2H, J_{app} = 5.8 Hz, NHCH_2), 5.90 (s, 1H, exchanges upon adding D_2O, vinyl), 6.40 (s, 1H, vinyl), 7.49 (t, 1H, J = 4.9 Hz, NH); ^{13}C NMR (DMSO-d_6) δ 27.4 (CH_3), 29.4 (CH_2), 37.2 (C), 38.0 (CH_2), 108.9 (CH), 130.4 (CH), 147.3 (C), 157.2 (C), 177.4 (C), 183.6 (C), 187.4 (C); HRMS calcd for $C_{13}H_{17}NO_4$ m/z 251.1158, found 251.1161 (M^+, 14%).

Catalytic and Stoichiometric Oxidative Deamination of $PhCH_2NH_2$ by TPQ Model 2. For the catalytic reactions, a solution of benzylamine (0.536 g, 5 mmol) in 75 mL H_2O–CH_3CN (2:1) was adjusted to pH 8.0 with 3 M HCl and diluted with additional H_2O to bring the volume to 82.5 mL. To this was added a solution of **2** (23.6 mg, 0.1 mmol) in 12.5 mL of CH_3CN and 5 mL of H_2O. The mixture was stirred with continuous O_2 bubbling for 17 h, during which time the pH dropped to 7.6. The resulting solution was concentrated to remove most of the CH_3CN, extracted with 50 mL of CH_2Cl_2, adjusted to pH 11 with 10% aqueous NaOH, and again extracted with 50 mL of CH_2Cl_2. Evaporation of the solvent left a mixture of $PhCH_2NH_2$ and $PhCH{=}NCH_2Ph$ as indicated by TLC and ^{1}H NMR (the latter imine exhibits the following signals in $CDCl_3$: δ 4.73 (s, 2H), 7.17–7.34 (m, 8H), 7.70 (m, 2H), 8.29 (s, 1H). Quantitation of the $PhCH{=}NCH_2Ph$ was either by integrating the ^{1}H NMR spectrum relative to the methyl singlet for weighed hexamethylbenzene added to the final extract or by treating with acidified 2,4-DNP and weighing the resulting benzaldehyde-derived 2,4-dinitrophenylhydrazone. The two methods agreed to within 4%. For the stoichiometric reactions, a solution of **2** (12.8 mg, 0.05 mmol) and $PhCH_2NH_2$ (26.8 mg, 0.25 mmol) in DMSO-d_6 through which had been bubbled N_2 before sealing in an NMR tube, was periodically monitored by ^{1}H NMR spectrometry. Over the course of 1 h, the distinct signals for the product $PhCH{=}NCH_2Ph$ grew in at δ 4.77 (s, 2H), 7.80 (m, 2H), 8.51 (s, 1H).

Kinetics of Reaction of TPQ Model 2 with $PhCH_2NH_2$ and $PhCD_2NH_2$. A 7.5 mM solution of the TPQ hydantoin **2** in CH_3CN–H_2O (2:1) and 37.5 mM solutions of either $PhCH_2NH_2$ or $PhCD_2NH_2$ in CH_3CN–H_2O (2:1) containing 15 mM H_3PO_4 and adjusted to pH 6.0 or 7.0 (with CH_3COOH) or pH 8.0 or 9.0 (with KOH) were each equilibrated under N_2 in a water bath at 40.0 °C. For the kinetic runs, 100 μL of the quinone solution and 200 μL of the amine solution were added to Teflon-stoppered 0.1-cm quartz cells which were equilibrated to 40.0 °C in the cell compartment of the spectrophotometer. The decrease in A_{485} was followed with time.

Reaction of (reduced) TOPA Model 5 with Amines. *N*-(2,4,5-Trihydroxyphenethyl)pivalamide (15 mg, 0.06 mmol), apparently contaminated with a trace of quinone **6** (see text), *tert*-butylamine (6.3 μL, 0.06 mmol) and 0.06 mmol of either benzylamine, phenethylamine, cyclopropylamine, or neopentylamine were dissolved in 0.5 mL of DMSO-d_6 in a 5-mm NMR tube, and N_2 was bubbled through the solution before closing the tube. The ^{1}H NMR spectrum was recorded immediately and periodically until no further changes were observed. In every case, the signals for the NHC(=O)C(CH_3)$_3$ fragment of **5** remained unaltered, whereas the signals for the $ArCH_2CH_2N$ fragment moved slightly downfield: the 2H t (J = 7.3 Hz) from 2.46 to 2.49 δ, and the 2H br q from 3.10 to 3.12 δ. The aryl signals at δ 6.27 and 6.37 and the signals for the respective amines changed as follows: **Benzylamine**: the two aryl 1H s moved to 6.17 and 6.30 δ, the CH_2 2H s moved from 3.69 to 4.19 δ, and the Ph 5H m at δ 7.25–7.30 was unchanged. **Phenethylamine**: the two aryl 1H s moved to 6.24 and 6.29 δ, the $PhCH_2$ 2H t moved from δ 2.61 (J = 6.5 Hz) to 2.80 (J = 6.9 Hz), and the NCH_2 2H t moved from δ 2.73 (J = 6.5 Hz) to 3.10–3.18 (overlapped with CH_2 of **5**). **Neopentylamine**: the two aryl 1H s moved to δ 6.21 and 6.28, the CH_3 9H s moved from 0.79 to 0.90 δ, and the CH_2 2H s moved from 2.25 to 2.70 δ. **Cyclopropylamine**: the two aryl 1H s moved to δ 6.26 and 6.50, the NCH 1H m moved from 2.13 to 2.19 δ, and the two CH_2 2H m moved from 0.11 and 0.29 δ to 0.33 and 0.56 δ. We made no attempt to isolate the substitution products **13** except in the case of phenethylamine.

4-(Phenethylamino)-6-(pivalamidoethyl)-1,3-benzenediol (13) (R = $PhCH_2CH_2$). A mixture of 76.5 mg (0.3 mmol)

(34) Startseva, N. V.; Frankovskii, Ch. S.; Ionin, B. I.; Chebotova, N. G. *Zh. Organ. Khim.* **1971**, *7*, 337.

(35) There is a literature report (Kokkinos, K.; Wizinger, R. *Helv. Chim. Acta* **1971**, *54*, 335) of mp 198 °C for this compound, but both Aldrich Chemical Co. 1992–1993 catalog and Pfaltz & Bauer 1991–1992 catalog report mp 185 °C dec.

of **5** and 38 μL (0.3 mmol) of phenethylamine in 0.5 mL of DMSO-d_6 was sealed under N_2 in an NMR tube. The reaction was shown by ^{1}H NMR to be complete in 17 h. At this time the series of NOE difference and ^{13}C NMR experiments described in the text and Table 1 were conducted. The solvent was removed under high vacuum and the residue was shown to consist of a single material by two TLC systems: HRMS calcd for $C_{21}H_{28}N_2O_3$ 356.2101, found 356.2100 (M^+, 79%); base peak at m/z 265.15 arising from loss of C_7H_7.

Kinetics of Autoxidation of TOPA Model 5 in Phosphate Buffer. The solvents used in the reactions were presaturated with O_2. Potassium phosphate buffer (1 mL of 300 mM) adjusted to the desired pH (5–7), 0.5 mL of H_2O, and 1.4 mL of CH_3CN were added to a 3-mL cuvette and preincubated in the spectrometer at 25.0 °C. A freshly prepared solution of *N*-(2,4,5-trihydroxyphenethyl)pivalamide (**5**) in CH_3CN (0.1 mL, 30 mM) was added, and the cuvette was shaken. The spectra were recorded from 650 nm to 350 nm every 5 min. In a second series of runs, the reaction solutions at pH 5.0 and 6.0 were bubbled with O_2 for 20 s between scans. The rate constants were calculated from the linear portion of first-order plots of the A_{490} increase (λ_{max} for **6** anion).

6-Hydroxy-7-(2-pivalamidoethyl)quinoxaline (14). A mixture of 0.4 mmol of either **5** or **6** and ethylenediamine (52 mg, 0.88 mmol) in 20 mL of CH_3CN was heated at 50 °C open to the atmosphere. After 26 h, the solvent was evaporated, and the residue was applied to a 2-mm thick silica gel plate which was eluted with MeOH–EtOAc (5:2). The major fast-moving material (R_f = 0.58) was obtained as a red solid: mp 93–95 °C; ^{1}H NMR ($CDCl_3$) δ 1.12 (s, 9H), 3.11 (t, 2H, J = 6.5 Hz), 3.71 (br q, 2H, J_{app} = 6.1 Hz, NHCH_2), 6.40 (t, 1H, J = 5.3 Hz, NH), 7.55 and 7.83 (2 s, 1H each, ArH), 8.67 and 8.68 (AB, 2H); ^{13}C NMR (DMSO-d_6) δ 27.39 (CH_3), 30.12 (CH_2), 37.92 (C), 38.27 (CH_2), 108.68 (CH), 129.53 (CH), 133.95 (C), 137.60 (C), 141.95 (CH), 144.51 (CH), 143.01 (C), 157.67 (C), 177.23 (C); HRMS calcd for $C_{15}H_{19}N_3O_2$ m/z 273.1479, found 273.1476 (M^+, 12%); base peak is m/z 172.06 for $C_{10}H_8N_2O^+$ arising from elimination of $(CH_3)_3$-CC(=O)NH_2.

The formation of **14** could also be followed by ^{1}H NMR in DMSO-d_6. For example, in the case of triol **5** (5-fold excess of $H_2NCH_2CH_2NH_2$) a spectral change occurred over a period of 20 min wherein the signals for the initial starting materials were replaced by those for the substitution product **13** (R = CH_2-CH_2NH_2: δ 1.05 (s, 9H), 2.69 and 2.89 (2t, 2H each, J = 5.9 Hz), 3.12 (br q, 2H, CH_2NHCO), 6.21 and 6.27 (2s, 1H each, ArH), 7.38 (br t, 1H, NH); ArCH_2 signal at δ ~2.49 is buried under the signal for unreacted $H_2NCH_2CH_2NH_2$. Upon exposure to air, these signals gradually disappear with the formation of new signals, of which those for **14** account for ~70% of the total integration.

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