Chemical Simulation of Biogenesis of the 2,4,5-Trihydroxyphenylalanine Quinone Cofactor of Copper Amine Oxidases: Mechanistic Distinctions Point toward a Unique Role of the Active Site in the *o*-Quinone Water Addition Step†

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Received August 9, 1999

Abstract: The biogenesis of the 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor from tyrosine at the active site of copper amine oxidases is believed to proceed along a pathway that includes a conjugate addition of water to the corresponding *o*-quinone intermediate, followed by autoxidation of the resulting benzenetriol to the hydroxyquinone cofactor. The water addition reaction has been presumed to occur not only in previous model studies reported for cofactor biogenesis starting with either catechol or *o*-quinone, but also for generation of the neurotoxin 6-hydroxydopamine during autoxidation of dopamine. We here report the surprising finding that water addition does not occur under solution chemistry conditions. The production of hydroxyquinone from catechol arises instead from reaction of the *o*-quinone with H_2O_2 generated during autoxidation of catechol. When starting with the *o*-quinone itself, production of hydroxyquinone still arises from autoxidation of the catechol, generated either by reduction of the *o*-quinone by its decomposition products at moderate pH, or by a novel base-mediated redox disproportionation of the *o*-quinone at high pH. These conclusions are supported by the behavior of independently studied *o*-quinone intermediates, the observed effects of added catalase, and ^{18}O-labeling studies utilizing both [^{18}O]H_2O and [^{18}O]O_2. The failure to observe water addition to the *o*-quinone has broad implications for aqueous *o*-quinone chemistry, and suggests that in TPQ biogenesis, this hydration is being catalyzed at the enzyme active site, possibly by the bound copper.

Introduction

The copper amine oxidases rely on posttranslational oxidation of an active-site tyrosine to a quinone moiety for mediating the transaminative conversion of primary amines to aldehydes. Most enzymes in this family contain the 2,4,5-trihydroxyphenylalanine quinone (topaquinone, TPQ) residue,[1] which exists as a resonance-stabilized anion at physiological pH. On the other hand, mammalian lysyl oxidase contains a derivative of this residue, involving substitution of a lysine ϵ-amino group at the 2-position, giving an aminoquinone referred to as lysine tyrosylquinone (LTQ).[2] The role of the single copper, also present at the active site, in the catalytic functioning of these enzymes has been controversial, although there is general consensus for its involvement in the dioxygen-dependent two-electron reoxidation of the aminoresorcinol (reduced) form of the cofactor back to the quinone, giving H_2O_2 and NH_3 as byproducts.[3]

Since peptidyl tyrosines are not routinely oxidized to hydroxyquinones, it is of great interest to determine the mechanism of the posttranslational conversion of the active-site tyrosine to the active quinone cofactor in these enzymes. Although one early possibility considered was initiation of TPQ biogenesis by action of a tyrosinase-like enzyme, converting the phenol side chain to the corresponding catechol or *o*-quinone, studies on cloned amine oxidase proteins in cell-free media demonstrated that the only factor needed for generation of the catalytically active TPQ cofactor was copper(II).[4] Presumably, binding of copper into its trihistidinyl coordination domain at the active site confers to it the property of mediating minimally a tyrosinase-like monooxygenation of the tyrosine phenol.

The consensus biotransformation mechanism has been represented as shown in Scheme 1.[5] Once the *o*-quinone is produced, biogenesis is completed by conjugate addition of water to give the reduced triol form (TOPA) of the TPQ cofactor, which can then undergo autoxidation to TPQ. Whether the active site copper participates in either of these latter steps is unknown. Although a stoichiometry of biogenesis requiring two molecules of O_2 has been demonstrated,[6] the source of the C2 oxygen has been shown to arise from water and not dioxygen.[7]

A number of previous model studies directed at mimicking

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† Presented in preliminary form: Sayre, L. M.; Purdy, M. M.; Lee, Y.; Nadkarni, D. V.; Mandel, S. *Abstracts of Papers*, 216th National Meeting of the American Chemical Society, Boston, MA, August 23−27, 1998, American Chemical Society, Washington, DC (ORGN 279).

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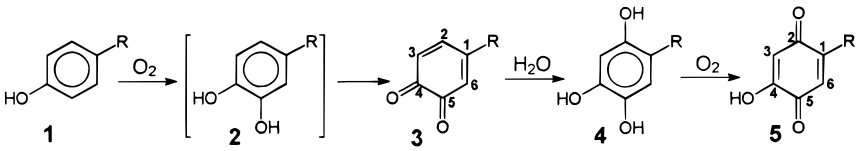
10.1021/ja992886g CCC: $19.00
Published on Web 04/05/2000

Scheme 1



tyrosinase, wherein various copper catalysts were shown to mediate *ortho* oxygenation of phenol to give catechols or *o*-quinones,[8−10] are relevant to the mechanism of TPQ biogenesis. Also, it has been known for some time that the catechol neurotransmitter dopamine (**2**, R = $CH_2CH_2NH_2$) can undergo autoxidation to the hydroxyquinone **5** (R = $CH_2CH_2NH_2$), presumably via the corresponding *o*-quinone **3** (R = CH_2CH_2-NH_2), which is itself also observed to be transformed to hydroxyquinone **5** under the autoxidation conditions.[11] However, the stoichiometries and mechanisms of these latter reactions have not been adequately characterized, and the *o*-quinone conjugate addition of water has always been assumed to occur without direct evidence.[12]

With renewed interest in this chemistry based on TPQ biogenesis, a series of papers by Sanjust, Rinaldi, and coworkers reported that 4-methylcatechol (**2**; R = Me)[13] or 4-*tert*-butylcatechol (**2**; R = *t*-Bu)[14] can be transformed in good yield to the corresponding hydroxyquinones **5** in the presence of O_2, as aided by Cu(II). These workers interpreted their observations in terms of following all but the first step of the consensus mechanism of Scheme 1, although again no direct evidence for the proposed conjugate addition of water to *o*-quinone **3** was presented. Also, no effective model system for the *complete* biogenetic transformation of tyrosine to TPQ has been achieved.

We have also been studying catechol autoxidation, as well as models for the tyrosinase-like copper-mediated monooxygenation of phenols.[15] The latter is achieved by oxygenation of Cu(I) phenolates in CH_3CN, and regardless of whether the *o*-quinones are generated directly or by oxidation of initially formed catechols, we considered that aqueous workup should, under the basic conditions, result in subsequent conjugate addition of water to give triol **4**, which in turn would be expected to easily autoxidize to hydroxyquinone **5**. Since such reaction sequence would represent the *complete biogenetic conversion* of **1** to **5**, we expended a considerable effort to observe this. However, following oxygenation of the Cu(I) phenolates **1** (**1**; R = *t*-Bu, Me) in CH_3CN, ultimate generation of hydroxyquinone **5** required raising the basicity of the final aqueous aerobic workup step to at least pH 12, and even then only traces of **5** could be detected. The apparent resistance of *o*-quinones **3** to conjugate addition of water at high pH seemed incompatible with the fact that high yields of **5** could be obtained from the autoxidation of catechols **2** at much lower pH, assuming the operation of Scheme 1.

In our attempts to understand this enigma, we have carefully examined possible differences in the oxidation pathways starting from *o*-quinones **3** as opposed to starting with catechols **2**. We describe here our findings and conclusions, which conflict with several presumptions expressed in the literature. Most importantly, we find no evidence for facile conjugate addition of water to *o*-quinones: the formation of hydroxyquinones **5** derives mainly if not entirely from reaction of *o*-quinones **3** with H_2O_2 that is generated either directly during autoxidation of catechol **2**, or indirectly when starting with *o*-quinones **3**. This finding has broad implications for understanding the mechanism of physiological autoxidation of catechols. In addition, the failure to witness chemical precedent for the C2 hydration step of TPQ biogenesis suggests that this step must be facilitated at the enzyme active site, possibly via electrophilic activation by the active-site copper.

Results and Discussion

Catechol autoxidation. Exposure of 4-methylcatechol **2** (R = Me) to aqueous base (pH 8−12) and oxygen generates hydroxyquinone **5** (R = Me) in the form of its anion (λ_{max} at 480 nm) as reported,[13] but the yields are variable, and the formation of one or more side-products is indicated by the appearance of a strong absorbance at 325 nm. The A_{325} side-product was judged to represent at least in part decomposition of hydroxyquinone **5**, based on what was observed when isolated **5** was exposed to the same conditions. By varying the pH, the solvent composition (water, water-methanol, or water-acetonitrile), the buffer (carbonate, phosphate, or maintaining pH by dropwise addition of aqueous NaOH), the reaction time, and the method of O_2 exposure, it was determined that optimal yields (50−55%) of hydroxyquinone **5** (R = Me) relative to the A_{325} side-products could be obtained when O_2 was bubbled through an unbuffered pH 10 solution of 4-methylcatechol (5−7.5 mM) for 10−11 min, with acid quenching prior to total consumption of starting 4-methylcatechol. Following extraction with CH_2-Cl_2, analysis of the material remaining in the aqueous layer by NMR and TLC indicated a complex mixture of polar compounds, suspected to represent at least in part carboxylic acid quinone cleavage products.

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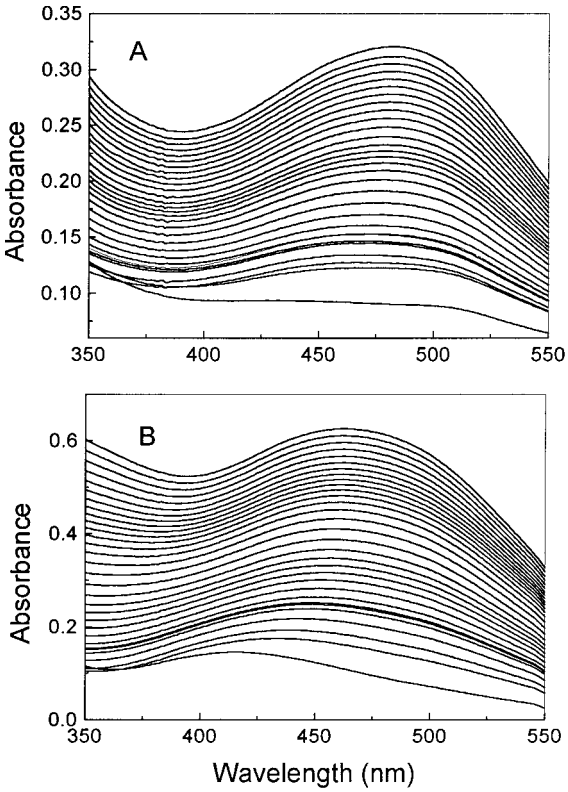


Figure 1. Spectral change during the autoxidation of 10 mM 4-methylcatechol in 0.1 M potassium phosphate buffer, pH 8, at 25 °C at 10 min intervals, starting from the bottom. (A) Contains no additive; (B) contains 0.5 mM $CuSO_4$.

In contrast to 4-methylcatechol, upon similar base-mediated autoxidation of 4-*tert*-butylcatechol, buildup of the intermediate *o*-quinone **3** (R = *t*-Bu) could be observed at early stages as reported,[14] but the reaction is much more complicated than is apparent from absorption spectroscopy. The corresponding hydroxyquinone **5** was obtained in at best 20−30% yield, and the CH_2Cl_2 extract obtained after neutralization of the reaction mixture contained nearly equivalent amounts of materials other than **5** and recovered **2**. As will be reported separately, we have isolated and characterized by NMR and mass spectrometry several of the side-products accompanying autoxidation of 4-*tert*-butylcatechol, and they absorb only weakly in the 300−600 nm range, thereby explaining why these products were missed by the earlier work based exclusively on absorption spectroscopy.[13,14] Needless to say, the autoxidation of catechols **2** in aqueous base does not afford clean conversion first to **3** and then to hydroxyquinones **5** as had been claimed.[13,14]

Effect of Copper(II) on Catechol Autoxidation. Rinaldi, Sanjust, and co-workers reported spectrophotometric evidence for a first-order Cu(II) catalysis of the autoxidation of 4-methylcatechol, suggesting that such may be important in TPQ biogenesis.[13] Indeed, transition-metal-catalyzed autoxidations are well-known. However, our attempt to verify Cu(II) catalysis of 4-methylcatechol autoxidation through spectrophotometric determination of the rate as a function of [Cu(II)], was complicated by Cu(II)-dependent spectral perturbations, whereas the peak for hydroxyquinone **5** (R = Me) at 480 nm was seen to grow steadily with time in the absence of Cu(II) (Figure 1A), reactions in the presence of Cu(II) exhibited a blue shift that was largest at the beginning of reaction and diminished at longer reaction times as the concentration of [**5**] relative to [Cu(II)] increased: Figure 1B shows that using 10 mM 4-methylcatechol and 0.5 mM Cu(II), the λ_{max} shifts from ~415 nm at the beginning of reaction to 465 nm at later stages of reaction. When higher concentrations of Cu(II) (1−2 mM) were used, the λ_{max} was seen to reach only 450 nm even at long reaction times. Furthermore, addition of increasing amounts of Cu(II) to samples of 4-methylcatechol that had undergone a 90 min pre-autoxidation at pH 8 in the absence of Cu(II), indicated that the blue shift is more pronounced, with enhanced ϵ, with increasing [Cu(II)]/[hydroxyquinone] ratios (Figure 2). We cannot explain the difference between our results and those of Rinaldi et al.,[13a] who reported no such λ_{max} complexities.

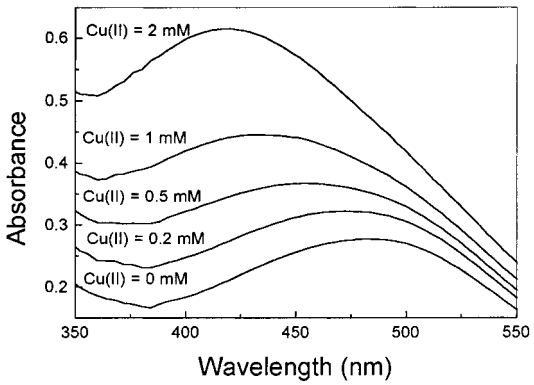


Figure 2. Spectral changes due to addition of different concentrations of $CuSO_4$ to aliquots of 10 mM 4-methylcatechol autoxidized for 90 min in 0.1 M potassium phosphate buffer, pH 8, at 25 °C.

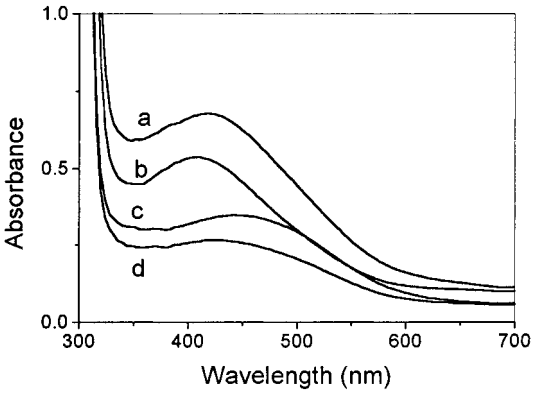


Figure 3. Spectra obtained for 10 mM 4-methylcatechol autoxidized for 90 min before adding Cu(II) (b,d) or in the presence of Cu(II) for 90 min (a, c), in 0.1 M potassium phosphate buffer, pH 8, at 25 °C: (a) 2 mM Cu(II) added at $t = 0$; (b) 2 mM Cu(II) added at $t = 90$ min; (c) 0.5 mM Cu(II) added at $t = 0$; (d) 0.5 mM Cu(II) added at $t = 90$ min.

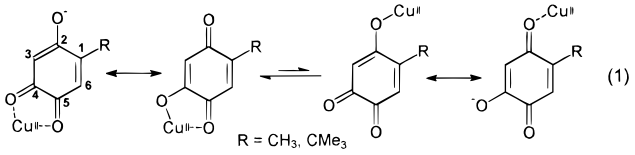
In lieu of a direct kinetic analysis, the effect of Cu(II) was estimated by comparing the spectral progress of 4-methylcatechol autoxidations in the presence of Cu(II) to the spectra obtained immediately following addition of the same concentration of Cu(II) to aliquots of 4-methylcatechol that had been pre-autoxidized in the absence of Cu(II). This approach should be independent of the Cu(II)-induced spectral shifts. As shown in the example (Figure 3), a larger spectral change indicative of greater reaction progress was seen in the former case, suggesting that Cu(II) does stimulate the reaction. In an effort to quantitate the effect of copper, we determined, as a function of [Cu(II)], how long it would take the reaction run in the presence of Cu(II) to match the state of completion of a 90-min pre-autoxidized sample (compared after adding the same [Cu(II)] to the latter). As shown in Table 1, it took successively less time to reach the same apparent state of completion with increasing [Cu(II)], although the spectral matching is inaccurate because (i) the λ_{max} values did not match and (ii) the reactions run in the presence

Table 1. Effect of Cu(II) on the Autoxidation of 4-Methylcatechol (10 mM) in Phosphate Buffer (0.1 M, pH 8.0) at 25 °C

[Cu(II)]	0.5 mM	1.0 mM	2.0 mM
λ_{max} (nm) upon adding Cu(II) following 90 min of pre-autoxidation	423	410	407
A at observed λ_{max}	0.264	0.372	0.527
time (min) needed to reach the same A_λ for autoxidations run in the presence of Cu(II)	70	50	38
actual λ_{max} (nm) at these times for autoxidations run in the presence of Cu(II)	450	433	417

of Cu(II) exhibited a growing shoulder at 350 nm (of increased intensity with increasing [Cu(II)]) that was not seen when Cu(II) was added to pre-autoxidized 4-methylcatechol. Although our data support the claim that Cu(II) increases the rate of conversion of 4-methylcatehol to hydroxyquinone **5** (R = Me),[13a] establishing the true kinetic order in Cu(II) would be difficult and would first require a careful product analysis in the presence and absence of Cu(II).

As far as why Cu(II) induces a spectral shift for hydroxyquinone **5** (R = Me), we initially considered that this likely reflected chelation of Cu(II) by **5** at the vicinal dioxy site as shown in eq 1. The associated neutralization might be expected to display a spectral change in the direction seen for protonation (the neutral hydroxyquinone absorbs at 372 nm in CH_3CN[16]). Interestingly, however, the addition of Cu(II) to solutions of the *tert*-butyl rather than methyl hydroxyquinone (**5**, R = *t*-Bu, 10% aqueous CH_3CN, pH 7) was not accompanied by a detectable spectral shift. Although the reasons for this are unclear, differing electronic effects or steric inhibition of solvation at the C2 oxygen adjacent to R = *t*-Bu may be responsible. Alternatively, the spectral shift may represent a minor equilibrium complex with Cu(II) coordinated to the C2 oxygen (eq 1), which is sterically inhibited in the case of R = *t*-Bu.



(1)

Effect of Chelating Agents on Catechol Autoxidation. Transition metal-catalyzed autoxidation of catechols has been extensively studied,[17,18] and most apparent metal-independent autoxidations invariably reflect contaminating trace metals.[19,20] However, the existence of a true metal-independent reaction has been demonstrated,[21] exhibiting distinct kinetics from what is observed in the presence of transition metals.[18] Metal-independent *base-mediated* autoxidations are thought to involve initial charge transfer of the electron-rich organic (phenolate anion in our case) to O_2 followed by collapse in the solvent cage to a radical pair (phenoxy radical and O_2^-).

To learn about the potential role of trace metal ion contaminants in the apparent base-mediated 4-methylcatechol autoxidations discussed above, the rate of generation of hydroxyquinone **5** (R = Me) was determined (pH 8.0 phosphate buffer) in the absence and presence of various chelating ligands (1.0 mM): bathocuproine disulfonate (BCS), bathophenanthroline disulfonate (BPS), iminodiacetic acid (IDA), EDTA, diethylenetriaminepentaacetic acid (DTPA), and deferoxamine, and some combinations thereof. Whereas BCS, BPS, and DTPA had no significant effect on the rate ($<\pm 4\%$), IDA, EDTA, and deferoxamine led to 13, 40, and 70% *increases*, respectively, over the background rate. Stimulatory effects of EDTA and deferoxamine on autoxidation reactions have been reported[20,22] and reflect in part an ability of these ligands to fine-tune the potentials of coordinated iron or copper to those optimal for redox catalysis. The rate increases suggest that despite the use of highly purified water, the normal buffer constituents (or ligands) contain traces of transition metal ions whose catalytic ability can be augmented by ligands.

Since BCS[23] and BPS[24] "lock" copper and iron in the Cu(I) and Fe(II) states, respectively, a combination of these ligands should inhibit the autoxidation process. Surprisingly, a combination of BCS and BPS (each at 0.5 mM) did not change the background rate, and in fact, in no case did addition of any chelator effect a significant reduction of the background autoxidation rate. DTPA was found to abrogate only the increase in rate caused by added Cu(II). We are forced to conclude either that there are trace transition metals present besides copper and iron that catalyze autoxidation or that the seemingly invariant background rate reflects primarily the metal-independent autoxidation process.

Conversion of *o*-Quinones to Hydroxyquinones. Noninvolvement of Conjugate Addition of Water. The consensus mechanism for biogenesis of hydroxyquinone **5** calls for conjugate addition of water to *o*-quinone **3** followed by autoxidation of the resulting triol **4** (Scheme 1). According to this reaction sequence, the yield of **5** starting with *o*-quinone **3** should be at least as high as that starting with catechol **2** under the same autoxidation conditions. To test this supposition and to investigate the key water conjugate addition step independently, 4-methyl-1,2-benzoquinone (MeBQ, **3** R = Me) and 4-*tert*-butyl-1,2-benzoquinone (*t*-BuBQ, **3** R = *t*-Bu) were prepared. Both quinones were evaluated, because MeBQ is known to give rise to several decomposition products[25,26] that might muddle the reaction of interest, whereas for the more stable *t*-BuBQ,[26] the *tert*-butyl group could sterically hinder conjugate addition. Transformation of **3** to **5** is readily detected under autoxidation conditions by virtue of the fact that at pH > 5, hydroxyquinones **5** are generated exclusively in the form of their red anions (absorption λ_{max} at 490 nm). For both MeBQ and *t*-BuBQ, high pH conditions were needed to generate significant levels of hydroxyquinones **5** at short reactions times (pH 10 for MeBQ, and pH 13 for *t*-BuBQ), as we had observed in the attempted "one-pot" biogenesis experiment described in the Introduction. By far the main identifiable product at lower

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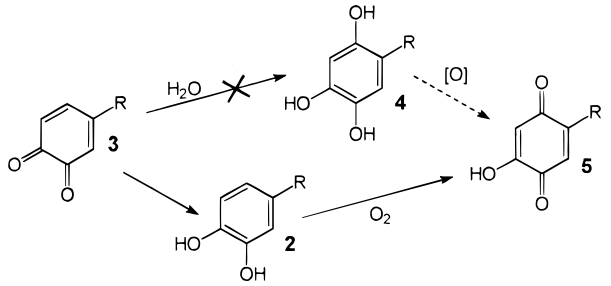
Scheme 2



pH conditions (pH 7−9 for MeBQ, and pH 10−12 for *t*-BuBQ) was that of *reduction* to the corresponding catechols **2** (*t*-BuBQ was mainly recovered at pH ≤ 9), though these were converted to hydroxyquinones **5** at longer reaction times, apparently by the same route as starting with the catechols themselves.

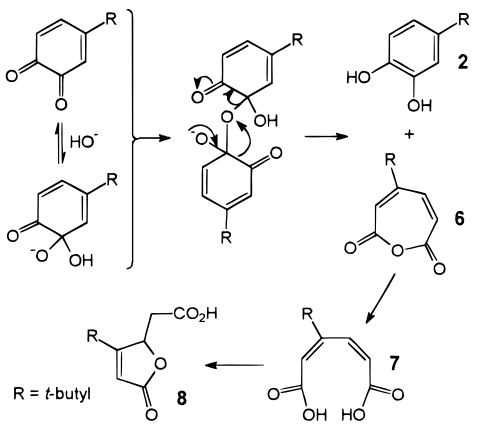
If conjugate addition of water to *o*-quinones **3** were occurring, it should be possible to isolate the corresponding triols **4** in the absence of subsequent autoxidation to **5**. In fact, bleaching of the *t*-BuBQ absorption in deaerated aqueous buffer was previously interpreted by Rinaldi and co-workers to represent addition of water to give triol **4** (R = *t*-Bu),[14] but no direct evidence for the latter (which has no visible absorption) was given. We found that substantial consumption of MeBQ occurred in aqueous CH_3-CN at pH 6.5 (phosphate buffer) in as little as 10 s, or at pH 5.6 (phthalate buffer) in 2 min, but only the respective catechols **2** and unidentified products were observed, with neither triol **4** nor hydroxyquinone **5** being detected.

To investigate the direct fate of *o*-quinones **3** at higher pH in the absence of any subsequent oxidation chemistry, the reactions of MeBQ and *t*-BuBQ were studied under argon. NMR analysis of the products from *t*-BuBQ revealed the complete absence of triol **4**: whereas only unidentified materials were seen at pH ≤ 11, the major products at pH 12−13 were 4-*tert*-butylcatechol and the corresponding muconic acid **7** (vide infra). For MeBQ (pH 10), the major identifiable product seen was again the corresponding catechol, with no trace of triol **4**. Thus, the fact that when the same high pH reactions were run *in the presence of* O_2, hydroxyquinones **5** *were* observed, it is clear that the latter cannot be arising from autoxidation of triols **4**.

The reluctance of *o*-quinones **3** to afford hydroxyquinones **5** was in marked contrast to the good yields of **5** obtained from the corresponding catechols **2** directly under the same conditions. As summarized in Scheme 2, these results indicate that the conversion of *o*-quinone **3** to hydroxyquinones **5** *does not proceed through conjugate addition of water as depicted in Scheme 1*, and instead follows a roundabout pathway involving reduction to catechol **2**, which must in turn undergo autoxidation to **5** *by a pathway independent of an o-quinone water addition step*. These data did not yet, however, provide an explanation for the apparent reduction of quinones **3** to catechols **2**.

Mechanism of Generation of Catechols from *o*-Quinones in Aqueous Base. Our finding that the main identifiable products obtained from attempted autoxidative conversion of quinones **3** were the *reduction* products **2**, might seem surprising. However, in previous model studies on transamination reactions catalyzed by TPQ models, we had periodically observed reduction of the TPQ moiety to the corresponding benzenetriol,[27] which was traced to the generation of byproducts with reducing capacity. We thus considered that generation of the catechols **2** from quinones **3** reflected reduction of the strongly oxidizing *o*-quinones by their own base-induced decomposition products.

Scheme 3



The possible generation of *o*-quinone decomposition products with reducing capability was assessed by investigating the fate of MeBQ or *t*-BuBQ exposed to aqueous base under argon. For the MeBQ reaction, although NMR spectral analysis indicated a mixture of products too complex to analyze (easily seen by counting the number of methyl singlets), it appeared reasonable that one or more such products could be reducing the quinone. NMR spectra obtained for the *t*-BuBQ reactions run at pH 10−11 were less complex, suggesting that the *tert*-butyl group was sterically inhibiting several of the "decomposition" pathways occurring in the case of MeBQ. In fact, at higher pH (12−13) for *t*-BuBQ, the extract obtained following acidic workup afforded a fairly clean NMR spectrum, indicating the presence of mainly 4-*tert*-butylcatechol, the corresponding muconic acid **7**, and a known cyclo-condensation product **8** of the latter.[28,29]

Assuming that the muconic acid could arise under the reaction conditions from hydrolysis of muconic anhydride, we thus propose the mechanism shown in Scheme 3, corresponding to a base-mediated disproportionation of the *o*-quinone, predicted to give a 1:1 mixture of catechol and muconic anhydride. The key step is a carbon shift to an electron-deficient center somewhat analogous to the Baeyer−Villiger reaction. Since electrophilic *o*-quinones are in equilibrium with their hydrates, reversible addition of a hydrate monoanion to a second *o*-quinone molecule is also likely, with the driving force for the ensuing disproportionation being reduction of the *o*-quinone.

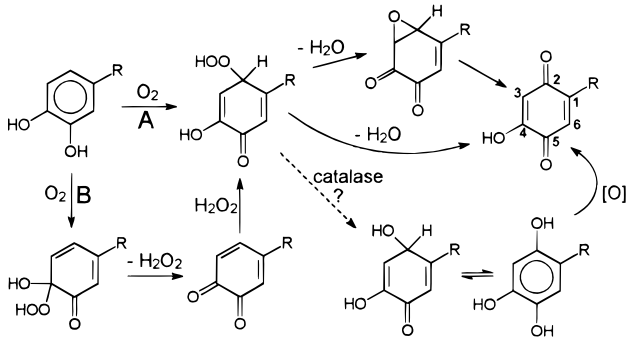
To obtain a precedent for Scheme 3, we carried out independent synthesis of 3-*tert*-butyl-*cis*,*cis*-muconic anhydride **6** and showed by NMR spectroscopy that it underwent stepwise conversion to **7** and then **8** under the basic reaction conditions in which it would be produced (see Experimental Section).

Hydroxyquinone Production from *o*-Quinones during Catechol Autoxidation. Effect of Catalase Implicates Involvement of H_2O_2. On the basis of the lack of evidence for conjugate addition of water to *o*-quinones **3**, we considered that the basic autoxidative conversion of catechols to hydroxyquinones could involve a direct four-electron oxidation by O_2 as shown in Scheme 4, path A. Charge transfer of a catechol monoanion with O_2 to give an aryloxy/superoxide radical pair, followed by spin inversion and radical combination in the cage, could lead to a 4-hydroperoxy-2,5-cyclohexadienone, which could in turn undergo dehydration either to the hydroxyquinone

(27) (a) Wang, F.; Bae, J.-Y.; Jacobson, A. R.; Lee, Y.; Sayre, L. M. *J. Org. Chem.* **1994**, *59*, 2409−2417. (b) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 11823−11828. (c) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 3096−3105.

(28) Demmin, T. R.; Rogic, M. M. *J. Org. Chem.* **1980**, *45*, 1153−1156.
(29) Gierer, J.; Imsgard, F. *Acta Chem. Scand.* **1977**, *B31*, 537−545.

Scheme 4



5 directly or to an epoxydione that would subsequently rearrange to the hydroxyquinone. Alternatively, autoxidation of the catechol could generate a 6-hydroperoxy-2,4-cyclohexadienone (Scheme 4, path B), followed by elimination of H_2O_2 that could in turn re-add to the *o*-quinone to give the same hydroperoxide isomer proposed in path A.

To distinguish between these two pathways, the catechol autoxidation reactions were run in the presence of catalase, with the aim of destroying any H_2O_2 that might be generated according to path B. Indeed, in the case of 4-*tert*-butylcatechol, the production of hydroxyquinone **5** (R = *t*-Bu) was abrogated by added catalase, and the main product was now *t*-BuBQ **3**. With 4-methylcatechol as starting reactant, formation of hydroxyquinone **5** (R = Me) was inhibited with increasing amounts of catalase, but never completely, perhaps because of the high competing reactivity of MeBQ toward H_2O_2.

The inhibition of hydroxyquinone formation by catalase appears to implicate path B, involving H_2O_2 as an obligatory intermediate. In fact, the conversion of *o*-quinone to hydroxyquinone by H_2O_2 was demonstrated to occur for dopamine quinone.[30] However, an alternative possibility for the observed inhibition that could not be excluded was that catalase was intercepting the hydroperoxide intermediate of path A. Alkyl hydroperoxides serve has oxygen atom donors for peroxidases, and catalase is known to be capable of exhibiting peroxidase activity.[31] If the hydroperoxide *did* serve as an oxygen atom donor to catalase, it would undergo reduction to the 4-hydroxy-2,5-cyclohexadienone, which would tautomerize to triol **4** as shown in the right-hand portion of Scheme 4. Since triol was not seen, only o-quinone **3** (for R = *tert*-butyl), it seemed clear that the inhibition of hydroxyquinone formation by catalase did indeed arise from its interception of H_2O_2 along path B. This further demonstrates that O_2 addition to catechols preferentially gives the 6-hydroperoxy-2,4-cyclohexadienone[32] (path B) rather than 4-hydroperoxy-2,5-cyclohexadienone (path A), probably because the latter is cross-conjugated.

Further evidence that hydroxyquinones **5** arise mainly from reaction of the *o*-quinones **3** with H_2O_2 was that for the reactions where MeBQ and *t*-BuBQ gave mainly the corresponding catechols and only traces of **5**, addition of 1−2 equivalents of H_2O_2 afforded catalase-inhibitable increases in yields of **5** that essentially matched those seen from autoxidation of the catechols themselves. Using greater amounts of H_2O_2 resulted in lower yields of **5** and instead mainly over-oxidation side-products.[25] Also, added H_2O_2 increased the yield of hydroxyquinones **5** when 4-*tert*-butylcatechol and especially 4-methylcatechol were used as substrates rather than the corresponding *o*-quinones.

Table 2. Predicted and Experimental[a] Results for ^{18}O Incorporation into Hydroxyquinone **5** from Solvent Exchange

	unlabeled	monolabeled	dilabeled	trilabeled
predicted 100% exchange[b]	67	33	0	0
experiment time 12.5 min	64 ± 1	33 ± 1	2.3 ± 0.4	0
experiment time 26 min	63 ± 3	34 ± 3	2.0 ± 1.0	0

[a] Hydroxyquinone **5** (R = Me) was dissolved in 33% $[^{18}O]H_2O$ at pH 9, maintained by NaOH, under O_2. [b] Assuming exclusive exchange at the C5 carbonyl.

Isotope Labeling Experiments. Finally, we desired direct evidence for the prediction of Scheme 4, path B, that the new oxygen introduced into the product hydroxyquinone **5** at C2 originates from O_2 via conjugate addition of H_2O_2 rather than from H_2O. Two seemingly straightforward approaches would be to carry out catechol oxidation (i) in an $^{18}OH_2$ medium with $^{16}O_2$ bubbling, in which case the C2 oxygen in the hydroxyquinone product should not incorporate the label or (ii) in an $^{16}OH_2$ medium with $^{18}O_2$ bubbling, in which case the C2 oxygen in the hydroxyquinone product should be labeled.

The former approach was implemented first. Since the TPQ hydroxyquinone is known to undergo carbonyl oxygen exchange at the electrophilic C5 position,[7,34] any solvent-based isotope labeling experiment would have to take into account such solvent exchange. In addition, the intermediate *o*-quinone would also undergo carbonyl exchange (at both positions) depending upon its lifetime and the rate of exchange. The relative degrees of exchange would be determined separately in control experiments.

4-Methylcatechol was used for the labeling experiments since its autoxidation gives a much better yield of hydroxyquinone than does 4-*tert*-butylcatechol. For an independent assessment of C5 carbonyl oxygen exchange that occurs at the product hydroxyquinone stage, **5** (R = Me) was placed in 33% ^{18}O-enriched water at pH 9 for 12.5 and 26 min. Mass spectral analysis (Table 2) indicated introduction of nearly exclusively one water-based oxygen at both time points, indicating that complete C5 oxygen exchange would occur in the hydroxyquinone product being formed during the time it existed in the pH 9 reaction mixture prior to workup. This exchange alone leads to the prediction of incorporation of one ^{18}O label according to Scheme 4, path B but two ^{18}O labels according to the water conjugate addition mechanism in Scheme 1.

In an attempt to obtain an independent assessment of solvent oxygen exchange at the *o*-quinone stage, MeBQ (**3**, R = Me) was allowed to react under argon atmosphere at pH 7.8 in aqueous-CH_3CN (using 54% ^{18}O-enriched water), for 10 s at 0 °C prior to acid quenching. As the stability of MeBQ is low even in slightly basic medium, we were unable to recover even a trace amount of quinone despite the short reaction time, but

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(31) Whitaker, J. R. Catalase and Peroxidase. In *Principles of Enzymology for Food Sciences*; Marcel Dekker: New York, NY, 1972; Chapter 24. Saunders, B. C. Peroxidases and Catalases. In *Inorganic Biochemistry*; Eichhorn, G. L., Ed.; Elsevier: Amsterdam, 1973; Chapter 28.

(32) We note that hydroperoxycyclohexadienones related to the intermediate in Scheme 4, path B, have been observed to undergo rearrangement to the corresponding muconic acids in the presence of transition metals[33] and may be responsible for production of water-soluble byproducts accompanying catechol autoxidation.

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Table 3. Predicted and Experimental Results for Mass Spectral Analysis of 2-Hydroxy-5-methyl-1,4-benzoquinone Derived from 4-Methylcatechol

	unlabeled	monolabeled	dilabeled	trilabeled
H_2O addition: 50% quinone exchange[a]	37.0	44.4	16.7	1.9
H_2O addition: 100% quinone exchange[a]	29.6	44.4	22.2	3.7
H_2O_2 addition: 50% quinone exchange[a]	55.5	38.9	5.6	0
H_2O_2 addition: 100% quinone exchange[a]	44.4	44.4	11.1	0
experimental results[b]	47 ± 2	42 ± 3	10 ± 2	0

[a] At C4, assuming 100% exchange occurs at C5 at the hydroxyquinone product stage. [b] 4-Methylcatechol was dissolved in 33% $[^{18}O]H_2O$ at pH 9, maintained by NaOH, under O_2 for 25 min, followed by acidification and extraction with CH_2Cl_2.

a substantial amount of catechol **2** from reduction of **3** byproducts of its decomposition was observed. As before, ^{1}H NMR analysis revealed the absence of both hydroxyquinone **5** and triol **4**. Conducting the reaction under argon eliminated any autoxidation of the catechol, which would then reflect the level of ^{18}O exchange that had occurred during the short lifetime of the *o*-quinone. Mass spectral analysis of the isolated 4-methylcatechol (**2**) showed the presence of three isotopomers $C_7H_8{}^{16}O_2$, $C_7H_8{}^{16}O^{18}O$, and $C_7H_8{}^{18}O_2$ with a relative % intensity ratio of 47.9:38.5:13.6, respectively. No ^{18}O incorporation was seen for 4-methylcatechol exposed to the same reaction conditions under argon, demonstrating that the exchange seen occurred prior to conversion of **3** to **2**. Focusing on the di-labeling, it can be shown that the predicted level of $^{18}O_2$ based on 50 or 100% exchange of the *o*-quinone oxygens with the 54% ^{18}O-enriched water is 7.3 and 29%, respectively. It thus appears that MeBQ undergoes between 50 and 100% exchange prior to its rapid reduction to 4-methylcatechol.

The instability of MeBQ led us to check the veracity of *o*-quinone carbonyl oxygen exchange with *t*-BuBQ, which should be recoverable from a short-term basic treatment. Indeed, a substantial amount of *t*-BuBQ was recovered from aqueous base (pH 11, under argon) even after 5 min exposure. The ^{1}H NMR spectrum of the organic extract following acid quenching showed the absence of triol **4** and corresponding hydroxyquinone **5**. Utilizing 64.5% ^{18}O-enriched water, mass spectral analysis of recovered *t*-BuBQ showed the presence of three isotopomers, $C_{10}H_{12}{}^{16}O_2$, $C_{10}H_{12}{}^{18}O^{16}O$, and $C_{10}H_{12}{}^{18}O_2$ with a relative % intensity ratio of 13.3:65.2:21.5, respectively. The predicted level of $^{18}O_2$-labeled quinone based on 50 or 100% exchange is 10 and 42%, respectively, indicating that *t*-BuBQ had undergone between 50 and 100% exchange in the 5 min time period at pH 11. The apparent slower exchange undergone by *t*-BuBQ relative to MeBQ most likely stems from its weaker electrophilicity, reflected in its greater stability.

Having demonstrated complete C5 oxygen exchange of product hydroxyquinone and greater than 50% exchange of both C4 and C5 oxygens (TPQ numbering) in the intermediate *o*-quinone, we were now in a position to interpret the $^{18}OH_2$-labeling during autoxidation of 4-methylcatechol. Incorporation of O_2-derived oxygen at C2 (Scheme 4, path B) would lead to no more ^{18}O in **5** than had been incorporated from the above exchange processes, whereas incorporation of H_2O-derived oxygen at C2 (Scheme 1) would lead to one additional ^{18}O label. Using 33% ^{18}O-enriched water, our experimental mass spectral data together with the prediction for either 50 and 100% exchange at C4 at the intermediate *o*-quinone stage (complete exchange occurs at C5 at the product stage), are given in Table 3 for the two possible mechanisms. The main differences are the prediction of relatively low unlabeled and relatively high di-labeled product in the case of the water addition as opposed to H_2O_2 addition mechanism, and the presence of trilabeled product only for the water addition mechanism. Our experimental finding of no trilabeling and levels of $^{18}O_0$- and $^{18}O_2$-labeling that are outside the range expected for water addition but in the range expected for the H_2O_2 addition mechanism, is consistent only with the autoxidation pathway shown in Scheme 4, path B. The results indicate a lifetime of the *o*-quinone intermediate that allows between 50 and 100% carbonyl oxygen exchange (but closer to the latter), consistent with that observed in the control studies.

As mentioned above, conduct of 4-methylcatechol **2** autoxidation under the conditions that maximize the yield of hydroxyquinone **5** (short reaction time), results in the recovery of some unreacted catechol that contaminates the product **5** in the initial CH_2Cl_2 extract. Thus, for the $^{18}OH_2$ experiment reported in Table 3, mass information for the recovered 4-methylcatechol was present in the same spectrum. Interestingly, the mass spectrum indicated nearly the same relative % intensities of $^{18}O_0$-, $^{18}O_1$-, and $^{18}O_2$-labeling (50:41:9) for the catechol as was seen for the hydroxyquinone. This indicates that when 4-methylcatechol is allowed to autoxidize to a point where apparently unreacted catechol is still present, this catechol is not actually unreacted *starting* material, but instead the product of reduction of the unstable *o*-quinone intermediate, that has already undergone nearly the level of ^{18}O exchange seen in the final product. Clearly, autoxidative consumption of *starting* 4-methylcatechol must be very rapid.

Despite the experimental evidence supporting conjugate addition of H_2O_2 rather than H_2O to the *o*-quinone intermediate, we wondered if the reaction could be channeled at least partly through the H_2O addition pathway by adding catalase so as to inhibit the H_2O_2-addition pathway. By using increasing amounts of catalase, the yield of hydroxyquinone **5** (R = Me) decreased, but within experimental error the relative % intensities of the various ^{18}O-labeled mass spectral peaks did not change from that shown in the last row of Table 3 (data not shown).

In an effort to further confirm the H_2O_2 addition mechanism, we carried out a labeling experiment using $^{18}O_2$ (92% enrichment) with 4-methylcatechol as starting substrate. Because of the small amount of $^{18}O_2$ available (due to the worldwide shortage), we could not conduct the experiment according to the standard protocol used above (O_2 bubbling with maintenance of pH) that maximized the yield of the hydroxyquinone product. Instead, the $^{18}O_2$ was admitted as a single bolus following vacuum degassing of the reaction mixture adjusted to pH 11 (final pH drops to 10.8), which gave the best yield of **5** in this case. The ^{1}H NMR spectrum of the organic extract following acid quenching indicated a substantial amount of hydroxyquinone **5** (~30%), no quinone **3** or triol **4,** and only a small amount of unreacted 4-methylcatechol, with the remainder being unidentified products. Mass spectral analysis of the crude product showed the presence of $^{16}O_3$- and $^{16}O_2{}^{18}O_1$-isotopomers of 2-hydroxy-5-methyl-1,4-benzoquinone **5**, whereas the recovered 4-methylcatechol exhibited no label incorporation, as expected. Although the ^{18}O incorporation into **5** (71%) was lower than predicted (92%) assuming strict observance of the Scheme 4, path B mechanism, we believe that this could reflect our inability to entirely eliminate ambient $^{16}O_2$ in the experimental setup. In any event, the high degree of labeling unambiguously confirms that O_2 (by way of H_2O_2) rather than H_2O is the source of the oxygen at C2 in hydroxyquinone **5** derived from autoxidation of 4-methylcatechol.

Conclusions and Biological Significance

In this study we investigated the mechanism of the autoxidative transformation of 4-alkylcatechols **2** to the corresponding 2-hydroxy-5-alkyl-1,2-benzoquinones **5** as a model for the known nonenzymatic biogenesis of the TPQ cofactor of copper amine oxidases. Our initial inability to generate **5** by aqueous base workup of the *o*-quinone product of copper-mediated 4-alkylphenolate **1** monooxygenation, which would ideally model the complete posttranslational modification of the active site Tyr residue, was a strong clue that the presumably straightforward biogenetic *o*-quinone water conjugate addition step (Scheme 1), demonstrated enzymatically,[7] was in fact intrinsically chemically unfavorable.

Several lines of evidence presented here demonstrate that the conjugate addition of water to *o*-quinones in aqueous base, previously assumed to rationalize the autoxidative formation of hydroxyquinones **5** from catechols **2**, does not occur or at least cannot achieve kinetic competitiveness with other reactions undergone by the intermediate *o*-quinone **3**. These are (i) the finding that anaerobic exposure of *o*-quinones **3** to aqueous base fails to give the required precursor triols **4**, (ii) the finding that the yield of **5** from aerobic autoxidation of catechol **2** is much greater than that starting with *o*-quinone **3** under the same reaction conditions, (iii) the implied involvement of H_2O_2, generated during autoxidation of **2**, in production of **5**, by observing the effect of catalase, (iv) the finding that generation of **5** from *o*-quinone **3** proceeds through pathways that convert **3** back to **2**, (v) the absence of evidence for conjugate addition of water to *o*-quinone with the use of ^{18}O-labeled solvent water, and (vi) the support for O_2-derived H_2O_2 addition to *o*-quinone with the use of ^{18}O-labeled dioxygen.

We demonstrated that in model reactions purported to involve conjugate addition of water to *o*-quinone **3**, the hydroxyquinone **5** formation which does occur is a consequence of addition *not* of water to *o*-quinone **3** but of H_2O_2 generated from autoxidation of the catechol formed in the base-induced transformations of the *o*-quinone. Thus, the generation of **5** *under model study conditions* does not reflect conjugate addition of water as believed, but instead conjugate addition of H_2O_2, giving an adduct which dehydrates to give hydroxyquinone **5** either directly or via an intermediate epoxide.

Not only is the transformation of *o*-quinone **3** to hydroxyquinone **5** important in TPQ biogenesis, but it has also been a reaction of considerable focus in studies on the autoxidation of the catecholaminergic neurotransmitter dopamine,[11] and other catechols.[12] The nonenzymatic autoxidation of dopamine to the neurotoxin 6-hydroxydopamine was presumed in the past to occur by the same series of steps (**2** → **3** → **4**, Scheme 1) as proposed for TPQ biogenesis. Our finding, consistent with the recent report by Prota and co-workers,[30] that hydroxyquinones arise most expeditiously from the reaction of the precursor *o*-quinones with H_2O_2 has broad importance to the chemical and biological properties of *o*-quinones. Although the question may be raised as to why the *o*-quinone undergoes conjugate addition of H_2O_2 and not H_2O, this can be attributed to the lower pK_a of the former (so that $[HOO^-] \gg [HO^-]$ at moderately basic pH) and the high α-effect nucleophilicity of HOO^-. It is also possible that addition of water reflects an unfavorable equilibrium, whereas the HOO^- adduct can undergo subsequent dehydration. It should be noted that the reactions of *o*-quinones with nucleophiles has taken on additional recent biological significance in studies on the generation of potential dopamine quinone-derived neurotoxins[35] and in studies on the reaction pathways undergone by estrogen quinones,[36] pathways in which soft nucleophiles (e.g., thiols) tend to add at the α- (1,6-addition) rather than β-position.

As far as TPQ biogenesis is concerned, a seminal resonance Raman study provided evidence that the C2 oxygen derives from solvent water rather than from O_2.[7] The failure of simple chemical model studies to reproduce this event suggests that the conjugate addition of water to the *o*-quinone intermediate is being aided by features of the amine oxidase active site. The obvious candidate is the tri-histidinyl-bound Cu(II) that must already be playing a key role in the initial tyrosine phenol side-chain monooxygenation. Nucleophilic attack of an active-site Cu(II)-bound hydroxide has been depicted in the latest consensus mechanisms for TPQ biogenesis[37] and appears to be consistent with conformational alternatives permitted for the tyrosine side chain. Since some previous model biogenetic conversions of 4-alkylcatechols to hydroxyquinones were conducted in the presence of Cu(II), we wondered if Cu(II) could be facilitating conjugate addition of water in these cases after all. Thus, the anaerobic reactions of MeBQ and *t*-BuBQ in aqueous base described above were repeated in the presence of added Cu(II) with or without one equivalent of added 2,2′-bipyridine to help solubilize Cu(II) and to mimic the histidine coordination of Cu(II) at the enzyme active site. The reaction outcomes were not altered (the products were mainly **2** and unidentified materials, see Experimental Section). However, if the active-site copper is indeed the mediator of water addition during TPQ biogenesis, this may take advantage of special proximity or stereoelectronic features not present in the solution model chemistry. It is also possible that active-site constituents other than the copper are responsible for mediating the conjugate addition of water.

Special features of the active-site of the copper amine oxidases have been well-recognized to contribute to certain key steps of the TPQ biogenetic mechanism, particularly the initial phenol oxygenation, but the step involving conjugate addition of water to the intermediate *o*-quinone has been assumed to be capable of occurring spontaneously. Our studies point to heretofore unappreciated evidence that the active site must be mediating this biogenetic step as well.

Experimental Section

General. Unless otherwise stated the solvents and reagents were of commercially available analytical grade quality. ^{18}O-water (96.8 atom %) from Isotec, Inc (Miamisburg, OH) and ^{18}O-oxygen (92%) from ICON Services, Inc. (Summit, NJ) were used as received. Catalase (20 000 units/mg) was from Sigma Chemical Co. (St. Louis, MO). 4-Methyl-1,2-benzoquinone and 4-*tert*-butyl-1,2-benzoquinone were prepared according to a literature procedure.[38] 2-Hydroxy-5-methyl-1,4-benzoquinone, 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone, 4-methyl-1,2,5-trihydroxybenzene, and 4-*tert*-butyl-1,2,5-trihydroxybenzene have been previously characterized.[16,27b] All reactions were carried out at 25 °C, using Millipore purified water with magnetic stirring unless

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otherwise noted, and all evaporations were carried out at reduced pressure with a rotary evaporator.

Spectroscopy. ^{1}H NMR spectra (300 MHz) were recorded on a Varian Gemini 300 instrument. In all cases, tetramethylsilane or the solvent peak served as an internal standard for reporting chemical shifts, expressed on the δ scale. Determination of yields by ^{1}H NMR was made on the basis of integrating either the methyl or *tert*-butyl signals. High-resolution mass spectra (HRMS) were obtained at 20 eV on a Kratos MS-25A instrument. Optical spectra were obtained with Perkin-Elmer model Lambda 3B or 20 spectrophotometers fitted with a water-jacketed multiple cell holder for maintaining constant temperature.

Autoxidation of 4-Alkylcatechols. Stock solutions of 4-methylcatechol in water were prepared freshly just before use. Autoxidation reactions of 4-methylcatechol (2−10 mM) were performed as a function of pH (using NaOH), reaction time, and mixed solvent system (25% aqueous MeOH or 25% aqueous CH_3CN), with or without various buffers (20−100 mM carbonate buffer, pH 8−10, and 50−100 mM phosphate buffer, pH 7.4−9), to optimize the yield of hydroxyquinone **5**. The reactions were followed spectrophotometrically in open cuvettes at 25 °C with repetitive scanning over the range 350−650 nm, or aliquots of reactions run with bubbling of O_2 at 25 °C were taken at different time intervals and scanned at 350−650 nm. Formation of hydroxyquinone **5** (R = Me) in the form of its anion was indicated by the appearance of a λ_{max} near 480 nm, whereas an absorption around 325 nm was considered to represent the product of decomposition of hydroxyquinone **5**. At a given pH, the yield of **5** (calculated from the ϵ = 2310 M^{-1} cm^{-1} at 480 nm) appeared to increase with time, but the purity decreased as indicated by the growth of A_{325} relative to A_{480}. The optimal yield/purity of **5** (R = Me) was obtained using NaOH to maintain pH = 10 in the absence of buffer, using O_2 bubbling with vigorous stirring for 10−11 min using 5.0−7.5 mM 4-methylcatechol. Reactions were quenched with dilute HCl to pH = 2, and the resulting mixture was extracted with CH_2Cl_2. The CH_2Cl_2 extract was dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. Trituration of the residue with CCl_4 selectively extracted the product hydroxyquinone **5** (R = Me) from unreacted 4-methylcatechol, affording a yield of 57% as judged by ^{1}H NMR ($CDCl_3$). Extension of the reaction time to reduce recovery of **2** resulted in reduced yields of **5**. To verify our assumption that the absorption near 325 nm arose from decomposition of **5**, we placed authentic hydroxyquinone **5** (R = Me) in the basic aqueous medium (pH 8−12) and found that A_{325} grew with time relative to A_{480}.

The effect of Cu(II) on autoxidation of 4-methylcatechol (10 mM) was investigated by following the spectral changes (350−650 nm) over time in 0.1 M pH 8 potassium phosphate buffer at 25 °C in the presence of 0−2 mM $CuSO_4$, in open-mouth 3 mL cuvettes (no O_2 bubbling). In another set of experiments, different amounts of Cu(II) (up to 5 mM) were added to aliquots of 4-methylcatechol (10 mM) pre-autoxidized for various periods of time in 0.1 M phosphate buffer, pH 8, and the spectra were immediately recorded. Spectra for a 90 min pre-autoxidation (Figure 2), representing about 30% conversion of 4-methylcatechol to **5** (R = Me), exhibit the shifts caused by addition of Cu(II). Spectra obtained at 5 and 18 h pre-autoxidation (at which times the 4-methylcatechol is completely consumed) still showed Cu(II)-induced shifts. To determine whether Cu(II) induces a shift in the spectrum of 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone (**5**, R = *t*-Bu), 0−40 μL aliquots of 0.3 M $CuSO_4$ were added to 3 mL of a solution of 1 mM **5** (R = *t*-Bu) in CH_3CN−sodium phosphate buffer (10 mM, pH 7.0) (1:9).

In another series of experiments, the autoxidation of 10 mM 4-methylcatechol in potassium phosphate buffer (0.1 M, pH 8) at 25 °C was followed spectrophotometrically (monitoring formation of hydroxyquinone **5** (R = Me) at 480 nm) in open-mouth 3 mL cuvettes, in the presence of 1.0 mM of various chelating ligands (BCS, BPS, DTPA, EDTA, deferoxamine, and iminodiacetic acid) and also in the presence of equimolar amounts (0.5 mM) of both BPS and BCS.

Autoxidation reactions of 4-*tert*-butylcatechol (**2**, R = *t*-Bu) were performed as in the case of 4-methylcatechol, with variation of buffer identity and strength, pH (9−11), reaction time, and solvent system (using up to 25% MeOH or CH_3CN). By following the reactions spectrophotometrically in open cuvettes, the yield of 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone (**5**, R = *t*-Bu) was estimated by the increase of the absorbance at 480 nm (ϵ = 2022 $M^{-1}cm^{-1}$ in water) and fell in the range of 10−25%, the latter using 3 mM **2** (R = *t*-Bu) in 167 mM pH 9 carbonate buffer. At lower basicity (pH 9), there was little initial increase in A_{480}, and instead an increase in absorbance at 380 nm was observed, corresponding to the intermediate quinone **3** (R = *t*-Bu). After about 10 min, this peak was detectable only as a shoulder on the growing peak at 480 nm, and was no longer apparent after 15 min.

Reaction of 2-*tert*-Butyl-*cis*,*cis*-muconic Anhydride in Alkaline Solution (pH 9). 2-*tert*-Butyl-*cis*,*cis*-muconic anhydride (**6**, 29 mg, 0.16 mmol), prepared as described,[28] was dissolved in 3 mL of CH_3CN, and then 27 mL of 100 mM carbonate buffer was added with stirring at 25 °C. Three 10 mL aliquots taken after 4, 16, and 33 min were immediately acidified with 4 M HCl to pH 2.1 and then extracted with EtOAc. The EtOAc extracts were dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The ^{1}H NMR spectrum ($CDCl_3$) revealed complete reaction of the muconic anhydride **6** even at the 4 min reaction time, and the presence instead of a mixture of 2-*tert*-butyl-cis,cis-muconic acid (**7**) and the corresponding lactone, 2,5-dihydro-5-oxo-3-*tert*-butylfuran-2-acetic acid (**8**), which have been previously characterized.[29] The relative yields of **7** and **8** (R = *t*-Bu) at 4, 16, and 33 min were 35 and 24%, 21 and 37%, and 6 and 57%, respectively. **6** (R = *t*-Bu): ^{1}H NMR ($CDCl_3$) δ 6.84 (dd, 1H, J = 2.0, 12.5 Hz), 6.44 (d, 1H, J = 12.6 Hz), 6.36 (d, 1H, J = 2.6 Hz), 1.20 (s, 9H). **7** (R = *t*-Bu): ^{1}H NMR ($CDCl_3$) δ 6.81 (d, 1H, J = 12 Hz), 6.0 (d, 1H, J = 12 Hz), 5.83 (s, 1H), 1.18 (s, 9H). **8** (R = *t*-Bu): ^{1}H NMR ($CDCl_3$) δ 5.91 (app s, 1H), 5.42 (dd, 1H, J = 2.9, 8.9 Hz), 3.12 (dd, 1H, J = 3.2, 16.4 Hz), 2.59 (dd, 1H, J = 9.2, 16.2 Hz), 1.27 (s, 9H).

Autoxidation of 4-*tert*-Butylcatechol in Alkaline Solution (pH 8.5 or 9.5) in the Absence or Presence of Catalase. Definition of Standard Workup. A solution of 4-*tert*-butylcatechol (83 mg, 0.5 mmol) in 100 mL of H_2O was stirred with O_2 bubbling, and NaOH solution was added to maintain the pH at either 8.5 or 9.5 (two experiments), which otherwise dropped over time. After 15 min, the pH drop ceased, and the reaction mixtures were subjected to *standard workup*: acidification to pH 2.0−2.5 by addition of 0.5 N HCl (no buffer used) or 2 N HCl (when using buffer), extraction with EtOAc, drying of the organic layers with Na_2SO_4, and evaporation of the organic solvent under reduced pressure. The ^{1}H NMR spectra ($CDCl_3$) of the residues showed that the starting 4-*tert*-butylcatechol had completely disappeared and the relative yield of hydroxyquinone **5** was 22% (pH 8.5) or 25% (pH 9.5).

The identical experiment (pH 9.5) performed in the presence of catalase (2 mg) revealed 4-*tert*-butyl-1,2-benzoquinone as a major identified product (45%), while even trace amounts of catechol **2** (R = *t*-Bu) or hydroxyquinone **5** (R = *t*-Bu) were not observed.

Autoxidation of 4-*tert*-Butylcatechol in Alkaline Solution (pH 9−9.5) with added H_2O_2. A mixture of 4-*tert*-butylcatechol (83 mg, 0.5 mmol) and H_2O_2 (110 μL, 1 mmol) was stirred with O_2 bubbling in 100 mL of H_2O maintained at pH 9.0−9.5 with NaOH. After 15 min of stirring, the reaction mixture was subjected to standard workup. The ^{1}H NMR spectrum ($CDCl_3$) of the resulting residue showed complete conversion of the starting catechol to hydroxyquinone **5** (R = *t*-Bu, 28%) and other unidentified *tert*-butyl-containing products.

Autoxidation of 4-*tert*-Butyl-1,2-benzoquinone in Aqueous Buffer (pH 9.5). 4-*tert*-Butyl-1,2-benzoquinone (82 mg, 0.5 mmol) dissolved in 5 mL of CH_3CN was diluted into 100 mL of sodium carbonate buffer (10 mM) with stirring. After 30 min, the reaction mixture was subjected to standard workup. The ^{1}H NMR spectrum ($CDCl_3$) of the residue showed a mixture of 4-*tert*-butyl-1,2-benzoquinone (20%), hydroxyquinone **5** (R = *t*-Bu, 2%), and other unidentified products.

Reaction of 4-*tert*-Butyl-1,2-benzoquinone in Aqueous Buffer (pH 9.5) with added H_2O_2. A mixture of 4-*tert*-butyl-1,2-benzoquinone (82 mg dissolved in 5 mL of CH_3CN, 0.5 mmol) and H_2O_2 (110 μL, 1 mmol) was added with stirring in air to 100 mL of sodium carbonate buffer (10 mM). After 5 min, the reaction mixture was subjected to standard workup. The ^{1}H NMR spectrum ($CDCl_3$) of the residue showed that the starting quinone **3** (R = *t*-Bu) was completely consumed and a mixture of 4-*tert*-butylcatechol (40%), hydroxyquinone **5** (R = *t*-Bu, 20%), and unidentified products were observed.

Reaction of 4-*tert*-Butyl-1,2-benzoquinone in Alkaline Solution (pH 9.0−9.5) in the Presence and Absence of Cu(II). A solution of 4-*tert*-butyl-1,2-benzoquinone (82 mg, 0.5 mmol) in 10 mL of CH_3CN was added to 90 mL of either H_2O, H_2O containing $Cu(ClO_4)_2 \cdot 6H_2O$ (19 mg, 0.05 mmol), or H_2O containing 0.05 mmol each of $Cu(ClO_4)_2 \cdot 6H_2O$ (19 mg) and 2,2′-bipyridine (8 mg). NaOH solution was then added to maintain the pH at 9.0−9.5. After 10 min stirring in air, the reaction mixtures were subjected to standard workup. The ^{1}H NMR spectra ($CDCl_3$) of the remaining residues were virtually identical, showing the presence of recovered 4-*tert*-butyl-1,2-benzoquinone (50%) and unidentified products.

Reaction of 4-*tert*-Butyl-1,2-benzoquinone in Alkaline Solution (pH 11.0, 12.0, or pH 12.5). 4-*tert*-Butyl-1,2-benzoquinone (41 mg, 0.25 mmol) dissolved in 5 mL of CH_3CN was added to 45 mL of H_2O. A solution of NaOH was added to maintain the pH at 11.0, 12.0, or 12.5 (three reactions). After 15 min of stirring, the reaction mixtures were subjected to standard workup. The ^{1}H NMR spectra ($CDCl_3$) of the residues revealed the presence of 4-*tert*-butylcatechol (40%, 50%, or 80%, respectively), hydroxyquinone **5** (R = *t*-Bu, 2% in all cases), and several unidentified compounds.

Reaction of 4-*tert*-Butyl-1,2-benzoquinone at pH 13 in the Presence or Absence of O_2. A solution of 4-*tert*-butyl-1,2-benzoquinone (41 mg, 0.25 mmol) in 20 mL of CH_3CN was diluted with 15 mL of H_2O, and adjusted to pH 13 (NaOH). After 3 min of stirring in air, the reaction mixture was subjected to standard workup. The ^{1}H NMR spectrum ($CDCl_3$) of the residue revealed the presence of hydroxyquinone **5** (R = *t*-Bu, 45%), muconic acid **7** (R = *t*-Bu, 35%), the corresponding lactone **8** (R = *t*-Bu, 10%), and unidentified compounds. In an identical reaction performed under argon, with a reaction time of 5 min, ^{1}H NMR spectral analysis of the final residue showed 4-*tert*-butylcatechol (40%), **7** (R = *t*-Bu, 35%), **8** (R = *t*-Bu, 10%), and unidentified compounds.

Oxidation of 4-Methylcatechol in Carbonate Buffer (pH 9.0) in the Presence or Absence of Catalase. A solution of 4-methylcatechol (62 mg, 0.5 mmol) in 100 mL of 50 mM sodium carbonate buffer (pH 9.0) was stirred with O_2 bubbling. After 15 min, the reaction mixture was subjected to standard workup. The ^{1}H NMR spectrum ($CDCl_3$) of the residue showed a mixture of starting 4-methylcatechol (65%), 2-hydroxy-5-methyl-1,4-benzoquinone (25%), and several unidentified compounds (totaling ∼10%). The identical experiment performed in the presence of catalase (2 mg) gave a mixture of starting 4-methylcatechol (80%), 2-hydroxy-5-methyl-1,4-benzoquinone (10%), and several unidentified products (totaling ∼10%).

Oxidation of 4-Methylcatechol at pH 8.0 with added H_2O_2. A solution (150 mL) of 4-methylcatechol (2 mM) in 100 mM sodium carbonate buffer (pH 8.0) containing either 0, 1, or 5 mM H_2O_2 (three experiments) was stirred with O_2 bubbling. The reaction mixtures were subjected to standard workup. The ^{1}H NMR spectra ($CDCl_3$) of the residues showed the presence of 2-hydroxy-5-methyl-1,4-benzoquinone in yields of 38, 55, or 66%, respectively, together with unidentified products.

Reaction of 4-Methyl-1,2-benzoquinone at pH 6.5 and pH 5.6. To a solution of 4-methyl-1,2-benzoquinone (24 mg, 0.2 mmol) in 5 mL CH_3CN was added 32 mL of pH 6.5 phosphate buffer (12.5 mM). After 10 s of stirring, the reaction was quenched by adding 2 mL of aqueous H_3PO_4 (10%,v/v) solution, and the resulting mixture was extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4), and the solvent was removed under reduced pressure. The ^{1}H NMR spectrum ($CDCl_3$) of the residue revealed a mixture of 4-methylcatechol (24%) along with other unidentified products, but not even a trace of triol **4**. When the same reaction was performed using phthalate buffer (pH 5.6) with a reaction time of 2 min, again no triol **4** was observed (we found 6% 4-methylcatechol, 47% recovered quinone **3**, and 47% unidentified products).

Reaction of 4-Methyl-1,2-benzoquinone in Aqueous Buffer (pH 8.0) in the Presence or Absence of H_2O_2 and Catalase, or Cu(II) and 2,2′-Bipyridine. A solution of 4-methyl-1,2-benzoquinone (0.2 mmol) in 10 mL of CH_3CN was added to 90 mL of 111 mM sodium phosphate buffer, pH 8.0, containing either nothing else, $CuSO_4$ and 2,2′-bipyridine (0.02 mmol each), 1 mmol H_2O_2, or 1 mmol H_2O_2 and 0.5 mg of catalase. After 6 min of stirring in air, the reaction mixtures were subjected to standard workup. Product analysis by ^{1}H NMR ($CDCl_3$) revealed a mixture of 4-methylcatechol (**2**, R = Me), 2-hydroxy-5-methyl-1,4-benzoquinone (**5**, R = Me), and several unidentified compounds: No additives: 20% **2** and 1% **5**; + (bipy)-Cu(II): 34% **2** and 2% **5**; + H_2O_2 − catalase: 11% **2** and 45% **5**; + H_2O_2 + catalase: 28% **2** and 6% **5**.

Reaction of 4-Methyl-1,2-benzoquinone in Alkaline Solution (pH 10) in the Presence or Absence of Cu(II) and 2,2′-Bipyridine under Argon. A solution of 4-methyl-1,2-benzoquinone (24 mg, 0.2 mmol) in 5 mL CH_3CN was diluted with 25 mL of water with argon bubbling, and the pH was adjusted to and maintained at pH 10 with NaOH. After 3 min, the reaction mixture subjected to standard workup. The ^{1}H NMR spectrum (acetone-d_6) of the residue revealed a mixture of 4-methylcatechol (35%) and other unidentified products, but not a trace of either triol **4** (R = Me) or hydroxyquinone **5** (R = Me). The same reaction performed in the presence of 0.1 mmol each of $Cu(ClO_4)_2 \cdot 6H_2O$ and 2,2′-bipyridine revealed a similar mixture, but with more 4-methylcatechol (45%).

Autoxidation of 4-Methylcatechol in [^{18}O]H_2O Medium (pH 9). Through a solution of 5 mM 4-methylcatechol in 3 mL of water (33% ^{18}O) adjusted to pH 9 with NaOH was bubbled O_2 with stirring at 25 °C. After 25 min and with the pH being maintained at 9 by the addition of trace volumes of concentrated NaOH, the reaction was quenched by addition of citric acid (3 mL, 0.2 M), and the resulting mixture was extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4), and the solvent was evaporated under reduced pressure. The crude product was subjected to mass spectral (EI) analysis as reported in Table 3.

Exchange of 2-Hydroxy-5-methyl-1,4-benzoquinone 5 in [^{18}O]-H_2O Medium (pH 9). Through a solution of **5** (R = Me) (0.7 mg, 5 μmol) in 3 mL of water (33% ^{18}O) adjusted to pH 9 with NaOH was bubbled O_2 with stirring at 25 °C. After either 12.5 or 26 min, the reaction was quenched by the addition of citric acid (3 mL, 0.2 M), and the resulting mixture was extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4), and the solvent was evaporated under reduced pressure. The crude product was subjected to mass spectral analysis (EI) as reported in Table 2.

Reaction of 4-Methyl-1,2-benzoquinone under Ar Atmosphere in [^{18}O]H_2O Medium. A mixture of 2.36 mL of CH_3CN, 3.57 mL of 54.5% ^{18}O-water, and 30 μL of 10 mM pH 7.8 potassium phosphate buffer was cooled to 0 °C, and argon was bubbled through for 10 min. A solution of 4-methyl-1,2 benzoquinone (35 mg, 0.287 mmol) in 0.040 mL of CH_3CN was then added to the reaction mixture with stirring under argon. The reaction was quenched after 10 s by adding 2 mL of 10% aqueous H_3PO_4 and then concentrated under reduced pressure at room temperature to a volume of 2 mL, followed by extraction with CH_2Cl_2 (3 × 10 mL). The combined CH_2Cl_2 extracts were dried over Na_2SO_4 and evaporated under reduced pressure at room temperature. The ^{1}H NMR spectrum ($CDCl_3$) of the crude residue revealed the absence of 4-methyl-1,2-benzoquinone, 4-methyl-1,2,4-benzenetriol, and 2-hydroxy-5-methyl-1,4-benzoquinone, but the presence of 4-methylcatechol (8%) along with other unidentified products. The crude product was subjected to preparative thin-layer chromatography (EtOAc−hexane, 1:1) to isolate the 4-methylcatechol, which was then subjected to mass spectral analysis: HRMS (EI) *m*/*z* (relative intensity) calcd for $C_7H_8{}^{16}O_2$, $C_7H_8{}^{16}O{}^{18}O$, and $C_7H_8{}^{18}O_2$ 124.0524, 126.0567, and 128.0610; found 124.0522 (100), 126.0564 (80.2), and 128.0606 (28.4), respectively.

Reaction of 4-*tert*-Butyl-1,2-benzoquinone under Ar Atmosphere in [^{18}O]H_2O Medium. A mixture of 1.48 mL of CH_3CN, 1.47 mL of 65.8% [^{18}O]H_2O, and 30 μL of potassium phosphate buffer (100 mM, pH 11) was degassed by bubbling with argon at 25 °C for 10 min. A solution of 4-*tert*-butyl-1,2-benzoquinone (0.020 g, 0.122 mmol) in 20 μL of CH_3CN was then added to the reaction mixture with stirring. The reaction was quenched after 5 min by adding 2 mL of aqueous 10% aqueous H_3PO_4, and the mixture was concentrated under reduced pressure at room temperature to a volume of 2 mL, followed by extraction with CH_2Cl_2 (3 × 10 mL). The combined CH_2Cl_2 extract was dried over Na_2SO_4 and evaporated under reduced pressure at room temperature. The ^{1}H NMR spectrum ($CDCl_3$) of the crude product showed the absence of 4-*tert*-butylcatechol, 4-*tert*-butyl-1,2,4-benzenetriol, and 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone, but the presence

of 4-*tert*-butyl-1,2-benzoquinone (65%) and unidentified products. The crude product was subjected to mass spectral analysis: HRMS (EI) *m*/*z* (relative intensity) calcd for $C_{10}H_{12}{}^{16}O_2$, $C_{10}H_{12}{}^{18}O^{16}O$, and $C_{10}H_{12}{}^{18}O_2$ 164.0838, 166.0881, and 168.0924; found 164.0840 (6.34), 166.0992 (31.2), and 168.1039 (10.3), respectively.

Autoxidation of 4-Methyl-catechol under $^{18}O_2$ Atmosphere. An aqueous solution of NaOH (10 mM, 24.75 mL) was deaerated by three freeze(−78 °C)−pump−thaw cycles under argon, with $^{18}O_2$ (92% enrichment) being admitted in a fourth cycle. A degassed solution of 4-methylcatechol (16 mg, 0.129 mmol) in 0.250 mL of water was then injected through the septum into the reaction system with stirring. The reaction was quenched after 7 min by injection of 0.3 mL of H_3PO_4 (85%), and the resulting mixture was extracted with CH_2Cl_2 (3 × 10 mL). The combined CH_2Cl_2 extracts were dried over Na_2SO_4, and the solvent was removed under reduced pressure. The ^{1}H NMR spectrum ($CDCl_3$) of the crude product showed the absence of 4-methyl-1,2-benzoquinone and 4-methyl-1,2,4-benzenetriol, but the presence of 2-hydroxy-5-methyl-1,4-benzoquinone (30%), a trace (<1%) of unreacted 4-methylcatechol, and other unidentified products. The crude product was subjected to mass spectral analysis: HRMS (EI) *m*/*z* (relative intensity) calcd for $C_7H_6{}^{16}O_3$ and $C_7H_6{}^{18}O^{16}O_2$, 138.0317 and 140.0360; found 138.0310 (29%) and 140.0361 (71%), respectively. A control experiment performed using $^{16}O_2$ instead of $^{18}O_2$ confirmed the absence of $C_7H_6{}^{18}O^{16}O_2$ by HRMS analysis.

Acknowledgment. We thank Drs. Durgesh V. Nadkarni and He Huang for preliminary studies on this project and the National Institutes of Health for funding of the work through Grant GM 48812.

JA992886G