Model Studies of Topaquinone-Dependent Amine Oxidases. 2. Characterization of Reaction Intermediates and Mechanism†

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Received October 21, 1994®

Abstract: The reaction of 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone (**1a**) and benzylamine in acetonitrile was studied under anaerobic conditions. Addition of benzylamine to the quinone **1a** solution generates the anionic form of the quinone (λ_{max} at 492 nm), followed by the formation of the product Schiff base **11** with λ_{max} at 368 nm and the aminoresorcinol **13** with λ_{max} at 304 nm. The rapid dissociation of the 2-hydroxyl proton was confirmed by the isolation of the amine salt **5a** in the reaction of *tert*-butylamine and **1a**. The substrate Schiff base **6a** was not spectrally detected due to its lower extinction coefficient and rapid conversion to the product Schiff base **11**. However, when α-methylbenzylamine was employed as a substrate, the formation of the substrate Schiff base **7** was detected by ^{1}H NMR and UV−vis spectroscopy. Cyclohexylamine, *n*-propylamine, and ammonia also gave the substrate Schiff bases **8**, **9**, and **10**, respectively. Both the steric bulk and the acidity of the C_α proton of the substrate are found to be factors controlling the further reaction (C_α proton abstraction). Detailed structural analysis of the substrate Schiff base was performed on **8** by 2D NMR spectroscopy, showing that **8** is in its amine salt form and has undergone nucleophilic addition at C_1, the carbonyl carbon next to the 2-hydroxyl group. UV−vis spectroscopy supports the view that **8** is not a solvent-separated ion pair (λ_{max} at 454 nm) but an intimate ion pair (λ_{max} at 352 nm) in CH_3CN. The latter λ_{max} value is very similar to λ_{max} observed for the Schiff base complex seen in bovine serum amine oxidase and different from a Schiff base complex with 4-methoxy-5-*tert*-butyl-1,2-benzoquinone **14**. The product Schiff base **11** was prepared by the reaction of the hydrochloride salt of the aminoresorcinol **13** and benzaldehyde. It has an ϵ value 10 times larger than that of the substrate Schiff base (**7**, **8**, or **9**) at 368 nm. Treatment of **11** with benzylamine yielded the aminoresorcinol **13** and the product, *N*-benzylidenebenzylamine ($PhCH{=}NCH_2Ph$). Comparison of these results to catalytic properties of the copper amine oxidases provides support for an aminotransferase mechanism from a Schiff base of topa in a localized *p*-quinone form (**B** in Scheme 1).

Introduction

Among topaquinone-dependent amine oxidases, bovine serum amine oxidase (BSAO) has been studied extensively and a transamination mechanism has been proposed on the basis of detailed kinetic studies (Scheme 1).[1-7] According to the mechanism, covalent addition of substrate to the cofactor forms a substrate Schiff base complex (**B**). A base-catalyzed proton abstraction from the substrate Schiff base complex produces a product Schiff base complex (**C**). Hydrolysis of the product Schiff base releases aldehyde and generates an aminoresorcinol form of the reduced cofactor (**D**). This reduced form of cofactor then undergoes oxidation by dioxygen to yield an iminoquinone form of the oxidized cofactor (**E**), which is converted back to the quinone form of the cofactor either by hydrolysis or directly to the substrate Schiff base complex by a transimination. Anaerobic incubation of BSAO with benzylamine has demonstrated that nitrogen is transferred from substrate to the cofactor, as required for a transamination reaction.[3] The intermediacy of a substrate Schiff base complex (λ_{max} at 340 nm) is supported by anaerobic rapid-scanning stopped-flow experiments.[1] The substrate Schiff base appears to accumulate to a significantly greater extent than the product Schiff base, based on results from reductive inactivation experiments using [^{14}C]benzylamine and [^{3}H]$NaBH_3CN$[5] and studies of pH-dependent isotope effects in the oxidation of benzylamine by BSAO.[6] It is proposed that a large change in pK_a upon cofactor reduction is the origin of the reduced stability of the product Schiff base complex, leading to a loss of electrostatic stabilization by the neighboring oxyanion which can present itself in the substrate Schiff base complex.[8]

In the preceding paper,[9] we report the first detailed study of the non-enzymatic oxidation of benzylamine by topaquinone analogs in an organic solvent (CH_3CN). It is shown that 2-hydroxy-1,4-benzoquinones bearing a bulky substituent at C_5 position (**1a**,**c**) can act as efficient turnover catalysts in the model system. The 2-hydroxyl group of the quinone is found to be an important component of its catalytic activity. It is proposed that the 2-hydroxyl group plays important roles in protecting the quinone ring from ring amination and in directing the reaction at C_1, as well as in mediating the stability and reactivity of various intermediates. The methoxy group at the C_2 position of the *p*-quinone (**2**) has the effect of directing the reaction to the C_1 carbonyl carbon, but at a greatly reduced rate relative to topa analogs. The *o*-quinone (**3**) shows reduced turnover relative to topa analogs, attributed in part to a less negative redox potential.

In this work, we examine the mechanism of interaction of 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone (**1a**) with amines in acetonitrile. Each of the intermediates postulated for the enzymatic reaction of topaquinone with amine substrate has been

† Supported by a grant from the National Institutes of Health (GM 39296).
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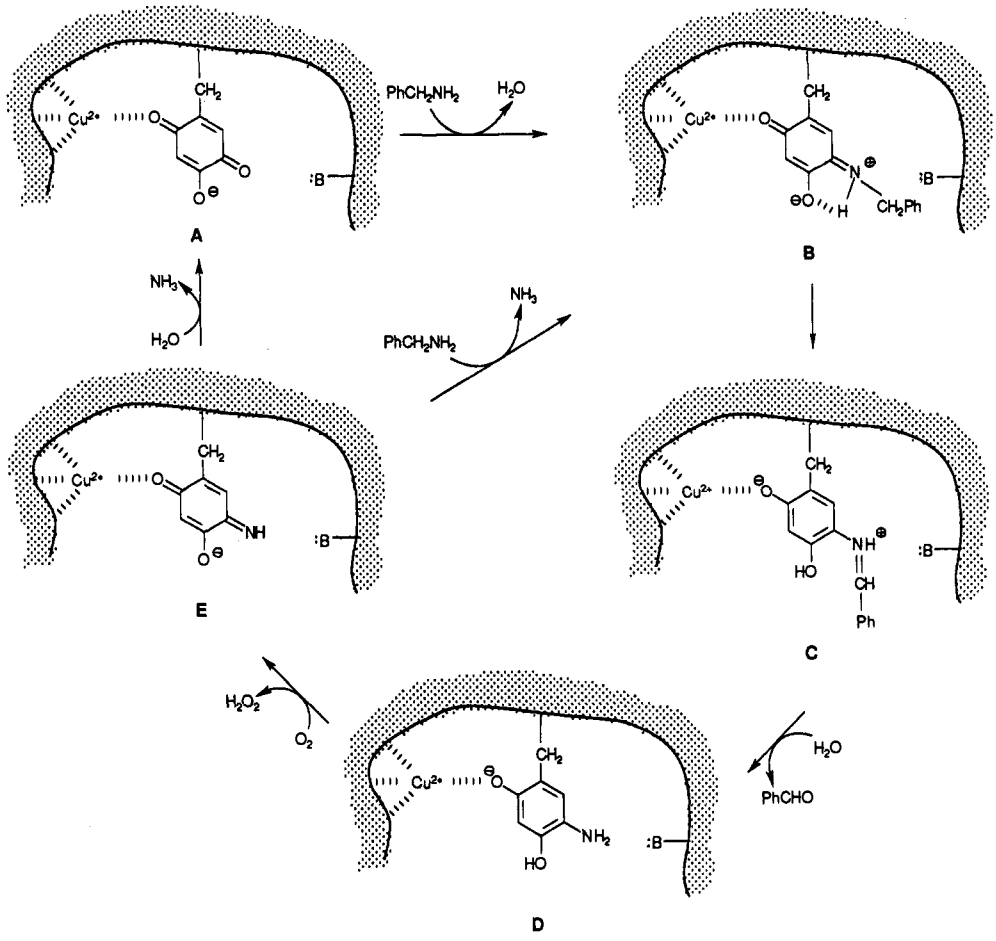
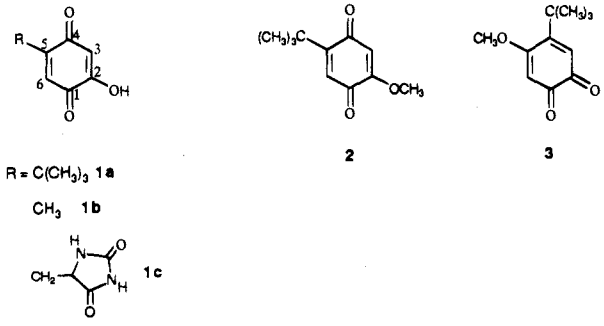
® Abstract published in *Advance ACS Abstracts*, July 1, 1995.
(1) Hartman, C.; Brzovic, P.; Klinman, J. P. *Biochemistry* **1993**, *32*, 2234.
(2) Hartmann, C.; Klinman, J. P. *Biochemistry* **1991**, *30*, 4606.
(3) Janes, S. M.; Klinman, J. P. *Biochemistry* **1991**, *30*, 4599.
(4) Grant, K. L.; Klinman, J. P. *Biochemistry* **1989**, *28*, 6597.
(5) Hartmann, C.; Klinman, J. P. *J. Biol. Chem.* **1987**, *262* (3), 962.
(6) Farnum, M.; Palcic, M.; Klinman, J. P. *Biochemistry* **1986**, *25*, 1898.
(7) Palcic, M.; Klinman, J. P. *Biochemistry* **1983**, *22*, 5957.
(8) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 7117.
(9) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 8698−8706 (preceding paper in this issue.)

0002-7863/95/1517-8707$09.00/0

Scheme 1. Proposed Mechanism for the Reaction Catalyzed by Bovine Serum Amine Oxidase (BSAO)[1]



synthesized and characterized. Anaerobic reaction of **1a** with benzylamine indicates the formation of similar intermediates, but with different rate-limiting steps. The data presented herein provide full support for a transamination reaction mechanism involving proton abstraction from the substrate Schiff base of topaquinone in a localized *p*-quinone form (cf. **B** in Scheme 1).



Experimental Section

2-Hydroxy-5-*tert*-butyl-1,4-benzoquinone (**1a**), 2-hydroxy-5-methyl-1,4-benzoquinone (**1b**), and 4-methoxy-5-*tert*-butyl-1,2-benzoquinone (**3**) were available from the previous study.[9] All the amine substrates except [1,1-$^2H_2$]benzylamine were obtained commercially and were purified by fraction distillation from CaH_2 under Ar. The ^1H NMR and ^{13}C NMR were performed on Bruker AM-400 MHz and AM-500 MHz spectrophotometers. UV−vis absorbance data were obtained on a HP8450A diode array spectrophotometer equipped with a thermostated cell holder at 25 ± 0.2 °C (path length of 1 cm). Mass spectra (MS) were obtained on a VG 70-SE or a VG ZAB 2-EQ instrument.

Synthesis of 4-*tert*-Butyl-6-nitrosoresorcinol. A solution of $NaNO_2$ (0.831 g, 0.0120 mol) in 4 mL of water was added dropwise to a stirred solution of 2 g (0.0120 mol) of 4-*tert*-butylresorcinol in 42 mL of 95% ethanol containing a 10-fold excess of HCl. The mixture was stirred at −5 to 5 °C for 15 min. The solution was concentrated to a small volume under vacuum, diluted with water, and extracted with CH_2Cl_2. The CH_2Cl_2 layer was dried over Na_2SO_4 and concentrated to give a red oil. The oil was treated with water to yield 4-*tert*-butyl-6-nitrosoresorcinol as an orange-red solid (1.638 g, 70%): ^1H NMR (DMSO-d_6) δ 1.208 (9H, s, *t*Bu), 5.595 (1H, s), 7.303 (1H, s).

Synthesis of 4-Amino-6-*tert*-butylresorcinol Hydrochloride Salt (13·HCl). Anhydrous ethanol (Aldrich) was deoxygenated by bubbling O_2-free Ar through it for 30 min. The O_2-free Ar was generated by passing Ar through an alkaline pyrogallol solution and two anhydrous EtOH solutions. A 3 mL aliquot of the O_2-free EtOH was added to the reaction vessel containing $SnCl_2{\cdot}2H_2O$ (1.1 g, 4.88 mmol) and 4-*tert*-butyl-6-nitrosoresorcinol (326.2 mg, 1.67 mmol). After the solution was stirred for 30 min at 70 °C under the stream of O_2-free Ar, a half-volume of the solvent was removed under vacuum, followed by the addition of 1.67 mmol of concentrated HCl. Removing the solvent under vacuum left a greenish white solid. This was dissolved in a small amount of H_2S-saturated 0.1 N HCl (ca. 3 mL), and H_2S gas was bubbled through it until all Sn^{2+} was converted to SnS. The solution was kept under a H_2S atmosphere overnight. SnS was removed by filtration and washed with H_2S-saturated 0.1 N HCl several times. The filtrate and the HCl solutions were combined and concentrated under O_2-free conditions to give 4-amino-6-*tert*-butylresorcinol hydrochloride salt (**13**·HCl) as a white solid, 120 mg (52%). **13**·HCl: ^1H NMR (CD_3CN) δ 1.272 (9H, s, *t*Bu), 6.717 (1H, s), 7.261 (1H, s), 7.8 (br s, exchangeable with D_2O), 8.9 (br s, exchangeable with D_2O). HRMS (EI) (M^+ − HCl) calcd for $C_{10}H_{15}NO_2$ 181.1103, obsd 181.1103. **13** can be generated *in situ* by treatment with an equimolar amount of *tert*-butylamine. **13**: ^1H NMR (CD_3CN) δ 1.284 (9H, s, *t*Bu), 6.249 (1H, s), 6.580 (1H, s).

Synthesis of the Product Schiff Base (11). A suspension of **13**·HCl (29.3 mg, 0.135 mmol) in anhydrous acetonitrile was treated with a slight excess (1.5 equiv) of benzaldehyde. The reaction mixture was stirred at room temperature for 3 h followed by addition of a small amount of anhydrous Et_2O to precipitate the product. A yellow solid was collected by centrifugation washed with anhydrous Et_2O, and dried in vacuo, 29.1 mg (80%): ^{1}H NMR (CD_3CN, poor solubility) δ 1.380 (9H, s, *t*Bu), 6.653 (1H, s), 7.471 (1H, s), 7.662 (2H, m), 7.793 (1H, m), 8.436 (2H, m), 8.923 (1H, s) (in the presence of $PhCH_2NH_2$) 1.374 (9H, s, *t*Bu), 6.392 (1H, s), 7.260 (1H, s), 7.316~7.471 (5H, m), 7.984 (2 H, m), 8.727 (1H, s); HRMS (EI) $C_{17}H_{19}NO_2$ calcd 269.1416, obsd 269.1412.

Reaction of *tert*-Butylamine and 2-Hydroxy-5-*tert*-butyl-1,4-benzoquinone (1a) under Anaerobic Conditions. 1a (31.3 mg, 0.174 mmol) was treated with a 5-fold excess of *tert*-butylamine in 10 mL of anhydrous acetonitrile under anaerobic conditions. After 1 h, a red precipitate was collected by centrifugation, washed with a small amount of hexane and dried in vacuo to give amine salt **5a** as a deep red solid, 30.5 mg (69%): HRMS (FAB) $C_{10}H_{13}O_3$ ($MH^+ - C_4H_9NH_3^+$) calcd 181.0865, obsd 181.0869; MS (FAB) 255.1 ($M^+ + 2$, 53%), 200.2 ($M^+ + 2 - C_4H_7^+$, 100%), 181.8 ($M^+ + 2 - C_4H_9NH_3^+$, 88%); ^{1}H NMR (DMSO-d_6) δ 1.210 (9H, s, *t*Bu), 1.225 (9H, s, *t*Bu), 5.045 (1H, s, exchangeable with D_2O), 6.012 (1H, s); ^{1}H NMR (CD_3CN) δ 1.237 (9H, s, *t*Bu), 1.252 (9H, s, *t*Bu), 5.430 (1H, exchangeable with D_2O), 6.273 (1H, s); ^{13}C NMR (DMSO-d_6) δ 27.1, 29.7, 34.9, 50.4, 105.6, 126.2, 158.8, 169.5, 184.4, 189.9.

Reaction of *tert*-Butylamine and 2-Hydroxy-5-methyl-1,4-benzoquinone (1b) under Anaerobic Conditions. 1b (50 mg, 0.362 mmol) was treated with a 5-fold excess of *tert*-butylamine in 5 mL of ethyl acetate under anaerobic conditions. After 5 min, a dark red precipitate was collected by centrifugation, washed with ethylacetate, and dried in vacuo to yield amine salt **5b** as a deep red solid, 77.4 mg (quantitative): ^{1}H NMR (DMSO-d_6) δ 1.229 (9H, s, *t*Bu), 1.855 (3H, d, J = 1.5 Hz, CH_3), 5.024 (1H, s, exchangeable with D_2O), 6.122 (1H, q, J = 1.5 Hz), 7.70 (3H, br s, exchangeable, NH_3).

Reaction of Cyclohexylamine and 2-Hydroxy-5-*tert*-butyl-1,4-benzoquinone (1a) under Anaerobic Conditions. 1a (30.3 mg, 0.168 mmol) was treated with a 5-fold excess of cyclohexylamine in 10 mL of anhydrous acetonitrile under anaerobic conditions. After 1 h, an orange-red precipitate was collected by centrifugation, washed with a small amount of hexane, and dried in vacuo to give the amine salt of the imine **8** as an orange-red solid, 52.3 mg (86%): MS (FAB) ($M^+ + 2 - C_4H_7^+$) 307.1 (31%), ($M^+ + 2 - C_6H_{12}N$) 264.2 (92%); ^{1}H NMR ($CDCl_3$) δ 1.02~1.852 (m, 20 H, $-CH(CH_2)_5 \times 2$), 1.272 (9H, s, *t*Bu), 2.612 (1H, m, $CH(CH_2)_5$), 3.594 (br s, exchangeable with D_2O), 3.847 (1H, m, $=NCH(CH_2)_5$), 5.821 (1H, s), 6.838 (1H, s); ^{1}H NMR (CD_3OD) δ 1.10~1.974 (m, 20 H, $-CH(CH_2)_5 \times 2$), 1.293 (9H, s, *t*Bu), 2.970 (1H, m, $CH(CH_2)_5$), 3.819 (1H, m, $=NCH(CH_2)_5$), 6.781 (1H, s); ^{13}C NMR ($CDCl_3$) δ 24.1, 25.1, 25.4, 25.6, 29.6, 34.0, 35.7, 36.6, 50.4, 58.9, 106.3, 116.6, 151.6, 153.2, 158.0, 188.1; ^{13}C NMR (CD_3OD) δ 25.6, 25.8, 26.1, 26.6, 30.4, 32.7, 35.1, 36.6, 51.5, 60.9, 107.0 (t, small intensity), 117.8, 154.0, 158.8, 175.2, 189.0.

NMR Assignment of the Substrate Schiff Base (8). All NMR spectra were acquired on a Bruker AM-500 spectrometer fitted with an inverse $^1H-^{13}C$ probe operating at a proton frequency of 500.13 MHz. ^{1}H chemical shifts were relative to a residual $CHCl_3$ signal set to 7.250 ppm. ^{13}C chemical shifts were relative to the ^{13}C signal of $CDCl_3$ set to 77.0 ppm. The long-range inverse proton carbon correlation (HMBC) spectra were processed in magnitude mode; 512 t_1 increments of 2K data points were corrected.[10] A delay of 60 ms was used in the pulse sequence to allow for the evolution of long-range couplings. The NOESY spectrum was obtained using 100 ms mixing time. All 2D spectra were zero fitted to twice their size in both dimensions prior to processing.

Reaction of *n*-Propylamine and 2-Hydroxy-5-*tert*-butyl-1,4-benzoquinone (1a). 1a (30.3 mg, 0.168 mmol) was treated with a 5-fold excess of *n*-propylamine in 5 mL of degassed anhydrous acetonitrile. After 1 h, an orange-red precipitate was collected by centrifugation, washed with a small amount of anhydrous acetonitrile, and dried in vacuo to give the imine **9** as a red solid, 31.2 mg (66%): ^{1}H NMR ($CDCl_3$) δ 0.878 (3H, t, J = 7.2 Hz, $CH_2CH_2CH_3$), 0.996 (3H, t, J = 7.1 Hz, $=NCH_2CH_2CH_3$), 1.271 (9H, s, *t*Bu), 1.439 (2H, m, $CH_2CH_2CH_3$), 1.785 (2H, m, $=NCH_2CH_2CH_3$), 2.627 (2H, t, J = 7.2 Hz, $CH_2CH_2CH_3$), 3.729 (2H, t, J = 7.1 Hz, $=NCH_2CH_2CH_3$), 5.836 (1H, s), 6.856 (1H, s).

Reaction of Ammonia and 2-Hydroxy-5-*tert*-butyl-1,4-benzoquinone (1a). 1 (50 mg, 0.278 mmol) was dissolved in 5 mL of anhydrous acetonitrile. NH_3 was bubbled through it. The ammonia salt of the quinone was first precipitated as a dark red solid (ca. 5 min), 34.2 mg (63%): ^{1}H NMR (DMSO-d_6) δ 1.217 (9H, s, *t*Bu), 5.667 (1H, br s), 6.353 (1H, s). With further stirring of the solution, the salt dissolved and the color of the reaction mixture turned to orange (λ_{max} 450 nm), indicating the formation of the iminoquinone **10**. It was not possible to isolate iminoquinone **10** since it was highly soluble and unstable during the isolation steps.

Reaction of *n*-Propylamine and 4-Methoxy-5-*tert*-butyl-1,2-benzoquinone (3) under Anaerobic Conditions. 3 (4.18 mg, 0.022 mmol) was dissolved in 0.5 mL of CD_3CN in a NMR tube and was degassed by flushing Ar through it for 30 min. *n*-Propylamine (2 mol equiv) was added to the tube, and the reaction was monitored by ^{1}H NMR. At 4 min, the formation of the product Schiff base **14** was detected along with that of the aminophenol **16**[9] and the product ($CH_3CH_2CH_2N=CHCH_2CH_3$). **14**: δ 1.388 (9H, s, *t*Bu), 3.858 (3H, s, OCH_3), 7.145 (1H, s), 7.505 (1H, s), 7.640 (1H, t, J = 5.2 Hz). The ethyl protons of **14** were not assigned due to their overlap with signals of the product ($CH_3CH_2CH_2N=CHCH_2CH_3$).

Reaction of α-Methylbenzylamine and 4-Methoxy-5-*tert*-butyl-1,2-benzoquinone (3) under Anaerobic Conditions. 3 (4.33 mg, 0.022 mmol) was dissolved in 0.5 mL of CD_3CN in a NMR tube and was degassed by flushing Ar through it for 30 min. α-Methylbenzylamine (2 mol equiv) was added to the tube, and the reaction was monitored by ^{1}H NMR. At 1 h, the formation the product Schiff base **15** was detected. **15**: δ 1.320 (9H, s, *t*Bu), 2.333 (3H, s, CH_3), 3.783 (3H, s, OCH_3), 6.455 (1H, s), 6.674 (1H, s), 7.35~7.48 (m), 8.03~8.05 (m). Although the reaction was slow, the formation of the aminophenol **16**[9] and the product [$Ph(CH_3)C=NCH(CH_3)Ph$] was detected overnight.

Reaction of *tert*-Butylamine and 4-Methoxy-5-*tert*-butyl-1,2-benzoquinone (3) under Anaerobic Conditions. 3 (50.2 mg, 0.26 mmol) was dissolved in 5 mL of CD_3CN. To the quinone solution was added 1 mL of *tert*-butylamine, and the reaction mixture was stirred at 40 °C under Ar. The reaction was monitored by ^{1}H NMR and UV−vis spectroscopy. At 5.3 h, the signals corresponding to the substrate Schiff base **17** were detected (65% conversion). **17**: δ 1.281 (9H, s, *t*Bu), 1.400 (9H, s, $=NC(CH_3)_3$), 3.792 (3H, s, OCH_3), 5.689 (1H, s), 6.713 (1H, s). **17** was easily hydrolyzed to **3** by addition of aqueous acid. UV−vis absorbance data of **17** are shown in Figure 4. The concentration of the UV−vis sample was determined by the absorbance of the regenerated quinone **3** at 436 nm (ϵ 1600) at pH 1.0. The concentrations of **17** and **3** in the sample were calculated from the ratio of the integration of their methoxy protons by ^{1}H NMR spectroscopy (**17**, 65%; **3**, 35%). The absorption spectrum of **17** was obtained by subtraction of that of **3** (4.77×10^{-5} M) from that of the mixture.

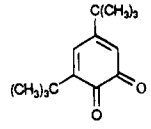
Reaction of Benzylamine and 2-Hydroxy-5-*tert*-butyl-1,4-benzoquinone (1a) under Anaerobic Conditions. A 3 mL of benzylamine solution (in CH_3CN) in a quartz cell with a 8 cm graded seal tube which was tightly closed wtih a septum rubber cap was degassed by bubbling oxygen-free Ar gas through it for 20 min. A 60 μL aliquot of the quinone stock solution (2.0×10^{-2} M in CH_3CN) was added to initiate the reaction ([**1a**] = 3.9×10^{-4} M, [amine] = $(1.93-7.54) \times 10^{-2}$ M), and the reaction was monitored spectrophotometrically.

Synthesis of [1,1-$^2H_2$]Benzylamine. [1,1-$^2H_2$]Benzylamine was prepared by reduction of benzonitrile (1.31 g, 0.0127 mol, Aldrich) with 1 mol equiv of lithium aluminum deuteride (98% D, ICN). After the addition of benzonitrile to lithium aluminum deuteride, a sufficient amount of H_2O was added dropwise, the pH of the aqueous layer was brought up to an alkaline value (>13), and the product was extracted with ether. The ether layer was dried over Na_2SO_4 and ether was removed by evaporation. The residue was purified by distillation. No undeuterated material was detected by ^{1}H NMR.

(10) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093.

Results and Discussion

Although the 4-methoxy *o*-quinone **3** does possess catalytic activity in the oxidation of benzylamine,[9] the role of topaquinone analogs in catalysis appears to involve a fundamentally different mechanism from that of *o*-quinones. For the reaction of 3,5-di-*tert*-butyl-1,2-benzoquinone (**4**) and *sec*-alkyl primary amines,



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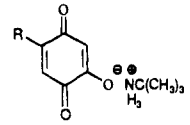
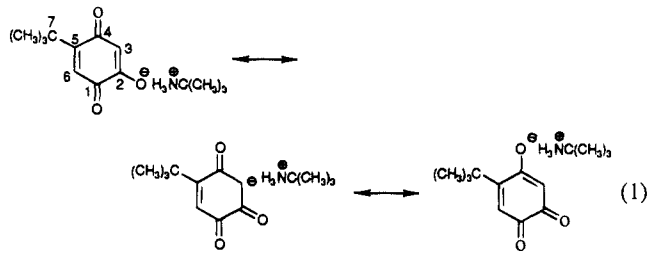
a transamination mechanism involving a non-base-catalyzed spontaneous rearrangement of the substrate Schiff base to the product Schiff base intermediate has been proposed.[11,12] By contrast, extensive studies of the mechanism of topa-containing enzymes have implicated a base-catalyzed C−H bond cleavage for the conversion of the substrate Schiff base complex (**B**) to the product Schiff base complex (**C**)[2] (Scheme 1). In order to carry out a detailed comparison of topaquinone analogs to *o*-quinones (**3** and **4**) and the enzyme system, the reaction intermediates and the products of topaquinone analog **1a** were prepared and characterized.

Amine Salt. The formation of an anionic form of **1a** by addition of an amine has been indicated from the pK_a value of its 2-hydroxyl group (4.1 in an aqueous solution) and a large red shift in λ_{max}.[8,9] When **1a** was treated with a 5-fold excess of *tert*-butylamine, a dark red precipitate was formed. The precipitate proved to be the amine salt **5a** by ^{1}H and ^{13}C NMR



R = C(CH$_3$)$_3$ **5a**
= CH$_3$ **5b**

and mass spectrometry. The ^{1}H NMR spectrum of **5a** in DMSO-d_6 showed two 1H singlets at δ 5.045 and 6.012, which were assigned to the ring protons at C_3 and C_6, respectively. The ^{13}C NMR spectrum of **5a** showed two signals with the typical chemical shift of the quinonoid carbonyl carbon. **1b** yielded a similar amine salt **5b** quantitatively. It is clear that the deprotonation of the 2-hydroxyl group by an amine precedes the amine addition reaction. UV−vis spectra of the amine salts in CH_3CN showed λ_{max} at 264, 372 and 492 nm; the red-shifted 492 nm band indicates a resonance stabilized anion, involving delocalization of electrons from the 2-oxyanion through the C_4 carbonyl group (eq 1). This deprotonation directs the nucleo-



(1)

philic addition of an amine to the C_1 carbonyl carbon, next to the hydroxyl group (see below). The steric bulk of *tert*-

(11) Klein, R. F. X.; Bargas, L. M.; Horak, V. *J. Org. Chem.* **1988**, *53*, 5994.
(12) Corey, E. J.; Achiwa, K. *J. Am. Chem. Soc.* **1969**, *91*, 1429.
(13) Reichardt, C. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 98.

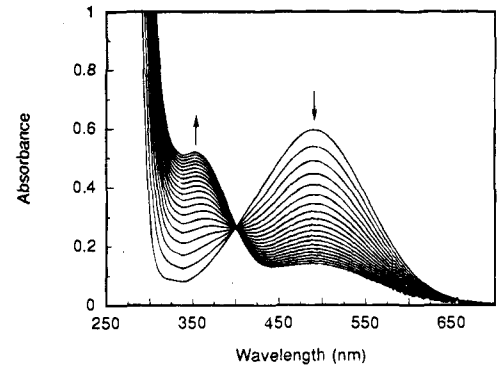
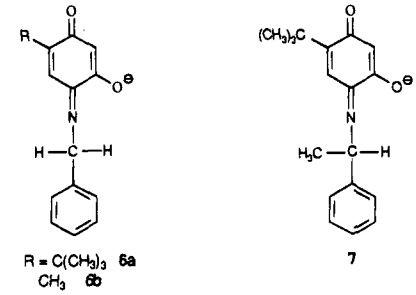


Figure 1. UV−vis spectra of the reaction of **1a** and α-methylbenzylamine in CH_3CN as a function of time: [**1a**] = 3.90 × 10^{-4} M, [α-methylbenzylamine] = 3.78 × 10^{-2} M.

butylamine appears to decrease its nucleophilicity sufficiently such that only the amine salt is obtained.

Substrate Schiff Base. While the formation of the dimer of the substrate Schiff base **6b** indicates the intermidacy of a substrate Schiff base in the catalytic oxidation of benzylamine by topaquinone analogs,[9] attempts to isolate or detect (by spectroscopic methods) **6a** in the reaction of **1a** and benzylamine proved unsuccessful. Presumably this is due to the rapid conversion of **6a** to a product Schiff base (**11**, discussed below). However, when α-methylbenzylamine was employed as a substrate, the formation of the substrate Schiff base **7** was detected by ^{1}H NMR and UV−vis spectroscopy. It was not possible to isolate **7** since it was highly soluble and unstable during the isolation steps.

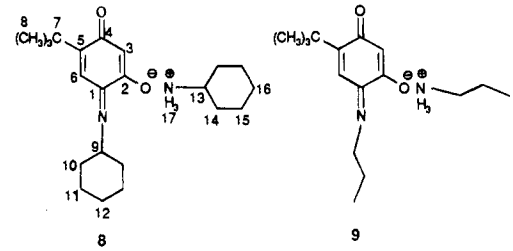


R = C(CH$_3$)$_3$ **6a**
CH$_3$ **6b**

7

The ^{1}H NMR of **7** in CD_3CN was characterized by a 3H doublet (J = 6.5 Hz) at δ 1.547 for α-methyl, a 1H quartet (J = 6.5 Hz) at δ 5.345 for the α-proton of the imino group, two 1H singlets at δ 5.738 and 7.103 for the ring protons, and a 9H singlet at δ 1.254 for *tert*-butyl protons. In Figure 1, the UV−vis spectral monitoring of the reaction of **1a** and α-methylbenzylamine is shown. At 10 s, the characteristic absorption band of the anionic form of the quinone at λ_{max} 488 nm was seen. During the next 200 min, this band disappeared with the appearance of a new band at λ_{max} 352 nm.

Similar substrate Schiff bases **8** and **9**, which showed identical spectra with λ_{max} at 352 nm, were isolated from the reaction of **1a** with cyclohexylamine (86% yield) and *n*-propylamine (66% yield), respectively. The substrate Schiff bases of the *sec*-alkyl



8 **9**

primary amines **7** and **8** were stable, and no spectral changes were observed even in the presence of the amine at room temperature for a few days or in boiling solvent (CH_3CN). In the case of **9**, a very slow change of the spectrum was observed in the presence of the amine. Since the reaction was very slow, it was not possible to identify the product. The color of the solution turned blue-purple over a few days, indicating the formation of dyes which were derived from the aminoresorcinol.[8,11] These results suggest that both steric bulk and the acidity of the C_1 proton of amines are the key factors which control the further reaction.

Detailed structural analysis of the substrate Schiff base was performed on **8** using 1D and 2D NMR techniques. The ^{13}C data of **8** in $CDCl_3$ showed a typical carbonyl carbon signal at 188.1 ppm, which was assigned to C_4. The ^1H NMR spectrum showed two ring protons with δ 5.821 and 6.838 chemical shifts. The proton at 5.821 ppm was assigned to H_3 on the basis of its upfield chemical shift and its absence in CD_3OD due to the proton−deuterium exchange (see the Experimental Section) as seen in **1b**[9] and **1c**$^-$Na$^+$.[8] There were two 1H multiplets at 2.612 (amine salt) and 3.847 ppm (imine) for two cyclohexyl α-protons. The signal at 3.847 ppm was assigned to the cyclohexyl α-proton on the imine moiety because of its downfield chemical shift of 1.235 ppm, reflecting the electronic environment adjacent to the quinone imine ring. To assign the carbons, we performed a multiple-bond heteronuclear correlation experiment (HMBC). Figure 2 (top, middle) shows two 2D cross sections with the ^1H and ^{13}C 1D spectra plotted along the horizontal and vertical axes, respectively. The imine carbon (C_1) was identified as the signal at 151.6 ppm by its strong $^3J_{C-H}$ correlations with H_3 (Figure 2, top) and H_9 (Figure 2, middle). In Figure 2 (top), the strong $^3J_{C-H}$ correlations are shown between H_6 (6.838 ppm) and C_4 (188.1 ppm), C_2 (158.0 ppm), and C_7 (35.7 ppm), and between H_3 (5.821 ppm) with C_1 (151.6 ppm) and C_5 (153.2 ppm). In addition, a small $^2J_{C-H}$ correlation between C_2 (158.0 ppm) and H_3 (5.821 ppm) was observed. A ^{13}C signal at 106.3 ppm was assigned to C_3, since in CD_3OD it became a triplet with small intensity due to the proton−deuterium exchange of H_3 (see the Experimental Section). Thus, the signal at 116.6 ppm was assigned to C_6. In a NOESY (nuclear Overhauser enhancement spectroscopy) experiment, positive cross peaks (NOE effect) between H_6 and H_9, supporting the substitution at C_1 with the cyclohexylimino group, were observed (Figure 2, bottom). Additionally, negative cross peaks indicating exchangeable protons with the amine (ν < 10 s) were detected between H_{17} and H_3. This corresponds to the observation that H_3 exchanges with D in CD_3OD or on addition of D_3O^+, as in the case of the quinone.[8,9] In addition, negative cross peaks were observed between the cyclohexyl protons within the imine and in the salt linkage. In Figure 2 (bottom), the negative cross peaks between H_9 and H_{13} are shown. This means that the two cyclohexyl groups are exchanging in the NMR sample. The integration of diagonal and exchange peaks indicates that the exchange rate is approximately 0.06 s^{-1}. We have not determined whether this exchange reaction is intermolecular or intramolecular.

From the detailed NMR data described above, we propose that **8** is the cyclohexylamine salt of the Schiff base. UV−vis spectroscopy was pursued, in an effort to examine the structure of the ion pair, in particular whether this is an intimate (M^+X^-) or a solvent-separated ($M^+|\ |X^-$) species. Figure 3 shows the effect of solvent on the UV−vis spectra of **8**, utilizing a series of solvents, which varies from apolar/aprotic (CH_2Cl_2) to polar/protic (H_2O) (cf. ref 13 for a discussion of scale of solvent polarity). In CH_2Cl_2, **8** indicates a λ_{max} at 352 nm (**A** in Figure 3, top). Changing the solvent to a polar aprotic one (CH_3CN) results in a slight difference of the spectrum (**B** in Figure 3, top). On the other hand, the use of a polar protic solvent (MeOH) generates a new band at λ_{max} 454 nm with a corresponding loss of the 352 nm band (**C** in Figure 3, top). In water (pH 10.0), the band at 352 nm has completely disappeared and **8** absorbs at 454 nm (**D** in Figure 3, top). These shifts in λ_{max} reflect the effect of solvent on **8** and its electronically excited state. We propose that **8** undergoes a shift from an intimate ion pair (M^+X^-) with λ_{max} 352 nm in an apolar/aprotic solvent to a fully ionized ion pair ($M^+|\ |X^-$) with λ_{max} at 454 nm in polar/protic solvents. The nature of the 100 nm red shift of λ_{max} is presumably the resonance delocalization of the anionic species as proposed for the amine salt (cf. eq 1). This view is supported by the observation that the ^{13}C signal of the imino carbon (C_1) of **8** in CD_3OD is shifted downfield (23.6 ppm) and has a chemical shift similar to that of the imino carbon of

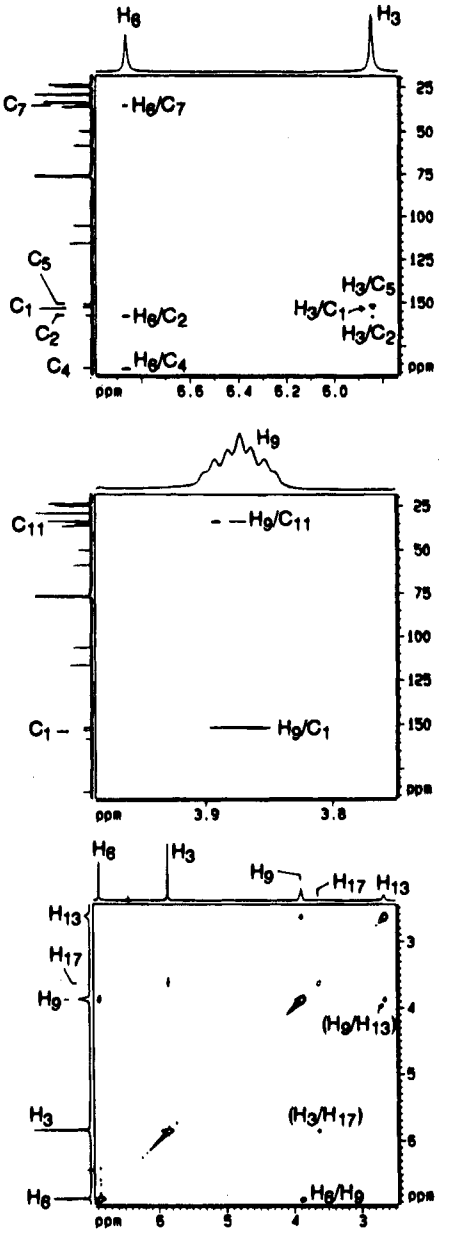
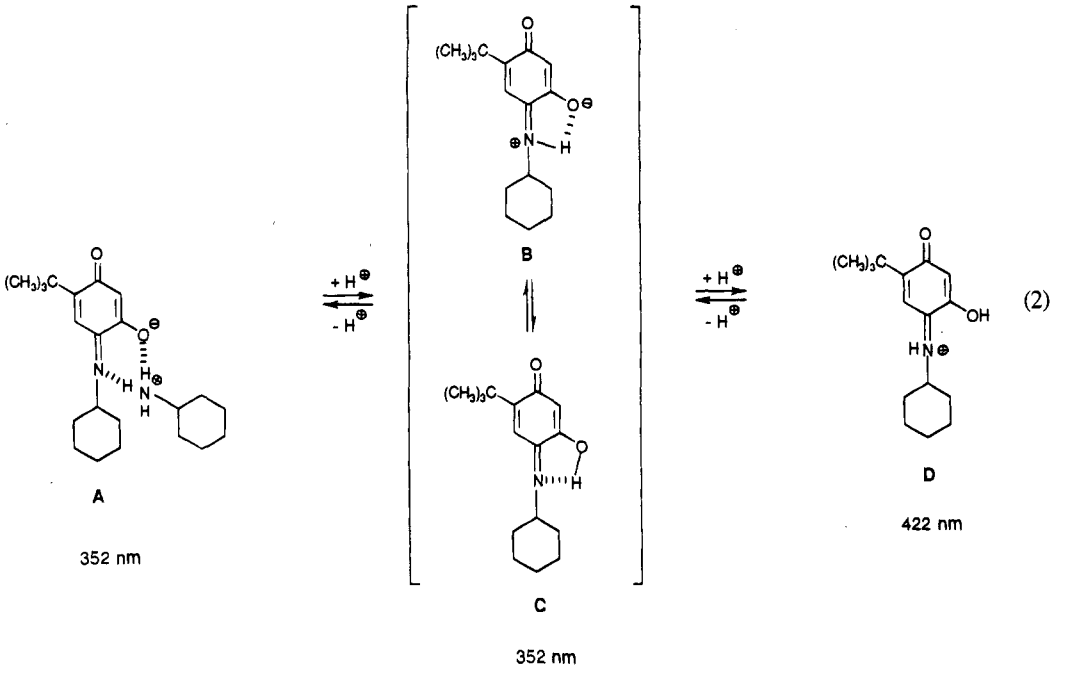


Figure 2. 2D NMR data of **8** in $CDCl_3$. (top) Parts of the HMBC spectrum of **8** covering correlations between the ring protons (H_3 and H_6) and carbons. (middle) Parts of HMBC spectrum of **8** covering correlations between the α-proton of the cyclohexylimino group (H_9) and carbons. (bottom) Parts of NOESY spectrum of **8** covering the ring protons (H_3 and H_6) and cyclohexyl α-protons (H_9 and H_{13}).



the (*tert*-butylimino)quinone derivative of PQQ (*o*-quinone).[14] It should be noted that such a resonance effect could be detected in CH_3CN, a polar aprotic solvent, in the case of the amine salt of quinone **5a** or **5b**. This implies that ion pairing of the amine salt of the imine (**7**, **8**, or **9**) is stronger than that of the amine salt of the quinone (**5a** or **5b**). For the amine salt of the imine, a hydrogen-bonding interaction to localize electrons (**A** in eq 2) is expected to be stronger than that for the amine salt of the quinone. Alternatively, in acetonitrile, **8** could exist as a neutral form where electrons are localized by the hydrogen-bonding interaction (**C** in eq 2). The pK_a of the 2-hydroxyl proton is expected to be elevated by this interaction. Although we cannot rule out this possibility, we prefer the intimate ion pair structure (**A** in eq 2) to account for the fact that a large excess of amine (ca. 100-fold) had no effect on the UV−vis property of **8** in acetonitrile.

When **8** was titrated with 0.1 N HCl in acetonitrile, a biphasic spectral change was observed (Figure 3, bottom). A small change was seen during the first titration with HCl up to 1.66 mol equiv (**A** to **B** in Figure 3, bottom). Further addition of aqueous HCl up to 2.1 mol equiv caused a pronounced 24 nm red shift of the λ_{max} at 274 to 298 nm and a 70 nm red shift of the λ_{max} at 352 to 422 nm (**B** to **C** in Figure 3, bottom). The first titration is assigned to the protonation of either the imine nitrogen (**B**) or 2-oxyanion (**C**) and the second titration to yield **D** (eq 2). The first protonation does not affect the spectroscopic property, significantly, suggesting similar electronic environments in structures **A** and **B** or **C**. In structures **B** or **C**, a hydrogen-bonding interaction which localizes electrons is expected.

The present results provide support for the previously proposed structure of the substrate Schiff base complex of the enzyme (**B** in Scheme 1). The observation of a λ_{max} at 340 nm for this species at the enzyme active site indicates a local electrostatic interaction which maintains the anionic electron density primarily in the 2-position. This could occur either through hydrogen bonding to the neighboring Schiff base in its protonated form (as shown in Scheme 1) or, alternatively, by interaction with an active site residue.

(14) Itoh, S.; Mure, M.; Ogino, M.; Ohshiro, Y. *J. Org. Chem.* **1991**, *56*, 6857. ^{13}C NMR (in DMSO-d_6): δ 171.74 (C=N), 183.19 (C=O).

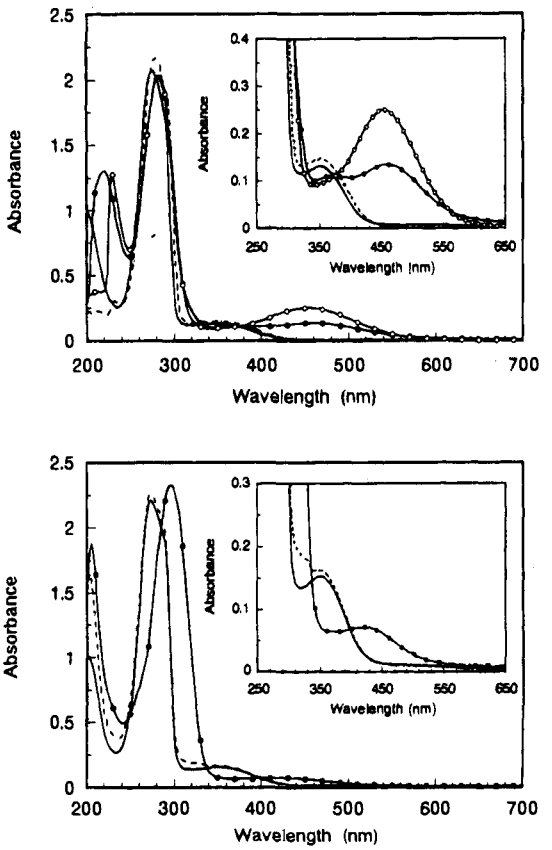


Figure 3. (top) Solvent effect for the UV−vis spectra of **8** ([**8**] = 1.0 × 10^{-4} M): **A** (···) CH_2Cl_2, **B** (—) CH_3CN, **C** (●) MeOH, **D** (○) pH 9.9 0.1 M carbonate buffer. (insertion) Expanded spectra. (bottom) Titration of **8** with acid in CH_3CN: **A** (—) [**8**] = 1.0 × 10^{-4} M, **B** (- - -) addition of [HCl] = 1.66 × 10^{-4} M, **C** (●) addition of [HCl] = 3.65 × 10^{-4} M. (insertion) Expanded spectra.

In the preceding paper,[9] we reported that the methoxy *o*-quinone **3** showed activity relatively close to that of topaquinone **1a** in anhydrous acetonitrile for the oxidation of benzylamine. However, no substrate Schiff base was detected in the reaction of **3** with α-methylbenzylamine or *n*-propylamine and only spectroscopic evidence of the corresponding product Schiff bases (**14** and **15**, respectively) was observed (see the

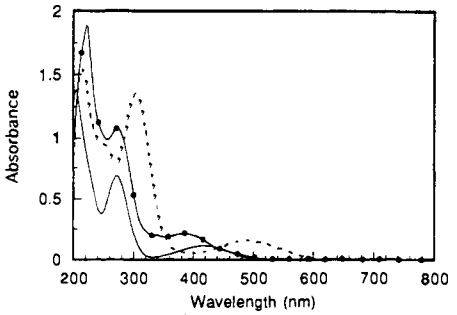
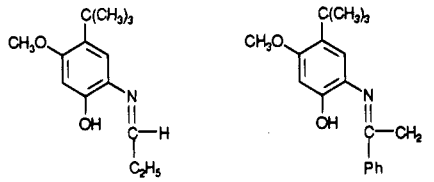


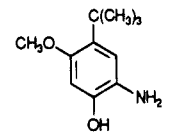
Figure 4. UV−vis spectra of **17** in CH_3CN: **A** (●) [**17**] = 9.0 × 10^{-5} M, **B** (- - -) addition of HCl, **C** (—) [**3**] = 9.0 × 10^{-5} M.

Experimental Section). In the reaction with *tert*-butylamine, we could detect the formation of the substrate Schiff base (**17**) *in situ*. It was not possible to isolate **17** since it was highly



14

15



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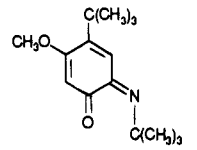
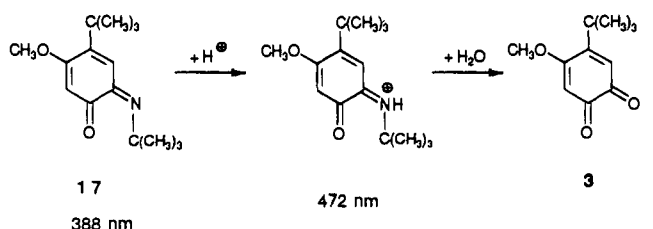


17

soluble and easily regenerated **3** during the isolation steps. The ^1H NMR of **17** in CD_3CN was characterized by two 9H singlets at δ 1.281 and 1.400, a 3H singlet at δ 3.792 for the methoxy protons, and two 1H singlets at δ 5.689 and 6.713 for the ring protons. The signal at 1.400 ppm was assigned to the *tert*-butyl protons on the imine moiety because of its downfield chemical shift, reflecting the electronic environment, adjacent to the quinone imine ring. The signal at δ 6.713 was assigned to the proton next to the imine moiety due to its downfield chemical shift of 1.026 ppm. The signal at 5.689 ppm was assigned as H_3 and the one at 6.713 ppm was assigned as H_6, since H_3 was not significantly affected (Δ0.074 ppm upfield shift) but H_6 was greatly influenced (Δ0.507 ppm downfield shift) by the imino moiety. As shown in Figure 4, **17** has λ_{max} at 274[15] and 388 nm (ϵ 2400) in CH_3CN (**A**). On addition of 1 equiv of acid, a red shift of λ_{max} (274 to 302 nm, 388 to 472 nm) was observed analogous to **8** (**B**) in the presence of excess acid. Dilution of the sample (**B**) with water readily generated the quinone **3** as shown in eq 3.



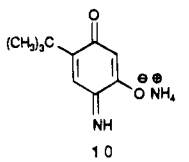
(3)

For the reaction of **4** with *sec*-alkyl primary amines,[11,12] Klein et al. proposed the non-base-catalyzed spontaneous rearrangement of the substrate Schiff base to the product Schiff base.[11] Topaquinone (**1a**) differs from *o*-quinones (**3** or **4**) in its inability to oxidize a *sec*-alkyl primary amine or a *n*-alkyl amine at significant rates. Characterization of the substrate Schiff base (**7**, **8**, or **9**) indicates that these will exist in aprotic/apolar solvents in a localized *p*-quinone-like structure (intimate ion pair). A general base-catalyzed ionization of a substrate Schiff base to the tautomeric product Schiff base has been proposed for *N*-alkylquinonemonoimines (*p*-quinone structure).[16] Thus the formation of the product Schiff base of topaquinone **1a** with reactive substrates is expected to occur *via* abstraction of the C_1 proton of the amine by a base (amine) rather than by a sigmatropic rearrangement. Steric hindrance between α-alkyl groups on substrate and the cofactor is anticipated with such complexes, consistent with the unreactivity of either topaquinone models or copper amine oxidases toward α-alkyl primary amines.

Topaquinone is distinguished from simple *p*-quinones in two important ways: First, it directs nucleophilic addition of an amine to the C_1 carbonyl carbon rather than the ring and second, it greatly facilitates the rate of the reaction. Since the 2-methoxy *p*-quinone **2** did give some evidence for a transamination reaction in boiling ethanol (17% of **2** converted to the corresponding product Schiff base),[9] it appears that either an oxyanion or a methoxy oxygen at the C_2 position can direct nucleophilic addition of an amine to the C_1 carbonyl carbon. For **2**, a hydrogen-bonding interaction in the carbinolamine has been proposed.[9]

In Scheme 2, several unique features of the topaquinone analog **1a** are addressed: (1) the deprotonation of the 2-hydroxyl group, which causes a resonance-stabilized structure, thereby preventing ring amination and directing the reaction at C_1; (2) the localization of the oxyanion of the substrate Schiff base by either an intramolecular (**C**) or an intermolecular electrostatic interaction (**D**). According to Scheme 2, the dehydration of the carbinolamine intermediate (**B**) is catalyzed by the protonated amine which is formed by an acid/base reaction with the 2-hydroxyl group on addition of the amine. As discussed above, the ionic interaction of the amine salt (**A**) is relatively weak.

Iminoquinone. In a previous paper,[8] we reported the formation of the iminoquinone complex with λ_{max} at around 448 nm (**E** in Scheme 1) in the reaction of the enzyme (BSAO) and ammonia at pH 9.1. We proposed the structure by comparison to the iminoquinone generated from air oxidation of the aminoresorcinol (4-amino-5-ethylresorcinol). In order to gain further information on the iminoquinone, we studied the reaction of **1a** and ammonia in acetonitrile. When ammonia gas was bubbled through a solution of **1a**, a formation of the iminoquinone **10** with λ_{max} at 454 nm was observed. It was not possible to isolate **10** since it was highly soluble and unstable



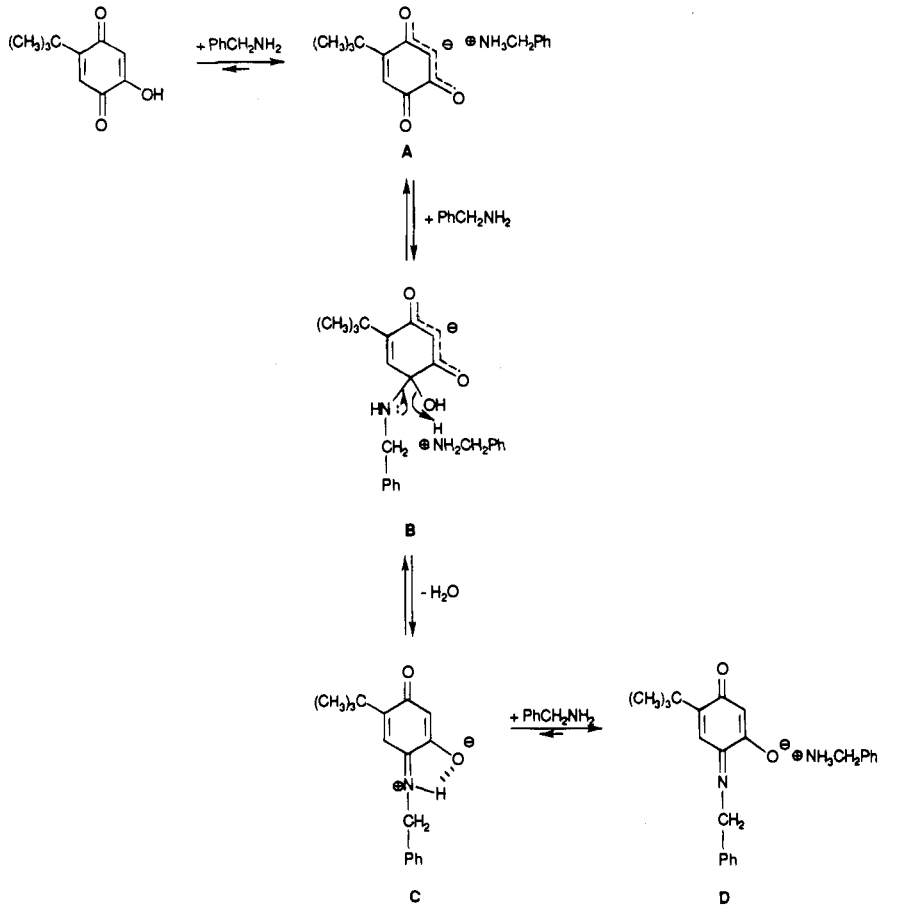
10

during the isolation steps. It should be noted that **10** has a spectroscopic property different from that of the amine salt of the substrate Schiff base. It shows a λ_{max} at around 450 nm in CH_3CN and in an alkaline aqueous solution (pH 9.1).[8] These

(15) The ϵ value of the λ_{max} at 274 nm of **17** was not obtained due to overlapping of the tail of the spectrum of the large excess *tert*-butylamine.

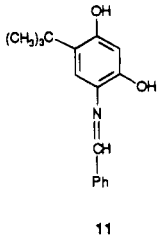
(16) Brown, E. R. in *The Chemistry of the Quinonoid Compounds*; Patai, S., Rappoport, Z., Eds.; Wiley: New York, 1988; Vol. II, Chapter 21.

Scheme 2. Proposed Mechanism for the Formation of the Substrate Schiff Base Intermediate

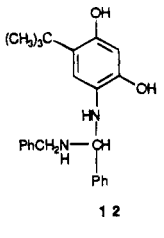


results indicate that the ion pairing of the ammonia salt of the iminoquinone **10** is not as strong as the substrate Schiff base, i.e. it exists most likely as a solvent-separated ion pair (M^+| $|X^-$). The structural difference between **10** and the other substrate Schiff bases (**7**, **8**, and **9**) is that **10** has a hydrogen on the imino nitrogen. However, if there were a hydrogen-bonding interaction between the 2-oxyanion and this hydrogen, electrons would be expected to be localized, generating a λ_{max} of ca. 350 nm. A more likely explanation for the difference between **10** and amine salts of substrate Schiff bases is that the large excess amount of ammonia required to produce **10** participates in solvation of the product salt.

Product Schiff Base. The product Schiff base **11** was prepared from the reaction of an equimolar amount of the hydrochloride salt of the aminoresorcinol **13** and benzaldehyde in anhydrous CH_3CN and isolated as a bright yellow solid in 80% yield. The ^1H NMR of **11** showed a 1H singlet at 8.923



11



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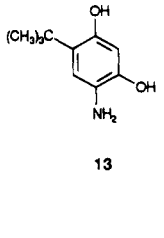


13

ppm, which was assigned to the imino proton (PhC**H**=N−) on the basis of its chemical shift. The two ring protons were at 6.653 and 7.471 ppm, respectively. The absorption spectrum of **11** in CH_3CN is shown in Figure 5 (**A**). It has a λ_{max} at 368 nm and an ϵ value about 10 times larger than that of the substrate

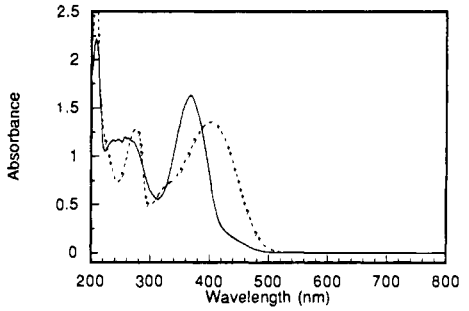


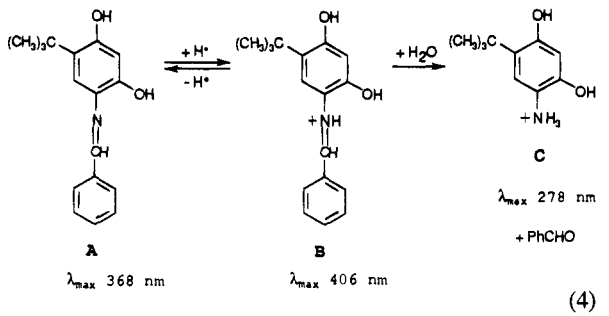
Figure 5. Titration of **11** with acid in CH_3CN: **A** (—) [**11**] = 1.0 × 10^{-4} M, **B** (- - -) addition of HCl = 1.66 × 10^{-4} M.

Table 1. UV−Vis Spectra Data of **8** and **11**

model compds[a]	λ_{max} (ϵ, M^{-1} cm^{-1})
8 (CH_3CN)	276 (20 800), 352 (1300)
8 (pH 10.0)	284 (20 300), 454 (2500)
11 (CH_3CN)	368 (13 500)
11 (pH 10.0)	ND

[a] Concentration = 1.0 × 10^{-4} M.

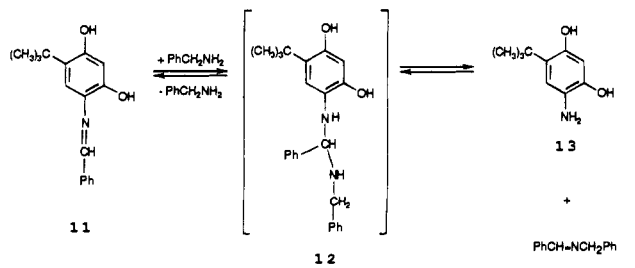
Schiff base (**7**, **8**, or **9**), reflecting the electronic environment of a double bond conjugated to the benzene rings (Table 1). When the solution of **11** was diluted with a carbonate buffer (pH 10.0), it was rapidly hydrolyzed to the aminoresorcinol **13** within 1 min. **11** was also not stable in MeOH. Upon titration of **11** with 0.1 N HCl in CH_3CN, a red shift of λ_{max} (368 nm to 406 nm) was observed (Figure 5 (**B**)) as in the case of **8**; however, the hydrolysis reaction (eq 4) took place concomitantly, resulting in the formation of the corresponding amount of the hydrochloride salt of the aminoresorcinol **13** with λ_{max} at 278 nm (**C**).



(4)

As described above, the substrate Schiff base **8** was relatively stable, since no hydrolysis to the quinone **1a** was observed during the acid titration in CH_3CN. In pH 10.2 buffer, it was slowly (ca. 1 h) hydrolyzed to **1a** (the anionic form). By contrast, the product Schiff base **11** was quite unstable, especially in an aqueous solution, and was rapidly hydrolyzed to aminoresorcinol **13** and benzaldehyde. These results fully support the earlier studies of the reductive $NaCNBH_3$ inactivation experiments on topaquinone-containing enzymes, where it was found that the substrate Schiff base complex had a longer lifetime than the product Schiff base complex.[5,17]

In the model reactions of topaquinone analogs (in CH_3CN), the conversion of the product Schiff base **11** to the aminoresorcinol **13** is expected to proceed *via* aminolysis (eq 5). When **11** was treated with a 1.5−4-fold excess of benzylamine in CD_3CN, time-dependent formation of the aminoresorcinol **13** and the product ($PhCH{=}NCH_2Ph$) was observed by ^1H NMR spectroscopy. Under these conditions, it was found that a new species **X** accumulates at long times (1 h), concomitant with the decrease in the intensity of signals from **13** and the product ($PhCH{=}NCH_2Ph$). Since it was not possible to isolate this species and fully assign its ^1H NMR signals, an unambiguous assignment was not possible. In principle, **X** could correspond to either **12** or a reduced secondary amine derived from **11**; these cannot be distinguished from the available NMR data due to an overlap of benzylic and phenyl hydrogens with unreacted benzylamine. Wang *et al.*[17] have proposed that the substrate Schiff base of a topa model with benzylamine can undergo reduction by aminoresorcinols to give iminoquinone and a secondary amine. We note that in this study we began with product Schiff base and, further, have seen no evidence for oxidation products of **13** (either as the iminoquinone or substrate Schiff base) in NMR spectra. The formation of **X** was also observed, together with **11**, in the reaction of **13** (generated *in situ* by treatment with an equimolar amount of **13**·HCl and *tert*-butylamine) and $PhCH{=}NCH_2Ph$. No corresponding oxazole formation was observed in this reaction.



(5)

Anaerobic Reaction of 1a and Benzylamine. The reaction of **1a** and benzylamine was monitored by ^1H NMR spectroscopy and UV−vis spectroscopy under anaerobic conditions. When

(17) Wang, F.; Bae, J.-Y.; Jacobson, A. R.; Lee, Y.; Sayre, L. M. *J. Org. Chem.* **1994**, *59*, 2409.

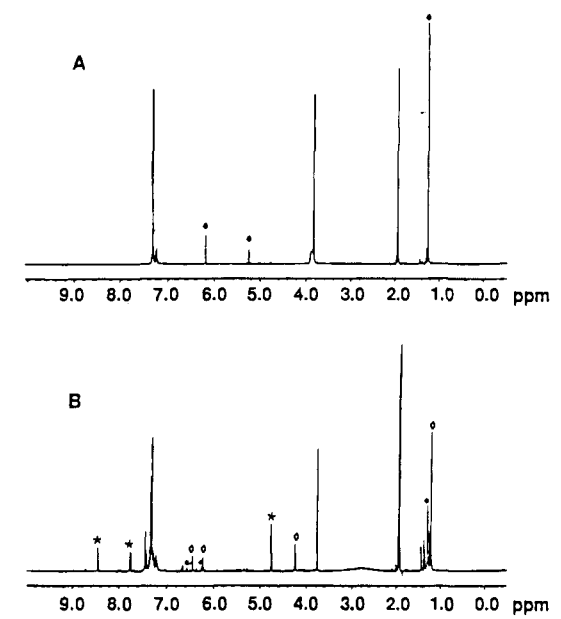
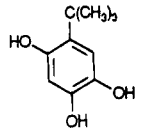


Figure 6. ^1H NMR spectra of the reaction of **1a** and benzylamine in CD_3CN ([**1a**]:[$PhCH_2NH_2$] = 1:3): (**A**) after 1 min, (**B**) after 30 min; (◆) deprotonated form of **1a**, (●) **13**, (*) $PhCH{=}NCH_2Ph$, and (◇) **X** (see text).

1a was treated with a 3-fold excess of benzylamine in CD_3CN, rapid dissociation of the 2-hydroxyl proton occurs. As shown in Figure 6, the NMR signals of this anionic quinone (marked ◆) decrease with the appearance of those of the aminoresorcinol (**13**, marked ●) and $PhCH{=}NCH_2Ph$ (marked *) as well as **X** (marked ◇). No spectroscopic evidence for the formation of the quinol **18** was obtained, ruling out an addition−elimination mechanism involving direct formation of the quinol **18** from the carbinolamine intermediate (**B** in Scheme 4).



18

Figure 7 shows the UV−vis spectral change of the anaerobic reaction of **1a** and benzylamine under pseudo-first-order conditions ([$PhCH_2NH_2$] = 3.84×10^{-2} M, [**1a**] = 3.95×10^{-4} M in a quartz cell with path length of 10 mm). When benzylamine was added to the quinone solution (λ_{max} at 372 nm), the instantaneous formation of the anionic species with λ_{max} at 492 nm was detected. During the first 14 min, a decrease in the absorption at 492 nm with an increase in absorption at 368 nm and at around 304 nm occurred, corresponding to the formation of the product Schiff base **11** and aminoresorcinol **13**, respectively (Figure 7 (top)). Over the next 7 h, the bands at 492 and 368 nm decreased with an increase in absorption at 304 nm (Figure 7 bottom)). Similar spectral changes were observed for the reaction with $PhCD_2NH_2$. The decrease of the 492 nm band (anionic form of **1a**) and the increase of 304 nm (the aminoresorcinol, **13**) followed a first-order rate law up to 80% conversion. Plotting the pseudo-first-order rate constants (k_{obsd}, s^{-1}) at A_{492} and A_{304} against the concentration of benzylamine ($(1.93-7.54) \times 10^{-2}$ M) yielded linear plots, with slopes corresponding to k_2 (a second-order rate constant). The values of k_2 at 492 and 304 nm are summarized together with those for the reaction of **1a** with $PhCD_2NH_2$ (Table 2). The isotope effect (k_H/k_D) for the reaction was found to be 1.09 (at 492 nm)

Table 2. Values of k_2 for the Decrease in A_{492} (the Anionic Form of **1a**) and Increase in A_{304} (**13**) in the Reaction of **1a** and Benzylamine or Benzylamine-d_2

	$PhCH_2NH_2$	$PhCD_2NH_2$	k_H/k_D
k_2 at A_{492}, M^{-1} s^{-1}	$2.08 \times 10^{-2} \pm 1.71 \times 10^{-4}$	$1.91 \times 10^{-2} \pm 1.38 \times 10^{-4}$	1.09
k_2 at A_{304}, M^{-1} s^{-1}	$2.13 \times 10^{-2} \pm 2.92 \times 10^{-4}$	$1.90 \times 10^{-2} \pm 2.38 \times 10^{-4}$	1.12

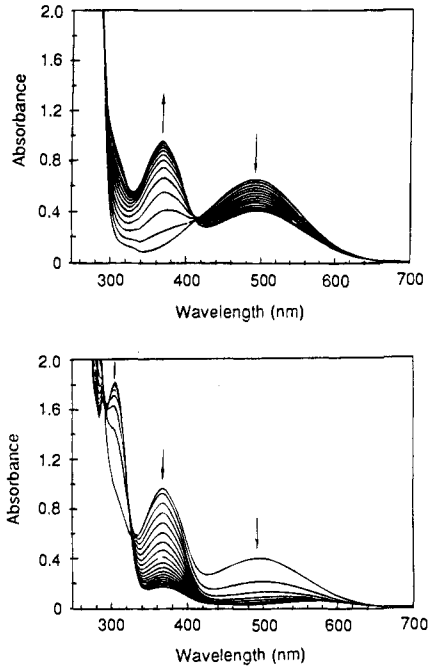


Figure 7. UV−vis spectra of the reaction of **1a** and benzylamine in CH_3CN as a function of time ([**1a**] = 3.95×10^{-4} M, [benzylamine] = 3.84×10^{-2} M): (top) During the period of 10 s to 13 min. (bottom) During the period of 14 min to 7 h.

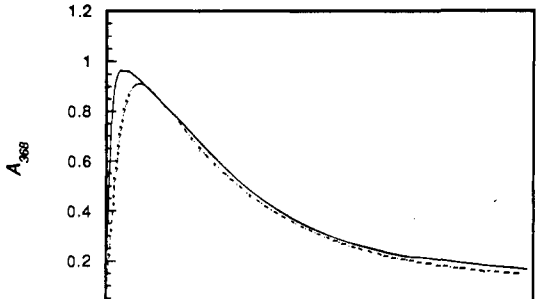


Figure 8. Single-wavelength absorbance traces comparing the rate of formation and decay of the product Schiff base intermediate **11** at 368 nm with 3.92×10^{-2} M of benzylamine (—) and 3.92×10^{-2} M of [1,1-$^2H_2$]benzylamine (- · -) and 3.90×10^{-4} M of **1a**.

and 1.12 (at 304 nm), respectively. The absence of a significant isotope effect in the oxidation of benzylamine by **1a** shows that C−H bond breaking is not occurring in the rate-determining step of the reaction. We propose that the formation and breakdown of the substrate and product Schiff base complexes are the rate-determining steps in the oxidation reaction. The band at 368 nm is characteristic of the product Schiff base **11**. During the first 14 min, increase in this band was observed up to 25% conversion which was calculated using the ϵ value (13 450) at 368 nm for the authentic sample of **11**. The kinetic constants for the changes in A_{368} were not analyzed since the kinetic processes were coupled. Figure 8 shows the single wavelength absorbance traces of the formation and decay of the product Schiff base at 368 nm with $PhCH_2NH_2$ and $PhCD_2NH_2$. Apparently, there is an isotope effect on the formation of the product Schiff base, whereas it is absent in the decay, consistent with α-proton abstraction from the substrate Schiff base to form **11**.

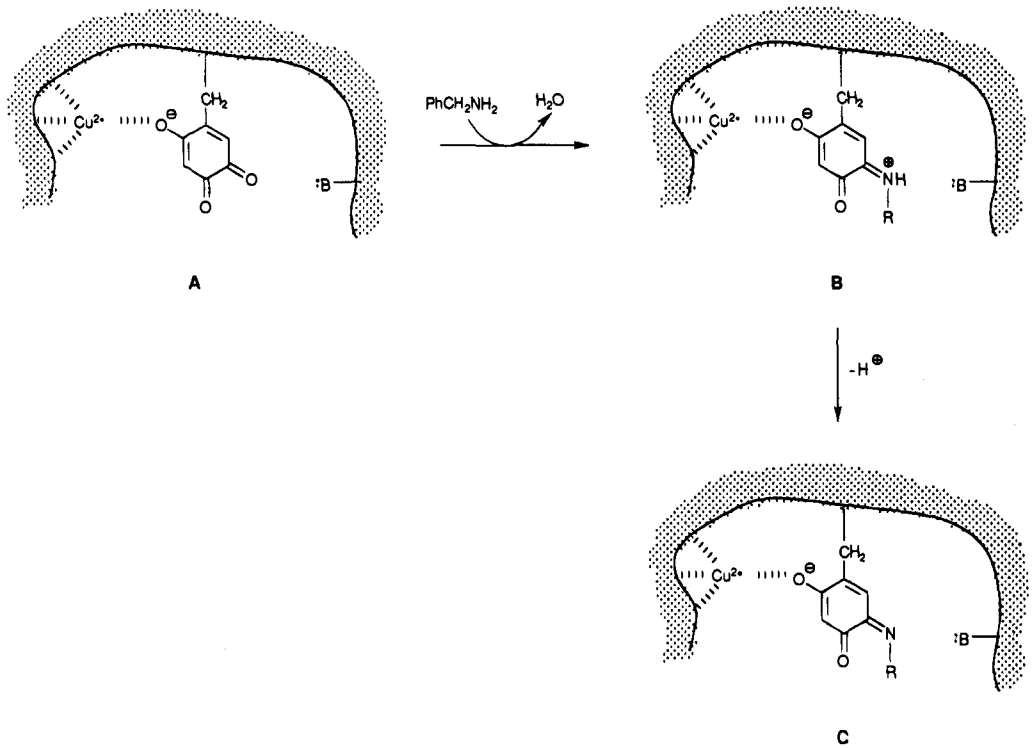
Comparison of the Model System and the Enzyme Reaction. In this study, 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone (**1a**) was employed as a model compound to study the mechanism of the oxidation of amine by topaquinone cofactor. In the reaction of **1a** with *tert*-butylamine, only the amine salt **5a** was isolated. This suggests that the deprotonation of the 2-hydroxyl group precedes the amine addition reaction to the quinone carbonyl carbon. The dissociation of the 2-hydroxyl proton generates a resonance hybrid with λ_{max} at 492 nm. This deprotonation appears to direct the nucleophilic addition of an amine to the C_1 carbonyl carbon, next to the hydroxyl group. In order to determine whether the resonance hybrid species takes on *p*-quinone or *o*-quinone character in the course of catalysis, the reaction with amines was studied.

1a is able to oxidize benzylamine but does not oxidize *sec*-alkyl primary amines such as α-methylbenzylamine and cyclohexylamine. This agrees with the substrate specificity of topaquinone-containing enzymes but differs from that of the *o*-quinone **3**, which efficiently oxidizes *sec*-alkyl primary amines. Only the substrate Schiff base analogs **7** and **8** were obtained in the reaction of **1a** with either α-methylbenzylamine or cyclohexylamine, and no further reaction to product Schiff base was observed. *n*-Propylamine also yielded the substrate Schiff base **9**. 2D NMR studies on **8** reveal that the nucleophilic addition of the amine is at the C_1 carbonyl carbon and UV−vis spectroscopic studies show that the substrate Schiff base (**7**, **8**, or **9**) is in a localized (intimate ion pair) form with λ_{max} at 352 nm in an apolar/aprotic solvent. There is no influence of the benzyl group of the amine on the spectroscopic properties. The fully ionized ion pair (M^+| |X^-) form with λ_{max} at 454 nm could be generated in polar/protic solvents. Titration with acid shows that the protonation of either the imine nitrogen (**B**) or 2-oxyanion (**C**) does not affect the spectroscopic property significantly, suggesting similar electronic environment in structures **A** and **B** or **C** (eq 2). On the other hand, the further protonation to form the protonated imine form (**D**) causes a pronounced red shift (70 nm) of the λ_{max} at 352 nm. Thus, structures **A**, **B**, and **C** (in eq 2) represent excellent models for the proposed substrate Schiff base intermediate (**B** in Scheme 1) in the enzymatic reaction. We propose a local electrostatic interaction which maintains the anionic electron density primarily in the 2-position either through hydrogen bonding to the neighboring Schiff base in its protonated form (as shown in Scheme 1 as **B**) or, alternatively, by interaction with an active site residue (as shown as **A** in eq 2).

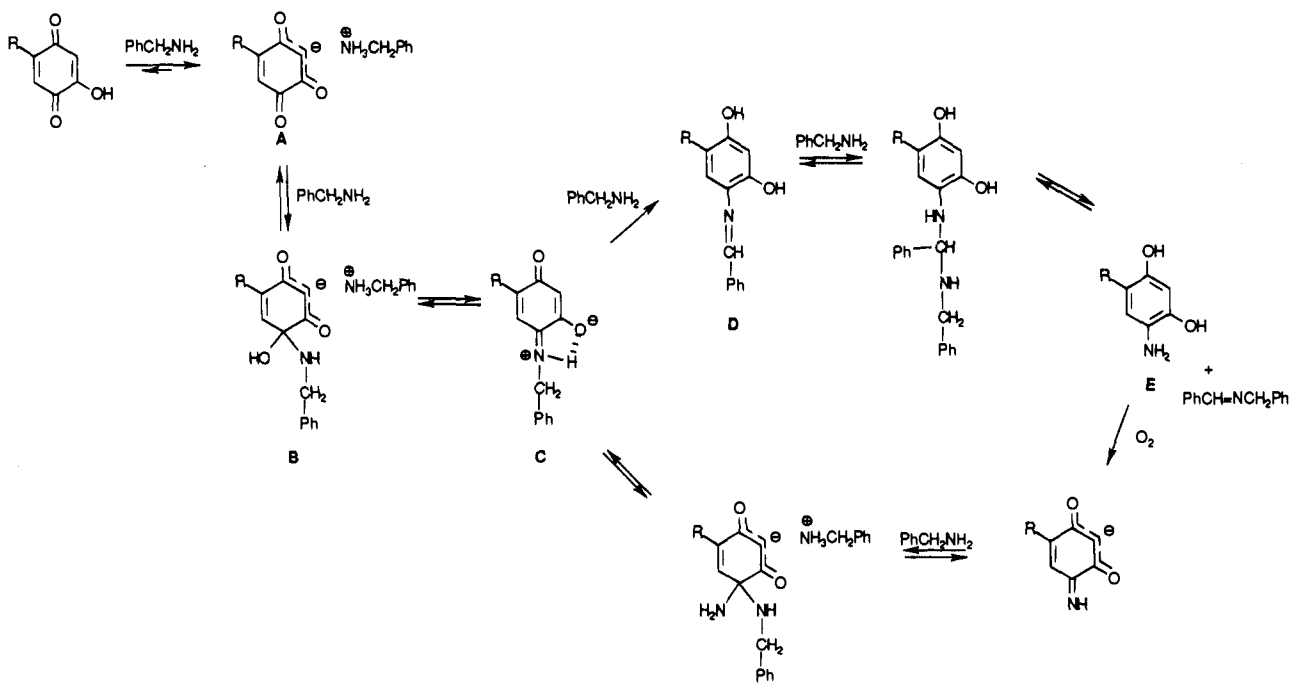
According to Scheme 1, Cu^{2+} lies in the vicinity of the C_4 carbonyl carbon of the cofactor, consistent with earlier distance mapping experiments.[18] If Cu^{2+} contributed a major stabilization interaction within the anionic form of the cofactor (localizing the electrons at the carbon adjacent to the methylene carbon position), the substrate Schiff base intermediate would

(18) (a) Williams, T. J.; Falk, M. C. *J. Biol. Chem.* **1986**, *261*, 15949. (b) Dooley, D. M.; McGuirl, M. A.; Cote, C. E.; Knowles, P. F.; Singh, I.; Spiller, M.; Brown, R. D., III; Knoenig, S. H. *J. Am. Chem. Soc.* **1991**, *113*, 154. (c) McGuirl M. A.; Brown, D. E.; McCahon, C. D.; Turawski, P. N.; Dooley, D. M. *J. Inorg. Biochem.* **1991**, *43*, 186.

Scheme 3. Proposed Mechanism Which Involves the *o*-Quinone Form of the Cofactor as an Active Species



Scheme 4. Proposed Mechanism for the Oxidation of Benzylamine Catalyzed by the Topaquinone Model in CH_3CN



be expected to be in the form of the *o*-quinone (**B** and **C** in Scheme 3). The 4-methoxy *o*-quinone **3** can serve as a good model for the electron-localized *o*-quinone and **17** as a model for a substrate Schiff base with *o*-quinone structure. The data in Figure 4 provide a λ_{max} at 472 nm for the protonated form of **17** (**B** in Scheme 3). We note that **B** would be unstable unless it were stabilized by active site residues. From the λ_{max} values of **17** (388 nm) and its protonated form (472 nm), neither **B** nor **C** (in Scheme 3) has a λ_{max} close to the substrate Schiff base complex detected under stopped-flow studies with bovine serum amine oxidase (λ_{max} = 340 nm).[1] These results provide further evidence for an enzymatic substrate Schiff base intermediate[1] with a *p*-quinone-like structure, with the caveat that they cannot rule out the participation of an *o*-quinone-like species (e.g., **A** in Scheme 3) in the initial nucleophilic attack by amine to form the Schiff base complex.

The product Schiff base intermediate was prepared from the aminoresorcinol **13** and benzaldehyde. It has a 10 times larger λ_{max} at 368 nm than the substrate Schiff base. In the model system (in acetonitrile), it undergoes aminolysis by the amine in a relatively slow step. In aqueous solution, it is very unstable and immediately hydrolyzes to the aminoresorcinol and benzaldehyde. The latter properly agrees with the noted instability of product Schiff base complexes in the enzymatic system,[5,19]

(19) Hartmann, C.; Klinman, J. P. *FEBS Lett.* **1990**, *261*, 441.

formally attributed to the loss of electrostatic stabilization in the course of cofactor reduction (cf. **C** in Scheme 1).

In the model system, the amine has several functions. As summarized in Scheme 4, it first deprotonates the 2-hydroxyl proton of the quinone (**A**). Subsequent attack at the C_1 carbonyl carbon leads to formation of a carbinolamine intermediate (**B**). The protonated amine may act as a general acid catalyst to form the substrate Schiff base intermediate (**C**). The formation of the product Schiff base (**D**) is catalyzed by the amine as a base. Finally **D** undergoes aminolysis to the aminoresorcinol (**E**).

Regarding the nature of base catalysis in the enzymatic reaction, previous studies of the pH dependence of substrate oxidation have implicated the participation of a group with pK_a = ca. 5.2.[6] This value is significantly higher than that for the hydroxyl group in enzyme-bound topaquinone (pK_a = 3.0).[8] However, the pK_a of the ring hydroxyl group could be elevated in the substrate Schiff base complex, raising the possibility of its role in general base catalysis. A mechanism of this type can, however, be eliminated from the identity of the pH dependence for substrate exchange and oxidation reactions, implicating a common base catalyst for both processes. The former reaction (exchange) occurs from within a reduced cofactor complex whereas the latter reaction (oxidation) involves oxidized cofactor.[6] Recent studies have established a very large difference in pK_a for the ring hydroxyl in oxidized and reduced forms of topaquinone.[8] The most satisfactory mechanism which can be written for copper amine oxidases involves general base catalysis by an active site side chain on an intimate ion-stabilized, *p*-quino structure of the substrate Schiff base (**B** in Scheme 1).

Acknowledgment. We acknowledge Dr. Graham Ball for running 2D NMR studies on **8** and for his helpful discussions. We also thank Dr. Larry M. Sayre for a critical reading of the manuscript.

JA943437D