BIOORGANIC CHEMISTRY 17, 443–453 (1989)

Unusual, Intramolecular Cyclization and Side Chain Desaturation of Carboxyethyl-*o*-benzoquinone Derivatives

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Received June 14, 1989

During the oxidation of dihydrocaffeic acid catalyzed by mushroom tyrosinase, the corresponding quinone formed undergoes an unusual nonenzymatic intramolecular nucleophilic addition reaction yielding dihydroesculetin. This compound was further converted to the *o*-quinone which accumulated in the reaction mixture. Chemically synthesized dihydrocaffeiyl quinone also exhibited the same lactonization reaction. The product of the reaction was isolated and characterized by uv, ir, and NMR spectral studies. One of the minor products of enzymatic oxidation was identified to be caffeic acid. Since intramolecular cyclization dominated side chain desaturation, methyl ester of the parent acid was substituted in the tyrosinase reaction in order to suppress the cyclization route and monitor the side chain desaturation. Such studies readily generated methyl caffeate as the major product of tyrosinase-catalyzed oxidation of dihydrocaffeate methyl ester. The formation of this product was accounted for by a novel nonenzymatic route involving tautomerization of enzymatically generated dihydrocaffeiyl quinone to its quinone methide and subsequent aromatization of the latter. The above results indicate that (a) even weak nucleophiles such as carboxylate can react with quinones to form esters and (b) suitably substituted quinones undergo spontaneous quinone methide formation and rearrange further to yield α,β-unsaturated catechols as stable products. The significance of these reactions for sclerotization of insect cuticle is discussed.

INTRODUCTION

The important role of *o*-dihydroxyphenols such as dopa, dopamine, and a variety of other catechols in diverse biological processes such as melanization (*1, 2*), sclerotization of arthropod cuticle (*3, 4*), oxidative browning of plant products (*5*), neurotransmission (*6*), hormone action (*6*), and siderophore formation (*7*) is well established. Tyrosinase, one of the key enzymes involved in the metabolism of *o*-diphenols, is of widespread occurrence in nature (*8, 9*). It is known to convert monophenols to *o*-diphenols and cause the oxidation of resultant *o*-diphenols to *o*-benzoquinones. The fates of enzymatically generated quinones could be diverse, but only a few reactions have been documented. Thus, suitably substituted quinones such as dopaquinone and dopamine quinone undergo intramolecular nucleophilic cyclization which constitutes the initial steps involved in melanogen-

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0045-2068/89 $3.00

esis (*1*). Alternatively, they can react with external nucleophiles as observed during pheomelanin biosynthesis (*2*), sclerotization of arthropod cuticle (*3, 4*), and oxidative browning of plant products (*5*). Compared to nucleophilic addition reactions, other reactions of quinones relevant to biological systems are less understood. Earlier from this laboratory, the production of quinone methides by insect cuticular phenol oxidases has been reported (*3, 4, 10–12*). Upon close scrutiny, this conversion turned out to be a two-step process involving oxidation of 4-alkylcatechols and enzymatic tautomerization of resultant quinones to quinone methides (*13–15*). We have also demonstrated the unique formation of quinone methides during oxidative decarboxylation of 3,4-dihydroxymandelic acid (*16*), oxidative dimerization of 1,2-dehydro-*N*-acetyldopamine (*17, 18*), and nonenzymatic side chain hydroxylation of 3,4-dihydroxyphenylacetic acid (*19*). From these studies, it became apparent that suitably substituted quinones undergo tautomerization to more reactive quinone methides. Therefore, we felt that it was essential to investigate, in detail, the fate of enzymatically generated quinones. Hence, we began to study the reactions of simple *o*-benzoquinone derivatives and report, in this paper, two unusual transformations of carboxyethyl-*o*-benzoquinone, viz., intramolecular cyclization and side chain desaturation.

EXPERIMENTAL PROCEDURES

Mushroom tyrosinase (sp act 4000 units/mg), dihydrocaffeic acid, and caffeic acid were purchased from Sigma Chemical Co. (St. Louis, MO).

Spectral studies associated with the enzymatic oxidation of catechols were carried out using a standard assay mixture (1 ml) containing 1–10 mM of substrate, 10 μg of mushroom tyrosinase in 50 mM sodium phosphate buffer, pH 6.0. The reaction was usually initiated by the addition of enzyme and followed by examining the visible spectral changes at room temperature in a spectrophotometer.

HPLC studies were carried out with an Altex HPLC system equipped with two Altex Model 100A pumps, a Model 420 controller, a Hitachi Model 110-10 spectrophotometer, and an Altex Model C-RIA integrator. Separations were achieved on a Beckman C_{18} ultrasphere reversed-phase column (5 μm, 4.6 $\times$ 150 mm) using the isocratic solvent system, 50 mM acetic acid containing 0.2 mM sodium octylsulfonate in 30% methanol at a flow rate of 1 ml/min for dihydrocaffeic acid reaction.

For separation of products formed in dihydrocaffeate methyl ester–tyrosinase reaction, a different HPLC program was used. The above column was equilibrated with 30% methanol in 50 mM acetic acid containing 0.2 mM sodium octylsulfonate at a flow rate of 1 ml/min. After the sample was injected, the same conditions were used to separate the mixture until 5 min, after which time the flow rate was increased to 1.5 ml/min and methanol was increased to 40% until 15 min. At 15 min, the methanol was brought back to 30% and at 20 min the flow rate was reduced back to 1 ml/min.

To isolate the products of tyrosinase-catalyzed dihydrocaffeic acid oxidation, a large-scale reaction mixture containing 75 mg of tyrosinase, 1 g of dihydrocaffeic acid in 500 ml of 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature for an hour. At the end of this period, the contents were treated with excess sodium borohydride and extracted with ethyl acetate twice. The organic layer was pooled, washed with dilute sodium bicarbonate followed by water, and dried over anhydrous magnesium sulfate. Removal of solvent on a rotary evaporator gave dihydroesculetin.

A reaction mixture containing 250 mg of dihydrocaffeate methyl ester, 25 mg of tyrosinase in 250 ml of 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature for about 2 h and lyophilized. The residue was chromatographed on a Sephadex LH-20 column (85 × 2.5 cm) in 50% methanol. The fractions containing methyl caffeate were collected, pooled together, evaporated to dryness on a flash evaporator, and used for spectral studies.

Synthesis of Dihydroesculetin

Dihydroesculetin was synthesized as follows: 0.9 g of dihydrocaffeic acid was treated with 2 g of *o*-chloranil in ether and the solution cooled to about −70°C. The precipitated 4-carboxyethyl-*o*-benzoquinone was filtered, washed with ice-cold ether, and suspended in 30 ml of water. The suspension was stirred at room temperature for 3 h. Following reduction with sodium dithionite, the product formed was extracted into ethyl acetate. The organic layer was successively washed with 1 M sodium bicarbonate and 1 M sodium chloride and dried over anhydrous magnesium sulfate. Removal of solvent on the rotary evaporator gave the required product—dihydroesculetin—as a white solid (from ether), 0.155 g; yield, 17%; mp 202–204°C. *Anal.* Cald for $C_9H_8O_4$: C, 60.00%; H, 4.44%. Found: C, 59.36%; H, 4.58%. ir spectra (nujol): $\bar{\nu}$ = 3280 (OH), 1720 (—O—C=O); 1160 (C–O) cm^{-1}. ^1H NMR (dimethyl sulfoxide-d_6) δ = 2.80 (multiplet, 4H, $-CH_2-CH_2$), 6.50 (singlet, 1H, ArH), 6.63 (singlet, 1H, ArH), 8.36–9.36 ppm (broad, 2H, OH) exchanged with D_2O.

Synthesis of Methyl Esters of Dihydrocaffeic Acid and Caffeic Acid

A solution of dihydrocaffeic acid (0.91 g; 5 mmol) in methanol (20 ml) was saturated with HCl gas and stirred at room temperature for 2 h. Removal of the solvent on a rotary evaporator gave a solid (1.0 g, quantitative yield; mp 44–45°C); single product by HPLC and TLC. ^1H NMR (d_6-DMSO): δ = 2.50–3.05 (multiplet, 4H, CH_2), 3.77 (singlet, 3H, $COOCH_3$), 6.30–6.80 (multiplet, 3H, ArH), 6.48 ppm (broad, 2H, OH) exchanged with D_2O.

A similar reaction of caffeic acid (0.9 g; 5 mmol) with methanol/HCl gave its methyl ester; single product by HPLC (1.0 g, quantitative yield; mp 127–128°C). ^1H NMR (d_6–DMSO): δ = 3.64 (singlet, 3H, $COOCH_3$), 6.19 (doublet, 1H, CH=CH), 6.60–7.10 (multiplet, 3H, ArH), 7.40 (doublet, 1H, CH=CH), 7.20 ppm (broad, 2H, OH) exchanged with D_2O.

RESULTS

Consistent with its wide substrate specificity (*9, 16, 17, 19*), mushroom tyrosinase oxidized a variety of catecholic compounds which included catechol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, dihydrocaffeic acid, caffeic acid, chlorogenic acid, 4-methyl catechol, *N*-acetyldopamine, *N*-acetylnorepinephrine, 3,4-dihydroxymandelate, 1,2-dehydro-*N*-acetyldopamine, dopa, dopamine, epinephrine, and norepinephrine. Enzymatic oxidation of 3,4-dihydroxymandelate resulted in the formation of 3,4-dihydroxybenzaldehyde (*9, 19*) and that of 1,2-dehydro-*N*-acetyldopamine, accompanied by the generation of the benzodioxan dimer (*17*), while 3,4-dihydroxyphenylacetic acid yielded 3,4-dihydroxybenzaldehyde via 3,4-dihydroxymandelic acid and 2,5,6-trihydroxybenzofuran (*19*). Oxidation of other catechols resulted in the production of their corresponding quinones in the reaction mixture. Figure 1A, for instance, shows

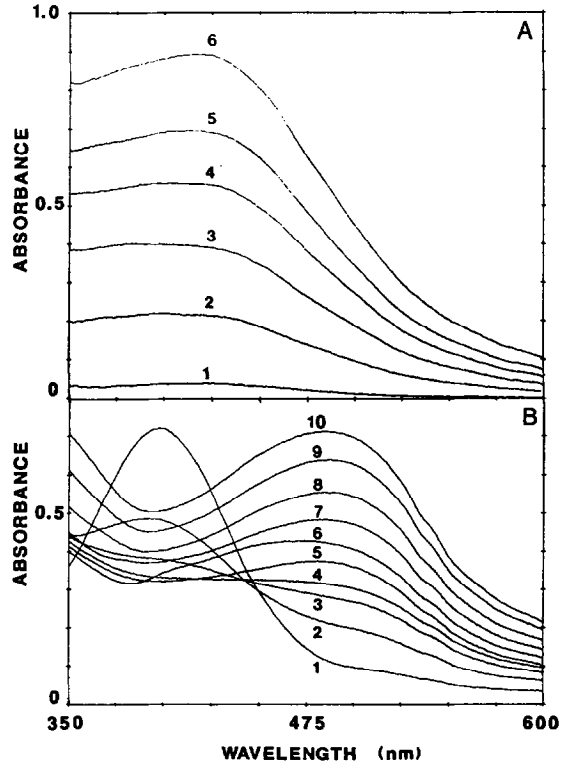

FIG. 1. Spectral changes associated with mushroom tyrosinase-catalyzed oxidation of (A) 3,4-dihydroxybenzoic acid and (B) dihydrocaffeic acid. A reaction mixture containing 10 mM substrate, 10 μg mushroom tyrosinase in 1 ml of 25 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature and the spectrum of the quinone formed was recorded at various time intervals. (A) Substrate used: 3,4-dihydroxybenzoic acid; spectra were recorded at (1) 0 time; (2) 3.5 min; (3) 6 min; (4) 8.5 min; (5) 11 min; (6) 13.5 min. (B) Substrate used: hydrocaffeic acid; spectra were recorded at (1) 30 s; (2) 3 min; (3) 5.5 min; (4) 8.0 min; (5) 10.5 min; (6) 20 min; (7) 30 min; (8) 45 min; (9) 60 min; (10) 75 min.

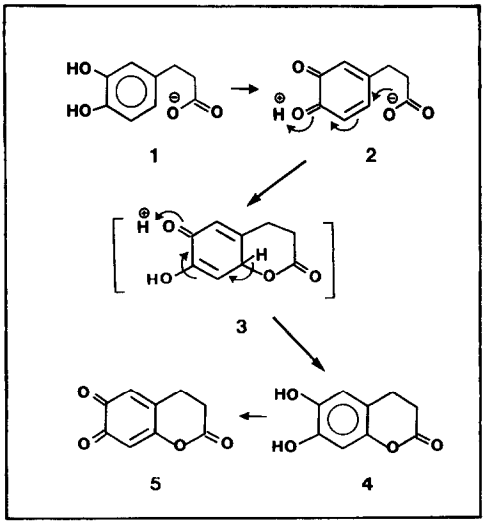

FIG. 2. Proposed mechanisms for the oxidative transformation of dihydrocaffeic acid. Mushroom tyrosinase catalyzes the oxidation of dihydrocaffeic acid (**1**) to 4-carboxyethyl-*o*-benzoquinone (**2**). The quinone formed, being unstable, undergoes rapid nonenzymatic intramolecular nucleophilic addition to yield the transient "dienone" (**3**). Dienone–phenol rearrangement of this intermediate produces dihydroesculetin (**4**) which is further oxidized by tyrosinase to the corresponding quinone (**5**) (λ_{max} 495 nm).

the visible spectral changes associated with tyrosinase-catalyzed oxidation of 3,4-dihydroxybenzoic acid. The enzymatic oxidation accompanied the generation and accumulation of 4-carboxy-*o*-benzoquinone, which exhibited an absorption maximum at around 400 nm in the visible region. The spectral changes associated with the oxidation of dihydrocaffeic acid shown in Fig. 1B also indicate the initial generation of quinone, which exhibits an absorption maximum at 400 nm in the visible region. However, the quinone formed in this case did not accumulate in the reaction mixture, but slowly converted into a product which exhibits an absorption maximum at around 495 nm. The time course study of spectral changes accompanying the oxidation indicated that the initially formed 4-carboxyethyl-*o*-benzoquinone was rapidly converted into a transient product which does not absorb in the visible region. With the progress of time, this intermediate was converted into the final product, which accumulated in the reaction mixture as evidenced by the increase in absorption at 495 nm. These spectral changes are consistent with the formation of a catechol as the product of 4-carboxyethyl-*o*-benzoquinone conversion and the oxidation of resultant catechol to its quinone derivative. Chemical considerations indicated the formation of dihydroesculetin as the ring-closed product of 4-carboxyethyl-*o*-benzoquinone and its oxidation to quinone (Fig. 2). To confirm this possibility, products formed in the reaction mixture were analyzed by HPLC. Figure 3 gives the HPLC analyses of reaction mixtures containing dihydrocaffeic acid and catalytic amounts of mushroom tyrosinase. At the start of the reaction, the only compound eluted out of the column is

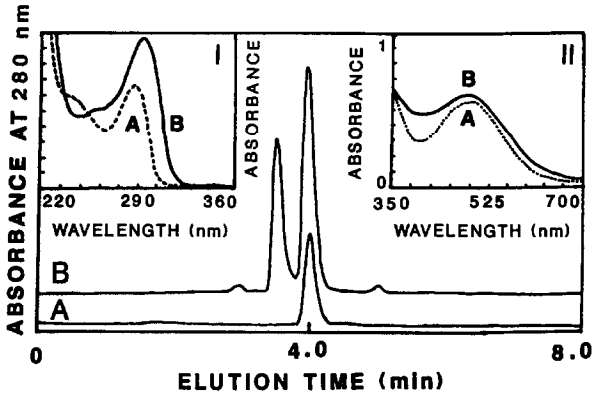

FIG. 3. HPLC analysis of reaction mixtures containing dihydrocaffeic acid and mushroom tyrosinase. A reaction mixture containing 1 mM dihydrocaffeic acid, 10 μg tyrosinase in 1 ml of 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature. An aliquot (10 μl) of the reaction mixture was mixed with 10 μl of ascorbic acid solution (5 mg/ml) and 10 μl of the sample was subjected to HPLC analysis as outlined under Materials and Methods. Trace A: authentic dihydrocaffeic acid. Trace B: reaction mixture at the end of 30 min. The peak at 3.49 min corresponds to dihydroesculetin. Inset I: The ultraviolet absorbance spectra of dihydroesculetin (A) in 0.2 M acetic acid and (B) in 5% sodium borate. Inset II: The visible spectra of (A) the quinone generated from dihydroesculetin by mushroom tyrosinase and (B) the quinone accumulating in the dihydrocaffeic acid–tyrosinase reaction mixture.

the starting material (Fig. 3, trace A). However, after a 30-min incubation with mushroom tyrosinase, formation of a new product could be witnessed (Fig. 3, trace B). This compound corresponded with synthetic dihydroesculetin and on cochromatography gave a singly symmetrical peak. The uv spectrum of the compound (Fig. 3, inset I) matched that of dihydroesculetin. Further, the quinone accumulating in the reaction mixture (Fig. 1B and Fig. 3, inset II, trace B) corresponded with the quinone generated from synthetic dihydroesculetin by mushroom tyrosinase oxidation (Fig. 3, inset II, trace A).

Although the above studies indicate the operation of the proposed pathway (Fig. 2), they do not conclusively prove the structure of the product. To meet this end, a large-scale reaction was conducted and the product formed was isolated as outlined under Materials and Methods. The isolated product exhibited uv spectral characteristics similar to those of synthetic dihydroesculetin. The ir spectrum (Fig. 4) of the compound exhibited absorptions at 3280 (OH), 1720 (—O—C=O), and 1160 (CO) cm^{-1} and matched peak-to-peak with that of synthetic sample, confirming that they are one and the same compound.

Examination of Fig. 3 also reveals that there are other minor products formed in the reaction. One of the products, eluting at 5.2 min, was tentatively identified to be caffeic acid, based on retention times and cochromatography. This reaction was quite unexpected from substituted quinones. Hence, attempts were made to optimize this reaction. Since the suitably substituted carboxyl group of the carboxyethyl-*o*-benzoquinone undergoes rapid intramolecular cyclization, it sup-

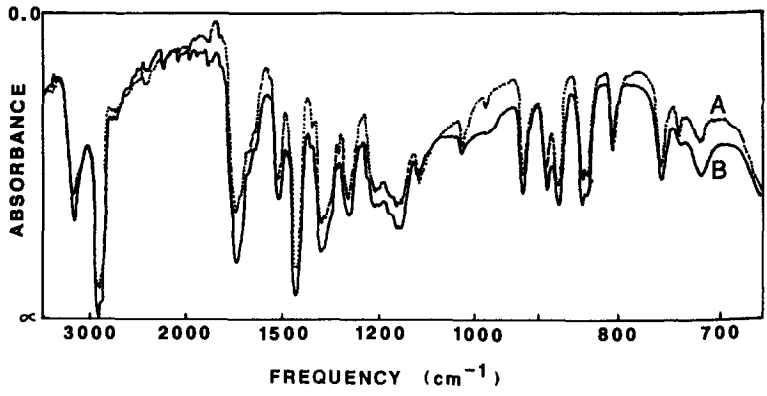

FIG. 4. Infrared spectrum of (A) synthetic dihydroesculetin and (B) the enzymatic product formed from the dihydrocaffeic acid–tyrosinase reaction mixture.

presses the other reactions of this compound. Therefore, the fate of this quinone was examined, taking the methyl ester of dihydrocaffeic acid, as esterification is expected to prevent any lactonization. Figure 5 (inset) shows the visible spectral changes accompanying the enzymatic oxidation of dihydrocaffeate methyl ester. From the figure it is evident that the quinone from the methyl ester is formed. However, it was found to be unstable and seemed to undergo further transformation(s). HPLC studies shown in Fig. 5 confirm this contention. When a reaction mixture containing dihydrocaffeate methyl ester and tyrosinase was subjected to HPLC analysis, formation of a new compound could be readily witnessed (Fig. 5, trace B). This compound corresponded with methyl caffeate (Fig. 5, trace C). On HPLC analysis in different solvent systems and under different conditions, both the isolated product and the methyl caffeate exhibited the same retention time and cochromatographed as a single symmetrical peak. Moreover, the enzymatic product exhibited the same uv spectrum as that of the synthetic compound (Fig. 6A). In addition, its fluorescence spectrum also matched with that of the synthetic compound, confirming the identity. The formation of methyl caffeate can be accounted for by the scheme of reactions shown in Fig. 7. The quinone formed from dihydrocaffeate methyl ester by the action of tyrosinase undergoes slow nonenzymatic tautomerization to its quinone methide analog. The quinone methide analog, being more reactive than the parent quinone, rapidly tautomerizes to generate methyl caffeate. Since the second tautomerization leads to aromatization of the quinonoid ring, it is highly favored.

DISCUSSION

Results presented in this paper clearly demonstrate the participation of the carboxyl group in the nucleophilic addition reaction with quinones. While carboxy-substituted quinones are generally expected to be stable, enzymatically generated 4-carboxyethyl-*o*-benzoquinone exhibited unusual lability and underwent

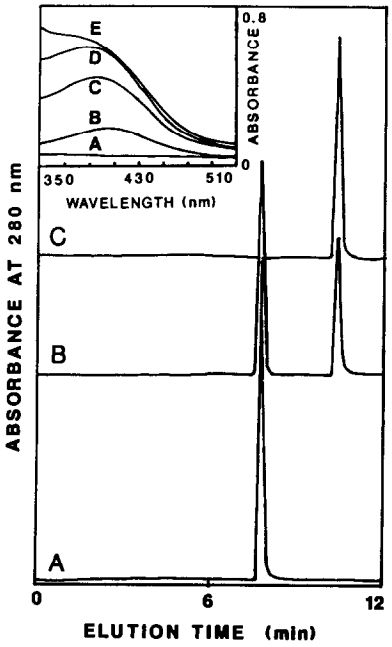

FIG. 5. HPLC analysis of the dihydrocaffeate methyl ester–tyrosinase reaction. A reaction mixture containing 1 mM dihydrocaffeate methyl ester, 10 μg mushroom tyrosinase, and 1 ml of 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature. An aliquot (10 μl) was mixed with a 10-μl solution of ascorbic acid (5 mg/ml) and a 10-μl sample was subjected to HPLC analysis as outlined under Materials and Methods. Trace A: reaction mixture at 0 time. Trace B: reaction mixture at 75 min. Trace C: authentic methyl caffeate. Inset: Visible spectral changes accompanying the enzymatic oxidation of dihydrocaffeate methyl ester. A reaction mixture prepared as outlined above was incubated at room temperature and the visible spectral changes occurring in the reaction mixture were monitored at (A) 0 time, (B) 2 min, (C) 4 min, (D) 6 min, and (E) 8 min after the start of the reaction. Further incubation did not cause any increase in absorbance at 400 nm, but the quinone formed slowly underwent further transformation.

the facile intramolecular cyclization reaction to give dihydroesculetin. Dihydroesculetin thus formed was further oxidized to its corresponding quinone derivative, which accumulated in the reaction mixture. Earlier, we have reported a similar lactonization reaction with carboxymethyl-*o*-benzoquinone (*19*). Thus, it appears that suitably substituted carboxy quinone can undergo a facile intramolecular cyclization reaction.

Although we have not been able to observe a similar nucleophilic addition reaction of carboxylate-containing compounds to quinones, the possible occurrence of such a reaction in insect cuticle cannot be ruled out. It is believed that during cuticular sclerotization, enzymatically generated quinones and quinone methides react with cuticular macromolecules to form quinonoid and catecholic adducts, respectively (*3, 4*). Cuticular proteins contain aspartic acid and glutamic acid at a level exceeding 20 to 30% of their total amino acid composition (*4*). While it is not known with certainty how many of these amino acids are present as their

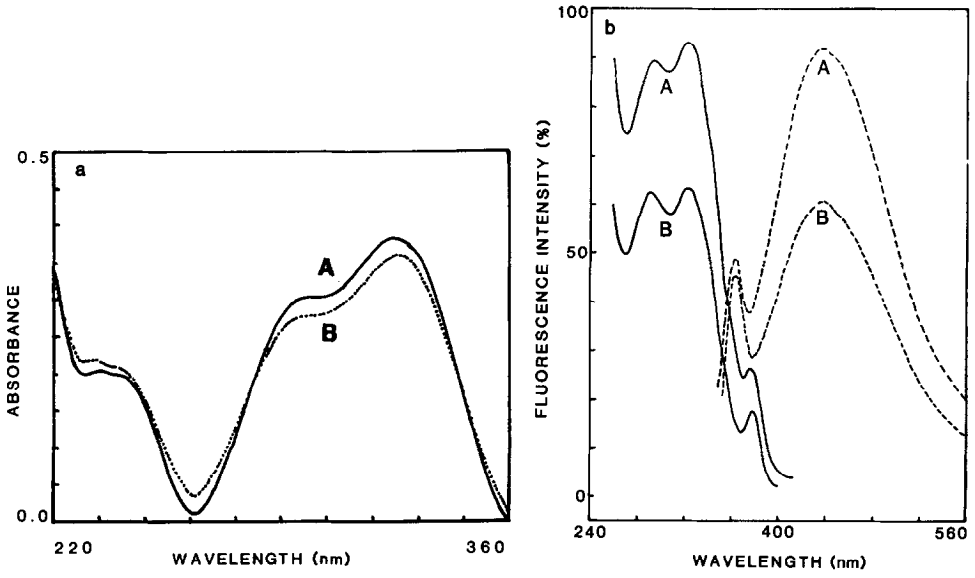

FIG. 6. (a) The ultraviolet absorbance spectrum of (A) the synthetic methyl caffeate (B) enzymatic product. (b) The fluorescence spectra of (A) the synthetic methyl caffeate (B) enzymatic product. Solid line, excitation spectrum; broken line, emission spectrum.

amide derivatives (asparagine and glutamine), the steric constraints imposed in cuticle may create appropriate conditions for the nucleophilic addition of carboxyl groups with enzymatically generated quinones. Such reactions will lead to ester formation. Usually, methods employed to study the crosslinks involve hydrolysis of cuticle and analysis of aryl conjugates and/or determination of the reduction in the level of potential crosslink-forming amino acids. Since such procedures destroy the ester linkage and liberate the parent amino acids from the adducts and

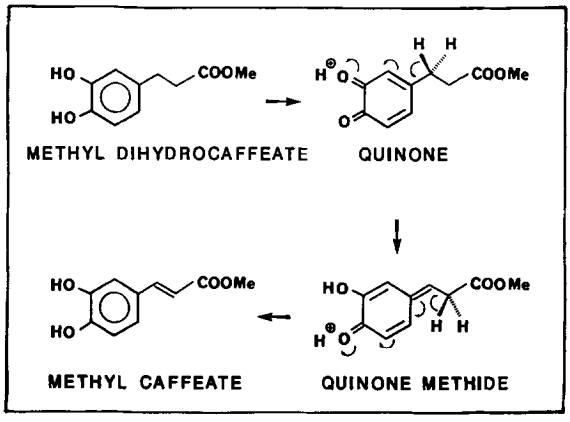

FIG. 7. Proposed mechanism for the generation of methyl caffeate. Mushroom tyrosinase oxidizes methyl caffeate to its quinone. The quinone formed, being unstable, undergoes slow nonenzymatic tautomerization to its quinone methide which rapidly aromatizes to yield methyl caffeate.

crosslinks, these crosslinks have never been detected. However, with the recent advances in nondestructive techniques, such as solid-state NMR spectroscopy, structural verification of such crosslinks will most certainly be possible in the near future.

The formation of methyl caffeate from the quinone of dihydrocaffeate methyl ester is quite unexpected and seems to occur by a double tautomerization. An initial tautomerization of quinone yields the quinone methide (Fig. 7). Earlier, we have reported a similar tautomerization of carboxymethyl quinone to its quinone methide (*19*). The quinone methide formed in this case reacted with water molecules yielding 3,4-dihydroxymandelic acid. However, the quinone methide derived from carboxyethyl-*o*-benzoquinone preferentially underwent another intramolecular rearrangement to generate methyl caffeate as the product (Fig. 7). We also proposed a similar double tautomerization route for the biosynthesis of dehydro-*N*-acetyldopamine from *N*-acetyldopamine quinone (*4, 12, 13*). Recently, we have demonstrated that this reaction is indeed enzyme catalyzed (*20*). Thus, *N*-acetyldopamine quinone : quinone methide isomerase converts the tyrosinase-generated *N*-acetyldopamine quinone to NADA quinone methide (*13, 14, 21*), while the *N*-acetyldopamine quinone methide : dehydro-*N*-acetyldopamine tautomerase produces dehydro-*N*-acetyldopamine from *N*-acetyldopamine quinone methide (*22*). The present studies with dihydrocaffeate methyl ester, however, indicate that such double bond formation can occur even nonenzymatically. Currently we are examining the fate of other simple quinones to unravel any new transformations.

ACKNOWLEDGMENTS

This work was supported in part by grants from National Institutes of Health (ROI-AI-14753) and the University of Massachusetts at Boston. We thank Professor J. P. Anselme for his generosity in allowing us to use his ir and NMR spectral facilities and Professor W. Hagar for the use of his fluorescence spectrophotometer.

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