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Novel Transformations of Enzymatically Generated Carboxymethyl-*o*-benzoquinone to 2,5,6-Trihydroxybenzofuran and 3,4-Dihydroxymandelic Acid

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The conversion of monophenols to *o*-diphenols and the oxidation of resultant *o*-diphenols to *o*-benzoquinones are two activities widely exhibited by tyrosinases. Accordingly, during the oxidation of 3,4-dihydroxyphenylacetic acid, mushroom tyrosinase produced 4-carboxymethyl-*o*-benzoquinone as the initial product. Carboxymethyl-*o*-benzoquinone thus formed was found to be extremely unstable as compared to its lower homolog 4-carboxy-*o*-benzoquinone and underwent novel transformations to produce 3,4-dihydroxymandelic acid and a new compound identified as 2,5,6-trihydroxybenzofuran, based on uv, ir, and NMR spectral and HPLC studies. Tyrosinase further oxidized the 3,4-dihydroxymandelate formed to 3,4-dihydroxybenzaldehyde, as reported earlier [Sugumaran, M. (1986) *Biochemistry* **25**, 4489–4492], while 2,5,6-trihydroxybenzofuran was oxidized to insoluble, unidentifiable dark pigments. Incubation of synthetic carboxymethyl-*o*-benzoquinone in dimethyl sulfoxide at room temperature resulted in the production of 3,4-dihydroxymandelic acid as the sole catecholic product. Based on these findings, possible mechanisms for the unusual conversion of carboxymethyl-*o*-benzoquinone to 3,4-dihydroxymandelic acid and 2,5,6-trihydroxybenzofuran are presented.

o-Dihydroxyphenols such as 3,4-dihydroxyphenylalanine, dopamine, norepinephrine, and a variety of their metabolites are of widespread occurrence in biological systems (*1, 2*). Central to their metabolism is their oxidation by the ubiquitously present phenoloxidase (*3*). Phenoloxidase, also known as tyrosinase, phenolase, or polyphenoloxidase, is a copper containing monooxygenase which is responsible for the conversion of certain monophenols to *o*-diphenols and the oxidation of resultant diphenols to quinone derivatives in nature. Its presence in higher animals, insects and other arthropods, plants, fungi, and certain bacteria has been demonstrated and its participation in key biological processes such as melanin biosynthesis (*2, 4*), oxidative browning of plant products (*5*), and sclerotization of insect cuticle (*6, 7*) has been well established. Based on the known reactivity of mushroom tyrosinase, it is usually assumed that phenoloxidases from various organisms catalyze the oxidation of catechols to *o*-benzoquinones. While this assumption is true in most cases, a few years ago we reported the characterization of a phenoloxidase from the cuticle of *Sarcophaga bullata* which converts 4-alkyl catechols to quinone methide derivatives rather

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than quinones (*7, 8*). While studying the comparative properties of this enzyme with mushroom tyrosinase, we discovered an unusual oxidative decarboxylation of 3,4-dihydroxymandelic acid to 3,4-dihydroxybenzaldehyde catalyzed by mushroom tyrosinase (*9*). Recently, we characterized yet another novel reaction catalyzed by tyrosinase viz. oxidative dimerization of 1,2-dehydro-*N*-acetyldopamine to a benzodioxan type compound (*10*). From these studies, it was clear that the oxidation products of catechols are not always quinone derivatives. Since the oxidation chemistry of catecholamine derivatives plays a central role in sclerotization of insect cuticle (*7*), oxidative browning of plant products (*5*), and melanin biosynthesis (*2, 4*), we decided to examine the mechanism of oxidation of simple catecholamine derivatives by well-characterized mushroom tyrosinase. In this paper we present novel transformations of enzymatically generated carboxymethyl-*o*-benzoquinone to 3,4-dihydroxymandelic acid and 2,5,6-trihydroxybenzofuran.

EXPERIMENTAL PROCEDURES

Mushroom tyrosinase (sp act 4000 units/mg), 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzoic acid, and 3,4-dihydroxymandelic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and solvents were procured from commercial sources.

Spectral studies associated with the enzymatic oxidation of *o*-dihydroxyphenols were carried out using a standard assay mixture (1 ml) containing 10 mM of specified *o*-dihydroxyphenol and 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 monitoring the visible spectrum in a spectrophotometer at room temperature. Oxygen uptake accompanying the oxidation was continuously measured on a similar reaction at 30°C using a Clark oxygen electrode (YSI Model 53) fitted with a strip chart recorder. The substrate concentration was reduced to 1 mM in this case. HPLC analyses of reaction mixtures were performed as outlined earlier (*11*). A large-scale reaction mixture containing 1 g of 3,4-dihydroxyphenylacetic acid and 25 mg of mushroom tyrosinase in 500 ml of 50 mM sodium phosphate buffer, pH 6.0, was used for isolation of the enzymatic products. It was incubated at 30°C for 30 min, arrested by the addition of 10 ml of glacial acetic acid, and lyophilized. The residue was taken up in a small amount of 0.2 M acetic acid and chromatographed on a Biogel P-2 column (90 × 2.5 cm) using the same solvent as the eluant at a flow rate of 60 ml/h. Under these conditions, 2,5,6-trihydroxybenzofuran eluted at around 640 ml; 3,4-dihydroxymandelic acid at 720 ml; unreacted substrate at 760 ml; and 3,4-dihydroxybenzaldehyde at 920 ml. The fractions containing the products were pooled, lyophilized, and used for structural elucidation.

Nonenzymatic conversion of carboxymethyl-*o*-benzoquinone to 3,4-dihydroxymandelic acid was achieved as follows: 3,4-dihydroxyphenylacetic acid (0.5 g; about 3.0 mmol) was dissolved in ether and oxidized with *o*-chloranil (1.0 g; about 4.0 mmol). The resultant *o*-benzoquinone was taken up in 20% dimethyl sulfoxide

solution in water and stirred at room temperature for 30 min. Following this period, the contents were concentrated by flash evaporation and chromatographed on a Biogel P-2 column as outlined earlier to obtain pure 3,4-dihydroxymandelic acid (yield about 5%).

Ultraviolet and visible spectra were recorded on a Gilford Model 2600 spectrophotometer. Infrared spectra were taken in a Perkin–Elmer Model 137 spectrophotometer and ^{1}H NMR spectra were obtained from a 60 MHz Perkin–Elmer Model R-24 spectrometer.

RESULTS AND DISCUSSION

In accordance with the published results (*8, 10, 12*), reaction mixtures containing different catechols and mushroom tyrosinase readily generated quinones as the initial products as evidenced by their typical visible absorption spectra. Thus Fig. 1A, for instance, illustrates the generation and accumulation of 4-carboxy-*o*-benzoquinone in the reaction mixture containing 3,4-dihydroxybenzoic acid and catalytic amounts of mushroom tyrosinase. Figure 1B shows the spectral changes associated with the oxidation of the higher homolog, 3,4-dihydroxyphenylacetic acid. The generation of *o*-benzoquinone can also be witnessed with this compound by the rapid appearance of an absorption peak at 400 nm in the visible region of the spectrum. However, unlike 4-carboxy-*o*-benzoquinone, this compound does not accumulate in the reaction mixture (Fig. 1B, inset). It was slowly converted into a product(s) which does not exhibit visible absorption. This observation was puzzling because, like 4-carboxy-*o*-benzoquinone, one expects the quinone from 3,4-dihydroxyphenylacetic acid also to be stable and accumulate in the reaction mixture. On the contrary, the higher homolog turned out to be extremely unstable in solution and underwent further reaction(s).

HPLC studies confirmed this contention. Figure 2 shows the HPLC analyses of reaction mixtures containing 3,4-dihydroxyphenylacetate and tyrosinase arrested at various time intervals. From the results, it is clear that three new aromatic compounds are formed in the assay mixture which eluted at 2.59, 3.75, and 7.59 min from the HPLC column. In order to characterize these three products, a large scale reaction mixture was conducted and the products formed were separated on a Biogel P-2 column as outlined under Experimental Procedures.

The 2.59 min peak (Fig. 2) exhibited a positive nitrite–molybdate reaction with Arnow's reagent (*13, 14*) indicating the presence of *o*-dihydroxyphenolic group. On treatment with tyrosinase, it was converted to 3,4-dihydroxybenzaldehyde, as shown in a previous publication (*9*). Based on its elution time, cochromatography with authentic sample, and ultraviolet and infrared spectra, the structure of this compound was confirmed to be 3,4-dihydroxymandelic acid.

HPLC studies of the 3.75 min peak (Fig. 2) indicated that this compound is different from any of the following structurally related authentic compounds: 3,4-dihydroxybenzyl alcohol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxybenzoylformic acid, or 3,4-dihydroxybenzaldehyde. The uv spectrum of this product exhibited a bathochromic shift in borate buffer, pH 8.6,

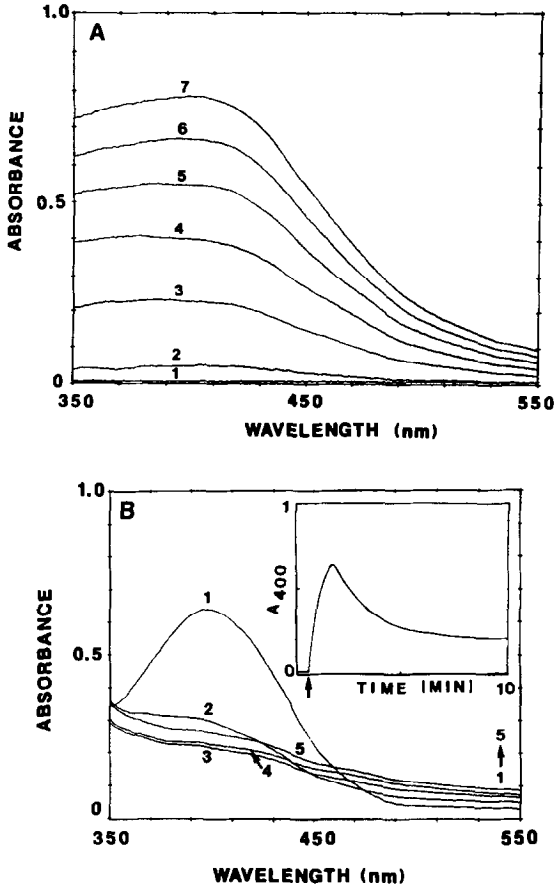

FIG. 1. Visible spectral changes associated with the enzymatic oxidation of (A) 3,4-dihydroxybenzoic acid and (B) 3,4-dihydroxyphenylacetic acid. A reaction mixture containing 10 mM indicated substrate, 10 μg of mushroom tyrosinase in 1 ml of 50 mM sodium phosphate buffer, pH 6.0, was incubated at room temperature and the spectral changes associated with the oxidation were monitored at various time intervals (for 3,4-dihydroxybenzoic acid: Curve 1, 0 time; Curve 2, 30 s; Curve 3, 3.5 min; Curve 4, 6 min; Curve 5, 8.5 min; Curve 6, 11.0 min; and Curve 7, 13.5 min after start of the reaction; for 3,4-dihydroxyphenylacetic acid: Curve 1, 45 s after start of the reaction. Curves 2–5 were recorded at 2.5 min interval after start of the reaction). Inset: Time course of formation and disappearance of 4-carboxymethyl-*o*-benzoquinone in the reaction mixture. The reaction was started at the time indicated by the arrow and monitored by the change in absorbance at 400 nm due to the quinone.

indicating the presence of *o*-dihydroxyphenolic group (Fig. 3). On treatment with nitrite–molybdate reagent of Arnow's (*13, 14*), it produced a red-colored complex which exhibited an absorption maximum at 500 nm confirming the presence of an *o*-diphenolic function (Fig. 3, inset). The ir spectrum of the compound in nujol (Fig. 4) exhibited absorptions at 3500–3100 cm^{-1} (broad, α,β-unsaturated OH and catecholic OH), 1620 and 1580 cm^{-1} (aromatic c-c-stretching), 1280 cm^{-1} (C–O–C), and 880 cm^{-1} (strong, isolated aromatic C–H). The NMR spectrum of the

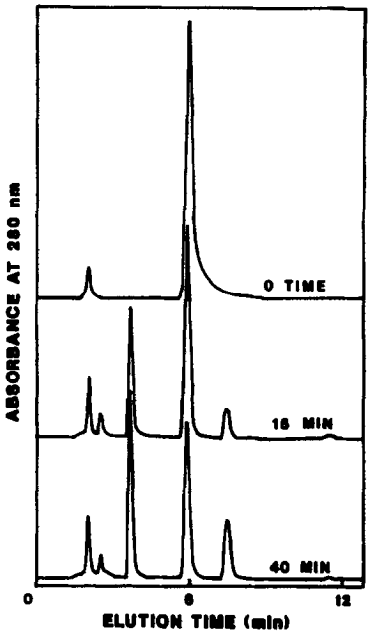

FIG. 2. HPLC of the reaction mixture containing tyrosinase and 3,4-dihydroxyphenylacetic acid. A reaction mixture containing 1 mM 3,4-dihydroxyphenylacetic acid and 10 μg of mushroom tyrosinase in 2 ml of 50 mM sodium phosphate buffer, ph 6.0, was incubated at room temperature. At indicated time intervals, 100 μl of aliquot of the above reaction mixture was withdrawn and mixed with 100 μl of 1 N acetic acid and 10 μl of this solution was subjected to HPLC analysis on a Waters' NovaPak C_{18} cartridge system. Separation of components was achieved with 50 mM acetic acid containing 0.2 mM sodium octylsulfonate in 20% methanol at a flow rate 0.6 ml/min. The void volume peak is due to salts; peaks at 2.59, 3.75, 5.98, and 7.59 min are due to 3,4-dihydroxymandelic acid, 2,5,6-trihydroxybenzofuran, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxybenzaldehyde, respectively.

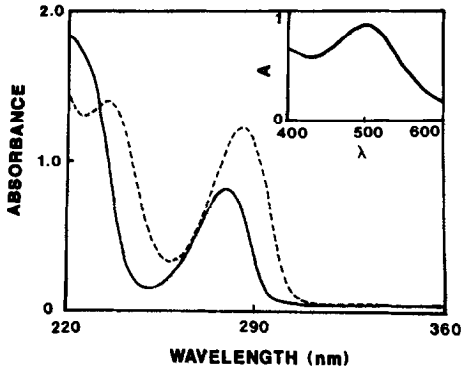

FIG. 3. Ultraviolet spectrum of 2,5,6-trihydroxybenzofuran in 0.2 M acetic acid (—) and 0.1 M sodium borate buffer, pH 8.6 (---). Inset: Visible spectrum of nitrite–molybdate complex of 2,5,6-trihydroxybenzofuran.

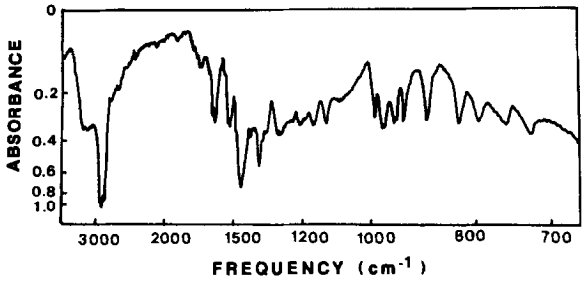

FIG. 4. Infrared spectrum of 2,5,6-trihydroxybenzofuran.

product in dimethyl sulfoxide-d_6 is shown in Fig. 5. The two catecholic hydroxyl protons exhibited broad absorption from δ = 8.00–9.40 ppm. The hydroxyl proton on the furan ring showed broad absorption at δ = 4.47–5.20 ppm. The signal due to these three protons disappeared after D_2O exchange confirming their assignment. The signal due to C–H (furan ring) appeared at δ = 4.20 as a singlet and the two aromatic protons exhibited signals at 6.40–6.78 as a multiplet. Based on these studies, the structure of this product was deduced to be 2,5,6-trihydroxybenzofuran.

The last peak (7.59 min; Fig. 2) had the same retention time as that of 3,4-dihydroxybenzaldehyde and eluted as a single symmetrical peak when cochromatographed with authentic sample under different HPLC conditions. Further, the uv and ir spectra of this product were indistinguishable from those of authentic 3,4-dihydroxybenzaldehyde. From these studies, this product was identified to be 3,4-dihydroxybenzaldehyde.

Tyrosinase-mediated 3,4-dihydroxyphenylacetate oxidation strictly required oxygen. Heat-inactivated enzyme did not catalyze the above conversions. Typical

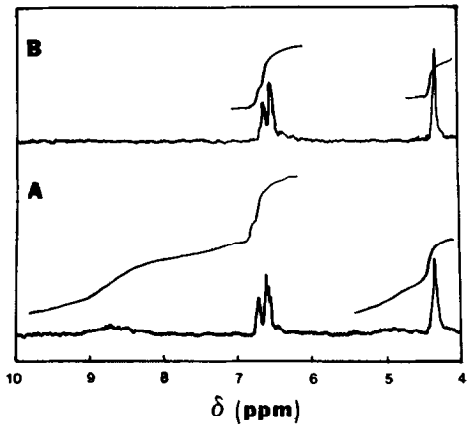

FIG. 5. NMR spectrum of 2,5,6-trihydroxybenzofuran (A) in dimethyl sulfoxide-d_6 and (B) in the same solvent after D_2O exchange.

TABLE 1

Effect of Various Phenoloxidase Inhibitors on the Enzymatic Oxidation of 3,4-Dihydroxyphenylacetic Acid

Compound added (Concn)	Inhibition (%)
None	0
Potassium cyanide (0.1 mM)	79
Potassium cyanide (0.5 mM)	97
Sodium azide (0.5 mM)	58
Sodium azide (5 mM)	95
Sodium fluoride (1 mM)	36
Sodium fluoride (5 mM)	42
Phenylthiourea (50 nM)	31
Phenylthiourea (0.5 μM)	87
Phenylthiourea (5.0 μM)	100

phenoloxidase inhibitors such as potassium cyanide, sodium azide, sodium fluoride, and phenylthiourea also inhibited the above conversions, indicating the participation of active site copper in the catalytic process (Table 1).

Generation of quinones as the major products of catechol oxidation by polyphenoloxidase is well established. However, the fate of various quinones formed has not been determined, with the exception of few compounds. Thus dopaquinone formed by the enzymatic oxidation of dopa undergoes a series of transformations to finally yield melanin pigments (*2*). Similar conversions also occur with quinones of dopamine, norepinephrine, and related compounds. In the case of other quinones, it is usually assumed that self-polymerization is their major fate of conversion. Contrary to this belief, present studies indicate that the quinone formed from 3,4-dihydroxyphenylacetate undergoes novel transformations in aqueous system to yield simple products such as 3,4-dihydroxymandelic acid and 2,5,6-trihydroxybenzofuran, which are further oxidized by tyrosinase present to 3,4-dihydroxybenzaldehyde and polymeric product(s), respectively.

The scheme shown in Fig. 6 accounts for the observed reactions. Tyrosinase-catalyzed oxidation of 3,4-dihydroxyphenyl acetate produces the initial product 4-carboxymethyl-*o*-benzoquinone which is characterized by its typical visible spectra (Fig. 1). The quinone thus formed, being unstable, undergoes further transformations. Intramolecular nucleophilic addition of the carboxylate group to the quinone yields the dienone **3** which on dienone–phenol rearrangement readily produces the lactone **4.** The lactone **4** undergoes rapid aromatization by a tautomerization reaction to yield the observed 2,5,6-trihydroxybenzofuran, **5.** The possibility of this lactone formation by an enzymatic hydroxylation of **1** at the 6 position followed by lactonization seems to be unlikely as tyrosinase can only hydroxylate a carbon adjacent to a hydroxyl or amine bearing aromatic carbon atom (*15*) and that lactonization in aqueous system by a nonenzymatic route is highly unlikely.

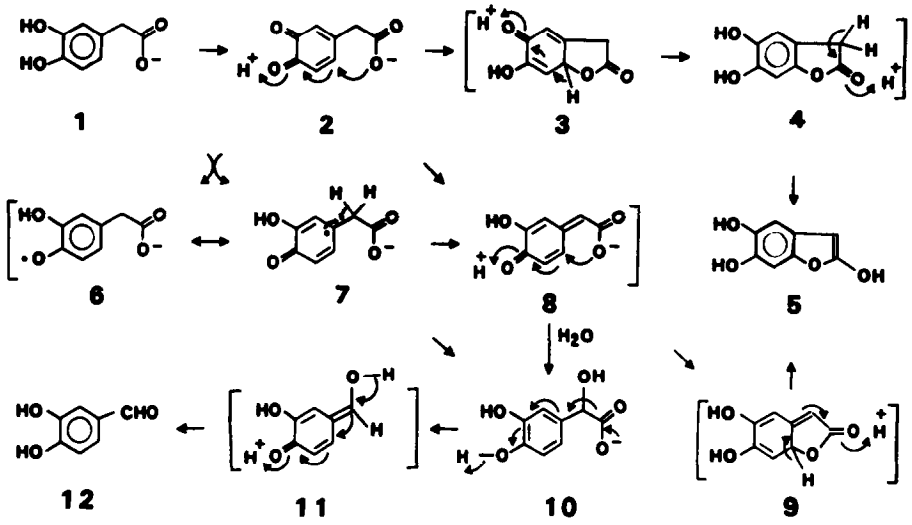

FIG. 6. Proposed mechanism for tyrosinase-catalyzed oxidation of 3,4-dihydroxyphenylacetic acid. Tyrosinase oxidizes 3,4-dihydroxyphenylacetate (**1**) to its quinone (**2**) which undergoes rapid intramolecular nucleophilic addition reaction to yield the dienone **3**. Dienone-phenol rearrangement of **3** produces the lactone **4** which readily tautomerizes to give observed 2,5,6-trihydroxybenzofuran (**5**). Isomerization of **2** to quinone methide **8** and cyclization of the latter can also produce **5** via **9**. Quinone methide formation by free radical pathway involving reverse dismutation of **1** and **2** to yield **6** and **7** and loss of electron from the radical is also likely. Hydration of **8** yields 3,4-dihydroxymandelic acid **10** which is oxidatively decarboxylated to 3,4-dihydroxybenzaldehyde **12** via **11** as outlined in an earlier publication (*9*).

The benzofuran can also be produced from the alternative route involving the quinone methide derivative **8** by cyclization reaction as shown in Fig. 6. The quinone methide itself can be formed from the quinone **2** by simple tautomerization. Such isomerization is facilitated by the presence of electron withdrawing quinone and carboxylate groups on the side chain methylene group. These substituents make the methylene group acidic and upon ionization the quinone transforms into the quinone methide. Another route for quinone methide **8** calls for the free radical intermediate **7** by the loss of a hydrogen radical. Since quinones are known to undergo reverse dismutation with parent catechols to produce semiquinone radicals (*16*), one cannot ignore the participation of free radical intermediates in quinone methide production. Once it is formed, quinone methide undergoes rapid hydration to yield 3,4-dihydroxymandelic acid. 3,4-Dihydroxymandelate formation could also occur by a direct enzymatic hydroxylation of the side chain of 3,4-dihydroxyphenylacetate similar to dopamine-β-hydroxylase reaction. However, tyrosinase is not known to perform such side chain hydroxylation. Besides, synthetic **2** upon stirring under anaerobic conditions afforded a single catecholic compound which exhibited the same properties as authentic 3,4-dihydroxymandelic acid. The uv and ir spectra of the compound were indistinguishable from those of authentic sample, confirming the identity of the product as 3,4-

dihydroxymandelic acid. The low yield was due to a number of side reactions of parent quinone such as (a) polymerization, (b) conversion to 2,5,6-trihydroxybenzofuran and its subsequent polymerization and (c) other unidentified reactions. Yet, the above experiment clearly demonstrates that quinone **2** can be nonenzymatically converted to 3,4-dihydroxymandelic acid under anaerobic conditions. Thus the quinone methide hydration route seems to be likely mechanism for the generation of 3,4-dihydroxymandelic acid from 3,4-dihydroxyphenylacetic acid. Tyrosinase oxidizes this compound further to 3,4-dihydroxybenzaldehyde as outlined in an earlier publication (*9*).

In general, carboxyl groups do not participate in Michael addition reaction with quinonoid compounds. However, in the case of carboxymethyl-*o*-benzoquinone (or its quinone methide), the internal carboxyl group seems to participate in such a reaction and hence its intramolecular cyclization readily yields the bicyclic product. Thus, the conversion of 3,4-dihydroxyphenyl acetic acid to 2,5,6-trihydroxybenzofuran represents a unique reaction and remarkably parallels the oxidative transformation of dopa and dopamine to 5,6-dihydroxyindole observed during eumalanin biosynthesis (*2, 4*). Like 5,6-dihydroxyindole, 2,5,6-trihydroxybenzofuran is also highly unstable and undergoes rapid oxidation to give polymeric pigments.

Cuticular polyphenoloxidase-catalyzed quinone methide formation was first reported by our group (*8*). Subsequently, we demonstrated that mushroom tyrosinase, which catalyzes the conversion of catechols to quinones, can also generate quinone methides provided suitable substrates are supplied to the enzyme. Thus, while oxidizing 1,2-dehydro-*N*-acetyldopamine to a novel benzodioxan compound, tyrosinase produced a quinone methide intermediate (*10*). The oxidative decarboxylation of 3,4-dihydroxymandelate to 3,4-dihydroxybenzaldehyde via quinone methide intermediate has already been reported from this laboratory (*9*) and is also observed in present study. Finally, as shown in Fig. 6, the formation of 3,4-dihydroxymandelate itself might proceed through a quinone methide intermediate. Currently we are investigating the mechanism of the newly discovered cyclization and side chain oxidation reactions.

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