

Structure of the Ring Cleavage Product of 1-Hydroxy-2-Naphthoate, an Intermediate of the Phenanthrene-Degradative Pathway of *Nocardioides* sp. Strain KP7

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1-Hydroxy-2-naphthoate (compound I) is a metabolite of the phenanthrene-degradative pathway in *Nocardioides* sp. strain KP7. This singly hydroxylated aromatic compound is cleaved by 1-hydroxy-2-naphthoate dioxygenase. In this study, the structure of the ring cleavage product generated by the action of homogeneous 1-hydroxy-2-naphthoate dioxygenase was determined upon separation by high-performance liquid chromatography at pH 2.5 by using nuclear magnetic resonance (NMR) and mass spectroscopic techniques. The ring cleavage product at this pH existed in equilibrium between two forms, 2-oxo-3-(3-oxo-1,3-dihydro-1-isobenzofuranyl)propanoate (compound III) and 2,2-dihydroxy-3-(3-oxo-1,3-dihydro-1-isobenzofuranyl)propanoate (compound IV). After the pH of the solution was raised to 7.5, the structure of the major species became (*E*)-4-(2-carboxylatophenyl)-2-oxo-3-butenate (compound II; common name, *trans*-2'-carboxybenzalpyruvate), which was in equilibrium with compound III. Direct monitoring of the enzymatic formation of the ring cleavage product by ¹H-NMR in a deuterated potassium phosphate buffer (pH 7.5) detected only compound II as a product, and the proton on carbon 3 of compound II was not exchanged with deuterium. Thus, compound II is likely to be the first stable product of dioxygenation of 1-hydroxy-2-naphthoate.

Biodegradation of polyaromatic hydrocarbons (PAHs) attracts much attention because they are contaminants frequently found in soils, aquifers, and sediments (2). Phenanthrene is a PAH which has often been used as a model for the biodegradation of PAHs (5). Two routes for phenanthrene degradation by bacteria have been described (1, 4, 8-10, 13). In one route, phenanthrene is converted via 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene, which is ring-cleaved by 1,2-dihydroxynaphthalene dioxygenase and further trans-

formed to salicylate. In the other route, phenanthrene is transformed to 1-hydroxy-2-naphthoate, which is ring cleaved by 1-hydroxy-2-naphthoate dioxygenase and further converted to *o*-phthalate.

The ring cleavage dioxygenases have an essential role in the decomposition of singly and doubly hydroxylated aromatic compounds (6, 7). Several ring cleavage dioxygenases have been purified and characterized, but few chemical structures of dioxygenase ring cleavage products have been characterized.

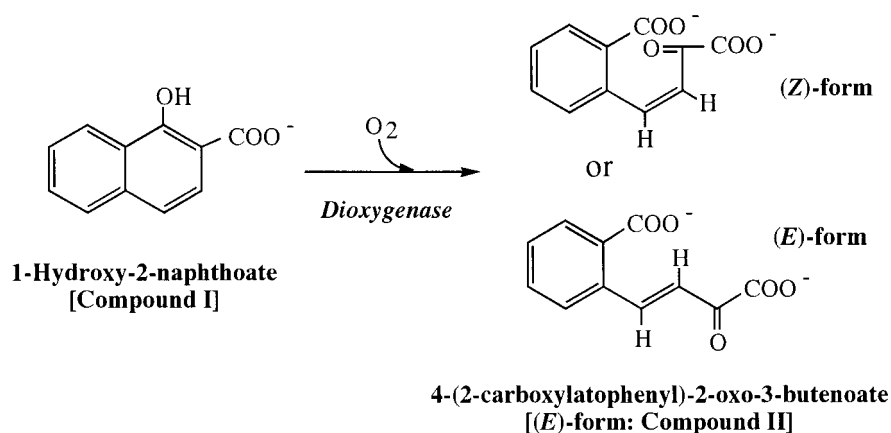


FIG. 1. Ring cleavage reaction of 1-hydroxy-2-naphthoate catalyzed by 1-hydroxy-2-naphthoate dioxygenase. The ring cleavage product is expected to be (*Z*)- or (*E*)-4-(2-carboxylatophenyl)-2-oxo-3-butenate (1, 8, 9, 14).

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TABLE 1. Purification of 1-hydroxy-2-naphthoate dioxygenase from *E. coli* containing pMKT290

Step	Vol (ml)	Protein concn (mg ml ⁻¹)	Total amt of protein (mg)	Sp act (μmol min ⁻¹ mg of protein ⁻¹)	Total activity (μmol min ⁻¹)	Yield (%)
Cell extract	65	17.2	1,118	12	13,304	100
First anion exchange ^a	10	8.0	80	86	6,880	52
Ammonium sulfate precipitation ^b	20	0.5	10	599	5,990	45
Second anion exchange ^c	2	1.7	3.4	1,567	5,328	40

^a Protein eluted on a TSKgel DEAE-5PW column (Tosoh).

^b Protein dissolved in a 10 mM Tris-H₂SO₄ buffer (pH 8.0) after precipitation by 2 M ammonium sulfate.

^c Protein eluted on a Mono Q HR5/5 column (Pharmacia).

The product of ring cleavage of 1,2-dihydroxynaphthalene by 1,2-dihydroxynaphthalene dioxygenase has been determined by nuclear magnetic resonance (NMR) analysis to be 2-hydroxychromene-2-carboxylate, which is further transformed by 2-hydroxychromene-2-carboxylate isomerase to *trans*-2'-hydroxybenzalpyruvate [IUPAC name, (*E*)-4-(2-hydroxyphenyl)-2-oxo-3-butenate] (3). In phenanthrene degradation, the aromatic ring of 1-hydroxy-2-naphthoate is cleaved by 1-hydroxy-2-naphthoate dioxygenase (10). The product of ring cleavage of 1-hydroxy-2-naphthoate by 1-hydroxy-2-naphthoate dioxygenase exhibits a high level of absorption at 300 nm, which is consistent with the presence of double bonds in a substituent conjugated with an aromatic ring (1, 14). Since pyruvate and 2-carboxybenzaldehyde have been detected as pathway intermediates, the chemical structure of the product of ring cleavage of 1-hydroxy-2-naphthoate by 1-hydroxy-2-naphthoate dioxygenase was suggested to be 2'-carboxybenzalpyruvate [IUPAC name, 4-(2-carboxylphenyl)-2-oxo-3-butenate] (1, 8, 9, 14) (Fig. 1). However, the geometrical structure of the ring cleavage product has not been investigated.

In the present study, we enzymatically produced the ring cleavage product of 1-hydroxy-2-naphthoate by using 1-hydroxy-2-naphthoate dioxygenase, and the geometrical structure of the ring cleavage product of 1-hydroxy-2-naphthoate was determined by NMR and fast atom bombardment-mass spectrometry (FAB-MS) analyses.

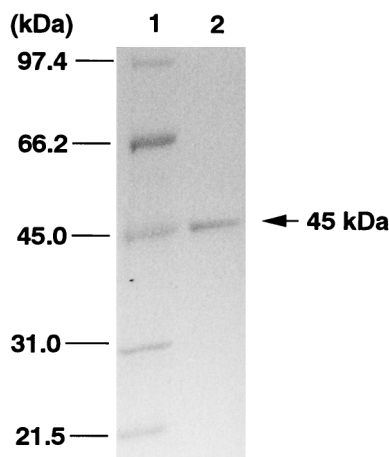


FIG. 2. SDS-PAGE of purified 1-hydroxy-2-naphthoate dioxygenase (45 kDa) from extracts of *E. coli* JM109(pMKT290). Lanes: 1, molecular mass markers; 2, pooled active fractions after the second anion-exchange column chromatography step (Mono Q column).

MATERIALS AND METHODS

Chemical. 1-Hydroxy-2-naphthoic acid was purchased from Tokyo Organic Chemicals (Tokyo, Japan).

Purification of 1-hydroxy-2-naphthoate dioxygenase. The pMKT290 plasmid is a derivative of pUC18 carrying the structural gene for 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. strain KP7 (10, 11). Cells of *Escherichia coli* JM109 containing pMKT290 were cultivated in 10 liters of L broth containing 50 μg of ampicillin ml⁻¹ (10). Cells from the overnight culture were pelleted by centrifugation at 10,000 × g for 20 min at 4°C, resuspended in 65 ml of a 10 mM Tris-H₂SO₄ buffer (pH 7.5), and disrupted by passage through a precooled French pressure cell (Ohtake Works, Tokyo, Japan) at a pressure of 800 kg/cm². The cell extract was centrifuged at 27,700 × g for 30 min at 4°C, and the

TABLE 2. NMR spectra for the products recorded in D₂O^a

Compound	Position	¹³ C δ	¹ H δ ^b
II (pH 7.5)	1	172.83	
	2	198.00	
	3	124.53	6.91 (d,16.4)
	4	148.99	8.05 (d,16.4)
	1'	131.40	
	2'	142.03	
	3'	128.22	7.51 (m)
	4'	131.99	7.50 (m)
	5'	130.04	7.49 (m)
	6'	128.03	7.81 (d, 7.1)
	2'-C=O	177.81	
III (pH 2.5)	1	168.69	
	2	201.44	
	3	43.67	3.60 (dd,18.3,4.4)
			3.40 (dd,18.3,7.9)
	1'	78.65	6.10 (dd,7.9,4.4)
	3'	173.96	
	4'	149.91	
	5'	125.94	7.93 (d,6.6)
	6'	130.23	7.65 (t,7.6)
	7'	135.86	7.82 (t,7.6)
8'	122.91	7.64 (d,7.6)	
9'	125.27		
IV (pH 2.5)	1	174.15	
	2	93.84	
	3	43.5	2.69 (dd,15.1,2.7)
			2.36 (dd,15.1,9.3)
			5.85 (dd, 9.3,2.7)
	1'	79.65	
	3'	175.05	
	4'	150.53	
	5'	125.85	7.91 (d,7.6)
	6'	130.11	7.65 (t,7.6)
	7'	135.72	7.83 (t,7.6)
8'	123.01	7.64 (d, 7.6)	
9'	125.00		

^a pH was adjusted with DCl and NaOD. Trimethylsilylpropionic acid sodium salt was used as an external standard.

^b Multiplicity (*J*), in hertz.

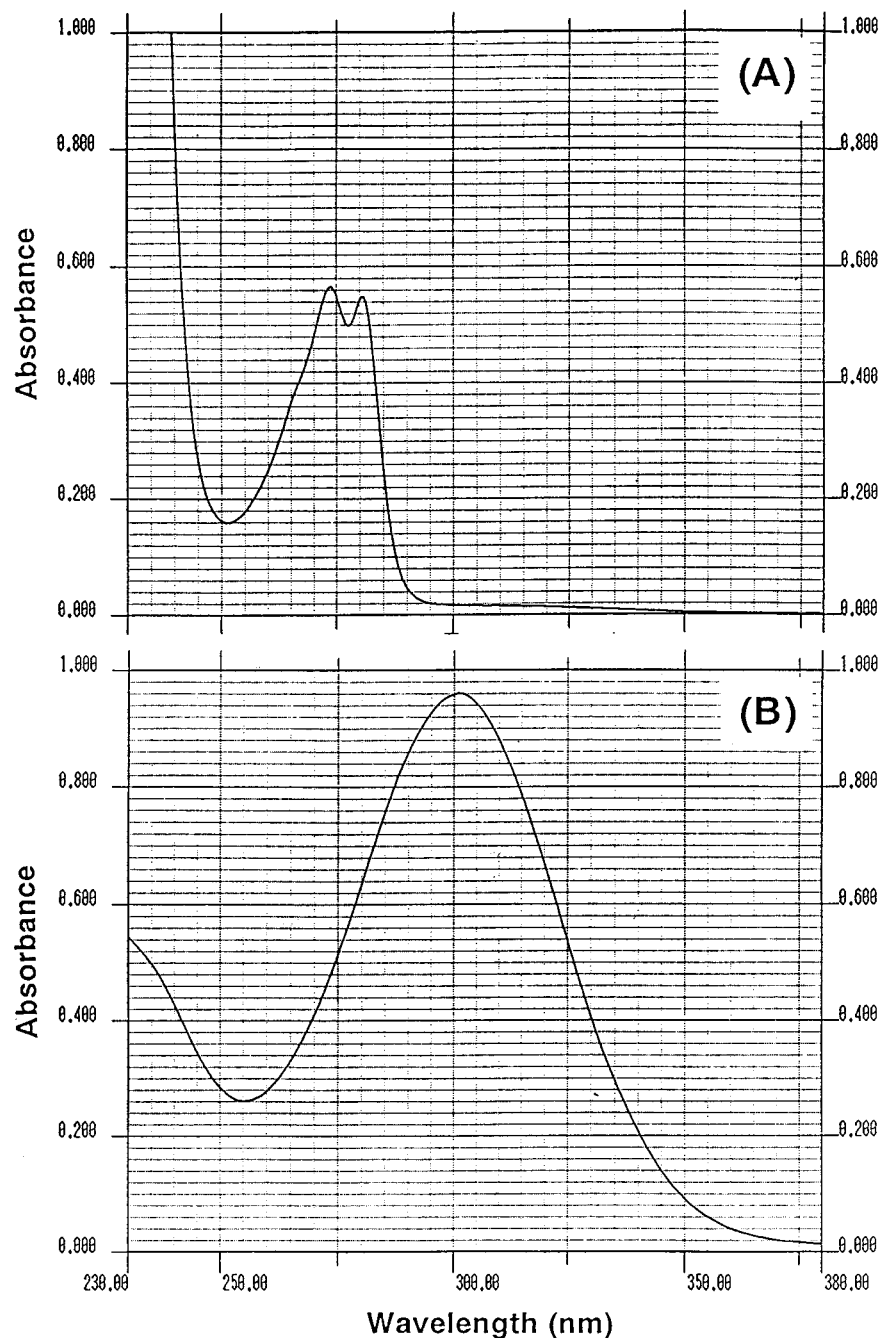


FIG. 3. UV spectra of the purified ring cleavage product at pH 2.5 (in a 50 mM potassium phosphate buffer acidified by 10 N HCl) (A) and pH 7.5 (in a 50 mM potassium phosphate buffer) (B).

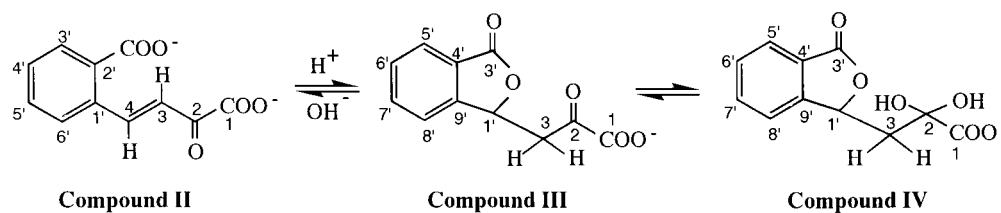


FIG. 4. Structures of the ring cleavage products identified by NMR and FAB-MS analyses. In a solution at pH 2.5, compounds III and IV were revealed. When the pH of the solution was raised to pH 7.5, compounds II and III were revealed, with compound II being the major species. Compound II, (*E*)-4-(2-carboxylatophenyl)-2-oxo-3-butenate (common name, *trans*-2'-carboxybenzalpyruvate); compound III, 2-oxo-3-(3-oxo-1,3-dihydro-1-isobenzofuranyl)propanoate; compound IV, 2,2-dihydroxy-3-(3-oxo-1,3-dihydro-1-isobenzofuranyl)propanoate.

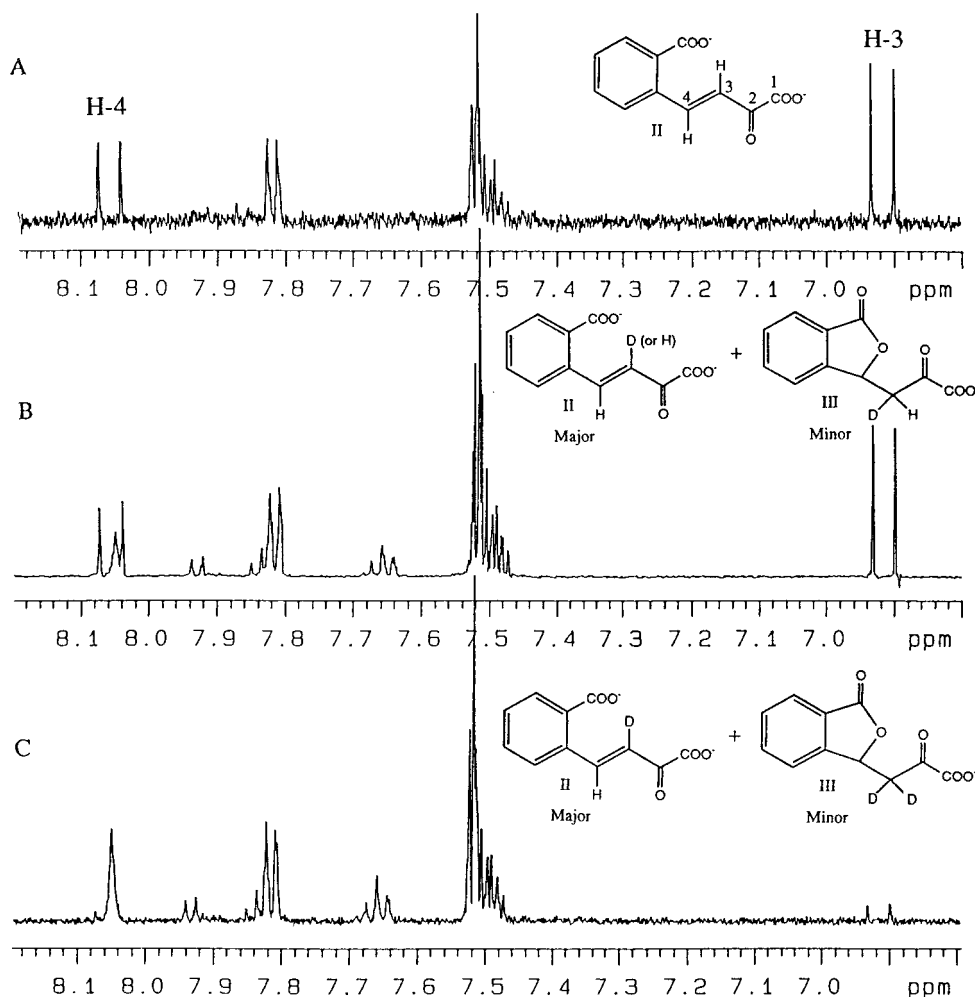


FIG. 5. ^1H -NMR signals of the freshly prepared ring cleavage product of 1-hydroxy-2-naphthoate (pH 7.5) (A), the ring cleavage product neutralized for 1 h at pH 7.5 after its purification at pH 2.5 (B), and the same sample after incubation in deuterated buffer for two more weeks at pH 7.5 (C).

supernatant was recentrifuged at $250,000 \times g$ for 60 min at 4°C . The supernatant was filtered through a Sterivex-HV filter (0.45- μm pore size; Millipore, Bedford, Mass.), and loaded into an anion-exchange column (TSKgel DEAE-5PW, 21.5 mm [inside diameter {i.d.}] by 150 mm; Tosoh, Tokyo, Japan) fitted to a high-performance liquid chromatography (HPLC) system (Tosoh). The protein was eluted by a linear gradient of 0 to 0.5 M Na_2SO_4 in 300 ml of a 10 mM Tris- H_2SO_4 buffer (pH 7.5) at a flow rate of 5 ml min^{-1} . The eluate was collected in 5-ml fractions on ice. Ammonium sulfate was added to the pooled fractions containing the 1-hydroxy-2-naphthoate dioxygenase activity to achieve a final concentration of 0.6 M at 4°C , and proteins which precipitated were removed by centrifugation at $27,700 \times g$ for 30 min at 4°C . The 1-hydroxy-2-naphthoate dioxygenase activity was recovered in the supernatant. The supernatant was filtered through a Millex-GV filter (0.45- μm pore size; Millipore) and loaded into a hydrophobic-interaction column (TSKgel Phenyl-5PW, 21.5 mm [i.d.] by 150 mm; Tosoh) pre-equilibrated with a 10 mM Tris- H_2SO_4 buffer (pH 7.5) containing 0.6 M ammonium sulfate. Proteins were eluted from the column by a linear gradient from 0.6 to 0 M ammonium sulfate in 60 ml of a 10 mM Tris- H_2SO_4 buffer (pH 7.5) at a flow rate of 1 ml min^{-1} . Ammonium sulfate was added to the pooled fractions containing the 1-hydroxy-2-naphthoate dioxygenase activity to achieve a final concentration of 2 M at 4°C , and precipitated proteins were dissolved in 20 ml of a 10 mM Tris- H_2SO_4 buffer (pH 8.0) at 4°C . The dissolved proteins were loaded on an anion-exchange column (Mono Q HR5/5, 5 mm [i.d.] by 50 mm; Pharmacia, Uppsala, Sweden) fitted to an HPLC system (Tosoh). Finally, purified protein was eluted by a linear gradient of 0 to 0.5 M Na_2SO_4 in 60 ml of a 10 mM Tris- H_2SO_4 buffer (pH 8.0) at a flow rate of 1 ml min^{-1} . Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a premade polyacrylamide slab gel (Multigel Gel 4/20; Dai-ichi Pure Chemicals, Tokyo, Japan), as described previously (10).

Purification and identification of the ring cleavage product. Purified recombinant 1-hydroxy-2-naphthoate dioxygenase (2.7 mg) was added to 1,600 ml of a

10 mM Tris-HCl buffer (pH 7.5) containing $150 \mu\text{M}$ 1-hydroxy-2-naphthoate. The progression and completion of the reaction at room temperature were monitored spectrophotometrically by measuring A_{300} (1, 10, 14). After acidification of the reaction mixture with approximately 2 ml of 10 N HCl to pH 2.5, the reaction mixture was extracted with 800 ml of chloroform and then with 800 ml of ethyl acetate. The chloroform and ethyl acetate phases were combined, and organic solvents were removed in vacuo. The dried sample was dissolved in 10 ml of 10% aqueous methanol containing 0.1% (vol/vol) H_3PO_4 and injected onto a C_{18} column (CapcellPak-C18 type SG120, 4.6 mm [i.d.] by 250 mm; Shiseido, Tokyo, Japan) fitted to an HPLC system (Tosoh) at 25°C . The elution profile was monitored at 280 nm. The ring cleavage product was eluted by a linear solvent gradient of 10 to 90% (vol/vol) methanol in 15 ml of ultrapure water containing 0.1% (vol/vol) H_3PO_4 at a flow rate of 1 ml min^{-1} . The reaction product was eluted at 50% (vol/vol) methanol. Methanol was removed from the purified fraction in vacuo, the dried sample was dissolved in D_2O , and its pH was adjusted with DCl or NaOD.

UV spectroscopy of the purified reaction product. UV spectra of the purified reaction product at different pH values were measured by using a UV spectrophotometer (UV2000; Shimadzu, Kyoto, Japan) at 25°C . The purified sample was dissolved in a 50 mM potassium phosphate buffer (pH 7.5) or a 50 mM potassium phosphate buffer acidified by 10 N HCl to pH 2.5.

NMR and MS. All NMR spectra were acquired on a Unity 500 spectrometer (Varian, Palo Alto, Calif.) at 30°C . Two-dimensional experiments such as ^1H , ^1H -correlated spectroscopy (COSY), heteronuclear single-quantum coherence spectroscopy (HSQC), and heteronuclear multibond correlation spectroscopy (HMBC) were used for structure determination of the product. FAB-MS was done with a JMS-SX102 mass spectrometer (JEOL, Tokyo, Japan).

Monitoring of enzymatic reaction by ^1H -NMR. Transformation of 1-hydroxy-2-naphthoate in the reaction catalyzed by 1-hydroxy-2-naphthoate dioxygenase was monitored by ^1H -NMR for 180 min at 30°C . Each data set was obtained after

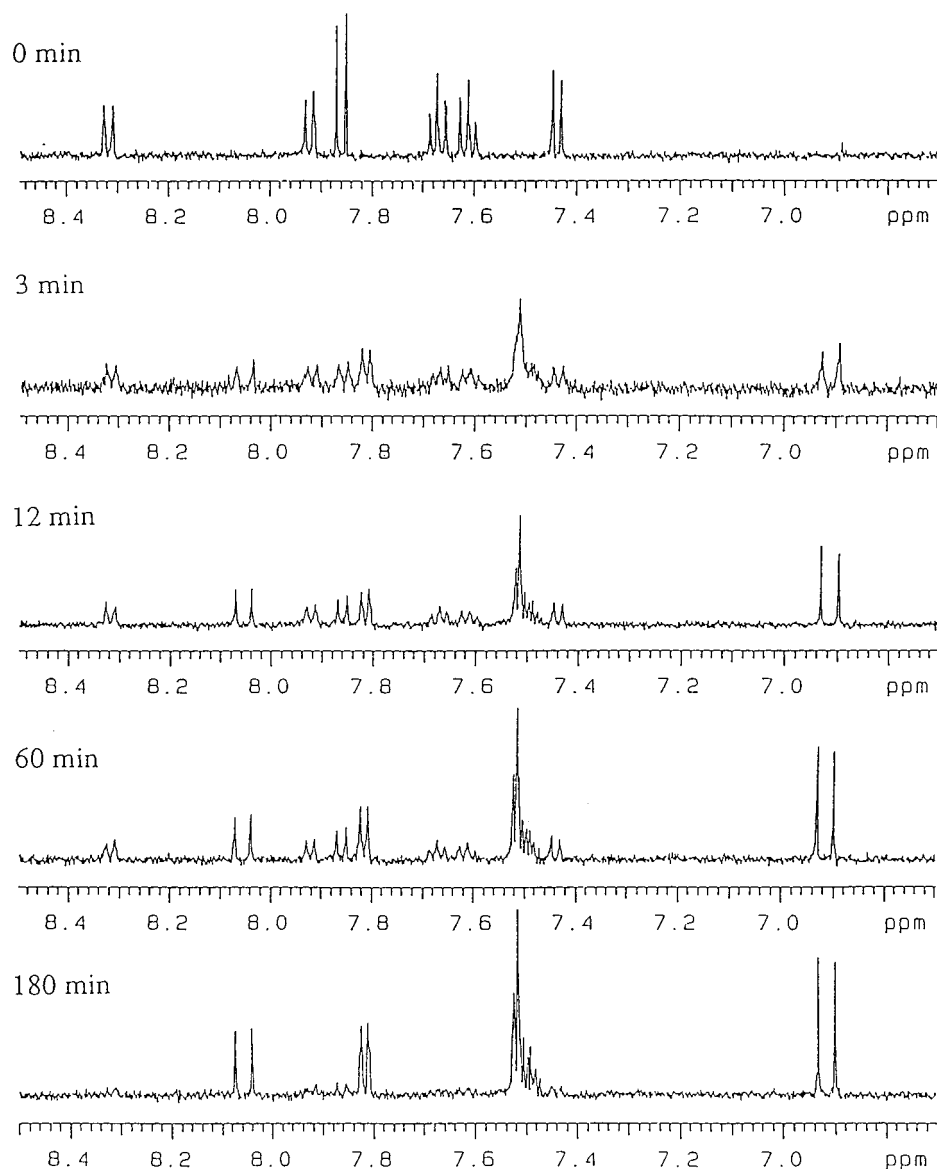


FIG. 6. Time course of the 1-hydroxy-2-naphthoate dioxygenase reaction monitored by $^1\text{H-NMR}$ at pH 7.5 in 50 mM deuterated potassium phosphate buffer.

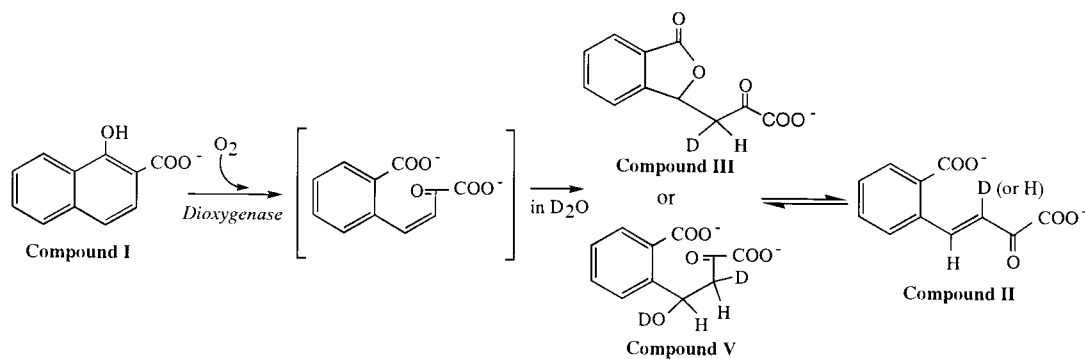


FIG. 7. Possible mechanisms of spontaneous isomerization of a hypothetical primary product [(Z)-4-(2-carboxylatophenyl)-2-oxo-3-butenate] to (E)-4-(2-carboxylatophenyl)-2-oxo-3-butenate (compound II) in D_2O . Compounds III and V are hypothetical intermediates in the nonenzymatic conversion from (Z)-4-(2-carboxylatophenyl)-2-oxo-3-butenate to (E)-4-(2-carboxylatophenyl)-2-oxo-3-butenate.

32 scans. The reaction was carried out in 700 μ l of 50 mM deuterated potassium phosphate buffer (pH 7.5) containing 0.56 mM 1-hydroxy-2-naphthoate and 0.5 mg of 1-hydroxy-2-naphthoate dioxygenase.

RESULTS AND DISCUSSION

Purification of 1-hydroxy-2-naphthoate dioxygenase from *E. coli*. An extract of *E. coli* JM109(pMKT290) contained 1-hydroxy-2-naphthoate dioxygenase activity at 12 μ mol min⁻¹ mg of protein⁻¹. This enzyme was purified from extracts of *E. coli* JM109(pMKT290) as shown in Table 1. The enzyme activity was eluted from the first anion-exchange column (DEAE column) by Na₂SO₄ at 0.11 M, and the activity was eluted from the second anion-exchange column (Mono Q column) by Na₂SO₄ at 0.2 M. The enzyme activity was broadly eluted from a phenyl column. After the second anion-exchange chromatography, the purified enzyme gave a single protein band at 45 kDa by SDS-PAGE (Fig. 2). The specific activity of the purified enzyme was 1.6 mmol min⁻¹ mg of protein⁻¹. The transformation of 1-hydroxy-2-naphthoate by the purified enzyme was spectrophotometrically examined, and the spectrum of 1-hydroxy-2-naphthoate changed immediately to one showing a maximal absorption at 300 nm (data not shown). Thus, it was concluded that the purified enzyme exhibits 1-hydroxy-2-naphthoate dioxygenase activity (1, 10, 14).

Chemical structures of the ring cleavage products of 1-hydroxy-2-naphthoate. The enzymatically prepared ring cleavage product of 1-hydroxy-2-naphthoate was purified by reverse-phase HPLC. The yield of the purified ring cleavage product was 70%, and the purity of the purified product was 94.4% by HPLC analysis. UV spectra of the purified product at pH 2.5 and 7.5 are shown in Fig. 3A and B, respectively. The UV spectrum at pH 7.5 showed a high level of absorption at 300 nm; however, the spectrum at pH 2.5 showed two absorptions at 274 and 281 nm (Fig. 3A and B). These results indicate that the reaction product has different forms at two pH values.

The ¹H- and ¹³C-NMR spectra of the product were recorded as described in Materials and Methods. The assignment of ¹H- and ¹³C-NMR signals of the ring cleavage product at two different pH values is shown in Table 2. The initial pH of the purified material was 2.5. At this pH, two molecular species, 2-oxo-3-(3-oxo-1,3-dihydro-1-isobenzofuranyl)propanoate (compound III in Table 2 and Fig. 4) and 2,2-dihydroxy-3-(3-oxo-1,3-dihydro-1-isobenzofuranyl)propanoate (compound IV in Table 2 and Fig. 4), were resolved by ¹H-NMR, ¹³C-NMR, and other two-dimensional NMR analyses including COSY, HSQC, and HMBC. These structures were supported by the negative FAB-mass spectrum at pH 2.5 showing molecular ions at *m/z* values of 219 and 237 (M-H)⁻. When the solution was neutralized to pH 7.5 with NaOD, compound IV was not detected and compound III became a minor species. Instead, (*E*)-4-(2-carboxylatophenyl)-2-oxo-3-butenate (compound II in Table 2, Fig. 1, and Fig. 4) was revealed as a major species. The (*E*) form of compound II was deduced from the coupling constant of (³J₃₋₄) 16.4 Hz (Table 2). The negative FAB-mass spectrum of this compound showed a molecular ion at an *m/z* of 219 (M-H)⁻. Thus, the ring cleavage product of 1-hydroxy-2-naphthoate took at least three different structures at different pH values. Two forms, compound III and compound IV, were in equilibrium under acidic conditions, while at neutral pH, compound III and compound II were in equilibrium, the latter compound being the major species (Fig. 5B).

Direct monitoring of the formation of the reaction product by ¹H-NMR. The transformation of 1-hydroxy-2-naphthoate to its ring cleavage product in a reaction catalyzed by 1-hydroxy-2-naphthoate dioxygenase was monitored in real time by ¹H-

NMR. The intensities of the signals corresponding to 1-hydroxy-2-naphthoate decreased as the reaction progressed. As shown in Fig. 6, a single product, (*E*)-4-(2-carboxylatophenyl)-2-oxo-3-butenate (compound II), was formed and was stable for at least 180 min under the experimental conditions. In this reaction, 95% of the substrate (1-hydroxy-2-naphthoate; compound I) was converted to the reaction product (compound II). Thus, compound II is either the primary product of the reaction catalyzed by 1-hydroxy-2-naphthoate dioxygenase or a stable derivative of a hypothetical primary product.

Deuterium exchange of the ring cleavage product. The ¹H-NMR spectrum of compound II (prepared as described above) is presented in Fig. 5A. In Fig. 5B, the ¹H-NMR spectrum of the mixture of compound II and compound III obtained after purification of the ring cleavage product at pH 2.5 and neutralization to pH 7.5 is presented. The H4 signals in compound II (δ , 8.05 ppm) are different between Fig. 5A and B. In Fig. 5A, H4 is a doublet, and in Fig. 5B a broad single peak has appeared inside the double peak for H4. This result indicates that the proton at position 3 of compound II was partly displaced by deuterium during the acidification-neutralization cycle (Fig. 5B). The sample whose spectrum is shown in Fig. 5B was kept in deuterated buffer at pH 7.5 for two more weeks at room temperature in the dark, and a ¹H-NMR spectrum was again recorded (Fig. 5C). The spectrum in Fig. 5C indicates that the proton at position 3 was exchangeable and was completely displaced by deuterium after 2 weeks.

From the structure of 1-hydroxy-2-naphthoate (compound I), it is reasonable to assume that the primary product is (*Z*)-4-(2-carboxylatophenyl)-2-oxo-3-butenate (Fig. 1), which is subsequently transformed into its (*E*) isomer (compound II) by a spontaneous or enzyme-catalyzed reaction. A model for (*Z*)-(*E*) nonenzymatic isomerization reactions in D₂O is depicted in Fig. 7. If the reaction in Fig. 7 occurs, the proton at position 3 of compound II should be exchanged. In fact, the transformation of compound III to compound II in the deuterated buffer displaced the proton at position 3 of the product (Fig. 5B). However, the proton at position 3 of compound II formed by the enzymatic reaction in the deuterated buffer was not displaced by deuterium (Fig. 5A and 6). From these observations, we eliminated the spontaneous nonenzymatic reactions following the enzymatic reaction with dioxygenase as shown in Fig. 7 from the possible (*Z*)-(*E*) isomerization mechanisms of the ring cleavage product. Probably, the (*Z*)-(*E*) isomerization reaction is a part of the catalytic mechanism of 1-hydroxy-2-naphthoate dioxygenase.

The chemical structures of the three different ring cleavage products have been determined. In the study of Eaton and Chapman (3), the product was isolated without changing the pH value, while in the study of Junker et al. (12), the product was isolated at pH 1.5. It is advisable to isolate such products without acidification, as this treatment may provoke the isomerization demonstrated in this study.

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