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## Metabolism of 2,2'-Dihydroxybiphenyl by *Pseudomonas* sp. Strain HBP1: Production and Consumption of 2,2',3-Trihydroxybiphenyl

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Cells of *Pseudomonas* sp. strain HBP1 grown on 2-hydroxy- or 2,2'-dihydroxybiphenyl contain NADH-dependent monooxygenase activity that hydroxylates 2,2'-dihydroxybiphenyl. The product of this reaction was identified as 2,2',3-trihydroxybiphenyl by <sup>1</sup>H nuclear magnetic resonance and mass spectrometry. Furthermore, the monooxygenase activity also hydroxylates 2,2',3-trihydroxybiphenyl at the C-3' position, yielding 2,2',3,3'-tetrahydroxybiphenyl as a product. An extradiol ring cleavage dioxygenase activity that acts on both 2,2',3-tri- and 2,2',3,3'-tetrahydroxybiphenyl was partially purified. Both substrates yielded yellow *meta*-cleavage compounds that were identified as 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid and 2-hydroxy-6-(2,3-dihydroxyphenyl)-6-oxo-2,4-hexadienoic acid, respectively, by gas chromatography-mass spectrometry analysis of their respective trimethylsilyl derivatives. The *meta*-cleavage products were not stable in aqueous incubation mixtures but gave rise to their cyclization products, 3-(chroman-4-on-2-yl)pyruvate and 3-(8-hydroxychroman-4-on-2-yl)pyruvate, respectively. In contrast to the *meta*-cleavage compounds, which were turned over to salicylic acid and 2,3-dihydroxybenzoic acid, the cyclization products are not substrates to the *meta*-cleavage product hydrolase activity. NADH-dependent salicylate monooxygenase activity catalyzed the conversions of salicylic acid and 2,3-dihydroxybenzoic acid to catechol and pyrogallol, respectively. The partially purified extradiol ring cleavage dioxygenase activity that acted on the hydroxybiphenyls also produced 2-hydroxymuconic semialdehyde and 2-hydroxymuconic acid from catechol and pyrogallol, respectively.

Biphenyl and 2-hydroxybiphenyl have been used extensively as volatile fungicides, especially for the control of postharvest diseases (1). Their fungal and mammalian metabolism has been previously investigated thoroughly (7, 11), and it was found that the most important transformation reactions proceed via hydroxylation of the aromatic ring, whereby various hydroxylated biphenyls are produced. Mono-, di-, and trihydroxylated chlorinated biphenyls may be generated by cometabolism of polychlorinated biphenyls with biphenyl-grown bacteria (6). In order to gain better insight into the metabolic fate of such compounds in bacteria, we have previously investigated bacterial growth on 2-hydroxy- and 2,2'-dihydroxybiphenyl (9). Subsequently, isolates that were able to grow on 3-hydroxy-, 3,3'-dihydroxy-, and 4-hydroxybiphenyl were described (8). Two major routes for the bacterial metabolism of hydroxybiphenyls were recognized. One route proceeds via hydroxylation of the already hydroxylated aromatic ring by an NADH-dependent monooxygenase followed by *meta* cleavage of 2,3-dihydroxybiphenyl. This route seems to be employed by the strains isolated on 2-hydroxy- and 3-hydroxybiphenyl (8, 9). The other route proceeds via dioxygenation of the nonhydroxylated aromatic ring, subsequent rearomatization, and *meta* cleavage. This pathway is utilized by *Pseudomonas* sp. strain FH23 (8), as 2,3,4'-trihydroxybiphenyl could be detected as an intermediate when the strain grew on 4-hydroxybiphenyl. *Pseudomonas testosteroni* B-356 (15), a biphenyl-chlorobiphenyl degrader, metabolized all three

monohydroxybiphenyls via the reactions of the second route.

We have previously suggested that 2,2',3-trihydroxybiphenyl is the first intermediate in the degradation of 2,2'-dihydroxybiphenyl by *Pseudomonas* sp. strain HBP1 (9). This was formulated as an analogy to the formation of 2,3-dihydroxybiphenyl from 2-hydroxybiphenyl by the NADH-dependent monooxygenase. So far, the identity of the product of the monooxygenase reaction with 2,2'-dihydroxybiphenyl has not been proven. Definitive proof of the suggested structure of the intermediate is important, because 2,2',3-trihydroxybiphenyl has been recently identified as the first detectable intermediate of the bacterial metabolism of dibenzofuran by *Pseudomonas* sp. strain HH69 (4) as well as by *Brevibacterium* sp. strain DPO 1361 (16) and it has been shown that it is the product of a novel degradation mechanism involving the angular dioxygenation of dibenzofuran (2). The fact that the two pathways, the one for 2,2'-dihydroxybiphenyl degradation and the one for dibenzofuran degradation, share the same intermediate makes comparative studies most important, because, in analogy to aerobic polychlorinated biphenyl transformation reactions, during which it was shown that biphenyl-oxidizing enzymes fortuitously oxidize chlorinated analogs, dibenzofuran was chosen as the model compound to elucidate the metabolic steps involved in the breakdown of polychlorinated dibenzofurans (3). Knowledge of the bacterial metabolism and the environmental fate of these compounds is desired because they are industrial by-products which have been identified as contaminants in almost every component of the global ecosystem (12, 14) and because they constitute a severe environmental hazard because of their high toxicity.

It has been proposed previously that in the course of the

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metabolic breakdown of dibenzofuran in *Brevibacterium* sp. strain DPO 1361 and *Pseudomonas* sp. strain HH69, 2,2',3-trihydroxybiphenyl is *meta* cleaved (4, 16). The investigators were able to identify a metabolite (metabolite M) as the cyclization product of the *meta*-cleavage product of 2,2',3-trihydroxybiphenyl, but direct evidence for the presence of the *meta*-cleavage product was not provided. Here we present, along with final proof of the formation of 2,2',3-trihydroxybiphenyl from 2,2'-dihydroxybiphenyl in *Pseudomonas* sp. strain HBP1, direct evidence for the *meta* cleavage of 2,2',3-trihydroxybiphenyl.

## MATERIALS AND METHODS

**Media and growth conditions.** The isolation and growth of *Pseudomonas* sp. strain HBP1 on 2-hydroxy- and 2,2'-dihydroxybiphenyl have been described in a previous article (9). The mineral salts medium used for growth consisted of 20 mM phosphate buffer ( $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , pH 7.2), 0.125 g of  $(\text{NH}_4)_2\text{SO}_4$ , 0.025 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.03125 g of  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  per liter of deionized water, supplemented with 1 ml of a trace element stock solution containing the following (in grams per liter):  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.00;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 1.00;  $\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$ , 0.25;  $\text{H}_3\text{BO}_3$ , 0.10;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.25;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25;  $\text{NH}_4\text{VO}_3$ , 0.10;  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 0.5; and  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ , 0.010. The stock solution also contained 5.0 ml of concentrated  $\text{H}_2\text{SO}_4$ . 2,2'-Dihydroxybiphenyl (500 mg/liter, if not indicated otherwise) was added after sterilization to minimize volatile losses.

**Chemicals.** 2,3-Dihydroxybiphenyl was obtained from Wako Chemicals GmbH, Neuss, Germany. 2-Hydroxymuconic acid was a gift from A. Cook, Institute for Microbiology, ETHZ, Zürich, Switzerland. All other chemicals used were purchased from Fluka Chemie AG, Buchs, Switzerland.

**Preparation of washed-cell suspensions and cell extract.** Cells were grown in 20-liter carboys equipped with a magnetic stirring bar and forced aeration. One drop of sterile polypropylene glycol was added after sterilization to prevent foaming. Cells were harvested in the late-exponential growth phase by centrifugation (15 min at  $6,000 \times g$ ) at  $4^\circ\text{C}$ , washed with an excess amount of phosphate buffer (20 mM, pH 7.2), and resuspended in the same buffer (0.2 g [wet weight]  $\text{ml}^{-1}$ ). For the preparation of crude cell extract, washed cells were broken by means of a French press (two passages,  $20,000 \text{ lb in}^{-2}$ ), followed by centrifugation (40 min at  $40,000 \times g$ ) at  $4^\circ\text{C}$ .

**Separation of enzyme activities.** The different enzymes were partially purified by protamine sulfate precipitation and ion-exchange chromatography. Protamine sulfate was added (0.05 mg/mg of protein, dissolved in 20 mM phosphate buffer, pH 7.2) to the crude cell extract and stirred for 30 min at  $4^\circ\text{C}$ . The precipitated biopolymers (DNA, basic proteins) were removed by centrifugation (30 min at  $40,000 \times g$ ) at  $4^\circ\text{C}$ . The supernatant was desalted on a Sephadex G-25M column (Pharmacia, Uppsala, Sweden) and subsequently fractionated by anion-exchange chromatography with a fast protein liquid chromatography system consisting of a 2152 LC controller, two 2150 HPLC pumps, and a 2212 helirac sample collector (all from Pharmacia, Uppsala, Sweden). The sample was applied to a Mono-Q anion-exchange column (HR5/5) and eluted with a linear gradient of 0 to 1 M NaCl in 40 ml of triethanolamine buffer (10 mM, pH 7.5, 1  $\text{ml min}^{-1}$ ). The protein profile was measured at 280 nm with a 655A variable-wavelength UV monitor (Hitachi, Tokyo,

Japan). After the fractions were collected, enzyme activities were determined as described below.

**Enzyme assays.** One unit of enzyme activity was defined as the amount of enzyme converting 1  $\mu\text{mol}$  of substrate per min.

NADH-dependent 2-hydroxybiphenyl monooxygenase was measured spectrophotometrically by monitoring NADH disappearance at 340 nm. The reaction mixture contained 20 mM phosphate buffer (pH 7.2), 0.2 mM NADH, 50  $\mu\text{l}$  of the appropriate protein fraction, and 0.1 mM substrate. The reaction was usually started by the addition of a substrate (10  $\mu\text{l}$  of a methanol solution).

Extradiol ring cleavage dioxygenase activity in phosphate buffer (20 mM, pH 7.5) was measured spectrophotometrically by monitoring the increase of absorption, which corresponded to the accumulation of the ring *meta*-cleavage products. The following extinction coefficients for various *meta*-cleavage products were used: 2-hydroxymuconic semialdehyde (*meta*-cleavage product of catechol) ( $\lambda_{\text{max}}$ , 375 nm),  $36,000 \text{ M}^{-1} \text{ cm}^{-1}$  (16), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta*-cleavage product of 2,3-dihydroxybiphenyl) ( $\lambda_{\text{max}}$ , 434 nm),  $22,000 \text{ M}^{-1} \text{ cm}^{-1}$  (16).

The *meta*-cleavage product hydrolase was assayed spectrophotometrically by monitoring the decrease in absorption of the different *meta*-cleavage products (extinction coefficients are described above).

Salicylate monooxygenase in cell extracts was measured spectrophotometrically by monitoring the appearance of 2-hydroxymuconic semialdehyde. This method was employed because, on the one hand, the activity of metapyrocatechase was present at such high levels that direct measurement of NADH at 340 nm was prevented by the interference of 2-hydroxymuconic semialdehyde and, on the other hand, the requirements for a coupled assay were set by the fourfold-higher activity of the coupling reaction (metapyrocatechase). With 2,3-dihydroxybenzoic acid as a substrate, direct measurement of NADH disappearance was possible, because the reaction product did not interfere with the  $A_{340}$ . The reaction mixture contained 20 mM phosphate buffer (pH 7.2), 0.2 mM NADH, 10  $\mu\text{l}$  of cell extract, and 0.1 mM substrate. The reaction was started by the addition of substrate (10  $\mu\text{l}$  of a methanol solution).

**Production of 2,2',3-tri- and 2,2',3,3'-tetrahydroxybiphenyl.** Five milliliters of the partially purified protein fraction (after ion exchange) containing 4.75 U of the monooxygenase activity, 90 ml of phosphate buffer (20 mM, pH 6.5) containing NADH (8 mM), and 35 mg of 2,2'-dihydroxybiphenyl (2 mM) were incubated at  $30^\circ\text{C}$ . After 45 min, the pH was adjusted to 1.5 with HCl (concentrated) and the reaction mixture was extracted with 250 ml of ethyl acetate. The organic fraction was evaporated to dryness and then dissolved in 5 ml of methanol. Products of the enzyme reaction were separated from one another and from the remaining educt with preparative high-performance liquid chromatography (HPLC) (50% methanol, 49.9% water, 0.1% trifluoroacetate). The fractions containing products were evaporated to dryness and subsequently used for gas chromatography-mass spectrometry (GC-MS),  $^1\text{H}$  nuclear magnetic resonance (NMR) and UV-VIS spectral characterization. Two incubations gave yields of 48.3 mg of 2,2',3-trihydroxybiphenyl (69%) and 8.8 mg of 2,2',3,3'-tetrahydroxybiphenyl (13%).

**Identification of metabolites generated with partially purified enzymes.** The metabolites were extracted from the enzymatic incubation mixtures (1 ml) with ethyl acetate (2 ml) after acidification with 1 N  $\text{H}_3\text{PO}_4$  (0.5 ml). The organic

fraction was dried over  $\text{Na}_2\text{SO}_4$  and subsequently treated with BSTFA [*N,O*-bis(trimethylsilyl)trifluoroacetamide], 0.5 ml/ml of ethyl acetate extract, to generate trimethylsilyl (TMS) derivatives (15 min at 70°C). The GC-MS analysis procedure and the instruments used were the same as those described below.

**HPLC.** The disappearance of 2,2'-dihydroxybiphenyl and the formation of metabolites were monitored by HPLC. Samples containing cells or protein were pretreated either by centrifugation (15 min at 20,000  $\times g$ ) or by the addition of trichloroacetic acid (0.2 ml of a 3 M solution per ml of sample) and subsequent centrifugation. The samples were analyzed by injecting 50  $\mu\text{l}$  onto a Waters-Millipore 625LC (Waters-Millipore, Milford, Mass.) high-performance liquid chromatograph consisting of a Rheodyne 9125 injector, a gradient controller, a low-pressure mixing fluid handling unit, a computer-controlled Waters 991 photodiode array detector, and a Pharmacia RediFrac fraction collector. Reverse-phase separation was achieved on a Spherisorb OD SII column (25 cm by 4.6 mm) (5- $\mu\text{m}$  particle size) from Bischoff GmbH (Leonberg, Germany) by applying a linear gradient of 60 to 70% B (A, 10 mM  $\text{H}_3\text{PO}_4$ ; B, 90% methanol, 10% 10 mM  $\text{H}_3\text{PO}_4$ ) with a flow rate of 1 ml  $\text{min}^{-1}$ . The column was equilibrated to its initial state before each injection.

**GC-MS.** Mass spectra were obtained with a Finnigan MAT ITD 800 (ion trap detection) mass spectrometer (Finnigan MAT, San Jose, Calif.) coupled with a Carlo Erba HRGC 5160 Mega Series gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a 10-m PS090 (80% dimethyl, 20% diphenyl) glass capillary column. Electron ionization was used. The injection (1  $\mu\text{l}$ ) occurred on-column at 80°C. The temperature program was run from 80 to 100°C at a rate of 20°C  $\text{min}^{-1}$  and from 100 to 220°C at a rate of 5°C  $\text{min}^{-1}$ . TMS derivatives were produced by silylating the hydroxy groups with BSTFA. Solid insertion probes were measured with a VG-Autospec Q (Fisons, Manchester, United Kingdom).

**$^1\text{H}$  NMR.**  $^1\text{H}$  NMR spectra were obtained with a 400-MHz Bruker AMX-400 spectrometer (Spektrospin, Fällanden, Switzerland). The probes were dissolved in  $\text{CD}_3\text{OD}-\text{C}_6\text{D}_6$  (100:15).

## RESULTS

**Enzyme activities in cell extracts.** NADH-dependent monooxygenase with activity for 2,2'-dihydroxybiphenyl was detected in cell extracts of 2,2'-dihydroxybiphenyl-grown cells of *Pseudomonas* sp. strain HBP1. Highly active extradiol ring cleavage dioxygenase was measured by monitoring the formation of the yellow *meta*-cleavage compound with 2,3-dihydroxybiphenyl as the substrate, because the product from 2,2',3-trihydroxybiphenyl did not remain stable in an aqueous solution. *meta*-Cleavage product hydrolase activity was detected by monitoring the disappearance of the *meta*-cleavage product of 2,3-dihydroxybiphenyl (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid). Additionally, NADH-dependent salicylate hydroxylase activity could be detected in these cell extracts. Specific activities of the enzymes detected in crude cell extracts are given in Table 1.

**2,2',3-Trihydroxybiphenyl is a product as well as a substrate of the monooxygenase reaction.** In order to characterize the product of the reaction catalyzed by the NADH-dependent monooxygenase, it was necessary to separate the monooxygenase activity from the extradiol ring cleavage dioxygenase activity. This was achieved by protamine sul-

TABLE 1. Specific catabolic enzyme activities in cell extracts of 2,2'-dihydroxybiphenyl-grown *Pseudomonas* sp. strain HBP1

Enzyme and assay substrate	Enzyme activity (nmol/min/mg of protein)
2-Hydroxybiphenyl monooxygenase	
2-Hydroxybiphenyl .....	197
2,2'-Dihydroxybiphenyl .....	96
Extradiol ring cleavage dioxygenase	
2,3-Dihydroxybiphenyl .....	8,417
Catechol .....	550
<i>meta</i> -Cleavage product hydrolase	
2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid ...	434
2-Hydroxybenzoic acid .....	0
Salicylate hydroxylase	
Salicylic acid .....	145
2,3-Dihydroxybenzoic acid .....	36

fate precipitation and anion-exchange chromatography as described in Materials and Methods. Partially purified monooxygenase was incubated with NADH and 2,2'-dihydroxybiphenyl. The course of the reaction was monitored by observing NADH consumption spectrophotometrically and by measuring 2,2'-dihydroxybiphenyl conversion with HPLC. The concentration of 2,2'-dihydroxybiphenyl (retention time, 13.4 min) decreased, and the appearance of a more hydrophilic compound, metabolite 1 (retention time, 9.8 min), was observed (Fig. 1). Upon prolonged incubation, metabolite 1 disappeared, and a second, even more hydrophilic compound, metabolite 2 (retention time, 6.9 min), appeared. Only when the 2,2'-dihydroxybiphenyl concentration reached a level close to zero could formation of metabolite 2 with a concomitant decrease in metabolite 1 be observed. The UV-VIS spectra of metabolites 1 and 2 had similar features. Both metabolites could be isolated according to the procedure described in Materials and Methods. Metabolite 1, a white crystalline powder, yielded the following data.

(i) **MS.** *m/e* (major fragment ions): 204, ( $M + 2$ )<sup>+</sup>; 203, ( $M + 1$ )<sup>+</sup>; 202 (base peak), ( $M$ )<sup>+</sup>; 185, loss of OH; 184, loss

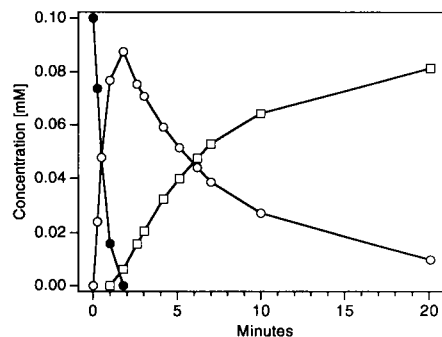


FIG. 1. Turnover of 2,2'-dihydroxybiphenyl (●) and formation of metabolite 1 (2,2',3-trihydroxybiphenyl) (○) and that of metabolite 2 (2,2',3,3'-tetrahydroxybiphenyl) (□), the products of incubation with partially purified monooxygenase. Incubation of 0.1 mM 2,2'-dihydroxybiphenyl with 0.3 ml (0.34 U) of partially purified monooxygenase took place in the presence of 0.2 ml of 10 mM NADH and 4.4 ml of 10 mM phosphate buffer (pH 7.5) at 30°C. Concentrations were calculated with the help of the following extinction coefficients:  $\epsilon_{280}$ , 6,780  $\text{M}^{-1} \text{cm}^{-1}$  for 2,2'-dihydroxybiphenyl and 6,194  $\text{M}^{-1} \text{cm}^{-1}$  for 2,2',3-trihydroxybiphenyl and 2,2',3,3'-tetrahydroxybiphenyl.

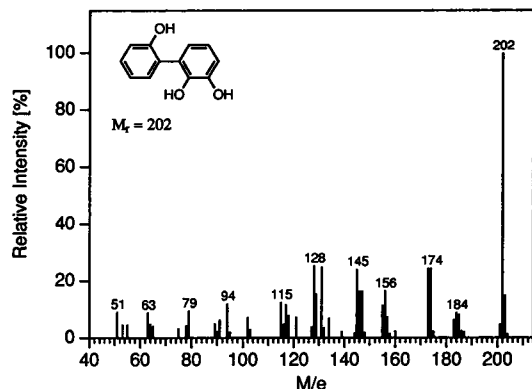


FIG. 2. Electron ionization mass spectrum of 2,2',3-trihydroxybiphenyl. See the text for a discussion of its fragmentation pattern.

of  $\text{H}_2\text{O}$ ; 174, loss of  $\text{CO}$ ; 173, loss of  $\text{CHO}$ ; 156, loss of  $\text{H}_2\text{O}$  and  $\text{CO}$ ; 147, loss of  $\text{C}_3\text{H}_4\text{O}$ ; 146, loss of  $\text{CO}$  and  $\text{CO}$ ; and 145, loss of  $\text{CHO}$  and  $\text{CO}$  (Fig. 2).

For the TMS derivative,  $m/e$  (major fragment ions): 418,  $(\text{M})^+$ ; 403, loss of  $\text{CH}_3$ ; 345, loss of  $\text{Si}(\text{CH}_3)_3$ ; 315 (base peak), loss of  $\text{CH}_3$  and  $\text{Si}(\text{CH}_3)_3$ .

(ii) **UV-VIS spectrophotometry.** Solvent (50% methanol, 50% 0.01 M  $\text{H}_3\text{PO}_4$ );  $\lambda_{\text{max}}$  (nm): 244 (sh), 282;  $\lambda_{\text{min}}$  (nm): 264;  $A_{244}/A_{282} = 1.5$ ;  $\epsilon_{280}$  (solvent, 50% methanol, 50% water with 0.1%  $\text{H}_3\text{PO}_4$ ) =  $6,194 \text{ M}^{-1} \text{ cm}^{-1}$ .

(iii) **Melting point.** A melting point of 119 to 120.5°C was observed.

(iv)  **$^1\text{H}$  NMR.** Solvent  $\text{CD}_3\text{OD}-\text{C}_6\text{D}_6$  (100:15), 400.14 MHz; ppm: 6.77 [dd,  $J = 7.66, 2.1$ , 1H, HC(4)]; 6.81 [t,  $J = 7.64, 7.53$ , 1H, HC(5)]; 6.86 [dd,  $J = 7.52, 2.1$ , 1H, HC(6)]; 6.94 [td,  $J = 7.32, 7.29, 1.7$ , 1H, HC(5')]; 6.97 [ddd,  $J = 7.80, 1.2, 0.4$ , 1H, HC(3')], partially overlapping with HC(5'); 7.20

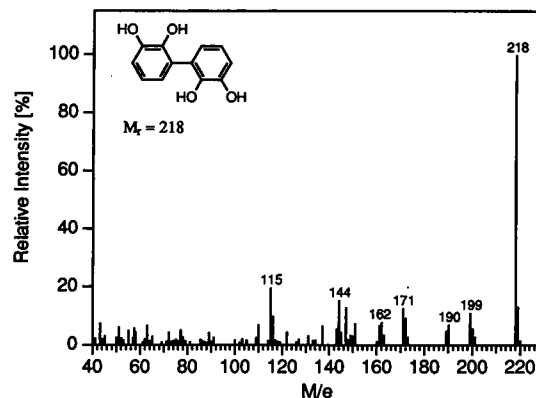


FIG. 4. Electron ionization mass spectrum of 2,2',3,3'-tetrahydroxybiphenyl. See the text for a discussion of its fragmentation pattern.

[ddd,  $J = 7.32, 7.29, 1.7$ , 1H, HC(4')]; 7.28 [ddd,  $J = 7.61, 1.7, 0.3$ , 1H, HC(6')] (Fig. 3). There are two coupling systems showing four and three adjacent aromatic protons. The four protons of the monohydroxylated ring appear at lower field than the three protons of the dihydroxylated ring. The spectrum agrees well with the one published by Fortnagel et al. (4) for 2,2',3-trihydroxybiphenyl.

All of the data presented above are consistent with metabolite 1 being 2,2',3-trihydroxybiphenyl.

Metabolite 2, a slightly yellow crystalline powder, yielded the following data.

(i) **MS.**  $m/e$  (major fragment ions): 220,  $(\text{M} + 2)^+$ ; 219,  $(\text{M} + 1)^+$ ; 218 (base peak),  $(\text{M})^+$ ; 201, loss of  $\text{OH}$ ; 200, loss of  $\text{H}_2\text{O}$ ; 199, loss of  $\text{H}_2\text{O}$ ; 190, loss of  $\text{CO}$ ; 189, loss of  $\text{CHO}$ ;

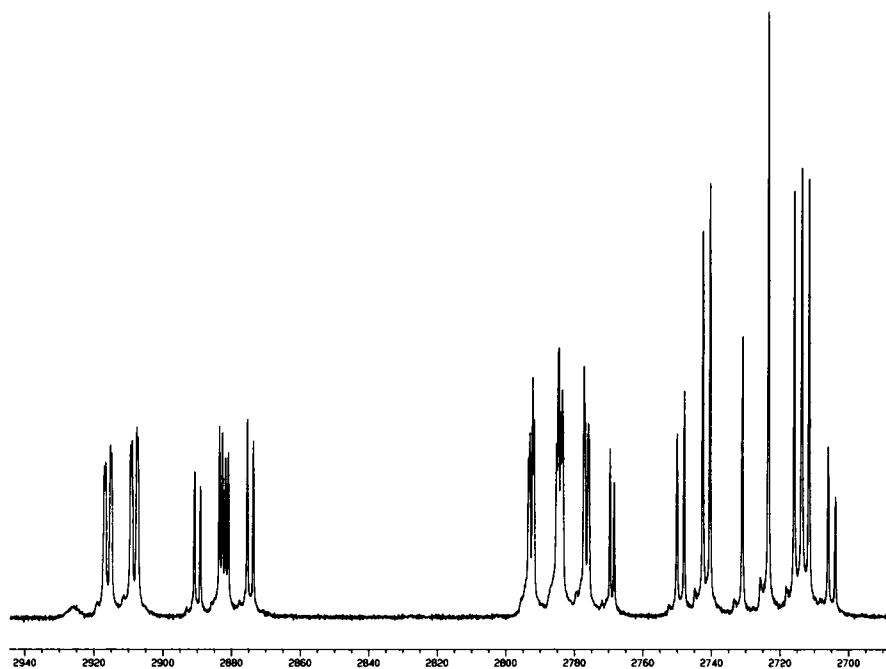


FIG. 3.  $^1\text{H}$  NMR spectrum (400 MHz) of 2,2',3-trihydroxybiphenyl. See the text for a detailed discussion of the chemical shifts. The x axis gives measurements in hertz.

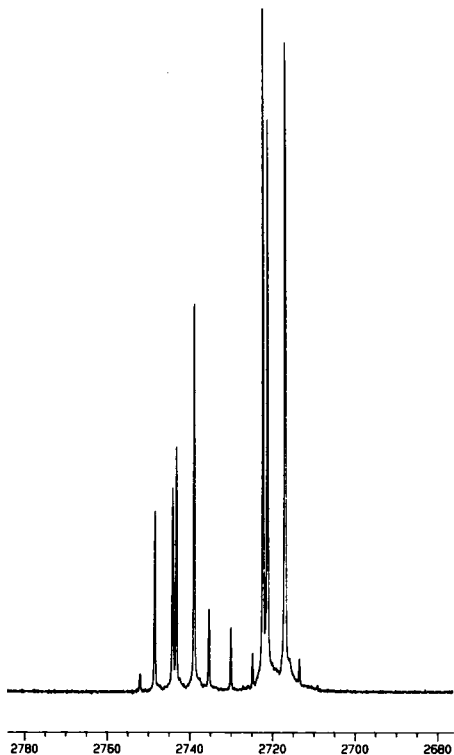


FIG. 5.  $^1\text{H}$  NMR spectrum (400 MHz) of 2,2',3,3'-tetrahydroxybiphenyl. See the text for a detailed discussion of the chemical shifts. The x axis gives measurements in hertz.

173, loss of OH and CO; 172, loss of  $\text{H}_2\text{O}$  and CO; 171, loss of  $\text{H}_2\text{O}$  and CHO; 115,  $\text{C}_9\text{H}_7^+$  (Fig. 4).

For the TMS derivative,  $m/e$  (major fragment ions): 506 (base peak),  $(\text{M})^+$ ; 491, loss of  $\text{CH}_3$ ; 433, loss of  $\text{Si}(\text{CH}_3)_3$ ; 403, loss of  $2\text{CH}_3$  and  $\text{Si}(\text{CH}_3)_3$ ; 315, loss of  $3\text{CH}_3$  and  $2\text{Si}(\text{CH}_3)_3$ .

(ii) **UV-VIS spectrophotometry.** Solvent (50% methanol, 50% 0.01 M  $\text{H}_3\text{PO}_4$ );  $\lambda_{\text{max}}$  (nm): 246, 285;  $\lambda_{\text{min}}$  (nm): 243, 273;  $A_{246}/A_{285} = 2.0$ .

(iii) **Melting point.** A melting point of 214 to 216.5°C was observed.

(iv)  **$^1\text{H}$  NMR.** Solvent  $\text{CD}_3\text{OD}-\text{C}_6\text{D}_6$  (100:15), 400.14 MHz; ppm: 6.88 to 6.84 (m, 1H); 6.82 to 6.78 (m, 2H) (Fig. 5). The  $^1\text{H}$  NMR spectrum of metabolite 2 is of a higher order, and direct assignments of the signals to the protons are not possible, but the spectrum agrees with the proposed structure, 2,2',3,3'-tetrahydroxybiphenyl, for this metabolite. All other data are consistent with this structure.

**2,2',3-Trihydroxy- and 2,2',3,3'-tetrahydroxybiphenyl are substrates of the partially purified extradiol ring cleavage dioxygenase activity.** When partially purified extradiol ring cleavage dioxygenase was incubated with 2,2',3-tri- or 2,2',3,3'-tetrahydroxybiphenyl, an intense yellow color immediately developed, but within 30 s the color disappeared completely, indicating that an unstable product was formed. Incubation mixtures were acidified at the moment the yellow color was most intense, quickly extracted with ethyl acetate, silylated with BSTFA, and subsequently analyzed by GC-MS. In this manner, one major metabolite was detected for each 2,2',3-trihydroxy- and 2,2',3,3'-tetrahydroxybiphenyl (Fig. 6 and 7). The molecular ions at  $m/z$  450 and 538 and the fragment ions at  $m/z$  435 (loss of  $\text{CH}_3$ ), 407 (loss of CO and

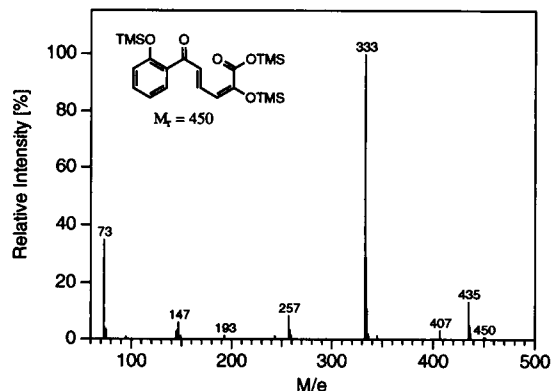


FIG. 6. Electron ionization mass spectrum of the TMS derivative of 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid, the *meta*-cleavage product of 2,2',3-trihydroxybiphenyl. See the text for a discussion of its fragmentation pattern.

$\text{CH}_3$ ), and 333 (loss of  $\text{COOTMS}$ ), and  $m/z$  523 (loss of  $\text{CH}_3$ ), 495 (loss of CO and  $\text{CH}_3$ ), and 421 (loss of  $\text{COOTMS}$ ) are in agreement with the notion that the metabolites are TMS derivatives of the *meta*-cleavage products of 2,2',3-trihydroxybiphenyl [2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid] (15) and 2,2',3,3'-tetrahydroxybiphenyl [2-hydroxy-6-(2,3-dihydroxyphenyl)-6-oxo-2,4-hexadienoic acid], respectively. In additional experiments, the incubation mixtures were acidified only after the yellow color had disappeared completely. Again, one major metabolite was detected in both cases (Fig. 8 and 9). The molecular ions at  $m/z$  378 and 466 and the fragment ions at  $m/z$  363 (loss of  $\text{CH}_3$ ) and 261 (loss of  $\text{COOTMS}$ ) and  $m/z$  451 (loss of  $\text{CH}_3$ ) and 349 (loss of  $\text{COOTMS}$ ) are indicative of the cyclization products of the *meta*-cleavage products of 2,2',3-trihydroxybiphenyl [3-(chroman-4-on-2-yl)pyruvate] and 2,2',3,3'-tetrahydroxybiphenyl [3-(8-hydroxychroman-4-on-2-yl)pyruvate], respectively.

**Salicylate and 2,3-dihydroxybenzoate are products of the hydrolase reaction.** Partially purified extradiol ring cleavage dioxygenase and *meta*-cleavage product hydrolase were used for the following experiments. First, 2,2',3-trihydroxybiphenyl was incubated with extradiol ring cleavage dioxygenase and *meta*-cleavage product hydrolase activity. The

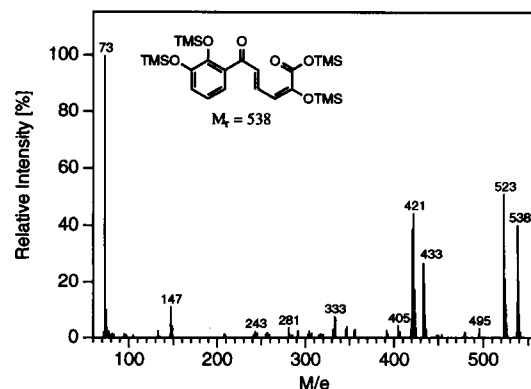


FIG. 7. Electron ionization mass spectrum of the TMS derivative of 2-hydroxy-6-(2,3-dihydroxyphenyl)-6-oxo-2,4-hexadienoic acid, the *meta*-cleavage product of 2,2',3,3'-tetrahydroxybiphenyl. See the text for a discussion of its fragmentation pattern.

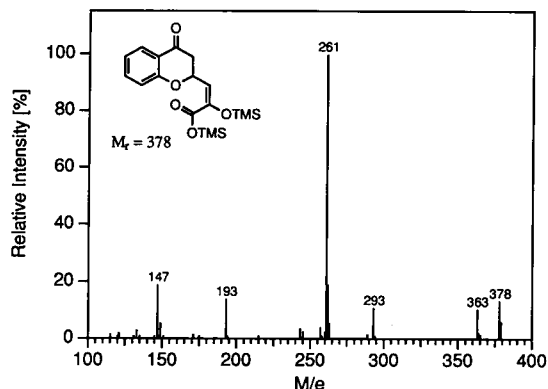


FIG. 8. Electron ionization mass spectrum of the TMS derivative of 3-(chroman-4-on-2-yl)pyruvate, the cyclization product of 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid. See the text for a discussion of its fragmentation pattern.

incubation mixture was extracted and derivatized with TMS as described above. The TMS derivative of the major metabolite cochromatographed with the TMS derivative of authentic salicylic acid, and both compounds produced identical mass spectra, which shows that salicylic acid was the major product of this reaction. An additional metabolite with the characteristics of the TMS derivative of salicylic methyl ester was found. Its mass spectrum showed the following fragmentation pattern.  $m/z$  (major fragment ions): 209 (base peak), loss of  $\text{CH}_3$ ; 193, loss of  $\text{OCH}_3$ ; 179, loss of  $\text{CH}_3$  and  $\text{CH}_2\text{O}$ ; 161, loss of  $\text{CH}_3$ ,  $\text{CH}_2\text{O}$ , and  $\text{H}_2\text{O}$ ; 151, loss of TMS; 135, loss of OTMS. Second, 2,2',3-trihydroxybiphenyl was incubated with extradiol ring cleavage dioxygenase, and only after the yellow color had disappeared completely was *meta*-cleavage hydrolase activity added to the incubation mixture. This time, the major metabolite was identified as the cyclization product of the *meta*-cleavage product (Fig. 8). Only trace amounts of salicylic acid could be detected. Therefore, only the *meta*-cleavage compound, but not its cyclization product, acts as a substrate for the *meta*-cleavage product hydrolase activity. The same experiments were carried out with 2,2',3,3'-tetrahydroxybiphenyl as a substrate. The concomitant incubation yielded 2,3-dihydroxybenzoic acid as a metabolite. Two additional me-

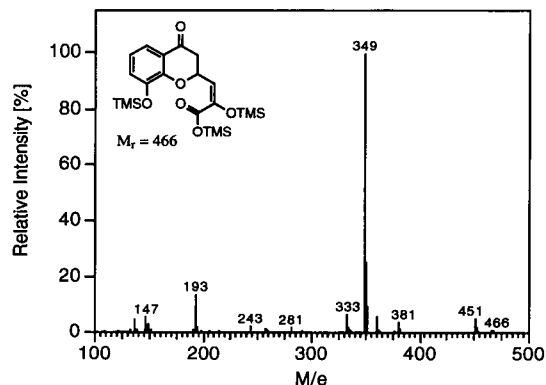


FIG. 9. Electron ionization mass spectrum of the TMS derivative of 3-(8-hydroxychroman-4-on-2-yl)pyruvate, the cyclization product of 2-hydroxy-6-(2,3-dihydroxyphenyl)-6-oxo-2,4-hexadienoic acid. See the text for a discussion of its fragmentation pattern.

tabolites were formed during the course of this incubation. The mass spectra of their TMS derivatives suggest that they were 2,3-dihydroxybenzoic acid methyl ester ( $m/z$  [major fragment ions]: 297 [base peak], loss of  $\text{CH}_3$ ; 281, loss of  $\text{OCH}_3$ ; 267, loss of  $\text{CH}_3$  and  $\text{CH}_2\text{O}$ ; 193) and 2,3-dihydroxybenzyl methyl ether [ $m/z$  (major fragment ions): 298, ( $\text{M})^+$ ; 283, loss of  $\text{CH}_3$ ; 267, loss of  $\text{OCH}_3$ ; 193].

**Salicylate monooxygenase activity catalyzes the conversions of salicylic acid and 2,3-dihydroxybenzoic acid to catechol and pyrogallol, respectively.** Partially purified NADH-dependent salicylate monooxygenase from *Pseudomonas* sp. strain HBP1 was incubated with salicylic acid and 2,3-dihydroxybenzoic acid, respectively. For incubations with salicylic acid as the substrate, GC-MS analysis revealed one major product. The TMS derivative of the metabolite produced a mass spectrum identical to that of authentic trimethylsilylated catechol, and it also had the same chromatographic behavior as the authentic sample. Therefore, catechol was the product of the above reaction. For incubations with 2,3-dihydroxybenzoic acid as the substrate, GC-MS analysis of the TMS derivatives revealed two major products. One was identified as the TMS derivative of pyrogallol, because its mass spectral characteristics and its chromatographical behavior were identical to those of the authentic sample. The other product was identical to the second side product formed in the course of the concomitant incubation of 2,2',3,3'-tetrahydroxybiphenyl with extradiol ring cleavage dioxygenase and hydrolase, i.e., 2,3-dihydroxybenzyl methyl ether.

**2-Hydroxymuconic semialdehyde and 2-hydroxymuconic acid are produced from catechol and pyrogallol, respectively, by the extradiol ring cleavage dioxygenase activity.** A yellow color appeared immediately after the addition of catechol to partially purified extradiol ring cleavage dioxygenase, indicating the production of *meta*-cleavage metabolites. The yellow metabolite was identified as 2-hydroxymuconic semialdehyde on the grounds of the mass spectrum of its TMS derivative;  $m/z$  (major fragment ions): 271, loss of  $\text{CH}_3$ ; 257, loss of  $\text{HCO}$ ; 243, loss of  $\text{CH}_3$  and  $\text{CO}$ ; 197, loss of OTMS; 169 (base peak), loss of  $\text{COOTMS}$ ; 147,  $\text{C}_5\text{H}_{15}\text{OSi}^+$ ; 73,  $\text{C}_3\text{H}_9\text{Si}^+$ . When pyrogallol was added to partially purified extradiol ring cleavage dioxygenase, formation of a yellow color could not be observed. MS analysis revealed a major metabolite. The electron impact mass spectrum of its TMS derivative showed the following fragment ions.  $m/z$  (major fragment ions): 359, loss of  $\text{CH}_3$ ; 331, loss of  $\text{CH}_3$  and  $\text{CO}$ ; 285, loss of OTMS; 257, loss of  $\text{COOTMS}$ ; 147,  $\text{C}_5\text{H}_{15}\text{OSi}^+$ ; 73,  $\text{C}_3\text{H}_9\text{Si}^+$ . The compound was identified as the TMS derivative of 2-hydroxymuconic acid because its mass spectrum was identical to that of the TMS derivative of an authentic sample of 2-hydroxymuconic acid.

## DISCUSSION

We have previously shown that 2-hydroxybiphenyl is degraded by *Pseudomonas* sp. strain HBP1 via a site-specific monooxygenase that hydroxylates aromatic compounds at the C-3 position when there is a hydroxy group at C-2 and an alkyl or phenyl rest at C-1. The same mechanism is operative in 2,2'-dihydroxybiphenyl-grown cells, and the product of the monooxygenase attack on 2,2'-dihydroxybiphenyl was proven to be 2,2',3-trihydroxybiphenyl. Interestingly, 2,2',3-trihydroxybiphenyl also serves as a substrate for the monooxygenase activity. We have demonstrated that the product of this reaction is 2,2',3,3'-tetrahydroxybiphenyl. Therefore, the monooxygenase also hydroxylates the

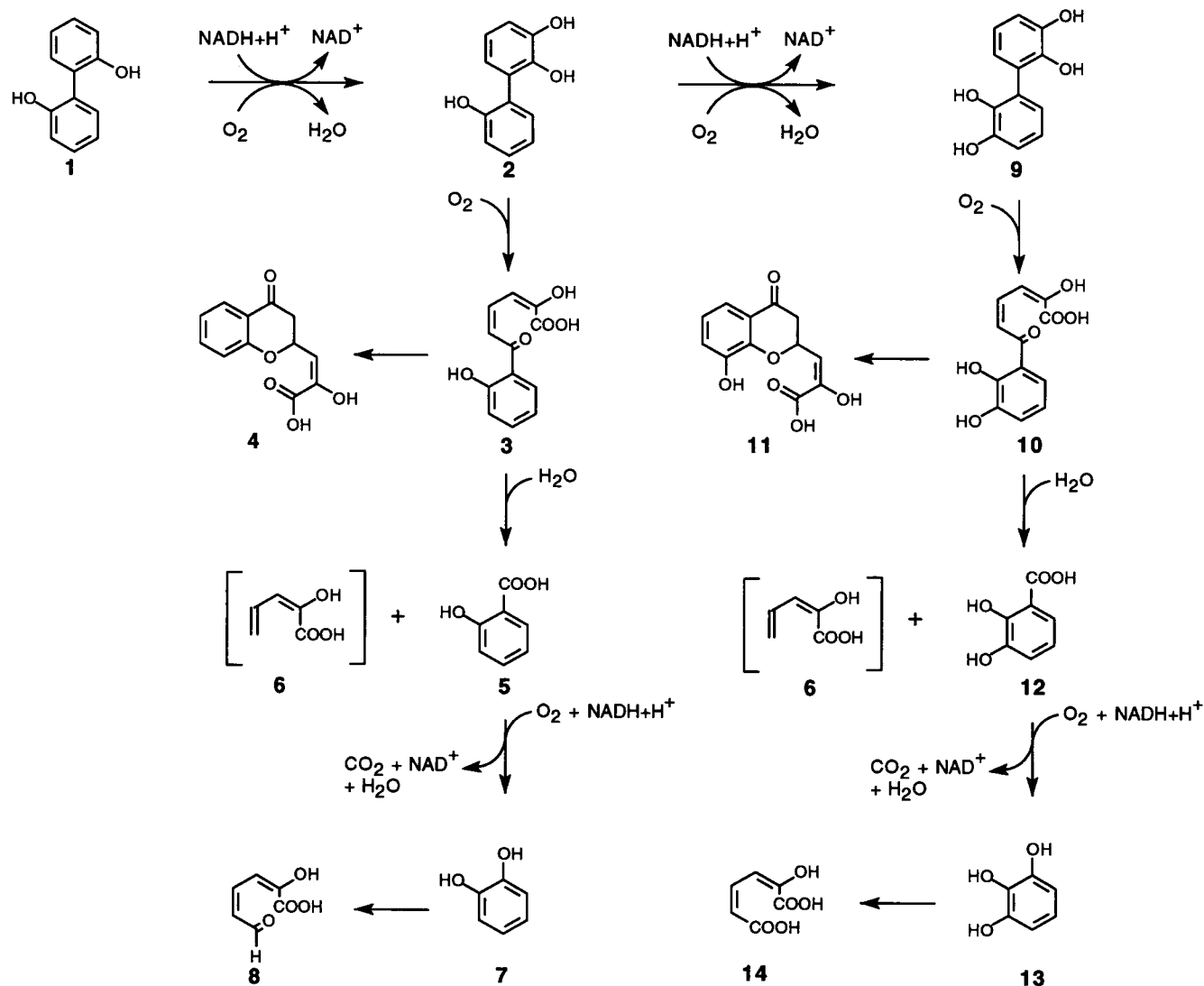


FIG. 10. Pathway proposed for the metabolism of 2,2'-dihydroxybiphenyl by *Pseudomonas* sp. strain HBP1. 1, 2,2'-Dihydroxybiphenyl; 2, 2,2',3-trihydroxybiphenyl; 3, 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid; 4, 3-(chroman-4-on-2-yl) pyruvate; 5, salicylic acid; 6, 2-hydroxy-2,4-pentadienoic acid; 7, catechol; 8, 2-hydroxymuconic semialdehyde; 9, 2,2',3,3'-tetrahydroxybiphenyl; 10, 2-hydroxy-6-(2,3-dihydroxyphenyl)-6-oxo-2,4-hexadienoic acid; 11, 3-(8-hydroxychroman-4-on-2-yl)pyruvate; 12, 2,3-dihydroxybenzoic acid; 13, pyrogallol; 14, 2-hydroxymuconic acid.

C-3' position of 2,2',3-trihydroxybiphenyl. This finding provides additional evidence for the previously suggested relaxed specificity of the monooxygenase with respect to the molecular rest at the C-1 position of the aromatic backbone structure (9). Incubations of biphenyl with strain KF274 carrying plasmid pMFB6 containing the *bphA* and *bphB* genes encoding for biphenyl dioxygenase and dihydrodiol dehydrogenase, respectively, also led to the final accumulation of 2,2',3,3'-tetrahydroxybiphenyl (5). For this case, it was proposed that 2,3-dihydroxybiphenyl was first produced and then its nonhydroxylated ring was further oxidized to form 2,2',3,3'-tetrahydroxybiphenyl, which then accumulated in the culture medium, because the construct KF274(pMFB6) did not contain a metapyrocatechase.

The proposed pathway for the metabolism of 2,2'-dihydroxybiphenyl is presented in Fig. 10. It can be seen that the first intermediate, 2,2',3-trihydroxybiphenyl, may be metab-

olized via two different routes. On one hand, it serves as a substrate to the extradiol ring cleavage dioxygenase, and on the other hand, it can be turned over by the monooxygenase. Figure 1 shows that formation of 2,2',3,3'-tetrahydroxybiphenyl starts only when 2,2'-dihydroxybiphenyl reaches a level close to zero. This could be an indication of a high  $K_m$  value with 2,2',3-trihydroxybiphenyl as a substrate. Since very high extradiol ring cleavage dioxygenase activity is also present in crude cell extract (Table 1), one can assume that in the course of normal metabolic breakdown most of the 2,2'-dihydroxybiphenyl is channeled via 2,2',3-trihydroxy- and not 2,2',3,3'-tetrahydroxybiphenyl.

The partially purified extradiol ring cleavage dioxygenase acts on both 2,2',3-tri- and 2,2',3,3'-tetrahydroxybiphenyl, producing yellow *meta*-cleavage products that are unstable in the aqueous incubation environment. Both *meta*-cleavage compounds and their cyclization products could be ex-



tracted, and their corresponding TMS derivatives could be prepared. When 2,2',3-tri- and 2,2',3,3'-tetrahydroxybiphenyl were incubated with extradiol ring cleavage dioxygenase and *meta*-cleavage product hydrolase together, salicylic acid and 2,3-dihydroxybenzoic acid, respectively, were formed, and only minor quantities of cyclization products could be detected. We have no explanations yet for the formation of the methyl esters of salicylic or 2,3-dihydroxybenzoic acid and for the formation of 2,3-dihydroxybenzyl methyl ether during these incubations. Our result, that 2,2',3-trihydroxybiphenyl is *meta*-cleaved, is in agreement with the findings of Strubel et al. (16). They described the formation of a yellow *meta*-cleavage product from 2,2',3-trihydroxybiphenyl catalyzed by crude extracts of dibenzofuran-grown cells of *Brevibacterium* sp. strain DPO 1361. This compound could not be analyzed directly, but it was transformed to a colorless metabolite identified as 3-(chroman-4-on-2-yl)pyruvate. The mass spectrum of the TMS derivative of 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid, the *meta*-cleavage product of 2,2',3-trihydroxybiphenyl, is identical to the one described by Sondossi et al. (15). They found this compound as one of many metabolites of 2-hydroxybiphenyl degradation in biphenyl- and chlorobiphenyl-degrading *P. testosteroni* B-356. Our direct analysis of the TMS derivative of 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid as a product of the reaction catalyzed by partially purified extradiol ring cleavage dioxygenase is final proof of the proposed *meta* cleavage of 2,2',3-trihydroxybiphenyl.

In contrast to 2-hydroxybiphenyl-grown cells of strain HBPI (9), 2,2'-dihydroxybiphenyl-grown cells induce an NADH-dependent salicylate monooxygenase activity. The partially purified enzyme activity catalyzed the formation of catechol from salicylic acid as well as the formation of pyrogallol from 2,3-dihydroxybenzoic acid. Both of these aromatic acids were previously shown to be substrates for salicylate monooxygenase (18).

The partially purified extradiol ring cleavage dioxygenase activity from *Pseudomonas* sp. strain HBPI, which acted also on the hydroxybiphenyls, catalyzed the conversion of catechol to 2-hydroxymuconic semialdehyde and that of pyrogallol to 2-hydroxymuconic acid. It has been shown previously that individual dioxygenases act on pyrogallol to form either 2-pyrone-6-carboxylic acid or 2-hydroxymuconic acid (13).

At the moment, we believe that the extradiol ring cleavage dioxygenase activity from strain HBPI is a broad-spectrum *meta*-cleavage dioxygenase because the same partially purified protein fraction is able to turn over various 3-alkylcatechols (10, 17); 2,3-di-, 2,2',3-tri-, and 2,2',3,3'-tetrahydroxybiphenyl; catechol; and pyrogallol and because under all growth conditions tested the activity always elutes as one homogenous peak from the ion-exchange column at the same salt concentration (data not shown). Additional work on the extradiol ring cleavage dioxygenase activity of *Pseudomonas* sp. strain HBPI will be needed in order for researchers to profoundly characterize the enzyme and to decide in which superfamily of the extradiol ring cleavage dioxygenases the enzyme must be grouped.

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