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Metabolism of Dibenzofuran by *Pseudomonas* sp. Strain HH69 and the Mixed Culture HH27

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A *Pseudomonas* sp. strain, HH69, and a mixed culture, designated HH27, were isolated by selective enrichment from soil samples. The pure strain and the mixed culture grew aerobically on dibenzofuran as the sole source of carbon and energy. Degradation proceeded via salicylic acid which was branched into the gentisic acid and the catechol pathway. Both salicylic acid and gentisic acid accumulated in the culture medium of strain HH69. The acids were slowly metabolized after growth ceased. The enzymes responsible for their metabolism showed relatively low activities. Besides the above-mentioned acids, 2-hydroxyacetophenone, benzopyran-4-one (chromone), several 2-substituted chroman-4-ones, and traces of the four isomeric monohydroxydibenzofurans were identified in the culture medium. 2,2′,3-Trihydroxybiphenyl was isolated from the medium of a dibenzofuran-converting mutant derived from parent strain HH69, which can no longer grow on dibenzofuran. This gives evidence for a novel type of dioxygenases responsible for the attack on the biarylether structure of the dibenzofuran molecule. A *meta*-fission mechanism for cleavage of the dihydroxylated aromatic nucleus of 2,2′,3-trihydroxybiphenyl is suggested as the next enzymatic step in the degradative pathway.

Increasing numbers and amounts of halogen-containing aromatic compounds have been produced commercially, and they are still being manufactured. Problems arise from the contamination of some of these products with halogenated dibenzo-*p*-dioxins (DD) and dibenzofurans (DF) because of the extreme toxicity of these compounds. These halogenated heterocycles are formed as undesired by-products during the synthesis of haloaromatic compounds or during their thermal destruction by incineration processes, leading to ubiquity (30). But not only the persistence of halogenated DD and DF cause problems; even the nonhalogenated carbon skeletons are difficult substrates for microorganisms to degrade.

Only scarce information is available concerning microbial attack on DF, DD, and their halogenated derivatives. Oxidation of DF by bacterial and fungal activities yielding the respective *cis*- and *trans*-isomers of dihydroxy-dihydrodibenzofuran has been described previously (7). Klecka and Gibson (16, 17) reported the cooxidation of DD and some of its chlorinated derivatives to *cis*-dihydrodiols and diols. Bumpus (5) as well as Foght and Westlake (9) described the microbial removal of DF from complex aromatic hydrocarbon mixtures. Recently, DF degradation via salicylic acid has been reported (10, 36). This paper describes the isolation and characterization of a strain capable of utilizing this compound as a carbon source. Metabolites have been identified which suggest a transformation of DF to 2,2′,3-trihydroxybiphenyl and its further degradation via an *ortho*-acyl phenol.

(This paper is based in part on a doctoral study by Hauke Harms in the Faculty of Biology and by Heinz Wilkes in the Department of Chemistry, the University of Hamburg.)

MATERIALS AND METHODS

Media and growth conditions. A mineral salts medium was used containing (per liter): 3.5 g of $Na_2HPO_4 \cdot 2H_2O$, 1 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 50 mg of $Ca(NO_3)_2 \cdot 4H_2O$, and 1 ml of a trace elements solution described by Pfennig and Lippert (27); EDTA was omitted. The final pH was 7.25. Solid media contained 10 g of agar no. 1 (Oxoid Ltd., Basingstoke, Hampshire, England) per liter. Carbon sources were added as stated in the text (usually 1 g/liter). For preparation of solid media, DF was aseptically added to the hot agar-containing medium just after it was autoclaved. Molten crystals were dispersed by sonication (two 20-W bursts for 2 min each) before the medium was poured into petri dishes. For growth of bacteria on nonaromatic organic compounds, sodium acetate (20 mM) or peptone (5 g/liter) and yeast extract (1 g/liter) were added to the above-described mineral salts medium. Bacteria were enriched and grown in Erlenmeyer flasks with baffles filled with the above-described medium at 20% of the nominal volume at 28°C on a rotary shaker at 175 rpm. The growth of cultures was measured by photometric determination of the turbidity (optical density) at 578 nm and, in the case of DF-utilizing cultures, after removal of substrate crystals by filtration as described below.

Determination of DF in the culture medium. For determination of substrate consumption, DF was separated from the spent culture by collecting residual crystals on Schleicher & Schuell (Dassel, Federal Republic of Germany) no. 597 1/2 filter paper. The crystals, after being washed with a defined amount of ice-cold mineral salts medium, were dried in a desiccator and weighed. Counting of cell numbers before and after filtration showed that no substantial numbers of bacteria were retained on filter paper. Amounts of DF obtained by this procedure were corrected with the aid of linear recovery rates, which were determined in the range from 50 to 1,000 mg of DF per liter of medium. The solubility of DF in water at 28°C was 5 mg/liter, and a solution of fine mortar-ground DF crystals (1 g of DF/liter) took about 25 to 30 min to reach 90% of the above-mentioned maximum solubility.

UV mutagenesis. Bacteria were grown to a turbidity (A_{578}) of 0.5 in mineral salts medium supplemented with 10 mM

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benzoate. Cells were diluted 10-fold with phosphate buffer, transferred to a rotating petri dish, and exposed to UV light of 254 nm for 5 min, sufficient for induction of an approximate 6-log kill. Subsequent procedures were carried out in the dark. After cells were grown on sodium acetate (20 mM)-containing medium for 24 h, they were twice washed with phosphate buffer and diluted into mineral salts medium containing DF (2 g/liter) and ampicillin (1 g/liter). Cells were incubated for 12 h, washed twice with buffer, and diluted onto solid medium containing sodium acetate (20 mM) as the carbon source. Colonies which arose from survivors were transferred to DF agar plates to test for growth on this compound. Appropriate clones were selected for further studies.

Preparation of cell-free extracts. Cell suspensions were freed from DF crystals by filtration through glass wool. Bacteria were harvested at the late logarithmic growth phase by centrifugation at 20,000 × *g* for 20 min. at 5°C and washed twice with 25 mM phosphate buffer, pH 7.2. Cells were suspended to a turbidity of about 10 to 15 in buffer containing a few crystals of deoxyribonuclease and 0.1 mM phenylmethylsulfonyl fluoride. Cells were broken by two passages through a French pressure cell at about 10,000 lb/in^2, and the extract was centrifuged at 5°C for 1 h at 100,000 × *g* before being used for enzyme assays.

Protein determination. The protein content of whole cells was estimated by the method of Spector (34) after cell lysis in the presence of NaOH (0.15 M) for 5 min at 95°C. The protein concentration of extracts was measured by the method of Bradford (4). Bovine serum albumin was used for calibration.

Estimation of enzyme activities. Enzyme activities were assayed by previously described procedures. Catechol 1,2-dioxygenase (EC 1.13.11.1) was measured by determining the formation of muconic acid (22). Catechol 2,3-dioxygenase (EC 1.13.11.2) was determined by measuring the formation of 2-hydroxymuconic acid semialdehyde (23). Gentisic acid dioxygenase activity was determined by the method of Wheelis et al. (38), and salicylaldehyde dehydrogenase was estimated by a previously published method (33). All assays were performed with an LKB Ultrospec II spectrophotometer (Pharmacia LKB GmbH, Freiburg, Federal Republic of Germany) at 25°C.

Isolation of metabolites. Metabolites were extracted from the supernatant of the spent stationary-phase culture medium with ethyl acetate (neutral fraction) and after acidification with concentrated phosphoric acid to pH 2.5 (acidic fraction). The solvent was dried with anhydrous magnesium sulfate. After evaporation of the solvent, residues were dissolved in methanol or in ethyl acetate and fractionated by preparative high-performance liquid chromatography (HPLC) or thin-layer chromatography under the conditions given below. Extracts and fractions were analyzed without further derivatization by coupled gas chromatography-mass spectroscopy and by nuclear magnetic resonance spectroscopy.

Analytical methods. Gas chromatographic analyses were carried out on a Carlo Erba Fractovap 2101 AC (Erba Science, Hofheim/Taunus, Federal Republic of Germany) by using a 50-m CP-Sil8 fused-silica column (Chrompack GmbH, Frankfurt, Federal Republic of Germany) with a temperature program from 80 to 300°C at a rate of 5°C/min. For GCMS investigations, a coupling system was used consisting of a HP 5890 gas chromatograph (Hewlett Packard GmbH, Bad Homburg, Federal Republic of Germany) linked to a VG 70-250S mass spectrometer (Vacuum Generators, Manchester, United Kingdom) operating at 70 eV. Separation conditions were the same as those described above. Identifications were based on the comparison of mass spectra and retention times with authentic reference compounds. ^{1}H NMR spectra of purified metabolites were recorded on a Bruker WM 400 instrument (Bruker GmbH, Karlsruhe, Federal Republic of Germany).

Formation of metabolites in the culture medium was routinely monitored by HPLC. The system consisted of a model 425 gradient former, a model 420 pump, a model 430 dual channel UV detector, and integration software from Kontron Instruments, Eching, Federal Republic of Germany, which was used on an AT-compatible personal computer. A 4- by 250-mm Spherisorb 5-μm ODS II column (Phase Separations Ltd., Deeside, United Kingdom) was used for separation. The mobile phase consisted of 10 mM H_3PO_4 in water (solvent A) and 10% (vol/vol) of solvent A in methanol (solvent B). The flow rate was set to 1 ml/min. Metabolites were identified by their retention times and in situ-scanned UV spectra, which were compared with those of authentic compounds. Results were routinely confirmed by separation of ethylacetate-extracted samples and authentic standards by thin-layer chromatography on silica gel G plates (Merck, Darmstadt, Federal Republic of Germany) in a system of diisopropyl ether-formic acid-water (200:7:3 [vol/vol/vol]).

Oxidation of aromatic compounds by washed cell suspensions was measured polarographically with a Clark-type oxygen electrode (DW 1 model from Bachofer GmbH, Reutlingen, Federal Republic of Germany) at 25°C. Substrates were dissolved in dimethylsulfoxide. The concentration in the assay corresponded to 1 mM unless stated otherwise. Specific uptake rates were corrected for endogenous oxygen consumption. The G+C content of bacterial DNA was determined photometrically by the method of Frank-Kamenetskii (11).

Chemicals. DF (purity >99%) and 2-hydroxydibenzofuran were purchased from Aldrich, Steinheim, Federal Republic of Germany. 2,3-Dihydroxybiphenyl was a product of Wako Chemicals GmbH, Neuss, Federal Republic of Germany. All other commercial chemicals were of the highest purity available and were recrystallized from an appropriate solvent, if necessary. 2-Acetoxydibenzofuran and 2-methoxydibenzofuran were obtained through derivatization of 2-hydroxydibenzofuran. For preparation of DD, a new synthetic method was applied. In analogy to a previously published procedure (8), 2-nitrodibenzo-*p*-dioxin was synthesized from catechol and 2,4-dinitrofluorobenzene. Subsequent reduction to DD followed the procedure of Saint-Ruf and Lobert (31). 2-Hydroxyphenylglyoxylic acid was prepared by using a known procedure (14). Reference samples of identified compounds were synthesized as follows (for numbers and structures, see Fig. 5). Starting from phenol or hydroquinone and succinic acid anhydride or succinic acid methylester chloride, syntheses of the two acylphenols XIII and XIV followed general methods described in the literature (18, 26, 29). Chromone (V) and 2-methylchromone (XII) were prepared by the methods of Hirao et al. (13) and Schmutz et al. (32). 2-Methylchroman-4-one (VI) was synthesized from 2-methylchromone (XII) through reduction with sodium borohydride and oxidation of the resulting 2-methylchroman-4-ol with manganese dioxide. Our first synthesis of (chroman-4-on-2-yl)-acetic acid (IV) and the methyl ester (III) was carried out through a Michael-type addition of dimethyl malonate to chromone and subsequent transformation by conventional procedures. Reduction of

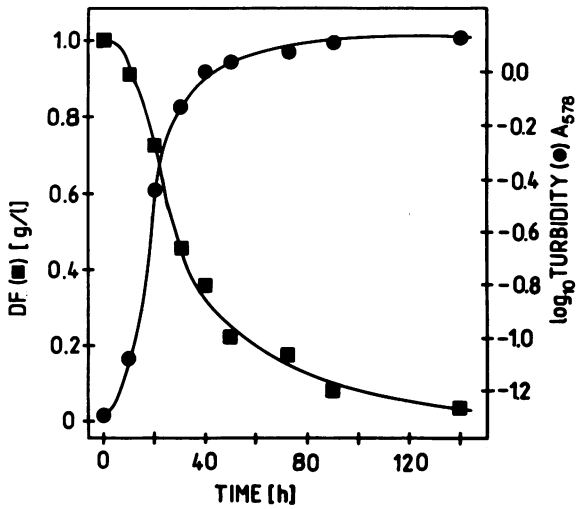

FIG. 1. Growth of *Pseudomonas* sp. strain HH69 with DF in parallel batch cultures. The concentration of DF and the growth of the microbial culture were determined as stated in Materials and Methods.

the ketoester III with lithium aluminumhydride furnished 2-(chroman-4-on-2-yl)-ethanol, while oxidation with pyridinium dichromate gave (chroman-4-on-2-yl)-acetaldehyde (XI). A second synthesis of compound IV followed the method of Iwasaki et al. (15). The latter strategy was also used to prepare methyl 3-(chroman-4-on-2-yl)-pyruvate (I) from chromone and methyl pyruvate. Reduction of I by sodium borohydride, followed by oxidation with manganese dioxide furnished methyl 3-(chroman-4-on-2-yl)-lactate (II).

RESULTS

Enrichment, isolation and characterization. A DF-utilizing bacterium and a mixed culture consisting of seven strains distinct from each other were isolated from batch enrichment cultures inoculated with a mixture of farmland and forest soil samples. DF crystals were added directly to the soil suspended in the mineral salts medium. Subcultures were transferred to fresh medium weekly. After 4 weeks, a yellow color which was accompanied by a high cell number appeared. After two subsequent transfers, samples were plated on mineral agar plates containing DF as the only carbon source. Colonies appearing on the plates were transferred to fresh plates and were checked for purity by being streaked on nutrient agar. After several transfers, strain HH69 was isolated and was tentatively identified as a *Pseudomonas* species on the basis of the following criteria. It was a strictly aerobic, gram-negative, oxidase- and catalase-positive rod, about 1.25 μm wide and 5 to 8 μm long. Capsules or spores were not formed. A single polar flagellum was present. The guanine-plus-cytosine content of the DNA was estimated to be 61 mol%.

Growth of bacteria with DF and other aromatic compounds. The ability of *Pseudomonas* sp. strain HH69 to grow with DF as the only source of carbon and energy is shown in Fig. 1. Growth was expressed as the increase of turbidity and was correlated with the removal of DF from the suspension in the culture medium. The doubling time of the bacterium during the exponential growth phase at 28°C was about 5 h, depending on the amount and the respective particle size (total surface) of DF. In the course of exponential growth, salicylic and gentisic acid were temporarily excreted at levels up to 0.1 to 0.2 mM. Salicylic acid disappeared from the culture medium during the late logarithmic and early stationary growth phase because of hydroxylation to gentisic acid at a slow rate. The latter compound disappeared very slowly from the culture medium. The mixed culture HH27 showed the same behavior as strain HH69 where growth parameters and excretion of the above-mentioned aromatic acids with DF as the substrate were concerned. The bacterium was able to grow on glucose and galactose. Aspartate and glutamate were the only amino acids used as carbon sources; tricarboxylic acid cycle intermediates other than acetic acid were not used as carbon sources. Growth on peptone and other complex media was observed. Further aromatic compounds utilized by *Pseudomonas* sp. strain HH69 were DF, biphenyl, catechol, 2,2',3-trihydroxybiphenyl, benzoic acid, salicylic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid (gentisic acid), phenylmalonic acid, phenylglyoxylic acid, and 2-hydroxyphenylglyoxylic acid. The ability to grow with biphenyl was lost after the strain was subcultured for 4 months with DF as the only source of carbon.

TABLE 1. Oxidation of DF, substituted DFs, structurally related compounds and potential metabolites by resting cells of *Pseudomonas* sp. strain HH69[a]

Substrate	Sp act (oxygen uptake) (nmol/min/mg) of HH69 cultures grown on:	
	Acetate	DF
DF	90	410
2-Hydroxydibenzofuran	83	298
2-Methoxydibenzofuran	73	256
2-Acetoxydibenzofuran	113	320
Dibenzothiophen	52	58
Carbazol	30	52
Dibenzo-*p*-dioxin	28	122
Biphenyl	54	79
4-Chlorobiphenyl	42	52
Diphenylether	36	53
3-Carboxydiphenylether	12	11
4-Carboxydiphenylether	15	21
2,3-Dihydroxybiphenyl	844	1,175
2,2',3-Trihydroxybiphenyl	200	520
Salicylic acid	18	129
Benzoic acid	10	27
Gentisic acid	10	11
2-Hydroxyphenylglyoxylic acid	160	138
Salicylaldehyde	78	151
Catechol	30	66
2-Hydroxyacetophenone	3	10
Chromone	15	6
2-Methylchromone	26	17
2-Methylchroman-4-one	35	35

[a] Absolute oxygen uptake rates are means of at least two independently performed experiments. The concentrations of 2-hydroxydibenzofuran and 2,2',3-trihydroxybiphenyl were reduced to 0.1 mM to prevent toxic effects to the cells.

Oxygen uptake by whole cells. Oxygen uptake rates of washed cells of strain HH69 after growth on DF or sodium acetate were determined with a number of aromatic compounds, i.e., derivatives of DF and related compounds and a number of potential degradation products (Table 1). Results indicated the induction of enzyme activities responsible for the oxidation of DF, some of its substituted derivatives, the structurally related DD, and of some metabolites of DF metabolism identified in the course of our studies. These

compounds were 2,2',3-trihydroxybiphenyl, salicylic acid, and catechol, the latter showing relatively low activities. Oxidation of gentisic acid was found to be extremely slow, while the highest rates were determined for 2,3-dihydroxybiphenyl; even acetate-grown cells exhibited relatively high constitutive levels for the oxidation of the latter compound and for the catabolite 2,2',3-trihydroxybiphenyl. 2-Hydroxyacetophenone, chromone, and 2-methylchroman-4-one, which were identified in the culture medium but which were not utilized by bacterial cells as a carbon source, were only slowly oxidized.

Determination of enzyme activities in cell extracts. We could not detect salicylic acid hydroxylases (salicylic acid-1-hydroxylase-decarboxylase [which produces catechol] and salicylic acid-5-hydroxylase [which produces gentisic acid]) in extracts by estimating NAD(P)H_2 oxidation. Catechol-2,3-dioxygenase activity was 3.6 nmol/min per mg, and the activity of the catechol-1,2-dioxygenase was always less than 1 nmol/min per mg when extracts from cells pregrown with DF were used in the assay. However, benzoic acid-grown cells exhibited an unexpectedly high catechol-1,2-dioxygenase activity of 2.7 μmol/min per mg. Gentisic acid dioxygenase and salicylaldehyde dehydrogenase activities were not detectable in extracts from DF-grown cells by published methods.

Isolation and characterization of 2,2',3-trihydroxybiphenyl. A clone obtained from the parent strain HH69 after UV mutagenesis which could no longer grow with DF as the carbon source (designated strain HH69-II) was grown with sodium acetate (20 mM) as a carbon source in 2 liters of the mineral salts medium. After the culture had reached the late logarithmic growth phase, 4 g of DF was added. Formation of a single product was monitored by HPLC until a maximum was reached. The medium was freed from DF and cells by filtration and centrifugation and extracted with ethyl acetate. The structure of the pure 2,2',3-trihydroxybiphenyl thus obtained was confirmed by ^{1}H NMR spectroscopy and mass spectroscopy (Fig. 2). Data obtained by ^{1}H NMR spectroscopy (400.13 MHz, CD_3OD/C_6D_6=100/15; tetramethylsilane [TMS] was the internal standard) were as follows (chemical shifts and respective coupling constants): δ = 6.78 (H-6), 6.81 (H-5, $J_{5,6}$ = 7.6 Hz), 6.87 (H-4, $J_{4,5}$ = 7.6 Hz, $J_{4,6}$ = 2.0 Hz), 6.94 (H-5'), 6.98 (H-3', $J_{3',5'}$ = 1.2 Hz), 7.20 (H-4', $J_{4',5'}$ = 7.4 Hz, $J_{3',4'}$ = 8.0 Hz) and 7.28 (H-6', $J_{4',6'}$ = 1.8 Hz, $J_{5',6'}$ = 7.7 Hz) ppm. The coupling systems of the aromatic protons clearly showed four adjacent and three adjacent asymmetrically arranged protons, respectively. According to a C,H-COSY-correlation and increment calculations (28), ^{13}C NMR data (100.62 MHz, CD_3OD/C_6D_6 = 100/15; TMS was the internal standard) are attributed as follows: C-2' = 154.60, C-2 = 147.23, C-3 = 143.11, C-6' = 132.62, C-4' = 129.61, C-1 = 128.33, C-1' = 127.52, C-6 = 123.27, C-5/C-5' = 121.54/121.52, C-3' = 117.20 and C-4 = 115.27 ppm.

DF- and sodium acetate-grown washed cells of the parent strain HH69 oxidized this new compound with high conversion rates (Table 1), causing an intense yellow coloring of the medium. A UV spectrum of the supernatant revealed an additional maximum at 446 nm.

Identification of further metabolites. The benzopyran-4-one derivative, (chroman-4-on-2-yl)-acetic acid [IV], represents a key compound for the identification of related metabolites in the acidified culture medium of strain HH69. Under the conditions described above, the compound could be isolated by HPLC and characterized by ^{1}H NMR spectroscopy and mass spectroscopy (Fig. 3). Data obtained

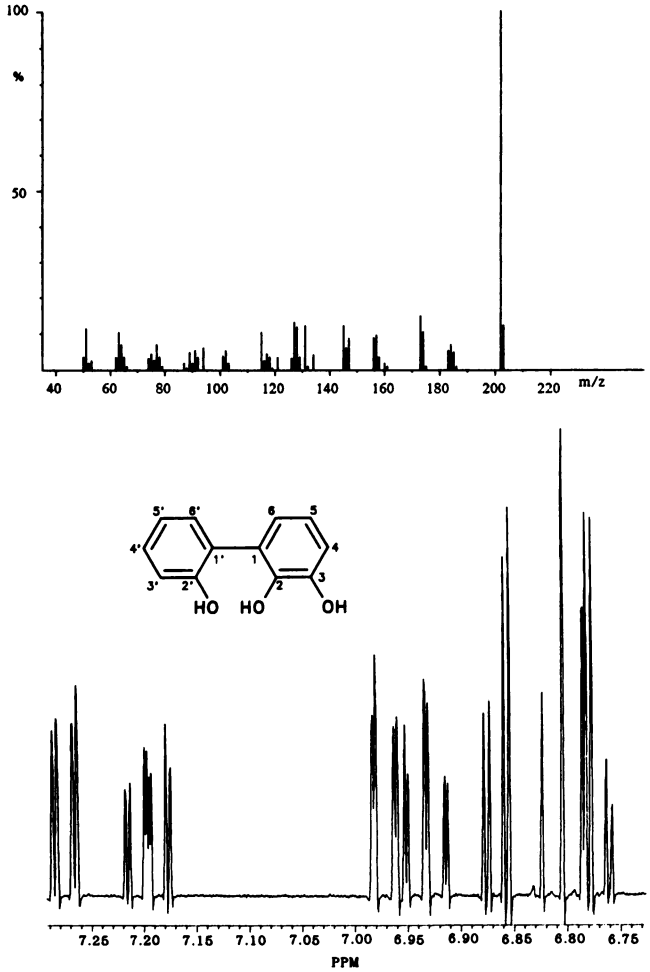

FIG. 2. 70 eV mass spectrum and ^{1}H NMR spectrum of 2,2',3-trihydroxybiphenyl (400.13 MHz; CD_3OD/C_6D_6 = 100/15; TMS was the internal standard).

upon ^{1}H NMR spectroscopy (400, 13 MHz, C_6D_6; TMS was the internal standard) were as follows (chemical shifts and respective coupling constants): δ = 1.94 (H-9, $J_{9,9'}$ = 16.4 Hz), 2.10 (H-3, $J_{3,3'}$ = 17.0 Hz), 2.19 (H-3'), 2.33 (H-9'), 4.36 (H-2, $J_{2,3'}$ = 1.4 Hz, $J_{2,3}$ = 12.6 Hz, $J_{2,9'}$ = 8.0 Hz, $J_{2,9}$ = 4.8 Hz), 6.64 (H-6), 6.75 (H-8, $J_{6,8}$ = 1.0 Hz), 6.95 (H-7, $J_{6,7}$ = 7.0 Hz, $J_{7,8}$ = 7.8 Hz), 8.04 (H-5, $J_{5,6}$ = 7.8 Hz, $J_{5,7}$ = 1.4 Hz) ppm. A typical gas chromatogram obtained from a spent culture medium of DF-grown *Pseudomonas* sp. HH69 is shown in Fig. 4. With the exception of 2,2',3-trihydroxybiphenyl, Fig. 5 presents all compounds which could be identified in the course of our studies (numbers in brackets refer to the compounds shown in Fig. 5): methyl 3-(chroman-4-on-2-yl)-pyruvate [I], methyl 3-(chroman-4-on-2-yl)-lactate [II], methyl (chroman-4-on-2-yl)-acetate [III], the free acid [IV], chromone [V], 2-methyl-chroman-4-one [VI], and the monocyclic compounds, 2-hydroxyacetophenone [VII], salicylic acid [VIII], and gentisic acid [IX]. Salicylic and gentisic acid were also identified by HPLC upon comparison of retention times and in situ UV data with authentic samples.

Traces of all four isomeric monohydroxydibenzofurans (molecular weight 184, $C_{12}H_8O_3$ upon high-resolution, coupled gas chromatography-mass spectroscopy) were sometimes found to be present in the culture medium of *Pseudo-*

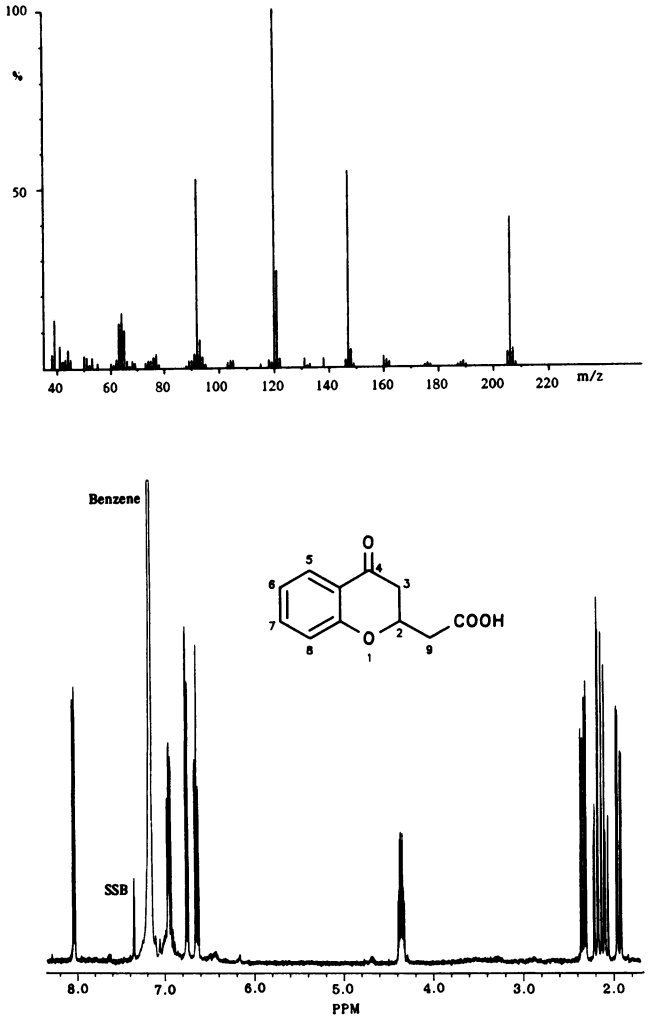

FIG. 3. 70 eV mass spectrum and ^1H NMR spectrum of (chroman-4-on-2-yl)-acetic acid (400.13 MHz; C_6D_6; TMS was as the internal standard).

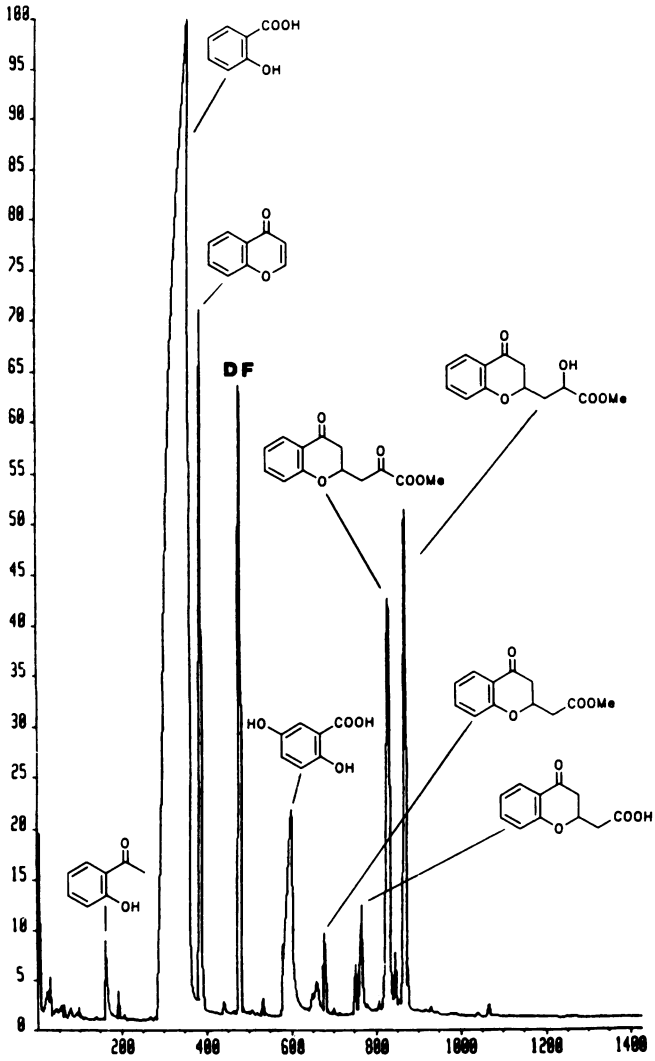

FIG. 4. Typical gas chromatogram of the acidified ethyl acetate extract of the spent culture medium of DF-grown *Pseudomonas* sp. HH69.

monas sp. strain HH69. The mixed culture HH27, also capable of degrading DF, produced compounds I through V and VII through IX along with small amounts of a dihydroxydibenzofuran, (chroman-4-on-2-yl)-acetaldehyde [X], the respective primary alcohol [XI], 2-methyl-chromone [XII], methyl 4-oxo-4-(2-hydroxyphenyl)-butyrate [XIII], methyl 4-oxo-4-(2,5-dihydroxyphenyl)-butyrate [XIV], and catechol [XV]. No degradation products of DF could be found in sterile, noninoculated control experiments analyzed after an appropriate period. Mass spectra of the chroman-4-one derivatives I, II, III, VI, X, and XI are shown in Fig. 6. Mass spectra and gas chromatographic retention times of compounds shown in Fig. 4 were identical to those obtained from samples synthesized in our laboratory (see Materials and Methods) and to those of commercially available compounds.

DISCUSSION

The presence of the isomers of monohydroxydibenzofuran in the culture medium, obviously derived from unstable *cis*-dihydrodiols, shows that known dioxygenation mechanisms are involved in the degradation of DF (7). However, the bulk of the substrate appears to be transformed to oxygenated products through a new and unique pathway via a trihydroxylated biphenyl as postulated in Fig. 7. During the initial step, DF is oxidized at positions 4 and 4a; this is followed by cleavage of the resulting obviously unstable hemiacetal and spontaneous dehydration to yield 2,2′,3-trihydroxybiphenyl as the product of rearomatization. During this process, the regeneration of NADH + H^+ seems to be impossible. Formation of the above trihydroxybiphenyl was recently proposed to be an intermediate in the degradation of 2-hydroxybiphenyl by dioxygenation and of 2,2′-dihydroxybiphenyl by a monooxygenase reaction which is also involved in the formation of 2,3-dihydroxybiphenyl from 3-hydroxybiphenyl (12, 19). In contrast, 4-hydroxybiphenyl was shown to be dioxygenated at the nonhydroxylated ring to yield 2,3,4′-trihydroxybiphenyl (12). The mass spectra of the latter compound and that of 2,3,3′-trihydroxybiphenyl, obtained from the oxidation of 3-hydroxy- and 3,3′-dihydroxybiphenyl (12), respectively, closely resemble the spectrum of our 2,2′,3-trihydroxybiphenyl. With respect to the dihydroxylated ring, NMR spectral data are almost

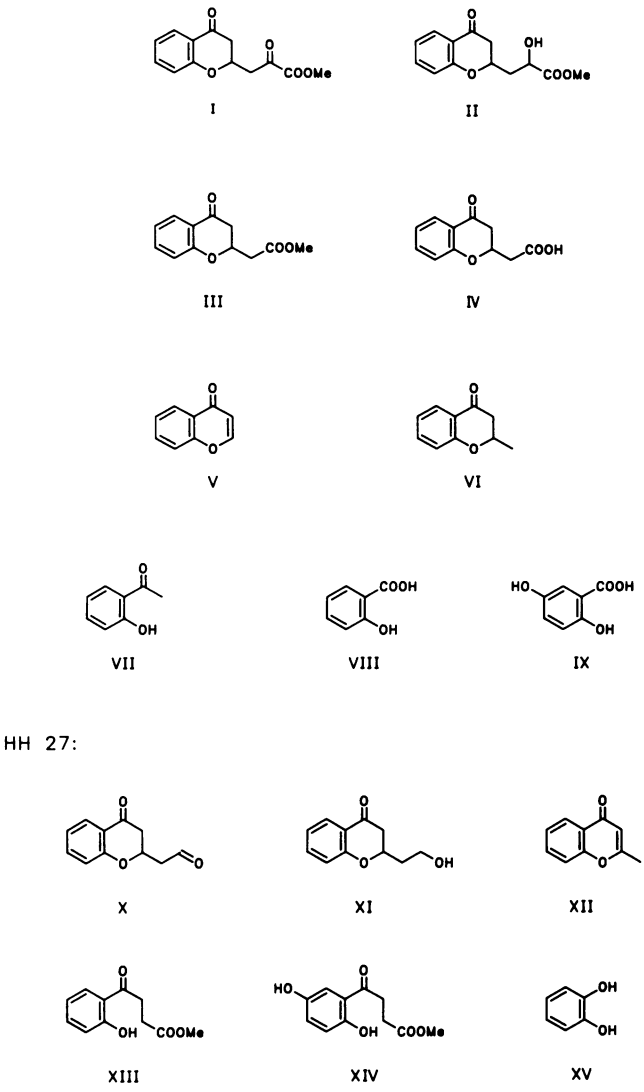

FIG. 5. Structures of identified metabolites detected in the culture medium of *Pseudomonas* sp. strain HH69 (top, I through IX) and those additionally found in the medium of the mixed culture HH27 (VI was not found in the medium of HH27). See Materials and Methods for details.

identical to those previously reported for 2,3-dihydroxybiphenyl (19).

Similar to the proposed route for the degradation of 2,3,4′-trihydroxybiphenyl (12), 2,2′,3-trihydroxybiphenyl (Fig. 7), formed from DF by strain HH69 and the mutant described in this paper, appears to be metabolized by a *meta*-cleavage reaction between C1 and C2 of the dioxygenated ring by strain HH69 to give 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoic acid, as could be assumed from the yellow color of the culture medium due to the production of a metabolite showing A_{446}. The corresponding 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, exhibiting a long wavelength absorption maximum of 435 nm, is known as the *meta*-cleavage product of 2,3-dihydroxybiphenyl (6), which is further metabolized to give benzoic acid and 2-hydroxypenta-2,4-dienoic acid (25).

The above-mentioned *ortho*-acylphenol shows a highly reactive vinylketone moiety in the side chain, and subsequent intramolecular addition of the phenolic hydroxyl group to the activated double bond would form a chroman-4-one with a side chain at position 2, as could be identified in 3-(chroman-4-on-2-yl)-pyruvic acid methylester (Fig. 5, I). [According to spectroscopic data, the unstable 3-(chroman-4-on-2-yl)-pyruvic acid should be the unidentified metabolite described by Strubel et al. (36).] We assume that the enzymatic cleavage of the postulated intermediate, 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)-hexa-2,4-dienoic acid (Fig. 7), and the above-mentioned addition of the hydroxyl residue to the vinylogous double bond will be competing reactions and that degradation via catechol is the preferred way. There are strong indications that the methyl esters shown in the scheme (Fig. 5) are produced during work up of the culture medium. Further transformations like reduction, decarboxylation, or β-oxidation then would lead to the compounds identified in the course of our studies (Fig. 5). Chain-shortening and -reducing reactions have also been discussed in the formation of 4-chlorobenzoic acid from 4-chlorobiphenyl via 2-hydroxy-6-oxo-6-(4-chlorophenyl)-hexa-2,4-dienoic acid (20) and with regard to biphenyl metabolism (24). On the other hand, and in analogy to the degradation mechanism of naphthalene (1), loss of an oxidized C4 unit from the initially formed acylphenol would directly lead to 2-hydroxyphenylglyoxylic acid or the respective aldehyde. The acid was found to be a growth substrate, and DF- and acetate-grown cells showed significant turnover and oxidation rates (Table 1). Subsequent decarboxylation would then lead to salicylaldehyde and/or the respective acid, similar to the bacterial metabolism of mandelic acid, which is degraded via phenylglyoxylic acid, benzaldehyde, benzoic acid, and catechol (35). However, we suggest a degradation sequence analogous to the above-described biphenyl pathway. Since chromone, 2-methylchromone, and 2-methylchroman-4-one were found not to support growth and not to be converted by washed-cell suspensions and since they did not significantly stimulate oxygen uptake, they were considered dead-end products of an unproductive branch line, together with 2-hydroxyacetophenone found in the culture medium of DF-grown strain HH69. This compound might be formed from decarboxylation of 3-(2-hydroxyphenyl)-3-oxopropanoic acid, which would represent a product of β-oxidation of chroman-4-on-2-acetic acid. 2-Hydroxyacetophenone was also detected as a dead-end product from xanthone degradation (37). Correspondingly, chloroacetophenones were identified from the media of bacterial cultures degrading chlorobiphenyls (2, 3).

The *ortho*-acylphenols XIII and XIV (Fig. 5), which were produced by the mixed culture HH27, are structurally close to the dihydrochromones; obviously, hydrogenation at the side chain had occurred before intramolecular trapping of the intermediate vinylketone. An analogous enzymatic reduction has been discussed for the biphenyl metabolism (24). Interestingly, 6-hydroxy-6-(4-chlorophenyl)-hexanoic acid was identified as a degradation product of 4-chlorobiphenyl (20). Its formation was attributed to a sequence starting from the initially produced 3,4-dihydroxy-4′-chlorobiphenyl (which was not detected in the culture medium); however, the mechanism postulated in our paper would offer a simple alternative. After the initial attack at positions 1 and 2 of 4-chlorobiphenyl (at the less-activated ring), the cleavage between the two hydroxyl groups would yield the above-mentioned same *para*-substituted chlorobenzene, which may be chain shortened by conventional steps.

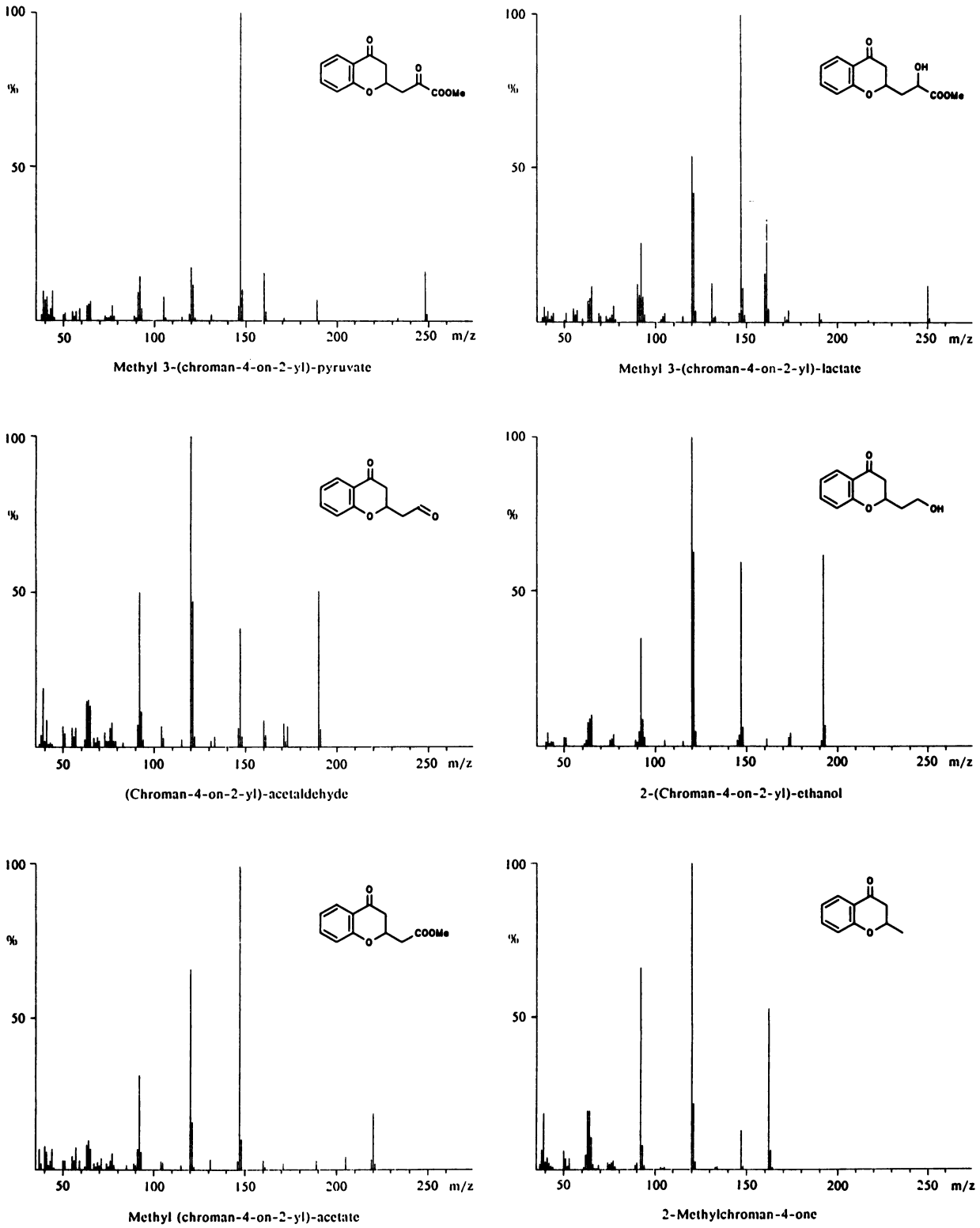

FIG. 6. 70 eV mass spectra of chroman-4-one derivatives identified from culture media. Note that in the spectrum of 2-methylchroman-4-one, relative intensities of the signals at M^+, *m/z* 120, and *m/z* 92 differ from data reported previously (21).

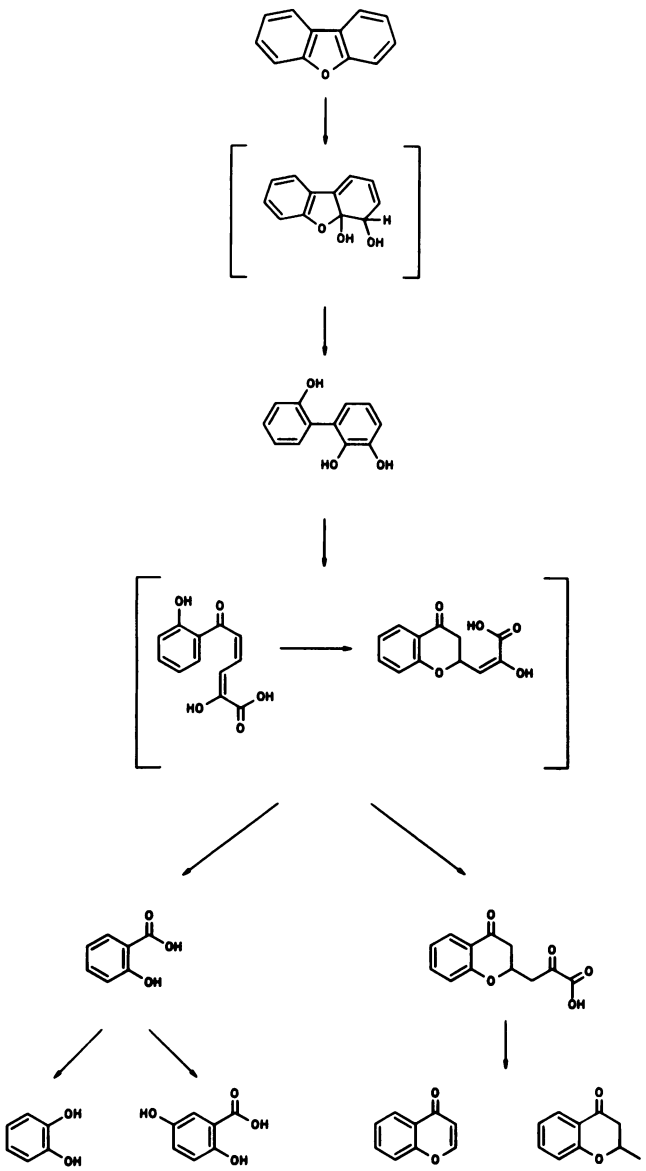

FIG. 7. Proposed pathway for the degradation of DF by *Pseudomonas* sp. strain HH69.

Research to clarify the metabolic route leading from 2,2',3-trihydroxybiphenyl to salicylic acid is in progress.

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