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Metabolism of Hydroxydibenzofurans, Methoxydibenzofurans, Acetoxydibenzofurans, and Nitrodibenzofurans by *Sphingomonas* sp. Strain HH69

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The metabolism of 11 substituted dibenzofurans by the dibenzofuran-degrading *Sphingomonas* sp. strain HH69 was investigated. Strain HH69 utilizes 2-, 3-, and 4-acetoxydibenzofuran as well as 2-, 3-, and 4-hydroxydibenzofuran as sole sources of carbon and energy. The degradation of acetoxydibenzofurans is initiated by hydrolysis of the ester bonds, yielding the corresponding hydroxydibenzofurans and acetate. Strain HH69 grew on 2-methoxydibenzofuran only after it was adapted to the utilization of 5-methoxysalicylic acid, whereas 3- and 4-methoxydibenzofuran as well as 2- and 3-nitrodibenzofuran were only cooxidized. During the breakdown of all eight hydroxy-, methoxy-, and nitrodibenzofurans studied here, the corresponding substituted salicylic acids accumulated in the culture broth. In the cases of 2- and 3-hydroxydibenzofuran as well as 2- and 3-nitrodibenzofuran, salicylic acid was also formed. Those four dibenzofurans which did not serve as carbon sources for strain HH69 were converted to a nonutilizable salicylic acid derivative. From turnover experiments with the mutant HH69/II, which is deficient in *meta*-cleavage, 2,2′,3,4′-tetrahydroxybiphenyl, 2,2′,3-trihydroxy-5′-methoxybiphenyl, 2,2′,3-trihydroxy-5′-nitrobiphenyl, and 2,2′,3-trihydroxy-4′-nitrobiphenyl were isolated as the main products formed from 3-hydroxydibenzofuran, 2-methoxydibenzofuran, and 2- and 3-nitrodibenzofuran, respectively. These results indicate significant regioselectivity for the dioxygenolytic cleavage of the ether bond of these monosubstituted dibenzofurans, with a preference for the nonsubstituted aromatic nucleus. Substituted trihydroxybiphenyls are converted further by *meta*-cleavage followed by the removal of the side chain of the resulting product. A stepwise degradation of this side chain was found to be involved in the metabolism of 2-hydroxydibenzofuran.

Dioxygenolytic cleavage of the ether bond is one of the crucial steps in the bacterial degradation of diaryl ethers such as dibenzofuran (12, 21, 29), dibenzo-*p*-dioxin (18, 34), diphenyl ether (26), various substituted derivatives (9, 17, 27), and the diaryl thioether dibenzothiophene (31). Gram-negative *Sphingomonas* spp. (12, 26, 34) as well as gram-positive bacteria (21, 29, 31) initiate the degradation by incorporating molecular oxygen adjacent to the ether bridge. This yields phenolic hemiacetals, which are subject to spontaneous cleavage. All strains described up to now that catalyze the dioxygenolytic cleavage of ether bonds share a broad substrate specificity which enables them to attack substituted diaryl ethers and structurally analogous compounds. Besides dibenzofuran, *Brevibacterium* sp. strain DPO 1361 (10) and *Staphylococcus auriculans* DBF63 (21) utilize the structural analog fluorene as the sole source of carbon and energy. It was shown that the substrate spectrum of *Sphingomonas* sp. strain SS3 originally enriched with 4-fluorodiphenyl ether can be extended by adaptation to 4-chloro-, 4-bromo- (26), and 3-methyldiphenyl ether (27). *Sphingomonas* sp. strain HH69, the strain used in the present study, attacks dibenzofuran, dibenzo-*p*-dioxin, 9-fluorenone, xanthone, xanthene, dibenzothiophene, and biphenyls (11, 13, 16, 18). Angular dioxygenation of dibenzofurans yielded 2,2′,3-trihydroxybiphenyls (10, 12, 17) which were further metabolized as described previously for 2,3-dihydroxybiphenyl (8). Since initial dioxygenation of monosubstituted dibenzofurans can take place either at the substituted or nonsubstituted ring, further degradation of substituted trihydroxylated biphenyls leads to either salicylic acid or to a substituted salicylic acid. This paper focuses particularly on the regioselectivity of the initial attack.

During dibenzofuran degradation by *Sphingomonas* sp. strain HH69, traces of all four monohydroxydibenzofurans were identified in the culture supernatants (12). 1-Hydroxy-2,3,7,8-tetrachlorodibenzo-*p*-dioxin was detected as a product formed from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by several microbial cultures (25). Hence, hydroxylated metabolites of diaryl ethers may also occur in natural environments as a result of biological processes. Therefore, in the search for effective bioremediation procedures, the existence of degradation routes not only for respective target compounds but also for emerging transformation products is required.

In the course of our investigations aiming at the aerobic degradation of diaryl ether compounds, we examined the breakdown of 11 hydroxy-, methoxy-, acetoxy-, and nitrodibenzofurans. Evidence for the mineralization of seven of these compounds and the cometabolic conversion of the remaining four dibenzofurans is presented here.

MATERIALS AND METHODS

Bacteria, media, and culture conditions. The isolation and characteristics of *Sphingomonas* sp. strain HH69, which is able to utilize dibenzofuran as its sole

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TABLE 1. Metabolites accumulated during degradation of dibenzofurans by *Sphingomonas* sp. strain HH69

Substrate	Metabolite(s)
Dibenzofuran[a]	2-Hydroxydibenzofuran
2-Hydroxydibenzofuran	5-Hydroxysalicylic acid, salicylic acid, 4-hydroxycoumarin, 2-hydroxyacetophenone, and 3-(2-hydroxyphenyl)-3-oxopropionic acid
3-Hydroxydibenzofuran	4-Hydroxysalicylic acid and salicylic acid
4-Hydroxydibenzofuran	3-Hydroxysalicylic acid
2-Methoxydibenzofuran	5-Methoxysalicylic acid
3-Methoxydibenzofuran	4-Methoxysalicylic acid
4-Methoxydibenzofuran	3-Methoxysalicylic acid
2-Acetoxydibenzofuran	2-Hydroxydibenzofuran, 5-hydroxysalicylic acid, salicylic acid, 4-hydroxycoumarin, and 2-hydroxyacetophenone
3-Acetoxydibenzofuran	3-Hydroxydibenzofuran, 4-hydroxysalicylic acid, and salicylic acid
4-Acetoxydibenzofuran	4-Hydroxydibenzofuran and 3-hydroxysalicylic acid
2-Nitrodibenzofuran	5-Nitrosalicylic acid and salicylic acid
3-Nitrodibenzofuran	4-Nitrosalicylic acid and salicylic acid

[a] Further products from dibenzofuran are reported in reference 12.

source of carbon and energy, have been described previously (12). The strain was formerly assigned to the paucimobilis group of the genus *Pseudomonas* and was reclassified on the basis of its pattern of fatty acids and ubiquinon (34). The mutant strain HH69/II lacks 2,3-dihydroxybiphenyl-1,2-dioxygenase and, therefore, converts dibenzofuran only to 2,2′,3-trihydroxybiphenyl, which is excreted (12). Bacteria were grown in a mineral salts medium containing (per liter) 1.75 g of $Na_2HPO_4 \cdot 2H_2O$, 0.5 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 (24). As a carbon source for strain HH69, either 2 g of acetate liter^{-1} or 1 g of crystalline dibenzofuran liter^{-1} was used. The mutant strain was grown on 0.5 g of sodium benzoate liter^{-1}. Unless stated otherwise, cells were grown, harvested, and washed as described previously (12).

Chemicals. Dibenzofuran; 2-hydroxydibenzofuran; salicylic acid; 3-, 4-, and 5-hydroxysalicylic acid; 3-, 4-, and 5-methoxysalicylic acid; 2-hydroxyacetophenone; and 4-hydroxycoumarin were purchased from Aldrich, Steinheim, Federal Republic of Germany. 3-Aminodibenzofuran was prepared by reduction of 3-nitrodibenzofuran (6) according to the general procedure of Balcom and Furst (3), while 4-aminodibenzofuran was synthesized from dibenzofuran in a method described by Gilman and Ingham (15). The aminodibenzofurans served as educts for the synthesis of 3- and 4-hydroxydibenzofuran by the method used by Butenandt et al. (7) for the preparation of 3-hydroxyacetophenone. Methylation and acetylation of the hydroxydibenzofurans by standard procedures furnished the corresponding methoxy and acetoxy derivatives, respectively. 2-Nitrodibenzofuran was synthesized by nitration of 3-acetaminodibenzofuran with nitroniumtetrafluoroborate according to the method of Oliver (22), which was followed by hydrolysis and removal of the amino group.

Substrate utilization. Substrate utilization was tested on solid media. Petri dishes containing mineral salts medium agar were inoculated. Subsequently, 5 mg of the crystalline substrate was put on the edge of the agar. The plates were then incubated for 1 week at 28°C. The toxicity of the substrates and metabolites was tested by growing strain HH69 in liquid media on dibenzofuran in the presence of various concentrations of the target compound.

Strain adaptation. In order to adapt strain HH69 to the utilization of methoxysalicylic acids as carbon sources, the strain was grown in a 300-ml Erlenmeyer flask containing 100 ml of mineral salts medium and 100 mg of salicylic acid as the carbon source on a rotary shaker at 28°C. After the postexponential phase was reached (approximately 48 h), 1 ml of the culture was transferred into fresh medium in which 10% of the salicylic acid was replaced by either 3-, 4-, or 5-methoxysalicylic acid. This shift was repeated every 48 h until methoxysalicylic acids were the sole organic carbon sources.

Production of metabolites. Strain HH69 or the *meta*-cleavage-deficient mutant HH69/II was grown in 2 liters of medium in a 5-liter bottle at 28°C. Aeration was achieved by stirring thoroughly with a triangular magnetic stir bar at 600 rpm. The carbon source was 2 g of acetate liter^{-1} (HH69) or 0.5 g of benzoate liter^{-1} (HH69/II). After 48 h, the organic substrates were almost consumed and had yielded biomasses corresponding to 0.5 g of protein liter^{-1} of wild-type cells or 0.15 g of protein liter^{-1} of mutant cells, respectively. Transformation was started by adding 50 mg of crystalline dibenzofuran liter^{-1} or substituted dibenzofurans together with 2 g of acetate liter^{-1} directly to the culture. Product formation was followed by high-pressure liquid chromatography (HPLC) and stopped at its maximum by pouring the incubation mixture through a paper filter in order to remove residual substrate crystals. Cells were removed by centrifugation, and the supernatant was kept at 4°C until further workup.

Isolation of metabolites. The supernatant of each incubation was extracted twice with half the volume of ethyl acetate (neutral fraction). Subsequently, it was adjusted to pH 2 and extracted two more times (acidic fraction). After the supernatant was dried over anhydrous sodium sulfate and then filtered through a paper filter, the solvent was evaporated. The residue of the neutral fraction was dissolved in a small volume of demineralized water, filtered through paper to remove the less soluble residual dibenzofurans, and extracted again. Residues of neutral and acidic fractions were dissolved in small volumes of methanol for the separation of metabolites by preparative reversed-phase HPLC with a Spherisorb 5-μm ODS II column (16 by 250 mm; Phase Separation Ltd., Deeside, United Kingdom) for the stationary phase. The mobile phase involved either water or 10 mM H_3PO_4 in water and 60% (vol/vol) methanol. The flow rate was set to 9.9 ml min^{-1}. Compounds were detected by means of their UV absorption at 210 nm. In some cases, extracts were incubated with identical volumes of BSTFA [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide] at 70°C for 15 min in order to form trimethylsilyl derivatives.

Analytical methods. HPLC, thin-layer chromatography, gas chromatography, gas chromatography-mass spectrometry, and nuclear magnetic resonance (NMR) spectroscopy were performed as described before (12). The assignment of protons in ^{1}H NMR spectra is based on H,H-COSY experiments (2) and increment calculations. Oxygen consumption was measured with a Clark-type electrode as described before (12). Protein was determined by the method of Spector (28).

RESULTS

Conversion products of dibenzofurans formed by *Sphingomonas* sp. strain HH69. These products are listed in Table 1. The acetate esters of all three acetoxydibenzofurans tested were hydrolytically cleaved, as could be seen from the appearance of the corresponding hydroxydibenzofurans. The conversion of hydroxy-, methoxy-, and nitrodibenzofurans yielded the corresponding substituted salicylic acids, which in the cases of 2- and 3-hydroxydibenzofuran as well as 2- and 3-nitrodibenzofuran were accompanied by salicylic acid. With the exception of 4-nitrosalicylic acid, the identification of theses metabolites, including 2-hydroxyacetophenone and 4-hydroxycoumarin (see below), is based on comparisons with authentic standards. After isolation from the culture broth and purification by recrystallization, 4-nitrosalicylic acid was identified by mass spectrometry and NMR spectroscopy. The mass spectrum showed a base peak at an *m*/*z* of 165, which is a result of the elimination of H_2O from the molecular ion at an *m*/*z* of 183 (35%). A predominant fragment ion was found at an *m*/*z* of 119 (47%) because of the loss of 46 atomic mass units (NO_2) from the base peak. NMR data were as follows. ^{1}H NMR [400.13 MHz, $(CD_3)_2CO$, tetramethylsilane as internal standard]: δ = 7.71 (H-3, $J_{3,5}$ = 2.0 Hz), 7.76 (H-5, $J_{5,6}$ = 8.7 Hz), and 8.15 (H-6) ppm. ^{13}C NMR (100.62 MHz, solvent and internal standard as above): δ = 112.3, 113.6, 117.8, 132.3, 152.5, 165.4, and 170.6 ppm. 5-Nitrosalicylic acid and salicylic acid were present in the extracts in a ratio of 20:1 (based on HPLC peak areas) upon incubation with 2-nitrodibenzofuran, whereas 3-nitrodibenzofuran as the substrate yielded 4-nitrosalicylic acid and salicylic acid in a ratio of 8:1. In addition to salicylic acid and 5-hydroxy-

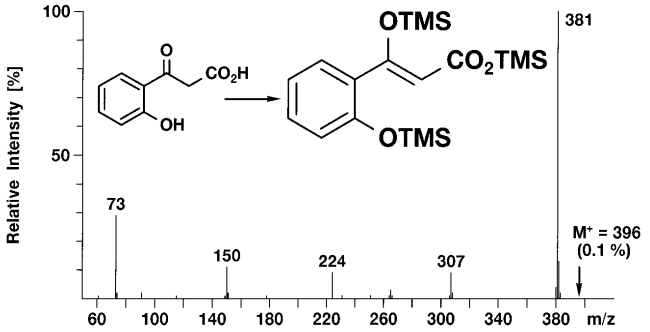

FIG. 1. Mass spectrum (EI, 70 eV) of the trimethylsilyl derivative of 3-(2-hydroxyphenyl)-3-oxopropionic acid.

salicylic acid, 2-hydroxyacetophenone and 3-(2-hydroxyphenyl)-3-oxopropionic acid were excreted during the degradation of 2-hydroxydibenzofuran. The identification of 3-(2-hydroxyphenyl)-3-oxopropionic acid is based on the mass spectrum of its trimethylsilyl derivative solely (Fig. 1), since no reference compound was available and the yield was insufficient for NMR analysis. The course of metabolite formation from 2-hydroxydibenzofuran is shown in Fig. 2. After a prolonged incubation, the concentrations of salicylic acid, 5-hydroxysalicylic (gentisic) acid, and 3-(2-hydroxyphenyl)-3-oxopropionic acid decreased again, whereas the concentration of 2-hydroxyacetophenone slightly increased. Mass spectrometric analysis of the extracts also revealed the presence of 4-hydroxycoumarin. Attempts to purify 3-(2-hydroxyphenyl)-3-oxopropionic acid by preparative HPLC resulted in partial conversion to 4-hydroxycoumarin. When cells of strain HH69 were added to such a mixture, 3-(2-hydroxyphenyl)-3-oxopropionic acid was slowly converted to 2-hydroxyacetophenone while the concentration of 4-hydroxycoumarin remained unchanged. In the absence of the cells, 3-(2-hydroxyphenyl)-3-oxopropionic acid was stable, but upon acidification, it was converted to 4-hydroxycoumarin. 2-Hydroxyacetophenone and 4-hydroxycoumarin were not transformed by whole cells and cell extracts of strain HH69. Small amounts of 2-hydroxydibenzofuran produced from dibenzofuran were observed by HPLC without a workup of the

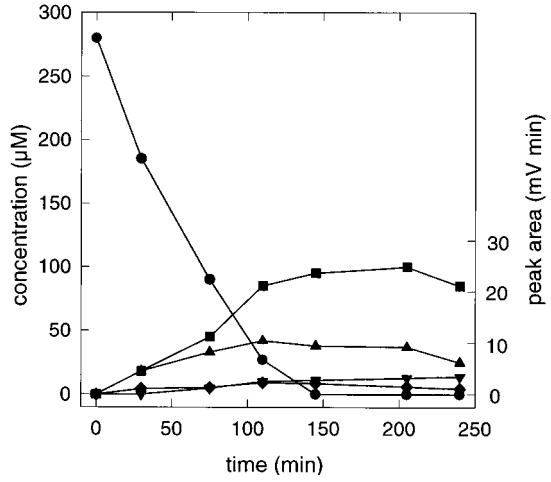

FIG. 2. Time course of the transformation of 2-hydroxydibenzofuran (●) to salicylic acid (▲), gentisic acid (■), 2-hydroxyacetophenone (▼), and 3-(2-hydroxyphenyl)-3-oxopropionic acid (◆). Concentrations of 3-(2-hydroxyphenyl)-3-oxopropionic acid are given as HPLC peak areas.

supernatant, whereas the other three hydroxydibenzofurans were not detected. All other metabolites mentioned so far were identified by comparison of their chromatographic and mass spectrometric behaviors with those of authentic standards.

Growth with substituted dibenzofurans. *Sphingomonas* sp. strain HH69 grew on solid media with dibenzofuran; 2-, 3-, and 4-hydroxydibenzofuran; 2-methoxydibenzofuran; 2-, 3-, and 4-acetoxydibenzofuran; salicylic acid; and 3-, 4-, and 5-hydroxysalicylic acid, whereas no growth was observed with 3- and 4-methoxydibenzofuran, 2- and 3-nitrodibenzofuran, 3- and 4-methoxydibenzofuran, 4- and 5-nitrosalicylic acid, 2-hydroxyacetophenone, and 4-hydroxycoumarin. 2-Methoxydibenzofuran was utilized as the sole source of carbon and energy after the adaptation of strain HH69 to 5-methoxysalicylic acid over a period of 20 days, whereas adaptation of strain HH69 to 3- and 4-methoxysalicylic acid was not achieved within 20 days. All hydroxydibenzofurans exhibited considerable toxicity, which could be seen from the large cell-free zones around substrate crystals adjacent to zones of considerable growth. As much as 271 μM of 2-hydroxydibenzofuran could be transformed by resting cells of strain HH69 (Fig. 2), while the presence of only 100 μM 2-hydroxydibenzofuran completely inhibited growth of strain HH69 on dibenzofuran in liquid medium. 2-Hydroxyacetophenone (1 mM) did not inhibit the growth of strain HH69 on dibenzofuran.

Conversion of substituted dibenzofurans by the mutant strain HH69/II. 2,2′,3-Trihydroxy-5′-methoxybiphenyl and 2,2′,3,4′-tetrahydroxybiphenyl were isolated as the products of a dioxygenolytic attack onto 2-methoxy- and 3-hydroxydibenzofuran, respectively. The ^{1}H NMR spectra of these compounds are shown in Fig. 3A and B. NMR data of the purified metabolites were as follows. 2,2′,3-Trihydroxy-5′-methoxybiphenyl. ^{1}H NMR (400.13 MHz, C_6D_6/CD_3CN = 4:1, tetramethylsilane as internal standard): δ = 3.47 (OCH_3), 6.78 (H-4′, $J_{4',5'}$ = 3.1 Hz), 6.85 (H-5, $J_{5,6}$ = 7.9 Hz), 6.89 (H-6), 6.94 (H-6′), and 6.99 to 7.02 (H-4/H-3′, $J_{4,5}$ = 7.9 Hz, $J_{4,6}$ = 1.8 Hz, $J_{3',4'}$ = 8.9 Hz) ppm. ^{13}C NMR (100.62 MHz, solvent and internal standard as above): δ = 55.4, 114.5, 115.1, 116.6, 117.6, 121.8, 122.5, 127.0, 127.2, 141.6, 146.5, 147.0, and 154.5 ppm. 2,2′,3,4′-Tetrahydroxybiphenyl. ^{1}H NMR (400.13 MHz, CD_3OD/C_6D_6 = 100:15, tetramethylsilane as internal standard): δ = 6.50 (H-5′, $J_{5',6'}$ = 8.3 Hz), 6.54 (H-3′, $J_{3',5'}$ = 2.4 Hz), 6.76 (H-6), 6.79 (H-5, $J_{5,6}$ = 7.6 Hz), 6.84 (H-4, $J_{4,5}$ = 7.6 Hz, $J_{4,6}$ = 2.1 Hz), and 7.12 (H-6′) ppm. ^{13}C NMR (100.62 MHz, solvent and internal standard as above): δ = 104.5, 109.4, 115.0, 119.3, 121.8, 123.4, 128.8, 133.5, 143.1, 147.5, 155.7, and 159.5 ppm. The mass spectra of the metabolites exhibited molecular ions at an *m/z* of 232 and an *m/z* of 218, corresponding to the molecular formulas $C_{13}H_{12}O_4$ and $C_{12}H_{10}O_4$, respectively. Acetic acid was identified in the acidic fraction of an incubation of strain HH69/II with 3-acetoxydibenzofuran.

Two products in a ratio of 4:1 (based on the HPLC peak areas) were formed from 2-nitrodibenzofuran, whereas the ratio of two products formed from 3-nitrodibenzofuran was 5:1. After isolation and purification, the main products obtained from 2- and 3-nitrodibenzofuran were identified as 2,2′,3-trihydroxy-5′-nitrobiphenyl and 2,2′,3-trihydroxy-4′-nitrobiphenyl, respectively. The mass spectra of both metabolites exhibited a molecular ion at an *m/z* of 247, corresponding to the molecular formula $C_{12}H_9NO_5$. ^{1}H NMR spectra of 2,2′,3-trihydroxy-5′-nitrobiphenyl and 2,2′,3-trihydroxy-4′-nitrobiphenyl are given in Fig. 3C and D. NMR data were as follows. 2,2′,3-Trihydroxy-5′-nitrobiphenyl. ^{1}H NMR [400.13 MHz, $(CD_3)_2CO$, tetramethylsilane as internal standard): δ = 6.79

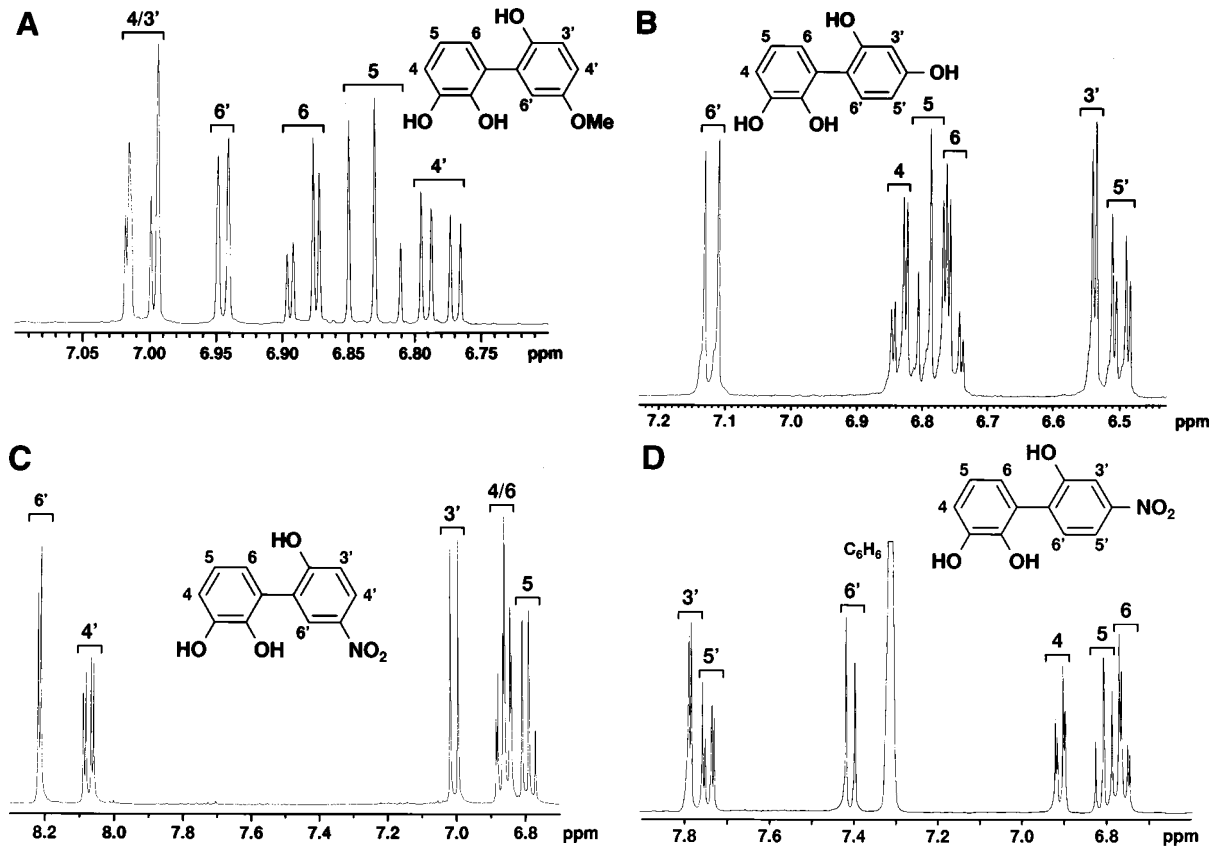

FIG. 3. ^{1}H NMR spectra of 2,2′,3-trihydroxy-5′-methoxybiphenyl (A; a singlet at 3.47 ppm representing the methoxy protons is not shown), 2,2′,3,4′-tetrahydroxybiphenyl (B), 2,2′,3-trihydroxy-5′-nitrobiphenyl (C), and 2,2′,3-trihydroxy-4′-nitrobiphenyl (D).

(H-5, $J_{5,6}$ = 7.8 Hz), 6.85 (4-H or 6-H, $J_{4,5}$ = 7.8 Hz, $J_{4,6}$ = 1.8 Hz), 6.88 (H-4 or H-6), 7.01 (H-3′, $J_{3',4'}$ = 9.1 Hz), 8.07 (H-4′, $J_{4',6'}$ = 2.9 Hz), and 8.21 (H-6′) ppm. 2,2′,3-Trihydroxy-4′-nitrobiphenyl. ^{1}H NMR (400.13, CD_3OD/C_6D_6 = 100:15, tetramethylsilane as internal standard): δ = 6.76 (H-6), 6.81 (H-5, $J_{5,6}$ = 7.7 Hz), 6.91 (H-4, $J_{4,5}$ = 7.7 Hz, $J_{4,6}$ = 1.8 Hz), 7.41 (H-6′), 7.74 (H-5′, $J_{5',6'}$ = 8.4 Hz), and 7.78 (H-3′, $J_{3',5'}$ = 2.3 Hz) ppm. The respective minor products formed during both experiments could not be isolated in amounts required for structure elucidation. Transformation rates of 2- and 3-nitrodibenzofuran were estimated to be <2 nmol mg of protein^{-1} min^{-1}, whereas the specific activity of cells from the same culture towards dibenzofuran was 26 nmol mg^{-1} min^{-1}.

Conversion of purified products from the mutant strain HH69/II. Purified 2,2′,3-trihydroxy-5′-nitrobiphenyl and 2,2′,3-trihydroxy-4′-nitrobiphenyl were used as substrates for further transformation experiments. Both whole cells and cell extracts of strain HH69 converted 2,2′,3-trihydroxy-5′-nitrobiphenyl to 5-nitrosalicylic acid and 2,2′,3-trihydroxy-4′-nitrobiphenyl to 4-nitrosalicylic acid. The reactions were accompanied by the transient appearance of an orange coloring. Since HH69 and cell extracts are yellow, this color was probably due to unstable red metabolites originating from *meta*-cleavage of the trihydroxynitrobiphenyls.

Substrate specific oxygen consumption rates. The consumption rates for acetate- and dibenzofuran-grown cells of HH69 and acetate-grown cells of HH69/II are given in Table 2. The specific oxygen consumption rates of acetate-grown cells were higher with acetoxydibenzofurans than with dibenzofuran, whereas dibenzofuran-grown cells consumed more oxygen with dibenzofuran than with acetoxydibenzofurans as the substrates. Considerable oxygen uptake with acetate as the substrate was detected only with acetate-grown cells.

DISCUSSION

The dibenzofuran-mineralizing *Sphingomonas* sp. strain HH69 (12) utilized 7 out of 11 substituted dibenzofurans tested as sole sources of carbon and energy. The degradation of

TABLE 2. Specific oxygen uptake rates by resting cells of *Sphingomonas* sp. strain HH69

Strain and substrate	Specific oxygen consumption (nmol of O_2 min^{-1} mg of protein^{-1}) of cells pregrown with:	
	Dibenzofuran	Acetate
Sphingomonas sp. strain HH69		
Dibenzofuran	410	90
2-Acetoxydibenzofuran	320	113
3-Acetoxydibenzofuran	238	159
Sodium acetate	38	285
Sphingomonas sp. strain HH69/II		
Dibenzofuran	–[a]	51
2-Acetoxydibenzofuran	–	139
3-Acetoxydibenzofuran	–	151

[a] Dibenzofuran does not serve as a growth substrate for *Sphingomonas* sp. strain HH69/II.

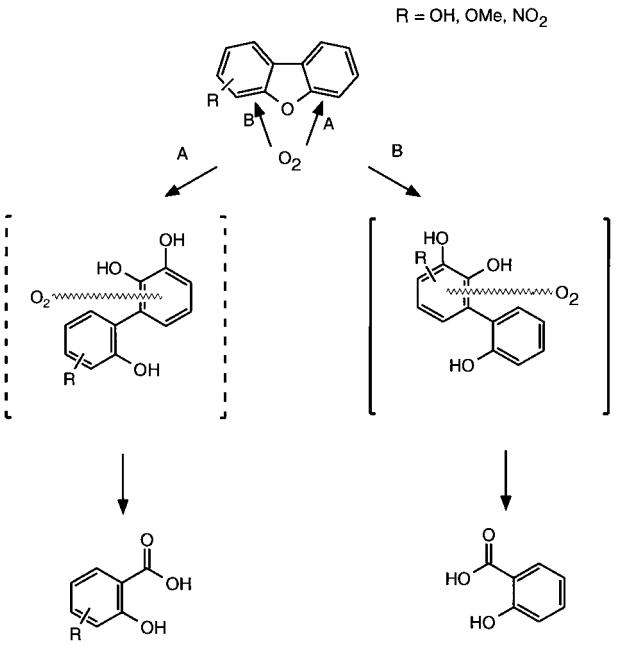

FIG. 4. Possible coexisting pathways for the degradation of substituted dibenzofurans.

acetoxydibenzofurans was initiated by hydrolysis of the ester bond, yielding the corresponding hydroxydibenzofurans and acetate. Therefore, their further degradation will be discussed together with that of the hydroxydibenzofurans. The appearance of hydroxy-, methoxy-, and nitrosubstituted salicylic acids from the correspondingly substituted dibenzofurans, accompanied by salicylic acid in the cases of 2- and 3-hydroxydibenzofuran and 2- and 3-nitrodibenzofuran, indicates that the degradation proceeds via the same principal pathway that has been described for dibenzofuran and 3-chlorodibenzofuran (12, 17). The crucial reactions leading from dibenzofuran to salicylic acid have been discussed in detail (10, 12). The two proposed coexisting pathways for the degradation of substituted dibenzofurans by strain HH69 are outlined in Fig. 4. For monosubstituted dibenzofurans, the regioselectivity of the initial angular dioxygenation yielding substituted 2,2′,3-trihydroxybiphenyls could be expected because of the steric and/or electronic effects of the respective substituents. The appearance of salicylic acid beside substituted salicylic acids indicates the dioxygenation of both the substituted and the nonsubstituted aromatic nucleus. Although salicylic acid was not detected, its formation as an intermediate in the degradation of 4-hydroxydibenzofuran and the methoxydibenzofurans cannot be excluded as a possibility, since salicylic acid is easily mineralized by strain HH69. The capability of strain HH69 to utilize substituted dibenzofurans was found to be strictly correlated with its ability to utilize emerging salicylic acid derivatives: dibenzofurans yielding substituted nonutilizable salicylic acid derivatives did not serve as carbon sources for strain HH69. After adapting to the utilization of 5-methoxysalicylic acid, strain HH69 grew also on 2-methoxydibenzofuran, whereas no growth on 4- and 3-methoxysalicylic acid and on 3- and 4-methoxydibenzofuran was achieved.

Upon transformation experiments with the *meta*-cleavage-deficient mutant strain HH69/II, 2,2′,3-trihydroxy-5′-methoxybiphenyl, 2,2′,3,4′-tetrahydroxybiphenyl, 2,2′,3-trihydroxy-5′-nitrobiphenyl, and 2,2′,3-trihydroxy-4′-nitrobiphenyl were identified as the major products of 2-methoxydibenzofuran, 3-hydroxydibenzofuran, and 2- and 3-nitrodibenzofuran, respectively. From this, we conclude that the degradation of these four dibenzofuran derivatives is predominantly initiated by dioxygenation of the nonsubstituted aromatic rings (pathway A in Fig. 4). There is no indication of the further transformation by mutant strain HH69/II of any of the dioxygenation products mentioned above. Therefore, we consider the obtained metabolite patterns to be well-suited to prove the regioselectivity of the dioxygenation. A strict regioselectivity is probable for 4-hydroxy- and 4-methoxydibenzofuran, whose occupied positions 4 may not be attacked by the enzyme for both steric and electronic reasons. For 2-hydroxydibenzofuran, the situation is more complicated, since 5-hydroxysalicylic acid could have originated either from the degradation of the nonsubstituted aromatic ring or from hydroxylation of salicylic acid (16). However, it should be noted that during our present study, 5-hydroxysalicylic acid was observed only as a metabolite of 2-hydroxydibenzofuran, which makes its formation from salicylic acid relatively improbable. Dioxygenation of the substituted ring, on the other hand, may play an important role in the degradation of this substrate by the pathway discussed below. In an earlier study, 3-chlorodibenzofuran was shown to be converted by strain HH69 into equal amounts of both possible dioxygenation products (17). A nonspecific attack of the dioxygenating enzyme system was also observed during the degradation of monohalogenated diphenyl ethers by *Sphingomonas* sp. strain SS3 (26). Similar results for several chlorinated dibenzofurans and dibenzodioxins were obtained with the dibenzo-*p*-dioxin-utilizing bacterium *Sphingomonas* sp. strain RW1 (33). In contrast, all results obtained so far for halogenated diarylketones indicated strict regioselectivity for the dioxygenation reaction (14). For most of the tested substrates, the oxidative attack took place at the substituted aromatic ring. On the overall basis of this available information on the dioxygenation of substituted diaryl ethers and structurally related compounds, two conclusions may be drawn: (i) the dioxygenolytic attack on individual substrates may or may not show significant regioselectivity, and (ii) at present, there is no evidence for any systematic influence of the substituents in terms of electronic and/or steric properties.

As outlined in Fig. 5, the appearance of 3-(2-hydroxyphenyl)-3-oxopropionic acid, 2-hydroxyacetophenone, and 4-hydroxycoumarin suggests that the breakdown of the substituted aromatic nucleus of 2-hydroxydibenzofuran has occurred. Assuming the existence of the generally accepted biphenyl pathway, *meta*-cleavage of 2,2′,3,5-tetrahydroxybiphenyl (compound B in Fig. 5) would yield 2,4-dihydroxy-6-(2-hydroxyphenyl)-6-oxohexa-2,4-dienoic acid (compound C). This metabolite contains an additional hydroxyl group at the side chain by comparison with the *meta*-cleavage product of 2,3-dihydroxybiphenyl, 2-hydroxy-6-oxo-6-phenyl-2,4-hexadienoic acid (8). The latter is cleaved by hydrolase activity to give benzoic acid and 2-oxo-4-pentenoic acid (23). In contrast, the hydrolytic split-off of pyruvate from 2,4-dihydroxy-6-(2-hydroxyphenyl)-6-oxohexa-2,4-dienoic acid (compound C in Fig. 5) would yield 3-(2-hydroxyphenyl)-3-oxopropanoic acid (compound D), which we found in high amounts as a transient product. Alternatively, this metabolite could be produced from 2,4-dihydroxy-6-(2-hydroxyphenyl)-6-oxohexa-2,4-dienoic acid by oxidative decarboxylation followed by the enzymatic loss of acetate. Unlike Tomasek and Crawford (30), we did not observe the decarboxylation of 4-hydroxycoumarin to give 2-hydroxyacetophenone. In our case, 2-hydroxyacetophenone (compound E in Fig. 5) was formed by decarboxylation of open chain 3-(2-hydroxyphenyl)-3-oxopropionic acid (compound D). The related decarboxylation of 3-oxo-3-phenylpropanoic acid to aceto-

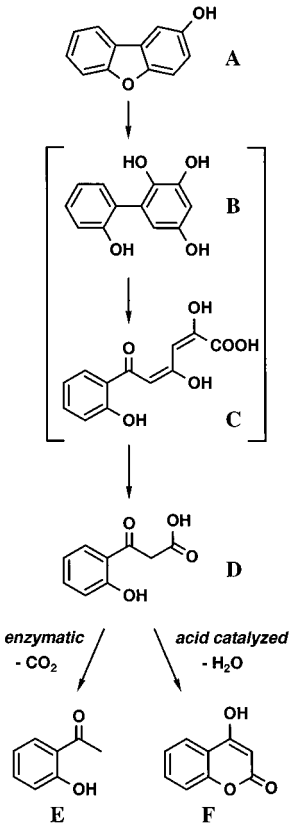

FIG. 5. Proposed byway in the degradation of 2-hydroxydibenzofuran. (A) 2-Hydroxydibenzofuran. (B) 2,2′,3,5-Tetrahydroxybiphenyl. (C) 2,4-Dihydroxy-6-(2-hydroxyphenyl)-6-oxohexa-2,4-dienoic acid. (D) 3-(2-Hydroxyphenyl)-3-oxopropionic acid. (E) 2-Hydroxyacetophenone. (F) 4-Hydroxycoumarin.

phenone by a *Pseudomonas* sp. has previously been proposed as a possibility (19). Acetophenone has also been detected as a by-product of the degradation of 2,3-dihydroxybiphenyl by strain HH69 (32). Chlorinated acetophenones have been identified as products of the microbial degradation of chlorinated biphenyls (4, 5). Excretion of several products shortened at the side chain has been reported to follow *meta*-cleavage of 2,2′,3-trihydroxybiphenyl (12) and 4′-chloro-2,3-dihydroxybiphenyl (1, 20). Complete degradation of biphenyl structures appears to generally proceed via 2,3-dihydroxybiphenyls. To our knowledge, no exceptions are reported. This might be due to the fact that only the 1,2-extradiol cleavage of 2,3-dihydroxybiphenyls yields products with an unbranched carbon chain, which seems to be substantial for efficient degradation. In contrast, Bedard et al. (5) propose 3,4-dioxygenation as an explanation of the appearance of chlorinated acetophenones during the degradation of chlorinated biphenyls. However, until now, no conclusive proof for this pathway has been given. Hence, at the present, the successive chain shortening of the *meta*-cleavage products of 2,3-dihydroxybiphenyls seems to be the most satisfying explanation for the variety of metabolites with different chain lengths that were observed during the degradation of dibenzofurans and biphenyls.

Sphingomonas sp. strain HH69 is able to mineralize dibenzofuran and substituted dibenzofurans. Degradative pathways leading from the substrate to carbon dioxide and water have been elucidated. These pathways, however, appear to coexist with metabolic byways which might lead to dead-end products. When the application of such bacteria for environmental purposes is intended, the consequences of the accumulation of these products have to be considered. From the biochemical point of view, this requires more information about (i) the involved side reactions, (ii) the extent of the formation of dead-end products, (iii) conditions minimizing the formation of dead-end products, and (iv) the effects of such by-products on the mineralization route.

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