Enantioselective Metabolism of Chiral 3-Phenylbutyric Acid, an Intermediate of Linear Alkylbenzene Degradation, by *Rhodococcus rhodochrous* PB1

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Rhodococcus rhodochrous PB1 was isolated from compost soil by selective culture with racemic 3-phenylbutyric acid as the sole carbon and energy source. Growth experiments with the single pure enantiomers as well as with the racemate showed that only one of the two enantiomers, (*R*)-3-phenylbutyric acid, supported growth of strain PB1. Nevertheless, (*S*)-3-phenylbutyric acid was cometabolically transformed to, presumably, (*S*)-3-(2,3-dihydroxyphenyl)butyric acid (the absolute configuration at the C-3 atom is not known yet) by (*R*)-3-phenylbutyric acid-grown cells of strain PB1, as shown by ¹H nuclear magnetic resonance spectroscopy of the partially purified compound and gas chromatography-mass spectrometry analysis of the trimethylsilyl derivative. Oxygen uptake rates suggest that either 3-phenylpropionic acid or cinnamic acid (*trans*-3-phenyl-2-propenoic acid) is the substrate for aromatic ring hydroxylation. This view is substantiated by the fact that 3-(2,3-dihydroxyphenyl)propionic acid was a substrate for *meta* cleavage in cell extracts of (*R*)-3-phenylbutyric acid-grown cells of strain PB1. Gas chromatography-mass spectrometry analysis of trimethylsilane-treated ethyl acetate extracts of incubation mixtures showed that both the *meta*-cleavage product, 2-hydroxy-6-oxo-2,4-nonadiene-1,9-dicarboxylic acid, and succinate, a hydrolysis product thereof, were formed during such incubations.

Chirality is a fundamental symmetry property of three-dimensional objects, and an object is said to be chiral if it cannot be superimposed upon its mirror image. In a chemical context, chirality is applied to three-dimensional structures of molecules (1). If a molecule is chiral, the mirror image must be a different molecule, since superimposability is the same as identity. In the most simple case—a molecule containing just one asymmetric carbon atom (defined as a carbon atom connected to four different groups)—there are two isomers for a chiral pure compound (Fig. 1), called enantiomers. Enantiomers have identical physical and chemical properties except in two important respects—they rotate the plane of polarized light in opposite directions, and they interact differently with other chiral compounds. In general, it may be said that enantiomers have identical properties in symmetrical (isotropic) environments but that their properties may differ in asymmetrical (anisotropic) environments (26) such as living systems.

A significant number of the organic chemicals regulated by the Environmental Protection Agency are chiral and are released into the environment as racemates (mixtures of equal amounts of the two enantiomers) (2, 3). Although several studies concerning the fate of environmentally relevant chiral compounds like secondary alkyl sulfates (11, 14), 2-(2,4-dichlorophenoxy)propionic acid (DCPP) (24), 2-(2-methyl-4-chlorophenoxy)propionic acid (34), α -hexachlorocyclohexane (10, 19, 25), and chlordane (9) demonstrate the utmost importance of treating enantiomers separately, most environmental regulations and scientific environmental studies treat racemates as though they consisted of one kind of molecule only (3).

Linear alkylbenzene sulfonates (LAS) and linear alkylbenzenes (LAB), as well as their degradation intermediates, are environmentally relevant chiral compounds. The former are important constituents of commercial detergents, with an annual production amounting to some 1 to 2 million metric tons worldwide (6), and the latter are unwanted residual educts of technical LAS synthesis found as low-percentage contaminants of commercial LAS (13, 17). LAB are reported to be present in natural waters, riverine and coastal sediments (16, 20, 30–32, 35), and sewage sludges (17). Some authors consider LAB to be more stable than LAS in the aquatic environment (20, 36).

The microbial degradation of LAB and LAS has been studied extensively since the early 1960s (11). When released in the environment, they are considered to biodegrade under aerobic conditions (29). There is a consensus (27) that degradation starts with ω -oxidation of the alkyl side chain. Chain shortening by β -oxidation seems to become hindered when the side chains are cut back to 4 or 5 carbon atoms from the point of attachment to the benzene ring, a phenomenon known as the distance principle (15, 18, 37). However, the fate of the resulting phenyl and sulfophenyl carboxylates—intermediates of LAB and LAS, respectively, usually excreted into the medium by the strains oxidizing the side chain—is still unclear. Regarding ring fission of such intermediates, Cain (11) suggested that once the side chain is oxidized, the compounds (even short-chain compounds, e.g., C₄-phenylalkanoates) undergo further side chain shortening before ring attack begins or alternatively simply accumulate. He further pointed out that direct fission of aromatic rings carrying a carboxylated side chain longer than C₃ is extremely rare. In contrast, Sariaslani et al. (28) concluded from their studies with a *Pseudomonas* sp. that 3-phenylbutyric acid is metabolized through both pathways—by initial oxidation of the benzene ring and by initial oxidation of the side chain. However, we must point out that in these studies

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FIG. 1. Structural formulas of the (*R*) and (*S*) enantiomers of 3-phenylbutyric acid.

pure enantiomers were not used and enantioselective separation techniques were not applied.

In this study, we examined the degradation of chiral 3-phenylbutyric acid, an intermediate of linear alkylbenzene degradation, by the gram-positive bacterium *Rhodococcus rhodochrous* PB1. Growth experiments with single pure enantiomers as well as with the racemate showed that only one of the enantiomers was used as a growth substrate. Evidence is presented that active cells were able to cometabolize the non-growth enantiomer to a dihydroxylated intermediate. We intend to show that it is imperative to deal with enantiomers exactly as they are—different molecules—when examining degradation of chiral compounds.

MATERIALS AND METHODS

Enrichment and isolation. Strain PB1 was isolated from a suspension of compost earth in minimal medium with racemic 3-phenylbutyric acid as the sole carbon and energy source. The gram-positive strain PB1 grows in a distinct growth cycle and was identified by the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as an *R. rhodochrous* strain.

Chemicals. When not explicitly stated otherwise, all chemicals were purchased from Fluka Chemicals, Buchs, Switzerland. 3-(*p*-Sulphophenyl)butyric acid was synthesized by a procedure described by Taylor and Nickless (33). 3-(2,3-Dihydroxyphenyl)propionic and 3-(2,5-dihydroxyphenyl)propionic acid were synthesized from 2,3-dimethoxycinnamic and 2,5-dimethoxycinnamic acids (Aldrich-Chemie, Steinheim/Albuch, Germany), respectively, by the method of Blakley and Simpson (7).

Media. (i) **Minimal medium.** The mineral salts medium used for growth of strain PB1 consisted of 20 mM phosphate buffer (KH_2PO_4 , Na_2HPO_4 [pH 7.2]) containing $(\text{NH}_4)_2\text{SO}_4$ (0.5 g/liter), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g/liter), and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.06 g/liter). The following trace elements were supplied from an acidified (5 ml of concentrated H_2SO_4 per 1,000 ml) stock solution to the indicated final concentrations: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0 mg/liter, 3.4 μM), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1.0 mg/liter, 5.9 μM), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.25 mg/liter, 1.0 μM), H_3BO_3 (0.10 mg/liter, 1.6 μM), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25 mg/liter, 1.0 μM), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 mg/liter, 0.9 μM), NH_4VO_3 (0.10 mg/liter, 0.9 μM), $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.50 mg/liter, 0.9 μM), and $\text{Ni}(\text{SO}_4) \cdot 6\text{H}_2\text{O}$ (0.01 mg/liter, 0.34 μM). The medium was supplemented with 10 mg of yeast extract (Merck, Darmstadt, Germany) per liter. The carbon sources were added to the mineral medium before autoclaving because no losses could be observed upon heating to 121°C for 30 min.

(ii) **Complex medium.** Phosphate buffer (20 mM; pH 7.2) containing 0.5 g of glucose and 0.3 g of yeast extract (Merck) served as the nonselective complex medium.

Growth conditions. When small amounts of biomass were sufficient, cells were grown in mineral medium (250 ml) supplemented with the appropriate substrate (250 mg/liter) in 1-liter Erlenmeyer flasks equipped with magnetic stirring bars. The flasks were equipped with a silicon tube that allowed easy sterile sampling. Incubations were started with a 2% (vol/vol) inoculum from an (*R*)-3-phenylbutyric acid-grown culture and were carried out in a water bath at 30°C with stirring held constant at 300 rpm. When large amounts of biomass were needed, cells were grown in mineral medium in 20-liter carboys. The appropriate carbon source was added in two portions of 500 mg/liter each, at the beginning and after 20 h of growth, respectively. In this case, 0.5 ml of polypropylene glycol was added to prevent foaming.

Screening for growth substrates. The carbon substrates to be screened were added to mineral medium in concentrations from 1 to 2 mM. Two 5-ml samples of the prepared medium were filtered through sterile filters (pore size, 0.2 μm)

into autoclaved 10-ml tubes. After inoculation with (*R*)-3-phenylbutyric acid-grown cells, the tubes were incubated for 3 days at 30°C and growth was assessed by measuring turbidity.

Determination of growth yields. Incubations for measurements of the growth yields ($Y_{X/S}$ or $Y_{X/C}$) were performed in mineral medium with a reduced Ca^{2+} concentration (0.025 mM) to avoid unwanted precipitations. The dry weight was measured by filtering defined volumes of cell cultures through tared 0.45- μm -pore-size polycarbonate filters (Nuclepore, Tübingen, Germany). Before being filtered, the cell suspensions were centrifuged at $12,000 \times g$ for 15 min and resuspended in 60 mM phosphate buffer (pH 7.2). Yields were calculated after determination of residual substrate and dissolved organic carbon.

Preparation of washed cell suspensions and cell extract. Cultures in the late exponential phase were harvested by centrifugation ($12,000 \times g$ for 15 min at 4°C) and washed with an excess amount of phosphate buffer (60 mM KH_2PO_4 – Na_2HPO_4 [pH 7.2]). Pellets were either frozen (–20°C) for later use or resuspended in the same buffer (approximately 1 to 5 mg of protein per ml of buffer) for oxygen uptake measurements. For preparation of cell extract, frozen cell paste (5 g) was allowed to thaw at room temperature. Cells were then mixed with 5 ml of 50 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.2), 5 g of glass beads, and 1.5 ml of acetone (approximately 10% [vol/vol]). The suspension was kept on ice and subjected to three 30-s bursts of vigorous shaking in a cell homogenizer (B. Braun Melsungen AG, Melsungen, Germany) with intermittent cooling on ice for several minutes. Cell debris was removed by centrifugation ($20,000 \times g$ for 30 min at 4°C).

Oxygen uptake by washed cell suspensions. Oxygen uptake was measured polarographically with an oxygen electrode (Rank Brothers, Cambridge, United Kingdom). The electrode was mounted on a reaction vessel (1 ml) whose temperature was held at 25°C. The assay mixture contained 1 ml of 60 mM phosphate buffer (pH 7.2) and 50 to 200 μl of cell suspension. Excess volume was allowed to escape through a small opening in the stopper. Reactions were started by the addition of 10 μl of a substrate as a concentrated methanol-water (50:50 [vol/vol]) solution (1 mM in the assay). Specific oxygen uptake rates were corrected for endogenous rates (with 10 μl of methanol added).

3-(2,3-Dihydroxyphenyl)propionate dioxygenase assay. 3-(2,3-Dihydroxyphenyl)propionate dioxygenase was assayed by monitoring the increase in A_{359} (12). The reaction mixture contained 1 ml of MOPS- Na^+ buffer (pH 7.2), 0.1 ml of crude cell extract, and 10 μl of a freshly prepared $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ solution (10 mM; 0.1 mM in the assay). The reaction was started by addition of 10 μl of a solution of 3-(2,3-dihydroxyphenyl)propionate (100 mM) in methanol-water (50:50 [vol/vol]). Samples for spectroscopic product analysis were extracted by the method described by Dagley et al. (12).

Analytical procedures. (i) **Dissolved organic carbon.** Samples were acidified with HCl (pH 2), centrifuged ($12,000 \times g$ for 15 min), freed from dissolved CO_2 by purging with N_2 for 10 min, and finally analyzed in a Toco 2 carbon analyzer (Maihak, Hamburg, Germany).

(ii) **HPLC.** High-pressure liquid chromatography (HPLC) analyses were performed on a 625 LC system with a WISP 700 autosampler and a photodiode array detector 901 (Waters Millipore Corp., Milford, Mass.). (*R*)- and (*S*)-3-phenylbutyric acids (retention times, 10.1 and 11.1 min, respectively) were separated on a Nucleodex- α -PM column (200 by 4.0 mm) with permethylated α -cyclodextrin as the stationary phase (Macherey-Nagel, Düren, Germany). The system was operated isocratically with an eluent consisting of 60% methanol and 40% NaH_2PO_4 (50 mM; pH 3) solution at a flow rate of 0.7 ml/min. When no separations of enantiomers were required, reverse phase separation was performed on a Spherisorb OD SII column (25 cm by 4.6 mm; Bischoff GmbH, Leonberg, Germany) with a 50:50 (vol/vol) methanol- NaH_2PO_4 (50 mM; pH 3) solution as the eluent at a flow rate of 1.0 ml/min. Metabolites were isolated from the culture media on a semipreparative C_{18} column (ET 250/21, Nucleosil 100-7; Macherey-Nagel) by isocratic elution with a 50:50 (vol/vol) methanol-water solution containing trifluoroacetic acid (1% [vol/vol]). Prior to analysis, samples were acidified with HCl to pH 2 and centrifuged at $12,000 \times g$ for 15 min.

(iii) **GC-MS.** Gas chromatography-mass spectrometry (GC-MS) analysis of metabolites and their trimethylsilyl (TMS) derivatives was performed as described elsewhere (22, 23). Exact masses were measured with an AutospecQ mass spectrometer (Fisons Instruments, Manchester, United Kingdom).

(iv) **^1H and ^{13}C NMR.** ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a 400-MHz Bruker AMX-400 NMR spectrometer (Spectrospin, Fällanden, Switzerland). Probes were dissolved in D_2O or CD_3OD .

(v) **Protein determination.** Protein contents of intact cells and cell extracts were determined by the Lowry and Bradford methods, respectively, with bovine serum albumin as the standard.

(vi) **UV-visible spectroscopy.** UV-visible spectra were obtained with a UVIKON 860 spectrophotometer (Kontron, Zürich, Switzerland).

RESULTS

Growth of strain PB1 with 3-phenylbutyric acid. Analytical techniques discriminating between the (*R*) and (*S*) enantiomers of 3-phenylbutyric acid revealed that *R. rhodochrous* PB1 was able to grow with the (*R*) enantiomer as the sole

FIG. 2. Growth experiments with *R. rhodochrous* PB1 incubated with (*R*)-3-phenylbutyric acid, (*S*)-3-phenylbutyric acid, and racemic 3-phenylbutyric acid. (A) Growth on the pure (*R*) enantiomer. (B) Incubation with the pure (*S*) enantiomer. (C) Growth on the racemate.

carbon and energy source but not with the (*S*) enantiomer (Fig. 2A and B). When cultures grew with racemic 3-phenylbutyric acid (Fig. 2C), they started to turn reddish brown after the (*R*) enantiomer was depleted. Some of the (*S*)-3-phenylbutyric acid was converted to an unknown metabolite by cells induced for growth on (*R*)-3-phenylbutyric acid, but no production of biomass could be observed in such a case. This situation is also reflected in the growth yield data. The growth yield ($Y_{X/C}$ and $Y_{X/S}$) determined for incubations with the (*R*) enantiomer alone were identical to the ones determined for incubations with the racemic mixtures, i.e., $Y_{X/C} = 1.2$ and $Y_{X/S} = 0.8$. When growing in minimal medium containing 1.6 mM (*R*)-3-phenylbutyric acid, strain PB1 grew at a rate of 0.16 h^{-1} and reached an optical density of 0.7 absorption unit at 546 nm. For reproducible growth, strain PB1 needed small amounts of yeast extract or vitamin mixture added to the growth medium. Growth after transfer from a complex medium to the minimal medium started with a lag phase of approximately 5 h. The lag phase was probably due to the time required for inducing the enzymes needed for growth on (*R*)-3-phenylbutyric acid, because it could be shown that it took about 4.5 h for cells grown on complex medium to reach an (*R*)-3-phenylbutyric acid-dependent specific oxygen consumption rate of 30 nmol/mg of protein per min upon incubation with (*R*)-3-phenylbutyric acid. Apart from (*R*)-3-phenylbutyric acid, strain PB1 was able to grow on 3-phenylpropionic acid, cinnamic acid, *o*-coumaric acid, coumarin, cinnamyl alcohol, *p*-hydroxyphenylacetic acid,

FIG. 3. Electron ionization mass spectrum of the TMS derivative of the dihydroxylated 3-phenylbutyric acid formed from (*S*)-3-phenylbutyric acid upon incubation with (*R*)-3-phenylbutyric acid-grown cells of *R. rhodochrous* PB1. The structural formula of the proposed compound has been drawn above the mass spectrum, and the chosen isomeric structure is supported by the NMR data (see Fig. 4). See the text for a discussion of the fragmentation pattern.

and benzoic acid and its *m*- and *p*-hydroxylated derivatives. No growth could be observed with 2-phenylbutyric acid, 3-(*p*-sulphophenyl)butyric acid, 2-phenylpropionic acid, phenylsuccinic acid, 3-phenyllactic acid, 3-(*p*-hydroxyphenyl)propionic acid, *m*- and *p*-coumaric acid, cinnamyl aldehyde, *o*- and *m*-hydroxyphenylacetic acid, mandelic acid, and *o*-hydroxybenzoic acid.

Conversion of (*S*)-3-phenylbutyric acid by cells induced for growth on (*R*)-3-phenylbutyric acid. Cultures incubated with racemic 3-phenylbutyric acid turned reddish brown after depletion of the (*R*) enantiomer. The same phenomenon could be observed when cells grown on (*R*)-3-phenylbutyric acid were incubated with (*S*)-3-phenylbutyric acid in resting-cell assays. When samples of such cultures were analyzed by HPLC, an additional prominent peak could be observed. This peak was detected only when (*R*)-3-phenylbutyric acid was exhausted, and its appearance was always accompanied by a decrease in the (*S*)-3-phenylbutyric acid concentration. GC-MS analysis of TMS-treated ethyl acetate extracts revealed one major additional metabolite. The electron impact mass spectrum of the TMS derivative is shown in Fig. 3. The metabolite was tentatively identified as a dihydroxylated phenylbutyric acid. The ions at m/z 397 and 412 are characteristic of a TMS-treated compound with a molecular ion at m/z 412. The ion at m/z 397 resulted from elimination of a methyl group. The fragment ions at m/z 323, 295, and 281 were most probably formed by loss of OTMS, COOTMS, and CH_2COOTMS , respectively, from the molecular ion. The exact mass of 412.226994 Da is in good agreement with $\text{C}_{19}\text{H}_{36}\text{O}_5\text{Si}_3$ for the structural formula of the molecular ion (the calculated mass is 412.192145 Da). The metabolite was purified by semipreparative HPLC. It eluted as one peak but did not remain completely stable and turned reddish brown during workup and green after being dried under nitrogen; there was another change to red after some days under laboratory conditions. In such probes, an additional peak with a mass spectrum indicative of the corresponding quinone could be observed in GC-MS traces of TMS-derivatized samples. The UV-visible spectra of fractions containing the unchanged metabolite showed an absorption maximum at 275 nm under acidic to neutral conditions. This is

FIG. 4. ^1H NMR spectrum (400 MHz) of dihydroxylated 3-phenylbutyric acid purified from (*S*)-3-phenylbutyric acid incubations with (*R*)-3-phenylbutyric acid-grown cells of *R. rhodochrous* PB1. The spectrum shows the aromatic region, and the x axis gives measurements in hertz as well as in ppm. See the text for a detailed discussion of the coupling pattern.

in agreement with the expected spectrum of a catechol derivative and closely resembles the absorption maximum of the aromatic region of 3-(2,3-dihydroxyphenyl)propionic acid, which was synthesized as described in Materials and Methods and, in contrast to the dihydroxylated 3-phenylbutyric acid, did not undergo oxidation to the quinone under the condition used. Samples of the purified fraction were subjected to NMR spectroscopy. Although an entirely perfect spectrum could not be obtained because of the instability of the compound, the spectrum could be interpreted. In the aromatic region of the spectrum, signals from two compounds could be recognized.

The signals at lower field (7.1 to 7.3 ppm)—assigned to the quinone—were of a higher order and could not be used for interpretation. The signals (Fig. 4) at higher field (6.72 to 7.00 ppm)—assigned to the dihydroxy compound—consisted of a triplet and two double doublets with an integral ratio of 1:2. This is in agreement with what would be expected for an aromatic compound with three protons attached to the ring. The coupling pattern (Fig. 4) of the three protons is compatible only with an asymmetrical aromatic structure that has the three protons adjacent to each other (21). Therefore, we conclude that the metabolite in question is 3-(2,3-dihydroxyphenyl)butyric acid.

Oxygen uptake by washed cell suspensions. The oxygen uptake by washed cells of strain PB1 grown on (*R*)-3-phenylbutyric acid was moderate for the (*R*) enantiomer but very low for the (*S*) enantiomer (Table 1). Such cells were highly induced, however, for oxygen uptake with 3-phenylpropionic and cinnamic acids. When the cells were grown on 3-phenylpropionic acid, oxygen consumption with (*R*)-3-phenylbutyric acid was low and the difference between the (*R*) and the (*S*) enantiomers diminished whereas the high rates observed with 3-phenylpropionic and cinnamic acids remained the same. These findings favor the idea that a compound structurally related to 3-phenylpropionic acid is the substrate for ring cleavage. When cells grown on (*R*)-3-phenylbutyric or 3-phenylpropionic acid were tested for oxygen consumption with dihydroxylated phenylpropionic acids, it became evident that only 3-(2,3-dihydroxyphenyl)propionic acid consumed oxygen whereas 3-(2,5-dihydroxyphenyl)propionic acid and 3-(3,4-dihydroxyphenyl)propionic acid did not. Cells grown on *o*-coumaric acid revealed an oxygen consumption pattern considerably different from the ones obtained with cells grown on (*R*)-3-phenylbutyric acid and 3-phenylpropionic acid. This result suggests that the pathway operative with *o*-coumaric acid is different from the other pathway. Other substrates tested but never found to contribute to oxygen consumption were 2-phenylpropionic acid, 2-phenylbutyric acid, ibuprofen, *o*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, phenylacetic acid, *o*-hydroxyphenylacetic acid, *m*-hydroxyphenylacetic acid, *p*-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, cin-

TABLE 1. Oxygen uptake rates with various compounds by resting cells of *R. rhodochrous* PB1 grown on different substrates

Substrate	Relative oxygen uptake rate (%) by cells grown on ^a :		
	(<i>R</i>)-3-Phenylbutyric acid	3-Phenylpropionic acid	<i>o</i> -Coumaric acid
3-Phenylpropionic acid	100 (90–480) ^b	100 (70–280) ^b	100 (10–60) ^b
Cinnamic acid	100	82	BD ^c
(<i>R</i>)-3-Phenylbutyric acid	26	10	BD
(<i>S</i>)-3-Phenylbutyric acid	5	6	BD
4-Phenylbutyric acid	BD	4	BD
<i>o</i> -Coumaric acid	12	18	446
Coumarin	BD ^d	13	1,262 ^d
Catechol	7	10	98
3-Methylcatechol	3	8	ND ^e
4-Methylcatechol	BD	2	ND
2,3-Dihydroxybenzoic acid	BD	BD	75 ^d
3,4-Dihydroxybenzoic acid	BD	BD	75
3-(2,3-Dihydroxyphenyl)propionic acid	11	11	170
3-(2,5-Dihydroxyphenyl)propionic acid	BD	BD	79
Pyrogallol	11	15	144 ^d

^a Data for each substrate represent means from at least two independent cultures. Other substrates tested but not showing oxygen uptake are mentioned in the text.

^b Ranges of absolute oxygen consumption rates of replicates in nanomoles of O₂ per milligram of protein per minute are given in parentheses.

^c BD, below detection limit.

^d Data from a single experiment.

^e ND, not determined.

FIG. 5. Electron ionization mass spectrum of the TMS derivative of the metabolite detected upon incubation of 3-(2,3-dihydroxyphenyl)propionic acid with cell extract of (*R*)-3-phenylbutyric acid-grown cells of *R. rhodochrous* PB1. The structural formula of the proposed compound (the *meta*-cleavage product, 2-hydroxy-6-oxo-2,4-nonadiene-1,9-dicarboxylic acid) has been drawn above the mass spectrum. See the text for a discussion and interpretation of the fragmentation pattern.

namyl aldehyde, cinnamyl alcohol, *m*-coumaric acid, *p*-coumaric acid, phenylsuccinic acid, 3-(*p*-hydroxyphenyl)propionic acid, 3-phenyllactic acid, caffeic acid, hydrocaffeic acid, mandelic acid, 2,3-dihydroxybiphenyl, and 3-(*p*-sulfophenyl)butyric acid.

3-(2,3-Dihydroxyphenyl)propionic acid dioxygenase activity in crude cell extracts. Upon addition of 1 mM 3-(2,3-dihydroxyphenyl)propionic acid to the enzyme assay mixture described in Materials and Methods, an increase in A_{359} could be observed. Simultaneously, a yellow color developed in the assay mixture. Addition of 1 mM EDTA salt to the assay stopped the reaction. The reaction rate increased with the amount of protein added and with higher substrate concentrations. The specific activity remained relatively low and was estimated to reach 5 to 10 nmol of substrate per mg of protein per min. Heating of the extract to 45°C for 5 min led to a 50% decrease in the initial reaction rate. The UV-visible spectra of samples that were extracted with ethyl acetate and taken up in aqueous solution after evaporation revealed absorption maxima at 397 and 394 nm at pH 11 and 7.2, respectively. The absorption maximum shifted to 317 nm at pH 1.5. These properties are in agreement with the ones of the *meta*-cleavage product described for *meta* cleavage of 3-(2,3-dihydroxyphenyl)propionic acid by an *Achromobacter* species (12) and *Escherichia coli* (8). GC-MS analysis of TMS-treated ethyl acetate extracts of incubations with cell extract and 3-(2,3-dihydroxyphenyl)propionic acid gave further proof of the view that the aromatic ring was opened through *meta* cleavage, because one of the product peaks was tentatively identified as the TMS derivative of 2-hydroxy-6-oxo-2,4-nonadiene-1,9-dicarboxylic acid—the *meta*-cleavage product of 3-(2,3-dihydroxyphenyl)propionic acid—on the grounds of the mass spectrum (Fig. 5); m/z (major fragment ions) 430 (M^+); 415, loss of CH_3 ; 357, loss of TMS; 341, loss of OTMS; 312, loss of COOTMS. Another metabolite formed during the course of this reaction was identified as succinate because the mass spectrum of its TMS derivative was identical to that of the TMS derivative of an authentic sample of succinate.

DISCUSSION

R. rhodochrous PB1, isolated on racemic 3-phenylbutyric acid, could grow only with the (*R*) enantiomer as the sole carbon and energy source and not with the (*S*) enantiomer. On the basis of data from growth measurements, oxygen consumption experiments, and enzyme assays with crude extract, we suggest that (*R*)-3-phenylbutyric acid is metabolized via a *meta*-cleavage pathway (Fig. 6) with 3-(2,3-dihydroxyphenyl)propionic acid as the ring cleavage substrate. The metabolic reactions involved in the conversion of (*R*)-3-phenylbutyric acid to 3-phenylpropionic acid are not known yet, but metabolism of 3-phenylpropionic acid seems to follow the same general pathway as described for an *Achromobacter* sp. by Dagley et al. (12). This view is substantiated by the fact that 2-hydroxy-6-oxo-2,4-nonadiene-1,9-dicarboxylic acid and succinate—the *meta*-cleavage product and one of its hydrolysis products, respectively—were detected as metabolites in crude cell extracts of strain PB1 upon incubation with 3-(2,3-dihydroxyphenyl)propionic acid.

Cells of strain PB1 grown on (*R*)-3-phenylbutyric acid were able to cometabolically dihydroxylate (*S*)-3-phenylbutyric acid, as shown by GC-MS of the TMS derivative and NMR spectroscopy of the partially purified metabolite. According to the NMR coupling pattern, the most likely structure of the metabolite is the one with the two hydroxy groups at C-2 and C-3 of the phenyl substituent (21, 28). We assume that the configuration at the stereogenic center stayed intact during the dihydroxylation, because the reaction did not involve the center, but there is no direct proof of this assumption at the moment. The product of this cometabolic dihydroxylation, (*S*)-3-(2,3-dihydroxyphenyl)butyric acid, was not very stable in aqueous solution. During incubation of (*S*)-3-phenylbutyric acid with resting cells, formation of a red color could already be observed. This finding is indicative of the nonenzymatic oxidation of the catecholic compound to the *ortho* quinone, because *ortho* quinones generally are dark red (5). The reversible conversion between quinones and hydroquinones is often accompanied by the intermediary formation of green quinhydrone (4). This explains the formation of the green color in samples of the extracted dihydroxy compound when kept under nitrogen. Accumulation of (*S*)-3-(2,3-dihydroxyphenyl)butyric acid during growth of strain PB1 on the racemate shows that the *meta*-cleavage enzyme could not use this compound as a substrate. Recently, the 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *E. coli* was overproduced and purified (8). Besides 3-(2,3-dihydroxyphenyl)propionic acid, the enzyme turned over 3-methylcatechol and catechol as alternate substrates but with reduced catalytic efficiency. 2,3-Dihydroxybenzoic acid was not a substrate, however, and showed only weak inhibition at 10 mM. The author concluded that there is some specificity for the propionate side chain. This is in agreement with the data from our oxygen consumption experiments. Catechol, as well as 3-methylcatechol, brought about low oxygen consumption rates in assays with induced cells, whereas 2,3-dihydroxybenzoic acid was inert.

The absolute oxygen consumption rates varied over a relatively large range of values (Table 1) between replicates. This was not the case for the relative oxygen consumption rates, whose values for replicates stayed within the acceptable error limits. On the one hand, this reflects the difficulty of repeatedly harvesting cells in the same physiological state (e.g., the level of enzyme expression). On the other hand, it shows that the relative enzyme activities with different substrates are independent of the absolute values.

Our usage of the term “enantioselective” is in accordance

FIG. 6. Pathway proposed for the metabolism of (*R*)- and (*S*)-3-phenylbutyric acid by *R. rhodochrous* PB1. Step 1, reactions that convert (*R*)-3-phenylbutyric acid to 3-phenylpropionic acid in a yet unknown but enantioselective manner. Step 2, dioxygenation of 3-phenylbutyric acid and (*S*)-3-phenylbutyric acid (cometabolically). Step 3, *meta* cleavage of 3-(2,3-dihydroxyphenyl)propionic acid. Step 4, hydrolysis of the *meta*-cleavage product.

with usage in the biology literature (9, 10, 34) but is not completely in agreement with that of organic chemists (1). For them, an enantioselective reaction is one in which an achiral substrate is converted to a chiral product such that possible enantiomers are formed in unequal amounts. In addition to that usage, biologists use the term "enantioselective" to describe biologically or enzymatically catalyzed reactions in which preferentially one enantiomer of a chiral substrate is reacted upon.

The data presented are consistent with the view that the enantioselective (see above) step discriminating between the

(*R*) and (*S*) enantiomers of 3-phenylbutyric acid is located at the beginning of the metabolic sequence (Fig. 6). At first, (*R*)-3-phenylbutyric acid is converted to 3-phenylpropionic acid in an unknown but enantioselective manner. 3-Phenylpropionic acid is then further metabolized via *meta* cleavage as described above. When present, (*S*)-3-phenylbutyric acid is cometabolically oxidized to (*S*)-3-(2,3-dihydroxyphenyl)butyric acid by the dihydroxylating dioxygenase and the dehydrogenase of the 3-phenylpropionic acid degradation pathway and is subsequently nonenzymatically oxidized to the *ortho* quinone.

Sariaslani et al. (28) suggested that 3-phenylbutyric acid was degraded by a *Pseudomonas* sp. via two different routes: (i) dioxygenation of 3-phenylbutyric acid to form 3-(2,3-dihydroxyphenyl)butyric acid followed by *meta* cleavage; and (ii) ω -oxidation with subsequent decarboxylation to produce 3-phenylpropionate, which was further degraded via phenylacetate and benzoate. Because the authors did not have available pure enantiomers or enantioselective analytical tools, they were not able to decide whether one of the enantiomers was preferentially metabolized through one of the pathways.

During the enantioselective metabolism of racemic 3-phenylbutyric acid by strain PB1, a reactive metabolite cometabolically produced from only one of the enantiomers accumulated in the culture medium. LAB entering the environment are racemates, and they will be degraded to racemic carboxylates. Here for the first time we have investigated the degradation of the enantiomers of such a compound, 3-phenylbutyric acid. Our data show that it is of the utmost importance to treat enantiomers separately when studying the degradation of chiral compounds. We believe that the microbial metabolism and environmental fate of many chiral compounds must be reevaluated by using enantiospecific techniques and analytical tools. Further work, especially on carboxylates of LAB and LAS, will be carried out in our laboratory.

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