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Degradation of 2-*sec*-butylphenol: 3-*sec*-butylcatechol, 2-hydroxy-6-oxo-7-methylnona-2,4-dienoic acid, and 2-methylbutyric acid as intermediates

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Key words: 2-*sec*-butylphenol, 3-*sec*-butylcatechol, biodegradation, *meta*-cleavage product, monooxygenase, metapyrocatechase

Abstract

Pseudomonas sp. strain HBP1 Prp, a mutant of strain HBP1 that was originally isolated on 2-hydroxybiphenyl, was able to grow on 2-*sec*-butylphenol as the sole carbon and energy source. During growth on 2-*sec*-butylphenol, 2-methylbutyric acid transiently accumulated in the culture medium. Its concentration reached a maximum after 20 hours and was below detection limit at the end of the growth experiment. The first three enzymes of the degradation pathway – a NADH-dependent monooxygenase, a metapyrocatechase, and a *meta*-fission product hydrolase – were partially purified. The product of the the monooxygenase reaction was identified as 3-*sec*-butylcatechol by mass spectrometry. This compound was a substrate for the metapyrocatechase and was converted to 2-hydroxy-6-oxo-7-methylnona-2,4-dienoic acid which was identified by gas chromatography-mass spectrometry of its trimethylsilyl-derivative. The cofactor independent *meta*-cleavage product hydrolase used 2-hydroxy-6-oxo-7-methylnona-2,4-dienoic acid as a substrate. All three enzymes showed highest activities for 2-hydroxybiphenyl and its metabolites, respectively, indicating that 2-*sec*-butylphenol is metabolized via the same pathway as 2-hydroxybiphenyl.

Introduction

Pseudomonas sp. strain HBP1 was isolated from municipal sewage sludge by selective culture enrichment with 2-hydroxybiphenyl. It was able to grow on 2-hydroxy- and 2,2′-dihydroxybiphenyl, as sole carbon and energy sources and was shown to employ a *meta*-cleavage pathway for utilization of these substrates. The first enzyme in the catabolic pathway, a NADH-dependent monooxygenase, was only induced when cells grew on 2-hydroxy- and 2,2′-dihydroxybiphenyl, respectively (Kohler et al. 1988). This enzyme showed strict regio-selectivity for the position of the hydroxyl group such that substrates had to be of the general structure 2-R-phenol. However, the structural requirements for R were quite relaxed and compounds with R = phenyl, 2-hydroxyphenyl, *sec*-butyl, propyl, ethyl, methyl were substrates of the enzyme (Kohler et al. 1988). Therefore, *ortho*-substituted alkylphenols were considered possible growth substrates for *Pseudomonas* sp. strain HBP1. Interest in the metabolism of this class of compounds arose, because nitrated analogs constitute structural backbones of

widely used pesticides such as dinoseb (Stevens et al. 1991), binapacryl, DNC, and dinocap (Gasiewicz 1991). Initial growth experiments with 2-alkylphenols as substrates failed, but after a selection period of several days, a mutant strain, that was designated as *Pseudomonas* sp. strain HBP1 Prp, with the ability to grow on 2-propylphenol was selected (Kohler et al. submitted). Here we report growth of the mutant strain HBP1 Prp on 2-*sec*-butylphenol and the catabolic pathway employed for its utilization.

Materials and methods

Media and growth conditions

Pseudomonas sp. strain HBP1 has been described in a previous paper (Kohler et al. 1988). Selection of the mutant strain HBP1 Prp is described elsewhere (Kohler et al. submitted). The mineral salts medium used for growth consisted of 20 mM phosphate buffer (KH_2PO_4-$Na_2HPO_4 \cdot 2H_2O$; pH 7.2), 0.125 g NH_4SO_4, 0.025 g $MgSO_4 \cdot 7H_2O$ and 0.03125 g $Ca(NO_3)_2 \cdot 4H_2O$ per liter of deionized water, supplemented with 1 ml of a trace element stock solution containing the following (in g l^{-1}): $FeSO_4 \cdot 7H_2O$, 1.00; $MnSO_4 \cdot H_2O$, 1.00; $Na_2MO_4 \cdot 2H_2O$, 0.25; H_3BO_3, 0.10; $CuSO_4 \cdot 5H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.25; NH_4VO_3, 0.10; $Co(NO_3)_2 \cdot 6H_2O$, 0.5; $NiSO_4 \cdot 6H_2O$, 0.010; H_2SO_4 concentrated, 5.00 ml.

2-Hydroxybiphenyl and 2-*sec*-butylphenol were added (500 mg l^{-1}, if not mentioned otherwise) after sterilization to minimize volatile losses.

Chemicals

2-*sec*-butylphenol, 3-methylcatechol, and 4-methylcatechol were obtained from Aldrich-Chemie, Steinheim/Albuch, Germany. 2,3-Dihydroxybiphenyl was obtained from Wako Chemicals GmbH, Neuss, Germany. Sodium benzoate was purchased from Merck, Darmstadt, Germany. All other chemicals used were purchased from Fluka Chemie AG, Buchs, Switzerland.

Preparation of washed cell suspensions and cell extract

Cells were grown in indented Erlenmeyer flasks (500 ml or 1000 ml capacity) on a rotary shaker (140 to 150 rpm) at 30° C. Alternatively, cells were grown in 20 liter carboys equipped with a magnetic stirring bar and forced aeration. In this case one drop of sterile polypropylene glycol was added after sterilization to prevent foaming. Cells were harvested in the late exponential growth phase by centrifugation (15 min at 6000 × g) at 4° C, washed with an excess amount of phosphate buffer (20 mM; pH 7.2) and resuspended in the same buffer (0.2 g wet weight ml^{-1}). For preparation of crude cell extract washed cells were broken by means of a French press (two passages, 20000 lb in^{-2}), followed by centrifugation (40 min at 40000 × g) at 4° C.

Oxygen uptake with washed cell suspension

Oxygen uptake was measured polarographically with an oxygen electrode (Rank Brothers, Cambridge, England). The assay mixture contained 1.79 ml phosphate buffer (pH 7.2, 20 mM) and 0.2 ml washed cell suspension (OD_{546} in the reaction vessel was approximately 1.9). Reactions were started by adding 10 µl of substrate as concentrated methanol or buffer solutions (0.2 mM in the assay). Specific oxygen uptake rates were corrected for endogenous rates.

Separation of enzyme activities

In order to separate the different enzyme activities, partial purification by protamine sulfate precipitation and ion exchange chromatography were necessary. 0.33 ml protamine sulfate solution (12 mg ml^{-1} in 20 mM phosphate buffer, pH 7.2) was added per ml of crude cell extract and stirred for 30 minutes at 4° C. The precipitated biopolymers were removed by centrifugation (30 min at 40000 × g) at 4° C. After desalting the protamine sulfate treated extract on a Sephadex G-25 M column (Pharmacia, Sweden) it was fractioned by anion exchange chroma-

tography. The FPLC system consisted of a 2152 LC controller, two 2150 HPLC pumps and a 2212 helirac sample collector (all from LKB-Pharmacia, Sweden). The samples were applied on a Mono-Q anion exchange column (HR5/5; Pharmacia, Sweden) and eluted with a linear gradient of NaCl, 0 to 1 M in 40 ml triethanolamine buffer (10 mM; pH 7.5; 1 ml min^{-1}). The protein profile was measured at 280 nm with a 655 A variable wavelength UV monitor (Hitachi, Japan).

Enzyme assays

One unit of enzyme activity was defined as the amount of enzyme converting 1 µmol of substrate per minute.

NADH-dependent monooxygenase activity was measured spectrophotometrically by monitoring NADH disappearance at 340 nm. The reaction mixture contained 20 mM phosphate buffer (pH 7.2), 0.2 mM NADH, 50 µl of the appropriate protein fraction, and 0.1 mM substrate. The reaction was started by addition of substrate (5 µl of a methanol solution).

Metapyrocatechase activity was measured spectrophotometrically by following the increase of absorption corresponding to the accumulation of ring *meta*-cleavage products. The following extinction coefficients for various *meta*-cleavage products were used: 2-hydroxymuconic semialdehyde (*meta*-cleavage product of catechol) (λ_{max}, 375 nm), 36000 M^{-1} cm^{-1} (Strubel et al. 1991); 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta*-cleavage product of 2,3-dihydroxybiphenyl) (λ_{max}, 434 nm), 22000 M^{-1} cm^{-1} (Strubel et al. 1991); in the case of 2-hydroxy-6-oxo-7-methylnona-2,4-dienoic acid (*meta*-cleavage product of 3-*sec*-butylcatechol) the molar absorption coefficient (13000 M^{-1} cm^{-1}) of the *meta*-cleavage product of 3-isopropylcatechol (Duggleby & Williams 1986) was used because no reference sample was available.

Meta-cleavage product hydrolase was assayed spectrophotometrically by monitoring the decrease in the absorption of the different *meta*-cleavage products. All assays were performed in 20 mM phosphate buffer at pH 7.5.

Analytical techniques

Disappearance of 2-*sec*-butylphenol (ε_{270} = 1797 M^{-1} cm^{-1}) and formation of metabolites (catechols) were monitored by HPLC. Samples containing cells and protein were pretreated either by centrifugation (15 min at 20000 × g) or by addition of trichloroacetic acid (0.2 ml of a 3 M solution per ml of sample) and subsequent centrifugation. The samples were analyzed by injecting 50 µl onto a Waters Millipore 625LC (Waters-Millipore, Milford, MA) high-performance liquid chromatograph, consisting of a Rheodyne 9125 injector, a gradient controller, a low pressure mixing fluid handling unit, a computer controlled Waters 991 photodiode array detector and a Pharmacia/LKB (Uppsala, Sweden) RediFrac fraction collector. Reverse phase separation was achieved on a 25 cm × 4.6 mm Spherisorb OD SII column of 5 µm particle size from Bischoff GmbH (Leonberg, Germany) applying a linear gradient of 60% B to 70% B (A: 10 mM H_3PO_4; B: 90% methanol, 10% 10 mM H_3PO_4) with a flow rate of 1 ml min^{-1}. The column was equilibrated at initial conditions before each injection.

2-Methylbutyric acid was analyzed on a HP 5890 Series II (Hewlett Packard Co., Palo Alto, CA) gas chromatograph equipped with a FID detector and an OV-351 capillary column (length, 30 m; inner diameter, 0.32 mm; film thickness, 0.25 µm; J&W, Carlo Erba Instruments, Italy). The temperature program ran from 80 to 140° C with an initial and final holding time of 2.0 and 4.74 min, respectively, and a rate of 15° C min^{-1}. The injector and detector temperatures were at 200 and 250° C, respectively. At the end of each run the oven was heated to 195° C and held for 2 min. Helium was the carrier gas with a linear flow velocity of 50 cm s^{-1} at 100° C. Splitless injection was done by using an automatic sampler (HP 7673A, Hewlett Packard). Samples were pretreated by centrifugation (15 min at 20000 × g) and were acidified (5% v/v formic acid final concentration) just prior to analysis.

Mass spectra of the catechol were obtained on a Carlo Erba QMD 1000 gas-chromatograph mass spectrometer (Carlo Erba Instruments, Italy) equipped with a SE-54 glass capillary column. Electron ionisation was used. The injection occurred on-

column at 58° C. The temperature program ran from 58 to 120° C with a rate of 21° C min^{-1} and from 120 to 200° C with a rate of 8° C min^{-1}. Mass spectra of the TMS-derivatives of the *meta*-cleavage compound were obtained on a Finnigan MAT ITD 800 (ion trap detection) mass spectrometer (Finnigan MAT, San Jose, CA) coupled to a Carlo Erba HRGC 5160 Mega Series gas-chromatograph (Carlo Erba Instruments, Italy) equipped with a 10 m PS090 (80% dimethyl, 20% diphenyl) glass capillary column. Electron impact ionisation and chemical ionisation with methanol as the reagent gas were used. The injection (1 µl) occurred on-column at 80° C. The temperature program ran from 80 to 100° C with a rate of 20° C min^{-1} and from 100 to 220° C with a rate of 5° C min^{-1}. TMS-derivatives were produced by silylating the hydroxy groups with BSTFA (N,O-*bis*(trimethylsilyl)trifluoro-acetamide).

UV-VIS spectra were taken with a Uvikon 860 spectrophotometer (Kontron AG, Zürich, Switzerland).

Protein contents in cell extracts were determined by the method of Bradford (Bradford 1976) with bovine serum albumin as a standard.

Production of 3-sec-butylcatechol

0.3 ml of the protein fraction containing the monooxygenase activity, 1.2 ml NADH (0.2 mM), 16.1 ml phosphate buffer (20 mM, pH 7.2), and 0.6 ml 2-*sec*-butylphenol (22 mM) were incubated at 30° C. After 30 minutes another 0.3 ml of the monooxygenase activity and another 1.2 ml NADH were added and the reaction mixture was reincubated for an additional 30 minutes. Then, pH was adjusted to 1 with H_3PO_4 (1 M) and the reaction mixture was extracted with 20 ml ethylacetate. The organic fraction was evaporated to dryness and taken up in 3 ml of methanol. This methanol solution was subsequently used for GC-MS analysis.

Production of 2-hydroxy-6-oxo-7-methylnona-2,4-dienoate

1 ml of 3-*sec*-butylcatechol (0.1 mM) was incubated with 10 µl of the protein fraction containing the *meta*-cleavage activity. After the intensity of the yellow color was at a maximum, the mixture was acidified (pH 1) with H_3PO_4 (1 M). The *meta*-cleavage product was extracted with ethylacetate. The ethylacetate extract was dried over Na_2SO_4, subsequently silylated with BSTFA, and used for GC-MS analysis.

Results

Growth on 2-sec-butylphenol

Pseudomonas sp. strain HBP1 Prp was able to grow on 2-*sec*-butylphenol as the sole carbon and energy source (Fig. 1). During growth the concentration of substrate decreased from 2.1 mM to a residual concentration of 0.08 mM. 2-Methylbutyrate (Fig. 1) and a yellow compound with an absorption maximum at 392 nm were formed as intermediates during growth. Both intermediates reached their highest concentrations (0.77 mM for 2-methylbutyrate) after 20 hours when approximately 70% of the substrate was consumed. A growth rate (µ) of 0.11 h^{-1} (t_d = 6.3 h) was calculated.

Oxygen uptake with washed cells

Oxygen uptake by cells grown on 2-*sec*-butylphenol with 2-methylbutyrate as substrate was significantly higher than with succinate or benzoate as substrates (Table 1). The oxidative enzymes for 2-methylbutyrate utilization are apparently induced during growth on 2-*sec*-butylphenol indicating that 2-methylbutyrate is an intermediate of the degradation of 2-*sec*-butylphenol. Oxygen uptake with 2-hydroxybiphenyl as substrate was always higher than with 2-*sec*-butylphenol as substrate. Cells were induced for oxygen uptake with 2-*sec*-butylphenol as a substrate when they were grown on 2-*sec*-butyl-

phenol as well as when they were grown on 2-methylbutyrate.

Conversion of 2-sec-*butylphenol to* 3-sec-*butylcatechol*

Crude cell extracts from 2-*sec*-butylphenol grown cells of *Pseudomonas* sp. strain HBP1 Prp exhibited NADH-dependent monoxygenase, metapyrocatechase and *meta*-fission product hydrolase activity when assayed with 2-hydroxybiphenyl and 2-*sec*-butylphenol, respectively, as substrates. Intermediates did not accumulate in crude cell extracts so that separation of the enzymes was necessary in order to characterize the metabolites. Therefore, for a detailed analysis of the 2-*sec*-butylphenol metabolizing enzyme system crude extract of 2-*sec*-butylphenol grown cells was applied to a Mono-Q-Sepharose column and eluted with a NaCl gradient (Fig. 2). Fractions containing the monooxygenase – well separated from the ones containing the metapyrocatechase – were pooled and used to analyse the reaction product with 2-*sec*-butylphenol as substrate. When 2-*sec*-butylphenol (HPLC-retention time, 16.5 min) was incubated with NADH and monooxygenase its concentration decreased and a more hydrophilic metabolite (HPLC-retention time, 11.4 min) appeared. The metabolite was enriched as described in Materials and methods and subsequently characterized by GC-MS. The MS-spectrum of the metabolite (Fig. 3) shows the following ion peaks (*m/e*) (major ion peaks): 167, $(M+1)^+$; 166

Table 1. Oxygen uptake rates by resting-cell suspensions of *Pseudomonas* sp. strain HBP1 Prp grown on 2-*sec*-butylphenol and 2-methylbutyrate.

Substrate	Oxygen uptake[a] of cells grown on:	
	2-*sec*-Butylphenol	2-Methylbutyrate
2-Hydroxybiphenyl	355	149
2-*sec*-Butylphenol	66	63
2-Methylbutyrate	37	22
Benzoate	0	10
Succinate	1	0

[a] Oxygen uptake rates are expressed as nmol O_2 min^{-1} (mg protein)$^{-1}$.

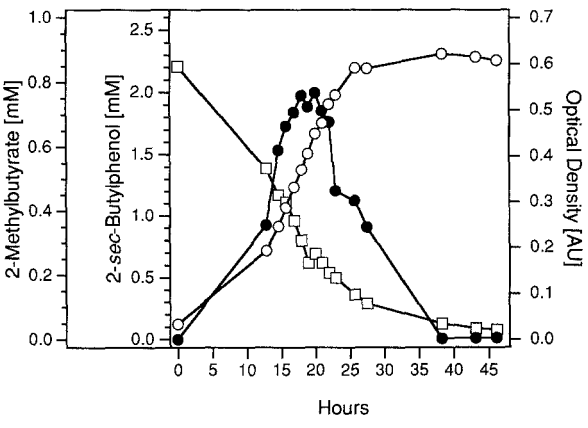

Fig. 1. Growth of *Pseudomonas* sp. strain HBP1 Prp on 2-*sec*-butylphenol. Symbols: ⇨, A_{546} (AU), □, 2-*sec*-butylphenol (mM), •, 2-Methylbutyrate (mM).

$(M)^+$; 151, loss of CH_3; 138, loss of CO; 137 (base peak) loss of CHO; 123, loss of C_3H_7; 119, loss of CHO and H_2O; 91, $C_7H_7^+$; 79, $C_6H_7^+$; 77, $C_6H_5^+$. The molecular ion $(M)^+$ of 166 and the fragmentation pattern agree well with this metabolite being 3-*sec*-butylcatechol. The UV/VIS spectrum (solvent 50% methanol, 50% 0.01 M H_3PO_4) shows a maximum at 275 nm and a minimum at 245 nm with a ratio $A_{275}/A_{245} = 8.2$. This is in agreement with a catechol structure.

Metapyrocatechase and meta-*fission product hydrolase activity*

With 2,3-dihydroxybiphenyl and catechol as substrates metapyrocatechase activity could be detected in four fractions of the anion exchange chromatography run (Fig. 2) eluting as a single peak at 0.5 M NaCl. Activities of the partially purified metapyrocatechase for a series of catechols are shown in Table 2. The enzyme exhibited very high activity for 2,3-dihydroxybiphenyl and rather low activity for catechol. 3-*sec*-Butylcatechol was turned over by the metapyrocatechase with a high rate (specific activity of 1.6 U/mg in crude extract) yielding as a product a yellow compound that was stable for several hours. This compound was enriched as described in Materials and Methods and subsequently confirmed to be 2-hydroxy-6-oxo-7-methylnona-2,4-dienoic acid on grounds of the mass

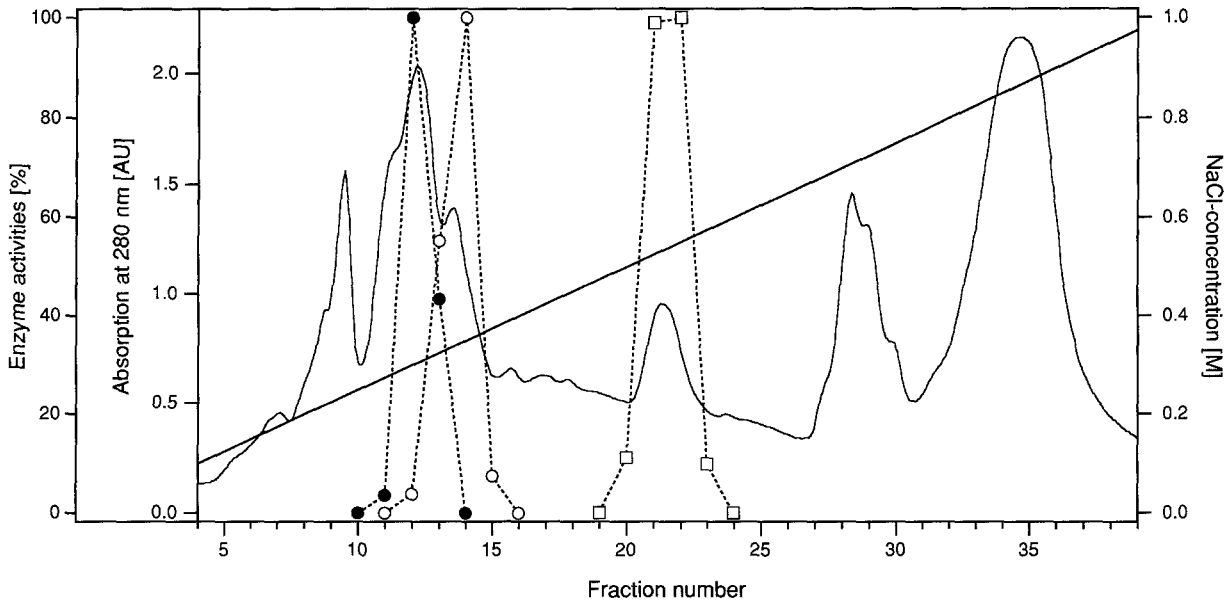

Fig. 2. Mono-Q ion exchange chromatography of a protamine sulfate percipitated and desalted cell extract of *Pseudomonas* sp. strain HBP1 Prp grown on 2-*sec*-butylphenol. The rates of enzyme activities in the eluted fractions were as follows: •, rate of monooxygenase (with 2-hydroxybiphenyl as assay substrate, 100% = 4.4 U ml^{-1}): ○, rate of *meta*-fission product hydrolase (with 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid as assay substrate, 100% = 9.7 U ml^{-1}); □, rate of metapyrocatechase (with 2,3-dihydroxybiphenyl as assay substrate, 100% = 60.1 U ml^{-1}).

spectra of its TMS-derivative. The electron impact mass spectrum (Fig. 4) shows the following ion peaks (*m/e*) (major ion peaks): 342, $(M)^+$; 327, loss of CH_3; 299, loss of CH_3 and CO; 285, loss of C_4H_9; 257, loss of C_5H_9O; 225 (base peak), loss of C_4H_9O-$_2$Si; 147, $C_5H_{15}OSi_2^+$; 95, $C_5H_3O_2^+$; 73, $C_3H_9Si^+$. The fragmentation pattern of the mass spectrum can be well rationalized. The neutral loss of CO from the $(M\text{-}15)^+$ ion is a characteristic mass spectral feature for α-hydroxy acid TMS-derivatives and has been reported for monosaturated analogs of *meta*-cleavage compounds (Massé et al. 1989). The ions at *m/e* 285 and 257 are formed through loss of the butyl side chain and the adjacent carbonyl group, respectively, and are very indicative of the molecular structure of the compound. The base peak at *m/e* 225 is formed by a loss of carboxytrimethylsilyl from the molecular ion. This loss has been observed for different *meta*-cleavage products (Furukawa et al. 1979; Furukawa et al. 1983; Massé et al. 1989) in case of electron ionisation. The relative abundance (0.46%) of the molecular ion $(M)^+$ in the electron impact mass spectrum was low such that chemical ionisation was employed to reconfirm the molecular weight of the TMS-derivative of the *meta*-cleavage compound. The spectrum showed the characteristic peak of $(M + H)^+$ at *m/e* 343 confirming the molecular weight to be 342.

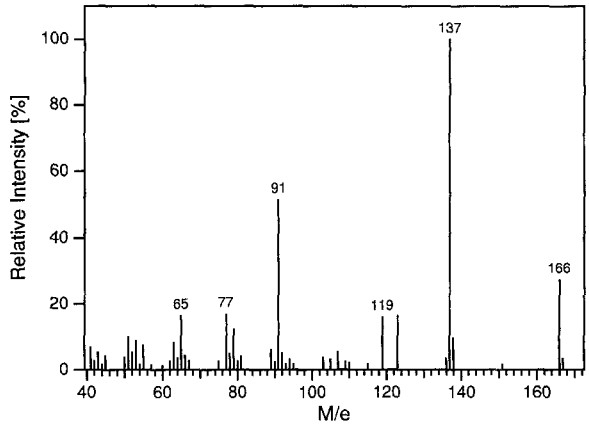

Fig. 3. Electron ionisation mass spectrum of 3-*sec*-butylcatechol, the product of the NADH-dependent monooxygenase activity with 2-*sec*-butylphenol as substrate. See text for discussion of fragmentation pattern.

Table 3 shows activities of the cofactor-independent *meta*-fission product hydrolase for a series of *meta*-fission products prepared from the corresponding catechols by use of the partially purified metapyrocatechase. The *meta*-cleavage products of catechol, 3-methylcatechol, and 4-methylcatechol were not substrates for the hydrolase. The measured activity of the hydrolase for the *meta*-fission product of 3-*sec*-butylcatechol gives rise to a flux of this metabolite that is higher than would be needed for growth of strain HBP1 Prp on 2-*sec*-butylphenol at the observed growth rates. Again, highest activity was measured with the metabolite of 2-hydroxybiphenyl degradation, that is the *meta*-cleavage product of 2,3-dihydroxybiphenyl.

Discussion

We have provided evidence that degradation of 2-*sec*-butylphenol in *Pseudomonas* sp. strain HBP1 Prp (Fig. 5) follows the same catabolic pathway as degradation of 2-hydroxybiphenyl (Kohler et al. 1988). 2-*sec*-Butylphenol is initially attacked by a NADH-dependent monooxygenase that hydroxylates C6 of the aromatic ring yielding 3-*sec*-butylcatechol (Fig. 3) as a product. This compound is further metabolized by a metapyrocatechase to a yellow *meta*-cleavage product that was identified as 2-hydroxy-6-oxo-7-methylnona-2,4-dienoic acid (Fig. 4) by mass spectrometry of its TMS-derivative.

The metapyrocatechase was very active with 2,3-dihydroxybiphenyl as a substrate indicating that the enzyme is best suited for turning over the metabolite of 2-hydroxybiphenyl, the compound strain HBP1 originally was isolated on. The enzyme catalyzed the formation of yellow *meta*-cleavage compounds from all the catechols tested and is therefore another example of a broad spectrum *meta*-cleavage dioxygenase (Kunz & Chapman 1981; Smith & Ratledge 1989). Recently, it was shown that the metapyrocatechase from *Pseudomonas* sp. strain NCIB 10643 had a marked preference for 2,3-dihydroxybiphenyl over numerous 3-alkylcatechols (Smith & Ratledge 1989). The activity for 3-butylcatechol was 28% of the activity for 2,3-dihydroxybiphenyl in cell extracts of n-butylbenzene-grown cells. In this respect the metapyrocatechase from strain HBP1 Prp resembles the one from strain NCIB 10643. The two enzymes differ only in their activity for catechol, 3-methylcatechol, and 4-methylcatechol. Activities of the metapyrocatechase from strain NCIB 10643 for 3-methylcatechol and catechol were in the range of the activity for 3-*sec*-butylcatechol whereas 4-methylcatechol was not a substrate at all. In contrast, the enzyme from strain HBP1 Prp had much lower activities for catechol and 3-methylcatechol than for 3-*sec*-butylcatechol. Additionally, 4-methylcatechol also served as a substrate. With respect to activity for these substituted catechols the enzyme from strain HBP1 Prp resem-

Table 2. Enzyme activities of partially purified metapyrocatechase from 2-*sec*-butylphenol grown *Pseudomonas* sp. strain HBP1 Prp.

Substrate	Activity of metapyrocatechase	
	U/mg	Relative (%)
2,3-Dihydroxybiphenyl	33.2	100.0
3-*sec*-Butylcatechol	7.1	21.3
Catechol	0.87	2.6
3-Methylcatechol	2.5	7.5
4-Methylcatechol	0.32	1.0

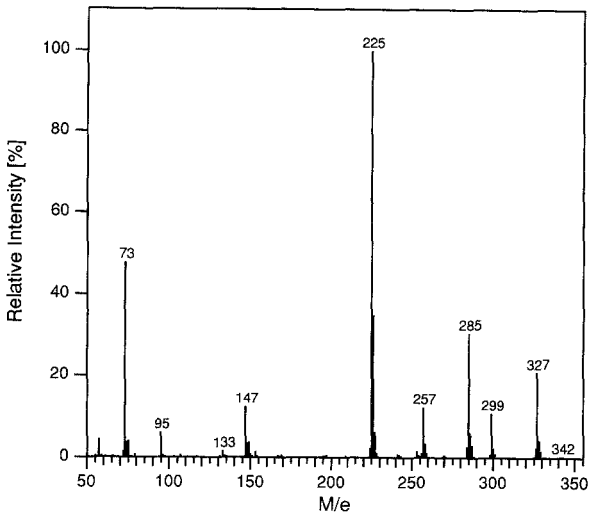

Fig. 4. Electron ionisation mass spectrum of the trimethylsilyl derivative of 2-hydroxy-6-oxo-7-methylnona-2,4-dienoic acid, the *meta*-cleavage product of 3-*sec*-butylcatechol. See text for discussion of the fragmentation pattern.

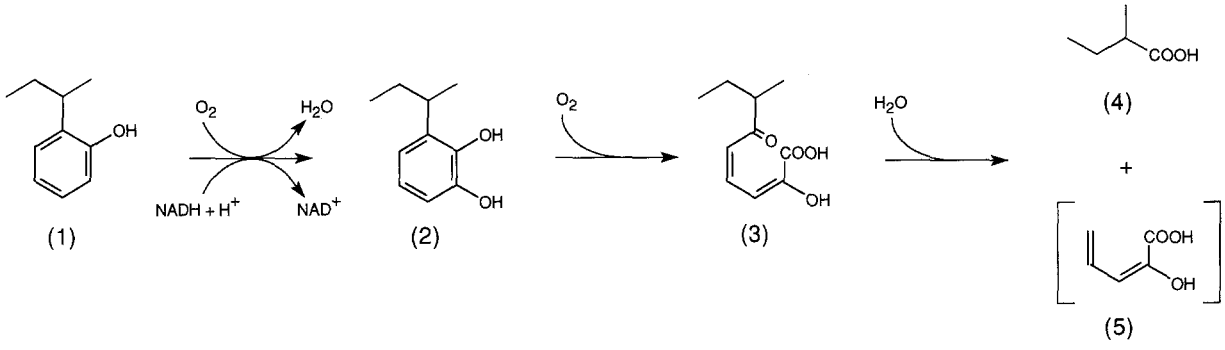

Fig. 5. Pathway proposed for the metabolism of 2-*sec*-butylphenol by *Pseudomonas* sp. strain HBP1 Prp. (1) 2-*sec*-butylphenol, (2) 3-*sec*-butylcatechol, (3) 2-hydroxy-6-oxo-7-methylnona-2,4-dienoic acid, (4) 2-methylbutyric acid, (5) 2-hydroxypent-2,4-dienoic acid.

bles the type I enzyme that has been found in dibenzofuran grown cells of *Brevibacterium* sp. strain DPO 1361 (Strubel et al. 1991).

The *meta*-fission product hydrolase from *Pseudomonas* sp. strain HBP1 only catabolized *meta*-fission products of higher alkylated catechols, but not the ones of catechol, 3-methylcatechol, and 4-methylcatechol. This is in agreement with what has been reported for the hydrolase from *Pseudomonas* sp. strain NCIB 10643 (Smith & Ratledge 1989), except that this enzyme shows some activity for the *meta*-cleavage product of 3-methylcatechol (6% of the activity for the *meta*-cleavage product of 2,3-dihydroxybiphenyl).

Pseudomonas sp. strain HBP1 Prp, a mutant of strain HBP1 that originally was isolated on 2-hydroxybiphenyl, seems to catabolize 2-*sec*-butylphenol in the same general fashion as *Pseudomonas* sp. strain NCIB 10643, originally isolated on biphenyl, catabolizes *sec*-butylbenzene (Smith 1990; Smith & Ratledge 1989). Both compounds are degraded via ring attack rather than side chain attack resulting in 3-*sec*-butylcatechol as the common intermediate. In both cases, 3-*sec*-butylcatechol is ring-cleaved by a metapyrocatechase between C1 and C2 resulting in the characteristic *meta*-cleavage product and further degradation occurs by hydrolytic attack yielding 2-methylbutyrate and, presumably, 2-oxopenta-4-enoic acid (Fig. 5). Both catabolic enzyme systems exhibit highest activities for the metabolites of the compounds the strains were originally isolated with, that is 2-hydroxybiphenyl and biphenyl for strain HBP1 Prp and strain NCIB 10643, respectively. The enzymes of 2-hydroxybiphenyl and biphenyl degrading pathways seem to have broad substrate spectra and are able to turn over various alkylanalogs – 2-alkylphenols in the case of strain HBP1 Prp and alkylbenzenes in the case of strain NCIB 10643, respectively.

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Table 3. Enzyme activities of partially purified *meta*-cleavage product hydrolase from 2-*sec*-butylphenol grown *Pseudomonas* sp. strain HBP1 Prp.

Substrate	Activity of hydrolase	
Meta-cleavage product of:	U/mg	Relative (%)
2,3-Dihydroxybiphenyl	1.5	100.0
3-*sec*-Butylcatechol	0.35	23.5
Catechol	0	0
3-Methylcatechol	0	0
4-Methylcatechol	0	0

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