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Transformation of difluorinated phenols by *Penicillium frequentans* Bi 7/2

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Abstract

The *Penicillium frequentans* strain Bi 7/2, using phenol as a sole source of carbon and energy, transformed the fluorinated phenols 2,3-, 2,4-, 2,5- and 3,4-difluorophenol rapidly. After growth on phenol, resting mycelia of the fungus converted the difluorophenols completely at an initial concentration of 0.5 mM within 6 hours. The corresponding difluorinated catechols were found to be intermediates of all difluorophenols investigated. A relatively unspecific phenol hydroxylase catalyzed this hydroxylation step and showed activities towards all difluorophenols tested. One difluorocatechol was formed from each difluorophenol substituted with fluorine in the *ortho*-position, whereas two catechols were formed from 3,4-difluorophenol, due to its two vacant *ortho*-positions. A partial defluorination (50-77%) was observed in all cases.

Abbreviations: DFC – difluorocatechol; DFP – difluorophenol; DFV – difluoroveratrol; IU – integration units; MFP – monofluorophenol

Introduction

Halogenated compounds are important environmental pollutants of soil, water and air. Detailed investigations were carried out on the degradation of chlorinated compounds, because they are the most widespread halogenated substances (Neilson 1990; Reinecke 1988; Rochkind-Dubinsky 1987). In addition, the production and the use of fluorinated substances (pesticides, pharmaceutics, polyfluorinated polymers and tensides) have been increased enormously in the recent years (Gerstenberger & Haas 1981; Naumann 1994; Cociglio et al. 1996; Key et al. 1997). Therefore, our interest has been focused on the microbial degradation of fluorinated aromatics, especially on the metabolism of fluorinated phenols by fungi.

Several studies have described the oxidative degradation of monofluorinated aliphatics and aromatics by bacteria, but polyfluorinated compounds were scarcely considered. Monofluoroacetate is the mostly investigated fluoroaliphatic compound, since it is produced and stored by certain plants in spite of its high toxicity (Goldman 1965; Wong et al. 1992; Visscher et al. 1994). The bacterial metabolism of *p*-fluorophenylacetic acid, 2-, 3- and 4-fluorobenzoic acid has been reported in detail by Harper and Blakley (1970; 1971), Oltmanns et al. (1989), Engesser et al. (1990) and Schlömann et al. (1990). A partial defluorination was observed, and in some cases the fluorobenzoic acids were used as sole sources of carbon and energy.

There are only few reports on the degradation of fluorinated compounds by fungi. Neujahr and Varga (1970) have described the utilization of phenol as carbon source by *Trichosporon cutaneum.* After growth on phenol, this yeast was also able to oxidize the MFPs. The fungal growth on MFPs has only been observed for the deuteromycetous mold *Penicillium simplicissimum* SK9117 (Marr et al. 1996). Hofrichter et al. (1993) have reported the utilization of phenol as sole source of carbon and energy by *Penicillium frequentans* Bi 7/2. Phenol pregrown resting mycelia of this

fungus metabolized the MFPs via the corresponding catechols and muconic acids. The initial hydroxylation in the *ortho*-position is effected by phenol hydroxylase and subsequently catechol dioxygenase cleaves the aromatic ring. The defluorination of MFPs occurres (Hofrichter et al. 1994). Until now, the transformation of polyfluorinated phenols has not been investigated in detail, neither for bacteria nor for fungi. Thus, we have focused our attempts on the degradation of DFPs by the fungus *P. frequentans*. The degradability, the extent of degradation, the release of fluoride and the primary enzymatic attack were studied.

Materials and methods

Chemicals

2,3-, 2,4-, 3,4-DFP, and 4,5-difluoroveratrol were obtained from Aldrich-Chemie, Steinheim/Germany. 2,5-DFP and 3,5-difluoro-2-hydroxyacetophenone were purchased from ABCR GmbH & Co., Karlsruhe/Germany. The purity of all chemicals was between 97 and 99%.

3,5-DFC was synthesized by Dakin oxidation of 3,5-difluoro-2-hydroxyacetophenone according to the prescription for 4-fluorocatechol of Corse and Ingraham (1951). Diazomethane was synthesized from N-nitroso-N-methylurea (Becker et al. 1996). HPLC grade methanol was obtained from Merck, Darmstadt/Germany.

Organism and culture conditions

The soil fungus *Penicillium frequentans* [Westling] strain Bi 7/2 (ATCC-number: 96048) was isolated from a soil contaminated with aromatic hydrocarbons as described previously (Hofrichter et al. 1993).

All investigations were carried out with resting mycelia precultivated on phenol in 0.05 M potassium-phosphate buffer (pH 7.8) (Hofrichter et al. 1993). The DFPs were added to a final concentration of 0.5 mM, the average dry weight of mycelia was about 1 g/l.

Cell free extracts were prepared from phenol grown washed mycelia by disruption with dry ice and a subsequent ultracentrifugation as reported earlier (Hofrichter et al. 1994).

The activity of phenol hydroxylase (E.C. 1.14.13.7.) was measured by following the disappearance of its specific cosubstrate NADPH at 340 nm (Neujahr & Varga 1970). Catechol-1,2-dioxygenase (E.C. 1.13.11.1.) activity was determined by measuring the increase in absorbance at 260 nm, due to the formation of *cis,cis*-muconic acid (Neujahr & Varga 1970). Molar absorption coefficients were published by Dorn and Knackmuss (1978).

Chemical and physical analysis

A Gynkotek high performance liquid chromatograph (HPLC) with a Gynkotek diode-array detector operated from 200 to 400 nm and fitted with a Sepserv Ultrasep ES RP-18 column (125 $\times$ 4.0 mm ID, 5 μm particle size) was used to determine the concentration of the phenols and to detect the metabolites using isocratic conditions (solvent: 0.05% phosphoric acid/methanol; 45: 55, v/v for 2,3-, 2,4-DFP and 55: 45, v/v for 2,5-, 3,4-DFP). As reference standards of 3,4-, 3,6- and 4,5-DFC were not available, concentrations are given as integration units.

To analyse the metabolites by gas chromatography/mass spectrometry (GC/MS), the organic substances were extracted with diethylether at pH 2. If it was necessary, the extracted metabolites were methylated with diazomethane. The extracts obtained were dried with Na_2SO_4 and used for GC/MS analysis carried out with a Hewlett/Packard GC/MS HP 5890 series II fitted with a HP 5 column.

A UV/VIS spectrometer Shimadzu UV-2102PC was used to determine the enzyme activities. The concentration of fluoride in the culture fluids was measured with a fluoride sensitive electrode, Orion Model 94-09.

Results

Transformation of difluorophenols

2,4-difluorophenol was completely converted within 6 hours. In contrast, the releasing of fluoride stopped after 23 hours and amounted about 50%. During the time course of degradation a metabolite with a typical catechol UV-spectrum was detected by HPLC/DAD analysis (Figure 1A). At the maximum of its concentration, the cultivation was stopped and the metabolite was extracted with diethylether. The neutralized extract was dried and divided in two fractions. The first fraction was directly analyzed by GC/MS. The mass spectrum of the metabolite showed a molecule peak of m/z 146, which corresponds to a compound with the composition $C_6H_4O_2F_2$. The following defragmentation peaks were determined: m/z 128 (M^+- H_2O),

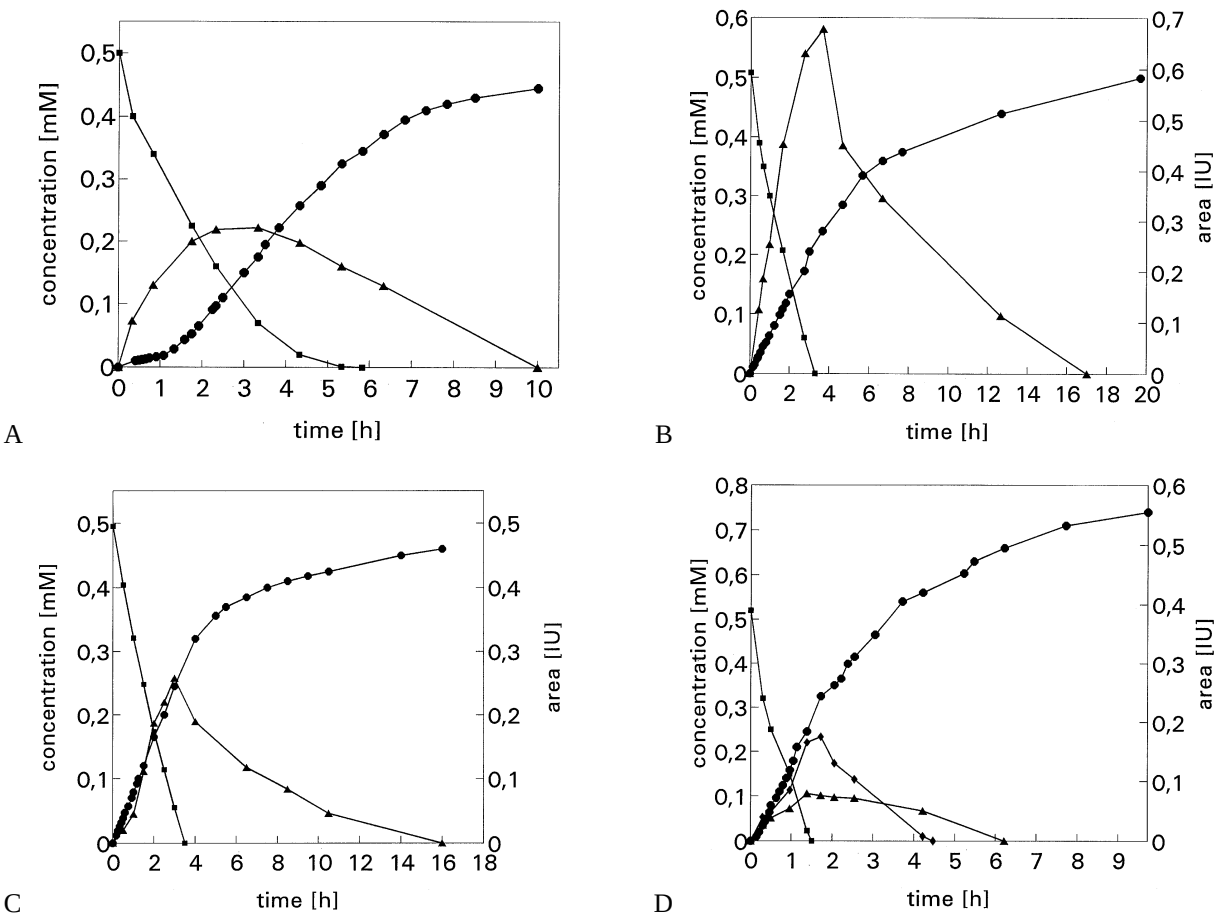

Figure 1. Elimination of DFPs (■) by resting mycelia of *P. frequentans* pregrown on phenol as sole source of carbon and energy (final dry weight: 1 g/l). The initial concentration amounted 0.5 mm DFP. During the degradation, the transient formation of the corresponding DFCs (▲, ♦) and a partial defluorination (●) were observed.
A). Conversion of 2,4-DFP [mM] (■) under formation of 3,5-DFC [mM] (▲) and fluoride [mM] (●).
B). Conversion of 2,3-DFP [mM](■) under formation of 3,4-DFC [IU] (▲) and fluoride [mM] (●).
C). Conversion of 2,5-DFP [mM](■) under formation of 3,6-DFC [IU] (▲) and fluoride [mM] (●).
D). Conversion of 3,4-DFP [mM](■) under formation of 3,4-DFC [IU] (▲), 4,5-DFC [IU] (♦) and fluoride [mM] (●).

m/z 117 (M^+- CHO). The other fraction was treated with diazomethane and also analyzed by GC/MS. The chromatogram showed a peak with a molecule peak of m/z 174, which corresponds to a compound with the elemental composition $C_8H_8O_2F_2$. The following defragmentation peaks were unequivocal: m/z 159 (M^+- CH_3), m/z 131 (M^+- CH_3 - CO), m/z 111 (M^+- CH_3- CO - HF). Therefore, the methylated product should be 3,5-DFV and the found metabolite was supposed to be 3,5-DFC. To substantiate this assumption, 3,5-DFC was synthesized. The comparison of both UV-spectra (HPLC/DAD), mass spectra (GC/MS), and retention times confirmed the identity of the metabolite formed. Thus, the extraction and methylation procedure used and the subsequent analysis by GC/MS have been shown to be a suitable method to detect the catecholic metabolites of the fungal degradation of the DFPs.

To get further information on the degradation pathway, the activity of catechol-1,2-dioxygenase was estimated in cell free extracts towards 3,5-DFC. The enzyme activity was about 2% of that detected for unsubstituted catechol. An exact determination of the enzyme activity, however, was difficult, as it was estimated by following the increase in absorbance at 260 nm and using the extinction coefficient of unsub-

Table 1. Release of fluoride and metabolites identified during the degradation of difluorinated phenols

compound	release of fluoride [%]	metabolites identified	phenolhydroxylase activity in the crude extract	
			[mU/mg]	[%] compared with phenol
2,3-DFP	50	3,4-DFC	107	23
2,4-DFP	50	3,5-DFC; 2,4-difluoro-*cis,cis*-muconic acid	131	28
2,5-DFP	50	3,6-DFC	86	18
3,4-DFP	77	3,4-DFC; 4,5-DFC	232	50

stituted *cis,cis*-muconic acid. This extinction coefficient was used as approximate value, since that of 2,4-difluoro-*cis,cis*-muconic acid was not available and no extinction coefficients were found in the literature. Controls omitting cell-free extract showed no decrease of 3,5-DFC concentration within this space of time. A direct qualitative determination of the metabolite was possible by HPLC/DAD analysis after treatment of 3,5-DFC with cell-free extract and a following acidification with HCl to pH 2. The UV-absorption maximum is 273 nm. 2,4-Difluoro-*cis,cis*-muconic acid can be assumed as an intermediate.

The degradation of 2,3-DFP was accompanied by a removal of 50% fluoride (Figure 1B) and the temporary accumulation of a metabolite having the typical UV-spectrum of catechols. As in the case of 2,4-DFP, 2,3-DFP has also one unsubstituted *ortho*-position and therefore only one difluorinated catechol is expected. To clarify this assumption by GC/MS analysis, methylation of the etheric extract of the culture filtrate was performed. The mass spectrum showed a molecule peak of m/z 174 corresponding to a composition of $C_8H_8O_2F_2$. The defragmentation is similiar to that described for 3,5-DFV. Therefore, the metabolite was proposed to be 3,4-DFC.

2,5-DFP was a degraded in a similar way to 2,3- and 2,4-DFP, due to its vacant *ortho*-position (Figure 1C). A release of 50% fluoride and a transient accumulation of a metabolite with a UV-spectrum typical for catechols were observed. The GC/MS analysis of the methylated intermediate showed also a molecule peak of m/z 174 corresponding to the composition $C_8H_8O_2F_2$ and the defragmentation mentioned above. These mass data are also in agreement with that found for 3,6-DFV by Ladd & Weinstock (1981). The metabolite was supposed to be 3,6-DFC.

3,4-DFP was transformed with the highest rate (within 2 hours) and the release of the highest amount of fluoride (77%) (Figure 1D). Since 3,4-DFP has two unsubstituted *ortho*-positions, the phenol hydroxylase of *P. frequentans* has two possible positions for attacking the molecule. Thus, the formation of two intermediary metabolites having UV-spectra typical for catechols was observed during the metabolism of 3,4-DFP. One of these intermediates has a UV-spectrum and retention time (HPLC/DAD) identical to that of 3,4-DFC found in the metabolism of 2,3-DFP. To confirm this finding, the metabolites were extracted and methylated as described above. GC/MS analysis demonstrated that the mass spectrum and the retention time of the methylated 3,4-DFC (3,4-DFV) detected during the degradation of 2,3-DFP also correspond to the data of one of both metabolites. Thus, 3,4-DFC is one of both metabolites in the degradation pathway. The other metabolite was identified by comparing its mass spectrum and retention time (GC/MS) with those of authentic 4,5-DFV. It was found to be 4,5-DFC.

Formation and disappearance of 4,5-DFC proceeded faster than of 3,4-DFC. Possibly the attack of phenolhydroxylase at the 6-position is preferential compared with the attack at the 2-position. The faster disappearance indicates that the *ortho*-cleavage of 4,5-DFC is easier than that of 3,4-DFC.

Table 1 shows the data of fluoride released, the metabolites identified during the degradation of the fluorophenols by the fungus *P. frequentans* as well as the activities of phenol hydroxylase towards the fluorinated phenols.

In Table 2, the most important data of the mass spectra of the different DFV and the relative maxima of UV-spectra of the DFC are summarized. The defragmentation patterns of the DFVs show a high similarity.

A brownish coloring was observed for all fluorophenol cultures tested due to autoxidation. This process causing oxidative polymerization of catechols (Ziechmann 1994) and leading to humic-acid like compounds (Bilton & Cain 1968, Hofrichter et al. 1994) probably gives also a small contribution to the release

Table 2. Relative maxima of the UV-spectra of the DFC and the mass spectra of the methylated metabolites (difluoroveratrols, DFV)

metabolite	UV-Spectra: relative maximum [nm]	mass spectra: m/z (% relative intensity) of the corresponding DFV
3,4- DFC	273	175 (9), 174 (100), 160 (4), 159 (46), 131 (14), 116 (16), 111 (15), 101 (16), 88 (25), 83 (48)
3,5-DFC	272	175 (9), 174 (100), 160 (6), 159 (74), 131 (30), 116 (13), 111 (30), 88 (23), 83 (39)
4,5- DFC	285	175 (9), 174 (100), 160 (4), 159 (52), 131 (33), 116 (14), 113 (33), 111 (30), 101 (20), 88 (46), 83 (58)
3,6- DFC	260	175 (9), 174 (100), 160 (3), 159 (38), 131 (6), 116 (17), 111 (23), 101 (17), 88 (18), 83 (36)

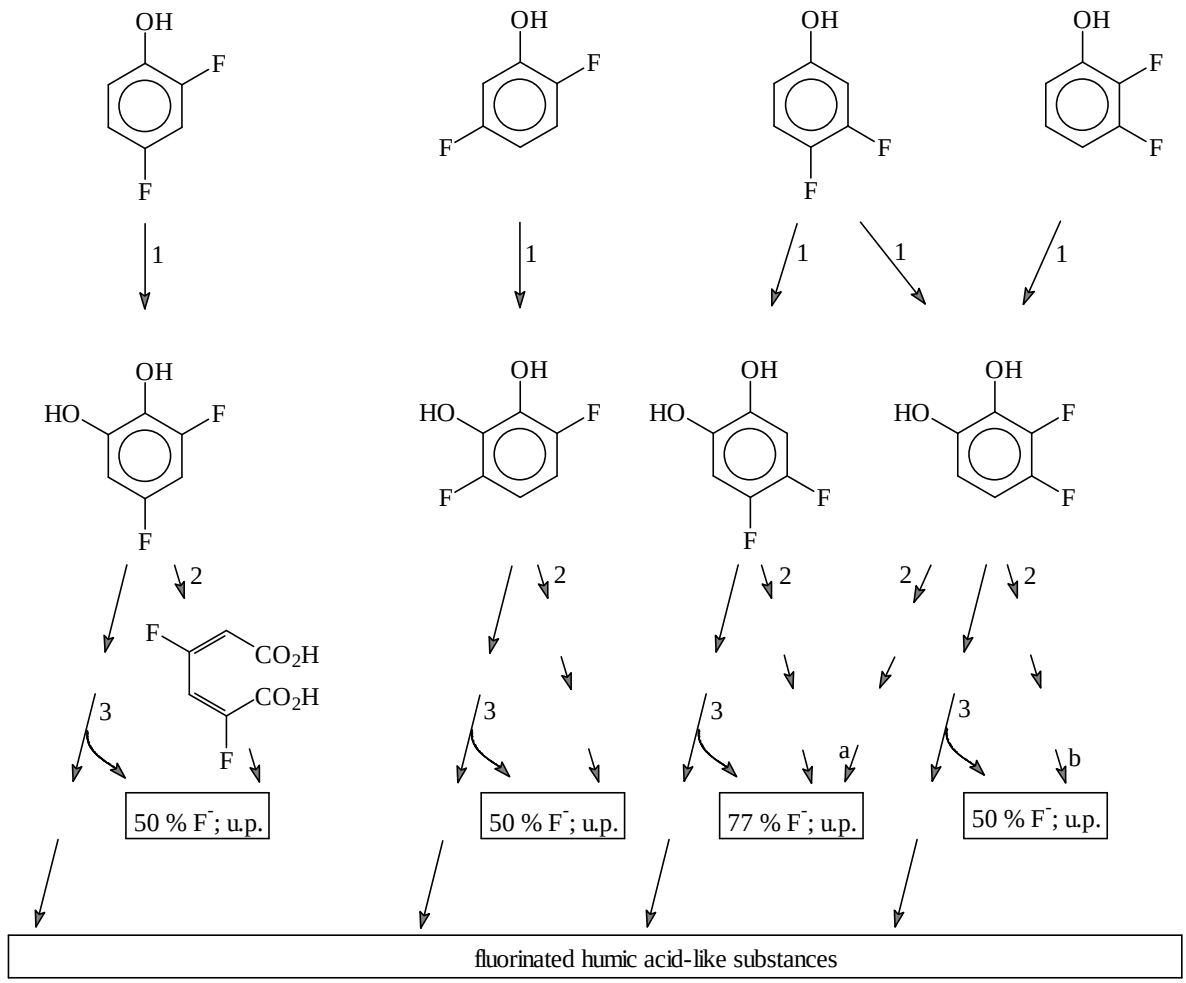

Figure 2. Proposed pathway for the metabolism of difluorophenols in *P. frequentans*. The first step is the formation of the corresponding DFCs by phenolhydroxylase. The release of fluoride proceeds via unknown metabolites probably initialized by the attack of catechol-1,2-dioxygenase (2). A part of the DFCs is transformed to fluorinated humic acid-like substances, which becomes visible by dark-brown coloring of the culture fluid and is probably accompanied by releasing of a small amount of fluorid. 1 - phenolhydroxylase, 2 - catechol-1,2-dioxygenase, 3 - spontaneous oxidative polymerization; u.p. - unknown products; a - is concerned to 3,4-DFP, b - is concerned to 2,3-DFP.

of fluoride (Figure 2). Investigations using monofluorocatechols demonstrated a spontaneous partial defluorination under degradation conditions in the absence of fungal mycelium. The rate depends on the position of the fluorine. 3-fluorocatechol is significantly more stable than 4-fluorocatechol. Information about the spontaneous defluorination of DFCs have not been found in the literature, but it can be assumed according to the behaviour of the monofluorocatechols.

Discussion

After precultivation on phenol, resting mycelia of the deuteromycetous soil fungus *Penicillium frequentans* metabolized, in addition to monofluorophenols (Hofrichter et al. 1994), also difluorophenols at an initial concentration of 0.5 mM rapidly. The degradation of all DFPs proceeded via formation of the corresponding difluorinated catechols. The subsequent degradation led, in all cases, to a partial defluorination. The identical data of defluorination (50%) of the *ortho*-substituted DFPs are conspicuous, but this value was reached only after about 1 day (not shown in Figure 1). In contrast, a removal of fluoride of about 77% took place in the case of 3,4-DFP.

On the other hand, this mold is unable to utilize fluorinated phenols as sole source of carbon and energy, since e.g. the defluorination product 4-carboxymethylenebut-2-ene-4-olide formed during the degradation of 3- and 4-MFP can not be further degraded (Hofrichter et al. 1994).

The potential for attacking fluorophenols is probably due to the low specificity of the phenol degrading enzyme system. Marr et al. (1996) have recently reported the utilization of monofluorinated phenols as carbon source by the soil fungus *Penicillium simplicissimum*, but the amounts of fluorophenols degraded and the biomass formed were comparatively low.

As far as we know, there is only one report in the literature on the microbial metabolism of DFP and TFP. Hofrichter et al. (1994) have described the cometabolic transformation and partial defluorination of 2,4- and 3,4-DFP and 2,3,4-TFP (initial concentration: 1 mM) by *P. frequentans*, but no intermediates were observed. Furthermore, activities of phenol hydroxylase were detectable towards these substrates. In our present experiments, the corresponding DFCs of all DFPs (2,3-, 2,4-, 2,5- and 3,4-DFP) tested were identified as major metabolites, their appearance, however, was transient. The formation of the corresponding catechols from dichlorophenols has been reported for the yeast *Candida maltosa* (Polnisch et al. 1993) and *P. frequentans* (Hofrichter et al. 1994), but no release of chloride was observed. Thus, it can be concluded that the same initial hydroxylation step (caused by phenol hydroxylase) took place both during the metabolism of difluoro- and dichlorophenols. In contrast to dichlorophenols, a subsequent dehalogenation was observed for DFPs. Information about the course of defluorination and the further degradation process were not obtained, since other metabolites than the catechols and 2,4-difluoro-*cis,cis*-muconic acid in the case of 3,5-DFC degraded by crude extract were not detectable (Figure 2). The activity of catechol-1,2-dioxygenase towards 3,5-DFC in the crude extract of *P. frequentans* indicates an intradiol ring cleavage to form the corresponding muconic acid, probably followed by the release of fluoride. Similiar findings have been made for various 4-monohalocatechols (metabolites of 4-monohalophenols), which are converted to the instable 3-halo-*cis,cis*-muconic acids and subsequently to dienlacton and halogenide (Schlömann et al. 1990; Polnisch et al. 1992; Hofrichter et al. 1994; Marr et al. 1996). Further studies will have to clarify the degradation process of other polyfluorinated phenols e.g. 2,6-DFP, tri- and tetrafluorophenols.

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