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Catabolism of 2,7-dichloro- and 2,4,8-trichlorodibenzofuran by *Sphingomonas* sp strain RW1

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Due to their physicochemical and toxicological properties, polychlorinated dibenzofurans are regarded as a class of compounds providing reason for serious environmental concern. While the nonhalogenated basic structure dibenzofuran is effectively mineralized by appropriate bacterial strains, its polychlorinated derivatives are not. To elucidate the ability of the strain *Sphingomonas* sp RW1 to metabolize some of these chlorinated derivatives, we performed turnover experiments using 2,7-dichloro- and 2,4,8-trichlorodibenzofuran. As indicated by the oxygen-uptake rates determined for these two chlorinated dibenzofurans, *Sphingomonas* sp RW1 can catabolize these chlorinated dibenzofurans yielding small quantities of oxidation products, which we isolated and subsequently characterized employing GC/MS and ^{1}H- as well as ^{13}C-NMR spectroscopy. In the case of 2,7-dichlorodibenzofuran, two metabolites accumulated, which we identified as 6-chloro- and 7-chloro-2-methyl-4*H*-chromen-4-one. The single metabolite isolated from the turnover experiments performed with 2,4,8-trichlorodibenzofuran was unequivocally identified as 6,8-dichloro-2-methyl-4*H*-chromen-4-one.

Keywords: biotransformation; *Sphingomonas*; 2,7-dichlorodibenzofuran; 2,4,8-trichlorodibenzofuran; 6-chloro-2-methyl-4*H*-chromen-4-one; 7-chloro-2-methyl-4*H*-chromen-4-one; 6,8-dichloro-2-methyl-4*H*-chromen-4-one

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are compounds that, although not produced on an industrial scale, have entered the global environment in significant amounts. Complex mixtures of congeners of PCDD/Fs are therefore frequently detected in environmental samples such as sediments [7,12,20]. These compounds are formed as unwanted by-products of industrial processes [4,15], during municipal incineration [18], or during recycling of plastics and metals [22], and are highly persistent under environmental conditions [27].

The well-documented toxicity of these compounds [2,23], especially of those derivatives with halogen substituents present in positions 2, 3, 7 and 8, has put these noxious substances into the focus of environmental research. Due to their catabolic versatility, bacteria are able to degrade a huge range of environmentally relevant aromatic compounds [6]. Accordingly, bacterial strains utilizing the nonchlorinated basic structures of polychlorinated dibenzo-*p*-dioxins and dibenzofurans have been isolated, and the catabolism of dibenzo-*p*-dioxin and dibenzofuran has been elucidated in detail [3,9,10,16,25,28]. However, although aerobic catabolism of monochlorinated derivatives by *Sphingomonas* spp has been reported [13,26], there is still a lack of knowledge concerning the bacterial catabolism of chlorinated derivatives carrying two or more chlorine substituents.

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Materials and methods

Organism, growth conditions and turnover experiments

Cultivation of *Sphingomonas* sp RW1 (DSM 6014) was done by employing a mineral salts medium [10] using dibenzofuran (1 g L^{-1}) as sole source of carbon and energy. For co-oxidation experiments, cells were grown in 1-L Erlenmeyer flasks containing 300 ml of the growth medium on an orbital shaker (120 rpm) at 28°C to the late-exponential growth phase. Cells were separated from dibenzofuran crystals by filtration, harvested and resuspended in 1/10 volume of phosphate buffer (pH 7.2, 20 mM). After incubating this suspension for about 30 min on an orbital shaker (120 rpm, 28°C) to allow for consumption of residual dibenzofuran, cells were washed twice and resuspended in phosphate buffer (pH 7.2, 20 mM) to an $OD_{578\,nm}$ of about 6.5. The selected substrate was then added to this suspension from a stock solution (prepared in N,N-dimethylformamide [DMF]) to a final concentration of about 100 mg L^{-1}. Similarly performed incubations employing poisoned cells (10 mM NaN_3) served as controls.

Oxygen uptake rates

Substrate-specific oxygen uptake rates were determined using a Clark-type electrode as described previously [10]. The substrates employed were added from stock solutions (100 mM) prepared in DMF.

Analytical methods

Optical densities at 578 nm and UV spectra were recorded using a Uvikon 922 spectrophotometer. Analytical HPLC

to check for formation of oxidation products was done as reported [19]. ^{1}H-NMR and ^{13}C-NMR spectra at 400 MHz were obtained using a Bruker WP 400 spectrometer (Bruker GmbH, Karlsruhe, Germany) with tetramethylsilan as internal standard. Mass spectra were obtained using a quadrupol mass spectrometer MD800 and a PLATFORM II system (Fisons Instruments, Manchester, UK) in EI or CI mode at 70 eV coupled to a Fisons gaschromatograph GC8000 using an Optima 5-MS GC-column (30 m, 0.25 mm i.d., 25 μm film thickness, Macherey-Nagel, Düren, Germany) and the following temperature programme: 60°C for 5 min → (5°C min^{-1}) → 320°C for 20 min. Extracts were purified by using column chromatography (silica gel, Mesh 240–400, Merck, Darmstadt, Germany) with mixtures of ethyl acetate, hexane and petrol ether as eluent. Products were detected by monitoring absorption at 254 nm.

Chemicals

2,7-Dichlorodibenzofuran was prepared from the corresponding diaminodibenzofuran by chlorination of its diazonium salt using the Sandmeyer reaction [5]. 2,7-Diaminodibenzofuran was obtained upon reduction of 2,7-dinitrodibenzofuran, which in turn was produced by nitration of dibenzofuran [13]. 2,4,8-Trichlorodibenzofuran was purchased from Aldrich (Steinheim, Germany).

6-Chloro-2-methyl-4*H*-chromen-4-one was synthesized from 5-chloro-2-hydroxyacetophenone according to the literature [26]. 7-Chloro- and 6,8-dichloro-2-methyl-4-*H*-chromen-4-one were obtained in an analogous manner starting from 4-chloro-2-hydroxyacetophenone and 3,5-dichloro-2-hydroxyacetophenone, respectively. Friedel–Crafts acylation of chlorophenols was employed to produce the required 4-chloro-2-hydroxyacetophenone from 3-chlorophenol and the 3,5-dichloro-2-hydroxyacetophenone from 2,4-dichlorophenol [11,14]. The synthetic references were used to verify the structures of isolated metabolites. All other compounds used were of the highest purity commercially available.

Results

Resting cells of *Sphingomonas* sp RW1 grown with dibenzofuran were able to oxidize 2,7-dichloro- and 2,4,8-trichlorodibenzofuran, albeit at low rates when compared to the nonsubstituted growth substrate, dibenzofuran (Table 1).

As shown by HPLC analysis, turnover experiments using 2,7-dichlorodibenzofuran led to the accumulation of μM-amounts of two metabolites. The bacterial suspensions were extracted with chilled ethyl acetate (4 × 1/4 vol) to remove excess substrate. After acidification of the remaining water layer with *ortho* phosphoric acid to pH 2.5 the extraction was repeated. The organic layer was dried over Na_2SO_4, and after the solvent had been removed under reduced pressure, the residue was purified by column chromatography using silica gel as described in Materials and Methods. The mass spectrum of the first metabolite (M^+194) was very similar to that reported by Wilkes *et al* [26] for 6-chloro-2-methyl-4*H*-chromen-4-one, which had been produced in co-oxidation experiments performed with *Sphingomonas* sp RW1 from 2,8-dichlorodibenzofuran. The corresponding ^{1}H-NMR spectrum [$CDCl_3$, 400.13 MHz, $\delta = 8.141$ (d, 1H, H5); 7.578 (dd, 1H, H7); 7.377 (d, 1H, H8); 6.178 (s, 1H, H3); and 2.393 (s, 3H, Me-H) ppm; $J_{H5-H7} = 2.54$ Hz; $J_{H7-H8} = 8.64$ Hz] and ^{13}C-NMR data [CDCL]$_3$, 400.13 MHz, $\delta = 176.972$; 166.480; 154.811; 133.645; 130.910; 125.167; 124.581; 119.543; 110.569; and 20.587 ppm] were identical to those of synthetic 6-chloro-2-methyl-4*H*-chromen-4-one, confirming the structure of the metabolite. The mass spectrum of the second metabolite resembled that of the first one, indicating the presence of a single chlorine (Figure 1). However, the ^{1}H-NMR spectrum [$CDCl_3$, 400.13 MHz, $\delta = 8.108$ (d, 1H, H5); 7.436 (d, 1H, H8); 7.341 (dd, 1H, H6); 6.164 (s, 1H, H3); and 2.381 (s, 3H, Me-H) ppm; $J_{H5-H6} = 8.65$ Hz; $J_{H6-H8} = 1.53$ Hz] and the ^{13}C-NMR spectrum [$CDCl_3$, 400.13 MHz, $\delta = 177.311$; 166.315; 156.588; 139.453; 127.082; 125.798; 122.158; 117.910; 110.847; and 20.530 ppm] revealed the structure of this compound to be the isomeric 7-chloro-2-methyl-4*H*-chromen-4-one.

Table 1 Relative oxygen uptake rates obtained for chlorinated dibenzofurans by resting cells of *Sphingomonas* sp RW1 after growth with dibenzofuran

Substrate	Oxygen uptake (%)[a]
Dibenzofuran	100 (250)
2-Chlorodibenzofuran	51
3-Chlorodibenzofuran	37
2,7-Dichlorodibenzofuran	9
2,4,8-Trichlorodibenzofuran	2

[a]Activities, determined as nmoles O_2 per min per mg of protein (means, $n > 3$, SD ≤ 6%) and corrected for endogenous respiration and autoxidation, are compared to that obtained for dibenzofuran (shown in brackets) which was taken as 100%.

The dead-end product (M^+228) isolated from the 2,4,8-trichlorodibenzofuran oxidation experiments performed with dibenzofuran-grown cells of *Sphingomonas* sp RW1, exhibited two distinct UV-maxima at λ^{MeOH}_{max} 227 nm (log ϵ 4.46) and λ^{MeOH}_{max} 310 nm (log ϵ 3.82). The isotope cluster in the mass spectrum (Figure 2) revealed the presence of two chlorines. Again, the spectrum showed a characteristic signal at $M^+-40 = M^+-C_3H_4$, formed upon retro cleavage of the pyrone ring. The ^{1}H-NMR spectrum [$CDCl_3$, 400.13 MHz, $\delta = 8.056$ (d, 1H, H5); 7.692 (d, 1H, H7); 6.221 (d, 1H, H3); and 2.459 (d, 1H, Me-H) ppm; $J_{H5-H7} = 2.55$ Hz] and the ^{13}C-NMR spectrum [$CDCl_3$, 400.13 MHz, $\delta = 176.263$; 166.653; 150.800; 133.612; 130.640; 125.428; 123.926; 110.771; and 20.523 ppm] clearly identified this compound as the novel metabolite, 6,8-dichloro-2-methyl-4*H*-chromen-4-one.

Experiments performed with poisoned controls showed no detectable formation of metabolites from 2,7-dichloro- or 2,4,8-trichlorodibenzofuran.

Discussion

Although the ability of bacteria to transform dichloro- and trichlorodibenzofurans under reducing conditions has been

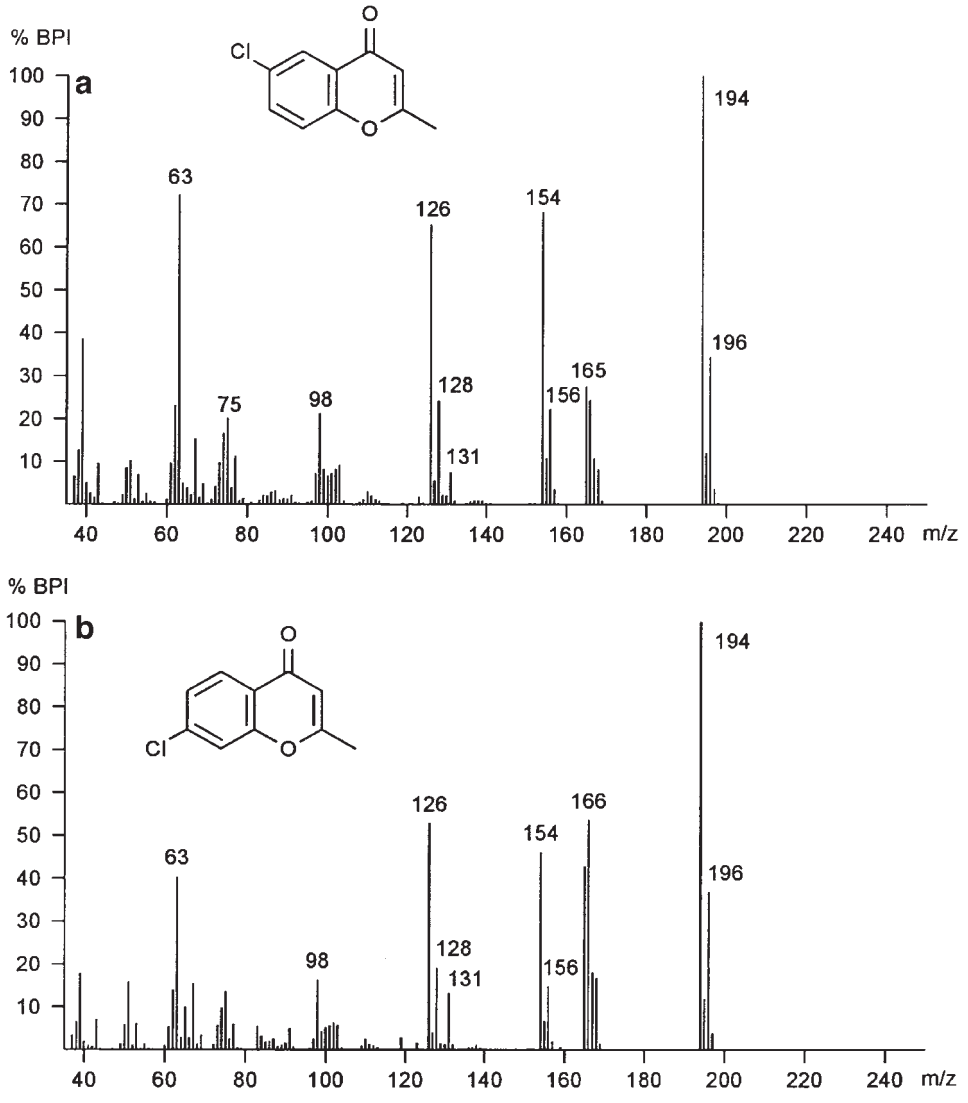

Figure 1 Mass spectra (70 eV) of (a) 6-chloro- and (b) 7-chloro-2-methyl-4*H*-chromen-4-one produced from 2,7-dichlorodibenzofuran by *Sphingomonas* sp RW1.

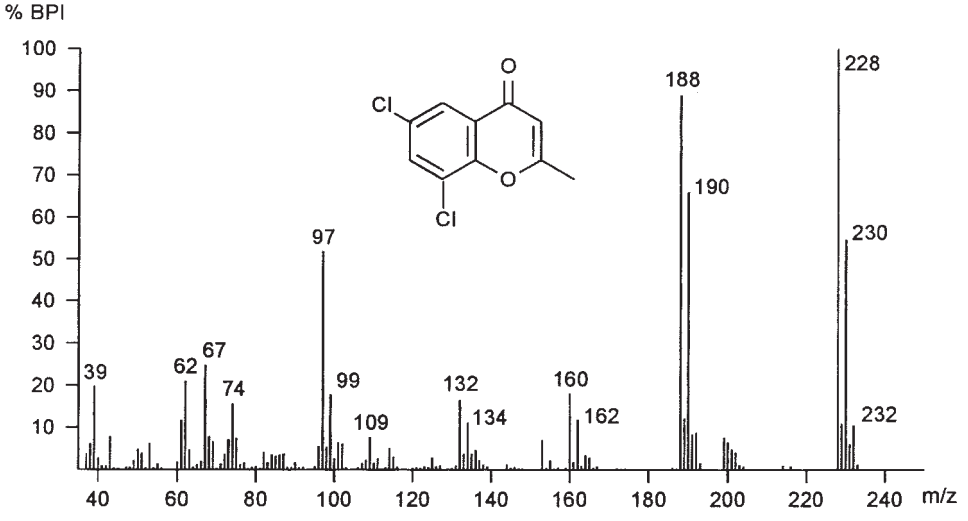

Figure 2 Mass spectrum (70 eV) of 6,8-dichloro-2-methyl-4*H*-chromen-4-one produced from 2,4,8-trichlorodibenzofuran by *Sphingomonas* sp RW1.

reported [1], there is need for more detailed information concerning the way these compounds are catabolized aerobically. By using *Sphingomonas* sp RW1 we showed recently that 2,3-dichlorodibenzofuran can be catabolized to 4,5-dichlorosalicylate and 2,8-dichlorodibenzofuran to 6-chloro-2-methyl-4*H*-chromen-4-one [26]. We employed 2,7-dichlorodibenzofuran which, in contrast to the symmetrically substituted 2,8-dichlorodibenzofuran, should yield two different monochlorinated 2-methyl-4*H*-chromen-4-ones if the initial dioxygenolytic attack takes place at both of the two rings. Indeed, the identification of 6-chloro- and 7-chloro-2-methyl-4*H*-chromen-4-one in our turnover experiments using 2,7-dichlorodibenzofuran, clearly revealed the ability of *Sphingomonas* sp RW1 to attack either of the rings (Figure 3).

The aerobic transformation of 2,4,8-trichlorodibenzofuran by several bacterial strains has been reported recently [21,26]; however, no details on isolation and structural determination of any catabolites were given. To enable reliable predictions on the environmental fate and impact of these compounds, elucidation of their potential metabolic pathways is important, as microbial metabolites can be more problematic than the parent compounds [17]. By employing *Sphingomonas* sp RW1 we were able to demonstrate for the first time its ability to oxidize a trichlorodibenzofuran and identified 6,8-dichloro-2-methyl-4*H*-chromen-4-one as a major catabolite. Probably due to the poor bioavailability of these chlorinated dibenzofurans, the transformation rates are rather low. Nevertheless, these findings demonstrate the amazing potential of *Sphingomonas* sp RW1 for the biodegradation of these xenobiotic compounds and confirm the biotechnological potential of the genus *Sphingomonas* [24]. When introduced into different soils, *Sphingomonas* sp RW1 quantitatively mineralized dibenzofuran and dibenzo-*p*-dioxin [8], which was not affected notably by the presence of additional, readily utilizable carbon sources. Having identified the degradation products it now remains to be seen, whether these compounds can be produced in contaminated soils and if known or not-yet-described bacterial strains have the potential to utilize the chlorinated methylchromenones we identified here as sources of carbon and energy.

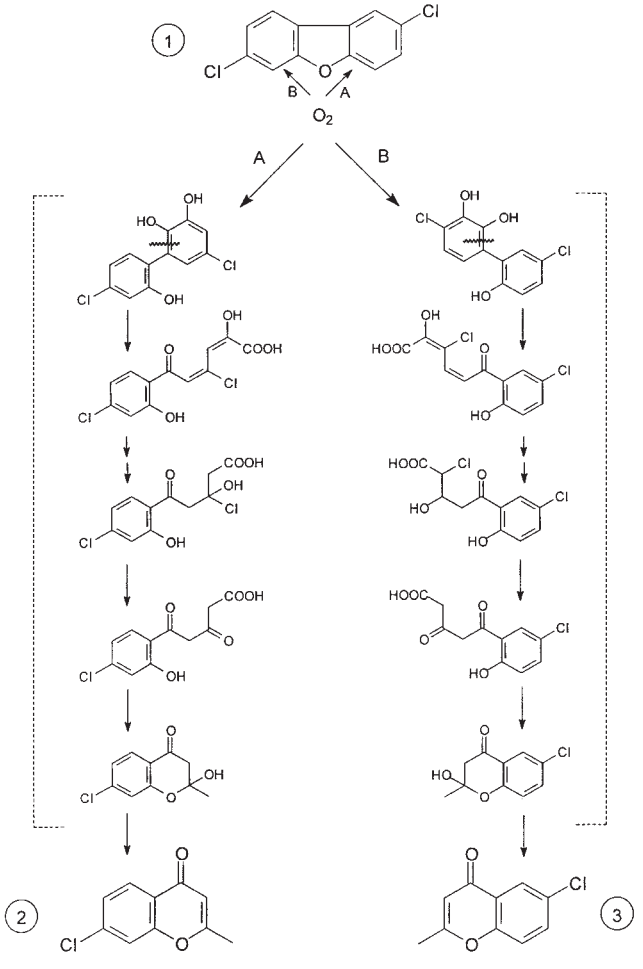

Figure 3 Proposed catabolism of 2,7-dichlorodibenzofuran by *Sphingomonas* sp RW1. 1 = 2,7-Dichlorodibenzofuran; 2 = 7-chloro-2-methyl-4*H*-chromen-4-one; 3 = 6-chloro-2-methyl-4*H*-chromen-4-one.

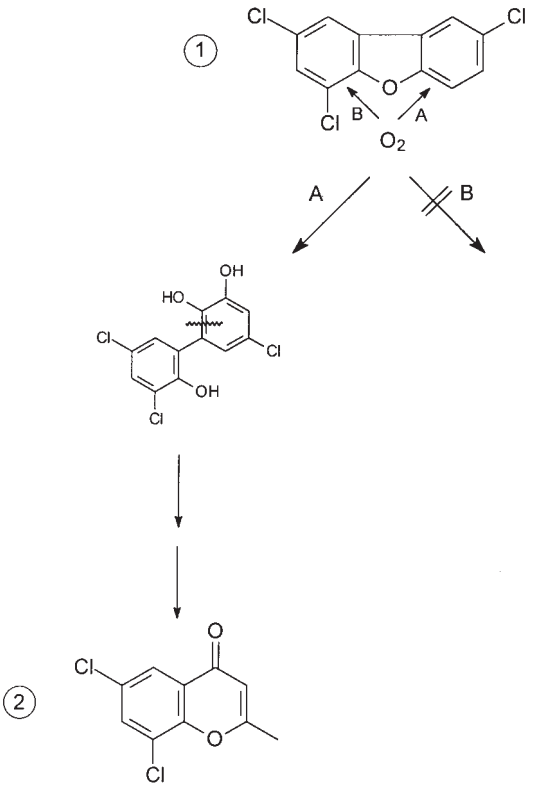

Figure 4 Catabolism of 2,4,8-trichlorodibenzofuran by *Sphingomonas* sp RW1. 1 = 2,4,8-Trichlorodibenzofuran; 2 = 6,8-dichloro-2-methyl-4*H*-chromen-4-one.

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