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Dioxygenolytic cleavage of aryl ether bonds: 1,10-dihydro-1,10-dihydroxyfluoren-9-one, a novel arene dihydrodiol as evidence for angular dioxygenation of dibenzofuran

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1. SUMMARY

Two dibenzofuran degrading bacteria, *Brevibacterium* strain DPO 1361 and strain DPO 220, were found to utilize fluorene as sole source of carbon and energy. Cells which were grown on dibenzofuran, transformed fluorene into a number of products. For five of the seven metabolites isolated, the structure could be established unequivocally. Accumulation of one metabolite, 1,10-dihydroxy-1,10-dihydrofluoren-9-one, indicated the presence of a novel type of dioxygenase, attacking polynuclear aromatic systems in the unusual angular position. Dibenzofuran degradation is proposed to likewise proceed via initial angular dioxygenation. One aryl oxygen ether bond, which normally is extremely stable, is thus transformed to a hemiacetal. After spontaneous cleavage and subsequent rearomatization by dehydration, 2,2′,3-trihydroxybiphenyl [3-(2-hydroxyphenyl)-catechol] thus results as the immediate product of the first enzymatic reaction in the degradation sequence.

2. INTRODUCTION

Dibenzofuran (DBF) and structurally related diaryl ethers have been employed in a general study of the mechanism of diaryl ether degradation [1–5]. This class of compounds has attracted special public attention since some halo derivatives, e.g. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), are among the most pernicious and at the same time persistent chemicals ever encountered [6–7]. Recently we proposed a new mechanism for the cleavage of aryl ether bonds [9]; according to this rationale, the aryl ether function is 'labilized' by formally transforming it to a hemiacetal (intramolecular alcohol aldehyde adduct). There is compelling evidence for a similar mechanism to be operative in dibenzofuran degradation.

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3. MATERIALS AND METHODS

Strain DPO 1361 and 220 have been described [1]. DPO 1361 has been characterized as a *Brevibacterium* species by Deutsche Sammlung von Mikroorganismen (DSM) Braunschweig, F.R.G.

Conditions for the cultivation of the strains and measurement of optical density were as given by Strubel et al. (1989) with the exception of Vitamin B 12 being added to the growth medium to a final concentration of 0.001%. Substrate turnover was monitored by HPLC as described elsewhere [1] with the following eluent compositions: acetonitrile/water 60/40 (v/v) for following substrate consumption; methanol/water 60/40 (v/v) for following metabolite accumulation (flow rates 1 ml/min). Mass and NMR spectra were recorded as described [1].

Isolation of the metabolites: After centrifugation the culture supernatant was extracted with chloroform and applied to Kieselgel-60 plates (Merck, Darmstadt, F.R.G.). Metabolite F2 had an R_f value of 0.45 (mobile phase ethylacetate). The other metabolites all showed an R_f value of about 0.9. They could be separated by a second chromatographic step using dichloromethane as a mobile phase. Metabolites F3, F4, and F5 in this system are charaterized by R_f values of 0.22, 0.62 and 0.81, respectively.

4. RESULTS AND DISCUSSION

4.1. Degradation of fluorene

Some dibenzofuran (DBF) degrading strains, previously described as cometabolizing fluorene, were investigated for growth with fluorene as sole source of carbon and energy. Both strains, DPO 220 in coculture with DPO 230, and DPO 1361, when previously incubated with DBF, degraded fluorene (5 mM) within 5 to 10 days attaining a difference in optical density (OD_{546nm}) of about 1.2 within this time. After prolonged growth and many transfers into fluorene containing medium, faster growing derivatives of both strains could be isolated.

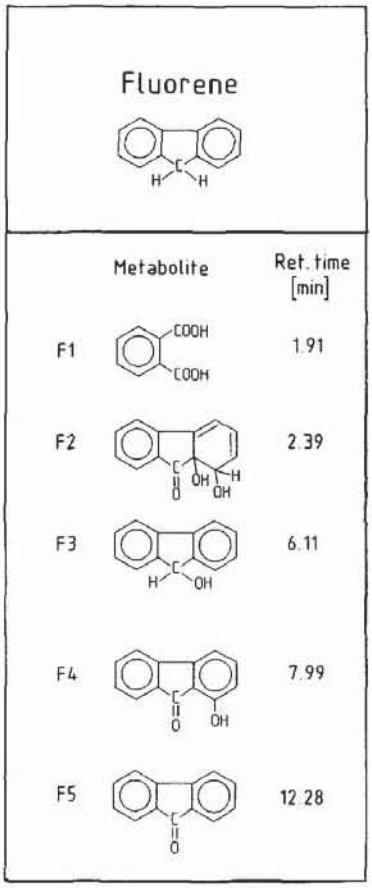

Fig. 1. Products of cometabolism of fluorene by dibenzofuran grown cells HPLC; methanol/water 60/40 (v,v); flow rate 1 ml/min.

4.2. Products of cometabolism of fluorene

Irrespective of the growth substrate, DPO 1361 cells, after centrifugation and resuspension (OD 5), cometabolized fluorene to seven products (F1 to F7). The retention times of F1 to F5 in reversed phase HPLC (methanol/water 60 : 40 (v/v) acidified with 0.1% H_3PO_4) were: 1.9, 2.4, 6.1, 8 and 12.3 min (see Fig. 1). Two products, F6 and F7, (ret. times 2.88 and 4.90 min.) were not further characterized. Cells grown with DBF showed higher initial rates of metabolism than fluorene grown cells (1.3 $mmol \cdot h^{-1}$ vs. 0.7 $mmol \cdot h^{-1}$; $OD_{546} = 5$). The metabolites were isolated in underivatized form as described in MATERIALS AND METHODS.

4.3. Characterization of fluorene cometabolism products

Metabolite F1 was readily identified as phthalic acid [benzene-*o*-dicarboxylic acid] by comparison

with authentic material, retention times on RP8 and UV spectra being identical.

The structure of the crucial metabolite F2 was established by three independent lines of spectroscopic reasoning. The exact mass of 214.0629 Dalton corresponded to a molecular formula of $C_{13}H_{10}O_3$, i.e. incorporation of three oxygens into the fluorene moiety. In the normal low-resolution EI-MS (20 eV, 310 K source temperature), F2 displayed a moderately intense molecular peak (*m/z* 214, 6%). The loss of HO from the molecular ion (*m/z* 197, 29%) was far less pronounced than the loss of H_2O (*m/z* 196, 100%) generating a polynuclear aromatic radical cation.

The ^{13}C-NMR spectrum of F2 showed one C = O resonance at 203.44 ppm, 10 ppm downfield from the value for fluorenone [23], and only three further quaternary sp^2 carbon signals pertaining to C-11,12,13. Two almost coincident signals for hydroxylated sp^3 carbon atoms, (70.85, 70.87 ppm) were revealed by an off-resonance spectrum to pertain to one quaternary and one methine (CH) carbon, respectively. Hydroxylation thus must have taken place in an angular fashion, forming the 1,10-dihydro-1,10-dihydroxy product. Taken together, the ^{13}C-NMR spectral evidence provided unequivocal proof of angular dihydroxylation. The corresponding dioxygenation product of naphthalene, analyzed in comparison, had two OH-bearing sp^3 carbon atoms which were clearly revealed as -CH(OH)-groups thus confirming 1,2-dihydroxylation (data will be published elsewhere). The ^{1}H-NMR spectrum showed a complex threepartite pattern between 7.4 and 7.9 ppm, pertaining to 1, 2, and 1 proton each, superimposable on the aryl proton spectrum of fluorene (unchanged left-hand ring of the metabolite). The remaining resonances at 6.575, 6.199, 5.937 and 4.736 ppm (1 H each) constitute one inter-correlated spin system; assignments of chemical shifts and coupling constants are given in Fig. 2. 2-H and 3-H appear coupled by 9.5 Hz, a typical value for olefinic (*cis*) coupling in cyclohexene structures [24] while the 5.1 Hz coupling between 3-H and 4-H is identical with the value reported for 1,3-cyclohexadiene itself [24]. The remaining signal is set off to higher field by almost 1.5 ppm, and appears split into a doublet of doublets of doublets by

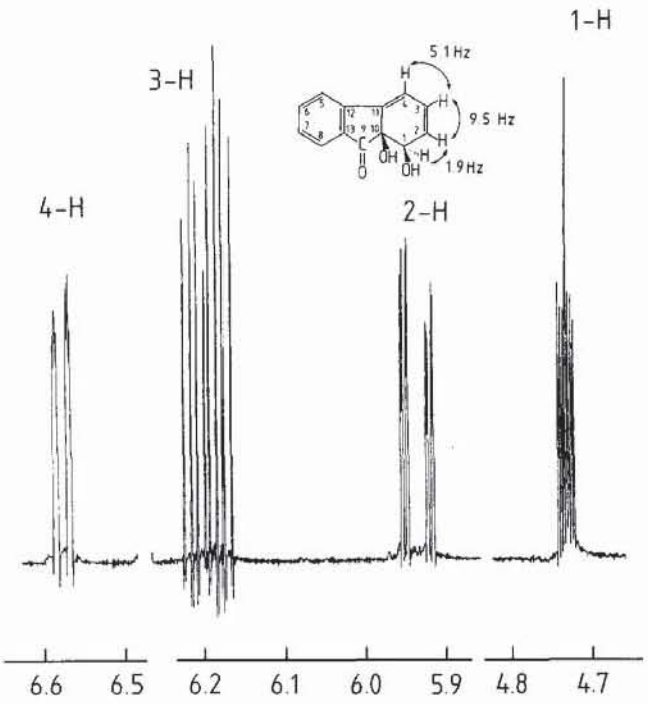

Fig. 2. Partial 300 MHz ^{1}H-NMR spectrum of 1,10-dihydro-1,10-dihydroxyfluoren-9-one (20 mg in 1 ml $CDCl_3$, 300K, TMS as internal standard, FID manipulated by Gaussian multiplication for improved resolution).

vicinal, allylic, and homoallylic coupling (see Fig. 2). In conjunction, the very large allylic and extremely small vicinal coupling constants (2.8 and 1.9 Hz, respectively) provide unrefutable proof of the proton at C-1 being in an almost perfect axial position, thus leaving the hydroxyl group at C-1 in equatorial orientation. Since the angular OH group at C-10 must be axially oriented for steric reasons, the cyclohexadiene ring has the two OH functions in e,a orientation, i.e. in *cis*-position — thus providing straightforward proof from an intermediate metabolite that the enzyme has indeed attacked the fluorene in a *cis* fashion. This stereochemistry definitely establishes the enzyme as a dioxygenase since monooxygenases inevitably produce *trans*-dihydro diols. Metabolites F3 and F5 were characterized by high-resolution MS, ^{1}H- and ^{13}C-NMR as 9-fluorenol and 9-fluorenone, respectilvely; the identification was verified by comparison with authentic material. Elemental composition of F4 was established by high resolution MS ($C_{13}H_8O_2$, 196.0526 Dalton); ^{1}H-NMR clearly

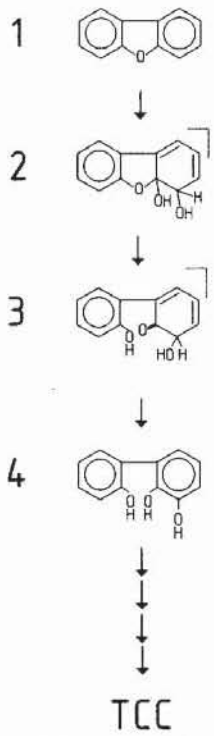

Fig. 3. Initial enzymatic step of dibenzofuran degradation pathway (for details see text).

showed the hydroxyl group being in 1-position and thus F4 to be 1-hydroxyfluoren-9-one (data of F3 to F5 will be published separately). Metabolite F4 presumably is a product of chemical rearomatization of metabolite F2. This type of reaction has frequently been described in the literature to occur with acid-labile diendiols [12–16]. The formation of C-9 oxygenated products of fluorene might be rationalized either by action of a fluorene monooxygenase or, more probably, of the dibenzofuran 1,10-dioxygenase performing like a monooxygenase. Wackett et al. [17] in the case of a toluene dioxygenase, and other authors [18,19] have shown that dioxygenases indeed can act as monooxygenases. The 2-chlorobenzoate-1,2-dioxygenase [9] was found in our laboratory to cooxidize 2-methyl to 2-hydroxymethyl benzoate [10,11].

4.4. Initial steps in DBF degradation

Applying the concept of angular dioxygenation likewise to DBF gives the degradation sequence outlined in Fig. 3. The sequence is triggered by dioxygenation to the chemically unstable diendiol (compound 2, Fig. 3). The stable diaryl ether thus is transformed to a hydrolytically labile hemiacetal which undergoes chemical cleavage to compound 3 (Fig. 3), followed by chemical rearomatization. A 2,3-dioxygenating enzyme for 3-(2-hydroxyphenyl)-catechol subsequently could cleave the ring liberating a substrate suitable for hydrolase action. Both types of enzyme are well established for classical arene degradation [20–22]. The ring fission substrate (HPC, compound 4, Fig. 3) was prepared by making use of mutants of the diphenyl pathway [25]. Indeed, high HPC turnover activities were found with crude extracts of DPO 1361, grown on DBF-containing medium. A transient yellow color was observed. Addition of 3-chlorocatechol, a well-known inhibitor for pyrocatechases [9], to cells transforming DBF resulted in accumulation of HPC (HPLC and GC/MS data not shown; results will be published elsewhere).

These preliminary findings strongly indicate DBF being degraded via a pathway analogous to that established for diphenyl. DBF may be considered as a diphenyl molecule substituted with an oxygen function in both 2- and 2′-position. Further experiments will have to clarify the potential of normal diphenyl degrading organisms to also degrade biaryl ethers after fortuitous initial dioxygenation. The metabolization of chlorinated dibenzodioxins by such diphenyl degrading bacteria will be of special importance. Preliminary experiments demonstrated the high metabolic potential of certain members of this bacterial group towards DBF, dibenzodioxin and even halodibenzodioxins.

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