

FEMSLE 04030

Dioxygenolytic cleavage of aryl ether bonds: 1,2-Dihydro-1,2-dihydroxy-4-carboxybenzophenone as evidence for initial 1,2-dioxygenation in 3- and 4-carboxy biphenyl ether degradation

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Received 15 February 1990
Revision received 2 March 1990
Accepted 4 March 1990

Key words: Biarylether; 4-Carboxybiphenylether; Dioxygenolytic ether bond cleavage; Phenol; Protocatechuate; *Pseudomonas* sp.

1. SUMMARY

A bacterial strain, *Pseudomonas* sp. POB 310, was enriched with 4-carboxy biphenyl ether as sole source of carbon and energy. Resting cells of POB 310 co-oxidize a substrate analogue, 4-carboxybenzophenone, yielding 1,2-dihydro-1,2-dihydroxy-4-carboxy-benzophenone. The ether bond of 3- and 4-carboxy biphenyl ether is cleaved analogously by initial 1,2-dioxygenation, yielding a hemiacetal which is hydrolysed to protocatechuate and phenol. These intermediates are degraded via an ortho and meta pathway, respectively. Alternative 2,3- and 3,4-dioxygenation can

be ruled out as triggering steps in carboxy biphenyl ether degradation.

2. INTRODUCTION

The biphenyl ether linkage is a salient structural feature of chlorinated dibenzofurans and dibenzodioxins. Within this class of xenobiotics, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most notorious.

The mechanism for the cleavage of alkyl-aryl ether bonds is well established [1-3]. Details on the mechanism of the cleavage of biaryl ether linkages have become available only recently [4-9]. The ether bond of dibenzofuran, for instance, is cleaved by initial dioxygenation; this was established unequivocally by co-metabolism of a substrate analogue [9]. The same mechanistic scheme has been advanced for the degradation of 4-

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carboxy biphenyl ether [8,10]. We now present proof for the 1,2-dioxygenation mechanism by structural assignment of intermediates from the metabolism of substrate analogues.

3. MATERIALS AND METHODS

The bacterial strain POB 310 was isolated with 4-carboxy biphenyl ether (4CBPE) as sole source of carbon and energy. 4 mM of the respective substrates were added per 1 liter of mineral medium [11] for growth in batch culture. The strain was characterized as described previously [3]. For growth of the organisms, preparation of cell extracts, measurement of whole cell activity, and analytical methods see Pieper et al. [12,13]. Enzymatic activities for both the phenol and the cumic acid pathway were determined as described [12,14]. Metabolites from structural analogues of 4CBPE were obtained by employing resting cells of strain POB310, grown with 4CBPE. The culture was centrifuged, the supernatant acidified with H_3PO_4 , extracted with ether, and the metabolites purified by preparative column chromatography (SiO_2 ; eluent cyclohexane/ethyl acetate/formic acid 120:80:1, v/v/v) or preparative HPLC (reversed phase). 1H - and ^{13}C -NMR spectra were recorded on a Bruker CXP 300 and AM 500 NMR spectrometer. Mass spectra were obtained on a Varian MAT 711 and a Finnigan 4500.

4. RESULTS

4.1. Isolation of strain

Soil and activated sludge from the area of Wuppertal, F.R.G., and water from the river Rhine were mixed with mineral medium containing 4-carboxy biphenyl ether (4CBPE), and percolated in a soil percolator system for several months. A mixed culture was isolated after this adaptation period which grew slowly with 4CBPE as main source of carbon and energy (0.1% yeast extract had to be added for supporting growth). This mixed culture was reinoculated into a percolator filled with fresh soil, and percolated for another 4

months. Two fast-growing strains could be isolated from the culture fluid after this procedure. One, strain POB 310, is investigated further in this paper.

4.2. Characterization of strain POB 310

Cells of strain POB 310 were short, mobile rods. They were oxidase and catalase positive, Gram negative and showed lysis after treatment with 3% KOH. A preliminary test (API 20 NE) tentatively identified this strain as a member of the genus *Pseudomonas*. It utilizes 3- and 4-carboxy biphenyl ether as sole source of carbon and energy (generation time ca. 3 h with 4CBPE).

4.3. Growth with 4-carboxy-4'-fluorobiphenyl ether

Cells of strain POB 310 were grown with 4CBPE (5 mM). When the substrate concentration had decreased below 0.5 mM, 4-carboxy-4'-fluorobiphenyl ether (2 mM) was added, and its turnover was monitored by HPLC. After total conversion, 4-fluorophenol was detected in the culture fluid in almost stoichiometric amount. During growth with 4CBPE, a transient accumulation of phenol was observed, though never exceeding 0.2 mM.

4.4. Degradation of phenol

4CBPE-grown cells of strain POB 310 showed a considerable oxygen uptake rate for phenol, moderate rates for cresols, and a very low rate for 4-fluorophenol, but a high rate for catechol and 4-methylcatechol. In crude extracts, the key enzymes of the meta cleavage pathway, 2-hydroxy-6-oxo-hepta-2,4-dienoate hydrolase and 2-hydroxy-muconic-semialdehyde dehydrogenase [15] were induced at high level (640 and 270 U/g protein in POB 310).

4.5. Degradation of protocatechuate

After growth with 4CBPE, both whole cells and crude extracts of strain POB 310 transformed protocatechuate at high rate. From 2-fluoro-4-carboxybiphenyl ether, 5-fluoroprotocatechuate was accumulated by resting cells, once more demonstrating protocatechuate as the pivot in CBPE degradation.

4.6. Cooxidation of substrate analogues

4-Carboxybiphenyl was transformed by 4CBPE grown cells into three products. One of these was characterized as 4-carboxy-2-hydroxybiphenyl (detailed MS and NMR data will be published elsewhere). 4-Carboxybenzophenone (4CBzP) was transformed into a single, fairly stable metabolite which was purified by preparative HPLC. The DCI mass spectrum gave a quasi-molecular ion MH^+ at 261 Da, compatible with a molecular formula $C_{14}H_{12}O_5$. Facile loss of H_2O from the MH^+ -ion (rearomatization) indicates a cyclohexadienediol partial structure. This was confirmed by a detailed NMR analysis.

4.7. NMR analyses

The 1H -NMR (300 MHz) spectrum of the metabolite displays 4 multiplets, besides the typical benzoyl pattern, corresponding to one proton each (δ 5.16, 6.12, 6.66, 6.92 ppm). This indicates a cyclohexadienediol structure with a total of 4 substituents, with the structural alternatives A, B

or C (Fig. 1). The 2,3-dihydroxylated structure B is ruled out, a priori, by the pronounced shift difference between each of these four protons. Two clearly separated groups of two protons each are expected for such a pseudo-symmetrical structure, as exemplified by the NMR data for a structurally related metabolite, 2,3-dihydro-2,3-dihydroxy-4-trifluoromethylbenzoic acid [16].

The two central multiplets in the metabolite spectrum (6.12, 6.66 ppm) are linked by a 10 Hz coupling constant and thence must be assigned to the vicinal olefinic protons 5-H,6-H common to all three structural alternatives A–C. This value is in accord with the *cis*-olefinic coupling in cyclohexenes and 1,3-cyclohexadienes [17], though far too large for the 3J coupling across the C-2/C-3 single bond in structure B (ca. 5 Hz; [17]). The 2 ppm shift difference between the two remaining resonances is incompatible with the comparable chemical environment for 2-H,3-H in structure B. Rather, the 6.92 ppm multiplet is in the shift range expected for an olefinic proton in β -position to a

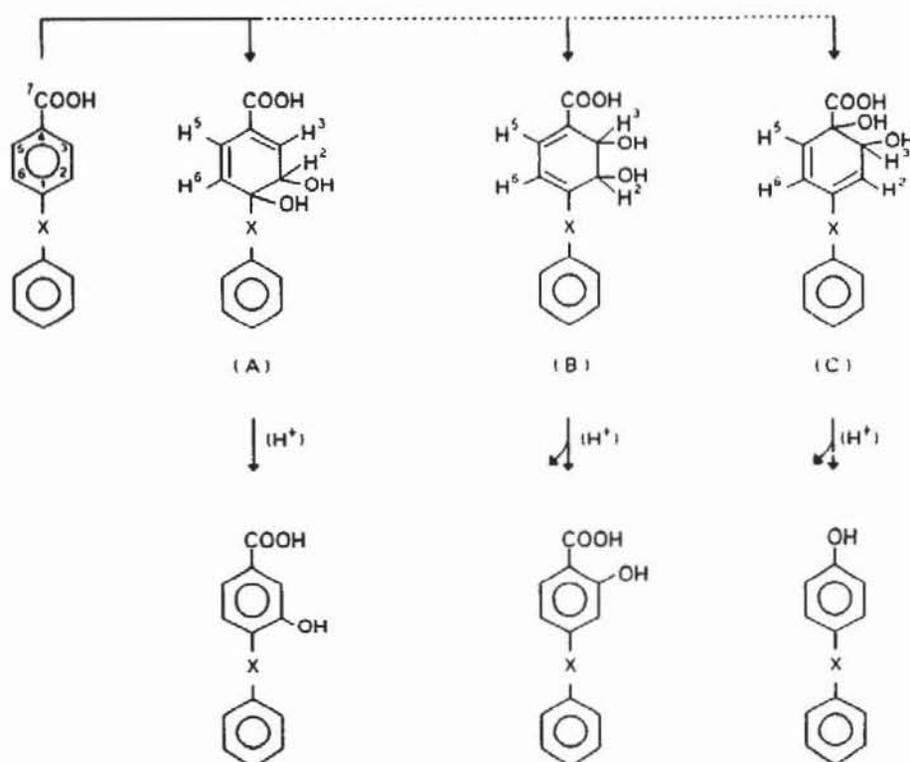


Fig. 1. 4-Carboxy-1,2-dihydro-1,2-dihydroxybenzophenone as product of the co-metabolism of 4-carboxybenzophenone by *Pseudomonas* sp. POB 310 (path A), together with the alternative metabolite structures for 2,3-dioxygenation (path B) and 3,4-dioxygenation (path C) (subsequent (chemical) rearomatization by treatment with acid).

carbonyl function, as in A or C. Also, the small 2.5 Hz coupling between this proton and the vicinical tertiary proton is in perfect accord with the close to 90° dihedral angle between an olefinic proton in a cyclohexadiene structure and a quasi-axial proton at an adjacent sp^3 carbon. All 4 protons appear coupled to each other; the respective long range coupling constants also being in agreement with both structures A and C.

Final assignment of structure A for the metabolite rests on a threefold, ^{13}C -NMR-derived argument. From turnover of 4-trifluoromethylbenzoic acid, 4-carboxy-1,2-dihydro-1,2-dihydroxy-1-trifluoromethylbenzene is isolated as a metabolite (Engesser et al., unpublished). This structure, analogous to A, is confirmed unequivocally by the $^2\text{J}(\text{C},\text{F})$ coupling of 26 Hz between the CF_3 fluorines and the quaternary sp^3 carbon (C-1). The carboxy carbon in this metabolite appears at 167.8 ppm, identical with the COOH carbon shift of the 4-carboxybenzophenone metabolite. Secondly, the $\text{C}=\text{O}$ carbon in structure C is bound to a phenyl and cyclohexadienyl moiety, i.e. its environment is more or less unchanged from benzophenone [$\delta(\text{C}=\text{O})$ 195.2 ppm]. The pronounced low field shift for the metabolite (δ 202.7 ppm) argues for interruption of the conjugation on one side and an additional β -effect, e.g. of a hydroxyl substituent at C-1. Finally, and conclusively, selective low-power decoupling of the methine proton at the tertiary hydroxyl function clearly establishes this proton to be coupled (by ≈ 5 Hz) to the 202 ppm, but not to the 167.8 ppm resonance. This proton thence is three bonds removed from the benzoyl

function and must be assigned to the 2-position. Taken together, these findings unequivocally establish structure A in Fig. 2 for the metabolite from 4CBzP.

5. DISCUSSION

Cleavage of the ether bond by oxidative mechanisms is well established for the degradation of dialkyl and alkyl aryl ethers [18–20]. Generally, attack at the alkyl moiety by *monooxygenases* generates chemically unstable hemiacetals which suffer spontaneous hydrolysis to the respective aldehydes and phenols. An alternative mechanism for alkyl aryl ether cleavage by *dioxygenation* has been proposed only recently [3,10].

Cleavage of biaryl ether bonds was reported for the degradation of xanthone [4] and biphenyl ether [7]. In both cases, the ether linkage is transformed into an ester function which is smoothly hydrolysed. A completely different mechanism, via 1,2-dioxygenation, has been advanced [3,8], but was experimentally established only quite recently [9]. According to this mechanism, dibenzofuran degradation is initiated by a dioxygenase attacking the angular position. The hemiacetal thus formed undergoes ring opening and rearomatization to 3-(2-hydroxyphenyl)-catechol [9]. A 1,2-dioxygenase likewise is operative in the degradation of 3- and 4-carboxybiphenyl ether, producing phenol and 4-carboxycatechol (protocatechuate) in the first enzymatically-triggered step. This was shown by co-metabolism of fluorinated analogues from

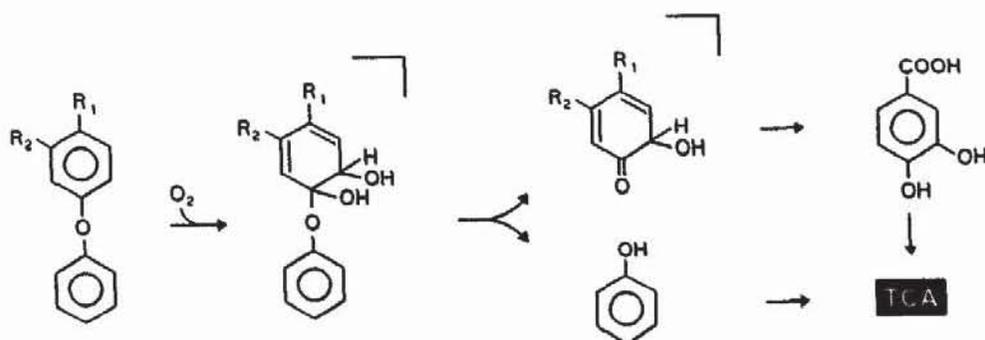


Fig. 2. Carboxy biphenylether degradation pathway in *Pseudomonas* sp. POB 310. $R_1=\text{COOH}$, $R_2=\text{H}$: 4-carboxybiphenylether; $R_1=\text{H}$, $R_2=\text{COOH}$: 3-carboxybiphenylether.

which 4-fluorophenol or 5-fluoroprotocatechuate, respectively, were accumulated in high yield.

Further proof of the 1,2-dioxygenation pathway comes from the isolation, and unequivocal structural characterization, of 4-carboxy-1,2-dihydro-1,2-dihydroxybenzophenone as a dead-end metabolite of 4-carboxybenzophenone. Initial 1,2-dioxygenation (path A, Fig. 1) likewise is indicated by exclusive excretion of 4-carboxy-2-hydroxybiphenyl after co-metabolism of 4-carboxy-biphenyl. In this case, immediate rearomatization by H₂O elimination [14] yields 2-hydroxy-4-carboxy biphenyl as *sole* product. Alternative 3,4-dioxygenation (path C) can be excluded since from the corresponding intermediate there is no way for chemical formation of 2-hydroxy-4-carboxybiphenyl. Furthermore, authentic 4-phenoxycatechol, the ring cleavage substrate in this pathway (C, Fig. 1) did not show substantial turnover with whole cells or crude extracts of 4CBPE grown cells of POB 310.

A 2,3-dioxygenation mechanism (B, Fig. 1) is also ruled out since, under identical conditions, biologically-synthesized 2,3-dihydroxy-4-carboxy-biphenyl ether (the ring cleavage substrate of pathway B) was not transformed substantially. Simultaneous adaptation experiments suggested only one enzyme to be involved in 4- and 3-CBPE metabolism. Therefore, the pathway shown in Fig. 2 is in agreement with all the data presented here. This stresses once more the importance of the novel mechanism for ether bond cleavage via initial dioxygenation. Further experiments will clarify the potential of CBPE-degrading strains to degrade related substrates, such as chloro- or nitro-substituted benzoates by the same mechanism.

NOTE ADDED IN PROOF

In the meantime this mode of initial cleavage of ether bonds has been proposed already for 4-

carboxybiphenylether degradation (R.M. Wittich et al. (1990) FEMS Microbiol. Lett. 67, 157-160). No detailed spectroscopic evidence for this mechanism, however, has been presented.

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