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Biotransformation of aromatic compounds

Monitoring fluorinated analogues by NMR

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The results presented here illustrate the power of NMR in the non-invasive analysis of microbial transformations. Whilst the definitive identification of products requires purification and full structural elucidation, NMR can provide rapid insights into the nature of these reactions and their regulation in vivo. In addition, once the products have been identified NMR methods allow rapid assessment of the effects of genetic and physiological manipulation, and on competing metabolic fluxes with mixed substrates and branched pathways.

^{19}F-NMR; Biotransformation; Chiral fluorinated compound; Fluorine metabolism; Difluorobenzoate metabolism

1. INTRODUCTION

The fates of fluoro analogues of metabolic intermediates are attracting considerable interest in view of their potential for probing enzyme mechanisms as well as their role as putative therapeutic agents [1-4]. In a recent review, Walsh [5] has surveyed the interactions of enzymes with a wide variety of fluorinated substrate analogues and has discussed how the fluorine atom ($r = 133$ pm) can replace either a proton (similar size, $r = 120$ pm) or a hydroxyl group (similar size, $r = 140$ pm, and electronegativity and hydrogen-bonding capacity). Our interest in the metabolism of fluoro-aromatic compounds arose from two research themes being actively pursued in this laboratory; on the one hand, we have a programme on the genetics, physiology and biochemistry of aromatic hydrocarbon microbiology, whilst on the other we are developing NMR methods to study microbial metabolism.

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The degradation of the aromatic ring by *Pseudomonas putida* proceeds via several possible pathways (fig.1). We are interested in the metabolite flux through the *ortho* and *meta* cleavage pathways (fig.1) and, in particular, the dioxygenation reaction that leads to *cis*-hydrodiols from several benzoates (step a). The dioxygenase that catalyses this reaction appears to be able to tolerate a range of substituents in the ring. Other bacterial strains are known to catabolize all three monofluorobenzoates and all four isomers of carboxyfluorohydrodiols have been isolated as metabolites [6-9]. We have now carried out a systematic study of the metabolism of all six isomers of the ring-substituted difluorobenzoates by mutants of *P. putida*. For this we have made use of the high NMR sensitivity of the ^{19}F nucleus to follow the fates of these compounds. A comprehensive account of the work will be published at a later date. We present here the results on the metabolism of 2,5- and 3,5-difluorobenzoate by *P. putida* PpJT103, a mutant unable to catalyse the rearomatisation reaction (step b in fig.1), which therefore accumulates the hydrodiol, to illustrate the power of the method.

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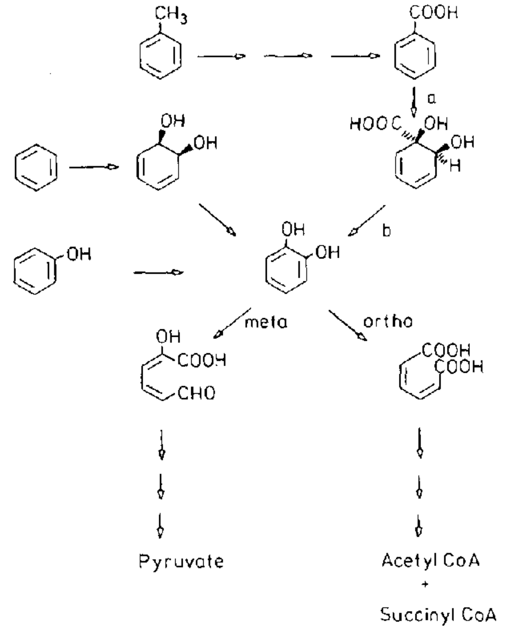

Fig.1. The branched pathways in *P. putida* for the oxidation of hydrocarbons, benzoates and phenols.

2. MATERIALS AND METHODS

The mutant PpJT103 was prepared as described [10] and grown overnight on a succinate/benzoate/salts medium. The cells were harvested by centrifugation, washed and resuspended in phosphate buffer (100 mM, pH 7.5), at 1/20 of the original culture volume. Substrate and succinate were added and samples taken over a period of several hours. After centrifugation the ^{19}F NMR spectra were recorded.

^{19}F NMR spectra were obtained on a Bruker AM 360 spectrometer with a 7.3 cm usable bore 8.5 T magnet. The ^{19}F frequency was 338.7 MHz and spectra were routinely ^{1}H-decoupled. Spectral accumulation parameters were: pulse width, 10 μs; pulse-to-pulse time, 2 s. All chemical shifts are referenced to external trifluoroacetic acid.

3. RESULTS AND DISCUSSION

We present here data on the metabolism of 2,5- and 3,5-difluorobenzoates. Fig.2 shows the time course for the conversion of 3,5-difluorobenzoate to new compounds. The initial spectrum shows a single resonance at −34.9 ppm as expected from the magnetic equivalence of the two fluorine atoms. Over a period of 160 min this resonance disappears and two major new resonances at −34 and −36.5 ppm appear along with a smaller resonance at −43.7 ppm (not shown). If this compound is oxygenated in the same way as benzoate, we would expect the product to be that shown in fig.3a. After work-up, conversion to the methyl ester with diazomethane, the mass spectrum (including accurate mass) and ^{1}H NMR were consistent with the proposed structure (fig.3a). In this molecule the fluorine atoms are no longer equivalent; analytical data are presented elsewhere [11].

The resonance at −43.7 ppm has the chemical shift expected for F^-, suggesting that the mutant is leaky, allowing a small proportion of the product to be further metabolised with cleavage of carbon–fluorine bonds. Monofluorobenzoates are known to release fluoride when they are degraded by *P. putida* [6-9].

In the case of the 2,5 isomer the reaction course appears to be considerably more complex as judged from the spectra shown in fig.4. The starting material has 2 resonances (−43.8 and −46.3 ppm) from the non-equivalent fluorines and over a period of 2 h these are lost and a new intense resonance at −43.7 ppm as well as 2 smaller resonances at −47.9 and −50 ppm are observed. The major resonance at −43.7 can again be assigned to F^-, although in this case a large proportion (about 85%) of the fluorine in the starting material has been lost. This is unlikely to be due to the 'leakiness' of the mutation since fluorines from both the 2- and 5-positions are mineralized. The minor product appears to be a non-metabolizable product retaining both fluorine atoms.

We explain these data by noting that there are two possible dioxygenation products depending upon whether the reaction occurs at a carbon–fluorine or carbon–hydrogen bond (fig.3b,c). If the oxygen atoms react as in fig.3b, then the resulting carboxyhydrodiol can rearomatise by decarboxylation and concomitant elimination of an F^-, to yield 4-fluorocatechol (fig.3d), thus chemically bypassing the genetic block of step b in fig.1. This reaction is formally analogous to that of the dehydrogenase that catalyses the rearomatisation of the hydrodiol derived from benzoate but, whereas the loss of hydride needs

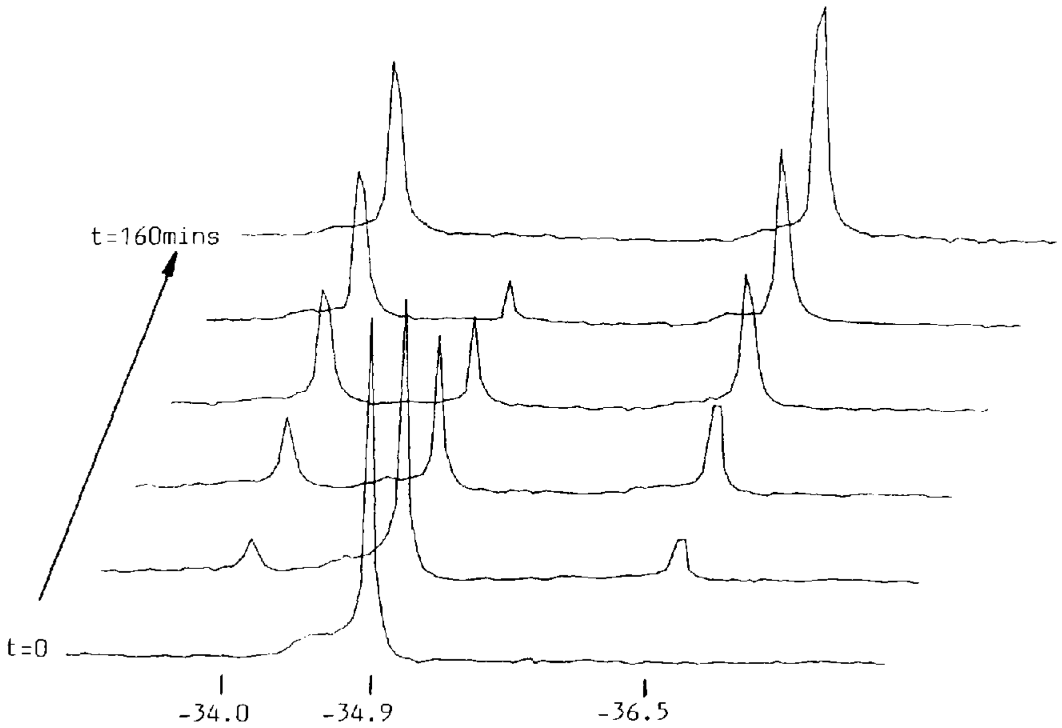

Fig.2. ^{19}F NMR spectra of cell-free supernatants from the microbial oxidation of 3,5-difluorobenzoate by *Pseudomonas* strain JT103 (chemical shifts in ppm).

both an acceptor (in this case NAD^+) and an enzyme, the loss of fluoride can occur spontaneously.

Although we have not quantified these spectra, it appears from the relative intensities of the starting material and products that the major hydrodiol is that which results from oxygenation at the carbon–fluorine bond (fig.3b). We cannot say as yet whether this is due to a preferential binding orientation at the enzyme active site or whether it is a consequence of the fluorine activating that position towards attack by the oxygenating species.

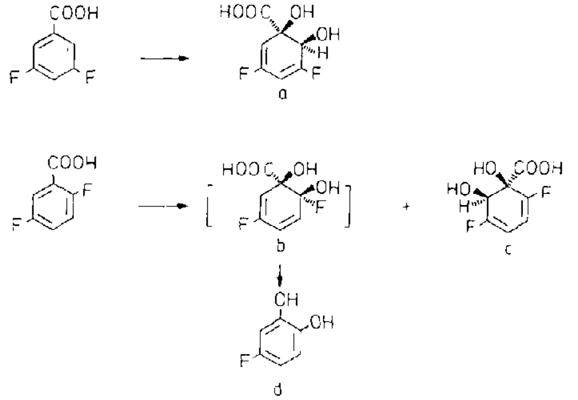

Fig.3. Proposed dioxygenation reactions for the oxidation of 3,5-difluorobenzoate and 2,5-difluorobenzoate by *P. putida* JT103.

One purpose of these experiments was to develop the practical requirements for monitoring in vivo aerobic catabolic processes by NMR spectroscopy. This was coupled with exploration of the substrate specificity and regio-selectivity of the benzoate 1,2-dioxygenase with a mutant of *P. putida* exposed to the six isomers of difluorobenzoate; none of these synthetic compounds has been the subject of previous metabolic studies. The amount of information gleaned from the 2,5-difluorobenzoate experiment described here is remarkable. Not only was it shown in a single experiment that 2,5-difluorobenzoate was a substrate with the formation of a small amount of a novel carboxydifluorohydrodiol product but also that most of the fluorine was eliminated from both

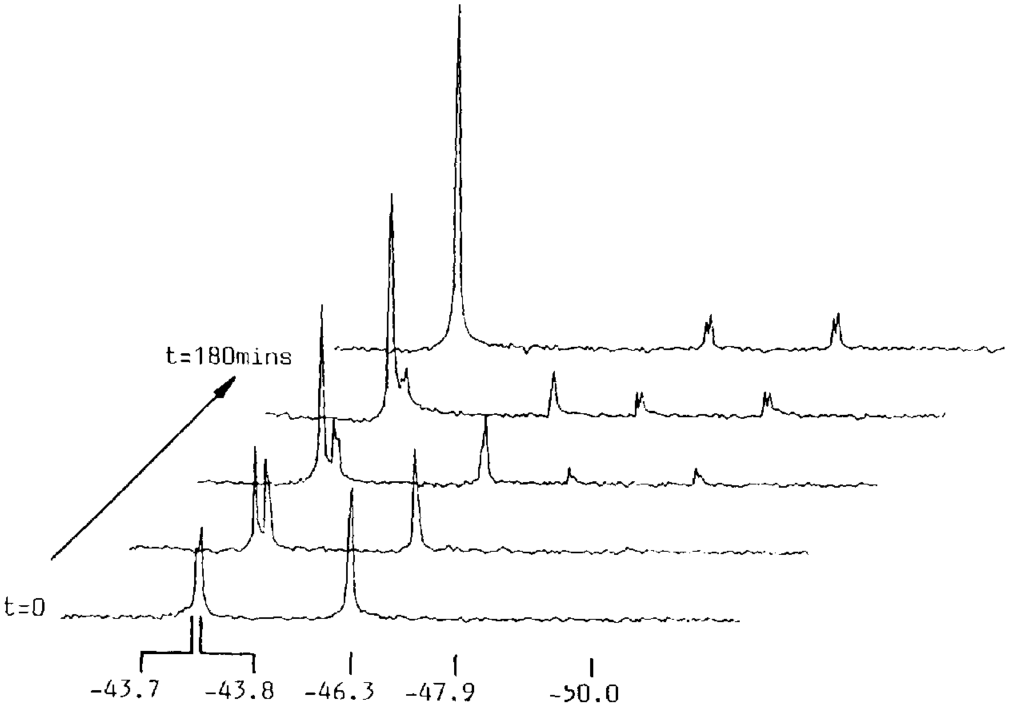

Fig.4. ^{19}F NMR spectra of cell-free supernatants from the microbial oxidation of 2,5-difluorobenzoate by *Pseudomonas* strain JT103 (chemical shifts in ppm).

positions as F$^-$. Clearly, the benzoate 1,2-dioxygenase of *P. putida* preferentially hydroxylates 2,5-difluorobenzoate in the 1,2- (not 1,6-) position allowing for spontaneous rearomatisation to 4-fluorocatechol with fluoride release from C-2, and overcoming the metabolic lesion. To account for the loss of fluorine from C-5, 4-fluorocatechol must be a substrate for basal levels of catechol 1,2-dioxygenase to give *cis,cis*-3-fluoromuconate which acts as: (i) a product inducer of the catechol 1,2-dioxygenase; (ii) a substrate inducer of the muconate cycloisomerase operon; (iii) a substrate for the cycloisomerase induced with almost exclusive regio-selectivity to form the putative 4-fluorobutenolide with spontaneous elimination of the second fluorine atom. The further metabolism of the carbon skeleton could not be ascertained from this experiment as only the fluorine nuclei had been monitored. The specificities of regulation by 3-fluoromuconate of the *ortho* cleavage pathway for catechol(s) in the mutant are deduced from the well-established regulatory roles of *cis,cis*-muconate as product inducer and substrate inducer of catechol 1,2-dioxygenase and *cis,cis*-muconate cycloisomerase, respectively, in *P. putida*.

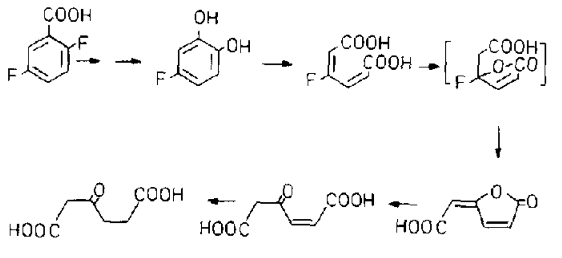

Fig.5. Proposed oxidation pathway of 2,5-fluorobenzoate by *P. putida* JT103 to account for the release of F$^-$ from both the 2- and 5-positions.

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