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On the Road to Bioremediation of "Dioxin"

Mohammadi and Sylvestre [1] report engineering of a dioxygenase to create an enzyme that attacks dibenzofuran in the lateral position. Subsequent oxidation and a second dioxygenation produced ring-open products. All metabolites were unambiguously identified by ^{1}H-NMR. This new pathway targets degradation of chlorinated dibenzofurans.

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (Figure 1) were contaminants of the defoliant Agent Orange, which was used in the Vietnam War from the early 1960s until 1971. Only after the accident in a chemical factory in the Italian city Sevoso on July 10, 1976, in which several kilograms of 2,3,7,8-tetrachorodibenzo-*p*-dioxin ("dioxin", Figure 1) were released into the environment did the public and the scientific community become aware of the extremely high toxicity of this class of chemicals. As a result of their hydrophobic and xenobiotic nature, polychlorinated organic compounds usually are very persistent in the environment. One promising mechanism for their detoxification is reductive dechlorination by anaerobic bacteria, a mechanism in which the perchlorinated substance acts as the terminal electron acceptor of a respiratory chain, a process called dehalorespiration [2, 3]. The resulting less-halogenated dibenzo-*p*-dioxins and dibenzofurans can then be attacked by aerobic bacteria, which initiate degradation by hydroxylation of the aromatic ring with molecular oxygen.

Several aerobic bacteria are known that grow on dibenzofuran and oxidize it completely via salicylic acid to carbon dioxide. In the primary hydroxylation step, two adjacent hydroxyl groups are added in the *angular* mode—i.e., one of the hydroxyl groups goes to the carbon, which already bears the bridging oxygen. Thus, carbons 4 and 4a or 5 and 5a are hydroxylated (numbering as for the chlorinated compounds in Figure 1). The hydroxylation at carbon 4a opens the ether linkage and the resulting 2,2′,3-trihydroxybiphenyl can be easily oxidized further. For the degradation of chlorinated dibenzofurans, however, it appears desirable to hydroxylate the aromatic ring in the *lateral* mode, e.g., at carbons 1 and 2, 2 and 3, or 3 and 4 (Figure 1), in order to obtain products that can be further degraded. Hydroxylation of dibenzofuran in this way would lead to three *cis*-dihydroxy isomers without opening the furan ring. Because no aerobic bacterium is yet known that uses the lateral mode for dibenzofuran hydroxylation or grows efficiently on chlorinated dibenzofuran, attempts have been made to engineer lateral-attacking enzymes from other sources by site-directed mutagenesis [4].

In order to obtain an enzyme working in the lateral mode, Mohammadi and Sylvestre chose biphenyl dioxygenase (BPDO) from *Burkholderia xenovorans* strain LB400 as a starting point for mutagenesis [1]. It comprises an iron-sulfur protein that catalyzes the addition of molecular oxygen, a flavoprotein reductase, and a ferredoxin that are involved in the transfer of electrons from NADH to the iron-sulfur protein [5]. Random mutagenesis of a stretch of seven amino acids in BPDO resulted in a variant (*p*4) with a slightly increased oxygenation rate of dibenzofuran as compared to the wild-type [4]. Because there was evidence that other residues contributed to the substrate specificity, this work [1] describes saturation mutagenesis that yielded, out of 60,000 transformants, one (RR41) that degraded dibenzofuran more efficiently. The His-tagged proteins were produced in *Escherichia coli*, and the cell-free extracts as well as the purified proteins were used in subsequent studies for the elucidation of the degradation pathway of dibenzofuran [1].

The results shown in Figure 2A of the paper by Mohammadi and Sylvestre [1] clearly demonstrate the large improvement obtained with the variants. The RR41 variant catalyzes oxygenation of dibenzofuran to *cis*-1,2-dihydro-1,2-dihydroxydibenzofuran about 3 times and 30 times better than the *p*4 and wild-type enzymes, respectively; similar results were obtained with the other product, the 3,4-diol, although only half of the amounts were generated. This important yield improvement paved the way to the unequivocal identification of the metabolites because enough material was now available for ^{1}H-NMR analysis. Whereas the structure of the stable 1,2-diol could be easily determined, the 3,4-diol eliminated the 4-hydroxyl group adjacent to the ether bridge, and this elimination resulted in 3-hydro-

2,3,7,8-Tetrachlorodibenzo[1,4]dioxin
"Dioxin"

2,3,6,7-Tetrachlorodibenzofuran

Figure 1. The Most Poisonous Perchlorinated Isomers of Dibenzo-*p*-Dioxin and Dibenzofuran

The article by Mohammadi and Sylvestre [1] describes the aerobic degradation of dibenzofuran, which carries only one or no chlorine substituents. These compounds are obtained by anaerobic dehalorespiration of tetrachlorodibenzofuran.

xydibenzofuran. Thus, the freshly prepared *cis*-3,4-dihydro-3,4-dihydroxydibenzofuran sample already contained the 3-hydroxydibenzofuran, but enough diol remained for solving its structure. The authors also noted that conversion of both diols to the trimethylsilyl (TMS) derivatives caused elimination to TMS-monohydroxydibenzofurans. Consequently, results from this otherwise powerful derivatization method have to be taken with caution.

Resting *E. coli* cells producing the RR41 variant also catalyzed the dioxygenation of 2-chlorodibenzofuran and 4-chlorodibenzofuran at roughly equal rates but about 4 times slower than unsubstituted dibenzofuran, whereas the wild-type enzyme was inactive with these chloro compounds. Interestingly, the 2-isomer was subject to *angular* oxidation leading to 2,2′,3-trihydroxy-6′-chlorobiphenyl, whereas the 4-isomer proceeded to the "normal" lateral path, probably to a mixture of *cis*-1,2- and *cis*-3,4-dihyrdrodihydroxy-5-chlorodibenzofurans. Thus, unexpectedly, the presence of a chlorine atom can change the enzyme's regiospecificity, reverting to the angular oxidation mode.

The availability of the more active RR41 variant enabled production of increased amounts of *cis*-diols for further metabolic studies. Both isomers, 1,2-dihydro-1,2-dihydroxydibenzofuran and 3,4-dihydro-3,4-dihydroxydibenzofuran, were oxidized by NAD^+ via rearomatization to 1,2-dihydroxydibenzofuran and 3,4-dihydroxydibenzofuran, respectively. Both reactions are catalyzed by a *B. xenovorans* enzyme called BphB, which was purified after production of the His-tagged version in *E. coli*. The subsequent ring opening by a second oxygenation is mediated by BphC from *B. xenovorans*. Whereas this enzyme takes only 1,2-dihydroxydibenzofuran as substrate, the ring opening of the 3,4-isomer is achieved by NahC from *Pseudomonas putida* G7.

In summary, the paper by Mohammadi and Sylvestre [1] provides a nice example of how to detour a catabolic pathway in order to avoid a cul-de-sac when chlorinated compounds are used. Surprisingly, it was shown that chlorination of the 2-position could revert the anticipated mechanistic change. With an eye to the future, the successful identification of the metabolites provides a firm basis for further studies.

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Selected Reading

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