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In conclusion, while nitrile hydratase exhibits enantioselectivity only to a limited number of substrates, amidase catalyzes highly enantioselective reactions of a wide range of amides.

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Introduction

Pseudomonas oleovorans alkane hydroxylase (EC 1.14.13.5) and xylene oxygenase (EC 1.14.13.-) from *P. putida* mt-2 are industrially relevant monooxygenases used for the production of optically active epoxides and (hetero)aromatic alcohols and acids [1]. Oxidation of aryl allyl ethers to (+)-aryl glycidyl ethers by alkane hydroxylase has thus provided a synthesis route towards the optically active β-blockers (–)-(*S*)-metoprolol and (–)-(*S*)-atenolol, developed by *Shell* and *Gist-Brocades* (Pat. EP 256586, US 49562843, and [2]). Heterocyclic aromatic acids produced by *Lonza* using these monooxygenases and subsequent enzymes (Pat. US 5242816, US 5236832, and [1]) are of use for the synthesis of pharmaceuticals such as the anti-hyperglycemia drug Glipizide.

The industrial application of these monooxygenases from *P. oleovorans* and *P. putida* mt-2 is limited to the use of the wild-type organisms, which fortuitously accumulate the desired products as non-metabolizable intermediates. Synthesis of 'up-stream' intermediates, such as heterocyclic aromatic alcohols, or of metabolizable intermediates (*e.g.* derivatives of benzyl alcohol or benzoic acid) is not feasible with these strains. Furthermore, the wild-type biocatalysts is grown on alkanes (*P. oleovorans*) or xylenes (*P. putida* mt-2), which are substrates that can compete with the desired starting compounds, thus reducing productivity.

We have constructed, by genetic engineering, biocatalysts that contain alkane hydroxylase or xylene oxygenase, regulated by inducers that do not cause competitive inhibition, that are devoid of undesired 'down-stream' metabolic activities. Furthermore, we have introduced *xyl*- and *alk*-based 'biotransformation cassettes' into the chromosome of *E. coli* and *Pseudomonas* strains in order to obtain

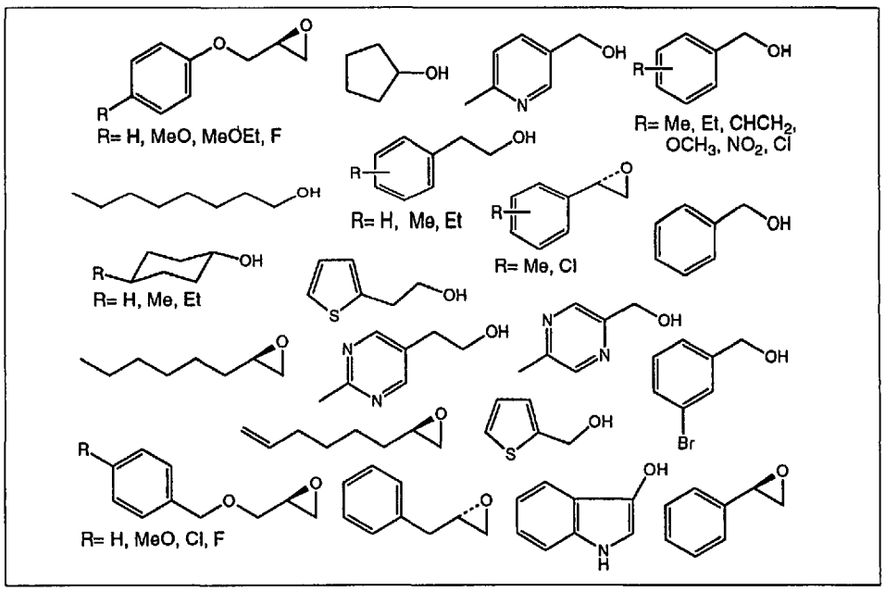

Figure. *Products of alkane-hydroxylase or xylene-oxygenase-mediated oxidation of aliphatic and (heterocyclic) aromatic compounds* [1][2][7–9]. Optically active epoxides without an assigned absolute configuration are represented with a dashed line. Substituents (R) that are accepted by the enzymes are indicated below each molecule.

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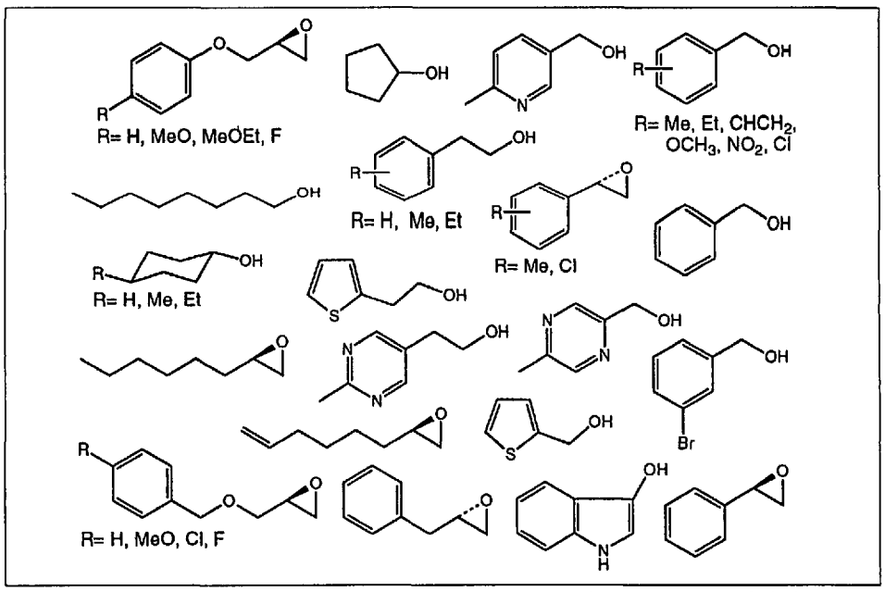

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production organisms that can be stably maintained in the absence of antibiotic selection using a mini-Tn*5* system developed by *de Lorenzo* and coworkers [3].

Construction of Specialized Biocatalysts

The OCT plasmid from *P. oleovorans* contains the genetic information for the degradation of alkanes. The molecular biology of alkane degradation by this organism has been evaluated in numerous studies (reviewed in [4]). *P. putida* mt-2 is capable of growth on xylenes by virtue of the TOL plasmid pWWO. As many as twenty structural and two regulatory genes have been reported to be involved in the metabolism of these aromatic hydrocarbons [5].

As a source of alkane hydroxylase, we made use of plasmid pGEc47 that contains all of the *alk* genes [6] and its derivative pGEc29, which is devoid of *alkJ* (alkanol dehydrogenase) [7]. Plasmid pBG63 (*xylMA*) was used to express xylene oxygenase in *E. coli* [8]. Using these plasmids, we have constructed *E. coli* and *P. putida* PpS81 recombinants. These strains, the latter by virtue of a chromosomal alcohol dehydrogenase mutation (*alcA81*), are not able to consume alkanols or substituted benzyl alcohols, which makes them suitable hosts to harbour the genes for alkane hydroxylase and xylene oxygenase.

Substrate Range of Alkane Hydroxylase and Xylene Oxygenase

In the absence of product consumption, we have been able to assess the substrate range of both alkane hydroxylase and xylene oxygenase using the above-mentioned recombinant strains [9]. Both enzymes catalyze the oxidation of terminal Me groups to the corresponding alcohols and also epoxidize terminal double bonds (see *Fig.*). Alkane hydroxylase has a broad substrate spectrum and substrates ranging from *n*-alkanes, branched alkanes, cyclic alkanes, and alkyl-substituted aromatic hydrocarbons can be oxidized stereo- and/or regioselectively. The substrate range of xylene oxygenase is limited to aromatic hydrocarbons with a Me side group, with indole (oxidized to indoxyl) and styrenes (oxidized to styrene oxides) as exceptions. The enzyme is very tolerant towards additional *meta*- and *para*-substituents at the aromatic ring of toluene derivatives, but does not allow any substituents in *ortho*-position relative to the Me group. Both alkane hydroxylase and xylene monooxygenase have been reported to oxidize heterocyclic aromatic hydrocarbons carrying Et or Me substituents, respectively, to heterocyclic alcohols, which are further metabolized to the corresponding acids [1].

The oxidation of vinylic end groups by both alkane hydroxylase and xylene oxygenase produces optically active epoxides, which are useful synthons in enantiospecific chemical synthesis. Alkane hydroxylase has the wider substrate range. Alkenes and alkadienes, but also allylbenzenes and aryl phenyl ethers can be oxidized to the corresponding optically active epoxides. Xylene monooxygenase does not oxidize alkenes or allylbenzene, but its activity is restricted to *meta*- and *para*-substituted styrenes, which are oxidized to the corresponding epoxides [8].

Application of Engineered Biocatalysts in Two-liquid-Phase Media

The substrates of alkane hydroxylase and xylene monooxygenase are typically water-insoluble and many of the substrates such as the lower alkanes, toluene, and styrene, but also products such as most of the epoxides, are toxic to the microorganisms.

We have used whole-cell biocatalysts of *Pseudomonas* and recombinant *E. coli* based on alkane hydroxylase as well as xylene monooxygenase in two-liquid-phase media with *n*-octane and higher *n*-alkanes as a semi-inert second liquid phase in addition to the aqueous phase. In such a system, product and substrate toxicity are diminished and product recovery is facilitated. The lab-scale production of epoxyalkanes, styrene oxides, octan-1-ol, and 1-octanoic acid has been realized in such two-liquid-phase systems in batch and continuous mode using specialized microorganisms [10].

Structure of Alkane Hydroxylase and Xylene Monooxygenases

The alkane hydroxylase system consists of the membrane monooxygenase AlkB, rubredoxin (AlkG), and rubredoxin reductase (AlkT), which channel electrons from NADH to the membrane monoxygenase. Xylene monooxygenase shows a similar architecture, although electron transfer takes place through only one protein (XylA). Genetic analyses demonstrated that AlkB and XylM are transmembrane polypeptides which span the membrane six times [11].

Both membrane monooxygenases require iron for activity and probably contain a heme-like Fe^{II} center or a di-iron-oxo center at the catalytic site [12]. We have obtained evidence by site-directed mutagenesis that the iron may well be bound to several histidine clusters (typically HxxxH) at the cytoplasmic membrane inner surface that occur at almost identical positions in both monooxygenases. Studying these enzymes in molecular detail may enable us to understand the mechanism of oxidation of non-heme iron monooxygenases and could provide and answer to the differences in substrate range of the enzymes.

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