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SUMMARY AND CONCLUDING REMARKS

Microbial sterol side chain cleavage

The selective microbial sterol side chain cleavage is an attractive way of converting cheap and abundantly available sterols into AD and ADD. These two compounds are excellent intermediates of high added value in the synthesis of nearly all steroid hormone drugs. Sterols of choice are the zoosterol cholesterol and phytosterols such as ß-sitosterol, campesterol and stigmasterol. However, the microbial side chain cleavage is characterized by some fundamental problems. Most organisms carry out side reactions such as the sterol nucleus degradation because they use sterols as a source of carbon. Therefore, there is a requirement for inhibitors of undesirable side reactions or for stable mutants lacking the genes responsible for such reactions. The majority of the side chain degrading microorganisms have low growth rates and a tendency to flocculate and float or adhere to vessel walls. Moreover, substrates and products are only very poorly soluble in aqueous media leading to hardly accessible substrates and to mixed crystal precipitation. Conversion in the presence of organic solvents which increase the reactant solubilities is often impossible as the microorganisms are not compatible with these reaction systems. As a consequence, the microbial sterol side chain cleavage often results in low conversion rates and poor yields.

In this thesis, I described the investigations into the conversion of cholesterol to AD(D) by *Mycobacterium* sp. NRRL-B 3683 side chain cleavage. This bacterium is a UV-mutagenized organism with reduced nucleus degradation activity. The aim of the project was the characterization and the improvement of the AD(D) production process by this strain in a broad sense. Therefore, the microbiology, biochemistry, genetics and the reactor performance of this process were studied.

Physiology and biochemistry

Mycobacterium sp. NRRL-B 3683 is a Gram positive rod shaped bacterium of ca. 0.7 x 1.7 µm. The bacterium is prototrophic, strictly aerobic and contains a yellow pigment. Cells grow mainly as free cells in liquid media. Optimum growth conditions in a 1 l fermentor include pH = 7, T = 32 °C and a stirrer speed of 200-300 rpm with a six bladed stirrer of 5.3 cm diameter. Taxonomically, *Mycobacterium* sp. NRRL-B 3683 resembled *M. phlei* and to a minor extent *M. smegmatis* and *M. vacaae*. However, no absolute correspondence with these genera was observed. *Mycobacterium* sp. NRRL-B 3683 showed remarkable metabolic versatility, being able to use many organic

Especially succinate was found to support rapid cell growth (t_d = 3.5-4.0 h) in the mineral E2-medium. Steroids did not support cell growth, with the exception of cholesterol which was metabolized only slowly.

Mycobacterium sp. NRRL-B 3683 was able to convert a variety of steroids to AD(D) with molar efficiencies ranging from 1 % with progesterone to 25-30 % with cholesterol or 47 % with testosterone as a substrate. Suitable substrates included various sterols, Δ^5- and Δ^4-cholestenone, lithocholate, Δ^5-cholenic acid, BNC and testosterone. A definite preference for the 3-hydroxy-Δ^5 structure and the relatively simply structured cholesterol side chain was observed. The steroid carboxylic acids were poorly converted whereas the C-21 steroids pregnenolone and progresterone were hardly degraded. The ratio of AD/ADD shifted towards more ADD at prolonged incubation times when cholesterol was converted.

The highest specific cholesterol side chain cleavage activity was observed during (early) exponential growth whereas the activity of the stationary phase cells was only 1-5 % of these values. In contrast, the steroid nucleus was mainly degraded by stationary phase cells. Consequently, a nearly threefold increase in AD(D) yield could be realized by keeping cells in their growth phase during the entire cholesterol fermentation by using a mixture of succinate and glucose as carbon sources. Cholesterol was needed to induce nucleus degradation; the presence of AD or ADD only did not result in any steroid losses. AD and ADD severely inhibited *Mycobacterium* cell growth rates while leaving the specific side chain cleavage activity unaffected.

Suppression of side reactions

Inhibitors of one of the nucleus degrading enzymes are widely applied in order to suppress the undesirable nucleus degradation. Attention has largely been focussed on the inhibition of the 9α-hydroxylase activity. This enzyme complex contains FeS clusters within an electron transport chain and is therefore susceptible to Fe^{2+} chelating agents, Fe^{2+} replacing metal ions, sulphydryl reagents and redox dyes. In contrast, we developed inhibitors of cholesterol oxidase, another enzyme involved in the steroid nucleus degradation. According to our interpretation of literature data and to some of our experiments, this reaction does not necessarily precede the process of sterol side chain cleavage. Several dimethylmorpholine fungicides were tested as microbial cholesterol oxidase inhibitors as we expected an analogy with the inhibition of sterol $\Delta^{8\rightarrow7}$-isomerase and C-14 reductase in higher plants and fungi by these compounds. We found that fenpropimorph, tridemorph and to a lesser extent fenpropidin were effective inhibitors of the isomerase activity of the cholesterol oxidases from *Nocardia erythropolis*, *Pseudomonas testosteroni*, *Streptomyes* sp. and *Schizophyllum commune in vitro*. These enzymes differed in reaction mechanism, steroid substrate specificity, cofactor required, optimal assay conditions and resistance to isopropanol, which was

used in enzyme assays to solubilize steroids and inhibitors. Inhibitor effectiveness with *N. erythropolis* cholesterol oxidase was greatly influenced by structural features. The most important of these included a tertiary nitrogen atom able to become a morpholinium ion and the presence of the two morpholine methyl groups, preferably in *cis*-configuration. Inhibition of cholesterol oxidase *in vivo* was also observed with *Arthrobacter simplex*, *Corynebacterium* sp. ATCC 31458, *N. erythropolis* and *N. restricta*, next to toxic side effects of the inhibitors on these strains. AD(D) accumulated maximally to 5 and 25 % molar conversion in experiments with *N. erythropolis* and *Mycobacterium* sp. NRRL-B 3683, respectively, suggesting that the cholesterol side chain cleavage itself was not inhibited by fenpropimorph or tridemorph. In addition, steroid nucleus degradation was completely inhibited. When no dimethylmorpholines were present, only trace amounts of AD(D) were formed in cholesterol fermentations by *N. erythropolis* and nucleus degradation caused severe steroid losses. In Gram negative strains, cell toxicity was absent, implying that application in steroid transformations by bacteria like engineered *Pseudomonas* strains might be feasible. A better separation of cell toxicity and cholesterol oxidase inhibition is expected to further increase the applicability of dimethylmorpholines in several other steroid transformations. Moreover, the resemblance between microbial cholesterol oxidase inhibition and the inhibition of sterol biosynthesis in higher plants and fungi was demonstrated. This might result in rapid and convenient fungicide screening systems for agronomic purposes when compared to the laborious and expensive *in vivo* test systems used at present.

Optimization of reactor performance

Steroids such as cholesterol have low solubilities and dissolution rates in aqueous media, resulting in low reactant transport rates to and from cells and in suboptimal biocatalyst performance. As the use of organic solvents was found to be incompatible with cholesterol side chain cleavage by *Mycobacterium* sp. NRRL-B 3683, we investigated the effects of the addition of several detergents and clathrating agents to aqueous fermentation broths.

A 1.7 - 3.0 fold increase of the specific side chain cleavage activity was observed when cyclodextrins were used in the optimized 1 : 2 molar ratio of sterol : cyclodextrin. For cholesterol, addition of ß-cyclodextrin containing 6 glucose residues was found to be optimal for AD(D) production, whereas the 7 glucose units containing γ-cyclodextrin was the best clathrant for ß-sitosterol, campesterol and Δ^4-cholestenone. The enhancement of steroid solubilities and transport rates was thought to be fully responsible for the increase in AD(D) production as cell growth was not affected by cyclodextrins. Moreover, nucleus degradation was decreased, resulting in over 85 % molar conversion of sterols to AD(D) after 140 h of incubation, in contrast to 35 % molar conversion in the absence of cyclodextrins.

Lecithins were effective enhancers of sterol side chain degradation by *Mycobacterium*. Especially egg-yolk lecithin increased the apparent solubilities of cholesterol and sitosterol and their transport rates to the biocatalyst. Thus, a rapid degradation of the cholesterol side chain and the sterol nucleus by *Mycobacterium* were observed. This resulted in a 1.5 - 2.0 fold increase in AD(D) yields. Fatty acid analysis indicated that the combined presence of a saturated and a mono-unsaturated fatty acid, preferably consisting of 12 or more carbon atoms, in the single lecithin molecules was optimal for this purpose.

A comparison of the stimulating effects on AD(D) production of some lecithins and various synthetic detergents including several Tweens, Spans and Brijs indicated that the type of polar head group of these detergents was more important than the length of the fatty acyl side chains. However, a minimal length of 16 or 12 carbon atoms was essential for AD(D) yields over 70 % with synthetic detergents and lecithins, respectively.

Genetics

Although *Mycobacterium* sp. NRRL-B 3683 degrades the cholesterol side chain, this strain has some undesirable characteristics such as low growth rates, sensitivity to product inhibition and incompatibility with organic solvents. Therefore, the total DNA was cloned in the broad host range cosmid pLAFRI and transferred to *E. coli*. This resulted in a gene bank of over 32,000 recombinants. When the pLAFRI constructs were introduced in *P. testosteroni*, 22 recombinants out of 2000 were able to grow on cholesterol as the sole source of carbon. During cholesterol fermentation, 8 of these 22 *P. testosteroni* recombinants produced an unidentified product, most probably a hydroxylated cholestenone, and 3 strains showed rapid nucleus degradation. Many of the 22 recombinant plasmids had common *Mycobacterium* DNA inserts. A provisional map could be constructed of a 12.45 kb total common insert which presumably contained the genes responsible for growth on cholesterol, rapid product formation and nucleus degradation.

Concluding remarks

This thesis describes the results of physiological, biochemical, genetic and engineering investigations in the field of microbial sterol side chain cleavage. New insights were developed and progress was made in the characterization and handling of *Mycobacterium* sp. NRRL-B 3683 as well as in its application as a biocatalyst in detergent or clathrant containing aqueous media. This resulted in much faster conversion rates of cholesterol or sitosterol to AD(D) and in considerably higher yields of this product. A novel class of inhibitors of the cholesterol oxidase activities was developed and proved

to be effective with cholesterol oxidases from a variety of sources, both *in vitro* and *in vivo*. This finding has potential applications in steroid biotransformations and in agronomic fungicide research. Finally, genes encoding steroid transforming enzymes in *Mycobacterium* were cloned and successfully expressed in *Pseudomonas*. This development of genetically engineered reactor compatible strains seems to be the most promising option for further improvement of the microbial sterol side chain cleavage and of many other steroid biotransformations. From the industrial point of view, the use of stable mutants which catalyse no side reactions and are compatible with organic solvents is to be preferred over the use of enzyme inhibitors or biotransformations in detergent containing aqueous media. The use of suitable organic solvents which increase reactant solubilities further than the addition of detergents to aqueous reaction systems is likely to result in higher biotransformation rates. Moreover, purification of hydrophobic products from organic solvents is generally much easier than from aqueous emulsions. Several *Pseudomonas* strains including *P. oleovorans* are well known for their capacity to carry out biotransformations of hydrophobic compounds in reaction systems containing over 99 % organic solvent. Information on their genetics, metabolism, regulation and handling as biocatalysts is rapidly becoming available. Therefore, application of the information described in this thesis in combination with thus genetically engineered strains should be interesting and worth while, both from a scientific and an economic point of view.