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Synthesis and Metabolism of Drugs by Means of Enzyme-Catalysed Reactions

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Abstract. The usefulness of enzyme catalysed-reactions is exemplified by recent results from research at *Roche*.

Sequences of enzyme reactions, well organised in metabolic pathways of selected microorganisms, lead to secondary metabolites with innovative chemical structures. An example is the pancreas-lipase inhibitor lipstatin produced by *Streptomyces toxytricini*. Hydrogenation of lipstatin yields tetrahydrolipstatin, the active substance of the anti-obesity drug *Xenical*™. The biosynthetic pathway has been elucidated and an improved fermentation process for the production of lipstatin has been developed.

Intermediates of the primary metabolism can be valuable building blocks for the chemical synthesis of drugs. Examples are quinic acid and shikimic acid, which are both suitable starting materials for the synthesis of the neuraminidase inhibitor GS 4104. Metabolic engineering of *Escherichia coli* with the goal to overproduce these two substances is briefly described

Microorganisms or enzymes derived thereof are used in drug synthesis to catalyse single, highly specific reaction steps (biotransformations). Three examples yielding chiral precursors of a protein-kinase inhibitor, a collagenase inhibitor, and an antifungal compound are discussed

Recombinant *Escherichia coli* strains expressing human drug-metabolising enzymes are suited to mimic drug metabolism and to produce intermediates of human drug metabolism. The desired hydroxylated drug derivatives could be obtained after incubation of drug substances with strains coexpressing one specific human cytochrome P450 isozyme together with human reductase.

1. Introduction

Microorganisms with their great variety of highly specific enzymes have proven to be useful catalysts in medicinal chemistry:

- Sequences of enzyme reactions, well organised in metabolic pathways, lead to a variety of secondary metabolites with innovative chemical structures. Many such secondary metabolites exhibit pharmaceutical activities, *e.g.* as antibiotics or enzyme inhibitors.
- Intermediates of the primary metabolism can be valuable building blocks for the chemical synthesis of drugs. Their overproduction in suitable microorganisms can be initiated by genetic engineering.
- Microorganisms or enzymes derived thereof are used in drug synthesis to catalyse single, highly specific reaction steps (biotransformations) difficult to perform by chemical methods.
- Recombinant microorganisms expressing human drug-metabolising enzymes are used to mimic drug metabolism and to produce intermediates of human liver drug metabolism.

Recent applications from the pharmaceutical research at *Hoffmann-La Roche* will be discussed.

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2. Lipstatin, the Precursor of *Xenical*™

Lipstatin is a potent, irreversible inhibitor of pancreas lipase, the key enzyme in dietary fat absorption. It has been detected in a screening for lipase inhibition in broths from soil microorganisms. Lipstatin is produced by *Streptomyces toxytricini* [1][2]. The molecule consists of a hydrocarbon backbone bearing two double bonds, a β-lactone structure essential for the biological activity and a hydroxy group substituted with *N*-formyl-L-leucine (*Fig.*).

Hydrogenation of lipstatin yields tetrahydrolipstatin, the active substance of the anti-obesity drug *Xenical*™. Tetrahydrolipstatin can be obtained by chemical synthesis [3] as well as by fermentative production of lipstatin followed by hydrogenation. However, original fermentation yields of lipstatin amounted only to a few milligrams per litre.

The biosynthesis of lipstatin was investigated in *Streptomyces toxytricini* by trace studies with crude (U-^{13}C)-lipid mixtures obtained from algal biomass cultured with $^{13}CO_2$. The data showed that the carbon backbone of lipstatin is built up by *Claisen* condensation of a C_{14}- and an C_8 moiety before the L-leucine residue is added [4].

The fermentation process for lipstatin could be significantly improved by establishing a fed-batch process with feeding of linoleic acid (which is readily degraded by twofold β-oxidation into the required C_{14} unit), caprylic acid (C_8 precursor) and L-leucine [5]. Feeding of these precursors to a typical run with 8 l of starting volume in a 14 l reactor was initiated after a growth phase of 47 h. In order to avoid inhibitory effects, fatty acids were added in a way that the concentration of each remained below 70 mg/l. Lipstatin concentration in

such a fed-batch experiment was 150 mg/l after an incubation period of 138 hours [5]. Further strain improvement and process optimisation is in progress.

3. Quinic Acid and Shikimic Acid as Starting Materials for the Synthesis of the Neuraminidase Inhibitor GS 4104

Neuraminidase is an enzyme essential for the propagation of the influenza virus. The sialic-acid analogue substance GS 4104 (Ro 64-0796; *Tamiflu*™) is a potent inhibitor of neuraminidase and therefore a promising drug substance for the prevention and treatment of influenza [6]. It is under advanced development jointly by *Hoffmann-La Roche Ltd.* and *Gilead Sciences Inc.*

Starting materials for the synthesis of GS 4104 [6-8] are either quinic acid (QA) or shikimic acid (SA) (*Scheme 1*) which are both available by extraction from plant sources.

QA and SA are both intermediates in the common biosynthetic pathway leading to aromatic amino acids (*Scheme 2*). Therefore, it was obvious to consider microbial fermentation as a production method.

Recombinant *Escherichia coli* strains which overproduce QA or SA have been engineered by streamlining carbon flow into the respective metabolic pathway, by repeated cloning of the genes encoding for rate-limiting key enzymes as well as by knocking out the genes encoding the two isozymes of shikimate kinase [9]. The latter step prevents further processing of the metabolic intermediate SA (or its precursor QA) to shikimic acid-3-phosphate and further towards aromatic amino acids (*Scheme 2*). The overproduced intermediates, QA or SA, are excreted and the strains become auxotrophic for aromatic amino acids. Resulting strains produced in a fed-batch process 60 g/l of QA within 60 h (side product: 3-dehydro-QA) or 27.2 g/l of SA in 42 h (side products: QA and 3-dehydro-SA) [9]. Further strain and process development is in progress.

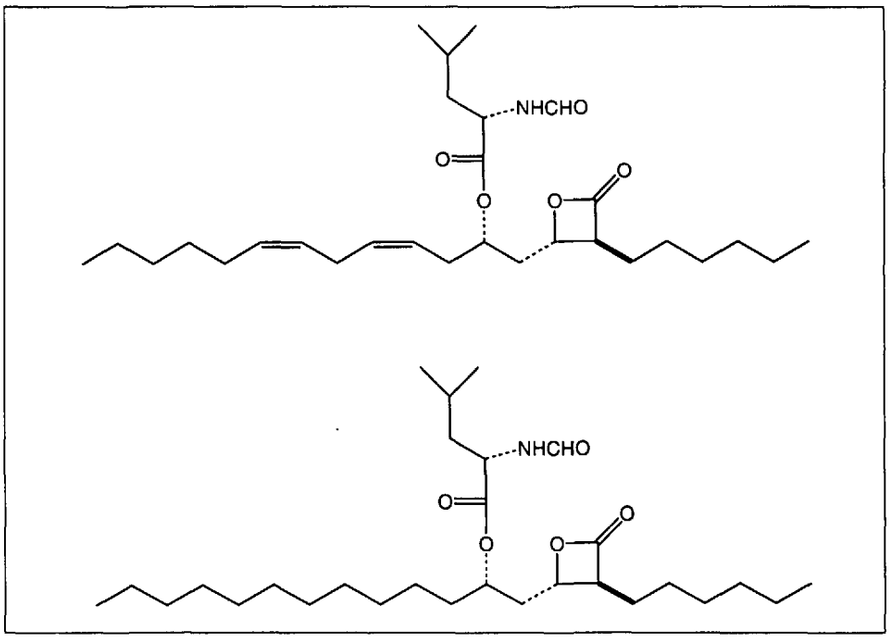
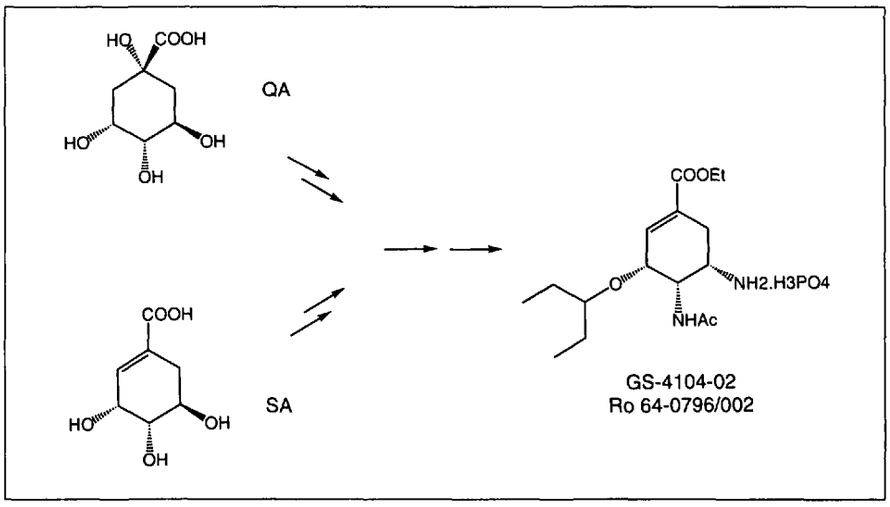


Figure. *Structures of the pancreas-lipase inhibitors lipstatin* (a fermentation product from *Streptomyces toxytricini*) *and tetrahydrolipstatin* (*Xenical*™)

Scheme 1. *(-)-Quinic Acid (QA) or (-)-Shikimic Acid (SA) as Precursors of the Neuraminidase Inhibitor GS-4104* (for details, see [6–8])



4. Biotransformations

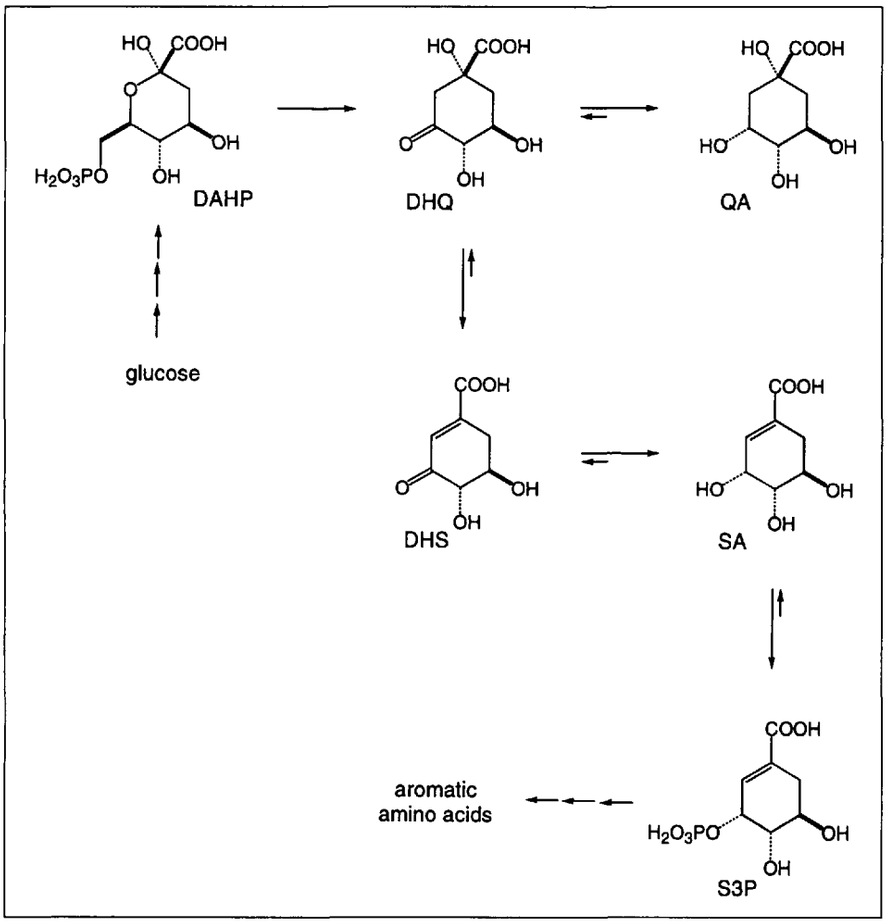
4.1. *Microbial Hydrogenation of Racemic β-Keto Ester* 1a *in the Synthesis of the Protein-Kinase Inhibitor Ro 43-2759*

A microbial hydrogenation with a useful combination of regio- and enantioselectivity was employed for the synthesis of a precursor of protein-kinase inhibitor Ro 43-2759. The synthetic pathway (designed by *Ulrich Zutter, Roche*, Basel [10]) is based on asymmetric microbial reduction of the β-keto ester **1a** to the respective *cis*-(3*R*,4*S*)-configured hydroxy ester **2** (*Scheme 3*). The theoretical yield is 100% because of *in situ* racemisation of substrate **1a**.

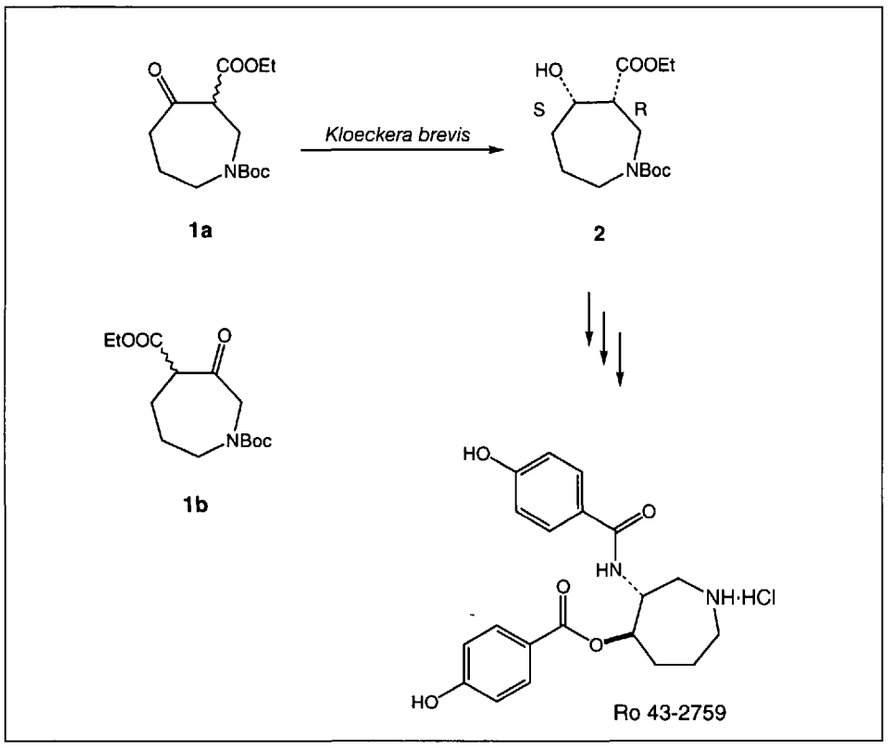
A microbial screening with various yeasts and fungi was carried out. Most of the strains tested afforded preferentially the *cis*-configured product. The best five strains generated both centres of asymmetry with excellent specificity. The enantiomeric excess as well as the *cis*/*trans*-purity was 97% or better. The best result was obtained with the yeast *Kloeckera brevis*, yielding the isolated product in 82% from regioisomerically pure substrate **1a**.

For economic reasons, the attempt was made to use directly the unpurified keto ester substrate containing **1a** and about 25% of the unwanted regioisomer **1b**. If the microorganism is able to reduce selectively the desired regioisomer **1a**, the remaining regioisomer **1b** can easily be removed by decarboxylation under alkaline conditions and subsequent extraction of the reaction product. *Kloeckera brevis* conveniently did exhibit this additional regioselectivity: only the desired isomer **1a** was reduced and afforded the *cis*-hydroxy ester in excellent steric purity. In a typical fermentation run, 29 g of **1** (3.5 g/

Scheme 2. *Metabolic Pathway Towards Aromatic Amino Acids.* DAHP: 3-Deoxy-D-arabino-heptulosonic acid-7-phosphate; DHQ: 3-Dehydro-quinic acid; DHS: 3-Dehydro-shikimic acid; S3P: Shikimic acid-3-phosphate



Scheme 3. *Microbial Hydrogenation of β-Keto Ester* **1a** *by* Kloeckera brevis. The chiral product **2** serves as a building block for the synthesis of the protein-kinase inhibitor Ro 43-2759.



l) were fed to a pre-grown stirred culture of *Kloeckera brevis* supplemented with 5% glucose. After 12 h of incubation at 27 °C, the culture broth was extracted with ethyl acetate. The yield of isolated product **2** was 80% (98% ee and 99% *cis*-purity). Thus, *Kloeckera brevis* exhibited excellent regio- and stereoselectivity in this hydrogenation reaction and afforded the desired product **2** in high chemical yield and steric purity.

4.2. *Enzymatic Resolution of Racemic Ester* 3 *Yielding a Chiral Precursor of the Collagenase Inhibitor Ro 32-3555*

Collagenase inhibitor Ro 32-3555 had to be prepared in larger amounts as a candidate for clinical trials. Among several synthetic approaches, the most advanced at that time proceeded *via* the benzyl-protected (*R*)-cyclopentyllactate (*R*)-**3**. Enzymatic kinetic resolution of racemic ester **3** with preferential hydrolysis of the (*S*)-ester was investigated as an approach to produce (*R*)-**3** (*Scheme 4*).

In a preliminary enzyme screening with cheap bulk enzymes, only subtilisin *Carlsberg* showed reasonable enantioselectivity (91% ee). The reproducibility of the reaction was, however, unsatisfactory and did not improve upon altering several chemical and physical parameters. Only the introduction of *tert*-butyl methyl ether as a biphasic co-solvent led to improved reproducibility as well as enantioselectivity (~96% ee at 53% conv.). Most interestingly, at higher substrate concentrations (20%), the enantioselectivity further increased, and the co-solvent could be omitted. The substrate was emulsified in aqueous buffer by vigorous stirring and the liquid commercial enzyme *Protease L660* was applied in 10% with respect to the substrate. The cheap bulk enzyme was discarded after use. Product (*R*)-**3** was obtained in 44% yield (>98% ee at 53% conv.) after extraction and subsequent distillation to remove formed benzylic alcohol. The reaction was – with some modifications - successfully carried out also on the multi-kg scale, and a total of more than 100 kg of (*R*)-**3** have been produced.

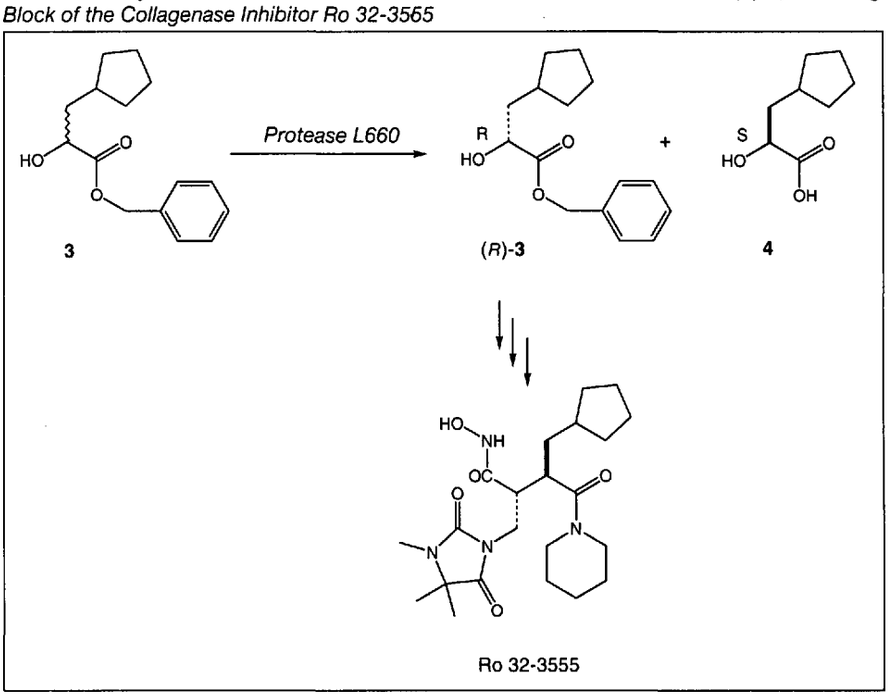
4.3. *Enzymatic Resolution of Racemic Ester* 5 *Yielding a Chiral Precursor of the Antifungal Compound Ro 09-3355*

(*R*)-Configured 2,4-difluorophenyl α-hydroxyethyl ketone **6** is a key intermediate in a synthetic pathway (designed by *Milan Soukup*, *Roche*, Basel) of the systemic antifungal Ro 09-3355. Two ap-

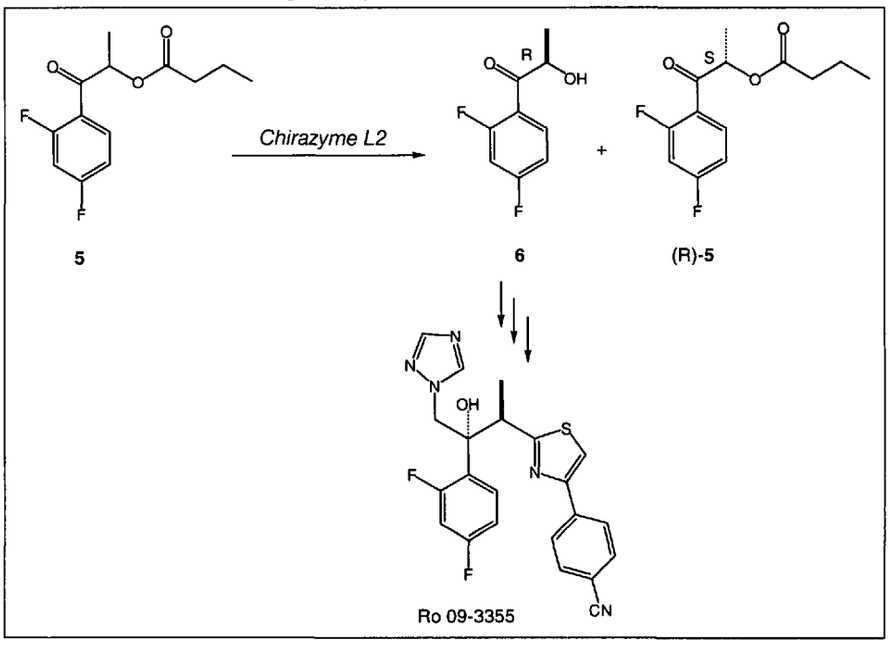
proaches were examined for its preparation (*Scheme 5*): enantioselective acylation of racemic 2,4-difluorophenyl α-hydroxyethyl ketone and enantioselective hydrolysis of its acylate. The first approach led to the (*R*)-acetate in excellent enantiomeric excess, however, since subsequent hydrolysis led to isomerisation, we switched to the alternative hydrolysis approach affording **6** directly (*Scheme 5*).

As hydrolysis experiments in monophasic organic systems (as well as alcoholysis experiments in various organic solvents) gave only poor results, biphasic hydrolysis was chosen. Butyrate **5** was selected as the substrate due to its superior discrimination (98% ee for **6**) as compared to the corresponding acetate (70% ee). In addition, the butyrate strongly facilitated work-up by allowing product separation by means of distillation. Hydrolysis with *Candida antarctica* lipase solution (*Chirazyme L2*, 15% with respect to the substrate) in a biphasic substrate/buffer system proceeded smoothly. At pH 8.5, a substrate concentration around 20% could be employed. The procedure was successfully carried out on the multi-kg scale: **5** was converted to give **6** in 42–44% yield after extraction and distillation. The procedure yielded 46 kg of enantiomerically highly pure product **6**.

Scheme 4. *Enzymatic Resolution of Racemic* **3** *with* Protease L660 *Yields Chiral (R)-***3**, *a Building Block of the Collagenase Inhibitor Ro 32-3565*



Scheme 5. *Enzymatic Resolution of Racemate* **5** *by* Chirazyme L2 *Yielding Optically Active* **6**, *a Chiral Precursor of the Antifungal Compound Ro 09-3355*



5. Recombinant *Escherichia coli* strains Expressing Human Cytochrome P450 Isozymes as Biocatalysts for the Preparation of Drug Metabolites

Drug metabolism in the liver is mainly dependent on cytochrome P450 monooxygenases. Thus, human liver microsome preparations are widely used to establish metabolic routes of drug candidates.

Larger amounts of drug metabolites are often required in order to assess their chemical structure and to evaluate activity/toxicity patterns or possible interferences with other drugs. In many cases, chemical syntheses of such drug metabolites are difficult and time consuming, and, therefore, a biotransformation approach is highly desirable. However, the availability of human liver preparations with cytochrome P450 activities is very limited. This dilemma has been overcome by using recombinant microorganisms expressing human cytochrome P450 isozymes [11][12].

A series of recombinant *E. coli* strains – each of them co-expressing one specific human cytochrome P450 isozyme as well as human reductase – has been obtained from the University of Dundee, Scotland. Fermentations with these strains have been carried out on the 10-l and 100-l scale. They yield between 3 and 18 g/l of wet cells. The amount of expressed cytochrome and reductase varies greatly within the diverse constructs: 30–1000 nmol for cytochromes and 2–750 nmol for the reductase.

Intact biomass (growing or resting cells in the broth or isolated wet cells), spheroplasts after lysozyme treatment, as well as partly or totally disrupted cell preparations have been evaluated for their biocatalytic potential and successfully used for the production of required human drug metabolites on the 10–100-mg scale. If necessary, a scaling up is possible.

In several examples with model compounds (midazolam, tacrine, diclofenac, dextromethorphane) and with drug candidates actually under development at *Roche*, the desired drug derivatives (*e.g.*, hydroxylation products) could readily be obtained in reasonable yield after biotransformation with wet cells from recombinant

Escherichia coli expressing the appropriate cytochrome P450 isozyme. The results show the usefulness of this technology for the production of human drug metabolites.

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[1] E.K. Weibel, P. Hadvary, E. Hochuli, E. Kupfer, H. Lengsfeld, *J. Antibiotics* **1987**, *40*, 1081.
[2] E. Hochuli, E. Kupfer, R. Maurer, W. Meister, Y. Mercadal, K. Schmidt, *J. Antibiotics* **1987**, *40*, 1086.
[3] P. Barbier, F. Schneider, *Helv. Chim. Acta* **1987**, *70*, 196.
[4] W. Eisenreich, E. Kupfer, W. Weber, A. Bacher, *J. Biol. Chem.* **1997**, *272*, 867.
[5] A. Bacher, P. Stohler, W. Weber, *Europ. Patentanmeldung* **1997**, 803 567.
[6] C.U. Kim, W. Lew, M.A. Williams, H. Liu, L. Zhang, S. Swaminathan, N. Bischofberger, M.S. Chen, D.B. Mendel, C.Y. Tai, W.G. Laver, R.C. Stevens, *J. Am. Chem. Soc.* **1997**, *119*, 681.
[7] J.C. Rohloff, K.M. Kent, M.J. Postich, M.W. Becker, H.H. Chapman, D.E. Kelly, W. Lew, M.S. Louie, L.R. McGee, E.J. Prisbe, L.M. Schultze, R.H. Yu, L. Zhang, *J. Org. Chem.* **1998**, *63*, 4545.
[8] M. Federspiel, R. Fischer, M. Hennig, H.J. Mair, T. Oberhauser, G. Rimmler, T. Albinez, J. Bruhin, H. Estermann, C.Gandert, V. Göckel, S. Götzö, U. Hoffmann, G. Huber, G. Janatsch, S. Lauper, O. Röckel-Stäbler, R. Tussardi, A.G. Zwahlen, *Org. Process Research & Development* **1999**, *3*, 266.
[9] K.M. Drahts, D.R. Knop, J.W. Frost, *J. Am. Chem. Soc.* **1999**, *121*, 1603.
[10] U. Zutter, P. Matzinger, M. Scalone, *Euop. Pat. Appl.* **1996**, No. 96 105998.7.
[11] G. Urban, R. Gasser, M. Pritchard, M. Bandera, T. Friedberg, *Exp. Toxic. Pathol.* **1996**, *48*, Suppl. II, 409.
[12] J.A.R. Blake, M. Pritchard, S. Ding, G.C.M. Smith, B. Burchell, C.R. Wolf, T. Friedberg, *FEBS Lett.* **1999**, *397*, 210.

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Sperm Cells as DNA Vectors for the Preparation of Transgenic Animals

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Abstract. Sperm-mediated DNA transfer strategy which has been used for the preparation of genetically modified, transgenic organisms is discussed, and sperm-mediated transgenesis of *Xenopus laevis* frogs with retroviral *Rous* sarcoma virus DNA is summarized.

1. Introduction

The process of transformation, both natural and artificial, continues to play an important role in molecular biology. It is generally accepted that natural genetic competence, the ability of cells to bind to and to take up exogenous DNA, is widespread among bacteria and might be an important mechanism for the horizontal transfer of genes. In recent years, an exciting progress has been made in characterizing the pathways and signals that regulate the development of bacterial competence as an active process [1]. Observations that DNA, RNA, and oligonucleotides may also naturally associate with the surface of eukaryotic cells and become internalized were much less frequently reported in the past, and mechanisms of the development of natural eukaryotic competence have been only rarely studied and are poorly understood until now. Nevertheless, transfection of eukaryotic cells *via* an artificially induced competence (*e.g.*, by chemical or electrical treatment) is a widely used experimental procedure in laboratories of molecular and cellular biology.

Transgenic animal and plant technologies represent some of the most powerful tools in functional studies of various genes and serve for the preparation of organisms with new properties. Furthermore, because of many potential benefits, the research into gene transfer has become a very rapidly developing field with a special focus on *1*) human gene therapy protocols and *2*) production of biologically important macromolecules by transgenic animals. There are still many problems in preparing a 'perfect' transgenic organism, and today's transgenic technology is far from being satisfactory. One of the crucial steps is to find a simple way to efficiently introduce exogenous DNA into one-cell embryos. Furthermore, it is difficult to ensure integration of the DNA into the host DNA in all cells of the transgenic organism or only in cells of some 'target' tissues. Finally, the controlled expression of exogenous DNA in the whole organism or only in target tissues is the last essential step. DNA Microinjection into fertilized egg cells has been so far the most popular and widely used method for the production of transgenic animals. However, it requires costly and sophisticated equipment and considerable skills in micromanipulation, while the efficiency of the method is rather low. It does not exceed more than 4–5% in mice, the most successfully used organisms, and it is much lower with other species such as livestock or marine animals. Moreover, microinjection is always a non-physiological process, which may damage the cell. On the other hand, if the DNA uptake by spermatozoa developed into a well-controllable and defined process, these cells might become the most potent and, at the same time, the most natural tool for the production of transgenic animals. Compared to microinjection, a

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