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Biocatalysis in the Fine Chemical and Pharmaceutical Industries

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1.1 Introduction

There are few areas of science where recent technological advances have had as great an impact as that in the area of biocatalysis and biotransformations. Arguably, in most synthetic laboratories, the biocatalysis vision of just 20 years ago extended no further than the use of a few simple hydrolases for esterification or hydrolysis to facilitate resolutions. There were certainly research groups around the world who were far more involved in this emerging science, using a much greater array of biocatalytic systems, but real industrial uptake of the work was often hindered by a single, recurring problem – availability of the enzyme(s).

More enzymes become available on a daily basis, available in greater quantities and with greater diversity than ever before. But what is the reason for this relatively recent change? The answer lies not only in consumer/scientific desire for new biocatalysts but in the advancement of three essential areas of science: bioinformatics, gene synthesis and enzyme evolution.

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Table 1.1.1 *Biocatalysis alignment with green chemistry.*

Green chemistry principle	Biocatalysis
1. Prevention (of waste)	Biocatalysis can enable new, more sustainable routes to APIs effectively reducing level of waste.
2. Atom economy	Biocatalysis often enables more efficient synthetic routes.
3. Less hazardous (less toxic reagents and intermediates) chemical syntheses	Generally low toxicity.
4. Designing safer (less toxic) chemicals	No impact.
5. Safer solvents and auxiliaries	Often performed in water; when solvents are used they are generally Class I or II.
6. Design for energy efficiency	Usually performed slightly above room temperature.
7. Use of renewable feedstocks	Biocatalysts are renewable.
8. Reduce derivatives (e.g., protecting groups)	Chemo-, regio-, enantio-selective nature of enzymes often obviates need for protecting groups.
9. Catalysis (preferred over stoichiometric reagents)	Catalytic.
10. Design for degradation (avoid environmental build-up)	No impact on design of products (although biocatalysts themselves are degradable in the environment).
11. Real-time analysis for pollution (and hazard) prevention	No impact.
12. Inherently safer chemistry for accident prevention	Biocatalysis is generally performed under mild conditions where risk of explosions/ run-away reactions is minimal.

Much of the drive towards biocatalysis is arising from the increasing awareness that our world's resources are finite and there is a need to husband these resources. The rise in interest of biotechnology in the last decade has, in many respects, progressed with clear strategic alignment to sustainability. Many biocatalytic processes are highly aligned with Anastas and Warners[1] enunciation of the twelve principles of green chemistry (Table 1.1.1).

A recent business report put the industrial enzyme market at ca. $3.3 billion with a prediction to grow at the rate of ca. 6% per annum ($4.4 billion in 2015).[2] While these figures clearly indicate an expectation of greater biocatalysis uptake across different business sectors they do not illustrate the shear number of new biocatalysts that are emerging and do not cover the increasing number of whole cell processes that are under investigation.

The use of lipases, esterases and proteases is now widely established throughout the chemical industry with alcohol dehydrogenases (ketoreductases) starting to become increasingly recognized as the pre-eminent method of choice for asymmetric ketone reduction to chiral alcohols. Other enzyme types are starting to become more familiar as they become commercially available; nitrilases, transaminases, enoate reductases, P450 monooxygenases, monoamine oxidases and carboxylic acid reductases to name a few. The practical methods sections of this book and the first volume of "Practical Methods for Biocatalysis and Biotransformations" provide excellent examples of how these different enzyme types can be employed.

One particular area that has seen considerable growth is that associated with P450 oxidations. Remote hydroxylations of a desired molecule can be difficult to achieve using 'traditional' chemical methods, often requiring an entirely new route to provide the desired molecules. This is particularly true for those molecules that need to be synthesized as a consequence of being first-pass metabolites. However, hydroxylation is a common consequence of cytochrome P450 catalyzed metabolism, and so there has been a growing interest and demand for P450s that can be used as scientific tools (catalysts). Recombinant versions of these enzymes (particularly when made self-sufficient by fusion to a reductase domain) is a growing research topic, and enzyme kits to allow rapid evaluation are now readily available.

Many of the enzymes which are starting to become popular research tools are often best applied in a host cell. These whole-cell approaches are increasingly being utilized as any co-factors which are required, e.g., ATP (adenosine triphosphate) or SAM (S-adenosyl-methionine) are already prepared within the cell as part of its normal operation. This makes whole cell approaches highly competitive from a cost perspective. The two key arguments against the use of whole cells are that the processes are generally dilute (low throughput) and can result in poor purity profiles due to the potentially large number of by-products and impurities that can arise. However, modern molecular biology allows the scientist to overexpress the desired enzymes to such a degree that the desired transformations are often very clean and although the processes are generally more dilute, the waste itself is typically an aqueous solution which can be easily and cheaply treated before disposal.

Handling the aqueous waste is often sited as a concern with biocatalytic approaches and waste treatment of aqueous waste prior to disposal is clearly essential. In some instances incineration is seen as expedient and this entails significant energy consumption – as higher aqueous volumes are often used in biocatalytic approaches the energy consumption, and carbon footprint, is likewise increased. Downstream processing often involves extraction of products from the aqueous stream using organic solvents. At this point the processing and issues of using organic solvents are similar to those encountered in 'chemical' approaches. Where infrastructure is in place for solvent recovery this can be a relatively green process but where this infrastructure is missing incineration is again a common form of disposal (for more detail, see Section 1.3.1).

Whole cell processes will become increasingly common moving forward as an inevitable consequence of the rise of synthetic biology, most particularly that aspect seeking to use multiple enzymes within a given cell to enable a cascade of reactions to occur (much like telescoping a traditional chemical process). There are a growing number of researchers seeking to achieve this aim and as the genes associated with new (either

recently discovered or 'designer') enzymes become known and understood so the potential number of reactions that can be performed within an organism will also grow.

Chemistry drove much of the growth in the life-sciences in the last century but our world is changing. It is the biosciences which will spur innovation in the coming century and this includes synthetic approaches to small molecules.

1.2 Biotrans Outsourcing – AstraZeneca

The successful design, development and execution of a synthetic route containing a biotransformation is an area that requires a truly interdisciplinary effort between organic chemistry, analytical, (bio)/chemical engineering, fermentation, molecular biology, etc. A few companies have all of these scientific skills and plant capabilities in house, but increasingly, many outsource part or all of this complex exercise.

A starting enzyme may be obtained from a propriety collection, or from an external public culture collection. Genomes can now rapidly be searched for novel enzymes using sequence homology. If a likely protein is identified, a gene can be rapidly and cheaply obtained through gene synthesis companies, cloned and over-expressed into a suitable producer host. A number of companies now offer screening services in this area, to identify a known or likely lead candidate enzyme.

For those who have access to a chemical processing plant but not fermentation capacity, many service companies exist who can produce biocatalysts from plasmids or cultures and can supply solid enzyme, solutions or whole cells which can be purchased and used in-house. If an enzyme cannot be identified that gives the correct stereoselectivity and purity, or good enough performance under process conditions, many companies offer rapid techniques for evolving proteins to produce 'designer' or tailor-made biocatalysts for a particular process. The boom in the uptake of biocatalysis over the past ten or so years has been driven by the rapid advances in proteomics, molecular biology and the use of techniques like directed evolution. This has made a wide range of enzymes available that have been specifically designed for organic synthesis. Many contract research organizations that have their own propriety enzymes now also work with these commercial enzyme suppliers like Almac, Codexis, Johnson-Matthey, Libragen and Syncozymes. Other companies such as ChiralVision, CLEA Technologies and Lentikats can supply supported enzymes or cells for use in organic synthesis. Larger companies from other sectors such as Amano, Genencor, Novozymes and others supply bulk enzyme products into other industries (food, detergents, garment processing, etc.). Many of these enzymes have been identified as useful biocatalysts for use in organic synthesis, and can be purchased and used in house or by a manufacturing partner.

Many fine chemical companies such as BASF, Cambrex, Daicel, Dr Reddy's, DSM, Kaneka and Lonza have in-house fully integrated organic chemistry and biotechnology groups. Whilst they are not commercial enzyme suppliers, they can develop and use biocatalytic reactions at all scales and supply material to c-GMP quality.

There is a large amount of skill and capacity in the biotransformation/molecular biotechnology arena that can be accessed via outsourcing rather than developing capability in house. However, technology outsourcing can lead to complex supply chains and intellectual property issues, so freedom to operate at commercial scale needs to be

considered at the commencement of any outsourcing relationship. This is particularly true when taking a bioprocess from concept to industrialization, where the need for multiple skills often necessitates the use of multiple CROs.

1.3 Biotrans Trends – Lonza

The pharmaceutical market is one of the most important drivers for innovation in biocatalysis. The number of small molecule 'new chemical entities' (NCEs) in the clinical pipeline is gradually decreasing, but the complexity of the molecules produced is increasing, as is demand for cheaper medicines in order to supply emerging markets. As a result, the demand for a broader range of enzymes is increasing (see Section 1.11).

This section will cover the trends in biocatalysis by using data gathered from the "Biotrans" conference, one of the most respected conferences in Europe and Lonza's own experience, which will include selected examples of Lonza's processes.

Table 1.3.1 gives a breakdown of contributions to the last four "Biotrans" conferences by enzyme class. It can be seen that the use of oxidoreductases and hydrolases still predominates in academic research, whereas the lowest interest appears to be in the use of isomerases and ligases (with a combined average of 3% of the research topics presented in each of the last six years). It is interesting to see that there has been a slight shift in academic interest away from hydrolytic enzymes accompanied by a similar increase in transferases. However, it should be noted that whereas there has been a shift away from the widely-used hydrolase "work-horses" such as lipases and proteases, which are typically used in resolution steps in organic syntheses, other members such as peptidases and glycosidases are gaining interest. In spite of the low representation of isomerase and lyase contributions, interesting work is being conducted with aminoacid racemases and sugar isomerases, the latter of which will offer new and efficient alternatives to prepare sugar analogs.

The distribution of biotransformation projects within Lonza over the last five years is shown in Table 1.3.2. This is influenced by the needs of Lonza's synthetic chemists, the state of development of the technology and by the need to integrate more biocatalysis into existing chemical processes to become even more competitive. Despite the important developments made over the last 10 years, the demand for ready to use oxidoreductases, hydroxylases, transaminases and enzymes with new activities is still increasing.

Table 1.3.1 *An overview of the enzyme classes presented as oral or poster presentations at the last four "Biotrans" conferences.*

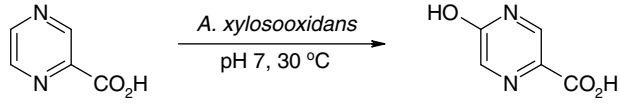
Enzyme class	2003	2005	2007	2009
Oxidoreductases	28%	24%	35%	32%
Transferases	3%	6%	8%	10%
Hydrolases	58%	55%	42%	46%
Lyases	10%	12%	12%	9%
Isomerases	1%	2%	2%	3%
Ligases	0%	1%	1%	0%

Table 1.3.2 *Distribution of projects handled at Lonza over the enzyme classes in the last five years.*

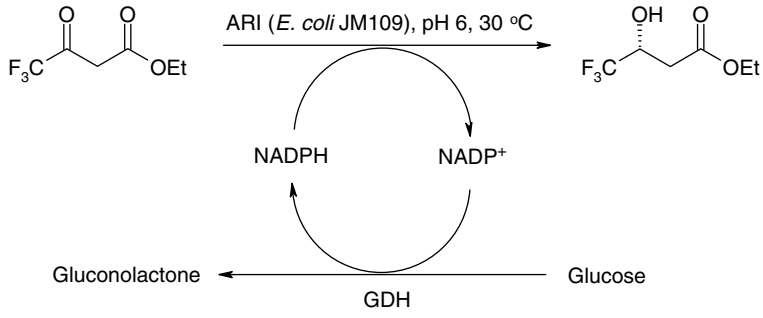
Enzyme class	2006	2007	2008	2009	2010
Hydrolases (nitrile hydratases, amidases, lipases, proteases, lactamases)	46%	40%	60%	38%	42%
Hydroxylases (dioxygenases, monooxygenases)	9%	10%	20%	23%	23%
Oxidoreductases (enoate reductases, ketoreductases)	36%	40%	10%	15%	23%
Transaminases (R- and S-specific ω-transaminases)	9%	10%	10%	24%	12%

Lonza entered the field of biocatalysis and biotechnology in 1983 and developed several large scale processes using biocatalysis for the production of small molecules like nicotinamide[3] and L-carnitine.[4] These pioneering processes used mostly wild-type whole cell systems and research focused mainly on the use of hydrolytic enzymes and the development of hydroxylation/oxidation reactions.[5] For example, a group of five strains from the genus *Pseudomonas* and *Achromobacter* were identified that catalyzed the hydroxylation of nicotinic acid to 6-hydroxynicotinic acid.[5,6] These bacteria use nicotinic acid as the sole source of carbon during their growth phase and through careful control of the residual concentration of the substrate, further degradation of the hydroxylated product can be avoided. Using *Achromobacter xylosooxidans* LK 1 a process for the hydroxylation of pyrazine-2-carboxylic acid was developed that enabled the production of 5-hydroxypyrazine-2-carboxylic acid on a 300 kg scale (Scheme 1.3.1).

With the discovery of new enzymes and the establishment of commercially available enzyme libraries, projects started to cover other reaction types. An impressive example of the discovery and adaptation of enzymes for use in industry is the ketoreductases which became commercially available approximately 10 years ago. This explains the high number of projects run with this class of enzyme in 2006 and 2007 (Table 1.3.2). Lonza is constantly expanding its biocatalytic toolbox through internal development as well as external collaborations. For example, through collaboration with Prof. Shimizu of Kyoto



Scheme 1.3.1 *Hydroxylation of pyrazine-2-carboxylic acid.*



Scheme 1.3.2 *Preparation of Ethyl (R)-trifluorohydroxybutyrate.*

University, Lonza developed a biocatalytic process for the preparation of ethyl (*R*)-trifluorohydroxybutyrate (Scheme 1.3.2).[7]

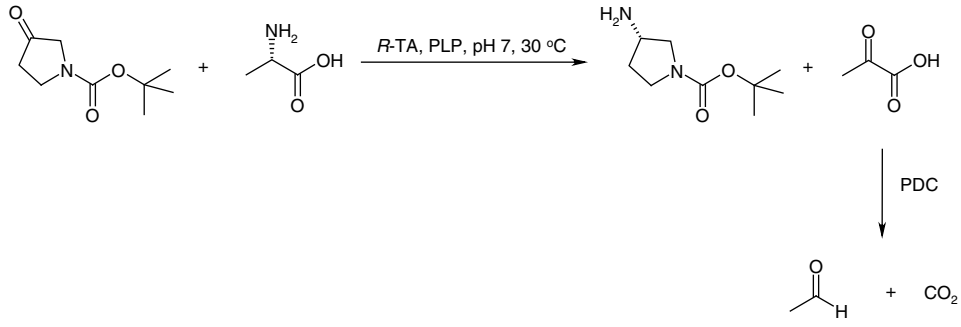
Aldehyde reductase I (ARI) from the red yeast *Sporobolomyces salmonicolor* AKU4429 was isolated by the Shimizu group during their work on the preparation of ethyl (*R*)-4-chloro-3-hydroxybutyrate from the corresponding ketone.[8,9] This NADPH-dependant aldehyde reductase was cloned into *E. coli* JM109 under control of the tac promoter. A second plasmid (pKKGDH),[8] which encodes a glucose dehydrogenase from *Bacillus megaterium*, was also transformed into the same host.[10] The *E. coli* harbouring both plasmids was then grown in 20 L fermenters at 22 °C to prevent insoluble protein formation. The biotransformation was carried out in a two-phase system at 8.8% substrate concentration in *n*-butylacetate and an aqueous mixture of *E. coli* JM109/pKAR, pKKGDH, 14 g/L of glucose and 0.56 g/L $NADP^+$ at pH 6.0 and 30 °C. A typical conversion reached 68% of the alcohol product with $>99\%$ *ee*.

Over the last few years ω-transaminases, which can catalyze the reversible transamination of a variety of simple ketones to amines, have become available to industry. This is reflected by the number of projects in this field handled by Lonza.

General hurdles to overcome when using ω-transaminases include the challenge of achieving suitable product enantioselectivity, which can sometimes be achieved through the correct choice of protecting group (substrate engineering); the unfavorable thermodynamic equilibrium, which needs to be overcome when used in the amination mode; the expression of these enzymes in an active form.

As part of a series of successful collaborations with the Bornscheuer group at the University of Greifswald, preliminary work at Lonza on the synthesis of chiral amines commenced with the preparation of 1-*N*-Boc-(*3R*)-aminopyrrolidine using the (*S*)-selective ω-transaminase from *Alcaligenes denitrificans* Y2k-2[11] by resolution of the corresponding racemic amine in 39% yield, 98% *ee*.[12]

The reverse reaction was then developed by shifting the thermodynamic equilibrium by using pyruvate decarboxylase (PDC) from *Zymomonas palmae* to deplete the pyruvate by-product. An example is the transamination of 1-*N*-Boc-3-oxopyrrolidine with L-alanine catalyzed by the (*S*)-selective ω-transaminase from *Vibrio fluvialis* supplemented with PDC which afforded 1-*N*-Boc-(*3S*)-aminopyrrolidine in 80% yield and 99% *ee* (Scheme 1.3.3).[13]



Scheme 1.3.3 *Preparation of 1-N-Boc-(3S)-aminopyrrolidine.*

Having understood the fundamentals of the reaction, a comprehensive search was carried out to identify less common (*R*)-ω-transaminases.[14] The lack of proper information was replaced by an interesting yet challenging approach; comprehensive protein sequence searches biased by the previous identification of key amino acid residues in similar enzymes. This resulted in the development of a platform of seventeen (*R*)-selective ω-transaminases which gave *R*-selective transamination towards a variety of substrates.[14] Expression of these new *R*-transaminases was improved by the use of different additives.[15]

1.3.1 Downstream Processing – Lonza

A candidate biocatalyst for an industrial process must be suited to the conditions required for the process such as high substrate and product tolerance, resistance to the pH, temperature and constituents of the reaction matrix. It must also possess high productivity and selectivity which maximizes production of the desired product and minimizes formation of side-products. However, these characteristics alone are not sufficient for a successful scale-up of the process. The down stream processing also needs to minimize losses, remove side-products and still be environmentally tenable.

Product isolation is often limited by factors such as solubility of the product and also the physico-chemical similarity of the product, substrate and side-products. Extraction methods and acid or base precipitation are typical methods used for product isolation.[5,6,10,12,13] In the examples described in Schemes 1.3.2 and 1.3.3 extraction was used for the isolation of ethyl-(*R*)-trifluorohydroxybutyrate and 1-*N*-Boc-3-aminopyrrolidine respectively. Multiple extractions were required to ensure maximal yield which means that in production large quantities of solvent would be required. Consequently an important aspect for such processes is solvent recycling in order to minimize the amount of solvent used in a campaign which reduces cost and alleviates the necessity to burn large quantities of solvent for waste management.

Another problem that can arise is inadequate phase separation caused by the presence of cells, enzymes or the products of cell lysis in the solution. These can accumulate at the interface between the aqueous and solvent phases as a stable emulsion. This in turn leads

to incomplete phase separation resulting in reduced yields. There are various methods of improving this separation such as centrifugation or slow stirring for prolonged periods of time, neither of which are realistic on scale. Other more innovative approaches are the addition of hydrolases or certain microorganisms that are capable of secreting such enzymes, in order to hydrolyze the bioemulsifiers responsible for the production of the stable emulsion.[16,17] In some cases the addition of cationic surfactants can reduce the time required for effective phase separation by agglomerating the bioemulsifiers.[18] Another novel method for phase separation and product isolation is the use of supercritical carbon dioxide.[19]

Less traditional inexpensive methods which are much more environmentally friendly can be implemented to avoid the problems mentioned above. These methods include membrane technologies such as ultrafiltration, nanofiltration, electrodialysis and perevaporation.[20] Ion-exchange resins, perevaporation and electrokinetic bioreactors[21] are suitable for *in situ* product removal (ISPR) which is especially attractive as the problem of product inhibition can be effectively addressed.[22] Unfortunately ISPR is not routinely used on a large scale, although Lonza has periodically tested various methods at pilot scale over the last 20 years.[23] Other possibilities are the use of ionic liquids as an alternative to organic solvents.[24] Bioprocesses using biphasic systems consisting of an aqueous phase and a suitable ionic liquid can overcome problems of water solubility of the substrate, product inhibition and product or substrate instability in the aqueous reaction mixture but require extraction from ionic liquids.[25]

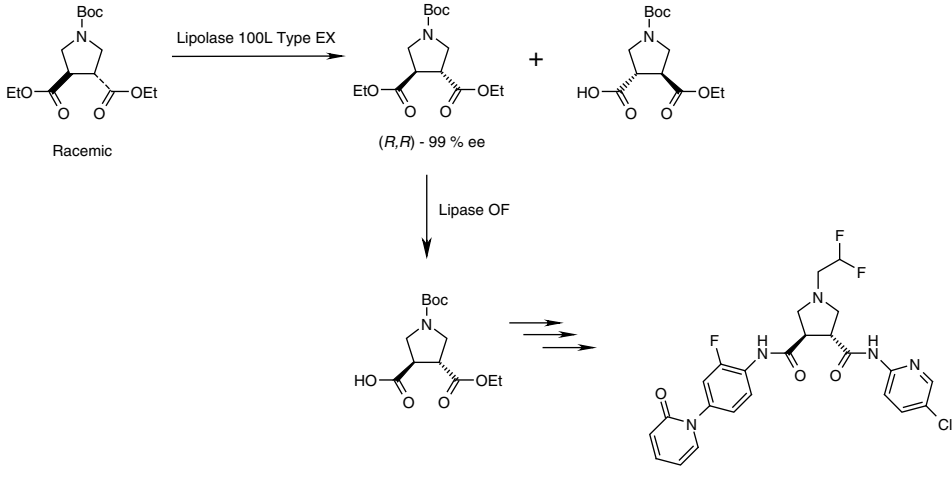
1.4 Biocatalysis in the Pharma Environment

Biocatalysis contributes significantly to the generation of APIs through the supply of chiral building blocks from the fine chemical industry. In contrast, there is a clear underutilization within the pharmaceutical industry, where biocatalysis could provide more efficient and less hazardous processes for pharmaceutical production. However, in recent years this has begun to change and this section briefly discusses how different companies have implemented biocatalysis in the different phases of pharmaceutical development and production.

1.4.1 Value Creation by Biocatalysis – Roche

Biocatalytic approaches can create value through a number of means. For example, it may offer the only viable approach to a desired API, as found in the synthesis of a factor Xa inhibitor (Scheme 1.4.1).[26]

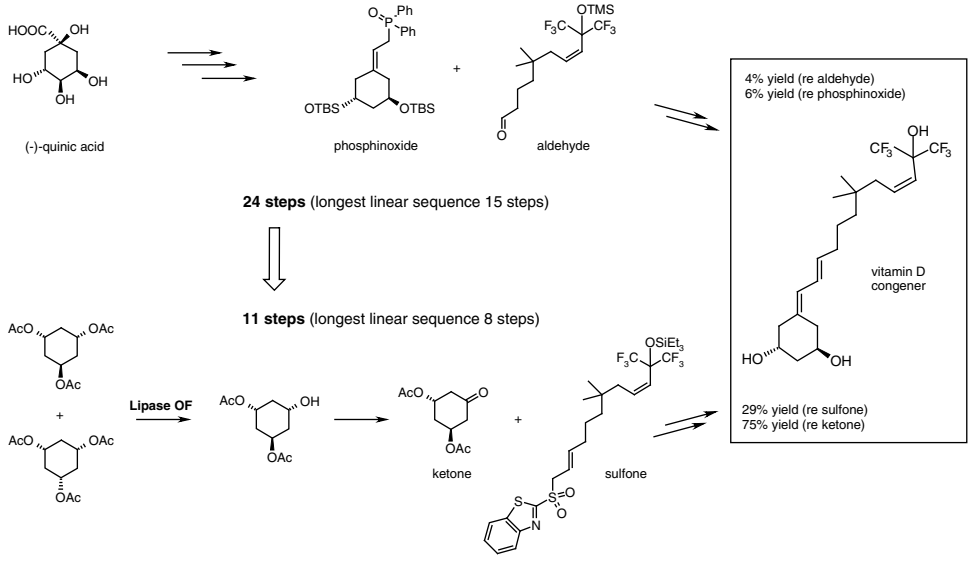
Alternatively, it may enable the development of synthetic routes that are significantly shorter than the competing chemical approaches, as illustrated in the synthesis of a vitamin D congener (Scheme 1.4.2)[27] and a collagenase inhibitor.[28] Biocatalysis can also sometimes help to meet short project timelines by offering temporary solutions for the production of 'first material', as was found for the kilogram scale synthesis of the building blocks for a glycine transporter inhibitor,[29] and an A2a receptor antagonist (see Chapter 8.4 for experimental details).[30] Of these benefits, enabling shorter synthetic routes might



Scheme 1.4.1 *Hydrolase resolution of a coagulation factor Xa inhibitor intermediate.*

be one of the most prominent contributions of biocatalysis to *Green Chemistry* in pharmaceutical synthesis.

The Centre of Excellence in Biocatalysis at Roche is part of the Chemical Synthesis Department and therefore is closely integrated in the design of chemical routes to drug candidates and chemical supply for Process Research & Development and related activities. The main task of the centre is to supply chiral building blocks to Discovery



Scheme 1.4.2 *Improved chemoenzymatic route to vitamin D congener.*

programs through to Chemical Development projects and to develop technically feasible process steps (mild reaction conditions offered by enzymes are only rarely requested). Being a tool to generate chirality, biocatalysis competes with a number of alternative technologies such as asymmetric chemical catalysis, chiral preparative chromatography, classical racemic resolution and the chiral pool. Particularly for the small amounts requested in Discovery Chemistry preparative chiral chromatography, HPLC and SFC are potent alternatives delivering both enantiomers for bioassays in a short time.

Because of the short time frames, the Roche biocatalyst toolbox focuses on reaction types having a high chance of success, using off the shelf catalysts (the major enzymatic and microbial libraries being on well plates). Among these, stereoselective hydrolysis/acylation using the well described hydrolase subclasses and asymmetric ketoreduction are clearly the most frequently applied and successful reaction types. In order to further improve the success rate, optimization of chemical and physical parameters in defined formats is routinely carried out. Routine screening of 1200 microbial strains is carried out in a well plate format.

The synthesis of *human drug metabolites* on a small scale (comprising hydroxylation, glucuronidation and sulfatation) for Drug Metabolism/Pharmacokinetics (DMPK) is also an integral part of our biocatalytic support. For this purpose, we have over 12 ready to use human CYP450 isozymes, co- and overexpressed with P450 reductase in *E. coli* together with a broad panel of proven microbial hydroxylating strains. In addition, we possess heterologously expressed human UGTs, efficiently supported by a number of animal liver homogenates for glucuronidation as well as a few sulfotransferases (including an efficient recycling system for the expensive cofactor 3′-phosphoadenosine-5′-phosphosulfate; PAPS). In this field it is essential to possess know-how and capacity in product isolation and purification.

Through Roche's continuing efforts to adopt green chemistry principles, asymmetric syntheses (involving for example, desymmetrizations or dynamic resolutions like those shown in Section 1.5.3) play an important role. Such key steps are attractive opportunities for biocatalysis, removing some of the aforementioned alternative methodologies from consideration.

There is a constant effort to extend our existing catalyst libraries and build up additional enzyme libraries in house, such as aminotransferases or enoate reductases, together with external collaboration. However, up to now the performance of these emerging platforms towards the often sterically demanding pharmacophoric substrates has been below expectations and will require continued effort. As a consequence, the need for more and more powerful platforms persists (see Section 1.10).

The emerging sciences (bioinformatics, gene synthesis, enzyme evolution) are also pursued but predominantly for projects at an advanced stage of development. These projects generally have increased synthetic constraints, importance and, at the same time, a higher survival rate, which keeps the financial frontloading on an acceptable level. It is not planned to integrate these technologies into our platform, but to utilize them via external collaboration with CMOs, CROs or academia (see Section 1.2). As the number of suitable advanced projects is comparatively low, activities such as creating tailor-made enzymes by means of directed evolution still remains a rare event (in two projects[26, 30] the respective activities had been initiated but were abandoned again when the respective projects were discontinued). In general, outsourcing is considered an important tool extension but

restricted to later phase projects. Examples are enzyme screenings for bulky substrates, the heterologous expression of various enzymes and assay development.

Another emerging trend is the application of *whole cells*. This is mainly determined by the need to use new, non-commercial enzymes and the ease of preparing the catalyst cost effectively. In addition, the cofactor regeneration issue can be elegantly addressed by the whole cell approach by coexpressing the relevant auxiliary enzymes, though up to the pilot scale this is not a burning issue in our pharma projects: A considerable number of cofactor-dependant ketoreductase (KRED) reactions together with various regenerating systems (depending on the particular issues, like enzyme stability, filterability, etc.) have been carried out in house on the multi-100 g to 100 kg scale without major problems.[29, 31, 32] In terms of usefulness the KREDs have caught up with the hydrolases and are on a par with the respective chemical catalysts.

When using whole-cells under GMP conditions to produce material for Entry into Human (EIH) the complete transmissible spongiform encephalopathy (TSE) certificates for 'biological' media components (if not 'synthetic') have to be collected from the suppliers, and the issues of bioburden and/or potential toxic media components addressed. This is comparatively straightforward when employing *E. coli* cells for orally administered APIs.

For the reasons already mentioned, enzyme immobilization for continuous or repeated batchwise operation is not normally a focus as the development work required to profit fully from the well known advantages (reuse of enzyme, *vs* disadvantages such as mass transfer limitation, stability) does not pay off with projects at an early stage.

In conclusion, biocatalysis at Roche is an indispensable tool in the synthesis of pharmaceutical compounds, of which the conventional platforms are clearly the most widely applied and most successful. There is undoubtedly a strong need for more and better catalysts to increase the synthetic versatility of biocatalysis (see Section 1.10). This is accomplished in a project-driven approach. In order to seize the manifold opportunities in biocatalysis, collaboration with external partners – industry or academia – will become even more important than at present.

1.4.2 Discovery Chemistry and Manufacturing in Pharma – Pfizer

Biocatalysis and biotransformations have a long history in Pfizer. Our efforts in this area stretch back to 1919, with the mass production of citric acid from sugar through mold fermentation, and in 1941 Pfizer became the first company to use fermentation technology for the production of penicillin. In the 1990s the importance of applying biocatalysis to the preparation of small molecule APIs began to be recognized, and biocatalysis resources were spread across multiple groups and sites. After the development of a highly efficient, biocatalysis-based process for the manufacture of Lyrica® in the mid-2000s, chemoenzymatic-based biocatalysis resources were combined with fermentation-based biotransformation resources to form the Chemical R&D Biocatalysis Center of Emphasis in 2006. Several processes, including one for Lipitor®, have been launched commercially. Early on, the importance of molecular biology was recognized and internal capabilities to perform enzyme engineering were developed, resulting in a number of engineered enzymes.

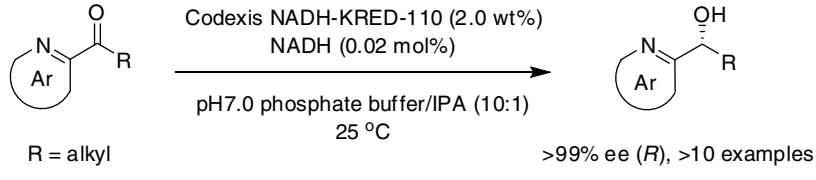
With respect to future developments, we see several major issues that affect the application of biocatalysis in the pharmaceutical industry. These include restricted freedom to operate (FTO) and intellectual property (IP) impediments with many commercial enzymes, lack of diversity in enzyme screening panels, the length of time required to perform enzyme engineering, and lack of easily scalable P450-type oxidation technologies. Our current efforts are focused on expansion of basic enzyme screening panels to cover broader aspects of substrate space for each type of reaction (i.e., panels that will make it possible to obtain a hit for nearly every reasonable, pharmaceutically relevant substrate for a particular reaction) and expansion of our enzyme panels to cover broader aspects of reaction space (i.e., panels for reaction types for which there are currently no panels available). We see increasing the speed of enzyme engineering as more of a project management/strategy/resource problem (as opposed to a technology issue) and development of scalable P450 oxidation technologies as a long-term project.

The timelines for delivery of chemical intermediates in early-stage development drug discovery programs are generally far shorter than in late-stage development, being measured in days and weeks rather than in months or even years. Options are therefore often limited to those employing pre-prepared and/or off the shelf biocatalysts, especially those with well understood protocols for preparative-scale use.

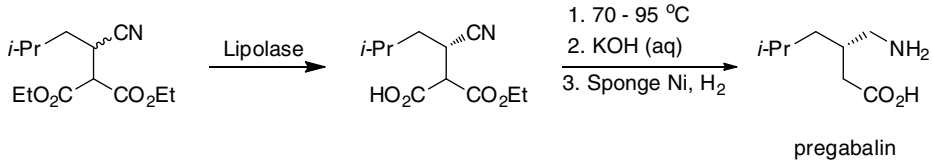
In order to minimize development times, well-plates containing sets of a particular class of enzyme can be prepared in large quantities and stored frozen, then thawed and screened as required.[33] We have found the hydrolases and ketoreductases to be particularly well-suited to this process, the hits from screening often being amenable to gram-scale synthesis with little or no need for process optimization. Along with target chiral alcohol synthesis, the ketoreductases have also shown particular utility for the generation of enantioenriched chiral alcohol libraries, displaying very high enantioselectivity against large sets of similar substrates (Scheme 1.4.3).[34]

It may be worth expending higher levels of effort to develop high-value "project-enabling" biotransformations. Examples include accessing novel chemical space through uniquely biocatalytic reactions and carrying out highly chemo-, regio- or stereoselective reactions when simple chemical methods prove inadequate. However, due to the high attrition rate of early development projects, it is our opinion that limited specialist biotransformation resources are, in general, better applied at a later stage of development, and that support should be given to early development chemists to enable them to develop biocatalytic processes independently.

Development and implementation of second generation manufacturing processes for pharmaceutical compounds requires substantial resources, and therefore must yield a satisfactory return on investment over the lifecycle of the process as well as achieving key



Scheme 1.4.3 *Synthesis of a library of (R)-alcohols from a set of 2-acyl-N-heterocycles.*



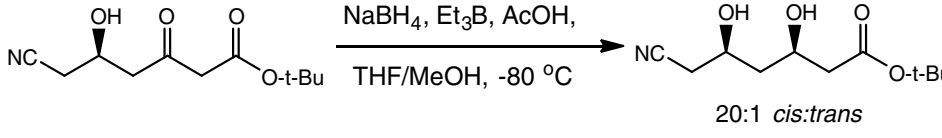
Scheme 1.4.4 *Biocatalytic synthesis of pregabalin.*

targets such as cost and throughput. Biocatalysis has enabled the successful implementation of second generation manufacturing processes for two of Pfizer's major drugs, Lipitor® (atorvastatin) and Lyrica® (pregabalin), which generated global sales of $10.7 and $3.06 billion, respectively, in 2010.

Pregabalin was launched in the United States in 2005 as Lyrica® for the treatment of neuropathic pain, post herpetic neuralgia, and epilepsy. The initial manufacturing process utilized a racemic synthesis of pregabalin followed by a classical resolution with (*S*)-mandelic acid. The inherent inefficiency of this resolution process in which the undesired isomer could not be recycled (E factor = 86), sparked efforts to develop more efficient synthetic routes. An asymmetric hydrogenation route[35] which reduced waste by a factor of two was developed and scaled up, but not implemented due to the development of a more efficient biocatalytic route (Scheme 1.4.4).

The biocatalytic route to pregabalin uses the same racemic diester starting material as the classical resolution route but employs a commercially available lipase (Lipolase, *Thermomyces lanuginosus* lipase), used in the detergent industry, to catalyze a kinetic resolution of the diester to (*S*)-monoester.[36] While the biocatalytic route still involved a resolution of enantiomers, earlier introduction of the resolution step combined with other improvements such as elimination of organic solvents from all reaction steps, and recycling of the undesired isomer resulted in a large improvement in process efficiency. Compared with the classical resolution route, the biocatalytic route reduced waste by a factor of seven (E factor = 12) and reduced energy usage by 82%.[37] This improved efficiency translated into higher yields of pregabalin from *rac*-diester, which increased from 25.8% for the classical resolution route to 33.4% for the enzymatic route without recycling and 42% for the enzymatic route with recycling of the undesired isomer.

Successful commercialization of the biocatalytic process for Lyrica® highlighted advantages of enzymatic synthesis and sparked a search for other opportunities to implement biocatalytic routes for Pfizer drugs. Reduction of the hydroxyketone to *cis*-diol (Scheme 1.4.5), a key step in the synthesis of atorvastatin, was an attractive target for process improvement due to drawbacks in the existing process. These drawbacks included



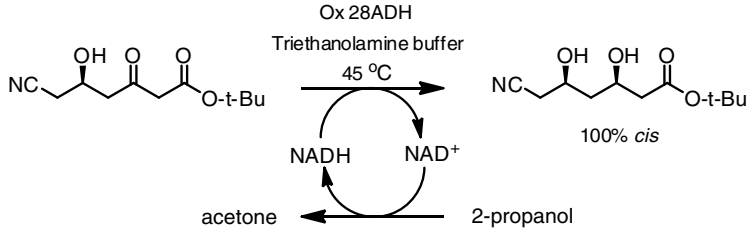
Scheme 1.4.5 *Chemical reduction process for atorvastatin intermediate.*

the use of hazardous reagents (triethylboron and sodium borohydride), cryogenic conditions, and mixed organic solvents (tetrahydrofuran, methanol, acetic acid). The process required multiple distillations to remove boron wastes resulting in long cycle times and produced the diol product as a 20:1 mixture of *cis:trans* isomers.

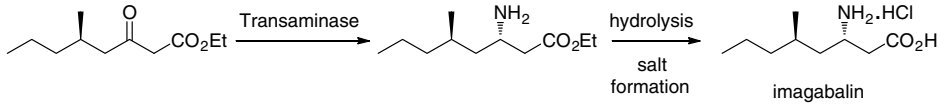
Enzymatic reduction of the hydroxyketone has been reported but biocatalysts mentioned in these reports were not readily available for evaluation.[38] However, a recombinant alcohol dehydrogenase (Ox 28, IEP, Weisbaden, Germany) was available, and preliminary evaluation showed that reduction of the hydroxyketone to the diol with complete selectivity for the desired *cis*-isomer was feasible. Efforts to develop a process for reduction of the hydroxyketone using Ox 28 were undertaken and several challenges specific to the use of enzymes were encountered.[39] One of these challenges was development of a manufacturing process for Ox 28, since this was a custom enzyme and not already in commercial production for other applications. This involved optimization of a fermentation process and identification of a suitable enzyme formulation that would meet performance and cost criteria. Initially, Ox 28, which was produced by a recombinant *E. coli* strain, was evaluated in the form of whole cells. This formulation met performance criteria but was considered impractical due to handling concerns such as the need to ship cells frozen and difficulties encountered in charging large masses of cells to reactors. A glycerol stabilized lysate, which could be readily charged into reactors, was also evaluated but gave lower yields due to the effect of glycerol on downstream chemistry. Finally, a lysate stabilized with isopropanol was evaluated and shown to meet performance and cost targets. Isopropanol served a dual purpose as it inhibited microbial contamination and served as the co-substrate for recycling NADH cofactor.

A manufacturing process developed using the isopropanol stabilized lysate (Scheme 1.4.6) was implemented at full scale for reduction of the hydroxyketone to the *cis*-diol. This process was operated in aqueous media at 45 °C and gave *cis*-diol with 100% diastereoselectivity. The enzymatic process reduced organic waste by 65% and eliminated drawbacks associated with the chemical reduction process, such as the use of hazardous reagents, cryogenic conditions, and mixed organic solvents.

Successful implementation of biocatalytic manufacturing processes for Lyrica® and Lipitor® has clearly demonstrated the advantages and value of this technology. To ensure that biocatalytic routes for new products are implemented at the beginning of their lifecycle, Pfizer has integrated biocatalysis into chemical process development so that biocatalytic routes are considered together with chemical routes and not only after attempts to develop chemical routes have been exhausted. This approach is illustrated by chemical process



Scheme 1.4.6 *Enzymatic reduction process for atorvastatin intermediate.*



Scheme 1.4.7 *Chemo-enzymatic synthesis of imagabalin.*

development for imagabalin, a drug for treatment of generalized anxiety disorder. A chemo-enzymatic route (Scheme 1.4.7) was projected to have lower cost of goods compared with two chemo-catalytic routes,[40] but showed only marginal feasibility in a screen of ω-transaminases, as the best hit, *V. fluvialis* aminotransferase gave barely detectable activity. Therefore, a program to improve *V. fluvialis* transaminase was undertaken using multiple techniques including homology modelling, bioinformatics, machine learning, crystal structure analysis, and site saturation mutagenesis of specific sites.[41] A total of fewer than 450 variants were designed and tested resulting in identification of a variant r414 with eight mutations that showed a 60-fold increase in activity compared to the wild-type enzyme and selectivity that favored the desired (3*S*,5*R*)-aminoester in 95% de.

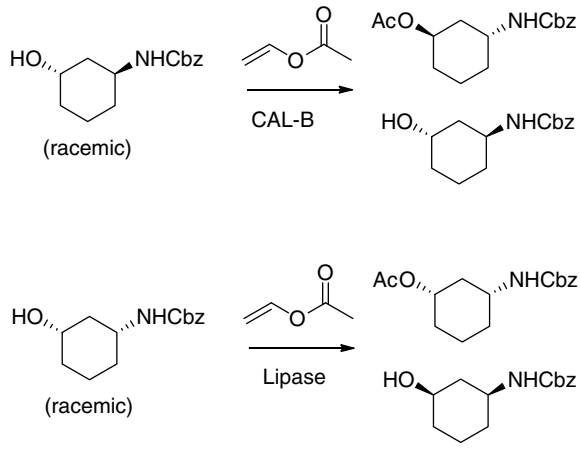
Further work to improve the activity and selectivity of r414 was required to yield a commercially useful enzyme, but not undertaken as development of imagabalin was halted. However, had the project continued, the program was on track to deliver a commercially useful variant enzyme. This would have been employed in reaction engineering studies and kinetic rate-based process optimization to develop a scalable synthetic procedure for regulatory filing and transfer to manufacturing plant.

1.4.3 Drug Metabolites and Building Blocks – Novartis

The "bioreactions" expertise within the Novartis Institutes for BioMedical Research applies biotransformations to support the drug discovery and drug development effort in multiple ways. Both isolated enzymes and whole cells (either wild type or recombinant) as well as liver homogenates are used as biocatalysts to produce chiral building blocks,[42–44] metabolites of drug candidates and natural product derivatives.[45–47] The expertise resides in an integrated team of chemists, biologists and engineers and covers the necessary steps from fermentation/catalyst supply to downstream processing of the product. A few recent examples from each category will be highlighted in this section.

For the synthesis of (chiral) building blocks both well established, commercially available biocatalysts as well as enzymes from internal screening campaigns are used. One recent example is the resolution of racemic *trans*-3-(hydroxy-cyclohexyl)carbamic acid benzyl ester (Scheme 1.4.8).[44]

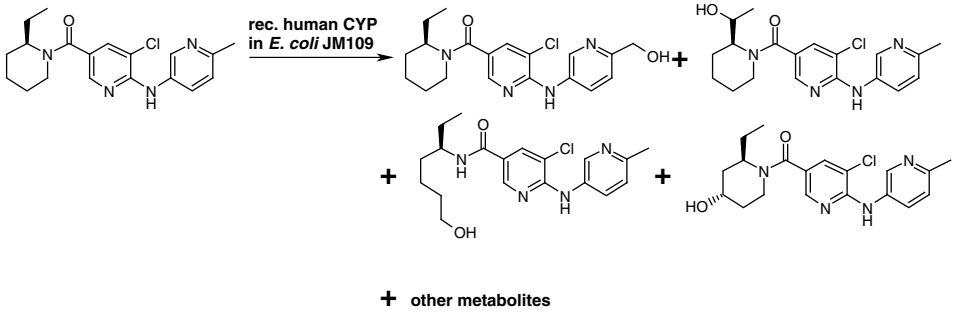
The resolution of the *cis*-racemate has also been carried out on a 660 g scale. The highest optical purities (99.9% *ee* for the alcohol) were obtained when using *Thermomyces lanuginosus* lipase. This illustrates the usefulness of hydrolytic enzymes in drug discovery, when timelines (which means the ready availability of a certain biocatalyst) are important. Moreover, resolution reactions give rise to both enantiomers and are thus sometimes preferable over asymmetric syntheses in early drug discovery phases.



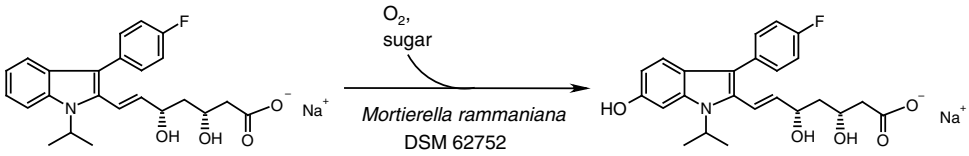
Scheme 1.4.8 *Resolution of 1,3 aminocyclohexanols.*

Metabolites of drug candidates are required throughout all phases of drug discovery and development, for structure elucidation, biological testing or as analytical references. A broad toolbox is applied for the synthesis of both Phase I (functionalization) as well as Phase II (conjugation) metabolites.[48] This toolbox comprises of, among others, recombinant *E. coli* overexpressing human cytochrome P450 monooxygenases (CYPs) and a collection of wild-type microorganisms preselected for their oxidative biotransformation capacity.

A recent example is the synthesis of some metabolites of a metabotropic glutamate receptor subtype 5 (mGluR5) antagonist drug candidate (Scheme 1.4.9; see Chapter 5.2 for experimental details).



Scheme 1.4.9 *Preparative scale synthesis of phase I metabolites using rec. human CYPs.*



Fluvastatin Na+

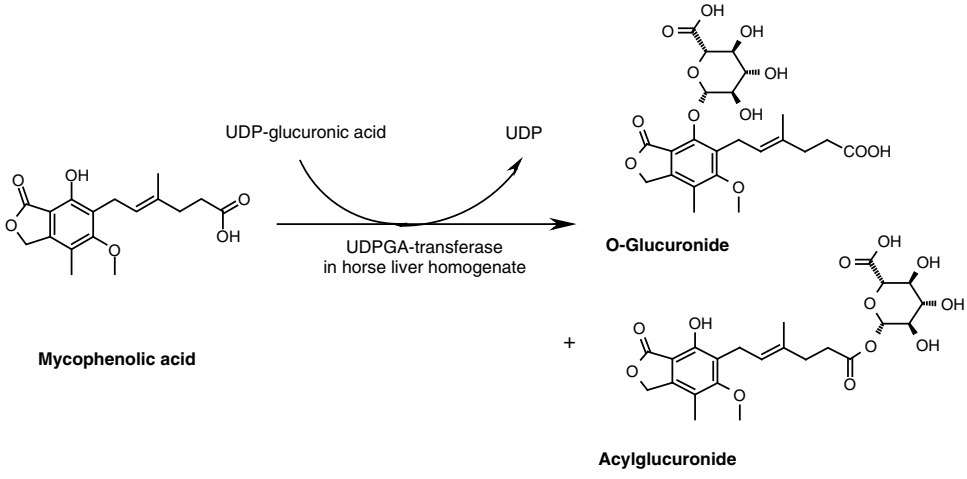
Scheme 1.4.10 *Preparative scale synthesis of phase I metabolites using microorganisms.*

In this case several hydroxylated metabolites of the drug candidate could be isolated and purified in multi-milligram amounts. As an alternative, microbial strains can also be used for the synthesis of hydroxylated derivatives, as demonstrated in the case of Fluvastatin (Scheme 1.4.10).[49]

The major reaction in Phase II metabolism is glucuronidation, which is catalyzed by UDP-glucuronosyltransferases (UGTs) in the liver. For a preparative scale application existing recombinant UGTs are too costly. Therefore, typically liver homogenates are used as biocatalysts for the synthesis of glucuronides. An example for such a reaction is the synthesis of an acyl glucuronide of mycophenolic acid, an immunosuppressant (Scheme 1.4.11).[50]

The synthesis of Phase II metabolites is typically less cumbersome than for the Phase I metabolites. In the case of mycophenolic acid, hundreds of milligram quantities of the metabolites could be synthesized and purified.

A major effort in the preparative scale synthesis of metabolites lies firstly in the analytical screening to identify the right biocatalyst for producing the desired metabolite and secondly in the method of purification used to isolate the right compound from a mixture of residual parent and multiple metabolites out of a complex matrix. Thus, support from analytical and purification experts is crucial in this effort, which needs to complement the already interdisciplinary biotransformation expertise. An integrated team for biotransformations concerned with all aspects of biotransformations as exemplified above is thus well suited to support drug discovery and development programs.



Mycophenolic acid

O-Glucuronide

Acylglucuronide

Scheme 1.4.11 *Synthesis of a phase II metabolite with liver homogenate.*

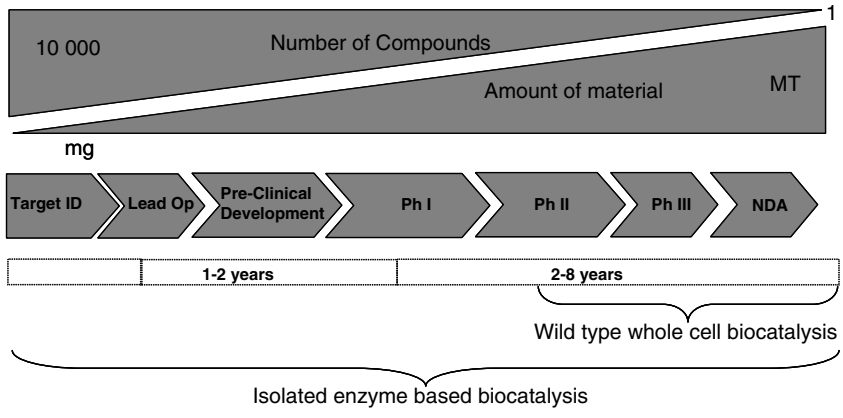
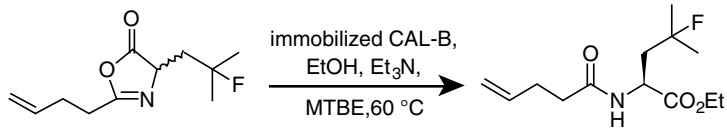


Figure 1.4.1 *Drug development timeline.*

1.4.4 Biotrans Using Isolated Enzymes – Merck

About 15 years ago, Merck made the decision to move away from whole cell biocatalysis and focus almost exclusively on isolated enzyme based processes (Figure 1.4.1). Long lead times due to complexities with the development of whole cell fermentation based processes relegated this work toward the end of the drug development timeline. This meant that the implementation of a whole cell biocatalysis step in a synthesis required supplanting existing chemistry that had been used well into human clinical trials. The switch to isolated enzyme biocatalysis changed that. Advances in enzyme evolution techniques allowed for the rapid improvement of enzyme activity and stability. Stable enzyme preparations could now be stored in the fridge and used to quickly deliver pharmaceutical intermediates at any stage of the drug development process. Implementing a biocatalytic step early on in route development was critical to solidifying the role of biocatalysis in synthesis. In fact, in the decade following the switch to isolated enzyme based processes, Merck's biocatalysis group had increased the number of deliveries made by >10-fold.

The move away from whole cell processes created an issue, the need for cofactor recycling. Early on, the vast majority of biotransformations utilized cofactor-independent hydrolases. The lack of need for cofactor recycling and the widespread availability of diverse enzymes with a broad range of substrate specificities made hydrolases uniquely well suited for large scale industrial applications. However, the ever increasing need to drive down the cost of manufacturing in the pharmaceutical industry has led Merck to evaluate additional methodologies for making more cost effective processes. Enzyme immobilization has the potential to be a transformative technology and enables the use of biocatalysts in organic solvent systems. This in turn facilitates reaction telescoping, facile enzyme recovery and reuse, and continuous processing. The ability to run the enzyme catalyzed transformation in solvents compatible with upstream and downstream chemistry eliminates many of the isolation and solvent switching steps required with conventional aqueous biocatalysis, providing for significantly more efficient processes with lower cost and less waste.



Scheme 1.4.12 *Enzyme catalyzed dynamic kinetic ring opening ethanolysis of azlactone.*

The total synthesis of Odanacatib relies on an enzyme mediated dynamic kinetic resolution for the production of a key chiral fluoroleucine intermediate. Early deliveries used Novozym 435 in a batch process for the enzyme catalyzed ring opening ethanolysis of azlactone to the desired (*S*)-γ-fluoroleucine ethyl ester (Scheme 1.4.12).[51] Significant yield and selectivity gains were achieved by minimizing side reactions and eliminating attrition of the solid enzyme support by switching to a continuous plug flow column reactor setup.[52,53] However, deactivation of the immobilized enzyme under the optimized reaction conditions remained a significant concern; and cost drivers led us to investigate the development of a more active and stable form of immobilized *Candida antarctica* lipase B (CAL-B) to arrive at an acceptable manufacturing route.

Five Sepabead resins from Mitsubishi were tested for the immobilization of CAL-B. The resins chosen represented various compositions and functional groups, and included both covalent and hydrophobic immobilization binding methodologies (Table 1.4.1).

After immobilization, each resin was tested for activity and 48 h stability under the optimized reaction conditions (Figure 1.4.2). Novozym 435 was included as a reference point for comparison to the standard process. Novozym 435 exhibited an activity of 2.2 g/L.h.g_{enzyme}. 50% higher specific activity was observed with the CAL-B immobilized on EXE120. Additionally, Novozym 435 retained only 6% of its initial activity after 48 h under process conditions compared to 94% activity retention for the EXE120 immobilized CAL-B (MRK-CALB-EXE120).

Novozym 435 and MRK-CALB-EXE120 were then compared head to head in continuous packed bed plug flow reactor mode. As expected from the batch reaction studies, MRK-CALB-EXE120 exhibited much greater (15×) stability compared to Novozym 435 (Figure 1.4.3). Additionally, higher product yield and ee was obtained using the higher specific activity MRK-CALB-EXE120 compared to Novozym 435 (95% yield vs 90% and 88% *ee* vs 86%). The greater stability of the new immobilized CAL-B preparation also provided for a significant reduction in enzyme to substrate loading, from 1:20 for Novozym 435 to $< 1:100$ for MRK-CALB-EXE120 (see Chapter 8.8 for experimental details).

Factoring in the reusability of the new immobilized CAL-B preparation along with cost to manufacture the immobilized enzyme, the MRK-CALB-EXE120 catalyzed process is

Table 1.4.1 *Immobilization resins and their composition and method of enzyme binding.*

Resin Name	Resin Composition	Resin Functional Group/Binding Method
EC-EP	polymethacrylate	epoxide/covalent
EC-HFA	polymethacrylate	amino epoxide/covalent
EXA252	styrene/DVB	porous structure/hydrophobic
EXE119	polymethacrylate	epoxide/covalent
EXE120	polymethacrylate	octadecyl/hydrophobic

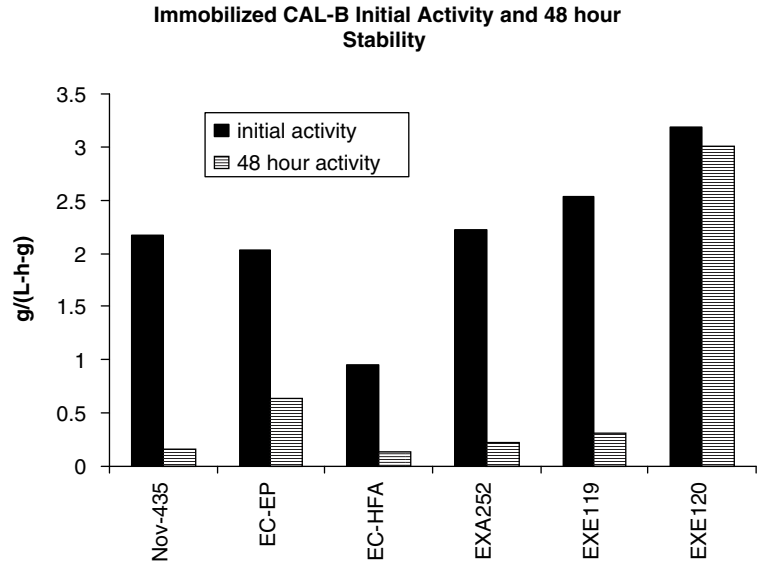


Figure 1.4.2 *Initial activity and 48-h stability of CAL-B immobilized on Sepabead resins compared to Novozym 435.*

99.9% less expensive than the process utilizing Novozym 435, with a threefold lower *E-factor*.[4] This process has been demonstrated at 100 kg scale (>90% yield and 88% *ee*).

Finally, we demonstrated the generality of this new immobilized CAL-B preparation via the resolution of a variety of alcohol and amine substrates. 20 g/L of each substrate was dissolved in MTBE with 5 equivalents of vinyl acetate and 50 g/L MRK-CALB-EXE120.

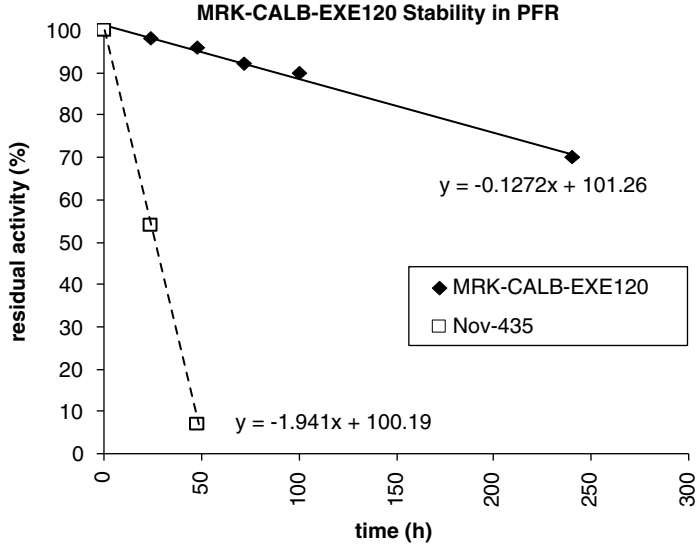
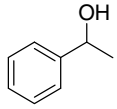
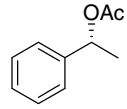
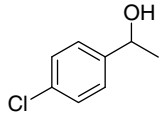
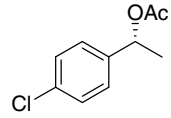
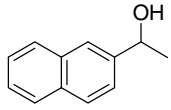
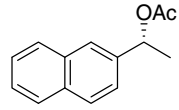
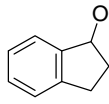
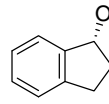
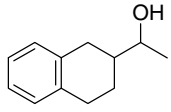
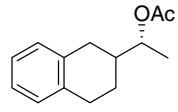
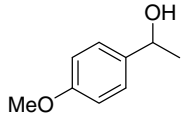
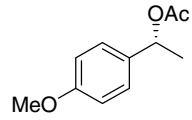
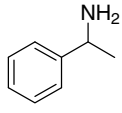
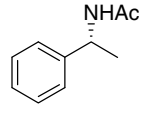


Figure 1.4.3 *Stability of MRK-CALB-EXE120 and Novozym® 435 in plug flow reactor.*

Table 1.4.2 *Resolution of alcohol and amine substrates using MRK-CALB-EXE120.*

Entry	Substrate	Product	conversion	% *ee*
1			47%	>99
2			48%	>99
3			50%	>99
4			49%	>99
5			48%	>99
6			45%	>99
7			48%	>99

The reactions were run overnight for 18 h. Excellent enantioselectivity (>99% *ee*) was obtained for each of the substrates tested (Table 1.4.2).

More recently, a growing majority of the work done in Merck's biocatalysis group has focused on the synthesis of chiral alcohols and amines using ketoreductases and transaminases respectively, which required the development of inexpensive cofactor recycling systems. The primary technology that has driven the success of biocatalysis has been the ability of enzyme evolution to quickly deliver highly active and selective biocatalysts for application towards more efficient routes to important APIs such as Montelukast (see Chapter 4.4 for experimental details) and Januvia (see Chapter 2.2 for experimental details) – also see Section 1.8.1 for more details on the directed evolution of enzymes for these processes.

One important benefit of enzyme evolution is that a range of mutants are generated that can be utilized as a platform for the progression of other projects that require chiral

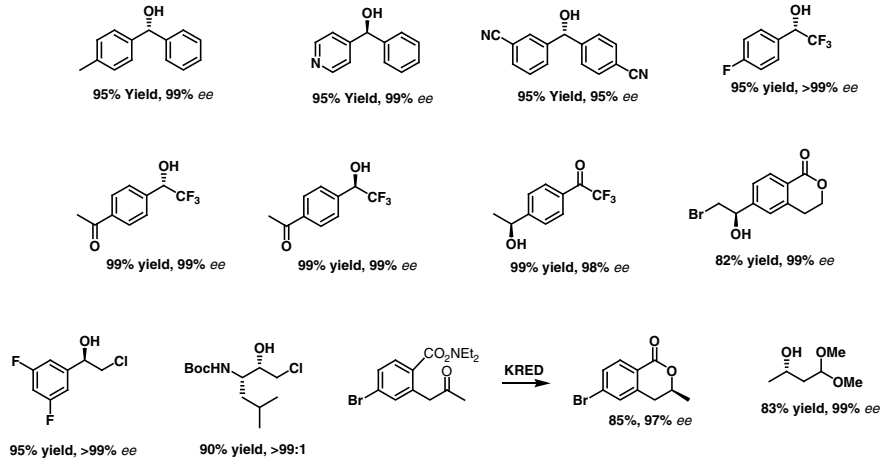


Figure 1.4.4 *Selected examples of ketoreductase products.*

alcohols (Figure 1.4.4) or amines (Figure 1.4.5). Success in this area has made biocatalysis the preferred synthetic method for installing these chiral functionalities.

The preparation of stable isolated enzymes has expanded the utility of biocatalysis across the breadth of the pharmaceutical development timeline. For biocatalysis to remain

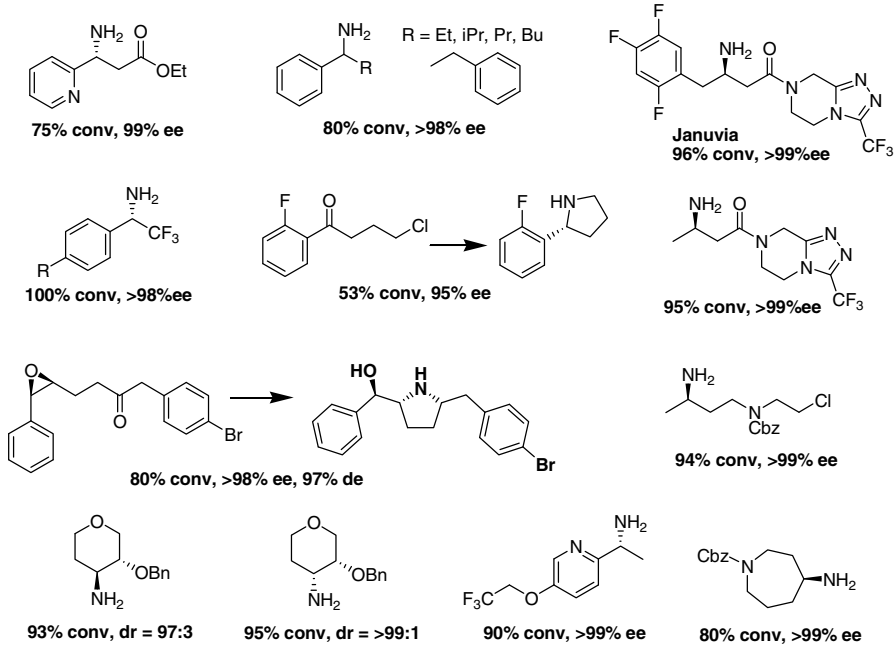


Figure 1.4.5 *Selected examples of transaminase products.*

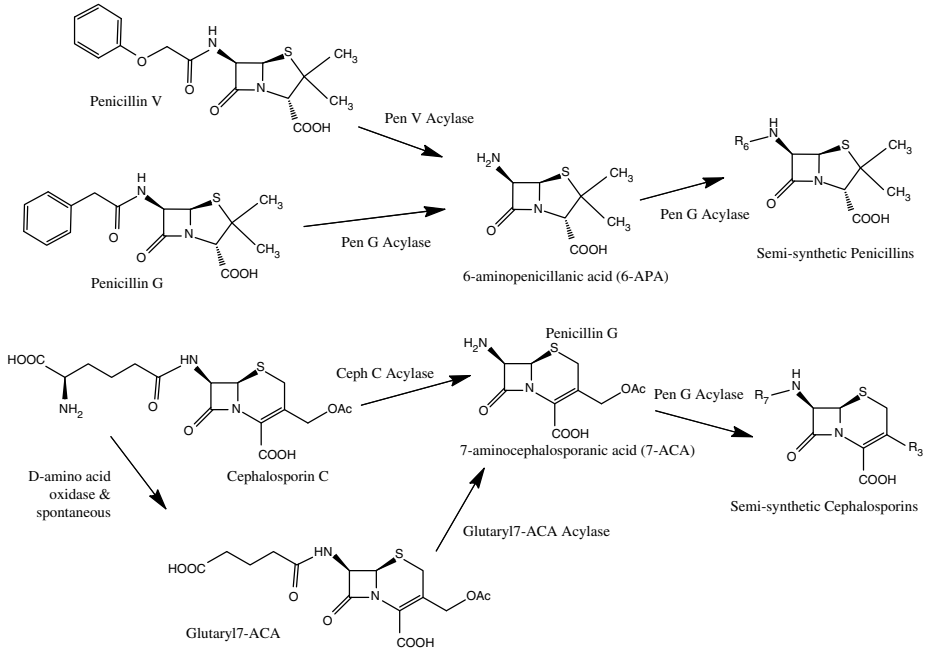
relevant and reach its full potential, innovation continues to be required. We must expand the practicality of useful chemistries through enzyme evolution (hydroxylations, epoxidations, reductive aminations, etc.). Finally, we must constantly evaluate and develop complimentary technologies that can enhance the positive impact of biocatalysis (enzyme immobilization, continuous processing, etc.).

1.5 Industrial Use of Hydrolases

Hydrolases continue to be the biocatalytic "work-horse" of the fine chemical and pharma industries in spite of the recent introduction of new enzyme activities that are also discussed in this chapter. This section briefly discusses some of the applications that make this such an industrially important class of catalyst.

1.5.1 β-Lactam Antibiotics Synthesis – GSK

Within GSK's pharmaceutical manufacturing business the highest volume applications for biocatalysis, now and for the foreseeable future, are semi-synthetic β-lactam antibiotics. Total annual production of penicillins and cephalosporins is thousands of tonnes worldwide, mainly generic medicines manufactured at competitively low cost. Starting materials for most are derived from the fermentation products penicillin G or cephalosporin C through enzymatic hydrolysis of the C-6 and C-7 side chains respectively (Scheme 1.5.1).[54]



Scheme 1.5.1 *Enzymes in β-lactam antibiotics manufacture.*

As GSK scientists embrace the current state of the art in biocatalysis, it is useful to reflect on the history of these apparently simple hydrolase reactions and consider: if these processes did not already exist, how would we now develop them? The challenge is to cleave a relatively bulky amide side chain in the presence of the labile β-lactam ring, using low cost and environmentally benign chemistry. From the introduction of the first semi-synthetic penicillin it was recognized that enzymatic cleavage should be the process of choice, and culture collections and environmental isolates were extensively screened for suitable enzymes. The original GSK process produced 6-aminopenicillanic acid from penicillin V rather than penicillin G, since penicillin V acylase was discovered first.[55]

As several companies independently isolated penicillin G acylases, penicillin G became the starting material of choice. An enzyme to remove the D-α-aminoadipoyl side chain of cephalosporin C proved to be far more elusive, and a compromise process was developed. Transformation of the side chain *via* D-amino acid oxidase and spontaneous reaction to glutaryl-7-ACA provides a substrate for a glutaryl-7ACA acylase.[56] This two-step process, requiring an air-sparged oxidase reaction, was originally deemed too complex and costly for practical use at GSK. Its application was ultimately driven by the need for a more benign process when replacing the original 7-ACA manufacturing plant, by which time several other manufacturers had commercialized the process and enzymes.[57]

It is unlikely that the commercial hydrolase screening kits available to synthetic chemists would provide an enzyme for any of these applications. Whether wild-types, or mutants "diversified" in substrate specificity through directed evolution, these are generally lipases, esterases and proteases originally selected for the needs of the food and detergent industries. Our penicillin and cephalosporin acylases were all first isolated through process specific screening, and later classified as members of the Ntn-hydrolase superfamily, characterized by distinctive structure and complex biology.[58] Although screening kits have some notable successes, failures to find "hits" for our desired reactions are rarely publicised. Established culture collections, environmental screening and the newer technologies of metagenomics are equally valuable sources for hydrolases to act on more complex structures. With the demise of natural products screening, such sources may no longer routinely be available within the pharmaceutical industry.

Despite reports of "beneficial" mutations of penicillin acylases, to the best of our knowledge the enzymes used for commercial production of 6-APA are well established wild-types, albeit now manufactured with high activity and purity as recombinant proteins.[59] Since yields and cycle times are acceptable from these "natural" enzymes, the main factor driving process economics is longevity and reusability of the biocatalyst, which has been addressed through the development of enzyme immobilization technologies.[60] A typical process will reuse the biocatalyst for many hundreds of cycles. The pH controlled reaction is run in a stirred tank, rather than a column, hence rapid recycling of the supported catalyst into the next batch is a challenge for the engineering and operation of the plant and process. We anticipate that any future high volume process to a low value product will take a similar form. Higher value and lower volume products may drive development of more highly active, single-use enzymes, avoiding cost and complexity of immobilization.

A more recent development is the use of penicillin acylases in reverse hydrolysis mode to manufacture β-lactam antibiotics such as cefalexin, ampicillin and amoxicillin. Although there is some evidence that synthesis/hydrolysis ratios may be improved through

protein engineering, we understand that existing commercial processes use wild-type enzymes.[61] The key to this synthesis process has been process optimization based on thorough understanding of the reaction kinetics, as well as optimized enzyme immobilization.[62]

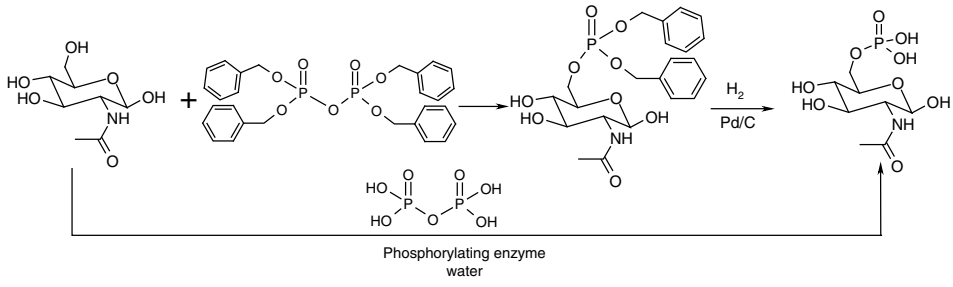
For production of 7-ACA from cephalosporin C, the situation has been somewhat different. Various industrial and academic research teams have pursued the goal of a simplified, lower cost, single enzyme step using a true cephalosporin C acylase. In addition to various programs to isolate such an enzyme from nature, attempts have been made to modify either penicillin G acylase or glutaryl-7-ACA acylase by directed evolution.[63,64] At GSK we isolated an enzyme with weak cephalosporin C acylase activity from a strain of *Pseudomonas vesicularis*, a result of screening over 100 000 environmental isolates.[65] By the time GSK ceased internal manufacture of 7-ACA the gene encoding this enzyme had been cloned and subjected first to site-directed mutagenesis, then directed evolution, to the point where yield and conversion rate approached the two step process (unpublished results). Today, at least one major producer finally claims to use a single enzyme, of undisclosed origin, for routine commercial manufacture of 7-ACA.

Although hydrolase reactions are hardly novel or exciting chemistry, they provide a valuable means to manipulate large and complex molecules without protection/deprotection. The role of Ntn hydrolases in β-lactams manufacture should remind us of the need for broader access to biological diversity, given the likely limitations of commercial hydrolases for manipulation of more complex molecules. Although protein engineering offers a means to diversify enzyme substrate range for a group of familiar enzymes, it is unlikely this will rival the full range of diversity to be found in nature. Compared with the lengthy development timescales of the past few decades, modern automated high throughput screening and molecular biology should allow rapid selection and development of enzymes from undefined natural sources. It is also useful to note the benefits that have been gained through efficient design and optimization of apparently simple biocatalytic processes, and in particular the key role of enzyme immobilization, a technology that appears to be going out of fashion in our industry.

1.5.2 Preparative Use of Phosphatases and Transglycosylases – LibraGen

LibraGen specializes in the development of innovative bioprocesses based on biocatalysis for the fine chemicals, cosmetics and pharmaceutical industries. One of LibraGen's key assets is its ability to look for high performance enzymes in bacterial populations that have not previously been explored and convert them into production tools. By combining the skills needed to go from laboratory-scale to pilot production, LibraGen is fulfilling a market need by giving its customers high performance and competitive synthesis solutions.

The chemical synthesis of N-Acetyl-D-glucosamine-6-phosphate (NAG-6P), the human biosynthetic precursor of hyaluronic acid, can be performed by the protection of N-Acetyl-D-glucosamine with tetrabenzyl pyrophosphate.[66] After debenzylation of the resulting dibenzyl phosphoryl moiety under hydrogenolysis conditions NAG-6P is obtained (Scheme 1.5.2). Although the synthesis proceeds smoothly and the product can be obtained in good yields and purity, this reaction is far from being environmental friendly.

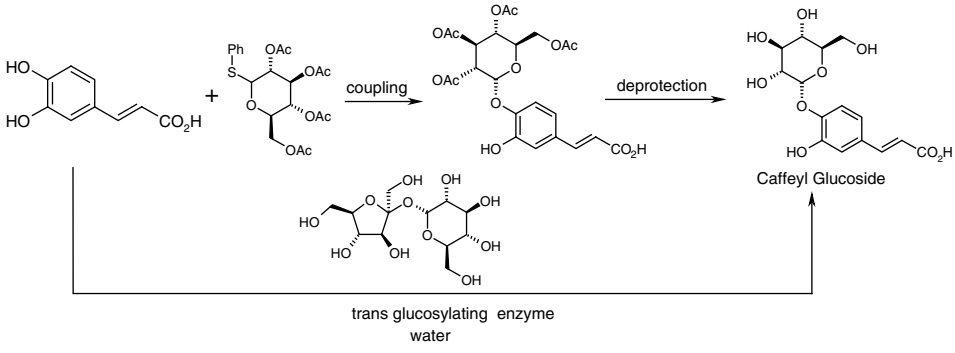


Scheme 1.5.2 *Comparison of chemical and enzymatic routes for the synthesis of N-acetyl-glucosamine-6-phosphate.*

Enzymatic synthesis of such phosphorylated aldohexoses can be performed with kinases (EC 2.7.1: phosphotransferases) that mainly use ATP as a source of phosphate, thus releasing ADP. Some processes for the production of D-glucose-6-phosphate with *in situ* ATP regeneration have been developed up to commercial scale,[67] but the main drawback of these approaches is the elevated price contribution of ATP to the total cost of the final product. Therefore we developed a biocatalytic alternative that uses a cheap source of phosphate (pyrophosphate) and a phosphotransferase enzyme (EC 2.7.1.63; Scheme 1.5.2). This results in a reduced environmental and economical impact when compared to the chemical procedure such as:

- The route is shorter: one-step conversion, without the need for a deprotection step.
- It uses a cheaper source of phosphate.
- Atom efficiency is increased: introduction of a phosphate moiety (molecular weight 97) requires a reagent with a molecular weight of 178 for the biocatalytic route compared to 538 for the chemical route.
- It can be performed in standard equipment whereas the chemical reaction needs special high-pressure equipment.
- It requires no hazardous chemicals or organic solvents that are required in the chemical route. For example, no residual palladium levels need to be checked to comply with health and environmental regulations.

The chemical manipulation of sugars is often heavily dependant on the use of protecting groups that leads to lengthy and mass-inefficient synthetic routes. In contrast, the highly selective nature of biocatalysts can obviate the need for protection, thus dramatically reducing the number of synthetic steps, timelines and waste. For example, the three-step chemical route to caffeic acid-alpha-D-O-glucoside, a skin anti-photo aging compound, includes a protection/deprotection sequence, followed by the purification of the desired anomer (Scheme 1.5.3). In contrast, a single step, stereo and regioselective synthesis could be performed in the presence of glycosyltransferases, able to transfer the sugar moiety of a sugar nucleotide (UDP-glucose) to an acceptor. These enzymes, which contribute in the synthesis of secondary metabolism in plants, have broad acceptor substrate specificities.[68] Nevertheless, this approach is impaired by the very high cost of the sugar nucleotides which require *in situ* regeneration.



Scheme 1.5.3 *Comparison of chemical and enzymatic routes for the synthesis of caffeic acid glucoside.*

To enable a cost-effective synthesis of caffeic acid-alpha-D-O-glucoside, a novel route was designed by Libragen with complete stereo- and regioselective control, by using a transglycosylating enzyme (EC 2.4.1.5) which uses sucrose as an inexpensive glucose donor (Scheme 1.5.3). Caffeic acid is recognized by the enzyme as a non-conventional substrate instead of glucose, and the reaction generates, in a single step, the desired diastereomer which can be easily purified from the non-glycosylated pyrocatechol (see Chapter 9.1 for experimental details).

The advantages of this biocatalytic route are numerous:

- Use of cheap sucrose instead of peracylated thioglucosides as glycosylating reagent[69]
- One-step process instead of two steps
- Perfect regioselectivity at the 4-position without need for protection
- Perfect stereoselectivity at the anomeric position
- Use of water as a solvent and no hazardous chemicals

These two examples, which are currently running at a scale of 10–100 kg per batch, clearly show that biocatalysis can offer a feasible alternative to chemical synthesis in terms of economics, environmental impact and ease of handling.

1.5.3 Biocatalytic Desymmetrization and Dynamic Kinetic Resolution (DKR) Processes – AstraZeneca

Biocatalysis is a widely used technology for the production of many varied targets; it will be obvious from the examples in this chapter, that a very large proportion of these biotransformations are directed at the production of high purity chiral molecules. This section describes two biotransformations used to make chiral APIs.

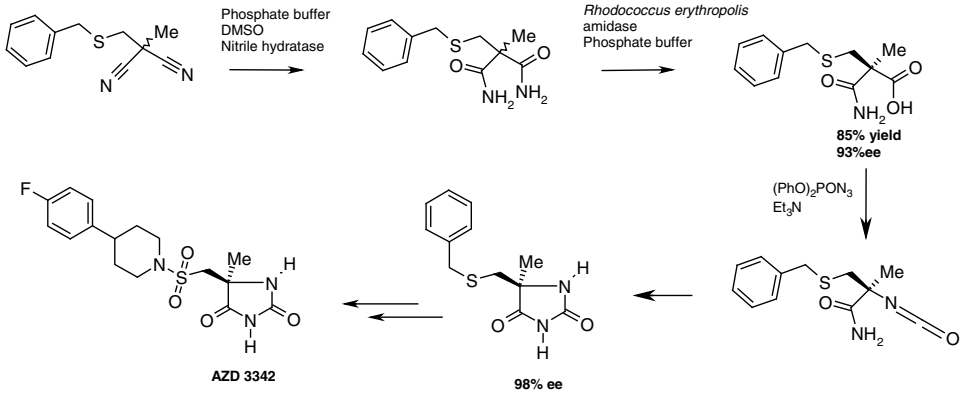
Whilst the use of hydrolytic enzymes for kinetic resolutions is still widely applied, it is becoming more common to see more productive processes being devised for the preparation of chiral materials. These typically revolve around the desymmetrization of prochiral starting materials[70], and the use of dynamic kinetic resolution.[71] In the former, the ability of an enzyme to recognize one face of a prochiral compound is employed.

In principle, a 100% yield of a single enantiomer can thus be obtained. Of course, in kinetic resolutions the unwanted enantiomer can sometimes be racemized offline and reused. This does however result in extra process steps, use of reagents and solvents and energy consumption. A more elegant solution is to devise reaction conditions under which the desired product enantiomer is configurationally stable, but the unwanted enantiomer spontaneously racemizes and can be eventually converted to a single enantiomer product in a yield much higher than the 50% maximum allowed by a kinetic resolution. Such processes are more commercially attractive than simple kinetic resolutions since they have better throughput, lower costs and generally have much better environmental metrics.

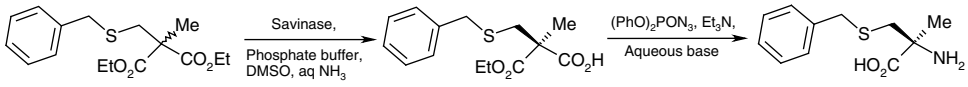
Chiral amino acids and hydantoins with quaternary chiral centres are common motifs in many drug candidates. Of course, with such chiral centres, synthesis by diastereomeric salt resolution, chromatography, or enzyme-catalyzed kinetic resolution leads to a situation where the unwanted enantiomer cannot be reused, thus rendering this synthetic strategy rather wasteful. These synthons are ideal candidates for preparation by enzymic desymmetrization of simple prochiral starting materials.

The example described here also highlights another important application of enzyme technology. Most synthetic organic chemists entering the field of biocatalysis view enzymes only as catalysts suitable for chiral transformations. Apart from chiral recognition, hydrolytic enzymes can be very useful for regio-selective transformations, manipulations under very mild conditions and highly selective transformations of functional groups. For example, the key chiral (*S*)-hydantoin synthons of AZD 3342, being developed by AstraZeneca as an MMP inhibitor useful in the treatment of COPD, could be prepared via the selective hydrolysis of some prochiral materials (Scheme 1.5.4).

A nitrile hydratase was used to control the chemoselective hydrolysis of a bisnitrile to give only the biscarboxamide, a transformation that is very difficult to achieve using standard chemical manipulation. This was then subjected to desymmetrization by an amidase from *Rhodococcus erythropolis* to give the intermediate carboxamide-acid in high yield and 93% *ee*. The *ee* reflects the imperfect enantioselectivity of this enzyme, but no attempt was made to find an alternative, since the ee was upgraded in downstream



Scheme 1.5.4 *Synthesis of chiral quaternary amino acids and hydantions via desymmetrization of simple meso compounds.*



Scheme 1.5.5 *Synthesis of chiral quaternary amino acids via desymmetrization of simple meso compounds.*

manipulations. This intermediate was surprisingly prone to decarboxylation, so was converted further as soon as prepared. Reaction under standard Curtius conditions yielded the intermediate isocyanate, which spontaneously cyclized to give the (*S*)-hydantoin in 93% *ee*. The product was crystallized up to 97–98% *ee* and could then be converted on to AZD 3342 (see Chapter 8.7 for experimental details).

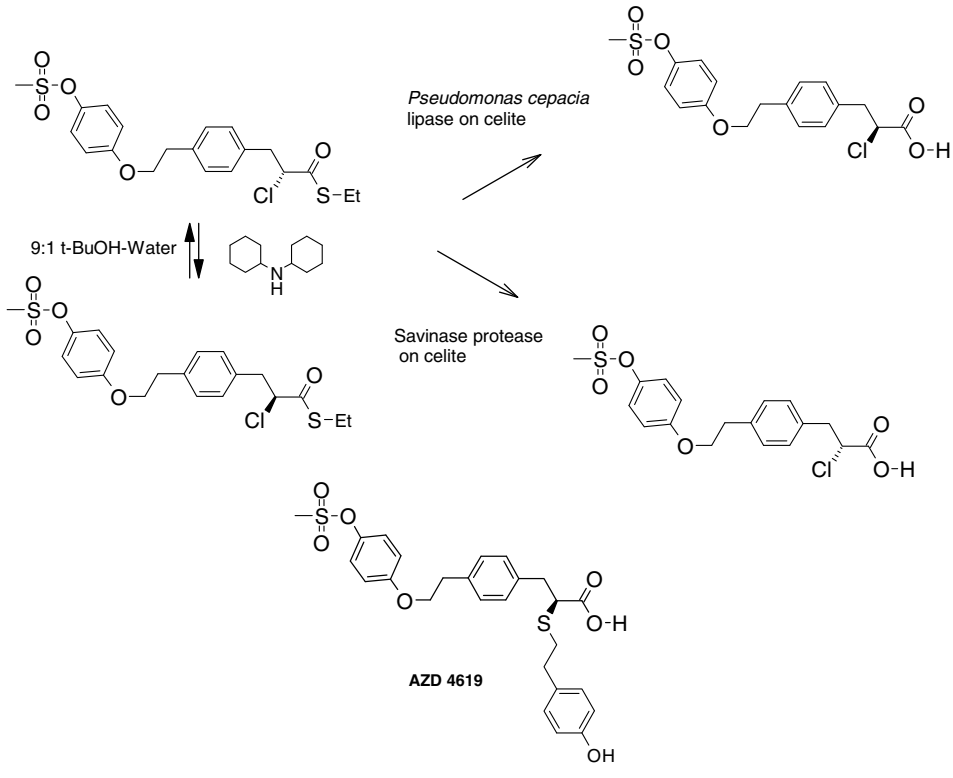
Since hydantoins and amino acids are readily interconverted, another prochiral substrate suitable for the synthesis of AZD 3342 is the corresponding diester. In this case, hydrolysis with a protease lead to the mixed acid-ester (Scheme 1.5.5), which could be converted to the desired amino acid via a Curtius rearrangement and hydrolysis as shown. Pig liver esterase produced the opposite configuration, (*R*)-enantiomer in ~80% *ee*.[72]

For the desymmetrization of both bis-amide and bis-ester, reaction times were quite long at ~4 days, and this reflects the slow rate of enzymic hydrolysis due to the bulky nature of these substrates. A really positive bonus for this approach to chiral quaternary amino acids and hydantoins would be the discovery or evolution of hydrolases that can convert this type of substrate at a faster rate. Unfortunately, this is not currently seen as an attractive area for research in academia.

DKR is another powerful tool for the efficient synthesis of single enantiomer molecules. For a successful DKR, the desired enantiomer product of the enzyme-catalyzed step must be configurationally stable, whilst the unreacted, undesired enantiomer is converted back to the racemate *in situ*. When the desired product is a chiral acid, hydrolysis of a racemic ester substrate with base-catalyzed racemization of the residual ester is an attractive option. Of course, the catalyst(s)/reagents chosen to effect the racemization have to favorably co-exist with the biocatalyst and, for example, not lower enantioselectivity or inhibit/denature the enzyme. This is illustrated by the DKR of α-chlorophenylpropionates to make intermediates for the PPAR agonist, AZD 4619 (Scheme 1.5.6).

Using α-chlorophenylpropionic esters, reasonably successful kinetic resolutions were found to produce the corresponding chiral acids. Typically the (*S*)-acid was afforded with lipases and the (*R*)-acid with protease enzymes. The residual esters could be racemized off-line and reused, however the strength of base needed for racemization was incompatible with a hydrolytic enzyme in the same vessel, and rapid enzyme deactivation was observed. Substitution of the ester OR for the corresponding thioester, SR, has been reported to greatly increase the acidity of the α-protons by 2–3 pK units.[73] This was observed with the α-chlorophenylpropionic thioethyl ester.

Crucial to the success of the DKR is selection of the correct base. This needs to be determined in the reaction solvent, *not by comparison of pKa data in water*. The results for the racemization of pure (*R*)-thioethyl ester are shown in Table 1.5.1. Bases like 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1,1,3,3-tetramethylguanidine (TMG) cause rapid chemical decomposition through elimination of HCl to give phenylacrylates. The rate of racemization by simple amine bases in the 9 : 1 t-BuOH-H_2O solvent mixture is



Scheme 1.5.6 *Synthesis of chiral α-chlorophenylpropionic acids via dynamic kinetic resolution of rac-thioesters.*

primary > secondary ≫ tertiary. However, primary amines could partake in a Sn2 displacement with the α-chloro substituent and so a secondary amine, dicyclohexylamine (DCHA), was selected as a good compromise (Table 1.5.1).

The results of the DKRs are shown in Scheme 1.5.6. The (*S*)-α-chloro acid is produced in high ee using *Pseudomonas cepacia* lipase on celite (Amano PS-D). This enzyme is highly enantioselective (98% *ee*; see Chapter 8.1 for experimental details).

The (*R*)-enantiomer was produced using the protease Savinase, where the ee value of 90% reflects the less than perfect enantioselectivity of this enzyme. Having a fast racemizaton step is crucial to always maximize the concentration of the desired enantiomer

Table 1.5.1 *Racemisation of (R)-thioethyl ester with various bases in 9 : 1 t-BuOH-H_2O.*

	% ee of thioester after treatment with different bases							
Time/h	No base	DABCO	Oct_3N	t-$BuNH_2$	DCHA	DBU or TMG	Proton sponge	$OctNH_2$
1	100	73	92	32	50	Decomp	100	0
18	100	0	29	0	0	N/A	96	0

ester, and hence overall *ee* of the process. If a less effective base is employed with Savinase, the *ee* of the resulting acid can drop to ~50%. When the project was terminated the search was on for a more (*R*)-selective biocatalyst.

1.6 Industrial Biooxidation and Reduction

Chiral secondary alcohols are important intermediates for the fine chemical and pharmaceutical industries. Asymmetric reduction of ketones using dehydrogenase and reductase enzymes represents an efficient and proven approach for producing chiral alcohols in high yield and chiral purity.[74] A wide range of reductase enzymes are present in nature and large numbers of isolated enzymes capable of reducing a broad range of substrates are now widely commercially available. Whilst addition of costly nicotinamide cofactors is necessary for the reaction to proceed to completion, methods of recycling these cofactors are now firmly established and represent no barrier to scaling up these reactions.[75]

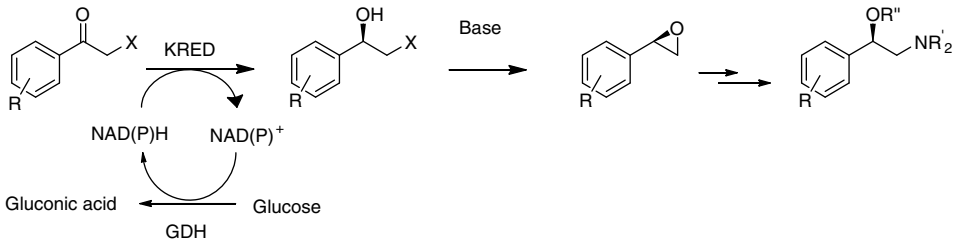
Biocatalytic oxidation approaches to chiral secondary alcohols have been adopted by industry to a lesser degree.[76] Biooxidations using fermentation processes are used in niche applications since they allow the direct introduction of oxygen into non-activated carbon atoms in a regio- and stereoselective manner, a reaction that is difficult or impossible to achieve by synthetic organic chemistry. Industrial biooxidation using isolated enzymes is at present mostly limited to oxidases and dehydrogenases. Whilst cytochrome P450 monooxygenases show great potential,[77] their functional complexity, low activity, and limited stability have so far limited their use industrially in high volume processing.

1.6.1 Approaches to Chiral Secondary Alcohols – Dr Reddy's, Chirotech

Chirotech has an established platform of complementary technologies to allow the efficient synthesis of secondary alcohols. Biocatalysis plays a key role here. Large collections of both in-house and commercially available enzymes such as ketoreductases (KREDs) have been built up, all arrayed in 96-well plates to facilitate fast screening. Numerous biocatalytic processes have been developed and applied at manufacturing scale using standard batch reactors. When deciding which particular technology to use we consider a number of factors including the cost, efficiency of the approach, freedom to operate the route, catalyst availability and the R&D/scale up effort required. Most importantly we have to comply with the timing expectations of our customers. The examples that follow describe processes to two single isomer secondary alcohol products that have been developed in our facilities – one utilizing a ketoreductase enzyme and one an oxidase enzyme.

Chiral 1,2-amino alcohol moieties are commonly found in clinical drug candidates, for example, adrenoreceptor modulators used for treatment of asthma and related respiratory disorders. These moieties are conveniently prepared by coupling an enantiomerically pure styrene oxide with an amine component. An attractive option for preparing the styrene oxide synthons is biocatalytic asymmetric reduction of an easily prepared phenacyl halide, followed by ring closure (Scheme 1.6.1).

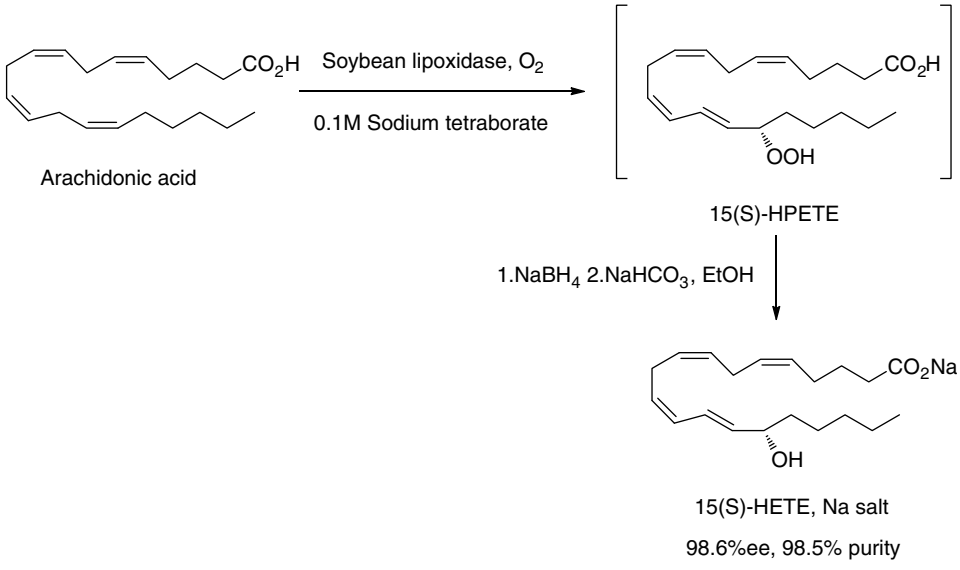
(*R*)-3-Chlorostyrene oxide is a potential synthon for a number of clinical candidates including Solabegron and Amibegron hydrochloride. Screening of 3-chlorophenacyl chloride against our collection of commercially available KRED enzymes revealed a



Scheme 1.6.1 *KRED catalysed preparation of amino alcohol intermediates.*

number of hits, the best of which was optimized to proceed at 100 g/L substrate concentration to give 2-chloro-1-(3-chlorophenyl)ethanol of >99.5% *ee* in >90% yield. Cofactor recycling was achieved using glucose dehydrogenase, allowing for the use of a concentration of 0.25 mM $NADP^+$ in the reaction. The halohydrin product was used directly in the next step and cyclized using base to give (*R*)-3-chlorostyrene oxide with >99% *ee*. This material was purified by distillation to give a final product of >99% purity. The suitability of this procedure to large scale manufacture along with the ready availability of the enzyme allowed the process to be quickly scaled up using standard equipment to 100 kg scale without any issues.

Chirotech was required to develop a robust synthesis of (5*Z*,8*Z*,11*Z*,13*E*)(15*S*)-15-hydroxyeicosa-5,8,11,13-tetraenoic acid (15(*S*)-HETE, Icomucret) to support clinical trials.[78] As this was potentially a highly potent treatment for dry eye syndrome the volumes of API required for the PIII trials were <100 g. After consideration of a number of potential routes, a robust synthesis of the single isomer secondary alcohol product was established, based on a previously reported biooxidation process (Scheme 1.6.2).



Scheme 1.6.2 *Biooxidation of arachidonic acid.*

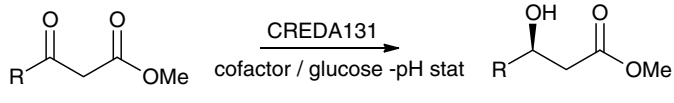
Treatment of readily available arachidonic acid with commercially available soybean lipoxidase in 0.1 M sodium tetraborate buffer under 35 psi oxygen pressure resulted in formation of the hydroperoxide, 15(*S*)-HPETE. The use of pressure in the reaction was key to obtaining decent volume efficiency (20 g/L), whilst running at low temperature (0–5 °C) was necessary to obtain the product in high enantiomeric excess. The unstable peroxide product was not isolated and addition of sodium borohydride to the reaction mixture reduced the hydroperoxide to 15(*S*)-HETE, an oil, which was then purified by column chromatography. 15(*S*)-HETE, sodium salt was prepared as a white solid by treatment of an ethanol solution of HETE with aqueous sodium hydrogen carbonate giving product with >98% enantiomeric excess and >98% chemical purity in an overall 51% yield from arachidonic acid.

1.6.2 Application of Alcohol Dehydrogenases and P450 Oxidation – Almac

The use of biocatalysis continues to dominate the news due to its green credentials, potential to reduce cost and increase purity profiles. Biocatalysis is certainly the technology of choice at Almac. Biocatalysis at Almac was born of industry stresses and the urgent need for solutions to chemical and chiral problems. It has been shown time and time again that speed of enzyme identification and scale-up is critical in demonstrating to customers that biocatalysis will compete with other technologies with respect to cost of goods and cost of development. It is these benefits that are accelerating the rise of biocatalyst acceptance into the chemist's toolbox at Almac. Key technologies in Almac's enzyme portfolio include hydrolase, oxidoreductase and transferase enzymes. As part of this discussion, highlights of carbonyl reductase and P450 enzymes will be included.

The advantages of carbonyl reductase (CRED) enzymes are plainly obvious; high substrate affinities translating to high activities, tolerance to organic co-solvents, tolerance to increased temperatures and the availability of practically applicable and economical cofactor recycle systems. Matsuda et al. recently published an exhaustive review, with 180 references, covering all aspects of carbonyl reductase technology.[79] Perhaps the most important advances made with this enzyme class have come through the various enzyme evolution approaches available which allow the tuning of a particular biocatalyst to a specific process.[80,81] Generally speaking, it is through these avenues that carbonyl reductase enzymes become most cost effective for large-scale industrial pharmaceutical manufacturing.

Almac uses proprietary CRED enzymes, discovered and developed in-house, for the reduction of ketones and both the two-enzyme and alcohol (isopropyl alcohol) systems for cofactor recycle have been applied in multi-kilogram synthetic processes. The reduction of the beta-keto ester in Scheme 1.6.3 using CRED A131, was developed to run in 5 vol



Scheme 1.6.3 *CRED reduction of a hydrophobic ketone using a two-enzyme system.*

buffer and 1 vol DMSO to aid in the mass transfer of the hydrophobic ketone into the aqueous media and interaction with the enzyme. Just 1 g of enzyme was sufficient to turn over 1 kg of product.

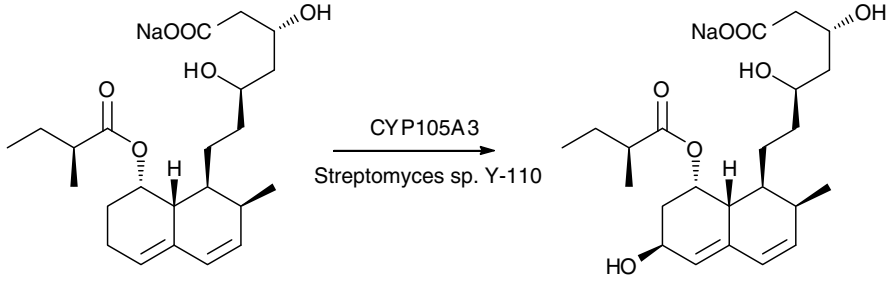
This process was stress-tested during process development, revealing that the enzyme was capable of tolerating a pH range of 6.3–7.8 at temperatures of up to 38 °C. The pH was adjusted continuously using an external circulation loop that added caustic *via* an automated pump.

This particular enzyme has since gone through several rounds of evolution to introduce isopropyl alcohol (IPA) tolerance. A key advantage of the one enzyme system is a drop in fermentation costs due to the preparation of a single enzyme. In addition, no pH adjustment is required and simple batch reactors with no modifications can be used. An extreme example is shown in Chapter 4.1, where Almac has developed an immobilization technique that encapsulates the enzyme and cofactor allowing the preparation of "woody alcohol" through bioreduction to be performed in neat IPA. This has additional advantages of facile processing and the minimization of the reaction volume. The product generated is a precursor to the high volume fragrance ingredient, woody acetate, whose current use worldwide is in the region of 1000 metric tonnes per annum.[82]

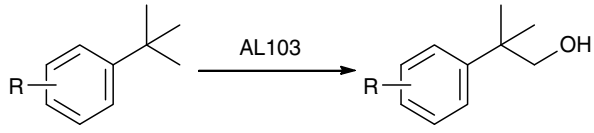
Cytochrome P450s are a superfamily of heme containing monooxygenases that can catalyze a wide range of synthetically challenging reactions. Their functions in nature range from carbon source degradation and metabolite elaboration for prokaryotes, lower eukaryotes and plants, to detoxification of xenobiotic compounds in insects and mammals, including humans.[83] Structurally, they consist of an NAD(P)H dependent heme containing oxygenase domain which is either loosely associated with, or physically linked to, a reductase domain which can be comprised of various electron transfer components.[84]

Cytochrome P450s have been an extremely attractive enzyme class to synthetic chemists for many years simply because the reactions catalyzed by P450s are very difficult, if not impossible, to carry out by traditional chemical means. Unactivated carbon hydroxylation reactions in particular offer great potential for accessing valuable metabolites or perhaps even NCEs for the pharmaceutical industry (Scheme 1.6.4).[85–87]

To date, unfortunately, their use has been limited for a myriad of reasons including low activity, low stability, limited organic solvent tolerance and expensive cofactor requirements. Despite increasing academic research focused on overcoming these issues in isolated enzyme models, the use of whole cell systems has proved to be the best method for the integration of these complex enzymes into organic synthesis.[88]



Scheme 1.6.4 *A prime hydroxylation example from BMS/Sankyo.*



Scheme 1.6.5 *P450 mediated oxidation using Almac's proprietary biooxidation technology.*

A typical example of Almac's work in this area is described in Scheme 1.6.5.

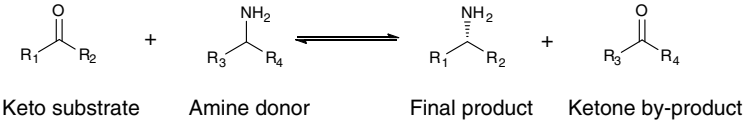
Following a successful screening project which identified an active P450 enzyme (AL-103) for the transformation shown in Scheme 1.6.5, Almac were contracted to generate 15 g of the API metabolite. Expression of the desired P450 in *E. coli* enabled the growth of the required biomass in a 150 L fermentation vessel after brief optimization at shake flask scale. A finding of particular note was the large beneficial effect of supplementing the growth medium with $Fe(III)Cl_3$ during the expression phase. Process development studies indicated that the dissolved oxygen level and the substrate addition rate were key parameters to control in order to facilitate the accumulation of acceptable product concentrations. Specifically, it was found that dissolved oxygen levels needed to be maintained above 70% while an addition rate of 25 $mL.h^{-1}$ of a 0.5 $g.L^{-1}$ solution of substrate in DMSO could not be exceeded. Higher addition rates resulted in the formation of an insoluble polymorph that was completely incompatible with the biocatalyst. The overall yield for this process following purification by filtration through a silica pad was 61%.

1.7 Industrial Application of Transaminases – Cambrex

ω-Transaminases are enzymes which catalyze the reversible interconversion of ketones to amines. Celgene developed transaminase technology in the early 1980s which can be used for gram to tonne scale production of chiral amines from ketones with a high degree of stereoselectivity (Scheme 1.7.1).

The technology, including specific enzyme strains and patents, was later purchased by Cambrex. It is used today as the method of choice when producing chiral amines in a cGMP manufacturing environment.

In contrast to classical chiral resolution, transaminase technology can, in theory, produce chiral amines in quantitative yield if the equilibrium is shifted toward the desired amine. For example, when inexpensive isopropylamine is used as an amine donor, the acetone by-product produced can easily be removed by distillation in an effort to drive the



Scheme 1.7.1 *General reaction scheme showing the conversion of a keto substrate to the corresponding chiral amine.*

equilibrium.[89] In some cases the equilibrium between ketone and amine is so unfavorable that the reaction stops at very low conversion. This can be the case even with excess amine donor and removal of the formed ketone, both of which are methods often implemented in an attempt to drive the equilibrium toward higher conversion.

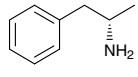
As the equilibrium cannot be changed by enzyme engineering another option is to run the reaction in resolution mode (see Section 1.3). In this case one starts with the racemic amine and removes the unwanted enantiomer. This can also prove useful when an enzyme selective for the desired enantiomer is not available, as out of all known ω-transaminases only a few show (*R*)-selectivity.

Although these ω-transaminases have been used in industry for many years there remain significant limitations both in the enzymes themselves and the processes which utilize them. As a result, work continues on developing improved enzyme variants and on optimizing process conditions. Early on, substrate concentration and thermostability were identified as major limitations to the utility of ω-transaminases. Enzyme engineering has therefore been explored for many years in order to develop enzyme variants that meet specific needs.[90,91] In those cases where the structure of the enzyme is known the required mutations can sometimes be predicted in order to reach improved enzyme characteristics. By using the known structures of related enzymes it is sometimes possible to provide a relatively good picture of the enzyme of interest even without a crystal structure. Using such homology structures, one can study the enzyme and predict which amino acids to target in the enzyme engineering work.

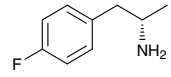
One example of a project where this strategy was used successfully was the development of a more selective ω-transaminase for the large scale production of D-amphetamine (Figure 1.7.1). The previous enzyme provided material with an *ee* around 98.5%. However, an enzyme was desired which could provide enantiomeric excess of greater than 99.0%.

An initial study found a thermostable variant which could be used at elevated temperature to provide higher reaction rates, however the selectivity for D-amphetamine was too low. This variant served as a starting point and molecular modelling was applied in a study where six amino acids were selected for mutagenesis work. As a result, 13 mutants (single and double) were prepared and tested. Out of these, one of the double mutants was found to give an *ee* of 99.3% and be stable at up to 40 °C. This body of work provided a commercially viable enzymatic process to produce D-amphetamine on a tonne scale (unpublished results).

In another project the aim was to develop a mutant of an (*S*)-selective ω-transaminase from *Arthrobacter citreus* (variant CNB05-01) to give higher stereoselectivity towards the preparation of 2-(4-fluorophenyl)-(*1R*)-methylethylamine from 4-fluorophenylacetone (>99.5% *ee* compared to 98% *ee* of the original variant; Figure 1.7.1). Studies of the



D-amphetamine



2-(4-fluorophenyl)-(*1R*)-methylethylamine

Figure 1.7.1 *Transaminase products.*

homology structure, followed by site-directed mutagenesis at three points and activity tests resulted in variants with higher stereoselectivity. Interestingly, alteration of one of the selected amino acids, V328, resulted in a mutant which showed reverse enantiopreference (58% *ee*). This change turned out to be substrate specific, giving reverse enantioselectivity towards 4-fluorophenylacetone but not 4-nitrophenylacetone. This result was also rationalized by molecular docking simulations.[92]

Depending on what is to be optimized it is not always possible to predict which position on the enzyme to target. In this case it is necessary to make a large number of mutants and screen each for improved variants. When this is the only alternative, a mutant library can be prepared by a random mutagenesis method such as ep-PCR and screened according to the method described in Matcham *et al.*,[90] where up to 5000 mutants can be screened per week without any extra equipment. The pool of selected mutants can be further narrowed through a second screening in microtiter plates where the specific reaction conditions can be tested and analyzed by HPLC.

1.8 Biocatalyst Discovery and Improvement

Widespread utilization of biocatalysis is still frequently relegated to the method of last resort, particularly in the earlier phases of drug development. Improved enzyme screening kits are beginning to change this view, but even the most established kits are still incapable of hitting the number of substrates that might be expected from a chemical reagent. Emerging biocatalyst classes hit even fewer substrates and so are seldom considered by chemists. Directed evolution technologies and metagenomic screening represent key technologies that are rapidly expanding the substrate spectrum of the biocatalyst toolbox.

1.8.1 Directed Evolution Technologies – Codexis

The exquisite selectivity of enzymes provides an inspiration for chemists pursuing the development of highly selective chemocatalysts. If enzymes had evolved in nature to be highly stable and active under chemical process conditions, the catalytic world as we know it might have been very different. However, the application of natural enzymes has been limited to only a few processes. The performance of natural enzymes is typically insufficient for chemical manufacturing processes where high substrate and product concentrations are required for economic reasons.[93] To overcome limitations in natural enzymes, biocatalytic process development has typically focused on enzyme stabilization and reuse via immobilization technology and reactor engineering. In the mid 1990s directed evolution technologies entered the biocatalysis world and customized enzymes are now routinely applied in various manufacturing processes.

Directed evolution technologies that are based on DNA shuffling formats[94] apply the principles of evolution at the laboratory scale as the genetic information that encodes enzymes of interest is modified iteratively to yield improved enzyme variants that are identified via high-throughput screening. By designing the best possible process and applying the corresponding reaction conditions to the HTP screens, catalysts are developed for practical, economic and 'green by design' manufacturing processes.

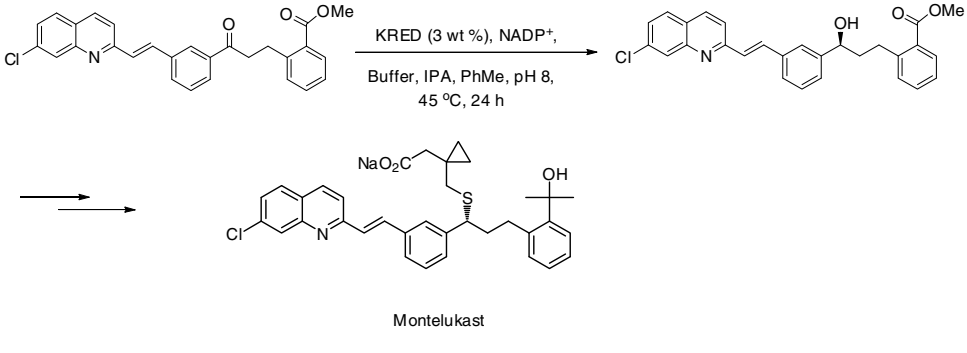
The development of directed evolution technologies over the past 15 years has been fast-paced. From hit-focused formats including error-prone PCR, as well as single and family shuffling formats, where the DNA of improved hits was either remutagenized or recombined, current formats are codon-focused. This change was made possible by the decreasing costs of DNA sequencing and DNA synthesis. As more detailed information became available from screening efforts higher quality enzyme libraries could be designed and generated. With better libraries, the screening throughput becomes less of an issue and more informative screens can be devised and implemented. This codon-based shuffling of mutations was first applied to an enzyme that is now used at commercial scale for the manufacture of an Atorvastatin intermediate.[95]

With more sequence data becoming available for hits (and non-hits) in each round of screening, the rapid and accurate synthesis of gene libraries became a bottleneck. Codexis developed technology to overcome this issue and enabled a new enzymatic process for the manufacture of Sitagliptin, a chiral amine for which no natural enzyme exhibited any activity.[96]

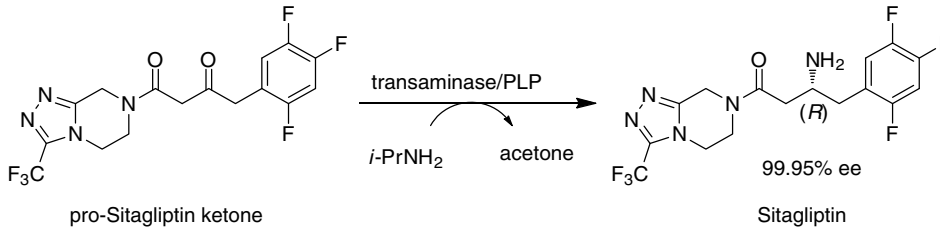
It is now apparent that any enzyme can be improved to function under chemical manufacturing conditions. In our laboratories we evolved enzymes to withstand isopropyl alcohol in concentrations up to 90%,[97] and temperatures up to 60 °C.[98] Enantioselectivity[80] is a highly evolvable trait as is regioselectivity.[99] The ability to engineer the substrate range of a single enzyme to accept very small substrates as well as very bulky ones, is truly remarkable and demonstrates how intricate the functioning of enzymes really is.[100]

We developed a range of ketoreductase-based (KRED) processes for the commercial scale manufacture of chiral alcohols in high enantiomeric excess. Many of these processes have been described previously and we emphasize two of these in this book. The ketone precursor to a chiral alcohol intermediate for Montelukast is only very poorly soluble. Nevertheless, when run under conditions where the rate of substrate solubilization is not-rate limiting and where the enzyme has a high affinity for the substrate, an economic process can be enabled via enzyme optimization (Scheme 1.8.1; see Chapter 4.4 for experimental details).[101]

In the second case, a small chiral primary alcohol was needed in high enantiopurity. After optimizing the enzyme, a kinetic resolution was enabled that gives the desired



Scheme 1.8.1 *Synthesis of the chiral alcohol for Montelukast.*



Scheme 1.8.2 *Biotransformation for converting pro-Sitagliptin ketone to Sitagliptin.*

product in high quality. Separation of the desired alcohol enantiomer from the undesired aldehyde enantiomer was achieved at high concentration on large scale (see Chapter 4.2 for experimental details).[102]

The direct synthesis of chiral amines from "bulky–bulky" ketones is a difficult but highly desirable reaction for process chemists.[103] In collaboration with scientists from Merck, we embarked on a program to develop a transaminase catalyst for the direct manufacture of Sitagliptin. After first creating a catalyst that exhibited barely detectable activity on this bulky substrate we then improved it over 25 000-fold to function efficiently under process conditions including 40+% DMSO and reaction temperatures $>50\,^{\circ}C$ (Scheme 1.8.2; see Chapter 2.2 for experimental details).[96]

Practical processes for the manufacture of chiral proline analogs using evolved amine oxidases have been developed.[104] In these processes, the enzyme needs to be not only highly resistant to the reactive imine product and trapping agents ($NaHSO_3$), it also needs to tolerate substantial gas-liquid interfaces resulting from air sparging, and the reactive oxygen species (H_2O_2) generated during the process. Similarly, Baeyer–Villiger monooxygenases (BVMOs) need to tolerate the oxidative environment as well as the presence of isopropyl alcohol which is used for co-factor regeneration.[105]

With these new enzyme engineering technologies all previous notions about the limitations of enzymes for large-scale use have been dispelled. The costs of enzyme optimization has decreased greatly over the past few years as with the new, codon-focused approach, the cycle time has decreased by 75% and the number of cycles, while still application dependent, has also decreased sharply. Advanced biocatalysts will continue to provide attractive options for the enablement of highly efficient processes that deliver economic advantages and environmental benefits.

1.8.2 Discovering Novel Enzymes from Untapped Biodiversity – LibraGen

Most enzymes that are currently used in biocatalysis were identified through cultivation of micro-organisms. However, it is estimated that only 1% of all existing micro-organisms can be cultivated using classical conditions.[106] Metagenomics offers the possibility to exploit the potential of non-cultivatable species by extracting the DNA from an environmental sample and subsequent cloning of the DNA in a suitable host-organism like *E. coli*.[107]

This approach has been used for the creation of a unique library of transaminases. DNA was extracted from different environmental samples. Purification and size fractioning gave

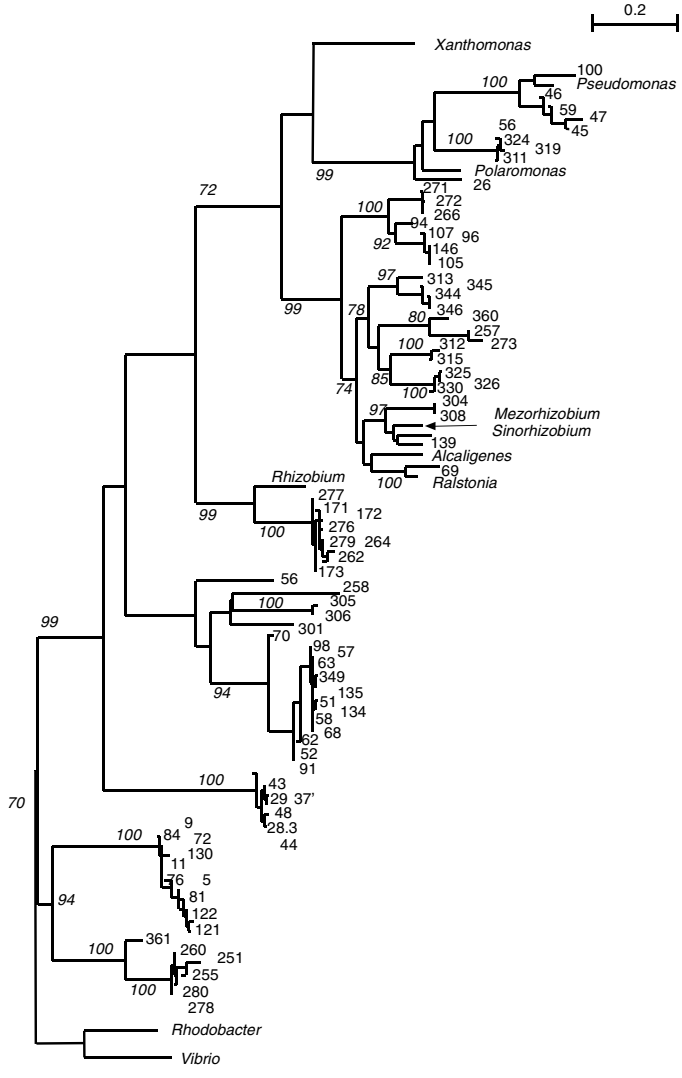


Figure 1.8.1 *Phylogenetic tree created with the sequences of the conserved domain beard by metagenomic transaminase genes.*[108]

30 kpb DNA inserts that were cloned into *E. coli*. The resulting metagenomic DNA library was screened by either using methylbenzylamine as sole nitrogen source or by using bioinformatic based degenerated primers. Fifty new transaminases were identified and analysis of their sequences showed that no redundancy was found with transaminases from public databases. The phylogenetic tree (Figure 1.8.1) exemplifies this variety: numbers represent the new transaminases, while names represent known transaminases in public databases.

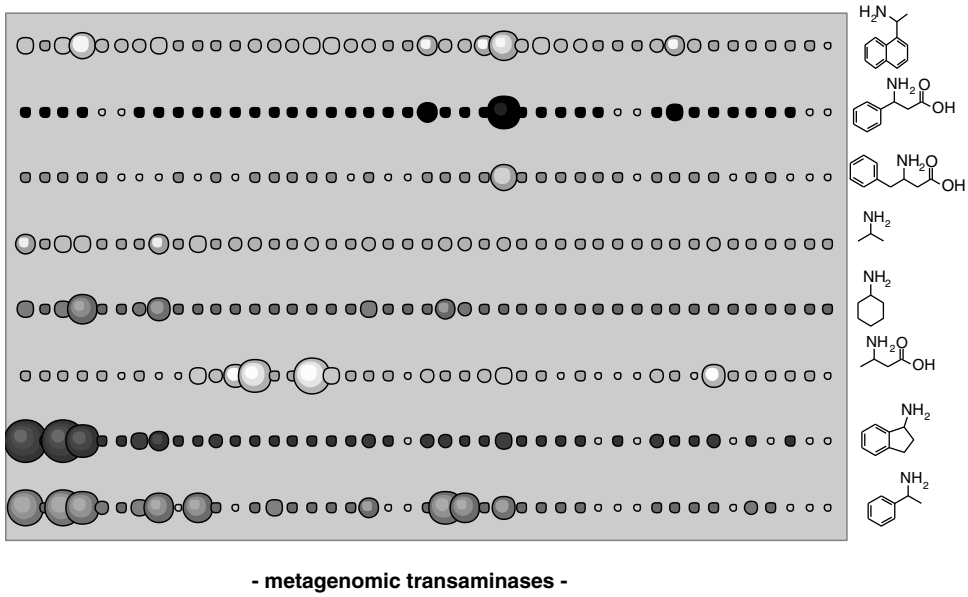


Figure 1.8.2 *Substrate mapping of new metagenomic transaminases on a variety of chemical substrates. Increased Bubble size correlates to higher activity of a metagenomic transaminase towards a given substrate.*

Having in hand this collection of transaminases, screening was performed in order to evaluate the activity of these enzymes for different substrates. As can be seen on the substrate map (Figure 1.8.2), for all tested substrates at least one suitable enzyme was found, showing that genetic diversity recovered from yet unexplored microbial diversity, can be converted into an efficient biocatalytic tool box for chemists.[108] This approach is a perfect example of synergies between molecular biologists and chemists in the design of more efficient production processes.

1.9 From Pathway Engineering to Synthetic Biology

Synthetic biology possesses enormous potential for the engineering of novel biological systems to provide sustainable and economically competitive production of a large array of compounds including chemicals, fuels, food, pharmaceuticals and polymers. With its roots within the established methodologies of classical pathway engineering in microbes, synthetic biology has now grown to represent the integration of biology, engineering and information technology, with the aim of increasing the speed and predictability in the design, construction and optimization of biosystems for practical industrial applications. Such biosystems may involve large engineered biosynthetic pathways or the co-ordinated expression of relatively few heterologous biocatalysts within a single microbial production system. Synthetic biology approaches also offer the potential to enable more rapid and efficient expression of single heterologous enzymes which to date cannot be easily expressed as active, soluble biocatalysts in a host organism of choice.

1.9.1 Pathway Engineering in Yeast – Sanofi

In the last 30 years, genomic sequencing, gene synthesis, molecular biology and pathway engineering has evolved from the artisanal to the industrial state.

Systematic genome sequencing (mammalian, insect, plant, fungal, bacterial genomes, etc.) has brought to light a wealth of new proteins (through their genes and cDNAs) interesting for the metabolic field with potential conversion activities. Gene synthesis has emerged and become faster, cheaper and able to prepare longer pieces of DNA up to the reconstitution of a bacterial genome.[109] Molecular biology has evolved from craftsman work in *Escherichia coli, Saccharomyces cerevisiae* and a few mammalian cells to cloning and expression in almost any kind of cells together with an immense set of tools.

Pathway engineering is benefiting from these advances. In the last decade, two complex molecules of pharmaceutical interest (hydrocortisone: a classical potent immuno suppressor and artemisinic acid: a precursor of Artemisinin an anti malarial compound) were biologically made in yeast (*S. cerevisiae*).[110] What makes these works important is that for the first time, an endogenous yeast substrate was transformed into an exogenous natural molecule using multiple conversion steps. Remarkably, the ergosterol pathway could be routed in two different directions, to a mammalian well known pathway or to a specific plant (*Artemisia annua*) pathway respectively (Figure 1.9.1).

In the hydrocortisone producing strains, the end product of yeast membrane synthesis, namely ergosterol is converted into a mammalian compatible sterol (campesterol) using a plant enzyme. This is then metabolized into hydrocortisone by a series of five reactions, including four challenging reactions dependent on mammalian P450s.

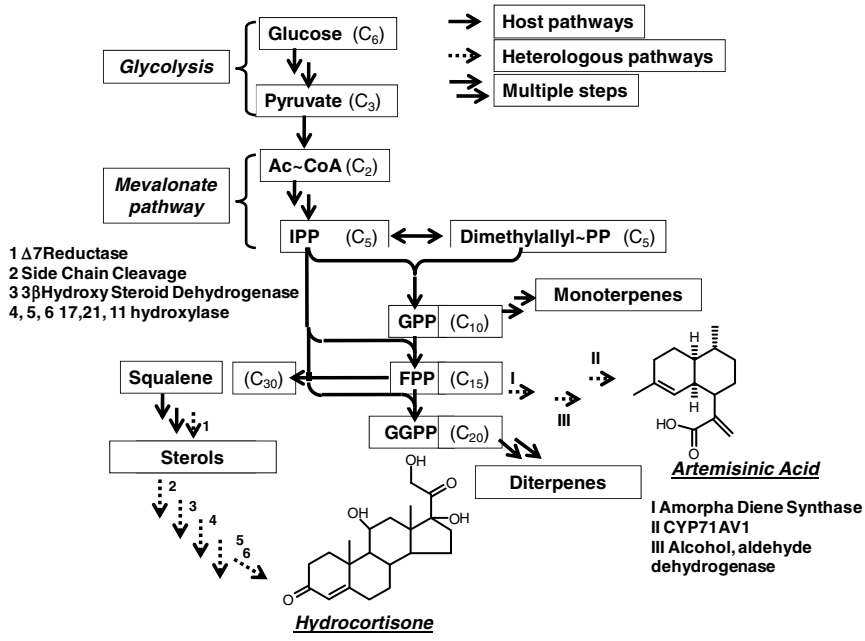


Figure 1.9.1 *Engineered routes to hydrocortisone and artemisinic acid in yeast.*

In the same vein, artemisinic acid is obtained from farnesyl pyrophosphate (FFP), a well known ergosterol biosynthetic intermediate. FPP accumulation is favored using elegant yeast genetics. Accumulated FPP is converted into artemisinic acid using two plant enzymes, namely amorphadiene synthase and a cytochrome P450 monooxygenase. The final steps to obtain Artemisinin are achieved photo chemically. In the two cases, success relied among other things on a correct and well known host organism, skilled molecular biology, presence of relatively abundant intermediate resembling the final molecule and a characterized pathway.

These innovative recombinant organisms are currently being developed for production of the two complex chemicals.

However, these two examples are atypical in two ways. Firstly, chemical entities accessible in yeast are limited to terpenoids, isoprenoids, flavonoids or alkaloids. Secondly, *S. cerevisiae* is not the preferred organism for pathway engineering due to its genomic complexity compared to a bacterial genome. Moreover bacterial genomes are much more accessible to synthetic biology due to the poly cistronic organization of their pathway (a single promoter can drive multiple genes that is not the case in yeast and mammals).[111]

A complex pathway coming from a multicellular organism was assembled into a microbial host. In other words, using the available genetic information it is possible to transfer an identified pathway into the appropriate host. The two examples could eventually be applied to different complex bioconversions, where a pathway will be transferred into the appropriate host grown on the identified substrate.

To get closer to synthetic biology, where industrial biological synthesis will be taking place mostly *in silico* before going into the real living organism, information should be gathered in databases connecting enzyme, DNA sequences, family activities, natural and artificial substrates. Already, Faulon and coworkers are developing software capable of recognizing enzyme signatures.[112] This software could be modified for biotechnological purposes to be capable of designing syntheses using available reaction databases.

In the long term, we can infer that it should be feasible to design new synthetic routes *in silico*.

1.9.2 Application of Synthetic Biology – Ingenza

Synthetic biology approaches involving the fermentation of engineered microbes not only offer lower cost methods to produce medicinal or industrial compounds at scale but can also offer greater stability and security of supply of natural products which derive from sources or regions threatened by environmental or political instability, such as morphine alkaloids[113] currently isolated from poppy seeds.

However, despite several decades of successful innovation in molecular biology and great advances in methods of gene isolation, gene synthesis, cell transformation and the adaptation of enzyme activities, the process to optimize expression of even a single heterologous gene remains largely empirical. Specific combinations of regulatory elements such as promoters, ribosome binding sites and transcription terminators which are found to enable efficient production of one target protein are generally not optimal for subsequent targets. Similarly, the redundancy of the genetic code permits an enormous number of DNA sequences to encode a typically sized bacterial enzyme. The relationship

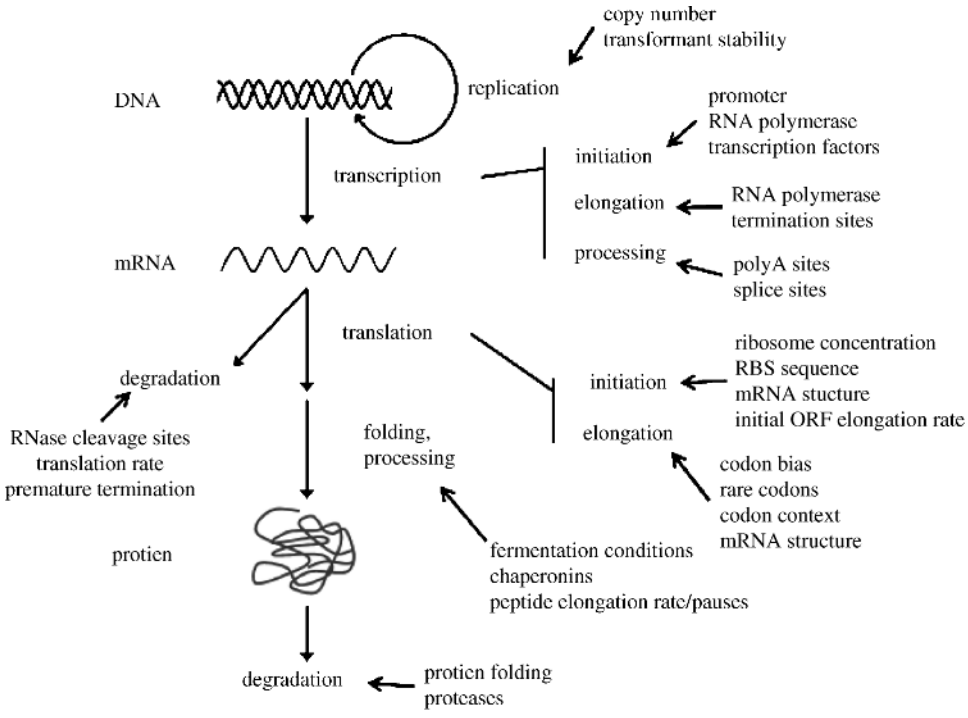


Figure 1.9.2 *Factors influencing protein expression.*[119]

between a particular gene sequence, the host organism and the resulting gene expression profile is complex. There are many distinct but interrelated factors to be considered, including codon bias, regulatory elements, RNA stability and protein folding. Determining the optimal DNA sequence to achieve efficient expression of a given protein in a particular host also remains a largely empirical process (Figure 1.9.2).

Therefore a number of strategies are underway to achieve step changes in the efficiency with which the biochemistry and genetics of organisms can be reprogrammed successfully to express new synthetic pathways. Principally amongst these strategies has been an initiative to establish a registry of freely available standardized biological "parts",[115] which includes protein coding regions, regulatory elements and plasmid vectors. These "BioBrick" parts are available to researchers to provide a systematic means to combine relevant fragments of DNA to conduct synthetic biology projects. This approach is critical to shorten the timeframe of the typically empirical methods of pathway engineering and requires systematic high-throughput combination of the key genetic elements. Ingenza now applies proprietary combinatorial gene and pathway assembly technology in most of its synthetic biology projects and partnerships to accelerate this process.

The international genetically engineered machine (iGEM) competition (http://igem.org) is encouraging the broader uptake of this modular approach. iGEM has resulted in the construction of a novel bioswitch for potential application in regulating cellular development[115] and a biosensor for detection of water contamination.[116]

Numerous software tools are now being developed to accelerate synthetic biology approaches. The extremely high rate, and concomitant low cost of automated DNA/gene synthesis now greatly exceeds the available know-how to design effective novel genetic systems. Likewise rapid DNA/RNA sequencing will allow detailed transcriptome analysis providing understanding at a genomic/proteomic level of the changes built into engineered organisms. Biological systems involve the interconnection of highly complex networks of reactions and so computational analysis is necessary in conjunction with experimental results to help construct models to predict the behavior of new genetic constructs.[117] Innovative design algorithms are also being applied in gene synthesis to try to introduce greater predictability in optimizing a particular gene for the greatest expression yield in a given context within a specific host organism.[118] Reliable criteria by which to design genes for expression in new biosynthetic pathways avoiding extensive trial and error are essential to future synthetic biology applications.

Two excellent and complex examples of the application of synthetic biology in the field of pharmaceuticals are cited above in the engineering of microbial systems for the production of hydrocortisone and artemisinin.

Biocatalytic approaches to manufacture fine chemicals which involve the concerted use of multiple enzymes can also benefit from the engineering principles now being applied in synthetic biology to reduce the development time to establish cost-competitive bioprocesses. Hydantoinase biocatalysts have proven highly successful in specific cases to manufacture non-proteinogenic amino acids[119] but require use with carbamoylase and in some cases racemase biocatalysts to be most effective. Flexible and co-ordinated high-level expression of all three biocatalysts in a single suitable[120] host strain is desirable but requires the design of complex gene expression systems which are time-consuming to develop for the reasons described above. Such approaches would benefit greatly from the advances in systematic gene and pathway assembly. Similarly, transaminase reactions can be enhanced using multi-enzyme systems[121] and require a synthetic biology strategy for rapid optimization.

The versatility and adaptability of synthetic biology is also highly suited to cellulosic biomass processing.[122] By combining useful enzymatic activities from a variety of sources it may enable the conversion of biomass to fuel, or other chemical products currently derived from petrochemicals, to become economically feasible in the most suitable host organisms. Cellulose degradation has been studied in many organisms and involves the co-ordinated activity of multiple classes of hydrolytic enzymes.[123] Furthermore, effective cellulose degrading organisms possess multiple enzymes of each class. In addition, hemicellulose can be enzymatically hydrolyzed with relative ease but the resulting monomer pentose sugars cannot be metabolized efficiently by the most suitable industrial organisms. Concerted production of an appropriate blend of cellulases in a suitable industrial host organism, along with the necessary metabolic engineering to overcome redox limitations of pentose sugar utilization is required to identify organisms with the potential to utilize these raw materials. To achieve this goal a synthetic biology strategy with a highly modular, interchangeable system of components is required to determine which combinations of enzymes are most effective for the degradation of different classes of biomass substrate.

The BioBricks initiative combined with high-throughput gene assembly tools and other synthetic biology approaches offer great potential to address the challenges in the rapid

construction of efficient, robust low cost genetic systems and bioprocesses for industrial biotechnology. Advances in the technology of DNA synthesis and assembly have now permitted the reconstruction of an entire chromosome.[124] However, the vast array of individual genetic components which must be considered, resulting from choice of codon usage and regulatory sequences, amongst other factors, along with the impact of directed evolution of individual enzymes, provides a bewildering number of options for researchers. The degree of optimization which any given system might require is also dependent on a number of critical variables. These variables include the commercial value of the end product, the starting point of the engineered pathway in terms of accessible intermediates and the basal levels of the required activities displayed by the necessary enzymes, or their progenitors. Therefore the highly integrated approach of synthetic biology, which draws upon disciplines including molecular biology, bioinformatics, engineering, computing and mathematics is essential to provide the systematic progression to predictability, required to implement new sustainable manufacturing processes in a realistic timeframe. Equally important is that these innovative methods are applied in a complementary fashion with very successful classical strategies such as the use of chemical analogs, chemical mutagenesis and robust screening protocols to achieve a new generation of industrially valuable microbes.

1.10 Prioritization of Future Biocatalysis and Synthetic Biology Needs

In 2007 the American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR) identified key green chemistry research areas of need for the industry.[103] The process began with a brainstorming exercise and the output was grouped into three categories:

1. Reactions currently used but better reagents preferred
2. More aspirational reactions
3. Other ideas outside of the reaction theme (e.g., ideas concerned with solvent use)

Following a cross-company debate, they concluded with a voting exercise, where each company had an equal vote, in order to prioritize the ideas.

Here, we have performed a similar exercise to generate an industry-wide consensus on the technical needs that would enable the widespread adoption of biotrans and synthetic biology in the fine chemical and pharmaceutical industries.

A total of 15 companies from the pharmaceutical industry (AstraZeneca, GlaxoSmithKline, Eli Lilly, Merck, Novartis, Pfizer, Roche and Sanofi), fine chemical industry (Cambrex, Dr Reddy's, Lonza) and specialist biocatalysis sector (Almac, Codexis, Ingenza, Libragen) took part in the voting process. Each company was asked to provide a list of issues that would enhance the adoption of biocatalysis or synthetic biology by industry, were they to be progressed. The received problem statements were then sorted into six categories and each company was asked to prioritize their five most important from each category by giving a score of five for their highest priority down to one for their lowest.

The tables below show the overall top three problem statements from each category as a percentage of the overall vote, with shading to aid visualization (dark = high priority and light = low priority). The percentage of the vote received by sector for the overall top three

problem statements from each category has also been included as there were significant differences in some cases. Each table is followed by some short comments on the findings and the complete data set, including all problem statements submitted and the vote received from each company, can be found in Appendix 1.

Category 1 Preparative biotransformations that have been widely reported but require new biocatalysts to broaden substrate ranges or biocatalysts with improved properties for efficient application on scale

Relevant issue	Pharma Subtotal/%	Fine Chemical Subtotal/%	Specialist Biocatalyst Subtotal/%	Overall Total/%
Reductive amination: More 'high-quality' transaminases to improve the already existing enzyme panels (e.g. improved acceptance of bulky substrates)	63	93	70	71
Hydroxylation: New monooxygenases with improved synthetic performance to improve existing enzyme panels	73	60	55	65
Hydrolysis/acylation: More 'high-quality' hydrolases to improve the already existing enzyme panels (improved success rate with e.g. tert. alcohols)	33	27	55	37

Agreement was fairly consistent between companies and across sectors. The top three issues were not surprising and correlate well with academic research activity that is shown in Table 1.3.1 as well as the chemical needs identified by Constable *et al.*[103] Chiral amines feature frequently in pharmaceutical molecules and transamination is still an emerging field, whereas hydroxylation is still an underutilized transformation due to a lack of suitable chemical reagents and biocatalysts. The use of hydrolases is seen as an established technology and yet there is still a lack of diverse, off the shelf biocatalysts that will provide the $>90\%$ hit rates required by chemists. There is a particular need in pharma for sets of hydrolases that will efficiently produce a diverse range of secondary and tertiary amides from esters and amines,[125] particularly in light of the issues with chemical coupling reagents.[126]

Category 2 Discovery and development of biocatalysts for preparative biotransformations with little or no precedent

Relevant issue	Pharma Subtotal/%	Fine Chemical Subtotal/%	Specialist Biocatalyst Subtotal/%	Overall Total/%
Enzymes for reductive amination to give primary, secondary and tertiary amines	48	87	60	59
New C-C and C-N bond forming activities (Knoevenagel, Suzuki, Heck, Strecker, Mannich…)	46	27	20	35
Fluorination of non-activated C	33	0	40	28

There was quite a wide disparity between the needs of the different companies and sectors for this category, where new imine reductases, of which there have been only sporadic reports

in the literature (and only towards the reduction of stabilized imines),[127] emerged as the only clear need across all sectors. These would open up the possibility of directly accessing chiral secondary and tertiary amines and avoiding the use of hazardous hydride reducing agents.

The need for a wider range of fluorinases was rated as a moderately high priority by the pharma and specialist biocatalysis sectors, but surprisingly not by the fine chemical sector. However, halogenation was scored particularly well by the fine chemical sector and so presumably fluorination was not distinguished from the need for other classes of halogenase by this group. A set of fluorinating agents would be particularly valuable given the hazardous nature of current fluorinating agents and the wide range of fluorine containing APIs, although it is recognized that this is a long term goal given that only one, with a very narrow substrate spectrum, has been identified to date.[128] Biocatalytic access to other halides, were it to be further developed, could also offer an attractive alternative to the hazardous classical approaches and advantages over more recent catalytic approaches such as greater selectivity and operation under physiological pH.

Other issues tended to be favored to a different extent according to sector and in fact, company. Of equal priority to imine reductases for the pharma sector was the need for new biocatalysts for C–C and C–N bond formation such as equivalents to the Suzuki, Heck, Mannich and Knoevenagel reactions, whereas amide and nitrile reduction rated highly among the specialist enzyme sector. In contrast, the fine chemical sector rated the need for dihydroxylases that act on non-activated olefins highly.

In summary, the wide variation between companies towards many of the issues in category 2 demonstrates that there is a general need for a more diverse toolbox of biocatalysts across all sectors. Certainly, when we at GSK were voting on this category, beyond our two issues of highest priority, it was difficult to further prioritize and may change depending on current company needs.

Category 3 Enabling technology/understanding needs for better biocatalyst identification and improvement

Relevant issue	Pharma Subtotal/%	Fine Chemical Subtotal/%	Specialist Biocatalyst Subtotal/%	Overall Total/%
Enzyme evolution strategies that are less labour intensive and much faster (an experiment rather than a project): · - Smarter approaches that use lower numbers of mutants · - Technologies that allow greater number of clones per round · - Self selecting, continuously evolving systems that don't require manual intervention.	70	100	40	68
Better understanding of sequence-structure-dynamics-function relationships, ultimately leading to complete in silico enzyme design, optimization and quantitative substrate scope/activity predictions.	58	70	70	63
Better understanding of transcription-translation-folding-secretion as a basis for rational (more predictive) enzyme expression approaches.	38	27	70	44

Faster methods of enzyme evolution that on average, usually take about nine months, was seen as a primary need by the pharma and fine chemical sectors, but less so by the

specialist biocatalyst sector. The specialist biocatalyst sector rated the need for rational expression approaches and better understanding of sequence-function relationships more highly and enhanced use of new bioinformatics platforms for structural alignment to a comparable extent. Presumably the latter two could lead to more reliable rational approaches that may ultimately obviate the need for the former, but are expected to take significantly longer to develop (>10 years).

Improved screening and cultivation of environmental samples and more metagenomics and sequencing of DNA all scored moderately well, demonstrating (as in Category 2) that there is a continued interest in the identification of improved enzymes and enzymes with novel activities.

Category 4 Enabling technology/understanding needs for biocatalyst use on scale

Relevant issue	Pharma Subtotal/%	Fine Chemical Subtotal/%	Specialist Biocatalyst Subtotal/%	Overall Total/%
• Efficient down-stream processes to remove biomaterials from API: Development of *in-situ* product recovery, and membrane technologies at industrial scale or other innovative methods • New/improved methods of product recovery from aqueous reaction mixture	43	100	50	58
Well defined and characterised, general purpose, free and easy to use, open access organisms as expression systems	55	47	25	45
Whole cell hosts that tolerate high solvent and substrate content (comparable to chemical reactions)	26	35	27	29

Downstream processing, which is often the most costly operation in bioprocessing, scored as the main issue in need of new technology for scale-up. Generic methods that allow the development of integrated bioprocesses for product purification and isolation, based on techniques such as those mentioned in Section 1.3.1, could greatly improve efficiency. It should be noted that two issues from the original vote have been combined into a single issue here, although there were mixed responses as to whether this should be done. One company that expressed a preference to keep the two issues separate viewed the first point as more about methods of controlling biomass-related impurities and thus satisfying regulators and the second to be related to extending methods of product recovery from aqueous solution.

The need for more expression systems that are easy to use and free of IP restrictions was also prioritized highly, as was host cells capable of tolerating high solvent concentrations.

The specialist biocatalyst sector also strongly prioritized the need for more efficient methods of recycling cofactors such as ATP, SAM and CoA. In fact, one of the pharma companies commented that this need might well have scored higher had it not also featured a number of times in Category 5 (i.e., the vote for this issue may have been diluted).

Better methods of enzyme immobilization and improved enzyme kits with a more diverse range of activities and sold as genes also individually scored well by the fine chemical and pharma sectors respectively.

Category 5: Enabling technology/understanding needs specific to synthetic biology

Relevant issue	Pharma Subtotal/%	Fine Chemical Subtotal/%	Specialist Biocatalyst Subtotal/%	Overall Total/%
Routine use of gene synthesis to directly synthesise genes/variants as identified by in silico methods quickly and at low cost	65	60	30	55
Better understanding of the substrate ranges of enzymes embedded in biosynthetic pathways	45	53	40	45
Better expression/ modification of complex protein structures (P450 monooxygenases, polyketide synthases ...)	53	40	25	43

The routine use of low cost gene synthesis attained the highest overall score as the issue that would most enhance the adoption of synthetic biology in industry by allowing the rapid optimization of gene expression in a chosen host, avoidance of the need to access the microorganism of origin and better control in rational enzyme design. Although the speed and cost of gene synthesis has dramatically reduced in recent years, there is a growing need for further reductions.

Better methods of heterologous expression and understanding of the substrate ranges of enzymes embedded in biosynthetic pathways were seen as the key priorities that would best enable uptake of synthetic biology by industry.

Category 6: Other

More biocatalysis in chemistry university courses was categorized separately, partly because it did not fit well into the other categories, but also because it is very apparent that the vast majority of chemists leaving university have little, if any, knowledge about biocatalysis. Given the widespread and increasing adoption of the technology across the chemical industry, there is a clear need to address this. As commented by one author, this should include the practical application of biocatalysis and the understanding of where a biotransformation might be incorporated into a synthetic route, as with any other chemical reagent.

In conclusion, the voting exercise revealed that a wider range of enzyme activities are required in order to further improve the uptake of biotrans and synthetic biology by industry. Both these and established transformations require the identification of sufficient enzymes or variants in order to allow coverage of $>90\%$ of substrates tested if biocatalysts are going to be considered as mainstream reagents rather than a niche technology.

Better methods of gene expression using a wider variety of free-to-use, robust hosts and wider use of gene synthesis are required in order to accelerate the discovery of new activities from the environment and provide cheaper ways to improve them towards process requirements.

Adoption of a wider variety of enzymes and the use of sequences of enzymes is expected to require an increase in the use of whole cell processes, key to which will be efficient downstream processes, particularly for the removal of biocontaminants and extraction of

APIs from aqueous solution. Currently very few biotransformations are employed in the final step of API production and then only using relatively expensive immobilized enzymes.

1.11 Concluding Remarks

As has been reflected in the recent literature and this book, it is apparent that green chemistry has become a vital driver in process development. Establishing a process with reduced use of solvents, reagents and energy, in short, one with minimal environmental impact, has become a focus of development chemists. No longer is it enough to merely eliminate the most toxic reagent, but to minimize all waste and maximize the use of every resource in the process. This is an admiral goal in terms of protecting the environment, but also comes with the frequent associated benefit of reduced cost and vice versa. This approach to efficient, optimized, environmentally responsible pharmaceutical manufacture is demonstrated, for example, by commitment from GSK to become carbon neutral by 2050.

Both environmental and economic returns are being realized in the industrial synthesis of complex APIs as a result of advances in protein engineering from academic and industrial laboratories. New technologies are resulting in reduced costs and timelines for enzyme optimization, providing highly active species which are being incorporated into large scale manufacturing routes. Progress in protein engineering is well documented by reports from academia and industry in this chapter and Chapters 4 and 8.

For the future successful collaboration between industry and academia, to ensure continuous progress and to coordinate needs from both sides, the voting exercise in Section 1.10 revealed interesting points and relatively good agreement between pharmaceutical, fine chemical and specialist biocatalysis companies. Some of the needs have already started to be addressed by academic researchers; e.g., broader panels of ω-transaminases and novel enzymes for reductive amination (Chapter 2), monooxygenases (Chapter 5) and decarboxylases (Chapter 10). However, investing significantly more effort and time is still necessary to achieve all of these goals.

With continued investment in the various "-omics" and interdisciplinary research programs where chemists, molecular biologists, engineers and bioinformatics come together, we hope to witness tremendous progress in the generation of improved and novel enzymes, that are practical to use, compatible with a wide range of reaction conditions and deliver environmental, as well as economic savings. Sophisticated prediction tools that can help deliver focused, "tailor-made" mutant libraries, for any reaction will greatly help early incorporation of enzymatic reactions into synthetic routes. Another application which could benefit from the recent advances in genomic sequencing, gene synthesis, molecular biology and pathway engineering is synthetic biology. Sequencing has revealed novel proteins with potentially interesting activities and also identified whole metabolic pathways of microbial, plant or even mammalian origin. These complex multicomponent biocatalytic entities can be embedded into the desired microbial host and give access to "microbial factories" where an endogenous substrate will be converted to exogenous product of interest via multi-step synthesis under benign conditions and without need for intermediate isolation. Pioneering work in

this area has shown the great potential of this application (Section 1.9). Nevertheless, further improvement of design and analysis software will be needed to better plan new synthetic routes *in silico*.

Cooperation between industry and academia in future years is certain to provide far reaching advances in reactivity and acceptance in biocatalysis. Commitment by industry to implement enzymatic routes early on in the research years of a projects life will provide the testing ground for new research in the field and the motivation to develop new techniques. The authors look forward to the day when classically trained organic chemists, working in any stage of the drug development cycle, the flavors and fragrance industry, the fine chemicals industry and even in bulk chemical manufacture, turn as readily to biocatalytic techniques as they do today to transition metal catalysis. Thirty years ago, very few people had heard of a Heck reaction, thirty years from today what fantastically useful, everyday biocatalytic reaction can you imagine we might be using?!

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