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Biotransformations in Small-molecule Pharmaceutical Development

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1.1 Introduction

The demand for medicines that treat illnesses formerly associated with the developed world is expanding at a time when some countries are becoming increasingly affluent. As a result, the global pharmaceutical market is predicted to grow to $800 billion by the year 2020.[1] However, as demand increases for products, the pharmaceutical industry is facing increasing pressures that can primarily be attributed to three factors:

1. As the global population ages and lifestyles become more sedentary, the cost of healthcare is becoming increasingly unsustainable. This is no more so than in the USA, where, although prescription products contribute only 10 % of healthcare costs, they are perceived to be much higher by the consumer and so represent an easy political target for cost cuts through price controls.
2. Erosion of product lifetimes as a result of greater generic competition means that a product can expect to lose the majority of its market in as little as 3 months after patent expiry.
3. Spiralling R&D costs. Typically, it takes 10 years at a cost of $500 million to bring a drug to market.[2] Fewer new molecular entities (NMEs) and biologics are reaching the market as a result of a shift of research focus away from already established and crowded therapeutic areas into new, unproven biological areas (Figure 1.1).[3]

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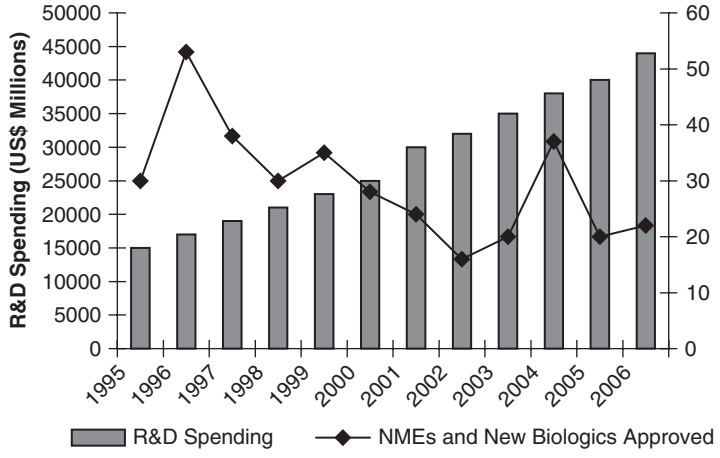


Figure 1.1 *R&D spending versus the number of NMEs and biologics approved by the US Food and Drug Administration (FDA). (Reprinted with permission from* Pharma 2020: The vision: Which path will you take?*, PricewaterhouseCoopers,* ***2007****.)*

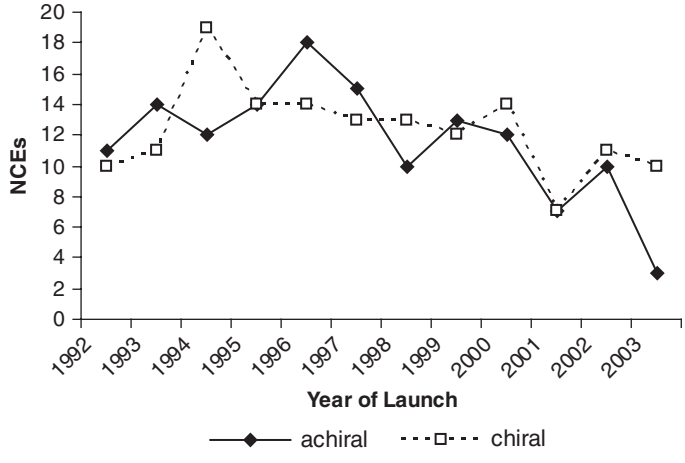


Figure 1.2 *Number of chiral and achiral marketed NCEs. (Reprinted with permission from Farina, V., Reeves, J.T., Senanayake, C.H. and Song, J.J. Asymmetric synthesis of active pharmaceutical ingredients.* Chem. Rev. ***2006****,* 106*, 2734–2793. Copyright 2006, American Chemical Society)*

Whereas new drugs reaching the market do not necessarily look any more complex or contain any more stereocentres than in the past (Figure 1.2),[3] the complexity of drug candidates under development has increased on average. In addition, following the FDA's 1992 policy statement on stereoisomers, it is now clearly more economical to progress an active pharmaceutical ingredient (API) in enantiopure form, as can be seen from the trend towards the launch of single-enantiomer new chemical entities (NCEs) (Figure 1.3).

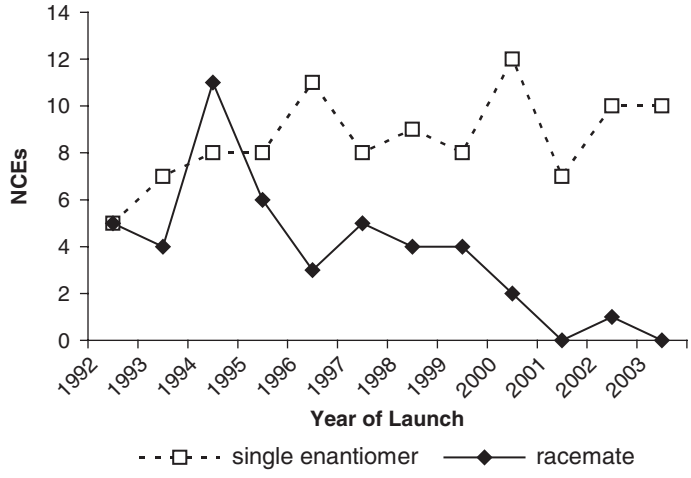


Figure 1.3 *Number of single enantiomer versus racemic NCEs. (Reprinted with permission from Farina, V., Reeves, J.T., Senanayake, C.H. and Song, J.J. Asymmetric synthesis of active pharmaceutical ingredients.* Chem. Rev. ***2006****,* 106*, 2734–2793. Copyright 2006, American Chemical Society)*

This trend seems likely to continue, with over 50 % of current drug candidates being developed as single enantiomers.[4] An obvious consequence has been an explosion of research activity into asymmetric synthetic methods.

These combined issues, on average, make pharmaceutical companies as much as 50 % riskier than other big industries.[5] Led by the FDA's Current Good Manufacturing Practices for the 21st Century initiative,[6] the pharmaceutical industry has begun to apply a risk management and quality systems approach, practiced in some other industries for decades, to products throughout their lifetimes.[7] The guidance from the FDA's Process Analytical Technologies regulatory framework, developed over the last decade, aims to build quality by design into pharmaceutical products through better process understanding and increased innovation. The ultimate goal is to minimize risk to the patient whilst encouraging the industry to cut operating costs.[8]

Encouraged by this more flexible regulatory approach, there is an increased willingness within the industry to adopt 'new' technologies.[9]

1.2 Current Status of Biocatalysis

A biotransformation, as defined by Straathof *et al*.,[10] is 'a process that describes a reaction or a set of simultaneous reactions in which a pre-formed precursor molecule is converted using enzymes and/or whole cells, or combinations thereof, either free or immobilised'. Fermentation processes, with *de novo* product formation from a carbon and energy source, such as glucose via primary metabolism, are outside the scope of this chapter and book unless employed in conjunction with a biotransformation.

Biocatalysis has long been known as a green technology, capable of delivering highly stereo-, chemo- and regioselective transformations that can sometimes allow

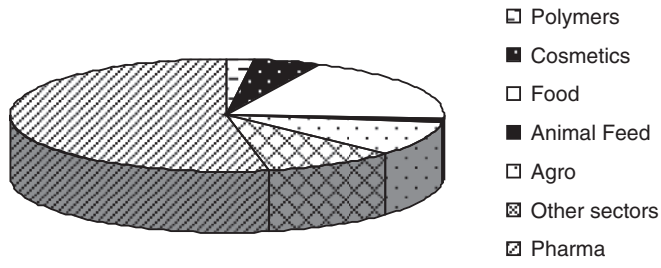


Figure 1.4 *Number of biotransformations used catagorised by industrial sector (based on 134 processes). (Reprinted from Straathof, A.J.J., Panke, S. and Schmid, A. The production of fine chemicals by biotransformations.* Curr. Opin. Biotechnol. ***2002****,* 13*, 548–556 with permission from Elsevier.)*

the number of steps in a synthetic route to be reduced. Numerous industrial biotransformations (announced to be commercialized at a scale of >100 kg per annum) are in operation worldwide, many of which have been described by Liese *et al.*[11] Most of these known biotransformations are used to produce building blocks that are subsequently supplied to the pharmaceutical industry (Figure 1.4).[10]

Biocatalysis is still an emerging field; hence, some transformations are more established than others.[12] Panke *et al.*[13] have performed a survey of patent applications in the area of biocatalysis granted between the years 2000 and 2004. They found that although hydrolases, which perform hydrolyses and esterifications, still command widespread attention and remain the most utilized class of enzyme (Figure 1.5), significant focus has turned towards the use of biocatalysts with different activities and in particular alcohol dehydrogenases (ADHs) – also known as ketoreductases (KREDs) – used for asymmetric ketone reduction.

Whereas the number of industrial biotransformations 'known' to be operating in 2002 was 134, the number of chiral drug candidates is much greater. Farina *et al.*[3] have estimated between 500 and 1000 single-enantiomer APIs to be in development each year in the global pipeline. This implies that biotransformations might supply only a small percentage of chiral centres. This might be partially attributable to the reluctance of the pharmaceutical industry to innovate in the absence of the recently established regulatory directives, or to the lack of commercial enzymes available on a large scale. However, the main factor lies in the strategy used to incorporate chirality into drug candidates.

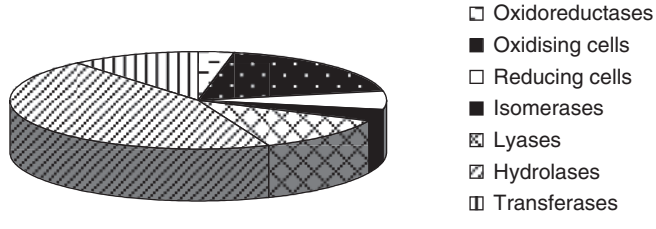


Figure 1.5 *Enzyme Types Used in Industrial Biotransformations (based on 134 processes). (Reprinted from Straathof, A.J.J., Panke, S. and Schmid, A. The production of fine chemicals by biotransformations.* Curr. Opin. Biotechnol. ***2002****,* 13*, 548–556 with permission from Elsevier.)*

In line with the construction of a target molecule from smaller, complex fragments, it is generally preferred to introduce chirality into a synthetic route at an early stage through the purchase of simple chiral starting materials from the fine chemical industry. This has been demonstrated by Carey *et al*.,[14] who performed a survey of 128 drug candidate syntheses, many of which were in an early phase of development. They found that, of the 69 chiral drug candidates considered, 55 % of the 135 chiral centres present were bought in from the fine chemicals industry. In cases where it was necessary to generate chirality in-house, the favoured method was racemate resolution (28 % of chiral centres – with classical salt formation employed in two-thirds of cases and dynamic kinetic resolution, chromatography and biocatalytic methods evenly distributed in the remainder) followed by chemical asymmetrization (10 % of chiral centres – see Section 1.3.4 for definition) and diastereoselective induction (7 % of chiral centres). Another important source of chirality (which was not exemplified in that article) is fermentation technology, which provides access to many of the important chiral scaffolds that have been employed by the industry in well-known classes of drug, such as β-lactam antibiotics and, more recently, first-generation statins.

Some 35 % of the chiral building blocks that are bought in from the fine chemical industry, such as both proteinogenic and non-proteinogenic amino acids, carboxylic acids, amines, alcohols and epoxides, are produced using generic biocatalytic technologies, and this is expected to increase to 70 % by 2010.[12] Far more chiral centres present in APIs are derived from industrial biotransformations than would be expected by counting the number of known processes.[15] This can also be noted from the procedures given in this book, the vast majority of which provide biocatalytic routes to chiral building blocks. These chiral building blocks, in turn, will be dictated by current drug candidates within the pharmaceutical industry's pipeline.

Given the wide utility of biocatalysis in the fine chemical industry, why is there such an in-house reliance on classical methods of enantioseparation? In fact, why is biocatalysis not applied more generally as a replacement for atom-inefficient or hazardous reactions that are intensively used in the pharmaceutical industry, such as amidation, reduction and oxidation?[16]

The sparse incorporation of biocatalysts into the process chemist's toolbox is at least in part due to a number of long-standing issues that differ depending on the drug development phase. At an early stage of development, where little resource is available for new route development, biocatalysis options are often neglected due to a lack of sufficient commercially available biocatalysts.[17] In contrast, classical salt formation regularly provides access to chiral material in $>$99.5 % enantiomeric purity; hence its widespread adoption. At a later phase biocatalysis may be considered, but the longer development times often needed and the more advanced state of competing chemical routes put it at a disadvantage.

Many of these issues have been or are being addressed. For example, with the continued expansion in the number of microorganisms whose genomes have been sequenced, the application of bioinformatics techniques is leading to a rapid expansion in the number of commercially available enzymes such as ADHs (ketone reduction), nitrilases (nitrile hydrolysis), enoate reductases (α,β-unsaturated olefin reduction) and transaminases (reductive amination).[18] Having identified a putative enzyme gene by sequence similarity, it can now be quickly and cheaply generated by using oligonucleotide synthesis services that are provided by a number of companies. However, it is predicted that about 99 % of

microorganisms are 'non-cultivable', and metagenomics – the extraction of environmental DNA – is proving highly successful in accessing novel biocatalysts from this untapped resource.[19]

Further expansion in the number of commercially available enzymes and the modification of hits to suit process requirements is being fuelled by advances in enzyme engineering[20] and high-throughput screening (HTS) technologies.[21] A particularly elegant approach is the protein sequence activity relationship (ProSAR) technology developed by Codexis.[22] By using a multivariate analysis approach, libraries of enzyme variants containing programmed mutations generated from different sources of diversity are screened against a given substrate and sequenced. Positive mutations and interactions between different mutations can then be ascertained, allowing more active variants to be predicted *in silico*, thus reducing the bottleneck often caused by screening. As the impact of individual mutations is understood, the possibility of missing important ones or carrying false hits through to the next round is reduced compared with traditional hit-based directed-evolution strategies. Similar multivariate techniques are used on a daily basis within the pharmaceutical industry (quantitative structure–activity relationships in medicinal chemistry and design of experiments to understand and optimize chemical reactions during process development) to interpret complex data sets. So powerful is this technique at producing ADHs suitable for process applications that one pharmaceutical company now considers biocatalysis as their first option for asymmetric ketone reduction.

There is also a greater appreciation within the biotech community that alternative routes to a drug candidate are always available and, although they are sometimes technologically inferior, will always be favoured if freedom to operate is at stake.

1.3 Application of Biocatalysis in the Pharmaceutical Industry

This section primarily focuses on examples of biotransformations that have been developed for the preparation of small-molecule APIs (molecular weight <1000), metabolites and late-stage intermediates. Particular emphasis will be given to the incorporation of biotransformation steps into synthetic routes and their advantages over competing technologies. Later sections will then expand on the key topics of 'enzymes in organic solvents,' 'enzyme immobilization' and 'green chemistry' that are introduced in earlier sections.

No attempt has been made to cover all drug classes or enzyme classes; instead, a flavour of the potential benefits that can be achieved by the adoption of biocatalytic methods as a compliment to chemical approaches is given. Biocatalytic methods of accessing chiral building blocks will only occasionally be discussed here and the reader is referred to a number of comprehensive reviews that have been published elsewhere.[15,23]

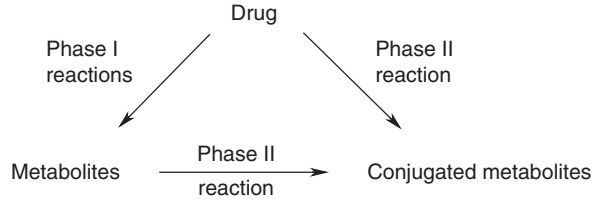
1.3.1 Drug Metabolites and Metabolic Transformations

Metabolites are generated by the body's own biochemical processes as a way to facilitate excretion of xenobiotics. The enzymes catalysing *in vivo* modification of drugs and drug-like molecules have a fundamental significance for the pharmaceutical industry. This was once primarily the field of the pharmacologist, but interest in metabolic reactions

increasingly extends to the synthetic chemist arising from the requirement to synthesize specific drug metabolites, as well as the realization that some of these enzymes could be exploited as general synthetic tools.

A thorough understanding of the metabolic fate of a drug candidate is essential in the assessment of its efficacy and toxicity and to safeguard patient welfare through the identification of potential drug–drug interactions (DDIs).[24] It is also an integral part of the drug discovery process, allowing molecular redesign based on the identification of active metabolites and an appreciation of how they arise. In fact, metabolism can lead to new structures that need to be covered in patent claims.

Metabolic reactions (also known as biotransformations) can be divided into two categories: functionalization, where a functional group is created or modified, and conjugation, where another molecule is transferred to the substrate.[25] These are also known as phase I and phase II metabolic reactions respectively (Scheme 1.1). Functionalization encompasses redox reactions, hydrolyses and hydrations, whereas conjugation can involve a wide variety of transformations, such as glucuronidation, methylation, sulfoxidation and phosphorylation (Table 1.1). Most of these drug-metabolizing enzymes are expressed intracellularly in the liver at comparatively high levels relative to the rest of the body.



Scheme 1.1 *Categories of metabolite*

The most prominent metabolic transformations are catalysed by oxidoreductases, hydrolases and transferases (glutathione transferases and glucuronyl transferases), oxidative transformations being quantitatively of greatest importance. Metabolic enzymes are often highly promiscuous, transforming a wide variety of xenobiotics. Given the number of different metabolic enzymes, their promiscuity and the multiple activities of some, it is not surprising that the metabolism of a xenobiotic often results in a soup of different compounds, all present in low abundance.

Direct isolation of sufficient quantities of each metabolite for structural characterization, assay validation and pharmacological or toxicological testing from *in vivo* studies using biological specimens is, therefore, often impossible, particularly from drugs with a low therapeutic index. Furthermore, many metabolites have structural modifications which are difficult to replicate by traditional chemical methods. A number of synthetic steps may be required to prepare such metabolites from the API, or, in the worst case, a completely new synthetic route may need to be developed.

It seems logical that drug metabolites should be prepared using the specific enzymes involved in their formation as biocatalysts for *in vitro* synthesis. This would overcome the problems inherent in the use of crude mammalian tissue extracts with their cocktails of

Table 1.1 *The main classes of mammalian metabolic transformations.[26] (Reprinted with kind permission of Springer Science + Business Media.)*

Reaction Type	Substrate	Enzyme	Product
Phase I Metabolism			
Hydrolysis	Esters Amides Epoxides	Esterases Amidases Epoxide hydrolases	Alcohols Amines Diols
Reduction	Ketones Alkenes Nitro and azo	Alcohol reductases Hydrogenases Nitro and azo reductases	Alcohols Alkanes Nitroso, oximes, amines
Hydroxylation	Aromatic, allylic, benzylic or saturated carbon containing	CYP450 or flavin monooxygenases	Phenols Alcohols
Epoxidation	Alkenes		Epoxides
N-, *O*-, *S*-Dealkylation	*N*-, *O*-, *S*-Alkyl		Amines, alcohols, thiols
C-Oxidation	Alcohols, aldehydes, ketones		Aldehydes, ketones, carboxylic acids
N-, *S*-Oxidation	Secondary and tertiary amines		*N*-Oxides
	S-Alkyl		Sulfoxides, sulfones
N-Hydroxylation	Secondary and tertiary amines		Oximes
Deamination	Primary amines	Monoamine oxidases	Aldehydes
Phase II Metabolism			
Glucuronidation	Alcohols/phenols Carboxylic acids Amines Thiols	Glucuronyltransferases	α- or β-glucuronides
Glycosylation	Alcohols/phenols Carboxylic acids Amines Thiols	Glycosyltransferases	α- or β-glycosides
Thiol conjugation	Epoxides	Glutathione-*S*-transferases	Glutathione or *N*-acetyl cysteine thioethers
Glycine conjugation	Carboxylic acids	*N*-Transferases	Glycinamide conjugates
Carbamoylation	Alcohols		*O*-Carbamoyl derivatives
Acetylation	Primary amines Hydrazines	Acetyltransferases	Acetamides
O-Methylation	Phenols	Methyltransferases	Methyl aryl ethers
Sulfation	Alcohols/phenols Amines	Sulfotransferases	Sulfate esters Sulfonamides

metabolic enzymes. However, such a system is rarely possible, as these enzymes present particular technical obstacles, being usually membrane bound rather than soluble and requiring a range of biochemical cofactors. Some examples are given in later sections.

The classes of reaction observed in drug metabolism are not exclusive to mammalian systems; hence, microorganisms can often be used to produce metabolites. Compared with mammalian enzyme preparations such as liver homogenates and other tissue preparations, microbial cultures provide low-cost maintenance, long-term stability and easier scale-up to prepare purified metabolites. Microbial systems tend to show higher tolerance of xenobiotics, allowing higher concentrations of metabolite to be produced, and greater regio- and stereo-specificity, limiting the number of metabolites to be separated for purification.

The ideal would be a collection of microorganisms, each mimicking a single metabolic reaction, together covering the full range of mammalian drug metabolism. Numerous different panels of microorganisms that allow the biotransformation of a wide variety of different substrates have been reported since the initial studies on these so-called 'microbial models of mammalian metabolism' by Smith and Rosazza in the 1970s.[27] The best of these systems will provide both phase I and phase II metabolites in milligram yields.

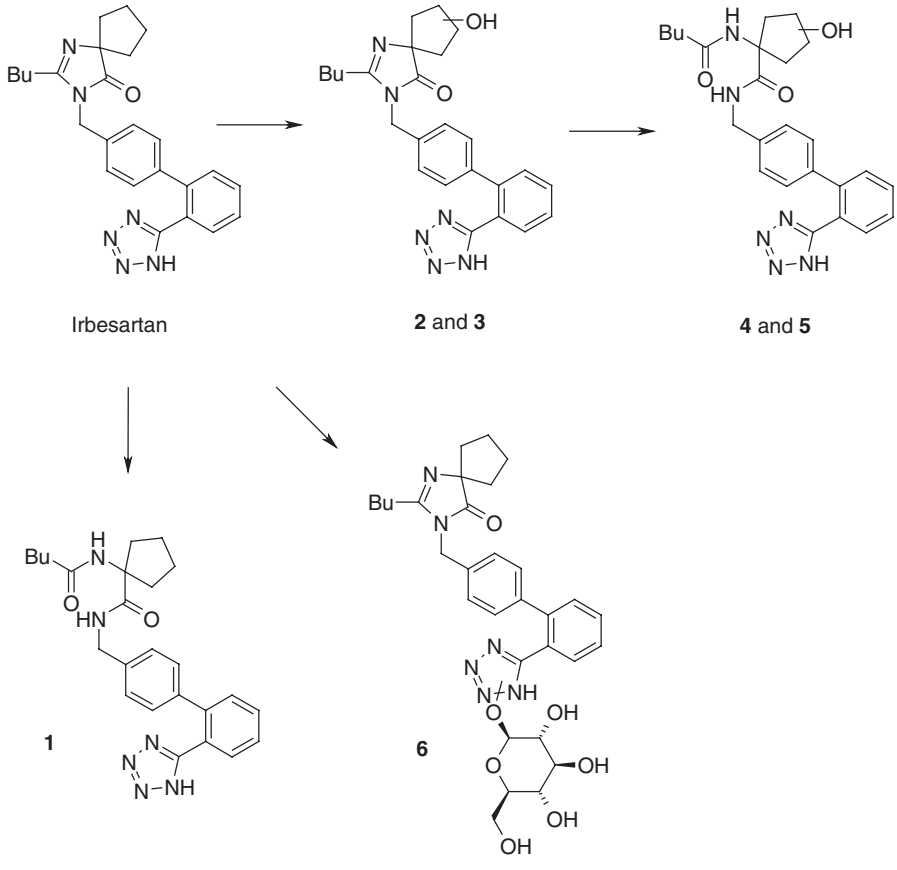
For example, the antihypertensive drug Irbesartan is known to give at least eight urinary metabolites in mammals and humans, which include hydroxylated, ring-opened and *N*-glucuronylated products. To provide sufficient quantities of these metabolites for further structural and stereochemical characterization, Azerad and co-workers[28] screened 10 fungal strains and 28 bacterial strains that are regularly used for drug hydroxylation within their laboratory for activity towards irbesartan. The hydrolysis product **1** was produced by three-quarters of the strains tested, whereas the hydroxylated products **2–5** were produced equally by about one-quarter of the strains (Scheme 1.2). The metabolite **6**, tentatively assigned as an *N*-glycosidic conjugate similar to the mammalian *N*-glucuronide metabolite, proved to be the least accessible metabolite, produced by only four strains.

Although small amounts of metabolites were detected in fungal incubations, actinomycetes or filamentous bacteria were found to be more productive both quantitatively and qualitatively. Thus, *Streptomyces* strains produced the highest levels of metabolites **2–5** and were the only strains capable of producing metabolite **6**. Some of these strains were then used to access 20–100 mg quantities of each metabolite. This mirrors the general findings that fungi and acinomycetes (including *Streptomyces*, *Nocardia*, *Actinoplanes*, *Mycobacteria* and *Corynebacteria*) are most useful for biotransformation. Other bacterial strains tend to consume the xenobiotic as a carbon or nitrogen source, making metabolite isolation problematic.[29]

1.3.1.1 Phase I Metabolic Transformations

One reaction characteristic of phase I metabolism is monooxygenase-catalysed hydroxylation at specific C–H bonds without chemical activation. Different enzymes show varying degrees of regio-, chemo-, and enantio-specificity, so the reaction is usually challenging for the synthetic chemist to reproduce once preparation of such a metabolite is required. It should be evident that biocatalysts with such capabilities would also be highly desirable as tools for chemical synthesis in general.

The vast majority of hydroxylations in mammalian systems result from the action of cytochrome P450s (CYPs – also known as P450s), which are a superfamily of

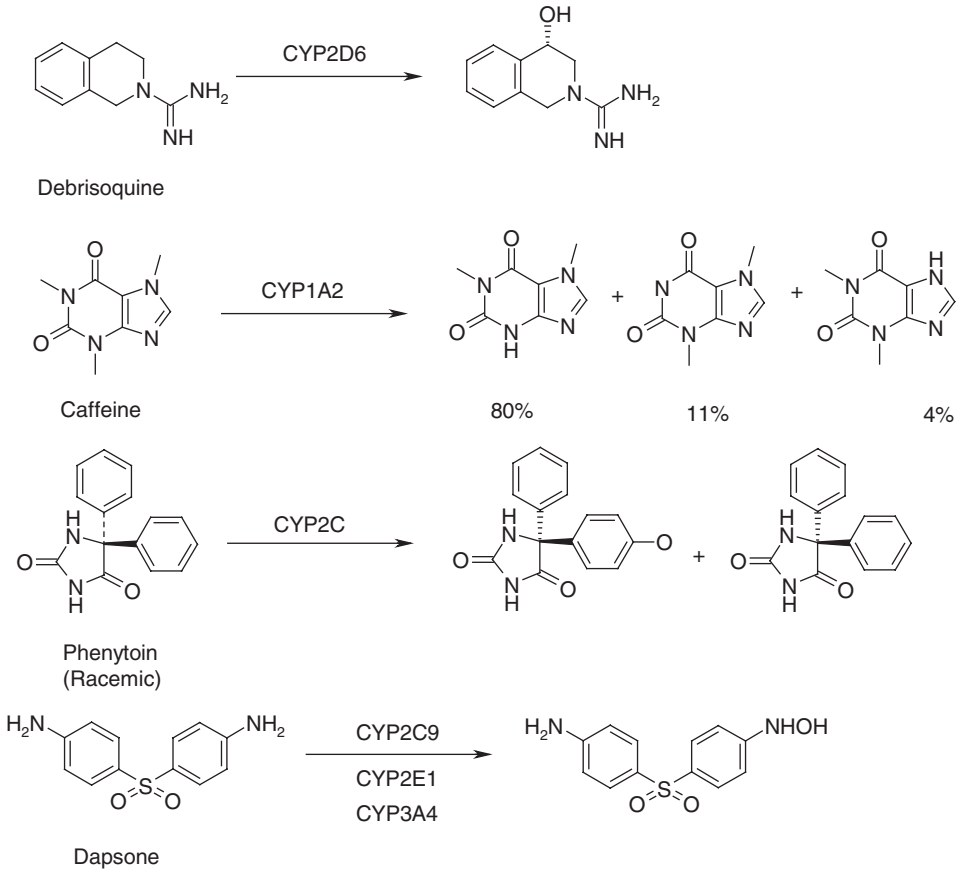


Scheme 1.2 *Metabolism of irbesartan*

monooxygenases catalysing an array of different reactions.[30] It is estimated that about 90 % of all marketed drugs and drug candidates are substrates for CYPs. Of more than 60 known human CYPs, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 are of particular significance in the metabolism of xenobiotics.[31]

These CYPs are isoenzymes (or isozymes), catalysing essentially the same reaction, but for different substrate ranges with specificity determined by their different amino acid sequences. CYPs (and other metabolic enzymes) often react with individual substrates in a highly regio-, chemo- or stereo-selective manner, each isozyme displaying its own unique selectivity. Some examples of selective CYP-catalysed transformations are shown in Scheme 1.3.

Although mammalian CYPs are attractive candidates for use as commercial biocatalysts, many functional characteristics limit the opportunities to exploit such a system. Association of the enzymes with membranes prevents easy extraction and purification and limits the opportunities to produce useful recombinant enzymes by cloning the relevant genes for expression in microbial systems. All P450s have a porphyrin-haem active site that requires a second protein to reduce the iron component, often cytochrome P450 reductase or



Scheme 1.3 *Selected examples of CYP-catalysed oxidations*[31]

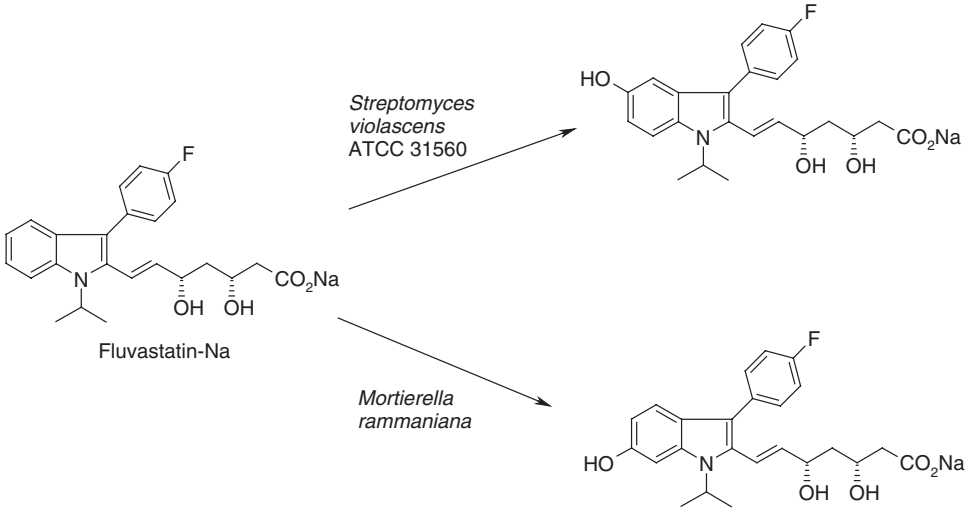
cytochrome b3. In addition, a reduced nucleotide cofactor (reduced-form nicotinamide adenine dinucleotide (NADH) or reduced-form nicotinamide adenine dinucleotide phosphate (NADPH)) must be provided in stoichiometric quantities. Despite these difficulties, a range of commercially available solutions exists for synthesis of small quantities of metabolites. The effort put into development of these systems reflects both their importance to the pharmaceutical industry and the limited availability of off-the-shelf microbial oxidations.

Liver microsomal preparations contain a spectrum of P450 isozymes with competing activities. Depending upon the source, these may or may not correspond to the specificities of the human CYPs. Such systems serve to evaluate the spectrum of potential metabolites from a given substrate molecule, but they have more limited value for synthesis of single metabolites in useful yield. To this end, considerable effort has been directed towards development of genetically engineered cell lines that express single specific CYPs.[32] These systems essentially provide a microsomal preparation of P450s expressed together with an appropriate reductase component in insect cells, yeasts or bacteria.

More recent developments include the modification of the genes encoding several human CYP genes and the corresponding reductase to allow expression as soluble proteins in *Escherichia coli*. This provides a water-soluble enzyme system for a limited number of pharmacologically relevant P450s, but retains the basic disadvantages of slow reaction rate, sensitivity to substrate concentration and the need for added reductase and reduced NAD(P) cofactors. All these systems remain very much a tool for synthesis of limited quantities of drug metabolites rather than scaleable synthetic tools.

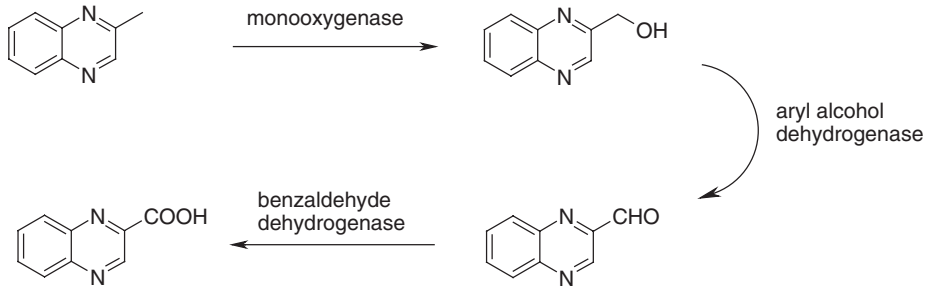
Although few of the P450s characterized from microbial sources have substrate specificity corresponding to that of the human liver CYPs, these enzymes may tolerate higher substrate concentrations, promising higher yields of metabolites. Of particular interest are enzymes such as cytochrome P450 BM-3 from *Bacillus megaterium* (CYP102), which is a natural fusion of monooxygenase with a reductase, and offers a system for biocatalysis with fewer components. Very recently, a kit of variants of P450 BM-3, developed by the Arnold group,[33] has become commercially available in a 96-well plate format. This collection of enzyme variants, generated by directed-evolution techniques, is claimed to accept a broader substrate range and offer greater potential for use at scale than human CYPs.

Currently, microbial whole cells must be considered the main option to produce larger quantities of phase I metabolites through biotransformation. These processes are generally regarded as scaleable through standard fermentation technology, although careful screening of microorganisms is required to achieve the reaction required in the absence of competing side reactions or catabolism of the substrate and product. Some microorganisms are particularly effective at mimicking phase I metabolic reactions such as hydroxylation, and a recent review on how these can be employed in the study of drug metabolism has been published by Ghisalba and Kittelmann.[34] The oxidation of fluvastatin by different microorganisms provides a good example of how these systems can be beneficial in selective metabolite preparation (Scheme 1.4).[35]



Scheme 1.4 *Selective microbial hydroxylation of fluvastatin*

For a manufacturing-scale process the requirement to maximize overall yield and eliminate side reactions is greater still, yet whole-cell biocatalysis remains the sole option for oxidative biotransformation. For example, a whole-cell monooxygenase-based oxidation of 2-methylquinoxaline has been reported by Wong *et al.*[36] that uses a strain of *Pseudomonas putida* possessing an aryl ADH and a benzaldehyde dehydrogenase that together generate 2-quinoxaline carboxylic acid in three steps at a reported 86 % overall yield (Scheme 1.5).



Scheme 1.5 *Biotransformation of 2-methylquinoxaline by* Pseudomonas putida *ATCC 33015*

This example shows the fortuitous interaction of multiple enzymes in a single microbial system. However, competing activities are more likely using a natural system, and there will be an ongoing desire to develop a scalable process using either an isolated stable and active P450 monooxygenase, or a recombinant whole-cell system based on a cloned and overexpressed P450. At the time of writing, neither system yet exists, although the recently characterized microbial enzymes with fused reductase clearly offer some potential for development. Until recently, the challenges of providing the necessary NADH or NADPH cofactors to permit the use of any oxidoreductase outside of a whole-cell system would have been considered a barrier to the development of a viable process, but these problems have been solved in response to the increasing application of enzymes for asymmetric ketone reduction (see Section 1.3.4.4).

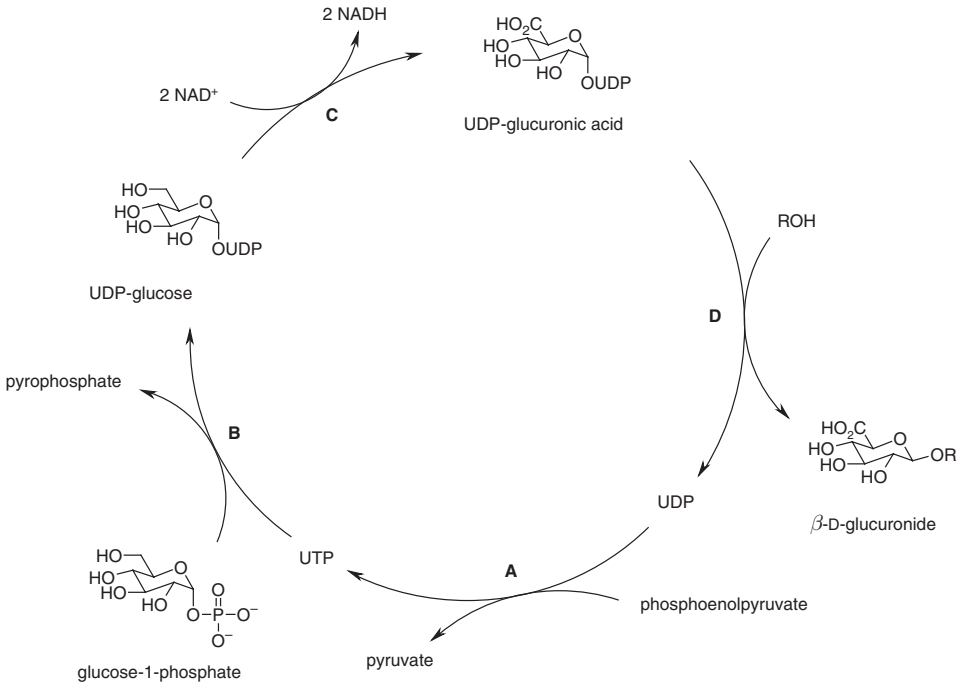
1.3.1.2 Phase II Metabolic Transformations

In phase II biotransformations, the conjugating functional group is generally transferred to the target molecule from an activated cofactor or 'coenzyme'. Most such reactions use transferase mechanisms found throughout biology; for example, acetyltransferases requiring acetylcoenzyme A or methyltransferases dependent on *S*-adenosylmethionine. Amongst phase II reactions, glucuronidation is a special case, as it is seldom observed outside of mammalian metabolism of xenobiotics.

Phase II drug metabolites are often the final form in which a xenobiotic is solubilized for release from the body, and many display significant biological activity. Synthesis of purified phase II metabolites, therefore, is a requirement of the drug development process.

Most of the enzymes involved in mammalian phase II metabolism are, like P450s, poor candidates for *in vitro* biocatalysis, being membrane associated and requiring activated

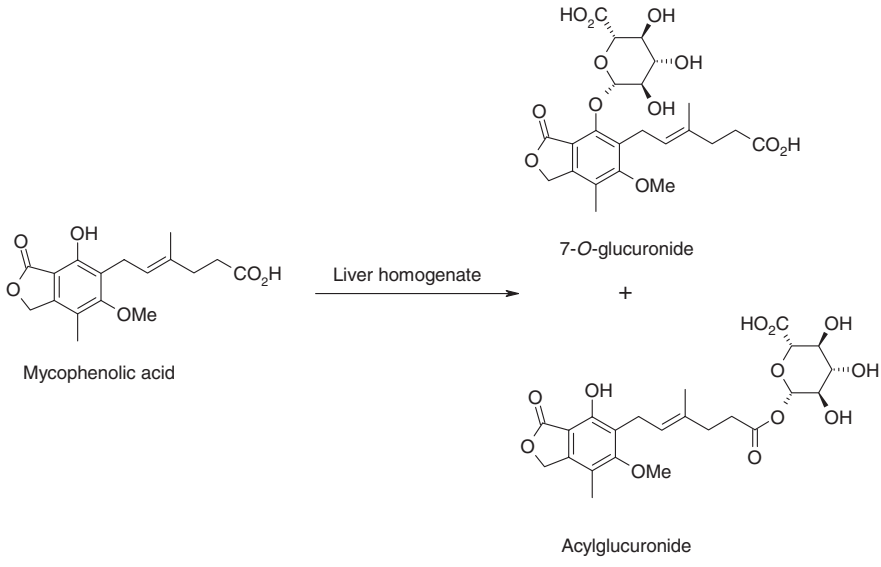
coenzymes that may be both costly and unstable. For example, glucuronidation using uridine diphosphate (UDP) cofactor is catalysed by UDP-glucuronyltransferases using the catalytic cycle shown in Scheme 1.6.



Scheme 1.6 *Uridine diphosphate glucuronide transferase cycle.[37] (Reproduced by permission of the Royal Society of Chemistry.)*

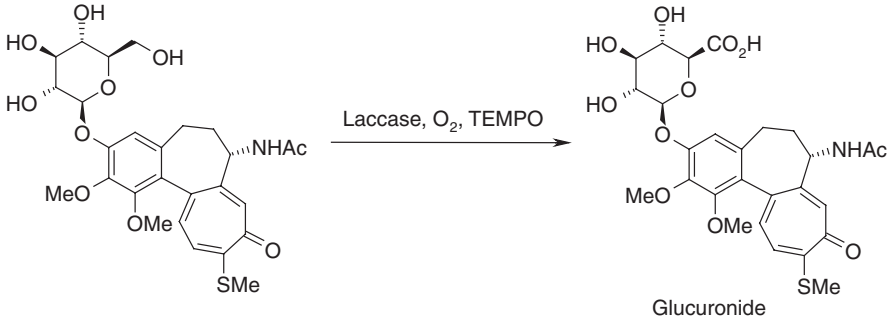
Currently, glucuronides that cannot be easily synthesized chemically are prepared using liver microsomes,[25] which can allow access to hundreds-of-milligram quantities of the desired metabolite after purification. Gene cloning for single mammalian UDP-glucuronosyltransferases has been less successful than for the human CYPs, or perhaps less rigorously attempted. Between mammalian species, the spectrum of glucuronyltransferase isoenzymes can vary significantly, particularly in the case of *N*-glucuronidation.[38] These differences may be exploited by the chemist seeking to prepare a particular glucuronide for pharmacological studies.

For example, Kittelmann *et al.*[39] were interested in the pharmacologically active acylglucuronide of mycophenolic acid, an immunosuppressant. In humans, this molecule is glucuronidated to afford a 1:80 mixture of acylglucuronide and inactive 7-*O*-glucuronide respectively (Scheme 1.7). By screening a range of liver homogenates from different mammalian species, the group was able to produce the two metabolites in a 1:1 mixture that allowed the preparation of multi-hundred-milligram quantities of the desired metabolite.



Scheme 1.7 *Glucuronidation of mycophenolic acid with liver homogenate.*

Glucuronidation specifically is rarely observed in microorganisms; however, enzymatic glycosylation as a general reaction is common to most living systems, the most common case being simple *O*- or *N*-glucosylation. Glucuronic acid is derived from glucose by oxidation at the 6-carbon; hence, it is worth considering glucosides as targets potentially accessible using microbial biotransformations and subsequently converting these to the corresponding glucuronides. Recent work by Baratto *et al.*[40] has shown that glucuronides can be readily accessed from glycosides by a mild laccase/2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) oxidation (Scheme 1.8).



Scheme 1.8 *Selective laccase/mediator oxidation of the natural glycoside thiocolchicoside*

Laccases are oxidoreductases, primarily secreted by fungi, available in industrial quantities for use in the fabrics industry. Their natural role is in the breakdown of

polyphenols (present in plants as lignin) using molecular oxygen as the oxidant. More recently, their substrate range has been dramatically increased to allow allylic and benzylic alcohol oxidation by the use of catalytic quantities of mediators such as 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), hydroxybenzotriazole (HOBt), *N*-hydroxyphthalimide (HPI) and TEMPO.[41] The mediator undergoes oxidation by the laccase, which in turn oxidizes the substrate of interest before returning to its original state. Any selectivity towards the substrate, therefore, is based on the chemical interaction between substrate and activated mediator and should be relatively broad. The feasibility of preparing a range of glucuronides using laccase/mediator oxidation may now be limited only by access to the required glycoside.

Of all the biotransformations involved in phase II metabolism it is glycosylation that potentially has the greatest significance as a tool in the synthesis of pharmaceutical molecules, extending well beyond the synthesis of glucuronides. The glycosylation reaction is involved in the synthesis of a range of molecules that is probably the most abundant in biology, from macromolecules such as polysaccharides and glycoproteins down to small-molecule glycosides, with functions ranging from structure and storage to signalling and detoxification.[42] Glycosylated molecules have increasing application both as active ingredients and in drug delivery. There are many opportunities for application of biocatalysis in an area where the chemistry is increasingly complex.

Many glycosylated natural products display potent pharmacological activity, including large molecules that are strictly beyond the scope of this review, although of immense significance to the pharmaceutical industry. Biopharmaceuticals represent a broad array of macromolecular natural products or natural product analogues whose development has been rapidly expanding since the introduction of recombinant insulin 20 years ago.[43] There are over 400 biopharmaceuticals currently under development, the majority being vaccines and monoclonal antibodies primarily targeting cancer, infectious and autoimmune diseases. Owing to their highly complex structures, biopharmaceutical drugs are generally produced by recombinant cell culture. The majority of such processes currently use mammalian cell culture rather than microbial fermentation as a system for protein expression owing to the requirement for biochemical modification of the protein, which may not be feasible in microbial cells. Post-translational modifications of proteins include glycosylation and other relatively complex conjugations that are often essential for biological activity.[44]

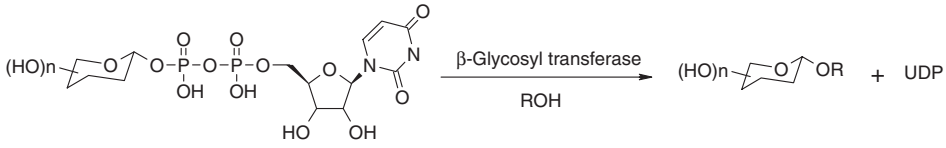
Many pharmacologically active glycosides of low or intermediate molecular weight are also produced as natural products, extracted from plants (digoxin), animals (heparin fractions) or microbial cultures (macrolide and aminoglycoside antibiotics). Currently, biocatalytic glycosylation is confined to relatively simple operations rather than *in vitro* synthesis of these more complex molecules, and natural products may be used as starting materials for semisynthetic molecules. Compared with proteins and nucleic acids, which are produced by template-driven biosynthesis, there is no equivalent to ensure the structural fidelity of carbohydrate macromolecules and other glycosylated products.

In the case of glycoprotein biopharmaceuticals, the consequence of the multiple glycosylated products is the need for strict control of the manufacturing process to maintain a reproducible spectrum of products with consistent therapeutic profile. That biopharmaceuticals often contain mixtures of related products can also be advantageous to the pharmaceutical industry in warding off generic competition, due to the challenge of

replicating an exact process to generate an equivalent product. The challenge of multiple glycosylation products, however, extends to much smaller molecules and is common to both chemical and biological methods of glycosylation.

The anticoagulant fondaparinux, a synthetic analogue of the terminal fragment of heparin, is synthesized using multiple protection/deprotection steps that result in a route of up to 50 steps. There is, as yet, no enzymatic system that approaches the capability to make such a molecule.[45] As this modified pentasaccharide is a natural product, it should, in theory, be accessible through a series of biotransformations, but we currently lack the biocatalytic tools to achieve more than a few steps and would still need to use some protection steps to avoid multiple products. Enzymatic synthesis *in vivo* depends largely on the levels and selectivities of glycosylating enzymes to achieve multistep reactions, a situation that has been mimicked *in vitro* for simpler systems.[46]

Most of the enzymes involved in the biosynthesis of glycosides are, like the UDP-glucuronyltransferases of phase II drug metabolism, members of the Leloir glycosyltransferase superfamily. Enzymes in this category catalyse transfer of the saccharide from a sugar nucleotide, usually a UDP or thymidine diphosphate glycoside, to an acceptor nucleophile such as an alcohol or amine (Scheme 1.9). The most abundant class of enzymes forming glycosidic bonds in nature, they are usually highly regioselective and enantioselective and have been widely applied in organic synthesis.[47,48]



Scheme 1.9 *General scheme for a UDP-β-glycosyltransferase-catalysed transformation (where ROH can be another sugar or any alcohol)*

Many of these enzymes are membrane associated, like the mammalian UDP-glucurosyltransferases, but there are also many soluble enzymes, such as the UDP-glucosyltransferases that serve to solubilize xenobiotics in plants by forming their glucosides. Plants appear to be particularly rich sources of glycosyltransferases: less than 30 such enzymes have been identified in the human genome,[49] yet there are 117 putative glycosyltransferases in the genome of *Arabidopsis thaliana*, a species used as a model system by molecular biologists due to the small size of its genome relative to that of other plants.[50] Lim *et al*.[51] successfully produced a panel of transgenic organisms where glycosyltransferases from *A. thaliana* are expressed in bacterial cells, potentially facilitating their use in chemical synthesis. Glycosyltransferases are generally employed in whole-cell biotransformations so that the required sugar nucleotide may be generated *in situ* by a mechanism similar to that outlined in Scheme 1.6.

'Non-Leloir' glycosyltransferases, using non-nucleotide donors such as simple sugar phosphates, are also found throughout biology and may be applied in biocatalysis; for example, in the *N*-transglycosylation reactions described in Section 1.3.3. There are, in addition, a large number of glycosidases which hydrolyse glycosidic bonds. Specificity for

these enzymes tends to be significantly lower than for the glycosyltransferases, potentially allowing for the development of a more general biocatalyst for glycosylation using the reverse reaction. Glycosides may be accessed through glycosidase-catalysed reverse hydrolysis under thermodynamic or kinetic control by using free or activated sugar donors respectively. However, this approach is hampered by low conversions.[52]

This limitation was overcome by Mackenzie *et al.*,[53] who developed glycosidase variants containing an amine in place of the active-site carboxylate nucleophile that is responsible for glycoside cleavage. This excellent example of rational enzyme modification resulted in a new class of artificial enzyme known as glycosynthases, which are capable of selectively producing oligosaccharides in high yield from glycosyl fluoride donors of opposite anomeric configuration to that of the desired products. This pioneering work led to the development of other artificial enzymes, such as retaining glycosynthases, where the anomeric configuration of the glycosyl donor is retained, and thioglycoligases and thioglycosynthases, which form *S*-glycosidic bonds.[52] Glycosynthases are now available for the formation of a diverse range of β-(1→3)-, β-(1→4)-, β-(1→6)- and α-(1→4)-linked oligosaccharides.[48]

The increasing availability of biocatalytic tools for glycosylation is applicable to the drug discovery process as well as to metabolite preparation and synthesis of established glycosidic active ingredients. There is growing interest in the discovery of new polysaccharide-containing drugs. Glycorandomization, a powerful biocatalytic approach to the generation of libraries of unnatural polysaccharides through the use of enzyme variants with relaxed substrate specificity, represents one important approach towards this end.[48]

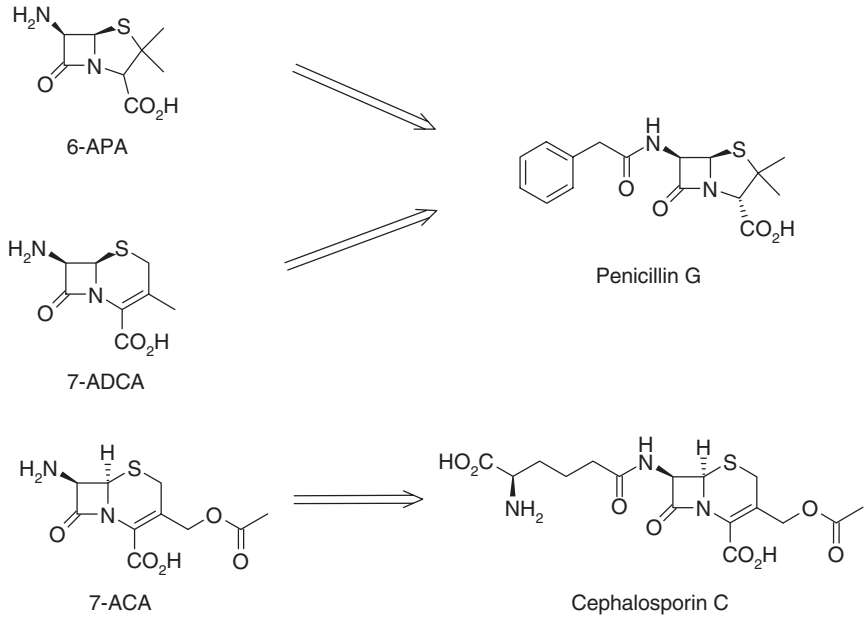
In summary, biocatalysis offers a number of alternatives to chemical synthesis for the selective preparation of metabolites. The use of recombinant human CYPs is an attractive method, as little screening is required and the enzymes catalyse a broad range of metabolic reactions. Unfortunately, owing to their instability and high cost, they are unlikely to prove suitable for the preparation of large quantities of a desired metabolite. In contrast, microorganisms offer a cheap and scalable alternative and their diversity can often allow the identification of a suitable system. However, microorganisms often contain competing activities, and so the availability of kits of microbial P450s and glycosylating enzymes, together with the development of new methodologies such as the laccase/mediator-catalysed oxidation of glycosides, offer distinct advantages.

1.3.2 Regioselective/Chemoselective Biotransformations

Selective reaction at only one position in a molecule that contains two or more of the same functionality, or different functionalities that react in a similar manner, can be difficult to achieve chemically without lengthy protection/deprotection strategies. In contrast, such regio- and chemo-selective transformations can frequently be realized surprisingly easily with a biocatalyst, as demonstrated by the following examples.

The β-lactams, mainly penicillins and cephalosporins, are by production volume the most important class of antibiotics worldwide, enjoying wide applicability towards a range of infectious bacteria. Most of the key molecules are semi-synthetic products produced by chemical modification of fermentation products. Production of these molecules has contributed significantly to the development of large-scale microbial fermentation technology, and also of large-scale biocatalytic processing.

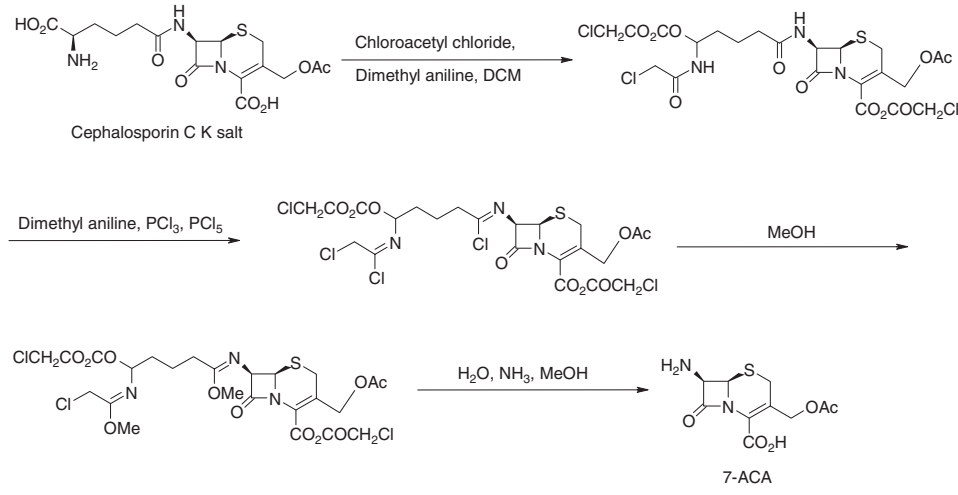
Semi-synthetic penicillins are accessed from 6-aminopenicillanic acid, (6-APA), derived from fermented penicillin G. Starting materials for semi-synthetic cephalosporins are either 7-aminodesacetoxycephalosporanic acid (7-ADCA), which is also derived from penicillin G or 7-aminocephalosporanic acid (7-ACA), derived from fermented cephalosporin C (Scheme 1.10). These three key building blocks are produced in thousands of tonnes annually worldwide. The relatively labile nature of these molecules has encouraged the development of mild biocatalytic methods for selective hydrolysis and attachment of side chains.



Scheme 1.10 *Key intermediates for the production of semi-synthetic cephalosporins*

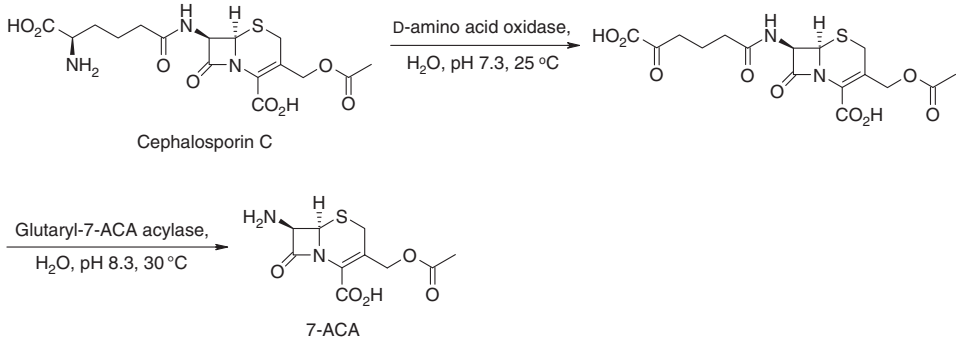
Penicillin acylases or amidohydrolases, which cleave the amide side chain of penicillin G, have been known for almost 50 years.[54] As one of the first enzymes to be developed for use at scale in the pharmaceutical industry, penicillin G acylase (PGA) has often been used as a model system for academic studies from molecular biology to biochemical engineering. Despite extensive screening, however, for decades there was no equivalent enzyme to generate 7-ACA by cleaving the polar D-α-aminoadipoyl side chain from cephalosporin C.

The traditional chemical approach to 7-ACA requires the protection of the amine and carboxylic acid groups of cephalosporin C by treatment with an acid chloride.[55] The resulting mixed anhydride is then converted to the imodyl chloride using phosphorus pentachloride, which is subsequently broken down to 7-ACA with methanol and water via a transient imodyl ether (Scheme 1.11). The use of subzero reaction temperatures and numerous hazardous reagents, required in order to avoid hydrolysis of the acetate and highly labile β-lactam moieties, have a significant cost and environmental impact on this high-tonnage product.



Scheme 1.11 *Chemical route to 7-ACA; DCM: dichloromethane*

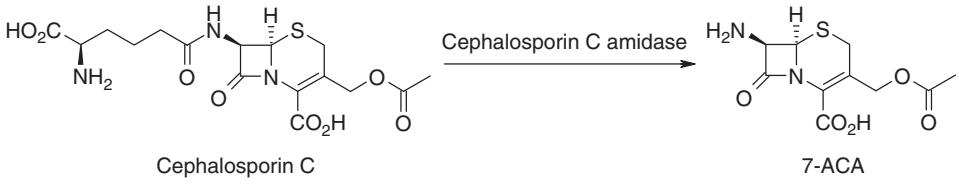
An alternative two-step biocatalytic route, first developed at Glaxo in the 1970s, utilized a D-amino acid oxidase and an amidase to provide 7-ACA under physiological conditions (Scheme 1.12).[56] This process has since been established in several companies, with minor modifications. In fact, 7-ACA was manufactured by GSK at Ulverston (Cumbria, UK) using both the chemical and biocatalytic processes in parallel for a period of 2 years during which time the environmental benefits of the biocatalytic process were assessed (see Section 1.6).



Scheme 1.12 *Two-enzyme route to 7-ACA*

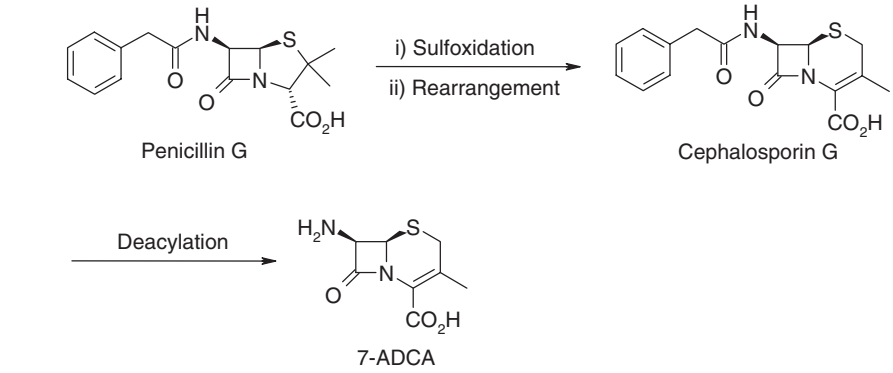
A single-step process using one biocatalyst might be expected to provide even greater environmental and cost benefits. In addition to providing a simplified process, the single-enzyme process affords D-α-aminoadipic acid as a co-product. Being an optically pure chiral product, α-aminoadipic acid is of potential commercial value in contrast to the ammonia and glutamic acid co-products resulting from the two-enzyme process. However,

this option proved to be somewhat elusive until the discovery of a cephalosporin C amidase from *Pseudomonas* sp. SE-83 (Scheme 1.13).[57] Cephalosporin C acylases have subsequently been found in other bacterial and fungal strains.[58]



Scheme 1.13 *Single-enzyme route to 7-ACA*

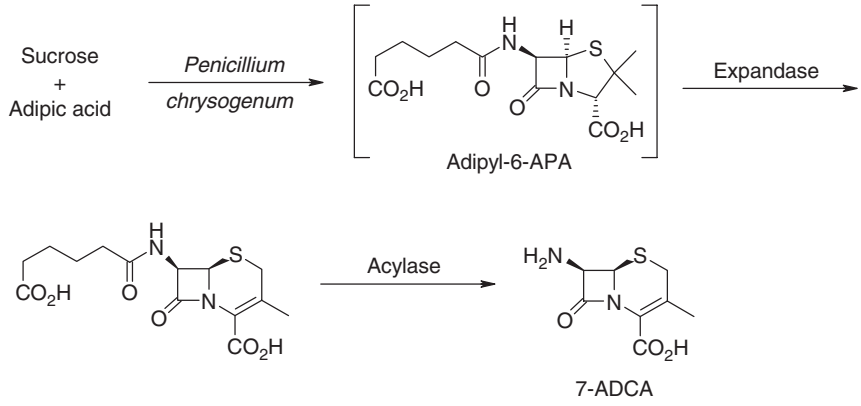
There has also been extensive activity towards the replacement of the entire chemical route to 7-ADCA (Scheme 1.14) with a biocatalytic one. This is somewhat more complex than the above example, as the penicillin fermentation product requires ring expansion as well as side-chain hydrolysis in order to arrive at the desired nucleus. The penicillin nucleus can be converted to the cephalosporin nucleus using expandase enzymes, a process that occurs naturally during the biosynthesis of cephalosporin C by *Acremonium chrysogenum* and cephamycin C by *Streptomyces clavuligerus* from isopenicillin N (6-APA containing a 6-L-α-aminoadipoyl side chain).[59]



Scheme 1.14 *Chemical route to 7-ADCA*

The expandase of cephalosporin C biosynthesis is fused with a hydroxylase acting on the 3-methyl group of the cephalosporin, these being two separate enzymes in cephamycin C biosynthesis. For this reason the *S. clavuligerus* expandase is used where 7-ADCA is the desired end product. Both expandases are highly specific for the 6-position amide: during the biosynthesis of cephalosporin C, isopenicillin N must be isomerized to the D-α-aminoadipoyl analogue, penicillin N, before ring expansion can be catalysed. Not surprisingly, cheap, commercially available penicillins, such as penicillin G or 6-APA, are not substrates for the expandase.

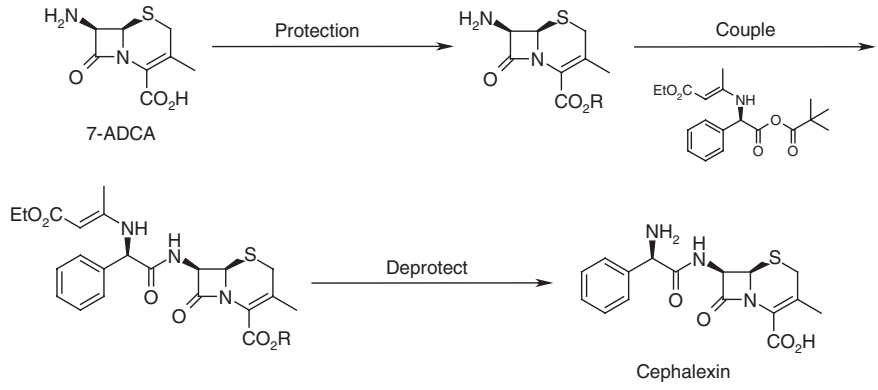
An elegant solution to production of 7-ADCA was achieved by Conder *et al.*[60] by combining modifications to the fermentation process, strain and downstream biocatalytic treatment. The expandase of *S. clavuligerus* was found to be active with adipoyl-6-APA as substrate. It was recognized that adipoyl-6-APA could be generated as a fermentation product by feeding adipic acid, in the same way that phenylacetic acid is fed to generate penicillin G. Feeding adipic acid to a *Penicillium chrysogenum* recombinant strain carrying a cloned expandase gene, adipoyl-7-ADCA can be directly obtained (Scheme 1.15). The side chain of adipoyl-7-ADCA can then be removed in a subsequent step by treatment with an acylase closely related to that used to remove the glutaryl side chain in the two-enzyme process for 7-ACA.



Scheme 1.15 *Biocatalytic route to 7-ADCA*

The extensive literature of β-lactam antibiotics biotechnology will show many further examples of genetic manipulation towards the formation of the three nuclei for semi-synthetics production; however, enzymatic methods have also been sought towards the synthesis of the final active antibiotics themselves. Further elaboration of 6-APA, 7-ACA or 7-ADCA requires the acylation of the 6- or 7-amino groups without affecting other sensitive functionality present in the molecules. Traditional approaches employ bulky coupling reagents, chlorinated organic solvents (such as dichloromethane) and atom-inefficient protection/deprotection strategies to achieve this goal. For example, in the production of cephalexin, 7-ADCA is esterified to protect the carboxylic acid functionality, prior to 7-aminoacylation using a heavily functionalized mixed anhydride derivative of (*R*)-phenylglycine (Scheme 1.16).[61]

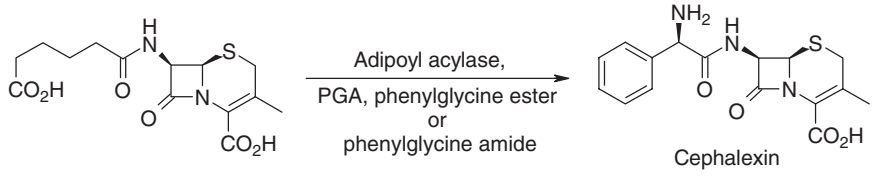
Given that hydrolysis is a reversible reaction, the principle of microscopic reversibility implies that biocatalytic aminoacylation should also be applicable as a mild and efficient alternative method of introducing the side chain of both penicillin- and cephalosporin-based antibiotics. This is the case, with PGAs proving to be particularly effective biocatalysts towards the aminoacylation of both penicillin and cephalosporin nuclei with a variety of carboxylic acids.[62] Amoxicillin and cephalexin, two of the most important β-lactam antibiotics, contain an (*R*)-phenylglycine side chain which cannot be directly introduced as the amino acid due to its zwitterionic nature at the moderate pH values at



Scheme 1.16 *Chemical route to cephalexin*

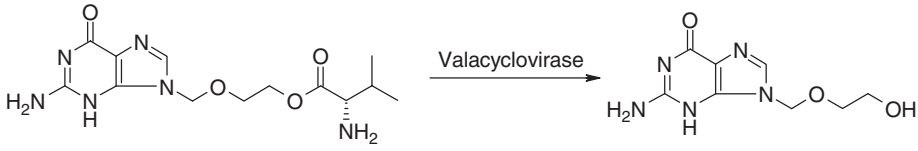
which PGAs operate. Amino acid esters and primary amides are not zwitterionic, and so (*R*)-phenylglycine, or other amino acids, can instead be chemoselectively introduced by a kinetically controlled PGA-catalysed reaction. PGAs are not stable in organic solvent, and so the aminoacylation reactions are performed in an aqueous environment at high substrate concentrations to minimize competing hydrolytic reactions.

During the some 40 years of development that have been devoted towards achieving current levels of efficiency in the production of β-lactam antibiotics, many contributions have been made towards our knowledge of biocatalytic processes, particularly enzyme immobilization techniques (see Section 1.5).[63] Even so, the biosynthesis of semi-synthetic antibiotics still holds further challenges. One limitation of the current cephalexin bioprocess is the inhibition of PGA by phenylacetic acid, which prevents the adoption of a single-pot side-chain exchange and, ultimately, a single-stage biosynthetic route. Schroen *et al*.[64] overcame this limitation by employing adipoyl-7-ADCA instead of penicillin G as starting material in the cephalexin process. PGA is not inhibited by adipic acid and so cephalexin can be accessed using an efficient tandem adipoyl-acylase-catalysed hydrolysis/PGA-catalysed aminoacylation procedure (Scheme 1.17).



Scheme 1.17 *Preparation of cephalexin using a tandem hydrolysis/amidation approach*

Prodrugs provide a vehicle by which the bioavailablility of a drug displaying poor water solubility can be enhanced or a method of targeting diseased areas of the body. Following uptake, the drug is frequently released by the action of metabolic enzymes. For example, the human enzyme believed to be primarily responsible for the rapid *in vivo* hydrolysis of valaciclovir to aciclovir has recently been isolated and characterized (Scheme 1.18).[65]



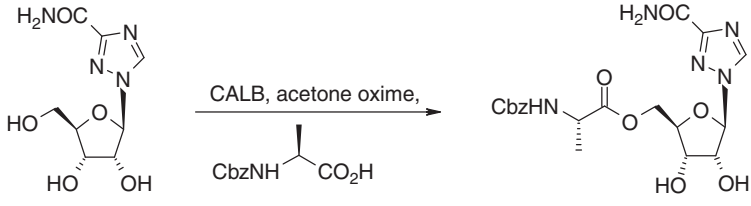
Scheme 1.18 *Hydrolysis of valaciclovir by a human hydrolase enzyme*

Given that many prodrugs are broken down by enzymic action, their enzymatic synthesis should also be feasible.

Unlike aciclovir, many other nucleoside analogues contain a number of hydroxyl groups, and so chemical synthesis of the desired ester prodrug with adequate regioselectivity can be challenging. For example, attempts to prepare the L-alanine prodrug of ribavirin, a powerful antiviral agent used to treat hepatitis C, by direct chemical esterification resulted in a mixture of products.[66] This could only be overcome by the use of a three-step procedure involving acetonide protection/deprotection of the secondary hydroxyl moieties.

At first sight, it appears that it should be feasible to prepare such esters regioselectively using a similar biocatalytic approach to that employed for the 6- and 7-amino acylation of 6-APA and 7-ADCA shown above. Unfortunately, owing to the poor nucleophilicity of alcohols, biocatalytic esterification in aqueous media is far more challenging than amidation. Therefore, it was not until the pioneering work of Klibanov and co-workers,[67] who first demonstrated the use of enzymes in neat organic solvents, that this option became viable (see Section 1.4).

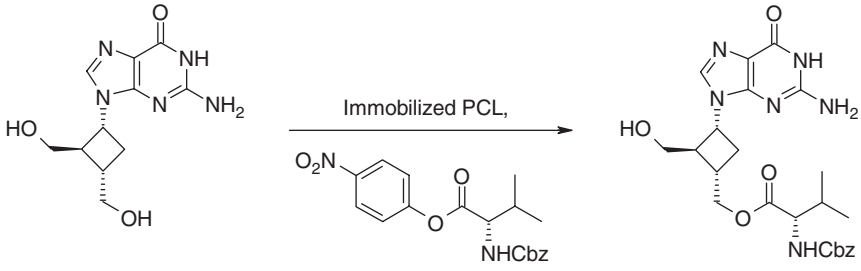
Employing methodology developed by the Gotor group,[68] Zaks and co-workers[69] were able to produce 5′-*N*-CBz-(*S*)-alaninyl ribavarin with complete selectivity using the widely utilized lipase B from *Candida antarctica* (CALB) and the oxime ester of *N*-CBz-protected L-alanine, an irreversible acyl donor used to shift the reaction equilibrium towards product formation (Scheme 1.19). After optimization, about 80 kg of the CBz-protected prodrug was produced in >80 % isolated yield by the treatment of ribavirin with 0.8 weight equivalents of CALB in tetrahydrofuran (THF) at 60 °C for 24 h. This approach has also been used to produce ester prodrugs of other nucleoside antivirals, such as nelarabine.[70]



Ribavirin

Scheme 1.19 *Regioselective preparation of 5′-N-CBz-(S)-alaninyl ribavarin*

The preparation of *N*-CBz-(*S*)-valinyl lobucavir provides a particularly challenging example, where only one of two primary alcohols is acylated with excellent regioselectivity (Scheme 1.20).[71]



Scheme 1.20 *Regioselective esterification of lobucavir (PCL: Pseudomonas cepacia lipase, now known as Burkholderia cepacia)*

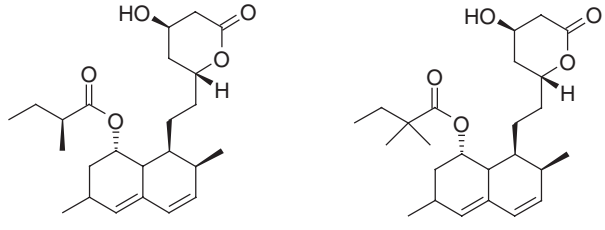


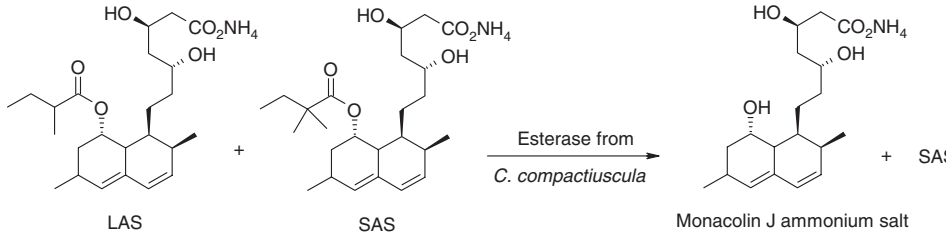
Figure 1.6 *Structures of lovastatin and simvastatin.*

Simvastatin is a semi-synthetic statin that is produced from the natural statin lovastatin.[72] Both are potent antihypercholesterolemic agents with simvastatin differing from lovastatin by just one additional methyl substituent residing on the 2-(*S*)-methylbutyrate side chain (Figure 1.6).

Lovastatin is produced by fermentation from the filamentous fungus *Aspergillus terreus* and can be converted to simvastatin by a single-step chemical methylation.[73] However, this transformation is hampered by low yields, which result in downstream processing issues resulting from difficulties in the separation of starting material and product. Simvastatin is instead produced using a lengthier protection/deprotection strategy.[74]

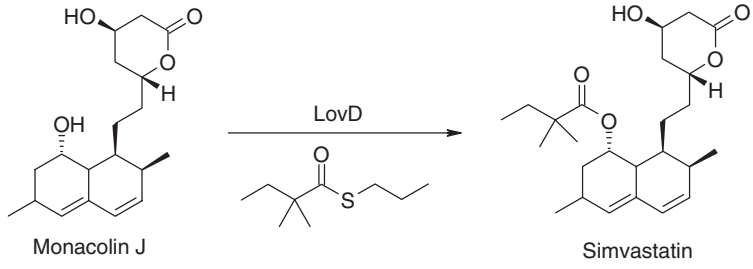
To overcome these separation issues, Schimmel *et al.*[75] sought a hydrolase enzyme capable of selectively hydrolysing the 2-(*S*)-methylbutyrate ester of lovastatin ammonium salt (LAS) whilst leaving the more hindered 2-dimethylbutyrate ester of the simvastatin ammonium salt (SAS) unchanged. After screening 150 microorganisms, the fungus *Clonostachys compactiuscula* was found to produce a suitable esterase. By applying this esterase to inseparable LAS/SAS mixtures resulting from the single-step chemical methylation, they were able to hydrolyse LAS selectively to the more polar, readily separable monacolin J ammonium salt, thus providing a two-step conversion of lovastatin to simvastatin (Scheme 1.21).

Regioselective esterification of the 8-hydroxyl group of accumulated monacolin J, produced using a truncated lovastatin biosynthetic pathway, could provide a viable biocatalytic route to simvastatin. With this aim, Xie and Tang[76] cloned and expressed the acyl transferase LovD from the lovastatin biosynthetic pathway into *E. coli*; they found



Scheme 1.21 *Selective enzymatic hydrolysis of LAS/SAS mixtures*

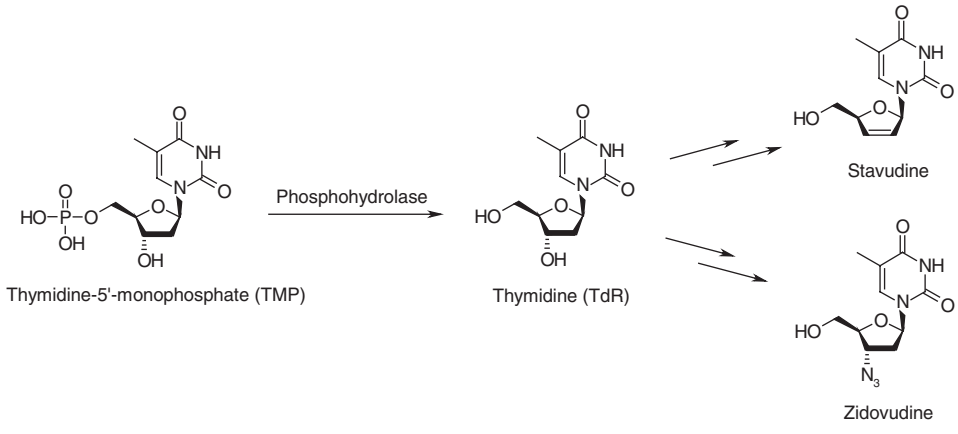
that LovD was not only active towards lovastatin synthesis, but also capable of producing simvastatin using simple α-dimethylbutyrate thioesters (Scheme 1.22). Whereas a biotransformation using partially purified LovD in aqueous solution gave a conversion of only 60 % due to competing hydrolysis, whole-cell reactions went to completion to afford 4–6 g L^{-1} product concentrations. The authors speculated that the superior results obtained from the whole-cell reactions might result from active transport of simvastatin out of the cells which are subsequently impermeable to re-entry, whereas the more polar monacolin J can diffuse in both directions. The efficiency of the transformation was later improved by knocking out the gene expressing the BioH enzyme which is responsible for competing thioester hydrolysis.[77]



Scheme 1.22 *Biocatalytic regioselective acylation of monacolin J*

Using molecular biology techniques to redirect primary metabolic pathways, microorganisms may be engineered to overproduce a wide range of biochemical intermediates, such as amino acids and vitamins.[78] This principle can be extended by introducing novel enzymes and, thereby, novel biotransformation steps into microbial hosts in order to generate unnatural products from natural precursors. Such a modification may be lethal for the host cell, requiring the application of techniques developed for controlled, conditional gene expression in the production of recombinant proteins.[79] This is illustrated by the engineered microbial production of the nucleoside thymidine (TdR), an important starting material for synthesis of the antiretrovirals zidovudine and stavudine (Scheme 1.23).

Although thymidine-5′-triphosphate is an almost universal component of DNA, it is exclusively derived from thymidine-5′-monophosphate (TMP). In contrast, TdR does not occur naturally and so it is impossible to manufacture TdR by manipulation of existing metabolic pathways as for most biochemical intermediates. This problem was addressed



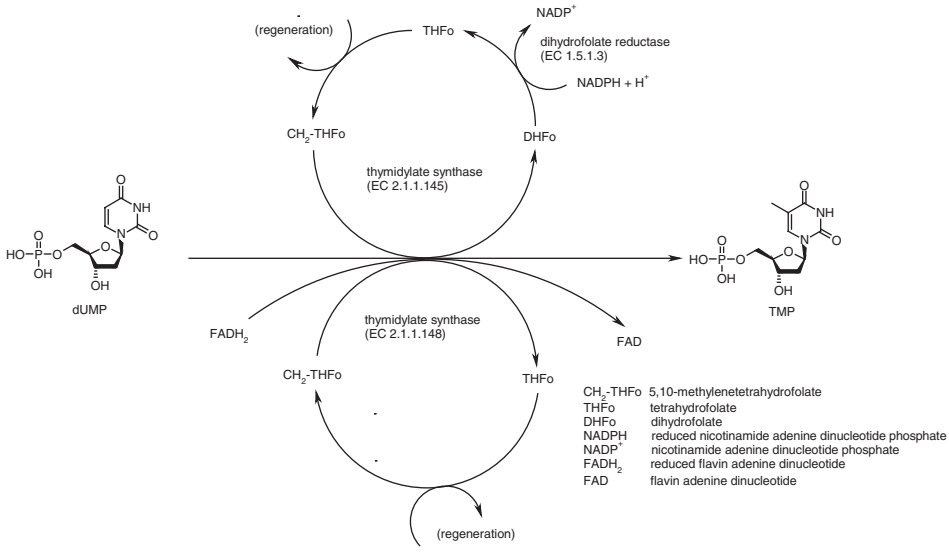
Scheme 1.23 *Enzyme-catalysed hydrolysis of thymidine-5′-monophosphate*

by McCandliss and Anderson[80] using a gene encoding TMPase, a phosphohydrolase acting on TMP to generate TdR. Such enzymes are found only in rare bacterial viruses with DNA incorporating uracil in place of thymine. This potentially lethal gene, capable of knocking out normal DNA synthesis, was coupled to an inducible promoter allowing strict control of its expression. Typical genetic refinements used in metabolic engineering were introduced, knocking out enzymes that would degrade TdR and enhancing pathways leading into TMP synthesis.

Deoxyribonucleotides are derived metabolically by reduction of the corresponding ribonucleotide, an arrangement that reflects the greater abundance of RNA compared with DNA. This reduction occurs at the level of the nucleoside diphosphates. TMP is derived by methylation of 2′-deoxyuridine-5′-monophosphate (dUMP), itself derived from the corresponding 2′-deoxyuridine-5′-diphosphate, which is generated by reduction of the analogous ribonucleotide uridine-5′-diphosphate. In order to enhance the process to commercial levels of productivity in an engineered strain of *E. coli*, Anderson *et al.*[81] added a number of recombinant genes encoding both a ribonucleotide reductase and the thioredoxin required to regenerate its reduced and active form.

TMPase acts to dephosphorylate both TMP and its precursor dUMP, forming a mixture of TdR and 2′-deoxyuridine (UdR). As a starting material for zidovudine synthesis, TdR must be essentially free of this impurity, which would pass through the manufacturing process to form a demethylated analogue of zidovudine. Separation of TdR and UdR requires difficult and costly downstream processing; hence, the key to a commercial process is metabolic engineering to minimize biosynthetic UdR.

Anderson *et al.*[82] investigated a range of solutions to this problem, each based on the principle of efficient methylation of dUMP to TMP to avoid the accumulation of a significant pool of free dUMP that could be converted to UdR. Various techniques were used to increase the efficiency of the methylation reaction itself using alternative forms of thymidylate synthase with enhanced catalytic activity and altered regulation. However, the most significant improvement was by enhancing activity of the enzymes recycling and replenishing the methyl donor 5,10-methylenetetrahydrofolate (Scheme 1.24).



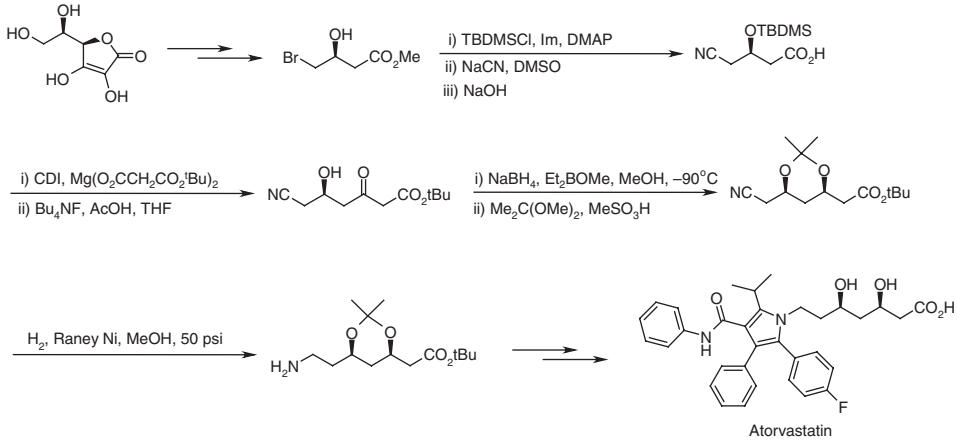
Scheme 1.24 *Methylation in TMP biosynthesis*

Biosynthetic production of thymidine is overall a complex process combining the controlled introduction of a novel biotransformation step into a biological system with selective enhancement or knock-out of a series of existing metabolic steps. Metabolic engineering to enhance cofactor recycling at both ribonucleotide reduction and dUMP methylation steps has important parallels in other systems, as whole-cell biotransformations are frequently employed as a means to supply, *in situ*, high-cost and usually labile cofactors.

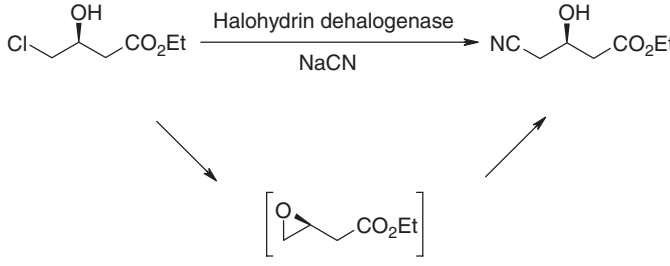
Atorvastatin, an antihypercholesterolemic agent, is a synthetic drug that was initially produced in kilogram quantities using an 11-step chemical route. The *syn*-1,3-diol-containing side chain was produced from the chiral starting material, isoascorbic acid (Scheme 1.25).[83]

Numerous biocatalytic routes to this challenging intermediate have been reported.[84] For example, Fox *et al*.[85] have recently developed an efficient regioselective cyanation starting from low-cost epichlorohydrin (Scheme 1.26). Initial experiments found that halohydrin dehydrogenase from *Agrobacterium radiobacter* expressed in *E. coli* produced the desired product, but inefficiently. To meet the projected cost requirements for economic viability, the product needed to be produced at 100 g L^{-1} with complete conversion and a 4000-fold increase in volumetric productivity. The biocatalyst needed to function under neutral conditions to avoid by-product formation, which causes downstream processing issues.

Using ProSAR technology (see Section 1.2), the group identified a variant halohydrin dehalogenase containing 37 mutations that gave the required volumetric productivity increase. This methodology is also applicable to other antihypercholesterolemic drugs, such as rosuvastatin and fluvastatin (Figure 1.7).



Scheme 1.25 *Chemical synthesis of the atorvastatin side chain*



Scheme 1.26 *Halohydrin-catalysed cyanation of epichlorohydrin*

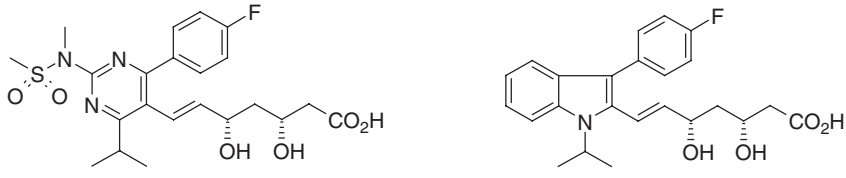


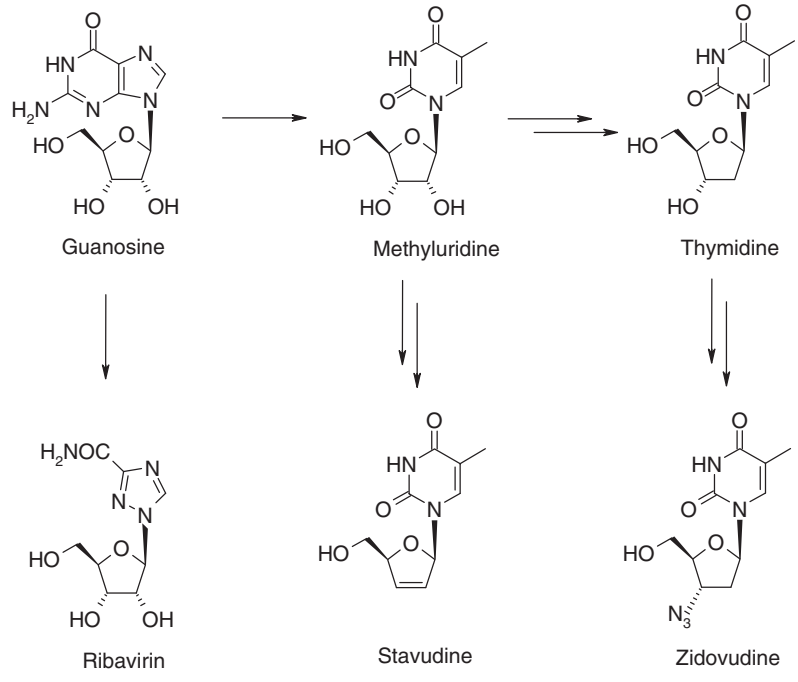
Figure 1.7 *Some other statins containing a 1,3-*syn*-diol side chain.*

In conclusion, regioselective biocatalysis has been extensively employed to access both semi-synthetic and synthetic pharmaceuticals. The methodology is particularly attractive for the streamlining of processes through the elimination of protecting-group strategies and to avoid the use of hazardous reagents.

1.3.3 Diastereoselective Biotransformations

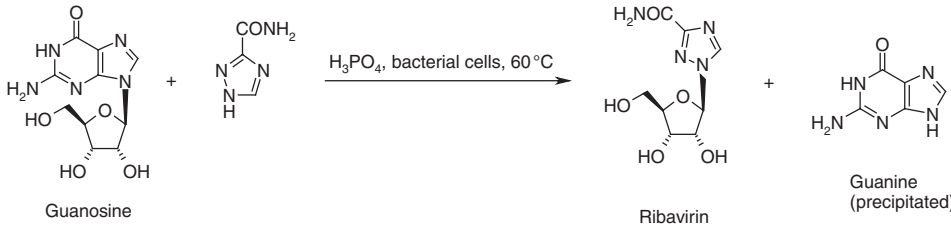
Diastereoselective reactions, where one or more chiral centres are generated in a selective manner within a molecule that already contains chirality, to produce single diastereoisomers (epimers) are very common in nature. Some examples of chemical processes which harness the properties of biocatalysts are shown below.

Highly diastereoselective enzyme-catalysed glycosylation reactions allow access to functionalized sugars and highly complex polysaccharides and provide an important pathway by which xenobiotics are metabolized (see Section 1.3.1). A similar transformation is the nucleoside phosphorylase (NP)-catalysed reversible cleavage of the *N*-glycosidic linkage of a nucleoside in the presence of phosphate to generate the corresponding pentose sugar phosphate and free nucleobase. NPs are ubiquitous in biology, and substrate ranges include deoxyribonucleosides and/or ribonucleosides with purine or pyrimidine nucleobases. *N*-Transglycosylation can be achieved by coupling cleavage of the sugar from a donor nucleoside to synthesis of a new nucleoside using a second, acceptor base. This reaction, which is completely regioselective towards the base and diastereoselective towards formation of the β-anomer at the sugar is a useful strategy for synthesis of nucleoside analogues, including many antiviral and anticancer agents, such as ribavirin or, indirectly via thymidine, zidovudine and stavudine (Scheme 1.27).[86]



Scheme 1.27 *Antiretroviral nucleosides accessible by NP catalysis*

Using guanosine or 2′-deoxyguanosine as starting material for the synthesis of ribonucleosides or deoxyribonucleosides respectively, the reaction can be driven towards completion by precipitation of the highly insoluble guanine co-product. This approach has



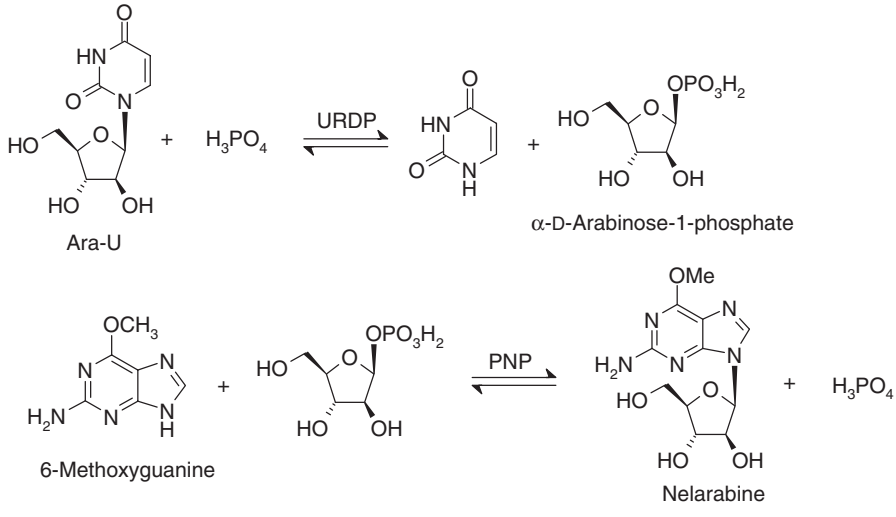
Scheme 1.28 *Enzymatic direct synthesis of ribavirin*

been used for direct synthesis of the antiviral ribavirin in approximately 75 % yield using bacterial cells (*Brevibacterium*) (Scheme 1.28).[87]

Like many reported *N*-transglycosylations, this reaction uses uncharacterized nucleoside phosphorylases from whole cells held at 50–60 °C, a temperature well above the range for viability of the parent microorganism. Remarkable temperature stability has been reported for three well-known NPs of *E. coli*: purine nucleoside phosphorylase (PNP), uridine phosphorylase (URDP) and thymidine phosphorylase.[88]

Certain NPs can use pentoses other than ribose or deoxyribose as substrates, enabling the synthesis of nucleoside analogues with unnatural sugar moieties: for example, in the synthesis of purine arabinonucleosides.[89] A convenient donor for transarabinosylation is 9-β-D-arabinofuranosyluridine (Ara-U), which can be accessed from uridine using a two-step chemical process to invert the 2′-hydroxyl group.[90] A general protocol for preparation of purine analogues using Ara-U with a mixture of purified URDP and PNP from *E. coli* is described by Averett *et al*.[95] The enzymes are used in varying proportions, depending upon the reaction rate for the required purine nucleoside synthesis, and are sufficiently robust for addition of water-miscible solvents to aid substrate solubility.

The URDP/PNP/Ara-U process is used to manufacture nelarabine, a water-soluble prodrug of 9-β-D-arabinofuranosylguanidine produced as a treatment for acute lymphoblastic leukaemia (Scheme 1.29).[70,92] The two-enzyme process is run at 200 g L^{-1}



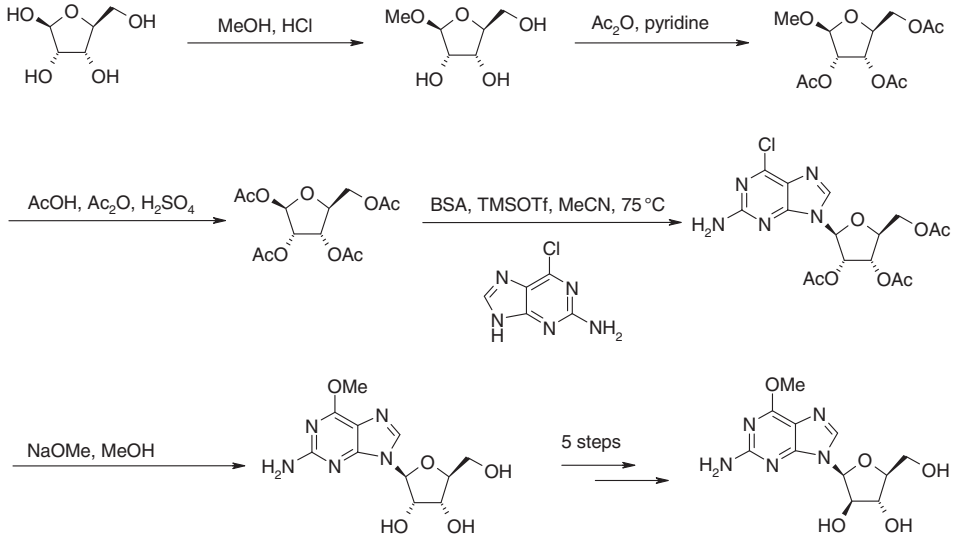
Scheme 1.29 *Preparation of nelarabine from Ara-U*

substrate concentration and can be driven to 90 % conversion over 50 h by using the correct ratio of the two enzymes. As with other NP-catalysed transformations, the process is run at 50 °C. To improve thermostability and facilitate reuse, the enzymes are co-immobilized onto the same support.

For design of a simple manufacturing process, the thermostability of the NP enzymes is a very useful feature. Although heat treatment can be used as part of a purification protocol to isolate the enzymes from contaminating materials, the high temperature of operation itself excludes undesired enzyme-catalysed side reactions. For example, in the synthesis of 9-β-D-arabinofuranosyladenine from Ara-U and adenine, using a wet cell paste of *Enterobacter aerogenes*, adenine and Ara-U mainly underwent deamination at lower temperatures to form hypoxanthine and uracil respectively.[93] At elevated temperature, deamination was completely eliminated and the rate of transarabinosylation increased.

One drawback of biocatalysis is that enzymes are not available in both enantiomeric forms. Particularly where a class of enzymes whose natural substrates are optically active, such as nucleosides, it can be difficult if not impossible to find an alternative enzyme that will accept the unnatural substrate enantiomer. This is not insurmountable if directed-evolution approaches are used, but it can be prohibitively expensive, especially when the desired product is in an early stage of development or required for use only as an analytical reference or standard.

During the development of nelarabine, the opposite enantiomer (*ent*-nelarabine) was required as an analytical marker.[94] The lengthy chemical route to *ent*-nelarabine and the fact that this chemical route is necessary illustrate both the advantages and disadvantages of biocatalytic approaches. The chemical synthesis of *ent*-nelarabine is not straightforward, commencing with a three-step global acetylation of the sugar (Scheme 1.30). As chemical glycosylation using arabinose results predominantly in formation of the undesired α-anomer, ribose is instead used as the starting sugar. The enhanced diastereoselectivity



Scheme 1.30 *Chemical synthesis of* ent*-nelarabine*

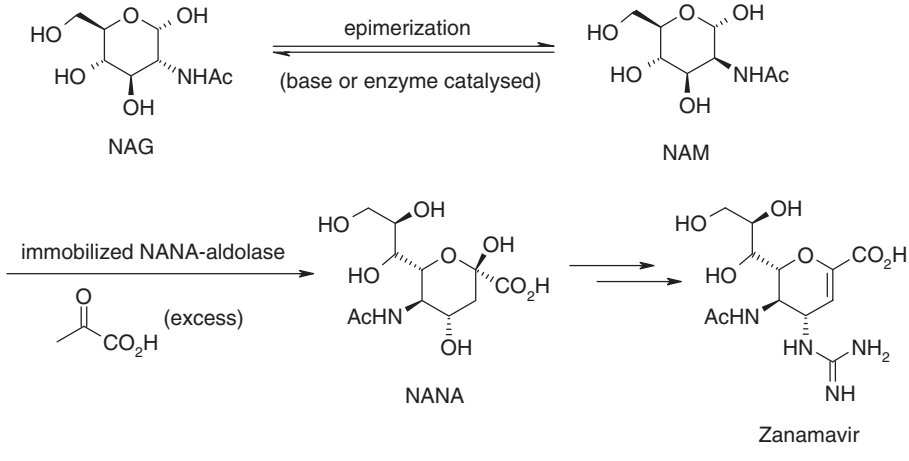
gained by the use of ribose in the glycosylation reaction also has a penalty, in that five additional steps are required in order to invert the 2′-alcohol of the resultant β-riboside. Furthermore, the unnatural 6-methoxyguanine base reacts chemically at N-7, as opposed to N-9 selectivity of the biocatalytic approach. This could be rectified by instead using 6-chloroguanine (to deactivate N-7) which could later be converted to the methoxide with concomitant acetate deprotection. Thus, *ent*-nelarabine was produced using an overall 10-step procedure.

Carbon–carbon bond lyases, used in the reverse, synthetic direction have also enjoyed significant application in the pharmaceutical industry. For example *N*-acetyl-D-neuraminic acid (NANA), an intermediate in the chemoenzymatic synthesis of the influenza virus sialidase inhibitor zanamavir, may be synthesized using NANA aldolase.

In nature, NANA arises through condensation of phosphoenolpyruvic acid with *N*-acetyl-D-mannosamine (NAM) catalysed by the biosynthetic enzyme NANA synthase.[95] Owing to the labile nature of phosphoenolpyruvate, the use of this reaction in the synthesis of NANA has been limited to whole-cell systems where this substance can be generated biosynthetically *in situ*.[96] Most recently, the NANA synthase reaction forms the basis of fermentation processes for total biosynthesis of NANA.[97]

Catabolic enzyme NANA aldolase catalyses cleavage of NANA to form NAM and pyruvic acid, the latter being a more attractive material for a chemoenzymatic process. It has long been known that the reverse reaction may be used for NANA synthesis.[98] However, this approach to a manufacturing process also has complications.

NAM is produced by base-catalysed epimerization of *N*-acetyl-D-glucosamine (NAG), generating an unfavourable 1:4 mixture of NAM:NAG. NAG, although not a substrate for the aldolase, inhibits the reaction. In addition, excess pyruvate is required to push the equilibrium in favour of product formation (Scheme 1.31). Although 90% yields can be obtained at laboratory scale using *E. coli* NANA aldolase using a NAG:NAM mixture, the NANA product is difficult to separate from the excess pyruvate required to achieve this.



Scheme 1.31 *Aldolase-catalysed preparation of NANA*

Cipolletti *et al.*[99] recently described a crystallization procedure to isolate NAM in >98 % purity from a 4:1 NAG:NAM epimerate, potentially enabling the use of a NAG-free process. However, Mahmoudian *et al.*[100] developed a scalable process using selective precipitation of NAG from aqueous solutions of NAG/NAM epimerate with isopropanol to generate a NAM-enriched solution as substrate for the enzymatic synthesis. Precipitated NAG could be recycled by base-catalysed epimerization. The NAM-enriched starting material allowed NANA product concentrations of 155 g L^{-1} to be attained by using just two equivalents of pyruvate. Because of the lower pyruvate content, NANA could be purified by a simple crystallization following removal of the Eupergit C-immobilized aldolase by filtration.

As an alternative to chemical epimerization, NAG epimerase may be used to maintain a constant NAM:NAG ratio in a one-pot reaction with pyruvate and NANA aldolase.[101] The epimerase is itself inhibited by pyruvate, which must, therefore, be added continuously or via aliquots to the reaction. In a refined version of this reaction at laboratory scale, Kragl *et al.*[102] produced NANA by a continuous process, using a membrane reactor to contain both enzymes in solution.

1.3.4 Asymmetric Biocatalysis

Asymmetric synthesis can refer to any process which accesses homochiral products. We will focus on asymmetric synthesis from racemic or prochiral starting materials in the presence of an enantioselective catalyst (enzyme). There are four general methodologies commonly applied: kinetic resolution, dynamic kinetic resolution, deracemization and asymmetrization.

The process of obtaining homochiral product from a racemate is known as kinetic resolution. Kinetic resolution functions by the transformation of two enantiomers of a racemic mixture at different rates. The objective is to effect a change in the physical properties of one enantiomer to such an extent that the resulting product is readily separable from the other. The technique suffers from the inherent inability to access >50 % of the desired enantiomer unless the unwanted enantiomer can be racemized and recycled or inverted.

Dynamic kinetic resolution (DKR) is an extension to the kinetic resolution process, in which an enantioselective catalyst is usually used in tandem with a chemoselective catalyst. The chemoselective catalyst is used to racemize the starting material of the kinetic resolution process whilst leaving the product unchanged. As a consequence, the enantioselective catalyst is constantly supplied with fresh fast-reacting enantiomer so that the process can be driven to theoretical yields of up to 100 %. There are special cases where the starting material spontaneously racemizes under the reaction conditions and so a second catalyst is not required.

An alternative method of obtaining theoretical yields of up to 100 % of homochiral product from racemic mixtures is known as deracemization. This process again employs two catalysts in tandem and so bears much similarity to the DKR process. However, here an enantioselective catalyst preferentially transforms one enantiomer of starting material into a prochiral product. The prochiral product is then converted back into racemic starting material using an achiral catalyst, resulting in an overall enrichment towards one enantiomer of starting material. Further enrichment results by allowing the process to run over multiple cycles, until only one enantiomer remains.

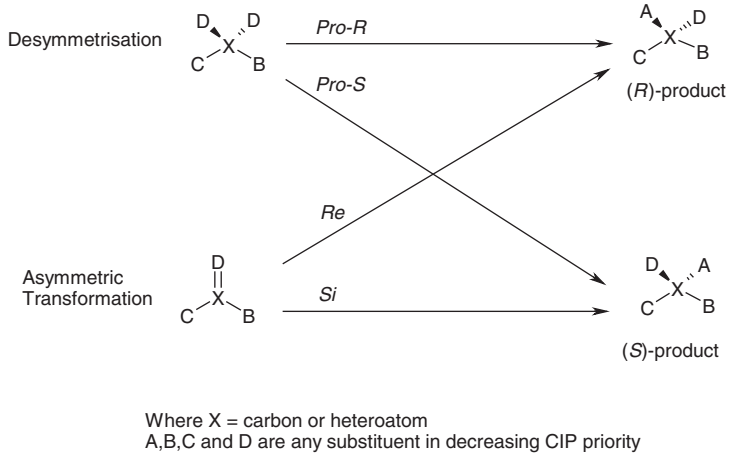


Figure 1.8 *Schematic representation of asymmetrization reactions.[103] (Reprinted with permission from the American Chemical Society Copyright (2005))*

The process of obtaining homochiral product from a prochiral starting material is known as asymmetrization. This encompasses reactions where a faster rate of attack of a reactive species occurs on one enantiotopic face of a prochiral trigonal biplanar system, or at one enantiotopic substituent of a C_2 symmetrical system, resulting in the preferential formation of one product enantiomer. The latter is also frequently referred to as the 'meso-trick' or 'desymmetrization'. These transformations can be more easily defined in pictorial form (Figure 1.8).

Unlike kinetic resolution, catalytic desymmetrization and asymmetrization can afford enantiopure products in theoretical yields of 100 % and are more generally applicable than DKR or deracemization techniques.

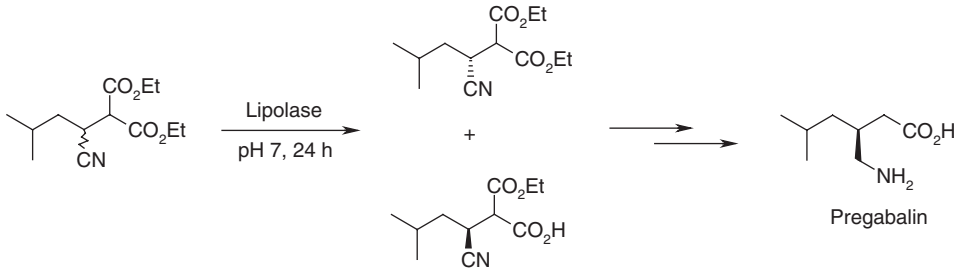
This section will only discuss examples of catalytic kinetic resolution, DKR, desymmetrization and asymmetrization. Deracemization will not be considered because, although an important developing technology, examples of its application to the production of chiral late-stage intermediates in API production have yet to appear.

1.3.4.1 Kinetic Resolution

This technique can allow the rapid development of processes for the separation of large quantities of enantiomers and can be ideal for early-stage 'fit for purpose' campaigns (where little resource is allocated to process development) in spite of the limitation in attainable yield. This can be useful in providing sufficient homochiral product for biological evaluation and the preparation of analytical standards of both enantiomeric forms.

Most kinetic resolutions of pharmaceutical intermediates that have been reported involve the use of hydrolases, particularly lipases and proteases. This is because many hydrolases are commercially available (in bulk and kit form),[104] do not require cofactors and are active in many organic solvents (see Section 1.4). Processes can therefore, often be developed rapidly, using high substrate concentrations and without specialist knowledge.

A second-generation manufacturing process involving a highly enantio- and diastereoselective lipase-catalysed kinetic resolution step has recently been reported for the production of pregabalin, a lipophilic γ-aminobutyric acid analogue that was developed for the treatment of several central nervous system disorders (Scheme 1.32).[105]

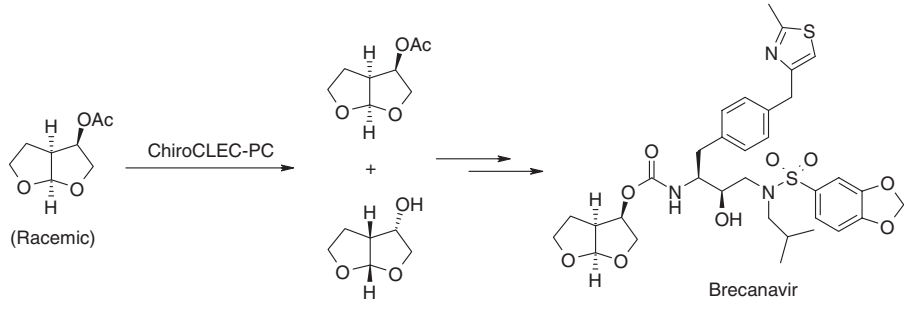


Scheme 1.32 *Kinetic resolution of a key intermediate to pregabalin*

Following a screen of hydrolase enzymes, the lipase from *Thermomyces lanuginosus* was selected based on its high activity and enantioselectivity. This enzyme is commercially available in industrial quantities as Lipolase, a cheap catalyst of importance to the detergents industry due to its high thermal stability and broad pH tolerance. Product inhibition was observed at concentrations over 1 M, and so divalent ion species were added as complexation agents. In the presence of calcium acetate, the reaction proceeded to completion at substrate concentrations up to 3 M, although only substoichiometric quantities were required, implying that the additive plays a more complex role than envisaged from the original rationale. A high concentration resulted in the added benefit of dramatically improved phase splitting during work-up, which facilitated product isolation and catalyst removal. The optimized biotransformation was successfully demonstrated in a manufacturing trial at 3.5 t scale in an 8000 L reactor.

(3*R*,3*aS*,6*aR*)-Hexahydrofuro[2,3-β]furan-3-ol (bisfuran alcohol), a key building block in the synthesis of human immunodeficiency virus (HIV) protease inhibitors such as brecanavir, can be accessed using a number of asymmetric approaches which include lipase resolution.[106] At first glance, lipase-catalysed acylation appears to be an attractive possibility for resolution, as there is the potential to remove the undesired alcohol through derivatization whilst leaving the desired enantiomer unchanged for subsequent chemical transformation. However, the desired alcohol is extremely water soluble, which eliminates the possibility of a simple extractive work-up.[107] In contrast, highly enantioselective hydrolytic resolution of the racemic acetate, using either PCL or CALB, affords the unwanted enantiomer as an alcohol that can be removed from the desired (*R*)-acetate on partition between dichloromethane and water.[108] During the separation process, a thick emulsion is formed if free enzyme is present. Emulsion formation can be avoided if an immobilized enzyme is used, but enzyme immobilization generally dilutes catalyst activity due to the large quantity of inert support that is required. Thus, high loadings of Novozym 435 (a commercially available form of CALB specifically designed for use in organic solvents) were required to perform the reaction at a reasonable rate, and this led to additional problems such as product absorption and catalyst swelling. By instead

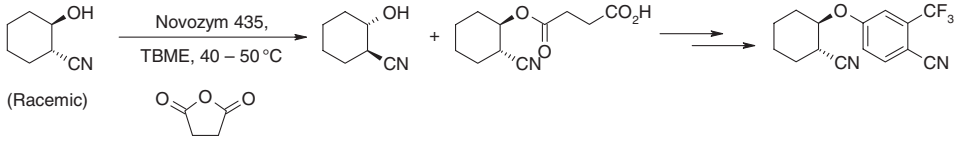
employing commercially available ChiroCLEC-PC (a cross-linked crystalline form of PCL), the reaction proceeded rapidly at low loadings (0.05 wt %) comparable to those of the free enzyme, whilst facilitating catalyst recovery and avoiding emulsion formation (Scheme 1.33) (T.C. Lovelace, personal communication).[109]



Scheme 1.33 *Kinetic resolution of a bisfuran intermediate of brecanavir*

An example where enzyme-catalysed acylation has been used to good effect was reported by Vaidyanathan *et al*.[110] for the preparation of an androgen receptor antagonist that was being developed as a treatment for alopecia and oily skin. The group were concerned that chromatographic separation of a racemic hydroxynitrile intermediate would afford ultrapure material with an impurity profile that would not be representative of a future commercial process. Enzymatic resolution could provide a practical solution, but enantioselective acylation with commonly used acyl donors like vinyl acetate would afford a neutral product that might be difficult to separate from the starting material and, therefore, also require chromatographic purification. The authors rationalized that, by employing succinic anhydride, previously demonstrated to be an effective acyl donor when used with some lipases,[111] an acidic product would result that could then be easily separated from the remaining alcohol by extraction with aqueous base.

By screening a variety of lipases in organic solvent for their ability to acylate the racemic hydroxynitrile with succinic anhydride, Novozym 435 was found to yield the best results, affording product in 94–95 % ee at conversions of 47–49 % (Scheme 1.34). After optimization, the reaction was successfully run at 22 kg scale. The immobilized catalyst could be easily isolated by filtration and reused.

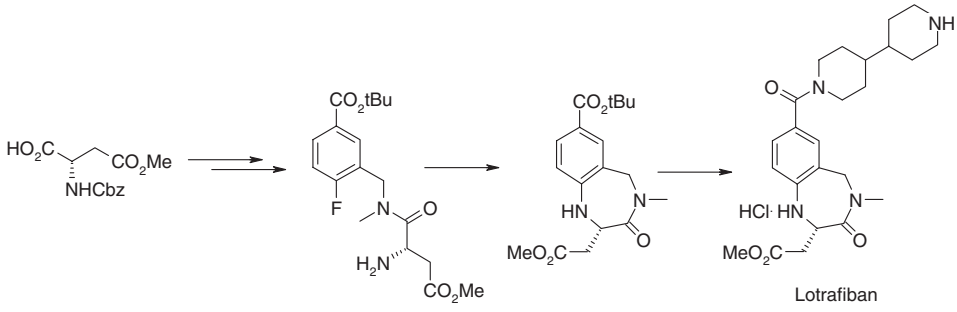


Scheme 1.34 *Lipase resolution of a key intermediate in the synthetic route to an androgen receptor antagonist. TBME:* tert*-butyl methyl ether*

Given that resolution can only achieve a maximum yield of 50 %, the approach is inherently inefficient. Additionally, classical resolution and simulated moving bed

chromatography can provide attractive alternatives, as development times are frequently shorter, there are no intellectual property or sourcing issues and the techniques are often more accessible to the process chemist. Some examples of where biotransformations have ultimately been replaced by alternative technologies are discussed below.

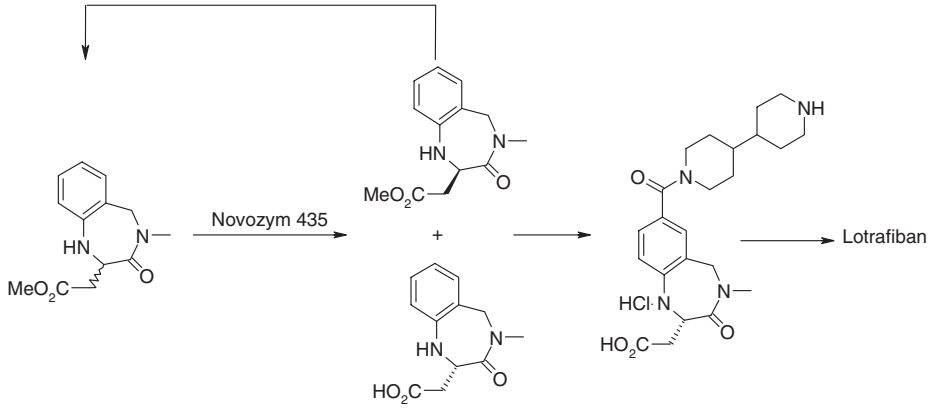
Lotrafiban, a nonpeptidic glycoprotein IIb/IIIa receptor anagonist that was under development as a treatment for the prevention of platelet aggregation and thrombus formation, was initially prepared using an 11-step linear sequence starting from methyl Cbz-L-aspartate (Scheme 1.35).[112] An overall yield of 9 % and issues with obtaining the product in sufficient enantiopurity led the group to look for an alternative route via the enzymatic



Scheme 1.35 *Medicinal chemistry approach to lotrafiban*

resolution of a racemic ester intermediate.

The ester was screened against a panel of enzymes for hydrolysis activity from which only Novozym 435 efficiently hydrolysed the desired (*S*)-enantiomer.[113] After significant optimization studies using Novozym 435, a process was established where a 100 g L^{-1} slurry of racemic ester in commercial *tert*-butanol (which is supplied as a mixture containing 12 % water – anhydrous *tert*-butanol could not be used due to its higher melting point), furnished the desired acid in 43 % yield and >99 % ee (Scheme 1.36). The reaction was performed at 50 °C as a compromise that gave satisfactory substrate concentration

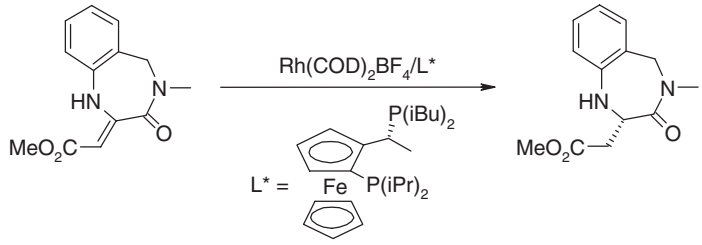


Scheme 1.36 *Kinetic resolution of an ester intermediate in the synthetic route to lotrafiban*

(2.4 g L^{-1}) whilst allowing the catalyst to be reused up to 10 times (running at 60 °C, a fivefold reduction in catalyst activity was observed after a single cycle). The undesired enantiomer, remaining as the ester, was separated from the acidic product by selective crystallization and was subsequently racemized and recycled. This route was ultimately run on scale at the site of primary manufacture.

In an attempt to find an improved reaction solvent, Roberts *et al.*[113] investigated a number of ionic liquids. Using [BMIM][PF_6], an eightfold increase in substrate concentration was observed compared with 88 % v/v *tert*-butanol, which resulted in a threefold increase in reaction rate and allowed the isolation of acid in comparable yield and enantiopurity to that obtained using the developed process.

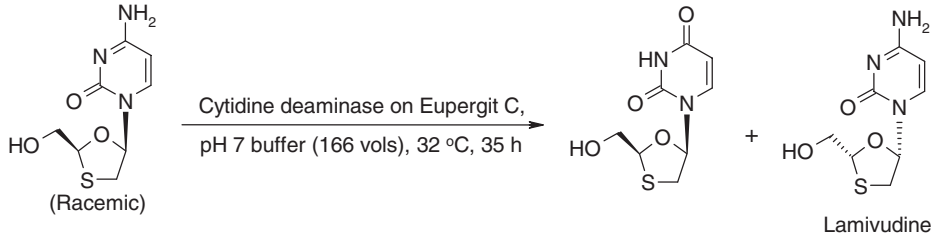
Ultimately, an alternative route based on asymmetric hydrogenation using a rhodium catalyst employing the Josiphos ligand was identified, but only demonstrated on a 10 g scale before the project was terminated (Scheme 1.37).[114]



Scheme 1.37 *Asymmetric synthesis of an ester intermediate in the synthetic route to lotrafiban*

Lamivudine (also known as Epivir and 3TC) is a potent antiviral drug used in the treatment of HIV and hepatitis B virus (HBV) infections. Although both enantiomers are equipotent antiviral agents, the unnatural enantiomer (with respect to natural nucleosides) is far less cytotoxic, and so a method of selectively accessing the single enantiomer was required.

Asymmetric routes to lamivudine have recently been reviewed.[115] A number of these are biocatalytic, the most elegant of which is a highly enantioselective kinetic resolution process based on the use of cytidine deaminase from *E. coli*.[116] The process is particularly impressive given that the reaction site is five atoms away from the nearest chiral centre (Scheme 1.38).



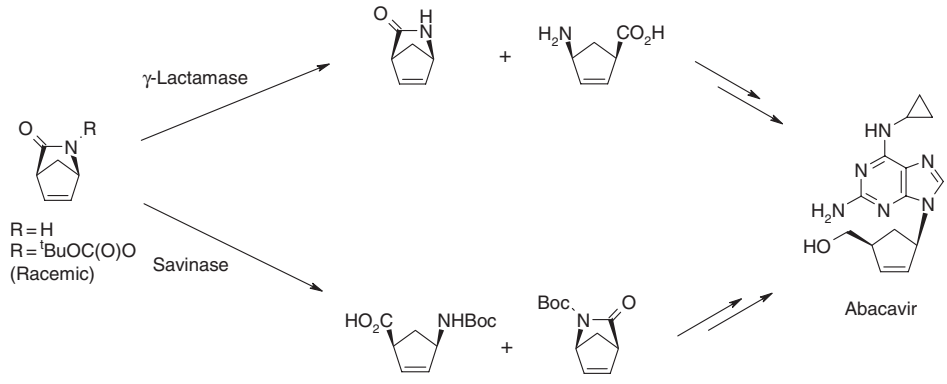
Scheme 1.38 *Cytidine-catalysed kinetic resolution of racemic lamivudine*

Cytidine deaminase was not commercially available, but it is produced by numerous microorganisms and can be induced at high levels in enteric bacteria, such as *E. coli*, in the presence of cytidine. To overcome the need to add cytidine, mutant strains that express the deaminase constitutively were sought through ultraviolet irradiation of the native microorganism. A selection process was developed to detect strains of interest that took advantage of the fact that both cytidine deaminase and uridine phosphorylase are induced by cytidine as they share the same repressor.[117] Thus, any mutant that grows well on uridine in the absence of cytidine is likely to have a defective repressor and express both enzymes constitutively. Using this procedure, the mutant *E. coli* strain 3732E was developed that gave high deaminase expression independent of cytidine concentration. However, a higher level of expression was required for pilot studies, and so a recombinant strain, overexpressing the deaminase gene from strain 3732E, was developed that produced 80 times more deaminase than the mutant strain. Crude cell extracts of the cytidine deaminase variant, immobilized on Eupergit C, were used on a multikilogram scale. The desired enantiopure product could be selectively extracted by adsorption onto an anion-exchange column and isolated in 40 % yield after subsequent recrystallization. The biocatalytic approach was ultimately replaced by the classical resolution of an early-stage intermediate in the final production route. Even so, the deaminase had proven valuable for achieving preclinical supplies.[118]

Other examples of efficient enzymatic resolutions by reaction at a remote position from stereocentres have been reported, such as the lipase-catalysed resolution of a synthetic intermediate of escitalopram.[119] This property of enzymes has also been effectively used to resolve sterically hindered compounds by the introduction of a tether so that the enzyme-catalysed reaction can be performed at an artificially created, but less hindered, remote location. An example is the resolution of tertiary alcohols by the introduction of a glyoxylate ester.[120]

Most of the examples encountered so far have employed cheap, commercially available enzymes or enzymes that can be readily produced in-house. When a proprietary enzyme, developed by a third party, is used, additional factors such as royalty payments, freedom to operate and single-source supply require consideration. An example is the production of the key (1*R*,4*S*)-azabicyclo[2.2.1]hept-5-en-3-one intermediate used in the manufacture of abacavir, another potent reverse transcriptase inhibitor used for the treatment of HIV and HBV infection. Enantiocomplimentary microorganisms (*Rhodococcus equi* NCIB 40213 and *Pseudomonas solanacearum* NCIB 40249) were first isolated from the environment under conditions to select for growth on *N*-acyl compounds as the sole source of carbon and energy.[121] Mutant strains of *Pseudomonas solanacearum* NCIB 40249, hyperexpressing γ-lactamase, resulted in a highly enantioselective kinetic resolution process using substrate concentrations of $>100\,\mathrm{g\,L^{-1}}$ (Scheme 1.39 where R = H). The process was initially run using whole cells, as the γ-lactamase was too unstable to isolate, but this resulted in complex downstream processing. Through further microbial screening, a new lactamase that was sufficiently stable to isolate was identified[122] and subsequently cloned (internal presentation from Dow). Using this recombinant lactamase, a highly efficient process was developed that uses $500\,\mathrm{g\,L^{-1}}$ substrate concentrations and a significantly improved workup.

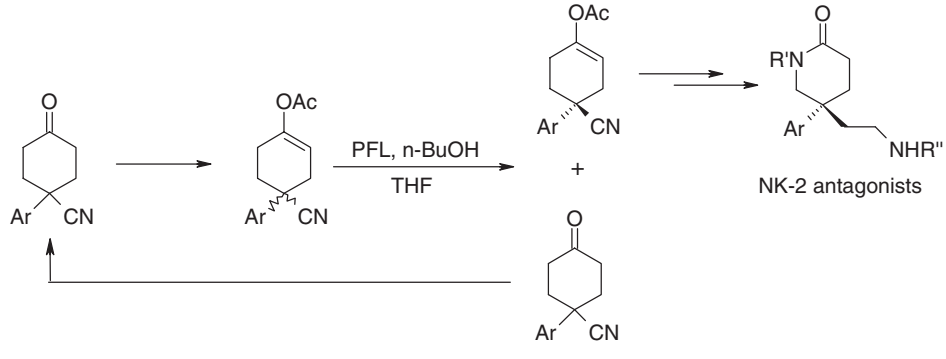
In a bid to find a process that employs a commercially available biocatalyst, Mahmoudian *et al*. rationalized that Boc-protection of the racemic lactam should activate



Scheme 1.39 *Enzymatic kinetic resolution approaches to abacavir*

the amide bond towards nucleophilic attack. After screening a variety of commercially available hydrolases towards hydrolysis of this substrate in 1:1 THF/buffer mixtures (to eliminate background hydrolysis), a number of hits were obtained. Of these hits, savinase (protease from *Bacillus lentus*) proved to be highly enantioselective towards hydrolysis of the undesired enantiomer, leaving the (1*R*,4*S*)-Boc-lactam in >99 % ee at 50 % conversion (Scheme 1.39 where R = ^{t}BuOC(O)O).[123] Savinase and other alkaline proteases are produced in industrial quantities for use in the detergent industry.[104b,c]

Carnell and co-workers have recently applied lipase-catalysed resolution to formally desymmetrize prochiral ketones that would not normally be considered as candidates for enzyme resolution, through enantioselective hydrolysis of the chemically prepared racemic enol acetate.[124] For example, an NK-2 antagonist was formally desymmetrized by this approach using *Pseudomonas fluorescens* lipase (PFL) (Scheme 1.40).[125] By recycling the prochiral ketone product, up to 82 % yields of the desired (*S*)-enol acetate (99 % ee) could be realized.[126] This method offers a mild alternative to methodologies such as base-catalysed asymmetric deprotonation, which requires low temperature, and biocatalytic Baeyer–Villiger oxidation, which is difficult to scale up.

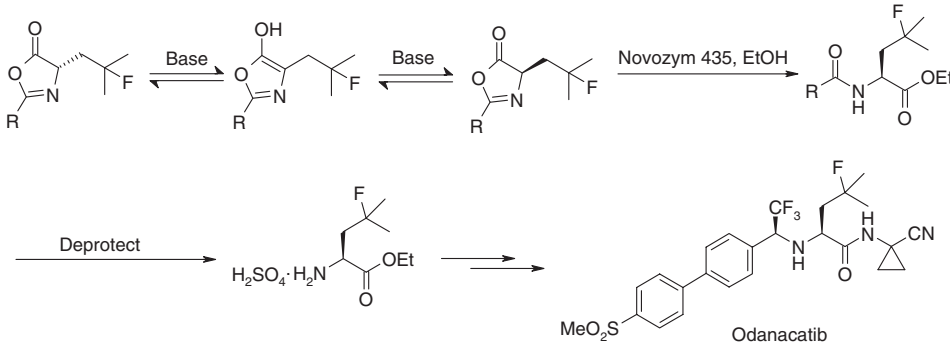


Scheme 1.40 *Access to NK-2 antagonists by the lipase-catalysed resolution of enol acetates*

1.3.4.2 Dynamic Kinetic Resolution

As seen in Section 1.3.4.1 (synthesis of lotrafiban), the recycling of an unwanted enantiomer resulting from a kinetic resolution allows theoretical yields of up to 100 % to be achieved, but it can also create a bottleneck in a production process. DKR, where a starting material undergoes racemization *in situ*, either spontaneously or through the action of a second catalyst, offers a more efficient approach. This technique has been applied, particularly in academia, to the preparation of a broad range of chiral building blocks, and a number of recent reviews are available.[127]

Odanacatib is currently under clinical development for the treatment of post-menopausal osteoporosis.[128] The medicinal chemistry route to the (*S*)-fluoroleucine moiety, requiring six synthetic steps from an expensive protected aspartic acid derivative and the use of numerous hazardous reagents, was not suitable for scale-up. A more efficient chemoenzymatic approach was instead sought, based on the enzyme-catalysed DKR of racemic 2-phenyl-4-substituted-5(4*H*)-oxazolones developed by Sih and co-workers.[129] The desired racemic azalactone, efficiently produced in a high-yielding, two-pot, four-step process underwent Novozym 435-catalysed ethanolysis in EtOH/TBME in the presence of 20 mol % of triethylamine to furnish ethyl *N*-Bz-(*S*)-γ-fluoroleucinate in 80 % isolated yield and 95 % ee (Scheme 1.41).[130] Unfortunately, benzoyl deprotection of the resultant product could not be effected without significant formation of the desfluoro compound. By instead using the 2-(3-butenyl)-oxazolone, the amino acid derivative was produced in comparable yields, but moderate enantioselectivity (78 % ee). However, deprotection of the 4-pentenamide by hydroxybromination using *N*,*N*′-dibromodimethylhydantoin and trifluoroacetic acid in water/MeCN afforded the desired product in high yield.[131] Recrystallization from TBME or isopropyl acetate with H_2SO_4 afforded the product as the hydrogen sulfate salt in 80 % yield and 97 % ee. This procedure was used to produce >250 kg of API.

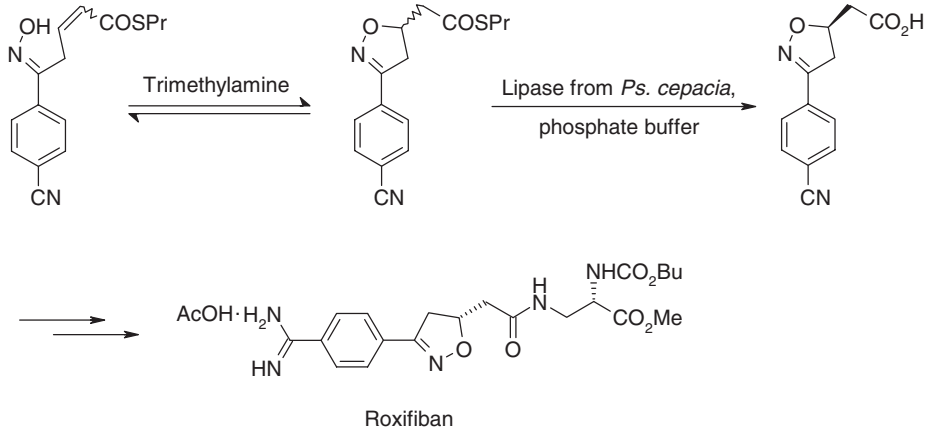


Scheme 1.41 *Preparation of a γ-fluoroleucinate intermediate of odanacatib by enzyme-catalysed DKR*

In addition to the moderate enantioselectivity, the DKR required one weight equivalent of catalyst to compensate for the background ethanolysis reaction. Furthermore, a significant quantity of hydrolysis product was produced, resulting from the water content of

the catalyst that is required for enzyme activity (see Section 1.4). By using a continuous flow format, the biotransformation was greatly improved.[132] Not only could the catalyst loading be substantially reduced to 0.05 weight equivalents, but catalyst lifetime was also increased 20-fold due to the absence of shear forces. Product was thus obtained in 90 % yields and 86 % ee in kilogram quantities. The yield of hydrolysis product was reduced, possibly as a result of the catalyst operating at suboptimal water activity due to stripping by solvent.

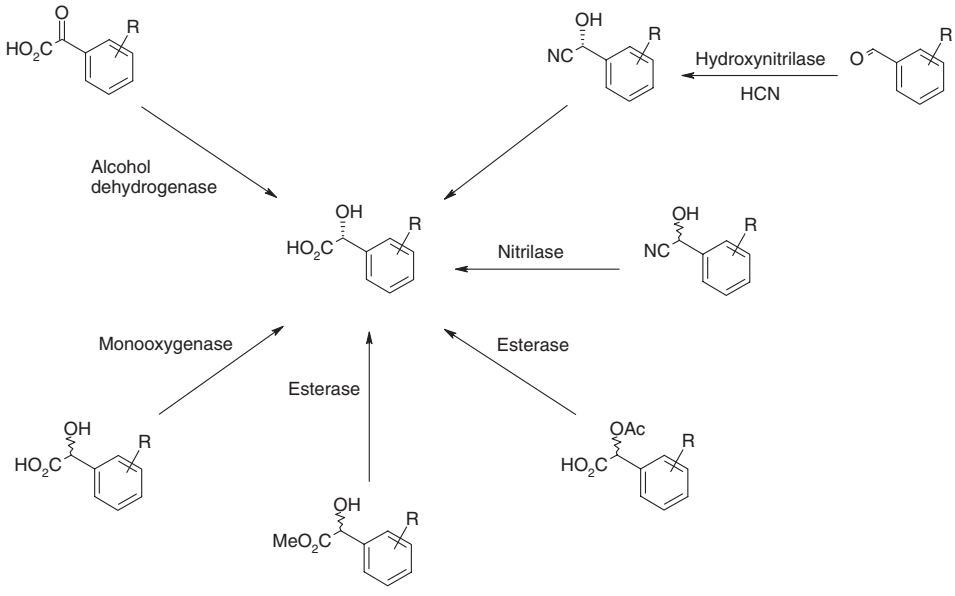
To provide a more efficient route to roxifiban, a drug candidate for the treatment of a range of cardiovascular disorders, Pesti *et al*. wanted to convert the hydrolytic kinetic resolution of an isoxazoline ester intermediate, using Amano PS30 (PCL), into a DKR.[133] Attempts to effect a DKR through adjustment of the reaction pH were unsuccessful even though the ester was prone to base-catalysed racemization via an intramolecular Michael/retro-Michael mechanism. Based on literature precedent for the DKR of α-thiophenyl esters, an efficient DKR process was finally established through Amano PS30-catalysed hydrolysis of the *n*-propyl thioester in triethylamine and aqueous pH 9 buffer solution to furnish the (*R*)-acid in 80 % yield and >99.9 % ee (Scheme 1.42).



Scheme 1.42 *Enzymatic DKR of a thioester intermediate of odanacatib*

Clopidogrel is a potent antithrombotic agent, the chiral portion of which can be accessed from (*R*)-2-chloromandelic acid. Mandelic acid derivatives are an important class of compound in their own right owing to their use as chiral resolving agents and as building blocks for pharmaceuticals. They can be accessed in enantiomerically pure form by a number of biocatalytic routes, such as nitrile hydrolysis, asymmetric cyanohydrin formation (see Section 1.3.4.5), ketoester reduction (see Scheme 1.53), ester hydrolysis/transesterification,[134] *O*-acetyl hydrolysis[135] or hydroxyacid oxidation (Scheme 1.43).[136]

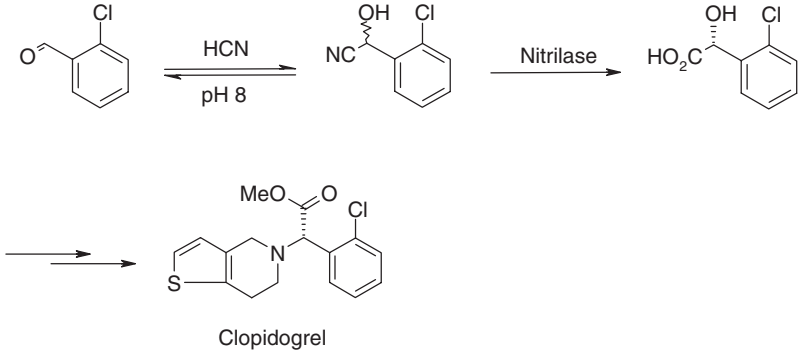
One of the most attractive biocatalytic options is the nitrilase-catalysed enantioselective hydrolysis of the racemic cyanohydrin. The hydroxyacid is produced directly without need for protection/deprotection steps and cyanohydrins racemize spontaneously at neutral or



Scheme 1.43 *Some potential biocatalytic approaches to optically pure mandelate derivatives*

high pH through the reversible loss of HCN. Another attractive aspect is that, like other hydrolases, nitrilase enzymes require no cofactor.

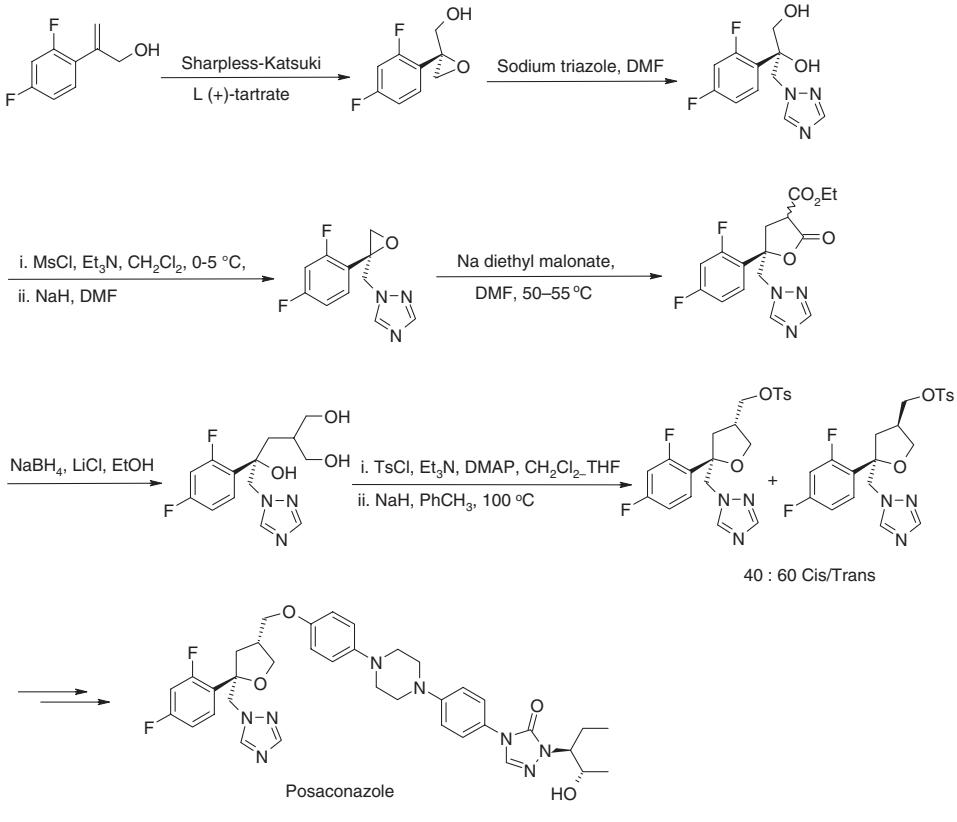
DeSantis *et al.*[137] have reported the discovery of new nitrilases through the screening of genomic libraries created by the extraction of DNA from various environments (metagenomics). In preliminary experiments, using 25 mM mandelonitrile in pH 8 buffer containing 10 % methanol and 0.12 g mL^{-1} of one of these nitrilases, the acid was produced quantitatively with 98 % ee within 10 min. The product was subsequently shown to be (*R*)-mandelic acid after isolation in 86 % yield. In a parallel reaction, (*R*)-2-chloromandelic acid was produced at a seventeenth of the rate (Scheme 1.44).



Scheme 1.44 *Nitrilase-catalysed preparation of a cyanohyrin intermediate to clopidogrel*

1.3.4.3 Desymmetrization

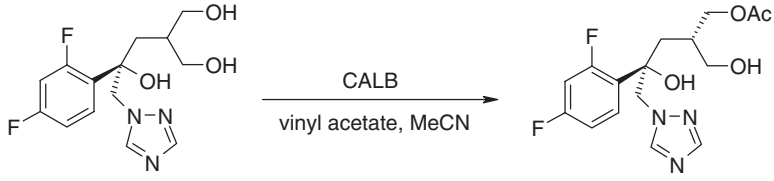
The initial synthetic route to the antifungal agent posaconazole employed an asymmetric Sharpless–Katsuki epoxidation to afford an (*R*)-epoxide intermediate in high yield and 88–92 % ee (Scheme 1.45).[138] The optical purity could satisfactorily be improved to >98 % ee after one recrystallization of the diol product obtained after ring opening of the epoxide, with retention of stereochemistry, by sodium triazole. Unfortunately, ditosylation and subsequent base-catalysed ring closure of a later triol intermediate gave an almost equimolar mixture of *cis*- and *trans*-THF products that required chromatographic separation.



Scheme 1.45 *Chemical synthesis of posaconazole*

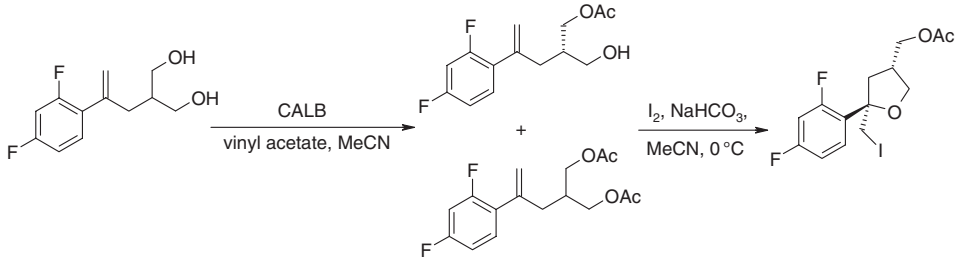
This was overcome by acetylation of the same triol intermediate, using Novozym 435 (immobilized CALB) in vinyl acetate and acetonitrile, to afford the monoacetate in 95 % yield and 97 % diastereoselectivity (Scheme 1.46).[139] The monoacetate was then readily converted to the desired *cis*-THF derivative by alcohol activation and cyclization as described above.

By performing the desymmetrization on a prochiral diol, a far more efficient asymmetric biocatalytic route was subsequently developed. Enzyme screening found that



Scheme 1.46 *Lipase-catalysed diastereoselective acetylation of a posaconazole intermediate*

CALB was again the favoured catalyst, selectively acetylating the *pro-S* alcohol (Scheme 1.47). To obtain the desired (*S*)-monoacetate in sufficient enantiopurity, the reaction was not terminated when all starting material had been consumed, but allowed to run a little further to transform a small portion of monoacetate to diacetate. This resulted in enantioenrichment of the desired (*S*)-monoacetate by the preferential acetylation of the unwanted (*R*)-monoacetate to prochiral diacetate.



Scheme 1.47 *Lipase-catalysed desymmetrization of a posaconazole intermediate*

This apparent swap of selectivity is a result of the predictable steric interactions of most commercially available lipases with primary and secondary alcohols and carboxylic acids. In fact, a simple predictive tool, known as the 'Kazlauskas rules', has been developed where attack is favoured towards substrates of configuration shown in Figure 1.9.[140] These rules are highly predictive for secondary alcohols and less reliable for primary alcohols and carboxylic acids.

In the case of the primary alcohols of Scheme 1.47, CALB operates in an anti-Kazlauskas fashion, resulting in anti-Kazlauskas diol acetylation to produce the (*S*)-monoacetate and anti-Kazlauskas acetylation of the (*R*)-monoacetate to produce diol (Figure 1.10). In contrast, CALB is observed to act in a Kazlauskas fashion toward the secondary alcohol shown in Scheme 1.34 and the ester shown in Scheme 1.36.

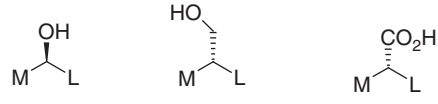


Figure 1.9 *Kazlauskas rules: preferential action of a lipase on alcohols and carboxylic acids (M and L indicate medium- and large-sized substituents respectively)*

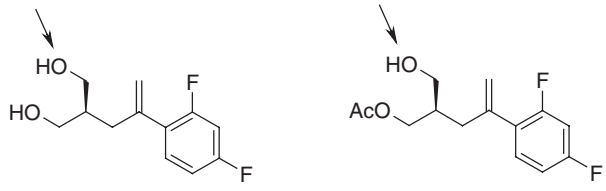
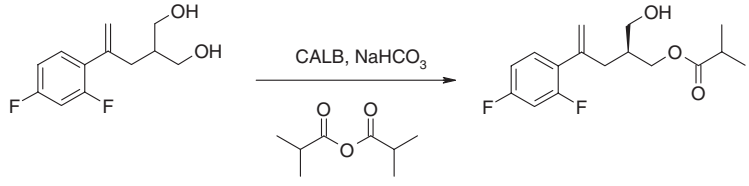


Figure 1.10 *Anti-Kazlauskas action of CALB on the primary alcohol intermediates of posaconazole*

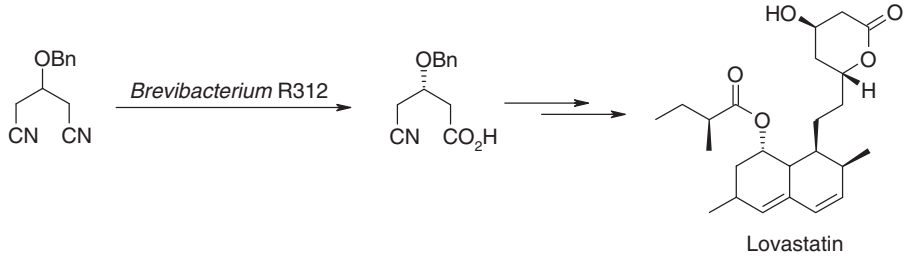
The desired *S*-monoacetate could thus be obtained in 81 % yield and 97 % ee at pilot-plant scale. The tertiary centre could then be constructed by diastereoselective iodocyclization of the resultant monoester, thus removing the need for the Sharpless–Katsuki epoxidation. Diacetate remained unchanged during this step and could be removed at a later stage.

Moderate yields of monoacylated product (74–81 %) were initially obtained using vinyl acetate as acylating agent as significant diacetylated by-product formation was necessary to achieve sufficiently high monoacetate enantiopurity. The ultimate route developed for the manufacture of multi-ton quantities of posaconazole used isobutyric anhydride as the acylating agent (Scheme 1.48).[141] This more bulky acylating agent proved to be superior, affording >90 % yields of the desired product at low temperature (−14 °C) in the presence of $NaHCO_3$ to suppress background reaction and acyl migration respectively.



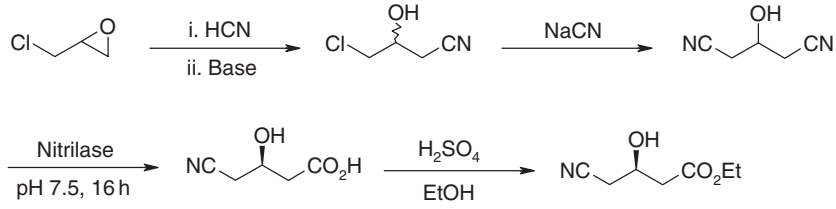
Scheme 1.48 *Industrial-scale desymmetrization of a posaconazole intermediate*

Desymmetrization is not restricted to a single class of enzyme. For example, Madrell *et al.*[142] reported the gram-scale preparation of a key intermediate of the lovastatin lactone through the desymmetrization of 3-(benzyloxy)glutaronitrile using whole cells from *Brevibacterium* R312. The transformation occurs via a dual nitrile hydratase/amidase-catalysed hydrolysis to afford acid in 65 % yield and 88 % ee (Scheme 1.49).



Scheme 1.49 *Synthesis of a key hydroxyacid intermediate of lovastatin*

Using a similar approach, Bergeron *et al.*[143] prepared the side chain of atorvastatin via a nitrilase catalysed desymmetrization of 3-hydroxyglutaronitrile. The dinitrile was prepared in two steps from epichlorohydrin, albeit in moderate yield. A highly enantioselective desymmetrization was then performed using the nitrilase BD9570, developed by Burk and co-workers,[144] expressed in a strain of *Pseudomonas fluorescens* (Scheme 1.50). The enzyme was obtained solely as a soluble, active multimer in excess of 25 g L^{-1} by fermentation, a quantity that represented >50 % of the total cell protein. An advantage of high-level protein expression is greatly simplified downstream processing of the enzyme, a contributing factor to the enzyme cost. In addition, if the enzyme is inexpensive there is no need to recycle, therefore potentially obviating the need for catalyst immobilization. However, reaction workup was problematic due to the high water solubility of the product and the presence of cell debris resulting from the use of crude catalyst.

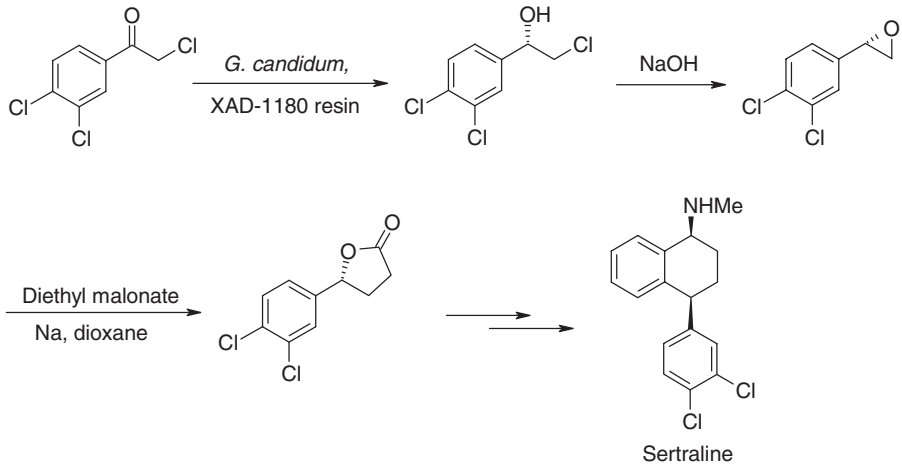


Scheme 1.50 *Nitrilase desymmetrization approach to the atorvastatin statin side chain*

1.3.4.4 Asymmetric Ketone Reduction

Microbial reduction has been recognized for decades as a laboratory method of preparing alcohols from ketones with exquisite enantioselectivity. The baker's yeast system represents one of the better known examples of biocatalysis, taught on many undergraduate chemistry courses. Numerous other microorganisms also produce the ADH enzymes (KREDs) responsible for asymmetric ketone reduction, and so suitable biocatalysts have traditionally been identified by extensive microbial screening. Homann *et al.*[145] have recently reported the identification of a subset of 60 ADH-producing microbial cultures that cut microbial screening time from weeks to days.

The advantage of using living microorganisms for bioreduction is that they can be readily sourced from the environment and the cofactors (necessary to regenerate the reduced form of the ADH enzyme and, thus, allowing catalyst turnover) are constantly generated by the intact cellular metabolic machinery. However, reduction using native microorganisms does have several drawbacks. Microorganisms often contain a number of ADHs that can display different or opposite enantioselectivities towards a given substrate. Also, enzymes displaying competing activities might be present or the desired enzyme might not be sufficiently active towards a chosen substrate or poorly expressed by the native organism. Furthermore, most living cells only tolerate low substrate and organic solvent concentrations. For example, Barbieri *et al.*[146] used whole cells from *Geotrichum candidum* to produce 2 g L^{-1} titres of (*S*)-chlorohydrin in 90 % yield and 93 % ee. The chlorohydrin can be used as a chiral building block in the synthesis of sertraline, an antidepressant and anorectic agent (Scheme 1.51). To overcome product inhibition, two



Scheme 1.51 *Chemoenzymatic approach to sertraline*

weight equivalents of the nonionic macroreticular resin Amberlite XAD-1180 was used for *in situ* product removal. This resulted in a twofold increase in product titre from an unoptimized reaction and both yield and enantioselectivity also increased.

Product extraction from large volumes of fermentation broth can be complex, requiring large volumes of organic solvent or solid-phase extraction techniques, which can sometimes greatly reduce or even cancel out the benefits of the biotransformation itself, such as shorter route and environmentally benign conditions.

Given the large capital investment required for specialist equipment, the fermentation needs to display considerable production cost benefits over the chemical process to be considered seriously as a route to API manufacture.

Partially purified or isolated ADHs offer several advantages:

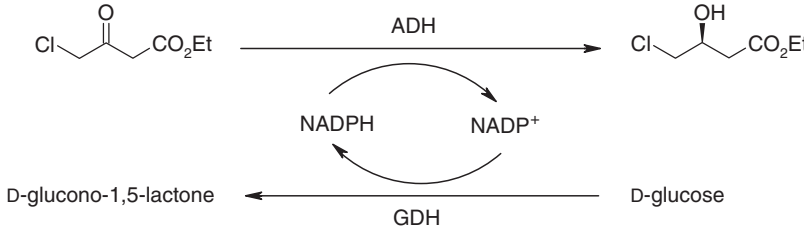
- higher substrate concentrations
- higher solvent tolerance
- simplified downstream processing.

Unlike the whole-cell system, enzymatic reductions require the addition of a hydride donating cofactor to regenerate the reduced form of the enzyme. Depending on the chosen ADH, the cofactor is usually NADH or NADPH, both of which are prohibitively expensive for use in stoichiometric quantities at scale. Given the criticality of cofactor cost, numerous methods of *in situ* cofactor regeneration, both chemical and biocatalytic, have been investigated. However, only biocatalytic regeneration has so far proven to be sufficiently selective to provide the cofactor total turnover numbers of at least 10^5 required in production.[147]

Biocatalytic approaches to cofactor regeneration can be divided into coupled-enzyme methods and coupled-substrate methods.[148] In the coupled-enzyme method, the oxidized cofactors (NAD^+ and $NADP^+$) are recycled *in situ* by performing an oxidation reaction using a second enzyme and an inexpensive auxiliary substrate. This second enzyme must employ the same cofactor, but neither enzyme should be able to accept the same substrate.

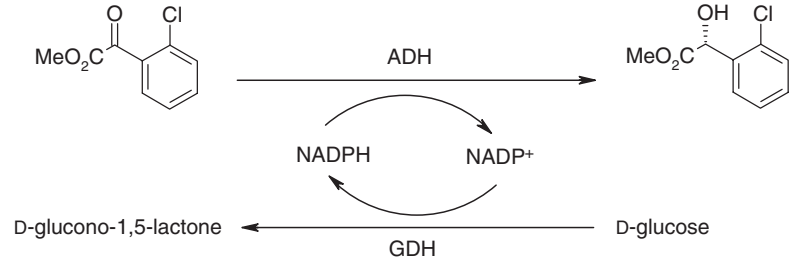
Furthermore, the oxidation reaction needs to be irreversible so as to drive the reduction reaction to completion. NAD^+ and $NADP^+$ are most frequently recycled using formate dehydrogenase (FDH) and glucose dehydrogenase (GDH) enzymes respectively as the second enzyme. By the introduction of formate and glucose as co-substrates, the oxidized forms of FDH and GDH irreversibly generate carbon dioxide and D-glucono-1,5-lactone respectively, thereby driving the reduction to completion. Alternatively, another ADH can be employed as the second enzyme in the presence of an inexpensive ketone so long as the resultant alcohol can be removed from the reaction mixture in some way as it forms.

Davis *et al.*[149] adopted the coupled-enzyme method to access the (*S*)-hydroxyester (Scheme 1.52) that is subsequently fed into the halohydrin-dehydrogenase-catalysed cyanation process shown in Scheme 1.26. Reaction workup using wild-type enzymes gave an emulsion that settled slowly, thus wasting valuable plant time. Modification of both ADH and GDH enzymes allowed improved separation as well as increased reaction rate and catalyst stability.



Scheme 1.52 *ADH reduction approach to the atorvastatin side chain*

Recombinant cells expressing a cloned ADH have also been used in a coupled enzyme method to efficiently produce the (*R*)-2-chloromandelate intermediate in the synthetic route to clopidogrel in 90 % yield and >99 % ee at 200 gL^{-1} substrate concentration (Scheme 1.53).[150] This procedure does not use hydrogen cyanide and, therefore, represents a less hazardous alternative to the nitrilase- and hydroxynitrilase (HnL)-catalysed approaches shown in Scheme 1.44 and Scheme 1.56 respectively.

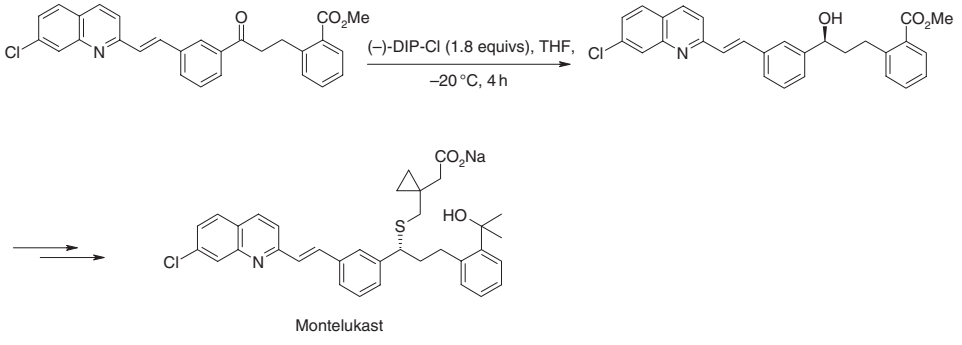


Scheme 1.53 *ADH approach to the (R)-2-chloromandelate intermediate to clopidogrel*

The coupled substrate method is perhaps the simplest approach to asymmetric ketone reduction, using a single recombinant ADH to perform the oxidation of a cheap auxiliary

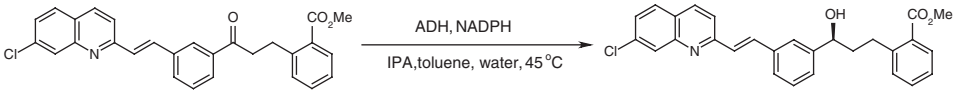
substrate (such as a low molecular weight alcohol) in addition to the desired reduction. By using a large excess of sacrificial alcohol, the reaction can be driven towards formation of the desired reduced product.

Montelukast, a leukotriene antagonist used for the treatment of asthma, is produced as a single enantiomer. Asymmetric reduction of the ketone with most hydrogenations and metal hydrides is precluded due to the presence of other sensitive functionality. Using (−)-β-chlorodiisopinocamphenylborane ((−)-DIP-Cl) as the reducing agent at −20 °C, the desired alcohol can be produced in 80 % isolated yield and 99.5 % ee,[151] but 1.8 equivalents of this moisture-sensitive and corrosive reagent are required (Scheme 1.54). In light of the need to use stoichiometric quantities of reagent, the development of more efficient catalytic methods has been the subject of extensive research.



Scheme 1.54 *Preparation of a montelukast intermediate using a chemical asymmetric catalyst*

Using a microbial screening strategy, Shafiee *et al.*[152] found that the chiral hydroxyester can be generated from *Microbacterium campoquemadoensis* in >95 % ee. The whole-cell reaction was optimized to produce 500 mg mL^{-1} product concentrations after 280 h. The ADH responsible was purified and found to be NADPH dependent and active in hexane or DMSO/aqueous mixtures, but no attempt to clone this enzyme has been reported.



Scheme 1.55 *Alcohol dehydrogenase preparation of a montelukast intermediate*

Ulijn *et al.* identified an enzyme, capable of enantioselectively reducing the ketone, from their extensive collection of ADH variants; further modification of the hit resulted in a biocatalyst that produces the desired (*S*)-alcohol in >99.9 % ee at concentrations of 100 gL^{-1} in a solid-to-solid biotransformation,[153] where both starting material and product display only sparing solubility in the reaction medium.[154] High conversions (>99 %) are achieved by the substrate-coupled method, using 50 % v/v isopropyl alcohol concentrations to drive the reaction by continuous acetone removal (Scheme 1.55). The product can be easily isolated by filtration and washing.

Both enantiomers of 1-[3,5-bis(trifluoromethyl)phenyl]ethan-2-ol are of importance in the pharmaceutical industry, and so considerable effort has been expended in their asymmetric synthesis. The (*R*)-enantiomer is a building block for aprepitant, a neurokinin-1 (NK-1) antagonist used for the treatment of chemotherapy-induced nausea (Figure 1.11).[155]

Gelo-Pujic *et al.*[156] recently reported the results of a comparison between enzymatic, microbial and chemocatalytic asymmetric reduction of 1-[3,5-bis(trifluoromethyl)phenyl]ethanone. Whereas both biocatalytic methods gave high product ees, both systems only functioned at low substrate concentrations and the enzymatic method gave inferior conversions to the whole-cell system. The chemocatalytic method gave moderate product ees but could be performed at high substrate concentrations and gave high yields. However, the enzymatic approach was only tested using the substrate-coupled method. In sharp contrast, Pollard *et al.*[157] efficiently prepared both alcohol enantiomers with different isolated ADHs using the enzyme-coupled method. For example, using the commercially available ADH from *Rhodococcus erythropolis* and a GDH cofactor recycling system they produced (*R*)-alcohol in >98 % yield and >99 % ee at 200 g L^{-1} concentrations on a 25 kg scale. Caution clearly needs to be taken in the proper choice of reaction conditions.

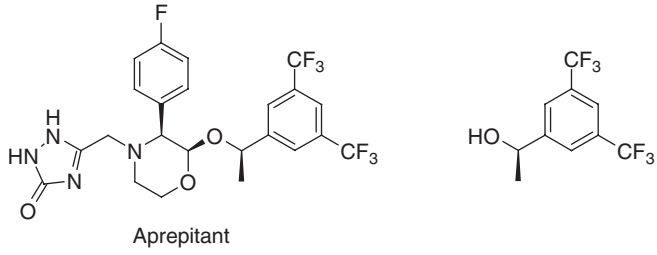
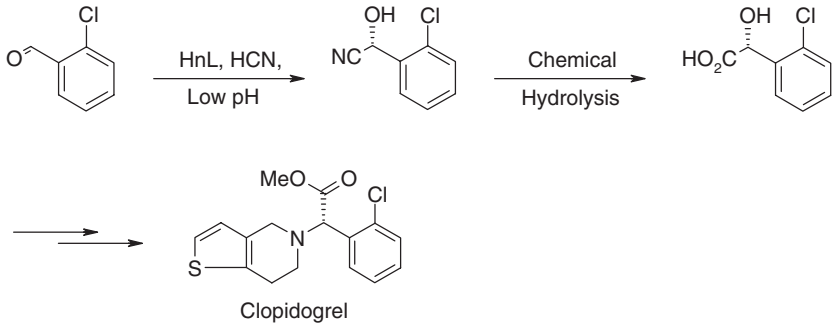


Figure 1.11 *Aprepitant and an (R)-1[3,5-bis(trifluoromethyl)phenyl]ethan-2-ol intermediate*

1.3.4.5 Asymmetrization Using Other Biocatalysts

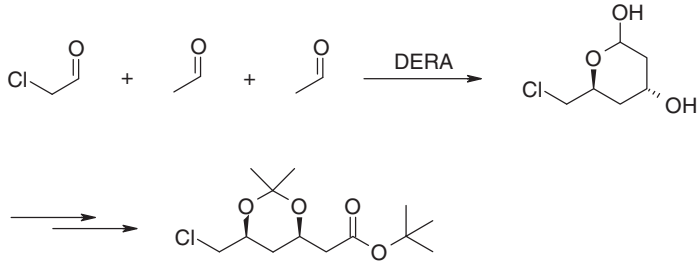
Another class of biocatalyst of great potential for the preparation of chiral intermediates through asymmetric carbon–carbon bond formation is the HnLs. A range of HnLs are commercially available which are enjoying increasing interest in the pharmaceutical industry. In addition to the nitrilase and ADH approach to the (*R*)-2-chloromandelate intermediate to clopidogrel discussed earlier (Schemes 1.44 and 1.53), asymmetric cyanation of 2-chlorobenzaldehyde using the crude HnL from *Prunus amygdalus* (almond meal) has also been reported.[158] The reaction is run at low pH (to slow the background reaction), to afford the cyanohydrin in 90 % ee (Scheme 1.56).

Several approaches to statin side-chain intermediates have so far been discussed. Whereas these chemoenzymatic approaches provide clear benefits over the chemical processes, they do not harness the true potential of biocatalysis as the biotransformations have simply been inserted into the existing chemical route. Wong and co-workers have developed a more biosynthetic-like approach by using a mutant 2-deoxyribose-5-phosphate aldolase (DERA)



Scheme 1.56 *Preparation of a clopidogrel hydroxyacid intermediates with HnL*

(Scheme 1.57).[159] Although the natural donor aldehyde is D-2-deoxyribose-5-phosphate, non-phosphorylated donor aldehydes are also tolerated and the enzyme displays some flexibility towards both donor and acceptor. Importantly, as both donor and acceptor substrates are aldehydes, the enzyme can perform sequential aldol reactions allowing the preparation of a key lactol intermediate to the atorvastatin side chain in a single step. Following substantial modification, this approach is now operated on an industrial scale to produce this intermediate in >100 gL^{-1} concentrations.[84]

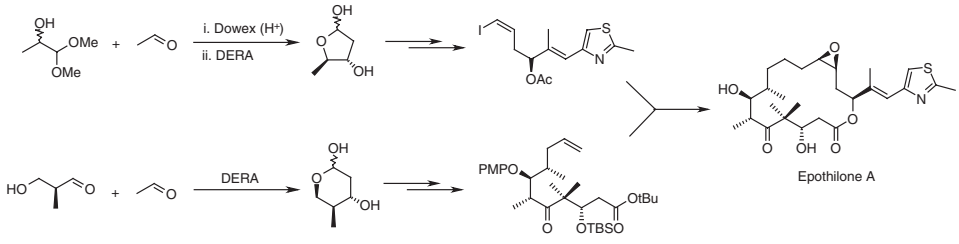


Scheme 1.57 *DERA approach to the atorvastatin side chain*

In a recent patent, Hu *et al*. reported a similar procedure where the acceptor aldehyde contains aminoalkyl substituents in place of chloride.[160] Subsequent to lactol oxidation and amine deprotection, these intermediates can directly undergo Paal–Knorr cyclization with the appropriate diketone to produce atorvastatin, thus avoiding the use of cyanide chemistry.

The flexibility of DERA enzymes makes them a valuable synthetic tool for the quick access to a range of polyoxgenated products, such as the cytotoxic agent epothilone A (Scheme 1.58).[161]

Of the known classes of aldolase, DERA (statin side chain) and pyruvate aldolases (sialic acids) have been shown to be of particular value in API production as they use readily accessible substrates.[162] Glycine-dependent aldolases are another valuable class that allow access to β-hydroxy amino acid derivatives. In contrast, dihydroxyacetone phosphate (DHAP) aldolases, which also access two stereogenic centres simultaneously,



Scheme 1.58 *DERA approach to the synthesis of epothilone A*

have only been of academic interest as they require expensive phosphorylated aldehyde donors and produce phosphorylated products that require subsequent deprotection. This is beginning to change with the discoveries of fructose-6-phosphate aldolase (FSA) that accepts dihydroxyacetone (DHA) as substrate[163] and that DHAP aldolase can accept DHA when used in borate buffer due to the transient formation of a borate ester that mimics phosphate.[164]

As more enzyme kits become commercially available, the screening for a suitable catalyst can now be performed in a matter of hours rather than days or weeks. Furthermore, both the screening and biotransformation can be performed by nonspecialists. This increases the likelihood of uptake of a biocatalytic process, as a proof of concept can be more readily obtained without the commitment of considerable resource. For these reasons, the use of ADHs by pharmaceutical companies has increased considerably in recent years.

1.4 Enzymes in Organic Solvent

Biocatalysis has traditionally been performed in aqueous environments, but this is of limited value for the vast majority of nonpolar reactants used in chemical synthesis. For a long time it was assumed that all organic solvents act as denaturants, primarily based on the flawed extrapolation of data obtained from the exposure of aqueous solutions of enzyme to a few water-miscible solvents, such as alcohols and acetone, to that of all organic solvents.[165]

This assumption has since been swept aside and it is now recognized that a broad range of enzymes retain their activity on exposure to organic solvents or organic solvent–water mixtures. The addition of organic solvent allows the coupling of the exquisite selectivities observed from traditional approaches with numerous other advantages, such as:

- increased concentrations of nonpolar reactants;
- enablement of reactions that have unfavourable thermodynamic equilibria in water;
- enhanced biocatalyst stability towards heat and autolysis;
- compartmentalization of substrate/product from enzyme (reduced substrate/product inhibition);
- modification of enzyme selectivity;
- selective inhibition of competing enzymes;

- reduced background reaction;
- improved workup;
- better integration into synthetic routes;
- greater potential for tandem chemoenzymatic processes.

The field of biocatalysis in organic media is now of considerable industrial importance, enjoying widespread application, particularly in the preparation of enantiopure intermediates.[166]

Enzyme catalysis in nonconventional media can be divided into a number of different categories depending on whether the aqueous and organic phases are miscible or immiscible and whether the biocatalyst is dissolved or not. In this section, only 'free' enzymes will be considered. Thus, the field can be simplified to just two categories, depending on whether the solvent is water miscible or immiscible (systems employing water-immiscible solvents, where water is present in quantities that are below its solubility limit, have been considered as monophasic):

1. monophasic biocatalysis
2. biphasic biocatalysis.

The state of the catalyst (homogeneous or heterogeneous) is dictated by the relative quantities of solvent and water used.

1.4.1 Monophasic Biocatalysis

The structural integrity of enzymes in aqueous solution is often compromised by the addition of small quantities of water-miscible organic solvents.[167] However, there are numerous examples, particularly using extremophiles,[168] where enzymes have been successfully employed in organic solvent–aqueous mixtures.[166b] A good example is the savinase-catalysed kinetic resolution of an activated racemic lactam precursor to abacavir in 1:1 THF/water (Scheme 1.39). The organic solvent is beneficial as it retards the rate of the unselective background hydrolysis.

The use of water-miscible organic solvent–water mixtures is a particularly attractive method for use with cofactor-dependent enzymes due to its simplicity. The high water content can allow dissolution of both enzyme and cofactor, whilst the water-miscible solvent can provide a dual role in both substrate dissolution and as a cosubstrate for cofactor recycling (substrate-coupled cofactor recycling).[148] The asymmetric reduction of a ketone intermediate of montelukast using an engineered ADH in the presence of 50 % v/v isopropanol offers a powerful demonstration of this methodology (Scheme 1.55).

It might be expected that in miscible organic solvent–water mixtures of increasing organic solvent content, the structural integrity of many enzymes will progressively diminish due to loss of essential hydrogen bonding. In fact, this is not the case, as demonstrated by Griebenow and Klibanov,[165] who used Fourier-transform infrared spectroscopy to assess the effect of acetonitrile–water mixtures (0–100 %) on the secondary structure of lysozyme. Rather than a gradual loss in secondary structure with increasing organic solvent content, they observed an inverse bell-shaped relationship, with maximum α-helicity occurring at both high water and high organic solvent content. Reduced enzyme solubility at high organic solvent content might have provided an attractive rationale, but this was not supported by the data. A similar trend was observed using *Bacillus subtilisin*

protease (also known as subtilisin Carlsberg) and other water-miscible organic solvents. The authors concluded that enzyme denaturation increases as the organic solvent content increases. At the same time, a decline in water content reduces conformational mobility so that the enzyme becomes kinetically trapped in an active conformation.

In addition to the retention of structural integrity in neat organic solvents, Klibanov and co-workers demonstrated that a diverse range of enzymes, from hydrolases and peroxidases to cofactor-dependent alcohol oxidases and ADHs, also retain activity.[67] This pioneering work single-handedly led to the popularization of biocatalysis in neat organic solvent.

Recent literature has shown that nonaqueous biocatalysis is not limited to traditional organic solvents, with examples that employ ionic liquids[169] and supercritical fluids[170] now widespread. Reaction in organic solvent has also led to the discovery that some enzymes display promiscuity towards reaction type as well as substrate type,[171] with the HnL-catalysed asymmetric Henry reaction,[172] and the lipase-catalysed Michael-type addition of thiols to α,β-unsaturated enones providing some recent examples.[173] Enhanced rigidity of enzymes in nonaqueous media also imparts greater thermostability, allowing reactions to be run at temperatures of up to 100 °C over prolonged time periods.[174] For example, the kinetic resolution of a key intermediate in the synthesis of lotrafiban using Novozym 435 as catalyst (Scheme 1.36) can be performed at temperatures of 70 °C over prolonged reaction times without enzyme degradation. However, a lower temperature of 50 °C was employed in the final production route due to limitations of the immobilization technique used rather than the enzyme. In the 88 % *tert*-butanol–12 % water solvent mixture, required to provide sufficient substrate solubility, substantial enzyme desorption from the support at higher temperatures limited reuse of this expensive catalyst.

Efficient biocatalysis in neat organic solvent depends on the careful choice of the method of 'dehydrated' enzyme preparation and solvent used. Optimization of these factors towards a given transformation is often known as 'catalyst formulation' and 'solvent, or medium, engineering' respectively, both of which will be briefly discussed below. 'Catalyst engineering' which also provides a powerful method of improving activity and stability, is discussed in Chapter 2.

1.4.1.1 Catalyst Formulation

A requirement of biocatalysis in neat organic solvent is the use of a dehydrated form of an enzyme that displays the desired activity. A number of techniques are available for the preparation of dehydrated enzymes, some of which are discussed in a recent review by Griebenow and Barletta.[175] The techniques that have been most commonly used are:

- lyophilization
- precipitation
- immobilization (see Section 1.5).

The resultant dehydrated enzyme preparations often display comparable activity to untreated enzyme when reconstituted in aqueous buffer. However, in the case of many enzymes, activity in a suitable neat organic solvent can be three to five orders of magnitude lower than in water. This was recognized by Klibanov early on in the

development of the field, and so many of the basic principles leading to reduced efficiency have been elucidated. These have been extensively reviewed and will only be briefly discussed here.[176,177]

A major cause of suboptimal activity in organic solvent results from the removal of 'essential water' during enzyme dehydration. All enzymes require some water in order to retain activity through the provision of conformational flexibility.[178] Particularly in the case of lipases, the amount of water can be so low that it appears that none is required. For example, following the development of suitable techniques to analyse low water concentrations,[179] it has been reported that the lipase from *Rhizomucor miehei* retains 30 % of its optimum activity with as little as two or three water molecules per molecule of enzyme.[180,181] Owing to the apparent absence of water in some exceptional cases, the term 'biocatalysis in anhydrous solvent' is commonly used, although in the vast majority of cases a monolayer of water is required for optimal activity (although this is often still well below its solubility limit in water-immiscible solvent).[67]

Numerous 'tricks' have been developed to retain activity of the dehydrated enzyme preparation. Activity can be dramatically enhanced by adding a small quantity of water to the enzyme prior to use,[182] but this can be detrimental in transformations where it can participate as a reactant, particularly where the reagents are expensive. Retention of activity without the need to partially rehydrate has, therefore, been the focus of intensive investigation. Some effective strategies, such as co-lyophilization in the presence of lyoprotectants (sugars or hydrophilic polymers) and the use of additives such as crown ethers, substrate or transition-state analogues (molecular imprinting) or inorganic salts, have recently been reviewed by Serdakowski and Dordick.[177] Some of these techniques can lead to dramatic changes in enantioselectivity and activity.[183]

The ionization state of polar (ionogenic) residues of the dehydrated enzyme preparation can also have a substantial impact on conformation and, hence, on activity in organic solvent. The ionization state can be optimized through pH control of the aqueous solution from which the enzyme was last in contact. Commonly referred to as the 'pH memory' effect, optimum activity in organic solvent is usually attained by preparing the dehydrated enzyme from an aqueous solution of optimal pH for enzyme activity in conventional media. In many cases, charged species are generated during the course of a transformation that can affect the enzyme ionization state. This can be controlled through the addition of solid-state buffers to the reaction mixture.[184]

Because enzymes are insoluble in organic solvent, mass-transfer limitations apply as with any heterogeneous catalyst. Water-soluble enzymes (which represent the majority of enzymes currently used in biocatalysis) have hydrophilic surfaces and so tend to form aggregates or stick to reaction vessel walls rather than form the fine dispersions that are required for optimum efficiency. This can be overcome by enzyme immobilization, as discussed in Section 1.5.

1.4.1.2 Solvent Engineering

Enzyme activity varies greatly depending on solvent choice, as illustrated by Zaks and Klibanov[185] for the transesterification of tributyrin and heptanol by three different lipases. Using these data, Laane *et al.*[186] found that enzyme activity correlates closely with solvent hydrophobicity (log *P*) for the lipases from *Mucor* sp. (MML) and *Candida cylindracea*

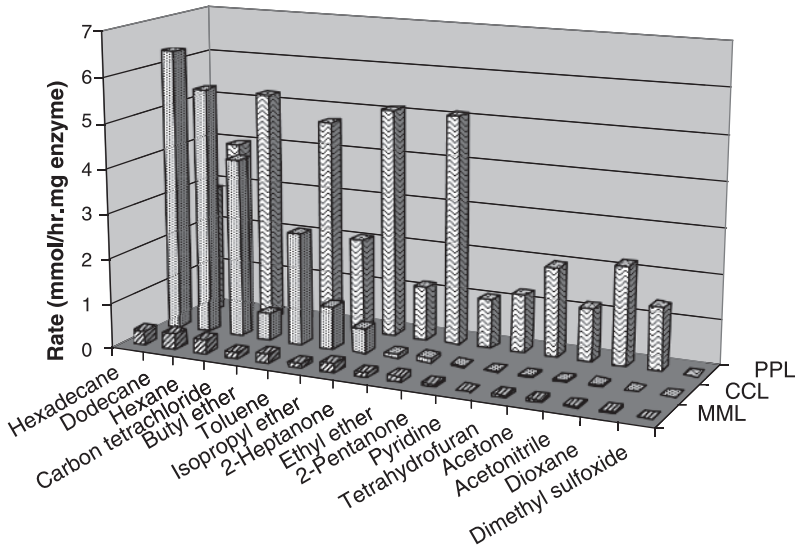


Figure 1.12 *Transesterification activity of PPL, CCL and MML in various organic solvents*

(CCL – now known as lipase from *Candida rugosa* (CRL)) but not porcine pancreatic lipase (PPL) (Figure 1.12).

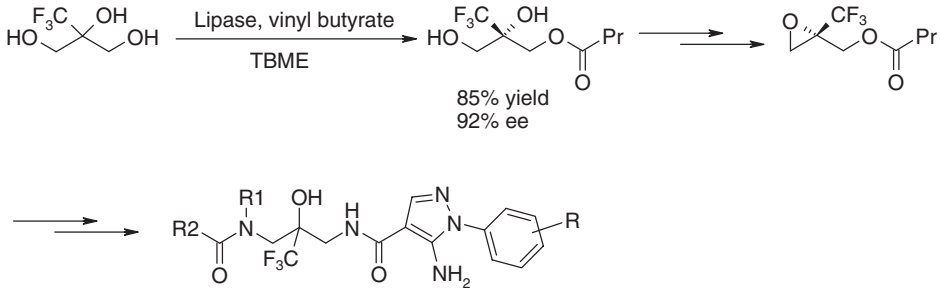
It was postulated that the differences in enzyme activity observed primarily result from interactions between enzyme-bound water and solvent, rather than enzyme and solvent. As enzyme-associated water is noncovalently attached, with some molecules more tightly bound than others, enzyme hydration is a dynamic process for which there will be competition between enzyme and solvent. Solvents of greater hydrophilicity will strip more water from the enzyme, decreasing enzyme mobility and ultimately resulting in reversible enzyme deactivation. Each enzyme, having a unique sequence (and in some cases covalently or noncovalently attached cofactors and/or carbohydrates), will also have different affinities for water, so that in the case of PPL the enzyme is sufficiently hydrophilic to retain water in all but the most hydrophilic solvents.

The impact of water on enzyme activity is powerfully demonstrated by the chymotrypsin-catalysed transesterification of ethyl *N*-acetyl-L-phenylalaninate with propanol. In dry acetone, the reaction is over 7000 times slower than in dry octane. However, by adding 1.5 % v/v water to acetone, the reaction rate dramatically increases to two-thirds the rate of that in dry octane.[67a] Zaks and Klibanov also demonstrated the effect of water stripping on enzyme activity by incubating chymotrypsin in various organic solvents and then assessing the resulting enzyme water content. Activity in the different organic solvents was found to correlate well with water retained by the enzyme. Halling was able to rationalize such findings by realizing that a given enzyme requires a defined quantity of water to attain optimal activity. This can be expressed in terms of thermodynamic water activity, which essentially describes the amount of water bound to the enzyme.[187] Thus, optimum chymotrypsin activity in acetone is realized at the same thermodynamic water activity as that in

octane even though the total water content of each system is very different. However, at comparable water activity, variations in optimum enzyme activity observed in each solvent show that the direct effect of solvent on the enzyme is also an important factor which may account for the activity deviations from the activity/log *P* relationship seen in Figure 1.12.

The choice of organic solvent can also have a dramatic effect on selectivity.[166a] In contrast to enzyme activity, in the majority of examples reported there appears to be no correlation between solvent physical properties and enantioselectivity. In fact, investigating the effect of various solvents towards a number of lipases, Secundo *et al.*[188] also found that the optimal solvent differed with both enzyme and substrate. A number of theories have been postulated in order to explain these effects in individual cases, but none has any general predictive value.[183b] This is somewhat intriguing given that differences in enantioselectivity simply relate to a change in the relative rate of conversion of each enantiomer.

Reaction in organic solvent can sometimes provide superior selectivity to that observed in aqueous solution. For example, Keeling *et al.*[189] recently produced enantioenriched α-trifluoromethyl-α-tosyloxymethyl epoxide, a key intermediate in the synthetic route to a series of nonsteroidal glucocorticoid receptor agonist drug candidates, through the enantioselective acylation of a prochiral triol using the lipase from *Burkholderia cepacia* in vinyl butyrate and TBME (Scheme 1.59). In contrast, attempts to access the opposite enantiomer by desymmetrization of the 1,3-diester by lipase-catalysed hydrolysis resulted in rapid hydrolysis to triol under a variety of conditions.



Scheme 1.59 *Synthesis of nonsteroidal GR agonists*

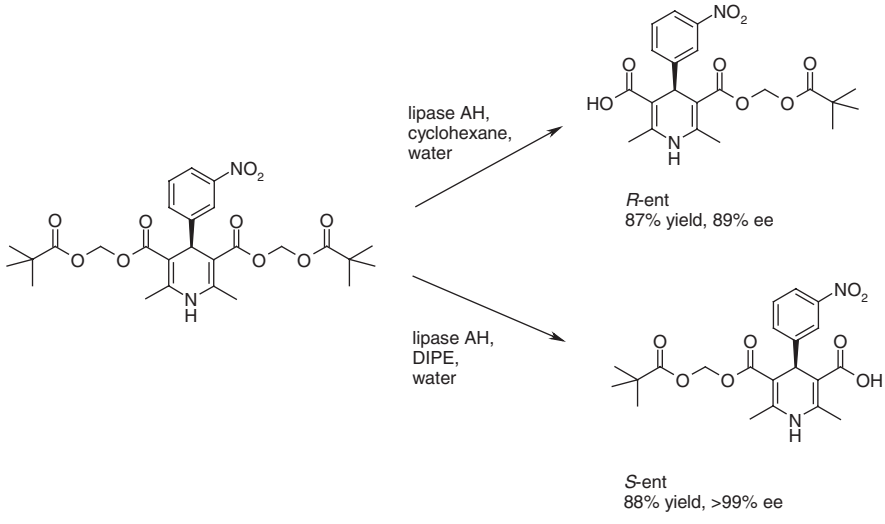
1.4.2 Biphasic Biocatalysis

Biocatalysis in biphasic mixtures of water-immiscible organic solvent and water involves the transfer of low concentrations of substrate from the organic to aqueous phase during agitation. The substrate then undergoes transformation before returning to the organic phase. The partition of substrate/product between the two phases is independent of their ratio and so the volume of the organic phase can be much greater than the aqueous phase, allowing high-intensity transformations to be achieved whilst simultaneously minimizing exposure of the enzyme to organic species. The technique is particularly valuable for transformations in which the enzyme is sensitive to inhibition by high concentrations of substrate or product and transformations where cofactor recycling is required.

Biphasic conditions can also be used to suppress background reaction. HnL-catalysed asymmetric addition of cyanide to aldehydes and ketones provides an important example,

allowing chiral intermediates to APIs such as clopidogrel to be accessed in excellent enantiopurity (Scheme 1.56). However, whereas the biphasic method of controlling background reaction works well with nonpolar substrates, it is less effective with polar, water-soluble substrates such as 3-pyridinecarboxaldehyde. Such substrates require transformation under nearly anhydrous conditions where, unfortunately, HnLs rapidly deactivate. Faced with this issue, Roberge *et al*.[190] have recently reported that HnLs, immobilized as cross-linked enzyme aggregates (CLEAs), retain their activity in nearly anhydrous conditions (see Section 1.5.2 for further details of CLEAs). Using two different commercially available HnL CLEAs they were able to produce either of the enantiomers of 3-pyridinecarboxaldehyde cyanohydrin in moderate to high yield and >90 % ee in dichloromethane containing just 0.18 % water.

The solvent present in biphasic reactions can still have an effect on the enzyme even though the enzyme functions primarily in an aqueous microenvironment. A particularly dramatic example is the lipase AH (lipase from *Burkholderia cepacia*)-catalysed desymmetrization of prochiral 1,4-dihydropyridine dicarboxylic esters, where either enantiomer can be accessed in high enantioselectivity by using either water-saturated cyclohexane or diisopropyl ether (DIPE) respectively (Scheme 1.60).[191] The acyl group used in acylation and deacylation can also have a dramatic effect on enantioselectivity.[134]



Scheme 1.60 *Resolution of a prochiral 1,4-dihydropyridine dicarboxylic ester with lipase AH in the presence of cyclohexane or DIPE*

In conclusion, by using organic solvents, biotransformations can achieve productivities suitable for pharmaceutical manufacture. Biocatalysis under organic solvent–aqueous conditions can be applied to a broad range of enzymes as the methodology is compatible with cofactor recycling, whereas biocatalysis in nearly anhydrous solvent facilitates numerous transformations that are thermodynamically disfavoured in the presence of water, although limited to use with enzymes that do not require cofactors, particularly hydrolases. In selecting an appropriate solvent, it is necessary to screen each new biotransformation on a case-by-case basis to ensure that optimum enzyme activity, stability and selectivity are

achieved. For optimal activity under nearly anhydrous conditions, attention should also be paid to water activity and the dehydrated enzyme formulation used. Water stripping is particularly important to consider when setting up a continuous process.

1.5 Enzyme Immobilization

Ballesteros *et al.*[192] defined immobilized biocatalysts as 'enzymes, cells or organelles (or combinations of these) which are in a state that permits their reuse'. Enzyme immobilization represents only a small part of this field, but is the most commonly employed in pharmaceutical production.

Immobilized enzymes are frequently used in biocatalysis to overcome limitations such as:

- insufficient stability towards temperature, pH, shear stress or autolysis;
- necessity to recycle the enzyme for economical reasons;
- biological contamination of the product causing complex downstream processing;
- emulsion formation during product extraction;
- poor catalyst dispersion in the reaction mixture;
- insufficient activity;
- inappropriate form if required for a continuous process.

Where immobilization is necessary, any resulting biocatalyst should be:

- toxicologically safe;
- low cost;
- sufficiently active and selective;
- chemically and thermally stable under process and storage conditions;
- insoluble towards the reaction solvent;
- mechanically strong;
- of uniform particle size;
- resistant to microbial attack;
- reusable.

Numerous different immobilization methods have been reported that take advantage of various enzyme properties such as size, chemically reactive functionality, ionic groups or hydrophobic domains.[193] Based on these properties, enzyme immobilization can be split into three main classes (which are also applicable to the immobilization of cell cultures):

- noncovalent attachment;
- covalent attachment and cross-linking;
- entrapment.

In spite of the immense quantity of available literature, it can still be a challenge to determine which immobilization technique is suitable for a particular application, and so it is usually necessary to test a number of options on a case-by-case basis.

1.5.1 Noncovalent Attachment

Noncovalent attachment is a popular method of immobilization, and numerous different support materials have been employed, ranging from organic supports, like cellulose,

chitin, ion-exchange resins and polyacrylamide, to inorganic supports, such as celite, salts, zeolites or even iron particles. However, the technique is disfavoured for industrial applications as the enzyme is weakly bound and, therefore, prone to leaching, potentially leading to product contamination and inefficient recycling.

Many lipases are commercially available in a noncovalently immobilized form either adsorbed onto celite, which aids dispersion in organic solvent, or onto a hydrophobic support such as accurel. As a result, noncovalently immobilized lipases are frequently employed, in spite of the above limitations, owing to their availability. Lipase immobilization on hydrophobic supports is particularly useful, as it takes advantage of the unique property of this enzyme class towards interfacial activation at the surface of oil droplets.[194] Unlike other enzymes, most lipases contain what is often referred to as a lid or flap that masks the active site. This lid is hydrophilic on the external surface and hydrophobic on the internal surface, so that in aqueous solution the lipase exists in an equilibrium lying primarily towards the inactive or closed form. On adsorption to an oil droplet, the flap undergoes a conformational change to the 'open form,' resulting in activation. As discussed in Section 1.4, enzymes are more rigid in organic solvent and so the lipase can be trapped in the form that was predominant in the aqueous solution from which it was last in contact.[195] On immobilization, the hydrophobic support itself can mimic an oil droplet, resulting in hyperactivation of the lipase. It is not uncommon for an immobilized lipase to display greatly enhanced activity over that of the free enzyme.

1.5.2 Covalent Attachment and Cross-linking

Immobilization of an enzyme through covalent attachment is a widely used technique, as the catalyst can be used in either aqueous or organic media without leaching and provides a suitable catalyst form for use in multipurpose apparatus or more specialized equipment such as a continuous reactor. Covalent attachment is usually achieved via attack from nucleophilic groups of the enzyme onto electrophilic moieties on the support (although the reverse has also been reported). Given that most enzymes have numerous reactive substituents (Table 1.2), multipoint attachment to the support can occur, which can have a significant stabilizing effect. A drawback of this technique can result from the formation of covalent linkages in or near to the enzyme active site, causing deactivation. However, this outcome can usually be circumvented by using another of the many alternative supports available.

Table 1.2 *Reactive functionality of amino acid residues frequently present in proteins*

Functional group	Amino acid
Primary amine	L-Lysine and N-terminus
Thiol	L-Cysteine
Carboxylic acid	L-Aspartate, L-glutamate and C-terminus
Phenol	L-Tyrosine
Guanidine	L-Argenine
Imidazole	L-Histidine
Disulfide	L-Cystine
Indole	L-Tryptophan
Thioether	L-Methionine
Alcohol	L-Serine, L-threonine

Eupergit C and, more recently, Sepabeads EC-EP are mesoporous supports that have proven to be of particular importance in pharmaceutical production. Both are highly hydrophilic macroporous resins, containing high densities of epoxide groups on the surface. Available as beads of 100–250 μm in diameter and 20–40 nm pore diameter, these resins display high chemical and mechanical stability, tolerating a wide range of pH and solvents.

About 60 mg of purified enzyme per gram of resin can generally be immobilized onto Sepabeads EC-EP, under extremely mild conditions, using enzyme dissolved in buffers of high salt concentration. An initial rapid adsorption takes place followed by slower covalent bond formation, after which the remaining epoxides (as much as 99 % of the original groups) can be opened or 'capped' using a nucleophilic species. Crude enzyme preparations can also be used, as other cell debris will either irreversibly bind to the support along with the enzyme or can be easily washed away after immobilization is complete. To exemplify the mildness and robustness of this technique, 85–90 % of PGA active sites have been reported to remain competent following immobilization to Eupergit C.[196] Furthermore, the immobilized catalyst lost only 40 % of its activity over >800 cycles.

Covalent enzyme attachment to an inert support is inherently inefficient, as enzyme activity is diluted and additional material costs are incurred. An attractive alternative that circumvents both of these issues is to cross-link enzyme molecules together using a bifunctional linker. This technique gained huge popularity with the emergence of cross-linked enzyme crystals (CLECs).[197] CLECs are produced by crystallization of purified enzyme and subsequent cross-linking, usually with glutaraldehyde, which is an FDA-approved fixing agent for the immobilization of glucose isomerase used in high-fructose corn syrup production.[198] CLECs proved to be excellent biocatalysts, displaying high activity, stability and separation properties, as demonstrated by their use in the resolution of the bisfuran alcohol intermediate of brecanavir (Scheme 1.33). Unfortunately, enzyme purification and crystallization can be labour intensive to develop and inefficient, resulting in an extremely active but highly expensive catalyst. This led to poor uptake of the technology and withdrawal of CLECs from the marketplace.

More recently, CLEAs have been introduced. They provide many of the positive attributes of CLECs but can be rapidly prepared from partially purified enzyme preparations with minimal technical expertise.[199] Essentially, their preparation involves enzyme precipitation (see Section 1.4.1.1) with *in situ* cross-linking, or vice versa. Glutaraldehyde is usually employed as the cross-linking agent, although bulkier linkers, such as dextran polyaldehyde, have been successfully used where cross-linking with the smaller reagent results in activity loss through interaction with the enzyme active site.[200]

1.5.3 Entrapment

Entrapment involves the physical confinement of an enzyme in a semipermeable matrix, in much the same manner as nature handles soluble enzymes.[201] This should represent an extremely mild method of immobilization, as the enzyme remains free, albeit confined to a small space. Two techniques, which at first sight appear unrelated, have been well utilized:

- entrapment in a polymer matrix;
- entrapment behind a membrane.

Entrapment in polymeric matrices is a variation of noncovalent attachment where the support is instead generated in the presence of the enzyme. A particularly popular entrapment technique is sol–gel encapsulation, where the enzyme is trapped within an SiO_2 matrix formed by acid- or base-catalysed hydrolysis of tetraalkoxysilanes in the presence of enzyme.[202] The technique can be tuned to provide the appropriate microenvironment for each enzyme in much the same way as can be done with other immobilization methods.[203]

Pharmaceutical production generally uses multipurpose equipment, and so entrapment behind a membrane would require significant capital expenditure on specialized equipment. In spite of this, the use of membrane reactors in biocatalysis represents an efficient method of enzyme immobilization, given the large molecular weight difference between enzymes (10–150 kDa) and most substrates (300–500 Da). The reader is referred to some recent reviews on the topic.[204]

In summary, enzyme immobilization is extremely important in the scale-up of many biocatalytic processes. The preferred method for pharmaceutical production involves covalent binding through cross-linking or attachment to a support. Noncovalent attachment is less attractive, but it is heavily utilized owing to the commercial availability of industrial quantities of some enzymes immobilized using this technique.

1.6 Green Chemistry

The use of biocatalysis in the manufacture of APIs can address some of the 12 principles of green chemistry set out by Anastas and Warner.[205] For example, biocatalytic processes can:

- increase atom efficiency;
- operate under mild conditions;
- reduce protection/deprotection steps;
- avoid the use of stoichiometric reagents;
- avoid the use of toxic/hazardous chemistry.

However, these statements are generalizations, and it is not necessarily true to say that all biotransformations will be greener than the chemical alternative. Therefore, it is important to analyse each comparison objectively on a case-by-case basis using a multivariate process to take into account the complexity of the analysis. Designing greener processes involves, for example:

- designing efficient processes that minimize the resources (mass and energy) needed to produce the desired product;
- considering the environmental, health and safety profile of the materials used in the process;
- considering the environmental life cycle of the process;
- considering the economic viability of the process;
- considering the waste generated in the process, both in nature and quantity, whether it is hazardous, benign, can be recycled or recovered and used in this or another process.

It is not easy or straightforward to determine how green a process is, and there have been a number of different approaches taken. Sheldon's *E*-factor was one of the first measures of greenness proposed in the 1980s, to highlight the amount of waste generated in order to

produce 1 kg of chemical product across different branches of the chemical industry.[206] Simply put, the higher the *E* number, the more waste is generated to produce 1 kg of product. Within the pharmaceutical industry there have been other variations of measuring the mass efficiency, such as the mass intensity proposed by Constable et al.[207] and the process mass intensity proposed by the ACS GCI Pharmaceutical Roundtable.[208]

Measuring greenness is not just about determining the quantity of waste; one must also consider the efficiency of the chemistry or biochemistry (atom efficiency, reaction mass efficiency) and the nature of the materials involved as reagents, solvents and as waste.[209] One should also consider the process conditions used, all within the context of the 12 principles of green chemistry. The next factor to take into account when trying to evaluate the greenness is the environmental life cycle impact of the materials used in the process. Determining the life cycle for every material used in a pharmaceutical synthetic process is a complex task, as often the life cycle data for every material is just not available. However, GSK have developed a methodology and a tool to enable good estimations of the life cycle impacts so that comparisons between different development options can be made.[210] Data have recently been added to the tool to enable life cycle comparisons for routes using enzymes as catalysts or involving a fermentation step. GSK have also developed a framework for analysing and comparing two processes based upon the suite of metrics discussed above.[211]

This framework was used as the basis for a comparison of the environmental, health, safety and life cycle (EHS and LCA) impacts of the chemical (Scheme 1.11) and two enzyme biocatalytic (Scheme 1.12) 7-ACA processes, recently reported by Henderson *et al*.[55] The measures used accounted for the chemical and process efficiencies, the nature of the materials used and waste generated, as well as determining the overall life cycle environmental impacts from 'cradle to gate' of each process. This analysis showed that the bioprocess could be classed as 'greener' when compared with the purely chemical process. The chemical process uses more hazardous materials and solvents, and requires about 25 % more process energy than the enzymatic process. When accounting for the cradle-to-gate environmental life cycle, the chemical process has a larger overall environmental impact, mainly derived from the production of raw materials. In comparison with the enzyme-catalysed process, the chemical process uses approximately 60 % more energy, about 16 % more mass (excluding water), has double the greenhouse gas impact and about 30 % higher photochemical ozone creation potential and acidification impact. Only the yield of the chemical process was higher, showing that yield is not a good measure of greenness, which reinforces the message that it is important to take a more holistic view, since assessing greenness is a multivariate and complex process. One of the aims of the analysis was to develop a methodology and framework for objective comparisons of two very different types of synthetic process, which could then be applied to other different systems. A secondary aim was to test the hypothesis that biotransformations are greener than chemical transformations. By the application of such rigorous and academic analyses one can test this hypothesis for a number of different systems, including once-through fermentations and enzyme-catalysed systems, where the amounts of waste generated will be significantly different.

To celebrate the fifteenth anniversary of his *E*-factor, Sheldon compared different measures of greenness[212] with the *E*-factor and reminds us of the value of the headline number, which challenges those in the pharmaceutical industry to improve the efficiency of

pharmaceutical processes by moving away from continually using stoichiometric reagents towards catalytic reagents. While it is true to say that the absolute volumes of waste are low compared with fine chemicals or petrochemicals, the challenge remains valid today that the pharmaceutical industry has the opportunity to embrace catalytic technology as one way to improve the mass efficiency of processes. The application of biocatalytic technology in the pharmaceutical industry is one way of addressing that challenge.

1.7 Future Perspectives

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 it could provide more efficient and greener methods of late-stage intermediate and API production.

The ACS GCI Pharmaceutical Roundtable recently set out to prioritize the areas of chemical synthesis where improved methodology would realize the greatest beneficial impact on pharmaceutical production. This resulted in the publication of a 'wish list' of currently utilized transformations that require better reagents and aspirational transformations that would provide shorter routes were they available (Table 1.3).[16]

A recent categorization of biotransformations by Pollard and Woodley[12] (Figure 1.13), based on the availability of commercial enzymes, together with the examples given in this book demonstrate that biocatalysis can meet many of these pharmaceutical needs as shown by the highlighted entries in Table 1.3.

Table 1.3 *List of key areas of green chemistry of importance to the pharmaceutical industry (in ascending order); areas where biocatalytic precedent exists are given in bold.*

Reactions currently used but better reagents preferred	More aspirational reactions
Amide formation avoiding poor atom economy reagents	***C–H activation of aromatics (cross coupling reactions avoiding the preparation of haloaromatics)***
OH activation for nucleophilic substitution	***Aldehyde or ketone + NH_3 + 'X' to give chiral amine***
Reduction of amides without hydride reagents	***Asymmetric hydrogenation of unfunctionalized olefins/enamines/imines***
Oxidation/epoxidation methods without the use of chlorinated solvents	***New greener fluorination methods***
Safer and more environmentally friendly Mitsunobu reactions	*N-Centred chemistry avoiding azides, hydrazine etc.*
Friedel–Crafts reaction on unactivated systems	***Asymmetric hydramination***
Nitrations	*Green sources of electophilic nitrogen (not TsN_3, nitroso, or diimide)* ***Asymmetric hydrocyanation***

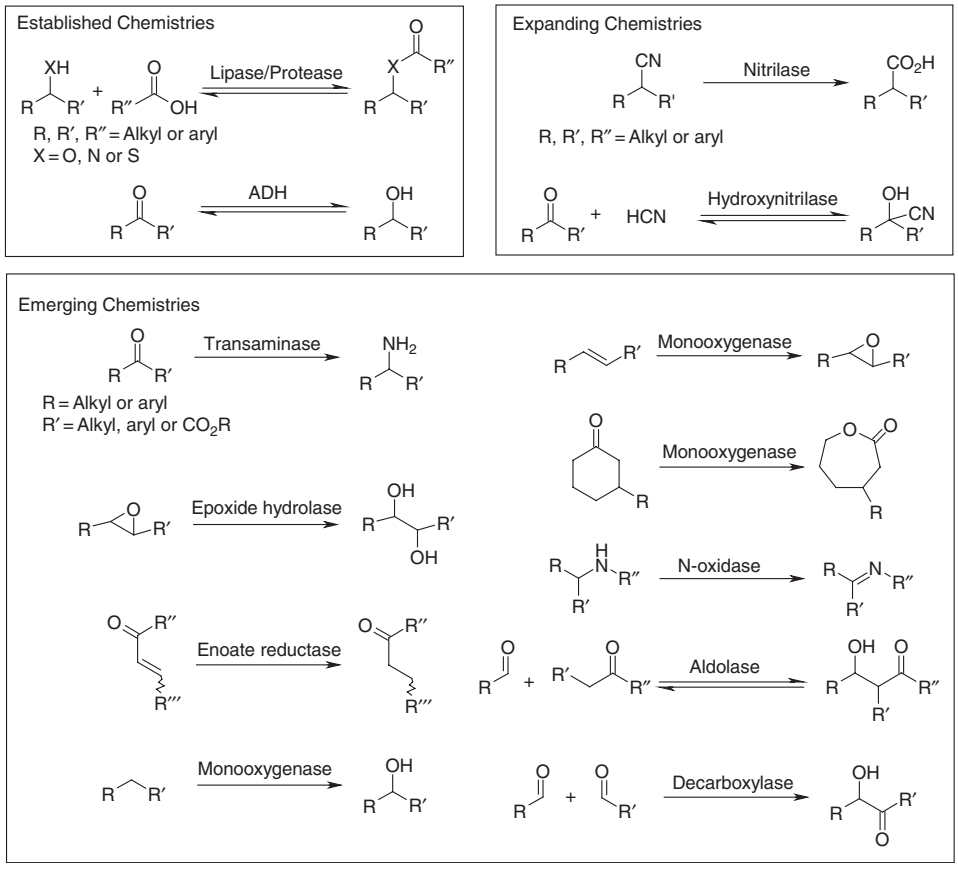


Figure 1.13 *Status of various biotransformations (not exhaustive). (Reprinted from Pollard, D.J. and Woodley, J.M. Biocatalysis for pharmaceutical intermediates: the future is now. Trends Biotechnol.* ***2007****,* 25*, 66–73 with permission from Elsevier.)*

Routes to APIs are predominantly designed by synthetic organic chemists who are well versed in the adoption of new technologies. To maximize uptake of biocatalytic techniques, the most efficient approach is to provide them with reasonably priced kits of enzymes that can easily be used without specialist knowledge. Greater availability of comprehensive commercial kits with diverse applications and better tools to predict improved biocatalyst properties *in silico* should diminish the current perception by many chemists that enzymes are exotic catalysts only to be used as a last resort. However, this expansion requires significant investment from specialist enzyme producers, many of whom subsequently base their business models on the generation of royalties from the use of their proprietary biocatalysts or biocatalytic processes. The use of proprietary enzymes in pharmaceutical production can be cost effective where a biocatalyst is involved in an asymmetric or regioselective transformation if traditional chemical approaches generate substantial waste or require additional steps, but is probably precluded for achiral transformations such as the replacement of an atom-inefficient coupling reagent for amide bond

formation. However, as the field matures and these enzymes become cheaper, such applications should become competitive.

The above applications consider biocatalysis from the perspective of the synthetic organic chemist rather than the biochemist. Slotting single-step biotransformations into chemical syntheses is unlikely to use biocatalysis to its full potential. Undoubtedly, isolated enzymes offer an attractive solution to rapid biocatalyst identification, and advances in molecular biology and biotransformation technology have provided a number of techniques by which hits can be modified to fit a required process, or vice versa. However, there is also a significant cost associated with the isolation of enzymes at scale. It is far more attractive to use crude lysates or whole cells; but, as shown in previous sections, these have their own disadvantages. The use of crude lysates can increase downstream processing complexity, and alleviation of this issue by immobilization adds extra costs associated with production time and additional materials. Whole-cell biocatalysis can also require complex downstream processing and is generally hampered by low substrate concentrations.

To realize the full potential of biocatalysis, a long-term approach might instead harness nature's tandem biocatalytic approach to the construction of complex secondary metabolites for the production of synthetic molecules.[213] Whilst product concentration is generally lower than that of a chemical process, this is offset by the ability to generate molecules of high complexity in a single step and to eliminate costly isolation steps. Fermentation scientists have been harnessing natural, highly selective biosynthetic pathways to produce complex pharmaceutical intermediates from cheap raw materials for decades. Some of the most important pharmaceutical core molecules, such as penicillins and cephalosporins, are economically produced in this way. The wide differences between biosynthetic and chemical approaches to a target API can be gleaned by comparison of the alternative routes that have been reported for the synthesis of orlistat ((−)-tetrahydrolipstatin), a potent gastrointestinal lipase inhibitor used in the treatment of obesity (Figure 1.14). Orlistat can be prepared by hydrogenation of the highly lipophilic secondary metabolite lipstatin. Lipstatin itself is produced by fermentation (or, more correctly, a tandem biotransformation) from linoleic acid via a key enzyme-catalysed Claisen condensation using *Streptomyces toxytricini* under aerobic conditions.[214] In contrast, the chemical approach to orlistat, based on the classical resolution or asymmetric synthesis of a highly functionalized six-membered ring lactone,[215,216] is considered to be one of the most complex in the pharmaceutical industry, requiring four isolation steps and a number of protection/deprotections.[217]

However, molecules currently produced by fermentation are usually natural products, whereas most current drug candidates are synthetic. If lipstatin was not known to be a

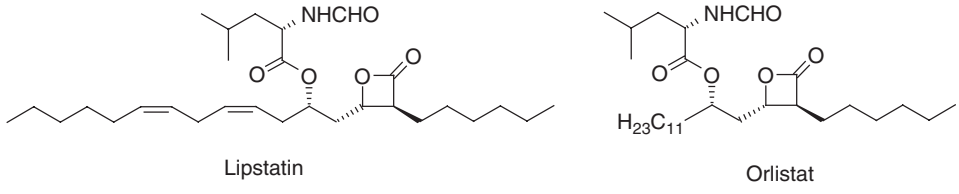
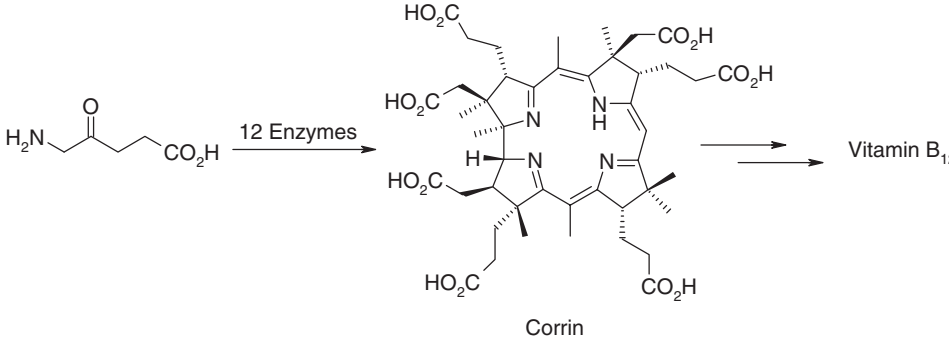


Figure 1.14 *Structures of lipstatin and orlistat*

natural product, would a biosynthetic approach have been developed? Most likely, biocatalytic approaches would be limited to the insertion of individual transformations into the current chemical route. In fact, lipase resolution of the six-membered lactone intermediate produced from the chemical approach to orlistat has been reported.[218] Metabolic engineering of unnatural biosynthetic pathways, by the insertion of non-native genes into a host organism, offers great hope in this respect but is currently still in its infancy.[214] The production of thymidine represents the first example of its successful implementation in pharmaceutical production (Scheme 1.23).

Thymidine, although a synthetic molecule, bears considerable resemblance to other natural products, whereas many drug candidates have no counterpart in nature and will likely require transformations for which there is no biocatalytic precedent. To build an entirely artificial biosynthetic pathway using genetically modified organisms would require a monumental screening effort, given that the vast majority of enzymes involved in biosynthetic pathways have not yet been characterized and their specificities remain unevaluated. Furthermore, should it be necessary to insert a chemocatalytic step into the middle of a biosynthesis, transport across cell membranes would also require consideration. An alternative approach might instead be to express the required enzymes together in a genetically modified microorganism and use partially purified isolates, perhaps in tandem with chemocatalysts. The one-pot synthesis of corrin, a biosynthetic intermediate of vitamin B_{12}, with 20 % unoptimized yield by 12 isolated enzymes demonstrates that complex tandem processes are feasible using isolated enzymes (Scheme 1.61),[219] and the numerous chemoenzymatic processes available in the literature (some of which appear later in the book) demonstrate that chemocatalysts can be efficiently inserted into biocatalytic processes.



Scheme 1.61 *Tandem biocatalytic synthesis of corrin*

1.8 Concluding Remarks

Biocatalysis has enjoyed widespread application in the preparation of chiral building blocks but has generally been employed on a limited basis for the production of more complex, late-stage pharmaceutical intermediates. Owing to pressure on the industry to develop more efficient and greener processes, along with rapid advances in the field of biocatalysis, this is beginning to change.[220]

The recent commercialization of more diverse ranges of enzymes, combined with a plethora of successful applications originating from both academia and the fine chemical industry, is placing biocatalysis in the mainstream as an addition to the chemist's toolbox rather than an exotic curiosity. It is likely that, as the field matures, a greater diversity of non-natural molecules of greater complexity will become accessible through the tandem use of biocatalysts and genetically modified microorganisms. Together with advances in chemocatalysis, this will significantly impact on pharmaceutical production by improving efficiency and reducing waste.

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