Application of biocatalysis and biotransformations to the synthesis of pharmaceuticals

Aleksey Zaks and David R. Dodds

The application of biologically derived catalysts to the synthesis of pharmaceuticals has grown rapidly in recent years. This review covers examples of isolated enzymes and microorganisms used as catalysts for the synthesis of pharmacologically valuable materials, on both a laboratory and a commercial scale.

The potential utility of biocatalysis in the pharmaceutical industry has been widely recognized for many years. Indeed, many fermentations have been instrumental in the synthesis of a variety of physiologically active compounds, including steroids, vitamins, alkaloids and antibacterials. Despite the enormous advances in synthetic chemistry over the past few decades, these biotransformations still remain the most cost-effective processes.

In contrast to the well-established fermentation technology, the application of isolated enzymes as catalysts in the synthesis of pharmaceuticals is more limited[1]. Considering the high catalytic power and exceptional stereo-, regio- and chemoselectivity of enzymes, the small number of their commercial applications in the pharmaceutical industry is surprising. Some factors that adversely affected the utilization of enzymes in the past were high cost, relative instability under industrial conditions, limited specificity and competition with established chemical processes carried out in equipment with fully depreciated capital cost.

Several new developments promise to address these drawbacks in the near future. In response to an ever-growing demand for new catalysts, more enzymes with different specificities have become available in recent years. Advances in fermentation, purification and immobilization techniques have resulted in the production of more stable biocatalysts at significantly reduced cost. New recombinant techniques[2] and automated screening[3] have led to the production of novel 'customized' industrial enzymes[4]. Recent progress in nonaqueous enzymology[5] has simplified both catalyst and product recovery, and has provided an additional tool to control substrate specificity and regio- and enantioselectivity of enzymes. Finally, immobilization technologies based on the cross-linking of crystalline proteins have resulted in the creation new types of highly active, stable, commercially available biocatalysts[6]. As a result of these developments the area of biotransformations is now experiencing a significant growth.

This review covers the application of biocatalysts to the preparation of pharmacologically active compounds. The first part describes processes that have been successfully demonstrated on a laboratory scale. The second part is devoted to biocatalytic processes that are actually practiced to provide material for clinical studies or commercial drug products.

Laboratory-scale synthesis

Non-steroidal anti-inflammatory drugs

Ibuprofen (**1**), naproxen (**2**), ketoprofen (**3**), flurbiprofen (**4**) and ketorolac (**5**) (Figure 1) are popular analgesic and

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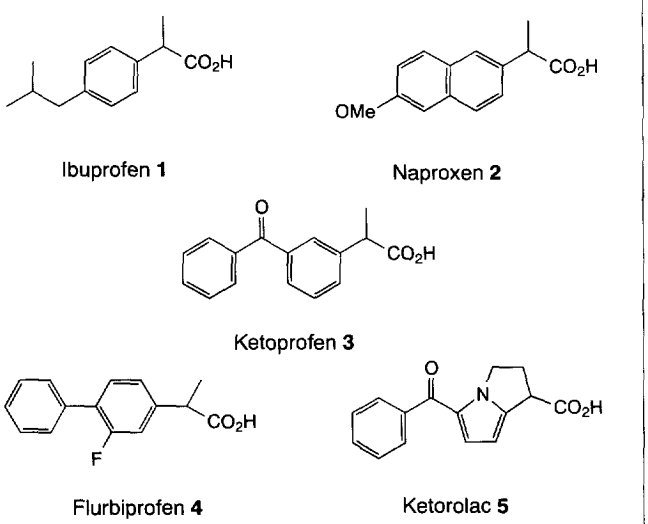

Figure 1. *Non-steroidal anti-inflammatory drugs.*

non-steroidal anti-inflammatory drugs that act by inhibiting prostaglandin biosynthesis. Numerous pharmacological studies on enantiomers of these compounds have shown that the (*S*)-isomer not only has a significantly greater therapeutic effect than the (*R*)-enantiomer[7] (28-fold in the case of ibuprofen) but also that it reaches therapeutic concentrations in blood faster than the racemate. Nevertheless, only naproxen is currently marketed in an optically active form. (*S*)-Naproxen ranked fourth in the sale of optically active pharmaceutical compounds in 1991; in 1995, sales reached $1.05 billion[8].

Although most of the naproxen currently on the market is made chemically, either by classical resolution of the corresponding racemic mixtures through selective crystallization of diastereoisomeric salts or by asymmetric synthesis, a number of biocatalytic approaches have been reported. For example, enantioselective hydrolysis of various esters of naproxen by a number of extracellular fungal lipases results in the production of the corresponding acids with enantiomeric excesses (e.e.'s) >98% (Refs 9–11). The rate of hydrolysis can be improved significantly without compromising enantioselectivity by utilizing activated esters. Enantiomerically enriched naproxen spontaneously precipitates from the aqueous reaction mixture and is easily recovered without the use of organic solvents.

(*S*)-Naproxen can also be produced in a continuous reactor via the hydrolysis of the corresponding racemic ethoxyethyl ester with *Candida cylindracea* lipase immobilized on Amberlite XAD-7. A 500 ml bioreactor operating continuously for 1,200 h at 35°C suffered only a 20% loss of activity and produced 1.8 kg of optically pure (*S*)-naproxen. It was suggested that the process can easily be scaled up for industrial application[12].

Lipase- and protease-catalyzed hydrolysis of esters has also been used for the resolution of other non-steroidal anti-inflammatory drugs, including ibuprofen (**1**), ketoprofen (**3**), flurbiprofen (**4**) and ketorolac (**5**), resulting in acid products in the (*S*)-configuration with varying degrees of enantiomeric purity[9,13,14]. Because an ester derivative of ketorolac is prone to *in situ* racemization under basic conditions, Sih *et al.* suggested performing the resolution at high pH (Ref. 9). Indeed, hydrolysis of the corresponding ethyl ester by *Streptomyces griseus* protease at pH 9.7 gave the desired (*S*)-ketorolac in 92% yield, which is significantly higher than the maximum yield in the conventional resolution process, which does not incorporate racemization[9].

Other lipase-catalyzed reactions

Captopril (**9**), the first orally active angiotensin-converting enzyme inhibitor, acts by preventing the conversion of angiotensin I to angiotensin II. This inhibition depends on the configuration of the mercaptoalkanoyl moiety, with the (*S*)-enantiomer being 100 times more potent than the corresponding (*R*)-enantiomer. Captopril is synthesized by coupling of 3-acylthio-(2*S*)-methylpropionyl chloride to L-proline, followed by deacylation[15], and therefore a lipase-catalyzed resolution of the corresponding methylpropionate is an appropriate strategy (Figure 2)[9,16]. While the hydrolysis of racemic methyl-3-acetylthio-2-methylpropionate (**6**) by numerous commercially available microbial lipases was not selective, hydrolysis by a cell-free extract of *Pseudomonas fluorescens* resulted in the formation of the corresponding (*S*)-acid in 49% yield and >97% e.e. (Ref. 16). Interestingly, *Aspergillus niger* lipase-catalyzed hydrolysis of sterically hindered (±)-methyl-3-benzoylthio-2-methylpropionate (**7**) also proceeded with a high degree of enantioselectivity (E >100) giving the corresponding (*S*)-acid with 98% e.e.

Another potentially useful lipase-catalyzed resolution was applied to the synthesis of taxol[17]. The total synthesis of taxol reported by Nicolaou *et al.* is based on achiral starting materials and utilizes a late-stage resolution to procuce a pure (–)-taxol enantiomer[18]. In order to improve yield, resolution of an earlier intermediate before coupling of the A-ring carbon framework has been suggested. Lipase

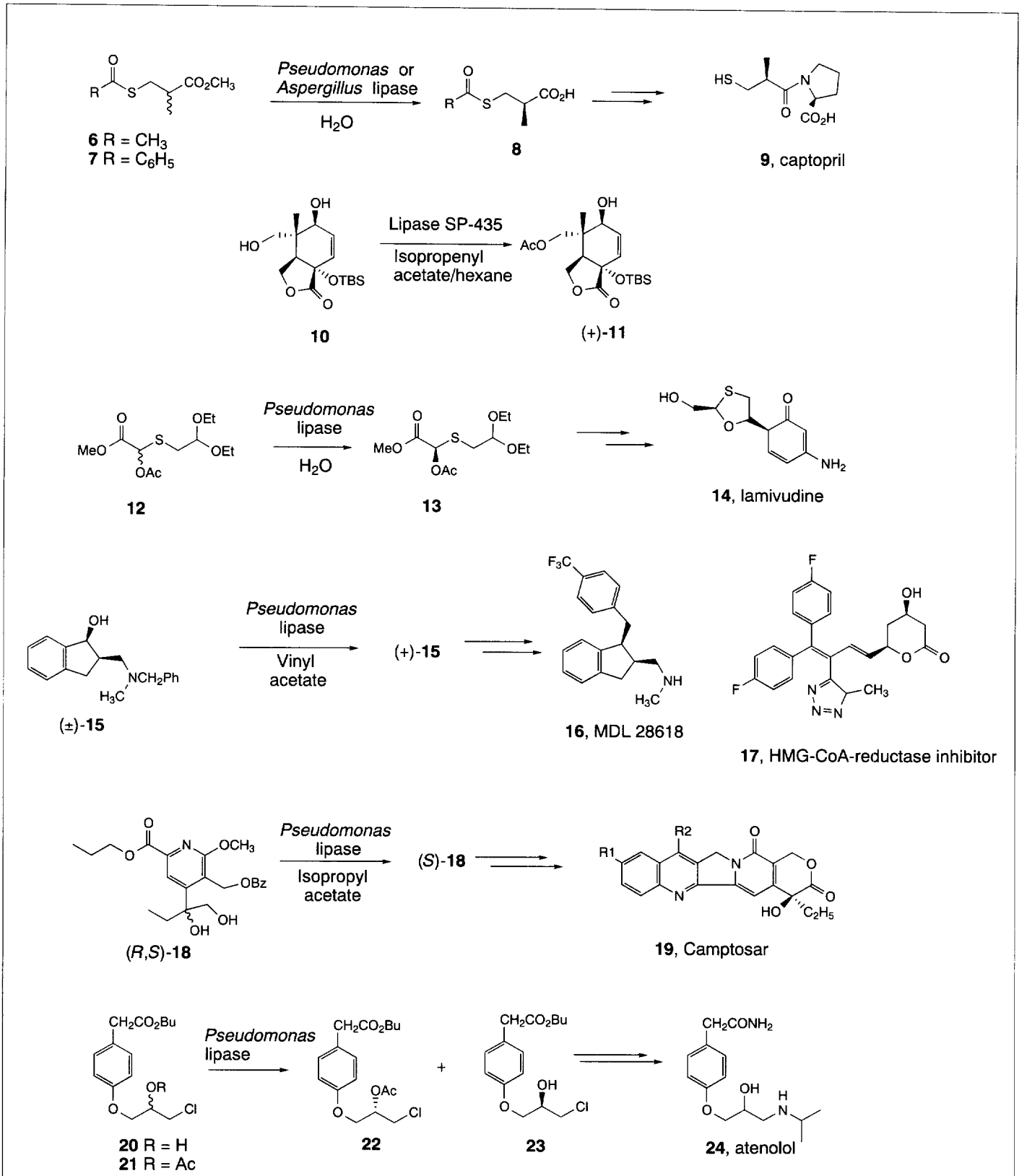

Figure 2. *Lipase-catalyzed resolution of enantiomers. HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.*

SP435-catalyzed acylation of **10** with isopropenyl acetate in hexane yielded (+)-**11** with 90% e.e. To increase the enantiomeric purity, the monoacetate was deacetylated with Hunig's base and the resulting alcohol was subjected to another transesterification with isopropenyl acetate. The procedure gives (+)-**11** in 47% yield and >99% e.e. (Figure 2).

A novel enzymatic resolution of α-acetoxysulfides has been developed as a key step in the synthesis of the important antiviral nucleoside analog lamivudin (**14**)[19]. As a result of the presence of two chiral centers sharing the same oxygen atom, the enantioselective synthesis of lamivudin is a formidable task. Resolution of **12** with a *Pseudomonas* sp. lipase was found to be highly enantioselective, giving the product **13** in 49% yield and >95% e.e. Concomitant cyclization followed by conventional nucleoside chemistry gave **14** in excellent yield.

In addition to hydrolysis, enzyme-catalyzed acylations in nonaqueous media have also proved to be a useful technique for resolving racemic compounds. Among numerous hydrolases used for this purpose a *Pseudomonas* sp. lipase proved to be one of the most efficient and versatile catalysts. It was successfully applied to the preparation of the (+)-enantiomer of the new serotonin uptake inhibitor **16**, which is at least 10-fold more active in preventing serotonin uptake than the (–)-enantiomer. The alcohol (+)-**15** was obtained in 46% yield and 98% e.e. via the lipase-catalyzed transesterification of its racemate with vinyl acetate in *tert*-butylmethyl ether (t-MBE) (Ref. 20).

Pseudomonas sp. lipase was also instrumental for the resolution of HMG CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitor, (±)-**17**, via acetylation of the undesired enantiomer. The resolution was scaled up to 640 liters with a substrate concentration of 4 g/l. The remaining enantiomer (**17**) was obtained in 48% yield and 98% e.e. (Ref. 21). The lipase was immobilized on Accurel polypropylene and reused five times without loss of activity. A *Pseudomonas* sp. lipase immobilized on Celite was used by Upjohn for the resolution of an intermediate (**18**) in the synthesis of the anticancer drug Camptosar (**19**). The acetylation with isopropyl acetate in t-BME gave the product in 46% yield and 93% e.e. (Ref. 22).

β-Blockers

β-Adrenoceptor antagonists used for the treatment of hypertension, angina and arrhythmia have a typical aryl(oxy)-propanolamine structure with one chiral center. Although β-blocking activity resides in the (*S*)-enantiomer, only three agents, (*S*)-timolol, (*S*)-penbutolol, and (*S*)-levobunolol, are marketed as single isomers. Numerous biocatalytic routes for the production of β-blockers have been suggested[23–33]. Most of them are based on the resolution of the corresponding racemates by enantioselective hydrolysis of esters, amides or oxazolidinones under aqueous conditions, or by acylation of alcohols under nonaqueous conditions. Yield and enantiomeric purity are usually good in most processes and vary between 40%–48% and 90%–96%, respectively. For example, in the synthesis of both enantiomers of atenolol (**24**) acylation of **20** with vinyl acetate in diisopropyl ether in the presence of *Pseudomonas cepacia* lipase stops at 50% conversion and results in the corresponding (*S*)-acetate (**22**) (e.e. >95%) and (*R*)-alcohol (**23**) (e.e. >95%)[34]. Alternatively, transesterification of **21** with butanol gives (*R*)-acetate (e.e. >95%) and (*S*)-alcohol. Both the alcohol and the acetate are then easily converted to enantiomerically pure (*S*)- and (*R*)-atenolol by treatment with aqueous isopropylamine followed by ammonium hydroxide[34].

Although there are fewer examples of enantioselective hydrolysis of lactams than there are of esters, Taylor *et al.* have reported an unusual and potentially useful hydrolysis of the racemic bicyclic lactam **25** used for the synthesis of the antiviral carbocyclic nucleoside carbovir (**27**) (Figure 3)[35]. By using a hydrolase from a *Rhodococcus* sp., the unreacted enantiomer of the lactam, (–)-**25**, and the amino acid product (**26**) have both been obtained with >98% enantiomeric purity.

Prochiral intermediates

The efficiency of all the enzyme-catalyzed kinetic resolutions described above is based on differences in the reactivities of two enantiomers. At the end of an ideal resolution the

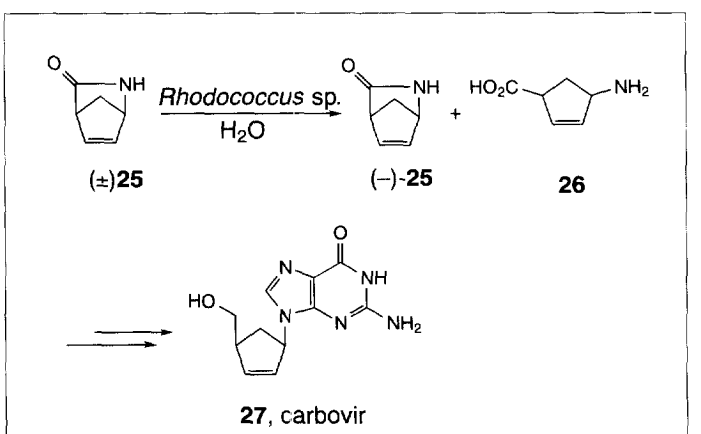

Figure 3. *Enzymatic hydrolysis of lactam.*

faster-reacting enantiomer is transformed, and the slower-reacting enantiomer is left behind unchanged. Thus, the maximum yield of a kinetic resolution cannot exceed 50%. The situation is different with prochiral or *meso* compounds. Since these molecules have a center or plane of reflective symmetry the chirality arises only as a result of the transformation. Hence, at least theoretically, these compounds can be converted to one enantiomer in 100% yield.

Hydrolytic enzymes such as esterases and lipases have proved particularly useful for the transformation of prochiral compounds because of their ability to discriminate between chemically identical, enantiotopic ester or hydroxyl groups. This characteristic was successfully utilized for the synthesis of a series of calcium channel blockers (Figure 4). This group of 4-aryl-1,4-dihydropyridine compounds (**28–30**) is widely used in the treatment of cerebrocirculatory disorders and hypertension. In order to separate the enantiomers, which have distinct biological activities[36,37], Hirose *et al.* carried out lipase-catalyzed hydrolysis of a series of prochiral 1,4-dihydropyridine esters in various solvents saturated with water[38]. They found that a *Pseudomonas* sp. lipase catalyzes the hydrolysis of a symmetrical nitrendipine derivative of **29** in water-saturated diisopropyl ether with a high degree of enantioselectivity, giving the (*S*)-monoester (e.e. >99%) in 87% yield. Remarkably, hydrolysis in water-saturated cyclohexane resulted in a complete reversal of enantioselectivity, giving the (*R*)-monoester in 88% yield and 89% e.e. Reversal of enantioselectivity was also achieved during the hydrolysis of a similar diester by a mutant *Pseudomonas* sp. lipase that bore three amino acid substitutions in the active site[39].

The prochiral selectivity of α-chymotrypsin was employed for the synthesis of both enantiomers of a popular antispastic drug baclofen (**33**) currently sold as a racemate (Figure 5). Because the pharmacological and toxicological properties of the enantiomers differ significantly[40], the chemoenzymatic synthesis of both enantiomers was undertaken. The enantioselective hydrolysis of the prochiral dimethyl 3-(*p*-chlorophenyl)glutarate (**31**) by chymotrypsin afforded the chiral half-ester in 85% chemical yield and 98% e.e. (Ref. 41). Similar hydrolysis of the corresponding diethyl ester by chymotrypsin produced the (*R*)-monoester in 92% yield and 97% e.e., which was later used as an auxiliary for the asymmetric synthesis of the cholesterol absorption inhibitor (+)-SCH54016 (Ref. 42).

An impressive illustration of the prochiral selectivity of lipases was observed during the synthesis of both enantiomers of a leukotriene D_4 antagonist (**34**)[43,44]. Although it is generally believed that selectivity of hydrolytic enzymes strongly depends on the proximity of the chiral center to the reacting carbonyl group, lipases from *Pseudomonas* and *Chromobacterium* sp. selectively hydrolyzed the dimethyl

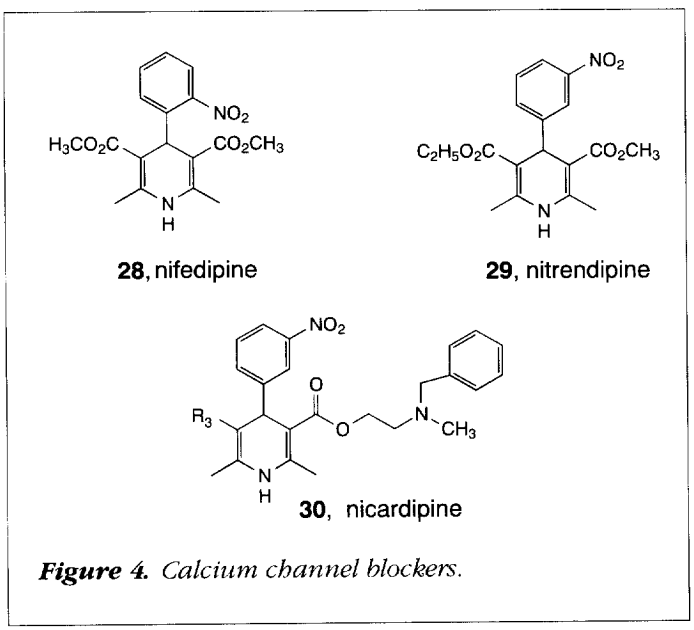

Figure 4. *Calcium channel blockers.*

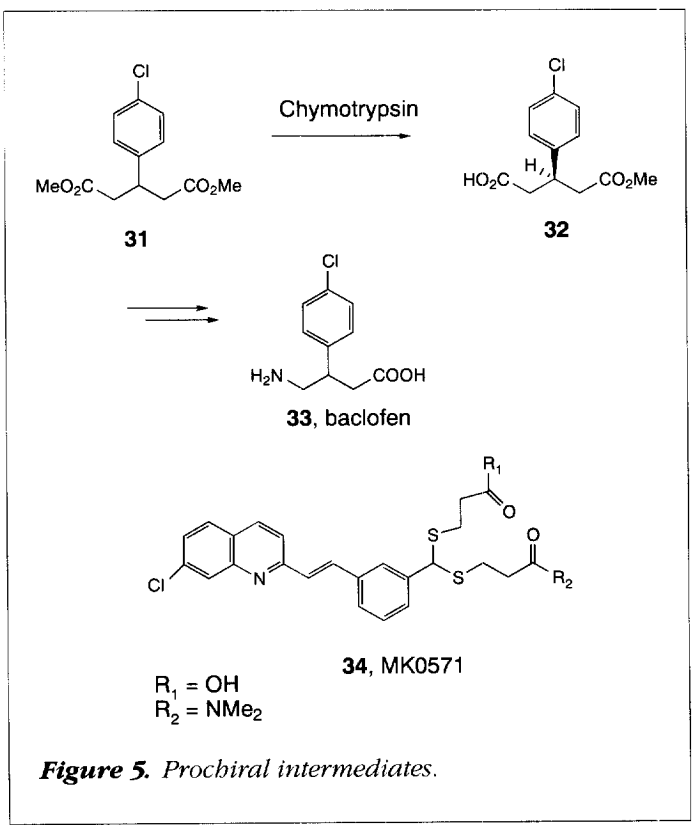

Figure 5. *Prochiral intermediates.*

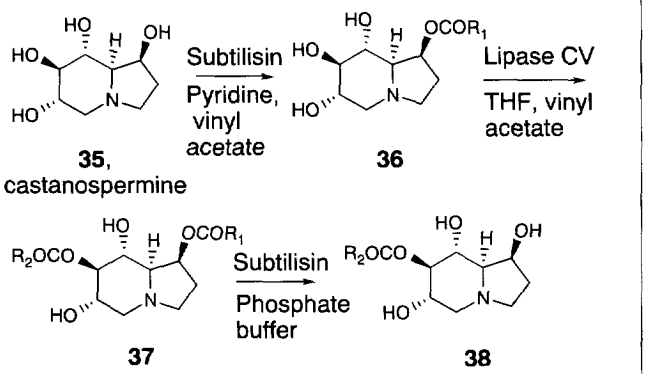

Figure 6. *Regioselective acylations. THF, tetrahydrofuran.*

ester derivative of **34** (R_1 = R_2 = OMe) which has a prochiral center four bonds away from the reactive carboxylate. Hydrolysis by *Chromobacterium* lipase gave the (*R*)-monoester in 95% yield and >98% e.e.

Regioselective acylations

The regioselectivity of enzymes has been widely utilized for selective protection and deprotection of a variety of polyfunctional molecules, including carbohydrates, steroids and nucleosides. It was also successfully applied to the chemoenzymatic synthesis of derivatives of castanospermine (**35**) (Ref. 45), a plant alkaloid that inhibits α-glucosidase I (Figure 6). It has been reported that certain *O*-acyl derivatives of **35** are up to 20 times more active than castanospermine itself in inhibiting HIV replication[46]. Because castanospermine contains four secondary hydroxyl groups with similar chemical reactivity, an enzymatic approach based on several hydrolytic enzymes has been developed as an alternative to a laborious chemical synthesis. Subtilisin-catalyzed acylation of castanospermine with vinyl acetate in pyridine was highly regioselective, providing 1-*O*-acylcastanospermine (**36**) in 91% yield. Subsequent acylation catalyzed by *Chromobacterium viscosum* lipase in tetrahydrofuran gave the diester (**37**) in 72% yield. The 7-*O*-acyl derivative (**38**) was then obtained in good yield by the subtilisin-catalyzed hydrolysis of the diester (**37**).

Synthesis of carbohydrates

The basic role of carbohydrates in a variety of biological recognition phenomena has raised an interest in carbohydrate-based pharmaceuticals. Indeed, carbohydrates act as binding sites for a wide range of bacteria, viruses, hormones and toxins. Cell-surface carbohydrates control cell adhesion and are involved in intracellular communication, governing cell growth and differentiation. In spite of their great potential, the development of carbohydrate-based pharmaceuticals has been slower than expected, partly because of their complicated chemical synthesis. Recent advances in chemoenzymatic synthesis of carbohydrates, in particular the application of aldolases and transferases, have simplified the synthetic methodology and allowed the economic synthesis of carbohydrate-based pharmaceuticals.

This progress is particularly evident in the synthesis of azasugars, which are typical inhibitors of glycoprotein synthesis and secretion (Figure 7)[47]. Two potent glycoside inhibitors, deoxynojirimycin (**39**) and deoxymannojirimycin (**40**), were readily prepared in three steps with the help of rabbit muscle aldolase[48]. Qualitatively similar results were obtained with the recombinant aldolase cloned in *Escherichia coli* – a superior enzyme that had 30-fold higher operational stability than the enzyme isolated from rabbit[49].

Synthesis of *N*-acetylneuraminic acid (NeuAc, **42**) and its derivatives is another example of the highly successful use of the aldolase-based technology for the synthesis of biologically active compounds[50–52]. NeuAc aldolase catalyzes the reversible condensation of pyruvate with D-*N*-acetylmannosamine (**41**). Although the equilibrium in this reaction

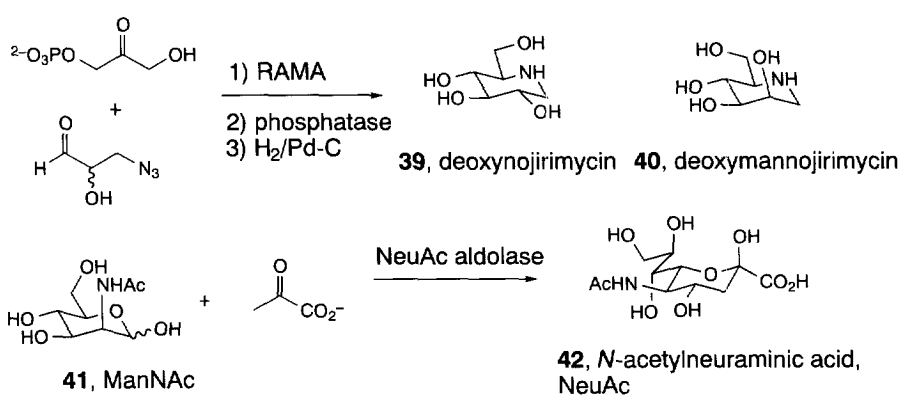

Figure 7. *Aldolase-catalyzed synthesis of carbohydrates. RAMA, rabbit muscle aldolase; NeuAc, N-acetylneuraminic acid.*

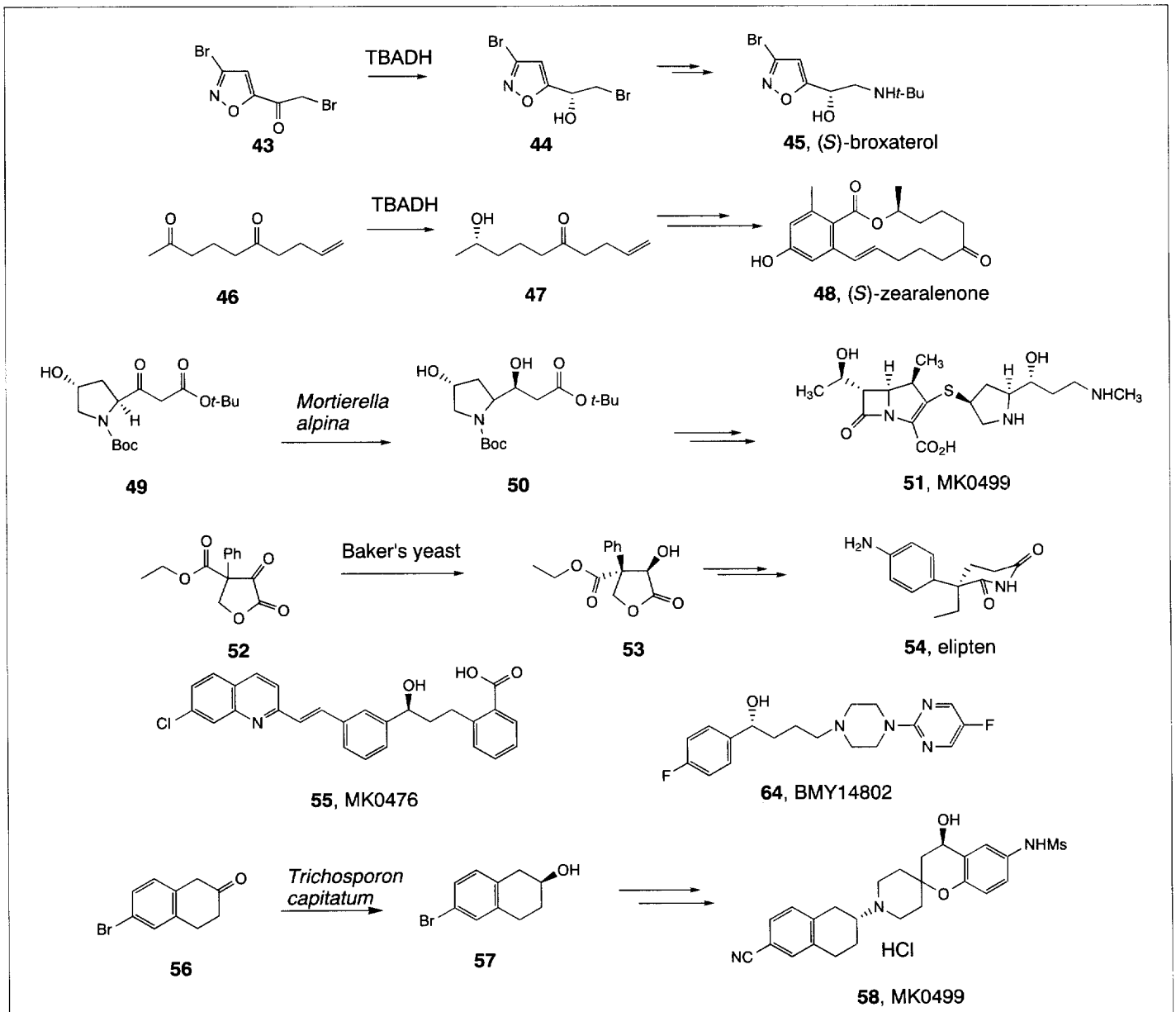

Figure 8. *Enzymatic and microbial reductions. TBADH, thermostable alcohol dehydrogenase from* Thermoanaerobium brokii.

is shifted towards the substrates, up to 90% yield of NeuAc can be achieved in the presence of 7 molar equivalents of pyruvate. To eliminate the need for excess pyruvate and simplify the isolation of products, the NeuAc synthesis can be coupled to a thermodynamically favorable process[53]. Although thus far pyruvate has been found to be the only donor, NeuAc aldolase takes a wide variety of acceptors, allowing the synthesis of many sialic acid derivatives[54]. NeuAc aldolase has been cloned, overexpressed[55] and is available commercially.

Enantioselective reductions

The vast majority of enantioselective reductions of ketones for the synthesis of chiral alcohols is carried out by intact microorganisms[56–58]. These biotransformations are easy to carry out as they do not require an external cofactor regeneration system and provide alcohols with excellent enantiomeric purity. However, utilization of whole-cell systems has some drawbacks, including competing reactions, mass transfer limitations and difficult downstream processing. These problems can be alleviated by the use of isolated

enzymes. The thermostable alcohol dehydrogenase from *Thermoanaerobium brokii* (TBADH) is, perhaps, the most popular enzyme used for enantioselective reductions of ketones. It has found utility in the synthesis of many biologically active compounds, including broxaterol (**45**), which is a potent and selective β_2-adrenergic stimulant (Figure 8)[59]. TBADH reduces isoxazole **43** to the corresponding (*S*)-alcohol with e.e. >98%. The alcohol is then converted chemically to the (*S*)-enantiomer of broxaterol, which is at least 100-fold more potent than the (*R*)-enantiomer. The use of TBADH was also instrumental in the synthesis of (*S*)-zearalenone (**48**)[60]. This mycotoxin, originally isolated from a fungus, exhibits anabolic, estrogenic and antibacterial activity. By taking advantage of the high regio- and enantioselectivity of the dehydrogenase, the diketone **46** was reduced to the corresponding keto alcohol **47** in 81% yield and >99% e.e. It was then converted by conventional chemistry to (*S*)-zearalenone with an optical purity of >99.5%.

A number of chiral reductions of pharmaceutical intermediates carried out by intact cells have been reported recently (Figures 8 and 9). For example, the fungus *Mortierella alpina* was used for the reduction of β-ketoester **49** in the synthesis of a side chain of a new broad spectrum β-methylcarbapenem antibiotic (**51**)[61]. The fermentation was scaled-up in a 23-liter bioreactor, allowing the production of gram quantities of the hydroxyester **50** with a diastereomeric excess (d.e.) >98%. Baker's yeast was successfully used for the reduction of ketolactone **52** to optically pure hydroxylactone **53**, an intermediate in the synthesis of the (*R*)-enantiomer of the antimetastatic drug aminoglutethimide (elipten, **54**)[62]. Following an extensive screening program, a *Microbacterium* sp. was identified as a suitable biocatalyst for the asymmetric reduction of the keto ester precursor of MK0476 (**55**), a potent cysteinyl leukotriene I receptor antagonist that is undergoing clinical studies for the treatment of asthma[63]. Following media optimization, preparative quantities of the (*S*)-hydroxy ester (**55**) with e.e. >95% were obtained. The yeast *Trichosporon capitatum* was successfully used for the asymmetric reduction of 6-bromo-β-tetralone (**56**) to the corresponding (*S*)-alcohol (**57**), which served as a precursor of the potent potassium channel blocker MK0499 (**58**)[64].

Another preparative-scale transformation process developed by Bristol-Myers Squibb utilizes *Nocardia salmonicolor* for the enantioselective reduction of ketone **61**. The corresponding alcohol (**62**) is a key intermediate in the total chemical synthesis of the potent calcium channel blocking agent SQ31765 (**63**). Since **61** exists predominantly in the achiral enol form **60**, which is in a rapid equilibrium with the two keto form enantiomers **59** and **61**, reduction of the ketone could give rise to four possible alcohol stereoisomers. Remarkably, the transformation catalyzed by the crude cell extracts gave only the *cis*-enantiomer **62** in 96% yield and >99.9% optical purity[65]. Finally, a single-stage fermentation process (fermentation–biotransformation) and a two-stage one (fermentation and subsequent biotransformation) were developed for the stereoselective reduction of the ketone derivative of **64** (Figure 8) by *Mortierella ramanniana*. The alcohol (**64**) is an effective antipsychotic drug under development at Bristol-Myers Squibb[66]. In both transformations a yield of up to 98% and e.e. of 99.4% were obtained.

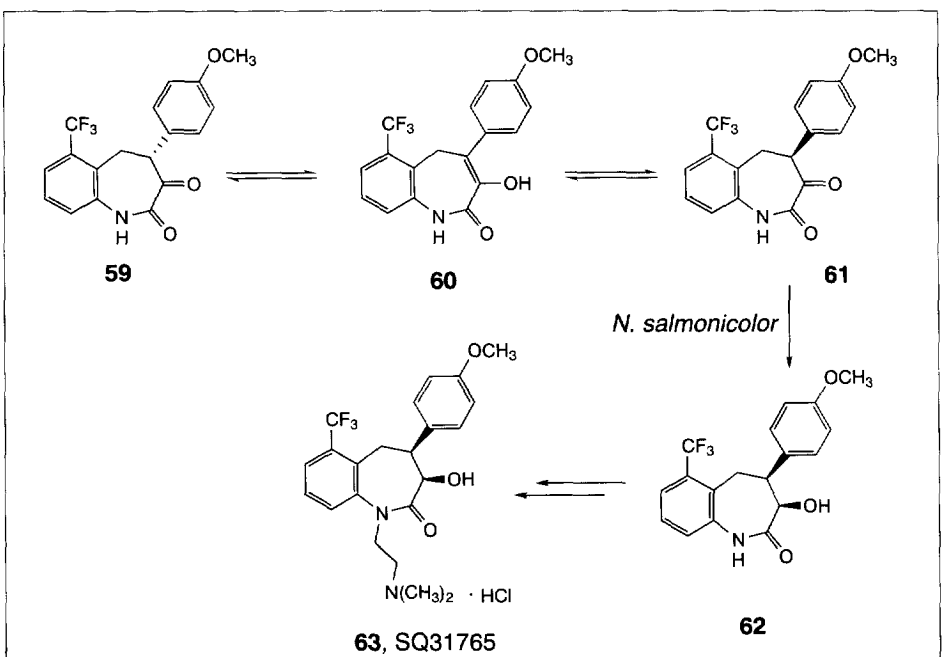

Figure 9. *Highly enantioselective reduction of ketone under racemization conditions.*

Enzymatic oxidations

Xanthine oxidase, an enzyme isolated from bacteria and cow's milk, catalyzes regioselective oxidations of numerous azaheterocycles. The enzyme's synthetic utility, however, was hampered by low

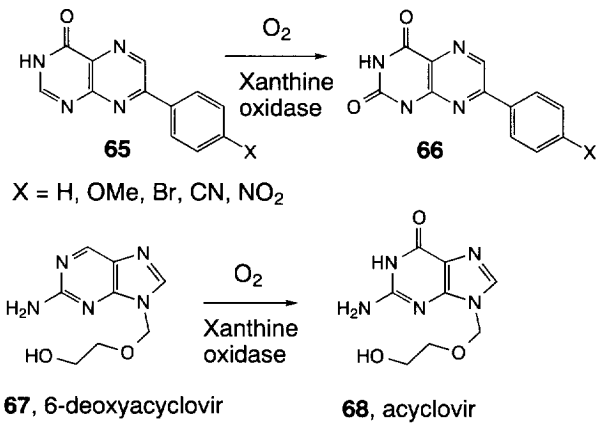

Figure 10. *Regioselective oxidation of azaheterocycles by xanthine oxidase.*

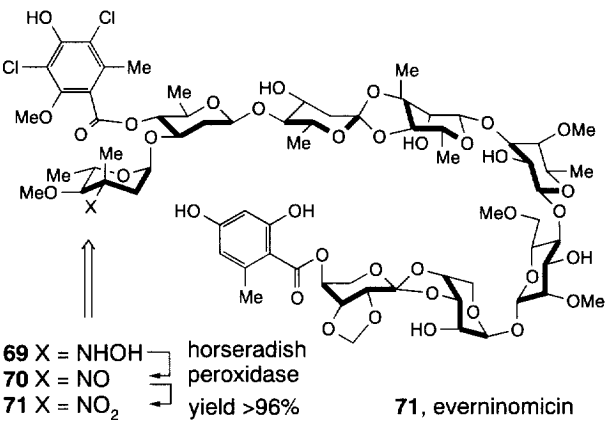

Figure 11. *Horseradish peroxidase catalyzed oxidation of everninomicin.*

stability under operating conditions. The development of a new gelatine-based immobilization technique helped to overcome this problem[67]. As a result, the oxidation of a number of 7-alkylpteridine-4-ones (**65**) with immobilized xanthine oxidase resulted in a series of biologically active lumazine derivatives (**66**) in about 90% yield (Figure 10)[68]. Similarly, 6-deoxyacyclovir (**67**) was efficiently oxidized to acyclovir (**68**), an acyclic nucleoside analog widely used as an antiherpetic agent[69].

Peroxidase, a protoporphyrin-containing enzyme, has also found utility in the synthesis of the antibiotic everninomicin (**71**). Everninomicin, a broad spectrum orthosomycin antibiotic, is produced by fermentation of *Micromonospora carbonaceae* and is excreted into the medium as a mixture of hydroxylamino-everninomicin (**69**) and nitroso-everninomicin (**70**) derivatives (Figure 11). They are then oxidized to a final product nitro-everninomicin (**71**) with *tert*-butylhydroperoxide in the presence of a vanadium catalyst. Interestingly, the same oxidation can be catalyzed by horseradish peroxidase in a process which employs environmentally friendlier hydrogen peroxide and does not require a metal catalyst[70]. The conversion of the hydroxylamino-everninomicin to nitro-everninomicin is quantitative and proceeds via the nitroso-everninomicin intermediate.

Synthesis of polyketides

Polyketides are a family of pharmaceutically important natural products that include antibiotics, anticancer agents and immunosuppressants. They are synthesized by multifunctional enzymes, polyketide synthases (PKSs), which catalyze a series of regio- and stereospecific condensations, reductions

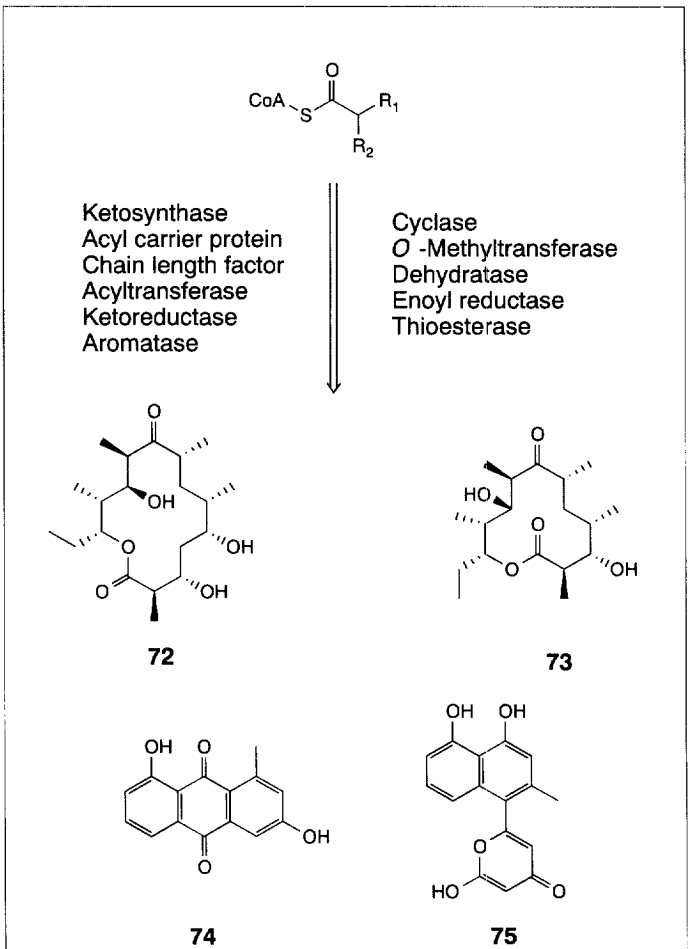

Figure 12. *Enzymatic synthesis of polyketides.*

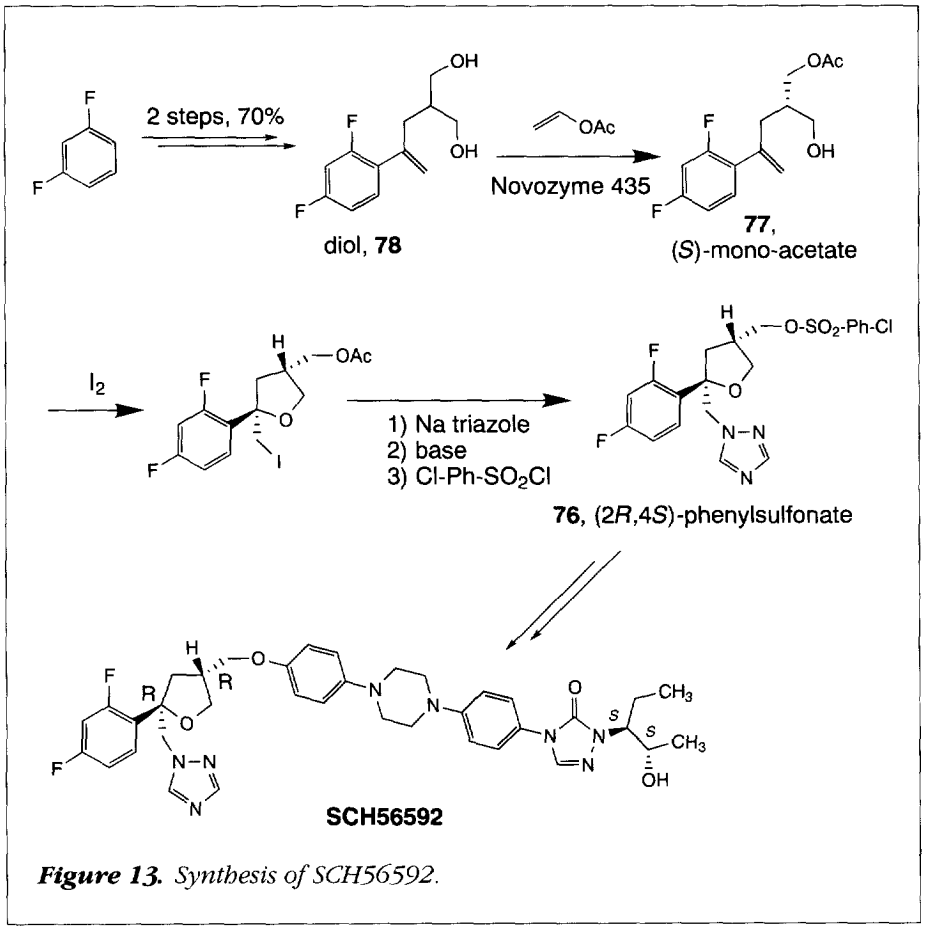

Figure 13. *Synthesis of SCH56592.*

and cyclizations (Figure 12)[71]. Carbon chains are built by successive decarboxylative condensation between coenzyme A thioesters of various organic acids. The enormous structural diversity of polyketides stems from a combination of variables including nature of structural units, number of condensations, extent of reduction, regiospecificity of cyclizations and others. Some PKSs are assemblies of large multifunctional proteins with a distinct active site for every catalyzed step. This modular organization, which allows separate units to operate successively and independently of one another, has provided the basis for the design of libraries of novel polyketides via combinatorial biosynthesis[72,73]. A *Streptomyces* host–vector system that has been developed recently allows the expression and mutagenesis of virtually any PKS gene clusters[74]. Structures **72–75** are just a few of numerous novel polyketides produced by engineered strains.

Large-scale synthesis

SCH56592

Schering-Plough's compound SCH56592 is an azole antifungal with enhanced activity against systemic *Candida* and pulmonary *Aspergillus* infections; it is now in Phase II clinical trials[75]. The increased activity of SCH56592 relative to other azole antifungals results from the tetrahydrofuran ring which replaces the 1,3-dioxolane ring present in other azole drugs[76]. The synthetic route to the final drug proceeds through a key intermediate, the (2*R*,4*S*)-phenylsulfonate **76** (Figure 13). The required stereochemistry at the 2- and 4-positions in the tetrahydrofuran ring of **76** is achieved via an iodocyclization reaction performed on the chiral monoester **77**, followed by displacement of the iodide by sodium triazolide[77,78]. In turn, **77** is synthesized by enzymatic acylation of the symmetric diol **78** under nonaqueous conditions. Using *Candida antarctica* lipase B (Novozyme 435) and vinyl acetate in acetonitrile, highly enantioselective acetylation of diol **78** occurs. By selectively blocking one of the two primary hydroxyl functions in diol **78**, the enzymatic reaction establishes the stereochemistry required to force the subsequent iodocyclization reaction to give a single product isomer.

LY300164

Another drug entering Phase II clinical trials is Eli Lilly's compound LY300164 (Ref. 79). An orally active benzodiazepine, this compound is being tested for efficacy in treating amylotropic lateral sclerosis. This compound possesses a single chiral center in the diazepine ring, and the (–)-isomer is the more active one[80,81]. The synthetic route to this compound was designed to avoid resolution of a racemate, and more efficiently introduce the desired stereochemistry in an asymmetric synthesis. It is accomplished via the stereoselective reduction of ketone **79** to chiral alcohol **80** as the first step in a seven-step synthesis. The yeast *Zygosaccharomyces rouxii* (ATCC 14462) was selected as the biocatalyst for this reduction (Figure 14).

Keeping the concentration of **79** below the observed toxic limit of 6 g/l (Ref. 77) without reducing volumetric productivity to impractically low levels is accomplished by introducing the substrate adsorbed on XAD-7 resin. A sufficient amount of resin is used to allow an effective substrate

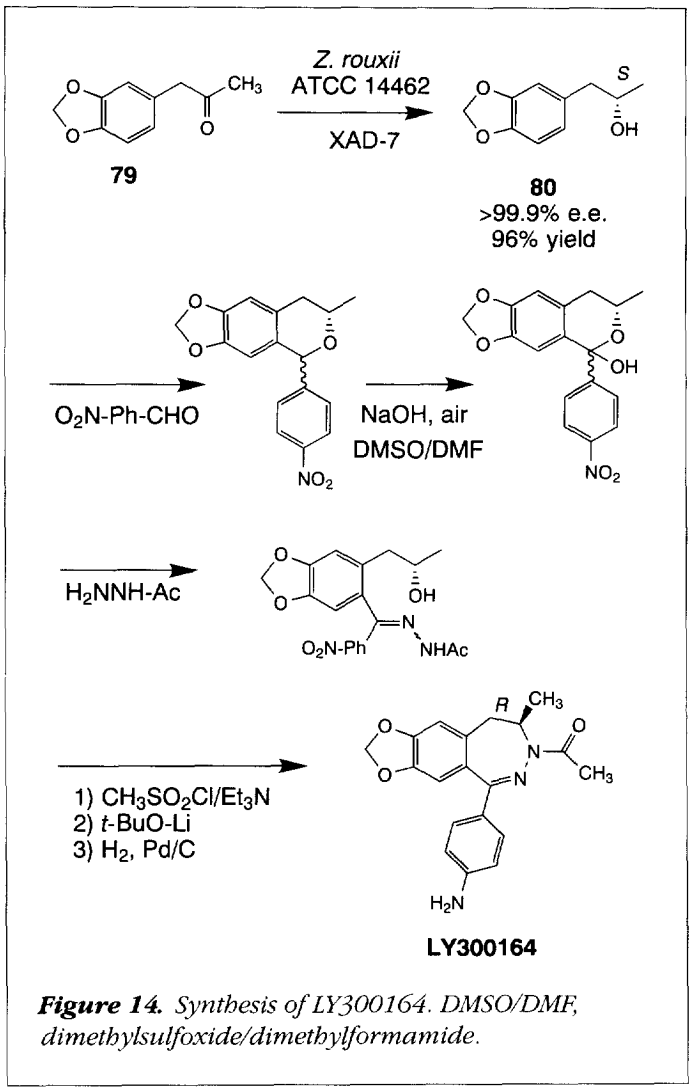

Figure 14. *Synthesis of LY300164. DMSO/DMF, dimethylsulfoxide/dimethylformamide.*

loading of 80 g/l of ketone **79** in broth. Desorption of the substrate ketone from the resin is limited to an equilibrium concentration of approximately 2 g/l in the aqueous phase, well below the level of toxicity. The resin also absorbs the product alcohol **80**, limiting its concentration below toxic levels as well. After ketone reduction is complete, the XAD-7 is separated from the *Zygosaccharomyces rouxii* cells, and the chiral alcohol product **80** is recovered by washing the resin with acetone. This procedure gives the product alcohol **80** in near optical purity (>99.9% e.e.) and in 96% yield. The stereochemical integrity of the chiral center in **80** is maintained over the additional six steps necessary to complete the synthesis of LY300164.

MK0507

A carbonic anhydrase inhibitor (CAI) for the treatment of glaucoma is marketed by Merck under the tradename Trusopt. This compound, previously designated MK0507, has sufficiently high water solubility that it can be applied directly to the eyes, thus minimizing the systemic side effects seen with orally administered CAIs (Ref. 82). MK0507 contains two chiral centers; the chirality at the 6-position of the dihydrothiopyran ring is directly introduced by using methyl-(*R*)-3-hydroxybutyrate as starting material (Figure 15). The chiral environment in this center was then used to induce chirality at the second center during $LiAlH_4$ reduction of the ketosulfide intermediate **81** (Ref. 83). However, the complete inversion of the *cis*-alcohol product to the desired *trans*-stereochemistry was not achieved.

The problem of incomplete epimerization was avoided by a biological reduction of the ketone function performed by Zeneca[84]. The ketosulfone **82** (Figure 16), which has greater water solubility than the ketosulfide intermediate, was chosen as the substrate for biological reduction. Microbial screening identified several microorganisms as possible candidates for reducing the ketone to the required (4*S*)-alcohol (**83**). However, the choice of the more water-soluble ketosulfone as substrate introduced complications not faced in the synthetic route of Merck. In aqueous media above pH 5, ketosulfone **82** undergoes epimerization via a ring-opened intermediate (Figure 16), which scrambles the stereochemistry of the (6*S*)-methyl group. Once the ketone has

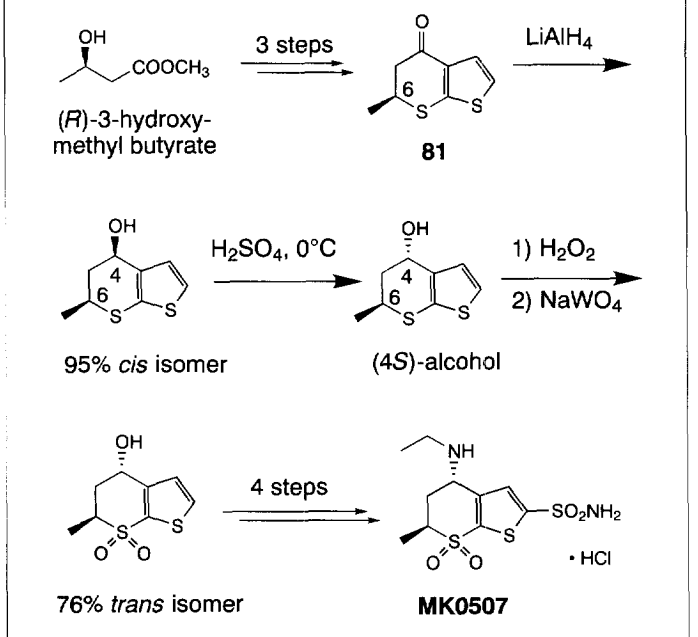

Figure 15. *Chemical synthesis of MK0507.*

been reduced, this epimerization is no longer possible. This problem was circumvented by choosing a microorganism that performs the reduction at a pH below 5, and adding the ketosulfone substrate slowly over the course of the reduction to keep its concentration as low as possible. The process developed at Zeneca uses the fungus *Neurospora crassa* (IMI 19419), grown in a medium the pH of which is adjusted to 4 before addition of the ketosulfone substrate. The substrate is added sufficiently slowly to keep its concentration in the fermentation broth below 200 mg/l (Refs 85,86). This process yields the desired (4*S*,6*S*)-hydroxysulfone in over 80% yield, and in 99.8% diastereomeric purity. The (6*R*)-methyl epimers are not detectable, and only 0.2% of the (4*R*)-alcohol epimer is present in the final product[87].

It should be noted that the chirality of the (6*S*)-methyl group is also derived from a biological source. A homopolymer of (*R*)-3-hydroxybutyrate is produced by Zeneca via fermentation for use in the manufacture of biodegradable plastics. The polymer is heated with acidic methanol to give methyl-(*R*)-3-hydroxybutyrate with >99.5% e.e. (Ref. 84). The Zeneca process to the (4*S*,6*S*)-hydroxysulfone (**83**) is currently practiced on a multi-ton scale (Figure 16).

Diltiazem

A recent notable success in the application of biocatalysis in the production of marketed pharmaceutical products is diltiazem, a substituted benzothiazepine. Diltiazem is a calcium channel blocker and coronary vasodilator indicated for some forms of angina, and is currently produced by Tanabe in Japan and DSM Andeno in the Netherlands; current combined production is over 100 tons annually. It has been known for some time that the desired pharmacological effect is attributed to only one of the enantiomers of diltiazem[88]; thus a chiral synthesis of the drug is required.

In an earlier process patented by Tanabe, the entire synthesis of diltiazem was completed on racemic material, and the final product resolved by diastereomeric crystallization with *l*-10-camphorsulfonic acid[89]. The first step in the current processes practised by Tanabe and DSM Andeno is the resolution of the racemic starting material *trans*-4-methoxyphenylglycidic acid methyl ester (**84**). This is accomplished via enzymatic hydrolysis of the undesired enantiomer by *Candida cyclindracea* lipase[90,91] (Figure 17). The remaining ester has the desired (2*S*,3*R*)-stereochemistry necessary for the synthesis of diltiazem. The free phenylglycidic acid produced in this hydrolysis is unstable, and decarboxylates spontaneously to give CO_2 and 4-methoxyphenyl acetaldehyde. This aldehyde rapidly reduces the activity of the enzyme, and forms an insoluble material which complicates downstream processing. These problems are overcome by running the enzymatic hydrolysis in an aqueous phase containing sodium bisulfite. The phenylacetaldehyde by-product forms the bisulfite adduct **86** which can be removed by filtration. This prevents both the build-up of undesirable solid material and damage to the enzyme. Bisulfite also serves as the buffer salt to maintain the desired pH for the enzymatic reaction[92,93]. The resolved (2*R*,3*S*)-4-methoxyphenylmethyl glycidate (**85**) is

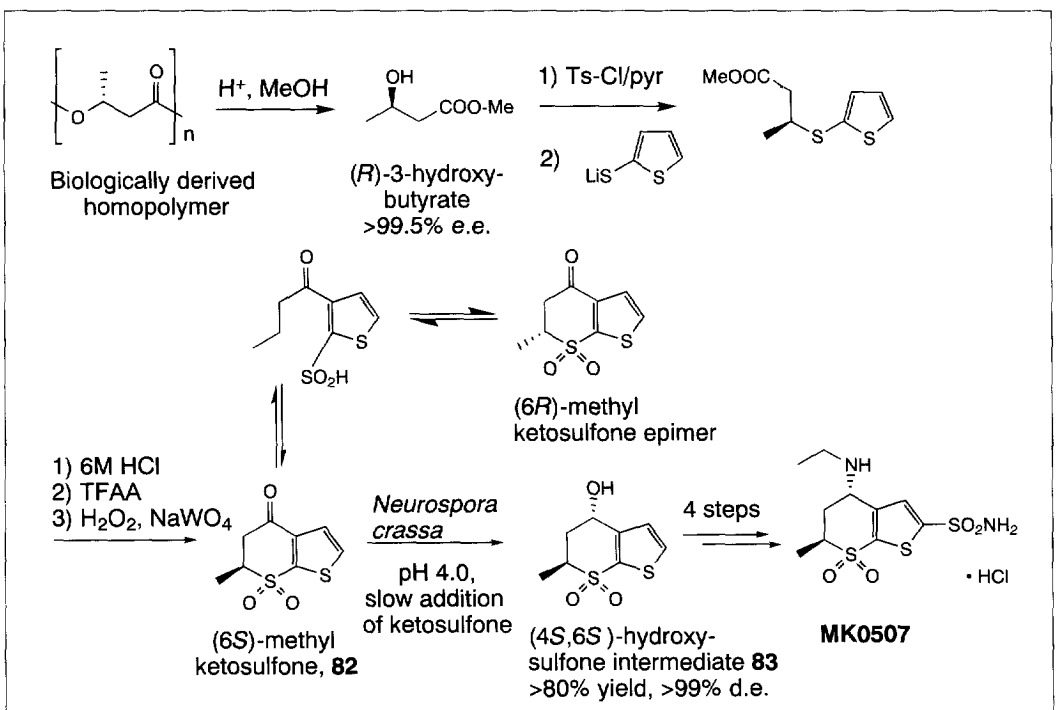

Figure 16. *Chemoenzymatic synthesis of MK0507. TFAA, trifluoroacetic anhydride.*

taken on to diltiazem via condensation with 2-aminothiophenol, followed by closure of the thiazepine ring, acetylation of the hydroxyl function and alkylation of the ring nitrogen (Figure 17).

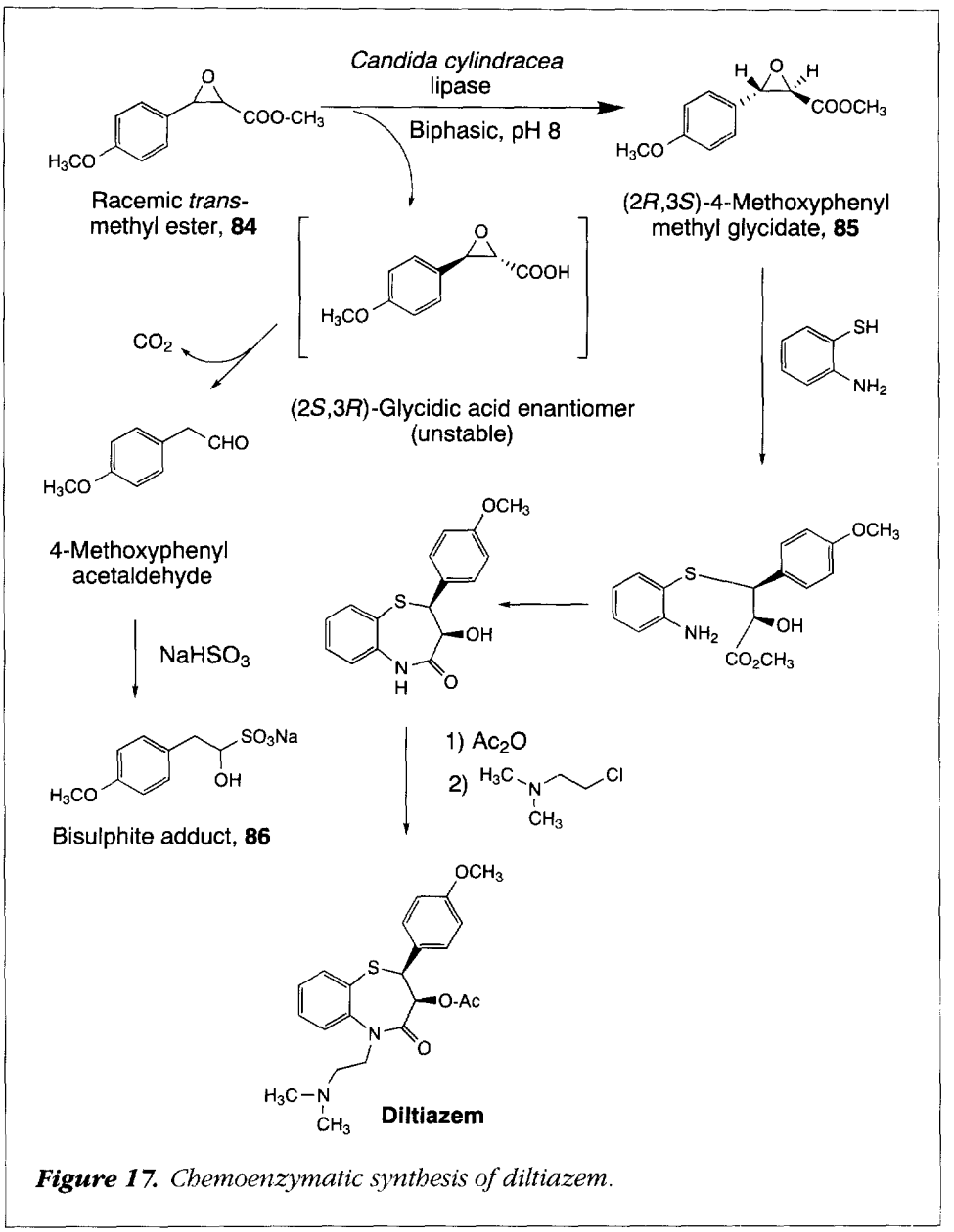

Figure 17. *Chemoenzymatic synthesis of diltiazem.*

Paclitaxel

Paclitaxel is currently marketed by Bristol-Myers Squibb under the tradename Taxol, and is indicated for the treatment of various cancers. The compound was initially isolated from the bark of the Pacific yew, in which it is present at very low levels. The tetracyclic diterpene nucleus of paclitaxel can be easily produced from 10-deacetylbaccatin III, available from the leaves of the European yew where it is present at a level of about 0.1% (Refs 94,95). In a semisynthetic approach the β-amido ester side chain, *N*-benzoyl-(2*R*,3*S*)-3-phenylisoserine, is synthesized following the lipase-catalyzed resolution of a racemic azetidinone precursor (**87**) (Figure 18). Work at Bristol-Myers Squibb demonstrated that lipase PS-30 (from *Pseudomonas cepacia*; Amano International) and a lipase isolated from *Pseudomonas* sp. SC13856 both hydrolyze the 3-acetate group of racemic *cis*-3-acetyloxy-4-phenyl-2-azetidinone (**87**) in a highly enantioselective manner, to leave the (3*R*)-acetate enantiomer. The enzyme is immobilized by adsorption to polypropylene beads, and reportedly can be reused under hydrolytic conditions up to 10 times without loss of activity[96,97]. Resolution of the azetidinone acetate and the subsequent chemistry required for coupling to the 10-deacetylbaccatin III moiety is shown in Figure 18 (Refs 98,99).

Glipizide

Glipizide is a sulfonylurea compound with hypoglycemic activity, used in the control of diabetes, and is now available as a generic drug. Lonza practices a fermentation process that supplies a precursor of glipizide for the European market[100–102]. In this process, the starting material, 2,5-dimethylpyrazine (2,5-DMP), is transformed via fermentation by *Pseudomonas putida* (ATCC 33015), which oxidizes only one of the symmetric methyl groups to a carboxylic acid. An inducer, such as xylene, is required in order to get the cells to express the desired enzymatic activity. The culture can be first grown in the presence of xylene, to which the 2,5-DMP substrate is later added, or as a continuously fed process, to which a 20–30% solution of 2,5-DMP in xylene is continuously added. In both cases, conversion of 2,5-DMP to the 2,5-dimethylpyrazine carboxylate occurs in 90% yield (Figure 19).

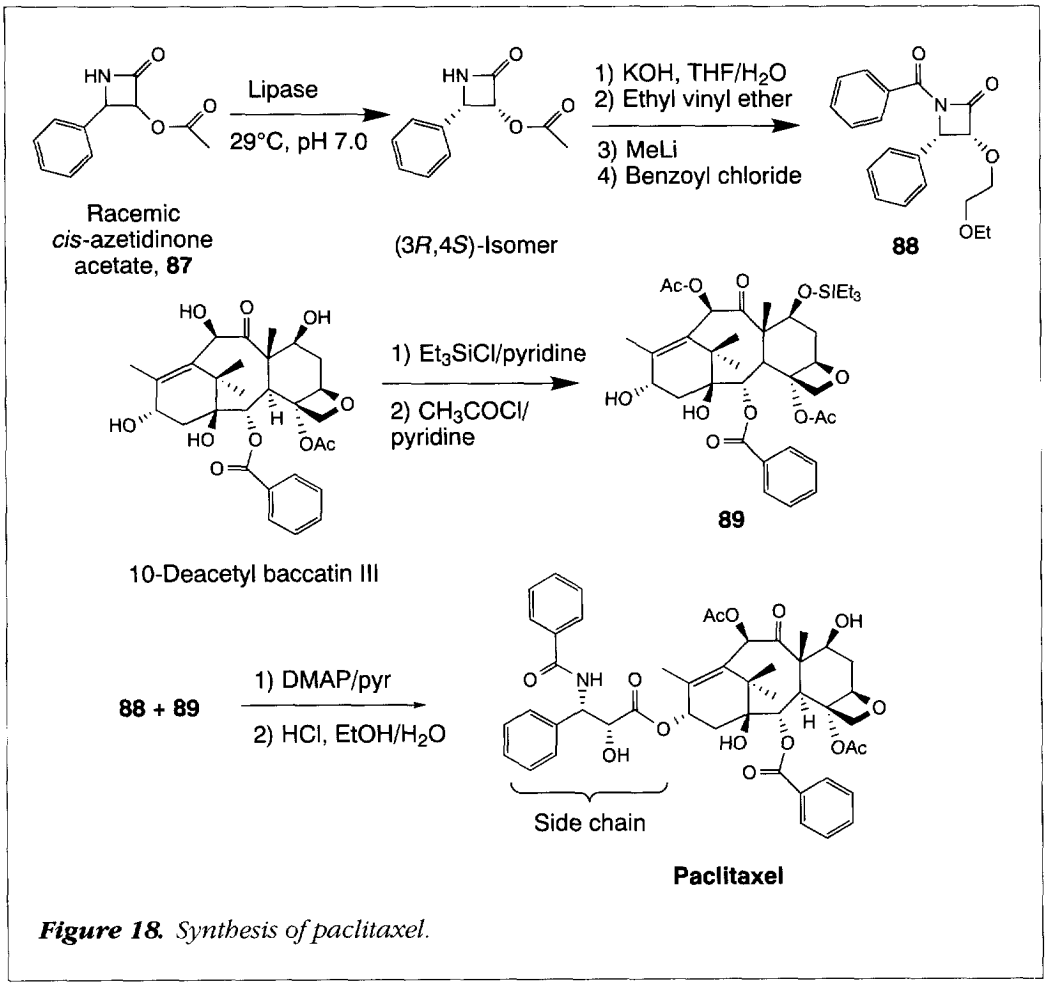

Figure 18. *Synthesis of paclitaxel.*

The isolation, crystallization, and characteristics of this enzyme have been reported[104]. Ajinomoto reports that L-DOPA is manufactured by mixing catechol and ammonium pyruvate in a fermentor with *Erwinia herbicola* (ATCC 21433) at a stationary phase[105,106]. In a variation of this process, the cells may be harvested and resuspended in buffer to which the catechol and ammonium pyruvate are then added[107].

L-Ephedrine and pseudoephedrine

These two related compounds are manufactured from a common starting material produced via a yeast-catalyzed condensation of pyruvate and benzaldehyde (Figure 21). This condensation has been practiced for over 60 years by Knoll[108,109] (now a division of BASF) to produce L-ephedrine, used in the treatment of asthma. In this process, formation of the key intermediate, L-phenylacetylcarbinol (L-PAC), is catalyzed by the pyruvate decarboxylase complex (EC 4.1.1.1). This activity has been reported generally in yeast, particularly in *Saccharomyces cerevisiae* and *Candida utilis*.

L-DOPA

The anti-Parkinson's drug 3,4-dihydroxy-L-phenylalanine (L-DOPA) is reported to be manufactured in Japan by Ajinomoto[103] using the enzyme tyrosine–phenol lyase (β-tyrosinase, EC 4.1.99.2) from *Erwinia herbicola* (Figure 20).

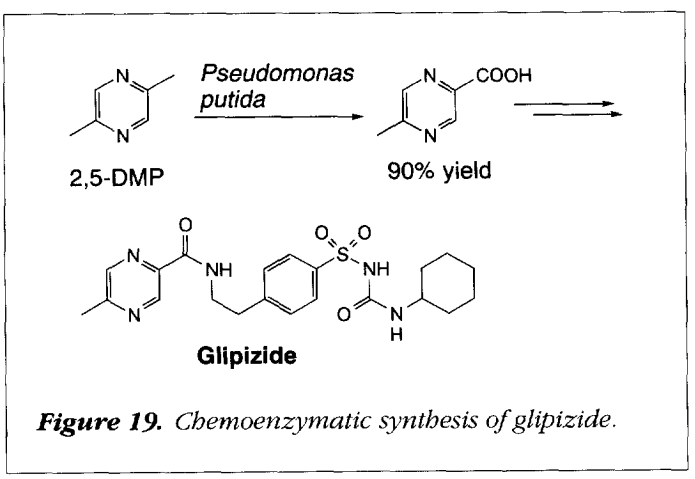

Figure 19. *Chemoenzymatic synthesis of glipizide.*

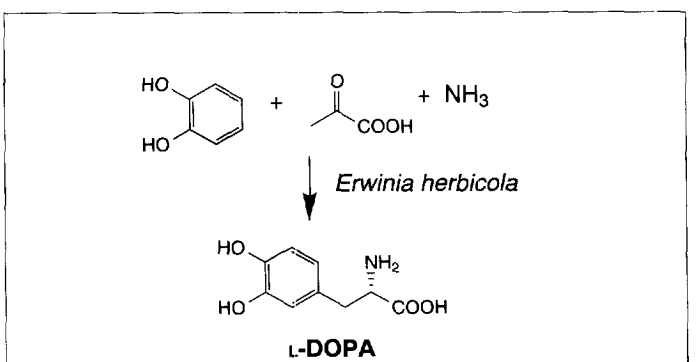

Figure 20. *Enzymatic synthesis of L-DOPA.*

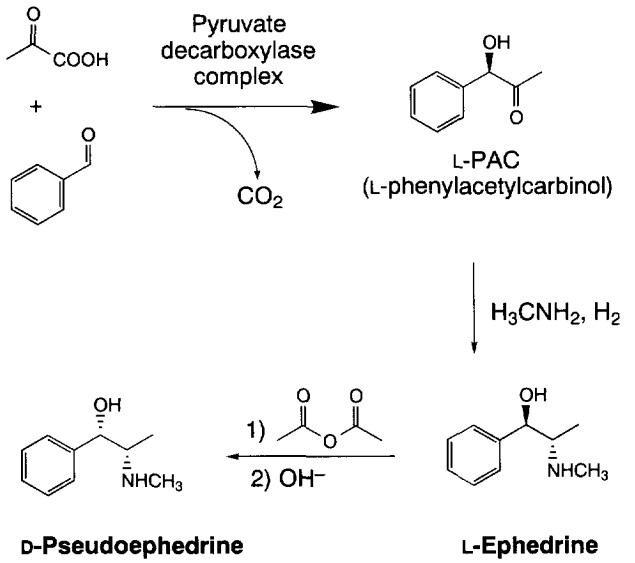

Figure 21. *Synthesis of L-ephedrine and D-pseudoephedrine.*

L-Ephedrine, (1*R*,2*S*)-2-methylamino-1-phenylpropanol, is synthesized from L-PAC via reductive amination with methylamine. The benzyl alcohol center may subsequently be inverted via acetylation and displacement by hydroxide to give the (1*S*,2*S*) isomer D-pseudoephedrine, a decongestant frequently co-formulated with antihistamines in cold and allergy medications[110] (Figure 21).

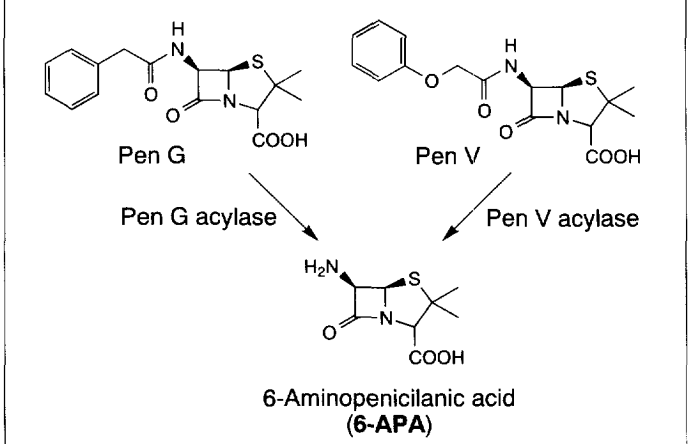

Figure 23. *Acylase-catalyzed hydrolysis of penicillin G (Pen G) and penicillin V (Pen V).*

Ribavirin

Antiviral drugs based on nucleoside analogs are relatively new. Yamasa is using a two-enzyme process for the commercial synthesis of the antiviral ribavirin. Two complementary phosphorylases are used sequentially to effect the net replacement of the pyrimidine base of either uridine or orotidine with the desired triazole carboxamide moiety[111,112] (Figure 22). Both the pyrimidine nucleoside phosphorylase and the purine nucleoside phosphorylase from *Erwinia carotovora* (AJ 2992) have been isolated and purified[113]. The synthesis of ribavirin from purine nucleosides (adenosine, inosine and guanosine) has also been reported[114,115], and the enzymes from *Brevibacterium acetylicum* (ATCC 954)[116] and *Bacillus megaterium* (AJ 3284)[117] have also been isolated and purified.

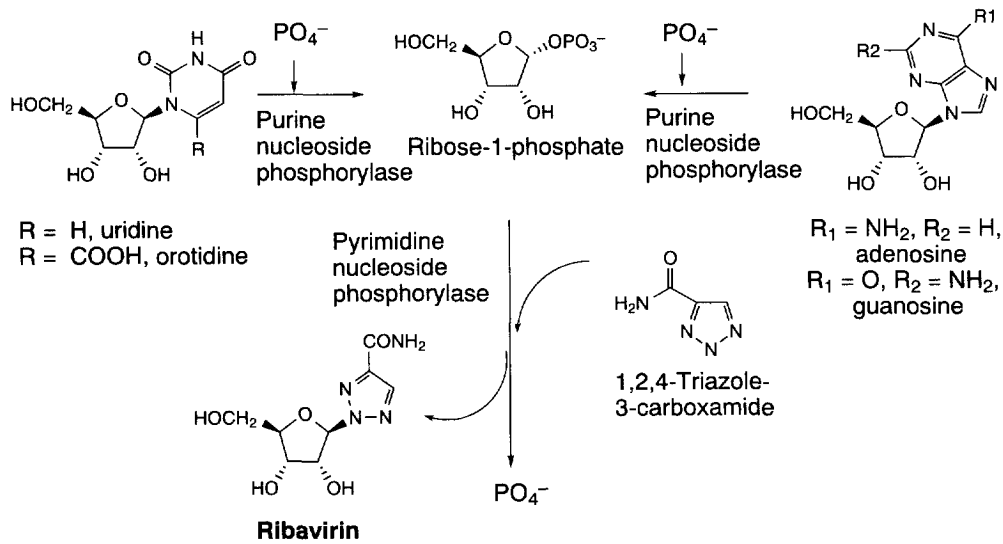

Figure 22. *Multienzymatic synthesis of ribavirin.*

Penicillin and cephalosporin

The *de novo* biosyntheses of penicillin G (Pen G), penicillin V (Pen V) and cephalosporin C (Ceph C) are well known and not within the scope of this

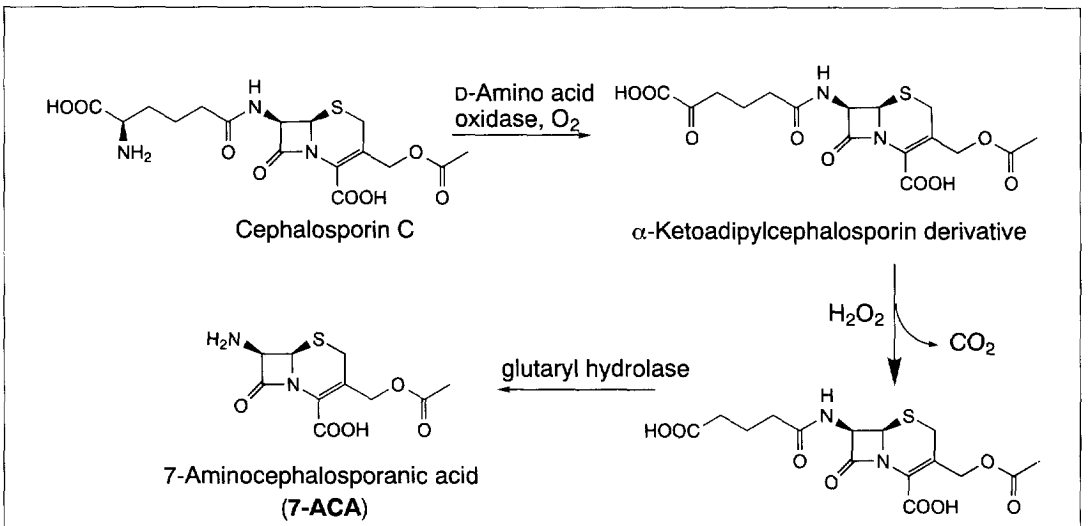

Figure 24. *Synthesis of 7-aminocephalosporanic acid (7-ACA).*

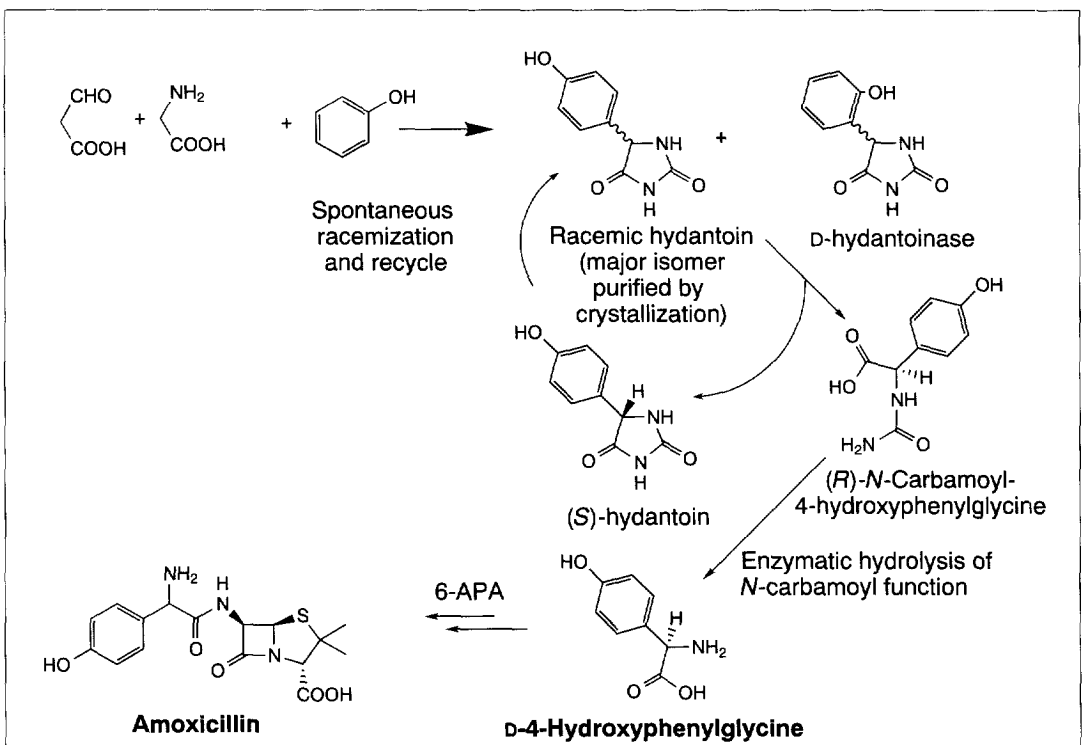

Figure 25. *Synthesis of amoxycillin. 6-APA, 6-aminopenicillanic acid.*

review. The use of enzymes to catalyze reactions in the subsequent chemistry following from Pen G, Pen V, and Ceph C does fall into the same category as the other examples given so far and is mentioned here.

The hydrolyses of the side chains of Pen G and Pen V are practiced commercially at very large scale for the production of 6-aminopenicillanic acid (6-APA), the β-lactam nucleus used in the production of semisynthetic penicillin antibiotics. These processes, including the production of the penicillin acylase (penicillin amidase) enzymes, are the subject of many extensive reviews[118–123] (Figure 23).

In earlier processes the removal of the side chains of Pen G and Pen V was performed via protection of the β-lactam with trialkylsilyl chloride and subsequent treatment with phosphorus pentachloride. The discovery of enzymes that specifically remove the side chains under mild, aqueous conditions was a major advance in the commercial production of 6-APA. Now the enzyme-based transformations have completely replaced the earlier chemical processes. The activity of the penicillin acylases is nearly mutually exclusive; the enzyme from *Escherichia coli* and *Bacillus megaterium* will hydrolyze the phenylacetic acid side chain of Pen G but not the phenoxyacetic acid side chain of Pen V. The penicillin acylase from *Fusarium oxysporum* is preferred for Pen V hydrolysis to 6-APA (Ref. 124). The phenylacetic and phenoxyacetic acids recovered are then recycled back into the Pen G and Pen V fermentations, respectively.

In a similar manner, enzymatic hydrolysis of a modified side chain of Ceph C has been used to produce the β-lactam

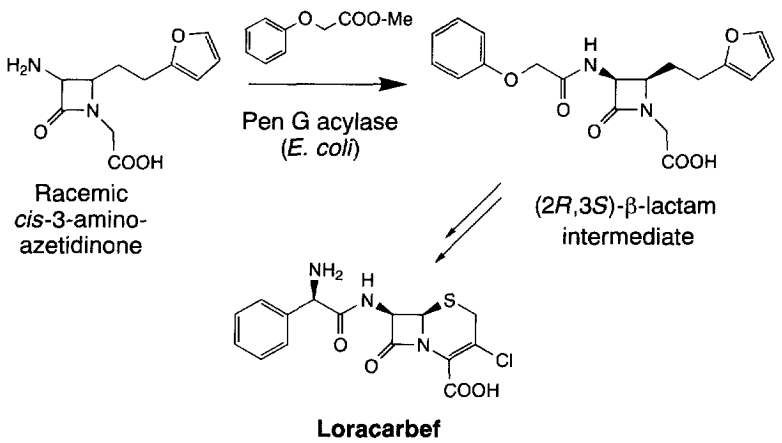

Figure 26. *Synthesis of Loracarbef.*

nucleus 7-aminocephalosporanic acid (7-ACA) for the production of semisynthetic cephalosporin antibiotics. A two-enzyme process is practiced by Antibioticos in Italy[125] (Figure 24). In this process, the side chain of Ceph C (the 'unnatural' isomer of the amino acid homoglutamate) is first oxidized to the α-ketoadipoyl analog by treatment with D-amino acid oxidase (from *Rhodotorula gracilis* ATCC 26217), which requires the presence of oxygen. Following this, oxidative decarboxylation with hydrogen peroxide leaves the glutaryl half-amide of cephalosporanic acid, which is then enzymatically hydrolyzed to leave the desired 7-ACA.

Syntheses of the 'unnatural' side chains that are condensed with 6-APA or 7-ACA to produce the semisynthetic antibiotics also utilize enzymes. Four widely prescribed semisynthetic antibiotics – the penicillins ampicillin and amoxicillin, and the cephalosporins cephalexin and cefadroxil – use D-phenylglycine or D-4-hydroxyphenylglycine in their side chains. Kaneka practices an efficient synthesis of D-4-hydroxyphenylglycine using the enzymatic resolution of a racemic hydantoin precursor, the undesired enantiomer of which then undergoes spontaneous racemization[126]. The hydrolysis of the hydantoin leaves the *N*-carbamoyl derivative of the 4-hydroxyphenylglycine. The carbamoyl group may then be removed enzymatically[127], and the two steps may be practised in a single process[128] (Figure 25).

The use of penicillin acylase to catalyze condensation reactions in the synthesis of β-lactams is illustrated in Eli Lilly's commercial route to their antibiotic sold under the tradename Loracarbef (Figure 26). Although Pen G acylase does not hydrolyze the phenoxyacetyl side chain of Pen V, Lilly found that the Pen G enzyme condenses the methyl ester of phenoxyacetic acid to the 3-amino function in very high yield and specificity (44% conversion, 97% e.e.)[129,130].

Steroids

The use of microorganisms in the commercial production of steroid pharmaceuticals has been practiced for decades. The review of this area is beyond the scope of this paper, but clearly this use of biocatalysis must be recognized. The reactions of commercial value performed by microorganisms and reviewed elsewhere are illustrated in Figure 27 (Refs 131–135). Since the oxidation of unactivated carbon is still difficult to perform with conventional chemistry, it is not surprising that in commercial processes

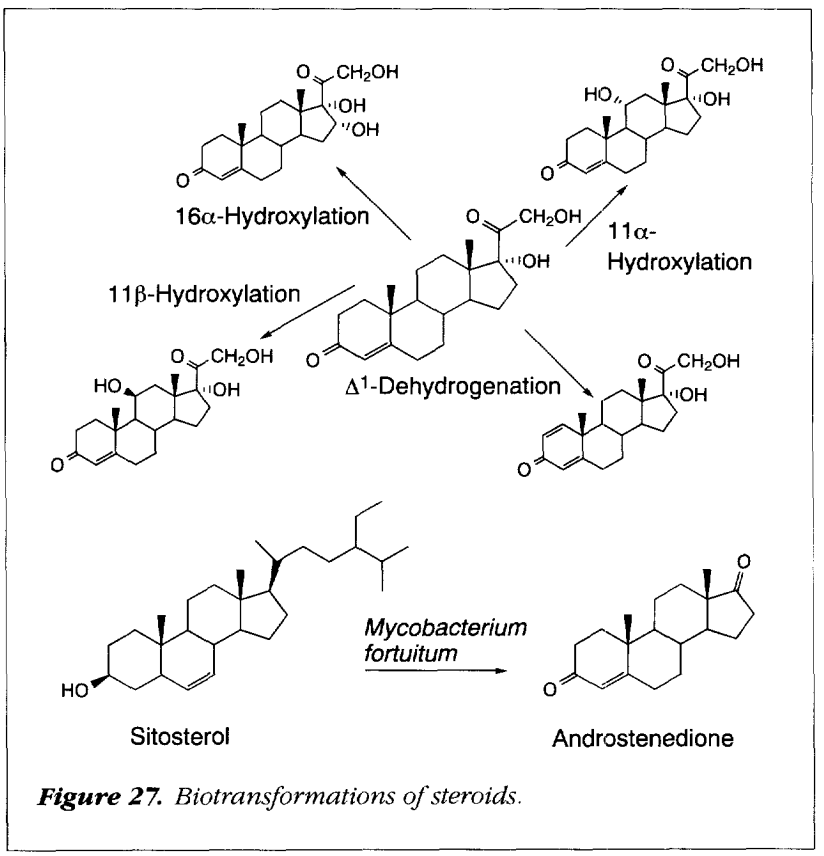

Figure 27. *Biotransformations of steroids.*

the 11α, 11β and 16α hydroxylations are still performed biologically.

Another major commercial process, established by Upjohn, is based on the degradation of sitosterol to androstenedione by a mutant of *Mycobacterium fortuitum*[136]. Sitosterol, which used to be of little commercial importance, is now a valuable starting material for a variety of steroid syntheses (Figure 27).

Despite its maturity, the area of bioconversions of steroids continues to grow. For example, the gene for the enzyme that performs the Δ^1 dehydrogenation has been cloned and patented recently[137]. Moreover, Gist-Brocades has patented the use of a recombinant microorganism with five introduced genes that allows the direct transformation of cholesterol into hydrocortisone[138].

Summary

Previously, much has been written about the potential use of biocatalysis in the pharmaceutical industry. In this review we have tried to depart from this format by listing not only transformations that have been tested at the laboratory scale, but also processes incorporating biocatalysis that are actually in use today for the manufacture of commercial drug products. The value of biocatalysis to the pharmaceutical industry need no longer be considered in terms of 'potential process technology', but should now be measured in terms of actual sales of pharmaceutical products on the market.

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From the Anderson Consulting drug discovery study...

The transition from discovery to development is highlighted as a major issue for the whole R&D organization. By focusing on improved integration of the two phases, one company surveyed has achieved a timeline of 18 months from lead optimization to the end of Phase IIA, with a goal of 12 months by 2000.

All participants in the study mentioned information management as a key factor in the future of drug discovery, yet 50% of companies surveyed reported no dedicated IT headcount within the discovery organization.

Copies of the executive briefing on the study (released 15 October 1997) are available from David Martin at Anderson Consulting (tel +44 171 304 8748).