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Biocatalysis: Synthesis of Key Intermediates for Development of Pharmaceuticals

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ABSTRACT: Chirality is a key factor for the safety and efficacy of many drug products. The production of single enantiomers of drug intermediates has become increasingly important in the pharmaceutical industry. There has been an enormous potential of microorganisms and enzymes derived from there for the transformation of synthetic chemicals with high chemo-, regio-, and enatioselectivities. Recent development in the area of directed evolution has led screen mutants under process conditions to increase activity and selectivity of biocatalysts, thus making the enzymatic process highly efficient and economically feasible. In this review, chemoenzymatic processes are described for the synthesis of chiral intermediates for the development of pharmaceuticals.

KEYWORDS: biocatalysis, enantioselective, regioselective, reduction, transamination, deracemization, reductive amination, desymmetrization, resolution, chiral drug intermediates

1. INTRODUCTION

The production of single enantiomers of chiral intermediates has become increasingly important in the development of drugs.[1] Single enantiomers can be produced by either chemical or biocatalytic synthesis. The advantages of biocatalysis over chemical synthesis are that enzyme-catalyzed reactions are often highly enantio- and regioselective. They can be carried out at ambient temperature and atmospheric pressure, thus avoiding the use of more extreme conditions, which could cause problems with isomerization, racemization, epimerization, and rearrangement of the compound. Microbial cells and enzymes derived therefrom can be immobilized and reused for many cycles. In addition, enzymes can be overexpressed to make biocatalytic processes economically efficient, and enzymes with modified activity can be tailor-made. Directed evolution of biocatalysts can lead to increased enzyme activity, selectivity, and stability. A number of review articles[2−19] have been published on the use of enzymes in organic synthesis. This review provides some examples and case studies on the use of enzymes for the synthesis of single enantiomers of key intermediates used in the development of pharmaceutical.

2. ENZYMATIC TRANSAMINATION: SYNTHESIS OF CHIRAL AMINE FOR SITAGLIPTIN

Sitagliptin **1** (sold under the trade name Januvia, Figure 1) is an oral antihyperglycemic (antidiabetic drug) of the dipeptidyl peptidase-4 (DPP-4) inhibitor class. It was developed and is marketed by Merck & Co. This enzyme-inhibiting drug is used either alone or in combination with other oral antihyperglycemic agents (such as metformin or a thiazolidinedione) for treatment of diabetes mellitus type 2.[20−22]

Pharmaceutical synthesis can benefit greatly from the selectivity gains associated with enzymatic catalysis. The current synthesis of sitagliptin[20−22] involves asymmetric hydrogenation of an enamine at high pressure using a rhodium-based chiral catalyst.[22] The chemistry suffers from inadequate stereoselectivity and a product stream contaminated with rhodium, necessitating additional purification steps at the expense of yield to upgrade both enantiomeric excess (ee) and chemical purity. By using a transaminase[23−28] scaffold and various protein engineering technologies, an enzymatic process has substantially improved the efficiency of sitagliptin manufacturing. Starting from an enzyme that had the catalytic machinery to perform the desired chemistry but lacked any activity toward the prositagliptin ketone, a substrate walking, modeling, and mutation approach to create a transaminase with marginal activity for the synthesis of the chiral amine, this variant was then further engineered via directed evolution for practical application in a manufacturing setting. The resultant biocatalysts showed broad applicability toward the synthesis of chiral amines that previously were accessible only via enzymatic resolution process.[29]

Under optimal conditions,[29] the best variant converted 200 g/L prositagliptin ketone **2** (Figure 1) to sitagliptin **1** of >99.95% ee by using 6 g/L enzyme in 50% DMSO with a 92% assay yield at the end of reaction. In comparison with the rhodium-catalyzed process, the biocatalytic process provides sitagliptin with a 10−13% increase in overall yield, a 53% increase in productivity (kg/L per day), a 19% reduction in total waste, the elimination of all heavy metals, and a reduction in total manufacturing cost. The enzymatic reaction is run in multipurpose vessels, avoiding the need for specialized high-pressure hydrogenation equipment.[29]

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3. ENZYMATIC REDUCTIVE AMINATION: SYNTHESIS OF CHIRAL (*S*)-AMINO ACID FOR SAXAGLIPTIN

Dipeptidyl peptidase 4 (DPP-4) is a ubiquitous proline-specific serine protease responsible for the rapid inactivation of glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide. To alleviate the inactivation of GLP-1, inhibitors of DPP-IV are being evaluated for their ability to provide improved control of blood glucose for diabetics.[30−32] Januvia developed by Merck is a marketed DPP-4 inhibitor.[31]

Saxagliptin **3** (Figure 2), a DPP-IV inhibitor[33,34] developed by Bristol-Myers Squibb, requires (*S*)-*N*-boc-3-hydroxyadamantylglycine **4** as a key chiral intermediate. A process for conversion of the keto acid **5** to the corresponding amino acid **4** using (*S*)-amino acid dehydrogenases was developed. A modified form of a recombinant phenylalanine dehydrogenase cloned from *Thermoactinomyces intermedius* and expressed in *Pichia pastoris* as well as in *Escherichia coli* was used for this process development and scale-up. NAD^+ produced during the reaction was recycled to NADH using formate dehydrogenase cloned and overexpressed in *E. coli*. The modified phenylalanine dehydrogenase contains two amino acid changes at the C-terminus and a 12 amino acid extension of the C-terminus.[33,34]

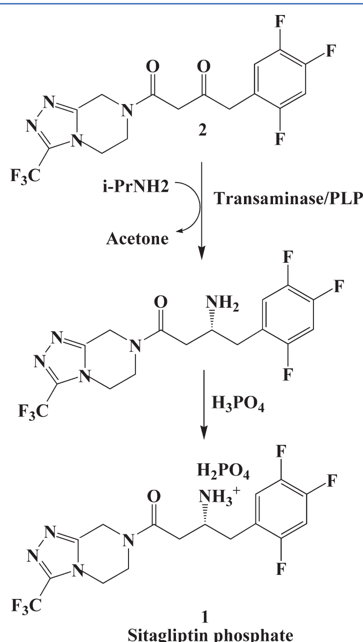

Figure 1. Enzymatic transamination: synthesis of chiral amine for sitagliptin.

Production of multikilogram batches was originally carried out with extracts of *P. pastoris* expressing the modified phenylalanine dehydrogenase from *T. intermedius* and endogenous formate dehydrogenase. The reductive amination process was further scaled up using a preparation of the two enzymes, formate dehydrogenase and phenylalanine dehydrogenase, expressed in single recombinant *E. coli*. The amino acid **4** was directly protected as its boc derivative without isolation to afford the intermediate. Yields before isolation were close to 98% with 100% ee.[33,34] This process has now been used to prepare several hundred kilograms of boc-protected amino acid **6** to support the development and manufacturing of Saxagliptin.

4. ENZYMATIC DERACEMIZATION: SYNTHESIS OF CHIRAL (*S*)-AMINO ACID FOR GLUCAGON-LIKE PEPTIDE (GLP-1)

(*S*)-Amino acids are useful intermediates for the synthesis of pharmaceuticals.[35] Many enzymatic approaches have been applied for their preparation, including (*S*)-hydantoinases combined with (*S*)-carbamoylases or HNO_2[36] (*S*)-acylases,[37] (*S*)-amidases,[38] (*S*)-transaminases,[39,29] (*S*)-amino acid dehydrogenases,[40,41] and dynamic resolution[42] has been developed.

The (*S*)-amino-3-[3-{6-(2-methylphenyl)}pyridyl]-propionic acid 7 (Figure 3) is a key intermediate required for synthesis of GLP-1 mimics or GLP-1 receptor modulators. Such receptor modulators are potentially useful for the treatment of type 2 diabetes treatment.[43,44]

(*S*)-Amino-3-[3-{6-(2-methylphenyl)}pyridyl]-propionic acid was prepared by enzymatic deracemization process[45] in 72% isolated yield with >99.4% ee from racemic amino acid **8** using combination of two enzymes, (*R*)-amino acid oxidase from *Trigonopsis variabilis* expressed in *E. coli* and (*S*)-aminotransferase from *Sporosarcina ureae* cloned and expressed in *E. coli*.

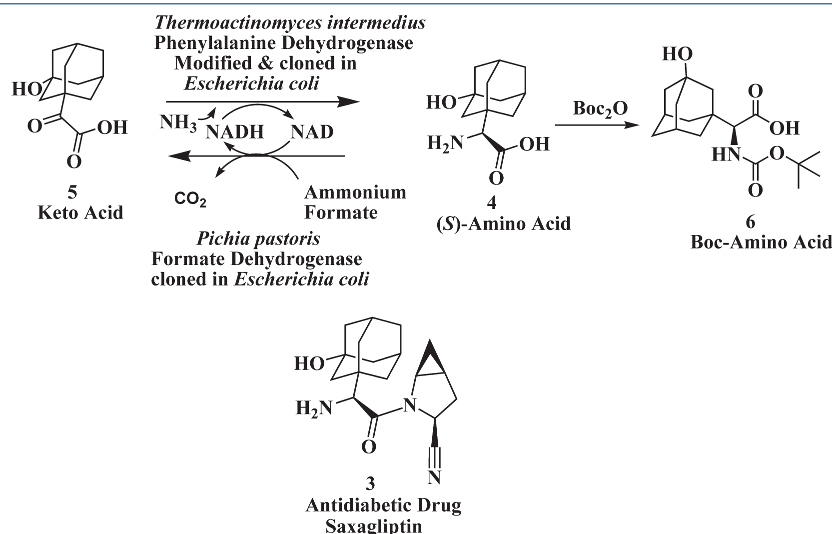

Figure 2. Enzymatic reductive amination: synthesis of chiral (*S*)-amino acid for saxagliptin.

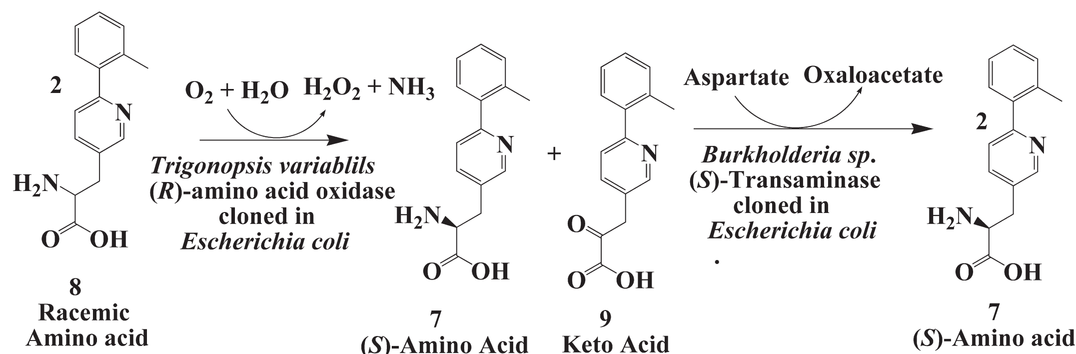

Figure 3. Enzymatic deracemization: synthesis of chiral (*S*)-amino acid for glucagon-like peptide (GLP-1).

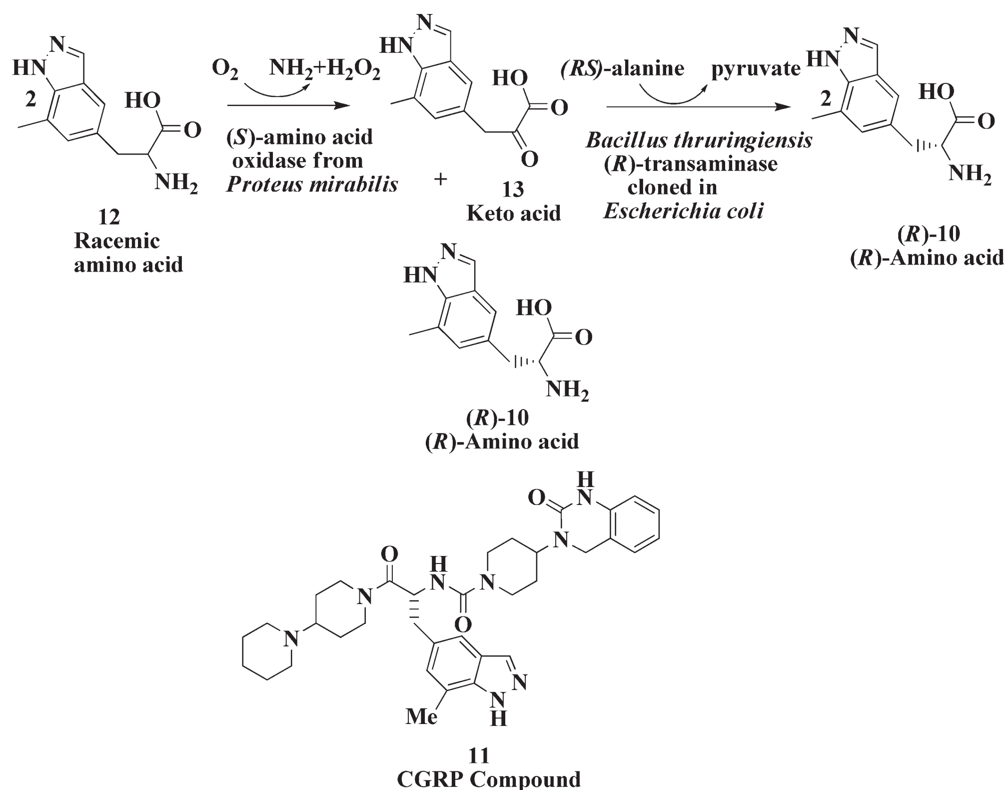

Figure 4. Enzymatic deracemization: synthesis of (*R*)-amino acid for calcitonin gene-related peptide.

(*S*)-aspartate was used as amino donor (Figure 3). A (*S*)-aminotransferase was also purified from a soil organism identified as *Burkholderia* sp. and cloned and expressed in *E. coli* and used in this process.[45] In the enzymatic process, racemic amino acid was first treated with (*R*)-amino acid oxidase for 4 h to convert racemic amino acid to a mixture of (*S*)-amino acid and keto acid **9**. Subsequently, in the same reaction mixture, (*S*)-aminotransferase was charged to convert keto acid **9** to (*S*)-amino acid **7** to get 85% yield at the end of the biotransformation process. This process was scaled up to 100 L scale at a substrate input of 1.5 kg.

In an alternate process, the enzymatic dynamic resolution of racemic amino acid **8** was also demonstrated. *R*-Selective oxidation with Celite-immobilized (*R*)-amino acid oxidase from *T. variabilis* expressed in *E. coli* in combination with chemical imine reduction with borane-ammonia gave a 75% in process yield and 100 ee of (*S*)-amino acid **7**.[45]

5. ENZYMATIC DERACEMIZATION: SYNTHESIS OF (*R*)-AMINO ACID FOR CALCITONIN GENE-RELATED PEPTIDE RECEPTORS (ANTIMIGRAINE DRUGS)

(*R*)-Amino acids are useful intermediates for the synthesis of β-lactam antibiotics and other pharmaceuticals.[46−48] Many enzymatic approaches have been applied for their preparation, in cluding (*R*)-hydantoinases combined with (*R*)-carbamoylases or HNO_2,[48,49] (*R*)-acylases,[50] (*R*)-amidases,[51] and (*R*)-transaminases,[46] and recently, an (*R*)-amino acid dehydrogenase has been developed.[52] Racemic amino acids have also been deracemized to give (*R*)-amino acids using an (*S*)-amino acid oxidase to selectively deplete the (*S*)-amino acid combined with an excess of reducing agent to recycle the imine product to the racemic amino acid.[53] The combination of an (*R*)-amino acid oxidase and an (*S*)-amino acid dehydrogenase to convert a racemic amino acid to an (*S*)-amino acid has been demonstrated.[54]

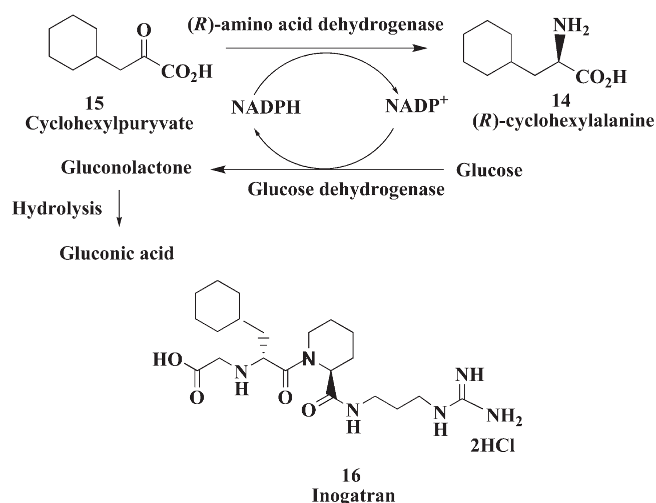

Figure 5. Enzymatic reductive amination: preparation of (R)-amino acid for Inogatran.

The (R)-amino acid (R)-2-amino-3-(7-methyl-1H-indazol-5-yl)-propanoic acid (R)-**10**, Figure 4) is a key intermediate needed for synthesis of antagonists of calcitonin gene-related peptide receptors **11**.[55] Such antagonists are potentially useful for the treatment of migraine and other maladies.[55,56]

(R)-Amino acid **10** was prepared in 68% isolated yield with >99% ee from racemic amino acid **12** using (S)-amino acid oxidase from *Proteus mirabilis* expressed in *E. coli* in combination with a commercially available (R)-transaminase using (R)-alanine as amino donor.[57] The R enantiomer was also prepared in 79% isolated yield with >99% ee from the corresponding keto acid **13** using the (R)-transaminase with racemic alanine as the amino donor. The rate and yield of this reaction could be accelerated by addition of lactate dehydrogenase (with NAD^+, formate, and formate dehydrogenase to regenerate NADH) to remove the inhibitory pyruvate produced during the reaction. An (R)-transaminase was identified and purified from a soil organism identified as *Bacillus thuringiensis* and cloned and expressed in *E. coli*. The recombinant (R)-transaminase was very effective for the preparation of **10** and gave a nearly complete conversion of **13** to **10** without the need for additional enzymes for pyruvate removal.[57]

6. ENZYMATIC REDUCTIVE AMINATION: PREPARATION OF (R)-AMINO ACID FOR INOGATRAN

(R)-Amino acids are increasingly becoming important building blocks in the production of pharmaceuticals and fine chemicals and as chiral directing auxiliaries and chiral synthons in organic synthesis. Applications of (R)-amino acids include their use as key components in β-lactam antibiotics, fertility drugs, and anticoagulants[58,59]

Two important (R)-amino acids used in semisynthetic antibiotics, (R)-phenylglycine (ampicillin), and p-hydroxy-(R)-phenylglycine (amoxicillin) are currently produced on a tons scale per year. In addition, there are more than 20 (R)-amino acids currently produced at pilot- or full-scale levels. In many of these cases, the R enantiomer is not only frequently more potent than the corresponding S enantiomer but also often more stable in vivo against enzyme degradation.[60,61]

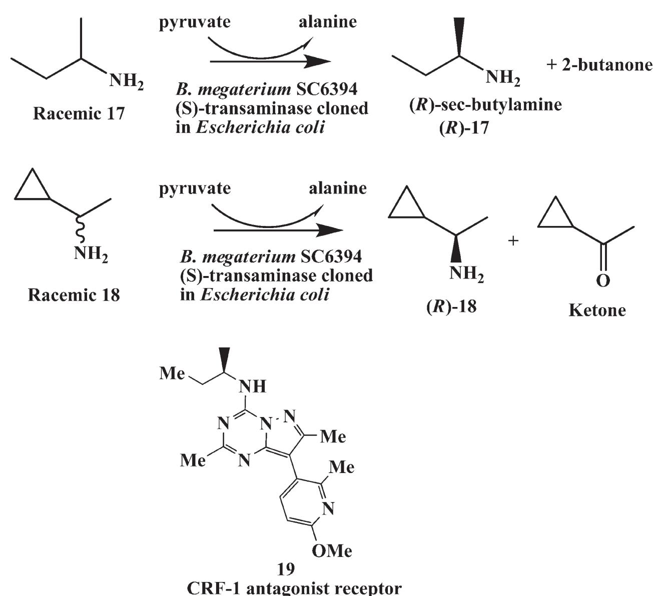

Figure 6. Enzymatic resolution by transaminase: (R)-amines synthesis for corticotropin releasing factor receptor antagonist.

Using both rational and random mutagenesis, Vedha-Peters et al.[52] have created the broad substrate range, nicotinamide cofactor dependent, and highly stereoselective (R)-amino acid dehydrogenase. This new enzyme is capable of producing (R)-amino acids via the reductive amination of the corresponding 2-keto acid with ammonia. This biocatalyst was the result of three rounds of mutagenesis and screening performed on the enzyme *meso*-diaminopimelate (R)-dehydrogenase from *Corynebacterium glutamicum*. The very high selectivity toward the R enantiomer (95 to >99% ee) was shown to be preserved after three rounds of mutagenesis and screening.[50] This new enzyme was active against a variety of amino acids and could complement and improve upon current methods for (R)-amino acid synthesis. The synthesis of (R)-cyclohexylalanine **14** (Figure 5) was developed by reductive amination of cyclohexylpyruvate **15** to yield (R)-**14** in 98% yield and >99% ee. (R)-**14** is a potential chiral intermediate for the synthesis of thrombin inhibitor Inogatran **16**.[62]

7. ENZYMATIC RESOLUTION BY TRANSAMINASE: (R)-AMINE SYNTHESIS FOR CORTICOTROPIN RELEASING FACTOR (CRF)-1 RECEPTOR ANTAGONIST

Anxiety and depression are psychiatric disorders that constitute a major health concern worldwide. Although numerous marketed treatments exit for both disorders, there continues to be a need for agents that have increased efficacy and reduced side-effect profiles.[63−65] CRF (1) receptor antagonists have been proposed as novel pharmacological treatments for depression, anxiety, and stress disorders.[65,66]

(R)-*sec*-Butylamine **17** and (R)-1-cyclopropylethylamine **18** (Figure 6) are key chiral intermediates for the synthesis of CRF-1 receptor antagonists, such as **19**.[67−69] Racemic amines have been resolved by acylation or deacylation reactions using lipases and proteases[70,71] or by using oxidases.[71] Enantioselective acylation combined with chemical racemization of amines[72] and enantioselective oxidation combined with nonselective chemical reduction of the imine product[73] have allowed deracemization reactions

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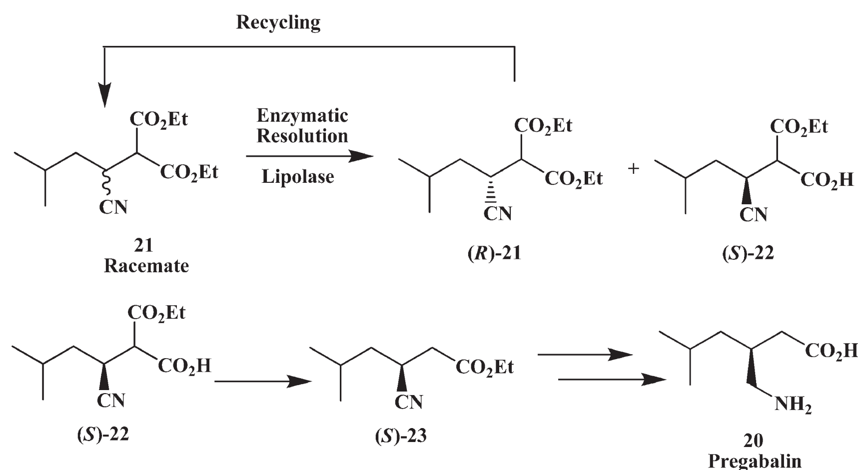

Figure 7. Enzymatic hydrolytic process: Synthesis of chiral intermediate for Pregabalin.

giving >50% yield of chiral amines. Omega-transaminases have provided another approach for resolution or synthesis of chiral amines.[74]

We have developed an enzymatic resolution process for the preparation of (*R*)-*sec*-butylamine and (*R*)-1-cyclopropylethylamine.[75] Screening was carried out to identify strains useful for the preparation of (*R*)-1-cyclopropylethylamine and (*R*)-*sec*-butylamine from the racemic amines with an *S*-specific transaminase. Several *Bacillus megaterium* strains as well as several soil isolates were found to have the desired activity for the resolution of the racemic amines to give the (*R*)-enantiomers. Using an extract of the best strain, *B. megaterium* SC6394, the reaction was shown to be a transamination requiring pyruvate as amino acceptor and pyridoxal phosphate as a cofactor. Initial batches of both amines were produced using whole cells of *B. megaterium* SC6394. The transaminase was purified to homogeneity to obtain N-terminal as well as internal amino acid sequences. The sequences were used to design polymerase chain reaction (PCR) primers to enable cloning and expression of the transaminase in *E. coli* SC16578. In contrast to using *B. megaterium* process, pH control and aeration were not required for the resolution of *sec*-butylamine, and an excess of pyruvate was not consumed by the recombinant cells. The resolution of *sec*-butylamine (0.68 M) using whole cells of *E. coli* SC16578 was scaled up to give (*R*)-*sec*-butylamine·$1/2H_2SO_4$ in 46.6% isolated yield with 99.2% ee. An alternative isolation procedure was also used to isolate (*R*)-*sec*-butylamine as the free base. Using the same recombinant (*S*)-transaminase, (*R*)-1-cyclopropylethylamine was obtained in 42% isolated yield (theoretical max. 50%) and 99% ee.[75]

8. ENZYMATIC HYDROLYTIC PROCESS: SYNTHESIS OF CHIRAL INTERMEDIATE FOR PREGABALIN

(*S*)-(+)-3-Aminomethyl-5-methylhexanoic acid (Pregabalin) **20** (Figure 7) is a lipophilic GABA (γ-aminobutyric acid) analogue that was developed for the treatment of several central nervous system disorders, including epilepsy, neuropathic pain, anxiety, and social phobia.[76,77] It has also been found effective for generalized anxiety disorder and is approved for this use in the European Union. Recent studies have shown that pregabalin is effective at treating chronic pain in disorders such as fibromyalgia and spinal cord injury. In June 2007, pregabalin became the first medication approved by the U.S. Food and Drug Administration specifically for the treatment of fibromyalgia.

During the initial process development for pregabalin, several routes were examined in detail.[78−80] The first generation manufacturing process was selected on the basis of overall yield, cost, and high process throughput and executed as a racemic synthesis, followed by resolution with (*S*)-(+)-mandelic acid. Although this route was cost-effective, there are possibilities for improvement in two areas: the use of an early- as opposed to a late-stage resolution and the introduction of a recycling step to avoid discarding the undesired enantiomer. Several new routes have been under development.[78] The use of asymmetric hydrogenation as the key step for the reduction of 3-cyano-5-methylhex-3-enoic acid[79] is an elegant potential manufacturing process but is surpassed in terms of cost effectiveness and environmental performance by the enzymatic route.[81]

A new manufacturing process for (*S*)-3-(aminomethyl)-5-methylhexanoic acid (Pregabalin) was developed using Lipolase, a commercially available lipase. *rac*-2-Carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester **21** (Figure 7) can be resolved to form 2-carboxyethyl-3-cyano-5-methylhexanoic acid **22**. A heat-promoted decarboxylation of **22** efficiently generates (*S*)-3-cyano-5-methylhexanoic acid ethyl ester **23**, a known precursor of Pregabalin.[81] This new route dramatically improved process efficiency compared with the first-generation process by setting the stereocenter early in the synthesis and enabling the facile racemization and reuse of (*R*)-**21**. The chemoenzymatic process also reduced organic solvent usage, resulting in a mostly aqueous process. Compared with the first-generation manufacturing process, the new process resulted in higher yields of pregabalin (40−45% after one recycle of (*R*)-**21**, and substantial 5-fold reductions of waste streams.

9. ENZYMATIC ASYMMETRIC HYDROLYSIS: PREPARATION OF CHIRAL INTERMEDIATE FOR NK1/NK2 DUAL ANTAGONISTS

Tachykinins are a group of biologically active neuropeptide hormones that are widely distributed throughout the nervous system. They are implicated in a variety of biological processes, such as pain transmission, inflammation, vasodilatation, and secretion.[82]The effect of tachykinins is modulated via specific G-protein coupled receptors, such as NK1 and NK2. Thus,

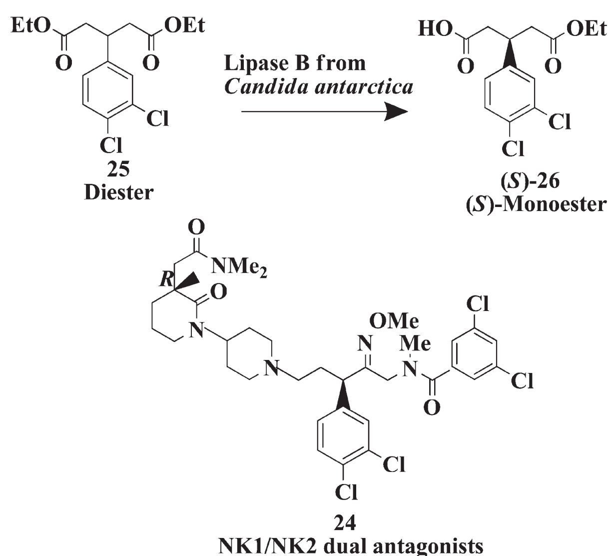

Figure 8. Enzymatic asymmetric hydrolysis: Preparation of chiral intermediate for NK1/NK2 dual antagonists.

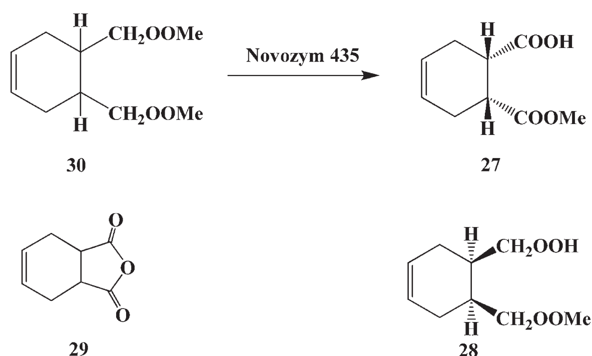

Figure 9. Enzymatic desymmetrization process: Preparation of (1*S*,2*R*)-2-(methoxycarbonyl)cyclohex-4-ene-1-carboxylic acid for chemokine receptor modulator.

nonpeptide NK-receptor antagonists are potentially useful in the treatment of variety of chronic diseases, including asthma, bronchospasm, arthritis, and migraine.[83]

The structure−activity relationship of several nonpeptide NK1/NK2 antagonists has led to the discovery of a new class of oxime-based NK1/NK2 dual antagonists,[84,85] such as compound **24** (Figure 8). The biological activity of **24** resides mainly in the *R,R* diastereomer. An enzymatic process for desymmetrization of the prochiral diethyl 3-[3′,4′-dichlorophenyl]glutarate **25** to the corresponding (*S*)-monoester **26** has been developed using lipase B from *Candida antarctica* with either the free or the immobilized enzyme. At 100 g/L substrate input, a reaction yield of 97% and an enantiomeric excess of >99% were obtained for the desired (*S*)-monoester. The process was scaled up to produce 200 kg of product in 80% overall isolated yield.[86]

DNA family shuffling was used to create a chimeric lipase B protein with improved activity toward diethyl 3-[3′,4′-dichlorophenyl]glutarate. Three homologous lipases from *C. antarctica* ATCC 32657, *Hyphozyma* sp. CBS 648.91, and *Cryptococcus tsukubaensis* ATCC 24555 were cloned and shuffled to generate a diverse gene library. Using a high-throughput screening assay, a

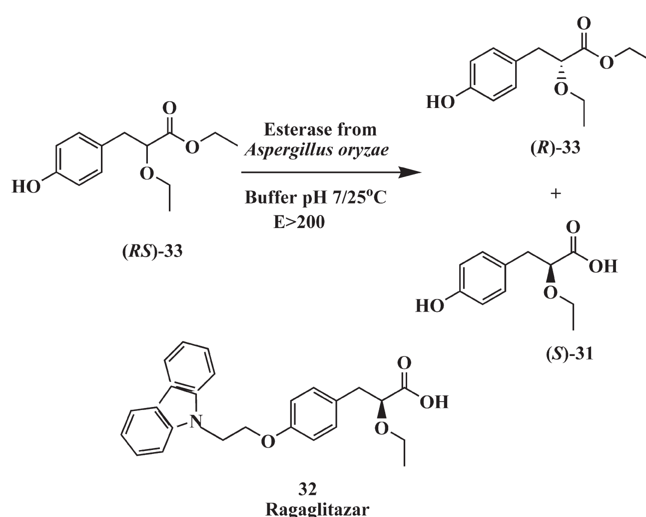

Figure 10. Enzymatic preparation of (*S*)-2-ethoxy-3-(4-hydroxyphenyl)-propanoic acid for Ragaglitazar.

chimeric lipase B protein having 20-fold higher activity toward the substrate was identified.[87] The thermostability of the lipase was also improved by directed evolution.[88]

10. ENZYMATIC DESYMMETRIZATION PROCESS: PREPARATION OF (1*S*,2R)-2-(METHOXYCARBONYL)CYCLOHEX-4-ENE-1-CARBOXYLIC ACID FOR CHEMOKINE RECEPTOR MODULATOR

The chiral monoester, (1*S*,2*R*)-2-(methoxycarbonyl)cyclohex-4-ene-1-carboxylic acid **27** (Figure 9) is a key chiral intermediate for the synthesis of a potential drug candidate for the modulation of chemokine receptor activity.[89,90] Both the (1*S*,2*R*)-monoester **27** and its enantiomer (1*R*,2*S*)-monoester **28** can be obtained by resolution[90] of the racemic acid with cinchonidine and ephedrine. However, the maximum theoretical yield of the resolution process is not more than 50%. A meso desymmetrization process has been published, affording either the (1*S*,2*R*)-monoester **27** or its enantiomer (1*R*,2*S*)-monoester **28** by desymmetrization of the meso-anhydride, *cis*-1,2,3,6-tetrahydrophthalic anhydride, by alcoholysis catalyzed by cinchona alkaloids. Cinchonine and quinine provided the (1*S*,2*R*)-monoester **27**, and cinchonidine and quinidine provided (1*R*,2*S*)-monoester **28**. The quinine-catalyzed alcoholysis of the anhydride **29** was used to prepare kilogram quantities of the (1*S*,2*R*)-monoester **27** with 90.8% ee.[91−93] There are several reports for the synthesis of the opposite enantiomer of the monoester, (1*R*,2*S*)-2-(methoxycarbonyl) cyclohex-4-ene-1-carboxylic acid, **28**, by porcine liver enzyme-catalyzed hydrolytic desymmetrization of the dimethyl ester **30**.[94,95]

An efficient process for the synthesis of the monoester, (1*S*,2*R*)-2-(methoxycarbonyl)cyclohex-4-ene-1-carboxylic acid **27** by *C. antarctica* lipase (Novozym 435)-catalyzed desymmetrization of the corresponding diester, dimethyl-cyclohex-4-ene-*cis*-1,2-dicarboxylate **30**, was developed. The process was optimized and scaled-up to prepare a total of 3.15 kg of the (1*S*,2*R*)-monoester from 3.42 kg of diester in two batches. The yield of the two batches ranged from 98.1to 99.8%, and the ee of the (1*S*,2*R*)-monoester was >99.9%.[96]

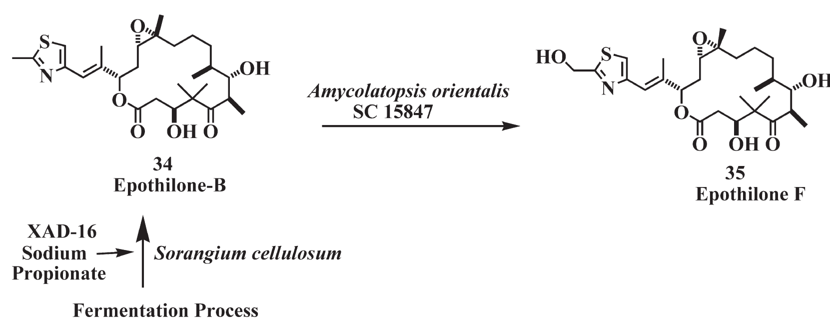

Figure 11. Epothilones: microbial hydroxylation of epothiolone B to epothilone F.

11. ENZYMATIC PREPARATION OF (*S*)-2-ETHOXY-3-(4-HYDROXYPHENYL)PROPANOIC ACID FOR RAGAGLITAZAR

(*S*)-2-Ethoxy-3-(4-hydroxyphenyl)propanoic acid, (*S*)-**31** (Figure 10), is a key intermediate in the synthesis of the new antidiabetic drug Ragaglitazar **32**. Compound **32** belongs to a novel therapeutic class of compounds of dual-acting PPARα and -γ agonists aimed for treatment of type-2 diabetes.[97,98] Ragaglitazar is designed to restore insulin sensitivity and to correct the dyslipidaemic disorders in people with type-2 diabetes.[99,100] The compound, in-licensed by Novo Nordisk from Dr. Reddy's Laboratories, is chemically and pharmacologically different from presently marketed PPAR agonists and is a member of a new class of insulin sensitizers.

The synthesis of the key intermediate, enantiomerically pure **(*S*)-31** was developed by a biocatalytic route. A novel large-scale production of (*S*)-2-ethoxy-3-(4-hydroxyphenyl)propanoic acid **(*S*)-31** was developed from its racemic ethylester ***rac*-33** by enantioselective hydrolysis using esterase from *Aspergillus oryzae*. The process was successfully run on a 44 kg pilot scale in 43−48% yields and with enantiomeric purity of 99.6%.[101]

12. EPOTHILONES: MICROBIAL HYDROXYLATION OF EPOTHIOLONE B TO EPOTHILONE F

The clinical success of paclitaxel has stimulated research into compounds with similar modes of activity in an effort to emulate its antineoplastic efficacy while minimizing its less desirable aspects, which include nonwater solubility, difficult synthesis, and emerging resistance. The epothilones are a novel class of natural product cytotoxic compounds derived from the fermentation of the myxobacterium *Sorangium cellulosum* that are nontaxane microtubule-stabilizing compounds that trigger apoptosis.[102,103] The natural product epothilone B **34** (Figure 11) has demonstrated broad spectrum antitumor activity in vitro and in vivo, including tumors with paclitaxel resistance.[104] The role of **34** as a potential paclitaxel successor has initiated interest in its synthesis, resulting in several total syntheses of **34** and various derivatives thereof.[105] The epothilone analogs were synthesized in an effort to optimize the water solubility, in vivo metabolic stability, and antitumor efficacy of this class of antineoplasic agents.[106−109]

A fermentation process was developed for the production of epothilone B, and the titer of epothilone B was optimized and increased by a continuous feed of sodium propionate during fermentation. The inclusion of XAD-16 resin during fermentation to adsorb epothilone B and to carry out volume reduction made the recovery of product very simple.[103] In addition, a high level of free epothilone B that is inhibitory to the growth of the producing culture was avoided by supplying XAD-16 resin during the fermentation process. A microbial hydroxylation process was developed for conversion of epothilone B **34** to epothilone F **35** by *Amycolatopsis orientalis* SC 15847. A bioconversion yield of 37−47% was obtained when the process was scaled up to 100−250 L with an intermittent feed of epothilone B (Patel et al., unpublished results). The reducing power NAD(P)H required for hydroxylation was generated internally during growth by the carbon source glucose that was used. Recently, the epothilone B hydroxylase along with the ferredoxin gene have been cloned and expressed in *Streptomyces rimosus* from *A. orientalis* SC 15847 and variants thereof. This cloned enzyme has been used in the hydroxylation of epothilone B to epothilone F to obtain even higher yields (80%) of product.[110]

13. ENZYMATIC REDUCTION PROCESS FOR SYNTHESIS OF MONTELUKAST INTERMEDIATE

With the discovery of the biological activity of the slow-reacting substance of anaphylaxis (SRS-A) and its relation to the leukotrienes (LTC4, LTD4, and LTE4) and asthma, the search for leukotriene antagonists has been intensive. As part of an ongoing program for the development of specific LTD4 antagonists for the treatment of asthma and other associated diseases, Merck has identified montelukast **36** (Figure 12) as an LTD4 antagonist.[111−114]

Merck has described the synthetic route for the production of montelukast, using a stereoselective reduction of a ketone **37** to the (*S*)-alcohol **38** as the key step. The alcohol subsequently undergoes a Sn2 displacement with a thiol to give the *R*-configured final product.[111−114] The key reduction step, the reduction of the ketone **37** to produce the chiral alcohol **38** (Figure 12), requires stoichiometric amounts of the chiral reducing agent (−)-B-chlorodiisopino campheylborane [(−)-DIP-chloride]. (−)-DIP-chloride is selective and avoids the side reactions, but it is corrosive and moisture-sensitive, causing burns if it is allowed to contact the skin. The reaction must be carried out at −20 to −25 °C to achieve the best stereoselectivity. The quench and extractive workup generate large volumes of waste solvent due to the product's low solubility. For several reasons, an enzyme-catalyzed process for reduction of the ketone **37** was developed by Codexis. A ketoreductase was developed by directed evolution by high-throughput screens using a slurry of the ketone substrate and high isopropanol concentration. Beneficial mutations among the various improved mutants were recombined in each round, and new mutations were made guided by ProSAR. The

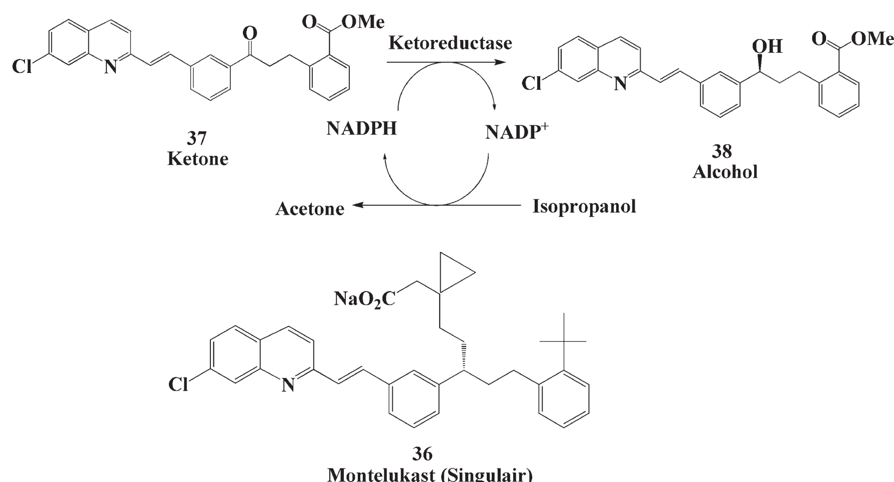

Figure 12. Enzymatic reduction process for synthesis of montelukast intermediate.

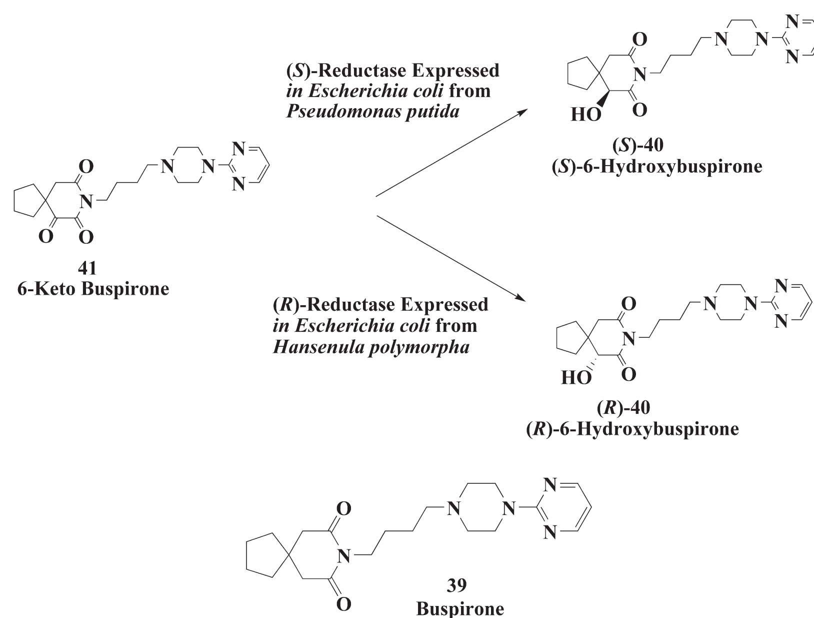

Figure 13. Enzymatic preparation of 6-hydroxybuspirone, antianxiety drug.

productivity of the final enzyme was improved 2000-fold, and stability was also substantially increased.

The final process was carried out as a slurry-to-slurry reaction at 45 °C, with the sparingly soluble ketone **37** being converted to an almost equally insoluble alcohol **38** at a concentration of 100 g/L of substrate in aqueous isopropyl alcohol and toluene. A reaction yield of 99.3% and enantiopmeric excess of 99.9% was obtained for alcohol **38**.[115]

14. ENZYMATIC PREPARATION OF 6-HYDROXYBUSPIRONE, ANTIANXIETY DRUG

Buspirone (Buspar, **39**, Figure 13) is a drug used for treatment of anxiety and depression that is thought to produce its effects by binding to the serotonin 5HT1A receptor.[116−118] Mainly as a result of hydroxylation reactions, it is extensively converted to various metabolites, and blood concentrations return to low levels a few hours after dosing.[119] A major metabolite, 6-hydroxybuspirone (**40**, Figure 13), produced by the action of liver cytochrome P450 CYP3A4, is present at much higher concentrations in human blood than buspirone itself. This metabolite has anxiolytic effects in an anxiety model using rat pups and binds to the human 5-HT1A receptor.[117−119] Although the metabolite has only about one-third of the affinity for the human 5HT1A receptor as buspirone, it is present in human blood at 30−40 times higher concentration than buspirone following a dose of buspirone and, therefore, may be responsible for much of the effectiveness of the drug.[118,119] For development of 6-hydroxybuspirone as a potential antianxiety drug, preparation and testing of the two enantiomers as well as the racemate was of interest. Both the *R* and *S* enantiomers, isolated by chiral HPLC, were effective in tests using a rat model of anxiety.[117−119] Whereas the *R* enantiomer showed somewhat tighter binding and specificity for the 5HT1A receptor, the *S* enantiomer had the advantage of being cleared more slowly from the blood.

An enantioselective microbial reduction of 6-oxobuspirone (**41**, Figure 13) to either (*R*)- or (*S*)-6-hydroxybuspirone **40** was

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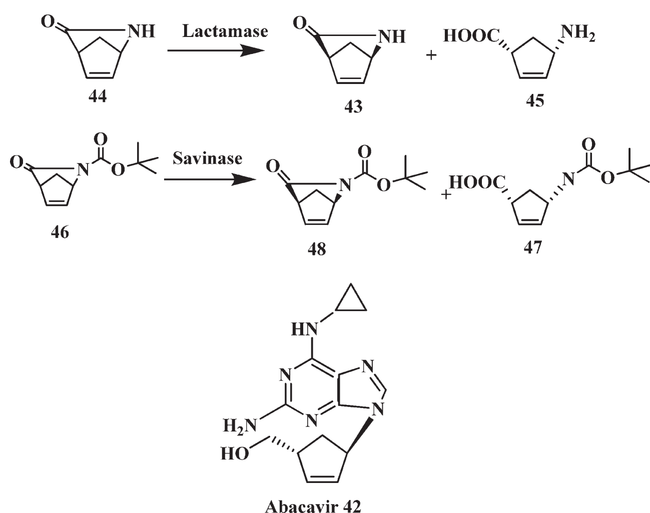

Figure 14. Enzymatic preparation of γ-lactam 2-azabicyclo[2.2.1]hept-5-en-3-one for Abacavir.

developed.[120,121] About 150 microorganisms were screened for the enantioselective reduction of **41**. The NADPH-dependent *R* reductase (RHBR), which catalyzes the reduction of 6-oxobuspirone to (*R*)-6-hydroxybuspirone **40**, was purified to homogeneity from cell extracts of *Hansenula polymorpha* SC 13845. *R* reductase from *H. polymorpha* SC 13845 was cloned and expressed in *E. coli*. To regenerate the cofactor NADPH required for reduction, glucose-6-phosphate dehydrogenase gene from *Saccharomyces cerevisiae* was also cloned and expressed in *E. coli*.[121]

The NADH-dependent *S* reductase (SHBR), which catalyzes the reduction of 6-ketobuspirone **41** to (*S*)-6-hydroxybuspirone **40**, was also purified to homogeneity from cell extracts of *Pseudomonas putida* SC 16269. The *S* reductase from *P. putida* SC 16269 was cloned and expressed in *E. coli*. To regenerate the cofactor NADH required for reduction, the formate dehydrogenase gene from *P. pastoris* was cloned and expressed in *E. coli*. Recombinant *E. coli* strains expressing *S* reductase and *R* reductase catalyzed the reduction of 6-ketobuspirone to (*S*)-6-hydroxybuspirone and (*R*)-6-hydroxybuspirone, respectively, in >98% yield and >99.9% ee.[121]

15. ENZYMATIC PREPARATION OF γ-LACTAM 2-AZABICYCLO[2.2.1]HEPT-5-EN-3-ONE FOR ABACAVIR (REVERSE TRANSCRIPTASE INHIBITOR)

Abacavir (Ziagen) **42** (Figure 14), a 2-aminopurine nucleoside analogue, is a selective reverse transcriptase inhibitor for the treatment of human HIV and hepatitis B viruses.[122] The γ-lactam 2-azabicyclo[2.2.1]hept-5-en-3-one **43** is a potential intermediate useful in the synthesis of Abacavir. A biocatalytic process was developed for the resolution racemic γ-lactam **44** (Figure 14) to yield the desired **43** and amino acid **45** using the γ-lactamase-containing organisms *Pseudomonas solonacearum* NCIMB40249 and *Rhodococcus* NCIMB40213.[123] However, because of the lack of a commercially available lactamase, an enzymatic process was developed for the enantioselective hydrolysis (in phosphate buffer, pH 8.0, containing 50% tetrahydrofuran) of racemic *tert*-butyl-3-oxo-2-azabicyclo-(2.2.1)hept-5-ene-2-carboxylate (**46**, Figure 14). A number of commercially available enzymes hydrolyzed the lactam bond of **46** to yield the corresponding *N*-acyl amino acid **47**, leaving unreacted the desired (1*R*,4*S*)-**48**. A reaction yield of 50% and an ee of 99% were obtained when the reaction was carried out at 100 g/L substrate input using savinase.[124]

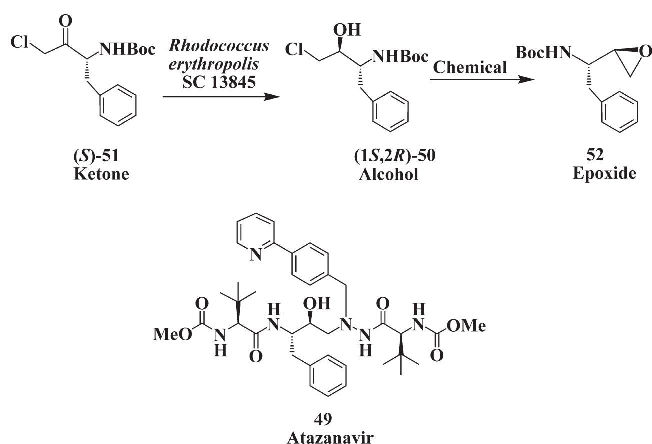

Figure 15. Enzymatic preparation of (1*S*,2*R*)-[3-chloro-2-hydroxy-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester for atazanavir.

16. ENZYMATIC PREPARATION OF CHIRAL INTERMEDIATES FOR ATAZANAVIR

Preparation of (1*S*,2*R*)-[3-Chloro-2-hydroxy-1-(phenylmethyl)propyl]-carbamic Acid, 1,1-Dimethylethyl Ester. Atazanavir (**49** Figure 15) is an acyclic aza-peptidomimetic, a potent HIV protease inhibitor[125,126] approved by the Food and Drug Adminstration for treatment of auto immune diseases (AIDs). An enzymatic process was developed for the preparation of (1*S*,2R)-[3-chloro-2-hydroxy-1-(phenylmethyl)propyl]-carbamic acid, 1,1-dimethylethyl ester (**50**, Figure 15), a key chiral intermediate required for the total synthesis of the HIV protease inhibitor atazanavir. The diastereoselective reduction of (1*S*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester **51**, was carried out using *Rhodococcus*, *Brevibacterium*, and *Hansenula* strains to provide **50**. Three strains of *Rhodococcus* gave >90% yield with a diastereomeric purity of >98% and an ee of 99.4%.[127] An efficient single-stage fermentation−biotransformation process was developed for the reduction of ketone **51** with cells of *Rhodococcus erythropolis* SC 13845 to yield **50** in 95% yield with a diasteromeric purity of 98.2% and an ee of 99.4% at a substrate input of 10 g/L. The reduction process was further improved by generating mutants and selection of desired mutant for conversion of **51** to (1*S*,2*R*)-**50** at a substrate input of 60 g/L. (1*S*,2*R*)-**50** was converted to epoxide **52** and used in the synthesis of atazanavir.[128] Chemical reduction of chloroketone **51** using $NaBH_4$ produces the undesired chlorohydrin diastereomer.[129]

Preparation of (*S*)-Tertiary Leucine. (*S*)-Tertiary leucine **53**, because of its bulky and hydrophobic side chain, is a key chiral amino acid required for the synthesis of number of drugs containing peptides such as atazanavir **49** and boceprevir.[125,126,130,131] Atazanavir **49** synthesis also required (*S*)-tertiary leucine as a key chiral intermediate. We also have developed the enzymatic reductive amination of ketoacid **54** to amino acid **53** (Figure 16) by recombinant *E. coli* expressing leucine dehydrogenase from *Thermoactinimyces intermedius*. The reaction required ammonia and NADH as a cofactor. NAD^+ produced during the reaction

was recycled back to NADH using recombinant *E. coli* expressing formate dehydrogenase from *P. pastoris*. A reaction yield of >95% with an ee of >99.5% was obtained for **53** at 100 g/L substrate input (R. Hanson, S. Goldberg, R. Patel, unpublished results). Leucine dehydrogenase from strains have also been cloned and expressed and used in a reductive amination process.[132−134]

17. CHLOESTEROL-LOWERING AGENTS: ENZYMATIC PREPARATION OF (3*S*,5*R*)-DIHYDROXY-6-(BENZYLOXY)HEXANOIC ACID, ETHYL ESTER

(3*S*,5*R*)-Dihydroxy-6-(benzyloxy) hexanoic acid, ethyl ester **55** (Figure 17) is a key chiral intermediate for cholesterol-lowering agents, such as **56**, atorvastatin **57**, and rosuvastatin, which acts by inhibiting hydroxyl methyl glutaryl CoA (HMG CoA) reductase inhibitor.[135−139] The enantioselective reduction of a diketone 3,5-dioxo-6-(benzyloxy)hexanoic acid, ethyl ester **58** to **55** was demonstrated by cell suspensions of *Acinetobacter calcoaceticus* SC 13876.[135] A reaction yield of 85% and an ee of 97% were obtained. Cell extracts of *A. calcoaceticus* SC 13876 in the presence of NAD^+, glucose, and glucose dehydrogenase reduced **58** to the corresponding monohydroxy compounds [3-hydroxy-5-oxo-6-(benzyloxy)-hexanoic acid ethyl ester **59** and 5-hydroxy-3-oxo-6-(benzyloxy)hexanoic acid ethyl ester **60**]. Both **59** and **60** were further reduced to the (3*S*,4*R*)-dihydroxy compound **55** in 92% yield and 99% ee by cell extracts. (3*S*,5*R*)-**55** were converted to **61**, a key chiral intermediate for the synthesis of **56** and **57**.[135,140] Three different ketoreductases were purified to homogeneity from cell extracts of *A. calcoaceticus* SC 13876, and their biochemical properties were compared. Reductase I only catalyzes the reduction of ethyl diketoester **58** to its monohydroxy products, whereas reductase II catalyzes the formation of dihydroxy products from monohydroxy substrates. A third reductase (III) was identified that catalyzes the reduction of diketoester **58** to *syn*-(3*R*,5*S*)-dihydroxyester **55**,[140] which now has been cloned and expressed in *E. coli*,[141] and the reduction of diketoester **58** to *syn*-(3*R*,5*S*)-dihydroxy-ester **55** was demonstrated by recombinant enzyme to give 99% yield.

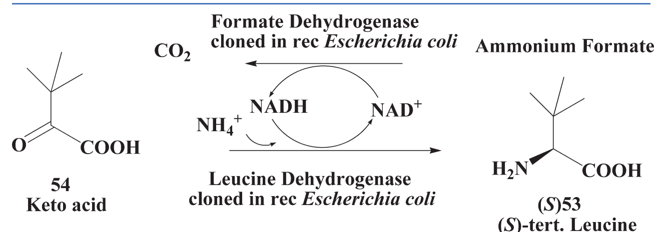

Figure 16. Enzymatic preparation of (*S*)-tertiary leucine for Atazanavir.

Enzymatic Preparation of a Chiral 2,4-Dideoxyhexose Derivative. In an alternate process, the chiral 2,4-dideoxyhexose derivative required for the HMG CoA reductase inhibitors has been prepared by aldol condensation reaction using 2-deoxy-ribose-5-phosphate aldolase (DERA). The reaction was initiated with a stereospecific addition of acetaldehyde (**62**, Figure 18) to a substituted acetaldehyde to form a 3-hydroxy-4-substituted butyraldehyde **63**, which reacts subsequently with another acetaldehyde to form a 2,4-dideoxyhexose derivative **64**. DERA has been expressed in *E. coli*.[142]

The above process has been improved and optimized. An increase of almost 400-fold in volumetric productivity relative to the published enzymic reaction conditions has been achieved, resulting in a attractive process that has been run on up to a 100 g scale in a single batch at a rate of 30.6 g/L/h. The catalyst load has been improved by 10-fold, as well, from 20 to 2.0 wt % DERA. These improvements were achieved by a combination of the discovery of a DERA with improved activity and reaction optimization to overcome substrate inhibition. The two stereogenic centers are set by DERA with an ee of >99.9% and a diastereomeric excess of 96.6%. In addition, downstream chemical processes have been developed to convert the enzymic product efficiently to versatile intermediates **65** applicable to the

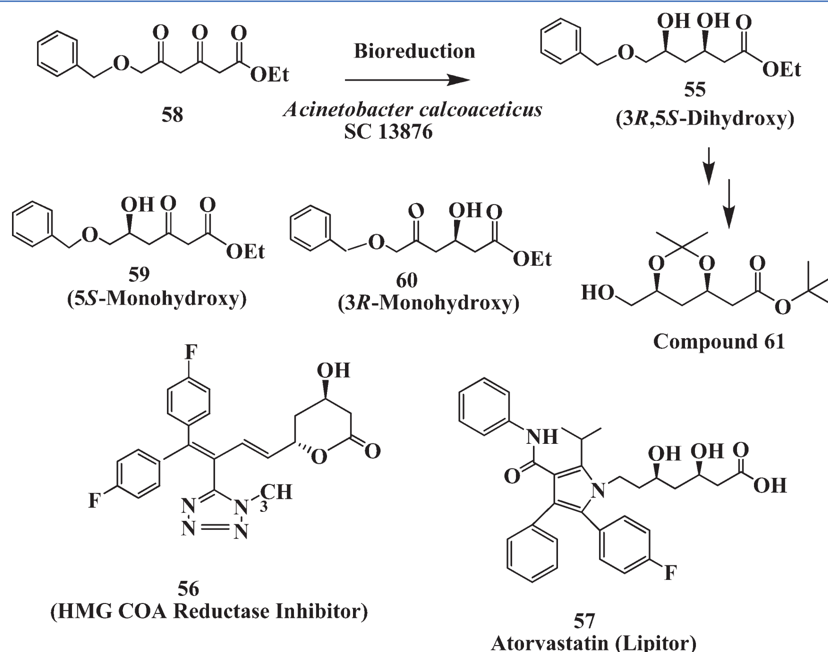

Figure 17. Chloesterol-lowering agents: Enzymatic preparation of (3*S*,5*R*)-dihydroxy-6-(benzyloxy)hexanoic acid, ethyl ester.

preparation of atorvastatin **57** and rosuvastatin.[143] DSM pharma also operates an aldolase-catalyzed synthesis of this key intermediate on an industrial scale.[144,145]

Enzymatic Preparation of (*R*)-4-Cyano-3-hydroxybutyrate. In another alternate enzymatic process, the preparation of ethyl (*R*)-4-cyano-3-hydroxybutyric acid **66** (Figure 19), a key intermediate for the synthesis of atorvastatin, was developed.[146] In this process, first, the enzymatic synthesis of ethyl (*S*)-4-chloro-3-hydroxybutyric acid derivatives **67** was carried out by ketoreductase-catalyzed conversion of 4-chloro-3-ketobutyric acid derivatives **68.**[147] The genes encoding halohydrin dehydrogenase from *Agrobacterium tumefaciens*, ketoreductase from *Candida magnoliae*, glucose dehydrogenase from *Bacillus subtilis*, and formate dehydrogenase from *Candida boidinii* were separately cloned into *E. coli* BL21. Each enzyme was then produced by fermentation, isolated, and characterized. Then ethyl (*R*)-4-cyano-3-hydroxybutyrate **66** (Figure 19) was prepared from ethyl 4-chloroacetoacetate **68** by the following procedure: Ethyl 4-chloroacetoacetate **68** was incubated at pH 7.0 with ketoreductase, glucose dehydrogenase, and $NADP^+$ for 40 h to produce ethyl (*S*)-4-chloro-3-hydroxybutyrate **67**, which was extracted with ethyl acetate, dried, filtered, and concentrated to yield ~97% pure ester. The dried ethyl (*S*)-4-chloro-3-hydroxybutyrate **67** was dissolved in phosphate buffer and mixed with halohydrin dehalogenase and sodium cyanide at pH 8.0. After 57 h, essentially pure ethyl (*R*)-4-cyano-3-hydroxybutyrate **66**, an intermediate used in HMG-CoA reductase inhibitors syntheses, was recovered.[147]

Enzymatic Synthesis of Chiral Carboxylic Acids by Nitrilase. Synthesis of a broad diversity of chiral carboxylic acid by nitrilases has been demonstrated.[148−153] Enzymatic desymmetrization of prochiral 3-hydroxyglutaronitrile **69** using a nitrilase[154,155] has been demonstrated (Figure 20). Following esterification of the resulting (*R*)-3-hydroxy-4-cyanobutyric acid **70**, an intermediate useful for the manufacture of the cholesterol-lowering drug Lipitor **57** (atorvastatin calcium) was produced. Nitrilases were identified in genomic libraries created by extraction of DNA directly from environmental samples and were expressed in *E. coli*. The resulting library was screened for highly enantioselective *R*-specific nitrilases.[154,155] (*R*)-3-Hydroxy-4-cyanobutyric acid was produced using a 100 mM initial nitrile concentration in 98% yield and 94.5% ee. The enantioselectivity of this wild-type nitrilase decreased with increasing nitrile concentration, and only 87.8% ee was obtained at 2.25 M substrate concentration. Mutagenesis of the nitrilase using a technique that combinatorially saturated each amino acid in the protein to each of the other 19 amino acids resulted in an improved variant (Ala190His) that was expressed in *E. coli*. This variant nitrilase gave an enantiomeric excess of 98.5% at 3 M substrate concentration with a volumetric productivity of 619 g/L/day.[155]

18. SAXAGLIPTIN: ENZYMATIC AMMONOLYSIS OF (5*S*)-4,5-DIHYDRO-1*H*-PYRROLE-1,5-DICARBOXYLIC ACID, 1-(1,1-DIMETHYLETHYL)-5-ETHYL ESTER

The synthesis of dipeptidyl peptidase-IV (DPP4) inhibitor, saxagliptin **3**[30−32] required (5*S*)-5-aminocarbonyl-4,5-dihydro-1*H*-pyrrole-1-carboxylicacid,1-(1,1-dimethylethyl)-ester **71** (Figure 21). Direct chemical ammonolyses were hindered by the requirement for aggressive reaction conditions, which resulted in unacceptable levels of amide racemization and side-product formation, whereas milder two-step hydrolysis−condensation protocols using coupling agents such as 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), were compromised by reduced overall yields.[156] To address this issue, a biocatalytic procedure was developed, based upon the *C. antarctica* lipase B (CALB)-mediated ammonolysis of (5*S*)-4,5-dihydro-1*H*-pyrrole-1,5-dicarboxylic acid, 1-(1,1-dimethylethyl)-5-ethyl ester **72** with ammonium carbamate to furnish **71** without racemization and with low levels of side-product formation.[157]

Experiments utilized process stream ester feed, which consisted of ~22% w/v (0.91 M) of the ester in toluene. Since the latter precluded the use of free ammonia due to its low solubility in toluene, solid ammonium carbamate was employed. Reactions

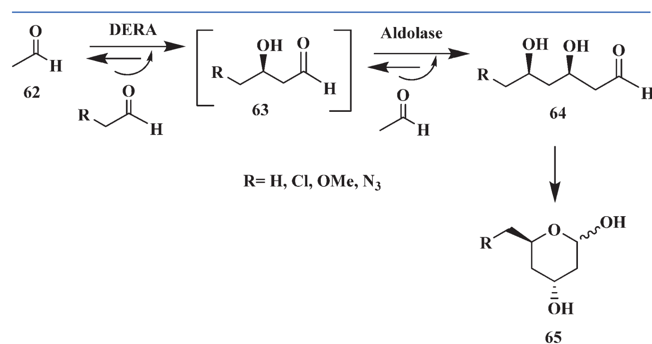

Figure 18. Chloesterol-lowering agents: Enzymatic preparation of a chiral 2,4-dideoxyhexose derivative.

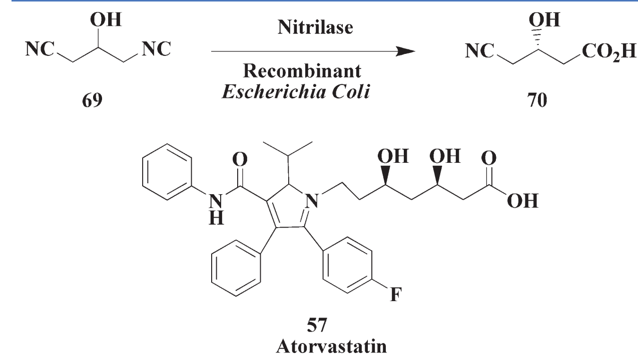

Figure 20. Chloesterol-lowering agents: enzymatic synthesis of chiral carboxylic acids by nitrilase.

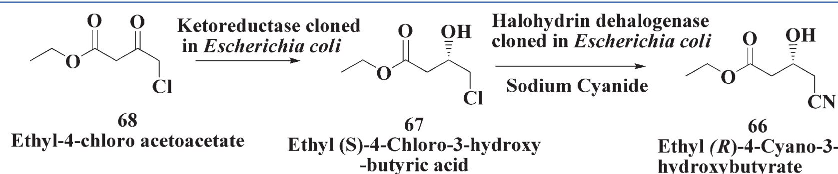

Figure 19. Chloesterol-lowering agents: Enzymatic preparation of (*R*)-4-cyano-3-hydroxybutyrate.

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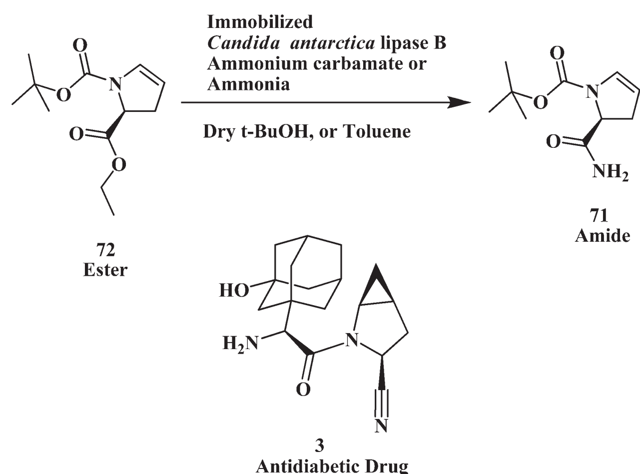

Figure 21. Saxagliptin: enzymatic ammonolysis of (5*S*)-4,5-dihydro-1*H*-pyrrole-1,5-dicarboxylic acid, 1-(1,1-dimethylethyl)-5-ethyl ester.

were performed using a mixture of neat process feed, ammonium carbamate (71 g/L, 2 mol equiv of ammonia), and biocatalyst (25 g/L) and shaken at 400 rpm, 50 °C. Under these conditions, CALB provided racemization-free amide with yields of 69%, together with 21% of side-products (by HPLC). The inclusion of various additives was investigated to solve potential inhibitory phenomena, shifting the equilibrium toward amide synthesis and reducing side-product formation. Drying agents such as calcium chloride gave significant improvement (79% amide and 13% side-products).The calcium chloride is known to complex alcohols as well as act as a desiccant, and its presumed binding of ethanol released during the course of amide formation may have served to mitigate any deleterious effects of this alcohol on CALB catalysis. A dramatic increase in amide yield to 84 and 95% was achieved by including sodalime and ascarite, respectively, at 200 g/L in the reaction headspace, this presumably by way of adsorption of carbon dioxide liberated from the decomposition of ammonium carbamate. A further increase in yield to 98% was attained via the combined use of 100 g/L of calcium chloride and 200 g/L of ascarite. A prep-scale reaction with the process ester feed was used. Ester (220 g/L) was reacted with 90 g/L (1.25 mol equiv) of ammonium carbamate, 33 g/L (15% w/w of ester input) of CALB, 110 g/L calcium chloride, and 216 g/L of ascarite (in the headspace) and run at 50 °C for 3 days. Complete conversion of ester was achieved, with the formation of 96% (182 g/L) of amide **71** and 4% of side-products, and after workup, 98% potency amide of >99.9% ee was isolated in 81% yield.[157]

19. ENZYMATIC DEAMINATION PROCESS FOR PREPARATION OF EPIVIR

(2′*R*-*cis*)-2′-deoxy-3-thiacytidine (3TC, Epivir, **73**; Figure 22), has been approved by the FDA and is marketed for the therapy of HIV (human immunodeficiency virus. Epivir is a potent and selective inhibitor of the reverse transcriptase enzyme, which catalyzes the conversion of the HIV RNA to a double-stranded DNA copy. Epivir is also active against HBV (hepatitis B virus) and is sold as Lamivudine.[158] In contrast to the majority of nucleoside analogues that display antiviral activity primarily residing in the "natural" ss-D-isomer, the enantiomers of ± **73** are equipotent in vitro against HIV-1 and HIV-2, but the "unnatural" ss-L-(−)-**73**

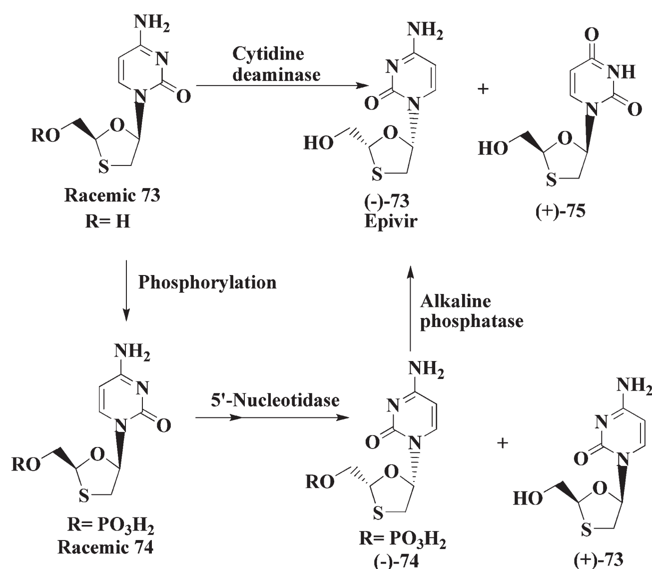

Figure 22. Enzymatic deamination process for preparation of Epivir.

isomer (Epivir, Figure 22) is substantially less cytotoxic than its corresponding "natural" ss-D-(+)-isomer[159]

One of the initial routes considered for the preparation of isomers was the enzymic resolution using 5′-nucleotidase and alkaline phosphatase that would allow access to both enantiomers of **73**. The chemically synthesized monophosphate derivative (±)-**74** was resolved using 5′-nucleotidase from *Crotalus atrox* venom, and the resulting mixture was separated by chromatography and purified on silica gel to give (+)-**73** (ee >99%, Figure 14). Hydrolysis of the remaining monophosphate (−)-**74** with alkaline phosphatase from *E. coli* afforded (−)-**73** (Epivir) in an optically pure form.[160] To produce much larger quantities of Epivir, a scaleable end-stage resolution, via enantioselective deamination of (±)-**73** with cytidine deaminase,[161] was developed to yield (−)-**73** (Epivir) and (+)-**75**.

To use *E. coli* as a source of large quantities of enzyme, the cloning and overexpression of enzyme, fermentation, and immobilization of cytidine deaminase for reuse, and development of an efficient isolation process was developed for large-scale manufacturing of Epivir.[161] About 1.15 kg of highly pure Epivir was recovered from each 3 kg batch. The purity was better than 97% by HPLC, and the enantiomeric excess was at least 99.8%. Using this approach, 20 kg of optically pure Epivir was isolated. This process was used on a manufacturing scale to prepare tons of Epivir using immobilized cytidine deaminase from the recombinant strain. The same batch of enzyme was used for at least 15 cycles.[161,162]

20. CONTINUOUS ENZYMATIC PROCESS FOR THE PREPARATION OF (*R*)-3-(4-FLUOROPHENYL)-2-HYDROXYPROPIONIC ACID FOR RHINOVIRUS PROTEASE INHIBITOR

(*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid **76** (Figure 23) is a building block for the synthesis of AG7088, a rhinovirus protease inhibitor **77**.[163,164] The preparation of **76** using a biocatalytic reduction was performed in a membrane reactor.[165] A continuous enzymatic process for an efficient synthesis of (*R*)-3-(4-fluorophenyl)-2-hydroxypropionic acid at multikilogram scale with a high space−time yield (560 g/L/day) using a membrane reactor was used. The

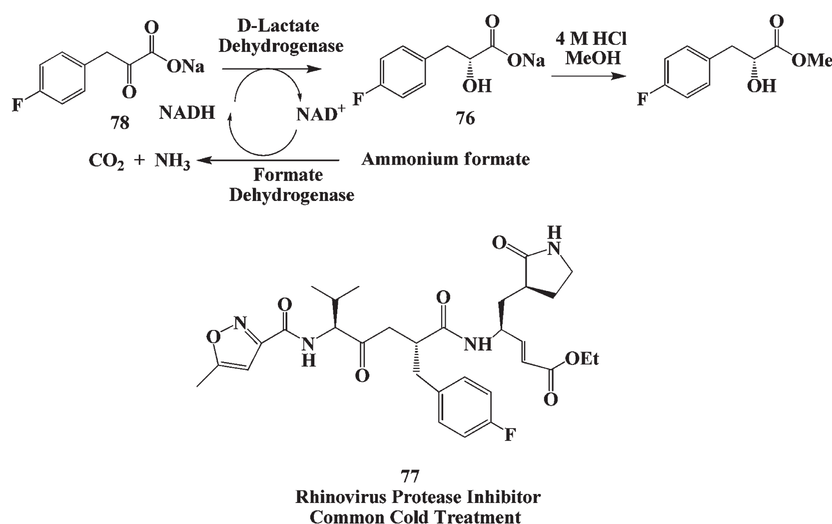

Figure 23. Continuous enzymatic process for the preparation of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid for rhinovirus protease inhibitor.

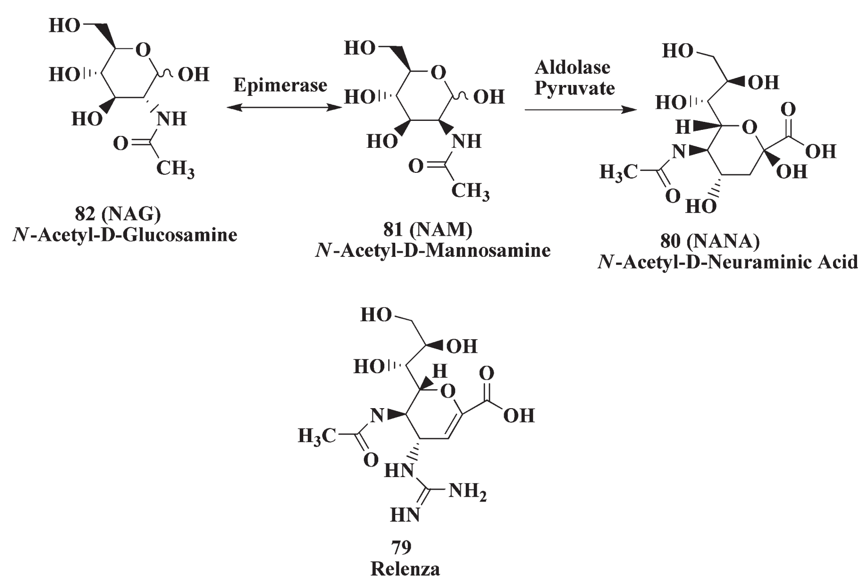

Figure 24. Enzymatic preparation of *N*-acetyl-D-neuraminic acid for zanamavir (Relenza).

product was generated in excellent enantiomeric excess (ee >99.9%) and good overall yield (68−72%). Using this method, an overall quantity of 23 kg of **76** was prepared.

The key step was an aqueous enzymatic reduction using D-lactate dehydrogenase (D-LDH) and formate dehydrogenase (FDH) (Figure 23). Mechanistically, the keto acid salt **78** is stereoselectively reduced to the corresponding (*R*)-hydroxy acid **76** in the presence of D-lactate dehydrogenase by NADH. The cofactor itself is oxidized to NAD^+ in the process. Subsequently, in the presence of formate dehydrogenase, NAD^+ is reduced back to NADH by ammonium formate, which was oxidized to CO_2 and NH_3. In this fashion, the expensive cofactor NAD^+ is regenerated by FDH, and only a catalytic amount of NAD^+ was required.[165]

21. ENZYMATIC PREPARATION OF *N*-ACETYL-D-NEURAMINIC ACID FOR ZANAMAVIR (RELENZA)

Relenza **79** (2,3-didehydro-2,4-dideoxy-4-guanidinyl-*N*-acetylneuraminic acid, Figure 24) is a potent and selective inhibitor of influenza virus sialidase (neuraminidase) and has been approved by the FDA for the treatment of type A and B influenza, the two types most responsible for flu epidemics.[167−169] *N*-Acetyl-D-neuraminic acid **80** (NANA) is the key intermediate for synthesis of Relenza.[168] The chemical synthesis of NANA is lengthy, requiring complex protection and deprotection steps. The most promising option was the use of the NANA aldolase. The synthesis of NANA using the aldolase either from *E. coli* or *Clostridium perfringens* has been reported.[169,170] These groups developed batch processes for the production of NANA from NAM **81** and pyruvate using free or immobilized NANA aldolase. To drive the equilibrium toward NANA, the cheaper pyruvate was generally used in large excess, making the downstream processing rather difficult. An elegant continuous process for NANA synthesis was developed by introducing the NANA-2-epimerase for epimerization of NAG **82** and integrating the epimerization with NANA synthesis in an enzyme−membrane reactor.[171] Subsequently, the enzyme from *E. coli* has been overexpressed in an inducible system (tac-promoter) at very

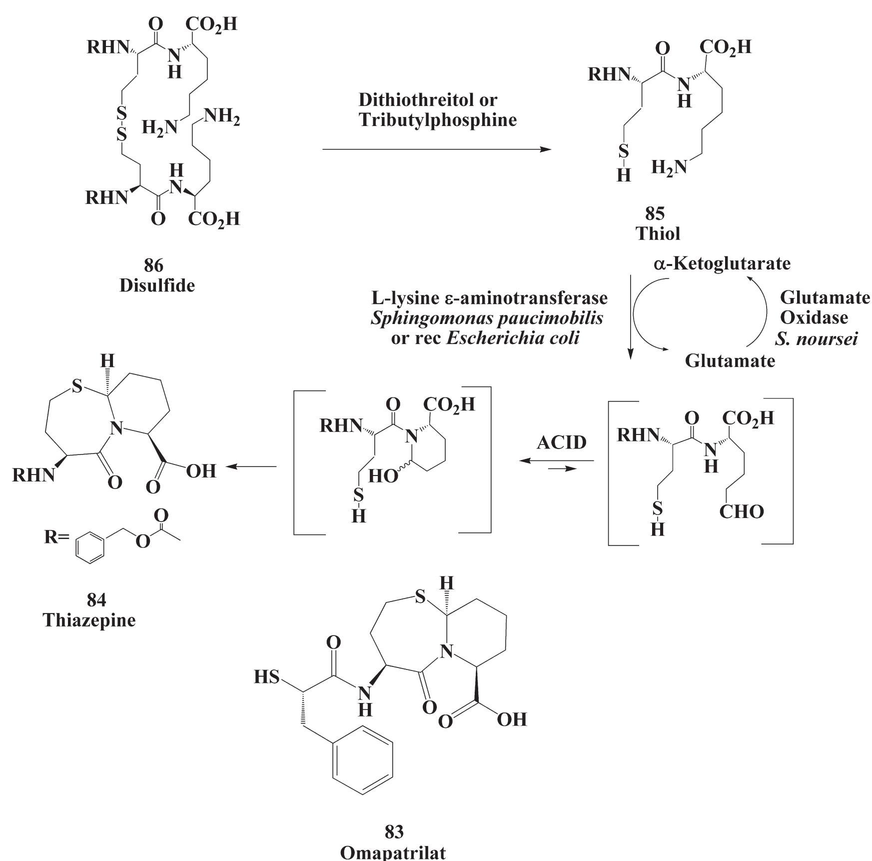

Figure 25. Enzymatic synthesis of thiazepine for ACE and NEP inhibitor.

high expression levels.[172,173] The homogenized bacterial cells were directly used to immobilize the enzyme from crude extracts onto Eupergit-C beads without any clarification, and the immobilized enzyme was used.

In the biotransformation process, a selective precipitation of NAG using isopropyl alcohol was developed to produce a NAM-enriched mixture. This was used in the reaction at a very high NAM concentration (up to 20% w/v) so that NAM itself drives the reaction, and it was not necessary to add a large molar excess of pyruvate. At the end of the reaction, NANA (155 g/L) was crystallized directly from the reaction mixture by the addition of acetic acid. In the manufacturing scale, the same batch of enzymes was reused >2000 cycles in batch column reactors, without any significant loss of activity, to produce multiton quantities of NANA.[172,173]

22. ENZYMATIC SYNTHESIS OF THIAZEPINE FOR ACE AND NEP INHIBITOR

Omapatrilat **83** (Figure 25) is an antihypertensive drug that acts by inhibiting angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP).[174] Effective inhibitors of ACE have been used not only in the treatment of hypertension but also in the clinical management of congestive heart failure. NEP, like ACE, is a zinc metalloprotease and is highly efficient in degrading atrial natriuretic peptide (ANP), a 28-amino acid peptide secreted by the heart in response to atrial distension. By interaction with its receptor, ANP promotes the generation of cGMP via guanylate cyclase activation, thus resulting in vasodilatation, natriuresis, diuresis, and inhibition of aldosterone. Therefore, simultaneous potentiation of ANP via NEP inhibition and attenuation of angiotensin II (AII) via ACE inhibition should lead to complementary effects in the management of hypertension and congestive heart failure.[175]

[(4*S*)-(4*a*,7*a*,10*ab*)]-1-Octahydro-5-oxo-4-[[(phenylmethoxy)-carbonyl]amino]-7*H*-pyrido-[2,1-*b*][1,3]thiazepine-7-carboxylic acid **84** is a key intermediate in the synthesis of omapatrilat **83**. An enzymatic process (Figure 25) was developed for the oxidation of the ε-amino group of (*S*)-lysine in the thiol **85** generated in situ from disulfide N^2-[*N*[[(phenylmethoxy)-carbonyl]-L-homocysteinyl]-L-lysine)-1,1-disulfide **86** to produce compound **84** using L-lysine ε-aminotransferase [LAT] from *Spingomonas paucimobilis* SC 16113.[176] This enzyme was overexpressed in *E. coli*, and a biotransformation process was developed using the recombinant enzyme. The aminotransferase reaction required α-ketoglutarate as the amine acceptor. Glutamate formed during this reaction was recycled back to α-ketoglutarate by glutamate oxidase [GOX] from *Streptomyces noursei* SC 6007.

A selective culture technique was used to isolate eight different types of microbial cultures able to utilize *N*-α-Cbz-(*S*)-lysine as the sole source of nitrogen. Cell extracts prepared from cell suspensions were evaluated for oxidation of the ε-amino group of (*S*)-lysine in the substrate **85** generated from compound **86** by treatment with DTT. Product **84** formation was observed with four cultures. One of the cultures, Z-2, was later identified as *S. paucimobilis* SC 16113. Because of the low activity of LAT in *S. paucimobilis* SC 16113 and to minimize **85** hydrolysis, the enzyme was overexpressed in *E. coli* strain GI724(pAL781-LAT).

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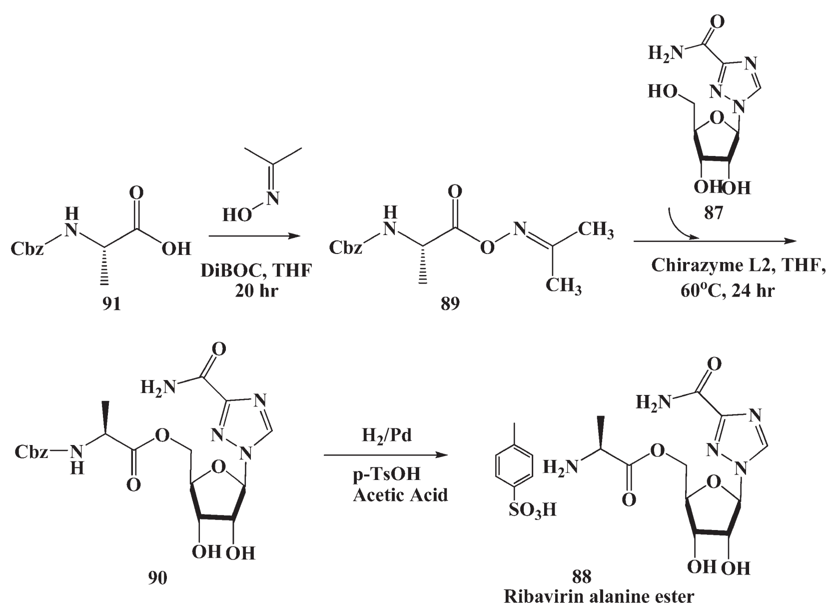

Figure 26. Enzymatic acylation reactions: preparation of chiral intermediate for Ribavirin.

Screening of microbial cultures led to the identification of *S. noursei* SC 6007 as a source of extracellular GOX. *S. noursei* SC 6007 was grown in 380 L fermentors. GOX activity correlated with growth of the culture in the fermentor and reached 0.75 unit/mL at harvest. Starting from the extracellular filtrate, the GOX was purified 260-fold to homogeneity, its amino terminal and internal peptide sequences determined, and expressed in *Streptomyces lividans*.

Biotransformation of compound **85** to compound **84** was carried out using LAT from *E. coli* GI724[pal781-LAT] in the presence of α-ketoglutarate and dithiothreitol (DTT or tributylphosphine) and glutamate oxidase. Reaction yields of 65−67 M % were obtained. To reduce the cost of producing two enzymes, the transamination reactions were carried out in the absence of GOX and with higher levels of α-ketoglutarate. The reaction yield in the absence of GOX averaged only about 33−35 M %; however, the reaction yield increased to 70 M % by increasing the α-ketoglutarate to 40 mg/mL of (10× increase in concentration) and conducting the reaction at 40 °C, equivalent to that in the presence of GOX.[176]

23. ENZYMATIC ACYLATION REACTIONS: PREPARATION OF CHIRAL INTERMEDIATE FOR RIBAVIRIN

Ribavirin **87** (Figure 26) is an antiviral agent used in combination with α-2β interferon to treat hepatitis C.[177,178] Although this therapy is effective against hepatitis C virus, it has several side effects.[179] To improve the pharmacokinetic profile and reduce side effects, a ribavirin prodrug was considered for development. In a series of preclinical evaluations, the alanine ester of ribavirin **88** showed improved bioavalibility and reduced side effects. The synthesis of **88** required the acylation of unprotected ribavirin. The chemical acylation gave a mixture of mono-, di-, and triacylated products. An enzymatic process was developed for the regioselective acylation of ribavirin **87** with the oxime ester of L-carbobenzyloxyalanine **89** to give the desired **90** using Novozym 435 (*C. antarctica* lipase B or Chirazyme L-2). Chemical deprotection of **90** gave **88**. On a preparative scale, the coupling of **91** with acetone oxime in the presence of di-*tert*-butyl dicarbonate in THF was carried out, giving **89** in >96% yield. At the end of the reaction, the reaction mixture was diluted 3-fold with THF, ribavirin was added, and the acylation was initiated by addition of the Novozyme 435. After 24 h at 60 °C, the product **88** was isolated in 85% yield.[180]

■ CONCLUSIONS

The production of single enantiomers of drug intermediates is increasingly important in the pharmaceutical and agrochemical industry. Biocatalysis provides organic chemists an alternate technology to prepare pharmaceutically important chiral compounds. The examples presented in this review are only from a few selected articles. Different types of biocatalytic reactions are capable of generating a wide variety of chiral compounds useful in the development of drugs. The use of hydrolytic enzymes, such as lipases, esterases, proteases, dehalogenases, acylases, amidases, nitrilases, epoxide hydrolases, and decarboxylases, for the resolution of variety of racemic compounds and in the asymmetric synthesis of enantiomerically enriched chiral compounds have been reported. Dehydrogenases and aminotransferases have been successfully used along with cofactors and cofactor-regenerating enzymes for the synthesis of chiral alcohols, amino alcohols, amino acids, and amines. Aldolases and decarboxylases have been effectively used in asymmetric synthesis by aldol condensation and acyloin condensation reactions. Monoxygenases have been used in enantioselective and regioselective hydroxylation, epoxidation, sulfoxidation, and Baeyer−Villiger reactions. Dioxygenases have been used in the chemo-enzymatic synthesis of chiral dihydrodiols. Several approaches have been described, such as enzymatic deracemization and dynamic resolution, to achieve >50% yield and high ee by combination of chemo- or biocatalysts (or both) in sequential reactions or by a single biocatalyst. Stereoinversion via an oxidation−reduction sequence has also been demonstrated in the literature. In the course of the past decade, progress in biochemistry, protein chemistry, molecular cloning, random and site-directed mutagenesis, directed evolution of biocatalysts under desired process conditions, and high cell density fermentation technology has

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opened up unlimited access to a variety of enzymes and microbial cultures as tools in organic synthesis for preparation of key intermediates and final drug products. The future of bicatalysis for synthesis of chiral compounds looks very promising.

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