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REVIEW PAPER

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Enzymatic synthesis of chiral intermediates for pharmaceuticals

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Abstract There has been an increasing awareness of the enormous potential of microorganisms and enzymes for the transformation of synthetic chemicals with high chemo-, regio- and enatioselective manner. Chiral intermediates are in high demand by pharmaceutical industries for the preparation bulk drug substances. In this review article, microbial/enzymatic processes for the synthesis of chiral intermediates for antihypertensive drugs, melatonin receptor agonists, and β3-receptor receptor agonists are described.

Keywords Biocatalysis · Chiral intermediates · Antihypertensive · Melatonin receptor agonists · β3-receptors agonists

Introduction

Chirality is a key factor in the efficacy of many drug products and agrochemicals, and thus the production of single enantiomers of intermediates has become increasingly important in the pharmaceutical industry [8]. Single enantiomers can be produced by chemical or chemo-enzymatic synthesis. The advantages of biocatalysis over chemical synthesis are that enzyme-catalyzed reactions are often highly enantioselective and regioselective. They can be carried out at ambient temperature and atmospheric pressure, thus avoiding the use of more extreme conditions that could cause problems with isomerization, racemization, epimerization, and rearrangement. 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. The preparation of thermostable and pH-stable enzymes by random and site-directed mutagenesis has led to the production of novel biocatalysts. A number of review articles [2, 11, 16, 18, 20, 21, 24, 30, 32] have been published on the use of enzymes in organic synthesis. This review provides examples of the use of enzymes for the synthesis of single enantiomers of key intermediates for drugs.

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Antihypertensive drug

Omapatrilat **1** (Fig. 1A) is an antihypertensive drug that acts by inhibiting angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) [25]. 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 [28].

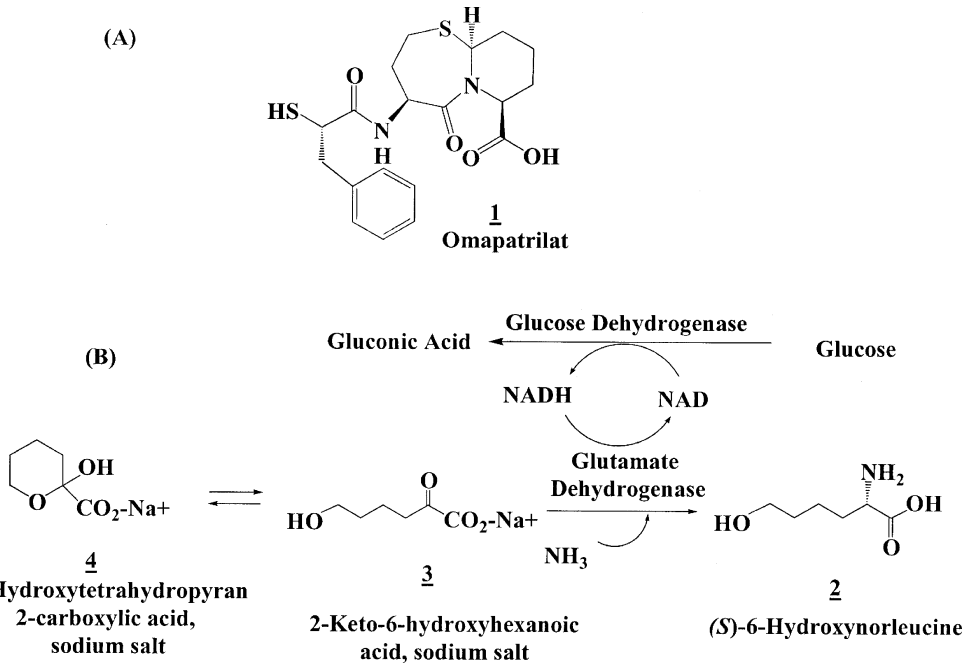


Fig. 1 **A** Structure of Omapatrilat **1**, an antihypertensive drug. **B** Enzymatic synthesis of chiral synthon for Omapatrilat **1:** reductive amination of sodium 2-keto-6-hydroxyhexanoic acid **3** to (*S*)-6-hydroxynorleucine **2** by glutamate dehydrogenase

Enzymatic synthesis of (*S*)-6-hydroxynorleucine

(*S*)-6-Hydroxynorleucine **2** (Fig. 1B) is a key intermediate in the synthesis of Omapatrilat. Reductive amination of ketoacids using amino acid dehydrogenases has long been known to be a useful method for the synthesis of natural and unnatural amino acids [15, 31]. The synthesis and complete conversion of 2-keto-6-hydroxyhexanoic acid **3** to (*S*)-6-hydroxynorleucine **2** by reductive amination using beef liver glutamate dehydrogenase has been demonstrated [12]. Beef liver glutamate dehydrogenase was used for preparative reactions at 100 g/l substrate concentration. As depicted, 2-keto-6-hydroxyhexanoic acid sodium salt **3**, in equilibrium with 2-hydroxy tetrahydropyran-2-carboxylic acid sodium salt **4**, was converted to (*S*)-6-hydroxynorleucine **2**. The reaction requires ammonia and NADH. NAD produced during the reaction was recycled to NADH by the oxidation of glucose to gluconic acid using glucose dehydrogenase from *Bacillus megaterium*. The reaction was complete in about 3 h with reaction yields of 92% and enantiomeric excess (e.e.) of >99% for (*S*)-6-hydroxynorleucine.

The synthesis and isolation of 2-keto-6-hydroxyhexanoic acid **3** required several steps. In a second, more convenient process (Fig. 2) the ketoacid was prepared by treatment of racemic 6-hydroxynorleucine **5** [produced by hydrolysis of 5-(4-hydroxybutyl)hydantoin **6**] with D-amino acid oxidase and catalase. After the e.e. of the remaining (*S*)-6-hydroxynorleucine had risen to >99%, the reductive amination procedure was used to convert the mixture containing 2-keto-6-hydroxyhexanoic acid **3** entirely to(*S*)-6-hydroxynorleucine **2** in 97% yield and 98% e.e. from racemic 6-hydroxynorleucine at 100 g/l. Porcine kidney D-amino acid oxidase and beef liver catalase or *T. variabilis* whole cells (source of both the oxidase and catalase) were used successfully for this transformation [12]. The (*S*)-6-hydroxynorleucine **2** prepared by the enzymatic process was converted chemically to Omapatrilat **1** as described previously [25].

Enzymatic synthesis of allysine ethylene acetal

(*S*)-2-Amino-5-(1,3-dioxolan-2-yl)-pentanoic acid (*S*)-allysine ethylene acetal **7** (Fig. 3) is one of three building blocks used in an alternative synthesis of Omapatrilat **1**. It had been previously prepared via an eight-step chemical synthesis from 3,4-dihydro[2H]pyran [26]. An alternate synthesis of **7** was demonstrated by reductive amination of ketoacid acetal **8** using phenylalanine dehydrogenase (PDH) from *Thermoactinomyces intermedius* [13]. The reaction required ammonia and NADH; NAD produced during the reaction was recyled to NADH by the oxidation of formate to CO_2 using formate dehydrogenase (FDH).

PDH activities and fermentor productivities in cells recovered from fermentations of various cultures are shown in Table 1. *T. intermedius* gave useful activity on a small scale (15 l) but lysed soon after the end of the growth period, making recovery of activity difficult or impossible on a large scale (4,000 l). This problem was solved by cloning and expressing the *T. intermedius* PDH in *Escherichia coli*, inducible by β-D-isopropylthiogalactoside (IPTG). Fermentation of *T. intermedius* yielded 185 units of PDH activity per liter of whole broth in 6 h. In contrast, *E. coli* BL21 (DE3) (pPDH155 K) produced over 19,000 units per liter of whole broth in about 14 h.

Candida boidinii [27] or *Pichia pastoris* [14] grown on methanol are useful sources of FDH.

Expression of *T. intermedius* PDH in *P. pastoris*, inducible by methanol, allowed both enzymes to be

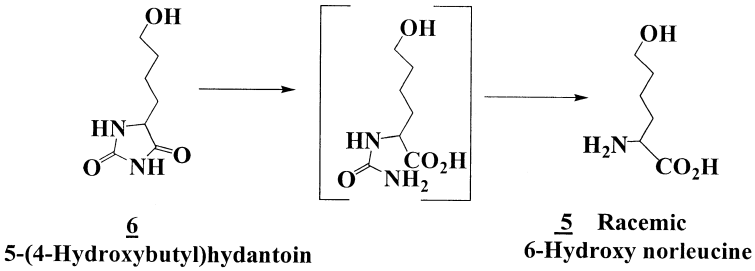
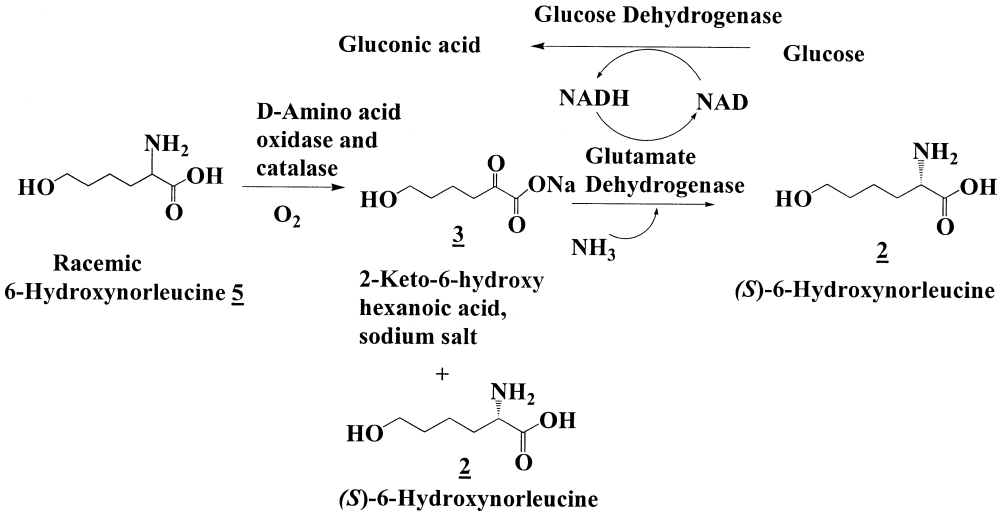


Fig. 2 Conversion of racemic 6-hydroxynorleucine **5** to (*S*)-6-hydroxynorleucine **2** by D-amino acid oxidase and glutamate dehydrogenase



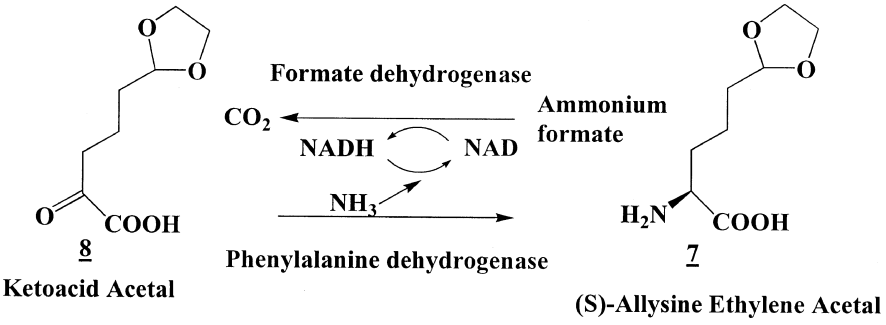


Fig. 3 Enzymatic synthesis of chiral synthon for Omapatrilat **1**: reductive amination of keto acid acetal **8** to (*S*)-allysine ethylene acetal acetal **7** by phenylalanine dehydrogenase. Regeneration of NADH was carried out using formate dehydrogenase

Table 1 Activities and productivities of phenylalanine dehydrogenase and formate dehydrogenase for microorganisms grown in a fermentor. *ND* Not determined

Enzyme	Strain	Specific activity (U/g wet cells)	Volumetric activity (U/l of broth)	Productivity (U/l/week)
Phenylalanine dehydrogenase	*Thermoactinomyces intermedius*	510	185	900
	Escherichia coli	10,000	24,000	94,000
	Pichia pastoris	ND	14,500	25,000
Formate dehydrogenase	*Candida boidinii*	9	120	350
	Pichia pastoris	26	1,950	3,200

obtained from a single fermentation. FDH activity/g wet cells in *P. pastoris* was 2.7-fold greater than for *C. boidinii* and fermentor productivity was increased by 8.7-fold compared to *C. boidinii*. Fermentor productivity for PDH in *P. pastoris* was about 28% of the *E. coli* productivity.

A procedure using heat-dried cells of *E. coli* containing cloned PDH and heat-dried *C. boidinii* was scaled up. A total of 197 kg of **7** was produced in three 1,600-l batches using a 5% concentration of substrate **8** with an average yield of 91 M % and e.e. >98%.

A second-generation procedure, using dried recombinant *P. pastoris* containing *T. intermedius* PDH inducible with methanol and endogenous FDH induced when *P. pastoris* was grown in medium containing methanol, allowed both enzymes to be produced during a single fermentation. The procedure with *P. pastoris* was also scaled up to produce 15.5 kg of **7** in 97 M % yield and >98% e.e. in a 180-l batch using 10% keto-acid **8** concentration. The (*S*)-allysine ethylene acetal **7** produced by the enzymatic process was converted to Omapatrilat **1** [23].

Enzymatic synthesis of thiazepine 9

[4*S*-(4a,7a,10ab)]-1-Octahydro-5-oxo-4-[[(phenylmethoxy) carbonyl]amino]-7H-pyrido-[2,1-b] [1,3]thiazepine-7-carboxylic acid **9** (Fig. 4) is a key intermediate in the synthesis of Omapatrilat **1** [25]. An enzymatic process was developed for the preparation of compound **9**. 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 **10** generated in situ from disulfide N^2-[N[[(phenylmethoxy) carbonyl] *S*-homocysteinyl] *S*-lysine)-1,1-disulphide **11** by treatment with dithiothreitol (DTT). Product **9** formation was observed in four cultures. One of the cultures, later identified as *S. paucimobilis* SC 16113, was used for process development. Due to the low activity of enzyme [*S*-lysine ε-aminotransferase (LAT)] in *S. paucimobilis* SC 16113 and to minimize **10** hydrolysis, LAT was overexpressed in *E. coli* strain GI724(pAL781-LAT) and a biotransformation process was developed [22]. 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.

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 units/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 the enzyme expressed in *Streptomyces lividans*.

Biotransformation of compound **11** to compound **9** was carried out using LAT from *Escherichia coli* GI724[pal781-LAT] in the presence of α-ketoglutarate and 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 (10× increase in concentration) and conducting the reaction at 40 °C, equivalent to that in the presence of GOX [22].

Melatonin receptor agonist

Enantioselective hydrolysis of racemic 1-{2′, 3′-dihydro benzo[b]furan-4′-yl}-1,2-oxirane

Epoxide hydrolase catalyzes the enantioselective hydrolysis of a epoxide to the corresponding enantiomerically enriched diol and unreacted epoxide [2, 17]. The (*S*)-epoxide **12** is a key intermediate in the synthesis of a number of prospective drug candidates [5]. The enantiospecific hydrolysis of the racemic 1-{2′, 3′-dihydro benzo[b]furan-

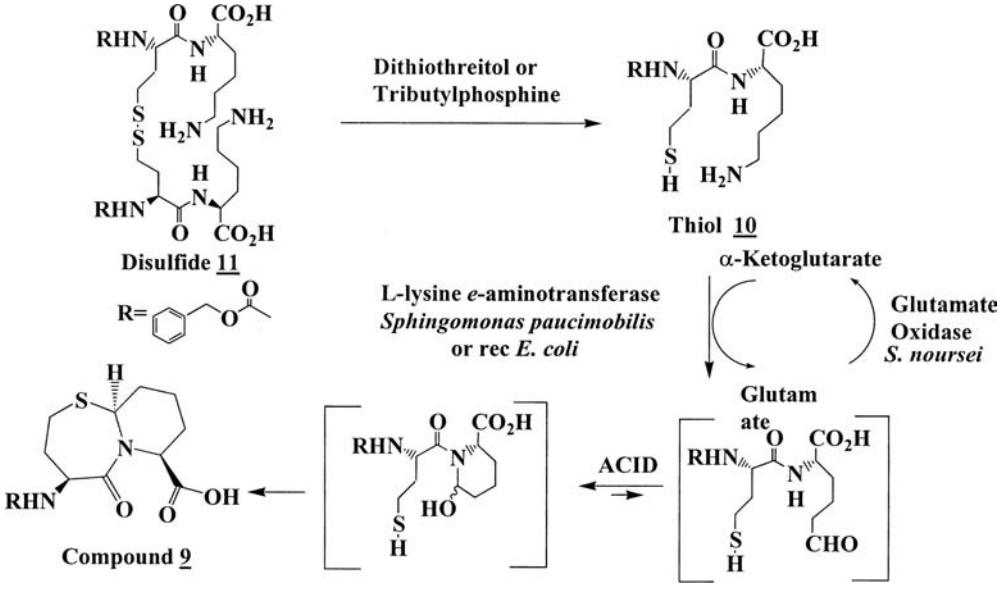


Fig. 4 Enzymatic synthesis of chiral synthon for Omapatrilat **1**: conversion of disulfide **11** to thiazepine **9** by L-lysine ε-aminotransferase

4′-yl}-1,2-oxirane **13** to the corresponding (*R*)-diol **14** and unreacted *S*-epoxide **12** (Fig. 5A) was demonstrated by Goswami et al. [9]. Among cultures evaluated, two *A. niger* strains (SC 16310, SC 16311) and *Rhodotorula glutinis* SC 16293 selectively hydrolyzed the (*R*)-epoxide, leaving behind the (*S*)-epoxide **12** in 95–96% e.e. and 45% yield (theoretical maximum yield is 50%).

Several solvents at 10% v/v were evaluated in attempts to improve the e.e. and yield. Solvents had significant effects on both the extent of hydrolysis and the e.e. of unreacted (*S*)-epoxide **12**. Most solvents gave a lower e.e. product and slower reaction rate compared to reactions without any solvent supplement. Methyl tertiarty butyl ether gave a reaction yield of 45% (theoretical maximum yield 50%) and an e.e. of 99.9% for unreacted (*S*)-epoxide **12**.

Biocatalytic dynamic kinetic resolution of (*R*,*S*)-1-{2', 3'dihydrobenzo[b]furan-4'-yl}-ethane-1,2-diol

Most commonly used biocatalytic kinetic resolution racemates often provide compounds with high e.e., the maximum theoretical yield of product is only 50%. In many cases, the reaction mixture contains a ca. 50:50 mixture of reactant and product that possess only slight differences in physical properties (e.g., a hydrophobic alcohol and its acetate), and thus separation may be very difficult. These issues with kinetic resolutions can be addressed by employing a "dynamic kinetic resolution" process involving a biocatalyst or biocatalyst with metal-catalyzed in-situ racemization [6, 29].

S-1-{2',3'-dihydrobenzo[b]furan-4'-yl}-ethane-1,2-diol **15** (Fig. 5B) is a potential precursor of *S*-epoxide **12** [9]. The dynamic kinetic resolution of the racemic diol **16** to the (*S*)-enantiomer **15** was demonstrated [10] . Seven cultures (*C. boidinii* SC 13821, SC 13822, SC 16115, *Pichia methanolica* SC 13825, SC 13860 and *Hansenula polymorpha* SC 13895, SC 13896) were found to be promising for dynamic kinetic resolution. During biotransformation, the relative proportion of (*S*)-diol **15** increased with time, and at the end of one week the e.e. was found to be in the range of 87–100% (yield 60–75%) with these microorganisms. Only two microorganisms, *Candida parapsilosis* SC 16346 and *Arthrobacter simplex* SC 6379, showed a higher yield of (*R*)-diol **17**. A new compound was formed during these biotransformations as evidenced by the appearance of a new peak in the HPLC of the reaction mixture. The identity of this compound was established as the hydroxy ketone **18** from an LC-MS peak at mass 178. The area of the HPLC peak for hydroxy ketone **18** first increased with time, reached a maximum, and then decreased, as expected for the proposed dynamic kinetic resolution pathway. *C. boidinii* SC 13822, *C. boidinii* SC 16115, and *P. methanolica* SC 13860 transformed the racemic diol **16** in 3–4 days, to *S*-diol **15** in yields of 62–75% and e.e.'s of 90–100%.

β3-Receptor agonist

β3-Adrenergic receptors are found on the cell surface of both white and brown adipocytes and are responsible for lipolysis, thermogenesis, and relaxation of intestinal

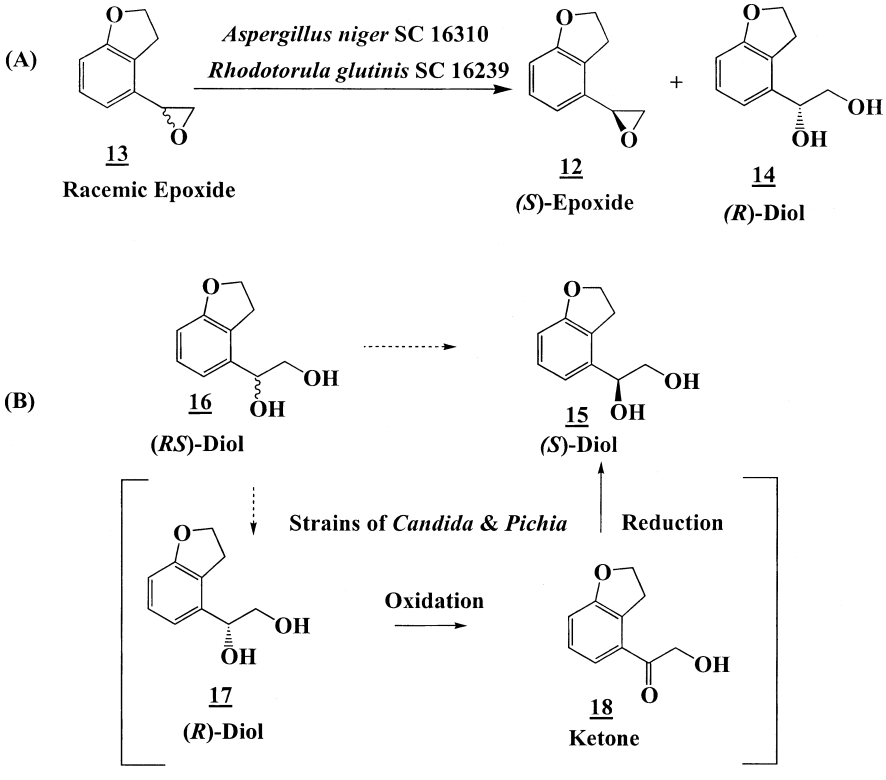


Fig. 5 **A** Synthesis of chiral intermediates for melatonin receptor agonist: enantioselective microbial hydrolysis of racemic epoxide **13** to the corresponding (*R*)-diol **14** and unreacted (*S*)-epoxide **12**. **B** Stereoinversion of racemic diol **16** to *S*-diol **15** by *Candida boidinii* and *Pichia methanolica*

smooth muscle [1]. Consequently, several research groups are engaged in developing selective β3 agonists for the treatment of gastrointestinal disorders, type II diabetes, and obesity [3, 7]. Three different biocatalytic syntheses of chiral intermediates required for the total synthesis of β3 receptor agonists **19** (Fig. 6A) have been investigated [19].

Microbial reduction of 4-benzyloxy-3-methane sulfonylamino-2′-bromoacetophenone

The microbial reduction of 4-benzyloxy-3-methanesulfonylamino-2′-bromoacetophenone **20** (Fig. 6B) to the corresponding (*R*)-alcohol **21** has been demonstrated [19] using *Sphingomonas paucimobilis* SC 16113. The growth of *S. paucimobilis* SC 16113 was carried out in a 750-l fermentor and cells (60 kg) harvested from the fermentor were used to conduct the biotransformation in 10-l and 200-l preparative batches. The cells were suspended in 80 mM potassium phosphate buffer (pH 6.0) at 20% (w/v, wet cells) concentration and supplemented with compound **20** (2 g/l) and glucose (25 g/l); the reduction was carried out at 37 °C. In some batches, the fermentation broth was concentrated three-fold by microfiltration, washed with buffer by diafiltration, and used directly in the bioreduction process. In all the batches, reaction yields of >85% and e.e.s of >98% were obtained. The isolation of alcohol **21** from the 200-l batch gave 320 g (80% yield) of product with an e.e. of 99.5%.

In an alternate process, frozen cells of *S. paucimobilis* SC 16113 were used with XAD-16 hydrophobic resin (50 g/l) adsorbed substrate at 10 g/l concentration. In this process, an average reaction yield of 85% and an e.e. of >99% were obtained for the alcohol **21**. At the end of the biotransformation, the reaction mixture was filtered on a 100-mesh (150 μm) stainless steel screen, and the resin retained by the screen was washed with water. The product was then desorbed from the resin with acetonitrile and crystallized in an overall 75 M% yield and 99.8% e.e.

Enzymatic resolution of racemic α-methyl phenylalanine amides

The chiral amino acids **22** and **23** (Fig. 7A) are intermediates for the synthesis of β3-receptor agonists [3, 7]. These are available via the enzymatic resolution of racemic α-methyl phenylalanine amide **24** and α-methyl-4-methoxyphenylalanine amide **25**, respectively, by an amidase from *Mycobacterium neoaurum* ATCC 25795 [19]. Wet cells (10% w/v) completed reaction of amide **24** in 75 min with a yield of 48 M% (theoretical max. 50%) and an e.e. of 95% for the desired (*S*)-amino acid **22**. Alternatively, freeze-dried cells were suspended in 100 mM potassium phosphate buffer (pH 7.0) at 1% concentration to give complete reaction in 60 min with a yield of 49.5 M% (theoretical max. 50%) and an e.e. of 99% for the (*S*)-amino acid.

Freeze-dried cells of *M. neoaurum* ATCC 25795 and partially purified amidase (amidase activity in cell extracts purified five-fold by diethyl aminoethyl celluose column chromatography) were used for the biotransformation of compound **25**. A reaction yield of 49 M% and an e.e. of 78% were obtained for the desired product **23** using freeze-dried cells. The reaction was completed in 50 h. Using partially purified amidase, a reaction yield of 49 M% and an e.e. of 94% were obtained after 70 h.

Enantioselective hydrolysis of diethyl methyl-(4-methoxyphenyl)-propanedioate

The (*S*)-monoester **26** (Fig. 7B) is a key intermediate for the syntheses of β3-receptor agonists. The enan-

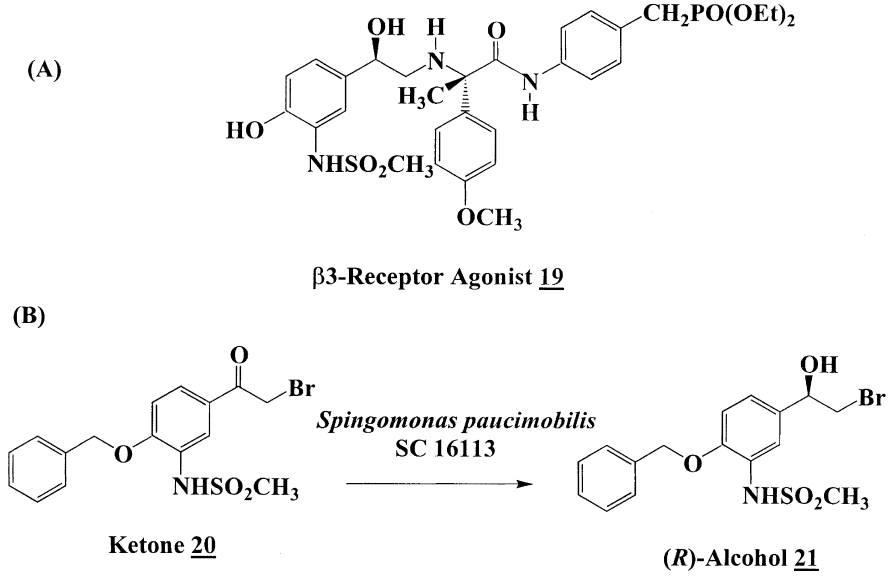


Fig. 6 A Enzymatic synthesis of chiral synthon for β-3-receptor agonist **19**. **B** Enantioselective reduction of 4-benzyloxy-3-methanesulfonylamino-2′-bromoacetophenone **20** to (*R*)-alcohol **21**

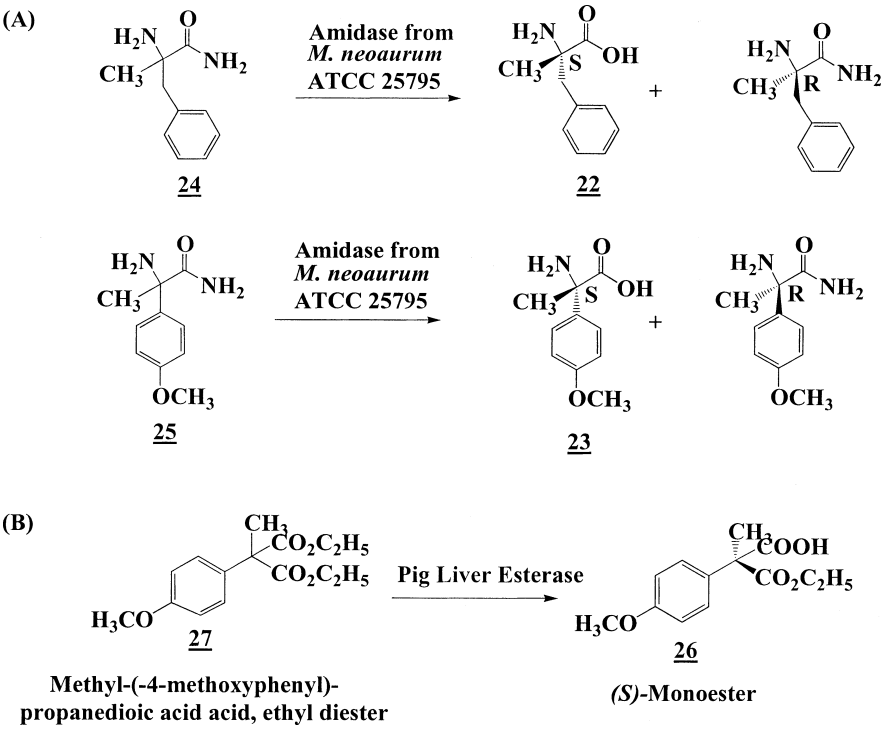


Fig. 7A Enantioselective hydrolysis of α-methyl phenylalanine amide *24* and α-methyl-4-hydroxyphenyl-alanine amide *25* by amidase. **B** Enantioselective enzymatic hydrolysis of methyl-(4-methoxyphenyl)-propanedioic acid ethyl diester *27* to (*S*)-monoester *26*

tioselective enzymatic hydrolysis of diester **27** to the desired acid ester **26** by pig liver esterase [19] has been demonstrated. In various organic solvents, the reaction yields and e.e. of monoester **25** were dependent upon the solvent used. High e.e.s (>91%) were obtained with methanol, ethanol, and toluene as a cosolvent. Ethanol gave the highest reaction yield (96.7%) and e.e. (96%).

It was observed that the e.e. of the (*S*)-monoester **25** was increased by decreasing the temperature from 25 °C to 10 °C when biotransformation was conducted in a biphasic system using ethanol as a cosolvent. A semi-preparative 30-g-scale hydrolysis was carried out using 10% ethanol as a cosolvent in a 3-l reaction mixture (pH 7.2) at 10 °C for 11 h. A reaction yield of 96 M% and an e.e. of 96.9% were obtained.

Conclusions

The production of single enantiomers of drug intermediates is increasingly important in the pharmaceutical industry. Organic synthesis is one approach to the synthesis of single enantiomers, and biocatalysis provides an added dimension and an enormous opportunity to prepare pharmaceutically useful chiral compounds. The advantages of biocatalysis over chemical catalysis are that enzyme-catalyzed reactions are stereoselecive and regioselective and can be carried out at ambient temperature and atmospheric pressure. The use of different classes of enzymes for the catalysis of many different types of chemical reactions is capable of generating a wide variety of chiral compounds. This includes the use of hydrolytic enzymes such as lipases, esterases, proteases, dehalogenases, acylases, amidases, nitrilases, lyases, epoxide hydrolases, decarboxylases, and hydantoinases in the resolution of racemic compounds and in the asymmetric synthesis of enantiomerically enriched chiral compounds. Oxido-reductases and aminotransferases have been used in the synthesis of chiral alcohols, aminoalcohols, 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 and epoxidation reactions and dioxygenases in the chemo–enzymatic synthesis of chiral diols. In the course of the last decade, progress in biochemistry, protein chemistry, molecular cloning, random and site-directed mutagenesis, directed evolution of biocatalysts and fermentation technology have opened up unlimited access to a variety of enzymes and microbial cultures as tools in organic synthesis.

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