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ORIGINAL ARTICLE

An intracellular esterase from *Bacillus cereus* catalyzing hydrolysis of 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol

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Abstract An esterase from *Bacillus cereus* that hydrolyzes 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol was purified to homogeneity. After purification, the molecular mass of the esterase was determined as 43 kDa by SDS-PAGE, and estimated as 45 kDa using gel filtration, suggesting that the enzyme is a monomer. The optimum pH and temperature for activity of the enzyme were 7.0 and 40°C respectively. The N-terminal sequence was determined. Whole cells of this strain were applied to the resolution of 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol.

Keywords *Bacillus cereus* · Esterase · 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol · Propranolol

Introduction

β-Adrenergic blocking agents such as (S)-propranolol are used mainly to treat angina pectoris, hypertension and other cardiac diseases (Ariens 1984); the desirable therapeutic activities reside mainly in the S-enantiomers of these compounds (Howe and Shanks 1968). Approaches to the preparation of (S)-propranolol can be based on chemical (Klunder et al. 1986; Sharpless et al. 1992; Hiroaki et al. 1993; Veloo and Koomen 1993; Chang and Sharpless 1996; Takuya et al. 2001) or chemoenzymatic (Noritada and Nobuo 1985; Fuganti et al. 1986; Bianchi et al. 1988; Yoshiyasu et al. 1988; Bevinakatti and Banerji 1991; Chiou et al. 1997; Pchelka et al. 2000; Subhash et al. 2000) methods.

1-Chloro-3-(1-napthyloxy-2-propanol) and its *O*-acetate derivative are key chiral intermediates in the chemo-enzymatic methods for the synthesis of propranolol. Resolution of the intermediates can be achieved in aqueous phase or organic solvents. Lipases of different origins, e.g., lipase from *Pseudomonas* species (Hsu et al. 1990), *Pseudomonas cepacia* (Bevinakatti and Banerji 1991), *Mucor miehei* (Ader and Schneider 1992), *Candida antarctica* (Kaimal et al. 1992), *Candida cylindracea*, *Mucor javanicus*, *Trichosporon* sp., *Bacillus* sp. (Kapoor et al. 2003) and *Pseudomonas fluorescens* (Di Nunno et al. 2000) have been successfully resolved. In this work, a *Bacillus cereus* strain expressing an intracellular esterase activity was identified using 1-chloro-3-(1-napthyloxy-2-acetoxypropanol) as the prochiral substrate. Whole cells of the strain were used for kinetic resolution of the substrate (Fig. 1). The esterase was also purified and characterized.

Materials and methods

Materials

The two enantiomers of propranolol and para-nitrophenyl fatty acid esters were purchased from Sigma (St, Louis, MO), 1-chloro-3-(1-napthyloxy)-2-propanol and 1-chloro-3-(1-napthyloxy)-2-acetoxy propanol were synthesized and purified in our laboratory according to described methods (Bevinakatti and Banerji 1991). The compound structures were confirmed by IR and NMR spectroscopy.

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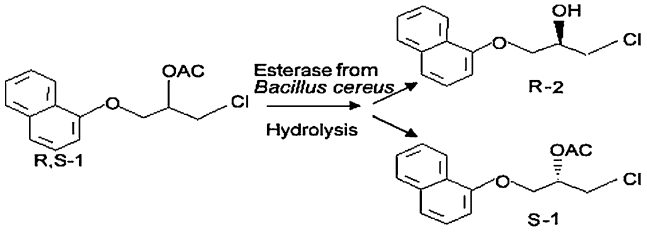

Fig. 1 Esterase-catalyzed resolution of racemic 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol. *S-1* 1-Chloro-3-(1-napthyloxy)-2-acetoxypropanol, *R-2* 1-chloro-3-(1-napthyloxy)-2-propanol

Microorganism

Strain WL 24 was isolated from soil samples from the suburbs of Beijing city employing a screening agar medium containing 1.0% olive oil, 2.0% yeast extract, 0.1% K_2HPHO_4, 0.05% $MgSO_4$, 0.05% $CaCl_2$, 0.5% Tween 80 and 1.5% agar. The strain was identified as *Bacillus cereus* using the 16S rRNA method (Fox et al. 1977). The strain was maintained in LB agar at 4°C or in LB containing 20% glycerol at −70°C.

Culture conditions

The microorganism was cultivated in 500 ml flasks with 100 ml medium containing 1.5% olive oil, 2.5% PEG-400, 0.5% soluble starch, 2% yeast extract $(NH)_4SO_4$, 0.1% K_2PO_4, 0.05% $MgSO_4$, 0.01% $CaCl_2$, and 0.001% $FeSO_4$. The initial pH of the culture medium was adjusted to 7.0 with 1 M NaOH. After inoculation with 10 ml of a preculture in the same medium, the culture was grown at 30°C and 220 rpm for 24 h.

Purification of esterase from *Bacillus cereus*

All procedures were performed at room temperature. Cells from a 24-h culture were harvested by centrifugation (8,000 rpm, 15 min). The cell pellet (10 g) was washed twice with 20 mM pH 7.2 Tris-HCl buffer and disrupted by sonication in same buffer with 1 mM EDTA. Cell debris was eliminated by high speed centrifugation (15,000 rpm, 25 min), one volume ethanol was added to the suspension, which was then left at room temperature for 30 min. The precipitate was eliminated by high speed centrifugation (15,000 rpm, 25 min), then another one volume ethanol was added and the suspension left at room temperature for 30 min. The suspension was centrifuged at 15,000 rpm for 25 min and the pellet was re-dissolved in a minimal volume of 20 mM pH 7.2 Tris-HCl buffer. The enzyme solution was dialyzed against the same buffer for 8 h then centrifuged to eliminate un-dissolved pellet. The enzyme solution was loaded onto a DEAE-Sepharose CL6B column (1.0×15 cm) equilibrated with 20 mM pH 7.2 Tris-HCl. The flow rate was set at 1.5 ml/min. After washing, a linear 0–1 M NaCl gradient in the same buffer was applied. The active fractions eluted were pooled, dialyzed against 20 mM pH 7.2 Tris-HCl with 1 M $(NH_4)_2SO_4$, and applied to a column of Phenyl Sepharose (1.0×5 cm), equilibrated in 20 mM pH 7.2 Tris-HCl containing 1 mM $(NH_4)_2SO_4$. After washing, a gradient from 1 M $(NH_4)_2SO_4$ to 0 M $(NH_4)_2SO_4$ was applied, the active fraction were pooled, and dialyzed against 10 mM pH 7.2 phosphate buffer. The enzyme solution was concentrated and re-dissolved in 0.5 ml 10 mM pH 7.2 phosphate buffer, and the solution was then loaded onto a Sephacryl S200 HR column (1.0×100 cm) equilibrated and eluted with 10 mM phosphate buffer (pH 7.2). Elution was performed at a rate of 1 ml/min and active fractions were pooled.

Molecular mass determination by size-exclusion chromatography

A Sephacryl S200 HR column (GE, Piscataway, NJ) was calibrated with the following proteins (Serva, Heidelberg, Germany): ribonuclease (13.7 kDa), ovalbumin (45 kDa), bovine serum albumin (67 kDa), and alcohol dehydrogenase (150 kDa).

Protein determination

Protein concentration was determined as described (Bradford 1976) with bovine serum albumin as standard protein.

Gel electrophoresis

SDS-PAGE of the purified enzyme was conducted as described by Laemmli (1970) using 12% gels at pH 8.8. The same procedure was used to set up a calibration curve with marker proteins from the LMW Electrophoresis Calibration Kit supplied by Sigma.

Determination of N-terminal amino acid sequence

The purified esterase on SDS-PAGE gel was transferred to a PVDF membrane (Bio-Rad, Richmond, CA). After blotting, the PVDF membrane was stained and the esterase band was excised. N-terminal Edman sequencing was performed on an Applied Biosystems Procise 419 sequencer (Foster City, CA).

Hydrolysis activity assay of racemic 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol

1-Chloro-3-(1-napthyloxy)-2-acetoxypropanol (0.2 mg) was dissolved in ethanol, and 0.85 ml 20 mM phosphate buffer (pH 7.2) was added to a flask with a lid. Enzyme (0.05 mg) was then added and the system was sealed and

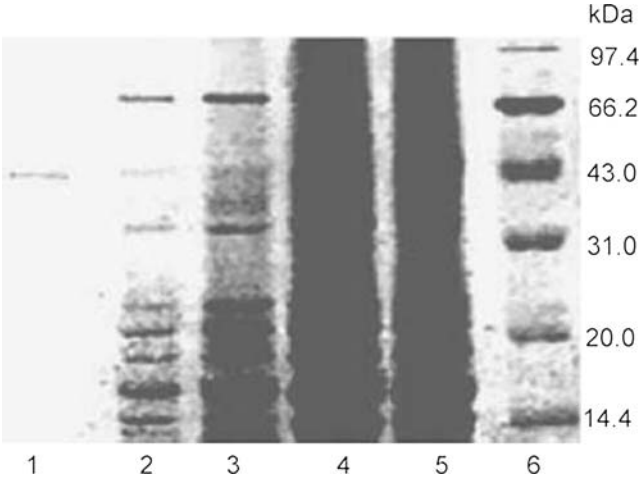

Fig. 2 SDS-PAGE analysis of purified esterase. Lanes: *1* Sephacryl S200 HR, *2* Phenyl sepharose, *3* DEAE-Sepharose CL6B, *4* ethanol precipitation, *5* sonication debris, *6* protein marker

shaken at 30°C, 200 rpm for 6 h. The reaction mixture was extracted with 1 ml ethyl acetate. The ethyl acetate extract (10 μl) was applied to a Phenomenex 3022 column (http://www.phenomenex.com) and was eluted with a mobile phase of 80% hexane/20% ethyl acetate (v/v). The UV absorption of 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol was monitored at 256 nm. One unit of enzyme activity is defined as the amount of enzyme hydrolyzing 1 μmol substrate per minute.

Determination of ee value by capillary electrophoresis

Solvent in the sample from the hydrolysis reaction was evaporated, and the residue was submitted to a silicon column mobile phase. The mobile phase was same as for the hydrolysis activity assay. Purified ester was reacted with 20 volumes of 10% NaOH and a two times molar ratio of isopropylamine for 12 h at room temperature. The product was then applied to a Beckman P/ACE SYSTEM-MDQ capillary electrophoresis system (Fullertin, CA) with an unmodified silicon capillary (75 μm I.D. × 375 μm O.D.). Capillary electrophoresis analysis was conducted as described (Wang et al. 2001). The enantioselectivity for the remaining substrate was expressed by enantiomeric excess (ee S) and enantiomeric ratio (*E* value) (Chen et al. 1982):

$$e.eS = \frac{S_S - S_R}{S_S + S_R} \times 100\%$$

$$E = \frac{\ln[(1-c)(1-eeS)]}{\ln[(1-c)(1+eeS)]}$$

where c stands for conversion, and S_S and S_R stand for S-propranolol and R-propranolol.

Esterase and activity assay

The standard assay system consisted of 10 mM para-nitrophenyl fatty acid esters, 100 mM NaH_2PO_4-citric acid buffer, pH 7.5, 0.1% (w/v) of sodium deoxycholate, and 10 μg enzyme, in a final volume of 0.2 ml. The mixture, without the substrate, was brought to 40°C. The reaction was started by the addition of substrate (10 μl), which had previously been prepared as a stock solution in isopropanol. Changes in absorbance at 405 nm were recorded for 20 min using a 721 model spectrophotometer (Kebo, Hangzhou, PR China). The reaction was stopped by adding two volumes of ethanol. The enzyme was replaced by water to generate a blank, and, in every measurement, the effect of non-enzymatic hydrolysis of substrates was taken into account and subtracted from the value measured when the enzyme was added. The molar extinction coefficients of para-nitrophenol were also determined under each reaction condition prior to the measurements. One unit of esterase activity is defined as the amount of enzyme forming 1 μmol substrate per minute.

Results

Screening

A collection of microorganisms screened from soil samples was used for the resolution of 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol. Whole cells of strain WL 24 were found to have the highest selectivity for resolution of the

Table 1 Summary of esterase purification

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	786	5.6	0.007	100	1
Ethanol precipitation	116	4.5	0.04	80.0	5.7
DEAE-Sepharose CL6B	9	2.2	0.24	39.2	34.2
Phenyl Sepharose	1.2	0.9	0.75	16	107.1
Sephacryl S200HR	0.1	0.32	3.2	5.7	457.1

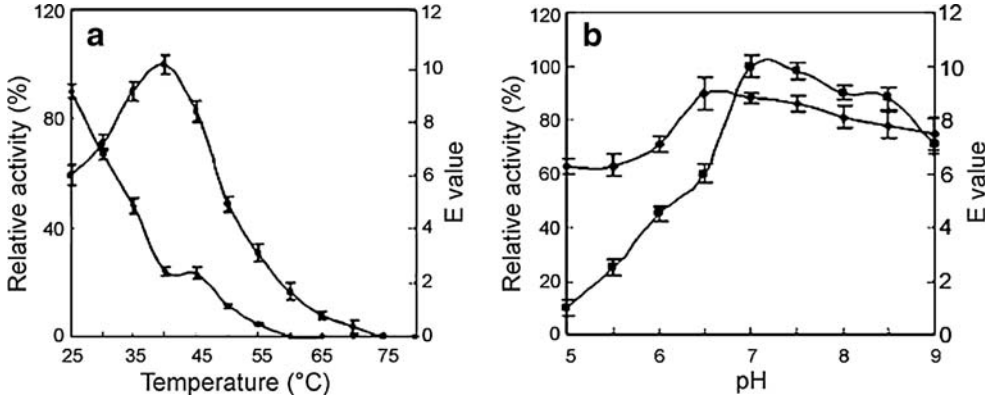

Fig. 3 Temperature (**a**) and pH (**b**) optima of purified esterase from *Bacillus cereus*. *Left axis* (■) relative activities, *right axis* (♦) E values. **a** Purified esterase (10 μg) was assayed under standard hydrolysis assay conditions at various temperatures. **b** Purified esterase (10 μg) was assayed under standard hydrolysis assay conditions with different buffers (100 mM NaH_2PO_4-citric acid buffer for pH 5.0–8.0; 100 mM glycine/NaOH for pH 8.5–9.0)

substrate, while the supernatant of the same culture exhibited no activity towards the substrate; therefore, the enzyme is an intracellular protein. Taxonomic studies identified the strain as *Bacillus cereus*.

Esterase purification

The esterase was purified 457 fold with a four-step purification procedure based on the hydrolysis activity assay for 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol. The purified fraction appeared homogenous on SDS gels (Fig. 2, Table 1).

Physical properties of the esterase

The molecular mass of the esterase was determined by SDS-PAGE as 43 kDa. The molecular mass of the native enzyme was determined as 42 kDa after calibration of the gel filtration (Sephacryl S200 HR) column. Maximum activity was found at pH 7.0 and 40°C. Maximum enantioselectivity was also found at pH 7.0, with the temperature optimum for enantioselectivity decreasing with increasing transformation temperature (Fig. 3).

Table 2 Specific activity for hydrolysis of various substrates. *p-NP* Para-nitrophenyl

Substrate (carbon chain length)	Specific activity (U/mg)
p-NP acetate (C2)	116.3 ± 9.4 × 10^3
p-NP butyrate (C4)	124.6 ± 7.8 × 10^3
p-NP caproate (C6)	215.6 ± 8.4 × 10^3
p-NP laurate (C12)	89.5 ± 2.3 × 10^3
p-NP tetradecanoate (C14)	64.6 ± 2.1 × 10^3
p-NP oleate (C18)	32.4 ± 3.5 × 10^3
p-NP stearate (C18)	21.1 ± 0.8 × 10^3

Substrate specificity of the esterase

Esterase specificity was tested on various p-nitrophenyl esters with increasing chain length. The results reported in Table 2 indicate that esterase activity is influenced strongly by the chain length of the acyl group. Enzyme activity displayed an increasing trend in terms of the rate of ester hydrolysis up to C6, and decreased as the chain length increased further.

N-terminal sequence analysis

The N-terminal amino acid sequence of the esterase from *Bacillus cereus* was determined as MILVTQTMKG. This sequence was compared with sequences of *Bacillus* esterase and lipase as deposited in GenBank. However, no signif-

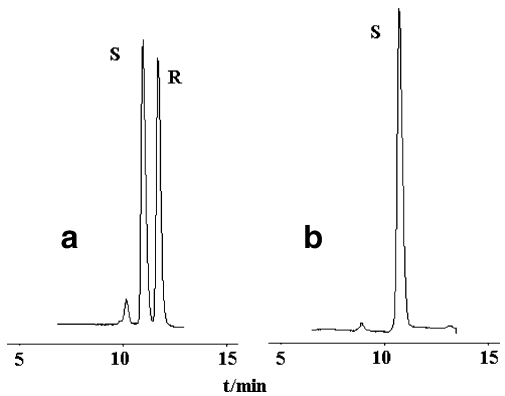

Fig. 4 a,b S-Propranolol produced by a chemoenzymatic process employing whole cells of *Bacillus cereus* WL 24. **a** Standard propranolol, **b** whole cell resolution: 100 mM, phosphate-trihydroxyethylamine buffer (pH 3.0), 30 mM hydroxypropyl-β-cyclodextrin, 50 cm unmodified capillary, 20 kV voltage. The conversion of the substrate was controlled beyond 73%; the ee value reached 99%

icant homology to other lipase or esterase sequences was found.

Discussion

The use of *Bacillus* carboxylesterase for the production of valuable chiral building blocks seems to be a promising approach. For example, a *Bacillus cereus* strain demonstrated hydrolysis activity towards prochiral 2-phenyl-1,3-propanediol diacetate and accumulated the corresponding chiral monoacetates in the reaction mixture (Mitsui et al. 2007); a novel carboxylesterase with high enantioselectivity toward racemic esters of 1,2-O-isopropylideneglycerol was isolated from *Bacillus coagulans* (Molinari et al. 1996; Romano et al. 2005); an extracellular esterase from *Bacillus sphaericus* hydrolyzed ethyl 2-hydroxyalkanoates with high enantioselectivity (Jackson et al. 1995); resolution of R, S-naproxen enantiomers by hydrolysis of the corresponding racemic ethyl esters was achieved by employing carboxylesterase from *Bacillus* sp. (Quax and Broekhuizen 1994), >while the stereospecific hydrolysis of racemic menthylacetate was achieved using esterase from *Bacillus subtilis* (Brookes and Lilly 1986).

There are many reports describing resolution of the same substrate by lipase, such as lipase from *Pseudomonas* sp., which possesses a high E value (around 100, Bevinakatti and Banerji 1991). The esterase from *Bacillus cereus* had a moderate E value (around 8 at 30°C). However, it was relatively economical when whole cells were used for kinetic resolution of the substrate. If conversion of the substrate was controlled beyond 73%, the ee value could reach 99% (Fig. 4). When 1 g dry cells was used, 73% conversion could be obtained for 0.8 g substrate in 12 h. To our knowledge, this is also the first report of the utilization of *Bacillus cereus* in the resolution of 1-chloro-3-(1-napthyloxy)-2-acetoxypropanol. The lack of homology of the N-terminal sequence to other known esterase sequences indicates that the esterase from *Bacillus cereus* might represent a novel esterase.

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References

Ader U, Schneider MP (1992) Enzyme assisted preparation of enantiomerically pure β-adrenergic blockers. I. A facile screening method for suitable biocatalysts. Tetrahedron: Asymmetry 3:201–204

Ariens E (1984) A basis for sophisticated nonsense in pharmacokinetics and clinical pharmacology. Eur J Clin Pharmacol 26:663–668

Bevinakatti HS, Banerji AA (1991) Practical chemoenzymic synthesis of both enantiomers of propranolol. J Org Chem 56:5372–5375

Bianchi D, Cesti P, Battistel E (1988) Anhydrides as acylating agents in lipase-catalyzed stereoselective esterification of racemic alcohols. J Org Chem 53:5531–5534

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quanties of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brookes IK, Lilly MP (1986) Stereospecific hydrolysis of d, l-menthyl acetate by *Bacillus subtilis*; mass-transfer interactions in a liquid–liquid system. Enzyme Microb Technol 8:53–57

Chang HT, Sharpless KB (1996) A practical route to enantiopure 1, 2-aminoalcohols. Tetrahedron Lett 37:3219–3222

Chen CS, Wu SH, Girdaukas GS, Sih CJ (1982) Quantitative analyses of biochemical kinetic resolution of enantiomers. J Am Chem Soc 104:7294–7299

Chiou TW, Cheng CC, Chung TL, Dar FT (1997) Kinetic resolution of propranolol by a lipase-catalyzed N-acetylation. Bioorg Med Chem Lett 7:433–436

Di Nunno L, Franchini C, Scilimati A, Sinicropi MS, Tortorella P (2000) Chemical and chemoenzymatic routes to 1-(benzothiazol-2-ylsulfanyl)-3-chloropropan-2-ol, a precursor of drugs with potential β-blocker activity. Tetrahedron Asymmetry 11:1571–1583

Fox GE, Pechman KR, Woese CR (1977) Comparative cataloguing of 16S ribosomal ribonucleic acid: molecular approach to procaryotic systematic. Int J Syst Bacteriol 27:44–57

Fuganti C, Grasselli P, Seneci PF, Servi S (1986) Immobilized benzylpenicillin acylase: application to the synthesis of optically active forms of carnitin and propranolol. Tetrahedron Lett 27:2061–2062

Hiroaki S, Takeyuki S, Noriie I, Koichi T, Tadamasa D, Kimio O, Masakatsu S (1993) Catalytic asymmetric nitroaldol reaction using optically active rare earth BINOL complexes, investigation of the catalyst structure. J Am Chem Soc 115:10372–10373

Howe R, Shanks RG (1968) β-adrenergic blocking agents. iii the optical isomers of pronethalol, propranolol, and several related compounds. J Med Chem 11:1118–1121

Hsu SH, Wu SS, Wang YF (1990) Lipase-catalyzed irreversible transesterification using enol esters: XAD-8 immobilized lipoprotein lipase-catalyzed resolution of secondary alcohols. Tetrahedron Lett 31:6403–6406

Jackson MA, Labeda DA, Becker LA (1995) Enantioselective hydrolysis of ethyl 2-hydroxyalkanoates by an extracelluar esterase from a *B. sphaericus* strain. Enzyme Microb Technol 17:175–179

Kaimal TNB, Prasad RBN, Rao TC (1992) A practical chemoenzymic route to (S)-(−)-propranolol. Biotechnol Lett 14:21–26

Kapoor M, Anand N, Koul S, Chimni SS, Manhas KS, Raina C, Parshad R, Taneja SC, Qazi GN (2003) Kinetic resolution of 1-chloro-3-(1-naphthyloxy)-2-propanol, an intermediate in the synthesis of β-adrenergic receptor blockers. Bioorg Chem 31:259–269

Klunder JM, Ko SY, Sharpless KB (1986) Asymmetric epoxidation of allyl alcohol: efficient routes to homochiral β-adrenergic blocking agents. J Org Chem 51:3710–3712

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacterial T4. Nature 227:680–685

Mitsui R, Shinya S, Ichiyama Y, Kudo K, Tsuno T, Tanaka M (2007) Site-specific and asymmetric hydrolysis of prochiral 2-phenyl-1, 3-propanediol diacetate by a bacterial esterase from an isolated strain. Biosci Biotechnol Biochem 71:1858–1864

Molinari F, Brenna O, Valenti M, Aragozzini F (1996) Isolation of a novel carboxylesterase from *Bacillus coagulans* with high enantioselectivity toward racemic esters of 1, 2-O-isopropylideneglycerol. Enzyme Microb Technol 19:551–556

Noritada M, Nobuo O (1985) Preparation of optically active 1-acetoxy-2-aryloxypropionitriles and its application to a facile synthesis of (S)-(−)-propranolol. Tetrahedron Lett 26:5533–5534

Pchelka BK, Loupy A, Plenkiewicz J, Blanco L (2000) Resolution of racemic 1-azido-3-aryloxy-2-propanols by lipase-catalyzed enantioselective acetylation. Tetrahedron Asymmetry 11:2719–2732

Quax WJ, Broekhuizen CP (1994) Development of a new *Bacillus* carboxyl esterase for use in the resolution of chiral drugs. Appl Microbiol Biotechnol 41:425–431

Romano D, Falcioni F, Mora D, Molinari F, Buth A, Ansorge-Schumacher M (2005) Enhanced enantioselectivity of Bacillus coagulans in the hydrolysis of 1, 2-O-isopropylidene glycerol esters by thermal knock-out of undesired enzymes. Tetrahedron Asymmetry 16:841–845

Sharpless KB, Amberg W, Bennani YL, Crispino GA, Hartung J, Jeong KS, Kwong HL, Morikawa K, Wang ZM, Xu D, Zhang XL (1992) The osmium-catalyzed asymmetric dihydroxylation: a new ligand class and a process improvement. J Org Chem 57:2768–2771

Subhash VD, Prashant NP, Manikrao MS (2000) Biotransformations with *Rhizopus arrhizus* and *Geotrichum candidum* for the preparation of (S)-atenolol and (S)-propranolol. Bioorg Med Chem 8:2067–2070

Takuya K, Naho A, Satoko N, Tsutomu I, Shizuo N (2001) Synthesis of enantiomeric 4-hydroxypropranolols from 1, 4-dihydroxynaphthalene. Tetrahedron Asymmetry 12:791–795

Veloo RA, Koomen GJ (1993) Synthesis of enantiomerically pure (S)-(−)-propranolol from sorbitol. Tetrahedron Asymmetry 4:2401–2404

Wang JJ, Zheng GJ, Wang JL (2001) Studies on chiral separation of β-blockers and their analogues by capillary electrophoresis. Chin J Anal Chem 65:782–787

Yoshiyasu T, Masakazu M, Kazuo A, Toshiyuki N, Minoru A, Minoru K (1988) Highly efficient lipase-catalyzed asymmetric synthesis of chiral glycerol derivatives leading to practical synthesis of S-propranolol. Tetrahedron Lett 29:5173–5176