Biocatalytic Synthesis of Some Chiral Pharmaceutical Intermediates by Lipases

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ABSTRACT: Chiral intermediates were prepared by biocatalytic processes for the chemical synthesis of three pharmaceutical drug candidates. These include (i) the synthesis of [(3*R-cis*)-3-(acetyloxy)-4-phenyl-2-azetidinone **2** for the semi-synthesis of paclitaxel (taxol) **5,** an anticancer compound; (ii) synthesis of chiral (exo,exo)-7-oxabicyclo [2.2.1] heptane-2,3-dimenthanol monoacetate ester **9** for the chemoenzymatic preparation of a thromboxane A_2 antagonist; (iii) the enzymatic synthesis of *S*-(–) 3-benzylthio-2-methylpropanoic acid, a key chiral intermediate for the synthesis of antihypertensive drugs captopril **10** or zofenopril **13**.

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KEY WORDS: Accurel PP, Captopril, chiral acetate, esterification, hydrolysis, immobilization, lipase, paclitaxel, stereoselective, thromboxane A_2 antagonist.

Currently, much attention is focused on the interaction of small molecules with biological macromolecules. The search for selective enzyme inhibitors and receptors, agonists, or antagonists is one of the keys for target-oriented research in the pharmaceutical industry. The increasing understanding of the mechanisms of drug interactions on a molecular level has led to an increasing awareness of the importance of chirality as the key to the efficacy of many drug products. It is now known that often only one stereoisomer of a drug substance is required for efficacy, and the other stereoisomer is either inactive or exhibits considerable reduced activity. Pharmaceutical companies are aware that, where appropriate, new drugs for the clinic should be homochiral to avoid the possibility of unnecessary side effects due to undesirable stereoisomers.

Chiral drug intermediates can be prepared by three different routes. One is to obtain them from naturally occurring chiral synthons, mainly by fermentation processes. Second is to resolve racemic compounds, which can be achieved by preferential crystallization of stereoisomers or diastereoisomers and by kinetic resolution of racemic compounds by chemical or biocatalytic methods. Finally, chiral synthons also can be prepared by asymmetric synthesis by either chemical or biocatalytic processes with microbial cells or enzymes derived therefrom. The advantages of microbial or enzyme-catalyzed reactions over chemical reactions are that they are stereoselective, can be carried out at ambient temperature, atmospheric pressure, and mostly in aqueous solution. Furthermore, microbial cells or enzymes derived therefrom can be immobilized and reused for many cycles. Lipases have been used extensively in the preparation of chiral compounds.

In this report, we describe biocatalytic processes for the synthesis of three chiral drug intermediate in (i) anticancer, (ii) thromboxane A_2 antagonist, and (iii) antihypertensive programs (1–3).

ANTICANCER DRUG

Among the antimitotic agents, paclitaxel (taxol) **5** (4,5), a complex polycyclic diterpene (6), exhibits a unique mode of action on microtubule proteins that are responsible for the formation of the spindle during cell division. In contrast to other "spindle-formation inhibitors," such as vinblastine or colchicine, both of which prevent the assembly of tubulin (7,8), paclitaxel is the only compound known to promote the assembly of microtubules and inhibit the tubulin disassembly process (9). Because of its biological nature and unusual chemical structure, paclitaxel may represent the prototype of a new series of chemotherapeutic agents. Various types of cancers have been treated with paclitaxel, and results with ovarian cancer are encouraging (10). In collaboration with the National Cancer Institute, Bristol-Myers Squibb is developing paclitaxel for treatment of various cancers. Paclitaxel was originally isolated from the bark of the Pacific yew tree, Taxus brevifolia (4), and also has been found in other Taxus species (11,12) in relatively low yields.

Currently, paclitaxel is produced from extracts of the bark of Pacific yew trees by a cumbersome purification process, which requires destruction of the trees. Alternative methods for production of paclitaxel by cell suspension cultures and by semi-synthetic processes are being evaluated (13–16). The enzymatic synthesis of a paclitaxel side-chain synthon is reported in this section. The stereoselective enzymatic hydrolysis of racemic acetate **1** (*cis*-3-acetyloxy-4-phenyl-2-azetidinone) to yield the desired (3*R-cis*)- acetyloxy-4-phenyl-2-azetidinone **2** and (3*S-cis*)-hydroxy-4-phenyl-2-azetidinone **3** by

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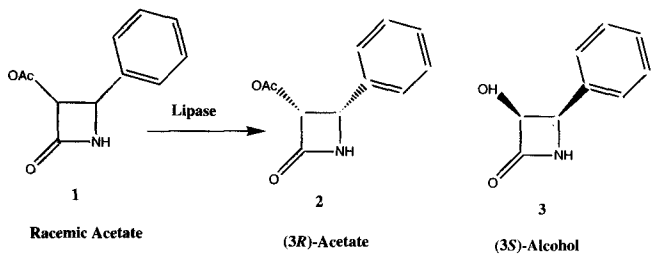


SCHEME 1

lipases has been demonstrated (Scheme 1). Product (3*R-cis*) acetate **2** is a key intermediate required for semi-synthesis of paclitaxel **5**. A patent application describing this process has been filed (17).

MATERIALS AND METHODS

Materials. Starting substrate **1** and reference compounds **2** and **3** were synthesized in the Chemical Process Research Department, Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ) as described previously (10). The physicochemical properties, including spectral characteristics [^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, mass spectra], were in full accord for all of these compounds.

Enzyme source. Lipases from *Pseudomonas cepacia* (lipase PS-30), *Geotrichum candidum* (lipase GC-20), *Candida cylindraceae* (lipase AY-30), *Rhizopus niveus* (lipase N), *Aspergillus niger* (lipase APF), and *Pseudomonas* sp. (lipase AK) were purchased from the Amano International Enzyme Company (Troy, VA). Porcine pancreatic lipase was purchased from the Sigma Chemical Company (St. Louis, MO). Bristol-Myers Squibb (BMS) lipase was made by fermentation as described below.

Enzymatic reaction. The enzymatic reaction mixture in 40 mL of 25 mM potassium phosphate buffer (pH 7.0) contained 1 g of crude lipase powder and 200 mg of substrate **1**. The reaction was carried out in a pH stat at pH 7.0, 200 rpm, and 29°C. The reaction yield and the optical purity were determined by high-performance liquid chromatography (HPLC).

(BMS) Lipase fermentation. Pseudomonas sp. SC 13856 was used in the fermentation. The inoculum was developed in a 25-L seed fermentor that contained 15 L of medium M (3% corn steep liquor, 1% glucose, 0.6% urea, 0.2% potassium dihydrogen phosphate, 0.05% potassium chloride, 0.05% magnesium sulfate, 1% soybean oil, and 0.1% antifoam). The culture was grown at 500 rpm agitation, 12 L/min aeration, and 26°C. Seed culture (12.5 L) was used to inoculate 250 L of medium M contained in a 380-L fermentor. The fermentation conditions were 250 rpm agitation, 250 L/min aeration, and 26°C. After 24 h of growth in a batch culture, additional soybean oil was supplied by adding 250 mL oil every hour from 24 to 48 or 72 h.

Lipase recovery. At the end of the fermentation, the culture broth was centrifuged through a Sharples (Alfa-Laval, Springfield, PA) centrifuge to remove cells. The supernatant was concentrated under vacuum at 50°C to one-tenth (25 L) volume in an ascending film evaporator. The filtrate was resuspended in 50 L cold ethanol (5°C), and the mixture was kept at 5°C for 6–8 h without agitation. The supernatant was decanted, and the slurry was filtered. The filtered precipitate was discarded. The filtrate was resuspended in 75 L cold ethanol, and the mixture was kept at 5°C for 18 h. The mixture was filtered, and the precipitated material was collected. The precipitate was washed with cold ethanol and dried under vacuum at 40°C to obtain about 1.7 kg of crude lipase powder (BMS lipase) with 140,000 units per g of lipase activity. This was immobilized on Accurel PP (Akzo Nobel Chemicals, Chicago, IL) as described below.

Lipase activity assay. The lipase activity was determined by titrimetric assay as recommended by Sigma Chemicals. In a suitable pH-stat (Metrohm pH-stat; Brinkmann Instrument Co., Westbury, NY) with a magnetic stirrer, 15 mL of reagent A (50 mL water, 50 mL Sigma lipase substrate, 20 mL of 3 M NaCl, 20 mL of 1.5% sodium taurocholate, and 20 mL of 0.075 M $CaCl_2$ $2H_20$), adjusted to pH 8.0 at 37°C, were pipetted into a titration vessel kept at 37°C. A suitable amount of enzyme was added, and the timer was started when the pH of the reaction mixture dropped to 7.7. The time (t) required for the consumption of 500 μL of 0.1 M NaOH to maintain the pH at 7.7 was recorded. Enzyme activity units/mg was defined as:

$$\text{units / mg} = \frac{0.1 \times 500}{\text{mg of lipase added} \times t\,(\text{min})} \qquad [1]$$

One unit under the above conditions hydrolyzes 1.0 microequivalent of fatty acids from a triglyceride in one minute at pH 7.7 and 37°C.

Immobilization and reusability of lipase PS-30 and BMS lipase. Crude lipase PS-30 (10 kg, 33,000 units per g) was immobilized on Accurel PP (nonionic polymeric adsorbant, 200–400 mesh polypropylene). The enzyme was dissolved in 50 L distilled water and centrifuged through a Cepa (Zentrifugenbau GmbH, Postfach, Germany) centrifuge to obtain a clear supernatant. Accurel PP (1.2 kg) resin was packed in a glass column at a packing density of 150 g/L of bed volume and with 25 L methanol. This was carried out by recirculating the methanol through the bottom of the column at a flow rate of 20 L per hour. After 2 h of washing with methanol, the resin was similarly washed in a column with 25 L Δ/1 water. After the water wash, the clarified enzyme solution was recirculated through the bottom of the column at a flow rate of 25 L/h for 16 h. Samples were taken periodically to check lipase activity and protein concentration. After 16 h of recirculation, the resin was further washed with 25 L Δ/1 water. Finally, the resin containing immobilized enzyme was removed from the column and dried under vacuum at 28°C for 48 h. BMS lipase (2.5 kg of 140,000 units per g) was also immobilized on Accurel PP (1.2 kg), as described above.

Immobilized BMS lipase and PS-30 lipase were evaluated for reusability in catalyzing the hydrolysis of racemic acetate **1**. The reaction was conducted in a 1-L jacketed re-

actor that contained 750 mL of 25 mM potassium phosphate buffer (pH 7.0), 7.5 g of substrate **1**, and 2.2 g of immobilized lipase PS-30. The reaction was carried out at 29°C, 250 rpm, and pH 7.0. After the end of each cycle (30–40 h), the reaction mixture was drained from the reactor, and the resin was washed twice with 100 mL of the same buffer. The reaction cycle was repeated under the same conditions. Reusability of BMS lipase was evaluated under similar conditions.

Preparative hydrolysis and recovery of product. Preparative-scale hydrolysis of racemic acetate **1** was carried out with lipase PS-30 (150-L vol) or BMS lipase (75-L vol). The reaction mixture in 150 L of 25 mM potassium phosphate buffer (pH 7.0) contained 10 g/L of **1** and 3 g/L of immobilized lipase PS-30. The reaction was conducted at 29°C and 200 rpm in a 250-L fermentor. At the end of the reaction, the reaction mixture was cooled to 5°C with slow agitation (50 rpm) and kept at that temperature for four hours to precipitate the product (3*R-cis*) acetate **2**. The immobilized enzyme, due to its hydrophobicity, remained floating at the top of the fermentor and was separated physically by draining the reaction mixture from the fermentor. The reaction mixture that contained precipitated **2** and soluble *S*(−)-alcohol **3** was filtered to recover 97–98% of **2**. The filtrate with **3** was also collected. The immobilized enzyme was washed with 10 L water, and the wash was kept in the cold room at 5°C for four hours. The remaining 2–3% of precipitated product **2** was recovered. The combined precipitate of **2** was washed with 10 L of cold water at 5°C and dried at 40°C under vaccum to a KF (Karl-Fischer) value of less than 0.3% to yield about 675 g of isolated product. By using the same procedure as for lipase PS-30, 331 g of product **2** was isolated from the 75-L reaction mixture with immobilized BMS lipase.

Analytical methods. The substrate and products (**1**, **2**, and **3**) were analyzed by HPLC. A Nova-Pak C_{18} (Waters, Milford, MA) reverse-phase column (3.9 × 150 mm) was used. The mobile phase was 15% acetonitrile in water, and the flow rate was 1 mL/min. The detection wavelength was 227 nm. The retention times for the alcohol and acetate were 2.7 and 14.9 min, respectively. The optical purity of chiral acetate **2** was determined by chiral HPLC. A chiralpak AS column was used. The mobile phase consisted of hexane/ethanol (96:4) and was used at 1 mL/min at ambient temperature. The detection wavelength was 210 nm. The retention times for the enantiomers of racemic **1** were 22.5 and 29.9 min, respectively. The retention times for the enantiomers of racemic alcohol were 36.9 and 51.4 min, respectively (Fig. 1).

RESULTS

Among commercially available enzymes evaluated, lipases from *Pseudomonas cepacia* (PS-30), *G. candidum* (GC-20), R. niveus (N), *Pseudomonas* sp. (AK), *A. niger* (APF), *C. cylindraceae* (AY-30), and porcine pancreatic lipase catalyzed the hydrolysis of the undesired enantiomer racemic acetate **1** to the corresponding *S*-(−)-alcohol **3**. The desired enantiomer

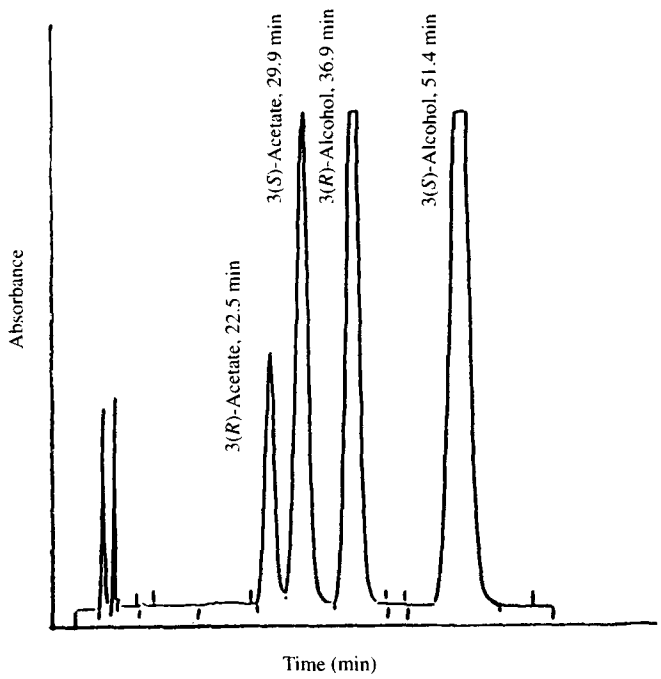


FIG. 1. The chromatogram of racemic *cis*-3-(acetyloxy)-4-phenyl-2-azetidinone and racemic *cis*-3-(hydroxy)-4-phenyl-2-azetidinone.

(3*R-cis*) acetate **2** remained unreacted. The reaction yield (40–96%) and the optical purity (94–99.6%) of chiral acetate **2** depended upon the enzyme used for resolution (Table 1).

For an in-house source of enzyme, a lipase fermentation and recovery process was developed with *Pseudomonas* sp. SC 13865. The highest lipase activity achieved in a fed-batch fermentation process with *Pseudomonas* sp. SC 13865 was 1500 units/mL. As shown in Figure 2, most of the glucose and

TABLE 1
Enzymatic Resolution of *cis*-3-Acetyloxy-4-Phenyl-2-Azetidinone[a]

Enzyme	Reaction time (h)	Yield of **2** (%)	Optical purity of **2** (%)
BMS lipase[b]	42	95	99.4
Pseudomonas lipase[c] (lipase PS-30)	48	96	99.6
Geotrichum candidum[c] (lipase GC-20)	48	84	99
Rhizopus niveus[c] (lipase N)	48	64	98.5
Aspergillus niger[c] (lipase APF)	48	40	99.5
Candia cylindraceae[c] (lipase AY 30)	48	90	94
Porcine pancreatic[d] lipase	48	90	99.2
Pseudomonas sp.[c] (lipase AK)	48	74	98.3

[a]Reactions were carried out as described in the Materials and Methods section. The reaction yields and optical purities were determined by high-performance liquid chromatography.
[b]Bristol-Myers Squibb (New Brunswick, NJ).
[c]From Amano International Enzyme Co. (Troy, VA).
[d]From Sigma Chemical Co. (St. Louis, MO).

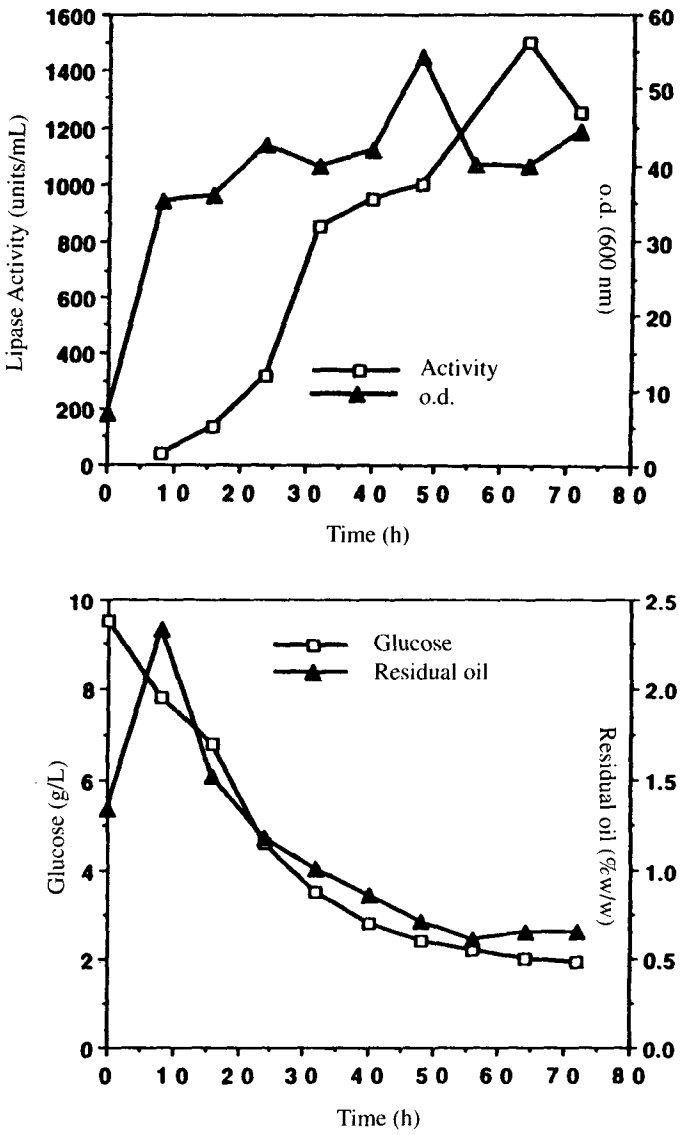


FIG. 2. Lipase fermentation with *Pseudomonas* sp. SC 13856 (Amano, Troy, VA).

soybean oil was consumed during the first 36 h, which corresponded to the period of rapid cell growth and lipase production. Crude BMS lipase (1.7 kg), which contained 140,000 units per g of lipase activity, was recovered from the fermentation broth by ethanol precipitation. BMS lipase was immobilized on Accurel PP as described in the Methods section with 98% adsorption efficiency. The immobilized BMS lipase was evaluated in the resolution of racemic acetate **1**. Substrate was used at 10 g/L, and enzyme was used at 3 g/L concentration. A reaction yield of 96 mol% and the optical purity of 99.5% was obtained for **2** after 40 h reaction time (Table 2).

For an alternative enzyme source, lipase PS-30 was also immobilized on Accurel PP in a similar manner. The kinetics of hydrolysis of racemic **1** was investigated independently with immobilized BMS lipase and PS-30 lipase. The reaction was conducted at 10 g/L substrate and 3 g/L enzyme concentrations. As shown in Table 2, hydrolysis of the undesired enantiomer of racemic **1** was observed, to enrich **1** with the desired **2** as the reaction progressed. The initial rate (0–24 h) of hydrolysis was faster than the later rate (24–40 h). The immobilized BMS lipase and PS-30 lipase were reused for 10 cycles without any loss of activity, productivity, or optical purity of the product. Results of reusability studies with PS-30 are shown in Table 3. An average reaction yield of 94% and optical purity of 99.3% were obtained for chiral acetate **2**. About 3.1 g of **2** was isolated from each cycle in an overall 62% yield. The rate of hydrolysis of **1** remained constant over 10 cycles (0.12 g/L/h). Similar results were obtained in reusability studies with BMS lipase, except that the reaction was completed in 36 h. Preparative-scale hydrolyses of racemic acetate **1** were carried out in 75 and 150-L volumes with immobilized BMS lipase and PS-30 lipase, respectively, as described in the Materials and Methods section. After 38 h, 94 mol% reaction yield and 99.4% optical purity of **2** was obtained in each batch. From the reaction mixtures, 331 and 675 g of chiral acetate **2** were isolated (overall yields of 88 and 90 mol%, respectively) from the two batches (Table 4). The isolated acetate **2** ($[\alpha]_D = -15.6°$) from both batches

TABLE 2
Kinetics of Hydrolysis of *cis*-3-Acetyloxy-4-Phenyl-2-Azetidinone by Lipase PS-30 and BMS Lipase[a]

Reaction time (h)	(3*R*)-Acetate (g/L)	(3*S*)-Acetate (g/L)	(3*S*)-Alcohol (g/L)	(3*R*)-Alcohol (g/L)	Conversion (%)	Optical purity (3*R*)-acetate (%)
Lipase PS-30						
0.5	5	4.5	0.4	0	8	54
16	4.95	2.5	2.5	0	50	75
24	4.92	1.2	3.8	0.013	72	86
32	4.89	0.4	4.3	0.07	92	96
40	4.82	0	4.95	0.14	96.4	99.6
BMS lipase						
0.5	5	4.2	0.8	0	14	57
16	4.98	2.48	2.49	0.02	50	75
24	4.96	1.5	3.6	0.04	66	86
32	4.9	0.26	4.7	0.1	95	97.4
40	4.8	0	4.92	0.16	96	99.5

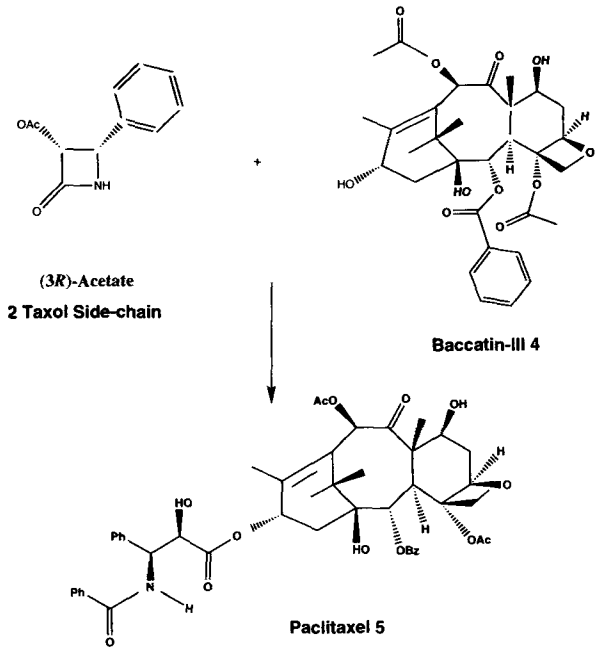
[a]The reaction mixture in 1 L of 25 mM potassium phosphate buffer (pH 7.0) contained 10 g of substrate **1** and 3 g of immobilized lipase. Reactions were carried out at 30°C, 200 rpm. Reaction yields and optical purities were determined by high-performance liquid chromatography. See Table 1 for abbreviations and company sources.

TABLE 3
Enzymatic Hydrolysis of *cis*-3-Acetyloxy-4-Phenyl-2-Azetidinone: Reusability of Immobilized Lipase PS-30[a]

Cycle number	Reaction time (h)	Reaction rate (g/L/h)	Product yield **2** (%)	Isolated product **2** (g)	Optical purity of product **2** (%)
1	40	0.123	94	3.1	99.4
2	35	0.121	94	3.1	99.4
3	36	0.12	96	3.05	99.5
4	37	0.17	95	3.09	99.4
5	37	0.15	94	3.1	99.5
6	40	0.121	96	3.2	99.5
7	40	0.124	93	3.12	99.4
8	41	0.126	94	3.2	99.3
9	42	0.12	95	3.12	99.2
10	42	0.121	95	3.1	99.3

[a]The reaction mixture in 750 mL of 25 mM potassium phosphate buffer (pH 7.0) contained 7.5 g of substrate **1**, and 2.2 g of immobilized lipase PS-30 or BMS lipase. Reactions were carried out at 29°C, 250 rpm agitation at pH 7.0. See Table 1 for abbreviations and company sources.

gave an optical purity of 99.5% by chiral HPLC and 99.9 area % purity by HPLC.



SCHEME 2

DISCUSSION

Paclitaxel **5** has become one of the most important anticancer compounds to emerge from the screening of natural products in recent years. Development of paclitaxel as a drug was slow because of the difficulty of isolating it from bark of the Pacific yew, *T. brevifolia*, and because of its lipophilicity, which made formulation difficult (18). It is in phase-II clinical trials for breast cancer and in phase-I trials for lung and colon cancers (5,10). The skeletal and stereochemical complexity of the taxanes provides a formidable synthetic challenge, and despite some outstanding synthetic work, total synthesis is an unattained goal. The partial synthesis **5** from the simpler diterpenoid baccatin III, **4**, derived from the renewable resource of yew leaves, as opposed to yew bark, has been reported (19,20). Recently, preparation of paclitaxel side-chain precursors by the lipase-catalyzed enantioselective esterification of methyl *trans*-β-phenylglycidate has been demonstrated (21). Brieva *et al.* (22) have also described the enantioselective hydrolysis of the N-benzoyl derivatives of **1**. In this report, we have described the stereoselective hydrolysis of racemic **1** to chiral alcohol **3** and chiral acetate **2** by lipases. Chiral **2** is a potential side-chain synthon for semi-synthesis of **5** from **4**, obtained from renewable resources (Scheme 2). We also have developed fermentation and recovery processes for production of lipase from *Pseudomonas* sp. SC 13865 that catalyze the enzymatic resolution process.

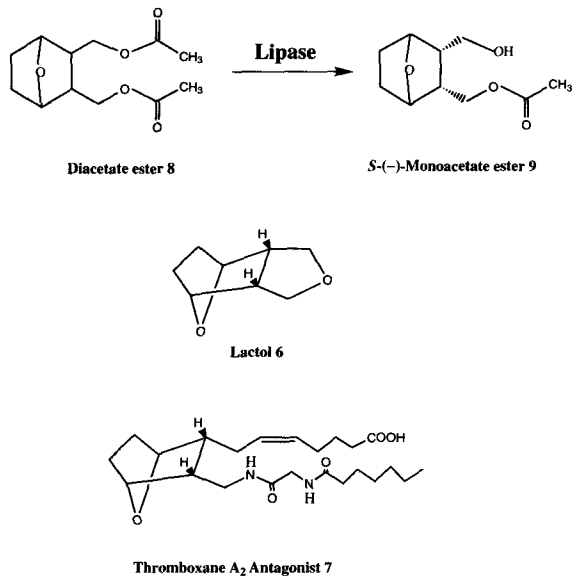
TABLE 4
Preparative-Scale Enzymatic Hydrolysis of *cis*-3-(acetyloxy)-4-Phenyl-2-Azetidinone[a]

Parameter	Batch number TAX-0892	TAX-2392
Volume (L)	75	150
Substrate input of racemic **1**	750	1500
Enzyme		
Name	BMS lipase	Lipase PS-30
Input (g)	225	450
Product output of (3*R*)-acetate (g)	331	675
Yield (mol%)	88	90
Optical purity of (3*R*)-acetate (%)	99.5	99.6

[a]See Table 1 for abbreviations and company sources.

THROMBOXANE A_2 ANTAGONIST

The lactol **6** [3a*S*-(3aa, 4a, 7a, 7aa)]-hexahydro-4,7-epoxy-isobenzo-furan-1-(3H)-one is a key chiral intermediate for the total synthesis of [1*S*-(1a, 2a (*Z*), 3a, 4a [[-7-[3-[[[[1-oxoheptyl)-amine] acetyl) methyl]-7-oxabicyclo-[2.2.1] hept-2-yl]-5-heptanoic acid **7** (a new cardiovascular agent, useful in the treatment of thrombolic disease) (23,24). TxA_2 is an exceptionally potent pro-aggregatory and vasoconstrictor substance, which is produced by the metabolism of arachidonic acid in blood platelets and other tissues. Together with the potent anti-aggregatory and vasodilator prostacyclin, it is thought to play a role in the maintenance of vascular homeostasis and may contribute to the pathogenesis of a variety of vascular disorders. Approaches toward limiting the effect of TxA_2 have focused on either inhibiting its synthesis or blocking its action at its receptor sites by means of an antagonist (25). The enzymatic synthesis of a key chiral intermediate (exo, exo)-7-oxabicyclo [2.2.1] heptane-2,3-dimethanol monoacetate ester **9** from the corresponding (exo,exo)-7-oxabicyclo [2.2.1] heptane-2,3-dimethanol diacetate ester **8** is



SCHEME 3

described in this section. Monoacetate ester **9** is converted to lactol **6,** which is required for the total synthesis of a new TxA_2 antagonist **7** (Scheme 3).

MATERIALS AND METHODS

Materials. Starting substrate **8** and reference compounds **6** and **9** were synthesized in the Chemical Process Research Department, Bristol-Myers Squibb Pharmaceutical Research Institute. The physicochemical properties, including spectral characteristics (^{1}H NMR, ^{13}C NMR, mass spectra), were in full accord for all of these compounds.

Enzyme source. P-30 lipase from *Pseudomonas* sp. (32,100 units/g) and lipase GC-20 from *G. candidum* (20,000 units/g) were purchased from Amano International Enzyme, Inc. *Pseudomonas fluorescens* (35,000 units/g) lipase was purchased from Biocatalysts Ltd (Pontybridd, United Kingdom). Lipase B from *P. fluorescens* was obtained from Enzymatics Ltd. (Cambridge, United Kingdom). Porcine pancreatic lipase (35,000 units/g), *C. cylindracea* (70,000 units/g) lipase and *Chromobacterium viscosum* (100,000 units/g) lipase were obtained from Sigma Chemical Co.

Enzymatic reaction. The enzymatic reaction mixture for the hydrolysis contained 36 mL of 50 mM potassium phosphate buffer pH 7.0, 4 mL toluene, 200 mg diacetate ester **8** and 1 g lipase. The reaction was carried out at 5°C in a pH stat with continuous mixing and was maintained at pH 7.0 with 1 N NaOH for 48 h.

Immobilization of lipase. Accurel PP (nonionic polymeric adsorbant, 200–400 mesh, polypropylene) obtained from Akzo, was used for the immobilization of lipase. Crude P-30 lipase (1.5 kg with 30,000 units/g activity) was dissolved in 8 L distilled water and centrifuged at 12,000 × *g* for 15 min at 4°C to obtain a clear supernatant. The supernatant solution (7 L with 6800 units/mL) was added to 169 g of resin, which was previously washed three times with 1 L methanol. The mixture was gently agitated on a gyratory shaker at room temperature for 48 h. Adsorption of enzyme to resin was checked periodically by determining the lipase activity and protein content that remained in the supernatant after centrifugation of the enzyme-resin mixture. After 48 h, the slurry was filtered, and the cake was washed with 40 L distilled water. The resins with immobilized enzyme were dried in vacuo for 24 h. About 190 g of lipase immobilized on Accurel PP was obtained.

Evaluation of immobilized lipase. Immobilized P-30 lipase was evaluated for reusability in catalyzing the asymmetric hydrolysis of diacetate ester **8** to the corresponding monoacetate ester **9**. The hydrolysis was conducted in a 1-L jacketed reactor that contained 450 mL of 0.05 M phosphate buffer (pH 7.0), 50 mL toluene, 2.5 g diacetate ester **8**, and 1.25 g immobilized enzyme, maintained at pH 7.0 with 2.5 N NaOH at 5°C. The reaction was carried out at 250 rpm. After the end of each cycle (16 h), the reaction mixture was drained from the reactor, and resins were washed twice with 500 mL of the same buffer. The reaction cycle was repeated under the same conditions.

Semi-preparative and preparative hydrolysis of diacetate ester **8**. The semi-preparative-scale hydrolysis was carried out in a 5-L jacketed reactor. The reaction mixture contained 2.7 L of 50 mM potassium phosphate buffer (pH 7.0), 300 mL toluene, 15 g diacetate ester **8**, and 7.5 g of immobilized P-30 lipase. The reaction was conducted at 5°C, 250 rpm, at pH 7.0 by using 6.25 N NaOH. Reaction mixtures from two batches were filtered to remove immobilized enzyme, which was then washed with 500 mL distilled water and filtered. The combined wash and reaction mixture filtrate (~6.5 L) was extracted three times with 15 L ethyl acetate. Separated ethyl acetate layers were combined and concentrated under reduced pressure and gave 27 g of oily residue. After overnight incubation at 5°C, the oily residue was washed three times with 100 mL *n*-hexane. The solids after hexane extraction were dissolved in 100 mL methylene chloride and, subsequently, 100 mL hexane was added. Crude crystals were formed upon concentration of the solution under reduced pressure. They were separated from the mother liquor, recrystallized in 50 mL of methylene chloride/hexane mixture (1:1 ratio) and yielded 11.9 g of white crystals. The mother liquor was further concentrated under reduced pressure to give three crops of crystals (1.98 g). A total of 13.88 g of chiral monoacetate ester **9** was isolated from the two preparative batches.

Preparative-scale hydrolysis of diacetate **8** was carried out in a 200-L jacketed reactor at 4.8–5.3°C (Fischer-Porter, Warminster, PA) control system. The reaction mixture was controlled with 1 N NaOH between pH 6.9–7.0 with a Markel Corporation (Norristown, PA) control unit. The reaction mixture contained 72 L of 50 mM phosphate buffer pH 7.0, 8 L of toluene that contained 400 g diacetate ester **8** and 200 g of immobilized enzyme. The reaction was monitored by in-process gas chromatographic (GC) analysis and was terminated after 24 h upon depletion of the substrate. The reaction

mixture was filtered, and the filtrate was extracted with 240 L of ethyl acetate in a 3″ CARR (CARR Separations, Inc., Easton, MA) column. The ethyl acetate concentrate (1.4 L), which contained crude crystals, was filtered, and the crystals were dissolved in 0.9 L ethyl acetate, which was filtered to remove the insolubles. The filtrate was then concentrated to an oily solid (158.3 g), which was redissolved in 450 mL ethyl acetate at 40°C. Crystallization of monoacetate ester was carried out by adding 950 mL cyclohexane with stirring at room temperature for 30 min and then in an ice bath for 30 min. The crystals were collected by filtration and washed with 300 mL of ice-cold cyclohexane, dried *in vacuo* overnight at room temperature. The yield was 127.8 g of **9**. The filtrate from 1.4 L of ethyl acetate concentrate was further concentrated under reduced pressure to yield 140 g of yellow solids. Solids were dissolved in 400 mL ethyl acetate at 40°C. Crystallization of **9** was carried out by the addition of 1.2 L cyclohexane with stirring for 20 min at room temperature and 30 min in an ice bath. The crystals were collected by filtration, washed with 300 mL of ice-cold cyclohexane, and dried overnight *in vacuo* at room temperature to obtain 105.6 g of **9**. Two preparations were combined as the first crop of 232.3 g of monoacetate ester **9**. The remaining product was crystallized by the same procedures to obtain second and third crops of monoacetate ester **9** (total 35.6 g). Overall, 268 g of product was isolated in 80.3 mol% yield.

Analytical methods. The hydrolysis of diacetate ester **8** to the corresponding monoacetate ester **9** was monitored by GC (flame-ionization detector) in a Hewlett-Packard (Palo Alto, CA) HP-5 capillary column (5% diphenyl, 95% dimethylpolysiloxane, 25 m × 0.2 mm, 0.11 mm film thickness) at 150°C oven, 250°C injection, and 250°C detector temperatures. Helium was used as carrier gas at 50 mL/min. Samples (2 mL) were extracted with 4 mL ethyl acetate. The separated ethyl acetate layer after centrifugation was filtered through an LID/X (Whatman, Inc., Fairfield, NJ) filter (0.2 mm) and used for GC analysis (1 μL injection). The retention times of diacetate ester **8** and monoacetate ester **9** were 6 and 4.13 min, respectively.

HPLC analysis. The optical purity of **9** was determined by chiral HPLC. The separation of its two enantiomers was achieved in a Bakerbond Chiralcel OB (Diacel Chemical Ind., Easton, PA) column at ambient temperature. Injection volume was 10 μL, mobile phase consisted of the 70% hexane and 30% isopropanol, the flow rate was 0.5 mL/min, and the detection wavelength was 220 mm. The retention time for the (−)-enantiomer is 8.5 min, and for the (+)-enantiomer it is 11.45 min.

TLC analysis. Diacetate ester **8** and monoacetate ester **9** were spotted on Kiesel 60 F254 (Merck, Darmstadt, Germany) TLC plate (20 cm × 10 cm) and developed in ethyl acetate/hexane 70:30 (vol/vol). The TLC plate was sprayed with a solution of 30% (vol/vol) phosphoric acid and 3% vanillin in ethanol. The compounds were visualized as brown-purple spots upon heating the plate at 110° for 5 min. R_f values for diacetate ester **8** and monoacetate ester **9** were 0.46 and 0.86, respectively.

NMR analysis. For NMR analysis, a Brucker (Karlsruhe, Germany) AP-300 MH2 spectrometer was used. Deuteriochloroform was used as solvent with 3% tetramethyl silane as an internal standard. A sample of 5 mg of chiral monoacetate ester **9** was dissolved in 0.8 mL $CDCl_3$, and 20 mg of the chiral shift reagent [*tris*-3-(heptafluoropropyl-hydroxymethylene)-(+)-camphorato] europium III was added. The split acetyl proton signal was measured with the addition of racemic monoacetate ester.

RESULTS AND DISCUSSION

Among the commercially available lipases evaluated, P-30 lipase and lipase B demonstrated efficient conversion of diacetate ester **8** to the corresponding chiral monoacetate ester **9**. Yields of 80 mol% and optical purities of 96–98% were obtained with both lipases (Table 5) when the reaction was conducted in a biphasic system of 10% toluene.

TABLE 5
Enzymatic Asymmetric Hydrolysis of Diacetate Ester 8 to Chiral Monoacetate Ester 9[a]

Enzyme	Diacetate ester **8** (mg/mL)	Monoacetate ester **9** (mg/mL)	Conversion (mol%)	Optical purity (%)
Pseudomonas fluorescens[b]	0.45	3.5	85	97
Pseudomonas lipase (Lipase PS-30)	0.33	3.75	90	98
Geotrichum candidum (Lipase GC-20)	1.6	1.8	44	60
Chromobacterium viscosum Lipase	1.8	2.1	51	78
Candia cylindraceae (Lipase AY-30)	1.2	2.5	61	81
Pseudomonas lipase[c]	0.52	3.42	82	75

[a]Reactions were carried out as described in the Materials and Methods sections. The reaction yields were determined by gas chromatography, and optical purities were determined by high-performance liquid chromatography. See Table 1 for company sources.
[b]From Biocatalysis Ltd. (Pontybridd, United Kingdom).
[c]From Enzymatics (Cambridge, United Kingdom).

Crude lipase P-30 was immobilized on Accurel PP with 98.5% adsorption efficiency. The immobilized enzyme was reused for six cycles without loss of activity and productivity in the hydrolysis of diacetate **8**. Each cycle gave the reaction yield of >85 mol% and optical purity of >99% of isolated monoacetate ester **9**.

A semi-preparative asymmetric hydrolysis of diacetate ester **8** was carried out in a 5-L jacketed reactor. Kinetics of the resolution process are as shown in Table 6. The reaction yield of 89.6 mol% was obtained after a 26-h reaction period. The optical purity was >99.5%. From two 3-L batches, 16.2 g of crystalline monoacetate ester **9** was isolated, which represents a 72 mol% yield.

A preparative-scale hydrolysis of diacetate ester **8** was carried out in 80-L volume. After 23 h, the reaction yield of 84 mol% was obtained. From the reaction mixture, after extraction, about 268 g of monoacetate ester **9** was isolated in crystalline form, at an overall yield of 80 mol%. The isolated monoacetate ester **9** had 99.3% optical purity, as determined by chiral HPLC (Fig. 3). The NMR analysis of chiral monoacetate ester **9** in $CDCl_3$ was carried out as described in the Methods section. The singlet, arising from the methyl protons of the acetyl group, was shifted from 2.05 to 2.80 ppm in the presence of chiral shift reagent. The same experiment was repeated in the presence of racemic monoacetate ester **9**. The methyl singlets of each enantiomer were separated by 0.15 ppm with baseline signal separation. The integration of the two singlets in chiral monoacetate ester **9** gave an optical purity of 99.4%. GC and TLC of isolated monoacetate ester **9** gave 99.5% HI. The mass spectrum indicated that the parent molecular ions $(M + H)^+$ [*m/z* 201] and $(M + NH_4)^+$ [*m/z* 218] were compatible with monoacetate ester **9**. The specific rotation was determined to be $[\alpha]_D = -7.6°$ (Cl, $CHCl_3$), $[\alpha]_{436}$, Hg = −16.3° (Cl, $CHCl_3$). The melting point of the isolated material was 80.5°C.

Recently, Lok *et al.* (26) have demonstrated that the stereoselective oxidation of exo- and endo-bridged [2.2.1] and [2.2.2] meso diols produces enantiomeric pure lactones. However, to oxidize 1 g of meso exo- and endo-7-oxabicyclo[2.2.1]heptanediol, it required 720 mg of NAD^+, 9.7 g of flavin mononucleotide (FMN), and 50 mg of horse liver alcohol dehydrogenase. We have demonstrated a simple and inexpensive way to hydrolyze diacetate ester **8** to the corresponding chiral **9**. The monoacetate ester **9** was oxidized to its corresponding aldehyde and subsequently hydrolyzed to give lactol **6** (27), a key chiral intermediate for the synthesis of thromboxane antagonist **7**.

TABLE 6
Kinetics of Hydrolysis of Diacetate Ester 8 to Monoacetate Ester 9 by Lipase PS-30: 3 L Semiprep Batch[a]

Reaction time (h)	Diacetate ester **8** (g/L)	(−)-Monoacetate ester **9** (g/L)	Conversion (mol%)	Optical purity (%)
4	2.2	1.8	43	
8	1.4	2.6	62	
16	0.6	3.3	79	
20	0.5	3.5	85	
24	0.4	3.7	89	
28	0.3	3.9	93	99.5

[a]The reaction mixture (3 L) contained 2.7 L of 50 mM potassium phosphate buffer (pH 7.0), and 300 mL toluene containing 15 g of substrate **8** and 7.5 g of immobilized lipase PS-30. The reaction was carried out at 5°C, 230 rpm. The pH was maintained at 7.0 with 6 M NaOH. Substrate and product concentrations were determined by gas chromatography, and the optical purity of the product was determined by chiral high-performance liquid chromatography.

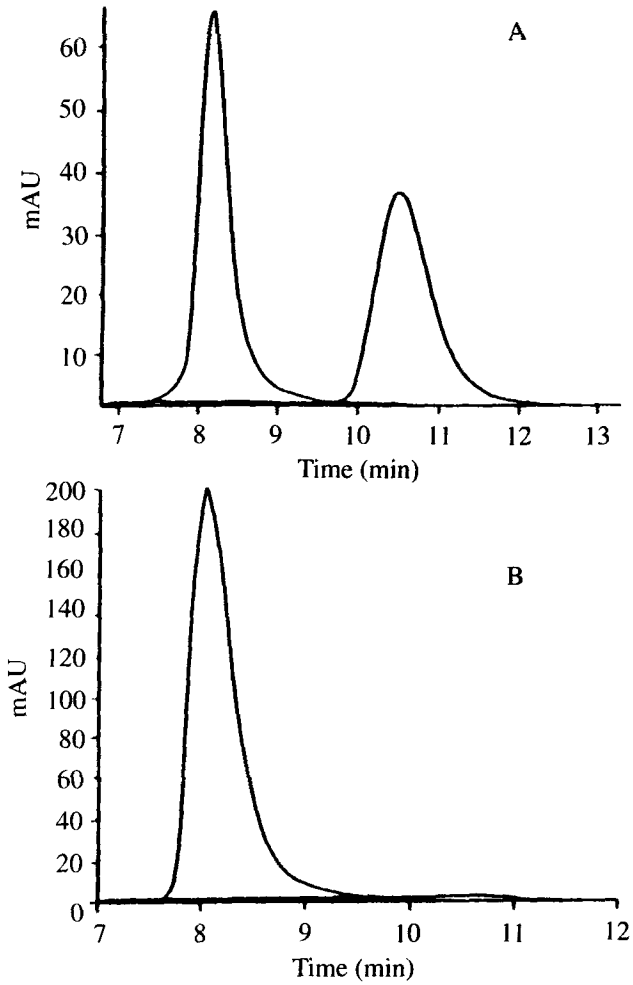
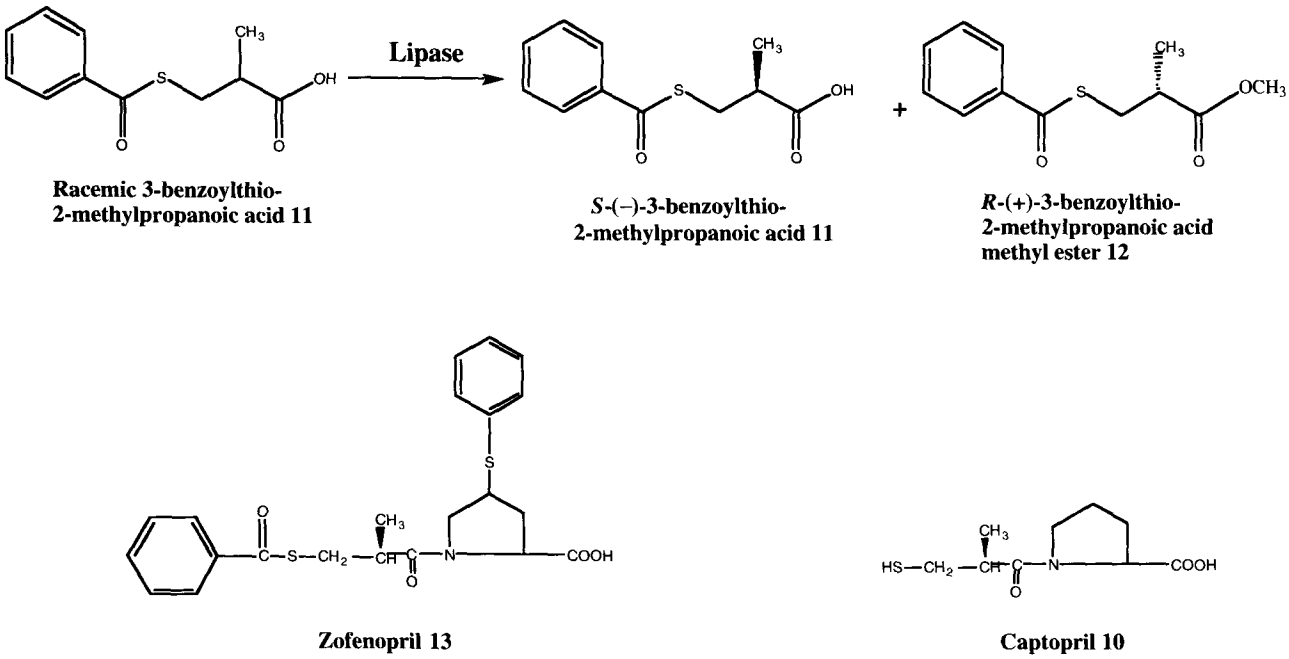


FIG. 3. Chiral high-performance liquid chromatographic analysis of (A) racemic (exo,exo)-7-oxabicyclo [2.2.1] heptane-2,3-dimethanol monoacetate ester and (B) enzymatically made chiral *S*-(−)-monoacetate ester **9**.

ANTIHYPERTENSIVE DRUG

Captopril is designated chemically as 1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline **10**. It is used as an antihypertensive agent through suppression of the renin–angiotensin–aldosterone system (28,29). Captopril prevents the conversion of angiotensin I to angiotensin II by inhibition of angiotensin-converting enzyme (30). The potency of captopril **10** as an inhibitor of an angiotensin-converting enzyme depends critically on the configuration of the mercaptoalkanoyl moiety;



SCHEME 4

the compound with the *S*-configuration is about 100 times more active than its corresponding *R*-enantiomer (31). The required 3-mercapto-(2*S*)-methylpropionic acid moiety has been prepared from the microbially derived chiral 3-hydroxy-(2*R*)-methylpropionic acid, which is obtained by hydroxylation of isobutyric acid (32–34). The use of extracellular lipases of microbial origin to catalyze the enantioselective hydrolysis of 3-acylthio-2-methylpropanoic acid ester in an aqueous system has been demonstrated to produce optically active 3-acylthio-2-methyl propanoic acid (35–37).

In this section, we describe the stereoselective esterification of racemic 3-benzoylthio-2-methylpropanoic acid **11** in an organic solvent system to yield *R*-(+) methyl ester **12** and unreacted acid enriched in the desired *S*-(−) enantiomer. *S*-(−) 3-benzoylthio-2-methylpropanoic acid [*S*-(−)-**11**] is a key intermediate for the synthesis of captopril (38) or zofenopril **13** (39). Both are antihypertensive drugs (Scheme 4).

MATERIALS AND METHODS

Enzyme source. Crude lipases AK, P-30, CES-30, and CE from *Pseudomonas* sp., lipase N from *Rhizopus* sp., lipase MAP from *Mucor* sp., lipase AY from *Candida* sp., lipase CE from *Humicola langinosa*, lipase G from *Penicillium* sp., and lipase APF-12 from *Aspergillus niger* were obtained from the Amano International Enzyme Co., Inc. Lipase B from *P. fluorescens* and lipase F9 from *Aspergillus* sp. were obtained from Enzymatics Ltd.

Lipase activity assay. The lipase activity was determined by titrimetric assay as recommended by Sigma Chemical Co. One unit hydrolyzes 1.0 microequivalent of fatty acids from a triglyceride in one minute at pH 7.7 and 37°C.

Immobilization of lipase. Three different resins, XAD-7 (Amberlite nonionic polymeric adsorbent, 20–60 mesh, polyacrylate; Rohm and Haas, Philadelphia, PA), XAD-2 (Amberlite nonionic polymeric adsorbent, 20–60 mesh, polystyrene; Rohm and Haas), and Accurel PP (nonionic polymeric adsorbant, 200–400 mesh, polypropylene) were used for immobilization of lipase. Crude Amano P-30 lipase from *Pseudomonas* sp. (30 g) was dissolved in 75 mL distilled water and centrifuged at 15,000 × *g* for 15 min at 4°C to obtain a clear supernatant. Twenty-five mL of the supernatant was added to 1.0 g each of resin that had been previously washed three times with 25 mL methanol. Resins and enzyme solutions were gently agitated on a gyratory shaker at room temperature for 24 h. Adsorption of enzyme to each resin was checked periodically by determining the lipase activity and protein content remaining in the supernatant, obtained after centrifugation of the enzyme–resin mixture. After 24 h, the slurry was filtered, and the cake was washed with 400 mL distilled water. The resins with immobilized enzyme were dried under vacuum for 24 h. Crude Amano P-30 *Pseudomonas* sp. lipase was also immobilized on Accurel PP on a preparative scale as described earlier.

Analytical methods. The esterification of 3-benzoylthio-2-methylpropanoic acid was monitored by GC (flame-ionization detector) of reaction mixture samples in a Hewlett-Packard fused-silica capillary column (cross-linked methyl silicone, 15-m long, 1.0-mm film thickness, 0.31-mm i.d., HP 190912-215) at 215°C oven temperature, 250°C injection temperature, and 250°C detection temperature. The retention time for 3-benzoylthio-2-methylpropanoic acid was 3.8 min, and for the corresponding methyl ester it was 3.1 min.

The optical purity of 3-benzoylthio-2-methylpropanoic

acid was determined by treating the samples with thionyl chloride and reacting the acid chloride formed with d-2-octanol to yield the diastereomers (40). In the procedure used, 1 mL of reaction mixture filtrate (3 mg of 3-benzoylthio-2-methylpropanoic acid) was evaporated to remove solvent. One mL of 10% thionyl chloride solution in *n*-hexane containing 2.5 mL of dimethylformamide was added to the vial, mixed thoroughly on a vortex mixer, and incubated at room temperature for at least 30 min. The reagent was subsequently evaporated under a gentle stream of nitrogen at 50°C. d-2-Octanol (0.3 mL) was added to the dried residue, mixed thoroughly on a vortex mixer, and incubated at 60°C for 30 min in a heating block. Octanol was evaporated under a stream of nitrogen at 50°C and the residue was dissolved in 0.5 mL methylene chloride. Diastereomers were analyzed by GC (flame-ionization detector) with a Hewlett-Packard fused-silica capillary column (HP #190912-215) at 215°C oven temperature, 250°C injection temperature, and 250°C detector temperature. The retention times for the esters of the L- and D-enantiomers were 14.8 and 16.0 min, respectively.

Enzymatic reaction. The enzymatic reaction mixture for the esterification contained 0.1 M (22.4 mg/mL) 3-benzoylthio-2-methylpropanoic acid, 0.4 M methanol, 0.1% water, and 1 g of crude lipase in 10 mL toluene. The reaction was conducted at 40°C and 280 rpm. Samples were taken periodically and analyzed by GC to determine the yield and optical purity of the product.

Evaluation of immobilized P-30 lipase. Immobilized crude Amano P-30 lipase on Accurel PP was evaluated in a 3-mL and 50-mL reactor volume. The reaction mixture contained 0.1 M 3-benzoylthio-2-methylpropanoic acid (22.4 mg/mL), 0.4 M methanol, 0.1% water, and 0.3 and 6 g immobilized enzyme in 3 or 50 mL toluene, respectively. The reaction was conducted at 28°C and 280 rpm.

Lipase fermentation. Pseudomonas sp. SC 13856 was used in the fermentation. Lipase fermentation, recovery of extracellular lipase, and immobilization of enzyme on Accurel PP were carried out as described earlier.

RESULTS AND DISCUSSION

Screening of lipases. Commercially available lipases were screened for the stereoselective esterification of racemic **11** to yield the acid *R*-(+) methyl ester **12** and unreacted acid enriched in the desired *S*-(−)-**11**. Lipases (P-30, AK, CES-30, B1) from various *Pseudomonas* sp. catalyzed the stereoselective esterification (Table 7). *S*(−)-**11** was obtained with a reaction yield of 37% (based on racemic substrate initially present) and an optical purity of 97.2% when Amano Lipase P-30 was used. Poor selectivity was observed with lipases from *C. cylindracea, Rhizopus* sp., *Mucor* sp., *Penicillium* sp. and *H. langinosa*. Further research was conducted with lipase P-30.

Screening of alcohols. Six different alcohols were screened in the esterification reaction with lipase P-30 (Table 8). Methanol exhibited the highest stereoselectivity. Benzyl alcohol and 1-octanol also exhibited comparable stereoselectivity. Trifluoroethanol and 2-amino-1-butanol exhibited poor selectivity.

TABLE 7
Evaluation of Lipases for the Stereoselective Esterification of 3-Benzylthio-2-Methyl Propanoic Acid 11[a]

Lipase source	Reaction time (h)	Conversion to **12** (%)	Yield of **11** (%)	Optical purity of *S*-(−)-**11** (%)
Candida cylindraceae	1.3	64	36	38.7
Pseudomonas lipase (Lipase PS-30)	27	62	38	97
Rhizopus sp.	26	58	42	56
Mucor sp.	0.5	78	22	58.4
Aspergillus niger	99	86	14	67
Pseudomonas sp.	27	68	32	96
Pseudomonas sp. (Enzymatics)	27	58	42	92
Pseudomonas sp. (Amano AK)	27	56	44	86.5
Pseudomonas sp. (Amano CES)	27	59	41	87
Penicillum sp. (Amano P)	99	61	39	42

[a]The reaction mixture in 5 mL toluene contained 0.025 M of **11** (5.6 mg), 0.1 M methanol, 0.1% water, and 1 g of crude lipase. The reaction was carried out at 40°C, 200 rpm. The reaction yields and optical purities were determined by gas chromatography. See Table 1 for abbreviations and company sources.

Immobilization and evaluation of lipase P-30. Crude lipase P-30 was immobilized on three different resins—XAD-7, XAD-2, and Accurel PP. The absorption efficiencies were about 68, 71, and 98%, respectively (Table 9). These im-

TABLE 8
Screening of Alcohols for the Stereoselective Esterification of 11[a]

Alcohol	Reaction time (h)	Conversion to **12** (%)	Yield of **11** (%)	Optical purity of *S*-(−)-**11** (%)
Methanol	112	50.3	49.7	91.7
	136	55.1	44.9	95.4
	162	63	37	97.3
2-Amino-1-ethanol	16	68	32	50
	40	93	7	50
Trifluoroethanol	162	29	71	62
	328	38	62	63
Isopropylidene glycerol	112	33	67	67
	280	58	42	87
Benzyl alcohol	112	39	61	72
	280	67	33	96
1-Octanol	112	34	66	68
	280	68	32	95

[a]The reaction mixture in 25 mL toluene contained 0.1 M of **11** (22.4 mg), 0.4 M methanol, 0.1% water, and 1 g of crude lipase PS-30. The reaction was carried out at 28°C, 280 rpm. The reaction yields and optical purities were determined by gas chromatography. See Table 1 for abbreviation and company source.

TABLE 9
Immobilization of Amano PS-30 Lipase on Various Supports[a]

Support	Reaction time (h)	Lipase activity in filtrate (units/mL)	Protein in filtrate (mg/mL)	Adsorption efficiency (%)
XAD-7[b]	0	3060	66	0
	2	1890	60	38
	24	960		68
XAD-2[b]	0	3950		0
	24	1145		71
Accurel PP	0	3950		0
	24	60		98

[a]Crude Amano PS-30 lipase (10 g) was immobilized on various supports (1.3-g each). The enzyme-support mixture was incubated at 28°C and 100 rpm. Periodically and after 24-h incubation, the immobilized enzyme was recovered by filtration, and the filtrate was analyzed for lipase activity. Immobilized lipase was dried in a vaccuum oven at room temperature. See Table 1 for abbreviation and company.
[b]From Rohm and Haas (Philadelphia, PA).
[c]From Akzo Nobel Chemicals (Chicago, IL).

mobilized lipases were evaluated for the ability to stereoselectively esterify racemic **11**. As shown in Table 10, enzyme immobilized on Accurel PP catalyzed efficient esterification, giving 46% reaction yield and 97% optical purity of *S*-(−)-**11**. The enzyme under identical conditions gave similar optical purity and yield of product in two additional reaction cycles.

Immobilized enzyme was evaluated for reusability in 3-mL and preparative 50-mL reactor volumes. Enzyme was reused over 23 cycles without loss of activity in a 3-mL reactor and for over 15 cycles in a 50-mL reactor without loss of activity or productivity. The optical purity of 97.7% and reaction yield of 40% was obtained for *S*-(−)-**11**.

Evaluation of solvents for esterification. Various solvents, such as heptane, cyclohexane, *n*-octane, and toluene, were used in the esterification reaction. Toluene was the best, based on the yield and optical purity of the product. Octane and cyclohexane gave products with high optical purities but in lower yields (Table 11).

Effect of temperature on esterification. The esterification was conducted at 28, 40, and 60°C. On increasing the reaction temperature from 28 to 40°C and to 60°C, the reaction time decreased from 18 to 4 h and to 2 h, respectively. The optical purity (*ca.* 82%) obtained at 40 and 60°C was inferior; however, a higher yield (46%) was obtained. A reaction yield of 37% and optical purity of 97.7% were obtained at 28°C.

Effect of substrate concentration on esterification. The racemic acid **11** was evaluated at 0.25, 0.5, and 1.0 M substrate concentrations with 1, 2, and 4 M methanol, respectively, in esterification reactions with 0.3 g of lipase P-30 immobilized on Accurel PP. The reaction yields (38–47%) were similar; however, optical purity decreased with increasing substrate concentration (Table 12).

Effect of methanol concentration. Various methanol concentrations (0.5, 1.0, 2.0, and 4.0 M), were evaluated at 0.5-M substrate concentration. The rate of esterification decreased as the methanol-to-substrate ratio was increased from 1:1 to 4:1 (Table 13). Higher methanol concentrations may inhibit the esterification reaction by stripping the essential water from the enzyme. Note the marked decrease in optical purity at >3 M methanol, indicating a marked change in the enzyme.

Lipase fermentations. The highest lipase activity achieved in fed-batch fermentation for *Pseudomonas* sp. SC13856 was 1500 units/mL. Crude BMS lipase (140,000 units per g) was immobilized on Accruel PP as described in an earlier section. Immobilized BMS lipase (batch #NWM-0589) and crude BMS lipase (batches #NWM-0506 and 0889) were evaluated in the esterification reaction. A yield of 40% and an optical purity of 96.5% were obtained with immobilized BMS lipase (Table 14). Reaction yields of 39 and 45% and optical purities of 95 and 93.6% were obtained for *S*-(−)-**11** using crude BMS lipase from batches NWM-0889 and NWM-0506, respectively.

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TABLE 10
Evaluation of Immobilized Lipase PS-30 for the Esterification Reaction[a]

Cycle number	Immobilized lipase on support	Reaction time (h)	Conversion to **12** (%)	Yield of **11** (%)	Optical purity of *S*-(−)-**11**
1	XAD-7	18	70	30	96.7
	XAD-2	18	28	72	
		41	50	50	82.5
	Accurel PP	18	64	36	97.5
		25	66	34	97.5
2	XAD-7	18	67	33	92
	Accurel PP	22	54	46	97.7
3	Accurel PP	22	55.6	46.4	96.7

[a]The reaction mixture in 3 mL toluene contained 0.1 M substrate **11**, 0.4 M methanol, 0.1% water, and 0.48 g of immobilized lipase PS-30. The reactions were carried out at 28°C and 280 rpm. The reaction yields and optical purities were determined by gas chromatography. See Tables 1 and 9 for abbreviation and company sources.

TABLE 11
Effect of Solvents on the Stereoselective Esterification of 11[a]

Solvent	Reaction time (h)	Conversion to **12** (%)	Yield of **11** (%)	Optical purity of *S*-(−)-**11** (%)
Heptane	16.5	82.9	17.1	92.2
Cyclohexane	16.5	90.6	9.4	97.8
n-Octane	16.5	91.4	8.6	97.3
Toluene	16.5	63	37	97.2

[a]The reaction mixture in 5 mL of solvent contained 0.1 M of **11** (22.4 mg), 0.4 M methanol, 0.1% water, and 0.48 g of immobilized lipase PS-30 on Accurel PP. The reaction was carried out at 40°C and 280 rpm. The reaction yields and optical purities were determined by gas chromatography. See Tables 1 and 9 for abbreviation and company source.

TABLE 13
Effect of Methanol Concentration on the Esterification of 11[a]

Methanol concentration (M)	Reaction time (h)	Conversion to **12** (%)	Yield of **11** (%)	Optical purity of *S*-(−)-**11**
0.5	5.5	55	45	93.6
1	8	61	39	96
1.5	21	60	40	96.5
2	30	62	38	95.5
3	64	13	87	56
4	64	6	94	56

[a]The reaction mixture in 10 mL toluene contained 0.5 M substrate **11** and methanol as indicated, 0.1% water and 1.0 g of immobilized lipase PS-30 on Accurel PP. The reactions were carried out at 40°C and 280 rpm. The reaction yields and optical purities were determined by gas chromatography. See Tables 1 and 9 for abbreviation and company source.

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TABLE 12
Effect of Substrate Concentration on the Esterification of 11[a]

Substrate concentration (M)	Methanol concentration (M)	Reaction time (h)	Conversion to **12** (%)	Yield of **11** (%)	Optical purity of *S*-(−)-**11**
0.1	0.4	6	55.7	44.3	97.1
0.25	1	17	61.7	38.3	96.5
0.5	2	23.5	53.8	46.2	92
1	4	48	52.6	47.4	90

[a]The reaction mixture in 10 mL toluene contained substrate **11** and methanol as indicated, 0.1% water and 1.2 g of immobilized lipase PS-30 on Accurel PP. The reactions were carried out at 40°C and 280 rpm. The reaction yields and optical purities were determined by gas chromatography. See Tables 1 and 9 for abbreviation and company source.

TABLE 14
Evaluation of Bristol-Myers Squibb Lipase for the Esterification of 11[a]

Batch number	Reaction time (h)	Conversion to **12** (%)	Yield of **11** (%)	Optical purity of *S*-(−)-**11**
NWM-0506	39	55	45	93.6
NWM-0889	39	65	39	95
NWM-0589 (immobilized)	39	52	40	96.5

[a]Lipase fermentations were carried out with *Pseudomonas* sp. SC 13856 by fed-batch process. Crude lipase recovered from the filtrate was used directly or immobilized on Accurel PP and used in the esterification reaction. The reaction mixture in 10 mL toluene contained 0.1 M substrate **11**, 0.4 M methanol, 0. 1% water, and 2 g of crude lipase or 0.5 g of immobilized lipase. The reactions were carried out at 40°C and 280 rpm. See Table 1 for company source.

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