Chemoenzymatic Approaches to SCH 56592, A New Azole Antifungal

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ABSTRACT: Chemoenzymatic approaches to the synthesis of two key chiral precursors of a new azole antifungal agent, SCH 56592, are described. In particular, the enzymatic diastereoselective acylation of 2-benzyloxy-3-pentanol (**7**) was developed to produce (*2S,3R*)-**7** in >97% diastereomeric excess (de) from otherwise unusable mixtures of (*2S,3R*)/(*2S,3S*) **7** (40–80% de). The selectivity and reactivity of commercially available *Candida rugosa* and *Mucor miehei* lipases are compared for the acylation of **7** and the hydrolysis of the corresponding butyrate **16a**. Of the 17 *C. rugosa* enzyme preparations that were examined for acylation of **7**, two purified enzyme preparations showed no reactivity, five enzymes showed high diastereoselectivity with preference for the (*2S,3R*)-isomer, and seven showed a slight preference for the (*2S,3S*)-isomer.
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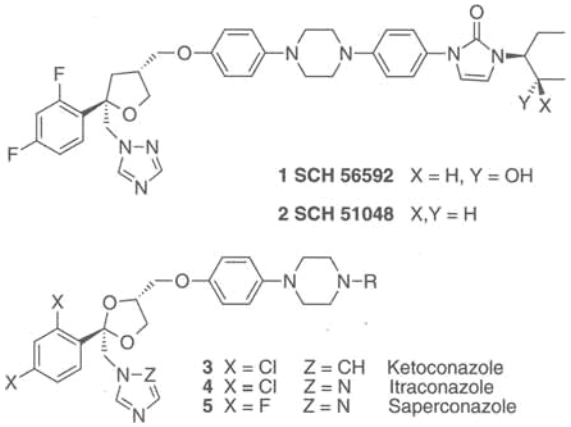
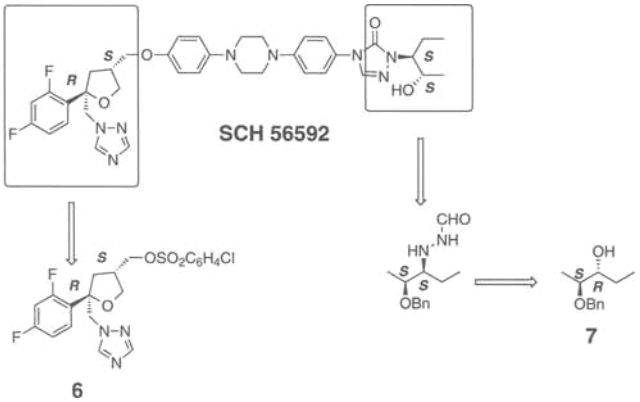
KEY WORDS: Acylation, azole antifungal, biocatalysis, *Candida rugosa*, diastereoselective, lipase, *Mucor miehei*, vinyl esters.

SCH 56592 is a new orally active azole antifungal, presently in Phase II clinical trials (1). It is superior to existing azole antifungals against a variety of fungal infections in both normal and immunocompromised infection models. In particular, it shows enhanced activity against *Candida* and *Aspergillus* infections. In common with other azole antifungals (Scheme 1), SCH 56592 (**1**) contains the azole ring, a dihalogenobenzene ring and a rigid side chain, distributed around a central five-member ring. Unlike Ketoconazole (**3**), Itraconazole (**4**) and Saperconazole (**5**), which contain a central 1,3-dioxolane ring, SCH 56592 contains a more robust tetrahydrofuran ring, which is believed to be responsible for its enhanced oral bioavailability (2). It also contains a hydroxylated side chain at the extreme right-hand side of the molecule, the presence of which enhances activity compared to SCH 51048 (**2**), an earlier nonhydroxylated version of the drug. The presence of these two moieties complicates the synthesis of SCH 56592 (Scheme 1).



SCHEME 1

Key intermediates. Enzymes have become increasingly popular in recent years for the synthesis of chiral building blocks (3–6). This is especially so in the pharmaceutical industry where regulatory pressure has encouraged the development and marketing of chiral drugs as single enantiomers (7,8). The two chiral subunits (**6** and **7**), necessary for the synthesis of optically pure SCH 56592, are shown retrosynthetically in Scheme 2.



SCHEME 2

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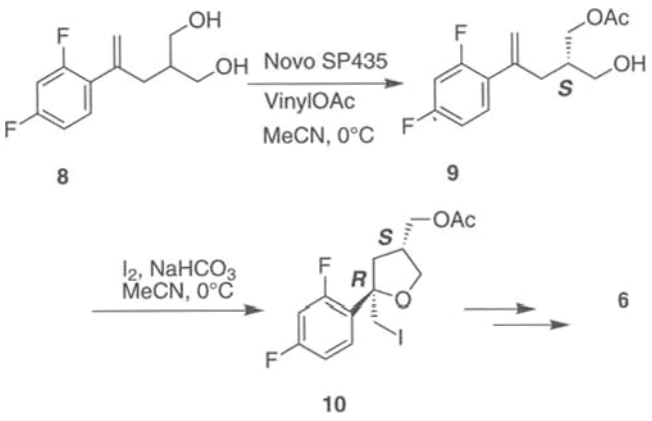


SCHEME 3

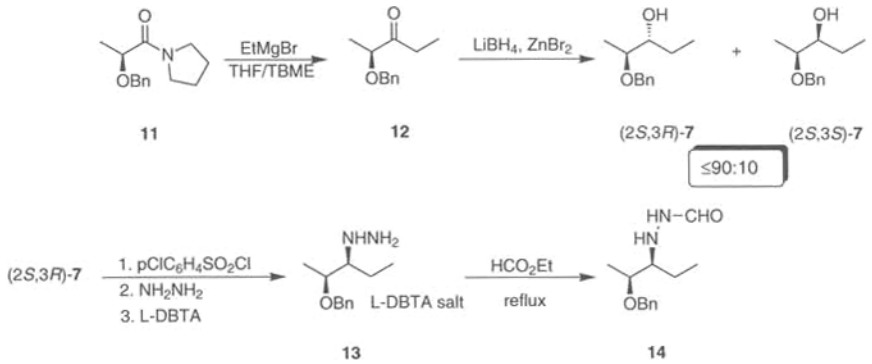
Trisubstituted tetrahydrofuran (6). Chemoenzymatic approaches to the 2,2,4-trisubstituted tetrahydrofuran (**6**) have been previously described (9–12). In particular, the enzymatic desymmetrization of diol (**8**) by *C. antarctica* lipase B (Novozyme 435) provides the (*S*)-monoacetate (**9**) in good yield with high enantiomeric excess [>98% enantiomeric excess (ee)] (Scheme 3). The stereoselectivity of the subsequent iodocyclization transfers the chirality generated by the enzyme to the newly formed benzylic center, to form the required (2*R*,4*S*)-tetrahydrofuran ring (**10**) (60–70% overall). Introduction of the triazole and replacement of the acetate with chlorobenzenesulfonate complete the synthesis of the key intermediate **6.**

(2S,3S)-Formylhydrazone (14). The required (2*S*,3*S*)-formylhydrazone (**14**) was prepared from ethyl lactate, which provided the C2 chiral center (Scheme 4). Subsequent chain elongation *via* the pyrrolidine amide provided ketone **12.** However, under all conditions tried, reduction of **12** resulted in a mixture (≤90:10) of the (2*S*,3*R*)- and (2*S*,3*S*) 2-benzyloxy-3-pentanol (**7**), with separation of the minor (2*S*,3*S*)-diastereomer at this or subsequent steps proving difficult. Displacement of the chlorobenzenesulfonate with hydrazine inverted the C3 chiral center to the required (*S*)-configuration and formylation completed the synthesis of **14** (Scheme 4).

Because the chemical reduction of ketone **12** to the alcohol **7** showed poor diastereoselectivity, various chemoenzymatic routes to pure (2*S*,3*R*)-2-benzyloxy-3-pentanol (**7**) were considered. These approaches to **7** could include microbial reduction of the ketone **12**, hydroxylation of 2-benzyloxypentane at the C3 position, or selective enzymatic acylation of 2-benzyloxy-3-pentanol (**7**). In this report, we describe our efforts to exploit enzyme diastereoselectivity to preferentially esterify a single isomer of 2-benzyloxy-3-pentanol (**7**) with a suitable acyl group to facilitate separation of the desired (2*S*,3*R*)-**7** from unwanted (2*S*,3*S*)-**7**.

MATERIALS AND METHODS

Analytical methods. High-performance liquid chromatography (HPLC) was carried out on a Waters 715 Ultra Wisp equipped with a Daicel Chiralcel OF column (0.46 × 25 cm) (Chiral Technologies Inc., Exton, PA) (1% *i*PrOH/hexane; 1 mL/min; room temperature (RT); detection at 215 nm; retention times: (2*S*,3*R*)/(2*S*,3*S*)-**16a**, 5.52/6.12 min and (2*S*,3*S*)/(2*S*,3*R*)-**7**, 12.01/13.49 min, respectively. Gas chromatography (GC) was performed on a Shimadzu GC 14A (Tokyo, Japan) equipped with a DB-1 capillary column (J&W Scientific) (15 m × 0.25 mm × 0.25 μm) (run conditions 100°C for 1 min; 10°/min to 250°C for 2 min; retention times: (2*S*,3*S*)/(2*S*,3*R*)-**7**, 6.15/6.25 min; the butyrates **16a**, propionates **16b**, and chloroacetates **16c**, at 9.63, 8.94 and 9.89 min, respectively, did not resolve). Optical rotations were determined on a Perkin-Elmer 243 B Polarimeter (Palo Alto, CA). Flash chromatography was carried out with Sorbisil C60 (40/60A) (Fisher Scientific, Pittsburgh, PA). Karl Fischer titrations were carried out on a Mettler DL37 KF Coulometer (Mettler Instrument Corp., Hightstown, NJ). Vinyl butyrate (VinylOBu), vinyl benzoate, vinyl hexanoate, vinyl propionate, vinyl chloroacetate, and butyric anhydride were obtained from TCI USA (Portland, OR), and vinyl acetate from Aldrich Chemical Company (Milwaukee, WI), and



SCHEME 4

were used as received. All other chemicals were obtained from Fisher Scientific and were used as received.

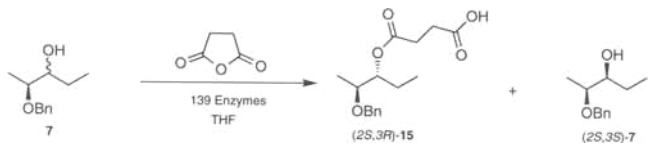
Enzyme source. Enzymes were obtained from the following commercial sources and were used as received: Lipase CR Analytical Grade 001, ChiroCLEC CR, ChiroCLEC CR (dry), and *C. rugosa* esterase from Altus Biologics Inc. (Cambridge, MA); Lipase AY-30 from Amano Enzyme USA Ltd. (Troy, VA); Lipase *C. cylindracea* from Biocatalysts Ltd. (Pontypridd, United Kingdom; Lipase 305, Chirazyme L-3, and cholesterol esterase from Boehringer-Mannheim Co. (Indianapolis, IN); Enzeco Lipase XX Concentrate and Enzeco S-4827 Esterase/Lipase from Enzyme Development Corp. (New York, NY); *Mucor miehei* esterase from Fluka Biochimika (Buchs, Switzerland); Lipase *C. cylindracea* from Genzyme Corp. (Cambridge, MA); Piccantase A from Gist-Brocades (Charlotte, NC); Lipase OF, OFG, OFC, and MY from Meito Sangyo Co., Ltd. (Tokyo, Japan); Lipozyme IM-20 and IM-60, Lipozyme 10,000L, SP-524 and SP-526 from Novo Nordisk Bioindustrials Inc. (Danbury, CT); Lipase *C. cylindracea* L-8525, Type VII and Type VIIA, Porcine Pancreatic Lipase (Type II) and Acylase I (*A. mellieus*) from Sigma (St. Louis, MO); Lipase G-100 from Solvay Enzymes, Inc. (Elkhart, IN); Toyobo LPL-701 from Shinko American Inc. (New York, NY). *Candida rugosa* esterase (Altus Biologics) (20 mL) was dialyzed against aqueous buffer (800 mL) (10 mM Tris; 1 mM $CaCl_2$; pH 6.7) at 4°C overnight and then against fresh buffer (200 mL) for 6 h. The contents of the dialysis bag were then frozen and lyophilized and the lyophilate (0.31 g) used as is.

(2S,3R)-2-Benzyloxy-3-pentyl butyrate, (2S,3R) (***16a***). A mixture of 4 Å molecular sieves (8 × 12 mesh) (1.0 g), vinyl butyrate (1.3 mL, 10.3 mmol), and Lipase OF (1.0 g) was suspended in *tert*-butyl methyl ether (*t*BME) (7.4 mL) and stirred at room temperature for 95 min. A solution of **7** (1.0 g, 5.15 mmol) (70% de) in *t*BME (2.6 mL) was added, and the mixture was stirred magnetically for 163 h. The reaction mixture was then filtered through a Celite pad, which was washed with *t*BME (10 mL), and the combined filtrate was evaporated. The residue was placed on a SiO_2 column (15 g) and eluted with hexane (50 mL), 2% (50 mL) and 4% (50 mL) *t*BME/hexane, and fractions of 15–20 mL were collected. The desired fractions were combined and evaporated to obtain a colorless liquid (0.97 g, 98.2% de; 60.4% based on total alcohol, 71.3% based on (2*S*,3*R*) starting material): $[\alpha]_D^{25}$ +16.7° (c 1.323, EtOH); infrared (IR) (neat) 1734 cm^{-1}; chemical ionization–mass spectrometry (CI–MS) (CH_4) 265 (M + 1); ^1H nuclear magnetic resonance (NMR) ($CDCl_3$) δ 0.87 (*t*, 3H), 0.93 (*t*, 3H), 1.15 (*d*, 3H), 1.63 (*m*, 4H), 2.82 (*t*, 2H), 3.55 (*m*, 1H), 4.53 (AB *quart*, 2H), 4.95 (*m*, 1H), 7.29 (*m*, 5H); ^{13}C NMR ($CDCl_3$) δ 10.1, 13.7, 15.7, 18.6, 22.9, 36.5, 70.9, 75.5, 76.3, 127.5, 127.6, 128.3, 138.6, 173.4. Anal., calc'd for $C_{16}H_{24}O_3$: C, 72.69; H, 9.15; found: C, 72.80; H, 9.27.

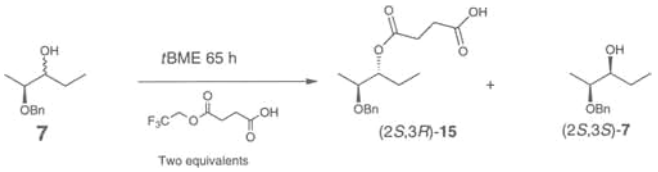
(2S,3R)-2-Benzyloxy-3-pentyl chloroacetate, (2S,3R)-(***16e***). This was prepared as above from a mixture of **7** (6.08 g, 31.3 mmol) (82.6% de), vinyl chloroacetate (6.34 mL, 62.6 mmol), and Novo Lipozyme IM (5.32 g) in *t*BME (60 mL) shaken at 250 rpm at RT for 160 h. While the Lipozyme IM-catalyzed acylation of **7** with vinyl chloroacetate is relatively fast, the reaction slows down considerably after ~80% conversion. This reaction was allowed to proceed for longer than usual to maximize conversion and allow isolation of the (2*S*,3*S*)-diastereomer with good de. Removal of the enzyme beads by filtration, followed by column chromatography, yielded (2*S*,3*R*)-**16e** (6.33 g, 98.4% de; 74.7% based on total alcohol, 90.4% based on composition of the starting mixture): $[\alpha]_D^{24}$+17.8° (c 0.202, EtOH); CI–MS (CH_4) 271 (M + 1); ^1H NMR ($CDCl_3$) δ 0.94 (*t*, 3H), 1.20 (*d*, 3H), 1.68 (*m*, 2H), 3.60 (*m*, 1H), 4.05 (*s*, 2H), 4.56 (*s*, 2H), 5.06 (*m*, 1H), 7.33 (*m*, 5H); ^{13}C NMR ($CDCl_3$) δ 10.0, 15.3, 22.7, 41.0, 70.9, 75.1, 127.6, 127.7, 128.4, 138.3, 164.2. Later fractions were eluted with 30% EtOAc/hexane to yield (2*S*,3*S*) **7** (0.85 g, 80.2% based on composition of the starting mixture) (95.7% de).

(2S,3R) 2-Benzyloxy-3-pentyl propionate (2S,3R)-(***16b***). This was prepared as above from a mixture of **7** (1.0 g, 5.1 mmol) (70% de), vinyl propionate (1.3 mL, 119 mmol), and Lipase OFG (3.0 g) in *t*BME (10 mL) at 250 rpm and RT for 125 h. After workup and column chromatography, the relevant fractions were combined to yield a slightly yellow liquid (0.91 g, 59.6% based on total starting material, 70.3% conversion of (2*S*,3*R*)-**7** (96.9% de): $[\alpha]_D^{24}$+21.1° (c 1.473, EtOH); CI–MS (CH_4) 251 (M + 1); IR (neat) 1738 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89 (*t*, 3H), 1.15 (*t*, 3H), 1.18 (*d*, 3H), 1.64 (*quin*, 2H), 2.35 (*q*, 2H), 3.59 (*m*, 1H), 4.56 (AB *quart*, 2H), 4.98 (*m*, 1H), 7.32 (*m*, 5H); ^{13}C NMR ($CDCl_3$) δ 9.3, 10.1, 15.6, 22.9, 27.8, 70.9, 75.4, 76.3, 127.5, 127.7, 128.3, 138.6, 174.3. Anal., calc'd. for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86; found: C, 71.78; H, 8.71.

(2S,3R)-2-Benzyloxy-3-pentanol (2S,3R)-(***7***). Four Lipozyme IM-catalyzed acylations [1.2 g of (2*S*,3*R*)-alcohol per reaction] were combined, and the butyrate (2*S*,3*R*)-(**16a**) (5.36 g, 20.3 mmol) obtained by column chromatography was dissolved in a mixture of *i*PrOH (30 mL) and 2 M NaOH (100 mL). The mixture was heated at reflux for 17.5 h, then cooled to RT, and *i*PrOH was removed. The residue was extracted twice with EtOAc (100 and 50 mL), and the combined organic extracts were washed with saturated aqueous NaCl (50 mL), dried ($MgSO_4$), filtered, and evaporated. The crude product was distilled in a Kugelrohr oven (oven temperature 100–105 C, 1.0 mm Hg) to obtain a colorless viscous liquid [3.70 g, 93.9%; 76.6% overall from starting (2*S*,3*R*)-alcohol] (97.4% de): $[\alpha]_D^{21}$ + 35.7° (c 1.181, EtOH):



SCHEME 5



SCHEME 6

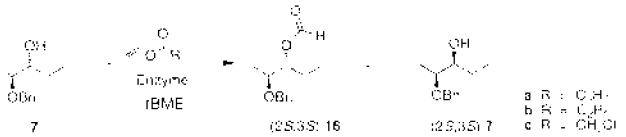
IR (neat) 3456 cm^{-1} (*br*); ^1H NMR ($CDCl_3$) δ 0.97 (*t*, 3H), 1.15 (*d*, 3H), 2.25 (*br s*, 1H), 3.51 (*m*, 1H), 3.66 (*m*, 1H), 4.55 (AB *quart*, 2H), 7.33 (*m*, 5H); ^{13}C NMR ($CDCl_3$) δ 10.5, 13.2, 25.1, 70.6, 74.4, 77.4, 127.6, 128.4, 138.5. Anal., calc'd. for $C_{12}H_{18}O_2$: C, 74.19, H, 9.34; found: C, 74.16, H, 9.32.

RESULTS AND DISCUSSION

Anhydride and amine acylating agents. The obvious strategy was to examine the enzymatic acylation of **7** with succinic anhydride as acylating agent because the product hemisuccinate (**15**) would be easily separated from the unreacted material by extraction with alkali (13–16) (Scheme 5).

A screen of 148 enzymes was undertaken with (**7**) (49% de) (50 mg), enzyme (50 mg), and succinic anhydride (five equivalents) in tetrahydrofuran (THF) (2.0 mL) at RT. After 67 h, thin-layer chromatography (TLC) showed that only eight enzymes displayed significant catalytic activity, and HPLC showed that only four displayed significant selectivity. The diastereomeric excesses of the hemisuccinate product (**15**) were poor (67–80% de). In terms of selectivity/reactivity, the best enzyme was Toyobo LPL-701, which acylated the (2*S*,3*R*)-diastereomer. With LPL-701, the acylation was examined in 14 solvents, ranging in polarity from ethanol to hexane. Best results were observed with *t*BME, 1,4-dioxane, *t*-amyl alcohol and *t*-butyl alcohol, but selectivity/reactivity was still low; de values of 72–77% were observed at 54–68% conversion after 64 h. A number of other anhydride acylating agents were examined. Glutaric anhydride (17,18) showed low reactivity (~12% conversion after 44 h), while maleic and phthalic anhydride and Meldrum's acid showed no signs of reaction. Hydrolysis of the hemisuccinate **15** (49% de) with Toyobo LPL-701 in 10 mM phosphate buffer/THF (10:2, mL/mL) (pH 7.0) was also slow. After 44 h, the reaction showed ~50% conversion with 50% de in the product.

TABLE 1
Enzymatic Butyrylation of 2-Benzyloxy-3-pentanol (7)[a]

Enzyme	mg	de of 7 (%)	Conversion (%)
			
1 Meito Lipase OF	63	97.8	71.9
2 Novo Lipozyme IM-20	63	99.0	60.0
3 Amano PS-30	57	98.0	39.5
4 Novo SP 524	52	99.1	60.7
5 Sigma Pork Pancreatic Lipase	96	96.9	24.2
6 Sigma Acylase I	98	98.4	29.3
7 Novo SP 526	60	98.2	22.9

[a]Conditions: Substrate, 50 mg; Vinyl OBu, five equivalents; *tert*-butyl methyl ether (*t*BME), 2 mL; room temperature; 25 h, except 57, 3 h; de, diastereomeric excess.

In a series of nitrogen-containing acylating agents was also examined, again for the purpose of effecting separation by acid/base extraction. However, treatment of (**7**) with ethyl dimethylaminopropionate, ethyl dimethyl aminoglycine, ethyl dimethylaminobenzoate, or sarcosine ethyl ester (five equivalents) in *t*BME (2 mL) in the presence of Toyobo LPL-701 (one weight equivalent) showed no sign of reaction after 62 h.

Vinyl ester acylating agents. Because it seemed unlikely that functionalized esters would be obtained enzymatically with high diastereoselectivity, we examined the enzyme-catalyzed acylation of 2-benzyloxy-3-pentanol (**7**) with vinyl butyrate as acylating agent. From a screen of 147 hydrolytic enzymes, a number of enzymes showed promising reactivity (Table 1). However, in all cases, the major diastereomer (2*S*,3*R*)-**7** reacted preferentially, resulting in a less convenient direct purification. Because of their high diastereoselectivity, reasonable reactivity, cost and commercial availability, Meito Lipase OF (*C. rugosa*) and Novo Lipozyme IM-20 (*M. miehei*) were chosen for further development. (*Candida rugosa* was formerly called *C. cylindracea*. *Candida rugosa* will be used throughout the text except when referring to manufacturers' labeling. Novo Lipozyme IM is Novo Lipo-

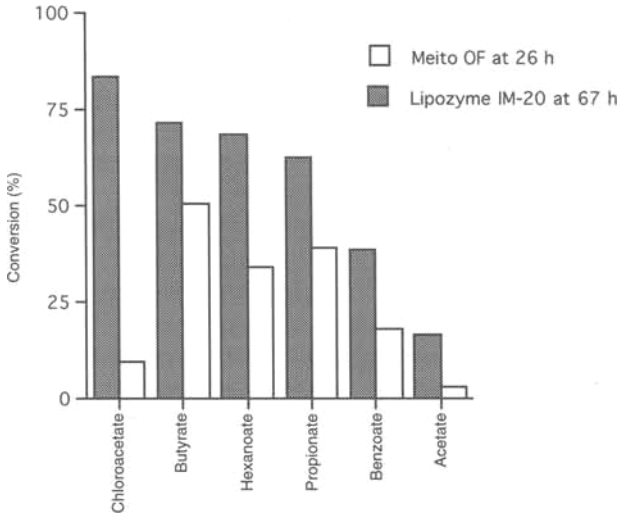


FIG. 1. The effect of acylating agent on the acylation of **7** in *tert* butyl methyl ether (*t*BME). Conditions: **7**, 0.2 g, 0.5 M, 50% de; enzyme 0.2 g; *t*BME, 2 mL; vinyl ester, two equivalents; room temperature. Conversion (%) was measured at 26 h for Meito OF and at 67 h for Lipozyme IM-20.

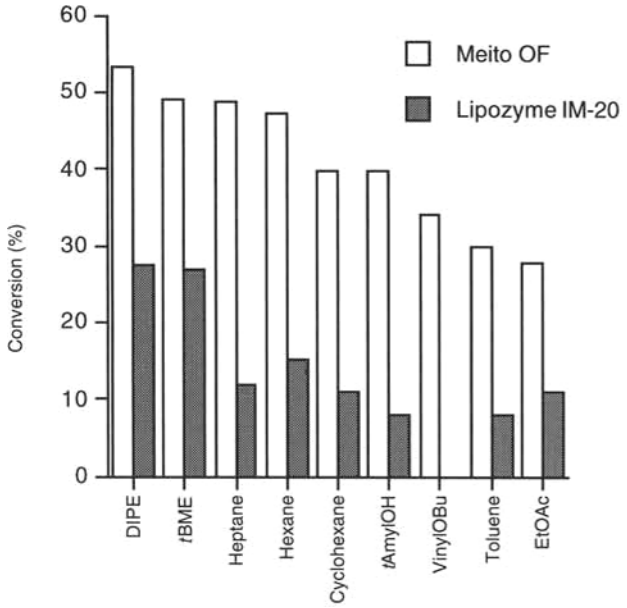


FIG. 2. The effect of solvent on the butyrylation of **7**. Conditions: **7**, 50 mg, 40% de; enzyme, 50 mg; solvent, 2 mL; vinylOBu, five equivalents; room temperature; 16 h. Abbreviation: DIPE, diisopropyl ether. For other abbreviations see Figure 1.

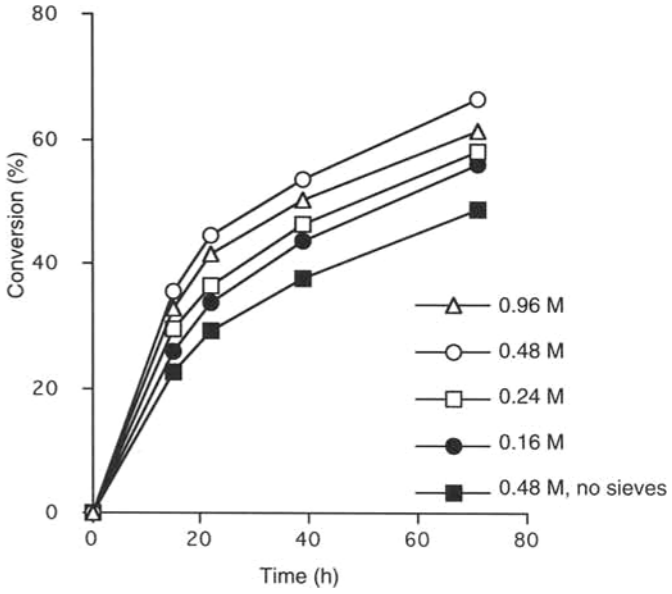


FIG. 3. The effect of concentration on Meito OF-catalyzed butyrylation of **7**. See Figure 1 for company source.

zyme immobilized on a macroporous resin; IM-20 and IM-60 are different loadings.)

The 14 best enzymes from the vinyl butyrate screen were also screened for their ability to use trifluoroethyl hemisuccinate as an acylating agent (Scheme 6). While three enzymes showed some selectivity [(2*S*,3*R*)-**15** 80–90% de], the reactivity was low (3–14% after 65 h).

Effect of acylating agent/solvent. The influence of acylating agent on the degree of conversion was examined, and both enzymes showed similar behavior (Fig. 1). Vinyl acetate was a poor acylating agent for both enzymes, and vinyl benzoate was sluggish. The vinyl esters of propionic, butyric, and hexanoic acid showed similar degrees of conversion, with vinyl butyrate performing best. The major difference was observed with vinyl chloroacetate, which performed best for Lipozyme IM-20, while reacting poorly with Meito OF. Acylation with butyric anhydride was slower than for vinyl butyrate with both enzymes, and diastereoselectivity was lower owing to chemical acylation. For a comparison of different enol esters in *C. rugosa* (Sigma Type VII)-catalyzed transesterification, see Reference 19, and for a demonstration of the effect of acid chainlength on the Novo Lipozyme-catalyzed esterification of alcohols, see Reference 20. For both enzymes, the enzymatic butyrylation was examined in 20 common solvents (the most promising solvents are compared in Fig. 2). The best results were obtained in ether and hydrocarbon solvents such as diisopropyl ether (DIPE), *t*BME, heptane, or hexane. *t*BME was the solvent of choice for further reactions. Generally reactions were faster with Meito OF than with Lipozyme IM-20.

Effects of molecular sieves/temperature/concentration. In an attempt to speed up the reaction, the effects of heating and molecular sieves on the Meito OF-catalyzed butyrylation were examined. The reaction was examined in *t*BME at ambient temperature, 42 and 55°C. Increasing temperature resulted in increased conversion without compromising selectivity [(2*S*,3*S*)-**7** > 0.97 de]. The presence of sieves (g/g) re-

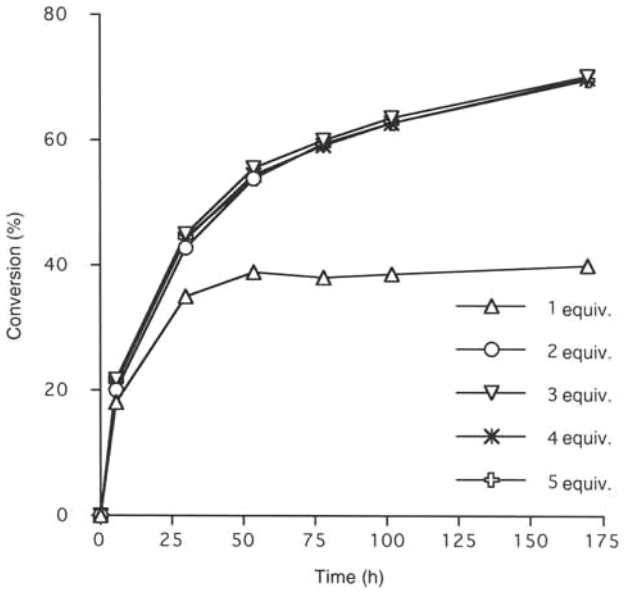


FIG. 4. The effect of vinyl butyrate concentration on Meito OF-catalyzed butyrylation of **7**. See Figure 1 for company source.

TABLE 2
Scale Up of Enzymatic Butyrylation of 2-Benzyloxy-3-pentanol 7[a]

		Yield			
	kg	(kg)	(%)	de (%)	ee[b] (%)
7	7.00			74.8	96.7
(2*S*,3*R*)-**16a**		6.67	69.1	99.0	99.4
(2*S*,3*R*)-**7**		4.88	61.7	98.8	99.4

[a]Conditions: **7**, 7 kg (74.8% de); Meito OF, 7 kg; VinylOBu, 8.2 kg; 4 Å sieves, 7 kg; *t*BME, 77 L; room temperature; 10 d.
[b]ee, enantiomeric excess. See Table 1 for other abbreviations and company sources.

sulted in a 10–20% higher conversion, compared to identical reactions without sieves, and the obvious explanation was that the sieves were removing water from the system. Karl Fischer titrations were carried out to determine if differences in moisture content could be measured. A mixture of substrate (10 g) and 3Å molecular sieves (10 g) in *t*BME (100 mL) was stirred for 1 h, and a sample was withdrawn. Meito OF (10 g) was added, the mixture was stirred for 0.5 h, and another sample was withdrawn. A parallel reaction in the absence of sieves was set up and analyzed simultaneously. No difference in moisture content was observed by Karl Fischer titration (0.17–0.20% before and 0.37–0.41% after addition of enzyme), and after addition of vinyl butyrate, the reaction in the absence of sieves was significantly slower. In two simultaneous reactions, with and without molecular sieves, the two reactions were pre-stirred in the absence of substrate for 5 h; we hoped that bulk water would be consumed in the hydrolysis of vinyl butyrate, and that the subsequent acylation on addition of substrate would not depend on the presence of sieves. On addition of substrate and stirring for a further 15.75 h, conversion in the presence and absence of molecular sieves was 26.8 and 17.1%, respectively (increasing to 67.8 and 45.3% after 89 h). It is possible that water in the reaction mixture, or the butyric acid or acetaldehyde resulting from hydrolysis of the acylating agent, adversely affects the enzyme's performance (21).

In parallel reactions, the best rate was observed at 0.48 M **7** with two equivalents of vinyl butyrate in the presence of sieves; again, the reaction was noticeably slower in the absence of sieves (Fig. 3). With 2–5 equivalents of acylating agent, there was no major difference in rate with either Meito OF or Lipozyme IM. However, with one equivalent, some batches of starting material showed incomplete reaction for both enzymes (Fig. 4). Variability in the performance of some batches of substrate was observed, and the possibility that some samples of **7** contained an enzyme inhibitor was briefly examined. Pyrrolidine, dimethoxyethane (DME), and residual pyrrolidine amide **11** were considered as potential contaminants, and their effect on the Meito OF-catalyzed acylation is shown in Figure 5. Under identical conditions, distilled purified (2*S*,3*R*)-**7** (97% de) reacted faster than crude material (78% de), even though the latter contains less active substrate. Of the three contaminants tested, only pyrrolidine showed a significant inhibitory effect on the enzymatic acylation: in the presence of 5% (vol/vol) (1.25 equivalent) pyrrolidine, only ~10% reaction was observed after 50 h. Guo and Sih (22) have shown that some chiral amines are effective inhibitors of *C. rugosa* lipase. However, the level of pyrrolidine in crude samples was not established. In addition, the presence of 5% of the enantiomeric (2*R*,3*S*)/(2*R*,3*R*)-**7** pair (86% de) had no effect on the rate of acylation of **7** (data not shown).

Scale-up. We still hoped that, after the enzyme-catalyzed butyrylation, the crude reaction mixture might be acylated chemically with succinic anhydride, and the (2*S*,3*S*)-hemisuccinate **15** so formed, containing all of the unwanted (2*S*,3*S*)-**7** diastereomer, could then be removed by alkaline

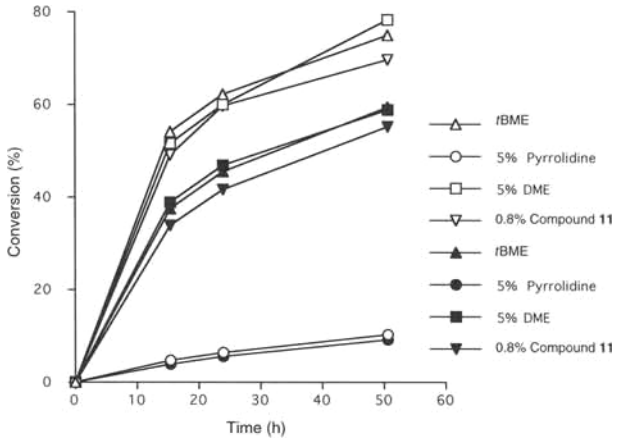


FIG. 5. The effect of potential contaminants on the enzymatic butyrylation of **7**; purified distilled (2*S*,3*R*)-**7** (97% de), open symbols; crude **7** (78% de), closed symbols. Conditions: **7**, 92 mg; Meito OF, 100 mg; VinylOBu, two equivalents; 4 Å sieves, 100 mg; *t*BME, 1.0 mL; room temperature. DME, dimethoxyethane. See Figure 1 for company source and other abbreviation.

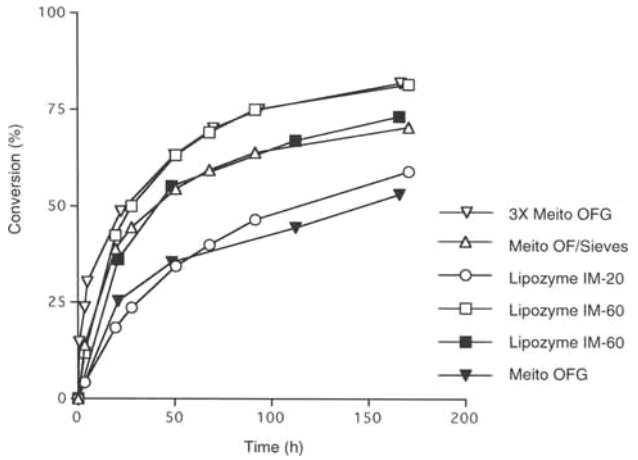
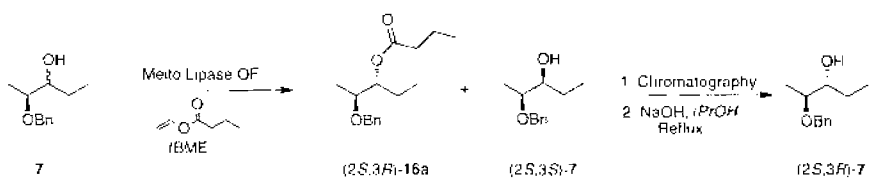


FIG. 6. Enzymatic butyrylation of **7** with immobilized enzymes. Conditions: (at) open symbols; **7**, 1.0 g (70% de); enzyme, 1 g, (except OFG, 3 g); VinylOBu, two equivalents; *t*BME, 10 mL; 4 Å sieves, 1 g (OF only); room temperature; (bt) closed symbols; **7**, 0.1 g (70% de); enzyme, 0.1 g; VinylOBu, two equivalents; *t*BME, 1.0 mL; room temperature. See Figure 5 for abbreviations; see Figure 1 for other company sources.

TABLE 3
Butyrylation of Compound 7 with Selected *Candida rugosa* and *Mucor miehei* Enzymes[a]

Entry	Vendor	Product	Wt (mg)	Time (h)	7 (% de)	16a (%de)	Conversion (%)
C. rugosa enzymes/vinyl butyrate							
1	Altus	Lipase CR Analytical Grade 001	36	89	43.8	n/d[b]	<1
2	Sigma	Lipase L8525 (purified)	19.8	93	45.6	n/d	1.2
3	Altus	ChiroCLEC CR (dry)	5.4	89	42.1	72.8	9.2
4	Altus	ChiroCLEC CR (dry)	81.1	93	42.6	68.6	13.1
5	Biocatalysts	Lipase *C. cylindracea*	94	89	11.8	91.0	41.0
6	Enzyme Development Corp.	Enzeco Lipase XX Concentrate	89	89	2.2	91.6	45.9
7	Meito	Lipase OF	107	89	5.4	90.2	44.7
8	Meito	Lipase OFG	141	89	25.8	89.1	57.3
9	Meito	Lipase OFC	110	89	13.4	90.7	40.9
10	Amano	Lipase AY-30	104	89	61.1	10.6	33.0
11	Boehringer-Mannheim	Lipase 305	46	89	51.2	1.3	14.8
12	Boehringer-Mannheim	Chirazyme L3	65	89	51.9	29.9	38.0
13	Genzyme	Lipase *C. cylindracea*	33	89	49.2	13.1	15.5
14	Meito	Lipase MY	100	89	50.8	21.0	26.2
15	Sigma	Lipase Type VII	122	89	57.6	23.2	40.3
16	Sigma	Lipase Type VII-A (immobilized)	145	89	54.4	6.7	20.3
17	Altus	*C. rugosa* esterase (lyophilate)	22.3	89	42.5	69.0	7.4
18	Boehringer-Mannheim	Cholesterol esterase	50	89	43.6	42.6	9.4
C. rugosa enzymes/trifluoroethyl butyrate							
19	Altus	Lipase CR Analytical Grade 001	63.6	93	44.6	48.1	0.3
20	Sigma	Lipase L8525 (purified)	15.6	93	45.0	20.0	0.3
21	Altus	ChiroCLEC CR (dry)	18	93	40.7	69.3	11.2
22	Meito	Lipase OI	88	93	27.6	90.5	29.2
23	Biocatalysts	Lipase *C. cylindracea*	89.9	93	42.1	90.3	23.9
24	Amano	Lipase AY-30	134.8	93	54.9	6.4	23.0
25	Boehringer-Mannheim	Chirazyme L3	106.8	93	55.7	14.3	28.3
26	Genzyme	Lipase *C. cylindracea*	26.9	93	46.6	2.7	7.1
27	Meito	Lipase MY	98.4	93	50.1	12.0	16.1
28	Sigma	Lipase Type VII	112.7	93	54.8	14.0	25.9
29	Sigma	Lipase Type VII-A (immobilized)	95.8	93	47.4	10.9	6.5
30	Altus	*C. rugosa* Esterase (lyophilate)	8	89	45.2	n/d	1.6
M. miehei enzymes/vinyl butyrate							
31	Novo	Lipozyme IM-20	96	89	21.3	92.8	35.0
32	Novo	Lipozyme IM-60	91	89	27.4	92.5	37.6
33	Novo	SP 524 Lipase (nonimmobilized IM-20/60)	72	89	25.0	92.5	30.9
34	Fluka	Esterase	37	89	40.1	93.0	10.3
35	Enzyme Development Corp.	S 4827 Enzeco Esterase/Lipase	104	89	42.3	n/d	<1
36	Gist-Brocades	Piccantase A	106	89	43.0	n/d	<1
37	Solvay	Lipase G-1000	174	89	44.0	n/d	<1

[a]Conditions: Substrate, 0.1 g, 0.26 M; vinyl butyrate, three equivalents, or trifluoroethyl butyrate, five equivalents; tBME, 2.0 mL; room temperature; 250 rpm. See Table 1 for abbreviations.
[b]n/d, not determined.



7 (2*S*,3*R*)-16a (2*S*,3*S*)-7 (2*S*,3*R*)-7

SCHEME 7

extraction. However, it proved difficult to cleanly and completely acylate all of the unreacted alcohol, so we decided to proceed with a convenient column chromatographic separation of the (2*S*,3*R*)-butyrate **16a** from the unreacted (2*S*,3*S*)-alcohol **7**. The reaction sequence was successfully scaled up in a pilot plant as shown by the results in Table 2 for a 7-kg reaction (Scheme 7). After complete acylation [10 d for >95% conversion of the (2*S*,3*R*)-isomer], the butyrate **16a** was isolated by column chromatography (5X SiO_2), then hydrolyzed to yield enantiomerically and diastereomerically pure (2*S*,3*S*)-**7**.

There were some process problems on scaling up the reaction: the high enzyme loading and the presence of sieves caused problems in filtration; the reaction required extended periods to approach completion; cost of the enzyme; and chromatography was required for isolation. Because an immobilized enzyme would greatly facilitate filtration, Meito Lipase OFG (*C. rugosa*, immobilized on granulated diatomaceous earth) was compared with Meito OF (acetone powder). The best results were observed with Lipozyme IM-60 (activity three times that of IM-20) or 3-g equivalents of Meito OFG (the activity of OFG is listed as one-third that of OF) (Fig. 6). The use of Lipozyme IM-60 with vinyl chloroacetate as acylating agent looks promising; the faster reaction, simplified and less odorous workup, and the possibility of enzyme reuse potentially outweigh the increased costs of enzyme and acylating agent.

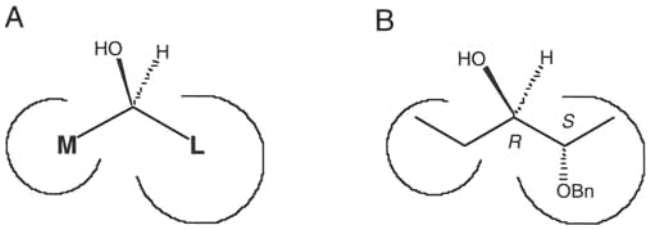


FIG. 7. (A) Empirical rule that predicts the faster reacting enantiomer of secondary alcohols with hydrolases, e.g., *Candida rugosa* (adapted from Ref. 16). (B) The faster reacting (2*S*,3*R*)-**7** obeys the model.

Enzyme source. Because of the low rate of enzymatic butyrylation, *Candida* and *Mucor* enzymes from different commercial sources were compared. While different reactivities were to be expected, given the different activities of the enzyme preparations, a surprisingly wide variation in selectivity was also observed. Of the seven *M. miehei* preparations examined, only Novo Lipozyme IM-20/60 and the corresponding nonimmobilized form, Lipase SP 524, showed any reactivity under acylating conditions. The 17 *C. rugosa* enzyme preparations that were surveyed fell into three groups (Table 3 for acylation conditions, and Table 4 for hydrolysis conditions). In general, the purified enzyme preparations showed poor activity (Table 3, entries 1–4). Altus Analytical Grade Lipase CR is a purified form of Meito OF and is known to be

TABLE 4
Hydrolysis of Compound 16a with Selected *Candida rugosa* and *Mucor miehei* Enzymes[a]

	Vendor	Product	Time (h)	Enzyme (mg)	**16a** (%de)	**7** (%de)	Conversion (%)
C. rugosa enzymes							
1	Altus	ChiroCLEC CR[b,c]	3.0	0.1 mL	43.4	62.7	24.7
4	Altus	Lipase CR Analytical Grade 001	3.0	51.7	42.0	62.0	26.5
2	Altus	*C. rugosa* Esterase (liquid)[d]	0.75	0.5 mL	46.6	44.6	17.1
3	Altus	*C. rugosa* (Dialyzed solid)[d]	3.0	57.1	49.7	46.6	93.1
5	Amano	Lipase AY-30	3.0	103.3	52.0	36.1	47.3
6	Boehringer-Mannheim	Cholesterol esterase	3.0	103.0	48.1	46.0	66.9
7	Boehringer-Mannheim	Lipase 305	1.0	11.6	45.7	43.4	20.4
8	Enzyme Development Corp.	Lipase XX Concentrate	20.0	99.8	31.2	56.9	75.7
9	Genzyme	Lipase *Candida cylindracea*	3.0	29.5	49.9	42.2	50.6
10	Meito	Lipase MY	3.0	100.0	54.3	34.1	45.3
11	Meito	Lipase OF	3.0	101.2	43.9	50.0	38.7
12	Sigma	L8525 (Purified)	4.0	15.4	31.8	34.8	42.7
M. miehei enzymes							
13	Enzyme Development Corp.	S-4827 Enzeco Esterase/Lipase	73.0	105.6	47.1	n/d[e]	0
14	Gist-Brocades	Piccantase A	73.0	108.1	44.4	n/d	0
15	Novo	Lipozyme 10,000L	47.0	3.5 mL	40.3	96.0	35.5
16	Novo	Lipozyme IM-20	23.5	120.5	45.2	n/d	0

[a]Conditions: Substrate, 91 mg (0.34 mmol); 50 mM phosphate buffer pH 6.8 (10 mL), room temperature.
[b]50 mM KCl (10 mL), pH stat.
[c]CLEC CR is a formulated suspension for hydrolytic use.
[d]*Candida rugosa* esterase is supplied as a solution; solid enzyme was obtained by dialysis and lyophilization.
[e]n/d, not determined.

inactive under acylation conditions. Similarly, purified Sigma lipase (L-8525) showed no activity in *t*BME. This may be due to the loss of constituent sugars during purification; the enzyme is 7% glycosylated (23). In contrast, the crude Sigma lipase preparation (Type VII), which contains 38% added lactose, showed 40% conversion after 89 h. Addition of carbohydrates to previously dialyzed *C. rugosa* has been shown to affect the enzyme's behavior under both hydrolytic and acylation conditions (24). The highly purified crosslinked enzyme crystal formulation [Altus ChiroCLEC-CR (dry)] also showed poor reactivity at 5% w/w loading. At higher loading (10–50% w/w) with vinyl butyrate as acylating agent, CLEC showed lower selectivity, and the reactions seemed to stall at <20% conversion in *t*BME and isooctane with pure (2*S*,3*R*)-**7** (data not shown). Similar behavior in the presence of vinyl acetate has recently been reported (25). CLEC-CR also showed poor reactivity with trifluoroethyl butyrate as acylating agent, and the reaction terminated at ~11% conversion. Optimal water activity and the presence of surfactants have been shown to be important determinants of the reactivity/selectivity of cross-linked enzyme crystals (26) and purified preparations of *C. rugosa* lipase (27) but were not addressed in the present study.

Candida rugosa Meito OF and its immobilized forms, OFG and OFC, showed 40–60% conversion after 89 h, as did Biocatalysts' *C. cylindracea* and EDC Lipase XX Concentrate (Table 3, entries 5–9). This set of enzymes also showed good diastereoselectivity and formed (2*S*,3*R*)-**16a** with 89–92% de [The de was lower than previously recorded above because an impurity coeluted on HPLC with (2*S*,3*S*)-**16a**, to depress the observed de]. The chiral preference shown by this group of enzymes agrees with the model proposed by Kazlauskas *et al.* and Franssen *et al.* (28,29) and is supported by X-ray crystal structures of the purified enzyme (30) if the medium and large groups are assigned to the ethyl and benzyloxy groups, respectively (Fig. 7).

A third group of enzymes, comprising Sigma Type VII, Meito MY, Amano AY-30, Boehringer-Mannheim Chirazyme L-3 and Lipase 305 (*Candida* sp.), and Genzyme *C. cylindracea*, all showed poor selectivity (Table 3, entries 10–16). In fact, the de of the product butyrate **16a** was less than that of the starting material (47% de), indicating a preference for acylation of the minor (2*S*,3*S*)-diastereomer. While Sigma Type VII has been shown to be heterogeneous (31–33), it is also reported to be free of contaminating enzymes that display opposite stereochemical preference for the enantioselective hydrolysis of racemic aryl- and (aryloxy)propionic esters (34). However, the present study suggests that some *C. rugosa* preparations contain fractions with opposing diastereoselectivity under acylating conditions.

A similar grouping of enzymes was observed when trifluoroethyl butyrate was used as acylating agent (Table 3, entries 19–30). The reactions were slower than with vinyl butyrate, but Meito OF and Biocatalysts' *C. cylindracea* acylated the (2*S*,3*R*)-isomer with 90–91% de, while Amano AY-30, Meito MY, Chirazyme L-3 and Genzyme *C. cylindracea* showed a preference for the minor (2*S*,3*S*)-isomer [de of the product (14–21%) was less than that of the starting material (47% de)]. While crude *C. rugosa* (Meito OF) reportedly consists of several hydrolases and at least one protease (35), the lack of reactivity of the esterases from Boehringer-Mannheim and Altus suggests that a contaminating esterase is not responsible for Meito OF's reactivity in the present study.

In contrast to their behavior under acylating conditions, all *Candida* enzymes were hydrolytically viable but showed uniformly poor selectivity (Table 4). As before, the increase in de of the unreacted starting material with increasing conversion indicates that Amano AY-30, Meito MY, and Sigma Type VII prefer the minor (2*S*,3*S*)-isomer. Of the *Mucor* enzymes tested, only Novo Lipozyme 10,000L (a liquid preparation) showed activity; the immobilized IM-60 showed no activity under similar conditions.

The disadvantages of using crude commercial enzyme preparations in synthetic reactions have long been recognized, and many attempts to improve the enantioselectivity of *C. rugosa* lipases have been reported (35,36). Our results reinforce the observation that an enzyme's performance may critically depend on commercial source and purity.

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