Enzymatic Esterification of Glycerol III. Lipase-Catalyzed Synthesis of Regioisomerically Pure 1,3-*sn*-Diacylglycerols and 1(3)-*rac*-Monoacylglycerols Derived from Unsaturated Fatty Acids

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ABSTRACT: Lipases that display high regioselectivities and broad substrate tolerance were used as catalysts for the efficient esterification of glycerol under the conditions of irreversible acyl transfer. A variety of unsaturated fatty acids, such as oleic, linoleic, erucic, ricinolic, hydroxystearic and coriolic acid, were used for this purpose in the form of their vinyl esters. Suitable biocatalysts were chosen on the basis of systematic screening experiments regarding their regioselectivities (RE) and substrate tolerances. Distinct differences were found and expressed in numerical RE values as a measure for differences of these biocatalysts as being specific, selective, and nonspecific. Based on these experiments, a variety of molecules were synthesized on a preparative scale (>150 mmol) in good yield (*ca.* 85%) and with high regioisomerical purities (>95% RE).
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KEY WORDS: Diacylglycerol, esterification, fatty acid vinyl ester, irreversible and reversible acyl transfer, lipase, monoacylglycerol, regioselectivity, RE value, substrate specificity, triacylglycerol.

Regioisomerically pure 1,3-*sn*-diacylglycerols and 1(3)-*rac*-monoacylglycerols are potentially attractive starting materials for numerous synthetic applications (1,2). They can be employed for the synthesis of defined triacylglycerols, phospholipids, glycolipids, lipoproteins and the preparation of numerous enzyme agonists and antagonists. They are useful as drug carriers and have been employed for the preparation of conjugates derived from various drugs (3–10). They also have been reported to be potent activators of enzymes and may take part in a variety of biological processes (11,12). Their use as emulsifiers for the preparation of processed foods is well documented, although impure product mixtures are often employed in such utilization (13,14). The considerable pharmaceutical and synthetic potential of these molecules has not been exploited to any great extent due to the inaccessibility of these molecules in synthetically useful quantities. Several chemical and biotechnological methods for the synthesis of regioisomerically pure 1,3-*sn*-diacylglycerols and 1(3)-*rac*-monoacylglycerols have been reported, which either involve multistep reaction sequences and/or lengthy isolation and purification steps to obtain the desired target molecules in regioisomerically pure form (15–21). A facile and direct method for the preparation of regioisomerically pure 1,3-*sn*-diacylglycerols and 1(3)-*rac*-monoacylglycerols, derived from saturated fatty acids, in synthetically useful quantities (>100 g) has been reported earlier from our laboratory (22–24). These target molecules were prepared by enzymatic esterification of glycerol, immobilized on a solid support (SiO_2), in the presence of various 1,3-selective lipases (*Chromobacterium viscosum, Rhizopus delemar, Rhizomucor miehei*) and a variety of different acyl donors (free fatty acids, fatty acid alkyl esters, fatty acid vinyl esters, and triacylglycerols). The reactions were carried out in aprotic organic solvents of low water content (*n*-hexane, diethyl ether, *t*-BuOMe or mixtures of these solvents). In the present paper, we describe a simple procedure by which regoisomerically 1,3-*sn*-diacylglycerols and 1(3)-*rac*-monoacylglycerols, derived from *unsaturated* fatty acids, can be obtained by esterification of glycerol with fatty acid vinyl esters as acyl donors in the presence of 1,3-selective lipases. For the synthesis of the required vinyl esters, synthetic procedures were developed (25). Suitable biocatalysts were chosen on the basis of systematic screening experiments regarding their regioselectivities (RE) and substrate tolerances. Distinct differences were found among the studied lipases, and RE were expressed as numerical RE values as a measure for these biocatalysts as being specific, selective or nonspecific. Based on these experiments, a variety of molecules were synthesized on a preparative scale (>150 mmol) in good yield (*ca.* 85%) and with high regioisomerical purities (>95%RE).

EXPERIMENTAL PROCEDURES

All solvents were of technical grade and distilled before use. Bracketed numbers refer to enzyme numbering in Tables 5 and 6. Lipase from *R. miehei* (Lipozyme) [10] was a gift from Novo Industri S/A (Bagsvaard, Denmark); lipases from *R.*

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oryzae [1], *R. delemar* (lipase D-10) [2], *R. niveus* (lipase N) [4], *Aspergillus niger* (lipase AP-6) [5], *Pseudomonas* species (SAM II, lipase CES) [7], *Humicola lanuginosa* (lipase CE) [11], *Pseudomonas* species (lipase P) [13], *Mucor javanicus* (lipase M-AP) [14], *Pseudomonas* species (lipase AK) [15], *C. lipolytica* (lipase L) [18], *Geotrichum candidum* (lipase GC-5) [19], *Penicillium cambertii* (lipase G) [20], *C. rugosa* (lipase AY) [21], and *Pseudomonas cepacia* (SAM I) [22] were gifts from Amano Pharmaceutical Co. (Nagoya, Japan); lipases from *R. arrhizus* [3], *Pseudomonas* species [6], *P. fluorescens* [8], *M. javanicus* [9], *P. roquefortii* [12], *Chromobacterium viscosum* [16], and *C. cylindracea* [17] were purchased from Fluka Chemie AG (Buchs, Switzerland). The RE of the lipases were determined as described previously (24). Other materials were purchased from Fluka Chemie AG.

Product compositions and purities were checked by gas–liquid chromatographic (GLC) analysis of the corresponding trimethylsilyl ethers (TMS-ethers) on a Shimadzu 14-Afs, equipped with a 25 m × 15 mm SE-3β-fs-column (Macherey-Nagel, Düren, Germany). On-column injection was employed, and hydrogen was used as carrier gas at 1.8 bar pressure. The detector and injector temperature was set at 250°C. The following temperature program was used: start temperature 140 to 165°C (10°C/min, 10 min isothermal), then to 170°C (2°C/min, 5 min isothermal), then to 240°C (10°C/min, 10 min isothermal), then to 270°C (30°C/min, 5 min isothermal), then to 320°C (30°C/min, 16 min isothermal), total time 60 min.

Preparation of TMS derivatives for GLC analysis: typical procedure. The corresponding anhydrous glyceride (1 mg) and 0.2 mL of a mixture consisting of *bis*(trimethysilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilan (TMCS) in a ratio of 10:1 was mixed and heated in a sealed vial at 80°C for 30 min. This mixture (0.2 µL) was filled up to 1 mL with *n*-hexane [high-performance liquid chromatography (HPLC)-grade] and analyzed by GLC (1% solution, on-column injection). When samples from liquid reaction mixtures were analyzed, the solvent was evaporated prior to the derivatization under a stream of dry nitrogen. The retention times of the relevant derivatives are summarized in Table 1.

Adsorption of glycerol onto solid support: typical procedure. Equal amounts of anhydrous glycerol and silica gel (mesh 200–400, Fluka) were mechanically mixed until the glycerol liquid was completely adsorbed and a free-flowing "dry" powder was obtained.

TABLE 1
Derivatives of Unsaturated Fatty Acids: Retention Times (min)

Derivative	Oleic 18:1	Linoleic 18:2	Erucic 22:1
Acid	17.73	17.27	27.44
Vinyl ester	13.40	13.02	19.68
1(3)-*rac*-Monoglyceride	29.31	29.19	38.44
2-Monoglyceride	28.95	28.75	38.08
1,3-*sn*-Diglyceride	49.77	49.28	50.42
1,2-Diglyceride	49.70	49.23	49.78

Synthesis of fatty acid vinyl esters: typical procedure (25). The fatty acid was dried over 3Å molecular sieves and then dissolved in vinyl acetate (Fluka) (10 mmol fatty acid/50 mL vinyl acetate). $Pd(OAc)_2$ (10 mg) and 1 mmol KOH (both per 10 mmol fatty acid) were added. The reaction mixture was stirred at room temperature in the dark under an inert gas atmosphere (Ar). The conversion was followed by thin-layer chromatography (TLC). After 24 to 72 h, the reaction mixture was filtered, and the unreacted vinyl acetate was evaporated under vacuum. The crude product (75–85% yield) was filtered over a short silica gel column and, if necessary, purified by flash chromatography on silica gel (eluent: heptane/*t*-BuOMe 9/1), leading the pure vinyl esters in yields of 63–80% (Table 2).

*Synthesis of 1(3)-*rac*-monoacylglycerols: irreversible acyl transfer.* Fatty acid vinyl ester (0.1 mol) was dissolved in 1 L of *t*-BuOMe to which 0.5 mol (46 g) glycerol (immobilized onto 92 g of silica gel) and 1 g lipase were added. The reaction mixture was stirred at room temperature, and reaction progress was monitored by TLC. After removal of the solid components (immobilized biocatalyst, silica gel), evaporation of solvent and unreacted vinyl ester, a crude mixture was obtained which contained about 70–90% of the desired 1(3)-*rac*-monoacylglycerols. The 1(3)-*rac*-monoacylglycerols (40–70%) were obtained chemically and regioisomerically pure by flash chromatography on silica gel (diethyl ether/*n*-hexane 3/2) (Table 3).

*Synthesis of 1,3-*sn*-diacylglycerols: irreversible acyl transfer.* Fatty acid vinyl ester (100 mmol) was dissolved in 1 L of *t*-BuOMe to which 0.05 mol (4.6 g) glycerol (immobi-

TABLE 2
Synthesis of Fatty Acid Vinyl Esters—Reaction Conditions and Properties

Vinyl ester	Reaction time (h)	Yield (%)	Properties, purity, % RE, % EE, $[\alpha]_D^{20}$
Oleic acid	24	85	Oil >99%
Linoleic acid	48	87	Viscous oil, >98%
Erucic acid	24	79	Viscous oil, >95%
(+)-(*S*)-Coriolic acid	48	67	Oil, 97%, 95% RE, 87% EE, $[\alpha]_D^{20}$ = +10.3°, c = 0.2, EtOH[a]
(+)-(*R*)-Ricinolic acid	24	58	Viscous oil, 93%, 99% EE, $[\alpha]_D^{20}$ = +4.9°, c = 1, EtOH[a]
(–)-(*R*)-Hydroxystearic acid	24	73	Solid, 95%, 99% EE, $[\alpha]_D^{20}$ = –0.9°, c = 1, EtOH[a]

[a]%EE, % regioselectivity (RE) of fatty acid residue and $[\alpha]_D^{20}$ of fatty acid vinyl ester; c = concentration in 1 g/100 mL.

TABLE 3
1(3)-*rac*-Monoacylglycerols of Unsaturated Fatty Acids

Product 1(3)-*rac*-Mono-	Biocatalyst, scale, reaction time	Yield[a] (%)	Properties
olein	1 g *Penicillium roquefortii* [12], 80 mmol, 12 d	85	m.p. 34°C, 97% RE[b]
linolein	500 mg *Rhizopus niveus* [4], 15 mmol, 6 d	79	Oil, 96% RE[b]
erucin	300 mg *Penicillium roquefortii* [12], 4mm, 48 h	71	m.p. 35°C, 98% RE[b]
(*S*)-coriolin	100 mg *R. niveus* [4], 4 mmol, 48 h	70	m.p. 43C, 95% RE[b] $[\alpha]_D^{20}$ = +7.24°, c = 0.2, MeOH[c]
(*R*)-ricinolein	1.4 g *R. niveus* [4], 40 mmol, 5 d	66	Oil, 96% RE[b], $[\alpha]_D^{20}$ = +4.05°, c = 1.85, MeOH[c]
(*R*)-hydroxystearin	100 mg *R. niveus* [4], 4 mmol, 4 d	56	m.p. 53°C, 98% RE[b], $[\alpha]_D^{20}$ = −0.51°, c = 1, MeOH[c]
linolein/-olein	500 mg, *R. niveus* [4], 32 mmol, 72 h	73	Oil, (65% linoleic acid-MG, 35% oleic acid-MG)
glycerols from sunflower oil	500 mg *R. niveus* [4], 200 mg Lipozyme [10], 32 mmol, 72 h	68	Oil, (71% linoleic acid-MG, 21% oleic acid-MG, 8% MG of saturated fatty acids)

[a]Yield calculated from vinyl ester; ratio vinyl ester/glycerol 1/5; colorless products; MG, monoglyceride.
[b]Regioisomerical purity of monoglyceride.
[c]EE, % RE of fatty acid residue and $[\alpha]_D^{20}$ of monoglyceride; compare Table 2; c = concentration in 1 g/100 mL.

lized on 9.2 g silica gel) and 1 g lipase were added. The reaction mixture was stirred at room temperature, and reaction progress was monitored by TLC. After removal of the solid components (immobilized biocatalyst, silica gel), evaporation of solvent and unreacted vinyl ester, crude mixtures were obtained which contained about 70–90% of the desired 1,3-*sn*-diacylglycerols. The products were obtained chemically and regioisomerically pure by flash chromatography on silica gel (diethyl ether/*n*-hexane 1/1) in yields of 40–70% (Table 4).

RESULTS AND DISCUSSION

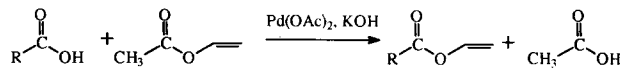
Synthesis of fatty acid vinyl esters. To synthesize the required vinyl esters, the corresponding fatty acid was reacted with vinyl acetate in the presence of $Pd(OAc)_2$ and KOH at room temperature (Scheme 1). After removal of the solid components and evaporation of unreacted vinyl acetate, the desired vinyl esters were prepurified by filtration over a short silica gel column (Table 2). Due to light, heat, and air sensitivity of vinyl esters derived from unsaturated fatty acids, the products were finally purified by flash chromatography on silica gel (*n*-heptane/*t*-BuOMe 9/1) rather than by distillation.

RE of lipases from different sources. Due to their high RE and broad substrate tolerance, lipases were used as mild catalysts for the irreversible acyl transfer of fatty acid vinyl ester onto glycerol. Thus, with lipases that displayed high RE, the resulting partial glycerides can be obtained regioisomerically pure. Depending on the employed ratio of glycerol and acyl donor, reactions can be carried out in such a way that the obtained 1(3)-*rac*-monoacylglycerols are free from di- or tria-

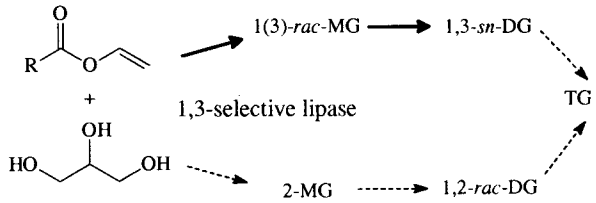
TABLE 4
1,3-*sn*-Diacylglycerols of Unsaturated Fatty Acids

Product: 1,3-*sn*-Di-	Lipase, scale, reaction time	Yield[a] (%)	Properties
olein	1 g *Penicillium roquefortii* [12], 150 mmol, 4 d	76	Oil, 90% RE[b]
linolein	500 mg *Rhizopus niveus* [4], 15 mmol, 5 d	70	Oil, 97% RE[b]
erucin	100 mg *P. roquefortii* [12], 4 mmol, 48 h	71	m.p. 38°C, 96% RE[b]
(*S*)-coriolin	100 mg *R. niveus* [4], 4 mmol, 48 h	65	m.p. 47°C, 98% RE[b], $[\alpha]_D^{20}$ = +8.62°, c = 0.2, MeOH[c]
(*R*)-ricinolin	1 g *R. niveus* [4], 15 mmol, 5 d	61	Oil, 97% RE[b], $[\alpha]_D^{20}$ = +4.4°, c = 1, MeOH[c]
(*R*)-hydroxystearin	100 mg *R. niveus* [4], 4 mmol, 4 d	52	m.p. 51°C, 96% RE[b], $[\alpha]_D^{20}$ = −0.42°, c = 1, MeOH[c]

[a]Yield calculated on vinyl ester; ratio vinyl acetate/glycerol 2/1; colorless products.
[b]Regioisomerical purity of diglyceride.
[c]% EE, % RE of fatty acid residue and $[\alpha]_D^{20}$ of diglyceride; compare Table 2; c = concentration in 1 g/100 mL.



SCHEME 1



SCHEME 2

cylated side products (Scheme 2—compare Fig. 1). Suitable lipases were chosen on the basis of systematic screening experiments. For this purpose, 1 mmol of the corresponding pure fatty acid vinyl ester (e.g., derived from oleic, linoleic, or erucic acid) and 5 mmol glycerol, immobilized onto 1.2 g silica gel, were suspended in 2 mL of *t*-BuOMe. The appropriate lipase (10 mg) was added to the reaction mixture. After reaction times of 24, 48 and 72 h, the compositions of the reaction mixtures were determined by GLC. A typical chromatogram, monitor the irreversible acyl transfer of linoleic acid vinyl ester onto glycerol is shown in Figure 1. All essential information, such as conversion rate, regioisomeric excess and product ratio, can be determined from one single experiment. The obtained results are summarized in Table 5. Comparable results were obtained with the vinyl esters of oleic, linoleic, or erucic acid as acyl donors. From these data,

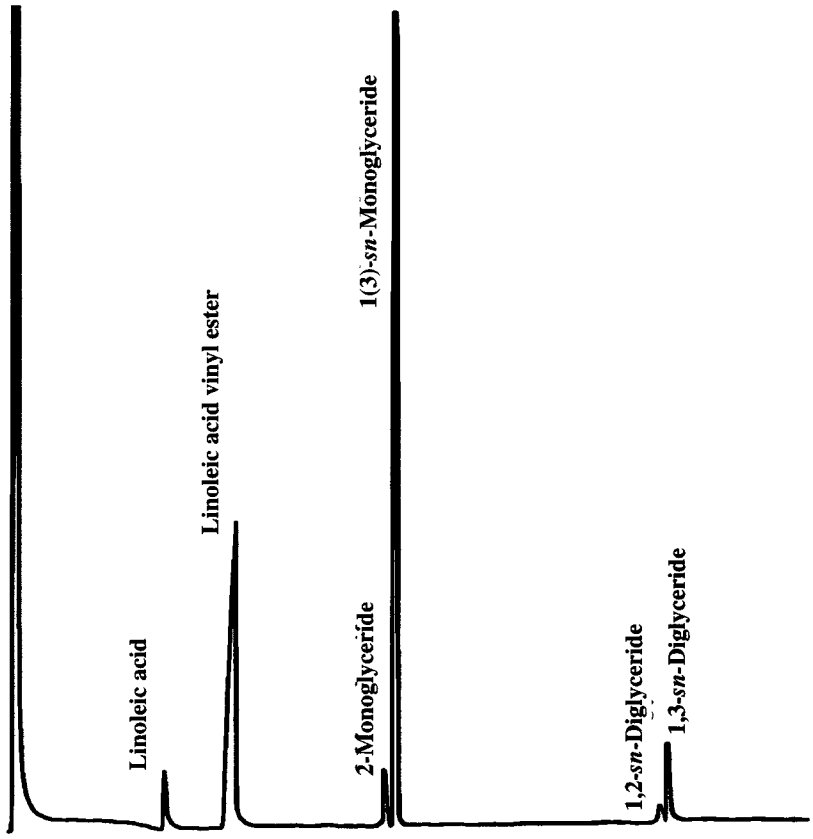


FIG. 1. Irreversible acyl transfer of linoleic acid vinyl ester onto glycerol in the presence of a lipase from *Rhizopus niveus*: GC-diagram after 24 h (compare Table 5, entry 4, 48 h).

based on earlier experiments (22–24), the RE of the investigated lipases can be calculated as RE values by using the formula:

$$\text{RE value} = \%\ 1(3)\text{-monoglyceride} - \%\ 2\text{-monoglyceride}$$

The thus obtained numerical RE values are summarized in Table 6. Based on these values and by subjective definition, the investigated lipases were classified as 1,3-specific (RE > 90), 1,3-selective (90 > RE > 70) and nonspecific (RE < 70). In Table 6, the RE values obtained for transformations of unsaturated fatty acids are listed together in comparison with the results obtained earlier with vinyl esters of saturated fatty acids. Although the numerical values are occasionally different, no remarkable differences were found in the general behavior of these groups of fatty acid derivatives. These experiments greatly facilitated the correct choice of a suitable biocatalyst and reliable planning of experiments aimed at the synthesis of regioisomerically pure 1(3)-*rac*-mono- and 1,3-*sn*-diglycerides. From Tables 5 and 6, it is obvious that the lipases derived from *R. niveus* [4], *H. lanuginosa* [11], and *P. roquefortii* [12] are the most useful biocatalysts for our purpose. Both the highest conversions and highest RE are associated with these enzymes.

Synthesis of mono- and diacylglycerols under the conditions of irreversible acyl transfer. Using lipases with high RE values as catalysts and vinyl esters of the unsaturated (functionalized) fatty acids, a variety of regioisomerically pure 1(3)-*rac*-mono- and 1,3-*sn*-diglycerides were synthesized by means of the previously published procedure (22–24) (Scheme 3). For synthesis of 1(3)-*rac*-monoacylglycerols, 1 mmol of the corresponding fatty acid vinyl ester was dissolved in 10 mL *t*-BuOMe and 5 mmol of glycerol immobilized on 460 mg silica gel were added together with the required amount of lipase (Table 3). The reactions were monitored by GLC and terminated after the desired conversions (Table 3) were achieved. The reaction mixtures were filtered, the solvent was evaporated, and the residues were purified by flash chromatography on silica gel. The desired 1(3)-*rac*-monoacylglycerols were obtained chemically pure in isolated yields ranging from 56 to 85% and isomeric purities from 95– 98% RE. The results are summarized in Table 3. For synthesis of 1,3-*sn*-diacylglycerols, 2 mmol of the corresponding fatty acid vinyl esters were dissolved in 10 mL *t*-BuOMe to which 1 mmol glycerol (immobilized on 920 mg silica gel) and the required amount of lipase were added. The reactions were monitored by TLC or GLC and terminated after the desired conversions were achieved (Table 4). The reaction mixtures were filtered, the solvent was evaporated, and the residues were purified by flash chromatography on silica gel. The desired 1,3-*sn*-diacylglycerols were obtained chemically pure in yields ranging from 52 to 76% and with high isomeric purity (90–98% RE). The results are summarized in Table 4. In summary, the described experiments—with 1,3-selective lipases and stoichiometric quantities of acyl donors—allow the convenient synthesis of multigram quantities of 1(3)-*rac*-monoglycerides and 1,3-*sn*-diglycerides from unsatu-

TABLE 5
Irreversible Acyl Transfer of Linoleic Acid Vinyl Ester onto Glycerol in the Presence of Various Lipases—Screening Results[a]

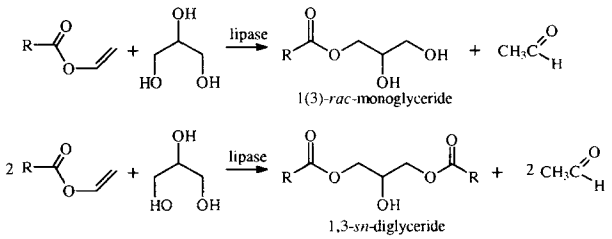
Number	Lipase from	Reaction time (h)	Conv. (%)	MG/DG	1(3)MG/2-MG
1	*Rhizopus oryzae*	72	47	91/9	96/4
2	*R. delemar*	48	62	98/2	96/4
3	*R. arrhizus*	72	53	95/5	97/3
4	*R. niveus*	**48**	**77**	**96/4**	**97/3**
5	*Aspergillus niger*	72	68	98/2	98/2
6	*Pseudomonas* species	<5	—	—	—
7	*Pseudomonas* species (SAM II)	43	43	97/3	79/21
8	*P. fluorescens*	72	<5	—	—
9	*M. javanicus*	72	66	89/11	98/2
10	*Mucor miehei* (Lipozyme)	72	35	90/10	90/10
11	*Humicola lanuginosa*	**72**	**83**	**94/6**	**98/2**
12	*Penicillium roquefortii*	**48**	**75**	**93/7**	**99/1**
13	Lipase P	72	31	84/16	89/11
14	Lipase M-AP	72	<5	—	—
15	Lipase AK	72	65	93/7	98/2
16	*Chromobact. viscosum*	48	82	88/12	97/3
17	*Candida cylindracea*	72	49	87/13	97/3
18	*C. lipolytica*	72	12	65/35	82/18
19	*Geotrichum candidum*	72	9	99/1	82/18
20	*Penicillium cambertii*	72	32	97/3	90/10
21	*C. rugosa*	72	23	91/9	89/11
22	*P. cepacia* (SAM I)	72	31	79/21	91/9

[a]MG, Monoglyceride; DG, diglyceride. Most useful lipases are printed in bold letters.

TABLE 6
RE Values of Different Lipases[a]

Number	Lipase from:	RE value (saturated fatty acids) (Ref. 24)	RE value (unsaturated fatty acids)[b]	Property
1	*Rhizopus oryzae*		91	1,3-Specific
2	*R. delemar*	98.1	83	1,3-Selective
3	*R. arrhizus*	88.0	84	1,3-Selective
4	*R. niveus*	98.1	92	1,3-Specific
5	*Aspergillus niger*		91	1,3-Specific
6	*Pseudomonas* species		96	1,3-Specific
7	*Pseudomonas* species (SAMII)	21.5	74	1,3-Selective
8	*Pseudomonas fluorescens*	79.0	68	Nonspecific
9	*Mucor javanicus*	92.8	89	1,3-Selective
10	*M. miehei* (Lipozyme)	83.7	88	1,3-Selective
11	*Humicola lanuginosa*		90	1,3-Specific
12	*Penicillium roquefortii*		94	1,3-Specific
13	*Pseudomonas* species		86	1,3-Selective
14	*M. javanicus* (Amano)		90	1,3-Specific
15	Lipase AK		95	1,3-Specific
16	*Chromobacterium viscosum*	92.7	87	1,3-Selective
17	*Candida cylindracea*		84	1,3-Selective
18	*C. lipolytica*		64	Nonspecific
19	*Geotrichum candidum*	59.1	65	Nonspecific
20	*Penicillium cambertii*		68	Nonspecific
21	*C. rugosa*	79.5	78	1,3-Selective
22	*P. cepacia* (SAM I)		84	1,3-Selective

[a]Regioselectivity (RE) value = % 1(3)-monoglyceride–% 2-monoglyceride, from three screening experiments RE > 90 = 1,3-specific, 90 > RE > 70 = 1,3-selective, RE < 70 = nonspecific.
[b]This work.



SCHEME 3

rated fatty acids in good chemical yields and with high isomeric purities.

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