Lipase-Catalyzed Synthesis and Properties of Estolides and Their Esters

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ABSTRACT: Eight lipases were screened for their ability to synthesize estolides from a mixture that contained lesquerolic (14-hydroxy-11-eicosenoic) acid and octadecenoic acid. With the exception of *Aspergillus niger* lipase, all 1,3-specific enzymes (from *Rhizopus arrhizus* and *Rhizomucor miehei* lipases) were unable to synthesize estolides. *Candida rugosa* and *Geotrichum* lipases catalyzed estolide formation at >40% yield, with >80% of the estolide formed being monoestolide from one lesquerolic and one octadecenoic acyl group. *Pseudomonas* sp. lipase synthesized estolides at 62% yield, but the product mixture contained significant amounts of monoestolide with two lesquerolic acyl groups as well as diestolide. Immobilized *R. miehei* lipase was chosen to catalyze the esterification of mono- and polyestolide, derived synthetically from oleic acid, with fatty alcohols or α,ω-diols. Yields were >95% for fatty alcohol reactions and >60% for diol reactions. In addition, the estolide linkage remained intact through the course of the esterification process. Esterification of estolides improved the estolide's properties—for example, lower viscosity and higher viscosity index—but slightly raised the melting point. Estolides and, particularly, estolide esters may be suitable as lubricants or lubricant additives.

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KEY WORDS: Esterification, estolides, hydroxy fatty acid, lesquerolic acid, lipases, supercritical fluid chromatography.

Estolides, depicted in Figure 1, are polymeric molecules that are composed mostly of hydroxy fatty acids. Acyl groups are bonded covalently *via* ester bonds between the hydroxyl moiety of one hydroxy acid and the carbonyl moiety of another acyl group. Estolides have potential use as lubricants, plasticizers, cosmetic additives, and printing ink components. Estolides have been reported to occur in nature (1) or to be synthesized at high pressure/temperature from castor oil or its hydroxy acid, ricinoleic ($18:1^{9}\text{-}OH^{12}$) acid (2). Recently, our research group invented a process for forming estolides from monounsaturated fatty acids by heterogeneous catalysis (3–5).

The primary goal of this investigation is to form estolide esters on a preparative scale so that the physical properties of the esters relative to the free estolides can be compared. Use of traditional oleochemical reactions to achieve this goal may not be desirable because such methods can lead to the cleavage of estolide bonds. As an alternative, we investigated lipase-catalyzed esterification. Lipases (EC 3.1.1.3) act at mild reaction conditions—for example, low temperatures and pressures and neutral pH—which would prevent degradation of estolides. In this investigation, we screened several lipases for their activity toward hydroxy fatty acids and estolides. Based on these results, we selected *Rhizomucor miehei* lipase as a suitable biocatalyst for estolide esterification. This lipase was employed for preparative-scale esterification between oleic acid estolides and fatty alcohols or α,ω-diols. Products were isolated *via* silica gel column chromatography, and their physical properties were determined.

EXPERIMENTAL PROCEDURES

Materials. Lipozyme, *R. miehei* lipase in free and immobilized form, and Palatase M (*R. miehei* lipase) were donated

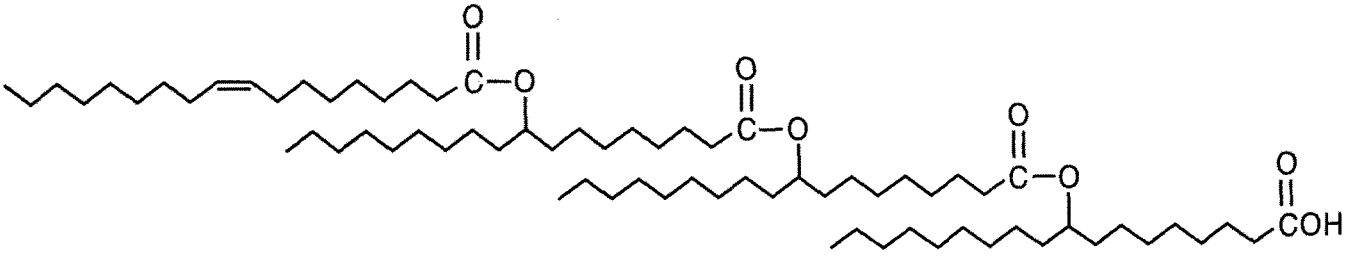

FIG. 1. Oleic acid polyestolide.

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by Novo-Nordisk (Danbury, CT). Lipase G (from *Penicillium cyclopium*), AP-12 (*Aspergillus niger*), GC-4 (from *Geotrichum candidum*), and PS-30 (from *Pseudomonas* sp.) were donated by Amano (Troy, VA). *Candida rugosa* (Type VII-S) and *Rhizopus arrhizus* (Type XI) lipase were purchased from Sigma (St. Louis, MO). Palatase M, "free" Lipozyme, and *R. arrhizus* lipase were supplied in aqueous solution. All lipases were used without further purification. Their specific activities, as assayed by 1-butanol-linoleic ($18:2^{9,12}$) acid esterification in reverse micelles (6), are listed in Table 1. A sample rich in monoestolide was provided by Dr. S.M. Erhan of our research center (National Center for Agricultural Utilization Research, Peoria, IL). Its preparation, from oleic acid as starting material, is described elsewhere (3,4). The sample contained 83.7% monoestolide, E^1 [molecular weight (MW) of 564], 10.6% diestolide, E^2 (MW = 844), and 3.3% free fatty acid (FFA) (or equivalently E^0). The vast majority of hydroxy acyl groups in E^1 and E^2 contain their hydroxyl moiety between the C_8 and C_{11} carbon, that is, near the C_9 double-bond position of the oleic acid substrate (7,8). Polyestolide, derived from oleic acid according to a method already outlined (5), was supplied by Dr. T.A. Isbell at our research center. Estolide molecules with 1 to 10 acyl groups were detected, with the majority of molecules containing 2–6 acyl groups (9). Most of the polyestolides' hydroxy acyl groups possessed hydroxyl moieties between C_8 and C_{12} (9). FFA samples containing hydroxy acid (HA) [lesquerolic ($20:1^{11}$-OH^{14}) and auricolic ($20:2^{11,17}$) acid] along with nonhydroxy FFA (NHA) (mostly oleic, linoleic, and linolenic acids) were derived *via* 1,3-specific lipolysis of lesquerella oil (10), followed by a saponification–solvent extraction technique (Hayes, D.G., R. Kleiman, and K.D. Carlson, submitted for publication). Sample 1 contained 83.2% FFA (77.4% of which was lesquerolic acid, 5.2% auricolic acid and 15.5% C_{18} unsaturates), 6.2% monoglyceride (MG), 9.4% diglyceride (DG), and 1.3% triglyceride (TG). The partial glycerides contained mostly C_{18} and C_{20}-hydroxy acyl groups. Sample 2 contained 80.2% FFA (52.1% lesquerolic acid, 3.1% auricolic acid, and 41.1% C_{18} unsaturates), 16.1% MG, 6.7% DG, and 5.0% TG. The surfactant AOT [sodium *bis* (2-ethylhexyl) sulfosuccinate, 99% pure], 1-decanol (98% pure) and molecular sieves (3Å) were purchased from Sigma. Oleyl alcohol (technical grade, 85% pure) and α,ω-diols, >95% pure, were purchased from Aldrich (Milwaukee, WI). Diols and Lipozyme IM20 were stored over $CaSO_4$ prior to use. Lesquerella oil was donated by Dr. K.D. Carlson at our center. Celite 545 was purchased from J.T. Baker (Phillipsburg, NJ). Silica gel 60 (230–400 mesh) was purchased from Aldrich. All organic solvents employed for operating reactions and conducting column chromatography were of high purity. Deionized water was used throughout.

TABLE 1
Specific Activities of Lipases

Lipase type	Activity[a]
Aspergillus niger[b]	2.29 units/mg
Candida rugosa	585 units/mg
Geotrichum candidum	0.00398 units/mg
Penicillium cyclopium	0.0209 units/mg
Pseudomonas sp.	0.0403 units/mg
Rhizopus arrhizus	55.4 units/μL
Rhizomucor miehei[c]	0.132 units/μL
R. miehei[d]	0.74 units/μL
R. miehei[e]	49.5[f] units/mg

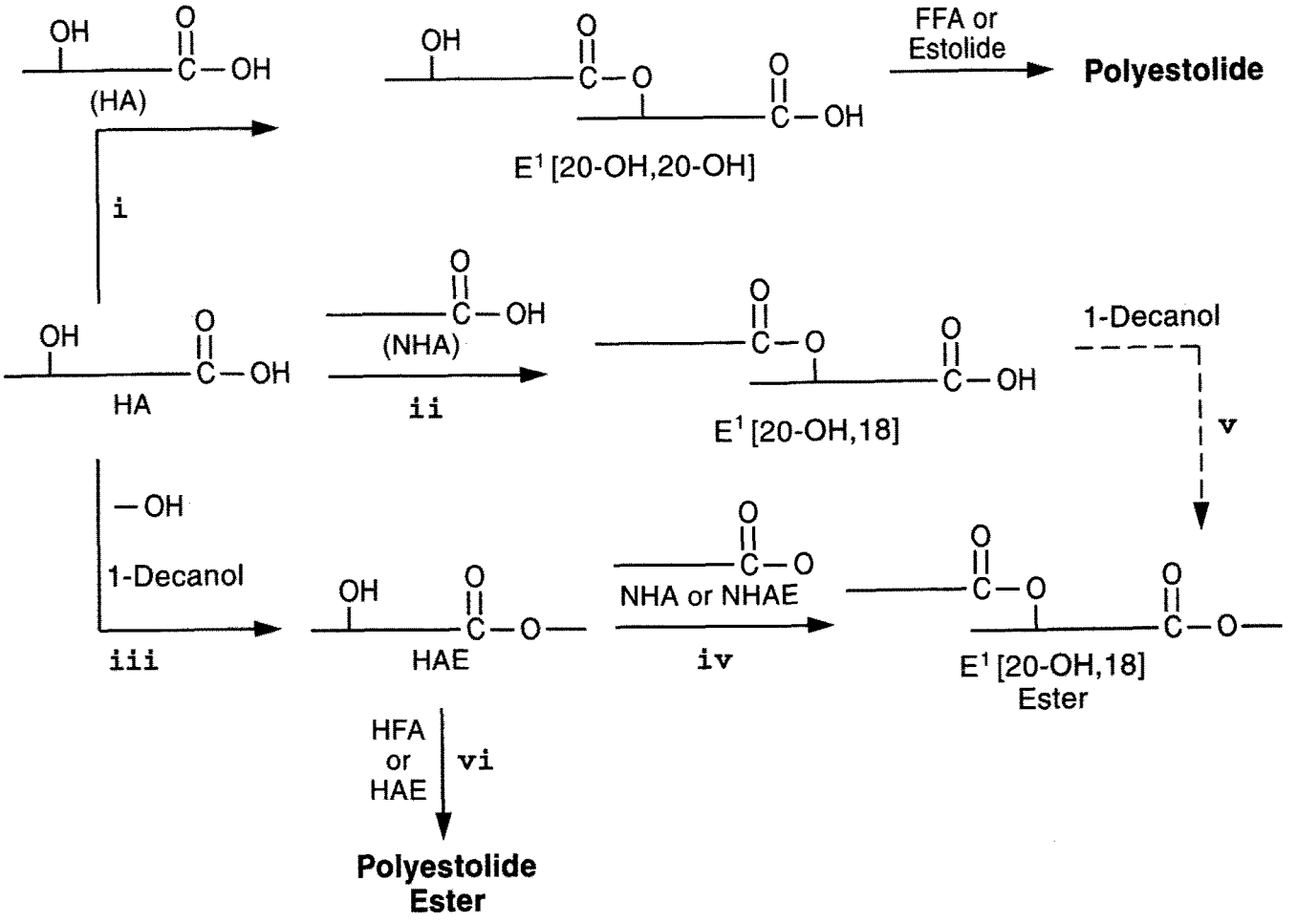
[a]Unit defined as the amount of lipase needed to produce 1 mM linoleic acid butyl ester per hour, according to assay conditions of Reference 6. Those species with activity defined in Units/μL were supplied by vendor in solution.
[b]Lipase AP-12.
[c]Palatase M.
[d]Lipozyme: free lipase.
[e]Lipozyme IM20: immobilized lipase.
[f]Activity is approximate; values changed slightly with stir rate and water content of medium and Lipozyme.

Enzyme immobilization. To immobilize *C. rugosa* lipase onto Celite 545, 22.4 mg lipase, dissolved in 3.0 mL 50 mM phosphate buffer at pH = 6.9 (PBS), was mixed with 1.0 g celite for 2 h. The suspension was dried at room temperature (22°C) *in vacuo* for approximately 12 h, then stored over $CaSO_4$ prior to use.

Reactions between C_{20}-hydroxy acid and octadecenoic acid (Scheme 1). Reactions in biphasic medium were conducted at 22°C by combining 2.5 mL each of isooctane and lesquerolic acid Sample 1 with 5.0 mL PBS, containing lipase at the following concentrations: *C. rugosa*, 1.12 mg/mL; *Pseudomonas* sp., 24.6 mg/mL; *R. miehei* (Palatase M), 400 μL/mL; *A. niger* (AP-12), 55.8 mg/mL; *G. candidum*, 11.6 mg/mL; and *P. cyclopium*, 12.7 mg/mL. Agitation was provided by magnetic stirring (300 revolutions/min). Reverse micellar medium consisted of 100 mM AOT, 5 vol% lesquerolic acid Sample 1, and 1000 mM H_2O in isooctane. It was formed by adding 90 μL aqueous lipase solution in PBS to 5.0 mL isooctane solution, followed by gentle agitation for approximately 1 min to allow for formation of reverse micelles (22°C). Aqueous enzyme concentrations (in PBS) were *C. rugosa*; 4.88 mg/mL; *R. arrhizus*, 100 μL/mL; and *R. miehei*, 1000 μL/mL. Reactions with immobilized lipases were conducted by suspending lipase in lesquerolic acid Sample 1/isooctane (1:1 vol/vol) at 22°C. Agitation was provided by a vortex-evaporator unit from Haake-Buchler (Saddlebrook, NJ). Overall enzyme concentrations were 60.2 mg/mL for Lipozyme IM20 and 26.4 mg/mL for immobilized *C. rugosa* lipase. All reactions were stopped after 65 h or when substrate and product concentrations remained unchanged.

For the competitive reaction between lesquerolic acid, octadecenoic acid, and 1-decanol at 22°C, 1.8 mL (1.7 g, 5.7 mmol acyl groups) lesquerolic acid Sample 2 was combined with 0.45 mL (0.38 g, 2.4 mmol) 1-decanol, 2.7 mL isooctane, and 5.0 mL PBS containing *Pseudomonas* sp. lipase at 60.8 mg/mL. The aqueous and organic phases were mixed *via* magnetic stirring at 300 revolutions/min.

Hydrolysis of monoestolide at 22°C. When immobilized *C. rugosa* lipase was used as biocatalyst, 0.151 g lipase was suspended in 5.0 mL PBS-saturated lesquerolic acid Sample 2/isooctane (1:1) *via* shaking. For biphasic media, 2.5 mL each isooc-



SCHEME 1

tane and Sample 1 were combined with 5.0 mL PBS containing lipase at 1.12 mg/mL (*C. rugosa*) and 25.3 mg/mL (*Pseudomonas* sp.). The stir rate was 300 revolutions/ min. Conditions for the reaction in reverse micelles were identical to those listed above (monoestolide replacing lesquerolic acid as substrate). The concentration of *C. rugosa* lipase in PBS was 4.88 mg/mL.

Synthesis of estolide esters. Estolide esters were synthesized on a preparative scale by Lipozyme IM20-catalyzed esterification between oleic acid mono- or polyestolide and alcohols. Typically, 20–30 mL each of estolide and fatty alcohol (oleyl alcohol or 1-decanol) were reacted with 0.01 g Lipozyme/g substrate in a stirred batch reactor (200 revolutions/min) at either 60°C (solventless) or 22°C (40 mL isooctane added). Estolide–diol esterification was conducted with 10 vol% estolide in ethyl ether that contained diol at saturating amounts (typically 0.2–0.5 mol/mol estolide). Reactions were operated in stirred batch reactors at 22°C. The major product at the end of reaction was typically monoester (ME). To produce diester (DE), the following additional steps were included. First, Lipozyme IM was separated *via* filtration, and ether was evaporated. Most of the remaining diol was insoluble in the resulting monoestolide/free estolide mixture. The diol precipitate was removed *via* centrifugation. The liquid mixture then was combined with additional estolide and Lipozyme IM and reacted in a stirred batch reactor in the solventless mode at 60°C. Ester products were then isolated and purified by silica gel column chromatography (25 cm × 4.5 cm i.d.) with hexane/ethyl acetate at varying proportions as eluate. Fractions were collected and analyzed *via* thin-layer chromatography and supercritical fluid chromatography (SFC).

Properties of estolide esters. Density, ρ, was estimated gravimetrically. Melting point (T_m) was determined in standard capillary melting point apparatus. Only values of T_m above −19°C could be determined due to limitation of the equipment. Kinematic viscosity (ν) was measured in a temperature-controlled oil bath with Cannon-Fenske viscometers from Cannon Instruments (State College, PA). Viscometer constants were determined with lesquerella oil as standard. Values of ν for lesquerella oil were derived from the data of Noureddini *et al.* (11,12). Values of viscosity index (VI) were determined by a standard procedure, which required values of ν at 40 and 100°C (13). These two values were estimated from the following equation and viscosity measurements at alternate temperatures:

$$\ln \nu = A + B \exp(T^{-1})$$

where T is the temperature in Kelvin and A and B are constants. Correlation coefficients were >0.99. This relationship did not fit data for monoestolide well. For monoestolides, values of v at 40 and 100°C were estimated *via* extrapolation.

Supercritical fluid chromatographic analysis. All reactions and silica gel column chromatographic separations were analyzed by SFC with high-purity carbon dioxide (Air Products, Tamaqua, PA) as carrier fluid and a model 600 chromatograph from Dionex (Salt Lake City, UT). A nonpolar capillary column (10 m × 50 μm i.d.) with SB-Methyl-100 stationary phase (from Dionex) was employed. Chromatographic conditions are described elsewhere (14). These nonpolar columns promote separation based on molecular weight (14).

RESULTS AND DISCUSSION

Screening of lipase activity toward hydroxy acids and estolides. Several lipases and reaction systems (free lipases and aqueous–organic biphasic medium, immobilized lipase and lipophilic medium, and free lipase and reverse micellar medium) were screened for the ability to form or hydrolyze estolide bonds. The literature contains several examples of estolide formation from ricinoleic ($18:1^{9}\text{-OH}^{12}$) acid, the C_{18} homolog of lesquerolic acid, and triricinolein from castor oil (15–21). Recent publications demonstrate that estolides can be formed from ω-hydroxy acids by using lipases (21,22). Results illustrate random lipases—that is, those lacking 1,3-positional specificity [for example, *C. rugosa* (16–19), *Chromobacterium viscosum* (16), *G. candidum* (15,19), and *Pseudomonas* sp. (17)]—were the most active in catalyzing estolide formation. [Porcine pancreatic lipase, a 1,3-positionally specific lipase, can form estolides from ω-hydroxy acids because their -OH groups are primary (21).] However, it would be doubtful that 1,3-positionally specific lipases would be able to form or break estolide bonds because they can rarely utilize secondary alcohols as substrate (23,24). In agreement, Okumura *et al.* (15) found that *Rhizopus delemar* lipase did not catalyze estolide formation during castor oil hydrolysis. Likewise, we found no evidence of estolide formation during lipolysis of *Lesquerella fendleri* oils by *R. arrhizus* or *R. miehei* lipases (Hayes, D.G., and R. Kleiman, unpublished results). Furthermore, Ghoshray and Bhattacharya (25) found that *R. miehei* lipase could not attack the hydroxyl group of 12-hydroxystearic acid during esterification.

Lipases were screened for estolide synthetic capability by using the reactions outlined in Scheme 1. The results for lipase-catalyzed esterification between lesquerolic and octadecenoic acids are contained in Figure 2 and Table 2. As expected, the 1,3-positionally specific enzymes (*R. arrhizus, R. miehei*, and *A. niger*) were not able to form estolides (Table 2), but they were able to hydrolyze glycerides (present at small percentages initially in the reaction media; data not shown). An exception to this rule is *A. niger* lipase, which formed a small amount (12%) of estolide. The random lipases (*C. rugosa, G. candidum*, and *Pseudomonas* sp.) with the exception of *P. cyclopium* lipase yielded significantly large percentages of estolide. *Penicillium cyclopium* lipase is unique among the random lipases of Table 1 for its specificity toward MG and DG over TG (26). Indeed, of the random lipases in Table 1, only *P. cyclopium* lipase and immobilized *C. rugosa* lipase, which was operated in nearly anhydrous medium, were not able to hydrolyze the TG present initially as substrate. Reported results are contradictory concerning *P. cyclopium* lipase's ability to utilize secondary alcohols (24,26).

Examination of product distribution yields important findings. Figure 2a demonstrates that *C. rugosa* lipase produced

TABLE 2
Estolide Formation from a Lesquerolic Acid/Octadecenoic Acid Mixture[a]

Lipase type	Medium	% Estolide[b]	Product distribution[c]		
			E^1(H,X)	E^1(H,H)	E^2
C. rugosa	Biphasic	41.3	83.9	13.5	2.6
	Immobilized	9.6	85.0[d]	15.0	0.0
	Reverse micelles	43.7	84.0	12.8	2.8
G. candidum	Biphasic	45.2	80.6	14.4	5.0
P. cyclopium	Biphasic	13.7	83.4	16.6	0.0
A. niger	Biphasic	12.3	65.7	30.2	4.1
Pseudomonas sp.	Biphasic	62.8	51.1	29.0	12.5
R. miehei[e]	Biphasic	0.0			
R. miehei	Immobilized	0.0			
R. miehei[f]	Reverse micelles	0.0			
R. arrhizus	Reverse micelles	0.0			

[a]Reaction conditions and duration described in the Experimental Procedures section. See Table 1 for "Lipase type" abbreviations.
[b]Mass percent of acyl groups belonging to estolides.
[c]H refers to hydroxy acyl groups, X refers to nonhydroxy acyl groups, En refers to estolide groups composed of n estolide bonds.
[d]Immobilized *C. rugosa* lipase did not hydrolyze diglyceride (DG). Due to DG peaks overlapping with E1 peaks, integration of supercritical fluid chromatography data was less accurate.
[e]Palatase M (Novo Nordisk, Danbury, CT).
[f]"Free lipozyme."

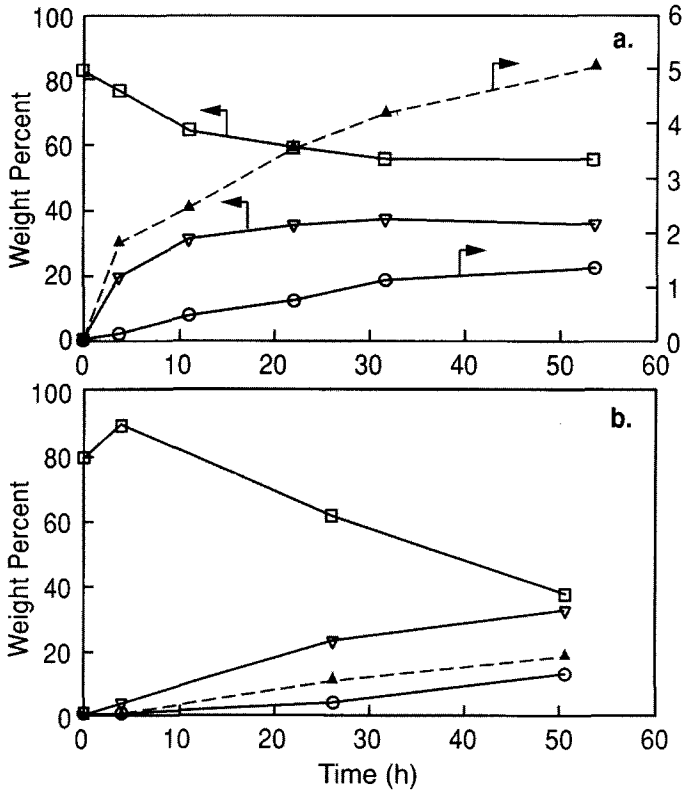


FIG. 2. Lipase-catalyzed formation of estolides from lesquerolic and C_{18} unsaturated acid in aqueous-organic biphasic medium using (a) *C. rugosa* lipase and (b) *Pseudomonas* sp. lipase. Medium composition and reactions conditions—listed in the Experimental Procedures section. (□) Free fatty acid; (△) E^1(20-OH,18); (▲) E^1(20-OH,20-OH), (●) E^2.

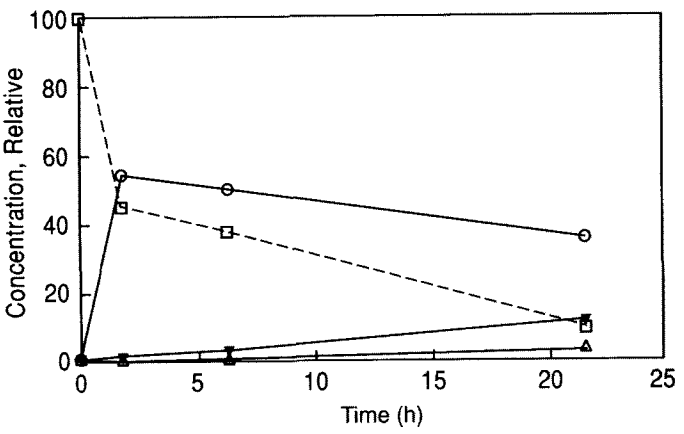


FIG. 3. Competitive reaction between lesquerolic acid, octadecenoic acid, and decanol, catalyzed by *Pseudomonas* sp. lipase in biphasic medium at 22°C. Medium composition and reaction conditions are described in the Experimental Procedures section. (□) Free fatty acid; (○) FAE; (f▲) E^1 ester, (△) E^2 ester.

mostly monoestolide that consisted of one C_{20}-hydroxy and one C_{18} acyl group (E^1[20–OH,18]), even though hydroxy acyl groups are in stoichiometric excess. E^1[20–OH,20–OH] and diestolide (E^2) were produced only in small amounts and at slow rates (Fig. 2a). Product distributions yielded by *G. candidum* and *P. cyclopium* lipases were similar to those obtained using *C. rugosa* lipase (Table 2). *Pseudomonas* sp. lipase also produced E^1[20–OH,18] as the major product but catalyzed E^1[20–OH,20–OH], E^2, and higher estolide synthesis at larger amounts (Fig. 2b). (The increase in FFA at 5.0 h in Figure 2b is due to the hydrolysis of MG + DG + TG present initially.) The reaction catalyzed by *A. niger* lipase proceeded similarly (Table 2). No other products—for example, lactones—were produced under the conditions employed here, as indicated by high-pressure liquid chromatographic analysis (4). Thus, it appears that lipases can be placed into three categories with respect to their ability to synthesize estolides: (i) lipases that form estolides between HA and NHA in strong preference over two HA (e.g., *C. rugosa*); (ii) lipases able to form several estolide species readily from both HA and NHA (e.g., *Pseudomonas* sp.), and (iii) lipases unable to catalyze estolide formation (1,3-specific lipases).

Pseudomonas sp. and *Candida rugosa* lipases were selected for further examination. First, these two lipases were subjected to conditions where formation of estolide and fatty acid wax ester could both occur (Scheme 1). Namely, the medium contained nearly equimolar amounts of HA, NHA, and 1-decanol as substrate. The results with *Pseudomonas* sp. are illustrated in Figure 3. They demonstrate that wax ester formation (reaction iii of Scheme 1) occurs quickly, while no free estolide (reactions i and ii) is formed. However, estolide esters do form slowly after the accumulation of large quantities of fatty acid wax ester, probably by using the latter as substrate (reaction iv of Scheme 1) rather than free estolide (reaction v). When *C. rugosa* lipase catalyzed the esterification under the same conditions, only wax esters were formed (data not shown). Similarly, two other reports demonstrate that, during *C. rugosa* lipase-catalyzed esterification between hydroxy acid and fatty alcohol, no esterification occurred at the hydroxyl moiety of the HFA (27,28). Furthermore, *C. rugosa* lipase was successfully employed to esterify primary alcohols and an estolide-like polymer without cleavage of the estolide bonds (29).

Of interest, HA–NHA esterification occurred at high yields for several lipases (Table 2). In general, the product distribution showed little dependence on the reaction system. The latter result was quite surprising, considering the large water content present in biphasic medium, which one would expect to promote hydrolysis and lessen the extent of esterification. In contrast, Yoshida and Kawase (19) discovered that increasing water content decreased the extent of esterification during lipase-catalyzed ricinoleic acid estolide formation. To further examine the effect of reaction system on equilibrium concentrations, lipase-catalyzed hydrolysis of oleic acid monoestolide was examined (Table 3). The results demonstrate that the extent of hydrolysis is low for both *C. rugosa* and *Pseudomonas* sp. lipases and, similar to lesquerolic–octadecenoic acid esterification, is independent of reaction system.

TABLE 3
Effect of Medium Type on Lipase-Catalyzed Hydrolysis of Oleic Acid Monoestolide[a]

Lipase type	Medium type	% Hydrolysis	% C_{18}-hydroxy FA/FFA
None	None	2.7	0.0
C. rugosa	Reverse micelles	15.7	27.1
C. rugosa	Biphasic	13.9	34.5
C. rugosa	Immobilized	9.8	32.2
Pseudomonas sp.	Biphasic	8.0	21.2

[a]Reaction conditions described in the Experimental Procedures section; FA/FFA, fatty acid/free fatty acid; see Table 1 for "Lipase type" abbreviation.

However, one could argue that the low extent of hydrolysis is due to the difference in location of the hydroxyl moieties on the monoestolides' acyl groups (C_8–C_{11}) compared to lesquerolic acid (C_{14}). However, the literature demonstrates that *C. rugosa* and *Pseudomonas* sp. lipases can utilize HA or estolide with hydroxyl moieties at several different positions (30–32).

Preparative-scale estolide ester synthesis. As established previously, 1,3-specific lipases (with the exception of *A. niger* lipase) cannot attack estolide bonds. For this reason and because of its established ability to catalyze reactions at high temperatures without inactivation and its reusability (33), immobilized *R. miehei* lipase (Lipozyme IM20) was employed for preparative-scale production of estolide esters. Reactions between estolide and fatty alcohols were operated in batch mode at either 60°C (solventless) or 22°C (isooctane added to lower viscosity). Figure 4 depicts oleic acid monoestolide–oleyl alcohol esterification at 22°C. Esterification is nearly 100% complete, with the initial rates of reaction, (V_0), being: $V_0(E^0) > V_0(E^1) > V_0(E^2)$. The course of reaction proceeded similarly when employing reverse micellar medium, or substituting oleyl alcohol with other fatty alcohols (data not shown). The ratio $E^1/E^2/E^3$ in the substrate nearly matches the ratio of the corresponding esters in the product, further indicating that estolide bonds were not broken. Oleic acid polyestolide and oleyl alcohol also were reacted. SFC results indicate for the lower estolides (E^1–E^5) that the conversions were high: >95%. The conversion of the higher estolides could not be determined because the SFC instrumentation employed cannot resolve analytes with MW over approximately 2200 (400 atm maximum pressure).

Esterification between estolide and α,ω-diols was conducted (at 22°C) in ethyl ether with saturating amounts of diol. Larger diol concentrations can slow the initial rate of esterification (Hayes, D.G., and R. Kleiman, unpublished data). As shown in Figure 5, the reaction proceeded readily, with the synthesis of ME being favored over DE. At 137 h, additional monoestolide was added, which led to a larger proportion of DE being produced. SFC chromatograms of polyestolide-1,6-hexanediol esterification, along with chromatograms of purified ME and DE product, are depicted in Figure 6. Free acid polyestolide (substrate) yielded broad SFC peaks, with the band width increasing as elution time (that is, carrier fluid density) increased. As a result, the largest analyte molecule to be resolved was pentaestolide (E^5) with a molecular weight of 1683. Reaction Step I consisted of diol–polyestolide esterification in ether at conditions similar to those employed for the reaction of Figure 5. The reaction was stopped after approximately one week. The reaction medium's chromatogram indicates that the majority of product formed was ME. Note that the resulting ME peaks are sharper and better resolved than those of free estolide. Ether was then evaporated away from the medium. Two phases formed: a lower, nearly solid phase, consisting of mostly diol,

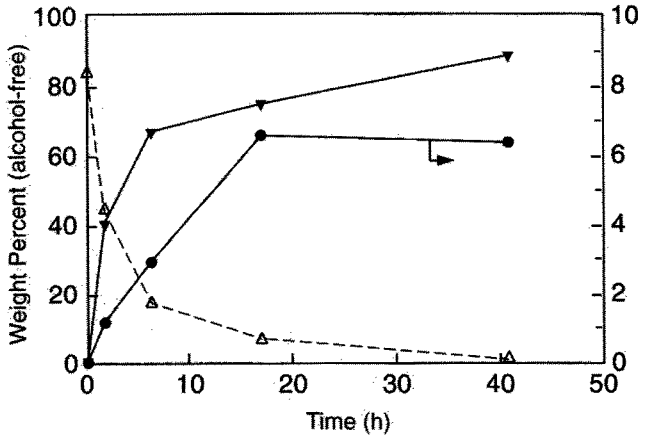


FIG. 4. Lipozyme IM20-catalyzed esterification between monoestolide sample and oleyl alcohol at 22°C. Medium: 25 mL (22.4 g, 38.6 mmol) oleic acid monoestolide, 25 mL (21.2 g, 79.1 mmol) oleyl alcohol, 50 mL isooctane; Lipozyme (recovered from previous experiments, then stored over $CaSO_4$ to reduce water content) present at 43.3 mg/mL. (△) E^1; (▲) E^1 ester; (●) E^2 ester.

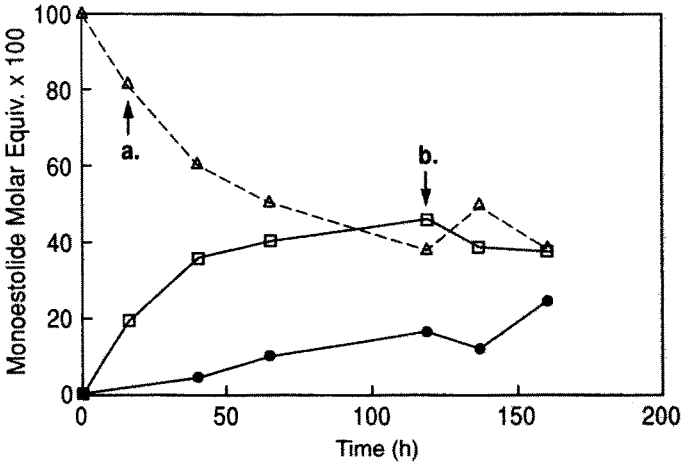


FIG. 5. Lipozyme IM20-catalyzed esterification between monoestolide sample and 1,10-decanediol at 22°C. Medium: 17.2 g oleic acid monoestolide, 120 mL ethyl ether; medium saturated with diol before reaction began. Medium shaken at 150–200 revolutions/min. Plotted are monoestolide molar equivalents in: (△) E1; (□), ME; (●) DE. (a) Medium resaturated with diol because >75% of diol present initially was consumed. (b) Approximately 8.7 g monoestolide added.

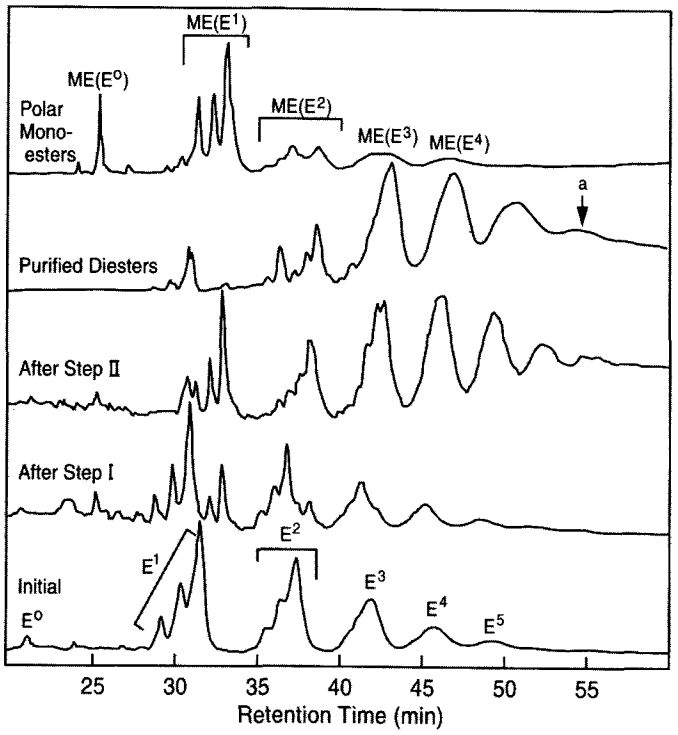


FIG. 6. Supercritical fluid chromatograms, depicting Lipozyme-catalyzed esterification between oleic acid polyestolide and 1,6-hexanediol. Steps I and II and peak a are described in the text; ME, monoester; E, ester.

and an upper phase that contained mostly polyestolide/esters. The upper phase was collected, additional free polyestolide and Lipozyme IM20 were added, and the resulting medium was reacted under magnetic stirring at 60°C (solventless mode) for 4 d (Step II). The chromatogram of the resulting reaction medium indicates that mostly DE was formed. The largest DE peak resolved (peak a of Fig. 6) has an apparent MW of approximately 2250 (SFC retention time–MW calibration curve). Calculations suggest that this peak represents estolide esters containing acyl groups; for example, DE(E^3,E^3), DE(E^4,E^2).

Physical properties and applications of estolide esters. Estolide esters were isolated and purified from the reaction media by column chromatography with a silica gel matrix. Physical properties of these esters and the free estolides from which they were derived are displayed in Table 4. Esterification greatly reduced viscosity and raised the melting point but had little effect on density. In addition, density values did not change appreciably between 23 and 60°C (data not shown). Estolide–diol ME differed less in properties from its parent compared to estolide–diol DE and wax ester. Such trends agree with results obtained for lesquerolic acid esters (Hayes, D.G., and R. Kleiman, unpublished data).

VI is a measure of a material's viscosity resistance to temperature. For lubricants, it is desirable that VI is above 150 (34). Thus, estolides and particularly estolide esters would be suitable lubricants or lubricant additives. Lubricants, especially when used for automobiles or aircraft, should remain liquid at low temperatures (34). With the exception of monoestolide–decanediol DE, all materials contained in Table 4 have low T_m, especially polyestolides and their esters. Diol DE of mono- and polyestolides are high MW materials that possess low viscosities relative to their lower MW parents. Such material may be desirable as lubricant additives, especially the polyestolide DE, which has a more suitable VI and T_m. Furthermore, it would be expected that these materials are biodegradable. The market for biodegradable lubricants is increasing in North America and western Europe as environmental regulations for chain saw oils, radio transmitter fluids, food machinery lubricants, and so on, increase (34). Biodegradation studies are underway in our laboratory (Erhan, S.M., unpublished data).

TABLE 4
Physical Properties of Estolides and Their Esters[a]

Material	Composition	T_m	ρ	*v* (25.3°C)	*v* (41.3°C)	*v* (58.0°C)	*v* (97.5°C)	VI
Monoestolide	83.7% E^1, 10.6% E^2	<(-19)	0.90	473.9[b]	126.3	61.08	23.11	191
Decyl ester	73.7% E(E^1), 17.5% E(E^2), 6.1% FAE	(-12.5) - (-11.5)	0.88	55.28[c]	30.43	18.82	8.16	204
Oleyl ester		(-19) - (-17.0)	0.87	49.92	37.20	21.96	10.88	302
Decanediol ME	88.7% ME(E^1), 4.3% ME(E^2), 4.1% ME(E^0)	<(-19)	0.90	273.0[c]	108.0[d]	47.77	18.32	165
Decanediol DE	75.0% DE(E^1,E^1) + DE(E^1,E^2), 20.4% DE(E^1,E^0)	(-4)-3	0.90	190.0	92.81	67.07	101	
Polyestolide	ND	<(-19)	0.92	700	329.6	170.4	39.13	150
Oleyl ester	ND	<(-19)	0.88	95.66	54.16	43.86	15.81	162
Decanediol DE	ND	<(-19)	0.87	540.5[c]	270.6[d]	117.6[e]	38.46	164

[a]T_m: melting point, °C; r: density at 25.3°C, g/mL; *n*: kinematic viscosity in CST at 25.3, 41.3, 58, and 97.5°C. VI, viscosity index.
[b]E^n refers to estolides containing *n* acyl groups (E^0 refers to C_{18} acyl groups), E ( ) refers to esters, and ME ( ) and DE ( ) refer to diol mono- and diesters, respectively.
[c]Measured at 23.9°C.
[d]Measured at 42.9°C.
[e]Measured at 60.0°C.

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REFERENCES

1. Hayes, D.G., and R. Kleiman, *J. Am. Oil Chem. Soc. 72:*559 (1995).
2. Lakshminarayana, G., R. Subbarao, Y. Sita Rama Sastry, V. Kale, C. Rao and A. Gangadhar, *Ibid. 61*:1204 (1984).
3. Burg, D.A., R. Kleiman and S.M. Erhan, U.S. Patent Application 07/662606 (1991).
4. Erhan, S.M., R. Kleiman and T.A. Isbell, *J. Am.. Oil Chem. Soc. 70*:461 (1993).
5. Isbell, T.A., R. Kleiman and B.A. Plattner, *Ibid. 71:*169 (1994).
6. Hayes, D.G., and R. Kleiman, *Ibid. 70*:555 (1993).
7. Kleiman, R., S.M. Erhan and D.A. Burg, *INFORM 3*:482 (1992).
8. Isbell, T.A., R. Kleiman and S.M. Erhan, *J. Am. Oil Chem. Soc. 69*:1177 (1992).
9. Isbell, T.A., and R. Kleiman, *Ibid. 71:*379 (1994).
10. Hayes, D.G., and R. Kleiman, *Ibid. 70*:1121 (1993).
11. Noureddini, H., B.C. Teoh and L.D. Clements, *Ibid. 69*:1184 (1992).
12. Noureddini, H., B.C. Teoh and L.D. Clements, *Ibid. 69*:1189 (1992).
13. American Society for Testing and Materials, *1981 Annual Book of ASTM Standards*, Philadelphia, 1981, p. 277.
14. Hayes, D.G., and R. Kleiman, in *Supercritical Fluid Extraction and Chromatography in Lipid Analysis and Processing,* edited by T. Lee and J.M. Snyder, AOCS Press, Champaign, in press.
15. Okumura, S., M. Iwai and Y. Tsujisaka, *Yukugaku 32*:271 (1983).
16. Matsumura, S., and J. Takahashi, *Makromol. Chem. Rapid Comm. 7*:369 (1986).
17. Yamaguchi, C., M. Akita, S. Asaoka and F. Osada, Japanese Patent 8916591 (1988).
18. Yamaguchi, C., A. Tooyama, S. Asaoka and F. Osada, Japanese Patent 9013387 (1989).
19. Yoshida, Y., and M. Kawase, *INFORM 3*:483 (1992).
20. Yamane, T., *Ibid. 4*:481 (1993).
21. Pavel, K., and H. Ritter, *Makromol. Chem. 192:*1941 (1991).
22. Knani, D., A.L. Gutman and D.H. Kohn, *J. Polym. Sci., Part A, Polym. Chem. 31*:1221 (1993).
23. Sonnet, P.E., *J. Am. Oil Chem. Soc. 65*:900 (1988).
24. Vorderwulbecke, T., K. Kieslich and H. Erdmann, *Enzyme Microb. Technol. 14*:631 (1992).
25. Ghoshray, S., and K.K. Bhattacharya, *J. Am. Oil Chem. Soc. 69*:85 (1992).
26. Amano International Enzyme Co., Inc., Lipase G Amano, *Technical Bulletin PR-9*, Troy.
27. Lang, S., R. Multzsch, A. Passeri, A. Schmeichel, B. Steffen, F. Wagner, D. Hamann and H.K. Cammenga, *Acta Biotechnol. 11*:379 (1991).
28. Chattopadhyay, S., and V.R. Mamdapur, *Biotechnol. Lett. 15*:245 (1993).
29. Pavel, K., and H. Ritter, *Makromol. Chom 194:*3369 (1993).
30. Honig, H., P. Seufer-Wasserthal and H. Webber, *Tetrahed. Lett. 31*:3011 (1990).
31. Scilimati, A., T.K. Ngooi and C.J. Sih, *Ibid. 29*:4927 (1988).
32. Zhi-Wei, G., T.K. Ngooi, A. Scilimati, G. Fulling and C.J. Sih, *Ibid. 29*:5583 (1988).
33. Hansen, T.T., and P. Eigtved, *Proceedings of the World Conference on Emerging Technologies in the Fats and Oils Industry,* edited by A.R. Baldwin, American Oil Chemists' Society, Champaign, 1986, p. 365.
34. *Synthetic Lubricants and High-Performance Functional Fluids,* edited by R.L. Shubkin, Marcel Dekker, New York, 1992.

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