Lipase-Catalyzed Synthesis of Chiral Triglycerides

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ABSTRACT: Under certain reaction conditions, the acidolysis of tripalmitin with oleic acid using immobilized lipase from *Rhizomucor miehei* resulted in a higher level of monosubstituted oleoyldipalmitoyl (OPP) triglycerides than had been predicted according to kinetic modeling. The reaction products were subjected to chiral analysis by high-performance liquid chromatography (HPLC), which indicated that the enzyme was more active at the *sn*-1 position of the triglyceride than at the *sn*-3 position, resulting in synthesis of the chiral triglyceride 1-oleoyl-2,3-dipalmitoyl-*sn*-glycerol. A kinetic model was developed and was correlated with the HPLC method to provide a simple means to predict the stereoselectivity of lipase-catalyzed reactions. By using the model, the stereoselectivity of immobilized *Rhizomucor miehei* lipase was found to depend strongly on the initial water activity (a_w) of the reaction mixture, with greater selectivity occurring at lower a_w. The *sn*-1 selectivity was essentially maintained using various solvents, or without solvent, when a_w was kept constantly low. Variation in the fatty acid composition of the triglyceride indicated that shorter-chain fatty acids result in greater stereoselectivity, while variation of the chainlength of the free fatty acid indicated an enhancement by the longest chainlength. The stereoselectivity of this lipase was confirmed using a new ^{13}C nuclear magnetic resonance method. By using immobilized *R. miehei* lipase at low a_w, approximately 80% of the chiral triglyceride found in the reaction mixture was the *sn*-1 enantiomer, at high reaction conversion. *JAOCS 75*, 1513–1518 (1998).

KEY WORDS: Acidolysis, chain length, chiral, free fatty acid, lipase, solvent type, source organism, stereoselectivity, triglyceride, water activity.

Lipases have been used extensively as stereoselective catalysts in organic synthesis, especially in the fine chemicals and pharmaceutical fields as reviewed by Roberts (1), but little attention has been given to the synthesis of chiral triglycerides. Recent studies have focused on the evaluation of lipases for the presence of stereoselectivity and their *sn*-preference using triglycerides or triglyceride analogs. A survey of 26 lipases by Verger and co-workers (2,3) revealed that in triglyceride hydrolysis reactions, all but one of the lipases exhibited some degree of stereoselectivity, most commonly at the *sn*-1 position, and that the *sn* selectivity could reverse depending on the acyl chain length of the triglyceride used. Unfortunately, the degrees of conversion used in this study were less than 6%, which is impractical from the point of view of synthesis of chiral triglycerides. Other studies with triglycerides confirmed the stereoselectivity of several lipases in hydrolysis and triglyceride–triglyceride interesterification (4–7). Although useful for lipase evaluation, the interesterification approach resulted in complex mixtures of products not amenable to isolation of specific enantiomers. The use of triglyceride analogs, for example containing ether linkages to the glycerol molecule, is also a useful approach for evaluation of lipase selectivity (8,9) but is not appropriate for synthesis of enantiomerically enriched triglycerides.

The purpose of the work described here is to develop a practical, high-yield method for the synthesis of triglycerides enriched in particular enantiomers. The method is based on lipase-catalyzed acidolysis of triglycerides, a process which has already been commercialized for the production of tailor-made triglycerides for food applications (10).

MATERIALS AND METHODS

Materials. Triglyceride and free fatty acid reagents were purchased in the highest available purity from Sigma-Aldrich Ltd. (Poole, United Kingdom). Lipases (EC 3.1.1.3) from *Rhizopus delemar* [Lipase D (650,000 LU/g)], *R. niveus* [Lipase N (4,500 LU/g)], and *Pseudomonas cepacia* [Lipase PS (22,000 LU/g)] were purchased from Amano Pharmaceutical Co. (Nagoya, Japan). Lipases from *Humicola lanuginosa* [Lipolase® (100,000 LU/mL)], *Rhizomucor miehei* [Lipozyme® (10,000 LU/mL)], and immobilized lipase from *R. miehei* supported on Duolite resin [Lipozyme® IM (100,000 LU/g)] were gifts from Novo Nordisk A/S (Bagsvaerd, Denmark). Macroporous poly-propylene enzyme support (Accurel EP100, particle size distribution 200–400 μm) was purchased from Akzo Nobel GmbH. (Obernburg, Germany). All other solvents and chemicals were of analytical reagent (AR) quality and purchased from Sherman Chemicals Ltd. (Sandy, United Kingdom) or Sigma-Aldrich Ltd.

Immobilization of lipases onto Accurel EP100. The method described by Bosley and Peilow (11) was followed. Lipase activity was measured in Lipase Units (LU); 1 LU is defined as the amount of enzyme which liberates 1 μmol titratable butyric acid per minute from an emulsion of tributyrin at pH 7.0. Accurel EP100 (1 g) was wetted with absolute ethanol (6 mL) and 10 mM sodium phosphate buffer, pH 7.0

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(94 mL). A solution of lipase of known concentration (50 mL) was added to the support and mixed for 16 h. The catalyst was recovered by filtration under vacuum, washed with buffer (350 mL), and dried at 20°C under vacuum. The lipase adsorbed onto the catalyst was calculated as the difference in solution lipase activity before and after immobilization, assuming no other loss due to inactivation of lipase.

Enzyme-catalyzed reactions of triacylglycerol and free fatty acid. All reactants and solvents were equilibrated to a known water activity (a_w) at ambient temperature (20°C) in a sealed vessel for at least 7 d, as described by Valivety *et al.* (12). The salts used for equilibration were lithium chloride (a_w 0.11 at 20°C), lithium bromide (0.07), potassium acetate (0.23), magnesium chloride (0.32), magnesium nitrate (0.55), and sodium chloride (0.75). Molecular sieve (3 Å) was used to dehydrate materials to an a_w of less than 0.02. It is assumed that, provided sufficient time is allowed for equilibration, the a_w of the sample equals that of the salt solution. Tripalmitin (200 mg; 250 μmol) and oleic acid (212 mg; 750 μmol) were mixed in petroleum ether (5 mL; 100–120°C bp) at 60°C. Immobilized *R. miehei* lipase on Duolite support (Lipozyme, 10 mg; pre-equilibrated to a_w of 0.11 over saturated lithium chloride solution at 20°C) was added to catalyze the acidolysis reaction with stirring. Reactions of other substrates were of a 1:3 molar ratio of triglyceride to fatty acid. Samples of reaction mixture (25 μL) were periodically removed from the bulk mixture. The sample volume was diluted by a factor of 25 with hexane and analyzed by gas–liquid chromatography (GC). From the carbon number data at each timepoint a degree of conversion (dc) between 0 and 1 was calculated according to Equation 1.

$$\text{dc} = \frac{\%\text{ oleic acid in total triglyceride}}{\%\text{ oleic acid in total triglyceride at equilibrium}} \quad [1]$$

The reaction was stopped by removing the solid enzyme catalyst by filtration after the required degree of conversion had been obtained. The reaction composition was analyzed by carbon number. The free fatty acid was removed from the converted triglyceride by saponification with basic aluminium oxide and elution with hexane.

GC. A Perkin-Elmer 8400 series gas–liquid chromatograph was used for all separations of triglycerides. Separation was achieved on the basis of triglyceride carbon number. The column fitted was a DB1 10-m bonded-phase fused-silica capillary column, 0.53 mm i.d., 0.1 μm methyl 5% phenylsilicone film from Quadrex (Weybridge, United Kingdom). The temperature program began with an initial temperature of 180°C, which was held for 4 min then raised 15°C/min to 350°C, and held for a further 2 min (total run time 18.8 min). Helium carrier gas was used with flame-ionization detection. Appropriate response factors were applied to the peak areas of the triglycerides.

Stereospecific analysis of triacylglycerol by high-performance liquid chromatography (HPLC). The method described by Christie *et al.* (13) for the stereospecific analysis of triglycerides was followed, with minor modifications. The basis of the analysis is the generation of the enantiomeric mono- and diacylglycerols from the parent triglyceride by Grignard reaction; they are then converted into diastereomeric 3,5-dinitrophenylurethane derivatives. Subsequent analysis by HPLC results in separation of the *sn*-1,2 and *sn*-2,3 diasteromers. HPLC was carried out with an ACS Model 352 pumping system connected to a Varian 9050 ultraviolet detector set at 280 nm. Separation was achieved on a single Nucleosil 100 3 μ silica 25 cm × 4.6 mm column from Jones Chromatography (Glamorgan, United Kingdom) with a mobile phase of 0.25% vol/vol 1-propanol (containing 2% vol/vol water) in hexane at a flow rate of 1.0 mL/min. The time for one HPLC run was 90 min. The ratio of stereoisomers can be estimated by comparison of the percentages of the diastereomers from the HPLC trace. However, this is not always satisfactory, as overlap between peaks (in particular those derived from the *sn*-1,3 isomers) can cause errors. The fatty acid composition of each triglyceride *sn* position can be determined by GC analysis of the separated diastereomers (supplemented by separate analysis of the *sn*-2 position if required). The distribution of triglyceride stereoisomers can then be calculated from the *sn*-1, *sn*-2, and *sn*-3 fatty acid compositions by use of the 1-random-2-random-3-random distribution method, first described by Hilditch and Williams (14), in which the content of a given triglyceride can be calculated as: $\%sn\text{-XYZ} = [\text{mol}\% \times \text{at } sn\text{-1}] \times [\text{mol}\% \text{ Y at } sn\text{-2}] \times [\text{mol}\% \text{ Z at } sn\text{-3}] \times 10^{-4}$.

Development of a kinetic model. Figure 1 shows the acidolysis reaction scheme, catalyzed by a 1,3-selective lipase which was developed to separate reactions at *sn*-1 from reactions at *sn*-3. Compositional data determined by GC were input into the parameter estimation facility of the computational package SPEEDUP from Aspen Technologies (Cambridge, MA) in order to estimate the rate constants for the reaction at the *sn*-1 and *sn*-3 positions of the triglyceride, i.e. $k_{sn\text{-}1}$ and $k_{sn\text{-}3}$, respectively. The ratio of $k_{sn\text{-}1}/k_{sn\text{-}3}$ is defined as the stereoselectivity ratio and is a measure of the stereoselectivity of the enzyme. A higher ratio indicates a greater preference for the *sn*-1 position of the triglyceride, which leads to an accumulation of one triglyceride stereoisomer at degrees of conversion below 100%.

RESULTS AND DISCUSSION

The present study demonstrates that, in microaqueous reactions of triglycerides, *R. miehei* lipase can exhibit a high degree of stereoselectivity for the *sn*-1 position under conditions of low a_w and a degree of reaction conversion of less than 100%. The stereoselectivity of lipases in synthetic-type reactions involving its natural triglyceride substrate has not been widely reported previously.

Effect of the initial a_w. Substrates and immobilized *R. miehei* lipase, pre-equilibrated to a particular initial a_w over saturated salt solutions, were reacted at 60°C in *n*-hexane. The stereoselectivity ratio ($k_{sn\text{-}1}/k_{sn\text{-}3}$) was calculated from the compositional data as described in the Materials and Methods section and the effect of initial a_w on the ratio is shown in

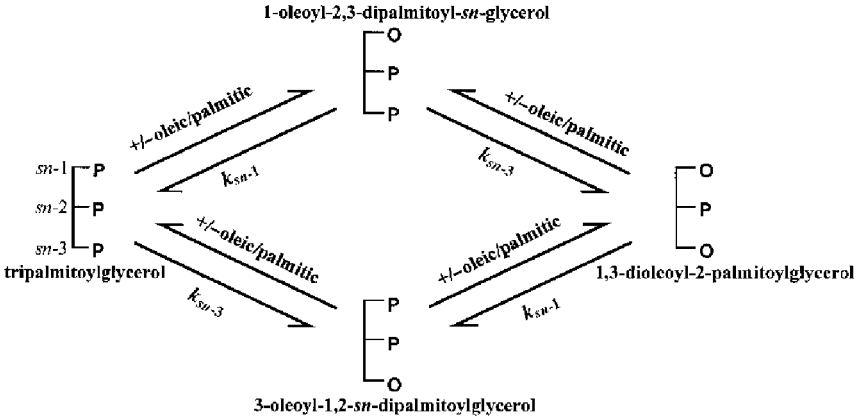

FIG. 1. Simplified acidolysis reaction pathway for *sn*-1,3 selective lipases with independent rate constants at positions *sn*-1 and *sn*-3.

Figure 2. At an initial a_w of approximately 0.4 or greater, the stereoselectivity ratio was close to 1, indicating that the immobilized lipase does not exhibit stereoselectivity. However, on reduction of the a_w to 0.3 or less, a dramatic increase in stereoselectivity ratio was observed. At the lowest a_w (close to 0), the lipase is most stereoselective and strongly favors the *sn*-1 position of the glyceride. The greatly enhanced stereoselectivity at the lower a_w may be explained by a reduction in the flexibility of the lipase molecule, a phenomenon which has previously been suggested as the cause of selectivity and stability changes in several types of enzyme (15–17).

Effect of lipase source on stereoselectivity under identical reaction conditions. To determine if the observed selectivity of *R. miehei* lipase is inherent to the lipase or is dependent only on a_w or the immobilization process, a selection of lipases was studied under the same initial a_w (0.11 at 20°C) and different immobilization conditions. The lipase sources and immobilization supports are shown in Table 1, along with the calculated stereoselectivity ratios for the acidolysis reaction with tripalmitin performed at an a_w of 0.11 (20°C).

In the case of *R. miehei* lipase, a consistently high stereoselectivity ratio (*ca.* 4) was obtained regardless of the immobilization supports evaluated or whether the lipase was cloned or native. In contrast, all other lipases evaluated exhibited no significant stereoselectivity (stereoselectivity ratio = 1) when immobilized on Accurel or Duolite. These results strongly indicate that the stereoselectivity of *R. miehei* is due to an inherent property of the enzyme not shared by the other lipases evaluated under similar reaction conditions. The effect is also independent of the immobilization support, where the lipase is adsorbed to the support by either ionic (Duolite) or hydrophobic (Accurel) interactions. However, as the reaction carried out here was optimized for immobilized *R. miehei* only, it is possible that improved stereoselectivity results could be obtained for other lipases under different reaction conditions or at very low degrees of reaction conversion.

Effect of the triglyceride acyl chain length on stereoselectivity under identical reaction conditions. Earlier reports indi-

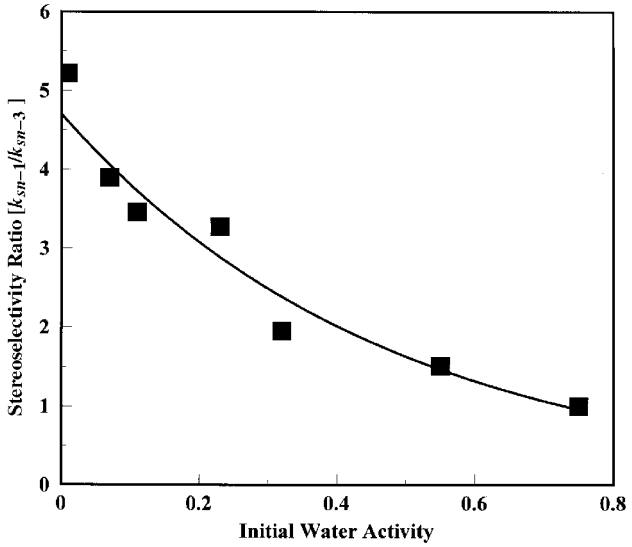

FIG. 2. Effect of the initial water activity on the apparent ratio of rate constants, $k_{sn\text{-}1}/k_{sn\text{-}3}$.

TABLE 1
Effect of the Lipase Source on the Apparent Ratio of Rate Constants

Lipase source organism	Support	Ratio of estimated rate constants (k_{sn-1}/k_{sn-3}) (± errors)
Rhizomucor miehei[a]	Duolite	4.2 (± 0.2)
R. miehei[b]	Duolite	3.8 (± 0.2)
R. miehei[b]	Accurel EP100	3.6 (± 0.2)
Humicola lanuginosa[a]	Duolite	1.1 (± 0.1)
H. lanuginosa[a]	Accurel EP100	1.1 (± 0.1)
Rhizopus niveus[b]	Accurel EP100	1.0 (± 0.05)
Rhizopus delemar[b]	Accurel EP100	1.0 (± 0.05)
Pseudomonas cepacia[b]	Accurel EP100	1.0 (± 0.1)

[a]These lipases were prepared using recombinant DNA technology: the genetic coding for the lipase was transferred from the source organism to a host, *Aspergillus niger*.
[b]These lipases were expressed by the native organism.

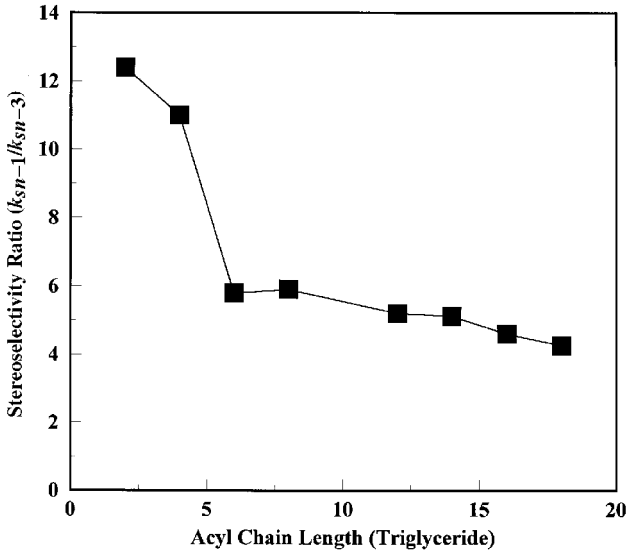

FIG. 3. Effect of the substrate triglyceride acyl chainlength on the ratio of apparent stereoselectivity.

cated that stereoselectivity of lipases may be dependent on the chainlength of the substituent fatty acids on the triglyceride substrate, in particular the substituent in position *sn*-2 (3,8). This observation was further investigated in the present work with immobilized *R. miehei* by reacting a series of monoacid triglycerides with oleic acid at an a_w of 0.11. The calculated stereoselectivity ratios shown in Figure 3 indicate little chainlength effect between C_6 and C_{18}. However, the short-chain fatty acids (C_2 and C_4) exhibited a dramatic enhancement in the stereoselectivity of the lipase. This is in general agreement with the findings of Rogalska *et al.* (3), but in this work it was not possible to determine any change in the *sn* position preference, owing to the impracticalities of analyzing the shorter-chain stereoselective products. The effect on stereoselectivity of only altering the acyl group chainlength in the *sn*-2 position of the triglyceride has not been evaluated.

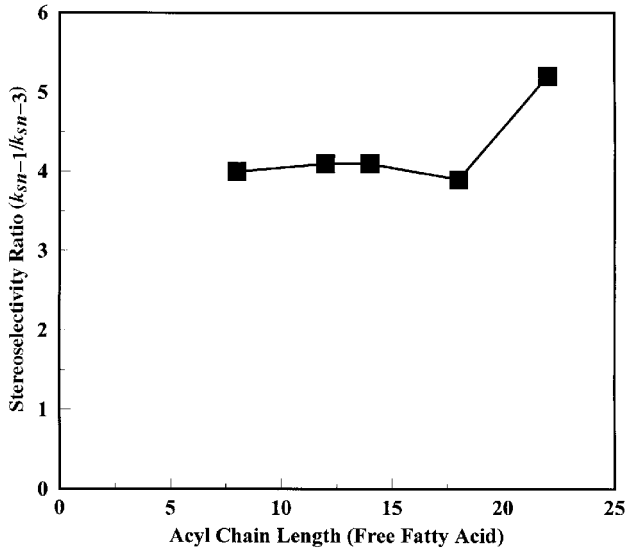

FIG. 4. Effect of the substrate free fatty acid chainlength on the ratio of apparent stereoselectivity.

TABLE 2
The Effect of the Reaction Solvent on the Apparent Ratio of Rate Constants

Solvent	Log *P*	Ratio of estimated rate (k_{sn-1}/k_{sn-3}) (± errors)
No solvent	Not determined	3.2 (± 0.4)
n-Pentane	2.4	3.4 (± 0.3)
n-Hexane	3.5	4.0 (± 0.3)
n-Heptane	4.0	2.9 (± 0.2)
n-Octane	4.5	3.2 (± 0.1)
n-Decane	5.5	3.0 (± 0.2)
n-Dodecane	6.6	2.6 (± 0.1)
n-Hexadecane	8.8	2.0 (± 0.1)
n-Octadecane	9.9	1.0 (± 0.0)
Toluene	2.5	2.8 (± 0.1)
Cyclohexane	2.5	2.8 (± 0.3)
2-Methylpentane	Not determined	3.1 (± 0.2)
2-Methylhexane	Not determined	3.2 (± 0.4)
1-Hexene	Not determined	1.7 (± 0.6)
Diethyl ether	0.85	1.3 (± 0.1)

[a]These lipases were prepared using recombinant DNA technology: the genetic coding for the lipase was transferred from the source organism to a host, *Aspergillus niger.*
[b]These lipases were expressed by the native organism.

Effect of free fatty acyl chain on stereoselectivity under identical reaction conditions. As the chainlength of the triglyceride fatty acyl substituent had an effect on lipase stereoselectivity, the effect of the free fatty acid chainlength was also evaluated. Figure 4 shows the stereoselectivity ratio for immobilized *R. miehei* lipase during acidolysis of tripalmitin with selected fatty acids. Little effect of free fatty acid chainlength was observed, with the exception of behenic acid ($C_{22:0}$) which resulted in a slightly higher stereoselectivity. Fatty acids shorter than C_8 could not be evaluated with the system described here.

With the available data, it can be concluded that only the fatty acid substituent on the triglyceride is important in the selectivity of the lipase, indicating that it is the formation of the triglyceride–enzyme complex within the active site of the lipase during catalysis that determines stereoselectivity. A hypothesis for the effect that acyl chainlength has on stereoselectivity was discussed by Rogalska *et al.* (3). The preferred stereospecific orientation of the triglyceride–enzyme intermediate is dictated by the size of the active site-binding pockets which accommodate the acyl chains, in particular, that for the *sn*-2 substituent. Reducing the chainlength of the acyl chains can therefore potentially affect the energetics of substrate binding in the active site and alter the extent of stereopreference, especially if the lipase has reduced flexibility through a lowering of the a_w. The positive effect that free behenic acid has on stereoselectivity is presumed to be a result of the greater steric demand of this long-chain acid, which may be preferentially accepted by one orientation of the complex.

Solvent effects on the stereoselectivity of immobilized R. miehei *lipase*. Several reports in the literature have claimed

TABLE 3
Results from Stereospecific Analysis of Lipase-Catalyzed Reactions Products and Calculation of Enantiomeric Ratios at 60% Reaction Conversion

Enantiomeric triacylglycerol species	Species in reaction product (wt%)	Ratio of *R/S* triacylglycerol enantiomers
Lipase from *Rhizomucor miehei*		
1,3-Dipalmitoyl-2-oleoylglycerol (achiral)	34.5	
1-Palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol (*S*)	9.0	4.8
1-Stearoyl-2-oleoyl-3-palmitoyl-*sn*-glycerol (*R*)	43.4	
1,3-Distearoyl-2-oleoylglycerol (achiral)	13.1	
Lipase from *Rhizopus delemar*		
1,3-Dipalmitoyl-2-oleoylglycerol (achiral)	37.7	
1-Palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol (*S*)	23.6	1.0
1-Stearoyl-2-oleoyl-3-palmitoyl-*sn*-glycerol (*R*)	23.7	
1,3-Distearoyl-2-oleoylglycerol (achiral)	15.0	

that the polarity of the solvent has an effect on stability and selectivity of lipases (12,18,19). The effect of the partition coefficient log *P* on the stereoselectivity of immobilized *R. miehei* lipase during acidolysis of tripalmitin with oleic acid was investigated. Table 2 shows that with solvents at the highest log *P*, the stereoselectivity tends to decrease from 4 to 2. At a log *P* of 5.5 or less, little variation in stereoselectivity is evident, with stereoselectivity ratios varying from 3 to 4. For optimal stereoselectivity, *n*-hexane was found to be the best solvent.

Confirmation and direct quantification of stereoselectivity by HPLC and ^{13}C nuclear magnetic resonance (NMR). By using the method of Christie *et al.* (13), the relative proportions of the two stereoisomers obtained by the reaction of 1,3-dipalmitoyl-2-oleoylglycerol with stearic acid was determined by chiral derivatization and HPLC analysis (see Material and Methods section). The results obtained (Table 3) confirm that immobilized *R. miehei* lipase is strongly stereoselective at the *sn*-1 position of the triglyceride. In the reaction mixture under optimal conditions (carried out in hexane at 60°C, initial a_w of 0.11), over 80% of the stereoisomers present were found to be 1-stearoyl-2-oleoyl-3-palmitoyl-*sn*-glycerol. As this was found at 60% conversion, this procedure provides a practical means to produce specific stereoisomers of triglycerides at high yields. A reaction carried out under identical conditions but catalyzed by immobilized *R. delemar* lipase was found to produce a racemic mixture of triglyceride isomers at 60% conversion (Table 3). Further evaluation of the progress of the stereoselective reaction was performed using deuterium-labeled trilaurin and oleic acid, details of which are reported elsewhere (20). This ^{13}C NMR spectroscopic evaluation procedure also confirmed the *sn*-1 selectivity determined by the HPLC method and the indirect kinetic derivatizations.

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