Steryl and Stanyl Esters of Fatty Acids by Solvent-Free Esterification and Transesterification in Vacuo Using Lipases from *Rhizomucor miehei*, *Candida antarctica*, and *Carica papaya*

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Sitostanol has been converted in high to near-quantitative extent to the corresponding long-chain acyl esters via esterification with oleic acid or transesterification with methyl oleate or trioleoylglycerol using immobilized lipases from *Rhizomucor miehei* (Lipozyme IM) and *Candida antarctica* (lipase B, Novozym 435) as biocatalysts in vacuo (20−40 mbar) at 80 °C, whereas the conversion was markedly lower at 60 and 40 °C. Corresponding conversions observed with papaya (*Carica papaya*) latex lipase were generally lower. High conversion rates observed in transesterification of sitostanol with methyl oleate at 80 °C using Lipozyme IM were retained even after 10 repeated uses of the biocatalyst. Saturated sterols such as sitostanol and 5α-cholestan-3β-ol were the preferred substrates as compared to Δ^5-unsaturated cholesterol in transesterification reactions with methyl oleate using Lipozyme IM. Transesterification of cholesterol with dimethyl 1,8-octanedioate using Lipozyme IM in vacuo yielded methylcholesteryl 1,8-octanedioate (75%) and dicholesteryl 1,8-octanedioate (5%). However, transesterification of cholesterol with diethyl carbonate and that of oleyl alcohol with ethylcholesteryl carbonate, both catalyzed by Lipozyme IM, gave ethylcholesteryl carbonate and oleylcholesteryl carbonate, respectively, in low yield (20%). Moreover, cholesterol was transesterified with ethyl dihydrocinnamate using Lipozyme IM to give cholesteryl dihydrocinnamate in moderate yield (56%), whereas the corresponding reaction of lanosterol gave lanosteryl oleate in low yield (14%).

Keywords: *Candida antarctica lipase B; Carica papaya lipase; esterification and transesterification in vacuo; fatty acids; Rhizomucor miehei lipase; solvent-free esterification and transesterification; stanyl esters; steryl esters*

INTRODUCTION

Cholesteryl esters are widely used for technical applications such as liquid crystal display devices. Fatty acid esters of sterols and steroids are well-known ingredients of cosmetics, nutraceuticals, and pharmaceutical formulations (*1*). Plant steryl and stanyl esters have been recently found to be effective in lowering plasma cholesterol concentration by inhibiting the absorption of cholesterol from the small intestine (*2*−*4*). Special margarines fortified with steryl esters are now commercially available as functional foods with the ability to reduce both total and low-density lipoprotein (LDL) cholesterol levels (*5*).

Fatty acid esters of sterols, stanols, and steroids are usually prepared from the corresponding sterols by chemical esterification with fatty acids or interesterification with fatty acid methyl esters as well as by their reaction with fatty acid halogenides or anhydrides (*6*). Enzymatic procedures for the preparation of steryl esters using organic solvents and molecular sieves or other drying agents have been reported (*1*, *7*−*15*). Moreover, a method has been described for the preparation of steryl esters of polyunsaturated fatty acids in an aqueous system (*16*). In most of the above methods the exent of formation of steryl esters is rather moderate and the use of organic solvents limits the application of such products.

Recently, we described the enzymatic preparation of carboxylic acid esters, particularly fatty acid esters, of sterols, stanols, and steroids in high yield by esterification and transesterification of fatty acids and other carboxylic acid esters, respectively, with the 3-hydroxy group of sterols, stanols, or steroids in vacuo at moderate temperature using immobilized lipase from *Candida rugosa* as the catalyst (*17*). Neither an organic solvent nor a molecular sieve was required. Here we report the synthesis of steryl and stanyl esters of carboxylic acids, particularly fatty acids, in vacuo using immobilized lipases from *Rhizomucor miehei* (Lipozyme IM) and *Candida antarctica* (lipase B, Novozym 435) as well as papaya (*Carica papaya*) latex under various conditions.

EXPERIMENTAL PROCEDURES

Chemicals. Sitostanol (stigmastanol), methyl myristate, methyl stearate, oleic acid, methyl oleate, triolein, ethyl dihydrocinnamate, 1,8-octanedicarboxylic acid dimethyl ester (suberic acid dimethylester), diethyl carbonate, cholesteryl chloroformate, oleylcholesteryl carbonate, and oleyl alcohol were obtained from Sigma-Aldrich-Fluka (Deisenhofen, Germany). Cholesterol, 5α-cholestan-3β-ol (dihydrocholesterol), lanosterol (containing ∼30% dihydrolanosterol), and 1-methylimidazole were purchased from Merck (Darmstadt, Germany). *N*-Methyl-*N*-(trimethylsilyl)heptafluorobutyramide

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10.1021/jf0107407 CCC: $20.00
Published on Web 10/27/2001

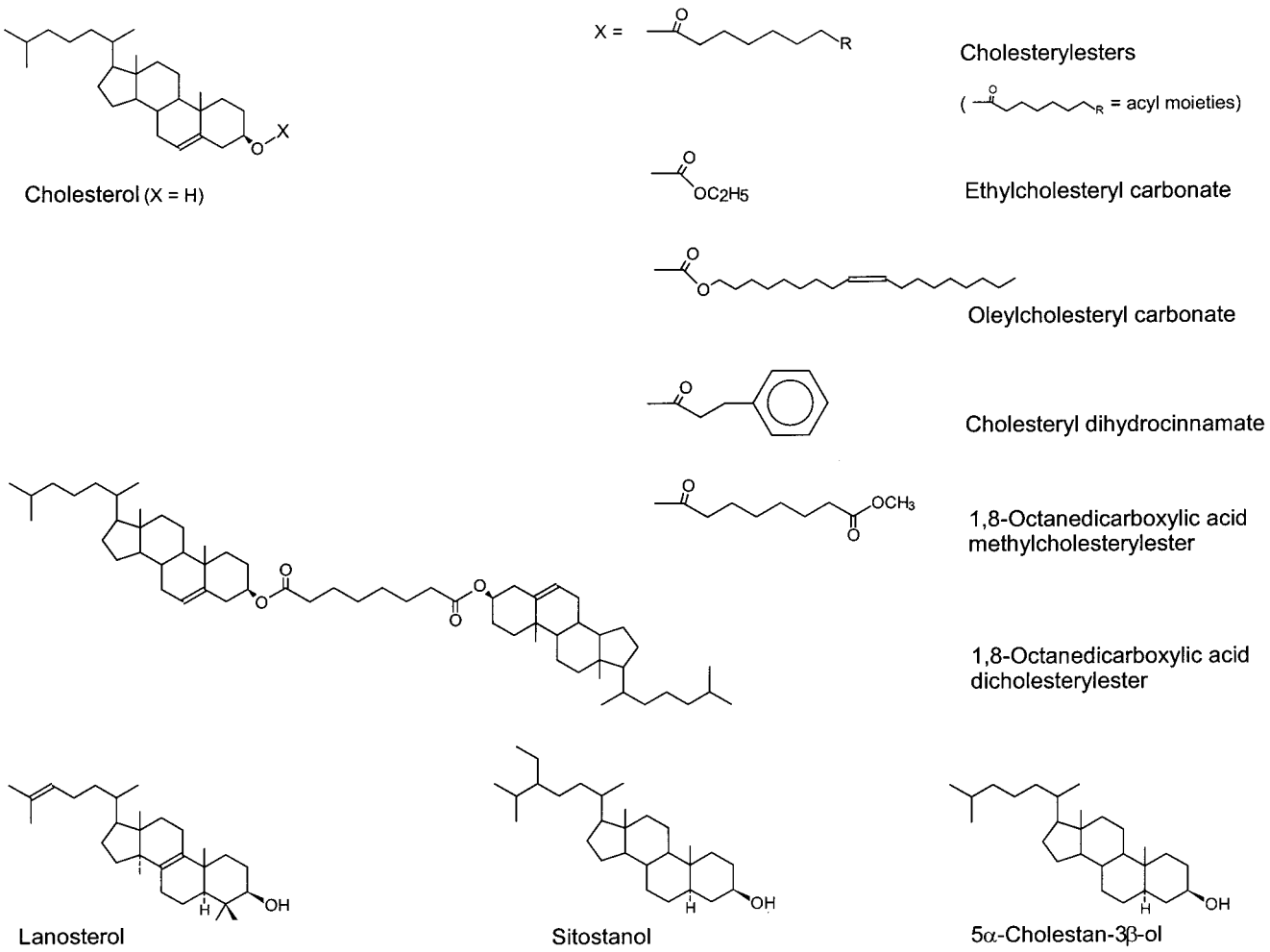

Figure 1. Chemical structures of steryl and stanyl esters.

(MSHFBA) was a product of Macherey-Nagel (Düren, Germany). The immobilized lipase preparations from *R. miehei* (Lipozyme IM 20; 23 batch interesterification units/g; 10% w/w water) and *Cd. antarctica* (lipase B, Novozym 435; 10500 propyl laurate units/g; 2% w/w water) were kindly provided by Novozymes (Bagsvaerd, Denmark). The granular papaya (*Cr. papaya*) latex preparation (Sigma-Aldrich-Fluka) was ground in a mortar with pestle to a fine powder to pass a 0.8 mm mesh width sieve. Ethylcholesteryl carbonate standard was prepared by reacting 0.5 g (1.1 mmol) of cholesteryl chloroformate dissolved in 3 mL of dichloromethane with 7 mL (150 mmol) of water-free methanol in the presence of 0.2 g of 4-(dimethylamino)pyridine (Sigma-Aldrich-Fluka) for 4 h at 45 °C. The reaction product was purified by column chromatography as described below.

Lipase-Catalyzed Reactions. Unless stated otherwise fatty acid or fatty acid methyl ester, 300 μmol each, or trioleoylglycerol (200 μmol) was esterified or transesterified with sitostanol or cholesterol, 100 μmol each, in the presence of 50 mg of each lipase preparation by magnetic stirring in a screw-capped tube in vacuo at 40, 60, and 80 °C for various periods with water (or methanol)-trapping in the gas-phase using KOH pellets. The vacuum used was 20−40 mbar, measured at room temperature.

In one set of experiments the reusability of the lipase preparations was evaluated in the transesterification of sitostanol with methyl oleate as described above. In each case the lipase was separated from the reaction mixture by repeated extraction with diethyl ether and centrifugation. Subsequently, the solvent was removed from the lipase in a stream of nitrogen followed by evacuation at 40 °C for 2 h and weighing. A fresh batch of reaction mixture was then added to the used lipase at a similar ratio of biocatalyst to substrate mixture as in the foregoing reaction.

Transesterification reactions of cholesterol (100 μmol) with ethyl dihydrocinnamate (initially 1 mmol, followed by additional 0.5 mmol after 32 h) and lanosterol (100 μmol) with methyl oleate (600 μmol) were performed in the presence of 100 mg of Lipozyme IM for 96 h in vacuo at 80 °C.

Transesterification of cholesterol (100 μmol) with dimethyl 1,8-octanedioate (400 μmol, two 200 μmol portions added initially and after 8 h) was carried out in the presence of 100 mg of Lipozyme IM for 72 h in vacuo at 80 °C. Transesterification of cholesterol (100 μmol) with diethyl carbonate (4.1 mmol) was carried out at 400 mbar and 60 °C in the presence of 50 mg of molecular sieve 4 Å and 50 mg of Lipozyme IM for 48 h. Transesterification of ethylcholesteryl carbonate with oleyl alcohol (300 μmol) was performed using 50 mg of Lipozyme IM at 20 mbar and 80 °C for 48 h.

Thin-Layer Chromatography (TLC). Aliquots of the reaction mixtures were checked for conversion by TLC on 0.3 mm layers of Silica Gel H (Merck, Darmstadt, Germany) using isohexane/diethyl ether (95:5, v/v); spots were located by iodine staining. Alternatively, the TLC plates were sprayed with 30% aqueous sulfuric acid and heated in an oven kept at 120 °C; Δ^5-sterols and their esters appeared as red spots (Liebermann-Burchard test). The R_f values of the various compounds were as follows: fatty acid steryl and stanyl esters, 0.6−0.7; methyl oleate, 0.4−0.5; trioleoylglycerol, 0.3−0.35; sterols and stanols, 0.1−0.15; oleic acid, <0.1.

Reaction mixtures containing steryl esters of 1,8-octanedicarboxylic acid and carbonic acid were analyzed by TLC on 0.3 mm layers of Silica Gel H using isohexane/diethyl ether (4:1, v/v). The R_f values of the various compounds

(Figure 1) were as follows: dicholesteryl 1,8-octanoate, 0.70−0.80; methylcholesteryl 1,8-octanedioate, 0.45−0.55; dimethyl 1,8-octanoate, 0.30−0.40; 1,8-octanedioic acid, <0.1; ethylcholesteryl carbonate, 0.60−0.65; oleylcholesteryl carbonate, 0.75−0.80.

Gas Chromatography (GC). In esterification reactions aliquots of reaction mixtures, ∼15 mg, were extracted twice with 2 mL of diethyl ether, each. The ether extract was concentrated and treated with a solution of diazomethane in diethyl ether to convert the unreacted fatty acids to methyl esters. The resulting mixture of methyl esters, unreacted sterols or stanols, and fatty acid steryl or stanyl esters was analyzed by GC. In transesterification reactions aliquots of products consisting of fatty acid methyl esters or triacylglycerols and unreacted sterols or stanols as well as carboxylic acid steryl or stanyl esters were analyzed without derivatization by GC. All GC samples, dissolved in dichloromethane, were filtered through a 0.45 μm syringe filter before injection into the gas chromatograph. A Hewlett-Packard (Böblingen, Germany) HP-5890 series II gas chromatograph equipped with a flame ionization detector was used. Separations were carried out on a 0.1 μm Quadrex 400-1HT (Quadrex Corp., New Haven, CT) fused silica capillary column, 15 m × 0.25 mm i.d., using hydrogen as the carrier gas (column pressure = 50 kPa) initially at 160 °C for 2 min, followed by linear programming from 160 to 180 °C at 5 °C·min^{-1} and from 180 to 410 °C at 20 °C·min^{-1}; the final temperature of 410 °C was held for 10 min. The split ratio was 1:10; the injector as well as flame ionization detector temperature was 350 °C. Peaks in gas chromatograms were assigned by comparison of their retention times with those of commercially available standards or those prepared by chemical or enzymatic synthesis. Peak areas and percentages were calculated using a Hewlett-Packard 3365 series GC ChemStation software and corrected using standard curves. The retention times of the various compounds were as follows: methyl myristate, 0.9 min; methyl stearate, 1.9 min; methyl oleate, 1.8 min; ethyl dihydrocinnamate, 1.0 min; cholesterol and 5α-cholestan-3β-ol, 8.9 min; sitostanol, 9.6 min; lanosterol, 9.5 min; dihydrolanosterol, 9.3 min; cholesteryl myristate, 12.9 min; cholesteryl stearate, 13.8 min; cholesteryl oleate, 13.9 min; cholesteryl dihydrocinnamate, 13.9 min; sitostanyl oleate, 14.3 min; lanosteryl oleate, 14.2 min; dihydrolanosteryl oleate, 14.1 min; ethylcholesteryl carbonate, 10.0 min; 1,8-octanedicarboxylic acid dimethyl ester (suberic acid dimethyl ester), 0.9 min; 1,8-octanedicarboxylic acid methyl cholesteryl diester (suberic acid methyl cholesteryl diester), 12.0 min. Percentage conversions were determined, unless stated otherwise, as the amount of product formed from the corrected peak areas of the chromatograms of the mixtures of steryl/stanyl esters and unreacted sterol/stanol.

Cholesterol and 5α-cholestan-3β-ol are not separated under the GC conditions described above. Therefore, products (∼5−10 mg) of Lipozyme IM-catalyzed transesterifications of methyl oleate with a mixture of cholesterol and 5α-cholestan-3β-ol, reacted under competitive conditions (i.e., both sterols present in the reaction mixture), were silylated for GC using 100 μL of MSHFBA reagent in the presence of 5 μL of 1-methylimidazole at 110 °C for 10 min. After cooling, the reagents were removed in a stream of nitrogen and the residual mixture was dissolved in dichloromethane for GC injection. Separations were carried out on a 0.25 μm SE-54 CB (CS-Chromatographie-Service, Langerwehe, Germany) fused silica capillary column, 25 m × 0.25 mm i.d., coupled with a 0.52 μm HP-1 (Hewlett-Packard) fused silica capillary column, 25 m × 0.32 mm i.d., using nitrogen as the carrier gas (column pressure = 80 kPa). Temperature was initially kept at 240 °C for 2 min, followed by linear programming from 240 to 300 °C at 5 °C·min^{-1}; the final temperature of 300 °C was held for 25 min. The retention times of the two silylated sterols were as follows: cholesterol derivative, 33.6 min; 5α-cholestan-3β-ol derivative, 34.2 min. The amounts of unreacted cholesterol and 5α-cholestanol-3β-ol were determined by GC to calculate the conversion.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). The products of lipase-catalyzed transesterification of ethylcholesteryl carbonate with oleyl alcohol contained the heat sensitive oleylcholesteryl carbonate (Figure 1); they were analyzed by RP-HPLC as follows. The HPLC system consisted of a Merck-Hitachi pump L-6200 (E. Merck) equipped with a Kontron (Kontron Instruments, Milan, Italy) UV−vis HPLC 332 detector (set to a wavelength of 210 nm) and an ACS (Applied Chromatography Systems, Macclesfield, U.K.) mass detector model 750/14 (thermostated to 55 °C), which were used in series. UV and mass traces were monitored and evaluated in a KromaSystem 2000 data acquisition unit (Kontron Instruments).

Diethyl carbonate was removed from aliquots of the reaction mixtures by evaporation in a stream of nitrogen at 45 °C. The remaining products were resuspended in 1 mL of acetone, and lipase catalyst was separated by centrifugation followed by filtration through a syringe filter (pore size = 0.35 μm). The resulting solution containing ethylcholesteryl carbonate, oleyl alcohol, and oleylcholesteryl carbonate was concentrated and analyzed using a 250 × 4 mm Hibar RP-18 colum, packed with 10 μm LiChrospher 100 (Merck), using acetonitrile/acetone (25:75, v/v) as eluent at a flow rate of 1 mL/min. Injections (∼1 mg of reaction mixture) were carried out with a Rheodyne 7161 sample injector (Cotati, CA) equipped with a 20 μL sample loop. Synthetic and commercial standards were used for comparison. The retention times of the various compounds were as follows: oleyl alcohol, 3.8 min; ethylcholesteryl carbonate, 7.3 min; oleylcholesteryl carbonate, 22.1 min.

Similarly, the products of lipase-catalyzed transesterification of cholesterol with 1,8-octanedicarboxylic acid dimethyl ester were analyzed by RP-HPLC (due to difficulties in the determination of high molecular weight 1,8-octanedicarboxylic acid dicholesteryl ester by high-temperature GC) using programmed elution starting with acetonitrile/acetone (25:75, v/v) for 15 min, followed by linear increase of the acetone concentration to a ratio of acetonitrile/acetone of 1:9 (v/v) within 5 min, followed by an isocratic period of 30 min. The retention times of the various compounds (Figure 1) were as follows: 1,8-octanedicarboxylic acid dimethyl ester, 2.1 min; cholesterol, 13.1 min; 1,8-octanedicarboxylic acid methylcholesteryl diester, 7.1 min; 1,8-octanedicarboxylic acid dicholesteryl ester, 36.1 min.

Purification of Steryl and Stanyl Esters by Column Chromatography. The reaction mixtures resulting from lipase-catalyzed esterification and transesterification of oleic acid and methyl oleate, respectively, with sitostanol were purified by column chromatography on silica gel using a 20 × 1.5 cm glass column and mixtures of isohexane/diethyl ether as eluents as described recently (*17*).

Similarly, the reaction mixtures resulting from Lipozyme IM-catalyzed esterification of 1,8-octanedicarboxylic acid dimethyl ester with cholesterol were purified by column chromatography as described above; 1,8-octanedicarboxylic acid dicholesteryl ester and 1,8-octanedicarboxylic acid methylcholesteryl diester eluted successively with 20 mL of isohexane/diethyl ether (4:1, v/v) followed by 20 mL of isohexane/diethyl ether (1:1, v/v). The two reaction products, that is, 1,8-octanedicarboxylic acid dicholesteryl ester and 1,8-octanedicarboxylic acid methylcholesteryl diester, present in the combined fractions were separated by silica gel column chromatography using three 10 mL fractions of isohexane/diethyl ether (4:1, v/v).

Oleyl cholesteryl carbonate and methyl cholesteryl carbonate were separated from the reaction mixture by column chromatography as described above using isohexane/diethyl ether (9:1, v/v) followed by isohexane/diethyl ether (4:1, v/v) as eluents.

Melting Points. Melting points (mp) of steryl esters determined with a Thermovar heating block (Reichert, Vienna, Austria) were as follows: sitostanyl oleate, 44−45 °C; 5α-cholestan-3β-yl oleate, 30−31 °C; cholesteryl myristate, 85 °C (86 °C) (*18*); cholesteryl stearate, 83−84 °C (82.5 °C) (*18*); cholesteryl oleate, 48−50 °C (51 °C) (*19*); cholesteryl dihydrocinnamate, 111−113 °C (Sigma-Aldrich Fine Chemicals catalog 2000/2001, Taufkirchen, Germany, 109.5−110.5 °C); 1,8-octanedicarboxylic acid methylcholesteryl diester, 83−84 °C; 1,8-octanedicarboxylic acid dicholesteryl ester, 188−191 °C

(179.5 °C) (*18*); oleyl cholesteryl carbonate 32−33 °C (mp of authentic sample = 34−35 °C).

RESULTS AND DISCUSSION

Esterification and interesterification reactions, catalyzed by lipases, have been widely used for bioorganic synthesis and biotransformation of fats and other lipids; however, little is known so far on the application of such reactions in the preparation of short- and long-chain cyclic or polyfunctional acyl esters of sterols, stanols, and steroids (*8*, *20*). We show here that esterification and, particularly, transesterification reactions, catalyzed by lipases from *R. miehei* (Lipozyme IM), *Cd. antarctica* (lipase B, Novozym 435), and papaya (*Cr. papaya*) latex in vacuo, provide fatty acyl esters of sterols and stanols in high to near-quantitative conversions. Neither an organic solvent nor water is added to the reaction mixtures, and no drying agent such as molecular sieve is used (with the exception of cholesteryl carbonate formation).

Figure 2 shows the time course of formation of sitostanyl oleate via esterification of oleic acid (Figure 2A) or transesterification of methyl oleate (Figure 2B) with sitostanol (molar ratio stanol/acyl donor = 1:3) catalyzed by various amounts (6.25, 12.5, 25 and 50 mg) of different lipases, that is, papaya lipase, Novozym 435, and Lipozyme IM, in vacuo at 40, 60, and 80 °C. It is evident from the results shown in Figure 2A that highest conversion by esterification (∼89%, cf. Table 1) is achieved at 80 °C in vacuo using Novozym 435 as catalyst, whereas conversions at 40 and 60 °C are markedly decreased. Slightly reduced conversions by esterification are observed in the presence of Lipozyme IM, whereas the conversions in the presence of papaya lipase are generally lower. It is also obvious from Figure 2A that the extent of conversion increases with increasing proportion of lipases and increasing temperature.

The results given in Figure 2B demonstrate that higher conversions are achieved by transesterification in vacuo using Lipozyme IM, Novozym 435, and papaya lipase than by the corresponding esterification reactions (Figure 2A). Maximum conversions (∼93−96% after 24 h) are observed using methyl oleate or triolein as acyl donors in the presence of Lipozyme IM (cf. Table 1). The data given in Figure 2B show that also in transesterification the extent of reaction strongly increases with increasing proportions of lipases and increasing temperature.

Figure 3 demonstrates the effects of various acyl donors and temperatures on the formation of sitostanyl oleate by esterification of sitostanol with oleic acid and by transesterification of sitostanol with methyl oleate or triolein, catalyzed by Lipozyme IM in vacuo at 40, 60, and 80 °C for various periods. Obviously, under these reaction conditions transesterification with methyl oleate gives the highest conversion rates at all three temperatures studied, followed by transesterification with triolein, whereas esterification reaction with oleic acid yields distinctly lower proportions of sitostanyl oleate.

The time course of the formation of sitostanyl oleate by transesterification of sitostanol with methyl oleate at various molar ratios, catalyzed by Lipozyme IM in vacuo at 80 °C, shows that increasing the molar ratio of methyl oleate from 1:1 to 1:3 and finally 1:5 does not markedly increase the extent of transesterification (Figure 4). Under similar conditions, esterification of sitostanol with oleic acid catalyzed by *Candida rugosa* lipase showed a steep increase in the extent of esterification with increasing molar ratio of oleic acid (*17*).

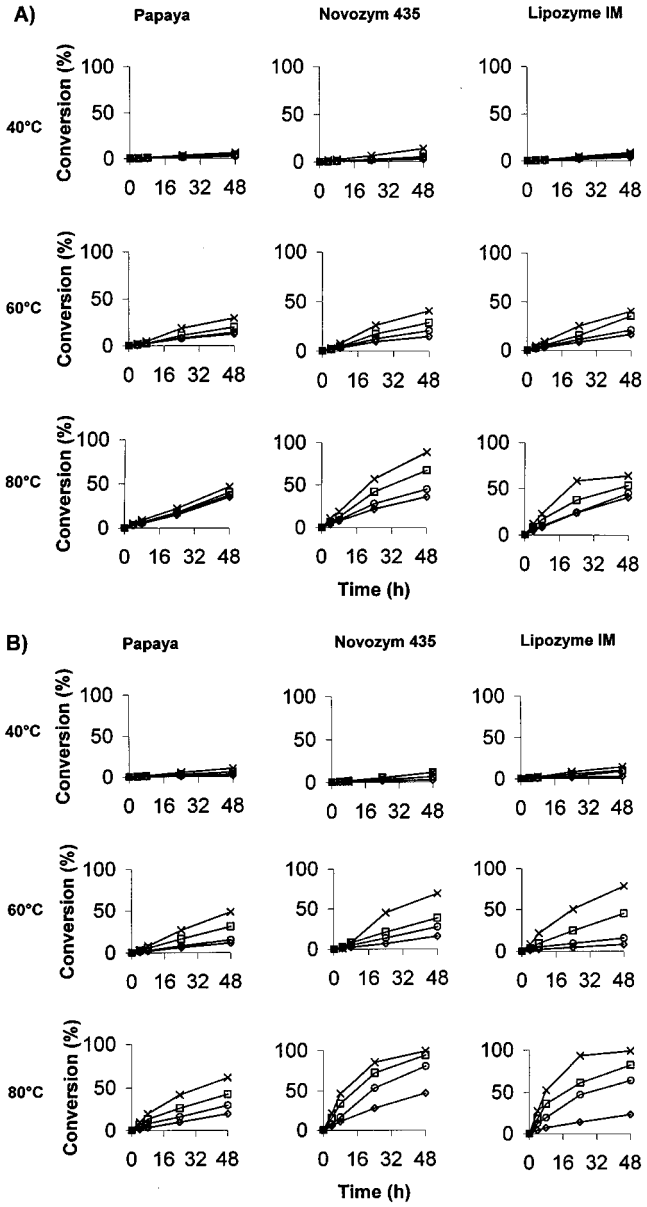

Figure 2. Time course of the formation of sitostanyl oleate via (A) esterification of 300 μmol of oleic acid or (B) transesterification of 300 μmol of methyl oleate with 100 μmol, each, of sitostanol catalyzed by various amounts (◇, 6.25 mg; ○, 12.5 mg; □, 25 mg; ×, 50 mg) of different lipases [left, papaya (*Cr. papaya*) latex; center, *Cd. antarctica* lipase B (Novozym 435); right, *R. miehei* lipase (Lipozyme IM)] in vacuo at 40, 60, and 80 °C for various periods.

The time course of the formation of cholesteryl myristate, cholesteryl stearate, and cholesteryl oleate by Lipozyme IM-catalyzed transesterification of methyl myristate, methyl stearate, and methyl oleate with cholesterol (molar ratio 3:1, each) in vacuo at 80 °C for various periods is shown in Figure 5. It is evident that transesterification with methyl oleate is slightly preferred as compared to the corresponding reactions with saturated long-chain fatty acid esters such as methyl myristate and methyl stearate. It is noteworthy that the transesterification of methyl myristate and methyl stearate with sitostanol, catalyzed by Lipozyme IM at 80 °C, leads by far to higher conversions than the esterification reaction of saturated fatty acids with

Table 1. Enzyme Activities of Various Lipases during Esterification and Transesterification of Sterols and Stanols in Vacuo at Different Temperatures[a]

sterol or stanol	enzyme	fatty acid or fatty acid ester	temp (°C)	time (h)	maximum conversion[b] (%)	enzyme activity[c] (units·g^{-1})
sitostanol	papaya[d]	oleic acid	40	48	6.1	0.08
sitostanol	papaya[d]	methyl oleate	40	48	10.1	0.06
sitostanol	Novozym 435	oleic acid	40	48	13.6	0.17
sitostanol	Novozym 435	methyl oleate	40	48	11.2	0.08
sitostanol	Lipozyme IM	oleic acid	40	48	8.6	0.08
sitostanol	Lipozyme IM	methyl oleate	40	48	14.0	0.08[g]
sitostanol	Lipozyme IM	triolein	40	48	13.9	0.08
sitostanol	papaya[d]	oleic acid	60	48	29.3	0.25
sitostanol	papaya[d]	methyl oleate	60	48	48.0	0.33
sitostanol	Novozym 435	oleic acid	60	48	40.2	0.25
sitostanol	Novozym 435	methyl oleate	60	48	69.0	0.63[f]
sitostanol	Lipozyme IM	oleic acid	60	48	40.0	0.4
sitostanol	Lipozyme IM	methyl oleate	60	48	78.3	1.0
sitostanol	Lipozyme IM	triolein	60	48	64.8	0.5
sitostanol	papaya[d]	oleic acid	80	48	46.6	0.42
sitostanol	papaya[d]	methyl oleate	80	48	61.6	0.83
sitostanol	Novozym 435	oleic acid	80	48	88.7	0.92
sitostanol	Novozym 435	methyl oleate	80	48	99.2	1.8
sitostanol	Lipozyme IM	oleic acid	80	48	63.8	1.3
sitostanol	Lipozyme IM	methyl oleate	80	24	93.2	3.2
sitostanol	Lipozyme IM	triolein	80	48	95.7	2.3
5α-cholestan-3β-ol	Lipozyme IM	methyl oleate	80	24	94.9	3.1
cholesterol	Lipozyme IM	methyl myristate	80	48	63.6	1.1[g]
cholesterol	Lipozyme IM	methyl stearate	80	48	74.9	0.96[g]
cholesterol	Lipozyme IM	methyl oleate	80	48	83.4	0.96[g]
cholesterol	Lipozyme IM	ethyl dihydrocinnamate	80	96	56.0	0.19[f]
lanosterol[e]	Lipozyme IM	methyl oleate	80	96	14.0	0.02[f]

[a] Experimental conditions: 100 μmol of sterol; molar ratio of sterol/fatty acid and sterol/fatty acid methyl ester, 1:3; molar ratio of sterol/trioleoylglycerol 2:3; amount of various lipases, 50 mg; 20−40 mbar. [b] Determined by GC. [c] Enzyme units were calculated as 1 μmol of steryl ester formed·min^{-1}·g^{-1} lipase from the initial rates (4 h) of esterification or transesterification as described under Experimental Procedures. [d] A fine powder obtained from crude granular papaya latex preparation by grinding in a mortar was used (cf. Experimental Procedures). [e] Commercial lanosterol preparation contained around 70% lanosterol and 30% dihydrolanosterol. The conversions were calculated as total amount of steryl esters formed. [f] Twenty-four-hour value was used for calculation of enzyme activity. [g] Eight-hour value was used for calculation of enzyme activity.

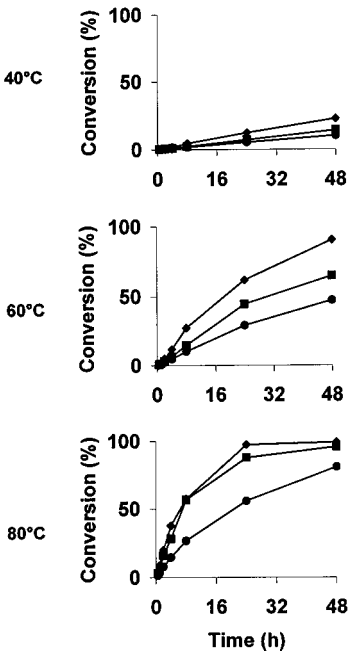

Figure 3. Effects of acyl donor and temperature on the time course of formation of sitostanyl oleate by esterification of 100 μmol of sitostanol with 300 μmol of oleic acid (●) and by transesterification of 100 μmol of sitostanol with 300 μmol of methyl oleate (◆) or 150 μmol of triolein (■) catalyzed by 50 mg of *R. miehei* lipase (Lipozyme IM) in vacuo at 40, 60, and 80 °C for various periods.

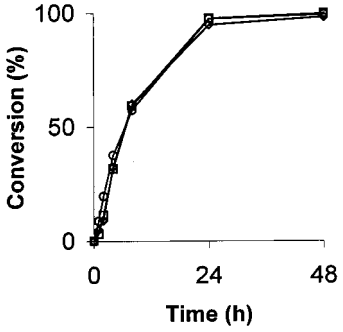

Figure 4. Time course of the formation of sitostanyl oleate by transesterification of sitostanol with methyl oleate at various molar ratios (◇, 1:1; ○, 1:3; □, 1:5), catalyzed by *R. miehei* lipase (Lipozyme IM, 50 mg/100 μmol of sitostanol) in vacuo at 80 °C for various periods.

cholesterol using *Cd. rugosa* lipase at 40 °C (*17*), which we attribute to the high melting points of these fatty acids and relatively low temperature used in the latter reaction.

Figure 6 summarizes the data on the formation of steryl oleates by Lipozyme IM-catalyzed transesterification of methyl oleate with sets of equimolar mixtures of (A) 5α-cholestan-3β-ol and cholesterol, (B) sitostanol and cholesterol, and (C) 5α-cholestan-3β-ol and sitostanol (molar ratio sterols/methyl oleate = 1:3). It is obvious from these experiments under competitive conditions that saturated stanols such as 5α-cholestan-3β-ol and sitostanol are the preferred substrates as compared to the Δ^5-unsaturated cholesterol in transesterification reaction with methyl oleate using Lipozyme IM as catalyst. In contrast, the enzyme activity of *Cd. rugosa* lipase was found to be higher in the esterification reaction of Δ^5-unsaturated cholesterol with oleic acid as

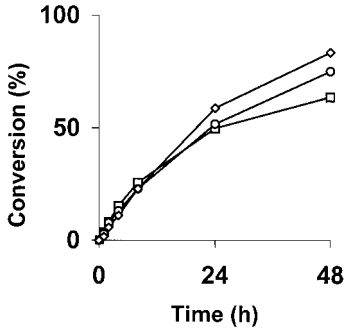

Figure 5. Time course of the formation of cholesteryl myristate, cholesteryl stearate, and cholesteryl oleate via transesterification of methyl myristate (□), methyl stearate (○), and methyl oleate (◇) with cholesterol (molar ratio 3:1, each), catalyzed by *R. miehei* lipase (Lipozyme IM, 50 mg/100 μmol cholesterol) in vacuo at 80 °C for various periods.

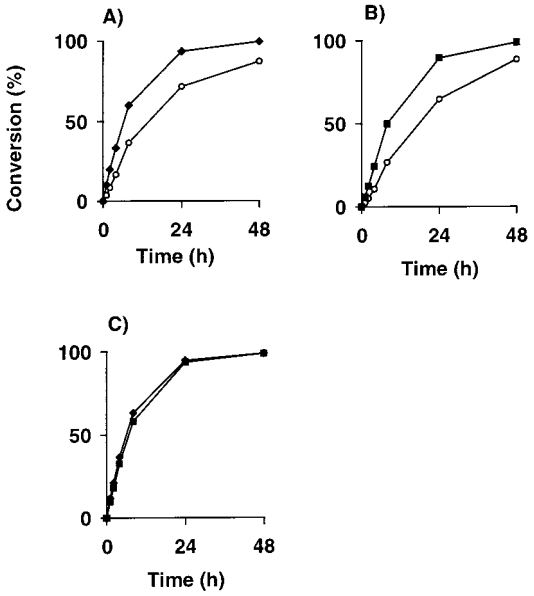

Figure 6. Time course of the formation of (A) 5α-cholestan-3β-yl oleate (◆) and cholesteryl oleate (○), (B) sitostanyl oleate (■) and cholesteryl oleate (○), and (C) 5α-cholestan-3β-yl oleate (◆) and sitostanyl oleate (■) via transesterification of 300 μmol of methyl oleate with 100 μmol, each, of equimolar mixtures of (A) 5α-cholestan-3β-ol and cholesterol, (B) sitostanol and cholesterol, and (C) 5α-cholestan-3β-ol and sitostanol, catalyzed by 50 mg of *R. miehei* lipase (Lipozyme IM) in vacuo at 80 °C under competitive conditions.

compared to the esterification of saturated sitostanol with the same fatty acid (*17*).

Figure 7 shows the time course of the formation of cholesteryl dihydrocinnamate (Figure 1) by Lipozyme IM-catalyzed transesterification of ethyl dihydrocinnamate with cholesterol (molar ratio 15:1) in vacuo at 80 °C for various periods. These results show that cholesteryl dihydrocinnamate is formed in moderate yield (56%, cf. Table 1) only after a prolonged time (96 h) using a large excess of ethyl dihydrocinnamate; at lower ratios of ethyl dihydrocinnamate to cholesterol the conversions were substantially lower (data not shown). Cholesteryl dihydrocinnamate may be used in liquid crystal devices or as an oil-soluble UV-B filter in suncare and cosmetic formulations.

The time course of the transesterification of methyl oleate with lanosterol, catalyzed by Lipozyme IM in vacuo at 80 °C for various periods (Figure 7), demonstrates that lanosteryl oleate is formed in low yield

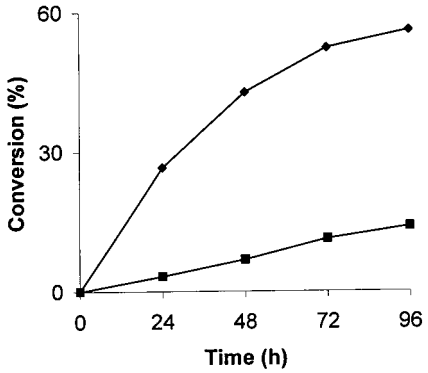

Figure 7. Time course of the formation of cholesteryl dihydrocinnamate (◆) and lanosteryl oleate (■) via transesterification of ethyl dihydrocinnamate with cholesterol (molar ratio ~10:1) and of methyl oleate with lanosterol (molar ratio 6:1), respectively, catalyzed by 50 mg of *R. miehei* lipase (Lipozyme IM) in vacuo at 80 °C for various periods.

(14%, cf. Table 1) only after prolonged time (96 h) using a large excess (molar ratio 6:1) of methyl oleate; at lower ratios of methyl oleate to lanosterol the conversions were substantially lower (data not shown). The results given in Figure 7 demonstrate that the extent of transesterification of cholesterol with ethyl dihydrocinnamate and of lanosterol with methyl oleate using Lipozyme IM as biocatalyst under the above conditions is rather low, which we attribute to the bulky structure of the dihydrocinnamoyl moiety and sterical hindrance by the 4,4-dimethyl substituents of lanosterol.

Cholesterol was also transesterified with 1,8-octanedicarboxylic acid dimethyl ester using Lipozyme IM as catalyst in vacuo (molar ratio of 1:4) for a prolonged time (72 h) to give 1,8-octanedicarboxylic acid methylcholesteryl diester in moderate yield (75%) and 1,8-octanedicarboxylic acid dicholesteryl ester in poor yield (5%; data not shown). Moreover, ethylcholesteryl carbonate and oleylcholesteryl carbonate (Figure 1) were formed in low yield (~20%, each) by transesterification of cholesterol with diethyl carbonate (molar ratio around 1:40; molecular sieve, 4 Å; 400 mbar; 60 °C; 48 h) and of ethylcholesteryl carbonate with oleyl alcohol (molar ratio of 1:3; 20 mbar; 80 °C; 48 h) using Lipozyme IM as catalyst (data not shown). These cholesterol derivatives having liquid crystalline properties may be used in liquid crystal devices.

Figure 8 shows the effect of repeated use of the lipase catalyst on the extent of transesterification of sitostanol with methyl oleate, catalyzed by papaya lipase, Novozym 435, and Lipozyme in vacuo. It is evident that both papaya lipase and Novozyme 435 lost some activity upon repeated use; however, between 20 and 40% of their activity was retained even after 10 repeated uses. Lipozyme IM retained most of its activity even after 10 repeated transesterification reactions.

Table 1 summarizes the data on maximum conversion and enzyme activity in esterification and transesterification reactions of sitostanol, 5α-cholestan-3β-ol, and cholesterol with different acyl donors, catalyzed by papaya lipase, Novozym 435, and Lipozyme IM in vacuo at various temperatures. From these results it is obvious that sitostanol was converted in high to near-quantitative yields to the corresponding long-chain acyl esters via esterification with oleic acid or transesterification with methyl oleate or trioleoylglycerol using Lipozyme IM and Novozym 435 as biocatalysts in vacuo (20−40 mbar) at 80 °C, whereas the conversion was markedly

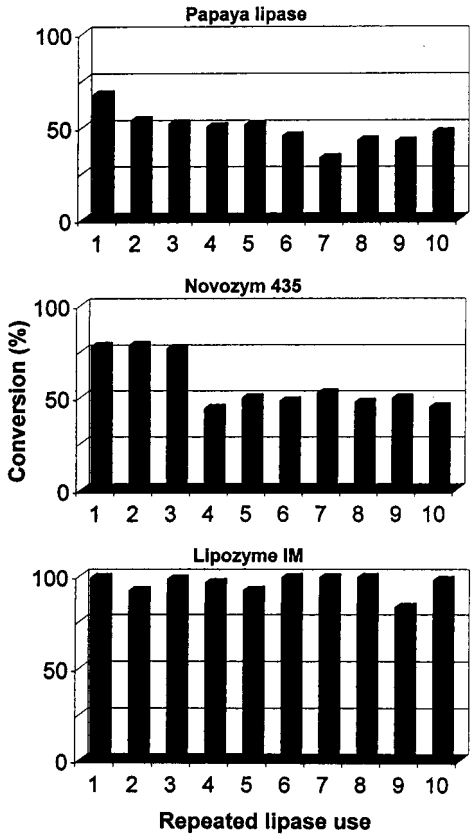

Figure 8. Percent conversion of sitostanol to sitostanyl oleate in the transesterification of sitostanol (100 μmol) with methyl oleate (300 μmol), catalyzed by papaya (*Cr. papaya*) latex lipase (50 mg), *Cd. antarctica* lipase B (Novozym 435, 50 mg), or *R. miehei* lipase (Lipozyme IM, 50 mg) after repeated use of the lipase catalyst as described under Experimental Procedures. The reactions were carried out in vacuo at 80 °C for 24 h.

lower at 60 °C and was very low at 40 °C. Conversions observed with papaya lipase were generally lower at all temperatures than those with Novozym 435 and Lipozyme IM. Highest conversion rates were observed in transesterification reactions of sterols and stanols with methyl oleate or triolein at 80 °C using Lipozyme IM as catalyst. When Lipozyme IM was used as catalyst, transesterification of cholesterol with ethyl dihydrocinnamate gave a moderate yield of cholesteryl dihydrocinnamate after prolonged reaction time using a large excess of ethyl dihydrocinnamate, whereas transesterification of lanosterol with methyl oleate resulted in very low yield of lanosteryl oleate.

It is interesting to note that enzyme activity of *Cd. rugosa* lipase in the esterification reaction of sitostanol with oleic acid yielding sitostanyl oleate is by far higher ($\sim$25 units$\cdot g^{-1}$) (*17*) than the enzyme activity of Lipozyme IM or Novozym 435 in the transesterification reaction of sitostanol with methyl oleate ($\sim$2−3 units$\cdot g^{-1}$) (Table 1); similar differences were obtained in the reaction of cholesterol with oleic acid and methyl oleate, respectively ($\sim$32 vs $\sim$1 unit$\cdot g^{-1}$; Table 1) (*17*).

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Received for review June 5, 2001. Revised manuscript received September 11, 2001. Accepted September 11, 2001.

JF0107407