Enzymatic Synthesis of 1,3-Dicapryloyl-2-eicosapentaenoylglycerol

Roxana Irimescu[a,b], Mamoru Yasui[a], Yugo Iwasaki[a], Nobuyoshi Shimidzu[b], and Tsuneo Yamane[a,*]

[a]Laboratory of Molecular Biotechnology, Graduate School of Bio- and Agro-Sciences, Nagoya University, Nagoya 464-8601, Japan, [b]Central Research Laboratory, Nippon Suisan Kaisha, Ltd., 559-6 Kitano-machi, Hachioji-shi, Tokyo 192-0906, Japan

ABSTRACT: 1,3-Dicapryloyl-2-eicosapentaenoylglycerol (CEC) was synthesized by interesterification of trieicosapentaenoylglycerol (EEE) with ethyl caprylate (EtC) catalyzed by Lipozyme™. After some of the reaction conditions were optimized, the maximal molar content of CEC in the glycerides of the reaction mixture was 91%. Among the parameters studied in the optimization, the critical ones were: (i) the water content, which influenced the conversion of EEE to CEC and 1-caprylyoyl-2-eicosapentaenoylglycerol (CEOH), and (ii) the timing of water removal under reduced pressure for the reesterification of CEOH to form CEC. The complete synthesis of CEC from ethyl eicosapentaenoate (EtE) was performed in three steps: (i) hydrolysis of EtE to free eicosapentaenoic acid (EPA), (ii) esterification of glycerol with EPA to form EEE, and (iii) interesterification of EEE with EtC under the optimized conditions. The first two steps were catalyzed by Novozym™ and the third by Lipozyme™. The total yield over all the steps was 88%, and no purification of the intermediates was necessary. The regioisomeric purity of the product was 100% by silver-ion high-pressure liquid chromatography.

Paper no. J9400 in *JAOCS* 77, 501–506 (May 2000).

KEY WORDS: 1,3-Dicapryloyl-2-eicosapentaenoylglycerol, esterification, ethyl caprylate, ethyl eicosapentaenoate, interesterification, Lipozyme™, Novozym™, trieicosapentaenoylglycerol.

Triglycerides containing a polyunsaturated fatty acid (PUFA), such as eicosapentaenoic or docosahexaenoic acid (EPA or DHA, respectively), at the second position of the glycerol backbone and two medium-chain acids (MCA) at the outer positions are a group of structured glycerides with proven therapeutic and nutritional properties. These lipids provide the essential fatty acids and retain the assimilation advantages of the medium-chain triglycerides (1,2).

The purpose of this paper is the establishment of a feasible method for the synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol (CEC) for pharmaceutical use. Chemically pure starting materials and intermediates as well as high regioisomeric purity of the product are basic requirements for the medical use of the product.

There are a few possible strategies for the synthesis of such triglycerides. Classical chemical esterification methods start either from the 1,3-diglyceride of MCA or the 2-monoglyceride of PUFA. These compounds are obtained chemically by multistep methods that use toxic reactants and catalysts. Tedious purification after each step employs large amounts of organic solvents. These partial glycerides are quite unstable and undergo acyl migration even at ambient temperature.

A straightforward method that combines an enzymatic with a chemical step was established in our laboratory (3). The symmetrical 1,3-diglyceride of caprylic acid (CA) was obtained enzymatically by direct esterification of glycerol with CA in a solvent-free system followed by chemical condensation with EPA. The product was a mixture of approximately 90% CEC and 10% 1,2-dicapryloyl-3-eicosapentaenoylglycerol, and the total yield was less than 40%.

A completely enzymatic process in two steps was recently reported (4,5). The 2-monoglyceride is obtained by ethanolysis of a triacylglyceride in an appropriate organic solvent. In the second step, after purification by crystallization, the 2-monoglyceride is reesterified with a fatty acid. However, the method cannot be applied for triglycerides containing EPA or DHA for two main reasons: (i) the low yields of the first step due to the low activity of the lipases on such PUFA, and the very difficult purification of their 2-monoglycerides, which have a very low melting point.

To our knowledge, a practical method for CEC production has not yet been reported, although several studies have concentrated on structured lipid syntheses by enzymatic acidolysis or interesterification (6–8).

In our approach to CEC synthesis, trieicosapentaenoylglycerol (EEE) was interesterified with ethyl caprylate (EtC) in a solvent-free system using 1,3-specific *Rhizomucor miehei* lipase as the catalyst. After the conditions for the interesterification reaction were optimized, the complete enzymatic synthesis of CEC in three steps starting from ethyl eicosapentaenoate (EtE) was performed.

EXPERIMENTAL PROCEDURES

Materials. Immobilized *Candida antarctica* lipase (Novozym 435) and *R. miehei* lipase (Lipozyme IM) were generous gifts from Novo Nordisk Bioindustry (Chiba, Japan). EtC (min. 99%) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). EtE (min. 99%) was donated by Nippon Suisan Kaisha, Ltd. (Tokyo, Japan). EEE (min. 99%) was ob-

*To whom correspondence should be addressed.
E-mail: yamanetu@agr.nagoya-u.ac.jp

tained by esterification of glycerol with the stoichiometric amount of EPA at 40°C and 3 mm Hg, followed by purification on a silica gel column.

Interesterification. EEE (0.284 g, 0.3 mmol) and EtC at a specified molar ratio were mixed with 10% Lipozyme in a flask under nitrogen atmosphere at 40°C and 300 rpm agitation speed. Different reaction conditions were employed in some cases and they are specified in the text where the experiments are discussed.

Reesterification at low pressure. The partial glycerides generated during CEC synthesis with water addition were reesterified by water removal at 3 mm Hg vacuum. At a predetermined time, the reaction vessel was connected to a vacuum pump through a reflux condenser. The condenser through which water at 5°C was circulated ensured the selective removal of water.

Complete CEC synthesis from EtE. EtE (0.992 g, 3 mmol), H_2O (0.540 g, 30 mmol), and Novozym (0.111 g, 10 wt% of the total reaction mixture) were mixed by a magnetic stirrer at 300 rpm and 40°C for 1 h at normal pressure and for 2 h under vacuum until the formed ethanol and water evaporated completely. The above procedure was repeated twice more after additions of 0.540 g H_2O, but the reaction time at normal pressure was shortened to 30 min. The degree of hydrolysis was 97.6%.

Glycerol (0.083 g, 0.9 mmol) and H_2O (0.021 g, 2 wt% of the total reaction mixture) were added directly to the flask with the final reaction mixture of hydrolysis. Esterification was performed for 24 h at 300 rpm agitation speed, 3 mm Hg, and 40°C. The yield of EEE was 92.6%. The enzyme (Novozym) was removed by filtration. The product composition by gas chromatography (GC) (wt %) was 89.9% EEE, 5.0% 1,2- and 1,3-diacylglycerols, and 5.1 % EPA + EtE.

A portion (0.292 g) of the resultant filtrate (containing 0.3 mmol EPA-glycerides) was used for CEC synthesis by interesterification with EtC (5.168 g, 30 mmol) and with Lipozyme (0.608 g, 10 wt% of the total reaction mixture). Water (0.094 g) was added to bring the water content of the reaction mixture up to 2%. The initial water contents of the reactants' mixture and Lipozyme were 0.19 and 3.15%, respectively. The reaction was performed at 40°C and 300 rpm agitation speed under nitrogen atmosphere at normal pressure for 10 h and then at 3 mm Hg for 3 h for the reesterification of the resulting partial glycerides.

Analyses. The analyses of the reaction mixtures of EEE and CEC syntheses were performed by high-temperature GC (9). A chromatograph (GC-14; Shimadzu Corporation, Kyoto, Japan) equipped with an on-column injector (OCI-14; Shimadzu Corporation) and an SGE-HT5 aluminum-clad fused-silica capillary column (6 m length, 0.53 mm internal diameter, and 0.1 μm film thickness; Supelco, Sigma-Aldrich Pty., New South Wales, Australia) was used. The temperature program of the column was heating at 50°C for 0.5 min, 30°C/min to 110°C, 10°C/min to 140°C, 20°C/min to 250°C, 10°C /min to 330°C, and, finally, 5°C/min to 360°C. The injector was heated from 50 to 370°C at 40°C/min and held at this temperature for 18 min.

The regioisomeric composition of the final product was analyzed by high-pressure liquid chromatography (HPLC) with a ChromSpher 5 Lipids silver ion chromatography column (250 × 4.6 mm × 1/4″; Chrompack, Middleburg, The Netherlands) (9). A binary solvent gradient made of solvent A (*n*-hexane/2-propanol/acetonitrile at 350:100:2 by vol), and solvent B (*n*-hexane/2-propanol/acetonitrile at 350:100:10 by vol) was used. The column was eluted, at a flow rate of 0.65 mL/min, isocratically with solvent A for 3 min, with a linear gradient of A to B for 22 min, and then held at B for 8 min. The lipid species were detected spectrophotometrically at 206 nm.

Water contents of the substrates and enzymes were determined with a Karl Fischer moisture meter (MKS-1; Kyoto Electronics, Kyoto, Japan).

RESULTS AND DISCUSSION

The reaction route used for CEC synthesis in this study contains three steps. After the enzymatic hydrolysis of EtE under controlled pressure (step 1), the resulting free EPA was used for esterification of glycerol with removal of water under vacuum (step 2). Immobilized *C. antarctica* lipase (Novozym), a nonspecific enzyme, was used in both reactions. The esterification product (EEE) was reacted with EtC using the 1,3-specific Lipozyme (immobilized *R. miehei* lipase) as the catalyst (step 3). The focus of this paper is on interesterification of EEE with EtC, which is the most difficult step of CEC synthesis. The complete CEC synthesis starting from EtE is described later.

Interesterification of EEE with EtC and acidolysis of EEE with caprylic acid (CA) catalyzed by *R. miehei* lipase were considered for CEC synthesis. The reaction rate and the final content of the CEC were much lower for acidolysis than for interesterification. For interesterification (EtC/EEE = 10:1 molar ratio), the molar content of CEC in the glyceride fraction at 24 h was approximately 64.8% (Table 1). The content of CEC was only 20.9% for acidolysis at the same molar ratio (CA/EEE = 10:1) and the same time. Another advantage of using EtC is the formation of EtE instead of EPA as the main by-product. Because the boiling point of an ethyl ester is always lower than that of its corresponding free acid, EtE is easier to remove from the final product by fractional distillation and to recycle in EEE synthesis.

The characteristics of the lipase employed are essential for high-yield CEC synthesis. A lipase suitable for this reaction should: (i) have very strict 1,3-specificity, (ii) be able to work well on PUFA glycerides, and (iii) maintain its activity at low water contents in the reaction media. Lipozyme satisfies all these requirements.

Optimization of EEE interesterification with EtC. Interesterification is a reversible reaction, and an excess of EtC is necessary to push the equilibrium toward a high content of CEC (Table 1). Molar composition of glycerides was used for the assessment of the system. The glycerides were separated according to their molecular weight by GC so that all the posi-

TABLE 1
Effect of Molar Ratio of EtC to EEE[a]

Molar ratio (EtC/EEE)	Initial reaction rate (mol% E acyl group liberated at 30 min)	Glyceride composition at 24 h (mol%)						
		CCOH	CCC	CEOH	CEC	EEOH	CEE	EEE
2	22.7	1.3	1.5	4.4	27.7	2.6	51.1	11.4
5	18.4	2.9	3.4	7.1	48.7	1.6	31.8	4.5
10	17.3	4.1	3.6	7.9	64.8	0	16.8	2.8
15	12.4	4.0	3.7	7.6	69.6	2.1	13.0	0
25	8.5	2.2	3.4	6.7	74.9	0	12.8	0
50	7.5	2.5	3.9	7.3	76.9	1.5	7.9	0
100	9.5	3.0	2.9	7.1	77.4	2.4	7.2	0

[a]The reaction temperature was 40°C. EtC, ethyl caprylate; EEE, trieicosapentaenoylglycerol; E, eicosapentaenoyl; CCOH, dicapryloylglycerol; CCC, tricapryloylglycerol; CEOH, 1-caprylol-2-eicosapentaenoylglycerol; CEC, 1,3-dicapryloyl-2-eicosapentaenoylglycerol; EEOH, 1,2-dieicosapentaenoylglycerol; CEE, 1-capryloyl-2,3-dieicosapentaenoylglycerol.

tional isomers (if formed) of each glyceride species with a specific molecular weight were included under the same name. The initial reaction rate was given as the mol% eicosapentaenoyl (E), acyl group liberated from glycerides at 30 min and was calculated from the glyceride composition. The maximal molar amount of E acyl groups was considered to be three times the molar amount of EEE. The reaction at the stoichiometric molar ratio had the highest initial rate, but the molar content of CEC at 24 h (close to equilibrium) was less than 28%. Higher molar ratios (EtC/EEE) increased the final content of CEC at the expense of increased reaction rates. Saturation was reached at 100:1 molar ratio with approximately 77% CEC obtained at 24 h.

In the reaction mechanism of the interesterification, 1,2-dieicosapentaenoylglycerol (EEOH) and 1-capryloyl-2-eicosapentaenoylglycerol (CEOH) are key intermediates for the enzymatic acyl exchange in triacylglycerols. Dicapryloylglycerol (CCOH) and tricapryloylglycerol (CCC) are byproducts created by acyl migration of EEOH and CEOH followed by acyl exchange. As shown in Table 1, certain amounts of CCOH and CCC were formed in all experiments. Further investigations to reduce the extent of acyl migration were performed for the improvement of the final yields.

Reaction temperature affects the reaction rates, including the rate of acyl migration. The content of CCOH and CCC in the reaction mixture is a measure of the degree of acyl migration. As expected, higher temperature increased the reaction rates, but also promoted acyl migration (Table 2). Although the reaction rate was higher, the final content of CCOH and CCC, combined, at 50°C increased compared to the content at 40°C, without any improvement of the CEC yield. The initial reaction rate at 30°C was less than half of the rate at 40°C, but CCC content at 24 h was only approximately 2%. As a compromise between a high reaction rate and reduced acyl migration, 40°C was used for the rest of our experiments.

High conversion of EEE and the intermediates, 1-capryloyl-2,3-dieicosapentaenoylglycerol (CEE) and EEOH, and low CCOH and CCC formation are desirable to simplify the downstream purification operations. Table 3 is an example of a reaction time course. The unreacted intermediates, CEE and EEOH, accounted for approximately 12% of the glycerides at 24 h. Further extension of the reaction time to 48 h did not improve the final yield of CEC due to the increase of CCC content. Shorter reaction time to limit CCC formation by acyl migration and high conversion of EEE, CEE, and EEOH was pursued.

The effect of water content of the reaction mixture was investigated with respect to the initial reaction rates and maximal yield of CEC and CEOH (Table 4). CEOH can be esterified later to CEC by removing the water under vacuum (Table 5) and, thus its formation is not a drawback. The initial reaction rate was almost unchanged in the range of 0.42 to 3% water content, but decreased at 5% (Table 4). The maximal yield of CEC and CEOH combined (approximately 92% at 12 h) was obtained with 1 and 2% water in the reaction mixture. The content of CCOH increased from 12 to 24 h and depended on the water content of the reaction mixture. Increased water content favored acyl migration and thus CCOH and CCC formation. The highest amount of CCOH and CCC (approximately 23% combined at 24 h) was formed in the reaction mixture with 3% water.

The partially hydrolyzed product CEOH was reesterified to CEC by removing the water under vacuum. The timing of

TABLE 2
Effect of Temperature[a]

Temperature (°C)	Initial reaction rate (mol% E acyl group liberated at 30 min)	Glyceride composition at 24 h (mol%)						
		CCOH	CCC	CEOH	CEC	EEOH	CEE	EEE
30	5.4	0	1.9	6.9	69.4	2.0	17.8	2.0
40	12.4	2.2	2.8	6.1	70.3	2.3	16.3	0
50	18.8	2.4	6.1	4.3	69.6	1.5	16.1	0

[a]The experiments were performed at EtC/EEE = 15:1 molar ratio. For abbreviations see Table 1.

TABLE 3
Glyceride Composition During the Reaction Time[a]

Time (h)	Glyceride composition at 24 h (mol%)						
	CCOH	CCC	CEOH	CEC	EEOH	CEE	EEE
0.5	0	0	0	5.2	4.2	14.0	76.6
1	0	0	0	4.8	5.3	23.2	66.7
2	0	0	2.5	10.7	3.9	40.2	42.7
3	0	0	5.0	16.7	3.5	45.8	29.0
5	0	0	5.0	30.2	3.7	47.8	13.3
8	0	0	5.8	47.1	3.3	38.0	5.8
12	0	2.3	8.0	70.3	2.2	17.2	0
24	1.7	2.2	5.8	78.6	2.6	9.1	0
48	0	9.4	7.2	78.4	1.7	3.3	0

[a]The experiments were performed at EtC/EEE =100:1 molar ratio. For abbreviations see Table 1.

water removal was essential for reaching the maximal content of CEC (Table 5). Experiments were performed for the reaction mixtures with 2 and 3% water and with the molar ratio 100:1 of EtE/EEE. The maximal yield of CEC was approximately 90% for both, but it was attained at different conditions: 10 h of reaction at normal pressure followed by 3 h of reesterification at 3 mm Hg for the former, and 8 h of normal pressure and 4 h of vacuum for the latter. Figure 1 depicts the time course of glyceride composition of the experiment at 2% water. The regioisomeric purity of the products was more than 99% CEC by silver-ion HPLC.

Complete CEC synthesis from EtE. The complete synthesis of CEC in three steps starting from EtE was performed using the optimized method for the interesterification of EEE with EtC. A simple and cheap method for the intermediate (EEE) synthesis is necessary for the industrial implementa-

TABLE 4
Effect of Water Content[a]

Water content (wt%)	Initial reaction rate (mol% E acyl group liberated at 30 min)	Time (h)	Glyceride composition (mol%)						
			CCOH	CCC	CEOH	CEC	EEOH	CEE	EEE
0.42[b]	9.5	12	0	2.3	8.0	70.3	2.2	17.2	0
		24	3.0	2.9	7.1	77.3	2.4	7.3	0
1	8.4	12	0	0	30.8	62.0	3.5	3.7	0
		24	13.7	5.2	23.1	52.4	2.4	3.2	0
2	9.2	12	0	0	51.4	41.1	2.5	5.0	0
		24	15.3	5.6	36.2	31.8	6.4	4.7	0
3	8.8	12	6.1	0	59.3	30.1	1.5	3.0	0
		24	18.2	4.6	43.2	27.6	3.5	2.9	0
5	4.7	12	4.7	2.2	59.9	27.3	1.6	4.3	0
		24	16.2	3.2	44.6	27.8	3.6	4.6	0

[a]The experiments were performed at EtC/EEE = 100:1 molar ratio. For abbreviations see Table 1.
[b]No extra water was added to the reaction mixture.

TABLE 5
Effect of Timing of Water Removal on the Final Yield of CEC[a]

Water content (wt %)	Time[b] (h)	Glyceride composition (mol%)						
		CCOH	CCC	CEOH	CEC	EEOH	CEE	EEE
2	8+0	4.6	0	46.6	35.6	6.7	6.5	0
	8+3	5.4	3.5	0	84.0	1.6	5.5	0
	10+0	6.8	2.3	46.2	37.4	2.7	4.6	0
	10+3	5.2	2.3	0	89.9	0.6	2.0	0
	12+0	6.1	2.2	34.8	48.7	3.3	4.9	0
	12+2	6.1	3.2	2.1	84.1	1.7	2.8	0
3	8+0	5.9	0	56.4	28.0	4.6	5.1	0
	8+4	4.4	2.3	0	90.7	0	2.6	0
	10+0	7.6	3.9	54.6	27.5	2.7	3.7	0
	10+4	6.4	3.3	0.9	86.6	1.1	1.7	0
	12+0	8.6	0	52.9	30.4	3.5	4.6	0
	12+4	7.6	4.4	1.4	84.1	1.0	1.5	0

[a]The experiments were performed at EtC/EEE =100:1 molar ratio. For abbreviations see Table 1.
[b](Reaction time at normal pressure) + (reaction time at 3 mm Hg), in hours.

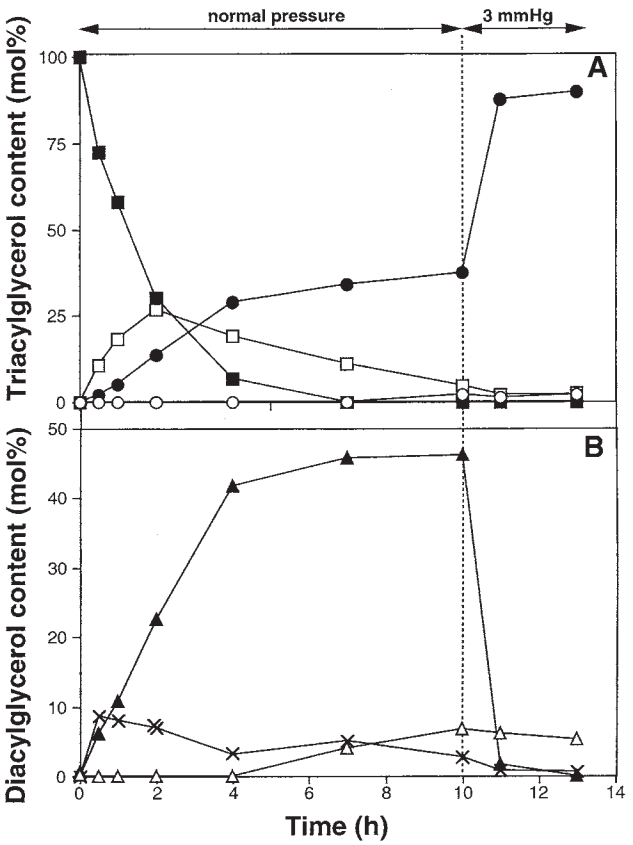

FIG. 1. Time course of glyceride composition during CEC synthesis. The reaction (molar ratio of EtE/EEE = 100:1) was performed with 2% initial water content at 40°C at normal pressure for 10 h, followed by 3 h at 3 mm Hg; (A) triacylglycerol content [CCC (-○-), CEC (-●-), CEE (-□-) and EEE (-■-)]; (B) diacylglycerol content [CCOH (-△-), CEOH (-▲-), and EEOH (-×-)]. Note that no monoacylglycerols were detected. EtE, ethyl eicosapentaenoate; EEE, trieicosapentaenoyl glycerol; CCC, tricapryloylglycerol; CEC, 1,3-dicapryloyl-2-eicosapenta-enoylglycerol; CEE, 1-capryloyl-2,3-dieicosapentaenoylglycerol; CCOH, dicapryloylglycerol; CEOH, 1-capryloyl-2-eicosapentaenoyl-glycerol; EEOH, 1,2-dieicosapentaenoylglycerol.

tion of this method. The enzymatic synthesis of EEE (10,11) is a good option as the reaction conditions are milder and the yield is higher than for the chemical methods. Product separation is done by filtration of the reaction mixture for the removal of the enzyme preparation.

Free EPA is a better acyl donor than EtE for enzymatic esterification (11) and, thus, hydrolysis of EtE is required. Hydrolysis was performed with immobilized *C. antarctica* lipase (Novozym), which was also used for EEE synthesis. The two steps were performed in the same flask without any separation. The removal of the resulting ethanol under reduced pressure was essential for attaining a high hydrolysis yield. As water was also removed, supplementary water addition was necessary. Glycerol was added directly to the flask after the hydrolysis was complete, and the extent of esterification was enhanced by the removal of the resulting water under vacuum. A water content of 2% was employed initially to enhance the enzyme activity. For this reaction, an enzyme that is nonspecific for the glycerol positions is necessary. *Candida antarctica* lipase has good specificity for EPA and has no positional specificity for glycerol. A molar excess of 10% EtE was used to ensure a high yield of EEE. The final content of EEE was high enough (89.9%) to use the reaction mixture after catalyst filtration without purification for CEC synthesis. Although the product contained 5.0% 1,2- and 1,3-diacylglycerols, the former is itself an intermediate in the interesterification step and the latter is transformed during the same reaction into 1,3-dicapryloylglycerol, which does not complicate the product purification. The unreacted EPA and EtE are not worth removing at this stage since they are also formed during the interesterification of EEE to CEC.

The molar glyceride composition of the product of interesterification was: 88.5% CEC, 1.4% EEOH, 3.0% CEE, 3.1% CCC, and 4.0% CCOH. The regiosomeric purity of CEC was 100%, as no positional isomer (1,2-dicapryloyl-3-eicosapentaenoylglycerol) was detected by silver-ion HPLC.

The excess of EtC can be removed easily at 5 mm Hg and 88–90°C and recycled in the interesterification step. The removal of EtE, CCC, and CCOH might be done fractionally by distillation. In a recent study, the product of borage oil acidolysis with CA was purified efficiently by molecular distillation (7). Separated EtE can then be reused for the subsequent synthesis of EEE. If such a purification method is established, the product purity might be more than 95% (molar). The rest of the impurities would be CEE and EEOH, which are difficult to remove by molecular distillation but can be separated by preparative reversed-phase column chromatography (12).

The molar ratio of the reactants (EtC/EEE = 100:1) was used as an experimental model taking advantage of the fact that lower quantities of EEE were necessary for the same experimental volume. After the strategy was established, fewer experiments were needed for scale-up. Therefore, the expensive substrate (EEE) could be used economically. At a 20:1 molar ratio and the same reaction conditions, 78% CEC yield was obtained after 10 h of reaction at normal pressure and 2 h of water removal.

The main aim of this study was to demonstrate the applicability of this synthetic strategy, but further work is necessary for the optimization of EEE synthesis.

ACKNOWLEDGMENTS

The authors are grateful to Novo Nordisk Bioindustry Ltd. for providing us with the enzymes.

REFERENCES

1. Hedeman, H., H. Brøndsted, A. Mullertz, and S. Frokjaer, Fat Emulsions Based on Structured Lipids (1,3-specific triglycerides): An Investigation of the *in Vivo* Fate, *Pharm. Res. 13*: 725–728 (1996).
2. Christensen, M.S., C.E. Høy, C.C. Becker, and T.G. Redgrave, Intestinal Absorption and Lymphatic Transport of Eicosapentaenoic (EPA), Docosahexaenoic (DHA), and Decanoic Acids: Dependence on Intramolecular Triacylglycerol Structure, *Am. J. Clin. Nutr. 61*:56–61 (1995).

3. Rosu, R., M. Yasui, Y. Iwasaki, and T. Yamane, Enzymatic Synthesis of Symmetrical 1,3-Diacylglycerols by Direct Esterification of Glycerol in Solvent-Free System, *J. Am. Oil Chem. Soc. 76*:839–843 (1999).
4. Soumanou, M.M., U.T. Bornscheuer, and R.D. Schmid, Two-Step Enzymatic Reaction for the Synthesis of Pure Structured Glycerides, *Ibid. 75*:703–710 (1998).
5. Schmid, U., U.T. Bornscheuer, M.M. Soumanou, G.P. McNeill, and R.D. Schmid, Optimization of the Reaction Conditions in the Lipase-Catalyzed Synthesis of Structured Triglycerides, *Ibid. 75*:1527–1531 (1998).
6. Shimada, Y., A. Sugihara, K. Maruyama, T. Nagao, S. Nakamura, H. Nakano, and Y. Tominaga, Production of Structured Lipids Containing Docosahexaenoic and Caprylic Acid Using Immobilized *Rhizopus delemar* Lipase, *J. Ferment. Bioeng. 81*: 299–303 (1996).
7. Shimada, Y., M. Suenaga, A. Sugihara, S. Nakai, and Y. Tominaga, Continuous Production of Structured Lipid Containing γ-Linolenic and Caprylic Acids by Immobilized *Rhizopus delemar* Lipase, *J. Am. Oil Chem. Soc. 76*:189–193 (1999).
8. Xu, X., S. Balchen, C.E. Høy, and J. Adler-Nissen, Pilot Batch Production of Specific-Structured Lipids by Lipase-Catalyzed Interesterification: Preliminary Study on Incorporation and Acyl Migration, *Ibid. 75*:301–308 (1998).
9. Han, J.J., Y. Iwasaki, and T. Yamane, Monitoring of Lipase-Catalyzed Transesterification Between Eicosapentaenoic Acid Ethylester and Tricaprylin by Silver Ion High Performance Liquid Chromatography and High Temperature Gas Chromatography, *Ibid. 76*:31–39 (1999).
10. Kosugi, Y., and N. Azuma, Synthesis of Triacylglycerol from Polyunsaturated Fatty Acid by Immobilized Lipase, *Ibid. 71*: 1397–1403 (1994).
11. Haraldsson, G.G., B.Ö. Gudmundsson, and Ö. Almarsson, The Synthesis of Homogenous Triglycerides of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase, *Tetrahedron 51*: 941–952 (1995).
12. Miura, S., A. Ogawa, and H. Konishi, A Rapid Method for Enzymatic Synthesis and Purification of the Structured Triacylglycerol, 1,3-Dilauroyl-2-oleoyl-glycerol, *J. Am. Oil Chem. Soc. 76*:927–931 (1999).

[Received September 21, 1999; accepted February 15, 2000]