Chemoenzymatic Synthesis of Structured Triacylglycerols Containing Eicosapentaenoic and Docosahexaenoic Acids[1]

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ABSTRACT: There are indications in the recent literature that the location of polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in triacylglycerols (TAG) may influence their oxidative stability. To address that question, two types of structured lipids were designed and synthesized: firstly, a TAG molecule possessing pure EPA or DHA at the mid-position with stearic acid at the outer positions; and secondly, a TAG molecule possessing pure EPA or DHA located at one of the outer positions with stearic acid at the mid-position and the remaining end position. The former adduct was synthesized in two steps by a chemoenzymatic approach. In the first step 1,3-distearoylglycerol was afforded in good yield (74%) by esterifying glycerol with two equivalents of stearic acid in ether in the presence of silica gel using Lipozyme™ as a biocatalyst. This was followed by a subsequent chemical esterification with pure EPA or DHA using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide as a coupling agent in the presence of 4-dimethylaminopyridine in dichloromethane in excellent yields (94 and 91%, respectively). The latter adduct was synthesized in two enzymatic steps. In the first step tristearoylglycerol was prepared in very high yield (88%) by esterifying glycerol with a stoichiometric amount of stearic acid under vacuum at 70–75°C using an immobilized *Candida antarctica* lipase without a solvent. That adduct was subsequently treated in an acidolysis reaction with two equivalents of EPA or DHA without solvent at 70–75°C or in toluene at 40°C in the presence of Lipozyme to afford the desired product in moderate yields (44 and 29%, respectively).

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The term structured lipids (1–3) usually refers to triacylglycerols (TAG) containing certain types of fatty acids or fatty acid composition at the end positions and different fatty acids or fatty acid composition at the mid-position of the glycerol backbone. They find use in various dietary and health supplements, such as functional food and infant formula. From a human nutritional point of view, structured lipids possessing long-chain n-3 polyunsaturated fatty acids (PUFA) located at the *sn*-2 position with medium-chain fatty acids (MCFA) at the *sn*-1 and *sn*-3 positions have received considerable attention recently (3–5). The reason is that the medium-chain fatty acids located at the end positions are rapidly hydrolyzed by pancreatic lipase, absorbed into the intestines, and rapidly carried into the liver where they are consumed as a quick source of energy. The remaining 2-monoacylglycerols (2-MAG), on the other hand, become a source of essential fatty acids after being absorbed through the intestinal wall (6). They are accumulated as TAG in the adipose tissues or as phospholipids in the cell membranes from where they can be released upon demand for their desired biological functions.

The beneficial health effects of the n-3 PUFA are now well established and have been almost exclusively attributed to *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) (7,8). The beneficial effects of EPA include reductions in heart diseases and various inflammatory disorders. Recently, there has been a dramatic shift of interest toward DHA and its beneficial effects on pregnancy, infants, and brain and nervous system development (9,10).

Numerous reports describe the preparation of structured TAG comprised of n-3 PUFA at the *sn*-2 position and MCFA at the *sn*-1 and *sn*-3 positions. Lipases, owing to their regioselectivity, are ideally suited as biocatalysts for producing such structured lipids. By acting preferentially at the primary *sn*-1 and *sn*-3 positions of the glycerol moiety, they can be used to incorporate fatty acids of a certain type or composition at these positions by esterification (11,12) or transesterification (13–16) processes. As a result of the mildness offered by lipases, they are also of great use when the highly labile n-3 type PUFA are involved (17,18). There are also numerous reports on the use of 1,3-regioselective lipases to introduce n-3 PUFA into the end positions of various TAG oil types (19,20).

The main objectives of the work described in this report were to synthesize structured TAG containing EPA or DHA with long-chain stearic acid rather than the more common MCFA. The interest for that type of structured TAG relates to its greater structural similarity to fish oil lipids, and the claimed observation of Endo and coworkers that oxidative

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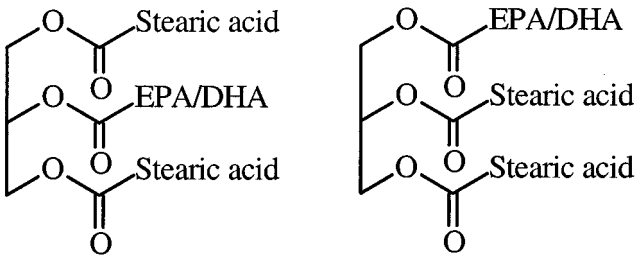


FIG. 1. Structure of the 2-eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA)-triacylglycerol (TAG) and 1-EPA/DHA-TAG adducts.

stability of n-3 PUFA may be influenced by their positions in TAG (21,22). The conclusion of their work was that n-3 PUFA including EPA and DHA located at the *sn*-2 position were more stable than when located at the *sn*-1 and *sn*-3 positions in fish oil TAG.

To address that question, two types of structurally labeled TAG were designed as possible models for oxidative stability studies. The synthesis of such model compounds by both enzymatic and chemoenzymatic means is described in this report. The first type comprises two stearyl groups at the outer positions and either pure EPA or DHA located at the *sn*-2 position, 2-eicosapentaenoyl-1,3-distearylglycerol (2-EPA-TAG) and 2-docosahexaenoyl-1,3-distearyl-glycerol (2-DHA-TAG), respectively. The second type possesses two stearyl groups with EPA or DHA located at the *sn*-1(3) position, 1-eicosapentaenoyl-2,3-distearylglycerol (1-EPA-TAG) and 1-docosahexaenoyl-2,3-distearyl-glycerol (1-DHA-TAG), respectively. The structure of these adducts is illustrated in Figure 1.

MATERIALS AND METHODS

Instrumentation. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC 250 NMR spectrometer (Karlsruhe, Germany) in deuterated chloroform as a solvent. The number of carbon nuclei behind each ^{13}C signal is indicated in brackets after each chemical shift value. Infrared (IR) spectra were collected on a Nicolet Avatar 360 FT-IR (E.S.P.) spectrophotometer (Madison, WI) on a KBr pellet. Analytical gas–liquid chromatography (GLC) was conducted on a PerkinElmer 8140 gas chromatograph (Norwalk, CT) according to a previously described procedure (23). Elemental analyses were performed on Carlo Erba Strumentazione Elemental Analyzer (model 1106; Milan, Italy). Melting points were determined on a Büchi 520 melting point apparatus and are uncorrected.

Materials and solvents. The immobilized *Rhizomucor miehei* (Lipozyme™) and *Candida antarctica* (Lipase SP 435, Novozyme™) lipases were supplied as a gift from Novo Nordisk A/S (Bagsvaerd, Denmark). EPA ethyl ester (98%) and DHA ethyl ester (>95%) were obtained from Norsk Hydro A/S (Porsgrunn, Norway). The purity of these products was confirmed by analytical GLC and high-field NMR spectroscopy. Glycerol (99%) and stearic acid (99%) were purchased from Sigma Chemicals (St. Louis, MO); 4-dimethylaminopyridine (DMAP; >99%) and reagent-grade NaOH, $Na_2EDTA{\cdot}2H_2O$, anhydrous $MgSO_4$, and HCl (37% wt/wt) were obtained from Merck (Darmstadt, Germany); and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI; >98%) was obtained from Aldrich Chemical Company (St. Louis, MO). They were all used without further purification. Solvents (*n*-hexane, petroleum ether, diethyl ether, chloroform, dichloromethane, toluene, ethyl acetate, acetic acid, and methanol) were obtained from Acros Organics (Geel, Belgium) and were of analytical grade and used without further purification. Molecular sieves (3Å), silica gel (Silica gel 60) and analytical thin-layer chromatography (TLC) plates (DC Alufolien Kieselgel 60 F_{254}) were obtained from Merck.

Hydrolysis of EPA ethyl ester. To a solution of NaOH (7.35 g, 185 mmol) and $Na_2EDTA{\cdot}2H_2O$ (104 mg; 0.28 mmol) in distilled water (150 mL) was added 96% ethanol (150 mL). After the solution had been de-aerated by blowing nitrogen through the solution for approximately 5 min, EPA ethyl ester (30.1 g; 91.0 mmol) was added and the resulting mixture heated at 65°C and magnetically stirred under argon. The progress of the reaction was monitored by analytical TLC (silica gel; eluted with 1:9 ethyl acetate/*n*-hexane). The reaction was completed in less than 90 min. The mixture was allowed to cool to 0–4°C in an ice bath and then acidified by carefully adding an aqueous 2 M HCl solution until slightly acidic as indicated by litmus paper. The resulting mixture was then extracted with *n*-hexane (2 × 100 mL), the combined organic layers were washed with water until they were at a neutral pH, dried over anhydrous $MgSO_4$, and the organic solvent was removed *in vacuo* on a rotary evaporator, followed by treatment under high vacuum (30 min) to remove traces of solvents. The product was afforded as a slightly yellowish liquid (26.7 g; 97% yield). ^{1}H NMR δ 11.91 (*br s*, 1H, –COOH), 5.46–5.26 (*m*, 10H, =C–H), 2.85–2.78 (*m*, 8H, =C–CH_2–C=), 2.36 (*t*, *J* = 7.7 Hz, 2H, –CH_2–COOH), 2.17–2.02 (*m*, 4H, =CH–CH_2–CH_2– and CH_3–CH_2–CH=), 1.77–1.65 (*m*, 2H, –CH_2–CH_2–COOH) and 0.97 (*t*, *J* = 7.5 Hz, 3H, –CH_3) ppm. ^{13}C NMR δ 180.3 (C=O), 132.0 (1), 129.0 (1), 128.7 (1), 128.5 (1), 128.2 (1), 128.1 (2), 128.0 (1), 127.8 (1), 127.0 (1), 33.4 (1), 26.4 (1), 25.6 (3), 25.5 (1), 24.4 (1), 20.5 (1), and 14.2 (1) ppm.

Hydrolysis of DHA ethyl ester. The procedure for the DHA ethyl ester (5.00 g; 14.0 mmol) hydrolysis was identical to the one for the EPA ethyl ester. The product was afforded as a slightly yellowish liquid (4.53 g; 98% yield). ^{1}H NMR δ 5.40–5.24 (*m*, 12H, =C–H), 2.85–2.77 (*m*, 10H, =C–CH_2–C=), 2.43–2.37 (*m*, A_2B_2, 4H, –CH_2–CH_2–COOH), 2.11–2.00 (*m*, 2H, CH_3–CH_2–CH=) and 0.95 (*t*, *J* = 7.5 Hz, 3H, –CH_3) ppm. ^{13}C NMR δ 179.5 (C=O), 132.0 (1), 129.6 (1), 128.5 (1), 128.3 (1), 128.2 (2), 128.1 (2), 127.9 (1), 127.8 (1), 127.5 (1), 127.0 (1), 33.6 (1), 25.6 (4), 25.5 (1), 22.4 (1), 20.5 (1) and 14.2 (1) ppm.

Preparation of 1,3-distearylglycerol. Glycerol (8.21 g, 89.1 mmol) was adsorbed onto the same weight of silica gel

by vigorously stirring for 30 min. To this preparation was added stearic acid (50.6 g; 178 mmol), immobilized *R. miehei* lipase (3.5 g), and molecular sieves (10 g). The mixture was suspended in diethyl ether (200 mL) and stirred on a magnetic stirrer hot-plate at room temperature for 48 h. The reaction was continuously monitored by analytical TLC (silica gel; eluted with 80:20:2 petroleum ether/diethyl ether/acetic acid). The lipase and silica gel were separated off by filtration, and the solvent was removed *in vacuo*. Recrystallization of the resulting solid material in methanol afforded the product as highly pure fine-powdered white crystals (82.3 g; 74% yield). M.p. = 77.8–78.4°C. ^{1}H NMR δ 4.21–4.08 (*m*, 5H, –CH_2–CH–CH_2–), 2.51 (*br s*, 1H, –OH), 2.35 (*t, J* = 7.4 Hz, 4H, –CH_2COO–), 1.62 (*m*, 4H, –C*H*$_2$–CH_2–COO–), 1.40–1.16 (*m*, 56H, –CH_2–), and 0.87 (*t, J* = 6.3 Hz, 6H, –CH_3) ppm. ^{13}C δ 173.9 (2; α C=O), 68.3 (1), 65.0 (2), 34.1 (2), 31.9 (2), 29.7 (10), 29.6 (4), 29.4 (6), 29.2 (2), 29.1 (2), 24.9 (2), 22.7 (2), and 14.1 (2) ppm. IR ν_{max} 3300–3600 (*br*, O–H), 2915 (*vs*, C–H), 2849 (*vs*, C–H), 1735 (*vs*, C=O) cm^{-1}. Elemental analysis: found: C, 74.87; H, 12.30. $C_{39}H_{76}O_5$ requires C, 74.94; H, 12.26%.

Preparation of tristearin. Immobilized *C. antarctica* lipase (1.50 g) was added to a mixture of glycerol (1.55 g, 16.8 mmol) and stearic acid (15.1 g; 53.1 mmol). The mixture was gently stirred on a magnetic stirrer hot-plate at 70–75°C under continuous vacuum of 0.05–0.1 Torr. The volatile water produced during the course of the reaction was continuously condensed into a liquid nitrogen cooled trap. After 48 h the reaction was discontinued, chloroform added, and the enzyme separated off by filtration. Pure product was afforded as a white crystalline material (13.2 g; 88% yield) after crystallization from chilled chloroform (0–4°C), filtration, and drying *in vacuo*. M.p. = 69.2–70.0°C. ^{1}H NMR δ 5.34–5.26 (*m*, 1H, –CH_2–C*H*–CH_2–), 4.30 (*dd*, *J* = 11.9 Hz, *J* = 4.3 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 4.14 (*dd, J* = 11.9 Hz, *J* = 6.0 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 2.31 (*t, J* = 7.3 Hz, 6H, –CH_2–COO), 1.63–1.58 (*m*, 6H, –C*H*$_2$–CH_2–COO–), 1.40–1.16 (*m*, 84H, –CH_2–), and 0.87 (*t, J* = 6.3 Hz, 9H, –CH_3) ppm. ^{13}C NMR δ 173.3 (2; α C=O), 172.9 (1; β C=O), 68.8 (1), 62.1 (2), 34.2 (1), 34.0 (2), 31.9 (3), 29.7 (15), 29.5 (6), 29.4 (6), 29.3 (3), 29.1 (6), 24.8 (3), 22.7 (3) and 14.1 (3) ppm. IR ν_{max} 2917 (*vs*, C–H), 2849 (*vs*, C–H), 1736 (*vs*, C=O) cm^{-1}. Elemental analysis: found: C, 76.89; H, 12.48. $C_{57}H_{110}O_6$ requires C, 76.79; H, 12.44%.

Preparation of 2-eicosapentaenoyl-1,3-distearylglycerol. To a solution of 1,3-distearylglycerol (33.0 g; 52.8 mmol) and EPA as a free acid (16.8 g; 55.4 mmol) in dichloromethane (300 mL) was added DMAP (3.22 g; 26.4 mmol) and EDCI (12.1 g; 63.0 mmol). The resulting solution was stirred on a magnetic stirrer hot-plate at room temperature for 24 h. The reaction was terminated by passing the reaction solution through a short column packed with silica gel after reduction of the volume to 50 mL by rotary evaporation *in vacuo*. Solvent removal *in vacuo* afforded a mixture of the crude product contaminated with some EPA which was purified by silica gel column chromatography using *n*-hexane as an eluent to afford the product as a white semicrystalline waxy material (41.1 g; 94% yield). It was possible to prepare crystalline material for melting point determination using ethyl acetate at −18°C. M.p. = 33.5–34.0°C. ^{1}H NMR δ 5.40–5.33 (*m*, 10H, =C–H), 5.34–5.25 (*m*, 1H, –CH_2–C*H*–CH_2), 4.29 (*dd, J* = 11.9 Hz, *J* = 4.3 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 4.13 (*dd*, *J* = 11.9 Hz, *J* = 5.9 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 2.85–2.77 (*m*, 8H, =C–CH_2–C=), 2.35–2.26 (2 × *t*, *J* = 7.6 Hz, 6H, –CH_2–COO–), 2.11–2.03 (*m*, 4H, =CH–C*H*$_2$–CH_2– and CH_3–C*H*$_2$–CH=), 1.72–1.65 (*m*, 2H, –C*H*$_2$–CH_2–COO– in EPA), 1.62–1.56 (*m*, 4H, –C*H*$_2$CH$_2$COO– in stearic acid), 1.40–1.16 (*m*, 56H, –CH_2–), 0.96 (*t, J* = 7.5 Hz, 3H, –CH_3 in EPA), and 0.87 (*t, J* = 6.4 Hz, 6H, –CH_3) ppm. ^{13}C δ 173.2 (2; α C=O), 172.5 (1; β C=O), 131.9 (1), 128.9 (1), 128.7 (1), 128.5 (1), 128.2 (1), 128.1 (2), 128.0 (1), 127.8 (1), 126.9 (1), 68.9 (1), 62.0 (2), 34.0 (2), 33.5 (1), 31.9 (2), 29.7 (10), 29.6 (4), 29.4 (4), 29.3 (2), 29.2 (2), 29.1 (2), 26.4 (1), 25.6 (3), 25.5 (1), 24.8 (2), 24.7 (1), 22.6 (2), 20.5 (1), 14.2 (1) and 14.1 (2) ppm. IR ν_{max} 3020 (*s*, C–H), 2914 (*vs*, C–H), 2849 (*vs*, C–H), 1732 (*vs*, C=O) cm^{-1}. Elemental analysis: found: C, 78.05; H, 11.58. $C_{59}H_{104}O_6$ requires C, 77.92; H, 11.53%.

Preparation of 2-docosahexaenoyl-1,3-distearylglycerol. The procedure was identical to the one for preparing the corresponding 2-EPA-TAG adduct using 1,3-distearin (1.00 g; 1.60 mmol), DHA (0.59 g; 1.80 mmol), DMAP (0.11 g; 0.90 mmol) and EDCI (0.35 g; 1.80 mmol) in dichloromethane (8 mL). The product was afforded as a semicrystalline waxy material (1.36 g; 91% yield), but crystals could be afforded from cold ethyl acetate treatment. M.p. = 35.0–35.5°C. ^{1}H NMR δ 5.42–5.30 (*m*, 12H, =C–H), 5.29–5.24 (*m*, 1H, –CH_2–C*H*–CH_2), 4.29 (*dd*, *J* = 11.9 Hz, *J* = 4.4 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 4.13 (*dd, J* = 11.9 Hz, *J* = 5.9 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 2.86–2.77 (*m*, 10H, =C–CH_2–C=), 2.38–2.35 (*m*, 4H, –C*H*$_2$–C*H*$_2$–COO– in DHA), 2.29 (*t, J* = 7.4 Hz, 4H, –CH_2–COO–), 2.12–2.00 (*m*, 2H, CH_3–C*H*$_2$–CH=), 1.62–1.54 (*m*, 4H, –C*H*$_2$CH$_2$COO– in stearic acid), 1.35–1.18 (*m*, 56H, –CH_2–), 0.96 (*t, J* = 7.5 Hz, 3H, –CH_3) and 0.86 (*t, J* = 6.3 Hz, 6H, –CH_3) ppm. ^{13}C NMR δ 173.1 (2; α C=O), 172.0 (1; β C=O) 131.9 (1), 129.4 (1), 128.5 (1), 128.2 (3), 128.0 (2), 127.9 (1), 127.8 (1), 127.5 (1), 126.9 (1), 69.0 (1), 61.9 (2), 34.0 (3), 31.9.(2), 29.7 (10), 29.6 (6), 29.4 (2), 29.3 (2), 29.2 (2), 29.1 (2), 25.6 (3), 25.5 (1), 24.8 (3), 22.6 (3), 20.5 (1), 14.2 (1) and 14.1 (2) ppm. IR ν_{max} 3020 (*s*, C–H), 2914 (*vs*, C–H), 2849 (*vs*, C–H), 1733 (*vs*, C=O) cm^{-1}. Elemental analysis: found: C, 78.27; H, 11.45. $C_{61}H_{106}O_6$ requires C, 78.32; H, 11.42%.

Preparation of 1-eicosapentaenoyl-2,3-distearylglycerol. Immobilized *R. miehei* lipase (2.2 g) was added to a mixture of tristearin (13.10 g; 14.7 mmol) and EPA as a free acid (8.90 g; 29.4 mmol). The mixture was gently stirred on a magnetic stirrer hot-plate at 75°C under continuous vacuum of 0.05–0.10 mm Hg for 20 h. The mixture was allowed to cool, chloroform added (25 mL), and the reaction stopped by filtering off the lipase. The chloroform was removed *in vacuo* on a rotary evaporator and the residue dissolved in diethyl ether

(100 mL). The ether solution was cooled and allowed to stand in a refrigerator (0–4°C) to precipitate stearic acid, which was filtered off. Treatment of the residue from the ether filtrate after solvent removal with cold chloroform (−18°C; 60 mL) resulted in the precipitation of pure tristearin starting material. After filtration the chloroform was removed *in vacuo* and the residue dissolved in *n*-hexane (may need a gentle warming). The hexane solution was allowed to stand overnight in a freezer (−18°C) during which the desired product precipitated together with some traces of EPA. EPA and 1,3-diEPA-TAG remained in the hexane solution. Column chromatography on silica gel using *n*-hexane as an eluent afforded the pure product as a semicrystalline waxy material (5.91 g; 44% yield), which could be crystallized from ethyl acetate as before. M.p. = 36.7–37.3°C. ^{1}H NMR δ 5.40–5.31 (*m*, 10H, =C–H), 5.27–5.25 (*m*, 1H, –CH_2–C*H*–CH_2–), 4.29 (*dd*, *J* = 11.9 Hz, *J* = 4.2 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 4.13 (*dd,* *J* = 11.9 Hz, *J* = 5.3 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 2.85–2.78 (*m*, 8H, =C–CH_2–C=), 2.34–2.26 (*m*, 6H, –CH_2–COO–), 2.11–2.03 (*m*, 4H, =CH–C*H*$_2$–CH_2– and CH_3–C*H*$_2$–CH=), 1.71–1.65 (*m*, 2H, –C*H*$_2$–CH_2–COO– in EPA), 1.62–1.57 (*t*, *J* = 6.8 Hz, 4H, –C*H*$_2$$CH_2$COO– in stearic acid), 1.38–1.19 (*m*, 56H, –CH_2–), 0.96 (*t,* *J* = 7.5 Hz, 3H, –CH_3) and 0.87 (*t,* *J* = 6.4 Hz, 6H, –CH_3) ppm. ^{13}C NMR δ 173.2 (1; α C=O), 172.9 (1; β C=O), 172.8 (1; α C=O, EPA), 131.9 (1), 128.9 (1), 128.7 (1), 128.5 (1), 128.2 (1), 128.1 (2), 128.0 (1), 127.7 (1), 126.9 (1), 68.8 (1), 62.1 (1), 62.0 (1), 34.1 (1), 34.0 (1), 33.3 (1), 31.9 (2), 29.7 (10), 29.6 (4), 29.4 (4), 29.3 (2), 29.2 (2), 29.0 (2), 26.4 (1), 25.6 (3), 25.5 (1), 24.8 (2), 24.6 (1), 22.6 (2), 20.5 (1), 14.2 (1), and 14.1 (2) ppm. IR ν_{max} 3020 (*s*, C–H), 2917 (*vs*, C–H), 2849 (*vs*, C–H), 1735 (*vs*, C=O) cm^{-1}. Elemental analysis: found: C, 77.90; H, 11.50. $C_{59}H_{104}O_6$ requires C, 77.92; H, 11.53%.

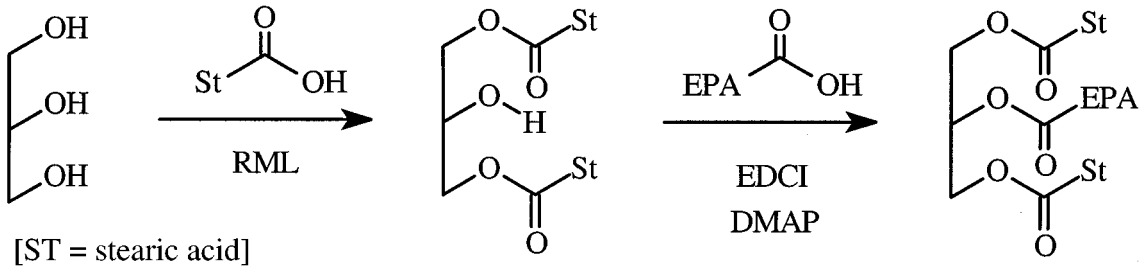
Preparation of 1-docosahexaenoyl-2,3-distearylglycerol. Immobilized *R. miehei* lipase (0.55 g) was added to a mixture of tristearin (2.22 g; 2.49 mmol) and DHA as free acid (3.20 g; 9.74 mmol) in toluene (6 mL). The resulting solution was stirred at 40°C for 48 h. The workup procedure was identical to the one for the 1-EPA-TAG described above. The crude reaction product was afforded as a semicrystalline waxy material (0.67 g; 28%), which could be crystallized by treatment with cold ethyl acetate as before. M.p. = 38.2–38.9°C. ^{1}H NMR δ 5.44–5.33 (*m*, 12H, =C–H), 5.32–5.21 (*m*, 1H, –CH_2–C*H*–CH_2–), 4.30 (*dd,* *J* = 11.9 Hz, *J* = 4.3 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 4.14 (2 × *dd,* *J* = 11.9 Hz, *J* = 6.0 Hz, 2H, –C*H*$_2$–CH–C*H*$_2$–), 2.86–2.79 (*m*, 10H, =C–CH_2–C=), 2.39–2.37 (*m*, 4H, –C*H*$_2$–C*H*$_2$–COO– in DHA), 2.34–2.27 (2 × *t*, *J* = 7.5 Hz, 4H, –CH_2–COO–), 2.12–2.01 (*m*, 2H, CH_3–C*H*$_2$–CH=), 1.63–1.58 (*m*, 4H, –C*H*$_2$$CH_2$COO– in stearic acid), 1.38–1.18 (*m*, 56H, –CH_2–), 0.97 (*t,* *J* = 7.5 Hz, 3H, –CH_3), and 0.87 (*t,* *J* = 6.6 Hz, 6H, –CH_3) ppm. ^{13}C NMR δ 173.3 (1; α C=O), 172.9 (1; β C=O), 172.6 (1; α C=O in DHA), 132.0 (1), 129.4 (1), 128.5 (1), 128.3 (1), 128.2 (2), 128.1 (2), 128.0 (1), 127.8 (1), 127.6 (1), 127.0 (1), 68.8 (1), 62.3 (1), 62.1 (1), 34.2 (1), 34.0 (1), 33.9 (1), 31.9 (2), 29.7 (10), 29.6 (2), 29.5 (4), 29.4 (2), 29.3 (4), 29.1 (2), 25.6 (3), 25.5 (1), 24.9 (2), 24.8 (1), 22.7 (2), 22.6 (1), 20.5 (1), 14.3 (1), and 14.1 (2) ppm. IR ν_{max} 3020 (*s*, C–H), 2917 (*vs*, C–H), 2849 (*vs*, C–H), 1735 (*vs*, C=O) cm^{-1}. Elemental analysis: found: C, 78.35; H, 11.59. $C_{61}H_{106}O_6$ requires C, 78.32; H, 11.42%.

RESULTS AND DISCUSSION

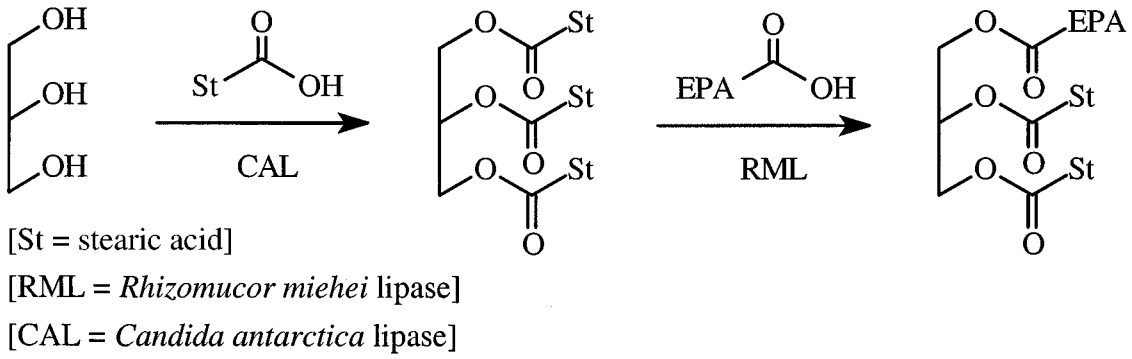
To enable investigation of the possibility that oxidative stability of n-3 PUFA may be related to location in fish oil TAG, two types of structured TAG molecules were designed and synthesized. The first type is comprised of a pure EPA or DHA acyl group at the *sn*-2 position with saturated stearyl groups at the *sn*-1 and -3 positions. The second type possesses two stearyl groups at the *sn*-2 and one of the *sn*-1 or -3 positions with pure EPA or DHA at the remaining *sn*-1 or -3 position. The reason for selecting stearic acid instead of the usual MCFA for structured lipids relates to the fact that in fish oils there are virtually no fatty acids present of the MCFA type. Stearic acid, on the other hand, is commonly found in fish oil TAG; it is saturated and thus less prone to oxidation, reducing possible interference of its own oxidation.

The former TAG were synthesized by a two-step chemoenzymatic route according to Scheme 1. In the first step the immobilized *R. miehei* lipase was used to esterify the end positions of glycerol with stoichiometric amount of stearic acid to afford 1,3-distearylglycerol in 75% yield after recrystallization from methanol. This step was based on a procedure of Berger and coworkers (24) with glycerol adsorbed on silica gel, in diethyl ether at room temperature in the presence of molecular sieves.

In the subsequent step EDCI was used as a chemical cou-



SCHEME 1



[St = stearic acid]
[RML = *Rhizomucor miehei* lipase]
[CAL = *Candida antarctica* lipase]

SCHEME 2

pling agent in the presence of 30-50% DMAP as based on the number of mol at room temperature for 24 h in dichloromethane to introduce EPA into the mid-position of the 1,3-diacylglycerol (DAG) adduct. Pure 2-EPA-TAG was afforded as a microcrystalline material in 94% yield after treatment on silica gel to get rid of a slight excess of EPA.

A similar strategy was used to accomplish the 2-DHA-TAG adduct, also as a microcrystalline material in excellent yield (91%) after purification on silica gel. Both adducts were obtained as crystalline material after recrystallization from cold ethyl acetate. Beyond much higher yields, EDCI offers the advantages over other coupling agents such as dicyclohexylcarbodiimide (12) of possessing a polar amino group and of water solubility, which is of benefit when working up the reaction and purifying the products. DMAP presumably serves as both a base and a catalyst for the acylation process.

The second TAG-type adducts were synthesized by an enzymatic approach based on two enzymatic steps according to Scheme 2. In the first step glycerol was fully esterified with a stoichiometric amount of melted stearic acid using an immobilized *C. antarctica* lipase as a biocatalyst. The method is based on a previously described procedure for preparing homogeneous TAG of pure EPA (triEPA-TAG) or DHA (triDHA-TAG) (25). The reaction was conducted under vacuum (0.05–0.10 mm Hg) at 70–75°C without a solvent using a 10% dosage of lipase as based on weight of substrates. The volatile co-produced water was condensed into a liquid-nitrogen cooled trap as the reaction proceeded, thus shifting the reaction toward completion. The progress of the reaction could be monitored by regularly withdrawing samples from the reaction mixture for high-field ^{1}H NMR analysis in a manner identical to the one previously described for synthesizing triEPA-TAG and triDHA-TAG (25). The reaction was completed after 48 h. The tristearin product was afforded in 88% yield after recrystallization from chloroform.

A subsequent treatment of tristearin with a twofold molar excess of pure EPA free acid using immobilized *R. miehei* lipase resulted in the 1-EPA-TAG adduct in moderate yield. The acidolysis reaction was performed under vacuum without solvent at 70–75°C for 20 h. It was evident that in order to optimize production of the desired 1-EPA-TAG adduct a compromise was needed between the desired monoadduct, the 1,3-diEPA-TAG adduct, and unreacted tristearin. After 20 h the reaction mixture constituted approximately 25–30% unreacted tristearin, 50–55% 1-EPA-TAG, and 20% 1,3-diEPA-TAG. A rather tedious workup was required for purifying that mixture, which also contained stearic acid and excessive EPA. Treatment with chilled diethyl ether resulted in precipitation of the bulk of the stearic acid, and a subsequent treatment in cold chloroform recovered unreacted tristearin by precipitation. Treatment with cold *n*-hexane resulted in elimination of the 1,3-diEPA-TAG adduct. Pure 1-EPA-TAG was afforded in 44% yield as a semicrystalline waxy material after purification on silica gel chromatography using *n*-hexane as an eluent, which could be recrystallized from chilled ethyl acetate. The chromatography was necessary for separating traces of EPA. The 1,3-diEPA-TAG adduct became the main product when using excess amounts of EPA and prolonged reaction time. That product was isolated and purified and characterized by high-field NMR.

When the reaction was conducted without any solvent according to the description above, traces of impurities related to EPA located at the *sn*-2 position were noticed in the product, presumably a result of intramolecular acyl-migration processes (23,25). Under the vacuum condition, there were hardly any signs of hydrolysis side-reactions. When the reaction was conducted under inert nitrogen at atmospheric pressure, a much faster reaction rate was observed, but a considerable extent of hydrolysis side-reaction was noticed together with acyl-migration, which resulted in a considerable amount of undesired 2-EPA adducts. Han and Yamane recently investigated the importance of water content control under comparable conditions (26). When performing the acidolysis reaction in toluene as solvent, there were virtually no signs of any of the 2-EPA or 1,2-diEPA adducts as indicated by ^{13}C NMR analysis, but signs of slight hydrolysis were noticed. However, yields were lower in toluene as a result of less favorable product composition, approximately 20% tristearin, and 40% each of 1-EPA-TAG and 1,3-diEPA-TAG under that condition.

In a similar manner, tristearin was converted into the corresponding 1-DHA-TAG adduct by a similar process using pure DHA. The reaction was conducted in toluene as a solvent at 35–40°C. As would be expected, the reaction involving DHA required a longer reaction time of 48 h. This is a

consequence of a higher preference of the *R. miehei* lipase for EPA than for DHA (27). The pure DHA adduct was obtained in only 29% yield after chromatographic purification and crystallization from chilled ethyl acetate. There were no signs of any of the 2-DHA-TAG adducts as indicated by the ^{13}C NMR analysis.

All intermediates and products were fully characterized by high-field ^{1}H and ^{13}C NMR and IR spectroscopic analysis, GLC analysis, and elemental analysis. Regarding the ^{1}H NMR spectroscopy, the protons belonging to the glyceryl backbone of the acylglycerols were highly useful for characterizing the products and evaluating their purity. As has been intensively studied, these protons resonate very characteristically in individual acylglycerols (1- and 2-MAG, 1,2- and 1,3-DAG, and TAG) (25). The ^{13}C NMR spectroscopy was of particular use to monitor the regiocontrol of the reactions. This is based on two distinctive resonance signals being obtained in the ^{13}C spectra for the carbonyl group carbon of each fatty acid depending on the location of their acyl groups at the end positions (α) or the mid-position (β) of the glyceryl backbone (25). This is shown in Table 1, which also contains data for the triEPA-TAG and triDHA-TAG adducts from previous studies (25).

From Table 1 it can be noticed that stearic acid located at the end position in tristearin resonates at 173.3 ppm, whereas stearic acid located at the mid-position resonates at 172.9 ppm. Similarly, EPA located at the end positions of triEPA-TAG resonates at 172.9 ppm, but at 172.6 ppm when located at the mid-position. DHA located at the end positions of triDHA-TAG resonates at 172.5 ppm, but 172.1 ppm when located at the mid-position.

There are some minor variations of the chemical shift values depending on the type of TAG structures. For instance, it is noticeable that there is an interesting shift of the α-carbonyl resonance of stearic acid depending on the type of fatty acid accommodating the β-position of the TAG. In tristearin the chemical shift was 173.3 ppm, with EPA in the β-position in 2-EPA-TAG it was 173.2 ppm, but when EPA was replaced with DHA in 2-DHA-TAG it was shifted further upfield to 173.1 ppm. In 1,3-distearylglycerol the value was further downfield at 173.9 ppm. Despite these minor variations and shifts this technique remained of enormous use to monitor the regioselectivity of these reactions.

TABLE 1
Location of ^{13}C NMR Peaks of Carbonyl Group Carbons of Stearic Acid (St) and n-3 PUFA in α and β Positions of Various Types of Structured Lipids[a]

	α–C=O (δ in ppm)		β–C=O (δ in ppm)	
Adduct	St	n-3	St	n-3
Tristearin	173.3	—	172.9	—
1,3-Distearin	173.9	—	—	—
1-EPA-TAG	173.2	172.8	172.9	—
2-EPA-TAG	173.2	—	—	172.5
1-DHA-TAG	173.3	172.6	172.9	—
2-DHA-TAG	173.1	—	—	172.0
Tri-EPA-TAG	—	172.9	—	172.6
TriDHA-TAG	—	172.5	—	172.1

[a]Abbreviations: NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; TAG, triacylglycerol; DHA, docosahexaenoic acid.

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