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Practical Syntheses of Triacylglycerol Regioisomers Containing Long-chain Polyunsaturated Fatty Acids

Benjamin H. Fraser · Patrick Perlmutter · Chakra Wijesundera

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Abstract Docosahexaenoic acid (DHA, 22:6n-3) is known to protect against a range of degenerative disease conditions and aid in the development of eye and brain function in infants. In dietary lipids DHA is found primarily in the triacylglycerol (TAG) form. However, the effects of the positional distribution of DHA in TAG on lipid functional properties such as bioactivity and oxidative stability are not clearly understood. Studies on this subject for the most part are limited by a lack of regioisomerically pure TAG model compounds containing DHA or similar long-chain (LC)-polyunsaturated fatty acids (PUFA). This paper reports on the development of a practical procedure, based on chemical and enzymatic reactions, for the syntheses of regioisomerically enriched, symmetrical and unsymmetrical TAG isomers containing two palmitic acid and one of linoleic acid, linolenic acid, or DHA. 1,3-Selective acylation of glycerol with vinyl esters of fatty acids catalyzed by *Candida antarctica* lipase and direct coupling with fatty acids in the presence of the coupling agents 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 4-dimethylaminopyridine furnished 1,3-dihexadecanoyl-2-docosahexaenoyl glycerol and its unsymmetrical isomer 1,2-dihexadecanoyl-3-docosahexaenoyl glycerol in 99 and 60% yield, respectively. Critical to the success of the unsymmetrical TAG synthesis is the demonstration that PUFA-containing glycerol acetonides can readily survive appropriately tailored acid-catalyzed conditions. In this way, sufficient quantities of highly regioisomerically enriched PUFA-containing unsymmetrical monoacylglycerols (MAG) and TAG have now become routinely accessible. The methods are amenable to scale-up and could be adopted for regioenriched synthesis of a wide range of TAG.

Keywords Docosahexaenoic acid · FA positional distribution · MAG · Regioisomers · Regiospecific syntheses · n-3 long-chain PUFA · TAG

B. H. Fraser · P. Perlmutter
School of Chemistry, Monash University,
Wellington Road, Clayton, VIC 3800, Australia

C. Wijesundera (✉)
CSIRO Food Futures National Research Flagship
and Food Science Australia, 671 Sneydes Road,
Werribee, VIC 3030, Australia
e-mail: Chakra.Wijesundera@csiro.au

Introduction

There is currently a significant interest in developing renewable food sources containing n-3 long-chain-polyunsaturated fatty acids (LC-PUFA; C_{20} and greater) such as docosahexaenoic acid (DHA) to replace the declining traditional fish sources. Oilseeds are an obvious choice as they are easily grown and processed into oils using conventional methods. Significant advances have already been made towards achieving the goal of producing n-3 LC-PUFA, including DHA, in plants [1, 2]. The continued interest in food sources of DHA stems from clinical trials showing that DHA assists in brain and vision development in infants [3] and protects against coronary heart disease [4]. There is also emerging evidence that DHA protects against other diseases, including diseases of the retina [5] and metabolic syndrome disorders such as type-2 diabetes and obesity [6, 7].

The beneficial health effects of DHA may be related not only to the amounts of these acids present in the diet but also to their positional distribution within the triacylglycerol (TAG) molecules [8–10]. By and large, digestion of dietary TAG takes place via hydrolysis with pancreatic lipase, which releases fatty acids (FA) regiospecifically from the sn-1 and sn-3 positions, resulting in enhanced absorption of FA attached to the sn-2 position of the TAG. This is widely believed to be the reason why palmitic acid in human milk fat, which is largely in the sn-2 position, is better absorbed by infants than it is from vegetable oil sources of similar FA composition where the saturated FA is mainly esterified to the sn-1,3 positions [11]. Apart from a few studies on stearic acid in cocoa butter, FA other than palmitic acid have not been studied systematically with respect to possible positional effects [10], and despite the growing interest in the health effects of DHA, little effort has been made to find out if the positional distribution of DHA in TAG affects bioactivity.

PUFA such as DHA are very susceptible to oxidative deterioration. For this reason, food products containing DHA may develop off-flavors, thereby limiting their useful shelf-life. Means of making oils containing DHA more resistant to oxidation would be extremely valuable. Consequently, a knowledge of the effects of positional distribution of DHA on its oxidative stability would help in designing oils for maximum stability. The few studies conducted to elucidate the effects of positional distribution on the oxidative stability of DHA have produced inconclusive and contradictory results.

A major impediment to elucidating the role of the positional distribution of DHA on oil functionality is the lack of regioisomerically pure TAG model compounds in amounts required for experiments. The synthesis of regioisomerically pure TAG containing *n*-3 LC-PUFA has recently been reviewed [12]. Although considerable efforts have been made to prepare structured lipids containing n-3 LC-PUFA such as DHA, with a few exceptions, these efforts have focused on symmetrical medium-chain (C_8–C_{12}) TAG with the DHA in the sn-2 position and the medium-chain FA in the sn-1 and sn-3 positions [13, 14]. Focus has been placed on the medium-chain TAG due to their usefulness as energy supplements for patients requiring remedial nutrition. In these applications, the medium-chain FA serve as a ready energy source and the DHA provide the essential FA [15]. In most cases, these structured lipids have been made by modifying natural oils, for example by lipase-mediated interesterification of fish oil with medium-chain FA using a 1,3-selective lipase. Such processes produce mixed TAG, and regioisomerically pure compounds are rarely obtained. Furthermore, as it is the symmetrical 1,3-TAG isomers that find applications as energy supplements, relatively little attention has been given to the preparation of the corresponding unsymmetrical 1,2-TAG.

We report here on practical procedures for the synthesis of regioisomerically enriched, symmetrical and unsymmetrical TAG containing two palmitoyl and one of linoleoyl, linolenoyl, or docosahexaenoyl residues.

Experimental Procedures

Materials

Most reactants were purchased from the Aldrich Chemical Company (Sydney, Australia) and were used as supplied. Vinyl palmitate was purchased from TCI (Tokyo, Japan). *Candida antarctica* (Novozym 435) and *Rhizomucor miehei* (Lipozyme RM IM) lipases were gifts of Novozymes Australia Pvt. Ltd (Sydney, Ausltralia). DHA (>99% purity) was purchased from Nu-Chek Prep (Elysian, Minn.). Drying agents and inorganic salts were purchased from AJAX or BDH chemicals. Solvents were purified as follows. Anhydrous diethyl ether was distilled from sodium/benzophenone ketyl prior to use. Dichloromethane (DCM) was distilled from calcium hydride. Hexane refers to the fraction boiling between 40° and 60°C.

Chromatography

The silica gel used for chromatography was a 40- to 63-μm (230–400 mesh) silica gel 60 (no. 9385; Merck, Darmstadt, Germany). Analytical thin-layer chromatography (TLC) was performed on Polygram Sil G/UV_{254} plastic sheets coated with silica gel containing a UV_{254} fluorescent indicator (Merck) and were visualized under UV light and/or dipped in an ammonium molybdate/cerium sulphate solution.

Proton NMR (^{1}H NMR) Spectroscopy

^{1}H nuclear magnetic resonance (NMR) spectra were recorded (Monash University, Melbourne, Australia) at 300 MHz on a Bruker AM 300 spectrometer, at 400 MHz on a Bruker Avance DRX 400 spectrometer, and at 800 MHz on a Bruker Avance 800 spectrometer (Bruker, Germany). Chemical shifts are given on the δ scale in parts per million (ppm). Unless otherwise stated, spectra were measured in deuterochloroform ($CDCl_3$) using the residual chloroform ($CHCl_3$,

7.26 ppm) signal as an internal reference. Each resonance is shown according to the following convention: chemical shift (δ ppm) [multiplicity, coupling constant(s) (Hz), number of hydrogens, assignment (where possible)]. Multiplicities are designated as 's' the singlet, 'd' the doublet, 't' the triplet, 'q' the quartet, 'p' the pentuplet, and 'm' the multiplet.

Carbon NMR (^{13}C NMR) Spectroscopy

^{13}C NMR spectra were recorded at 75 MHz on a Bruker AM 300 spectrometer or at 200 MHz on a Bruker Avance 800 spectrometer using $CDCl_3$, unless otherwise stated. The spectra were referenced using the solvent carbon signal ($CDCl_3$ = 77.16 ppm). Two-dimensional NMR techniques such as homonuclear correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) were used to aid the assignment of some NMR spectra.

Mass Spectrometry

Electrospray ionisation (ESI) was performed on a Micromass Platform Quadrupole Mass Spectrometet (Monash University, Melbourne, Australia). High-resolution mass spectra (HRMS) were recorded on a Bruker BioApex 47e FTMS using NaI for accurate mass calibration. M^+ refers to the molecular ion.

Infrared (IR) Spectroscopy

IR spectra were recorded on a Perkin Elmer 1600 Series Fourier Transform spectrometer (Perkin Elmer, Foster City, Calif.) as neat samples, $CHCl_3$ solutions, or as paraffin (Nujol) mulls of solids between NaCl plates. IR frequencies are reported in wave numbers (cm^{-1}), and intensities are reported qualitatively as strong (s), medium (m) or weak (w) and/or broad (b).

Melting Points (MP)

The MP were recorded on a Kofler hot stage apparatus and are uncorrected.

Lipase-based Positional Analysis

In addition to ^{1}H and ^{13}C NMR, a recently published lipase-mediated method [16] was used to determine regiopurity of the synthesized TAG. In brief, a mixture of the TAG in ethanol (1:10, w/w) was shaken with the 1,3-specific immobilized *Candida antarctica* lipase (4%, by weight of the ethanolic solution of TAG) at 30°C for 4 h. The products were separated on a silica solid phase extraction (SPE) cartridge to isolate 2-monoacylglycerol (MAG), which were trans-methylated to fatty acid methyl esters (FAME) and analyzed by gas chromatography (GC) for FA composition.

Synthesis of 1,3-Dihexadecanoylglycerol **2**

The procedure of Halldorsson et al. [17] was followed and enabled the synthesis of the title compound on a scale of approximately 30 g.

Synthesis of 1,3-Dihexadecanoyl-2-(9,12-octadecadienoyl)glycerol (PLP) **3**

1,3-dihexadecanoylglycerol **2** (5.3 g, 9.3 mmol) in DCM (100 ml) was mixed with 9,12-octadecadienoic acid (linoleic acid, 3.02 g, 11.2 mmol), 4-dimethylaminopyridine (DMAP, 0.45 g, 3.7 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 2.14 g, 11.2 mmol). The solution was stirred at ambient temperature overnight before most of the DCM was removed under reduced pressure and the residue passed through a short plug of silica (100 g silica, 100 ml of 20:80 ether/DCM by vol. eluent). The eluent was removed under reduced pressure, yielding the title compound **3** (97:3, PLP:PPL by ^{13}C NMR) as white crystals (4.81 g, 62%). MP 34–36°C. ^{1}H NMR (800 MHz, $CDCl_3$) δ 0.88 (t, *J* 7.3 Hz, 6H, 2× palmitic CH_3), 0.89 (t, *J* 7.8 Hz, 3H, linoleic CH_3), 1.22–1.33 (m, 64H, 32× CH_2), 1.58–1.63 (m, 6H, 3 × $CO_2CH_2CH_2CH_2$), 2.00–2.03–2.06 (apparent q, *J* 7.2 Hz 4H, $CH_2CH{=}CHCH_2CH{=}CHCH_2$), 2.31 (t, *J* 7.5 Hz, 2H, 2× palmitic $CO_2CH_2CH_2CH_2$), 2.32 (t, *J* 7.4 Hz, 2H, linoleic $CO_2CH_2CH_2CH_2$), 2.77 (apparent t, *J* 7.0 Hz, 2H, $CH{=}CHCH_2CH{=}CH$), 4.14 (dd, *J* 5.9, 11.9 Hz, 2H, one of each OCH_2CHCH_2O), 4.30 (dd, *J* 4.3, 11.9 Hz, one of each OCH_2CHCH_2O), 5.24–5.28 (m, 1H, OCH_2CHCH_2O), 5.30–5.40 (m, 4H, 4× *CH* alkene). ^{13}C NMR (200 MHz, $CDCl_3$) δ 14.22 (CH_3), 14.26 (CH_3), 22.84 (CH_2), 25.02 (CH_2), 25.03 (CH_2), 25.79 (CH_2) 27.35 (CH_2), 29.21 (CH_2), 29.28 (CH_2), 29.29 (CH_2), 29.50 (CH_2), 29.51 (CH_2), 29.64 (CH_2), 29.78 (CH_2), 29.82 (CH_2), 29.84 (CH_2), 29.85 (CH_2), 32.08 (CH_2), 34.21 (CH_2), 62.24 (OCH_2), 69.04 (OCH_2), 128.04 (*CH* alkene), 128.24 (*CH* alkene), 130.14 (*CH* alkene), 130.38 (*CH* alkene), 172.98 (*C*=O, 1C, 2-linoleic), 173.44 (*C*=O, 2C, 1,3-palmitic). IR ($CHCl_3$) υ_{max} 3010s, 2922s, 2854s, 1744s, 1465s, 1418m, 1378m, 1215s, 1170s, 1113s, 868w, 759s, 721m, 668w cm^{-1}. MS calculated for $C_{53}H_{98}O_6Na^+$ = 853.7; found: 853.7. HMS calculated for $C_{53}H_{98}O_6Na^+$ = 853.7261; found: 853.7257.

Synthesis of 1,3-Dihexadecanoyl-2-(9,12,15-octadecatrienoyl)glycerol (PLnP) **4**

A similar procedure as for **3** was followed. 1,3-dihexadecanoylglycerol **2** (4.00 g, 7.03 mmol) in DCM (100 ml) was reacted with 9,12,15-octadecatrienoic acid (linolenic acid) (2.28 g, 8.24 mmol), DMAP (0.2 g, 1.64 mmol), and EDCI (1.92 g, 9.88 mmol). The product was passed through a short plug of silica (100 g silica, 100 ml of 20:80 ether/DCM by vol eluent) to give the title compound **4** (98:2 PLnP:PPLn by ^{13}C NMR) as white crystals (5.60 g, 94%). MP 28–30°C. ^{1}H NMR (800 MHz, $CDCl_3$) δ 0.88 (t, *J* 7.2 Hz, 6H, 2× palmitic C*H*$_3$), 0.98 (t, *J* 7.5 Hz, 3H, linolenic C*H*$_3$), 1.22–1.38 (m, 56H, 28× C*H*$_2$), 1.59–1.62 (m, 6H, 3× $O_2CCH_2CH_2CH_2$), 2.03–2.10 (m, 4H, C*H*$_2$CH=CHCH$_2$CH=CHCH$_2$CH=CHC*H*$_2$), 2.31 (t, *J* 7.4 Hz, 4H, 2× palmitic $O_2CCH_2CH_2$), 2.32 (t, *J* 6.3 Hz, 2H, linolenic $O_2CCH_2CH_2$), 2.81 (apparent t, *J* 5.6 Hz, 4H, CH_2CH=CHC*H*$_2$CH=CHC*H*$_2$CH=CHCH$_2$), 4.14 (dd, *J* 5.9, 11.9 Hz, 2H, one each of OC*H*$_2$CHC*H*$_2$O), 4.29 (dd, *J* 4.3, 11.9 Hz, one each of OC*H*$_2$CHC*H*$_2$O), 5.25–5.28 (m, 1H, CH_2CHOCH_2), 5.30–5.42 (m, 6H, 6× C*H* alkene). ^{13}C NMR (200 MHz, $CDCl_3$) δ 14.26 (*C*H$_3$), 14.42 (*C*H$_3$), 20.70 (*C*H$_2$), 22.84 (*C*H$_2$), 25.02 (*C*H$_2$), 25.68 (*C*H$_2$), 25.77 (*C*H$_2$), 27.36 (*C*H$_2$), 29.20 (*C*H$_2$), 29.28 (*C*H$_2$), 29.35 (*C*H$_2$), 29.42 (*C*H$_2$), 29.51 (*C*H$_2$), 29.63 (*C*H$_2$), 29.77 (*C*H$_2$), 29.81 (*C*H$_2$), 29.85 (*C*H$_2$), 32.08 (*C*H$_2$), 34.21 (*C*H$_2$), 62.24 (O*C*H$_2$), 69.04 (O*C*H), 127.26 (*C*H alkene), 127.92 (*C*H alkene), 128.38 (*C*H alkene), 128.46 (*C*H alkene), 130.36 (*C*H alkene), 132.11 (*C*H alkene), 172.98 (*C*=O, 1C, 2-linolenic), 173.44 (*C*=O, 2C, 1,3-palmitic). I.R (neat) υ_{max} 2927s, 2855s, 1737s, 1466m, 1379w, 1216w, 1165w, 1099w, 908s, 734s, 651s cm^{-1}. MS calculated for $C_{53}H_{96}O_6Na^+$ = 851.7; found: 851.8. HMS calculated for $C_{53}H_{96}O_6H^+$ = 829.7285; found: 829.7268.

Synthesis of 1,3-Dihexadecanoyl-2-(3,6,9,12,15,18-docosahexaenoyl)glycerol (PDP) **5**

A similar procedure as for **3** was followed. 1,3-dihexadecanoylglycerol **2** (4.00 g, 7.04 mmol) in DCM (100 ml) was mixed with 3,6,9,12,15,18-DHA (2.89 g, 8.8 mmol), DMAP (0.36 g, 3.08 mmol), and EDCI (1.48 g, 7.72 mmol). The solution was stirred at ambient temperature overnight before the DCM was removed under reduced pressure. The residue was purified by flash chromatography (140 g silica, 2 l of 10:90 ethyl acetate/hexane by vol. eluent) to afford the title compound **5** (97:3, PDP:PPD, by ^{13}C NMR) as a white semi-crystalline solid (6.19 g, 99%). MP 24–26°C. ^{1}H NMR (800 MHz, $CDCl_3$) δ 0.88 (t, *J* 7.2 Hz, 6H, 2× palmitic C*H*$_3$), 0.97 (t, *J* 7.5 Hz, 3H, DHA C*H*$_3$), 1.21–1.33 (m, 50H, 25× C*H*$_2$), 1.59–1.62 (m, 4H, 2× palmitic $O_2CCH_2CH_2CH_2$), 2.03–2.13 (m, 2H, DHA CH=CHC*H*$_2$CH$_2$), 2.31 (t, *J* 7.6 Hz, 4H, 2× palmitic $O_2CCH_2CH_2$), 2.36–2.39 (m, 2H, O_2CCH_2 CH=CH$_2$) 2.80–2.87 (m, 10H, DHA 5× C=CC*H*$_2$C=C), 4.14 (dd, *J* 5.9, 11.9 Hz, 2H, one each of OC*H*$_2$CHCH$_2$O), 4.29 (dd, *J* 4.4, 11.9 Hz, 2H, one each of OC*H*$_2$CHC*H*$_2$O), 5.22–5.46 (m, 13H, 12× C*H* alkene and CH_2CHOCH_2). ^{13}C NMR (200 MHz, $CDCl_3$) δ 14.26 (*C*H$_3$), 14.42 (*C*H$_3$), 20.71 (*C*H$_2$), 22.8 (*C*H$_2$), 22.79 (*C*H$_2$), 22.84 (*C*H$_2$), 25.01 (*C*H$_2$), 25.70 (*C*H$_2$), 25.76 (*C*H$_2$), 25.79 (CH$_2$), 29.28 (*C*H$_2$), 29.42 (*C*H$_2$), 29.51 (*C*H$_2$), 29.63 (*C*H$_2$), 29.77 (*C*H$_2$), 29.81 (*C*H$_2$), 29.84 (*C*H$_2$), 32.08 (*C*H$_2$), 34.20 (*C*H$_2$), 62.18 (O*C*H$_2$), 69.26 (O*C*H), 127.17 (*C*H alkene), 127.79 (*C*H alkene), 128.03 (*C*H alkene), 128.13 (*C*H alkene), 128.23 (*C*H alkene), 128.43 (*C*H alkene), 128.44 (*C*H alkene), 128.49 (*C*H alkene), 128.73 (*C*H alkene), 129.61 (*C*H alkene), 132.19 (*C*H alkene), 172.29 (*C*=O, 1C, 2-DHA), 173.43 (*C*=O, 2C, 1,3-palmitic). IR (neat) υ_{max} 3014s, 2913s, 2849s, 1732s, 1655w, 1471s, 1418s, 1376m, 1280s, 1230s, 1156s, 1107s, 1064m, 1025m, 921w, 867w, 718s cm^{-1}. MS calculated for $C_{57}H_{98}O_6Na^+$ = 901.7; found: 901.8. HMS calculated for $C_{57}H_{98}O_6H^+$ = 879.7442; found: 879.7445.

Synthesis of 1,2-Acetonide-3-(9,12-octadecadienoyl)glycerol **7**

1,2-Glycerol acetonide (solketal, **6**) was synthesized according to the procedure of Kubiak and Bruzik [18]. Solketal (3.76 g, 28.5 mmol) in DCM (40 ml) was mixed with 9,12-octadecadienoic acid (linoleic acid) (8.00 g, 28.5 mmol), DMAP (1.36 g, 11.2 mmol), and EDCI (6.56 g, 20.9 mmol). The solution was stirred at ambient temperature overnight before most of the solvent was removed under reduced pressure, and the residue was passed through a short plug of silica (100 g silica, 200 ml of 20:80 ether/DCM by vol. eluent). The solvent was then removed under reduced pressure, yielding the title compound **7** as a colorless oil (11.2 g, 100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, *J* 7.0 Hz, 3H, linoleic C*H*$_3$), 1.27–1.41 (m, 16H, 8× C*H*$_2$), 1.36 (s, 3H, acetonide C*H*$_3$), 1.42 (s, 3H, acetonide C*H*$_3$), 1.58–1.69 (m, 2H, $O_2CCH_2CH_2CH_2CH_2$), 2.00–2.09 (m, 4H, C*H*$_2$CH=CHCH$_2$CH=CHC*H*$_2$), 2.33 (t, *J* 7.5 Hz, 2H, linoleic $CO_2CH_2CH_2CH_2$), 2.75 (apparent t, 5.7 Hz, 2H, CH=CHC*H*$_2$CH=CH), 3.72 (dd, *J* 6.2, 8.4 Hz, 1H, one of OC*H*$_2$CHC*H*$_2$O), 4.03–4.18 (m, 3H, thee of OC*H*$_2$CHC*H*$_2$O), 4.29 (m, 1H, CH_2CHOCH_2), 5.26–5.42 (m, 4H, alkene C*H*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.2 (*C*H$_3$), 22.7 (*C*H$_2$), 25.0 (*C*H$_2$), 25.5 (*C*H$_3$), 25.8

(CH_2), 26.8 (CH_3), 27.3 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.5 (CH_2), 29.7 (CH_2), 31.6 (CH_2), 34.2 (CH_2), 64.7 (OCH_2), 66.5 (OCH_2), 73.8 (OCH), 109.9 ($O_2C(CH_3)_2$ acetonide), 128.0 (CH alkene), 128.2 (CH alkene), 130.1 (CH alkene), 130.3 (CH alkene), 173.6 (C=O linoleic). MS calculated for $C_{24}H_{42}O_4Na^+$ = 417.3; found: 417.3. HMS calculated for $C_{24}H_{42}O_4Na^+$ = 417.2981; found: 417.2986.

Synthesis of 1,2-Acetonide-3-(9,12, 15-octadecatrienoyl)glycerol **8**

A similar procedure as for **7** was followed. 1,2-Acetonideglycerol **6** (3.76 g, 28.5 mmol) in DCM (40 ml) was reacted with 9,12,15-octadecatrienoic acid (linolenic acid) (8.36 g, 30.0 mmol), DMAP (1.44 g, 11.8 mmol), and EDCI (6.88 g, 22.3 mmol), and the product was purified by passage through a short plug of silica (100 g silica, 200 ml of 20:80 ether/DCM by vol. eluent) to obtain the title compound **8** as a colorless oil (10.7 g, 96%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.94 (t, J 7.5 Hz, 3H, linolenic CH_3), 1.22–1.31 (m, 8H, 4× CH_2), 1.33 (s, 3H, CH_3 acetonide), 1.39 (s, 3H, CH_3 acetonide), 1.54–1.61 (m, 2H, $CO_2CH_2CH_2CH_2$), 2.00–2.09 (m, 4H, CH_2CH=$CHCH_2CH$=$CHCH_2CH$=$CHCH_2$), 2.30 (t, J 7.7 Hz, 2H, linolenic $O_2CCH_2CH_2CH_2$), 2.77 (apparent t, 5.6 Hz, 4H, CH_2CH=$CHCH_2CH$=$CHCH_2$ CH=$CHCH_2$), 3.69 (dd, J 6.2, 8.4 Hz, 1H, one of OCH_2CHCH_2O), 4.00–4.11 (m, 3H, thee of OCH_2CHCH_2O), 4.25 (m, 1H, CH_2CHOCH_2), 5.26–5.33 (m, 6H, alkene CH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.2 (CH_3), 19.5 (CH_2), 23.8 (CH_2), 24.4 (CH_3), 24.5 (CH_2), 24.6 CH_2), 25.6 (CH_3), 26.2 (CH_2), 28.1 (CH_2), 28.6 (CH_2), 33.1 (CH_2), 63.5 (OCH_2), 65.4 (OCH_2), 72.7 (OCH), 108.8 ($O_2C(CH_3)_2$ acetonide), 126.1 (CH alkene), 126.7 (CH alkene), 127.2 (CH alkene), 127.3 (CH alkene), 129.2 (CH alkene), 130.9 (CH alkene), 172.5 (C=O linolenic). I.R (neat) υ_{max} 3010s, 2932s, 2856s, 1742s, 1654w, 1456m, 1380s, 1371s, 1214s, 1161s, 1086s, 1058s, 918w, 843m, 792w, 721m cm^{-1}. MS calculated for $C_{24}H_{40}O_4Na^+$ = 415.3; found: 415.4. HMS calculated for $C_{24}H_{40}O_4Na^+$ = 415.2824; found: 415.2829.

Synthesis of 1,2-Acetonide-3-(3,6,9,12,15, 18-docosahexaenoyl)glycerol **9**

A similar procedure as for **7** was followed. 1,2-Acetonide glycerol (solketal, **6**) (3.00 g, 22.7 mmol) in DCM (100 ml) was reacted with DHA (8.22 g, 25.0 mmol), DMAP (1.26 g, 10.0 mmol), and EDCI (5.76 g, 18 mmol). The product was purified by passage through a plug of silica (100 g silica, 100 ml of 20:80 ether/DCM by vol. eluent) to obtain the title compound **9** as a colorless oil (7.51 g, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.97 (t, J 7.5 Hz, 3H, DHA CH_3), 1.37 (s, 3H, CH_3 acetonide), 1.43 (s, 3H, CH_3 acetonide), 2.00–2.12 (m, 4H, CH=$CHCH_2CH_2CH_3$), 2.41 (d, J 4.9 Hz, 2H, DHA O_2CCH_2C=CH_2), 2.80–2.90 (m, 10H, DHA 5× CH=$CHCH_2CH$=C), 3.73 (dd, J 6.1, 8.4 Hz, 1H, one of OCH_2CHCH_2O), 4.04–4.20 (m, 3H, three of OCH_2CHCH_2O), 4.27 (m, 1H, CH_2CHOCH_2), 5.29–5.44 (m, 12H, alkene CH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4 (CH_3), 20.7 (CH_2), 22.9 (CH_2), 25.5 (CH_3), 25.7 (CH_2), 25.7 CH_2), 25.8 (CH_2), 25.8 (CH_2), 26.8 (CH_3), 34.1 (CH_2), 64.8 (OCH_2), 66.5 (OCH_2), 73.8 (OCH), 110.0 ($O_2C(CH_3)_2$ acetonide), 127.2 (CH alkene), 127.2 (CH alkene), 127.9 (CH alkene), 128.0 (CH alkene), 128.2 (CH alkene), 128.3 (CH alkene), 128.4 (CH alkene), 128.5 (CH alkene), 128.7 (CH alkene), 129.6 (CH alkene), 132.2 (CH alkene), 173.0 (C=O DHA). IR (neat) υ_{max} 3013s, 2964s, 2933s, 1715s, 1654w, 1455m, 1380s, 1371s, 1258s, 1215s, 1157s, 1084s, 1058s, 990m, 928m, 843m, 792w, 711s cm^{-1}. MS calculated for $C_{28}H_{42}O_4Na^+$ = 465.3; found: 465.3. HMS calculated for $C_{28}H_{42}O_4Na^+$ = 465.2981; found: 465.2977.

Synthesis of 3-(9,12-Octadecadienoyl)glycerol **10**

An adaptation of the procedure of Kubiac and Bruzik [18] was used. A solution of 1,2-acetonide-3-(9,12-octadecadienoyl)glycerol **7** (14.0 g, 33.5 mmol) in methanol/DCM (2:1 by vol., 150 ml) was mixed with Amberlyst-H$^+$ resin (5 g). The mixture was stirred at ambient temperature for 2 days before the resin was filtered off and the solvent removed under reduced pressure. The residual oil was purified by flash chromatography (150 g silica, 2 l of 60:40 ethyl acetate/hexanes by vol.) to yield the title compound **10** as a clear oil (9.75 g, 78%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.89 (t, J 6.8 Hz, 3H, linoleic CH_3), 1.19–1.46 (m, 14H, 7× CH_2), 1.55–1.76 (m, 2H, $CO_2CH_2CH_2CH_2$), 2.04 (m, 4H, CH_2CH=$CHCH_2CH$=$CHCH_2$), 2.35 (t, J 7.2 Hz, 2H, $CO_2CH_2CH_2CH_2$), 2.50 (bs, 2H, 2× OH), 2.77 (apparent t, J 5.1 Hz, 2H, CH=$CHCH_2CH$=CH), 3.58 (dd, J 5.8, 11.1 Hz, 2H, one each of OCH_2CHCH_2O), 3.70 (dd, 3.9, 11.5 Hz, 2H, one of each OCH_2CHCH_2O), 3.88–4.21 (m, 1H, CH_2CHOCH_2), 5.31–5.47 (m, 4H, alkene CH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.6 (CH_3), 22.7 (CH_2), 25.0 (CH_2), 25.4 (CH_2), 25.8 (CH_2), 27.3 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.5 (CH_2), 29.7 (CH_2), 31.7 (CH_2), 33.5 (CH_2), 34.3 (CH_2), 63.5 (OCH_2), 65.3 (OCH_2), 71.6 (OCH), 128.0 (CH alkene), 128.2 (CH alkene), 130.1 (CH alkene), 130.4 (CH alkene), 174.4 (C=O linoleic). IR (neat) υ_{max}

3421s, 3010s, 2929s, 2856s, 1731s, 1648w, 1466m, 1378w, 1247w, 1181w, 1120w, 1050w, 909s, 734s, 649m cm^{-1}. MS calculated for $C_{21}H_{38}O_4Na^+$ = 377.3; found: 377.3. HMS calculated for $C_{21}H_{38}O_4Na^+$ = 377.2668; found: 377.2670.

Synthesis of 3-(9,12,15-Octadecatrienoyl)glycerol **11**

A similar procedure as for **10** was used. A solution of 1,2-acetonide-3-(9,12,15-octadecatrienoyl)glycerol **8** (8.1 g, 20.6 mmol) in methanol/DCM (2:1 by vol., 120 ml) was mixed with Amberlyst-H^+ resin (3.5 g) and stirred at ambient temperature for 2 days. The product was recovered and purified by flash chromatography (140 g silica, 2 l of 60:40 ethyl acetate/hexanes by vol.) to yield the title compound **11** as a clear oil (5.07 g, 69%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.94 (t, *J* 7.5 Hz, 3H, linolenic CH_3), 1.21–1.41 (m, 8H, 4× CH_2), 1.54–1.68 (m, 2H, $CO_2CH_2CH_2CH_2$), 2.01–2.08 (m, 4H, CH_2CH=CHCH$_2$ CH=CHCH$_2$CH=CHCH_2), 2.33 (t, *J* 7.4 Hz, 2H, linolenic $O_2CCH_2CH_2CH_2$), 2.62 (bs, 1H, OH), 2.79 (apparent t, 5.6 Hz, 4H, CH_2CH=CHCH_2CH=CH CH_2CH=CHCH_2), 2.98 (bs, 1H, OH), 3.57 (dd, *J* 5.9, 11.5 Hz, 1H, one of OCH_2CHCH_2O), 3.67 (dd, *J* 3.8, 11.5 Hz, 1H, one of OCH_2CHCH_2O), 3.90 (m, 1H, CH_2C*H*OCH_2), 4.10–4.20 (m, 2H, two of OCH_2CH-CH_2O), 5.28–5.40 (m, 6H, alkene C*H*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.4 (*C*H_3), 20.7 (*C*H_2), 25.0 (*C*H_2), 25.6 (*C*H_2), 25.7 (*C*H_2), 27.3 *C*H_2), 29.2 (*C*H_2), 29.3 (*C*H_2), 29.7 (*C*H_2), 34.2 (*C*H_2), 34.4 (*C*H_2), 63.5 (O*C*H_2), 65.2 (O*C*H_2), 70.4 (O*C*H), 127.2 (*C*H alkene), 127.9 (*C*H alkene), 128.4 (*C*H alkene), 128.4 (*C*H alkene), 130.3 (*C*H alkene), 132.1 (*C*H alkene), 174.4 (*C*=O linoleic). IR (neat) υ_{max} 3396s, 3011s, 2930s, 2855s, 1739s, 1654w, 1462s, 1392m, 1244m, 1178s, 1120s, 1056s, 932w, 866w, 714m cm^{-1}. MS calculated for $C_{21}H_{36}O_4Na^+$ = 375.3; found: 375.4. HMS calculated for $C_{21}H_{36}O_4Na^+$ = 375.2511; found: 375.2511.

Synthesis of 3-(3,6,9,12,15, 18-docosahexaenoyl)glycerol **12**

A similar procedure as for **10** was used. A solution of 1,2-acetonide-3-(3,6,9,12,15,18-docosahexaenoyl)glycerol **9** (4.50 g, 10.2 mmol) in 2:1 MeOH/DCM (90 ml) was mixed with Amberlyst-H^+ resin (2 g). The mixture was stirred at ambient temperature overnight before the resin was filtered off, and solvent was removed under reduced pressure. The residual oil was purified by flash chromatography (ethyl acetate/hexane, 60:40 by vol.) to yield the title compound **12** as a clear oil (3.60 g, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.97 (t, *J* 7.5 Hz, 3H, DHA CH_3), 2.02–2.12 (m, 2H, CH=CHCH$_2$CH_2CH$_3$), 2.26 (bs, 1H, OH), 2.39–2.45 (m, 4H, DHA O_2CCH_2C=CH$_2$ and CH=CHCH_2CH$_2$CH$_3$), 2.68 (bs, 1H, OH), 2.79–2.89 (m, 10H, DHA 5× CH=CHCH_2CH=C), 3.58 (dd, *J* 5.8, 11.5 Hz, 1H, one of OCH_2CHCH_2O), 3.68 (dd, *J* 3.9, 11.5 Hz, 1H, one of OCH_2CHCH_2O), 3.88–3.95 (m, 1H, one of OCH_2CH CH_2O), 4.08–4.23 (m, 2H, one of OCH_2CHCH_2O and OCH$_2$C*H*CH$_2$O), 5.28–5.45 (m, 12H, alkene C*H*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.2 (*C*H_3), 19.5 (*C*H_2), 21.7 (*C*H_2), 24.5 (*C*H_3), 24.6 (*C*H_2), 24.6 *C*H_2), 33.0 (*C*H_2), 62.3 (O*C*H_2), 64.3 (O*C*H_2), 69.2 (O*C*H), 126.0 (*C*H alkene), 126.7 (*C*H alkene), 126.9 (*C*H alkene), 126.9 (*C*H alkene), 127.1 (*C*H alkene), 127.3 (*C*H alkene), 127.3 (*C*H alkene), 127.4 (*C*H alkene), 127.6 (*C*H alkene), 128.6 (*C*H alkene), 131.0 (*C*H alkene), 172.5 (*C*=O DHA). IR (neat) υ_{max} 3045s, 3013s, 2963s, 2932s, 1740s, 1654w, 1442m, 1391m, 1266m, 1161m, 1120m, 1053m, 988m, 928m, 792w, 708s cm^{-1}. MS calculated for $C_{25}H_{38}O_4Na^+$ = 425.3; found: 425.5. HMS calculated for $C_{25}H_{38}O_4Na^+$ = 425.2668; found: 465.2667.

Synthesis of 1,2-Dihexadecanoyl-3-(9,12-octadecadienoyl)glycerol (PPL) **13**

A similar procedure as for **3** was followed. 3-(9,12-ctadecadienoyl)-Glycerol **10** (4.00 g, 10.8 mmol) in DCM (80 ml) was reacted with hexadecanoic acid (palmitic acid) (5.52 g, 21.6 mmol), DMAP (1.04 g, 8.6 mmol), and EDCI (4.96 g, 25.9 mmol). The product was purified by passage through a short plug of silica (100 g silica, 100 ml of 20:80 ether/DCM by vol. eluent) to obtain the title compound **13** (92:8 PPL:PLP by ^{13}C NMR) as a white solid (7.88 g, 88%). MP 25–28°C. ^{1}H NMR (800 MHz, $CDCl_3$) δ 0.88 (t, *J* 7.3 Hz, 6H, 2× CH_3 palmitic), 0.89 (t, *J* 7.8 Hz, 3H, CH_3 linoleic) 1.23–1.38 (m, 64H, 32× CH_2), 1.57–1.63 (m, 6H, 3× $CO_2CH_2CH_2CH_2$), 2.03–2.06 (m, 4H, CH_2CH=CH CH$_2$CH=CHCH_2), 2.31 (t, *J* 7.2 Hz, 6H, 2× CO_2CH_2 CH_2CH_2), 2.32 (t, *J* 7.4 Hz, 3H, $CO_2CH_2CH_2CH_2$), 2.77 (apparent t, *J* 7.0 Hz, 2H, CH=CHCH_2CH=CH), 4.15 (dd, *J* 6.0, 11.9 Hz, one of each of OCH_2CH CH_2O), 4.29 (dd, *J* 4.1, 11.9 Hz, one of each of OCH_2CHCH_2O), 5.25–5.28 (m, 1H, OCH$_2$C*H*CH$_2$O), 5.30–5.41 (m, 4H, alkene C*H*). ^{13}C NMR (200 MHz, $CDCl_3$) δ 14.21 (*C*H_3), 14.26 (*C*H_3), 22.72 (*C*H_2), 22.84 (*C*H_2), 25.79 (*C*H_2), 27.35 (*C*H_2), 29.24 (*C*H_2), 29.28 (*C*H_2), 29.50 (*C*H_2), 29.64 (*C*H_2), 29.66 (*C*H_2), 29.77 (*C*H_2), 29.78 (*C*H_2), 29.82 (*C*H_2), 29.86 (*C*H_2), 31.68 (*C*H_2), 32.08 (*C*H_2), 34.38 (*C*H_2), 62.27 (O*C*H_2), 69.03 (O*C*H), 128.05 (*C*H alkene), 128.22 (*C*H alkene), 130.16 (*C*H alkene), 130.37 (*C*H alkene), 173.03 (*C*=O,

1C, 2-palmitic), 173.40 (*C*=O, 1C, 3-linoleic), 173.44 (*C*=O, 1C, 1-palmitic). IR (neat) υ_{max} 3009m, 2925s, 2854s, 1746s, 1466s, 1378m, 1236s, 1163s, 1116m, 722w cm^{-1}. MS calculated for $C_{53}H_{98}O_6Na^+$ = 853.7; found: 853.7. HRMS calculated for $C_{53}H_{98}O_6Na^+$ = 853.7261; found: 853.7265.

Synthesis of 1,2-Dihexadecanoyl-3-(9,12,15-octadecatrienoyl)glycerol (PPLn) **14**

A similar procedure as for **3** was followed. A solution of 3-(9,12,15-octadecatrienoyl)-Glycerol **11** (5.07 g, 14.4 mmol) in DCM (90 ml) was reacted with hexadecanoic acid (palmitic acid) (8.25 g, 31.8 mmol), DMAP (1.59 g, 12.9 mmol), and EDCI (7.44 g, 38.7 mmol). The product was purified by passage through a short plug of silica (100 ml of 20:80 ether/DCM by vol.) to yield the title compound **14** (88:12 PPLn:PLnP by ^{13}C NMR) as a white solid (10.3 g, 86%). MP 23–25°C. ^1H NMR (800 MHz, $CDCl_3$) δ 0.88 (apparent t, *J* 7.0 Hz, 6H, 2× palmitic CH_3), 0.98 (t, *J* 7.5 Hz, 3H, linolenic CH_3), 1.16–1.31 (m, 56H, 28× CH_2), 1.56–1.58 (m, 6H, 3× $O_2CCH_2CH_2CH_2$), 2.03–2.10 (m, 4H, CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2), 2.28–2.33 (m, 4H, 2× palmitic $O_2CCH_2CH_2$), 2.81 (apparent t, *J* 5.6 Hz, 4H, CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2), 4.14 (dd, *J* 6.0, 12.0 Hz, 2H, one each of OCH_2CHCH_2O), 4.29 (dd, *J* 7.8, 12.0 Hz, one each of OCH_2CHCH_2O), 5.25–5.41 (m, 7H, 6× C*H* alkene and CH_2CHOCH_2). ^{13}C NMR (200 MHz, $CDCl_3$) δ 14.26 (*C*H_3), 14.42 (*C*H_3), 20.70 (*C*H_2), 22.84 (*C*H_2), 24.99 (*C*H_2), 25.02 (*C*H_2), 25.06 (*C*H_2), 25.68 (*C*H_2), 25.77 (*C*H_2), 27.36 (*C*H_2), 29.24 (*C*H_2), 29.27 (*C*H_2), 29.33 (*C*H_2), 29.43 (*C*H_2), 29.45 (*C*H_2), 29.52 (*C*H_2), 29.63 (*C*H_2), 29.66 (*C*H_2), 29.74 (*C*H_2), 29.78 (*C*H_2), 29.82 (*C*H_2), 29.86 (*C*H_2), 32.08 (*C*H_2), 34.18 (*C*H_2), 34.21 (*C*H_2), 34.38 (*C*H_2), 62.27 (O*C*H_2), 69.03 (O*C*H), 127.27 (*C*H alkene), 127.90 (*C*H alkene), 128.40 (*C*H alkene), 128.45 (*C*H alkene), 130.39 (*C*H alkene), 132.11 (*C*H alkene), 173.03 (*C*=O, 1C, 2-palmitic), 173.40 (*C*=O, 1C, 3-linolenic), 173.44 (*C*=O, 1C, 1-palmitic). IR (neat) υ_{max} 3011s, 2926s, 2854s, 1747s, 1465w, 1377w, 1236m, 1162s, 1115m, 721w cm^{-1}. MS calculated for $C_{53}H_{96}O_6Na^+$ = 851.7105; found: 851.8. HMS calculated for $C_{53}H_{96}O_6H^+$ = 829.7285; found: 829.7270.

Synthesis of 1,2-Dihexadecanoyl-3-(3,6,9,12,15,18-docosahexaenoyl)glycerol (PPD) **15**

A similar procedure as for **3** was followed. A solution of 1-(3,6,9,12,15,18-docosahexaenoyl)glycerol **12** (3.60 g, 8.94 mmol) in DCM (100 ml) was reacted with hexadecanoic acid (palmitic acid) (5.10 g, 19.7 mmol), DMAP (0.99 g, 7.98 mmol) and EDCI (4.62 g, 24.0 mmol). The product was purified by passage through a short plug of silica (100 ml of 20:80 ether/DCM by vol.) to obtain the title compound **15** (90:10 PPL:PLP by ^{13}C NMR) as a white/yellow solid (7.01 g, 89%). MP 21–23°C. ^1H NMR (800 MHz, $CDCl_3$) δ 0.88 (apparent t, *J* 7.2 Hz, 6H, 2× palmitic CH_3), 0.97 (t, *J* 7.3 Hz, 3H, DHA CH_3), 1.22–1.34 (m, 50H, 25× CH_2), 1.58–1.63 (m, 4H, 2× palmitic $O_2CCH_2CH_2CH_2$), 2.06–2.10 (m, 2H, DHA CH=CHCH_2CH$_2$), 2.31 (t, *J* 7.4 Hz, 2H, palmitic $O_2CCH_2CH_2$), 2.32 (t, *J* 7.3 Hz, 2H, palmitic $O_2CCH_2CH_2$), 2.38 (d, *J* 2.8 Hz, 2H, O_2CCH_2CH=CH$_2$) 2.80–2.87 (m, 10H, DHA 5× C=CCH_2C=C), 4.14 (m, 2H, one each of OCH_2CH-CH_2O), 4.29 (dd, *J* 4.2, 11.9 Hz, 2H, one each of OCH_2CHCH_2O), 5.26–5.43 (m, 13H, 12× C*H* alkene and CH_2CHOCH_2). ^{13}C NMR (200 MHz, $CDCl_3$) δ 14.26 (*C*H_3), 14.42 (*C*H_3), 20.71 (*C*H_2), 22.77 (*C*H_2), 22.85 (*C*H_2), 25.02 (*C*H_2), 25.05 (*C*H_2), 25.70 (*C*H_2), 25.75 (*C*H_2), 25.79 (*C*H_2), 29.24 (*C*H_2), 29.28 (*C*H_2), 29.43 (*C*H_2), 29.44 (*C*H_2), 29.52 (*C*H_2), 29.64 (*C*H_2), 29.66 (*C*H_2), 29.78 (*C*H_2), 29.80 (*C*H_2), 29.82 (*C*H_2), 29.85 (*C*H_2), 32.0 (*C*H_2), 34.05 (*C*H_2), 34.20 (*C*H_2), 34.37 (*C*H_2), 62.24 (O*C*H_2), 62.44 (O*C*H_2), 68.99 (O*C*H), 127.17 (*C*H alkene), 128.14 (*C*H alkene), 128.03 (*C*H alkene), 128.15 (*C*H alkene), 128.24 (*C*H alkene), 128.26 (*C*H alkene), 128.41 (*C*H alkene), 128.43 (*C*H alkene), 128.54 (*C*H alkene), 128.73 (*C*H alkene), 129.63 (*C*H alkene), 132.19 (*C*H alkene), 172.70 (*C*=O, 1C, 3-DHA), 173.02 (*C*=O, 1C, 2-palmitic), 173.43 (*C*=O, 1C, 1-palmitic). I.R (neat) υ_{max} 3014s, 2925s, 2854s, 1747s, 1466s, 1377w, 1235m, 1154s, 1159m, 721m cm^{-1}. MS calculated for $C_{57}H_{98}O_6Na^+$ = 901.7; found: 901.8. HMS calculated for $C_{57}H_{98}O_6H^+$ = 879.7442; found: 879.7448.

Results and Discussion

Limited studies have been conducted on regiospecific synthesis of TAG containing n-3 LC-PUFA in combination with FA of a chain length C_{16} or greater [19–21]. Using a modification of the method of Awl et al. [20], Endo et al. [19] prepared approximately 1-g quantities of several TAG isomers containing DHA and eicosapentaenoic acid (EPA) by acylation of the appropriate MAG and diacylglycerol (DAG) in the presence of 1,1-dicyclohexylcarbodiimide (DCC) and 4-DMAP. Although excellent regioisomeric purity was obtained by this method, it is not suitable for the preparation of larger amounts required for functionality studies as the starting MAG and DAG are expensive and not readily

available in large quantities. Fauconnot et al. [21] also recently published syntheses of structured TAG pairs containing DHA and arachidonic acid using the (benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate (PyBOP) coupling reagent. Again, the regioisomeric purity appeared to be excellent, but the synthesis requires MAG and DAG starting materials that are not readily available. The coupling reactions were carried out on small scales (<100 mg) and gave variable yields (20–60%). The results disclosed in the present paper address this issue of availability of MAG and DAG on larger scales (5–10 g) for functionality studies.

Symmetrical TAG Synthesis

We synthesized the symmetrical TAG isomers **3–5** (Fig. 1) using the chemoenzymatic procedure of Halldorsson et al. [17]. Regioselective acylation of glycerol **1** with vinyl palmitate in the presence of *Candida antarctica* lipase (CAL) gave the corresponding 1,3-dipalmitate **2.** It was then coupled, using EDCI, to the desired PUFA to give the symmetrical 1,3-TAG isomers **3–5** in excellent yields (94–99%, with the exception of PLP which was obtained in 62% yield) and regiopurity (97–98% according to ^{13}C NMR; Table 1). The starting materials for this synthetic sequence are readily available, and the intermediates and products are easily isolated by crystallization or silica gel chromatography. In this manner, symmetrical TAG isomers containing linoleic, linolenic, and DHA acids were prepared on scales of up to 10 g.

Unsymmetrical TAG Synthesis

For the synthesis of the unsymmetrical TAG isomers **13–15** (Fig. 1), glycerol was protected, under standard conditions, as the 1,2-acetonide **6**. The unprotected sn-3 hydroxyl was then coupled, using EDCI, with the desired PUFA to give esters **7–9** in excellent yields. At this stage we experimented with suitable conditions for efficient removal of the acetonide protecting group. A number of groups have investigated this reaction. Working with saturated FA chains, Bornscheuer and Yamane [22] reported that acetonide hydrolysis catalyzed by boric acid produced MAG in good yield but

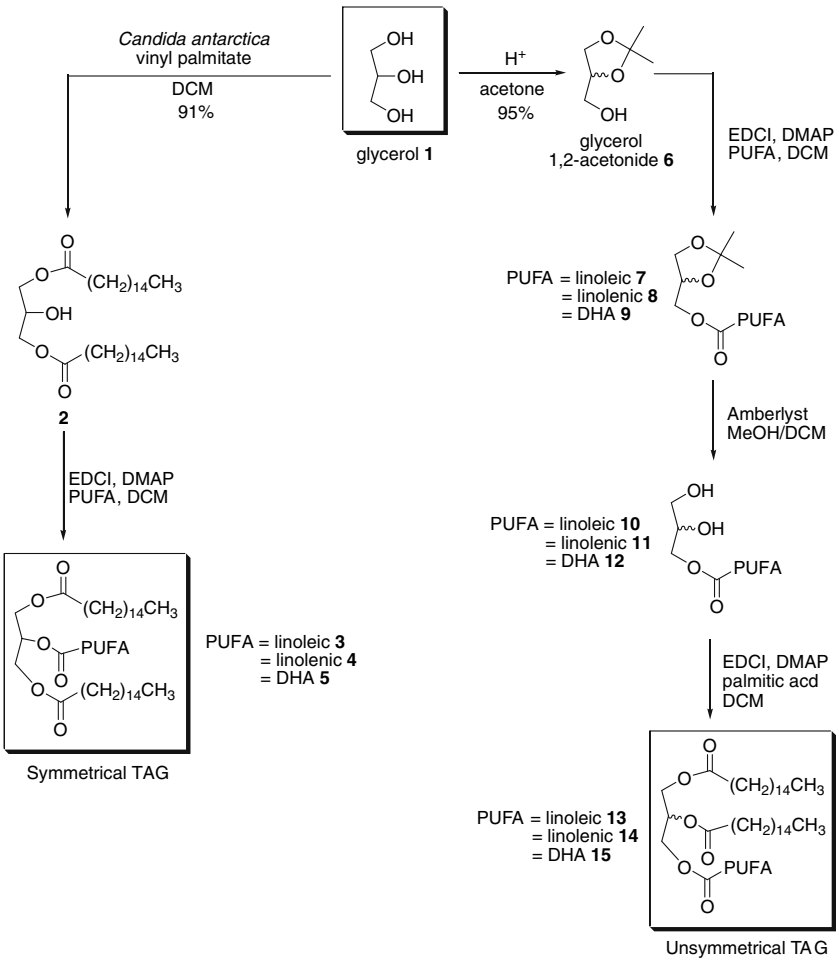

Fig. 1 Regiospecific syntheses of symmetrical and unsymmetrical triacylglycerols (TAG)

Table 1 Yields, regiopurities, melting points, and characteristic ^{13}C NMR data for synthetic triacylglycerol (TAG) regioisomers

TAG[a]	Yield (%)[b]	MP (°C)	^{13}C C=O shifts (ppm)	Regiopurity by enzymatic analysis[c] (%)	Regiopurity by high field ^{13}C NMR (%)
PLP	62	34–36	172.98, 173.44	94.8	97
PLnP	94	28–30	172.98, 173.42	98.0	98
PDP	99	24–26	172.29, 173.43	96.6	97
PPL	69	25–28	173.03, 173.40, 173.44	86.7	92
PPLn	57	23–25	173.03, 173.40, 173.44	84.1	88
PPD	60	21–23	172.70, 173.02, 173.43	82.2	90

[a] P, Palmitic acid; L, linoleic acid; Ln, linolenic acid; D, docosahexaenoic acid

[b] Overall yield from PUFA

[c] According to the method of Shimada et al. [16]

that the reaction was slow. Aqueous HCl (50%) is reported to be unsuitable because it results in high concentrations of DAG and TAG in the reaction mixture. In our experience, strong acids (e.g. HCl) are unsuitable for this purpose, particularly for compounds containing PUFA because of significant by-product formation, including the cleavage of the PUFA ester linkage and acyl migration and reesterification. Kodali [23] synthesized 1-MAG using dimethylboron bromide at –50°C to cleave the acetonide group, but we considered this inappropriate for larger scale synthesis because of the high cost of the reagent and the low reaction temperature required. The method of Kubiak et al. [18] was an attractive option for larger scale syntheses. It involves cleavage of acetonide groups with H^+-amberlyst resin/methanol at ambient temperature. Thus, the treatment of esters **7–9** with H^+-amberlyst resin/methanol gave MAG **10–12** in good yields, with no evidence of ester cleavage nor a reaction with the highly sensitive methylene interrupted alkene functionality. Evidence of some acyl migration in the ^{13}C NMR was observed and is discussed in detail later. A final coupling of two palmitic acid units onto the sn-1 and sn-2 positions of MAG was achieved with EDCI and afforded regioenriched unsymmetrical TAG **13–15** (Fig. 1) in good yields (57–69%) and high regiopurity (82–92% according to ^{13}C NMR; Table 1).

Melting Properties of TAG Regioisomers

The melting points of the various TAG isomers followed a predictable trend. The 1,3-symmetrical TAG with the PUFA in the middle position had slightly higher melting points than the corresponding unsymmetrical 1,2-TAG isomers where the PUFA are located in one of the outer positions. This concurs with the fact that randomized oils have a lower solid fat content than the parent oil because the PUFA predominantly located in the 2-position of glycerol in the parent oil is randomly distributed in the three glycerol positions in the randomized oils. We also observed that with increased unsaturation from linoleic, linolenic to DHA corresponded to a decrease in the melting point of the TAG.

^{13}C NMR Spectra

The regioassignment of the symmetrical TAG (PLP, PLnP, PDP) and unsymmetrical TAG (PPL, PPLn, PPD) targets were primarily made from high-field (200 MHz) ^{13}C NMR spectroscopy. For the symmetrical TAG **3–5** only two carbonyl resonances were observed, as expected, which included a signal integrating for one carbon in the range 172.29–172.98 ppm (1C, sn-2 PUFA carbonyl) and a single signal further downfield integrating for two carbons in the range 173.42–173.44 ppm [2C, sn-1(3) palmitic carbonyls] (Table 1). For each unsymmetrical TAG **13–15**, three distinct carbonyl resonances were observed for each, as expected. The sn-2 carbonyl (palmitic) resonances came in the upfield range of 172.70–173.03 ppm, while the two distinct sn-1,3 (PUFA or palmitic) carbonyl resonances were in the range of 173.02–173.44 ppm (Table 1). This carbonyl data is in agreement with that reported by Haraldsson and co-workers [17, 24] and Bergana and Lee [25] for similarly structured TAG.

The ^{13}C NMR alkene region was also useful when assigning the regioposition of the unsaturated carbon chain on the glycerol backbone. Specifically, for the PLP/PPL pair, the C-9 (130.14) and C-12 (128.04) PLP resonances were lower than that for the C-9 (130.16) and C-12 (128.05) resonances of PPL, whereas the C-10 (128.24) and C-13 (130.38) PLP resonances were higher than that for the C-10 (128.22) and C-13 (128.24) carbons of PPL. A similar pattern was observed for the PLnP/PPLn pair. This trend in difference of alkene carbon shifts for an unsaturated chain in

the sn-2 versus sn-3 position is in agreement with the work of Wollenberg [26] on TAG-containing linoleic and linolenic acid.

Regiopurity

The regiopurity of each TAG was first determined by ^{13}C NMR (Table 1). The regiopurities of the PLP and PPL isomers were 97 and 92% based on the ratio of the carbonyl signals at 173.44 ppm (PLP) and 173.4 ppm (PPL), respectively. Similarly, the regiopurities of PLnP and PPLn were determined to be 98 and 88%, respectively, and those of PDP and PPD, 97 and 90%, respectively. For the PLnP/PPLn pair the carbonyl resonances at 172.98 (PLnP) and 173.03 (PPLn) were used, and for the PDP/PPD pair, the resonances at 172.29 (PDP) and 172.7 (PPD) were used.

Further evidence for the high regiopurity of the synthesized TAG was provided by enzyme-based positional analysis. Lipase-based methods are generally considered to be unsuitable for positional analysis of EPA and DHA because they have been shown to resist hydrolysis by lipase [24]. However, Shimada et al. [16] recently reported that immobilized CAL acts on saturated and unsaturated C_{14}–C_{24} FA to a similar degree, subsequently proposing that this lipase could be used for positional analysis of DHA-containing oils. Analysis of the synthetic TAG according to the method of Shimada et al. [16] gave regiopurities of 82–98% (Table 1). The actual purities of the compounds were probably better than those indicated by the lipase-based measurements, particularly for the unsymmetrical isomers, considering the likelihood of a small amount of acyl migration during isolation of the 2-MAG by chromatography on silica. Furthermore, we have recently shown (unpublished) that CAL lipase releases DHA from TAG more slowly than other FA, resulting in an underestimation of the regiopurity of TAG isomers in which the DHA is located at sn-1(3).

Acyl Migration

Acyl migration is a major problem in the synthesis and purification of regiopure structured TAG [17, 23, 27–29]. The rate of migration depends upon many factors, including temperature, solvent type, and the presence of trace acid or base impurities. ^{13}C NMR analysis and enzyme-based positional analysis revealed that the symmetrical TAG (PLP, PLnP and PDP) showed very little acyl migration, which is consistent with the findings of Halldorsson et al. [17]. For the unsymmetrical TAG (PPL, PPLn and PPD), acyl migration in the range of 8–12% was observed. This can be rationalized in the context that 3-MAG intermediates **10–12** are precursors to TAG **13–15** (Fig. 1) and that MAG are known to isomerize faster than 1,2- or 1,3-DAG [30]. ^{13}C and ^{1}H NMR analysis of the glycerol 1,2-acetonide-3-PUFA intermediates **7–9** showed that they were free from the corresponding 1,3-isomers, but following amberlyst-catalyzed methanolysis a small amount of the 2-MAG isomer could be detected. Acyl migrations catalyzed by H^+-amberlyst resin have been observed previously [31]. Equilibrium ratios of MAG are known to be approximately 90:10 in favor of the 1-MAG isomer [32], which suggests something near equilibrium was reached during the acetonide cleavage reaction. An equilibrium ratio of 1,3- and 1,2-DAG is generally 60:40 favoring the 1,3-DAG [32], which explains the relatively low amount of migration during syntheses of the symmetrical 1,3-TAG.

The synthesis of unsymmetrical TAG via glycerol 1,2-acetonide and the removal of the protecting group by hydrolysis with H^+-amberlyst resin/methanol is readily amenable to scale-up. This affords a practical method for preparing unsymmetrical TAG containing PUFA in quantities required for functionality studies. The synthesis has the potential of being made enantioselective, as both enantiomers of glycerol 1,2-acetonide (solketal) are readily available from sugar precursors.

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