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Large-scale synthesis of both symmetrical and unsymmetrical triacylglycerols containing docosahexaenoic acid

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ABSTRACT

A large-scale (~100 g) synthesis of symmetrical and unsymmetrical triacylglycerols containg docosahexaenoic acid (D) and two of either lauric (L), palmitic (P) or stearic acid (S) is described. Key improvements in purification of synthetic intermediates, in addition to a more efficient acetonide cleavage reaction affords the six TAGs (LaDLa, LaLaD, PDP, PPD, SDS, SSD) in yields of 80–90% and in regioisomeric purities greater than or equal to 90%.

1. Introduction

Currently there is significant interest in the manufacture of food products fortified with oils containing *n*−3 long-chain (C_{20} and greater) polyunsaturated fatty acids (*n*−3 LC-PUFAs) such as docosahexaenoic acid (DHA). This interest stems from mounting scientific evidence showing beneficial health effects of increased dietary intake of *n*−3 LC-PUFAs.[1] However, oils containing *n*−3 LC-PUFAs, particularly DHA, are extremely susceptible to oxidation leading to the formation of off-flavours, which severely compromise the flavour quality and shelf-life of the product. It is possible that the oxidative stability as well as the bioactivity of DHA-containing oils is related not only to the amounts of DHA but also the position of DHA on the triacylglycerol (TAG). However, only limited studies have been conducted to elucidate DHA positional effects on oil functionality. A major impediment to such investigations is the lack of access to DHA-containing TAGs positional isomers in the required purity and quantity. Although numerous methods have been published for the enzymatic synthesis of symmetrical TAGs,[2,4] there is substantially less published work on unsymmetrical TAGs[3] and in general, these procedures give moderate yields and are not suitable for larger-scale syntheses. We now report on a practical synthesis of regioisomerically enriched, symmetrical and unsymmetrical TAGs containing DHA in yields of 80–90% and in regioisomeric purities above 90%.

2. Results and discussion

2.1. Preparation of symmetrical TAGs

The first step in the synthesis of the symmetrical TAGs **4**–**6** is a 1,3-selective enzyme mediated acylation of glycerol. Preparation of up to 100 g each of the 1,3-diacylgylcerols **1**–**3** is achieved through adaptation of the procedure of Haraldsson.[4] This involved adding the appropriate vinyl ester (~90 g) in CH_2Cl_2 (50 mL) at 5 °C to a suspension of glycerol and solid supported lipase B Novozyme 435. Reactions were run for 12–18 h and yields of >90% were obtained (Scheme 1). We found this reaction is well suited for large-scale synthesis as only a relatively small amount of CH_2Cl_2 is required for a 100-g scale reaction. We also found care must be taken to keep the reaction temperature at 5 °C when using vinyl laurate (C12) as significant amounts (up to ~25% contamination) of the trilauroylglycerol results if the reaction warms to room temperature overnight. This undesired side reaction in the case of shorter chain vinyl esters was previously noted by Haraldsson.[4]

The synthesis of symmetrical TAGs **4**–**6** is completed by coupling of DHA to the 2-position of 1,3-diacylgylcerols **1**–**3** by treatment with EDCI and DMAP in CH_2Cl_2 (Scheme 1). The major advantage, on large scales (~100 g), of using EDCI is that there is a substantial difference in R_f values on silica gel plates between the TAG products **4**–**6** (R_f 0.95) and the polar coupling by-products (R_f<0.05). This allows for easy separation on a 12.5 cm×10 cm silica plug using CH_2Cl_2 as an eluent (Fig. 1). We have found the less expensive coupling reagent DCC unsuitable as it makes purification difficult on large scales due to the less polar nature of coupling by-products. This advantage was noted by Haraldsson and co-workers in their

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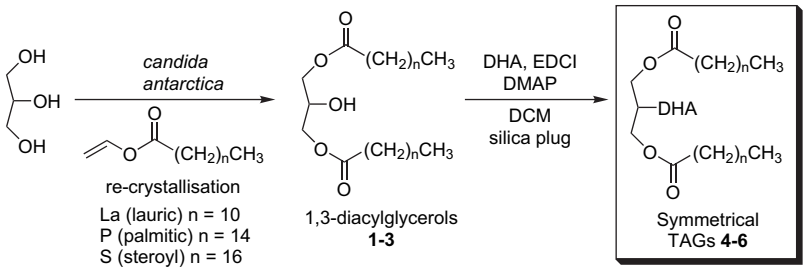


Scheme 1. Preparation of symmetrical TAGs **4**–**6**.

small scale synthesis of TAGs[4] and becomes of even greater significance on larger scales because it reduces substantially the amount of silica gel required for purification.

2.2. Preparation of unsymmetrical TAGs

Synthesis of the unsymmetrical TAGs **10**–**12** begins by coupling the hydroxyl group in the 3 position of glycerol-1,2-acetonide **7** to DHA using EDCI in CH_2Cl_2 (Scheme 2).

The resulting acetonide protected MAG **8** is then purified on a large scale (~60 g) by passage through a 12.5 cm×10 cm silica plug. Again the significant R_f difference between the acetonide protected MAG **8** and the polar coupling by-products allows for efficient separation.

The second step in the synthesis of the unsymmetrical TAGs **10**–**12** is the removal of the acetonide protecting group. This is performed through acid catalysed methanolysis of MAG **8** (step 2) and is the key step in this synthesis. The challenges are to (i) efficiently remove the acetonide group without oxidising the sensitive methylene interrupted alkene functionality of DHA, (ii) avoid ester cleavage and (iii) minimise acyl migration. Previously we found[3b] Amberlyst-15 in methanol gave reasonable yields (~60%) of the desired MAG **9**. In an attempt to improve this yield we carried out the methanolysis of the acetonide group in HPLC grade methanol (as trace metal impurities are known to catalyse the oxidation of DHA[5]) and followed the reaction progress by ^{1}H NMR spectroscopy. Shown below is the acetonide cleavage reaction monitored over a period of 40 h (Fig. 2).

After 33 h, the reaction is essentially complete and the yield of MAG **9** is greater than 90%. Reactions that are allowed to continue for longer than 40 h decrease in yield significantly due to the competing ester cleavage and oxidation of the DHA chain. The same reaction performed with reagent grade methanol also gives a substantially lower yield (70%) when performed over a period of 33 h. Identifying this optimal reaction time (33 h), and using HPLC grade methanol, has allowed us to increase the yield of MAG **9** to 90%.

The purification of MAG **9** was carried out on a silica plug using 1:1 EtOAc/hexane eluent (Fig. 3). Minor amounts of unreacted acetonide MAG **8** (<5%) elute first with an R_f of 0.9 (1:1 EtOAc/hexane) and can be collected and re-cycled. Free DHA, which is also formed in minor amounts through competing ester cleavage, elutes second (R_f 0.7 1:1 EtOAc/hexane) and can also be collected and re-cycled. This is a significant cost saving exercise, for larger-scale reactions, given the expense of commercially available DHA (~$65 per gram).[6]

The synthesis of the unsymmetrical TAGs **10**–**12** is completed by the coupling of 2 equiv of saturated fatty acid to the two free hydroxyl groups of MAG **9** (Scheme 2). To achieve this we again employed EDCI, and again, the polarity of the EDCI coupling by-products allows purification of the resulting unsymmetrical TAGs **10**–**12** on large scales (~100 g) in an identical manner to that shown earlier (Fig. 1).

2.3. Acyl migration and ^{13}C NMR spectroscopy

Acyl migration is a major problem in the synthesis and purification of regioisomerically pure TAGs. The rate of migration depends upon many factors including temperature, solvent and the presence of trace acid or base impurities.[7] In this work, the regioisomeric purity of each TAG (**4**–**6**, **10**–**12**) was determined, as previously,[3b] by integration of carbonyl signals in the ^{13}C NMR spectra (Table 1). Each of the symmetrical TAGs **4**–**6** contained the expected two carbonyl resonances in a ratio of 2:1 while the unsymmetrical TAGs **10**–**12** contained the expected three resonances

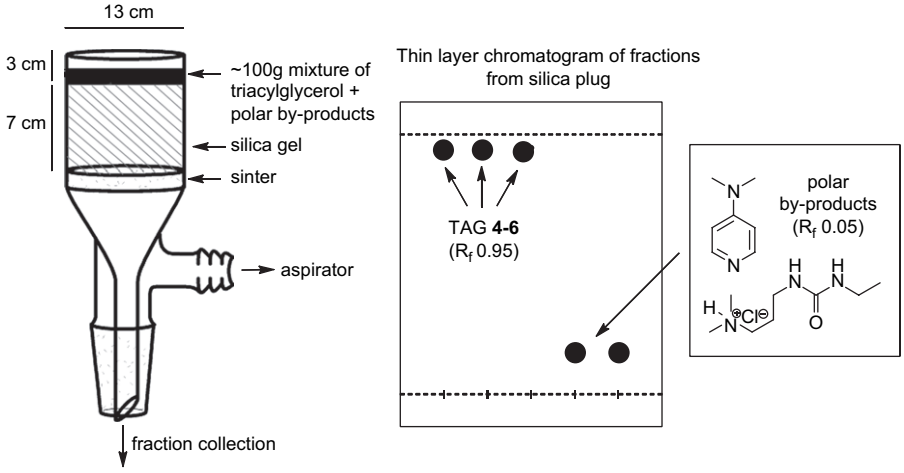
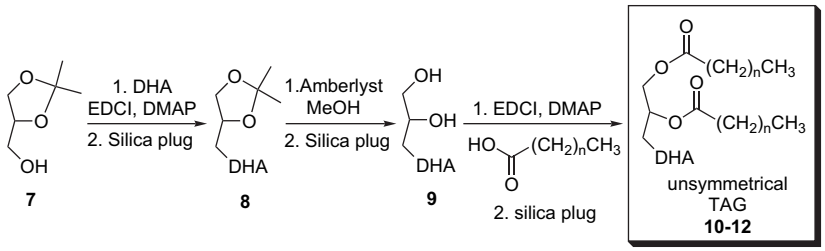


Figure 1. Silica plug purification of TAGs **4**–**6**.



Scheme 2. Preparation of unsymmetrical TAGs **10**–**12**.

in a 1:1:1 ratio. The symmetrical pairs **4**–**6** all had regio-purities of greater than 95%. As the equilibrium ratio of 1,3-DAG to 1,2-DAG isomers is known to be approx. 60:40[8] this suggests that very little acyl migration occurred during the enzymatic 1,3-acylation step, and also, the final coupling step. For the unsymmetrical TAGs **10**–**12** their regioisomeric purity ranged from 90% to 92%. Given that these TAG were all derived from MAG **8** and that the equilibrium ratio of 1-MAG to 2-MAG isomers is known to be approx. 90:10[8] this suggests that a near equilibrium state was obtained for the acetonide cleavage reaction. This is not unexpected given Amberlyst-15 has been noted previously to catalyse acyl migrations of TAGs.[9]

2.4. Melting points

The melting points of the synthesised TAGs (**4**–**6**, **10**–**12**) were measured and are reported in Table 1. As expected the TAGs containing lauric acid (**4**, **10**) had the lowest melting points (liquids at room temperature). As the length of the saturated fatty acid increased (**5**, **11**) the melting points increased, expectedly, to 29–31 °C and 25–27 °C, respectively. The TAGs containing the stearic acid (**6**, **12**) had the highest melting points at 39–40 °C and 37–39 °C, respectively. Interestingly there was little difference between SDS and SSD suggesting the length of alkyl chain becomes the significant factor, and not its position on the glycerol backbone, in determining the melting point.

3. Summary

The large-scale synthesis of approx. 100 g each of symmetrical TAGs (**4**–**6**) was achieved through a scaled up adaptation of the procedure of Haraldsson.[4] In a complementary outcome the synthesis of approx. 100 g each of unsymmetrical TAGs (**10**–**12**) was achieved through improving and refining our previously reported method.[3b] Both the adapted and revised synthetic procedures utilise the EDCI coupling reagent, and the advantages of this reagent are most apparent on larger scales, as significant amounts (100 g) of product can be purified on a moderately sized (7 cm tall, 13.5 cm wide) plug of silica gel. For the unsymmetrical TAGs, the yield of the key acetonide cleavage reaction was improved from 60% to 90% by, firstly changing the solvent to HPLC grade methanol to decrease DHA oxidation, and secondly by optimising the reaction time to 33 h. Careful purification also allowed re-cycling of DHA, which contributes significantly to cost savings for large-scale synthesis of TAGs. The products were obtained in yields of 80–90% from DHA, and in regioisomeric purities above 90%. To the best of our knowledge, this is the first efficient and practical method for the large-scale laboratory synthesis of TAGs containing DHA. Its simplicity and reliability will enable the large-scale synthesis of TAGs, which are required for animal feeding studies and other larger-scale studies.

4. Experimental

4.1. General procedures

Materials. Most reactants were purchased from the Aldrich Chemical Company (Sydney, Australia) and were used as supplied. Vinyl palmitate was purchased from TCI (Japan). Vinyl laurate and vinyl stearate were purchased from Sigma–Aldrich. *Candida antarctica* (Novozym® 435) was a gift of Novozymes Australia Pty. Ltd (Sydney, Australia). DHA (>99% purity) was purchased from Nu-Chek Prep, Inc. (MN, USA). Drying agents and inorganic salts were purchased from AJAX or BDH chemicals. Solvents were purified as follows. Dichloromethane was distilled from calcium hydride. Hexanes were distilled prior to use and refer to the fraction boiling between 40–60 °C. Ethyl acetate (Reagent grade) was purchased from Merck and used without any further purification. *Chromatography*. Silica gel used for chromatography was 40–63 μm (230–400 mesh) silica gel 60 (Merck No. 9385). Analytical TLC was performed on Polygram Sil G/UV_{254} plastic sheets coated with silica gel containing UV_{254} fluorescent indicator and visualised under UV

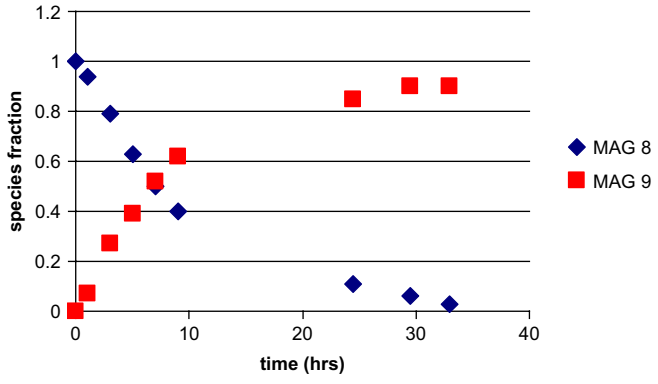


Figure 2. Reaction profile for acetonide cleavage of MAG **8**.

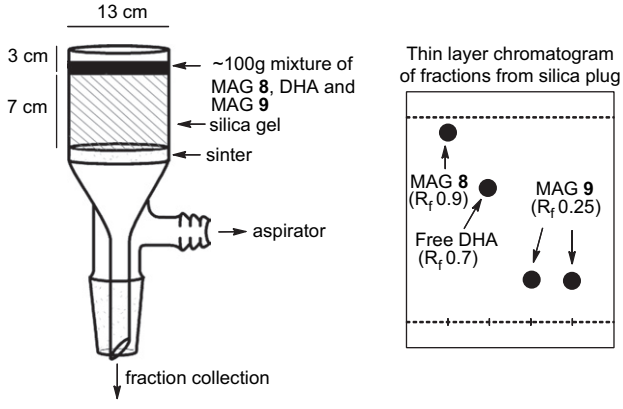


Figure 3. Silica plug purification of MAG **9**.

Table 1
Yields, regioisomeric purities, melting points and characteristic ^{13}C NMR data for TAG regioisomers

TAG	Yield[a] (%)	Mp (°C)	^{13}C C=O shifts (ppm)	Regiopurity by high field ^{13}C NMR (%)
LaDLa **4**	90	—	172.31, 173.44	95
PDP **5**	89	29–31	172.25, 173.38	97
SDS **6**	92	39–40	172.20, 173.32	98
LaLaD **10**	81	—	172.71, 173.04, 173.44	92
PPD **11**	84	25–27	172.70, 173.02, 173.43	90
SSD **12**	79	37–39	172.65, 172.98, 173.38	92

La, P, S and D refer to lauric, palmitic, stearic and docosahexaenoic acid, respectively.
[a] Overall yield from DHA.

light and/or dipped in an ammonium molybdate/cerium sulfate solution. *Proton NMR (^1H NMR) spectroscopy*. ^1H NMR spectra were recorded at 300 MHz on a Bruker AM 300 spectrometer or 400 MHz on a Bruker Avance DRX 400 spectrometer. 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. *Carbon NMR (^{13}C NMR) spectroscopy*. ^{13}C NMR spectra were recorded at 75 MHz on a Bruker AM 300 spectrometer using $CDCl_3$ unless otherwise stated. The spectra were referenced using the solvent carbon signal ($CDCl_3$=77.16 ppm). 2D NMR techniques such as homonuclear COrrelation SpectroscopY (COSY), Heteronuclear Multiple Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Coherence (HMBC) were used to aid assignment of some NMR spectra. *Mass spectrometry*. ESI was performed on a Micromass Platform QMS spectrometer. 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 spectroscopy*. IR spectra were recorded on a Perkin Elmer 1600 Series Fourier Transform spectrometer 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 reported qualitatively as strong (s), medium (m) or weak (w) and/or broad (br). *Melting points*. Mps were recorded on a Kofler hot stage apparatus and are uncorrected.

4.2. Synthesis

*4.2.1. Synthesis of 1,3-didodecanoylgylcerol (LaOHLa) **1**, 1,3-dihexadecanoylglycerol (POHP) **2** and 1,3-di octadencanoyl-gylcerol (SOHS) **3***

The procedure of Haraldsson and co-workers[4] was followed and allowed synthesis of the title compounds on scales up to 100 g.

*4.2.2. Synthesis of 1,3-didodecanoyl-2-(3,6,9,12,15,18-docosahexaenoyl)glycerol (**4**)*

To a solution of 1,3-didodecanoylglycerol (LaOHLa) **1** (61 g, 142 mmol) in CH_2Cl_2 (300 mL) were added *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (32.7 g, 170 mmol), docosahexaenoic acid (56.1 g, 170 mmol) and *N*,*N*-dimethylaminopyridine (1.37 g, 11.2 mmol). The solution was stirred at room temperature overnight before CH_2Cl_2 was removed under reduced pressure and the crude material purified by passage through a plug of silica gel (diameter 13 cm, height 9 cm, 3 L CH_2Cl_2 eluent) to give the title compound (LaDLa) **4** as a white/yellow solid (94.5 g, 90%). Mp 35–41 °C. ^1H NMR (400 MHz, $CDCl_3$) δ 0.86 (t, 3H, *J* 6.4 Hz, lauric C*H*$_3$), 0.97 (t, 3H, *J* 7.5 Hz, DHA C*H*$_3$), 1.16–1.38 (m, 36H, 18×C*H*$_2$), 1.55–1.65 (m, 4H, 2×lauric $O_2CCH_2CH_2CH_2$), 2.0–2.10 (m, 2H, DHA CH=CHC*H*$_2$CH$_2$), 2.30 (t, 4H, *J* 7.6 Hz, 2×lauric $O_2CCH_2CH_2$), 2.36–2.40 (m, 2H, $O_2CCH_2CH{=}CH_2$), 2.76–2.89 (m, 10H, DHA 5×C=CC*H*$_2$C=C), 4.13 (dd, 2H, *J* 5.9, 11.9 Hz, one each of OC*H*$_2$CHCH$_2$O), 4.28 (dd, 2H, *J* 4.3, 11.9 Hz, one each of OC*H*$_2$CHCH$_2$O), 5.21–5.41 (m, 13H, 12×C*H* alkene and CH_2CHOCH_2). ^{13}C NMR (200 MHz, $CDCl_3$) δ 14.29 (*C*H$_3$), 14.45 (*C*H$_3$), 20.75 (*C*H$_2$), 22.88 (*C*H$_2$), 25.06 (*C*H$_2$), 25.11 (*C*H$_2$), 25.74 (*C*H$_2$), 25.84 (*C*H$_2$), 29.32 (*C*H$_2$), 29.46 (*C*H$_2$), 29.53 (*C*H$_2$), 29.67 (*C*H$_2$), 29.81 (*C*H$_2$), 32.11 (*C*H$_2$), 34.24 (*C*H$_2$), 62.23 (OCH$_2$), 69.32 (OCH), 127.22 (*C*H alkene), 127.84 (*C*H alkene), 128.07 (*C*H alkene), 128.18 (*C*H alkene), 128.27 (*C*H alkene), 128.48 (*C*H alkene), 128.53 (*C*H alkene), 128.77 (*C*H alkene), 129.65 (*C*H alkene), 132.21 (*C*H alkene), 172.31 (*C*=O, 1C, 2-DHA), 173.40 (*C*=O, 2C, 1,3-lauric). IR (neat) ν_{max} 3013 w, 2954 m, 2916 s, 2851 s, 1738 s, 1468 w, 1417 w, 1390 w, 1287 w, 1265 w, 1239 w, 1205 m, 1177 m, 1147 m, 717 w cm^{-1}. MS calculated for $C_{49}H_{82}O_6Na^+$=789.6; found: 789.5. HRMS calculated for $C_{49}H_{82}O_6Na^+$=789.6009; found: 789.6007.

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

To a solution of 1,3-dihexadecanoylglycerol (POHP) **2** (52 g, 96 mmol) in CH_2Cl_2 (250 mL) were added *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (22.1 g, 115 mmol), docosahexaenoic acid (38 g, 115 mmol) and *N*,*N*-dimethylaminopyridine (2.8 g, 23 mmol). The solution was stirred at room temperature overnight before CH_2Cl_2 was removed under reduced pressure and the crude material purified by passage through a plug of silica gel (diameter 13 cm, height 7 cm, 2 L CH_2Cl_2 eluent) to give the title compound **5** as a white/yellow soild (75.3 g, 89%). Mp 25–27 °C. ^1H NMR (800 MHz, $CDCl_3$) δ 0.88 (t, 6H, *J* 7.2 Hz, 2×palmitic C*H*$_3$), 0.97 (t, 3H, *J* 7.5 Hz, 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, 4H, *J* 7.6 Hz, 2×palmitic $O_2CCH_2CH_2$), 2.36–2.39 (m, 2H, $O_2CCH_2CH{=}CH_2$) 2.80–2.87 (m, 10H, DHA 5×C=CC*H*$_2$C=C), 4.14 (dd, 2H, *J* 5.9, 11.9 Hz, one each of OC*H*$_2$CHCH$_2$O), 4.29 (dd, 2H, *J* 4.4, 11.9 Hz, one each of OC*H*$_2$CHCH$_2$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 (*C*H$_2$), 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 (OCH$_2$), 69.26 (OCH), 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) ν_{max} 3014 s, 2913 s, 2849 s, 1732 s, 1655 w, 1471 s, 1418 s, 1376 m, 1280 s, 1230 s, 1156 s, 1107 s, 1064 m, 1025 m, 921 w, 867 w, 718 s cm^{-1}. MS calculated for $C_{57}H_{98}O_6Na^+$=901.7; found: 901.8. HRMS calculated for $C_{57}H_{98}O_6H^+$=879.7442; found: 879.7445.

*4.2.4. Synthesis of 1,3-dioctadecanoyl-2-(3,6,9,12,15,18-docosahexaenoyl)glycerol (SDS) (**6**)*

To a solution of 1,3-dioctadecanoylglycerol (SOHS) **3** (56 g, 94 mmol) in CH_2Cl_2 (250 mL) were added *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (21.6 g, 113 mmol), docasahexaenoic acid (37.1 g) and *N*,*N*-dimethylaminopyridine (2.76 g, 22.6 mmol). The solution was stirred at room temperature overnight before CH_2Cl_2 was removed under reduced pressure and the crude material purified by passage through a plug of silica gel (diameter 13 cm, height 7 cm, 2 L CH_2Cl_2 eluent) to give the title compound (SDS) **6** as a white/yellow soild (78.5 g, 92%). Mp 39–40 °C. ^1H NMR (200 MHz, $CDCl_3$) 0.87 (t, 3H, *J* 6.6 Hz, DHA C*H*$_3$), 0.97 (t, 6H, *J* 7.5 Hz, 2×stearic CH$_3$), 1.11–1.41 (m, 58H, 29×C*H*$_2$), 1.50–1.71 (m, 4H, 2×stearic $O_2CCH_2CH_2CH_2$), 2.06–2.08 (m, 2H, DHA CH=CHC*H*$_2$CH$_2$), 2.30 (t, 4H, *J* 7.6 Hz, 2×stearic $O_2CCH_2CH_2$), 2.34–2.40 (m, 2H, $O_2CCH_2CH{=}CH_2$), 2.77–2.84 (m, 10H, DHA 5×C=CC*H*$_2$C=C), 4.14 (dd, 2H, *J* 5.8, 11.9 Hz, one each of OC*H*$_2$CHCH$_2$O), 4.29 (dd, 2H, *J* 4.4, 11.9 Hz, one each of

OCH_2CHCH_2O), 5.26–5.42 (m, 13H, 12×CH alkene and CH$_2$CHOCH$_2$). ^{13}C NMR (50 MHz, CDCl$_3$) δ 14.22 (CH_3), 14.37 (CH_3), 20.68 (CH_2), 22.81 (CH_2), 24.98 (CH_2), 25.66 (CH_2), 25.75 (CH_2), 29.24 (CH_2), 29.39 (CH_2), 29.49 (CH_2), 29.60 (CH_2), 29.82 (CH_2), 32.05 (CH_2), 34.15 (CH_2), 62.14 (OCH_2), 69.24 (OCH), 127.14 (CH alkene), 127.76 (CH alkene), 127.98 (CH alkene), 128.09 (CH alkene), 128.19 (CH alkene), 128.38 (CH alkene), 128.45 (CH alkene), 128.68 (CH alkene), 129.57 (CH alkene), 132. 11 (CH alkene), 172.20 (C=O, 1C, 2-DHA), 173.32 (C=O, 2C, 1,3-stearic). IR (neat) ν_{max} 3012 w, 2961 m, 2914 s, 2849 m, 1733 s, 1471 s, 1255 m, 1255 w, 1195 w, 1179 w, 1156 w, 1106 w cm^{-1}. MS calculated for $C_{61}H_{106}O_6Na^+$=957.8; found: 957.5. HRMS calculated for $C_{61}H_{106}O_6Na^+$=957.7887; found: 957.7883.

4.2.5. Synthesis of 1,2-acetonide-3-(3,6,9,12,15,18-docosahexaenoyl)glycerol (**8**)

To a solution of 1,2-acetonide glycerol (**7**) (16 g, 121 mmol) in CH_2Cl_2 (200 mL) were added docosahexaenoic acid (43 g, 131 mmol), *N*,*N*-dimethylaminopyridine (3.2 g, 26.2 mmol) and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (25.1 g, 131 mmol). The solution was stirred at room temperature overnight before the crude material was purified by passage through a plug of silica (diameter 13 cm, height 7 cm, 2 L CH_2Cl_2 eluent) to obtain the title compound **8** as a colourless oil (51.9 g, 97%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 0.97 (t, 3H, *J* 7.5 Hz, 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, 2H, *J* 4.9 Hz, DHA O$_2$CCH_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), 4.21–4.29 (m, 1H, CH$_2$CHOCH$_2$), 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$_2$C(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) ν_{max} 3013 s, 2964 s, 2933 s, 1715 s, 1654 w, 1455 m, 1380 s, 1371 s, 1258 s, 1215 s, 1157 s, 1084 s, 1058 s, 990 m, 928 m, 843 m, 792 w, 711 s cm^{-1}. MS calculated for $C_{28}H_{42}O_4Na^+$=465.3; found: 465.3. HRMS calculated for $C_{28}H_{42}O_4Na^+$=465.2981; found: 465.2977.

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

To a solution of 1,2-acetonide-3-(3,6,9,12,15,18-docosahexaenoyl)glycerol **8** (38 g, 86 mmol) in HPLC grade methanol (300 mL) was added Amberlyst-H$^+$ resin (5 g). The mixture was stirred at room temperature for 33 h before the resin was filtered off and solvent removed under the reduced pressure. The residual oil was then purified by passage through a plug of silica (diameter 13 cm, height 7 cm, 2 L of 3:2 EtOAc/hexane eluent) to yield the title compound **9** as clear oil (31 g, 90%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 0.97 (t, 3H, *J* 7.5 Hz, DHA CH_3), 2.02–2.12 (m, 2H, CH=CHCH$_2$CH_2CH$_3$), 2.26 (br s, 1H, OH), 2.39–2.45 (m, 4H, DHA O$_2$CCH_2C=CH$_2$ and CH=CHCH_2CH$_2$CH$_3$), 2.68 (br s, 1H, OH), 2.79–2.89 (m, 10H, DHA 5×CH=CHCH_2CH=C), 3.58 (dd, 1H, *J* 5.8, 11.5 Hz, one of OCH_2CHCH_2O), 3.68 (dd, 1H, *J* 3.9, 11.5 Hz, one of OCH_2CHCH_2O), 3.88–3.95 (m, 1H, one of OCH_2CHCH_2O), 4.08–4.23 (m, 2H, one of OCH_2CHCH_2O and OCH$_2$CHCH$_2$O), 5.28–5.45 (m, 12H, alkene CH). ^{13}C NMR (75 MHz, CDCl$_3$) δ 13.2 (CH_3), 19.5 (CH_2), 21.7 (CH_2), 24.5 (CH_3), 24.6 (CH_2), 24.6 (CH_2), 33.0 (CH_2), 62.3 (OCH_2), 64.3 (OCH_2), 69.2 (OCH), 126.0 (CH alkene), 126.7 (CH alkene), 126.9 (CH alkene), 126.9 (CH alkene), 127.1 (CH alkene), 127.3 (CH alkene), 127.3 (CH alkene), 127.4 (CH alkene), 127.6 (CH alkene), 128.6 (CH alkene), 131.0 (CH alkene), 172.5 (C=O DHA). IR (neat) ν_{max} 3045 s, 3013 s, 2963 s, 2932 s, 1740 s, 1654 w, 1442 m, 1391 m, 1266 m, 1161 m, 1120 m, 1053 m, 988 m, 928 m, 792 w, 708 s cm^{-1}. MS calculated for $C_{25}H_{38}O_4Na^+$=425.3; found: 425.5. HRMS calculated for $C_{25}H_{38}O_4Na^+$=425.2668; found: 425.2667.

4.2.7. Synthesis of 1,2-didodecanoylgylcerol-(3,6,9,12,15,18-docosahexaenoyl)glycerol (LaLaD) (**10**)

To a solution of 1-(3,6,9,12,15,18-docosahexaenoyl)glycerol **9** (56 g, 140 mmol) in CH_2Cl_2 (200 mL) were added dodecanoic acid (lauric acid) (68 g, 336 mmol), *N*,*N*-dimethylaminopyridine (6.8 g, 56 mmol) and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (64.4 g, 336 mmol). The solution was stirred at room temperature overnight before being purified by passage through a plug of silica (diameter 13 cm, height 7 cm, 2 L CH_2Cl_2 eluent) to give the title compound (LaLaD) **10** as a white/yellow solid (99.8 g, 93%). Mp 37–41 °C. ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.86 (apparent t, 6H, *J* 7.0 Hz, 2×lauric CH_3), 0.97 (t, 3H, *J* 7.5 Hz, DHA CH_3), 1.21–1.47 (m, 36H, 18×CH_2), 1.58–1.65 (m, 4H, 2×lauric O$_2$CCH$_2$CH_2CH$_2$), 2.04–2.11 (m, 2H, DHA CH=CHCH_2CH$_2$), 2.29 (t, 2H, *J* 7.7 Hz, lauric O$_2$CCH_2CH$_2$), 2.31 (t, 2H, *J* 7.4 Hz, lauric O$_2$CCH_2CH$_2$), 2.38 (d, 2H, *J* 2.8 Hz, O$_2$CCH_2CH=CH$_2$) 2.80–2.86 (m, 10H, DHA 5×C=CCH_2C=C), 4.13–4.18 (m, 2H, one each of OCH_2CHCH_2O), 4.29 (dd, 2H, *J* 4.3, 11.9 Hz, one each of OCH_2CHCH_2O), 5.26–5.42 (m, 13H, 12×CH alkene and CH$_2$CHOCH$_2$). ^{13}C NMR (50 MHz, CDCl$_3$) δ 14.25 (CH_3), 14.41 (CH_3), 20.70 (CH_2), 22.83 (CH_2), 22.87 (CH_2), 25.06 (CH_2), 25.09 (CH_2), 25.74 (CH_2), 25.78 (CH_2), 25.83 (CH_2), 29.27 (CH_2), 29.31 (CH_2), 29.46 (CH_2), 29.53 (CH_2), 29.66 (CH_2), 29.81 (CH_2), 32.10 (CH_2), 34.10 (CH_2), 34.24 (CH_2), 34.40 (CH_2), 62.80 (OCH_2), 62.47 (OCH_2), 69.06 (OCH), 127.22 (CH alkene), 127.85 (CH alkene), 128.06 (CH alkene), 128.18 (CH alkene), 128.28 (CH alkene),128.46 (CH alkene), 128.51 (CH alkene), 128.75 (CH alkene), 129.67 (CH alkene), 132.20 (CH alkene), 172.71 (C=O, 1C, 3-DHA), 173.04 (C=O, 1C, 2-lauric), 173.43 (C=O, 1C, 1-lauric). IR (neat) ν_{max} 3012 m, 2955 s, 2919 s, 2851 s, 1741 s, 1647 m, 1551 m, 1454 m, 1377 m, 1146 s, 1110 m, 1034 m, 719 w cm^{-1}. MS calculated for $C_{49}H_{82}O_6H^+$=767.6; found: 766.6. HRMS calculated for $C_{49}H_{82}O_6H^+$=767.6190; found: 767.6192.

4.2.8. Synthesis of 1,2-dihexadecanoyl-3-(3,6,9,12,15,18-docosahexaenoyl)glycerol (PPD) (**11**)

To a solution of 1-(3,6,9,12,15,18-docosahexaenoyl)glycerol **9** (41 g, 104 mmol) in CH_2Cl_2 (200 mL) were added hexadecanoic acid (palmitic acid) (64 g, 250 mmol), *N*,*N*-dimethylaminopyridine (3 g, 12.5 mmol) and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (48 g, 250 mmol). The solution was stirred at room temperature overnight before being purified by passage through a plug of silica (diameter 13 cm, height 7 cm, 2 L CH_2Cl_2 eluent) to give the title compound (PPD) **11** as a white/yellow solid (87.8 g, 96%). Mp 22–25 °C. ^{1}H NMR (800 MHz, CDCl$_3$) δ 0.88 (apparent t, 6H, *J* 7.2 Hz, 2×palmitic CH_3), 0.97 (t, 3H, *J* 7.3 Hz, DHA CH_3), 1.22–1.34 (m, 50H, 25×CH_2), 1.58–1.63 (m, 4H, 2×palmitic O$_2$CCH$_2$CH_2CH$_2$), 2.06–2.10 (m, 2H, DHA CH=CHCH_2CH$_2$), 2.31 (t, 2H, *J* 7.4 Hz, palmitic O$_2$CCH_2CH$_2$), 2.32 (t, 2H, *J* 7.3 Hz, palmitic O$_2$CCH_2CH$_2$), 2.38 (d, 2H, *J* 2.8 Hz, O$_2$CCH_2CH=CH$_2$) 2.80–2.87 (m, 10H, DHA 5×C=CCH_2C=C), 4.14 (m, 2H, one each of OCH_2CHCH_2O), 4.29 (dd, 2H, *J* 4.2, 11.9 Hz, one each of OCH_2CHCH_2O), 5.26–5.43 (m, 13H, 12×CH alkene and CH$_2$CHOCH$_2$). ^{13}C NMR (200 MHz, CDCl$_3$) δ 14.26 (CH_3), 14.42 (CH_3), 20.71 (CH_2), 22.77 (CH_2), 22.85 (CH_2), 25.02 (CH_2), 25.05 (CH_2), 25.70 (CH_2), 25.75 (CH_2), 25.79 (CH_2), 29.24 (CH_2), 29.28 (CH_2), 29.43 (CH_2), 29.44 (CH_2), 29.52 (CH_2), 29.64 (CH_2), 29.66 (CH_2), 29.78 (CH_2), 29.80 (CH_2), 29.82 (CH_2), 29.85 (CH_2), 32.0 (CH_2), 34.05 (CH_2), 34.20 (CH_2), 34.37 (CH_2), 62.24 (OCH_2), 62.44 (OCH_2), 68.99 (OCH), 127.17 (CH alkene), 128.14 (CH alkene), 128.03 (CH alkene), 128.15 (CH alkene), 128.24 (CH alkene),128.26 (CH alkene), 128.41 (CH alkene), 128.43 (CH alkene), 128.54 (CH alkene), 128.73 (CH alkene), 129.63 (CH alkene), 132.19 (CH alkene),

172.70 (*C*=O, 1C, 3-DHA), 173.02 (*C*=O, 1C, 2-palmitic), 173.43 (*C*=O, 1C, 1-palmitic). IR (neat) ν_{max} 3014 s, 2925 s, 2854 s, 1747 s, 1466 s, 1377 w, 1235 m, 1154s, 1159 m, 721 m cm^{-1}. MS calculated for $C_{57}H_{98}O_6Na^+$=901.7; found: 901.8. HRMS calculated for $C_{57}H_{98}O_6H^+$=879.7442; found: 879.7448.

*4.2.9. Synthesis of 1,2-dioctadecanoyl-3-(3,6,9,12,15,18-docosahexaenoyl)glycerol (SSD) (**12**)*

To a solution of 1-(3,6,9,12,15,18-docosahexaenoyl)glycerol **9** (52 g, 130 mmol) in CH_2Cl_2 (200 mL) were added octadecanoic acid (stearic acid) (83.2 g, 292 mmol), *N*,*N*-dimethylaminopyridine (14.2 g, 58.4 mmol) and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (55.6 g, 292 mmol). The solution was stirred at room temperature overnight before being purified by passage through a plug of silica (diameter 13 cm, height 7 cm, 2 L CH_2Cl_2 eluent) to give the title compound (SSD) **12** as a white/yellow solid (110 g, 91%). Mp 47–49 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (apparent t, 6H, *J* 6.9 Hz, 2×stearic C*H*$_3$), 0.97 (t, 3H, *J* 7.5 Hz, DHA C*H*$_3$), 1.15–1.38 (m, 58H, 29×C*H*$_2$), 1.52–1.69 (m, 4H, 2×stearic $O_2CCH_2CH_2CH_2$), 2.01–2.13 (m, 2H, DHA CH=CHC*H*$_2$CH$_2$), 2.30 (t, 2H, *J* 7.7 Hz, stearic $O_2CCH_2CH_2$), 2.31 (t, 2H, *J* 7.4 Hz, stearic $O_2CCH_2CH_2$), 2.37 (d, 2H, *J* 2.6 Hz, $O_2CCH_2CH{=}CH_2$) 2.75–2.90 (m, 10H, DHA 5×C=CC*H*$_2$C=C), 4.10–4.17 (m, 2H, one each of OC*H*$_2$CHC*H*$_2$O), 4.29 (dd, 2H, *J* 4.3, 11.9 Hz, one each of OC*H*$_2$CHC*H*$_2$O), 5.24–5.48 (m, 13H, 12×C*H* alkene and CH_2CHOCH_2). ^{13}C NMR δ 14.23 (*C*H$_3$), 14.39 (*C*H$_3$), 20.70 (*C*H$_2$), 22.77 (*C*H$_2$), 22.82 (*C*H$_2$), 25.00 (*C*H$_2$), 25.04 (*C*H$_2$), 25.68 (*C*H$_2$), 25.73 (*C*H$_2$), 25.77 (*C*H$_2$), 29.22 (*C*H$_2$), 29.26 (*C*H$_2$), 29.42 (*C*H$_2$), 29.50 (*C*H$_2$), 29.63 (*C*H$_2$), 29.84 (*C*H$_2$), 32.06 (*C*H$_2$), 34.16 (*C*H$_2$), 34.18 (*C*H$_2$), 34.24 (*C*H$_2$), 62.22 (O*C*H$_2$), 62.41 (O*C*H$_2$), 68.99 (O*C*H), 127.16 (*C*H alkene), 127.79 (*C*H alkene), 128.00 (*C*H alkene), 128.12 (*C*H alkene), 128.22 (*C*H alkene), 128.39 (*C*H alkene), 128.41 (*C*H alkene), 128.45 (*C*H alkene), 128.70 (*C*H alkene), 129.61 (*C*H alkene), 132.15 (*C*H alkene), 172.65 (*C*=O, 1C, 3-DHA), 172.98 (*C*=O, 1C, 2-stearic), 173.38 (*C*=O, 1C, 1-stearic). IR (neat) ν_{max} 3014 w, 2957 m, 2918 s, 2849 s, 1732 s, 1472 m, 1462 m, 1418 w, 1386 w, 1315 w, 1293 w, 1273 w, 1255 w, 1236 w, 1214 w, 1193 w, 1171 s, 1069 w, 1032 w, 981 w, 720 w, 633 w cm^{-1}. MS calculated for $C_{61}H_{106}O_6Na^+$=957.8; found: 957.7. HRMS calculated for $C_{61}H_{106}O_6Na^+$=957.7887; found: 957.7888.

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