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Chemoenzymatic synthesis of symmetrically structured triacylglycerols possessing short-chain fatty acids

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ABSTRACT

Synthesis of symmetrically structured triacylglycerols possessing bioactive *n*−3 polyunsaturated fatty acids (eicosapentaenoic acid or docosahexaenoic acid) at the 2-position and a short-chain fatty acid (C_2, C_4, C_6) located at the end-positions by a highly efficient two-step chemoenzymatic process is described. Full regiocontrol devoid of any acyl-migration side reactions was obtained in both a lipase promoted step to introduce the short-chain fatty acids exclusively into the primary alcohol positions of glycerol using activated vinyl esters at low temperature and a subsequent coupling reaction involving free EPA and DHA using EDAC as a coupling agent.

1. Introduction

The long-chain *n*−3 polyunsaturated fatty acids (PUFA) are characteristic of marine fat, the two most prevalent being eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[1,2] Numerous beneficial effects of EPA and DHA on human health have been firmly established.[3–5] The strongest evidence relates to reductions in the incidence of cardiovascular and heart diseases by both EPA and DHA. Besides that, the beneficial effects of EPA have been linked to various inflammatory disorders, and DHA to pre- and postnatal nutrition and brain and nervous system development.[6,7] Currently there is general interest among scientists in the beneficial effects of EPA and DHA on various mental disorders, such as schizophrenia,[8,9] Alzheimer's dementia[10] and depression.[11,12]

Structured triacylglycerols (TAG) containing saturated medium-chain fatty acids (MCFA) at the terminal positions and long-chain biologically active polyunsaturated fatty acids (PUFA) at the 2-position of the glycerol backbone have gained increased attention of scientists as dietary and health supplements.[13–15] Recently, a highly efficient synthesis of such symmetrically structured ABA type lipids was reported by a two-step chemoenzymatic process. This includes structured MLM (medium–long–medium) type TAG comprised of a range of a pure saturated MCFA (C_8, C_{10} and C_{12}) at the 1- and 3-positions and pure EPA or DHA at the 2-position as well as similarly structured TAG possessing longer-chain saturated fatty acids (C_{14}, C_{16} and C_{18}).[16–18]

Synthesis of positionally labelled structured TAG by traditional synthetic organic chemistry methods requires a full regiocontrol and can hardly be undertaken without multi-step protection–deprotection processes. Owing to their regioselectivity, lipases are ideally suited as biocatalysts for preparing structured TAG of the ABA type. By acting preferentially or exclusively at the primary alcoholic end-positions of the glycerol backbone they may be employed to introduce fatty acids of selected type at these positions by esterification or transesterification processes.[16–22] An immobilized *Candida antarctica* lipase (CAL-B) was observed to display excellent regioselectivity towards the end-positions of glycerol at 0–4 °C when using vinyl esters as acylating agents. The *n*−3 fatty acids were subsequently introduced into the remaining 2-position highly efficiently using EDAC as a chemical coupling agent.[16]

The current work completes the task of preparing all variations of symmetrically structured ABA type TAG compounds containing all saturated even-numbered fatty acids from C_2–C_{18} at the end-positions with bioactive EPA and DHA located at the 2-position. This means synthesis of SLS (short-long-short) type structured TAG possessing the shortest-chain fatty acids C_2 and C_4 and the shortest-chain MLM type variant possessing C_6. An example is provided in Figure 1 for DHA and caproic acid (C_6).

The resulting well-defined 'library' of pure ABA type structured TAG may become useful for studying the effects of structured TAG and to compare biological effects of individual fatty acids by a range of screening experiments. The methodology may also be useful when introducing isotopically labelled fatty acids to predetermined positions of the TAG glycerol backbone as well as other types of bioactive fatty acids or potent drugs possessing carboxylic group to prepare prodrugs.

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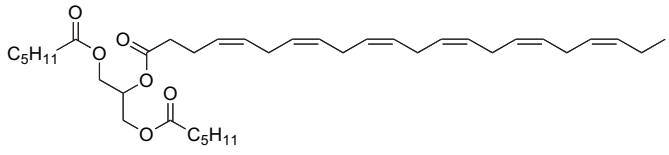
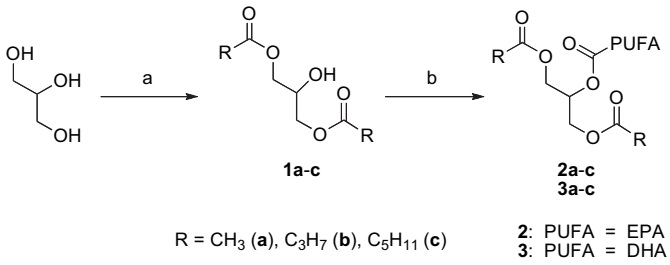


Figure 1.

2. Results and discussion

The synthesis of the shortest-chain (C_2, C_4 and C_6) symmetrically structured TAG was accomplished by following the two-step chemoenzymatic approach described in a previous report starting from glycerol to synthesize symmetric MLM type structured TAG.[16] In the first step, lipase regioselectivity was exploited to synthesize the symmetric 1,3-diacylglycerols (DAG) intermediate adducts of the shortest-chain fatty acids (SCFA). This was followed by introduction of the *n*−3 PUFA into the mid-position by chemical coupling. The overall process is displayed in Scheme 1.



Scheme 1. Reagents and conditions: (a) *C. antarctica* lipase, SCFA as vinyl esters, CH_2Cl_2, 0–4 °C, 4 h; (b) PUFA, EDAC, DMAP, CH_2Cl_2, rt 4 h.

As in the previous report the immobilized *C. antarctica* lipase was observed to display superb performance in terms of regiocontrol and yields of the enzymatic step.[16] This is based on rapid, irreversible transesterification of glycerol using 2.5-fold stoichiometric amount of vinyl esters of the SCFA in dichloromethane or chloroform at 0–4 °C. The lipase acted exclusively on the glycerol end-positions to afford the 1,3-DAG adducts **1a–c** as colourless oils, respectively, for C_2, C_4 and C_6. On this occasion, flash chromatography on silica gel was needed to purify the products that were obtained in excellent yields (90–92%) after purification. The yields are revealed in Table 1.

Table 1
Yields and type of products and 1,3-DAG intermediates (**1a**,**b**,**c**) from the chemoenzymatic synthesis of ABA type structured TAG constituting pure SCFA and EPA (**2a**,**b**,**c**) or DHA (**3a**,**b**,**c**)

Compound	SCFA	PUFA	Yield (%)
1a	$-CH_3$	—	90
1b	$-C_3H_7$	—	91
1c	$-C_5H_{11}$	—	92
2a	$-CH_3$	EPA	90
2b	$-C_3H_7$	EPA	92
2c	$-C_5H_{11}$	EPA	91
3a	$-CH_3$	DHA	90
3b	$-C_3H_7$	DHA	92
3c	$-C_5H_{11}$	DHA	94

As for the MCFA, only 1-monoacylglycerol (1-MAG) intermediate was detected in small quantities during the progress of the reaction. It took the reaction only 4 h to proceed to completion resulting in quantitative conversion into the desired 1,3-DAG. Only traces of the 1-MAG intermediate remained and there were no signs of undesired 1,2-DAG and 2-MAG regioisomers and TAG present and thereby no indications of any acyl-migration[16,23–25] side reactions or the lipase acting at the mid-position.

A full regiocontrol was secured by mild conditions offered by the lipase acting at low temperature. To enable the lipase to act at such a rather unusual temperature activated vinyl esters were used. The use of vinyl esters also renders the transesterification reaction system irreversibility by keto–enol tautomerism that the enolic leaving group undergoes upon release.

The subsequent chemical coupling reaction to introduce pure EPA and DHA into the mid-position of the 1,3-DAG adducts **1a–c** was performed at room temperature in dichloromethane. EDAC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; 1.2 equiv) was used as a coupling agent in the presence of DMAP (dimethylaminopyridine; 20% as based on mole) with an exact stoichiometric amount of EPA or DHA as based on the 1,3-DAG adduct. The reactions were completed within 3–4 h, much faster than the 12–15 h previously stated.[16] It was confirmed that only 4 h were also required for the coupling reaction involving C_8–C_{16} to proceed to completion. Chemically and regioisomerically pure structured TAG **2a–c** (for EPA) and **3a–c** (for DHA), respectively, for C_2, C_4 and C_6, were afforded as yellowish oils in excellent yields (90–94%) after purification by chromatography treatment on silica gel. The yields are shown in Table 1.

There were no indications of any losses in regiocontrol during this reaction as was established after careful collection of samples from the progressing reaction and their analysis by 400 MHz ^{1}H NMR spectroscopy and ^{13}C NMR on the final product. This was observed not only for C_2–C_6, but also C_8–C_{16}. This prompted further investigations on the stability of the 1,3-DAG adducts under the conditions of the coupling reaction and to what an extent a possible acyl-migration to the corresponding 1,2-DAG might occur. During 4 h stirring of 1,3-DAG under the exact background conditions, in the absence of free fatty acids, no acyl-migration was detected, whereas after 24 h the acyl-migration levels had reached a maximum of 0.5%. Very similar results were obtained when the 1,3-DAG was stirred in the solvent in the presence of free fatty acid, this time with EDAC and DMAP absent. It is important to bear in mind that the initial 1,3-DAG concentration remained unchanged throughout in the experiment. This, together with the fact that the coupling reaction proceeded to completion in 3–4 h, unequivocally supports our previous claims (based on 250 MHz ^{1}H NMR studies)[16] that no detectable acyl-migration was taking place during the coupling reaction between 1,3-DAG and the *n*−3 PUFA. All this firmly confirms the full regiocontrol of both reactions and that both the intermediate 1,3-DAG adducts and the final structured TAG products were virtually 100% regiopure in all cases.

As was described earlier, all individual acylglycerols potentially involved in the reactions, 1-MAG and 2-MAG, 1,3-DAG and 1,2-DAG, as well as the TAG adduct, display quite characteristic ^{1}H NMR spectra in their glyceryl moiety proton region (5.30–3.50 ppm).[16,26] This makes it quite straightforward to track down all individual acylglycerol constituents present in the reaction mixture at a time and to quantify them with reasonable or good accuracy. Therefore, this became an excellent tool to monitor the progress of the reaction as it proceeds, as well as monitoring the regiocontrol of the reactions. ^{13}C NMR spectroscopy was also useful to aid with monitoring the regiocontrol of the reactions.[16,18]

The limit of quantification as detected by 400 MHz ^{1}H NMR spectroscopy for a possible acyl-migration of 1,3-DAG into 1,2-DAG as well as 1-MAG to 2-MAG has been determined by careful intensive studies. Freshly prepared standard solutions of pure 1,2-DAG and 2-MAG in $CDCl_3$ were added in portions to accurately weighed 1,3-DAG and 1-MAG dissolved in $CDCl_3$ in NMR tube and the ^{1}H NMR spectra immediately recorded for the resulting solutions. The results indicate that the levels of 2-MAG and 1,2-DAG

acyl-migration products can be accurately quantified down to 0.106 mg/ml. This corresponds to 0.25 mol % for a sample concentration of 40 mg/ml, which is a practical concentration level to prepare in the current studies for this type of investigation. The levels of detection are well below that. This will be reported in more detail later. It may be borne in mind that an equilibrium composition between 1(3)-MAG and 2-MAG is roughly 10% 2-MAG and 90% 1(3)-MAG.[24] The corresponding equilibrium composition for 1,3-DAG and 1(3),2-DAG is roughly 70% 1,3-DAG and 30% 1(3),2-DAG.[25]

3. Conclusions and summary

The pure compounds, both the structured TAG products and the 1,3-DAG intermediate adducts, may become highly useful as standards for various analytical purposes, as fine chemicals and, last, but not least, for various drug formulations, as drug carriers or potential drugs. The 1,3-DAG intermediate adducts ranging all fatty acids from C_2–C_{18} may offer excellent possibilities in studying acyl-migration[16,23–25] and how the rate of such processes may depend upon acyl chain-length. Such investigations are now under way in our group.

4. Experimental

4.1. General

^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on a Bruker Avance 400 spectrometer in deuteriated chloroform as a solvent at 400.12 and 100.61 MHz, respectively. Chemical shifts (δ) are quoted in parts per million (ppm) and the coupling constants (J) in Hertz (Hz). The following abbreviations are used to describe the multiplicity: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet. The number of carbon nuclei behind each ^{13}C signal is indicated in parentheses after each chemical shift value, when there is more than one carbon responsible for the peak. Infrared spectra were conducted on a Nicolet Avatar 360 FTIR (E.S.P.) Spectrophotometer on neat liquids. The high-resolution mass spectra (HRMS) were acquired on a Bruker micrOTOF-Q mass spectrometer equipped with an atmospheric pressure chemical ionization chamber. All data analysis was done on Bruker software.

The immobilized *C. antarctica* lipase (Novozym 435; CAL-B) was supplied as a gift from Novozyme A/S (Bagsvaerd, Denmark). All chemicals and solvents were used without further purification unless otherwise stated. Glycerol (99%) was purchased from Sigma Chemicals (St. Louis, Missouri), vinyl acetate (≥99%) from Sigma–Aldrich (Steinheim, Germany) and vinyl butyrate (>97%) and vinyl hexanoate (>97%) from TCI Europe (Zwindrecht, Belgium). EDAC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) was obtained of commercial grade from Sigma–Aldrich (Steinheim) and 4-dimethylaminopyridine (99%) from Acros Organics (Geel, Belgium). EPA (98%) and DHA (≥95%) were obtained as ethyl esters from Pronova Biocare (Sandefjord, Norway) and were hydrolysed to their corresponding free acids.[18] Toluene (dried over CaH_2), dichloromethane (dried over CaH_2) and ethyl acetate were all obtained HPLC grade from Sigma–Aldrich (Steinheim, Germany). Silica gel (Silica gel 60) and analytical TLC plates (DC Alufolien Kieselgel 60 F_{254}) were obtained from Merck (Darmstadt, Germany).

4.1.1. 1,3-Diacetylglycerol ***1a***. Immobilised *C. antarctica* lipase (67 mg) was added to a 10 ml round-bottomed flask containing a mixture of glycerol (200 mg, 2.17 mmol) and vinyl acetate (468 mg, 5.42 mmol) in dry dichloromethane (0.6 ml). The resulting mixture was gently stirred at 0–4 °C (ice-bath) for 4 h under nitrogen atmosphere. The lipase was separated off by filtration, the solvent removed in vacuo on a rotary evaporator and the excess vinyl acetate and by-produced acetic acid removed on a Kugelrohr apparatus (40 °C; 0.01 Torr) to afford the pure product as a colourless oil (344 mg, 90% yield). ^{1}H NMR δ 4.21–4.06 (m, 5H, CH_2CHCH_2), 2.44 (br d, J=4.0 Hz, 1H, OH) and 2.10 (s, 6H, CH_3) ppm. ^{13}C NMR δ 171.1 (2), 68.2, 65.2 (2) and 20.8 (2) ppm. IR ν_{max} 3300–3600 (br, O–H), 2958 (vs, C–H) and 1724 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_7H_{12}O_5$–OH m/z 159.0652; found 159.0650 amu.

4.1.2. 1,3-Dibutanoylglycerol ***1b***. The same procedure was followed as described for **1a** using immobilized *C. antarctica* lipase (76 mg), glycerol (200 mg, 2.17 mmol) and vinyl butanoate (545 mg, 4.77 mmol) in dry dichloromethane (0.6 ml). The pure product was afforded as a colourless oil (458 mg, 91% yield) after removal of excessive vinyl butanoate and butyric acid by distillation on a Kugelrohr apparatus (60 °C; 0.01 Torr). ^{1}H NMR δ 4.21–4.05 (m, 5H, CH_2CHCH_2), 2.53 (br s, 1H, OH), 2.33 (t, J=7.5 Hz, 4H, CH_2COO), 1.66 (sextet, J=7.5 Hz, 4H, CH_2CH_2COO) and 0.95 (t, J=7.5 Hz, 6H, CH_3) ppm. ^{13}C NMR δ 173.7 (2), 68.3, 65.0 (2), 35.9 (2), 18.3 (2) and 13.6 (2) ppm. IR ν_{max} 3300–3600 (br, O–H), 2965 (vs, C–H), 2878 (vs, C–H) and 1736 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_{11}H_{20}O_5$–OH m/z 215.1278; found 215.1285 amu.

4.1.3. 1,3-Dihexanoylglycerol ***1c***. The same procedure was followed as described for **1a** using immobilized *C. antarctica* lipase (44 mg), glycerol (100 mg, 1.09 mmol) and vinyl hexanoate (340 mg, 2.40 mmol) in dry dichloromethane (0.3 ml). The pure product was afforded as a colourless oil (290 mg, 92% yield) after removal of excessive vinyl hexanoate and hexanoic acid by distillation on a Kugelrohr apparatus (70 °C; 0.01 Torr). ^{1}H NMR δ 4.21–4.06 (m, 5H, CH_2CHCH_2), 2.35 (t, J=7.6 Hz, 4H, CH_2COO), 1.64 (quintet, J=7.6 Hz, 4H, CH_2CH_2COO), 1.38–1.26 (m, 8H, CH_2) and 0.89 (t, J=6.8 Hz, 6H, CH_3) ppm. ^{13}C NMR δ 173.9 (2), 68.3, 65.0 (2), 34.0 (2), 31.2 (2), 24.5 (2), 22.2 (2) and 13.9 (2) ppm. IR ν_{max} 3300–3600 (br, O–H), 2957 (vs, C–H), 2873 (vs, C–H) and 1736 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_{15}H_{28}O_5$–OH m/z 271.1904; found 271.1891 amu.

4.1.4. 1,3-Diacetyl-2-eicosapentaenoylglycerol ***2a***. To a solution of EPA as free acid (172 mg, 0.568 mmol), DMAP (14 mg, 0.114 mmol) and EDAC (131 mg, 0.686 mmol) in dry dichloromethane (2 ml) was added 1,3-diacetylglycerol **1a** (100 mg, 0.568 mmol). The resulting solution was stirred on a magnetic stirrer hot-plate at rt for 4 h under nitrogen atmosphere. After solvent removal in vacuo on a rotary evaporator the reaction mixture was purified by silica gel chromatography on a short column by use of dichloromethane–ethyl acetate (95:5). Solvent removal in vacuo afforded the pure product as a yellowish oil (236 mg, 90% yield). ^{1}H NMR δ 5.43–5.32 (m, 10H, =CH), 5.30–5.24 (m, 1H, CH_2CHCH_2), 4.28 (dd, J=12.0, 4.4 Hz, 2H, CH_2CHCH_2), 4.15 (dd, J=12.0, 6.0 Hz, 2H, CH_2CHCH_2), 2.85–2.76 (m, 8H, $=CCH_2C=$), 2.35 (t, J=7.5 Hz, 2H, CH_2COO), 2.14–2.00 (m, 4H, $=CCH_2CH_2$ and $=CCH_2CH_3$), 2.06 (s, 6H, CH_3COO), 1.71 (quintet, J=7.5 Hz, 2H, CH_2CH_2COO) and 0.97 (t, J=7.5 Hz, 3H, CH_3 in EPA) ppm. ^{13}C NMR δ 172.6, 170.4 (2), 132.0, 129.0, 128.8, 128.6, 128.3, 128.2, 128.1, 128.0, 127.8, 127.0, 68.8, 62.3 (2), 33.5, 26.4, 25.6 (3), 25.5, 24.7, 20.7 (2), 20.5 and 14.3 ppm. IR ν_{max} 3012 (vs, C–H), 2963 (s, C–H), 2874 (s, C–H), 1740 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_{27}H_{40}O_6$+H m/z 461.2898; found 461.2895 amu.

4.1.5. 1,3-Dibutanoyl-2-eicosapentaenoylglycerol ***2b***. The procedure was similar to that for preparing **2a** using EPA (195 mg, 0.646 mmol), DMAP (14 mg, 0.114 mmol) and EDAC (161 mg, 0.840 mmol) in dry dichloromethane (3 ml) to which was added 1,3-dibutanoylglycerol **1b** (150 mg, 0.646 mmol). After 4 h the reaction was disconnected by passing the reaction mixture in

dichloromethane through a short column packed with silica gel. The product was afforded as a yellowish oil (307 mg, 92% yield). ^{1}H NMR δ 5.44–5.33 (m, 10H, =CH), 5.33–5.24 (m, 1H, CH_2CHCH_2), 4.30 (dd, J=12.0, 4.4 Hz, 2H, CH_2CHCH_2), 4.15 (dd, J=12.0, 6.0 Hz, 2H, CH_2CHCH_2), 2.88–2.78 (m, 8H, $=CCH_2C=$), 2.34 (t, J=7.5 Hz, 2H, CH_2COO in EPA), 2.30 (t, J=7.5 Hz, 4H, CH_2COO), 2.14–2.04 (m, 4H, $=CCH_2CH_2$ and $=CCH_2CH_3$), 1.70 (quintet, J=7.5 Hz, 2H, CH_2CH_2COO in EPA), 1.69–1.60 (m, 4H, CH_2CH_2COO), 0.97 (t, J=7.5 Hz, 3H, CH_3 in EPA) and 0.94 (t, J=7.5 Hz, 6H, CH_3) ppm. ^{13}C NMR δ 173.1 (2), 172.6, 132.0, 129.0, 128.8, 128.6, 128.3, 128.2, 128.1, 128.0, 127.8, 127.0, 69.0, 62.0 (2), 35.9 (2), 33.6, 26.4, 25.6 (3), 25.5, 24.7, 20.5, 18.3 (2), 14.3 and 13.6 (2) ppm. IR ν_{max} 3012 (s, C–H), 2964 (vs, C–H), 2876 (s, C–H), 1736 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_{31}H_{48}O_6$+H m/z 517.3524; found 517.3515 amu.

4.1.6. 2-Eicosapentaenoyl-1,3-dihexanoylglycerol ***2c****.* The procedure was identical to that for preparing **2b** using EPA (210 mg, 0.693 mmol), DMAP (17 mg, 0.139 mmol) and EDAC (159 mg, 0.832 mmol) in dry dichloromethane (3 ml) to which was added 1,3-dihexanoylglycerol **1c** (200 mg, 0.693 mmol). The product was afforded as a yellowish oil (360 mg, 91% yield). ^{1}H NMR δ 5.43–5.32 (m, 10H, =CH), 5.33–5.24 (m, 1H, CH_2CHCH_2), 4.30 (dd, J=12.0, 4.4 Hz, 2H, CH_2CHCH_2), 4.15 (dd, J=12.0, 6.0 Hz, 2H, CH_2CHCH_2), 2.88–2.76 (m, 8H, $=CCH_2C=$), 2.34 (t, J=7.3 Hz, 2H, CH_2COO in EPA), 2.31 (t, J=7.5 Hz, 4H, CH_2COO), 2.14–2.04 (m, 4H, $=CCH_2CH_2$ and $=CCH_2CH_3$), 1.70 (quintet, J=7.5 Hz, 2H, CH_2CH_2COO in EPA), 1.65–1.58 (m, 4H, CH_2CH_2COO), 1.37–1.25 (m, 8H, CH_2), 0.97 (t, J=7.5 Hz, 3H, CH_3 in EPA) and 0.89 (t, J=6.8 Hz, 6H, CH_3) ppm. ^{13}C NMR δ 173.2 (2), 172.6, 132.0, 128.9, 128.8, 128.6, 128.3, 128.2, 128.1, 128.0, 127.8, 127.0, 69.0, 62.1 (2), 34.0 (2), 33.6, 31.2 (2), 26.5, 25.6 (3), 25.5, 24.7, 24.5 (2), 22.3 (2), 20.5, 14.3 and 13.9 (2) ppm. IR ν_{max} 3013 (s, C–H), 2958 (vs, C–H), 2872 (s, C–H), 1739 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_{35}H_{56}O_6$+H m/z 573.4150; found 573.4135 amu.

4.1.7. 1,3-Diacetyl-2-docosahexaenoylglycerol ***3a****.* The procedure was identical to that for preparing **2a** using DHA (186 mg, 0.568 mmol), DMAP (14 mg, 0.114 mmol) and EDAC (131 mg, 0.686 mmol) in dry dichloromethane (2 ml) to which was added 1,3-diacetylglycerol **1a** (100 mg, 0.568 mmol). The product was afforded as a yellowish oil (248 mg, 90% yield). ^{1}H NMR δ 5.44–5.30 (m, 12H, =CH), 5.30–5.24 (m, 1H, CH_2CHCH_2), 4.29 (dd, J=12.0, 4.4 Hz, 2H, CH_2CHCH_2), 4.15 (dd, J=12.0, 6.0 Hz, 2H, CH_2CHCH_2), 2.88–2.80 (m, 10H, $=CCH_2C=$), 2.40–2.36 (m, 4H, CH_2CH_2COO), 2.11–2.04 (m, 2H, $=CCH_2CH_3$), 2.07 (s, 6H, CH_3COO) and 0.97 (t, J=7.5 Hz, 3H, CH_3 in DHA) ppm. ^{13}C NMR δ 172.1, 170.3 (2), 131.9, 129.3, 128.4, 128.2, 128.1 (2), 127.9 (2), 127.8, 127.7, 127.5, 126.9, 68.8, 62.1 (2), 33.9, 25.5 (4), 25.4, 22.5, 20.6 (2), 20.4 and 14.2 ppm. IR ν_{max} 3013 (vs, C–H), 2964 (s, C–H), 2875 (s, C–H), 1740 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_{29}H_{42}O_6$+H m/z 487.3054; found 487.3073 amu.

4.1.8. 1,3-Dibutanoyl-2-docosahexaenoylglycerol ***3b****.* The procedure was identical to that for preparing **2b** using DHA (250 mg, 0.761 mmol), DMAP (19 mg, 0.152 mmol) and EDAC (175 mg, 0.913 mmol) in dry dichloromethane (3 ml) to which was added 1,3-dibutanoylglycerol **1b** (177 mg, 0.761 mmol). The product was afforded as a yellowish oil (380 mg, 92% yield). ^{1}H NMR δ 5.44–5.32 (m, 12H, =CH), 5.32–5.25 (m, 1H, CH_2CHCH_2), 4.30 (dd, J=12.0, 4.4 Hz, 2H, CH_2CHCH_2), 4.15 (dd, J=12.0, 6.0 Hz, 2H, CH_2CHCH_2), 2.88–2.79 (m, 10H, $=CCH_2C=$), 2.40–2.38 (m, 4H, CH_2CH_2COO in DHA), 2.30 (t, J=7.5 Hz, 4H, CH_2COO), 2.11–2.04 (m, 2H, $=CCH_2CH_3$), 1.64 (sextet, J=7.5 Hz, 4H, $CH_3CH_2CH_2COO$), 0.97 (t, J=7.5 Hz, 3H, CH_3 in DHA) and 0.95 (t, J=7.5 Hz, 6H, CH_3) ppm. ^{13}C NMR δ 173.1 (2), 172.1, 132.0, 129.4, 128.5, 128.3, 128.2, 128.0 (2), 127.9 (2), 127.8, 127.6, 127.0, 69.0, 62.0 (2), 35.9 (2), 34.0, 25.6 (4), 25.5, 22.6, 20.5, 18.3 (2), 14.3 and 13.6 (2) ppm. IR ν_{max} 3013 (s, C–H), 2964 (vs, C–H), 2876 (s, C–H), 1736 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_{33}H_{50}O_6$+H m/z 543.3680; found 543.3655 amu.

4.1.9. 2-Docosahexaenoyl-1,3-dihexanoylglycerol ***3c****.* The procedure was identical to that for preparing **2b** using DHA (236 mg, 0.718 mmol), DMAP (18 mg, 0.144 mmol) and EDAC (15 mg, 0.862 mmol) in dry dichloromethane (3 ml) to which was added 1,3-dihexanoylglycerol **1c** (207 mg, 0.718 mmol). The product was afforded as a yellowish oil (404 mg, 94% yield). ^{1}H NMR δ 5.44–5.32 (m, 12H, =CH), 5.31–5.24 (m, 1H, CH_2CHCH_2), 4.30 (dd, J=12.0, 4.4 Hz, 2H, CH_2CHCH_2), 4.15 (dd, J=12.0, 6.0 Hz, 2H, CH_2CHCH_2), 2.88–2.78 (m, 10H, $=CCH_2C=$), 2.40–2.38 (m, 4H, CH_2CH_2COO in DHA), 2.31 (t, J=7.5 Hz, 4H, CH_2COO), 2.11–2.04 (m, 2H, $=CCH_2CH_3$), 1.65–1.58 (m, 4H, CH_2CH_2COO), 1.37–1.25 (m, 8H, CH_2), 0.97 (t, J=7.5 Hz, 3H, CH_3 in DHA) and 0.89 (t, J=6.8 Hz, 6H, CH_3) ppm. ^{13}C NMR δ 173.3 (2), 172.1, 132.0, 129.4, 128.6, 128.3 (2), 128.2, 128.1 (2), 127.9, 127.8, 127.6, 127.0, 69.0, 62.0 (2), 34.0 (3), 31.2 (2), 25.6 (4), 25.5, 24.5 (2), 22.6, 22.3 (2), 20.5, 14.3 and 13.9 (2) ppm. IR ν_{max} 3013 (s, C–H), 2959 (vs, C–H), 2872 (s, C–H), 1740 (vs, C=O) cm^{-1}. HRMS (API): Calcd for $C_{37}H_{58}O_6$+NH_4 m/z 616.4572; found 616.4572 amu.

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