Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 4, pp. 345-348, 1996

0960-894X/96 $15.00 + 0.00

S0960-894X(96)00020-0

Two step synthesis of 1,3-acetylated, -butyroylated and -caproylated triglycerides from a microorganism oil rich in Docosahexaenoic Acid (DHA)

Yves Bayon [a], and René Dolmazon [b*]

[a] *IMEDEX, Z.I. Les Troques, BP 38, F-69630 Chaponost - France*

[b] *Laboratoire de Chimie Organique, B 403, Institut National des Sciences Appliquées, 20 Avenue A. Einstein, F-69621 Villeurbanne Cédex - France*

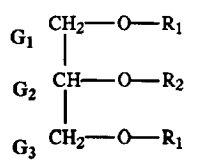
Abstract: 1,3-acetylated, -butyroylated and -caproylated triglycerides were prepared in two steps by hemi-synthesis from microorganism triglycerides rich in DHA. The triglycerides were first enzymatically transesterified into 2-monoacyl-glycerols which were then acylated with saturated short acid derivatives without significant migration of the β-long chain fatty acyl groups to the α position.

Since the first epidemiological studies showing the prevention of cardiovascular diseases by fish oils rich in eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic acids (22:6n-3, DHA)[1,2], ω3 polyunsaturated fatty acids (PUFA) have been the focus of extensive studies in recent years. It has been clearly proved that docosahexaenoic acid played a crucial role in the development of visual and brain functions[3,4] and reduced platelet agregation, in part by inhibiting thromboxane A_2/prostaglandin H_2 receptors in human[5,6]. In addition, it appears to have preventive or therapeutic activities in atherosclerotic, inflammatory and immunologic processes[7].

Natural sources of DHA often contain significant amounts of other PUFA, such as EPA. Most DHA preparations are obtained by HPLC purification from fish oil fatty acid ethyl esters, which are non physiological derivatives of fatty acids. Although the enteral bioavailability of DHA seems to be as good for ethyl esters as for natural triglycerides, DHA in the last form is more rapidly absorbed and recovered in lymph in rats[8]. Indeed, some observations suggest that ethyl esters are hydrolyzed to a lower degree than triglycerides *in vitro*[9].

The intestinal absorption of DHA in its triglyceride forms is still dependent on the distribution of DHA among the α and β positions since DHA is resistant to the hydrolysis by pancreatic lipase, an 1,3-specific lipase[10]. It is also well-known that the short chain and medium chain triglycerides esterified with C2:0 to C6:0 and C6:0 to C12:0, respectively, are absorbed more readily than long chain triglycerides, short and medium chain fatty acids being the preferred substrates of the intestinal lipases[11,12]. Moreover, it can be speculated that 2-monoacylglycerols, acylated with short chain fatty acids at α position, may be also absorbed without being hydrolyzed by digestive lipases as well as 2-monoacylglycerols. Considering these different points, the absorption of DHA may be optimal when this fatty acid is esterified at β position with short chain fatty acids at α positions in triglycerides (TGs).

Figure. Symmetrical (TGs).



Compound	α chain	β chain
2a	C4:0	DHA
2b	C4:0	oleic
3a	C6:0	DHA
3b	C6:0	oleic
4a	C2:0	DHA
4b	C2:0	oleic

The present work describes the synthesis of 1,3-acetylated, butyroylated and caproylated TGs (see figure) from an inexpensive microorganism oil rich in DHA and exempt of other polyinsaturated fatty acids including EPA (IMX oil). These TGs may be theoretically obtained by enzymatic interesterification with immobilized 1,3-positional specific lipases[13,14]. However, we preferred to transesterify first the triglycerides from the microorganism oil into 2-monoacylglycerols and then to acylate the α positions. This synthetic pathway presents theoretically the advantage to obtain low cost TGs with poor lipase substrates - *e.g.* very short chain and branched fatty acyls, unfatty acyl groups - at the α positions. The 2-monoacylglycerols were acylated in presence of *p*-dimethylaminopyridine (DMAP) without significant acyl migration from the β to the α positions although it was previously suggested that DMAP catalyzed acylations in the α position of phosphatidylcholines is accompanied by some acyl migration[15].

The fatty acyl identification and distribution of TGs between α and β positions were analysed by ^{13}C NMR. In ^{13}C NMR spectroscopy, the slight chemical shift difference observed in carbonyl ester, carbon glyceryl backbone and end methyl (Cω1) of saturated, mono and polyunsaturated fatty acids (PUFA) in the α and β-acyl positions of triglycerides have been used for qualitative and quantitative determinations[16]. It is known that for saturated acids and unsaturated acids such as oleic or DHA, the chemical shifts for C-1 atoms (carbonyl) in the α and β chains differ by about 0.4 ppm from the α chain having the higher chemical shift[16a,c]. There are also differences in the acyl chains themselves of which the most important features seem to be the extent of unsaturation and its relation to the ester group or the end methyl group. In our case, the chemical shift difference for C-1 atoms allowed to distinguish DHA from oleic acid and saturated long chain or short chain acids. Moreover, the slight chemical shift difference ($\Delta\delta$ = 0.22 ppm) observed in carbon G-2[17] of the glyceryl backbone (see figure) allowed to distinguish DHA and oleic acid in β position. The intensities associated to the several C-1 signals of the hydrocarbon chains, or G-2 signals or end methyl signals can be used to determine the percentage of DHA, oleic acid and saturated short chain acids. Good agreement was obtained between the NMR and GC-derived data.

Experimental section

2-monoacyl-glycerols (1): Typically, 1.10 g of IMX oil (1.065 mmol of triglycerides containing 1.035 mmol of DHA) in 5.50 ml of 96:4 v:v butanol:water solution were transesterified into **1** with 550 mg of Lipozyme IM, an immobilized 1,3-specific lipase from Novo Nordisk. After 48 h the solution was filtered and concentrated. It was then chromatographed on silica pre-soaked in an 1:1 ethanol:water boric acid solution. Butylesters of fatty acids, di- and triglycerides were removed with 7:3 hexane:diethylether and 0.71 mmol of **1** (yield of 66 %) was then selectively eluted with 2:1 $CHCl_3$:CH_3OH. The molar proportion of the main fatty acids, DHA and oleic acid of **1**, determined by gas-liquid chromatography[18], were 72-78 % and 8-18 % according to the different batches of **1**. The compounds **1** were identified and their purity was checked by thin layer chromatography on silica gel pre-soaked in boric acid and developed with 96:4 $CHCl_3$:acetone (R_F of **1** : 0.35)[18].

1,3-dibutyroyl-2-acyl-glycerols (2): **1** (0.71 mmol) was dissolved in 4.0 ml of anhydrous $CHCl_3$ and 14.2 mmol of butyric anhydride with stirring. After 5 minutes, 0.55 mmol of DMAP was added every 5 minutes, during 20 minutes. After 1 h, the mixture was treated with 100 ml of an aqueous molar solution of sodium hydrogen carbonate for 45 minutes. It was then added 240 ml of 1:1 $CHCl_3$:CH_3OH and the mixture was shaked. The $CHCl_3$ phase was concentrated to 50 ml and further washed twice with 90 ml of 1:1.2 aqueous 1M HCl:CH_3OH. It was evaporated and finally chromatographed on silica to give 336 mg of **2** (90 %; 0.636 mmol containing 78.2 and 13.1 mol % of DHA and oleic acid respectively) after their selective elution from the column with 80/20 hexane:diethylether. The purity of **2** was checked by thin layer chromatography on silica gel developed with 80:20 hexane:diethylether (R_F of **2** : 0.70). The compounds **2** exibited IR bands for ester group (1745 cm^{-1}) and a vinyl moiety (3050 cm^{-1}). Electrospray ionization mass spectrometric analysis[19] showed the

presence of two major compounds 1,3-dibutyryl-2-DHA-glycerol (C4:0/C22:6n-3/C4:0) (**2a**) [m/z = 565.6 $(M+Na)^+$, 30 %] and 1,3-dibutyryl-2-oleoyl-glycerol (C4:0/C18:1n-9/C4:0) (**2b**) [m/z = 519.3 $(M+Na)^+$, 100 %] which were identified by ^{13}C NMR data in comparison with known TGs[16a,b], fatty acid methyl esters[16b,f] and tributyrin[20a] :

1,3-dibutyroyl-2-DHA-glycerol (C4:0/C22:6n-3/C4:0) (**2a**): ^{13}C NMR ($CDCl_3$), δ (ppm) : 173.01 (αCO butyroyl), 172.11 (βCO DHA), 132.00, 129.45, 128.57, 128.33, 128.28, 128.09, 128.00, 127.89, 127.68, 127.05, 69.15 (G-2), 62.03 (G-1, G-3), 35.91 ($\alpha CO\underline{C}H_2$), 34.05 ($\beta CO\underline{C}H_2$), 25.66, 25.63, 25.57, 22.67, 20.59 (βCω2), 18.37 (αCω2), 14.30 (βCω1), 13.64 (αCω1).

1,3-dibutyroyl-2-oleoyl-glycerol (C4:0/C18:1n-9/C4:0) (**2b**): ^{13}C NMR ($CDCl_3$), δ (ppm): 173.01 (αCO butyroyl), 172.81 (βCO oleic)[21], 130.02 (CH=CH) ; 129.70 (CH=CH), 68.93 (G-2), 62.09 (G-1, G-3), 35.91 ($\alpha CO\underline{C}H_2$), 34.20 ($\beta CO\underline{C}H_2$); 31.93 (βCω3), 29.79, 29.73, 29.55, 29.35, 29.20, 29.13, 29.05, 27.24, 27.19, 24.90, 22.67 ($\beta C\omega_2$), 18.37 ($\alpha C\omega_2$), 14.14 (βCω1), 13.64 (αCω1).

1,3-dicaproyl-2-acyl-glycerols (3): Prepared from 0.450 mmol of **1**, 15.8 mmol of caproic anhydride, and 1.58 mmol of DMAP similarly to **2**. Yield of **3**, 0.230 g (88 %; 0.396 mmol containing 71,7 and 17,9 mol % of DHA and oleic acid respectively). The compounds **3** exibited IR bands for ester group (1745 cm^{-1}) and a vinyl moiety (3050 cm^{-1}). Electrospray ionization mass spectrometric analysis [19] showed the presence of two major compounds 1,3-dicaproyl-2-DHA-glycerol (C6:0/C22:6n-3/C6:0) (**3a**) [m/z = 621.8 $(M+Na)^+$, 100 %] and 1,3-dicaproyl-2-oleoyl-glycerol (C6:0/C18:1n-9/C6:0) (**3b**) [m/z = 575.6 $(M+Na)^+$, 44 %] which were identified by ^{13}C NMR data in comparison with known TGs[16a,b], fatty acid methyl esters[16b,f] and methyl caproate[20b]:

1,3-dicaproyl-2-DHA-glycerol (C6:0/C22:6n-3/C6:0) (**3a**): ^{13}C NMR ($CDCl_3$), δ (ppm): 173.20 (αCO caproyl), 172.10 (βCO DHA), 132.00, 129.46, 128.57, 128.33, 128.28, 128.09, 127.99, 127.89, 127.67, 127.05, 69.15 (G-2), 62.05 (G-1, G-3), 34.06 ($\beta CO\underline{C}H_2$), 34.01 ($\alpha CO\underline{C}H_2$), 31.27, 25.66, 25.63, 25.57, 24.57 (αCω3), 22.68, 22.32, 20.59 (βCω2), 14.30 (βCω1), 13.91 (αCω1).

1,3-dicaproyl-2-oleoyl-glycerol (C6:0/C18:1n-9/C6:0) (**3b**) : ^{13}C NMR ($CDCl_3$), δ (ppm): 173.20 (αCO caproyl), 172.79 (βCO oleic)[21], 130.02 (CH=CH) ; 129.70 (CH=CH), 68.93 (G-2), 62.11 (G-1, G-3), 34.21 ($\beta CO\underline{C}H_2$), 34.01 ($\alpha CO\underline{C}H_2$), 31.94 (βCω3), 31.27, 29.78, 29.71, 29.51, 29.34, 29.21, 29.14, 29.07, 27.24, 27.19, 24.91, 24.57 (αCω3), 22.71 (βCω2), 22.32 (αCω2), 14.13 (βCω1), 13.91 (αCω1).

1,3-diacetyl-2-acyl-glycerols (4): eight hundred mg of IMX oil (0.78 mmol of triglycerides containing 0.76 mmol of DHA) in 4.16 ml of 96:4 butanol:water were converted into 2-monoglycerides with 400 mg of Lipozyme IM. After 72 h, the Lipozyme IM was discarded. The IMX oil solution was then desolvented and solubilized in 4 ml of anhydrous $CHCl_3$ and 7.0 mmol of acetic anhydride with stirring. After 5 minutes, 1.40 mmol of DMAP was added to the solution which was agitated for 10 minutes. The mixture was then concentrated and chromatographed on silica gel. The most apolar byproducts (butylesters of fatty acids, monoacylated and unmodified IMX triglycerides) were discarded with 90:10 hexane:diethylether and 0.140 g of **4** (yield : 37 % ; 0.290 mmol containing 85.4 and 7.9 mol % of DHA and oleic acid respectively) was selectively eluted with 85:15 hexane:diethylether. The purity of **4** was examinated by thin chromatography on silica gel developed with 85:15 hexane:diethylether (R_F of **4** : 0.20). The compounds **4** exibited IR bands for ester group (1745 cm^{-1}) and a vinyl moiety (3050 cm^{-1}). Electrospray ionization mass spectrometric analysis[19] showed the presence of two compounds 1,3-diacetyl-2-DHA-glycerol (C2:0/C22:6n-3/C2:0) (**4a**) [m/z = 509.3 $(M+Na)^+$, 100 %] and 1,3-diacetyl-2-oleoyl-glycerol (C2:0/C18:1n-9/C2:0) (**4b**) [m/z = 463.4 $(M+Na)^+$, 4.5 %] which were identified by ^{13}C NMR data in comparison with known TGs[16a,b], fatty acid methyl esters[16b,f] and triacetin[20c]:

1,3-diacetyl-2-DHA-glycerol (C2:0/C22:6n-3/C2:0) (**4a**): ^{13}C NMR ($CDCl_3$), δ (ppm): 172.14 (βCO DHA); 170.37 ($\alpha CO\underline{C}H_3$) , 131.98, 129.46, 128.55, 128.32, 128.26, 128.08, 127.98, 127.88, 127.65, 127.05, 69.00 (G-2), 62.27 (G-1, G-3), 34.04 ($\beta CO\underline{C}H_2$), 25.65, 25.62, 25.55, 22.68, 20.66 ($CO\underline{C}H_3$), 20.57 (βCω2), 14.29 (βCω1).

1,3-diacetyl-2-oleoyl-glycerol (C2:0/C18:1n-9/C2:0) (**4b**): ^{13}C NMR ($CDCl_3$), δ (ppm): 172.83 (βCO oleic) [21]; 170.37 ($\alpha CO\underline{C}H_3$) ; 130.01 (CH=CH) ; 129.69 (CH=CH), 68.78 (G-2), 62.27 (G-1, G-3),

34.17 (βCOCH2); 31.92 (βCω3), 29.77, 29.70, 29.53, 29.33 (2 C), 29.17, 29.10, 29.01, 27.22, 27.17, 24.89, 22.68 (βCω2), 20.66 (COCH3), 14.13 (βCω1).

References and Notes

1 Kromann, N.; Green, A. *Acta Med. Scand.* **1980**, *208*, 401-406.

2 Burr, M.L.; Gilbert, J.F.; Holliday, R.M.; Elwood, P.; Fehily, A.M.; Rogers, S.; Sweetnam, P.M.; Deadman, N.M. *Lancet* **1989**, *2*, 757-761.

3 Carlson, S.E.; Werkman, S.H.; Rhodes, P.G.; Tolley, E.A. *Am. J. Clin. Nutr.* **1993**, *58*, 35-42.

4 Uauy, R.; Birch, E.; Birch, D.; Peirano, P. *J. Pediatr.*, **1992**, *120*, S168-S180.

5 Swann, P.G.; Parent, C.A.; Croset, M., Fonlupt, P.; Lagarde, M.; Venton, D.L.; LeBreton, G.C. *J. Biol. Chem.* **1990**, 21692-21697.

6 Parent, C.A.; Lagarde, M.; Venton, D.L.; LeBreton, G.C. *J. Biol. Chem.* **1992**, *267*, 6541-6547.

7 Leaf, A. *Circulation* **1990**, *82*, 624-628.

8 Ikeda, I.; Imasato, Y.; Nagao, H.; Sasaki, E.; Sugano, M.; Imaizumi, K., Yazawa, K. *Life Sciences* **1993**, *52*, 1371-1379.

9 Krokan, H.E.; Bjerve, K.S.; Mork, E. *Biochim. Biophys. Acta* **1993**, *1168*, 59-67.

10 Bottino, N.R.; Vandenburg, G.A., Reiser, R. *Lipids* **1967**, *2*, 489-493.

11 Yang L.Y.; Kuksis, A.; Myher, J.J. *J. Lipid Res.* **1990**, *31*, 137-148.

12 Bach, A.C.; Babayan, V.K. *Am. J. Clin. Nutr.* **1982**, *36*, 950-962.

13 Haraldsson, G.G. In *The Chemistry of The Functional Groups, Supplement B2: The chemistry of Acid derivatives*; Patai, S. Ed.; Vol. 2; John Wiley and sons: Chichester, 1992; pp. 1395-1473.

14 Haraldsson, G.G.; Thorarensen, A. *Tetrahedron Letters* **1994**, *35*, 7681-7684.

15 Plückthun, A.; Dennis, E.A. *Biochemistry* **1982**, *21*, 1743-1750.

16 (a) Gunstone, F. D.; Seth, S.; *Chem. Phys. Lipids* **1994**, *72*, 119-126; (b) Sacchi, R.; Medina, I.; Paolillo, L. and Addeo, F. *Chem. Phys. Lipids* **1994**, *69*, 65-73; (c) Sacchi, R.; Medina, I.; Aubourg, S. P.; Giudicianni, I.; Paolillo, L. and Addeo, F. *J. Agr. Food Chem.* **1993**, *41*, 1247-1253; (d) Gunstone, F.D. *J. A. O. C. S.*, **1993**, *10*, 965-970 ; (e) Aursand, M.; Rainuzzo, J. R. and Grasdalen, H. *J.A.O.C.S.*, **1993**, *10*, 971-981; (f) Gunstone, F. D., *High-resolution ^{1}H and ^{13}C NMR in Lipids Analysis, A practical Approach*, Ed R. J. Hamilton and S. Hamilton, Irl Press, Oxford 1992, pp. 243-262; (g) Gunstone, F. D. *Chem. Phys. Lipids* **1991**, *59*, 83-89.

17 Abbreviations : the carbons of glycerol backbone are designated α, β (NMR spectroscopy while not distinguish between the two α chains); Carbons are designated as follows : G-1-3, glyceryl carbons; αCi, α chain carbons ; βCi, β chain carbons.

18 Gunstone, F.D.; Kates, M.; Harwood, J.L. In *The Lipid Handbook*; Gunstone, F.D.; Harwood, J.L.; Padley, F.B. Eds.; Chapman & Hall Chemical Database: London, 1994; pp. 229-243.

19 Duffin, K. L.; Henion, J. D. and Shieh, J. J. *Anal. Chem.* **1991**, *63*, 1781-1788.

20 (a) Pourchert, C. J. and Behnke, J. *The Aldrich Library of 13C and 1H FTNMR Spectra* Ist Edn, 1993;Vol. I, p. 950 B; (b) ibid p. 918 A; (c) ibid. p. 949 C.

21 Oleic acid and saturated long chain acids are not distinguished.

(*Received in Belgium* 26 *September* 1995; *accepted* 6 *January* 1996)