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The Synthesis of Homogeneous Triglycerides of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase

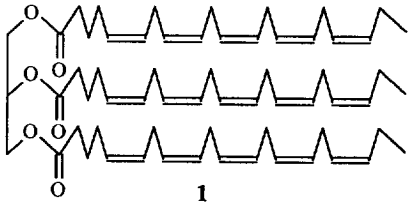
Gudmundur G. Haraldsson*, Birgir Ö. Gudmundsson and Örn Almarsson

Science Institute, University of Iceland, Dunhaga 3, 107 Reykjavik, Iceland.

Abstract: A highly efficient synthesis of homogeneous triglycerides of either pure eicosapentaenoic acid, **1**, or docosahexaenoic acid, **2**, by an immobilized non-regiospecific yeast lipase from *Candida antarctica* is described. Two methods were used: Interesterification of tributyrin and direct esterification of glycerol with stoichiometric amount of 99 % EPA or DHA as ethyl esters and free fatty acids, respectively. Both processes were performed under vacuum at 65 °C in the absence of any solvent. The volatile co-products were condensed into a cooled trap, thus shifting the equilibrium toward completion. Complete incorporation was reached in less than 72 h for all cases, but the direct esterification was found to proceed considerably faster than the transesterification as were both processes involving EPA as compared to DHA. High-field ^{1}H NMR spectroscopy analysis offered detailed investigation of the intermediate glycerides during the direct esterification reaction. The purity of the resulting crude products which were afforded in excellent yields was very high.

INTRODUCTION

The various beneficial biochemical and pharmacological effects of marine fat on human health are now well established[1-3]. They are attributed to the long-chain n-3 type polyunsaturated fatty acids characteristic of marine fat, notably *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA). The most common source for these fatty acids is fish oil in which they are bound into triglycerides usually within the range of 10 - 25 %[4]. They are also widely abundant in phospholipids of fish and related marine species[5]. The pharmaceutical industry has displayed increasing interest in these fatty acids in the natural triglyceride form[6,7]. But due to the great variety of combinations of fatty acids in marine triglyceride oils it is difficult to prepare triglycerides containing much higher than 30 % EPA and DHA directly from fish oil, without splitting the fat. Traditional chemical esterification processes for triglyceride preparation from free fatty acid and monoester concentrates of the highly labile long-chain n-3 polyunsaturated acids are not really feasible either[8], as their all-*cis* n-3 framework will be partially destroyed by oxidation, *cis-trans* isomerization or double-bond migrations. They are also quite susceptible to polymerization as a result of the rather drastic conditions involved in terms of extremes of pH and high temperature.



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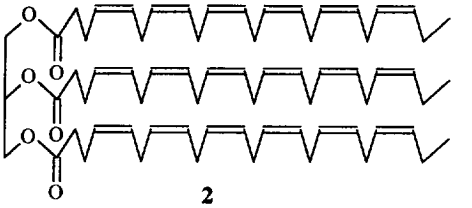

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Recently, there has been a tremendous growth in the application of enzymes in organic synthesis and enzymes are now commonly recognized as practical catalysts for asymmetric synthesis[9-11]. Lipases are among the most widely applied and versatile biocatalysts in organic synthesis[12,13]. Their scope of applicability

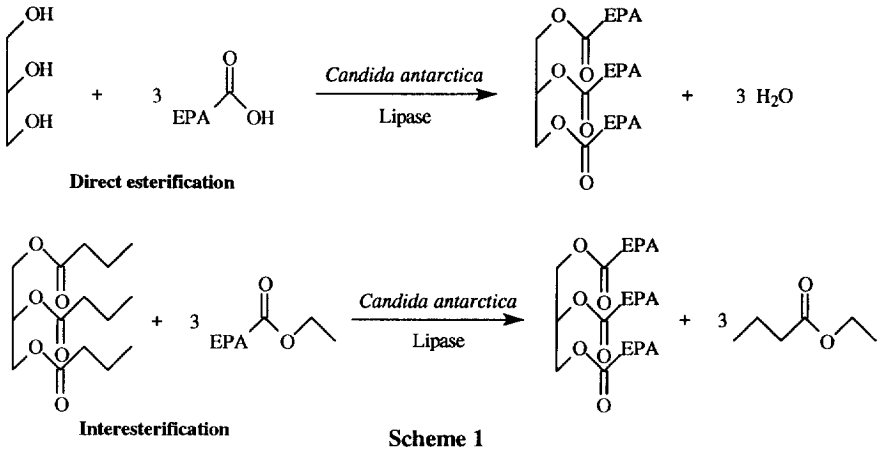
extends far beyond their natural glyceryl ester substrates, toward which they exhibit either 1,3- or non-regiospecificity. Upon the discovery that lipases and other enzymes successfully catalyse reactions under almost anhydrous conditions in organic medium, the range of possible chemical reactions involving lipases widened far beyond hydrolysis, and various esterification reactions became readily accessible. Their mildness certainly renders them ideally suited as catalysts for transformations involving the highly unstable n-3 polyunsaturated fatty acids[8,13]. However, many of the commercially available lipases are rather reluctant to the long-chain n-3 polyunsaturated fatty acids[8]. This has enabled their exploit to generate n-3 concentrates by hydrolysis and alcoholysis of fish oil[14,15]. Nearly all lipases that display any significant activity toward these fatty acids discriminate between EPA and DHA in favour of the first one. This has allowed several groups to prepare concentrates of EPA and DHA by discrimination between these two by hydrolysis[16-18] and various esterification[18-20] reactions of fish oil. Recently, a lipase has been reported to display resistance to hydrolysing triglycerides containing DHA[21]. Another lipase of different selectivity was subsequently employed to prepare triglycerides enriched with DHA by reesterification of the resulting glyceride mixture[22]. Lipases have also been employed for the enrichment of fish oil[23,24] and vegetable oil[25] triglycerides with n-3 concentrates by transesterification reactions.

A few years ago we reported on the efficient preparation of triglycerides highly enriched with n-3 polyunsaturated fatty acids (above 70 %)[23]. The catalytic effect of an immobilized 1,3-regiospecific fungal lipase from *Mucor miehei* was exploited to catalyse transesterification reactions of cod liver oil with concentrates of EPA and DHA as free fatty acids (acidolysis) or ethyl esters (interesterification) in a solvent-free environment. As a consequence of acyl migrations the fatty acids were equally and randomly distributed among positions of the triglycerides despite the 1,3-regiospecificity of the lipase[26]. By that method the extent of enrichment of the n-3 fatty acids into the triglycerides was limited to a weighted average of the fatty acid composition of the initial triglycerides and the concentrates at an equilibrium.

In this report an improvement of that method which resulted in the highly efficient generation of triglycerides homogeneous with EPA, **1**, or DHA, **2**, is described[27]. In the light of the discussion above regarding lability of the n-3 polyunsaturated fatty acids and their inaccessibility as highly concentrated triglycerides this is quite an achievement. Such homogeneous triglycerides may serve of great importance in pharmacological studies of EPA and DHA aiming at discrimination between the effect of those two fatty acids in their natural triglyceride form. Usually EPA and DHA come together in concentrates and most certainly interfere with each other in such studies. Triglycerides of any composition identical to the concentrates being used can be generated by the reported method.

RESULTS AND DISCUSSION

The homogeneous triglyceride synthesis was accomplished by an immobilized non-regiospecific yeast lipase from *Candida antarctica* which was provided by Novo Nordisk A/S in Denmark. Two methods were successfully employed: Direct esterification of glycerol and interesterification (ester-ester interchange) of tributyrin with stoichiometric amount of 99 % pure EPA or DHA as free fatty acids and ethyl esters, respectively, according to Scheme 1 (only shown for EPA). The reactions were conducted under vacuum (0.01 - 0.1 Torr) at 65 °C in the absence of any solvent with 10 % dosage of lipase as based on the weight of substrates. The volatile co-products, water or ethyl butyrate, depending on reaction type, were condensed into a liquid nitrogen cooled trap as the reactions proceeded, thus shifting the equilibrium toward completion. The traps were weighed during the progress of the reactions and their weights used as an aid to monitor the progress of the reactions supplementary to the more accurate ^{1}H NMR spectroscopy analysis. These reactions were observed to be superior to the corresponding interesterification of triacetin with n-3 enriched ethyl esters as well as the glycerolysis of n-3 enriched ethyl esters which were conducted under similar conditions. Furthermore, the *Candida antarctica* lipase was found superior to the immobilized 1,3-regiospecific *Mucor miehei* lipase also provided by Novo Nordisk as Lipozyme™ under these conditions.



Scheme 1

Direct Esterification of Glycerol

Progress of Reactions. In the direct esterification reactions an exact stoichiometric ratio of the 99 % EPA or DHA free fatty acids and glycerol was used. This means 3 mol equivalents based on the glycerol molecule or 1 equivalent as based on the number of hydroxyl group equivalents participating in the reaction. The progress of the reactions of EPA and DHA is shown in Tables 1 and 2, respectively, and graphically presented in Figures 1 and 2, respectively, as determined by the aid of high-field ^{1}H NMR spectroscopy analysis and based on mol % incorporation of free fatty acid equivalents into glycerides. The difference of the time scales in the graphs should be noted. Although less accurate due to relatively small scale of the reactions the weight measurements of the cooling traps offered results in reasonably good agreement with those provided by the NMR spectroscopy.

For both reactions 97 % incorporation was obtained in 24 h, but after 72 h both reactions had proceeded to completion and reached 100 % conversion. The extended time required for the incorporation of the very last few percentages is noteworthy and warrants a comment. It is believed to be related to the purity of the substrates and the fact that an exact stoichiometric ratio was used in both reactions. This may be improved by using a slight excess (1 - 2 %) of the fatty acid substrates.

Table 1. Percentage incorporation of EPA into glycerides during the direct esterification.

Time (h)	Total	TG	1,3-DG	1,2-DG	1-MG
0.5	38	0	32	3	3
1	60	17	36	4	3
2	74	33	32	6	3
4	89	66	17	6	0
6	91	74	12	5	0
12	96	86	7	3	0
24	97	93	3	1	0
30	98	94	3	1	0
48	99	98	1	0	0
72	100	99	1	0	0

Table 2. Percentage incorporation of DHA into glycerides during the direct esterification.

Time (h)	Total	TG	1,3-DG	1,2-DG	1-MG
0.5	14	0	14	0	0
1	24	0	20	2	2
2	45	8	30	5	2
4	67	25	32	6	4
6	77	44	25	5	3
12	91	74	13	4	0
24	97	91	5	1	0
30	98	94	3	1	0
48	99	98	1	0	0
72	100	99	1	0	0

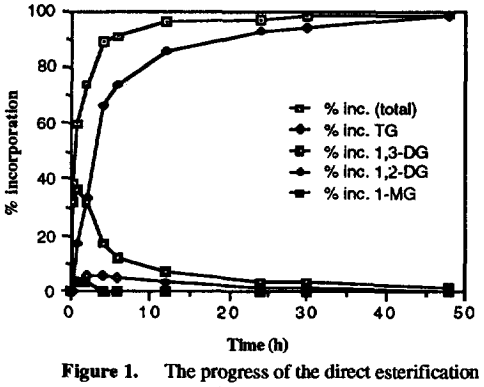


Figure 1. The progress of the direct esterification of glycerol and EPA

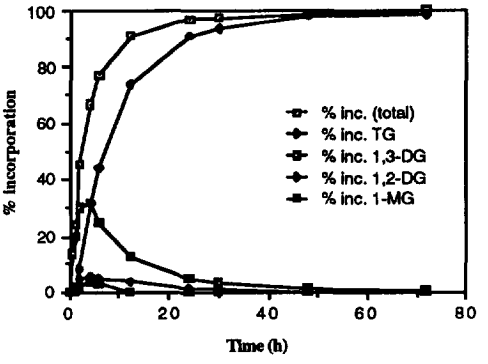


Figure 2. The progress of the direct esterification of glycerol and DHA.

Fatty Acid Selectivity. It is evident that EPA is a considerably better substrate for this lipase as compared to DHA. This was not surprising and appears to be a common feature of the already existing commercially available lipases that display moderate or high activity toward the long-chain n-3 polyunsaturated fatty acids. The reason is believed to be related to the carbon-carbon double bond in the closest proximity of the carboxyl group being located one bond closer in DHA[28]. This presumably adds some strain to the active site of these enzymes to accommodate DHA properly[29].

Water-content Control. In biotransformations involving lipases and ester synthesis the water content control in the organic medium is crucial[13]. A compromise must be kept between enzyme stability, enzyme activity and hydrolysis side-reactions in terms of water content. Moisture-free lipase, apart from the essential water for the lipase to maintain its integrity, was used, since under these conditions all additional water, including the co-produced water, escaped into the cooled traps as became evident from the weight measurements. Despite the consequential low water content the enzyme retained sufficient activity surprisingly well and the extent of hydrolysis side-reaction was obviously disappearingly low.

Analytical Aspects. The high-field ^{1}H NMR spectroscopy analysis was found extremely valuable as a probe for monitoring the progress of the reaction as well as enabling us to follow the incorporation of EPA and DHA into the various intermediate glycerides participating in the direct esterification process. In order to make that possible each individual participating glyceride, i.e. triglyceride (TG), 1(3),2-diglyceride (1,2-DG), 1,3-diglyceride (1,3-DG), 1(3)-monoglyceride (1-MG) and 2-monoglyceride (2-MG) had to be obtained in a pure form and studied individually by high-field ^{1}H NMR spectroscopy. This was carried out on the corresponding 99 % pure palmitates as commercially available reference samples. Each individual glyceride compound displays a characteristic ^{1}H NMR spectrum for the protons which belong to the glyceryl backbone of these molecules. The corresponding segments of the spectra are shown in Figure 3 for each individual palmitate glyceride. It can be noticed that the 1,2-diglyceride sample was contaminated with the triglyceride. Since these protons resonate virtually unaffected of the type of acyl moiety attached to the glyceryl backbone it became easy to identify individual glycerides in the reaction mixture and elucidate information about the composition of the reaction mixture as the direct esterification reactions proceeded. The results are revealed in Tables 1 and 2 and Figures 1 and 2. The incorporation results are based on initial total number of mol equivalents of EPA and DHA free fatty acids or hydroxyl groups present at the beginning of the reactions.

After each glyceride component had been unequivocally assigned in the spectrum the intensity of an adequate signal was used to calculate and quantify each component as a percentage of the initial amount of free EPA or DHA acid equivalents, by taking into account that the monoglycerides, diglycerides and triglycerides contain one, two and three equivalents, respectively. At the beginning, however, before all the glycerol had

reacted, the combined intensities of these protons were matched with the intensity of the methylene protons in positions 19 and 21, respectively, for EPA and DHA, to determine the total incorporation. This became necessary by reason of glycerol neither being very soluble in chloroform nor miscible with the EPA and DHA free fatty acids at the beginning of the reactions. When it became evident that all the glycerol had reacted, the protons belonging to the glyceryl backbone could be used directly for determining the incorporation into the various glycerides and thus the total incorporation.

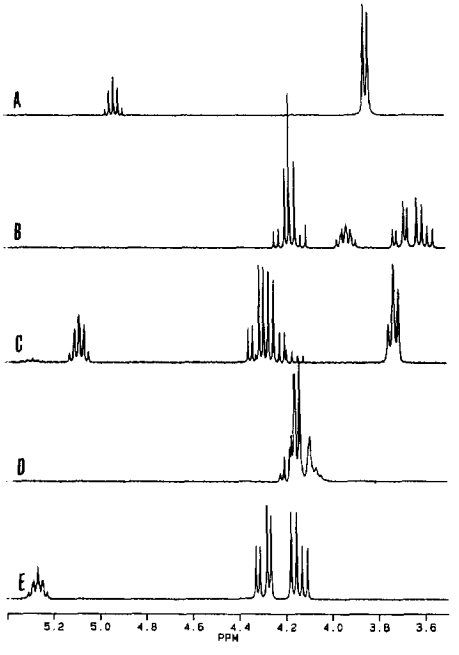
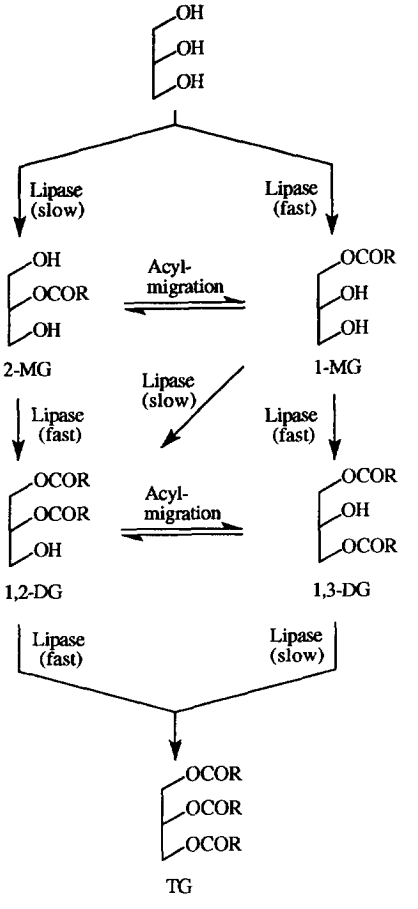


Figure 3. 250 MHz ^{1}H NMR spectra of the glyceryl backbone of A) 2-MG; B) 1-MG; C) 1,2-DG; D) 1,3-DG and E) TG as palmitates.



Scheme 2

Glyceride Interrelationship. As can be seen from Table 1 and Figure 1 for the esterification of EPA, the reaction mixture was dominated by the 1,3-diglyceride during the first 2 h of the reaction, into which the maximum of 36 % incorporation had taken place after 1 h at 60 % conversion. 1,2-Diglycerides reached the incorporation maximum at 6 % after 2 h and 1-monoglycerides remained at the 3 % incorporation level during the first 2 h, but 2-monoglyceride was not detected throughout the reaction. At 74 % conversion after 2 h the triglyceride had caught up with the 1,3-diglyceride. It is evident from these results that all the glycerol had reacted after 2 h reaction time.

The behaviour for DHA was observed to be similar, but as can be noticed in Table 2 and Figure 2 the DHA esterification reaction proceeded considerably slower than the EPA reaction. After 0.5 h only 1,3-diglyceride was present with none of the 1-monoglyceride precursor detected at 14 % conversion. As for the EPA reaction 1,3-diglyceride dominated the reaction mixture during the first few hours of the reaction reaching a maximum of 32 % after 4 h at 67 % conversion. At that stage both 1,2-diglycerides and 1-monoglycerides also reached a maximum and it is evident that all the glycerol had reacted. As for the EPA reaction no 2-monoglyceride was detected throughout the reaction.

Rationalization and Mechanistic Aspects. The apparently rather complicated interrelationship of the glycerides may be rationalized in terms of the general assumption that lipases normally display strong preferences for primary rather than secondary alcoholic substrates due to increased bulkiness of the secondary alcohols rendering them less nucleophilic. This means that acylations at the end-positions (α) should be considerably faster than acylations at the mid-position (β), and the results appear to fit into this perspective rather nicely. The rate of formation of 1,3-diglycerides is higher than that of triglycerides. In order to form triglycerides from 1,3-diglycerides either the non-regiospecific lipase will have to act directly at the mid-position, or alternatively, an intramolecular acyl-migration of the 1,3-diglyceride into 1,2-diglyceride (or 2,3-diglyceride) must take place together with a subsequent acylation of the resulting more prone 1,2-diglyceride. Such intramolecular acyl-migration isomerizations are well established[30] and take place via a cyclic five-membered ortho ester type intermediate. Both the enzymatic acylation of the mid-position and the acyl-migration are believed to be considerably slower than the enzymatic acylation at the end-positions, the latter presumably being accelerated by the presence of the free fatty acid substrates. This also provides an explanation why the formation of 2-monoglycerides was not detected during the process and why 1,2-diglycerides are less abundant than 1,3-diglycerides. The precursor to the 1,3-diglyceride is 1-monoglyceride which follows the favoured route of end-position acylation of glycerol. The formation of the corresponding 2-monoglyceride is certainly possible for the non-specific lipase from which acyl migration could also lead to the 1-monoglyceride. The overall process is summarized in Scheme 2.

The stereodifferentiation of the enzyme during the formation of the chiral glyceride intermediates is another important aspect, which certainly may influence the rate of formation of various intermediates and thus add to the complexity of the overall process described in Scheme 2. This relates to questions as to which enantiomer is more readily formed during the acylation of the prochiral glycerol, the *sn*-1- or the *sn*-3-monoglyceride, and the enantiopreference of the enzyme for the *sn*-1,2- or *sn*-2,3-diglycerides formed after a non-enzymatic acyl migration of the 1,3-diglyceride.

Finally, it may be of great interest to examine the kinetics involved in this rather complicated process under equilibrium conditions. That has recently been undertaken for the direct esterification of glycerol with oleic acid employing the 1,3-regiospecific *Mucor miehei* lipase[31]. Isomerization of 1,3-diolein into 1,2-diolein by intramolecular acyl migration and the subsequent reaction of 1,2-diolein with oleic acid, catalysed by the enzyme, is responsible for the largest part of the triolein synthesis catalysed by that lipase. The presence of free oleic acid was also found to speed up the acyl migration isomerization processes. Recently, the employment of high-field ^{1}H NMR has been described to investigate the lipase-catalysed isomerization of 1,2-diglycerides into 1,3-diglycerides (as palmitates) in a similar manner as described in the current paper[32].

Product Characteristics. Both products were afforded as an oil in nearly quantitative yield after aqueous alkaline treatment to free them of traces of free fatty acids, and in high purity as indicated by NMR and iatroscan[33] (TLC with flame ionization detection) analysis. Pure triglycerides of both EPA and DHA were afforded as colourless oils after preparative HPLC treatment. All spectroscopic data were in full harmony with the proposed structure of the products. The mass spectrometry analysis provided molecular ions consistent with the molecular weight of the products, satisfactory accurate mass determination and the fragmentation spectra were fully consistent with their structure as were their high-field ^{1}H and ^{13}C NMR spectra. In both compounds the acyl moieties belonging to the mid-position and the end-positions of the glyceryl backbone had identical ^{1}H and ^{13}C NMR spectra with no apparent line-broadening, apart from the methylene protons in

position 2 of EPA and the carbonyl group carbons and the carbons in position 2 of both EPA and DHA, in which two distinctive resonance signals were obtained in the ^{13}C spectra. This has recently been utilized for analysing the distribution of EPA and DHA into positions of triglycerides from fish muscle[34,35]. In the ^{1}H NMR spectra the methylene protons of the end-positions of the glyceryl backbone displayed first order behaviour and afforded two regular sets of doublet of doublets with the integration in perfect harmony with the remaining protons of the acyl moieties, demonstrating the 1:3 stoichiometric ratio and fully establishing the nature and purity of the products. The protons belonging to the mid-position of the glyceryl backbone resonated as a multiplet together with the vinylic protons of the acyl groups.

Both compounds were stored under argon at -25 °C for three years without any disintegration as was established by ^{1}H NMR spectroscopy studies. However, some samples containing DHA triglycerides appeared to polymerize in a few months intimating that great care must be undertaken when handling such compounds. This may, however, be related to the preparation of such compounds with the transesterification method described below, since DHA samples prepared by that method seem to deteriorate much faster.

Interesterification with Tributyrin

Progress of Reactions. In the interesterification reaction involving tributyrin and 99 % pure EPA or DHA as ethyl esters, the volatile co-product is ethyl butyrate. Since ethyl butyrate is several times heavier than water, the weight measurements of the entrapped co-product for monitoring the progress of the reaction were obviously more accurate than those of the direct esterification involving water. The progress of the transesterification reactions is demonstrated in Table 3 and Figure 4 for both EPA and DHA as determined by high-field ^{1}H NMR spectroscopy. As can be noticed from these results this reaction proceeded significantly slower than the direct esterification reaction. Also, as before, DHA incorporation was significantly slower as compared to EPA. After 72 h, 96 % incorporation had been reached for EPA and 95 % for DHA. After extended reaction time of 96 h, 97 % incorporation was reached for both EPA and DHA. As for the direct esterification, and even more so in this case, the extended time required for the incorporation of the very few last percentages is noteworthy and, again, must be related to the purity of the substrates and the fact that an exact stoichiometric ratio of substrates was used.

Table 3. The incorporation of EPA and DHA into tributyrin during interesterification.

Time (h)	% inc. EPA	% inc. DHA
2	64	33
4	73	63
8	82	78
12	86	85
24	91	89
48	93	94
72	96	95
96	97	97

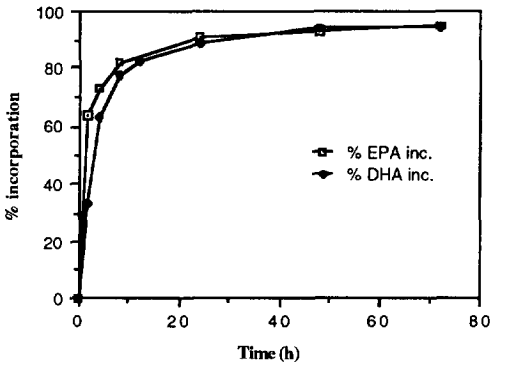
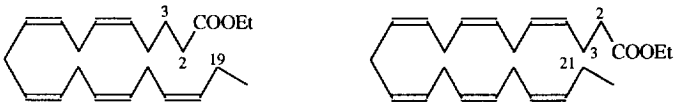


Figure 4. The incorporation of EPA and DHA into tributyrin during interesterification.

Water-content Control. In this reaction it was found beneficial to use lipase containing 10 % water, but only for speeding up the initial reaction rate. The weight measurements of the co-produced ethyl butyrate, which were in good agreement with the more accurate ^{1}H NMR spectroscopy analysis, indicated that under these vacuum conditions all additional water escaped into the cooled traps, which also was easily observed from the appearance of the biphasic content of the traps. In spite of the resulting low water content the enzyme maintained sufficient activity surprisingly well. Unlike the direct esterification no water co-production occurs

during the interesterification process which means that the enzyme must retain its essential water content required for maintaining its catalytic function very effectively. That content varies among lipases[13] and may be especially low for this particular lipase. It must also be borne in mind that the essential water content is very much dependent upon the polarity of the reaction medium, which should be relatively high in this case since no solvent was used and more water thus required. Under these virtually water-deficient conditions the extent of hydrolysis side-reaction was very low as would be expected and was established by the NMR analysis and titration experiments (less than 1 %). It is important to maintain the pressure between 0.1 and 0.01 Torr, since fluctuations in the vacuum affect the progress of the reaction. Too high vacuum may strip off too much or all the essential water from the lipase which leads to loss of activity, whereas too low vacuum may lead to slowing-down of the overall reaction rate and increase the amount of hydrolysis related side-products.

Details of Analysis. In the EPA reaction the intensities of the methyl protons of the ethyl group of the monoester and the methylene proton pair in position 3 of the EPA moiety were matched and used to monitor the progress of the reaction. These protons resonated as a triplet at δ1.22 and a multiplet at 1.75 - 1.61 ppm, respectively. In the DHA reaction, on the other hand, the ratio of the intensities of the corresponding methyl protons of the ethyl group of the monoester and the methylene group at carbon 21 of the DHA moiety, were used, resonating as a triplet at δ1.21 and a multiplet at 2.13 - 2.02 ppm, respectively (Scheme 3).



Scheme 3

Product Characteristics. Crude products of very high purity were afforded in nearly quantitative yield. As indicated by ^{1}H NMR spectroscopy analysis only traces of the butyric acid moiety remained in the triglycerides. This may sound a bit surprising in light of the 97 % incorporation extent mentioned above, since residual butyric acid to the extent of 3 % should still be present. The explanation for this is presumably that the purity of the tributyrin which was used in these experiments was only 98 - 99 %. The glyceryl proton region of the ^{1}H NMR spectra revealed that very little or no mono- and diglyceride products related to hydrolysis side-reactions were present as was also confirmed by titration and iatroscan analysis. As in the glycerol case preparative HPLC was used to purify the products, eluting with 10 % diethyl ether in n-hexane, to afford 100 % pure triglycerides. Both products had spectroscopic properties identical to the products from the direct esterification reaction. There remains a question as to whether the homogeneous DHA triglyceride produced by this method was more prone to polymerization.

CONCLUSION

The highly successful preparation and full characterization of homogeneous triglycerides of both EPA and DHA by two procedures involving the catalytic effect of an immobilized non-regiospecific *Candida antarctica* lipase has been accomplished. In both procedures, direct esterification of glycerol with free fatty acids, and interesterification of tributyrin with ethyl esters, exact stoichiometric amounts were used. The direct esterification reaction was observed to proceed considerably faster than the interesterification reaction. Very high extent of incorporation was obtained, close to 100 %, but in order to reach completion the purity of the substrates appeared to be the crucial factor. The ^{1}H NMR spectroscopy not only proved enormously powerful as a probe for monitoring the progress of the reaction, but also enabled us to look into the composition of the participating intermediate glycerides as the reaction proceeded. Under these water-deficient conditions the lipase retained its essential water content to maintain its activity remarkably well. Finally, it is believed that such pure homogeneous triglycerides of EPA and DHA may prove important in clinical studies of EPA and DHA in their natural triglyceride form for discriminating between them in terms of their claimed beneficial health effects.

EXPERIMENTAL

General. ^1H and ^{13}C nuclear magnetic resonance spectra were recorded on a Bruker AC 250 NMR spectrometer in deuterated chloroform as a solvent. Infrared spectra were conducted on a Perkin-Elmer 283 Infrared Spectrophotometer on the neat liquid. HPLC separations were carried out by a PrepLC™ System 500A instrument from Waters using the PrepPak® 500/Silica Cartridge column from Millipore, eluting with 10 % diethyl ether in petroleum ether. Analytical GLC was conducted on a Perkin-Elmer 8140 Gas Chromatograph according to a previously described procedure[26]. Mass spectrometric analysis were kindly performed at Liverpool University in England, U.K. Iatroscan (TLC-FID) analysis were performed at Novo Nordisk in Bagsværd, Denmark.

The immobilized *Candida antarctica* lipase was provided by Novo Nordisk in Denmark as lipase SP 382. It was used moisture-free in the esterification experiments, but in the transesterification experiments the moisture content was 10 %. Analytical grade diethyl ether purchased from Merck was used without any purification, but synthetic grade n-hexane also from Merck was freshly distilled prior to use in extractions and HPLC chromatography. Glycerol (99 %) and tributyrin (98-99 %) were purchased from Sigma and Aldrich Chemical Companies, respectively and were used without further purification. Sodium hydroxide, ethylenediaminetetraacetic acid disodium salt dihydrate and anhydrous magnesium sulfate were obtained from Merck. 99 % pure reference samples of 1-monoglyceride, 2-monoglyceride, 1,2-diglyceride, 1,3 diglyceride and triglyceride as palmitates were purchased from Sigma Chemical Company. EPA ethyl ester (99 %; a colourless liquid) and DHA ethyl ester (99 %; a yellowish liquid) were obtained from Toyo Fine Products Co., Ltd. in Tokyo, Japan. The purity of these products was confirmed by analytical GLC and high-field NMR spectroscopy. EPA: 250 MHz ^1H NMR ($CDCl_3$) δ 5.40-5.22 (m, 10 H, =C-H), 4.09 (q, J = 7.12 Hz, 2 H, -O-CH_2-CH_3), 2.84-2.76 (m, 8 H, =C-CH_2-C=), 2.28 (t, J = 7.37 Hz, 2 H, -CH_2-COOEt), 2.10-2.02 (m, 4 H, =CH-CH_2-CH_2- and CH_3-CH_2-CH=), 1.73-1.61 (m, 2 H, -CH_2-CH_2-COOEt), 1 .22 (t, J = 7.12 Hz, 3 H, -O-CH_2-CH_3) and 0.94 ppm (t, J = 7.52 Hz, 3 H, CH_3-CH_2-CH=); ^{13}C NMR ($CDCl_3$) δ 173.4 (C=O), 131.9 (CH), 128.9 (CH), 128.7 (CH), 128.4 (CH), 128.1 (CH), 128.1 (CH), 128.0 (CH), 128.0 (CH), 127.7 (CH), 126.9 (CH), 60.0 (CH_2), 33.6 (CH_2), 26.4 (CH_2), 25.5 (CH_2), 25.5 (CH_2), 25.5 (CH_2), 25.4 (CH_2), 24.7 (CH_2), 20.4 (CH_2), 14.1 (CH_3) and 14.1 ppm (CH_3). DHA: 250 MHz ^1H NMR ($CDCl_3$): δ 5.42-5.22 (m, 12 H, =C-H), 4.09 (q, J= 7.13 Hz, 2 H, -O-CH_2-CH_3), 2.83-2.75 (m, 10 H, =C-CH_2-C=), 2.39-2.27 (m, A_2B_2, 4H, -CH_2-CH_2-COOEt), 2.10-1.98 (m, 2H, CH_3-CH_2-CH=), 1.21 (t, J = 7.13 Hz, 3 H, -O-CH_2-CH_3) and 0.94 ppm (t, J = 7.52 Hz, 3 H, CH_3-CH_2-CH=); ^{13}C NMR ($CDCl_3$) δ 172.9 (C=O), 131.9 (CH), 129.1 (CH), 128.4 (CH), 128.1 (CH), 128.1 (CH), 128.1 (CH), 128.0 (CH), 128.0 (CH), 128.0 (CH), 127.9 (CH), 127.8 (CH), 126.9 (CH), 60.2 (CH_2), 34.1 (CH_2), 25.5 (CH_2), 2 5.5 (CH_2), 25.5 (CH_2), 25.5 (CH_2), 25.4 (CH_2), 22.7 (CH_2), 20.5 (CH_2), 14.2 (CH_3) and 14.2 ppm (CH_3).

Hydrolysis of EPA ethyl ester. To a solution of sodium hydroxide (1.35 g, 33.8 mmol) and ethylenediaminetetraacetic acid, disodium salt dihydrate ($Na_2EDTA{\cdot}2H_2O$; 0.013 g, 0.035 mmol) in distilled water (25 ml) was added 96 % ethanol (25 ml). After the solution had been aerated by blowing argon through the solution for approximately 15 minutes the 99 % EPA ethyl ester (M.wt. 330.5 g/mol; 5.00 g, 15.1 mmol) was added and the resulting mixture heated at 60 - 65 °C and magnetically stirred under argon. The progress of the reaction was monitored by analytical TLC (silica gel; eluted with 1:9 ethyl acetate/n-hexane; the ethyl esters had R_f value of 0.5, whereas the free fatty acid product did not move on the plate). The rection was completed in less than 2 h. The mixture was allowed to cool to 0-4 °C in an ice-bath and then acidified by carefully adding an aqueous 2 M HCl solution until slightly acidic as indicated by litmus paper (about 30 ml required). The resulting mixture was then extracted with n-hexane (two times 100 ml), the combined organic layers washed with water until neutral pH, dried over anhydrous magnesium sulfate and the organic solvent removed *in vacuo* on a rotary evaporator followed by high-vacuum to remove traces of solvents. NMR spectroscopy indicated an absolutely pure product (4.50 g, 98 - 99 % yield) which was almost a colourless liquid. 250 MHz ^1H NMR ($CDCl_3$) δ 5.42-5.24 (m, 10 H, =C-H), 2.85-2.77 (m, 8 H, =C-CH_2-C=), 2.35 (t, J =

7.65 Hz, 2 H, -C*H*$_2$-COOH), 2.16-2.00 (m, 4 H, =CH-C*H*$_2$-CH$_2$- and CH$_3$-C*H*$_2$-CH=), 1.76-1.64 (m, 2 H, -C*H*$_2$-CH$_2$-COOH) and 0.96 ppm (t, J = 7.53 Hz, 3 H, -CH$_3$); ^{13}C NMR ($CDCl_3$) δ 180.1 (C=O), 132.0 (CH), 129.0 (CH), 128.7 (CH), 128.5 (CH), 128.2 (CH), 128.1 (CH), 128.1 (CH), 128.0 (CH), 127.8 (CH), 127.0 (CH), 33.4 (CH$_2$), 26.4 (CH$_2$), 25.6 (CH$_2$), 25.6 (CH$_2$), 25.6 (CH$_2$), 25.5 (CH$_2$), 24.4 (CH$_2$), 20.5 (CH$_2$) and 14.2 ppm (CH$_3$).

Hydrolysis of DHA ethyl ester. The procedure was identical to the one for the 99 % EPA ethyl ester using 5.00 g of 99 % DHA ethyl ester (M.wt. 356.6 g/mol; 14.0 mmol). Other components were used in exactly the same amounts as in the corresponding EPA ethyl ester procedure above. NMR spectroscopy indicated an absolutely pure product (4.53 g, 98 - 99 % yield) which was afforded as a slightly yellowish liquid. 250 MHz ^{1}H NMR ($CDCl_3$) δ 5.40-5.24 (m, 12 H, =C-H), 2.85-2.77 (m, 10 H, =C-CH$_2$-C=), 2.43-2.37 (m, A_2B_2, 4H, -C*H*$_2$-C*H*$_2$-COOH), 2.11-2.00 (m, 2 H, CH$_3$-C*H*$_2$-CH=) and 0.95 ppm (t, J = 7.53 Hz, 3 H, -CH$_3$); ^{13}C NMR ($CDCl_3$) δ 179.5 (C=O), 132.0 (CH), 129.6 (CH), 128.5 (CH), 128.3 (CH), 128.2 (CH), 128.2 (CH), 128.1 (CH), 128.1 (CH), 127.9 (CH), 127.8 (CH), 127.5 (CH), 127.0 (CH), 33.6 (CH$_2$), 25.6 (CH$_2$), 25.6 (CH$_2$), 25.6 (CH$_2$), 25.6 (CH$_2$), 25.5 (CH$_2$), 22.4 (CH$_2$), 20.5 (CH$_2$) and 14.2 ppm (CH$_3$).

The preparation of EPA triglyceride (1) by direct esterification. Immobilized *Candida antarctica* lipase (0.50 g; moisture-free) was added to a mixture of glycerol (0.44 g, 4.78 mmol) and 99 % EPA as free fatty acid (M.wt. 302.5 g/mol; 4.40 g, 14.54 mmol). The mixture was gently stirred on a magnetic stirrer hot-plate at 65 °C under continuous vacuum of 0.01 - 0.1 Torr. The volatile water produced during the progress of the reaction was continuously condensed into a liquid nitrogen cooled trap, which could be separated and weighed regularly during the process by disconnecting the reaction by replacing the vacuum with dry oxygen-free nitrogen atmosphere. After 30 h the reaction was discontinued, n-hexane added and the enzyme separated off by filtration. The organic solvent was removed *in vacuo* on a rotary evaporator followed by high-vacuum treatment to afford the crude product as a slightly yellowish oil (4.18 g, 93 %). Weight measurements indicated a complete incorporation, but NMR spectroscopy indicated 98 % incorporation, which had increased to 99 % after additional 18 h reaction time. Titration by standardized sodium hydroxide was applied to determine the free fatty acid content of the crude reaction product (less than 1 % free fatty acid content as based on number of mol of ester groups, corresponding to at least 99 % incorporation, which is equivalent to the minimum of 97 % triglyceride content). The crude product was directly introduced into HPLC eluting with 10 % diethyl ether in n-hexane to afford 100 % pure triglyceride as a colourless oil (3.51 g, 84 % recovery from HPLC) which was confirmed by iatroscan studies. 250 MHz ^{1}H NMR ($CDCl_3$) δ 5.41-5.26 (m, 31 H, =C-H and -CH$_2$-C*H*-CH$_2$-), 4.30 (dd, J = 11.90 Hz, J = 4.34 Hz, 2 H, -C*H*$_2$-CH-C*H*$_2$-), 4.14 (dd, J = 11.90 Hz, J = 5.93 Hz, 2 H, -C*H*$_2$-CH-C*H*$_2$-), 2.90-2.78 (m, 24 H, =C-CH$_2$-C=), 2.33 (t, J = 7.34 Hz, 2 H, β -CH$_2$-COO-), 2.32 (t, J = 7.36 Hz, 4 H, α -CH$_2$-COO-), 2.15-2.01 (m, 12 H, =CH-C*H*$_2$-CH$_2$- and CH$_3$-C*H*$_2$-CH=), 1.75-1.61 (m, 6 H, -C*H*$_2$-CH$_2$-COO-) and 0.97 ppm (t, J = 7.52 Hz, 9 H, -CH$_3$); ^{13}C NMR ($CDCl_3$) δ 172.9 (α C=O), 172.6 (β C=O), 132.0 (CH), 128.9 (CH), 128.7 (CH), 128.5 (CH), 128.2 (CH), 128.1 (CH), 128.1 (CH), 128.0 (CH), 127.8 (CH), 127.0 (CH), 68.9 (CH), 62.1 (CH$_2$), 33.5 (β CH$_2$), 33.3 (α CH$_2$), 26.4 (CH$_2$), 25.6 (CH$_2$), 25.6 (CH$_2$), 25.5 (CH$_2$), 24.7 (CH$_2$), 24.6 (CH$_2$), 20.5 (CH$_2$) and 14.2 ppm (CH$_3$); IR (neat liquid) ν_{max} 3020 (vs, C=C-H), 2970 (s, CH$_3$), 2935 (s, CH$_2$), 2875 (s, CH$_3$), 2850 (w, CH$_2$), 1745 (vs, C=O) and 1645 cm^{-1} (m, C=C); m/e (EI) 945 (M$^+$, 100 %); Found: 944.68784, $C_{63}H_{92}O_6$ requires 944.68939 amu.

The preparation of DHA triglyceride (2) by direct esterification. A procedure identical to the one described above for the direct esterification of EPA was used in which immobilized *Candida antarctica* lipase (0.50 g; moisture-free) was added to a mixture of glycerol (0.41 g, 4.45 mmol) and 99 % DHA as free fatty acid (M.wt. 328.5 g/mol; 4.43 g, 13.48 mmol). The crude product was afforded as a slightly yellowish oil (4.36 g, 95 %). Weight measurements indicated a complete incorporation which remained constant from 24 to 72 h, whereas NMR spectroscopy indicated 97 % incorporation after 24 h which had increased to 100 % after 72 h. The crude product was directly introduced into HPLC eluting with 10 % diethyl ether in n-hexane to afford pure triglyceride as a colourless oil (3.46 g, 80 % recovery from HPLC) which was confirmed by iatroscan studies.

250 MHz ^{1}H NMR ($CDCl_3$) δ 5.44-5.25 (m, 37 H, =C-H and -CH_2-C*H*-CH_2-), 4.30 (dd, J = 11.90 Hz, J = 4.36 Hz, 2 H, -C*H*$_2$-CH-C*H*$_2$-), 4.15 (dd, J = 11.90 Hz, J = 5.89 Hz, 2 H, -C*H*$_2$-CH-C*H*$_2$-), 2.90-2.79 (m, 30 H, =C-CH_2-C=), 2.39-2.38 (m, A_2B_2, 12 H, -C*H*$_2$-C*H*$_2$-COO-), 2.13-2.02 (m, 6 H, CH_3-C*H*$_2$-CH=) and 0.97 ppm (t J = 7.53 Hz, 9 H, -CH_3); ^{13}C NMR ($CDCl_3$) δ 172.5 (α C=O), 172.1 (β C=O), 132.0 (CH), 129.5 (CH), 128.5 (CH), 128.3 (CH), 128.3 (CH), 128.2 (CH), 128.2 (CH), 128.0 (CH), 127.9 (CH), 127.8 (CH), 127.6 (CH), 127.0 (CH), 69.0 (CH), 62.2 (CH_2), 34.0 (β CH_2), 33.8 (α CH_2), 25.6 (CH_2), 25.6 (CH_2), 25.6 (CH_2), 25.6 (CH_2), 25.5 (CH_2), 22.6 (CH_2), 20.5 (CH_2) and 14.2 ppm (CH_3); IR (neat liquid) ν_{max} 3020 (vs, C=C-H), 2970 (s, CH_3), 2930 (s, CH_2), 2870 (s, CH_3), 2850 (w, CH_2), 1750 (vs, C=O) and 1650 cm^{-1} (m, C=C); m/e (EI) 1023 (M^+, 100 %); Found: 1022.7340, $C_{69}H_{98}O_6$ requires 1022.7363 amu.

The preparation of EPA triglycerides (1) by interesterification. Immobilized *Candida antarctica* lipase (0.65 g; 10 % moisture-content) was added to a mixture of tributyrin (1.48 g, 4.89 mmol) and 99 % EPA ethyl ester concentrate (M.wt. 330.5 g/mol; 4.87 g, 14.7 mmol). The mixture was gently stirred at 65 °C on a magnetic stirrer hot-plate under a continuous vacuum of 0.01 - 0.1 Torr. The volatile ethyl butyrate product was continuously condensed into a liquid nitrogen cooled trap, which could be separated and weighed regularly during the process by disconnecting the reaction by replacing the vacuum with a dry oxygen-free nitrogen atmosphere. After 72 h the reaction was discontinued, n-hexane added and the enzyme separated off by filtration. After solvent removal *in vacuo* on rotary evaporator followed by high-vacuum treatment the crude reaction product was afforded (4.43 g, 95 %). Hydrolysis was found to occur only to a minimum extent as indicated by titration, which demonstrated less than 1 % free fatty acid content of the product (0.7 %), and further established by iatroscan studies. The weight measurements indicated 95 % incorporation, iatroscan studies 97 % and NMR spectroscopy 95 %. The product was freed from the residual ethyl ester and purified by the aid of prep. HPLC eluting with 10 % diethyl ether in n-hexane to afford 100 % pure triglyceride (3.80 g, 86 % recovery from HPLC) as established by iatroscan studies. NMR spectroscopy indicated very pure material with only traces of the butyric acid moiety left in the triglycerides. The product exhibited all spectroscopic data identical to the corresponding product of the direct esterification of EPA and glycerol.

The preparation of DHA triglyceride (2) by interesterification. The procedure was identical to the one for EPA interesterification described above, adding immobilized *Candida antarctica* lipase (0.65 g; 10 % moisture-content) to a mixture of tributyrin (1.38 g, 4.56 mmol) and 99 % DHA ethyl ester concentrate (M.wt. 356.6 g/mol; 4.87 g, 13.6 mmol). The crude reaction product was afforded (4.40 g, 94 %). Hydrolysis was found to occur only to a minimum extent as indicated by titration, which demonstrated about 1 % free fatty acid content of the product (1.2 %), and further established by iatroscan studies. The weight measurements indicated 95 % incorporation, iatroscan 96 %, but NMR spectroscopy indicated 94 %. The product was freed from the residual ethyl ester and purified by the aid of prep. HPLC eluting with 10 % diethyl ether in n-hexane to afford 100 % pure triglyceride (3.56 g, 81 % recovery from HPLC) as established by iatroscan studies. NMR spectroscopy indicated very pure material with only traces of the butyric acid moiety left in the triglyceride. The product exhibited all spectroscopic data identical to the corresponding product of the direct esterification of DHA and glycerol.

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