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The Generation of Glyceryl Ether Lipids Highly Enriched with Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase

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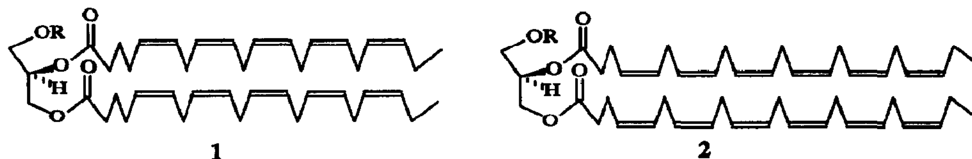
Abstract: Methods for generating non-polar glyceryl ether lipids highly enriched with EPA or DHA or preparing them highly efficiently homogeneous with EPA, **1**, or DHA, **2**, by immobilized lipases is described.

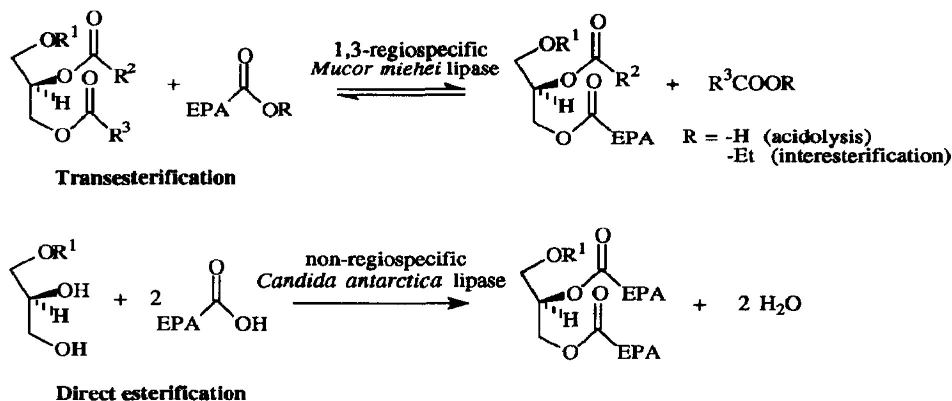
Non-polar glyceryl ether lipids of the 1-O-alkyl-2,3-diacyl-*sn*-glycerol type are major constituents in liver oil of certain species of shark, such as the Greenland shark (*Somniosus microcephalus*), making up to 30 - 60 % of the oil[1,2]. Recently, they have been claimed to display various beneficial effects on human health[3,4]. The close structural resemblance between the ether lipids and the well-known platelet activating factors[3,4], both possessing the 1-O-alkyl-*sn*-glycerol framework, should be pointed out. The advantageous health effects of marine oil are also well established and are attributed to the long-chain n-3 type polyunsaturated fatty acids characteristic of marine fat, *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), in particular[5-7]. In order to possibly combine the claimed beneficial effects of fish oil and the ether lipids it was decided to prepare such lipids highly enriched with EPA and DHA by the lipase catalysis procedures already described[8-10]. The main objective was to prepare ether lipids homogeneous in terms of EPA and DHA such as **1** and **2**, respectively.



Lipases are now among the most widely applied and versatile biocatalysts in organic synthesis[11]. They exhibit high tolerance for variation in substrate structure and their active site can be expected to readily accommodate the ether lipids, which are closely related to their natural triglyceride substrates. Lipases are ideally suited as catalysts for transformations involving the highly labile long-chain polyunsaturated fatty acids. The mildness they offer most certainly protects them from partial destruction of their natural all-*cis* n-3 framework by oxidation, double-bond migrations or *cis-trans* isomerization during traditional chemical processes involving extremes of pH and high temperature[12].

Ether lipids were isolated in a pure state, as established by high-field NMR, from shark liver oil concentrates[13] by preparative HPLC employing ethyl ether in pet. ether (1:16) as an eluent. There are three major fatty alcohol constituents present in the ether moiety, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, the last one being the most abundant. Fatty acid analysis by GLC revealed that EPA and DHA make up only 0.4 and 2.5 % of the fatty acid composition of the ether lipids, respectively, with palmitic acid ($C_{16:0}$) and, primarily, oleic acid ($C_{18:1}$) as the most abundant fatty acids, 14 and 46 %, respectively. Pure 1-O-alkyl-*sn*-glycerols were obtained in good yield from the diacyl ether lipids by sodium methoxide catalyzed methanolysis. The fatty acid methyl esters were separated by extraction into hexane, whereas the glycerol ethers (GLE) remained in the methanol phase.

In the first approach to accomplish ether lipids enriched with n-3 type polyunsaturated fatty acids two types of transesterification reactions were investigated: Acidolysis involving fatty acid exchange between the initial ether lipids and concentrates of EPA and DHA as free fatty acids; interesterification involving ester-ester interchange reactions with ethyl ester concentrates of EPA and DHA. This is demonstrated in Scheme 1 with R = -H and -Et, respectively, for the acidolysis and interesterification reactions (only shown for pure EPA). Reaction conditions identical to those previously described for cod liver oil triglycerides were applied[8,9]. Based on the weight of substrates 10 % dosage of the immobilized 1,3-regiospecific *Mucor miehei* lipase[14] containing 10 % water was employed under solvent-free conditions at 60 - 65 °C with a three-fold molar excess of the concentrates as based on the number of mol of ester equivalents present in the glyceride backbone of the ether lipids.



Scheme 1

Ether lipids of various composition containing up to 57 % EPA, 25 % DHA and 43 % EPA+DHA (34 % EPA; 9 % DHA) were obtained from concentrates of 98 % EPA, 88 % DHA and 85 % EPA+DHA (55 % EPA; 30 % DHA), respectively[15]. As for the cod liver oil triglycerides the interesterification was considerably faster than the acidolysis reaction. Also, the DHA incorporation rate was much slower than for EPA, and partial hydrolysis side-reaction (5 - 10 %) occurred depending on moisture content of the lipase preparation as well as reaction type. Reaction time from 24 - 72 h was required for reaching an equilibrium depending upon type of reaction and type of concentrate. From these results it is evident that the mid-position of the glyceride moiety of the ether lipids is much more reluctant to participate in these reactions as compared to the corresponding triglycerides[9]. In that case it did not make difference whether a 1,3-regiospecific or a non-regiospecific lipase

was used since acyl migrations occur to afford a total randomization involving all positions. The ether lipids with one of the end-positions locked into an ether function are apparently far less prone to acyl migration as compared to the triglycerides under these conditions. It is obviously of interest to get a comparison with a non-regiospecific lipase such as the *Candida antarctica* lipase, which should offer higher enrichment of these fatty acids into the ether lipids.

By the methodology described above the EPA/DHA fatty acid composition of the product at an equilibrium is determined by weighted average of the initial fatty acid composition of the ether lipids and the composition of the n-3 concentrates. To avoid that limitation and obtain ether lipids of composition identical to the concentrates the direct esterification procedure recently reported[10] for glycerol and free fatty acids of pure EPA and DHA was employed. Stoichiometric amounts of substrates were mixed together and stirred without any solvent at 65 °C under vacuum (0.01 - 0.1 Torr) with 10 % dosage of the immobilized non-regiospecific *Candida antarctica* lipase[14] as based on the weight of substrates. The co-produced water was condensed into a liquid nitrogen cooled trap during the progress of the reaction, thus driving the reaction to completion. Virtually homogeneous ether lipids in terms of EPA were accomplished by direct esterification of the 1-O-alkyl-*sn*-glycerols (glycerol ether, GLE) with stoichiometric amount of 98 % pure EPA as free fatty acids. Similarly, a free fatty acid concentrate of 88 % DHA afforded ether lipids of that composition. As for the corresponding esterification of glycerol it is evident that EPA is a considerably better substrate as compared to DHA, but after 72 h both reactions had proceeded to completion and reached 100 % incorporation (see Figures 1. and 2.) to afford the ether lipid products (EL) highly pure in nearly quantitative yields. From these results and previous results for glycerol[10] there is no reason to believe that similar homogeneous DHA ether lipids **2** cannot be prepared by this method with a pure DHA concentrate, which was not available at the time when these experiments were carried out.

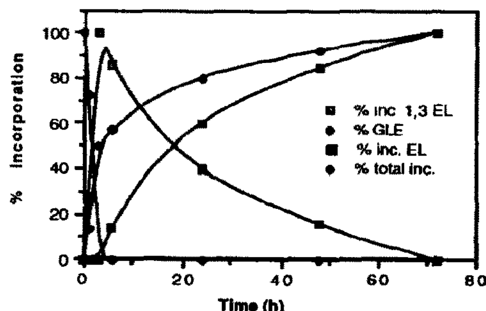


Figure 1. The incorporation of 98 % EPA into various ether lipids during the direct esterification of glycerol ethers.

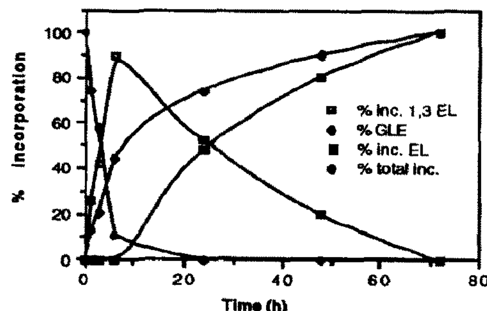


Figure 2. The incorporation of 88 % DHA into various ether lipids during the direct esterification of glycerol ethers.

250 MHz ^{1}H NMR spectroscopy enabled the monitoring of the incorporation of EPA and DHA into various glyceryl ethers during the progress of the direct esterification reaction[16]. This is graphically demonstrated in Figure 1. for pure EPA, and as can be seen 100 % incorporation had been obtained after 72 h. After 3 h all the glycerol ethers (GLE) had disappeared and 100 % intermediate 1,3-ether lipids (1-O-alkyl-3-acyl-*sn*-glycerol; 1,3-EL) were present. After 6 h 14 % ether lipids (EL) had formed at 57 % conversion. 1,2-Ether lipids (1,2-EL) were not detected during the reaction. Very similar behaviour was obtained for the DHA

concentrate, although the overall process was significantly slower, but after 72 h 100 % conversion had been reached leading to pure ether lipids of 88 % DHA content. After 6 h 11 % glycerol ethers were still present at 44 % conversion, no ether lipids had formed, but 89 % 1,3-ether lipids. After 24 h all the glyceryl ethers had reacted at 74 % conversion and 48 % ether lipids had formed with 52 % 1,3-ether lipids present.

As far as we know, this is the first report of non-polar ether lipids to be enriched with n-3 polyunsaturated fatty acids. It is assumed that the natural *sn*-1 configuration of the ether lipids (R for the glyceryl ether lipids; S for the glycerol ethers) was preserved during the chemical and enzymatic transformations involved in these processes.

Acknowledgement.

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13. Encapsulated shark liver oil concentrates were purchased as "Ecomer" from Halsoprodukter AB in Sweden.
14. The immobilized 1,3-regiospecific *Mucor miehei* lipase (Lipozyme™) and the immobilized non-regiospecific *Candida antarctica* lipase (SP 382) were kindly provided by Novo Nordisk in Denmark.
15. The concentrates were kindly supplied by Norsk Hydro in Norway as ethyl esters. They were converted into free fatty acids of identical fatty acid composition by a previously described procedure (see ref. 9).
16. All intermediate glyceryl ether derivatives involved were prepared by treating the diacyl glyceryl ethers with a Grignard reagent (methyl magnesium bromide), followed by separation on prep. TLC to afford each of them in a pure state. In the reaction mixture they were quantified by integration of protons belonging to the glyceryl moiety of the ether lipid molecules.

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