Trans Fatty Acids: Chemical Synthesis of Eicosapentaenoic Acid Isomers and Detection in Rats Fed a Deodorized Fish Oil Diet

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Trans fatty acids: chemical synthesis of eicosapentaenoic acid isomers and detection in rats fed a deodorized fish oil diet

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Supporting Information Placeholder

ABSTRACT: Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid present in fish oils used for omega-3 enriched diets. The natural cis double bond geometry can be transformed to the trans configuration during the deodorization process utilized in food industry. The analytical discrimination of the possible five mono-trans regioisomers represents a limiting step for recognition and structure-activity relationship in connection with the harmful effects of trans fatty acids in health. We carried out a dual synthetic strategy, providing a new access to mono-trans EPA isomers and valuable information on GC and NMR characteristics for further applications in metabolomics and lipidomics. This small library was used as analytical reference for the isomer determination in deodorized fish oils and the follow-up of rats fed fish oil diets, evidencing for the first time that mono-trans EPA isomers are incorporated in liver mitochondria membranes after dietary intake.

INTRODUCTION

The cis geometry is the ubiquitous structural feature of natural unsaturated fatty acids in mammals. It is provided by the activity of desaturase enzymes which work regio- and stereospecifically, preserving the position and configuration of double bonds in lipid structures and functions.[1] Trans fatty acids, either as geometrical and positional isomers with unshifted and shifted double bond positions compared to the natural compounds, were first identified as side-products of industrial oil processing that can enter the food chain. In particular, partial hydrogenation and deodorization processes used in food manufacturing are the most frequent and well known causes of double bond alteration.[2] On the other hand, geometrical trans fatty acids can derive from a free radical isomerization process, and this pathway has been recognized for the endogenous transformation of natural lipids under stressful cellular conditions.[3-5] The reaction mechanism is an addition-elimination of the isomerizing radical species to one of the double bonds of polyunsaturated fatty acids (PUFA), forming mono-trans isomers in the first stage. In this process only geometrical trans isomers can be obtained, since there is no possibility of double bond shift.[6] The number of geometrical isomers is equal to 2^n, where n is the number of double bonds present in the molecule. The full identification of trans PUFA isomers represents an analytical challenge, which is acquiring more and more relevance due to the food safety regulations active in most of the industrialized countries.[7] Indeed, trans fatty acids are considered to be toxic, since they can have health consequences,[8] therefore it is important to monitor structures and effects connected to the various isomer structures. The case of arachidonic acid was the first to be studied in details, underlining also the discrimination between dietary and endogenous origins of trans fatty acids. [9-11]

Due to its important nutritional and health correlations,[12-14] 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid (EPA, Figure 1) a long-chain polyunsaturated fatty acid (PUFA) of the omega-3 series (Figure 1) found in fish oils is worthy of study, both in chemical and biological aspects.

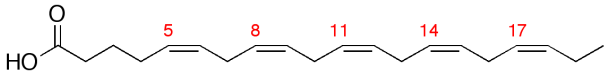


Figure 1. Structure of EPA (5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid)

Fish oils usually undergo an industrial deodorization process, in order to provide an odourless material used for functional foods or nutraceutical formulations. However, the heat treatment converts EPA to geometrical trans isomers. Among all possible isomers (2^5 = 32 isomers), the major by-products to be formed are mono-trans isomers, together with a small amount of di-trans isomers.[15-17] Separation and recognition of these isomers was carried out by gas chromatography after transformation of fatty acid-containing molecules (usually triglycerides in oil sources) to fatty acid methyl esters. In treated oils only two out of the five mono-trans isomers are recognized so far, by comparison with synthetically available references, e.g., (17E)-**1** and (11E)-**1** isomers (Figure 1).[18-20]

Metabolic toxicity of EPA isomers has been indirectly studied following-up the intake of partially hydrogenated or heated linseed oil. In these cases EPA containing the trans double bond was detected in rats, after dietary supplementation and *in vivo* enzymatic elongation and desaturation of trans isomers of α-linolenic acid (9Z,12Z,15Z-18:3) present in the processed oil.[8, 20-23]

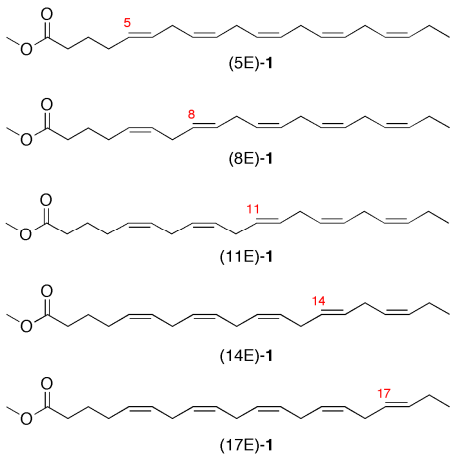


Figure 2. Structures of the five of mono-trans EPA methyl ester isomers.

Direct toxic effects of EPA isomers were evaluated in endothelial and retina cultured cells.[24,25] So far, the identification of the five EPA mono-trans isomers is not available and there is no information on the correlation between intake of deodorized fish oils and specific incorporation of trans EPA isomers *in vivo*. It is worth noting that the availability of only two references out of the five mono-trans EPA isomers can represent a limiting step for the study of structure-activity relationships.

With these premises, we approached the study of EPA isomers in natural sources and in biological samples by a chemical-biology approach. We performed a dual synthetic strategy in order to build-up and fully characterize the mono-trans EPA library. Then, we used this library for isomer recognition in deodorized fish oils and the follow-up of rats fed fish oil diets. We report the full characterization of the five mono-trans EPA isomers and the usefulness of this small library for the follow-up of rats fed diets at two different percentages of deodorized fish oil (5% and 30%), evidencing for the first time that EPA isomers can be metabolized and incorporated in liver mitochondrial phospholipids.

EXPERIMENTAL PROCEDURES

Materials and Methods (see Supporting Information)

Epoxidation of methyl all-(Z)-5,8,11,14,17-eicosapentaenoate (EPA-Me, 1). A 0.054 M acetone solution of DMD (2.6 mL corresponding to 0.142 mmol) was added to a solution of methyl ester of EPA (100 mg; 0.316 mmol) in acetone (2 mL) at 0 °C. The reaction was monitored by spectrophotometry (335 nm) for the consumption of the dioxirane reagent and TLC for the formation of products (Figure 3).[26] Eluent: 1:2 diethyl ether/*n*-hexane; starting material **1** R_f = 0.74, mix (**4** and **5**) R_f = 0.54, mix (**3** and **6**) R_f = 0.48, pure (**2**) R_f = 0.39, bis-epoxide $R_f \leq 0.26$]. Reaction time of 10 minutes was the best compromise for the formation of mono-epoxides with traces of di-epoxides, therefore the reaction was stopped and the solvent was removed in vacuum. The crude was purified by preparative TLC (eluent: 1:2 diethyl ether/*n*-hexane) to give **2** (colourless oil; 6.6 mg; 0.02 mmol; 6.3% yield), an inseparable mixture of **3** and **6** (colourless oil; 17.7 mg; 0.053 mmol; 17% yield) and an inseparable mixture of **4** and **5** (colourless oil; 14.1 mg; 0.043 mmol; 13% yield). Starting EPA-Me was also recovered (52 mg; 0.18 mmol; 52% recovered yield).

Methyl (Z)-5,6-epoxy-all-(Z)-8,11,14,17-eicosatetraenoate (**2**). ^{1}H and ^{13}C NMR spectra were identical with those reported in the literature.[27,28]

Mono-epoxides (**3-6**) in the mixtures were assigned analyzing the NMR spectral data in comparison with those reported in the literature (see Table S1 in Supporting Information).[27]

Methyl (Z)-8,9-epoxy-all-(Z)-5,11,14,17-eicosatetraenoate and methyl (Z)-17,18-epoxy-all-(Z)-5,8,14,17-eicosatetraenoate as an inseparable mixture (**3** and **6**). ^{1}H NMR, δ 0.97 (t, J = 7.4 Hz), 1.05 (t, J = 7.6 Hz), 1.47-1.76 (m), 2.01-2.12 (m), 2.18-2.29 (m), 2.31 (t, J = 7.6 Hz), 2.32 (t, J = 7.4 Hz), 2.34-2.48 (m), 2.77-2.99 (m), 5.26-5.55 (m). ^{13}C NMR, δ 10.60, 14.23, 20.54, 21.05, 24.66, 24.76, 25.53, 25.59, 25.62, 25.79, 26.16, 26.21, 26.54, 26.71, 33.36, 33.42, 51.46, 56.33, 56.36, 56.49, 58.30, 124.24, 124.51, 125.00, 126.87, 127.50, 127.82, 127.97, 128.31, 128.39, 128.77, 128.82, 128.97, 130.37, 130.65, 131.36, 132.10, 174.02.

Methyl (Z)-11,12-epoxy-all-(Z)-5,8,11,17-eicosatetraenoate and methyl (Z)- 14,15-epoxy-all-(Z)-5,8,11,14-eicosatetraenoate as an inseparable mixture (**4** and **5**). ^{1}H NMR, δ 0.97 (t, J = 7.6 Hz), 0.98 (t, J = 7.6 Hz), 1.70 (tt, J = 7.2 Hz, J = 7.2 Hz), 2.02-2.14 (m), 2.18-2.32 (m), 2.32 (t, J = 7.4 Hz), 2.36-2.49 (m), 2.77-2.87 (m), 2.93-2.99 (m), 5.26-5.57 (m). ^{13}C NMR, δ 14.16, 14.22, 20.56, 20.74, 24.73, 24.75, 25.61, 25.70, 25.77, 25.79, 26.06, 26.18, 26.20, 26.54, 33.42, 51.46, 56.34, 56.50, 123.09, 124.01, 124.27, 124.40, 126.59, 127.72, 128.43, 128.49, 128.70, 129.02, 129.21, 130.52, 130.63, 130.97, 132.29, 134.42, 173.99, 174.02.

Transformation of mono-epoxides to mono-trans EPA-Me isomers. Step I (Bromination of epoxide). To a freshly prepared solution of triphenylphosphine dibromide (16 mg; 0.04 mmol) in dry dichloromethane (100 µL) was added a solution of **2** (6.6 mg; 0.02 mmol) in dry dichloromethane (200 µL) and pyridine (2 µL, 0.025 mmol) at 0 °C under argon atmosphere. The mixture was left stirring overnight at 5 °C, then was quenched with a 1M aqueous solution of hydrochloric acid (0.5 mL) and extracted three times with chloroform/ethanol (4/1). The organic layers were collected, dried over Na_2SO_4 and evaporated under vacuum. Finally, the crude was purified by flash chromatography (eluent: 1:3 *n*-hexane/diethyl ether) to afford methyl erythro-5,6-dibromo-(8Z,11Z,14Z,17Z)-eicosatetraenoate (6.4 mg, 0.013 mmol, yield 68%). The same reaction was performed also on the other mixtures: for the mixture of **3** and **6** the dibromide adducts were obtained in a 82% yield (20.8 mg; 0.044 mmol), for the mixture of **4** and **5** they were obtained in a 75% yield (15.1 mg; 0.032 mmol). These crude intermediates were used directly in the next step.

Step II (Elimination). To an ice-cooled slurry of activated zinc (10 mg, 0.15 mmol), acetic acid (2 µL) and *N*,*N*-dimethylformamide (150 µL) was added a solution of methyl erythro-5,6-dibromo-(8Z,11Z,14Z,17Z)-eicosatetraenoate (6.4 mg, 0.013 mmol) in *N*,*N*-dimethylformamide (100 µL) and the mixture was left stirring at 0 °C. After 10 hours, a 1M aqueous hydrochloric acid solution (1 mL) was poured into the mixture and the precipitate was filtered off. The filtrate was then extracted three times with a mixture of chloroform/ethanol (3/1). The organic layers were collected, dried over Na_2SO_4 and evaporated under vacuum to give a crude that was finally purified by flash chromatography (eluent: 1:3 *n*-hexane/diethyl

ether). (5E)-**1** was obtained as a colourless oil (3.4 mg, 0.0107 mmol, yield 80%; purity >80% by GC analysis).

The debromination reaction occurred also to the other dibromide intermediates affording (8E)-**1**/(17E)-**1** (colourless oil; 10.9 mg, 0.0344 mmol, yield 79%; purity >78% by GC analysis) and (11E)-**1**/(14E)-**1** (colourless oil; 6.3 mg, 0.0199 mmol, yield 63%; purity >83% by GC analysis).

NMR spectra of 11E-**1** and 17E-**1** were identical to literature (see Supporting Information).[18,20]

Methyl (5E,8Z,11Z,14Z,17Z)-eicosapentaenoate. ^{1}H NMR, δ 0.98 (t, 3H, *J* = 7.6 Hz), 1.69 (tt, 2H, *J* = 7.6 Hz, *J* = 7.6 Hz), 2.00-2.06 (m, 2H), 2.08 (dt, 2H, *J* = 7.6 Hz, *J* = 7.6 Hz), 2.30 (t, 2H, *J* = 7.6 Hz), 2.74-2.78 (m, 2H), 2.78-2.88 (m, 6H), 3.68 (s, 3H), 5.27-5.44 (m, 10H). ^{13}C NMR, δ 14.25, 20.55, 24.62, 25.55, 25.61, 30.31, 30.38, 31.85, 33.39, 51.45, 127.02, 127.91, 127.95, 128.16 (overlap of two C), 128.36, 128.53, 129.25, 129.53, 132.03, 174.14.

Methyl (5Z,8E,11Z,14Z,17Z)-eicosapentaenoate and methyl (5Z,8Z,11Z,14Z,17E)-eicosapentaenoate as mixture. ^{1}H NMR, δ 0.96 (t, *J* = 7.4 Hz), 0.97 (t, *J* = 7.4 Hz), 1.71 (tt, 2H, *J* = 7.6 Hz, *J* = 7.6 Hz), 1.96-2.15 (m), 2.32 (t, *J* = 7.6 Hz), 2.70-2.89 (m), 5.27-5.53 (m). ^{13}C NMR, δ 13.79, 14.24, 20.53, 24.76, 25.52, 25.55, 25.59, 25.60, 26.43, 26.54, 30.31, 30.34, 30.38, 33.42, 51.45, 126.95, 127.04, 127.86, 127.95, 128.02, 128.11, 128.14, 128.17, 128.25, 128.30, 128.39, 128.44, 128.61, 128.68, 128.84, 128.92, 129.06, 132.01, 132.57, 174.03.

Methyl (5Z,8Z,11E,14Z,17Z)-eicosapentaenoate and methyl (5Z,8Z,11Z,14E,17Z)-eicosapentaenoate as mixture. ^{1}H NMR, δ 0.96 (t, *J* = 7.6 Hz), 0.97 (t, *J* = 7.6 Hz, 1.70 (tt, 2H, *J* = 7.6 Hz, *J* = 7.6 Hz), 1.71 (tt, 2H, *J* = 7.6 Hz, *J* = 7.6 Hz, 1.98-2.14 (m), 2.32 (t, *J* = 7.6 Hz), 2.70-2.86 (m), 5.26-5.45 (m). ^{13}C NMR, δ 14.25, 20.43, 20.52, 24.76, 25.44, 25.54, 25.58, 26.54, 30.24, 30.30, 30.34, 30.36, 33.43, 51.46, 126.83, 127.09, 127.65, 127.89, 128.02, 128.11, 128.19, 128.26, 128.37, 128.59, 128.71, 128.82, 128.88, 128.93, 129.06, 131.96, 132.24, 174.05.

GC/MS (m/z) analysis coupled with mass spectrometry gave a common fragmentation pattern for the mono-trans isomers: 316 (M^+), 315, 278, 266, 253, 247, 220, 201, 180, 175, 161, 156, 147, 133, 119, 105, 91, 79 (base peak) 67, 55, 41. Subtle changes of fragmentation patterns can be appreciated by a careful examination of the MS spectra of each isomer; in particular, the 11E isomer shows an increase of the fragmentation peak at 91 m/z, which becomes the base peak in the spectrum registered at 49-071 min (see Supporting Information).

Synthesis of mono-trans EPA-Me isomers by photolysis. A 15 mM solution of **1** (20 mg, 63 μmol) in 2-propanol (4.2 mL) was placed in a quartz photochemical reactor, then a 7 mM solution of 2-mercaptoethanol in 2-propanol was added. The reaction mixture was flushed with argon for 20 minutes to evacuate oxygen, and UV-irradiation with a 5.5 W low-pressure mercury lamp was carried out at (22 ± 2) °C temperature for 5 minutes. The reaction course was monitored by Ag/TLC in order to evidence the formation of the mono-trans fraction (see Figure 4). The solvent was removed in vacuum, the crude was taken up with *n*-hexane (1 mL) and loaded onto a preparative Ag/TLC plate. After elution (eluent: 3:5 *n*-hexane-diethyl ether), the area corresponding to the mono-trans isomer fraction was scraped off. Silica was washed with absolute ethanol (3 × 5 mL). The solvent was evaporated to give a solid material which is the Ag-fatty acid complex insoluble in *n*-hexane. This material was dissolved with aqueous ammonia (5% NH_4OH solution) and was vigorously stirred (600 rpm) for 15 minutes. The basic water phase was extracted with *n*-hexane (3 × 5 mL). Finally the organic phase was treated with dry Na_2SO_4 and filtered, affording the mono-trans EPA methyl ester isomer mixture as a colourless oil (5.4 mg; 17 μmol; yield 27%).

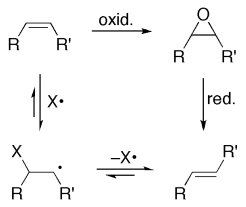
Mitochondrial phospholipid preparation and GC analysis. For the rat dietary intervention and isolation of mitochodria see Supporting Information. Total lipids for TLC were extracted from the mitochondrial suspension with 2:1 chloroform/methanol (v/v) in the presence of an antioxidant (BHT, 50 mg/L).[29] The chloroform/methanol lipid extract was used in order to isolate mitochondrial PL from other neutral lipids (cholesterol, free fatty acids and triglycerides) by thin layer chromatography. The total PL fraction was obtained as part of silica gel fraction and directly treated by transesterification with KOH/MeOH (0.5 M) for 10 min at room temperature. The fatty acids methyl esters (FAME) were subsequently extracted with *n*-hexane. In particular, FAME extracts of ca. 1.2-1.3 mg from rats fed a 30% omega-3 rich diet (n=6) were used and dissolved in 50 μL of *n*-hexane for gas chromatography analysis. 1 μL of these solutions was injected and the results as described in the Supporting Information.

RESULTS

Synthesis of mono-trans EPA-Me isomers. Among the five mono-trans isomers shown in Figure 2, the synthesis of two specific regioisomers, namely the 11E and 17E isomers, was previously reported by a multistep procedure with overall yield of ca. 20%. The key step was a Wittig coupling between an unsaturated aldehyde containing the trans double bond and an ylide of the remaining carbon frame.[18-20]

In order to have a simple approach for the mono-trans isomer identification, we planned the dual synthetic strategy shown in Scheme 1 starting from the natural cis geometry: the free radical isomerization process, and a parallel trans-alkene synthesis *via* mono-epoxide formation followed by ring opening and elimination. Geometrical trans isomers are produced in both methods, with the advantage that separation and identification of the five regioisomers can be performed at the level of mono-epoxides, as recently reported.[27] The preparation of the mono-epoxide derivatives (Figure 3) was carried out by dimethyldioxirane (DMD),[30] and separation by preparative TLC on silica gel (1:2 diethyl ether/*n*-hexane as the eluent) provided three fractions of epoxide regioisomers (Scheme 2). Under our experimental conditions the 5-monoepoxide **2** (Figure 3) was obtained in a pure form, and it was also possible to characterize the other regioisomers of the remaining fractions also by comparison with the reported NMR data (see Table S1 in Supporting Information).[27]

The subsequent transformations by ring opening as dibromides and elimination to alkenes were performed in one-step for each of the three fractions. The latter reaction sequence was adapted for EPA-Me from a procedure described for arachidonic acid methyl ester[31] (see Experimental).



Scheme 1. Dual synthetic strategy for E-alkene synthesis

Further purification of each cis/trans fraction was performed by silver-thin layer chromatography (Ag-TLC)[32] with an overall isolated yield of 25-28% of the five mono-trans isomers.

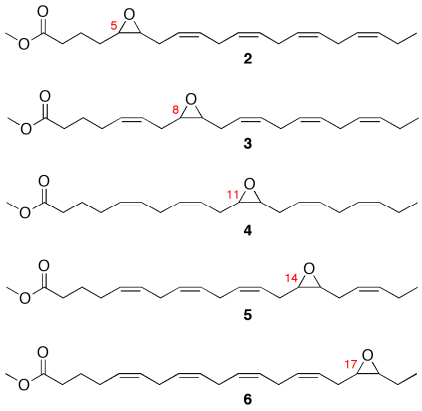
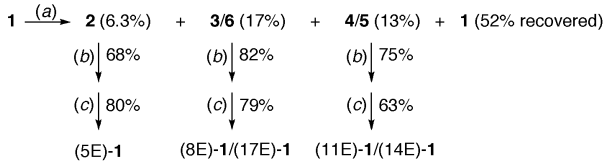


Figure 3. The five mono-epoxide regioisomers of EPA methyl ester

In parallel, free radical isomerization of EPA-Me (**1**) was carried out, by irradiation of an alcoholic solution with a low-pressure mercury lamp in the presence of 2-mercaptoethanol (0.5 equiv) in analogy to previous reports.[9]



Scheme 2. (a) DMD, acetone, 0 °C, 10 min; quantitative conversion based on the recovery of starting material; (b) dry CH_2Cl_2, pyridine, Ph_3PBr_2, 5 °C. overnight; (c) DMF, activated Zn, AcOH, 0 °C, 10 h.

The isomerization course was carefully monitored by silver-thin layer chromatography (Ag-TLC), showing that photolysis can be stopped at an early stage favouring the mono-trans isomer formation. The Ag-TLC procedure can be also used to isolate the mono-trans isomer fractions and recover the starting cis isomers (Figure 4). The latter can undergo repetitive isomerization rounds, leading to an overall yield of 25%, similar to that obtained from the previously described route through the mono-epoxide formation.

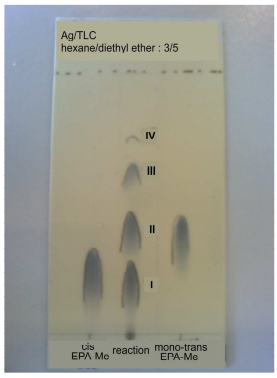


Figure 4. Ag/TLC after 5 minutes of isomerization under photolysis in the presence of 2-mercaptoethanol. Left: standard all-cis EPA-Me (I). Center: reaction mixture. Right: reference of 5E EPA-Me isolated as reported in the Experimental. (II). Also di-trans (III) and tri-trans (IV) isomers of EPA-Me are visible.

Analysis of mono-trans EPA isomers using ^{13}C-NMR and GC. Identification of the mono-trans isomers can be carried out by carbon nuclear magnetic resonance (^{13}C NMR), in particular based on the ethylenic carbon atoms, as previously reported in the case of arachidonic acid.[10] The ^{13}C NMR analysis focused in particular on the C-17 resonance, which is found to have a diagnostic value for the double bond position of EPA isomers. Four mono-trans EPA isomers out of five gave separated C-17 resonance (Figure 5a, traces III and IV), with the 17E isomer at the lowest field (132.6 ppm). The 5E isomer was superimposed with cis-EPA isomer (Figure 5a, trace V; see Supporting Information for the whole spectra).

It is worth underlining that the strategy of the synthetic route via mono-epoxides was expedient for the assignment of the five regioisomers. In fact, it was possible to separate and characterize the mono-epoxides, recognizing at this level their structures also by comparison with literature data,[27] thus facilitating the assignment of the corresponding mono-trans isomers formed after bromination-elimination. From the pure 5-monoepoxide, the (5E)-**1** was isolated and characterized as pure compound and its GC analysis gave satisfactory separation from all-cis-EPA (Figure 5b, trace V). Also the other regioisomers were satisfactorily separated, matching with the products obtained by the free radical isomerisation route (Figure 5b, trace II vs. traces III, IV, V).

Considering NMR and GC data and combining with the mono-epoxide assignment and the previously reported retention times for some mono-trans EPA,[16] the following GC order of elution could be assigned: 17E<14E<all-cis<5E<11E≈8E. In Figure 5 it can be clearly appreciated how useful is the combination of two techniques in order to get a complementary and overall view of trans isomer distribution.

Having assigned the elution order of the synthetic mono-trans EPA isomer, a careful examination of the corresponding GC/MS data was also carried out in order to envisage possible differences in fragmentation patterns. This is described for the omega-3 precursor, α-linolenic acid, although using low energy electron ionization mass spectrometry.[33]

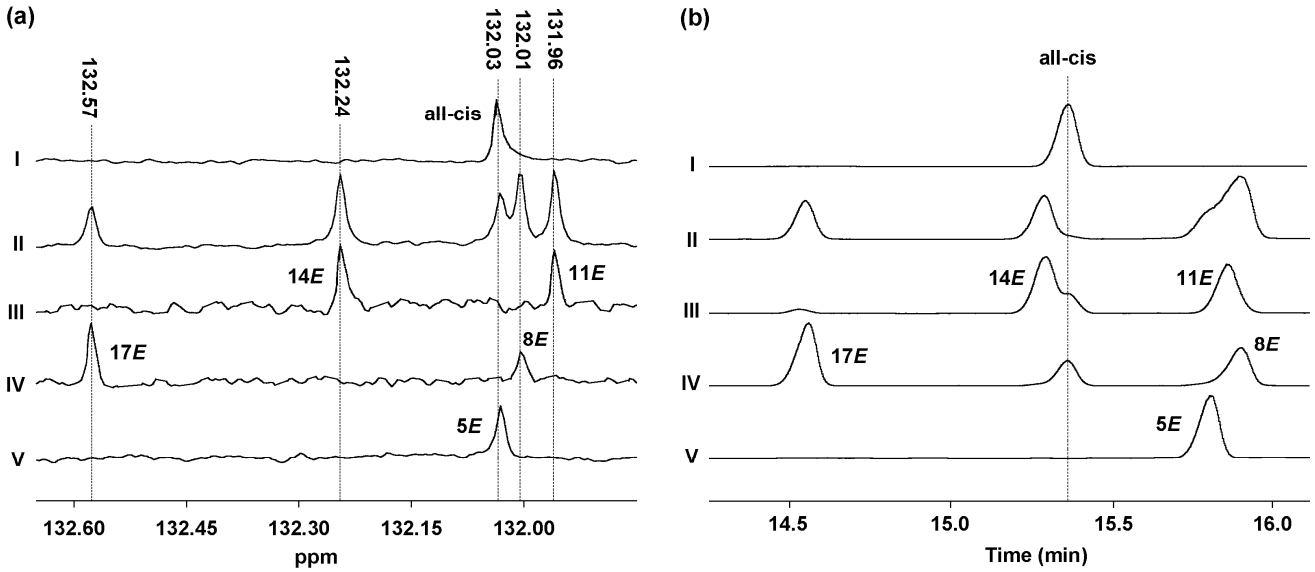


Figure 5. (a) Regions of ^{13}C NMR spectra (CDCl3) and (b) GC chromatograms of: (I) all cis EPA methyl ester; (II) mixture of mono-trans EPA methyl ester isomers obtained after free radical isomerization by photolysis; (III) 14E and 11E isomers; (IV) 17Eand 8E; (V) 5E

In the spectra of the isomers, variance of the *m/z* intensities was observed and in particular, in case of the 11E isomer the base peak was at *m/z* 91 instead at *m/z* 79 peak as in all the other isomers (see Experimental and Supporting Information). These preliminary observations confirmed the applicability of mass spectrometry techniques to the geometrical trans isomer recognition for fatty acid derivatives.

It should be added at this point that the development of trans isomer libraries can be useful to implement other analytical tools for trans fatty acid separation, such as silver-high performance liquid chromatography (Ag-HPLC), already used with partial success for trans isomers of EPA and DHA.[16]

EPA-rich dietary regimes and incorporation in rat liver mitochondrial phospholipids. Deodorization is a common practice in food industry to eliminate the unpleasant odour of fish oils. Analysis of EPA isomers carried out after deodorization showed that protocols at 220 °C cause the 17E isomer formation, accompanied by another group of two isomers, identified as 11E and 14E mono-trans EPA.[16] The biological fate of mono-trans EPA from deodorized fish oils was not yet explored. Taking into account that the trans geometry is unnatural for unsaturated fatty acids, it is very important to study the possibility of incorporation in cell structures, such as mitochondria where the effects of a structural change can influence the core of the cell metabolism. Recently, we reported the impact of different nutritional patterns of dietary lipids on liver mitochondrial functions of rats.[29] In that study we reported the presence of trans isomers derived from oleic acid (18:1), linoleic (18:2) and arachidonic acid (20:4) in the mitochondrial phospholipids, recognized by previously developed trans fatty acid libraries. Trans isomers increased with lard diet vs. basal and fish oil diets, whereas decreased using a 30% fat-diet compared with 5% fat-diet, thus indicating the influence of lipid intake on the trans isomer levels. We also reported that mitochondrial membrane functionality is equally affected at low (5%) and high (30%) fish oil diets. Considering that rats were fed a deodorized fish oil diet, we were interested to investigate whether dietary trans EPA isomers could reach the mitochondrial level and might be, therefore, involved in the observed metabolic changes. The fish oils are described as deodorized and refined from the producer and, having the library of EPA mono-trans isomers in our hands, we proceeded to their analysis. In tuna oil trans isomers were found in a 0.27% content over a content of ca. 9% EPA, corresponding to a 3% isomerization of the natural cis content (Figure 6, trace II; see Figure S1B and Table S2 in Supporting Information). Sardine oil gave similar results (data not shown). It is worth noting that a careful evaluation of different GC conditions is needed in order to have a safe separation of omega-6 and omega-3 fatty acids and their trans isomers. In fact, with the GC column used in our analysis an overlap of cis and trans EPA isomers with 20:4 ω3 and 22:0 and 22:1 fatty acids can occur. Optimal conditions are described as Method B in Supplementary Information, in particular the choice of hydrogen as the carrier gas contributed for the best separation (see Methods and Figures S1A and S1B in Supporting Information).

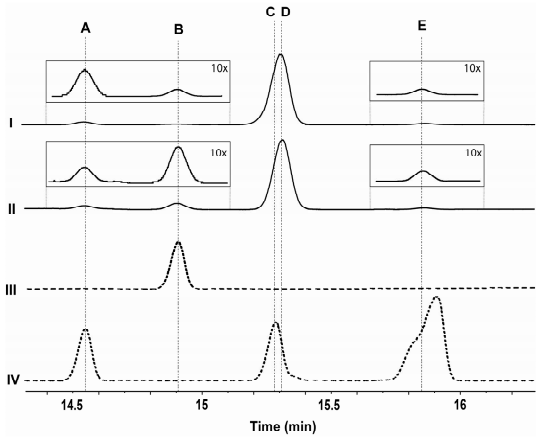


Figure 6. Region of the GC chromatogram of: (I) rats fed with the ω3 PUFA rich diet with 30% fats; (II) tuna oil; (III) 20:4 ω3 standard reference; (IV) mixture of mono-trans EPA-Me isomers. Peaks correspond to: (A) 17E; (B) 20:4 ω3; (C)14E; (D) EPA-Me;

(E) 5E+8E+11E. GC analytical conditions are described in Supporting Information (Method B).

In the oil also the other omega-3 component DHA (docosahexaenoic acid), and minor peaks were detected, likely corresponding to trans-DHA isomers. The full recognition of these isomers represents another challenging analytical target, which will be addressed by further work.

It is worth underlining that IR analysis of tuna oil indicated the presence of trans unconjugated double bonds with an absorbance at 967 cm^{-1} (see Figure S2 in Supporting Information).[34] However, this analysis can provide only the sum of isomer content, since the peak corresponds to all the trans fatty acid isomers contained in the sample.

In order to understand the influence of trans isomers from the diet, rats fed deodorized fish oil were sacrificed after 12 weeks and phospholipids from liver mitochondria were isolated and analyzed, as previously described.[29] The complete fatty acid analysis of the different diets has been previously reported as relative percentages of the total fatty acid content,[29] finding that the natural EPA content was higher in fish oil diets than in rats fed the control diets, i.e., diet with an equilibrated blend of different plant oils. We also found that the cis-EPA incorporation varied also in the rats fed 5% and 30% fish oil diets, and was found to be less for a high fat diet (Figure 7, upper graphic). In the mean time, we evidenced that mono-trans EPA isomers were present in quantities almost identical for both diets (Figure 7, lower graphic), being the same present in the dietary oil, i.e., 17E and, more likely, the 5E regioisomer, with the former more abundant than the other (Figure 6, peak A vs. peak E in trace I and trace II; the full GC analysis and results are reported in Figure S3 and Table S3 of the Supporting Information). The relative percentage of incorporated trans EPA isomers resulted to be ca. 0.050% of the total fatty acids of the samples, with the amount of cis-EPA being ca. 4.2 and 2.1%, respectively Significant differences were found both in all-cis and mono-trans EPA incorporation compared to controls ($p<0.0001$).

Using calibration from the reference compounds, the total content of mono-trans EPA in liver mitochondria lipids was found to be 11.2 ± 2.1 ng/mL, i.e., ca. 35 nM (Table S4 in the Supporting Information). This is the first report showing that mono-trans EPA isomers can be processed as triglyceride constituents of the dietary oil and incorporated as phospholipid residues in mitochondrial membranes of living organisms.

DISCUSSION

The dual synthetic strategy followed by our protocol allowed the full characterization of the five geometrical mono-trans isomers of EPA methyl ester (Figure 2). Indeed, the identification and separation can be performed through the mono-epoxide route, and the isomer pattern is identical to the mono-trans mixture deriving from the free radical catalyzed isomerisation of EPA methyl ester (traces II in Figures 5a and 5b). With this identification is now possible to fully address the presence of mono-trans EPA residues in biological samples and use the thiyl radical catalysed isomerization route as a rapid and convenient access of trans libraries, without any contamination with positional isomers.[6,35] GC showed characteristic retention times and the examination of the ^{13}C NMR spectral region corresponding to the ethylenic carbon atoms evidenced the C-17 resonance to be a diagnostic signature for the mono-trans isomers. Indeed, the chemical shift of the C-17 position is very sensitive to the geometry of the double bond, as shown in Figure 5a. These data provide valuable information for applications in food analysis as well as in metabolomics and lipidomics.

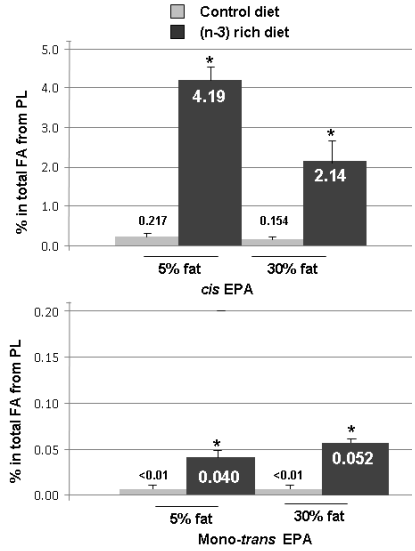


Figure 7. EPA (upper) and mono-trans EPA isomer (lower) content in liver mitochondria of rats fed 5% and 30% fish oil diets compared with control diet (n=6). The statistical significance is indicated with the asterisk ($p<0.0001$), see ref. 29 and Supporting Information

Deodorized fish oils are used for functional foods and nutraceuticals with the health claim derived from the beneficial effects of omega-3 fatty acids.[12-14] However, trans fatty acids are well known toxic compounds which must be accurately controlled for their presence in foods.[2,8] In our opinion, the content of trans isomers in fish oils used for nutraceutical purposes can be considered harmful at any content, due to possible incorporation and metabolic interferences of these isomers after dietary intake. Indeed, they can reach the level of liver mitochondria as demonstrated in rats fed a fish oil diet, and cause impairment of crucial cellular functions in correlation with metabolic diseases.[29] A 0.27% trans-EPA content in fish oil has been found to correlate with an almost constant percentage of incorporation in liver mitochondria, as shown in Figure 7, which is independent from the high or low fish oil diets. This fact suggests the hypothesis of a limit value of isomer incorporation in mitochondrial phospholipids. It is worth mentioning that in a previous paper we measured other trans fatty acid isomers, such as monounsaturated (9*t*-18:1, 11*t*-18:1), linolelaidic acid (9*t*,12*t*-18:2) and mono-trans arachidonic acid isomers (14*t*-20:4, 8*t*-20:4, 5*t*-20:4) in rat liver mitochondrial phospholipids obtained by the same experiments (see also Tables S3 and S4 in Supporting Information for rats fed a 30% fish oil diet).[29] Among these isomers, it is important to evidence that mono-trans isomers of arachidonic acid such as 5*t*-20:4 and 8*t*-20:4 are known markers of an endogenous free radical isomerization,[11,35] which is likely to occur to rats for the stressful life conditions of the stabularium. Indeed, the presence of trans arachidonic acid isomers was first evidenced in tissue phospholipids of rats fed a trans-free diet, therefore unambiguously attributed to free radical isomerization.[36] In case of arachidonic acid isomers, it was observed that a 30% fat rich diet induces a diminution of this trans isomer content compared to controls, suggesting that a wash-out of endogenously formed trans lipids could occur by replacement with

natural fats during turnover, especially under abundant dietary conditions. Conversely, in the present work the dietary EPA isomers were incorporated reaching ca 35 nM for a 30% fish oil diet. This quantity corresponds to a 2% EPA isomer content relative to the natural cis-EPA in mitochondrial membrane lipids (1.7 μM, see Table S4 in Supporting Information). Since the trans EPA content in the oil is a 3% of the natural cis EPA (Table S2), it can be extrapolated that one third of the isomeric dietary content is able to reach the liver mitochondrial membrane phospholipids. It is worth noting that rats fed the fish oil-rich diets showed mitochondrial impairments, such as a decrease of the activity of respiratory chain complexes II+III and IV, and a significant decrease of cardiolipins (CL) proportions, the inner mitochondrial membrane constituents, (data not shown) in comparison to control rats, whatever the quantity of fat in the diets is.[29] A few data are available on the trans isomer incorporation in CL concerning 9*c*,12*c*,15*t*-18:3 acid, a compound commonly found in deodorized edible linolenic acid-containing oils (linseed oil).[38] In the same study, 17*t*-EPA metabolically derived from 15*t*-18:3 (n-3) was not incorporated to any significant extent into CL, whereas was observed in rat tissue phospholipids.[21-23] Further investigations are needed on the selectivity of lipid incorporation and the meaning for health. Nutritional and biological investigations can surely take advantage of the synthetic mono-trans EPA library built-up by the present work.

Fatty acid isomerism affects membrane structures, lipid metabolism and enzymatic reactions,[2,8,39] and has not yet been connected to the impairment of mitochondrial respiratory chain or liver nutritional associated pathologies as non-alcoholic fatty liver disease (NAFLD) or steatosis. More attention to the trans fatty acid content related to the diet and biodistribution is needed in order to evaluate the influence at molecular level on health. Our work indicates the usefulness of the synthetic trans lipid library for biological applications, and raises concern on the fate of processed fish oils used in the diet or as nutraceutical component, affecting the cis content in the crucial mitochondrial compartment by inserting the trans geometry, which is unnatural for eukaryotic cells.

ASSOCIATED CONTENT

Supporting Information. Additional data and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; DMD, dimethyl dioxirane; DMF, *N*,*N*-dimethyl formamide; Ag-TLC, silver-thin layer chromatography; GC, gas chromatography,; NMR, nuclear magnetic resonance; CL, cardiolipins; NAFLD, non-alcoholic fatty liver disease.

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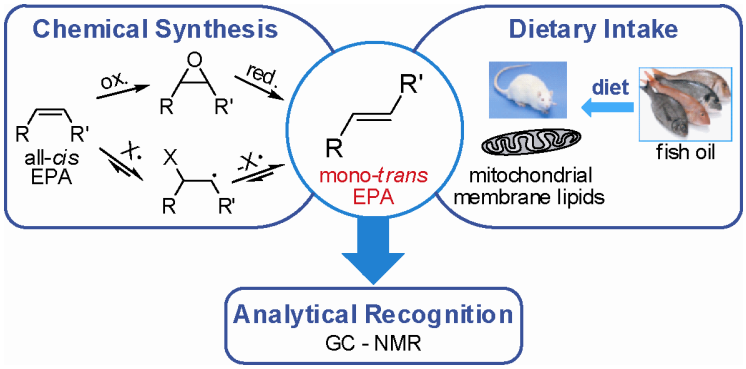
Chemical Synthesis
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fish oil
mitochondrial
membrane lipids
Analytical Recognition
GC - NMR