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 the food industry. The analytical discrimination of the possible five monotrans regioisomers represents a limiting step for the 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 new access to monotrans EPA isomers and valuable information on GC and NMR characteristics for further applications in metabolomics and lipidomics. This small library was used as an analytical reference for isomer determination in deodorized fish oils and the follow-up of rats fed fish oil diets, evidencing for the first time that monotrans EPA isomers are incorporated in liver mitochondrial 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 However, 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 monotrans 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 2ⁿ, 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 detail, also underlining the discrimination between dietary and endogenous origins of trans fatty acids.9−11

Because of 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.

Fish oils usually undergo an industrial deodorization process, in order to provide an odorless 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 byproduct to be formed are monotrans isomers, together with a small amount of dirans isomers.25-27 Separation and recognition of these isomers were 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 monotrans isomers are recognized so far, by comparison with synthetically available references, e.g., (17E) and (11E) isomers.18

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

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 monotrans 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 monotrans 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 monotrans EPA isomers and the usefulness of this small library 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 monotrans 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 monotrans EPA isomers and the usefulness of this small library in natural sources and in biological samples by a chemical-biology approach.

## Experimental Procedures

### Materials and Methods

See Supporting Information.

**Epoxidation of Methyl All-(Z)-5,8,11,14,17-eicosapentaenoate (EPA-Me).** 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.25 Eluent: 1:2 diethyl ether/n-hexane; starting material R = 0.74, mix (4 and 5) R = 0.54, mix (3 and 6) R = 0.48, pure (2) R = 0.39, bis-epoxide R ≤ 0.26. Reaction time of 10 min was the best compromise for the formation of monoepoxides with traces of diepoxides; 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 (colorless oil; 6.6 mg; 0.02 mmol; 63% yield), an inseparable mixture of 3 and 6 (colorless oil; 17.7 mg; 0.053 mmol; 17% yield), and an inseparable mixture of 4 and 5 (colorless 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-Epoxo-all-(Z)-8,11,14,17-eicosatetraenoate (2).** 1H and 13C NMR spectra were identical with those reported in the literature.

**Methyl (Z)-8,9-Epoxo-all-(Z)-5,11,14,17-eicosatetraenoate and Methyl (Z)-17,18-Epoxo-all-(Z)-5,8,14,17-eicosatetraenoate as an Inseparable Mixture (3 and 6).** 1H 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 (s, J = 5.4 Hz), 2.34–2.46 (m), 2.77–2.99 (m), 5.26–5.55 (m). 13C 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, 124.30, 124.51, 125.00, 126.87, 127.50, 127.82, 127.97, 128.31, 128.77, 128.82, 130.37, 130.65, 131.36, 132.10, 174.02.

**Transformation of Monoepoxides to Monotrans 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 1 M aqueous solution of hydrochloric acid (8/1). The organic layers were collected, dried over Na2SO4 and evaporated under vacuum. Finally, the crude was purified by flash chromatography (eluent: 1:3 diethyl ether/hexane) to afford methyl ethy thro-5,6-dibromo-(8Z,11Z,14Z,17Z)-eicosatetraenoate (64 μg; 0.013 mmol, yield 60%). The same reaction was performed also on the other epoxide for the mixture of 3 and 6, the dibromide adducts were obtained in 82% yield (20.8 μg; 0.044 mmol); for the mixture of 4 and 5, they were obtained in a 75% yield (15.1 μg; 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 ethylthreo-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 h, a 1 M 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 (4/1). The organic layers were collected, dried over Na2SO4 and evaporated under vacuum to give a crude that was finally purified by flash chromatography (eluent: 1:3 n-hexane/diethyl ether) to afford methyl ethy thro-5,6-dibromo-(8Z,11Z,14Z,17Z)-eicosatetraenoate (6.4 μg; 0.013 mmol, yield 60%). The same reaction was performed also on the other epoxide for the mixture of 3 and 6, the dibromide adducts were obtained in 82% yield (20.8 μg; 0.044 mmol); for the mixture of 4 and 5, they were obtained in a 75% yield (15.1 μg; 0.032 mmol). These crude intermediates were used directly in the next step.

**NMR spectra of (11E)-Methyl (5Z,8E,11Z,14Z,17Z)-Eicosapentaenoate.** 1H NMR, δ 0.97 (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). 13C 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, 26.24, 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, 132.44, 173.92, 174.02.

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For the rat dietary intervention and isolation of mitochondria, see Supporting Information.

Mitochondrial Phospholipid Preparation and GC Analysis.

For the rat dietary intervention and isolation of mitochondria, 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). 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. One microliter of these solutions was injected and the results described in the Supporting Information.

Results

Synthesis of Monotrans EPA-Me Isomers. Among the five monotrans 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 27%.

Further purification of each cis/trans fraction was performed by silver-thin layer chromatography (Ag-TLC) with an overall isolated yield of 25–28% of the five monotrans isomers.
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 The isomerization course was carefully monitored by silver-thin layer chromatography (Ag-TLC), showing that photolysis can be stopped at an early stage favoring the monotrans isomer formation. The Ag-TLC procedure can be also used to isolate the monotrans 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 monoepoxide formation.

Analysis of Monotrans EPA Isomers Using 13C NMR and GC. Identification of the monotrans isomers can be carried out by carbon nuclear magnetic resonance (13C NMR), in particular based on the ethylenic carbon atoms, as previously reported in the case of arachidonic acid.10 The 13C 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 monotrans 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 the cis-EPA isomer (Figure 5a, trace V; see Supporting Information for the whole spectra).

It is worth emphasizing that the strategy of the synthetic route via monoepoxides was expedient for the assignment of the five regioisomers. In fact, it was possible to separate and characterize the monoepoxides, recognizing at this level their structures also by comparison with literature data,27 thus facilitating the assignment of the corresponding monotrans 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 isomerization route (Figure 5b, trace II vs traces III, IV, V).

In Figure 5, it can be clearly appreciated how useful the combination of two techniques is to get a complementary and overall view of trans isomer distribution.

Having assigned the elution order of the synthetic monotrans 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 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 Procedures 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 the food industry to eliminate the
unpleasant odor 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 monotrans EPA. The biological fate of monotrans EPA from deodorized fish oils is 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. In that study, we reported the presence of trans isomers derived from oleic acid (18:1), linoleic acid (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 they 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 monotrans isomers in our hands, we proceeded to analyze them. In tuna oil, trans isomers were found in 0.27% content over a content of ca. 9% EPA, corresponding to 3% isomerization of the natural cis content (Figure 6, trace II; see Figure S1B and Table S2 in Supporting Information). 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 Supporting 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).

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 emphasizing 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). 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. The complete fatty acid analysis of the different diets has been previously reported as relative percentages of the total fatty acid content, 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 monotrans EPA isomers were present in quantities almost identical for both diets (Figure 7, lower graphic), the same being 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 were 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 $11.2 \pm 2.1 \, \text{ng/mL}$, 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 monoepoxide route, and the isomer pattern is identical to the mono-trans mixture deriving from the free radical catalyzed isomerization of EPA methyl ester (traces II in Figures 5a and 5b). With this identification, it is now possible to fully address the presence of mono-trans EPA residues in biological samples and use the thyl radical catalyzed isomerization route as a rapid and convenient access of trans libraries, without any contamination with positional isomers. 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.

Deodorized fish oils are used for functional foods and nutraceuticals with the health claim derived from the beneficial effects of omega-3 fatty acids. However, trans fatty acids are well-known toxic compounds which must be accurately controlled for their presence in foods. 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. 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 (9t-18:1, 11t-18:1), linolelaidic acid (9t,12t-18:2), and mono-trans arachidonic acid isomers (14t-20:4, 8t-20:4, 5t-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). Among these isomers, it is important to evidence that mono-trans isomers of arachidonic acid such as 5t-20:4 and 8t-20:4 are known markers of an endogenous free radical isomerization, which is likely to occur in rats due to the stressful life conditions in 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. In the case of arachidonic acid isomers, it was observed that a 30% fat rich diet induces a diminution of this trans isomer content compared to that in 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).
Information). Since the trans-EPA content in the oil is a 3% of the natural cis-EPA (Table S2, Supporting Information), it can be extrapolated that two-thirds 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 that in control rats, whatever the quantity of fat in the diets. A few data are available on the trans isomer incorporation in CL concerning 9c,12c,15t-18:3 acid, a compound commonly found in deodorized edible linolenic acid, whereas it was observed in rat liver mitochondrial membranes (data not shown) in comparison to that in control rats, whatever the quantity of fat in the diets. In the same study, 17t-EPA metabolically derived from 15t-18:3 (n-3) was not incorporated to any significant extent into CL, whereas it was observed in rat tissue phospholipids. 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 trans-EPA library built-up by the present work.

Fatty acid isomerism affects membrane structures, lipid metabolism, and enzymatic reactions, and has not yet been connected to the impairment of mitochondrial respiratory chain or liver nutritional associated pathologies as nonalcoholic fatty liver disease (NAFLD) or steatosis. More attention to the trans fatty acid content related to diet and biodistribution is needed in order to evaluate the influence at the molecular level on health. Our work indicates the usefulness of the synthetic trans EPA library built-up by the present work.

ASSOCIATED CONTENT

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

AUTHOR INFORMATION

Corresponding Author
*E-mail: cferreiri@isof.cnr.it (C.F.); chrys@isof.cnr.it (C.C.).

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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, nonalcoholic fatty liver disease.

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