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# Lipid Markers of "Geometrical" Radical Stress: Synthesis of Monotrans Cholesteryl Ester Isomers and Detection in Human Plasma

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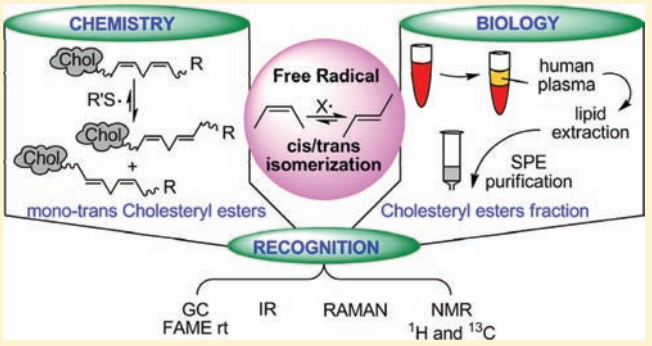
Supporting Information

**ABSTRACT:** Heteroatom-centered free radicals are able to transform cis unsaturated fatty acids to the thermodynamically more stable, but unnatural, trans configuration. The "geometrical" radical stress can be estimated in biological samples using trans fatty acid isomers as lipid markers. Regioselectivity is an important feature of the "geometrical" radical stress, because the supramolecular organization of the polyunsaturated fatty acid moieties of phospholipids can lead to preferential monotrans isomer formation. The regioisomer recognition is a crucial step, which is helped by appropriate molecular libraries available through radical-based synthetic methodologies. Cholesteryl linoleate and arachidonate esters are interesting targets for their biochemical connection with membrane phospholipid turnover and their well-known roles in cardiovascular health. The synthesis of monotrans isomers of PUFA cholesteryl esters was achieved by a thiyl radical-catalyzed cis−trans isomerization. Valuable NMR, IR, and Raman spectroscopic data have been collected for promising application in metabolomics and lipidomics. The identification of monotrans cholesteryl ester isomers was carried out in human plasma by GC, Raman, and IR analyses, demonstrating for the first time the presence of specific regiosiomers connected to free radical stress. This work helps to highlight the chemical biology approach for the access to molecular libraries applicable to biomarker development, and the cis−trans isomerization as a relevant process to be integrated in the puzzling scenario of free radical-mediated lipid modifications.




## INTRODUCTION

Free radical reactivity is an important issue in biological systems,[1] and the corresponding products can be studied as markers, with signaling or damaging roles in living organisms.[2] The "geometrical" radical stress is due to the isomerization of the natural cis geometry of unsaturated fatty acids to the trans configuration induced by free radical species.[3,4] Using biomimetic models as well as in vitro and in vivo studies, the formation of geometrical trans isomers was studied in detail, and it was demonstrated that this process can occur for polyunsaturated fatty acid (PUFA) residues of membrane phospholipids in a regiospecific manner. The cis−trans conversion is also correlated with cellular stress and the reactivity of sulfur-containing compounds.[5−9] Figure 1a shows the general chemical mechanism, consisting of a thiyl radical reversible addition to the double bonds of a polyene (linoleic acid; 9*cis*,12*cis*-octadecadienoic acid). This is a step-by-step process carried out by catalytic amounts of thiyl radicals, producing $\beta$-thiyl-substituted alkyl radicals as intermediates, whose subsequent fragmentation affords two monotrans isomers as the first products. They can undergo a second isomerization step with formation of the ditrans isomer.[10] The trans isomers obtained by this mechanism are exclusively geometrical isomers, because no shift of double bonds occurs during the process.

Early observations of trans fatty acids in biological samples were made during nutritional studies, because these isomers can be ingested with foods containing partially hydrogenated or deodorized oils.[11] To discriminate dietary intake from the endogenous free radical path, two crucial points can be considered: (i) in trans fats derived from the diet, positional trans isomers are present, recognizable by the double bonds in shifted positions compared to natural structures; (ii) when free radical processes occur for natural lipids, the regiospecificity is an important feature, because of the nonequivalent reactivity of the double bonds toward free radical species diffusing through lipid structures. This is the case of membrane phospholipids, as depicted in Figure 1b, and it can be followed up by a PUFA moiety of linoleic acid where the double bond in position 9 of the fatty acid chain is found to be more exposed than position 12.[7] This is also true for positions 5 and 8 of the arachidonic acid chain (5*c*,8*c*,11*c*,14*c*-20:4).[7,10] To facilitate the isomer recognition, molecular libraries of the different regioisomers need to be used as references for the examination of complex biological samples.

Lipid geometry is an important issue in biology and medicine. Several health effects are connected with trans fat-containing diets, in particular a cardiovascular risk for a corresponding increase of LDL-cholesterol and lipoprotein levels, decrease of HDL levels,

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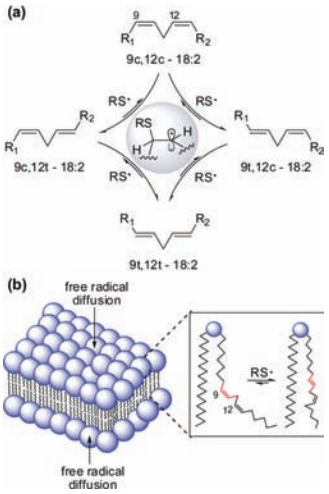



**Figure 1.** (a) Step-by-step cis−trans isomerization of linoleic acid (9*c*,12*c*-18:2) catalyzed by thiyl radicals. Thiyl radicals add reversibly to the double bond, forming a carbon-centered radical as the intermediate. (b) Free radical diffusion in organized lipid assembly that can preferentially isomerize the double bond in position 9 of a linoleic acid moiety.

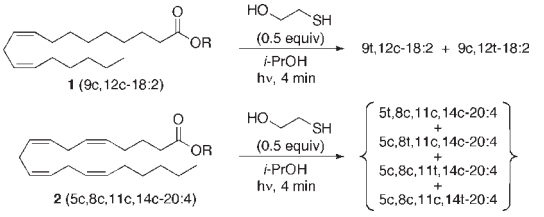
and of cholesterol transfer from HDL and LDL.[12,13] Cholesteryl esters represent a well-known fraction of plasma lipids, strictly involved in lipoprotein metabolism. The ester linkage between fatty acids and cholesterol is obtained largely by the transfer of fatty acids from position 2 of the glycerol moiety of phosphatidylcholine to cholesterol, a step catalyzed by the enzyme lecithin cholesterol acyl transferase (LCAT).[14] From this mechanism of biosynthesis, plasma cholesteryl esters are strictly connected to membrane turnover and contain relatively high proportions of the polyunsaturated fatty acids (PUFA) typically present in phosphatidylcholine, i.e., linoleic and arachidonic acids. They are further used for the assembly of high-density lipoproteins (HDL) and are transferred to the other lipoprotein fractions, LDL (low density lipoprotein) and VLDL (very low density lipoprotein), by a reaction catalyzed by cholesteryl ester transfer protein (CETP). These compounds and the LCAT and CEPT activities have a close relationship with the onset of atherosclerosis and vascular inflammation.[15] Free radicals are implied with inflammation; therefore, cholesteryl esters become interesting targets to be studied as markers because they are organized in supramolecular assemblies,[16] mainly containing linoleic and arachidonic moieties. Their susceptibility to oxidative damage has been already investigated,[17,18] whereas to our knowledge the "geometrical" radical stress and the formation of trans isomers of cholesteryl esters have never been addressed.

Here we describe a multidisciplinary approach coupling the synthetic access and full analytical characterization of monotrans cholesteryl linoleate and arachidonate with the identification and quantification of cholesteryl ester regioisomers in human plasma. This work aims at contributing to the debate on free radical transformations in the complex biological scenario and in biomarker discovery.

## RESULTS

**Synthesis and Characterization of Monotrans Cholesteryl Esters.** The photoisomerization reaction represents a flexible methodology to prepare geometrical trans isomers. A degassed

**Scheme 1. Synthesis of the Monotrans PUFA Cholesteryl Esters Library**



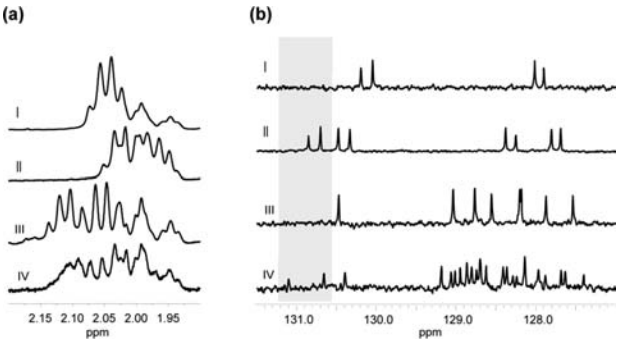




**Figure 2.** (a) The allyl hydrogen resonance in the $^{1}$H NMR spectra ($CDCl_3$) of (I) all-cis cholesteryl linoleate, (II) monotrans isomer fraction of cholesteryl linoleate, (III) all-cis arachidonate, (IV) monotrans isomer fraction of cholesteryl arachidonate. (b) The corresponding ethylenic carbon atom region of the $^{13}$C NMR spectra. The gray region shows diagnostic resonances for the carbon atoms in position 13 of the linoleate moiety and in position 15 of the arachidonate moiety that are typical for monotrans cholesteryl esters and can be used for investigation of the trans content in biological lipid samples.

solution of cholesteryl ester **1** or **2** in 2-propanol was irradiated in the presence of 50% mol equiv of 2-mercaptoethanol. The reaction course is carefully monitored by silver-thin layer chromatography (Ag-TLC),[19] and photolysis can be stopped at an early stage, favoring monotrans isomer formation (Scheme 1). The Ag-TLC procedure can be used to isolate the monotrans isomer fractions and recover the unreacted cis isomers. The latter can undergo subsequent isomerization rounds, leading to overall yields of 80% in the case of monotrans cholesteryl linoleate isomers and 30% in the case of monotrans cholesteryl arachidonate isomers.

These isomers were characterized by an array of analytical methodologies, also in view of scrutinizing the most convenient tools for biological applications. Information obtained from appropriate molecular libraries can be very important for analysis of complex mixtures.

Figure 2 shows NMR resonances that have a diagnostic potential for the cis and trans structures of cholesteryl esters. In the $^{1}$H NMR spectra of the all-cis isomer and monotrans isomers of cholesteryl linoleate, the region around 2.0 ppm, corresponding to the allylic hydrogen atoms, was found to be characteristic for the different geometry of the adjacent trans and cis double bonds. Figure 2a shows that these signals are mainly localized at 2.04 ppm with less contributions at 1.94 and 1.98 ppm for the cis isomer (trace I), whereas the monotrans isomer fraction has a different distribution of two multiplets centered at 1.97 and 2.03 ppm (trace II). Analogously, the cis and monotrans cholesteryl arachidonate isomers have a diagnostic region in the 2.10−2.16 ppm interval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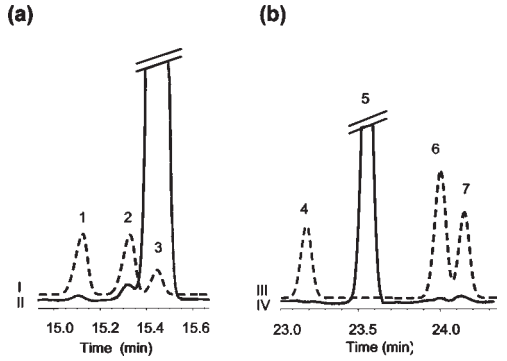


**Figure 3.** GC analysis of fatty acid methyl esters obtained from standard references (dashed line, traces I and III) and from transesterification of cholesteryl esters isolated from human plasma (solid line, traces II and IV). (a) The peak of trace I corresponds to the following: (1) 9*c*,12*t*-18:2; (2) 9*t*,12*c*-18:2; (3) 9*c*,12*c*-18:2, obtained by photolytical isomerization of 15 mM of methyl linoleate with 7 mM of 2-mercaptoethanol in 2-propanol (reaction time: 5 min); comparison with the GC chromatogram section of a sample obtained after transesterification of plasma cholesteryl esters (trace II). (b) Monotrans isomers obtained by photolytical isomerization of 15 mM methyl arachidonate with 7 mM 2-mercaptoethanol in 2-propanol and separated as described in ref 11 (trace III); comparison with the GC chromatogram section of a sample obtained after transesterification of plasma cholesteryl esters (trace IV) (4, 5*c*,8*c*,11*c*,14*t*-20:4; 5, 5*c*,8*c*,11*c*,14*c*-20.4; 6, 5*c*,8*t*,11*c*,14*c*-20:4 + 5*c*,8*c*,11*t*,14*c*-20:4; 7, 5*t*,8*c*,11*c*,14*c*-20:4).

(traces III and IV). In the $^{13}$C NMR spectrum (Figure 2b), an optimal distinction of the ethylenic carbon atom resonances can be appreciated, and diagnostic signals are found in the case of cholesteryl linoleate monotrans isomers (*cfr.*, traces I and II) and cholesteryl arachidonate monotrans isomers (*cfr.*, traces III and IV). The 130−131.5 ppm region corresponding to C-13 and C-15 carbon atom resonances of cis and trans linoleic and arachidonic moieties, respectively, can have a diagnostic potential (gray area in Figure 2b) for analysis of lipid mixtures.[20−22] In fact, chemical shifts higher than 130.5 ppm can be attributed only to ethylenic carbon atoms of trans isomers; in particular, the resonance at 131.1 ppm refers to the carbon atom in position 15 of the 14-trans arachidonic acid isomer.[7]

Gas chromatography (GC) is a very efficient methodology as demonstrated by the optimal resolution achieved with fatty acid methyl esters (FAME) and represents the best technique that allows for the distinction among different types of fatty acids, including the cis and trans positional and geometrical isomers.[23] Mass spectrometry, which is a powerful tool for lipidomic analysis, is not able to distinguish cis and trans geometrical isomers, whereas HPLC methodology has recently shown some progress using silver-ion stationary phases.[24] GC analysis requires that the fatty acid-containing lipids be converted to fatty acid methyl esters (FAME) by transesterification. In the case of cholesteryl esters, previous data on transesterification methods warned about the sensitivity of these compounds to oxidation upon prolonged heating.[25,26] With the synthetic monotrans isomers in our hands, we could establish the best conditions for a quantitative transesterification, free of oxidation and alteration of double bond geometry. The resulting FAME mixture was analyzed using previously described protocols.[7,10] In the case of linoleic acid, the monotrans isomer separation (9*t*,12*c* and 9*c*,12*t* isomers) is successfully achieved, as shown in Figure 3a (trace I, dashed line, peaks 1 and 2).

This FAME isomer mixture is also commercially available, whereas the arachidonate monotrans isomer mixture is available only through synthesis.[7] Figure 3b shows the separation of the 14-monotrans and 5-monotrans isomer of arachidonic acid methyl ester (trace III, dashed line, peaks 4 and 7, respectively), whereas the 8-monotrans and 11-monotrans isomer peaks are superimposed (peak 6).[7,8] The efficient separation of these isomers by GC is very important for the examination of biological samples to individuate a specific regioisomer, which can be correlated with the free radical path and considered as a biomarker of "geometrical" radical stress.[5,7,27]

Vibrational spectroscopic techniques have a very interesting application for lipid analysis, because they can be performed directly on the lipid extracts without derivatization reactions, and the cis and trans geometrical configurations have distinct vibrational bands. These techniques can convey the total trans content, without specifying each fatty acid type. They are also reported as official methods for the trans unsaturated fatty acid determination in edible oils and fats.[23] For the cis and trans isomer analysis, Raman spectroscopy uses the frequency of the C═C stretching vibrations. The C═C stretching band is known to appear in the wavenumber range 1680−1665 cm$^{-1}$ for the nonconjugated trans configuration, while it is in the 1660−1650 cm$^{-1}$ range for the nonconjugated cis.[28,29] Raman spectra of the reference compounds, cis and monotrans isomers of cholesteryl linoleate, in the 1750−1600 cm$^{-1}$ range (spectra I and II, respectively) are shown in Figure 4a. The band at about 1740 cm$^{-1}$ is due to the C═O double bond vibrations, whereas the bands in the 1700−1600 cm$^{-1}$ range reflect the contribution from the double bonds present in the molecule. The peak associated to cis double bond geometry in the fatty alkyl chain appears at 1659 cm$^{-1}$ both for cholesteryl linoleate (spectrum I) and for cholesteryl arachidonate (not shown), whereas the isolated trans double bond is clearly identifiable by the peak at 1670 cm$^{-1}$ (spectrum II for the monotrans cholesteryl linoleate isomers; see Supporting Information for the Raman spectrum of monotrans cholesteryl arachidonate). The latter band and the band at ∼1660 cm$^{-1}$ have been also used for determining the trans/cis isomer content of edible vegetable oils.[30]

In addition to double bonds in the alkyl chain, cholesteryl esters possess one double bond in ring B of cholesterol; the Raman sprectrum of cholesteryl linoleate shows a component, very visible as a shoulder, at 1668 cm$^{-1}$ (Figure 4a, spectrum I).[31,32] The reference spectra of cis and monotrans isomers were subjected to a curve-fitting analysis (Figure 4b) which revealed the contribution of the various $\nu$C═C and was preparatory for the examination of plasma cholesteryl ester samples.

In the IR spectrum, the isolated trans double bonds absorbs between 976 and 956 cm$^{-1}$ because of deformation of the C−H bond adjacent to the trans double bond.[33] Figure 4c shows the relevant IR spectral regions (1800−880 cm$^{-1}$ range) of cis and monotrans isomers of cholesteryl linoleate (spectra A and B). The band at 966 cm$^{-1}$ is characteristic for the isolated trans double bond[23] and is observed in the same position in the monotrans isomers of cholesteryl linoleate and arachidonate (Figure 4c, spectra B and C, respectively; see also Supporting Information). Other bands observed in this fingerprint region are related to $CH_3$ and $CH_2$ deformations and C−O or C−C stretching. The property of the C−H bond adjacent to the trans double bond to absorb in the 974−969 cm$^{-1}$ IR region is used for determining trans levels, i.e., in partially hydrogenated oils. This represents a method used directly on fats or oils that has

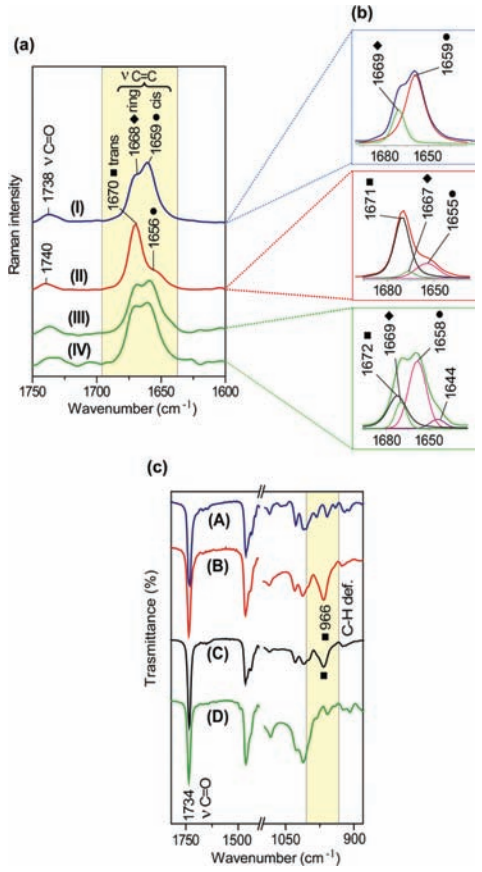


**Figure 4.** Raman and IR spectra of cis, monotrans isomers and human plasma cholesteryl esters. (a) The 1760−1600 $cm^{-1}$ Raman region of cis (I) and monotrans (II) isomers of cholesteryl linoleate, and two samples of plasma cholesteryl ester (traces III and IV). (b) The curve-fitting analyses of the 1700−1620 $cm^{-1}$ Raman region (evidenced by the light yellow area in Figure 3a) of cis cholesteryl linoleate (blue box), monotrans fraction of cholesteryl linoleate (red box), and the plasma cholesteryl ester samples (green box). (c) The 1800−880 $cm^{-1}$ IR region of cis (A) and monotrans (B) isomers of cholesteryl linoleate, (C) monotrans cholesteryl arachidonate, and (D) plasma cholesteryl esters. The IR region where the characteristic band for the isolated trans double bonds appears is evidenced by the light yellow area.

high reproducibility for middle or high trans fatty acids levels, but it is known to have a limited accuracy when the amounts of trans isomers approach quantities as low as 1−2%.[33]

**Identification of Monotrans Cholesteryl Esters in Human Plasma.** Human sera were obtained from routine blood samples that arrived at a clinical laboratory facility involved in lipidomic analysis. A total of 15 samples were processed, and at this stage no information was gathered about the health status of the donor subjects. Our aim was to individuate the presence of monotrans cholesteryl esters; therefore, correlation with health conditions is not within the scope of this work. The cholesteryl ester fraction was isolated from each human serum according to a known protocol, followed by analytical characterization (see Experimental Section).[34] The fatty acid composition was estimated in each sample after the transesterification procedure previously optimized with the reference cholesteryl esters. GC analysis of the corresponding FAME mixture was found to be similar to literature data.[34] Linoleic acid was the most abundant fatty acid (~50% of the total fatty acids), whereas arachidonic acid moieties were ~6% of the total fatty acid content (Figure S1 and Table S1 in Supporting Information). The GC chromatogram region of standard references of methyl linoleate and its monotrans isomers (Figure 3a, trace I, dashed line) were compared with that of FAME mixtures obtained from plasma samples. Figure 3a (trace II, solid line) shows a representative plasma sample where the monotrans linoleate isomers are recognizable (peaks 1 and 2) and are in different amounts. The 9*t*,12*c*-18:2 isomer is prevalent over the 9*c*,12*t*-18:2 isomer. The sample reported in Figure 3a contained ~0.8% monotrans linoleic isomers of the total FAME composition. In the 15 plasma samples, a relative percentage of 0.6 ± 0.2% monotrans linoleic isomers was found (Table S1 in Supporting Information). The plasma cholesteryl ester fraction also contains monounsaturated fatty acids (MUFA; see Supporting Information). In particular oleic acid (9*c*-18:1) and its geometrical trans isomer (elaidic acid, 9*t*-18:1) were found (see Table S1 and Figure S1 in Supporting Information). However, the connection of this MUFA isomer with an endogenous free radical isomerization cannot be addressed in the absence of dietary information.[11,35] More interestingly, GC chromatograms showed the presence of methyl arachidonate and its monotrans isomers (Figure 3b; see also Figure S1 in Supporting Information) by comparison with standard references available through synthesis (Figure 3b, trace III, peaks 4, 6, and 7).[7] It was gratifying to find two monotrans peaks in plasma samples, in particular the 5-monotrans isomers (Figure 3b, peak 7), which is strictly correlated with an endogenous free radical transformation, as previously described.[5,8,27] In the representative sample reported in Figure 3, monotrans 20:4 isomers were present in a relative percentage of ~0.3% of the total fatty acid content. In the 15 plasma samples, a relative percentage of 0.3 ± 0.1% was found (see Table S1 in Supporting Information).

Based on the GC calibration with synthetic standard references, the quantification of trans isomers detected in the biological samples was also performed. A mean value of monotrans linoleic isomers equal to 8.7 ± 2.2 nM and a mean value of monotrans arachidonic isomers equal to 4.1 ± 1.3 nM summed to a total trans PUFA content of 12.8 ± 2.1 nM (see Supporting Information for the conversion factors).

Raman analysis was performed on plasma cholesteryl ester samples in comparison with the library of cis and monotrans cholesteryl ester isomers. In Figure 4a the 1600−1750 $cm^{-1}$ spectral region of two representative plasma samples (spectra III and IV) is reported, in comparison with cis and monotrans linoleate isomers (spectra I and II). The arachidonate isomers gave similar spectra in that region (see Supporting Information). Because both the steroid ring C═C resonance and the double bond of trans isomers contribute to this spectral region, a curve-fitting analysis was needed. Figure 4b shows the corresponding curve-fitting analyses on the two reference samples and one of the plasma samples. The cis isomer spectrum exhibited two components resulting from the cis C═C bonds in the alkyl chain and the double bond in the cholesterol ring (Figure 4b, blue box). The spectrum of the trans isomers showed these two components slightly shifted toward lower wavenumbers, and an additional component at 1671 $cm^{-1}$, attributable to the trans C═C configuration (Figure 4b, red box). It was gratifying to see that plasma samples showed the two components at 1659 and 1668 $cm^{-1}$ as expected for the presence of the cis cholesteryl esters, in addition to the contribution of a higher wavenumber component at 1672 $cm^{-1}$, clearly indicating unconjugated trans geometry (Figure 4a, spectra III and IV, and the curve-fitting

analysis in 4b, green box). A small component at about 1644 $cm^{-1}$ was found in plasma samples, which corresponds to the C═C stretching vibration in conjugated systems.[36]

An estimation of the integrated intensities of the Raman components allowed the total amount of trans unconjugated isomers present in the 15 plasma samples to be evaluated in the 0.8−1.6% range. This is in agreement with the total amounts estimated by GC analysis and confirms the potential of Raman spectroscopy in this field.

In the IR spectrum of the cholesteryl ester fraction from plasma samples the band at 966 $cm^{-1}$, a marker of isolated trans double bonds, was not clearly identifiable (Figure 4c, spectrum D). In fact, its visibility depends on the amount of trans fatty acids present and whether the band is masked partially or completely by other peaks nearby. Thus, to verify the presence or absence of this band in the samples, the curve-fitting analysis of the region was performed taking the linoleate esters as a reference. The analysis revealed the presence of eight to nine components almost at the same position in all samples (see Table S2 in Supporting Information); in particular, a weak peak at about 969−974 $cm^{-1}$ attributable to ═C−H out-of-plane deformation band was present in all spectra with the exception of the cis cholesteryl linoleate. This component, characteristic of isolated double bonds with trans configuration, allowed the presence of a low amount of trans isomers to be assessed also by IR, in agreement with the Raman data. Although the ATR-FTIR method is claimed to have the same potentiality of GC analysis for the detection limit,[33] the problems of superimposition of other peaks can represent a drawback for application of infrared spectroscopy to plasma cholesteryl esters.

As far as NMR analysis is concerned, the results with plasma cholesteryl ester samples were not satisfactory because of detection limits under our experimental conditions (see Supporting Information). However, NMR data gathered on the cholesteryl ester library evidenced distinctive resonances for cis and monotrans isomers (see Figure 2), which can be certainly implemented by high resolution techniques utilized in metabolomics.[20−22,37]

## ■ DISCUSSION

Our results show the effectiveness of a chemical biology approach starting from the synthesis and characterization of monotrans isomers of cholesteryl linoleate and arachidonate, which form a small library for specific applications such as biomarker discovery. The identification and quantification of trans cholesteryl ester isomers are here proposed for the first time in human plasma and can straightforwardly suggest health application, because cholesteryl esters have been clearly associated with atherosclerosis and vascular inflammation.[13,15] The deleterious effects of trans isomers are known, but research has not yet clearly distinguished between dietary and free radical contributions.[38−41] This work focused on the PUFA moieties (linoleic and arachidonic acids) of cholesteryl esters in connection with the regiospecificity feature of the free radical-based isomerization process. In fact, the double bonds of PUFA belonging to supramolecular structures such as membranes have a precise organization and are not equivalent in reactivity as they are in solution toward diffusing radical species (see Figure 1b). The regioisomer prevalence can help assign the origin of trans isomers, distinguishing the dietary and free radical paths, as it has been shown in cellular lipids.[5,27] Indeed, a trans-free diet was useful to show the occurrence of an endogenous pathway, highlighting the role of the 5- and 8-monotrans isomers of arachidonic acid as products of endogenous isomerization.[27]

In plasma samples, monotrans cholesteryl linoleate isomers in different amounts (peaks 1 and 2 in Figure 3a) and the 5-monotrans arachidonate isomer (peak 7 in Figure 3b) were present, indicating a regioselectivity that can be correlated with an endogenous free radical isomerization. Recall that the biosynthetic formation of the double bond in position 5 of 8*c*,11*c*,14*c*-eicosatrienoic acid by the desaturase enzyme can provide only the cis geometry; therefore, the 5-monotrans arachidonic isomer is a strong indicator for an endogenous free radical pathway.[5,27,42] Conditions used for GC analysis are well-known to avoid superimposition by positional trans isomers, such as those deriving from the diet.[11,23] At the moment, the presence of MUFA isomers, in particular elaidic acid (see Table S1 and Figure S1 in Supporting Information), cannot be directly correlated to the isomerization process in the absence of information on the dietary intake (milk, diary products) of the subjects.[11,35] Further work using the cis and trans lipid library for lipidomic analyses of biological samples under different conditions is required, to achieve a comprehensive scenario of fatty acid geometrical changes related to nutrition and stress.

The idea of trans fatty acid isomers as "geometrical" stress markers stemmed from the chemical reactivity of sulfur-centered or nitrogen-centered radical species, which can be generated in the biological environment, toward double bonds (see Scheme 1).[6−10,42,43] Whether the isomerization can occur directly in cholesteryl esters, or in other tissues and then transferred to plasma lipids, will be considered further. As a matter of fact, thiyl radicals can be formed by several processes, which involve repair reactions or hydrogen abstraction from thiols during oxidative stress.[9] The thiol concentrations in plasma reach micromolar levels either for glutathione[44] or for $H_2S$,[45] the latter generating a highly diffusible isomerizing species.[46] Moreover, diffusible thiyl radicals can derive from the desulfurization of disulfide and methionine residues of proteins, as recently shown for albumin, which is indeed the most representative protein in human plasma.[47]

Plasma is a relevant site with enormous potential for lipidomic studies.[48] Cholesteryl ester analysis can be extended to different health and dietary conditions to fully address the significance of these lipid markers, also in comparison with other plasma lipid fractions. This work underscores the efficiency of vibrational spectroscopy for biological samples. Analyses can be performed without derivatization, directly on the isolated cholesteryl ester lipid fraction from human serum. In particular, Raman spectroscopy was very useful for cholesteryl ester analysis in the 1700−1600 $cm^{-1}$ region. In fact, the comparison of the cis and monotrans cholesteryl linoleate isomers (spectra I and II, respectively in Figure 4a) with two representative plasma samples (spectra III and IV), complemented with the curve-fitting analyses (Figure 4b), revealed all components of the bands. The component at 1671 $cm^{-1}$ is related to the trans unconjugated C═C configuration in plasma cholesteryl esters (Figure 4b, red box). The detection of a small component at about 1645 $cm^{-1}$, corresponding to peroxidation products, known to be formed in nanomolar levels from PUFA cholesteryl esters,[49] can be further exploited for a quantitative relationship between oxidation and isomerization processes, thus contributing to an integrated view of the free radical damage to unsaturated fatty acid moieties. The interconnection between the two processes have been recently addressed using a biomimetic model of lipid micelles under irradiation, showing that oxidative stress conditions in the presence of thiols are able to induce both

events.[50] Only after a curve-fitting analysis was the presence of unconjugated trans double bonds with a band at ~970 $cm^{-1}$ detectable in the IR spectra of plasma cholesteryl esters (Figure 4c). This points out the superior performance of Raman analysis for this lipid class.

Finally, new perspectives for testing the biological activity of cholesteryl ester isomers can be envisaged, because it is known that trans fatty acids in humans can affect the transfer of cholesterol from HDL to LDL by CEPT,[51] but a connection with the monotrans isomer formation by free radical stress is still unknown. This work helps to highlight the chemical biology approach for the access to molecular libraries and biomarker development, and the cis−trans isomerization as a relevant process to be integrated in the puzzling scenario of free radical-mediated lipid modifications.

## EXPERIMENTAL SECTION

**Synthesis of Monotrans Isomers of Cholesteryl Linoleate (cholesteryl 9*c*,12*c*-octadecadienoate).** Cholesteryl linoleate **1** (28.3 mg; 0.043 mmol) was dissolved in 2-propanol (2.89 mL; 15 mM solution) using ultrasound for 15 min. The solution was transferred to a quartz photochemical reactor, and then a 7 mM solution of 2-mercaptoethanol in 2-propanol was added. The reaction mixture was flushed with argon for 20 min to evacuate oxygen, and UV-irradiation with a 5.5 W low-pressure mercury lamp was carried out at (22 ± 2) °C for 4 min. The reaction course was monitored by Ag-TLC to evidence the formation of the monotrans fraction. The solvent was removed by a rotary evaporator, and the crude product was taken up with hexane (1 mL) and loaded onto a preparative Ag-TLC plate. After elution (eluent: 9:1 *n*-hexane−diethyl ether), the area corresponding to the monotrans 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 hexane. This material was dissolved with aqueous ammonia (5% $NH_4OH$ solution) and was vigorously stirred (600 rpm) for 15 min. The basic water phase was extracted with hexane (3 × 5 mL). Finally, the organic phase was treated with dry $Na_2SO_4$ and filtered, affording the monotrans cholesteryl linoleate isomer fraction as a gummy white solid (11.3 mg; 0.017 mmol; 40% yield). $^1$H NMR ($CDCl_3$) $\delta$ 0.68 (s, 3H), 0.86−0.92 (m, 12H), 0.94−1.04 (m, 6H), 1.06−1.17 (m, 7H), 1.25−1.36 (m, 17H), 1.43−1.62 (m, 10H), 1.79−1.87 (m, 3H), 1.93−2.05 (m, 6H), 2.24−2.32 (m, 4H), 2.72 (t, $J$ = 5.8 Hz, 2H), 4.57−4.65 (m, 1H), 5.33−5.47 (m, 5H). $^{13}$C NMR ($CDCl_3$, APT) $\delta$ 11.8, 14.1, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.3, 25.0, 27.1, 27.8, 28.0, 28.2, 28.9−29.7, 30.4, 31.4, 31.5, 31.8, 31.9, 32.5, 32.6, 34.7, 35.8, 36.2, 36.6, 37.0, 38.2, 39.5, 39.7, 42.3, 50.0, 56.1, 56.7, 73.7, 122.6, 127.7, 127.8, 128.2, 128.4, 130.3, 130.5, 130.7, 130.8, 139.7, 173.3. HRMS ($C_{45}H_{76}O_2$ ; mw 648) calcd for $C_{45}H_{80}NO_2$ $[M + NH_4]^+$ 666.6189, found 666.6182. IR (neat; $cm^{-1}$): 3015; 2949 ($\nu$CH); 2920 ($\nu$CH), 2852 ($\nu$CH),1735 ($\nu$C═O), 1462 ($\delta CH_2$), 1440 ($\delta CH_2$), 1375, 1256, 1169 ($\omega CH_2$), 1136, 1010, 965 ($\delta$═C−H), 922. RAMAN (neat; $cm^{-1}$): 3011; 2966 ($\nu$CH); 2934 ($\nu$CH), 2906 ($\nu$CH), 2867($\nu$CH), 2851 ($\nu$CH),1738 ($\nu$C═O), 1670 ($\nu$C═C), 1656 ($\nu$C═C),1466 ($\delta CH_2$), 1439 ($\delta CH_2$), 1305 ($tCH_2$), 1258. The starting cholesteryl linoleate was also isolated from the preparative Ag-TLC (17 mg; 0.026 mmol; 63,6% recovery) and reutilized for two reaction rounds. In this way the efficiency of the synthesis reached ~80% overall yield.

**Synthesis of Monotrans of Cholesteryl Arachidonate (cholesteryl 5,8,11,14-eicosatetraenoate).** Cholesteryl arachidonate **2** (24 mg; 0.035 mmol; 15 mM solution) and 2-mercaptoethanol (7 mM) in 2-propanol reacted as described above for cholesteryl linoleate. The workup was done as described above. Ag-TLC purification was performed using 9:1:0.1 *n*-hexane−diethyl ether−acetic acid, as the eluent. The monotrans fraction was obtained as a gummy white solid (3.6 mg; 0.0053 mmol; 15% yield). $^1$H NMR ($CDCl_3$) $\delta$ 0.68 (s, 3H), 0.85−0.92 (m, 12H), 0.95−1.04 (m, 6H), 1.06−1.17 (m, 7H), 1.25−1.36 (m, 9H), 1.43−1.60 (m, 8H), 1.66−1.72 (m, 2H), 1.82−1.89 (m, 3H), 1.95−2.12 (m, 6H), 2.26−2.32 (m, 4H), 2.72−2.83 (m, 6H), 4.57−4.65 (m, 1H), 5.34−5.43 (m, 9H). $^{13}$C NMR ($CDCl_3$, APT) $\delta$ 11.9, 14.1, 18.7, 19.3, 21.0, 22.6, 22.8, 23.8, 24.3, 24.9, 25.6−27.2, 27.8, 28.0, 28.2, 29.2−31.9, 32.5, 34.1, 35.8, 36.2, 36.6, 37.0, 38.2, 39.5, 39.7, 42.3, 50.0, 56.1, 56.7, 73.8, 122.6, 127.4−129.2, 130.4, 130.7, 131.1, 139.7, 173.0. HRMS ($C_{47}H_{76}O_2$; mw 672) calcd for $C_{47}H_{76}O_2Na$ $[M + Na]^+$ 695.5743, found 695.5740. IR (neat; $cm^{-1}$) 3012; 2949 ($\nu$CH); 2929 ($\nu$CH), 2867, 2852,1735 ($\nu$C═O), 1466 ($\delta CH_2$), 1440 ($\delta CH_2$), 1375, 1366, 1256, 1170 ($\omega CH_2$), 1029, 1006, 965 ($\delta$═C−H), 923. RAMAN (neat; $cm^{-1}$) 3014; 2936 ($\nu$CH); 2907 ($\nu$CH), 2896 ($\nu$CH), 2866 ($\nu$CH), 2854 ($\nu$CH), 1739 ($\nu$C═O), 1670 ($\nu$C═C), 1658 ($\nu$C═C),1461 ($\delta CH_2$), 1439 ($\delta CH_2$), 1308 ($tCH_2$), 1261. The starting cholesteryl arachidonate was also isolated by preparative Ag-TLC (15 mg; 0.022 mmol; 63,7% recovery) and reutilized for two reaction rounds. In this way the efficiency of the synthesis reached ~31% overall yield.

**Treatment of Human Serum for Isolation of Cholesteryl Ester Fraction.** Human blood plasma (0.4 mL) was diluted with water (1 mL), and the lipid fraction was partitioned with 2:1 chloroform: methanol (3 × 10 mL). The organic layers were collected, washed with water and brine, and dried ($Na_2SO_4$). After solvent evaporation under vacuum, a yellow oily solid was obtained (10 mg), which was taken up with hexane (1 mL) and loaded on a silica gel cartridge (1 g silica gel) conditioned with *n*-hexane. Using 9:1 *n*-hexane−diethyl ether as the eluent, the fractions containing pure cholesteryl esters were isolated (1.8 mg). This procedure was applied to a total number of 15 human plasma samples obtained from a clinical laboratory facility. The samples were anonymous; therefore, no information on the subject health status was known.

**Transesterification of Cholesteryl Esters Isolated from Human Serum.** A 0.5 mL amount of a 1 M solution of sodium hydroxide in 2:3 benzene−methanol was added to the cholesteryl ester (2 mg) placed in the dark in a small vial under argon. The mixture was stirred at room temperature for 90 min and monitored by TLC (eluent: 8:2:0.1 *n*-hexane−diethyl ether−acetic acid) until disappearance of the starting material. Careful reaction monitoring is needed because once the corresponding methyl esters are formed, the reaction must be stopped, to avoid degradation and formation of side products. TLC shows the quantitative formation of methyl esters. The reaction mixture is quenched with brine (2 mL), extracted with *n*-hexane (3 × 2 mL), and analyzed without further purification. The reaction and analysis were carried out for 15 samples of cholesteryl esters isolated from human plasma. The FAME composition was determined by GC (Table S1 in Supporting Information). Trans isomers were identified and calibrated by comparison with appropriate standard references.

## 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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