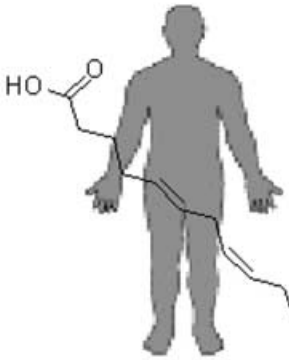
TRANS LIPIDS AND HEALTH



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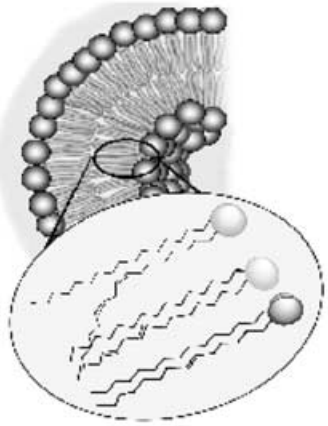

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Geometrical *trans* Lipid Isomers: A New Target for Lipidomics

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Evidence that lipids play different roles in the biological environment, particularly in dealing with metabolic regulation and cell signaling, has led to a growing interest in these molecules, and nowadays the research field of lipid structures and functions is called lipidomics. The term describes diverse research areas, from mapping the entire spectrum of lipids in organisms to describing the function and metabolism of individual lipids. Recent investigations on geometrical trans *isomers of fatty acid derivatives, which have the double bonds in the same position as the natural compounds but with the* trans *instead of the naturally occurring* cis *geometry, highlighted these compounds as a new target for lipidomics. In addition to the identification of their structures and functions, research in a multidisciplinary context aims at understanding the biochemical significance of* cis *and* trans *lipid geometry, and a chemical biology approach can be envisaged to explore the role of the geometry change as either an alteration or a signal that can perturb a biological system and induce a cellular response.*

1. Introduction

The last decade has marked the awakening of interest in lipids, with the main goal being the mapping of the entire spectrum of lipids in a biological system, namely the lipidome.

Lipids (from the Greek *lipos*, meaning fat) are "old" molecules with established roles as the structural backbones of cell membranes and as sources of metabolic energy. Since ancient times they have been linked to human health, as testified by the Hippocratic writings on obesity stating: "*Sudden death is more common in those who are naturally fat than in the lean*" (circa 460 B.C.). The recently emerging roles of lipids as cell signaling molecules and as the regulators of a myriad of cellular processes have produced a renaissance for research, thereby forming the nascent field of lipidomics, which deals with "the full characterization of lipid molecular species and their biological roles with respect to the expression of proteins involved in lipid metabolism and function, including gene regulation".[1] The fact that lipids are nowadays in the spotlight is clearly indicated by the USA's massive investment in research, with more than $45 million granted to a consortium of 10 American universities, named Lipid MAPS,[2] and the foundation of a Lipidomics and Pathobiology Research Center of Excellence, named COBRE.[3] In Europe, there is the ELIfe initiative, involving research teams that are organizing lipidomic research in order to be competitive for the next Framework Program of the European Community in 2007,[4] but actually research funds in the '-omics' are still more directed toward genomics and proteomics, rather than lipidomics. We can foresee that funds will be certainly reoriented, since it is now clear that lipids do not take backstage to other biomolecules with relation to cell functions.

Due to the wide diversity in both structure and function of these molecules, which have the common feature of being insoluble in water, different aspects of research and perspectives in the chemistry, biochemistry, and molecular biology of lipids and lipoproteins have been dealt with in several books and reviews.[5] Despite the fact that lipid research dates back several decades, it should be underlined that the advances of analytical techniques and the use of an interdisciplinary approach in recent times have allowed new insights to be gained into lipid characterizations and functions. The aim of providing a comprehensive view of the lipidome for each organism, with its connection to the corresponding functioning of the genome and proteome, seems more attainable. To achieve this goal, lipidomic research has taken different directions.

Analytical chemistry was the first area of development, with methods that enable the sensitive and specific determination of lipid classes in biological samples, with as little manipulation as possible. The introduction of "soft" ionization techniques, such as electrospray (ES), also associated with liquid chromatography (LC), and matrix-assisted laser desorption/ionization (MALDI) facilitated the analysis of lipid extracts or fractions by mass spectrometry and enabled the detection and identification of phospholipids.[6] The analytical approach is used to map the lipidome of specific organelles, for example, the mouse membrane mitochondria[7] or macrophages,[2] thereby also evaluating reproducible changes of lipid regions or levels upon specific receptor stimulation. It should be added that the comprehension of the role played by lipids in biological processes is strictly related to the availability of a technology for monitoring dynamic changes, as well as for acquiring large, relevant sets of measurements from cellular phenomena. Therefore, mathematical analysis of mass spectrometry data has been implemented, and *computational lipidomics* has been proposed as a novel analytical technique, which couples mass

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spectrometry with statistical algorithms to facilitate the comprehensive examination of hundreds of lipid species from cellular extracts.[8]

Another aspect is related to the clinical impact of lipid classes on human health, since it is now understood that lipids play important roles as secondary messengers in signal-transduction processes, as well as participating in membrane topology and creating specialized domains of specific lipid–protein complexes, both in normal and diseased cells. Abnormal lipid levels are also associated with several diseases, including cancer.[9] Therefore, a wealth of information can be gathered by comparing lipid profiles of plasma or tissues obtained from diseased patients with those from healthy controls. In this context, *functional lipidomics* aims at understanding the functional implications of lipid diversity. This approach combines mass spectrometry analysis both with cell biology, in order to establish localization, trafficking, and interaction partners of specific lipids, and with the manipulation of specific lipid levels followed by phenotypic analysis, to reveal lipid functionality.[10] Again, the study of signaling pathways can be greatly supported by a computational approach, and models for understanding the complexity of cell networks are continuously in development.[11]

Carla Ferreri was born in Napoli, Italy. She graduated at the University of Napoli in 1979 and started working in the field of organic synthesis at the same university. After a training period in the group of Prof. E. Wenkert at the University of San Diego, California, she was appointed as a researcher in the Organic and Biological Chemistry Department at the University of Napoli in 1984. In 2001 she moved to Bologna, where she has a permanent research position at the Consiglio Nazionale delle Ricerche. Her present research focuses on lipidomics of cellular stress, including biomimetic models for the study of protein–lipid tandem radical damage, and chemical biology studies related to cis/trans *isomerization.*

Chryssostomos Chatgilialoglu was born in 1952 in Greece and studied at the University of Bologna, Italy. He was a postdoctoral fellow (1977–79) at the University of York, England, and a research associate (1979–82) at the National Research Council of Canada in Ottawa. He currently works for the Consiglio Nazionale delle Ricerche, Bologna, where he has been Director of Research since 1991. His research focuses on free radical chemistry with current interests in mechanistic studies of biological processes.

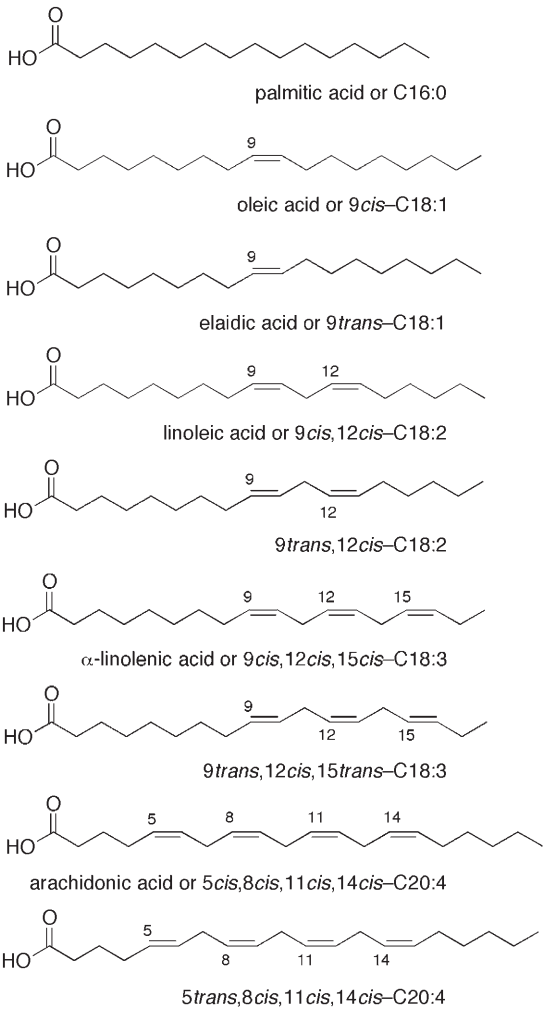
Lipidomic research is also aimed at studying lipid modifications occurring in a biological environment. In this area, radical-based transformation of biomolecules has attracted a lot of interest.[12] For example, in apoptosis, the free-radical oxidative injury of cellular unsaturated lipids has been demonstrated to influence membrane composition and function,[13] and the role of antioxidants, including thiol compounds like glutathione, in this context has been discovered to be either preventive or repairing.[14] Oxidatively modified lipids also have a functional role, for example, activating or disturbing signaling pathways, and *oxidative lipidomics* is a new and exciting research focus involving free radical chemistry and biology.[15]

This review will cover the recent achievements related to a class of lipid molecules, the geometrical *trans* isomers of fatty acid derivatives, which have the double bonds in the same position as the natural compounds but with the *trans* instead of the naturally occurring *cis* geometry. It will be shown that this is a very important research area carried out in a multidisciplinary context and, in particular, that these molecules are connected with a free-radical-catalyzed transformation in biomimetic and biological systems. The latter aspect has been one of the research targets of our laboratory for the last six years,[16] and geometrical *trans* lipid isomers have emerged as new targets for lipidomics. Together with the identification of their structures and functions, the research addresses a more general goal concerning the biochemical significance of the *cis* into *trans* conversion of lipid geometry, as either an alteration or a signal that can perturb a biological system and induce a cellular response.

2. The Double Bond of Lipid Fatty Acid Residues in Biology

Among the most important building blocks of lipid molecules are the fatty acids. These carboxylic acids possess a long hydrocarbon chain (up to 31 carbon atoms) and they can be saturated, or monounsaturated, or polyunsaturated (containing up to 6 double bonds) compounds. Examples of mono- and polyunsaturated fatty acid (MUFA and PUFA, respectively) structures and also of some *trans* isomers are shown in Scheme 1, with the common names and the abbreviations describing the position and geometry of the double bonds (for example, 9*cis* or 9*trans*), as well as the notation for the carbon chain length and the total number of unsaturations (for example, C18:1).

Two initial considerations have to be made: 1) the *cis* and *trans*, instead of the unambiguous *Z* and *E*, notations are still frequently used among lipid chemists to designate stereoisomers and 2) we cannot label *trans* geometry as "unnatural" in lipid structures, because *trans* compounds such as sphingolipids and isoprene lipids, are naturally occurring. As we will see, *trans* lipids are also natural products of bacterial transformations. Nevertheless, when considering MUFA and PUFA structures present in eukaryotic glycerol-based phospholipids—the major type of lipid found in biological membranes—the naturally occurring double-bond geometry is *cis*, and PUFA double bonds have the characteristic methylene-interrupted motif.



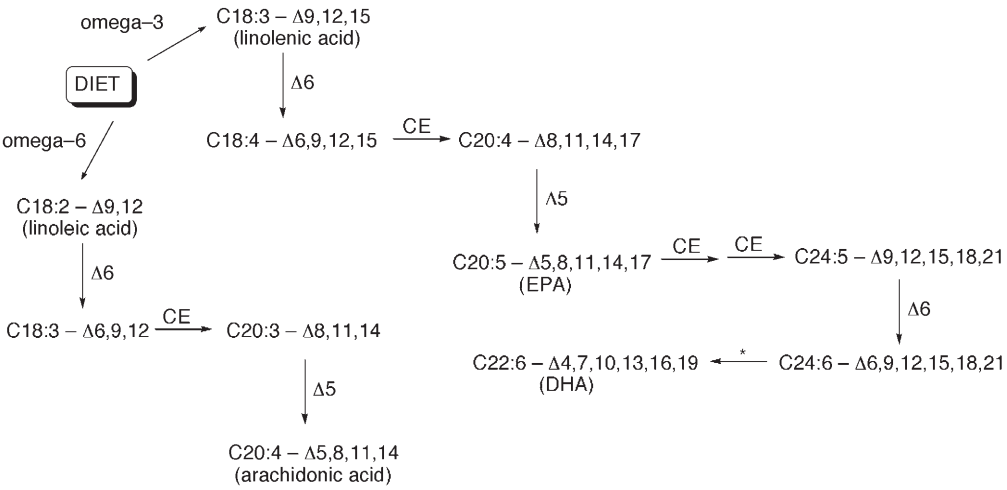
Scheme 1. Examples of structures and common names of natural fatty acids and the abbreviations adopted to describe the number of carbon atoms and the position and geometry of the double bonds.

The *cis* geometry is needed for the biological activities of the compounds and it is provided by the regiospecific and stereoselective enzymatic activity of desaturases.[17] The interplay of desaturase and elongase enzymes in lipid biosynthesis is shown in Scheme 2 for the transformation of linoleic and linolenic acids. It is worth recalling that both compounds are essential fatty acids for mammals; therefore, they have to be acquired from foods as precursors of the omega-6 (or n-6) and omega-3 (or n-3) fatty acid series, which always have the *cis* requisite. An exception is represented by a specific class of dietary conjugated fatty acids, the conjugated linoleic acid isomers (CLAs; for example, the 9*cis*,11*trans*, 9*trans*,11*trans*, 10*trans*,12*cis*, and 10*trans*,12*trans* isomers rather than the 9*cis*,12*cis* isomer of linoleic acid), found in foods derived from ruminant animals.

The geometric *trans* isomers of naturally occurring *cis*-unsaturated fatty acids were almost ignored until a few years ago. Then, beginning in the early 1990s, a constant scientific interest can be documented, with an average of 200 papers per year in the last 7 years.[18] The research covers a wide range of disciplines, such as lipid and membrane properties, microbiology, nutrition, and health, and a brief overview of the recent achievements in these areas will be given below.

2.1. Geometric isomerism as a natural adaptation system

As previously shown, during fatty acid biosynthesis eukaryotic cells form unsaturated fatty acids as *cis* isomers. Prokaryotic cells also biosynthesize *cis* lipids; however, a few years ago it was noticed that some bacteria also contained the corresponding geometrical *trans* isomers.[19] Those compounds did not derive from a de novo synthesis, were also present in non-growing cells, and increased if chemical or environmental stress was applied to the bacterial cultures. It has to be said that microbiologists accurately determine the structure of the *trans* isomers present in bacterial lipids by preparation of the dimethyldisulfide derivatives, which have typical GC/MS spectra depending on where the double bond is positioned along the hydrocarbon chain.[20] As a matter of fact, it was established that geometrical, and not positional, isomers were present in bacteria of the *Pseudomonas* and *Vibrio* species and they were obtained by a biological path carried out by a specific enzyme, the *cis–trans* isomerase (cti).[21] Gene cloning and sequencing from *Pseudomonas putida* P8 and *P. putida* DOT-T1E showed that the isomerase has an N-terminal hydrophobic signal sequence, which is cleaved off after targeting the



Scheme 2. Major biosynthetic pathways of omega-3 and omega-6 fatty acids in animal tissues. The double bonds induced by desaturation (Δ5, Δ6) are in the *cis* geometric configuration and are indicated by vertical arrows. Horizontal arrows indicate chain elongation (CE) and 2-carbon chain shortening (*). EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.

enzyme to the periplasmic space.[21] Comparison among several cti protein sequences identified them as heme-containing proteins of the cytochrome *c* type.[22] The isomerase activity for the transformation of palmitoleic acid (9*cis*-C16:1) into its geometrical isomer palmitelaidic acid (9*trans*-C16:1) was activated in the solvent-tolerant bacterium *P. putida* S12 by the addition of 3-nitrotoluene and gave a final *cis*/*trans* ratio of 32:68.[21b] Recently, through a carbon-isotope fractionation experiment it has been established that the structure of the *cis* double bond is involved in the transition state. A mechanism has been proposed where the heme domain is involved in the sp^2–sp^3 transition by providing an electrophilic iron (Fe^{3+}), which removes an electron from the double bond.[21a,b] The transformation occurs directly on phospholipids, and the cti activity is independent of additional factors, such as adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide (NADPH), or oxygen.[21d] Destruction of the heme-binding motif on the cti of *P. putida* P8 in site-directed mutagenesis experiments caused the loss of isomerization activity.[23]

The enzymatic conversion occurring in some bacterial membranes is an adaptation mechanism, which works when the environmental conditions become unfavorable due to the presence of toxic compounds, increases in the ambient temperature,[19–23] or hypoosmotic shock.[24] Upon restoration of the normal conditions, the same enzyme can work in the opposite sense to regenerate the membrane *cis* geometry. Indeed, this is one of the bacterial short-term adaptation responses with an immediate effect on cell-membrane permeability and fluidity, based on the conversion of the naturally present *cis* fatty acid structures to their *trans* isomers and vice versa. As will be seen in a further section, lipid bilayers made of two monounsaturated glycerophospholipid geometrical isomers have different structural and functional features.

The enzymatic isomerization mechanism active in prokaryotic cells is unknown for eukaryotes, and the majority of unsaturated fatty acids in eukaryotes are provided stereospecifically with only *cis* double bonds during biosynthesis. Such diversity has a still unexplored meaning with relation to the preservation of geometry during the evolution of different organisms. But it can certainly have a more general meaning, for example, with relation to yet unknown surveillance systems on the thermodynamically less stable *cis* isomers. Geometric isomerism is a subject worthy of further investigation in the fields of evolutionary biology, in connection with microbiology, and of biochemistry in eukaryotic cells.

2.2. *trans* Fatty acids in eukaryotic cells: the exogenous origin and biological effects

A great deal of interest has been aroused by the fact that *trans* fatty acid isomers can be present in foods. For example, the level of *trans* isomers in human adipose tissue was found to be in the range of 0.5–3.8% of the main fatty acid residues, and correlations with dietary intakes in different countries were determined.[25] Since enzymatic *cis*–*trans* isomerization in eukaryotic cells is unknown, it was assumed that these compounds could derive from exogenous sources. Indeed, a microbial biohydrogenation occurring in the first stomach of ruminants determines the 2–8% *trans* fatty acid content of dairy products.[26,27]

Other sources of *trans* isomers have also been identified, particularly in industrialized countries that have a high consumption of those foods containing fats and oils manipulated through the partial hydrogenation or deodorization processes[26] or foods that undergo a frying process at high temperatures.[28] The leading countries, the USA and Northern European countries, reach as much as $12\,g\,day^{-1}$ consumption of these fats. At the same time, a series of panels in several countries ascertained the harmful effects on health attributable to the *trans* fatty acid isomers, effects spanning from the inhibition of lipid metabolic pathways to an increase in coronary artery diseases, risk factors of heart attack, and the impairment of fetal and infant growth. Detailed information on these effects can be found in several studies, reviews, and books.[29–32] After these studies and the increase in public concern, a new regulation in the USA has established that by 2006 foods must show the *trans*-isomer content in the nutritional facts.[33] The European regulations do not yet take this issue into account, although European producers have been the first to put *trans*-isomer-free margarine on the market. However, it must be pointed out that *trans* isomers coming from hydrogenation or deodorization processes are only made up to a minor extent of geometrical isomers, with the major part being isomers with a shifted position of the double bond, that is, positional isomers. This positional shift is particularly relevant for linoleic acid that is converted into its conjugated isomers, which are naturally occurring *trans* isomers (see above). Indeed, whether the biological consequences of *trans* isomers can always be considered from a negative perspective is matter of a debate since the discovery that CLAs can exert beneficial effects on health. Conjugated *trans* isomers are far from the objective of this review, and readers can find a summary of recent achievements in this area elsewhere.[34]

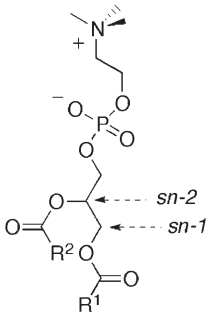
At this point, one could question whether it is enough to know the total *trans* fatty acid content or whether the type and concentration of each *trans* isomer should be ascertained instead. When the case of geometrical isomers only is considered, the number of isomers for each compound is equal to 2^n, where n is the number of the double bonds present in the molecule, and for PUFA derivatives this number can be very high (for example, for a C22:6 fatty acid, $2^6 = 64$). Therefore, the separation and identification of all possible isomers is a challenge for analytical techniques, and the achievements are still few, as we will see later. The situation becomes worse if one also considers the case of positional isomers, as they are present in chemically manipulated dietary fats. As far as the biological effects of geometrical *trans* fatty acid derivatives are concerned, it is evident that the double-bond position is important for the enzymatic interaction. In early reports that appeared during the 1960s and 1970s, it was already noted that changing the geometry of linoleic acid results in the loss of essential fatty acid activity.[35] Now, other information has been gathered and shows that the main fate of *trans* lipids during cell metabolisms can lie along one of two paths:

1) interaction with lipid enzymes and conversion, similar to that of the natural isomers, so that the lipids are incorporated into membranes or enter the lipid cascades but give rise to different molecules, which can influence cell properties and functions.[36] Generally speaking, *trans*-monoenoic acids are recognized as a distinct class with properties intermediate between saturated and *cis*-monounsaturated acids, particularly in specificity for esterification to phospholipids.[5c] Other examples come from polyunsaturated substrates, such as the mono-14*trans* isomer of arachidonic acid (Scheme 1), which has been found to react with cytochrome P450 epoxygenase, a monooxygenase enzyme present in rat liver microsomes, thereby resulting in the corresponding epoxide; this shows that the natural pathway can work and produce an unnatural compound.[37] Metabolism of *trans* fatty acids through the β-oxidation pathway can be important in order to evaluate the potential harmful effects due to an impairment of degradation systems. *trans*-Unsaturated fatty acids are oxidized preferentially in peroxisomes,[38] which are single-membrane-limited organelles present in all eukaryotic cells examined. The yeast *Saccharomyces cerevisiae* is a good model since peroxisomes are their sole site for fatty acid β oxidation. Recently with this model, efficient cell growth with elaidic acid and the need of β-oxidation auxiliary enzymes, to metabolize the *trans* double bond in the odd-numbered position of linolelaidic acid (that is, position 9), have been reported.[39]
2) inhibition of lipid enzymatic pathways. The mono-14*trans* isomer of arachidonic acid can also inhibit the synthesis of thromboxane B_2 and, therefore, prevent rat platelet aggregation.[40] Assays on the in vitro desaturation or elongation of mono-*trans* isomers of linoleic acid by rat liver microsomes showed that the 9*cis*,12*trans* isomer was better desaturated, whereas the 9*trans*,12*cis* isomer (Scheme 1) was better elongated.[41] A general inhibition of the metabolic conversion of linoleic acid into arachidonic acid and other omega-6 PUFAs has been confirmed by a recent study on dietary supplementation of hydrogenated fats given to piglets.[42] A recent study of the activity of the all-*trans* isomer of arachidonic acid (that is, 5*trans*,8*trans*,11*trans*,14*trans*-C20:4) in rabbit platelet aggregation showed that this unnatural isomer can specifically inhibit the response induced by the platelet aggregating factor.[43]

These data indicate that geometrical lipid isomers could be ideal for a chemical biology approach that utilizes small molecules to explore biology.[44] Indeed, a systematic study on the influence of the double bond could contribute to the understanding of lipid diversity for the properties and functions of lipids and for the biological role of geometrical structural change.

2.3. The influence of lipid geometry on membrane properties

An evident difference between the *cis* and the *trans* geometry is that the first confers a kink in the lipid hydrocarbon chain, whereas the latter gives a straight molecular shape, more similar to that of saturated compounds. Since phospholipids are the main components of membranes, their structures regulate the supramolecular organization and properties of the bilayer, and many details of this regulation are known.[45] The general structure of the L-α-phosphatidylcholines (PCs) is shown in Scheme 3. The backbone given by L-glycerol presents the R^1



egg yolk lecithin

palmitoyl	32.0%
stearoyl	14.1%
oleoyl	27.0%
vaccenoyl	1.2%
linoleoyl	20.0%
arachidonoyl	4.8%

Scheme 3. Structure of L-α-phosphatidylcholines (PCs) and the composition of fatty acid residues in egg yolk lecithin.

and R^2 substituents, which are two fatty acyl chains, in positions 1 and 2, whereas a phosphate group esterified with choline is the polar substituent in position 3. In PCs the two fatty acid hydrophobic tails can be different; in naturally occurring lecithins they are saturated or unsaturated residues, as shown in the table of Scheme 3, where the percentage composition of a commercial egg yolk lecithin is reported.

With exclusion of the contributions from variations of polar heads and fatty acid chain lengths and with the focus on the role of unsaturation, a general rule regulating lipid assembly is that the lower the number of the double bonds, the higher the packing order of the lipids. Therefore, the rigidity of the lipid assembly follows the order saturated > *trans*-unsaturated > *cis*-unsaturated. The gross membrane properties of "fluidity" and also permeability are in the inverted order, so that *cis*-unsaturated residues make a relevant contribution to keeping the "ideal" values. The different behavior can also stem from physical properties of saturated and geometrical phospholipid isomers, such as phase-transition temperatures (T_m), that is, the temperature at which the change between the gel and liquid-crystal phases occurs. For example, in the case of PCs (Scheme 3) where the R^1 fatty acid substituent is fixed as C16:0 (palmitic acid) and the R^2 varies through the series C18:0 (stearic acid), 9*trans*-C18:1 (elaidic acid), and 9*cis*-C18:1 (oleic acid; Scheme 1), the T_m values are 41.5, 35, and −3 °C, respectively.[45] Therefore, at a physiological temperature, the *cis*-unsaturated residue ensures the most "fluid" state for the lipid assembly. Polyunsaturated residues can produce an even more pronounced effect, by efficiently lowering the temperature of the gel to liquid-crystal phase transition in natural membranes and ensuring a lipid-chain motion due to their double-bond arrangement.[46]

The permeability and "fluidity" of vesicles made of PCs with saturated and unsaturated fatty acid residues were compared

and, in particular, vesicles were used where the *cis*-monounsaturated components underwent a thiyl-radical-catalyzed transformation into the corresponding geometrical *trans* isomers,[47] a process that will be discussed in the next section. The phospholipid "fluidity" was studied by the fluorescent probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) inserted in the bilayer at temperatures of 8–48 °C.[47b] Figure 1 shows the results obtained with vesicles

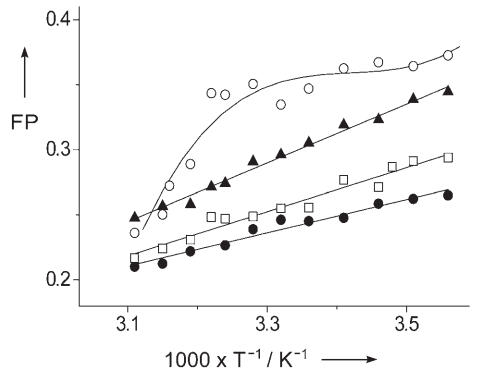


Figure 1. Polarization of TMA-DPH fluorescence (FP) as a function of temperature in DPPC (○) and DOPC (●) vesicles, as well as in DPPC/DOPC (1:1; □) and DPPC/DEPC/DOPC (5:4:1; ▲) vesicles. DPPC = dipalmitoylphosphatidyl choline, DOPC = dioleoylphosphatidyl choline, DEPC = dielaidoyl phosphatidyl choline.

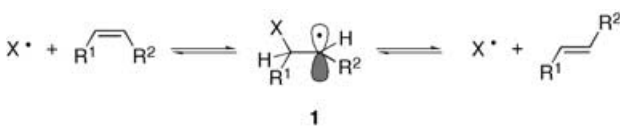
made of different phospholipid compositions. In particular, vesicles made of a saturated PC (open circles), that is, DPPC, where the R^1 and R^2 substituents are both C16:0 fatty acid chains (Scheme 3), were compared with: a) *cis* lipid vesicles (solid circles) made of DOPC, that is, with R^1 and R^2 substituents that are both 9*cis*-C18:1, b) lipid vesicles (triangles) that contain a *trans* PC, DEPC, that is, with R^1 and R^2 substituents that are 9*trans*-C18:1, in a mixture with DPPC and DOPC in the DPPC/DEPC/DOPC ratio 5:4:1, and c) vesicles containing a 1:1 ratio of DPPC/DOPC (open squares). It was clearly shown that a 40% *trans*-lipid content gives higher polarization values, as compared to the values for vesicles where only *cis* residues are present and also for vesicles having a mixture of saturated and *cis* residues, thereby demonstrating the relevant contribution of the *trans* geometry to decreasing the membrane "fluidity".

The alteration in the physical properties of membranes due to the presence of *trans* fatty acid residues has been investigated in connection with some biological effects. In particular, a possible "antioxidant " effect has been advanced, since PC vesicles containing geometrical isomers of PUFA residues are less efficiently oxidized than the corresponding *cis* lipids.[48] The different properties, in terms of molecular dynamics, lateral lipid packing, thermotropic phase behavior, "fluidity", lateral mobility, and permeability, were also evaluated in a study of PC vesicles containing saturated (C18:0) residues and *cis* or *trans* isomers of C18:1 and C18:2 residues.[49] Again, all experiments confirmed the effect of *trans* fatty acid residues to be giving membrane properties more similar to those of saturated chains and indicated that *trans* double bonds induce a more rigid packing than *cis* residues. Another investigation on model membranes containing different *trans*-monounsaturated fatty acid residues (C14:1, C16:1, and C18:1) considered their affinity for cholesterol, as determined from the cholesterol partition coefficient.[50] It was shown that a 40–80% higher affinity is displayed by *trans* membranes as compared to that of their *cis* analogues, probably due to a better interaction between the straight *trans* acyl chain and the cholesterol molecule. In the same report, the behavior of rhodopsin, a prototypical member of the G-protein-coupled receptor family, was evaluated as influenced by the *trans* geometry. In *trans* membrane models the level of rhodopsin activation was diminished, in particular at lower temperatures (5 °C) where *trans* isomers are in the gel state but *cis* isomers are in the fluid state. Other indications of some functional effects come from studies of influence on protein activity[51] and ionic transport.[51c]

All these data indicate that, when a cell membrane made by natural *cis* lipids incorporates *trans* isomers, the lipid assembly can survive but a permanent modification is introduced. Which *trans* isomers, the minimum concentrations at which they affect membrane properties of eukaryotes, and what the perturbation of a biological system and its response is are all matters that need to be thoroughly investigated. The design of more complex membrane models, made of lipids and other components, will probably be useful for the evaluation of the effective contribution of a geometrical change. Moreover, it can be noted that information is still lacking on some basic properties, such as the critical aggregation concentration, that is, the minimum lipid concentration to form a vesicle, and the *trans*-isomer vesicle dimensions, in comparison with the known values for *cis* phospholipids.[45] We can foresee that progress in studies of vesicles including *trans* isomers will provide important support for biology, in particular for individuating the minimum *trans* isomer content at which a perturbation of the state or function of a biological membrane occurs.

3. The Double Bond of Lipid Fatty Acid Residues in Chemistry

For a long time it has been known that *cis–trans* isomerization of double bonds is promptly carried out by free-radical attack.[16,52] Scheme 4 shows the reaction mechanism that consists of a reversible addition of radical X• to the double bond to form the radical adduct **1**. The reconstitution of the double bond is obtained by β elimination of X• and the result is in favor of *trans* geometry, the most thermodynamically favorable disposition. Indeed, the energy difference between the two geometrical isomers of prototype 2-butene is 1.0 kcal mol^{-1}. It is worth noting that 1) the radical X• acts as a catalyst for *cis–*



Scheme 4. Reaction mechanism for the *cis–trans* isomerization catalyzed by free radicals.

trans isomerization and 2) positional isomers cannot be formed as reaction products because the mechanism does not allow a double-bond shift.

Due to the straightforward nature of the method, this approach can successfully support the lipidomic approach for the study of geometrical *trans* isomers. Two aspects relevant to the isomerization protocol will be discussed below in some detail, that is, the choices of isomerizing agent and convenient starting materials.

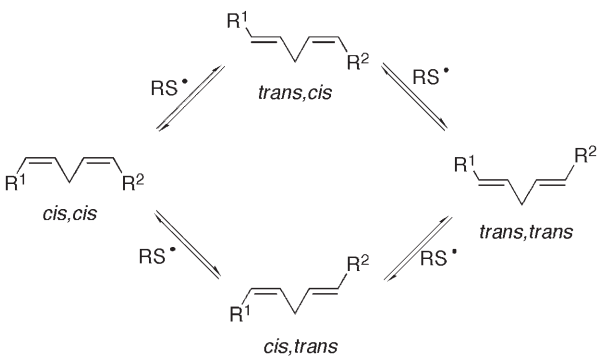
3.1. Radical-based *cis–trans* isomerization processes: what are the isomerizing species and possible inhibitors?

Many free radicals (for example, RS$^\bullet$, RSe$^\bullet$, $RSO_2^\bullet$, $NO_2^\bullet$, $R_3Sn^\bullet$, or $(Me_3Si)_3Si^\bullet$) and atoms (such as Br$^\bullet$ or I$^\bullet$) are known to induce *cis–trans* isomerization of double bonds by addition–elimination steps.[16,52] However, the efficiency of the isomerization process strongly depends on the characteristics of the attacking radicals. The most biologically relevant species that are known to induce isomerization are the thiyl radical (RS$^\bullet$) and nitrogen dioxide ($NO_2^\bullet$).

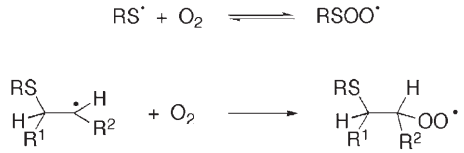
Thiyl-radical-catalyzed *cis–trans* isomerizations with different monounsaturated fatty acid residues under a variety of experimental conditions have been carried out in recent years. The addition rate constants (k_a) for $HOCH_2CH_2S^\bullet$ to methyl oleate (*cis*) and methyl elaidate (*trans*) were found to be rather similar ($k_a^{trans}/k_a^{cis} = 1.8$; where $k_a^{trans} = 2.9\times10^5\ M^{-1}s^{-1}$), whereas the rate constants for the fragmentation step (k_f) were substantially different ($k_f^{trans}/k_f^{cis} = 9.4$; where $k_f^{trans} = 1.6\times10^8\ s^{-1}$).[53,54] The large preference of fragmentation to the *trans* isomer was attributed to different barriers for the formation of the two transition states from the equilibrium radical structure. Therefore, the formation of the *trans* isomer was also favored from a kinetic point of view. However, the *cis/trans* ratio of 13:87 corresponds to a thermodynamic equilibrium of the two geometric isomers at 22 °C.[47b,55]

When polyunsaturated substrates are considered, the isomerization mechanism occurs as a step-by-step process, as depicted in Scheme 5 for linoleate moiety, that is, each isolated double bond behaves independently as discussed above.[55]

Interestingly, the *cis–trans* isomerization by thiyl radicals attacking unsaturated fatty acid residues is also effective in the presence of molecular oxygen up to 0.3 mM concentration, which is a few times higher than the molarity of well-oxygenated tissues. As shown in Scheme 6, under these conditions the equilibrium of the well-known reaction of thiyl radicals with oxygen[56] is shifted to the left (for glutathione, $K_{eq} = 3200\ M^{-1}$), whereas the reaction of the radical adduct with molecular oxygen is unimportant because of the very fast β elimination of the thiyl radical from the adduct, that is, $k_f^{trans} \gg k_f^{cis} \gg k_{oxygen}\,[O_2]$.



Scheme 5. *cis–trans* Isomerization of the linoleate residue catalyzed by thiyl radicals.



Scheme 6. The reactions of oxygen with radical intermediates generated in the isomerization process.

The effectiveness of *cis–trans* isomerization in the presence of the most common antioxidants has also been addressed.[47a] The high efficiency of all-*trans* retinol and ascorbic acid as anti-isomerizing agents in the lipophilic and hydrophilic compartments, respectively, parallel the well-assessed high reactivity of RS$^\bullet$ radicals towards these two antioxidants.[56]

Nitrogen dioxide $NO_2^\bullet$, is an emerging species in biology[57] and was reported to produce *trans* arachidonic acid isomers in human platelets.[58] However, based on the kinetic data available on the various processes carried out by the $NO_2^\bullet$ radical, it emerges that it cannot be as efficient as thiyl radicals as an isomerizing species, and in a biological environment this reaction should not play a role.[16] In fact, the rate constants at 25 °C for the addition of $NO_2^\bullet$ to monounsaturated olefins are $k_a^{cis} = 0.18\ M^{-1}s^{-1}$ and $k_a^{trans} = 0.038\ M^{-1}s^{-1}$, whereas the fragmentation of the adduct radical occurs with $k_f \approx 8\times10^4\ s^{-1}$ for both isomers.[59] Furthermore, hydrogen-atom abstraction from the allylic position is of the same order of magnitude as the addition step, whereas the abstraction of bisallylic hydrogen atoms in polyunsaturated moieties is expected to be faster. Therefore, in the presence of 0.1 mM of oxygen the *cis–trans* isomerization becomes unimportant, since the various carbon-centered radicals will react with O_2 to give peroxyl radicals that propagate peroxidation. Indeed, the induction of lipid peroxidation has been observed in human plasma.[60]

It should be added that thiols are known to be the dominant "sink" for $NO_2^\bullet$ in cells/tissues [Eq. (1)], with the rate constant being close to $2\times10^7\ M^{-1}\ s^{-1}$ and with generation of thiyl radicals.[61] Therefore, in the biological environment thiyl radicals are likely to be the most relevant isomerizing species.

$$NO_2^\bullet + RSH \rightarrow NO_2^- + RS^\bullet + H \qquad (1)$$

3.2. A chemical biology approach through the *trans*-lipid library and the all-*trans* PUFA strategy

Lipid isomerization has all the favorable features for a chemical biology approach: 1) chemical synthesis that can also be performed in biochemical laboratories, because of the simple

choice of the most suitable conditions in terms of sources of initiating radicals, isomerization agents, and solvents, 2) applicability to a variety of easily available natural lipids, 3) purity of the products, due to the absence of positional isomers, and 4) the significance of the modification produced on lipid structures for inducing perturbation of biological systems.

From the library of *cis* lipid molecules available from natural sources, a *trans* lipid library can be easily planned. In this synthetic approach, polyunsaturated compounds represent a challenge because of the complexity of the isomeric trends and the wealth of analytical data that can be gathered. In this context, as further described in the case of arachidonate residues,[62] NMR spectroscopy can be very useful for double-bond identification by using the ethylenic carbon atom resonances, which are known to differ for *cis* and *trans* geometries and for their positions along the chain.[63]

A strategy for building-up the *trans* lipid library by starting from natural oils and lecithins rich in unsaturated fatty acid residues has been proposed. In particular, vegetable oils can be a cheap and convenient material for accessing a variety of *trans* lipid derivatives.[64] The formation of *trans* triglycerides is very efficient by a radical-catalyzed isomerization carried out in alcoholic solution, with thiyl radicals generated by photolysis. The isomerized oil can be further enriched in its *trans* content by the winterization process, that is, by separating the triglycerides rich in *trans* fatty acid residues from the residual *cis*-containing material by crystallization at low temperatures in *n*-hexane. Oils with a high *trans* content can be prepared. NMR spectra of triglycerides with a high *trans* content form a library of data that can be a useful tool for the investigation of biological samples by ^{13}C NMR spectroscopy and comparative studies of health conditions with diseases.[65] A tandem chemical–enzymatic methodology was applied to the protocol of oil isomerization, and the two corresponding classes of *trans* fatty acids and esters were also obtained. *Candida antarctica* lipase was used; this enzyme did not show selectivity, as it could transform both isomers equally well. However, further development can be foreseen for the substrate–enzyme selectivity as well as for the application of the chemical–enzymatic protocol to phospholipids.

Based on the efficiency of the isomerization process, the all-*trans* PUFA strategy has recently been implemented for chemical biology studies. The first all-*trans* long-chain PUFA synthesis has been successfully carried out by using the naturally occurring arachidonic acid,[43] and this protocol can be extended to the preparation of several all-*trans* analogues for various biochemical and biological assays, including pharmacological applications, and also to support lipidomic research.

4. Biomimetic Models for Lipid Isomerization

The first report highlighting the lipid isomerization mechanism as a biologically meaningful process was from our group in 1999.[66] By using biologically relevant compounds and phospholipids, the occurrence of such a transformation was modeled under biomimetic conditions. The subject was of interest to other research groups, and all work done in this area showed that thiyl radicals are efficient and effective isomerizing agents.[67] A recent review summarizes the subject of the thiyl radical production in biosystems and the effects on lipid metabolism.[68]

With inspiration taken from the lipid peroxidation process extensively studied in liposomes,[69] unsaturated lipid vesicles were envisaged as a good biomimetic model for the double-bond isomerization. Indeed, early reports on the use of glutathione, or other thiol compounds, as an effective protective agent against radiation-induced lipid peroxidation did not mention the stability of the double-bond geometry.[70] Generally speaking, thiol compounds are still regarded as radioprotective agents against the various types of damage on lipids, DNA, and proteins,[71] despite the reported activation of PUFA peroxidation, which forwarded the hypothesis that thiols can be a double-edged sword in the biological environment.[72] An overview of the main features of the isomerization reaction studied in the biomimetic models is given below, that is, the role of diffusible thiyl radicals combined with the reactivity of arachidonate residues and also the radical degradation of sulfur-containing proteins, which results in a tandem protein–lipid damage. Finally, the first evidence of formation of *trans* lipids in models will be described, as monitored during normal cell metabolism in the absence of a *trans* fat dietary source.

4.1. Diffusible thiyl radicals and isomerization of arachidonate residues in model membranes

Liposomes such as multilamellar (MLV) and small or large unilamellar (SUV or LUV) vesicles are widely accepted as models of membrane lipid assembly. After an initial use of MLV vesicles, the choice was oriented toward large unilamellar vesicles obtained by an extrusion technique (LUVET),[73] because of their close relationship with cell membranes due to the presence of a single bilayer. They can be prepared with a quite uniform diameter depending on the polycarbonate filter size used for the extrusion. Vesicles of 100 nm diameter form an almost transparent suspension, which is also suitable for studies under photolytic conditions.

The aqueous and lipid phases are the two distinct compartments of this nonhomogeneous system. There are several features to be taken into account for examining the reactivity of this system towards free radicals: 1) the characteristic supramolecular arrangement of the lipid assembly, with the fatty acid chains of phospholipid molecules that form the hydrophobic core of the model membrane, and the polar heads that face the aqueous internal and external phases, 2) the partition coefficient of compounds added into the system, which influences the distribution of the reactive species in the two compartments, and 3) particularly, the location of the initiation step, that is, where the formation of an initial radical species, able to abstract the hydrogen atom from the thiol group, occurs.

As far as the lipid organization is concerned, there is a precise arrangement of the hydrophobic core, which can influence the position of the double bonds in the layer and the reactivity of the different fatty acids to radical attack. This was found to

be the case in the double-bond isomerization, studied with an amphiphilic thiol, 2-mercaptoethanol, that is, a compound able to diffuse without restriction from the aqueous phase to the lipid bilayer and vice versa. A regioselective process resulted where the double bonds were not involved to the same extent in the radical isomerization. In particular, by using vesicles made of egg yolk lecithin it was possible to demonstrate that the double bonds located closest to the membrane polar region are the most reactive towards the attack of diffusing thiyl radicals.[55,74] In the case of linoleic acid residues in vesicles, the double bond in position 9 was more reactive than that in position 12. Also, arachidonic acid residues in vesicles were more reactive than oleic and linoleic acids, and two positions, the double bonds at positions 5 and 8, were transformed preferentially over the others present in this compound. The scenario could be different for other long-chain PUFAs depending on their supramolecular arrangement, and isomerization by diffusible thiyl radicals could act in this context as a reporter, indirectly informing on the double-bond disposition in the bilayer. From the studies carried out so far, arachidonic acid residues in membrane phospholipids emerge as very important elements to be investigated, in order to distinguish endogenous *trans* isomers, formed by radical processes, from the exogenous *trans* isomers, derived from dietary contribution. In particular, investigation could focus on erythrocyte membrane phospholipids, which are the preferential storage for arachidonic acid after biosynthesis. As shown in a previous section, nutritional investigations indicated that *trans* fatty acids are incorporated in cell membranes, because the *trans* dietary precursor is processed in vivo. In the case of arachidonic acid, as shown in the biosynthetic pathways of Scheme 2, two double bonds (positions 11 and 14) originate from linoleic acid, the precursor taken from the diet, whereas the other two double bonds (positions 5 and 8) are formed by desaturase enzymes, which selectively produce the *cis* unsaturation. It is evident that the double bonds at positions 5 and 8 of arachidonic acid, stored in membrane phospholipids, can only have a *cis* configuration, unless these positions are involved in an isomerization process by diffusible thiyl radicals and transformed into *trans* isomers.[62]

A careful identification of membrane lipids containing arachidonic residues may be important for functional lipidomics, in order to achieve a clear understanding of the contribution from endogenous or exogenous processes. Moreover, if one considers the close relationship established between free radical processes and human pathologies and aging,[75] the functional lipidomic approach involving arachidonate geometrical isomers could provide additional useful information on the role of radical stress conditions in health and diseases.

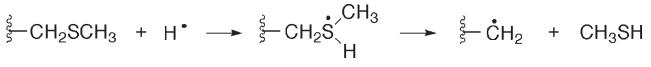
4.2. *trans* Lipids as markers of protein damage

The partition coefficient of sulfur-containing compounds used in the vesicle model and the compartment where the radical initiation step occurs have a role in the isomerization outcome. Hydrophilic, lipophilic, and amphiphilic compounds have different behaviors, and the combination between the thiol and the radical initiator can be a crucial step. In biomimetic models, a variety of biologically relevant sulfur-containing compounds, such as cysteine, glutathione, methionine, and lipoic and dihydrolipoic acids, were examined under different radical-initiating conditions.[47,55] A radical initiation exclusively occurring in the aqueous compartment was obtained by two methodologies: 1) thermal decomposition of a hydrophilic azo compound,[47b,55] which can also have biological significance, simulating the "repair" hydrogen-donation reaction of thiols, such as cysteine and glutathione, towards carbon-centered radicals;[76] 2) γ irradiation of aqueous systems, which gives water primary radicals and solvated electrons as the initiating species [Eq. (2)] and has been widely used for studying damage to biomolecules, with the possibility of selection of the reacting radical species under appropriate conditions.[77]

$$H_2O \xrightarrow{\gamma} e_{aq}^- + HO^{\cdot} + H^{\cdot} \qquad (2)$$

By examining different combinations of thiols and initiators, it was established that hydrophilic compounds, such as cysteine or methionine, combined with a hydrophilic initiator, did not give any isomerization of the lipid bilayer. This behavior was easily explained by the fact that hydrophilic thiols are not able to enter the lipid bilayer, so their corresponding thiyl radicals cannot reach the lipid double bonds.[47b] On the other hand, when initiation was obtained from primary water radicals generated under appropriate γ-radiolysis conditions [Eq. (2)], lipid isomerization occurred. This highlighted the different operating mechanisms that can take place to form thiyl radicals from water-soluble sulfur-containing compounds, and it was of relevance in the case of sulfur-containing proteins, particularly methionine-containing proteins.

Indeed, in the early sixties, radical damage caused by hydrogen atoms, H$^{\cdot}$, to the ribonuclease A protein (RNase A) from bovine pancreas was studied in detail.[79] The mechanism of the degradation is shown in Scheme 7 for the thioether function and starts from the preferential attack of H$^{\cdot}$ atoms on the sulfur moieties, thus forming an intermediate sulfuranyl radical species. This species can give a β fragmentation of the C–S bond with the release of the low-molecular-weight thiol moiety.



Scheme 7. Formation of sulfuranyl radical species by attack of H$^{\cdot}$ atoms on the thioether functionality of a methionine residue, followed by β fragmentation with release of a low-molecular-weight thiol compound.

The relevance of this degradation path with the formation of low-molecular-weight thiol compounds lay dormant, until the hypothesis of tandem radical damage, which involves protein and lipid domains, was advanced.[80] Indeed, tandem damage is much more harmful than single damage in a biological environment, since it can involve more sites or molecules at the same time, with serious risk of impairment of the cell's defence or repair systems. Moreover, the possibility that two

apparently distinct compartments, that is, the aqueous and the lipid phases, could be involved at the same time, has been tested a few times, for example, by isolation of coupling products between the lipid peroxidation derivative 4-hydroxynonenale and protein or amino acid residues[81a,b] or nucleic acid components.[81c]

A biomimetic model composed of a *cis*-unsaturated lipid vesicle (DOPC) and a sulfur-containing protein, such as bovine RNase, was designed for the detection of damage mediated by thiyl radicals.[80] The protein was at μM concentration levels and the lipid concentration was at mM levels. The system underwent γ irradiation, which induces the release of a low-molecular-weight thiol from the methionine residues, as previously described (Scheme 7). Under radical conditions, the reactive and highly diffusible thiyl radical species $CH_3S^{\bullet}$ could be formed from the thiol; this radical species rapidly diffused in the lipid bilayer, thereby causing isomerization of the double bonds. Indeed, the formation of *trans* residues in the vesicles has proven to be a very sensitive tool for detecting protein damage, even at nanomolar levels, that is not easily detectable with other techniques. The design of such models for several sulfur-containing proteins and the use of different lipid/protein ratios will allow the tandem radical damage to be fully evaluated, including in the presence of other components acting as competitors or inhibitors at various stages of the process.

The conclusive picture emerging from the chemical studies under biomimetic conditions is that thiyl radicals are efficient catalysts for *cis–trans* isomerization of lipids in bilayers, and this process cannot be ignored when considering radical damage to biological components. Instead, it can represent a sensitive tool for detecting radical stress occurring in the cell compartments at a very early stage.

4.3. In vitro models: the groundwork of lipid isomerization in living systems

The results obtained from chemical and biomimetic studies suggest the extension of the investigation to biological systems, in order to prove the "endogenous" *trans* lipid formation under strictly physiological conditions. It is important to deal with "*trans*-free" conditions, which means that the presence of any external source of *trans* fatty acid isomers is carefully checked. The cell-membrane lipid composition of human leukemia cell lines (THP-1) was monitored during incubation in the absence and presence of thiol compounds, thereby ensuring that no contribution of *trans* compounds could come from the medium.[82] The experiments were based on the hypothesis that the normal cell metabolism includes several radical-based processes.[68] Therefore, the intracellular level of sulfur-containing compounds could have produced a certain amount of thiyl radicals and, consequently, caused lipid isomerization. In parallel experiments, some thiol compounds were added in mM levels to the cell cultures during incubation, and a comparison of isomeric trends was done. A basic content of *trans* lipids in THP-1 cell membranes was found during their growth before thiol addition, and the content was increased up to 5.6% of the main fatty acid residues by addition of amphiphilic 2-mercaptoethanol. Even greater *trans*-lipid formation was obtained by radical stress artificially produced in the cell cultures with thiol added; for example, a 15.5% *trans* content in membrane phospholipids was reached by γ irradiation. The fatty acid residues most involved in this transformation were arachidonate moieties, and this result confirmed that these are the most important residues to be monitored in cells.

The *trans* arachidonate content determined in THP-1 membrane phospholipids provides the first indication of the occurrence of an endogenous isomerization processes, not to be confused with a dietary contribution, as previously explained. This opens new perspectives for the role of *trans* lipids in the lipidome of eukaryotic cells.

5. Perspectives and Future Research

The work done so far on *trans* lipids provides the framework for development in several directions, as indicated from time to time in this review. Analytical improvements for detection of *cis* and *trans* PUFA isomers are certainly needed, as well as for facilitating the characterization of these isomers in biological samples. Besides the total *trans* content, which can be determined by infrared spectroscopy with a very easy method,[83] there are no satisfactory analyses by gas chromatography or HPLC for PUFA isomers with chains of more than 18 carbon atoms. In fact, we have approached the separation and identification of arachidonic acid isomers, but further work is needed to achieve the goal of characterizing a complete *trans* PUFA library. Also, the development of computational lipidomics, which can facilitate the analyses of biological samples and the construction of a large database of geometric isomerism, would be of great help for understanding implications of *trans* lipids in human health and diseases.

Geometrical *trans* lipid isomers are a new target for lipidomic research. This survey has outlined the fields to be developed with an inter- and multidisciplinary approach, spanning from chemistry, biochemistry, biology, and medicine to engineering and computer science, with the ultimate goal of understanding the role of geometrical *cis*/*trans* lipid conversions in cell structures and signaling activities.

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Keywords: fatty acids · isomerization · lipidomics · lipids · phospholipids

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