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Regioselective Cis−Trans Isomerization of Arachidonic Double Bonds by Thiyl Radicals: The Influence of Phospholipid Supramolecular Organization

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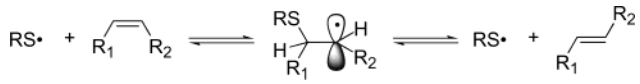
Abstract: Trans unsaturated fatty acids in humans may be originated by two different contributions. The exogenous track is due to dietary supplementation of trans fats and the endogenous path deals with free-radical-catalyzed cis−trans isomerization of fatty acids. Arachidonic acid residue (5c,8c,11c,14c−20:4), which has only two out of the four double bonds deriving from the diet, was used to differentiate the two paths and to assess the importance of a radical reaction. A detailed study on the formation of trans phospholipids catalyzed by the $HOCH_2CH_2S^{\bullet}$ radical was carried out on L-α-phosphatidylcholine from egg lecithin and 1-stearoyl-2-arachidonoyl-L-α-phosphatidylcholine (SAPC) in homogeneous solution or in large unilamellar vesicles (LUVET). Thiyl radicals were generated from the corresponding thiol by either γ-irradiation or UV photolysis, and the reaction course was followed by GC, Ag/TLC, and ^{13}C NMR analyses. The isomerization was found to be independent of cis double bond location (random process) in *i*-PrOH solution. In the case of vesicles, the supramolecular organization of lipids produced a dramatic change of the isomerization outcome: (i) in egg lecithin, the reactivity of arachidonate moieties is higher than that of oleate and linoleate residues, (ii) in the linoleate residues of egg lecithin, the 9t,12c−18:2 isomer prevailed on the 9c,12t−18:2 isomer (3:1 ratio), and (iii) a regioselective isomerization of SAPC arachidonate residues occurred in the 5 and 8 positions. This effect of "positional preference" indicates that thiyl radicals entering the hydrophobic region of the membrane bilayer start to isomerize polyunsaturated fatty acid residues having the double bonds nearest to the membrane surfaces. We propose that arachidonic acid and its trans isomers can function as biomarkers in membranes for distinguishing the two trans fatty acid-forming pathways.

Introduction

Polyunsaturated fatty acids (PUFA) are ubiquitous biological molecules, which have established roles as metabolic fuels and essential components of cell membrane lipids and have emerging roles as precursors of several classes of signal molecules and regulators of transcriptional factors.[1−4] In the last 20 years, the degradation of PUFA has been the subject of intense chemical, biochemical, and medical research, which has highlighted the importance of lipid peroxidation in several diseases and the aging process.[5,6] More recently, PUFA structural change due to the conversion of the naturally occurring cis configuration into the corresponding trans geometry by $NO_2^{\bullet}$ or $RS^{\bullet}$ radicals has been evidenced.[7−10] Scheme 1 shows the process of cis−trans isomerization by attack of thiyl radicals to an isolated double bond.[11]

Scheme 1



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Biomimetic studies of the radical-catalyzed cis−trans isomerization performed with liposomes and human blood have correlated this process with the possibility of trans PUFA formation in vivo.[7,9,10] It is worth noting that the geometrical trans isomer still possesses a methylene-interrupted unsaturated skeleton, but the configurational change confers distinct physical properties, compared to the corresponding cis isomer. Moreover, since the trans lipid geometry resembles that of saturated lipids, the event of a geometrical isomerization does not cause lipid degradation, but a permanent modification. In this respect, cis−trans isomerization can be considered similar to a decrease of the number of unsaturations in the lipid bilayer.[12] The different properties of trans lipids determine a more rigid packing of the bilayer, which is a well-known expedient in nature. In fact, some bacteria utilize the enzymatic cis−trans geometrical conversion of phospholipids to harden their membrane as a short-term adaptation response to environmental stresses or as an expedient to tolerate toxic compounds.[13] Mammalian cells do not form trans lipids via enzymatic pathways; therefore, trans fatty acids detected in tissues during nutritional investigations have been attributed to dietary consumptions of meat, milk, and partially hydrogenated fats and oils.[14] It is worth pointing out that in these cases the structures of trans mono- and polyunsaturated fatty acids consist of geometrical and positional isomers having unshifted and shifted double bonds compared to natural cis compounds.[15] A public concern has arisen regarding the consumption of altered fats which can cause deleterious effects on health,[14,16−18] and the need for lipid identification in foods has now been claimed.[19] From epidemiological studies, it is clear that the health risk depends on the quality rather than the quantity of fats, but it is remarkable that only exogenous (dietary) sources of trans fatty acids have been considered thus far.[20]

The distinction between the endogenous formation of trans lipids by free radical attack and the dietary supplementation is an open subject, involving the new field of lipidomics, which aims at providing a comprehensive analysis of membrane lipid composition and all lipid pattern and modifications in living organisms.[21] Although the total number of PUFA geometrical isomers is easily calculated (equal to 2^n where n is the number of double bonds) and their total concentration in a sample is measured by infrared spectroscopy,[22] the chemical identification for each trans isomer is a difficult task. This is especially true for eicosenoates, which have a high number of unsaturations (up to six double bonds), and being one of the most important classes of lipid mediators,[23] each isomer is expected to display a different biological activity. As a matter of fact, two isomers of arachidonic acid (**1**), namely the mono trans isomers in positions 5 and 14 prepared by total synthesis, were used for enzymatic assays (Scheme 2).[24,25] The 14-trans isomer decreased platelet aggregation and inhibited prostaglandin biosynthesis,[26,27] whereas the 5-trans isomer did not cause any enzymatic inhibition and could be transformed to 5-trans PGE_2.[24]

Each mono-trans isomer of arachidonic acid is now available by stereospecific synthesis,[28] which is a barrier for those not experienced in organic synthesis. In this context, the thiyl radical-catalyzed isomerization process can help to provide a more flexible access to geometrical trans isomers of mono- and polyunsaturated structures.[9,11] An example with long-chain PUFA (LC-PUFA) has been recently reported by us, concerning the isomerization of methyl arachidonate in alcoholic solution.[10] Since it is reported by pulse radiolysis studies that $RS^•$ radicals[29] and $Br^•$ atom[30] react with arachidonic acid both via abstraction of bisallylic hydrogen atom and via addition to the double bonds, these species could act also as potential initiators for lipid peroxidation. Knowledge on the isomerization of LC-PUFA has still to be gained, in particular under biomimetic conditions, which could help to understand the balance when competition between isomerization and oxidation processes occurs in vivo.

Another relevant aspect of the study on the arachidonic acid isomerization in biomimetic models is that it could provide useful information to distinguish between exogenous (dietary) supplementation and endogenous formation due to radical-catalyzed isomerization. Indeed, the four double bonds of arachidonic acid originate from different pathways in vivo: (i) two enzymatic desaturation steps provide the double bonds of arachidonic acid in positions 5 and 8 which rigorously have the cis configuration and (ii) the double bonds in the 11 and 14 positions derive from linoleic acid (**2**); therefore, they can be cis, trans, or both, depending on the dietary contribution (Scheme 2).[31] On the other hand, no data are available on the

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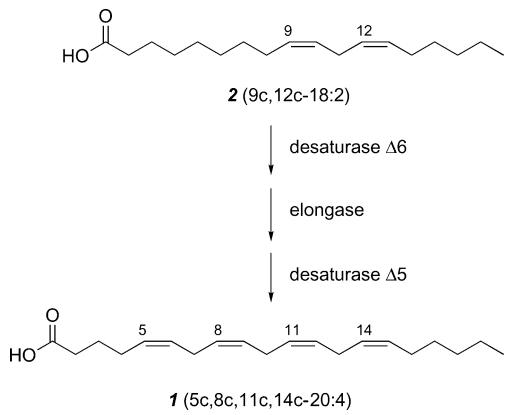
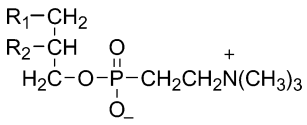
Scheme 2



selectivity of the isomerization process by thiyl radical in vivo and which of the four double bonds of arachidonic acid is involved.

On the basis of our biomimetic studies performed with liposomes and human blood,[9−11] we thought that the phospholipid supramolecular organization could indeed induce a regioselective process related to the mode of radical diffusion into the bilayer and the relative availability of the double bonds.[37] Herein we report a detailed study of the thiyl radical-catalyzed isomerization of phospholipids containing arachidonic acid residues in solution and in large unilamellar vesicles (LUVET). Both 1-stearoyl-2-arachidonoyl-L-α-phosphatidylcholine (**3** or SAPC) and L-α-phosphatidylcholine from egg lecithin were used in the reaction with thiyl radical species generated from 2-mercaptoethanol. SAPC vesicles represented the simplest model containing only arachidonate and stearate residues. On the other hand, the mixture of fatty acid components in egg lecithin offered a model closest to cell membranes, where phospholipids have indeed different acyl chains as their hydrophobic tails.

This study aims at giving information on the structural changes induced by the thiyl radical stress to arachidonate residues in cell membranes and on the origin and positions of the trans double bonds in this important molecule. The overall objective of this chemical study is to contribute to the research on the identification of LC-PUFA trans isomers, in connection



R_1 = stearoyl (C18:0)
R_2 = arachidonoyl (C20:4)

3 (SAPC)

with the biochemical and medical relevance of membrane lipid modifications.

Results and Discussion

Generation of Thiyl Radicals in *i*-PrOH. Thiyl radicals were produced by γ-irradiation of N_2O-saturated *i*-PrOH solutions containing 2-mercaptoethanol ($HOCH_2CH_2SH$). Radiolysis of *i*-PrOH mainly led to solvated electrons and alkyl radicals, as shown in eq 1.[38,39] In N_2O-saturated solutions, e^-_{sol} was transformed into the $HO^\bullet$ radical (eq 2). Hydrogen abstraction from *i*-PrOH by $HO^\bullet$ produced $(CH_3)_2C(^\bullet)OH$ (eq 3, $k_3 = 1.9 \times 10^9$ M^{-1} s^{-1}).[40] The alkyl radicals in turn reacted with the thiol to give the corresponding thiyl radical (eqs 4 and 5, $k_4 = 5.1 \times 10^8$ M^{-1} s^{-1}, $k_5 = 7.4 \times 10^7$ M^{-1} s^{-1}).[40]

$$(CH_3)_2CHOH \rightsquigarrow e^-_{sol} \quad (CH_3)_2C(^\bullet)OH \quad H_3C^\bullet \qquad (1)$$

$$e^-_{sol} + N_2O \xrightarrow{H^+} N_2 + HO^\bullet \qquad (2)$$

$$(CH_3)_2CHOH + HO^\bullet \rightarrow (CH_3)_2C(^\bullet)OH + H_2O \qquad (3)$$

$$(CH_3)_2C(^\bullet)OH + RSH \rightleftharpoons (CH_3)_2CHOH + RS^\bullet \qquad (4)$$

$$H_3C^\bullet + RSH \rightarrow CH_4 + RS^\bullet \qquad (5)$$

Thiyl radicals were also generated by means of direct photolysis of 2-mercaptoethanol in *i*-PrOH solutions.[43] The solutions were flushed with argon prior to irradiation. In this condition, the generation of thiyl radicals and H atoms occurred as shown in eq 6, and the H atoms were efficiently quenched by the solvent (eq 7, $k_7 = 7.4 \times 10^7$ M^{-1} s^{-1}).[41] The $(CH_3)_2C(^\bullet)OH$ radical in turn reacted with the thiol to give the thiyl radical (eq 4).

$$RSH \xrightarrow{h\nu} RS^\bullet + H^\bullet \qquad (6)$$

$$(CH_3)_2CHOH + H^\bullet \rightarrow (CH_3)_2C(^\bullet)OH + H_2 \qquad (7)$$

It is worth emphasizing that reaction 4 is reversible.[29b,44] Forward and reverse rate constants for the reaction of

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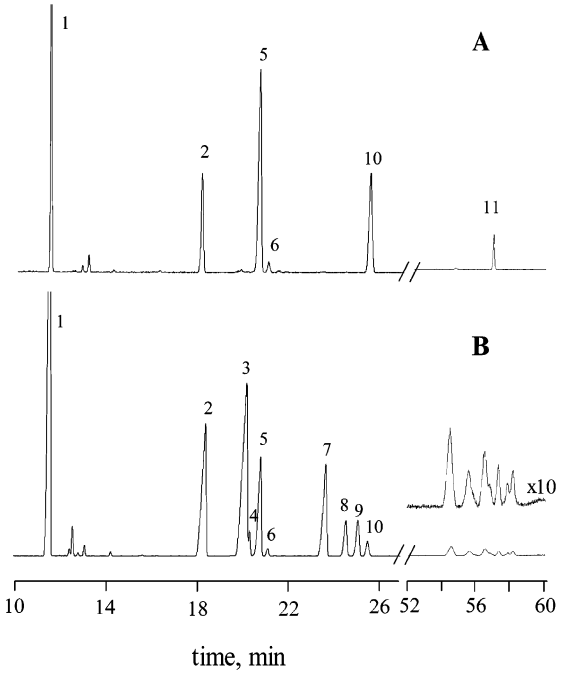


Figure 1. GC run using Rtx-2330 column: (A) starting fatty acid composition of egg lecithin obtained after transesterification and (B) fatty acid composition after 25 min of photolysis (5.5 W low-pressure mercury lamp) of 15 mM egg lecithin in the presence of 7 mM 2-mercaptoethanol at 22 °C. Peak labels: (1) methyl palmitate (16:0), (2) methyl stearate (18:0), (3) methyl elaidate (9t−18:1), (4) methyl trans vaccenate (11t−18:1), (5) methyl oleate (9c−18:1), (6) methyl vaccenate (11c−18:1), (7) methyl linolelaidate (9t,12t−18:2), (8) 9c,12t−18:2 methyl ester, (9) 9t,12c−18:2 methyl ester, (10) methyl linoleate (9c,12c−18:2), and (11) methyl arachidonate (5c,8c,11c,14c−20:4). Inset: The cluster of peaks related to arachidonate residue and its geometrical isomers was increased by a factor of 10 for a better resolution.

$(CH_3)_2C(^{\bullet})OH$ radical with penicillamine were $k_4 = 1.2 \times 10^8$ and $k_{-4} = 1.2 \times 10^4\ M^{-1}\ s^{-1}$, respectively, which corresponded to an equilibrium constant of $K = 1 \times 10^4$. Under our experimental conditions, i.e., [*i*-PrOH] = 13.06 M and [$HOCH_2CH_2SH$] = 0.007 M, the equilibrium was still shifted to the right but the forward reaction was only 5 to 6 times faster than the reverse reaction.

Isomerization of L-α-Phosphatidylcholines in *i*-PrOH. A first set of experiments dealing with cis−trans isomerization of double bonds in L-α-phosphatidylcholine from egg lecithin was carried out to find the best experimental conditions. Indeed, this material was relatively cheap and contained a substantial amount of arachidonate residues. Run A in Figure 1 shows the initial fatty acid composition obtained by transesterification[45] of L-α-phosphatidylcholine from egg lecithin followed by GC analysis by a 90% biscyanopropyl-10% phenylcyanopropyl polysiloxane capillary column (Rtx-2330): methyl palmitate (32%), methyl stearate (14.1%), methyl oleate (27%), methyl vaccenate (1.2%), methyl linoleate (20%), and methyl arachidonate (4.8%).

The reaction of thiyl radicals with egg lecithin was carried out by UV- or γ-irradiation in *i*-PrOH solutions.[46] A PC/chloroform solution (55 mg dissolved in ca. 3 mL) was evaporated in a test tube under an argon stream. The thin film formed was kept under vacuum for 30 min, and then a 4.3 mL *i*-PrOH was added to reach a concentration of about 15 mM fatty acid content. After saturation of the solution with N_2O, the $HOCH_2CH_2SH$ (0.030 mmol, corresponding to 7 mM) was added, and the solution was irradiated using a ^{60}Co-Gammacell (dose = 20 Gy/min) at (22 ± 2) °C. Alternatively, the solution was placed in a quartz photochemical reactor and flushed with argon for 20 min. Then, $HOCH_2CH_2SH$ (0.030 mmol, corresponding to 7 mM) was added and UV-irradiation with a 5.5 W low-pressure mercury lamp was carried out at (22 ± 2) °C temperature, maintained by means of a refrigerating solution which also cut the UV light below 240 nm.

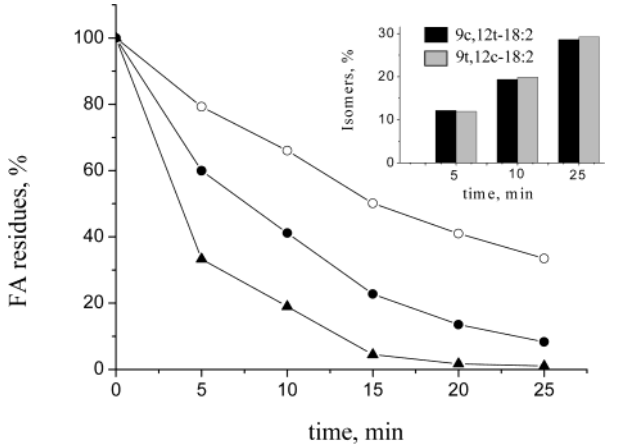


Figure 2. Time profiles of disappearance of cis fatty acid residues obtained by photolysis of PC from egg lecithin in *i*-PrOH (15 mM fatty acid contents) with 7 mM 2-mercaptoethanol at 22 °C. (○) Oleate (9c−18:1), (●) linoleate (9c,12c−18:2), and (▲) arachidonate (5c,8c,11c,14c−20:4). Inset: Time courses of the formation of two mono-trans isomers 9c,12t−18:2 and 9t,12c−18:2.

Aliquots (100 μL) of the reaction mixture were processed at different times for both set of experiments. After transesterification of the phospholipid,[45] the fatty acid composition was obtained by GC analysis. Run B in Figure 1 is relative to the photolysis experiment, and the excellent separation using Rtx-2330 column of all possible geometrical isomers of 18:1 and 18:2 components formed in the reaction can be appreciated. The retention times were compared with authentic samples which are commercially available. On the other hand, the cluster of peaks related to arachidonate residues isomerization was more complex (see inset of Figure 1B). The all-cis isomer eluted in the middle of the cluster and had an almost identical retention time with some mono-trans and di-trans isomers. Indeed, we reported that GC analysis using a cross-linked 5% phenylsilicone capillary column (HP-5) was preferable with respect to the quantification of arachidonate, since the all-cis isomer eluted as the first peak without overlapping, although these conditions were not useful to resolve each of the trans isomers (vide infra).[10] Taking the saturated fatty acid moieties of egg lecithin as the internal standards, the time courses of disappearance of cis mono- and polyunsaturated fatty acid residues were monitored, as reported in Figures 2 and 3, for UV- and γ-irradiated experiments, respectively. The content of each unsaturated residue is normalized to 100% for a better comparison.

It was gratifying to see that linoleate residue (having two double bonds) isomerized 2 times faster than oleate residue and arachidonate residue (having four double bonds) isomerized 2

(45) Kramer, J. F. K.; Fellner, V.; Dugan, M. E. R.; Sauer, F. D.; Mossoba M. M.; Yurawecz, M. P. *Lipids* **1997**, *32*, 1219−1228.
(46) It is known that phospholipids do not aggregate in this solvent. See: Barclay, L. R. C.; McNeil, J. M.; VanKessel, J.; Forrest, J. B.; Porter, N. A.; Lehman, L. S.; Smith, K. J.; Ellington, J. C., Jr. *J. Am. Chem. Soc.* **1984**, *106*, 6740−6747.

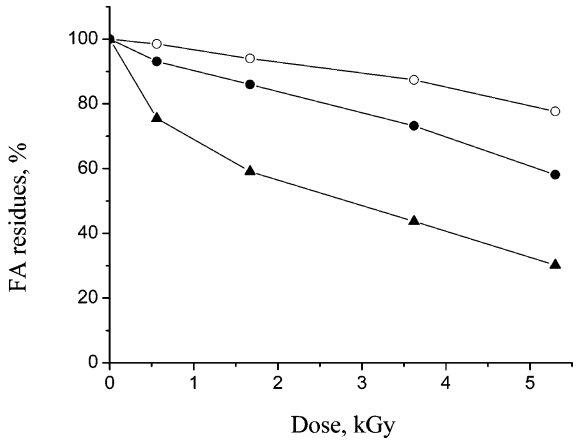
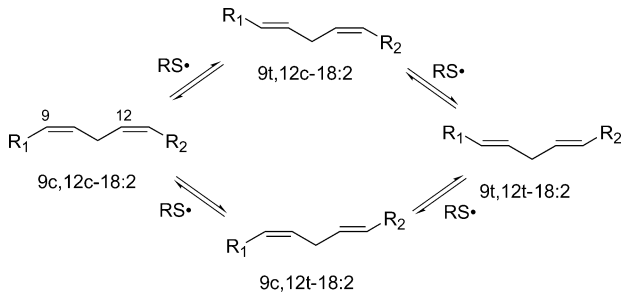


Figure 3. Dose dependence of the disappearance of cis fatty acid residues in the isomerization of PC from egg lecithin by γ-irradiation in *i*-PrOH (15 mM fatty acid contents) at 22 °C in the presence of 7 mM 2-mercaptoethanol. (○) Oleate (9c−18:1), (●) linoleate (9c,12c−18:2), and (▲) arachidonate (5c,8c,11c,14c−20:4).

Scheme 3



times faster than linoleate residue in the initial stage of the reaction. Therefore, all the cis double bonds isomerized with the same efficiency independently of their location.

Figures 2 and 3 show that the disappearance of oleate, linoleate, and arachidonate residues after 5 min of photolysis or 5.3 kGy irradiation was about in the same percentages. The efficiency of the photochemical method with respect to the γ-irradiation approach was probably due to the cleaner source and to higher concentrations of thiyl radicals.[47] It is worth noting that the reaction led to the geometrical isomers of the starting substrate, and the formation of conjugated dienes and thiol-arachidonate adducts did not occur at an appreciable extent.[48] The conversion of the initial material to the final mixture can be considered quantitative, since the overall yield of about 97% was found in all cases.

It is envisaged from the isomerization profile of mono-trans and di-trans isomers of 18:2 (not shown) that the initial disappearance of linoleate residues was replaced by two mono-trans isomers, which in turn were the precursors of the di-trans isomer, i.e., a step-by-step isomerization (Scheme 3). Furthermore, the two mono-trans isomers of linoleic acid residues were formed in the same amount (see inset of Figure 2), which confirmed the unimportance of the double bond location.

(47) In the photochemical method, the concentration of thiyl radicals is estimated to be 2 to 3 times higher than in γ-radiolysis, which is calculated to be $\sim 10^{-8}$ M.[11c]

(48) In our previous article on the isomerization of methyl arachidonate carried out by γ-irradiation, we used equimolar quantities (150 mM) of methyl ester and 2-mercaptoethanol. Under these conditions, after 5 kGy irradiation the all-cis isomer was halved, whereas the overall geometrical isomer yield was found to be ca. 75%. A fraction of ca. 20% yield was characterized as a mixture of thiol-methyl arachidonate adducts. To avoid the adduct formation, in this work we dropped the fatty acid contents of PC to 15 mM (10 times lower), which in terms of double bond molarity means a decrease of ca. 50 times. Furthermore, we decreased the concentration of thiol from 150 mM to 7 mM, i.e., ca. 20 times.

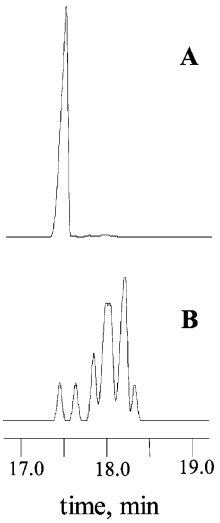


Figure 4. Partial GC runs using a HP-5 column. Peaks related to arachidonate residue and its geometrical trans isomers obtained (A) before and (B) after 25 min photolysis of SAPC in *i*-PrOH in the presence of 7 mM 2-mercaptoethanol at 22 °C.

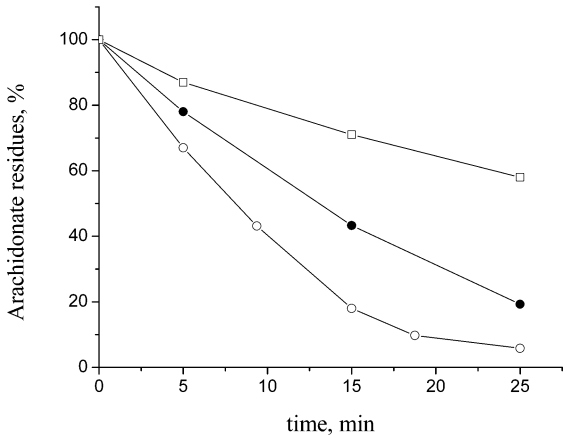


Figure 5. Time profile of disappearance of arachidonate residues obtained by $HOCH_2CH_2S^\bullet$ radical-catalyzed isomerization of SAPC using the photolytic method at 22 °C. (○) In Ar-saturated *i*-PrOH, (□) in *i*-PrOH with 1×10^{-4} M oxygen, and (●) in Ar-saturated vesicles.

The photolytic method has been chosen for the isomerization studies of SAPC. The phospholipid was dissolved in *i*-PrOH to reach a final fatty acid residue concentration of 15 mM, which corresponds to 7.5 mM of arachidonic residues. The solution was placed in the photochemical reactor and flushed with argon for 20 min prior to the addition of 7 mM $HOCH_2CH_2SH$. Aliquots (100 μL) of the irradiated solution were withdrawn and processed at different times. GC analysis was performed using a HP-5 column. Figure 4 shows the chromatograms where the all-cis isomer eluted as the first peak of the isomer group and almost disappeared after 25 min of photolysis, whereas Figure 5 (open circles) shows the time course of the disappearance of the all-cis isomer contained in SAPC, which was reduced up to a 6% yield within 25 min. The efficiency of this reaction allowed us to prepare SAPC lecithin containing up to 47% of fatty acids as the trans isomers in a 97% yield, after workup under alkaline conditions and purification by column chromatography.

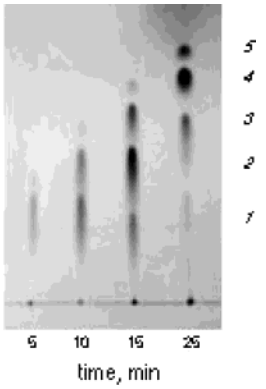


Figure 6. Ag/TLC monitoring of SAPC isomerization in solution by UV irradiation in the presence of 7 mM 2-mercaptoethanol. Spot labels: (1) all-cis isomer, (2) mono-trans isomers, (3) di-trans isomers, (4) tri-trans isomers, and (5) all-trans isomer, obtained by transesterification of SAPC reaction mixtures.

The arachidonate isomerization was also monitored by thin layer silica gel chromatography, by pretreating the plate with a silver ion solution (Ag/TLC), which is a well-known method to separate the trans isomers, based on the number of trans double bonds.[49]Ag/TLC monitoring of the reaction course, after transesterification of the aliquots, is shown in Figure 6, and it can be clearly seen that together with the all-cis isomer (spot 1), four new spots 2−5 were progressively formed, which corresponded to the mono-trans, di-trans, tri-trans, and all-trans isomers, respectively.[50]

Using these chromatographic conditions, we could isolate mono-trans isomers formed in the early stage of the SAPC isomerization in solution as the corresponding methyl esters and compare with those obtained by methyl arachidonate isomerization.[10] C-13 NMR spectroscopy, and in particular the resonances relative to the carbon atom in position 15, revealed that the four mono-trans isomers were formed in similar amounts (see below for a detailed NMR treatment). Therefore, in SAPC as well as in egg lecithin, the isomerization occurred by a step-by-step isomerization mechanism (cf. Scheme 3), in which the mono-trans isomers were the precursors of di-trans, and so on. An interesting feature of the lecithin isomerization protocol was that the total trans isomer content of the final product could vary by simply stopping the reaction at different times. Therefore, the thiyl radical-catalyzed isomerization represented an easy approach for inducing selective structural changes to natural phospholipids and for studying the differences in physical and aggregation properties, as already described for lecithins after partial hydrogenation.[51]

The reaction of polyunsaturated fatty acids with a variety of thiyl radicals has been studied by pulse radiolysis technique.[29] Kinetic data were obtained based on the buildup of pentadienyl-type radical formed by hydrogen abstraction by thiyl radicals. Rate constants of 3.1×10^7 and 6.8×10^7 M^{-1} s^{-1} have been reported for the reactions of $HOCH_2CH_2S^\bullet$ radicals with linoleic and arachidonic acids, respectively. On the basis of several assumptions, the authors proposed that ca. 50% of thiyl radicals abstracted the bisallylic hydrogen, whereas the remaining $RS^\bullet$ was added reversibly to the double bond to form the radical adduct. In the present work, the bisallylic hydrogen abstraction by $HOCH_2CH_2S^\bullet$ radical seemed to be unimportant, in the sense of product formation. To intercept pentadienyl radicals, the thiyl radical-catalyzed cis−trans isomerization of SAPC was carried out in *i*-PrOH saturated by 4% oxygen, which corresponded to ca. 1×10^{-4} M.[52] This amount of oxygen was 2 to 3 times higher than that of typical well-oxygenated tissues. Figure 5 (open squares) shows the time profile of the disappearance of the all-cis isomer in the presence of oxygen. In comparison with the deoxygenated solution experiment (open circles), the reaction was much slower, although the yield of all geometrical isomers after 25 min was 99%, i.e., there was no consumption of arachidonic moieties due to peroxidation. These findings are easily explained by assuming a smaller concentration of $RS^\bullet$ in the presence of molecular oxygen. Indeed, both radicals involved in equilibrium 4/−4 were added quickly to molecular oxygen with a similar rate constant ($k_8 \approx k_9 \approx 2 \times 10^9$ M^{-1} s^{-1}), with the thiyl radical addition being reversible (for $GS^\bullet$ radical in water $k_{-9} = 6.2 \times 10^5$ s^{-1}).[53] In fact, in the presence of 1×10^{-4} M oxygen, the reaction 4, which is also reversible, was only 4 times faster than reaction 8. In other words, the effectiveness of the cis−trans isomerization with or without oxygen is the same.[54]

$$(CH_3)_2C(^\bullet)OH + O_2 \rightarrow (CH_3)_2C(OO^\bullet)OH \qquad (8)$$

$$RS^\bullet + O_2 \rightleftharpoons RSOO^\bullet \qquad (9/-9)$$

Generation of Thiyl Radicals in Vesicles. In the heterogeneous system (vesicles), thiols can either be incorporated into the bilayer or be dissolved in the aqueous phase. The use of an amphiphilic thiol, such as 2-mercaptoethanol, eliminated the concern about the partition between hydrophobic and hydrophilic regions.[55] Indeed, in all experiments the thiol was added to the aqueous compartment immediately before starting the reaction. γ-Radiolysis of neutral water led to the transient species shown in eq 10.[38] The presence of N_2O efficiently transformed hydrated electrons into the $HO^\bullet$ radical (eq 11, $k_{11} = 9.1 \times 10^9$ M^{-1} s^{-1}). Hydrogen abstraction from 2-mercaptoethanol by $HO^\bullet$ and $H^\bullet$ directly produced thiyl radicals (eqs 12 and 13, $k_{12} = 6.8 \times 10^9$ M^{-1} s^{-1} and $k_{13} = 1.7 \times 10^9$ M^{-1} s^{-1}).[41] Generation of thiyl radicals by photolysis of thiol in degassed water was essentially the same process examined in alcoholic solution (eq 6).[43] The $H^\bullet$ atoms reacted directly with the thiol and produced other thiyl radicals (eq 13).

$$H_2O \rightsquigarrow e^-_{aq}, \quad HO^\bullet, \quad H^\bullet \qquad (10)$$

(49) Williams, C. M.; Mander, L. N. *Tetrahedron* **2001**, *57*, 425−447.
(50) Under these conditions, the saturated fatty acids (stearate moieties) are not evidenced. Stearic acid moieties could be detected by heating the plate sprayed with a 40% sulphuric acid aqueous solution.
(51) (a) Lang, J.; Vigo-Pelfrey, C.; Martin, F. *Chem. Phys. Lipids* **1990**, *53*, 91−101. (b) Wratten, M. L.; van Ginkel, G.; van't Veld, A. A.; Bekker, A.; van Faassen, E. E.; Sevanian, A. *Biochemistry* **1992**, *31*, 10901−10907. (c) Preston, M. R.; Walter, M. F.; Mason, P. E. *Free Radical Biol. Med.* **1997**, *23*, 419−425.
(52) The solubility of molecular oxygen in *t*-BuOH is determined to be 2.34 mM at room temperature (see: Cipollone, M.; di Palma, C.; Pedulli, G. F. *Appl. Magn. Reson.* **1992**, *3*, 98−102).
(53) Wardman, P. In *S-Centered Radicals*; Alfassi, Z. B., Ed.; Wiley: Chichester, U.K., 1999; pp 289−309.
(54) A mechanistic study that settles differences between the product studies and the pulse radiolysis results as well as the competition kinetics between isomerization and peroxidation processes is beyond the scope of this paper. Work is in progress to address properly the reaction mechanism.
(55) Newton, G. L.; Aguilera, J. A.; Kim, T.; Ward, J. F.; Fahey, R. C. *Radiat. Res.* **1996**, *146*, 206−215 and references therein.

$$e_{aq}^- + N_2O \xrightarrow{H^+} N_2 + HO^\bullet \quad (11)$$

$$HO^\bullet + RSH \rightarrow H_2O + RS^\bullet \quad (12)$$

$$H^\bullet + RSH \rightarrow H_2 + RS^\bullet \quad (13)$$

Isomerization of PC in LUVET. As far as the model membranes are concerned, LUVET made by the extrusion technique[56] was prepared using either SAPC (**2**) or L-α-phosphatidylcholine from egg lecithin. The choice of unilamellar vesicles was important in order to work with a model as close as possible to the membrane bilayer and to observe the effect of diffusion through two leaflets. The phospholipids were dissolved in chloroform, evaporated to a thin film in a test tube under an argon stream, and then kept under vacuum for 30 min. A degassed phosphate-buffered saline solution, pH 7.2, was added, and multilamellar vesicles (MLV) were formed by vortex stirring for 7 min under an argon atmosphere. From this suspension, LUVET was prepared by membrane extrusion with LiposoFast, using a 100-nm polycarbonate membrane filter.[57] The suspension was placed in a quartz reactor and flushed with argon for 20 min prior to UV photolysis. The choice of a small liposome diameter was also important for this experiment, since the suspension was nearly transparent. To the degassed suspension, 7 mM 2-mercaptoethanol was added. The reaction progress was monitored by extraction and transesterification of lipids, as already described.

First, we considered egg lecithin vesicles, and the disappearances of cis fatty acid residues are shown in Figure 7.[58] By comparing the egg lecithin experiments in solution and in LUVET (cf. Figures 2 and 7), the effect of a slower isomerization process in vesicles was evident, mainly due to a certain degree of opalescence that did not allow the passage of light as in a transparent solution. More importantly, it was possible to see that in vesicles the lipid supramolecular organization had a profound effect on the isomerization progress for the differentiation of the various double bonds. The isomerization rates of oleate and linoleate were more similar than in solution, where linoleate residues isomerized 2 times faster than oleate (cf. Figure 2). On the other hand, the arachidonate moiety disappearance was much faster than linoleate when it was compared with the solution. It appeared that thiyl radicals, initially formed in the aqueous compartment, first entered the hydrophobic region of the bilayer, reached, and isomerized the 5,6-double bond of arachidonate. Depending on the supramolecular arrangement of lipids in the vesicle, the 9,10-double bonds of oleate and linoleate residues were less available. This concept of "positional preference" could be further supported by the differentiation observed with the two double bonds in the linoleate moiety as well as with the four double bonds of arachidonate residues.

The inset of Figure 7 shows the time courses relative to the formation of the two mono-trans isomers of linoleate residues, namely 9t,12c−18:2 and 9c,12t−18:2 isomers. Indeed, the two isomers in vesicles were formed in different amounts, with the

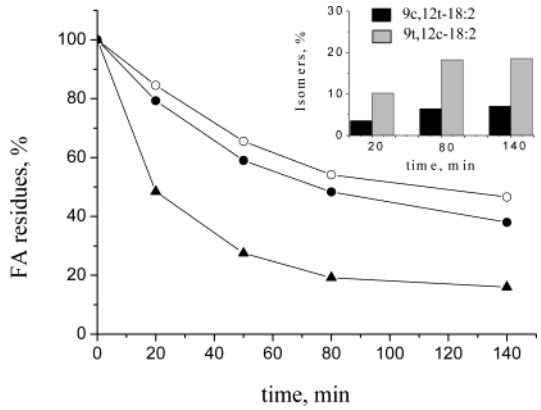


Figure 7. Time profiles of disappearance of cis fatty acid residues obtained by photolysis of PC from egg lecithin in LUVET (15 mM fatty acid contents) with 7 mM 2-mercaptoethanol at 22 °C. (○) Oleate (9c−18:1), (●) linoleate (9c,12c−18:2), and (▲) arachidonate (5c,8c,11c,14c−20:4). Inset: time course of the formation of two mono-trans isomers 9c,12t−18:2 and 9t,12c−18:2.

9t,12c−18:2 isomer being at least 3 times more abundant than 9c,12t−18:2 during the course of isomerization. The comparison of LUVET and solution experiments (see insets in Figures 2 and 7) provided compelling evidence for the influence of supramolecular structure on the regioselectivity of radical addition to fatty acid double bonds. In egg lecithin vesicles, the arachidonate moiety was difficult to be analyzed by ^{13}C NMR spectroscopy because it was only 4.8% of the total fatty acid content. Therefore, we proceeded with SAPC vesicles to investigate the "positional preference" of the four double bonds of arachidonic moiety.

SAPC vesicles were prepared as described for egg lecithin LUVET and the photolytic method was used. The time profile of disappearance of arachidonate residues in SAPC is shown in Figure 5 (solid circles). Figure 5 also shows that the isomerization rate was slightly slower than in solution. For example, after 25 min of photolysis the arachidonic residues were 6 and 20% in solution and in vesicles, respectively. This again can be attributed to the liposome suspension and to its turbidity. We stopped the reaction at an early stage to form prevalently mono-trans isomers. After workup and transesterification, the isomeric trend was analyzed by GC, and Figure 8 shows the profiles before and after 12 min of photolysis (profiles A and B, respectively). Moreover, to N_2O-saturated suspensions, 7 mM 2-mercaptoethanol was added prior to γ-irradiation.[58] Figure 8C shows the GC profile after 1 kGy of irradiation.

The comparison of the GC analysis with the isomeric trend obtained in solution (cf. Figure 4) evidenced that two geometrical isomers of arachidonate were formed preferentially in vesicles, in an overall yield of about 20%. The analysis by Ag/TLC, under the conditions described for SAPC in solution, showed that these isomers belonged to the mono-trans fraction. It has been recently reported that C15 resonances relative to the four mono-trans isomers obtained either from methyl arachidonate isomerization[10] or from ring opening of the corresponding mono-epoxides[28] were well separated and specifically assigned to each isomer.[59] The isomeric mono-trans

(56) *Liposomes: A Practical Approach*; New, R. R. C., Ed.; IRL Press: Oxford, U.K., 1990.
(57) The average diameter of the unilamellar vesicles was found to be ca. 90 nm. See: Fiorentini, D.; Cipollone, M.; Pugnaloni, A.; Biagini, G.; Landi, L. *Free Radical Res.* **1994**, *21*, 329−339.
(58) No isomerization was detected upon UV- or γ-irradiating samples without thiol.
(59) It is worth recalling that the significance of ^{13}C NMR for the identification of cis/trans isomers is well-documented.[60,61] Early work on the spectra of arachidonic acid methyl ester[60] and its mono-5-trans[24] and mono-14-trans isomers[25] evidenced that the ethylenic carbon atom resonance in position 15 is indeed meaningful for these isomers.

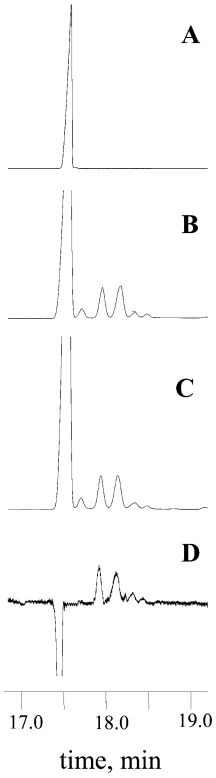


Figure 8. Partial GC runs using a HP-5 column. Peaks related to arachidonate residue and its geometrical trans isomers obtained from SAPC in LUVET in the presence of 7 mM 2-mercaptoethanol at 22 °C. (A) Before irradiation, (B) after 12 min of photolysis, and (C) after 1 kGy of γ-irradiation. (D) The resulting trace derived from subtracting the GC run of eicosenoic fatty acids present in erythrocyte membranes from the run obtained after γ-irradiation (20 kGy) of human blood in the presence of 2.8 μM of β-mercaptoethanol (cf. ref 10 for the individual runs).

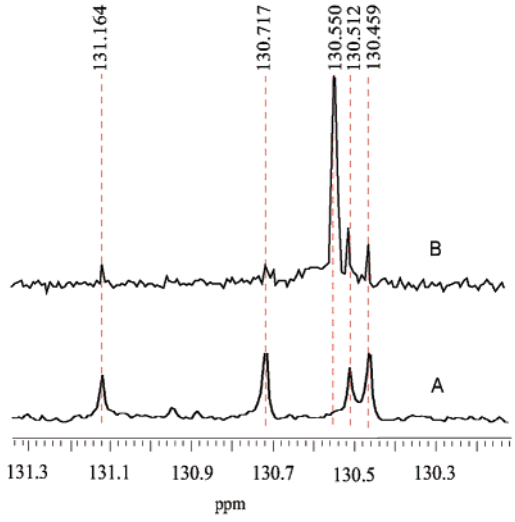


Figure 9. ^{13}C NMR spectral region relative to C15 resonance of all-cis and mono-trans arachidonate isomers. (A) The four mono-trans arachidonate isomers isolated by preparative Ag/TLC, after isomerization of SAPC in solution and (B) the all-cis (130.55 ppm) and mono-trans isomers obtained after isomerization and workup of SAPC in LUVET.

mixture derived from SAPC in solution was compared with the two mono-trans obtained in vesicles. Profile A in Figure 9 represents the ^{13}C NMR spectral region containing the resonances relative to the ethylenic carbon atom in the position 15, as detected from the mono-trans isomeric fraction isolated from the SAPC isomerization in solution, after workup and transesterification. From right to left, the resonances are assigned to mono-trans in positions 5, 8, 11, and 14, respectively, as previously reported in the isomerization of methyl arachidonate.[10]

Profile B in Figure 9 is the spectrum obtained from the reaction mixture of SAPC in LUVET, after workup and transesterification. It was gratifying to see in profile B that the two isomers preferentially formed in the vesicle experiment matched very well with the mono-trans in positions 5 and 8.

On the basis of these results, the overall picture of the fatty acid isomerization in model membranes is proposed as follows: the radical species, entering the hydrophobic region of the membrane bilayer, starts to isomerize the double bonds nearest to the surfaces, and the transformation is driven by both the supramolecular arrangement of the hydrocarbon tails and the highly defined lateral diffusion,[62] which favor positions 5 or 8 of arachidonic acid moieties. Therefore, polyunsaturated fatty acids with the double bond positions closest to the membrane surfaces are the first to be involved in the configurational change due to a radical-catalyzed cis−trans isomerization. The "positional preference" based on the lipid organization is indeed more evident in the case of fatty acid mixtures, such as oleate, linoleate, and arachidonate residues of egg lecithin, since the double bonds in positions 9 and 12 are located deeper in the bilayer and, although present in high percentage, they are isomerized with less efficiency compared to arachidonic moieties. Whether this positional preference could be detected in a radical isomerization process occurring in vivo is an intriguing question. Recently, we reported the analysis of fatty acid residues of erythrocyte membranes before and after γ-irradiation (20 kGy) in the presence of 2.8 μM β-mercaptoethanol.[10] In particular, the eicosenoic fatty acid residues of membrane phospholipids were found to be meaningful for the cis−trans isomerization. By subtracting the GC of the starting membrane lipids from the GC of the γ-irradiated sample, the trace shown in Figure 8D can be obtained. It is gratifying to see that the composition of the new peaks is similar to that obtained in model vesicles (cf. Figures 8B and C).

These findings suggest for the first time that the endogenous formation of trans isomers can be distinguished from exogenous supplementation of altered (trans) fats by a careful monitoring of geometrical isomers of arachidonate moieties present in cell membranes.[63] As described in the Introduction, exogenous arachidonate isomers contain trans double bonds in positions 11 and 14, whereas we have demonstrated that in model membranes, endogenous isomers, derived from thiyl radical attack, contain trans double bonds in positions 5 and 8. The feasibility of radical-based transformation of arachidonic acid points out the need for further investigations related to biological mechanisms and enzyme bindings,[64] which could be influenced by this structural change.

(60) Gunstone, F. D.; Pollard, M. R.; Scrimgeour, C. M.; Vadanayagam, H. S. *Chem. Phys. Lipids* **1977**, *18*, 115−129.
(61) Bus, J.; Sies, I.; Lien Ken Jie, M. S. F. *Chem. Phys. Lipids* **1977**, *18*, 130−144.
(62) Zachowski, A. *Biochem. J.* **1993**, *294*, 1−14.
(63) Evidence that cis−trans isomerization of unsaturated fatty acids can occur endogenously both in membrane phospholipids and fat depots has recently been gathered. Indeed, rats fed a diet completely deprived of trans fatty acids showed trans isomers of 18:1, 18:2, and 20:4 in different percentages depending on the kinds of tissue extracts (manuscript in preparation).
(64) (a) Malkowski, M. G.; Ginell, S. L.; Smith, W. L.; Garavito, R. M. *Science* **2000**, *289*, 1933−1937. (b) Peng, S.; Okeley, N. M.; Tsai, A.-L.; Wu, G.; Kulmacz, R. J.; van der Donk, W. A. *J. Am. Chem. Soc.* **2002**, *124*, 10785−10796.

Conclusions

The thiyl radical-catalyzed cis−trans isomerization of double bonds in homogeneous solutions is a flexible methodology, which leads to lecithins containing trans fatty acid moieties in good yields. This result is relevant for application in lipidomics and for studying new phospholipid interaction and structures due to a conformational change.[65] Another aspect of phospholipid reactivity has been evidenced which is correlated to the biochemical field. We have shown that in unilamellar vesicles, the mode of thiyl radical diffusion through the lipid assembly and the lipid supramolecular organization drive the preferential isomerization of PUFA double bonds in the closest proximity of the bilayer borders.

The regioselective cis−trans isomerization of arachidonate residues in membranes has a biochemical and medical relevance, involving the monitoring of lipids in biological samples and trans fatty acid recognition in diseases.[66] Arachidonate residues emerge as the most representative components of cell membranes in order to determine the origin of trans fatty acids in human tissues, and this analysis can be easily performed by using a blood sample and isolating erythrocyte membranes since they are rich in arachidonic acid moieties.[67]

Experimental Section

Materials. $HOCH_2CH_2SH$, L-α-phosphatidylcholine-1-stearoyl-2-arachidonoyl (SAPC) and L-α-phosphatidylcholine from egg lecithin, and all reference fatty acid methyl esters (methyl oleate, methyl elaidate, methyl linoleate, linoleic acid methyl ester isomer mix, linolelaidic acid methyl ester, conjugated octadecadienoic acid methyl esters, methyl palmitate, and methyl stearate) were commercially available from Aldrich, Fluka, or Sigma and used without further purification. Chloroform, methanol, *n*-hexane, and *i*-PrOH were purchased from Merck (HPLC grade) and used without further purification. Phosphate saline buffer (PBS) was prepared (Na_2HPO_4 10 mM, NaCl 0.14 M) at pH 7.2.

General Methods. GC analyses for the determination of the isomeric ratio of the unsaturated fatty acids were performed after transesterification of lecithins, as further described, and run by two different protocols. The first protocol has taken into account the reported GC conditions for lipid analyses.[68] We used a Varian CP-3800 gas chromatograph equipped with a flame ionization detector and a Rtx-2330 column (90% biscyanopropyl−10% phenylcyanopropyl polysiloxane capillary column; 60 m, 0.25 mm i.d., 0.20-mm film thickness). Temperature started from 160 °C held for 55 min, followed by an increase of 5 °C/min up to 195 °C, held for 10 min, followed by a second increase of 10 °C/min up to 250 °C. A constant pressure mode (29 psi) was chosen. For the analysis of arachidonic acid isomers, the second GC protocol was performed on an Agilent 5860 gas chromatograph, equipped with a flame ionization detector and a HP-5 column (cross-linked 5% phenylsilicone capillary column; 30 m, 0.25 mm i.d., 0.25-mm film thickness). Temperature started from 150 °C, held for 1 min, followed by an increase of 8 °C/min up to 250 °C, and held for 5 min. A constant pressure mode (13 psi) was chosen. Methyl esters were identified by comparison with the retention times of authentic samples (trans isomers were identified by comparison with authentic samples, which were commercially available or obtained as previously described).[8,9,11] When necessary, GC/MS spectra were recorded on a Hewlett-Packard GC 5890 (series II) coupled to a Hewlett-Packard mass selective detector model 5971A.

Continuous radiolyses were performed at room temperature (22 ± 2 °C) using a ^{60}Co-Gammacell at different dose rates. The exact absorbed radiation dose was determined with the Fricke chemical dosimeter by taking $G(Fe^{3+}) = 1.61$ μmol J^{-1}.[69]

Photolysis was carried out in a quartz photochemical reactor (Sigma Aldrich) equipped with a 5.5 W low-pressure mercury lamp. The temperature was maintained at (22 ± 1) °C by means of a thermostat bath, composed of $NiSO_4 \cdot 7H_2O$ and $CoSO_4 \cdot 7H_2O$ at pH 1, which allows the UV light (240−350 nm) to pass through.[70]

Chromatography of lecithins was performed on Florisil (TLC grade, Aldrich) by using the eluents specified in the protocols, under an argon stream to avoid lipid degradation. Silica gel thin-layer chromatography (analytical and preparative) was performed on Merck silica gel 60 plates (0.25 and 2 mm thickness, respectively). For the reaction monitoring and separation of cis and trans isomers, argentation of silica gel plates was done by a reported procedure.[49] The isomers can be eluted on the Ag/TLC plates after transesterification of lecithins, as the corresponding methyl esters. In particular, arachidonate isomer fractions were monitored and separated by preparative Ag/TLC eluting three times with 7:3 (v/v) *n*-hexane/diethyl ether. The isomer fractions were detected by spraying the plate with cerium ammonium sulfate/ammonium molybdate reagent.[50] When preparative Ag/TLC plates were used, isomer fractions were detected by spraying a small portion of the plate with the above indicated reagent. For the isolation of the isomer fractions, the bands were then scraped from the plates, and silica gel was repeatedly washed with 2:1 (v/v) chloroform/methanol. Filtrates were evaporated, and in all cases white powders were obtained which were insoluble in *n*-hexane and consisted of the Ag−fatty acid methyl esters complexes. The complexes were dissolved in diluted aq NH_4OH solution and extracted with *n*-hexane. A quantitative recovery of the methyl esters was obtained from the lecithin isomerizations. Lecithin GC analyses for cis and trans isomer content were performed after transesterification with 0.5 M KOH/MeOH for 10 min at room temperature,[45] thus obtaining the corresponding fatty acid methyl esters. ^{13}C NMR spectra were recorded on a Varian VXR 400 MHz instrument using $CDCl_3$ as the solvent and the reference peak at 77.0 ppm.

Isomerization of L-α-Phosphatidylcholine from Egg Lecithin in *i*-PrOH by UV Photolysis or γ-Radiolysis. A solution of egg lecithin (55 mg; ca. 0.072 mmol) in *i*-PrOH (4.3 mL) was placed in a quartz photochemical reactor and bubbled with argon for 20 min. Then, 2-mercaptoethanol (2.73 mg; 0.035 mmol) was added, and the solution was irradiated by low-pressure mercury lamp (5.5 W). Alternatively, the above-described solution containing 15 mM egg lecithin in *i*-PrOH was saturated with N_2O, and 7 mM 2-mercaptoethanol was added prior to γ-irradiation. To follow the formation of trans fatty acid residues, 100 μL aliquots were withdrawn at different times. The aliquots were processed by partitioning between *n*-hexane (or 2:1 chloroform/methanol in case of vesicles) and brine, extracting and collecting the organic phases dried over anhydrous sodium sulfate, and evaporating the solvent under vacuum at room temperature. The residue containing the phospholipids was treated with 0.5 M KOH/MeOH for 10 min at ambient temperature and then poured into the brine and extracted with *n*-hexane. The organic layer containing the corresponding fatty acid methyl esters was analyzed by GC in comparison with the retention times of authentic samples. Isolation of the isomerized egg lecithin was done as described in the following experiment with SAPC.

Isomerization of 1-Stearoyl-2-arachidonoyl-L-α-phosphatidylcholine (SAPC) in *i*-PrOH by UV Photolysis. A chloroform solution of SAPC (5.5 mL of a 10 mg/mL chloroform solution corresponding to 55 mg; 0.068 mmol) was evaporated in a test tube and kept under

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vacuum for 20 min. Lecithin was dissolved in *i*-PrOH (4.5 mL), placed in a quartz photochemical reactor, and bubbled with argon for 20 min. Then, 2-mercaptoethanol (2.5 mg; 0.032 mmol) was added, and the solution was irradiated, following the above-reported procedure for egg lecithin. GC monitoring was effected in the conditions reported above. Also Ag/TLC monitoring was done as described in the general methods. Workup of the reaction can be done as follows: when the isomerization reaches the desired amount of trans fatty acid residues, the reaction can be stopped by transferring the reaction crude to a flask and evaporating the reaction solvent under vacuum. The crude reaction mixture was partitioned between 2:1 chloroform/methanol and aqueous NaOH (0.01 M) to eliminate the thiol. After washing the organic layer with brine and drying over anhydrous sodium sulfate, the solvent was evaporated, and the crude was dissolved in chloroform for purification on a Florisil column. After elution with a 80:20 chloroform/methanol mixture, the lecithin was collected in one fraction by elution with a (20:10:0.4:0.2) (chloroform/methanol/water: 25% aq NH_4OH) mixture. The purity of the material was checked by TLC on silica gel, using the above specified eluent ($R_f = 0.51$). The purified material corresponded to 53 mg of isomerized lecithin (0.066 mmol; 97% yield), which was analyzed by GC and Ag/TLC for the trans isomer content, as already described. The same experiment was repeated by flushing the reaction mixture with 4% O_2 in N_2 during the photolysis.

Isomerization of PC in LUVET. Lecithin (0.068 mmol) dissolved in chloroform was evaporated to a thin film in a test tube under an argon stream and under vacuum for 30 min. Degassed PBS (4.5 mL) was added, and multilamellar vesicles were formed by vortex stirring for 7 min under an argon atmosphere. To obtain LUVET, the lipid emulsion was transferred into a LiposoFast and extruded 19 times back and forth through two polycarbonate membranes with a pore diameter of 100 nm.[56,57] The suspension was then transferred to a vial equipped with an open-top screw cap and a Teflon-faced septum where it was saturated with N_2O prior to γ-irradiation, or it was transferred to the photochemical reactor and saturated with argon prior to photolysis. 2-Mercaptoethanol (0.032 mmol) was added to the suspension and irradiation started. Aliquots of 100 μL were withdrawn and processed at different times as already described. GC analyses and NMR spectra are reported in the figures.

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