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Rate Constants for Peroxidation of Polyunsaturated Fatty Acids and Sterols in Solution and in Liposomes

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Abstract: Rate constants for autoxidation propagation of several unsaturated lipids in benzene solution at 37 °C and in phosphatidylcholine liposomes were determined by a linoleate radical clock. This radical clock is based on competition between hydrogen atom abstraction by an intermediate peroxyl radical derived from linoleic acid that leads to a *trans,cis*-conjugated hydroxyoctadecadienoic product and β-fragmentation of the same peroxyl that gives the *trans,trans*-product hydroxyoctadecadienoic acid. Rate constants determined by this approach in solution relative to linoleic acid ($k_p = 62\ M^{-1}\ s^{-1}$) were: arachidonic acid ($k_p = 197 \pm 13\ M^{-1}\ s^{-1}$), eicosapentaenoic acid ($k_p = 249 \pm 16\ M^{-1}\ s^{-1}$), docosahexaenoic acid ($k_p = 334 \pm 37\ M^{-1}\ s^{-1}$), cholesterol ($k_p = 11 \pm 2\ M^{-1}\ s^{-1}$), and 7-dehydrocholesterol ($k_p = 2260 \pm 40\ M^{-1}\ s^{-1}$). Free radical oxidations of multilamellar and unilamellar liposomes of various mixtures of glycerophosphatidylcholine molecular species were also carried out. In some experiments, cholesterol or 7-dehydrocholesterol was incorporated into the lipid mixture undergoing oxidation. A phosphatidylcholine bearing a linoleate ester at *sn-2* was a component of each liposome peroxidation reaction and the ratio of *trans,cis/trans,trans* (*t,c/t,t*)-conjugated diene oxidation products formed from this phospholipid was determined for each oxidation reaction. This *t,c/t,t*-product ratio from linoleate was used to "clock" liposome constituents as hydrogen atom donors in the lipid bilayer. Application of this lipid bilayer radical clock gives relative autoxidation propagation rate constants of arachidonate (20:4), eicosapentaenoate (20:5), docosahexaenoate (22:6), and 7-dehydrocholesterol to be 115 ± 7, 145 ± 8, 172 ± 13, and 832 ± 86, respectively, a reactivity trend that parallels the one in solution. We also conclude from the liposome oxidations that linoleate peroxyl radicals at different positions on the eighteen-carbon chain (at C-9 and C-13) have different kinetic properties. This is in contrast to the results of solution oxidations of linoleate in which the C-9 and C-13 peroxyl radicals have similar reactivities. We suggest that peroxyl radical β-scission depends on solvent polarity and the polarity of the local environment of peroxyl radicals in liposomal oxidations depends on the position of the peroxyl radical on the 18-carbon chain.

Introduction

Autoxidation of polyunsaturated fatty acids and esters (PUFAs) and sterols, a process known as lipid peroxidation, has attracted research attention over the last few decades due to its involvement in the pathophysiology of common diseases like atherosclerosis,[1,2] asthma,[3,4] neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease,[5−8] and metabolic disorders such as the Smith−Lemli−Opitz syndrome (SLOS).[9−12] PUFAs are important membrane constituents and the highly unsaturated members of the group of linoleic (18:2, $\omega-6$), arachidonic (20:4, $\omega-6$), eicosapentaenoic (20:5, $\omega-3$) and docosahexaenoic (22:6, $\omega-3$) are extremely prone to reaction with molecular oxygen. Cholesterol free radical oxidation has been studied in great detail and some of its peroxidation products have potent biological activities.[13−15] Cholesterol is also an important constituent of biological membranes and 7-dehydrocholesterol (7-DHC) is a biosynthetic precursor of cholesterol as well as vitamin D_3.[16−18] 7-DHC is present in

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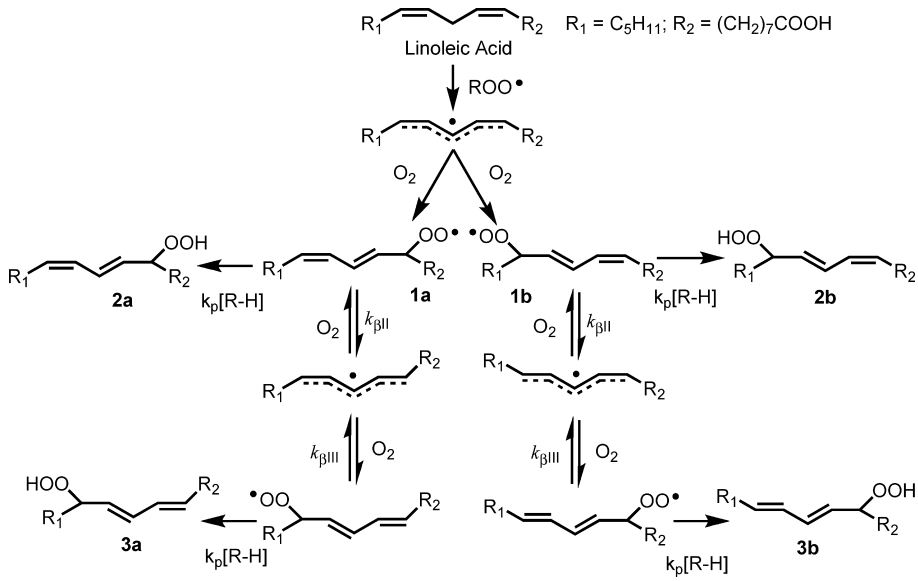
(1) Esterbauer, H.; Gebicki, J.; Puhl, H.; Jurgens, G. *Free Radical Biol. Med.* **1992**, *13*, 341–390.
(2) Berliner, J. A.; Heinecke, J. W. *Free Radical Biol. Med.* **1996**, *20*, 707–727.
(3) Antczak, A.; Nowak, D.; Shariati, B.; Krol, M.; Piasecka, G.; Kurmanowska, Z. *Eur. Respir. J.* **1997**, *10*, 1235–1241.
(4) Montuschi, P.; Corradi, M.; Ciabattoni, G.; Nightingale, J.; Kharitonov, S. A.; Barnes, P. J. *Am. J. Respir. Crit. Care. Med.* **1999**, *160*, 216–220.
(5) Simonian, N. A.; Coyle, J. T. *Annu. Rev. Pharmacol. Toxicol.* **1996**, *36*, 83–106.
(6) Sayre, L. M.; Zelasko, D. A.; Harris, P. L. R.; Perry, G.; Salomon, R. G.; Smith, M. A. *J. Neurochem.* **1997**, *68*, 2092–2097.
(7) Fahn, S.; Cohen, G. *Ann. Neurol.* **1992**, *32*, 804–812.
(8) Yoritaka, A.; Hattori, N.; Uchida, K.; Tanaka, M.; Stadtman, E. R.; Mizuno, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2696–2701.
(9) Gaoua, W.; Chevy, F.; Roux, C.; Wolf, C. *J. Lipid. Res.* **1999**, *40*, 456–463.
(10) Richards, M. J.; Nagel, B. A.; Fliesler, S. J. *Exp. Eye. Res.* **2006**, *82*, 538–541.
(11) Valencia, A.; Rajadurai, A.; Carle, A. B.; Kochevar, I. E. *Free Radical Biol. Med.* **2006**, *41*, 1704–1718.
(12) Valencia, A.; Kochevar, I. E. *Free Radical Biol. Med.* **2006**, *40*, 641–650.
(13) Smith, L. L. *Cholesterol Autoxidation*; Plenum Press: New York, 1981.
(14) Murphy, R. C.; Johnson, K. M. *J. Biol. Chem.* **2008**, *283*, 15521–15525.
(15) Girotti, A. W. *J. Lipid Res.* **1998**, *39*, 1529–1542.

Scheme 1



relatively high concentrations in skin where it is exposed to exogenous radical sources and oxygen, and it is of additional interest because of syndromes in which high levels of this sterol are associated with severe developmental defects, SLOS being one example. Oxidative stress and peroxidation have been associated with SLOS in several studies and the oxidative instability of 7-DHC has been implied in more than one instance.[11,12]

The slow step in free radical chain oxidation is the step in which a hydrogen atom is transferred from an organic reactant, R−H, to an intermediate peroxyl radical, R−OO•. The overall rate of oxidation depends directly on the rate constant for this propagation step, k_p, the concentration [R−H], and the rate constants for radical initiation and radical−radical termination. We have recently made use of a peroxyl radical clock to determine propagation rate constants for organic compounds undergoing free radical chain oxidation. The radical clock is based on the unimolecular β-fragmentation rate constants of peroxyl radicals derived from linoleic acid (Scheme 1) and these clocks have been applied in the measurement of a number of propagation rate constants for autoxidation in solution.[19] Two *trans*,*cis*-conjugated diene peroxyl radicals, **1a** and**1b**, are formed in linoleate oxidation and trapping of these radicals by hydrogen atom transfer with a rate of k_p[R−H][ROO•] gives rise to *trans*,*cis*-conjugated diene oxidation products, **2a** and **2b**.[20−23] β-Fragmentation of the peroxyl radicals **1a** and**1b**, leading to *trans*,*trans*-conjugated diene products, competes with hydrogen atom transfer and this competition can be used to "clock" the bimolecular atom-transfer reaction.[19]

Based upon the mechanism shown in Scheme 1, the ratio of *trans*,*cis/trans*,*trans*-products from linoleate, **[(2a + 2b)/(3a +3b)]**, can be related to the concentrations and rate constants of all hydrogen atom donors present in the reaction mixture. Experiments to determine important fragmentation rate constants and oxygen partitioning parameters for processes shown in Scheme 1 have been carried out for reactions in benzene at 37 °C and this permits the formulation of a simple kinetic expression for peroxidation under these standard conditions, relating the *trans,cis/trans,trans*-product ratio and kinetic rate constants, as shown in eq 1.[19,24] The boundary limit of eq 1 as k_p^i[R_i − H]approaches zero corresponds to oxidation conditions in which poor H-atom donors are present or good donors are present at very low concentrations. Under these conditions, the *trans*,*cis/trans*,*trans* product ratio approaches 0.16 and the reaction is effectively under thermodynamic control. Under conditions in which good H-atom donors such as α-tocopherol are present, the reaction is under kinetic control and the *trans,cis* isomers dominate the product mixture.

$$\frac{(\mathbf{2a} + \mathbf{2b})}{(\mathbf{3a} + \mathbf{3b})} = \frac{trans, cis}{trans, trans} = \sum_{i=1-n} \frac{k_p^i[R_i - H]}{214\ s^{-1}} + 0.16 \quad (1)$$

Peroxidation of phospholipids in lipid bilayers follows the same kinetic rate law as in homogeneous systems.[25−31] However, generally applicable radical clocks in membranes have not been developed due to the complexity of autoxidation reactions in heterogeneous systems and the difficulty in defining necessary parameters. Barclay and co-workers determined k_{inh}

(16) Kresge, N.; Simoni, R. D.; Hill, R. L. *J. Biol. Chem.* **2005**, *280*, e7.
(17) Javitt, N. B. *Steroids* **2008**, *73*, 149–57.
(18) Herman, G. E. *Hum. Mol. Genet.* **2003**, *12* (Spec No 1), R75–88.
(19) Roschek, B.; Tallman, K. A.; Rector, C. L.; Gillmore, J. G.; Pratt, D. A.; Punta, C.; Porter, N. A. *J. Org. Chem.* **2006**, *71*, 3527–3532.
(20) Porter, N. A.; Weber, B. A.; Weenen, H.; Khan, J. A. *J. Am. Chem. Soc.* **1980**, *102*, 5597–5601.
(21) Porter, N. A.; Lehman, L. S.; Weber, B. A.; Smith, K. J. *J. Am. Chem. Soc.* **1981**, *103*, 6447–6455.
(22) Tallman, K. A.; Roschek, B.; Porter, N. A. *J. Am. Chem. Soc.* **2004**, *126*, 9240–9247.
(23) Pajunen, T. I.; Johansson, M. P.; Hase, T.; Hopia, A. *Lipids* **2008**, *43*, 599–610.
(24) Davis, T. A.; Gao, L.; Yin, H.; Morrow, J.; Porter, N. A. *J. Am. Chem. Soc.* **2006**, *128*, 14897–14904.
(25) Antunes, F.; Barclay, L. R. C.; Vinqvist, M. R.; Pinto, R. E. *Int. J. Chem. Kinet.* **1998**, *30*, 753–767.
(26) Barclay, L. R. C.; Baskin, K. A.; Kong, D.; Locke, S. J. *Can. J. Chem.* **1987**, *65*, 2541–2550.
(27) Barclay, L. R. C. *Can. J. Chem.* **1993**, *71*, 1–16.
(28) Barclay, L. R. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1980**, *102*, 7792–7794.
(29) Barclay, L. R. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6478–6485.
(30) Barclay, L. R. C.; Locke, S. J.; Macneil, J. M.; Vankessel, J.; Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1984**, *106*, 2479–2481.
(31) Barclay, L. R. C.; Vinqvist, M. R.; Antunes, F.; Pinto, R. E. *J. Am. Chem. Soc.* **1997**, *119*, 5764–5765.

Table 1. Autoxidation Propagation Rate Constants Determined by the Linoleic Radical Clock[a]

fatty acid or sterol, R−H	k_p in solution at 37 °C ($M^{-1}s^{-1}$)
linoleic (18:2)	62 (ref 34)
arachidonic (20:4)	197 ± 13
eicosapentaenoic (20:5)	249 ± 16
docosahexaenoic (22:6)	334 ± 37
cholesterol	11 ± 2
7-dehydrocholesterol	2260 ± 40

[a] errors are 2σ

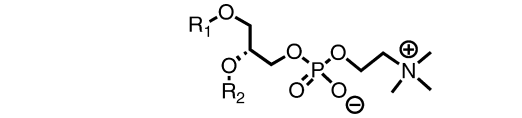
of α-tocopherol in model membranes by applying the linoleate peroxyl radical clock, but this approach has not been widely applied.[31,32]

We report here the propagation rate constants of PUFAs and sterols in solution and also an expanded study of the linoleate peroxyl radical clock in lipid bilayers such as those found in biomembranes with a goal being to predict and/or interpret peroxidation reactions *in vivo* in a more substantive way.[33] One of our aims is to develop a clocking equation for reactions in lipid bilayers where the ratio of *trans,cis/trans,trans*-products from linoleate is expressed as a function of k_p, the propagation rate constants of hydrogen atom donors and the bilayer mole fraction of those donors.

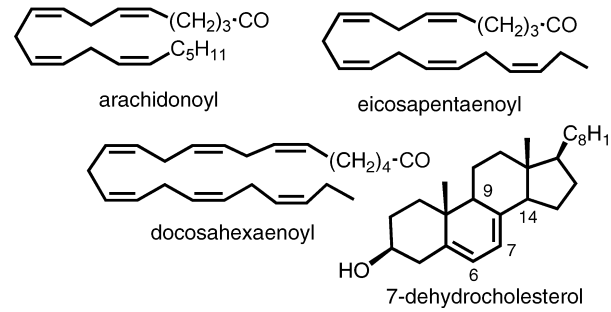
Results

Propagation Rate Constants for Autoxidation of Important Lipids in Solution. We first sought to determine propagation rates for the important fatty acids and sterols in homogeneous solution by the use of the linoleate clock.[19] The experiments require determining the *trans*,*cis/trans*,*trans*-product ratio from linoleate as a function of the concentration of other oxidizable substrates. In a typical experiment, 0.2 to 0.4 M solutions of linoleic acid or methyl linoleate in the presence of 0.1 to 0.6 M of the compound under study (e.g., arachidonic acid etc.) in benzene or chlorobenzene at 37 °C were oxidized for 30 to 90 min in reactions initiated by 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVN, < 0.5 mol %). Less than a percent of linoleate or substrate were consumed in a typical experiment. The linoleate *trans,cis/trans,trans*-product ratio was determined by HPLC/MS or HPLC/UV at various substrate concentrations and k_p for the substrate was then determined by the application of Equation 1 (Figure S1−S3, Supporting Information). Typically, duplicate analyses of triplicate experiments were carried out for six or seven concentrations of substrate between 0.1 and 0.6 M. Table 1 gives the propagation rate constants determined by this approach for five substrates. Note that all of the rate constants are based upon the propagation rate constant for autoxidation of linoleic acid, a rate constant that has been determined by the rotating sector method.[25,34] We find that rate constants determined using either linoleic acid or methyl linoleate are within experimental error under the conditions described.

Calibration of the Linoleate Radical Clock in Liposomes. Liposomes of multilamellar vesicles (MLV) from phosphatidyl cholines were the colloids studied in all experiments unless otherwise noted. Oxidation of unilamellar vesicles (ULV) gave similar results to experiments carried out in MLV (Table 2; also see Figure S4, Supporting Information). 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) was used as the linoleate oxidation substrate in all experiments and it was diluted by mixing with non- or poorly oxidizable PCs to determine the effect of linoleate mole fraction in the bilayer on the *trans*,*cis/trans*,*trans*-product ratio.[20,33] The peroxidation chain reactions were initiated with a catalytic amount of the water-soluble azo initiator 2,2′- azobis(2-(2-imidazolin-2-yl) propane) dihydrochloride (AIPH) at 37 °C and were quenched at an appropriate time to avoid overoxidation of linoleate (in all cases <5%). Conjugated diene products were analyzed by HPLC-UV after reduction with PPh_3 and hydrolysis of the phosphatidylcholine under basic conditions.



DPPC, R_1= $CO(CH_2)_{14}CH_3$; R_2= $CO(CH_2)_{14}CH_3$
DMPC, R_1= $CO(CH_2)_{12}CH_3$; R_2= $CO(CH_2)_{12}CH_3$
PLPC, R_1= $CO(CH_2)_{14}CH_3$; R_2= linoleoyl
POPC, R_1= $CO(CH_2)_{14}CH_3$; R_2= oleoyl
DOPC, R_1= oleoyl; R_2= oleoyl
PAPC, R_1= $CO(CH_2)_{14}CH_3$; R_2= arachidonoyl
PEPC, R_1= $CO(CH_2)_{14}CH_3$; R_2= eicosapentaenoyl
PDPC, R_1= $CO(CH_2)_{14}CH_3$; R_2= docosahexaenoyl



The *trans*,*cis/trans*,*trans*-product ratio, **[(2a + 2b)/(3a +3b)]**, for oxidation of PLPC liposomes is presented in Figure 1. Dilution experiments of PLPC with different nonoxidizable phospholipids are presented in the Figure in different colors. The data in the figure show that the ratio of *trans,cis/trans,trans*

$$\frac{(\mathbf{2a} + \mathbf{2b})}{(\mathbf{3a} + \mathbf{3b})} = \frac{trans,cis}{trans,trans} = a \sum_{i=1-n} k_p^i n_{R-H}^i + b \qquad (2)$$

products from linoleate depends on the mole fraction of linoleate in the lipid bilayer. In eq 2, n_{R-H}^i is the mole fraction of any H-atom donor in the liposome, k_p^i is the propagation rate constant for a given H-atom donor in the liposome,[35] b is a constant that is essentially the thermodynamic product ratio in a given lipid bilayer and a is a constant having units of time. Equation 2 is based on the analogy with eq 1, and the assumption that it is the bilayer concentration of H-atom donors and not the solution molarity that effectively controls the linoleate product ratio. The bilayer mole fraction can, of course, be converted to a bilayer concentration by making assumptions about bilayer density and lipid average molecular weight, but for purposes of our analysis the mole fraction analysis seems more straightforward and just as instructive. Statistical analyses suggest that the various series of data shown in Figure 1 are not significantly

(32) Culbertson, S. M.; Antunes, F.; Havrilla, C. M.; Milne, G. L.; Porter, N. A. *Chem. Res. Toxicol.* **2002**, *15*, 870–876.
(33) Weenen, H.; Porter, N. A. *J. Am. Chem. Soc.* **1982**, *104*, 5216–5221.
(34) Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1967**, *45*, 793–802.

(35) Mole fraction is unitless but we define a unit n for calculation convenience. Therefore, the unit of the propagation rate constant (k_p^i) in mole fraction will be $n^{-1}s^{-1}$, while the unit of the pseudo-first order rate constant ($k_p^i\, n_{R\text{-}H}^i$) will be s^{-1}.

Table 2. Analysis of Linoleate Product Ratio **2a/3b** or **2b/3a** = $a\,\Sigma k_p n_{R\text{-}H} + b$ (eq 2) for Liposomal Oxidations and Co-Oxidations[a]

R−H	series[b]	slope	intercept	Δ_{slope}	$\Delta_{intercept}$	$k_p(n^{-1}s^{-1})$ [d]
PLPC	1	0.76 ± 0.06	0.34 ± 0.02	0.12 ± 0.13	0.09 ± 0.04	35 (ref 25)
MLVs	2	0.64 ± 0.07	0.25 ± 0.02			
PLPC	1	0.79 ± 0.11	0.40 ± 0.04	0.03 ± 0.20	0.10 ± 0.07	
ULVs	2	0.76 ± 0.09	0.30 ± 0.03			
PLPC	1	2.79 ± 0.22	0.40 ± 0.05	0.65 ± 0.39	0.13 ± 0.09	115 ± 7
PAPC (20:4)	2	2.14 ± 0.17	0.27 ± 0.04			
PLPC	1	3.47 ± 0.30	0.36 ± 0.07	0.54 ± 0.57	0.10 ± 0.13	145 ± 8
PEPC (20:5)	2	2.93 ± 0.27	0.26 ± 0.06			
PLPC	1	4.04 ± 0.43	0.39 ± 0.09	0.65 ± 1.01	0.07 ± 0.21	172 ± 13
PDPC (22:6)	2	3.39 ± 0.58	0.32 ± 0.12			
PLPC	1	28 ± 3	0.59 ± 0.14	18 ± 6	0.06 ± 0.29	832 ± 86
7-DHC	2	10 ± 3	0.53 ± 0.15			
MeLin	1	0.27 ± 0.02	0.17 ± 0.01	0.02 ± 0.03	0 ± 0.02	
in solution [c]	2	0.25 ± 0.01	0.17 ± 0.01			

[a] Errors are 2σ. [b] Series 1: **2a/3a**, series 2: **2b/3b**. [c] Data for experiments in solution are presented in molar units, not mole fraction. [d] Mole fraction propagation rate constants obtained from (**2a** + **2b**)/(**3a** +**3b**) vs $n_{R\text{-}H}$ (see Figure 2).

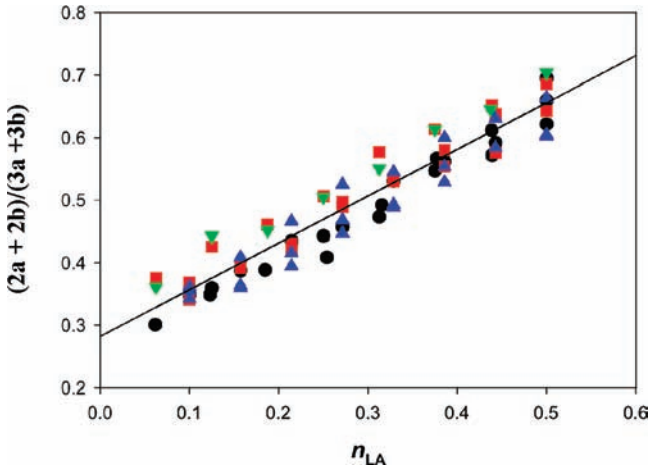


Figure 1. Values **[(2a + 2b)/(3a + 3b)]** from oxidation of mixtures of PLPC and nonoxidizable PCs in lipid bilayers at 37 °C vs linoleate mole fraction in the bilayer. Different mixtures are shown in different colors: PLPC/POPC (black), PLPC/DMPC (red), PLPC/DOPC (blue), and PLPC/POPC/DPPC (green). The line shows the fit all of the data in the Figure to eq 2.

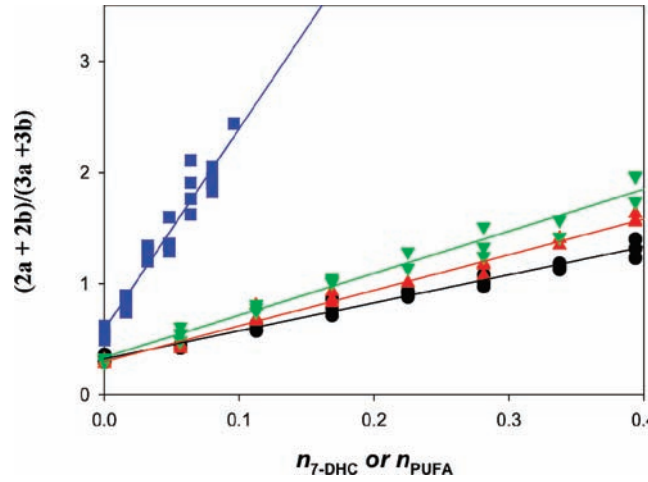


Figure 2. Values of **[(2a + 2b)/(3a + 3b)]** from oxidation of mixtures of PLPC/POPC and oxidizable PCs in lipid bilayers at 37 °C vs oxidizable lipid mole fraction in the bilayer. Different mixtures are shown in different colors: PLPC/PAPC (black), PLPC/PEPC (red), PLPC/PDPC (green), and PLPC/7-DHC (blue). The lines show the fit to eq 2. POPC was used as a nonoxidizable bilayer diluent.

different ($p > 0.05$). Therefore, all data in Figure 1 were fit to eq 2, which gave $a = 0.022 \pm 0.002$ s and $b = 0.28 \pm 0.02$.[25]

Co-Oxidation of Linoleate Liposomes with Other Oxidizable Substrates. To obtain the propagation rate constants of other oxidizable lipids compared to linoleate, liposomes containing a fixed *mole fraction* of PLPC and variable amounts of other oxidizable phospholipid and sterol substrates were oxidized at 37 °C in reactions initiated with the water-soluble initiator AIPH. Phospholipid mixtures generally contained $n_{LA} = 0.1-0.4$ and varaiable amounts of another oxidizable phospholipid, for example, PAPC, and a nonoxidizable phospholipid, POPC. Analysis by HPLC/MS rather than HPLC/UV was required for these oxidations since products arise from both PLPC and the other oxidizable lipids but only the linoleate products are used in the clocking experiments. Data from the experiments with mixtures of PLPC, POPC and several co-oxidants are presented in Figure 2, and propagation rate constants of various hydrogen donors in the liposome can be calculated from the slopes according to eq 2 (see Table 2). Cholesterol acts, in effect, as an inert diluent when it is a constituent of MLVs, a result that is not surprising based upon its relative reactivity as an H-atom donor compared to linoleate (Figure S5, Supporting Information).

The data obtained from oxidation of PLPC with nonoxidizable substrates can be plotted such that the chemistry associated with peroxyl radicals **1a** and **1b** are differentiated. In solution, these two regioisomeric peroxyl radicals follow identical reaction paths at virtually the same rates, the result of this being that peroxidation of linoleic acid or linoleate esters in solution gives rise to essentially a symmetrical distribution of products, that is, [**2a**] = [**2b**] and [**3a**] = [**3b**].

It is possible that a nonsymmetrical product distribution may be obtained for peroxidation reactions in liposomes since the peroxyl radicals at different chain positions experience different local environments in the lipid bilayer. We therefore considered differentiation of the two peroxyls according to the radical clock framework presented in Scheme 1. In the clock mechanism, peroxyl radical **1a** is trapped by hydrogen atom donors to give product **2a**, but fragmentation of **1a** leads to the *trans,trans*-product **3a**. The clock product ratios that provide differential information about peroxyl radicals **1a** and **1b** formed from oxidation in a lipid bilayer are therefore **[2a]/[3a]** and **[2b]/[3b]**. Analysis of the data plotted in Figure 1 in two plots of linoleate mole fraction vs **[2a]/[3a]** or **[2b]/[3b]** leads to Figure 3. It seems clear from the Figure that the 9-peroxyl radical **1a** gives rise to more *trans,cis*- or less *trans,trans*-product than

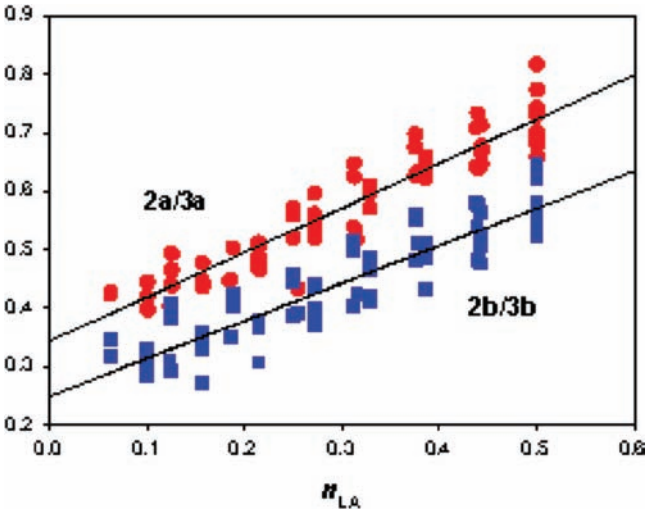


Figure 3. Values of 9-*trans*,*cis*/13-*trans*,*trans*(red) and 13-*trans*,*cis*/9-*trans*,*trans*(blue) vs linoleate mole fraction in the bilayer for oxidation of PLPC in liposomes with various nonoxidizable substrates as shown in Figure 1.

does the corresponding 13-peroxyl radical, **1b** (Figure S10−S11, Supporting Information). Statistical analysis of the data leads to the conclusion that the intercepts for the two plots are significantly different ($P < 0.0001$) while the slopes of the two plots do not differ ($P > 0.05$). Data from the experiment with mixtures of PLPC, PAPC and POPC are presented according to the analysis of **[2a]/[3a]** and **[2b]/[3b]** in Figure 4A and data from a similar set of experiments with 7-dehydrocholesterol is presented in Figure 4B. Analogous plots of **[2a]/[3a]** and **[2b]/[3b]** for co-oxidants PEPC (Figure S6) and PDPC (Figure S7) are presented in Supporting Information. Analysis of data from a series of oxidations of linoleic acid in homogeneous solution is also presented in Supporting Information (Figure S9).

Discussion

Propagation Rate Constants of PUFAs and Sterols in Solution. The rate of oxygen and substrate consumption in radical chain oxidation depends on the propagation and termination rate constants k_p and k_t, as well as the rate of radical initiation, R_i, Equation 3.[34,36] Oxidizability is the inherent propensity to undergo oxidation, defined as $k_p/(2k_t)^{1/2}$, and this parameter can be determined by measurement of oxygen consumption at a known rate of initiation. The determination of k_p has typically involved a set of experiments requiring measurement both of oxidizability and k_t, the latter usually by means of a spectroscopic probe. The radical clock method, described in Scheme 1 and eq 1, permits an independent and straightforward determination of k_p based on the competition of unimolecular β-fragmentation of a linoleate peroxyl radical and hydrogen atom transfer to that radical.[19]

$$-\mathrm{d}[O_2]/\mathrm{d}t = \{k_p/(2k_t)^{1/2}\}[R - H]R_i^{1/2} \qquad (3)$$

Table 1 shows the results obtained for application of the clock method to determine k_p for a number of important polyunsaturated fatty acids and sterols in solution. The propagation rate constant for fatty acid oxidation depends, as expected, on the number of oxidizable bis-allylic $-CH_2-$ centers in the molecule. Linoleate has one such center; arachidonate, 3; eicosapentaenoate, 4 and docosahexaenoate, 5 and the relative propagation rate constants determined for these compounds are in a ratio of 1, 3.2, 4.0, and 5.4. Cosgrove, Church and Pryor reported that the oxidizability ratios of linoleate, arachidonate and docosahexaenoate were 1, 2.8 and 5.0, in good agreement with the clock values of k_p reported here.[36]

The rate constant determined by the clock method for propagation of cholesterol autoxidation, 11 M^{-1} s^{-1}, falls in line with those determined for other cycloalkenes; cyclohexene and cyclopentene both having rate constants of about 6 M^{-1} s^{-1}.[37] The propagation rate constants for these cycloalkenes are considerably larger than those measured for simple acyclic olefins such as methyl oleate, which has a value of 0.8 M^{-1} s^{-1}.[38] The increased reactivity of cycloalkenes as H-atom donors compared to acyclic alkenes is undoubtedly due to conformational effects that orient the reactive C−H bond so that maximum orbital overlap between the alkene π bond and the developing radical center is possible in the transition state.

The propagation rate constant determined for autoxidation of 7-DHC, 2260 M^{-1} s^{-1}, is surprisingly large. 1,4-Cyclohexadiene, considered to be an excellent H-atom donor that is frequently used for mechanistic studies, has a k_p of only 265 M^{-1} $s^{-1}$and arachidonic acid, which is normally considered to be a highly oxidizable lipid is over an order of magnitude less reactive than 7-DHC, Table 1.[19] Indeed, the propagation rate constant for 7-DHC makes this substrate competitive for peroxyl radicals with some hindered phenols that are used as antioxidants. 2,6-Di-*tert*-butylphenol, for example, has a rate constant for H-atom transfer to peroxyl radicals of 3100 M^{-1} s^{-1}.[19] Molecular mechanics calculations indicate that the torsion angles C(7)−C(8)−C(9)−H(9) and C(7)−C(8)−C(14)−H(14) are close to 90°, suggesting that H(9) and H(14) are aligned for removal from the open α face of the steroid so that maximum delocalization stabilization is experienced in the transition state for H-atom transfer, see Figure 5.

Propagation Rate Constants of PUFAs and 7-DHC in Lipid Bilayers. The data presented in Figure 1 shows that the ratio of linoleate products **[(2a + 2b)/(3a +3b)]** depends directly on the mole fraction of linoleate, n_{LA}, in lipid multilamellar vesicles. These experiments were used to calibrate the constants a and b in eq 2 since the rate constant for autoxidation propagation in these aggregates, k_p^{LA}, was determined to be 16.6 M^{-1} s^{-1} by Antunes et al.[25] This molar rate constant can be converted to a mole fraction propagation rate constant, 35 $n^{-1}s^{-1}$, based upon the authors' assumption that the average molecular weight of phospholipid in their MLVs was ~800 and the density approximately 0.8.[25,28,39] In this way, the data in Figure 1 was fit to eq 2 with $k_p^{LA} = 35$ $n^{-1}s^{-1}$, which leads to $a = 0.022 \pm 0.002$ s and $b = 0.28 \pm 0.02$.

Having determined a and b from the experiments in which linoleate is the only oxidizable lipid, Equation 2 can then be used to provide rate constants for other H-atom donors in the lipid bilayer. The data for arachidonate, eicosapentaenoate, docosahexaenoate and 7-DHC are plotted in Figure 2 and the

(36) Cosgrove, J. P.; Church, D. F.; Pryor, W. A. *Lipids* **1987**, *22*, 299–304.
(37) Korcek, S.; Chenier, J. H. B.; Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1972**, *50*, 2285–2897.
(38) Porter, N. A.; Mills, K. A.; Carter, R. L. *J. Am. Chem. Soc.* **1994**, *116*, 6690–6696.
(39) The pseudo first-order rate constant for a given hydrogen donor at a given concentration can be expressed as $k_p^M[R-H] = k_p^n n_{R\text{-}H}$, where k_p^M is the propagation rate constant in units of $M^{-1}s^{-1}$ and k_p^n is the constant in units of $n^{-1}s^{-1}$. Thus, k_p^n can be expressed as a function of k_p^M as follows: $k_p^n = (k_p^M[R-H])/(n_{R\text{-}H})$.

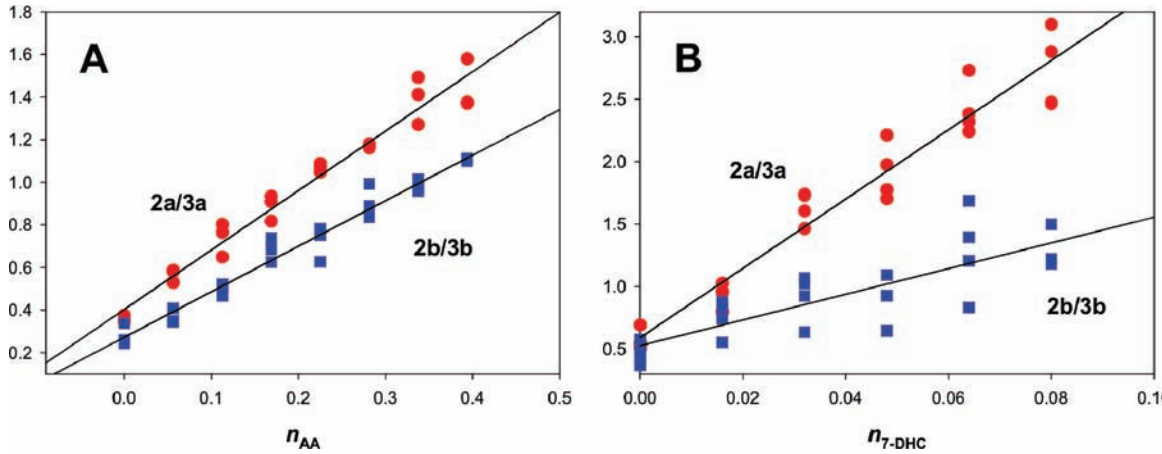


Figure 4. Values of 9-*trans*,*cis*/13-*trans*,*trans* (red circle) and 13-*trans*,*cis*/9-*trans*,*trans* (blue square) vs in **A**, arachidonate mole fraction in the bilayer for oxidation of PLPC in liposomes containing PAPC as an oxidizable cosubstrate and nonoxidizable POPC and in **B,** 7-dehydrocholesterol (7-DHC) mole fraction in the bilayer for oxidation of PLPC in liposomes containing 7-DHC as an oxidizable cosubstrate and nonoxidizable POPC.

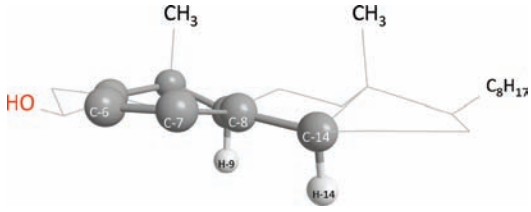


Figure 5. 7-Dehydrocholesterol low-energy conformation (MM2) showing reactive H-9 and H-14 and the B-ring conjugated diene frame.

values for k_p determined by Equation 2 are presented in the last column in Table 2. The relative reactivities of 18:2, 20:4, 20:5, 22:6 and 7-DHC in vesicles determined in this way are 1:3.3:4.1:4.9:24 while in solution the relative reactivities are 1:3.2:4.0:5.4:62. The fatty acids have remarkably similar reactivity in solution and in the lipid bilayer while 7-DHC appears to be somewhat less reactive as an H-atom donor in vesicles as compared to isotropic media.

Differential Kinetic Behavior of the Regioisomeric 9- and 13-Peroxyl Radicals in Lipid Bilayers. The data plotted in Figure 3 suggest that the two *trans,cis*-peroxyl radicals **1a** and **2a** (see Scheme 1) have distinctly different reaction dynamics in oxidation reactions carried out in vesicles. The consequences of the kinetic differences between these two radicals are expressed in the ratios of products **2a/3a** (series 1 in Figure 3) and **2b/3b** (series 2). Significant differences between the two radicals were also observed in the series of reactions in which arachidonate or 7-DHC was the co-oxidant in the clocking experiments (Figure 4A and B), while the differences were smaller for eicosapentaeonate and they were negligible for the docosahexaenoate clocking experiments (see Supporting Information). The data shown in Figures 3 and 4 are in stark contrast to the results obtained in isotropic media. The radicals **1a** and **2a** undergo chemistry in benzene solution, for example, that is virtually identical. There is no difference in the distribution of products derived from these radicals in reactions carried out in solution (Figure S9, Supporting Information).

We speculate that differentiation of peroxyl radicals **1a** and **1b** in lipid bilayers is due to the nonisotropic nature of these colloids. The *9-trans,cis*-peroxyl radical occupies a position in the bilayer that is closer to the aqueous interface and this radical is therefore exposed to a more polar environment than is the 13-*trans*,*cis*-peroxyl. The rate constant for β-fragmentation of peroxyl radicals is known to decrease in polar solvents and being closer to the aqueous interface, the *9-trans*,*cis*-peroxyl may well have a slower rate of fragmentation (k_β) than the *13-trans*,*cis*-species (k_β).[40] Recently, Lucarini and co-workers reported that peroxyl radicals can be stabilized by hydrogen bonding, which is another factor that could result in a lower k_β for the *9-trans*,*cis*-peroxyl radical.[41] Radical β-fragmentation is the basis of the radical clock competition and any factor that would differentially affect the fragmentation rates of **1a** and **1b** would differentiate the **2a/3a** and **2b/3b** product ratios.

The slopes of the two series are roughly parallel in Figure 3, but the intercept of the plot in the Figure is statistically different for the two series ($p < 0.0001$). This intercept is dependent on processes that do not involve H-atom transfer$-\beta$-fragmentation of the peroxyls, and the data presented in Figure 3 suggest that these processes occur at different rates for the two radicals, **1a** and **1b**. On the other hand, the slope difference between **2a/3a** and **2b/3b** can be given by eq 4 (Figure 4), where (k_β) is the β-fragmentation rate constant of **1a** and (k_β) is that of **1b**, k_p is xthe propagation rate constant to 9-peroxyl for a given H-atom donor and k_p' is that to 13-peroxyl, and a' is a constant that is determined by the

$$\text{Slope Difference} = a'\left(\frac{k_p}{k_\beta} - \frac{k\prime_p}{k\prime_\beta}\right) \qquad (4)$$

partitioning of oxygen to different sites on the pentadienyl radical.[19] Thus, when hydrogen donors are equivalent to 9- or 13-peroxyls, the *slope difference* would be proportional to the propagation rate constant.

Studies of the co-oxidations of PLPC and 7-DHC, presented in Figure 4b, give results in striking contrast to those for oxidations of PLPC alone or PLPC/PAPC co-oxidations. For these experiments, the slope of the **2a/3a** (series 1) and **2b/3b** (series 2) are substantially different. This indicates that for these co-oxidations, 7-DHC is a better H-atom donor to radical **1a** than it is to radical **2a** ($k_p > k_p'$). The results suggest that in the local bilayer environment, the reactive hydrogen atoms at C-9 and C-14 of 7-DHC are better positioned for transfer to the 9-peroxyl radical than to the 13-peroxyl. In addition, no statistically significant slope differences were observed for

(40) Jha, M.; Pratt, D. A. *Chem. Commun.* **2008**, 1252–1254.
(41) Mugnaini, V.; Lucarini, M. *Org. Lett.* **2007**, *9*, 2725–2728.

PLPC/PEPC or PLPC/PDPC co-oxidation experiments. (Figure S6−S7, Supporting Information).

Different β-fragmentation rate constants and propagation rate constants to 9- and 13-peroxyl radicals also lead to differences in the total amounts of 9- and 13-hydroperoxides (Figure S10, Supporting Information). As indicated in Figure S10, more 9-products than 13-products were consistently observed in the co-oxidation experiments of PLPC with nonoxidizable PCs, PAPC, and 7-DHC, while they are about the same for oxidation of linoleate in solution. Barclay and co-workers reported a difference between 9- and 13-product formation in a study of linoleate oxidation in micelles, more 13-product being formed than 9.[42] The different regioselectivities observed in bilayer and micellar oxidations of linoleate are not readily explained but the source of the selectivity must result from different exposures of intermediate peroxyl radicals to the aqueous medium in the micelle and bilayer environments.

Implications of the Exceptional Reactivity of 7-Dehydrocholesterol in Biology. The unusual reactivity of 7-DHC toward peroxyl radicals in solution and in bilayer aggregates calls attention to the importance of this steroid in biology. 7-DHC is the immediate biosynthetic precursor of cholesterol,[16,18,43,44] with the enzyme 7-dehydrocholesterol reductase catalyzing the reduction of the C7═C8 double bond of 7-DHC. The Smith−Lemli−Opitz syndrome (SLOS) is a recessive disorder caused by mutations of the DHCR7 gene. Impaired reductase activity for individuals having this syndrome leads to a build-up of 7-DHC and lower than normal levels of cholesterol in tissues and plasma.[18,43,45−47] The experiments that we report here show that 7-DHC is not just oxidatively unstable, but it is one of the best chain-carrying molecules that has been evaluated in free radical oxidation. This property of 7-DHC should serve as an alert for the possibility of oxidative stress whenever elevated levels of the compound are found, SLOS being such an instance.[48] Clearly, the compound should be added to the list of lipids that are susceptible to peroxidation since our studies show that 7-DHC is greater than ten times more reactive than arachidonic acid, a polyunsaturated fatty acid that is normally thought of as being particularly prone to peroxidation. Products of 7-DHC peroxidation are also of interest because of their potential as markers of oxidative stress *in vivo*. Furthermore, there are a host of oxysterol natural products[17,48−63] and some have been shown to have interesting and diverse biological activities.[14,17,49] Because of this, we are in the process of defining the reaction pathways and describing the products of 7-DHC peroxidation. The results of those studies will be reported in due course.

Experimental Section

General Procedure for Calibration Experiments and Co-Oxidation with Cholesterol in Liposomes. PLPC and nonreactive PCs or cholesterol (in chloroform; $[PC]_{final} = 50-80$ mM) were added to a 2 mL vial. The solution was dried by a flow of N_2, and was kept under vacuum for 10 min. PBS buffer (190 μL, 50 mM, pH = 7.4) was then added and the mixture was sonicated for 10−20 s. The resulting milky suspension was incubated at 37 °C for 10 min, which is followed by sonication for an additional 10−20 s. After addition of AIPH (10 μL, 0.02 M in PBS buffer), the milky suspension was kept at 37 °C for 2 h. The oxidation reaction was then quenched by adding BHT (100 μL, 0.1 M in ethanol) and PPh_3 (100 μL, 0.1 M in ethanol/CH_2Cl_2 = 4/1). The mixture was vortexed, and was then kept at room temperature for 30 min. Upon addition of ethanol (500 μL) and LiOH (500 μL, 3 M), the resulting mixture was stirred at 37 °C for 1 h, followed by acidification with HCl (600 μL, 3 M). After extraction with hexanes (2 mL × 3), the organic layers were combined and dried over $MgSO_4$. The upper clear solution was collected after centrifugation, and was blown dry by a flow of N_2. The residue remaining was dissolved in benzene (300 + 100 μL) and was further stabilized by addition of BHT in benzene (50 μL, 0.1 M). Samples were analyzed on normal phase HPLC-UV (solvent, hexane/1.4% 2-propanol/0.1% acetic acid; column 5 μm silica, 4.6 mm × 25 cm column, 1.0 mL/min; detection UV 234 nm).

General Procedure for Clocking Experiments of PUFAs and 7-DHC in Liposomes. One co-oxidation experiment of PLPC/POPC/PAPC with $n_{AA} = 0.1125$ in the liposome is used as an illustration: Chloroform solutions of PLPC (1.7 μmol), POPC (4.5 μmol) and PAPC (1.8 μmol) were added to a 2-mL vial and the solution was dried by a flow of N_2, after which it was kept under vacuum for 10 min. Liposomes from this mixture were then prepared by a procedure similar to that described in the previous section. Thus, 95 μL of PBS buffer (50 mM, pH = 7.4) was added and the mixture was sonicated for an additional 10−20 s. The resulting milky suspension was incubated at 37 °C for 10 min, which was followed by another 10−20 s of sonication. After adding the initiator AIPH (5 μL, 0.02 M in PBS buffer), the milky suspension was kept at 37 °C for 1 h, and the reaction was quenched by addition of BHT (100 μL, 0.1 M in ethanol) and PPh_3 (150 μL, 0.1 M in ethanol/CH_2Cl_2 = 4/1). The mixture was kept at room temperature for 30 min and it was then hydrolyzed and worked up in a procedure as described in the preceding section. Samples were analyzed on normal phase HPLC-APCI-MS/MS (hexane/1.4% 2-propanol/0.1% acetic acid, 4.6 mm × 25 cm column, 5 μ, 1.0 mL/min). Selective reaction monitoring (SRM) was employed for detection (*m*/*z* 295 → 195 for 13-substituted products **2b** and **3a**,

(42) Barclay, L. R. C.; Crowe, E.; Edwards, C. D. *Lipids* **1997**, *32*, 237–245.
(43) Haas, D.; Kelley, R. I.; Hoffmann, G. F. *Neuropediatrics* **2001**, *32*, 113–22.
(44) Waterham, H. R. *Clin. Genet.* **2002**, *61*, 393–403.
(45) Opitz, J. M.; Gilbert-Barness, E.; Ackerman, J.; Lowichik, A. *Pediatr. Pathol. Mol. Med.* **2002**, *21*, 153–81.
(46) Kelley, R. I. *Adv. Pediatr.* **2000**, *47*, 1–53.
(47) Kelley, R. I.; Hennekam, R. C. *J. Med. Genet.* **2000**, *37*, 321–35.
(48) Gaoua, W.; Chevy, F.; Roux, C.; Wolf, C. *J. Lipid Res.* **1999**, *40*, 456–63.
(49) Javitt, N. B. *Curr. Opin. Lipidol.* **2007**, *18*, 283–8.
(50) Töröcsik, D.; Szanto, A.; Nagy, L. *Mol. Aspects Med.* **2009,** electronic ahead of print.
(51) Akihisa, T.; Franzblau, S. G.; Tokuda, H.; Tagata, M.; Ukiya, M.; Matsuzawa, T.; Metori, K.; Kimura, Y.; Suzuki, T.; Yasukawa, K. *Biol. Pharm. Bull.* **2005**, *28*, 1117–1119.
(52) Akihisa, T.; Nakamura, Y.; Tagata, M.; Tokuda, H.; Yasukawa, K.; Uchiyama, E.; Suzuki, T.; Kimura, Y. *Chem. Biodiversity* **2007**, *4*, 224–231.
(53) Ishizuka, T.; Yaoita, Y.; Kikuchi, M. *Chem. Pharm. Bull.* **1997**, *45*, 1756–1760.
(54) Kawagishi, H.; Katsumi, R.; Sazawa, T.; Mizuno, T.; Hagiwara, T.; Nakamura, T. *Phytochemistry* **1988**, *27*, 2777–2779.
(55) Luo, X.; Li, F.; Shinde, P. B.; Hong, J.; Lee, C.-O.; Im, K. S.; Jung, J. H. *J. Nat. Prod.* **2006**, *69*, 1760–1768.
(56) Mansoor, T. A.; Hong, J.; Lee, C. O.; Bae, S. J.; Im, K. S.; Jung, J. H. *J. Nat. Prod.* **2005**, *68*, 331–336.
(57) Mansoor, T. A.; Lee, Y. M.; Hong, J.; Lee, C.-O.; Im, K. S.; Jung, J. H. *J. Nat. Prod.* **2006**, *69*, 131–134.
(58) Yaoita, Y.; Amemiya, K.; Ohnuma, H.; Furumura, K.; Masaki, A.; Matsuki, T.; Kikuchi, M. *Chem. Pharm. Bull.* **1998**, *46*, 944–950.
(59) Yaoita, Y.; Endo, M.; Tani, Y.; Machida, K.; Amemiya, K.; Furumura, K.; Kikuchi, M. *Chem. Pharm. Bull.* **1999**, *47*, 847–851.
(60) Yaoita, Y.; Matsuki, K.; Iijima, T.; Nakano, S.; Kakuda, R.; Machida, K.; Kikuchi, M. *Chem. Pharm. Bull.* **2001**, *49*, 589–594.
(61) Yaoita, Y.; Yoshihara, Y.; Kakuda, R.; Machida, K.; Kikuchi, M. *Chem. Pharm. Bull.* **2002**, *50*, 551–553.
(62) Yue, J. M.; Chen, S. N.; Lin, Z. W.; Sun, H. D. *Phytochemistry* **2001**, *56*, 801–806.
(63) Albro, P. W.; Bilski, P.; Corbett, J. T.; Schroeder, J. L.; Chignell, C. F. *Photochem. Photobiol.* **1997**, *66*, 316–325.

and m/z 295 → 171 for 9-substituted products **2a** and **3b**; the collision energy was 21 eV; see Supporting Information). The elution order of the four products is 13-*t*,*c* (**2b**), 13-*t*,*t* (**3a**), 9-*t*,*c* (**2a**), and 9-*t*,*t* (**3b**).[2] Response factors relative to the 13-*t*,*c*-product were determined before, during, and after each series of HPLC analyses using a standard mixture with known ratios of four products.

For a set of experiments with a given PUFA, arachidonate in this example, the mole fraction of linoleic acid (n_{LA}, from PLPC) was kept at the same value. the mole fraction of arachidonic acid (n_{AA}, from PAPC) was varied, and the mole fraction of oleic acid (n_{OA}, from POPC) was adjusted to compensate for the change in n_{AA}.

Clocking experiments on other fatty esters (PEPC and PDPC) and 7-DHC was carried out with a similar procedure to that described here for PAPC. In the case of 7-DHC, the oxidation reactions were carried out for 30 min to avoid overoxidation of 7-DHC due to its high oxidizability.

Oxidation of PLPC/POPC in Liposomes Made of ULV. MLV liposomes were prepared in the same way as described the Calibration Experiments. The MLV liposomes were then passed through a LiposoFast Basic extruder (Avestin, Inc., Ottawa, Canada) 19 times to give ULV liposomes as a transparent emulsion. After addition of the initiator AIPH (10 μL, 0.02 M in PBS buffer), the reaction mixture was kept at 37 °C for 1 h. The oxidation reaction was worked up and analyzed in a manner similar to the Calibration Experiments.

Abbreviations: PUFA, polyunsaturated fatty acid; 7-DHC, 7-dehydrocholesterol; MeOAMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); HPLC, high performance liquid chromatography; MS, mass spectrometry; MLV, multilamellar vesicles; ULV, unilamellar vesicles; PLPC, 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphotidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphotidylcholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphotidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphotidylcholine; PAPC, 1-Palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine; PEPC, 1-Palmitoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphatidylcholine; PDPC, 1-Palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphatidylcholine; AIPH, 2,2′-azobis(2-(2-imidazolin-2-yl) propane) dihydrochloride; MeLin, methyl linoleate; SLOS, Smith−Lemli−Opitz syndrome; PC, phosphotidylcholine; BHT, butylated hydroxytoluene; APCI, atmospheric pressure chemical ionization; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

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Supporting Information Available: General methods and materials, clocking experiments in solutions, figures, typical chromatogram, etc. This material is available free of charge via the Internet at http://pubs.acs.org.

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