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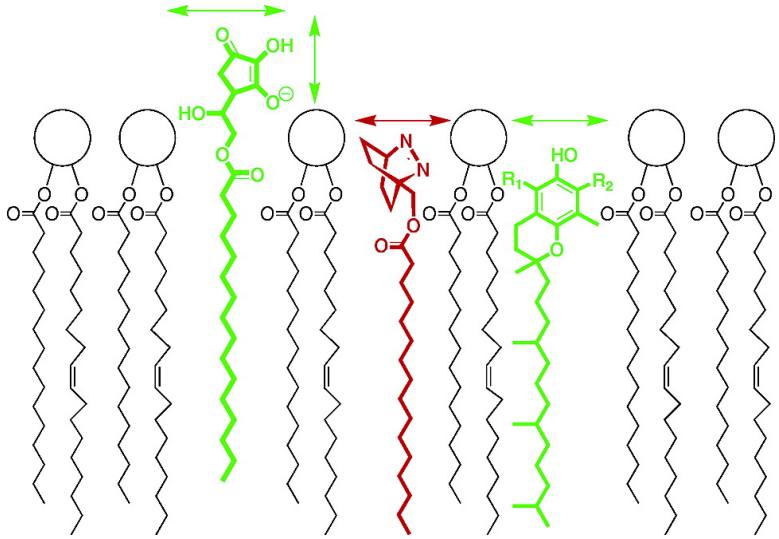

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Effect of Temperature, Cholesterol Content, and Antioxidant Structure on the Mobility of Vitamin E Constituents in Biomembrane Models Studied by Laterally Diffusion-Controlled Fluorescence Quenching

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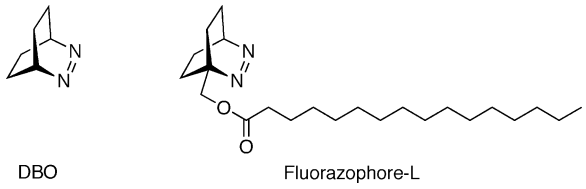
Abstract: Kinetic parameters relevant for the antioxidant activity of the vitamin E constituents (α, β, γ, and δ homologues of tocopherols and tocotrienols) and of an amphiphilic vitamin C derivative, L-ascorbyl 6-palmitate, were determined. Fluorescence quenching experiments of 2,3-diazabicyclo[2.2.2]oct-2-ene in homogeneous acetonitrile−water mixtures afforded reactivity trends in terms of intermolecular quenching rate constants, while the quenching of Fluorazophore-L in liposomes provided the lateral diffusion coefficients relevant for understanding their biological activity in membranes. The reactivity in homogeneous solution was not influenced by the nature of the isoprenoid tail (tocopherol versus tocotrienol), but was dependent on the methylation pattern. The resulting order ($\alpha > \beta = \gamma > \delta$) was found to be in line with their reactivities toward peroxyl radicals as well as the phenolic O−H bond dissociation energies. The mutual lateral diffusion coefficient in POPC liposomes was the same, within error, for different tocopherols and tocotrienols ($D_L = (1.6 \pm 0.2) \times 10^{-7}$ cm^2 s^{-1}). L-Ascorbyl 6-palmitate exhibited a reactivity similar to that of δ-tocopherol in homogeneous solution, but displayed a 1 order of magnitude lower fluorescence quenching efficiency in liposomes than the vitamin E constituents. Temperature effects on the laterally diffusion-controlled fluorescence quenching were large, with activation energies of 44 ± 6 kJ mol^{-1}. The addition of cholesterol (0−30%) to POPC liposomes resulted only in slightly reduced diffusion coefficients. The combined results demonstrate that Fluorazophore-L can provide important physicochemical parameters for the understanding of antioxidant activity in biological environments.

Introduction

Antioxidants play a vital role in medicine, biology, and polymer chemistry as well as the cosmetics and food industries. Antioxidant activity in lipids, in particular, is of prominent interest for the understanding of lipid peroxidation in membranes[1,2] and low-density lipoproteins,[3−5] which is being associated with oxidative stress, a cause of a multitude of diseases,[6,7] and degradation of food, in particular the rancification of fats.[8] Knowledge of the bimolecular reaction kinetics by which antioxidants intercept reactive oxidizing species, as studied herein, is of utmost importance for modeling their actual activity and understanding the mechanisms by which antioxidants act and interact.



DBO Fluorazophore-L

Fluorophores based on the azoalkane 2,3-diazabicyclo[2.2.2]-oct-2-ene (DBO), for which we have coined the name fluorazophores,[9] display a pronounced radical-like reactivity in their singlet excited state. Owing to their exceedingly long fluorescence lifetimes, they have been introduced as fluorescent probes for antioxidants in homogeneous solution[10−13] and microheterogeneous systems.[14−16] The amphiphilic palmitoyl derivative Fluorazophore-L, in particular, has recently been used as a model for reactive *membrane-bound* radicals to investigate its

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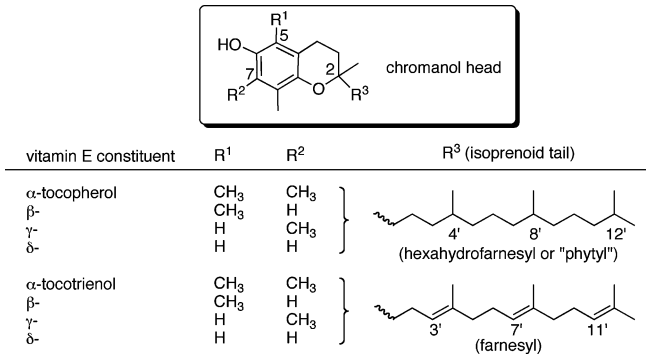
(1) Niki, E.; Noguchi, N. *Acc. Chem. Res.* **2004**, *37*, 45−51.
(2) Burton, G. W.; Ingold, K. U. *Acc. Chem. Res.* **1986**, *19*, 194−201.
(3) Noguchi, N.; Niki, E. *Free Radical Res.* **1998**, *28*, 561−572.
(4) Alessi, M.; Paul, T.; Scaiano, J. C.; Ingold, K. U. *J. Am. Chem. Soc.* **2002**, *124*, 6957−6965.
(5) Bowry, V. W.; Ingold, K. U. *Acc. Chem. Res.* **1999**, *32*, 27−32.
(6) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*; Clarendon Press: Oxford, 1985.
(7) Sies, H.; Murphy, M. E. *J. Photochem. Photobiol. B: Biol.* **1991**, *8*, 211−218.
(8) Parker, R. S. In *Advances in Food and Nutrition Research*; Kinsella, J. E., Ed.; Academic Press Inc.: New York, 1989; Vol. 33, pp 157−232.
(9) Nau, W. M.; Wang, X. *ChemPhysChem* **2002**, *3*, 393−398.

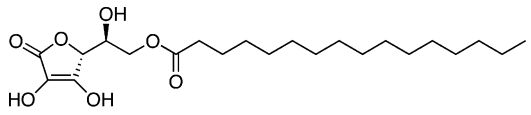
interaction with the water-soluble antioxidant vitamin C at varying pH,[15] and with the lipid-soluble antioxidant α-tocopherol.[16] These studies have afforded evidence for an unexpectedly enhanced reactivity of vitamin C at low pH, as well as the reaction kinetics in membrane-mimetic lipidic environments.[16] The size and type of the lipidic assembly (small anionic versus large neutral micelles versus phospholipid bilayer liposomes) proved to be critical for selecting the appropriate diffusion model. The diffusion coefficient of α-tocopherol in POPC liposomes, which was appropriately analyzed by a lateral diffusion model, could be deduced for the first time from a direct time-resolved measurement,[16] thereby correcting previous estimates based on indirect methods.

In fact, the fluorescence quenching of Fluorazophore-L presents a novel approach to study diffusion of membrane additives in real time, which complements alternative approaches based on NMR,[17−22] or fluorescence recovery after photobleaching.[23−25] The latter two methods are restricted in that they afford only time-averaged information on unreactive membrane constituents or require labeling with usually large aromatic fluorescent probes, thereby modifying the diffusion characteristics to an unknown extent. In the present study, we apply the unconventional fluorescence quenching method for a comprehensive investigation of the diffusive properties of several lipid-soluble antioxidants in liposomes in order to address some standing questions in antioxidant and in particular vitamin E research.

Vitamin E is the most important and effective chain-breaking antioxidant in biological membranes,[26−29] although undesirable prooxidant effects have been described under certain circumstances, e.g., in low-density lipoproteins.[5,30,31] One important question is related to the variation in antioxidant activity of the different constituents of vitamin E. Although α-tocopherol is deemed to be the most active compound,[32−35] vitamin E is actually a generic term, which includes four tocopherols and four tocotrienols as individual constituents (cf. Chart 1). All of them are potentially active as chain-breaking antioxidants in cellular membranes.[2] The chromanol headgroup of tocopherols and tocotrienols is located at the lipid/water phase boundary[36−38] and terminates lipid peroxidation by donating a hydrogen atom to a peroxyl radical from its phenolic hydroxyl group.[10,34,39] The isoprenoid side chain is situated in the lipid bilayer and contains, in the case of tocotrienols, isolated *trans* double bonds at positions 3′, 7′, and 11′ with an *E* stereochemistry at positions 3′ and 7′.[40] Depending on the methyl substitution pattern on the C5 and C7 positions of the chromanol bicycle, α, *β*, *γ*, and *δ* homologues can be further distinguished.[41] Not surprisingly, the structural differences between the individual tocopherol and tocotrienol homologues manifest themselves in different in vivo and in vitro activities,[42] but to which degree these can be related to differential diffusional properties in membranes has not been scrutinized.

Chart 1





L-ascorbyl 6-palmitate

A second question extends to L-ascorbyl 6-palmitate, which is frequently used as an antioxidant additive instead of or in combination with vitamin E. Although this artificial amphiphilic vitamin C derivative is broadly employed to increase the shelf life of foods,[43−49] and for cosmetic and pharmacological

(10) Nau, W. M. *J. Am. Chem. Soc.* **1998**, *120*, 12614−12618.
(11) Zhang, X. Y.; Erb, C.; Flammer, J.; Nau, W. M. *Photochem. Photobiol.* **2000**, *71*, 524−533.
(12) Zhang, X. Y.; Nau, W. M. *J. Inf. Rec.* **2000**, *25*, 323−330.
(13) Erb, C.; Nau-Staudt, K.; Flammer, J.; Nau, W. M. *Ophthalmic Res.* **2004**, *36*, 38−42.
(14) Gramlich, G.; Zhang, J.; Winterhalter, M.; Nau, W. M. *Chem. Phys. Lipids* **2001**, *113*, 1−9.
(15) Gramlich, G.; Zhang, J.; Nau, W. M. *J. Am. Chem. Soc.* **2002**, *124*, 11252−11253.
(16) Gramlich, G.; Zhang, J.; Nau, W. M. *J. Am. Chem. Soc.* **2004**, *126*, 5482−5492.
(17) Scheidt, H. A.; Pampel, A.; Nissler, L.; Gebhardt, R.; Huster, D. *Biochim. Biophys. Acta* **2004**, *1663*, 97−107.
(18) Aisenbrey, C.; Bechinger, B. *J. Am. Chem. Soc.* **2004**, *126*, 16676−16683.
(19) Ferrage, F.; Zoonens, M.; Warschawski, D. E.; Popot, J. L.; Bodenhausen, G. *J. Am. Chem. Soc.* **2003**, *125*, 2541−2545.
(20) Hoff, B.; Strandberg, E.; Ulrich, A. S.; Tieleman, D. P.; Posten, C. *Biophys. J.* **2005**, *88*, 1818−1827.
(21) Lindblom, G.; Wennerstrom, H. *Biophys. Chem.* **1977**, *6*, 167−171.
(22) Fukuzawa, K.; Ikebata, W.; Shibata, A.; Kumadaki, I.; Sakanaka, T.; Urano, S. *Chem. Phys. Lipids* **1992**, *63*, 69−75.
(23) Yamazaki, V.; Sirenko, O.; Schafer, R. J.; Groves, J. T. *J. Am. Chem. Soc.* **2005**, *127*, 2826−2827.
(24) Wu, E.; Jacobson, K.; Papahadjopoulos, D. *Biochemistry* **1977**, *16*, 3936−3941.
(25) Saxton, M. J.; Jacobson, K. *Annu. Rev. Bioph. Biom.* **1997**, *26*, 373−399.
(26) Burton, G. W.; Joyce, A.; Ingold, K. U. *Lancet* **1982**, *320*, 327.
(27) Burton, G. W.; Ingold, K. U. *Ann. NY Acad. Sci.* **1986**, *570*, 7−22.
(28) Schwarz, K. *Fed. Proc.* **1965**, *24*, 58−67.
(29) Halliwell, B.; Chirico, S. *Am. J. Clin. Nutr.* **1993**, *57*, 715S−725S.
(30) Upston, J. M.; Terentis, A. C.; Stocker, R. *FASEB J.* **1999**, *13*, 977−994.
(31) Witting, P. K.; Mohr, D.; Stocker, R. *Method. Enzymol.* **1999**, *299*, 362−375.
(32) Farrel, P. M. In *Vitamin E: A Comprehensive Treatise*; Machlin, L. J., Ed.; Decker: New York, 1980; Vol. 1, pp 520−620.
(33) Diplock, A. T. In *Fat-Soluble Vitamins*; Diplock, A. T., Ed.; Technomic Publ.: Lancaster, 1985; pp 154−224.
(34) Burton, G. W.; Doba, T.; Gabe, E. J.; Hughes, L.; Lee, F. L.; Prasad, L.; Ingold, K. U. *J. Am. Chem. Soc.* **1985**, *107*, 7053−7065.
(35) Burton, G. W.; Hughes, L.; Ingold, K. U. *J. Am. Chem. Soc.* **1983**, *105*, 5950−5951.
(36) Kagan, V. E.; Quinn, P. J. *Eur. J. Biochem.* **1988**, *171*, 661−667.
(37) Perly, B.; Smith, I. C. P.; Hughes, L.; Burton, G. W.; Ingold, K. U. *Biochim. Biophys. Acta* **1985**, *819*, 131−135.
(38) Urano, S.; Matsuo, M.; Sakanaka, T.; Uemura, I.; Koyama, M.; Kumadaki, I.; Fukuzawa, K. *Arch. Biochem. Biophys.* **1993**, *303*, 10−14.
(39) DeDuve, C.; Hayaishi, O. *Tocopherol, Oxygen and Biomembranes*; Elsevier/North-Holland Medical Press: Amsterdam, 1978.
(40) Kamal-Eldin, A.; Appelqvist, L.-A. *Lipids* **1996**, *31*, 671−701.
(41) Wang, X.; Quinn, P. J. *Prog. Lipid Res.* **1999**, *38*, 309−336.
(42) Packer, L. In *Bioradicals Detected by ESP Spectroscopy*; Ohya-Nishigushi, H., Packer, L., Eds.; Birkhäuser Verlag: Basel/Switzerland, 1995.
(43) Cort, W. M. *J. Am. Oil. Chem. Soc.* **1974**, *51*, 321−325.
(44) Baardseth, P. *Food Addit. Contam.* **1989**, *6*, 201−207.
(45) McMullen, L. M.; Hawrysh, Z. J.; Lin, C.; Tokarska, B. *J. Food Sci.* **1991**, *56*, 1651−1654.
(46) Kaitaranta, J. K. *J. Am. Oil. Chem. Soc.* **1992**, *69*, 810−813.
(47) Hamilton, R. J.; Kalu, C.; McNeill, G. P.; Padley, F. B.; Pierce, J. H. *J. Am. Oil. Chem. Soc.* **1998**, *75*, 813−822.
(48) Satyanarayana, A.; Giridhar, N.; Joshi, G. J.; Rao, D. G. *J. Food Lipids* **2000**, *7*, 1−10.
(49) Beddows, C. G.; Jagait, C.; Kelly, M. J. *Food Chem.* **2001**, *73*, 255−261.

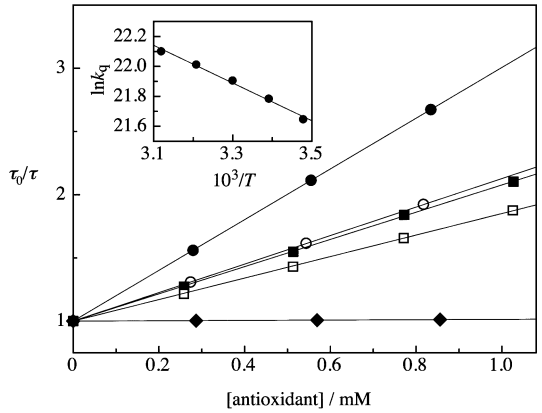


Figure 1. Fluorescence quenching plots for DBO with (all-*rac*)-α-tocopherol (●), (all-*rac*)-*β*-tocopherol (○), (all-*rac*)-*γ*-tocopherol (■), (all-*rac*)-*δ*-tocopherol (□), and (*S*,*E*,*E*)-α-tocotrienyl acetate (◆) as quenchers in acetonitrile−water (9:1). Shown in the inset is the corresponding Arrhenius plot for the variation of the bimolecular quenching rate constant of (all-*rac*)-α-tocopherol versus temperature.

applications,[50] it is not accurately known to which degree it is able to intercept lipid peroxyl radicals, although it has been suggested to protect and recycle α-tocopherol in erythrocyte membranes.[51,52] Additional open problems are how temperature and the presence of cholesterol, a membrane phase-modulator abundantly found in natural membranes, affect the efficiency by which lipid peroxyl radicals can be intercepted by lipid-soluble antioxidants. This will also be addressed in the present study from the viewpoint of variations of diffusional properties by using fluorescence quenching of Fluorazophore-L as a model reaction.

Results

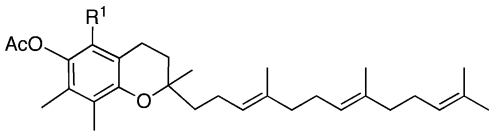
Quenching in Homogeneous Solution. For the determination of the bimolecular quenching rate constants, which provide a measure of the hydrogen donor propensity of additives,[10,15] we selected degassed acetonitrile−water (9:1) as solvent, in keeping with a previous study.[10,53] All investigated antioxidants were found to quench DBO fluorescence efficiently, with close to diffusion-controlled rates, and the quenching plots were strictly linear ($r > 0.99$, $n = 5$, Figure 1). Hydrogen atom abstraction from the phenolic hydroxyl group is the quenching mechanism, as was previously demonstrated through the observation of a deuterium kinetic isotope effect and the tocopheroxyl radical produced in the quenching process.[10,54] It should be noted that electron transfer as quenching mechanism can be ruled out on an energetic basis, which has recently been scrutinized for a series of substituted phenols and alkylbenzenes as quenchers.[55]

Strikingly, when the phenolic hydroxyl group was acetylated and therefore deactivated (see structure below), fluorescence quenching of DBO became negligible (Figure 1), which provides independent experimental support for the quenching mechanism. Quenching of DBO proceeds thus via hydrogen atom transfer,[54,56,57] as does the scavenging of reactive peroxyl or alkoxyl radicals by vitamin E in vitro[2,58] and in vivo.[26,27,59,60]

Table 1. Quenching Rate Constants of DBO by Racemic and Enantiopure Tocopherol and Tocotrienol Homologues as well as L-Ascorbyl 6-Palmitate in Homogeneous Solution

homologue	antioxidant type	stereochemistry[a]	purity[b]	$k_q/(10^9\ M^{-1}\ s^{-1})$[c]
α	tocopherol	all-*rac*	99.6	3.0
		R,*R*,*R*	99.5	2.9
	tocotrienol	*R*,*E*,*E*	>99.9	2.6
	tocotrienyl acetate	*S*,*E*,*E*	99.0	0.02 ± 0.01
β	tocopherol	all-*rac*	98.7	1.7
		R,*R*,*R*	99.7	1.6
	tocotrienol	*R*,*E*,*E*	97.9	1.5
γ	tocopherol	all-*rac*	96.8	1.6
		R,*R*,*R*	96.6	1.7
	tocotrienol	*R*,*E*,*E*	99.4	1.6
	tocotrienyl acetate	*S*,*E*,*E*	99.8	0.02 ± 0.01
δ	tocopherol	all-*rac*	96.7	1.3
		R,*R*,*R*	99.0	1.3
	tocotrienol	*R*,*E*,*E*	99.0	1.3
	ascorbyl 6-palmitate	L	≥99.0	1.4

[a] Absolute stereochemistry at position 2 of the chromanol ring, and 4′ and 8′ of the isoprenoid tail. [b] In % according to GC for tocopherols and according to HPLC for tocotrienols and acetate derivatives. [c] In acetonitrile−water, 9:1; error in data is 5%, unless explicitly stated.



α-tocotrienol acetate ($R^1 = CH_3$)
γ-tocotrienol acetate ($R^1 = H$)

The absolute quenching rate constants were obtained from the slopes of the quenching plots and are listed in Table 1. The value for α-tocopherol ($k_q \approx 3.0 \times 10^9\ M^{-1}\ s^{-1}$) was consistent with that determined in a previous study.[10] The *δ* homologues had the lowest reactivity ($k_q \approx 1.3 \times 10^9\ M^{-1}\ s^{-1}$), similar to L-ascorbyl 6-palmitate ($k_q = 1.4 \times 10^9\ M^{-1}\ s^{-1}$, lowest entry in Table 1). The rate constant of L-ascorbyl 6-palmitate in the acetonitrile−water mixture was found to be comparable to that of ascorbic acid in water.[15] Noteworthy, the rate constant for each tocopherol was the same, within error, as that for the corresponding tocotrienol; that is, the reactivity did not depend on the isoprenoid tail. Moreover, the quenching efficiency of each (all-*rac*)-tocopherol homologue was the same, within error, as that obtained for the enantiomerically pure (*R*,*R*,*R*) stereoisomer. While this is immediately expected, considering that the reactive site is remote from the chirality centers, one must recall that the different samples had been independently

(50) Dunham, W. B.; Zuckerkandl, E.; Reynolds, R.; Willoughby, R.; Marcuson, R.; Barth, R.; Pauling, L. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7532−7536.
(51) Ross, D.; Mendiratta, S.; Qu, Z.-C.; Cobb, C. E.; May, J. M. *Free Radical Biol. Med.* **1999**, *26*, 81−89.
(52) May, J. M.; Qu, Z.-C.; Cobb, C. E. *Free Radical Biol. Med.* **1996**, *21*, 471−480.
(53) The fluorescence lifetime in this mixture is also particularly long (660 ns, degassed), the presence of water allows for the possibility of deuterium exchange (not presently explored), and it provides a polar environment similar to that sensed by the headgroups of Fluorazophore-L as well as the amphiphilic antioxidants when immersed in liposomes, cf. ref 16.
(54) Nau, W. M.; Greiner, G.; Rau, H.; Olivucci, M.; Robb, M. A. *Ber. Bunsen-Ges. Phys. Chem.* **1998**, *102*, 486-492.
(55) Pischel, U.; Patra, D.; Koner, A. L.; Nau, W. M. *Photochem. Photobiol.* **2005**, in press.
(56) Nau, W. M.; Greiner, G.; Wall, J.; Rau, H.; Olivucci, M.; Robb, M. A. *Angew. Chem., Int. Ed.* **1998**, *37*, 98−101.
(57) Sinicropi, A.; Pischel, U.; Basosi, R.; Nau, W. M.; Olivucci, M. *Angew. Chem., Int. Ed.* **2000**, *39*, 4582−4586.
(58) Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6472−6477.
(59) Burton, G. W.; Ingold, K. In *Handbook of Free Radicals and Antioxidants in Biomedicine*; Miquel, J., Ed.; CRC Press: Boca Raton, 1986; Vol. 2, pp 29−43.
(60) Porkony, J. In *Autoxidation of Unsaturated Lipids*; Chan, H. W. S., Ed.; Academic Press: London, 1987; pp 141−206.

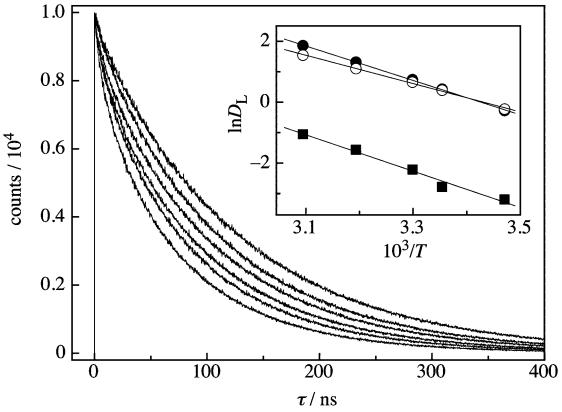


Figure 2. Fluorescence decay traces of Fluorazophore-L in POPC liposomes at different (*R*,*E*,*E*)-α-tocotrienol concentrations at 25 °C, from top to bottom: 0, 7.94×10^{15}, 1.59×10^{16}, 3.17×10^{16}, 4.76×10^{16}, and 6.35×10^{16} molecules/m^2 POPC. Shown in the inset are the Arrhenius plots for the variation of the mutual lateral diffusion coefficients of Fluorazophore-L with (all-*rac*)-α-tocopherol (●), (*R*,*R*,*R*)-*δ*-tocopherol (○), and L-ascorbyl 6-palmitate (■) with respect to temperature.

prepared and purified,[61−64] such that the similar reactivity of the enantiopure versus all-racemic compounds corroborates sample purity and excludes also that differential impurities are responsible for the observed quenching effects.

The activation energy of bimolecular fluorescence quenching of DBO was determined for α-tocopherol as a representative case. For this purpose, the quenching rate constants were determined in the temperature range from 15 to 50 °C. The resulting Arrhenius plot (inset of Figure 1) afforded an activation energy of 10 ± 2 kJ mol^{-1} in homogeneous acetonitrile−water solution.

Lateral Diffusion Coefficients in POPC Liposomes. Fluorazophore-L was included into POPC liposomes, which were prepared by the injection method[65−67] to allow a homogeneous distribution and accurate adjustment of the concentration of the respective tocopherol or tocotrienol homologue. The fluorescence quenching in liposomes was analyzed according to a lateral diffusion model.[16] In contrast to fluorescence quenching in homogeneous solution (see above), a time-independent quenching rate constant cannot be defined in this case. Instead, the kinetics of fluorescence quenching in liposomes can be characterized by a mutual lateral diffusion coefficient (D_L), which can be obtained by global fitting of the decay data according to eq 1.[16,68] An example of the decays, which were obtained by time-correlated single photon counting, is shown for (*R*,*E*,*E*)-α-tocotrienol in Figure 2.

$$I(t) = I_0 \exp[-(k_0 t + 2.31 D_L N_a [Q_{2D}] t + 7.61 \sqrt{D_L} R N_a [Q_{2D}] \sqrt{t})] \quad (1)$$

The additional variables in eq 1 are defined as follows: R is the intermolecular distance at which quenching occurs (assumed to be 8.0 Å for the employed antioxidants, cf. ref 16), and $[Q_{2D}]$ is the two-dimensional concentration of the quencher (antioxidant) on the surface of the liposome. $[Q_{2D}]$ ranged generally from ca. 0.8×10^{16} to 5×10^{16} molecules/m^2 (corresponding to 0.5−3 mol %) and was calculated by assuming an arrangement with all headgroups oriented toward the aqueous environment. For this purpose, the area per molecule of POPC was taken to be 70 Å^2,[14] and that of cholesterol to be 38.5 Å^2.[69] I_0 and $I(t)$ are the fluorescence intensities at time 0 and t, and k_0 is the unquenched fluorescence decay rate, i.e., the inverse lifetime in the absence of additives ($1/\tau_0$). It should be noted that the empirical eq 1 was originally devised to apply to lifetimes around 100 ns.[16,68] The lifetimes in POPC liposomes (which vary from ca. 70−180 ns depending on temperature and also on cholesterol content, see below) present an ideal match in this respect.

We selected α-tocopherol homologues as the most reactive antioxidants and *δ*-tocopherol homologues as the least reactive ones for further study in the experimentally highly demanding liposome fluorescence quenching experiments. Their lateral diffusion coefficients (Table 2) were found to be on the order of 10^{-7} cm^2 s^{-1}, similar to those of lipids.[70,71] As was found to be the case in homogeneous solution (Table 1), the stereochemistry of the isoprenoid tail has no effect on the lateral diffusion coefficient in the membrane model (entry 1 versus 2 and entry 4 versus 5). Notably, the methylation pattern of the chromanol moiety, which modulates the vitamins' reactivity in homogeneous solution, does not manifest itself in a sizable difference in the lateral diffusion coefficient (entries 1−3 versus 4−6). Interestingly, no marked influence of the nature of the isoprenoid chain on the diffusion of tocotrienols and tocopherols could be observed either (entry 1 versus 3 and entry 4 versus 6); tocotrienols did not display significantly higher diffusion coefficients than the respective tocopherol homologues.

Temperature Dependence of Fluorazophore-L Fluorescence Quenching. The temperature dependence of vitamin E diffusion in POPC liposomes was investigated by determining the mutual lateral diffusion coefficient of the α and *δ* homologues at five temperatures ranging from 15 to 50 °C (Table 2). The diffusion of the antioxidants increased steeply with temperature, also explaining the significant difference in D_L between the α-tocopherol value at 25 °C (1.54×10^{-7} cm^2 s^{-1}, Table 2), the previously reported value at 27 °C (1.8×10^{-7} cm^2 s^{-1}),[16] and the value at 30 °C (2.10×10^{-7} cm^2 s^{-1}, Table 2). The activation energies for fluorescence quenching by the tocopherols in the POPC membrane could be extracted from an Arrhenius plot of the mutual lateral diffusion coefficient versus temperature (inset of Figure 2).[72] The activation energies for α- and *δ*-tocopherol were found to be 47 ± 5 and 39 ± 4 kJ mol^{-1}, respectively (Table 2).

Quenching in Cholesterol-Containing Membranes. Fluorazophore-L quenching by α-tocopherol was also studied in POPC membranes containing cholesterol to better mimic the more heterogeneous composition of biomembranes. Cholesterol is a weak quencher of DBO fluorescence ($k_q = (2.0 \pm 0.2) \times$

(61) Mazzini, F.; Alpi, E.; Salvadori, P.; Netscher, T. *Eur. J. Org. Chem.* **2003**, 2840−2844.
(62) Netscher, T. *Recent Res. Dev. Org. Chem.* **2003**, *3*, 71−83.
(63) Walther, W.; Netscher, T. *Chirality* **1996**, *8*, 397−401.
(64) Netscher, T. *Chimia* **1996**, *50*, 563−567.
(65) Batzri, S.; Korn, E. D. *Biochim. Biophys. Acta* **1973**, *298*, 1015−1019.
(66) Domazou, A. S.; Luisi, P. L. *J. Liposome Res.* **2002**, *12*, 205−220.
(67) Kremer, J. M. H.; van der Esker, M. W.; Pathmamanoharan, C.; Wiersema, P. H. *Biochemistry* **1977**, *16*, 3932−3935.
(68) Razi Naqvi, K.; Martins, J.; Melo, E. *J. Phys. Chem. B* **2000**, *104*, 12035−12038.
(69) Takano, E.; Ishida, Y.; Iwahashi, M.; Araki, T.; Iriyama, K. *Langmuir* **1997**, *13*, 5782−5786.
(70) Johnson, M. E.; Berk, D. A.; Blankschtein, D.; Golan, D. E.; Jain, R. K.; Langer, R. S. *Biophys. J.* **1996**, *71*, 2656−2668.
(71) Köchy, T.; Bayerl, T. M. *Phys. Rev. E* **1993**, *47*, 2109−2116.
(72) Filippov, A.; Orädd, G.; Lindblom, G. *Langmuir* **2003**, *19*, 6397−6400.

Table 2. Mutual Lateral Diffusion Coefficients and Activation Energies Obtained by Fluorescence Quenching of Fluorazophore-L by Membrane-Bound Antioxidants in POPC Liposomes with Varying Cholesterol Content at Different Temperatures

				$D_L/(10^{-7}$ cm^{2}s$^{-1})^a$					
				T/°C					
entry	antioxidant type	stereochemistry	cholesterol content/mol %	15	25	30	40	50	E_A/(kJ mol^{-1})
1	α-tocopherol	all-*rac*	0	0.76	1.54	2.10	3.73	6.42	47 ± 5
2	α-tocopherol	*R*,*R*,*R*	0		1.53				
3	α-tocotrienol	*R*,*E*,*E*	0		1.58				
4	δ-tocopherol	all-*rac*	0		1.48				
5	δ-tocopherol	*R*,*R*,*R*	0	0.79	1.48	1.93	3.01	4.67	39 ± 4
6	δ-tocotrienol	*R*,*E*,*E*	0		1.53				
7	ascorbyl 6-palmitate[b]	L	0	0.04	0.06	0.11	0.21	0.35	49 ± 5
8	α-tocopherol	all-*rac*	5		1.57				
9	α-tocopherol	all-*rac*	15		1.49				
10	α-tocopherol	all-*rac*	30		1.36				

[a] Error in lateral diffusion coefficient is 10%. [b] The data for L-ascorbyl 6-palmitate are apparent values, since the condition of laterally diffusion-controlled quenching may not be fulfilled, cf. text.

10^7 M^{-1} s^{-1}), similar to alkenes and alcohols,[73,74] which resulted also in a slight lifetime shortening of Fluorazophore-L in cholesterol-containing liposomes; that is, τ_0 dropped from 125 ns to 115, 99, and 81 ns upon incorporation of 5, 15, and 30 mol % cholesterol, respectively. Because the lifetimes remained sufficiently long, i.e., in the recommended range of 100 ns,[16] and because the fluorescence decays remained monoexponential, no additional experimental modifications were necessary, such that the fluorescence quenching in cholesterol-containing POPC liposomes could be studied according to the established protocol. The mutual lateral diffusion coefficients for the probe/α-tocopherol pair were determined at 25 °C for liposomes containing 0, 5, 15, and 30 mol % cholesterol (Table 2, entries 1 and 8−10). Upon incorporation of 5 mol % cholesterol, D_L increased insignificantly. Higher cholesterol contents (15−30%), however, led to a significant decrease, e.g., $D_L = 1.36 \times 10^{-7}$ cm^2 s^{-1} for 30 mol % cholesterol.

L-Ascorbyl 6-Palmitate. The reactivity of L-ascorbyl 6-palmitate was also investigated by fluorescence quenching in POPC liposomes. Recall that the bimolecular reaction rate constant of L-ascorbyl 6-palmitate with DBO was virtually the same as that for δ-tocopherol and δ-tocotrienol (Table 1). In contrast, the apparent lateral diffusion coefficients of L-ascorbyl 6-palmitate, studied in POPC liposomes with respect to temperature by Fluorazophore-L quenching, were more than 1 order of magnitude smaller than those of the investigated vitamin E homologues at the respective temperatures (Table 2, entry 7 versus entries 1−6). The apparent lateral diffusion coefficients increased again steeply from 0.04×10^{-7} cm^2 s^{-1} at 15 °C to 0.35×10^{-7} cm^2 s^{-1} at 50 °C to afford an apparent activation energy for lateral diffusion of 49 ± 5 kJ mol^{-1}, similar to that of α-tocopherol and α-tocotrienol (inset of Figure 2).

Liposome Size Determination. The liposome size was determined by dynamic light scattering measurements. A narrow monomodal size distribution with a polydispersity index < 0.2 and an average diameter of 70 ± 10 nm was obtained for POPC liposomes containing different amounts of vitamin E constituents and also different percentages of cholesterol. For the self-aggregates containing L-ascorbyl 6-palmitate an average diameter of 70 ± 5 nm was found. Importantly, the size was not systematically dependent on Fluorazophore-L, antioxidant, or cholesterol incorporation.

Discussion

As set out in the Introduction, knowledge about the fundamental kinetics underlying the oxidative processes in membranes, in particular tocopherol and tocotrienol-mediated radical scavenging, is quintessential for the mechanistic understanding and modeling of the antioxidant action of vitamin E from first principles. The purpose of the present study was therefore to investigate the relative hydrogen donor propensities and the diffusional behavior of lipophilic peroxyl radical-trapping antioxidants in membrane models, both of which are deemed to be important factors in determining antioxidant activity.

Structural Effects on Hydrogen Donor Propensity. The strongly fluorescent n,π*-excited state of DBO resembles reactive free radicals in that it is selectively quenched by chain-breaking antioxidants via hydrogen atom transfer.[54] The exceedingly long-lived fluorescence lifetime of DBO (e.g., 325 ns in aerated water) provides a large time window for collisional encounters, allowing for time-resolved analysis and direct, highly sensitive determination of antioxidant reaction kinetics at physiologically relevant concentrations (μM−mM range).[10−16] The bimolecular fluorescence quenching rate constant of DBO by an additive provides a measure of its hydrogen donor potency. Indeed, the decrease in rate constant in the order $\alpha > \beta \approx \gamma > \delta$ for the tocotrienol and tocopherol homologues determined in the present study (Table 1) resembles that observed for peroxyl radicals[2,34,75−79] and is consistent with the influence of the chromanol ring methylation pattern on the O−H bond dissociation energy.[80] The variation in rate constants for Fluorazophore-P (factor 2.3) falls short of that observed for peroxyl radicals (factor 7.3),[2,34] which is nicely in line with the reactivity−selectivity principle; that is, the 3 orders of magnitude less reactive peroxyl radicals behave as more selective reactive species. Note that the quenching rate constants of *acetylated*

(73) Nau, W. M.; Greiner, G.; Rau, H.; Wall, S. N.; Olivucci, M.; Scaiano, J. C. *J. Chem. Phys. A* **1999**, *103*, 1579−1584.
(74) Nau, W. M.; Pischel, U. In *Organic Photochemistry and Photophysics*; Ramamurthy, V., Schanze, K., Eds.; 2005; pp 75−129.
(75) Yoshida, Y.; Niki, E.; Noguchi, N. *Chem. Phys. Lipids* **2003**, *123*, 63−75.
(76) Pryor, W. A.; Strickland, T.; Church, D. F. J. *J. Am. Chem. Soc.* **1988**, *110*, 2224−2229.
(77) Niki, E.; Tsuchiya, J.; Yoshikawa, Y.; Yamamoto, Y.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 497−501.
(78) Mukai, K.; Kageyama, Y.; Ishida, T.; Fukuda, K. *J. Org. Chem.* **1989**, *54*, 552−556.
(79) Neely, W. C.; Martin, J. M.; Barker, S. A. *Photochem. Photobiol.* **1988**, *48*, 423−428.
(80) Wayner, D. D. M.; Lusztyk, E.; Ingold, K. U.; Mulder, P. *J. Org. Chem.* **1996**, *61*, 6430−6433.

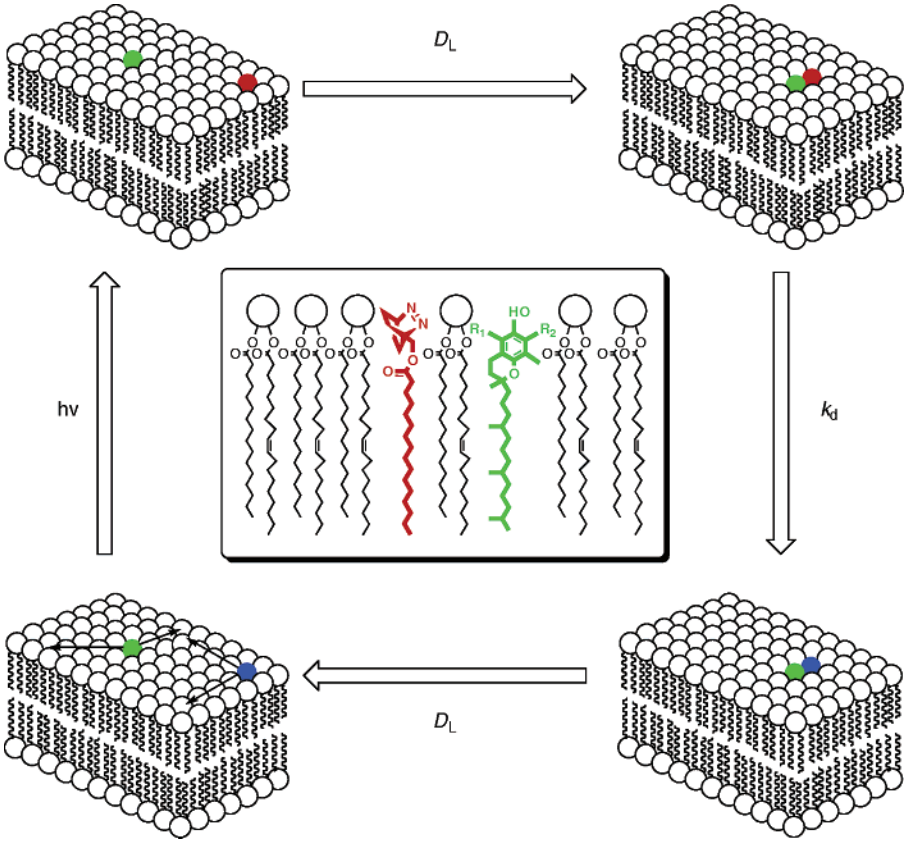


Figure 3. Schematic view of the quenching of Fluorazophore-L in a membrane via lateral diffusion. The long-lived fluorescent singlet excited state produced by excitation of Fluorazophore-L (blue to red) can form an encounter complex with added antioxidant (green), the rate of which is determined by the mutual lateral diffusion coefficient (D_L). Once the encounter complex is formed, the antioxidant quenches the fluorescence (k_d). The inset shows the presumed transversal immersion patterns of probe and quencher.

vitamin E constituents (tocotrienyl acetates) were 2 orders of magnitude lower (Table 1), which demonstrates that the isoprenoid tail does expectedly not contribute to radical scavenging,[58,81,82] but rather the chromanol ring does, with its active hydroxyl group.

The combined data therefore reveal substantial variations in the hydrogen donor propensities for the different vitamin E constituents, but the variation of the isoprenoid tail or stereochemistry has no significant effect (cf. Results). Despite the higher reactivity toward peroxyl radicals of recently reported synthetic vitamin E mimics,[83−86] α-tocopherol and α-tocotrienol remain the most active *natural* chain-breaking antioxidants known to date,[34] and their high hydrogen donor propensity is once more reflected in the present reactivity data in Table 1.

Structural Effects on Lateral Diffusion. Differences in the diffusion behavior of the antioxidants in membranes could be studied through a modification of the fluorescence quenching method, which involves Fluorazophore-L as a previously designed[14] amphiphilic DBO derivative. The principle of measurement is depicted in Figure 3: Both the fluorescent probe (blue) and the antioxidant (green) are immersed in a membrane, employing liposomes as models. Upon excitation with a short near-UV laser pulse, the fluorescent probe is promoted to its singlet excited state (red), which has the important photophysical property of being sufficiently long-lived (70−180 ns, depending on temperature and cholesterol content), to allow diffusional encounter complex formation with an antioxidant molecule. This diffusion occurs in a lateral manner,[14] because the lipid tail is anchored in the hydrophobic region of the lipid leaflet (see inset of Figure 3).

The formation rate of the encounter complex is limited by the mutual lateral diffusion coefficient of the probe/antioxidant pair (D_L). Owing to the favorable positioning of the uncharged reactive headgroups of both Fluorazophore-L and the chromanol ring of the vitamin E constituent near the lipid/water interface (see inset of Figure 3), and owing to the high reactivity of the antioxidant, immediate quenching occurs once the encounter complex is formed (k_d in Figure 3), such that the fluorescence quenching reports ultimately on the rate of lateral diffusion. The response of the time-resolved fluorescence decay to varying antioxidant concentrations, which is recorded after the excitation laser pulse, can be employed to extract the mutual lateral diffusion coefficient (D_L). It should be emphasized at this point that the liposome size distributions were narrow and monomodal, and no systematic effect of antioxidant addition on the liposome size was noticed (cf. Results). Also important, the custom-made vitamin E constituents[61−64] had purities far above commercial qualities (generally > 99%, Table 1), such that

(81) Burlakova, E. B.; Kukhtina, E. N.; Ol'khovskaya, I. P.; Sarycheva, I. K.; Sinkina, E. B.; Khrapova, N. G. *Biofizika* **1979**, *24*, 965−969.
(82) Ingold, K. U.; Burton, G. W.; Foster, D. O.; Hughes, L. *Free Radical Biol. Med.* **1990**, *9*, 205−210.
(83) Ingold, K. U.; Burton, G. W.; Foster, D. O.; Zuker, M.; Hughes, L.; Lacelle, S.; Lusztyk, E.; Slaby, M. *FEBS Lett.* **1986**, *205*, 117−120.
(84) Pratt, D. A.; DiLabio, G. A.; Brigati, G.; Pedulli, G. F.; Valgimigli, L. *J. Am. Chem. Soc.* **2001**, *123*, 4625−4626.
(85) Valgimigli, L.; Brigati, G.; Pedulli, G. F.; DiLabio, G. A.; Mastragostino, M.; Arbizzani, C.; Pratt, D. A. *Chem. Eur. J.* **2003**, *9*, 4997−5010.
(86) Wijtmans, M.; Pratt, D. A.; Valgimigli, L.; DiLabio, G. A.; Pedulli, G. F.; Porter, N. E. *Angew. Chem., Int. Ed.* **2003**, *42*, 4370−4373.

neither differences in sample purity or sizable artifacts due to impurities nor liposome size variations have to be considered further.

Interestingly, when the most reactive (α) and least reactive (δ) vitamin E constituents were immersed in liposomes, they showed, within error, the same efficiency of fluorescence quenching. From a structural (Chart 1) and reactivity (Table 1) point of view, the properties of the β and γ homologues should fall between those of the α and δ ones, such that we suggest comparable rates of lateral diffusion ($D_L = (1.6 \pm 0.2) \times 10^{-7}$ cm^2 s^{-1}) for all vitamin E constituents. The fact that the homologues display significantly different reactivity in nonviscous homogeneous solution (Table 1), but exhibit the same efficiency (near an apparently limiting maximum value) of fluorescence quenching when immersed in lipid membranes (Table 2), provides an important experimental manifestation of the diffusion-controlled nature of the quenching process in liposomes. Note that chemical reactions displaying some selectivity near the diffusion-controlled limit in nonviscous solution (like those of the tocopherols and tocotrienols with DBO in the acetonitrile−water mixtures, Table 1) can easily become diffusion-controlled in somewhat more viscous media, like lipids, such that any remaining differentiation in hydrogen donor propensity is eventually masked.

Intuitively, one expects similar diffusion coefficients for amphiphiles with only one hydrophobic, membrane-anchoring alkyl chain,[16] and the same should apply to the tocopherol and tocotrienol homologues, which are structurally even more closely related. It is therefore not surprising that any minor differences in the diffusional behavior become insignificant in liposomes. This means that the addition of methyl groups on the chromanol skeleton does not significantly decrease the diffusion coefficient of the tocopherol (as might be expected from a steric effect), while the change from a saturated hexahydrofarnesyl (or "phytyl") to an unsaturated farnesyl chain does not result in a significant change in diffusion of the tocotrienols versus tocopherols either. On the basis of this important result, differential diffusion of tocopherols and tocotrienols does not appear to be an important factor in discriminating their antioxidant activity in membranes and organisms.[42]

Relevance to Radical Scavenging and Antioxidant Activity. With respect to the characterization of the antioxidant potencies of the vitamin E constituents, it is first important to recall (see above) that we have moved from an at least partially reaction-controlled system (fluorescence quenching in nonviscous homogeneous solution, Table 1) to a diffusion-controlled system (fluorescence quenching in the more viscous membrane model, Table 2). This allows one to characterize both the relative hydrogen donor propensity and the diffusion coefficient of the antioxidant by means of two independent experiments. Which of the two factors is more relevant to radical scavenging in membranes depends largely on the reactivity of the reactive free radical that is involved. If free radicals formed in the membrane were highly reactive, akin to the singlet excited state of Fluorazophore-L, a diffusion-controlled reaction with a chain-breaking lipophilic antioxidant would be expected (unless a faster reaction with the lipid itself competes), such that the efficiency of radical scavenging would be limited by diffusion. If less reactive lipid radicals or oxidizing species are involved, e.g., the biologically most relevant peroxyl radicals, the selectivity will necessarily increase, and the relative efficacy of the antioxidant to donate a hydrogen atom should then become critical.

Second, while the hydrogen donor propensity and diffusion coefficient are jointly of utmost importance to understand and model antioxidant activity in membranes mechanistically and kinetically, the actual biological activity of an antioxidant is dependent on several additional factors affecting its bioavailability, biokinetics, and transport, including resorption, cellular conditions, enzymatic degradation, etc.[27,87] For instance, the hepatic α-tocopherol transfer protein selectively incorporates α-tocopherol into membranes and lipoproteins to result in a pronounced plasma preference.[88,89] A cytosolic α-tocopherol-binding protein has also been identified, which facilitates incorporation of α-tocopherol into and its transfer between membranes of intracellular organelles.[90]

In view of the described limitations, any interpretation of the reactivity data in Tables 1 and 2 with previously documented antioxidant effects of different tocopherols and tocotrienols must be made with the necessary caution. For example, the antioxidant potencies of tocopherols and tocotrienols toward peroxyl radicals were shown to increase with the extent of methyl substitution on the chromanol ring system.[2,34,75−79] In contrast, homologous tocopherols and tocotrienols displayed the same antioxidant activity in homogeneous solution,[75,81,91,92] in blood plasma,[91] in low-density lipoproteins,[91] and in liposomes.[75] However, other studies in biomembrane models and cellular systems have suggested that tocotrienols are more effective antioxidants than their tocopherol counterparts[92−96] and that α-tocotrienol could even provide a better protection against free radical-induced diseases than α-tocopherol,[94] for example against lipid peroxidation in supplemented heart tissue.[97]

The above outlined experimental results are generally in line with the present reactivity data (Table 1) and diffusion coefficients (Table 2), which suggest an enhanced reactivity with increasing methylation, but the same reactivity and diffusional properties for the different isoprenoid chain types. The important contrast that has emerged from the present study is that the lateral diffusion coefficients (Table 2) do *not* give rise to the expectation of an enhanced antioxidant activity of tocotrienols relative to tocopherols:[92−97] Neither does the hydrogen donor propensity differ, nor do the absolute diffusion coefficients suggest a significantly higher mobility of tocotrienols in membranes.

The higher antioxidant activity of tocotrienols versus tocopherols observed in some studies has been attributed by Packer

(87) Burton, G. W.; Ingold, K. U. *Annu. Rev. Nutr.* **1990**, *10*, 357−382.
(88) Burton, G.; Dutton, P.; Behrens, W.; Hodgkinson, B.; Foster, D.; Ingold, K. *FASEB J.* **1991**, *5*, A918−A918.
(89) Hosomi, A.; Arita, M.; Sato, Y.; Kiyose, C.; Ueda, T.; Igarashi, O.; Arai, H.; Inoue, K. *FEBS Lett.* **1997**, *409*, 105−108.
(90) Duttaroy, A. K.; Gordon, M. J.; Campbell, F. M.; Duthie, G. G.; James, W. P. T. *J. Nutr. Biochem.* **1994**, *5*, 562−570.
(91) Suarna, C.; Hood, R. L.; Dean, R. T.; Stocker, R. *Biochim. Biophys. Acta* **1993**, *1166*, 163−170.
(92) Suzuki, Y.; Tsuchiya, M.; Wassall, S. R.; Choo, Y. M.; Govil, G.; Kagan, V. E.; Packer, L. *Biochemistry* **1993**, *32*, 10692−10699.
(93) Serbinova, E.; Kagan, V. E.; Han, D.; Packer, L. *Free Radical Biol. Med.* **1991**, *10*, 236−275.
(94) Komiyama, K.; Iizuka, K.; Yamaoka, M.; Watanabe, H.; Tsuchiya, N.; Umezawa, I. *Chem. Pharm. Bull.* **1989**, *37*, 1369−1371.
(95) Noguchi, N.; Hanyu, R.; Nonaka, A.; Okimoto, Y.; Kodama, T. *Free Radical Biol. Med.* **2003**, *34*, 1614−1620.
(96) Saito, Y.; Yoshida, Y.; Akazawa, T.; Takahashi, K.; Niki, E. *J. Biol. Chem.* **2003**, *278*, 39428−39434.
(97) Serbinova, E. A.; Packer, L. *Method. Enzymol.* **1994**, *234*, 354−366.

and co-workers to a higher membrane reorientational dynamics induced by tocotrienol as opposed to a higher ordering effect of tocopherols on the lipidic phase.[92,93] Close inspection of the results that have led to this conclusion reveals, however, that the corresponding experiments were performed at unnaturally high antioxidant concentrations (20 mol % in DPPC[92] or 5 mol % in DMPC lipids), such that the implications for lateral diffusion in the context of antioxidant activity remain highly questionable.[93] For comparison, the present experiments were performed in a concentration range of 0.5−3 mol % vitamin E in POPC lipids, which compares better with the common natural range (0.1 mol % in most organelles[98] and up to 1.5 mol % in lysosomal membranes of the liver). As exemplified herein for cholesterol (see below and Table 2), additives may well have pronounced effects on membrane properties at high concentration (15−30 mol %), but insignificant ones at lower concentration (≤5 mol %). The effects of α-tocopherol and α-tocotrienol on membrane fluidity may therefore be entirely negligible at physiological concentrations, an opinion that is shared by others.[75] Moreover, their effects on the membrane surface, where the reactive lipid peroxyl radical sites are presumed to reside, were shown to be essentially the same.[75] A more "uniform" distribution of α-tocotrienol as compared to α-tocopherol was put forward as an alternative underlying reason for the (putative) higher antioxidant potency,[93,99] but such reasoning appears to be as much hypothetical as the fluidity effects, and it is not supported by the present fluorescence quenching data. Apart from these in part speculative arguments on the higher antioxidant activity of tocotrienols, one must also keep in mind that most in vitro experiments have *not* confirmed a higher antioxidant activity of tocotrienols.[75,81,91,92] In light of the present reactivity and diffusion data, we contend that other, "biological" factors (see above) and not the hydrogen donor propensity and membrane mobility (or related fluidity or uniformity effects) are responsible for the structural dependence of antioxidant activity of tocotrienols versus tocopherols in vivo.[94−97] For example, recent studies suggest that α-tocotrienol has a higher cellular uptake rate than α-tocopherol.[95,96]

Temperature Effects. We realized that the temperature dependence of fluorescence quenching of Fluorazophore-L in POPC liposomes could provide information on the temperature dependence of the lateral diffusion coefficients and thereby on the activation energies of diffusion in phospholipid membranes. Indeed, dramatic temperature effects on the quenching efficiency of Fluorazophore-L by antioxidants in liposomes were observed, which had several implications. From an experimental point of view, a very precise temperature control with a feedback cycle from a temperature sensor directly positioned in the cuvette was compulsory. From a mechanistic point of view, the large temperature effects implied large activation energies for the diffusion-controlled quenching process, which could be determined by measurements of the lateral diffusion coefficient at different temperatures (Table 2). The resulting Arrhenius plots, which are linear in the investigated temperature range ($r > 0.98$, $n = 5$, 15−50 °C, inset of Figure 2), led to activation energies of 47 kJ mol^{-1} for α-tocopherol, 39 kJ mol^{-1} for δ-tocopherol, and 49 kJ mol^{-1} for L-ascorbyl 6-palmitate (see below), all in POPC liposomes (10% error, Table 2). Considering possible sources of uncertainty, we interpret these values conservatively as being the same within error, such that we assign an activation energy of 44 ± 6 kJ mol^{-1} for the fluorescence quenching of Fluorazophore-L by the investigated antioxidants.

The measured activation energy for fluorescence quenching by α-tocopherol in homogeneous solution (10 ± 2 kJ mol^{-1}, in acetonitrile−water, 9:1) was found to be much lower than that in the lipid bilayer system. In terms of formal kinetics, the bimolecular rate constant of a diffusion-controlled reaction depends inversely on the solvent viscosity, such that the experimentally determined activation energies should coincide with the temperature dependence of solvent viscous flow in the different environments. The activation energy in acetonitrile−water lies only marginally above the activation energy for solvent viscous flow in neat acetonitrile (ca. 7 kJ mol^{-1}),[100] as expected for a close to diffusion-controlled reaction in nonviscous solution. Accordingly, the actual activation energy for fluorescence quenching within the encounter complex should be about 10 kJ mol^{-1} or less. This is consistent with high-level CAS-SCF-calculated activation energies for photoinduced hydrogen abstraction by DBO,[54,56,57] which constitutes the elementary photoreaction responsible for quenching of fluorazophores by hydrogen donors (k_d in Figure 3). The fact that the activation energies for Fluorazophore-L quenching in lipid membranes are much higher than this "reaction-controlled" limit, and more close to the activation energies for viscous flow projected from FRAP and NMR studies in POPC or egg-PC membranes (27−41 kJ mol^{-1}),[24,72,101,102] provides an important piece of experimental evidence that the fluorescence quenching in liposomes is limited by lateral diffusion, and not by the hydrogen transfer step. Jointly with the observed insensitivity toward the hydrogen donor propensity (methylation pattern) of the tocopherol homologues (see above) and the circumstantial evidence reported in our exploratory study (similar positioning in membrane, appropriate fitting according to lateral diffusion model, magnitude of the obtained diffusion coefficients, etc.),[16] we consider the experimental evidence in favor of a laterally diffusion-controlled quenching of Fluorazophore-L by vitamin E homologues in POPC lipid bilayers to be compelling.

While the small differences between the activation energies determined from the present fluorescence-quenching experiments and those from the previous FRAP and NMR studies may be related to either the investigated diffusing species, the different types of membrane models, or the techniques themselves, the immediate conclusion is as follows: *Lateral diffusion of antioxidants increases quite strongly with temperature, even within a small physiological range of 36−40 °C, which may potentially modulate their antioxidant activity.*

Effect of Cholesterol. To gain more realistic insights into the lateral diffusion kinetics of lipophilic antioxidants, we also studied the effect of cholesterol to better model the situation in natural membranes. We have studied a cholesterol concentration range of 0−30 mol %, to allow comparison with previous diffusion studies by alternative techniques,[72] and to cover the biologically most relevant concentration range; for example, Golgi apparatus membranes contain approximately 15 mol %

(98) Buttriss, J. L.; Diplock, A. T. *Biochim. Biophys. Acta* **1988**, *963*, 61−69.
(99) Nakano, M.; Sugioka, K.; Nakamura, T.; Ogi, T. *Biochim. Biophys. Acta* **1980**, *619*, 274−286.
(100) Saltiel, J.; Shannon, P. T.; Zafiriou, O. C.; Uriarte, A. K. *J. Am. Chem. Soc.* **1980**, *102*, 6799−6808.
(101) Kuo, A.; Wade, C. G. *Biochemistry* **1979**, *18*, 2300−2308.
(102) Lindblom, G.; Johansson, L. B.-A.; Arvidson, G. *Biochemistry* **1981**, *20*, 2204−2207.

cholesterol.[103] As can be deduced from the data in Table 2 (entries 1 and 8−10), the diffusion coefficient of α-tocopherol decreases slightly at high cholesterol content to result in a decrease by up to 15%. The data do therefore reveal the expected effect of cholesterol, which causes a rigidification and reduced fluidity or increased microviscosity of the membranes;[104,105] this limits diffusion of membrane additives, including antioxidants. Accordingly, judging on the basis of diffusional mobility alone, the activity of lipophilic antioxidants could be slightly higher in membranes with lower cholesterol content.

The effect of cholesterol on the mutual diffusion coefficient of the Fluorazophore-L/α-tocopherol probe/quencher pair compares well with the effect reported for the self-diffusion of lipids in the respective macroscopically aligned bilayers by pulsed-field gradient ^{1}H NMR.[72] Close inspection reveals that the decrease in diffusion coefficients is somewhat less pronounced for the fluorescence-based method, which is most likely due to differences in the diffusing species. Thus, while it is commonly accepted that lateral diffusion is slowed by addition of cholesterol, there are also some studies suggesting an enhanced diffusion, e.g., for lecithins,[72,101,102] which indicates a differential response of the diffusing species. Filippov et al. have also observed an apparent fluidizing effect at *low* cholesterol concentrations (2−3 mol %), which was attributed to a reduction in lipid chain entanglements.[72] Also Lemmich et al. reported on a softening of lipid bilayers upon incorporation of small amounts (<3%) of cholesterol.[106] At similarly low concentrations (5 mol %), the diffusion of α-tocopherol in POPC liposomes was in fact not significantly affected (entry 1 versus 8 in Table 2).

The putative patch or raft formation of cholesterol[107−109] presents another complication, which could lead to either an enhanced or reduced diffusion, depending on whether probe or quencher or both are included in or excluded from the cholesterol patches. The presently applied fluorescence-based technique has distinct advantages, since it is direct and time-resolved and does not rely on time-averaged spectroscopic observations. It may therefore be relevant to mention that our results do not provide evidence for such patch or raft formation; for example, the distribution of fluorescence lifetimes in the absence of antioxidant remains monomodal, and the lateral diffusion model can be identically applied in the presence and absence of cholesterol. Notably, epifluorescence microscopy measurements suggest a domain formation in POPC monolayers only above a concentration of 40 mol % cholesterol.[110] Regardless of those complications and contrasting results, all studies performed on lateral diffusion in cholesterol-containing membrane models, including the present fluorescence quenching work, demonstrate that the addition of cholesterol has no dramatic effects on the mobility of the additives, i.e., their lateral diffusion coefficients. *In particular and most important, the cholesterol effect is smaller than that caused by changing the temperature by 2−3 °C (Table 2), such that we rate the presence of cholesterol in natural concentrations as less significant in the context of the rate of diffusion of vitamin E in biomembranes and its consequences for antioxidant activity.*

Fluorescence Quenching by L-Ascorbyl 6-Palmitate. L-Ascorbyl 6-palmitate, the lipid-soluble palmitic acid derivative of vitamin C, can penetrate biomembranes,[52] exposing the ascorbyl moiety toward the aqueous environment.[51] It frequently provides a protection against lipid peroxidation,[111,112] because it is able to protect α-tocopherol from oxidation in lipidic environments[49] and to recycle endogeneous α-tocopheroxyl radicals during oxidative stress.[51,52] In contrast to unesterified ascorbic acid, L-ascorbyl 6-palmitate may also scavenge radicals in the core region of low-density lipoprotein particles.[113]

Within the present set of experiments, L-ascorbyl 6-palmitate was an exceptional case since its efficiency of fluorescence quenching in liposomes fell far below the expectations from the quenching rate constants measured in homogeneous solution. Thus, while its reactivity in homogeneous solution is similar to that of δ-tocopherol (Table 1), it becomes more than 1 order of magnitude less reactive in liposomes (Table 2). The immediate conclusion from this contrast, drawn by comparison with the data for the various vitamin E constituents (Table 2), is that the fluorescence quenching of L-ascorbyl 6-palmitate in liposomes is no longer laterally diffusion controlled.

Interestingly, the activation energy for fluorescence quenching is similar to that for the more efficient tocopherols as quenchers (inset of Figure 2), which reveals a major difference in preexponential factors as the underlying reason for the decreased reactivity of L-ascorbyl 6-palmitate. In other words, the reaction is entropically or statistically disfavored compared to tocopherols. We therefore assume that the mutual lateral diffusion coefficient for the Fluorazophore-L/L-ascorbyl 6-palmitate couple is comparable to those of the Fluorazophore-L/vitamin E pairs (cf. activation energies), but that the reaction efficiency upon encounter is very low. This can be rationalized by comparing the charge status. The chromanol headgroup of tocopherols is uncharged near neutral pH, such that a transversal position similar to that of the reactive uncharged headgroup of Fluorazophore-L can be presumed (inset of Figure 3). L-Ascorbyl 6-palmitate has the same alkyl tail as Fluorazophore-L, but its headgroup bears a negative charge (at neutral pH), which unquestionably displaces it more into the aqueous phase, away from the region where the azo chromophore would need to undergo collision-induced quenching and also away from the putative region where peroxyl radical groups would float.[16] Such a scenario would require L-ascorbyl 6-palmitate to undergo an up-and-down transversal motion in order to achieve the proper geometry for quenching, which imposes a statistical limit to this reaction within a membrane according to the Collins−Kimball model.[114] A more detailed investigation into the transversal requirements for fluorescence quenching of fluorazophores will require the design of additional derivatives with shorter and longer alkyl tails, of fluorazophore-labeled phos-

(103) Bigay, J.; Gounon, P.; Robineau, S.; Antonny, B. *Nature* **2003**, *426*, 563−566.
(104) Stockton, G. W.; Smith, I. C. P. *Chem. Phys. Lipids* **1976**, *17*, 251−263.
(105) Socaciu, C.; Jessel, R.; Diehl, H. A. *Chem. Phys. Lipids* **2000**, *106*, 79−88.
(106) Lemmich, J.; Mortensen, K.; Ipsen, J. H.; Hønger, T.; Bauer, R.; Mouritsen, O. G. *Eur. Biophys. J.* **1997**, *25*, 293−304.
(107) Bergelson, L. O.; Gawrisch, K.; Ferretti, J. A.; Blumenthal, R. *Mol. Membr. Biol.* **1995**, *12*, 1−162.
(108) Simons, K.; Ikonen, E. *Nature* **1997**, *387*, 569−572.
(109) Rietveld, A.; Simons, K. *Biochim. Biophys. Acta* **1998**, *1376*, 467−479.
(110) Worthman, L. A.; Nag, K.; Davis, P. J.; Keough, K. M. *Biophys. J.* **1997**, *72*, 2569−2580.
(111) Marinova, E. M.; Yanishlieva, N. V. *Fett Wiss. Technol.* **1992**, *94*, 448−452.
(112) Bowry, V. W.; Mohr, D.; Cleary, J.; Stocker, R. *J. Biol. Chem.* **1995**, *270*, 5756−5763.
(113) Gotoh, N.; Noguchi, N.; Tsuchiya, J.; Morita, K.; Sakai, H.; Shimasaki, H.; Niki, E. *Free Radical Res.* **1996**, *24*, 123−134.
(114) Collins, F. C.; Kinmball, G. E. *J. Colloid Sci.* **1949**, *4*, 425−437.

pholipids, and the tethering of charged residues, which are subject to future work.

In comparison to the vitamin E constituents studied herein, the present results imply a poorer chain-breaking antioxidant activity of L-ascorbyl 6-palmitate toward reactive lipid radicals as a consequence of a less favorable positioning within membranes. This notion, obtained from fluorescence-based measurements and manifested in an *apparently* reduced lateral diffusion coefficient, is in fact supported by several independent experimental observations. For example, in membranes containing both α-tocopherol and L-ascorbyl 6-palmitate, it is the tocopherol that is predominantly involved in the primary antioxidant reaction (scavenging of the reactive oxygen species), while the major role of L-ascorbyl 6-palmitate is a recycling of the tocopheroxyl radical product; that is, it is only involved in a secondary process.[51,52] Tocopheroxyl radicals have a much longer lifetime than peroxyl radicals, such that a favorable position of L-ascorbyl 6-palmitate is less critical. In fact, the recycling is otherwise known to be performed by *water-soluble* ascorbate,[2,115−118] which statistically resides even less often near the membrane surface.[15]

Conclusions

Fluorazophores are versatile and convenient fluorescent probes for antioxidants. The bimolecular fluorescence quenching rate constants of antioxidants in homogeneous solution provide a useful measure of their hydrogen donor propensity, which has been established first for glutathione, ascorbic acid, uric acid, and α-tocopherol,[10] subsequently for melanin-related metabolites,[11] and now for differently methylated tocopherols and tocotrienols. The ease of hydrogen atom abstraction from the phenolic O−H bond increases with increasing methylation degree of the chromanol ring ($\alpha > \beta \approx \gamma > \delta$), and this is reflected in the efficiency of fluorescence quenching in homogeneous solution. In other words, the more active antioxidant (in terms of radical-scavenging reactivity) causes more fluorescence quenching. The synthetic antioxidant L-ascorbyl 6-palmitate is a similarly reactive hydrogen donor as δ-tocopherol in homogeneous solution.

Fluorazophore-L is an amphiphilic derivative of DBO, which can be employed to monitor the interaction with antioxidants near the lipid−water interface. The fluorescence quenching in POPC liposomes as membrane models, which needs to be treated according to the formalism of two-dimensional diffusion and affords a mutual lateral diffusion coefficient instead of a time-independent rate constant, revealed no differences in the quenching efficiency for the different vitamin E constituents. Accordingly, the mutual lateral diffusion coefficient does not vary significantly with the methylation pattern or with the isoprenoid chain type (tocopherol versus tocotrienol). This contrasts with previous suggestions according to which a higher in vivo antioxidant activity of tocotrienols was related, among others, to a membrane fluidizing effect.[92,93,119] While the effect of structural variation among the tocopherols and tocotrienols has little effect on the efficiency of fluorescence quenching in POPC liposomes, L-ascorbyl palmitate displayed an unexpectedly low reactivity in liposomes, which is presumably due to its less favorable positioning within the membrane. As a particularly important result, lateral diffusion and therefore fluorescence quenching are strongly affected by temperature, and even a variation of 2−3 °C easily overwhelms the effect of added cholesterol, which decreased the lateral diffusion coefficient by merely 15% even at high concentration (30 mol %).

In summary, Fluorazophore-L allows one to conveniently monitor antioxidants in micro-heterogeneous biomimetic systems in real time by fluorescence spectroscopy and, in limiting cases such as for the vitamin E constituents, to determine the lateral diffusion coefficients of lipid-soluble antioxidants in membrane models. In combination with the fluorescence quenching of DBO in homogeneous solution, which affords relative hydrogen donor propensities, it is therefore possible to routinely and systematically "assay" two complementary kinetic parameters relevant for antioxidant activity.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(115) Doba, T.; Burton, G. W.; Ingold, K. U. *Biochim. Biophys. Acta* **1985**, *835*, 298−303.
(116) Niki, E.; Kawakami, A.; Yamamoto, Y.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1985**, *58*, 1971−1975.
(117) Packer, J. E.; Slater, T. F.; Willson, R. L. *Nature* **1979**, *278*, 737−738.
(118) Bisby, R. H.; Parker, A. W. *Arch. Biochem. Biophys.* **1995**, *317*, 170−178.
(119) Packer, L.; Weber, S. U.; Rimbach, G. *J. Nutr.* **2001**, *131*, 369−373.