Unsymmetrical Azo Initiators Increase Efficiency of Radical Generation in Aqueous Dispersions, Liposomal Membranes, and Lipoproteins

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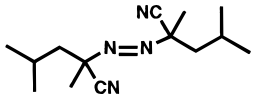
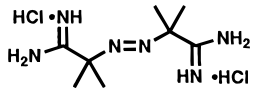
Abstract: Lipid peroxidation studies often employ the use of azo initiators to produce a slow, steady source of free radicals, but the lack of initiators capable of efficiently generating radicals in lipid aggregates such as micelles and membranes has created persistent problems in these investigations. We report here the synthesis and study of unsymmetrically substituted (hydrophilic/hydrophobic) azo initiators **C-8**, **C-12**, and **C-16** that increase the efficiency of radical generation in lipophilic regions of aqueous emulsions such as micelles and liposomes. Radical generation from these initiators was monitored in micelles, liposomes, and lipoproteins by the use of two radical scavengers, one that scavengers lipophilic peroxyl radicals and one that scavenges hydrophilic peroxyls. The lipophilic radical scavenger used was the well-known antioxidant α-tocopherol and the hydrophilic radical scavenger used was uric acid. Two peroxyl radicals are trapped by each of these scavengers, tocopherol presumably being biased toward reacting with lipid soluble radicals, uric acid presumably reacting preferentially with water-soluble radicals. In Triton X-100 micelles the unsymmetrical initiators **C-8** and **C-16** display an increase in both α-TOH (α-tocopherol) trapping and in overall radical generation efficiency compared to the symmetrical initiators **C-0** (hydrophilic) and **MeOAMVN** (lipophilic). The unsymmetrical azo initiators performance in liposomes was excellent (increased cage escape with lipid compartment access). In low-density lipoprotein oxidations, the initiators **C-8**, **C-12**, and **C-16** also provided advantages over **C-0** and **MeOAMVN**. The hydrophilic/hydrophobic character of the two radicals generated from the unsymmetrical initiators is an important factor for separating the geminate radical pair. These initiators, when compared to the widely used symmetrical azo initiators, provide an advantage of free radical production, lipophilic access, and constant radical generation in the investigation of lipid peroxidation in various media.

Lipid peroxidation has been increasingly implicated in a number of pathological events including atherosclerosis, liver disease, tumorigenesis, and various neurological disorders, such as Alzheimer's disease.[1] Initiation and antioxidant defenses are of key importance to the mechanism(s) of lipid peroxidation, and α-tocopherol (α-TOH) has attracted attention as a peroxidation chain breaker since it is a naturally abundant and potent lipophilic antioxidant.[2]

Lipid peroxidation studies often employ the use of azo initiators to produce a slow, steady source of free radicals, but the lack of initiators capable of efficiently generating radicals in lipid regions has created persistent problems in these investigations.[3] The azo compounds **AMVN** (2,2′-azobis(2,4-dimethylvaleronitrile)) and **AAPH** (2,2′-azobis(2-amidinopropane) dihydrochloride), frequently used to investigate these oxidations, present difficulties.[4,5] For example, the increased viscosity of the lipid environment in lipid bilayers and the macromolecular "cage" nature of a lipoprotein significantly prevents the cage escape of the lipophilic radicals formed in the decomposition of **AMVN**.[3] Studies of the peroxidation of low-density lipoproteins (LDL), for example, include the use of high concentrations of **AMVN** to produce initiation at a reasonable rate. Such high initiator concentrations may compromise the integrity of the lipoprotein.



AMVN



AAPH

While the hydrophilic radicals derived from **AAPH** exhibit efficient cage escape in water, the transfer of these radicals into the lipid region of a molecular aggregate is dependent upon some transport mechanism(s). In fact, it has been demonstrated that peroxyl radicals derived from **AAPH** in the aqueous phase do not initiate low-density lipoprotein (LDL) oxidation in the absence of α-TOH.[6] Indeed, there is an increasing body of evidence suggesting that subtle changes in the initiator source, rate of decomposition, or location of radical formation can influence mechanistic pathways of peroxidation.[4]

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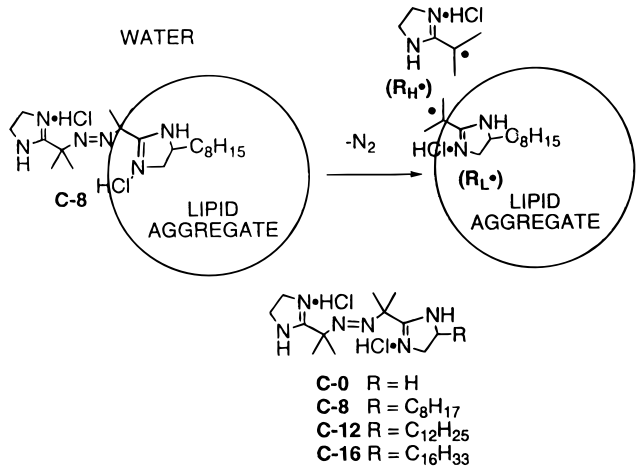
Scheme 1



Given the limitations of existing initiators, there is a need for new compounds that efficiently generate radicals in lipid aggregate structures such as lipoproteins, liposomes, and aqueous dispersions (or micelles).[5] We report here the synthesis and the study of the new unsymmetrical azo compounds, **C-8**, **C-12**, and **C-16** (Scheme 1), that decompose at convenient temperatures with an increased lipophilic radical generation efficiency. These new initiators generate one amphiphilic radical, $\mathbf{R_L^{\bullet}}$, and one hydrophilic radical, $\mathbf{R_H^{\bullet}}$, in the geminate radical pair. The results of this study suggest that the amphiphilic radical associates with the lipid aggregate while the hydrophilic radical escapes to the aqueous environment. The use of the "hydrophobic effect" to separate the geminate radical pair apparently overcomes the difficulties of cage escape and lipid compartment access that plagues the use of the symmetrical initiators **AMVN** and **AAPH**.

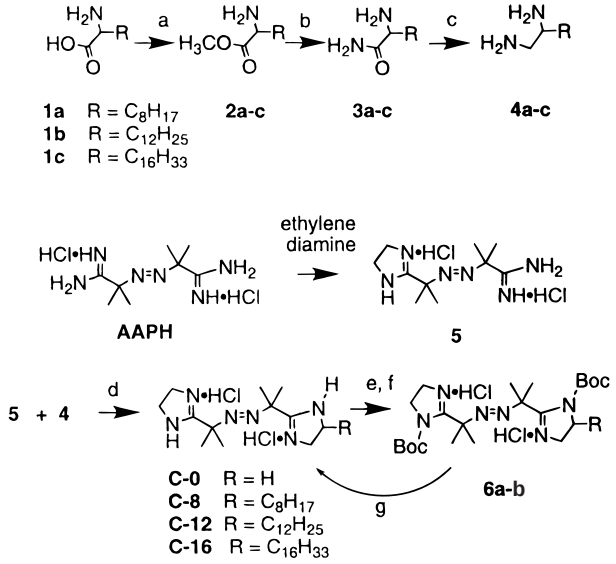
Results and Discussion

Initiator Synthesis. Initiator structures based on the imidazoline functional group were chosen since the parent compound, **C-0** (2,2′-azobis(*N*,*N*′-dimethyleneisobutyramidine) dihydrochloride), was previously shown to have a substantially shorter half-life for decomposition than that of the acyclic counterpart, **AAPH**.[7] Thus, **C-0** has a rate constant for decomposition in water at 50 °C five times that of **AAPH**.

The synthesis of **C-8**, **C-12**, and **C-16** was based on the displacement of ammonia from amidines by 1,2-diamines.[8] The general approach is outlined in Scheme 2. The 1,2-diamine **4** was prepared by LAH reduction of the amide and **4** was then reacted with the unsymmetrical azo compound **5**. The compound **5** was prepared by exchange of ethylenediamine on to **AAPH**. The bis hydrochloride salt of **5** crystallizes from ethanol−ether solvent mixtures.

Purification of the unsymmetrical initiators, **C-8**, **C-12**, and **C-16**, proved to be difficult. Recrystallization was successful for purification of **C-16** while the other two initiators proved to be contaminated with azo compound **5** as an impurity under all recrystallization conditions attempted. Chromatography of the compounds was also unsuccessful. A purification method for use with each of the initiators was developed that involved their conversion to the bis-BOC protected compounds **6**. The protected imidazoline chromatographs well on silica gel and

Scheme 2



a. MeOH, $SOCl_2$. b. MeOH, NH_3, c. $LiAlH_4$ d. MeOH, NaOMe, e. NaOMe, f. $Na_2CO_3/(Boc)_2O$, g. TFAA

the pure unsymmetrical initiator was generated, after chromatography, by BOC removal with trifluoroacetic acid.

Initiator Decomposition. The unsymmetrical initiators were designed for use in lipid emulsions or molecular aggregates and determining the rates for decomposition of the compounds in a variety of media was therefore of critical importance. Anionic SDS (sodium dodecyl sulfate), neutral Triton X-100, and cationic TTAB (tetradecyltrimethylammonium bromide) micelles were chosen for study since micelles from these surfactants are in common usage. Micelles generated from these surfactants are appropriate to test micellar charge effects on the azo initiators since SDS and TTAB micelles bear negative and positive charges respectively while Triton micelles are uncharged. Liposomal bilayer membranes were also chosen for study and multilamellar vesicle (MLV) aggregates were prepared from plant phosphatidyl choline (PC) as previously described.

Kinetics for initiator decomposition were determined by measurement of the disappearance of the azo absorption in the UV at 366 nm.[7] Experiments with **C-0** in methanol solvent gave good first-order kinetics over 3 half-lives. Rate constants for **C-0** decomposition, k_d, were determined at 65 °C, 2.3×10^{-4} s^{-1}, $\tau_{1/2} = 0.8$ h; 58 °C, 1.1×10^{-4} s^{-1}, $\tau_{1/2} = 1.8$ h; and 50 °C, 3.9×10^{-5} s^{-1}, $\tau_{1/2} = 5.0$ h. Extrapolation of these rate constants to 37 °C gives a value of $k_d = 7.1 \times 10^{-6}$ s^{-1}, $\tau_{1/2} =$ 27 h at this temperature. Rate constants determined for **C-8**, **C-12**, and **C-16** were similar to those measured for **C-0** in methanol. Rates of decomposition of **C-0** could be determined in phosphate saline buffer at pH 7.4 as well as in this buffer containing SDS, TTAB, and Triton X-100 micelles. The unsymmetrical initiators **C-8**, **C-12**, and **C-16** are themselves amphiphiles and it was impossible to monitor the loss of the azo chromophore for these initiators in micellar media. Rate constants could not be determined by UV for any of the initiators in PC multilamellar liposomes since these emulsions are milky white and not conducive to UV analysis. Rate constants for decomposition of **C-0** in pH 7.4 buffer, k_d, were determined at 80 °C, 7.3×10^{-4} s^{-1}, $\tau_{1/2} = 0.3$ h; 65 °C, 1.4×10^{-4} s^{-1}, $\tau_{1/2}$ $= 1.4$ h; 50 °C, 2.4×10^{-5} s^{-1}, $\tau_{1/2} = 8$ h; and 37 °C, $4.7 \times$ 10^{-6} s^{-1}, $\tau_{1/2} = 41$ h. Rate constants for decomposition of **C-0** in pH 7.4 buffer micelles at 37 °C were as follows: 0.5 M SDS,

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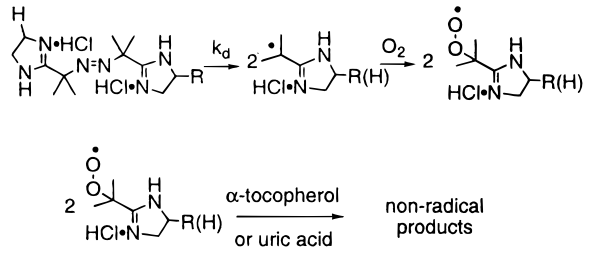
(8) Dougherty, T. J. *J. Am. Chem. Soc.* **1961**, *83*, 4849−4853.

Scheme 3



3.3×10^{-6} s^{-1}, $\tau_{1/2} = 58$ h; 0.1 M Triton X-100, 4.5×10^{-6} s^{-1}, $\tau_{1/2} = 43$ h; and 0.1 M TTAB, 4.5×10^{-6} s^{-1}, $\tau_{1/2} = 43$ h. Surfactant concentrations were chosen such that the experiment was carried out at least 2 orders of magnitude above the critical micelle concentration and such that the initiator concentration was approximately 1% of the surfactant concentration.

Free Radical Generation and Scavenging. Radical generation was monitored by the use of two antioxidants, one lipophilic, α-tocopherol (α-TOH) and one hydrophilic, uric acid (UA).[9] Both α-TOH and UA are efficient radical chain breakers that scavenge two free radicals per antioxidant molecule.[9,10] The widely used aqueous antioxidant ascorbic acid is known to reduce α-tocopheroxyl radicals (α-TO•) and regenerate α-TOH across the interface of micelles, liposomes, and LDL.[11,12] Uric acid does not exhibit this regenerating capability with α-TOH in any system so far investigated.[9] The experiments carried out monitored the rate of disappearance of α-tocopherol and uric acid during the decomposition of a given initiator. Since we monitor radical consumption by the use of two radical scavengers, α-TOH and UA, the sum of rates of consumption for the two scavengers should give information about the efficiency of radical generation from a given initiator under given conditions of decomposition.

If the efficiency of radical generation from an initiator were 100%, then the sum of the rates of scavenger consumption would be equal to the rate of initiator decomposition since the initiator generates two radicals and both antioxidants (α-tocopherol and uric acid) consume two radicals. Furthermore, the partitioning of the radical formed from the initiator between α-TOH and UA presumably gives information about the locus of the scavenged radical in the emulsion. Lipophilic radicals would presumably be scavenged by α-TOH while hydrophilic radicals would be trapped by UA. The chemistry described in these studies is outlined in Scheme 3. Values for observed initiator free radical flux, as measured by consumption of scavengers, are given by $2e_T \cdot k_d$[initiator], where e_T is the total efficiency of radical escape from the initial radical pair formed in the decomposition (100•escape fraction), and k_d[initiator] is the rate of geminate radical pair formation. Two radicals are generated per initiator decomposition accounting for that factor in eq 1; an efficiency (e_T) of 100% means that all radicals formed from an initiator escape from the solvent cage and react with a radical scavenger. Equations 2 and 3 separate the total efficiency of radical generation into lipophilic and hydrophilic components (e_L and e_H). The hydrophobic term, e_H, represents radicals that have escaped the cage and that are trapped by uric acid while the lipophilic term, e_L, reports the efficiency of escape of radicals that leads to scavenging by the lipophilic scavenger α-tocopherol.

$$\text{Rate of free radical formation from initiator} = 2\, e_T \times k_d\,[\text{initiator}] \quad (1)$$

$$2\, e_T \times k_d\,[\text{initiator}] = 2\, d[\alpha\text{-tocopherol}]/dt + 2\, d[\text{uric acid}]/dt \quad (2)$$

$$e_T = e_L + e_H = \frac{d[\alpha\text{-tocopherol}]/dt}{k_d\,[\text{initiator}]} + \frac{d[\text{uric acid}]/dt}{k_d\,[\text{initiator}]} \quad (3)$$

To determine the values of e_T, e_L and e_H, the rate of consumption of both α-tocopherol and uric acid was determined. Dividing these rates by the k_d[initiator] appropriate for the medium of study gives e_L and e_H; the sum of these two efficiencies is e_T.[9,13] Rates of consumption of α-tocopherol and uric acid were determined by monitoring the disappearance of these compounds by HPLC. In a typical experiment in micelles or liposomes, initiator (∼2 mM), surfactant (0.1−0.5 M), α-tocopherol (∼0.1 mM), uric acid (∼0.1 mM), and methyl linoleate (∼5 mM)[14] were vortexed in pH 7.4 phosphate buffer and kept at 37 °C for several hours (<0.1 half-lives) during which time aliquots were taken and analyzed for α-tocopherol and uric acid. Rates of tocopherol and urate consumption were determined and e_T, e_L, and e_H were calculated by the use of eq 3.

Scavenging Efficiencies. Table 1 and Figures 1−3 summarize the results obtained from the azo initiators tested in TTAB, SDS, and Triton-X100 micelles. The following general trends are noted: (a) In TTAB micelles, a general trend for nearly an equal split between lipophilic and hydrophilic scavenging, e_L and e_H, was observed for all of the intiators and e_T• was high. (b) In SDS micelles α-TOH was the primary antioxidant consumed for **C-0** and the unsymmetrical initiators, **C-8** and **C-16** ($e_L > e_H$). (c) In Triton X-100, radical generation from **C-0** was significantly less than that observed for the unsymmetrical initiators. The e_L term for the unsymmetrical initiators was very high. (d) The symmetrical initiator **MeOAMVN** (2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile)) has a low efficiency of radical generation in Triton X-100 micelles (e_T = 9%), consistent with earlier reports that symmetrical initiators such as **AMVN** and **MeOAMVN** have low efficiencies of radical generation in surfactant emulsions.[5,11] Some of the e_T's determined were greater than 100%, a result that is obviously in error. These errors are likely due to the assumptions made in the calculations, particularly with regard to the extrapolation of k_d values from **C-0** to the unsymmetric compounds.[15]

The anionic, neutral, and cationic micelles each interact differently with the cationic azo initiators tested. Of the micelles investigated, TTAB shows the highest consumption of uric acid, which may be due to the surfactant's cationic character.[16] The charge on the micelle has an effect of gathering scavenger to

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(13) 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.

(14) In TTAB micelles the presence of methyl linoleate with α-TOH and no initiator caused significant antioxidant consumption. Thus, methyl linoleate was excluded from these experiments.

(15) Preliminary experiments have been carried out measuring the rates of initiator decomposition in molecular aggregates such as micelles and liposomes by nitrogen evolution. These preliminary studies suggest that the assumption of a common rate of decomposition for **C-0**, **C-8**, **C-12**, and **C-16** is reasonable, To be submitted for publication: S. M. Culbertson, N. A. Porter, L. R. C. Barclay, and M. Vinqvist.

(16) If the micelle captures urate because of charge effects, urate will compete effectively for lipophilic peroxyl radicals. **C-0** decomposes in the aqueous phase where UA can scavenge a large fraction of the free radicals before partitioning into the lipid phase.

Table 1. Radical Generation Efficiency of Azo Initiators in Micelles

initiator	surfactant	d[αToc]/d*t* [initiator]	d[urate]/d*t* [initiator]	k_d	e_L	e_H	e_T
C-0	SDS	3.4×10^{-6}	2.5×10^{-7}	3.3×10^{-6}	102	7	109
	X-100	1.5×10^{-6}	1.2×10^{-6}	4.5×10^{-6}	34	27	61
	TTABS	1.5×10^{-6}	1.8×10^{-6}	4.5×10^{-6}	33	40	73
C-8	SDS	1.5×10^{-6}	4.5×10^{-7}	3.3×10^{-6}	44	14	58
	X-100	4.0×10^{-6}	1.3×10^{-6}	4.5×10^{-6}	88	29	117
	TTABS	2.1×10^{-6}	1.8×10^{-6}	4.5×10^{-6}	47	40	87
C-16	SDS	1.7×10^{-6}	2.0×10^{-8}	3.3×10^{-6}	52	1	53
	X-100	4.1×10^{-6}	9.0×10^{-7}	4.5×10^{-6}	90	20	110
	TTABS	8.2×10^{-7}	1.4×10^{-6}	4.5×10^{-6}	18	32	50
MeOAMVN	X-100	2.2×10^{-6}	6.4×10^{-7}	3.2×10^{-5}	7	2	9

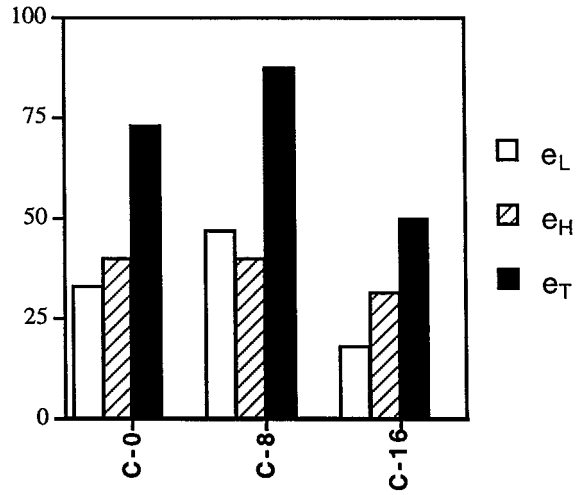

Figure 1. Efficiencies for scavenging by α-tocopherol (e_L), uric acid (e_H), and total (e_T) in TTAB. The e's are as defined in the text. Efficiencies based on α-TOH and uric acid (UA) consumption rates at 37 °C in air. The k_d values for **C-0** were measured in 10 mM PBS (pH 7.4) for liposomes and LDL, and in each respective micelle. The k_d values for the unsymmetrical initiators were estimated by using **C-0** values. Micelles composed of TTAB (0.1 M) containing initiator 2 mM, α-TOH ≤ 140 μM, and UA ≤ 150 μM.

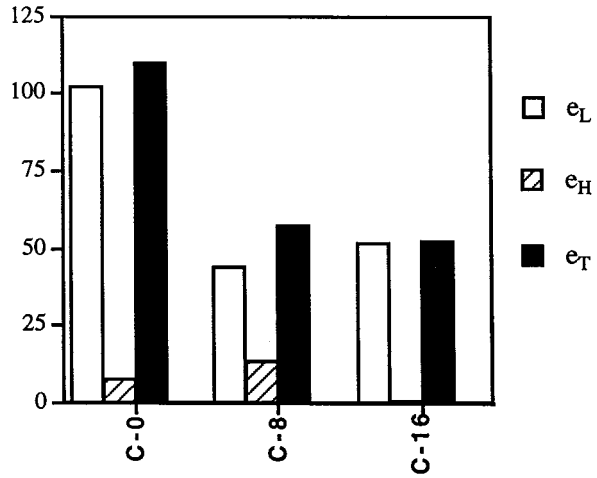

Figure 2. Efficiencies for scavenging by α-tocopherol (e_L), uric acid (e_H), and total (e_T) in SDS. See caption for Figure 1. Micelles composed of SDS (0.5 M) and methyl linoleate (5.0 mM).

the micellar surface. Urate is an anion at pH 7.4,[10] and it presumably associates with the cationic micelle surface where it can scavenge a higher percentage of radicals generated near the aqueous interface. The total efficiency of scavenging of radicals in SDS micelles (e_T) is ~100% for the water-soluble symmetric initiator **C-0** and approximately 50% for the unsymmetric initiators. The acyclic analogue of **C-0, AAPH**, has a high efficiency of radical generation, consistent with the results

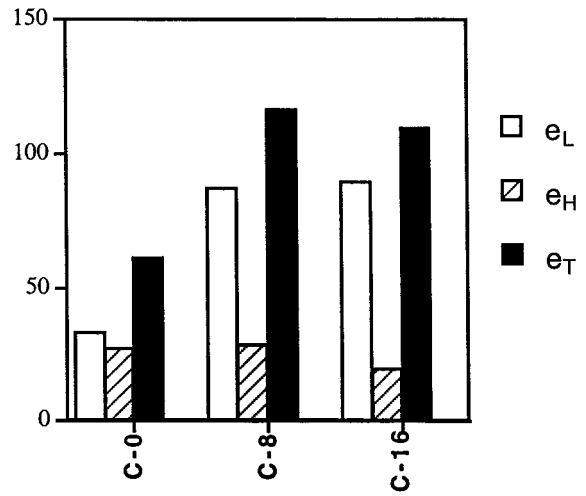

Figure 3. Efficiencies for scavenging by α-tocopherol (e_L), uric acid (e_H), and total (e_T) in Triton-X100. See caption for Figure 1. Micelles composed of Triton X-100 (0.1 M) and methyl linoleate (5.0 mM).

from **C-0**. What is nevertheless surprising is the fact that nearly all of the radicals generated by the water-soluble intitiator are trapped by the hydrophobic inhibitor, α-TOH. Indeed, α-TOH is the dominant radical scavenger for **C-8** and **C-16** as well. We suggest that the anionic surface of SDS micelles acts to trap cationic azo initiator or their derived peroxyl radicals by salt exchange, significantly favoring scavenging by α-TOH near the micelle surface. In short, the cationic initiators or the radicals formed from each initiator associate with negatively charged SDS micelles as does lipophilic α-TOH. The radicals and the scavenger are associated with the same aggregate leading to a bias for consumption of α-TOH. Association of cations with negatively charged micelles is the basis of surfactant catalysis; the phenomenon is well-known.[17] We speculate that the differences in overall efficiency between **C-0** and the unsymmetrical intitiators (**C-8** and **C-16**) in SDS is due to exchange of the radicals out of the initial geminate radical pair micelle aggregate for **C-0**. This exchange process would be expected to be slower for the more lipophilic radical derived from **C-8** or **C-16**. An alternate explanation for the high efficiency observed from **C-0** is the possibility that its decompostion occurs outside of the aggregate while **C-8** and **C-16** decompose while associated with the micelle. Radicals derived from **C-0** will likely associate with different micelles while radicals derived from **C-8** and **C-16** are confined, at least initially, to the same micelle. This leads to a low e_T for the unsymmetrical initiators. In Triton X-100 micelles, no ion association with the micelle is expected. The hydrophilic/hydrophobic character of the two radicals generated from the unsymmetrical initiators should be the dominant factor leading to differences between **C-0** and the

(17) See, for example: Fendler, J. H.; Fendler, E. J. *Catalysis in Micellar and Macromolecular Systems*; Academic Press: New York, 1975.

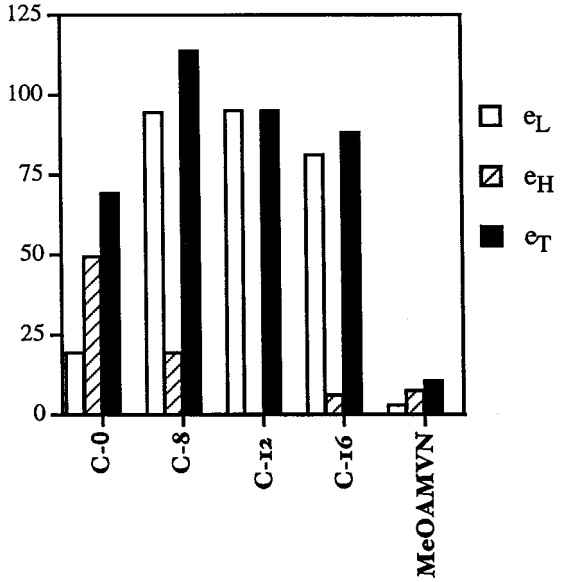

Figure 4. Efficiencies for scavenging by α-tocopherol (e_L), uric acid (e_H), and total (e_T) in phosphatidyl choline liposomes. See caption for Figure 1. Liposomes prepared from plant PC (10.3 mM) containing initiator (2 mM), α-TOH ≤140 μM, and UA (150 μM).

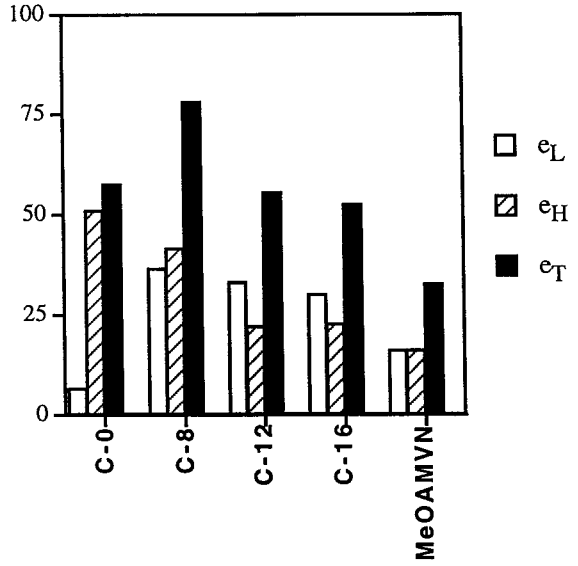

Figure 5. Efficiencies for scavenging by α-tocopherol (e_L), uric acid (e_H), and total (e_T) in low-density lipoproteins (LDL). See caption for Figure 1. Oxidation of LDL (0.75 mg protein/mL) containing endogenous α-TOH (12 μM), added UA (60 μM), and 0.5 mM initiator, except 0.25 mM for **MeOAMVN**.

amphiphilic initiators, **C-8** and **C-16**. The results suggest that the hydrophobic effect is an important factor leading to separation of the geminate radical pair for these initiators. We also note the observed high efficiencies of radical generation for **C-8** and **C-16** ($e_T \sim 100\%$) as compared to **MeOAMVN** (9%, see Table 1) in Triton X-100 micelles. **MeOAMVN** is a symmetric lipophilic initiator and the geminate radical pair separates with very low efficiency in the micellar media.

The greatest effect on lipophilic radical generation observed was in phosphatidyl choline liposomes. The data for studies in liposomes are presented in Figure 4. Overall efficiency for **C-0** was good, but the fraction of radicals scavenged in the lipophilic phase was low. **MeOAMVN** efficiency in phosphatidyl choline liposomes was very low overall with a negligible consumption of α-TOH. In contrast, the unsymmetrical azo initiators performance in liposomes was excellent. The high overall efficiency observed suggests that most radicals derived from these initiators escaped the initial cage. The high α-TOH consumption also supports the conclusion that most radicals had access to the lipid phase of these multilamellar vesicles.[18]

Unsymmetrical azo initiators also provided increased lipophilic radical generation in LDL, Figure 5. The low lipophilic scavenging (e_L) of **C-0** suggests that the aqueous peroxyl radicals it generates do not have efficient access to the lipid core of LDL.[6,11] Each of the unsymmetrical azo initiators tested displayed significantly greater lipophilic scavenging e_L than did **C-0**. The addition of the alkyl chain to one radical in the pair apparently allows greater cage escape and access to LDL lipid regions. It was surprising that the symmetrical **MeOAMVN** initiator showed a higher overall efficiency in LDL than was observed in either micelles or liposomes.[19] We point out that the rate constant for decomposition of all of the initiators in LDL and liposomes is assumed to be comparable to rate constants determined in pH 7.4 buffer and this assumption may lead to errors in the calculated *e*'s.[15] By the same token, the rate constant for decomposition of **MeOAMVN** in LDL was assumed comparable to rates of decompostion in acetonitrile to calculate *e*'s.[5] The calculated efficiencies may reflect the fact that this assumption is not valid. Experiments are in progress to determine initiator k_d values in lipoproteins and other molecular aggregates which would avoid the necessity of any rate constant assumptions.[15]

The experiments presented here demonstrate that unsymmetrical azo initiators increase free radical generation efficiency in the lipid phase of neutral micelles, PC liposomes, and LDL. These initiators, when compared to the widely used symmetrical azo initiators, provide an advantage of free radical production, lipophilic access, and consistent radical generation in the investigation of lipid peroxidation in various media. They may provide valuable tools in the investigation of lipid peroxidation, antioxidant protection, or even emulsion polymerization. Furthermore, the results show that charge on a radical and molecular aggregate host can have a significant effect on the radical chemistry. Negatively charged micelles such as SDS gather cationic radicals derived from the initiators and this has impact on the ultimate fate of the radicals.

Experimental Section

α-Aminodecanoic Acid (1a).[20] Diethyl acetamidomalonate (24.3 g, 0.11 mol) and 1-bromooctane (29 g, 0.15 mol) were heated under reflux in a solution of sodium (2.5 g, 0.11 mol) in EtOH (85 mL) for 24 h. Upon cooling, the mixture was poured onto ice/water (160 mL) and the precipitate filtered and washed with water. The crude solid was placed into a 500 mL round-bottom flask, concentrated HCl (180 mL) and DMF (20 mL) were added, and then the mixure was heated at reflux for 24 h. The mixture was allowed to cool, poured into a solution of EtOH/water (3:1), and neutralized with concentrated aqueous NH_3. The precipitate was filtered and washed with EtOH/water; crude yield of **1a** 14.6 g (71%). Crude product **1b** was carried directly onto the next step. Crude yield of **1c** 29.5 g (80%).

Methyl α-Aminodecanoate Hydrochloride (2a).[20] The crude amino acid **1a** (25.9 g, 0.14 mol) was heated at reflux in a mixture of thionyl chloride (11.2 mL, 0.15 mol) and MeOH (60 mL) for 24 h. Ninety

(18) The initiators **C-8**, **C-12**, and **C-16** primarily decompose in the lipid aggregate where both free radicals generated can be quickly scavenged by α-TOH in the immediate vicinity.

(19) It has been reported that **MeOAMVN** peroxyl radical formation in LDL as measured by the single, lipophilic antioxidant DPPD gave an $e \times k_d$ value of 4.14×10^{-6} (or % $e = 13\%$).[5] Our experience with the antioxidant DPPD implicated it did not function the same as α-TOH in micelles and liposomes tested. In fact, results suggest that there is an interconnection (possibly a regeneration of uryl radical) between UA and DPPD across the aqueous/lipid interface (data not shown).

percent of the solvent was removed and the precipitated product filtered and recrystallized from MeOH. **2a:** Yield 29.0 g (88%). ^{1}H NMR (300 MHz, CD_3OD) δ 4.05 (t, 1 H); 3.85 (s, 3 H); 1.90 (m, 2 H); 1.35 (m, 12 H); 0.90 (t, 3 H). **2b:** Crude yield 25.4 g (overall 79%). ^{1}H NMR (300 MHz, CD_3OD) δ 4.05 (t, 1 H); 3.85 (s, 3 H); 1.90 (m, 2 H); 1.35 (br m, 20 H); 0.90 (t, 3 H). **2c:** Yield 37.2 g (82%). Mp 103 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 4.05 (t, 1 H); 3.85 (s, 3 H); 1.90 (m, 2 H); 1.35 (br m, 28 H); 0.90 (t, 3 H). MS (FAB) calcd for $[C_{19}H_{40}NO_2]^+$ 314.3, found 314.3.

α-Aminodecanamide (3a).[21] The amino methylester hydrochloride **2a** (29.0 g, 0.12 mol) was dissolved in 250 mL of methanol and saturated with NH_3 at 0 °C. The solution was resaturated with NH_3 at 0 °C after 5 h and once more after 2 d. After 4 d the solvent was removed. The resulting amide, amide hydrochloride, and NH_4Cl were treated with 10% NaOH and extracted with $CHCl_3$. Recrystallization from EtOH yielded 15.1 g (66%) of solid α-amino amide **3a**. ^{1}H NMR (300 MHz, CD_3OD) δ 3.85 (t, 1 H); 1.85 (m, 2 H); 1.30 (br m, 12 H); 0.90 (t, 3 H). **3b:** Yield 16.5 g (79%). Mp 95 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 3.84 (t, 1 H); 1.85 (m, 2 H); 1.30 (br m, 20 H); 0.90 (t, 3 H). **3c:** Yield 10.4 g (45%). Mp 98 °C.[22] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.10 (s, 2 H); 5.50 (s, 2 H); 3.35 (m, 1 H); 1.85 (m, 2 H); 1.25 (m, 28 H); 0.90 (m, 3 H).

1,2-Diaminodecane (4a).[22,23] The α-amino amide **3a** (12.0 g, 64.5 mmol) was added in small portions to a suspension of $LiAlH_4$ (5.39 g, 142 mmol) in 140 mL of anhydrous THF. The reaction mixture was stirred and heated under reflux for 24 h. It was then cooled to 5 °C and cautiously quenched by adding, successively and dropwise, water (5.4 mL), 10% NaOH (8.1 mL), and finally water (13.5 mL). The precipitated, granular alumina was filtered, washed with hot THF, and then extracted three times with boiling THF. The combined filtrates were taken to dryness in vacuo at 50 °C. Crude yield: **4a** 80%, **4b** 92%, **4c** 92%. A portion of the crude 1,2-diaminodecane (5.8 g, 34 mmol) was dissolved in MeOH (125 mL) and treated with $(BOC)_2O$ (18.2 g, 83 mmol) and triethylamine (11.65 mL, 83 mmol). After the mixture was stirred for 2 h, the solvent was evaporated to give a crude oil. Gradient FC (5%, 10%, 20%, 40%, 80%, 100% EtOAc in hexanes with 0.2% TEA) gave the BOC-protected diaminodecane as a white solid, 9.2 g (73%). The solid was dissolved in MeOH and treated with excess HCl gas. Recrystallization from MeOH/Et_2O provided a purified dihydrochloride that was converted to the free base by treatment with aqueous NaOH and extraction with EtOAc. **4a:** Yield 4.42 g (60%) of a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.74 (dd, 1 H); 2.65 (m, 1 H); 2.44 (dd, 1 H); 1.28 (br m, 14 H); 0.89 (t, 3 H). **4b:** Yield colorless, semisolid (41%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.75 (dd, 1 H); 2.65 (m, 1 H); 2.45 (dd, 1 H); 1.30 (br m, 22 H); 0.90 (t, 3 H). **4c:** Yield white powder (56%). Mp 59 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.74 (dd, 1 H); 2.65 (m, 1 H); 2.45 (dd, 1 H); 1.28 (br m, 30 H); 0.89 (t, 3 H).

2,2′-Azobis[2-(2-imidazolin-2-yl)propane] Dihydrochloride (C-0).[24] In a 500 mL three-necked round-bottom flask was dissolved a sample of AAPH (10 g, 0.037 mol) in 200 mL of anhydrous MeOH which was treated with ethylenediamine (12.5 mL, 0.185 mol) at room temperature. One neck of the flask was connected to an argon line that had a hollow glass tube long enough to bubble Ar directly into the solvent. The second neck served as the exhaust over the surface of the solvent. The exhaust line was submersed into an erlynmeyer flask of water containing a small amount of the indicator methyl red. The remaining neck of the flask was stoppered after the addition of the reactants. The reaction was followed to 87% completion after 36 h by titration of the trapped NH_3 in water.[26] Ninety percent of the solvent was evaporated and the precipitated product filtered and recrystallized first from $CHCl_3$ and then from MeOH. The free base was then dissolved in MeOH saturated with HCl gas, stirred for 1 h, evaporated, and then recrystallized from MeOH/Et_2O to give a white powder. Mp 198 °C dec. ^{1}H NMR (300 MHz, CD_3OD) δ 4.05 (s, 8 H); 1.55 (s, 12 H). MS (FAB) calcd for $[C_{12}H_{23}N_6]^+$ 251.20, found 251.22.

2,2′-Azo(2-amidinopropane)[2-(2-imidazolin-2-yl)propane] Dihydrochloride (5). The unsymmetrical initiator **5** was synthesized similiarly to **C-0** with a few modifications. In a 500 mL three-necked flask were dissolved AAPH (12.0 g, 44.3 mmol) and sodium methoxide (1.44 g, 26.7 mmol) in 450 mL of anhydrous MeOH at room temperature. After 30 min, ethylenediamine (1.48 mL, 22.2 mmol) was added and argon bubbled vigorously through the solution. NH_3 in the exhaust was trapped in water containing methyl red indicator. Titration of the NH_3 showed the reaction to be complete after 52 h. The solvent was evaporated and the resulting solid was washed with 125 mL of EtOH at 40 °C. The filtrate was treated with HCl gas and then evaporated after 30 min. The solid was washed with30 mL of EtOH at room temperature and then rinsed with 30 mL of Et_2O. The remaining residue was dissolved in 125 mL of EtOH at 40 °C, filtered, and recrystallized with the addition of Et_2O. **5:** Yield 2.43 g (37%). Mp 143 °C dec. ^{1}H NMR (300 MHz, d_6DMSO) δ 10.49 (s, 2 H); 9.40 (s, 2 H); 9.10 (s, 2H); 3.90 (s, 4 H); 1.50 (d, 12 H).

2,2′-Azo[2-(2-imidiazolin-2-yl)propane]{2-[2-(4-*n*-octyl)imidazolin-2-yl]propane} Dihydrochloride(C-8). In a three-necked flask was dissolved azo initiator **5** (6.5 g, 22 mmol) and sodium methoxide (0.65 g, 12 mmol) in 200 mL of anhydrous MeOH. After the mixture was stirred for 30 min, 1,2-diaminiodecane (1.88 g, 11 mmol) was added as argon bubbled through the solution and exhausted into a water trap. The reaction was followed for 48 h (84% completion) by titration of trapped NH_3. The argon bubbling was stopped and sodium methoxide (1.31 g, 24.2 mmol) was added. After 1 h the solvent was evaporated and the solid washed with $CHCl_3$. The filtrate was saved and evaporated to dryness in vacuo. The crude solid and anhydrous sodium carbonate (25 g, 0.30 mol) were suspended in 80 mL of EtOH.[25] With vigorous stirring, $(BOC)_2O$ (33.3 g, 0.15 mol) was added and reacted for 3 d. After the first 3 d additional $(BOC)_2O$ (9.5 g, 0.04 mol) was added and reacted another 24 h. Evaporation of the solvent and extraction of the solid with EtOAc yielded a crude BOC protected initiator. Gradient FC (5%, 10%, 20%, 30% EtOAc in hexanes) provided purified BOC protected initiator **6**, yield 2.3 g (37%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.94 (m, 1 H); 3.82 (m, 1 H); 3.79 (s, 4 H); 3.40 (dd, 1 H); 1.58 (m, 12 H); 1.42 (s, 18 H); 1.30 (br m, 12 H); 0.89 (t, 3 H). HRMS (FAB) calcd for $[C_{30}H_{55}O_4N_6]^+$ 563.4285, found 563.4294. Deprotection with trifluroacetic acid gave a TFA salt that was dissolved in water, treated with 50% NaOH until strongly basic, and extracted with $CHCl_3$. The resulting free base was then treated with HCl in MeOH to provide the desired dihydrochloride. Recrystallization from MeOH/Et_2O yielded pure **C-8** (0.63 g, 13% overall) as a white powder. Mp 156 °C dec. ^{1}H NMR (400 MHz, CD_3OD) δ 4.41 (m, 1 H); 4.13 (t, 1 H); 4.05 (s, 4 H); 3.71 (dd, 1 H); 1.75 (br m, 2 H); 1.55 (s, 12 H); 1.35 (br m, 12 H); 0.90 (t, 3 H). HRMS (FAB) calcd for $[C_{20}H_{39}N_6]^+$ 363.3236, found 363.3252. Anal. calcd for $C_{20}H_{40}N_6Cl_2$: C, 55.14; H, 9.26; N, 19.31. Found: C, 55.34; H, 9.20; N, 19.24.

2,2′-Azo[2-(2-imidiazolin-2-yl)propane]{2-[2-(4-*n*-dodecyl)imidazolin-2-yl]propane} Dihydrochloride (C-12). The unsymmetrical azo initiator **C-12** was synthesized and purified the same way as **C-8**. **C-12**: Yield 0.62 g (19% overall) of a purified white powder. Mp 160 °C dec. ^{1}H NMR (400 MHz, CD_3OD) δ 4.40 (m, 1 H); 4.13 (t, 1 H); 4.05 (s, 4 H); 3.71 (dd, 1 H); 1.75 (br m, 2 H); 1.55 (s, 12 H); 1.35 (br m, 20 H); 0.90 (t, 3 H). HRMS (FAB) calcd for $[C_{24}H_{47}N_6]^+$ 419.3862, found 419.3857. Anal. calcd for $C_{24}H_{48}N_6Cl_2$: C, 58.62; H, 9.85; N, 17.10. Found: C, 58.75; H, 9.77; N, 17.04.

2,2′-Azo[2-(2-imidiazolin-2-yl)propane]{2-[2-(4-*n*-hexadecyl)imidazolin-2-yl]propane} Dihydrochloride (C-16). The unsymmetrical azo initiator **C-16** was synthesized the same way as **C-8**, but was purified by fractional recrystallization rather than protection and chromatography. Breifly, the crude reaction product was first treated with HCl gas in MeOH, evaporated, then washed with a small amount of EtOH at room temperature. Four successive recrystallizations from MeOH/Et_2O yielded **C-16** (1.7 g, 44%) as a pure white powder. Mp

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150 °C dec. ^{1}H NMR (400 MHz, CD_3OD) δ 4.40 (m, 1 H); 4.13 (t, 1 H); 4.05 (s, 4 H); 3.71 (dd, 1 H); 1.75 (br m, 2 H); 1.56 (s, 12 H); 1.35 (br m, 28 H); 0.90 (t, 3 H). ^{13}C NMR (75.4 MHz, CD_3OD) δ 174.4, 173.3, 71.6, 59.3, 51.0, 46.0, 45.9, 36.0, 35.9, 35.9, 35.9, 33.0, 30.7, 30.7, 30.6, 30.6, 30.5, 30.5, 30.4, 28.3, 25.6, 25.5, 23.7, 23.6, 23.6, 19.5, 19.4, 14.4 ppm. HRMS (FAB) calcd for $[C_{28}H_{55}N_6]^+$ 475.4490, found 475.4510. Anal. calcd for $C_{228}H_{56}N_6Cl_2$: C, 61.38; H, 10.31; N, 15.35. Found: C, 61.66; H, 10.36; N, 15.06.

Determination of Rate Constants for Azo Decomposition. The k_d values were measured by following the loss of azo chromophore at 366 nm.[7,24] The k_d values for the initiators **C-8**, **C-12**, and **C-16** were measured in MeOH containing 0.2% concentrated HCl. Values for k_d in methanol were the following: **C-0** (7.1×10^{-6}), **C-8** (8.3×10^{-6}), **C-12** (8.0×10^{-6}), and **C-16** (7.4×10^{-6}). MeOH experiments were conducted at three temperatures, 50, 58, and 65 °C, over the course of several half-lives, then extrapolated to calculated k_d at 37 °C. k_d values for **C-0** were also measured in PBS (phosphate buffered saline) buffer pH 7.4 (4.7×10^{-6}), SDS (3.3×10^{-6}), Triton X-100 (4.5×10^{-6}), and TTAB micelles (4.5×10^{-6}). SDS micelles were 0.5 M in PBS buffer pH 7.4; Triton X-100 and TTAB micelles were both 0.1 M diluted in PBS buffer pH 7.4. Aqueous experiments for **C-0** were conducted at 37 °C and followed the decomposition for the first 2 h. Inititial rates were used because interfering absorbances began to grow in and result in nonlinear rate analysis for extended decomposition studies. The UV initial rate method was not successful for k_d analysis of unsymmetrical azo initiators, **C-8**, **C-12**, and **C-16**, in aqueous dispersions. The k_d values for compounds **C-8**, **C-12**, and **C-16** were only measured in methanol. However, considering the unsymmetrical azo initiators are structurally similar to **C-0** and also show similiar decomposition rates in MeOH, the k_d values obtained in buffer and micelles were assumed to be comparable.

Free Radical Generation Efficiency In Micelles. The generation of free radicals in aqueous dispersions was carried out at 37 °C in air using either SDS (0.5 M), Triton X-100 (0.1 M), or TTAB (0.1 M). In a typical experiment α-TOH (150 μL, ~4.5 mM in MeOH) and methyl linoleate (8.3 μL (neat); no methyl linoleate was added for TTAB micelles)[14] were mixed in a 5 mL flask. Next, **C-8** (150 μL, 0.065 M in MeOH) was added, swirled, and followed by addition of surfactant (4 mL;either 0.625 or 0.125 M in PBS pH 7.4 depending on desired final concentration). (Note that in the case of water-soluble **C-0**, the initiator was added in PBS pH 7.4 after the addition of surfactant, and pure MeOH (150 μL) was added in place of the initiator solution in methanol.) Finally, uric acid (600 μL, 1.25 mM in PBS pH 7.4) and PBS (100 μL) were added bringing the total volume to 5 mL. The dispersions were thoroughly mixed by vortexing 2 min.

Oxidation and Extraction of Antioxidants. The flask containing all components of the experiment was incubated at 37 °C for 5 min before the initial timepoint was removed. Aliquots were taken for α-TOH analysis (200 μL) and UA analysis (20 μL, or 50 μL) and placed directly on ice. Typically, 4 to 6 timepoints were taken during the first 3 h of oxidation. To the 200 μL α-TOH aliquot was added BHT (30 μL, 3 mM in MeOH) and a known amount of the internal standard, δ-TOH (100 μM in MeOH). Extraction of all the aliquots was performed with ice-cold MeOH (1 mL) and ice-cold hexanes (5 mL) in sequence, vortexed vigorously after the addition of each solvent (ca. 15 s) and then centrifuged at 1700 rpm for approximately 1 min with the use of an Adams analytical centrifuge.[27] The hexane phase was removed by pipet and concentrated under argon, and then stored at −78 °C until analysis. The UA aliquots were simply stored at −78 °C until analysis by HPLC.

α-TOH Analysis.[28]Samples of α-TOH were re-suspended in 0.5−1.5 mL of HPLC solvent and analyzed by HPLC with electrochemical detection. The mobile phase was composed of methanol/reagent alcohol (60:40) and lithium perchlorate (20 mM). Electrochemical detector settings were +0.60 to +0.65 V with a range setting of 0.5 μA. The flow rate was 1.0 or 1.5 mL/min, which provided good separation of δ-TOH and α-TOH within 7 min. External standard curves were created by injecting 5−25 μL of working standard (α-TOH (9.0 μM) and δ-TOH (12.0 μM) in the mobile phase) intermittently between sample injections of 15−75 μL. Columns were flushed with MeOH/H_2O (50:50) after each use.

Urate Analysis.[29] Uric acid standards were prepared from a stock solution (1.25 mM UA in PBS pH 7.4). The stock solution was diluted with a mobile phase to produce a working standard of 2.25 μM UA. The mobile phase consisted of filtered and degassed sodium acetate buffer (40 mM, pH 4.75), Na_2EDTA (0.54 mM), dodecyltriethylammonium phosphate (1.5 mM, Regis Chemical Co., Morton Grove, IL), and 7.5% MeOH. Electrochemical detector settings were +0.60 to +0.65 V with a range setting of 50 nA and the flow rate was 1.5 mL/min. The standard curves were generated by intermittently injecting 5−25 μL of working standard between sample injections. Samples of UA were diluted with 1.0−2.5 mL of mobile phase and 15 μL was injected. Columns were flushed with MeOH/H_2O (50:50) after each use.

Free Radical Generation Efficiency in Liposomes. The generation of free radicals in MLV liposomal membranes was carried out at 37 °C in air using plant PC (10.3 mM). PC and lipid soluble components (α-TOH and initiator, except for **C-0**) were dissolved in MeOH and placed in a 5 mL flask. The MeOH was evaporated in vacuo to obtain a thin film. PBS buffer (4.4 mL) and UA (0.6 mL, 1.25 mM in PBS) were added and the PC film was slowly peeled off by shaking and then vortexing to obtain white, milky liposome suspensions.[30] Aliquots for α-TOH and UA were removed at various intervals, and treated as described above.

Free Radical Generation Efficiency in Lipoproteins. (a) Lipoprotein Isolation.[31] Whole blood from fasting, normolipidemic healthy subjects was collected in a 450 mL ACD blood collection bag (Baxter) containing the following: 1.61 g of dextrose; 1.66 g of sodium citrate dihydrate; 188 mg of anhydrous citric acid; and 140 mg of monobasic sodium phosphate monohydrate. The bag containing blood was centrifuged at 4200 rpm for 10 min at 22 °C and the plasma was collected. The low-density lipoprotein (LDL) was isolated from plasma over 15 h by density gradient sequential ultracentrifugation at 14 °C using a Beckman Optima LE-80K centrifuge and a Ti 70 rotor. Each spin was preformed at 504 000 g for 5.5 h. Lipoproteins were dialyzed extensively against 10 mM PBS, sterilized by passage through a Millex-HA 0.45 μM filter, and stored at 4 °C under argon. Protein concentrations of the LDL preparations were determined by the method of Lowry.[32] LDL isolation was confirmed with the use of SDS PAGE separation of associated apoproteins and Beckman LIPO Gel electroproesis of intact lipoproteins.[33]

(b) Oxidation of LDL. Lipoprotein concentrations were adjusted to 0.75 mg of protein/mL with PBS containing 60 μM UA and allowed to equilibrate to 37 °C for 5 min in a 10 mL flask. To the stirred solution was added initiator (0.065 M) in MeOH (except **C-0** was in PBS) to give a final **C-0**, **C-8**, **C-12**, or **C-16** concentration of 0.5 mM, or a MeOAMVN concentration of 0.25 mM. Following addition of initiator (time zero) aliquots for α-TOH and UA were removed at various intervals and treated as described above.

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

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