Oxidation of Vitamin E: Evidence for Competing Autoxidation and Peroxyl Radical Trapping Reactions of the Tocopheroxyl Radical

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Abstract: Peroxyl radicals generated by thermolysis of azobis(2,4-dimethylvaleronitrile) (AMVN) in oxygenated acetonitrile reacted with *d*-α-tocopherol (vitamin E, **1**) to produce 8a-[(2,4-dimethyl-1-nitrilopent-2-yl)dioxy]tocopherone (**3a**), 4a,5-epoxy-8a-hydroperoxytocopherone (**4a**), and 7,8-epoxy-8a-hydroperoxytocopherone (**5a**). When formed in acetonitrile/water (6:4, v/v), **4a** and **5a** hydrolyzed quantitatively to 2,3-epoxy-α-tocopherylquinone (**6**) and 5,6-epoxy-α-tocopherylquinone (**7**), respectively, whereas **3a** underwent limited hydrolysis to α-tocopherylquinone (**8**). Peroxyl radicals further oxidized **3a** and its homolog, 8a-hydroperoxy-α-tocopherone (**3b**), which was formed in small amounts during the oxidation of **1**. However, neither **3a** nor **3b** were consumed by peroxyl radicals when the reaction mixture contained **1**. Formation of epoxytocopherones **4a**/**5a** therefore does not proceed by oxidation of **3a** or **3b**. Epoxytocopherones **4a** and **5a** may be formed instead by epoxidation of the tocopheroxyl radical (**2**), followed by reaction of the epoxy **2** with oxygen and a H• donor. Thus, **2** either may trap peroxyl radicals to yield 8a-(alkyldioxy)tocopherones or produce epoxytocopherones by peroxyl radical-dependent autoxidation.

The biological antioxidant α-tocopherol (**1**, Figure 1) prevents the spread of free-radical damage in cellular membranes by trapping reactive peroxyl radicals.[1,2] Compound **1** reacts with peroxyl radicals to form the tocopheroxyl radical (**2**), an unusually stable phenoxyl radical that does not readily propagate radical chains.[3] Biochemical reductants, such as ascorbic acid, regenerate **1** from **2**[4] in a reaction thought to explain the antioxidant synergism between **1** and ascorbic acid in model systems and that may maintain **1** in living tissues.[5,6] When regeneration does not occur, kinetic evidence indicates that **2** traps a second peroxyl radical to form nonradical products.[3] Peroxyl radicals are the chain-carrying radicals in lipid peroxidation,[7] and their reactions with **1** are postulated to lead to its oxidative turnover in biological membranes.

Peroxyl radicals generated from azobis[(*n*-butylcarboxy)-propane] or azobis(isobutyronitrile) in acetonitrile oxidized **1** to 8a-(alkyldioxy)tocopherones **3c**/**3d** in approximately 50% yield.[8] 8a-(Alkyldioxy)tocopherones may be considered reversibly oxidized products of **1** because their reduction by ascorbic acid to **1** completes a two-electron redox cycle.[9] In a similar experiment, **1** reacted with peroxyl radicals generated by AMVN in ethanol to yield 4a,5-epoxy-8a-ethoxytocopherones (**4b**), 7,8-epoxy-8a-ethoxytocopherones (**5b**), and 8a-hydroperoxytocopherone **3b**, in addition to **3a**, although product yields were not reported.[10] The authors proposed that peroxyl radicals oxidized **1** to the tocopherone cation (T^+), which then reacted with either the resulting peroxide anion or the solvent to form tocopherones. They proposed that subsequent epoxidation of the 8a-ethoxytocopherones yields epoxytocopherone products (**4b** and **5b**) but did not find either the putative 8a-ethoxytocopherone intermediates or epoxides of 8a-(alkyldioxy)tocopherones (**3a**).

Matsuo et al. found that the *tert*-butylperoxyl radical oxidized **1** in benzene/*tert*-butyl hydroperoxide without producing an 8a-(alkyldioxy)tocopherone product.[11] Instead, the reaction yielded two diastereomers of **4a** in 28% yield after a short (5 h) incubation and two 4a,5-epoxy-8a-(*tert*-butyldioxy)tocopherone (**4c**) diastereomers in 20% yield after an extended (40 h) incubation. The authors proposed that oxygen addition to **2** yielded a tocopherone 8a-peroxyl radical, which then abstracted H• from *tert*-butyl hydroperoxide to produce 8a-hydroperoxytocopherone (**3b**). Subsequent epoxidation of **3b** was proposed to yield the corresponding epoxide **4a**. This mechanism results in no net consumption of peroxyl radicals and is difficult to reconcile with the well-documented role of **1** as a peroxyl radical trap[3] and with the apparent lack of reactivity of **2** with O_2.[12]

The purpose of this investigation was to identify all of the products formed by peroxyl radical oxidation of **1** in a single system and to determine whether more than one product-forming pathway exists. Here we report that **1** reacts with AMVN-derived peroxyl radicals in homogeneous solution to form the 8a-(alkyldioxy)tocopherone **3a** and epoxy-8a-hydroperoxytocopherones **4a** and **5a** as the principal products and that small amounts of the 8a-hydroperoxytocopherone **3b** also were formed. Neither **3a** nor **3b** are precursors to epoxytocopherones **4a**/**5a** in mixtures that contain **1**. Our data suggest that two different reactions of **2** with peroxyl radicals form the 8a-(alkyldioxy)tocopherone **3a** and epoxytocopherones **4a**/**5a**.

Results

Oxidation of 1 and [^{14}C]-1. Peroxyl radicals were generated by thermolysis of AMVN at 50 °C in oxygen-saturated acetonitrile. The radicals oxidized [^{14}C]-**1** to two groups of products, which were resolved by reverse-phase high-performance liquid chromatography (HPLC) on a Spherisorb octyl column (Figure 2). Repeated analyses over a 2-h incubation indicated a disappearance of the [^{14}C]-**1** peak, which eluted at 12 min, concomitant with the appearance of a more polar product fraction A eluting at 8 min and a less polar product fraction B eluting at 19 min. At each incubation time point, 90–98% of the radioactivity present could be accounted for by these three fractions.

HPLC analysis with diode array detection (DAD-HPLC) of the reaction products after 120 min of incubation is depicted in

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(1) *Vitamin E: Biochemistry and Health Implications*; *Ann. N.Y. Acad. Sci.* Diplock, A. T., Machlin, L. J., Packer, L., Pryor, W. A., Eds.; 1989; Vol. 570.
(2) *Vitamin E: A Comprehensive Treatise*; Marcel Dekker: Machlin, L. J., Ed.; New York, 1980.
(3) Burton, G. W.; Ingold, K. U. *Acc. Chem. Res.* **1986**, *19*, 194–201.
(4) Packer, J. E.; Slater, T. F.; Willson, R. L. *Nature* **1979**, *278*, 737–738.
(5) McCay, P. B. *Ann. Rev. Nutr.* **1985**, *5*, 323–340.
(6) Niki, E. *Chem. Phys. Lipids* **1987**, *44*, 227–253.
(7) Porter, N. A. *Acc. Chem. Res.* **1986**, *19*, 262–268.
(8) Winterle, J.; Dulin, D.; Mill, T. *J. Org. Chem.* **1984**, *49*, 491–495.
(9) Liebler, D. C.; Kaysen, K. L.; Kennedy, T. A. *Biochemistry* **1989**, *28*, 9772–9777.
(10) Yamauchi, R.; Matsui, T.; Satake, Y.; Kato, K.; Ueno, Y. *Lipids* **1989**, *24*, 204–209.
(11) Matsuo, M.; Matsumoto, S.; Iitaka, Y.; Niki, E. *J. Am. Chem. Soc.* **1989**, *111*, 7179–7185.
(12) Doba, T.; Burton, G. W.; Ingold, K. U.; Matsuo, M. *J. Chem. Soc., Chem. Commun.* **1984**, 461–462.

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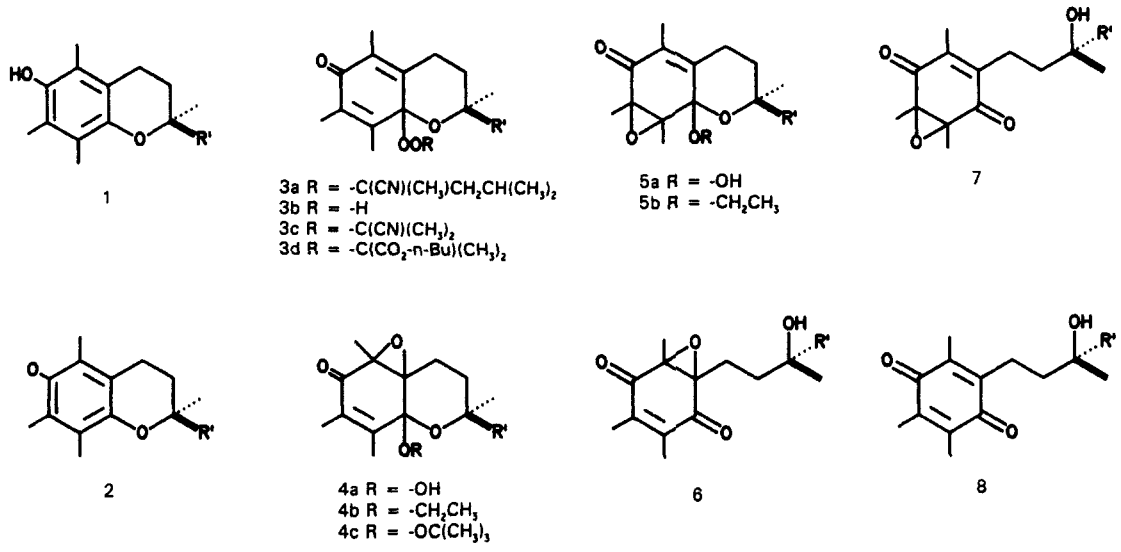

Figure 1. Structures of compounds referred to in the text. R′ = -phytyl, $-C_{16}H_{33}$.

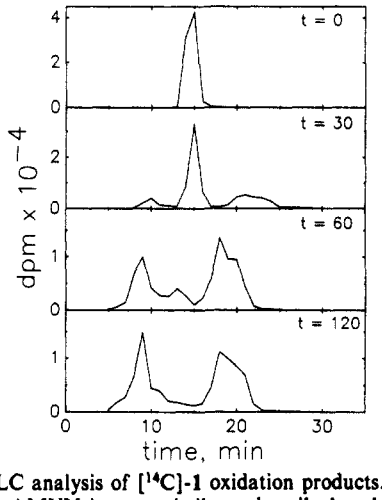

Figure 2. HPLC analysis of [^{14}C]-**1** oxidation products. [^{14}C]-**1** was incubated with AMVN in acetonitrile as described under the Experimental Section. Although the time scales are identical, the chromatograms are offset for clarity.

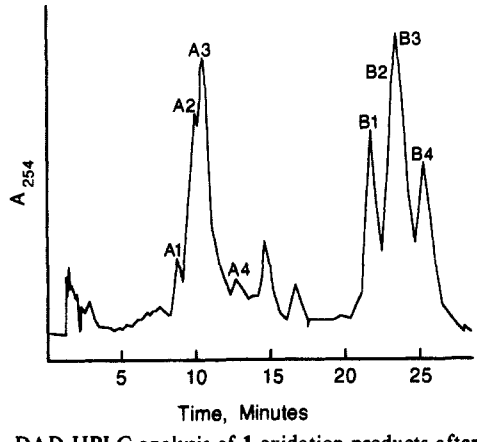

Figure 3. DAD-HPLC analysis of **1** oxidation products after 120-min incubation with AMVN in acetonitrile. Annotations identify peaks in the A and B fractions.

Figure 3. Fraction A consisted of at least four peaks eluting between 8.5 and 12.5 min with UV absorbance maxima at approximately 255 nm. Fraction B consisted of our peaks eluting between 21.5 and 25.5 min with identical UV spectra and absorbance maxima at 240 nm. Although the second and third peaks eluting at approximately 23–24 min appear in Figure 3 as a single peak, partial separation of these was achieved by increasing the polarity of the mobile phase. All four peaks were resolved by normal phase HPLC (see Experimental Section). Two other peaks that eluted between the A and B fractions were detected by HPLC-DAD analysis but not by radiochromatography. The first eluted at 14.5 min and had a retention time and UV spectrum identical with that of authentic **3b**. Levels of **3b** observed were approximately 2–5% of **3a** levels and increased linearly with time (data not shown). Although this product was not further characterized, the role of **3b** as a potential precursor to other products was examined (see below). The second peak eluted at 16.6 min and displayed a UV spectrum identical with **3b**, but its identity was not further investigated.

Oxidation Products of 1: Fraction A. Purification of products in fraction A was hindered by their facile hydrolysis to secondary products, which subsquently were identified as α-tocopherylquinone-2,3-oxide (**6**) and α-tocopherylquinone-5,6-oxide (**7**) (see below). Two of the product peaks (A2 and A3) were purified together by reverse-phase HPLC on Spherisorb ODS-2 and identified as diastereomers of the 4a,5-epoxy-8a-hydroperoxytocopherone (**4a**). ^{1}H resonance assignments for **4a** were based on phase-sensitive ^{1}H double quantum filtered correlation spectroscopy (DQFCOSY)[13,14] of the sample (not shown). During the five-hour ^{1}H-DQFCOSY acquisition, the resonances attributable to **4a** gradually were replaced by a separate set of ^{1}H resonances, which corresponded to the two epimers of the quinone epoxide **6**, a hydrolysis product of **4a** (see below). Products A2 and A3 were thus epimeric epoxides, but their stereochemistry was not further investigated. Hydrolysis of **4a** to **6** during NMR analysis may have been caused by trace moisture present in the NMR tube or in the C_6D_{12} solvent.

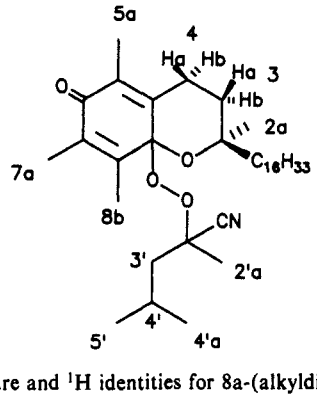

Figure 4. Structure and ^{1}H identities for 8a-(alkyldioxy)tocopherones **3a**.

(13) Marion, D.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967–974.
(14) Rance, M.; Sorensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479–485.

Table I. ^{1}H Chemical Shift Assignments for **2a** Diastereomers

^{1}H assignment[b]	chemical shift[a]			
	B1 [8a(*S*),2′(*S*)]	B2 [8a(*R*),2′(*R*)]	B3 [8a(*S*),2′(*R*)]	B4 [8a(*R*),2′(*S*)]
2′a	1.47 s[c]	1.48 s	1.52 s	1.52 s
3′	1.56 m	1.52, 1.57 m	1.51 m	1.52 (1.44, 1.76) m
4′	1.77 m	1.78 m	1.76 (2.05) m	1.76 (2.05) m
4′a	0.91 d	0.91 d	0.89 (1.01) d	0.86 (1.10) d
5′	0.82 d	0.82 d	0.75 (1.01) d	0.78 (1.10) d
2a	1.10 s	1.31 s	1.08 s	1.28 s
3a	2.27 m	1.73 m	2.38 m	1.73 m
3b	1.58 m	2.05 m	1.60 m	2.15 m
4a	2.58 m	2.58 m	2.95 m	2.56 m
4b	2.58 m	2.62 m	2.55 m	2.96 m
5a	1.82 s	1.81 s	1.82 s	1.85 s
7a	1.81 s	1.82 s	1.80 s	1.80 s
8b	1.97 s	1.96 s	1.90 s	1.90 s

[a] Chemical shifts are in parts per million downfield from tetramethylsilane. [b] Refer to Figure 4 for ^{1}H assignments in **2a**. [c] Multiplicity of H resonances is indicated by s (singlet), d (doublet), and m (multiplet).

Table II. Effects of Solvent and Reaction Time on Oxidation Products of **1**[a]

run	solvent	reaction time, min	recovery of **1**, %	yield, %					
				3a	**3b**	**4a/5a**	**6**	**7**	**8**
1	CH_3CN	60	20	51	3	22	nd[b]	nd	nd
2	CH_3CN	120	nd	39	6	33[c]	nd	nd	9
3	CH_3CN/H_2O (6:4)	60	40	32	1	6	8	5	2
4	CH_3CN/H_2O (6:4)	120	nd	26	2	nd	47	12	4

[a] [^{14}C]-**1** was oxidized with AMVN, and products were analyzed by HPLC on Spherisorb ODS-2 as described in the Experimental Section. [b] Not detected. [c] DAD-HPLC analyses of incubations with unlabeled **1** indicated the presence of small amounts (<10%) of **6** and **7**, which were incompletely resolved from **4a/5a**.

Oxidation Products of 1: Fraction B. Product peaks from fraction B were identified as four diastereomers of 8a-(alkyldioxy)tocopherone **3a** by UV spectroscopy, ^{1}H-NMR spectroscopy, and mass spectrometry. One-dimensional ^{1}H-NMR, phase-sensitive ^{1}H-DQFCOSY, and phase-sensitive ^{1}H nuclear Overhauser enhancement spectroscopy (^{1}H-NOESY)[15] analyses (not shown) were employed to assign ^{1}H shifts and couplings. The absolute configurations and ^{1}H resonances of the four diastereomers of **3a** are listed in Table I. The numbering scheme for protons in **3a** is depicted in Figure 4. The absolute configuration at C-8a was assigned based on the chemical shift of the 2a-methyl protons.[10] In the C-8a(*S*) configuration, the 8a-alkyldioxy moiety and the 2a-methyl group are trans with respect to the ring system and the 2a-methyl protons occupy a shielding environment above the dienone ring and resonate at approximately δ1.1. In the C-8a(*R*) configuration, the 2a-methyl protons are forced away from the dienone ring by the adjacent 8a-alkyldioxy group and resonate at approximately δ1.3. The absolute configuration at C-2′ was deduced by observing that for each pair of C-2′ epimers, one exhibited a downfield shift in one of the H4 resonances to δ2.95–δ2.96, whereas the other exhibited a downfield shift of the 8b-methyl protons to δ1.96–δ1.97. This was attributed to preferred conformations of the 8a-alkyldioxy group that minimize steric crowding between it and the tocopherone moiety. In B3 [8a-(*S*),2′(*R*)], the 1′-nitrilo group is positioned over H4a, which resonates at δ2.95. In B4 [8a(*R*),2′(*S*)], where the 8a-alkyldioxy moiety is on the other side of the ring system, the nitrile is positioned over H4b, which resonates at δ2.96. In B1 [8a(*S*),2′(*S*)], the 2′ epimer of B3, the 1′-nitrilo group lies over the 8b-methyl protons instead of H4a, and they resonate at δ1.97. Similarly, in B2, the 2′ epimer of B4, the 1′-nitrilo group causes the 8b-methyl protons to resonate at δ1.96. In all four products, the 5a-methyl protons were distinguished by weak coupling to H4a or H4b and by NOEs detected between these nuclei in the ^{1}H-NOESY experiments. The distinct differences in the H4 and 8b-methyl proton resonances for each pair of C-2′ epimers were not observed in a previous study, in which the [8a(*R*),2′(*RS*)] pair was not separated prior to analysis but produced nearly identical spectra.[10] Interpretation of spectra of B1 and B3 was complicated somewhat by the presence of a minor conformer of each, in which two sets of resonances were detected for H3′, H4′, H4a′, and H5′. These additional resonances are listed in parentheses in Table I.

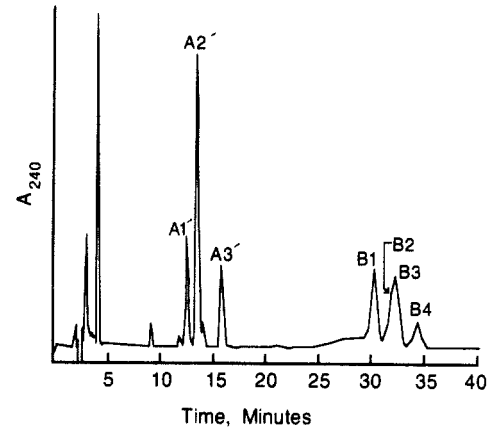

Figure 5. DAD-HPLC analysis of **1** oxidation products after 120-min incubation with AMVN in acetonitrile/water (60:40, v/v).

Effect of Water on Oxidation Products of 1. When **1** was incubated with AMVN in an acetonitrile/water (60:40, v/v) mixture rather than in acetonitrile alone, 8a-(alkyldioxy)tocopherones B1–B4 (**3a**) were formed as in the absence of water, but the fraction A products were not (Figure 5). Instead, three other products, designated A1′, A2′, and A3′, were detected. Peaks A2′ and A1′ were identified as the quinone epoxides **6** and **7**.

The quinone epoxide **6** was formed by hydrolysis of the epoxytocopherone **4b** (see above), presumably through a S_N1 hydrolysis and rearrangement reaction sequence that forms quinone **8** from the 8a-(alkyldioxy)tocopherones **3a**.[9] Quinone epoxide **7**, which was present at approximately 20% of the level of **6**, is presumably formed from the corresponding epoxytocopherone **5a**. The presence of quinone epoxide **7** in the product mixture suggests that epoxytocopherone **5a** was present in fraction A but was not identified because it was formed at low levels and hydrolyzed to **7** during product workup.

Peak A3′ was identified as α-tocopherylquinone (**8**), which is formed by hydrolysis of the 8a-(alkyldioxy)tocopherones **3a**.[9] Peak A3′ coeluted with authentic **8** and also displayed UV and mass

(15) Derome, A. *Modern NMR Techniques for Chemistry Research*; Pergamon Press: Oxford, 1987; pp 239–244.

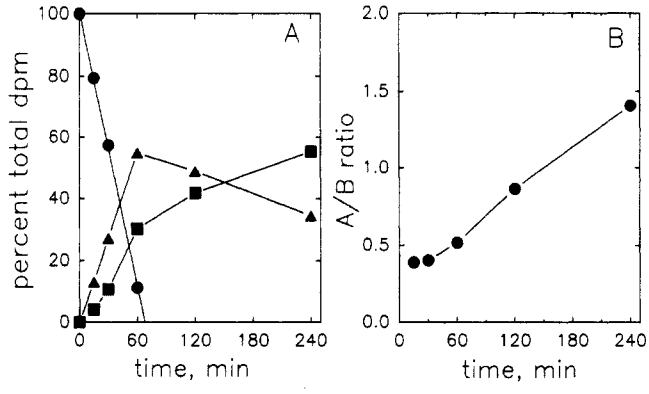

Figure 6. (A) Depletion of [^{14}C]-**1** (circles) and appearance of fraction A products (squares) and fraction B products (triangles) with time during [^{14}C]-**1** oxidation depicted in Figure 2. Amounts are expressed as percent of the total radioactivity eluted from the column during HPLC analysis. (B) Ratio of fraction A products to fraction B products versus time during and after [^{14}C]-**1** depletion depicted in Figure 2.

spectra identical with those of authentic **8**. The effects of reaction time and solvent on the distribution of oxidation products of **1** are summarized in Table II.

Kinetics of Product Formation from 1. To determine whether 8a-(alkyldioxy)tocopherones **3a** and epoxytocopherones **4a/5a** were formed concomitantly or sequentially, the kinetics of [^{14}C]-**4a/5a** and [^{14}C]-**3a** formation from [^{14}C]-**1** were compared. Figure 6A depicts the depletion of [^{14}C]-**1** and the accumulation of radiolabel in fractions A and B as monitored by HPLC analysis of the incubation mixture. Compound **1** was depleted at virtually a constant rate in the presence of a large (18-fold) excess of AMVN and was completely consumed within 60 min. Radiolabel accumulated in both fractions A and B for 60 min. After 60 min, **1** was completely depleted, and a loss of radiolabel from fraction B ensued, which was paralleled by a further accumulation of products in fraction A. The fraction A/fraction B ratio increased gradually during the first 60 min of **1** oxidation and more sharply thereafter (Figure 6B). DAD-HPLC analyses indicated that fraction A contained only epoxytocopherones **4a/5a** during the first 60 min of reaction; peaks for quinone epoxides **6** and **7** were detected at 120 and 240 min. Fraction B contained only **3a** at all incubation times. Both **3a** and **4a/5a** were therefore produced concomitantly during the oxidation of **1**, but further oxidation of **3a** after **1** was depleted yielded products with retention characteristics and UV spectra identical with epoxytocopherones **4a/5a**.

Evaluation of 3a and 3b as Precursors to Epoxytocopherone Products 4a/5a. Since **3a** was converted to more polar products after complete consumption of **1** (Figure 6A), it seemed possible that further oxidation of **3a** also could occur during depletion of **1**. This reaction could account for epoxytocopherone **4a/5a** formation that occurred as **1** was oxidized. In reaction mixtures containing AMVN, [^{14}C]-**3a** was almost completely consumed within 4 h (Figure 7). The products of this reaction were not characterized. However, DAD-HPLC analyses after 18 h of a similar reaction mixture with unlabeled **1** revealed products with retention times and UV spectra identical with compounds **4a**, **6**, and **7**. Other unidentified products also were present. The initial amount of [^{14}C]-**3a** used (0.11 μmol) was equal to the amount of fraction B products (**3a**) present after 60 min (complete depletion of **1**) in Figure 6A. Accordingly, [^{14}C]-**3a** was consumed at about the same rate when incubated alone with AMVN (Figure 7) as when present with other oxidation products of **1** (Figure 6A). Consumption of [^{14}C]-**3a** was peroxyl radical dependent; no loss occurred when AMVN was omitted from the reaction. Addition of **1** (0.235 μmol) to the reaction completely prevented [^{14}C]-**3a** consumption until after 2 h, when **1** had been depleted. Thus, **1** prevented the oxidation of **3a** to other products.

Because small amounts of **3b** also were detected in the reaction mixture, we tested the hypothesis[11] that **3b** serves as a precursor to epoxytocopherones **4a/5a**. The initial amount of **3b** (0.129 μmol) was selected to equal the **4a/5a** production from 0.235 μmol

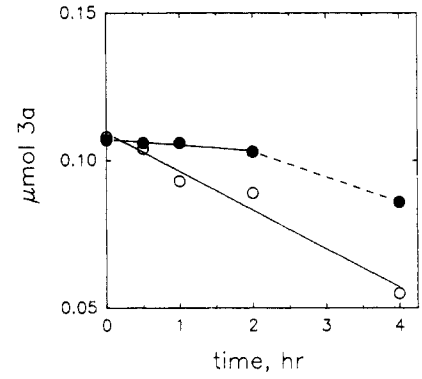

Figure 7. AMVN-dependent consumption of 8a-(alkyldioxy)tocopherones **3a** in acetonitrile in the presence (filled circles) and absence (open circles) of **1**. The dashed line segment indicates [^{14}C]-**3a** consumption that occurred after **1** depletion was complete (approximately 2 h).

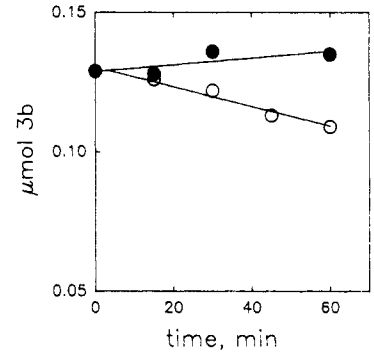

Figure 8. AMVN-dependent consumption of 8a-hydroperoxytocopherone (**3b**) in acetonitrile in the presence (filled circles) and absence (open circles) of **1**. Levels of **3b** in incubations with **1** (filled circles) are corrected for **3b** produced by **1** oxidation.

1 in 60 min under identical experimental conditions (Figure 6A). When incubated with AMVN, approximately 0.02 μmol (15%) of **3b** was consumed in 60 min (Figure 8). However, when 0.235 μmol **1** was included, **3b** was not consumed. These data indicate that **1** protects both **3a** and **3b** against further oxidation and that neither can produce other products in systems containing **1**.

Discussion

Peroxyl radicals react with **1** to produce hydroperoxides and **2**, which is thought to trap a second peroxyl radical to produce a stable product and break a second radical chain.[3] Although this scheme is largely consistent with kinetic evidence,[8,16,17] the oxidative fate of **2** remains poorly understood in mechanistic terms. The overall objective of this investigation was to account for the disposition of **2** by identifying its reaction products with peroxyl radicals. Our first aim was to identify all of the products formed when **1** reacts with AMVN-derived peroxyl radicals. The 8a-(alkyldioxy)tocopherones **3a** and the epoxytocopherones **4a/5a** accounted for at least 96% of the products formed. Although the complete product distribution is reported here for the first time, **3a** and **4a** were identified previously as oxidation products of **1** in separate studies.[10,11] Four diastereomers of 8a-(alkyldioxy)-tocopherone **3a** were formed in similar amounts, which suggests that racemic AMVN-derived peroxyl radicals apparently added easily to either face of **2**. Each epoxytocopherone **4a/5a** may likewise exist in four diastereomeric forms, but epimeric epoxides of **4a** were the major epoxytocopherones formed. Although acetonitrile was the solvent for the oxidations described here, an essentially identical product distribution was observed by DAD-HPLC analysis of oxidations in hexane.

(16) Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6472–6477.
(17) Niki, E.; Tanimura, R.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 1551–1555.

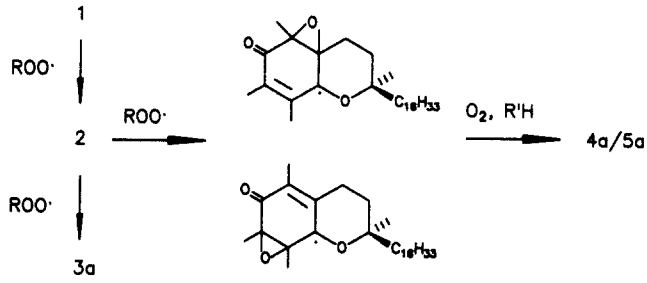

Figure 9. Proposed mechanisms for oxidation of **2** by peroxyl radicals to 8a-(alkyldioxy)tocopherone **3a** and epoxytocopherones **4a** and **5a** (see text for Discussion).

One notable aspect of the observed product distribution is that dimer and trimer oxidation products of **1** were not formed in this system. Recent work by Southwell-Keeley and co-workers indicates that alkoxyl and hydroxyl radicals formed by homolysis of *tert*-butyl hydroperoxide in $CHCl_3$/alcohol mixtures oxidize **1** either to the neutral quinone methide or to a tocopherol cation analogous to **2**.[18,19] The quinone methide may either polymerize or undergo solvolysis in the presence of alcohols, whereas the tocopherol cation undergoes solvolysis exclusively. This scheme also accounts for other products not observed here but reported previously from oxidation of **1** in polar solvents by ferric salts, Br_2, benzoyl peroxide, 2,3,5,6-tetrachlorobenzoquinone, and *N*-bromosuccinimide.[20,21] Oxidants other than peroxyl radicals evidently oxidize **1** to products that are distinctly different from those formed in our system.

The distribution of products from **1** changed with time. Epoxytocopherones **4a/5a** and 8a-(alkyldioxy)tocopherones **3a** initially were formed in an approximately 3:7 ratio until **1** was depleted (Figure 6B). The ratio then increased sharply as **3a** underwent peroxyl radical-dependent conversion to more polar products that appeared in fraction A. DAD-HPLC analyses suggest that **3a** was converted to more polar products through a combination of oxidative and hydrolytic reactions, since quinone epoxides **6** and **7** began to appear in fraction A as **3a** was lost. Epoxytocopherone hydrolysis, which appears to be a very facile process, may easily have occurred since no attempt was made to exclude ambient moisture from the reaction. Epoxides **6** and **7** could result from hydrolysis of **4a/5a** or from epoxidation of **3a** followed by hydrolysis, but further work will be required to clarify the chemistry involved.

Our second aim was to determine whether epoxytocopherones **4a/5a** were formed by oxidation of either **3a** and **3b** or directly from **2** via a separate reaction pathway. Matsuo et al.[11] proposed that *tert*-butylperoxyl radicals oxidized **1** in benzene/*tert*-butyl hydroperoxide to **4a** via 8a-hydroperoxytocopherone intermediate **3b**. In our system, **3b** apparently was formed in small amounts, but it was not further oxidized when **1** also was present. Thus, **3b** could not be a precursor to epoxytocopherones **4a/5a**, since these were formed while the reaction mixture contained **1**. This interpretation also is consistent with our observation that **3b** levels steadily increased during depletion of **1** (data not shown). Moreover, **3a** also was not further oxidized in reaction mixtures that contained **1**. The ability of **1** to protect tocopherones against peroxyl radical oxidation argues strongly that epoxytocopherones **4a/5a** were not derived from tocopherone intermediates.

These data suggest that epoxytocopherones **4a/5a** and 8a-(alkyldioxy)tocopherone **3a** are formed by competing reactions of peroxyl radicals with **2a** as outlined in Figure 9. Peroxyl radical addition to C-8a of **2** yields **3a**, as proposed previously.[8] Alternatively, epoxidation of **2** by a peroxyl radical would yield either 4a,5-epoxy-**2** or 7,8-epoxy-**2**. These intermediates would then add oxygen at the 8a-position to form peroxyl radicals. Epoxy-**2** would be less resonance stabilized than **2** and would have a greater tendency to add oxygen at C-8. The resulting 8a-peroxyl radicals would then abstract H• to form epoxytocopherones **4a/5a**. Although **1** is certainly the best H• donor in this system, different oxidizable substrates in other systems also could donate H•, particularly if **1** were present at low levels. Further work is needed to establish the exact mechanism of epoxidation. Nevertheless, it seems likely that introduction of a hydroperoxy group at C-8 involves an intermediate peroxyl radical. Oxidation of **1** to epoxytocopherones therefore results in no net trapping of peroxyl radicals, and **1** oxidation by this route would yield no antioxidant effect. Peroxyl radical-dependent autoxidation of **2** would also be expected to reduce *n*, the stoichiometric ratio of radicals trapped to **1** consumed, to values below 2. Reported values for *n*, based on kinetic measurements, range from 1.4 to 2.0.[8,16,17] This variation of stoichiometry with experimental conditions suggests that the partitioning of **2** between competing reaction pathways can vary with different peroxyl radicals and reaction environments. Investigations into the oxidative fate of **1** in lipid bilayers and in biological membranes are in progress in our laboratory.

Conclusion

Peroxyl radicals in homogeneous solution oxidize **1** to 8a-(alkyldioxy)tocopherones and epoxytocopherones. The latter readily hydrolyze to quinone epoxides **6** and **7**. Epoxytocopherones are not formed by further oxidation of corresponding tocopherone precursors and appear to be formed instead by a reaction pathway separate from that leading to tocopherones. The data suggest that **2** is a less efficient radical trap than **1** and that epoxytocopherone formation involves radical chain transfer rather than a radical-trapping reaction.

Experimental Section

Chemicals and Instrumentation. AMVN was purchased from Polysciences, Inc., Warrington, PA; [^{14}C]formaldehyde (52.5 mCi mmol^{-1}) was purchased from New England Nuclear, and *d*-α-tocopherol was purchased from Kodak. Acetonitrile (Omni-Solv; EM Science, Cherry Hill, NJ) was used as received. All other reagent chemicals were of the highest quality available. Compound **1** (99+% *d*-α-tocopherol) was generously provided by Henkel Corporation, Fine Chemicals Division, La Grange, IL.

HPLC analyses and preparative work were done with Spectra Physics Model 8800 or 8810 pumps and a Kratos Spectroflow SF-757 UV-visible detector. Prepacked HPLC columns containing Whatman and Spherisorb packings were purchased from Alltech (Deerfield, IL). DAD-HPLC analyses were done with a Hewlett-Packard Model 1040A diode array detector system controlled by a HP 300 (Series 9000) computer with HP Chem-Station software. MS was done with a Finnegan MAT-90 instrument equipped with a Micro PDP-11/73 computer (U.S. Design, Palo Alto, CA) and a Finnegan ICIS data system. Samples were introduced by direct probe insertion, and electron impact MS samples were ionized with a 70-eV electron beam. Analyses were made in the electron impact mode unless otherwise specified. Analyses in the chemical ionization and desorption chemical ionization modes were done with methane as the reagent gas. ^{1}H-NMR spectra were obtained with a Bruker-AM-500 instrument equipped with an Aspect 3000 computer and operating at 500.13 MHz for ^{1}H. Spectra were recorded in C_6D_{12} (Cambridge Isotope Laboratories, Woburn, MA) with tetramethylsilane as a reference standard. The 8a-(alkyldioxy)tocopherones, which are acid-sensitive, decomposed in $CDCl_3$, possibly because the solvent contained traces of DCl.

Synthesis of [5-^{14}CH$_3$]-1, [5-^{14}CH$_3$]-3a, and Unlabeled 3b. [5-^{14}CH$_3$]-**1** ([^{14}C]-**1**) was synthesized by reductive alkylation of *d*-γ-tocopherol with [^{14}C]formaldehyde as described.[22] [^{14}C]-**1** was separated from unreacted γ-tocopherol on a Spherisorb ODS-2, 5 μm, 4.6 × 250 mm column eluted with methanol at 2 mL min^{-1}. [5-^{14}CH$_3$]-**3a** ([^{14}C]-**3a**) was prepared from [^{14}C]-**1** by oxidation with AMVN as described below. The [^{14}C]-**3a** product was purified by reverse-phase HPLC on a Hamilton PRP-1 10 μm, 4.6 × 250 mm analytical column (Hamilton, Reno, NV) eluted with methanol/2-propanol (3:1, v/v) at 2 mL min^{-1}. The four **3a** diastereomers eluted as a single peak. Unlabeled **3b** was synthesized by photochemical oxidation of **1** as described previously.[23]

(18) Suarno, M.; Atkinson, E.; Suarna, C.; Saunders, J. K.; Cole, E. R.; Southwell-Keeley, P. T. *Biochim. Biophys. Acta* **1987**, *920*, 247–250.
(19) Suarna, C.; Southwell-Keeley, P. T. *Lipids* **1989**, *24*, 56–60.
(20) Goodhue, C. T.; Risley, H. A. *Biochemistry* **1965**, *4*, 854–858.
(21) Durckheimer, W.; Cohen, L. *J. Am. Chem. Soc.* **1964**, *86*, 4388–4393.
(22) Urano, S.; Hattori, Y.; Yamanoi, S.; Matsuo, M. *Chem. Pharm. Bull.* **1980**, *28*, 1992–1998.

Oxidation of 1. In a typical experiment, 1.0 mg (2.35 μmol) of **1** and 10.8 mg (43.6 μmol) of AMVN were dissolved in 7 mL of oxygen-saturated acetonitrile and heated in a screw-cap test tube at 50 °C. Large-scale oxidations contained up to ten times higher reactant concentrations at the same mole ratio. Incubations with [^{14}C]-**1** contained 0.238 μCi (0.235 μmol) and 4.36 μmol AMVN in 0.7 mL of oxygen-saturated acetonitrile. Aliquots for product analysis were removed to glass tubes and rapidly chilled by immersion in ice. [^{14}C]-**1** oxidation products were analyzed by reverse-phase HPLC on a Spherisorb octyl 5 μm, 4.6 × 250 mm column eluted with methanol/1 N sodium acettae, pH 4.25 (9:1, v/v). Radioactivity in fractions of the effluent was quantitated by liquid scintillation counting. Improved resolution of oxidation products for DAD-HPLC analysis was obtained by HPLC on Spherisorb ODS-2, 5 μm, 4.6 × 250 mm column eluted with methanol/1 N sodium acetate, pH 4.25 (93:7, v/v) at a flow rate of 1.5 mL min^{-1}. Products for NMR and mass spectral analysis were extracted from the mobile phase with hexane.

4a,5-Epoxy-8a-hydroperoxytocopherone (4a). The diastereomeric 4a,5-epoxy-8a-hydroperoxytocopherones (**4a**) were purified on Spherisorb ODS-2 as peaks A2-3 as described above. The following analytical data were obtained for the peak A2/A3 mixture: UV_{max}(hexane) 246 nm; MS *m/z* 478 (M^+, 0.5%), 444 (3.4), 430 (3.4), 419 (60.3), 402 (17.7), 237 (95.4), 194 (50.0), 167 (100.0); DCIMS *m/z* 479 (MH^+, 1.7), 463 (9.3), 445 (100.0), 429 (10.0); CIMS (peak matching vs PFK *m/z* 454.9727) MH^+ 479.3732 (calcd $C_{29}H_{50}O_5 + H^+ = 479.3736$); ^{1}H NMR ($C_6D_{12}$) δ1.23 (s, H2a), δ1.43 (s, H5a), δ1.68 (s, H7a), δ1.43, 1.74 (2 m, H3a,b), δ1.83 (s, H8b), δ1.99, 2.72 (2 m, H4a,b).

8a-[(2,4-Dimethyl-1-nitrilopent-2-yl)dioxy]tocopherone (3a). The four diastereomers of **3a** (peaks B1–4, Figure 2) were purified together by reverse-phase HPLC on Spherisorb ODS-2 (see above). The diastereomeric mixture was analyzed by CIMS (peak matching vs tetrakis(trimethylsilyl)adenosine (MH^+ = 556.263) MH^+ 572.469 (calcd $C_{36}H_{61}O_4N + H^+$ 572.479). Diastereomers B1–4 were completely resolved by normal-phase HPLC on a Spherisorb silica, 5 μm, 4.6 × 250 mm column eluted with hexane/isopropyl alcohol (99.5:0.5, v/v) at a flow rate of 1 mL min^{-1}: **3a** (B1) UV_{max} (hexane) 237 nm; MS *mz* 445 (2.6), 430 (76.3), 417 (3.0), 401 (3.6), 203 (28.1), 165 (100). **3a** (B2) UV_{max} (hexane) 237 nm; MS *m/z* 445 (5.9), 430 (97.2), 419 (5.5), 417 (4.1), 401 (4.2), 203 (16.7), 165 (100). **3a** (B3) UV_{max} (hexane) 237 nm; MS *m/z* 445 (3.0), 430 (86.8), 417 (1.7), 401 (2.1), 205 (14.1), 165 (100). **3a** (B4) UV_{max} (hexane) 236 nm; MS *m/z* 445 (2.2), 430 (100), 417 (2.7), 401 (3.4), 205 (11.8), 165 (89.3).

Oxidation of 1 in Acetonitrile/Water. AMVN (10.8 mg, 43.6 μmol) and **1** (1.0 mg, 2.35 μmol) were dissolved in 7 mL of oxygen-saturated acetonitrile/water (6:4, v/v) and heated for 2 h in a screw-cap test tube at 50 °C. The mixture was extracted with hexane, and the extracts were evaporated in vacuo and then redissolved in acetonitrile. Products were purified by reverse-phase HPLC on Spherisorb ODS-2 as described above and extracted from the mobile phase with hexane. α-Tocopherylquinone-2,3-oxide **6** and α-tocopherylquinone-5,6-oxide **7** were purified as peaks A1′ and A2′ by reverse-phase HPLC on Spherisorb ODS-2 (see above). **6**: UV_{max}(hexane) 274 nm; MS/*mz* 462 (M^+, 0.4), 447 (2.5), 444 (3.2), 430 (16.1), 419 (20.4), 402 (8.6), 237 (55.7), 195 (100.0); ^{1}H NMR (C_6D_{12}) δ2.61 (m, H7), δ2.33 (m, H7), δ1.94 (s, 2-CH_3), δ1.54 (m, H8), δ1.52 (s, 5,6-CH_3), δ1.35 (m, H8), δ1.14 (s, 9-CH_3). **7**: UV_{max}(hexane) 272 nm; MS *m/z* 462 (M^+, ND), 447 (2.9%), 444 (3.0), 430 (17.2), 419 (59.9), 402 (24.8), 237 (100), 194 (48.9), 167 (89.5); ^{1}H NMR (C_6D_{12}) δ2.32 (m, H7), δ1.89 (s, 5,6-CH_3), δ1.73 (m, H7), δ1.59 (m, H8), δ1.55 (s, 2-CH_3), δ1.51 (m, H8), δ1.15, 1.14 (2 s, 9-CH_3, 2 epimers).

Oxidation of [^{14}C]-3a and 3b. AMVN (1.08 mg, 4.36 μmol) and [^{14}C]-**3a** (0.11 μmol, 0.11 μCi) with or without **1** (0.235 μmol) were dissolved 0.7 mL of oxygen-saturated acetonitrile and heated in a screw-cap tube at 50 °C. Aliquots of the reaction mixture were analyzed by reverse-phase HPLC on Spherisorb ODS-2 as described above. Radioactivity in effluent fractions was quantitated by liquid scintillation counting. AMVN (1.08 mg, 4.36 μmol) and **3b** (0.129 μmol) with or without **1** (0.235 μmol) were heated in oxygen-saturated acetonitrile in a screw cap tube at 50 °C. Aliquots of the reaction mixture were analyzed at various times for remaining **3b** by reverse-phase HPLC on Spherisorb ODS-2 as described above. Quantitation of **3b** was by absorbance at 240 nm.

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Registry No. 1, 59-02-9; [5-$^{14}CH_3$]-**1**, 128442-17-1; **2**, 23531-69-3; **3a** (isomer 1), 121250-09-7; **3a** (isomer 2), 121250-10-0; **3a** (isomer 3), 121186-95-6; **3a** (isomer 4), 121250-11-1; [^{14}C]-**3a** (isomer 1), 128359-84-2; [^{14}C]-**3a** (isomer 2), 128442-22-8; [^{14}C]-**3a** (isomer 3), 128442-23-9; [^{14}C]-**39** (isomer 4), 128442-24-0; **3b** (isomer 1), 121250-08-6; **3b** (isomer 2), 80876-63-7; **4a**, 128442-18-2; **5a**, 128359-82-0; **6** (isomer 1), 128442-19-3; **6** (isomer 2), 128442-20-6; **7** (isomer 1), 128359-83-1; **7** (isomer 2), 128442-21-7; **8**, 7559-04-8; AMVN, 4419-11-8; H_2O, 7732-18-5.

Supplementary Material Available: ^{1}H NMR spectra, phase-sensitive ^{1}H-DQFCOSY spectra, and phase-sensitive ^{1}H-NOESY spectra of compounds B1–B4 (diastereomers of product **3a**) from Table I (12 pages). Ordering information is given on any current masthead page.

(23) Clough, R. L.; Yee, B. G.; Foote, C. S. *J. Am. Chem. Soc.* **1979**, *101*, 683–686.