Tocotrienols from Palm Oil: Electron Spin Resonance Spectra of Tocotrienoxyl Radicals

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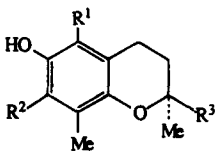
The major vitamin E components present in palm oil, viz. α-tocopherol, α-, γ- and δ-tocotrienols, have been isolated and their structures verified by the NMR spectra of their acetate and succinate derivatives. Oxidation of γ- and δ-tocotrienols with alkaline $K_3Fe(CN)_6$ gave isolable dimeric species, which were studied by ^{13}C NMR. Free radicals generated from the monomeric and dimeric tocotrienols were investigated using ESR spectroscopy. The distinction between antioxidant activity and antioxidant capacity of vitamin E isomers is discussed.

The tocopherols and tocotrienols, 1-8 (See chart below.), collectively known as vitamin E, are lipid-soluble natural antioxidants often present as minor components of vegetable oils. Most vegetable oils provide α-tocopherol, 1, but palm oil is unique in that it contains relatively large concentrations of the tocotrienols, 5, 7 and 8, which together account for about 72-80% of the total vitamin E content (600-1000 ppm). While there have been many studies of the tocopherols, the tocotrienols have received comparatively little attention. However, our interest in the tocotrienols was aroused by reports of their medicinal and nutritional values. For instance, 5 has been shown to be anticholesterogenic (1). Possibly it suppresses



Vitamin E	R^1	R^2	R^3
α-tocopherol (1)	Me	Me	$(CH_2CH_2CH_2CH(Me))_3Me$
β-tocopherol (2)	Me	H	
γ-tocopherol (3)	H	Me	
δ-tocopherol (4)	H	H	
α-tocotrienol (5)	Me	Me	$(CH_2CH_2CH=C(Me))_3Me$
β-tocotrienol (6)	Me	H	
γ-tocotrienol (7)	H	Me	
δ-tocotrienol (8)	H	H	

HMGCoA (3-hydroxy-3-methylglutaryl-Coenzyme A) reductase activity, leading to the inhibition of cholesterol biosynthesis. Recently, it has been shown that 5 has antitumor activity in laboratory animals (2). γ-Tocotrienol, 7, also impedes the aggregation of blood platelets (Holub, B.L., University of Guelph, Canada, private communication to Palm Oil Research Institute of Malaysia, 1987), which may explain the reported anti-thrombotic effect of palm oil (3).

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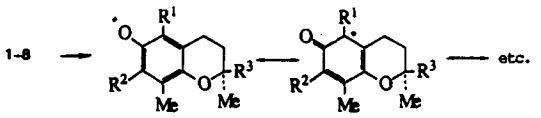
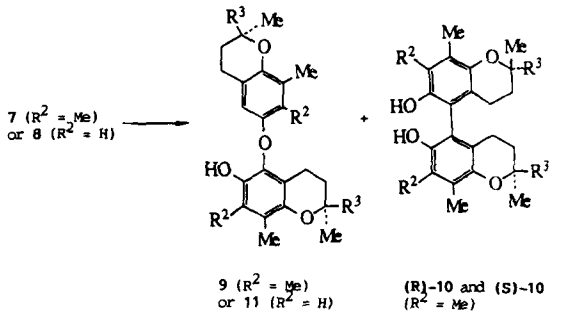
Vitamin E has been shown to be an excellent chain-breaking antioxidant, being more effective than other synthetic *o*- and *p*-substituted phenolics (4). As antioxidants, 1-8 are important for the protection of unsaturated lipids against peroxidation, particularly in biomembranes (5-7). Their effectiveness in this role depends on the fact that they react with chain-propagating free radicals, especially peroxyl radicals, with the formation of relatively stable phenoxyl radicals. These phenoxyl radicals are conjugatively stabilized, as suggested by the following scheme:



The oxygen atom in the para position of a vitamin E free radical formed in this way has an important stabilizing influence, since its fully occupied p orbitals can interact with the aromatic π system (4). The tetrahydropyran moiety ensures that the p orbitals are in an orientation which allows some overlap with the π system, but even more effective overlap appears to be possible in a synthetic analogue of 1 in which the tetrahydropyran ring is replaced by a tetrahydrofuran ring (8). In general, it may be expected that structurally similar chromanols, tocopherols and tocotrienols, can prevent tissue and cellular damage associated with radical peroxidation.

There have been several studies of the ESR (electron spin resonance) and ENDOR (electron nuclear double resonance) spectra of the tocopheroxyl radicals, 1•-4• (9-13). In this paper we give details of the ESR spectra of the analogous tocotrienoxyl radicals, 5• and 8• are susceptible to radical coupling reactions, via their unsubstituted aromatic positions. Following oxidation of the substrate 7, we have successfully isolated the dimers 9 and 10. In 10, internal rotation about the bond linking the phenyl groups is restricted, with the result that this bond is a center of chirality. Since, in our experiments, the starting natural material, 7, was enantiomerically pure with 2R configuration, two chromatographically separable diastereomers of 10 were formed, corresponding to the two possible chiralities, R and S biphenyl-type configurations. We have not unambiguously determined the absolute stereochemistry of these diastereomers, and labeled them simply (R)-10 and (S)-10. For compound 8, a number of dimers could, in principle, be formed by oxidation and radical combination, but the only major species identified in our work was the diphenyl ether dimer, 11.

It seems reasonable to suppose that dimers such as 9-11 are also effective antioxidants, and we have observed the ESR spectra of the expected radicals (9• and 11•) derived from 9 and 11. These considerations lead us to



draw a distinction, in our discussion, between antioxidant activity and antioxidant capacity of the vitamin E constituents.

EXPERIMENTAL

Isolation of the tocotrienols. "Palm fatty acid distillate" is a by-product from the refining of palm oil, and has a considerable high content (about 1000–4000 ppm) of vitamin E. Vitamin E concentrates, obtained from palm fatty acid distillate (14,15), were subjected to preliminary purification by column chromatography using Merck silica gel (230–400 mesh) as the stationary phase and n-hexane containing 5–15% of ethyl acetate as the eluant. Further purification of the tocotrienols to give pure samples of **5**, **7** and **8** was achieved by preparative HPLC, using a Waters Associates liquid chromatograph equipped with a HP SI-100 column (25 cm × 8 mm) and using 2–10% ethyl acetate in n-hexane as the eluant. Detection using either a R401 differential refractometer or a 440 UV-absorbance detector at 254 nm was found to be satisfactory.

Preparation of the tocotrienol esters. Acetates and succinates were prepared by reacting the pure tocotrienols with acetic and succinic anhydrides, respectively, in the presence of pyridine.

Preparation and isolation of the tocotrienol dimers. **7** and **8** were oxidized by alkaline $K_3Fe(CN)_6$, the reaction products were subjected to essentially the same combination of column chromatography and HPLC as described above for the purification of the precursors, except that, for the HPLC, elution using 1–2% ethyl acetate in n-hexane was used.

Spectral characterization of compounds **5**, **7** *and* **8**, *and their derivatives.* Nuclear magnetic resonance was recorded on a JEOL-FX 100 FT-NMR spectrometer. Spectral details are given in Tables 1–3. EI mass spectra for the tocotrienols, their derivatives and dimers were recorded on Kratos AEI MS3074 mass spectrometer.

ESR studies. ESR spectra were recorded at room temperature using a Bruker B-R 70 X-band spectrometer, employing 100 kHz magnetic field modulation. G-values were estimated by comparison with polycrystalline DPPH (1,1-diphenyl-2-picrylhydrazyl), for which a g-value of 2.0036 was assumed (16). Hyperfine splitting constants were invariably estimated by computer simulation of the ESR spectra. This was done using a program written in ASYST, which forms part of a system described elsewhere (17). Free radicals were generated from the tocotrienols and their dimers by oxidation with alkaline potassium ferricyanide. Ca. 1 mg of the tocotrienol was dissolved in 2 ml of toluene, then shaken for 2 min with 1 ml of 0.1M $K_3Fe(CN)_6$ and 1 ml of 0.1M NaOH. The mixture was immediately centrifuged, and about 0.2 ml of the organic phase was transferred to an ESR sample tube (i.d. 3 mm). All operations were carried out under nitrogen, vessels were capped by rubber septa and transfers were by gas-tight syringes.

A second method was also used to generate the tocotrienoxyl radicals. A solution containing radical **11**$^\bullet$ generated from **8** was transferred to an ESR tube containing ca. 1 mg of **5** or **7**. From the exchange reactions, ESR spectra recorded indicated the presence of radicals **5**$^\bullet$ or **7**$^\bullet$ only.

TABLE 1

^{1}H NMR Spectral Data of Tocotrienols and Their Acetates[a]

	Chemical shift/ppm[b]					
Proton	5	5-A	7	7-A	8	8-A
5-H (aromatic)	—	—	6.39	6.62	6.45	6.67
7-H (aromatic)	—	—	—	—	6.51	6.70
3′,7′,11′-CH (olefinic)	5.23	5.17	5.22	5.2	5.20	5.16
6-OH (phenolic)	4.34	—	4.90	—	5.68	—
4-CH_2	2.69	2.66	2.72	2.76	2.72	2.77
CH_3-C(O)-	—	2.36	—	2.32	—	2.26
5a-CH_3	2.19	2.03	—	—	—	—
7a-CH_3	2.19	2.03	2.2	2.07	—	—
8b-CH_3	2.19	2.16	2.2	2.18	2.17	2.20
1′,2′,5′,6′,9′,10′-CH_2	2.0	2.0	2.0	2.0	2.0	2.0
3-CH_2	1.8	1.8	1.8	1.8	1.8	1.8
13′-CH_3	1.77	1.74	1.77	1.73	1.74	1.73
4′a,8′a,12′a-CH_3	1.68	1.65	1.68	1.66	1.66	1.65
2a-CH_3	1.33	1.31	1.34	1.32	1.31	1.32

[a] Acetate denoted by A.
[b] In $CDCl_3$ solution.

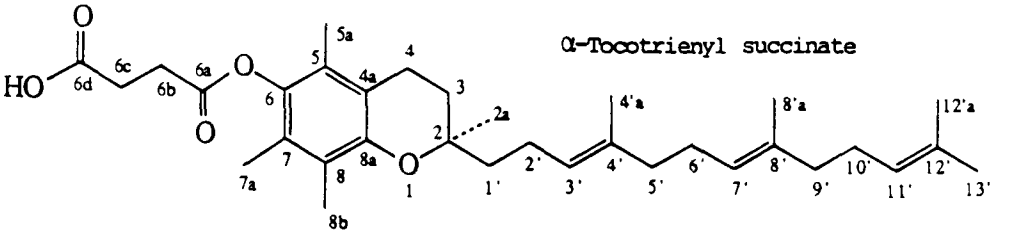
RESULTS AND DISCUSSION

In the refining of palm oil, vitamin E components are partially co-distilled into the palm fatty acid distillate from which they may be recovered. The relative concentrations of vitamin E components in the sample of vitamin E concentrate (used in our experiments) were **1** (25%), **5** (22%), **7** (38%) and **8** (12%). Minor amounts of **2**, **3** and **6** totalled about 3%. The major tocotrienols are characterized by their proton and carbon NMR spectral data which are given in Tables 1 and 2, respectively. The spectral assignments for the aromatic and chroman parts are according to assignments for the corresponding tocopherols (18,19). Assignments for the unsaturated isoprenyl chain are based on the work of Tanaka *et al.* for polyprenol homologues (20).

Oxidation of **1** leads to **1**$^\bullet$, which has been reported (21) to slowly undergo further oxidation to quinones, dimers, and trimers. Our observations suggest that **7** and **8** undergo more facile dimerization under oxidizing conditions, involving bond formation at the unsubstituted 5 (in **7**) or 5 and 7 (in **8**) positions. **7** gives the expected dimers **9** and **10** (two diastereomers). **8** has the possibility to form three biphenyl-type dimers (each with two diastereomers) and two diphenyl ether dimers; however, preferential coupling at position 5 (rather than at position 7) is expected, both on the basis of the known (21,22)

TABLE 2

^{13}C NMR Spectral Data[a] of Palm Oil Tocotrienols and Their Derivatives[b]



Carbon	5	7	8	5-A	7-A	8-A	5-S	7-S
6d	—	—	—	—	—	—	177.88	177.59
6a	—	—	—	169.49	169.77	169.65	170.86	171.25
6	144.54 (s)	146.26 (s)	147.74 (s)	149.30	149.41	149.45	149.36	149.40
8a	145.94 (s)	145.63 (s)	145.87 (s)	140.56	141.62	142.43	140.48	141.51
4′	134.91 (s)	134.99 (s)	135.03 (s)	134.95	134.95	134.87	135.07	135.22
8′	134.91 (s)	134.87 (s)	134.91 (s)	134.79	134.75	134.60	134.88	134.95
12′	131.13 (s)	131.13 (s)	131.13 (s)	131.01	130.93	130.78	131.80	131.80
4a	122.51 (s)	125.71 (s)	127.23 (s)	126.60	126.92	126.96	127.08	127.07
3′	124.42 (d)	124.38 (d)	124.42 (d)	124.38	124.34	124.63	124.39	124.39
7′	124.42 (d)	124.38 (d)	124.34 (d)	124.27	124.15	124.63	124.25	124.20
11′	124.23 (d)	124.23 (d)	124.23 (d)	124.19	124.15	124.27	124.25	124.20
8	121.26 (s)	121.85 (s)	121.80 (s)	122.94	118.81	120.56	123.03	118.79
7	118.69 (s)	118.14 (s)	115.84 (d)	117.21	118.18	118.89	117.32	118.40
5	117.17 (s)	112.30 (d)	112.80 (d)	124.81	125.70	120.99	124.93	125.85
2	74.20 (s)	75.22 (s)	75.30 (s)	74.71	75.65	75.61	74.81	75.83
1′	39.67 (t)	39.78 (t)	39.70 (t)	39.66	39.97	39.74	39.70	40.04
5′	39.67 (t)	39.70 (t)	39.70 (t)	39.66	39.62	39.54	39.70	39.70
9′	39.67 (t)	39.70 (t)	39.70 (t)	39.66	39.62	39.54	39.70	39.70
3	31.63 (t)	31.47 (t)	31.43 (t)	31.08	30.97	30.89	31.12	31.07
6b	—	—	—	20.52	20.64	20.72	28.98	28.91
6c	—	—	—	—	—	—	28.59	28.91
10′	26.76 (t)	26.76 (t)	26.76 (t)	26.72	26.68	26.60	26.73	26.78
6′	26.60 (t)	26.60 (t)	26.60 (t)	26.56	26.52	26.40	26.59	26.64
13′	25.66 (q)	25.66 (q)	25.66 (q)	25.62	25.59	25.47	25.66	25.71
2a	23.68 (q)	23.95 (q)	23.99 (q)	23.79	24.03	23.91	23.76	24.10
2′	22.23 (t)	22.23 (t)	22.19 (t)	22.16	22.12	22.23	22.20	22.20
4	20.75 (t)	22.23 (t)	22.47 (t)	20.40	22.12	22.00	20.54	22.20
12′a	17.67 (q)	17.64 (q)	17.64 (q)	17.60	17.55	17.48	17.66	17.71
8′a	15.96 (q)	15.96 (q)	16.00 (q)	15.92	15.88	15.88	15.86	15.96
4′a	15.88 (q)	15.84 (q)	16.00 (q)	15.84	15.80	15.80	15.86	15.96
7a	11.79 (q)	11.86 (q)	—	12.84	12.57	—	12.84	12.64
8b	11.79 (q)	11.86 (q)	15.84 (q)	11.98	11.86	15.69	12.01	12.01
5a	11.32 (q)	—	—	11.75	—	—	11.81	—

[a]This is ppm in $CDCl_3$ solution. s = singlet, d = doublet, t = triplet, q = quartet.

[b]Acetate denoted by -A and succinate by -S.

directing effect of the heterocyclic ring, and on the basis of the hyperfine splitting constants observed for **4**• (Table 4). Therefore, it is not surprising that the major dimer we could isolate from the oxidation products of **8** was **11**.

As an antioxidant, each vitamin E constituent can readily donate its phenoxy proton to an oxidant or radical to give rise to a relatively unreactive phenoxy radical. ESR spectra of the radicals **5**• and **7**•, generated by oxidation of the substrates **5** and **7** respectively, were recorded. A solution containing **11**• was found to be suitable to generate radicals **5**• and **7**• by simple H-atom transfer reactions. The spectrum of **5**• is shown in Figure 1, and, as expected, does not differ noticeably from spectra of the saturated analogue **1**• which may also be found in the literature (11–13). New ESR spectral data for the tocotrienoxyl radicals are reported in Table 4, along with literature data for the corresponding tocopheroxyls. The influence of unsaturation in the side chain, as expected from its distance from the radical center, is insignificant.

ESR spectra of the radicals **9**• and **11**•, formed by oxidation of the diphenyl ether dimers (or derived *in situ* from **7** and **8** respectively), were also recorded. Details are reported in Table 4.

Spectra from uncontrolled ferricyanide oxidation of γ- and δ-tocotrienols were recorded usually as broad 9-line and 5-line signals, respectively, which were due to γ- and δ-tocotrienol diphenyl ether dimer radicals (**9**• and **11**•), respectively, with the computer-simulated hyperfine coupling constants given in Table 4. A reasonably well

TABLE 3

^{13}C NMR Spectral Data of Tocotrienol Dimers

Assignment[a]	7	(R)-10	(S)-10	9	8	11
6	146.26 (s)	145.95 (s)	145.90 (s)	145.05 (s)	147.74 (s)	145.63 (s)
6	—	145.95 (s)	145.90 (s)	146.65 (s)	—	147.15 (s)
8a[b]	145.63 (s)	144.66 (s)	144.77 (s)	140.17 (s)	145.87 (s)	141.26 (s)
8a[b]	—	144.66 (s)	144.77 (s)	137.13 (s)	—	137.09 (s)
4a	125.71 (s)	126.92 (s)	126.92 (s)	126.18 (s)	127.23 (s)	127.70 (s)
4a	—	126.92 (s)	126.92 (s)	123.56 (s)	—	123.41 (s)
8	121.85 (s)	122.28 (s)	122.28 (s)	121.89 (s)	121.80 (s)	121.38 (s)
8	—	122.28 (s)	122.28 (s)	121.65 (s)	—	115.34 (s)
7[b]	118.14 (s)	117.40 (s)	117.33 (s)	118.14 (s)	115.84 (d)	115.26 (d)
7[b]	—	117.40 (s)	117.33 (s)	111.91 (s)	—	115.10 (d)
5	112.30 (d)	115.22 (s)	115.22 (s)	148.13 (s)	112.80 (d)	149.73 (s)
5	—	115.22 (s)	115.22 (s)	109.84 (d)	—	111.95 (d)
2	75.22 (s)	74.98 (s)	75.02 (s)	75.41 (s)	75.30 (s)	75.61 (s)
2	—	74.98 (s)	75.02 (s)	75.02 (s)	—	75.10 (s)
3	31.47 (t)	31.42 (t)	31.42 (t)	31.36 (t)	31.43 (t)	31.31 (t)
3	—	31.42 (t)	31.42 (t)	30.81 (t)	—	30.69 (t)
2a[b]	23.95 (q)	23.79 (q)	23.91 (q)	24.03 (q)	23.99 (q)	24.10 (q)
2a[b]	—	23.79 (q)	23.91 (q)	23.79 (q)	—	23.87 (q)
4,*4*	22.23 (t)	20.68 (t)	20.68 (t)	22.43 (t)	22.47 (t)	22.66 (t)
7a	11.86 (q)	12.29 (q)	12.29 (q)	12.10 (q)	—	—
7a	—	12.29 (q)	12.29 (q)	11.63 (q)	—	—
8b,*8b*	11.86 (q)	12.02 (q)	12.02 (q)	11.98 (q)	15.84 (q)	16.23 (q)

[a] In $CDCl_3$ as internal standard. Carbons for tocotrienoxy (in **9** or **11**) and one of the tocotrienyl fragments (in **10**) are italic. Multiplicity: (s) = singlet, (d) = doublet, (t) = triplet, (q) = quartet.

[b] Distinguishable peaks were observed for **(R)-10** and **(S)-10**.

TABLE 4

ESR Parameters of Tocopheroxyl and Tocotrienoxyl Radicals

Free radical	Ref.	g-factor (± 0.0002)	Hyperfine coupling constants (Gauss) 5-R_1	7-R_2	8-CH_3	4-CH_2
5•	*	2.0046	6.11	4.58	0.97	1.53
1•	11	2.0046	6.07	4.55	0.98	1.52
	12	—	6.02	4.58	0.94	1.48
	13	—	5.98	4.57	0.94	1.47
7•	*	2.0048	6.00	4.80	1.08	1.21
3•	11	2.0047	6.03	4.82	1.12	1.30
	12	—	5.95	4.77	1.16	1.16
	13	—	5.93	4.77	1.17	1.17
4•	11	2.0049	6.19	4.82	1.03	1.45
	12	—	5.92	4.60	1.11	1.11
	13	—	6.21	4.72	1.04	1.42
9•	*	2.0047	—	3.92	0.34	1.86 & 2.06
11•	*	2.0047	—	3.70	0.28	1.74 & 1.95

*This study.

resolved spectrum of **9**• generated from **7** or its diphenyl ether dimer is shown in Figure 2, where the effect of slightly nonequivalent methylene hydrogens at position 4 may be seen.

Generally, on standing at room temperature for 24 hr, the spectrum will broaden to the 9-line signals. The line broadening in the spectra, not due to concentration or

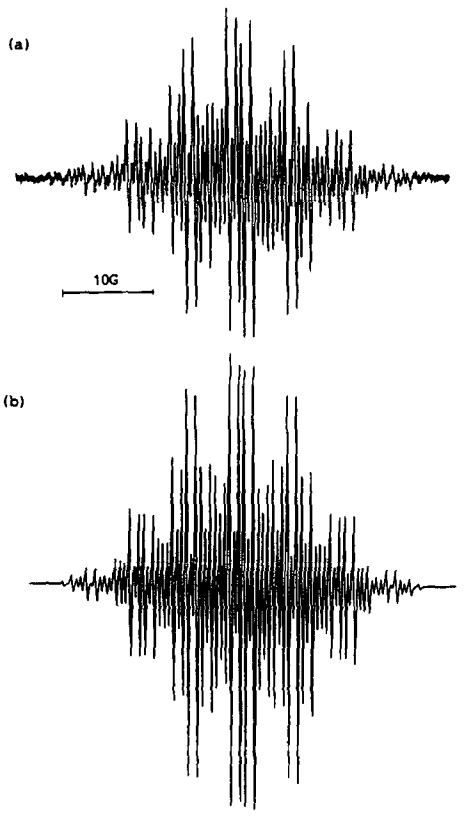


FIG. 1. (a) ESR spectrum of α-tocotrienoxyl generated from H-abstraction by 11• and (b) the simulated spectrum.

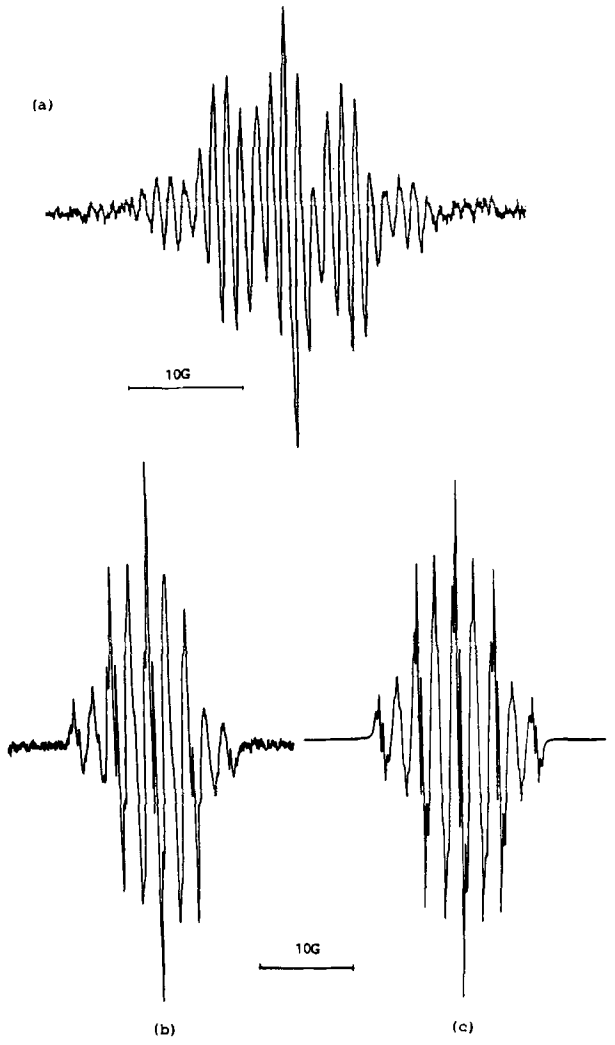


FIG. 2. ESR spectra of (a) 7$^{\bullet}$ and (b) 9$^{\bullet}$, (c) the simulated spectrum of 9$^{\bullet}$.

modulation effects alone, may be caused by impurity radicals of other structurally similar oxidation products (e.g. trimers or tetramers).

There has been considerable interest in the antioxidant activity of tocopherols and model compounds. When activity is based on kinetic measurements (e.g. H-transfer in the termination of peroxy-induced radical chain reactions), the order of decreasing activity among the tocopherols is generally accepted as $\alpha > \beta > \gamma > \delta$ (23-25), and this order is in agreement with their biopotencies (26,27). On the other hand, when the capacity of tocopherols to inhibit oxidation is considered, a reverse order may be found (28); indeed, other workers have found **3** to be more effective than **1** in preventing *in vitro* lipid peroxidation (29,30). This can be rationalized on the basis that **3** not only acts as an antioxidant itself, but also forms dimers which are also antioxidants. In fact, it has been shown that the γ-tocopherol diphenyl ether dimer is more effective than **1** in preventing the oxidation of lard (30). It is clear that apparent discrepancies will exist for the order of antioxidant activity among the isomeric tocopherols and tocotrienols depending on whether the reactivity is measured directly (kinetic) or the antioxidant capacity is being determined. In other words, although trimethylated chromanol (α) is more reactive as a H-atom donor than the 7,8-dimethylated chromanol (γ), the ready formation of diphenyl ether dimer (also a H-atom donor) from the latter will enhance the γ-isomer's antioxidant capacity.

To explore these ideas further, we subjected equal amounts of **5** and **7**, dissolved in toluene, to ferricyanide oxidation. The ESR spectrum recorded after 10 min indicated that **5**$^{\bullet}$ was the principal paramagnetic species presnt. After 24 hr at room temperature, the spectrum of **5**$^{\bullet}$ was completely replaced by the spectrum of **9**$^{\bullet}$, which persisted for several days. We concluded that, although the α-chromanols (**1** and **5**) may be more active as antioxidants, the other vitamin E constituents may have greater antioxidant capacities, as a result of their ready formation of dimers which are antioxidants.

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