Products Formed by Peroxyl Radical Oxidation of β-Carotene

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β-Carotene was reacted with alkylperoxyl radicals at 37 °C in benzene. 2,2′-Azobis(2,4-dimethylvaleronitrile) was used to generate the alkylperoxyl radicals. The reaction products were isolated by reversed-phase and normal-phase high-performance liquid chromatography, and their structures were characterized by ultraviolet–visible, infrared, ^1H and ^{13}C nuclear magnetic resonances and mass spectrometry. They were identified as a mixture of 12-formyl-11-nor-β,β-carotene (**1a**) and 15′-formyl-15-nor-β,β-carotene (**1b**), 19-oxomethyl-10-nor-β,β-carotene (**2**), 5,6-epoxy-5,6-dihydro-β,β-carotene (**3**), 13,15′-epoxyvinyleno-13,15′-dihydro-β,β-carotene (**4a**), a mixture of (13*R*,15′*R*)- and (13*R*,15′*S*)-15′,13-epoxyvinyleno-13,15′-dihydro-β,β-carotenes (**4b**), a mixture of (13*R*,15′*S*)- and (13*S*,15′*R*)-15′,13-epoxyvinyleno-13,15′-dihydro-β,β-carotenes (**4c**), and 11,15′-cyclo-12,15-epoxy-11,12,15,15′-tetrahydro-β,β-carotene (**5**). These products were formed together from the initial stages of the reaction. As the reaction progressed, compounds **4** and **5** were somewhat stable and remained in the reaction mixture.

INTRODUCTION

Carotenoids are widely distributed in nature, where they play an important role in protecting cells and organisms. Due to their highly conjugated double-bond system, carotenoids are extremely efficient quenchers of singlet molecular oxygen which is generated by photochemical reactions (Foote and Denny, 1968; Foote et al., 1970). In addition, carotenoids may participate in free-radical reactions. β-Carotene and related carotenoids have been demonstrated to possess antioxidant activity in homogeneous solutions (Burton and Ingold, 1984; Terao, 1989; Polazza and Krinsky, 1991), liposomes (Krinsky and Deneke, 1982; Kennedy and Liebler, 1992; Lim et al., 1992), microsomal membranes (Polazza et al., 1992), and lipoproteins (Jialal et al., 1991). Evidence has been presented that the antioxidant effectiveness of β-carotene is markedly enhanced at low oxygen tensions (Burton and Ingold, 1984; Stoker et al., 1987; Burton, 1989; Kennedy and Liebler, 1992). Though carotenoids have been known to function as antioxidants, little is known about the mechanism of their antioxidant activity. It has been suggested that the chain-propagating peroxyl radical is trapped by addition to the conjugated polyene system of β-carotene, forming a resonance-stabilized, carbon-centered β-carotene radical (Burton and Ingold, 1984).

To know the mechanism of antioxidant activity of β-carotene, it is important to analyze its oxidation products that may arise during its action as an antioxidant. The characterization of oxidation products of β-carotene has been examined in systems using high-temperature treatment (Marthy and Berset, 1986, 1990; Onyewu et al., 1986; Kanasawud and Crouzet, 1990). Because of the presence of long and conjugated double bonds, β-carotene is an excellent substance for free-radical attack, and the resulting products are very complex (El-Tinay and Chichester, 1970; Kennedy and Liebler, 1991; Handelman et al., 1991; Mordi et al., 1991). The reaction products of β-carotene with peroxyl radicals in model systems have been reported to be apocarotenals and epoxy compounds (Kennedy and Liebler, 1991; Handelman et al., 1991; Mordi et al., 1991).

In this study, the reaction products of β-carotene with alkylperoxyl radicals in benzene solution were isolated and characterized. The alkylperoxyl radicals were generated by thermolysis of a free-radical initiator, 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN).

MATERIALS AND METHODS

Materials. *all-trans*-β-Carotene and AMVN were purchased from Wako Pure Chemical Industries (Osaka, Japan), and β-carotene was recrystallized from benzene. 5,8-Epoxy-5,8-dihydro-β,β-carotene was prepared by the oxidation of β-carotene with monoperphthalic acid (Tsukida and Zechmeister, 1958). All other chemicals were obtained from common laboratory suppliers. All experimental procedures were done under reduced light.

Apparatus. High-performance liquid chromatography (HPLC) was performed with a Jasco Model 880 pump (Japan Spectroscopic Co., Tokyo, Japan) and a Model 830 differential refractometer. Ultraviolet–visible (UV–vis) spectra were measured with a Jasco Ubest-30 spectrophotometer. Infrared (IR) spectra of samples in liquid film were taken on a Jasco A-302 IR spectrometer. Electron impact mass spectrometry (EI-MS) was done with a Shimadzu QP-1000 instrument (Shimadzu Co., Kyoto, Japan). Samples were introduced by a direct probe insertion and ionized with a 70-eV electron beam. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Jeol GX-270 instrument with $CDCl_3$ as the solvent and with tetramethylsilane as the internal standard. ^1H NMR was performed at 270.17 MHz, and the ^1H–^1H chemical shift-correlated (COSY) NMR technique was employed to assign ^1H shifts and couplings. ^{13}C NMR was a 67.94 MHz with proton decoupling. For carbon discrimination in the ^{13}C NMR data, the distortionless enhancement by polarization transfer (DEPT) technique was employed.

Reaction Procedure. β-Carotene (0.75 g, 1.4 mmol) and AMVN (3.48 g, 14 mmol) were dissolved in 700 mL of benzene. The mixture was shielded from light and incubated at 37 °C for 2 h under air. After the solvent was evaporated to dryness in vacuo, the oily residue was dissolved in 500 mL of petroleum ether, washed 10 times with 90% methanol (20 mL each) to remove AMVN, and evaporated to dryness. The reaction mixture was then dissolved in chloroform, methanol was added, and the mixture was allowed to stand at −20 °C for 2 h. The resulting crystals were removed by filtration. The filtrate contained most of the reaction products, and the crystals contained β-carotene and one product, respectively. The products in the filtrate were separated by preparative HPLC. Reversed-phase HPLC was done with a Bio-Sil C_{18} HL 90-10 column (10 × 250 mm, Nihon Bio-Rad Laboratory Co., Tokyo, Japan) developed with methanol–ethyl acetate (8:2 v/v) at a flow rate of 6.0 mL/min. Normal-phase HPLC was done with a Wakosil 5Sil (10 × 300 mm, Wako Pure Chemical Industries) developed with hexane–2-propanol

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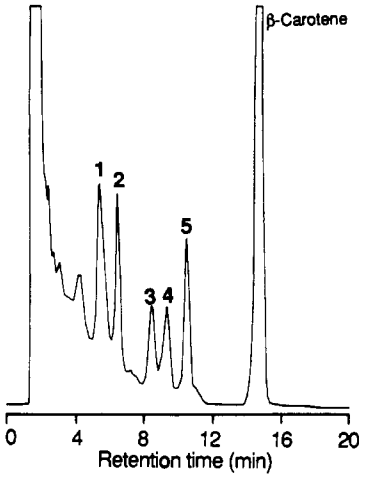


Figure 1. Reversed-phase HPLC analysis of incubation mixture of β-carotene and AMVN in benzene for 2 h. HPLC was done with a μBondasphere 5-μm C_{18} column (3.9 × 150 mm) developed with methanol–ethyl acetate (8:2 v/v) at a flow rate of 0.8 mL/min. The eluent was monitored by a refractive index.

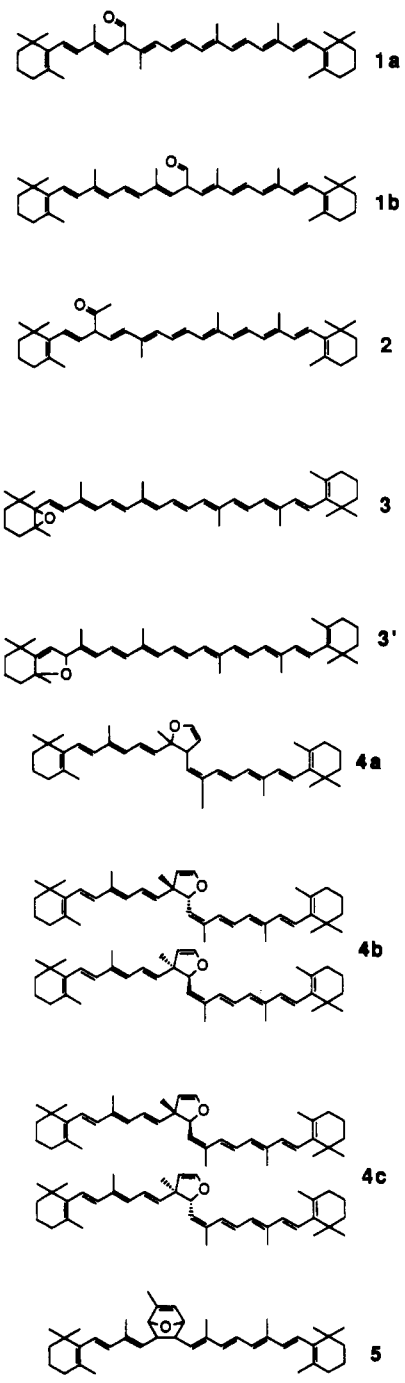
(100:0.2 v/v) at a flow rate of 5.0 mL/min. The eluent was monitored by a refractive index. A product in the crystalline mixture was separated by preparative TLC (silica gel 60 PF_{254}, 1.25 mm thick, Merck, Darmstadt, Germany) using benzene–petroleum ether (1:1 v/v) as the solvent. The purities of isolated compounds were checked by silica gel TLC (precoated silica gel 60 plate, 0.25 mm thick, Merck) using the same solvent system.

Quantification of Product Distribution. β-Carotene (2 mM) and AMVN (20 mM) in benzene (100 mL) were incubated as described above. Periodically, 5.0 mL of the reaction mixture was taken and benzene was removed in vacuo. The residue was dissolved in 1.0 mL of chloroform. A 10-μL aliquot of the sample solution was injected onto HPLC. A μBondasphere 5-μm C_{18} 100-Å column (3.9 × 150 mm, Nihon Waters Co., Tokyo, Japan) was developed with methanol–ethyl acetate (8:2 v/v) at a flow rate of 0.8 mL/min. The eluent was monitored by a refractive index. The quantities of β-carotene and its reaction products were determined by their peak areas calibrated against known amounts of β-carotene.

RESULTS

AMVN decomposes at 37 °C to form alkyl radicals which, in turn, react with molecular oxygen to form alkylperoxyl radicals (Niki et al., 1984). The resulting alkylperoxyl radicals attack β-carotene. The reaction products of β-carotene with AMVN were analyzed by reversed-phase HPLC (Figure 1). Five major peaks, 1–5, besides several minor peaks and a peak corresponding to β-carotene, appeared on the chromatogram. Due to the poor solubility of β-carotene to the HPLC mobile phase (Craft and Soares, 1992), the removal of unreacted β-carotene from the reaction mixture was necessary to isolate the reaction products. The reaction mixture was dissolved in chloroform–methanol to obtain the crystalline and soluble fractions. The soluble fraction contained most of the reaction products (**1**, **2**, **4**, and **5**) which were separated by reversed-phase HPLC. The crystalline fraction contained β-carotene and **3**, which were separated by preparative TLC.

Compound **1** was obtained as an orange solid (R_f 0.70, 41.0-mg yield). Compound **1** was identified as a mixture of 12-formyl-11-nor-β,β-carotene (**1a**) and 15′-formyl-15-nor-β,β-carotene (**1b**) by the following spectral data. Compound **1** displayed UV–vis maxima at λ 358 (ε 52 300), 374 (49 100), and 399 nm (41 800) in hexane; IR (film) maxima at ν 2950, 1730 (C=O), 1450, 1380, 1360, and 965 cm^{-1}; and EI-MS at *m/z* 552 (M^+, 26%), 415 (11), 349 (6), 177 (27), 159 (22), 145 (27), 133 (28), 119 (51), 105 (59), 95



(74), 81 (58), and 69 (100). ^1H NMR ($CDCl_3$) of **1** gave resonances at δ 1.02 (m, 12 H, H-16,16′,17,17′), 1.43–1.48 (m, 4 H, H-2,2′), 1.58–1.65 (m, 4 H, H-3,3′), 1.70 (s, 3 H, H-18), 1.71 (s, 3 H, H-18′), 1.81 (s, $^3/_2$ H, **1a** H-19), 1.82 (s, $^3/_2$ H, **1a** H-20), 1.89 (s, $^3/_2$ H, **1b** H-20), 1.96 (s, $^{15}/_2$ H, **1a** H-19′,20′, **1b** H-19,19′,20′), 2.01 (m, 4 H, H-4,4′), 3.99 (d, *J* = 8.6 Hz, $^1/_2$ H, **1a** H-12), 4.34 (m, $^1/_2$ H, **1b** H-15′), 5.49 (d, *J* = 8.6 Hz, $^2/_2$ H, **1b** H-14,14′), 5.65 (d, *J* = 8.6 Hz, $^1/_2$ H, **1a** H-10), 6.07–6.70 (m, $^{21}/_2$ H, **1a** H-7,7′,8,8′, 10′,11′,12′,14,14′,15,15′, **1b** H-7,7′,8,8′,10,10′,11,11′,12,12′), 9.40 (d, *J* = 2.1 Hz, $^1/_2$ H, **1b** C*H*O), and 9.49 (d, *J* = 2.6 Hz, $^1/_2$ H, **1a** C*H*O). Peak assignments were confirmed by ^1H–^1H COSY analysis (not shown). The spectrum contained several cross-peaks: cross-peaks corresponding to **1a** appeared between resonances at δ 3.99 (H-12) and 5.65 (H-10) and at δ 3.99 (H-12) and 9.49 (C*H*O), respectively; cross-peaks corresponding to **1b** appeared between resonances at δ 4.34 (H-15′) and 5.49 (H-14,14′) and at δ 4.34 (H-15′) and 9.40 (C*H*O), respectively. The ^{13}C NMR

($CDCl_3$) data showed signals due to 40 carbon atoms: δ 12.76 ($^1/_2$ C, CH_3), 12.81 (1 C, CH_3), 13.1 ($^1/_2$ C, CH_3), 13.4 ($^1/_2$ C, CH_3), 14.1 (1 C, CH_3), 16.2 ($^1/_2$ C, CH_3), 19.3 (2 C, C-3,3′), 20.7 ($^1/_2$ C, CH_3), 21.7 ($^1/_2$ C, CH_3), 21.8 ($^1/_2$ C, CH_3), 22.7 ($^1/_2$ C, CH_3), 28.9 (2 C, C-16,16′), 29.0 (2 C, C-17,17′), 31.6 ($^1/_2$ C, CH_2), 33.0 ($^1/_2$ C, CH_2), 33.10 ($^1/_2$ C, CH_2), 33.13 ($^1/_2$ C, CH_2), 34.2 (1 C, C-1), 34.3 (1 C, C-1′), 39.6 (1 C, C-2), 39.7 (1 C, C-2′), 53.0 ($^1/_2$ C, **1b** C-15′), 61.5 ($^1/_2$ C, **1a** C-12), 122.1 ($^1/_2$ C, CH), 124.7 (1 C, CH), 125.2 ($^3/_2$ C, CH), 126.76 ($^1/_2$ C, CH), 126.80 (1 C, CH), 126.9 ($^1/_2$ C, CH), 128.6 ($^1/_2$ C, CH), 129.2 ($^1/_2$ C, C), 129.4 ($^1/_2$ C, C), 130.0 (1 C, C), 130.1 ($^3/_2$ C, CH), 130.7 ($^1/_2$ C, CH), 131.6 ($^1/_2$ C, CH), 133.0 ($^1/_2$ C, C), 136.0 (1 C, C), 136.1 ($^1/_2$ C, C), 136.5 (1 C, C), 136.7 ($^1/_2$ C, C), 136.8 ($^1/_2$ C, CH), 137.1 ($^1/_2$ C, CH), 137.4 ($^1/_2$ C, C), 137.66 (1 C, CH), 137.77 ($^1/_2$ C, CH), 137.85 (1 C, CH), 137.93 ($^1/_2$ C, C), 138.5 (1 C, C), 138.7 ($^1/_2$ C, C), 196.0 ($^1/_2$ C, CHO), 197.9 ($^1/_2$ C, CHO). The DEPT spectrum (not shown) of **1** showed signals of two aldehydes (δ 196.0 and 197.9) and two tertiary carbons (δ 53.0 and 61.5) in addition to other signals as shown above. The structures shown represent one of the most likely conformations.

Compound **2** was obtained as an orange solid (R_f 0.35, 46.2-mg yield). Compound **2** displayed UV–vis maxima at λ 380 (sh, ε 44 900), 402 (57 600), and 424 nm (49 000) in hexane; IR (film) maxima at ν 2950, 1720 (C=O), 1450, 1360, and 965 cm^{-1}; and EI-MS at *m/z* 552 (M^+, 24%), 509 ($[M - CH_3CO]^+$, 6), 347 (4), 257 (5), 197 (11), 171 (15), 157 (23), 145 (30), 133 (28), 119 (53), 105 (55), 95 (56), 81 (53), and 65 (100). ^1H NMR ($CDCl_3$) of **2** gave resonances at δ 0.989 and 0.994 (s, 6 H, H-16,17), 1.03 (s, 6 H, H-16′,17′), 1.43–1.48 (m, 4 H, H-2,2′), 1.58–1.63 (m, 4 H, H-3,3′), 1.68 (s, 3 H, H-18), 1.72 (s, 3 H, H-18′), 1.91 (s, 3 H, H-20), 1.93–2.04 (m, 4 H, H-4,4′), 1.97 (s, 6 H, H-19′,20′), 2.21 (s, 3 H, H-19), 3.93 (dd, *J* = 7.7, 8.1 Hz, 1 H, H-9), 5.48 (dd, *J* = 8.1, 15.8 Hz, 1 H, H-8), 5.84 (dd, *J* = 7.7, 15.8 Hz, 1 H, H-11), 6.03 (d, *J* = 15.8 Hz, 1 H, H-7), 6.12–6.24 (m, 5 H, H-7′,8′,10′,14,14′), 6.24 (d, *J* = 15.4 Hz, 1 H, H-12), 6.34 (d, *J* = 15.0 Hz, 1 H, H-12′), 6.60 (m, 1 H, H-11′), and 6.65 (dd, *J* = 11.1, 15.0 Hz, 2 H, H-15,15′). The ^1H–^1H COSY spectrum (not shown) gave cross-peaks between resonances at δ 3.93 (H-9) and 5.48 (H-8), at δ 3.93 (H-9) and 5.84 (H-11), at δ 5.48 (H-8) and 6.03 (H-7), and at δ 5.84 (H-11) and 6.24 (H-12), respectively. ^{13}C NMR ($CDCl_3$) of **2** yielded resonances at δ 12.78 (C-19′), 12.84 (C-20′), 12.88 (C-20), 19.28 (C-3), 19.32 (C-3′), 21.5 (C-18), 21.8 (C-18′), 28.3 (C-19a), 28.77 (C-16), 28.80 (C-17), 29.0 (2 C, C-16′,17′), 32.7 (C-4), 33.1 (C-4′), 34.1 (C-1), 34.3 (C-1′), 39.4 (C-2), 39.7 (C-2′), 61.3 (C-9), 125.2 (C-11′), 125.5 (C-7), 126.8 (C-7′), 129.2 (C-5), 129.4 (C-5′), 129.5 (C-15), 129.8 (C-8), 130.3 (C-15′), 130.8 (C-10′), 131.7 (C-11), 132.0 (C-14), 132.1 (C-14′), 135.1 (C-13), 136.1 (C-9′), 136.6 (C-13′), 137.0 (C-6), 137.2 (C-12), 137.3 (C-12′), 137.8 (C-8′), 138.0 (C-6′), and 207.4 (C-19, C=O). The DEPT spectrum (not shown) indicated that **2** had a ketone carbon (δ 207.4) and a tertiary carbon (δ 61.3) in addition to other carbon atoms that were consistent with the assigned structure. Thus, compound **2** was identified as 19-oxomethyl-10-nor-β,β-carotene. The structure shown represents one of the most likely conformations.

Compound **3** in the crystalline fraction was subjected to preparative TLC. This chromatography resulted in the rearrangement of **3** to compound **3′**. Compound **3′** was obtained as a red solid (R_f 0.27, 28.4-mg yield). The spectral data of **3′** were essentially identical with those of authentic 5,8-epoxy-5,8-dihydro-β,β-carotene (Tsukida and Zechmeister, 1958): UV–vis (hexane) λ_{max} 405 (ε 83 200), 426 (121 000), and 452 nm (109 000); IR (film) ν_{max} 2850, 1450, 1370, 1305, 1220, 1170, 1125, 1070, 995, and 960 cm^{-1}; EI-MS *m/z* 552 (M^+, 20%), 472 (24), 336 (19), 205 (59), 165 (45), 119 (48), 105 (65), 91 (69), 81 (45), and 69 (100); ^1H NMR ($CDCl_3$) δ 1.03 (s, 6 H, H-16′,17′), 1.01 and 1.11 (s, 3 H, H-16), 1.15 and 1.18 (s, 3 H, H-17), 1.42 and 1.46 (s, 3 H, H-18), 1.44–1.48 (m, 4 H, H-2,2′), 1.54–1.64 (m, 4 H, H-3,3′), 1.72 (s, 3 H, H-18′), 1.74 and 1.80 (s, 3 H, H-19), 1.90–2.04 (m, 4 H, H-4,4′), 1.94 and 1.95 (s, 3 H, H-20), 1.97 (s, 6 H, H-19′,20′), 5.07 and 5.15 (s, 1 H, H-8), 5.17 and 5.24 (s, 1 H, H-7), 6.15 (m, 4 H, H-7′,8′,10,10′), 6.21 (m, 2 H, H-14,14′), 6.31 (d, *J* = 15.4 Hz, 1 H, H-12), 6.35 (d, *J* = 15.0 Hz, 1 H, H-12′), and 6.45–6.70 (m, 4 H, H-11,11′,15,15′). A peak fraction corresponding to compound **3** was subjected to the reversed-phase HPLC several times. Compound **3** displayed UV–vis maxima at λ 421, 444, and 473 nm in hexane and EI-MS at *m/z* 552 (M^+, 32%), 472 (26), 336 (18), 205 (51), 165 (31), 119 (50), 105 (60), 91 (60), 81 (43), and 69 (100). Treatment of **3** in hexane with a few drops of concentrated formic acid led to a hypsochromatic shift in its UV–vis spectrum consistent with rearrangement to **3′** (Kennedy and Liebler, 1991). Thus, compound **3** was identified as 5,6-epoxy-5,6-dihydro-β,β-carotene.

Compound **4** was further resolved into three peaks (**4a–c**) by normal-phase HPLC. Compounds **4a–c** were obtained as yellow solids (**4a**, R_f 0.88, 7.9-mg yield; **4b**, R_f 0.85, 12.2-mg yield; **4c**, R_f 0.80, 7.8-mg yield). Compound **4a** showed UV–vis maxima at λ 323 (ε 68 400) and 333 nm (70 300) in hexane; IR (film) maxima at ν 2950, 1615, 1450, 1360, 1135, 1030, and 965 cm^{-1}; and EI-MS at *m/z* 552 (M^+, 13%), 379 (11), 378 (24), 349 (11), 321 (6), 255 (7), 197 (17), 185 (13), 171 (18), 157 (23), 145 (30), 133 (32), 119 (63), 105 (60), 95 (55), 81 (47), and 69 (100). ^1H NMR ($CDCl_3$) of **4a** yielded resonances at δ 1.01 and 1.02 (s, 12 H, H-16,16′,17,17′), 1.30 (s, 3 H, H-20), 1.43–1.48 (m, 4 H, H-2,2′), 1.57–1.64 (m, 4 H, H-3,3′), 1.70 (s, 3 H, H-18), 1.71 (s, 3 H, H-18′), 1.83 (s, 3 H, H-20′), 1.93 (s, 3 H, H-19), 1.95 (s, 3 H, H-19′), 1.99–2.03 (m, 4 H, H-4,4′), 3.76 (dt, *J* = 2.1, 10.3 Hz, 1 H, H-15′), 4.75 (t, *J* = 2.6 Hz, 1 H, H-15), 5.42 (d, *J* = 10.3 Hz, 1 H, H-14′), 5.86 (d, *J* = 15.0 Hz, 1 H, H-12), 5.97–6.18 (m, 6 H, H-7,7′,8,8′,10,10′), 6.30 (d, *J* = 15.0 H, 1 H, H-12′), 6.38 (dd, *J* = 2.1, 2.6 Hz, 1 H, H-14), and 6.50–6.64 (m, 2 H, H-11,11′). The ^1H–^1H COSY spectrum (not shown) showed cross-peaks between resonances at δ 3.76 (H-15′) and 4.75 (H-15), at δ 3.76 (H-15′) and 5.42 (H-14′), and at δ 4.75 (H-15) and 6.38 (H-14), respectively. ^{13}C NMR ($CDCl_3$) of **4a** yielded 40 carbon atoms: δ 12.7 (2 C, C-19,19′), 12.9 (C-20′), 19.3 (2 C, C-3,3′), 21.72 (C-18), 21.75 (C-18′), 28.97 (3 C, C-16,20,16′), 29.00 (2 C, C-17,17′), 33.05 (C-4), 33.12 (C-4′), 34.28 (C-1), 34.32 (C-1′), 39.6 (C-2), 39.7 (C-2′), 50.3 (C-15′), 89.1 (C-13), 103.5 (C-15), 123.8 (C-11), 124.2 (C-11′), 126.5 (C-7), 126.9 (C-7′), 129.0 (CH), 129.1 (C-5), 129.2 (C-5′), 130.3 (CH), 131.4 (CH), 135.7 (2 C, C-9,9′), 136.2 (C-13′), 137.0 (CH), 137.6 (CH), 137.8 (CH), 137.90 (C-6), 137.95 (C-6′), 138.1 (CH), and 144.7 (C-14). The DEPT spectrum (not shown) indicated that **4a** had a secondary carbon (δ 50.3) and one tertiary ether (δ 89.1). Thus, compound **4a** was identified as 13,15′-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotene. The stereochemistry of the 13- and 15′-carbon atoms could not be resolved.

Compounds **4b** and **4c** had similar spectral data and were identified as stereoisomers of 15′,13-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotene. Compound **4b** was a mixture of (13*R*,15′*R*)- and (13*S*,15′*S*)-15′,13-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotenes: UV–vis (hexane) λ_{max} 300 (sh, ε 54 500), 323 (64 500), and 330 nm (65 000); IR (film) ν_{max} 2950, 1615, 1450, 1360, 1135,

1030, and 965 cm^{-1}; EI-MS m/z 552 (M^+, 11%), 379 (9), 378 (25), 349 (13), 321 (7), 255 (8), 197 (18), 185 (13), 171 (18), 159 (22), 145 (30), 133 (32), 119 (63), 105 (60), 95 (56), 81 (47), and 69 (100); ^1H NMR ($CDCl_3$) δ 1.01 and 1.02 (s, 12 H, H-16,16′,17,17′), 1.09 (s, 3 H, shielded H-20), 1.43-1.48 (m, 4 H, H-2,2′), 1.55–1.64 (m, 4 H, H-3,3′), 1.70 (s, 3 H, H-18), 1.71 (s, 3 H, H-18′), 1.84 (s, 3 H, shielded H-20′), 1.91 (s, 3 H, H-19), 1.95 (s, 3 H, H-19′), 1.99–2.03 (m, 4 H, H-4,4′), 4.91 (d, J = 2.6 Hz, 1 H, H-14), 5.00 (d, J = 9.0 Hz, 1 H, H-15′), 5.63 (d, J = 9.0 Hz, 1 H, H-14′), 5.78 (d, J = 15.0 Hz, 1 H, H-12), 6.03–6.15 (m, 6 H, H-7,7′,8,8′,10,10′), 6.34 (d, J = 15.4 Hz, 1 H, H-12′), 6.38 (d, J = 2.6 Hz, 1 H, H-15), 6.44 (dd, J = 11.1, 15.4 Hz, 1 H, H-11), and 6.62 (dd, J = 11.1, 15.4 Hz, 1 H, H-11′). ^1H–^1H COSY spectrum of **4b** (not shown) yielded cross-peaks between resonances at δ 4.91 (H-14) and 6.38 (H-15) and at δ 5.00 (H-15′) and 5.63 (H-14′), respectively. Since the stereochemistry of the 13- and 15-carbon atoms was R,R or S,S, **4b** had a shielded methyl proton (δ 1.09). ^{13}C NMR ($CDCl_3$) δ 12.7 (C-19), 12.8 (C-19′), 13.5 (C-20′), 19.3 (2 C, C-3,3′), 21.3 (C-20), 21.72 (C-18), 21.75 (C-18′), 29.0 (4 C, C-16,17,16′,17′), 33.05 (C-4), 33.12 (C-4′), 34.28 (C-1), 34.32 (C-1′), 39.65 (C-2), 39.70 (C-2′), 52.3 (C-13), 87.2 (C-15′), 111.0 (C-14), 125.4 (2 C, CH), 126.6 (CH), 126.7 (C-7), 126.9 (C-7′), 129.1 (C-5), 129.3 (C-5′), 129.5 (C-10), 130.1 (C-10′), 135.2 (C-9), 136.37 (CH), 136.44 (C-9′), 136.66 (CH), 137.70 (CH), 137.9 (2 C, C-6,6′), 138.7 (C-13′), 140.1 (CH), and 145.0 (C-15). The DEPT spectrum (not shown) showed signals of a quaternary carbon (δ 52.3) and a secondary ether (δ 87.2) in addition to other carbon signals. Compound **4c** was a mixture of (13R,15′S)- and (13S,15′R)-15′,13-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotenes: UV–vis (hexane) λ_{max} 302 (ε 63 200), 317 (62 200), and 330 nm (61 000); IR (film) ν_{max} 2950, 1615, 1450, 1360, 1135, 1030, and 965 cm^{-1}; EI-MS m/z 552 (M^+, 11%), 379 (8), 378 (28), 349 (13), 321 (7), 255 (7), 197 (17), 185 (11), 171 (19), 159 (22), 145 (31), 133 (31), 119 (62), 105 (57), 95 (55), 81 (45), and 69 (100); ^1H NMR ($CDCl_3$) δ 1.01 and 1.02 (s, 12 H, H-16,16′,17,17′), 1.30 (s, 3 H, deshielded H-20), 1.43–1.48 (m, 4 H, H-2,2′), 1.56–1.65 (m, 4 H, H-3,3′), 1.70 (6 H, H-18,18′), 1.90 (s, 3 H, deshielded H-20′), 1.91 (s, 3 H, H-19), 1.95 (s, 3 H, H-19′), 1.98–2.01 (m, 4 H, H-4,4′), 4.91 (d, J = 2.6 Hz, 1 H, H-14), 4.93 (d, J = 11.1 Hz, 1 H, H-15′), 5.52 (d, J = 11.1 Hz, 1 H, H-14′), 5.58 (d, J = 15.4 Hz, 1 H, H-12), 6.00–6.14 (m, 6 H, H-7,7′,8,8′,10,10′), 6.31 (d, J = 15.4 Hz, 1 H, H-12′), 6.36 (dd, J = 11.1, 15.0 Hz, 1 H, H-11), 6.43 (d, J = 2.6 Hz, 1 H, H-15), and 6.61 (dd, J = 11.1, 15.0 Hz, 1 H, H-11′). The ^1H–^1H COSY spectrum (now shown) showed cross-peaks between resonances at δ 4.91 (H-14) and 6.43 (H-15) and at δ 4.93 (H-15′) and 5.52 (H-14′), respectively. Since the stereochemistry of the 13- and 15-carbon atoms is R,S or S,R, **4c** has a deshielded 20-methyl group (δ 1.30). ^{13}C NMR ($CDCl_3$) δ 12.7 (C-19), 12.8 (C-19′), 13.3 (C-20′), 19.3 (2 C, C-3,3′), 21.8 (2 C, C-18,18′), 25.7 (C-20), 29.0 (4 C, C-16,17,16′,17′), 33.05 (C-4), 33.12 (C-4′), 34.28 (C-1), 34.32 (C-1′), 39.7 (2 C, C-2,2), 51.8 (C-13), 88.1 (C-15′), 109.6 (C-14), 125.4 (C-11), 125.8 (C-11′), 126.4 (C-7), 126.8 (C-7′), 127.5 (CH), 129.0 (C-5), 129.3 (C-5′), 129.6 (C-10), 130.1 (C-10′), 134.9 (C-9), 136.4 (C-9′), 136.6 (CH), 137.3 (CH), 137.7 (CH), 137.8 (2 C, C-6,6′), 137.9 (C-13′), 138.3 (CH), and 145.2 (C-15). The DEPT spectrum (not shown) showed signals of a quaternary carbon (δ 51.8) and a secondary ether (δ 88.1) in addition to other signals. Compounds **4a**, **4b**, and **4c** gave each the same fluorescent signal for emission at 482 nm with excitation at 350 nm in hexane, respectively.

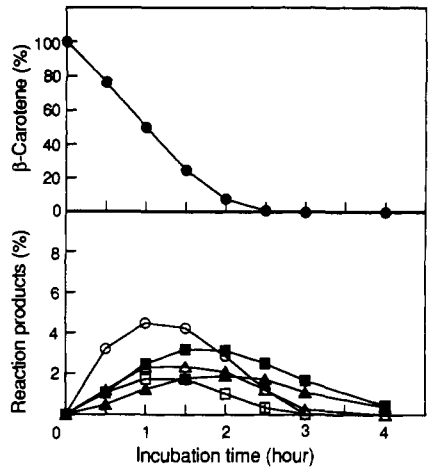


Figure 2. Kinetics of AMVN-dependent reaction of β-carotene and formation of compounds **1**–**5**. A reaction mixture containing 2.0 mM β-carotene and 20 mM AMVN in benzene was incubated at 37 °C: β-carotene (●); compound **1** (○); compound **2** (△); compound **3** (□); compound **4** (▲); compound **5** (■).

Compound **5** was obtained as a yellow solid (R_f 0.95, yield 52.2 mg). Compound **5** showed UV–vis maximum at λ 330 nm (ε 52 600) in hexane; IR (film) maxima at ν 2950, 1680, 1655, 1450, 1385, 1360, 1310, 1165, and 965 cm^{-1}; and EI-MS at m/z 552 (M^+, 12%), 509 (6), 379 (8), 378 (19), 349 (6), 255 (6), 225 (6), 197 (11), 183 (11), 171 (15), 157 (22), 145 (28), 133 (29), 119 (58), 105 (59), 95 (57), 81 (49), and 69 (100); ^1H NMR ($CDCl_3$) of **5** gave resonances at δ 0.99 and 1.02 (s, 12 H, H-16,16′,17,17′), 1.42–1.48 (m, 4 H, H-2,2′), 1.57–1.63 (m, 4 H, H-3,3′), 1.67 (s, 3 H, H-18), 1.70 (s, 3 H, H-18′), 1.76 (s, 3 H, H-20), 1.802 and 1.805 (s, 3 H, H-19), 1.82 (s, 3 H, H-20′), 1.92 (s, 3 H, H-19′), 1.95–2.03 (m, 4 H, H-4,4′), 3.27 (m, 2 H, H-11,15′), 4.64 (dd, J = 5.6, 7.7 Hz, 1 H, H-15), 4.73 (d, J = 6.0 Hz, 1 H, H-12), 5.34 (d, J = 9.4 Hz, 1 H, H-14′), 5.38 (d, J = 9.0 Hz, 1 H, H-10), 5.97 (s, 2 H, H-8,8′), 6.06 (d, J = 11.1 Hz, 1 H, H-10′), 6.10 (s, 2 H, H-7,7′), 6.19 (d, J = 8.1 Hz, 1 H, H-14), 6.24 (d, J = 15.0 Hz, 1 H, H-12′), and 6.49 (dd, J = 11.1, 15.0 Hz, 1 H, H-11′). ^1H–^1H COSY spectrum (not shown) showed cross-peaks between resonances at δ 3.27 (H-15′) and 4.64 (H-15), at δ 3.27 (H-11) and 4.73 (H-12), at δ 3.27 (H-15′) and 5.34 (H-14′), at δ 3.27 (H-11) and 5.38 (H-10), and at δ 4.64 (H-15) and 6.19 (H-14), respectively. ^{13}C NMR ($CDCl_3$) δ 12.65 (C-19), 12.72 (C-19′), 13.0 (C-20′), 19.3 (2 C, C-3,3′), 21.65 (C-20), 21.72 (C-18), 21.9 (C-18′), 28.9 (2 C, C-16,17), 29.0 (2 C, C-16′,17′), 32.9 (C-4), 33.1 (C-4′), 34.2 (C-1), 34.3 (C-1′), 39.6 (C-2), 39.7 (C-2′), 41.9 (C-15′), 42.7 (C-11), 107.1 (C-15), 109.3 (C-12), 123.7 (C-7), 125.1 (C-11′), 126.1 (C-7′), 128.5 (C-5), 129.1 (C-5′), 130.54 and 130.57 (C-10′), 133.0 (C-13′), 133.3 (C-9), 133.7 (C-14), 135.0 (C-9′), 136.0 and 136.1 (C-12′), 137.4 (C-14′), 137.79 (C-10), 137.85 (C-8′), 137.9 (C-6), 138.0 (C-6′), 141.1 (C-8), and 150.8 (C-13). The DEPT spectrum (not shown) indicated that **5** had two tertiary carbons (δ 41.9 and 42.7) and two secondary ethers (δ 107.1 and 109.3) in addition to other carbon atoms that were consistent with the assigned structure. Thus, compound **5** was identified as 11,15′-cyclo-12,15-epoxy-11,12,15,15′-tetrahydro-β,β-carotene. Compound **5** gave a fluorescent signal for emission at 482 nm, with 350-nm excitation in hexane.

Aliquots of the reaction mixture were taken during the reaction and analyzed by reversed-phase HPLC to determine the distribution of products and the relative rates of formation of each product (Figure 2). Compounds **1**–**5** accumulated with the decrease of β-carotene at the first hour. Thereafter, compounds **1**–**3** disappeared with the disappearance of β-carotene, while compounds **4** and **5**

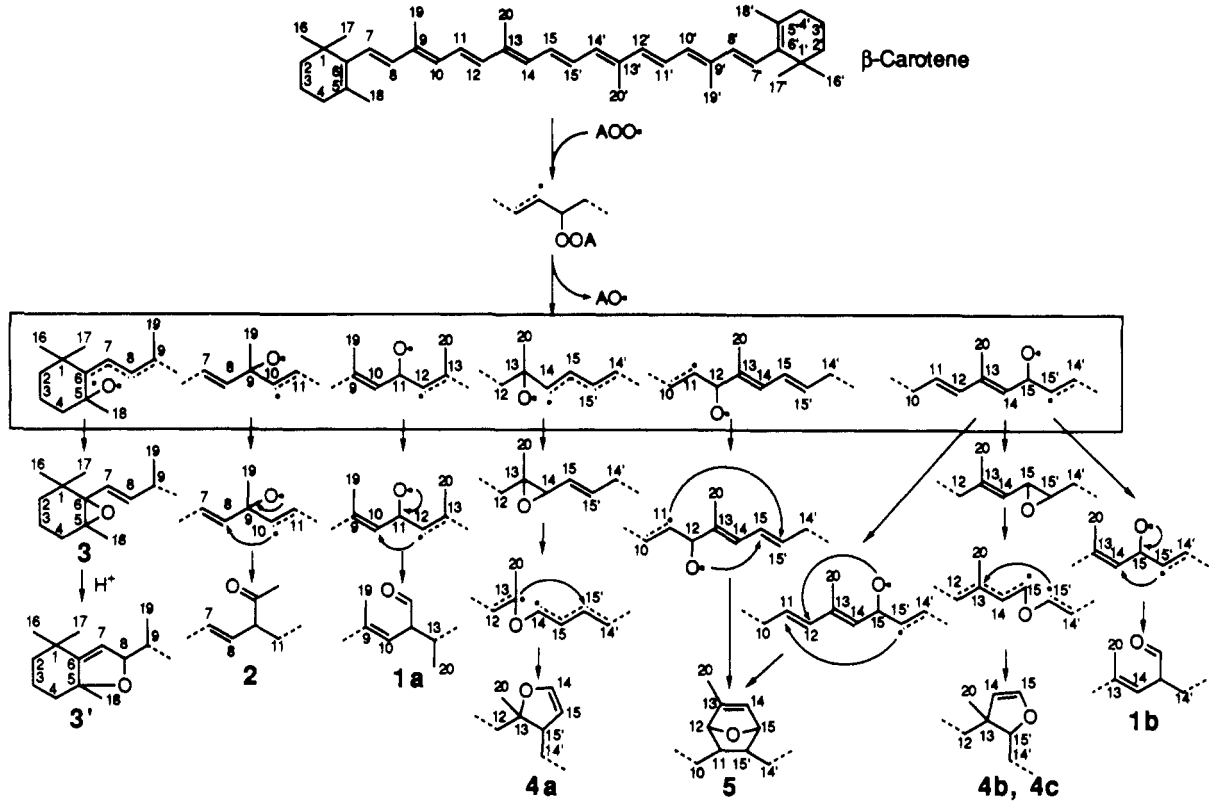


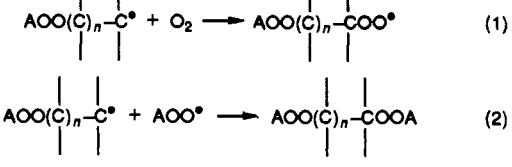
Figure 3. Proposed reaction pathway for the peroxyl radical reaction of β-carotene.

were somewhat stable and remained in the reaction mixture at that time. Compounds **1**–**5** accounted for only 25% of the consumed β-carotene in the 1.0-h reaction mixture, and the rest were polar unknown products.

DISCUSSION

Carotenoids such as β-carotene are considered to be effective inhibitors of lipid peroxidation (Krinsky, 1989). These compounds seem to exert antioxidant activities by a mechanism in which the chain-propagating peroxyl radical is trapped by addition to the conjugated polyene system (Burton and Ingold, 1984). The peroxyl radical addition reactions of β-carotene resulted in formation of some epoxy compounds and apocarotenals (Kennedy and Liebler, 1991; Handelman et al., 1991). We have isolated and characterized the reaction products of β-carotene with the alkylperoxyl radical from AMVN. The products are a mixture of 12-formyl-11-nor-β,β-carotene (**1a**) and 15′-formyl-15-nor-β,β-carotene (**1b**), 19-oxomethyl-10-nor-β,β-carotene (**2**), 5,6-epoxy-5,6-dihydro-β,β-carotene (**3**), 13,15′-epoxyvinyleno-13,15′-dihydro-β,β-carotene (**4a**), stereoisomers of 15′,13-epoxyvinyleno-13,15′-dihydro-β,β-carotene (**4b** and **4c**), and 11,15′-cyclo-12,15-epoxy-11,12,15,15′-tetrahydro-β,β-carotene (**5**). These products except **3** were first identified in this study. Kennedy and Liebler (1991) reported that 5,6-epoxy-β,β-carotene and 15,15′-epoxy-β,β-carotene in addition to several unidentified polar products were formed by the peroxyl radical oxidation of β-carotene. However, we could not detect the 15,15′-epoxide in our reaction system. Handelman et al. (1991) characterized the apocarotenal series and several nonpolar compounds, which have not been previously identified, as the autoxidation products of β-carotene. Their nonpolar compounds included β-carotene-5,6-epoxide and three unknowns, referred to as peaks 6, 8, and 9. From the comparison of the data of spectral and fluorescent properties and the relative position on the HPLC chromatogram, we assumed that the unknown compounds **6**, **8**, and **9** by Handelman et al. corresponded to our compounds **2**, **4**, and **5**, respectively. Compounds **1**–**3** were unstable and decomposed into another polar products during the peroxyl radical reaction. On the other hand, compounds **4** and **5** were somewhat stable in the reaction mixture (Figure 2), so that it might be expected to accumulate during the reaction of peroxyl radical scavenging (Handelman et al., 1991).

Lipid peroxidation principally involves the abstraction of allylic and bisallylic hydrogen atoms by peroxyl radicals (Frankel, 1980). In contrast, the processes in the peroxyl radical reaction of β-carotene appeared not to give products in which an allylic position had become functionalized. This implies that peroxyl radical addition to the conjugated system is strongly favored over abstraction. Figure 3 shows a possible reaction pathway for the reaction of β-carotene with peroxyl radicals. The first step is peroxyl radical (AOO•) addition to the polyene chain (Mayo, 1968; Burton and Ingold, 1984). Addition can be followed by the unimolecular decomposition of the carbon-centered radicals, affording a carbonyl compound (**1** or **2**) or an epoxide (**3** or **5**) and an alkoxyl radical (AO•). Furthermore, we propose that the formation of five-membered cyclic ethers (**4a**–**4c**) occurs by the scission of epoxides. Alternatively, there may be other routes by which some of the carbon-centered radicals are reacted (Samokyszyn and Marnett, 1990; Kennedy and Liebler, 1992).



At the atmospheric oxygen concentration, oxygen adds reversibly to the carbon-centered radical to form a new β-carotene-peroxyl radical (eq 1), which may then oxidize

another β-carotene molecule. At low oxygen tensions, on the other hand, the carbon-centered radical traps a second peroxyl radical to form nonradical products (eq 2). The evidence that the antioxidant activity of β-carotene is markedly potentiated at low oxygen tensions (Burton, 1989; Kennedy and Liebler, 1992) indicates that eq 2 may occur in that condition. However, such nonradical products have not yet been detected.

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LITERATURE CITED

Burton, G. W. Antioxidant action of carotenoids. *J. Nutr.* **1989**, *119*, 109–111.

Burton, G. W.; Ingold, K. U. β-Carotene: An unusual type of lipid antioxidant. *Science* **1984**, *224*, 569–573.

Craft, N. E.; Soares, J. H., Jr. Relative solubility, stability, and absorptivity of lutein and β-carotene in organic solvents. *J. Agric. Food Chem.* **1992**, *40*, 431–434.

El-Tinay, A. H.; Chichester, C. O. Oxidation of β-carotene. Site of initial attack. *J. Org. Chem.* **1970**, *35*, 2290–2293.

Foote, C. S.; Denny, R. W. Chemistry of singlet oxygen. VIII. Quenching by β-carotene. *J. Am. Chem. Soc.* **1968**, *90*, 6233–6235.

Foote, C. S.; Chang, Y. C.; Denny, R. W. Chemistry of singlet oxygen. X. Carotenoid quenching parallels biological protection. *J. Am. Chem. Soc.* **1970**, *92*, 5216–5218.

Frankel, E. N. Lipid oxidation. *Prog. Lipid Res.* **1980**, *19*, 1–22.

Handelman, G. J.; Van Kuijk, F. J. G. M.; Chatterjee, A.; Krinsky, N. I. Characterization of products formed during the autoxidation of β-carotene. *Free Radical Biol. Med.* **1991**, *10*, 427–437.

Jialal, I.; Norkus, E. P.; Cristol, L.; Grundy, S. M. β-Carotene inhibits the oxidative modification of low-density lipoprotein. *Biochim. Biophys. Acta* **1991**, *1086*, 134–138.

Kanasawud, P.; Crouzet, C. Mechanism of formation of volatile compounds by thermal degradation of carotenoids in aqueous medium. 1. β-Carotene degradation. *J. Agric. Food Chem.* **1990**, *38*, 237–243.

Kennedy, T. A.; Liebler, D. C. Peroxyl radical oxidation of β-carotene. *Chem. Res. Toxicol.* **1991**, *4*, 290–295.

Kennedy, T. A.; Liebler, D. C. Peroxyl radical scavenging by β-carotene in lipid bilayers: Effect of oxygen partial pressure. *J. Biol. Chem.* **1992**, *267*, 4658–4663.

Krinsky, N. I. Antioxidant functions of carotenoids. *Free Radical Biol. Med.* **1989**, *7*, 617–635.

Krinsky, N. I.; Deneke, S. M. Interaction of oxygen and oxyradicals with carotenoids. *J. Natl. Cancer Inst.* **1982**, *69*, 205–210.

Lim, B. P.; Nagao, A.; Terao, J.; Tanaka, K.; Suzuki, T.; Takama, K. Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. *Biochim. Biophys. Acta* **1992**, *1126*, 178–184.

Marthy, C.; Berset, C. Degradation of *trans*-β-carotene during heating in sealed glass tubes and extrusion cooking. *J. Food Sci.* **1986**, *51*, 698–702.

Marthy, C.; Berset, C. Factors affecting the thermal degradation of all-*trans*-β-carotene. *J. Agric. Food Chem.* **1990**, *38*, 1063–1067.

Mayo, F. R. Free-radical autoxidation of hydrocarbons. *Acc. Chem. Res.* **1968**, *1*, 193–201.

Mordi, R. C.; Walton, J. C.; Burton, G. W.; Hughes, L.; Ingold, K. U.; Lindsay, D. A. Exploratory study of β-carotene autoxidation. *Tetrahedron Lett.* **1991**, *32*, 4203–4206.

Niki, E.; Saito, T.; Kawakami, A.; Kamiya, Y. Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. *J. Biol. Chem.* **1984**, *259*, 4177–4182.

Onyewu, P. N.; Ho, C.-T.; Daun, H. Characterization of β-carotene thermal degradation products in a model food system. *J. Am. Oil Chem. Soc.* **1986**, *63*, 1437–1441.

Palozza, P.; Krinsky, N. I. The inhibition of radical-initiated peroxidation of microsomal lipids by both α-tocopherol and β-carotene. *Free Radical Biol. Med.* **1991**, *11*, 407–414.

Palozza, P.; Moualla, S.; Krinsky, N. Effects of β-carotene and α-tocopherol on radical-initiated peroxidation of microsomes. *Free Radical Biol. Med.* **1992**, *13*, 127–136.

Samokyszyn, V. M.; Marnett, L. J. Inhibition of liver microsomal lipid peroxidation by 13-*cis*-retinoic acid. *Free Radical Biol. Med.* **1990**, *8*, 491–496.

Stocker, R.; Yamamoto, Y.; McDonagh, A. F.; Glazer, A. N.; Ames, B. N. Bilirubin is an antioxidant of possible physiological importance. *Science* **1987**, *235*, 1043–1046.

Terao, J. Antioxidant activity of β-carotene-related carotenoids in solution. *Lipids* **1989**, *24*, 659–661.

Tsukida, K.; Zechmeister, L. The stereoisomerization of β-carotene epoxides and the simultaneous formation of furanoid oxides. *Arch. Biochem. Biophys.* **1958**, *74*, 408–426.

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