Bioorganic & Medicinal Chemistry 18 (2010) 777–786

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of α-tocohexaenol (α-T6) a fluorescent, oxidatively sensitive polyene analogue of α-tocopherol

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ARTICLE INFO

Article history:
Received 27 October 2009
Revised 20 November 2009
Accepted 21 November 2009
Available online 27 November 2009

Keywords:
Vitamin E
α-Tocopherol
Fluorescent membrane probe
Lipid peroxidation

ABSTRACT

A polyunsaturated analogue of α-tocopherol was synthesized that is both fluorescent and sensitive to peroxidative chemistry that occurs in phospholipid membranes. α-Tocohexaenol **1**, [(*S*)-2,5,7,8-tetramethyl-2-((1*E*/*Z*,3*E*,5*E*,7*E*,9*E*)-4,8,12-trimethyltrideca-1,3,5,7,9,11-hexaenyl)chroman-6-ol, α-T6] was prepared by condensing a known triene fragment triphenyl-(2,6-dimethyl-octa-2,4,6-trienoic acid methyl ester)-phosphonium bromide with a protected chromanol aldehyde, (2S)-6-{[*tert*-butyl(dimethyl)silyl]oxy}-2,5,7,8-tetra-methyl-3,4-dihydro-2*H*-chromene-2-carbaldehyde. The full side chain was then completed with isopentyl(tri-*n*-butyl)phosphonium bromide to give **1**. The geometry of the C1′−C2′ alkene appears to be Z (*cis*) although the coupling constants of the olefinic protons are intermediate between values normally assigned to *E* and *Z*-isomers. In ethanol, α-T6 has a maximum absorption at 368 nm with an absorption coefficient of 45,000 M^{-1} cm^{-1}, and displays a maximum fluorescence emission at 523 nm. The susceptibility of α-T6 to peroxidative chemistry was dependent on the concentration of azo-initiators of lipid oxidation in acetonitrile solution as well as in phospholipid vesicles. A loss of fluorescence at 520 nm was observed when α-T6 (vesicles or α-T6-lipid mixtures) was exposed to peroxidative conditions, and this loss mirrored the production of conjugated dienes and trienes during the peroxidation of bulk phospholipids. Addition of natural α-tocopherol during the AMVN induced oxidation of 4 μM α-T6 and 0.5 mg/ml soybean PC induced a characteristic lag phase, after which the fluorescence of α-T6 began to lessen. Thus, α-T6 may be a useful reporter not only of tocopherol location in cells, but also of the extent of peroxidative events.

1. Introduction

The absorption and distribution of the lipophilic antioxidant vitamin, α-tocopherol, has been studied for many years by using mass spectrometry to follow deuterium containing synthetic tocopherols.[1–9] [For reviews see Refs. 10–13.] However, such isotopically labeled compounds are less useful at the cellular level because identification of the labeled tocopherol and its metabolites would normally require extraction from tissue samples, and this precludes any time and spatial resolution of vitamin E movement in a cell. It is advantageous, therefore, to use fluorescent molecular probes that report cellular locations directly at low concentrations. As vitamin E is only weakly fluorescent, we have prepared a series of fluorescent analogues of α-tocopherol[14] one of which (NBD-α-tocopherol) has been successfully used in the study of intracellular trafficking of vitamin E.[15–20]

We envisioned that a new fluorescent analogue of α-tocopherol could be designed and synthesized that would be less bulky and less polar than the NBD moiety. This new molecule, α-tocohexaenol (α-T6) (Fig. 1), keeps much of the structure of α-tocopherol: it has the same chromanol head and the skeleton of α-tocopherol, and the same (*R*)-stereochemistry at C-2. Furthermore, the inclusion of six conjugated double bonds in the side chain will make it fluorescent. Using the Fieser–Kuhn rules, α-T6 is calculated to have an absorption maximum of 366 nm and we predicted that the fluorescence emission of α-T6 would be >500 nm based on its similarity to a reported fluorescent didehydrofarnesol analogue[22] a pentaene that fluoresces at 465 nm. Compared with α-tocopherol, which has an absorption maximum of 292 nm and emission maximum of 325 nm, the fluorescent properties of α-T6 will make it a useful fluorescent reporter not interfered with by endogenous cellular chromophores.

A similar molecule named FeAOX-6 has been made and characterized by the group of Palozza et al.[21,23,24] FeAOX-6 is missing one double bond at C9′ and the lack of full conjugation leads to a shortening of the wavelength of absorption (to ~300 nm), which is not that different from natural α-tocopherol. Also, the lesser degree of conjugation was insufficient for our intended use as a fluorophore.

Polyenes such as retinoids[25] and carotenoids[26] are susceptible to oxidation. Indeed, both retinoic acid[27] and β-carotene[28–30] have been studied as inhibitors of lipid peroxidation and they have been

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doi:10.1016/j.bmc.2009.11.051

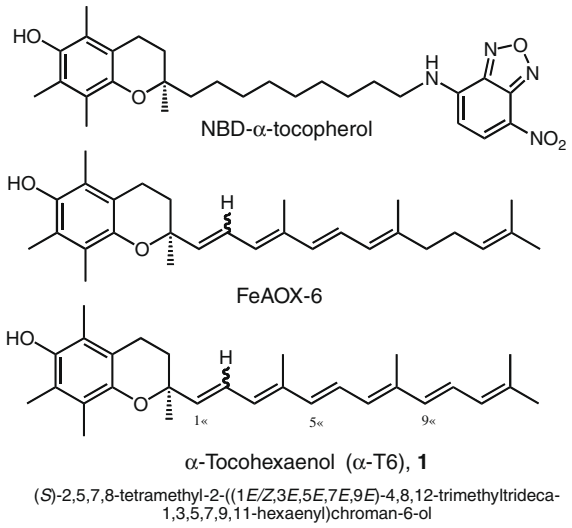
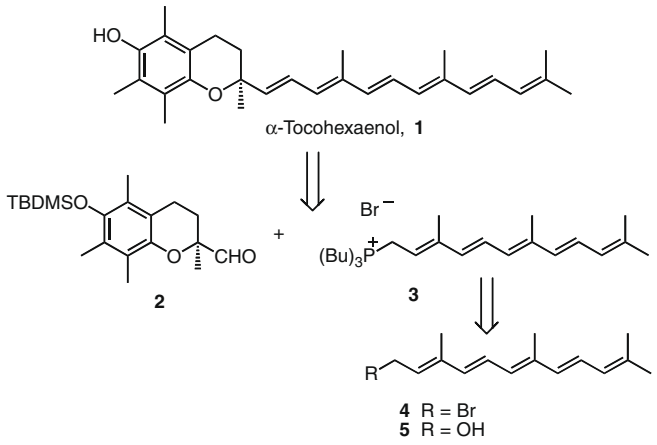


Figure 1. Structure of the fluorescent tocopherol derivative NBD-α-tocopherol[14], FeAOX-6[21] and the α-tocohexaenol (α-T6) prepared in this work.

shown to be capable of reacting with lipid hydroperoxides. If the fully conjugated side chain of α-T6 is susceptible to oxidation in a manner similar to carotenoids[31] then oxidation should destroy the π-conjugation and thus the fluorescence intensity. We hoped that the fluorescence signal of α-T6 would act as an oxidatively sensitive membrane probe whose structure would mimic the membrane location and behavior of α-tocopherol[32], but whose fluorescent signal would diminish when local oxidative stress overwhelmed antioxidant defenses. In effect, α-T6 would act as a fluorescent-reporting tocopherol until it was consumed by oxidative events. This would represent a unique manner of visualizing not only the location of tocopherol within a cell, but also potentially the differential loss of tocopherol-specific fluorescence in those parts of the cell where oxidative events are greatest. Admittedly, the polyene tail of α-tocohexaenol will make it more rigid than α-tocopherol so that it may not have the exact same properties when embedded in a membrane.

1.1. Synthesis of α-tocohexaenol (α-T6), 1

Our synthetic strategy first pursued a convergent synthesis (chromanol + C15 fragment) based on our past use of a chroman aldehyde derived from the available starting material Trolox and a pentaene phosphonium salt which would form the final double bond in a Wittig reaction. Scheme 1 illustrates this approach.



Scheme 1.

We planned to convert alcohol **5** to the bromide **4** and eventually the phosphonium salt **3**, which would be coupled with Trolox aldehyde **2**[14,33] to provide α-T6, **1**. We followed reported synthetic methods for preparation of pentaenol **5**[22,34] with minor modifications, the most important of which was the substitution of LiHMDS for *n*-BuLi during the synthesis of the intermediate 8-(*tert*-butyldimethylsilanoxy)-2,6-dimethylocta-2,4,6-trienoic acid methyl ester (compound **7** in their numbering[22]) which we found to give higher yields.

Unfortunately, all our efforts to transform the alcohol **5** to the corresponding phosphonium salt **3** failed. Treatment of **5** with brominating reagents, such as HBr, PBr_3, or CBr_4 and PPh_3, followed by reaction with PPh_3 or PBu_3, did not provide the desired phosphonium salt. Treatment of alcohol **5** with triphenylphosphine hydrobromide proceeded in only 10% yield despite the results of Furuhata et al.[35] who reported a one-pot Wittig reaction with a similar conjugated allylic alcohol as starting material. Reacting alcohol **5** with triphenylphosphine hydrobromide followed by adding aldehyde **2** and KOH in methanol, did not produce an olefin product, only aldehyde **2** was recovered. Alcohol **5** was presumably lost to polymerization to form unrecoverable products. In an attempt to circumvent this failure we tried to prepare a phosphonium salt from the bromochromanol **8**, however, **8** did not react with triphenylphosphine, even when refluxed in toluene for 5 days (Scheme 2), presumably due to the sterically congested nature of this neopentylic position.

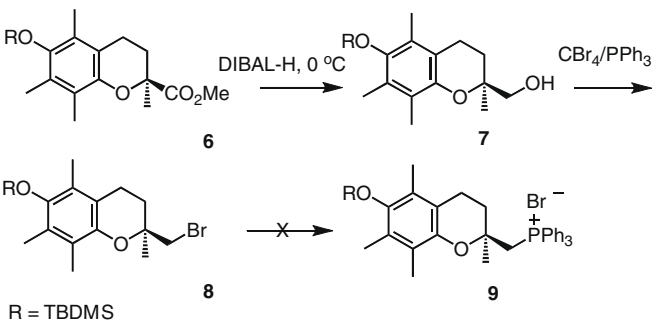
1.2. Synthesis of α-T6, 1 (C_{14} + C_{10} + C_5 strategy)

Our final and successful approach involved the coupling of aldehyde **2** with a smaller phosphonium salt **12** that was available from an intermediate we had already prepared in the synthesis of **5**, followed by side chain extension (Scheme 3).

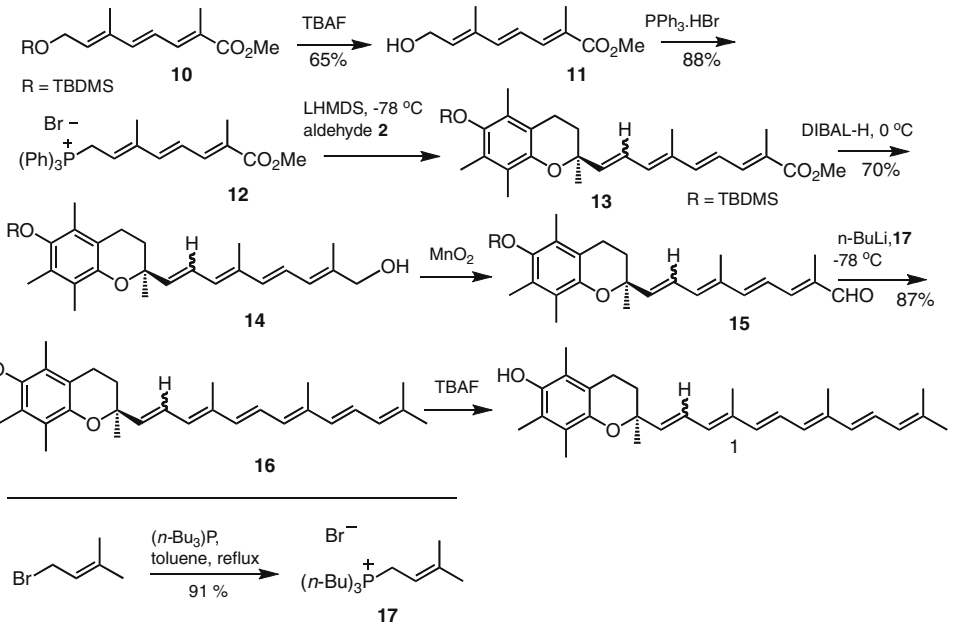
Deprotection of silyl ether **10** gave alcohol **11**. The conversion of alcohol **11** to bromide **12** gave only fair yields (~40%) using hydrobromic acid solution, PBr_3, or CBr_4/PPh_3. However, good yields (88%) of **12** were obtained using triphenylphosphine hydrobromide. Treatment of **12** with LiHMDS and coupling to aldehyde **2** gave ester **13**. Reduction of ester **13** with DIBAL-H at 0 °C afforded **14** (70%), which was oxidized by active MnO_2 to yield aldehyde **15**. A final olefination using isopentyl(tri-*n*-butyl)phoshphonium bromide **17** provided the silyl protected phenol **16** that, following deprotection, gave the target α-T6, **1** (Fig. 2).

1.3. Establishment of the alkene geometry

The geometry of the double bonds in intermediate **10** were confirmed as *trans* based on the comparison to published spectra.[22] Only the alkenes at C1′–C2′ and C9′–C10′ were created after the use of intermediate **10**. Palozza et al. report the chemical shift of the proton on C1′ in FeAOX-6 (similar to α-T6 but lacking the al-



Scheme 2. Attempted synthesis of protected chromanol phosphonium salt.



Scheme 3.

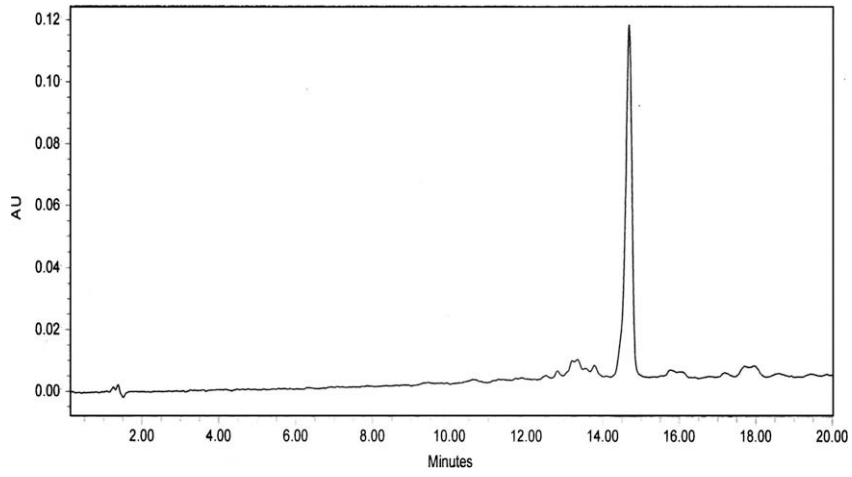
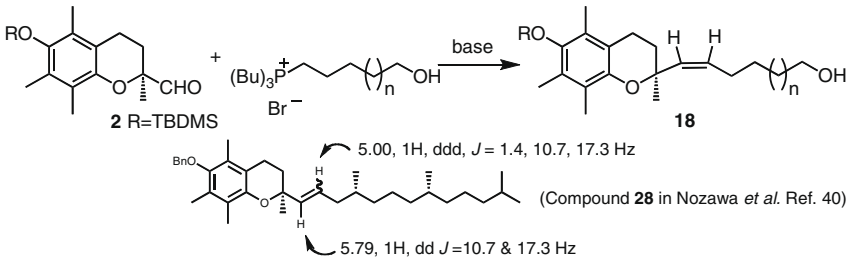


Figure 2. HPLC chromatogram of α-T6. The column was a Synergi 4 μm Hydro-RP (C18), 150 × 4.6 mm (Phenomenx) running on a Waters 626 pump with 990 photodiode array with detection at 525 nm. The solvent system was 100% methanol at 1.0 ml/min.

kene between C9′ and C10′) as δ 6.82 with a coupling constant (*J*-value) of 12 Hz to H-C2′. The analogous C1′ proton in tetraene aldehyde **15** was a doublet at δ 6.89 (J = 12.1 Hz) and in α-T6 is found at δ 6.90 (J = 12.3 Hz). The proton at C2′ is not easily discernable in the 1D spectrum of α-T6 due to the multiplicity of signals between δ 6.6–6.2, but the 2D COSY of α-T6 clearly shows a correlation between the C1′ signal at δ 6.90 and a doublet centered at δ 6.37 with J = 12.3 Hz. This value of the coupling constant is intermediate between those normally expected for *cis*- or *trans*-alkenes: J_3 H–H coupling of 8–12 Hz for *cis* and 14–17 Hz for *trans*. By way of comparison, our previous preparation of the alkenes **18** (Scheme 4)[14,33,36] used a similar triphenylphosphonium salt and clearly produced a *cis*-alkene with a *J*-value for an overlapped doublet of doublets at δ 5.3 of ~6 Hz. Similar monoalkenes have been made previously using triphenylphosphonium salts,[37–39] and in one case[40] (Scheme 4) the product was reported to be a 14:1 mixture of *cis*:*trans*, but only coupling constants for the *trans* isomer were discernible: the C1′ proton at δ 5.79 was a doublet of doublets with *J*-values of 10.7 and 17.5 Hz. We conclude, therefore, that the C1′–C2′ double bond in the α-T6 we prepared likely has the *cis*-geometry. The structures of C1′-*cis* and C1′-*trans* α-T6 were investigated by molecular mechanics minimization using Spartan (Wavefunction, Inc.). When the alkenyl π-systems were kept in the same plane, the distance between the C2 of the pyran ring and C12′ was reduced from 14.84 Å in the all-*trans*-α-T6 to 13.82 Å in the C1′–C2′ *cis*-α-T6. No attempt was made to photoisomerize this bond.

1.4. Fluorescence characterization of α-T6

The absorbance spectrum of α-T6 includes several maxima. In ethanol these occur at 349, 368, and 390 nm. The maximum absorption at 368 nm in ethanol shifts slightly to 367 nm in hexane (Fig. 3). The absorption coefficients at 368 nm are ~45,000 and 24,000 M^{-1} cm^{-1} in ethanol and hexane, respectively. The maximum fluorescence emission is at 523 nm in EtOH and 520 nm in



Scheme 4.

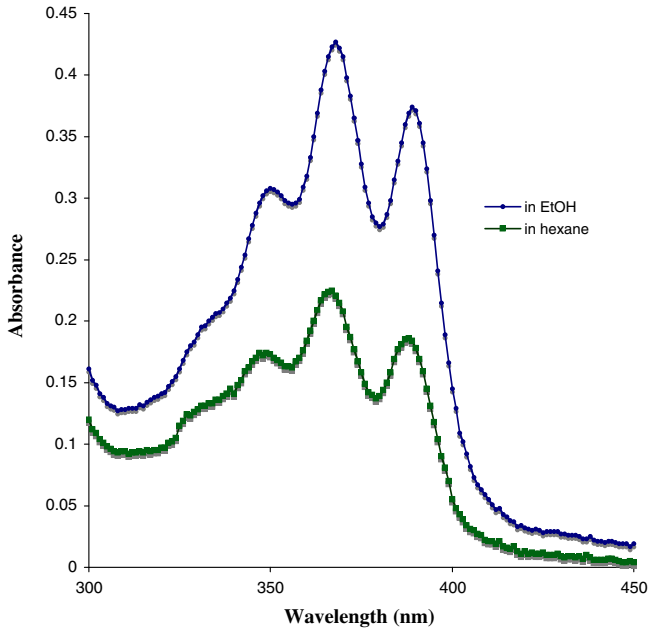


Figure 3. Absorption spectrum of 9.2 μM α-T6 in ethanol and hexane.

hexane (Fig. 4). Fluorescence intensity in ethanol–water solutions decreases with increasing water content and is almost absent below 10% (vol/vol) ethanol concentrations.

α-T6 was designed to be fluorescent and yet oxidatively sensitive. Since the structure has been kept as similar to α-tocopherol as possible, the expectation is that α-T6 will mimic α-tocopherol's location and orientation in phospholipid bilayers. If α-T6 is exposed to conditions that initiate and support polyunsaturated phospholipid peroxidation, one should be able to observe a loss of fluorescence that parallels peroxidative chemistry. Several other fluorescent probes are available that accomplish the same goal, such as *cis*-parinaric acid[41–49] and BODIPY-(581/591),[48,50–55] but these are global lipid sensors and may not share the same cellular membrane location and dynamics as α-tocopherol.

To get a preliminary sense of whether α-T6 meets these criteria we measured the loss of fluorescence of this compound in both organic solution and phospholipid vesicles when subjected to various common initiators of lipid peroxidation. Figure 5 shows a representative time course of fluorescence traces when 4 μM α-T6 was combined with 0.5 mg/ml soy PC and 1.25 mM 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) in acetonitrile at 37 °C. The fluorescence maxima at 520 nm slowly decayed over several hours, while the maxima at 485 nm initially rose slightly and then dropped. Figure 6 shows fluorescence decay curves observed when acetonitrile solutions of α-T6, soy PC, and AMVN were followed over time. The rate of fluorescence loss at 520 nm was dependent on the concentration of AMVN and was inhibited by the addition of 10 μM α-tocopherol, exhibiting the typical lag phase seen with these sorts of assays.[56–59] Importantly, α-T6 is stable in aerated solution when no initiator is present.

Niki has shown that, in benzene, β-carotene is 32 times less reactive than α-tocopherol towards peroxyl radicals generated from AMVN in the presence of methyl linoleate.[29] However, when α-tocopherol and β-carotene are combined in the same soybean PC liposomal membrane both are consumed at the same rate. These authors noted that, '…β-carotene can be as or even more effective than α-tocopherol toward lipophilic radicals present in the membrane.'

This is thought to be due to the position of α-tocopherol in the membrane where the phenol hydroxyl is near the head group region and the isoprenoid side chain is anchored in the core of the membrane.[32] β-Carotene has no similar polar functional group and is thus likely buried in the bilayer core.[60] Thus, when hydrophobic AMVN is the radical initiator in solution, peroxyl radicals have easy access to the phenolic hydrogen of α-tocopherol and this spares the β-carotene. In a membrane the reaction is slowed by the greater difficulty of the peroxyl radical reaching the tocopherol phenol, that is, oriented away from the center of the bilayer.

In the case of α-T6 the structural elements of both α-tocopherol (phenol) and β-carotene (polyene) reside in the same molecule. We

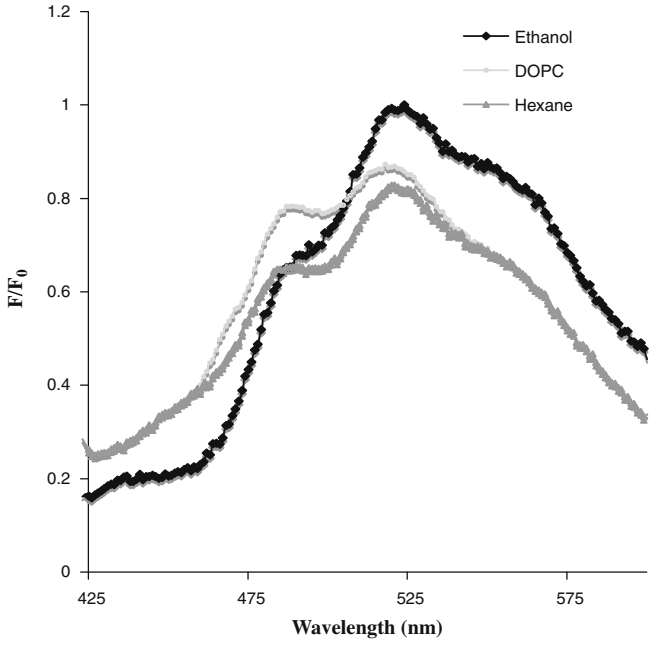


Figure 4. Fluorescence spectra of 6.0 μM α-T6 in ethanol, 100 nm DOPC unilamellar vesicles, and hexane. The excitation wavelength was at 368 nm. The fluorescence intensities are normalized to the maximal fluorescence at 523 nm in ethanol.

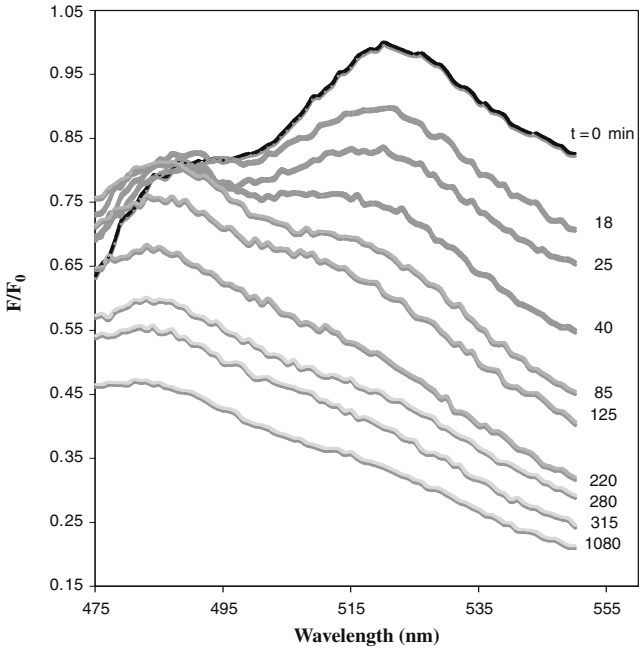


Figure 5. Fluorescence decay of α-T6 (4.0 μM) combined with 0.5 mg/ml soy PC and 1.25 mM AMVN in acetonitrile at 37 °C. Fluorescent spectra were normalized based on the maximal fluorescence at 520 nm (F_0) at the beginning of the reaction ($t = 0$ min). Spectra were recorded at various intervals throughout the reaction where spectra representative of the progressive changes in the fluorescence as oxidation proceeds are shown in the figure.

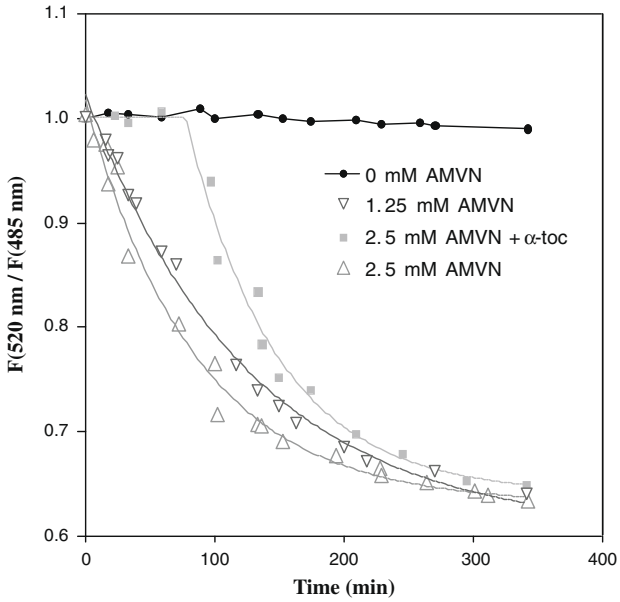


Figure 6. AMVN induced oxidation of 3.0 μM α-T6 and 0.5 mg/ml soy PC in the presence and absence of 10 μM α-tocopherol in acetonitrile at 37 °C. α-T6 was monitored as the ratio of fluorescence at 520 nm/485 nm (λ_{ex} 368 nm) since over the course of oxidation the 520 nm emission decreases and the 485 nm emission increases slightly before decreasing.

therefore expect that it is able to act as a hydrogen atom donating antioxidant like α-tocopherol as well as have peroxyl radicals add to the polyene like β-carotene. The lack of a substantial lag phase in fluorescence loss when only α-T6 is present suggests that the phenoxyl radical formed by H-atom donation delocalizes to the polyene chain where it disrupts conjugation and destroys fluorescence. When α-tocopherol is present in approximately threefold excess over α-T6 there appears to be some sparing of α-T6 which delays the loss of fluorescence. This would also occur if the polyene tail of α-T6 did not react as quickly with peroxyl radicals as does β-carotene—a reasonable assumption given the greater degree of conjugation of β-carotene. Should α-T6 be used as a probe to detect peroxidative events in cells it will be easy to dose α-T6 in concentrations equivalent to or more than endogenous α-tocopherol thus eliminating any sparing effect.

Figure 7 confirms that α-T6 is susceptible to oxidative loss of fluorescence and that this occurs simultaneously with lipid peroxidation and the production of conjugated dienes as monitored at 234 nm. Importantly, the absorption of α-T6 at 368 nm also decreased over time, mirroring the loss of fluorescence, showing that α-T6 was being consumed. That α-T6 is present in the bilayer of vesicles is strongly suggested by the half-lives for fluorescence loss being more sensitive to the concentration of the hydrophobic AMVN than the more polar ABAP (Fig. 8). At dilute concentrations of either initiator (0.5 mM) the half-life of α-T6 was approximately the same, but rates of α-T6 disappearance rapidly diverged as the amount of each initiator was increased.

Other commonly used initiators of lipid peroxidation also promoted loss of α-T6. When α-T6 in egg PC vesicles was treated with 50 μM $CuSO_4$, 250 μM ascorbate and 1.5 mM H_2O_2, the half-life for loss of fluorescence was 83 min (data not shown). The half-life of α-T6 in egg PC vesicles when exposed to 125 μM $Fe(ClO_4)_2$ and 1.0 mM H_2O_2 was 280 min. Treatment of lipid samples with 1 mM hydrogen peroxide or 100 μM inorganic superoxide (as KO_2) did not decrease α-T6 fluorescence.

Titration of a solution of TTP with aliquots of α-T6 provided the binding curve shown in Figure 9. A dissociation constant, K_d, was obtained by fitting to a one-site binding model and was found to be 540 ± 35 nM. We attempted to illustrate the specificity of this binding by first equilibrating α-T6 and TTP to a constant fluorescence signal, followed by sequential addition of increasing amounts of α-tocopherol as competitive ligand. Unfortunately, the fivefold excess of α-T6 used to assure high occupancy on TTP

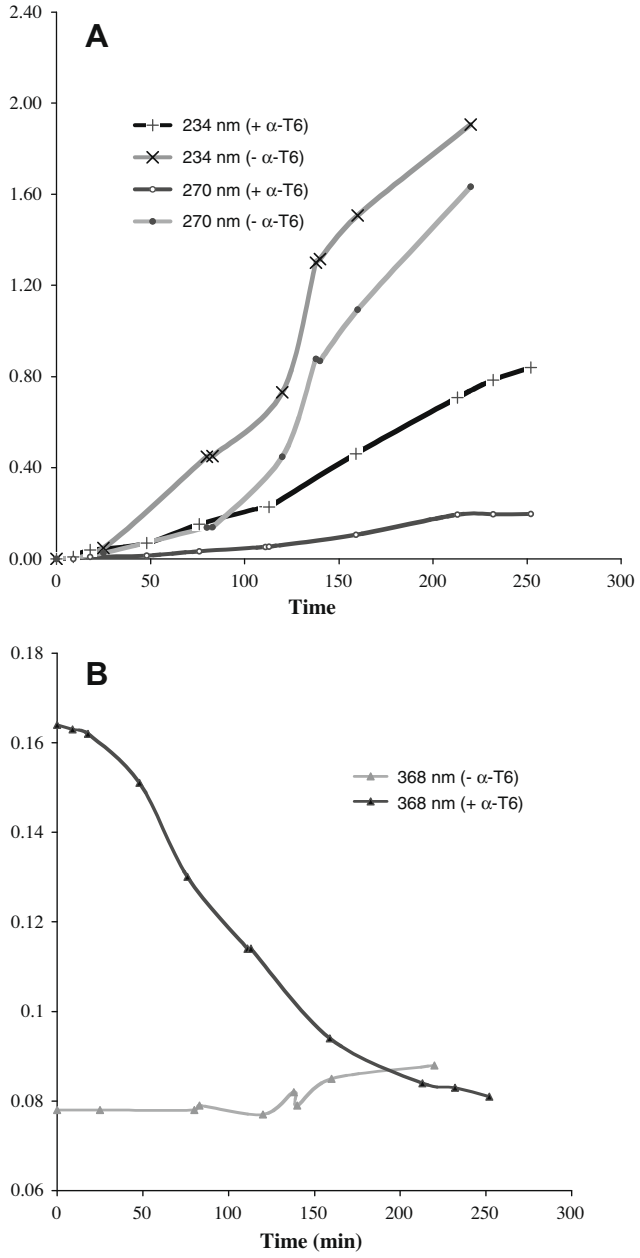


Figure 7. UV absorbance changes during AMVN induced oxidation of 4 μM α-T6 and 0.5 mg/ml soybean PC by 2.5 mM AMVN in acetonitrile at 37 °C. (A) Lipid oxidation was monitored by the absorbance of conjugated dienes at 234 and 270 nm for dienes and trienes, respectively. (B) Oxidation of α-T6 was followed by the loss of absorbance at 368 nm.

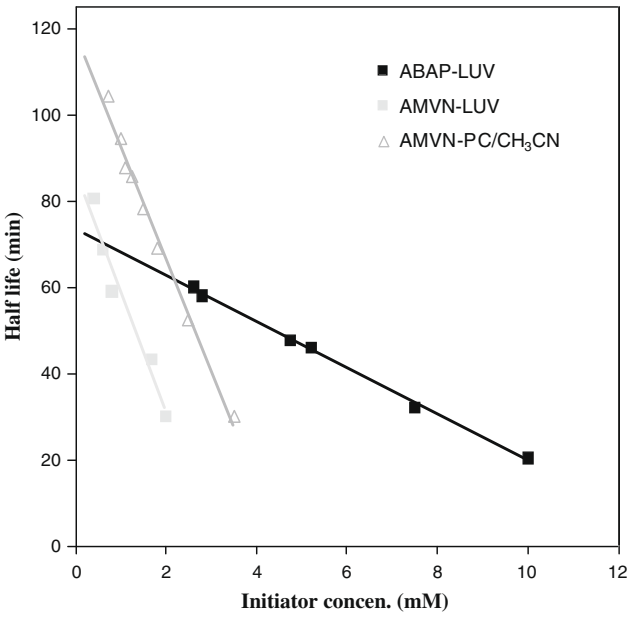


Figure 8. Changes in the observed half-lives of α-T6 fluorescence when oxidations were performed with water soluble (ABAP) and lipid soluble (AMVN) initiators in 100 nM large unilamellar vesicles (LUV) composed of egg PC or in solutions of soy PC in acetonitrile at 37 °C.

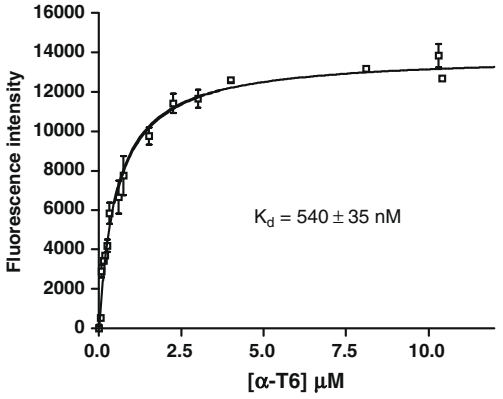


Figure 9. Titration of 0.2 μM TTP with α-T6. Values have been corrected for fluorescence measured when α-T6 was added at the same concentrations to buffer solution containing no TTP. See Section 2 for full details.

supported an increase in fluorescence when tocopherol was added, presumably due to interaction of the two hydrophobic compounds in free solution.

Thus, the polyene tocopherol analogue α-T6 is sensitive to conditions of polyunsaturated lipid peroxidation and its consumption over time can be followed by the loss of fluorescence. In future work we plan to explore the utility of α-T6 as a cellular marker of peroxidative events.

2. Experimental

2.1. General

All reagents were purchased from Sigma–Aldrich Chemical Co., Oakville, Ontario. All glassware was dried in an oven of 90 °C overnight, and then cooled in desiccators before use. Cooling baths were prepared with acetone/liquid nitrogen for −78 °C and −60 °C, and ice/water for 0 °C. Air or moisture sensitive reactions were performed under N_2. THF (tetrahydrofuran) was distilled with sodium/benzophenone immediately before use, benzene was distilled over sodium metal, dichloromethane was distilled over CaH_2, methanol was distilled from Mg/iodine. *n*-Butyllithium was titrated with diphenylacetic acid or *N*-benzylbenzamide to a yellow or a blue endpoint, respectively, immediately before use. Reagent grade solvents were used for extractions. Distilled water was used for all aqueous workups.

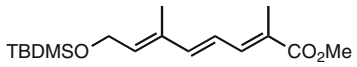
Analytical thin-layer chromatography (TLC) was performed on Merck 0.25 mm pre-coated Silica Gel 60 Å F-254 aluminum plates and visualized under UV light, or stained in 5% $KMnO_4$ solution. Column chromatography was carried out on silica gel (200–300 mesh) purchased from Aldrich.

^1H NMR and ^{13}C NMR spectra were measured on a Bruker Advance DPX-300 digital FT NMR spectrometer (300 and 75 MHz, respectively) in $CDCl_3$ with residual chloroform as internal

reference (7.281 ppm for ^{1}H and 77.0 ppm for ^{13}C) unless otherwise noted. Chemical shifts are reported as δ values and coupling constants (J) are reported in hertz (Hz). The following abbreviations are used: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Mass spectra (MS) were recorded on a Carlo Erba/Kratos GC/MS Concept 1S double focusing mass spectrometer interfaced to a Kratos DART acquisition system and a Sun SPARC workstation. Samples were introduced through a direct inlet system. Ions are generated using electron impact (EI) or Fast Atom Bombardment (FAB) and were reported in m/z values. Fluorescence was recorded on a Photon Technologies International QuantaMaster Model QM-2001 L-format, equipped with double-grating monochromators, a 150 W xenon lamp, and Felix 32 software.

2.2. Synthesis

2.2.1. 8-(*tert*-Butyldimethylsilanyloxy-2,6-dimethyl-octa-2,4,6-trienoic acid methyl ester (10)



To a solution of a 3:2 mixture of (*E*)-methyl 4-(diethoxyphosphoryl)-2-methylbut-2-enoate and (*Z*)-methyl 2-((diethoxyphosphoryl)methyl)but-2-enoate[61] (6.75 g, 27 mmol) in dry THF (100 ml) was added LiHMDS (27 ml, 1 M in THF) at 0 °C. After stirring for 30 min, 4-(*tert*-butyldimethylsilanyloxy)-2-methyl-but-2-enal[34] (1.89 g, 8.83 mmol) in THF (80 ml) was added dropwise. After 3 h, the reaction was quenched by water and extracted with ethyl acetate (4 × 50 ml). The combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated. Purification on SiO_2 (ethyl acetate/hexane 1:9) afforded 2.36 g (86%) of **10** as a light yellow oil. TLC R_f = 0.4 (hexane/ethyl acetate = 9:1).

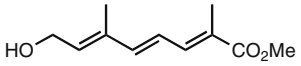
^{1}H NMR δ 7.28 (d, J = 8.2 Hz, 1H HC=C), 6.53 (d, J = 15.3 Hz, 1H, CH=CH), 6.44 (m, 1H, CH=CH), 5.78 (t, J = 6.2 Hz, 1H, CH=), 4.38 (d, J = 6.2 Hz, 2H O–CH2), 3.78 (s, 3H, OCH3), 2.74 (s, 3H, CH3), 1.99 (s, 3H, CH3), 0.93 (s, 9H), 0.10 (s, 6H).

^{13}C NMR δ 168.94, 143.7, 138.84, 135.92, 134.05, 126.24, 122.71, 60.39, 51.79, 25.94, 25.84, 18.6, 12.79, 12.61, −5.14, −5.29.

Mass spectra [EI+] m/z 310 (M+, 6.2%), 253 (46.9%), 221 (49.1%), 178 (58.9%), 147 (71.5%), 119 (73.9%), 73 (100%).

HRMS calcd: 310.19642, found: 310.19487.

2.2.2. 8-Hydroxy-2,6-dimethyl-octa-2,4,6-trienoic acid methyl ester (11)



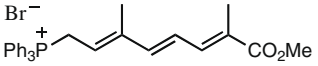
To a solution of compound **10** (2.23 g, 7.19 mmol) in THF (250 ml) was added TBAF (33 ml, 1 M in THF) under N_2. After 4 h the reaction was quenched with water followed by extraction with ethyl acetate (4 × 50 ml). The combined organic extracts was dried over anhydrous $MgSO_4$ and concentrated. Purification on SiO_2 (hexane/ethyl acetate 1:1) afforded 962 mg (68%) of pale yellow crystal. Mp 58–60 °C. TLC R_f = 0.27 (hexane/ethyl acetate = 9:1).

^{1}H NMR δ 7.28 (m, 1H, =CH), 6.6–6.45 (m, 2H, =CH), 5.84 (t, J = 6 Hz, 1H, =CH), 4.35 (d, J = 6.6 Hz, 2H, CH_2), 3.78 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 1.87 (s, 3H, CH_3).

^{13}C NMR δ 168.62, 143.29, 138.63, 135.92, 134.12, 126.74, 123.45, 59.51, 51.84.

Mass spectra [EI+] m/z 196 (53.3%), 119 (91.7%), 107 (96.3%), 91 (100%), 41 (59.1%).

2.2.3. Triphenyl-(2,6-dimethyl-octa-2,4,6-trienoic acid methyl ester)-phosphonium bromide (12)



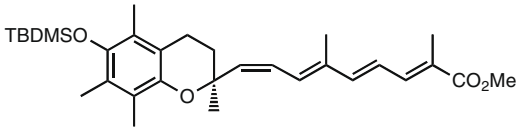
The mixture of alcohol **11** (312 mg, 1.59 mmol) and triphenylphosphine hydrobromide (657 mg, 1.91 mmol) in DCM and ethyl ether (10 ml, 3:2) was stirred for 4 h at room temperature under N_2. After the solvent was removed, the residue was washed with hexane (5 × 10 ml), and dried under vacuum to give 865 mg of **12** as an orange foam. This was used directly in next step without further purification. Mp 122–124 °C.

^{1}H NMR δ 7.9–7.6 (m, 15H), 7.18 (d, J = 9.6 Hz, 1H, =CH), 6.36 (m, 2H, =CH), 5.58 (virtual quartet, J = 7.5 Hz, 1H, =CH), 4.86 (dd, J_1 = 8.1 Hz, J_2 = 16.2 Hz, 2H, P^+–CH_2), 3.67 (s, 3H, OCH_3), 1.89 (s, 3H, CH_3), 1.46 (d, J = 3.6 Hz, 3H, CH_3).

Mass spectra [FAB] m/z 441 (M−Br), 100%), 262 (38.3%), 55 (58%).

HRMS calcd: 441.19834, found: 441.20047.

2.2.4. (*S*)-6-(*tert*-Butyldimethylsilanyloxy)-2,5,7,8-tetramethyl-chroman-2(4,8-dimethyl-1,3,5,7-tetraenoic acid methyl ester) (13)



To a solution of phosphonium bromide **12** (410 mg, 0.79 mmol) in THF (10 ml) was added LiHMDS (0.27 ml, 0.27 mmol) at 0 °C under N_2. The resulting red colored solution was stirred for 30 min, then cooled to −78 °C. The Trolox aldehyde **2**[33] (92 mg, 0.26 mmol) in THF (2 ml) was added via syringe. The mixture was allowed to warm up to room temperature and stirred overnight. Water was then added, followed by extraction with ethyl acetate (4 × 20 ml). The combined organic extracts was dried over anhydrous $MgSO_4$ and concentrated. Purification on SiO_2 (DCM/hexane 3:1) afforded 55.6 mg of **13** (40%) as a light yellow oil. TLC R_f = 0.42 (DCM/hexane = 3:1).

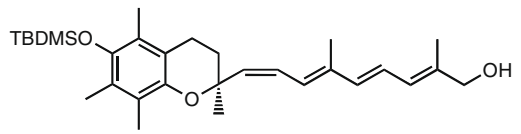
^{1}H NMR δ 7.32 (d, J = 12 Hz, 1H, =CH), 7.06 (d, J = 12 Hz, 1H, =CH), 6.60–6.48 (m, 2H, =CH), 6.34 (t, J = 12 Hz, 1H, =CH), 5.59 (d, J = 12 Hz, 1H, =CH), 3.80 (s, 3H, CH_3), 2.60 (m, 1H, CH_2), 2.19 (s, 3H, Ar-CH_3), 2.11 (s, 3H, Ar-CH_3), 2.05 (s, 3H, Ar-CH_3), 2.01 (s, 3H, CH_3), 1.88 (s, 3H, CH_3), 1.56 (s, 3H, CH_3), 1.05 (s, 9H, $3CH_3$), 0.13 (s, 6H, $2CH_3$). Protons from the chroman C3–CH_2 are partially obscured by the multiple CH_3 signals.

^{13}C NMR δ 144.47, 139.04, 137.25, 135.89, 130.73, 126.09, 125.69, 123.60, 123.25, 117.68, 51.81, 33.73, 27.51, 26.10, 21.24, 18.60, 14.37, 13.43, 12.84, 12.44, 11.88, −3.30.

Mass spectra [EI+] m/z 510 (M+, 23.9%), 378 (23.1%), 319 (58.4%), 279 (42.3%), 73 (100%).

HRMS calcd: 510.31645, found: 510.31621.

2.2.5. (2*Z*,4*E*,6*E*,8*E*)-9-(6-(*tert*-Butyldimethylsilanyloxy)-(*S*)-2,5,7,8-tetramethylchroman-2-yl)-2,6-dimethylnona-2,4,6,8-tetraen-1-ol) (14)



To a solution of ester **13** (270 mg, 0.53 mmol) in THF was added DIBAL-H (1.6 ml, 1 M in DCM) at 0 °C. The resulting solution was stirred for 2 h under N_2. The reaction was quenched with methanol

and water, followed by extraction with ethyl acetate (4 × 20 ml). The combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated. Purification on SiO_2 (ethyl acetate/hexane 1:3) afforded 180 mg (71%) of **14** as a clear oil. TLC R_f = 0.33 (hexane/ethyl acetate = 3:1).

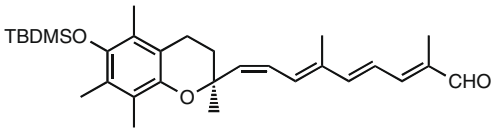
^{1}H NMR δ 6.91 (d, J = 12 Hz, 1H, =CH), 6.49–6.28 (m, 3H, =CH), 6.18 (d, J = 10.8 Hz, 1H, =CH), 5.47 (d, J = 12 Hz, 1H, =CH), 4.13 (m, 2H, OH–CH_2), 2.60 (m, 2H, CH_2), 2.18 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 1.88–1.85 (m, 6H, 2CH_3), 1.55 (s, 3H, CH_3), 1.05 (s, 9H, 3CH_3), 0.13 (s, 6H, 2CH_3). Protons from the chroman C3–CH_2 are partially obscurred by the multiple CH_3 signals at ~2.1–2.0.

^{13}C NMR δ 136.30, 135.29, 127.66, 127.40, 125.92, 125.76, 122.57, 76.58, 69.40, 68.67, 63.18, 33.71, 27.83, 27.47, 27.05, 29.09, 21.26, 21.06, 20.44, 20.37, 18.60, 14.32, 12.36, 12.01, −3.32, −3.37.

Mass spectra [EI+] m/z 482 (M+, 4%), 86 (81%), 84 (100%).

HRMS calcd: 482.32162, found: 482.30629.

2.2.6. (2*Z*,4*E*,6*E*,8*E*)-9-(6-(*tert*-Butyldimethylsilanyloxy)-(*S*)-2,5,7,8-tetramethylchroman-2-yl)-2,6-dimethylnona-2,4,6,8-tetraenal) (15)



A mixture of alcohol **14** (180 mg, 0.38 mmol), MnO_2 (600 mg, 6.9 mmol) and Na_2CO_3 (200 mg, 1.89 mmol) in DCM (15 ml) was stirred for 3 h at room temperature. After filtration and concentration, the residue was purified on SiO_2 (ethyl acetate/hexane 1:3) to give 69 mg (39%) of **15** as a yellow oil. TLC R_f = 0.58 (hexane/ethyl acetate = 3:1).

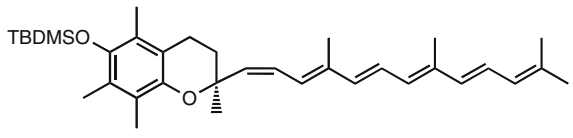
^{1}H NMR δ 9.49 (s, 1H, CHO), 7.16 (d, J = 12 Hz, 1H, =CH), 6.97 (d, J = 9.6 Hz, 1H, =CH), 6.72–6.65 (m, 2H, =CH), 6.36 (t, J = 12 Hz, 1H, =CH), 5.62 (d, J = 12 Hz, 1H, =CH), 2.60 (t, J = 6.6 Hz, 2H, CH_2), 2.20 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 1.98–1.85 (m, 8H, CH_2 and 2 × CH_3), 1.56 (s, 3H, CH_3), 1.05 (s, 9H, 3CH_3), 0.13 (s, 6H, 2CH_3).

^{13}C NMR δ ppm 194.68, 149.25, 146.41, 138.25, 137.02, 135.73, 132.41, 126.10, 125.55, 123.70, 122.85, 122.36, 117.68, 76.09, 33.72, 27.48, 26.10, 21.23, 18.60, 14.39, 13.44, 12.45, 11.87, 9.64, −3.31.

Mass spectra [EI+] m/z 480 (M+, 21.6%), 221 (27.9%), 149 (39.7%), 95 (55.2%), 73 (100%).

HRMS calcd: 480.30597, found: 480.30605.

2.2.7. *tert*-Butyldimethylsilanyloxy ((*S*)-2,5,7,8-tetramethyl-2-((1*Z*,3*E*,5*E*,7*E*,9*E*)-4,8,12-tetramethyltrideca-1,3,5,7,9,11-hexaenyl)chroman-6-yoxyl)silane (16)



To a solution of tri-*n*-butyl(3-methylbut-2-enyl)phosphonium bromide **17** (160 mg, 0.46 mmol) in THF (12 ml) was added *n*-BuLi (0.33 ml, 1.39 M in hexane) at 0 °C. The resulting pale yellow solution was stirred for 30 min under N_2. The aldehyde **15** (81.7 mg, 0.17 mmol) in THF (2 ml) was added after the reaction mixture was cooled to −78 °C. The mixture was allowed to warm up to room temperature and stirred overnight. Water was added followed by extraction with ethyl ether (4 × 15 ml). The combined organic extracts was dried over anhydrous $MgSO_4$ and concentrated. Purification on SiO_2 (ethyl acetate/hexane 1:9) afforded 79.3 mg (88%) of **16** as an orange oil. TLC R_f = 0.67 (hexane/ethyl acetate = 9:1).

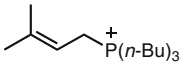
^{1}H NMR δ 6.90 (d, J = 12 Hz, 1H, =CH), 6.60–6.18 (m, 6H, =CH), 5.97 (d, J = 10.8 Hz, 1H, =CH), 5.48 (d, J = 12 Hz, 1H, =CH), 2.60 (m, 2H, CH_2), 2.20 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 1.87–1.83 (m, 14H, CH_2 and 4 × CH_3), 1.57 (s, 3H, CH_3), 1.06 (s, 9H, 3CH_3), 0.14 (s, 6H, 2CH_3).

^{13}C NMR δ ppm 145.88, 144.37, 137.70, 136.74, 136.01, 135.74, 135.19, 134.98, 131.28, 127.43, 126.06, 125.10, 124.80, 123.54, 122.61, 117.79, 75.96, 33.74, 27.52, 26.29, 26.11, 21.30, 18.61, 18.58, 14.37, 13.43, 12.90, 12.37, 11.98, −3.30.

Mass spectra [EI+] m/z 532 (M+, 9.5%), 319 (39.3%), 279 (30.5%), 221 (24.9%), 91 (44.5%), 73 (100%), 57 (50.2%), 43 (83.8%).

HRMS calcd: 532.37366, found: 532.37321.

2.2.8. Tri-*n*-butyl-(3-methyl-but-2-enyl)-phosphonium bromide (17)[62]



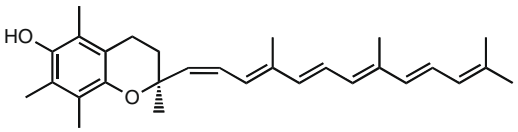
A mixture of tri-*n*-butylphosphine (4.25 ml, 17.0 mmol) and 1-bromo-3-methyl-2-butene (2.0 ml, 17.3 mmol) in toluene (30 ml) was heated at reflux for 5 h. After the toluene was removed under reduced pressure, hexane (60 ml) was added and the solution was heated at reflux for 1 h. After cooling to room temperature, the solid that formed was filtered and dried under vacuum to give a white crystal (5.48 g, 91%). Mp 61–63 °C.

^{1}H NMR δ 4.95 (m, =CH), 3.28 (q, J = 7.8 Hz, 2H), 2.28–2.38 (m, 6H), 1.68–1.71 (m, 6H), 1.36–1.55 (m, 12H), 0.83–0.89 (m, 9H).

^{13}C NMR δ 109.08, 108.96, 25.9, 24.00, 23.80, 23.74, 23.68, 19.19, 18.57.

Mass spectra [FAB] m/z 271 (M^+−Br, 100%).

2.2.9. (*S*)-6-Hydroxy-2,5,7,8-tetramethyl-2-((1*Z*,3*E*,5*E*,7*E*,9*E*)-4,8,12-tetramethyl-trideca-1,3,5,7,9,11-hexaenyl)chromane (1)



To a solution of compound **16** (50 mg, 0.09 mmol) in THF (5 ml) was added TBAF (0.2 ml, 1 M in THF) at room temperature under N_2. After stirring for 2 h, the reaction was quenched with water and extracted with ethyl acetate (4 × 10 ml). The combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated. Purification on SiO_2 (ethyl acetate/hexane 1:3) afforded 14.3 mg (38%) of compound **1** as an orange oil.

TLC R_f = 0.45 (hexane/ethyl acetate = 3:1).

^{1}H NMR δ 6.90 (d, J = 12 Hz, 1H, =CH), 6.63–6.21 (m, 6H, =CH), 5.95 (d, J = 10.8 Hz, 1H, =CH), 5.47 (d, J = 12 Hz, 1H, =CH), 4.19 (b, 1H), 2.65 (m, 2H, CH_2), 2.22 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 1.98–1.85 (m, 14H, 1CH_2 and 3CH_3), 1.57 (s, 3H, CH_3).

^{13}C NMR δ 145.47, 144.84, 137.62, 136.92, 136.10, 135.81, 134.94, 131.21, 127.25, 126.33, 126.03, 125.19, 124.84, 121.13,

118.52, 117.65, 75.89, 34.66, 33.74, 31.58, 27.59, 26.27, 25.27, 21.16, 12.88, 12.23, 12.16, 12.00, 11.29.

Mass spectra [EI+] *m*/*z* 418 (M+, 1.6%), 205 (3.8%), 149 (13.6%), 75 (100%).

HRMS calcd: 418.28718, found: 418.28669.

2.3. Controlled oxidation of 1

Fluorescence spectra were recorded on a Photon Technology International (PTI) QuantaMaster 2000 fluorimeter using Felix 32 software. All solvents were degassed prior to use. Oxidation trials of α-tocohexaenol were conducted with α-tocohexaenol incorporated into dioleoyl phosphatidylcholine (DOPC), and egg yolk phosphatidylcholine (EYPC) large unilamellar vesicles, and with soybean PC dispersions in acetonitrile. All preparations consisted of approximately 1 mol % α-tocohexaenol per mol lipid. The oxidizing agents tested included (1) potassium superoxide, KO_2, (2) iron perchlorate with hydrogen peroxide, H_2O_2, (3) copper sulfate, ascorbate and H_2O_2 and the radical initiators (4) AMVN, and (5) ABAP. All oxidizing agents were prepared fresh before use as approximately 1000-fold stock solutions. Oxidation of α-tocohexaenol was examined in DOPC and egg yolk vesicles by 100 μM KO_2, 125 μM $FeClO_4$/1.0 mM H_2O_2, 150 μM $CuSO_4$/150 μM ascorbate/1.5 mM H_2O_2 and 1 mM H_2O_2 in addition to 4–10 mM 2,2′-azobis-(2-amidinopropane) dihydrochloride (ABAP) and 0.2–4 mM 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN). AMVN was also used to induce oxidation of α-tocohexaenol soybean PC dispersions in acetonitrile at a range of 0.2–8 mM.

Assays were initiated by adding an appropriate volume of oxidizing agent stock solution to a quartz cuvette containing the α-tocohexaenol liposomes in 10 mM phosphate 0.1 M NaCl pH 7.4 buffer or α-tocohexaenol: soy PC in acetonitrile. The solution was mixed and the fluorescence spectrum was recorded using an excitation wavelength of 368 nm and the UV absorbance at 215, 234, 270, and 368 nm were measured. Subsequently, the sample was incubated at 37 °C in the dark and measured at various intervals over 2–5 h time spans.

2.3.1. Oxidation of lipid vesicles

Hundred-nanometer vesicles were prepared from chloroform stock solutions of DOPC, EYPC, and soybean PC from Avanti Lipids using standard methods that we have reported previously.[20] α-T6 was added from an ethanolic stock for integration into liposomal membranes. Due to the light sensitivity of α-T6, all manipulations were performed shielding the samples from direct irradiation using opaque glassware in dim lighting. Briefly, aliquots of the phospholipid stocks with or without added α-T6 were evaporated under N_2 and dried for 1.5 h under vacuum. The dried films were re-suspended in 10 mM phosphate, 0.1 M NaCl pH 7.4 and vortexed for full dispersion of the phospholipids into solution. Unilamellar vesicles were prepared from the suspension by extruding through 100 nm polycarbonate membranes. These liposome stock solutions consisted of 6.2 mM DOPC with 0.16 mM α-T6 or 25 mg/ml egg yolk PC with 0.236 mM α-T6 for dilution prior to measurements. Liposomes were stored at 4 °C under N_2.

2.3.2. Oxidation in solution

Soybean phosphatidylcholine in chloroform with or without added α-tocohexaenol was dried as above for subsequent resuspension into degassed HPLC grade acetonitrile. These solutions contained 15 mg/ml soybean PC and 0.13 mM α-T6 and were stored at 4 °C under N_2.

2.3.3. Data analysis

The ratio of fluorescence at 520 nm versus 485 nm for every spectrum throughout each time course were calculated and plotted in Prism Graphpad 4.0. The data was analyzed using an exponential one phase decay model for derivation of the rate constants and half-lives for each condition of oxidizing agent. Plateau values were taken as the maximal oxidation from the 520/485 nm ratio of the spectra obtained after incubation of each sample overnight at room temperature.

2.3.4. Fluorescence titration of TTP with α-T6

Binding of α-T6 to the tocopherol transfer protein (TTP) was investigated using methods previously reported.[14] Briefly, the baseline fluorescence from 515 to 535 nm (λ_{ex} = 366 nm) of 0.2 μM α-TTP in SET buffer (250 mM sucrose, 100 mM KCl, 50 mM Tris, 1 mM EDTA, pH 7.4) in a quartz cuvette. Subsequently, an aliquot of α-T6 was added from an ethanolic stock solution and equilibrated on a rotating mixer for 15 min at room temperature in the dark prior to measurement of fluorescence intensity. Further additions of α-T6 continued up to a final concentration of 17 μM α-T6 while never exceeding 1% ethanol in the sample. Control titrations with buffer only (no TTP) were conducted as above. The fluorescence at 520 nm at each α-T6 concentration was plotted after subtracting the fluorescence of the buffer control. The data was fit using a one-site saturation model by non-linear regression in Prism GraphPad 4.0. Each titration curve was performed in duplicate in three independent experiments.

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