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Design, synthesis, and evaluation of an α-tocopherol analogue as a mitochondrial antioxidant

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ABSTRACT

An efficient synthesis has provided access to a novel α-tocopherol analogue (**2**), as well as its trifluoroacetate salt and acetate ester. An annulation reaction was used to establish the pyridinol core structure and a Stille coupling reaction was employed for conjugation with the tocopherol side chain. This analogue was shown to suppress the levels of reactive oxygen species in cultured cells, and to quench peroxidation of mitochondrial membranes.

1. Introduction

Mitochondria are organelles found in eukaryotic cells.[1] They produce much of the ATP employed by eukaryotic cells. Mitochondria are also a major source of intracellular reactive oxygen species (ROS); as such, they are potentially vulnerable to oxidative stress. When ROS production exceeds the capacity for detoxification and repair, oxidative damage to proteins, DNA, and phospholipids can occur, disrupting mitochondrial oxidative phosphorylation and potentially leading to impairment of cell function and death. In addition to this pathological role, ROS can also act as redox signaling molecules.[2,3] Accordingly, mitochondrial ROS production and oxidative damage are attractive targets for pharmacological intervention.[4–8]

Increasing evidence suggests that oxidative stress and mitochondrial dysfunction can play crucial roles in neurodegenerative diseases.[9–19] It seems logical to anticipate that reducing mitochondrial oxidative stress may blunt the progression of these neurodegenerative disorders. α-Tocopherol (α-TOH, **1**) is among the most potent natural lipophilic antioxidants studied, exhibiting excellent properties in quenching lipid peroxidation.[20–22] Suppression of lipid peroxidation results from the ability of α-tocopherol to transfer its phenolic H atom to an incipient lipid carbon-centered radical, and thereby quench the free radical chain reaction that occurs in the presence of O_2.[23–25] The development of synthetic radical-scavenging antioxidants with better properties than α-TOH has been a goal of a number of studies. Strategies for the targeting of antioxidants to mitochondria have also been considered.[26–28]

Recently Porter, Pratt and co-workers have described a new antioxidant (**4**) reported to be ~90-fold more potent than α-tocopherol as an antioxidant, as judged by suppression of the autoxidation of methyl linoleate in benzene solution (Figure 1).[23–25] We reasoned that conjugation of the pyridinol antioxidant core to a lipophilic side chain might facilitate delivery of the antioxidant to membranes, including mitochondrial membranes, and thereby confer cytoprotective properties at the level of mitochondrial function. The phytyl side chain of α-tocopherol seemed to be a logical choice for the requisite lipophilic side chain. Herein, we report the synthesis of a novel tocopherol analogue (**2**) incorporating the pyridinol core of compound **4**. Also reported is the evaluation of the ability of **2** to protect mitochondrial membranes from AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride)-induced lipid peroxidation, and to diminish reactive oxygen species (ROS) in cultured CEM leukemia cells pretreated with diethyl maleate.

2. Results and discussion

The retrosynthetic analysis of compound **2** is outlined in Figure 2. Preparation of the desired compound was envisioned by the coupling of pyridinol **17** and a side chain such as alkyne **9** or its tributyltin derivative **10**. Alkyne **9** should be accessible from commercially available (*R*)-phytol.[29,30] Pyridinol derivative **17** could be assembled from **13** through functional group transformations. Compound **13**, in

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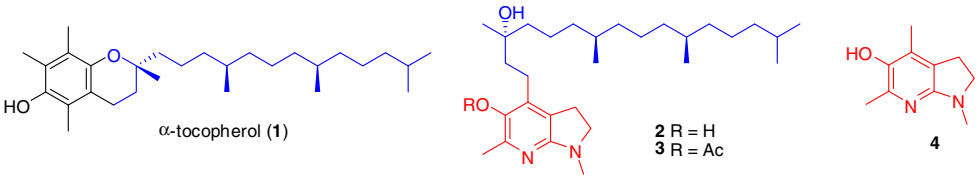


Figure 1. Structures of α-tocopherol (**1**), novel antioxidants **2** and **3**, as well as the pyridinol core antioxidant **4**.

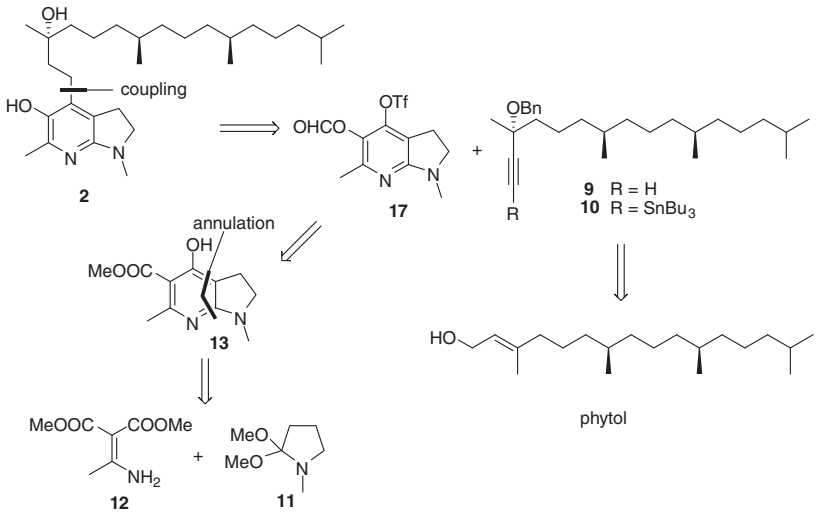
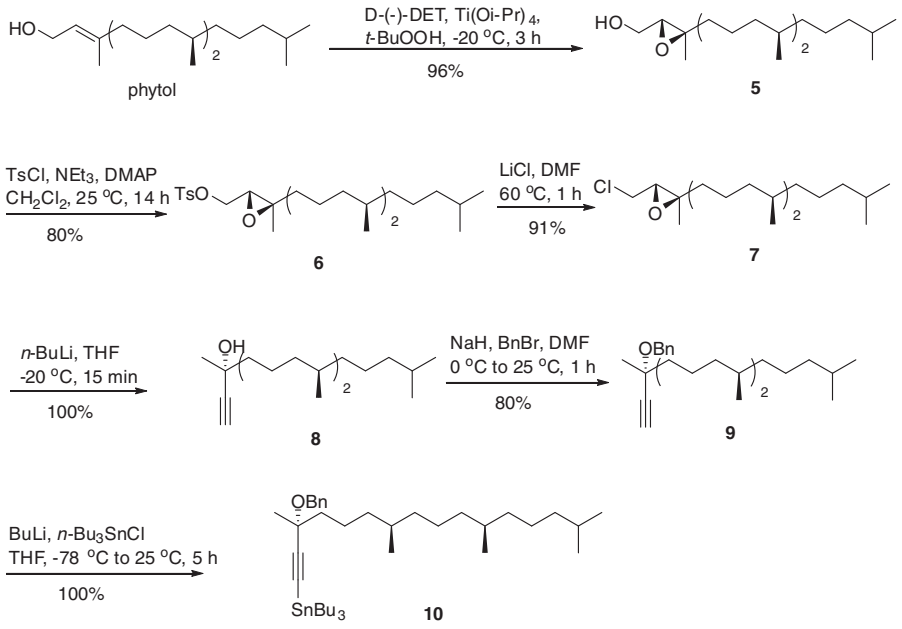


Figure 2. Retrosynthetic analysis of compound **2**.

turn, could be obtained by the annulation reaction of **11**[31] and **12**.[32] Compounds **11** and **12** themselves were easily prepared from *N*-methylpyrrolidine and dimethyl malonate, respectively.

2.1. Synthesis of side chain derivatives 9 and 10

The synthesis of the side chain derivatives is outlined in Scheme 1. The configuration of the stereogenic centers in the C-16 chain can be obtained from the natural product phytol, which has the same configuration as the α-tocopherol side chain. The third chiral center was established by Sharpless epoxidation.[33] Thus, by the use of D-(−)-diethyl tartrate, the desired (*R*,*R*)-isomer of **5** was prepared in 96% yield. The primary alcohol could be converted to the chloride by heating in carbon tetrachloride in the presence of triphenylphosphine.[29,30] Although the primary alcohol **5** could be converted directly to chloride **7**, a large amount



Scheme 1. Synthesis of side chain derivative **10**.

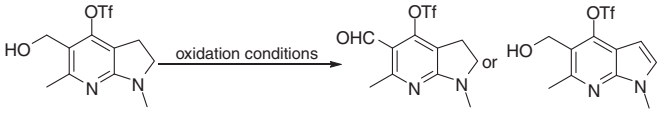
of triphenylphosphine by-product made **7** difficult to purify. This problem could be resolved by tosylation of the primary alcohol followed by conversion to the chloride by treatment with lithium chloride. This two-step process was more efficient and cleaner than the direct route, and is better suited to larger scale preparation. Chloride **7** was then treated with *n*-butyllithium and converted quantitatively into alkyne alcohol **8**.[29,30,34,35] Finally, the alcohol functionality was protected with a benzyl group in 80% yield to form **9**. Although alkyne **9** itself could be used in the coupling reaction, its tin derivative **10** was found to be more efficient in the coupling reaction with formate ester **17**. The tin derivative **10** was prepared by treating alkyne **9** with butyllithium followed by exchange with tributyltin chloride in quantitative yield.

2.2. Synthesis of pyrrolo[2,3-*b*]pyridine core structure 17

The core structure methyl 1,6-dimethyl-4-hydroxy-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxylate (**13**) could be prepared readily by an annulation reaction of **11** and **12** (Scheme 2). The conversion of **13** to its triflate was accomplished easily using $PhNTf_2$ in the presence of potassium carbonate in dry DMF; triflate **14** was obtained in 94% yield. Direct reduction of the methyl ester to the corresponding aldehyde by using 1 equiv of DIBAL-H was attempted but found to be unsuccessful. Thus the methyl ester in **14** was reduced to the corresponding alcohol by the use of DIBAL-H at low temperature in dichloromethane, providing alcohol **15** in 95% yield.

The oxidation of alcohol **15** to aldehyde **16** required some optimization. Several oxidization methods were studied for this purpose (Table 1), including Swern oxidization[36] and Corey–Kim oxidization.[37] While these methods are widely used for the preparation of aldehydes from primary alcohols, **16** was formed only in low yield from alcohol **15**. Dess–Martin periodinane is a mild and effective reagent, but in this case provided only 18% of aldehyde **16** with >50% of an unidentified by-product. Pyridinium chlorochromate (PCC)[38] provided ~20% of desired product and the TPAP/NMO (tetra-*n*-propylammonium perruthenate (VII)/4-methylmorpholine *N*-oxide) system[35] gave multiple products. MnO_2 oxidized the pyrrolidine moiety selectively to form 7-azaindole derivative **22** in 52% yield. *o*-Iodoxybenzoic acid (IBX)[39] was finally found to be an excellent oxidant for the conversion of **15** → **16** in high yield.

Table 1
Conditions studied for oxidation of alcohol **11** to aldehyde **12**



15 **16** **22**

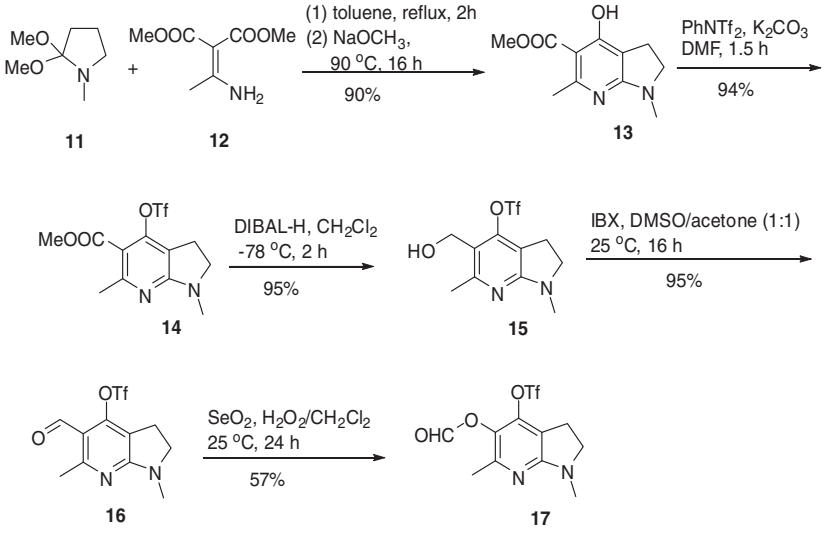
Experiment	Oxidation conditions	Product[a] (yield)
1	Pyridinium chlorochromate (PCC)	**16** (20%)
2	Swern oxidation	**16** (<10%)
3	Corey–Kim oxidation	**16** (<10%)
4	MnO_2	**22** (52%)
5	Dess–Martin periodinane	**16** (18%)
6	*o*-Iodoxybenzoic acid (IBX)	**16** (95%)
7	NMO/TPAP[b]	**16** (<10%)

[a] Isolated yield.
[b] Tetra-*n*-propylammonium perruthenate/4-methylmorpholine *N*-oxide.

The oxidation of aldehyde **16** to the corresponding formate ester **17** was also challenging (Table 2). Peracids such as *m*-chloroperbenzoic acid or AcOOH did not effect the desired conversion. Fortunately, the modified selenium catalyzed Baeyer–Villiger reaction (Table 2, experiment 4) worked reasonably well.[40]

2.3. Preparation of acetate ester 3 and tocopherol analogue 2

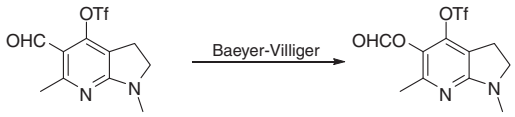
With pyridinol ester **17** and side chains **9** and **10** in hand, the coupling reaction of the side chains **9**/**10** and **17** was investigated (Scheme 3). Direct coupling of side chain **9** with ester **17** in the presence of $Pd(PPh_3)_4$, CuI, and *i*-Pr_2NEt in DMF was successful, but proceeded in low yield either at room temperature or at 80 °C (Table 3). Changing the ligand to tri-cyclohexylphosphine or tri-*t*-butylphosphine resulted in little or no product. Likewise, activating the side chain as a zinc derivative resulted in only trace amounts of the desired product. In comparison, when the side chain derivative **10** was employed in this coupling reaction using $Pd(PPh_3)_4$ as catalyst and *N*-methylpyrrolidin-2-one as solvent, the reaction proceeded smoothly at 105 °C for 2 h to give the desired product **18** in 88% yield (Table 3). DIBAL-H reduction of the formate ester and O-acetylation then provided **19** in 78% yield. Treatment of **19** with Pearlman's catalyst in methanol overnight then gave compound **3** in 71% yield. Removal of the acetyl group from pyridinol **3** was carried out at low temperature (−78 °C) to



11 **12** **13** **14** **15** **16** **17**

Scheme 2. Synthesis of pyrrolo[2,3-*b*]pyridine core structure **17**.

Table 2
Conditions studied for the Baeyer–Villiger reaction



16 17

Experiment	Oxidation conditions	Yield of **17**[a]
1	*m*-CPBA	—[b]
2	AcOOH/AcOH	—
3	$Na_2CO_3{\cdot}1.5H_2O_2$/TFAA	<10%
4	SeO_2/H_2O_2	57%
5	$PhSeSePh/H_2O_2$	20%

[a] Isolated yield.
[b] No desired product.

avoid decomposition. DIBAL-H reduction afforded **2** in quantitative yield. The final product was found to be unstable, decomposing completely in one day. This problem was resolved by forming the trifluoroacetate salt of **2**, which proved to be reasonably stable. Compounds **2** and **4** were purified by reversed-phase HPLC prior to bioassay, and were tested as their trifluoroacetate salts.

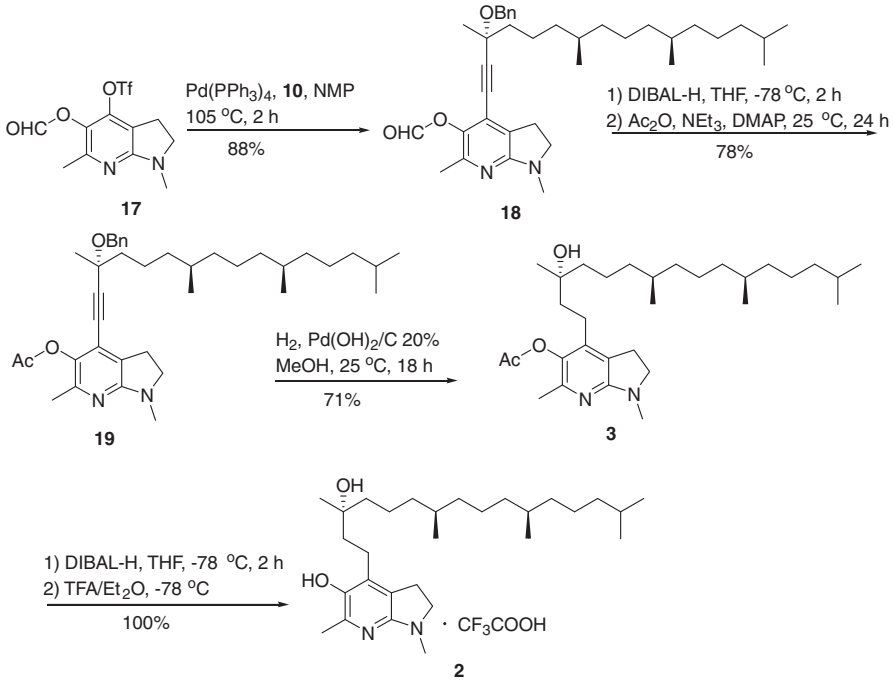
Another possible route for the preparation of **2** was also tried (Scheme 4). In this route, the coupling reaction was carried out before the Baeyer–Villiger reaction. The coupling reaction was quite successful using alkyne **9**; the yield was almost quantitative. However, in the subsequent Baeyer–Villiger reaction, aldehyde **20** reacted with *m*-CPBA in dichloromethane to give only acid **21** as a product in 49% yield (Scheme 4). Several different substrates were also examined in the coupling reaction with alkyne **9** (Table 4). When there was an electron withdrawing group such as methyl carboxylate or aldehyde in the *o*-position, the coupling reaction could be carried out in high yield. However, weaker electron withdrawing groups hindered the reaction; using a formyloxy substituent, only a 22% yield of the product was achieved and no desired product was obtained using an acetoxy substrate.

2.4. Biochemical and biological evaluation of the tocopherol analogue

To investigate the ability of tocopherol analogue **2** to inhibit lipid peroxidation, bovine mitochondria were incubated with *cis*-parinaric acid (Figure 3). This fatty acid is incorporated into the mitochondrial membrane and fluoresces within this lipid environment. The conjugated double bond of the fluorophore is susceptible to oxidation by oxygen radicals; consequently, the disappearance of fluorescence provides a measure of lipid peroxidation. As shown in Figure 3, compound **2** was found to be slightly more effective in blocking the oxidation of *cis*-parinaric acid by peroxyl radicals than was α-tocopherol (naturally occurring isomer).

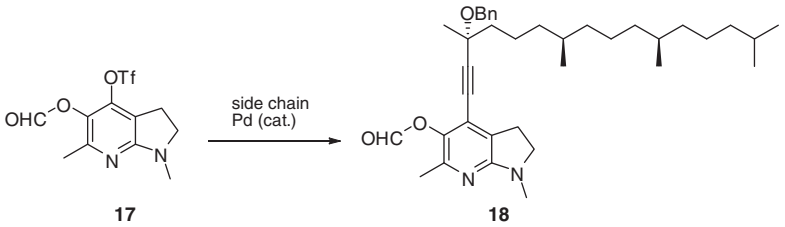
Cellular ROS production was determined in CEM leukemia cells by monitoring the fluorescence derived from the oxidant-sensitive probe DCFH-DA. DCFH-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed by esterases to the non-fluorescent polar derivative DCFH and thereby trapped within the cell. In the presence of an appropriate oxidant, DCFH is oxidized to the highly fluorescent DCF (dichlorofluorescein). In the present case, cellular oxidative stress was induced by pharmacological depletion of glutathione (GSH) using the chemical diethyl maleate (DEM).

Increased DCF fluorescence, a measure of intracellular ROS production, was determined by a shift in DCF fluorescence (FL1-H channel) to the right on the *x*-axis of the FACS histogram stained with DCF (Figure 4, panel A). DEM treatment caused the DCF fluorescence to shift right on the *x*-axis of the FACS histogram, indicating increased ROS production as a result of glutathione depletion. Pretreatment of CEM cells with compound **2** was able to afford significantly better protection than α-tocopherol or compound **4** against the DEM-induced oxidative stress. Again, the fluorescence microscopy analysis (Figure 4, panel B) shows that compound **2** provided the best protection against ROS in the glutathione-depleted CEM cells. Also shown in panel C is the bright image (differential interference contrast, (DIC)) for the treated cells. Thus both the pyridinol core and phytyl side chain of **2** are important to its demonstrated ability to suppress cellular ROS levels.



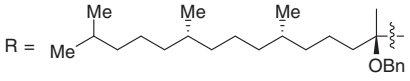
Scheme 3. Preparation of acetate ester **3** and tocopherol analogue **2**.

Table 3
Conditions studied for coupling reaction with formate ester **17**

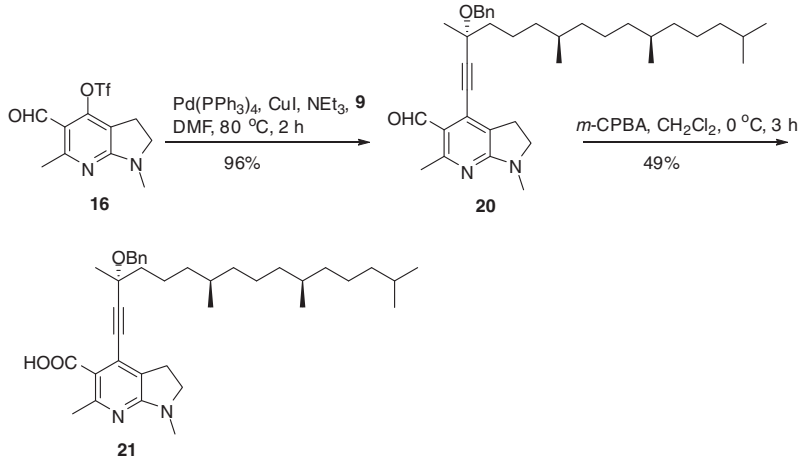


17 **18**

Experiment	Side chain	Catalyst and conditions	Yield of **18**[a]
1	R—≡—H	$Pd(PPh_3)_4$, CuI, *i*-Pr_2NEt, DMF, 25 °C, 20 h	31%
2	R—≡—H	$Pd(PPh_3)_4$, CuI, *i*-Pr_2NEt, DMF, 80 °C, 2 h	22%
3	R—≡—H	$Pd_2(OAc)_2$, PCy_3,*i*-Pr_2NEt, DMF, 25 °C, 20 h	2%
4	R—≡—H	$Pd_2(CH_3CN)_2Cl_2$, P*t*-Bu_3, CuI, *i*-Pr_2NH, 25 °C, 24 h	—[b]
5	R—≡—ZnCl	$Pd(PPh_3)_4$, THF, 25 °C, 20 h	Trace
6	R—≡—$SnBu_3$	$Pd(PPh_3)_4$, NMP, 105 °C, 2 h	88%

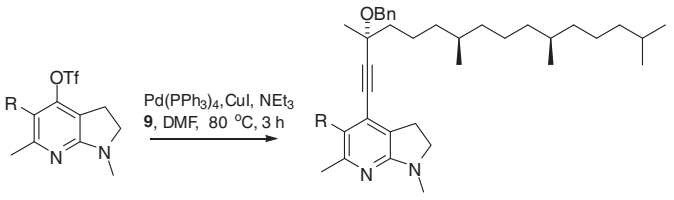


[a] Isolated yield.
[b] No desired product.



Scheme 4. Alternative route studied for the preparation of **2**.

Table 4
Sonogashira coupling using different substrates



Experiment	R	Product[a] (yield)
1	–COOMe	**23** (95%)
2	–CHO	**20** (96%)
3	–OCHO	**18** (22%)
4	–OAc	**24** (—[b])

[a] Isolated yield.
[b] No desired product.

Tocopherol analogue **2** afforded significant protection of mitochondrial membranes against lipid peroxidation and suppressed ROS levels in oxidatively stressed cultured CEM cells. More detailed biochemical experiments comparing compounds **2** and **4** have reinforced the importance of the phytyl side chain of **4** in conferring these protective effects.[41] These findings parallel in a general way those in earlier reports that utilized lipophilic pyridinol[42] and naphthyridinol analogues.[43]

3. Conclusions

A novel antioxidant analogue of α-tocopherol (**2**) has been prepared. The pyridinol core of this analogue was obtained via an annulation reaction. Functional group transformation of a primary alcohol to the aldehyde was carried out efficiently using IBX. The Baeyer–Villiger reaction was found to be workable using H_2O_2 catalyzed by SeO_2. Stille coupling was the most efficient method for

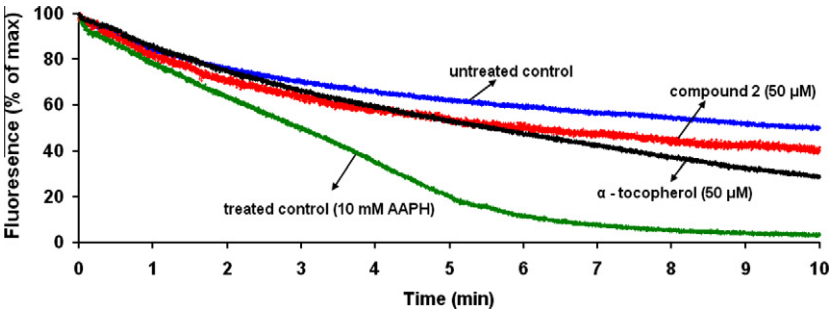


Figure 3. Time course of AAPH-induced oxidation of *cis*-parinaric acid incorporated into bovine heart mitochondrial membranes (1 mg/mL) detected by fluorescence decay (*cis*-parinaric acid oxidation) in presence and absence of compound **2** or α-tocopherol (**1**).

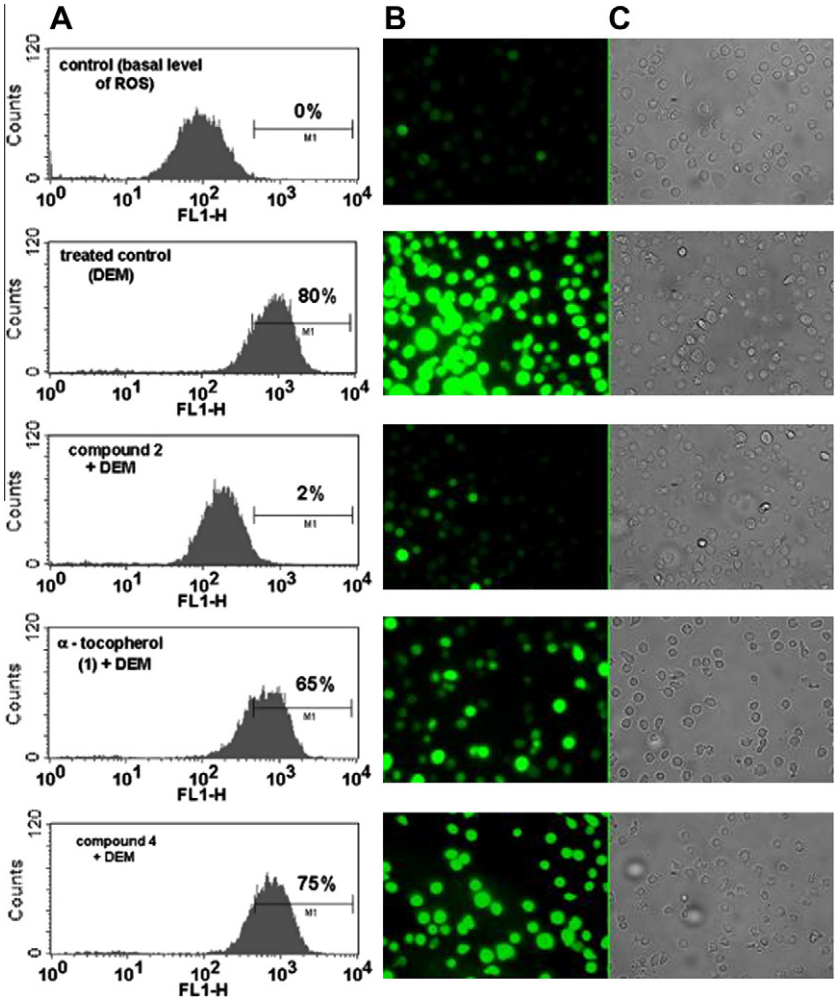


Figure 4. Effect of treatment with compound **2** on intracellular ROS accumulation induced by glutathione depletion with 5 mM diethyl maleate (DEM) in cultured CEM leukemia cells. Following pretreatment of CEM cells with 5 μM compound **2**, α-tocopherol or compound **4** for 1 h, the medium was changed, followed by treatment with 5 mM DEM for 30 min to deplete glutathione. Intracellular ROS accumulation was determined by measuring the DCF-derived fluorescence after incubation of the stressed cells with 5 μM DCFH-DA for an additional 20 min. Left panel (A) represents the flow cytometric histograms of ROS production in CEM cells. The green fluorescence (DCF) was measured by flow cytometry using the FL1-H channel. The histogram panel shows a representative example of three independent experiments. In each analysis, 10,000 events were recorded. Increased DCF fluorescence, a measure of intracellular oxidation and ROS production, was determined by a shift in DCF fluorescence to the right on the *x*-axis of the FACS histogram. Center and right panel images are the imaging conditions (green emission (DCF fluorescence) (B) and bright image (differential interference contrast (DIC)) (C) with an exposure time of 250 ms showing fluorescence microscopy analysis of CEM cells upon treatment with DEM (5 mM) (highly fluorescent) and in the presence or absence of 5 μM compound **2**, α-tocopherol (**1**) or compound **4**.

conjugation of the phytyl side chain. The final product was found to be unstable, but could be stabilized as an acetate ester or as its TFA salt. Tocopherol analogue **2** was found to quench the AAPH-mediated peroxidation of mitochondrial membranes more effectively than α-tocopherol, and to strongly diminish ROS levels in cultured cells depleted of glutathione.

4. Experimental section

4.1. Chemistry

All chemicals and solvent were of reagent grade and were used without further purification, except as noted below. Anhydrous

tetrahydrofuran (THF) was distilled from sodium/benzophenone under dry argon. Dichloromethane (CH_2Cl_2) was distilled from calcium hydride under argon. Thin layer chromatography (TLC) was performed on Merck pre-scored Silica Gel 60 F-254 plates; visualization employed 254 nm UV light, or staining with iodine, ceric ammonium molybdate, or *p*-anisaldehyde. Silica gel (200–400 mesh, Silicycle) was used for flash chromatography. ^{1}H NMR spectra were recorded in chloroform-*d*, CD_3OD, acetone-d_6, DMSO-d_6 or D_2O on Varian 400 or 500 MHz spectrometers. Chemical shifts were reported in parts per million (ppm, δ) referenced to the residual ^{1}H resonance of the solvent ($CDCl_3$, 7.26 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77 ppm). Splitting patterns were designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. The low resolution mass spectrum was obtained on a Voyager DE-STR, MALDI-TOF instrument at Arizona State University. The sample was prepared by mixing droplets of the sample solution in chloroform with 2,5-dihydroxybenzoic acid and 4-hydroxybenzylidenemalononitrile solution in acetonitrile, where the latter served as the matrix. High resolution mass spectra were obtained at the Ohio State University Mass Spectrometry Facility.

4.1.1. ((2*R*,3*R*)-3-Methyl-3-((4*R*,8*R*)-4,8,12-trimethyltridecyl)-oxiran-2-yl)methanol (5)[29,30]

To a solution of 4.70 g (22.0 mmol) of (−)-diethyl D-tartrate and 3.50 g of 4 Å molecular sieves in 300 mL of dichloromethane was added 13.7 g (20.0 mmol) of Ti(*i*-PrO)$_4$ and 5.5 mL (30 mmol) of *t*-BuOOH (5.5 M in nonane), at −20 °C under argon. After 20 min a solution of 5.93 g (20.0 mmol) of phytol in 80 mL of dichloromethane was added. The reaction mixture was stirred at −20 °C for 3 h. The absence of starting material was confirmed by silica gel TLC. The reaction was quenched by adding 250 mL of a 1 N NaOH–brine mixture, then the mixture was allowed to warm to room temperature. The mixture was filtered through Celite then the mother liquor was separated into layers and the solid was washed with two 400-mL portions of ethyl acetate. The combined organic layer was dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 3.2 cm). Elution with 10:1 hexanes/ethyl ether gave the product (*R*)-**5** as colorless oil: yield 5.98 g (96%); $[\alpha]_D^{25}$ +4.50 (*c* 0.20, EtOH), lit.[30] $[\alpha]_D^{26}$ +4.88 (*c* 2.80, EtOH), lit.[44] $[\alpha]_D^{20}$ +4.39 (*c* 2.78, EtOH); silica gel TLC R_f 0.26 (1:1 ether/hexanes); ^{1}H NMR ($CDCl_3$) δ 0.83–0.87 (m, 12H), 1.00–1.65 (m, 21H), 1.21 (s, 3H), 2.27 (br s, 1H), 2.97 (dd, 1H, *J* = 4.8, 3 Hz), 3.68 (dd, 1H, *J* = 8.1, 5.4 Hz) and 3.83 (br d, 1H, *J* = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 17.0, 19.9, 20.0, 22.8, 22.9, 23.0, 24.7, 25.1, 26.1, 28.2, 37.2, 37.5, 37.6, 37.7, 39.1, 39.6, 61.65, 61.7, 63.3, and 80.9.

4.1.2. (2*R*,3*R*)-3-Methyl-3-((4*R*,8*R*)-4,8,12-trimethyltridecyl)-oxiran-2-ylmethyl *p*-toluenesulfonate (6)[30]

To a solution of 0.93 g (3.00 mmol) of epoxy alcohol **5** in 20 mL of dichloromethane was added 0.75 g (7.43 mmol) of triethylamine and a catalytic amount of *N*,*N*-dimethylaminopyridine. Then 0.68 g (3.58 mmol) of *p*-toluenesulfonyl chloride was added portionwise to the reaction mixture. The reaction mixture was stirred at room temperature overnight, then quenched by the addition of 30 mL of water. The organic layer was washed with brine and dried over Na_2SO_4. The solvent was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (25 × 3.2 cm). Elution with 100:1 → 10:1 hexanes/ethyl ether gave **6** as colorless oil: yield 1.12 g (80%); $[\alpha]_D^{25}$ +22.6 (*c* 0.97, $CHCl_3$), lit.[30] $[\alpha]_D^{27}$ +15.92 (*c* 1.06, $CHCl_3$); silica gel TLC R_f 0.20 (5:1 hexanes/ether); ^{1}H NMR ($CDCl_3$) δ 0.82–0.86 (m, 12H), 0.99–1.92 (m, 21H), 1.19 (s, 3H), 2.44 (s, 3H), 2.96 (t, 1H, *J* = 6.0 Hz), 4.09 (dd, 1H, *J* = 11.0, 6.0 Hz), 4.15 (dd, 1H, *J* = 11.0, 5.5 Hz), 7.34 (d, 2H, *J* = 8.0 Hz), and 7.80 (d, 2H, *J* = 6.8 Hz).

4.1.3. (2*R*,3*S*)-3-Methyl-3-((4*R*,8*R*)-4,8,12-trimethyltridecyl)-oxiran-2-yl)methyl chloride (7)[30]

To a solution of 1.12 g (2.40 mmol) of tosylate **6** in 20 mL of DMF was added 0.12 g (2.6 mmol) of lithium chloride. The reaction mixture was stirred at 60 °C for 1 h. The cooled reaction mixture was poured into 40 mL of water and extracted with three 30-mL portions of EtOAc. The combined organic layer was washed with satd aq $NaHCO_3$ and brine, then dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 3.2 cm). Elution with 100:1 hexanes/ether gave **7** as a colorless oil: yield 0.72 g (91%); $[\alpha]_D^{25}$ −7.69 (*c* 0.77, $CHCl_3$), lit.[30] $[\alpha]_D^{27}$ −9.47 (*c* 1.07, $CHCl_3$); silica gel TLC R_f 0.66 (5:1 hexanes/ether); ^{1}H NMR ($CDCl_3$) δ 0.83–0.88 (m, 12H), 1.00–1.70 (m, 21H), 1.31 (s, 3H), 3.03 (t, 1H, *J* = 6.6 Hz), 3.45 (dd, 1H, *J* = 11.4, 7.2 Hz), and 3.70 (dd, 1H, *J* = 11.7, 6.0 Hz).

4.1.4. (3*R*,7*R*,11*R*)-3,7,11,15-Tetramethylhexadec-1-yn-3-ol (8)[30]

To a solution of 720 mg (2.20 mmol) of epoxy chloride **7** in 10 mL of dry THF was added 4.4 mL (11.0 mmol, 2.5 M in hexane) of *n*-butyllithium at −20 °C under argon. After 20 min the reaction was quenched by adding 10 mL of satd aq ammonium chloride and allowed to warm to room temperature. The reaction mixture was extracted with three 10-mL portions of ethyl acetate. The combined organic layer was washed with brine and dried over $MgSO_4$. The solvent was removed under diminished pressure and the residue was purified by flash chromatography on a silica gel column (20 × 3.2 cm). Elution with 5:1 hexanes/ether gave **8** as a colorless oil: yield 640 mg (100%); $[\alpha]_D^{25}$ +1.88 (*c* 0.87 $CHCl_3$), lit.[30] $[\alpha]_D^{27}$ +1.37 (*c* 0.73 $CHCl_3$); silica gel TLC R_f 0.16 (1:1 hexanes/ether); ^{1}H NMR ($CDCl_3$) δ 0.82–0.87 (m, 12H), 0.99–1.72 (m, 21H), 1.49 (s, 3H), 2.12 (br s, 1H), and 2.42 (s, 1H).

4.1.5. 3-Benzoxy-(3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadec-1-yne (9)

To a solution of 71.8 mg (0.24 mmol) of **8** in 2 mL of dry DMF was added 16.0 mg (0.40 mmol) of sodium hydride (60% mixture in mineral oil). The reaction mixture was stirred at 0 °C for 20 min, then 68.0 mg (0.40 mmol) of benzyl bromide was added. The reaction mixture was allowed to warm to room temperature and stirred for 1 h. Two milliliters of satd aq ammonium chloride solution and 2 mL of water were added to quench the reaction. The reaction mixture was extracted with three 5-mL portions of ethyl acetate. The combined organic layer was washed with brine and dried ($MgSO_4$) then the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 3.2 cm). Elution with 100:1 hexanes/ether gave **9** as a colorless oil: yield 75 mg (80%); $[\alpha]_D^{25}$ +3.08 (*c* 1.32, $CHCl_3$); silica gel TLC R_f 0.95 (20:1 hexanes/ether); ^{1}H NMR ($CDCl_3$) δ 0.83–0.88 (m, 12H), 1.02–1.81 (m, 19H), 1.50 (s, 3H), 2.48 (s, 3H), 4.63 (ABq, 2H, $\Delta\nu_{AB}$ = 25.2 Hz, J_{AB} = 11.2 Hz), 7.24–7.27 (m, 1H) and 7.30–7.37 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 19.7, 19.8, 21.7, 22.65, 22.7, 24.5, 24.8, 26.3, 26.4, 28.0, 32.7, 32.8, 37.1, 37.3, 37.4, 39.4, 41.8, 66.2, 73.7, 85.5, 127.3, 127.6, 128.3, and 139.1; mass spectrum (ESI), *m*/*z* 385.3471 $(M+H)^+$ ($C_{27}H_{45}O$ requires *m*/*z* 385.3470).

4.1.6. 3-Benzoxy-((3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadec-1-ynyl)tri-*n*-butylstannane (10)

To a solution of 424 mg (1.40 mmol) of alkyne **9** in 6 mL of dry THF was added 1.12 mL (2.8 mmol, 2.5 M in hexane) of *n*-butyllithium at −78 °C. The reaction mixture was allowed to warm to 0 °C and stirred for 20 min, then 470 mg (1.40 mmol) of *n*-Bu_3SnCl was added. The reaction mixture was allowed to warm to room temperature and stirred for 4 h. The reaction mixture was poured into 10 mL of satd aq NH_4Cl, then extracted with three 10-mL portions of ethyl ether. The combined organic layer was washed with brine,

dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 3.2 cm). Elution with hexanes gave **10** as a colorless oil: yield 768 mg (100%); $[\alpha]_D^{25}$ +2.25 (*c* 0.80, $CHCl_3$); silica gel TLC R_f 0.96 (20:1 hexanes/ether); ^1H NMR ($CDCl_3$) δ 0.78–0.92 (m, 39H), 0.96–1.84 (24H), 4.64 (ABq, 2H, $\Delta\nu_{AB}$ = 25.2 Hz, J_{AB} = 12.0 Hz) and 7.24–7.36 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 8.8, 11.2, 13.75, 13.8, 19.8, 22.2, 22.7, 22.8, 24.7, 24.9, 27.0, 27.5, 28.1, 29.0, 29.4, 29.9, 32.9, 37.6, 39.5, 66.2, 73.3, 74.6, 87.4, 127.1, 127.7, 128.2, 128.4, and 139.8; mass spectrum (ESI), *m*/*z* 675.4536 $(M+H)^+$ ($C_{39}H_{71}OSn$ requires *m*/*z* 675.4527).

4.1.7. 2,2-Dimethoxy-1-methylpyrrolidine (11)[31]

A mixture of 52.0 mL (546 mmol) of *N*-methyl-2-pyrrolidinone and 52.0 mL (548 mmol) of dimethyl sulfate was stirred and heated at 90 °C for 1.5 h, then allowed to cool to room temperature. A solution containing 130 mL of 25% methanolic sodium methoxide and 370 mL of methanol was added at −10 °C under Ar over a period of 1 h. The precipitated solid was filtered and the solvent was concentrated under diminished pressure. The residue was dissolved in 500 mL of ether and stirred for 1 h, then the precipitated solid was filtered. The solid was washed with two 50-mL portions of ether. After the ether was concentrated, the residue was distilled in vacuo to give **11** as a yellow liquid: yield 31.0 g (40%); ^1H NMR ($CDCl_3$) δ 1.62–1.74 (m, 2H), 1.83 (t, 2H, *J* = 7.8 Hz), 2.28 (s, 3H), 2.78 (t, 2H, *J* = 6.6 Hz), and 3.15 (s, 6H).

4.1.8. 2-(1-Aminoethylidene)malonic acid dimethyl ester (12)[32]

To a solution of 1.32 g (10.0 mmol) of dimethyl malonate in 20 mL of 1,2-dichloromethane was added 0.41 g (10.0 mmol) of acetonitrile and 20.0 mL (20.0 mmol, 1 M in dichloromethane) of tin chloride under Ar at room temperature. The reaction mixture was heated at reflux and stirred for 3 h. The solvent was concentrated under diminished pressure. The residue was dissolved in 20 mL of acetone and treated slowly with satd aq sodium carbonate. After filtration, the solid was washed with ethyl acetate and the mother liquor was extracted with three 20-mL portions of ethyl acetate. The combined organic layer was washed with brine and dried ($MgSO_4$). The solvent was concentrated under diminished pressure, and the residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 10:1 hexanes/ethyl acetate gave **12** as a colorless solid: yield 0.66 g (38%); mp 82–83 °C, lit.[32] mp 83–84 °C; silica gel TLC R_f 0.30 (1:1 hexanes/ethyl acetate); ^1H NMR ($CDCl_3$) δ 2.15 (s, 3H), 3.71 (s, 3H), 3.75 (s, 3H), 5.14 (br s, 1H), and 9.01 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 22.4, 51.3, 51.9, 92.8, 164.1, 169.0, and 169.2.

4.1.9. Methyl 1,6-dimethyl-4-hydroxy-2,3-dihydro-1*H*-pyrrolo-[2,3-*b*]pyridine-5-carboxylate (13)

A solution containing 100 mg (0.57 mmol) of 2-(1-aminoethylidene)malonic acid dimethyl ester (**12**) and 168 mg (1.20 mmol) of 2,2-dimethoxy-1-methylpyrrolidine (**11**) in 2 mL of toluene was heated at reflux for 3 h, then cooled to 90 °C and treated with 160 mg (1.67 mmol) of sodium methoxide in one portion. The resulting reaction mixture was heated at 90 °C and stirred overnight. The cooled solution was treated with 5 mL of satd aq ammonium chloride and was then extracted with three 10-mL portions of ethyl acetate. The combined organic layer was washed with brine and dried ($MgSO_4$). The solvent was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 20:1 → 10:1 hexane/ethyl acetate gave **13** as a colorless solid: yield 115 mg (90%); mp 131–132 °C; silica gel TLC R_f 0.46 (4:1 hexanes/ethyl acetate); ^1H NMR ($CDCl_3$) δ 2.62 (s, 3H), 2.93 (t, 2H, *J* = 8.4 Hz), 2.98 (s, 3H), 3.55 (t, 2H, *J* = 9.0 Hz), 3.90 (s, 3H) and 11.79 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 22.5, 27.8, 31.9, 52.0, 52.4, 101.1, 102.2, 162.2, 163.8, 165.6 and 172.4; mass spectrum (ESI), *m*/*z* 223.1082 $(M+H)^+$ ($C_{11}H_{15}N_2O_3$ requires *m*/*z* 223.1083).

4.1.10. Methyl 1,6-dimethyl-4-(trifluoromethylsulfoxy)-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxylate (14)

To a solution of 74 mg (0.33 mmol) of methyl 1,6-dimethyl-4-hydroxy-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxylate (**13**) and 150 mg of potassium carbonate in 2.5 mL of dry DMF was added 180 mg (0.50 mmol) of *N*-phenyl-bis-trifluoromethanesulfonimide at room temperature. The reaction mixture was stirred at room temperature for 1.5 h then quenched by adding 5 mL of satd aq ammonium chloride. The reaction mixture was extracted with three 5-mL portions of ethyl acetate. The combined organic layer was washed with brine and dried ($MgSO_4$). The solvent was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 40:1 hexane/ethyl acetate gave **14** as a colorless solid: yield 110 mg (94%); mp 56–58 °C; silica gel TLC R_f 0.30 (1:1 hexanes/ethyl acetate); ^1H NMR ($CDCl_3$) δ 2.54 (s, 3H), 3.01 (s, 3H), 3.11 (t, 2H, *J* = 8.5 Hz), 3.62 (t, 2H, *J* = 8.0 Hz), and 3.85 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 23.5, 24.4, 31.8, 51.3, 52.0, 108.5, 110.0, 111.9, 149.3, 161.8, 164.9, and 165.6; mass spectrum (ESI), *m*/*z* 355.0566 $(M+H)^+$ ($C_{12}H_{14}N_2O_5SF_3$ requires *m*/*z* 355.0576).

4.1.11. 1,6-Dimethyl-5-(hydroxymethyl)-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-4-yl trifluoromethanesulfonate (15)

To a solution of 170 mg (0.48 mmol) of methyl ester **14** in 2 mL of dichloromethane was added dropwise 1.43 mL (1.43 mmol, 1 M in toluene) of DIBAL-H at −78 °C under Ar. The reaction mixture was stirred at −78 °C for 2 h then 5 mL of satd aq sodium potassium tartrate was added by syringe. The reaction mixture was allowed to warm to room temperature and stirred for an additional 2 h. After separation, the aqueous layer was extracted with three 5-mL portions of dichloromethane. The combined organic layer was washed with brine and dried over $MgSO_4$. The solvent was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 2:1 hexanes/ethyl acetate gave **15** as a colorless solid: yield 148 mg (95%); mp 67–70 °C (decomp.); silica gel TLC R_f 0.20 (1:1 hexanes/ethyl acetate); ^1H NMR ($CDCl_3$) δ 1.72 (br s, 1H), 2.52 (s, 3H), 2.96 (s, 3H), 3.08 (t, 2H, *J* = 8.4 Hz), 3.54 (t, 2H, *J* = 8.4 Hz), and 4.58 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 22.0, 24.0, 32.3, 51.8, 76.7, 111.5, 114.1, 120.0, 149.8, 159.9, and 164.7; mass spectrum (ESI), *m*/*z* 327.0622 $(M+H)^+$ ($C_{11}H_{14}N_2O_4SF_3$ requires *m*/*z* 327.0626).

4.1.12. 1,6-Dimethyl-5-formyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]-pyridin-4-yl trifluoromethanesulfonate (16)

To a solution of 134 mg (0.41 mmol) of alcohol **15** in 4 mL of DMSO and 4 mL of acetone was added 116 mg (0.41 mmol) of *o*-iodoxybenzoic acid (IBX). The reaction mixture was stirred at room temperature for 16 h, then cooled to 0 °C. Five milliliters of a 1:1 mixture of 5% aq $NaHCO_3$ and 5% aq $Na_2S_2O_3$ was added slowly by syringe. After stirring for an additional 20 min, the mixture was extracted with three 5-mL portions of ethyl acetate. The combined organic layer was washed with brine and dried over $MgSO_4$. The solvent was concentrated under diminished pressure, and the residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 10:1 hexanes/ethyl acetate gave **16** as colorless needles: yield 126 mg (95%); mp 96–98 °C (decomp.); silica gel TLC R_f 0.20 (4:1 hexanes/ethyl acetate); ^1H NMR ($CDCl_3$) δ 2.70 (s, 3H), 3.09 (s, 3H), 3.18 (t, 2H, *J* = 8.5 Hz), 3.72 (t, 2H, *J* = 8.5 Hz) and 10.09 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 23.2, 24.1, 31.5, 51.2, 112.3, 112.4, 120.0, 151.2, 165.5, 166.5, and 185.2; ^{19}F NMR ($CDCl_3$) δ −73.5 (s, 3F); mass spectrum (ESI), *m*/*z* 325.0465 $(M+H)^+$ ($C_{11}H_{12}N_2O_4SF_3$ requires *m*/*z* 325.0470).

4.1.13. 1,6-Dimethyl-4-(trifluoromethylsulfoxy)-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-5-yl formate (17)

To a solution of 80 mg (0.25 mmol) of aldehyde **16** in 3 mL of dichloromethane was added 1 mg of SeO_2 and 1.5 mL of aq 30% H_2O_2. The reaction mixture was stirred at room temperature for 16 h then cooled to 0 °C. Twelve milliliters of 5% aq $NaHCO_3$ was added slowly. The mixture was stirred for an additional 20 min then extracted with three 15-mL portions of ethyl acetate. The combined organic layer was washed with brine and dried over Na_2SO_4. The solvent was concentrated under diminished pressure, and the residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 10:1 hexanes/ethyl acetate afforded **17** as a light yellow solid: yield 47.6 mg (57%); mp 59–61 °C; silica gel TLC R_f 0.28 (4:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H), 2.94 (s, 3H), 3.08 (t, 2H, J = 8.4 Hz), 3.57 (t, 2H, J = 8.4 Hz), and 8.23 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 19.2, 23.7, 32.4, 52.1, 113.4, 116.8, 120.0, 126.7, 150.2, 158.2, and 162.8; ^{19}F NMR ($CDCl_3$) δ −73.8 (s, 3F); mass spectrum (ESI), m/z 340.0413 $(M)^+$ ($C_{11}H_{11}N_2O_5SF_3$ requires m/z 340.0419).

4.1.14. 4-((3*R*,7*R*,11*R*)-3-Benzoxy-3,7,11,15-tetramethylhexadec-1-ynyl)-1,6-dimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-5-yl formate (18)

To a solution of 28.0 mg (0.08 mmol) of **17** in 2 mL of *N*-methylpyrrolidin-2-one (NMP) was added 9.1 mg (0.008 mmol) of $Pd(PPh_3)_4$ and 66.0 mg (0.10 mmol) of **10**. The reaction mixture was heated at 105 °C and stirred for 2 h then cooled to 0 °C. Five milliliters of 5% aq $NaHCO_3$ was added slowly. The mixture was stirred at 0 °C for additional 20 min, and then extracted with three 8-mL portions of ethyl acetate. The combined organic layer was washed with brine and dried over Na_2SO_4. The solvent was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 10:1 hexanes/ethyl acetate gave **18** as a colorless oil: yield 42 mg (88%); $[\alpha]_D^{25}$ +21.1 (c 0.38, CH_3OH); silica gel TLC R_f 0.38 (4:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.84–0.89 (m, 12H), 1.05–1.83 (m, 21H), 1.56 (s, 3H), 2.47 (s, 3H), 2.92 (s, 3H), 2.96 (t, 2H, J = 6.4 Hz), 3.50 (t, 2H, J = 6.4 Hz), 4.65 (ABq, 2H, $\Delta\nu_{AB}$ = 24.3 Hz, J_{AB} = 11.5 Hz), 7.27–7.39 (m, 5H), and 8.16 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 19.1, 19.7, 19.7, 21.9, 22.6, 22.7, 24.5, 24.8, 25.5, 26.3, 28.0, 32.75, 32.77, 32.8, 37.1, 37.3, 37.5, 39.4, 41.9, 52.3, 66.5, 74.5, 101.0, 105.0, 106.4, 124.4, 127.4, 127.5, 128.3, 130.2, 135.2, 139.0, 146.4, and 159.2; mass spectrum (ESI), m/z 575.4244 $(M+H)^+$ ($C_{37}H_{55}N_2O_3$ requires m/z 575.4213).

4.1.15. 4-((3*R*,7*R*,11*R*)-3-Benzoxy-3,7,11,15-tetramethylhexadec-l-ynyl)-2,3-dihydro-1,6-dimethyl-1*H*-pyrrolo[2,3-*b*]pyridin-5-yl acetate (19)

To a solution of 25.0 mg (0.04 mmol) of formate ester **18** in 2 mL of CH_2Cl_2 was added 160 μL (0.16 mmol, 1 M in toluene) of DIBAL-H at −78 °C. The reaction mixture was stirred at −78 °C for 1.5 h, then 300 μL of triethylamine, 150 μL of acetic anhydride and a catalytic amount of *N*,*N*-dimethylaminopyridine were added. The reaction mixture was allowed to warm to room temperature and stirred for 24 h. The reaction was quenched with 2 mL of satd aq NH_4Cl and extracted with three 5-mL portions of ethyl acetate. The combined organic layer was washed with brine, dried (Na_2SO_4) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 50:3 hexanes/ethyl acetate gave **19** as a yellowish oil: yield 20 mg (78%); $[\alpha]_D^{25}$ +11.2 (c 0.20, CH_3OH); silica gel TLC R_f 0.48 (4:1 hexane/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.83–0.88 (m, 12H), 1.02–1.99 (m, 21H), 1.55 (s, 3H), 2.21 (s, 3H), 2.22 (s, 3H), 2.89 (s, 3H, J = 7.6 Hz), 2.94 (t, 2H, J = 8.0 Hz), 3.46 (t, 2H, J = 8.0 Hz), 4.76 (ABq, 2H, $\Delta\nu_{AB}$ = 21.2 Hz, J_{AB} = 10.4 Hz) and 7.24–7.36 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 19.0, 19.70, 19.74, 19.8, 20.5, 22.0, 22.65, 22.7, 24.5, 24.8, 25.5, 26.5, 28.0, 32.79, 32.83, 32.9, 37.2, 37.3, 37.5, 39.4, 42.0, 52.5, 66.5, 74.4, 78.0, 100.1, 120.9, 124.3, 127.4, 127.6, 128.3, 136.1, 139.0, 146.4, and 169.2; mass spectrum (ESI), m/z 589.4383 $(M+H)^+$ ($C_{38}H_{57}N_2O_3$ requires m/z 589.4369).

4.1.16. 1,6-Dimethyl-4-((3*R*,7*R*,11*R*)-3-hydroxy-3,7,11,15-tetramethylhexadecyl)-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-5-yl acetate (3)

To a solution of 25 mg (0.04 mmol) of acetate ester **19** in 5 mL of methanol was added 13 mg of Pearlman's catalyst. Hydrogen was bubbled through the stirred reaction mixture for 2 h, then the reaction mixture was maintained under a hydrogen atmosphere overnight. The reaction mixture was filtered through a Celite pad and the pad was washed with methanol. The solvent was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 5:1 hexanes/ethyl acetate gave **3** as a colorless oil: yield 15.7 mg (71%); $[\alpha]_D^{22}$ +17.5 (c 0.80, CH_3OH); silica gel TLC R_f 0.10 (4:1 hexane/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.83–0.87 (m, 12H), 1.00–1.56 (m, 23H), 1.20 (s, 3H), 2.20 (s, 3H), 2.30 (s, 3H), 2.38 (t, 2H, J = 7.2 Hz), 2.87 (t, 2H, J = 8.4 Hz), 2.89 (s, 3H) and 3.44 (t, 2H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 19.64, 19.67, 20.5, 21.4, 22.3, 22.6, 22.7, 24.3, 24.4, 24.7, 26.57, 26.60, 27.9, 29.6, 32.7, 33.2, 37.2, 37.36, 37.39, 37.5, 39.3, 40.1, 42.1, 52.8, 72.4, 118.9, 128.5, 136.3, 146.3, and 169.9; mass spectrum (ESI), m/z 503.4213 $(M+H)^+$ ($C_{31}H_{55}N_2O_3$ requires m/z 503.4207).

4.1.17. ((3*R*,7*R*,11*R*)-3-Hydroxy-3,7,11,15-tetramethylhexadecyl)-1,6-dimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-5-ol (2) and its trifluoroacetic acid salt

To a solution of 10.3 mg (20.5 μmol) of acetyl ester **3** in 2 mL of CH_2Cl_2 was added 84.0 μL (84.0 μmol, 1 M in toluene) of DIBAL-H at −78 °C. The reaction mixture was stirred at same temperature for 2 h then 2 mL of aq satd sodium potassium tartrate was added slowly. The reaction mixture was allowed to slowly warm to room temperature and stirred for 3 h, then the layers were separated. The aqueous layer was extracted with two 5-mL portions of ethyl acetate. The combined organic layer was washed with brine, dried (Na_2SO_4) and concentrated under diminished pressure to give **2** as an orange oil: yield 9.44 mg (100%). This product was analyzed by C_8 reversed-phase HPLC column (150 mm × 4.6 mm) using a gradient of methanol and water. Linear gradients were employed using 1:4 methanol/water → 4:1 methanol/water over a period of 20 min, and then 4:1 methanol/water → methanol over a period of 40 min, both at a flow rate of 1.5 mL/min (monitoring at 260 nm); compound **2** eluted at 21.8 min, and was judged to be >90% pure; mass spectrum (MALDI-TOF), m/z 461.4 $(M+H)^+$ (theoretical m/z 461.4). This compound decomposed after storage for one day at 25 °C.

Freshly prepared compound **2** was dissolved in 1 mL of ether and cooled to −78 °C. To this solution was added 0.3 mL of trifluoroacetic acid (0.1 M in ether solution). The mixture was stirred at −78 °C for 1 h, then the solvent was concentrated under diminished pressure. The residue was transferred in portions to small vials as a CH_3CN solution, cooled to −78 °C and lyophilized to give **2** as a trifluoroacetic acid salt: yield 11.5 mg (97%); $[\alpha]_D^{25}$ −0.86 (c 0.14, CH_3CN); ^{1}H NMR (CD_3CN) δ 0.89–0.92 (m, 12H), 1.03–1.53 (m, 19H), 1.20 (s, 3H), 1.53–1.61 (m, 1H), 1.67 (t, 2H, J = 8.0 Hz), 2.37 (s, 3H), 2.64 (t, 2H, J = 7.5 Hz), 3.09 (t, 2H, J = 8.5 Hz), 3.09 (s, 3H), 3.79 (t, 2H, J = 8.0 Hz) and 12.35 (br s, 1H); ^{13}C NMR (CD_3CN) δ 19.1, 21.2, 21.9, 21.9, 22.0, 24.1, 24.2, 24.5, 25.9, 27.8, 32.4, 32.5, 32.5, 37.0, 37.0, 37.1, 37.1, 37.3, 38.5, 39.1, 42.0, 53.2, 72.3, 125.8, 127.7, 140.4, 145.9, 152.1, 162.5, and 165.0; mass spectrum (ESI), m/z 461.4106 $(M+H)^+$ ($C_{29}H_{53}N_2O_2$ requires m/z 461.4107).

4.1.18. 4-((3*R*,7*R*,11*R*)-3-Benzoxy-3,7,11,15-tetramethylhexadec-1-ynyl)-1,6-dimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-5-carbaldehyde (20)

To a solution of 74 mg (0.23 mmol) of aldehyde **16** in 2 mL of DMF was added 26 mg (0.023 mmol) of $Pd(PPh_3)_4$, 4.3 mg (0.23 mmol) of CuI, 0.6 mL of *i*-Pr_2NEt and 136 mg (0.35 mmol) of **9**. The reaction mixture was stirred at 80 °C overnight. The cooled reaction mixture was treated with 5 mL of 1 N NaOH in satd aq NaCl. The mixture was extracted with three 8-mL portions of ether. The combined organic layer was washed with brine and dried over $MgSO_4$. The solvent was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 20:1 hexanes/ethyl acetate afforded **20** as a reddish oil: yield 122 mg (96%); $[\alpha]_D^{22}$ +28.0 (*c* 0.05, CH_3OH); silica gel TLC R_f 0.55 (4:1 hexanes/ethyl acetate); ^1H NMR ($CDCl_3$) δ 0.83–0.88 (m, 12H), 1.04–1.57 (m, 19H), 1.60 (s, 3H), 1.74–1.87 (m, 2H), 2.70 (s, 3H), 3.01 (t, 2H, *J* = 8.4 Hz), 3.04 (s, 3H), 3.62 (t, 2H, *J* = 8.4 Hz), 4.70 (q, 2H, *J* = 11.2 Hz), 7.25–7.38 (m, 5H), and 10.34 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 19.66, 19.69, 19.7, 22.6, 22.7, 24.5, 24.79, 24.87, 24.93, 26.4, 28.0, 31.4, 32.7, 32.8, 37.1, 37.3, 37.4, 37.5, 39.4, 41.9, 51.2, 66.6, 74.5, 79.1, 103.0, 117.7, 123.8, 127.4, 128.3, 128.4, 139.0, 163.04, 163.07, and 189.8; mass spectrum (ESI), *m*/*z* 559.4259 $(M+H)^+$ ($C_{37}H_{55}N_2O_2$ requires *m*/*z* 559.4264).

4.1.19. 4-((3*R*,7*R*,11*R*)-3-Benzoxy-3,7,11,15-tetramethylhexadec-1-ynyl)-1,6-dimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-5-carboxylic acid (21)

To a solution of 20 mg (0.036 mmol) of aldehyde **20** in 2 mL of dichloromethane was added 8 mg (77% purity; 0.036 mmol) of *m*-chloroperbenzoic acid and a catalytic amount of *p*-TsOH·H_2O. The reaction mixture was stirred at room temperature overnight. The cooled (0 °C) reaction mixture was treated slowly with 5 mL of a 1:1 mixture of 5% aq $NaHCO_3$ and 5% aq $Na_2S_2O_3$. After stirring for an additional 20 min, the mixture was extracted with three 5-mL portions of dichloromethane. The combined organic layer was washed with brine and dried over Na_2SO_4. The solvent was concentrated under diminished pressure to afford a residue, which was purified by preparative silica gel TLC. Development with 4:1 hexanes/ethyl acetate gave **21** as a colorless oil: yield 10 mg (49%); $[\alpha]_D^{25}$ −30.0 (*c* 0.04, $CHCl_3$); silica gel TLC R_f 0.47 (4:1 hexanes/ethyl acetate); ^1H NMR ($CDCl_3$) δ 0.83–0.88 (m, 12H), 1.03–1.61 (m, 19H), 1.64 (s, 3H), 1.79–1.98 (m, 2H), 2.73 (s, 3H), 2.80–3.20 (m, 4H), 3.00 (s, 3H), 4.70 (q, 2H, *J* = 6.8 Hz), 7.25–7.35 (m, 5H), and 10.55 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 19.62, 19.65, 19.7, 21.9, 22.6, 22.7, 24.1, 24.8, 25.2, 26.3, 28.0, 32.7, 32.8, 37.3, 37.5, 39.4, 41.9, 46.9, 54.1, 66.7, 74.6, 78.0, 108.1, 114.8, 124.0, 127.4, 127.5, 128.4, 137.4, 138.6, 158.7, 163.5, 190.90, and 190.94; mass spectrum (ESI), *m*/*z* 575.4210 $(M+H)^+$ ($C_{37}H_{55}N_2O_3$ requires *m*/*z* 575.4213).

4.2. Biochemical and biological evaluation

4.2.1. Lipid peroxidation assay

Peroxidation of isolated mitochondrial membrane can be estimated kinetically from the fluorescence quenching of incorporated *cis*-parinaric acid, as described previously.[45,46] Oxidation can be elicited by oxygen radicals generated at a constant rate in aqueous buffer by thermal decomposition of 10 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C. To measure *cis*-parinaric acid oxidation, bovine heart mitochondria isolated as described previously[47] were suspended (1 mg/mL) in 100 mM KCl and 10 mM Tris–HCl, pH 7.5, at 37 °C. Compound **2** or α-tocopherol (**1**) were preincubated where indicated, 1 μM *cis*-parinaric acid (Molecular Probes, Eugene, OR) was added and its fluorescence decay was monitored spectrofluorometrically (λ_{ex} 324 nm; λ_{em} 413 nm) after initiation with 10 mM AAPH.

4.2.2. Reactive oxygen species (ROS) assay

Intracellular ROS production was measured using the oxidant-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR). One milliliter (1×10^6 cells/mL) of CEM leukemia cells were plated in 12-well plates (RPMI with 10% fetal bovine serum (FBS), 2 mM glutamine and 1% penicillin–streptomycin mix antibiotics supplements), treated with the tested compounds (final concentration 5 μM), and incubated at 37 °C for 1 h in a humidified atmosphere of 5% CO_2 in air. Cells were treated with 5 mM diethyl maleate for 30 min and collected by centrifugation at 300*g* for 3 min, then washed twice with phosphate-buffered saline (PBS). The cells were resuspended in Hanks' Balanced Salt Solution buffer and incubated at 37 °C in the dark for 20 min with 5 μM DCFH-DA. The cells were collected by centrifugation at 300*g* for 3 min and then washed twice with phosphate-buffered saline. The samples were analyzed immediately by flow cytometry (FACS Caliber instrument, Becton–Dickinson; Cell Quest software, BD Biosciences) using 488 nm excitation laser and FL1-H channel 538 nm emission filter. In each analysis, 10,000 events were recorded. The results obtained were verified by repeating the experiments in triplicate. For fluorescence microscopy analysis, the cells were treated as described above and transferred to culture dishes (glass bottom SensoPlate™, 24 well, Greiner Bio-One) and analyzed with a Zeiss Axiovert 200 M inverted microscope, equipped with a 40× oil immersion objective and AxioCam MR. The generation of reactive oxygen species was detected as a result of the oxidation of DCFH (λ_{ex} 488 nm; λ_{em} 515–540 nm).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.030. These data include MOL files and InChiKeys of the most important compounds described in this article.

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