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Simplified bicyclic pyridinol analogues protect mitochondrial function

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ABSTRACT

Bicyclic pyridinol antioxidants have been reported to suppress the autoxidation of methyl linoleate more effectively than α-tocopherol in benzene solution. A few novel lipophilic analogues have recently been synthesized by conjugating a pyridinol core with the phytyl side chain of α-tocopherol; these have been shown to possess potent antioxidant activity. However, the complexity of the synthetic routes has hampered their further development. Herein, we describe a facile approach, involving only five synthetic steps, to simplified analogues (**1a**–**1c**) and their acetate ester precursors (**2a**–**2c**). Simple alkyl chains of different lengths were attached to the 6-methyl group of the antioxidant core via regioselective metalation. These analogues were found to retain biological activity and exhibit protective behaviour under conditions of induced oxidative stress, which could lead to the development of more readily accessible analogues as potential antioxidants capable of preserving mitochondrial function.

1. Introduction

In aerobic organisms, oxygen is an indispensable element for life. When cells use oxygen as part of the process of ATP generation in mitochondria, reactive oxygen species (ROS) are inevitably created.[1,2] The uncontrolled accumulation of ROS physiologically results in a phenomenon termed oxidative stress, which is linked to many human degenerative ailments, such as aging, cancer and neurodegenerative diseases.[3–7] Mitochondria are recognized as one of the key targets for the toxic effects of ROS.[8] These oxidizing radicals can disrupt the mitochondrial electron transport chain, diminish ATP synthesis, and thereby impede energy metabolism in cells.[9] Natural lipophilic antioxidants, such as ubiquinol and vitamin E, have attracted increasing attention recently as potential agents to prevent free radical damage of mitochondria. In particular, α-tocopherol (α-TOH), a major component of vitamin E, is a chain-breaking antioxidant which quenches peroxyl radicals by transferring a phenolic hydrogen atom.[10] Extensive effort has also been expended in seeking synthetic antioxidants with activity superior to α-TOH.

Recently, compounds **8** and **9** have been reported to be highly effective antioxidants, exhibiting 88-fold and 28-fold greater potency, respectively, than α-TOH in suppressing the autoxidation of methyl linoleate (Fig. 1).[11,12] These novel antioxidants have been incorporated into more lipophilic species by conjugating the pyridinol core structures with the phytyl side chain of α-TOH (e.g., compounds **10**, **11** and **12**).[13–17] The lipophilic phytyl group in α-TOH is believed to facilitate cellular uptake and to provide an anchor within membranes, thereby conferring protection from oxidative stress near the surface of the cellular membranes.[16–19] It was shown by Burton and Ingold that removal of phytyl side chain did not diminish the in vitro antioxidant potential of α-tocopherol but completely abolished antioxidant function in vivo.[20] Although these pyridinol-based α-TOH analogues have promising biological profiles,[13–17] their reported syntheses have not been straightforward; all have required 15 or more steps. The lengthy syntheses represent a limitation to the further development of these analogues. Thus, the design of simpler and more readily available analogues which retain biological activity has become an important goal.

In a recent report, we described a short and scalable route to synthesize bicyclic pyridinol antioxidants **8** and **9**.[21] The novel feature of this synthesis is the preparation of the bicyclic structures by a cyclocondensation reaction of a lactam acetal with an enaminone (Scheme 1). Based on this strategy, a lipophilic side chain can readily be introduced onto the pyridinol core in a subsequent step via regioselective metalation of the 6-methyl group. The function of the phytyl group is thought to be related to its lipophilicity;[16,18,22] however; synthetic incorporation of this side chain requires multiple steps due to its three chiral centers. Accordingly, we chose to replace the phytyl group with simple linear alkyl chains of varying length, to assess their ability to mimic the function of the phytyl chain. Presently, we describe the synthesis of three simplified α-TOH analogues (**1a**–**1c**) and their corresponding acetate esters (**2a**–**2c**) (Fig. 2); these have been prepared in five and four steps, respectively, and also evaluated for their antioxidant activities. These analogues were found to retain the ability to suppress ROS levels in cultured cells, and to quench the peroxidation of mitochondrial membranes.

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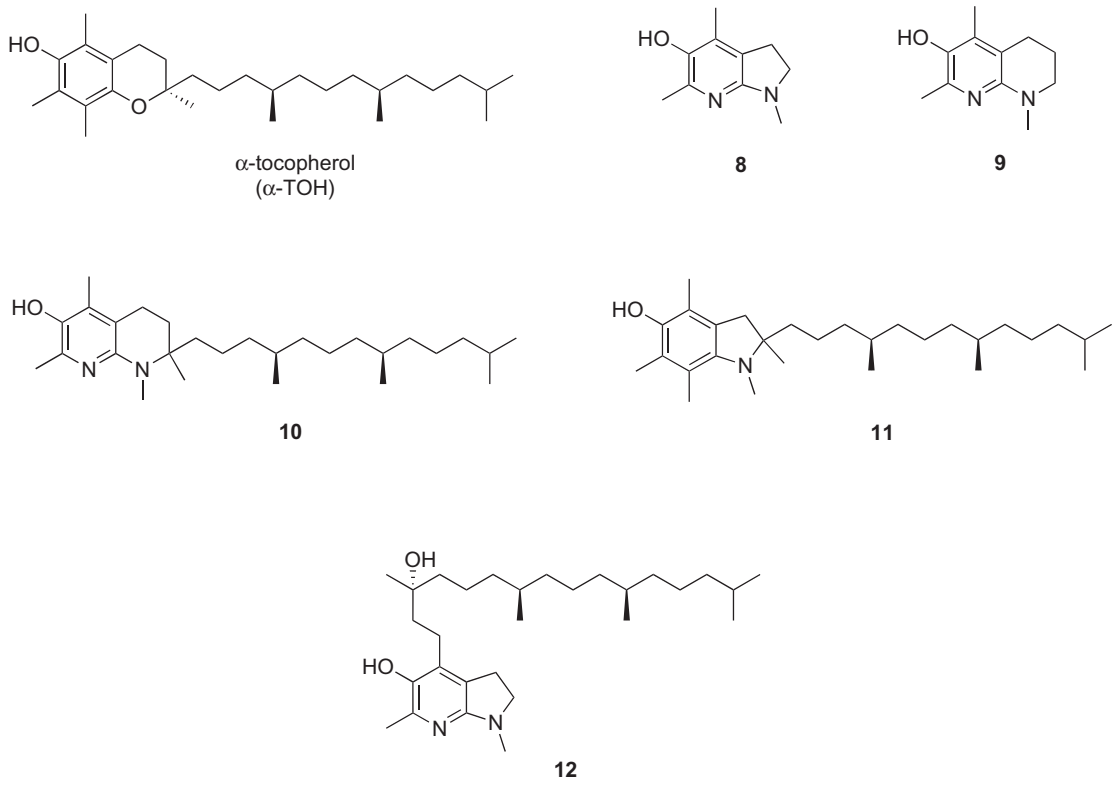


Figure 1. Structures of α-tocopherol, bicyclic pyridinol antioxidants (**8** and **9**) and recently reported aminopyridinol analogues (**10**–**12**).

2. Results and discussion

2.1. Synthesis of novel pyridinol analogues

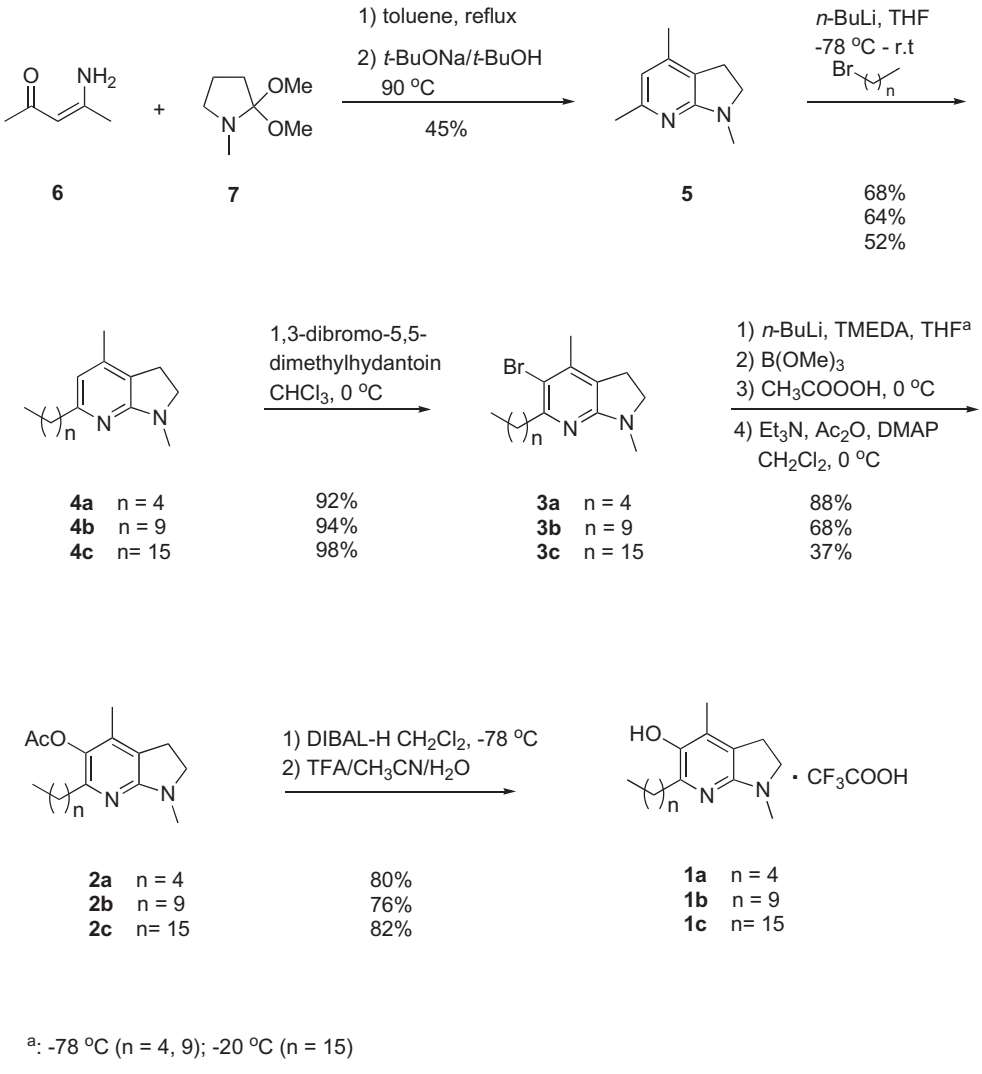
Our goal was to develop simplified and easily accessible analogues of α-tocopherol bearing a lipophilic side chain on an appropriate antioxidant core. All of the analogues were designed to possess a pyridinol structure with a fused five-membered ring due to its superior radical-scavenging ability relative to the phenol ring.[11,12,23,24] α-TOH may be regarded as having a modular structure, in which the phenolic group is responsible for its antioxidant activity, while the phytyl side chain is responsible for anchoring the molecule to cellular membranes. This led us to design analogues having simpler lipophilic side chains attached to a synthetically more accessible position on the pyridine ring. Since the 6-methyl group can be selectively metalated, the introduction of an alkyl chain to this position seemed feasible. This coupling strategy is more straightforward than previous approaches, which involved the functionalization of the pyridine ring either with an aldehyde group followed by a Wittig reaction,[13–15] or with a triflate group followed by a metal-catalyzed cross coupling reaction.[16]

A retrosynthetic analysis, shown in Figure 3, suggested that the target compounds could be prepared from bicyclic aminopyridine **5** by functional group transformations. Conjugation of alkyl side chains to compound **5** was envisioned by initial metalation of the 6-methyl group, followed by a subsequent nucleophilic substitution reaction on alkyl bromides. Based on the synthetic studies described previously,[21] compound **5** could be constructed by a cyclocondensation reaction of enaminone **6** and amide acetal **7**.

The synthesis of the desired antioxidants **1a**–**1c** and **2a**–**2c** is illustrated in Scheme 1. Accordingly, 1,4,6-trimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridine (**5**) was prepared in 45% yield by the cyclocondensation of enaminone **6**[25] and amide acetal **7**.[26] Aliphatic side chains were introduced by regioselective metalation of the 6-methyl group of azaindoline **5** in the presence of *n*-BuLi, followed by treatment with 1-bromobutane to afford **4a** in 68% yield. In similar fashion, treatment with 1-bromononane afforded **4b** in 64% yield and treatment with 1-bromopentadecane gave **4c** in 52% yield. Azaindolines **4a**, **4b** and **4c** were brominated using 1,3-dibromo-5,5-dimethylhydantoin in chloroform at 0 °C to afford the brominated azaindolines **3a**, **3b** and **3c** in 92%, 94% and 98% yields, respectively. Bromides **3a**, **3b** and **3c** were hydroxylated using *n*-BuLi and tetramethylethylenediamine followed by treatment with trimethoxy boron and finally with peracetic acid. Due to the instability of the pyridinols toward air oxidation, we protected the phenolic hydroxyl groups as their acetate esters **2a**–**2c**. The reactions were carried out in the presence of NEt_3, DMAP followed by the addition of Ac_2O at 0 °C and afforded **2a**, **2b** and **2c** in 88%, 68% and 37% yields, respectively. This strategy would permit the easy scale-up and storage of the acetate ester intermediates prior to conversion to the more reactive pyridinol products **1a**–**1c**. Additionally, the acetate esters could potentially act as prodrugs for the corresponding pyridinols, as we have demonstrated for compound **12**.[17] The removal of the acetyl group was achieved by treatment with DIBAL-H in CH_2Cl_2 at −78 °C. Due to their sensitivity toward oxidation, the final products **1a**, **1b** and **1c** were converted to their trifluoroacetate salts. Compounds **1a**, **1b** and **1c** were further purified by reversed-phase HPLC prior to bioassay, and were tested as their trifluoroacetate salts. Although not intended to support a detailed study of the relationship between structure and function, it was felt that the bicyclic pyridinol analogues having three different side chain lengths would provide an initial indication of the structural requirements for protection of mitochondrial function.

2.2. Biochemical and biological evaluation

Significant efforts have been devoted to the design of synthetic tocopherol analogues with the aims of increasing activity and stability, and also to achieve better insight into the structure–activity



[a]: -78 °C (n = 4, 9); -20 °C (n = 15)

Scheme 1. Synthetic route employed for the synthesis of analogues **1a–c** and **2a–c**.

relationships for such molecules.[13,15–17,27–30] In this study, a simplified synthesis of pyridinol-based tocopherol analogues possessing a modified redox core and a simple aliphatic side chain has been achieved. The protective effect of these tocopherol analogues against oxidative stress-mediated toxicity were investigated and compared with that of α-TOH in cultured mammalian leukemia cells. The thiol-reactive compound diethyl maleate (DEM) was used to deplete glutathione pharmacologically resulting in the production of ROS and lipid peroxidation in CEM leukemia cells.[31–34] While diethyl maleate induces greater oxidative stress than would normally be encountered physiologically, it was felt that compounds capable of mitigating the effects of DEM would likely function well under conditions of oxidative stress more typical of those that occur physiologically.

Cellular ROS production was determined in glutathione depleted CEM leukemia cells by monitoring the fluorescence derived from the oxidant-sensitive probe dichlorodihydrofluorescein diacetate (DCFH-DA)[35] as described previously.[17,31–33] DCFH-DA is a cell permeable probe that readily diffuses into the cell and is cleaved intracellularly by nonspecific esterases to form the polar, non-fluorescent derivative DCFH, which is further oxidized by ROS to form the fluorescent compound DCF (dichlorofluorescein).[35] Increased DCF fluorescence in CEM cells depleted of glutathione is analyzed by flow cytometry. Figure 4 shows representative DCF overlay of FACS histograms of CEM cells stained with DCFH-DA and analyzed as described in the experimental section. DEM treatment caused the DCF fluorescence (FL1-H) to shift right on the *x*-axis of the FACS histogram, indicating increased ROS production as a result of glutathione depletion.[34] Pretreatment of CEM cells with analogues **1b** and its acetate **2b** afforded significantly better protection than α–tocopherol, while **1a** and **2a** afforded poor protection and **1c** and **2c** gave intermediate protection. The results clearly show a significant decrease in the intensity of DCF fluorescence in a dose-dependent manner for the analogues having a simple aliphatic side chain with 10 carbon atoms (compounds **1b** and **2b**). While the shorter side chain having a five-carbon length (compounds **1a** and **2a)** showed little protection against ROS, the analogues having a linear 16-carbon side chain afforded protection almost comparable to that of α-TOH itself (Fig. 4). While the foregoing assay has been used extensively to measure ROS, it has been criticized for lack of specificity under certain experimental conditions.[36,37] Accordingly, in addition to the data shown in Figure 4, we also demonstrated that the increase in DCF fluorescence resulting from treatment with DEM was completely reversed by treatment with superoxide dismutase + catalase (not shown).[38,39] The antioxidant *N*-acetylcysteine (5 mM) also reversed the effects of DEM. This established that the effects in Figure 4 were due to hydrogen peroxide and superoxide.

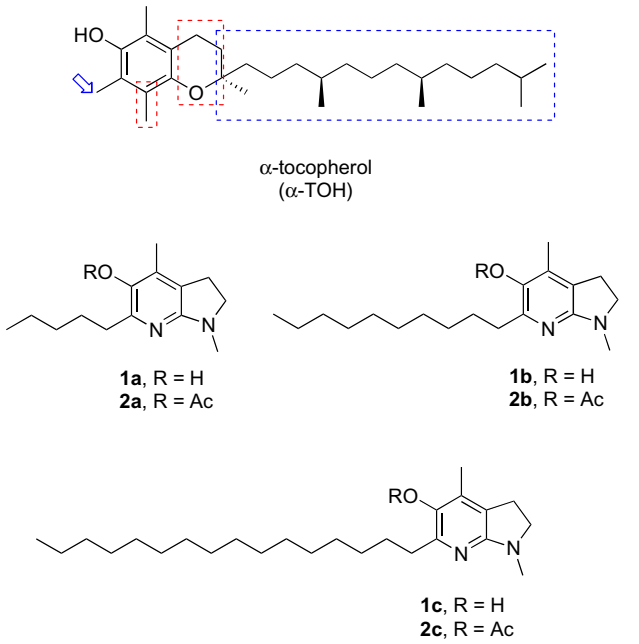


Figure 2. α-Tocopherol with areas targeted for modification (in boxes and arrow) and simplified bicyclic pyridinol antioxidants (**1a**–**1c**) and their acetate ester precursors (**2a**–**2c**).

Lipid peroxidation was determined in CEM leukemia cells by monitoring the fluorescence of the peroxidation-sensitive fatty acid-conjugated dye C_{11}-BODIPY$^{581/591}$, which incorporates into membranes and is useful for assessing membrane oxidation since its oxidized and reduced forms fluoresce at different wavelengths.[40,41] Once oxidized, both the absorption and emission maxima of the probe are shifted with good spectral separation of the non-oxidized (red fluorescent) and oxidized (green fluorescent) forms that can be quantitatively measured fluorometrically. Increased C_{11}-BODIPY$^{581/591}$—green (oxidized) fluorescence was assessed using FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA). Figure 5 shows representative C_{11}-BODIPY$^{581/591}$—green (oxidized) FACS histograms overlay of CEM leukemia cells stained with BODIPY$^{581/591}$—red (reduced) and analyzed using the FL1-H channel, as described in the experimental section. DEM treatment caused the BODIPY$^{581/591}$—green fluorescence to shift right on the *x*-axis of the FACS histogram, indicating increased membrane peroxidation as a result of glutathione depletion.[17] Pretreatment of CEM cells with the synthesized α-tocopherol analogue **1b** afforded significantly better protection than was achieved with α-tocopherol, while **2b** gave results comparable to α-TOH. Weaker protection was observed for compounds **1c** and **2c**, and no protection was obtained using compounds **1a** and **2a**. These findings indicate the importance of side chain length, where a simple 10 carbon side chain acts as an excellent anchor to membranes and produces more efficient protection than α-TOH.

In normally functioning mitochondria, oxidative phosphorylation maintains an electrochemical proton gradient and, therefore, a mitochondrial membrane potential ($\Delta\psi_m$), which drives ATP synthesis. Reduced $\Delta\psi_m$ can result from increased mitochondrial inner membrane permeabilization and uncouplers of oxidative phosphorylation.[42] Previously, we have successfully used TMRM to monitor mitochondrial function during DEM-induced oxidative stress in CEM leukemia cells.[17,43] The effect of these pyridinol-based tocopherol analogues on mitochondrial membrane integrity and depolarization caused by treatment of CEM leukemia cells with DEM was assessed by measuring mitochondrial membrane potential ($\Delta\psi_m$) using tetramethylrhodamine methyl ester (TMRM) probe as described previously.[17,43] TMRM, a fluorescent cationic indicator, accumulates across polarized mitochondrial membranes with a Nernstian distribution in a potential-dependent manner, and the opening of the mitochondrial permeability transition pore and depolarization leads to diminished labeling of the mitochondria by the fluoroprobe.[44] Figure 6 (panel a) illustrates representative two-dimensional density dot plots of TMRM-stained cells showing the percentage of cells with intact $\Delta\psi_m$ (TMRM fluorescence in top right quadrant) vs the percentage of cells with depolarized $\Delta\psi_m$ (TMRM fluorescence in bottom left and right quadrants). Figure 6 (panel b) shows a bar graph of the percentage (mean ± SE) of CEM cells with intact $\Delta\psi_m$. The results show that DEM treatment reduced the percentage of cells with TMRM fluorescence in the top right quadrant, indicating that DEM treatment caused loss of $\Delta\psi_m$. Pretreatment of the cells for 16 h (panel a) with RPMI medium (untreated control), pyridinol analogues **1a**–**1c**, and their esters **2a**–**2c** prevented the loss of $\Delta\psi_m$ induced by DEM. Once again $\Delta\psi_m$ protection was dose-dependent and compound **1b** and its ester (**2b**) were the most effective in preventing mitochondrial depolarization, consistent with the ROS and lipid peroxidation results. Again, the analogues with shorter side chains (**1a** and **2a**) and longer side chains (**1c** and **2c**) were less effective in preventing mitochondrial depolarization under oxidative stress (Fig. 6). While the limited number of

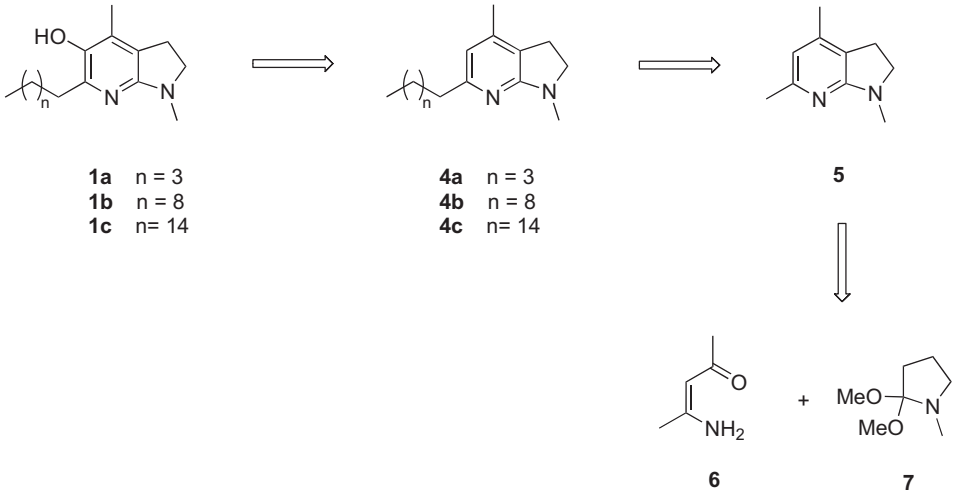


Figure 3. Retrosynthetic analysis of the simplified bicyclic pyridinol analogues.

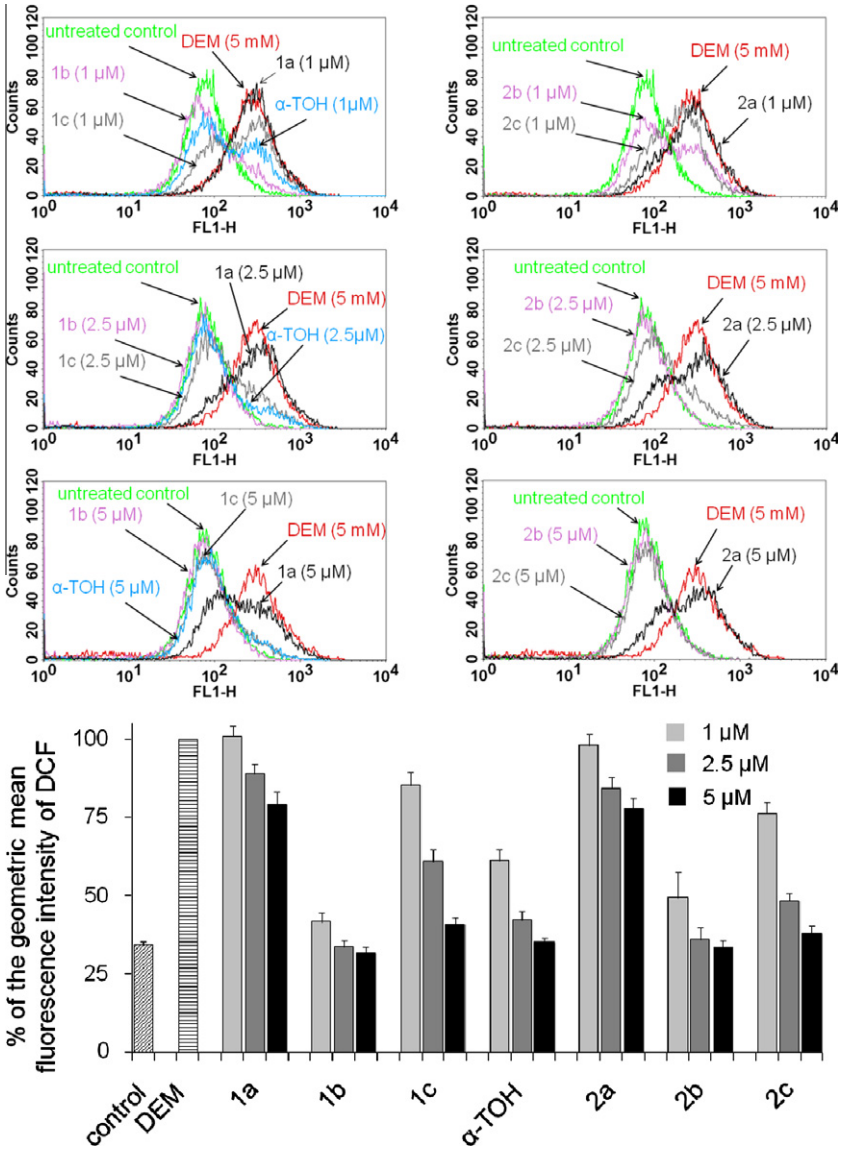


Figure 4. Representative flow cytometric histograms overlay showing ROS production in CEM cells. Following pretreatment with the indicated compounds (1, 2.5 and 5 μM) for 16 h, the cells were treated with 5 mM diethyl maleate (DEM) for 50 min to deplete glutathione. The cells were washed in phosphate-buffered saline, and suspended in phosphate-buffered saline containing 20 mM glucose. Cells were loaded with 10 μM dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min, and the green fluorescence (DCF) was measured by flow cytometry using the FL1-H channel. The figure shows a representative example of three independent experiments. A total of 10,000 events were recorded for each sample and analyzed with the CellQuest software (BD Biosciences). 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. The bottom panel shows a bar graph of the percentage of the geometric mean fluorescence intensity (GMFI) of DCF fluorescence relative to a DEM-treated control. Data are expressed as the mean ± SEM ($n = 3$).

analogues employed does not define the side chain optimal for preventing mitochondrial depolarization, it does clearly establish side chain length dependency.

The effect of the test compounds on the viability of DEM-treated CEM leukemia cells was assessed by the trypan blue exclusion method,[45] as presented in Figure 7. Glutathione depletion caused significant reduction of cell viability after 5.5 h of DEM treatment. Pretreatment of cells with α-tocopherol and the pyridinol based analogues significantly reduced cell death. Compound **1b** and its ester form **2b** were more effective in cytoprotection than was α-TOH (Fig. 7). With the exception of **1c**, all of the analogues afforded significant cytoprotection at 5 μM concentration, and varying levels of protection when used at 1 μM concentration.

The results of this study strongly suggest that the antioxidant efficiency of bicyclic pyridinol analogues can be improved by designing analogues that possess a simpler side chain than recently described analogues.[13–17] The best derivatives among those investigated in the present study were those having a 10-carbon linear side chain (**1b** and **2b**). It is important to note that the activity of α-TOH in animals must reflect the action of α-tocopherol transfer protein, which is responsible for the retention of α-TOH from dietary vitamin E.[46] Since it does not seem likely that the bicyclic pyridinol analogues described here will be substrates for this protein, the bicyclic pyridinols may well compare less favorably with α-TOH in vivo.

3. Conclusions

A series of simplified bicyclic pyridinol analogues (**1a**–**1c**) and their corresponding acetate esters (**2a**–**2c**) have been prepared in five and four steps, respectively. Despite significant simplification, including the relocation of the side chain and the removal of all asymmetric centers, the compounds retain high efficiency in quenching lipid peroxidation and suppressing reactive oxygen spe-

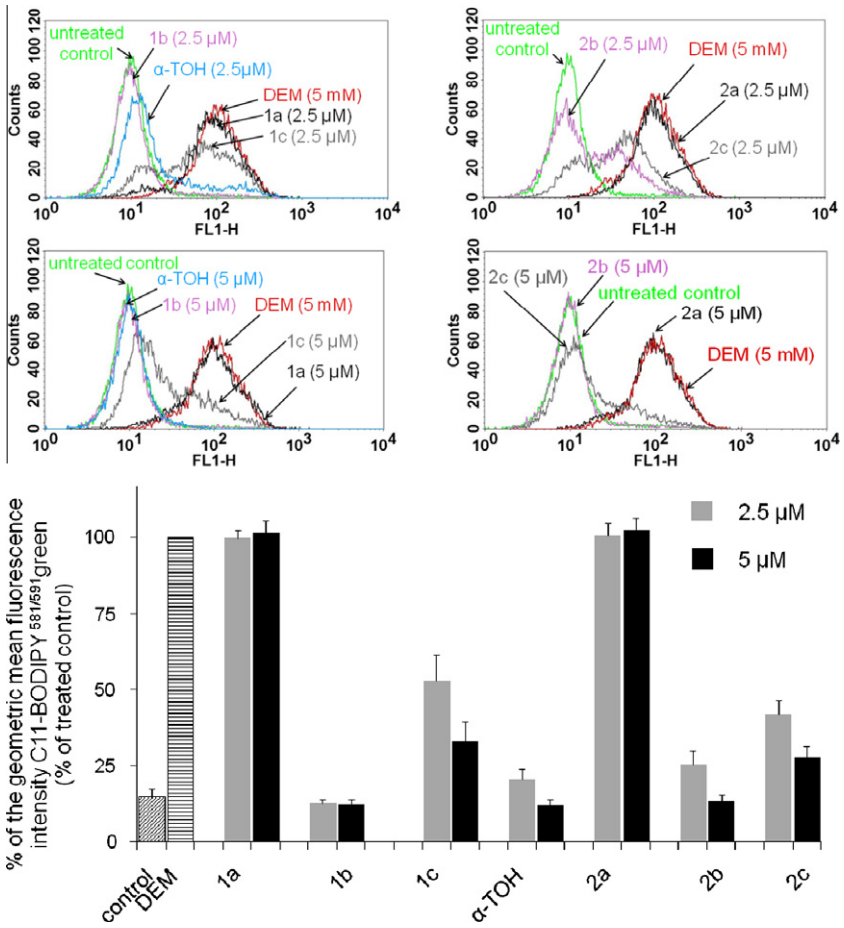


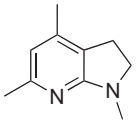
Figure 5. Lipid peroxidation in CEM leukemia cells depleted of glutathione was detected by utilizing the oxidation-sensitive fatty acid probe C_{11}-BODIPY$^{581/591}$ using flow cytometry. Increased C_{11}-BODIPY-green fluorescence (oxidized form), a measure of intracellular lipid peroxidation, was determined by increasing the geometric mean fluorescence intensity (GMFI) of C_{11}-BODIPY-green relative to the untreated control. A bar graph representing the percentage of the geometric mean fluorescence intensity (GMFI) of C_{11}-BODIPY-green fluorescence relative to a treated control is shown. Data are expressed as the mean ± SEM (n = 3).

cies in cells under oxidative stress. As anticipated, these properties were strongly dependent on the length of the introduced side chain. These results further confirm that the bicyclic pyridinol structure can act as a highly effective antioxidant core. Analogues bearing a lipophilic side chain at the 6-position still exhibited antioxidant behavior. Further, at least for those protective functions studied here, it appears that the phytyl side chain can be replaced by a simple alkyl group. Analogue **1b** and its ester (**2b**) were found to strongly diminish ROS levels, quench lipid peroxidation, preserve mitochondrial membrane potential ($\Delta\psi_m$) and prevent cell death in cultured cells placed under oxidative stress. These findings should contribute to the development of synthetically more accessible antioxidants as potential therapeutic agents.

4. Experimental section

4.1. Chemistry

Chemicals and solvents were of reagent grade and were used without further purification. All reactions involving air or moisture-sensitive reagents or intermediates were performed under an argon atmosphere. Flash chromatography was carried out using Silicycle 200–400 mesh silica gel. Analytical TLC was carried out using 0.25 mm EM silica gel 60 F_{250} plates that were visualized by irradiation (254 nm) or by staining with *p*-anisaldehyde stain. HPLC separations were performed on a Waters 600 series HPLC multi-solvent delivery system using a Kratos 747 UV detector. ^{1}H and ^{13}C NMR spectra were obtained using Inova 400 or 500 MHz Varian instruments. 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 ($CDCl_3$, 77.0 ppm). Splitting patterns were designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were obtained in the Arizona State University CLAS High Resolution Mass Spectrometry Laboratory.



4.1.1. 1,4,6-Trimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridine (5)[47]

To a solution of 2.36 g (23.8 mmol) of enaminone **6** in 8 mL of toluene was added 5.88 g (40.5 mmol) of lactam acetal **7**. The reaction mixture was heated at reflux and stirred for 2 h, cooled to 90 °C, and then treated with 4.65 g (48.4 mmol) of *t*-BuONa and 4 mL of *t*-BuOH. The reaction mixture was stirred at 90 °C for another 16 h. The cooled reaction mixture was quenched by the addition of 20 mL of satd aq NH_4Cl. The mixture was extracted with EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue

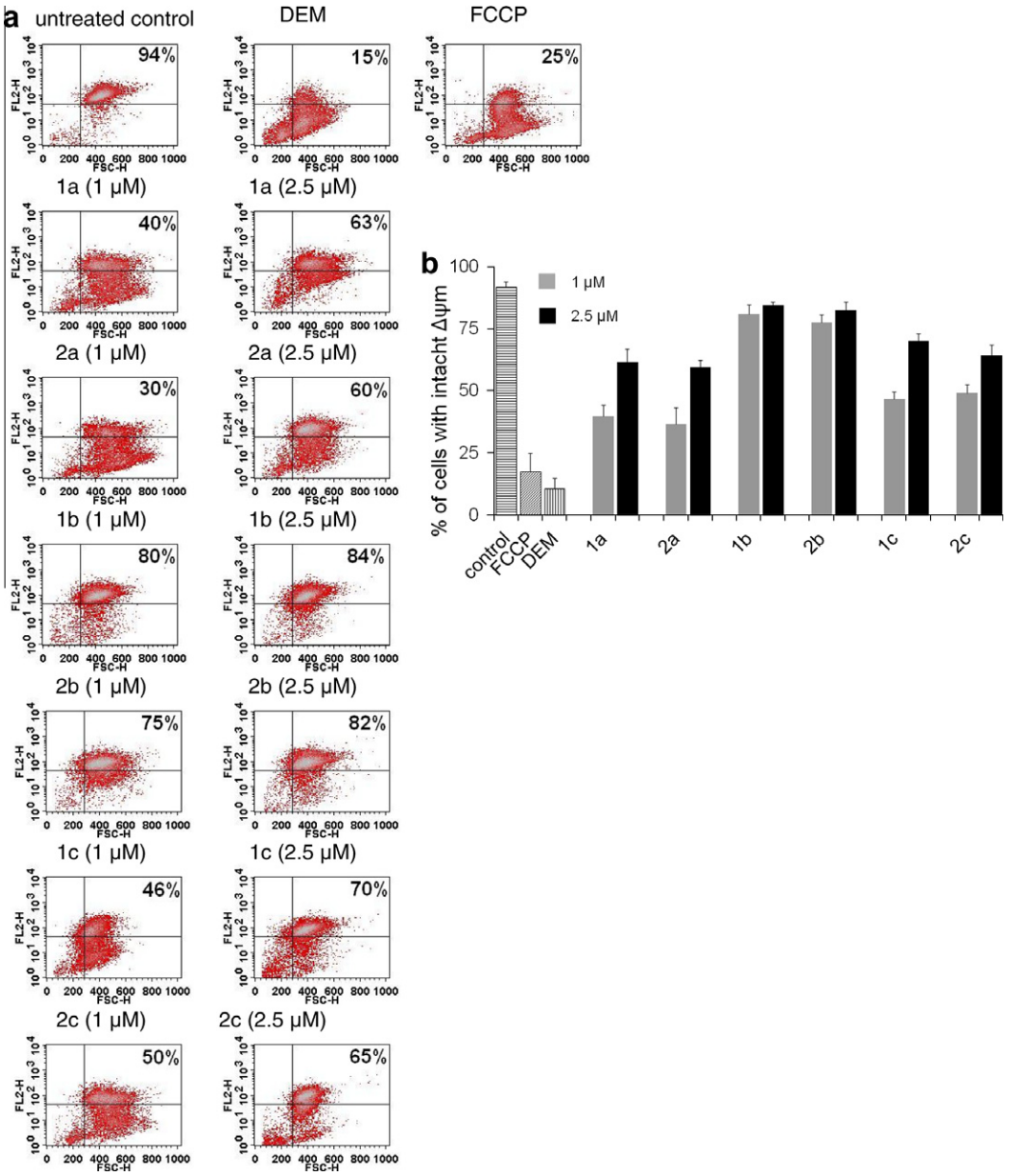


Figure 6. Panel a. Representative flow cytometric two-dimensional color density dot plot analyses of mitochondrial membrane potential ($\Delta\Psi_m$) in leukemia CEM cells stained with 250 nM TMRM and analyzed using the FL2-H channel as described in the experimental section. The percentage of cells with intact $\Delta\Psi_m$ is indicated in the top right quadrant of captions. Representative example from at least three independent experiments is shown. A total of 10,000 events were recorded for each sample and analyzed with the CellQuest software (BD Biosciences). Panel b shows a bar graph of the means of the percentage of cells with intact $\Delta\Psi_m$ recorded by FACS. Data are expressed as means ± SEM of three independent experiments run in duplicate.

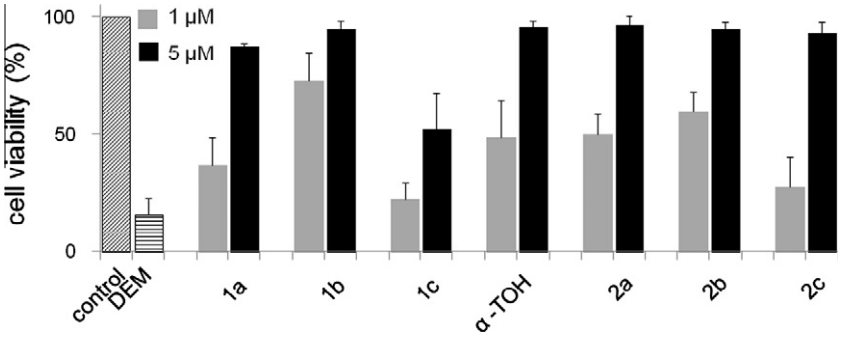
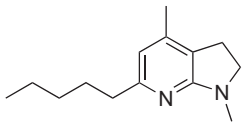


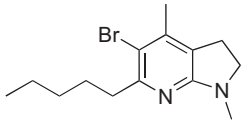
Figure 7. Trypan blue exclusion assay of CEM cells treated with test compounds for 16 h before exposure to 5 mM diethyl maleate for 5.5 h. At the time of assay, >80% of the DEM-treated cells were trypan blue positive, whereas in the non-DEM treated control, >95% of the cells were viable. Cell viability was expressed as the percentage of control. Results are an average of three independents trials.

was purified by flash chromatography on a silica gel column (25 × 2.6 cm). Elution with 10:1 hexanes/ethyl acetate gave the product **5** as a yellow oil: yield 1.60 g (42%); silica gel TLC R_f 0.15 (4:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H), 2.32 (s, 3H), 2.76 (t, 2H, J = 8.4 Hz), 2.86 (s, 3H), 3.34 (t, 2H, J = 8.4 Hz) and 6.10 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 17.8, 24.0, 24.3, 33.1, 52.4, 113.1, 118.1, 141.2, 154.5 and 163.7; mass spectrum, m/z 162.1 (M^+) (theoretical 162.1).



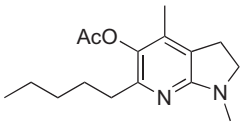
4.1.2. 1,4-Dimethyl-6-pentyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*] pyridine (4a)

To a solution of 225 mg (1.39 mmol) of compound **5** in 4 mL of THF was added 1.39 mL (2.22 mmol, 1.6 M in hexanes) of *n*-BuLi followed by 157 μL (1.46 mmol) of 1-bromobutane at −78 °C. The reaction mixture was slowly warmed to room temperature and stirred for another 16 h. The reaction mixture was quenched by the addition of 10 mL of satd aq NH_4Cl at 0 °C. The mixture was extracted with EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) 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 acetate gave the product **4a** as a yellow oil: yield 0.20 g (66%); silica gel TLC R_f 0.15 (8:1:1 hexanes/ethyl acetate/MeOH); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 7.2 Hz), 1.30–1.36 (m, 4H), 1.66 (quint, 2H, J = 7.6 Hz), 2.08 (s, 3H), 2.55 (t, 2H, J = 8.0 Hz), 2.81 (t, 2H, J = 8.0 Hz), 2.88 (s, 3H), 3.38 (t, 2H, J = 8.0 Hz) and 6.13 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 18.0, 22.6, 24.4, 29.6, 31.8, 33.3, 38.0, 52.5, 112.4, 118.2, 141.1, 159.1 and 163.7; mass spectrum, m/z 218.2 (M^+) (theoretical 218.3); mass spectrum (APCI), m/z 219.1864 $(M+H)^+$ ($C_{14}H_{23}N_2$ requires 219.1861).



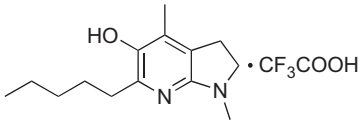
4.1.3. 5-Bromo-1,4-dimethyl-6-pentyl-2,3-dihydro-1*H*-pyrrolo [2,3-*b*]pyridine (3a)

To a solution of 108 mg (0.50 mmol) of **4a** in 3 mL of chloroform was added 70.7 mg (0.25 mmol) of 1,3-dibromo-5,5-dimethylhydantoin in portions at 0 °C. The reaction mixture was stirred at 0 °C for 30 min. The reaction was quenched by the addition of 5 mL of satd aq $NaHCO_3$. The mixture was extracted with chloroform and the combined organic layer was washed with brine and dried over $MgSO_4$. The concentrated residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 100:1 hexanes/ethyl acetate gave **3a** as a yellow oil: yield 136 mg (92%); silica gel TLC R_f 0.65 (8:1:1 hexanes/ethyl acetate/methanol); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 3H, J = 7.2 Hz), 1.33–1.38 (m, 4H), 1.66 (quint, 2H, J = 7.6 Hz), 2.16 (s, 3H), 2.77 (t, 2H, J = 8.0 Hz), 2.82–2.87 (m, 5H) and 3.40 (t, 2H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1, 19.5, 22.6, 25.2, 28.4, 31.8, 33.0, 38.1, 52.4, 110.9, 120.4, 141.1, 156.8 and 161.8; mass spectrum, m/z 297.1 and 299.1 $(M+H)^+$ (theoretical 297.1 and 299.1); mass spectrum (APCI), m/z 297.0968 and 299.0937 $(M+H)^+$ ($C_{14}H_{22}N_2Br$ requires 297.0966 and 299.0946).



4.1.4. 1,4-Dimethyl-6-pentyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*] pyridinyl acetate (2a)

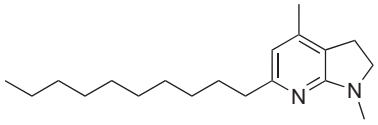
To a solution of 33.0 mg (0.11 mmol) of **3a** in 1 mL of THF was added 16.5 μL (0.11 mmol) of tetramethylethylenediamine (TMEDA) at −78 °C followed by 139 μL (0.22 mmol, 1.6 M in hexanes) of *n*-BuLi. After 30 min, 27.2 μL (0.24 mmol) of trimethoxy boron was added and the resulting mixture was stirred for another 1 h. To the reaction mixture was added slowly 51.4 μL (0.24 mmol, 32 wt%) of peracetic acid and the solution was then warmed to 0 °C over a period of 30 min. The mixture was diluted with 5 mL of H_2O and extracted with three 5-mL portions of EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) and the solvent was concentrated under diminished pressure. The resulting oil was dissolved in 2 mL of CH_2Cl_2 at 0 °C, followed by the addition of 92.8 μL (0.66 mmol) of triethylamine, 1.40 mg (0.01 mmol) of DMAP and 31.4 μL (0.33 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 1 h and quenched by the addition of 2 mL of satd aq NH_4Cl. The solution was then extracted with three 5-mL portions of EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 100:1 hexanes/ethyl acetate gave **2a** as a yellow oil: yield 26.7 mg (88%); silica gel TLC R_f 0.1 (3:7 EtOAc/hexanes); ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, J = 7.0 Hz), 1.32–1.37 (m, 4H), 1.64 (quint, 2H, J = 7.5 Hz), 1.95 (s, 3H), 2.31 (s, 3H), 2.47 (t, 2H, J = 8.0 Hz), 2.85 (t, 2H, J = 8.0 Hz), 2.89 (s, 3H) and 3.44 (t, 2H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 14.0, 20.6, 22.5, 24.7, 28.4, 29.6, 31.8, 32.5, 33.3, 52.4, 120.1, 134.7, 136.5, 149.7, 161.2 and 169.9; mass spectrum, m/z 276.2 $(M)^+$ (theoretical 276.2); mass spectrum (APCI), m/z 277.1910 $(M+H)^+$ ($C_{16}H_{25}N_2O_2$ requires 277.1916).



4.1.5. 1,4-Dimethyl-6-pentyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*] pyridine-5-ol and its trifluoroacetic acid salt (1a)

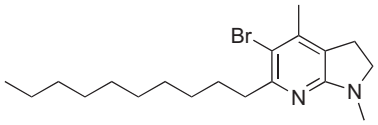
To a solution of 20.0 mg (72.4 μmol) of **2a** in 1 mL of CH_2Cl_2 was added 217 μL (84.0 μmol, 1.0 M in hexanes) of DIBAL-H at −78 °C. The reaction mixture was stirred at −78 °C for 1 h and then 2 mL of satd aq sodium potassium tartrate was added slowly. The reaction mixture was slowly warmed to room temperature over a period of 30 min. The solution was extracted with three 5-mL portions of ethyl acetate. The combined organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure to give the crude product as a yellow oil: silica gel TLC R_f 0.12 (1:9 MeOH/CH_2Cl_2). The residue was then dissolved in 1 mL of CH_3CN and 1%

aq TFA, frozen and lyophilized. The crude product was purified on a Prepex C_8 reversed phase semi-preparative (250 mm x 10 mm) HPLC column 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, at a flow rate of 3.5 mL/min (monitoring at 260 nm). Fractions containing the desired product eluted at 21.6 min, and were collected, frozen, and lyophilized to give **1a** as a light yellow solid: yield 13.5 mg (80%); ^{1}H NMR (CD_3CN) δ 0.88 (t, 3H, J = 6.8 Hz), 1.27–1.32 (m, 4H), 1.54 (quint, 2H, J = 7.6 Hz), 2.13 (s, 3H), 2.69 (t, 2H, J = 8.0 Hz), 2.99 (t, 2H, J = 8.4 Hz), 3.06 (s, 3H) and 3.72 (t, 2H, J = 8.4 Hz); ^{13}C NMR (CD_3CN) δ 13.0, 13.3, 22.1, 24.4, 27.2, 28.3, 31.1, 32.4, 53.1, 117.3, 126.1, 132.1, 140.4, 141.6 and 152.0; mass spectrum, m/z 234.2 $(M)^+$ (theoretical 234.2); mass spectrum (APCI), m/z 235.1806 $(M+H)^+$ ($C_{14}H_{23}N_2O$ requires 235.1810).



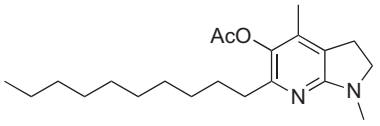
4.1.6. 6-Decyl-1,4-dimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*] pyridine (4b)

To a solution of 181 mg (1.12 mmol) of compound **5** in 3 mL of THF was added 1.19 mL (1.90 mmol, 1.6 M in hexanes) of *n*-BuLi followed by 255 μL (1.34 mmol) of 1-bromononane at −78 °C. The reaction mixture was slowly warmed to room temperature and stirred for another 16 h. The reaction mixture was quenched by the addition of 10 mL of satd aq NH_4Cl at 0 °C. The mixture was extracted with EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) 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 acetate gave the product **4b** as a yellow oil: yield 210 mg (66%); silica gel TLC R_f 0.18 (8:1:1 hexanes/ethyl acetate/MeOH); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.8 Hz), 1.24–1.30 (m, 16H), 1.65 (quint, 2H, J = 7.6 Hz), 2.08 (s, 3H), 2.54 (t, 2H, J = 8.0 Hz), 2.81 (t, 2H, J = 8.0 Hz), 2.90 (s, 3H), 3.38 (t, 2H, J = 8.0 Hz) and 6.13 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 18.1, 22.8, 24.5, 29.5, 29.68, 29.73, 29.75, 29.77, 30.1, 32.1, 33.4, 38.2, 52.6, 112.6, 118.4, 141.3, 159.3 and 163.9; mass spectrum, m/z 288.3 (M^+) (theoretical 288.3); mass spectrum (APCI), m/z 289.2653 $(M+H)^+$ ($C_{19}H_{33}N_2$ requires 289.2644).



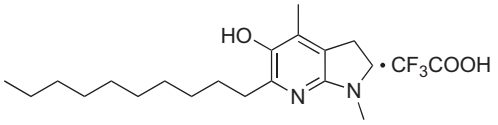
4.1.7. 5-Bromo-1,4-dimethyl-6-decyl-2,3-dihydro-1*H*-pyrrolo [2,3-*b*]pyridine (3b)

To a solution of 286 mg (0.99 mmol) of **4b** in 4 mL of chloroform was added 143 mg (0.50 mmol) of 1,3-dibromo-5,5-dimethylhydantoin in portions at 0 °C. The reaction mixture was stirred at 0 °C for 30 min. The reaction was quenched by the addition of 5 mL of satd aq $NaHCO_3$. The mixture was extracted with chloroform and the combined organic layer was washed with brine and dried over $MgSO_4$. The concentrated residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 100:1 hexanes/ethyl acetate gave **3b** as a yellow oil: yield 342 mg (94%); silica gel TLC R_f 0.7 (8:1:1 hexanes/ethyl acetate/methanol); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 6.8 Hz), 1.25–1.39 (m, 16H), 1.66 (quint, 2H, J = 7.2 Hz), 2.17 (s, 3H), 2.77 (t, 2H, J = 8.0 Hz), 2.83–2.87 (m, 5H) and 3.40 (t, 2H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1, 19.5, 22.7, 25.2, 28.7, 29.4, 29.54, 29.59, 29.64, 29.64, 31.9, 33.0, 38.1, 52.4, 110.9, 120.4, 141.1, 156.8 and 161.8; mass spectrum, m/z 366.2 and 368.2 $(M)^+$ (theoretical 366.2 and 368.2); mass spectrum (APCI), m/z 367.1747 and 369.1673 $(M+H)^+$ ($C_{19}H_{32}N_2Br$ requires 367.1749 and 369.1728).



4.1.8. 6-Decyl-1,4-dimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*] pyridin-5-yl acetate (2b)

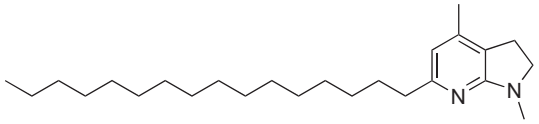
To a solution of 43.0 mg (0.12 mmol) of **3b** in 1 mL of THF was added 17.4 μL (0.12 mmol) of tetramethylethylenediamine (TMEDA) at −78 °C followed by 146 μL (0.23 mmol, 1.6 M in hexanes) of *n*-BuLi. After 30 min, 28.7 μL (0.26 mmol) of trimethoxy boron was added and the resulting mixture was stirred for another 1 h. To the reaction mixture was added slowly 54.2 μL (0.26 mmol, 32 wt%) of peracetic acid and the solution was then warmed to 0 °C over a period of 30 min. The mixture was diluted with 5 mL of H_2O and extracted with three 5-mL portions of EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) and the solvent was concentrated under diminished pressure. The resulting oil was dissolved in 2 mL of CH_2Cl_2 at 0 °C, followed by the addition of 97.9 μL (0.70 mmol) of triethylamine, 1.4 mg (0.01 mmol) of DMAP and 33.1 μL (0.35 mmol) of acetic anhydride. The reaction was stirred at room temperature for 1 h and quenched by the addition of 2 mL of satd aq NH_4Cl. The solution was then extracted with three 5-mL portions of EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 100:1 hexanes/ethyl acetate gave **2b** as a yellow oil: yield 28.0 mg (68%); silica gel TLC R_f 0.15 (3:7 EtOAc/hexanes); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.8 Hz), 1.24–1.28 (m, 16 H), 1.60 (quint, 2H, J = 7.6 Hz), 1.92 (s, 3H), 2.28 (s, 3H), 2.44 (t, 2H, J = 8.0 Hz), 2.82 (t, 2H, J = 8.4 Hz), 2.87 (s, 3H) and 3.41 (t, 2H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$) δ 12.9, 14.1, 20.5, 22.7, 24.6, 28.7, 29.3, 29.5, 29.6, 28.6, 31.9, 32.5, 33.3, 52.8, 120.1, 134.7, 136.5, 149.7, 161.2 and 169.9; mass spectrum, m/z 346.3 $(M)^+$ (theoretical 246.3); mass spectrum (APCI), m/z 347.2696 $(M+H)^+$ ($C_{21}H_{35}N_2O_2$ requires 347.2699).



4.1.9. 6-Decyl-1,4-dimethyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*] pyridine-5-ol and its trifluoroacetic acid salt (1b)

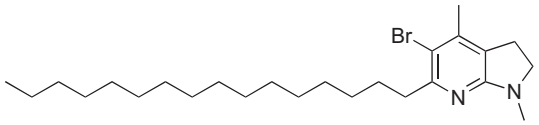
To a solution of 19.0 mg (54.8 μmol) of **2b** in 1 mL of CH_2Cl_2 was added 165 μL (165 μmol, 1.0 M in hexanes) of DIBAL-H at

−78 °C. The reaction mixture was stirred at −78 °C for 1 h and then 2 mL of satd aq sodium potassium tartrate was added slowly. The reaction mixture was slowly warmed to room temperature over a period of 30 min. The solution was extracted with three 5-mL portions of ethyl acetate. The combined organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure to give the crude product as a yellow oil: silica gel TLC R_f 0.16 (1:9 MeOH/CH_2Cl_2). The residue was then dissolved in 1 mL of CH_3CN and 1% aq TFA, frozen and lyophilized. The crude product was purified on a Prepex C_8 reversed phase semi-preparative (250 mm x 10 mm) HPLC column 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, at a flow rate of 3.5 mL/min (monitoring at 260 nm). Fractions containing the desired product eluted at 28.2 min, and were collected, frozen, and lyophilized to give **1b** as a light yellow solid: yield 13.0 mg (76%); ^{1}H NMR (CD_3CN) δ 0.89 (t, 3H, J = 6.8 Hz), 1.27–1.31 (m, 16H), 1.54 (quint, 2H, J = 7.6 Hz), 2.13 (s, 3H), 2.70 (t, 2H, J = 8.0 Hz), 2.99 (t, 2H, J = 8.4 Hz), 3.06 (s, 3H) and 3.72 (t, 2H, J = 8.4 Hz); ^{13}C NMR (CD_3CN) δ 13.0, 13.4, 22.4, 24.41, 27.3, 28.6, 28.9, 29.00, 29.04, 29.3, 29.3, 31.6, 32.4, 53.1, 117.3, 126.1, 132.1, 140.3, 141.5 and 152.1; mass spectrum, m/z 304.3 $(M)^+$ (theoretical 304.3); mass spectrum (APCI), m/z 305.2590 $(M+H)^+$ ($C_{19}H_{33}N_2O$ requires 305.2593).



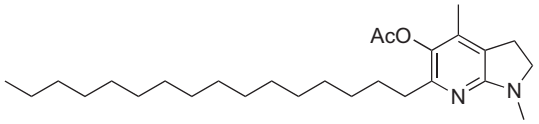
4.1.10. 1,4-Dimethyl-6-hexadecyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridine (4c)

To a solution of 277 mg (1.71 mmol) of compound **5** in 5 mL of THF was added 1.71 mL (2.73 mmol, 1.6 M in hexanes) of *n*-BuLi followed by 518 μL (1.79 mmol) of 1-bromononane at −78 °C. The reaction mixture was slowly warmed to room temperature and stirred for another 16 h. The reaction mixture was quenched by the addition of 10 mL of satd aq NH_4Cl at 0 °C. The mixture was extracted with EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) 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 acetate gave the product **4c** as a white solid: yield 330 mg (52%); mp 43–45 °C; silica gel TLC R_f 0.25 (8:1:1 hexanes/ethyl acetate/MeOH); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 7.2 Hz), 1.25–1.31 (m, 28H), 1.66 (quint, 2H, J = 7.6 Hz), 2.08 (s, 3H), 2.55 (t, 2H, J = 8.0 Hz), 2.80 (t, 2H, J = 8.4 Hz), 2.90 (s, 3H), 3.38 (t, 2H, J = 8.4 Hz) and 6.13 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 18.0, 22.7, 24.4, 29.38, 29.38, 29.57, 29.62, 29.65, 29.68, 29.72, 29.72, 29.72, 29.72, 29.72, 29.72, 30.0, 32.0, 33.2, 38.1, 52.4, 112.4, 118.2, 141.0, 159.1 and 163.7; mass spectrum, m/z 372.3 (M^+) (theoretical 372.4); mass spectrum (APCI), m/z 373.3574 $(M+H)^+$ ($C_{25}H_{45}N_2$ requires 373.3583).



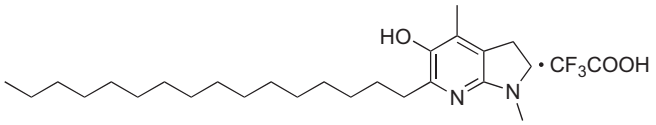
4.1.11. 5-Bromo-1,4-dimethyl-6-hexadecyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridine (3c)

To a solution of 278 mg (0.75 mmol) of **4c** in 4 mL of chloroform was added 108 mg (0.38 mmol) of 1,3-dibromo-5,5-dimethylhydantoin in portions at 0 °C. The reaction mixture was stirred at 0 °C for 30 min. The reaction was quenched by the addition of 5 mL of satd aq $NaHCO_3$. The mixture was extracted with chloroform and the combined organic layer was washed with brine and dried over $MgSO_4$. The concentrated residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 100:1 hexanes/ethyl acetate gave **3c** as a white solid: yield 331 mg (98%); mp 49–50 °C; silica gel TLC R_f 0.78 (8:1:1 hexanes/ethyl acetate/methanol); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 7.2 Hz), 1.25–1.31 (m, 28H), 1.66 (quint, 2H, J = 7.6 Hz), 2.08 (s, 3H), 2.55 (t, 2H, J = 8.0 Hz), 2.80 (t, 2H, J = 8.4 Hz), 2.90 (s, 3H), 3.38 (t, 2H, J = 8.0 Hz) and 6.13 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 19.5, 22.7, 25.2, 28.7, 29.4, 29.57, 29.61, 29.65, 29.68, 29.71, 29.71, 29.71, 29.71, 29.71, 29.71, 31.9, 33.0, 38.1, 52.4, 110.9, 120.3, 141.1, 156.8 and 161.8; mass spectrum, m/z 451.3 and 453.2 $(M+H)^+$ (theoretical 451.3 and 453.3); mass spectrum (APCI), m/z 451.2680 and 453.2697 $(M+H)^+$ ($C_{25}H_{44}N_2Br$ requires 451.2688 and 453.2667).



4.1.12. 1,4-Dimethyl-6-hexadecyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*]pyridin-5-yl acetate (2c)

To a solution of 43.5 mg (0.10 mmol) of **3c** in 1 mL of THF was added 14.2 μL (0.10 mmol) of tetramethylethylenediamine (TMEDA) at −20 °C followed by 119 μL (0.19 mmol, 1.6 M in hexanes) of *n*-BuLi. After 30 min, 23.4 μL (0.21 mmol) of trimethoxy boron was added and the resulting mixture was stirred for another 1 h. To the reaction mixture was added slowly 44.1 μL (0.21 mmol, 32 wt%) of peracetic acid and the solution was then warmed to 0 °C over a period of 30 min. The mixture was diluted with 5 mL of H_2O and extracted with three 5-mL portions of EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) and the solvent was concentrated under diminished pressure. The resulting oil was dissolved in 2 mL of CH_2Cl_2 at 0 °C, followed by the addition of 79.6 μL (0.57 mmol) of triethylamine, 1.2 mg (0.01 mmol) of DMAP and 27.0 μL (0.29 mmol) of acetic anhydride. The reaction mixture was stirred at room temperature for 1 h and quenched by the addition of 2 mL of satd aq NH_4Cl. The solution was then extracted with three 5-mL portions of EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$) and the solvent was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 100:1 hexanes/ethyl acetate gave **2c** as a yellow oil: yield 15.0 mg (37%); silica gel TLC R_f 0.20 (3:7 EtOAc/hexanes); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.8 Hz), 1.23–1.28 (m, 28H), 1.60 (quint, 2H, J = 7.6 Hz), 1.93 (s, 3H), 2.28 (s, 3H), 2.44 (t, 2H, J = 8.0 Hz), 2.82 (t, 2H, J = 8.0 Hz), 2.87 (s, 3H) and 3.41 (t, 2H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 12.9, 14.1, 20.5, 22.7, 24.6, 28.7, 29.4, 29.51, 29.59, 29.64, 29.68, 29.68, 29.68, 29.68, 29.68, 29.68, 29.68, 31.9, 32.5, 33.3, 52.8, 120.1, 134.7, 136.5, 149.7, 161.2 and 169.9; mass spectrum, m/z 430.3 $(M)^+$ (theoretical 430.4); mass spectrum (APCI), m/z 431.3637 $(M+H)^+$ ($C_{27}H_{47}N_2O_2$ requires 431.3638).



4.1.13. 1,4-Dimethyl-6-hexadecyl-2,3-dihydro-1*H*-pyrrolo[2,3-*b*] pyridin-5-ol and its trifluoroacetic acid salt (1c)

To a solution of 15.0 mg (34.8 μmol) of **2c** in 1 mL of CH_2Cl_2 was added 105 μL (105 μmol, 1.0 M in hexanes) of DIBAL-H at −78 °C. The reaction mixture was stirred at −78 °C for 1 h and then 2 mL of satd aq sodium potassium tartrate was added slowly. The reaction mixture was slowly warmed to room temperature over a period of 30 min. The solution was extracted with three 5-mL portions of ethyl acetate. The combined organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure to give the crude product as a yellow oil: silica gel TLC R_f 0.22 (1:9 MeOH/CH_2Cl_2). The residue was then dissolved in 1 mL of CH_3CN and 1% aq TFA, frozen and lyophilized. The crude product was purified on a Prepex C_8 reversed phase semi-preparative (250 mm x 10 mm) HPLC column 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, at a flow rate of 3.5 mL/min (monitoring at 260 nm). Fractions containing the desired product eluted at 37.5 min, and were collected, frozen, and lyophilized to give **1c** as a light yellow solid: yield 11.0 mg (82%); ^{1}H NMR (CD_3CN) δ 0.89 (t, 3H, J = 6.4 Hz), 1.27–1.36 (m, 28H), 1.55 (quint, 2H, J = 7.2 Hz), 2.13 (s, 3H), 2.70 (t, 2H, J = 8.0 Hz), 3.00 (t, 2H, J = 8.4 Hz), 3.06 (s, 3H) and 3.73 (t, 2H, J = 8.4 Hz); ^{13}C NMR (CD_3CN) δ 13.0, 13.4, 22.4, 24.4, 27.2, 28.6, 28.7, 28.99, 29.06, 29.07, 29.2, 29.30, 29.33, 29.36, 29.39, 29.39, 29.39, 31.6, 32.4, 53.1, 117.3, 126.2, 131.9, 140.2, 141.4 and 152.1; mass spectrum, m/z 388.3 $(M)^+$ (theoretical 388.3); mass spectrum (APCI), m/z 389.3526 $(M+H)^+$ ($C_{25}H_{45}N_2O$ requires 389.3532).

4.2. Biochemical and biological evaluation

4.2.1. Reactive oxygen species (ROS) assay

Intracellular ROS levels in CEM leukemia cells were monitored using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA)[35] according to the procedures described previously.[17] The freely cell-permeable DCFH-DA is readily converted to dichlorodihydrofluorescein which is able to interact with peroxides to form the highly fluorescence DCF. Cellular oxidative stress was induced by depletion of glutathione (GSH) using diethyl maleate (DEM). Briefly, CEM cells (5×10^5 cell/mL) were plated (1 mL in 24-well plates) and treated with the test compounds at final concentrations of 1, 2.5 and 5 μM, then incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 5 mM DEM for 50 min, collected by centrifugation at 300×g for 3 min, and then washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline containing 20 mM glucose and 10 μM DCFH-DA and incubated at 37 °C in the dark for 20 min. Cells were collected by centrifugation at 300×g for 3 min, then washed with phosphate buffered saline. The samples were analyzed immediately by flow cytometry (Becton-Dickinson FACS Caliber instrument equipped with Cell Quest software, BD Biosciences, San Jose, CA, USA) using a 488 nm excitation laser and FL1-H channel 538 nm emission filter. A total of 10,000 events were recorded for each sample and analyzed with the CellQuest software (BD Biosciences). The results were verified by repeating the experiments in duplicate. Results are expressed as the relative percentage of treated control (DEM).

4.2.2. Lipid peroxidation assay

A quantitative analysis of lipid peroxidation of CEM leukemia cells, treated with 5 mM DEM in the presence or absence of the test compounds, was carried out by flow cytometry measurement using a lipophilic conjugated C_{11}-BODIPY$^{581/591}$ probe, as described previously.[17] Briefly, CEM cells (5×10^5 cell/mL) were plated (1 mL in 24-well plates) and treated with the test compounds at final concentrations of 2.5 and 5 μM and incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 1 μM C_{11}-BODIPY$^{581/591}$ in phenol red-free RPMI-1640 media and incubated at 37 °C in the dark for 30 min. Lipid peroxidation was induced with 5 mM DEM in phenol red-free RPMI-1640 media for 90 min. Cells were collected by centrifugation at 300×g for 3 min and then washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline and were analyzed immediately by FACS (FACS Caliber flow cytometer, Becton-Dickinson) to monitor the change in intensity of C_{11}-BODIPY$^{581/591}$—green (oxidized) fluorescence signal. A total of 10,000 events were recorded for each sample and analyzed with the CellQuest software (BD Biosciences). The results were verified by repeating the experiments in duplicate. Results are expressed as the relative percentage of treated control (DEM).

4.2.3. Mitochondrial membrane potential ($\Delta\psi_m$) assay

ΔΨm was assessed by flow cytometry using the cationic-lipophilic tetramethylrodhodamine methyl ester (TMRM) (Molecular Probes, Eugene, Oregon), an indicator dye that accumulates inside the mitochondria in proportion to their membrane potential.[44] Briefly, CEM leukemia cells were pretreated with or without the test compounds for 16 h. The cells were treated with 5 mM DEM for 120 min, collected by centrifugation at 300×g for 3 min, and then washed with phosphate buffered saline. The cells were resuspended in phosphate buffered saline and incubated at 37 °C in the dark for 15 min with 250 nM TMRM. Cells were collected by centrifugation at 300×g for 3 min, and then washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline supplemented with 20 mM glucose and were analyzed immediately by FACS (FACS Caliber flow cytometer, Becton-Dickinson) using 488 nm excitation laser and the FL2-H channel. For each analysis 10,000 events were recorded and the percentage of cells exhibiting a high level of TMRM uptake, which reflects normal mitochondrial membrane potential ($\Delta\psi_m$), was determined and analyzed with the CellQuest software (BD Biosciences). The protonophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone) was used at 10 μM concentration to dissipate the chemiosmotic proton gradient and set a threshold of fluorescence intensity for those cells with intact $\Delta\psi_m$. The results were verified by repeating the experiments in duplicate.

4.2.4. Cell viability

Cell viability was determined by trypan blue exclusion assay.[45] The viability of DEM-treated CEM cells is distinguished by their ability to exclude the dye trypan blue, whereas the non-viable cells take up the dye and stain blue. Briefly, CEM lymphoblasts (CCL-119) grown in RPMI medium 1640 supplemented with 10% FBS, with 1% penicillin-streptomycin, were seeded at density of 5×10^5 cells/mL and treated with different concentrations of the indicated compounds. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 16 h. After pre-incubation, the cells were treated with 5 mM DEM for 5.5 h. Cell viability was determined microscopically by staining cells with 0.4% trypan blue. At least 500 cells were counted in each experimental group. At the time of assay, >80% of DEM-treated cells were trypan blue

positive, whereas in non-DEM-treated control cell cultures >95% of the cells were viable. Cell viability was expressed as the percentage of control. Results are an average of three independent trials.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.075.

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