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Optimization of pyrimidinol antioxidants as mitochondrial protective agents: ATP production and metabolic stability

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A B S T R A C T

Previously we described a novel series of pyrimidinol antioxidants and their structural optimization as potential therapeutic agents for neurodegenerative and mitochondrial disorders. Our initial lead compound was a potent antioxidant *in vitro*, but was subsequently found to exhibit poor stability to oxidative metabolism. The current study focused on balancing potency with metabolic stability through structural modification, and involved modifications at position 2 and 6 of the pyrimidinol redox core, likely sites of oxidative metabolism. Eight new analogues have been prepared and their ability to suppress lipid peroxidation and reactive oxygen species (ROS), and to preserve mitochondrial membrane potential ($\Delta\psi_m$) and support ATP production, has been investigated. The metabolic stability of the prepared compounds was also assessed *in vitro* using bovine liver microsomes to obtain preliminary insight on this class of compounds. This study revealed the complexity of balancing reasonable metabolic stability with efficient antioxidant properties. While a few analogues appear promising, especially in terms of metabolic stability, a 4-isopropoxy derivative conserved the favorable biological activity and exhibited good metabolic stability. The favorable metabolic stability conferred by the combination of the azetidine and isopropoxy moieties in analogue **6** makes this compound an excellent candidate for further evaluation.

1. Introduction

Mitochondria are organelles found in most eukaryotic cells which fulfill essential cellular functions.[1-7] Although adenosine-5′-triphosphate (ATP) synthesis is often considered their primary function, they also participate in critical cellular processes, such as programmed cell death and neoplastic transformation.[2,3] Mitochondria are central to energy metabolism; they use oxidative phosphorylation (OXPHOS) to convert the energy stored in cellular nutrients into ATP through the action of the mitochondrial electron transport chain (ETC) complexes. Electrons removed from oxidizable substrates transit a series of electron carriers (complexes I-IV) in the respiratory chain, resulting in the reduction of oxygen to water. The flow of these electrons through these complexes also results in the transport of protons from the inner mitochondrial membrane into the intermembrane space. These protons are used by a fifth complex, ATP synthase (complex V), to convert ADP to ATP.[2-4]

Defects in ETC protein complexes can have a major impact on mitochondrial coupling efficiency and the production of reactive oxygen species (ROS).[8,9] The delicate balance between ROS production and elimination is necessary for normal cellular homeostasis. Ordinarily, mammalian cells employ a ubiquitous antioxidant defense system to cope with by-product ROS production accompanying aerobic metabolism.[10,11] These ROS scavenging systems include antioxidant enzymes, the glutathione redox cycle, and the pool of coenzyme Q_{10} (CoQ_{10}).[10,11] Oxidative stress is considered to result from an imbalance of ROS and antioxidants; excess ROS generation can overwhelm endogenous antioxidant capacity, expose cellular macromolecules to oxidative damage, and impair the ability of mitochondria to make ATP and carry out other functions. The decline in mitochondrial function is well recognized in aging, neurodegenerative diseases and many complex mitochondrial disorders.[12-16]

Metabolic stability is a key property of compounds employed therapeutically; it is important for a drug to reach and maintain therapeutic concentrations following administration, and drug

degradation can contribute to toxicity. Oxidative dealkylations are commonly observed metabolic reactions for compounds containing heteroatoms, such as secondary and tertiary amines as well as ethers.[17] Structural modification of such functional groups can minimize oxidative metabolism. We previously reported the discovery of compound **1** as a potent multifunctional radical quencher (MRQ),[18] i.e. a compound which functions in part by quenching radicals involved in oxidative stress (Figure 1). This compound was subsequently shown to lack sufficient metabolic stability to function well *in vivo*. Presently, this issue is addressed by identifying structurally related pyrimidinol antioxidants which retain good efficacy but exhibit reduced liability to oxidative metabolism. A recent study of related pyridinols established enhanced metabolic stability by replacing the *N,N*-dimethylamino moiety with an exocyclic azetidine.[19] This functional group tends to exhibit a defined, rigid conformation and can confer superior metabolic stability.[19-21]

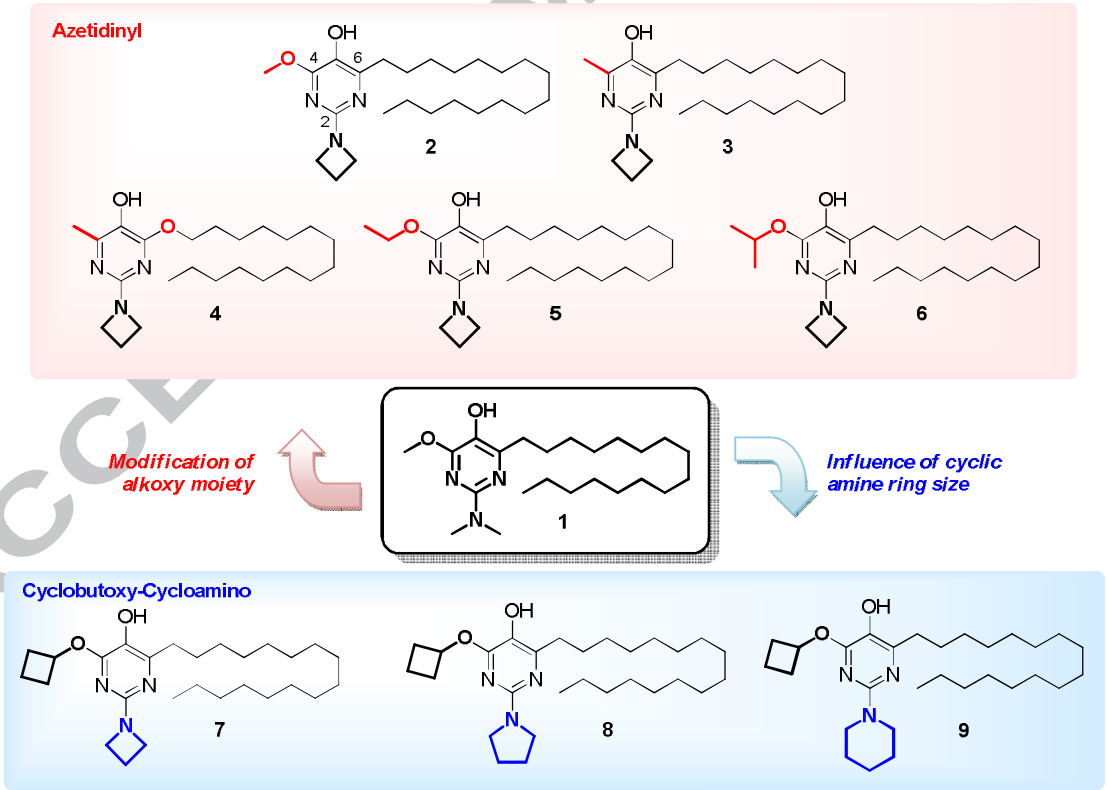


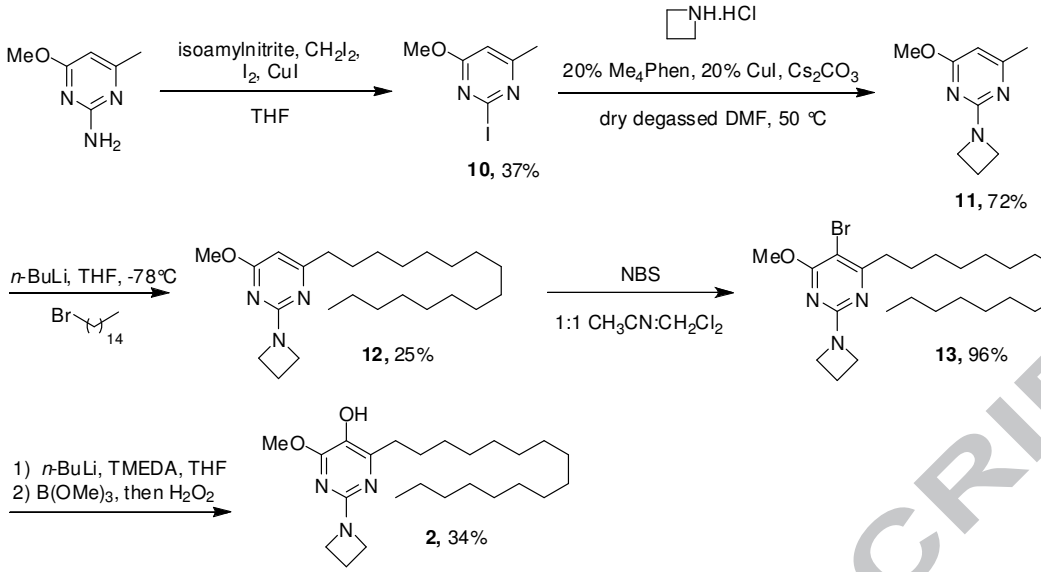
Figure 1. Chemical structures of compounds **1**–**9**.

We describe the preparation and study of several novel pyrimidinol derivatives (**2**–**9**) (Figure 1), which exhibit improved metabolic stability relative to **1**, and some of which exhibit improved cellular antioxidant properties. Strategies for diminution of metabolic *N*- and *O*-demethylation were employed; the pyrimidinol derivatives were evaluated for their stability in bovine liver microsomes. Metabolic stability was achieved by exploiting the relative resistance of the azetidine moiety to oxidative attack, and by the presence of a sterically hindered cyclobutoxy or isopropoxy moiety, blocking another site of possible oxidative attack. The compounds were evaluated for their ability to suppress ROS and lipid peroxidation, and for their effects on mitochondrial membrane potential, and their ability to protect cultured FRDA lymphocytes from oxidative stress induced by glutathione depletion. The compounds were also evaluated for their ability to increase ATP levels in FRDA lymphocytes.

2. Results and discussion

2.1. Design and synthesis of pyrimidinol analogues of MRQ based on the compound 1 scaffold

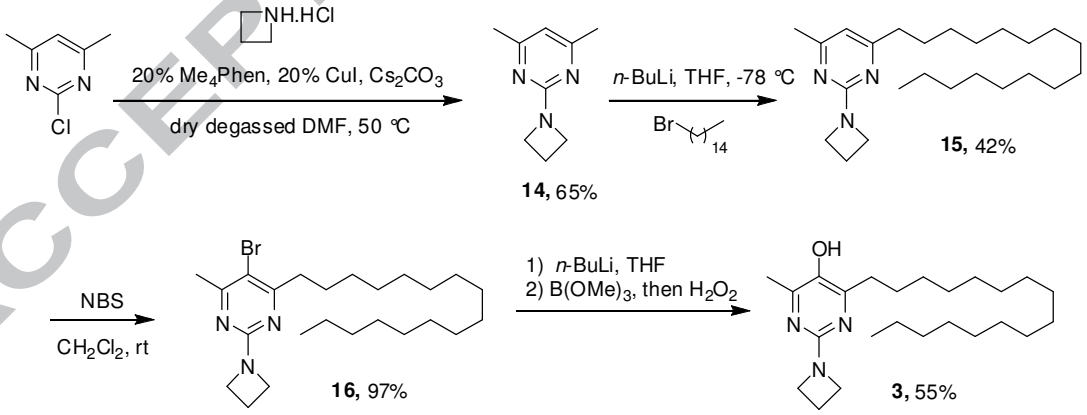
The objective of this work was to determine what modifications of compound **1** could both conserve its favorable biological activities and improve metabolic stability. In a previous study of pyridinol antioxidants, it was found that introduction of a cyclic amine in place of the exocyclic dimethyl amine improved metabolic stability. Accordingly, the preparation of compound **2**, having an exocyclic azetidine moiety, was envisioned. For this synthesis, aryl iodide **10** was prepared starting from commercially available 4-methoxy-6-methylpyrimidin-2-amine in 37% yield (Scheme 1). The introduction of iodine was accomplished using Cu(I) as catalyst, and subsequent treatment with azetidine furnished the requisite heterocyclic core structure **11**. Compound **11** was then alkylated by deprotonation of the benzylic proton using *n*-BuLi, then treated with pentadecyl bromide to obtain intermediate **12** in 25% yield.



Scheme 1. Synthetic route employed for the preparation of compound **2**.

Bromination of the 5-position of the pyrimidine ring using *N*-bromosuccinimide provided compound **13** in 96% yield, the latter of which was transformed to the respective 5-hydroxypyrimidine (**2**) by halogen-metal exchange, conversion to the boronate ester, and oxidation in 34% overall yield.

The influence of the 6-alkoxy moiety was also investigated. An initial assessment of the need for an alkoxy group was made by replacing this substituent by a methyl group. As shown in Scheme 2, the requisite compound (**3**) was accessible starting from commercially available

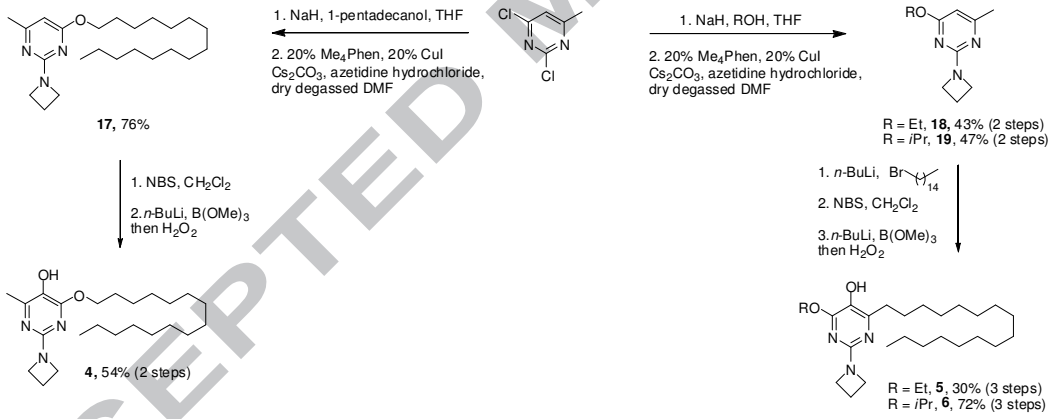


Scheme 2. Synthetic route employed for the preparation of compound **3**.

2-chloro-4,6-dimethylpyrimidine, which was treated with azetidine to provide **14** in 65% yield. Following the same strategy employed for the synthesis of **2**, the benzylic anion derived

from **14** was treated with pentadecyl bromide to afford **15**; following bromination to provide **16**, compound **3** was then obtained from the intermediate bromide in 55% yield as a yellowish oil.

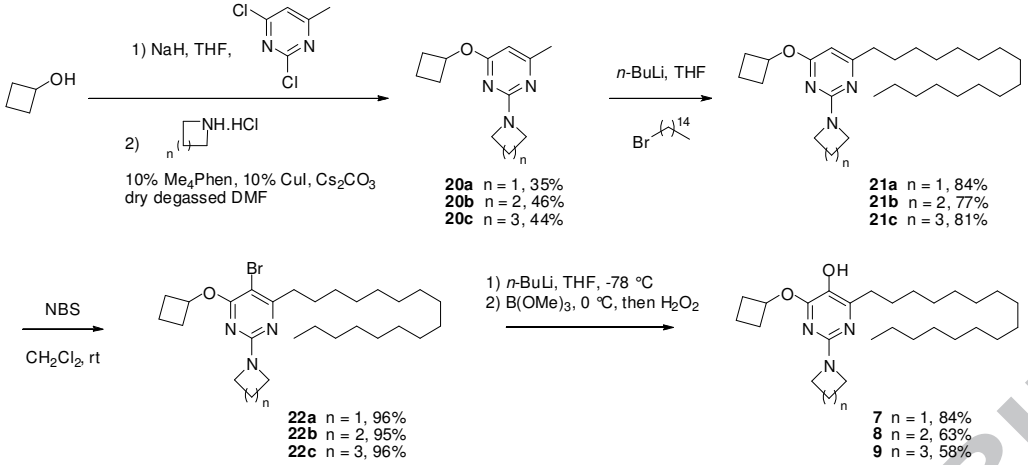
The possibility of altering the position of the exocyclic oxygen atom was also investigated by including the oxygen atom within the 16 atom linear side chain. Accordingly, compound **4** was prepared starting from commercially available 2,4-dichloro-6-methylpyrimidine. Initial nucleophilic replacement of the chlorine atom in position 4 was performed using the anion produced from 1-pentadecanol, followed by the introduction of the azetidinyl moiety in position 2 to obtain **17** in 76% yield (Scheme 3). In analogy with Schemes 1 and 2, successive bromination and hydroxylation afforded compound **4** as a colorless powder in 54% overall yield. The 4-ethoxy (**5**) and 4-isopropoxy (**6**) analogues were also prepared starting from 2,4-dichloro-6-methylpyrimidine, as outlined in Scheme 3. The transformations outlined in



Scheme 3. Synthetic routes employed for the preparation of compounds **4**, **5** and **6**.

Schemes 1–3 thus provided a series of five new potential mitochondrial antioxidants incorporating a cyclic amine in position 2.

The introduction of a second cyclic moiety was also considered as a means of increasing metabolic stability. As outlined in Scheme 4, a cyclobutoxy moiety was introduced by nucleophilic substitution of the chlorine atom in position 4 of 2,4-dichloro-6-



Scheme 4. Synthetic routes employed for the preparation of compounds **7**, **8** and **9**.

methylpyrimidine, followed by the introduction of cycloamine moieties in position 4. In parallel, three substituted pyrimidine cores (**20a-c**) were prepared incorporating azetidinyl,pyrrolidinyl and piperidinyl groups in position 2 to explore the influence of amine ring size on activity and metabolic stability. For each of these cores, the same sequence of alkylation, bromination and hydroxylation was applied and analogues **7, 8** and **9** were obtained in 27%, 21% and 20% overall yields, respectively. As described below, compounds **2** – **9** were evaluated in comparison with **1** in a panel of biochemical and biological assays, enabling their antioxidant and cytoprotective properties to be understood, and especially to determine whether these compounds were more stable metabolically than compound **1** in a microsomal assay.

2.2. Biochemical and biological evaluation

2.2.1. Inhibition of lipid peroxidation

The ability of the new pyrimidinol analogues to quench lipid peroxidation was studied in FRDA lymphocytes that had been depleted of glutathione by treatment with diethyl maleate (DEM) as described previously.[18,19] The fluorescent lipid peroxidation-sensitive fatty acid-conjugated dye (C_{11}-BODIPY$^{581/591}$) was used to probe peroxyradical-induced lipid oxidation by flow cytometry as described previously.[18,19] This lipophilic fluorophore changes its fluorescence from red to green when it interacts with peroxyradicals.[22] C_{11}-BODIPY-green

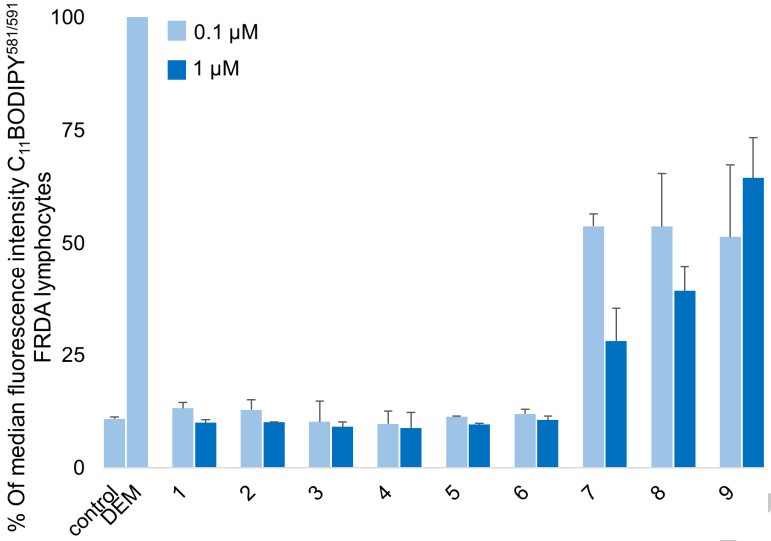


Figure 2. Lipid peroxidation in FRDA lymphocytes cells depleted of glutathione was determined by utilizing the oxidation-sensitive fatty acid probe C_{11}-BODIPY$^{581/591}$ using fluorescence activated cell sorting (FACS). Increased C_{11}-BODIPY-green fluorescence (oxidized form), a measure of intracellular lipid peroxidation, was determined by an increase in the median mean fluorescence intensity of C_{11}-BODIPY-green relative to the untreated control. A bar graph representing the percentage of the median mean fluorescence intensity of C_{11}-BODIPY-green fluorescence relative to a treated control is shown. Statistical analyses were performed with a two-tailed Student's t-test, and data are mean values ± SEMs (n = 3). P values ≤ 0.0005 were considered to be statistically significant as compared to treated control (DEM). Compounds **7– 9** were considered to be statistically significant ($P \leq 0.001$) as compared to untreated control.

fluorescence (oxidized form), a measure of intracellular lipid peroxidation, was determined by increasing the median mean fluorescence intensity of C_{11}-BODIPY-green relative to the untreated control. The results presented in Figure 2 clearly show a significant ($P \leq 0.001$) decrease in protection from lipid peroxidation for cyclobutoxy-cycloamino analogues **7–9** as compared to untreated control. The lipid peroxidation quenching activity decreased as the ring size of the 2-cycloamino moiety increased. Introducing a non-cyclic alkoxy side chain at position 4 did not have any significant negative effect on the ability of these quenchers to protect against lipid peroxidation; they were as proficient as compound **1** ($P \leq 0.0005$) as compared to DEM-treated control.

2.2.2. Suppression of ROS

The ability of pyrimidinol analogues **1**–**9** to suppress ROS induced by depletion of glutathione by diethyl maleate (DEM) treatment was evaluated in FRDA lymphocytes. Cellular ROS production was monitored using dichlorodihydrofluorescein diacetate (DCFH-DA), a membrane permeable and a non-fluorescent probe which, upon oxidation by ROS is converted to the highly fluorescent derivative 2,7-dichlorofluorescein (DCF).[23] This increase of green fluorescence intensity resulting from ROS production induced by DEM was measured by FACS as described before.[18,19] The results obtained in FRDA lymphocytes are presented in Figure 3, and show that the compound **1** and analogues **2**–**6** were all effective at

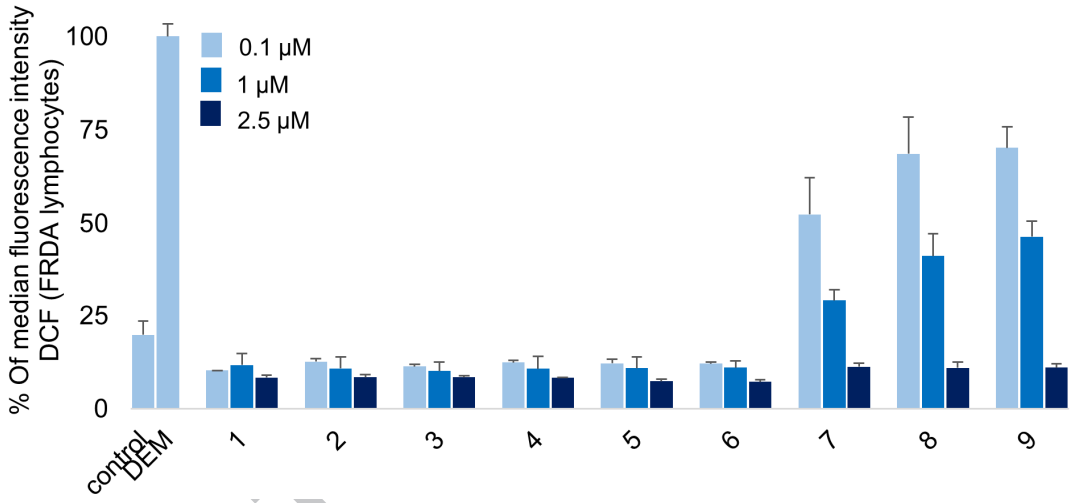


Figure 3. Flow cytometric analysis of ROS in FRDA lymphocyte cells pretreated with compounds **1**–**9** at 0.1 µM, 1 µM and 2.5 µM concentrations for 16 h, and then treated with diethyl maleate (DEM) for 80 min to induce the production of ROS. The cells were stained with 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) for 15 min prior to analysis. The samples were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA). Increased DCF fluorescence is a measure of intracellular oxidation and ROS production. A bar graph of the percentage of the median mean fluorescence intensity of DCF fluorescence relative to a DEM-treated control. Statistical analyses were performed with a two-tailed Student's test, and data are mean values ± SEMs (n = 2). P values ≤ 0.0005 were considered to be statistically significant as compared to treated control (DEM). Compounds **7**– **9** were considered to be statistically significant ($P \leq 0.001$) as compared to untreated control at low concentrations (0.1 and 1 µM).

suppressing ROS and did so at low concentration, reducing ROS to the same levels as controls not treated with DEM. In comparison, 2-cyclobutoxy analogues **7**–**9** gave dose dependent suppression of ROS, with effective suppression apparent only at the highest concentration employed (2.5 μM), and poor protection at low (100 nM) concentration. As the ring size of the cycloamino moiety increased among these three analogues, there was a slight decrease in ROS scavenging activity. This trend was analogous to the results obtained for the lipid peroxidation experiments.

2.2.3. Preservation of mitochondrial inner membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential ($\Delta\psi_m$) is critical for maintaining the ability of the respiratory chain to produce ATP. Oxidative stress can induce the modification of the inner mitochondrial membrane and lead to loss of $\Delta\psi_m$, and trigger a series of mitochondria-associated events including apoptosis and cell death. Compounds **1**–**9** were assessed for their ability to preserve mitochondrial membrane potential following induction of oxidative stress in FRDA lymphocytes. A lipophilic cationic fluorescent dye, tetramethyl rhodamine methyl ester (TMRM) was used to assess $\Delta\psi_m$. This dye distributes passively between the cytosol and

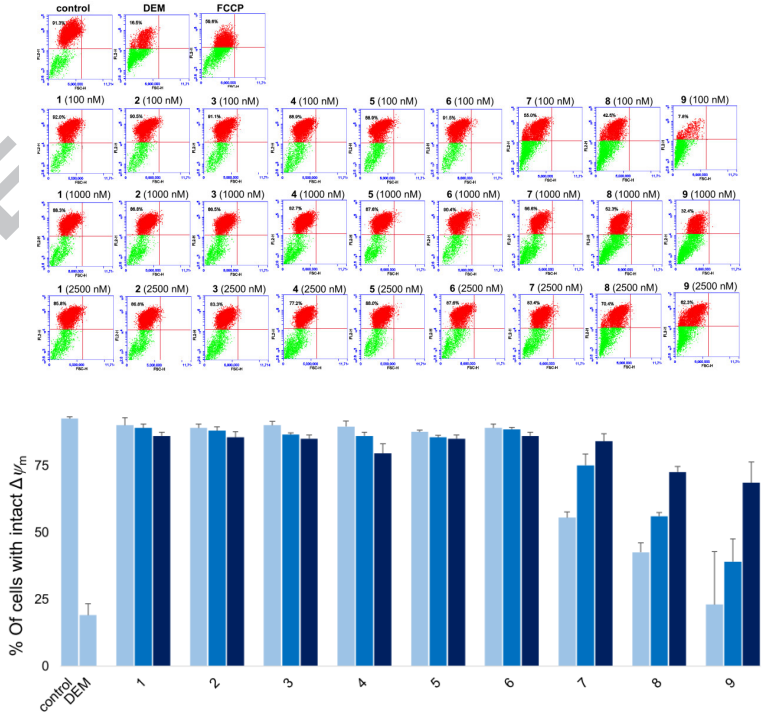


Figure 4. Representative flow cytometric two-dimensional color density dot plot analyses of the ability of compounds **1** – **9** to maintain mitochondrial membrane potential ($\Delta\psi_m$) in DEM-treated FRDA lymphocytes cells stained with 250 nM TMRM and analyzed using the FL2-H channel as described in the experimental. The percentage of cells with intact $\Delta\psi_m$ is indicated in the top left quadrant of captions (red) and the cells with depolarized $\Delta\psi_m$ in the bottom left quadrant of captions (green). Representative examples from at least two independent experiments are shown. A total of 10,000 events was recorded for each sample and analyzed using C6 Accuri software (BD Biosciences). A bar graph representing the percentage of the cells with intact $\Delta\psi_m$ is shown. Data are expressed as means ± S.E.M. of two independent experiments run in duplicate. Statistical analyses were performed with a two-tailed Student's t-test, and data are mean values ± SEMs (n = 2). $^{*}P \leq 0.003$ and $^{**}P \leq 0.03$, as compared to untreated control.

mitochondria according to the Nernst equation.[24] A collapse of mitochondrial potential causes the redistribution of the probe from mitochondria to the cytosol, a process that can be followed readily using fluorescence imaging techniques. Upon collapse of $\Delta\psi_m$, a reduction in red fluorescence intensity occurs. Figure 4 (top panel) illustrates representative two-dimensional density dot plots of TMRM-stained lymphocyte cells showing the percentage of cells with intact $\Delta\psi_m$ (TMRM fluorescence in top left quadrant/red) versus the percentage of cells with reduced $\Delta\psi_m$ (TMRM fluorescence in bottom left quadrant/green). Figure 4 (bottom panel) shows a bar graph of the percentage (means ± SEM) of FRDA lymphocytes with intact $\Delta\psi_m$. Treatment with 5 mM DEM decreased the percentage of cells with TMRM fluorescence in the top left quadrant, revealing a depolarization of the $\Delta\psi_m$. These data indicate that modification of the alkoxy side chain, as in compounds **2**–**6**, did not diminish the ability to block oxidative stress-induced collapse of $\Delta\psi_m$ completely; **2**–**6** were clearly as good as compound **1** in this regard. In comparison cyclobutoxy compounds **7**–**9** were all less efficient in conferring protection against loss of mitochondrial membrane potential, although all functioned in a dose dependent fashion. As noted above for suppression of lipid peroxidation and ROS, increasing the cycloamine substituent ring size led to increasing loss

of efficiency in maintaining polarizable and healthy $\Delta\psi_m$ under oxidative stress in FRDA lymphocytes. Treatment with compounds **1–9** in the absence of DEM treatment had no effect on mitochondrial membrane potential (data not shown). Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), a commonly used uncoupler of oxidative phosphorylation in mitochondria, was employed as a positive control to dissipate the chemiosmotic proton gradient, which resulted in lowering of TMRM fluorescence as a result of the depolarization of mitochondrial inner membrane potential.

2.2.4. Cellular ATP levels

Coenzyme Q_{10} serves as an electron carrier within the mitochondrial respiratory chain, where it is integrally involved in oxidative phosphorylation, and consequently in ATP production.[25,26] It also functions as an antioxidant.[25,26] CoQ_{10} levels have been reported to decrease with age and to be reduced in patients with mitochondrial and neurodegenerative diseases.[27,28] An optimal, exogenously available CoQ_{10} analogue could well have the potential to improve energy production in mitochondria, e.g. by bypassing lesions in the respiratory chain, and by reducing the effects of oxidative stress. Previously, we have illustrated the importance of an optimized (hexadecyl) side chain on the properties of exogenously supplied analogues (such as compound **1**) in FRDA and CoQ_{10} deficient lymphocytes.[18] Since such analogues are significantly less hydrophobic than CoQ_{10} itself, their bioavailability following exogenous administration should be more favorable. Modified analogues **2–9** were evaluated for their ability to support electron transport through the respiratory chain as judged by an increase in ATP levels. Culturing FRDA cells in galactose as the sole sugar source forces mammalian cells to rely on mitochondrial OXPHOS and is a strategy previously used to diagnose human mitochondrial disorders or drug toxicity.[29,30] Since cells grown in galactose rely mostly on OXPHOS to produce their ATP, they become more sensitive to mitochondrial respiratory chain inhibitors than cells grown in glucose medium. In the present study,

compounds **1**–**9** were evaluated for their ability to enhance ATP levels in FRDA lymphocytes (Table 1). As noted in the Table, with the exception of compound **4**, all of the analogues increased ATP levels at one or two of the tested (1, 5 and 20 μM) concentrations. Among the analogues having an acyclic 4-substituent, **1**, **2**, **3** and **6** increased ATP levels at the lowest tested concentration, while **1**, **2**, **5** and **6** increased ATP when employed at 5 μM concentration. Among the analogues having a 4-cyclobutoxy substituent (**7** – **9**), increased ATP levels in the FRDA lymphocytes were observed for all three compounds when tested at 5 μM concentration, and to a lesser extent for **7** and **8** at 20 μM concentration. Significantly

Table 1
Total ATP concentration in FRDA lymphocytes following incubation with compounds **1**–**9** for 48 h[a]

Compound	Total ATP level (% control) FRDA lymphocytes		
	1 μM	5 μM	20 μM
Untreated control	100	100	100
1	115 ± 5[b]	107 ± 2[b]	88 ± 3[b]
2	102 ± 5	109 ± 4[b]	74 ± 3[b]
3	109 ± 3[b]	98 ± 1	60 ± 3[b]
4	96 ± 4	68 ± 13[b]	11 ± 8[b]
5	100 ± 1	108 ± 4[b]	96 ± 1[b]
6	106 ± 2[b]	109 ± 2[b]	95 ± 3
7	98 ± 4	112 ± 3[b]	103 ± 2
8	98 ± 4	107 ± 5	102 ± 1
9	99 ± 3	106 ± 4	100 ± 4

[a] Determined from intracellular ATP levels using the luciferin-luciferase reaction.
[b] Two-tailed Student's t-test, with $p \leq 0.05$ considered as statistically significant compared to vehicle control.

diminished ATP levels in the treated cells were observed following treatment at 20 μM for compounds **1**–**6**. Interestingly, the movement of the oxygen atom position from the 4-methoxy group in compound **2** to the lipophilic side chain at position 6 in analogue **4** resulted in a compound which did not increase ATP levels above the basal level when used at low (1 μM) concentration, and strongly diminished ATP levels in a concentration-dependent fashion in FRDA lymphocytes when employed at higher concentrations. The reduction in ATP levels

in the treated cells likely reflects the ability of test compounds to inhibit complex I activity in the mitochondrial electron transport chain, as we have shown previously in bovine heart mitochondria.[31]

Interestingly, the addition of a cyclobutoxy moiety enhanced ATP production at higher applied concentrations; for compounds **7**–**9**, the ATP level was elevated at 5 μM concentration. This phenomenon was also observed for compounds **5** and **6**. It may also be noted that the ring size of the cyclic amine, which was important in the previous assays, was negligible for ATP concentration.

2.2.5. Cytoprotection

The ability of the test compounds (**1**–**9**) to confer cytoprotection was evaluated in cultured FRDA lymphocytes that had been treated with diethyl maleate to induce oxidative stress through depletion of glutathione. As is clear from Figure 5, compounds **2**–**6** exhibited good

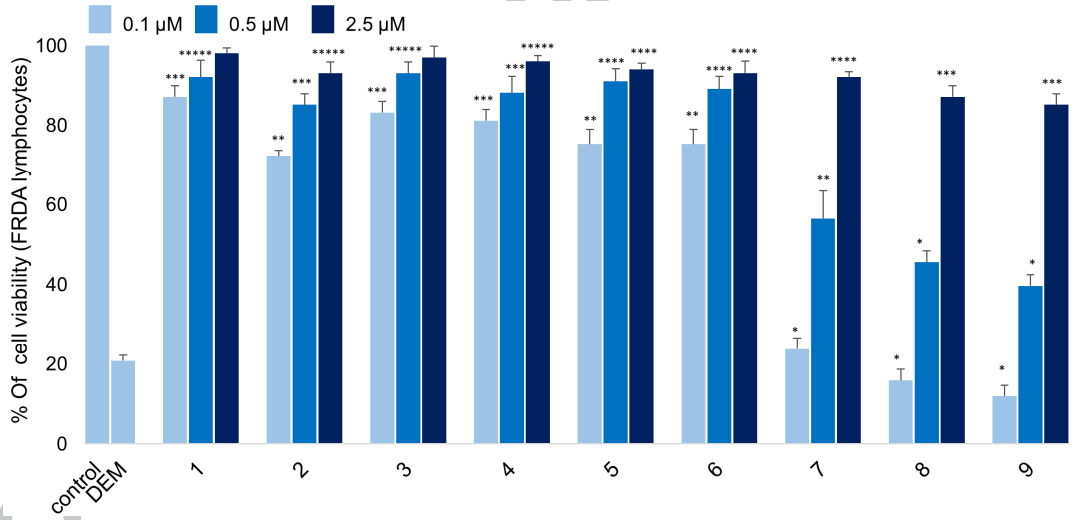


Figure 5. Viability of Friedreich's ataxia lymphocytes following pretreatment with the test compounds for 16 h and then treatment with DEM (5 mM) for 6 h to induce oxidative stress**.** Flow cytometric determination of cell viability by fluorescence labeling was used, employing calcein acetoxy-methyl ester and ethidium homodimer-1 (EthD-1) as live and dead cell stains. Cell viability was expressed as the percentage of cells relative to control. Results are an average of two independent trials run in duplicate. Statistically significant differences, where observed, are also indicated. Significant p-values are indicated with * for extremely significant ($p \leq 0.0001$), ** for very significant

($p \leq 0.0005$), *** for ($p \leq 0.0025$), **** for ($p \leq 0.005$), ***** for significant ($p \leq 0.05$), as compared to untreated control.

cytoprotective activity, even when used at 0.5 μM concentration. Their cytoprotective activity was clearly superior to that of the cyclobutoxy analogues **7**–**9**, none of which conferred significant cytoprotection at 0.1 μM concentration, and which were also not strongly cytoprotective when used at 0.5 μM concentration. Again, the size of the cyclic amine ring was correlated with decreased cytoprotection as the ring size increased.

2.2.6. Metabolic stability assays

The main objective of this study was to identify an efficient MRQ structurally related to **1**, but having improved metabolic stability. As shown in Table 2, following incubation in the presence of activated bovine liver microsomes for 30 minutes, the recovery of compound **1** was only about 46%. Compounds **2**–**9** were subjected to the same 30-minute incubation, and recovered starting material was analyzed by C_{18} reversed phase HPLC. Replacing the *N,N*-dimethylated amino moiety in **1** with the azetidine moiety in **2** increased the recovery of starting material to 58%. These results are consistent with those of an earlier metabolic study

Table 2
In vitro microsomal stability of the compounds **1**–**9** following incubation with bovine liver microsomes.

Compound	Recovery (%)[a]
1	46 ± 8
2	58 ± 11
3	57 ± 8
4	62 ± 6
5	54 ± 11
6	76 ± 7
7	65 ± 12
8	72 ± 9
9	72 ± 8

[a] Results expressed as % of compound recovered after reaction with activated microsomes. Microsomal stability values represent means ± SD.

of similar compounds in the pyridinol series.[19] The two analogues methylated at the 4-position (**3** and **4**) were recovered in 57 and 62% yields, respectively, following incubation with the microsomes. Replacing the 4-methoxy substituent with an ethoxy moiety (compound **5**) resulted in similar metabolic stability to that of compound **2** (54 vs 58%). Surprisingly, introducing an isopropoxy group at position 4 resulted in a substantial improvement in metabolic stability (76% recovery).

Compounds **7** – **9**, all of which contained a cyclobutoxy group at position 4 and a cycloamine at position 2, exhibited significantly enhanced metabolic stability with recoveries of unmetabolized starting material in the 65-72% range after 30-minute microsomal incubations. Despite their metabolic stability, analogues **7**–**9** lack the potent biological activities exhibited by compound **6.** Because of its favorable cellular potency and *in vitro* metabolic stability, compound **6** is a good candidate for further pharmacokinetic and *in vivo* evaluation.

3. Conclusions

Eight new multifunctional radical quenchers based on a pyrimidinol scaffold have been prepared using straightforward synthetic methods. Two types of modification of the prototype compound have been explored, one involving the use of a cyclic amine at the 2-position to confer metabolic stability. This strategy worked well to increase metabolic stability without compromising biological activity when an azetidine moiety was introduced, but not for larger cyclic amines. A cyclobutoxy moiety was introduced at the 4-position, and significantly increased metabolic stability. Unfortunately, while this group did confer increased metabolic stability, it also substantially compromised the properties of the derived compounds as antioxidants and cytoprotective species. The introduction of an azetidine moiety at the 2-position and an isopropoxy group at the 4-position provided an analogue (**6**) with good metabolic stability and excellent cytoprotective properties. This compound appears to be a

good candidate for further study, and should be helpful in furthering our understanding of neurodegenerative and mitochondrial diseases, and developing strategies for therapeutic intervention.

4. Experimental Section

4.1. Chemistry

Anhydrous grade solvents were purchased from Sigma–Aldrich Inc. (St. Louis, MO) and from Fisher Scientific. Most of the chemical reagents were purchased from Sigma–Aldrich and used without further purification. $ImPrPh_2HCl$, morpholine and iodine were purchased from TCI America. Azetidine hydrochloride was purchased from Combi-Blocks. All glassware and needles were pre-dried in an oven at 120 °C prior to use. Tetrahydrofuran was distilled from sodium/benzophenone. All reactions were performed under a stream of argon. Flash column chromatography was carried out using silica gel (Silicycle R10030B, 60 Å particle size, 230– 400 mesh), applying a low pressure stream of nitrogen. Analytical thin layer chromatographic separations were carried out on silica gel (60 Å particle size, 250 lm thickness, F-254, Silicycle) coated glass plates. Spots were visualized with UV light, or developed by using iodine vapor, or by immersing the plates in 2.0% anisaldehyde in ethanol/sulfuric acid/acetic acid, followed by heating with a heat gun. The NMR spectra were recorded using a 400 MHz Varian Inova instrument. Chemical shifts were reported in parts per million (ppm, δ) relative to the residual ^{1}H resonance of the solvent $CDCl_3$ or CD_3OD at 7.26 ppm or 3.31 ppm, respectively. ^{13}C NMR chemical shifts were reported relative to the central line of $CDCl_3$ or CD_3OD at 77.16 ppm or 49.00 ppm, respectively. Splitting patterns were designated as follows: s, singlet; br s, broad singlet; d, doublet; t, triplet; m, multiplet; quint, quintet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Laboratory.

4.1.1. 2-Iodo-4-methoxy-6-methylpyrimidine (10)

To a stirred solution containing 3.00 g (21.6 mmol) of 2-amino-4-methoxy-6-methylpyrimidine, 5.46 g (21.6 mmol) of iodine, 4.31 g (22.6 mmol) of CuI and 2.50 mL (30.9 mmol) of CH_2I_2 in 120 mL of anhydrous THF was added 10.5 mL (78.2 mmol) of isoamylnitrite. The reaction mixture was stirred at reflux for 3 h. The cooled reaction mixture was filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 5 cm). Elution with hexane followed by 95:5 hexane/Et_2O and then 80:20 hexane/Et_2O afforded **10** as a yellowish solid: yield 2.01 g (37%); mp 43-44 °C; silica gel TLC R_f 0.35 (4:1 hexane/Et_2O); ^{1}H NMR ($CDCl_3$) δ 2.37 (s, 3H), 3.93 (s, 3H) and 6.50 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 23.7, 54.6, 106.5, 127.4, 169.0 and 169.1; mass spectrum (APCI), *m/z* 250.9675 $(M+H)^+$ ($C_6H_8N_2OI$ requires *m/z* 250.9682).

4.1.2. 2-(Azetidin-1-yl)-4-methoxy-6-methylpyrimidine (11)

To a stirred solution containing 560 mg (5.98 mmol) of azetidine hydrochloride, 76.0 mg (0.39 mmol) of CuI, and 3.90 g (11.9 mmol) of Cs_2CO_3 in 10 mL of dry, degassed DMF was added sequentially 1.00 g (3.99 mmol) of **10** and 95.0 mg (0.39 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline. The reaction mixture was stirred at 50 °C for 5 h. The cooled reaction mixture was filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 95:5 hexane/EtOAc and then 85:15 hexane/EtOAc afforded **11** as a yellowish oil: yield 515 mg (72%); silica gel TLC R_f 0.26 (3:2 hexane/EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.25 (s, 3H), 2.30 (quint, 2H, J = 8.0 Hz), 3.84 (s, 3H), 4.11 (t, 4H, J = 7.6 Hz) and 5.83 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz)

δ 16.3, 24.1, 50.2, 53.0, 95.0, 163.2, 168.0 and 170.7; mass spectrum (APCI), *m/z* 180.1136 $(M+H)^+$ ($C_9H_{14}N_3O$ requires *m/z* 180.1137).

4.1.3. 2-(Azetidin-1-yl)-6-hexadecyl-4-methoxypyrimidine (12)

To a stirred solution containing 261 mg (1.45 mmol) of **11** in 7 mL of anhydrous THF at –78 °C was added 870 µL (2.17 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 15 min and then 300 µL (1.03 mmol) of 1-bromopentadecane was added. The reaction mixture was stirred at 0 °C for another 30 min, then quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 95:5 hexane/Et_2O afforded **12** as a yellowish solid: yield 142 mg (25%) and 87 mg (33%) of the starting material was recovered; mp 45-46 °C; silica gel TLC R_f 0.32 (4:1 hexane/Et_2O); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, *J* = 7.2 Hz), 1.18-1.35 (m, 26H), 1.62 (quint, 2H, *J* = 7.2 Hz), 2.29 (quint, 2H, *J* = 7.2 Hz), 2.48 (t, 2H, *J* = 7.6 Hz), 3.82 (s, 3H), 4.10 (t, 4H, *J* = 7.6 Hz) and 5.83 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 16.3, 22.8, 28.7, 29.46, 29.5, 29.6, 29.7, 29.78, 29.8, 32.0, 37.9, 50.2, 52.9, 94.3, 163.3, 170.7 and 172.2; mass spectrum (APCI), *m/z* 390.3481 $(M+H)^+$ ($C_{24}H_{44}N_3O$ requires *m/z* 390.3484).

4.1.4. 2-(Azetidin-1-yl)-5-bromo-4-methoxy-6-hexadecylpyrimidine (13)

To a stirred solution containing 106 mg (0.27 mmol) of **12** in 4 mL of 1:1 CH_2Cl_2/acetonitrile was added 58.0 mg (0.33 mmol) of *N*-bromosuccinimide in the dark. The reaction mixture was stirred for 30 min at room temperature in the dark, then diluted with 50 mL of CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 95:5 hexane/Et_2O afforded **13** as a colorless solid: yield 121 mg (96%); mp 82-83 °C; silica gel TLC R_f 0.55 (4:1 hexane/Et_2O); ^{1}H NMR ($CDCl_3$, 400

MHz) δ 0.88 (t, 3H, *J* = 7.2 Hz), 1.19-1.37 (m, 26H), 1.64 (quint, 2H, *J* = 7.2 Hz), 2.32 (quint, 2H, *J* = 7.2 Hz), 2.69 (t, 2H, *J* = 7.6 Hz), 3.93 (s, 3H) and 4.10 (t, 4H, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$) δ 14.3, 16.3, 22.8, 28.0, 29.5, 29.6, 29.7, 29.8, 29.9, 32.1, 37.0, 50.5, 54.3, 92.7, 161.2, 165.7 and 169.6; mass spectrum (APCI), *m/z* 468.2589 $(M+H)^+$ ($C_{24}H_{43}N_3OBr$ requires *m/z* 468.2589).

4.1.5. 2-(Azetidin-1-yl)-6-hexadecyl-4-methoxypyrimidin-5-ol (2)

To a stirred solution containing 93.0 mg (0.19 mmol) of **13** in 2 mL of anhydrous THF at –5 °C was added 30.0 μL (0.19 mmol) of TMEDA and 198 μL (0.49 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –5 °C for 15 min and then 66.0 μL (0.59 mmol) of trimethoxyborane was added. The reaction mixture was stirred for 30 min at room temperature followed by the addition of 426 μL (4.35 mmol) of H_2O_2 (35% v/v). The reaction mixture was stirred for an additional 30 min and then poured into 20 mL of water, neutralized with dilute aq HCl and extracted with 100 mL of EtOAc. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 90:10 hexane/EtOAc afforded **2** as a yellowish solid: yield 27.0 mg (34%); mp 59-60 °C; silica gel TLC R_f 0.22 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 7.2 Hz), 1.19-1.37 (m, 26H), 1.64 (quint, 2H, *J* = 7.2 Hz), 2.27 (quint, 2H, *J* = 7.2 Hz), 2.61 (t, 2H, *J* = 8.0 Hz), 3.92 (s, 3H), 4.04 (t, 4H, *J* = 7.6 Hz) and 4.61 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.3, 16.3, 22.8, 28.1, 29.5, 29.7, 29.72, 29.8, 29.82, 29.9, 31.5, 32.1, 51.0, 53.6, 128.3, 155.2, 157.6 and 158.6; mass spectrum (APCI), *m/z* 406.3436 $(M+H)^+$ ($C_{24}H_{44}N_3O_2$ requires *m/z* 406.3434).

4.1.6. 2-(Azetidin-1-yl)-4,6-dimethylpyrimidine (14)

To a stirred solution containing 655 mg (6.99 mmol) of azetidine hydrochloride, 133 mg (6.99 mmol) of CuI, and 3.42 g (10.5 mmol) of Cs_2CO_3 in 10 mL of dry, degassed DMF was

added sequentially 500 mg (3.49 mmol) of 2-chloro-4,6-pyrimidine and 165 mg (6.99 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline. The reaction mixture was stirred at 50 °C for 4 h. The reaction mixture was allowed to warm to room temperature and was then filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 4:1 hexane/EtOAc and then 1:1 hexane/EtOAc afforded **14** as yellowish solid: yield 372 mg (65%); mp 51-52 °C; silica gel TLC R_f 0.22 (3:2 hexane/EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.20 (s, 6H), 2.24 (t, 2H, J = 7.6 Hz), 4.05 (t, 4H, J = 7.2 Hz) and 6.19 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2, 23.9, 50.1, 109.1, 163.2 and 167.0; mass spectrum (FAB), *m/z* 164.1192 $(M+H)^+$ ($C_9H_{14}N_3$ requires *m/z* 164.1188).

4.1.7. 2-(Azetidin-1-yl)-6-hexadecyl-4-methylpyrimidine (15)

To a stirred solution containing 321 mg (1.96 mmol) of **14** in 10 mL of anhydrous THF at –78 °C was added 1.02 mL (2.56 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 15 min and then 398 μL (1.37 mmol) of 1-bromopentadecane was added. The reaction mixture was stirred at 0 °C for another 30 min, then quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 96:4 hexane/EtOAc and then 90:10 hexane/EtOAc afforded **15** as a colorless solid: yield 307 mg (42%); mp 63-64 °C; silica gel TLC R_f 0.45 (3:2 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.8 Hz), 1.18-1.37 (m, 26H), 1.62 (quint, 2H, J = 7.6 Hz), 2.27 (s, 3H), 2.29 (quint, 2H, J = 7.6 Hz), 2.49 (t, 2H, J = 7.2 Hz), 4.11 (t, 4H, J = 7.2 Hz) and 6.24 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 16.4, 22.8, 24.2, 28.8, 29.46,

29.5, 29.6, 29.64, 29.75, 29.8, 32.0, 37.9, 50.3, 108.6, 163.4, 167.0 and 171.2; mass spectrum (FAB), *m/z* 374.3545 $(M+H)^+$ ($C_{24}H_{44}N_3$ requires *m/z* 374.3535).

4.1.8. 2-(Azetidin-1-yl)-5-bromo-6-hexadecyl-4-methylpyrimidine (16)

To a stirred solution containing 290 mg (0.77 mmol) of **15** in 5 mL of CH_2Cl_2 was added 152 mg (0.85 mmol) of *N*-bromosuccinimide in the dark. The reaction mixture was stirred for 30 min at room temperature under dark, then diluted with 20 mL of CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 96:4 hexane/EtOAc afforded **16** as a colorless solid: yield 338 mg (97%); mp 74-75 °C; silica gel TLC R_f 0.45 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, *J* = 7.2 Hz), 1.18-1.37 (m, 26H), 1.65 (quint, 2H, *J* = 7.6 Hz), 2.31 (quint, 2H, *J* = 7.6 Hz), 2.44 (s, 3H), 2.71 (t, 2H, *J* = 7.6 Hz) and 4.09 (t, 4H, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 16.3, 22.8, 25.3, 27.8, 29.5, 29.6, 29.7, 29.8, 29.84, 32.1, 37.4, 50.5, 108.6, 161.3, 165.7 and 168.8; mass spectrum (FAB), *m/z* 454.2611 $(M+H)^+$ ($C_{24}H_{43}N_3O^{81}Br$ requires *m/z* 454.2620).

4.1.9. 2-(Azetidin-1-yl)-4-hexadecyl-6-methylpyrimidin-5-ol (3)

To a stirred solution containing 57.0 mg (0.13 mmol) of **16** in 2 mL of anhydrous THF at –5 °C was added 84.0 µL (0.75 mmol) of trimethoxyborane and 156 µL (0.39 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at 23 °C for 30 min followed by the addition of 221 µL (3.25 mmol) of H_2O_2 (50% v/v). The reaction mixture was stirred for additional 30 min and poured into 20 mL water, neutralized with dilute aq HCl and then extracted with 100 mL of EtOAc. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 95:5 hexane/EtOAc followed by 80:20 hexane/EtOAc afforded **3** as a yellowish oil: yield 28.0 mg (55%); silica gel TLC R_f 0.27 (3:2 hexane/EtOAc); ^{1}H NMR (CD_3OD) δ 0.90 (t, 3H, *J* = 6.8 Hz), 1.27-1.32 (m, 26H), 1.64 (m,

2H), 2.25-2.34 (m, 5H), 2.65 (m, 2H), 4.04 (t, 4H, J = 7.6 Hz) and 4.28 (br s, 1H); ^{13}C NMR (CD_3OD) δ 14.5, 17.0, 18.6, 23.8, 29.1, 30.5, 30.6, 30.7, 30.8, 30.81, 30.83, 32.8, 33.1, 52.2, 140.7, 157.6, 159.9 and 161.6; mass spectrum (FAB), *m/z* 390.3480 $(M+H)^+$ ($C_{24}H_{44}N_3O$ requires *m/z* 390.3484).

4.1.10. 2-(Azetidin-1-yl)-4-methyl-6-(pentadecyloxy)pyrimidine (17)

To a stirred solution containing 1.01 g (6.13 mmol) of 2,4-dichloro-6-methylpyrimidine in 20 mL of anhydrous THF was added 620 mg (25.8 mmol) of NaH (60% suspension in oil) and 1.47 g (6.44 mmol) of 1-pentadecanol. The reaction mixture was stirred for 24 h at room temperature and then poured slowly into 100 mL of water. The crude was extracted with two 200-mL portions of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure to afford crude 790 mg of 2-chloro-4-methyl-6-pentadecyloxy)pyrimidine.

To a round bottom flask containing 350 mg (0.99 mmol) of crude 2-chloro-4-methyl-6-(pentadecyloxy)pyrimidine, 139 mg (1.49 mmol) of azetidine hydrochloride, 19.0 mg (0.09 mmol) of CuI, 23.0 mg (0.09 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline and 806 mg (2.48 mmol) of Cs_2CO_3 was added 15 mL of dry, degassed DMF. The reaction mixture was stirred at 50 °C for 5 h. The cooled reaction mixture was filtered through Celite and the Celite pad was washed with portions of CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 19:1 hexane/EtOAc followed by 9:1 hexane/EtOAc afforded **17** as a colorless solid: yield 282 mg (76%); mp 40-41 °C; silica gel TLC R_f 0.27 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.8 Hz), 1.21-1.38 (m, 24H), 1.70 (quint, 2H, J = 7.2 Hz), 2.24 (s, 3H), 2.29 (quint, 2H, J = 7.6 Hz), 4.09 (t, 4H, J = 7.6 Hz), 4.21 (t, 2H, J = 6.8 Hz) and 5.81 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.3, 16.4, 22.9, 24.2, 26.2, 29.1, 29.5, 29.7, 29.76, 29.8, 29.9,

32.1, 50.3, 65.9, 95.3, 163.2, 168.0 and 170.6; mass spectrum (FAB), *m/z* 376.3317 $(M+H)^+$ ($C_{23}H_{42}N_3O$ requires *m/z* 376.3328).

4.1.11. 2-(Azetidin-1-yl)-5-bromo-4-methyl-6-(pentadecyloxy)pyrimidine

To a stirred solution containing 145 mg (0.39 mmol) of **17** in 4 mL CH_2Cl_2 was added 72.0 mg (0.41 mmol) of *N*-bromosuccinimide in the dark. The reaction mixture was stirred for 30 min at room temperature in the dark, then diluted with CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 96:4 hexane/EtOAc afforded 2-(azetidin-1-yl)-5-bromo-4-methyl-6-(pentadecyloxy)pyrimidine as a colorless solid: yield 159 mg (90%); mp 71-72 °C; silica gel TLC R_f 0.53 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, *J* = 6.8 Hz), 1.21-1.47 (m, 24H), 1.75 (quint, 2H, *J* = 7.6 Hz), 2.30 (quint, 2H, *J* = 7.2 Hz), 2.40 (s, 3H), 4.07 (t, 4H, *J* = 7.6 Hz) and 4.30 (t, 2H, *J* = 6.8 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 16.2, 22.8, 24.5, 26.1, 28.9, 29.45, 29.5, 29.7, 29.72, 29.8, 29.83, 32.1, 50.4, 67.1, 93.3, 161.0, 165.3 and 166.0; mass spectrum (FAB), *m/z* 454.2421 $(M+H)^+$ ($C_{23}H_{41}N_3OBr$ requires *m/z* 454.2433).

4.1.12. 2-(Azetidin-1-yl)-4-methyl-6-(pentadecyloxy)pyrimidin-5-ol (4)

To a stirred solution containing 130 mg (0.28 mmol) of 2-(azetidin-1-yl)-5-bromo-4-methyl-6-(pentadecyloxy)pyrimidine in 3 mL of anhydrous THF at –5 °C was added 229 μL (0.57 mmol) of a 2.5 M solution of *n*-BuLi in hexane and 94.0 μL (0.84 mmol) of trimethoxyborane. The reaction mixture was stirred at 23 °C for 30 min followed by the addition of 419 μL (6.16 mmol) of H_2O_2 (50% v/v). The reaction mixture was stirred for an additional 30 min, poured into 20 mL $NaHCO_3$ and then extracted with 100 mL of CH_2Cl_2. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 95:5 hexane/EtOAc afforded **4** as a colorless powder: yield 66.0 mg (60%); mp

83-85 °C; silica gel TLC R_f 0.21 (3:2 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, *J* = 6.8 Hz), 1.05-1.41 (m, 24H), 1.70 (quint, 2H, *J* = 6.8 Hz), 2.15-2.32 (m, 5H), 4.01 (t, 4H, *J* = 7.2 Hz), 4.30 (t, 2H, *J* = 6.8 Hz) and 5.11 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 16.3, 17.8, 22.8, 26.1, 29.0, 29.5, 29.7, 29.74, 29.8, 29.83, 32.1, 50.9, 66.6, 128.6, 151.1, 157.3 and 158.5; mass spectrum (FAB), *m/z* 392.3286 $(M+H)^+$ ($C_{23}H_{42}N_3O_2$ requires *m/z* 392.3277).

4.1.13. 2-Chloro-4-ethoxy-6-methylpyrimidine

To a stirred solution containing 2.01 g (12.3 mmol) of 2,4-dichloro-6-methylpyrimidine in 40 mL of anhydrous THF was added 927 mg (38.6 mmol) of NaH (60% suspension in oil) and 392 μL (12.9 mmol) of EtOH. The reaction mixture was stirred at room temperature for 5 h and then poured slowly into 200 mL of water. The crude reaction mixture was extracted with two 300-mL portions of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 6 cm). Elution with 19:1 hexane/EtOAc afforded 2-chloro-4-ethoxy-6-methylpyrimidine as a colorless solid: yield 2.16 g (51%); mp 37-38 °C; silica gel TLC R_f 0.41 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.38 (t, 3H, *J* = 7.2 Hz), 2.42 (s, 3H), 4.42 (d, 2H, *J* = 7.2 Hz) and 6.46 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.4, 23.8, 63.5, 105.7, 159.8, 169.8 and 170.9; mass spectrum (APCI), *m/z* 173.0477 $(M+H)^+$ ($C_7H_{10}N_2OCl$ requires *m/z* 173.0482).

4.1.14. 2-(Azetidin-1-yl)-4-ethoxy-6-methylpyrimidine (18)

To a round bottom flask containing 600 mg (3.48 mmol) of 2-chloro-4-ethoxy-6-methylpyrimidine, 489 mg (5.22 mmol) of azetidine hydrochloride, 131 mg (0.69 mmol) of CuI, 164 mg (0.69 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline and 2.83 g (8.70 mmol) of Cs_2CO_3 was added 15 mL of dry, degassed DMF. The reaction mixture was stirred at 50 °C for 3 h. The cooled reaction mixture mixture was filtered through Celite and the Celite pad was washed with portions of CH_2Cl_2. The combined organic phase was washed with water

and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 19:1 hexane/EtOAc followed by 9:1 hexane/EtOAc afforded **18** as a colorless solid: yield 565 mg (84%); mp 42-43 °C; silica gel TLC R_f 0.29 (3:2 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.24 (t, 3H, *J* = 7.2 Hz), 2.16 (s, 3H), 2.20 (quint, 2H, *J* = 7.6 Hz), 4.01 (t, 4H, *J* = 7.6 Hz), 4.20 (q, 2H, *J* = 7.2 Hz) and 5.73 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.4, 16.1, 23.9, 49.9, 61.2, 95.0, 163.0, 167.7 and 170.1; mass spectrum (APCI), *m/z* 194.1289 $(M+H)^+$ ($C_{10}H_{16}N_3O$ requires *m/z* 194.1293).

4.1.15. 2-(Azetidin-1-yl)-4-ethoxy-6-hexadecylpyrimidine

To a stirred solution containing 450 mg (2.32 mmol) of **18** in 20 mL of anhydrous THF at –78 °C was added 1.02 mL (2.56 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 15 min and then 475 μL (1.63 mmol) of 1-bromopentadecane was added. The reaction mixture was stirred at 0 °C for another 30 min, then quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 19:1 hexane/EtOAc afforded 2-(azetidin-1-yl)-4-ethoxy-6-hexadecylpyrimidine as a colorless solid: yield 421 mg (45%); mp 40-41 °C; silica gel TLC R_f 0.42 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.84 (t, 3H, *J* = 6.8 Hz), 1.18-1.33 (m, 29H), 1.60 (quint, 2H, *J* = 6.8 Hz), 2.24 (quint, 2H, *J* = 7.6 Hz), 2.44 (t, 2H, *J* = 7.6 Hz), 4.05 (t, 4H, *J* = 7.6 Hz), 4.26 (q, 2H, *J* = 7.2 Hz) and 5.78 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 14.5, 16.2, 22.7, 28.6, 29.39, 29.42, 29.55, 29.61, 29.7, 29.8, 32.0, 37.8, 50.1, 61.3, 94.4, 163.2, 170.2 and 172.0; mass spectrum (FAB), *m/z* 404.3632 $(M+H)^+$ ($C_{25}H_{46}N_3O$ requires *m/z* 404.3641).

4.1.16. 2-(Azetidin-1-yl)-5-bromo-4-ethoxy-6-hexadecylpyrimidine

To a stirred solution containing 464 mg (1.15 mmol) of 2-(azetidin-1-yl)-4-ethoxy-6-hexadecylpyrimidine in 10 mL of CH_2Cl_2 was added 209 mg (1.17 mmol) of *N*-bromosuccinimide in the dark (round bottom flask was wrapped with aluminum foil). The reaction mixture was stirred in the dark for 30 min at room temperature, then diluted with CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 96:4 hexane/EtOAc afforded 2-(azetidin-1-yl)-5-bromo-4-ethoxy-6-hexadecylpyrimidine as a colorless solid: yield 522 mg (94%); mp 69-70 °C; silica gel TLC R_f 0.56 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, *J* = 7.2 Hz), 1.18-1.40 (m, 29H), 1.64 (quint, 2H, *J* = 7.6 Hz), 2.29 (quint, 2H, *J* = 7.6 Hz), 2.69 (t, 2H, *J* = 7.6 Hz), 4.06 (t, 4H, *J* = 7.6 Hz) and 4.37 (q, 2H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 14.5, 16.2, 22.8, 27.9, 29.5, 29.56, 29.58, 29.7, 29.78, 29.83, 32.1, 37.0, 50.3, 62.8, 92.9, 161.1, 165.2 and 169.4; mass spectrum (FAB), *m/z* 482.2753 $(M+H)^+$ ($C_{25}H_{45}N_3OBr$ requires *m/z* 482.2746).

4.1.17. 2-(Azetidin-1-yl)-4-ethoxy-6-hexadecylpyrimidin-5-ol (5)

To a stirred solution containing 400 mg (0.83 mmol) of 2-(azetidin-1-yl)-5-bromo-4-ethoxy-6-hexadecylpyrimidine in 10 mL of anhydrous THF at –5 °C was added 663 μL (1.66 mmol) of a 2.5 M solution of *n*-BuLi in hexane and 278 μL (2.49 mmol) of trimethoxyborane. The reaction mixture was stirred at 23 °C for 30 min followed by the addition of 1.2 mL (18.3 mmol) of H_2O_2 (50% v/v). The reaction mixture was stirred for additional 30 min, poured into 20 mL $NaHCO_3$ and then extracted with 100 mL of CH_2Cl_2. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 95:5 hexane/EtOAc afforded **5** as a colorless powder: yield 250 mg (72%); mp 79-80 °C; silica gel TLC R_f 0.33 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 7.2 Hz), 1.19-1.39 (m, 29H), 1.63 (quint, 2H, *J* = 7.6 Hz), 2.26 (quint, 2H, *J* = 7.2 Hz), 2.61 (t, 2H, *J* = 7.6 Hz), 4.02

(t, 4H, J = 7.6 Hz), 4.37 (q, 2H, J = 7.2 Hz) and 4.89 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.3, 14.7, 16.3, 22.8, 28.1, 29.5, 29.71, 29.73, 29.77, 29.81, 29.85, 31.5, 32.1, 50.9, 62.3, 128.3, 155.1, 157.6 and 158.3; mass spectrum (FAB), *m/z* 420.3578 $(M+H)^+$ ($C_{25}H_{46}N_3O_2$ requires *m/z* 420.3590).

4.1.18 2-(Azetidin-1-yl)-4-isopropoxy-6-methylpyrimidine (19)

To a stirred solution of 1.00 g (16.6 mmol) of isopropanol in 100 mL of freshly distilled THF under argon was added slowly 1.30 g (33.2 mmol) of NaH (60% in paraffin) and the reaction mixture was stirred at room temperature for 30 min. The cooled (0 °C) reaction mixture was treated dropwise with 2.70 g (16.6 mmol) of the 2,4-dichloro-6-methylpyrimidine in 10 mL of distilled THF. The reaction mixture was allowed to warm to room temperature and was kept under argon for 4 h. After the reaction was complete, as judged by silica gel TLC, the reaction mixture was poured slowly into 100 mL of deionized water. The aqueous layer was extracted with three 100-mL portions of ethyl acetate. The combined organic phase was dried ($MgSO_4$) and concentrated to dryness under diminished pressure. The crude product was recovered as a yellowish oil and was used directly for the next step. To 1.00 g (5.36 mmol) of the crude mixture was added 3.50 g (10.7 mmol) of Cs_2CO_3 and 1.00 g (10.7 mmol) of azetidine hydrochloride in 30 mL of dry, degassed DMF. The suspension was stirred under argon at room temperature for 10 min and 127 mg (0.54 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline and 102 mg (0.54 mmol) of copper (I) iodide were added successively to the reaction mixture. The reaction mixture was then warmed to 50 °C and maintained under argon for 12 h. After the reaction was complete as judged by silica gel TLC, the reaction mixture was diluted in 30 mL of ethyl acetate and filtered through Celite. The filtrate was concentrated to dryness. The crude residue was purified by flash chromatography on a silica gel column (15 × 4 cm). Elution with 95:5 hexane/EtOAc to afford **19** as a colorless oil: yield 526 mg (47%); silica gel TLC R_f 0.25 (9:1

hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.24 (s, 3H), 1.26 (s, 3H), 2.18 (s, 3H), 2.25 (quint, 2H, J = 7.5 Hz), 4.05 (t, 4H, J = 7.5 Hz), 5.20 (quint, 1H, J = 6.1 Hz) and 5.74 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 16.2, 21.9, 23.9, 50.1, 68.0, 95.8, 163.11, 167.7 and 169.7; mass spectrum (APCI), *m/z* 208.1447 $(M+H)^+$ ($C_{11}H_{18}N_3O$ requires *m/z* 208.1450).

4.1.19. 2-(Azetidin-1-yl)-6-hexadecyl-4-isoproxypyrimidine (19a)

A stirred solution containing 207 mg (1.00 mmol) of **19** in 10 mL of freshly distilled THF was cooled under argon at –78 °C and kept under argon for 15 min. A solution containing 656 µL (1.05 mmol) of 1.6 M solution of *n*-BuLi in hexane was added dropwise and the resulting reaction mixture was stirred at –78 °C for 1 h. A solution of 306 mg (1.05 mmol) of 1-bromopentadecane in 500 µL of distilled THF was then added dropwise and the reaction mixture was then warmed to 0 °C and stirred for 1 h. The reaction was quenched by adding 30 mL of satd aq NH_4Cl, and was extracted with two 25-mL portions of CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 98:2 to 95:5 hexane/EtOAc afforded 2-(azetidin-1-yl)-6-hexadecyl-4-isoproxypyrimidine as a colorless solid: yield 414 mg (99%); mp 36 °C; silica gel TLC R_f 0.15 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.7 Hz), 1.17-1.29 (m, 26H), 1.29 (s, 3H), 1.30 (s, 3H), 1.61 (quint, 2H, J = 7.5 Hz), 2.28 (quint, 2H, J = 7.5 Hz), 2.46 (m, 2H), 4.08 (t, 4H, J = 7.5 Hz), 5.24 (quint, 1H, J = 6.1 Hz) and 5.78 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 16.3, 22.1, 22.8, 28.7, 29.5, 29.6, 26.7, 29.77, 29.79, 29.81, 32.0, 37.9, 50.2, 68.0, 95.1, 163.4, 169.8 and 172.1, ; mass spectrum (APCI), *m/z* 418.3795 $(M+H)^+$ ($C_{26}H_{48}N_3O$ requires *m/z* 418.3797).

4.1.20. 2-(Azetidin-1-yl)-5-bromo-6-hexadecyl-4-isopropoxypyrimidine

To a stirred solution containing 200 mg (0.49 mmol) of 2-(azetidin-1-yl)-6-hexadecyl-4-isoproxypyrimidine in 7 mL of freshly distilled CH_2Cl_2 at room temperature in the dark was

added 89 mg (0.50 mmol) of recrystallized *N*-bromosuccinimide. The reaction mixture was stirred under argon in the dark at room temperature for 1 h. The solvent was concentrated under diminished pressure and the resulting residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 99:1 to 98:2 hexane/EtOAc afforded 2-(azetidin-1-yl)-5-bromo-6-hexadecyl-4-isopropoxypyrimidine as a colorless solid: yield 240 mg (99%); mp 49 °C; silica gel TLC R_f 0.35 (95:5 hexane/AcOEt); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz), 1.17-1.32 (m, 26H), 1.34 (s, 3H), 1.36 (s, 3H), 1.64 (quint, 2H, J = 7.5 Hz), 2.30 (quint, 2H, J = 7.5 Hz), 2.69 (m, 2H), 4.07 (t, 4H, J = 7.5 Hz) and 5.29 (quint, 1H, J = 6.1 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 16.2, 22.0, 22.8, 28.0, 29.5, 29.61, 29.63, 26.7, 29.81, 29.85, 32.1, 37.2, 50.4, 70.0, 93.4, 161.3, 164.9 and 169.5; mass spectrum (APCI), *m/z* 499.2911 $(M+H)^+$ ($C_{26}H_{47}{}^{79}BrN_3O$ requires *m/z* 496.2902), *m/z* 498.2886 $(M+H)^+$ ($C_{26}H_{47}{}^{81}BrN_3O$ requires *m/z* 498.2886).

4.1.21. 2-(Azetidin-1-yl)-6-hexadecyl-4-isopropoxypyrimidin-5-ol (6) A stirred solution containing 150 mg (0.30 mmol) of 2-(azetidin-1-yl)-5-bromo-6-hexadecyl-4-isopropoxypyrimidine in 3 mL of freshly distilled THF was cooled to –78 °C and maintained under argon for 10 min. To the resulting suspension was added 196 µL (0.31 mmol) of a 1.6 M solution of *n*-BuLi in hexane and the resulting reaction mixture was stirred at –78 °C for 1 h, resulting to a clear, yellowish solution. Then 67 µL (0.6 mmol) of trimethyl borate was added slowly and the reaction mixture was maintained at 0 °C for 1 additional hour. A solution of 150 µL of H_2O_2 (30% v/v) was then added and the reaction mixture was allowed to warm to room temperature and was stirred for 30 min. The reaction mixture was diluted by the addition of 50 mL of satd aq NH_4Cl and extracted with two 30-mL portions of CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 98:2 to 9:1 hexane/EtOAc afforded compound **6** as a colorless solid: yield

96 mg (74%); mp 78 °C; silica gel TLC R_f 0.45 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 6.7 Hz), 1.17-1.31 (m, 26H), 1.31 (s, 3H), 1.33 (s, 3H), 1.63 (quint, 2H, J = 7.5 Hz), 2.25 (quint, 2H, J = 7.5 Hz), 2.61 (m, 2H), 4.02 (t, 4H, J = 7.5 Hz), 4.95 (br s, 1H) and 5.31 (quint, 1H, J = 6.1 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 16.3, 22.1, 22.8, 28.1, 29.5, 29.7, 29.76, 29.80, 29.85, 31.6, 32.1, 50.9, 69.5, 128.4, 155.0, 157.5 and 157.9; mass spectrum (APCI), *m/z* 434.3728 $(M+H)^+$ ($C_{26}H_{48}N_3O_2$ requires *m/z* 434.3747).

General procedure for the preparation of compounds 20a-c

To a stirred solution of 1.40 g (19.4 mmol) of cyclobutanol in 100 mL of freshly distilled THF under argon was slowly added 1.55 g (38.8 mmol) of NaH (60% in paraffin) and the reaction mixture was stirred at room temperature for 30 min. The cooled (0 °C) reaction mixture was treated dropwise with 3.00 g (18.5 mmol) of the 2,4-dichloro-6-methylpyrimidine in solution in 10 mL of distilled THF. The reaction mixture was allowed to warm to room temperature and was maintained under argon for 4 h. After the reaction was complete, as judged by silica gel TLC, the reaction mixture was poured slowly into 100 mL of deionized water. The aqueous layer was extracted with three 100-mL portions of ethyl acetate. The combined organic phase was dried over $MgSO_4$ and concentrated to dryness under diminished pressure. The crude product was recovered as a yellowish oil and was used directly for the next step.

To 1.00 g (5 mmol) of the crude mixture was added 3.25 g (10.0 mmol) of Cs_2CO_3 and 10.0 mmol of the appropriate cyclic amine in 30 mL of dry, degassed DMF. The suspension was stirred under argon at room temperature for 10 min and 118 mg (0.50 mmol) of 3,4,7,8-tetramethyl-1, 10-phenanthroline and 95.0 mg (0.50 mmol) of copper (I) iodide were added successively to the reaction mixture. The reaction mixture was then warmed to 50 °C and maintained under argon for 12 h. After the reaction was completed as judged by silica gel TLC, the reaction mixture was diluted in 30 mL of ethyl acetate and filtered through Celite.

The filtrate was concentrated to dryness. The crude residue was purified by flash chromatography on a silica gel column (15 × 4 cm).

4.1.22. 2-(Azetidin-1-yl)-4-cyclobutoxy-6-methylpyrimidine (20a)

Compound **20a** was prepared using 936 mg (10.0 mmol) of azetidine hydrochloride. The crude residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 9:1 hexane/EtOAc afforded **20a** as a colorless solid: yield 390 mg (35%); mp 60-61 °C; silica gel TLC R_f 0.22 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.58–1.70 (m, 1H), 1.76-1.84 (m, 1H), 2.05-2.17 (m, 2H), 2.24 (s, 3H), 2.29 (qt, 2H, J = 7.4 Hz), 2.38 (m, 2H), 4.08 (t, 4H, J = 7.5 Hz), 5.04 (qt, 1H, J = 7.4 Hz) and 5.77 (s, 1H) ; ^{13}C NMR ($CDCl_3$) δ 13.6, 16.3, 24.2, 30.8, 50.2, 70.1, 95.0, 163.2, 168.2 and 169.6; mass spectrum (APCI), *m/z* 220.1445 $(M+H)^+$ ($C_{12}H_{18}N_3O$ requires *m/z* 220.1450).

4.1.23. 4-Cyclobutoxy-6-methyl-2-(pyrrolidin-1-yl)pyrimidine (20b)

Compound **20b** was prepared using 711 mg (10.0 mmol) of pyrrolidine. The crude residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 95:5 to 9:1 hexane/EtOAc afforded **20b** as a colorless solid: yield 540 mg (46%); mp 47-48°C; silica gel TLC R_f 0.2 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.58-1.7 (m, 1H), 1.75-1.83 (m, 1H), 1.91 (m, 4H), 2.06-2.18 (m, 2H), 2.24 (s, 3H), 2.35-2.45 (m, 2H), 3.53 (m, 4H), 5.08 (qt, 1H, J = 7.5 Hz) and 5.74 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 24.3, 25.6, 30.9, 46.6, 69.9, 94.0, 160.6, 168.0 and 169.2; mass spectrum (APCI), *m/z* 234.1605 $(M+H)^+$ ($C_{13}H_{20}N_3O$ requires *m/z* 234.1606).

4.1.24. 4-Cyclobutoxy-6-methyl-2-(piperidin-1-yl)pyrimidine (20c)

Compound **20c** was prepared using 851 mg (10.0 mmol) of piperidine. The crude residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 98:2 to 95:5 hexane/EtOAc afforded **20c** as a colorless solid: yield 542 mg (44%); mp 49-50°C; silica gel TLC R_f 0.45 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.52-1.7 (m, 7H), 1.75-1.86 (m,

1H), 2.06-2.18 (m, 2H), 2.23 (s, 3H), 2.35-2.45 (m, 2H), 3.74 (m, 4H), 5.08 (qt, 1H, J = 7.4 Hz) and 5.73 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 24.3, 25.1, 25.9, 30.8, 44.9, 69.8, 94.2, 161.8, 168.1 and 169.4; mass spectrum (APCI), *m/z* 248.1766 $(M+H)^+$ ($C_{14}H_{22}N_3O$ requires *m/z* 248.1763).

4.1.25. 2-(Azetidin-1-yl)-4-cyclobutoxy-6-hexadecylpyrimidine (21a)

A stirred solution containing 242 mg (1.07 mmol) of **20a** in 10 mL of freshly distilled THF was cooled under argon at –78°C and maintained under argon for 15 min. A solution of 739 µL (1.18 mmol) of 1.6 M *n*-BuLi in hexane was added dropwise and the resulting reaction mixture was stirred at –78 °C for 1 h. A solution of 319 mg (1.07 mmol) of 1-bromopentadecane in 500 µL of distilled THF was then added dropwise and the reaction mixture was allowed to warm to 0 °C and was stirred for 1 h. The reaction was quenched by the addition of 30 mL of satd aq NH_4Cl, and extracted with two 25-mL portions of CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 98:2 to 95:5 hexane/EtOAc afforded compound **21a** as a colorless solid: yield 389 mg (84%); mp 39-40°C; silica gel TLC R_f 0.5 (9:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 6.6 Hz), 1.20-1.35 (m, 26H), 1.58-1.70 (m, 3H), 1.76-1.85 (m, 1H), 2.07-2.18 (m, 2H), 2.25-2.32 (m, 2H), 2.35-2.45 (m, 2H), 2.70 (t, 2H, J = 7.6 Hz), 4.08 (t, 4H, J = 7.5 Hz), 5.06 (qt, 1H, J = 7.4 Hz) and 5.78 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.2 16.3, 22.8, 28.8, 29.5, 29.5, 29.6, 29.7, 29.8, 29.8, 29.8, 30.8, 32.1, 38.0, 50.2, 70.1, 94.3, 163.4, 169.6 and 172.5; mass spectrum (FAB), *m/z* 430.3786 $(M+H)^+$ ($C_{25}H_{48}N_3O$ requires *m/z* 430.3797).

4.1.26. 4-Cyclobutoxy-6-hexadecyl-2-(pyrrolidin-1-yl)pyrimidine (21b)

A stirred solution containing 200 mg (0.86 mmol) of **21a** in 9 mL of freshly distilled THF was cooled under argon at –78 °C and maintained under argon for 15 min. A solution of 562

μL (0.90 mmol) of 1.6 M *n*-BuLi in hexane of was added dropwise and the resulting reaction mixture was stirred at –78 °C for 1 h. A solution of 262 mg (0.90 mmol) of 1-bromopentadecane in 500 μL of distilled THF was then added dropwise and the reaction mixture was allowed to warm to 0 °C and was then stirred for 1 h. The reaction was quenched by adding 30 mL of satd aq NH_4Cl, and extracted with two 25-mL portions of CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 98:2 to 95:5 hexane/EtOAc afforded compound **21b** as a colorless solid: yield 289 mg (77%); mp 57-58°C, silica gel TLC R_f 0.45 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.9 Hz), 1.18-1.35 (m, 26H), 1.58-1.7 (m, 3H), 1.75-1.85 (m, 1H), 1.92 (m, 4H), 2.08-2.18 (m, 2H), 2.35-2.45 (m, 2H), 2.45-2.52 (m, 2H), 3.54 (m, 4H), 5.10 (qt, 1H, J = 7.5 Hz) and 5.75 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.2, 22.8, 25.6, 28.7, 29.5, 29.66, 29.70, 29.80, 29.84, 30.9, 32.1, 38.02, 46.6, 69.9, 93.2, 160.6, 169.2 and 172.2; mass spectrum (APCI), *m/z* 444.3963 $(M+H)^+$ ($C_{28}H_{50}N_3O$ requires *m/z* 444.3974).

4.1.27. 2-(Piperidin-1-yl)-4-cyclobutanoxy-6-hexadecylpyrimidine (21c)

A stirred solution containing 200 mg (0.81 mmol) of **20c** in 9 mL of freshly distilled THF was cooled under argon at –78 °C and maintained under argon for 15 min. A solution of 530 μL (0.84 mmol) of 1.6 M *n*-BuLi in hexane was added dropwise and the resulting mixture was stirred at –78 °C for 1 h. A solution of 245 mg (0.84 mmol) of 1-bromopentadecane in 500 μL of distilled THF was then added dropwise and the reaction mixture was allowed to warm to 0 °C and was then stirred for 1 h. The reaction was quenched by the addition of 30 mL of satd aq NH_4Cl, and extracted with two 25-mL portions of CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 98:2 hexane/EtOAc afforded compound **21c** as a colorless solid: yield 298 mg (81%);

mp 42-43°C; silica gel TLC R_f 0.65 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 7.0 Hz), 1.2-1.37 (m, 26H), 1.53-1.7 (m, 9H), 1.75-1.86 (m, 1H), 2.08-2.19 (m, 2H), 2.37-2.43 (m, 2H), 2.43-2.51 (m, 2H), 3.75 (m, 4H), 5.09 (qt, 1H, *J* = 7.4 Hz) and 5.73 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.2, 22.8, 25.1, 25.9, 28.6, 29.5, 29.6, 29.7, 29.82, 29.85, 30.8, 32.1, 38.0, 44.9, 69.8, 93.6, 161.9, 169.4 and 172.2; mass spectrum (APCI), *m/z* 458.4110 $(M+H)^+$ ($C_{29}H_{52}N_3O$ requires *m/z* 458.4110).

4.1.28. 2-(Azetidin-1-yl)-5-bromo-4-cyclobutoxy-6-hexadecylpyrimidine (22a)

To a stirred solution containing 340 mg (0.79 mmol) of **21a** in 8 mL of freshly distilled CH_2Cl_2 at room temperature in the dark was added 147 mg (0.83 mmol) of recrystallized *N*-bromosuccinimide. The reaction mixture was stirred under argon for 1 h at room temperature. The solvent was removed under diminished pressure and the residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 99:1 to 98:2 hexane/EtOAc afforded compound **22a** as a colorless solid: yield 389 mg (96%); mp 84 °C silica gel TLC R_f 0.25 (9:1 hexane/EtOAc); mp 71-73°C silica gel TLC R_f 0.5 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 6.6 Hz), 1.2-1.35 (m, 26H), 1.58-1.70 (m, 3H), 1.78-1.86 (m, 1H), 2.13-2.22 (m, 2H), 2.25-2.33 (m, 2H), 2.39-2.46 (m, 2H), 2.67-2.71 (m, 2H), 4.06 (t, 4H, *J* = 7.5 Hz) and 5.13 (qt, 1H, J = 7.4Hz); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.2, 16.2, 22.8, 28.0, 29.5, 29.6, 29.7, 29.8, 29.8, 29.81, 29.9, 30.8, 32.1, 37.0, 50.3, 71.3, 92.7, 161.1, 164.7 and 169.5; mass spectrum (FAB), *m/z* 508.2897 $(M+H)^+$ ($C_{25}H_{47}BrN_3O$ requires *m/z* 508.2902).

4.1.29. 5-Bromo-4-cyclobutoxy-6-hexadecyl-2-(pyrrolidin-1-yl)pyrimidine (22b)

To a stirred solution containing 280 mg (0.63 mmol) of **21b** in 8 mL of freshly distilled CH_2Cl_2 at room temperature in the dark was added 113 mg (0.63 mmol) of recrystallized *N*-bromosuccinimide. The reaction mixture was stirred under argon for 1 h at room temperature. The solvent was removed under diminished pressure and the residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 99:1 to 98:2 hexane/EtOAc

afforded compound **22b** as a colorless solid: yield 313 mg (95%); mp 84 °C silica gel TLC R_f 0.25 (9:1 hexane/EtOAc); mp 70-71°C silica gel TLC R_f 0.55 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.9 Hz, 3H), 1.18-1.35 (m, 26H), 1.59-1.7 (m, 3H), 1.83 (m, 1H), 1.94 (m, 4H), 2.15-2.26 (m, 2H), 2.40-2.48 (m, 2H), 2.67-2.72 (m, 4H), 3.50 (m, 4H) and 5.16 (qt, J = 7.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.3, 22.8, 25.7, 27.9, 29.5, 29.59, 29.64, 29.74, 29.81, 29.83, 29.86, 30.9, 32.1, 37.0, 46.8, 71.2, 91.3, 158.4, 164.3 and 169.3; mass spectrum (APCI), *m/z* 522.3046 $(M+H)^+$ ($C_{28}H_{49}BrN_3O$ requires *m/z* 522.3059).

4.1.30. 5-Bromo-4-cyclobutoxy-6-hexadecyl-2-(piperidin-1-yl)pyrimidine (22c)

To a stirred solution containing 200 mg (0.44 mmol) of **21c** in 5 mL of freshly distilled CH_2Cl_2 at room temperature in the dark was added 80.1 mg (0.45 mmol) of recrystallized *N*-bromosuccinimide. The reaction mixture was stirred under argon for 1 h at room temperature. The solvent was removed under diminished pressure and the residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 99:1 to 98:2 hexane/EtOAc afforded compound **22c** as a colorless solid: yield 226 mg (96%); mp 60-61°C. silica gel TLC R_f 0.7 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 7.0 Hz), 1.22-1.39 (m, 26H), 1.53-1.59 (m, 4H), 1.60-1.71 (m, 5H), 1.79-1.88 (m, 1H), 2.15-2.25 (m, 2H), 2.40-2.48 (m, 2H), 2.68 (m, 2H), 3.71 (m, 4H) and 5.14 (qt, 1H, J = 7.4 Hz); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.3, 22.8, 25.0, 25.8, 27.7, 29.57, 29.63, 29.76, 29.86, 30.8, 32.1, 37.0, 45.1, 71.1, 91.3, 159.6, 164.4 and 169.2; HRMS (APCI+), *m/z* 536.3216 $(M+H)^+$ ($C_{29}H_{51}BrN_3O$ requires *m/z* 536.3215).

4.1.31. 2-(Azetidin-1-yl)-4-cyclobutoxy-6-hexadecylpyrimidin-5-ol (7)

A stirred solution containing 340 mg (0.67 mmol) of **22a** in 7 mL of freshly distilled THF was cooled to –78 °C and maintained under argon for 10 min. To the resulting suspension was added 458 µL (0.73 mmol) of 1.6 M *n*-BuLi in hexane and the reaction mixture was stirred at –78 °C for 1 h resulting in a clear, yellowish solution. Then 120 µL (1.34 mmol) of trimethyl

borate was added slowly and the reaction was maintained at 0 °C for 1 additional hour. A solution of 300 µL of H_2O_2 (30% v/v) was then added and the reaction mixture was allowed to warm to room temperature and stirred for 30 min. The reaction mixture was diluted by the addition of 50 mL of satd aq NH_4Cl and extracted with two 30-mL portions of CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 1 cm). Elution with 98:2 to 9:1 hexane/EtOAc to afford compound **7** as a colorless solid: yield 248 mg (84%); mp 95-97°C; silica gel TLC R_f 0.42 (4:1 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 6.6 Hz), 1.2-1.35 (m, 26H), 1.55-1.70 (m, 3H), 1.83 (m, 1H), 2.06-2.16 (m, 2H), 2.26 (quint, 2H, *J* = 7.2 Hz), 2.37-2.45 (m, 2H), 2.61 (m, 2H), 4.01 (t, 4H, *J* = 7.2 Hz), 4.76 (br s, 1H) and 5.17 (qt, 1H, *J* = 7.4Hz); ^{13}C NMR ($CDCl_3$) δ 13.6, 14.3, 16.3, 22.8, 28.2, 29.5, 29.71, 29.73, 29.8, 29.81, 29.86, 29.9, 30.9, 31.5, 32.1, 50.9, 70.8, 128.1, 155.2, 157.6 and 157.8; mass spectrum (FAB), *m/z* 446.3742 $(M+H)^+$ ($C_{25}H_{48}N_3O_2$ requires *m/z* 446.3747).

4.1.32. 4-Cyclobutanoxy-6-hexadecyl-2-(pyrrolidin-1-yl)pyrimidin-5-ol (8)

A stirred solution containing 150 mg (0.29 mmol) of **22b** in 4 mL of freshly distilled THF was cooled to –78 °C and maintained under argon for 10 min. To the resulting suspension was added 200 µL (0.31 mmol) of 1.6 M *n*-BuLi in hexane and the reaction mixture was stirred at –78 °C for 1 h resulting in a clear, yellowish solution. Then 70 µL (0.62 mmol) of trimethyl borate was added slowly and the reaction was maintained at 0 °C for 1 additional hour. A solution of 300 µL of H_2O_2 (30% v/v) was then added and the reaction mixture was allowed to warm to room temperature and stirred for 30 min. The reaction mixture was diluted by the addition of 50 mL of satd aq NH_4Cl and extracted with two 30-mL portions of CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 1 cm). Elution with 98:2 to 9:1 hexane/EtOAc afforded compound **8** as a colorless solid: yield 82 mg (63%);

mp 74-76°C; silica gel TLC R_f 0.2 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 6.9 Hz), 1.18-1.38 (m, 26H), 1.60-1.70 (m, 3H), 1.83 (m, 1H), 1.92 (m, 4H), 2.07-2.20 (m, 2H), 2.38-2.49 (m, 2H), 2.62 (m, 2H), 3.48 (m, 4H), 4.51 (br s, 1H) and 5.19 (qt, 1H, *J* = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 13.7, 14.3, 22.8, 25.8, 27.8, 28.1, 29.5, 29.73, 29.78, 29.81, 29.86, 31.0, 31.5, 32.1, 46.9, 70.6, 126.9, 154.5, 155.3 and 157.4; mass spectrum (APCI), *m/z* 460.6176 $(M+H)^+$ ($C_{28}H_{50}N_3O_2$ requires *m/z* 460.6176).

4.1.33. 4-Cyclobutanoxy-6-hexadecyl-2-(piperidin-1-yl)pyrimidin-5-ol (9)

A stirred solution containing 150 mg (0.28 mmol) of **22c** in 4 mL of freshly distilled THF was cooled to –78 °C and maintained under argon for 10 min. To the resulting suspension was added 193 µL (0.31 mmol) of 1.6 M *n*-BuLi in hexane and the reaction mixture was stirred at –78 °C for 1 h resulting in a clear, yellowish solution. Then 67 µL (0.62 mmol) of trimethyl borate was added slowly and the reaction was maintained at 0 °C for 1 additional hour. A solution of 300 µL of H_2O_2 (30% v/v) was then added and the reaction mixture was allowed to warm to room temperature and stirred for 30 min. The reaction mixture was diluted by the addition of 50 mL of satd aq NH_4Cl and extracted with two 30-mL portions of CH_2Cl_2. The combined organic phase was dried over $MgSO_4$ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 1 cm). Elution with 98:2 to 9:1 hexane/EtOAc afforded compound **9** as a colorless solid: yield 83 mg (58%); mp 78-79°C; silica gel TLC R_f 0.35 (95:5 hexane/EtOAc); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, *J* = 7.0 Hz), 1.22-1.39 (m, 26H), 1.52-1.70 (m, 9H), 1.79-1.88 (m, 1H), 2.09-2.20 (m, 2H), 2.39-2.48 (m, 2H), 2.60 (m, 2H), 3.63 (m, 4H), 4.46 (br s, 1H) and 5.18 (qt, 1H, *J* = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 13.6, 14.3, 22.8, 25.1, 25.8, 27.8, 29.52, 29.68, 29.70, 29.78, 29.82, 29.86, 30.9, 31.5, 32.1, 45.6, 70.6, 127.3, 155.0, 155.6 and 157.2; mass spectrum (APCI), *m/z* 474.4039 $(M+H)^+$ ($C_{29}H_{52}N_3O_2$ requires *m/z* 474.4060)

4.2. Biochemical and biological evaluation of pyrimidinol analogues

4.2.1. Cell lines and culture conditions

A human mitochondrial disease cell line, Friedreich's ataxia lymphocytes (GM15850), was obtained from Coriell Cell Repositories (Camden, NJ). Lymphocytes were cultured in RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY) with 15% fetal calf serum, 2 mM glutamine (HyClone, South Logan, UT) and 1% penicillin–streptomycin antibiotic supplement (Cellgro, Manassas, VA). Cells were passaged every other day to maintain them in log phase growth and kept at a nominal concentration of 5–10 × 10^5 cell/mL. A nutrient sensitized screening strategy to identify CoQ_{10} analogues that function within the mitochondrial respiratory chain was used by growing the CoQ_{10}-deficient lymphocyte in galactose-containing media to force energy production predominantly through oxidative phosphorylation rather than glycolysis. The lymphocytes were cultured in RPMI 1640 glucose free medium (Gibco, Grand Island, NY) supplemented with 25 mM galactose, 2 mM glutamine and 1% penicillin–streptomycin, and 10% dialyzed fetal bovine serum (FBS) (<0.5 μg/mL) (Gemini Bio-Product, West Sacramento, CA).

4.2.2. Lipid peroxidation assay

Lipid peroxidation was measured by a quantitative FACS assay using the oxidation-sensitive fatty acid probe C_{11}-BODIPY$^{581/591}$ (Molecular Probe) as described.[17, 18] The degree of probe oxidation was followed using flow cytometry. Briefly, FRDA lymphocytes (5 × 10^5 cell/mL) were plated (1 mL in 24-well plates), treated with the test compounds and incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2 in air. The following day, cells were treated with 1 μM of C_{11}-BODIPY$^{581/591}$ probe in phenol red-free media and incubated at 37 °C in the dark for 30 min. Oxidative stress was induced with 5 mM DEM in phenol red-free RPMI-1640 media for 120 min. Cells were collected by centrifugation at 300 × *g* for 3 min and then washed with phosphate buffered saline (PBS). Cells were resuspended in phosphate buffered saline and were analyzed immediately by

FACS (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the FL1-H channel 530 ± 15 nm emission filter. The generation of lipid peroxide was detected as a result of the oxidation of the polyunsaturated butadienyl portion of the dye, resulting in a shift of the fluorescence emission peak from red to green. In each analysis, 10,000 events were recorded after cell debris was electronically gated out. Data are reported as means ± S.E.M. (n = 3). Results were expressed as a percentage of the median mean fluorescence intensity of C_{11}-BODIPY-green relative to the treated control.

4.2.3. Reactive oxygen species (ROS) assay

Quantitative analysis of intracellular ROS levels in FRDA lymphocytes, challenged with 5 mM diethyl maleate (DEM) in presence or absence of the test compounds, was obtained by FACS analysis using a dichlorodihydrofluorescein diacetate probe (DCFH-DA), as described previously.[19,31] Briefly, 1 mL of FRDA lymphocytes (5×10^5 cells) was plated in a 24-well plate, treated with the test compounds and incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 5 mM diethyl maleate (DEM) for 80 min, collected by centrifugation at 300 × g for 3 min and then washed with phosphate buffered saline (Life Technologies). Cells were resuspended in PBS containing 20 mM glucose and incubated at 37 °C in the dark for 25 min with 10 μM DCFH-DA. Cells were collected by centrifugation at 300 × g for 3 min and then washed with PBS. The samples were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the FL1-H channel 530 ± 15 nm emission filter. The generation of ROS, mainly peroxides, was detected as a result of the oxidation of DCFH. In each analysis, 10,000 events were recorded after cell debris was electronically gated out. Results obtained were verified by running duplicates and repeating experiments in three independent runs. Results were expressed as a percentage of the median mean fluorescence intensity of DCF relative to the treated control.

4.2.4. Preservation of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential of FRDA lymphocytes was assessed using the fluorescence probe Mitotracker TMRM (tetramethylrhodamine methyl ester; Molecular Probes, Portland, OR) as described previously.[18,19] TMRM is a lipophilic potentiometric dye which partitions between the mitochondria and cytosol in proportion to the negative membrane potential across the inner mitochondrial membrane, in accordance with the Nernst equation.[23] Therefore, the accumulation of dye in the mitochondria and the intensity of the signal is a direct function of mitochondrial membrane potential. Mitochondrial depolarization then causes the redistribution of dye from mitochondria into the cytosol, causing a change in signal intensity. The detection of mitochondrial depolarization using TMRM was accomplished by flow cytometry as described before.[18,19] Briefly, FRDA lymphocytes cells (5×10^5 cells) were pre-treated 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 washed with phosphate buffered saline. The cells were resuspended in PBS containing 20 mM glucose 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 washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline supplemented with 20 mM glucose and were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA), using a 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, was determined and analyzed using C6 Accuri software (BD Biosciences). The results obtained were verified in three independent experiments. FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), a mitochondrial uncoupler, was used to produce a negative control. The results were verified by repeating the experiments in duplicate.

4.2.5. Cellular ATP concentration assay

The intracellular ATP content was determined by a bioluminescence assay measuring the light output from the luciferin-luciferase reaction as described previously.[18,19] Briefly, FRDA lymphocytes (2×10^5 cell/mL) were plated (1 mL in 24-well plates) in glucose-free media supplemented with galactose and treated with the test compounds at final concentrations of 1, 5 and 25 μM, and then incubated at 37 °C for 48 h in a humidified atmosphere containing 5% CO_2 in air. Wells were mixed and cells in each well were transferred (100 μL) to 96-well microtiter white-walled cell culture plates (Costar, Corning, NY). The total intracellular ATP level was measured in a luminator (Clarity™ luminescence microplate reader) using an ATP bioluminescence assay kit (ViaLight-Plus ATP monitoring reagent kit, Lonza, Walkersville, MD) following the manufacturer's protocol. The total ATP level was expressed as a percentage of untreated control. Data are reported as the mean of at least three independent runs.

4.2.6. Cytoprotection (FACS analysis Live/ Dead® Viability/Cytotoxicity assay)

The cytoprotection conferred by the pyridinol derivatives (**1**–**9**) was determined in FRDA lymphocytes by using a simultaneous staining with a two-color fluorescence FACS assay, the Live/ Dead® Viability/Cytotoxicity Kit (Molecular Probes). This assay is used to measure two recognized parameters of cell viability, intracellular esterase activity and plasma integrity. The membrane-impermeant DNA dye ethidium homodimer-1 (EthD-1) was used to identify dead cells whose plasma membrane integrity was disrupted. The membrane-permeant dye calcein-AM was used to label live cells. It penetrates into the cells, where it is metabolized by cytoplasmic esterases and becomes a fluorescent but membrane-impermeant probe which is retained in viable cells. Briefly, FRDA lymphocyte cells were seeded at a density of 5×10^5 cells/mL and treated with different concentrations of the test compounds. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 16 h. Oxidative stress was

then induced by incubation with 5 mM DEM for 6 h, followed by evaluation of cytoprotection. Cells were collected by centrifugation at 300 × *g* for 3 min and washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline containing 25 mM galactose. The cell suspension was stained with 0.1 µM calcein AM and 0.1 µM EthD-1 and incubated in the dark at 37 °C for 15 min. Cells were collected by centrifugation at 300 × *g* for 3 min and then washed with PBS. The samples were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the FL1-H channel 530 ±15 nm emission filter and the FL2-H channel 585 ±15 nm. For each analysis 10,000 events were recorded and analyzed using C6 Accuri software (BD Biosciences). Cytoprotection by the test compounds was assessed with respect to the untreated controls. Cells not treated with DEM had >90% cell viability whereas DEM treatment reduced cell viability to <20%. The cell viability was expressed relative to the vehicle control (DMSO only) group (n = 3).

4.2.7. Microsomal stability assay

Bovine liver microsomes were prepared from the liver of a freshly slaughtered animal as described previously.[19] *In vitro* metabolic stability was determined in bovine liver microsomes at a protein concentration of 1 mg/mL in 50 mM phosphate buffer mixture, pH 7.4, containing 5 mM $MgCl_2$ in a final incubation volume of 0.5 mL. Each test compound was added to a final concentration of 25 µM. This mixture was pre-warmed to 37 °C prior to starting the reaction by the addition of β-NADPH to 1 mM final concentration. After incubation for 30 min at 37 °C, the reaction was quenched by the addition of 1 mL of propanol, vortexed for 2 min and centrifuged at 15,000 × g for 10 min to pellet the precipitated protein. The resulting supernatant was pipetted out and then concentrated under diminished pressure. A parallel incubation of the test compound with deactivated microsomes (quenched immediately with propanol) lacking β-NADPH served as a control and was run for

each test agent to detect microsome-independent degradation. The sample was reconstituted in 130 μL of MeOH and centrifuged again at 15,000 × g for 3 min. The supernatant was removed and 4 μM fluorene was added as an internal standard before HPLC analysis. HPLC analyses were performed on a Zorbax SB-phenyl reversed phase analytical (150 × 4.6 mm, 5 μm) HPLC column using a mobile phase consisting of MeOH/H_2O. A linear gradient of (50:50 MeOH/H_2O → 100:0 MeOH/H_2O) was employed over a period of 14 min at a flow rate of 1 mL/min. Metabolic stability was expressed as percent of control remaining. The experiments were carried out in quadruplicate to verify the results.

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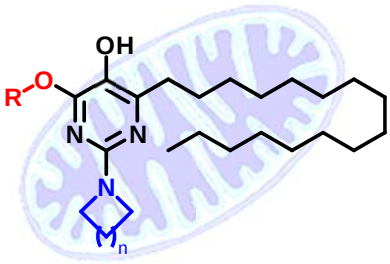
Graphical abstract



- ✓ Protection from lipid peroxidation
- ✓ ROS suppression
- ✓ Preservation of $\Delta\psi_m$
- ✓ Support ATP production
- ✓ Cytoprotective
- ✓ Metabolically stable