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Analysis of the structural and mechanistic factors in antioxidants that preserve mitochondrial function and confer cytoprotection

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ABSTRACT

Selected pyridinol analogues of the experimental neuroprotective drug idebenone have been synthesized and evaluated as antioxidants capable of preserving mitochondrial function. The compounds, having a different redox core but the same side chain as idebenone, exhibited a range of potencies, reflecting differences in their structures. The results obtained provide guidance in the design of such analogues with improved properties. Analogues were identified that have significantly improved antioxidant activity compared with idebenone in cultured lymphocytes, and which exhibit lesser inhibition of the electron transport chain.

1. Introduction

Mitochondria play a central role in the cellular production of ATP, and in a range of other metabolic processes, including signaling, cell growth, differentiation and death.[1–3] Mitochondria are also involved in the production of reactive oxygen species through electron carriers in the respiratory chain;[4–6] and the mitochondrion itself is quite susceptible to oxidative stress.[7,8] A number of human pathologies result from some mitochondrial dysfunction, including hereditary mitochondrial cytopathies, diabetes, cardiovascular disease and neurodegenerative diseases.[9–16] One of the neurodegenerative diseases, Friedreich's ataxia (FRDA), is the most common autosomal recessive ataxia in the Caucasian population,[17] and has been estimated to affect ~1 in 50,000 people in the United States. There is currently no effective cure or treatment available. Friedreich's ataxia disease is believed to arise from reduced synthesis of the iron chaperone frataxin due to impaired gene transcription.[17,18] This leads to mitochondrial iron accumulation, dysfunction of mitochondrial Fe–S containing enzymes, and increased Fenton-mediated reactive oxygen species (ROS) production.[19,20] An actively pursued strategy for FRDA treatment at the present time involves the use of antioxidants to slow the progression of the mitochondrial degradation resulting from oxidative stress.[21–23] While not all such compounds are targeted to the mitochondria, they have been shown to preserve the function of mitochondria under conditions of oxidative stress. Idebenone (Catena®), a synthetic analogue of coenzyme Q_{10}, is currently the only compound that has reached Phase III clinical trials for the treatment of FRDA.[24] The pharmacological and pharmacokinetic behavior of idebenone has been described.[25] However, the clinical efficacy of idebenone is uncertain,[24,26–28] and the degree of improvement may be limited by the dose currently administered, suggesting that higher doses may be necessary to have a beneficial effect on neurological function.[29] Unfortunately, biochemical studies indicate that idebenone is a significant inhibitor of both the electron transport and proton pumping activities of mitochondrial respiratory chain complex I, and could thus potentially stimulate oxygen radical production in mitochondria if used at high doses.[30,31] Accordingly, alternative approaches may have to be considered. Recently, a few nitrogen heterocycles, including pyrimidinols and pyridinols, have been investigated and have exhibited strong antioxidant activity in vitro, as judged by their ability to scavenge peroxyl radical formation in benzene solution. Some of these have relatively high stability in air, consistent with potential therapeutic utility.[32]

To facilitate the delivery of such molecules to the mitochondria of cells, and enable their permeability in plasma, we have designed antioxidants bearing lipophilic side chains and having pyrimidinol or pyridinol redox cores.[33,34] An earlier study focused on one pyrimidinol derivative (compound **6**, Fig. 1)[34] showed encouraging results, suggesting that further optimization of the redox core might afford improved efficacy. Currently, we describe the preparation and characterization of several novel pyridinol derivatives having the same side chain as idebenone. This includes an analysis of structural factors among these pyridinol analogues that afford

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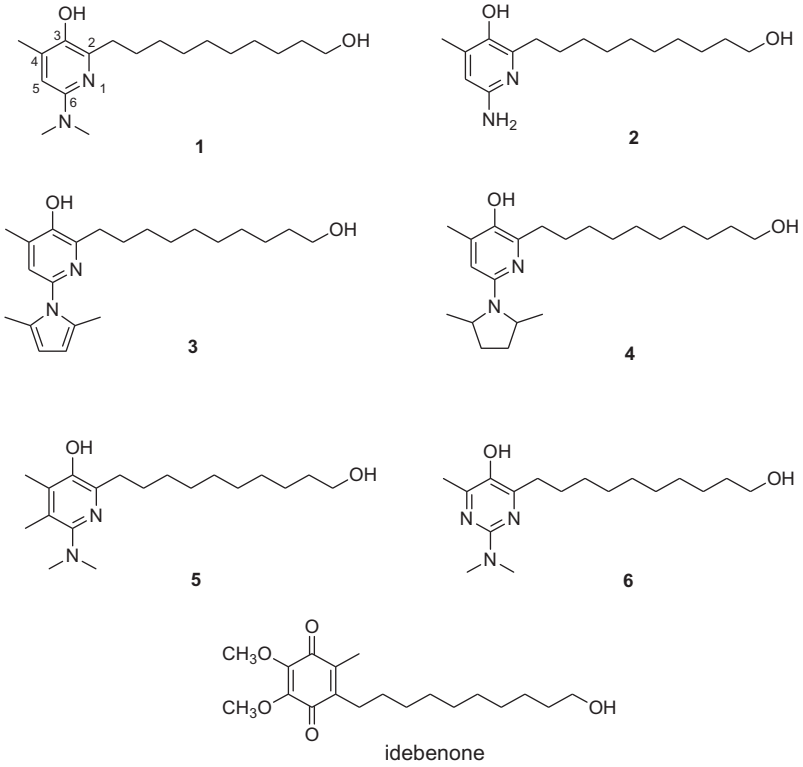


Figure 1. Chemical structures of compounds **1–6** and idebenone.

effective cytoprotection, in comparison with the results obtained with idebenone and compound **6**.

2. Results and discussion

The approach used for the preparation of this series of antioxidants followed the same general strategy employed for the synthesis of compound **6**.[34]

2.1. Synthesis of pyridinol analogue 1

6-(Dimethylamino)-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (**1**) was synthesized in 8 steps (Scheme 1). First, 6-amino-3-bromo-2,4-dimethylpyridine (**8**) was prepared in 84% yield by treating commercially available 6-amino-2,4-dimethylpyridine with *N*-bromosuccinimide. Compound **8** was then treated with 2,5-hexanedione in presence of *p*-toluenesulfonic acid to afford pyrrole-substituted pyridine **9** in 62% yield. Replacement of the halide moiety by successive treatments with KOH/$Pd_2(dba)_3$ and phosphine ligand L_1, and then with benzyl bromide, afforded **10** in 82% yield. The fully protected pyridinol core, 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4-dimethylpyridine (**10**), was then treated with phenyllithium in the presence of 1-bromo-9-(methoxymethoxy)nonane (**7**) to afford fully protected **11** in 60% yield. None of the regioisomer resulting from alkylation of the other pyridine methyl group was observed. Compound **11** was deprotected by treatment with hydroxylamine hydrochloride in the presence of KOH to obtain 6-aminopyridinol **12** in 56% yield. This compound was treated with methyl iodide in presence of sodium hydride to obtain 3-(benzyloxy)-6-(dimethylamino)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (**13**) in 21% yield. Pyridinol analogue **1** was then obtained in 77% yield by treating **13** successively with HCl and then with palladium hydroxide under a H_2 atmosphere.

2.2. Synthesis of pyridinol analogue 2

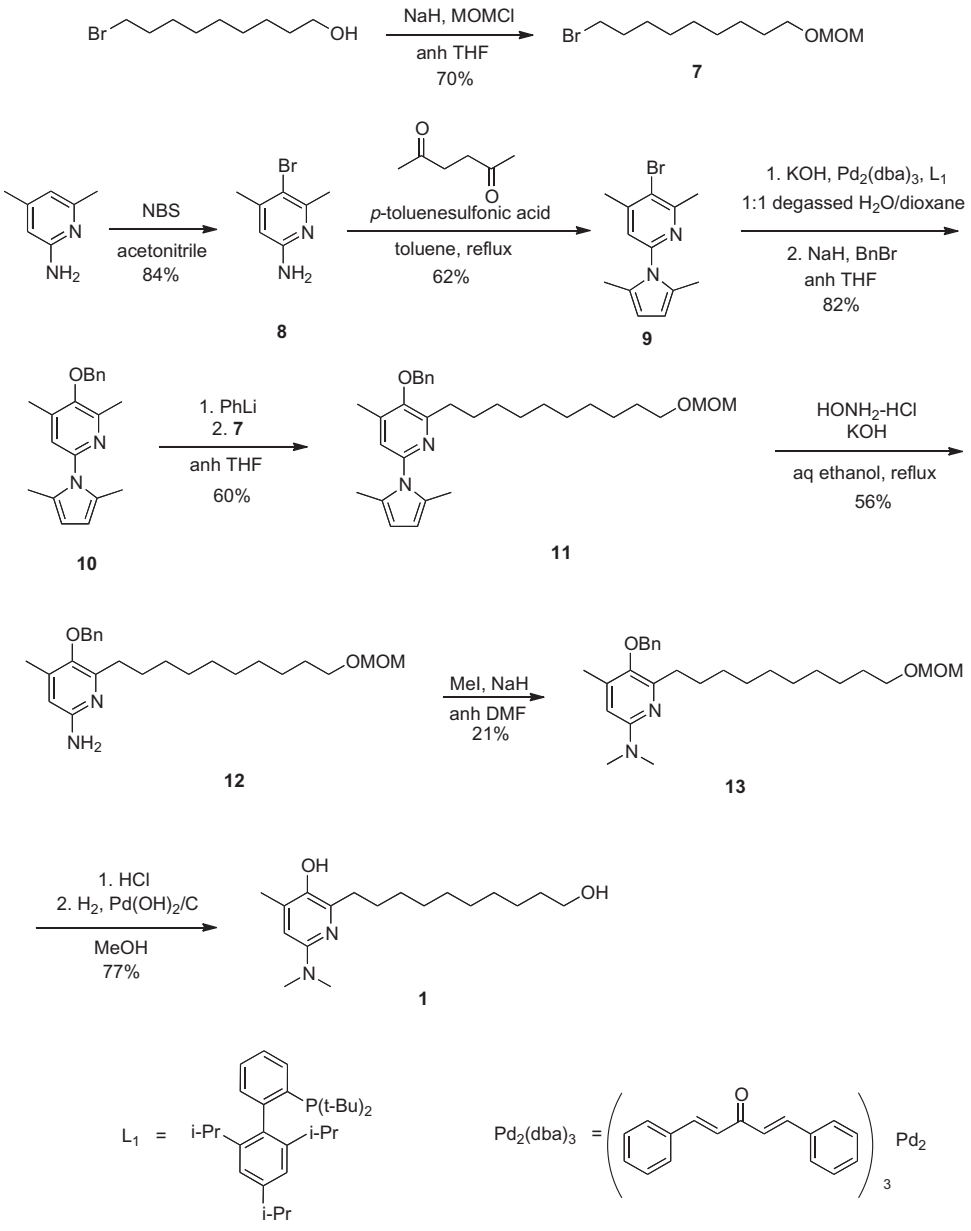
Analogue **2** was prepared from 6-amino-3-(benzyloxy)-6-[10-(methoxymethoxy)decyl]-4-methylpyridine (**12**) by deprotection of the aliphatic hydroxyl group under acidic conditions, followed by hydrogenolysis in 29% yield (Scheme 2).

2.3. Synthesis of pyridinol analogues 3 and 4

Pyridinol analogues **3** and **4** were both synthesized from 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (**11**) (Scheme 2). Treatment under acidic conditions effected removal of the MOM protecting group. Hydrogenolysis over 20% palladium hydroxide on carbon in MeOH at room temperature for 15 min afforded **3** in 17% yield. Hydrogenolysis under the same conditions for 16 h afforded **4**, albeit only in low (5%) yield.

2.4. Synthesis of pyridinol analogue 5

6-(Dimethylamino)-2-(10-hydroxydecyl)-4,5-dimethylpyridin-3-ol (**5**) was synthesized in seven steps as shown in Scheme 3. First, 6-amino-2,4,5-trimethylpyridin-3-ol (**14**) was synthesized by a literature procedure.[35] The exocyclic amine of **14** was then protected by treatment with 2,5-hexanedione in presence of *p*-toluenesulfonic acid to afford pyrrole **15** in 75% yield. *O*-Benzylation using NaH/BnBr gave **16** in 78% yield. Subsequent treatment of **16** with *n*-BuLi in the presence of the 1-bromo-9-(methoxymethoxy)nonane (**7**) gave fully protected analogue **17** in 41% yield. Compound **17** was deprotected by treatment with hydroxylamine hydrochloride in presence of KOH, affording **18** in 13% yield. Treatment of **18** with formaldehyde solution and sodium cyanoborohydride in presence of acetic acid gave **19** in 77% yield. Deprotection of **19** to afford **5** was accomplished in 79% yield by successive



Scheme 1. Route employed for the synthesis of compound **1**.

treatments with HCl and then with palladium hydroxide under a hydrogen atmosphere.

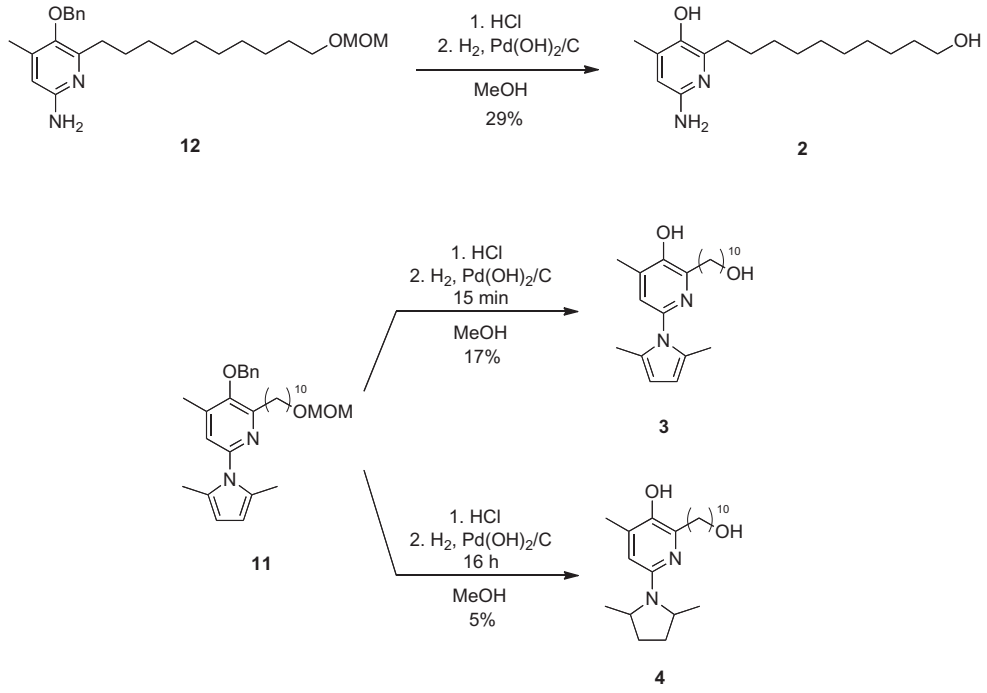
2.5. Biochemical and biological evaluation of the pyridinol analogues

2.5.1. Inhibition of lipid peroxidation

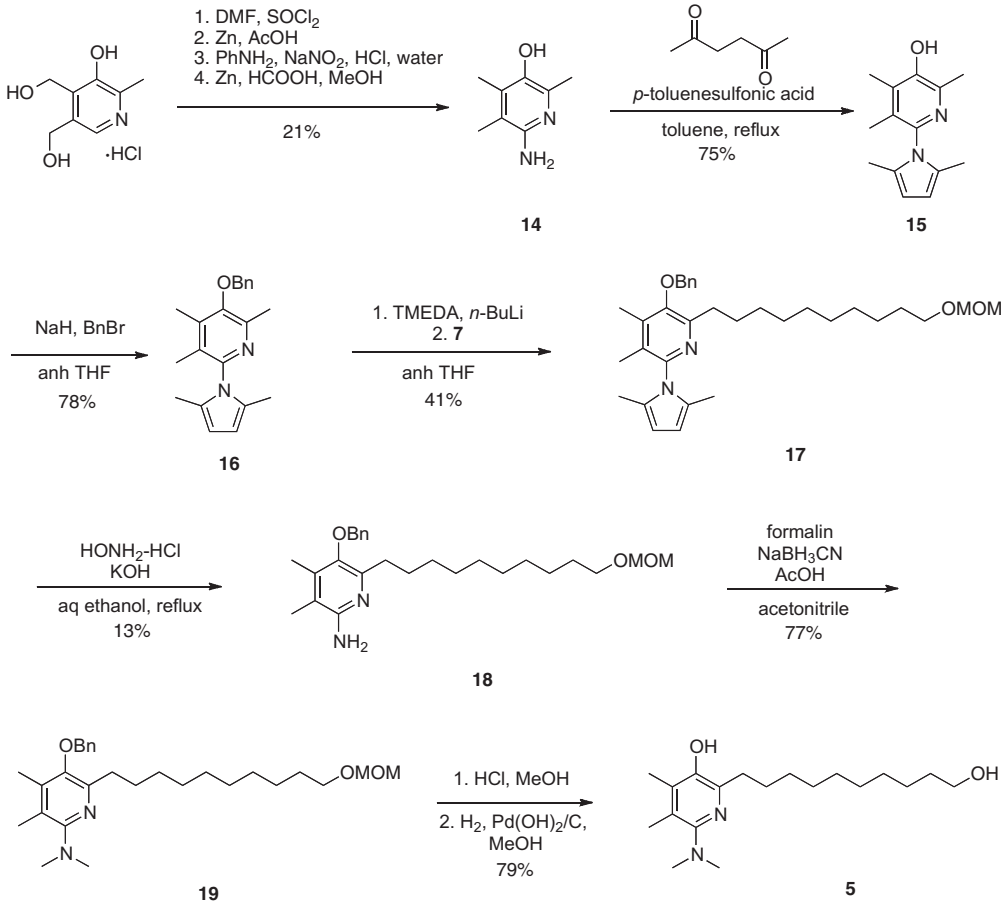
The ability of the pyridinol analogues to quench lipid peroxidation has been studied in FRDA lymphocytes depleted of glutathione by treatment with diethyl maleate (DEM). The cells were pre-incubated with the test compounds for 16 h prior to DEM treatment. Lipid peroxidation was measured by a quantitative FACS analysis using the fluorescent probe, C_{11}-BODIPY$^{581/591}$, reported to be highly accurate in measuring lipid peroxidation.[36] The results, presented in Table 1, show the most potent activity for compounds **4** and **5**. Both of these compounds were more potent than pyrimidinol **6**. Compound **1**, lacking a methyl group at position 6, was slightly more potent than idebenone, and better than compound **2**. However, the redox core of **1** lacked any activity. Finally, compound **3** had the lowest activity for suppressing lipid peroxidation among the analogues having a 10-hydroxydecyl side chain.

2.5.2. Suppression of reactive oxygen species

The ability of the pyridinol analogues to suppress ROS induced by depletion of glutathione was evaluated in CEM leukemia cells. ROS was measured in a quantitative FACS experiment, using dichlorofluorescein diacetate (DCFH-DA) as a substrate for determining intracellular oxidant production. DCFH-DA is hydrolyzed by esterases to afford 2,7-dichlorodihydrofluorescein (DCFH), the latter of which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by the action of cellular oxidants. The results are presented in Figure 2, and show that analogue **4** was the most potent in suppressing ROS, and did so in a concentration-dependent manner. Compounds **5** and **6** also had quite good activity; interestingly, all of the compounds tested, with the exception of compound **3**, were better



Scheme 2. Routes employed for the syntheses of compounds **2**, **3** and **4**.



Scheme 3. Route employed for the synthesis of compound **5**.

ROS scavengers than idebenone. Because the use of DCF fluorescence as an endpoint has been shown to lack specificity under certain circumstances,[37,38] it was shown in the present study that the increase in DCF fluorescence induced by DEM was completely

Table 1
Suppression of lipid peroxidation by pyridinol antioxidants in cultured FRDA lymphocytes treated with diethyl maleate (DEM)[a]

Compound	Scavenging activity (%)	
	2.5 μM	5 μM
Untreated control[b]	100	100
Treated control[c]	0	0
1	71 ± 6.0	82 ± 3.7
2	62 ± 6.4	74 ± 4.7
3	20 ± 10	39 ± 7.6
4	90 ± 2.9	97 ± 2.3
5	87 ± 5.1	97 ± 3.1
6	83 ± 4.2	91 ± 3.6
Idebenone	68 ± 6.4	77 ± 2.4
Redox core **1**	3.5 ± 2.3	3.9 ± 3.0

[a] Values have been calculated as [(100–% mean)/(100–% mean of the untreated control)] x 100.
[b] No DEM treatment.
[c] DEM treatment.

reversed by superoxide dismutase + catalase, or by the antioxidant N-acetylcysteine (not shown).

2.5.3. Preservation of mitochondrial membrane potential

The ability of the pyridinol analogues to preserve mitochondrial membrane potential under conditions of oxidative stress was studied. Mitochondrial membrane potential, $\Delta\psi_m$, is an important parameter of mitochondrial integrity and is essential for maintaining the physiological function of the respiratory chain in ATP synthesis.[39] $\Delta\psi_m$ was estimated using the cationic fluorescent probe tetramethylrhodamine methyl ester (TMRM), which preferentially accumulates in the mitochondria due to the negative membrane potential across the inner mitochondrial membrane, in accordance with the $\Delta\psi_m$ Nernst potential.[40] The red fluorescent signal in mitochondria decreases when $\Delta\psi$m is impaired, and can be measured using flow cytometry. A representative flow cytometric two-dimensional color density dot plot analyses of the mitochondrial membrane potential measurements with and without incubation in the presence of selected compounds are shown in the upper panel of Figure 3. The percentage of cells with intact mitochondrial membrane potential appears in the top right quadrant of individual treatments. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a commonly used uncoupler of oxidative phosphorylation in mitochondria, was employed to dissipate the chemiosmotic proton gradient ($\Delta\mu H^+$). The lower levels of TMRM fluorescence resulting from FCCP treatment reflect the depolarization of mitochondrial inner membrane potential. The bar graph, in the lower panel of Figure 3, summarizes the relative geometric mean fluorescence intensity (GMFI) of the flow cytometric profiles. It shows clearly that compounds **4** and **6** had the greatest potency, and acted in a dose-dependent manner; compound **5** was almost as good although not strictly dose-dependent in this experiment. These three compounds were clearly better than idebenone, and better than compounds **1** and **2,** which had activity in the same range as idebenone. Finally, compound **3** had the lowest activity and the redox core of compound **1** was completely inactive. The flow cytometric two-dimensional color density dot plot analyses for compounds **2**, **3**, **6** and the redox core of **1** are shown in Figure 1 of the Supplementary data.

2.5.4. Cytoprotection

Cytoprotection was measured initially using cultured CEM leukemia cells (ATCC CCL-119) that were treated with diethyl maleate to induce oxidative stress through depletion of glutathione. As shown in Table 2, compounds **4** and **6** were the most effective at the concentrations tested. Compounds **1** and **5** were also effective at 2.5 μM concentration, but not at the two lower concentrations tested. The ability of the best pyridinols to protect cultured Friedreich's ataxia lymphocytes from cell death induced by oxidative stress was also measured (Fig. 2 of the Supplementary data). The half maximal effective concentrations (EC_{50}) for the best pyridinol analogues were determined using FRDA lymphocytes; they are shown in Table 3. Compound **4** was by far the most efficient, having an EC_{50} value of 0.15 ± 0.05 μM. Compounds **5** and **6** were both more efficient than idebenone. Finally, compound **1** had the lowest potency, with an EC_{50} value of 0.84 ± 0.05 μM.

2.5.5. Mitochondrial electron transport chain function

The inhibitory effects of the test compounds on bovine heart mitochondrial complexes I, III and IV were evaluated using submitochondrial particles (SMP), by measuring NADH oxidase activity. The results are presented in Table 4, and show that compounds

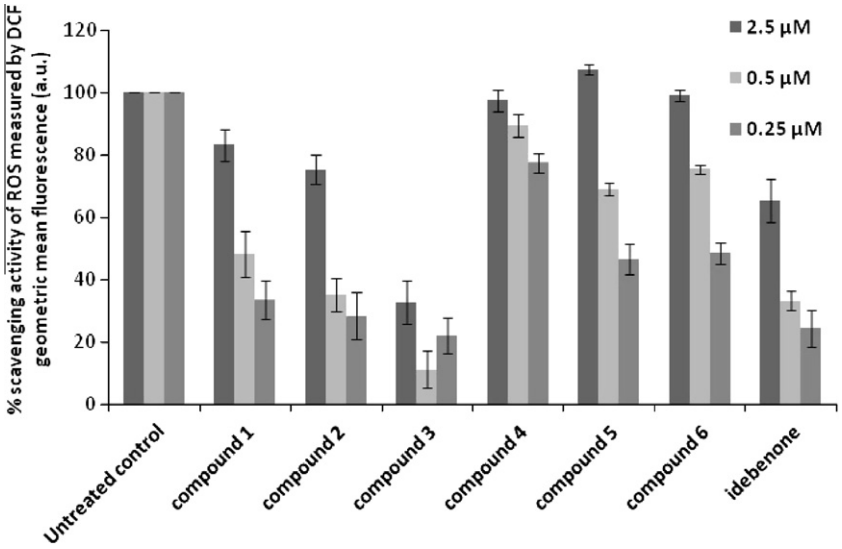


Figure 2. Flow cytometric analysis of CCRF-CEM leukemia cells pre-treated with compounds **1**–**6** or idebenone at 2.5, 0.5, and 0.25 μM concentrations for 15 h, and then treated with diethyl maleate (DEM) for 1 h to induce the production of ROS. The cells were stained with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) for 25 min prior to analysis. Data shown represent the mean ± SEM of three different experiments run as duplicates. Results are expressed as % ROS scavenging activity.

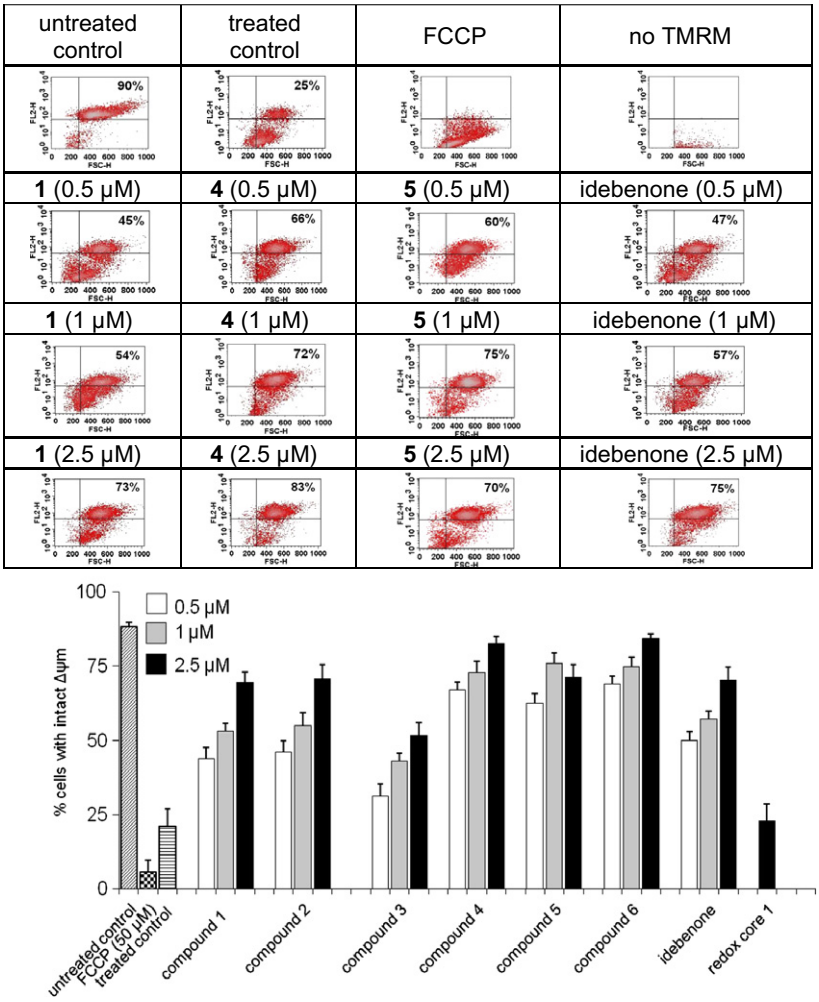


Figure 3. Effect of pyridinol antioxidants on mitochondrial membrane potential of DEM-treated FRDA lymphocytes. The upper panel shows representative flow cytometric two-dimensional color density dot plot analyses of mitochondrial membrane potential ($\Delta\psi_m$) in FRDA lymphocyte cells stained with 250 nM TMRM and analyzed using 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 each treatment. Representative example from at least three independent experiments. In each analysis, 10,000 events were recorded. Bottom panel shows a bar graph of 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. The bar graph shows the relative geometric mean fluorescence intensity (GMFI) of the above flow cytogram profiles calculated using CellQuest software. Data are expressed as means of the SEs ($n = 3$).

Table 2
Cytoprotective effects of pyridinol antioxidants on the viability of cultured CEM leukemia cells treated with DEM[a]

Compound	Viable cells (%)		
	0.1 μM	0.5 μM	2.5 μM
1	41 ± 9.7	59 ± 14	94 ± 3.4
2	28 ± 13	38 ± 12	78 ± 14
3	18 ± 5.6	23 ± 6.6	26 ± 7.5
4	88 ± 10	90 ± 6.6	93 ± 2.4
5	42 ± 5.4	47 ± 4.0	92 ± 1.6
6	76 ± 9.9	93 ± 5.1	94 ± 4.1
Idebenone	34 ± 13	49 ± 12	88 ± 4.3

[a] The viability of untreated cells was defined as 100%; cells treated with DEM alone had 18 ± 10% viability.

Table 3
Effect of pyridinol antioxidants on the cellular viability of FRDA lymphocytes treated with DEM

Compound	EC_{50} (μM)
1	0.84 ± 0.05
4	0.15 ± 0.05
5	0.56 ± 0.06
6	0.38 ± 0.1
Idebenone	0.73 ± 0.1

1, **2**, **3**, and **4** were all less inhibitory to respiratory chain function with complexes I, III and IV than compound **6** or idebenone. Compound **5** was the most inhibitory of the tested compounds.

2.5.6. Cyclic voltammetric analysis

Cyclic voltammetric analyses of the redox cores of compounds **1** and **5** are presented in Figure 4A in comparison with CoQ_0. The figure shows that the redox cores of **1** and **5** have similar redox potentials and that they are both more easily reduced than CoQ_0, consistent with their enhanced ability to suppress ROS (cf Fig. 2). Figure 4B shows that the redox core of compound **1** was unstable to repetitive redox cycles while that of compound **5** was completely stable.

2.5.7. Electron spin resonance analysis

The ability of compound **1** to transfer its phenolic hydrogen atom and form a stabilized radical has been tested by the use of ESR. Figure 5A shows the signal obtained from the radical

Table 4
Inhibitory effects of compounds **1**–**6**, and idebenone on bovine heart mitochondrial NADH oxidase activity

Compound	NADH oxidase activity[a] (%)		
	10 μM	5 μM	1 μM
Idebenone	15 ± 2.5	53 ± 3.5	65 ± 2.5
1	25 ± 5.2	42 ± 5.8	85 ± 9.1
2	48 ± 2.7	52 ± 3.6	74 ± 2.2
3	43 ± 5.5	49 ± 3.5	71 ± 4.5
4	27 ± 4.0	59 ± 3.8	78 ± 4.0
5	6 ± 0.2	9 ± 0.3	27 ± 0.8
6	22 ± 6.4	26 ± 6.7	49 ± 4.3

[a] Relative to untreated control.

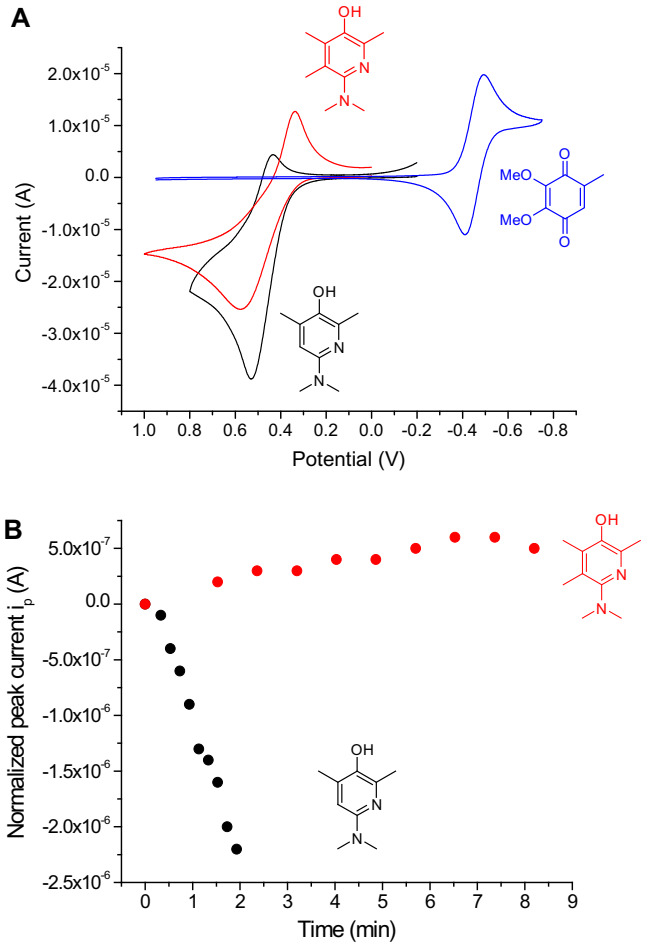


Figure 4. (A) Cyclic voltammetric analysis of redox cores **1** (scan rate 0.1 V s^{-1}, 5 mM concentration) and **5** (scan rate 0.04 V s^{-1}, 5 mM concentration) versus CoQ_0 (scan rate 0.04 V s^{-1}, 1 mM concentration). (B) Normalized peak current ($i_{p(n)}-i_{p(1)}$) plotted against time in continuous cyclic voltammetric analysis of core **1** versus core **5**, where $i_{p(n)}$ is the peak current for the nth scan while $i_{p(1)}$ is the peak current for the first scan.

activation of compound **1** in the presence of illuminated di-*tert*-butyl peroxide. Figure 5B shows the resonance contributors of the stabilized 3-pyridinoxyl radical formed.

3. Discussion

New pyridinol analogue **1** has been synthesized using a strategy similar to that used to synthesize its aza analogue **6** (Scheme 1).[34] Compounds **2**, **3**, and **4** were prepared from intermediates on the pathway to **1** (Scheme 2), while the preparation of compound **5** involved a new strategy (Scheme 3). These six analogues all have the same side chain as idebenone at the 2-position of the aromatic ring, have an amino group at the 6-position, and a methyl group at the 4-position (Fig. 1). Compounds **1**–**5** were of interest as new examples of multifunctional radical quenchers, a type of coenzyme Q analogue capable of conferring cytoprotection to cells under oxidative stress by a few different mechanisms that operate concurrently.[33,34]

The antioxidant properties of the new pyridinol analogues were tested and analyzed in selected biological and biochemical assays. Cultured CEM leukemia cells and Friedreich's ataxia lymphocytes were challenged with diethyl maleate, which depletes cellular glutathione.[41–43] Diethyl maleate induces greater oxidative stress than is likely to be encountered physiologically, permitting the identification of compounds anticipated to function robustly under pathophysiological conditions. Compounds **1**–**5** showed a range of potencies in blunting the effects of the resulting oxidative stress. In addition, an experiment was carried out to determine whether, and to what extent, compounds **1**–**5** inhibited the function of the mitochondrial electron transport chain (ETC). This was done because quinone derivatives are known to bind to the ETC, and thereby inhibit mitochondrial respiration. Many compounds have been reported to inhibit mitochondrial complexes I[44–47] and III[48,49] and these invariably strongly diminish cell viability. Since idebenone has been found to significantly inhibit complex I,[30,31] it seemed logical to consider that this property might limit its useful antioxidant properties. Accordingly, we evaluated compounds **1**–**6** for this property using a biochemical assay that measures the activity of NADH oxidase, which encompasses mitochondrial complexes I, III, and IV. In fact, compounds **1**–**4** were found to be superior both to idebenone and compound **6** in their lesser inhibition of NADH oxidase activity (Table 4). While these experiments do not definitively establish the biochemical locus of inhibition by compounds **1**–**5**, complex I seems likely to be the relevant locus.

Summarizing the results obtained for compounds **1**–**6** in the several assays studied, it appears that compounds **1**, **4**, **5**, and **6** have superior antioxidant activity as compared with idebenone. Compounds **4**, **5** and **6** were also more potent cytoprotective agents than idebenone (Tables 2 and 3). The greater potency of pyridinols **4** and **5** in suppressing ROS in cultured CEM leukemia cells as compared with idebenone is entirely consistent with the reduction of their redox core at more oxidizing potentials than the redox core of idebenone (and coenzyme Q_{10}) (Fig. 4A). Further, introduction of N-atoms into the quinone moiety has been shown to lower the bond dissociation energy of the phenolic OH group of the reduced form of the cofactor (analogues),[32] consistent with the superior activities of most of the pyridinols in quenching lipid peroxidation (Table 1).

All the new compounds, other than compound **5**, exhibited a lesser inhibitory effect on complexes I, III and IV of the mitochondrial electron transport chain than idebenone (Table 4), which seems likely to be related to their greater potency as cytoprotective agents (Tables 2 and 3). The diminished potency of **1** is likely related to its lack of stability under redox cycling conditions (vide infra).

From Table 1 and Figure 3, we can easily conclude from the data for the redox core of compound **1**, which lacked any antioxidant activity, that the lipophilic side chain is crucial for compound efficacy. Interestingly, it appears that compound **6** had better antioxidant activity than **1**, despite the fact that it was more inhibitory to NADH oxidase (Table 4). This is undoubtedly due to the low stability of compound **1** to the redox cycling (Fig. 4B), which we posit to be essential to its antioxidant function.[34] This is also consistent with the higher EC_{50} value obtained with **1** (Table 3). One strategy to increase the stability of compound **1** was to synthesize compound **5**, having a C-methyl group at position 5 of the pyridinol

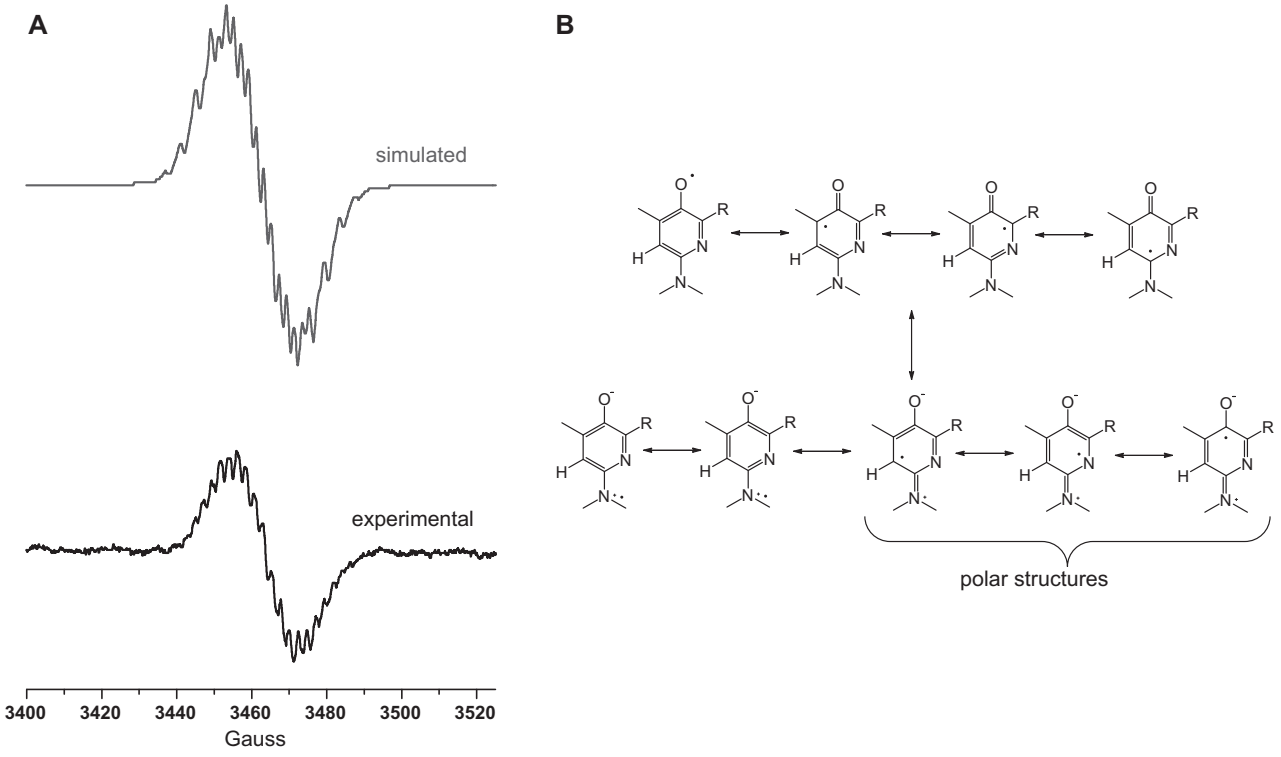


Figure 5. (A) ESR signal and simulation of compound **1** (20 mM) in dry de-aerated benzene at 25 °C during continuous illumination, in the presence of di-*tert*-butyl peroxide. The hyperfine splitting constants are as follows: a_H (3H, *o*-methyl) = 3.5 G, a_H (2H, *o*-carbon chain) = 3.3 G, a_H (1H, *o*-carbon chain) = 3.4 G, a_H (1H, *m*-ring) = 1.9 G, a_H (6H, *p*-NMe_2) = 4.2 G, a_N (1 N, ring) = 1.1 G, a_N (1 N, *p*-NMe_2) = 5.9 G, and *g* = 2.00493. The simulation corresponds to the experimental signal with a relative error of *R* = 0.983. (B) Resonance stabilized 3-pyridinoxyl radical resulting from abstraction of the O–H hydrogen atom.

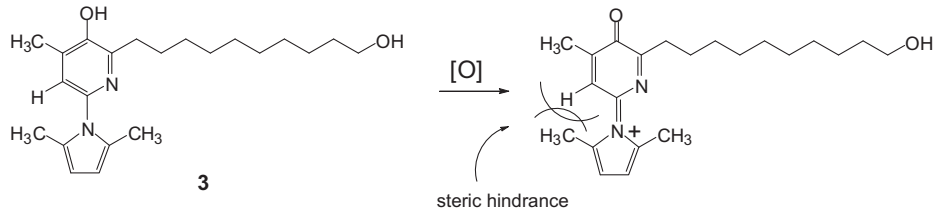


Figure 6. Steric interactions putatively arising from the oxidation of compound **3**.

ring (Fig. 4B). This resulted in a compound having increased antioxidant activity, equal or superior to **6** in the various assays used for their evaluation. Unfortunately the increased stability was also accompanied by an increase in NADH oxidase inhibition, resulting in an EC_{50} of 0.56 ± 0.06 μM for **5** compared to 0.38 ± 0.1 μM for compound **6** in the FRDA lymphocyte protection assay (Tables 3 and 4).

Another possible way to improve compound **1** stability involved changing the electronic character and steric bulk of the substituted group at the 6-position. Toward that end, compounds **2**, **3** and **4** were synthesized and tested. It appears from the data that compound **4** was the most effective of these compounds. This presumably reflects the steric effects of the C-methyl groups of the pyrrolidine moiety in preventing side reactions at the unsubstituted position of the pyridine ring. Additionally, the more favorable properties of **4** as compared with **3** is consistent with the possibility that electron-donating substituents at the 6 position of the pyridine ring may stabilize the radical putatively formed upon quenching of lipid peroxidation by **4**. It may be noted that the modest effects of compound **3** as an antioxidant may also be related to the steric interactions that would be anticipated for the oxidized form (Fig. 6).

As suggested in our previous report,[34] and predicted for this family of molecules,[32,50] the mechanism for the scavenging of ROS and lipid radicals by these new pyridinols is through the formation of a pyridinoxyl radical. To test this hypothesis, we studied the ability of compound **1** to form a radical upon hydrogen abstraction of the hydroxyl group at position 3. As described above, the radical derived from compound **1** was formed by excitation of di-*tert*-butyl peroxide, and the signal obtained was measured using an ESR spectrophotometer (Fig. 5). The signal was characterized by its g factor of 2.0049 and the small hyperfine coupling of the pyridine ring nitrogen (1.1G). In agreement with the work of Pratt and co-workers[50] this is attributed to the electron donating character of $N(CH_3)_2$ responsible for an increase in contribution to stability arising from the higher energy polar structures (Fig. 5B).

It is interesting that the best compounds described in this report function more effectively than idebenone as regards the several parameters studied, and were more potent overall as cytoprotective agents. Idebenone has also been reported to form a hydroquinone in situ via the action of NAD(P)H: quinone reductases, which enables the transfer of electrons to complex III.[51] Augmentation of ATP production was not evaluated for compounds

1–**5** in the present study, but optimization of this property could plausibly further enhance the favorable properties of the pyridinols studied here. Likewise, cellular distribution of the pyridinols, especially as regards mitochondrial localization, could also materially affect their biological function, and is worthy of characterization.

4. Conclusions

In the present study, the effects of several structural changes have been shown to influence both the antioxidant activities of the compounds as well as inhibition of the mitochondrial ETC. The conclusions include the requirement that a lipophilic side chain is essential for good antioxidant activity, and a redox core that undergoes electron transfer at more oxidizing potentials than CoQ_0 is preferable. Also, good stability at the pyridinoxyl-radical level is important and can be achieved by full substitution of the 3-pyridinol redox core. Finally, resonance delocalization increasing the resonance contribution due to polar structures of the radical is observed; these are likely stabilized by increasing the electron donating character of the substituted 6-amino group. The data obtained for inhibition of NADH oxidase activity do not provide any simple strategy for achieving low inhibition. However, they do underscore the importance of minimizing such inhibitory effects.

Finally, it can be noted that compound **4** has the best antioxidant activity among the tested compounds, including idebenone and compound **6**. Compound **4** has the lowest EC_{50} value (0.15 ± 0.05 μM) in FRDA lymphocytes, and was the most potent in conferring cytoprotection to cultured CEM leukemia cells (Tables 2 and 3). It also has the best ROS and lipid peroxidation scavenging properties, as well as the best ability to maintain mitochondrial membrane potential. Importantly, compound **4** is also less inhibitory to NADH oxidase than compound **6** and idebenone, making this compound a good candidate for further evaluation.

While the focus of the current study is Friedreich's ataxia, there are several mitochondrial diseases involving a significant component of energy dysfunction (e.g. Alzheimer's disease, Parkinson's disease, LHON, and Leigh syndrome).[10,11,15,52–54] The underlying biochemistry at the level of mitochondrial dysfunction is likely similar for these diseases. Therefore, patients with these disorders may well benefit from therapeutic agents designed by employing the concepts that arise from the present results.

5. Experimental section

5.1. Chemistry

^{1}H NMR spectra were recorded on a Varian Inova 400 MHz, using chloroform-*d*, methanol-d_4 or acetonitrile-d_3. ^{1}H NMR chemical shifts were reported relative to residual chloroform at 7.26 ppm, or to residual methanol at 3.31 ppm, or to residual acetonitrile at 2.10 ppm. ^{13}C NMR chemical shifts were reported relative to residual chloroform at 77.1 ppm, or to residual methanol at 49.0 ppm, or to residual acetonitrile at 1.89 ppm and 116.43 ppm. All solvents were analytical grade and were used without further purification. All chemicals were purchased from Aldrich Chemical Company and were used without further purification. The reactions were carried out under an argon atmosphere unless specified otherwise. Column chromatography was carried out using silica gel (Silicycle R10030B, 60 particle size, 230–240 mesh). Analytical thin layer chromatography separations were carried out on glass plates coated with silica gel (60, particle size F254, E. Merck 5608/7). The TLC chromatograms were developed using UV irradiation or by immersing the plates in 2.5% potassium permanganate in ethanol or 2% anisaldehyde + 5% sulfuric acid + 1.5% glacial acetic acid in ethanol, followed by heating. Melting points were recorded on a MelTemp apparatus and are uncorrected. High resolution mass spectra were obtained in the Arizona State University CLAS High Resolution Mass Spectrometry Laboratory.

5.1.1. 1-Bromo-9-(methoxymethoxy)nonane (7)[34]

To a stirred solution containing 5.00 g (22.4 mmol) of 9-bromo-1-nonanol in 60 mL of anh THF was added 5.10 mL (5.41 g; 67.2 mmol) of MOMCl followed by 1.79 g (44.8 mmol) of a 60% suspension of NaH in mineral oil. The reaction mixture was stirred at 23 °C overnight. The reaction mixture was carefully quenched with satd aq sodium bicarbonate, poured into 200 mL of water and extracted with two 150-mL portions of ether. The combined organic solution was washed with 200 mL of brine, dried ($MgSO_4$) and then concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 5 cm). Elution with 9:1 hexanes/ethyl acetate afforded the expected product as a colorless oil: yield 4.21 g (70%); silica gel TLC R_f 0.45 (9:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.28–1.39 (br, 10H), 1.56 (quint, 2H, J = 7.2 Hz), 1.82 (quint, 2H, J = 7.2 Hz), 3.33 (s, 3H), 3.37 (t, 2H, J = 6.8 Hz), 3.48 (t, 2H, J = 6.8 Hz), and 4.59 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 26.1, 28.1, 28.7, 29.28, 29.33, 29.7, 32.8, 34.0, 55.0, 67.7, and 96.4; mass spectrum (APCI), m/z 267.0953 $(M+H)^+$ ($C_{11}H_{24}O_2Br$ requires 267.0960).

5.1.2. 6-Amino-3-bromo-2,4-dimethylpyridine (8)[55]

To a stirred solution containing 2.00 g (16.3 mmol) of 2-amino-4,6-dimethylpyridine in 25 mL of acetonitrile was added 2.90 g (16.3 mmol) of *N*-bromosuccinimide. The reaction mixture was stirred at room temperature for 5 h. The formed precipitate was filtered and dried to afford 6-amino-3-bromo-2,4-dimethylpyridine (**8**) as a colorless solid: yield 2.76 g (84%); mp 143–145 °C; silica gel TLC R_f 0.15 (2:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H), 2.48 (s, 3H), 4.39 (br s, 2H), and 6.22 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 23.3, 25.1, 108.1, 112.3, 148.6, 155.2, and 156.3; mass spectrum (APCI), m/z 201.0032 $(M+H)^+$ ($C_7H_{10}N_2Br$ requires 201.0027).

5.1.3. 3-Bromo-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4-dimethylpyridine (9)

To a stirred solution containing 2.76 g (13.8 mmol) of 6-amino-3-bromo-2,4-dimethylpyridine (**8**) in 25 mL of toluene were added 2.02 mL (1.98 g; 17.2 mmol) of 2,5-hexanedione followed by 130 mg (0.68 mmol) of *p*-toluenesulfonic acid. The cooled reaction mixture was stirred at reflux for 14 h. The cooled reaction mixture was poured into 150 mL of water and then extracted with 200 mL of ethyl acetate. The organic phase was washed with 150 mL of brine, dried ($MgSO_4$) and then concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 6 cm). Elution with 6:1 hexanes/ethyl acetate afforded 3-bromo-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4-dimethylpyridine (**9**) as an orange oil: yield 2.37 g (62%); silica gel TLC R_f 0.70 (6:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.12 (s, 6H), 2.47 (s, 3H), 2.70 (s, 3H), 5.88 (s, 2H), and 6.93 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.1, 23.4, 25.6, 106.9, 121.16, 121.24, 122.5, 128.4, 149.6, and 157.4; mass spectrum (APCI), m/z 279.0502 $(M+H)^+$ ($C_{13}H_{16}N_2Br$ requires 279.0497).

5.1.4. 3-(Benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4-dimethylpyridine (10)

To a stirred solution containing 3.13 g (11.2 mmol) of 3-bromo-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4-dimethylpyridine (**9**) in 50 mL of 1:1 degassed dioxane/water was added 612 mg (0.67 mmol) of $Pd_2(dba)_3$ followed by 284 mg (0.67 mmol) of 2-di-*tert*-butylphosphino-2′,4′,6′-triisopropylbiphenyl (L_1) and 1.88 g (33.6 mmol) of KOH. The reaction mixture was stirred at 100 °C for 3 h. The reaction mixture was poured into 200 mL of

water and extracted with two 100-mL portions of ethyl acetate. The aqueous layer was acidified with HCl (to pH 2–3) and then extracted with two 100-mL portions of ethyl acetate. The combined organic layer was washed with a 100-mL portion of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was dissolved in 50 mL of anh THF and 1.99 mL (2.86 g; 16.8 mmol) of benzyl bromide followed by 807 mg (22.4 mmol) of 60% sodium hydride suspension in mineral oil were added. The reaction mixture was stirred at 23 °C for 16 h. The reaction mixture was poured into 150 mL of water and extracted with two 100-mL portions of diethyl ether. The combined organic layer was washed with 100 mL of 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 hexanes (removal of unreacted benzyl bromide) and then with 4:1 hexanes/diethyl ether afforded 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4-dimethylpyridine (**10**) as a yellowish oil: yield 2.82 g (82%); silica gel TLC R_f 0.65 (5:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.17 (s, 6H), 2.37 (s, 3H), 2.58 (s, 3H), 4.94 (s, 2H), 5.91 (s, 2H), 6.98 (s, 1H), and 7.40–7.50 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.1, 16.2, 19.5, 74.6, 106.4, 121.9, 127.9, 128.31, 128.34, 128.6, 136.7, 142.0, 146.6, 151.0, and 152.0; mass spectrum (APCI), *m*/*z* 307.1801 $(M+H)^+$ ($C_{20}H_{23}N_2O$ requires 307.1810).

5.1.5. 3-(Benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (11)

To a stirred solution at −78 °C containing 906 mg (2.95 mmol) of 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4-dimethylpyridine (**10**) in 20 mL of anh THF was added 789 mg (2.95 mmol) of 1-bromo-9-(methoxymethoxy)nonane (**7**) followed by 1.99 mL (3.54 mmol) of a 1.80 M solution of PhLi in hexane. The reaction mixture was stirred at −78 °C for 30 min then the reaction mixture was allowed to warm to 23 °C slowly; the reaction mixture was then stirred at 23 °C for 30 min. The reaction was quenched with satd aq ammonium chloride and then poured into 80 mL of water. The mixture was then extracted with two 80-mL portions of ethyl acetate. The combined organic layer was then washed with 100 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 5 cm). Elution with 4:1 hexanes/ethyl acetate afforded 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (**11**) as a light yellow oil: yield 877 mg (60%); silica gel TLC R_f 0.30 (9:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.18–1.22 (br m, 12H), 1.50 (quint, 2H, *J* = 6.8 Hz), 1.65 (m, 2H), 2.06 (s, 6H), 2.26 (s, 3H), 2.75 (dd, 2H, *J* = 7.6 and 7.6 Hz), 3.28 (s, 3H), 3.43 (t, 2H, *J* = 6.8 Hz), 4.53 (s, 2H), 4.82 (s, 2H), 5.78 (s, 2H), 6.79, (s, 1H) and 7.29–7.40 (m, 5H);^{13}C NMR ($CDCl_3$) δ 13.3, 16.4, 26.2, 28.9, 29.40, 29.48, 29.50, 29.55, 29.6, 29.7, 32.1, 55.1, 67.9, 75.2, 96.4, 106.4, 121.7, 127.7, 128.3, 128.6, 128.9, 138.8, 142.0, 146.8, 150.8, and 155.9; mass spectrum (APCI), *m*/*z* 493.3426 $(M+H)^+$ ($C_{31}H_{45}N_2O_3$ requires 493.3430).

5.1.6. 6-Amino-3-(benzyloxy)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (12)

To a stirred solution containing 240 mg (0.49 mmol) of 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-[10-(methoxymethoxy) decyl]-4-methylpyridine (**11**) in 10 mL of 9:1 ethanol/water was added 338 mg (4.87 mmol) of hydroxylamine hydrochloride followed by 273 mg (4.87 mmol) of KOH. The reaction mixture was stirred at reflux for 6 h, then a second portion of 338 mg (4.87 mmol) of hydroxylamine hydrochloride was added, followed by 273 mg (4.87 mmol) of KOH. The reaction mixture was stirred at reflux for 16 h. The cooled reaction mixture was poured into 50 mL of water and extracted with two 50-mL portions of dichloromethane. The combined organic layer was washed with 60 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 3 cm). Elution with 9:1 dichloromethane/methanol afforded 6-amino-3-(benzyloxy)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (**12**) as a yellowish oil: yield 113 mg (56%); silica gel TLC R_f 0.45 (9:1 dichloromethane/methanol); ^{1}H NMR ($CDCl_3$) δ 1.24–1.31 (br m, 12H), 1.53–1.63 (m, 4H), 2.16 (s, 3H), 2.62 (dd, 2H, *J* = 8.0 and 8.0 Hz), 3.33 (s, 3H), 3.48 (t, 2H, *J* = 6.8 Hz), 4.24 (br s, 2H), 4.59 (s, 2H), 4.71 (s, 2H), 6.16 (s, 1H), and 7.31−7.43 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 16.7, 29.2, 29.40, 29.46, 29.48, 29.6, 29.7, 29.8, 32.2, 53.4, 55.1, 67.9, 75.4, 96.3, 108.1, 127.7, 128.0, 128.5, 137.4, 142.5, 144.9, 153.5, and 154.1; mass spectrum (APCI), *m*/*z* 415.2957 $(M+H)^+$ ($C_{25}H_{39}N_2O_3$ requires 415.2961).

5.1.7. 3-(Benzyloxy)-6-(dimethylamino)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (13)

To a stirred solution containing 218 mg (0.53 mmol) of 6-amino-3-(benzyloxy)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (**12**) in 3 mL of anh DMF was added 100 μL (228 mg; 1.59 mmol) of methyl iodide followed by 64.0 mg (1.59 mmol) of a 60% suspension of NaH in mineral oil. The reaction mixture was stirred at 23 °C for 3 h. The reaction was quenched with 0.5 mL of water and concentrated under diminished pressure. The residue was dissolved in 50 mL of dichloromethane and washed with 50 mL of brine. The organic solution was dried ($MgSO_4$) and then concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (8 × 3 cm). Elution with 3:1 hexanes/ethyl acetate afforded 3-(benzyloxy)-6-(dimethylamino)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (**13**) as a yellowish oil: yield 27 mg (21%); silica gel TLC R_f 0.30 (3:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.22–1.34 (br m, 12H), 1.56 (quint, 2H, *J* = 6.8 Hz), 1.71 (quint, 2H, *J* = 7.6 Hz), 2.21 (s, 3H), 2.68 (dd, 2H, *J* = 7.6 and 7.6 Hz), 3.01 (s, 6H), 3.43 (s, 3H), 3.49 (t, 2H, *J* = 6.8 Hz), 4.60 (s, 2H), 4.71 (s, 2H), 6.16 (s, 1H), and 7.32–7.45 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 16.7, 26.2, 28.7, 29.4, 29.5, 29.6, 29.73, 29.76, 31.6, 32.0, 38.3, 55.0, 67.9, 75.3, 96.4, 104.9, 127.8, 127.9, 128.5, 137.7, 141.2, 143.4, 152.9, and 155.7; mass spectrum (APCI), *m*/*z* 443.3290 $(M+H)^+$ ($C_{27}H_{43}N_2O_3$ requires 443.3274).

5.1.8. 6-(Dimethylamino)-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (1)

To a stirred solution containing 27.0 mg (0.09 mmol) of 3-(benzyloxy)-6-(dimethylamino)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine (**13**) in 3 mL of methanol was added one drop of concentrated aq HCl. The reaction mixture was stirred at reflux for 16 h. Then 3 mg of 20% palladium hydroxide on carbon (Degussa type E101 NE/N) was added to the cooled reaction mixture, which was stirred at 23 °C under a H_2 atmosphere for 15 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 9:1 dichloromethane/methanol afforded 6-(dimethylamino)-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (**1**) as a colorless oil: yield 10 mg (77%); silica gel TLC R_f 0.50 (9:1 dichloromethane/methanol); ^{1}H NMR (methanol-d_4) δ 1.28–1.34 (br m, 12H), 1.49 (m, 2H), 1.63 (m, 2H), 2.18 (s, 3H), 2.66 (dd, 2H, *J* = 7.6 and 7.6 Hz), 2.93 (s, 6H), 3.51 (t, 2H, *J* = 6.4 Hz) and 6.31 (s, 1H); ^{13}C NMR (methanol-d_4) δ 15.5, 25.5, 28.2, 29.16, 29.20, 29.22, 29.24, 29.3, 31.2, 32.2, 38.1, 67.9, 106.0, 138.1, 141.0, 147.1 and 154.2; mass spectrum (APCI), *m*/*z* 309.2553 $(M+H)^+$ ($C_{18}H_{33}N_2O_2$ requires 309.2542).

5.1.9. 6-Amino-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (2)

To a stirred solution containing 113 mg (0.27 mmol) of 6-amino-3-(benzyloxy)-2-[10-(methoxymethoxy)decyl]-4-methylpyridine

(**12**) in 10 mL of methanol was added two drops of concentrated HCl. The reaction mixture was stirred at reflux for 16 h. To the reaction mixture was added 5 mg of 20% palladium hydroxide on carbon (Degussa type E101 NE/N). The reaction mixture was stirred at 23 °C under a H_2 atmosphere for 15 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 1 cm). Elution with 17:3 dichloromethane/methanol afforded 6-amino-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (**2**) as a colorless solid: yield 22 mg (29%); mp 138–139 °C; silica gel TLC R_f 0.20 (17:3 dichloromethane/methanol); ^{1}H NMR (methanol-d_4) δ 1.28–1.35 (m, 12H), 1.48–1.58 (m, 4H), 2.13 (s, 3H), 2.60 (dd, 2H, J = 7.6 and 7.6 Hz), 3.51 (t, 2H, J = 6.8 Hz) and 6.25 (s, 1H); ^{13}C NMR (methanol-d_4) δ 15.2, 25.5, 28.8, 29.15, 29.20, 29.22, 29.3, 31.5, 32.2, 61.6, 108.4, 139.2, 141.7, 146.9 and 152.5; mass spectrum (APCI), m/z 281.2231 $(M+H)^+$ ($C_{16}H_{29}N_2O_2$ requires 281.2229).

5.1.10. 6-(2,5-Dimethyl-1*H*-pyrrol-1-yl)-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (3)

To a stirred solution containing 321 mg (0.65 mmol) of 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-[10-(methoxymethoxy) decyl]-4-methylpyridine (**11**) in 5 mL of methanol was added two drops of concentrated HCl. The reaction mixture was stirred at reflux for 16 h. To the cooled reaction mixture was added 10 mg of 20% palladium hydroxide on carbon (Degussa type E101 NE/E) and the reaction mixture was then stirred at 23 °C under a H_2 atmosphere for 15 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (13 × 3 cm). Elution with 1:1 hexanes/ethyl acetate afforded 6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (**3**) as a colorless solid: yield 40 mg (17%); mp 98–99 °C; silica gel TLC R_f 0.42 (1:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.29–1.35 (m, 12H), 1.53 (quint, 2H, J = 6.8 Hz), 1.68 (quint, 2H, J = 6.8 Hz), 2.03 (s, 6H), 2.24 (s, 3H), 2.77 (dd, 2H, J = 7.6 and 7.6 Hz), 3.60 (t, 2H, J = 6.8 Hz), 5.80 (s, 2H), and 6.80 (s, 1H), ^{13}C NMR ($CDCl_3$) δ 12.9, 15.9, 25.6, 28.2, 29.24, 29.25, 29.30, 29.36, 29.38, 32.0, 32.6, 63.0, 106.0, 121.8, 128.5, 134.4, 143.5, 147.9, and 148.3; mass spectrum (APCI), m/z 359.2697 $(M+H)^+$ ($C_{22}H_{35}N_2O_2$ requires 359.2699).

5.1.11. 6-(2,5-Dimethyl-1*H*-pyrrolidin-1-yl)-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (4)

To a stirred solution containing 479 mg (0.65 mmol) of 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-[10-(methoxymethoxy) decyl]-4-methylpyridine **(11)** in 5 mL of methanol was added two drops of concentrated HCl. The reaction mixture was stirred at reflux for 16 h. To the cooled mixture was added 10 mg of 20% palladium hydroxide on carbon (Degussa type E101 NE/E) and the reaction mixture was then stirred at 23 °C under a H_2 atmosphere for 16 h. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (13 × 3 cm). Elution with 1:1 toluene/ethyl acetate afforded 6-(2,5-dimethyl-1*H*-pyrrolidin-1-yl)-2-(10-hydroxydecyl)-4-methylpyridin-3-ol (**4**) as a colorless oil: yield 17 mg (5%); silica gel TLC R_f 0.42 (1:1 hexanes/ethyl acetate); ^{1}H NMR (CD_3CN) δ 1.24–1.34 (m, 18H), 1.46 (m, 2H), 1.68 (m, 4H), 2.01 (m, 2H), 2.25 (s, 3H), 2.59 (t, 2H, J = 7.2 Hz), 3.47 (t, 2H, J = 6.4 Hz), 3.87 (m, 2H), and 6.11 (s, 1H); ^{13}C NMR (CD_3CN) δ 16.1, 16.8, 19.6, 22.5, 22.6, 26.6, 29.0, 30.2, 30.24, 30.27, 30.33, 30.9, 32.6, 32.8, 33.6, 56.1, 62.6, 106.5, 136.9, 141.3, 143.6 and 152.9; mass spectrum (APCI), m/z 363.3018 $(M+H)^+$ ($C_{22}H_{39}N_2O_2$ requires 363.3012).

5.1.12. 6-Amino-2,4,5-trimethylpyridin-3-ol (14)[35]

To a stirred solution containing 10.0 g (48.6 mmol) of 4,5-bis(hydroxymethyl)-2-methylpyridin-3-ol hydrochloride in 37 mL of thionyl chloride was added 370 μL (372 mg; 0.51 mmol) of DMF. The reaction mixture was stirred at reflux for 2 h. The cooled reaction mixture was treated with 20 mL of diethyl ether. The resulting suspension was stirred for 1 h and the formed precipitate was filtered and washed with 20 mL of ether. The solid obtained was dissolved in 42 mL of glacial acetic acid and 9.80 g (150 mmol) of zinc dust was added in three portions. The resulting suspension was stirred and heated at reflux for 2 h. The cooled reaction mixture was filtered and washed with glacial acetic acid. The filtrate was neutralized with 6 M NaOH and the formed precipitate was filtered and washed with a small amount of brine. The orange precipitate was dissolved in 10 M HCl solution and the HCl adduct was salted out with NaCl. The solid so obtained was dissolved in 200 mL of saturated aq. sodium bicarbonate. Then a diazonium salt, freshly prepared by slowly mixing 4.00 mL (4.09 g; 43.9 mmol) of aniline in 40 mL of 6 M HCl at 0 °C with a solution containing 3.00 g (44.0 mmol) of $NaNO_2$ in 15 mL of water, was added to the reaction mixture dropwise. After 1 h, a red precipitate that had formed was filtered. The diazo intermediate was dissolved in 80 mL of 1:1 methanol/formic acid and 14.1 g (220 mmol) of zinc dust was added in three portions. The reaction mixture was then stirred at reflux for 2 h. The cooled reaction mixture was then filtered and the filtrate was washed with hot MeOH. The MeOH was concentrated and the resulting white precipitate was filtered, washed with ether, dried, and then dissolved in hot water and adjusted to pH 8.0 with 6 M NaOH. Upon cooling the solution, a white precipitate formed. The precipitate was filtered, dissolved in EtOH and filtered through a silica gel pad. The filtrate was concentrated under diminished pressure to afford 6-amino-2,4,5-trimethylpyridin-3-ol (**14**) as a pale orange solid; yield 1.54 g (21%); mp 169–171 °C, lit[35] mp 170–172 °C; silica gel TLC R_f 0.20 (9:1 dichloromethane/methanol); ^{1}H NMR (DMSO-d_6) δ 1.88 (s, 3H), 2.00 (s, 3H), 2.12 (s, 3H), 4.86 (br s, 2 H), and 7.40 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 12.9, 13.4, 19.4, 112.7, 135.0, 140.2, 141.4, and 151.3.

5.1.13. 6-(2,5-Dimethyl-1*H*-pyrrol-1-yl)-2,4,5-trimethylpyridin-3-ol (15)

To a stirred solution containing 1.36 g (8.94 mmol) of 6-amino-2,4,5-trimethylpyridin-3-ol (**14**) in 50 mL of toluene was added 1.32 mL (1.28 g; 11.2 mmol) of 2,5-hexanedione followed by 86.0 mg (0.45 mmol) of *p*-toluenesulfonic acid. The reaction mixture was stirred and heated at reflux for 14 h using a Dean-Stark apparatus. The reaction mixture was poured into 80 mL of water and then extracted with 100 mL of ethyl acetate. The organic solution was washed with 80 mL of 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 2:1 hexanes/ethyl acetate afforded 6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4,5-trimethylpyridin-3-ol (**15**) as colorless needles: yield 1.54 g (75%); mp 218–219 °C; silica gel TLC R_f 0.25 (2:1 hexanes/ethyl acetate); ^{1}H NMR (methanol-d_4) δ 1.75 (s, 3H), 1.86 (s, 6H), 2.24 (s, 3H), 2.38 (s, 3H), and 5.77 (s, 2H); ^{13}C NMR (methanol-d_4) δ 12.4, 12.8, 13.6, 18.6, 106.7, 129.1, 131.1, 136.7, 142.7, 144.0, and 151.2; mass spectrum (APCI), m/z $(M+H)^+$ 231.1492 ($C_{14}H_{19}N_2O$ requires 231.1497).

5.1.14. 3-Benzyloxy-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4,5-trimethylpyridine (16)

To a stirred solution containing 1.54 g (6.69 mmol) of 6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4,5-trimethylpyridin-3-ol (**15**) in 50 mL of anh THF was added 1.19 mL (1.71 g; 10.0 mmol) of benzyl bromide followed by 803 mg (20.1 mmol) of 60% sodium hydride sus-

pension in mineral oil. The reaction mixture was stirred at 23 °C for 16 h. The reaction mixture was poured into 150 mL of water and extracted with two 100-mL portions of ether. The combined organic layer was washed with 100 mL of 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 hexanes (separation of unreacted benzyl bromide) and then with 4:1 hexanes/diethyl ether afforded 3-benzyloxy-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4,5-trimethylpyridine (**16**) as yellowish crystals: yield 1.67 g (78%); mp 64–65 °C; silica gel TLC R_f 0.60 (2:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.83 (s, 3H), 1.95 (s, 6H), 2.27 (s, 3H), 2.52 (s, 3H), 4.89 (s, 2H), 5.87 (s, 2H), and 7.40–7.50 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 12.5, 13.3, 13.8, 19.5, 74.9, 105.8, 128.0, 128.1, 128.5, 128.8, 129.7, 136.7, 141.2, 145.8 149.5, and 151.6; mass spectrum (APCI), *m*/*z* $(M+H)^+$ 321.1969 ($C_{21}H_{25}N_2O$ requires 321.1967).

5.1.15. 3-Benzyloxy-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)- 2-[10-(methoxymethoxy)decyl]-4,5-dimethylpyridine (17)

To a stirred solution at −78 °C containing 670 mg (2.09 mmol) of 3-(benzyloxy)-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2,4,5-trimethylpyridine (**16**) in 30 mL of anh THF was added 314 μL (243 mg; 2.09 mmol) of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) followed by 1.00 mL (2.51 mmol) of a 2.50 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at −78 °C for 5 min and then 615 mg (2.30 mmol) of 1-bromo-9-(methoxymethoxy)nonane (**7**) was added. The reaction mixture was stirred at −78 °C for 30 min, then the reaction mixture was warmed to 23 °C slowly and stirred for 30 min. The reaction was quenched with satd aq ammonium chloride and then poured into 50 mL of water. The mixture was then extracted with 80 mL of ether. The combined organic layer was then washed with 100 mL of 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 4:1 hexanes/ether afforded 3-benzyloxy-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-[10-(methoxymethoxy)decyl]-4,5-dimethylpyridine (**17**) as a colorless oil: yield 432 mg (41%); silica gel TLC R_f 0.25 (4:1 hexanes/ether); ^{1}H NMR ($CDCl_3$) δ 1.21–1.32 (br m, 12H), 1.55 (m, 2H), 1.68 (m, 2H), 1.81 (s, 3H), 1.92 (s, 6H), 2.25 (s, 3H), 2.82 (dd, 2H, *J* = 8.0 and 7.6 Hz), 3.33 (s, 3H), 3.48 (t, 2H, *J* = 6.7 Hz), 4.59 (s, 2H), 4.85 (s, 2H), 5.85 (s, 2H), and 7.38–7.50 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 12.6, 13.4, 13.8, 26.3, 29.45, 29.54, 29.63, 29.65, 29.68, 29.7, 29.9, 32.4, 55.2, 68.0, 75.6, 96.5, 105.8, 127.8, 128.1, 128.4, 128.8, 129.4, 137.0, 141.2, 146.0, 151.3, and 153.4; mass spectrum (APCI), *m*/*z* $(M+H)^+$ 507.3590 ($C_{32}H_{47}N_2O_3$ requires 507.3587).

5.1.16. 6-Amino-3-benzyloxy-2-[10-(methoxymethoxy)decyl]-4,5-dimethylpyridine (18)

To a stirred solution containing 849 mg (1.68 mmol) of 3-benzyloxy-6-(2,5-dimethyl-1*H*-pyrrol-1-yl)-2-[10-(methoxymethoxy) decyl]-4,5-dimethylpyridine (**17**) in 10 mL of 9:1 ethanol/water was added 2.34 g (33.6 mmol) of hydroxylamine hydrochloride followed by 2.07 g (37.0 mmol) of potassium hydroxide. The reaction mixture was stirred and heated at reflux for 16 h. The reaction mixture was then poured into 50 mL of water and extracted with two 50-mL portions of ethyl acetate. The combined organic layer was washed with 60 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 3 cm). Elution with 9:1 dichloromethane/methanol afforded 6-amino-3-benzyloxy-2-[10-(methoxymethoxy)decyl]-4,5-dimethylpyridine (**18**) as a colorless oil: yield 96 mg (13%); silica gel TLC R_f 0.45 (9:1 dichloromethane/methanol); ^{1}H NMR ($CDCl_3$) δ 1.21–1.32 (br m, 12H), 1.55 (m, 2H), 1.67 (m, 2H), 2.01 (s, 3H), 2.18 (s, 3H), 2.64 (dd, 2H, *J* = 8.0 and 8.0 Hz), 3.34 (s, 3H), 3.50 (t, 2H, *J* = 6.7 Hz), 4.45 (br s, 2H), 4.60 (s, 2H), 4.69 (s, 2H), and 7.32–7.45 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 12.9, 13.1, 21.9, 26.3, 29.46, 29.53, 29.54, 29.63, 29.66, 29.79, 29.87, 32.2, 55.1, 67.9, 96.4, 113.6, 127.8, 128.0, 128.6, 137.5, 140.1, 144.9, 150.1, and 152.8; mass spectrum (APCI), *m*/*z* $(M+H)^+$ 429.3114 ($C_{26}H_{41}N_2O_3$ requires 429.3117).

5.1.17. 3-Benzyloxy-6-dimethylamino-2-[10-(methoxymethoxy)decyl]-4,5-dimethylpyridine (19)

To a stirred solution containing 96.0 mg (0.22 mmol) of 6-amino-3-benzyloxy-2-[10-(methoxymethoxy)decyl]-4,5-dimethylpyridine (**18**) in 3 mL of 1:1 formalin/acetonitrile was added 83.0 mg (1.32 mmol) of sodium cyanoborohydride followed by 46.0 μL (48.2 mg; 0.80 mmol) of glacial acetic acid. The reaction mixture was stirred at 23 °C for 16 h and then poured into 20 mL of satds aq. sodium bicarbonate. The mixture was then extracted with 20 mL of ether. The organic phase was washed with 20 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 hexanes/ether afforded 3-benzyloxy-6-dimethylamino-2-[10-(methoxymethoxy)decyl]-4,5-dimethylpyridine (**19**) as a colorless oil: yield 78 mg (77%); silica gel TLC R_f 0.30 (4:1 hexanes/diethyl ether); ^{1}H NMR ($CDCl_3$) δ 1.21–1.32 (br m, 12H), 1.55 (m, 2H), 1.73 (m, 2H), 2.18 (s, 3H), 2.19 (s, 3H), 2.64 (m, 8H), 3.36 (s, 3H), 3.50 (t, 2H, *J* = 6.8 Hz), 4.62 (s, 2H), 4.73 (s, 2H), and 7.32–7.45 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.2, 15.0, 26.4, 28.8, 29.6, 29.70, 29.74, 29.8, 29.9, 32.0, 42.7, 55.2, 68.0, 75.4, 96.5, 121.5, 127.9, 128.1, 128.7, 137.7, 140.3, 147.4, 149.6, and 158.2; mass spectrum (APCI), *m*/*z* $(M+H)^+$ 457.3437 ($C_{28}H_{45}N_2O_3$ requires 457.3430).

5.1.18. 6-Dimethylamino-2-(10-hydroxydecyl)-4,5-dimethylpyridin-3-ol (5)

To a stirred solution containing 76.0 mg (0.17 mmol) of 3-benzyloxy-6-dimethylamino-2-[10-(methoxymethoxy)decyl]-4,5-dimethylpyridine (**19**) in 3 mL of methanol was added 1 drop of concentrated HCl. The reaction mixture was stirred at reflux for 2 h. The cooled reaction mixture was concentrated under diminished pressure and the residue was partitioned between 20 mL of satd aq $NaHCO_3$ and 20 mL of ethyl acetate. The organic layer was washed with 20 mL of brine, dried over $MgSO_4$ and concentrated under diminished pressure. The residue was dissolved in 2 mL of methanol, then 2 mg of 20% palladium hydroxide on carbon (Degussa type E101 NE/N) was added and the reaction mixture was stirred at 23 °C under a H_2 atmosphere for 15 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure to afford 6-dimethylamino-2-(10-hydroxydecyl)-4,5-dimethylpyridin-3-ol (**5**) as a yellowish oil: yield 41 mg (79%); silica gel TLC R_f 0.50 (9:1 dichloromethane/methanol); ^{1}H NMR (methanol-d_4) δ 1.28–1.34 (br m, 12H), 1.51 (m, 2H), 1.65 (m, 2H), 2.15 (s, 3H), 2.17 (s, 3H), 2.66 (s, 6H), 2.72 (dd, 2H, *J* = 8.0 and 7.2 Hz) and 3.51 (t, 2H, *J* = 6.8 Hz); ^{13}C NMR (methanol-d_4) δ 12.9, 14.6, 26.9, 29.5, 30.46, 30.56, 30.59, 30.66, 30.69, 30.74, 32.7, 33.7, 43.4, 63.0, 123.7, 136.9, 145.8, 146.4 and 156.3; mass spectrum (APCI), *m*/*z* 323.2694 $(M+H)^+$ ($C_{19}H_{35}N_2O_2$ requires 323.2699).

5.1.19. Cyclic voltammetry

Cyclic voltammetry (CV) studies were carried out using a Model 1030 multi-potentiostat from CH Instruments. The platinum working electrode 2 mm diameter (model CHI102), platinum wire counter electrode (model CHI115), and Ag/AgCl reference electrode (model CHI111) were obtained from CH Instruments. Cyclic voltammetry measurements were performed in a precut 20 mL vial at room temperature in 2 mL of a 0.1 M tetrabutylammonium perchlorate solution in acetonitrile containing the pyridinol analyte at a 1 mM concentration. The analytes were dissolved in acetonitrile.

The vial was covered with a Teflon cap (CHI223) and sealed using parafilm. All samples were purged with nitrogen for 5 min to remove any oxygen. The substrate was then added with a syringe and stirred for 1 min. Stirring was stopped and the nitrogen was moved above the sample so that only the headspace was streamed with nitrogen. The cell was allowed to sit for 1 min to allow for the diffusion layer to set up and come to equilibrium. The parameters used for the starting and vertex potential were −1.5 V and 1.5 V, respectively, and the scan rate was 100 mV/s. The cyclic voltammagram of CoQ_0 was recorded using an initial reductive sweep; those of the pyridinols involved initial oxidative sweeps. Between each CV experiment, the electrodes were rinsed with dichloromethane. The platinum working electrode was then rinsed with water and polished with 0.05 micron aluminum powder and distilled water on a polishing pad (CHI120). Immediately before each CV was performed, the electrodes were rinsed with acetonitrile.

5.1.20. Electron spin resonance (ESR) study

The compounds were dissolved in deoxygenated benzene solution for analysis to a final concentration of 10–20 mM. Then di-*tert*-butylperoxide (98%, Aldrich) was added to a final concentration of 0.1 M. The sample was inserted in the ESR cavity and the mercury lamp was turned on. Illumination was done directly in the cavity by inserting an optic fiber that was connected to the lamp. The ESR spectra were recorded at regular intervals on a X-band Bruker ELEXSYS E580 spectrometer using the following settings: microwave frequency 9.70–9.77 GHz, power 64 mW, modulation amplitude 0.1G, center field 3462 G, sweep time 168 s, and time constant 40 ms. The simulations were done using the Winsim software. Control experiments (dark sample, and sample without test compounds) were measured and gave no signal.

5.2. Biochemical and biological evaluation

5.2.1. Cell culture experiments

Friedreich's ataxia lymphocytes (Coriell, GM158150) were cultured in RPMI (Gibco, Grand Island, NY, USA) with 15% fetal bovine serum (Fisher Scientific, TX, USA), 2 mM glutamine (HyClone, South Logan, Utah, USA) and 1% penicillin–streptomycin mix (Cellgro). Cells were maintained in log phase at a concentration between 1×10^5 and 1×10^6 cells/mL.

CCRF-CEM leukemia lymphocytes (ATCC, CRL-119) were cultured in RPMI (Gibco) with 10% fetal bovine serum (Fisher Scientific), 2 mM glutamine (HyClone) and 1% penicillin–streptomycin mix (Cellgro). Cells were maintained in log phase at a concentration between 1×10^5 and 1×10^6 cells/mL. For the experiments involving cell viability, the media did not contain glutamine.

5.2.2. Lipid peroxidation assay

A quantitative FACS analysis of lipid peroxidation of FRDA lymphocytes, which had been treated with diethyl maleate following incubation in the presence and absence of the test compounds, was measured as described previously.[56] Briefly, FRDA lymphocytes (5×10^5 cells/mL) were 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. Oxidative stress was induced with 5 mM DEM in phenol red-free RPMI-1640 media for 2 h. Treated cells were collected by centrifugation at 300×*g* for 3 min and then washed with phosphate buffered saline. Cells were resuspended in 250 μL of phosphate buffered saline and analyzed by FACS (FACS Calibur flow cytometer, Becton Dickinson) to monitor the change in intensity of the C_{11} BODIPY$^{581/591}$-green (oxidized) fluorescence signal. In each analysis, 10,000 events were recorded. Results obtained were verified by running duplicates and repeating experiments in three independent experiments.

5.2.3. NADH oxidase activity assay

Mitochondria were prepared as described.[57,58] One beef heart was ground and blended in sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.8, containing 0.2 mM EDTA) at 4 °C. Cell debris was removed by centrifugation at 1200×*g* for 20 min. The supernatant was filtered through two layers of cheesecloth. Mitochondria were harvested by centrifugation at 26,000×*g* for 15 min and then homogenized in the same buffer with a Dounce homogenizer. Mitochondria were harvested by centrifugation at 12,000×*g* for 30 min, and stored at −80 °C in sucrose buffer. Submitochondrial particles (SMPs) were prepared as described by Linnane and Titchener.[59] Mitochondria were sonicated with a sonic dismembrator (Fisher Scientific) in 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM ATP, 10 mM $MnCl_2$, 1 mM sodium succinate, 10 mM Tris–HCl, pH 7.8, at 4 °C. Cell debris was pelleted by centrifugation at 20,000×*g* for 7 min at 4 °C. SMPs were harvested at 152,000×*g* for 30 min at 4 °C and stored at −80 °C in 10 mM Tris–HCl, pH 7.5, containing 0.25 M sucrose, 5 mM $MgCl_2$, 2 mM ATP, 2 mM glutathione, and 1 mM sodium succinate. The protein concentration was determined by BCA titration (Pierce) and the sample was diluted as described below.

The inhibitory effects of the test compounds on bovine heart mitochondrial complexes I, III and IV were evaluated. The compounds were dissolved in dimethylsulfoxide (DMSO), and then used to make serial dilutions. Maximal DMSO concentrations never exceeded 2% and had no influence on the control enzymatic activity. Bovine heart SMPs were diluted to 0.5 mg/mL. The enzymatic activities were assayed at 30 °C and monitored spectrophotometrically with a Beckman Coulter DU-530 (340 nm, ε 6.22 mM^{-1} cm^{-1}). NADH oxidase activity was determined in a reaction medium (2.5 mL total volume) containing 50 mM Hepes, pH 7.5, and 5 mM $MgCl_2$. The final mitochondrial protein concentration was 30 μg. After the pre-equilibration of SMP with inhibitor for 5 min, the initial rates were calculated from the linear portion of the traces.

5.2.4. Cell viability (trypan blue exclusion assay)

A hemocytometer-based assay was used to determine the number of viable cells present in the cell suspensions. Briefly, CEM leukemia or FRDA 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 17 h. Oxidative stress was then induced by incubation with 5 mM DEM for 6 h followed by evaluation of cytoprotection. Cell viability was determined by the use of 0.4% trypan blue. 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 ability of the test compounds to protect the cells against the effects of DEM was determined. Cell viability was expressed as the percentage of untreated control. Data are expressed as means ± S.E.M. (n = 3).

5.2.5. Scavenging of reactive oxygen species (ROS)

Intracellular ROS production was measured using the oxidant-sensitive fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR, USA). One mL of CEM lymphocytes (2.5×10^5 cell/mL) were plated in a 24-well plate, treated with the test compounds and incubated for 16 h at 37 °C, in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 5 mM diethyl maleate (DEM) for 1 h, collected by centrifugation at 300×*g* for 3 min and then washed twice with phosphate buffered saline (PBS) (Invitrogen, NY, USA). Cells were

resuspended in PBS containing 10 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 twice with PBS. The samples were analyzed immediately by flow cytometry using a 488 nm excitation laser and FL1-H channel 538 nm emission filter. In each analysis, 10,000 events were recorded after cell debris was electronically gated. The generation of ROS, mainly peroxides, was detected as a result of the oxidation of DCFH (λ_{ex} 488 nm; λ_{em} 515–540 nm). Results obtained were verified by running duplicates and repeating experiments in three independent experiments. Hydrogen peroxide was used to produce the positive control.

5.2.6. Maintenance of mitochondrial membrane potential ($\Delta\psi_m$)[40,60,61]

Briefly, cells were pre-treated with or without the test compounds for 16 h. The cells were treated with 5 mM DEM for 140 min, collected by centrifugation at 300×*g* for 3 min and then washed twice with phosphate buffered saline. The cells were resuspended in PBS containing 20% 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 were then washed with phosphate buffered saline. The samples were analyzed immediately by flow cytometry using a 488 nM excitation laser and the FL2-H channel. The results obtained were verified in three independent experiments. FCCP, a mitochondrial uncoupler, was used to produce a negative control. In each analysis, 10,000 events were recorded.

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Supplementary data

Supplementary data (flow cytometric analysis of the ability of selected analogues to maintain mitochondrial membrane potential, and of their ability to confer cytoprotection, as well as ^{1}H and ^{13}C NMR spectral data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.07.005.

References and notes

1. Duchen, M. R. *Diabetes* **2004**, *53*, S96.
2. McBride, H. M.; Neuspiel, M.; Wasiak, S. *Curr. Biol.* **2006**, *16*, R551.
3. Mandal, S.; Lindgren, A. G.; Srivastava, A. S.; Clark, A. T.; Banerjee, U. *Stem Cells* **2011**, *29*, 486.
4. McLennan, H. R.; Esposti, M. D. *J. Bioenerg. Biomembr.* **2000**, *32*, 153.
5. Liu, Y.; Fiskum, G.; Schubert, D. *J. Neurochem.* **2002**, *80*, 780.
6. Rhoads, D. M.; Umbach, A. L.; Subbaiah, C. C.; Siedow, J. N. *Plant Physiol.* **2006**, *141*, 357.
7. Murakami, K.; Kondo, T.; Kawase, M.; Li, Y.; Sato, S.; Chen, S. F.; Chan, P. H. *J. Neurosci.* **1998**, *18*, 205.
8. Lenaz, G.; Bovina, C.; Formiggini, G.; Parenti Castelli, G. *Acta Biochim. Pol.* **1999**, *46*, 1.
9. Santos, D. L.; Palmeira, C. M.; Seica, R.; Dias, J.; Mesquita, J.; Moreno, A. J.; Santos, M. S. *Mol. Cell. Biochem.* **2003**, *246*, 163.
10. Battisti, C.; Formichi, P.; Cardaioli, E.; Bianchi, S.; Mangiavacchi, P.; Tripodi, S. A.; Tosi, P.; Federico, A. *J. Neurol. Neurosurg. Psychiatry* **2004**, *75*, 1731.
11. Emerit, J.; Edeas, M.; Bricaire, F. *Biomed. Pharmacother.* **2004**, *58*, 39.
12. Lin, M. T.; Beal, M. F. *Nature* **2006**, *443*, 787.
13. Pieczenik, S. R.; Neustadt, J. *Exp. Mol. Pathol.* **2007**, *83*, 84.
14. Friederich, M.; Hansell, P.; Palm, F. *Curr. Diabetes Rev.* **2009**, *5*, 120.
15. Fernandez-Checa, J. C.; Fernandez, A.; Morales, A.; Mari, M.; Garcia-Ruiz, C.; Colell, A. *CNS Neurol. Disord. Drug Targets* **2010**, *9*, 439.
16. Rocha, M.; Apostolova, N.; Hernandez-Mijares, A.; Herance, R.; Victor, V. M. *Curr. Med. Chem.* **2010**, *17*, 3827.
17. Koenig, M. *Encycl. Life Sci.* **2005**, 1.
18. Campuzano, V.; Montermini, L.; Molto, M. D.; Pianese, L.; Cossee, M.; Cavalcanti, F.; Monros, E.; Rodius, F.; Duclos, F.; Monticelli, A.; Zara, F.; Canizares, J.; Koutnikova, J.; Bidichandani, S. I.; Gellera, C.; Brice, A.; Trouillas, P.; De Michele, G.; Filla, A.; De Frutos, R.; Palau, F.; Patel, P. I.; Di Donato, S.; Mandel, J. L.; Cocozza, S.; Koenig, M.; Pandolfo, M. *Science* **1996**, *271*, 1423.
19. Rotig, A.; de Lonlay, P.; Chretien, D.; Foury, F.; Koenig, M.; Sidi, D.; Munnich, A.; Rustin, P. *Nat. Genet.* **1997**, *17*, 215.
20. Koutnikova, H.; Campuzano, V.; Foury, F.; Dolle, P.; Cazzalini, O.; Koenig, M. *Nat. Genet.* **1997**, *16*, 345.
21. Calabrese, V.; Lodi, R.; Tonon, C.; D'Agata, V.; Sapienza, M.; Scapagnini, G.; Mangiameli, A.; Pennisi, G.; Stella, A. M.; Butterfield, D. A. *J. Neurol. Sci.* **2005**, *233*, 145.
22. Sparaco, M.; Gaeta, L. M.; Santorelli, F. M.; Passarelli, C.; Tozzi, G.; Bertini, E.; Simonati, A.; Scaravilli, F.; Taroni, F.; Duyckaerts, C.; Feleppa, M.; Piemonte, F. *J. Neurol. Sci.* **2009**, *287*, 111.
23. Armstrong, J. S.; Khdour, O. M.; Hecht, S. M. *FASEB J.* **2010**, *24*, 2152.
24. Lynch, D. R.; Perlman, S. L.; Meier, T. *Arch. Neurol.* **2010**, *67*, 941.
25. Zs.-Nagy, I. *Arch. Gerontol. Geriatr.* **1990**, *11*, 175.
26. Rustin, P.; Von Kleist-Retzow, J. C.; Chantrel-Groussard, K.; Sidi, D.; Munnich, A.; Rotig, A. *Lancet* **1999**, *354*, 477.
27. Lagedrost, S. J.; Sutton, M. S.; Cohen, M. S.; Satou, G. M.; Kaufman, B. D.; Perlman, S. L.; Rummey, C.; Meier, T.; Lynch, D. R. *Am. Heart. J.* **2011**, *161*, 639.
28. Meier, T.; Perlman, S. L.; Rummey, C.; Coppard, N. J.; Lynch, D. R. *J. Neurol.* **2012**, *259*, 284.
29. Di Prospero, N. A.; Baker, A.; Jeffries, N.; Fischbeck, K. H. *Lancet Neurol.* **2007**, *6*, 878.
30. Esposti, M. D.; Ngo, A.; Ghelli, A.; Benelli, B.; Carelli, V.; McLennan, H.; Linnane, A. W. *Arch. Biochem. Biophys.* **1996**, *330*, 395.
31. Briere, J. -J.; Schlemmer, D.; Chretien, D.; Rustin, P. *Biochem. Biophys. Res. Commun.* **2004**, *316*, 1138.
32. Wijtmans, M.; Pratt, D. A.; Valgimigli, L.; DiLabio, G. A.; Pedulli, G. F.; Porter, N. A. *Angew. Chem., Int. Ed.* **2003**, *42*, 4370.
33. Lu, J.; Khdour, O. M.; Armstrong, J. S.; Hecht, S. M. *Bioorg. Med. Chem.* **2010**, *18*, 7628.
34. Arce, P. M.; Khdour, O. M.; Goldschmidt, R.; Armstrong, J. S.; Hecht, S. M. *ACS Med. Chem. Lett.* **2011**, *2*, 608.
35. Serwa, R.; Nam, T. G.; Valgimigli, L.; Culbertson, S.; Recto, C. L.; Jeong, B. S.; Pratt, D. A.; Porter, N. A. *Chem. Eur. J.* **2010**, *16*, 14106.
36. Drummen, G. P.; Van Liebergen, L. C.; Op den Kamp, J. A.; Post, J. A. *Free Radical Biol. Med.* **2002**, *33*, 473.
37. Bonini, M. G.; Rota, C.; Tomasi, A.; Mason, R. P. *Free Radical Biol. Med.* **2006**, *40*, 968.
38. Karlsson, M.; Kurz, T.; Brunk, U. T.; Nilsson, S. E.; Frennesson, C. I. *Biochem. J.* **2010**, *428*, 183.
39. Bernardi, P.; Krauskopf, A.; Basso, E.; Petronilli, V.; Blachly-Dyson, E.; Di Lisa, F.; Forte, M. A. *FEBS J.* **2006**, *273*, 2077.
40. Ehrenberg, B.; Montana, V.; Wei, M. D.; Wuskell, J. P.; Loew, L. M. *Biophys. J.* **1988**, *53*, 785.
41. Miccadei, S.; Kyle, M. E.; Gilfor, D.; Farber, J. L. *Arch. Biochem. Biophys.* **1988**, *265*, 311.
42. Tirmenstein, M. A.; Nicholls-Grzemski, F. A.; Zhang, J.-G.; Fariss, M. W. *Chem. Biol. Interact.* **2000**, *127*, 201.
43. Armstrong, J. S.; Whiteman, M.; Rose, P.; Jones, D. P. *J. Biol. Chem.* **2003**, *278*, 49079.
44. Esposti, M. D.; Ghelli, A.; Ratta, M.; Cortes, D.; Estornell, E. *Biochem. J.* **1994**, *301*, 161.
45. Zharova, T. V.; Vinogradov, A. D. *Biochim. Biophys. Acta* **1997**, *1320*, 256.
46. Watabe, M.; Nakaki, T. *Mol. Pharmacol.* **2008**, *74*, 933.
47. Fato, R.; Bergamini, C.; Leoni, S.; Lenaz, G. *Biofactors* **2008**, *32*, 31.
48. Yagi, M.; Hatefi, Y. *J. Biol. Chem.* **2001**, *276*, 19006.
49. Monzote, L.; Stamberg, W.; Patel, A.; Rosenau, T.; Maes, L.; Cos, P.; Gille, L. *Chem. Res. Toxicol.* **2011**, *24*, 1678.
50. Valgimigli, L.; Brigati, G.; Pedulli, G. F.; DiLabio, G. A.; Mastragostino, M.; Arbizzani, C.; Pratt, D. A. *Chem. Eur. J.* **2003**, *9*, 4997.
51. Haefeli, R. H.; Erb, M.; Gemperli, A. C.; Robay, D.; Fruh, I. C.; Anklin, C.; Dallman, R.; Gueven, N. *PLoS One* **2011**, *6*, e17963.
52. Taylor, R. W.; Morris, A. A. M.; Hutchinson, M.; Turnbull, D. M. *Eur. J. Hum. Genet.* **2002**, *10*, 141.
53. Ohta, S.; Ohsawa, I. *J. Alzheimers Dis.* **2006**, *9*, 155.
54. Henchcliffe, C.; Beal, M. F. *Nat. Clin. Pract. Neurol.* **2008**, *4*, 600.
55. Wijtmans, M.; Pratt, D. A.; Brinkhorst, J.; Serwa, R.; Valgimigli, L.; Pelludi, G. F.; Porter, N. A. *J. Org. Chem.* **2004**, *69*, 9215.
56. Khdour, O. M.; Lu, J.; Hecht, S. M. *Pharmaceut. Res.* **2011**, *28*, 2896.
57. Smith, A. L. *Methods Enzymol.* **1967**, *10*, 81.
58. Palotti, F.; Lenaz, G. *Methods Cell Biol.* **2007**, *80*, 3.
59. Linnane, A. W.; Titchener, E. B. *Biochim. Biophys. Acta* **1960**, *39*, 469.
60. Armstrong, J. S.; Yang, H.; Duan, W.; Whiteman, M. *J. Biol. Chem.* **2004**, *279*, 50420.
61. Lu, C.; Armstrong, J. S. *Biochem. Biophys. Res. Commun.* **2007**, *363*, 572.