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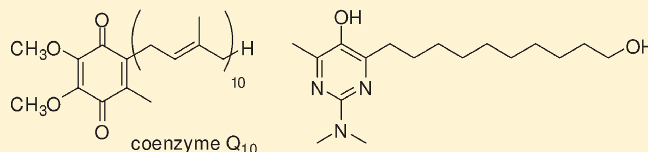
A Strategy for Suppressing Redox Stress within Mitochondria

Pablo M. Arce, Omar M. Khdour, Ruth Goldschmidt, Jeffrey S. Armstrong, and Sidney M. Hecht*

Center for BioEnergetics, Biodesign Institute, and Department of Chemistry and Biochemistry, Arizona State University, 1001 South McAllister Avenue, Tempe, Arizona 85287, United States

Supporting Information

ABSTRACT: An aza analogue (**1**) of the experimental neuroprotective drug idebenone has been prepared and evaluated. The compound quenches lipid peroxidation more effectively than α-tocopherol and potently suppresses reactive oxygen species in cells under oxidative stress. It is thought to do so via a catalytic cycle in which both forms of oxidative stress are suppressed simultaneously. Consequently, the compound effectively protects cultured CEM leukemia cells and Friedreich's ataxia fibroblasts from oxidative stress more effectively than idebenone or idebenol.



KEYWORDS: Reactive oxygen species, lipid peroxidation, electron transport chain, mitochondria

The production of ATP in eukaryotic cells occurs predominantly within the mitochondrial respiratory chain.[1,2] Unsurprisingly, mitochondrial dysfunction is strongly linked to the pathogenesis of a number of neurodegenerative diseases including Alzheimer's disease, MELAS, and Friedreich's ataxia.[3−7] Mitochondria are a major source of reactive oxygen species (ROS); while these are normally detoxified by antioxidant enzymes in the mitochondria and cytosol,[8,9] disruptions of the mitochondrial electron transport chain can undoubtedly lead to increased ROS, exhausting the capacity for detoxification and exposing cellular macromolecules to oxidative damage.[3−7,10,11]

Idebenone (Figure 1) is an analogue of coenzyme Q_{10} designed to function as a neuroprotective agent. Idebenone has been shown to accept electrons from mitochondrial complex I and to restore respiration in ubiquinone-deficient mitochondria.[12] The compound has been the subject of a number of clinical trials for the treatment of inherited disorders of the respiratory chain.[13−15]

In a recent study, we demonstrated that idebenone analogues having one or both OCH_3 groups replaced with CH_3 groups retained the ability to support O_2 consumption within mitochondrial complex IV.[16] The finding that a redox center analogous to those in tocopherol (hydro)quinone could function within the mitochondrial respiratory chain prompted the study of a lipophilic pyridinol analogue of tocopherol. This analogue quenched the peroxidation of mitochondrial membranes more effectively than tocopherol and conferred protection to CEM leukemia cells that had been placed under oxidative stress.[17] Presently, we extend our studies of idebenone/idebenol by synthesizing and evaluating an aza analogue (**1**) designed to blunt the effects of oxidative stress resulting from excess ROS production within the respriatory chain. Compound **1** incorporates a structural motif similar to one recently shown to suppress the autoxidation of methyl lineolate.[18−20]

Compound **1** was prepared as illustrated (Scheme 1). 2-Amino-4,6-dimethylpyrimidine (**2**) was prepared by the condensation of guanidine and 2,4-pentanedione[21,22] and then brominated with

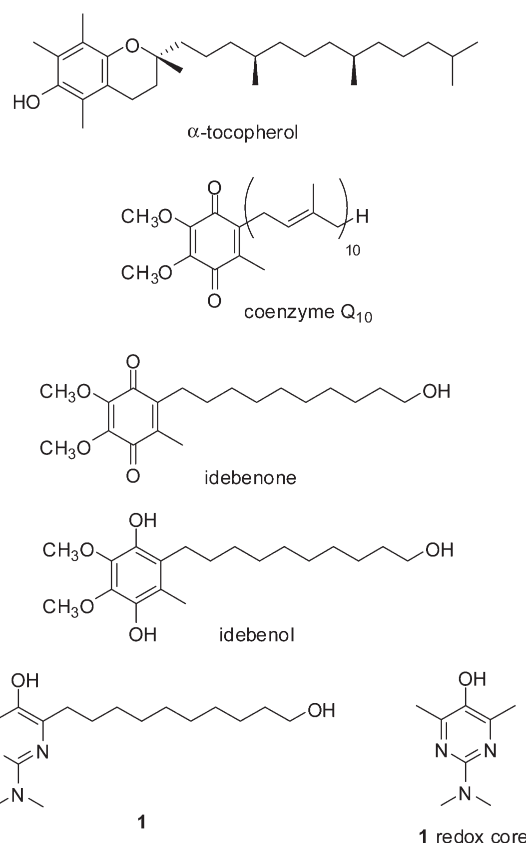


Figure 1. Chemical structures of α-tocopherol (α-TOH), coenzyme Q_{10}, idebenone, idebenol, an aza analogue of idebenone/idebenol (**1**), and its redox core.

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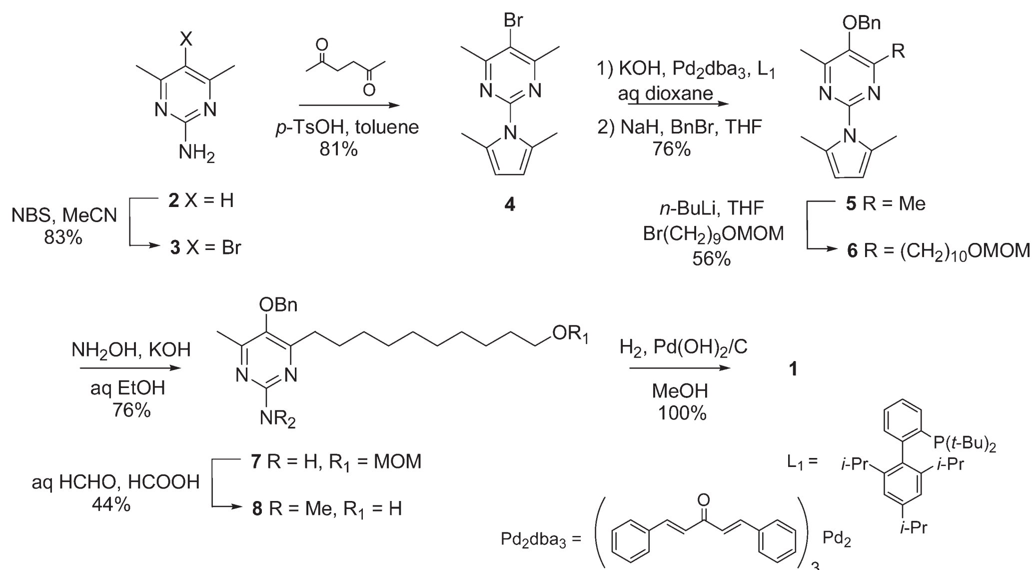
Scheme 1. Synthesis of an Aza Analogue (1) of Idebenone



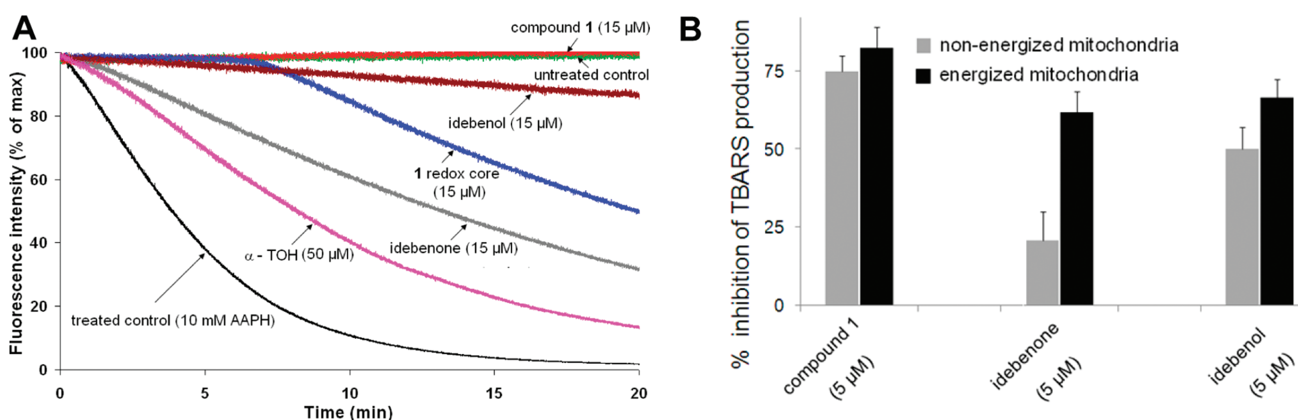


Figure 2. (A) Effect of compound **1** on lipid peroxidation induced by 10 mM AAPH in phospholipid liposomes containing C_{11}-BODIPY$^{581/591}$ in Tris-HCl buffer at 40 °C. Relative fluorescence units are normalized to 100% intensity. Identical results were obtained in replicate experiments. (B) Percent inhibition of iron-dependent TBARS formation in nonenergized (gray bar) and energized (black bar) mouse liver mitochondria in the presence of **1** or idebenone and its reduced form (idebenol). Mouse liver mitochondria (2 mg of protein/mL) were suspended in 10 mM Tris-HCl, pH 7.6, containing 100 mM KCl at 37 °C. Aliquots (0.8 mL) were preincubated with the test compounds at 37 °C for 5 min in presence of 5 mM succinate and 10 μM rotenone (energized mitochondria) or 20 mM malonate (nonenergized mitochondria). Ascorbic acid (300 μM) and 100 μM ferric ammonium sulfate were then added to induce lipid peroxidation, and TBARS formation was quantified 40 min later as described in the Supporting Information. Data are expressed as % of control samples lacking any test compound and expressed as means ± SEs (n = 3).

N-bromosuccinimide to afford the brominated pyrimidine **3** in an overall 79% yield. Following protection of the amine with 2,5-hexanedione,[22] bromide **4** was converted to the respective pyrimidinol [KOH, Pd_2dba_3, di-*tert*-butylphosphino-2′,4′,6′-triisopropylbiphenyl (L_1)][23] and then *O*-benzylated (NaH, BnBr) to provide **5** in 76% yield. The MOM-protected side chain was introduced by alkylation of a formed (*n*-BuLi, anh THF) methyl group anion to afford fully protected derivative **6**.[24] Following removal of the pyrrole protecting group (NH_2OH, KOH), treatment of **7** with aqueous HCHO/HCOOH at reflux afforded **8**, the *O*-benzylated derivative of **1**. Compound **1** was then obtained as a colorless oil by hydrogenolysis over $Pd(OH)_2$/C.

As shown (Figure 2A), compound **1** suppressed lipid peroxidation in a model membrane system consisting of unilamellar phospholipid vesicles containing the unsaturated phospholipids 1,2-dilinoleoylphosphatidylcholine (DLPC) and 1-stearoyl-2-oleylphosphatidylcholine (SOPC), and the embedded fluorophore C_{11}-BODIPY$^{581/591}$.[25] When treated with the free radical generator 2,2′-azo-bis(2-amidinopropane) (AAPH), the fluorescence of the fluorophore diminished with time, reflecting peroxidation of the (lipid) fluorophore. As shown, **1** effectively suppressed this lipid peroxidation and was more effective than the natural antioxidant α-tocopherol, as well as idebenone or idebenol.

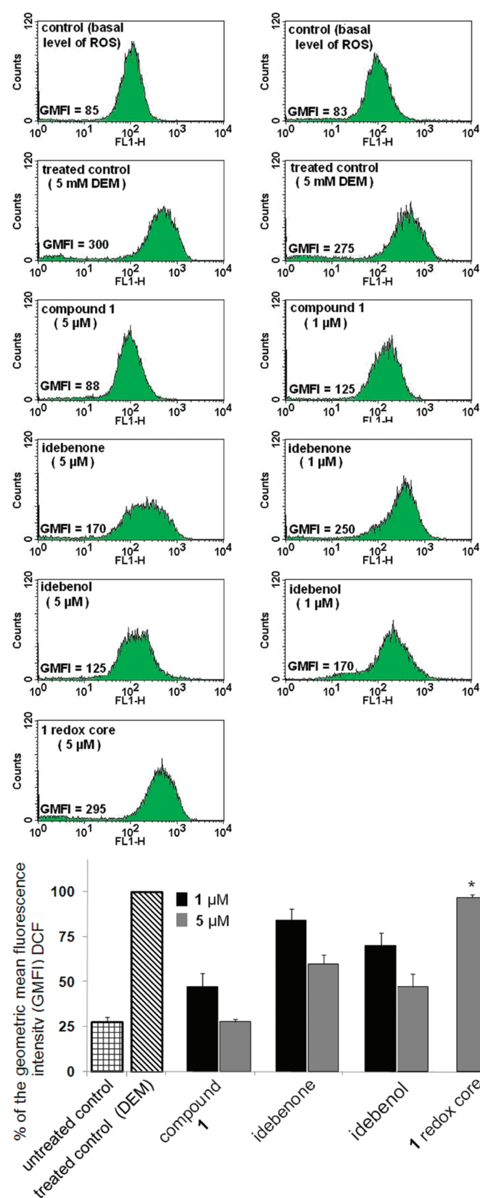


Figure 3. Representative flow cytometric analysis of ROS production in CEM cells stained with DCFH-DA and analyzed using the FL1-H channel. Results are plotted as counts (number of cells) vs log DCF fluorescence intensity. Following pretreatment with the indicated compounds (at 1 or 5 μM concentration) for 3 h, the cells were treated with 5 mM diethyl maleate (DEM) for 40 min to induce oxidative stress by depleting cellular glutathione. The cells were washed twice with PBS and suspended in phosphate-buffered saline containing 20 mM glucose. Cells were loaded with 10 μM dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min, and the green fluorescence (DCF) was measured by flow cytometry using the FL1-H channel. The figure shows a representative example of three independent experiments. In each analysis, 10000 events were recorded. Increased DCF fluorescence, a measure of intracellular oxidation and ROS production, was determined by a shift in DCF fluorescence to the right on the *x*-axis of the FACS histogram. 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 ± SEs ($n = 3$), statistically significant ($P < 0.01$) with respect to the treated control; Student's *t* test. *Not significant with respect to the treated control.

Separately, **1** was also shown to protect mitochondrial membranes from peroxidation. The content of thiobarbituric acid-reactive substances (TBARS), an indicator of mitochondrial lipid peroxidation, was determined in presence and absence of **1** in mouse liver mitochondria. Ascorbic acid + Fe^{2+} were used to induce lipid peroxidation.[26] To compare compound **1** to both idebenone and its reduced form (idebenol), we carried out the experiment in succinate-energized and nonenergized intact mitochondria. Reduction of idebenone was facilitated by the respiratory chain by including succinate in energized mitochondria and was prevented by including malonate and rotenone in nonenergized mitochondria.[27]

A significant increase in the amount of TBARS was observed in intact mouse liver mitochondria following incubation with ferrous iron. Compound **1** was found to be more effective in preventing the accumulation of TBARS than both idebenone and idebenol in succinate-energized and nonenergized mitochondria (Figure 2B). Idebenone did not block lipid peroxidation effectively in nonenergized mitochondria, while its reduced form did.

Diethyl maleate (DEM) depletes cellular glutathione,[30] and this has been used to induce oxidative stress in cellular systems with the production of ROS,[31,32] providing a model system for studying oxidative stress. Compound **1** suppressed ROS production, as illustrated (Figure 3). Cultured CEM leukemia cells were pretreated with 5 μM **1** for 3 h and then treated with 5 mM diethyl maleate (DEM), which depletes cellular glutathione. In the absence of antioxidant, depletion of glutathione placed the cells under oxidative stress, as shown by the shift in the DCF fluorescence to the right on the *x*-axis of the FACS histogram (upper panel). This enhanced ROS production was completely suppressed by 5 μM **1** and to a significant extent by concentrations of **1** as low as 1 μM. In comparison, 5 μM concentrations of idebenone[12] or its hydroquinone idebenol afforded only partial suppression of ROS.[33] Likewise, the redox core of compound **1** lacking the lipophilic side chain (**1** redox core, Figure 1) had no effect on cellular ROS levels.

Compound **1** was tested for its ability to maintain mitochondrial membrane potential in DEM-treated CEM cells. As shown (Figure S2 in the Supporting Information), the protection conferred by **1** was dose-dependent, consistent with its demonstrated ability (Figure 3) to suppress DEM-induced ROS.

The viability of the cultured CEM cells was measured by trypan blue exclusion and reflected the trends apparent in the FACS histogram. The cytoprotective effects of **1** were much greater than those realized with idebenone or idebenol. In CEM cells, **1** had an EC_{50} value of 250 ± 35 nM, while those of idebenone and idebenol were 820 ± 50 and 720 ± 45 nM, respectively (Figure 4). The ability of compound **1** to protect cultured Friedreich's ataxia fibroblasts from oxidative stress was also studied (Figure 4). The cells were pretreated overnight with idebenone, compound **1**, α-tocopherol, and compound **1** redox core and then subjected to L-buthionine (*S*,*R*)-sulfoximine (BSO), an inhibitor of glutathione biosynthesis.[38] After incubation for another 24 h, cell viability was measured by the membrane impermeable dye ethidium homodimer-1 (EthD-1)[39,40] and the membrane permeable dye calcein-AM.[40] As shown (Figure 4), **1** was superior to idebenone and much more effective than its redox core. It was also superior to α-tocopherol. Once again, **1** mediated its effects at nanomolar concentrations.

The foregoing results may be rationalized as outlined in Figure 5. In common with α-tocopherol and other potent phenolic antioxidants, it is likely that **1** quenches lipid peroxidation by

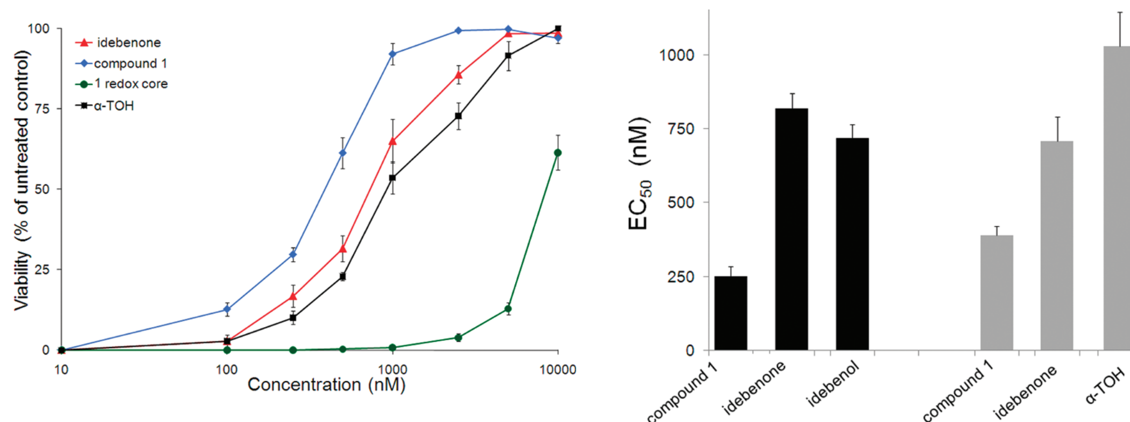


Figure 4. GSH depletion-induced death of FRDA fibroblasts and CEM cells were blocked by **1**. (left panel) Cellular viability of FRDA cells after BSO treatment was measured with a dual fluorescence cell viability assay using ethidium homodimer-1 and calcein-AM. Viability of FRDA cells (expressed as percentage of untreated cells) preincubated for 12 h with increasing concentrations of **1**, idebenone, or α-TOH, followed by BSO treatment. (right panel) The EC_{50} values for **1**, idebenone, and α-TOH were 390 ± 28, 710 ± 80, and 1030 ± 116 nM, respectively (gray bars). Data are from a typical experiment, repeated at least three times, and each point represents the mean ± SD of three determinations. Effects on cell viability were also determined by a trypan blue exclusion assay of the treated CEM cells. The CEM cells were incubated in RPMI medium (control) with **1**, idebenone, or idebenol for 3 h, followed by treatment with 5 mM DEM. Cell viability was determined by trypan blue exclusion analysis 4 h after DEM treatment. At the time of assay, >90% of the DEM-treated cells were trypan blue positive, whereas in non-DEM-treated control cell cultures, >95% of the cells were viable. The EC_{50} values for **1**, idebenone, and idebenol were 250 ± 35, 820 ± 50, and 720 ± 45 nM, respectively (black bars). Data shown are from a typical experiment, repeated at least three times, and each point represents the mean ± SD of three determinations. The cell viability was expressed as the percentage of control. Data are expressed as means ± SEMs (n = 3).

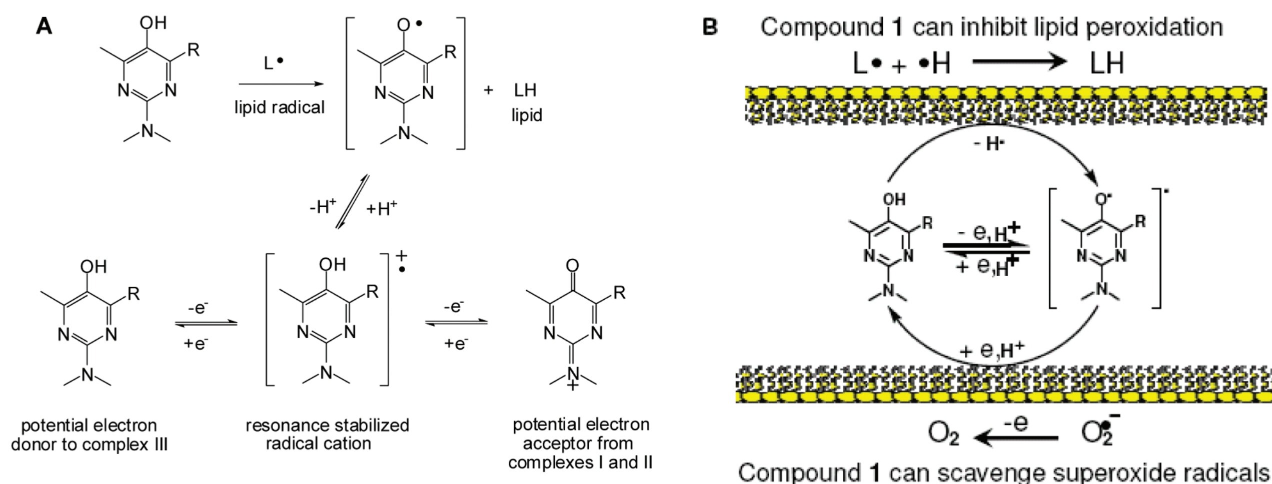


Figure 5. (A) Resonance-stabilized 5-pyrimidinoxyl radical resulting from the reaction of **1** with lipid radicals and its relationship to the oxidized and reduced forms of **1**. (B) Proposed catalytic mode of action of **1** as a quencher of lipid radicals and superoxide.

donation of an H atom to carbon-centered lipid radicals. The radical resulting from donation of $H^•$ from **1** would be expected to be resonance stabilized; as shown in Figure 5A, the protonated form of this radical is analogous to the semiquinone radical that is an intermediate between the oxidized and the reduced forms of coenzyme Q_{10}. Thus, quenching of lipid peroxidation by **1** would be expected to be facile (as it leads to a resonance-stabilized product), and the product should potentially be competent to participate in electron transfer reactions within the mitochondrial respiratory chain.

One facet of the results shown in Figures 3 and 4 and Figure S2 in the Supporting Information is particularly worthy of comment. In these experiments, chemical treatments were employed to deplete glutathione, which is typically present in cells at ∼1−10 mM concentration to protect cells against oxidative stress.[41] All three figures document that nanomolar−low micromolar concentrations of **1** are sufficient to suppress ROS and maintain mitochondrial membrane potential and cell viability. Given that glutathione protects cells by acting stoichiometrically as an antioxidant, the dramatically lower concentrations of **1** required to achieve the same effects are remarkable. The most logical explanation is that **1** may function *catalytically* to suppress ROS, protect lipid membranes, and maintain cell viability. A working hypothesis consistent with the putative catalytic function of **1** is outlined in Figure 5B. Quenching of lipid peroxidation by **1** would result in a radical that should be a strong electron acceptor. This species should be capable of reacting with superoxide, the form of ROS that is expected to be produced initially in partially dysfunctional

mitochondria through the leakage of electrons onto oxygen. The cycle in Figure 5B posits the simultaneous quenching of ROS and lipid radicals, in a fashion that should be catalytic. Additional experimental data, including those involving cyclic voltammetry, should be helpful in providing evidence for the proposed cycle. The behavior of **1** suggests a strategy for preparing optimized analogues of coenzyme Q_{10} capable of quenching oxygen and lipid radicals, as well as supporting electron transfer between mitochondrial complexes. We suggest the descriptor multifunctional radical quenchers for such species.

■ ASSOCIATED CONTENT

Supporting Information. Procedures and characterization data for all compounds, procedures for biochemical assays, and assay data for the effect of **1** on superoxide production and mitochondrial membrane potential in CEM cells under induced oxidative stress. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 480-965-6625. Fax: 480-965-0038. E-mail: sid.hecht@asu.edu.

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experiment was carried out using antimycin A (AMA), which inhibits succinate oxidase and NADH oxidase, and also inhibits mitochondrial electron transport between cytochromes *b* and *c* (complex III).[34] The inhibition of electron transport causes a collapse of the proton gradient across the mitochondrial inner membrane, thereby diminishing mitochondrial membrane potential.[34,35] This causes the production of superoxide,[36] measured in this work using dihydroethidium (DHE), a fluorogenic probe that is highly selective for superoxide among ROS.[37] As shown (Figure S1 in the Supporting Information), compound **1** also effected dose-dependent reduction of this oxidative stress.

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