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Synthesis and Biological Activities of *N*-(3-Carboxylpropyl)-5-amino-2-hydroxy-3-tridecyl-1,4-benzoquinone and Analogues

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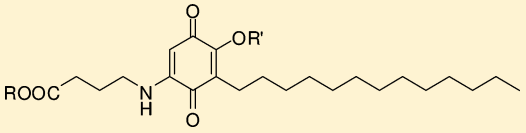
Supporting Information

ABSTRACT: The synthesis of benzoquinone natural product **2** and three analogues is described. The synthesized compounds were tested for their ability to protect Friedreich's ataxia (FRDA) lymphocytes from induced oxidative stress. One of the analogues (**3**) conferred cytoprotection in a dose-dependent manner in FRDA lymphocytes at micromolar concentrations. The biological assays suggest that the modification of the 2-hydroxy and *N*-(3-carboxypropyl) groups in the natural product can improve its antioxidant activity and significantly enhance its ability to protect mitochondrial function under conditions of oxidative stress.



Mitochondria are intracellular organelles that play a critical role in a number of metabolic pathways.[1] A primary function of the mitochondria is the generation of sufficient ATP to meet the energy requirements of the cell.[1] Mitochondrial damage can play a critical role in the etiology and progression of several neurodegenerative diseases, including Friedreich's ataxia (FRDA).[2] In normal cells, the generation of reactive oxygen species (ROS) and free radicals in the mitochondria is controlled by cellular antioxidant enzymes. In diseased cells, disruptions in the mitochondrial electron transport chain can cause enhanced oxidative stress and damage, challenging the intrinsic cellular antioxidant capacity.[2]

Natural products have been an important source for the development of small-molecule therapeutic agents for the treatment of many diseases, including neurodegenerative diseases.[3] Quinones such as coenzyme Q_{10} (CoQ_{10}) are an important class of natural products and play a key role in cellular respiration.[4] The pharmacological and toxicological properties of quinones have been well documented.[5] Natural product **2**, first isolated from *Embelia ribes* Burm. (Myrsinaceae), which is a species used in traditional Chinese medicine, has been synthesized previously, but its biological activity has not been well studied.[6,7]

Compound **2** has a structure similar to the redox-active quinone core of the natural product geldanamycin. Geldanamycin is a benzoquinone ansamycin that has been widely studied for its antiproliferative activities against a wide panel of human tumor cell lines.[8] Interestingly, it has been shown experimentally that an analogue of geldanamycin (17-AAG) undergoes reduction in normal epithelial cells under physiological conditions and that this reduction significantly enhances its affinity for the cellular protein target Hsp90.[9] Given the structural similarity between the redox cores of **2** and geldanamycin, it seemed likely that **2** and its structural analogues would also undergo reduction in situ to afford the corresponding hydroquinones under physiological conditions, potentially enabling them to protect cells from oxidative stress.

Herein we describe the synthesis of natural product **2** and analogues **1**, **3**, and **4** (Figure 1). The synthesized compounds **1**−**4** were studied for their ability to confer cytoprotection to FRDA lymphocytes placed under conditions of induced oxidative stress. Compound **3** showed promising antioxidant activity and maintained cell viability in a dose-dependent manner (Table 1) at micromolar concentrations in oxidatively stressed FRDA lymphocytes. Compound **3** was also shown to

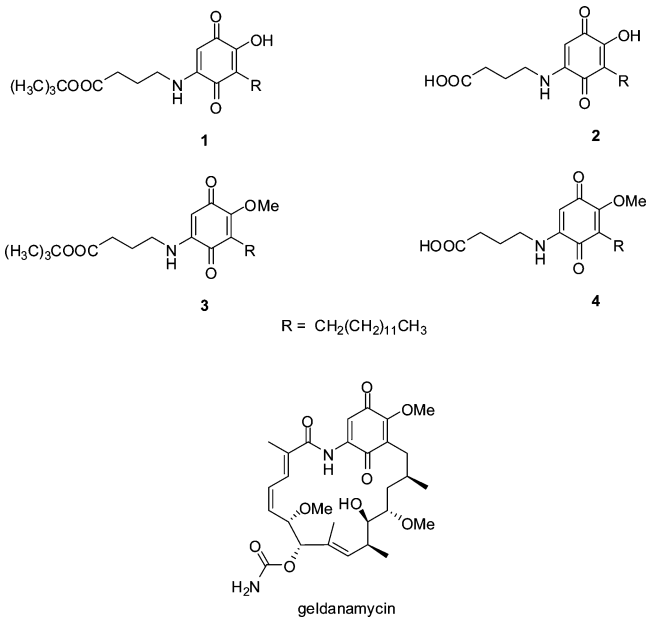


Figure 1. Chemical structures of 5-[*N*-(3-carboxypropyl)amino]-2-hydroxy-3-tridecyl-1,4-benzoquinone (**2**), three derivatives (**1**, **3** and **4**) and geldanamycin.

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Table 1. Cytoprotection of Cultured FRDA Cells from the Effects of Oxidative Stress[a]

	viable cells (%)			
compound	5 μM	2.5 μM	0.25 μM	0.125 μM
1	50 ± 2.9			
2	36 ± 7.3			
3	93 ± 4.0	84 ± 5.0	58 ± 4.4	48 ± 5.6
4	74 ± 5.5			

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

suppress ROS production and lipid peroxidation and to maintain mitochondrial membrane potential in cultured FRDA lymphocytes that had been subjected to oxidative stress. These findings are consistent with the possible use of this class of compounds as therapeutic agents for the management of oxidative stress.

RESULTS AND DISCUSSION

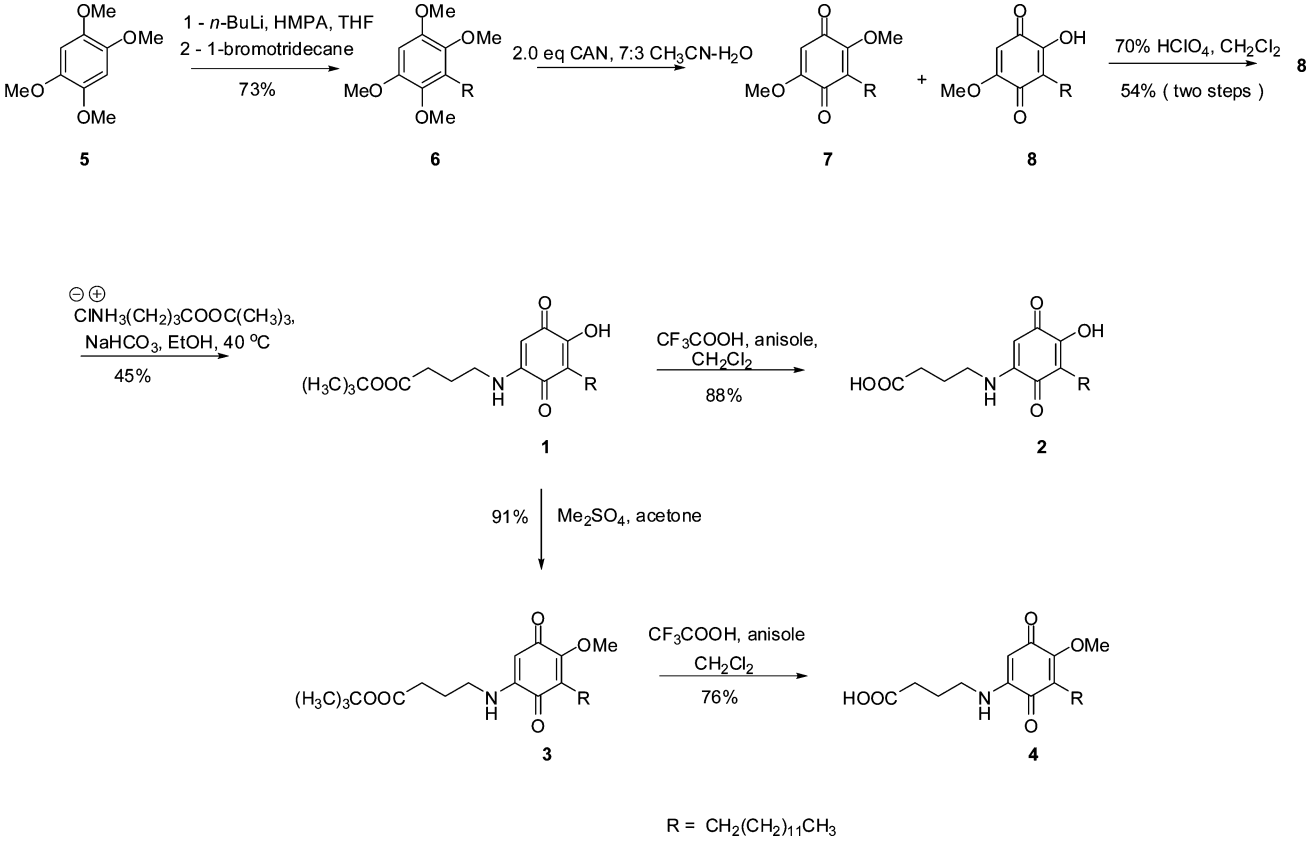
The synthesis of **2** (Scheme 1) began with the *n*-butyllithium-mediated alkylation of compound **5**[10,11] with purified 1-bromotridecane to yield **6** in 73% yield.[11] The alkylated tetramethoxybenzene **6** was then subjected to oxidation with cerium(IV) ammonium nitrate to give a crude mixture containing quinones **7** and **8**, which underwent selective perchloric acid-catalyzed demethylation to afford hydroxyquinone **8** in 54% yield over two steps.[11] Regioselective demethylation took place with the removal of the more hindered methoxy group.[11]

The synthesis of **2** was attempted by treatment of **8** with sodium bicarbonate and γ-aminobutyric acid, which would involve conjugate addition of the amino acid to the vinylogous methyl ester.[7,12] The reaction did not proceed as expected, possibly due to γ-butyrolactam formation. Therefore, **8** was treated with γ-aminobutyric acid *tert*-butyl ester hydrochloride salt in the presence of a large excess of sodium bicarbonate to afford *tert*-butyl ester **1** in 45% yield. Finally the *tert*-butyl ester was cleaved by treatment with trifluoroacetic acid in the presence of anisole;[13] compound **2** precipitated from methanol as a red amorphous solid in 88% yield. Alternatively, *tert*-butyl ester **1** was methylated with dimethyl sulfate in dry acetone to yield **3** in 91% yield, which, upon treatment with trifluoroacetic acid in the presence of anisole, afforded acid **4** in 76% yield.

Biochemical and Biological Evaluation. *Cytoprotection.* The ability of the test compounds to confer cytoprotection was evaluated in cultured Friedreich's ataxia lymphocytes. These cells were treated with diethyl maleate to induce oxidative stress through depletion of glutathione. As shown in Table 1, compound **3** was the most efficient, exhibiting 84% cytoprotection at 2.5 μM concentration. The cytoprotection afforded by this compound increased at higher doses up to 5 μM concentration. Benzoquinone analogue **4** afforded greater cyoprotection to FRDA lymphocytes at 5 μM concentration than did the *tert*-butyl ester **1** (74% vs 50%). The natural product **2** afforded the least protection when tested at this concentration.

Inhibition of Lipid Peroxidation. The ability of compound **2** and its analogues to quench lipid peroxidation was evaluated in FRDA lymphocytes. These cells were placed under oxidative stress by depleting them of glutathione (GSH) using diethyl

Scheme 1. Synthesis of Natural Product 2 and Analogues



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maleate (DEM).[14−16] The extent of lipid peroxidation was quantified using the fatty acid sensitive fluorescent reporter C_{11}-BODIPY$^{581/591}$ (Molecular Probes).[17,18] Upon oxidation of the phenylbutadiene moiety of the fluorophore, the red-emitting form of the dye (595 nm) is converted into a green-emitting form (520 nm). Increased C_{11}-BODIPY$^{581/591}$-green (oxidized) fluorescence, a measure of peroxyl radical production, was determined by flow cytometric analysis, which is expressed as percent scavenging activity (Table 2). The results in Table 2 show that analogue **3** was very effective in suppressing lipid peroxidation at 5 and 10 μM concentrations (72% and 86% suppression of lipid peroxidation), while the natural product **2** was much less active (24% suppression at 10 μM concentration). Compounds **1** and **4** also exhibited concentration-dependent suppression of lipid peroxidation, affording 37% and 51% suppression, respectively, at 10 μM concentration.

Table 2. Suppression of Lipid Peroxidation by Compounds 1−4 in Cultured FRDA Lymphocytes Treated with Diethyl Maleate[a]

	scavenging activity (%)	
compound	5 μM	10 μM
untreated control[b]	100	100
treated control[c]	0	0
1	26 ± 6.7	37 ± 1.4
2	8.0 ± 6.6	24 ± 7.4
3	72 ± 1.8	86 ± 1.8
4	41 ± 7.2	51 ± 5.0

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

Suppression of Reactive Oxygen Species. The ability of compounds **1**−**4** to suppress ROS production was determined in FRDA lymphocytes and CEM leukemia lymphocytes in the presence and absence of the test compounds by monitoring the fluorescence of the ROS-sensitive dye dichlorofluorescein (DCF). Increased DCF fluorescence, a measure of intracellular oxidation and ROS production, was determined by flow cytometry. These cells were placed under oxidative stress by depleting them of GSH using DEM.[14−16] The results in Figure 2 show that analogue **3** was very effective in suppressing ROS in the stressed cells at 5 μM concentration, while the natural product **2** was only very weakly active at the same concentration. Compounds **1** and **4** were less effective than compound **3**.

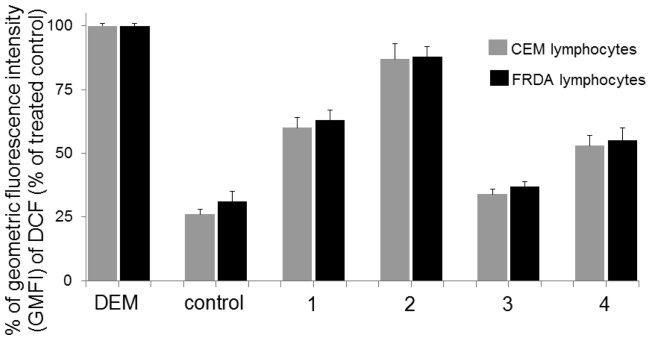

Figure 2. Flow cytometric analysis of CEM leukemia lymphocytes (gray bars) and FRDA lymphocytes (black bars) stained with dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min, following pretreatment with the test compounds at 5 μM concentration for 16 h, and subsequent treatment with diethyl maleate (DEM) for 40 or 80 min to induce the production of ROS in CEM and FRDA lymphocytes, respectively. Data shown represent the mean ± SEM of two different experiments run as duplicates.

Preservation of Mitochondrial Membrane Potential ($\Delta\psi_m$). The ability of the test compounds to preserve mitochondrial membrane potential under conditions of oxidative stress was studied. Assessment of $\Delta\psi_m$ is an important indicator of cellular function during stress-induced cell death. Changes in mitochondrial membrane potential were measured using two different fluorescent dyes, tetramethylrhodamine methyl ester (TMRM) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1). TMRM is a potentiometric, cell-permeable fluorescent indicator that accumulates in the highly negatively charged interior of the mitochondrial inner membrane in a Nernstian manner.[19] The fluorescence signal of TMRM can be directly correlated to $\Delta\psi_m$ across the inner mitochondrial membrane. Therefore, the accumulation of dye in the mitochondria and the intensity of the signal are direct functions of mitochondrial potential. Loss of mitochondrial membrane potential is indicated by a reduction in TMRM red fluorescence. The detection of mitochondrial depolarization using TMRM was accomplished by flow cytometry. Figure 3 illustrates representative two-dimensional density dot plots of TMRM-stained lymphocyte cells showing the percentage of cells with intact $\Delta\psi_m$ (TMRM fluorescence in top right quadrant) vs the percentage of cells with reduced $\Delta\psi_m$ (TMRM fluorescence in bottom left and right quadrants). Figure 3 (bottom panel) shows a bar graph of the percentage (mean ± SE) of FRDA lymphocytes with intact $\Delta\psi_m$. The results show that treatment with 5 mM DEM decreased the percentage of cells with TMRM fluorescence in the top right quadrant, indicating that DEM treatment caused depolarization of $\Delta\psi_m$. Compound **3** preserved mitochondrial membrane polarization the most effectively when used at 5 μM concentration (85%), while compound **2** was the least effective (27%). Intermediate values were determined for compounds **1** and **4** (40% and 60%, respectively).

These results were further confirmed using JC-1 dye in primary FRDA fibroblasts treated with buthionine sulfoximine (BSO) (Figure 4). BSO was used in this cellular model to induce an oxidative insult by inhibiting de novo glutathione synthesis.[20] JC-1 is a lipophilic, cationic dye that can selectively enter into mitochondria and reversibly change color from green to red as the membrane potential increases by forming aggregates.[21] The dye exhibits red fluorescence when it aggregates in the matrix of healthy energized mitochondria, whereas it has green fluorescence in cells with depolarized $\Delta\psi_m$. In untreated FRDA cells and cells treated with 5 μM compound **3** in addition to 1 mM BSO, the JC-1 dye was mainly in the aggregated state (red−orange) (Figure 4A and D), indicating that compound **3** preserved the mitochondrial membrane potential in spite of the presence of BSO. Treatment with 1 mM BSO prevented JC-1 mitochondrial accumulation in the cells, resulting in a pronounced green fluorescence due to complete loss of mitochondrial membrane potential (Figure 4C). A significant mitochondrial membrane depolarization was observed with natural product **2** in BSO-treated cells (Figure 4E). 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 pronounced green fluorescence resulting from FCCP treatment reflects

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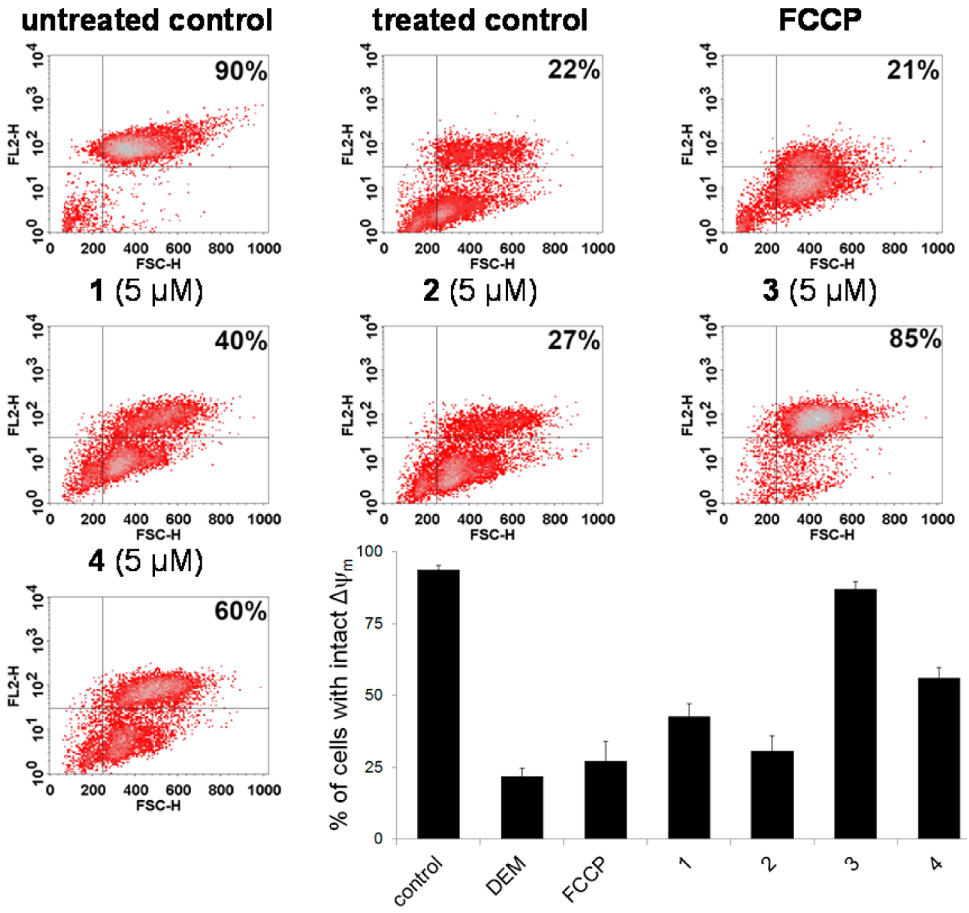

Figure 3. Effect of nitrogen-containing 1,4-benzoquinone derivatives on preservation of mitochondrial membrane potential of cultured FRDA cells following treatment with the test compounds. Representative flow cytometric two-dimensional color density dot plot analyses of mitochondrial membrane potential $\Delta\psi_m$ in FRDA lymphocytes stained with TMRM and analyzed using the FL2-H channel as described in the Experimental Section. The cells were washed twice in phosphate-buffered saline and suspended in phosphate-buffered saline containing 20 mM glucose. The percentage of cells with intact $\Delta\psi_m$ is indicated in the top right quadrant of the panels. In each analysis, 10 000 events were recorded. Data are expressed as means ± SEM of three independent experiments run in duplicate. The bar graph shows the percentage of cells with intact $\Delta\psi_m$ calculated using CellQuest software.

the depolarization of mitochondrial inner membrane potential (Figure 4B). These data indicate that compound **3** is able to prevent oxidative stress induced collapse of $\Delta\psi_m$, an event associated with the disruption of mitochondrial function that occurs prior to cell death.

Recently, we have described three structural series of compounds that are capable of protecting cells with suboptimal mitochondrial function resulting from the effects of induced oxidative stress.[22] These compounds suppress lipid peroxidation and reactive oxygen species and confer cytoprotection to cultured mammalian cells under oxidative stress. The present study extends these findings to a fourth structural class, providing an additional pharmacophore of potential utility for the design of therapeutic agents for the treatment of mitochondrial disorders.

In conclusion, we have prepared natural product **2** and three structural analogues and tested them for their ability to prevent ROS-induced damage of cellular lipid membranes, maintain mitochondrial membrane potential, and confer cytoprotection to FRDA lymphocytes despite severe induced oxidative stress.

EXPERIMENTAL SECTION

General Experimental Procedures. All the chemicals were purchased from Sigma Aldrich and Chem-Impex International. The chemicals were all ACS reagent grade and were used without further purification, except for 1-bromotridecane, which was purified by silica gel flash column chromatography prior to use. The reactions were carried out under an atmosphere of argon. Flash column chromatography was carried out using silica gel (Silicycle R10030B, 60 particle size, 230−400 mesh), applying a low-pressure stream of nitrogen. Analytical thin-layer chromatographic separations were carried out on glass plates coated with silica gel (60 particle size F254, SiliCycle TLG-R10011B-323). The TLC chromatograms were developed 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, or else visualized by UV irradiation (254 nm). Melting points were recorded on a MelTemp apparatus and are uncorrected. Tetrahydrofuran was distilled from sodium/benzophenone ketyl and dichloromethane from calcium hydride. ^{1}H and ^{13}C NMR spectra were recorded on a Gemini 300 or Varian Inova 400 or on a Varian Inova 500 spectrometer, using $CDCl_3$ as solvent and internal standard, unless otherwise indicated. ^{1}H NMR chemical shifts were reported relative to residual $CDCl_3$ at 7.26 ppm or to residual DMSO-d_6 at 2.50 ppm; ^{13}C NMR shifts were reported relative to the central line of $CDCl_3$ at 77.16 ppm or to residual DMSO-d_6 at 39.51 ppm. Splitting patterns are designated as s, singlet; d, doublet; dd, doublet of doublets; m, multiplet; q, quartet; quin, quintet; br, broad. High-resolution mass spectrometric data was obtained at the Michigan State Mass Spectrometry Facility or at the Arizona State University CLAS High Resolution Mass Spectrometry Facility.

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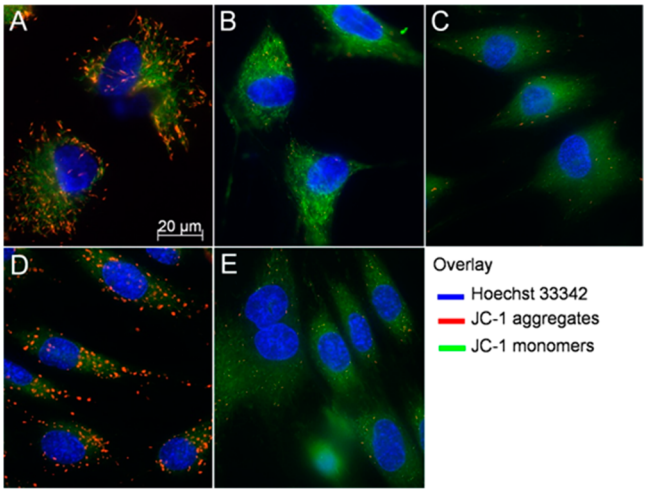

Figure 4. Representative fluorescence microscopy images of JC-1-stained primary FRDA fibroblasts were examined under a Zeiss fluorescent microscope. Red indicates JC-1 aggregates, which are formed in the mitochondria when a sufficiently high membrane potential is reached. When the $\Delta\psi_m$ collapses as a result of glutathione depletion, the reagent (JC-1) no longer accumulates inside the mitochondria, and instead, it is distributed throughout the cell in the monomeric form, which fluoresces green. Hoechst 33342 was used to identify all nuclei. (A) Untreated primary FRDA fibroblasts, (B) FRDA fibroblasts treated for 2 h with a 25 μM concentration of the uncoupler FCCP, (C) FRDA fibroblasts treated for 24 h with 1 mM BSO, (D) FRDA fibroblasts pretreated for 12 h with 5 μM compound **3** and then treated for 24 h with 1 mM BSO, and (E) FRDA fibroblasts pretreated for 12 h with 5 μM compound **2** and then treated for 24 h with 1 mM BSO.

Chemistry. Compounds **5** and **6** were synthesized according to literature procedures.[10,11]

2,3,5,6-Tetramethoxyphenyl-1-tridecylbenzene (6) (ref 11). To a solution containing 1.0 g (5.0 mmol) of 1,2,4,5-tetramethoxybenzene (**5**)[11] and 87 μL (90 mg, 0.5 mmol) of hexamethylphosphoramide in 25 mL of dry THF was added 3.4 mL (1.6 M in hexanes, 5.5 mmol) of *n*-butyllithium dropwise at −40 °C over a period of 5 min. The reaction mixture was warmed to 0 °C over a period of 2 h; then 1.4 mL (1.4 g, 5.5 mmol) of purified 1-bromotridecane was added, and the reaction mixture was stirred at room temperature under argon for 15 h. The reaction mixture was quenched with 20 mL of saturated NH_4Cl and extracted with five 10 mL portions of ether. The organic layer was washed with distilled water and brine, then dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (6 × 3 cm). Elution with 1:9 ethyl acetate−hexanes afforded **6** as a colorless solid: yield 1.4 g (73%); mp 31−32 °C; lit.[11] mp 31−32 °C. A 0.20 g (20%) amount of unreacted 1,2,4,5-tetramethoxybenzene (**5**) was recovered: silica gel TLC R_f 0.45 (1:1 ethyl ether−hexanes); ^{1}H NMR ($CDCl_3$) δ 0.87 (3H, t, J = 6.8 Hz), 1.14−1.46 (20H, m), 1.47−1.58 (2H, m), 2.61 (2H, dd, J = 8.8 and 6.9 Hz), 3.76 (6H, s), 3.82 (6H, s), and 6.40 (1H, s); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.7, 24.7, 29.4, 29.5, 29.6, 29.70, 29.75, 29.76, 30.0, 30.8, 32.0, 56.2, 60.4, 60.9, 96.7, 131.1, 141.1, and 148.8.

2-Hydroxy-5-methoxy-3-tridecyl-1,4-benzoquinone (8) (ref 11). To a solution containing 0.10 g (0.26 mmol) of 2,3,5,6-tetramethoxyphenyl-1-tridecylbenzene (**6**) in 2.6 mL of acetonitrile was added dropwise a solution containing 0.28 g (0.52 mmol) of cerium(IV) ammonium nitrate in 2.6 mL of 7:3 acetonitrile−water at −7 °C (salt−ice bath) over a period of 30 min. The reaction mixture was stirred at room temperature for 3 h and diluted with 10 mL of ether. The organic layer was washed successively with distilled water and brine, then dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a mixture of quinones **7** and **8**. To a solution of this mixture in 2.6 mL of dichloromethane was added 1.1 mL (13 mmol) of 70% perchloric acid dropwise at 0 °C. The reaction mixture was stirred at 0 °C for 9 h, diluted with 10 mL of dichloromethane, then washed successively with distilled water and brine, and dried ($MgSO_4$). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (7 × 2 cm). Elution with 1:4 ethyl acetate−hexanes gave **8** as a yellow-orange solid: yield 48 mg (54%); mp 90−92 °C; lit.[23] mp 90−91 °C; silica gel TLC R_f 0.58 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 0.85 (3H, t, J = 6.8 Hz), 1.17−1.33 (20H, m), 1.39−1.49 (2H, m), 2.41 (2H, t, J = 8 Hz), 3.84 (3H, s), 5.82 (1H, s), and 7.32 (1H, s); ^{13}C NMR ($CDCl_3$) δ 14.2, 22.7, 22.8, 28.1, 29.48, 29.54, 29.68, 29.69, 29.77, 29.78, 29.79, 29.80, 32.0, 56.9, 102.3, 119.4, 151.7, 161.2, 181.8, and 183.0.

4-(4-Hydroxy-3,6-dioxo-5-tridecylcyclohexa-1,4-dienylamino)butyric Acid *tert*-butyl Ester (1) (ref 7). To a solution of 42 mg (0.13 mmol) of 2-hydroxy-5-methoxy-3-tridecyl-1,4-benzoquinone (**8**) and 1.0 g (13 mmol) of sodium bicarbonate in 9.7 mL of ethanol was added 39 mg (0.19 mmol) of γ-aminobutyric acid *tert*-butyl ester hydrochloride salt. The reaction mixture was stirred at 45 °C for 27 h under an argon atmosphere. The reaction mixture was then diluted with 5 mL of water and extracted with seven 2 mL portions of dichloromethane. The combined organic layer was washed successively with water and brine and then dried (Na_2SO_4). The solvent was concentrated under diminished pressure to afford a crude residue. The residue was applied to a silica gel column (5 × 2 cm). Elution with dichloromethane gave **1** as a dark red solid: yield 27 mg (45%); mp 96−97 °C; lit.[7] mp 82−85 °C; silica gel TLC R_f 0.38 (dichloromethane); ^{1}H NMR ($CDCl_3$) δ 0.86 (3H, t, J = 6.5 Hz), 1.20−1.32 (20H, m), 1.38−1.46 (11H, m), 1.94 (2H, quin, J = 6.9 Hz), 2.31 (2H, t, J = 7.0 Hz), 2.34−2.40 (2H, m), 3.21 (2H, dd, J = 12.9 and 6.6 Hz), 5.35 (1H, s), 6.58 (1H, s), and 8.10 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 14.3, 22.79, 22.84, 23.5, 28.23, 28.24, 29.5, 29.6, 29.73, 29.75, 29.81, 29.83, 29.84, 32.1, 32.8, 42.4, 81.2, 91.9, 115.9, 149.9, 155.1, 172.1, 179.0, and 182.6; mass spectrum (LCT electrospray), m/z 486.3181 $(M + Na)^+$ ($C_{27}H_{45}NO_5Na$ requires m/z 486.3195).

5-[*N*-(3-Carboxylpropyl)amino]-2-hydroxy-3-tridecyl-1,4-benzoquinone (2). To a solution containing 28 mg (60 μmol) of **1** in 0.4 mL of dichloromethane was added 6.5 μL (60 μmol) of anisole and 400 μL (5.4 mmol) of trifluoroacetic acid. The reaction mixture was stirred for 24 h at room temperature under an argon atmosphere. The reaction mixture was concentrated under diminished pressure, and the excess trifluoroacetic acid removed by co-evaporation three times with cyclohexane to afford a crude residue. The residue was precipitated from methanol to give **2** as a red, amorphous solid: yield 21 mg (88%); mp 194−195 °C; lit.[7] mp 177−180 °C; ^{1}H NMR (DMSO-d_6) δ 0.85 (3H, t, J = 6.8 Hz), 1.15−1.42 (22H, m), 1.74 (2H, quin, J = 14.4 and 7.2 Hz), 2.26 (4H, q, J = 6.9 Hz), 3.14 (2H, dd, J = 13.8 and 6.7 Hz), 5.32 (1H, s), 7.78 (1H, t, J = 6.2 Hz), 10.49 (1H, br s), and 12.2 (1H, br s); ^{13}C NMR (DMSO-d_6) δ 14.0, 22.1, 22.2, 22.8, 27.6, 28.8, 28.9, 29.0, 29.02, 29.06, 29.08, 29.10, 30.9, 31.3, 41.4, 91.8, 115.6, 149.3, 156.7, 174.2, 178.5, and 182.5; mass spectrum (LCT electrospray), m/z 430.2564 $(M + Na)^+$ ($C_{23}H_{37}NO_5Na$ requires m/z 430.2569).

4-(4-Methoxy-3,6-dioxo-5-tridecylcyclohexa-1,4-dienylamino)butyric Acid *tert*-Butyl Ester (3). To a solution containing 22 mg (47 μmol) of **1** and 0.25 g (1.8 mmol) of potassium carbonate in 1.2 mL of dry acetone was added 23 μL (0.23 mmol) of dimethyl sulfate. The reaction mixture was heated at reflux overnight, allowed to cool to room temperature, and concentrated under diminished pressure. The crude mixture was redissolved in 10 mL of dichloromethane and washed with 5 mL of 1 N HCl, and the aqueous layer was extracted with three 10 mL portions of dichloromethane. The combined organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash column chromatography on a silica gel column (24 × 2 cm). Elution with 1:5 ethyl acetate−hexane gave **3** as a bright red, amorphous solid: yield 21 mg (91%); silica gel TLC R_f 0.60 (1:2 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 0.87 (3H, t, J = 6.8 Hz), 1.16−1.42 (22H, m), 1.45

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(9H, s), 1.82−2.03 (2H, quin, J = 9 Hz), 2.31 (2H, t, J = 7.2 Hz), 2.35−2.39 (2H, m), 3.14 (2H, dd, J = 13.0 and 6.8 Hz), 4.10 (3H, s), 5.28 (1H, s), and 5.94 (1H, t, J = 5.6 Hz); ^{13}C NMR ($CDCl_3$) δ 14.3, 22.8, 23.1, 23.6, 28.20, 28.24, 28.8, 29.5, 29.6, 29.7, 29.81, 29.83, 32.1, 32.9, 42.1, 61.8, 81.1, 96.1, 127.6, 146.9, 158.5, 172.18, 172.20, 181.8, and 183.9; mass spectrum (APCI), m/z 478.3532 $(M + H)^+$ ($C_{28}H_{48}NO_5$ requires m/z 478.3532).

5-[*N*-(3-Carboxypropyl)amino]-2-methoxy-3-tridecyl-1,4-benzoquinone (4). To a solution containing 9.0 mg (19 μmol) of **3** in 0.12 mL of dichloromethane were added 2.0 μL (19 μmol) of anisole and 0.13 mL (1.7 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 24 h under an atmosphere of argon. The reaction mixture was co-evaporated with six 5 mL portions of cyclohexane, and the solvent was concentrated under diminished pressure to afford a crude residue. The residue was purified by flash column chromatography on a silica gel column (22 × 2 cm). Elution with 100:1 chloroform−methanol gave **4** as a red, amorphous solid: yield 6.0 mg (76%); silica gel TLC R_f 0.32 (1:1 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.9 Hz), 1.22−1.41 (22H, m), 1.98 (2H, quin, J = 6.9 Hz), 2.33−2.40 (2H, m), 2.47 (2H, t, J = 6.9 Hz), 3.20 (2H, q, J = 6.6 Hz), 4.11 (3H, s), 5.29 (1H, s), and 5.97 (1H, s); ^{13}C NMR ($CDCl_3$) δ 14.3, 18.5, 22.8, 23.1, 23.2, 28.8, 29.5, 29.6, 29.7, 29.81, 29.84, 31.3, 32.1, 42.0, 51.0, 58.6, 61.8, 96.2, 127.7, 146.9, 158.5, 176.6, 181.8, and 184.0; mass spectrum (APCI), m/z 422.2898 $(M + H)^+$ ($C_{24}H_{40}NO_5$ requires 422.2906).

Biological Evaluation. *Cytoprotection.* Cell viability was determined by the use of a trypan blue exclusion assay[22c] in Friedreich's ataxia lymphocyte cell line GM15850 (Coriell Institute). This technique was used to assess the cytoprotective effects of the tested compounds in cultured cells treated with DEM to induce cell death by GSH depletion. The viability of DEM-treated FRDA cells was determined by their ability to exclude the dye trypan blue. Viable cells exclude trypan blue, whereas nonviable cells take up the dye and are stained blue. Briefly, FRDA lymphocytes were grown in RPMI 1640 medium (Gibco) supplemented with 15% fetal calf serum, 2 mM glutamine (HyClone), and 1% penicillin−streptomycin mix (Cellgro). Cells were seeded at a density of 5 × 10^5 cells/mL and treated with different concentrations of the indicated compounds. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 17 h. After preincubation, the cells were treated with 5 mM DEM and maintained for an additional 6 h. Cell viability was determined after staining cells with 0.4% trypan blue by the use of a hemacytometer. At least 500 cells were counted in each experimental group. At the time of assay, <20% of DEM-treated cells were viable (trypan blue negative), whereas in the non-DEM-treated control, >90% cells were viable. Cell viability was expressed as the percentage of control. Data are expressed as means ± SEM (n = 3).

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.[16] Briefly, FRDA lymphocytes (5 × 10^5 cell/mL) were treated with the test compounds at final concentrations of 5 and 10 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 medium 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 medium for 2 h. Treated cells were collected by centrifugation at 300g for 3 min and then washed with phosphate-buffered saline (PBS). Cells were resuspended in PBS 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 the assays in three independent experiments. Results are expressed as percent scavenging activity.

Scavenging of Reactive Oxygen Species. Changes in cellular ROS in CEM leukemia or FRDA lymphocytes were measured by the ROS reactive fluorescent indicator 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) using flow cytometry (BD FACS Calibur). Briefly, 1 mL of cells (5 × 10^5 cells) was plated in a 24-well plate, treated with the test compounds at 5 μM concentration, and incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 5 mM diethyl maleate (97%, Sigma-Aldrich) for 40 or 80 min to induce oxidative stress by glutathione depletion in the treated cells. The cells were collected by centrifugation at 300g for 3 min and then washed with PBS (Invitrogen). Cells were resuspended in PBS containing 20 mM glucose and incubated at 37 °C in the dark for 20 min with 10 μM DCFH-DA. Cells were collected by centrifugation at 300g for 3 min and then washed with PBS. The samples were analyzed immediately by flow cytometry (BD FACS Calibur) using a 488 nm excitation laser and FL1-H channel 538 nm emission filter. In each analysis, 10 000 events were recorded. The results obtained were verified in two independent experiments.

Assessment of Mitochondrial Membrane Potential. Mitochondrial membrane potential was measured using two different fluorescent dyes, TMRM and JC-1. $\Delta\psi_m$ was determined as previously described by staining FRDA lymphocyte cells with TMRM (Molecular Probes) and analyzing fluorescence emission by flow cytometry in detection channel 2 (FL2-H).[16] Briefly, FRDA lymphocytes were pretreated with or without the test compounds for 16 h. The cells were then treated with 5 mM DEM for 140 min, collected by centrifugation at 300g for 3 min, and then washed twice with phosphate-buffered saline. The cells were resuspended in PBS containing 20% glucose and incubated with 250 nM TMRM at 37 °C in the dark for 15 min. Cells were collected by centrifugation at 300g 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 to dissipate $\Delta\psi_m$. In each analysis, 10 000 events were recorded. We qualitatively examined the mitochondrial membrane potential using JC-1 dye in primary FRDA fibroblasts GM04078 (Coriell Institute) after treatment with 1 mM buthionine sulfoxime, in the presence and absence of the tested compounds (5 μM concentration). JC-1 is a cationic dye that is accumulated in mitochondria according to membrane potential. In polarized mitochondria, it accumulates in aggregated form and appears as red punctate staining, whereas in cells having depolarized mitochondria, JC-1 diffuses throughout the cell and appears as green diffused monomeric staining. Briefly, FRDA fibroblasts (2 × 10^5 cells/mL) were seeded in coverslips (Corning) in six-well plates. The plates were incubated at 37 °C overnight in a humidified atmosphere of 5% CO_2 in air to allow attachment of the cells to the coverslips. The following day, cells were treated with the test compounds and incubated for an additional 12 h before treatment with 1 mM BSO. $\Delta\psi_m$ was assessed after 24 h using JC-1 Mitochondrial Membrane Potential Detection Kit (Biotium, Inc.) following the manufacturer's protocol. Glass coverslips were rinsed with phosphate-buffered saline and mounted onto slides, and images were recorded and analyzed with a Zeiss AxioCam MRm and AxioVision 3.1 software (Carl Zeiss) on a Zeiss Axiovert 200 M inverted microscope, equipped with a 40× oil immersion objective.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **1**−**4**, **6**, and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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