Journal of Medicinal Chemistry Article

J. Med. Chem. **2010,** *53,* 4849–4861 **4849**
DOI: 10.1021/jm100212x

Amphiphilic Amide Nitrones: A New Class of Protective Agents Acting as Modifiers of Mitochondrial Metabolism

Grégory Durand,*[,†] Burkhard Poeggeler,[‡,§] Stéphanie Ortial,[†] Ange Polidori,[†] Frederick A. Villamena,[∥] Jutta Böker,[§] Rüdiger Hardeland,[§] Miguel A. Pappolla,[⊥] and Bernard Pucci*[,†]

[†]*Laboratoire de Chimie BioOrganique et des Systèmes Moléculaires Vectoriels, Université d'Avignon et des Pays de Vaucluse, Faculté des Sciences, 33 Rue Louis Pasteur, 84000 Avignon, France,* [‡]*Department of Dermatology, University of Luebeck, Ratzeburger Allee 160, D-23538 Luebeck, Germany,* [§]*Abteilung fuer Stoffwechselphysiologie, Institut fuer Zoologie, Anthropologie und Entwicklungsbiologie der Georg August Universität Göttingen, Berliner Strasse 28, D-37073 Göttingen, Germany,* [∥]*Department of Pharmacology and Center for Biomedical EPR Spectroscopy and Imaging, The Davis Heart and Lung Research Institute, College of Medicine, The Ohio State University, Columbus, Ohio 43210, and* [⊥]*Department of Neurology, Medical University of South Carolina, Charleston, South Carolina 29425*

Received February 17, 2010

Our group has demonstrated that the amphiphilic character of α-phenyl-*N*-*tert*-butyl nitrone based agents is a key feature in determining their bioactivity and protection against oxidative toxicity. In this work, we report the synthesis of a new class of amphiphilic amide nitrones. Their hydroxyl radical scavenging activity and radical reducing potency were shown using ABTS competition and $ABTS^{\bullet+}$ reduction assays, respectively. Cyclic voltammetry was used to investigate their redox behavior, and the effects of the substitution of the PBN on the charge density of the nitronyl atoms, the electron affinity, and the ionization potential were computationally rationalized. The protective effects of amphiphilic amide nitrones in cell cultures exposed to oxidotoxins greatly exceeded those exerted by the parent compound PBN. They decreased electron and proton leakage as well as hydrogen peroxide formation in isolated rat brain mitochondria at nanomolar concentration. They also significantly enhanced mitochondrial membrane potential. Finally, dopamine-induced inhibition of complex I activity was antagonized by pretreatment with these agents. These findings indicate that amphiphilic amide nitrones are much more than just radical scavenging antioxidants but may act as a new class of bioenergetic agents directly on mitochondrial electron and proton transport.

Introduction

Under normal conditions, most of the intracellular reactive oxygen species (ROS[a]) are produced within the mitochondria with the superoxide radical anion ($O_2^{-\bullet}$) being the first molecule generated during the respiration process. The ability of $O_2^{-\bullet}$ to chemically or enzymatically self-dismutate to H_2O_2 and subsequently to form $HO^{\bullet}$ via the Fenton reaction as well as its selective reactivity toward transition metal ions accounts for its high toxicity. Furthermore, the higher reactivity of $O_2^{-\bullet}$ at lower pH is due to the formation of the hydroperoxyl radical ($HO_2^{\bullet}$) ($pK_a(HO_2^{\bullet}/O_2^{-\bullet}) = 4.8$), which is more reactive than $O_2^{-\bullet}$. The overproduction of ROS can greatly overwhelm the endogenous antioxidant system for their detoxification and hence can lead to an accumulation of deleterious species. This unregulated production of ROS has been associated with critical pathological events such as myocardial ischemia−reperfusion or stroke as well as with the etiology of the aging process and that of many diseases.

Therefore, there has been extensive research in the development of effective molecules that can scavenge ROS and can be used either as a probe or therapeutic agents; among them are nitrone spin-traps. Previous efforts have been mainly focused on the design of intrinsically more potent compounds, but recently, the selective targeting of antioxidants has been developed as an alternative approach. Because of the inner membrane potential of the mitochondria (160−185 mV), the Skulachev cations consisting of lipophilic triphenylphosphonium cations accumulate in the negatively charged compartment. Various antioxidants or probes have been grafted through their aliphatic chain to this phosphonium carrier.[1,2] It has been reported that the PBN conjugate, MitoPBN (Figure 1), reaches 2.2−4.0 mM within the mitochondria and blocks the oxygen-induced activation of uncoupling proteins.[3] However, toxicity of triphenylphosphonium derivatives was found at low concentration which may considerably limit their use as therapeutic agents.[4] Another approach is the use of cell penetrating peptides (CPPs) as molecular carriers. Highly cationic CPPs have been engineered for transport into mitochondria, demonstrating that lipophilicity and positive charge in peptidic sequences induce

*To whom correspondence should be addressed. For G.D.: phone, +33 4 9014 4445; fax, 33 4 9014 4491; e-mail, gregory.durand@univ-avignon.fr. For B.P.: phone, +33 4 9014 4442; fax, 33 4 9014 4499; e-mail, bernard.pucci@univ-avignon.fr.

[a] Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BHT, butylated hydroxytoluene; cmc, critical micellar concentration; CPP, cell penetrating peptides, CV, cyclic voltammetry; EA, electron affinity; ETC, electron transport chain; FCCP, *p*-trifluoromethoxy carbonylcyanide phenylhydrazone; FOX, ferrous oxidation of xylenol orange; IP, ionization potential; MMP, mitochondrial membrane potential; NARP, neurogenic ataxia retinitis pigmentosa; NBT, nitro blue tetrazolium; NPA, natural population analysis; PCM, polarized continuum model; PBN, α-phenyl-*N*-*tert*-butylnitrone; ROS, reactive oxygen species; SOD, superoxide dismutase.

Published on Web 06/09/2010

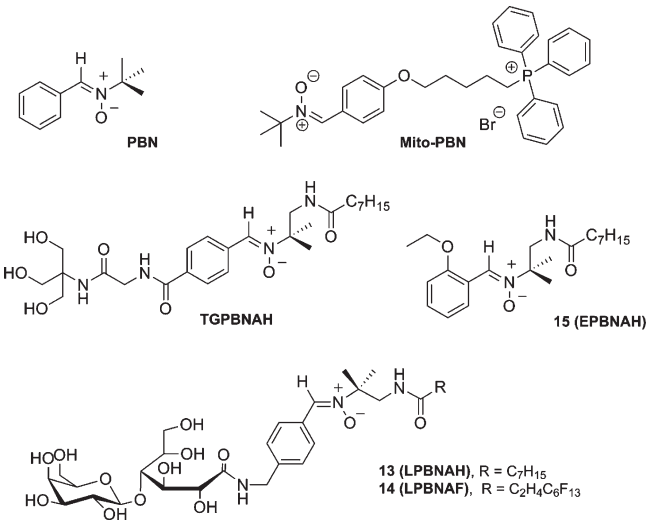


Figure 1. Chemical structures of PBN, mito-PBN, and amide nitrones previously developed.

mitochondrial accumulation.[5] For instance, the tetrapeptide SS-31, consisting of alternating aromatic and basic amino acids, was able to prevent apoptosis of neurons at nanomolar concentration because of its 5000-fold accumulation in the mitochondria.[6] Gramicidin S segments also exhibit specific mitochondrial compartmentalization and exert antiapoptotic properties when conjugated to nitroxides.[7] Another approach that recently attracted attention is to attach a nitrone group to the (−)-(*R*)-carnitine, which is used in the transport of fatty acids into the matrix of the mitochondria.[8]

With the expectation that amphiphilic compounds possessing both a hydrophilic polar head and a lipophilic group would exhibit improved bioavailability and membrane crossing ability, our work over the past 10 years has been devoted to the design and the synthesis of amphiphilic antioxidants with particular attention to nitrone derivatives. Two original series of amphiphilic compounds, bearing α-phenyl-*N*-*tert*-butylnitrone (PBN) as synthetic antioxidant moiety, have been developed in our lab. In the first series, the PBN moiety is grafted onto a fluorinated glycolipidic carrier through an amide bond. The first amphiphilic nitrone of this series[9] was found to readily diminish superoxide dismutase (SOD) induction and apoptosis in cultured skin fibroblasts with an isolated complex V deficiency of the respiratory chain, caused by the neurogenic ataxia retinitis pigmentosa (NARP) mutation.[10] A more general amino acid based amphiphilic carrier consisting of a hydrophilic head derived from lactobionic acid and a perfluoroalkyl tail was then developed later.[11] An amino acid, that is, lysine or aspartic acid, is used as scaffold upon which hydrophobic and hydrophilic parts are linked respectively by carboxyl and amino groups and upon which PBN is grafted to functional side chain groups.[12] This amphiphilicity mediated delivery strategy was then extended to a broad spectrum of antioxidants such as lipoic acid, indole-3-propionic acid, Trolox, and very recently the cyclic nitrone DMPO.[13]

We also reported the synthesis of a second series of amphiphilic PBN derivatives in which the nitrone function is fitted into the core of the molecule[14] bridging the polar head grafted onto the aromatic function and the hydrophobic moiety, bound to the *tert*-butyl group. This convergent synthetic pathway allows modification of the nature, length, and type of grafted hydrophobic moiety, as well as the ionic or nonionic character of the polar head.[15] For both series of amphiphilic PBN, the preliminary biological evaluations showed that these amphiphilic compounds were in general far more potent than the parent antioxidant, PBN. This clearly demonstrates that the amphiphilicity of nitrones is a key feature in determining their bioactivity and protection against the oxidative toxicity in vitro and in vivo[14,16,17] as exemplified by compound **13** (LPBNAH) exhibiting exceptionally high antioxidant activity.[18,19] In order to extend and explore the importance of the amphiphilicity of nitrone derivatives in determining their antioxidant potency, we synthesized hydrophilic, amphiphilic, and lipophilic amide analogues of **13**. Although these new analogues exerted good protective effects both in vitro and in vivo, exceeding the antioxidant activity of many other compounds, nitrone **13** was found once again to be the most potent compound.[20]

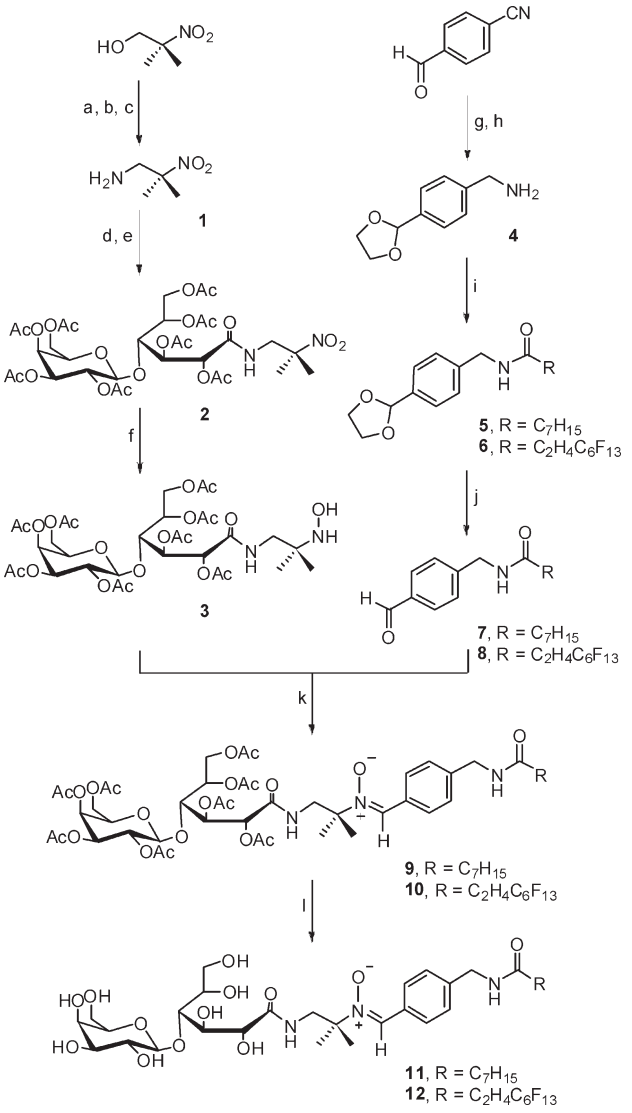
We report herein the synthesis of an amphiphilic amide nitrone **11** (LPBNH15) in which the polar headgroup derived from lactobionic acid and the C7 alkyl chain are both linked via an amide bond to the *N*-*tert*-butyl group and the phenyl ring of the PBN moiety, respectively. Nitrones **11** and **13** are structural isomers because they are differentiated only from the position of the polar head and the hydrophobic chain. To explore the importance of amphiphilicity, the fluorinated analogue **12** (LPBNF13) bearing a 1*H*,1*H*,2*H*,2*H*-perfluorooctyl chain has also been synthesized. Indeed, while amphiphilic compounds endowed with a hydrocarbon chain can induce a destabilization of cell membranes when used above their critical micellar concentration (cmc), fluorinated amphiphilic analogues are not detergent-like. However, from our findings with amphiphilic nitrones, the usefulness of fluorinated tails compared to hydrogenated ones is still unclear, as our lead compound **13** was found to be much more potent than its fluorinated analogue **14** (LPBNAF).[20] Moreover, because of its relatively short alkyl chain, no cmc was observed for compound **13**[14] and it was found to be devoid of any detergent properties or toxicity even at very high concentrations.[18]

The hydroxyl radical scavenging activity and the radical reducing potency of these newly designed amphiphilic amide nitrones were explored using the ABTS competition and an $ABTS^{\bullet+}$ reduction assay, respectively. The effect of the substitution on the electrochemical properties of the nitronyl group was investigated by cyclic voltammetry and was correlated with theoretically calculated electron affinities and ionization potentials using B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory. Their protective effects in primary cortical mixed cell cultures exposed to three oxidotoxins, that is, hydrogen peroxide, peroxynitrite, and doxorubicin, were investigated. An in vivo model, using aquatic invertebrate microorganisms (i.e., rotifers) exposed to lethal concentrations of H_2O_2 and doxorubicin oxidotoxins, was also employed. Finally, the ability of amphiphilic amide nitrones to interact with the mitochondrial energy metabolism at nanomolar concentration was further investigated. For the sake of comparison, compound **13** and its fluorinated analogue **14** were also included in the physical−chemical and biological assays as well as in the computational studies.

Chemistry

Synthesis. The convergent synthetic pathway of the amphiphilic nitrones is based on three key steps (Scheme 1): (i) synthesis of a polar lactobionamide *N*-*tert*-butylhydroxylamine from 2-methyl-2-nitropropanol; (ii) synthesis of the

Scheme 1[a]



[a] Reagents and conditions: (a) TsCl, Pyr, DCM, 80%; (b) NaN_3, DMF, 89%; (c) PPh_3, THF followed by NaOH 2 N; (d) lactobionic acid, TEA, 2-methoxyethanol; (e) Ac_2O/Pyr 1:1, 67% in two steps; (f) Zn, NH_4Cl, THF/H_2O 3:1 (v/v), 80%; (g) $HOCH_2CH_2OH$, apts, toluene, 90%; (h) $LiAlH_4$, THF, 62%; (i) $C_7H_{15}COOH$, BOP, DIEA, DCM, 85% or $C_6F_{13}CH_2CH_2COOH$, DCC, HOBt, DIEA, DCM, 52%; (j) CH_3CHO, *p*-toluenesulfonic acid, 88−96%; (k) THF/AcOH 3:2 (v/v), dark, 76−80%; (l) MeONa, MeOH, 75−90%.

hydrogenated and perfluorinated hydrophobic *N*-4-formylbenzylamides from 4-cyanobenzaldehyde; (iii) formation of the nitronyl function by condensation of the hydroxylamine to the benzaldehyde derivatives under inert atmosphere.

Lactobionamide-based *N*-*tert*-butylhydroxylamine **3** was synthesized in six steps from the commercially available 2-methyl-2-nitropropanol. First, the hydroxyl group was converted to the amino group following our previously reported procedure leading to compound **1**.[14] For convenience, compound **1** was usually converted to its ammonium chloride form, allowing its storage for months. After regeneration of the amino group, compound **1** was grafted onto lactobionolactone, followed by acetylation of the hydroxyl groups leading to **2** after purification by flash chromatography. Finally, the reduction of the nitro group was mildly carried out using a mixture of zinc/ammonium chloride in THF at room temperature without any observable over reduction. After purification by flash chromatography, the hydroxylamine **3** was obtained in 31% overall yield from the starting 2-methyl-2-nitro propanol. It has to be noted that the hydroxylamine was found to be stable for months at −20 °C.

4-Cyanobenzaldehyde was used as starting material for the synthesis of the hydrophobic benzaldehyde derivatives. Protection of the carbonyl group followed by the reduction of the cyano group led to compound **4** as previously reported.[21] The resulting amino group was directly condensed with octanoic acid or 1*H*,1*H*,2*H*,2*H*-perfluorononanoic acid in the presence of DCC/HOBT leading to compounds **5** and **6**, respectively, in moderate yields (∼50%). One can note that the yield of the amide bond formation was significantly improved using BOP as coupling agent instead of DCC/HOBT. Then transacetalization with acetaldehyde afforded the benzaldehydic compounds **7** and **8** in ∼45% overall yield from 4-cyanobenzaldehyde.

The nitrone functional group was finally synthesized by reaction of the hydroxylamine **3** with the hydrophobic benzaldehyde derivatives **7** and **8**, using a 3:2 (v/v) THF/AcOH mixture under argon atmosphere and in the absence of light. It is important to note that such acidic conditions greatly increase the kinetics of the reaction which is usually reported to be long as well as giving a low yield of coupling. O-Acetylated amphiphilic nitrone amides were extensively purified by flash chromatography and by size exclusion chromatography. Finally, after purification by aqueous size exclusion chromatography, the Zemplén de-O-acetylation procedure led to compounds **11** and **12**. The amphiphilic nitrones **11** and **12**, as well as their acetylated derivatives **9** and **10**, were fully characterized by ^1H, ^{13}C, ^{19}F, DEPT, and HMQC NMR as well as mass spectrometry (ESI+). The purity of **11** and **12** was confirmed by C18 reverse phase HPLC and was higher than 95% (see Supporting Information).

Physical−Chemical Measurements. All the amphiphilic amide nitrones were soluble in water, and their solubility was found to be significantly higher than that of PBN as shown in Table 1. No significant difference was observed in the solubility of **11** and **13**, with values of about 500 $g \cdot L^{-1}$ for both compounds. However, because of the high hydrophobicity of the perfluorinated chain, compound **12** exhibited lower solubility than its hydrogenated derivative **11**.

Relative Lipophilicities. Relative lipophilicities ($\log k'_W$) of the amphiphilic amide nitrones were measured by a chromatographic technique previously used.[12,15,20] Despite their higher water solubility, all the amphiphilic amide nitrones were also found to be more lipophilic than the parent compound PBN. Finally, because of the peculiar nature of their chain, fluorinated derivatives exhibited the highest values of lipophilicity, which is consistent with our previous finding.[22] Such a behavior demonstrates the interest in providing amphiphilic character to nitrones, as the water solubility and the lipophilicity are both increased compared to that of the parent compound, in agreement with our finding with the amphiphilic amino acid carriers.[12] One can also note that the grafting of the chain onto the aromatic ring slightly increased the lipophilicity of the nitrone, as **11** and **12** exhibited higher values of $\log k'_W$ compared to those of their substituent-reversed analogues **13** and **14**.

Self-Aggregation Properties. Self-aggregation properties were specified using surface tension measurement. Results

Table 1. Physical−Chemical, Radical Scavenging, and Reduction Properties of Nitrone Agents

			self-aggregation				
nitrones	water solubility (g/L)	$\log k'_W$	cmc[f]	γ[h]	quenched ABTS$^{\bullet+}$ (nmol/30 min)[i]	reduced ABTS$^{\bullet+}$ (nmol/30 min)[i]	$E_{1/2}$ (red)[j] (V)
PBN	26.5	1.62[b] 1.64−1.75[c, d, e]	*g*	*g*	18.2 ± 0.9[d]	7.8 ± 0.3[d]	−1.93 ± 0.02
13	515	2.67−2.76[c, e]	*g*	*g*	75.9 ± 0.9[e]	12.4 ± 0.5[e]	−1.76 ± 0.03
11	530	2.86[b]	*g*	*g*	96.8 ± 0.9[b]	49.1 ± 1.1[b]	−1.64 ± 0.04
14	*a*	4.44−4.65[c, e]	0.213[c]	20	65.9 ± 1.1[e]	5.9 ± 0.3[e]	*a*
12	270	4.71[b]	0.180[b]	23	90.7 ± 1.3[b]	42.6 ± 0.9[b]	*a*

[a] Not determined. [b] This work. [c] Data from ref 15. [d] Data from ref 12. [e] Data from ref 20. [f] Critical micellar concentration in mM. [g] No cmc. [h] γ, limit surface tension, in mN/m. [i] The findings are presented as the mean ± SEM ($N = 10$). All results are statistically significant different from control (vehicle) at $p < 0.01$ (ANOVA followed by Bonferroni *t* test). [j] 0.15 M NaCl in water, glassy carbon electrode, sweep rate of 0.15 V s^{-1}. Average of three to four measurements.

show that only the fluorinated nitrones were able to form micelles in pure water, in agreement with the literature in which these perfluorinated surfactants exhibit very low critical micellar concentration (cmc) as well as very low surface tension at the cmc (γ_{cmc}).[23] No micelle formation was observed with nitrone **11**, suggesting the absence of detergent properties and related toxicity of this C7 hydrogenated derivative as it was previously demonstrated with compound **13**.[15] According to the slightly higher hydrophobic character of **12** compared with its substituent-reversed analogue **14**, the cmc of the former compound was found to be slightly lower.

ABTS Competition Assay. Radical scavenging was investigated by means of a scavenger competition assay based on the formation of the intensely green 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ABTS cation radical initiated by a Fenton reaction.[12,24] Radical scavengers suppress time dependent ABTS cation radical formation by competition. Because of the micellar aggregation of the fluorinated derivatives, the measurements were done in the presence of DMSO to allow a comparison of compounds having a different degree of amphiphilicity. All amphiphilic nitrones showed considerable effects on ABTS oxidation. Compound **11** and its fluorinated analogue **12** acted as very potent radical scavengers and inhibited almost completely the formation of ABTS cation radicals. Their analogues, **13** and **14**, also inhibited the formation of ABTS cation radical but to a lesser extent. This suggests that the position of the substituent on the nitrone moiety may affect the reactivity of the nitronyl group toward radical trapping. High ability in reducing the burstlike formation of ABTS cation radicals was already reported for fluorinated amphiphilic amino acid PBN derivatives, while that of PBN was found to be limited.[12] Moreover, we found that a fluorinated amphiphilic amino acid carrier devoid of PBN derivative was also able to reduce the ABTS cation radical formation (unpublished results). Such a result clearly shows that the lactobionamide polar head may act as a radical trap because of its many reactive hydroxyl groups.

ABTS$^{\bullet+}$ Reduction Assay. We also investigated the ability of amide nitrones to act as electron donors in an ABTS$^{\bullet+}$ reduction assay.[25] Surprisingly, while PBN itself and the first generation of amide nitrones were found to be very limited reductants and electron donors, amide nitrones **11** and **12** proved to be good reductants (Table 1). The fact that a simple reversal of the position of the polar head and the hydrophobic chain changes the radical reducing potency dramatically is surprising and suggests that the nature of the substituents at the aromatic ring system and the *N-tert*-butyl group may exert a decisive role in determining antioxidant capacity of nitrone compounds. The effects of para-substitution of PBN on the charge density of the nitronyl group and on the reactivity with superoxide radical anion and hydroperoxyl radical were theoretically rationalized by us in a recent effort, demonstrating the importance of the polar effect on the spin-trapping and antioxidant properties of linear nitrone.[26]

Cyclic Voltammetry. Cyclic voltammetry (CV) was used to investigate the redox behavior of the amphiphilic amide nitrones (200 μM) in aqueous solution. Nitrone spin traps are known to be reduced in unbuffered aqueous medium through an ECE process (a two-electron transfer reaction with an intervening chemical reaction).[27] First, a two-electron reduction of the nitrone leads to a disubstituted hydroxylamine, which is further dehydrated to an imine compound. The resulting imine is subsequently two-electron-reduced to an amine compound. The reduction potentials of PBN and compounds **13** and **11** were measured and are reported in Table 1. The pronounced shifts in reduction potentials of nitrones **11** (−1.64 V) and **13** (−1.76 V) relative to that of PBN (−1.93 V) reflect the effect of substituents at the aromatic ring and the *N-tert*-butyl group[27,28] and the fact that **11** and, to a lesser extent, **13** are more easily reduced than PBN. The slight difference in reduction potential between **11** and **13** may arise from steric hindrance and electronic effects due to the presence of a lactobionic group or an alkyl chain on the *N-tert*-butyl group. As previously reported, electron withdrawing groups on the *N-tert*-butyl group induce a slight decrease in the reduction potentials.[27] According to this, one can expect that the lactobionic group of **11** should exert an electron-withdrawing effect on the nitronyl group while the alkyl chain of **13** would exert an electron-donating effect. The higher degree of hydration of the two amphiphilic compounds, due to the presence of the very polar lactobionamide group, compared to PBN might also play a role in the reduction of the nitronyl function.

Our preliminary EPR spin trapping experiments with **13** in the presence of a nonaqueous generating system of $O_2^{\bullet-}$ such as pyridine/H_2O_2 or DMSO/KO_2 gave strong EPR signals, confirming the spin trapping ability of the amide nitrones (data not shown). However, because of its amphiphilic character and formation of secondary products, anisotropic and complex spectra were observed. The spin trapping properties of amphiphilic amide nitrones and their voltammetric behavior in organic solvent will be further investigated.

Computational Studies. The electron affinities (EAs), ionization potentials (IPs), and charge densities of the nitronyl atoms of compounds **11**−**13** were calculated at the B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level of theory. The bulk

Table 2. Calculated Electron Affinities, Ionization Potentials, and Charge Densities of the Nitronyl Atoms of **13**, **12**, **11**, and PBN at the B3LYP/6-31G+(d,p)//B3LYP/6-31G(d) Level of Theory[a]

			charge densities of the nitronyl atoms (*e*)		
nitrones	electron affinity (eV)	ionization potential (eV)	C	N	O
13	0.79 (1.68)	7.09 (5.78)	−0.024 (0.024)	−0.030 (0.061)	−0.539 (−0.601)
12	0.76 (2.15)	7.21 (5.74)	0.012 (0.027)	0.062 (0.059)	−0.551 (−0.605)
11	0.68 (2.14)	6.90 (5.75)	0.016 (0.014)	0.075 (0.078)	−0.559 (−0.607)
PBN	0.12 (2.05)	7.37 (5.75)	−0.007 (0.009)	0.077 (0.073)	−0.548 (−0.606)

[a] Values in parentheses are PCM(water)/B3LYP/6-31G+(d,p) B3LYP/6-31G(d).

Table 3. Inhibition of Induced Cell Death in Mixed Cortical Cultures and Percentage of Viable Rotifer after Treatment with Nitrone Agents[a]

	% inhibition of cell death in mixed cortical cultures[a]			% viable rotifers[b]	
compd (10 μM)	H_2O_2 (200 μM)	peroxynitrite (200 μM)	doxorubicin (200 μM)	H_2O_2 (200 μM)	doxorubicin (200 μM)
control[c]				11.9 ± 0.4	15 ± 0.6
PBN[c]	25.0 ± 0.9	20.6 ± 0.7	24.2 ± 0.8	25.6 ± 0.8	18.7 ± 0.5[f]
13[d]	81.0 ± 1.0	61.0 ± 1.3	82.0 ± 1.3	83.0 ± 1.1	85.8 ± 1.7
11[e]	93.0 ± 1.1	90.1 ± 1.9	93.0 ± 0.7	87.6 ± 1.4	96.5 ± 0.7
14[d]	63.4 ± 1.1	40.1 ± 1.0	34.9 ± 0.8	40.6 ± 1.2	30.6 ± 1.2
12[e]	42.6 ± 1.0	30.3 ± 1.1	32.8 ± 0.8	30.1 ± 1.5	46.7 ± 1.3

[a] Cultures were treated for 24 h with oxidotoxins at 200 μM. Nitrones were simultaneously added at 10 μM. Shown is the percentage inhibition of trypan blue absorbance indicating enhanced survival of the cells. [b] Rotifers were treated for 24 h with oxidotoxin at 200 μM. Nitrones were simultaneously added at 10 μM. Shown is the percentage of viable organisms after exposure to the oxidotoxins. The findings are presented as the mean ± SEM ($N = 10$). Unless otherwise indicated, all results were statistically significantly different from results of the control (vehicle) at $p < 0.01$ (ANOVA followed by Bonferroni *t* test). [c] Data from ref 12. [d] Data from ref 20. [e] This work. [f] Nonsignificant versus doxorubicin with vehicle.

dielectric effect of water on the energetics was also investigated at the PCM/B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level, and the results are shown in Table 2. Results indicate that PBN exhibits the lowest electron affinity but no significant difference was observed in the EAs among the amphiphilic nitrones in both gaseous and aqueous phases. This suggests that PBN substitution facilitates reduction of the nitrone, consistent with the experimentally observed reduction potentials as determined by cyclic voltammetry. The IP value was also highest for PBN in the gaseous phase, indicating that PBN is harder to reduce compared to the amphiphilic nitrones, but no significant difference in the IPs was observed in aqueous phase. This is in agreement with the $ABTS^{\bullet+}$ reduction assay demonstrating that amphiphilic amide nitrones are better reductants and electron donors than the PBN parent compound. However, only a slight difference was observed in the IP values between **11** and **13** while the $ABTS^{\bullet+}$ reduction assay showed the superiority of the reverse-analogue **11**. The NPA charges in aqueous phase show the following order of increasing charge on the nitronyl C: PBN < **11** < **13** < **12**, demonstrating that the substitution of the nitronyl function alters its charge density which in turn may affect its reactivity toward free radicals. Finally, both experimental and theoretical data showed that substitution of the PBN by hydrophilic and lipophilic groups alters its redox properties, with the amphiphilic amide nitrones being easier to oxidize and reduce than the parent PBN. Moreover, the position of the substituent on the nitronyl group is also of importance for the redox behavior, with the reverse analogue **11** also exhibiting at the same time better reducing capacity and electron donating properties than **13**.

Biological Results

Primary Cortical Mixed Cell Cultures. We examined the prevention of cell death in three paradigms of lethal oxidative stress in primary cortical mixed cell cultures, and the results are shown in Table 3. Cultures were treated for 24 h with three different oxidotoxins (viz. hydrogen peroxide, peroxynitrite, or doxorubicin) at 200 μM. While hydrogen peroxide induces damage to the whole cell mainly because of its ability to cross membranes and so is usually referred to as a global and nonselective toxin, doxorubicin is a mitochondria-selective oxidotoxin also referred to as a specific mitotoxin.[29,30] The cytotoxicity of the doxorubicin is likely due to its accumulation in the mitochondria lipid membrane and its high affinity toward binding to cardiolipin, an inner mitochondrial-membrane specific lipid. At physiological pH, peroxynitrite decomposes through a proton-dependent mechanism to form the hydroxyl radical ($HO^{\bullet}$) and the nitrite radical ($NO_2^{\bullet}$). Peroxynitrite was also found to be able to induce oxidative damages to the mitochondrial electron transport chain (ETC). It is produced by reaction of superoxide with nitric oxide in the mitochondria but can also diffuse through cell membranes or anion channels to induce mitochondrial damage.[31] Its ability to induce apoptosis was also reported[32] as well as its oxidant property which leads to reaction with biomolecules.

No surviving neuronal cells in vehicle oxidotoxin treated cultures were detected. Under such conditions of lethal toxicity (200 μM oxidotoxin) only very potent protective agents are able to rescue the cells and prevent their degeneration and death.[12] Nitrones were added simultaneously with the oxidotoxin at only 10 μM to evaluate their putative cytoprotective effects. Regardless of the nature of the oxidotoxin, all the amphiphilic nitrones exhibited significant neuroprotective activity and were significantly more effective than the parent compound PBN. Both hydrogenated derivatives exhibited very high protective activity against the toxicity induced by the three stressors, with compound **11** being more potent than **13**. Worth noting is the very pronounced cytoprotection against the mitochondria-selective toxin doxorubicin. This may indicate an enhanced affinity of these amphiphilic amide nitrones for the mitochondria compartment. Fluorinated derivatives also exhibited good protection of cells against hydrogen peroxide but to a lesser extent than their hydrogenated analogues. One can note that the protection toward the mitochondria selective oxidotoxin

was significantly lower, suggesting that these fluorinated compounds are less prone to reduce endogenous oxidative stress. Surprisingly, fluorinated amphiphilic amino acid based PBN derivatives have also been shown to reduce oxidative stress and to exert a higher efficacy than nitrones **12** and **14** toward mitochondria selective oxidotoxins such as doxorubicin.[12] This suggests that the nature of the fluorinated chain is not the only parameter that accounts for the affinity to the mitochondrial compartment but also indicates that the chemical structure of nitrone molecules may equally affect their compartmentalization.

Rotifer Cultures. The antioxidant properties of amphiphilic amide nitrones were confirmed in in vivo experiments using rotifers, a well established animal model in experimental gerontology.[18] This animal model allows for a large scale analysis of the protective effects of antioxidant drugs tested on individually housed organisms of defined and uniform age and has been used successfully in aging studies by other investigators.[33−37] Since rotifers of the genus *Philodina* are aquatic organisms, single housed animals can be exposed to exactly the same toxin and drug regime in a controlled environment, a great advantage over models requiring dosing of the test compounds by food and water administration or injection.

Rotifers were treated 24 h with oxidotoxin at 200 μM in the presence or absence of nitrones at 10 μM to evaluate their putative protective effect (Table 3). The fluorinated nitrones **12** and **14** exerted substantial protection against the toxicity of hydrogen peroxide and doxorubicin toward these organisms, whereas only a limited protection by PBN was observed for the rotifers exposed to hydrogen peroxide. Once again, these two compounds exhibited a lower efficacy than the fluorinated amphiphilic amino acid based PBN derivatives.[12] On the contrary, the two hydrogenated nitrones **11** and **13** were very effective against the lethal toxicity of the nonselective agent hydrogen peroxide and against the mitochondria-selective oxidotoxicity of doxorubicin, these agents being ~3 and ~5 times more active than the parent compounds in the two oxidative stress paradigms, respectively. The almost complete protection by the new hydrogenated amphiphilic amide nitrone **11** confirms previous findings that enhanced bioavailability is a key feature in determining antioxidant protection in cells and organisms.[12,15,18,20] To study the molecular mechanisms of the antioxidant protection of the two very efficient hydrogenated amphiphilic amide nitrones **11** and **13**, we next carried out a series of biochemical experiments on mitochondrial preparations as described below.

Mitochondrial Preparation. Determination of Soluble Hydroperoxides Formation. The ability of amide nitrones to reduce peroxide formation in respiring mitochondria under physiological conditions was first investigated. We used the xylenol orange method (also referred to as the FOX assay) to determine hydroperoxides in mitochondria as described by Hermes-Lima et al.[38] A microassay variant of this method was adapted to measure soluble hydroperoxides as described by Lyras et al.[39] Because of protein precipitation during homogenization, the assay as applied does not detect total peroxides but only the soluble hydroperoxides and thus almost exclusively the abundant hydrogen peroxide (at least 98% in the mitochondrial preparations, data not shown). As shown in Figure 2, both amide nitrones were found to reduce the peroxide formation in respiring mitochondria under physiological conditions at submicromolar concentrations;

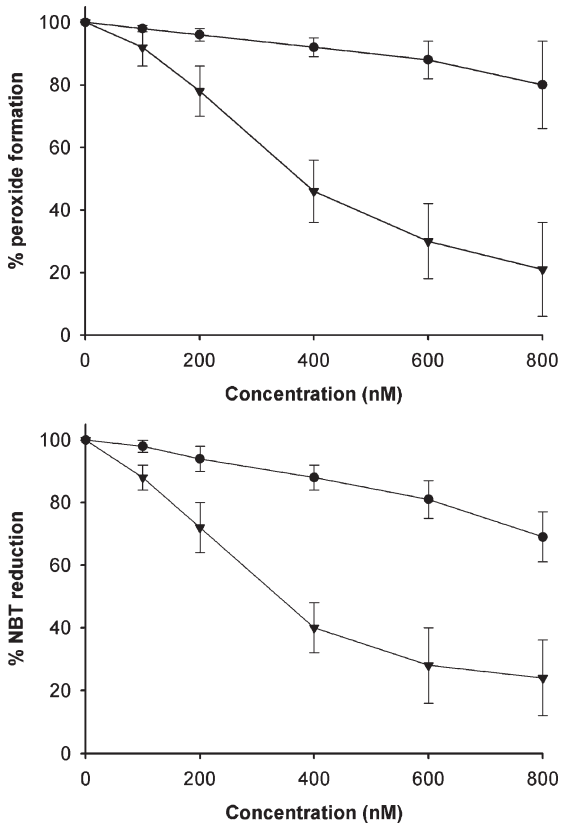


Figure 2. Inhibition of the water-soluble peroxide formation (top) and inhibition of the NBT reduction (bottom) by **11** (▼) and **13** (●) in isolated rat brain mitochondria. Glutamate and succinate concentrations were 6 mM. Mitochondrial preparations were harvested 30 min after incubation with nitrones. The findings are presented as the mean ± SEM of at least four independent mitochondrial preparations.

however, compound **11** was much more potent than **13**. For instance, at only 0.4 μM, **13** decreases peroxide formation by ~10%, while at the same concentration **11** leads to more than 50% of inhibition. As the concentration of nitrones increases, the trend remains the same with 20% and 80% of inhibition of peroxide formation by 0.8 μM **13** and **11**, respectively. This result demonstrates that **11** and to a lesser extent **13** can prevent oxidant formation with extremely high efficacy. Interestingly, both nitrones prevent the time dependent increase in hydroperoxide formation in rotifers cultures when maintained over a period of 4 weeks (unpublished results). These findings indicate the role for antioxidant protection in mediating at least some of the beneficial effects of amphiphilic amide nitrones in promoting survival, thereby protecting the mitochondria, cells, and organisms against oxidant formation.

Determination of Electron Leakage. Amphiphilic amide nitrones and mitochondrial complex I interaction was studied in rat brain mitochondrial preparations. The determination of electron leakage induced by rotenone in mitochondrial preparations was assayed by the NBT reduction test.[40] Nitro blue tetrazolium is an alternative electron acceptor, which is reduced by active respiring mitochondria to a formazan dye. Specificity of the NBT method to quantify electron leakage from the mitochondrial electron transport chain was rigorously examined and confirmed as described by Hensley et al.[40] As shown in Figure 2, the nitrones greatly reduced electron leakage in rat brain

mitochondrial preparations respiring on physiological substrates. Compound **11** was much more effective than **13** in this regard, but both compounds acted at nanomolar concentration to reduce formazan formation in respiring mitochondria at physiological glutamate and malate concentrations. After treatment with only 0.8 μM nitrones, the inhibition of the NBT reduction reached 30% and 75% for **13** and **11**, respectively. This indicates that both nitrones may safeguard electron flow by preventing leakage, probably acting as alternative electron acceptors being reduced to active nitroxides or even to the fully reduced hydroxylamines. Hensley et al. reported that PBN interacts with mitochondrial flavin dehydrogenases to alter the dynamics of electron transit and to prevent leakage from these enzymes.[40] More recently, Genova et al. suggested Fe−S cluster N2 as the site of electron leakage within complex I to direct reaction with oxygen and with water-soluble quinone.[41,42] The iron sulfur cluster N2 is localized in an amphiphatic ramp extruding from the inner membrane to the mitochondrial matrix, and because of their amphiphilic nature, amide nitrones **11** and **13** may target this specific site and act as alternative electron acceptors. This confirms previous findings where **13** efficiently prevented the inhibition of the activity of Fe−S cluster N2 in submitochondrial particles after incubation with oxidotoxins (viz. H_2O_2, peroxynitrite, and doxorubicin), while PBN only exerted limited protection.[20] By preventing electron leakage, amphiphilic amide nitrones may reduce superoxide radical formation, which in turn may reduce hydrogen peroxide and hydroperoxide formation. The data observed with the FOX assay further support this hypothesis.

Increase of Mitochondrial Membrane Potential. Oxidative stress can induce a depolarization of the mitochondrial membrane potential (mmp) by inhibiting the activities of Krebs cycle enzymes. Therefore, maintaining intact mitochondria is of importance for the survival of cells exposed to oxidative stress, as the collapse of mmp has been identified as a critical event in cell injury during ischemia.[43] Mitochondrial membrane potential restoration after protonophore FCCP-induced partial collapse was measured directly by recording rhodamine 123 fluorescence,[44,45] and results are shown in Figure 3. FCCP at 0.2 μM induced a 50% decrease of the mmp, resulting in a dequenching of rhodamine 123 and thereby increasing the detectable fluorescence in the mitochondrial preparations. Compound **11** almost completely prevented this loss of mmp at 0.8 μM. Compound **13** was also very potent in preserving mmp under these conditions. This demonstrates that pretreatment with extremely low concentrations of amide nitrones prevents FCCP-induced decrease of proton potential and results in reenergized mitochondria that are able to sustain ATP production. These findings suggest a direct effect of the amide nitrones on mitochondrial energy metabolism by preventing uncoupling. Lee et al. reported that the inhibitory effect of ciclopirox (a synthetic antifungal agent) on H_2O_2-induced depolarization of mmp is not caused by its antioxidant properties because it does not scavenge H_2O_2, hydroxyl, and superoxide radicals or peroxynitrite.[45] These findings indicate that amphiphilic amide nitrones are much more than just radical scavenging antioxidants and may act directly on mitochondrial electron and proton transport.

Determination of Complex I Activity. The mechanisms by which dopamine inhibits complex I are not fully identified and characterized. Yet dopamine is believed to interfere with

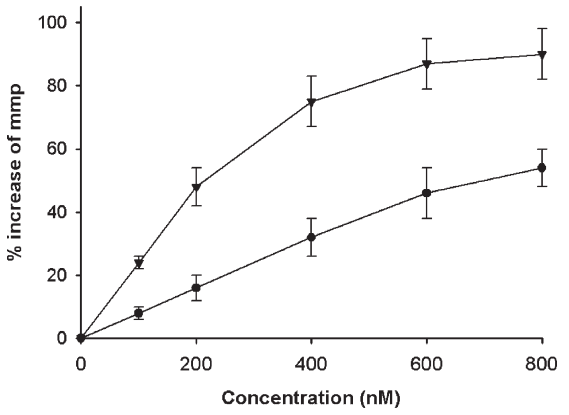


Figure 3. Increase of the membrane potential in isolated rat brain mitochondria after protonophore-induced collapse by treatment with **11** (▼) and **13** (●). Mitochondrial preparations were harvested 30 min after incubation with nitrones. The protonophore FCCP was added 15 min after incubation with nitrones. The findings are presented as the mean ± SEM of at least four independent mitochondrial preparations.

Table 4. Complex I Activity in Rat Cortical Mitochondria[a]

compd (10 μM)	complex 1 activity ((nmol/mg protein)/min)
control	99 ± 1.2
PBN	107 ± 1.1
13	116 ± 1.3
11	124 ± 1.9
dopamine	24 ± 0.4
dopamine + PBN	60 ± 1.3
dopamine + **13**	79 ± 1.2
dopamine + **11**	110 ± 1.6
15	11 ± 0.3
15 + PBN	13 ± 0.4
15 + **13**	19 ± 0.5
15 + **11**	31 ± 0.7

[a] Complex I activity in rat cortical mitochondrial preparations was determined by measuring the reduction of ferric cyanide in the presence of antimycin A and azide as previously published.[20,50] Submitochondrial particles were incubated for 30 min with nitrone agents and dopamine. The findings are presented as the mean ± SEM (N = 10).

mitochondrial respiration in a dual manner, via the formation of ROS or by direct interaction with complex I. Ben-Shachar et al. have shown the ability of dopamine to inhibit mitochondrial function through its interaction with complex I in disrupted brain mitochondria[46] as well as in intact viable human neuroblastoma SH-SY5Y cells.[47−49] Indeed, in intact cells while dopamine inhibits respiration through complex I, no inhibition was observed through complexes II and III. Moreover, the ability of dopamine to specifically inhibit complex I activity in disrupted mitochondria, possibly interacting at a site located between the binding site for NADH and the iron−sulfur clusters but not that of complexes II, IV, and V, further supports this hypothesis.

The relief of dopamine-induced inhibition of complex I activity by nitrone amides **11** and **13** was assayed by the ferric cyanide reduction method[20,50] and compared to that of PBN (Table 4).[20] The two amphiphilic nitrones and the parent compound PBN induce a slight increase of the complex activity compared to the control, demonstrating the nontoxicity of these agents on the complex I. Dopamine inhibits complex I activity immediately, leading to a strong toxicity, and this inhibition is relieved by pretreatement with the

nitrone compounds but fully only by the amide nitrone **11**. This shows that nitrone agents are able to prevent dopamine-induced complex I toxicity, with the amphiphilic amide nitrones being more efficient. Once again, we observed that the positions of the hydrophilic and lipophilic substituents on the nitronyl group play an important role in the bioactivity of this series of amide nitrones, with the reverse analogue **11** being ~40% more potent than its analogue **13** in preventing dopamine toxicity. Interestingly, dopamine did not induce an increase of hydroperoxide levels (data not shown). These findings indicate that the effects of such mitochondrial metabolism modifiers not only are mediated by their antioxidant properties but also may be related to many other effects like direct interactions with a key component of the mitochondrial respiratory chain, the complex I. Indeed, Ben-Shachar et al. found that neither the antioxidant BHT nor the monoamine oxidase A and B inhibitors prevented dopamine-induced inhibition of complex I activity.[47] Moreover, the dopamine-induced dissipation of mitochondrial membrane potential in intact cells, as demonstrated by confocal microscopy and flow cytometry, was not associated with the production of ROS and was without any effect on cell survival.[48,49] This suggests that dopamine oxidation is not involved in the inhibition of complex I activity and further confirms the nonantioxidant mediated protection by amphiphilic amide nitrones.

Previous work by Singer et al. demonstrated that dopamine inhibitory effect is detected in the presence of ferrycyanide, which accepts electrons from Fe−S clusters.[51,52] This suggests that dopamine may interact at a site that lies between the binding site for NADH and the Fe−S cluster N1, before the more hydrophobic part of the complex.[47] This hypothesis is further supported by the work by Harmon and Crane who observed that NADH dehydrogenase activity is inhibited by iron chelators, likely by displacing the labile sulfur of the Fe−S cluster and chelating the iron.[53] As shown by Lenaz et al., capsaicin, a class B inhibitor, completely prevents ROS production from complex I.[54] Like capsaicin, dopamine may bind to Fe−S clusters in complex I blocking the reducing center or inducing a conformational change making it less accessible, which in turn could prevent electron escape from complex I. Moreover, preliminary findings suggest that nitrones might displace [^{3}H]dopamine from its mitochondrial complex I binding site (unpublished results). This would result in a reversal of complex I inhibition by nitrones as observed in Table 4, acting as ligands of the Fe−S centers at the same sides also targeted by dopamine and capsaicin. By increasing and preserving complex I activity, amide nitrones and to a lesser extend PBN may increase energy metabolism efficacy. In this regard, the effects of the nitrones on mitochondrial respiration and oxidative phosphorylation have to be investigated further in future studies involving a broad spectrum of additional methods such as oxygen consumption and ATP formation. As specific mitochondrial metabolism modifiers, amphiphilic nitrones may constitute a new unique class of bioenergetic agents that target mitochondria and mediate their effects by directly modulating the activity of the respiratory chain and its key components such as complex I.

Very surprisingly the lipophilic amide nitrone **15** (EPBNAH)[20] (Figure 1) was found to strongly inhibit complex I activity, thereby disrupting the rate-limiting step of mitochondrial respiration (Table 3), while the other nitrones were found to be devoid of any toxicity. This indicates, in agreement with a previous work,[20] that lipophilic nitrone agents may induce acute toxicity. Moreover, none of the PBN and nitrones **11** and **13** were able to significantly relieve the **15**-induced complex I inhibition, as it was observed with the dopamine. This suggests that the inhibition of complex I activity induced by treatment with the lipophilic **15** may proceed through a different mechanism than that of dopamine. The understanding of such toxicity will be further investigated.

Conclusion

We have synthesized a novel series of amphiphilic amide nitrones in which a lactobionic acid based polar headgroup and an alkyl chain are both linked via an amide bond to the *N-tert*-butyl group and the phenyl ring of the PBN moiety, respectively. To explore the importance of amphiphilicity, the hydrogenated derivative, called **11**, and its fluorinated analogue, called **12**, were synthesized. For the sake of comparison, physical−chemical and biological properties of **11** and **12** were compared to those previously synthesized structural isomers, **13** and **14**, respectively, in which the positions of the polar head and the alkyl chain on the PBN moiety are reversed. Experimental and theoretical data showed that substitution of the PBN by hydrophilic and lipophilic groups alters its redox properties, with the amphiphilic amide nitrones being easier to oxidize and reduce than the parent PBN. Moreover, we found that the position of the substituents on the nitronyl group is also of importance for their redox behavior, with the reverse analogue **11** exhibiting better reducing properties than **13**. Very high protective effects were observed in in vitro and in vivo experiments for the hydrogenated derivatives **11** and **13**, while the fluorinated compounds were found to be less potent. Specific interactions of hydrogenated amide nitrones with the mitochondria were also demonstrated. Compounds **11** and **13** decreased peroxide formation, reduced electron leakage, and maintained mitochondrial membrane potential at nanomolar concentration. These findings indicate that amphiphilic amide nitrones are more than just radical scavenging antioxidants but may act as a new class of bioenergetic agents targeted to the mitochondrial electron and proton transport chain. Thus, by increasing and preserving complex I activity, they may increase energy and oxygen metabolism efficacy and hence protect the cell against oxidative insults maintaining its function and viability.

Experimental Section

Synthesis. All starting materials were purchased from either Sigma-Aldrich or Acros except 1*H*,1*H*,2*H*,2*H* perfluorooctyl iodide from Elf Atochem. All solvents were distilled and dried according to standard procedures. TLC analyses were performed on aluminum sheets precoated with silica gel 60F254 (Merck). Compound detection was achieved by exposure to UV light (254 nm), by spraying a 5% sulfuric acid solution in methanol or 5% ninhydrin solution in ethanol, and then by heating at 150 °C. Flash chromatography was carried out on Merck silica gel Geduran Si 60 (0.063−0.200 mm), and size exclusion chromatography was carried out on Sephadex LH 20 or Sephadex G-15 (Amersham Biosciences). Optical rotations were determined with a Perkin-Elmer MC241 polarimeter. Mass spectra were recorded on a triple quadripolar spectrometer API III Plus Sciex for ESI+. Melting points were measured on an Electrothermal IA9100 apparatus and have not been corrected. The purity of all final amphiphilic amide

nitrones used in biological testing was assessed by reverse phase HPLC with UV detection, confirming >95% purity. HPLC analysis were performed on a Varian apparatus equipped with a Microsorb C18 column (5 μm, 4.6 mm ×250 mm i.d.) at 0.8 mL/min. UV/vis spectra were recorded on a Cary Win Varian spectrophotometer with a double-compartment quartz cell of 10 mm length (Suprasil). The ^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded on a Brüker AC-250 spectrometer at 250, 66.86, and 235 MHz, respectively. Chemical shifts are given in parts per million (ppm) relative to the solvent residual peak as a heteronuclear reference for ^{1}H and ^{13}C. Abbreviations used for signal patterns are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublet.

***N*-(Octa-*O*-acetyllactobionyl)-2-methyl-2-nitropropanamide (2).** A solution of compound **1**[14] (0.97 g, 6.27×10^{-3} mol, 1 equiv) and lactobionolactone (2.72 g, 7.99×10^{-3} mol, 1.2 equiv) in methoxyethanol with TEA (pH ~9) was stirred at 55 °C for 20 h. The solvent was evaporated under vacuum, and a solution of Ac_2O/pyridine (1:1 v/v) was added dropwise to the residue at 0 °C. After 12 h of being stirred at ambient temperature, the mixture was poured into cold 1 N HCl and extracted with CH_2Cl_2 (3×). The organic layer was washed with brine (3×), dried over Na_2SO_4, and concentrated in vacuum. Purification by flash chromatography and elution with EtOAc/cyclohexane (4:6 v/v), gave compound **2** (3.23 g, 4.06×10^{-3} mol, 65% yield) as a white foam. R_f = 0.18 in EtOAc/cyclohexane (6:4 v/v). ^{1}H NMR ($CDCl_3$) δ 6.64 (1H, t, J = 6.2 Hz), 5.55 (2H, m), 5.40 (1H, m), 5.25−4.90 (3H, m), 4,64 (1H, d, J = 7.7 Hz), 4.49 (1H, m), 4.35−3.85 (5H, m), 3.73 (2H, m), 2.30−1.90 (24H, m), 1.58 (3H, s), 1.57 (3H, s). ^{13}C NMR ($CDCl_3$) δ 170.5, 170.5, 170.2, 170.1, 169.9, 169.7, 169.6, 169.2, 168.0 (CO), 101.8 (CH), 88,5 (C), 77.6, 71.8, 71.1 71.0, 69.8, 69.1, 68.9, 66.8 (CH), 61.6, 61.0, 46,0 (CH_2), 24.0, 20.8, 20.8, 20.7, 20.7, 20.6, 20.6 (CH_3).

***N*-(Octa-*O*-acetyllactobionyl)-2-methyl-2-hydroxylaminopropanamide (3).** At 0 °C, compound **2** (3.32 g, 4.18×10^{-3} mol, 1 equiv) and NH_4Cl (0.32 g, 5.98×10^{-3} mol, 1.4 equiv) were dissolved in a THF/H_2O (3:1 v/v) mixture while stirring. Zinc powder (1.08 g, 1.65×10^{-2} mol, 4 equiv) was added as a small fraction in order to keep the temperature below 30 °C. After 1 h of being stirred at room temperature, the mixture was filtered over a pad of Celite, the zinc salt was washed with hot THF, and the solvents were concentrated under vacuum. Purification by flash chromatography and elution with EtOAc/cyclohexane (8:2 v/v) gave compound **3** (2.60 g, 3.33×10^{-3} mol, 80% yield). R_f = 0.52 in EtOAc/CH_3OH (9:1 v/v). $[\alpha]_D^{20}$ +27.0 (c, l, CH_2Cl_2). ^{1}H NMR ($CDCl_3$) δ 6.62 (1H, t, J = 6.0 Hz), 5.65−5.50 (2H, m), 5.39 (1H, d, J = 3.2 Hz), 5.25−4.95 (3H, m), 4.68 (1H, d, J = 8.0 Hz), 4.60−4.45 (2H, m), 4.35 (1H, dd, J = 3.3 Hz and J = 6.8 Hz), 4.30−3.85 (5H, m) 3.27 (2H, m), 2.25−1.90 (24, m), 1.06 (6H, s). ^{13}C NMR (DMSO-d_6) δ 170.7, 170.3, 170.1, 170.1, 170.0, 169.6, 169.6, 166.7 (CO), 101.1, 78.4, 72.1, 70.8, 70.2, 70.0, 69.5, 69.3, 67.6 (CH), 61.7, 61.4, 57.4 (CH_2), 45.4 (C), 22.8, 22.7, 21.1, 21.0, 20.9, 20.8 (CH_3).

***N*-[4-(1,3-Dioxacyclopent-2-yl)benzyl]octanamide (5).** Octanoic acid (1.74 g, 12.08×10^{-3} mol, 1.2 equiv), compound **4** (1.80 g, 10.17×10^{-3} mol, 1 equiv), and BOP (5.75 g, 13.01×10^{-3} mol, 1.3 equiv) were dissolved in DCM with DIEA (pH ~9). After 48 h of being stirred at room temperature, the solvent was concentrated under vacuum. Purification by flash chromatography and elution with EtOAc/cyclohexane (2:8 v/v) gave compound **5** (2.60 g, 8.51×10^{-3} mol, 84% yield) as a beige powder. R_f = 0.47 in EtOAc/cyclohexane (6:4 v/v). ^{1}H NMR ($CDCl_3$) δ 7.47 (2H, d, J = 8.0 Hz), 7.30 (2H, d, J = 8.0 Hz), 5.81 (2H, m), 4.45 (2H, d, J = 5.7 Hz), 4.2−3.9 (4H, m), 2.21 (2H, t, J = 7.6 Hz), 1.63 (2H, m), 1.29 (8H, m), 0.88 (3H, t, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$) δ 173.0 (CO), 139.7, 137.0 (C), 127.9, 126.8, 103.5 (CH), 65.3, 43.3, 36.8, 31.7, 29.3, 29.0, 25.8, 22.6 (CH_2), 14,1 (CH_3).

***N*-[4-(1,3-Dioxacyclopent-2-yl)benzyl]-2*H*,2*H*,3*H*,3*H*-perfluorononamide (6).** 1*H*,1*H*,2*H*,2*H*-Perfluorononanoic acid (1.71 g, 4.36×10^{-3} mol, 1.1 equiv), compound **4**[21] (0.70 g, 3.95×10^{-3} mol, 1 equiv), DCC (0.97 g, 4.70×10^{-3} mol, 1.2 equiv), and a catalytic amount of HOBt were dissolved in DCM with DIEA (pH ~9). After 48 h of being stirred at room temperature, the mixture was filtered over a pad of Celite and the solvent was concentrated under vacuum. Purification by flash chromatography and elution with EtOAc/cyclohexane (2:8 v/v) gave compound **6** (1.13 g, 2.04×10^{-3} mol, 52% yield) as a white powder. R_f = 0.57 in EtOAc/cyclohexane (6:4 v/v). ^{1}H NMR ($CDCl_3$) δ 7.47 (2H, d, J = 8.0 Hz), 7.30 (2H, d, J = 8.0 Hz), 5.97 (1H, m), 5.81 (1H, s), 4.45 (2H, d, J = 5.7 Hz), 4.25−3.90 (4H, m), 2.70−2.30 (4H, m). ^{13}C NMR ($CDCl_3$) δ 169.7 (CO), 138.9, 137.5 (C), 128.1, 127.0, 103.4 (CH), 65.3, 43.6, 33.9, 26.4 (CH_2). ^{19}F NMR ($CDCl_3$) δ −80.7 (3F, CF_3), −114.1 (2F, CF_2), −121.8 (2F, CF_2), −122.8 (2F, CF_2), −123.5 (2F, CF_2), −126.1 (2F, CF_2).

***N*-(4-Formylbenzyl)octanamide (7).** At 10 °C, compound **5** (0.92 g, 3.01×10^{-3} mol) and a catalytic amount of *p*-toluenesulfonic acid were dissolved in 15 mL of ethanol. After 14 h of being stirred at 10 °C the solvent was concentrated under vacuum and the crude mixture was dissolved in EtOAc. The organic phase was washed with saturated $NaHCO_3$ solution (2×), brine (2×), dried over Na_2SO_4, then concentrated under vacuum to give compound **7** (0.75 g, 2.87×10^{-3} mol, 95% yield) as a beige powder. R_f = 0.47 in EtOAc/cyclohexane (6:4 v/v). ^{1}H NMR ($CDCl_3$) δ 9.98 (1H, s), 7.84 (2H, d, J = 8.1 Hz), 7.42 (2H, d, J = 8.0 Hz), 6.18 (1H, m), 4.50 (2H, d, J = 5.9 Hz), 2.26 (2H, t, J = 7.6 Hz), 1.63 (2H, m), 1.29 (8H, m), 0.88 (3H, t, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$) δ 191.9, 173.0 (CO), 145.7, 135.5 (C), 130.1, 128.1 (CH), 43.1, 36.8, 31.7, 29.3, 29.0, 25.8, 22.6 (CH_2), 14.1 (CH_3).

***N*-(4-Formylbenzyl)-2*H*,2*H*,3*H*,3*H*-perfluorononamide (8).** The synthetic procedure was essentially the same as for compound **7**. Compound **6** (1.13 g, 2.04×10^{-3} mol) was reacted to give compound **8** (0.97 g, 1.90×10^{-3} mol, 93%) as a white powder. R_f = 0.57 in EtOAc/cyclohexane (6:4 v/v). ^{1}H NMR ($CDCl_3$) δ 10.02 (1H, s), 7.86 (2H, d, J = 8.2 Hz), 7.45 (2H, d, J = 8.2 Hz), 6.05 (1H, m), 4.56 (2H, d, J = 6.0 Hz), 2.58 (4H, m). ^{13}C NMR ($CDCl_3$) δ 191.8, 169.9 (CO), 144.8, 135.8 (C), 130.2, 128.1 (CH), 43.5, 33.9, 26.7 (CH_2). ^{19}F NMR ($CDCl_3$) δ −80.7 (3F, CF_3), −114.1 (2F, CF_2), −121.8 (2F, CF_2), −122.8 (2F, CF_2), −123.5 (2F, CF_2), −126.1 (2F, CF_2).

***N*-[1,1-Dimethyl-1-(octa-*O*-acetyl-lactobionamidomethyl)]-α-[4-octanamidomethyl)phenyl]nitrone (9).** Compound **3** (0.66 g, 8.45×10^{-4} mol, 0.6 equiv) and compound **7** (0.368 g, 1.41×10^{-3} mol, 1 equiv) were dissolved in THF/AcOH mixture (3:2 v/v) under argon. After the mixture was stirred for 2 h at 60 °C under argon, compound **3** (0.443 g, 5.68×10^{-4} mol, 0.4 equiv) was added and the mixture was heated overnight. Then more of the compound **3** (0.110 g, 1.41×10^{-4} mol, 0.1 equiv) was added and the heating was continued for 2 h. The solvent was concentrated under vacuum. Purification by flash chromatography and elution with EtOAc/cyclohexane (7:3 v/v) followed by size exclusion chromatography using CH_2Cl_2/CH_3OH (1:1 v/v) gave compound **9** (0.98 g, 9.57×10^{-4} mol, 67% yield) as a white powder. R_f = 0.25 in EtOAc. $[\alpha]_D^{20}$ +44.6 (c, l, CH_2Cl_2). ^{1}H NMR ($CDCl_3$) δ 8.25 (2H, d, J = 8.3 Hz), 7.49 (1H, s), 7.35 (2H, d, J = 8.3 Hz), 7.17 (1H, m), 5.96 (1H, m), 5.51 (2H, m), 5.29 (1H, m), 5.15 (1H, m), 4.98 (2H, m), 4.68 (1H, d, J = 7.9 Hz), 4.55−4.40 (3H, m), 4.33 (1H, m), 4.25−3.85 (4H, m), 3.85−3.50 (2H, m), 2.26 (2H, t, J = 7.6 Hz), 2.20−1.85 (24H, m), 1.67 (2H, m), 1.59 (3H, s), 1.57 (3H, s), 1.29 (8H, m), 0.87 (3H, t, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$) δ 173.2, 170.4, 170.2, 169.8, 168.7, 169.6, 169.2, 167.7 (CO), 141.5 (C), 131.9 (CH), 129.4 (C), 129.4 127.7, 101.8, 77.7 (CH), 73.4 (C), 72.1, 71.1, 70.9, 69.8, 68.9, 66.8 (CH), 61.6, 60.9, 47.1, 43.2, 36.8, 31.7, 29.3, 29.0, 25.8 (CH_2), 25.1, 24.3 (CH_3), 22.6 (CH_2), 20.9, 20.8, 20.7, 20.6, 20.5, 20.4, 14.1 (CH_3). MS (ESI+, m/z): 1024.8 $[M + H]^+$, 1041.8 $[M + NH_4]^+$, 1046.7 $[M + Na]^+$, 1062.7 $[M + K]^+$.

***N*-[1,1-Dimethyl-1-(octa-*O*-acetyllactobionamidomethyl)]-α-[4-(2*H*,2*H*,3*H*,3*H*-perfluorononamidomethyl)phenyl]nitrone (10).** The synthetic procedure was essentially the same as for compound **9**. Compound **3** (1.03 g, 1.32×10^{-3} mol, 1.1 equiv) and compound **8** (0.615 g, 1.21×10^{-3} mol, 1 equiv) were reacted to give compound **10** (1.162 g, 9.13×10^{-4} mol, 75% yield) as a white powder. $R_f = 0.27$ in EtOAc. $[\alpha]_D^{20}$ +36.2 (*c*, l, CH_2Cl_2). ^{1}H NMR ($CDCl_3$) δ 8.24 (2H, d, $J = 8.3$ Hz), 7.50 (1H, s), 7.34 (2H, d, $J = 8.3$ Hz), 7.22 (1H, m), 6.35 (1H, m), 5.60−5.40 (2H, m), 5.29 (1H, d, $J = 2.9$ Hz), 5.25−5.10 (1H, m), 5.05−4.85 (2H, m), 4.68 (1H, d, $J = 8.0$ Hz), 4.50 (2H, d, $J = 5.7$ Hz) 4.45−4.25 (2H, m), 4.22−3.85 (6H, m), 3.85−3.45 (2H, m), 2.56 (2H, m), 2.20−1.90 (24H, m), 1.59 (3H, s), 1.57 (3H, s). ^{13}C NMR ($CDCl_3$) δ 170.5, 170.4, 170.2, 170.1, 169.9, 169.8, 169.7, 169.3, 167.6 (CO), 140.8 (CH), 131.7 (CH), 129.6 (C), 129.4, 127.7, 101.8, 77.9 (CH), 73.4 (C), 72.6, 71.9, 71.2, 70.8, 69.8, 68.9, 66.8 (CH), 61.6, 61.0 (CH_2), 47.2, 43.4, 26.9, 26.7 (CH_2), 25.1, 24.3, 20.8, 20.7, 20.6, 20.5, 20.4 (CH_3). ^{19}F NMR ($CDCl_3$) δ −80.7 (3F, CF_3), −114.1 (2F, CF_2), −121.8 (2F, CF_2), −122.8 (2F, CF_2), −123.5 (2F, CF_2), −126.2 (2F, CF_2). MS (ESI+, *m*/*z*): 1272.6 $[M + H]^+$, 1289.6 $[M + NH_4]^+$, 1294.6 $[M + Na]^+$, 1310.6 $[M + K]^+$.

***N*-[1,1-Dimethyl-1-(lactobionamidomethyl)]-α-[4-(octanamidomethyl)phenyl]nitrone (11).** Compound **9** (0.500 g, 4.88×10^{-4} mol) and a catalytic amount of sodium methoxide (pH ∼8) were dissolved in MeOH. After the mixture was stirred for 4 h, 1 N HCl solution was added dropwise to neutralize the mixture and the solvents were concentrated under vacuum. Purification by size exclusion chromatography using water followed by lyophilization gave compound **11** (0.25 g, 3.63×10^{-4} mol, 75% yield) as a white and hygroscopic foam. $R_f = 0.38$ in EtOAc/MeOH/H_2O (7:2:1 v/v/v). $[\alpha]_D^{20}$ +10.6 (*c*, l, DMSO). UV (CH_3OH): $\lambda_{max} = 298$ nm. ^{1}H NMR (DMSO-d_6) δ 8.35 (3H, m), 7.82 (1H, s), 7.57 (1H, t, $J = 6.2$ Hz), 7.29 (2H, d, $J = 8.3$ Hz), 5.45−3.10 (25H, m), 2.15 (2H, t, $J = 7.3$ Hz), 1.49 (8H, m), 1.25 (8H, s), 0.87 (3H, t, $J = 6.6$ Hz). ^{13}C NMR (CD_3OD) δ 174.4, 174.2 (CO), 142.4 (C), 135.1, 129.9 (CH), 129.2 (C), 127.1, 104.3, 81.7, 75.8 (CH), 73.8 (C), 73.3, 72.5, 71.7, 71.3, 71.1, 68.9 (CH), 62.3, 61.3, 45.7, 42.4, 35.7, 31.5, 28.9, 28.7, 25.7 (CH_2), 23.5, 23.4 (CH_3), 22.3 (CH_2), 13.0 (CH_3). MS (ESI+, *m*/*z*): 688.6 $[M + H]^+$, 705.5 $[M + NH_4]^+$, 710.6 $[M + Na]^+$, 726.5 $[M + K]^+$. HR-MS (ESI+, *m*/*z*) for $[C_{32}H_{53}N_3O_{13} + H]^+$: calcd 688.3651, found 688.3630. RP-HPLC t_R = 10.05 min (CH_3OH/H_2O 60:40 v/v).

***N*-[1,1-Dimethyl-1-(lactobionamidomethyl)]-α-[4-(2*H*,2*H*,3*H*,3*H*-perfluorononamidomethyl)phenyl]nitrone (12).** The synthetic procedure was essentially the same as for compound **11**. Compound **10** (0.5 g, 3.93×10^{-4} mol) was reacted to give compound **12** (0.33 g, 3.53×10^{-4} mol, 90% yield) as a white and hygroscopic foam. $[\alpha]_D^{20}$ +6.1 (*c*, l, DMSO). UV (CH_3OH): $\lambda_{max} = 298$ nm. ^{1}H NMR (DMSO-d_6) δ 8.60 (1H, m) 8.32 (2H, d, $J = 8.3$ Hz), 7.83 (1H, s), 7.56 (1H, m), 7.31 (2H, d, $J = 8.3$ Hz), 5.40−3.20 (25H, m), 2.80−2.40 (4H, m), 1.48 (6H, s). ^{13}C NMR (DMSO) δ 173.1, 169.9 (CO), 141.6 (C), 131.0 (CH), 130.4 (C), 129.2, 127.4, 105.1, 83.5, 76.1 (CH), 73.8 (C), 73.7, 72.4, 71.8, 71.5, 71.0, 68.7 (CH), 62.8, 61.1, 46.2, 42.6, 26.3 (CH_2), 24.8, 24.7 (CH_3). ^{19}F NMR (DMSO) δ −80.2 (3F, CF_3), −113.5 (2F, CF_2), −121.7 (2F, CF_2), −122.6 (2F, CF_2), −123.1 (2F, CF_2), −125.7 (2F, CF_2). MS (ESI+, *m*/*z*): 936.5 $[M + H]^+$, 958.5 $[M + Na]^+$, 974.5 $[M + K]^+$. RP-HPLC t_R = 11.99 min (CH_3OH/H_2O 70:30 v/v).

Determination of $\log k'_W$ Values. Methanol solutions of the nitrones (1.0 mg/mL) were injected into an HPLC equipped with a Microsorb C18 column (250 mm × 4.6 mm, 5 μm). The nitrones were eluted using a MeOH/water mixture (9:1 to 7:3 v/v) using a flow rate of 0.8 mL/min. The column temperature was 23 °C, and the UV detector wavelength was $\lambda = 298$ nm. The $\log k'$ values were calculated by using the equation $\log k' = \log((t - t_0)/t_0)$, where t is the retention time of the nitrone and t_0 is the elution time of MeOH, which is not retained on the column. Linear regression analysis ($r^2 > 0.997$) was performed on the three data points for each nitrone and the resulting line extrapolated to 100% aqueous to give the $\log k'_W$ values listed in Table 1.

Surface Tension Measurements. Nitrone solutions were prepared by using Milli-Q water (resistivity of 18.2 MΩ·cm; surface tension of 72.8 mN/m). All measurements were carried out at 25 °C. The surface tensions of nitrone solutions were measured with a Krüss K12ST tensiometer controlled by the Krüss Labdesk software (Krüss, Germany) by the Wilhelmy plate technique. The critical micelle concentration (cmc) and the surface tension (γ_{cmc}) were determined from the breakpoint of the surface tension and logarithm of concentration curve.

Radical Scavenging. Radical scavenging was investigated using a scavenger competition assay based on the burstlike formation of the intensely green colored 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ABTS cation radical initiated by a Fenton reaction.[55] Measurements were made at 420 nm and monitored for a period of 30 min in the absence or presence of scavenging competitor at 1 mM dissolved in DMSO. The reaction system consisted of 0.9 mL of distilled water, 0.15 mL of ABTS at 1 mM, 0.15 mL of $FeSO_4$ at 0.5 mM, 0.15 mL of the test compound at 1 mM dissolved in DMSO, and 0.15 mL of H_2O_2 at 10 mM. Controls received the DMSO vehicle only, with the solvent reaching a final concentration of 10% in the assay. The reaction was started with the addition of H_2O_2 and the rapidly increasing concentration of the ABTS cation radicals was determined every minute. Radical scavengers suppressed this time dependent ABTS cation radical formation by competition.

Radical Reduction. ABTS cation radical reduction was assayed according to Re et al.[25] with the following modifications:[24] assay concentration of the ABTS cation radical was 105 μM, final concentration of the test compounds was 0.5 mM. Controls received DMSO vehicle only, reaching a final concentration of 2.5% in the assay. The reactions were started with the addition of the nitrone agent, and reduction of the ABTS cation radical was monitored every minute at 420 nm for 30 min.

Cyclic Voltammetry. Cyclic voltammetry was performed on a potentiostat and computer-controlled electroanalytical system. Electrochemical measurements were carried out in a 10 mL cell equipped with a glassy carbon working electrode, a platinium-wire auxiliary electrode, and a Ag/AgCl reference electrode. Solutions were degassed by bubbling with nitrogen gas before recording the voltammograms at a scan rate (ν) of 0.15 $V \cdot s^{-1}$. Nitrone concentration was 1 mM in doubly distilled water, and the supporting electrolyte was 0.15 M NaCl.

Computational Methods. All calculations were performed at the Ohio Supercomputer Center. The minimization of initial structures using MMFF94[56] was performed with MacroModel 9.6.[57] Conformational search was then carried out using MMFF94[56] via the Monte Carlo multiple minimum method coupled with generalized Born/surface area (GB/SA) continuum solvation model using water as the solvent[58] as implemented in the MacroModel package. Density functional theory[59,60] was further applied in this study to determine the optimized geometry of each species[61−64] at the B3LYP/6-31G(d) level using Gaussian 03.[65] Electron spin densities (populations) were obtained from a natural population analysis (NPA) approach using single-point energies evaluated at the B3LYP/6-31G+(d,p) level with 6d functions.[66] The effect of solvation on the gas-phase calculations was also investigated using the polarized continuum model (PCM).[67−70] Only the bottom-of-the-well energies were considered in this study, since the size of the molecules limits the calculation of the vibrational frequencies.

Primary Cortical Mixed Cell Cultures. Mixed cortical cell cultures from E15 Sprague−Dawley rat embryos were prepared, cultured, and treated as described by Law et al.[71] The cultures were exposed to toxins (200 μM) and antioxidant agents (10 μM) for 24 h. Cell death was quantified by the trypan blue absorbance assay.[72] The absorbance of the dye was measured at 590 nm.

Rotifers. Survival of rotifers was investigated in standardized viability assays as described previously.[18] Experiments on the controls receiving vehicle only resulted in zero percentage survival and were carried out in parallel on animals of the same stock culture to ensure absolutely identical exposure conditions for all animals.

Rat Brain Mitochondrial Preparations. The mitochondrial preparations from the forebrain of 3-month-old Sprague−Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) fed at libitum and housed under a 12−12 h light−dark cycle. The mitochondrial preparations were isolated and prepared as previously described.[40] The rat brain mitochondrial preparations containing at least 80% of structurally fully conserved and intact mitochondria with very little debris present were resuspended to allow for respiration with the physiological substrates glutamate and malate in phosphate buffered Tris-KCl medium: 10 mM Tris-HCl, pH 7.4, and 5 mM potassium phosphate buffer, pH 7.4, with 100 mM KCl and 1.5 mM $MgCl_2$.

Incubation Procedures Using Rat Brain Mitochondrial Preparations. Incubation with the nitrones and respiration in the mitochondrial preparations were started with following substrates: 6 mM glutamate and 6 mM malate at 37.5 °C. In the experiment on the mitochondrial membrane potential (mmp), the protonophore FCCP was added at 0.2 μM after 15 min to induce a 50% reduction (partial collapse of mitochondrial membrane potential). Electron leakage, hydrogen peroxide formation, and mitochondrial membrane potential were assayed after 30 min of incubation by adding extraction solvent (NBT), acid (hydroperoxides) or doing direct measurements on quenching of rhodamine 123 fluoresence by mitochondria in the preparations themselves to determine the recovery of the mmp.

Determination of Soluble Hydroperoxides Formation in Mitochondrial Preparations from Rat Forebrain by the FOX Assay. Xylenol orange method (FOX assay) was used to determine soluble hydroperoxides in mitochondria as described by Hermes-Lima et al.[38] The sensitivity and specifity of the peroxide measurements was increased by adding 100 mM modifier sorbitol as described by Butterfield et al.[73] Mitochondrial preparations were harvested after 30 min of incubation and collected on ice. Then 800 μL of the mitochondrial preparations was added to 200 μL of 0.35 M H_2SO_4 and homogenized in a Potter−Elvehjem homogenizer with a tightly fitting glass pestle. The acidic homogenate was centrifuged for 3 min at 10000*g*. Soluble hydroperoxides in supernatants were determined by a microassay variant[39] to increase yield, sensitivity, and specificity by 1 order of magnitude; 0.07 M final assay concentration of H_2SO_4 was used to ensure stability and reproducible measurements by obtaining an optimal pH with 250 μL of homogenate or reagent blank containing 0.07 M H_2SO_4 and 750 μL of reagent solution containing 2.5 mM $FeSO_4$, 1 mM xylenol orange, 100 mM sorbitol, and 0.07 M H_2SO_4. Additional homogenate blanks contained a reagent solution devoid of xylenol orange. After being vortexed, mixtures were incubated for 30 min at 25 °C in the dark. After another centrifugation for 3 min at 10000*g* the Fe(III)−xylenol orange complex was determined at 570 nm. Because of protein precipitation by the acid used already during homogenization, the assay as applied does not detect total peroxides but only the soluble hydroperoxides, which are mainly hydrogen peroxide (at least 98% in mitochondrial preparations).

Determination of Electron Leakage in Mitochondrial Preparations from Rat Forebrain by the Nbt Reduction Assay. Nitro blue tetrazolium (NBT) reduction was measured as described by Hensley et al.[40] in the presence of 3 μM rotenone and 3 mM azide with the following modifications: incubation temperature was 37.5 °C, and formazan was extracted according to the method described by Vrablic et al.[74]

Increase of Mitochondrial Membrane Potential in Mitochondrial Preparations from Rat Forebrain. Mitochondrial membrane potential was measured directly in the preparations by recording the quenching of rhodamine 123 fluorescence due to the accumulation of the dye in respiring mitochondria and the dequenching after addition of the protonophore FCCP due to a partial collapse of the membrane potential, release of rhodamine 123, and a resulting increase in detectable rhodamine 123 fluorescence according to Lee et al.[45] with the modifications as described above in the paragraph on incubation procedures. Mitochondrial membrane potential were calculated according to the methods as described by Scaduto and Grotyohann.[44]

Complex I Activity. Ferric cyanide reductase activity in submitochondrial particles was measured by monitoring the reduction of ferric cyanide from the wavelength difference at 440 and 490 nm in the presence of antimycin and azide[20,50] at an incubation temperature of 37.5 °C. This recently developed method allows for a specific and sensitive measurement of mitochondrial iron sulfur cluster N2 activity in complex I, as it is completely abolished in the presence of the specific ligand and inhibitor capsaicin when added at 50 μM. Dopamine added at equimolar concentration was almost as effective as capsaicin in acting as a specific antagonist at this site of the respiratory chain. Preparations were preincubated for 30 min with the nitrone agent or the dopamine or the addition of both compounds prior to the measurement of ferric cyanide reduction. Basal activity was 1200 (nmol/min)/mg protein.

Acknowledgment. This work was supported by Association Française contre les Myopathies (AFM) Grant No. 12674 (2005−2007). S.O. was the recipient of a fellowship from the "Région Provence Alpes Côte d'Azur" and from Targeting System Pharma Company. B.P. was supported by the grant PO 662/1−1 of the German Research Foundation ("Deutsche Forschungsgemeinschaft—DFG"). F.A.V. was supported by the Ohio Supercomputer Center and NIH Grant HL81248.

Supporting Information Available: NMR and MS spectra of compounds **9**−**12**; HPLC chromatograms of **11** and **12**; cyclic voltammograms of **11** and **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Coulter, C. V.; Kelso, G. F.; Lin, T.-K.; Smith, R. A. J.; Murphy, M. P. Mitochondrially targeted antioxidants and thiol reagents. *Free Radical Biol. Med.* **2000**, *28*, 1547–1554.
(2) Muratovska, A.; Lightowlers, R. N.; Taylor, R. W.; Wilce, J. A.; Murphy, M. P. Targeting large molecules to mitochondria. *Adv. Drug Delivery Rev.* **2001**, *49*, 189–198.
(3) Murphy, M. P.; Echtay, K. S.; Blaikie, F. H.; Asin-Cayuela, J.; Cocheme, H. M.; Green, K.; Buckingham, J. A.; Taylor, E. R.; Hurrell, F.; Hughes, G.; Miwa, S.; Cooper, C. E.; Svistunenko, D. A.; Smith, R. A. J.; Brand, M. D. Superoxide activates uncoupling proteins by generating carbon-centered radicals and initiating lipid peroxidation: studies using a mitochondria-targeted spin trap derived from α-phenyl-*tert*-butylnitrone. *J. Biol. Chem.* **2003**, *278*, 48534–48545.
(4) Covey, M. V.; Murphy, M. P.; Hobbs, C. E.; Smith, R. A. J.; Oorschot, D. E. Effect of the mitochondrial antioxidant, mito vitamin E, on hypoxic−ischemic striatal injury in neonatal rats: a dose-response and stereological study. *Exp. Neurol.* **2006**, *199*, 513–519.
(5) Horton, K. L.; Stewart, K. M.; Fonseca, S. B.; Guo, Q.; Kelley, S. O. Mitochondria-penetrating peptides. *Chem. Biol.* **2008**, *15*, 375–382.
(6) Zhao, K.; Luo, G.; Giannelli, S.; Szeto, H. H. Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by *tert*-butyl hydroperoxide in neuronal cell lines. *Biochem. Pharmacol.* **2005**, *70*, 1796–1806.
(7) Wipf, P.; Xiao, J.; Jiang, J.; Belikova, N. A.; Tyurin, V. A.; Fink, M. P.; Kagan, V. E. Mitochondrial targeting of selective electron scavengers: synthesis and biological analysis of hemigramicidin−TEMPO conjugates. *J. Am. Chem. Soc.* **2005**, *127*, 12460–12461.

(8) El Fangour, S.; Marini, M.; Good, J.; McQuaker, S. J.; Shiels, P. G.; Hartley, R. C. Nitrones for understanding and ameliorating the oxidative stress associated with aging. *Age* **2009**, *31*, 269–276.
(9) Ouari, O.; Polidori, A.; Pucci, B.; Tordo, P.; Chalier, F. Synthesis of a glycolipidic amphiphilic nitrone as a new spin trap. *J. Org. Chem.* **1999**, *64*, 3554–3556.
(10) Geromel, V.; Kadhom, N.; Cebalos-Picot, I.; Ouari, O.; Polidori, A.; Munnich, A.; Rötig, A.; Rustin, P. Superoxide-induced massive apoptosis in cultured skin fibroblasts harboring the neurogenic ataxia retinitis pigmentosa (NARP) mutation in the ATPase-6 gene of the mitochondrial DNA. *Hum. Mol. Genet.* **2001**, *10*, 1221–1228.
(11) Périno, S.; Contino-Pépin, C.; Jasseron, S.; Rapp, M.; Maurizis, J.-C.; Pucci, B. Design, synthesis and preliminary biological evaluations of novel amphiphilic drug carriers. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1111–1114.
(12) Ortial, S.; Durand, G.; Poeggeler, B.; Polidori, A.; Pappolla, M. A.; Boeker, J.; Hardeland, R.; Pucci, B. Fluorinated amphiphilic amino acid derivatives as antioxidant carriers: a new class of protective agents. *J. Med. Chem.* **2006**, *49*, 2812–2820.
(13) Durand, G.; Prosak, R. A.; Han, Y.; Ortial, S.; Rockenbauer, A.; Pucci, B.; Villamena, F. A. Spin trapping and cytoprotective properties of fluorinated amphiphilic carrier conjugates of cyclic versus linear nitrones. *Chem. Res. Toxicol.* **2009**, *22*, 1570–1581.
(14) Durand, G.; Polidori, A.; Salles, J.-P.; Pucci, B. Synthesis of a new family of glycolipidic nitrones as potential antioxidant drugs for neurodegenerative disorders. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 859–862.
(15) Durand, G.; Polidori, A.; Ouari, O.; Tordo, P.; Geromel, V.; Rustin, P.; Pucci, B. Synthesis and preliminary biological evaluations of ionic and nonionic amphiphilic alpha-phenyl-*N*-*tert*-butylnitrone derivatives. *J. Med. Chem.* **2003**, *46*, 5230–5237.
(16) Durand, G.; Polidori, A.; Salles, J.-P.; Prost, M.; Durand, P.; Pucci, B. Synthesis and antioxidant efficiency of a new amphiphilic spin-trap derived from PBN and lipoic acid. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2673–2676.
(17) Asanuma, T.; Yasui, H.; Inanami, O.; Waki, K.; Takahashi, M.; Iizuka, D.; Uemura, T.; Durand, G.; Polidori, A.; Kon, Y.; Pucci, B.; Kuwabara, M. A new amphiphilic derivative, *N*-{[4-(lactobionamido)methyl]benzylidene}-1,1-dimethyl-2-(octylsulfanyl)ethylamine *N*-oxide, has a protective effect against copper-induced fulminant hepatitis in Long−Evans cinnamon rats at an extremely low concentration compared with its original form alpha-phenyl-*N*-(*tert*-butyl) nitrone. *Chem. Biodiversity* **2007**, *4*, 2253–2267.
(18) Poeggeler, B.; Durand, G.; Polidori, A.; Pappolla, M. A.; Vega-Naredo, I.; Coto-Montes, A.; Boeker, J.; Hardeland, R.; Pucci, B. Mitochondrial medicine: neuroprotection and life extension by the new amphiphilic nitrone LPBNAH acting as a highly potent antioxidant agent. *J. Neurochem.* **2005**, *95*, 962–973.
(19) Tanguy, S.; Durand, G.; Reboul, C.; Polidori, A.; Pucci, B.; Dauzat, M.; Obert, P. Protection against reactive oxygen species injuries in rat isolated perfused hearts: effect of LPBNAH, a new amphiphilic spin-trap derived from PBN. *Cardiovasc. Drugs Ther.* **2006**, *20*, 147–149.
(20) Durand, G.; Poeggeler, B.; Boeker, J.; Raynal, S.; Polidori, A.; Pappolla, M. A.; Hardeland, R.; Pucci, B. Fine-tuning the amphiphilicity: a crucial parameter in the design of potent alpha-phenyl-*N*-*tert*-butylnitrone analogues. *J. Med. Chem.* **2007**, *50*, 3976–3979.
(21) Ouari, O.; Chalier, F.; Bonaly, R.; Pucci, B.; Tordo, P. Synthesis and spin-trapping behavior of glycosylated nitrones. *J. Chem. Soc., Perkin Trans. 2* **1998**, 2299–2308.
(22) Lebaupain, F.; Salvay, A. G.; Olivier, B.; Durand, G.; Fabiano, A. S.; Michel, N.; Popot, J. L.; Ebel, C.; Breyton, C.; Pucci, B. Lactobionamide surfactants with hydrogenated, perfluorinated or hemifluorinated tails: physical-chemical and biochemical characterization. *Langmuir* **2006**, *22*, 8881–8890.
(23) Kissa, E. *Fluorinated Surfactants: Synthesis, Properties, Applications*; Dekker: New York, 1994; Vol. 7, pp 264−282.
(24) Poeggeler, B.; Thuermann, S.; Dose, A.; Schoenke, M.; Burkhardt, S.; Hardeland, R. Melatonin's unique radical scavenging properties—roles of its functional substituents as revealed by a comparison with its structural analogs. *J. Pineal Res.* **2002**, *33*, 20–30.
(25) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231–1237.
(26) Durand, G.; Choteau, F.; Pucci, B.; Villamena, F. A. Reactivity of superoxide radical anion and hydroperoxyl radical with α-phenyl-*N*-*tert*-butylnitrone (PBN) derivatives. *J. Phys. Chem. A* **2008**, *112*, 12498–12509.
(27) McIntire, G. L.; Blount, H. N.; Stronks, H. J.; Shetty, R. V.; Janzen, E. G. Spin trapping in electrochemistry. 2. Aqueous and nonaqueous electrochemical characterizations of spin traps. *J. Phys. Chem.* **1980**, *84*, 916–921.
(28) Tuccio, B.; Bianco, P.; Bouteiller, J. C.; Tordo, P. Electrochemical characterisation of [beta]-phosphorylated nitrone spin traps. *Electrochim. Acta* **1999**, *44*, 4631–4634.
(29) Gille, L.; Nohl, H. Analyses of the molecular mechanism of adriamycin-induced cardiotoxicity. *Free Radical Biol. Med.* **1997**, *23*, 775–782.
(30) Kotamraju, S.; Konorev, E. A.; Joseph, J.; Kalyanaraman, B. Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species. *J. Biol. Chem.* **2000**, *275*, 33585–33592.
(31) Radi, R.; Rodriguez, M.; Castro, L.; Telleri, R. Inhibition of mitochondrial electron transport by peroxynitrite. *Arch. Biochem. Biophys.* **1994**, *308*, 89–95.
(32) Estévez, A. G.; Radi, R.; Barbeito, L.; Shin, J.; Thompson, J. A.; Beckman, J. S. Peroxynitrite-induced cytotoxicity in PC12 cells: evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. *J. Neurochem.* **1995**, *65*, 1543–1550.
(33) Meadow, N. D.; Barrows, C. H. J. Studies on aging in a bdelloid rotifer. I. The effect of various culture systems on longevity and fecundity. *J. Exp. Zool.* **1971**, *176*, 303–313.
(34) Meadow, N. D.; Barrows, C. H. J. Studies on aging in a bdelloid rotifer. II. The effects of various environmental conditions and maternal age on longevity and fecundity. *J. Gerontol.* **1971**, *26*, 302–309.
(35) Enesco, H. E.; Verdone-Smith, C. α-Tocopherol increases lifespan in the rotifer philodina. *Exp. Gerontol.* **1980**, *15*, 335–338.
(36) Sawada, M.; Carlson, J. C. Association between lipid peroxidation and life-modifying factors in rotifers. *J. Gerontol.* **1987**, *42*, 451–456.
(37) Sawada, M.; Carlson, J. C. Biochemical changes associated with the mechanism controlling superoxide radical formation in the aging rotifer. *J. Cell. Biochem.* **1990**, *44*, 153–165.
(38) Hermes-Lima, M.; Willmore, W. G.; Storey, K. B. Quantification of lipid peroxidation in tissue extracts based on Fe(III) xylenol orange complex formation. *Free Radical Biol. Med.* **1995**, *10*, 271–280.
(39) Lyras, L.; Cairns, N. J.; Jenner, A.; Jenner, B.; Halliwell, B. An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. *J. Neurochem.* **1997**, *68*, 2061–2069.
(40) Hensley, K.; Pye, Q. N.; Maidt, M. L.; Stewart, C. A.; Robinson, K. A.; Jaffrey, F.; Floyd, R. A. Interaction of α-phenyl-*N*-*tert*-butyl nitrone and alternative electron acceptors with complex 1 indicates a substrate reduction site upstream from the rotenone binding site. *J. Neurochem.* **1998**, *71*, 2549–2557.
(41) Genova, M. L.; Pich Merlo, M.; Bernacchia, A.; Bianchi, C.; Biondi, A.; Bovina, C.; Falasca, A. I.; Formiggini, G.; Parenti Castelli, G.; Lenaz, G. The mitochondrial production of reactive oxygen species in relation to aging and pathology. *Ann. N.Y. Acad. Sci.* **2004**, *1011*, 86–100.
(42) Genova, M. L.; Ventura, B.; Giuliano, G.; Bovina, C.; Formiggini, G.; Parenti Castelli, G.; Lenaz, G. The site of production of superoxide radical in mitochondrial complex I is not a bound ubisemiquinone but presumably iron−sulfur cluster N2. *FEBS Lett.* **2001**, *505*, 364–368.
(43) Murakami, K.; Kondo, T.; Kawase, M.; Li, Y.; Sato, S.; Chen, S. F.; Chan, P. H. Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J. Neurosci.* **1998**, *18*, 205–213.
(44) Scadoto, R. C.; Grotyohann, L. W. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys. J.* **1999**, *76*, 469–477.
(45) Lee, S. J.; Jin, Y.; Yoon, H. Y.; Choi, B.-O.; Kim, H. C.; Oh, Y.-K.; Kim, H.-S.; Kim, W.-C. Ciclopirox protects mitochondria from hydrogen peroxide toxicity. *Br. J. Pharmacol.* **2005**, *145*, 469–476.
(46) Ben-Shachar, D.; Zuk, R.; Glinka, Y. Dopamine neurotoxicity: inhibition of mitochondrial respiration. *J. Neurochem.* **1995**, *64*, 718–723.
(47) Ben-Shachar, D.; Zuk, R.; Gazawi, H.; Ljubuncic, P. Dopamine toxicity involves mitochondrial complex I inhibition: implications to dopamine-related neuropsychiatric disorders. *Biochem. Pharmacol.* **2004**, *67*, 1965–1974.
(48) Brenner-Lavie, H.; Klein, E.; Zuk, R.; Gazawi, H.; Ljubuncic, P.; Ben-Shachar, D. Dopamine modulates mitochondrial function in viable SH-SY5Y cells possibly via its interaction with complex I: relevance to dopamine pathology in schizophrenia. *Biochim. Biophys. Acta* **2008**, *1777*, 173–185.

(49) Brenner-Lavie, H.; Klein, E.; Ben-Shachar, D. Mitochondrial complex I as a novel target for intraneuronal DA: modulation of respiration in intacts cells. *Biochem. Pharmacol.* **2009**, *78*, 85–95.
(50) Moreno-Sanchez, R.; Hogue, B. A.; Bravo, C.; Newman, A. H.; Basile, A. S.; Chiang, P. K. Inhibition of substrate oxidation in mitochondria by the peripheral-type benzodiazepine receptor ligand AHN 086. *Biochem. Pharmacol.* **1991**, *41*, 1479–1484.
(51) Gutman, M.; Singer, T. P. EPR studies on the iron sulfur centers of DPNH dehydrogenase during redox cycle of the enzyme. *Biochem. Biophys. Res. Commun.* **1973**, *44*, 1572–1578.
(52) Lusty, C. J.; Machinist, J. M.; Singer, T. P. Studies on the respiratory chainlinked nicotinamide adenine dinucleotide dehydrogenase. VII. Labile sulfide group in the dehydrogenase and related proteins. *J. Biol. Chem.* **1965**, *240*, 1804–1810.
(53) Harmon, H. J.; Crane, F. L. Inhibition of mitochondrial electron transport by hydrophilic metal chelators. Determination of dehydrogenase topography. *Biochim. Biophys. Acta* **1976**, *440*, 45–48.
(54) Lenaz, G.; Fato, R.; Genova, M. L.; Bergamini, C.; Bianchi, C.; Biondi, A. Mitochondrial complex I: structural and functional aspects. *Biochim. Biophys. Acta* **2006**, *1757*, 1406–1420.
(55) Poeggeler, B.; Reiter, R. J.; Hardeland, R.; Tan, D.-X.; Barlow-Walden, L. R. Melatonin and structurally-related endogenous indoles act as potent electron donors and radical scavengers in vitro. *Redox Rep.* **1996**, *2*, 179–184.
(56) Halgren, T. A. Merck molecular force field. I. Basis, form, scope, parametrization and performance of MMF94. *J. Comput. Chem.* **1996**, *17*, 490–519.
(57) *MacroModel*, version 9.6; Schrödinger, LLC: New York, 2005.
(58) Still, W. C.; Tempczyk, A.; Hawlely, R. C.; Hendrickson, T. Semianalytical treatment of solvation for molecular mechanics and dynamics. *J. Am. Chem. Soc.* **1990**, *112*, 6127–6129.
(59) Labanowski, J. W.; Andzelm, J. *Density Functional Methods in Chemistry*; Springer: New York, 1991.
(60) Parr, R. G.; Yang, W. *Density Functional Theory in Atoms and Molecules*; Oxford University Press: New York, 1989.
(61) Becke, A. D. Density-functional exchange-energy approximation with correct asymptotic behavior. *Phys. Rev. A* **1988**, *38*, 3098–3100.
(62) Becke, A. D. Density-functional thermochemistry. III. The role of exact exchange. *J. Chem. Phys.* **1993**, *98*, 5648–5652.
(63) Hehre, W. J.; Radom, L.; Schleyer, P. V.; Pople, J. A. *Ab Initio Molecular Orbital Theory*; John Wiley & Sons: New York, 1986.
(64) Lee, C.; Yang, W.; Parr, R. G. Development of the Colle−Salvetti correlation-energy formula into a functional of the electron density. *Phys. Rev. B* **1988**, *37*, 785–789.
(65) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03*, revision B.04; Gaussian, Inc.: Pittsburgh PA, 2003.
(66) Freed, J. H. *Theory of Slow Tumbling ESR Spectra for Nitroxides*; Academic: New York, 1976.
(67) Cossi, M.; Barone, V. Analytical second derivatives of the free energy in solution by polarizable continuum models. *J. Chem. Phys.* **1998**, *109*, 6246–6254.
(68) Cossi, M.; Barone, V.; Cammi, R.; Tomasi, J. Ab initio study of solvated molecules: a new implementation of the polarizable continuum model. *Chem. Phys. Lett.* **1996**, *255*, 327–335.
(69) Tedeschi, A. M.; D'Errico, G.; Busi, E.; Basosi, R.; Barone, V. Micellar aggregation of sulfonate surfactants studied by electron paramagnetic resonance of a cationic nitroxide: an experimental and computational approach. *Phys. Chem. Chem. Phys.* **2002**, 2180–2188.
(70) Tomasi, J.; Persico, M. Molecular interactions in solution: an overview of methods based on continuous distributions of the solvent. *Chem. Rev.* **1994**, *94*, 2027–2094.
(71) Law, A.; Gauthier, S.; Quirion, R. Neuroprotective and neurorescuing effects of isoform-specific nitric oxide synthase inhibitors, nitric oxide scavenger, and antioxidant against beta-amyloid toxicity. *Br. J. Pharmacol.* **2001**, *133*, 1114–1124.
(72) Uliasz, T. F.; Hewett, S. J. A microtiter trypan blue absorbance assay for the quantitative determination of excitoxic neuronal injury in cell culture. *J. Neurosci. Methods* **2000**, *100*, 157–163.
(73) Butterfield, D. A.; Martin, L.; Carney, J. M.; Hensley, K. $A_{\beta}(25-35)$ peptide displays H_2O_2-like reactivity towards aqueous Fe^{2+}, nitroxide spin probes, and synaptosomal membrane proteins. *Life Sci.* **1996**, *58*, 217–228.
(74) Vrablic, A. S.; Albright, C. D.; Craciunescu, C. N.; Salganik, R. I.; Zeisel, S. H. Altered mitochondrial function and overgeneration of reactive oxygen species precede the induction of apoptosis by 1-*O*-octadecyl-2-methyl-rac-glycero-3-phosphocholine in p53-defective hepatocytes. *FASEB J.* **2001**, *15*, 1739–1744.