Fluorinated Amphiphilic Amino Acid Derivatives as Antioxidant Carriers: A New Class of Protective Agents

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Received January 10, 2006

The use of classical antioxidants is limited by their low bioavailabilities, and therefore, high doses are usually required to display significant protective activity. In a recent article (*J. Med. Chem.* **2003**, *46*, 5230) we showed that the ability of the α-phenyl-*N*-*tert*-butylnitrone (PBN) to restore the viability of ATPase-deficient human skin fibroblasts was greatly enhanced by grafting it on a fluorinated amphiphilic carrier. With the aim of extending this concept to other antioxidants, we present here the design, the synthesis, and the physicochemical measurements of a new series of fluorinated amphiphilic antioxidant derivatives. The hydroxyl radical scavenging activity and the radical reducing potency of these newly designed compounds were respectively demonstrated in an ABTS competition and an $ABTS^{\bullet+}$ reduction assay. We also showed that the protective effects of amphiphilic antioxidants derived from PBN, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or lipoic acid (5-[1,2]-dithiolan-3-ylpentanoic acid) in primary cortical mixed cell cultures exposed to oxidotoxins are greatly improved compared to their parent compounds in the following rank-order: (1) PBN, (2) Trolox, and (3) lipoic acid. In contrast, the protective activity of indole-3-propionic acid was slightly decreased by grafting it on the amphiphilic carrier. Similar observations were made in in vivo experiments using aquatic invertebrate microorganisms, called rotifers, which were exposed to lethal concentrations of nonselective (H_2O_2) and mitochondria-selective (doxorubicin) oxidotoxins. The conclusion of these studies is that fluorinated amphiphilic PBN, Trolox, and lipoic acid derivatives exhibit very potent protective activities in in vitro and in vivo experiments. The findings demonstrated herein therefore strongly suggest that the amphiphilic character enhances the bioavailability of the antioxidants and allows for a selective targeting of mitochondria.

Introduction

For many years, reactive oxygen species (ROS) have been known to be implicated in the etiology of a large number of diseases and disorders.[1] Among them are atherosclerosis, cancers, diabetes, ischaemia-reperfusion injury, and neurotrauma. Enhanced free radicals production and oxidative damage are also implicated in aging[2] and related neurodegenerative diseases such as Alzheimer's disease,[3] Parkinson's disease,[4] and Huntington's disease.[5] The direct consequence of an accumulation of ROS is an imbalance of the cellular redox homeostasis, which leads to the commonly called *oxidative stress* state. Thus, therapeutic strategies using antioxidants have been extensively developed. In animal models promising results have been reported with natural antioxidants such as vitamin E,[6] vitamin C,[7] and flavonoids.[8] However, clinical trials assessing the efficiency of antioxidant supplementation have provided inconsistent results.[9]

Usually many natural antioxidants exhibit poor bioavailability and cannot easily cross biological barriers such as membranes. Lipophilic compounds are insoluble in water and are located almost exclusively within membranes. Hydrophilic compounds cannot penetrate many biological compartments enclosed by membranes through passive diffusion and largely remain in extracellular compartments. Since oxidative stress is frequently associated with mitochondrial dysfunction,[10] antioxidants able to penetrate intracellular compartments and ensure protection close to the ROS production site should exhibit a higher bioavailability than those remaining in the extracellular compartment (i.e., the hydrophilic ones) or in cytoplasmic membranes (i.e., the lipophilic ones).

According to this, we have previously developed new amphiphilic compounds bearing α-phenyl-*N*-*tert*-butylnitrone (PBN)[11] as the synthetic antioxidant moiety. We have developed a glycolipidic nitrone called TA1PBN as shown in Figure 1.[12] The nitronyl group, responsible for antioxidant properties, was grafted to a tris amino group by a peptidic spacer arm. TA1PBN readily diminished 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.[13] We have also designed a new family of PBN-derived amphiphilic compounds in which structural modifications can easily be performed.[14] LPBNSF (Figure 1), the most hydrophobic compound of this new series, reduced apoptosis in NARP skin fibroblasts but to a lesser extent than TA1PBN.[15]

These studies have consistently demonstrated that fluorinated amphiphilic nitrones are more potent antioxidants than the parent

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10.1021/jm060027e CCC: $33.50 © 2006 American Chemical Society
Published on Web 04/08/2006

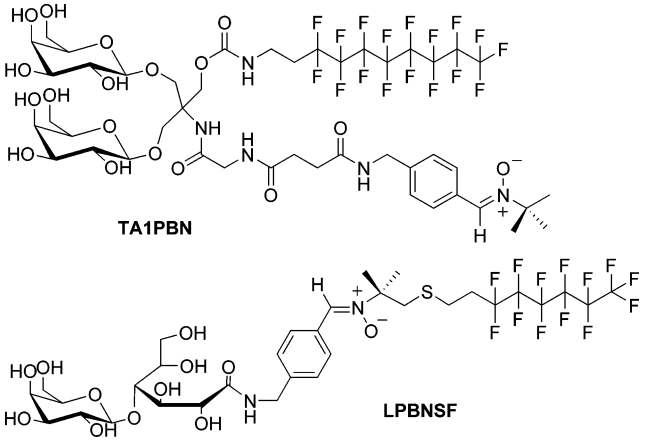


Figure 1. Fluorinated amphiphilic PBN derivatives previously developed.

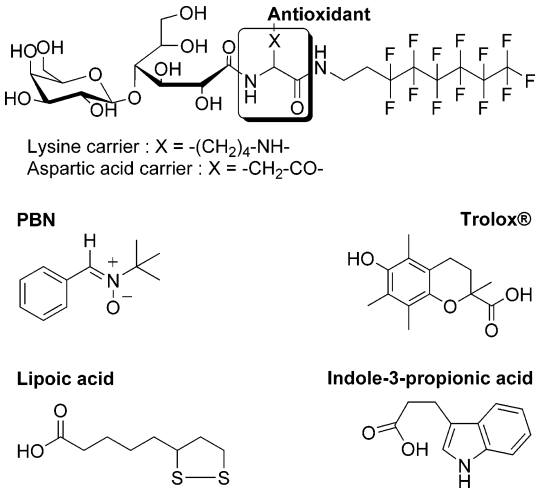
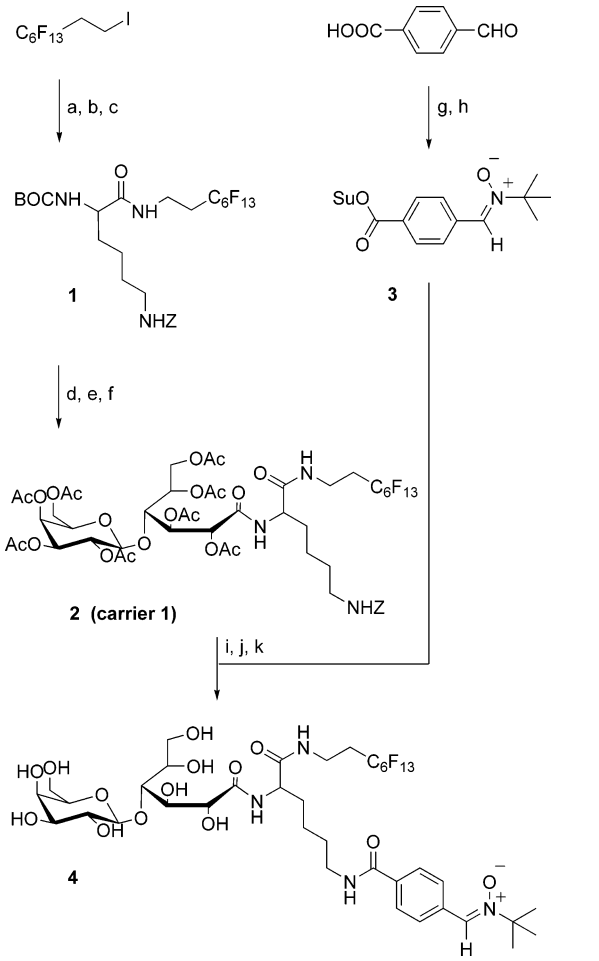


Figure 2. Fluorinated amphiphilic carriers and antioxidants grafted.

compound PBN and possibly because they are able to penetrate cell compartments including mitochondria, thereby providing on-site protection against ROS.

As a continuation of our efforts focused on the synthesis of potent mitochondria targeted antioxidants with enhanced bioavailability, we have designed and developed a new series of fluorinated amphiphilic carriers (Figure 2). This new fluorinated amphiphilic carrier exhibits good bioavailability in vivo and does not seem to show any prohibitive toxicity after iv administration up to 500 mg/kg on mice.[16] The glycosidic hydrophilic head derived from lactobionic acid provides the solubility in water and biological fluids. On the other hand, the perfluoroalkyl tail provides the hydrophobic character and therefore enables the membrane crossing ability without inducing any cell membrane destabilization.[17] Lysine and aspartic acid are used as a scaffold upon which hydrophobic and hydrophilic parts are linked respectively by carboxyl and amino groups and upon which PBN moieties are grafted to functional side chain groups through amide bonds. To confirm that this targeting strategy is generally applicable to a broad spectrum of antioxidant agents, we have chosen to graft three other antioxidants with different mechanisms of action. Lipoic acid (LA), a "metabolic antioxidant", is an effective scavenger of numerous ROS and an effective protective agent.[18] Indole-3-propionic acid (IPA, OXIGON), an endogenous antioxidant, is a potent hydroxyl radical scavenger and an effective neuroprotective agent and in contrast to many antioxidants does not generate prooxidant intermediates.[19] Trolox, a water-soluble vitamin E analogue, is one of the most

Scheme 1. Synthesis of PBN Lysine Derivative **4**[a]



[a] Reagents: (a) NaN_3, DMF; (b) H_2, 7 bar, Pd/C, Et_2O; (c) BocLys(Z)OH, DCC/HOBt, CH_2Cl_2; (d) TFA/ CH_2Cl_2 3:7 (v/v); (e) lactobionic acid, TEA, methoxyethanol; (f) Ac_2O/pyridine 1:1 (v/v); (g) *N*-*tert*-butylhydroxylamine, ethanol; (h) HOSu, DCC, CH_2Cl_2; (i) H_2, 7 bar, Pd/C, ethanol/AcOH 99:1 (v/v); (j) **3**, TEA, CH_2Cl_2; (k) MeONa, methanol.

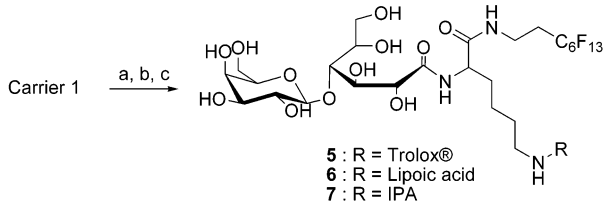
powerful antioxidant agents known[20−23] and is usually used as a standard in biological studies on free radical reactions.

Chemistry

Synthesis. The synthetic pathway of amphiphilic antioxidants is based on three key steps: (i) introduction of a perfluorocarbon chain on a suitably protected amino acid; (ii) condensation of a lactobionamide polar group on the amino acid derivatives; (iii) fixation of antioxidant moieties on an amino acid side chain.

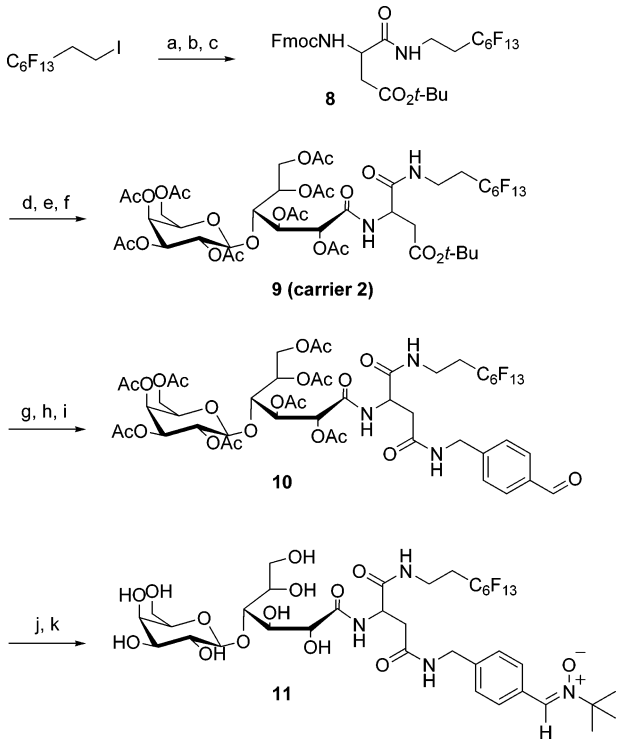
Perfluorocarbon amino acid **1** was easily obtained in three steps from 1*H*,1*H*,2*H*,2*H*-perfluorooctyl iodide as starting material (Scheme 1). The iodine group was quantitatively converted into an azido derivative by substitution with a large excess of sodium azide in DMF. Reduction of the azido group and condensation on N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysine in the presence of dicyclohexylcarbodiimide (DCC)/hydroxybenzotriazole (HOBt) led to **1** with good yield. After *N*-Boc group removal under acidic conditions, the resulting lysine amino intermediate was grafted onto a lactobionic acid following our well-established procedure.[24] Then acetylation of the hydroxyl groups led to compound **2**, also called carrier 1, with 40% yield from perfluorooctyl iodide as starting material. Benzyloxycarbonyl group removal was achieved by catalytic hydrogenolysis, and the resulting amino compound was added to *N*-hydroxysuccimide PBN derivative **3** obtained in three steps

Scheme 2. Synthesis of Antioxidant Lysine Derivatives **5**−**7**[a]



[a] Reagents: (a) H_2, 7 bar, Pd/C, ethanol/AcOH 99:1 (v/v); (b) Trolox, DCC/HOBt, DIEA, CH_2Cl_2 or *N*-hydroxysuccinimide activated antioxidants, TEA, CH_2Cl_2 for lipoic acid and IPA; (c) MeONa, methanol.

Scheme 3. Synthesis of PBN Aspartic Acid Derivative **11**[a]



[a] Reagents and conditions: (a) NaN_3, DMF; (b) H_2, 7 bar, Pd/C, Et_2O; (c) FmocAsp(tbu)OH, DCC/HOBt, CH_2Cl_2; (d) DEA, CH_3CN 1:9 (v/v); (e) lactobionic acid, TEA, methoxyethanol; (f) Ac_2O/pyridine 1:1 (v/v); (g) TFA/ CH_2Cl_2 4:6 (v/v); (h) 4-(1,3-dioxacyclopent-2-yl)benzylamine, BOP, TEA, CH_2Cl_2; (i) CH_3CHO, apts; (j) *N-tert*-butylhydroxylamine, THF/AcOH 3:2 (v/v); (k) MeONa, methanol.

from commercially available 4-carboxybenzaldehyde. Then the Zemplen de-O-acetylation procedure led, after purification through Sephadex LH-20 size exclusion chromatography, to amphiphilic PBN **4**.

The same procedure was followed for the synthesis of the amphiphilic lysine antioxidant derivatives **5**−**7** (Scheme 2). Lipoic acid and indole-3-propionic acid were converted to *N*-hydrosuccinimide derivatives according to a general procedure[25] and were grafted onto an amino deprotected carrier 1. Trolox was grafted on carrier 1 by direct condensation in the presence of DCC/HOBt. Removal of acetyl groups from the sugar moiety followed by suitable purification led to amphiphilic compounds **5**−**7** in quantitative yields.

Aspartic acid derivative **11** was prepared by a connected synthetic pathway (Scheme 3). 1*H*,1*H*,2*H*,2*H*-Perfluorooctylamine was condensed on N^{α}-(9-fluorenylmethyloxycarbonyl-L-aspartic acid β-*tert*-butyl ester in the presence of DCC/HOBt to lead to compound **8** with 70% yield. The *N*-Fmoc protective group of compound **8** was removed under basic conditions, and the resulting amino compound was linked to lactobionic acid.

Table 1. Physicochemical Data of Antioxidants **4**−**7** and **11** and Parent Compounds

compd	cmc[a]	γ_{cmc}[b]	A_{cmc}[c]	log k'_W
PBN	*d*	*d*	*d*	1.69
4	0.047	26.2	42.3	5.18
11	0.347	24.1	59.5	5.07
Trolox	*d*	*d*	*d*	1.34
5	0.025	25.5	58.9	*d*
lipoic acid	*d*	*d*	*d*	1.61
6	*d*	*d*	*d*	6.19
IPA	*d*	*d*	*d*	1.36
7	0.019	26.3	41.3	5.69

[a] cmc, critical micellar concentration, in mM. [b] γ, limit surface tension, in mN/m. [c] Occupied area per molecule at cmc. [d] Not determined.

After acetylation of hydroxyl groups and subsequent purification by silica gel chromatography, compound **9**, also called carrier 2, was obtained in 22% yield from perfluorooctyl iodide as starting material. The ester bond of the *tert*-butoxycarbonyl protective group was cleaved under acidic conditions, and 4-(1,3-dioxacyclopent-2-yl)benzylamine[24] was linked to the carboxylic group in the presence of benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP). Then transacetalization with acetaldehyde afforded benzaldehyde **10**. The nitrone moiety was obtained by condensing *N-tert*-butylhydroxylamine with **10** under an inert atmosphere following our general procedure.[12] The kinetics of this coupling reaction was dramatically increased by using acetic acid as a cosolvent instead of using pure THF. The Zemplén de-O-acetylation procedure led, after purification through Sephadex LH-20 size exclusion chromatography, to amphiphilic PBN **11**.

All amphiphilic compounds were fully characterized by ^1H, ^{13}C, and ^{19}F NMR, distortionless enhancement by polarization transfer (DEPT), and heteronuclear multiple-quantum coherence (HMQC) NMR sequences. Mass spectrometry (positive fast atom bombardment (FAB)) allowed for the observation of characteristic adducts (see Experimental Section). The purity of samples was checked by C18 reverse phase HPLC and was higher than 96%.

Physicochemical Measurements. All amphiphilic antioxidants obtained were freely soluble in water except for the lipoic acid derivative, which exhibit very low solubility. To specify their surfactant properties, critical micellar concentrations were determined using surface tension measurements. In agreement with the literature,[26,27] these perfluorinated surfactants exhibit very low critical micellar concentration (cmc) and very low surface tension at the cmc (γ_{cmc}). By comparing the cmc values of the two PBN derivatives **4** and **11**, we showed that the nature of these amino acid derivatives can modulate the physicochemical properties. The lysine derivative with its four-methylene side chain led to a lower cmc than the aspartic acid derivative. The nature of these antioxidants grafted on carriers was also of importance in determining their properties as surfactants.

The relative lipophilicities (log k'_W) of all these compounds were measured by a chromatographic technique we used in previous work.[15] All parent antioxidants and PBN were also included for the sake of comparison. The values obtained were in good agreement with data resulting from superficial tension measurements. The nature of these antioxidants has a significant influence on the physicochemical properties of amphiphilic compounds. For instance, the cmc and the lipophilicity of lysine derivatives were enhanced with increasing lipophilicity of the antioxidant agent (Table 1).

The radical scavenging ability was investigated by means of a scavenger competition assay based on the formation of the intensely green 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic

Table 2. Radical Scavenging and Reduction by the Antioxidant Agents As Determined by the ABTS Competition and Reduction Assay[a]

compd (1 μmol/mL)	quenched ABTS$^{\bullet+}$ (nmol/30 min)	reduced ABTS$^{\bullet+}$ (nmol/30 min)
PBN	18.2 ± 0.9	7.8 ± 0.3
4	83.4 ± 0.8	9.9 ± 0.5
11	85.4 ± 1.0	19.3 ± 0.7
Trolox	87.3 ± 1.1	93.4 ± 0.8
5	85.9 ± 1.2	86.1 ± 1.2
lipoic acid	−18.6 ± 0.9	44.1 ± 1.2
6	10.8 ± 0.7	87.9 ± 1.2
IPA	95.7 ± 0.7	96.5 ± 0.4
7	94.8 ± 0.9	92.7 ± 1.5

[a] Inhibition of hydroxyl radical initiated ABTS cation radical formation (quenched ABTS cation radicals in nmol over 30 min) and reduction of this organic radical (reduced ABTS cation radicals in nmol over 30 min) by different antioxidant agents. The findings are presented as the mean ± SEM ($N = 10$). All results are statistically significantly different from control (vehicle) at $p < 0.01$ (ANOVA followed by Bonferroni *t*-test). Lipoic acid is prooxidant (−18.6) in the ABTS competition assay as determined by enhanced ABTS cation radical formation.

acid) (ABTS) cation radical initiated by a Fenton reaction.[28] Radical scavengers suppressed the burstlike formation of ABTS cation radicals, which reached a stable plateau after 3 min of incubation. Measurements were made at 420 nm and monitored for 30 min in the absence and presence of 1 mM scavenging competitor dissolved in DMSO. Because of their very low cmc values, all the amphiphilic derivatives were dissolved in DMSO to prevent the formation of micellar aggregation at 1 mM. When their uniform distribution in the incubation medium was ensured, the use of DMSO allowed for a comparison of the scavenging potency of compounds with a different degree of hydro-, amphi-, and lipophilicity. Radical scavenging was indirectly determined by competition of the antioxidants with ABTS. Previous studies[28,29] have demonstrated that hydrophilic compounds have an advantage over lipophiphilic ones in protecting the highly polar ABTS molecule against oxidation by free radicals formed in the Fenton reaction in pure aqueous systems, if DMSO is not used as a solvent and intermediate.

Radical scavenging activity by parent antioxidants and by amphiphilic derivatives **4**−**7** and **11** was easily demonstrable by the ABTS competition assay and resulted in significant suppression of ABTS cation radical formation. All compounds showed considerable effects on ABTS oxidation (Table 2). As previously shown,[19a] IPA acted as a very potent hydroxyl radical scavenger and inhibited almost completely the formation of ABTS cation radicals. Trolox exhibited high scavenging activity but was less potent than IPA. In contrast, PBN was a hydroxyl radical scavenger of low potency and reduced only slightly the formation of ABTS$^{\bullet+}$. Lipoic acid even enhanced ABTS oxidation in this competition assay, indicating significant prooxidant activity. Such prooxidant activity in the presence of redox active iron has been previously demonstrated to be a potentially dangerous property of many other antioxidant agents of similar reactivity.[19b] Grafting the antioxidants on an amphiphilic perfluorinated amino acid derivative fully preserves the radical scavenging activity of the Trolox and the IPA analogue. The prooxidant activity of the parent compound lipoic acid is turned into a slight antioxidant effect by derivatization. Surprisingly, the amphiphilic PBN derivatives **4** and **11** acted as very effective radical scavengers and reduced the formation of ABTS$^{\bullet+}$ with a potency increased by more than 4-fold when compared to their parent compound PBN.

In the presence of excess DMSO, the chemistry of the ABTS competition assay is highly complex and possible reactions may involve secondary methyl and peroxyl radicals, other reactive intermediates formed upon oxidation of the scavengers, and oxygen and iron present in the incubation medium. Thus, oxygen consumption or iron chelation could contribute to a reduced ABTS cation radical formation and lead to artifactual results under the experimental conditions employed. Nonspecific and unselective reactions of the carrier structures with reactants present in the medium or formed during incubation are possible and are now investigated in detail.

Direct reduction of the organic ABTS cation radicals by the antioxidants was negligible under the current assay conditions. If iron or hydrogen peroxide were omitted from the incubation medium, no significant ABTS cation radical formation can be seen using the present incubation protocol, indicating that a Fenton reaction specifically initiated the oxidation of the chromogen. The burstlike formation of the ABTS cation radicals and the immediate inhibition of this ABTS oxidation by all compounds except lipoic acid also indicate that competition is the main principle of action exerted by the radical scavengers tested in this assay.

We also investigated the ability of antioxidants and amphiphilic derivatives to act as electron donors in an ABTS$^{\bullet+}$ reduction assay as previously described.[29] IPA and Trolox proved to be much better reductants than lipoic acid. As in the competition assay, PBN was the least efficient compound and led to a very poor reduction of the ABTS$^{\bullet+}$ cation radical. Amphiphilic antioxidant derivatives **5**−**7** showed high antioxidant activity, as demonstrated by their extensive reduction of cation radical. However, for PBN derivatives **4** and **11**, significant differences were observed between the inhibition and the reduction of ABTS$^{\bullet+}$, showing that there were potent hydroxyl scavengers but limited reductants and electron donors in the ABTS assay.

With the exception of IPA and Trolox, all amphiphilic derivatives were more potent radical scavengers and reducers than their parent compounds, as judged by the ABTS competition and reduction assays. The 4-fold increase in reactivity of the amphiphilic derivatives **4** and **11** toward reactive radicals compared to PBN and the change from pro- to antioxidant activity after derivatization of lipoic acid as also demonstrated in the ABTS competition assay may be due to direct scavenging by the carriers, unspecific effects of these groups, or their modulation of certain physicochemical properties of the active moieties. Since IPA and Trolox react at a diffusion-controlled rate with the hydroxyl radical and the highly reactive ABTS cation radical,[19a,b,28] it is not surprising that derivatization of the compounds using carriers cannot enhance their scavenging and electron-donating activity.

We are currently investigating the effects of the carriers and their possible interactions with the different active moieties in great detail. In biological compartments, highly reactive radicals can only be detoxified by scavengers that can provide on-site protection, since these short-lived intermediates would otherwise immediately react with other organic compounds to produce secondary radicals.[28] Since secondary and even tertiary radicals can mediate oxidative stress, damage, and death,[26] the superiority of the amphiphilic derivatives compared to their parent compounds in mediating antioxidant protection is very likely caused by their enhanced bioavailability and not by their enhanced reactivity as demonstrated in the competition and reduction assays.

Biological Results

Primary Cortical Mixed Cell Cultures. To determine the antioxidant properties of our amphiphilic derivatives, we

Table 3. Inhibition of Hydrogen Peroxide, Peroxynitrite, and Doxorubicin Induced Cell Death in Mixed Cortical Cultures[a]

compd (10 μM)	% inhibition		
	hydrogen peroxide (200 μM)	peroxynitrite (200 μM)	doxorubicin (200 μM)
PBN	25.0 ± 0.9	20.6 ± 0.7	24.2 ± 0.8
4	52.6 ± 1.2	42.2 ± 1.2	64.5 ± 0.7
11	54.6 ± 1.3	53.7 ± 1.5	73.2 ± 1.0
Trolox	22.2 ± 0.7	16.8 ± 0.6	15.5 ± 0.5
5	25.7 ± 0.7	25.9 ± 0.8	23.8 ± 0.8
lipoic acid	15.1 ± 0.5	13.1 ± 0.5	14.8 ± 0.4
6	28.6 ± 0.7	23.4 ± 0.7	32.4 ± 0.8
IPA	87.7 ± 1.1	83.8 ± 1.5	76.1 ± 0.9
7	47.6 ± 1.2	43.9 ± 1.4	61.6 ± 0.8

[a] Cultures were treated for 24 h with oxidotoxin at 200 μM. Antioxidants were added at 10 μM to evaluate their putative cytoprotective effects. Shown is the percentage inhibition of trypan blue absorbance indicating enhanced survival of the cells. The findings are presented as the mean ± SEM (N = 10). All results were statistically significantly different from control (vehicle) at $p < 0.01$ (ANOVA followed by Bonferroni t-test).

examined the prevention of cell death in three paradigms of lethal oxidative stress in primary cortical mixed cell cultures. There was no survival of neuronal cells in vehicle treated cultures after exposure to the lethal concentrations of hydrogen peroxide, peroxynitrite, and doxorubicin. Almost all cells were stained with trypan blue after exposure to these oxidotoxins, indicating that the oxidative stress was very severe. Under such conditions only very potent protective agents are able to rescue the cells and prevent their degeneration and death. Although all compounds exhibited significant neuroprotective activity against the toxicity of hydrogen peroxide, peroxynitrite, and doxorubicin, all other amphiphilic antioxidants, with exception of the IPA derivative, were significantly more effective than their corresponding parent compounds (Table 3) in enhancing neuronal survival. The fact that derivatization of IPA reduced its protective potency was surprising, since this single exception contrasted with the observation that all other amphiphilic antioxidants were superior compared to their parent compounds. Interestingly, the amphiphilic nitrone derivatives were more than twice as potent as their parent compound PBN in protecting the neuronal cells against the toxicity of hydrogen peroxide, peroxynitrite, and doxorubicin. All amphiphilic antioxidants demonstrated highly significant protective effects against the mitochondria-selective oxidotoxin doxorubicin. These findings are very promising and may be of great importance indicating a potential advantage and superiority of such novel compounds in neuroprotection,[14,15] because mitochondria are considered to be the primary target and origin of free radical mediated oxidative stress.[30] Since the lethal toxicity of all oxidotoxins at the very high concentrations used resulted in a complete loss of viable cells in nonprotected cultures, even the least potent compounds have a significant neuroprotective activity. A similar degree of neuroprotection against the lethal toxicity of such oxidotoxins has been only demonstrated after pretreatment with certain indole and nitrone antioxidants.[19b,30] When lower concentrations of the toxins were used, even conventional antioxidant agents such as Trolox can exert profound neuroprotective activity. These observations are in good agreement with previous findings of others.[21] We used the unusually high concentrations of the toxins to demonstrate the differences in potency of protection. The extremely high potency of the compounds is also demonstrated by the fact that all agents exhibited significant neuroprotection at only 10 μM, antagonizing cell death induced by oxidative stress at a 20-fold lower concentration than that used for employing the toxins. At the low micromolar concentration used, none of the compounds themselves exhibited any toxicity, with the indole and nitrone agents even showing some neurotrophic activity. The findings presented herein confirm the extremely high efficacy of the amphiphilic antioxidants in enhancing cellular survival and the superiority of these new derivatives compared to their parent compounds.

Table 4. Hydrogen Peroxide and Doxorubicin Toxicity and Antioxidant Protection: Percentage of Viable Rotifers after Treatment with Antioxidant Agents[a]

compd (10 μM)	% viable rotifers	
	hydrogen peroxide (200 μM)	doxorubicin (200 μM)
control	11.9 ± 0.4	15.0 ± 0.6
PBN	25.6 ± 0.8	18.7 ± 0.5[c]
4	66.0 ± 1.0	69.0 ± 1.0
11	76.6 ± 1.1	80.7 ± 0.9
Trolox	13.0 ± 0.5[b]	13.1 ± 0.5[c]
5	18.0 ± 0.6	26.8 ± 0.9[c]
lipoic acid	14.1 ± 0.5[b]	13.3 ± 0.5[c]
6	23.2 ± 0.6	24.1 ± 0.8
IPA	67.9 ± 1.0	53.8 ± 0.7
7	60.3 ± 1.1	41.1 ± 1.2

[a] Rotifers were treated for 24 h with oxidotoxin at 200 μM. Antioxidants were added at 10 μM to evaluate their putative protective effects in vivo. Shown is the percentage of viable organisms after exposure of the aquatic animals to the oxidotoxins hydrogen peroxide and doxorubicin. The findings are presented as the mean ± SEM (N = 10). Results were statistically significantly different from control (vehicle) at $p < 0.01$ (ANOVA followed by Bonferroni t-test) unless otherwise indicated. [b] Statistically nonsignificant versus hydrogen peroxide with vehicle. [c] Statistically nonsignificant versus doxorubicin with vehicle.

Rotifer Cultures. The in vitro experiments on the neuronal cells were repeated in in vivo experiments using a well-established animal model for the large-scale analysis of the protective effects of antioxidant drugs on whole organisms of a defined and uniform age.[30] Again, all amphiphilic derivatives showed protective effects, increasing rotifer survival in both experimental models of lethal oxidative stress: the nonselective toxin hydrogen peroxide and the mitochondria-selective toxin doxorubicin (Table 4). The most pronounced effects were observed with the nitrone derivatives and the amphiphilic indole compound. Since IPA itself is a very potent protective compound in vivo, it is very important to note that the amphiphilic derivatives almost matched its efficacy. The degree of protection against the lethal toxicity of hydrogen peroxide and doxorubicin by both indole compounds is indeed very remarkable. In this context, it is important to note that the amphiphilic nitrones **4** and **11** protected more efficiently against the mitochondrial oxidotoxin doxorubicin than IPA and its derivative **7**.

The high concentrations of hydrogen peroxide and doxorubicin killed almost all test animals treated with vehicle, and so even a survival rate of 20% or less may reflect a significant protective effect. Thus, even PBN itself exhibited some antioxidant activity in these animal models of severe oxidative stress. It is important to note that the amphiphilic nitrone derivatives were 3−4 times more active in promoting survival than the parent compound. The best outcome with almost complete protection was achieved after treatment with doxorubicin, clearly demonstrating the superiority of the new amphiphilic derivatives. Moreover, amphiphilic PBN **4** was slightly more potent than the IPA derivative **7** in protecting the organisms against the toxicity of hydrogen peroxide and doxorubicin, whereas amphiphilic PBN **11** was much more effective when compared to the indole compound. The extremely high potency of these amphiphilic PBN derivatives in vivo confirms previous findings using such agents in vitro and in vivo.[14,15,30] Our observations indicate that enhanced bioavail-

ability is a key feature in determining the antioxidant activity in cells and organisms. All compounds were well tolerated when used at the low concentration of only 10 μM. At a 10-fold higher concentration for all compounds tested in this study, only Trolox and lipoid acid showed some minor toxicity at longer incubation periods but not if the animals were exposed for only 24 h to both antioxidants as in these experiments. These findings indicate that amphiphilic antioxidants have a great potential as protective agents in preventing death of organisms exposed to enhanced oxidative stress and damage. The development of such agents may lead to more effective treatments of diseases associated with free radical mediated pathologies.

Conclusion

We synthesized new glycolipidic amphiphilic antioxidant derivatives possessing a lactobionic polar head, a perfluorinated hydrophobic tail, and an amino acid as scaffold upon which antioxidants (i.e., PBN, Trolox, lipoic acid, and indole-3-propionic acid) were grafted. Our results confirm and extend previous findings demonstrating that the amphiphilic character of antioxidant drugs is a very important parameter determining the biological efficacy of these compounds. The best results were obtained with the nitrone derivatives, but our findings indicate that the protective activity of other antioxidants can also be greatly improved. The most pronounced improvements by derivatization were observed after challenge with lethal concentrations of the mitochondria-selective oxidotoxin doxorubicin. A certain amphiphilicity is a necessity to ensure on-site protection in this intracellular compartment against oxidative stress. The design, synthesis, and development of mitochondria-targeted amphiphilic antioxidant agents is very promising and may lead to more safe and effective treatments of diseases associated with enhanced oxidative stress and damage. Amphiphilic antioxidants have a great potential in preventing death of cells and organisms exposed to increased levels of ROS by targeting the mitochondria, which are their primary source.

Experimental Section

Synthesis. All starting materials were purchased from Sigma-Aldrich except for the amino acids, which were purchased from Iris Biotech, and 1*H*,1*H*,2*H*,2*H*-pefluorooctyliodide, which were from Elf Atochem. All solvents were distilled and dried according to standard procedures. TLC analyses were performed on 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 heating at 150 °C. Flash chromatography experiments were carried out on Merck silica gel Geduran Si 60 (0.063−0.200 mm), and size exclusion chromatography experiments were carried out on Sephadex LH 20 (Amersham Biosciences). Optical rotations were determined with a Perkin-Elmer MC241 polarimeter. Mass spectra were recorded on a DX 300 JEOL apparatus for FAB+ experiments and on a Triple quadripolar spectrometer API III Plus Sciex for ESI+ experiments. Melting points were measured on an Electrothermal IA9100 apparatus and have not been corrected. HPLC analyses were performed on a Varian Microsorb C18 column (5 μm, 4.6 mm × 250 mm i.d.). UV spectra were recorded on a Shimadzu A160 apparatus. The ^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded on a Brüker AC-250 spectrometer. Chemical shifts are given in ppm relative to tetramethylsilane using the deuterium signal of the solvent ($CDCl_3$) as a heteronuclear reference for ^{1}H and ^{13}C. For the ^{19}F NMR spectra, the internal reference is $CFCl_3$. Abbreviations used for signal patterns are as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet.

***N*-(*tert*-Butyloxycarbonyl)-N^{ϵ}-(benzyloxycarbonyl)-L-lysinyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (1).** Sodium azide (10.4 g, 16 × 10^{-2} mol) was added to a solution of 1*H*,1*H*,2*H*,2*H*-perfluorooctyl iodide (13 g, 2.7 × 10^{-2} mol) in DMF. After 72 h of being stirred at room temperature, the mixture was poured into cold water and extracted with Et_2O (3×). The organic layer was washed with brine (2×), dried over Na_2SO_4, filtered, and concentrated in vacuo. The resulting yellow oil was dissolved in Et_2O with 0.31 g of 10% Pd/C and submitted to a hydrogen atmosphere for 12 h (7 bar). Filtration of the catalyst through a pad of Celite and evaporation of the solvent gave 1*H*,1*H*,2*H*,2*H*-perfluorooctylamine (8.1 g, 2.2 × 10^{-2} mol, 80% yield). A mixture of amine (8.1 g, 2.2 × 10^{-2} mol), BocLys(Z)OH (8.8 g, 2.3 × 10^{-2} mol), DCC (5.4 g, 2.6 × 10^{-2} mol), and HOBt in dry CH_2Cl_2 was stirred for 24 h at room temperature and then concentrated in vacuo. Purification by flash chromatography, eluting with EtOAc/cyclohexane (5/5 v/v) followed by crystallization from EtOAc/*n*-heptane, gave compound **1** (12.27 g, 1.7 × 10^{-2} mol, 77% yield). R_f = 0.36 in EtOAc. Mp 89 °C (dec). $[\alpha]^D_{20}$ −9.4° (*c* l, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.36 (5H, s), 6.80 (1H, m), 5.27 (1H, d), 5.11 (2H, s), 4.97 (1H, t), 4.06 (1H, m), 3.59 (2H, q, J = 6.5 Hz), 3.20 (2H, q, J = 6.5 Hz), 2.35 (2H, m), 1.89−1.29 (6H, m), 1.44 (9H, s). ^{19}F NMR ($CDCl_3$, 235 MHz) δ −80.7 (3F, CF_3), −114.0 (2F, CF_2), −121.8, −122.8, −123.5 (6F, 3CF_2), −126.1 (2F, CF_2). ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 172.5, 156.7 (CO), 136.5 (C), 128.5, 128.1 (CH), 80.3 (C), 66.7 (CH_2), 54.3 (CH), 31.8, 31.4, 30.6, 29.5 (CH_2), 28.2 (CH_3), 22.4 (CH_2).

***N*-(2,3,4,6,2′,3′,4′,6′-*O*-Acetyllactobionyl)-N^{ϵ}-(benzyloxycarbonyl)-L-lysinyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (2).** At 0 °C, compound **1** (4 g, 5.5 × 10^{-3} mol) was dissolved in a TFA/CH_2Cl_2 (3/7 v/v) mixture. After 4 h, the solution was concentrated in vacuo to give the corresponding amino derivative as a yellow oil. Lactobionolactone, prepared according to a published procedure,[24] was added to a solution of amino compound in 2-methoxyethanol with TEA (pH 8−9). The mixture was stirred at 65 °C under argon for 24 h until complete consumption of the amino derivative. Then the solvent was evaporated in vacuo and the residue was added to a solution of Ac_2O/pyridine (40 mL, 1/1 v/v) at 0 °C. After 12 h, the mixture was poured into cold 1 N HCl and extracted three times with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification by flash chromatography, eluting with EtOAc/cyclohexane (4/6 v/v), gave compound **2** (4.65 g, 3.57 × 10^{-3} mol, 65% yield) as a white foam. R_f = 0.25 in EtOAc/cyclohexane (6/4 v/v). Mp 56 °C (dec). $[\alpha]^D_{20}$ +2.7° (*c* l, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.36 (5H, s), 6.66 (1H, m), 6.58 (1H, m), 5.51 (1H, m), 5.39 (1H, m), 5.33 (1H, m), 5.11−5.03 (5H, m), 4.60 (1H, d), 4.58 (1H, m), 4.22−3.90 (6H, m), 3.57 (2H, q), 3.18 (2H, m), 2.35 (2H, m), 2.07−1.40 (6H, m), 2.24−2.04 (24H, m). ^{19}F NMR ($CDCl_3$, 235 MHz) δ −80.7 (3F, CF_3), −114.1 (2F, CF_2), −121.8, −122.8, −123.5 (6F, 3CF_2), −126.1 (2F, CF_2). ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 171.1, 170.6, 170.1, 169.8, 169.3, 168.0, 156.7 (CO), 136.7 (C), 128.5, 128.1, 128.0, 101.6, 77.9, 72.6, 71.2, 70.9, 69.9, 69.4, 68.9, 66.9 (CH), 66.6, 61.6, 61.0 (CH_2), 52.7 (CH), 40.3, 32.0, 30.9, 30.5, 29.1, 22.2 (CH_2), 20.6 (CH_3). MS (FAB+, *m*/*z*) 1302 [$(M + H)^+$, 5%], 1324 [$(M + Na)^+$, 14%].

***N*-*tert*-Butyl-α-4-(2,5-dioxopyrrolidin-1-ylphenylester)]nitrone (3).** A solution of 4-formylbenzoic acid (0.41 g, 4.02 × 10^{-3} mol) and *N*-*tert*-butylhydroxylamine (0.6 g, 4.02 × 10^{-3} mol) was stirred at 65 °C in ethanol under argon and in the dark for 48 h. Every 12 h, 0.1 equiv of hydroxylamine was added to complete the reaction. The mixture was concentrated in vacuo, purified by flash chromatography eluting with EtOAc/cyclohexane (8/2 v/v), and crystallized from MeOH/Et_2O to afford *N*-*tert*-butyl-α-(4-carboxyphenyl)nitrone (0.38 g, 1.72 × 10^{-3} mol, 43% yield) as a white powder. The nitrone was added to a solution of NHS (0.237 g, 2.06 × 10^{-3} mol) and DCC (0.425 g, 2.06 × 10^{-3} mol) in dry THF. After 12 h, the solvent was removed in vacuo and the mixture was purified by flash chromatography, eluting with EtOAc/cyclohexane (5/5 v/v) to give **3** (0.40 g, 1.26 × 10^{-3} mol, 93% yield) as a white powder. R_f = 0.72 in EtOAc/MeOH (9/1 v/v). Mp 177 °C (dec). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 8.41 (2H, d, J = 8.5 Hz), 8.17 (2H, d, J = 8.5 Hz), 7.68 (1H, s), 2.93 (4H, s), 1.63

(9H, s). ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 169.3, 161.3 (CO), 136.8, 130.7, 128.5 (CH), 128.3, 125.4, 72.1 (C), 28.4 (CH_3), 25.7, 25.0 (CH_2).

***N*-Lactobionyl-*N*$^{\epsilon}$-(*N*-*tert*-butyl-α-carboxyphenylnitrone)-L-lysinyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (4).** At 0 °C, compound **2** (1.24 g, 9.51×10^{-4} mol) was dissolved in ethanol/acetic acid (99/1 v/v) and 0.060 g of 10% Pd/C was slowly added. The reaction mixture was submitted to a hydrogen atmosphere for 12 h (7 bar). After filtration of the catalyst through a pad of Celite and evaporation of the solvent in vacuo, the resulting amino compound was added to a solution of **3** (0.030 g, 9.4×10^{-4} mol) in dry CH_2Cl_2 with TEA (pH 8−9) at room temperature under argon. After 24 h, the solvent was removed in vacuo and the crude mixture was purified by flash chromatography eluting with EtOAc/cyclohexane (4/6 v/v) and by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1/1 v/v) to give the acetylated derivative **4a** (0.800 g, 5.84×10^{-4} mol, 69% yield) as a white foam. A catalytic amount of sodium methoxide was added under argon to a solution of compound **4a** in MeOH. The mixture was stirred for 4 h, and 1 N HCl was added dropwise to neutralize the solution. Purification by size exclusion chromatography eluting with MeOH and precipitation in Et_2O afforded **4** (0.544 g, 5.25×10^{-4} mol, 90% yield) as a white powder. $R_f = 0.49$ in EtOAc/MeOH/H_2O (7/2/1 v/v). Mp 124 °C (dec). $[\alpha]^D_{20}$ +8.4° (*c* l, DMSO). UV λ_{max}: 300 nm. ^{1}H NMR (CD_3OD, 250 MHz) δ 8.40 (2H, d, $J = 8.6$ Hz), 7.98 (1H, s), 7.88 (2H, d, $J = 8.6$ Hz), 4.48 (1H, d, $J = 7.3$ Hz), 4.39 (2H, m), 4.20 (1H, m), 3.94−3.69 (7H, m), 3.57−3.39 (7H, m), 2.41 (2H, m), 2.10−1.67 (4H, m), 1.61 (9H, s), 1.47 (2H, m). ^{19}F NMR (CD_3OD, 235 MHz) δ −80.2 (3F, CF_3), −113.4 (2F, CF_2), −121.7, −122.6, −123.2 (6F, $3CF_2$), −125.7 (2F, CF_2). ^{13}C NMR (CD_3OD, 62.86 MHz) δ 174.1, 172.8, 167.9 (CO), 135.9, 133.3 (C), 132.3, 129.2, 126.9, 104.4, 81.9, 75.9, 73.4, 72.8, 71.8 (CH), 71.4 (C), 71.3, 68.9 (CH), 62.4, 61.2 (CH_2), 53.0 (CH), 39.3, 31.4, 31.1, 29.9, 28.6 (CH_2), 27.0 (CH_3), 22.9 (CH_2). MS (ESI+, *m*/*z*) 1305 [$(M + H)^+$], 1052 [$(M + NH_4)^+$], 1057 [$(M + Na)^+$], 1073 [$(M + K)^+$]. Anal. ($C_{38}H_{51}F_{13}N_4O_{14}\cdot 1H_2O$) C, H, N.

***N*-Lactobionyl-*N*$^{\epsilon}$-(6-hydroxy-2,5,7,8-tetramethylchroman-2-oyl)-L-lysinyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (5).** The synthetic procedure was essentially the same as for compound **4**. Compound **2** was submitted to hydrogenolysis, and the resulting amino compound was added to Trolox (0.127 g, 5.07×10^{-4} mol), DCC (0.114 g, 5.53×10^{-4} mol), and a catalytic amount of HOBt in CH_2Cl_2 with DIEA (pH 8−9) at room temperature. After 24 h, the solvent was removed in vacuo and the mixture was purified by flash chromatography eluting with EtOAc/cyclohexane (4/6 v/v) and by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1/1 v/v) to give the acetylated derivative **5a** (0.260 g, 1.86×10^{-4} mol, 40%) as a white foam. After deacetylation, acid resin (IRC 50) was added to neutralize the solution followed by filtration. Purification by size exclusion chromatography eluting with MeOH and precipitation in Et_2O afforded **5** (0.162 g, 1.53×10^{-4} mol, 82%) as a white powder. $R_f = 0.61$ EtOAc/MeOH/H_2O (7/2/1 v/v). Mp 95 °C (dec). $[\alpha]^D_{20}$ +11.6° (*c* l, DMSO). UV λ_{max}: 289 nm. ^{1}H (CD_3OD, 250 MHz) δ 7.24 (1H, m), 4.52 (1H, d, $J = 7,5$ Hz), 4.50−4.20 (3H, m), 3.97−3.52 (12H, m), 3.05 (2H, m), 2.39 (5H, m), 2.18 (6H, s), 2.09 (3H, s, CH_3), 1.75 (1H, m), 1.51 (3H, s), 1.90−1.17 (6H, m). ^{19}F NMR (CD_3OD, 235 MHz) δ −80.1 (3F, CF_3), −113.4 (2F, CF_2), −121.7, −122.6, −123.2 (6F, $3CF_2$), −125.7 (2F, CF_2). ^{13}C NMR (DMSO-d_6) δ 173.6, 172.8, 172.0 (CO), 146.3, 144.4, 123.2, 121.6, 120.8, 117.5 (C), 105.3 (CH), 83.9 (C), 83.8, 77.6, 76.2, 73.7, 72.6, 71.8, 71.6, 68.6 (CH), 62.7, 61.0 (CH_2), 52.6 (CH), 38.7, 31.1, 30.7, 29.9, 29.3, 29.0 (CH_2), 24.4 (CH_3), 22.8, 20.5 (CH_2), 13.2, 12.5, 12.2 (CH_3). MS (ESI+, *m*/*z*) 1065 [$(M + H)^+$], 1082 [$(M + NH_4)^+$], 1087 [$(M + Na)^+$], 1103 [$(M + K)^+$]. Anal. ($C_{40}H_{54}F_{13}N_3O_{15}\cdot 1H_2O$) C, H, N.

***N*-Lactobionyl-*N*$^{\epsilon}$-(5-[1,2]-dithiolan-3-ylpentanoyl)-L-lysinyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (6).** The synthetic procedure was essentially the same as for compound **4**. Compound **2** was submitted to hydrogenolysis, and the resulting amino compound was added to *N*-hydroxysuccinimide activated lipoic acid (0.154 g, 5.07×10^{-4} mol) prepared following the same procedure as for compound **3**. The crude mixture was purified by flash chromatography eluting with EtOAc/cyclohexane (4/6 v/v) and by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1/1 v/v) to give the acetylated derivative **6a** (0.437 g, 3.22×10^{-4} mol, 70% yield) as a slightly yellow foam. After deacetylation, acid resin (IRC 50) was added to neutralize the solution followed by filtration. Purification by size exclusion chromatography eluting with MeOH and precipitation in Et_2O afforded **6** (0.262 g, 2.57×10^{-4} mol, 80%) as a slightly yellow powder. $R_f = 0.61$ EtOAc/MeOH/H_2O (7/2/1 v/v). Mp 106 °C (dec). $[\alpha]^D_{20}$ +14.2° (*c* l, DMSO). UV λ_{max}: 330 nm. ^{1}H NMR (CD_3OD, 250 MHz) δ 8.01 (1H, m), 4.52 (1H, d, $J = 7.5$ Hz), 4.49−4.20 (3H, m), 3.98−3.49 (13H, m), 3.18 (4H, m), 2.56 (4H, m), 2.21 (2H, t), 1.95 (2H, m), 1.90−1.44 (10H, m). ^{19}F NMR (DMSO-d_6, 235 MHz) δ −80,1 (3F, CF_3), −113.1 (2F, CF_2), −121.5, −122.4, −123.0 (6F, $3CF_2$), −125.5 (2F,CF_2). ^{13}C NMR (CD_3OD, 62.86 MHz) δ 174.6, 174.2, 172.8, (CO), 104.4, 81.8, 75.9, 73.4, 72.8, 71.8, 71.7, 71.4, 68.9 (CH), 62.3, 61.3 (CH_2), 56.2, 53.0 (CH), 39.9, 38.7, 38.0, 35.5, 34.4, 31.4, 31.0, 29.8, 28.6, 28.5, 25.4, 22.9 (CH_2). MS (ESI+, *m*/*z*) 1020 [$(M + H)^+$], 1037 [$(M + NH_4)^+$], 1042 [$(M + Na)^+$], 1058 [$(M + K)^+$]. Anal. ($C_{34}H_{50}F_{13}N_3O_{13}S_2\cdot 1H_2O$) C, H, N.

***N*-Lactobionyl-*N*$^{\epsilon}$-[3-(1*H*-indol-3-yl)propionyl)]-L-lysinyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (7).** The synthetic procedure was essentially the same as for compound **4**. Compound **2** was submitted to hydrogenolysis, and the resulting amino compound was added to *N*-hydroxysuccinimide activated indole-3-propionic acid (0.171 g, 6×10^{-4} mol) prepared following the same procedure as for compound **3**. The crude mixture was purified by flash chromatography eluting with EtOAc/cyclohexane (4/6 v/v) and by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1/1 v/v) to give the acetylated derivative **7a** (0.567 g, 4.23×10^{-4} mol, 78% yield) as a white foam. After deacetylation, acid resin (IRC 50) was added to neutralize the solution followed by filtration. Purification by size exclusion chromatography eluting with MeOH and precipitation in Et_2O afforded **7** (0.348 g, 3.47×10^{-4} mol, 82% yield) as a white powder. $R_f = 0.51$ EtOAc/MeOH/H_2O (7/2/1 v/v). Mp 137 °C (dec). $[\alpha]^D_{20}$ +11.8° (*c* l, DMSO). UV λ_{max}: 281 nm. ^{1}H NMR (CD_3OD, 250 MHz) δ 7.57 (1H, d, $J = 7.75$ Hz), 7.34 (1H, d, $J = 7.75$ Hz), 7.04 (3H, m), 4.52 (1H, d, $J = 7.5$ Hz), 4.49−4.20 (3H, m), 3.98−3.49 (12H, m), 3.13 (4H, m), 2.56 (2H, t, $J = 7.25$ Hz), 2.45 (2H, m), 1.90−1.20 (6H, m). ^{19}F NMR (DMSO-d_6, 235 MHz) δ −80.1 (3F, CF_3), −113.1 (2F, CF_2), −121.7, −122.6, −123.2 (6F, $3CF_2$), −125.7 (2F, CF_2). ^{13}C NMR (CD_3OD, 62.86 MHz) δ 173.0, 172.6, 171.4 (CO) 135.2, 125.6 (C), 120.2, 119.4, 116.6, 116.4 (CH), 112.0 (C), 109.3, 102.9, 80.4, 74.3, 71.9, 71.2, 70.2, 69.8, 67.4 (CH), 60.8, 59.7 (CH_2), 51.5 (CH), 37.1, 35.5, 29.9, 29.4, 28.3, 26.9, 21.2, 19.8 (CH_2). MS (ESI+, *m*/*z*) 1004 [$(M + H)^+$], 1021 [$(M + NH_4)^+$], 1025 [$(M + Na)^+$], 1042 [$(M + K)^+$]. Anal. ($C_{37}H_{47}F_{13}N_4O_{13}\cdot 1H_2O$) H, N. C: calcd, 43.53; found, 41.86.

***N*-(9-Fluorenylmethyloxycarbonyl)-β-(*tert*-butyloxycarbonyl)-L-aspartyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (8).** The synthetic procedure was essentially the same as for compound **1**. A mixture of 1*H*,1*H*,2*H*,2*H*-perfluorooctylamine (2 g, 5.5×10^{-3} mol), FmocAsp(O^{t}Bu)OH (2.4 g, 5.78×10^{-3} mol), DCC (1.36 g, 6.60×10^{-3} mol), and HOBt in dry CH_2Cl_2 was stirred for 24 h at room temperature and then concentrated in vacuo. Purification by flash chromatography, eluting with EtOAc/cyclohexane (1/9 v/v) followed by crystallization from EtOAc/*n*-heptane, gave compound **8** (2.9 g, 3.83×10^{-3} mol, 70% yield) as a white powder. $R_f = 0.68$ EtOAc/cyclohexane (4/6 v/v). Mp 123 °C (dec). $[\alpha]^D_{20}$ +11.4° (*c* l, CH_2Cl_2). ^{1}H NMR ($CDCl_3$) δ 7.79 (2H, d, $J = 7.25$ Hz), 7.60 (2H, d, $J = 7.25$ Hz), 7.46−7.29 (4H, m), 6.82 (1H, m), 5.97 (1H, d, $J = 7.75$ Hz), 4.48 (3H, m), 4.24 (1H, t, $J = 6.5$ Hz), 3.59 (2H, m), 2.99−2.57 (2H, m), 2.35 (2H, m), 1.47 (9H, s). ^{19}F NMR ($CDCl_3$, 235 MHz) δ −80.7 (3F, CF_3), −114.1 (2F, CF_2), −121.8, −122.8, −123,6 (6F, $3CF_2$), −126.1 (CF_2). ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 171.3, 170.8, 156.1 (CO), 143.5, 141.4 (C), 127.8, 127.1, 125.0, 124.9, 120.1 (CH), 82.1 (C), 67.2 (CH_2), 51.0, 47.2 (CH), 37.2, 32.1, 30.6 (CH_2), 28.0 (CH_3).

***N*-(2,3,4,6,2′,3′,4′,6′-*O*-Acetyllactobionyl)-*β*-(*tert*-butyloxycarbonyl)-L-aspartyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (9).** The synthetic procedure was essentially the same as for compound **2**. Compound **8** (1 g, 1.32×10^{-3} mol) was dissolved in a DEA/CH_3-CN mixture (1/9 v/v) at room temperature. After 1 h, the solvent was removed in vacuo, the resulting amino derivative was added to a solution of lactobionlactone (0.571 g, 1.58×10^{-3} mol) in 2-methoxyethanol with TEA, and the mixture was stirred at 65 °C under argon. After acetylation of the crude mixture and convenient workup, purification by flash chromatography eluting with EtOAc/cyclohexane (4/6 v/v) gave compound **9** (0.640 g, 5.3×10^{-4} mol, 40% yield) as a white foam. $R_f = 0.22$ EtOAc/cyclohexane (6/4 v/v). Mp 54 °C (dec). $[\alpha]^{D}_{20}$ +48.1° (*c* l, CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.58 (1H, d, $J = 8.5$ Hz), 7.00 (1H, t, $J = 5.25$ Hz), 5.45 (1H, m), 5.41 (2H, m), 5.15 (2H, m), 5.03 (1H, m), 4.68−4.58 (3H, m), 4.15−3.92 (5H, m), 3.57 (2H, m), 3.03 (1H, dd, $J =$ 6.5 Hz, 10.75 Hz), 2.37 (3H, m), 2.22−2.00 (24H, m), 1.46 (9H, s). ^{19}F NMR ($CDCl_3$, 235 MHz) δ −80.7 (3F, CF_3), −114.1 (2F, CF_2), −121.8, −122.8, −123.5 (6F, $3CF_2$), −126.1 (2F, CF_2). ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 172.1, 170.8, 170.5, 170.1, 169.8, 169.5, 169.2, 168.5 (CO), 101.6 (CH), 82.1 (C), 77.9, 72.1, 71.2, 70.9, 70.0, 69.8, 68.9, 66.9 (CH), 61.6, 61.0 (CH_2), 49.0 (CH), 35.5, 32.2, 30.2 (CH_2), 27.9, 20.6 (CH_3). MS (FAB+, *m*/*z*) 1211 [(M + H)$^+$], 1233 [(M + Na)$^+$].

***N*-(2,3,4,6,2′,3′,4′,6′-*O*-Acetyllactobionyl)-*β*-(4-amidomethylbenzaldehyde)-L-aspartyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (10).** At 0 °C, compound **9** (1.78 g, 1.47×10^{-3} mol) was dissolved in a TFA/CH_2Cl_2 (4/6 v/v) mixture. After 2 h, the solution was concentrated in vacuo. The acid derivative obtained (1.5 g, 1.30×10^{-3} mol, 88%) was added to a solution of BOP (0.69 g, 1.56×10^{-3} mol) and 4-[1,3]-dioxolan-2-ylbenzylamine[24] (0.25 g, 1.43×10^{-3} mol) in THF with TEA. The mixture was stirred for 12 h at room temperature, and the crude product was purified by column flash chromatography, eluting with EtOAc/cyclohexane (6/4 v/v). The white foam obtained (1.57 g, 1.19×10^{-3} mol, 92%) was then dissolved with a catalytic amount of apts in CH_3CHO cooled at 10 °C. After 12 h, the mixture was evaporated in vacuo, washed with $NaHCO_3$ and brine, and dried over Na_2SO_4. Concentration in vacuo gave **10** (1.25 g, 9.83×10^{-4} mol) as a white foam in 89% yield. $R_f = 0.42$ EtOAc. Mp 52 °C (dec). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 10.01 (1H, s), 8.05 (1H, d, $J = 7.25$ Hz), 7.86 (2H, d, $J = 8.25$ Hz), 7.56 (1H, t, $J = 7.75$ Hz), 7.45 (2H, d, $J = 8.25$ Hz), 6.73 (1H, t, $J = 7.75$ Hz), 5.51 (1H, m), 5.44 (1H, m), 5.37 (1H, m), 5.16−5.03 (2H, m), 4.65−4.51 (5H, m), 4.30−3.92 (6H, m), 3.55 (2H, m), 2.95 (1H, m), 2.42 (3H, m), 2.21−2.00 (24H, m). ^{19}F NMR ($CDCl_3$, 235 MHz) δ −80.7 (3F, CF_3), −114.1 (2F, CF_2), −121.9, −122.9, −123.6 (6F, $3CF_2$), −126.2 (2F, CF_2). ^{13}C NMR ($CDCl_3$, 62.86 MHz) δ 191.7, 171.4, 170.9, 170.7, 170.4, 170.1, 169.9, 169.8, 169.2, 168.6 (CO), 144.6, 135.7 (C), 130.1, 127.9, 101.6, 77.9, 72.6, 71.2, 70.9, 69.9, 69.4, 68.9 (CH), 61.6, 61.0 (CH_2), 49.6 (CH), 43.3, 36.3, 32.1, 30.5 (CH_2), 20.7 (CH_3).

***N*-Lactobionyl-*β*-[*N*-*tert*-butyl-4-amidomethylphenylnitrone)-L-aspartyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (11).** A solution of **10** (0.262 g, 2.06×10^{-4} mol) and *N*-(*tert*-butyl)hydroxylamine acetate (0.03 g, 2.06×10^{-4} mol) in THF/AcOH (3/2 v/v) was stirred 48 h at 60 °C under argon and in the dark. Every 12 h, 0.2 equiv of hydroxylamine was added to complete the reaction. The mixture was then concentrated in vacuo and purified by flash chromatography eluting with EtOAc/cyclohexane (9/1 v/v) followed by size exclusion chromatography eluting with CH_2Cl_2/MeOH (1/1 v/v) to give the acetylated derivative **11a** (0.152 g, 1.13×10^{-4} mol, 55% yield) as a white foam. After deacetylation, 1 N HCl was added dropwise to neutralize the solution. Purification by size exclusion chromatography eluting with MeOH and precipitation in Et_2O afforded **11** (0.091 g, 9.04×10^{-5} mol, 80%) as a white powder. $R_f = 0.57$ EtOAc/MeOH/H_2O (7/2/1 v/v). Mp 157 °C (dec). $[\alpha]^{D}_{20}$ +22.7° (*c* l, MeOH). UV λ_{max}: 296 nm. ^{1}H NMR (CD_3OD, 250 MHz) δ 8.29 (2H, d, $J = 8.25$ Hz), 7.88 (1H, s), 7.39 (2H, d, $J = 8.25$ Hz), 4.52 (1H, d, $J = 7.5$ Hz), 4.49−4.20 (3H, m), 3.98−3.49 (12H, m), 3.18 (2H, m), 2.80 (2H, m), 2.56 (2H, m), 1.61 (9H, s). ^{19}F NMR (CD_3OD, 235 MHz) δ −82.3 (3F, CF_3), −115.0 (2F, CF_2), −122.6, −123.6, −124.3 (6F, $3CF_2$), −127.1 (2F, CF_2). ^{13}C NMR (CD_3OD) δ 174.0, 171.9, 171.2 (CO), 141.9 (C), 133.5, 129.7(CH), 129.4 (C), 127.0, 104.4 (CH), 81.9 (C), 75.9, 73.4, 72.8, 72.3, 72.1, 71.8 (CH), 71.4 (C), 68.9 (CH), 62.3, 61.2 (CH_2), 49.8 (CH), 42.4, 36.0, 31.5, 29.8 (CH_2), 27.0 (CH_3). MS (ESI+, *m*/*z*) 1007 [(M + H)$^+$], 1024 [(M + NH_4)$^+$], 1029 [(M + Na)$^+$], 1045 [(M + K)$^+$]. Anal. ($C_{36}H_{47}F_{13}N_4O_{14}\cdot 1H_2O$) C, H, N.

Determination of log k'_W Values. Compounds were dissolved in MeOH at 1.0 mg/mL, injected onto a Microsorb C18 column (250 mm × 4.6 mm, 5 μm), and eluted with various mixtures of MeOH and water (9:1 to 6:4 (v/v), 3 concentrations/compound). The flow rate was 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 ($r^2 > 0.999$) was performed on the three data points for each nitrone, and the resulting line was extrapolated to 100% aqueous to give the log k'_W values listed in Table 1.

Surface Tension Measurement. All the surfactant 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 surfactant solutions were measured with a Krüss K12ST tensiometer controlled by the Krüss Labdesk software (Krüss, Germany) by the Wilhelmy plate technique. The critical micellar concentration (cmc) and the surface tension (γ_{cmc}) were determined from the break point of the surface tension and logarithm of the concentration curve. The occupied area per molecule at the cmc, A_{cmc}, was determined by the equation $A_{cmc} = 1/(N\Gamma_{cmc})$, where N is the Avogadro number and Γ is the adsorption amount of surfactant calculated by the Gibbs adsorption isotherm equation.

Radical Scavenging. Radical scavenging was investigated using a scavenger competition assay based on the burstlike formation of the intensely green 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) ABTS cation radical initiated by a Fenton reaction.[28] Measurements were made at 420 nm, and the mixture was monitored for a period of 30 min in the absence and presence of scavenging competitor at 1 mM dissolved in DMSO. The reaction system consisted of 0.9 mL of distilled water, adjusted to pH 5.0, 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. Scavenging was determined by quenching ABTS cation radical formation due to the trapping of the secondary radicals formed in the presence of DMSO.

Radical Reduction. ABTS cation radical reduction was assayed according to Re et al.[31] with the following modifications:[29] assay concentration of the ABTS cation radical was 105 μM; final concentration of the test compounds was 0.5 mM. The reaction was followed for 30 min. Controls received DMSO vehicle only, reaching a final concentration of 2.5% in the assay. Measurements were started with the addition of the test agent, and reduction of the ABTS cation radical was monitored every minute at 420 nm.

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.[21] The cultures were exposed to toxins and antioxidant agents for 24 h. Cell death was quantified by the trypan blue absorbance assay.[32] The absorbance of the dye was measured spectrophotometrically at 590 nm. The percentage of inhibition of trypan blue absorbance is a highly reproducible and reliable method to determine prevention of cell death and to evaluate cytoprotection by antioxidant agents.

Rotifers. The animal model using rotifers allowed for a fast large-scale analysis of the protective effects of antioxidant drugs tested on organisms of defined age and uniform character. Survival

of rotifers was investigated in standardized viability assays as described previously.[30] Experiments on the controls receiving vehicle only resulted in very low survival and were carried out in parallel on animals of the same stock culture to ensure absolutely identical exposure conditions for all animals.

Acknowledgment. Stéphanie Ortial received a grant from the research council of "Région Provence Alpes Côte d'Azur" and from the company TS Pharma. Burkhard Poeggeler was supported by a grant of the German Research Foundation ("Deutsche Forschungsgemeinschaft—DFG").

Supporting Information Available: Chemical data of compounds **4a**−**7a** and **11a**; ^{1}H, ^{19}F, ^{13}C, DEPT 135, DEPT 90, and DEPT 45 NMR spectra of acetylated compound **4a**; ^{1}H, ^{19}F, and ^{13}C NMR spectra and analytical RP-HPLC chromatogram of deacetylated compound **4**; analytical data for the final compounds **4**−**7** and **11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM060027E