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Synthesis of a New Family of Glycolipidic Nitrones as Potential Antioxidant Drugs for Neurodegenerative Disorders

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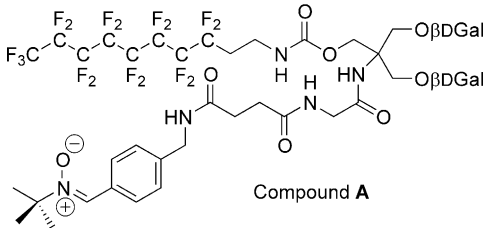
Abstract—This paper deals with the synthesis of a novel series of amphiphilic glycosylated spin-traps derived from α-Phenyl-*N*-*tert*-butyl nitrone (PBN) and an initial characterization of their anti-caspase-3 activity. Preliminary investigation of their anti-apoptosis effect showed they dramatically inhibit the activity of caspase-3 in cultured neuronal cells following induction of apoptosis by hydrogen peroxide.

Oxygen free radicals are produced in numerous and varied pathological states such Alzheimer's disease,[1] chronic neurodegenerative disorders,[2] ischemia-reperfusion or stroke.[3] Agents that can trap these free radicals to form more stable adducts may be useful as therapeutic agents. α-Phenyl-*N*-*tert*-butyl nitrone (PBN) has been used extensively for decades in electron spin resonance spectroscopy as an efficient spin-trap. More recently, it has been shown that PBN can minimize oxidative damage in various biological processes[4] and disease models[5] in which free radical species have been implicated. For example, PBN has been used to protect against a variety of agents that promote the formation of brain lesions by disrupting mitochondrial energetics.[6,7]

With the ultimate goal of improving its bio-availability, several studies have focussed on the measurement of the hydrophilic-lipophilic balance (HLB) of PBN, subsequent to mainly small modifications of either the aromatic or N-terminal portion of PBN.[8,9] These studies showed that amphipathic spin-traps exhibit better membrane-crossing ability than PBN itself and may provide protection against free radicals in both the cytosol and in cell membranes.[10]

In this context, a previous work described the synthesis of a new compound, glycosylated amphiphilic nitrone PBN (Compound **A**).[11] We showed that this compound exhibits the same trapping activity as PBN (using the RPE technique) and is efficiently recognized by galactose-specific yeast lectins.



Compound **A**

Moreover, it appears that the amphiphilic character of compound A enhances its membrane-crossing ability.[12] Whereas PBN shows no protective activity, this compound readily diminishes SOD production and apoptosis in cultured skin fibroblasts with an isolated complex V deficiency of the respiratory chain (NARP mutation). However, such a structure was relatively complex and not easy to prepare in large quantities. To simplify the preparation of these amphiphilic compounds, while maintaining their spin-trap properties, we chose to graft a hydrophobic moiety onto the *t*-butyl group of PBN and a hydrophilic head onto its aromatic group.

Here we report a new, efficient and convergent synthetic route to amphiphilic PBN analogues. To provide a

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molecule with significant hydrophilicity, a lactobionamide group was grafted onto the aromatic portion of PBN. The hydrophobic moiety was an alkyl or perfluoroalkyl chain linked to the *tert*-butyl group through a thioether, a carbamate or an amide bond. In order to maintain good water solubility of the nitrone analogues, we chose to graft only short hydrocarbon chains of 6 to 8 carbon atoms. Furthermore, in order to minimize the detergent properties (and thus the cytotoxicity), essentially due to the hydrocarbon chain of these substrates, while maintaining their amphiphilic properties, we also used a perfluorocarbon chain as the hydrophobic moiety. In this new amphiphilic PBN family, the nitronyl function is thus located at the heart of the surfactant, between the hydrophobic tail and the polar head. Such a structure allows us to modulate either the nature and the length of the lipophilic portion or the hydrophilic head easily and to determine the impact of the HLB of these traps on their biological activity.

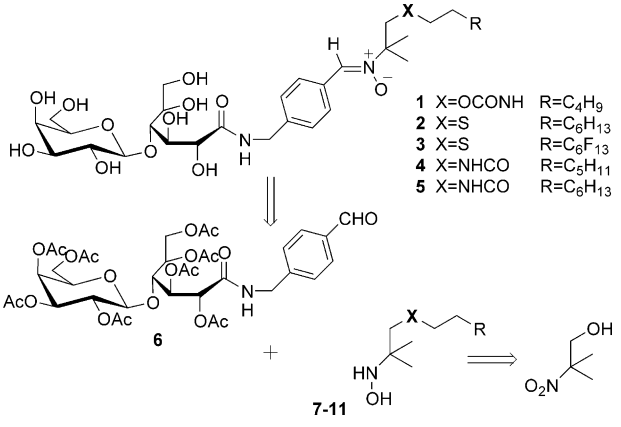
The retrosynthetic strategy (Scheme 1) is based on two key steps: (i) the synthesis of substituted long chain *N*-*tert*-butyl hydroxylamines from 2-methyl-2-nitropropanol and (ii) the condensation of these hydroxylamines on *N*-4-formyl benzyl lactobionamide **6** to obtain nitronyl function. The synthesis of this synthon **6** has been previously described.[13]

The five steps leading to **6** were achieved with a 26% overall yield using the 4-cyanobenzaldehyde pathway. Hydrophobic chains were grafted onto the 2-methyl-2-nitropropanol through three different bonds: carbamate, amide or thioether bonds. First, the carbamate derivative **14** was obtained in quantitative yield by reaction of hexyl isocyanate on 2-methyl-2-nitropropanol, in the presence of diazabicyclooctane (DABCO) as a catalyst, in refluxing toluene under an atmosphere of argon (Scheme 2). To obtain the thioether compounds **15**–**16**, a tosylation reaction of the hydroxyl function of 2-methyl-2-nitropropanol was first carried out by treatment with tosyl chloride and pyridine in dry CH_2Cl_2. Compound **12** was isolated with a yield of 83% after recrystallization in AcOEt/hexane. Substitution of the tosyl group in dry DMF by the hydro or perfluorocarbon thiol led to thioether derivatives **15**–**16**. A partial degradation of the perfluorocarbon thiol in the strong basic conditions used for this reaction (sodium methylate) could explain the difference in yield observed between the hydrocarbon (97%) and the perfluorocarbon (58%) series.

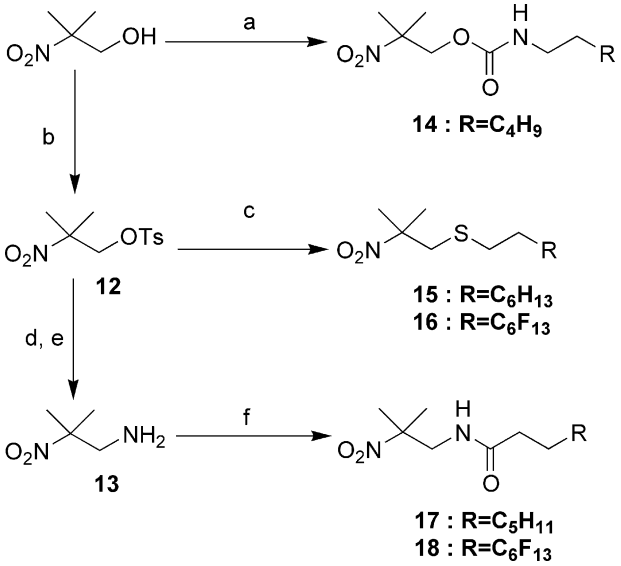
To prepare the amide derivatives, using the Vaultier et al. synthetic pathway,[14] **12** was first converted into the amine compound **13** by the Staudinger reaction. It is noteworthy that the substitution kinetics of the tosyl groups by sodium azide was increased by ultrasonic activation. Condensation of octanoyl chloride at −20 °C with **13**, in a mixture of CH_2Cl_2 and triethylamine (TEA) (3 equiv), led to the hydrocarbon amide derivative **17** with a yield of 77%. The perfluorocarbon analogue **18** was obtained in good yield (90%) by reacting **13** in dry CH_2Cl_2 with the corresponding perfluorocarbon carboxylic acid in the presence of dicyclohexylcarbodiimide(DCC)/hydroxybenzotriazole (HOBt) as a catalyst.

Reduction of the nitro group of compounds **14**–**17** under mild conditions, using Kende and Mendoza's method,[15] led to the hydroxylamines **7**–**11** in good yield (Scheme 3, Table 1).

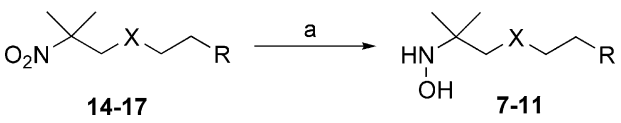
Nitrones were finally obtained following our well-established procedure by condensation of the sub-



Scheme 1. Retrosynthetic strategy.



Scheme 2. (a) 1.3 Equiv hexyl isocyanate, toluene, DABCO, reflux, 3 h, 100%; (b) 1.2 equiv TsCl, pyridine, CH_2Cl_2, 48 h, 83%; (c) 2 equiv octanethiol, *t*BuOK, DMF, 50 °C, 4 h, 97% for **15** or 2 equiv 1H, 1H, 2H, 2H perfluorooctanethiol, MeONa, same conditions, 58% for **16**; (d) NaN_3, DMF, ultrasounds, 30 min, 85%; (e) PPh_3, THF, 2 h, then NaOH 2N, 24 h, 82%; (f) octanoyl chloride, TEA, DMAP, −15 °C, 2 h, 77% for **17** or 2H, 2H, 3H, 3H perfluorononanoic acid, DCC/HOBt, 36 h, 90% for **18**.



Scheme 3. Controlled reduction of nitroalkanes to alkyl hydroxylamines (a) 4 equiv SmI_2, THF/MeOH, 15 min.

Table 1. Reduction yields of nitro compounds

Hydroxylamines	X–CH_2–CH_2–R	Yields (%)[a]
7	O–CO–NH–C_6H_{13}	73 (100)[b]
8	S–C_8H_{17}	70 (88)[b]
9	S–CH_2–CH_2–C_6F_{13}	85 (98)[b]
10	NH–CO–C_7H_{15}	100
11	NH–CO–CH_2–CH_2–C_6F_{13}	50 (70)[b]

[a]After purification by flash chromatography.
[b]Non-reduced nitro compounds were recovered and re-used to complete the reaction.

Table 2. Physico-chemical data of nitrones **1–5**

Compd	X-CH_2-CH_2-R	Yield %[a]	CMC[c]	γ[d]
1	O–CO–NH–C_6H_{13}	65	No CMC	—
2	S–C_8H_{17}	68	0.41	30
3	S–CH_2–CH_2–C_6F_{13}	56	0.035	20
4	NH–CO–C_7H_{15}	63	No CMC	—
5	NH–CO–CH_2–CH_2–C_6F_{13}	44 (70)[b]	0.228	20

[a]Coupling reaction yields, measured after purification on silica gel and on Sephadex resin.
[b]Calculated with the residual starting benzaldehyde.
[c]CMC = critical micellar concentration, in mM.
[d]γ = limit surface tension, in mN/m.

stituted *N-tert*-butyl hydroxylamine on compound **6** in dry THF (Scheme 4). In order to avoid the possible oxidation of hydroxylamine via a free radical process, the reaction was carried out in the dark under argon atmosphere and small amounts of hydroxylamine were added to the mixture every three days. The coupling reaction was slow and regular addition of molecular sieves increased the kinetics of the condensation reaction. After 15 days of reaction, acetylated amphiphilic nitrones were isolated in good yields, after chromatography on silica gel and filtration on Sephadex LH-20, with small amounts of starting aldehyde (less than 5%).[16] Hydrolysis of the acetyl groups using the Zemplen method with catalytic amounts of MeONa in dry MeOH, led to the production of amphiphilic nitrones **1**–**5** in quantitative yields. All compounds were purified by C18 reversed-phase HPLC (eluant methanol–water) and were obtained as a pure white powder without a residual ESR signal.

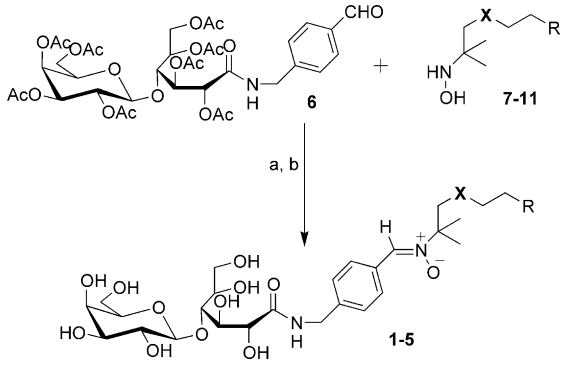
The main physico-chemical properties of these amphiphilic spin-traps is reported in Table 2. The critical micellar concentration (CMC) was determined by surface tension measurement. As expected, perfluorocarbon nitrone **3** exhibited lower CMC than its hydrocarbon analogue **2**. Due to their relatively short tail nitrones **1** and **4** presented no CMC, and were probably less detergent than compound **2**.

Preliminary biological assays were carried out with amphiphilic nitrone **2**.[17] The cytotoxicity of compound **2** in cultured neuronal cells was specified using the MTT viability assay (Fig. 1).[18] The cytotoxicity of this compound appeared at a concentration of 400 μM, close to its CMC (0.41 mM). This toxicity could thus be due to the detergent property of this amphiphilic compound, which could lead to the dissolving of cell membranes at this concentration.

The ability of compound **2** to reduce the level of caspase-3 activity in cultured neuronal cells was then evaluated and compared to that of PBN and DMPO, two commercially available nitrones. The level of caspase-3 activity, an enzyme that plays a crucial role in apoptosis, is a good quantitative parameter for this phenomenon in neurons.[19] (Fig. 1)

The data presented in Figure 2 shows that nitrone **2** at 0.2 mM level dramatically inhibits the activity of caspase-3 in cultured neuronal cells following exposure to 0.05 mM hydrogen peroxide. Furthermore, the activity of this nitrone at 200 μM appears much greater than those observed with commercially available nitrones PBN (α-phenyl-*N-tert*-butyl nitrone) and DMPO (5,5-dimethylpyrroline-*N*-oxyde). However, at lower concentration (10 and 100 μM), these last spin-traps exhibit a better efficiency. Further experiments with the other amphiphilic spin-traps should allow us to explain this behavior.

These preliminary results clearly show the ability of amphiphilic nitrone **2** to decrease the apoptosis in



Scheme 4. (a) 2 Equiv of hydroxylamines, THF, 55 °C, molecular sieves 4 Å, dark, 15 days; (b) MeONa, MeOH, 1 h, quantitative yields.

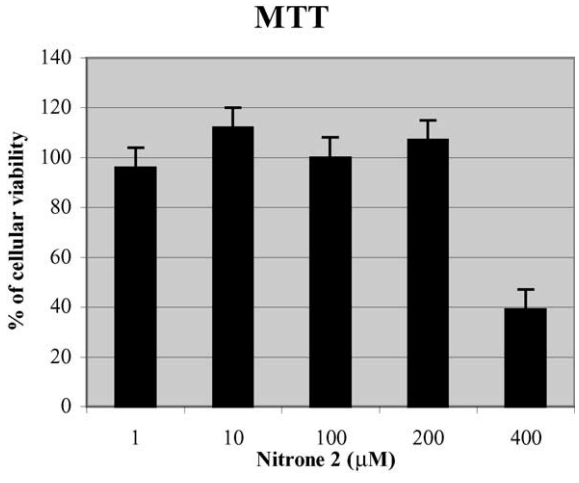


Figure 1. Cytotoxicity of compound **2** in cultured neuronal cells. MTT staining after 20 h exposure to different concentration of nitrone **2**. Values are expressed as % control value and are mean ± SD ($N = 4$–6).

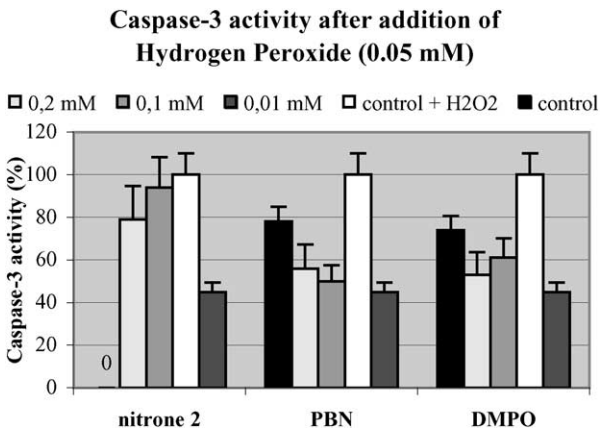


Figure 2. Caspase-3 activities (kit Sigma CASP3C) in cultured neuronal cells after 20 h exposure to variable concentrations of nitrone **2**, PBN and DMPO (after 8 days of cells culture). After incubation with nitrones, apoptosis was induced by the addition of 0.05 mM hydrogen peroxide. Values are mean ± SD of four experiments.

cultured neuronal cells at a threshold of concentration. Further analysis of all compounds of this new spin traps family is currently underway in order to determine the impact of both the nature of the hydrophobic chain (hydro or perfluorocarbon), and that of the bond between the hydrophobic chain and the nitrone moiety, on their antiapoptosis activity.

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17. Nitrone **2** analysis: R_f: 0.52 (ethyl acetate/methanol/water 7:2:1). Mp: 115 °C (dec). $[\alpha]_D = +17.2$ (0.25c, 1, CH_3OH). ^{1}H NMR (250 MHz, CD_3OD) δ 8.28 (2H, d, $J = 8.25$ Hz, H arom.), 7.82 (1H, s, CH = N(O)), 7.42 (2H, d, $J = 8.25$ Hz, H arom.), 4.65–4.35 (4H, m, CH_2-NH, H-1′, H-2), 4.25 (1H, m, H-3), 4.00–3.35 (10H, m, H-4, H-5, CH_2-OH, H-4′, H-5′, H-3′, H-2′), 3.01 (2H, s, C^{IV}–CH_2–S), 2.43 (2H, t, $J = 7.3$ Hz, CH_2–S), 1.61 (6H, s, CH_3 *tert*-butyl), 1.44 (2H, m, CH_2–CH_2–S), 1.30–1.10 (10H, m, CH_2), 0.87 (3H, t, $J = 6.9$ Hz, CH_3 chain). ^{13}C NMR (62.86 MHz, CD_3OD) δ 175.3 (CO–NH), 143.4 (C^{IV} arom.), 136.0 (CH = N(O)), 131.1 (CH arom.), 130.6 (C^{IV} arom.), 128.3 (CH arom.), 105.8 (CH-1′), 83.3 (CH-4), 77.2 (CH-5′), 74.8 (C^{IV}), 74.6 (CH-3′ or CH-2′), 74.1 (CH-2), 73.2 (CH-5), 72.8 (CH-3′ or CH-2′), 72.5 (CH-3), 70.4 (CH-4′), 63.8, 62.7 (CH_2-6, CH_2-6′), 43.5, 43.0 (CH_2–NH, CH_2–S), 34.2 (CH_2–S), 32.9, 31.0, 30.3, 30.2, 29.7 (CH_2), 26.0 (CH_3 *tert*-butyl), 23.7 (CH_2), 14.4 (CH_3). UV (MeOH, nm): $\lambda_{max} = 298.8$. FABMS (m-nitrobenzyl alcohol matrix): $m/z = 729$ $[M+K]^+$; 713 $[M+Na]^+$. Elemental analysis $C_{32}H_{54}N_2O_{12}S$, $3H_2O$ (744.9): calcd C, 51.60; H, 8.12; N, 3.76; S, 4.30; found C, 51.83; H = 7.66; N = 3.70; S = 4.25.
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