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# *N-tert*-Butyl and *N*-methyl nitrones derived from aromatic aldehydes inhibit macromolecular permeability increase induced by ischemia/reperfusion in hamsters

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ABSTRACT

*N*-Alquil nitrones **1c** and **3–6** were prepared from aromatic aldehydes and *N-tert*-butylhydroxylamine or *N*-methylhydroxylamine in good yields and soft conditions. Their protective effect against microvascular damages caused by ischemia/reperfusion in 'hamster cheek pouch' assay was investigated and compare with that observed for nitrones **1a**,**b** and **2**, previously studied. Nitrones **3b**, **4b** and **4c** were the most active ones in inhibiting macromolecular permeability increase induced by ischemia/reperfusion when administered by gavage and intravenous, while **3a** and **4a** were active only after intravenous administration. *N-tert*-butylhydroxylamine and *Nt*-methylhydroxylamine, products of the hydrolysis of these nitrones, were weakly active when administered by gavage or intravenous. Nitrone (**4a**) was the most potent in inhibiting macromolecular permeability increase induced by histamine. In this case, *N-tert*-butylhydroxylamine was as active as **4a**. The lypophylicity in nitrones, specially in *N*-methyl nitrones, play an important role on the protective action when compounds were administered by gavage.



## 1. Introduction

Nitrones have been extensively used for detection and identification of transient radical species in chemistry and biology.[1] Phenyl-*t*-butyl nitrone (PBN) and derivatives act as effective protective agents in rat models of transient and permanent focal ischemia and stroke model in rodents (Fig. 1).[2]

Ames and co-workers showed, for the first time, that old solutions of PBN were more effective than fresh ones in delaying senescence of IMR90 cells.[3] Taking into account that benzaldehyde, *N-t*-butyl hydronitroxide and *N-t*-butylhydroxylamine are metabolites derived from PBN and that hydroxylamine delays senescence at concentrations as low as 10 μM compared with 200 μM PBN needed to produce a similar effect, it has been suggested that *N-t*-butylhydroxylamine is the active form of PBN.

Knowing that shelf life of NXY-059, a PBN like nitrone, is short and in parenteral solutions it can be hydrolyzed as much as 1% per day, Proctor and Tamborello postulated that hydrolysis products are the true neuroprotectors against acute stroke.[4]

Recently we have reported on protective effects of a series of PBN-derivatives against microvascular damages induced by ischemia/reperfusion in the hamster cheek pouch assay.[5] Compounds **1a**,**b** and **2** were very active administered by gavage and

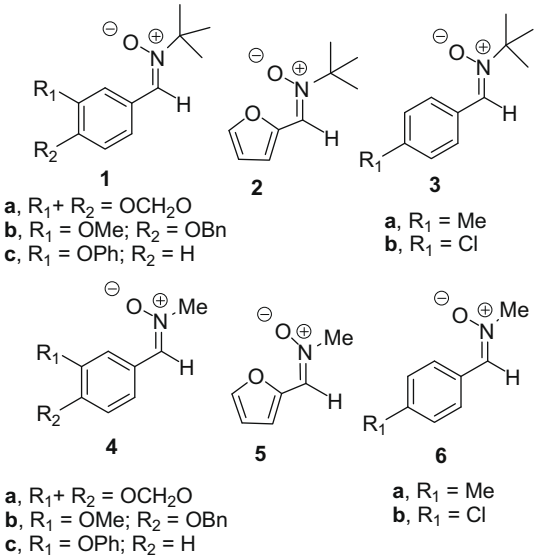


**Figure 1.** PBN-like nitrones **1–6**.

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were selected as the most promising ones. In this paper we describe synthesis of new *N*-*t*-butylnitrones **1c** and **3a**,**b** and corresponding less expensive *N*-methylnitrones **4a**–**c**, **5** and **6a**,**b**. The protective effect of these compounds on microvascular damage induced by ischemia/reperfusion and on inhibition of macromolecular permeability increase induced by histamin in hamsters was evaluated and the results compared with those observed for **1a**,**b** and **2**.[5]

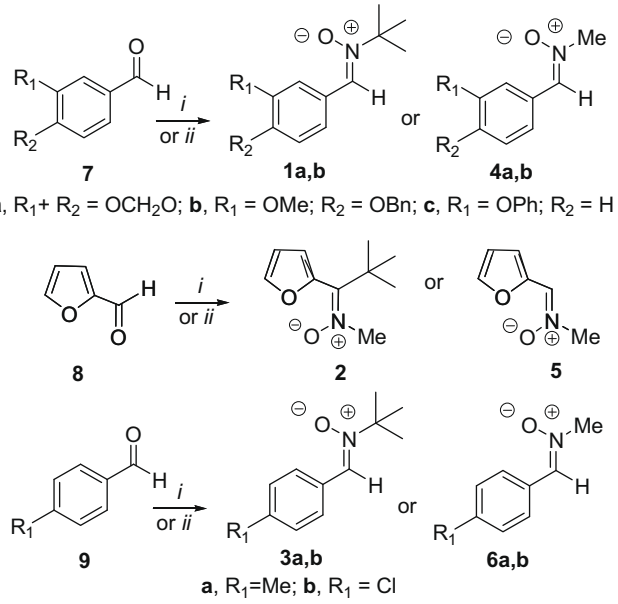
## 2. Results

### 2.1. Chemistry

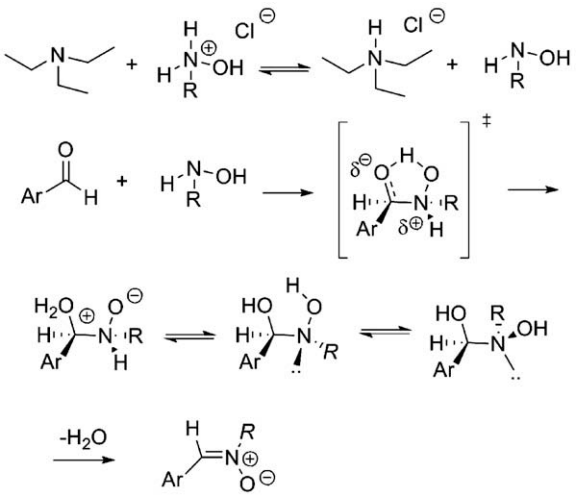
Several methods are reported to prepare nitrones and we choose the protocol described by Dondoni et al.[6] While the synthesis of **1a**–**c**, **2**[5] and **3a**,**b** required the use of 2 equiv of *N*-*t*-Butylhyroxyl amine, an expensive reagent, and long reaction times to go to completion (scheme 1, entries 1–6), nitrones **4a**–**c**, **5** and **6a**,**b** were prepared in excellent yields (entries 7–12) by the reaction between the corresponding aromatic aldehydes **7** and **8** with of 1 equiv of MeNHOH, in only 4 h of reaction at room temperature.

Only one stereoisomer was formed in all cases, as shown by the analysis of their $^{13}C$ NMR spectra. The *Z*-configuration was confirmed for **3a** on the basis of NOE experiments. An increase of 3% in the signal of the olefinic proton was observed after irradadiation at *N*-Me group, as previously reported by Dondoni et al.[5,6] The configuration of others nitrones were assumed to be Z.

Only scattered reports on the mechanism of nitrone formation through the addition of hydroxyl amines to aldehyde are described in the literature.[7] When studied in water in pH 1–11, the reaction showed to be reversible, being the dehydration the rate determining step. However these finds are not applicable to our reaction conditions. The mechanistic rationalization proposed in Scheme 2 explains our results. Once the reaction rate was very dependent on the steric hindrance around the nucleophilic nitrogen atom on the hydroxyl amine (MeNHOH reacts faster than *t*BuNHOH) and is not reversible under the condition studied (no hydrolysis of **1a** and **1b** was observed when the solvent was saturated with water),




**Scheme 1.** Synthesis of nitrones **1**–**6** from aldehydes **7**–**9**.




**Scheme 2.** Mechanistic rationalization for the reaction of hydroxyl amines with aldehydes in $CH_2Cl_2$.

it is reasonable to accept that the nucleophilic addition is the rate determining step.

The first step is the deprotonation of hydroxyl amine chloridrate by $Et_3N$, generating the neutral hydroxyl amine, the nucleophilic specie. The nucleophilic addition of these species to the aldehydes is proposed to occur through a cyclic transition state in which, to minimize the steric interaction, the R group in the hydroxyl amine and the Ar group in the aldehyde are at opposite sides. Prototropism in the resulting intermediate followed by an *anti*-elimination of water lead to the nitrones with the observed *Z*-geometry. According to this mechanism a partial negative charge is generated at the benzylic position during the nucleophilic addition step and nitrones bearing substituents at the aromatic ring able to stabilize this charge should react faster. In fact, we previously found that aromatic aldehydes substituted by electron donors substituents react slower than those substituted by electron withdrawing groups with *N*-*t*-butylhyroxyl amine.[5] For example **1b,c** required reflux while **1c**, **2**, 3a,b reacted at room temperature. On the other hand, the reactions with the less bulky MeNHOH are faster and less sensitive to the nature of the substituent at the aromatic ring, and all aldehydes studied were completely consumed after 4 h of reaction.

## 3. Pharmacology

In the present study we used the cheek pouch preparation of male hamsters to observe macromolecular permeability increase [measured by number of leakage sites per unit area (leaks/cm$^2$)], one of the earliest signs of microvascular dysfunction, induced by ischemia/reperfusion (dependent on formation of reactive oxygen species) or topical application of histamine (dependent on increased $Ca^{2+}$ permeability on endothelial cells; 1 μM). The thin distal part of the cheek pouch is highly vascularized and well suited to in vivo intravital microscopy studies. Reactive oxygen species formed after reperfusion to an ischemic area initiates a complex cascade of events leading to enhanced vascular macromolecular leakage.[5] Compounds were given to the animals either by gavage or intravenously to observe the biodisponibility as well as the effect of metabolites.

In Table 2 are shown the inhibition of macromolecular permeability increase induced by ischemia/reperfusion provided by nitrones **1**–**6**. *N*-*tert*-Butylhydroxylamine and *N*-metylhydroxylamine, products of the hydrolysis of *N*-*tert*-butylhydroxylnitrones and *N*-

**Table 1**
Yields for nitrones **1–6**

| Entry | Aldehyde | Nitrone | Time | Yields (%) |
|---|---|---|---|---|
| 1 | **7a** | **1a** | 3 days* | 51 |
| 2 | **7b** | **1b** | 3 days* | 66 |
| 3 | **7c** | **1c** | 3 days | 71 |
| 4 | **7a** | **4a** | 4 h | 98 |
| 5 | **7b** | **4b** | 4 h | 96 |
| 6 | **7c** | **4c** | 4 h | 98 |
| 7 | **8** | **2** | 7 days | 80 |
| 8 | **8** | **5** | 3 days | 68 |
| 9 | **9a** | **3a** | 3 days | 84 |
| 10 | **9b** | **3b** | 3 days | 63 |
| 11 | **9a** | **6a** | 4 h | 87 |
| 12 | **9b** | **6b** | 4 h | 93 |

* In this case reflux was necessary.

**Table 2**
Inhibition of macromolecular permeability increase induced by ischemia/reperfusion by nitrones **1–6**

| Entry | Compound | Oral | Intrav. | Log *P* theorical |
|---|---|---|---|---|
| 1 | MeNHOH | 23 | 33 | — |
| 2 | *t*BuNHOH | 10 | 7 | — |
| 3 | **1a** | 65 | 38 | 0.66 |
| 4 | **1b** | 68 | 36 | 2.93 |
| 5 | **1c** | 12 | 18 | 3.41 |
| 6 | **2** | 61 | 43 | 0.04 |
| 7 | **3a** | 14 | 55 | 1.71 |
| 8 | **3b** | 63 | 53 | 2.40 |
| 9 | **4a** | 3 | 50 | −0.57 |
| 10 | **4b** | 72 | 51 | 1.70 |
| 11 | **4c** | 46 | 61 | 2.18 |
| 12 | **5** | 15 | 59 | −1.19 |
| 13 | **6a** | 24 | 21 | 0.48 |
| 14 | **6b** | 31 | 27 | 1.17 |
| 15 | α-Tocopherol[1] | 50 | — | — |
| 16 | Shark cartilage[1] | 50 | — | — |
| 17 | Fish oil[2] | 60 | — | — |

Number of leaks in the absence of nitrone (control) = 123.3 ± 4.7 leaks/cm$^2$ observed at 10 min after the onset of reperfusion. The number of leaks observed in the presence of nitrones was transformed in percentage of protection. Total ischemia of 30 min was obtained by placing a tourniquet made of a soft rubber tubing underneath the metal around the preparation as soon as it leaves the mouth of the animal. The rubber tubing was connected to a syringe and its pressure was increased until the blood flow in the preparation was completely stopped. After 30 min, the tube was evacuated in a similar but inverse procedure. Male golden hamsters *(Mesocricetus auratus)*, 7–10 weeks old, weighing approximately 100 g, were obtained from ANILAB (Campinas, SP, Brazil). Experiments were performed according to protocols approved by the Ethical Committee of UERJ (protocol CEA 215/2007). Animals received appropriate laboratory diet (Nuvital, Nuvilab, Paulinia, PR, Brazil) and water ad libitum. All drugs tested were administered by gavage, 30 min before the induction of anesthesia or intravenously 10 min before the onset of reperfusion.[1]10 days treatment,[2] 15 days treatment. The log *P* was determined by ACD Labs/Log *P* dB 3.5 and chemsketch 3.5 program.

metylhydroxylnitrones, respectively, were weakly active (entries 1 and 2). The protective effect observed for *N*-*t*-butyl nitrones after gavage administration seemed to be dependent on the substitution pattern at the aromatic ring. Among new synthesized derivatives, **3b** was equipotent to **1a**,**b** and **2** (entries 1–6). Except for **4b**,**c**, (entries 7 and 8) the corresponding *N*-Methyl nitrones were less potent.

Surprisingly, except for **3a**, the protective effect observed for *N*-*t*-butyl nitrones after intravenous administration decreased (entries 1–6). In methyl series (entries 7–12) **4b**,**c** were the more potent nitrones.

Since hystamine is also involved in the inflamatory process, we decided to study some nitrones on this asssay, Table 3. The best results were obtained for nitrone **4a** and *N*-*t*-butylhydroxylamine (entries 3 and 9).

**Table 3**
Inhibition of macromolecular permeability increase induced by histamine by nitrones **1–6**

| Entry | Compound | Oral |
|---|---|---|
| 1 | **1b** | 32 |
| 2 | **2** | 18 |
| 3 | **4a** | 42 |
| 4 | **4b** | 15 |
| 5 | **4c** | 13 |
| 6 | **5** | 32 |
| 7 | **6b** | 29 |
| 8 | MeNHOH | 11 |
| 9 | *t*BuNHOH | 40 |
| 10 | α-Tocopherol[1] | 40 |
| 11 | Shark cartilage[1] | 50 |

Number of leaks in the absence of nitrone (control) = 450.6 ± 20.6 leaks/cm$^2$observed 5 min after topical application of histamine (1 μM). The number of leaks observed in the presence of nitrones was transformed in percentage of protection. Male golden hamsters *(Mesocricetus auratus)*, 7–10 weeks old, weighing approximately 100 g, were obtained from ANILAB (Campinas, SP, Brazil). Experiments were performed according to protocols approved by the Ethical Committee of the State UERJ (protocol CEA 215/2007). Animals received appropriate laboratory diet (Nuvital, Nuvilab, Paulinia, PR, Brazil) and water ad libitum. All drugs tested were administered by gavage, 30 min before the induction of anesthesia.[1] 10 days,[2] 15 days treatment.

## 4. Discussion

The mechanism of action of nitrones is not fully understood. The protective effect of PBN against MPTP toxicity in mice was initially attributed to an antioxidant activity, but results have shown that it acts only as moderate retarder of oxygen uptake at relatively high concentrations.[8] This action is independent of hydroxyl radical trapping and seems to be associated with carbon-centered radical trapping.

Firstly we discuss results showed on Table 2. Ames,[3] as well as Proctor and Tamborello[4] suggested that *N*-*t*-butylhydroxylamine can be an effective neuroprotector agent against acute stroke. However, in our model, occlusion-induced isquemia, neither methyl hydroxylamine nor *N*-*t*-butylhydroxylamine showed important protective effect (Table 1, entries 1 and 2). Two hypotheses could be considered to explain these results: the organic moiety is the carrier of hydroxylamine species or the biological action is associated with the nitrone itself.

The low activity observed for some nitrones administrated by gavage could be associated with hydrolyzes at the stomach. So we decided to investigate the stability of this family of compounds under low pH, to mimic physiological conditions in the stomach. We expected that, due to the steric hindrance around the carbon atom attached to the nitrogen, *N*-*t*-Butyl nitrones would be more resistant to hydrolysis than *N*-methyl series (microscopic reversibility principle).[9] In fact, **1a** was not hydrolyzed when allowed in water at pH 1 for 15 min, while under these conditions the corresponding *N*-methyl nitrone **4a** was completely hydrolyzed.

In agreement with this proposal all *N*-*t*-butyl nitrones, except **1c**, were active after gavage administration. These nitrones were also active after intravenous administration (Table 1, entries 1–6). However, since these nitrones are more resistant to hydrolysis, no correlation could be found between log *P* and the protective effect. On the other hand, for *N*-methyl nitrones the hydrolysis is fast and in this case the protective effect depended on log *P*.[10] Only those nitrones (**4b** and **4c**), having high log *P* values were active by gavage, suggesting that they are rapidly absorbed. In agreement with this rationalization, nitrones **4a** and **5**, which were weakly active by gavage administration, showed to be very active by intravenous administration (entries 7 and 10).

Nitrones **1a**, **1b**, **2** and **3b** were more potent than α-tocopherol and shark cartilage (entries 15 and 16) and as potent as fish oil (entry 17), used as references.

In contrast with results showed on Table 2, in which hydroxylamines presented low potency in histamine-induced microvascular damages in the cheek pouch microcirculation, *N*-*t*-Butyl nitrone **4a** was as active as *N*-*t*-butylhydroxyl amine (Table 3, entries 3 and 9). These compounds were as potent as α-tocopherol but less active than shark cartilage (entries 10 and 11).

## 5. Conclusions

In conclusion, we demonstrated for the first time the activity of less expensive *N*-methyl nitrones as protectors against microvascular damage induced by occlusion-induced ischemia-reperfusion in the hamster cheek pouch preparation. In these cases, liposolubility seems to be important for the biological action. In contrast with results of Ames,[3] and Proctor and Tumbarello[4] pointing to higher activity of *N*-*t*-butylhydroxylamine compared to PBN, in our case nitrones were more active on occlusion-induced ischemia-reperfusion. On the other hand, on histamine-induced microvascular damage, *N*-*t*-butylhydroxylamine was as active as **4a**, the best nitrone in this assay. Selected nitrones were as active as or more active than α-tocopherol, shark cartilage and fish oil, used as references.

We previously showed that *N*-*t*-butyl nitrones type 1 and 2 react with methyl radical leading to stable spin adducts. However, a clear correlation between the ability of these nitrones as spin trappers and the protective effect in the hamster cheek pouch preparation could not be found.[5] The results showed herein for *N*-methyl nitrones suggest again that this property is not the main factor for the proctective effect, once these nitrones are poor spin trappers.[11]

## 6. Materials and methods

Chemicals were purchased from Sigma–Aldrich–Fluka Co. Column chromatography was carried out using 200–400 mesh chromagel were determined on an Electrothermal 9300 capillary melting point apparatus and are uncorrected. $^1$H NMR spectra were recorded on an AC Bruker spectrometer at 200 and 400 MHz using $CDCl_3$ as solvent, chemical shifts ($\delta$) are reported in ppm relative to tetramethylsilane) and the following multiplicity abbeviations were used: s, singlet; d, doublet; t, triplet; q, quatruplet; m, multiplet; dd, double doublet; dt, double triplet. $^{13}$C NMR spectra were recorded on a Bruker spectrometer at 75 and 100 MHz (Bruker, Wissembourg). Mass spectra were obtained on a MS-Nermag R10-10 spectrometer. IR spectra were recorded on a Perkin–Elmer PARAGON 1000 FT-IR spectrometer. UV–visible spectra were recorded on an Uvikon 931 Kontron spectrometer.

### 6.1. Synthesis of *N*-aryl *N*-methyl nitrones

To a solution of aromatic aldehydes **7**–**9** (5.0 mmol), triethylamine (5.0 mmol; 1532 μL) in $CH_2Cl_2$ (40 mL), in the presence of dry $Na_2SO_4$ (2 g), was added *N*-*methyl*tylhydroxylamine hydrochloride (5.0 mmol, 1256 mg). The mixture was stirred at room temperature for 4 h and after this time the reaction mixture was filtered and $CH_2Cl_2$ was evaporated under reduced pressure. The crude product was purified by column chromatography (solvent: cyclohexane/ethyl acetate 60/40).

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